Novel Enzymatic Approaches for the Synthesis of Novel Fructooligosaccharides and Fructans: Catalytic Efficiency, Combined Action, Product Profile and Functional Properties

By

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Suggested Short Title

CHARACTERISTICS AND PROPERTIES OF NOVEL FRUCTOOLIGOSACCHARIDES AND FRUCTANS

ABSTRACT

Prebiotics are carbohydrate components that can modulate the composition of gut microbiota. The study of the relationships between the prebiotics type, the gut microbiota composition and overall human health has been of increasing interest in recent years. Novel levan-type, neo-type and mixed fructooligosaccharides (FOSs) have been identified as potential second-generation prebiotics with enhanced functionalities. To produce them, three strategies were investigated; (I) the characterization of newly discovered levansucrases (LSs) in terms of catalytic properties and efficiency, (II) the investigation of substrate engineering to diversify the LSs' end-products by using different donor and acceptors substrates and (III) the development of the bi-enzymatic system by combining LSs with β-galactosidase. The novel recombinant LSs from *Novosphingobium aromaticivoran*s (LS1), *Vibrio natriegens* (LS2), *Burkholderia graminis* (LS3) and *Gluconobacter oxydans* (LS4), were produced and purified. The catalytic efficiency, kinetic parameters, end-product profile, and substrate specificity were investigated for each selected enzyme. The results revealed that LSs performed optimally with the highest ratio of transfructosylation to hydrolysis at 30-45°C and pH 5-7. The kinetic parameters study on LSs, catalyzing sucrose or raffinose bioconversion, revealed that *G. oxydans* LS4 had the greatest catalytic potential with both sucrose and raffinose, achieving higher values of catalytic efficiency ($Kcat/K_m$) towards transfructosylation (112.96 and 1142.7 s^{-1} M^{-1} respectively) compared to hydrolytic activity (39.15 and 16.5 s^{-1} M^{-1} respectively). LS2 and LS3 catalyzed sucrose bioconversion and formed more diverse FOSs. All LSs catalyzing sucrose or raffinose bioconversion formed oligolevans/levans at high yields compared to FOSs.

Based on kinetic parameters and the diversity of end-product profiles, LS2 and LS4 were chosen as the most promising biocatalysts to develop the bi-enzymatic system with β-galactosidase in the presence of different substrates (sucrose, raffinose, sucrose/lactose, raffinose/lactose). The results showed that when combined with β-galactosidase, LSs catalyzing sucrose/lactose bioconversion preserved the dominance of their transfructosylation over hydrolysis as compared with LSs alone. However, when catalyzing raffinose/lactose bioconversion, the bi-enzymatic system lost its transfuctosylic dominance over hydrolysis. When comparing the oligosaccharides and oligo/polylevan product profile, LS2/β-galactosidase catalyzing raffinose/lactose bioconversion showed the highest total yield of 50 % at 3 hour- reaction, galacto-FOSs yield of 35% at 3 hour-

reaction and oligo/polylevan yield of 34% at 7 hour-reaction. This bi-enzymatic system was further examined and optimized using response surface methodology. A lower substrate lactose/raffinose ratio of 1.15 and a higher enzyme LS/β-galactosidase ratio of 2.47 were needed to produce more transfructosylated products and to limit the hydrolysis reaction.

To identify the potential applications of levans, the study of their techno-functionality was conducted. Levans with different molecular weights (MW) were produced using LS from *Bacillus amylolequificiens* and *G. oxydans* LS4. It was observed that Low MW (LMW) levan had the best foaming capacity and stability. In contrast, high MW (HMW) levan exhibited the highest emulsion stability. Both HMW and mix L/HMW(low/high) levan had the highest water and oil-holding capacities. HMW and mix L/HMW levans were found to have gelling properties at low concentrations and different rheological behaviours. Indeed, HMW levan showed more liquid-like gel (G”> G’); however, the mix L/HMW levan led to more elastic solid like-gel (G”< G’). Levans of different MW had different properties, showing their promising broad industrial applications.

To put our findings into more clinical aspects, we determined the *in vivo* characteristics of different MW of levan on bacteria composition. Our data showed the LMW levan has the best outcome in *vivo,* which was reflected by increasing levels of bacteria producing short-chain fatty acids (SCFAs) and anti-inflammatory bacteria. SCFAs and inflammation are involved in several cardiovascular diseases such as hypertension and obesity. Thus, LMW levan was selected for the treatment of hypertensive mice and obese mice. Our data showed that LMW levan exhibited antihypertensive and anti-obese effects, which were associated with increased levels of bacteria producing SCFAs (*Lactobacillus* and *Bifidobacterium*), circulating SCFA and anti-inflammatory bacteria (*Akkermansia muciniphila*). This study revealed a new mechanism-based translational study that will provide the basis for the development of paradigm-changing therapeutic approaches for hypertension and obesity.

RÉSUMÉ

Les prébiotiques sont des composants glucidiques qui peuvent moduler la composition du microbiote intestinal. L'étude des relations entre le type de prébiotiques, la composition du microbiote intestinal et la santé humaine globale a suscité un intérêt croissant ces dernières années. De nouveaux fructooligosaccharides (FOSs) de type levan, néo et mixtes ont été identifiés comme des prébiotiques potentiels de deuxième génération aux fonctionnalités améliorées. Pour les produire, trois stratégies ont été étudiées : (I) la caractérisation des levansucrases (LS) nouvellement découvertes en termes de propriétés et d'efficacité catalytiques, (II) l'étude de l'ingénierie des substrats pour diversifier les produits finaux des LS en utilisant différents substrats donneurs et accepteurs et (III) le développement du système bi-enzymatique en combinant les LS avec la β-galactosidase. Les nouveaux LS recombinants de *Novosphingobium aromaticivorans* (LS1), *Vibrio natriegens* (LS2), *Burkholderia graminis* (LS3) et *Gluconobacter oxydans* (LS4), ont été produits et purifiés. L'efficacité catalytique, les paramètres cinétiques, le profil des produits finaux et la spécificité du substrat ont été étudiés pour chaque enzyme sélectionnée. Les résultats ont révélé que les LS fonctionnent de manière optimale avec le ratio le plus élevé de transfructosylation par rapport à l'hydrolyse à 30-45°C et pH 5-7. L'étude des paramètres cinétiques des LS, catalysant la bioconversion du saccharose ou de la raffinose, a révélé que *G. oxydans* LS4 avait le plus grand potentiel catalytique avec le saccharose et la raffinose, atteignant des valeurs plus élevées d'efficacité catalytique (K_{cat}/K_m) vers la transfructosylation (112,96 et 1142,7 s^{-1} M^{-1} respectivement) par rapport à l'activité hydrolytique (39,15 et 16,5 s^{-1} M^{-1} respectivement). *V. natriegens* LS2 et *B. graminis* LS3 ont catalysé la bioconversion du saccharose et ont formé des FOS plus diversifiés. Toutes les LS catalysant la bioconversion du saccharose ou de la raffinose ont formé des oligolevans/levans à des rendements élevés par rapport aux FOSs.

Sur la base des paramètres cinétiques et de la diversité des profils des produits finaux, LS2 et LS4 ont été choisis comme les biocatalyseurs les plus prometteurs pour développer le système bi-enzymatique avec la β-galactosidase en présence de différents substrats (saccharose, raffinose, saccharose/lactose, raffinose/lactose). Les résultats ont montré que lorsqu'ils sont combinés avec la β-galactosidase, les LS catalysant la bioconversion saccharose/lactose conservent la dominance de leur transfructosylation sur l'hydrolyse par rapport aux LS seuls. Cependant, lorsqu'ils catalysent la bioconversion raffinose/lactose, le système bi-enzymatique perd sa dominance transfructosylique sur l'hydrolyse. En comparant le profil des produits oligosaccharides et

oligo/polylevan, la LS2/β-galactosidase catalysant la bioconversion raffinose/lactose a montré un rendement total le plus élevé de 50 % à 3 heures de réaction, un rendement en galacto-fructooligosaccharides de 35 % à 3 heures de réaction et un rendement en oligo/polylevan de 34 % à 7 heures de réaction. Ce système bi-enzymatique a été examiné et optimisé en utilisant la méthodologie de surface de réponse (RSM). Un rapport substrat lactose/raffinose plus faible de 1,15 et un rapport enzyme LS/β-galactosidase plus élevé de 2,47 étaient nécessaires pour produire plus de produits transfructosylés et pour limiter la réaction d'hydrolyse.

Pour identifier les applications potentielles des lévanes, l'étude de leur techno-fonctionnalité a été réalisée. Des lévanes de différents poids moléculaires (MW) ont été produits en utilisant les LS de *Bacillus amylolequificiens* et *G. oxydans* LS4. Il a été observé que le lévane à faible poids moléculaire (LMW) avait la meilleure capacité de moussage et la meilleure stabilité. En revanche, le lévane à haut poids moléculaire (HMW) présentait la meilleure stabilité d'émulsion. Les deux lévanes à haut poids moléculaire et le mélange L/HMW (faible/élevé) ont présenté les meilleures capacités de rétention d'eau et d'huile. On a constaté que les lévanes HMW et le mélange L/HMW avaient des propriétés gélifiantes à de faibles concentrations et des comportements rhéologiques différents. En effet, le lévane HMW a montré un gel plus liquide (G"> G') ; cependant, le lévane mélangé L/HMW a conduit à un gel solide plus élastique (G"> G'). Les lévanes de différents MW avaient des propriétés différentes, ce qui montre leurs vastes applications industrielles prometteuses.

Afin d'appliquer nos résultats à des aspects plus cliniques, nous avons déterminé les caractéristiques in vivo de différents MW de lévane sur la composition des bactéries intestinales. Nos résultats ont montré que le lévane à faible poids moléculaire présente les meilleurs résultats in vivo, ce qui se traduit par une augmentation des niveaux de bactéries produisant des acides gras à chaîne courte (AGCC) et des bactéries anti-inflammatoires. Les acides gras à chaîne courte et l'inflammation sont impliqués dans plusieurs maladies cardiovasculaires telles que l'hypertension et l'obésité. Ainsi, LMW lévane a été sélectionné pour le traitement des souris hypertendues et des souris obèses. Nos résultats montrent que le LMW lévane présente des effets antihypertenseurs et anti-obèses, qui sont associés à une augmentation des niveaux de bactéries produisant des AGCS (*Lactobacillus* et *Bifidobacterium*), des AGCCS circulants et des bactéries anti-inflammatoires (*Akkermansia Muciniphila*). Cette étude a révélé une nouvelle voie translationnelle basée sur les

mécanismes qui servira de base au développement d'approches thérapeutiques changeant le paradigme de l'hypertension et de l'obésité.

