BIO-GENERATION OF POLY/OLIGOSACCHARIDES AND THEIR FERULOYLATED DERIVATIVES FROM COCOA BEAN SHELLS FOR POTENTIAL USE AS SWEETENER PREBIOTICS

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ABSTRACT

Cocoa bean shell, the outermost layer of the cocoa bean, is a heavily accumulated biomass product of the chocolate industry. Efforts to valorize this biomass are of high interest, in terms of their nutritional benefits, in agricultural, feedstuff, bioremediation, and food applications. Based on the proximate compositional analysis, cocoa bean shells from two varieties (Nacional (NAC) and CCN) were characterised. Overall, cocoa bean shells were found to be a great source of carbohydrates, specifically dietary fiber (50.1- 61.4% w/w), protein (12.7-19.7% w/w), ash, and polyphenols, namely quercetin, epicatechin, and catechin. There were noticeable differences in the CCN variety where the degree of processing accounted for a variation in the chemical composition. For example, is the protein content has shown a noticeable increase ($p < 0.05$) from its fermented (13%, CCN) to dried (18.9%, CCN) states, followed by a decrease in its roasted state (12.8%, CCN). Similarly, the fat content increases ($p < 0.05$) with the processing of cocoa beans from fermentation to drying and roasting, which may be attributed to subjection of elevated temperatures in the drying and roasting processes.

The cell wall material of cocoa bean shells was isolated and characterised as being primarily composed of pectic polysaccharides (80.6-86%), as indicated by the presence of uronic acids (35.1-50.5 %), galactose (22.7-29.2%), arabinose (13.6-17.2%), and rhamnose (5.1-6.9%) residues. Additionally, the extraction of cell wall polysaccharides from the cell wall material of cocoa bean shells was explored. The alkaline extractions (0.5 and 4M potassium hydroxide) were successful in the recovery of polysaccharide predominantly enriched with pectic polysaccharide regions (80.6-86 % w/w polysaccharide extract), rhamnogalacturonan and arabinogalactan. Increasing the alkali concentration accounts in a shift in the recovery from high to low molecular weight fractions, due to a higher degree of defragmentation; where the 4M extract accounted for two MW main populations (24.0, and 6.3 kDa – NAC; 11.0 and 3.2 kDa - CCN) compared to the three populations found in the less concentrated extracts (3.7, 12.0, and 130 kDa – NAC; 4.5, 9.1, and 32.5 kDa - CCN).

Enzymatic hydrolysis of cocoa bean shell cell wall polysaccharides by arabinase, galactanase and five multi-enzymatic products was investigated for the bio-generation of oligosaccharides. The multi-enzymatic products expressed varied levels of polysaccharide-degrading activities. Viscozyme® L (76.4%, NAC) and Depol™ 670L (20.72%, CCN) attained the highest hydrolysis

extents, while also generating a diverse complex of oligosaccharides with degrees of polymerization between 3 and 10. Most of the oligosaccharides (50.99 – 99.51%) generated by multi-enzymatic biocatalysts had a degree of polymerization between 2 and 5. The prebiotic activities were assessed using cell wall polysaccharides (NAC, CCN) as well as NAC- and CCN-oligosaccharides bio-generated by Viscozyme® L and Depol™ 670L, respectively. For the promotion of the growth of selected probiotic strains (*Lactobacillus rhamnosus GG* and *Bifidobacterium* longum), cell wall polysaccharides and bio-generated oligosaccharides performed similarly or more than inulin and rhamnogalacturonan as it was shown by the prebiotic activity scores. The short chain fatty acid profiles were mostly characterized by high amounts of lactic and acetic acid. However, oligosaccharides also contributed to a significant production of butyric and propionic acids, possibly attributed to their smaller degree of polymerization.

Enzymatic esterification of generated oligo- and polysaccharides from NAC and CCN cocoa bean shells with ferulic acid was investigated. Feruloyl esterases, from *Humicola insolens* and rumen microorganism, were immobilized on iminodiacetic acid-copper modified epoxy-polyacrylic supports. The specific esterifying activity of free and immobilized feruloyl esterases was assessed on raffinose in surfactant-less microemulsions, made of n-hexane/butanone/water (49.4/44.6/6, v/v/v). Although high immobilization yields were attained, the free feruloyl esterases were found to be most active. For the esterification of cocoa bean shell oligo- and polysaccharides, free feruloyl esterases were used. Feruloyl esterase from rumen microorganism led to the highest feruloylation efficiencies with polysaccharides (56.9-60.1%), while the feruloyl esterase from *H. insolens* resulted in the highest feruloylation efficiencies (30.8-50.5%) with oligosaccharides. Based on DPPH, ORAC, and ABTS assays, feruloylated polysaccharides prompted significantly notable increases in the scavenging and antioxidant activity compared to their native forms as well as to ferulic and ascorbic acid.

The extraction techniques explored for the recovery of oligo- and polysaccharides from CCN variety of cocoa bean shells were scaled-up in a pilot plant facility, to compare their efficiencies and extracts recovered, in terms of their monosaccharide profile and molecular weight distribution. One process was based on the isolation of cell wall polysaccharides from cocoa bean shells using 0.5M alkali concentration, while the second process was based on the recovery of bio-generated oligosaccharides using commercially available Depol™ 670L. As expected, the recovery of pilot-scale polysaccharides (3.69%) was lower than those recovered at the lab-scale (29.1%). The

monosaccharide profile of pilot-scale polysaccharides were similar to those recovered at the lab-scale with the exception of a higher proportion of total neutral sugars, namely galactose (17.4%) and glucose (13.9%). Based on the degree of polymerization, polysaccharides ranged between DP 2-5 (85.2% mg/L), DP 10-15 (10.8% mg/L) and DP > 40 (4.0% mg/L); while oligosaccharides ranged between DP 6-9 (57.6% mg/L), DP 2-5 (39.25 % mg/L), and DP 16-40 (3.2% mg/L). In terms of the MW distribution, both extracts showed four main populations at 0.87, 1.1, 1.6, and 3.3 kDa for oligosaccharides and at 0.83, 2.1, 320, and 2400 kDa for polysaccharides.

Selected chocolate-based formulations were enriched with pilot-scale polysaccharides for the assessment of their consumer acceptability and purchase intent, where a sensory panel was held to compare chocolate formulations with and without polysaccharide enrichment. The design was evaluated by 60 untrained panelists on a 9-point hedonic scale for mean quantitative descriptive scores and mean liking based scores. The relationships between the consumers liking scores and the perceived complexity were assessed to understand the degree of complexity inflicted upon consumers. Overall, consumers generally preferred the enriched chocolate formulations.

RÉSUMÉ

La coque de la fève de cacao, la couche la plus externe de la fève de cacao, est une biomasse fortement accumulée par l'industrie du chocolat. Les efforts pour valoriser cette biomasse sont d'un grand intérêt, en raison de leurs avantages nutritionnels, dans les applications agricoles, alimentaires, de biorémédiation et d'alimentation animale. Sur la base de l'analyse de la composition proximale, les coques de fèves de cacao de deux variétés (Nacional (NAC) et CCN) ont été caractérisées. Dans l'ensemble, les coques de fèves de cacao se sont révélées être une excellente source de glucides, en particulier de fibres alimentaires (50,1 à 61,4 % p/p), de protéines (12,7 à 19,7 % p/p), de cendres et de polyphénols, à savoir la quercétine, l'épicatéchine et la catéchine. Des différences notables ont été constatées dans la variété CCN où le degré de transformation explique une variation de la composition chimique. Par exemple, la teneur en protéines a montré une augmentation notable ($p < 0,05$) de l'état fermenté (13%, CCN) à l'état séché (18,9%, CCN), suivie d'une diminution à l'état de torréfaction (12,8%, CCN). De même, la teneur en graisse augmente ($p < 0,05$) avec le traitement des fèves de cacao, de la fermentation au séchage et à la torréfaction, ce qui peut être attribué à la soumission à des températures élevées dans les processus de séchage et de torréfaction.

La paroi cellulaire des coques de fèves de cacao a été isolée et caractérisée comme étant principalement composée de polysaccharides pectiques (80,6-86 %), comme l'indique la présence d'acides uroniques (35,1-50,5 %), du galactose (22,7-29,2 %), d'arabinose (13,6-17,2 %) et de rhamnose (5,1-6,9 %). Les extractions alcalines (hydroxyde de potassium 0,5 et 4M) ont permis de récupérer des polysaccharides principalement enrichis en régions pectiques (80,6-86 % p/p), en rhamnogalacturonan et en arabinogalactan. L'augmentation de la concentration d'alcali entraîne un déplacement de la récupération des fractions de poids moléculaire élevé vers les fractions de poids moléculaire faible, en raison d'un degré plus élevé de défragmentation ; où l'extrait 4M représentait deux populations principales de MW (24,0 et 6,3 kDa - NAC ; 11,0 et 3,2 kDa - CCN) par rapport aux trois populations trouvées dans les extraits moins concentrés (3,7, 12,0 et 130 kDa - NAC ; 4,5, 9,1 et 32,5 kDa - CCN).

L'hydrolyse enzymatique des polysaccharides de la paroi cellulaire de la coque de la fève de cacao par l'arabinase, la galactanase et cinq produits multi-enzymatiques a été étudiée pour la bio-génération d'oligosaccharides. Les produits multi-enzymatiques ont exprimé des niveaux variés

d'activités de dégradation des polysaccharides. Viscozyme® L (76,4 %, NAC) et Depol™ 670L (20,72 %, CCN) ont atteint les plus hauts degrés d'hydrolyse, tout en générant un complexe diversifié d'oligosaccharides avec des degrés de polymérisation entre 3 et 10. La plupart des oligosaccharides (50,99 - 99,51%) générés par les biocatalyseurs multi-enzymatiques avaient un degré de polymérisation entre 2 et 5. Les activités prébiotiques ont été évaluées en utilisant des polysaccharides de la paroi cellulaire (NAC, CCN) ainsi que des oligosaccharides NAC- et CCN-générés par Viscozyme® L et Depol™ 670L, respectivement. Pour la promotion de la croissance des souches probiotiques sélectionnées (*Lactobacillus rhamnosus* GG et *Bifidobacterium longum*), les polysaccharides de la paroi cellulaire et les oligosaccharides biogénérés ont eu des performances similaires ou supérieures à celles de l'inuline et du rhamnogalacturonane, comme le montrent les scores d'activité prébiotique. Les profils des acides gras à chaîne courte étaient principalement caractérisés par des quantités élevées d'acides lactique et acétique. Cependant, les oligosaccharides ont également contribué à une production significative d'acides butyrique et propionique, probablement attribuée à leur plus faible degré de polymérisation.

L'estérification enzymatique d'oligo- et polysaccharides bio-générés à partir de coques de fèves de cacao NAC et CCN avec de l'aide férulique a été étudiée. Des féruloyl estérases, provenant de *Humicola insolens et* de microorganismes du rumen, ont été immobilisées sur des supports époxy-polyacryliques modifiés par de l'acide iminodiacétique et du cuivre. L'activité estérifiante spécifique des féruloyl estérases libres et immobilisées a été évaluée sur le raffinose dans des microémulsions sans tensioactif, composées de n-hexane/butanone/eau (49,4/44,6/6, v/v/v). Bien que des rendements élevés d'immobilisation aient été atteints, les féruloyl estérases libres se sont avérées les plus actives. Pour l'estérification des oligo- et polysaccharides de la coque de fèves de cacao, des féruloyl estérases libres ont été utilisées. La féruloyl estérase provenant d'un micro-organisme du rumen a conduit aux efficacités de féruloylation les plus élevées avec les polysaccharides (56,9-60,1%), tandis que la féruloyl estérase provenant de H. insolens a conduit aux efficacités de féruloylation les plus élevées (30,8-50,5%) avec les oligosaccharides. Les capacités antioxydantes des polysaccharides féruloylés ont été évaluées. D'après les tests DPPH, ORAC et ABTS, les polysaccharides féruloylés ont présenté des augmentations significatives et notables de l'activité antioxydante et de piégeage par rapport à leurs formes natives ainsi qu'à l'acide férulique et ascorbique.

Les techniques d'extraction explorées pour la récupération des oligo- et polysaccharides de la variété CCN de coques de fèves de cacao ont été mises à l'échelle dans une installation pilote, afin de comparer leurs efficacités et les extraits récupérés, en termes de profil de monosaccharides et de distribution de poids moléculaire. Un procédé était basé sur l'isolement des polysaccharides de la paroi cellulaire des coques de fèves de cacao en utilisant une concentration alcaline de 0,5 M, tandis que le second procédé était basé sur la récupération des oligosaccharides biogénérés en utilisant le Depol™ 670L disponible dans le commerce. Comme prévu, la récupération des polysaccharides à l'échelle pilote (3,69 %) était inférieure à ceux récupérés à l'échelle du laboratoire (29,1 %). Le profil des monosaccharides des polysaccharides de l'échelle pilote était similaire à ceux récupérés à l'échelle du laboratoire, à l'exception d'une proportion plus élevée de sucres neutres totaux, à savoir le galactose (17,4 %) et le glucose (13,9 %). Selon le degré de polymérisation, les polysaccharides variaient entre DP 2-5 (85,2 % mg/L), DP 10-15 (10,8 % mg/L) et DP > 40 (4,0 % mg/L) ; tandis que les oligosaccharides variaient entre DP 6-9 (57,6 % mg/L), DP 2-5 (39,25 % mg/L) et DP 16-40 (3,2 % mg/L). En termes de distribution des MW, les deux extraits ont montré quatre populations principales à 0,87, 1,1, 1,6, et 3,3 kDa pour les oligosaccharides et à 0,83, 2,1, 320, et 2400 kDa pour les polysaccharides.

Des formulations à base de chocolat sélectionnées ont été enrichies avec des polysaccharides à l'échelle pilote pour l'évaluation de leur acceptabilité par les consommateurs et de leur intention d'achat. Un panel sensoriel a été organisé pour comparer les formulations de chocolat avec et sans enrichissement en polysaccharides. Les formulations ont été évaluées par 60 panélistes non formés utilisant une échelle hédonique à 5 points pour les scores descriptifs quantitatifs moyens et les scores moyens basés sur le goût. Les relations entre les scores d'appréciation des consommateurs et la complexité perçue ont été évaluées afin de comprendre le degré de complexité infligé aux consommateurs. Dans l'ensemble, les consommateurs ont généralement préféré les formulations de chocolat enrichies.

STATEMENT FROM THE THESIS OFFICE

According to the regulations of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contribution of Authors" as a preface of the thesis.

When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

PREFACE AND CONTRIBUTION OF AUTHORS

This thesis includes six chapters.

Chapter I provides a general introduction to the valorization of cocoa bean shells, the extraction of their cell wall polysaccharides as well as their current and potential food applications as a functional ingredient.

Chapter II comprises a literature review of the studies relevant to cocoa bean shells' cell wall polysaccharide composition and characterization their methods of cell wall poly- and oligosaccharide extraction, especially chemical and enzymatic approaches.

Chapter III discusses the results pertaining to the extraction, characterization of the cocoa bean shell cell wall polysaccharides as well as the assessment of their prebiotic activity.

Chapter IV discusses the results pertaining to the enzymatic generation of cell wall oligosaccharides via multi-enzymatic products, the assessment of their prebiotic activity and the production of short chain fatty acid metabolites. This chapter will also discuss the results pertaining to the feruloylation of oligo- and polysaccharides using feruloyl esterases as well as the assessment of their antioxidant activities using selected antioxidant assays.

Chapter V discusses the pilot-plant extraction of cocoa bean shell carbohydrate and hydrolyzed carbohydrate extracts through alkaline isolation and multi-enzymatic hydrolysis, respectively. This chapter also discusses the results pertaining to chocolate-based formulation trials along with their sensory evaluation for consumer acceptability and purchase intentions.

Finally, **Chapter VI** covers an overall summary of the current research results

Amalie Younes was responsible for the experimental work and the preparation of the manuscripts for the publications and thesis.

Dr. Mingqin Li, the second author of Chapter II, reviewed the manuscript prior to submission.

Dr. Lan Liu, the third author of Chapter III, contributed to the research and experimental work related to the characterization of the phenolic compounds by mass spectrometry.

Dr. Amanda Waglay, the second author of Chapter V, contributed towards the facilitation of the pilot-plant scale-up trial related to extraction of cocoa bean shell carbohydrate extracts as well as reviewed the manuscript prior to submission.

Dr. Asma Mdimagh, the third author of Chapter V, contributed towards the statistical analysis of the sensory evaluation datasets of chocolate-based formulations.

Dr. Salwa Karboune, the supervisor of the current M.Sc. Research project, guided the entire research framework and reviewed all the presented chapters in this thesis prior to the submission.

RESEARCH CONTRIBUTIONS

PUBLICATIONS

1 **Younes, A.,** Li, M., & Karboune, S. (2022). Cocoa bean shells: a review into the chemical profile, the bioactivity, and the biotransformation to enhance their potential applications in foods. Critical reviews in food science and nutrition, 1–25. https://doi.org/10.1080/10408398.2022.2065659

2 **Younes, A.,** Karboune, S., Liu, L., Andreani, E.S., & Dahman, S. Extraction and Characterization of Cocoa Bean Shell Cell Wall Polysaccharides. *Polymers* 2023, *15*, 745. https://doi.org/10.3390/polym15030745

3 **Younes, A.,** & Karboune, S. (2023). Enzymatic generation of oligosaccharides from cocoa bean shells polysaccharides using multi-enzymatic products and their feruloylation (Publication under review)

4 **Younes, A.,** Razmjooyhassankhani, N., Waglay, A., Mdimagh, A., & Karboune, S. (2023). Pilot plant extraction of cocoa bean shell oligo- and polysaccharides and its incorporation into chocolate-based formulations as a prebiotic sugar alternative (Publication under review).

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NOMENCLATURE/LIST OF ABBREVIATIONS

AJS ESI	Agilent jet stream electrospray ionization
AnFaeA	Feruloyl esterase from *Aspergillus niger*
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AXE	Acetyl xylan esterase
CBS	Cocoa bean shells
CDTA	Cyclohexanediaminetetraacetic acid
CFU	Colony forming units
CHO	Carbohydrate
CWM	Cell wall material
CWP	Cell wall polysaccharides
Da	Dalton
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
FAE	ferulic acid esterase
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
HC	Hydrodynamic cavitation
HCl	Hydrochloric acid
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
HSD	Honest significant difference
KOH	Potassium hydroxide
LC-MS	Liquid chromatography mass spectrometry
LDL	Low-density lipoprotein
m/z	mass to charge ratio
MAE	Microwave-assisted extraction

MANOVA	Multi-analysis of variance
MES-NAOH	2-Morpholinoethanesulfonic acid - sodium hydroxide
mM	Millimolar
MRS	de Mann regosa
MS	Mass spectrometry
MW	Molecular weight
Na_2CO_3	Sodium carbonate
$NaBH_4$	Sodium borohydride
$NaClO_2$	Sodium chlorite
NaOH	Sodium hydroxide
NcFaeD	Feruloyl esterase from *Neurospora crassa*
PA	Prebiotic activity score
PAW	Phenol: Acetic acid :water
PDCAAS	Protein digestibility-corrected amino acid score
PEF	Pulsed electric field
PLE	Pressurized liquid extraction
PS	Polysaccharide
Q-TOF-MS	Quadrupole time of flight mass spectrometry
qPCR	Quantitative polymerase chain reaction
RGI	Rhamnogalacturonan I
SCFA	Short chain fatty acids
StFaeC	Type C FAE from *Sporotrichum thermophile*
SWE	Subcritical water extraction
TBARS	thiobarbituric acid reactive substance assay
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor alpha
TsFaeC	Feruloyl esterase from *Talaromyces stipitatus*
UAE	Ultrasound-assisted extraction

CHAPTER I. GENERAL INTRODUCTION

Theobroma cacao L., also known as the cacao tree, is natively grown in tropical climate countries on the equatorial belt such as Ivory Coast, Ghana, Nigeria, Cameroon, Mexico, Brazil, Ecuador, and Indonesia. It has been cultivated for use ever since the Aztec and Mayan era in South America (Motamayer et al., 2008). Cocoa beans are seeds of the *T. cacao* L. in which undergo a sequence of post-harvesting practices, namely fermentation, drying and roasting to obtain cocoa, the main ingredient used to produce chocolate. However, throughout these post-harvesting conditions, cocoa biomass is generated. In fact, cocoa bean production generates an estimated 15% cocoa biomass (International Cocoa Organization, 2017). One of these by-products are the cocoa bean shells, the outermost layer of the bean, removed in the dehulling or deshelling stage to retrieve the cocoa beans (Younes et al., 2022). Efforts to valorize cocoa bean shells have been explored in several applications, such as in agricultural, feedstuff, and bioremediation practices (Yajima et al., 2016; Magistrelli et al., 2016; A. Adeyina et al., 2010; Soeharsono et. al., 2017).

Indeed, the valorization of biomass for their rich dietary fiber composition is of high interest. Cocoa bean shells account for a high dietary fiber (13.86 – 60.6 %, w/w) , and varies depending on the applications of roasting and drying (Agus et al., 2018; Bamba et al., 2015; Fakhlaei et al., 2019; Martínez et al., 2012; Okiyama et al., 2017; Rojo-Poveda et al., 2019, 2020; Younes et al., 2022), and polyphenol composition (1.45-94.95 mg GAE/g) (Delgado-Ospina et al., 2020; Lessa et al., 2018; Martínez et al., 2012; Mellinas et al., 2020; Nsor-Atindana et al., 2012; Rojo-Poveda et al., 2020; Utami et al., 2016; Younes et al., 2022). Cocoa bean shell cell-wall polysaccharides, a sub-class of dietary fiber, comprise of 40–45% pectin, 34% cellulose, and 21–26% hemicellulose (Redgwell et al., 2003). Pectic regions are mostly attributed the presence of rhamnogalactouronan and arabinogalactan. Hemi-cellulosic fractions comprise of 5–7% xyloglucan, 7–10% galactoglucomannan, and 4% glucuronoarabinoxylan of non-cellulosic cell wall polysaccharides in cocoa bean shells (Redgwell et al., 2003; Vojvodić et al., 2016; Acosta et al., 2018). However, the origin, variety and processing conditions can affect the overall composition of cocoa bean shells. As far as we are aware, this has not been assessed so far. The assessment of cocoa bean shells' nutritional and cell wall compositions between two varieties at three different processing conditions, namely fermentation, drying, and roasting, are to be evaluated in the present study.

Considering the structural complexity of cell wall polysaccharides, the single enzymatic hydrolysis for the retrieval of isolated oligosaccharides can be challenging. Alternatively, commercially available multi-enzymatic products, have been previously explored for their favorable and efficient isolation enzymatically generated oligomers and oligosaccharides (Waglay et al., 2019; Spadoni Andreani et al., 2021; Khodaei & Karboune, 2016). The prebiotic potential of cell wall oligo- and polysaccharides derived from cocoa bean shells has yet to be reported. Therefore, a comprehensive assessment of the prebiotic capabilities of cocoa bean shells would complement their techno-functional properties for potential use in food formulations.

With a generic structure of one or two ferulic acid moieties esterified to a mono-, oligo- and polysaccharide chains, the generation of feruloylated oligo- and polysaccharides through feruloyl esterase (EC 3.1.1.73)-catalysed esterification reaction has been reported (Tamayo-Cabezas & Karboune, 2019; Spadoni Andreani et al., 2021). Feruloylated oligo- and polysaccharides have attracted scientific attention due to the prebiotic, antimicrobial, anti-inflammatory and antioxidant functions inherent to their structural features (Gálvez Ranilla et al., 2017; Wang et al., 2020). For instance, *in vitro* research showed that feruloylated arabinoxylooligosaccharides are fermented by cultured human colon microbiota (Snelders et al., 2014), while feruloylated oligosaccharides isolated from corn bran showed higher protective effect than ferulic acid against oxidative stress in H_2O_2-injured pheochromocytoma cells (Yao et al., 2014). Currently, the assessment of feruloylated oligo- and polysaccharides from cocoa bean shells has not been conducted.

The overall objective of the present research is to assess the nutritional and cell wall composition of cocoa bean shells as well as investigate the isolation of cell wall polysaccharides from cocoa bean shells by alkaline treatment, the enzymatic generation of oligosaccharides using commercial multi-enzymatic products and the feruloylation of these carbohydrates. The assessment of prebiotic and antioxidant activities of the generated cocoa bean shells carbohydrates (oligo/polysaccharides) and of their contribution to chocolate formulations as prebiotic sweeteners were also carried out in order to gain insight of their properties as functional food ingredients. The specific objectives are as follows:

1) Proximate compositional analysis and the evaluation of the polyphenolic profile of cocoa bean shells

2) Isolation of cell wall materials/polysaccharides from cocoa bean shells through alkaline treatments and the characterization of their monosaccharide profile and molecular weight distribution.
3) Enzymatic generation of oligosaccharides from cell wall polysaccharides using selected commercial multi-enzymatic products and the evaluation of their monosaccharide profile and molecular weight distribution.
4) Investigation of the prebiotic potential and the production of short chain fatty acids of cell wall polysaccharides and enzymatic generated oligosaccharides
5) Study of the feruloylation efficiency of free and immobilized feruloyl esterases and the assessment of their antioxidant capacities through various assays.
6) Pilot-plant extraction trial for the isolation of cell wall oligo- and polysaccharides for use as a functional food ingredient in chocolate-based products.
7) Elucidation of the relationship between the sensory attributes and the perceived complexity of panelists of selected chocolate-based formulations enriched with cell wall polysaccharides.

CHAPTER II. LITERATURE REVIEW

2.1 Introduction

Theobroma cacao L., also known as the cacao fruit, is a heavily utilized commodity in the confectionary industry. Cacao beans are cultivated in an acclimatized belt within 20 degrees latitude of the equator where cacao plants are subjected to relatively high temperatures, such as in the Ivory Coast, Ghana, Nigeria, Brazil, and Ecuador (Aprotosoaie et al., 2016). Several varieties of *T. cacao* exist in nature; however only three varieties, Criolla, Forastero and Trinitario, are widely cultivated, with the Forastero variety being the most extensively planted around the world (Panak Balentić et al., 2018).

During production, raw cacao beans are subjected to an extensive set of processing methods and conditions, such as roasting to render an end-product known as cocoa. It should be noted that the composition of cacao beans and its by-products vary depending on the clone variety, climate, harvest location, fruit maturity as well as post-harvesting conditions (Agus et al., 2018). In 2017, 4.7 million tons of cocoa beans were produced globally, where 700,000 tons of cocoa waste were generated in return (International Cocoa Organization, 2017). Cacao by-products are discarded as waste which negatively influence the environment and economic value of cocoa beans. Cocoa bean shells (CBS), also referred to as bean husk, hull, or skin, are the outer layer of the cocoa bean, which is removed in the de-hulling step to retrieve cacao nibs. CBS are considered as one of the heavily discarded by-products of cacao which constitutes around 17-20% of the total cacao bean weight.

CBS valorization is being explored in agricultural and bioremediation fields, such as animal feed, fertilizer, use as a bio adsorbent as well as a source of biofuel (Table 2.6). However, in the case of animal applications, serious caution is advised given that the presence of polyphenols, such as theobromine, are considered harmful to animals. Nonetheless, Adamafio et al., (2013) investigated the in-situ purification of cocoa pod husks with *Candida krusei* to reduce theobromine and other phenolic compounds for use in animal feed. The authors indicated a significant reduction in theobromine (62.5%) as in the total phenolic content (41.5%). Considering that animals digest polyphenols at a slower rate, in-situ approach treatments shows promising approach towards enhancing polyphenolic digestibility in animals.

In addition, researchers have also explored its use in several food applications. Adamafio et al., (2013) incorporated CBS powder as a clean label functional ingredient in baked goods to increase their fiber content. Other CBS food explorations include its use as a fat replacer in functional cakes and chocolate muffins (Martínez-Cervera et al., 2011), a stabilizer and color enhancer in pork sausages (Choi et al., 2019), as well as a sugar and fat replacer in chocolate (Barišić et al., 2019).

There is some concern pertaining to the stability of CBS when incorporated into food systems due to the subjection of fermentation, roasting and other processes that involve exposure to temperature changes. As a result, researchers have proposed the idea of CBS extract encapsulation to enhance its stability (Jokić et al. 2020). Nevertheless, it is of great importance to ensure CBS bioavailability upon consumption as the rich content of phytochemicals can promote anti-carcinogenic and anti-diabetic activity, protection against neuro-degenerative diseases, as well as enable immunomodulatory activity (Rojo-Poveda et al., 2020). From an economic perspective, the chocolate industry can greatly benefit from the valorization of the bean by-product, which would ultimately reduce their production, manufacturing, and transportation costs. CBS can be used as a low-cost, clean label, functional ingredient to substitute cocoa as well as serve as a cocoa flavoring agent (Rojo-Poveda et al., 2020). Furthermore, CBS is a rich source of dietary fibre due to the presence of various cell-wall polysaccharides (CWP) such as, cellulose, pectin, 1,3 and 1,4 β-glucans, and arabinoxylans. During processing, these non-starch functional components will migrate from within the bean to the outer regions of the shell (Harris & Smith, 2006; Anderson et al., 2009; Zhu, 2018).

For years, researchers have explored the interaction of CWP with polyphenols and/or proteins in food-food ingredients. These studies primarily explore the synergistic effects between these biopolymers, in terms of their antioxidant and prebiotic activities, as well as their potential in disease prevention. Studies have also investigated the ionic and hydrophobic interactions of polyphenol-CWP and feruloylation as possible approaches for modulating the health promoting properties of cell wall polysaccharide type functional ingredients (Padayachee et al., 2013; Tamayo-Cabezas & Karboune, 2019). There are several factors to consider that may impact the interaction between polyphenols, proteins and CWP which include 1) polyphenol structure, 2) CWP structure, 3) environmental factors, i.e. temperature and pH, 4) polysaccharide-polyphenol-protein interactions, and 5) bioavailability (Zhu, 2018). To the best of our knowledge, chemical

and enzymatic extraction methods on CBS and the modification of CBS oligosaccharides for enhanced functionality have not been investigated. The scope of this review will therefore highlight the chemical and bioactive compositions of CBS, bioactive chemical changes by means of processing as well as methods of extraction and isolation of the cell wall polysaccharides from CBS and of their biotransformation, in particular for the generation of feruloylated oligo/polysaccharides.

2.2 Chemical composition of cacao bean shell

Proximate composition analyses of CBS have been previously explored for its use in food, animal feed and other applications (Rojo-Poveda et al., 2020; Panak Balentić et al., 2018). CBS chemical composition was found to vary according to several production factors, such as genotype, soil, climate, harvest conditions, quality. As well as several processing factors such as fermentation, roasting and winnowing conditions (Agus et al., 2018). Table 2.1 summarizes CBS proximate analysis under various conditions, such as unprocessed, fermented, roasted, etc. Several studies explored certain macro and micro-components in CBS that promote its bioactive functionality to human health.

The carbohydrate content in cocoa beans ranges between 3.62-55.85% w/w (Table 2.1) which is related to the flavor development and profiles of cocoa bean products upon fermentation, drying, and roasting (Agus et al., 2018). As seen in Table 2.1, roasted CBS were found to have a higher proportion of carbohydrates compared to unroasted CBS and roasted cocoa beans. This could be attributed to the degradation of mucilaginous pulp and the migration of carbohydrates towards outer shell layer during processing (Agus et al., 2018). In contrast, protein, a macronutrient that contributes to cocoa's flavor profile, was observed to decrease upon roasting (El-Saied et al., 1981) suggests that approximately 10% of the protein content, is present in its unbound form while the remainder, in the form of alpha-amino nitrogen, is bound to oxidized polyphenols, which upon roasting transform into polyphenol-quinones. Additionally, the fat content will fluctuate depending on the degree of roasting applied on the material, as cocoa butter is inclined to migrate from its cotyledon layer to the cocoa shell (El-Saied et al., 1981). Comparing the fat content in CBS to cocoa butter, El-Saied et al. (1981) reported that CBS accounted for a greater percentage of fat content, lower melting point, higher saponification value, as well as lower iodine value. Furthermore, CBS was also found to be richer in short-chain unsaturated fatty acids compared to

cocoa butter (Table 2.2). In terms of the vitamin composition, CBS are found to contain vitamins B1, B2, B6, D, and E. Knapp and Coward (1935) predicted that vitamin D is produced as a result of the activation of ergosterol, a vitamin D precursor, upon exposure to sun drying. On the other hand, Oracz et al. (2014) concluded that the roasting process is known to reduce the tocopherol content, a class of compounds promoting vitamin E activity (Table 2.2). Alternatively, the ash composition in CBS contains a great source of calcium, phosphorus, potassium and more (Fakhalei et al., 2019; Agus et al., 2018; Martinez et al., 2012; Mellinas et al., 2020; Okiyama et al., 2017). Minerals in CBS continuously accumulate with increasing cocoa bean processing time, further reinforcing its use as low-cost fertilizer.

2.3 Bioactive/functional chemical composition of cacao bean shells

2.3.1 Dietary fibre

Dietary fibre is the edible part of plants or analogous carbohydrate that is resistant to digestion and absorption in the small intestine, with complete or partial fermentation in the large intestine (American Association of Cereal Chemists, 2001). In CBS, the dietary fibre content accounts for 13.86-60.6 (w/w %), and varies depending on the applications of roasting and drying (Fakhlaei et al., 2019; Martínez et al., 2012; Agus et al., 2018; Okiyama et al., 2017). In roasted CBS, dietary fibre is likely present in the form of complexes with protein from Maillard reaction (Redgwell et al., 2003). Cell-wall polysaccharides (CWP) are a sub-class of dietary fibres that are heavily constituted in CBS, roughly comprising of 40-45% pectin, 34% cellulose, and 21-26% hemicellulose (Redgwell et al., 2003). As shown in Table 2.3, the CBS monosaccharide profile of CWP material comprises of glucose (63%), galactose (12%), mannose (7.5%), arabinose (6.6%) rhamnose (3%), and xylose (4.6%) (Vojvodić et al., 2016; Acosta et al.,2018; Grillo et al., 2019). Comparing the monosaccharide profiles, CBS have a relatively lower glucose content than cocoa beans. However, CBS contains more rhamnose, galactose, mannose, xylose, and uronic acid content than cocoa beans, indicating less cellulosic polysaccharides and greater pectic and non-cellulosic CWP . According to Redgwell et al. (2003), increased rhamnose content in CBS was a result of increased rhamnosylation of the pectin backbone compared to its content in cocoa beans. Furthermore, the neutral sugar residues of the non-cellulosic region were predominantly mannose, whereas cocoa beans were predominantly arabinose and galactose (Redgwell et al., 2003). As seen in Table 2.3,, the monosaccharide profile of CBS is comparable to coffee silver-skin, a by-product of the coffee industry used as a potential dietary fibre supplement, with enriched xylose in CBS

(Mussatto et al., 2011; Ballesteros et al., 2014; Vojvodić et al., 2016; Acosta et al., 2018; Grillo et al., 2019; Gemechu, 2020). Sánchez et al. (2010) suggests that fibre from the cocoa cell wall was shown to exhibit health-promoting properties, by means of decreasing total cholesterol, triglyceride, plasma glucose and insulin levels in Zucker rats. This indicates the ability of soluble dietary fibers to counter balance cardiovascular diseases and other metabolic syndromes (Sánchez et al. 2010).

2.3.1.1 Pectin

Pectin is a polysaccharide comprised of (1-4)-linked α-D-galactopyranosyluronic acid units that is primarily used as a gelling agent in food formulations (Biliaderis & Izydorczyk, 2007). Alternatively, pectin has been explored in terms of its ability to interact with polyphenols for use as a microencapsulation agent to promote bio-functionality in foods (Noh et al., 2018). In CBS, pectin constitutes around 7.62-15.59 % (dry weight) depending on the method of extraction (Rojo-Poveda et al., 2020). Pectin is known for its colloidal properties that help support the structure and texture of the products. The techno-functional properties of pectin can be modulated by interactions with other biopolymers, varying degrees of esterification, oxidation and enzymatic degradation (Le Bourvellec & Renard, 2005; Le Bourvellec et al., 2005; Simonsen et al., 2009; Watrelot et al., 2013; Zhu, 2018). Additionally, cross-linking has been shown to greatly impact the interaction of pectin with polyphenols, where Le Bourvellec et al. (2005) observed that the apparent affinity constant of pectin and the procyanidin molecular weight were directly proportional. A higher pectic apparent affinity would facilitate a stronger gel network as well as lead to the formation of hydrophobic pockets to enhance complexation and encapsulation with procyanidins.

2.3.1.2 Cellulose

β-1,4 D-glucan linked units comprise the basic structure of cellulose, a primary component in plant cell walls (Aspinal, 1980). Depending on the method of isolation and extraction, cellulose constitutes around 15.1-34% w/w (Table 2.3) in CBS (Acosta et al., 2018; Grillo et al., 2019; Gómez Hoyos et al., 2020). Similarly, to pectin, processing, including cross-linking and harsh drying, has shown to impact the structural integrity of cellulose. This can result in decreased

porosity, increased apparent saturation and affinity levels, which enhances pectin's binding affinity with polyphenols (Le Bourvellec et al., 2005).