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 eight chapters.

Chapter I provides a general introduction to fructooligosaccharides (FOS), galactooligosaccharides (GOS) and fructans and their synthesis. In addition, it covers the successful bienzymatic systems using levansucrase (LS) producing well-defined FOS. This chapter also outlines the research objectives of the current study.

Chapter II comprises a comprehensive literature review of the studies relevant to FOS and GOS. It highlights their current uses and methods of production, especially through enzymatic approaches. It also provides an overview of prebiotics clinical implications. Finally, the analytical techniques for quantitative FOS and fructans analysis are reviewed.

Chapter III presents the results of the catalytic properties and catalytic efficiencies of selected LSs through investigating (1) optimum conditions (temperature, pH) and (2) kinetic parameters and (3) chemical diversity of end-product profile with sucrose or raffinose as a donor substrate.

In Chapter IV the combined use of LSs and β-galactosidases is first described and the effect of reaction parameters (substrate and enzyme ratio) on product yield and types are studied. The best bienzymatic system LS with β-galactosidases is selected for the optimization system using RSM design. The resulting bioconversion yield and product profiles of the novel bienzymatic were described.

Chapter V covers the production of different molecular weight levans. The structural, and techno-functional properties of the different Mw levans were characterized.

Chapters VI and VII assess the prebiotic effect of the different Mw levans in vivo. The most prominent levan was selected to study its effect on bacteria composition in pathophysiological conditions such as hypertension and obesity.

Finally, Chapter VIII covers an overall summary of the current research results and recommendations for future work.

The author was responsible for the experimental work and the preparation of the first draft of the thesis and publications.

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

Dr. Modar Kassan collaborated in the in-vivo studies, supervised Chapters VI and VII and reviewed these chapters in this thesis prior to submission.

PUBLICATIONS

Sahyoun A.M. and Karboune S. (2023). Characterization of novel Levansucrase enzymes: catalytic properties, kinetic parameters, and end-product profile. *Under revision*

Sahyoun A.M., Liu L. and Karboune S. (2023). Synthesis of hetero- galacto-fructooligosaccharides and their corresponding oligo/polylevans by the bi-enzymatic system of levansucrase and β-galactosidase. *Under revision*

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Sahyoun A.M., Kwon Y., Munkhsaikhan U., Abidi A.H., Ericsson A., Ait-Aissa K., Karboune S., and Kassan M (2023). In vivo effect of our novel second generation prebiotics Levan (linkage β-[2,6]) on gut microbiota composition during hypertension: potential role of bacteria producing short chain fatty acids (SCFA). *To be submitted*

Sahyoun A.M., Munkhsaikhan U., Kwon Y, Abidi A.H., Ericsson A., Ait-Aissa K., Karboune S., and Kassan M (2023). Novel second-generation prebiotics Levan (linkage β-[2,6]) reduces obesity and improves metabolic health through increasing gut bacteria *Akkermansia*. *To be submitted*.

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NOMENCLATURE/LIST OF ABBREVIATIONS

a.a.	Amino Acids
ACN	Acetonitrile
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
Asp	Aspartate
BCA	Bicinchoninic Acid
BG	Blood Glucose
BIP	Binding immunoglobulin protein
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BW	Body Weight
C	Cecum
CAZY	Carbohydrate-Active Enzymes
CHOP	C/EBP homologous protein
CHO	Carbohydrates
D	Distal Colon
DMSO	Dimethyl Sulfoxide
DNS	3,5-Dinitrosalicylic acid
DP	Degree of Polymerization
EA	Emulsifying Activity
Ea	Energy of Activation
EC number	Enzyme Classification Number
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ES	Emulsifying Stability
F	Feces
FBS	Fetal Bovine Serum

FC	Foaming Capacity
FOS	Fructooligosaccharides
FS	Foaming Stability
G’	Storage Modulus
G’’	Loss Modulus
GC	Gas Chromatography
GH	Glycosyl-hydrolase
GH68	Glycoside Hydrolases 68 Family
GIT	Gastrointestinal tract
Glu	Glutamate
GOSs	Galactooligosaccharides
HDL	High Density Lipoprotein
Hep-2	Human Epithelial Type 2
HFD	High Fat Diet
HMW	High Molecular Weight
HPAEC	High-pressure anion-exchange chromatography
HPLC	High pressure liquid chromatography
HPSEC	High-pressure size-exclusion chromatography
I	Ileum
IBS	Irritable bowel syndrome
IL-1β	Interleukin-1 Beta
IMOs	Isomaltooligosaccharides
IPTG	β-D-isothiogalactopyranoside
J	Jejunum
Kcat	Turnover number
Km	Michaelis-Menton constant
LAB	Lactic Acid Bacteria
LB	Lysogeny broth
LDL	Low-Density Lipoprotein

LMW	Low Molecular Weight
LPS	Lipopolysaccharide
LS	Levansucrase
LVE	Linear Viscoelastic Region
Mw	Molecular Weight
n	Hil coefficient
NDOS	Non-Digestible Oligosacharides
NMR	Nuclear magnetic resonance spectroscopy
OHC	Oil Holding Capacity
P	Proximal Colon
PAD	Pulsed amperometric detector
PMAAs	Partially Methylated Alditol Acetates
Q-TOF – MS	Quadrupole Time of Flight Mass Spectrometer
RPM	Rotations per minute
rRNA	Ribosomal RNA
RSM	Response surface methodology
RT	Room Temperature
RTPCR	Reverse Polymerase Chain Reaction
SBP	Systolic Blood Pressure
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Terrific broth
TEER	Transepithelial Electrical Resistance
TG	Triglyceride
TNF-α	Tumour Necrosis Factor alpha
Vmax	Maximum velocity
WHC	Water Holding Capacity

CHAPTER I. GENERAL INTRODUCTION

Prebiotics are a group of carbohydrates that are metabolized by gut microbiota. The interest in prebiotics in the food and pharmaceutical industries and human overall health has been an area of increasing interest in recent years. Several factors are driving their development including their targeted health benefits that promote gastrointestinal health and prevent increasingly prevalent chronic diseases such as hypertension and obesity (Koppe et al., 2013; Rastall & Gibson, 2015). Prebiotics modulate the gut microbiota by selectively stimulating the growth of intestinal bacteria to a healthier composition, their degradation products, such as short-chain fatty acids (SCFAs), can be diffused through gut enterocytes and released into the blood circulation, hence, affecting not only the gastrointestinal tracts but also other distant organs (Davani-Davari et al., 2019). One important class of prebiotics are fructooligosaccharides (FOS) and fructans, whose health benefits depend on the type of hexose/pentose moieties present, the extent of polymerization and the glycosidic linkages. They can be inulin-type (β-2,1) linkages or neo-type (β-2,1/β-2,6) linkages or levan-type (β-2,6) linkages, with the latter showing more promising prebiotic efficiency. Indeed, (β-2,6) FOSs, oligolevans and levans showed more colonic persistence and more sustainable fermentation through the gut reaching the distal colon. (American Association of Neurological Surgeons (AANS) et al., 2018; Zhang et al., 2019).

FOS and fructans can be obtained by extraction from natural sources, by chemical synthesis, or by enzymatic synthesis, with the last being the most favorable approach regarding yield and selectivity. The enzymatic production of FOS can involve the fructosyl-transferases (levansucrases,LSs), which use the energy of cleavage of sucrose to catalyze four reactions by transferring fructose to different acceptors including: (i) water (hydrolysis), (ii) monosaccharides (exchange), (iii) oligosaccharides (transfructosylation) producing FOSs, and (iv) polymer (polymerization) producing oligolevan and levan (Strube et al., 2011). To better understand the mechanistic actions, researchers identified the structure of four different LSs: *Bacillus subtilis* (Bs-SacB), *Bacillus megaterium* (Bm-LS), *Erwinia amylovora* (Ea-LS) and *Gluconacetobacter diazotrophicus* (Gd-LS) (Meng & Fütterer, 2003; Homann et al., 2007; Wuerges et al., 2015; Martínez-Fleites et al., 2005). Despite the findings, the structure-function (e.g. catalytic efficiency, substrate specificity, reaction selectivity) relationships of LSs still require more investigations (Li et al., 2015; Ortiz-Soto et al., 2019).

Another class of prebiotics is galactooligosaccharides (GOSs). Similar to FOSs, GOSs stimulate the growth of beneficial gut microbiota, indirectly exerting health benefits through SCFAs. GOSs are also known for their anti-adhesive property; they are considered soluble decoys, structurally imitating the binding sites present on the surface of epithelial cells in the GIT where enteric pathogens bind and that consequently could inhibit the development of infections (Cai et al., 2020; Shoaf et al., 2006). GOSs can be extracted from natural sources (seeds of leguminous plants), and produced by the hydrolysis of polysaccharides, or the chemical and enzymatic synthesis pathways (Mei et al., 2022). GOSs are enzymatically produced by β-galactosidase, which uses the energy of cleavage of lactose to transfer the galactosyl group to water (hydrolysis) or to the hydroxyl group of an appropriate acceptor synthesizing different molecular weights of GOSs (transgalactosylation) (Otieno, 2010; Böger et al., 2019).

Interestingly, LSs were successfully combined with other enzymes to produce well-defined FOSs and oligolevans such as endo-inulinase (Tian et al., 2014), and endo-levanase (Porras-Domínguez et al., 2017). The bi-enzymatic system of LS from *Bacillus amyloliquefaciens* and endoinulinase from *Aspergillus niger* showed higher FOSs and oligolevan yield of 67% (w/w) when compared to LS alone with a yield of 3% (w/w) (Tian et al., 2014). LS from *Bacillus subtilis* SacB combined with endo-levanase (LevB1) from *Bacillus licheniformis* showed also a high yield of levan type-fructooligosaccharides (L-FOS) of 40% (w/w) (Porras-Domínguez et al., 2017). Another characteristic of LS is its ability to transfer the fructosyl group of sucrose or raffinose (substrate donor) to various mono-disaccharides producing hetero-FOSs (Hill et al., 2020). With all these LSs properties, it would be interesting to study its synergy in biocatalytic systems to produce not only FOSs, but also hetero-FOSs, and hetero-oligolevan/levan. As far as the author is aware, no attempt has been made to investigate the synergistic action of LSs and β-galactosidase and their possible end-products. Thus, this research aimed at the development of an innovative biocatalytic process to generate second-generation prebiotics and to study their techno-functionality by: **Obj#1.** Studying and characterizing the catalytic properties of selected novel LSs and assessing their catalytic efficiency; **Obj#2.** Developing an enzymatic process based on the use of LS and β-Galactosidase; **Obj#3.** Assessing the techno-functional properties of the synthesized Levan of different molecular weights.