2.3.1.3 Hemicellulose

Hemicellulose constitutes 11.1% (w/w) of CWP in CBS (Gómez Hoyos et al., 2020), residing with cellulose and pectin, primarily comprised of 5-7% xyloglucan, 7-10% galactoglucomannan, and 4% glucuronoarabinoxylan of non-cellulosic cell wall polysaccharides (Redgwell et al., 2003; Vojvodić et al., 2016; Acosta et al.,2018). Xyloglucan is present in CBS in its fucosylated form, similarly to other cell wall materials of dicotyledons (Bacic et al., 1988; Redgwell & Hansen, 2000). Galactoglucomannan in CBS was found to consist of a β-1,4 glycosyl (13%) and mannosyl (42%) that are substituted at O-6 to provide 2-linked galactose side chain residues as the internal sugar. Other sugar residues were found to bind with 2-linked galactose as the terminal sugar, such as galactose, arabinose or xylose (Redgwell & Hansen, 2000). Relative to cocoa beans, CBS galactoglucomannan appears more dispersed due to the degree of molecular branching and therefore, is more abundant (Redgwell & Hansen, 2000). Glucuronoarabinoxylan consists of β-1,4 xylosyl residues, where every seventh xylosyl residue is substituted at O-4 with glucuronosyl, arabinosyl, and xylosyl residues (Redgwell & Hansen, 2000).

2.3.1.4 Lignin

Lignin is composed of monomer linkages of p-coumaryl, guiacyl, and sinapyl-propane, which have been studied to possess antioxidant properties, which enhance their application in food systems (Redgwell et al., 2003). The lignin content is generally estimated by the insoluble fraction obtained by removal of ash concentrates via acid hydrolysis of the plant tissues, termed as "Klason lignin" (Chen, 2015). As seen in Table 2.3, lignin varies between 15.1-35 % (w/w) (Gomez Hoyos et. al, 2020; Acosta et. al, 2018). Lignin can also form tannin-protein complexes, where Redgwell et al. (2003) concluded it accounts for roughly 30% (dry weight) in non-defatted roasted CBS.

Table 2.1 Proximate Analysis of Cocoa beans and its by-product, CBS

	Component	% w/w dry material	Reference
*Cocoa Bean**	Crude Protein	6.3-10.6 / 7.0-29.0	Sotelo & Alvarez, 1991; Agus et al., 2018; Adamafio, 2013; Olubamiwa et al., 2006; Martín-Cabrejas et al., 1994; Pèrez Sira 2015
	Carbohydrate	3.62-55.85	Sotelo & Alvarez, 1991; Agus et al., 2018; Adamafio, 2013; Olubamiwa et al., 2006; Martín-Cabrejas et al., 1994; Pèrez Sira 2015
	Dietary Fibre	3.13-17.8 / 6.0-19.4	Sotelo & Alvarez , 1991; Grillo et al. 2019; Agus, et al., 2018; Olubamiwa et al., 2006; Martín-Cabrejas et al., 1994; Pèrez Sira, 2015
	Crude Fat	5.46-13.93/ 6.0-17.3	Sotelo & Alvarez , 1991; Grillo et al. 2019; Agus et al., 2018; Olubamiwa et al., 2006; Martín-Cabrejas et al., 1994; Pèrez Sira , 2015
	Ash	1.41-11.67	Sotelo & Alvarez , 1991; Grillo et al. 2019; Agus et al. , 2018; Olubamiwa et al., 2006; Martín-Cabrejas et al., 1994; Pèrez Sira , 2015
Unroasted CBS	Crude Protein	18	Fakhlaei et al., 2019
	Carbohydrate	44.63	Agus et al., 2018
	Dietary Fibre	13.86	Agus et al., 2018
	Crude Fat	4.09-4.87	Fakhlaei et al., 2019; Agus et al., 2018
	Ash	9.93-10.22	Fakhlaei et al., 2019; Agus et al., 2018
Roasted CBS	Crude Protein	16.35	Fakhlaei et al., 2019; Agus et al., 2018
	Carbohydrate	55.85	Agus et al., 2018
	Dietary Fibre	16.06-32.01	Fakhlaei et al., 2019; Agus et al., 2018
	Crude Fat	3.09-6.46	Fakhlaei et al., 2019; Agus et al., 2018
	Ash	10.44-11.67	Fakhlaei et al., 2019; Agus et al., 2018
Dried CBS	Crude Protein	6.2-18.6	Martínez et al., 2012; Mellinas et al., 2020; Okiyama et al.,2017
	Carbohydrate	17.8-23.17	Martínez et al., 2012
	Dietary Fibre	50.4-60.6	Martínez et al. 2012; Okiyama et al., 2017
	Soluble Fibre	14.53-16.24	Martínez et al., 2012
	Insoluble Fibre	35.64-42.17	Martínez et al., 2012
	Crude Fat	2.02-2.05	Martínez et al. 2012; Okiyama et al.,2017
	Ash	6-11.42	Martínez et al., 2012; Mellinas et al., 2020; Okiyama et al.,2017

Table 2.2. Fatty acids, Vitamins and Crude Minerals Composition of CBS

Component		% w/w dry material	Reference
Crude Fat		1.8-6.87	Vojvodić et al., 2016; Nsor-Atindana et al., 2012; Gómez Hoyos et al., 2020
Fatty Acids	Fatty Acid 16:0	15.6-19.6	Lessa et al., 2018
	Fatty acid 17:0	0.32-1.12	Lessa et al., 2018
	Fatty acid 18:0	24.23-41.67	Lessa et al., 2018
	Fatty acid 20:0	1.24-1.29	Lessa et al., 2018
	Fatty acid 22:0	1.1-3.64	Lessa et al., 2018
	Fatty acid 24:0	0.6-0.83	Lessa et al., 2018
	MUFA 18:1n9c	28.7-35.8	Lessa et al., 2018
	PUFA 18:2n6	2.6-5.4	Lessa et al., 2018
	PUFA 18:3n6	2.3-8.26	Lessa et al., 2018
	PUFA 20:5n-3	1.13-1.73	Lessa et al., 2018
Vitamins	Vitamin B1	0.00007-0.00031	Rojo-Poveda et al., 2020
	Vitamin B2	0.00009-0.00031	Rojo-Poveda et al., 2020
	Vitamin B6	traces	Rojo-Poveda et al., 2020
	Vitamin D	traces - 0.000053	Rojo-Poveda et al., 2020
	Vitamin E	0.000102	Rojo-Poveda et al., 2020
Crude Minerals		9.1	Vojvodić et al., 2016
	Calcium	0.23-0.44	Rojo-Poveda et al., 2020
	Chromium	0.67-4.86	Rojo-Poveda et al., 2020
	Cobalt	0.1	Rojo-Poveda et al., 2020
	Copper	2.35-6.62	Rojo-Poveda et al., 2020
	Magnesium	0.48-1.29	Rojo-Poveda et al., 2020
	Manganese	4.53	Rojo-Poveda et al., 2020
	Phosphorus	0.58-1.00	Rojo-Poveda et al., 2020
	Potassium	1.25-1.82	Rojo-Poveda et al., 2020
	Selenium	0.21	Rojo-Poveda et al., 2020
	Sodium	16.00-192.20	Rojo-Poveda et al., 2020
	Zinc	2.75-19.00	Rojo-Poveda et al., 2020

2.4 Proteins and bioactive peptides

Table 2.3 shows that CBS crude protein constitutes 0.7-28.1 % (w/w) depending on the degree of processing, where the protein content decreases with increased processing (Fakhlaei et al., 2019). Albumins and globulins were the major fractions (79%, w/w) of total protein from CBS (Bonvehí & Coll, 1999).The essential amino acids in cocoa husk represent 44.75% of total amino acids, predominately lysine (28.4%), tyrosine (18.7%), phenylalanine (14.1%), and histidine (7.7%) in roasted CBS (Bamba et al., 2015). In comparison, Iriondo-DeHond et al. (2019) reported coffee silver-skin constitutes 11-19 % (w/w) where its essential amino acid profile is predominantly phenylalanine (14.6%), valine (12.7%), lysine (12.6%), and threonine (12.6%) (Table 2.3). Additionally, Bonvehí and Coll (1999) reported an amino acid score of 73 for CBS, comparable to rice (73) and soybean protein (80), as well as a low energetic value of 21.9J/kg further supporting its incorporation in food formulations.

Bioactive peptide fractions, namely albumin (11.5%) and vicilin-like (3.9%), were identified in cocoa beans by means of spontaneous fermentation. To date, the presence of bioactive peptides in CBS have not been explored, however, current studies using cocoa beans suggest that oligopeptides are released through three main pathways, which include 1) cocoa bean autolysis (Sarmadi et al., 2012; Sarmadi et al., 2011; Domínguez-Pérez et al., 2020), 2) exogenous enzymatic hydrolysis (Tovar-Pérez et al., 2019; Martorell et al., 2013; Domínguez-Pérez et al., 2020) and 3) after cocoa bean fermentation (Preza et al., 2010; Marseglia et al., 2019; Marseglia et al., 2014; Ryan et al., 2017; Domínguez-Pérez et al., 2020). Typically, oligopeptides contribute to the flavor profile of cocoa, serving as flavor precursors. Additionally, Domínguez-Pérez et al. (2020) suggest they may provide health benefits as potential antioxidant, anti-diabetic, and anti-obesogenic agents.

2.5 Phenolic compounds

Phenolic acids are plant secondary metabolites that are released upon exposure of the plant material to environmental stress. They exist as lignin oligomers/polymers or form hetero-conjugates with CWP (Mandal et al., 2010). Similarly, to the cocoa bean itself, CBS contain a great source of phenolic compounds, aiding to its potential use as a functional ingredient. Studies (Table 2.4) indicate the total flavonoid and phenolic contents in CBS to be within 1.65-40.72 mg

catechin equivalent per gram of dry CBS (Rojo-Poveda et al., 2020) and 17.21-41.82 mg gallic acid equivalent per 100 gram of dry CBS, respectively (Lessa et al., 2018). Sub-classes of flavonoids such as anthocyanins and catechins were identified and explored in terms of their content and functionality in CBS.

CBS contains caffeic (0.004-0.89 mg/g), chlorogenic (0.76 mg/g) , ferulic (0.51 mg/g), gallic (0.015-0.48 mg/g) and p-coumaric (0.017-0.04 mg/g), phytic (6g/kg), protocatechuic (0.98-1.32 mg/g) and uronic (82.7 -115.2 mg galacturonic acid/g) acids were identified among several studies (Okiyama et al., 2017; Jokić et al., 2020; Barišić et al., 2019; Mellinas et al., 2020). These phenolic compounds are valued for their potential health-promoting effects, such as inhibiting LDL oxidation, inhibiting pro-inflammatory cytokines as well as promoting radical scavenging, antimicrobial and anti-diabetic activities (Meyer et al., 1998; Călinoiu & Vodnar, 2018; Kumar & Goel, 2019). Flavanols have been identified in CBS as another major class of polyphenols, including 0.03-6.16 mg/g catechin, 0.059-17.7 mg/g epicatechin, and 0.007- 0.07 mg/g epicatechin gallate. Flavanols have also shown optimal antioxidant activity by means of "modulating inflammatory mediators", decreasing LDL oxidation levels, as well as providing antiatherogenic properties (Goya et al., 2016; Martín & Ramos, 2017). Similarly, procyanidin A (83 mg/mL) and B types (B1: 0.55-0.85 mg/g; B2: 0.23-1.38 mg/g) were found in CBS extracts (Okiyama et al., 2018; Rossin et al., 2019; Rojo-Poveda et al., 2020). Other flavonoids present in CBS include quercetin, kaempferol, myricetin, and isorhamnetin, at concentration of 4.24 mg/g of dry matter, condensed tannins at 2.36-12.9 mg catechin equivalent per gram of dry matter and anthocyanins at 0.4-0.5 mg per gram of dry matter (Table 2.4).

2.5.1 Phenolic content and protein digestibility

Alternatively, excess polyphenol content may interfere with protein digestibility from CBS. Efforts were made to explore both *in vitro* and *in vivo* protein digestibility of foods from various poly-phenol rich extracts from cocoa powder, black and green tea, coffee, faba bean hulls, and more (Eggum et al., 1983; Jansman et al., 1994; Bohn, 2014; Cirkovic Velickovic & Stanic-Vucinic, 2018). The general consensus obtained from *in vitro* studies concluded that both covalent and non-covalent interaction between polyphenols and proteins decreased protein digestibility. Charlton et al. (2002) suggested that the key factors influencing protein affinity to polyphenols were the amino acid composition and their structures. A green coffee extract rich in chlorogenic

acid was found to slightly decrease protein digestion upon supplementation in bread (Budryn et al., 2013; Cirkovic Velickovic & Stanic-Vucinic, 2018). However, coffee polyphenol extracts supplemented with β-lactoglobulin increased protein digestion (Stojadinovic et al., 2013; Cirkovic Velickovic & Stanic-Vucinic, 2018). Świeca et al. (2013) observed decreased protein digestion upon supplementation of dry onion skin, as the primary source of phenols, in bread.

In vivo studies primarily focus on the protein digestibility-corrected amino acid score (PDCAAS) to determine the protein value in human nutrition (Cirkovic Velickovic & Stanic-Vucinic, 2018). Jansman et al. (1994) supplemented fava bean hull extracts, abundant in condensed tannins, in a casein-rich diet. The outcome resulted in decreased protein digestibility in rats. Similarly, green coffee also showed a negative correlation with protein digestibility upon supplementation in soy-bean meal and barely diets in rats. Alternatively, this was not the case for cocoa supplementation, as findings suggested there was no impact on protein digestibility (Eggum, et al., 1983).

2.5.2 Phenolic content and antioxidant ability

Due to the rich polyphenolic content in CBS, the antioxidant ability of this by-product should be explored in order to determine its potential use as a functional ingredient. Table 2.5 summarizes the antioxidant capacity of CBS at various conditions using different assay methods. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity was measured at 78.5-79.2% for unprocessed CBS, 19.4% for unfermented CBS, and 7.881.3% for fermented CBS (Utami et al., 2016; Agus et al., 2018; Lessa et al., 2018). The large variation in DPPH activity for fermented CBS is due to several factors, such as fermentation time, pH, and roasting time. The ferric reducing antioxidant power (FRAP) activity was estimated for dried CBS samples between 47-84 mM Trolox/g, while undetermined conditions of CBS were estimated at 0.67-72.32 mM Trolox/g, depending on the method of CBS extraction prior (Lecumberri et al., 2007; Martínez et al., 2012; Okiyama et al., 2018; Mellinas et al., 2020). The ABTS assay, also known as Trolox equivalent antioxidant capacity (TEAC) assay, was estimated between 2.56-7.73 mM Trolox/g, with no mention of the degree of processing applied on CBS (Lecumberri et al., 2007; Martínez et al., 2012). Lastly, thiobarbituric acid reactive substance assay (TBARS), was assessed for dried CBS between 0.17-0.53 mM Trolox/g (Choi et al., 2019). It is important to note that the antioxidant capacity of CBS will also vary drastically depending on the condition and mode of processing. Generally, processed

CBS samples accumulate a higher antioxidant activity compared to unprocessed samples (Agus, et al., 2018).

2.6 Phytosterols

Referred to as plant lipids, phytosterols identified in CBS were composed of stigmasterol (4.8-15.5%), campsterol (0.12%), cholesterol (1.72%) and β-sitosterol (0.0078-0.21%). Phytosterols in CBS were shown to be less abundant as compared to those in cocoa beans in both roasted and unroasted forms (El-Saied et al., 1981; Agus et al., 2018). Nonetheless, they are viewed as a critical class of compounds serving anti-inflammatory, anti-bacterial, anti-fungal, anti-tumoral activities (Sánchez-Machado et al., 2004). Additionally, phytosterols were found to reduce cholesterol absorption in the intestine, further reducing blood cholesterol levels in humans (Agus et al., 2018; Cantril, 2008).

Table 2.3. Protein and dietary fiber comparison between CBS and coffee silver-skin

Component		% w/w dry material (relative %)	Reference
Cocoa bean shells			
Crude Protein		0.7 - 28.1	Okiyama et al., 2018; Agus et al., 2018; Vojvodić et al., 2016; Bamba et al., 2015; Nsor-Atindana et al., 2012; Aregheore, 2002
Amino Acids	Lysine	1.45 (28.4%)	Bamba et al., 2015
	Arginine	0.48 (9.4%)	Bamba et al., 2015
	Histidine	0.39 (7.7%)	Bamba et al., 2015
	Phenylalanine	0.72 (14.1%)	Bamba et al., 2015
	Tyrosine	0.95 (18.7%)	Bamba et al., 2015
	Leucine	0.35 (6.9%)	Bamba et al., 2015
	Isoleucine	0.27 (5.3%)	Bamba et al., 2015
	Valine	0.22 (4.3%)	Bamba et al., 2015
	Threonine	0.19 (3.73%)	Bamba et al., 2015
	Methionine	0.07 (1.4%)	Bamba et al., 2015
Dietary Fibres*		13.86 - 60.6	Agus et al., 2018; Fakhlaei et al., 2019; Rojo-Poveda et al., 2020; Rojo-Poveda et al., 2019; Martínez et al., 2012; Okiyama et al., 2017
	Total Monosaccharides	25.9	Grillo et al., 2019
	Glucose	16.5 (63%)	Grillo et al., 2019
	Galactose	3.1 (12%)	Grillo et al., 2019
	Mannose	2.6 (7.5%)	Grillo et al., 2019
	Rhamnose	0.8 (3%)	Grillo et al., 2019
	Xylose	1.2 (4.6%)	Grillo et al., 2019
	Arabinose	1.7 (6.6%)	Grillo et al., 2019
	Cellulose	15.1- 34	Grillo et al., 2019; Gómez Hoyos et al., 2020; Acosta et al., 2018
	Hemicellulose	11.1**	Gómez Hoyos et al., 2020
	Lignin	15.6 -35	Gómez Hoyos et al. 2020; Acosta et al., 2018
	Holocellulose	49	Acosta et al., 2018
	Pectin	7.62 - 15.59	Rojo-Poveda et al., 2020
	Uronic acid	3.48 - 7.13	Lecumberri et al., 2007
	Galacturonic acid	6.9	Vojvodić et al., 2016
Coffee Silver-skin			
Crude Protein		11-19	
Amino Acids	Lysine	0.177 (12.6%)	Iriondo-DeHond et al., 2019
	Arginine	0.114 (8.1%)	Iriondo-DeHond et al., 2019

		0.092 (6.5%)	Iriondo-DeHond et al., 2019
	Histidine		
	Phenylalanine	0.205 (14.6%)	Iriondo-DeHond et al., 2019
	Tyrosine	0.148 (10.5%)	Iriondo-DeHond et al., 2019
	Leucine	0.154 (10.9%)	Iriondo-DeHond et al., 2019
	Isoleucine	0.104 (7.4%)	Iriondo-DeHond et al., 2019
	Valine	0.179 (12.7%)	Iriondo-DeHond et al., 2019
	Threonine	0.178 (12.6%)	Iriondo-DeHond et al., 2019
	Methionine	0.057 (4.0%)	Iriondo-DeHond et al., 2019
Dietary Fibers		54-74	Ballesteros et al., 2014; Gemechu 2020; Borrelli et al., 2004; Costa et al., 2018; Iriondo-DeHond et al., 2019; Jiménez-Zamora et al., 2015; Toschi et al., 2014)
	Total Monosaccharides	not reported	
	Glucose	17.8	
	Galactose	3.8	Mussatto et al., 2011; Ballesteros, et al., 2014; Gemechu, 2020
	Mannose	2.6	Mussatto et al., 2011; Ballesteros et al.,,2014; Gemechu, 2020
	Rhamnose	not reported	
	Xylose	4.7-7.61	Mussatto et al., 2011; Ballesteros et al., 2014; Gemechu, 2020
	Arabinose	2.0 - 7.61	Mussatto et al., 2011; Ballesteros et al., 2014; Gemechu, 2020
	Cellulose	18 -23.8	Ballesteros et al., 2014; Gemechu , 2020
	Hemicellulose	13 -16.7	Ballesteros et al., 2014; Gemechu , 2020
	Lignin	28.6	Ballesteros et al., 2014; Gemechu, 2020
	Holocellulose	not reported	
	Pectin	not reported	
	Uronic acid	10	Furusawa et al., 2011
	Galacturonic acid	not reported	

*specific to roasted and dried CBS

** reported as % w/w of total cell wall polysaccharides in CBS

Table 2.4. Phytochemical contents of cocoa bean shells in terms of phenolic compounds, phytosterols, and alkaloids

Component		Extraction Method	Value*	Reference
Total Flavonoids content		Acetone, Ethanol, Methanol, Water	1.65-40.72 mg CE/g	Rojo-Poveda et al., 2020; Nsor-Atindana et al., 2012
Total Phenolic content		Methanol-Acetone/Water/Soxhlet/Microwave Assisted/Ethanol/Hexane-Acetone/Methanol/Acetone/Hydroethanol/Acetone-Water-Acetic Acid	1.45-94.95 mg GAE/ g	Nsor-Atindana et al., 2012; Mellinas et al., 2020; Utami et al., 2016; Lessa et al., 2018; Martínez et al., 2012; Delgado-Ospina et al., 2020; Rojo-Poveda et al., 2020
Total Tannin content		Ethanol-Water/ Ethanol-Methanol/Ethanol Acetone	85-359.7 mg CE / L	Rojo-Poveda et al., 2020; Rossin et al., 2019
Total Condensed Tannins		Water/Ethanol/Methanol/Acetone	2.36-12.9 mg CE/g	Nsor-Atindana et al., 2012
Carotenoids		Hydro-ethanol	0.2-0.8 mg b-carotene/100g	Lessa et al., 2018
Phenolic Acids	Caffeic acid	Methanol/Subcritical Water/Microwave Assisted Extraction	0.0004-0.002136 g/100g	Mellinas et al., 2020; Jokić et al., 2020; Barišić et al., 2019
	Chlorogenic acid	Water/Microwave Assisted Extraction	0.001824 g/100g	Mellinas et al., 2020
	Ferulic acid	Water/Microwave Assisted Extraction	0.001224 g/100g	Mellinas et al.,2020
	Gallic acid	Subcritical Water	0.0037-0.0048 g/100g	Jokić et al., 2020
	p-coumaric acid	Methanol/Subcritical Water	0.0002 g/100g	Jokić et al., 2020
	Phytic acid	N.D.	0.6 g/100g	Okiyama et al., 2017; Arlorio et al., 2005
	Protocatechuic acid	Microwave Assisted	0.002353-0.003168 g/100g	Mellinas et al., 2020
	Galacturonic acid	n.a (raw material)	6.9 g/100g	Vojvodić et al., 2016
	Uronic acid	Microwave Assisted/Soxhlet	82.7-115.2 mg GlcA/g	Mellinas et al., 2020
Anthocyanins		In natura/Hydro-ethanol	0.4-0.5 mg QE/100g	Lessa et al., 2018
Flavanols		Water	0.424g/100g	Soares et al., 2020
	Procyanidin A-type pentoside	Ethanol/Acetone	0.166 g/100g	Rossin et al., 2019

	Procyanidin B1	Water	55-70 g/100g	Rojo-Poveda et al., 2020; Papillo et al., 2019
	Procyanidin B2	Accelerated solvent extraction/Ethanol-Methanol	0.0138-0.03728 g/100g	Rojo-Poveda et al., 2020; Papillo et al., 2019; Rossin et al., 2019; Okiyama et al., 2018
	Catechin	Methanol/Hexane/Subcritical Water	0.0088-0.024g/100g	Rojo-Poveda et al., 2020; Okiyama et al., 2018; Papillo et al., 2019; Jokić et al., 2020; Barišić et al., 2019
	Epicatechin	Methanol/Subcritical Water/Pulsed Electric Field/Water/Hexane	0.00022-0.0568 g/100g	Barbosa-Pereira et al., 2018; Rojo-Poveda et al., 2019; Delgado-Ospina et al., 2020; Jokić et al., 2020; Barišić et al., 2019
	Epicatechin gallate	Methanol/Subcritical Water	0.00027-0.0007 g/100g	Jokić et al., 2020; Barišić et al., 2019
Phytosterol	Stigmasterol	N.D.	4.803 -15.54 g/100g	Agus et al., 2018
	Campsterol	N.D.	0.1224 g/100g	Romancyzk & McClelland , 2004
	Cholesterol	N.D.	1.712 g/100g	Agus et al., 2018
	b-sitosterol	N.D.	0.0078-0.211 g/100g	Agus et al., 2018
Alkaloids	Caffeine	N.D.	0.04-0.42 g/100g	Rojo-Poveda et al., 2019
	Theobromine	N.D.	0.39-1.83 g/100g	Rojo-Poveda et al., 2019
	Theophylline	N.D.	0.0058-0.0188 g/100g	

*values will drastically vary depending on the method of extraction and degree of processing.

Table 2.5 Antoxidant activity of cocoa bean shells at various conditions

Antioxidant Activity Assay	Condition	Value**	Method of CBS extraction	Reference
DPPH activity (%)	*In natura*	78.5-79.2	N.D.	Lessa et al., 2018
	unfermented	19.38	N.D.	Utami et al., 2016
	fermented*	7.77-81.3	N.D.	Lessa et al., 2018; Utami et al., 2016
	unroasted	14.97	N.D.	Agus et al., 2018
	roasted*	59.3	N.D.	Agus et al., 2018
FRAP activity (mM Trolox/g)	N.D.	0.67-72.32	Ethanol/Methanol-Acetone/Soxhlet/Microwave assisted extraction/N.D.	Lecumberri et al., 2007; Martínez et al., 2012; Mellinas et al., 2020
	Dried	47-84	Pressurized liquid extraction	Okiyama et al., 2018
TEAC (mM Trolox/g)	N.D.	2.56-7.73	Ethanol/Methanol-Acetone/N.D.	Lecumberri et al., 2007; Martínez et al., 2012;

*duration of fermentation and roasting and pH are variable

**values will drastically vary as a result of the methods of extraction

2.7 Effect of processing on cocoa bean shell by-products

The quality of CBS starting materials is determined by the cacao variety and post-harvesting processing conditions of the cacao beans. Upon harvest, cacao beans undergo fermentation, drying and roasting to favor the retrieval of cocoa nibs, as well as serve as the main modes for cocoa flavor development. These processes, especially fermentation and roasting, impact the overall quality, flavor, and composition of cocoa beans and their by-products, such as CBS.

2.8 Fermentation

Initially, cacao beans are covered in a mucilaginous pulp, namely rich in glucose and saccharose (Anvoh et al., 2009). Spontaneous fermentation is induced by subjecting the pulp-covered beans into an anaerobic environment, where endogenous microorganisms such as yeast, lactic acid bacteria and acetic acid bacteria, easily degrade the pulp exposing the bean (Schwan & Wheals, 2004; Agus et al., 2018). Ultimately, fermentation and drying processes consume the carbohydrate content of cocoa beans, while increasing the crude protein content (Cirkovic Velickovic & Stanic-Vucinic, 2018; Agus et al., 2018; Aremu et al., 1995). Fermentation also promotes the migration of small phenolic compounds from the bean to the shell (Roelofsen, 1958; Kim & Keeney, 1984; Utami et al., 2016). Utami et al. (2016) suggested that partial fermentation (24 hours) resulted in the highest total phenolic content and antioxidant activity in CBS. On the other hand, longer fermentation times surpassing 24 hours led to decreased phenolic content and antioxidant activity due to oxidation caused by polyphenol oxidase, and complexation between phenolic compounds and proteins/peptides (Brito et al., 2001; Camu et al., 2008; Utami et al., 2016). Effects of fermentation on the bioactivity of cocoa proteins have also been studied (Flint et al., 1998; Preza et al., 2010; Addison & Aguilar, 2011; Ryan et al., 2017; Marseglia et al., 2019). Studies suggested that fermentation of cocoa bean enhances *in vitro* proteolytic hydrolysis of cocoa protein, whereas subsequent processes, such as roasting and chocolate formulation, may reverse such effects (Marseglia et al., 2019; Carbonaro et al., 2000; Giami et al., 2001; Pushparaj & Urooj, 2011; El Hag et al., 2013; Cirkovic Velickovic & Stanic-Vucinic, 2018). In addition, fermentation was shown to positively correlate with the quantity and varieties of peptides of cocoa beans, favor the release of angiotensin converting enzyme inhibitory di- or tri-peptides and increase the anti-diabetic, anti-tumor properties of the cocoa products/by-products (Marseglia et al., 2019; Kumari et al., 2018; Preza et al., 2010; Flint et al., 1998; Addison & Aguilar, 2011; Ryan et al., 2017).

These were found to be mediated by the amino acid profile of the fermented cocoa products, as opposed to their antioxidant properties (Preza et al., 2010; Flint et al., 1998; Addison & Aguilar, 2011; Ryan et al., 2017).

Solid-state fermentation is viewed as a method to valorize waste-products, such as CBS, which can be carried out using fungi such as *Aspergillus* (Santos et al., 2011)*, Rhizopus* (Santos et al., 2017) and *Penicillium* (Lessa et al., 2018). Lessa et al. (2018) observed that solid-state fermentation of CBS using *Penicillium roqueforti* led to an increase in the phenolic, carotenoid and saponin content, which contributed to the antioxidant properties of CBS (Mioso et al., 2015; Lessa et al., 2018). In addition, Lessa et al. (2018) reported that anthocyanins accounted for 0.4-0.5 mg quercetin equivalent per 100 gram CBS both pre- and post-solid-state fermentation, where its complexing capabilities can be enhanced with increasing pH to bind with pectic and cellulosic materials (Lin et al., 2016; Zhu, 2018). Moreover, a 25% increase in mono-unsaturated fatty acid, specifically in terms of oleic, linoleic and gamma-linolenic acids was observed in CBS upon fermentation (Lessa et al., 2018).

2.9 Roasting

Following fermentation and drying, roasting is initiated and is regarded as the key processing step involved in generating the characteristic and distinctive cocoa flavors and aromas. Cocoa beans are subjected to high temperatures where enzymatic and non-enzymatic, like Maillard reactions are induced due to the low moisture content present in cocoa beans. As a result, compositional changes in the bean and shell vary depending on the roasting temperature, between 120-150˚C and typically 20-40 minutes (Pèrez Sira, 2015). Oracz et al. (2014) implied that the fat, tocopherol, phytosterol contents and antioxidant capacity of cocoa beans, from Brazil, Ecuador and Indonesia, are reduced with increased roasting. Another study, conducted by Agus et al. (2018), observed an increase in fat, crude fiber, crude protein, carbohydrate, phenolic and phytosterol content in roasted CBS samples at 140˚C for 40 minutes. The discrepancy reported in the literature is due to variations in the roasting condition, including temperature and relative humidity, as well as the type and variety of the cultivar (Oracz et al., 2014). Several authors concluded comparable findings in terms of the protein content upon roasting (Carbonaro et al., 2000; Giami et al., 2001; Pushparaj & Urooj, 2011; El Hag et al., 2013; Cirkovic Velickovic & Stanic-Vucinic, 2018). In fact, the roasting process resulted in decreased protein digestibility in

cocoa beans, however, this can be avoided by establishing a pre-soaking treatment in a sodium bicarbonate solution (Zia-Ur-Rehman & Shah, 2001; Cirkovic Velickovic & Stanic-Vucinic, 2018). Typically, the roasting process is known to decrease the phytochemical content. However, Bitzer et al. (2015) observed that the high-molecular weight and degree of polymerization of pro-anthocyanidins have also shown to prevent loss of gut barrier function and epithelial inflammation. Similarly, Stanley et al. (2018) observed that a roasting condition of 150˚C increased catechin and high-molecular weight pro-anthocyanidin concentrations, inducing significant inhibition of pancreatic lipase.

2.10 Extraction methods

In addition to the means of processing applied on cocoa beans and their by-products, various methods of extraction of the bioactive components will also have a significant impact on the composition and functional properties. These chemical, physical (through microwave, pulsed-electric field, subcritical water, ultrasound, hydrodynamic cavitation and pressurized liquid) and enzymatic methods.

2.11 Alkaline/acid-assisted extraction

Chemical treatment, via acid or alkaline solutions, is commonly used for the extraction and characterization of plant cell wall polysaccharides or phenolic compounds occurs by means of disrupting the plant cell wall. Acidic extraction, using tartaric, malic, citric acids and others, is a more commonly performed technique due to its ability to yield commercial pectin with a high degree of esterification; however, this approach may lead to side-chain degradation (Harris & Smith, 2006; Karboune & Khodaei, 2016; Levigne et al., 2002). Alkaline treatment ensures the solubilization of lignin by means of ester-linkage cleavage between the lignin-polysaccharide complexes and the release of phenolic acids (Kondo et al., 1992). Alkaline hydrolysis has been applied on cell wall materials from corn cobs, brewer's spent grain, sugarcane bagasse, and cocoa bean shells for the generation of polysaccharides and phenolic acids (Li et al., 2020; Mussatto et al., 2007; Redgwell et al., 2003; Torre et al., 2008). In the alkaline extraction of corn cobs, Torre et al. (2008) obtained an optimal solubilization of ferulic acid and *p*-coumaric acid at 1171 mg/L and 2156 mg/L, respectively, using 0.5N NaOH treatment for 6 hours, while retaining most of the hemicellulosic and cellulosic fractions. In another study, brewer's spent grain was first subjected to acid hydrolysis, using 72% sulfuric acid, for the removal of hemicelluloses, followed by 2%

NaOH treatment at 120˚C for 90 minutes to achieve a release of 145.3 mg/L of ferulic acids and 138.8 mg/L of *p*-coumaric acids (Mussatto et al., 2007). Furthermore, both studies suggested a positive correlation between phenolic acid release and the NaOH concentration, temperature, and reaction time (Torre et al., 2018; Mussatto et al., 2007). Redgwell et al (2003) investigated a sequential alkaline hydrolysis treatment of cocoa bean shell cell wall material using 0.05M CDTA, 0.05M Na_2CO_3, and 4M KOH paired with 20mM $NaBH_4$. In the CDTA fraction, a high proportion of soluble pectic polysaccharides rich in uronic acid (87.2%mol) were observed; indicating the presence of a homogalacturonan-type structure. In the Na_2CO_3 fraction, the authors observed high proportions of rhamnose (13.%mol) , galactose (14.9%) and uronic acid (66.6%mol), indicating the presence of rhamnogalacturonan. The authors also observed the presence of xylose (23% mol), mannose (9.9%mol), glucose (21.8%mol), and uronic acid (26.2%mol), suggesting the 4M KOH fraction containing a combination of hemicellulose and pectic polysaccharides (Redgwell et al., 2003). Chemical extraction via alkaline/acid hydrolysis was observed to yield higher efficiency in the extraction of phenolic acids than enzymatic approaches using ferulic acid esterases (FAEs) isolated from *A. niger,* xylanase from *T. viride,* or commercial enzyme preparations (Bartolomé et al., 1997; Bartolomé et al., 2003; Bartolome & Gomez-Cordoves, 1999; Mussatto et al., 2007). Furthermore, sequential chemical treatments can be applied for the generation of well-defined cell wall polysaccharides for characterization and quantification purposes (Pan et al., 1998; Xu et al., 2005; Li et al., 2020). Mateos-Aparicio et al. (2010) assessed the cell wall material composition of okara, bean pod, and pea pod samples upon sequential treatment using CDTA/ammonium oxalate, 0.05M NaOH, 1M KOH, 4M KOH and $NaClO_2$/acetic acid. Their findings suggested that okara and bean pod samples are mostly comprised of pectic-rich regions, specifically slightly methylated rhamnogalacturonan substituted with arabinans, galactans, and/or arabinogalactans. On the other hand, the cell wall compositions of pea pods are predominately insoluble fibre fractions, namely cellulose, xylogalacturonan and xylan (Mateos-Aparicio et al., 2010). Moreover, hot water, 2% EDTA and 4M NaOH at 30˚C followed by an increase in temperature to 70˚C were applied sequentially to hydrolyze coffee (*Coffea arabica L)* leaves to study the effect of salt stress on the cell wall composition. Results showed that salt stress during plant growth resulted in significant increase in negatively charged cell wall polysaccharides and lignin content (de Lima et al., 2014).