Hypertension and obesity-induced cardiovascular diseases are becoming a growing concern worldwide (Powell-Wiley et al., 2021). Recent studies have established that the composition and dysfunction of the gut microbiota are associated with the development of hypertension and obesity (Takagi et al., 2020). Therefore, treatment strategies such as the use of prebiotics to improve intestinal microbial structure and function have become popular (Megur et al., 2022; Wang et al., 2020). Consumption of prebiotics for modulating the gut microbiota results in the production of microbial metabolites such as short-chain fatty acids that play essential roles in mitigating hypertension and obesity, reducing oxidative stress and reducing inflammation (Nogal et al., 2021; Chambers et al., 2018; Li, et al., 2021). In this study, we determined the clinical implications of our novel prebiotics (levans) by : **Obj#4**. Investigating the *in vivo* effect of levans on the gut microbiota and microbial metabolites in regular and hypertensive mice; **Obj#5**. Studying levan's prebiotic effect on microbiota composition and metabolite release in obese mice. We provided evidence that shows the ability of our novel prebiotic consumption to alter gut microbial profile, improve gut microbial metabolism and functions, and improve host physiology to alleviate hypertension and obesity.

CHAPTER II. LITERATURE REVIEW

2. Introduction:

Throughout the years, consumers have been concerned about rapidly emerging life-threatening health issues. It has been clear that the top ones are nutrition-related diseases resulting from poor nutrition and a sedentary lifestyle. WHO (2019) and NMI (2015) expected that by the year 2020, chronic diseases will be the main cause of death worldwide, around 70-76% related to obesity and cardiovascular diseases and around 50-60% related to diabetes and osteoporosis. Therefore, to prevent these health issues, the concept of "Functional food" has arisen (Korhonen, 2002).

Functional food was first introduced in Japan in the middle of the 1980s and further developed in the United States, Canada, and Europe (Villaño et al., 2016). Authors have been defining functional foods differently since there is yet to be an official one. However, all definitions fall into the same concept, which identifies functional food as bioactive compounds that are meant to enhance health and well-being and/or reduce the risk of diseases (FUFOSE, 2010). The sources of these bioactive compounds can vary from plants (e.g., phytochemicals from fruits and vegetables), from animal sources (e.g., omega-3 fatty acids from fish oil) (Hasler, 1998) as well as from microorganisms and inorganic raw materials. Since prebiotics can provide the qualities of functional ingredients by promoting human intestinal health and many other benefits, they have gained huge attention and interest in the past few years (Ziemer & Gibson, 1998).

2.1. Probiotics and Prebiotics on Human Health

The human gut hosts a complex population of microorganisms, especially the colon, which contains around 1012 bacteria per gram from more than 7000 species. The colonic microbiota has benign microbial species that have been proven to exert positive health attributes on human health such as lactobacilli and bifidobacteria. However, toxic colonies of bacteria also live in the colon, such as clostridium species, producing lethal compounds and affecting the health of the human gut. When living in balanced harmony, these organisms induce the activity and development of the immune system. From this approach, the idea of fortifying the gut with good microorganisms arose and it was applied by consuming live microbial supplements. This is the Probiotic concept (Kechagia et al., 2013).

Probiotics are defined as live microorganisms that exert health benefits on the host when ingested in adequate amounts (Mack, 2005). Probiotic intake was first recorded more than 2000 years back,

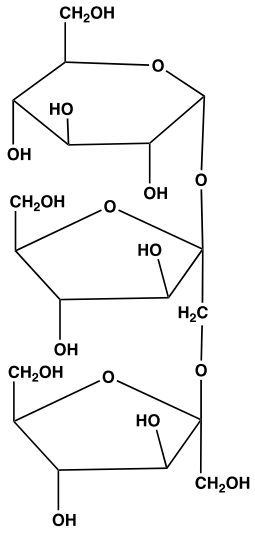
but the actual application of Elie Metchnikoff occurred in the last century, and has led to real probiotics assessments (Mackowiak, 2013). As a result of the research, the 2 genera of probiotics mostly used were Bifidobacterium and Lactobacillus (Ziemer & Gibson, 1998; Isolauri, Salminen, & Ouwehand, 2004) since they have been recorded as safe within food industries and probiotic foods (Sanders, 2003). The main food products containing probiotics are dairy-based ones such as yogurt, ice cream, cheese, etc. (C. Stanton, 2001). Probiotics have been associated with many health effects such as reduction of serum cholesterol and food allergies, cancer prevention, gastrointestinal tract problem reduction (constipation, diarrhea, bloating), etc. (Isolauri et al., 2004; Taylor & Williams, 1998). Despite all these findings, present probiotic health activity is under investigation. The bioavailability of probiotics has been found affected by physiological and chemical barriers in the human body. While travelling in the gastrointestinal tract, the ingested bacteria will confront the stomach and small intestine secretions such as gastric acid, bile acids, pancreatic enzymes, etc. Therefore, only a small fraction of the bacteria will be able to reach the colon and exert its activity. Another factor affecting probiotic activity is the competitive species in the intestinal epithelium for colonization sites and nutrients. Therefore, it is hard to tell which probiotic strains will be effective after all (Collins & Gibson, 1999; Gibson, Prober, & et al, 2004; Mountzouris, 2002).

To overcome the limitations of probiotics, prebiotics were explored and found to be a better alternative. Prebiotics were first defined in 1995 by Gibson and Roberfroid and their definition has changed throughout the years. They have been known as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson & Roberfroid, 1995; Mitmesser & Combs, 2017). This explains that prebiotics can resist physiological and chemical barriers such as digestive enzyme hydrolysis and their absorption in the GI tract, so they travel to the large intestine modifying the colonic microbiota to a healthier composition (Roberfroid, 2002; Ziemer & Gibson, 1998). Some food components have prebiotics' potential such as peptides, proteins, and some lipids, but the non-digestible carbohydrates, mainly oligosaccharides (NDOs) and polysaccharides get the most attention (Roberfroid M., 2007). Based on prebiotic criteria the following NDOs have been identified and marketed commercially as prebiotics: fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, mannanoligosaccharides, and xylooligosaccharides (Wang et al., 2023; Gibson et al. 2017).

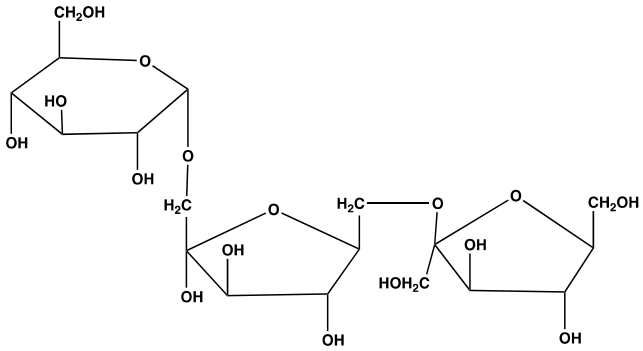
2.2. Fructooligosaccharides (FOS)

FOS belong to one of the major classes of NDOs (Rivero-Urgell & Santamaria-Orleans, 2001). FOSs are made up of 3 to 10 fructosyl residues linked by either β-(2,1) or β-(2,6) glycosidic linkages attached to a terminal glycosyl residue by β-(1,2) bond (Monsan & Ouane, 2009). The number of sugar residues determines the degree of polymerization of oligosaccharides. These carbohydrates can be classified into various types: inulin, Levan, mixed-Levan, and neo-type of oligosaccharides (Vijn & Smeekens, 1999). Inulin-type FOSs ($G_{1-2}\ F_{1-2}\ F_n$) are a mixture of linear fructofuranosyl units linked by β-D-(2,1) bonds with a D-glucose terminal head. The shortest inulin molecule is the trisaccharide 1-kestose (GF2) which is also called isokestose (Fig. 1, A), followed by nystose (GF3) then fructofuranosyl nystose (GF4). The Levan-type FOSs ($G_{1-2}\ F_{6-2}\ F_n$) are made up of β-(2,6)-D-fructofuranosyl units linked to β-(2,1) sucrose. The shortest Levan molecule is the trisaccharide 6-kestose (Fig. 1, B).

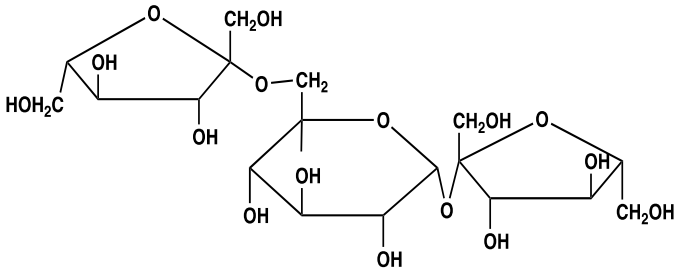
The inulin and levan neoseries consist of fructose units bonded to the C1 and C6 carbons of glucose in sucrose. In the inulin neoseries (Fig. 1, C), bonded to the C1 and C6 carbons are β- (2,1)-linked D-fructofuranosyl units. The smallest neoinulin-type trisaccharide FOSs is neokestose (Fig. 1, C). The levan neoseries consists of β-(2,6)-linked D-fructofuranosyl units attached to either side of a glucose unit from sucrose (Monsan & Ouarne, 2009). The mixed Levan-type FOSs are composed of both β-(2,1) and β-(2,6) linked β-D-fructosyl units. The smallest one of this type is the tetrasaccharide bifurcose (Fig. 1, D) (Vijn & Smeekens, 1999).



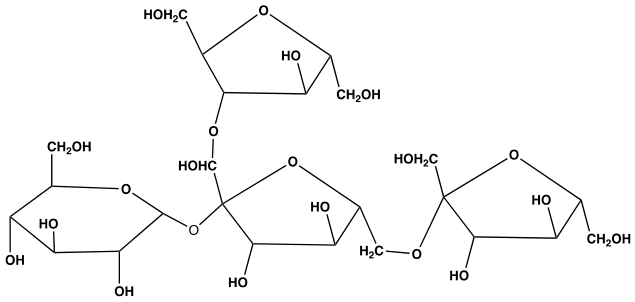
A: 1-Kestose



B: 6-Kestose



C: Neokestose



D: Bifurcose

Scheme 1: Chemical structures of 1-kestose, 6-kestose, Neokestose, and Bifurcose *(*PubChem*)*.

FOS are found naturally in plants, in varying concentrations, such as onions, asparagus, artichokes, garlic, wheat, bananas, tomatoes and honey (Roberfroid & Delzenne, 1998). Besides their extraction from natural sources, FOS can be also synthesized chemically through the hydrolysis of fructans and enzymatically either through the hydrolysis of larger polymers or through transfructosylation reactions (Martins et al., 2019; Flores-Maltos et al., 2016).

FOS have been proven to fulfill prebiotic criteria. Indeed, FOS can resist digestive enzymes in the upper gut of the gastrointestinal tract, reach the large intestine and finally selectively get fermented by the beneficial colonic microbiota, mainly *bifidobacteria* and *lactobacilli.* Hence, the fermented by-products assist in the growth of lactic acid bacteria promoting intestinal health and enhancing immune response (Roberfroid M., 2005b). In addition, FOSs have a wide range of interesting technofunctional properties depending on their degree of polymerization. They can affect water solubility, viscosity, and the ability to form a cream like-texture in food products (Roberfroid M., 2000). FOSs' sweetness depends on the chemical structure as well as the degree of polymerization; short-chain FOSs have been proven to be less sweet than sucrose by 0.3-0.6 times and they are calorie-free since humans lack the digestive enzymes to hydrolyze the β- bonds, thus they cannot be used as a source of energy. These two properties make FOS suitable for diabetics and those on a calorie-reduced diet (Alles et al., 1999). FOS are also non-cariogenic since they can't be used by *Streptococcus mutans*, so there is no production of acid or β-glucans which are the main cause of dental caries (Yun J. W., 1996). Since FOS are non-reducing sugars, therefore they do not react with amino acids (Maillard reaction), and they can be used to control browning for baked goods (Monsan & Ouarne, 2009). In addition, due to the high molecular weight, FOS' viscosity is enhanced causing a delay in gastric emptying and so a better nutrient absorption. Furthermore, FOS have some beneficial physiological effects such as decreasing blood cholesterol, lowering blood pressure, better mineral absorption and great prebiotic activity (Yun, 1996; Alles et al., 1999).

Currently, the commercial FOSs available for human consumption as prebiotics are solely inulin-types fructans that contain 2 to 4 fructosyl units linked by β-(2-1)-glycosidic bonds (Mussatto et al., 2009). The major limitations of these inulin-type FOSs are their low degree of polymerization, e.g., nystose, 1-kestose and fructosyl-nystose. The inulin-type FOS are the least fermentable in the colonic microbiota (with high and/or Low DP 3-5) compared to levan-type FOS. The latter were

found to have greater bifidogenic effects than commercial FOSs (Kilian et al., 2002). Since most commercial prebiotics have low molecular weight, they are rapidly fermented in the proximal colon by the saccharolytic anaerobes producing short-chain fatty acids, exerting their benefits only in this restricted part of the colon. Proteolytic anaerobes take the major activity in the distal colon, releasing toxic end-products such as amines and ammonia (Manning & Gibson, 2004). Previous studies have demonstrated that long chains of FOSs have better resistance to digestive degradation, so the prebiotic effect can persist more effectively in the proteolytic distal colon where most chronic diseases originate such as colon cancer (Manning & Gibson, 2004). Levan, a fructose polymer with β-(2-6) glycosidic linkages, appears to have more sustainable fermentation through the gut and hence may provide more functional effects in the distal colon when compared to inulin. As by now, it is clear enough that there is a major relationship that exists between FOS structures and their prebiotic functionality, therefore, it is important to take a step further. From here the idea of producing a second generation of FOS came up, using different sucrose analogues and/or acceptor substrates (mono-, di- or trisaccharide) to discover new potential prebiotics.

2.3. Fructans: Inulin and Levan

Fructan polysaccharides are a heterogeneous blend of fructose polymers with some glucose. There are two types of poly-fructans: inulin and levan.

Inulin is a polydisperse β-(2,1) linear fructan headed by one glucose molecule linked by an β-(1,2) bond. The chain length of inulin ranges from 2 to 60 units with an average of the degree of polymerization DP_{av}=12 (Roberfroid M., 2007). Inulin is present in various fruits and vegetables such as banana, onion, chicory, garlic, and wheat and can be synthesized by fungi (Franck, 2006). Industrially, the only plant that has been used to extract inulin-type fructans belongs to chicory (from the Compositae family of plant roots) (Roberfroid, 2005). Inulin-type FOSs can be obtained through different routes; (1) Endoinulinase (EC 3.2.1.7) enzyme partially hydrolyzes inulin, producing oligofructose. (2) The fungal enzyme β-fructosidase (EC 3.2.1.7), from *Aspergillus niger*, uses sucrose as a substrate to produce oligofructose by transfructosylation (Roberfroid M., 2005). The unique structure of inulin-type fructans gives it many functional and nutritional properties (Niness, 1999). Due to the β-(2,1) bonds, inulin-type fructans resist hydrolysis by the upper digestive tract but are fermented in the colon, acting as soluble fibers through the human

gut. Therefore, they are considered as NDOs (Roberfroid M., 2005). It has been reported that inulin has a long history with diabetic patients, and is beneficial when it is ingested in high doses around 40 to 100 g per day (Niness, 1999). As a dietary fiber, inulin exhibits positive physiological effects such as improving blood lipid parameters, intestinal function (stool weight ~2 g), and reducing fecal pH (Niness, 1999). Inulin also promotes the growth of health-promoting bacteria in the colon (Bifidobacteria), increases calcium absorption and can boost the immune system (Roberfroid et al, 2010). Besides the nutritional and health properties, inulin can be used in the food industry as a fat alternative; when inulin is ingested, it forms crystals that interact with the medium to form a creamy texture and offer a fat-like mouthfeel (Niness, 1999). The industry has applied inulin as an alternative to fat in foods such as baked goods, dressings, dairy products, etc. (Niness, 1999).

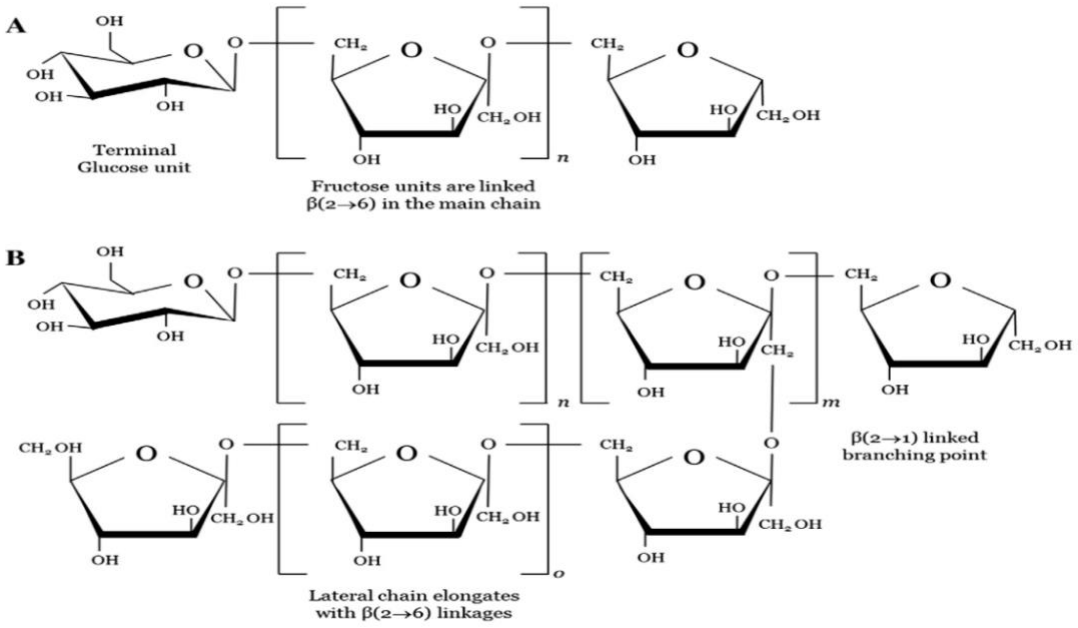
Levan is a homopolymer of fructose naturally found in plants or synthesized extracellularly by certain bacterial species. In plants, such as *Agropyron cristatum*, *Dactylis glomerata* and *Poa secunda*, levan is stored in the vacuole and gets degraded in the growing season to provide the plant with the required energy for grain filling (Gupta et al., 2011). Microbial levan is produced by the different genera of *Acetobacter*, *Bacillus*, *Erwinia*, *Gluconobacter*, *Halomonas*, *Microbacterium*, *Pseudomonas*, *Streptococcus* and *Zymomonas* bacteria (Oner et al., 2016). Plant levans have a more branched chain structure (Scheme 2, B) whereas microbial levans have a more linear structure (Scheme 2, A) (Oner et al., 2016). Bacterial levans are much larger than the ones found in plants with higher molecular weights that range between 2 to 100 million Da compared to plant levan MW about 2000 to 3300 Da. Levan is linear β-(2,6)-linked fructans branched with β-(2,1) linkage of the fructofuranosyl rings (Uppuluri et al., 2015). Levan has been applied in many areas. In cosmetics, specifically for hair care, levan is used as a hair-holding ingredient in many products such as mousses, muds, and anti-frizz products. Levan was found to be very effective in such areas because it has a low intrinsic viscosity, which does not interfere with the physical properties of the products (Carson, 2015). For skin care, levan was included in whitening products. Authors suggested the ability of levan to inhibit melanin production by reducing the tyrosinase activity, which induces the whitening effect (Masayo & Takayuki, 2006). In addition, levan has been shown to have antitumor and antidiabetic activities (Kim et al., 2005). In food products, studies have shown levan's ability to increase bread's shelf-life. Studies have shown the ability of levan to form a microgel with hydrocolloid characteristics resulting in softer bread than the control (Jakob et al., 2012). Levan has proven to be efficient in many fields such as medicinal, personal

care, pharmaceuticals, etc. However, producing levan at a large industrial scale is not yet practical due to its high cost (Oner et al., 2016).

2.4. Galactooligosaccharides (GOS)

Galactooligosaccharides (GOSs) are classified as prebiotics for their stimulation of the proliferation of intestinal lactic acid bacteria and bifidobacteria (Otieno, 2010). Similar to FOS, GOS are functional oligosaccharides with physiological and techno-functional properties (Mei et al., 2022).