2.12 Physical extraction

2.12.1 Microwave-assisted extraction

Microwave-assisted extraction (MAE) was utilized for extracting bioactive compounds from plant cell wall materials such as citrus peels, peanut skins, grape seeds, spent filter coffee, walnut leaves, defatted canola seed cake, brown seaweed, *solanum nigrum,* cranberry pomace and CBS (Hayat et al., 2009; Ballard et al., 2010; Li et al., 2011; Pavlović et al., 2013; Kaderides et al., 2019; Mellinas et al., 2020; Spadoni Andreani & Karboune, 2020). Microwave energy assists the release of bioactive compounds from plant materials by inducing molecular friction, which triggers the dipolar rotation of polar solvents and the conductive migration of dissolved ions (Eskilsson & Björklund, 2000; Srogi, 2006; Rodriguez-Jasso et al., 2011; Pandit et al., 2015; Mirzadeh et al., 2020). The advantages of MAE include less solvent consumption, shorter extraction times, while improving extraction yields compared to conventional extraction methods, like Soxhlet techniques (Proestos & Komaitis, 2008). The parameters that highly influence the overall extraction yield include the extraction time, solvent type and proportion, as well as microwave power. These parameters are modulated depending on the target compounds of extraction as well as the nature of plant material. For example, Mellinas et al. (2020) observed that the MAE operating conditions of 5 minutes, pH 12, 97˚C, and a solid: liquid ratio of 0.04 g/mL resulted in improved polysaccharide (370.5 mg glucose/g) and polyphenol (35.9 GAE/g) extraction yields from cocoa bean shells. Chen et al. (2005) suggested that high extraction yields of *Solanum nigrum* were achieved at MAE conditions of a 15-minute extraction time, 455W microwave radiation powder, and a 1:20 process ratio. Interestingly, the study observed that extraction times exceeding 15 minutes resulted in decreased polysaccharide extraction yields (Chen et al., 2005). Proestos and Komaitis (2008) noted that acetone is a better extraction solvent than methanol due to its low absorptivity against microwave energy. However, the authors observed comparably high phenolic acid extraction yields using acetone/water (60:40, v/v) and methanol/water (60:40, v/v), ranging between 11.2-23.8 mg gallic acid/g dry sample and 10.3-21.2 mg gallic acid/g dry sample from a variety of plants, namely *Styrax officinalis, Origaanum dictamnus, Rosmarinus officinalis, Origanum majorama, Teurcrium polium, Vitex aganus-cactus* (Proestos and Komaitis, 2008). Using MAE (855W power, 30 seconds extraction time) on peanut skins (1.5g), a high recovery of phenolic compounds was achieved, measuring 143.6mg gallic acid equivalent/g skins. In addition, ORAC levels significantly increased with a longer extraction time (150 seconds) and higher

temperature (Ballard et al., 2010). Kaderides et al. (2019) concluded similar findings with total phenolic content extraction yields from pomegranate peels (199.4 mg GAE/g dry peel), with optimal MAE conditions at 600W, 0.0167 g/mL solvent (50 and 70% aqueous ethanol and methanol) for 4 minutes.

Microwave-assisted extraction (MAE) can also be paired with an alkaline solvent to improve the release of higher molecular weight oligosaccharides by means of loosening the CWP bonds (Khodaei et al., 2016; Müller-Maatsch et al., 2016; Spadoni Andreani & Karboune, 2020). Spadoni Andreani and Karboune (2020) showed that a low microwave power (85 W/g) paired with a high alkaline molarity (0.5M KOH) yielded 20% of pectic oligosaccharides, of homogalacturonan and rhamnogalacturonan origin, from cranberry pomace, which have a higher degree of polymerization (7-10) than those obtained from enzymatic extractions. Alternatively, Davis et al. (2021) recommend the use of high microwave power would favor the release of high molecular weight polysaccharides (200-700kDa). Nevertheless, MAE can be coupled with other analytical techniques such as chromatography to have a more complete picture on the bioactive compounds of plant cell wall materials (Mirzadeh et al., 2020; Xia et al., 2011).

2.12.2 Pulsed electric field

Pulsed electric field (PEF) treatment is an emerging non-thermal extraction technique involving the application of moderate to high voltage pulses for a short period of time. PEF is currently commercially available for food processing and has the potential to revalorize agro-industrial by-products, such as CBS, for the development of novel products or ingredients (Poojary et al., 2017). Barbosa-Pereira et al. (2018) explored the extraction yield of CBS polyphenols using PEF treatment and observed increases in the extraction of phenolics and epicatechin up to 1.8-19.5% and 30% from CBS compared to supercritical carbon dioxide extraction (Arlorio et al., 2005) and solid-liquid extraction (Martínez et al., 2012). Furthermore, the authors highlighted that higher recovery and radical scavenging activity of flavonoids from CBS were achieved, compared to other thermal treatments. In agreement with Barbosa-Pereira et al. (2018), other studies reported PEF as an efficient method to extract certain polyphenols, specifically for anthocyanins (Luengo et al., 2013; Medina-Meza & Barbosa-Cánovas, 2015; Puértolas & Barba, 2016). The factors influencing the PEF extraction yield include the extraction time, solvent concentration, as well as the material's compound characteristics (Puértolas & Barba, 2016; Barbosa-Pereira et al., 2018).

2.12.3 Sub-critical water extraction

Subcritical water extraction (SWE) is another environmentally friendly extraction method. This technique uses water as the solvent along with the application of high pressure and temperature to extract both polar and non-polar compounds (Muñoz-Almagro et al., 2019). In theory, SWE would provide a high recovery for active compounds, such as phenols. SWE (121˚C, 103.4 bar, 30 minutes) with and without supercritical fluid extraction resulted in higher pectic yields and purity (10.9%) from cacao pod husks compared to conventional extraction, using 4% (w/v) citric acid (8.3%) (Muñoz-Almagro et al., 2019). This was explained by high reactivity of the ions generated as a consequence of water dissociation under subcritical conditions (Muñoz-Almagro et al., 2019). SWE was further coupled with micro-encapsulation and spray-drying techniques to ensure the high yield and stability of the bioactive phytochemicals in the CBS extracts (Jokić et al., 2020). Using response surface methodology, Getachew et al. (2018) and Tomšik et al. (2017) investigated the optimal SWE operating conditions to mediate a high polysaccharide and phenolic extraction yield from spent coffee grounds and wild garlic samples. Getachew et al. (2018) suggested SWE conditions set at 178.85˚C, 20 bar for 5 minutes would provide a high polysaccharide, namely galactomannans, recovery, (18.25%), while Tomšik et al. (2017) suggested that optimal phenolic recovery (4.11 g gallic acid equivalent/100 g) would be achieved at 179.0˚C for 10 minutes with a 1.09% HCl acidifier.

2.12.4 Cavitation extraction techniques

2.12.4.1 Ultrasound-assisted extraction

Following the principles of acoustic cavitation, ultrasound-assisted extraction (UAE) will ensure cell wall disruption by applying low-frequency, high-intensity ultrasonic waves (5-400W), providing the release of phenolic compounds, oligo- and polysaccharides from the plant material (Yusof et al., 2019; Pavlović et al., 2019; Grillo et al., 2019; Leong et al., 2011). Grillo et al. (2019) compared the extraction efficiency of UAE on CBS starting materials pre-treated with a ternary mixture of hexane/ethanol/water (30:49:21). With UAE (15 min, 150W, 19.9kHz, 40˚C) yielded higher proportions of hexane (2.5%) and hydro-alcoholic phases (13.9%). Similarly, Yusof et al. (2019) reported UAE (55˚C, 45min) coupled with an 80% ethanol pre-treatment yielded a maximum level of flavonoids (7.41mg rutin equivalent / g) in CBS extracts. In terms of the extraction efficiency of alkaloids, Pavlović et al. (2019) proposed UAE (30 min, 35kHz, 25-60˚C)

using 50% aqueous ethanol is ideal for caffeine recovery (994.60 mg/kg) while providing a 91.87% DPPH radical scavenging activity from CBS extracts.

2.12.4.2 Hydrodynamic cavitation

Hydrodynamic cavitation (HC) is a low-cost, yet advanced extraction method in situations where industrial scale up is desired. Also paired with a pre-treatment, this technique generates cavitation by producing high pressure turbulences that is reduced below liquid vapor pressure, further, disrupting the cell wall matrix, increasing mass transfer and providing better contact between reactants (Rinaldi et al., 2017). Similar to UAE, HC extraction has been explored for by-product valorisation, such as in CBS (Grillo et al., 2019). Using hexane/ethanol/water (30:49:21) for CBS pre-treatment, HC (3000 rpm, 11 min, 25˚C) yielded greater proportions of hydrophilic extracts (14.6% vs 13.9%) and lipid fractions (10.1% vs 2.5%) than UAE. Furthermore, HC provided a two-fold increase in theobromine recovery than UAE (16.02% vs 9.14% w/w) (Grillo et al., 2019). Comparably, Lohani et al. (2016) explored the extraction of sorghum flour and apple pomace using hydrodynamic cavitation, in which increases in their total phenolic content (39.5% and 42%) and antioxidant activity (38.6% and 97%) were observed. HC has shown its ability to extract protein from soybeans at much greater yields compared to acoustic cavitation, through UAE (Preece et al., 2017).

2.12.5 Pressurized-liquid extraction

Heavily utilized for its successful polyphenol recovery, pressurized-liquid extraction (PLE) involves the use of liquid solvents, namely ethanol or water, at a high temperature and pressures for a short period of time (Mustafa & Turner, 2011; Colivet et al., 2016; Okiyama et al., 2018; Fernández-Ponce et al., 2016). Okiyama et al. (2018) explored the effects of PLE (60-90˚C, 10.35MPa, 5-50 minutes, EtOH), on the total flavonol and alkaloid content in CBS extracts. Their findings suggested that the catechin (26-135 µg flavonols/g CBS) and epicatechin (1.36-3.26 µg flavonols/g CBS) concentrations increased with increasing temperature and static time. Similar observations were made with the caffeine and theobromine concentrations where equilibrium was achieved after a 30-minute static time (Okiyama et al., 2018). Jacques et al. (2008) suggests that elevated temperatures promote the release of high molecular weight compounds, however, the static time may negatively influence the extraction yield. This was observed for CBS' procyanidin B2 concentrations as they decreased with increasing static time (0.973 µg flavonol/ g CBS at 30

minutes vs 0.73µg flavonols/ g CBS at 50 minutes) (Okiyama et al., 2018). Another study conducted by Mazzutti et al. (2018) compared PLE (70˚C, 10MPa, 20 minutes, EtOH) to Soxhlet extraction (EtOH) to recover polyphenols from CBS. Both, techniques provided similar extraction yields (PLE: 7.2% vs Soxhlet: 6.8%), however, PLE yielded a lower total phenolic content (0.6 mg gallic acid equivalent/g vs 49.4 mg gallic acid equivalent/g) than Soxhlet, which was a result of using a non-defatted CBS sample as well as a short PLE static time (Mazzutti et al., 2018). Nevertheless, PLE ensures less solvent consumption, reduced extraction time, improved solation of target compounds, namely phenolic compounds, lignin, and carotenoids (Mazzutti et al., 2018; Okiyama et al., 2018; Mustafa & Turner, 2011).

2.13 Enzymatic-based approaches

Plant by-product valorization using enzymatic-based approaches involve disrupting the cell wall matrix by means of breaking down lignocellulosic material while supporting the release and recovery of selected bioactive compounds. In plant cell wall structures, the lignin is tightly bound around cellulose fractions via phenolic-glycoside or di-ferulic covalent linkages that forms a physical barrier to prevent glycoside hydrolase activity. Gartaula et al. (2019) used ferulic acid esterase from *Clostridium thermocellum* to release trans-ferulic acid, 5-5' diferulic acid and 8-O-4' diferulic acid from popped endosperm and wheat cell walls. The synergistic effects of FAEs and different glycosyl hydrolases were often evaluated in terms of cell wall disruption and the release of phenolic compounds. The works of de Oliveira et al. (2016) observed the use of ferulic acid esterase (40 µg/mL), from *Aspergillus clavatus,* in combination with xylanase (25 U/mL) which contributed to a significant release of ferulic acid (0.55 mg/g) using the enzyme cocktail compared to FAE (0.1 mg/g) and xylanase (0.03 mg/g) alone, as a result of xylanases producing short chain feruloylated xylooligosaccharides with less steric hindrance (Vardakou et al., 2004; de Oliveira et al., 2016). Enzymatic hydrolysis of natural sugar cane fractions was investigated by Várnai et al. (2014) using xylanases, ß-xylosidase, acetyl xylan esterase, and three feruloyl esterases (AnFaeA, TsFaeC, and NcFaeD, respectively) in cellulase mixtures. Acetyl xylan esterase (AXE) improved the yield of cellulose and xylan hydrolysis by removing substitutions and enhancing the accessibility of cellulose/xylan structure (Tenkanen et al., 1997; Várnai et al., 2011). In terms of the FAE-cellulase mixtures, the TsFaeC-cellulase mixture enabled the release of *p*-coumaric acid (20.9% in sugar cane pith) while the AnFaeA and NcFaeD mixtures effectively

released ferulic acid (18.3% from sugar cane pith). Both studies observed the synergistic effects of FAE and cellulase where the extraction product profile of phenolic acids is related to type of FAEs (Gottschalk et al., 2010; Várnai et al., 2014). Alternatively, Gottschalk et al. (2010) assessed the enzymatic efficiency of fungal biomass *Trichoderma reesei* and *Aspergillus awamori* in the hydrolysis of steam pre-treated sugarcane bagasse. In *T. reesei* biomass, carboxymethyl-cellulase and exoglucanase activities were predominant, while xylanase and ß-glucosidase activities were predominant in that of *A. awamori.* Their blend resulted in synergistic enhancement of exoglucanase and carboxymethyl-cellulase activities by 73-110% and promoted the hydrolysis of celluloses and xylans to over 80%. The enhanced effectiveness of the cellulase and xylanase blend was attributed to the cleavage between xylan and lignin, further creating more hydrolyzing sites in glucan/xylan (Gottschalk et al., 2010).

Treatments with glycosyl hydrolase at controlled conditions were developed for the generation of polysaccharides with potential functionalities from cell wall materials. In order to effectively release polysaccharides from CBS, enzymatic treatment was carried out with a cocktail comprised of Pentopan®MonoBG, Viscozyme® L and Pectinex® 5XL and the total sugar release was attained at 47.1-50.7g/kg (dry matter). The authors recommended enzyme concentrations of 0.6% , 0.8% and 1% (w/w) would enable optimal polysaccharide release from CBS (Alemawor et al., 2009). The extraction of pectin from lime peels using Laminex C2K from *Penicillum funiculosum* (cellulase, xylanase, and arabinoxylanse) was reported to be around 23% (w/w) (Dominiak et al., 2014). Other studies have suggested enzymatic extraction at pH conditions of 4-7 with assistance using organic solvents, acids or hot water extraction for improved efficiency in extracting high molecular weight cell wall polysaccharides (Wu et al., 2013; Kilpeläinen et al., 2014; Reisinger et al., 2014; Martínez-Abad et al., 2016). Using a multi-enzymatic approach to yield a variety of low and high oligosaccharides and oligomers, Khodaei and Karboune (2018), observed that equal proportions of Depol™ 670L and Gamanase 1.5L reacted with potato rhamnogalacturonan I (RGI), both chosen for its high RG I specificity, attained yields of low (0.1-13.9%) to high molecular-weight oligosaccharides (0.0-37.5%) as well as oligomers (0.0-75.7%).

2.14 Cell wall feruloylated oligo- and polysaccharides: biotransformation and functionalities

Feruloylated oligosaccharides, a hydroxycinnamic acid derivative, exist in nature within the plant cell wall. Typically, their recovery from the plant cell wall is limited due to structural inconsistencies as well as the prevalence of ferulic acid in the phenolated moiety (Tamayo-Cabezas & Karboune, 2019). In order to better exploit the plant cell wall extracts, different approaches have been developed to achieve desired functionality profiles via the modulation of the structural and physicochemical properties of the main compounds. Potential modifications of cell wall feruloylated oligo- and polysaccharides are highlighted in this section to support the development of multi-functional and bioactive ingredients derived from CBS.

2.14.1 Hydrolysis of cell wall polysaccharides for the generation of feruloylated oligosaccharides

Acid hydrolysis can serve as a technique to recover feruloylated oligosaccharides. Previously, successful extraction of feruloylated oligosaccharides were recovered from maize bran (Allerdings et al., 2005; Saulnier et al.,1995), sugar beet pulp (Ralet et al., 1994; Ishii, 1997) and rice bran (Li et al., 2008) using a mild acid hydrolytic treatment with trifluoroacetic acid (TFA). Furthermore, a food-grade treatment, using oxalic acid, was also investigated for the extraction of feruloylated oligosaccharides from maize bran, which increased the production of feruloylated oligosaccharides as 52.2% of ferulic acid was released (Ou & Sun, 2014; Huang & Ou, 2012). Response-surface modeling suggested that optimal acid hydrolysis conditions for rice bran were at 193mM TFA, 1.36 hours hydrolysis time at 100 ºC, in which 916.12µg feruloylated oligosaccharides/g were recovered, accounting for 54.08% of the total acyl ferulic content (Li et al., 2008). Alternatively, Ralet et al. (1994) suggested that high temperatures or a two-step TFA hydrolytic treatment would support a more complete hydrolytic approach, recovering 90-95% of feruloylated oligosaccharides. The product from acid hydrolysis derived from sugar beet pulp was characterized by its enriched arabinose-type sidechains, attributing to high susceptibility of the regions with arabinose and their α-(1→5) linkages towards acid hydrolysis than other regions (Ralet et al. 1994). The ferulic acid-glycoside linkages from cell wall materials of maize bran were identified to be 5-O-(trans-feruloyl)-L-arabinose *f*, O-ß-D-xylose *p*-(1→2)-[5-O-(*trans*-feruloyl)-L-arabinose-*f]* and O-L-Gal-*p*(1→4)-O-D-xyl-*p-(*1→2)-[5-O-(*trans* feruloyl*)*-L-Ara *f]*, by nuclear

magnetic resonance spectroscopy (Saulnier et al.,1995). The feruloyl groups were found to be linked to the arabino-furanosyl residues of the main core α-(1→ 5)-linked arabinan chains and to the galacto-pyranosyl residues of the main core of ß-(1→4)-linked type 1 galactan chains of the pectic polysaccharide of cell wall materials (Colquhoun et al., 1994; Fry, 1982; Ralet et al., 1994). As compared to acid hydrolysis, enzymatic hydrolysis has proven to recover higher yields in feruloylated oligo- and polysaccharides (Ralet et al., 1994).

2.15 Feruloylation of cell wall oligo- and polysaccharides

Efforts to enhance the degree of feruloylation of cell wall oligo and polysaccharides have been achieved using an enzymatic approach. Successful transesterification of methyl ferulates to L-arabinose was mediated using type C FAEs from *Sporotrichum thermophile* (StFaeC), feruloyl esterase paired with xylanase (Topakas et al., 2005; Vafiadi et al., 2007). The authors suggested the modified glycosides are biocompatible, biodegradable as well as exhibit anti-microbial activity (Topakas et al., 2005). Electrospray ionization mass spectrometry (ESI-MS) was used to characterized the chemoenzymatic synthesized feruloylated arabino-oligosaccharide; the results suggested that feruloylation occurred on the hydroxyl group of thenon-reducing ring of each L-arabino-oligosaccharide (Vafiadi et al. 2007). Several studies have reported increases in bioconversion yields using surfactant-less microemulsions for enzymatic synthesis of feruloylated (Couto et al., 2010; Coutu et al., 2011; Topakas et al., 2005; Vafiadi et al., 2006). Couto et al. (2011) used a n-hexane/1,4-dioxane/MES-NaOH buffer mixture, where a 26.8% feruloylated galactobiose bioconversion yield was achieved. Similarly, a n-hexane/2-butanone/MES-NaOH buffer mixture, resulted in feruloylated sugars of raffinose, arabinobiose, xylobiose bioconversions yield were achieved at 11.0%, 7.9% and 9.0% (Couto et al., 2011). The microemulsion composition will impact the overall esterification efficiency of FAE, where it is highly dependent on the structure of the glycoside substrate (Couto et al., 2011). Moreover, Couto et al. (2011) suggested ideal bioconversion yields would be achieved at 35˚C, with a 3:1 ferulic acid to raffinose molar ratio, 3% water content (v/v) as well as an enzyme concentration of 345 FAE units.

Furthermore, enzyme immobilization can serve as commercial application of the biosynthesis of feruloylated oligosaccharides, through optimizing enzyme activity at specific macroenvironment and ensuring enzyme stability and reusability (Thörn et al., 2011; He et al., 2015; Tamayo-Cabezas & Karboune, 2019). Using FAE, from *Humicola insolens,* (Tamayo-

Cabezas & Karboune, 2020) suggest that the pH, buffer molarity, and the enzyme-to-support ratio served as the key parameters to modulate the immobilization activity yield to mediate the bioconversion of feruloylated oligosaccharides. By means of modulating the optimal conditions, the authors reported that as much as 92% of the feruloylation efficiency was retained upon immobilization of FAE, from *H. insolens* (Tamayo-Cabezas & Karboune, 2020). Similarly, Spadoni Andreani et al. (2021) observed as much as a six-fold increase in the feruloylation efficiency of cranberry polysaccharides using immobilized FAE, from *H. insolens;* as compared to free FAE. Additionally, this yielded feruloylated polysaccharides with a greater phenolic content (Spadoni Andreani et al., 2021). Other works explored similar effects of immobilization on the feruloylated oligosaccharide bioconversion yield (Couto et al., 2011; He et al., 2015; Tamayo-Cabezas & Karboune, 2019; Thörn et al., 2011; Topakas et al., 2005).

2.16 Functional properties of cell wall feruloylated oligosaccharides

Feruloylated oligosaccharides are functionally bioactive compounds that have shown to promote antioxidant (Couto et al., 2011; Fang et al., 2012), anticarcinogenic, antimicrobial and anti-inflammatory activities (Ou & Kwok, 2004; Couto et al., 2010), as well as promoting microflora proliferation and pathogen suppression in the gastrointestinal tract (Ou et al., 2016).

2.16.1 Antioxidant activity

Ferulic acid glycoside esters were shown to be stronger antioxidants than their free forms, as indicated by radical scavenging activity against DPPH (Kylli et al., 2008). Couto et al. (2010) suggested this was attributed to the enhanced ability of ferulic acid to donate hydrogen to DPPH and to stabilize the phenoxy radical upon its conformational modification with di- and oligosaccharides. Similar findings were observed with feruloylated oligosaccharides from maize bran, wheat bran, wheat flour, and barley; providing improved radical scavenging activities compared to free ferulic acid (Ohta et al., 1994; Katapodis et al., 2003; Szwajgier et al., 2005; Zhang et al., 2005; Shen et al., 2012; Lin et al., 2014). In contrast, feruloylated xylo-oligosaccharides and arabino-xylooligosaccharides showed less radical scavenging specificity towards DPPH compared to free ferulic acid (Couto et al., 2011; Katapodis et al., 2003). However, feruloylated xylo-oligosaccharides were viewed as " the most potent scavenger" as it retained a radical scavenging yield of 94% (Couto et al., 2011). The study also reported that the antioxidant activity was greater for acylated ferulic acid with hexoses than pentoses (Couto et al., 2011). Ou

and Sun (2014) suggests that the antioxidant activity of feruloylated oligosaccharides is related to the structure, and ferulic acid content. Silva et al. (2000) proposed that the steric hindrance of the glycosidic substituents on the rotation degree of the phenyl moiety can impact the scavenging activity.

Chigorimbo-Murefu et al. (2009) stated that lipase catalyzed feruloylated arbutin ferulates (23%) obtained from lipase-catalyzed reactions showed enhanced capability in inhibiting LDL oxidation as compared to ferulic acid (16%) and arbutin (13%) alone. Ou and Sun (2014) investigated the feruloylated oligosaccharides antioxidant activity in human erythrocytes which suggested that feruloylated oligosaccharides prevented hydrogen peroxide cell damage (Ou and Sun, 2014). In addition, no signs of cytotoxicity or genotoxicity were observed after this intervention against hydrogen peroxide exposure (Wang et al., 2008; Zhang et al., 2005; Yuan et al., 2005; Shen et al., 2012). Other biological property of feruloylated oligosaccharides associated with their antioxidant activity were reported, including increased the cell viability, superoxide dismutase activity, decreasing the release of lactate dehydrogenase and malondialdehyde and inhibiting glycation reaction (Boušová et al., 2005; Shen et al., 2012; Silván et al., 2011; Vlassara & Palace, 2002; Wang et al., 2009; Wu et al., 2009).

2.16.2 Prebiotic potential

Similarly, to non-digestible oligosaccharides, feruloylated oligosaccharides may serve as prebiotics to enhance the intestinal microbiome. Feruloylated oligosaccharides extracted from wheat bran have shown to stimulate the growth of *Bifidobacterium bifidum* (Yuan et al., 2005). However, these carbohydrates showed lower fermentability in the colon compared to xylo-oligosaccharides. In order to improve the rate of fermentation, Ou and Sun (2014) suggested ferulic acid to be released from oligosaccharides via microbial ferulic acid esterases to increase their bioavailability.

2.16.3 Immunomodulatory activity

Since feruloylated oligosaccharides were deemed as viable antioxidants, one may investigate their impact on immune responses. *In vitro* analysis with feruloylated oligosaccharides extracted from rice bran suggested the suppression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) , interleukin-1ß, interleukin-6, nitric oxide, and prostaglandin E2 (Fang et

al., 2012). As a result, Fang et al. (2012) concluded these findings are the first signs of the immunomodulatory effects associated with feruloylated oligosaccharides. Therefore, one could correlate the anti-inflammatory response of feruloylated oligosaccharides to that of ß-glucan, arabinogalactan, pectin, arabinan, heteroglycan, inulin, agaro-oligosaccharides, chitosan oligosaccharides as well as ferulic acid (Hirabayashi et al., 1995; Sakai et al., 1999; Chen & Yan, 2005; Yamada & Kiyohara, 2007; Yoon et al., 2007)

2.17 Conclusion

CBS are de-hulled from cocoa beans during processing, later discarded as waste or used in animal feed and fertilizer. Nonetheless, chemical and bioactive composition analysis studies conclude an abundant source of dietary fiber and phenolic compounds, providing functional properties that can serve use in food formulations. Many techniques that are developed for the valorization of plant cell wall materials can be adapted towards cocoa bean shells to support its exploitation. This review explored the effects of cocoa processing, especially fermentation and roasting, where observed differences were found in terms of chemical and bioactive compositions. Physical, chemical and enzymatic methods in bioactive component extraction from the cell wall matrix of CBS were discussed. Depending on the desired target compound, either individual approaches or their combined uses can be explored. Future research should focus on the isolation, bio-generation and potential formulation of feruloylated oligo- and polysaccharides derived from CBS. These bio-products will be analyzed for the functional properties that can be used in food formulations such as a sugar alternative in chocolate manufacturing. Although non-specific to CBS, studies were compiled to investigate efforts done to modify or extract feruloylated oligosaccharides from the cell wall material. Provided that CBS has both a rich phenolic and cell wall polysaccharide content, the potential to generate feruloylated oligosaccharides is promising where one can further investigate its bioactive properties of cell wall polysaccharides, such as its prebiotic, antioxidant, and immunomodulatory activity. With this, future research should assess the use of CBS-derived feruloylated oligosaccharides in food formulations and their techno-functional property as well as investigate health-related functional properties.

Table 2.6. Applications of cocoa bean shells among several industries

Field	***Use***	***Application***	***Outcome***	***Reference***
Agriculture		Barn bedding material	Lower bacterial counts and plasma cortisol levels as well as decreased ammonia concentrations from 6-21% were observed using CBS bedding for lactating Holstein cows to promote udder health	Yajima et al., 2016
	Swine intestinal microbiota	Prebiotic	7.5% CBS substitution with conventional cereal-based diets led to a decrease in *Firmicutes* and an increase in *Bacteroidetes* in pigs. Also, reduced fecal populations of *Lactobacillus-Enterococcus* and *Clostridium histolyticum* were observed suggesting CBS' potential for stabilizing swine intestinal microbiota.	Magistrelli et al., 2016
Feedstuff	Cockerel Feed	Protein and fibre supplement	a 10% CBS supplementation in Cockerel feed led to a decrease in packed cell volume, haemoglobin and white blood cell counts as well as increased Broiler weights.	Adeyina et al., 2011
	Goat Feed	Fibre supplement	CBS supplementation with Dried Brewer's grain led to a 5.3kg increase among goats over 56 days	Aregheore, 2002
	Laying hens feed	Feed supplement	Up to 15% CBS supplementation in laying hens feed results in an increase in spleen, kidney and ovary weights as well as an increase in egg production per week.	Emiola et al., 2011
	Laying hens feed	Feed supplement	Enzyme-treated CBS supplementation (10%) in replacement of maize was effectively incorporation in hens' diets without adverse effects on the performance and egg quality characteristics.	Olumide et al., 2014
	Rabbit feed	Feed supplement	Hot water treated CBS (200g/kg) in rabbit feed increased feed intake and led to significant weight gain and optimal performance.	Adeyina et al., 2010
	Cattle feed	Feed supplement	60% rice bran with 40% CBS as a protein supplement resulted in optimal daily weight gain and feed efficiency among post-weaning cattle than rice bran supplementation alone.	Soeharsono et al.,2017
Food	Biscuits	Microencapsulated polyphenol extract	Maltodextrin (stabilizing agent)-microencapsulated CBS polyphenol (80:20) provided a stable powder without affecting the total polyphenol content during the baking process.	Papillo et al., 2019

Gluten-free bread	Fibre-enrichment and textural enhancement during storage	Fibrous material led to a coarser structure, reduced specific volume and poor moisture absorption. Also, led to a desirable color as well as a crumb and crust texture (hardness)	Rinaldi et al., 2020
Functional Beverage	Antioxidant and anti-diabetic agents	CBS polyphenol extracts contributed to the high antioxidant activity and alpha-glucosidase inhibition capacity in the home-made functional beverage while achieving consumer desirability in terms of appearance and odor.	Rojo-Poveda et al., 2019
Pork Sausages	Color enhancer, stabilizer, textural enhancer	Pork sausages supplemented with CBS (0.75-1%) showed ideal emuslion stability and increased apparent viscosity, color enhancement (less redness), and flavor acceptability. Also, CBS improved refrigerated storage properties through prolonging the onset of lipid oxidation.	
Cookies	Fiber enriched flour substitute	Supplementing wheat flour with 20% of CBS flour with led to high acceptability in terms of texture, taste, crunchiness, hardness, and after-taste	Handojo et al., 2019
Chocolate muffins	Fat replacer	20% soluble cocoa fibre replaced with oil showed similar textural (cohesiveness and chewiness) properties while imparting a nice color compared to the control,	Martínez-Cervera et al., 2011
Cooked Beef	Antioxidant enrichment	CBS extracts reduced lipid oxidation as a result of its high antioxidant and radical scavenging activity as compared to synthetic antioxidants (BHT and ß-tocopherol) for cooked beef stored at 4˚C for 14 days.	Ismail & Yee, 2006
Extra virgin olive oil jam	Antioxidant enrichment	Encapsulated CBS extract prolonged onset of rancidity	Hernández-Hernández et al.,2019
Dairy drink	Antioxidant enrichment	Dairy drink using CBS, coffee silverskin and orange peels attained a desirable antioxidant activity (against DPPH) by 82.20% and a total phenolic content of 5.74 gallic acid equivalent mg/g while retaining consumer acceptability.	Quijano-Aviles et al., 2016
Yoghurt Drink	Polyphenol and antioxidant enrichment	Encapsulated CBS phenolic extract *in-vitro* bioaccessbility was 5-fold higher in the liposomal powder where catechin and ferulic acid bioaccessibilty accounted for 50 and 80% during 15 days of storage	Altin et al., 2018
Extruded snacks	Protein and fibre fortifier	Extruded snacks using corn flips and CBS achieved harder textures with increased retrogradation	Jozinović et al., 2019

			tendencies while increasing the proportions of resistant starch, polyphenols, and antioxidant activity	
	Pound cake	Fat replacer	50% vegetable oil replacement with leached or raw CBS in pound cake formulation improved the chemical, physical, and sensory properties compared to the control	Öztürk & Ova, 2018
Packaging		Antioxidant bioelastomer	Active packaging material (CBS + PDMS) exhibited good moisture barrier properties and optimal antioxidant activity against DPPH and ABTS	Tran et al., 2017
	Biocomposite packaging films		Biocomposite films of polylactic acid and CBS produced showing increases in Young's modulus by 80% as well as exhibiting moisture-barrier, antioxidant and swelling properties that allow these films to be biodegradable in aquatic environments	Papadopoulou et al., 2019
	Biocomposite packaging films		Polycaprolactone and CBS (30%) composites produced and exhibited enhanced rigidity as well as optimal composite biodegradability	Puglia et al., 2016
Industrial	Concrete	Biomass ash partial concrete replacement	CBS biomass ash led to decreased water absorption, porosity and capillary indexes, resulting in increased concrete mechanical strength and durability.	Fontes et al., 2019
		Bioadsorbent	Grafted CBS with diazonium salts experienced increased chemical and mechanical resistance that would facilitate as a bioadsorbent to entrap pollutants such as heavy metals, gases, or industrial dyes.	Fioresi et al., 2017
		Bioadsorbent	Plasma-treated CBS effectively removed Azur II and Reactive Red dyes in the basic and acidic conditions after 40 and 240 min serving as a multi-purpose sorbent for the removal of coexisting pollutants from aqueous solutions	Takam et al., 2017
		Bioadsorbent	Developed active carbons with high meso- and microporosity	Pérez-Cadenas et al., 2018
		Bioadsorbent	Developed binderless carbon monoliths with a high micropore volume and good mechanical performance	Plaza-Recobert et al., 2017
	Biofuel	Liquid smoke and charcoal production	CBS pyrolysis (400-550˚C) achieved a liquid smoke (18-23%) and charcoal (39%) while showing faster heating rates for the production of ash and water contents of 16.5-19% and 6.5-8.5%	Handojo et al., 2020

Biofuel	Biogas production	A 14% increase in the biomethane yield was achieved using pre-treated CBS as a source of lignocellulosic material.	Mancini et al., 2016
Biofuel	Bioethanol production	An 8.46% bioethanol yield was achieved using hydrolyzed CBS functioning as biomass alternative	Awolu & Oyetuji, 2015
Automotive brake pads	Composite filler additive	Observed increases in the wear rate, tensile and compressive strength as well as increases in the coefficient of friction using CBS as an asbestos filler replacement	Olabisi et al., 2016
	Bioadsorbent	Using CBS as an activated-carbon precursor where Methylene Blue dye was adsorbed and controlled by film diffusion	Ahmad et al., 2012
	Bioadsorbent	CBS (20g) with coffee grind residues (10g) effectively lead concentrations in contaminated waters 98.53%	Diaz et al., 2018

CONNECTING STATEMENT I

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In the literature review, presented in Chapter II**,** the structural characterization, composition and functional properties of cocoa bean shells are discussed. The characterization of cocoa bean shells composition and the corresponding cell wall material, together with the assessment of the isolation of polysaccharides using alkaline treatment are reported in Chapter III. Proximate analysis, dietary fiber, and polyphenol quantification were performed on cocoa bean shells and their cell wall material. The monosaccharide profile of the cell wall material was determined by high performance anion exchange chromatography. Alkaline treatment, using two concentrations, was applied to cocoa bean shells cell wall material, for the isolation of their polysaccharides, which were characterized and compared in terms of the molecular weight and composition.

CHAPTER III. EXTRACTION AND CHARACTERIZATION OF COCOA BEAN SHELL CELL WALL POLYSACCHARIDES

3.1 Abstract

Cocoa bean shells are the outermost layer of the cocoa bean and a heavily accumulated by-product of the cocoa industry. In this study, cocoa bean shells were characterized in terms of their chemical composition where they were found to be a great source of carbohydrates, specifically dietary fiber, protein, ash, and polyphenols, namely quercetin, epicatechin, and catechin. Cell wall polysaccharides were isolated through sequential extraction (ethanol, chloroform:methanol, and phenol:acetic acid:water) followed by alkaline extraction (0.5M or 4M KOH) for their characterization. Polysaccharide extracts were found to be primarily rich in pectic polysaccharides (80.6-86%) namely rhamnogalacturonan and arabinogalactan as well as hemi-cellulosic polysaccharides (13.9-19.4%). Overall, 0.5M KOH polysaccharides were favored having provided a diverse profile of neutral sugars and uronic acids. When tested for the promotion of the growth of selected probiotic strains, CBS cell wall polysaccharides performed similarly or more than inulin and rhamnogalacturonan based on the prebiotic activity scores. The short chain fatty acid profiles were characterized by high amounts of lactic acid, followed by acetic and propionic acid.

3.2 Introduction

The generation of Cacao (*Theobroma cacao)* by-products are estimated at 700,000 tons annually, which negatively contribute to the environmental and economic value of cocoa processing industry. Among these by-products, cocoa bean shells (CBS) are the most abundant and are the outermost layer of the cacao bean that encases cacao nibs. Efforts to valorise CBS have been explored in agricultural and bioremediation fields such as its use in animal feeds and fertilizers (Adeyina et al., 2010; Soeharsono et al., 2017). In food applications, CBS has been used as a functional ingredient for fiber enrichment and fat replacement in baked goods and as a stabilizer and color enhancer in pork sausages (Bernaert & Ruysscher, 2016; Choi et al., 2019; Martínez-Cervera et al., 2011). Although there has been an increasing industrial interest in effective valorization of CBS into functional food ingredients, only the pilot-scale extraction of polyphenols from CBS has been explored (Grillo et al., 2019). The extraction of cell wall polysaccharides from CBS has yet to be reported. To the authors' knowledge, only few studies have characterized the cell wall polysaccharide profile of CBS in terms of monosaccharide profile,

molecular weight distribution and linkage types (Lecumberri et al., 2007; Redgwell et al., 2003; Redgwell & Hansen, 2000; Vojvodić et al., 2016). According to Redgwell et al. (2003), CBS holds a reliable source of dietary fibers as its cell-wall polysaccharide profile comprises of 45% pectic polysaccharides, 35% cellulose as well as 20% hemicelluloses, namely gluconoarabinoxylan, xyloglucan, and galactoglucomannans. As far as the authors are aware, the extent of the variability in the cell wall polysaccharide profile of CBS, depending on the cacao variety and the processing conditions, has not been studied.

Several approaches have been reported for the isolation of cell wall polysaccharides from cell wall, including chelating agent, acid, microwave-assisted alkaline, and ultrasound-assisted alkaline extractions (Aguiló-Aguayo et al., 2017; Khodaei & Karboune, 2013; Redgwell et al., 2003; Spadoni Andreani & Karboune, 2020). The selection of an appropriate extracting agent or approach can help target the isolation of polysaccharides with well-defined structures. As an example, acidic extraction, using hydrochloric acid, targets the isolation of homogalacturonan (Khodaei & Karboune, 2013). Alternatively, alkaline extraction of cell wall polysaccharides, using sodium or potassium hydroxides paired with sodium borohydride, has proven to be effective for isolating and understanding the distribution of pectic and hemi-cellulosic polysaccharides within the cell wall materials of potato, cranberries, bamboo, maize and more (Khodaei & Karboune, 2013; Li et al., 2015; Spadoni Andreani et al., 2021; Zhu et al., 2022). This approach enables the release of rhamnogalacturonan I through ß-elimination and oxidative peeling of homogalacturonan (Bonnina et al., 2001; Khodaei & Karboune, 2013; Zykwinska et al., 2006).

There is an increasing interest in the cell wall-rich by-products as their polysaccharide content can act as a beneficial source of dietary fibers and prebiotics that can modulate the gut microbiota and hence promote the intestinal health (Spadoni Andreani et al., 2021). Cell wall polysaccharides of by-products derived from pearl millet fibre, tangerine peels, apple pomace, and coconut residues have been previously reported to support the effective growth of beneficial gut microorganisms (Farooq et al., 2017; Islamova et al., 2017; Mohd Nor et al., 2017; Spadoni Andreani et al., 2021). However, no study on the fermentability of CBS cell wall polysaccharides by beneficial lactic acid bacteria has been reported so far.

The aim of the present study was to assess the nutritional composition of CBS at various processing conditions. To the authors' knowledge, this has not been previously investigated for

cocoa beans. In addition, the present study offers novel insight pertaining to the isolation and characterization of CBS cell wall materials from two cacao varieties obtained after selected processing conditions (fermentation, drying, roasting). The cell wall polysaccharides were, thereafter, extracted by alkaline treatments, structurally characterised, and subjected to fermentation by selected lactic acid bacteria. The investigation of the compositional, structural, and functional properties of CBS cell wall polysaccharides would provide novel insight useful to the development of a highly effective approach that valorizes cocoa bean shells as a source of carbohydrate-based functional ingredients in food applications.

3.3 Materials and Methods

3.3.1 Materials

Fresh cacao pods (CCN 51) were supplied by Smart Natural Circle (Montreal, QC, Canada). Commercial samples of CBS (Nacional/Arriba (NAC) and CCN 51) were supplied by Cocoa processing industry (Guayaquil, Ecuador). The bacterial strains, *Lactobacillus rhamnosus GG* (RO343 AR) and *Bifidobacterium Longum* (ATCC 15707™) strains were supplied from Harmonium International (Mirabel, QC, Canada) and ATCC©. 1-Kestose, nytose, and 1F-fructofuranoysl-nystose were obtained from Wake Pure Chemical (Osaka, Japan). The analytical grade reagents were from Sigma- Aldrich Co., St. Louis, MO, USA and salts were obtained from Fisher Scientific (Fair Lawn, NJ).

3.3.2 Preparation of cocoa bean shells

Fresh cacao pods (CCN 51) were subjected to lab-scale fermentation and drying processes to retrieve the cacao bean shells (CBS) as described by Romanens et al. (2018). The inner contents of cacao pods, containing the mucilage-pulp and beans, were placed in tightly sealed containers (4L) to provide an anaerobic environment in an incubator (Excella E24 incubator, New Brunswick Scientific, Canada), for 117 hours, where the temperature was adjusted in 6-24hr intervals, 28˚C at 0hr, 30˚C at 6hr, 32˚C at 21hr, 35˚C at 30hr, 38˚C at 45hr, 42˚C at 54hr, and 45˚C at 69hr. The pulp-bean mass was mixed every 24 hours to ensure proper removal of the pulp. After fermentation, the beans were subjected to drying in an oven for 72 hr at 45˚C until a final water content of 6-7%. The beans were de-shelled manually. Commercial samples of cocoa bean shells were from two varieties (Nacional/Arriba (NAC) and CCN 51). Both freshly prepared and

commercial CBS samples were blended using a Model 7011C commercial blender (Conair corporation, Stamford, CT, USA) and analysed for their proximate compositions.

3.3.3 Proximate compositional analysis of cocoa bean shells

Moisture content of CBS was determined using a variation of AOAC method 927.05 by drying for 18 hr at 70 °C in vacuum oven. Total fat content of blended CBS was determined using a modified AOAC method 945.16. In this last method, Soxhlet extraction of CBS (1 g with 150 ml) was carried out using petroleum ether as solvent for 6 hr. Ash content was measured by AOAC method 942.05, charring 2 g of blended CBS for 30 min, followed by incineration in a muffle furnace (5 hr at 525 °C). Crude protein content was determined on 0.1 g of blended CBS by Dumas method (Saint-Denis and Goupy 2004) with a FP 628 analyser (LECO Corporation, St Joseph, MI, USA). Carbohydrate content (% dry weight) of blended CBS will be determined as:

$$100 - (crude\ fat\ +\ ash\ +\ crude\ protein)$$

Dietary fiber content was determined on 1 g of blended CBS using the total dietary fiber assay kit (Sigma-Aldrich Co., USA) and a modified AOAC method 985.29 (Latimer and AOAC International 2016), in which α-amylase, protease and amyloglucosidase activities were used to digest the starting material. The remaining fiber was precipitated with ethanol, washed with ethanol and acetone, filtered and, once dry, its protein and ash content were determined and subtracted from the dry weight. All analyses were performed in triplicate.

3.3.4 Isolation of cell wall materials from cocoa bean shells

The cell wall material (CWM) was sequentially isolated as described by Redgwell et al. (2003). Using de-fatted CBS (4g), the isolation proceeded as follows: 1) 80% ethanol (2hr, 50 rpm), 2) chloroform: methanol (1:1, 1.5 hr, 50 rpm), 3) chloroform: methanol (1:1, 1.5 hr, 50 rpm), 4) phenol: acetic acid: water (PAW, 2:1:1, overnight), 5) phenol: acetic acid: water (PAW, 2:1:1, 2hr). Each treatment was followed by centrifugation (7,000xg, 15mins) and vacuum filtration prior to the following step. The recovered CWM was dialyzed (5-8kDa cut-off) against distilled water and freeze-dried. The isolation of CWM was performed in triplicate.

3.3.5 Alkaline extraction of cocoa bean shell cell wall material polysaccharides

Extraction of CBS polysaccharides was performed as described by Khodaei and Karboune (2013) where CWM material (2% w/v) was suspended in KOH solutions (0.5 and 4M) containing 0.02M $NaBH_4$. Mixtures were incubated (60˚C, 24 hr), thereafter centrifuged (10,000 x g for 15 mins) and recovered via filtration (0.45µm). Recovered polysaccharides were neutralized with HCl, dialyzed (5-8 kDa cut-off) and freeze-dried (-50˚C, 0.003 mbar).

3.3.6 Structural characterisation of carbohydrates

3.3.6.1 Determination of total neutral sugars and uronic acid contents

Uronic acid content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid colorimetric assay was used for the determination of neutral sugar content (DuBois et al., 1956).

3.3.6.2 Monosaccharide profile

CWM and CBS polysaccharides were subjected to acid hydrolysis according to the method of Khodaei and Karboune (2013). Briefly, 1 ml of a mixture of 37% HCl/methanol (1:4 v/v) was added to the sample (200 µl of liquid extract, or 5% (w/v) of solid in water). The mixtures were incubated at 70 °C for 24 hr and dried by heating in a convection incubator (Excella E24 incubator, New Brunswick Scientific, Canada). To complete hydrolysis, 3 ml of water and 1 ml of trifluoroacetic acid were added and heated at 100 °C for 1 hr. After evaporating the trifluoroacetic acid, the samples were neutralized with NaOH and centrifuged (8,000 rpm, 5 min). The supernatants (0.02 ml) were analyzed by high performance anion exchange chromatography (HPAEC) on a Dionex ICS 3000 system equipped with pulsed amperometric detection using a Carbopac PA-20 column (Dionex Co., Sunnyvale, CA, USA) at a temperature of 30 ˚C. Mobile phase was 20mM NaOH at flow rate of 0.4 ml/min. Rhamnose, arabinose, glucose, xylose, galactose, and mannose were used at varying concentrations (2.5 – 50µM) as standards.

3.3.6.3 Molecular weight distribution

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525 binary HPLC pump, equipped with a Model 2414 refractive index detector, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution of the carbohydrates. Analysis was determined using a TSK G5000 PWXL (Tosoh Co., Yamaguchi, Japan) column, with dextrans

(50– 670 kDa), soybean rhamnogalacturonan (0.125-1 g/l) and galactoglucomannan (0.125-1g/l) standards for molecular weight and concentration calibration. The temperature of the system was 30 °C, the eluent was 0.1 M NaCl, and the flow rate was set at 0.4 ml/min.

3.3.7 Extraction and determination of polyphenols content

The polyphenolic compounds were extracted using accelerated solvent extraction (Dionex ASE 350, Dionex, USA) from CBS and CWM. 0.5g samples were placed in 22ml cells containing Ottawa sand and Diatomaceous Earth. Samples were subjected to three consecutive repeated extractions using 80% methanol (40˚C,1600 psi) to exhaust the material. The total polyphenolic content was determined using the folin-ciocalteu assay as described by Singleton, Orthofer, and Lamuela-Raventós (1999). A gallic acid calibration curve (0.001to 0.1mM) was constructed (R = 0.95). The total polyphenolic content was expressed as gram of gallic acid equivalent per gram of sample. All measurements were performed in triplicate.

3.3.8 Determination of phenolic compounds via LC-MS

CBS and CWM were analyzed by LC-MS using an Agilent 1290 Infinity II LC system coupled to the 6560-ion mobility Q-TOF-MS (Agilent Technologies, Santa Clara, USA). The LC separation was conducted on a Poreshell120 EC-C18 analytical column (Agilent Technologies; 2.7 µm × 3 mm × 100 mm) connected with a Poreshell120 EC-C18 guard column (Agilent Technologies; 2.7 µm × 3 mm × 5 mm). The mobile phase A was HPLC water with 0.1% formic acid and the mobile phase B was acetonitrile with 0.1% formic acid. HPLC parameters were as follows: injection volume was 1 µl, the flow rate was 0.3 ml/min, and the column temperature was set to 30°C. The mobile phase profile used for the run in negative ion mode was 2% B (0 to 1.0 min), 2% - 20% B (1.0 to 4.0 min), 20%-100% B (4.0 to 8.0 min), 100% B (4.0 to 8.0 min), hold at 100% B (8.0-13.0 min), decrease to 2% B (13.0.0 to 13.5 min), and hold 2% B (13.5 to 14 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions were as follows: for ESI-, the drying gas temperature was 200 °C, drying gas flow rate was 12 l/min, sheath gas temperature was 250°C, sheath gas flow rate was 12 l/min, the pressure on the nebulizer was 35 psi, the capillary voltage was 4000 V, the fragmentor voltage was 240 V, and the nozzle voltage was 1000 V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 100 and 1100 at a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. Reference ions (*m/z* at 112.9856 and 1033.9881for ESI-) were used for

automatic mass recalibration of each acquired spectrum. Data treatment was conducted using Quantitative Analysis B.07.01 from Agilent MassHunter Workstation Software.

3.3.9 Prebiotic activity assay

The fermentability of CBS polysaccharides (0.5M KOH extracts, roasted CCN and NAC) by *B. longum* (ATCC 15707™) and *L. rhamnosus GG* (Harmonium AR) was investigated and compared to that of inulin and rhamnogalacturonan, using a modification of the method reported by Yang et al. (2013). The selected strains, *B. longum* and *L. rhamonosus GG,* were maintained in an anaerobic chamber at 37˚C. Pre-reduced reinforced clostridial broth was used for the reanimation of *B. longum* from a freeze-dried stock, and MRS broth was used for *L. rhamnosus GG*. An aliquot (0.5ml) of the primary cultures was then added into tubes containing 5ml of pre-reduced, carbohydrate-free MRS broth, supplemented with 0.4% glucose. Bacterial growth was measured using colony counting on Tryptic soy agar supplemented with 5% sheep blood for *B. longum* and pre-reduced MRS agar for *L. rhamnosus GG.* Tubes containing 5mL of pre-reduced carbohydrate-free MRS broth supplemented with 0.2% glucose, inulin, rhamnogalacturonan, and cell wall polysaccharides (CCN and NAC varieties) were inoculated with 0.1ml of secondary culture. Colonies were counted on Tryptic soy agar supplemented with 5% sheep blood for *B. longum and* pre-reduced MRS agar for *L. rhamnosus GG* after 6, 24, 48, 72 and 96 hr incubation intervals. Microbial counts were measured as colony forming units (CFU)/ml, and the resulting averaged data were transformed to log CFU/ml. A prebiotic activity score (PA) was calculated for each combination of bacterium and polysaccharide as follows:

$$PA = \frac{\left(Log\frac{CFU}{ml}\,on\ polysaccharide\ at\ x\ hr - Log\frac{CFU}{ml}\,on\ polysaccharide\ at\ 0hr\right)}{Log\frac{CFU}{ml}\,on\ glucose\ at\ x\ hr - Log\frac{CFU}{ml}\,on\ glucose\ at\ 0hr}$$

3.3.10 Determination of short chain fatty acid catabolites

An aliquot of cultures (0.5ml) was taken at 24 and 48hr. Bacteria were precipitated by centrifugation at 1400xg for 5min at 37˚C in a Minispin Plus centrifuge (Eppendorf AG, Hamburg, Germany). The supernatants were filtered using 0.22µm polytetrafluorethylene syringe filters (Thermo Fischer Scientific) and stored at -80˚C for a maximum of 2 weeks. Freshly thawed samples (0.02mL) were analyzed using the HPLC Model 1525 system (Waters) equipped with a refractive index detector and a Zorbax SB-C18 column (4.6x 250mm) (Agilent Technologies).

Elution was done with 0.005N sulfuric acid at a constant flow rate of 0.6ml/min. Standard curves were constructed using lactic, acetic, propionic, and butyric acids as standards (R = 0.95).

3.3.11 Statistical analysis

Statistical analyses were performed using XLSTAT software (Addinsoft, New York, NY, USA) in Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$).

3.4 Results and Discussion

3.4.1 Characterization of cocoa bean shells and its cell wall material

The chemical composition of fermented (CCN), dried (CCN) and roasted (CCN and NAC) cocoa bean shells was determined based on AOAC standard protocols. Overall, the composition of CBSs comprised of 12.7-19.7% protein, 0.3-7.6% fat, 6.1-7.1% ash, 65.5-80.7% carbohydrates, and 50.1- 61.4% fibers (Table 3.1). These findings are within the ranges reported in the literature (Fakhlaei et al., 2019; Agus et al., 2018; Martínez et al., 2012; Mellinas et al., 2020; Okiyama et al.,2017), which found CBS to comprise of 6.2-18.6% protein, 2.02-6.46% fat, 6-11.67% ash, 17.8-55.85% carbohydrates, and 13.86-60.6% fibers . However, it should be noted that the broad range in the composition is due to variations in their origin, variety, processing condition, and assessment method.

The protein content has shown a noticeable increase ($p < 0.05$) from its fermented (13%, CCN) to dried (18.9%, CCN) states, followed by a decrease in its roasted state (12.8%, CCN). According to Agus, Mohamad, and Hussain (2018), the observed increase in the protein content from fermented to dried conditions is attributed to the increased microbial mass which produce nitrogen during growth". The decrease in protein content, following roasting, may be a result of the elevated temperatures involved in the roasting process (120-150˚C). This mediates the Maillard reaction, which involves the interaction between the carbonyl group of a reducing sugar and a free amino acid from protein, thereby decreasing the protein content (Adeyeye et al., 2010; Agus et al., 2018). Interestingly, Okiyama et al. (2017), reported that only 1% of the protein content in cocoa bean shells exists in its free form. The remainder is strongly bound to oxidized polyphenols, further converting into polyphenol-quinones. Similarly, the fat content increases ($p < 0.05$) with the processing of cocoa beans from fermentation to drying and roasting. The variation in the fat content

can be explained by the subjection of elevated temperatures involved in the drying and roasting processes (70-150˚C), further inducing the migration of cocoa butter, from its cotyledon layer to the cocoa bean shells (El-Saied et al., 1981; Younes et al., 2022). The fatty acid composition in CBS has been reported to predominantly contain palmitic, oleic and linoleic acids (El-Saied et al., 1981).The ash content ($p < 0.05$) is relatively stable amongst the four samples, fermented, dried, and roasted CBS. Overall, cocoa bean shells have shown to contain higher ash contents than other fruit by-products, namely passion fruit seeds (1.34%), and apple pomace (0.5%) (Chau & Huang, 2004; Martínez et al., 2012; Sudha et al., 2007).

On average, the fiber content in cocoa bean shells accounts for around 50% of the entire material. Our findings are consistent with the literature (Martin-Cabrejas et al. 1994; Grillo et. al 2019), in which all four CBS samples contain 50.1-64.1% fiber. Using the sequential isolation approach, involving the use of chloroform and methanol, ethanol, followed by phenol, acetic acid, and water (2:1:1), to retrieve the cell wall material, we observed an overall cell wall material yield between 40.6 - 48.1%; higher than previously reported by Redgwell et. al (2003). The overall ratio between neutral sugar and uronic acid between the fermented and dried shells (0.82 - 0.97) compared to the roasted shells (1.4-1.8), indicate a higher proportion of neutral sugars upon subjection to roasting. Xiao et al. (2018) observed a reduction in the cell wall polysaccharide composition of apple chips, specifically fucose, rhamnose, arabinose, and xylose, with increased drying temperatures (60-90˚C); further supporting that temperature conditions significantly influence the neutral sugar and uronic acid profile of foodstuff.

Based on the findings, as determined by anionic exchange chromatography (Table 3.1), all four sets of CBS CWM, regardless of their processing conditions, were predominantly rich in pectic polysaccharides (80.6-86%), comprised of uronic acids (35.1-50.5 %), galactose (22.7-29.2%), arabinose (13.6-17.2%), and rhamnose (5.1-6.9%) residues. The remainder consists of hemi-cellulosic polysaccharides (13.9-19.4%), comprised of glucose (3.3-7.1%), fucose (2.4-3.6%), xylose and mannose (0.9-1.9%) residues. These are consistent with the findings of Redgwell et al. (2003) and Grillo et al. (2019) which suggested that CBS contain a greater proportion of pectic and non-cellulosic cell wall polysaccharides than cellulosic polysaccharides. Upon extracting the cell wall material, the protein content (12.9-16.6%) remained unchanged upon extraction, likely

indicating the complexes between dietary fiber and protein from the Maillard reaction, especially in the roasted samples (Redgwell et al. 2003).

Table 3.1. Proximate composition of Cocoa Bean Shells (CBS) and saccharide profile of their corresponding Cell Wall Materials (CWM)

Proximate Analyses of CBS				
Components	Content (%, w/dry w)			
	Fermented CCN51	Dried CCN 51	Roasted CCN 51	Roasted NAC
Protein	13±2[b]	18.9±1.02[a]	12.8±1.1[b]	19.8±1.9[a]
Fat	0.3±0.04[c]	2.3±0.4[b]	2.1±0.4[b]	7.6±0.4[a]
Ash	6.1±0.07[b]	7.1±0.13[a]	6.5±0.5[ab]	7.1±0.3[a]
CHO	80.6±2.1[a]	71.7±1.6[b]	78.7±1.1[a]	65.5±1.6[c]
Fiber	61.4±8.3[a]	50.2±7.3[a]	50.1±0.3[a]	54.3±2.8[a]
Polyphenol content*	0.52±0.08 [a]	0.63±0.13 [a]	0.63±0.05 [a]	0.44±0.02 [a]
Composition of CWM of CBS				
CWM yield	40.6±12.6	41.6±2.6	49.4±0.04	48.1±3.2
Polyphenol content*	0.05±0.002[c]	0.14±0.01[a]	0.1±0.01[b]	0.11±0.01[b]
Protein content*	16.6±0.8[a]	17.6±1.6[a]	17.3±1.4[a]	12.9±1.1 [a]
Neutral Sugar content*	31.4±5.3[c]	29.9±7.6[bc]	41.9±5.8[a]	40.9±8.0[ab]
Uronic acid content*	32.5±7.6 [a]	36.5±14.7 [a]	22.8±2 [a]	29.8±2.7[a]
Monosaccharide profile (%, g/100g rel. proportion)				
Fucose	2.6±0.03 [a]	3.6±2.4[a]	2.4±0.05[a]	2.8±1.6[a]
Rhamnose	6.2±0.9[ab]	5.1±1.1[b]	6.9±0.4[ab]	6.4±0.1[a]
Arabinose	13.8±2.1[a]	13.6±1.26[a]	17.2±1.1[a]	14.2±0.9[a]
Galactose	22.7±2.5[a]	23.7±3.9[a]	29.2±1.4[a]	27.2±0.7[a]
Glucose	3.3±0.1[b]	5.2±0.7[b]	7.1±0.8[a]	5.9±0.3[a]
Xylose+ Mannose	0.9±0.3[a]	1.3±0.12[a]	1.9±0.15[a]	1.5±0.2[a]
Uronic acids	50.5±5.9[a]	47.5±1.1[a]	35.1±2.0[a]	42.1±2.2[a]
Hemi-cellulosic PS*	13.9±0.8[a]	19.4±6.1[a]	17.8±1.9[a]	17.5±2.9[a]
Pectic PS*	86.0±0.8[a]	80.6±6.1[a]	82.2±1.9[a]	82.5±2.9[a]
Polyphenol Profile of CBS and CWM (ng/Kg CBS / CWM CBS)				
Gallic acid	0.2±0.01[ab]/0.3±0.03[a]	0.2±0.06[a]/0.2±0.02[ab]	0.1±0.02[bc]/n.d.**[c]	n.d.**[c]/n.d.**[c]
Protocatechuic acid	7.2±1.9[c]/9.3±0.03[c]	23.2±3.9[b]/34.6±5.2[a]	7.0±0.5[cd] /5.3±0.3[cd]	1.3±0.1[d]/7.5±1.1[c]
Catechin	4.1±0.4[cd] /6.4±0.1[cd]	24.3±6.7[a]/20.2±0.9[ab]	0.3±0.06[d]/0.4±0.04[d]	13.0±1.3[bc]/2.1±0.4[d]
Epicatechin	37.4±0.1[bc]/65.8±1.7[ab]	122.8±7.5[a]/124.4±26.9[a]	6.1±0.5[bc]/10.1±0.7[bc]	0.9±0.1[c]/19.8±2.6[bc]
Caffeic acid	0.1±0.03[b]/0.4±0.1[b]	0.3±0.1[b]/0.4±0.1[b]	0.1±0.003[b]/9.6±0.9[b]	1.1±0.1[a]/0.3±0.1[b]
p-Coumaric acid	0.05±0.01[b]/ 0.05±0.0005[b]	0.1±0.01 [ab] /0.1±0.02[ab]	n.d.**[b]/ n.d.**[b]	0.1±0.01[ab]/0.2±0.04[a]
Ferulic acid	0.2±0.01[a]/0.2±0.04[a]	0.1±0.04[a]/0.05±0.0001[a]	0.4[a]/0.3±0.2[a]	0.2±0.1[a]/1.2±0.1[a]
Quercetin	2.1±0.1[bc]/4.6±0.5[a]	3.1±0.3[ab]/6.0±1.1[a]	0.4±0.04[d]/0.5±0.03[d]	0.8±0.1[cd]/n.d.** [d]
Caffeine	261.6±1.2[a]/257.6±7.9[a]	139.4±3.9[a]/169.5±13.1[a]	4.5±0.2[a]/19.3±0.4[a]	17.7±0.1[a]/22.3±5.0[a]

Values reported as average ± standard deviation
*Units expressed as % weight per weight CWM
**not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$

3.4.2 Polyphenolic profile of cocoa bean shells and its cell wall material

Polyphenolic compounds are naturally present within the cotyledons of the cocoa seeds; however, compounds can migrate towards the shell through diffusion, further enriching the by-product (Andres-Lacueva et al., 2008; Forsyth et al., 1958; Okiyama et al., 2017). The CBS total polyphenol content comprised between 0.44-0.63% (Table 3.1) similar to the content found in cocoa beans (Hernández-Hernández et al., 2018; Rojo-Poveda et al., 2020). In addition, after isolating its CWM, the total polyphenol content was obtained around 0.05-0.14%; directly influenced by the use of solvents in the sequential isolation process used to retrieve the CWM. In general, the polyphenol content is expected to increase with further processing. Our findings showed no significant differences in the polyphenol content of CBS at different processing conditions of fermentation, drying and roasting. However, overall, the roasted CBS samples (NAC and CCN) hold a greater total polyphenol content as compared to their fermented and dried states. Alternatively, dried CBS polyphenols may be more tightly bound to cell wall materials than fermented and roasted CBS. Subjection of heat from the drying and roasting process can mediate not only the Maillard reaction, but also promote condensation and polymerization. Given our findings showed a great amount of crude fiber (non-digestible carbohydrates and lignin), one may suggest that lignin is pyrolyzed slowly with the onset of roasting conditions allowing for the release of polyphenols entrapped within its network, otherwise known as the polyphenol complex (Agus et al., 2018; Brebu & Vasile, 2010; Hečimović et al., 2011).

Based on our findings, as determined by LC-MS ($p < 0.05$) in Table 3.1, the most abundant class of polyphenols found in CBS and its CWM are the flavan-3-ols, which include epicatechin (122.8-0.9 ng/kg material or extract) and catechin (24.3-0.3 ng/kg material or extract; this is consistent with the literature (Miller et al., 2006; Payne et al., 2010). Other polyphenols were detected in both CBS and the corresponding CWM, which include flavonoids, such as quercetin, as well as phenolic acids, such as gallic, protocatechuic, p-coumaric, ferulic, and caffeic acids. Nevertheless, cocoa and its by-products are known for its richness in methylxanthines, such as theobromine and caffeine (Okiyama et al., 2017). Our findings detected considerable amounts of caffeine among all samples of CBS and its CWM; with the highest amounts found among the fermented and dried types (139.4-261.6 ng/kg material). Interesting, the caffeine content was significantly lower after the roasting process. According to Hečimovic et. al (2011), caffeine and

other polyphenols were found in greater amounts in their raw and lightly roasted forms (160˚C); further indicating the effect of processing conditions on the polyphenolic profile.

3.4.3 Extraction of cell wall polysaccharides and their characterisation

To extract and characterize the CBS cell wall polysaccharides, alkaline extraction was performed using two concentrations of KOH, 0.5M and 4M. The polysaccharide yield (Table 3.2) ranged between 23.7-35.4 % (w/w) using 0.5M KOH depending on the starting materials (fermented, dried, roasted) and variety. Interestingly, as the alkaline solution increased, the roasted samples (CCN and NAC) saw an increase in their polysaccharide yields (32.9-53.6%, w/w). Such findings are consistent with the findings assessed on pectic polysaccharides derived from potato peel and cranberry pomace (Khodaei & Karboune, 2013; Spadoni Andreani et al., 2021). In addition, the results show the proportion of neutral sugars decreased as the alkaline solution was increased, whereas the molar proportion of uronic acid remained relatively unchanged. As a result, the uronic/neutral sugar ratio increased, possibly indicating the onset of debranching or defragmentation of neutral sugar regions.

The monosaccharide composition (Table 3.2) reveals the main sugars in the extracted CBS cell wall polysaccharides were uronic acids (42.9-58.2%), arabinose (7.8-16.4%), and galactose (8.1-22.4%) for the 0.5M alkaline treatment, signifying the presence of arabinan, galactan and rhamnogalacturonan. As for the 4M alkaline treatment, the main sugars were uronic acids (56.4-66.7%), galactose (9.9-15.9%), xylose and mannose (2.6-15.9%), indicating a higher proportion of hemicellulose as compared to the latter. We can estimate the proportion of pectic polysaccharides by determining the pectic neutral sugars content, calculated as the ratio of rhamnose, arabinose and galactose to total neutral sugars. The results suggest the polysaccharide profile of CBS alkaline extracts are predominantly pectic polysaccharides (56.4-88.8%) with the remainder being hemi-cellulosic polysaccharides, as signified by the presence of xylose, mannose, and glucose. Using a 4M KOH extraction treatment, Redgwell et al. (2003) reported the presence of a mixture of hemicelluloses and pectic polysaccharides based on the levels of uronic acid (26.2%) xylose (23.5%), glucose (21.8%), and mannose (9.9%). In addition, the study reported rhamnogalacturonan as the main pectic polysaccharide, as well as three hemi-cellulosic polysaccharides, namely galactoglucomannan, xylogalacturonan, and glucoarabinoxylan (Redgwell et al., 2003). These findings are supported by the fact that pectic neutral sugars are of a

lower proportion compared to 0.5M alkaline extracts, comprising of 55.6-76.1% (Table 3.2). Considering all samples (using both treatments) showed high proportions of pectic polysaccharides, the ratio of arabinose and galactose to rhamnose provides an estimate of the level of branching of rhamnogalacturonan I. As seen in Table 3.2, the 4M extracts showed the highest ratios, indicating the presence of more abundant arabinan and galactan side chains. Furthermore, by increasing the alkaline solution concentration, the degree of branching increased by two to five-folds, depending on the processing condition. Based on the rhamnose molar proportions with the 0.5M alkaline treatment, it can be assumed that rhamnogalacturonan I is more abundant in Fermented and Roasted CCN 51 samples. As the alkaline solution increased, the molar proportion had decreased to 2.3-3.7%. All in all, the NAC variety attained a higher polysaccharide yield (35.4-53.6%) among the two alkaline treatments compared to the CCN variety (19.5-32.9%). In addition, the polysaccharide profiles of both varieties showed similarities, with the exception that the CCN variety expressed a richer profile of galactose (10.7-15.9% μM) and xylose and mannose (4.2-10.6% μM) while the NAC variety expressed a richer content of arabinose (13.4% μM).

Roasted samples (NAC and CCN varieties) were analyzed for their molecular weight (MW) distribution. The results showed a significant impact of increasing the alkaline solution from 0.5 to 4M KOH. In the 0.5M extracts, three main MW populations were found at 3.7, 12, and 130, kDa (NAC variety) and 4.5, 9.1, and 32.5 kDa (CCN variety) (Figure 3.1). Increasing the alkaline solution resulted in a shift from high to low molecular weight fractions, where the 4M extract main populations were found at 24, and 6.3 kDa (NAC variety) and 11, and 3.2 kDa (CCN variety) (Figure 3.1). This is a result of increasing the alkaline solution which allowed for a higher degree of debranching or defragmentation of the pectic polysaccharides present in CBS. As per the author's knowledge, no studies have been reported on the MW populations of CBS. However, Redgwell and Hansen (2000) previously reported that a high MW population (2000 kDa) was detected in cell wall polysaccharides extracted from unfermented and fermented cocoa beans. The MW populations of cranberry pomace (1.5-1500 kDa), potato peel protein (1-600 kDa), and olives (260-400 kDa) have also been reported, showing a profile mixture of both low and high MW fractions (Jiménez et al., 1994; Khodaei & Karboune, 2013; Spadoni Andreani et al., 2021). The presence of low MW fractions may be due to the disassociation of non-covalently bound oligosaccharides during the dialysis purification steps (Spadoni Andreani et al., 2021).

Table 3.2. Alkaline extraction of polysaccharides from the cell wall material (CWM) of cocoa bean shells (CBS)

	Fermented CCN51	Dried CCN 51	Roasted CCN 51	Roasted NAC
0.5M KOH				
PS yield (%, w/w)	23.7±0.01	24.4±0.05	29.1±0.4	35.4±4.3
Neutral sugar content*	27.1±1.9[a]	25.5±3.2[a]	20.6±0.7[b]	13.5±2.3[b]
Uronic acid content*	54.5±9.3[a]	55.1±0.9[a]	58.3±5.2[a]	49.8±3.02[a]
Uronic/Neutral Sugar ratio	2.01	2.16	2.83	3.69
Monosaccharide Composition (%, g/100g - relative proportion)				
Fucose	0.9±0.2[b]	0.7±0.07[b]	1.8±0.02[a]	1.8±0.1[a]
Rhamnose	11.9±0.6[a]	9.9±0.8[a]	12.4±0.3[a]	6.8±1.1[b]
Arabinose	16.4±0.9[a]	13.9±0.8[a]	8.6±0.01[b]	7.8±0.6[b]
Galactose	22.4±0.9[a]	18.5±0.4[b]	8.1±0.2[c]	17.5±1.3[b]
Glucose	1.1±0.1[b]	3.7±1.1[a]	5.2±0.5[a]	2.9±0.1[ab]
Xylose+ Mannose	4.4±0.9[b]	8.5±1.9[ab]	12.6±2.0[c]	5.0±0.4[b]
Uronic acids	42.9±2.5[b]	44.9±2.6[b]	51.4±3.1[ab]	58.2±2.8[b]
Pectic neutral sugars*	88.8±1.7[a]	76.8±4.2[b]	59.9±2.8[c]	76.8±1.8[b]
Branching	3.3	3.3	1.3	3.7
4M KOH				
PS yield (%, w/w)	19.5±0.05	21.1±0.07	32.9±0.08	53.6±8.1
Neutral sugar content*	13.9±2.1[a]	12.3±0.7[a]	11.4±1.4[a]	10.7±0.2[a]
Uronic acid content*	47.9±5.1[a]	60.3±9.3[a]	60.8±3.6[a]	51.6±1.4[a]
Uronic/Neutral Sugar ratio	3.44	4.89	5.31	4.84
Monosaccharide Composition (%, g/100g - relative proportion)				
Fucose	1.2±0.2[b]	1.8±0.1[b]	4.3±0.1[a]	3.4±0.4[a]
Rhamnose	3.5±0.1[ab]	2.3±0.4[b]	3.7±0.3[a]	3.7±0.5[a]
Arabinose	5.9±0.7[c]	4.2±0.3[c]	10.6±0.6[b]	13.4±0.6[a]
Galactose	14.9±1.8[a]	15.9±0.4[a]	10.7±0.4[b]	9.9±0.2[b]
Glucose	2.2±0.7[a]	4.2±2.2[a]	1.5±0.1[a]	2.03±0.04[a]
Xylose+ Mannose	15.9±0.6[a]	7.1±1.8[b]	2.6±0.04[c]	3.03±0.2[c]
Uronic acids	56.4±1.0[a]	64.6±4.9[a]	66.7±1.4[a]	64.5±1.02[a]
Pectic neutral sugars*	55.6±4.5[b]	63.6±5.9[ab]	74.85±0.62[a]	76.1±1.3[a]
Branching	6	8.9	5.8	6.3

Values represented as averages ± standard deviation
Pectic neutral sugars calculated as (rhamnose + arabinose + galactose) x 100 / total neutral sugars
Branching calculated as (rhamnose + arabinose / galactose)
*Units expressed as % gram per 100 g sample
[a] Within the same row, means with different letters are significantly different at $P < 0.05$.

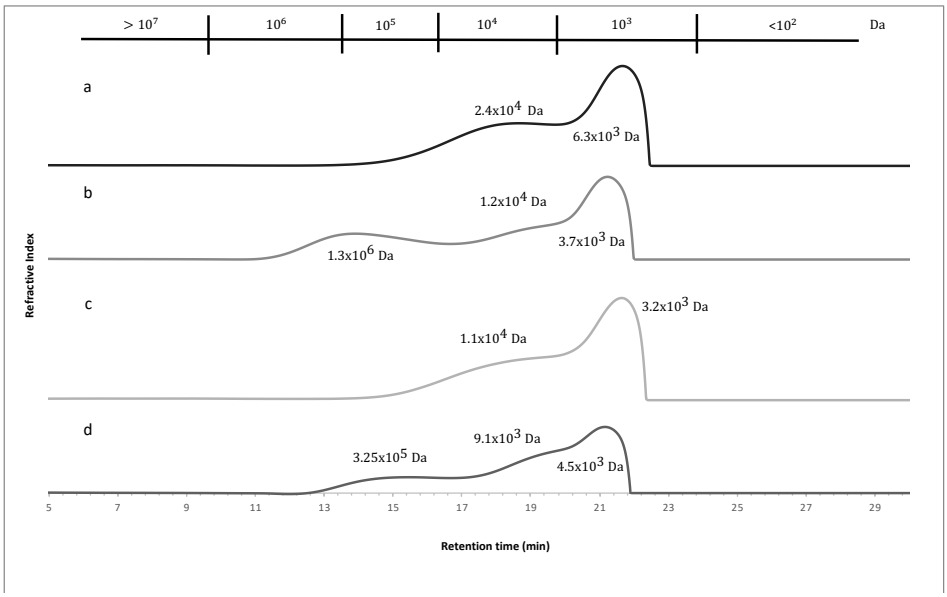

Figure 3.1. Molecular weight distribution of CWP alkaline extracts. Letters are denoted by the different varieties (NAC, CCN) subjected to different alkaline solution treatments (0.5M, 4M KOH) A: NAC 4M, B: NAC 0.5M, C: CCN 4M, D: CCN 0.5M, respectively.