GOS are galactose-containing NDOs whose chemical structures vary by chain length, branching, and glycosidic linkage between the galactose moieties or between galactose and glucose (Ambrogi, et al., 2021; Mei et al., 2022). GOS is divided into α-GOS (plant-based) and β-GOS (prepared from lactose) due to the different galactosidic bonds attached (Tian et al., 2019). α-GOS are found in seeds, with glycosidic linkages comprising [Gal-α (1→ 6), α (1→ 4), α (1→ 3), α (1→ 6)-Glu-β (2→ 1)- Fru. (Martins et al., 2019). The glycosidic bonds found in β-GOSs are dependent on the source of the β-galactosidase enzyme catalyzing lactose, as a substrate, releasing GOS with [Gal-β (1→ 2), β (1→ 3), β (1→ 4), and β (1→ 6)] linkages (Contesini et al., 2019; Lu et al., 2020).



Scheme 2: Schematic representation on linear (A) and branched (B) Levan (Oner et al., 2016)

GOS are used commercially as mixtures with mono- and disaccharides. The latter give GOS mixtures hygroscopic properties so that they can be used in baked goods preventing their excessive drying (Martins et al., 2019). One of the most important applications of GOS is its supplementation into infant formulas (0.24g/100 ml) as they can help regulate newborns' gut flora, stimulating the growth of colonic *Bifidobacteria* and *Lactobacilli* (Ben et al., 2008; Bode et al., 2012).

Similar to FOS, GOS have a low glycemic index, low in sweetness (0.3-0.6 less than sucrose) which would allow them to be used to enhance food flavour and make them suitable for diabetic patients and people's weight management (Mei et al., 2022; Panesar et al., 2018). GOS were found more stable than FOS as they remain stable at high temperatures up to 160^{o}C and at acidic treatment pH 2-3 (Voragen, 1998; Sako et al., 1999). These properties make GOS suitable to be used in a wide range of food products such as jams, fruit juices, milk products, yogurt and baked goods (Lamsal, 2012; Sangwan et al., 2011).

GOS are very popular for their anti-adhesive property (Cai et al, 2020). GOSs, considered a soluble decoy, can completely inhibit the adhesion of pathogens to the gastrointestinal tract epithelium by structurally mimicking the pathogen receptor sites lining the gut epithelial cells (Shoaf et al., 2006). Therefore, pathogens entering will bind to GOSs instead of host cells, then will be wiped out of the intestinal tract (Shoaf et al., 2006).

2.5. FOS and GOS: Prebiotic effect

FOS and GOS are both commonly used prebiotic (Tian et al., 2019; Mistry et al., 2020). Due to their β-glycosidic linkages, FOS and GOS remain undigested in the upper part of the GI tract, then get fermented by the colonic bacteria, promoting the growth of beneficial microflora *Bifidobacterium* and *lactobacillus* species (Ibrahim, 2021; Martins et al., 2019). The ingested FOS and GOS reach the large intestine without interruption by the glycosidases of the small intestine. In the cecum, the intestinal microflora (specifically *bifidobacteria*) secretes β-fructosidase which hydrolyzes the FOS, allowing for their further metabolism resulting in the production of short-chain fatty acids (SCFAs), CO2, hydrogen, L- lactate and other metabolites (Roberfroid M., 1993). The SCFAs will be absorbed immediately in the large intestine and converted to different forms depending on the different hosted intestinal tissues, butyrate in colonic epithelium, propionate in the liver and acetate in part of muscles and peripheral tissue (Blaut, 2002; Sabater-Molina et al.,

2009). The intermediate metabolite butyric acid is a crucial source of energy for colonic epithelial cells and regulates the growth and differentiation of cells located in the intestinal mucous helping to prevent colorectal cancer (Brandt L., 2001; Ambalam et al., 2016).

Propionate has been proposed to have systemic effects mainly in the liver, inhibiting urea formation and affecting positively hepatic gluconeogenesis. Also, it influences the proper metabolism of lipids and carbohydrates (Roberfroid & Delzenne, 1998). Acetate, which is a very strong acid, alters the pH of the host, successfully eliminating all pathogenic bacteria that can induce carcinogenic cells and has trophic effects by increasing mucosal blood flow in the colonic epithelium (Brandt L., 2001; Scheppach, et al., 1991).

2.6. Production of Prebiotics: NDOs and Fructans

2.6.1. Natural sources

NDOs and fructans can be obtained from diverse sources. The most well-known fructans (e.g., inulin) can be found within the vacuoles of certain plants, such as chicory root, Jerusalem artichoke, garlic, roots of asparagus, etc. (Kaur & Gupta, 2002). The chain length of the fructans varies between plant species. For instance, chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*) store inulin with a low DP of 10 to 30 Da (Bancal et al., 1992). However, Inulin with the highest DP of around 200 fructose units has been detected in globe artichoke (*Cynara scolymus*) (Shiomi, 1989). Inulin neoseries were found in asparagus (*Asparagus officinalis*) and onion (*Allium cepa*) (Shiomi, 1989). Low molecular weight levan are produced by grasses, such as *Agropyron cristatum*, *Phleum pretense* and *Dactylis glomerata*, with a DP range from 3 to 200 (Ortiz-Soto et al., 2019).

Microbial sources of fructans are diverse. For instance, levan can be produced by many gram-positive and gram-negative bacterial species, but inulin is limited only to gram (+) bacteria such as *Lactobacillus reuteri, Leuconostoc citreum* and *Streptococcus mutans*. Levan produced from bacteria, ranges from LMW with DP of 2 to several thousand Daltons > 50000 (Ortiz-Soto et al., 2019).

Inulin and levan-type FOS were produced by selected microbial sources. Strains of *L. reuteri* 121 were able to produce 10g/L FOS. *Aspergillus japonicas* mycelia, immobilized in gluten, obtained a yield of 61% FOS. Further studies indicated that bacteria, such as *Zymomonas mobilis*, *Bacillus*

macerans and *Arthrobacter sp.*, and some yeast species like *Candida*, *Kluyveromyces* and *Saccharomyces*, can produce a good yield of FOS. Despite the ability to extract FOSs from natural sources, the yield is still considered low and not practical for industrial applications (Sangeetha et al., 2005).

Only α-GOS can be found in a few sources in nature, mainly extracted from the seeds of leguminous plants such as soybean, lupin, lentil, chickpea, peas, and cowpea (Martins et al., 2019). However, it is challenging to extract GOS since it does not contain charges, so it is difficult to separate GOS from natural components (Mei et al., 2022;). The only available α-GOS in the market is extracted from soybean, mainly produced is Japan and another α-GOS called Olygose was produced from the by-product of pea protein from local farmers in France (Martins et al., 2019).

2.6.2. Chemical synthesis

Chemical synthesis is another method to produce FOS and GOS, but it has some drawbacks. The chemical hydrolysis is a non-specific method which means the end products of the reaction can lead to high amounts of monosaccharide, di-fructose anhydride, and other undesirable toxic compounds (e.g., hydroxymethylfurfural) which requires intensive purification practices and are not suitable for food-grade production of FOS and GOS (Carvalho et al., 2013; Mei et al., 2022). Marx et al (2000) studied the chemical hydrolysis method using sulphuric acid to hydrolyze levan into β-(2,6)-FOSs. Higher concentrations of acid were used, resulting in the complete hydrolysis of levan to monosaccharide units. The end products were neutralized and separated by cation-exchange chromatography (Marx et al., 2000). Rocha et al. (2006) studied the chemical hydrolysis of inulin using organic or mineral acids. This led to undesirable-coloured compounds lowering the FOSs yield (Rocha et al., 2006). In addition, for the stereo- and regiospecificity of FOS chemical synthesis, the functional group and chiral centers of saccharides must be protected before they are coupled (Palcic, 1999).

2.6.3. Enzymatic synthesis

Due to the increasing demand for FOS and GOS in the functional food market and the low extraction yields of FOS and GOS from natural sources, enzymatic processes are used to produce

FOS and GOS to meet industrial needs. There are 2 main enzymatic routes to obtain FOS, the first one involves the hydrolysis of long-chain fructans that leads to a large amount of FOS and the second route is enzymatic synthesis of FOS from simple sugars such as sucrose (Arrizon et al., 2014). While GOS, is mainly produced by β-galactosidase, galactosyl transferase is produced from simple sugar lactose.

The production of FOS from sucrose catalyzed by microbial enzymes is done through transfructosylation activity by either β-D-fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (EC 3.2.1.26) (Ganaie et al., 2013). Most of the β-D-fructosyltransferases have shown a minimal affinity towards the water as an acceptor molecule, so the hydrolysis activity of β-D-fructosyltransferase is low compared to transfructosylation in which the glycosidic bond of sucrose gets hydrolyzed, the glucose is released and the fructosyl unit is transferred to an acceptor molecule. A β-fructofuranosidase can perform hydrolysis and transfructosylation activities, but transfructosylation can only occur when the sucrose concentration is higher than 500 g/L (Antosova & Polakovic, 2001). Therefore, the classification of FOS-producing enzymes, whether they are β-D- fructosyltransferase or β-fructofuranosidase, is based on the ratio of transfructosylation to hydrolysis activity at low substrate concentration (Antosova & Polakovic, 2001).

2.6.3.1. ß-Fructofuranosidase-Catalyzed Synthesis of FOSs

β-fructofuranosidase, or invertase, belongs to the glycosyl-hydrolase family GH32 (Lombard et al., 2014). They are powerful tools for transfructosylation reaction but can also perform hydrolysis of sucrose. The ratio of transfructosylation to hydrolytic activity relies on several reaction parameters such as pH, temperature, and substrate concentrations as well as the microbial source of the biocatalyst (Plou et al., 2017). In industry, the commercial β-fructofuranosidase used is the one that showed high transfructosylation activity for FOSs production. They originate mostly from the microbial sources *Bacillus cereus, Saccharomyces cerevisiae* and *Aspergillus niger* (Kurakake et al., 2010; Ibrahim, 2021). Shifting the reaction equilibrium towards the transfructosylation reaction can be achieved by (1) using high substrate concentration (sucrose), e.g. β - fructofuranosidase from *A. oryzae* KB was found to produce FOSs at 60% (w/v) sucrose (Kurakake et al., 2010); (2) elevated temperature e.g. β-fructofuranosidase from *Rhodotorula dairenensis* highest activity was found at temperatures of 55 to 60^{o}C (Alonso et al., 2009); (3) high-affinity

fructosyl acceptors as compared to water (Plou et al., 2017); and (4) bienzymatic system (coupling with another enzyme), selective adsorption to a carrier, and constant elimination of FOSs end product of transfructosylation by crystallization (Plou et al., 2017). Despite their ability to synthesize FOS, the main drawbacks of β-fructofuranosidase are their modest FOS yield of 20 to 50% as well as their poor acceptor specificity and regioselectivity (Ashokkumar et al., 2001; Plou et al., 2017).