3.4.4 Prebiotic activity

The prebiotic activity scores of CBS polysaccharides, rhamnogalacturonan, and inulin, a known prebiotic polysaccharide (Shoaib et al., 2016), were compared using them as carbon sources for the anaerobic growth of two probiotic bacteria strains, *L. rhamnosus GG* and *B. longum.* Both strains were handled and grown in anaerobic conditions. While *L. rhamnosus GG* is oxygen-tolerant, the anaerobic conditions were selected to simulate the intestinal environment more accurately. In general, *Lactobacillus* species show a significant heterogeneity in their abilities to ferment carbohydrates (Boguta et al., 2014; Broadbent et al., 2012; Kim et al., 2010; Spadoni Andreani et al., 2021). Figure 3.2 shows that CBS polysaccharides (NAC and CCN varieties) showed a prebiotic activity score similar to that of inulin and rhamnogalacturonan for *L. rhamnosus GG* (Figure 3.2). At 24hr of fermentation, the prebiotic activity scores of CBS polysaccharides were significantly higher ($P \leq 0.05$) than that of inulin and rhamnogalacturonan. For *B. longum* (Figure 3.3), CBS polysaccharides showed statistically significant ($P \leq 0.05$) prebiotic activity scores at 48hr of fermentation than that of inulin and rhamnogalacturonan; however, the prebiotic activity scores decreased at the 72hr fermentation period. Therefore, it appears that CBS polysaccharides, primarily rich in pectin, galactan, and arabinan, effectively stimulate the growth of both strains. Dietary fibers, rich in pectin, undergo bacterial fermentation in the ileum and colon, leading to acidification of the colonic contents as well as the production of short-chain fatty acids (Ashaolu et al., 2021; Koropatkin et al., 2012; Slavin, 2013). Studies reported effective growth of *L. rhamnosus GG* with pectic polysaccharides and galacto-oligosaccharides derived from pearl millet fibre, and *F. kuhistanic* leaves (Farooq et al., 2017; Islamova et al., 2017) and *B. longum* with pectic polysaccharides derived from cranberry pomace, pumpkin peel, and citrus pectin (Bianchi et al., 2018; Jun et al., 2006; Spadoni Andreani et al., 2021).

Interestingly, gene expression analysis of *B. longum* indicate the presence of over 40 glycosyl hydrolases, which include 2 xylanases, 9 arabinosidases, 2 α-galactosidases and more, whose predicted substrates cover a wide range of di, tri- and higher order oligo-saccharides, indicating the strain's high affinity for oligosaccharides with a degree of polymerization less than 8 (Schell et al., 2002).

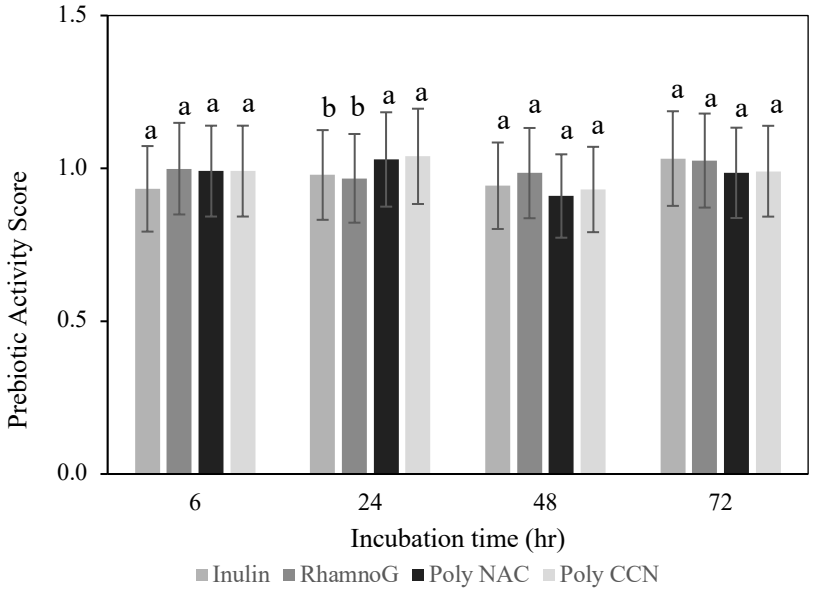

Figure 3.2. Prebiotic activity scores of *Lactobacillus rhamnosus* GG (R0343 AR) on polysaccharide extracts, inulin and rhamnogalacturonan, measured at 6,24, 48, 72 and 96h of incubation. Values represented as an average ± standard deviation. Poly NAC: polysaccharide NAC variety, Poly CCN: polysaccharide CCN variety, RhamnoG: Rhamnogalacturonan, respectively. For each incubation time, bars with different letters represent scores significantly different at $P < 0.05$.

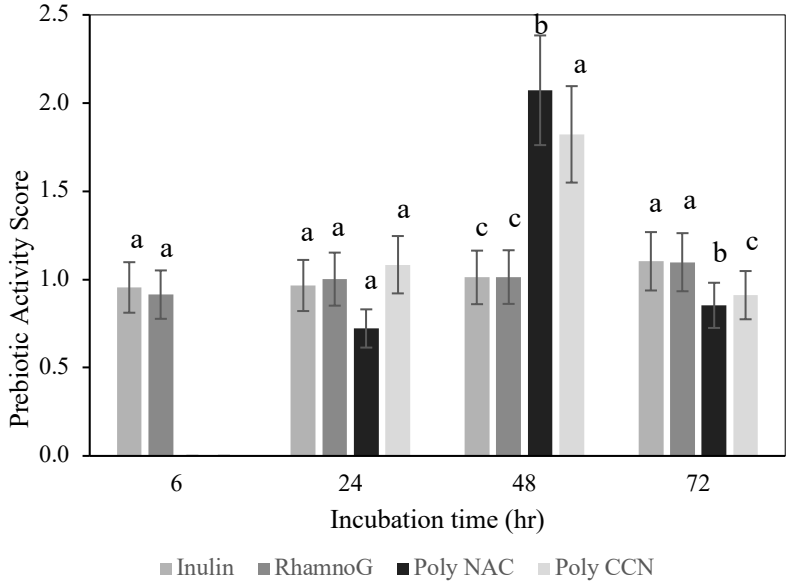

Figure 3.3. Prebiotic activity scores of *Bifidobacterium longum* (ATCC® 15707) on polysaccharide extracts, inulin and rhamnogalacturonan, measured at 6, 24, 48, 72 and 96h of incubation. Values represented as an average ± standard deviation. Poly NAC: polysaccharide NAC variety, Poly CCN: polysaccharide CCN variety, RhamnoG: Rhamnogalacturonan, respectively. For each incubation time, bars with different letters represent scores significantly different at $P < 0.05$.

Regarding, *L. rhamnosus GG*, this strain has shown the highest affinity towards fucosylated oligo- and polysaccharides as it has been found to express the fucose permease, fucose isomerase, fucolose kinase, fucose mutarotose, and fucolose-1-phosphate aldolase, promoting the fucose catabolic pathway to produce 1,2-propanediol or lactate (Becerra et al., 2015). According to one study assessing the probiotic activities of *B. longum* and *L. rhamnosus GG* using qPCR showed that both strains indirectly utilize pectic polysaccharides as a source of prebiotics. The polysaccharides undergo metabolization prior by other gut microbiota, namely *E. coli* H, resulting in metabolic end products that are then metabolized by *B. longum* and *L. rhamnosus GG* (Li et al., 2020). In addition, studies suggest the degree of methylation of PS may affect the fermentation rate. Based on FTIR and NMR analysis, CBS pectic fractions have been characterized as highly acetylated low methyoxyl homogalacturonan and type I rhamnogalacturonan with galactan or arabinogalactan side chains (Vriesmann et al., 2011). In addition, Larsen et al. (2019) reports that *Lactobacillus* strains showed higher fermentation rates with polysaccharides with a low degree of methylation. This may explain the faster fermentation rates observed with *L. rhamnosus GG* with CBS polysaccharides. Studies also found conflicting correlations between the degree of esterification and bacterial taxa in stimulating their growth (Chengxiao et al., 2021; Ferreira-Lazarte et al., 2019; Larsen et al., 2019). Further investigation on the structural linkage, degree of methylation and degree of esterification is needed to assess whether they could result in the differential promotion of the growth of selected bacteria.

SCFA are produced upon the fermentation of dietary fiber by gut microbiota (Lin et al., 2016; Spadoni Andreani et al., 2021). Prebiotic production of SCFA can be used to complement *L. rhamnosus GG* and *B. longum* fermentation of CBS polysaccharides. Table 3.3 and Table 3.4 show the concentration of four SCFA after 24 and 48 hr of fermentation of *L. rhamnosus GG* and *B. longum* with glucose, CBS polysaccharides (NAC and CCN varieties) and positive controls, inulin and rhamnogalacturonan. In the culture of *L. rhamnosus GG* (Table 3.3), the main increase was seen in the lactic acid followed by acetic acid. Although unabundant, the significance ($P < 0.05$) was observed with the production of propionic acid for CBS polysaccharides from both varieties (NAC and CCN) at 48hr of fermentation. *L. rhamnosus GG* is known for its ability to produce lactic and propionic acid during fermentation, which can promote anti-inflammatory and anti-microbial properties in our gastrointestinal tract (Al-Lahham et al., 2010; Markowiak-Kopeć & Śliżewska, 2020). The production of butyric acid was prevalent in glucose, inulin, and

rhamnogalacturonan, indicating that CBS polysaccharides do not favor the production of butyrate in the *L. rhamnosus* fermentation pathway. However, the presence of butyric acid can be attributed to the metabolization of lactate, further explaining the decrease in the lactate concentration with increased incubation time (Cummings et al., 1987; Karboune et al., 2022).

Similarly, to the SCFA profile of *L. rhamnosus GG,* the culture of *B. longum* was characterized by the greatest increase in lactic acid, followed by acetic acid predominantly after 48hr of fermentation; with the except of CCN polysaccharides. The acetic acid content of the medium relatively remained unchanged for the polysaccharides. The lactic acid concentrations produced by polysaccharides were higher at 24hr of fermentation than that of glucose, inulin, and rhamnogalacturonan. At 48hr of fermentation, the lactic acid concentrations decreased and were surpassed by their positive controls. However, propionic, and butyric acid concentrations either decreased or were not detected after 24 hr of *B. longum* fermentation. Overall, the total SCFA profile of polysaccharides were higher at 24 hr than 48 hr, indicating possible SCFA degradation. Studies suggest that *Bifidobacterium spp.*, specifically. *B. longum,* are producers of lactate, acetate and propionate in the human intestinal tract (Liu et al., 2020; Markowiak-Kopeć & Śliżewska, 2020). However, only the production of lactic and acetic acids was observed with CBS polysaccharides. In addition, when carbohydrates are in excess, *Bifidobacterium* utilize the fermentation pathway, to produce two molecules of acetate with 1 molecule of lactate (LeBlanc et al., 2017; Macfarlane & Macfarlane, 2003; Markowiak-Kopeć & Śliżewska, 2020). However, our findings suggest a higher proportion of lactic than acetic acid.

Overall, comparing the total SCFA released at 48hr revealed that CBS polysaccharides positively contributed towards effective growth of both strains, producing high proportions of lactic and acetic acid, compared to the growth initiated by glucose, inulin and rhamnogalacturonan. New research suggests that multi-strain probiotics can contribute to more enhanced health benefits compared to single-strain probiotics (Kwoji et al., 2021; Puvanasundram et al., 2021). Further investigation can assess the effect of multi-culturing, using *L. rhamnosus GG* and *B. longum,* to determine whether a synergistic approach can promote higher prebiotic activity scores and SCFA production at faster fermentation rates.

Table 3.3. Concentration (10^{-3}mol/L) of short chain fatty acids released in the 0.5M alkaline treated cell-wall polysaccharide samples (NAC, CCN) as compared to glucose, inulin and rhamnogalacturonan standards which were obtained from *Lactobacillus rhamnosus* GG (R0343 AR) fermentation using different carbon sources. Values determined as difference with concentrations detected immediately after inoculation.

	Time (h)	Glucose	Inulin	RhamnoG	NAC	CCN
Lactic acid	24	313.0±4.0[a]	212.5±15.5[a]	508.8±10.3[a]	427.6±62.0[a]	343.0±85.9[a]
	48	386.0±16.2 [a]	315.7±14.8[a]	293.5±26.0[a]	354.7±41.1[a]	393.0±44.2[a]
Acetic acid	24	58.1±8.4[a]	46.4*[a]	112.2±nd*[a]	42.5±0.9[a]	71.9*[a]
	48	111.7*[a]	66.4*[a]	39.0±8.5[a]	35.3±3.0[a]	96.8*[a]
Propionic acid	24	4.7*[a]	1.0±0.04[a]	1.8±0.03[a]	1.1±0.1[a]	1.2*[a]
	48	0.7*[b]	0.4*[b]	0.6±0.06[b]	1.1±0.2[ab]	1.9±0.3[a]
Butyric acid	24	1.8*[a]	0.3*[a]	0.4±0.04 [a]	-	-
	48	-	-	-	-	-
Total	24	377.6	260.2	623.2	471.2	416.1
	48	498.4	382.5	333.1	391.1	491.7

Values represented as average ± standard deviation
RhamnoG; Rhamnogalacturonan
* Standard deviation is zero or not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$.

Table 3.4. Concentration (10^{-3}mol/L) of short chain fatty acids released in the 0.5M alkaline treated cell-wall polysaccharide samples (NAC, CCN) as compared to glucose, inulin and rhamnogalacturonan standards which were obtained from *Bifidobacterium longum* (ATCC 15707®) fermentation using different carbon sources. Values determined as difference with concentrations detected immediately after inoculation.

	Time (h)	Glucose	Inulin	RhamnoG	NAC	CCN
Lactic acid	24	31.3±5.1[a]	56.3*[a]	178.0* [a]	169.0±2.4 [a]	273.2±66.4 [a]
	48	404.4±96.6[a]	281.3±1.8 [a]	327.9±2.1 [a]	299.8±18.3[a]	216.2±1.5 [a]
Acetic acid	24	44.4±3.9[a]	43.7±10.3[a]	52.6*[a]	39.5*[a]	53.9*[a]
	48	230.1±11.3[a]	55.3±3.6[b]	50.5±1.5[b]	28.6±2.9[b]	53.3*[b]
Propionic acid	24	1.9±0.4[a]	1.3±0.2[a]	2.6±0.4[a]	1.9±0.2[a]	1.7*[a]
	48	0.6*[a]	0.5±0.1[a]	0.5±0.03[a]	0.3* [a]	2.0* [a]
Butyric acid	24	0.3*	0.3*	-	-	-
	48	-	-	-	-	-
Total	24	77.9	101.6	233.3	210.4	328.8
	48	635.1	337.1	378.9	328.7	271.5

Values represented as average ± standard deviation
RhamnoG; Rhamnogalacturonan
* Standard deviation is zero or not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$.

3.5 Conclusion

The chemical composition of CBS (fermented, dried, and roasted) is predominantly carbohydrates (65.5-80.7%), of which around 50% is dietary fiber. CBS was also found to contain a great source of protein (12.7-19.7%), ash (6.1-7.1%). As expected, the fat content (0.3-7.6%) was less the amounts reported for cocoa beans; however, fat migration from the bean to the outer shell is induced upon subjection to high temperatures involved in the drying and roasting processes. Upon isolation of the CBS CWM, the polysaccharide profile was found to contain both pectic (80.6-86%) and hemi-cellulosic (13.9-19.4%) polysaccharides. Furthermore, the protein content of CWM remained unchanged relative to their raw states, indicating possible interactions with dietary fiber. The cell wall polysaccharides were isolated from CWM using two concentrations of KOH, 0.5M and 4M. The less concentrated extract, although had a lower recovery yield, was favored as it provided a better neutral sugar and uronic acid profile. By increasing the alkaline concentration, defragmentation and debranching occurred. Overall, the nature of the CBS cell wall composition was found to be predominantly rich in pectic polysaccharides, such as rhamnogalacturonan. When tested for the promotion of the growth of selected probiotic strains, CBS cell wall polysaccharides performed similarly or more than inulin and rhamnogalacturonan based on the prebiotic activity scores. The short chain fatty acid profiles were characterized by high amounts of lactic acid, followed by acetic and propionic acid. Further investigation of the use of these polysaccharides using other single-strain and possibly multi-strain probiotics is needed to assess the effect of CBS polysaccharides on the selectivity of colonic fermentation.

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CONNECTING STATEMENT II

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In chapter III, the composition of cocoa bean shells was analyzed, and the extraction of their cell wall polysaccharides was investigated using alkaline treatments. The obtained extracts were characterized by the presence of monosaccharide residues associated to both pectic and hemicellulosic polysaccharides, in variable proportions. The prebiotic properties of polysaccharides and the short chain fatty acid composition were determined, showing optimal prebiotic activity. The enzymatic generation of oligosaccharides has been assessed on other valorized by-products; however, those of CBS have received little attention. CBS polysaccharides were hydrolyzed into respective oligosaccharides of varying degrees of polymerization using a selection of multi-enzymatic products based on their galactanase activity. The oligosaccharide extracts were characterized in terms of their degrees of polymerization, prebiotic activity, and short chain fatty acid production. Furthermore, the feruloylation of oligo- and polysaccharide extracts from cocoa bean shells was assessed and characterized in terms of their feruloylation efficiency as well as their antioxidant capacities, based on three antioxidant assays.

The results of this study have been submitted for publication.

CHAPTER IV. ENZYMATIC GENERATION OF OLIGOSACCHARIDES FROM COCOA BEAN SHELLS USING MULTI-ENZYMATIC PRODUCTS AND THE FERULOYLATION OF OLIGO/POLYSACCHARIDES

4.1 Abstract

Cell wall polysaccharides isolated from two varieties (NAC, CCN) of cocoa bean shell (CBS) consisted of high pectic regions, primarily rhamnogalacturonan, and hemicelluloses. Enzymatic hydrolysis of CBS polysaccharides was performed using bi-enzymatic (arabinase/galactanase) and multi-enzymatic systems. Viscozyme® L led to the highest yield (76.64%) of NAC-based oligosaccharides of DP 3-5 (42.04%, mol) and 6-10 (36.78%, mol). The hydrolysis of CCN-based polysaccharides by DepolTM 670L led to 20.72% yield, while generating a diverse complex of oligosaccharides of DP up to 10. When tested for the promotion of the growth of selected probiotic strains, Lactobacillus rhamnosus GG and Bifidobacterium longum, oligosaccharides performed similarly or more than inulin and rhamnogalacturonan based on the prebiotic activity scores. In addition, the production of lactic, acetic, butyric and traces of propionic were prominent. Free feruloyl esterases from rumen microorganism provided best feruloylation efficiencies with CBS polysaccharides (56.9-60.1%), while that from H. insolens provided best feruloylation efficiencies with the oligosaccharides (30.8-50.5%). The antioxidant activity of feruloylated polysaccharides were expressed using three assays, DPPH, ORAC, and ABTS. Feruloylated CBS polysaccharides prompted significantly notable increases in the scavenging and antioxidant activity compared to their native forms as well as ferulic and ascorbic acid. Overall, CBS oligo- and feruloylated polysaccharides show great potential for use as optimal functional food ingredients.

4.2 Introduction

Cocoa bean shells (CBS) contain an abundant source of dietary fibers, consisting of about 45% pectic, 35% cellulosic, and 20% hemi-cellulosic polysaccharides (Redgwell et al., 2003). As a result, CBS' cell wall composition makes it a desirable source for the extraction and generation of non-digestible oligosaccharides. Oligosaccharides are naturally present in foods in very low amounts and can be produced through chemical or enzymatic approaches (Du et al., 2011; Holck, Hjernø, et al., 2011; Lama-Muñoz et al., 2012; Martínez Sabajanes et al., 2012). For instance, the use of multi-enzymatic systems, which comprise of a mixture of selected glycosyl-hydrolases working in synergy, has been proven as a potential approach for the generation of non-digestible oligosaccharides from complex biomasses, including cranberry pomace, potato pulp, sugar beet pulp, orange peel (Spadoni Andreani & Karboune, 2020; Waglay & Karboune, 2017; Khodaei & Karboune 2016; Holck, Hjernø, et al., 2011; Martínez Sabajanes et al., 2012). Multi-enzymatic systems, namely Pectinex® Ultra Mash, Depol™ 670L, Pectinex® Ultra Clear, Viscozyme® L, Viscomyl® Flow, have been used for a variety of commercial applications, such as juice clarification, viscosity reduction, and maceration (Waglay & Karboune, 2017).

Furthermore, the enzymatically generated non-digestible oligosaccharides from cell wall polysaccharides have shown to function as great prebiotics. From previous findings in par 3.4.4 when tested for the promotion of the growth of *Lactobacillus rhamnosus GG* and *Bifidobacterium. longum* probiotic strains, CBS cell wall polysaccharides showed significant prebiotic activity and hence ability to produce short chain fatty acids, namely lactic and acetic acid. In addition, Du et al. (2011) found that non-digestible oligosaccharides, prompted both higher solubility and prebiotic responses compared to their polysaccharide fractions from pumpkin pulp. We hypothesize that CBS cell wall non-digestible oligosaccharides would also act as prebiotics, while favoring faster metabolization by probiotic strains and contributing to the sweetness ability and techno-functionalities.

Feruloylated oligo- and polysaccharides exist in nature within the plant cell wall; however, their recovery is limited due to their low yield and structural inconsistencies (Tamayo-Cabezas & Karboune, 2019). Alternatively, enzymatic acylation of ferulic acid with cell wall oligo- and polysaccharides has shown great potential, as it has exhibited higher bioconversion yields as well as the generation of reproducible structures with phenolated moieties (Couto et al., 2011; Spadoni

Andreani et al., 2021; Tamayo-Cabezas & Karboune, 2019). This reaction, otherwise, known as feruloylation, can be catalyzed by feruloyl esterases (E.C. 3.1.1.73), which are a subclass of carboxylic ester hydrolases known for their catalytic efficiency for the release of hydroxycinnamic acids from the plant cell wall (Spadoni Andreani et al., 2021; Tamayo-Cabezas & Karboune, 2019). Feruloylation of oligo- and polysaccharides or their isolation from cell wall materials have been previously explored with cranberry pomace (Spadoni Andreani et al., 2021), sugar beet (Zaidel et al., 2011), and cultured rice (Mastihubová et al., 2006). To the author's knowledge, feruloylation of CBS oligo- and polysaccharides has yet to be investigated . One approach to enhance the feruloylation efficiency is enzyme immobilization. This technique can enhance the enzymatic synthesis of feruloylated compounds, through optimizing enzyme activity in specific macroenvironments while also ensuring enzyme stability and reusability (He et al., 2015; Spadoni Andreani et al., 2021; Tamayo-Cabezas & Karboune, 2019; Thörn et al., 2011). Ultimately, feruloylated oligo- and polysaccharides have been idealized for their combined prebiotic and antioxidant activities, as ferulic acid serves as a functional and bioactive phenolic acid (Couto et al., 2010).

In the present study, the enzymatic generation of CBS oligosaccharides using bi-enzymatic and multi-enzymatic systems, was investigated. The generated oligosaccharides were structurally characterized, and their prebiotic activity was assessed and correlated with the production of short-chain fatty acids. To our knowledge, studies on the recovery of oligosaccharides from CBS using multi-enzymatic systems have not been reported so far. In addition, this study inquired the feruloylation efficiencies of CBS polysaccharides and their corresponding enzymatically generated oligosaccharides using feruloyl esterases from different microbial sources, rumen microorganism and *Humicola insolens.* The assessment of their antioxidant activities was determined using DPPH, ORAC, and ABTS assays.

4.3 Materials and Methods

4.3.1 Materials

Commercial samples of CBS (Nacional/Arriba (NAC) and CCN 51) were supplied from Cocoa processing industry (Guayaquil, Ecuador) and blended using a Model 7011C commercial blender (Conair corporation, Stamford, CT, USA). Depol™ 670L (from *Trichoderma reesei*) and Feruloyl esterase (from *H. insolens*) were provided by Biocatalysts Ltd. (Mid Glamorgan, UK).

Pectinex® Ultra Mash, Viscozyme® L, and Viscomyl® Flow were from Novozymes, endo-1,4-ß-galactanse, endo-1,5-α-arabinanase, soybean RG, and Feruloyl Esterase (from rumen microorganism) were from Megazyme (Wicklow, Ireland). 1-Kestose, nytose, and 1F-fructofuranoysl-nystose were obtained from Wake Pure Chemical (Osaka, Japan). The bacterial strains, *L. rhamnosus GG* and *B. longum* (ATCC 15707™) strains were supplied from Lallemand and ATCC©. Pectinex® Ultra Clear and the analytical grade reagents were from Sigma- Aldrich Co., St. Louis, MO, USA. Salts were obtained from Fisher Scientific (Fair Lawn, NJ). 2,2-diphenyl-1-picrylhydrazyl (DPPH) (>90%), α-tocopherol (96%), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein sodium salt, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from MilliporeSigma (Burlington, Massachusetts, United States).

4.3.2. Alkaline extraction of CBS polysaccharides

Extraction of CBS polysaccharides was performed as described by Khodaei and Karboune (2013) where CBS cell wall material (CWM, 2% w/v) was suspended in KOH solutions (0.5M) containing 0.02M $NaBH_4$. Mixtures were incubated (60˚C, 24 hr), centrifuged (10,000 x g for 15 mins), and recovered by filtration (0.45µm). Recovered polysaccharides were neutralized with HCl, dialyzed (cut-off 5-8 kDa) and freeze-dried (-50˚C, 0.003 mbar) for further analysis.

4.3.3. Bio-generation of oligosaccharides from CBS polysaccharides

The time course for the hydrolysis of CBS polysaccharides was carried out over reaction times of 1, 3, 7 and 24hr as described by Khodaei and Karboune (2016). Pectic polysaccharide suspensions in sodium acetate buffer (50mM, pH 5.0) at a concentration of 1% (w/v) were prepared. The multi-enzymatic preparations (Depol™ 670L, Viscozyme® L, Pectinex® Ultra Mash, Pectinex® Ultra Clear, Viscomyl® flow), and arabinase:galactanase bienzymatic system were added to the CBS polysaccharide suspensions to yield final enzyme/substrate ratio of 50 and 100 U galactanase /g substrate. After incubation at 40˚C, the reaction mixtures were boiled for 5 min to inactivate the enzyme and subsequently centrifuged (10,000g, 10 min). Oligosaccharides (DP of 2–12) and oligomers (DP of 3–70) were quantified by HPSEC. Yield was defined as the number of generated oligosaccharides/oligomers over the initial amount of RG I-type pectic polysaccharides. The MW distribution of generated oligosaccharides was also determined by high

performance size exclusion chromatography (HPSEC), and their monosaccharide profile was determined by high performance anionic exchange chromatography (HPAEC).

4.3.4. Analytical methods for the quantification of oligo- and polysaccharide extracts

4.3.4.1. Determination of total neutral sugars and uronic acid contents

Uronic acid content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid colorimetric assay was used for the determination of neutral sugar content (DuBois et al., 1956).

4.3.4.2. Molecular weight distribution profile

A HPSEC system (Model 1525 binary HPLC pump, equipped with a Model 2414 refractive index detector, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution of the carbohydrates. Oligosaccharide analysis was determined using a TSK G-Oligo-PW column (Tosoh Co., Yamaguchi, Japan) column. Galactose, sucrose, 1-kestose, nystose and 1F-fructofuranosyl nystose standards were used for molecular weight and concentration calibration. The temperature of the system was set at 30 °C, the eluent was 0.2 M NaCl, and the flow rate was set at 0.4 ml/min. Polysaccharide analysis was determined using a TSK G5000 PWXL (Tosoh Co., Yamaguchi, Japan) column, with dextrans (50– 670 kDa), soybean rhamnogalacturonan (0.125-1 g/l) and galactoglucomannan (0.125-1g/l) standards for molecular weight and concentration calibration. The temperature of the system was 30 °C, the eluent was 0.1 M NaCl, and the flow rate was set at 0.4 ml/min.

4.3.5. Preparation of feruloyl esterase and feruloylation of oligo- and polysaccharides

4.3.5.1. Immobilization of feruloyl esterase

Prior to immobilization, epoxy-activated Sepabeads® EC-EP R were modified by suspending them (0.2g/l) in a solution of 1.8M iminodiacetic acid (IDA) brought to alkaline pH with sodium hydroxide (pH 9) and shaken for 5h**r** at 55 rpm in Excella E24 orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 25˚C. Excess reagents were removed by washing the supports (0.2g/**l**) eight times with water. After each wash, the supports were vacuum filtered on a fritted glass filter. The supports were then suspended (0.17g/**l**) in cupric sulfate (0.13M) for 2h**r** under the same conditions as the previous step. Activated supports were then washed (0.2g/l) eight times in water and five times in 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (0.02M, pH 6) and

stored in MOPS buffer at 4˚C. Modified IDA-Cu-Sepabeads® EC-EP R support was washed with 0.2 M potassium phosphate buffer at pH 8 according to the optimized conditions for immobilization determined in our previous works (Tamayo-Cabezas and Karboune, 2019). The supports were then suspended (0.2 g/l) in the same buffers containing 0.01 g of enzyme per gram of support. The suspensions were kept for 28 to 36 hr at 6 °C under gentle shaking. Supernatants were recovered by centrifugation at 2400 × g for 5 min, and the supports, containing immobilized enzyme, were washed (0.2 g/l) five times with MOPS buffer (0.02 M, pH of 6) and stored at 4 °C. Immobilization blanks were prepared by incubating the enzyme solutions in the absence of support. Protein contents of the initial enzyme solutions, the supernatants and the washing solutions were measured by Bradford assay. Hydrolytic activities of the initial enzyme suspension, the immobilization blanks, the supernatants and of the washing solutions were determined using methyl ferulate substrate as described by Tamayo-Cabezas and Karboune (2019). Immobilization yield was calculated as the difference between activity in the immobilization blank and the activities in supernatant and washing solutions, divided by the activity of the initial enzyme solution.

4.3.5.2. Esterification activity of free and immobilized feruloyl esterase

The esterification activity of feruloyl esterase (from rumen microorganism and *H. insolens)* was carried out according to the method reported by Spadoni Andreani et al. (2021) with raffinose and ferulic acid as substrates at a concentration of 1.5 and 4.5 mM, respectively. A surfactant-less microemulsion made of n-hexane:butanone:water was evaluated as a reaction medium at the selected ratio of 49.4:44.6:6 (v/v/v). In vacuum sealed flasks, free or immobilized feruloyl esterase were added to each emulsion to reach 10 g/l. The reaction mixtures were incubated for 24 hr at 25 °C under 70 rpm. Negative controls without enzyme and without raffinose were run in parallel. Reaction progress was monitored through the consumption of ferulic acid, determined by high performance liquid chromatography (HPLC) on Beckman 32 Karat system (Beckman Coultier) equipped with a binary pump (Model 1525), and a photodiode array detector (Model 2998). The reaction mixture was loaded on a Zorbax SB-C18 reversed-phase column (5 µm, 250×4.6 mm, Agilent Technologies Canada Inc., Mississauga, Ca) and eluted with a linear gradient starting with 100% mobile A (water:acetonitrile:formic acid, 75.6:20:4.4 v/v/v) to 50:50 mobile A: mobile B (100% acetonitrile) for 20 min, followed by a linear gradient for 10 min until 100% mobile A is

reached. A constant flow rate of 0.7 ml/min was applied, and the absorbance was continually monitored at 320 nm. Ferulic acid standard was used to construct the calibration curve (R= 0.95). One unit of esterification activity was defined as the amount of enzyme that consumes one micromole of ferulic acid per minute.

4.3.5.3. Feruloylation of oligo- and polysaccharides

The feruloylation of CBS oligo- and polysaccharides was carried out according to the method described by Spadoni Andreani et al. (2021) in the n-hexane:butanone:water surfactant-less microemulsion at the ratio of 49.4:44.6:6 (v/v/v). The enzymatic feruloylation reaction was initiated by adding the free feruloyl esterase (2 U/l) into the microemulsion containing ferulic acid (1.5 g/l) and CCN or NAC polysaccharide extracts and their corresponding oligosaccharide extracts (0.3 g/l) generated using Depol™ 670L (CCN variety) and Viscozyme L (NAC variety) . The enzymatic reactions were conducted in vacuum sealed flasks at 25 °C for 144 hr, with continuous shaking at 70 rpm. The mixtures were then centrifuged at 2400 × g for 3 min and the feruloylated oligo- and polysaccharides (FA-OCCN, FA-ONAC,FA-PCCN, FA-PNAC) were isolated from the supernatants by extraction with water. The aqueous phase was dialyzed (MWCO 5-8 kDa) and freeze dried. The reaction yield was calculated from the decrease of ferulic acid concentration in the reaction supernatants, measured by HPLC.

4.3.6. Functional properties

4.3.6.1. Prebiotic activity assay

CBS Oligosaccharides (CCN-Depol 670L and NAC-Viscozyme L) were investigated as a carbon source for *B. longum* (ATCC 15707™) and *L. rhamnosus GG* (Harmonium AR) growth compared to growth on inulin and rhamnogalacturonan, using the method reported by Yang et al. (2013), modified to consider the growth requirements of the selected strains. *B. longum* and *L. rhamnosus GG* were maintained in an anaerobic chamber at 37˚C. Pre-reduced reinforced clostridial broth was used for the reanimation of *B. longum* from a freeze-dried stock, and MRS broth was used for *L. rhamnosus GG*. An aliquot (0.5 ml) of the primary cultures was then added into tubes containing 5 ml of pre-reduced, carbohydrate-free MRS broth, supplemented with 0.4% glucose. Bacterial growth was measured using colony counting on Tryptic soy agar supplemented with 5% sheep blood for *B. longum* and pre-reduced MRS agar for *L. rhamnosus GG*. Tubes containing 5 ml of pre-reduced carbohydrate-free MRS broth supplemented with 0.2% glucose,

inulin, rhamnogalacturonan, and cell wall oligosaccharides (CCN and NAC varieties) were inoculated with 0.1ml of secondary culture. Colonies were counted on Tryptic soy agar supplemented with 5% sheep blood for *B. longum* and pre-reduced MRS agar for *L. rhamnosus GG* after 6, 24, 48, 72 and 96 hr incubation intervals. Microbial counts were measured as colony forming units (CFU)/ml, and the resulting averaged data were transformed to log CFU/ml. A prebiotic activity score (PA) was calculated for each combination of bacterium and oligosaccharide as follows:

$$\mathrm{PA} = \frac{\left(\mathrm{Log}\frac{\mathrm{CFU}}{\mathrm{ml}}\ \text{oligosaccharide at x hr} - \mathrm{Log}\frac{\mathrm{CFU}}{\mathrm{ml}}\ \text{on oligosaccharide at 0hr}\right)}{\mathrm{Log}\frac{\mathrm{CFU}}{\mathrm{ml}}\ \text{on glucose at x hr} - \mathrm{Log}\frac{\mathrm{CFU}}{\mathrm{ml}}\ \text{on glucose at 0hr}}$$

4.3.6.2. Determination of short chain fatty acid catabolites

An aliquot of cultures (0.5ml) was taken at 24 and 48hr. Bacteria were precipitated by centrifugation at 1400xg for 5min at 37˚C in a Minispin Plus centrifuge (Eppendorf AG, Hamburg, Germany). The supernatants were filtered using 0.22µm polytetrafluorethylene syringe filters (Thermo Fischer Scientific) and stored at -80˚C for a maximum of 2 weeks. Freshly thawed samples (0.02ml) were analyzed using the HPLC Model 1525 system (Waters) equipped with a refraction index detector and a Zorbax SB-C18 column (4.6x 250mm) (Agilent Technologies). Elution was carried out with water containing 0.005N sulfuric acid at a constant flow rate of 0.6ml/min. Standards were prepared by serial dilution of stock solutions of lactic, acetic, propionic, and butyric acids in water (R = 0.95).

4.3.6.3. Total phenolic content

CBS polysaccharides (NAC and CCN) were characterized in terms of total phenolic content by Folin- Ciocalteu colorimetric method (Singleton et al., 1999). To 0.8 ml of aqueous solution of the sample, 0.15 ml of sodium bicarbonate (250 g/l) and 0.05 ml of Folin-Ciocalteu reagent were added. The mixtures were heated at 40 °C for 30 min, and their absorbance was measured at 765 nm. Gallic acid standard solutions (0.001 M to 0.1 M) were used for calibration (R = 0.95).

4.3.6.4. Antioxidant Activity

4.3.6.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH assay was carried out according to the method reported by Srikanth et al. (2015) with modifications. 25 µl aliquots of varying concentrations of feruloylated (in 50% methanol) and native polysaccharides (in water, pH 7.4) were mixed with 25ul of DPPH (0.1mM), which were then incubated in the dark for 30 min at 25˚C. In preliminary trials, 30 min was identified as the appropriate time at which the steady state of scavenging was achieved. Following incubation, the absorbance of each sample was read at 517nm using a Synergy HTX Multi-Mode Reader. Water was used as the blank. 50% methanol in water was used as the negative control. Ascorbic and ferulic acids prepared in 100% methanol were used as positive controls. The inhibition ratio (%) was calculated as follows:

$$\text{Inhibition ratio (\%)} = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

Where: Ac is the absorbance of the control; As is the absorbance of the sample
The different concentration of feruloylated and native oligo- and polysaccharides were plotted against the estimated inhibition ratio. The IC_{50} was defined as the concentration required to reduce DPPH by 50%. The IC_{50} was expressed as ug sample/ml DPPH.

4.3.6.4.2. Oxygen Radical Absorption Capacity (ORAC) Assay

The ORAC assay was carried out according to the method outlined by Cao et al. (1993), with modifications. AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was used as a free-radical generator and varying concentrations of selected feruloylated and native polysaccharides were used to prevent the decay of Fluorescein Sodium Salt. Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (100, 50, 25, 12.5, 6.25, and 0mM) was used as a standard and phosphate buffer (75mM, pH 7.4) was used as a blank. Ascorbic and ferulic acids prepared in 100% methanol were used as positive controls. Aliquots of 25µl of feruloylated and native polysaccharides at different concentrations (Phosphate buffer pH 7.4) and 150µl of 16 mM fluorescein (prepared in PBS 10mM pH 7.4) was pipetted into a 96-well black-walled plate. Following incubation at 37^{o}C for 30 min in a Synergy HTX Multi-Mode Reader, all wells were injected with 25µl of freshly prepared AAPH (79.65mmol/l). Fluorescence readings were taken for 1 hr at 485nm (excitation wavelength) and 520nm (emission wavelength) every 60 seconds.

The AUC (area under the curve) and Net AUC of the standards and samples were determined using Gen5 Data Analysis Software using the following equations respectively:

$$\text{AUC} = \left(\frac{R_1}{R_1}\right) + \left(\frac{R_2}{R_2}\right) + \left(\frac{R_3}{R_3}\right) + \ldots\ldots + \left(\frac{R_n}{R_n}\right)$$

Where: R_1 is the fluorescence reading at the initiation of the reaction; R_n is the last measurement

$$\text{Net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}$$

The standard curve consisted of the Net AUC of different Trolox concentrations plotted against their concentration. Finally, to calculate the Trolox Equivalents (TE) of each sample range, the following equation was used:

$$\text{TE (range of concentrations)} = m_{\text{compound}}/m_{\text{Trolox}}$$

Where: m_{compound} is the slope of the linear regression analysis of the compound; m_{Trolox} is the slope of the linear regression analysis of Trolox. Results are expressed as μmol Trolox Equivalents (TE)/g sample.

4.3.6.4.3. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay

The ABTS assay was carried out according to the method described by Re et al. (1999) with modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 hr before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 25°C. Stock solutions of feruloylated and native polysaccharides were prepared in 50% ethanol and phosphate buffered saline (PBS, pH 7.4). After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution ($A_{734nm} = 0.700 \pm 0.020$) to 10 μl of sample or Trolox standards (final concentration 0–15 μM) in ethanol or PBS the absorbance reading was taken at room temperature immediately after mixing and up to 6 min. Appropriate solvent blanks were run in each assay, ascorbic and ferulic acid were used as positive controls. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

4.3.7. Statistical analysis

Statistical analyses were performed using XLSTAT software (Addinsoft, New York, NY, USA) in Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$).

4.4 Results and Discussion

4.4.1 Bio-generation of oligosaccharides from cocoa bean shells

4.4.1.1 Composition of cocoa bean shell polysaccharides

Two polysaccharide extracts, NAC and CCN, were isolated from CBS CWM and analysed. As shown in Table 4.1, the extracts were recovered at a yield of 29.1 and 35.4% (w/w) for the NAC and CCN varieties, respectively. Both polysaccharide extracts showed three main populations of poly- and oligomers with different molecular weight distributions (NAC - 3.7, 12, 1300 kDa; CCN - 4.5, 9.1, 325 kDa). As for their monosaccharide profile, the NAC extract is predominantly comprised of pectic polysaccharides due to the presence of uronic acid (58.24 % mol), galactose (17.54 % mol), arabinose (7.75 % mol), and rhamnose (6.81 % mol). Alternatively, the CCN extract, showed a monosaccharide profile abundant in uronic acid (51.44 % mol), mannose and xylose (12.56 % mol), rhamnose (12.38 % mol), and galactose (8.09 % mol), indicating the presence of both pectic and hemi-cellulosic polysaccharides. Furthermore, both extracts contain a notable total phenolic amount (79.7-113.6 $x10^{-6}$ mol GAE /g), higher than the amounts reported for cranberry extracts (31.9 - 80.8 $x10^{-6}$ mol GAE /g, Spadoni Andreani et al., 2021) as well as other by-products, namely coffee silver-skin (76.4 $x10^{-6}$ mol GAE /g, Ballesteros et al., 2014), and purple potato peel (42.3 $x10^{-6}$ mol GAE /g, Albishi et al., 2013). Based on previous findings in par 3.4.2, the class of polyphenols predominantly present in CBS CWM extracts were flavan-3-ols, namely epicatechin, catechin. Other phenolics compounds were found which include quercetin and phenolic acids, such as gallic acid, protocatechuic, p-coumaric, ferulic, and caffeic acids.

Table 4.1. Characterization of cocoa bean shell polysaccharide extracts

	NAC	CCN
Polysaccharide yield (%, w/w)	35.4±4.3	29.1±0.4
Molecular weight of polysaccharide (PS) (kDa)	3.7, 12, 1300	4.5, 9.1, 325
Monosaccharide profile (% mol)		
Fucose	1.76 ± 0.14[a]	1.75 ± 0.02[a]
Rhamnose	6.81 ± 1.05[b]	12.38 ± 0.31[a]
Arabinose	7.75 ± 0.56[b]	8.58 ± 0.01[b]
Galactose	17.54 ± 1.26[b]	8.09 ± 0.2[c]
Glucose	2.86 ± 0.13[ab]	5.19 ± 0.5[a]
Mannose, Xylose	5.04 ± 0.37[b]	12.56 ± 2.04[c]
Uronic acids	58.24 ± 2.76[b]	51.44 ± 3.06[ab]
Total phenolic content (10^{-6} mol GAE/g)	113.36±0.01[a]	79.69±0.01[b]

Values reported as average ± standard deviation
*Standard deviation zero or not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$

4.4.2 Enzymatic generation of oligosaccharides from cocoa bean shells polysaccharides

Enzymatic hydrolysis of cell wall pectic polysaccharides from CBS was investigated in order to generate oligosaccharides. Polysaccharides in CBS were reported to be namely rhamnogalacturonan, xylogalacturonan, and glucuronoxylan heavily branched and linked to other polysaccharides (Redgwell et al., 2000). Six biocatalysts, including the bi-enzymatic system (arabinase/galactanase, 50:50 v/v) and multi-enzymatic preparations (Viscozyme® L, Pectinex® Ultra Mash, Pectinex ® Ultra Clear, Viscomyl ® Flow, Depol™ 670L) with varied levels of polysaccharide-hydrolyzing activities (including cellulase, polygalacturonase, endogalactanase, arabinase, galactanase, rhamnogalacturonase, xylanase), were screened for their ability to release oligosaccharides from CBS polysaccharides.

Provided that the cell wall polysaccharides were predominantly pectic ones, the selection of the multi-enzymatic preparations was based on their galactanase and pectinolytic activities (50 and 100U/g) as previously reported by Khodaei and Karboune (2016) and Spadoni Andreani and Karboune (2020). Table 7 and Table 4.8 show the hydrolysis extents as well as the MW distribution (DP 2-10 and DP > 10) of enzymatically generated oligosaccharides after 7 hr of hydrolysis. The highest hydrolysis extents were observed with Viscozyme® L (76.64%, 50U/g) for NAC-based oligosaccharides (Table 4.2). For CCN-based oligosaccharides, the highest hydrolysis extents were achieved with Pectinex® Ultra Mash (21.31%, 50U/g), followed by Depol® 670L (20.72%, 50U/g) and Viscomyl® Flow (20.31%, 50U/g) (Table 4.3).The enzymatically generated oligosaccharides from the bi-enzymatic system and the multi-enzymatic products varied in terms of their degrees of polymerization (DP). As seen in Table 7, all biocatalysts achieved a high proportion of NAC-based oligosaccharides of DP $\lesssim$ 2 (95.55-99.51%, mol) with the exception of Viscozyme® L (20.86%, mol). Indeed, Viscozyme® L generated diverse complexity of oligosaccharides of varying DP, where the highest proportion of oligosaccharides were of DP 3-5 (42.04%, mol). The results also show that NAC-based oligosaccharides of DP 6-10 were generated by Viscozyme® L (50 U/g, 36.78% ,mol), while those of DP greater than 10 were only generated by Pectinex® Ultra Mash (1.00%, mol), Pectinex® Ultra Clear (100 U/g, 0.49%, mol) and Viscozyme® L (0.32%, mol).

Table 7. Efficiency of bi and multi-enzymatic systems used for the enzymatic generation of oligosaccharides from the NAC cell wall polysaccharides after 7hr reaction time

	DP> 10	DP 6-10	DP 3-5	DP ≲ 2	Hydrolysis extent (%)
Arabinase/Galactanase [g]	0.00[d]	2.08[b]	2.37[b]	95.55[f]	17.10
Arabinase/Galactanase [h]	0.00[d]	0.00[f]	2.35[b]	97.65[c]	43.79
Viscozyme® L [g]	0.32[c]	36.78[a]	42.04[a]	20.86[g]	76.64
Pectinex® Ultra Mash [h]	1.00[a]	0.72[e]	0.99[d]	97.30[e]	64.68
Pectinex® Ultra Clear[h]	0.49[b]	0.00[f]	0.00[e]	99.51[a]	24.38
Viscomyl® Flow [g]	0.00[d]	1.35[c]	1.13[c]	97.53[d]	21.81
Depol™ 670L [g]	0.00[d]	0.92[d]	0.00[e]	99.08[b]	29.06

Values reported as mean (%, mol)
DP – Degree of polymerization
[a-f] Within the same column, means with different letters are significantly different at $P < 0.05$
[g] Enzyme concentration of 50U/g (galactanase units)
[h] Enzyme concentration of 100U/g (galactanase units)

Table 4.8. Efficiency of bi and multi-enzymatic systems used for the enzymatic generation of oligosaccharides from the CCN cell wall polysaccharides after 7hr reaction time

	DP>10	DP 6-10	DP 3-5	DP ≲ 2	Hydrolysis extent (%)
Arabinase/Galactanase [g]	1.20[d]	47.8[a]	0.00[f]	50.9[e]	2.15
Viscozyme® L[g]	1.31[c]	5.93[c]	16.1[b]	76.6[d]	20.19
Pectinex® Ultra Mash [g]	1.97[b]	0.17[f]	0.24[e]	97.6[b]	21.31
Pectinex® Ultra Clear® [g]	2.02[a]	4.23[d]	11.9[c]	81.8[c]	1.36
Viscomyl® Flow [g]	0.21[e]	1.00[e]	0.49[d]	98.3[a]	20.31
Depol™ 670L [g]	0.23[e]	16.4[b]	38.5[a]	44.7[f]	20.72

Values reported as mean (%, mol)
DP – Degree of polymerization
[a-f] Within the same column, means with different letters are significantly different at $P < 0.05$
[g] Enzyme concentration of 50U/g (galactanase units)

As seen in Table 4.8, CCN-based oligosaccharides hydrolyzed by Depol® 670L generated the most diverse oligosaccharides, with DP ≲ 2 (44.77%, mol), followed by DP 3-5 (38.56%, mol), DP 6-10 (16.44%, mol), and DP greater than 10 (0.23%, mol). Oligosaccharides of DP 6-10 were recovered more upon the use of the bi-enzymatic system (50 U/g, 47.81%, mol). In addition, compared to those from NAC, CCN-based oligosaccharides of DP >10 were more prevalent among the bi-enzymatic system and all multi-enzymatic products, ranging from 0.21 to- 2.02% mol. Overall, CCN-based polysaccharides were hydrolyzed less in comparison to the NAC variety, which may be attributed to enzyme low affinity and/or inaccessibility to hemicellulosic polysaccharides and to the branching. Pectinex® Ultra Mash, Viscozyme® L and Depol™ 670L multi-enzymatic products all share specific enzyme activity towards pectinases, namely endo-galacturonase and polygalacturonase. This may explain the higher hydrolysis extents favored towards NAC based polysaccharides using Pectinex® Ultra Mash, Viscozyme® L and Depol™ 670L. Khodaei and Karboune (2018) suggested other possible explanations of this occurrence may include enzyme inhibition by substrate excess, occurrence of substrate diffusional limitations, as well as the presence of substrate/substrate interactions hindering their access to the enzyme's active site. This is an agreement with Emaga et al. (2012) as well as Efrinalia et al. (2022), where the strong interactions between neutral sugars and acid fractions had a significant effect on the enzymatic release of total sugar residues from flaxseed mucilage. In summary, Viscozyme® L was favored for the generation of NAC based oligosaccharides, while Depol 670®L was selected for the production of CCN-based oligosaccharides. In addition, arabinase/galactanase bienzymatic system was selected for both varieties in order to compare its efficiency to a multi-enzymatic system along the hydrolysis time course. The time courses for the hydrolysis extent by the three biocatalysts (arabinase/galactanase, Viscozyme® L and Depol™ 670L) are reported in Figure 4.1A. With NAC based polysaccharides, the hydrolysis extent by the bi-enzymatic system and Viscozyme® L remained more or less constant over the first 7 hr reaction and increased thereafter at 24 hr to reach 66.0 and 100%, respectively. The results also show that the hydrolysis of CCN based polysaccharides by bi-enzymatic system did not exceed 8.9% over the 24 hr time course, while the hydrolysis extent by Depol™ 670L increased throughout the time course to reach 42.4%.

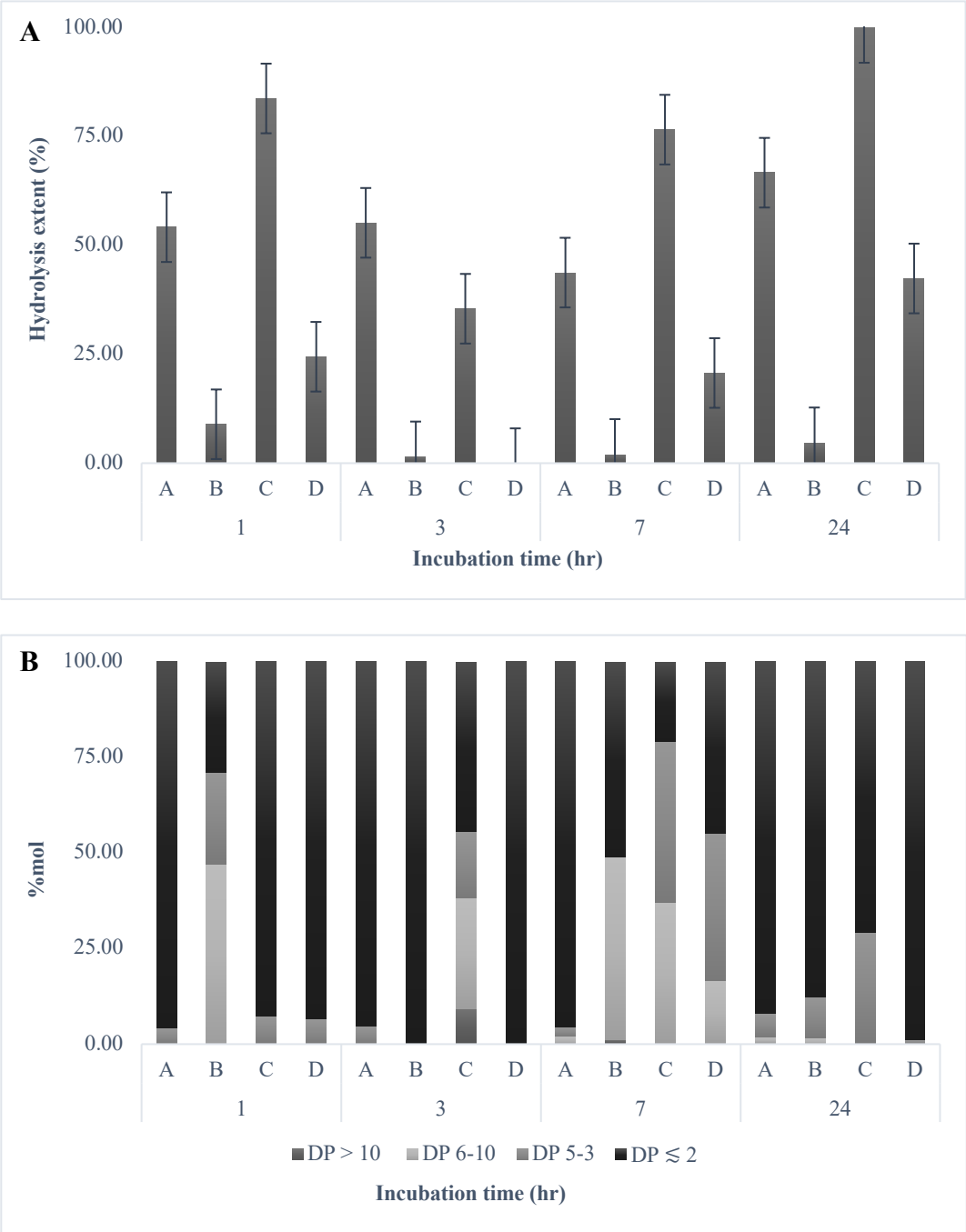

Figure 4.1. A) Hydrolysis extent obtained with selected multi-enzymatic products (50U/g); B) the corresponding MW distribution of enzymatically generated oligosaccharides; A- Arabinase: Galactanase NAC, B- Arabinase:Galactanase CCN, C – Viscozyme L® NAC, D – Depol™ 670L CCN, at various reaction times (1,3,7,24hr).

In Figure 4.1B, the MW distribution of oligosaccharides shows a shift from high to low DP over time, which is observed with the bi-enzymatic system and Depol™ 670L for the generation CCN-based oligosaccharides as well as with Viscozyme® L for the production of NAC-based oligosaccharides. The results also show that Depol™ 670L with CCN oligosaccharides and the bi-enzymatic system with NAC oligosaccharides generated a high proportion of DP ≲ 2 within 1hr of reaction, likely indicating that the substrates were more accessible towards those biocatalysts. CCN oligosaccharides hydrolyzed by Depol™ 670L after 3hr allowed for the generation of a diverse complex of oligosaccharides including DP ≲ 2 (44.5%), DP 3 to 5 (17.33%), DP 6 to 10 (28.87%), and DP > 10 (9.30%). Similarly, at 7hr, NAC oligosaccharides hydrolyzed by Viscozyme® L generated a profile comprised DP ≲ 2 (44.77%), DP 3 to 5 (38.56%), DP 6 to 10 (16.44%), and DP > 10 (0.23%). All in all, the results indicate the generation of a versatile and complex oligosaccharide profile with the use of multi-enzymatic products, Depol™ 670L and Viscozyme® L, compared to the bi-enzymatic system, arabinase: galactanase. Future investigation towards optimizing the hydrolysis reaction parameters, in terms of substrate/enzyme ratio, pH, reaction time would be needed to determine the favorable conditions to achieve maximal hydrolysis extents along with a diverse oligosaccharide profile. In addition, structural linkage analysis of the enzymatically generated CBS oligosaccharides would help provide a more complete visualization towards the enzyme cleavage specificity for each of the multi-enzymatic products.

4.4.3 Feruloylation of oligo and polysaccharides from cocoa bean shells

4.4.3.1 Feruloyl esterase immobilization

Immobilization of feruloyl esterases, from rumen microorganism and *H. insolens,* was investigated using an epoxy-based support, modified IDA-Cu-Sepabeads® EC-EP R and assessed for the hydrolytic efficiency compared to their free forms. The selection of the Sepabeads ® EC-EP R support was based on previous studies, which reported high immobilization yields with feruloyl esterase (Spadoni Andreani et al., 2021; Tamayo-Cabezas & Karboune, 2019). In addition, the use of this support applies the chelation immobilization approach, offering both an ease in the support regeneration as well as restricting enzyme leakage (Afaq & Iqbal, 2001). Table 9 shows the immobilization yields and the retained activities of feruloyl esterases from rumen microorganism and *H. insolens.*

Table 9. Comparison of the hydrolytic and esterification activities of feruloyl esterases in their free and immobilized (modified IDA-Cu-Sepabeads EC-ER®) forms.

	Hydrolytic activity (U/mg enzyme)	Immobilization Yield (%)	Retention of Activity (%)	Esterification activity (U/mg protein)
Free enzyme from rumen microorganism	8.66±0.72[a]	-	-	0.17±0.03[b]
Immobilized enzyme from rumen microorganism	0.23±0.03[b]	99.30+10.31[a]	2.66±0.27[a]	n.d.*
Free enzyme from *H. insolens*	2.07±0.04[c]	-	-	1.36±0.22[a]
Immobilized enzyme from *H. insolens*	0.015±0.0001[c]	56.9±0.55[b]	59.13±0.57[b]	0.0029±0.001[b]

Values reported as average ± standard deviation
*n.d.- not detected
[a] Within the same column, means with different letters are significantly different at $P < 0.05$

Table 10. Feruloylation extent (%) of cocoa bean shell oligo- and polysaccharides (FA-OCCN, FA-ONAC,FA-PCCN, FA-PNAC enzymatically generated oligosaccharides using free feruloyl esterases (2U/l) from rumen microorganism and H. insolens at 144hr.

	FA-PNAC (%)	FA-ONAC (%)	FA-PCCN (%)	FA-OCCN (%)
Free enzyme from rumen microorganism	56.9±10.5[ab]	38.2±0.3[bc]	60.1±6.3[a]	38.7±2.1[bc]
Free enzyme from *H. insolens*	47.3*[abc]	50.5±8.4[ab]	49.5±8.6[abc]	30.8±7.9[c]

Values reported as average ± standard deviation
*Standard deviation zero or not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$

Feruloyl esterase from rumen microorganism attained a higher immobilization yield (99.3%) than feruloyl esterase from *H. insolens* (56.9%). Based on previous findings, the immobilization yields are higher than those previously reported, ranging between 66.2-72.5% (Spadoni Andreani et al., 2021; Tamayo-Cabezas & Karboune, 2019).

Although higher immobilization yield was attained with feruloyl esterase rumen microorganism, the retention of activity was poor (2.66%) in comparison to feruloyl esterase from *H. insolens* (59.13%). According to the manufacturer, the Sepabeads® EC-EP R support has a pore size of 10-20nm. The large pore size may have impacted the efficiency of the enzyme loading onto the support (Kang et al., 2007; Miao et al., 2022). In addition, He et al. (2015) reported that the immobilization time affected the immobilisation eficiency and resulted in both lower enzyme and specific activities of feruloyl esterases. This may be attributed to the saturated adsorption on the supports, thereby distributing the conformation of the biocatalyst (He et al., 2015; Juang et al., 2014). Furthermore, the hydrolytic and esterification activities of free and immobilized feruloyl esterases from rumen microorganism and *H. insolens* were compared. As seen in Table 9, free feruloyl esterases expressed significant ($P < 0.05$) hydrolytic activities (2.07-8.66 U/mg enzyme) compared to their immobilized forms (0.015-0.23 U/mg enzyme). The findings of the esterification of feruloyl esterases with raffinose show higher activities attained with free enzyme (0.17-1.36 U/mg protein) than immobilized enzyme (0-0.00029 U/mg protein). One study reported similar results where free feruloyl esterase attained higher raffinose esterification activity than its immobilized form by 54.2% (Spadoni Andreani et al. 2021). Based on our findings, the use of free feruloyl esterase, from rumen microogranism and *H, insolens,* was selected for further analysis.

4.4.3.2 Feruloylation cocoa bean shell oligo- and polysaccharide extracts

The feruloylation of oligo- and polysaccharides from CBS using free feruloyl esterases from rumen microorganism and *H. insolens* was investigated using a surfactant-less microemulsion comprised of n-hexane:butanone:water (49.4:44.6:6 % v/v/v). CBS oligosaccharides were obtained from multi-enzymatic hydrolysis with Viscozyme® L (NAC variety) and Depol 670™ L (CCN variety), while polysaccharides (both varieties, NAC and CCN) were prepared from alkaline extraction.

As shown in Table 10, FA-PCCN and FA-PNAC using free feruloyl esterase from rumen microorganism attained significantly ($P < 0.05$) higher feruloylation extents than with

oligosaccharides. Having a closer look between the two polysaccharide varieties, FA-PCCN (60.1%) were feruloylated at a slightly, yet significantly, higher extent than FA-PNAC (56.9%). On the other hand, free feruloyl esterase from *H. insolens* attained a significantly ($P < 0.05$) higher feruloylation extent (50.5%) with FA-ONAC, compared to the extent achieved with FA-OCCN (30.8%). Indeed, feruloyl esterase from *H. insolens* has previously shown large substrate specificity towards mono, di, and oligosaccharides (Couto et al., 2011). The latter authors suggested a correlation between the feruloylation extent and the degree of polymerization, where the feruloyl esterase-catalyzed reaction is dependant on the structural characteristics, linkage, and length of glycosides. According to the manufacturer, feruloyl esterase from rumen microorganism has a substrate specificity towards arabinose. In addition, studies have reported natural occurring pectic feruloylation confined to O2 or O3-positions of arabinan and the O6-position of galactans (Holck et al., 2011; Ralet et al., 1994; Sato et al., 2013; Spadoni Andreani et al., 2021). This may explain the higher feruloylation extents attained with FA-PCCN and FA-PNAC, which were found to be primarily composed of pectic regions (56.4-88.8%), with arabinose (7.8 to 16.4%) and galactose (8.1-22.4%) as one of the predominant monosaccharides.

The feruloylation efficiency was found to be dependent on the structural characteristics of the carbohydrate substrate (Couto et al., 2011; Tamayo-Cabezas & Karboune, 2019). To the author's knowledge, this is the first study on the feruloylation of CBS oligo- and polysaccharides by feruloyl esterases. The observed feruloylation extents are above the range reported for analogous reaction systems on non-digestible oligosaccharides from sugar-beet pulp and wheat bran (27.4%), pectic polysaccharides from cranberry pomace (4.2-18%), arabinobiose (10-18%), xylobiose (12.8%), xylooligosaccharides (10.6%) (Tamayo-Cabezas & Karboune, 2019; Spadoni Andreani et al., 2021; Vafiadi et al., 2006).

4.4.4 Functional Properties

4.4.4.1 Prebiotic Activity

The prebiotic activity scores of enzymatically generated oligosaccharides by selected multi-enzymatic products, Viscozyme® L for NAC variety and Depol™ 670L for CCN variety, rhamnogalacturonan, and inulin, a known prebiotic polysaccharide (Shoaib et al., 2016), were compared by using them as carbon sources for the anaerobic growth of two probiotic bacteria strains, *L. rhamnosus GG* and *B. longum.* Studies reported effective growth of *L. rhamnosus GG*

with oligosaccharides derived from pearl millet fibre, *F. kuhistanic* leaves, wheat fibre, and mulberries (Farooq et al., 2017; Islamova et al., 2017; Li et al., 2022; Soukoulis et al., 2014) and *B. longum* with oligosaccharides derived from wheat flour, apple, and sugar beet pectin (Holck et al., 2011; Van Laere et al., 2000). In terms of specific oligosaccharide affinity, *L. rhamnosus GG* has been most analyzed for its affinity towards galacto-oligosaccharides. A study conducted by Li et al. (2022) reported that the proliferation of *L. rhamnosus GG* was significant due to up-regulated genes associated with galactose metabolism as well as those involved with glycolysis. In addition, *L. rhamnosus GG* has been previously known to express key enzymes involved in the fucose pathway, further promoting it affinity towards fucosylated oligosaccharides (Becerra et al., 2015). On the other hand, *B. longum* was found to exhibit excellent affinity towards fructo-oligosaccharides, arabino-furanosyl containing oligosaccharides, as well as α-galactosyl-oligosaccharides, such as raffinose and stachyose (Garro et al., 1999; Minami et al., 1983; van den Broek et al., 2008; Van Laere et al., 2000). Furthermore, *B. longum* has been found to possess over 40 glycosyl hydrolases which may indicate the strain's high affinity towards a wide range of oligosaccharides with a degree of polymerization less than 8 (Schell et al., 2002).

CBS oligosaccharides (NAC and CCN) showed a prebiotic activity score similar to that of inulin and rhamnogalacturonan for *L. rhamnosus GG* (Figure 4.2 A). At 24hr of fermentation, the prebiotic activity scores of oligosaccharides were slightly higher ($P \leq 0.05$) than that of inulin and rhamnogalacturonan. For *B. longum* (Figure 4.2 B), CBS oligosaccharides

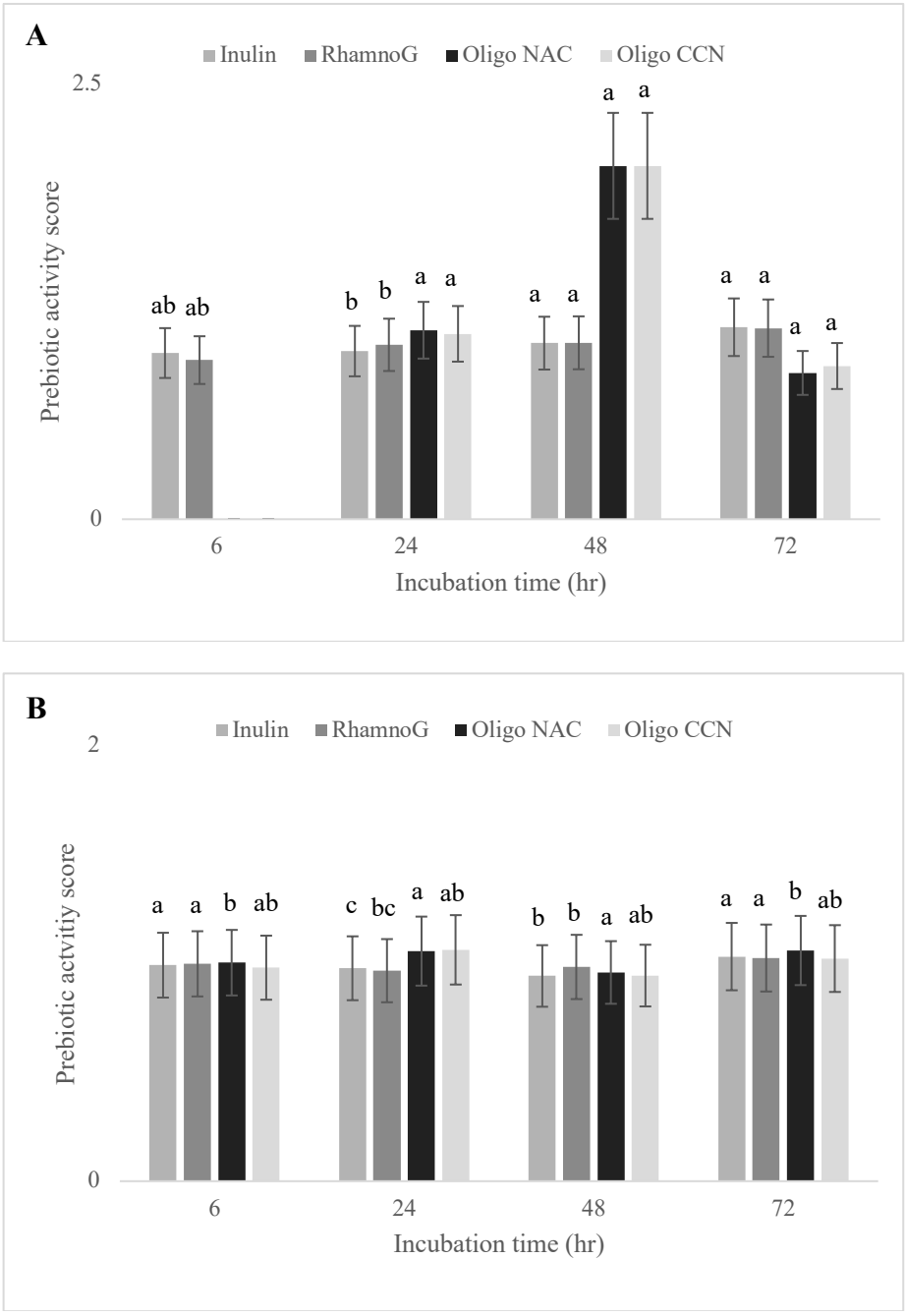

Figure 4.2. A) Prebiotic activity scores of *Lactobacillus rhamnosus GG* (Harmonium R0343 AR) on oligosaccharide extracts prepared from multi-enzymatic hydrolysis, inulin and rhamnogalacturonan, measured at 6,24, 48, 72 hr of incubation. B) Prebiotic activity scores of *Bifidobacterium longum* (ATCC ® 15707) on oligosaccharide extracts prepared from multi-enzymatic hydrolysis, inulin and rhamnogalacturonan, measured at 6, 24, 48, 72 hr of incubation. Values represented as an average ± standard deviation. Oligo NAC: oligosaccharide NAC variety, Oligo CCN: oligosaccharide CCN variety, RhamnoG: Rhamnogalacturonan, respectively. For each incubation time, bars with different letters represent scores significantly different at $P < 0.05$

showed statistically significant ($P \leq 0.05$) prebiotic activity scores as early as 6hr of fermentation than that of inulin and rhamnogalacturonan, while maintaining a relatively constant score throughout the 72 hr fermentation period. Therefore, it appears that CBS enzymatically generated oligosaccharides (Viscozyme® L and Depol™ 670L, 50U/g, 7hr) effectively stimulate the growth of both strains.

One study suggested that pectic polysaccharides used as a source of prebiotics underwent metabolization prior to consumption by *B. longum* and *L. rhamnosus GG* (Li et al., 2020). With that being said, one can assume that using enzymatically generated oligosaccharides offered faster and more direct consumption from both strains, while also providing an increased production of SCFA than those from cell wall polysaccharides. SCFA are produced upon the fermentation of dietary fiber by gut microbiota (Lin et al., 2016; Spadoni Andreani et al., 2021). Table 11 shows the concentration of four SCFA after 24 and 48 hr of fermentation of *L. rhamnosus GG* and *B. longum* with glucose, enzymatically generated oligosaccharides (NAC and CCN varieties) and positive controls, inulin and rhamnogalacturonan. In the culture of *L. rhamnosus GG* (Table 11), the main increase was seen in the lactic acid followed by acetic acid, similar to the trend observed with CBS polysaccharides in par 3.4.4 . For oligosaccharides of CCN variety, the significance ($P < 0.05$) was observed with the increased production of propionic acid from 24 to 48 hr of fermentation. *L. rhamnosus GG* is known for its ability to produce lactic and propionic acid during fermentation, which can promote anti-inflammatory and anti-microbial properties in our gastrointestinal tract (Al-Lahham et al., 2010; Markowiak-Kopeć & Śliżewska, 2020). The production of butyric acid was prevalent in glucose, inulin, and rhamnogalacturonan, as well as CBS enzymatically generated oligosaccharides of NAC variety (24hr), indicating the favored production of butyrate in the *L. rhamnosus* fermentation pathway. This can be attributed to the metabolization of lactate as observed in Table 11, further explaining the decrease in the lactate concentration with increased incubation time (Cummings et al., 1987; Karboune et al., 2022).

Similarly, to the SCFA profile of *L. rhamnosus GG,* the culture of *B. longum* was characterized by the greatest increase in lactic acid, followed by acetic acid predominantly after 48 hr of fermentation. The acetic acid content of the medium relatively remained unchanged for the CBS oligosaccharides. The lactic acid concentrations produced by oligosaccharides were significantly comparable to their positive controls, glucose, inulin, and rhamnogalacturonan, at 24 hr of fermentation. Interestingly, at 48hr of fermentation, the lactic acid concentrations continuously

increased with their positive controls, contrary to the behavior of CBS polysaccharides as described in par 3.4.4. Furthermore, CBS oligosaccharides, showed a notable significant ($P < 0.05$) production of butyric and propionic acids at 24hr of fermentation, either surpassing or similar to the positive controls. According to studies assessing the prebiotic effects of plant-derived oligosaccharides, smaller oligosaccharides, with a degree of polymerization less than 4, have shown to induce a "bifidogenic effect" as well as contribute to higher production of acetate and butyrate (Sanchez et al., 2009; Snelders et al., 2014; Van Craeyveld et al., 2008). However, the onset of this "bifidogenic effect" was not observed after 24 hr of *B. longum* fermentation, as butyric acid concentrations were decreased or undetected depending on the CBS variety.