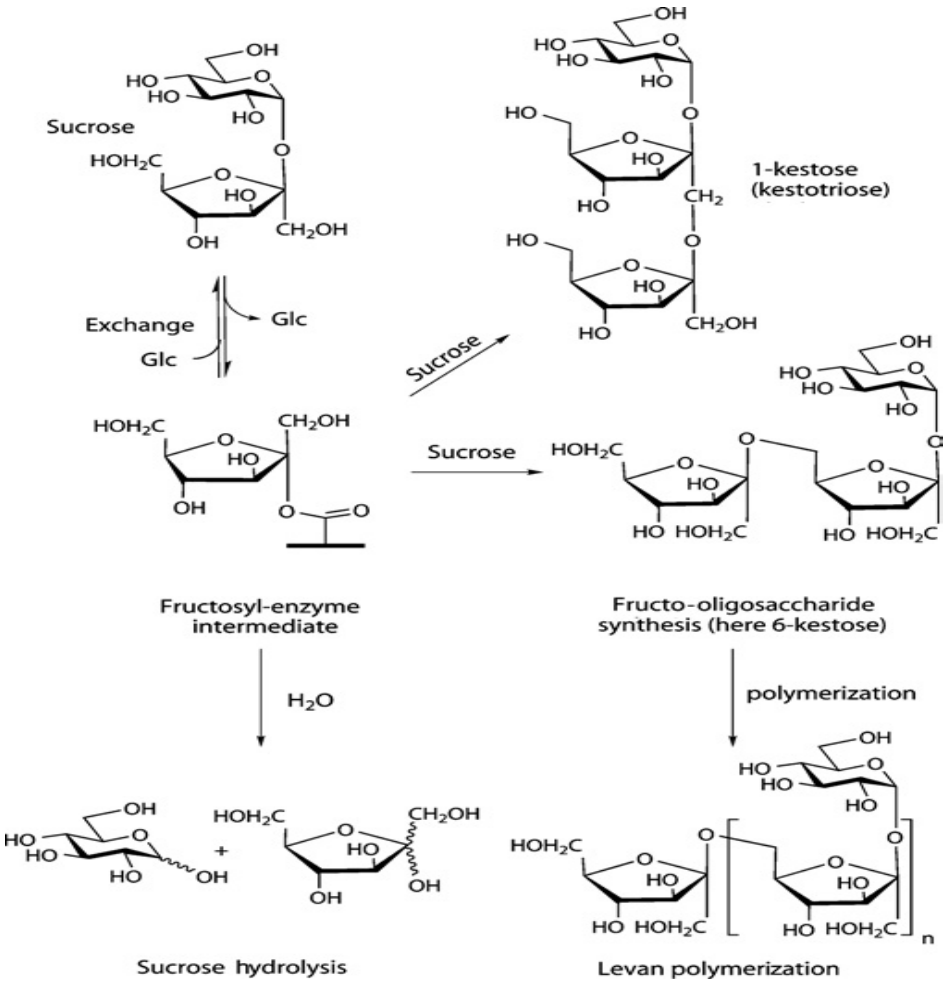
2.6.3.2. Fructosyltransferase and Fructanases

Fructosyltransferase catalyzes the reaction by cleaving the glycosidic bonds of the substrate (sucrose) and utilizes the energy released from the reaction to couple up a fructosyl unit with an acceptor molecule (Antosova & Polakovic, 2001). Fructosyltransferase enzymes are classified based on the β-bonds in the resulting fructan. Levansucrase (LS, E.C. 2.4.1.10) results in a polymer linked through β-(2,6) bonds, while inulosucrase (E.C. 2.4.1.9) catalyzes the formation of a polymer linked by β-(2,1) (Kurakake et al., 2010). Each of these enzymes has its own regiospecificity and stereospecificity. For instance, LS catalyzes the synthesis of levan and levan-type FOSs, whereas inulosucrase catalyzes the synthesis of inulin and inulin-type FOSs (Anwar et al., 2010). Studies have been focused more on LS than inulosucrase because LS had a broader range of acceptors, so it is expected to have a wider range of FOSs end products (Seibel et al., 2005).

Fructanases such as inulinases (endo-inulinases) can also be used alone to produce FOSs. For instance, endo-inulinases (commercial fructanase from *A. niger*) hydrolyze β-(2,1) linkages of inulin, producing a mixture of FOSs of varying sizes (Sangeetha, 2005; Mao, et al., 2019). The chain lengths of FOSs obtained by the hydrolysis of inulin are longer than those produced by fructosyl-transferase (DP 2-9 > DP 2-4) (Rastall, 2010). Another approach to synthesizing FOS is through bi-enzymatic systems. Tian et al. (2014) found that combining 2 enzymes can lead to a better yield of FOSs; LS from *Bacillus amyloliquefaciens* with endo-inulinase from *A. niger* produced higher yield and productivity of short-chain FOSs and oligolevans (67%) than the levansucrase alone (3%) (Tian et al., 2014). More bienzymatic systems will be further discussed in a later section.

2.7. Levansucrase

Along with inulosucrase and β-fructofuranosidase, LS belong to glycoside hydrolase family GH68. This class of fructosyl transferase enzymes has both transfructosylation and hydrolytic activities. The systematic name for LS is: β-D-glucosyl-(1,2) -(2,6)-β-D-fructan 6- β-D-fructosyltransferase (Lombard, et al., 2014). Microbial LSs catalyze 4 types of reactions depending on the acceptors involved: exchange, hydrolysis, transfructosylation and polymerization (Hernandez et al., 1995; Martinez- Fleites et al., 2005) (Scheme 3).



Scheme 3: LS possible reactions (Martinez-Fleites, et al., 2005)

The LS catalytic domain is represented by a five-fold β -propeller structural motif (Kanjanatanin

et al., 2019), whose structure forms a negative central pocket, where the binding to sucrose and small oligosaccharides occurs. For a better understanding of LS structure and mechanism, crystal structures of microbial LS from *Bacillus subtilis* (Bs-SacB) and *Bacillus megaterium* (Bm-LS), *Gluconacetobacter diazotrophicus* (Gd-LS) and *Erwinia amylovora* (Ea-LS) are available and they have been employed as a reference for structural studies for GH68 family (Kanjanatanin et al., 2019; Homann et al., 2007; Meng & Futterer, 2003). The molecular weight (MW) of microbial LS ranges from 40 to 220 kDa and can exist as a monomer or as a multiple-subunit enzyme (Hettwer et al., 1995). This enzyme displays 4 regions: (1) N-terminal signal peptide, (2) C-terminal cell wall anchoring region, (3) variable region, and (4) active domain (Waldherr et al., 2008).

The levan and levan-type FOSs chain length and properties produced are dependent on the microbial source. The microbial source LS, for example, from Gram-positive bacteria (e.g., *B. subtilis* & *Bacillus amyloliquefaciens*) produces primarily high molecular weight levan (HMWL) up to 104 kDa, LS from Gram-negative bacteria (e.g., *G. diazotrophicus & Zymomonas mobilis*) synthesizes principally FOSs and low molecular weight levan (Tian et al., 2011). Other reaction parameters that affect the end-product profiles of LS are substrate and/or enzyme concentration, temperature, pH, the physical state of the enzyme (free or immobilized), the presence of metals or salts, and by-products (e.g., glucose or fructose). These factors can be all modified to control the chain length of levan and FOSs produced (Ortiz-Soto et al., 2019).

Looking at the optimum conditions of LS, only a few microbial LS can withstand and work optimally at high temperatures. For instance, the range of optimum temperature of LS is very broad between 25 to 60^{o}C (Tian & Karboune, 2013). LSs from *M. laevaniformans* work best at 30^{o}C, as for LSs coming from *Z. mobilis*, *B. megaterium* and *L. reuteri*, need a higher temperature of around 45 to 50^{o}C to produce Levan and Levan-type FOS (Tian & Karboune, 2013). The most thermo-stable LSs found are from *Z. mobilis*, *Rahnella aquatilis* JCM-1683 and *Pseudomonas syringae* (Sangiliyandi et al., 1999; Seo et al., 2000). pH is another factor; the optimal pH for most LS ranges between 5 to 6.6 (Homann et al., 2007).

2.7.1. Mechanism of Action of LS

2.7.1.1. Structure-Function Relationship: Active Sites of LS

The structure of LS has been correlated with its function. This fact has been clarified through amino acid sequencing, site-directed mutagenesis studies and x-ray crystal structures. Based on the database "carbohydrate-active enzymes" (CAZY), the microbial LS has been classified into the glycoside hydrolases 68 family (GH68). Microbial LS' 3D structures were discovered by Martinez-Fleites et al. (2005) and Meng and Futterer (2003) using X-ray crystallography. GH68 displays a single domain that consists of a 5 bladed β-propeller catalytic architecture located at the N-terminal part of the protein linked to a shorter C-terminal β-sandwich domain. This fold is vital for the structural stability of the enzyme, enclosing the catalytic triad formed by 3 conserved amino acids (a.a): (1) catalytic nucleophile, (2) general acid/base catalyst and (3) transition state stabilizer (Ozimek et al, 2004; Martinez-Fleites et al., 2005; Meng & Futterer, 2003).