Overall, the total SCFA profile of oligosaccharides continuously increased throughout 48 hr of fermentation with *B. longum*, contrary to those with *L. rhamnosus GG*. Studies suggest that *Bifidobacterium spp.*, specifically. *B. longum,* are producers of lactate, acetate and propionate in the human intestinal tract, as observed in Table 11 (Liu et al., 2020; Markowiak-Kopeć & Śliżewska, 2020). In addition, when carbohydrates are in excess, *Bifidobacterium* utilize the fermentation pathway, to produce two molecules of acetate with one molecule of lactate (LeBlanc et al., 2017; Macfarlane & Macfarlane, 2003; Markowiak-Kopeć & Śliżewska, 2020); on the contrary, our findings suggest a higher proportion of lactic than acetic acid.

4.4.4.2 Antioxidant Activity

The antioxidant activities of native and feruloylated polysaccharides were assessed using assays based on both single electron transfer (SET), which include DPPH and ORAC antioxidant assays, and hydrogen atom transfer (HAT), which include 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant assay. The selection of different antioxidant mechanisms will help in providing a complete and precise evaluation of the antioxidant activity. The DPPH assay relies on the radical quenching capabilities of antioxidant by reducing DPPH in the presence of the antioxidant extract in a concentration dependant manner (Baumann et al., 1979; Rahman et al. 2015). Results from the DPPH assay were expressed as the IC_{50} value, the concentration of the antioxidant needed to inhibit radical scavenging by 50% inhibition. As seen in Table 12, feruloylated polysaccharides led to a statistically significant ($P < 0.05$) decrease, by as much as 92-98%, in their IC_{50} values as compared to native polysaccharides.

Table 11. Concentration (10^{-3} mol/l) of short chain fatty acids released in the cell-wall oligosaccharides samples (NAC, CCN) prepared from multi-enzymatic hydrolysis as compared to glucose, inulin and rhamnogalacturonan (RhamnoG) standards which were obtained from *Lactobacillus rhamnosus GG* (R0343 AR) and *Bifidobacterium longum* (ATCC® 15707) fermentation using different carbon sources. Values determined as difference with concentrations detected immediately after inoculation.

		hr	Glucose	Inulin	RhamnoG	NAC	CCN
Lactic acid	*L. rhamnosus GG*	24	312.9±4.0^{c}	212.5±15.5^{c}	508.8±10.3^{a}	458.6±37.3^{ab}	393.7±42.7^{bc}
		48	385.7±16.2^{a}	315.6±14.8^{a}	293.5±25.9^{a}	337.3±67.7^{a}	383.9±29.2^{a}
	B. longum	24	31.3±5.1^{a}	56.3*^{a}	178.0*^{a}	275.3±15.5^{a}	174.2±21.3^{a}
		48	404.4±96.6^{a}	281.3±1.8^{a}	327.9±2.1^{a}	369.8±21.8^{a}	320.9±32.5^{a}
Acetic acid	*L. rhamnosus GG*	24	58.1±8.4^{a}	46.4*^{a}	112.2*^{a}	38.9±3.6^{a}	35.6±3.9^{a}
		48	111.7*^{a}	66.4*^{a}	38.9±8.5^{a}	42.1*^{a}	36.8±3.5^{a}
	B. longum	24	44.4±3.9^{a}	43.7±10.3^{a}	52.62*^{a}	43.9±3.4^{a}	30.1±3.1^{a}
		48	230.1±11.3^{a}	55.3±3.6^{b}	50.5±1.5^{b}	37.6±1.7^{b}	33.8±6.3^{b}
Propionic acid	*L. rhamnosus GG*	24	4.71*^{a}	0.9±0.04^{a}	1.8±0.03^{a}	1.3±0.3^{a}	1.7*^{a}
		48	0.66*^{ab}	0.4*^{ab}	0.6±0.1^{ab}	-	2.55*^{a}
	B. longum	24	1.9±0.4^{a}	1.3±0.2^{a}	2.6±0.4^{a}	1.75*^{a}	0.8±0.2^{a}
		48	0.6*^{a}	0.5±0.1^{a}	0.5±0.03^{a}	0.91*^{a}	-
Butyric acid	*L. rhamnosus GG*	24	1.8*^{a}	0.33*^{a}	0.4±0.04^{a}	3.48*^{a}	-
		48	-	-	-	-	-
	B. longum	24	0.3*^{b}	0.33*^{b}	-	5.33*^{a}	-
		48	-	-	-	3.05±0.3^{a}	-
Total	*L. rhamnosus GG*	24	377.6	220.2	623.2	502.4	430.9
		48	498.1	382.4	332.9	379.4	423.2
	B. longum	24	77.8	101.6	233.3	326.2	205.1
		48	635.2	337.1	378.8	411.3	355.5

Values reported as average ± standard deviation
*Standard deviation zero or not detected
[a] Within the same row, means with different letters are significantly different at $P < 0.05$

Table 12. Antioxidant activity as determined by the DPPH, ORAC, and ABTS assays, expressed as IC50 µg/ml for DPPH, µmol TE /mg sample for ORAC and ABTS of native and feruloylated polysaccharides (FA-PCCN and FA-PNAC) as compared to ferulic and ascorbic acids

	DPPH (IC50, ug/mL)	**ORAC (µmol TE/mg sample)**	**ABTS (µmol TE/ mg sample)**
NAC Native	6417.6±931.4^{a}	202.6±0.2^{c}	312.6±97.5^{c}
FA-NAC	39.3*^{c}	252.3±0.7^{b}	1542.7±264^{c}
CCN Native	4100.8±45^{b}	204.1±1.3^{c}	211.6±49.6^{c}
FA-CCN	170.6±17^{c}	266.8±2.5^{a}	1517.7±292.7^{c}
Ferulic Acid	37.4±0.8^{c}	202.4±0.9^{c}	15291.7±3279.1^{a}
Ascorbic Acid	57.6±16.1^{c}	168.2±1.7^{d}	9773.1±2239.6^{b}

Values reported as average ± standard deviation
*Standard deviation zero or not detected
[a] Within the same column, means with different letters are significantly different at $P < 0.05$

In addition, that FA-PNAC and FA-PCCN attained IC_{50} values in proximity to their positive controls, ferulic and ascorbic acid. The decrease in the IC_{50} indicates that a lower concentration of the FA-NAC and FA-PCCN (39.3-170.6 µg/ml) is needed to inhibit and scavenge free radicals as compared to native polysaccharides (4100.8-6417.6 µg/ml). Comparing the feruloylated polysaccharides from two CBS varieties, FA-PNAC attained lower IC_{50} (4 fold) compared to FA-PCCN. As compared to the literature, FA-PNAC and FA-PCCN showed stronger antioxidant activity than feruloylated hemicelluloses and oligosaccharides from wheat bran (IC_{50} 53.6 -1174.3 µg/ml, Ruthes et al., 2017) and feruloylated xylo-oligosaccharides from pearl millet fibre (IC_{50} 51.2 µg/ml, Singh & Eligar 2021). Furthermore, CBS FA-PCCN and FA-PNAC showed a higher antioxidant capacity than those reported by Khodaei et al. (2021) as the best overall antioxidant capacities of essential oils which include Pimento berries ($1.14x10^5$ µg/ml), Ceylon cinnamon ($1.76x10^5$ µg/ml), and clove bud oil ($1.90x10^5$ µg/ml).

The ORAC assay relies on fluorescence-time based radical scavenging using peroxyl radicals to provide a better antioxidant reaction model as well as providing a continuous generation of radicals on a time-scale (Schaich et al., 2015). The findings from the ORAC assay exhibited a similar trend to that observed with the DPPH assay. The results are expressed in terms of TE, where a higher TE mediates a higher antioxidant activity. As seen in Table 12, the findings suggested that FA-PNAC and FA-PCCN (252.3-266.8 µmol TE/mg) achieved a higher TE ($P < 0.05$) by as much as 10.9-13.3% as compared to CBS native polysaccharides (202.6-204.1µmol TE/mg). Furthermore, a significant ($P < 0.05$) difference between the feruloylated polysaccharides from the two varieties was observed, where FA-PCCN polysaccharides were superior. In addition, that FA-PNAC and FA-PCCN also showed a significant ($P < 0.05$) increase as compared to ferulic and ascorbic acids. Furthermore, the antioxidant activities of that FA-PNAC and FA-PCCN are higher than those reported for roasted cocoa beans (0.25 µmol TE/mg), cloves (3.14 µmol TE/mg), and cinnamon (2.67 µmol TE/mg) (Jolić et al., 2011; Schaich et al., 2015).

The ABTS assays adopts the principles of hydrogen atom transfer antioxidant mechanism in which test the ability of antioxidant extracts to intercept initial oxidation and prevent production of $ABTS^{\bullet+}$ (Schaich et al., 2015). Our findings, in Table 12, suggest that that FA-PCCN and FA-PNAC (1517.7-1542.7µmol TE/mg) attained a higher TE by as much as 66.3-75.5% ($P < 0.05$) compared to native polysaccharides (211.6-312.6 µmol TE/mg). Between FA-PNAC and FA-

PCCN, no significant ($P<0.05$) differences were observed. These results are higher than those reported for raw CBS (0.0135-0.046 μmol TE/mg, Botella-Martínez et al., 2021), native and feruloylated arabinoxylans from maize dried grains (39-67 nmol TE/mg, Marquez-Escalante & Carvajal-Millan 2019), feruloylated non-starch polysaccharides isolated from barley and wheat (58.0-105.0 μM TE/g, Malunga & Beta 2015).

As an overall, CBS polysaccharides exhibited stronger antioxidant capacities upon feruloylation. Studies suggest that specific linkages between polysaccharides and ferulic acid moieties may promote a higher antioxidant activity (Cos et al., 2002; Kylli et al., 2008; Ou & Sun, 2014). One study conducted by Kylli et al. (2008) found that the 6-O-feruloyl-glucoside, 2-O- and 3-O-feruloyl-glycoside linkages exhibited high antioxidant activities higher or comparable to free ferulic acid. As mentioned earlier, CBS native polysaccharides contain a high phenolic content, which may provide an early indication of high antioxidant activity. There are conflicting agreements concerning the correlation between phenolic content and antioxidant activities. Using electron spin resonance spectroscopy to assess the antioxidant capacity of cocoa fibre found a positive correlation ($R=0.87$) between total phenolic compounds and antioxidant activity (Lecumberri et al., 2007). On the other hand, other studies assessed on cocoa beans and flaxseed found no correlation among the two attributes, as the antioxidant activities due to the high content of reducing agents, such as ascorbic acid (Agus et al., 2018; Bruna et al., 2012; Georgé et al., 2005; Ikram et al., 2009; Othman et al., 2007).

4.5 Conclusion

Two CBS polysaccharide extracts were assessed in terms of the cell wall composition and MW distribution. Both varieties contained high proportion of pectic polysaccharides, identified to be primarily rhamnogalacturonan. CCN based polysaccharides also contained a shared proportion of hemi-cellulosic polysaccharides, including galactomannan and xylan. Additionally, the two CBS varieties showed high contents of phenolic compounds compared to other by-products, such as cranberry pomace. Of the six biocatalysts, Viscozyme® L led to the highest hydrolysis extent of NAC based polysaccharides and resulted in oligosaccharides with DP 3-5 and 6-10. As for CCN based polysaccharides, Depol® 670L had the highest hydrolysis extent and generated a mixture rich in oligosaccharides with DP up to 10, likely from the degradation of pectic and hemi-cellulosic polysaccharides.

The raffinose esterification efficiency using free and immobilized feruloyl esterases, from rumen microorganism and *H. insolens,* was also explored. Although feruloyl esterases attained high immobilization yields (56.9-99.3%), their hydrolytic and retention of activities were minimal, further impacting their esterification activity. Based on the results, the selection of free feruloyl esterases was deemed favorable for feruloylation reaction with CBS oligo- and polysaccharides. Overall, feruloyl esterase from rumen microorganism provided best feruloylation efficiencies with CBS polysaccharides (56.9-60.1%), while the enzyme from *H. insolens* provided best feruloylation efficiencies with the oligosaccharides (30.8-50.5%).

Enzymatically generated oligosaccharides as well as feruloylated polysaccharides were assessed for their prebiotic and antioxidant activities, respectively. When tested for the promotion of the growth of selected probiotic strains, select CBS oligosaccharides (Viscozyme® L for NAC and Depol® 670L for CCN) performed similarly or more than inulin and rhamnogalacturonan based on the prebiotic activity scores. The short chain fatty acid profiles were characterized by high amounts of lactic acid and acetic. In addition, a significant production of butyric and propionic acids were observed with CBS oligosaccharides. Based on the DPPH, ORAC, and ABTS assays, the feruloylated CBS polysaccharides prompted significantly notable increases in the scavenging and antioxidant activity compared to their native forms as well as ferulic and ascorbic acid.

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Supplementary Data

Table I. Multi-enzymatic product profile and commercial specification according to indicated providers. PGase- polygalacturonase, EndoGlnase – endoglucanase, Arase- arabinase, Galase – galactanase, RGase – rhamnogalacturonase, XYase - xylanase

Product name	Provider	PGase	Endo-Glnase	Arase	Galase	RGase	XYase	Amylase	Commercial specification
Pectinase A. Aculeatus	Sigma Aldrich	**1689.2**							Pectinase
endo- 1,5, Arabinanase	Megazyme			**52.9**					Arabinanase
Viscozyme L	Novozyme			175.9	**1493.1**	91.9	115.1		beta-glucanases (107.1 U/g), pectinases, hemicellulases and xylanases
Pectinex Mesh	Novozyme	**5474.6**	170.5	181.6	0.7	285.8	404.2		Pectinases (10031 U/g) and hemicellulases and viscosity reduction
Viscomyl Flow	Dupont	14.3	**8964.4**	2.8	1.3	0.8	2028.1		Cellulase, 6200-7580 IU/g

* One unit of enzyme was defined as μmol of reduced sugar was generated per min, measured using sodium acetate 0.1M, pH 5.5 as buffer, at 40 ˚C.

CONNECTING STATEMENT III

The research in Chapter IV focused on the enzymatic generation of cocoa bean shell oligosaccharides using bi-enzymatic and commercially available multi-enzymatic products. The assessment of the structural components and MW distribution were performed. Similarly, to Chapter III, the prebiotic potential of enzymatically generated oligosaccharides were determined. Chapter IV also explores enzymatic esterification of ferulic acid, otherwise known as feruloylation, to cocoa bean shell oligo- and polysaccharides, using free and immobilized feruloyl esterases from *Humicola insolens* and rumen microorganism. The antioxidant activity of feruloylated polysaccharides were explored using three assays, DPPH, ABTS, and ORAC, and compared to the scavenging activity of ferulic and ascorbic acids.

Chapter V explores the pilot plant scale up for the isolation and enzymatic generation of cocoa bean shell oligo- and polysaccharides accounted for a large quantity with a good understanding of their structural components. Chapter V also examines the incorporation of the scaled-up cocoa bean shell polysaccharides for the development of functionally enriched chocolate-based formulations. Sensory evaluation of the chocolate-based formulations were assessed for their consumer acceptability and purchase intent.

The results from this study have been submitted for publication.

CHAPTER V. PILOT PLANT EXTRACTION OF OLIGO/POLYSACCHARIDES FROM COCOA BEAN SHELLS AND THEIR INCORPORATION INTO CHOCOLATE BASED FORMULATIONS

5.1 Abstract

A pilot plant extraction of cocoa bean shell oligo- (hCHO) and polysaccharide (CHO) extracts using alkali isolation (0.5M KOH) and commercially available multi-enzymatic product, Depol™ 670L, was performed. Compared to the recovery yield attained at a laboratory scale (29.1%), pilot-scale CHO extracts were recovered by as much as 3.69%. However, both laboratory and pilot scale extracts contained similar monosaccharide profiles; showing a predominant composition of pectic polysaccharides followed by hemicellulosic polysaccharides as indicated by the presence of uronic acid (55.9%µM), galactose (17.4%µM), glucose (13.3%µM), arabinose (5.8%µM), mannose and xylose (4.7%µM) contents. The MW distribution of the extracts showed four main populations of 0.83, 2.1, 320, and 2400 kDa for the CHO extract, while 0.87, 1.1, 1.6, and 3.3 kDa for the hCHO extract. CHO extracts were utilized for the enrichment of chocolate-based formulations for its use as a functional food ingredient without altering the flavor. Sensory panels consisted of 60 untrained panelists per session, evaluating the formulations before and after enrichment rating the intensity and 9-point hedonic likeness for the following sensory attributes: chocolate flavor, sweetness, sourness, bitterness, and melting in mouth, as well as a binary question for overall acceptance and purchase intent. Overall, chocolate formulations enriched with CHO extracts accounted for higher overall acceptance and purchase intent consumer ratings. Through factor analysis, the correlation and interaction between intensity and likeness attributes were assessed. In addition, boxplot analysis was performed to gain insight regarding the relationship between the perceived complexity of the sensory attributes and the mean quantitative descriptive responses specified by consumers.

5.2 Introduction

Cocoa bean shells (CBS), the outermost layer of the cocoa bean, are one of many by-products heavily accumulated in the cocoa industry. CBS by-product is rich in dietary fiber (13.86-60.6 % w/w) and polyphenols (1.45-94.95 mg GAE/g) (Delgado-Ospina et al., 2020; Lessa et al., 2018; Martínez et al., 2012; Mellinas et al., 2020; Nsor-Atindana et al., 2012; Rojo-Poveda et al., 2020; Utami et al., 2016; Younes et al., 2022). This composition makes CBS a potential biomass for its

valorized use as a source of low-cost, clean label, functional ingredients such us fiber enhancer, stabilizer, and color enhancer (Adamafio et al., 2013; Barišić et al., 2019; Choi et al., 2019; Jozinović et al., 2019; Martínez-Cervera et al., 2011; Okiyama et al., 2017; Panak Balentić et al., 2018; Rojo-Poveda et al., 2020; Younes et al., 2022). However, studies have yet to assess isolated CBS cell wall polysaccharides in food applications. From our previous findings in par 3.4.3 alkaline treatment served as an optimal process for the isolation of cell wall polysaccharides, which was predominantly comprised of pectic regions, namely rhamnogalacturonan, arabinan, and arabinogalactan. In addition, the enzymatic generation of oligosaccharides of diverse degrees of polymerization (DP), ranging from 2 to 10, from CBS cell wall polysaccharides can be achieved using a commercially available multi-enzymatic system namely, Depol™ 670L. Our previous findings also suggested that CBS oligo- and polysaccharides can be classified as a prebiotic and possess antioxidant properties, thereby broadening its industrial applications (Younes et al., 2022).

Lab-scale practices were optimal towards efficient recovery for structural characterization and compositional analysis yet rendered minimal quantities. Therefore, CBS scale-up efforts were performed to account for a high-volume recovery of CBS polysaccharide extracts. To the author's knowledge, no study has assessed the recovery of CBS oligo- and polysaccharides in a pilot plant scale. The present study investigates the efficiency of two approaches using alkaline extraction and commercially available multi-enzymatic system (Depol™ 670L) for the recovery of CBS carbohydrate (polysaccharide) and hydrolyzed carbohydrate (oligosaccharide) extracts at a pilot-scale level. In addition, this study also explores the enrichment of chocolate-based formulations composed of cocoa, carob at selected ratios with CBS carbohydrate extracts. This study will generate consumer predictive models thereby establishing a link between key sensory properties of enriched chocolate formulations and consumer acceptability and purchase intent.

5.3 Materials and Methods

5.3.1 Materials

Commercial samples of CBS (CCN 51) were supplied by (Guayaquil, Ecuador). Commercial multi-enzymatic system Depol™ 670L from *Trichoderma reesei*, was provided by Biocatalyst Ltd (IL, USA). 1-Kestose, nytose, and 1F-fructofuranoysl-nystose were obtained from Wake Pure Chemical (Osaka, Japan). Food grade potassium hydroxide was purchased from Spectrum Chemical Mfg Group (New Brunswick, NJ, USA). Food grade hydrochloric acid as well as

analytical grade reagents were from Sigma- Aldrich Co., St. Louis, MO, USA. Salts were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

5.3.2 Lab-scale extraction of cocoa bean shell polysaccharides

The cell wall material (CWM) was sequentially isolated as described by Redgwell et al. (2003). Using de-fatted CBS (4g), the isolation proceeded as follows: 1) 80% ethanol (2hrs, 50 rpm), 2) chloroform: methanol (1:1, 1.5 hrs, 50 rpm), 3) chloroform: methanol (1:1, 1.5 hrs, 50 rpm), 4) phenol: acetic acid: water (PAW, 2:1:1, overnight), 5) phenol: acetic acid: water (PAW, 2:1:1, 2hrs). Each treatment was followed by centrifugation (7,000xg, 15mins) and vacuum filtration prior to the following step. The recovered CWM was dialyzed (5-8kDa cutoff) against distilled water and freeze-dried (-50˚C, 0.003 mbar). Extraction of CBS polysaccharides was performed as described by Khodaei and Karboune (2013) where CWM material (2% w/v) was suspended in 0.5M KOH solution containing 0.02M $NaBH_4$. Mixtures were incubated (60˚C, 24 hrs), thereafter centrifuged (10,000 x g for 15 mins) and recovered via filtration (0.45µm). Recovered polysaccharides were neutralized with HCl, dialyzed (5-8 kDa cutoff) and freeze-dried (-50˚C, 0.003 mbar).

5.3.3 Pilot-plant extraction of cocoa bean shell oligo- and polysaccharides

CBS of CCN 51 variety were suspended in 75l KOH solution (0.5M) to reach a concentration of 2% (w/v) with constant stirring for 3 hr at 60˚C. The suspension was then left with constant stirring for 18hr at 25˚C. The solution was neutralized with HCl to a desired pH between 6 to 8. The resulting suspension was subjected to two rounds of centrifugation, basket centrifugation using the Western States STM-2000 (3600 rpm, MWCO basket 100um) followed by centrifugation with a speed of 11,000 rpm, feed rate 3PSI using the Dexter MiSR 1010 (SRS A USI Company, MI, USA). The filtrate was collected and subjected to subsequent ultra- and diafiltration (1 time) steps, on the recovered precipitate, using a Koch Hollow Fiber Cartridge (with a MWCO of 5000 Da). A fraction of the retentate was collected and lyophilized (Sublimator 50-3 EKS, Zirbus Technology). The freeze-dried powder of pilot-scale cocoa bean shell polysaccharides is further abbreviated as CHO extract. The remaining fraction was taken further for enzymatic-based hydrolysis for the generation of oligosaccharides. The pH of the solution was adjusted to pH 5.5 to achieve the optimal conditions of the multi-enzymatic product, Depol™ 670L (135 galactanase units/ml) which were suspended with the CHO extract with constant stirring for 3hr at 40˚C. The

hydrolysate was collected and lyophilized. The freeze-dried powder of pilot-scale cocoa bean shell oligosaccharides is further abbreviated as hydrolyzed carbohydrate extract, hCHO extract.

5.3.4 Analytical methods for the quantification and characterization of carbohydrate extracts

5.3.4.1 Determination of total neutral sugars and uronic acid contents

Uronic acid content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid colorimetric assay was used for the determination of neutral sugar content (DuBois et al., 1956).

5.3.4.2 Monosaccharide profile

The carbohydrate extracts were subjected to acid hydrolysis according to the method of Spadoni Andreani et al. (2021) . Briefly, 1 ml of a mixture of HCl/methanol (1:4 v/v) was added to the sample (200 µl of liquid extract, or 5% (w/v) of solid in water). The mixtures were incubated at 70 °C for 24 hr and dried by heating in a convection incubator (Excella E24, New Brunswick Scientific, Canada). To complete hydrolysis, 3 ml of water and 1 ml of trifluoroacetic acid were added and heated at 100 °C for 1 hr. After evaporating the trifluoroacetic acid. The samples were neutralized with NaOH and centrifuged (8,000 rpm, 5 min). The supernatants (0.02 ml) were analyzed by high performance anion exchange chromatography (HPAEC) on a Dionex ICS 3000 system equipped with pulsed amperometric detection using a Carbopac PA-20 column (Dionex Co., Sunnyvale, CA, USA) at a temperature of 30 ˚C. Mobile phase was 20mM NaOH at flow rate of 0.4 ml/min. Rhamnose, arabinose, glucose, xylose, galactose, and mannose were used at varying concentrations (2.5 – 50µM) as standards.

5.3.4.3 Molecular weight profile

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525 binary HPLC pump, equipped with a Model 2414 refractive index detector, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution of the carbohydrates. Analysis was determined using a TSK G5000 PWXL (Tosoh Co., Yamaguchi, Japan) column, with dextrans (50– 670 kDa), soybean rhamnogalacturonan (0.125-1 g/l) and galactoglucomannan (0.125-1g/L) standards for molecular weight and concentration calibration. The temperature of the system was 30 °C, the eluent was 0.1 M NaCl, and the flow rate was set at 0.4 ml/min.

5.3.5 Preparation of chocolate

Chocolate formulations, containing 40-65% cocoa mass with and without 25-50% (w/w) carob powder, were prepared according to CODEX STAN 87 -1981 (Codex Alimentarius, 1981) with slight modifications. The organoleptic properties of chocolate formulations were enhanced with the addition of cane sugar (10.5-11.25%, w/w) and soy lecithin (0.05%, w/w). Formulations were prepared by combining cocoa butter (15-19.5%), carob powder (25-50%), and soy lecithin (0.05%) together. In a separate mixture, cocoa mass (40-65%) and cane sugar (10.5-11.25%) were combined. Batters were melted in the microwave, with mixing and scraping of bowl sides at uniform intervals. Once a uniform mixture consistency was achieved, the two batters were blended using a Robot Coupe Food Processer (R100) with blade attachment for 10 minutes with scraping of bowl sides after each minute, this allowed for the development of a uniform emulsion. Batters were then tempered to 32˚C before molding. Three selected chocolate formulations were enriched with CHO extract from CBS (1%), namely 40% Cocoa/Carob/CHO (75:24:1, w/w/w), 40% Cocoa/Carob/CHO (50:49:1), and 65% Cocoa/Carob/ CHO (75:24:1, w/w/w). Table 1 shows the proportion of ingredients for formulations with and without CHO extract. Each chocolate bar consisted of 30 grams of combined batter and were left to cool at 4˚C. Following cooling, chocolates were analyzed for sensory properties. Measurements were done the same day as baking.

5.3.6 Sensory evaluation of chocolate formulations

The chocolates were analyzed for their intensity and likeness using untrained panelists consisting of 60 McGill University students, staff, and faculty. The sensory attributes were evaluated using the Compusense software (Compusense Inc, On, CA) and a mean quantitative descriptive 9-point hedonic scale (extremely dislike/weak intensity, moderately dislike/weak intensity, dislike/weak intensity, slightly dislike/weak intensity, neutral liking/intensity, slightly like/strong intensity, like/strong intensity, moderately like/strong intensity, extremely like/strong intensity).

Table 5.13. Chocolate formulation composition before and after addition of carbohydrate extract

			% Chocolate Proportion (w/w)			
	Formulation	**Chocolate:Carob:CHO**	**Cocoa mass**	**Cocoa butter**	**Sugar**	**Lecithin**
Before addition	40% Cocoa	100:0:0	40	39	20.9	0.1
	40% Cocoa + Carob	75:25:0	40	39	20.9	0.1
	65% Cocoa + Carob	75:25:0	65	19.9	15	0.1
	40% Cocoa + Carob	50:50:0	40	39	20.9	0.1
	65% Cocoa + Carob	50:50:0	65	19.9	15	0.1
After addition	40% Cocoa + Carob + CHO	75:24:1	40	39	20.9	0.1
	40% Cocoa + Carob + CHO	50:49:1	40	39	20.9	0.1
	65% Cocoa + Carob + CHO	75:24:1	65	19.9	15	0.1

The panelists were considered untrained as they were not familiarized with the attributes prior or during testing. The sensory attributes consisted of chocolate flavor, sweetness, sourness, bitterness, and melting in mouth. Moreover, panelists were asked three binary choice questions (yes/no) regarding if the attributes were too complex to assess, overall acceptance, and purchase intent of the product.

5.3.7 Statistical Interpretation

Statistical analysis of the saccharide profile was performed using XLSTAT software (Addinsoft, New York, NY, USA) in Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$). Regarding the sensory evaluation data, Statistical Analysis System (SAS version 9.4) was used. The Proc Means was used to calculate the average and standard deviation of quantitative descriptive scores and liking scores. The Proc Can was used to assess the multivariate analysis of variance and canonical results, which were used to interpret the relationship between attributes and formulations. Consumer data were coded with a positive response denoted as 1 and a negative response as 2. The Proc Logistic was used to relate the consumer response for overall acceptance and purchase intent to the liking scores of attributes according to the following logistic function:

$$P\left(\frac{1}{x}\right) = \exp(\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n)/\{1 + \exp(\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n)$$

Where, P (1/x) is the probability of overall acceptance or purchase intent; x_1, x_2,..., x_n are the intensities of attributes; and α, β_1, β_2,..., β_n are parameter estimates associated with the model terms (Malundo et al., 2001). Additionally, factor analysis was performed on the mean scores using RStudio (RStudio, Inc., Version 4.0.2) for all samples to visually observe sample grouping and differentiation. To assess the correlations between sensory attributes, a heatmap was created using RStudio (RStudio, Inc., Version 4.0.2).

5.4 Results and Discussion

5.4.1 Recovery of carbohydrate extracts using laboratory and pilot-plant scale up isolation

CBS were used in a continuous flow pilot-plant facility for the isolation of their CHO (enriched with polysaccharides) and hCHO (enriched with oligosaccharides) extracts using alkaline and

commercial multi-enzymatic treatment, respectively. Conversely, the lab-scale process was performed as an individual batch system. For batch comparisons, lab-scale extraction commenced using 100 mL total volume and 2% (w/v) CBS cell wall material, while pilot-plant extraction consisted of 88.31 L total volume and 2% (w/v) CBS starting material. Figure 5.5 shows the pilot-plant process, where CBS (2%, w/v) was suspended in potassium hydroxide (0.5 M) with constant stirring for 3 hours at 60˚C followed by overnight incubation at 25˚C. Two consecutive rounds of centrifugation were necessary for retentate and precipitate separation, one using a basket centrifuge, followed by a pilot-scale centrifugation step. In pilot-scale the CBS suspension mimicked a gelatinous consistency, making it difficult to separate the retentate from the precipitate; this was attributed to the rich pectin content in CBS. This phenomenon was encountered at the lab-scale, however to a lesser extent. This can be attributed towards the use of $NaBH_4$. as a reducing agent, at the lab-scale level. Without a reducing agent, as is the case at the pilot-scale, accelerated hydrolysis rates are facilitated, contributing to a higher degree of debranching (Minkina et al., 2012). The suspension was then subjected to ultra- and diafiltration steps to isolate and concentrate, which yielded an extract rich in pectic and hemi-cellulosic polysaccharides (CHO). Diafiltration steps were performed to remove interfering salts, accumulated from the alkaline extraction and neutralization steps (Resende et al., 2012). That being said, a balance should be established between recurring rounds of diafiltration as they are attributed to significant losses due to carbohydrate adhesion to the membrane filter. In addition, membrane fouling, indicated by irreversible loss of membrane permeability is possible which can prompt further column washing and chemical cleaning (Pu et al., 2012). The second process commenced with CHO extract, enriched with polysaccharides, in which Depol™ 670L multi-enzymatic system was added; the enzymatic hydrolysis for the efficient release of oligosaccharides was carried out with constant gentle stirring for 3 hrs at 40˚C (Figure 1).

Table 5.14 shows the recovery yield of the CHO extract on the lab- and pilot-scales. The crude extract, containing mono-, di- and saccharides greater than 5 kDa, was recovered by 78.4% (w/w), with some loss attributed to the sequential centrifugation processes as they follow a continuous flow. Following ultra/diafiltration and freeze drying steps, the CHO extract recovery yield was achieved at 3.69% (w/w) at the pilot scale level. This yield is significantly ($P < 0.05$) lower than that recovered at the lab-scale level (29.1%, w/w).

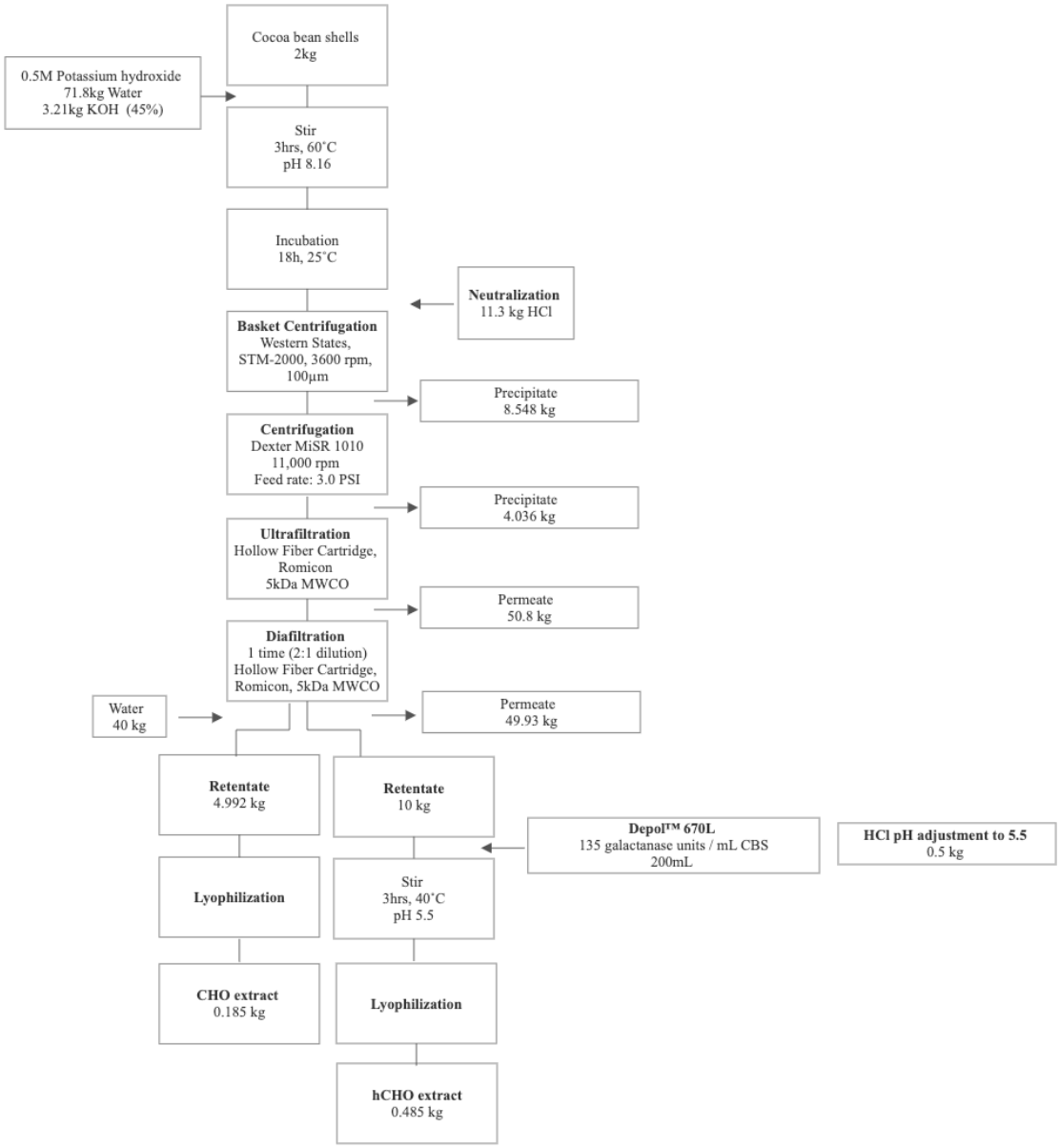

Figure 5.5. Flow diagram of pilot-scale production of cocoa bean shell (CBS) oligo- and polysaccharides by alkaline extraction (CHO extract) and upon commercial multi-enzymatic system Depol™ 670L treatment (hCHO extract).

The low recovery can be explained by the high proportion of saccharides lower than 5 kDa contained within the extract, removed during ultra- and diafiltration, as the focus was on the recovery of polysaccharides (> 5kDa). Waglay et al. (2019) reported similar findings where lower yields were attained at the pilot-scale level (21.6-44.6%) for the recovery of potato proteins when compared to laboratory extraction (50.9-74.4%). On the contrary, the total carbohydrate content at the pilot-scale level (90.6% w/w) was higher than at the lab-scale level (78.9% w/w), where significantly ($P < 0.05$) higher proportion of neutral sugars were recovered. This was attributed to the interference of proteins present within the extracts at the lab-scale level as reported in our previous study.

The monosaccharide profile of the extracts, recovered at the lab-scale level, showed a distribution of both pectic and hemicellulosic regions, indicated by the high proportion of uronic acid (51.4%), rhamnose (12.4%), mannose and xylose (12.6%), and arabinose (8.6%). Alternatively, the extract recovered at the pilot-scale level, resulted in higher proportions of uronic acid (55.9%), galactose (17.4%) and glucose (13.3%) indicating the presence of rich pectic regions. It is important to note that lab-scale alkaline extraction incorporated a reducing agent, sodium borohydride ($NaBH_4$). This may explain the higher pectic regions recovered at the pilot-scale level, where the exclusion of $NaBH_4$ mediated faster hydrolysis rates than at the lab-scale level (Minkina et al., 2012).

Figure 5.3 shows the MW distribution of CHO and hydrolyzed CHO extracts retrieved at pilot-scale as determined by size exclusion chromatography. The presence of four main populations are observed for both extracts, where CHO populations were found at 0.83, 2.1, 320, and 2400 kDa; similar to those reported in par 3.4.1 for hydrolyzed CHO populations were found at 0.87, 1.1, 1.6, and 3.3 kDa. This shows that high enzymatic hydrolytic activity was expressed by Depol™ 670L, which led to the enzymatic generation of oligosaccharides of varying DP compared to isolated polysaccharides in the CHO extract.

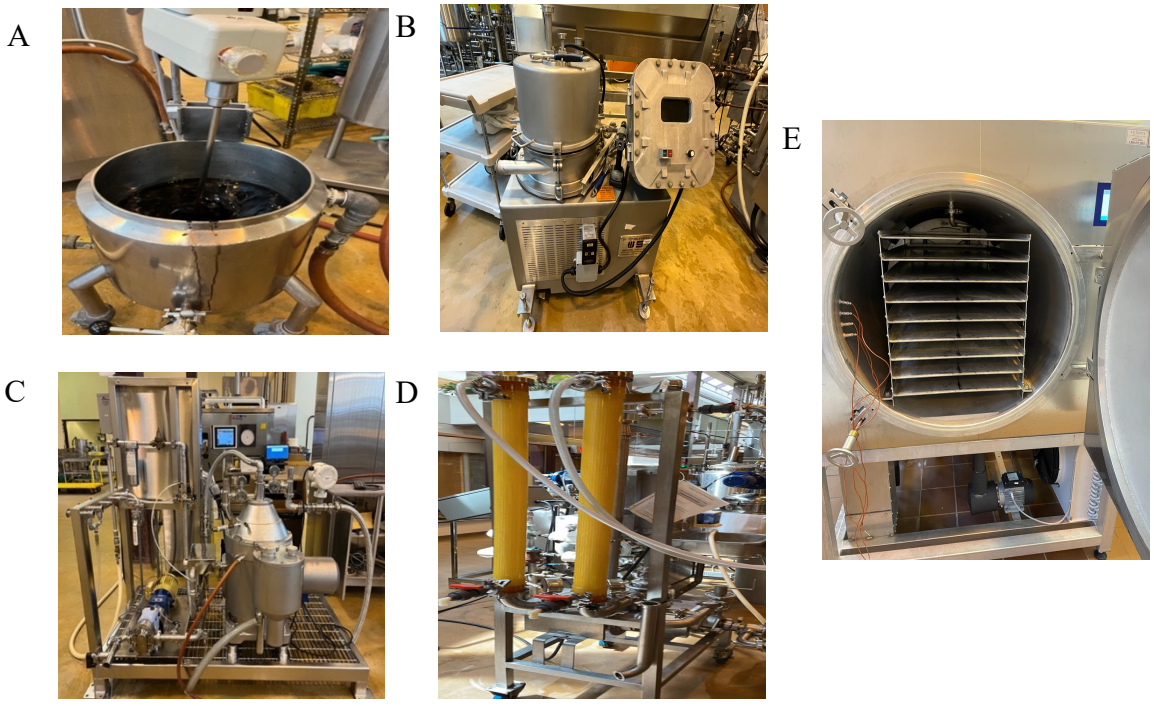

Figure 5.6. Images of equipment utilized in the pilot-plant extraction process, A - 250L double jacketed vessel equipped with an immersion mixer; B – Basket centrifugation (Western States STM-2000) equipped with 100µm MWCO; C – Centrifuge (Dexter MiSR 1010); D – Ultra- and diafiltration with Koch Hollow Fiber Cartridge (5000 Da MWCO); E – Lyophilizer (Sublimator 50-3 EKS, Zirbus Technology).

Table 5.14. Effects of extraction process scale-up of cocoa bean shell polysaccharides (carbohydrate extraction) on the recovery yield, total carbohydrate content, neutral sugar and uronic acid contents, and the relative proportion of saccharide profile.

		Carbohydrate extract	
		Laboratory Scale	*Pilot Scale*
Crude extract recovery yield (%, w/w)*		-	78.4
Extract recovery yield (%, w/w)		29.1[a]	3.69[b]
Total CHO content (%, w/w)		78.9	90.6
Neutral Sugar (%, w/w)		20.6±0.7[a]	30.5±7.6[b]
Uronic content (%, w/w)		58.3±5.2[a]	60.1±22.3[a]
Saccharide Profile (%, rel. proportion)	Fucose	1.8±0.02[a]	1.8±0.1[b]
	Rhamnose	12.4±0.3[a]	1.2±0.1[b]
	Arabinose	8.6±0.01[a]	5.8±1.1[a]
	Galactose	8.1±0.2[a]	17.4±1.1[b]
	Glucose	5.2±0.5[a]	13.3±2.9[b]
	Mannose, Xylose	12.6±2.0[a]	4.7±0.4[b]
	Uronic acids	51.4±3.1[a]	55.9±1.3[b]

Values reported as mean ± standard deviation

*Calculated as recovery yield prior to ultra- and diafiltration

[a-b] Within the same row, means with different letters are significantly different at $P < 0.05$

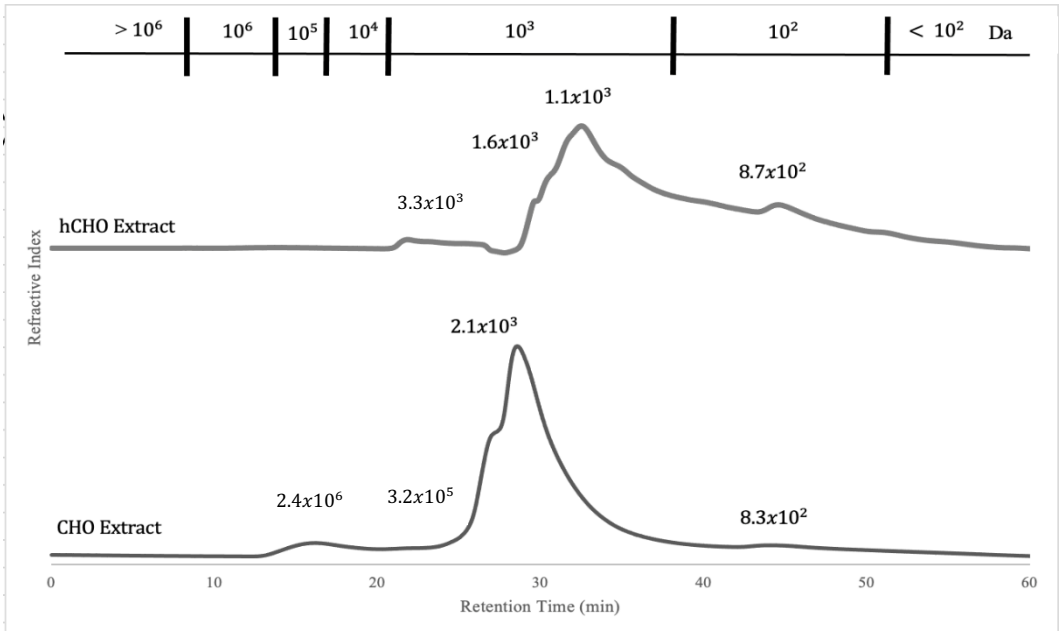

Figure 5.3. Size exclusion chromatogram of carbohydrate extract recovered in pilot scale by alkaline extraction and hydrolyzed carbohydrate extract recovered using commercial multi-enzymatic product (Depol™ 670L)

5.4.2 Chocolate-based formulations enriched with carbohydrate extract

5.4.2.1 Statistical interpretation of chocolate-based formulations

In order to assess multiple attributes on a single set of chocolate formulation design, multivariate statistics analysis was used (Lawless & Heymann, 2010). Table 5.3 shows the multivariate analysis of variance (MANOVA) results, represented by *F-values* greater than 1 and *P values* less than 0.05, which indicate that the chocolate formulations were perceived differently by the panelists before addition of the CHO extract. Alternatively, this was not that case for chocolate formulations upon enrichment, suggesting that the addition of CHO prompted consumers to perceive no significant differences between the samples. Canonical discriminant analysis can be used to compliment MANOVA by determining the multi-dimensional mean separation of the attributes, otherwise known as the canonical coefficient (CAN), allowing us to determine the attribute(s) responsible for the group differences (Lawless & Heymann, 2010). Our results indicate that chocolate flavor intensity (0.7278), melting in mouth intensity (0.7123), bitterness intensity (0.7155), chocolate flavor likeness (0.4664), and bitterness likeness (0.4536) are the attributes most responsible for group differences before addition of CHO extract.

Figure 5.4 outlines the sensorial responses of 60 panelists to the following intensity and likeness sensory scores of chocolate flavor, sweetness, bitterness, sourness and melting in mouth before and after the addition of CHO extract in chocolate-based formulations containing carob. Overall, the results show that higher likeness scores for all attributes were obtained with the enrichment of CHO extract in chocolate-based formulations. On the other hand, intensity scores of chocolate flavor, sweetness, bitterness, and sourness were decreased with the addition of CHO. This suggests that CBS polysaccharides play a role on masking attribute intensities while achieving desirable flavor profiles of chocolate-based formulations. It is possible that reduced intensities, with accompanying higher likeness scores for bitterness, are attributed to the enrichment with carob. There is an opposite trend for the 40% cocoa + carob (50:50) formulation, where an increase in the intensity is observed, further revealing the contribution of CBS CHO extract to the sweetening and chocolate flavor masked by the use of higher proportion of carob. García-Díez et al. (2022) reported carob contributed to reduced bitterness scores due to its rich dietary fibre (55.67 g/100g) and soluble sugar content (7.87 g/100g), namely glucose and fructose. Interestingly, only one attribute, namely, melting in the mouth showed increases in both intensity and likeness scores.

Table 5.15. Statistical interpretation of chocolate formulation design prepared with carob powder before and after addition of pilot-scale carbohydrate (CHO) extract

MANOVA						
	Statistic	**Value**	**F Value**	**Num DF**	**Den DF**	**Pr > F**
Before addition	Wilks' Lambda	0.660	3.37	30	664.03	<.0001
	Pillai's Trace	0.370	3.21	30	684.00	<.0001
	Hotelling-Lawley Trace	0.470	3.52	30	508.55	<.0001
	Roy's Greatest Root	0.356	8.12	10	228.00	<.0001
After addition	Wilks' Lambda	0.887	0.93	30	664.03	0.581
	Pillai's Trace	0.117	0.92	30	684.00	0.5881
	Hotelling-Lawley Trace	0.124	0.93	30	508.55	0.5724
	Roy's Greatest Root	0.087	1.99	10	228.00	0.0353

Canonical Analysis			
	Variable	**Can1**	**Can2**
Before addition	Chocolate Flavor Intensity	0.727821	0.10073
	Sweetness Intensity	0.604509	-0.032326
	Bitterness Intensity	0.71553	0.169553
	Sourness Intensity	0.564908	0.361457
	Melting in Mouth Intensity	0.712296	-0.218191
	Chocolate Flavor Likeness	0.466456	0.388913
	Sweetness Likeness	0.115789	-0.093993
	Bitterness Likeness	-0.453639	0.219391
	Sourness Likeness	-0.319053	0.515693
	Melting in Mouth Likeness	-0.166455	-0.00337

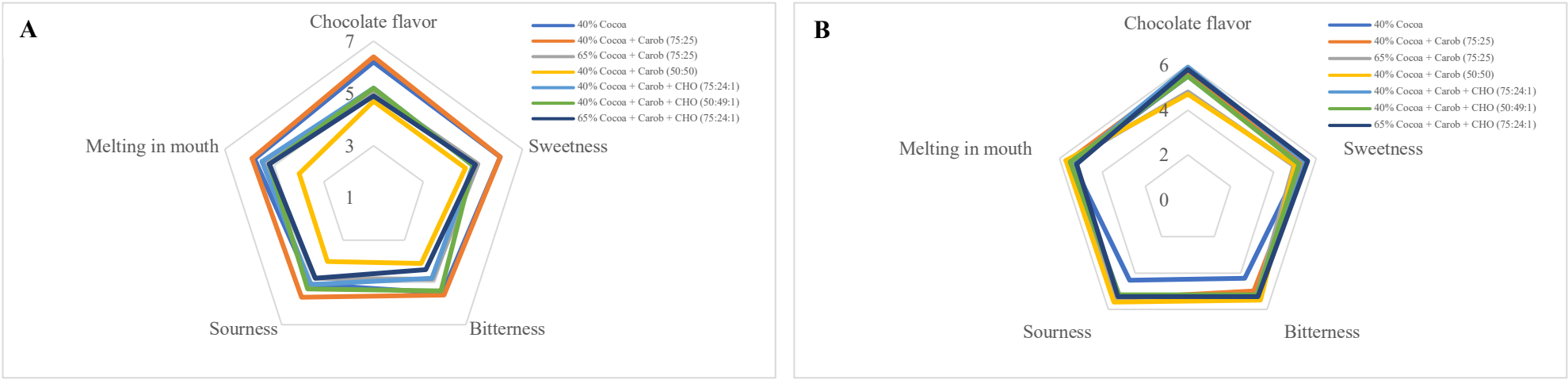

Figure 5.4. Average mean quantitative descriptive scores of selected chocolates enriched with and without carob and carbohydrate extract from pilot scale based on intensity (A) or likeness (B): 40% cocoa (blue); 40% cocoa + carob (75:25, orange); 65% + carob (75:25, gray); 40% cocoa + carob (50:50, yellow); 40% cocoa + carob + CHO (75:24:1, light blue); 40% cocoa + carob + CHO (50:49:1, green); 65% cocoa + carob + CHO (75:24:1, dark blue).

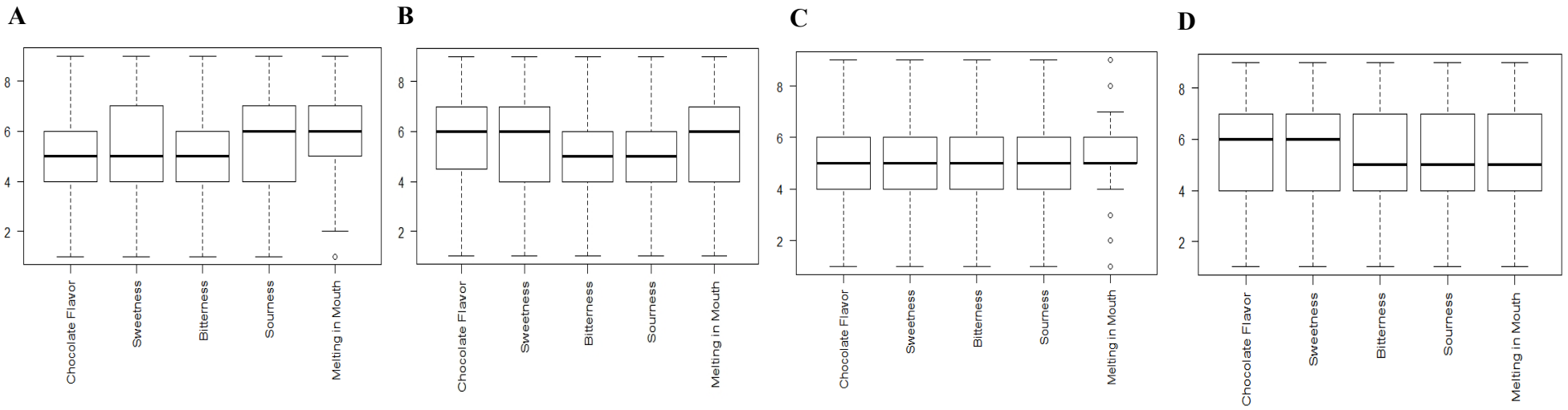

Figure 5.5. Boxplot analysis for perceived complexity of sensory attributes (chocolate flavor, sweetness, bitterness, sourness, melting in mouth) chocolates enriched with carob and carbohydrate extract from pilot scale based on before addition - intensity (A) likeness (B) and after addition (right) - intensity (C) and likeness (D) .

As seen in Table 5.4, the proportion of overall acceptance and purchase intent responses increased by as much as 16% and 14% likely suggesting that consumers favored formulations enriched with CBS CHO extract. Logistic regression analysis (LRA) can be applied using dependant responses (probability of overall acceptance and purchase intent of chocolate formulation) with independent variables (sensory attributes) to behave according to a multivariate normal distribution (Malundo et al., 2001; Waglay & Karboune, 2020). Determinants impacting the overall acceptance and purchase intent are identified based on the lowest *P-value* and highest odds ratio scores. According to LRA results (Table 5.4), chocolate flavor intensity and sweetness likeness were found to be the most significant ($\alpha = 0.05$) and determinant factors for the overall acceptance and purchase intent of chocolate-based formulations enriched with CBS carbohydrate extract.

Consumers' preferences are influenced by several factors, one being the perceived complexity of sensory attributes. Berlyne (1967) states that perceived complexity and hedonic measurement are interconnected, where an individual's hedonic response to a stimulus increases with its complexity until an optimal threshold is achieved followed by a decline, resembling an inverted U-curve. Figure 5.5 depicts the boxplot analysis of perceived complexity of sensory attributes in terms of chocolate flavor, sweetness, bitterness, sourness, and melting in mouth of chocolate-based formulations before (Figures 5.5A and 5.5B) and after enrichment with CBS CHO extract (Figures 5.5C and 5.5D). Indication of differences in the perceived complexity of attributes is identified by mean scores (bold line) residing outside the comparison boxplots. Prior to enrichment, there lies a divide between the perceived complexity of intensity attributes, in which chocolate flavor, sweetness and bitterness attained lower degree of perceived complexity compared to sourness and melting in mouth. Interestingly, in terms of the complexity of likeness attributes, bitterness and sourness are perceived with a lower degree of complexity compared to chocolate flavor, sweetness, and melting in mouth. Upon enrichment of the chocolate-based formulations, boxplot analysis suggests no differences among the intensity and likeness attributes in terms of their perceived complexity. According to Köster & Mojet (2007a, 2007b), exposure to a lower complexity will have no effect on the consumer's optimal complexity level. This may explain the account of no observed differences in the perceived complexity of intensity and likeness attributes upon enrichment.

Table 5.4. Percentage overall acceptance and purchase intent of selected chocolate-based formulations and the statistical interpretation of chocolate-based formulation prepared with carob and pilot-scale CHO extract based on logistic regression analysis ($P < 0.05$).

	% Overall Acceptance	% Purchase Intent
40% Cocoa + Carob + CHO (75:24:1)	89	68
40% Cocoa + Carob + CHO (50:49:1)	82	69
65% Cocoa + Carob + CHO (75:24:1)	86	68
65% Cocoa + Carob (50:50:0)	73	55

Logistic Regression Analysis

	Overall Acceptance			Purchase Intent		
	Estimate	Pr > χ^2	Odds Ratio	Estimate	Pr> χ^2	Odds Ratio
Chocolate Flavor Intensity	0.48	<.0001	1.62	0.35	<.0001	1.42
Sweetness Intensity	0.10	0.30	1.11	0.01	0.91	1.01
Bitterness Intensity	-0.01	0.93	0.99	0.02	0.83	1.02
Sourness Intensity	0.05	0.63	1.05	-0.06	0.49	0.94
Melting in Mouth Intensity	0.17	0.22	1.18	0.23	0.03	1.26
Chocolate Flavor Likeness	-0.15	0.38	0.86	0.12	0.30	1.13
Sweetness Likeness	0.59	0.00	1.80	0.31	0.01	1.37
Bitterness Likeness	0.02	0.86	1.02	0.13	0.16	1.14
Sourness Likeness	-0.13	0.30	0.88	-0.05	0.61	0.95
Melting in Mouth Likeness	0.44	0.00	1.56	0.09	0.40	1.10

In addition, enrichment may have imparted a synergistic blend among sensory attributes towards the consumers experience. Furthermore, studies suggest that perceived complexity are influenced by several factors, namely the number of attributes assessed, the consumers level of expertise, familiarity, or gender (Lawless & Heymann, 2010; Livermore & Laing, 1998; Palczak et al., 2019). Humans have a limited capacity to distinguish many attributes, suggesting that sensory attributes should be confined to no more than four at a time (Lawless & Heymann, 2010).

5.4.2.2 Correlation of sensory attributes

A correlation study of intensity and likeness sensory attributes was performed to determine the strongest correlations impacting panelists' responses. Figure 5.6 presents the correlation coefficients of intensity and likeness attributes before and after enrichment of CHO extract in chocolate-based formulations. The results prior to enrichment suggest that chocolate flavor likeness with sweetness likeness ($R = 0.69$), melting in mouth likeness with chocolate flavor likeness ($R = 0.64$), and melting in mouth likeness with sweetness likeness ($R = 0.61$) were viewed as the strongest positive correlations with significance ($P < 0.001$). In addition, sourness intensity with melting in mouth intensity ($R = 0.49$), bitterness intensity with sourness intensity ($R= 0.43$), and bitterness intensity with melting in mouth intensity ($R = 0.34$) were viewed as moderately positive correlations. Interestingly, only one correlation between intensity and likeness attributes was observed, being chocolate flavor intensity with melting in mouth likeness ($R = 0.34$). Contrary findings were reported by (Leite et al., 2013), who found a negative correlation ($R = -0.89$) between melting quality, defined as the length of time for chocolate to melt in the mouth, with chocolate flavor, defined as the residual of dark chocolate flavor. The length of melting time is directly proportional to the particle size distribution of non-fat solids within chocolates, where studies have shown the relationship significantly contributing towards the sensory quality (Beckett, 1994; Lee et al., 1992; Pangborn et al., 1973; Ziegler et al., 2001). Indeed, according to several studies, the particle size can impact the perceived intensities and duration of the perception of flavor, sweetness and other attributes (Beckett, 1994; Lee et al., 1992; Pangborn et al., 1973; Ziegler et al., 2001).

Enrichment of chocolate-based formulations provided a higher extent of correlations with significance ($P < 0.001$) between intensity and likeness attributes, which include chocolate flavor likeness with chocolate flavor intensity ($R = 0.65$), chocolate flavor intensity with sweetness likeness ($R = 0.53$), melting in mouth likeness with melting in mouth intensity ($R = 0.5$), chocolate

flavor intensity with sourness likeness (R = 0.46), and chocolate flavor intensity with bitterness likeness (R = 0.44). Overall, chocolate flavor intensity showed moderate to strong correlations among all likeness attributes, suggesting that CBS polysaccharide enrichment may have played a role in the development of a complex chocolate matrix, due to the increase in intensity and likeness attribute interactions. One study conducted by (Al Sheraji et al., 2017) observed similar responses with polysaccharides, derived from mango peels, in non-fat yoghurt, which can suggest that polysaccharide enrichment can impart better mouthfeel consistencies in even fat-free food formulations

To show an overview of the sample variation in relation to perceived complexity and the correlation of intensity and likeness sensory attribute individually, factor analysis was applied. Figure 5.7 presents the two-dimensional (MR1, MR2) biplots generated for intensity and likeness sensory attributes before and after enrichment with CHO extract. Before enrichment, the cumulative variance accounts for 42.5% and 69% of the dataset; where MR1 accounts for 23.7% and 43.7% of the variance for intensity and likeness biplots. Each attribute is represented by a vector, where the vector length expresses the degree of variance; suggesting that a longer vector would indicate higher variance. In addition, the angle of the vector would indicate the degree of correlation between the attributes, where angles smaller than 90˚ indicate strong correlations.

As seen in Figure 5.7A, all intensity attributes show positive correlations, where the strongest correlations are observed for both, sourness and melting in mouth with bitterness. Moreover, sourness shows the highest level of variance, suggesting that this attribute is most responsible for the differences within the dataset. Alternatively, weaker correlations, signified by angles smaller than 90˚, are observed for both chocolate flavor with sweetness and sourness. Furthermore, chocolate flavor and sweetness attributes have a lower contribution towards the dataset, considering their low level of variance as determined by their small vector lengths.

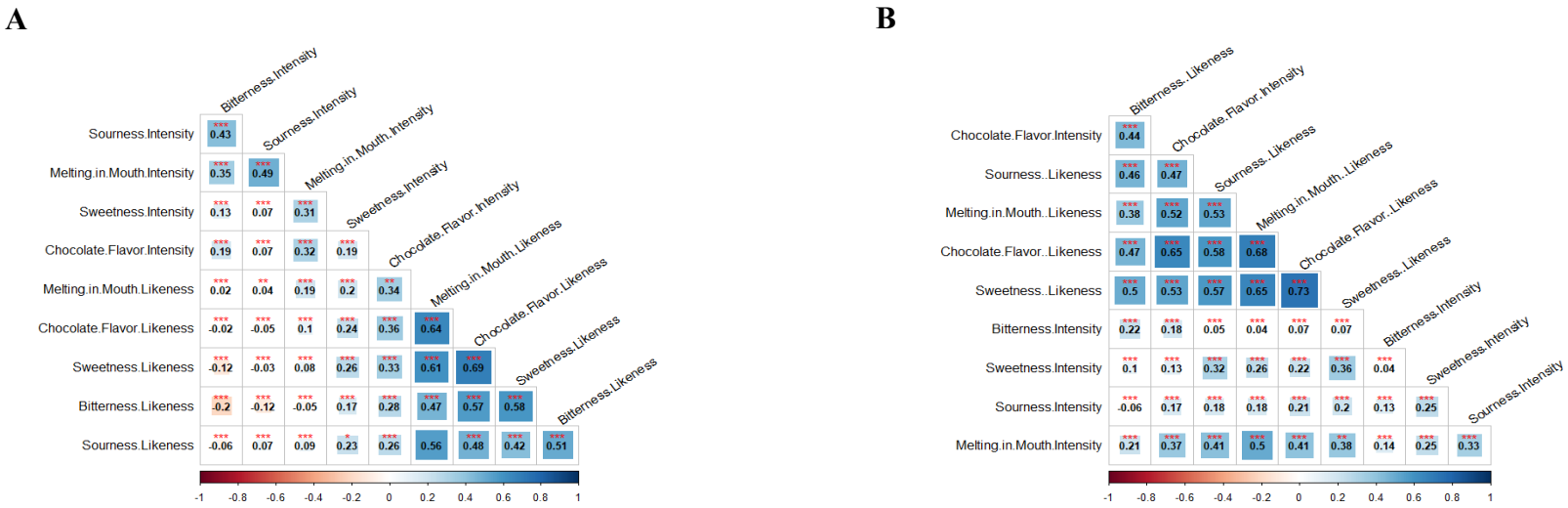

Figure 5.6. Correlation between intensity and likeness scores of each attribute, chocolate flavor, sweetness, bitterness, sourness, and melting in mouth, before (A) and after (B) addition of CHO extract in chocolate formulations; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

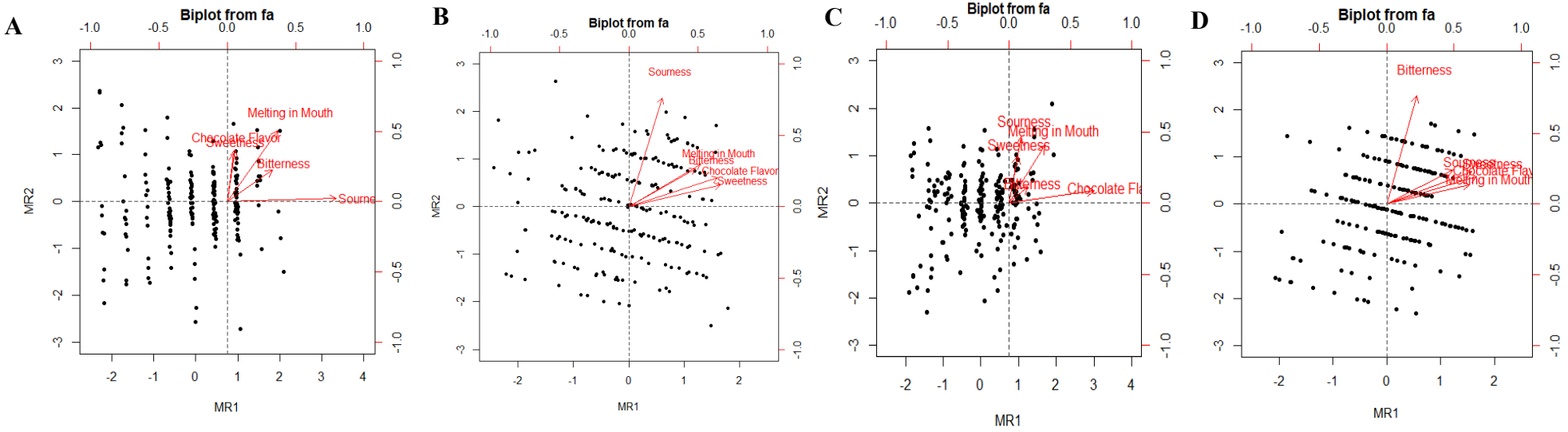

Figure 5.7. Bi-plot factor analysis of sensory attributes (chocolate flavor, sweetness, bitterness, sourness, melting in mouth) chocolates enriched with carob and carbohydrate extract from pilot scale based on before addition - intensity (A) likeness (B) and after addition (right) - intensity (C) and likeness (D).

In Figures 5.7C and 5.7D, the cumulative variance of the two dimensions represents 34.8% and 70.9%, with MR1 which explains the 18.9% for intensity and MR2 which explains the 47.3% for likeness. Similar to the intensity biplot, sourness is observed as the attribute responsible for differences within the dataset, while a shared level of variance is observed for the remaining attributes for likeness (Figure 5.7B). Strong positive correlations are observed between chocolate flavor with sweetness and melting in mouth with bitterness; while weak correlations were found for sourness with sweetness. Based on the high proportion of variance, where chocolate flavor (for intensity) and bitterness (for likeness) were responsible for the differences after enrichment (Figures 5.7C and 5.7D). All attributes expressed positive correlations, with strongest interactions being observed between chocolate flavor with bitterness for intensity. On the other hand, likeness attributes only showed weak positive correlations between chocolate flavor, sweetness, melting and sourness.

5.5 Conclusion

A pilot-plant scale up process for the isolation of CBS oligo- and polysaccharides using commercially available multi-enzymatic system and an alkali treatment was developed. As expected, the recovery yields were lower than those encountered at lab-scale. However, the saccharide profile was comparable; showing that pilot plant CBS polysaccharides contained a combination of pectic and hemi-cellulosic polysaccharides based on the uronic acid (55.9%µM), galactose (17.4%µM), glucose (13.3%µM), arabinose (5.8%µM), as well as the mannose and xylose (4.7%µM) contents. Saccharides found in both extracts were predominantly of DP 2-5 (85.2% w/v) for CHO and DP 6-9 (57.6% w/v) for hCHO extracts. Based on the MW distribution profile, four main populations were found for each CHO (0.83, 2.1, 320, and 2400 kDa) and hCHO (0.87, 1.1, 1.6, and 3.3 kDa) extract. CBS polysaccharides were used for the enrichment of chocolate-based formulations, where sensory evaluation panels were held to assess the intensity and likeness of the chocolate flavor, sweetness, bitterness, sourness, and melting in mouth attributes. Based on the canonical discriminant analysis before enrichment, the formulations were perceived as significantly different from one another with chocolate flavor (intensity and likeness), bitterness (intensity and likeness), melting in mouth (intensity) which were responsible for group differences. Spider-plot analysis suggested that higher likeness responses were attained after enrichment, with a significant increase observed for melting in mouth. Furthermore, the perceived

complexity of the intensity and likeness attributes varied before and after enrichment. The results suggested that enrichment of chocolate-based formulations mediated no differences between the perceived complexity of the attributes. According to the correlation heatmaps, more interactions between intensity and likeness of the attributes was observed after enrichment, likely indicating that CBS polysaccharides contributed towards the development of a diverse and complex formulation matrix. Overall, all three enriched formulations attained highest overall acceptance (82-89%) and purchase intent responses (68-69%).

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CHAPTER VI. GENERAL CONCLUSION AND FUTURE WORK

This research focuses primarily on the isolation and characterization of cocoa bean shell oligo- and polysaccharides, respectively. These cell wall components are of high interest for their valorization as a potential functional food ingredient. Insight pertaining to the nutritional composition of cocoa bean shells, from two varieties (NAC and CCN) was explored. Cocoa bean shells were found to be an abundant source of carbohydrates, specifically dietary fiber, protein, ash, and polyphenols. Their chemical profile showed alterations upon various degrees of processing, namely fermentation, drying and roasting. This was primarily attributed to the subjection of fluctuating temperatures throughout the process. The isolation of the cell wall material was evaluated according to their total sugar composition, monosaccharide profile, and molecular weight distribution. The cell wall material extracts were found to contain mostly pectic polysaccharides and smaller amounts of hemicellulose. Pectic polysaccharides in the extract were characterized by the presence of uronic acid, galactose, arabinose, and rhamnose.

Diluted and concentrated alkali treatments, involving potassium hydroxide, were investigated for the isolation of polysaccharides from cell wall material of cocoa bean shells from both varieties (NAC and CCN). Both alkali extracts were successful in the recovery of polysaccharide predominantly enriched with pectic polysaccharide regions, possibly indicating the presence of rhamnogalacturonan and arabinogalactan. Increasing the alkali concentration resulted in a shift in the recovery from high to low molecular weight fractions, due to a higher degree of defragmentation. The more concentrated extracts were found to contain four main molecular weight populations, compared to the three main molecular weight fractions found in the diluted alkali extracts.

Enzymatic hydrolysis of cocoa bean shell cell wall polysaccharides using pure enzymes and five multi-enzymatic products was investigated for the generation of oligosaccharides. The multi-enzymatic products expressed varied levels of polysaccharide-degrading activities. Viscozyme® L and Depol™ 670L attained the highest hydrolysis extents for NAC and CCN cocoa bean shell varieties, while also generating a diverse complex of oligosaccharides with degree of polymerization between 3 and 10. A majority of the oligosaccharides (generated by multi-enzymatic biocatalysts had a degree of polymerization between 2 and 5. To explore their potential

functional properties, the prebiotic activities were assessed using cell wall polysaccharides (diluted alkali extracts) and oligosaccharides bio-generated by Viscozyme® L and Depol™ 670L, respectively. For the promotion of the growth of selected probiotic strains, *Lactobacillus rhamnosus GG* and *Bifidobacterium longum*, cell wall polysaccharides and enzymatically generated oligosaccharides performed similarly or more than inulin and rhamnogalacturonan based on their prebiotic activity scores. The short chain fatty acid profiles were mostly characterized by high amounts of lactic and acetic acid. In addition, enzymatically generated oligosaccharides also contributed towards a significant production of butyric and propionic acids.

Feruloylation of enzymatically generated oligosaccharides and diluted alkali polysaccharides was achieved by the esterifying activity of free feruloyl esterases, from *Humicola insolens* and rumen microorganism, using a surfactant-less microemulsion made of n-hexane/butanone/water (49.4/44.6/6, % v/v/v). Feruloyl esterase, from rumen microorganism, contributed towards the highest feruloylation efficiencies for polysaccharides, while that from *H. insolens* contributed more towards a higher feruloylation extent for enzymatically generated oligosaccharides. The antioxidant activity of feruloylated polysaccharides were assessed to gain insight on their radical scavenging functionalities. Based on three antioxidant assays, DPPH, ORAC and ABTS, significantly notable increases in the scavenging and antioxidant activity of feruloylated polysaccharides were observed compared to their native forms, i.e., non-feruloylated polysaccharides, as well as to ferulic and ascorbic acids.

The isolation of polysaccharides and the enzymatic generation of oligosaccharides were further scaled-up in a pilot plant facility. Comparing the extracts recovered at the laboratory-scale, those recovered in the pilot plant were similar in terms of their monosaccharide profile and total neutral sugar content, with the exception of a slightly higher recovery of hemicellulose. The molecular weight distributions for each of the oligo- and polysaccharide extracts showed four main populations. Taking advantage of the functional properties the extracts exhibited, the development of a chocolate-based product was investigated. Cocoa bean shell polysaccharide extracts produced select chocolate products which were evaluated sensorially to be more desirable than those without their enrichment. Sensory evaluation consisted of five attributes related to the intensity and likeness of the chocolate product, namely, chocolate flavor, sweetness, sourness, bitterness, and melting in mouth. The attributes were evaluated according to mean quantitative descriptive scores

and mean liking scores on a 9-point hedonic scale. According to the statistical analysis, the chocolate flavor (intensity and likeness), bitterness (intensity and likeness), melting in mouth (intensity) were responsible for the significant group differences prior to enrichment. Overall, higher likeness responses were obtained with enrichment, specifically in terms of the melting in mouth attribute. The relationships between the consumers liking scores and the perceived complexity were assessed to understand the degree of complexity inflicted upon consumers. Enrichment of the chocolate products did not contribute to differences between the perceived complexity of the sensory attributes. An understanding of the correlation between the intensity and likeness attributes indicated that polysaccharide enrichment contributed towards the development of a diverse and complex formulation matrix. All in all, consumers sought approval for the enriched chocolate products based on the overall acceptance and purchase intent responses.

Overall, this research contributes to the scientific knowledge providing a detailed description of the abundance and functional properties of cocoa bean shell oligo- and polysaccharides. It also offers a look at the feasibility of the generation of these extracts on an industrial scale, which may broaden the spectrum of their applications as a functional food ingredient.

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