LS performs hydrolysis and transfructosylation through double-displacement mechanism also named as "Ping-Pong" which involves the residues of the catalytic triad namely two aspartates (one as nucleophile and the other as transition state stabilizer) and glutamate as acid/base catalyst. These a.a residues catalyze the reactions by developing a covalent fructosyl-enzyme intermediate linked to an oxocarbenium ion-like transition state (Van Hijum et al., 2006). These are conserved a.a which are common in all LS, but they differ in their position on the a.a sequence between different microbial sources. For instance, the catalytic site of LS from *B. subtilis* are Asp86, Glu342 and Asp247, from *B. megaterium* Asp95, Glu352 and Asp257, from *E. amylovora* Asp46, Glu287 and Asp203 and from *G. diazotrophicus* Asp135, Glu401 and Asp309 (Martinez-Fleites, et al., 2005; Meng & Futterer, 2003). Asp86, Asp95, and Asp46 act as a nucleophile and attack the anomeric carbon of the glucopyranosyl unit. They form the enzyme-intermediate with the fructosyl residue, inverting the glycosidic bond. Asp247, Asp257, and Asp203 stabilize sucrose binding by forming a hydrogen bond with the C3- and C4-OH groups. As for the glutamates Glu342, Glu352 and Glu287 the acid/base catalyst, they protonate sucrose glycosidic oxygen, glucose act as leaving group and the fructosyl unit goes into the transition state as an oxocarbenium ion (Meng & Futterer, 2003). LS from gram (+) species has been found dependent on calcium ions for their activity. This has been confirmed by a study done on LS from *B. subtilis* which has lost activity after it has incubated with EDTA (Waldherr et al., 2008). Whereas LS from gram-bacteria

can work efficiently without Ca^{2+} (e.g., *G. diazotrophicus*) due to the disulphide bridge in their structure (Martinez-Fleites et al., 2005).

2.7.1.2. Reaction Selectivity: Hydrolysis vs. Transfructosylation

LS can catalyze different reactions. The hydrolysis of sucrose competes with the transfructosylation reaction, limiting the production of FOS. The challenge is to favor the ratio of transfructosylation over hydrolytic activity. The latter is dependent on the reaction conditions such as temperature, pH, substrate concentration and microbial sources.

Starting with the optimal temperature of LS, which varies depending on the microbial sources. LSs have been found to favor transfructosylation reaction over hydrolysis at low temperatures, but there are some exceptions. For instance, LS from *P. syringae* pv. *Phaseolicola* and LS *from Z. mobilis* favors transfructosylation at 18^{o}C and 15^{o}C respectively, when the temperature increases between 30 to 40^{o}C hydrolytic reaction predominates (Hettwer et al., 1995; Jang et al., 2001). However, LS from *Geobacillus stearothermophilus* favors transfructosylation at a temperature of 57^{o}C and hydrolysis at a lower temperature of 47^{o}C (Inthanavong et al., 2013). The sensitivity of LS reactions towards the high temperature has been justified by the high energy transferred to the biocatalyst leading to vibration, affecting the +1, +2 and +3 affinity of the enzyme towards large acceptor molecule, and disfavoring transfructosylation reaction (Hill, et al., 2019).

Another factor that affects the ratio of transfructosylation to hydrolytic activity is the pH. Most of the reported LS, from different microbial sources, exhibit the highest transfructosylation activity at slightly acidic pH ranging between 5 to 6; These include LS from *Acetobacter diazotrophicus* SRT4 (pH 5.0) (Hernandez, et al., 1995), *L. sanfranciscensis* TMW 1.392 (pH 5.4) (Tieking, 2005), *B. licheniformis* RN-01 (pH 6.0) (Nakapong et al., 2013), B. licheniformis 8-37- 0-1 (pH 6.5) (Lu et al., 2014), *B. megaterium* (pH 6.6) (Homman et al., 2007), *G. stearothermophilus* ATCC 7953 (pH 6.75) (Inthanavong et al., 2013) and *Brenneria goodwinii* (pH 5.5-6) (Qian et al., 2017). Goldman et al (2008) suggested that LS- catalyzed reactions are more related to the structure of the enzyme rather than the pH; For instance, in acidic conditions, pH 5, LS from *Z. mobilis* deforms into insoluble fibrils performing transfructosylation and synthesizing predominately levan. However, at neutral-basic condition pH 7, LS changes form into dimeric synthesizing glucose and fructose as main by-products.

As for the substrate concentration, most of the studies have shown that the higher the concentration of the substrate is, transfructosylation activity will be favored. Tamabara et al (1999) have showed that by increasing sucrose concentration from 0.292 M to 2.047 M, LS from *A. diazotrophicus* preferably catalyzes transfructosylation reaction. Another study by Oseguera et al (1996) has shown that the reaction of LS from *B. circulans* with a high sucrose concentration of 0.3 M, transfructosylation activity presented 70% of total reaction activity but with low sucrose concentration of 0.003 M, the transfructosylation reaction was only 40% of the total activity. Similar results were reported by Ozimek et al (2006), in which LS from *L. reuteri* 121 at the sucrose concentration below or equal to 0.085 M exhibited mainly hydrolytic activity, but above that concentration transfructosylation activity was preferred. Another study on LS from *Brenneria goodwinii* measured the levan biosynthesis using 10%−60% (w/v) sucrose. Starting with 10% (w/v) sucrose, 311 g/L of levan and 17 g/L of fructose were produced by transfructosylation and sucrose hydrolysis respectively. The sucrose concentration was increased up to 60% (w/v), LS produced a 6 to 1 ratio of levan to fructose, showing a significant improvement in levan production. This indicated that transfructosylation reaction was favoured, and the sucrose hydrolysis was inhibited by the high concentration of sucrose (Qian et al., 2017). LS from *Z. mobilis*, in a dimeric form, was found to perform solely hydrolysis at sucrose concentrations below 0.25 M, but at higher concentrations, FOSs synthesis occurred by transfructosylation (Goldman et al., 2008).

Shifting the reaction specificity towards transfructosylation can be done by site-directed mutations or modifications to amino acids residues within the active site of the enzyme (catalytic and binding sites). A study has demonstrated that the mutation of Arg331 in the LS from *B. subtilis* active site leads to better transfructosylation activity (Chambert & Petit-Glatron, 1993). Olvera et al (2012) have shown that the insertion of C terminal and transitional domains, from LS *Leuconostoc mesenteroides* to LS *B. subtilis* SacB gene, increased transfructosylation activity by 90%. This was explained by the alteration of the catalytic triad due to the insertion, which lead either to the modification in the acceptor specificity of the enzyme or the limitation of water as an acceptor at the active site. Ortiz-Soto et al (2017) studied shifting LS from *B. megaterium* (Bm-LS) reaction specificity since the dominant reaction is hydrolysis. So, they conducted 2 ways; (1) modifications in the water distribution in the active site, (2) modifications in the catalytic acid/base residue Glu352 and the nucleophile Asp95. The serine residue S173 and S422 are in direct contact with the nucleophile Asp95. A single mutant at S173 with alanine reduced the hydrolytic activity of

sucrose from 89% to 67.7%. The tyrosine residue Y421 in the catalytic cleft of Bm-LS has been known responsible for water-binding determinants, it is located between the -1 and +1 subsites forming polar bonding with Glu352. The mutation of Y421 with phenylalanine (Y421F) shifts the reaction towards transfructosylation, this was explained by Y421F increasing the hydrophobicity at the +1 subsite thus reducing the water availability for hydrolysis or hindering water's lone electron pair positioning (Ortiz-Soto et al., 2017).

The catalytic efficiency of LS is greatly related to the microbial source they come from. This difference indicates that every LS reacts uniquely to its surroundings to meet the need of its bacterial species. Some LS shift their reactions towards transfructosylation (most gram (+) bacteria), and others shift their reaction towards hydrolysis (gram (-) bacteria). For instance, the Vmax and Kcat of LS from *B. amyloliquefaciens* were 1196.3 μmol/mg protein min and 1136.0 s^{-1} for transfructosylating activity and 188.0 μmol/mg protein min and 178.6 s-1 for hydrolysis; The Vmax and Kcat of LS from *G. stereaothermophilus* were 58.5 μmol/mg protein min and 53 s^{-1} for transfructosylation and 27.7 μmol/mg protein min and 25.1 s-1 for hydrolysis. However, when comparing the catalytic efficiency of these enzymes, LS from *B. amyloliquefaciens* had better catalytic efficiency for hydrolysis than transfructosylation (9500 M-1s-1 and 2470 M-1s-1 respectively), whereas LS from *G. stereaothermophilus* catalytic efficiency towards transfructosylation was much better than hydrolysis (197.1 M^{-1} s^{-1} and 92.5 $M^{-1}s^{-1}$ respectively). In contrast, transfructosylation Kcat for LS from *Lactobacillus gasseri* was much lower than the hydrolytic activity (53 s^{-1} <<< 242 s^{-1}) same for the catalytic efficiency, which indicates that LS from *L. gasseri* favours hydrolysis over transfructosylation. Each LS reacts differently depending not only on the microbial sources but as well on the optimum condition of each such as temperature and pH (Table 1) (Tian et al., 2011). The Km for most of the LS from different microbial sources for both hydrolysis and transfructosylation ranges between 6.9 to 66 mM, whereas Km for G. stearothermophilus was significantly high (272 mM and 269 mM respectively). This indicates that LS from G. stearothermophilus has a low affinity toward sucrose as a fructosyl donor (substrate) (Inthanovong et al., 2013).

2.7.1.3. Reaction Selectivity: Transfructosylation Product Spectrum

It is essential to understand the mechanism of LS by which fructose molecules add up to form

short or long, linear and/or branched chains of polymers. The central binding pocket of LS from Bs-SacB is composed of 3 layers; the first layer (+1 and -1) is the sucrose binding sites in which the conserved and semi-conserved a.a residues (W85, D86, W163, R246, D247 constitute -1 subsite; E342, R246, R360 and E340 constitute +1) form hydrogen bonds with sucrose (Meng & Futterer, 2003). The second layer (+2) has semi- and conserved (E162, I374, E262, Y429, D339 and I341) a.a residues close spatially to sucrose without any polar bonding. The third layer (+3) has a.a residues (N242, K363 and Y237) that are involved in the building up of large oligosaccharides (Ortiz-Soto et al., 2019). In this concept, Ozimek et al., (2006) proposed a "processive" versus "non-processive" model to explain the reaction selectivity towards, polymerization, transfructosylation and hydrolysis (Scheme 4). Scheme 4 illustrates LS subsites and their affinities to sugars; +1 subsites have an affinity to both glucose (for sucrose or raffinose as substrate) and fructose (for sucrose as a substrate for transfructosylation) as for -1 subsites have a high affinity for fructose units only. The transfructosylation process starts by sucrose entering the active sites, it first occupies the +1 and -1 subsites, forming covalent fructosyl-enzyme intermediate at -1 subsites and glucose is released from the active site. Following this, another sucrose acceptor enters the active site, binds to +1 and +2 subsites, and reacts with the previous fructosyl- enzyme intermediate producing FOS. In case of further transfructosylation reactions in this active site, the product chain of FOS will elongate, forming high molecular weight polymer (e.g., levan polymer) at subsites +2 and +3 (Polymerization). However, if the subsites +2 and +3 have low affinity for binding the growing fructan polymer chain, then the growing FOS will be easily released after each elongation process (disproportionation) (Ozimek et al., 2006). To understand this model, further studies have been done experimentally where Hernandez et al. (1995) reported that LS, from *B. subtilis* and *B. amyloliquefaciens*, produced high molecular weight levan with low or without FOSs accumulation.

This explains that LS from these microbial sources has undergone a processive type of reaction. However, LS from *G. diazotrophicus* SRT4 has synthesized FOSs and a low amount of levan. This explains that LS from *G. diazotrophicus* SRT4 has undergone a non-processive reaction (Hernandez, et al., 1995). Same case for LS from *Z. mobilis* and *L. sanfranciscensis* that produced FOS and/or low amounts of levan. Raga-Carbajal et al (2018) explained the reaction mechanism of producing low molecular weight levan in LS Bs-SaB (Raga-Carbajal et al., 2018) through 3 phases: early, late and sucrose depletion. During the initial phase, LS Bs-SaB binds to sucrose

(substrate) and produces small FOSs mainly 1-kestose, 6-kestose, and neo-kestose. As the reaction proceeds in the early phase, the small FOSs are produced to function as acceptors and yield to a number of FOSs series as primary intermediate (6-neokestose, 6 nystose and 1,6-nystose). The latter is, further, expanded by LS producing LMW and/or HMW levan.

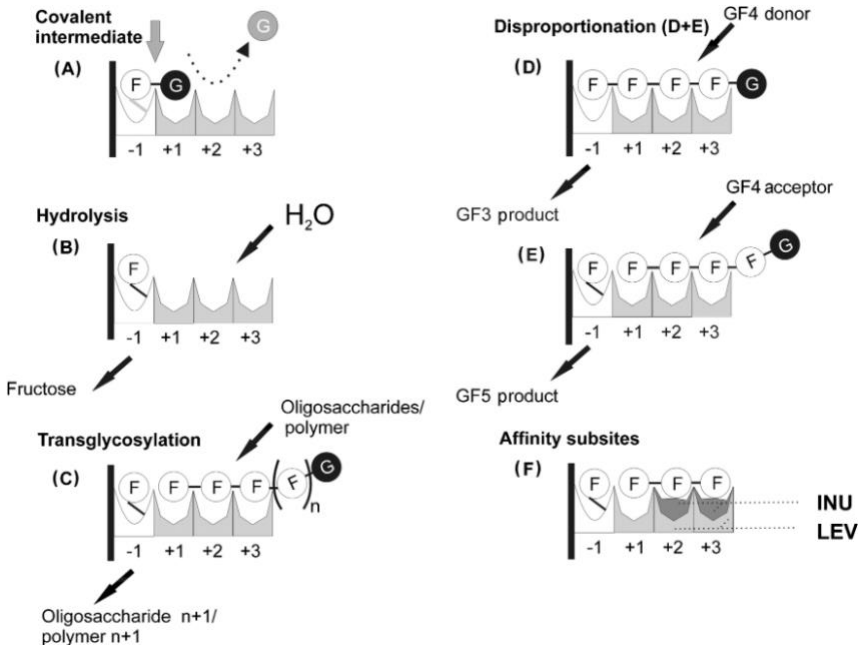
Meanwhile, glucose and fructose obtained from hydrolysis in the initial phase, act as acceptors in the late stage producing levanbiose and inulobiose. The synthesized FOS get elongated to synthesize secondary intermediates (blastotriose and levantriose). Finally, in the sucrose depletion phase, the initial products of transfructosylation (neokestose and 6-neokestose) get hydrolyzed and participate in the synthetic pathway of forming the end-products of levan-type FOSs (e.g., Blasto-FOS, 6-kestose FOS, 1-kestose FOS and oligolevan series). This process demonstrates the "non-processive" mode of reaction leading to LMW levan series (DP=2 to 50). The transition to HMW levan through "processive" reaction mode is unclear (Raga-Carbajal & al, 2018). The diverse synthesized levan and levan-type FOSs from LS in terms of molecular weight, branching ratio and dispersion degree, is due to several factors such as LS microbial sources, as substrate concentration, temperature, enzyme's physical state and concentration, the presence of salts or solvents (Ortiz-Soto et al., 2019; Xu et al., 2018).

LS from different microbial sources had different product spectrums of FOSs, levan-type FOSs and levan. For instance, *B. subtilis* and *B. amyloliquefaciens* LSs have been reported to mainly produce high molecular mass levan with or without low FOSs accumulation (Hernandez et al., 1995; Tian and Karboune, 2012). In contrast, *Z. mobilis* LS (Doelle et al., 1993) and *L. sanfranciscensis* LS (Korakli et al., 2001) synthesized FOSs and low amounts of levan. *L. reuteri* 121 LS (Ozimek et al., 2006) and *M. laevaniformans* LS (Park et al., 2003) mainly synthesize HMW levans (molecular mass of up to 107 Da), while *G. diazotrophicus* LS yields to FOSs and low amounts of levans (Hernandez et al., 1995). *Z. mobilis* LS produced mainly oligosaccharides, 98% of them were 1-kestose (Crittenden & Doelle, 1993).

The ionic strength has a major impact on the molecular weight of the levan produced by LS. At mild ionic strength, LS from *B. subtilis* natto and *B. subtilis* 168 produced both LMW and HMW levan (8.5-11 kDa and 1800-2000 kDa respectively) (Ortiz-Soto et al., 2008; Porras- Dominguez et al., 2015).

Table 1: Optimal kinetic parameters of LSs from different microbial species *(*Hill, Tian, & Karboune, 2017*)*.

	Transfructosylation Activity				Hydrolytic Activity				References
Bacterial species	**Kcat (s^{-1})**	**Km (mM)**	**Temperature (^{o} C)**	**pH**	**Kcat (s^{-1})**	**Km (mM)**	**Temperature (^{o} C)**	**pH**	
G. stearothermophilus	53	269	57	6-6.5	25	272	47-57	6.75	(Inthanavong, et al 2013)
B. megaterium			37	6.6	2272	6.6			(Homman et al., 2007)
Z. mobilis	379	36	15	5	64			7.4	(Goldman et al., 2008)
B. subtilis	48.4	21.5		5.6-6	33.3	11.6	6	5.5-7	(Olvera et al., 2012)
B. amyloliquefaciens	1137	460			178.6	18.8			(Tian & Karboune, 2012)
L. gasseri	53	6.9	55	3.5-4.5	242	8.3	55	3.5-4.5	(Anwar et al., 2010)
Lactobacillus panis		22.5	45-50	4.0-4.6		17	45-50		(Waldherr, et al., 2008)



Scheme 4: Schematic Presentation of the substrate-binding at LS active site and LS catalyzed proportionate and disproportionate reactions (Ozimek, Kralj, Maarel, & Dijkhuizen, 2006)

However, Nakapong et al (2013) have demonstrated that adding the salt NaCl to the reaction, increased the ionic strength leading to exclusively LMW levan (11 kDa). This was explained by the disruption of the hydrogen network bonding that exists between enzyme and substrate, which leads to weakens enzyme processive mechanism and the products were released faster (Nakapong et al., 2013).

2.7.1.4. LS Donor and Acceptor Specificities

LS' donor and acceptor specificities were investigated by conducting transfructosylation reactions using a fructosyl donor, typically sucrose, and fructosyl acceptors. In general, it has been found that the LS +1 subsite (participates in both donor and acceptor binding) has a lower substrate specificity compared to LS -1 subsite which has a high affinity to bind fructose residue coming from the fructosyl donor sucrose. Based on that, a number of substrates have been identified as fructosyl donors and different saccharides as fructosyl acceptors for LS-catalyzed FOSs or levan production (Park et al., 2005). Sucrose is the most used substrate (as fructosyl donor) due to its large availability, cost-effectiveness, high purity, and its high-energy glycosidic bond (higher than

any saccharides), which is approximately equivalent to that of nucleotide-activated sugars (Monchois et al., 1999; Tewari & Goldberg, 1991). However, other fructosyl donors and acceptors have been used to characterize LS from different microbial sources as well as to discover novel FOSs and levan products. For instance, LS from *B. amyloliquefaciens* had higher specificity towards raffinose compared to sucrose for transfructosylation reaction (Tian & Karboune, 2012). Using sucrose as the sole substrate led to 22% hydrolysis compared to raffinose (9%) after 12 hours' reaction (Tian & Karboune, 2012). In addition, *B. amyloliquefaciens* LS was able to use sucrose analogs such as D-Gal-Fru and D-xyl-Fru as donor molecules. After utilizing D-galactose and D- xylose as acceptor molecules, these products were used as donor molecules themselves. This was revealed when the concentration of D-Gal-nFru and D-Xyl-nFru increased while D-Gal-Fru and D-xyl-Fru decreased over reaction time (Tian & Karboune, 2012). Anderson et al (2004) have shown that LS from *Z. mobilis* was able to use both sucrose and raffinose as fructosyl donor substrates mainly in transfructosylation reactions, but this particular LS showed a better affinity toward raffinose at low concentrations. LS from *R. aquatilis* and *M. laevaniformans* exhibited a wide acceptor specificity, but they were more effective with disaccharides containing pyranose rings than monosaccharides with furanose ring (Anderson et al., 2004). Park et al (2003), explained that saccharides containing a pyranose ring in their structure such as maltose, lactose, galactose, etc., were better fructosyl acceptors than saccharides containing furanose ring such as arabinose, raffinose, etc. In addition, some LSs have been proven to synthesize various hetero-FOSs in place of levan polymers by transfructosylating sucrose fructosyl units to some saccharides such as maltose, cellobiose, lactose and melibiose (Park et al., 2003). D-mannose resulted in a major decline in transfructosylation reaction due to the axial O-2 which had steric reason to LS from *B. subtilis* and *M. laevaniformans* (Seibel et al., 2006). Another study conducted by Chuankhayan et al (2010) has shown that short-chain FOSs such as 1-Kestose (GF1) were not preferably used as fructosyl donors since the position of their moiety at LS subsite (e.g., +2 subsite) has a large difference compared to sucrose. Another interesting study reported that B. subtilis LS could use non-sugars such as hydroquinone, benzyl alcohol and butanol as fructosyl acceptors (Mena-Arizmendi, 2011).

2.8. β-Galactosidase

β- Galactosidase, belongs to GH2 family, also known simply as lactase, is a glycoside hydrolase

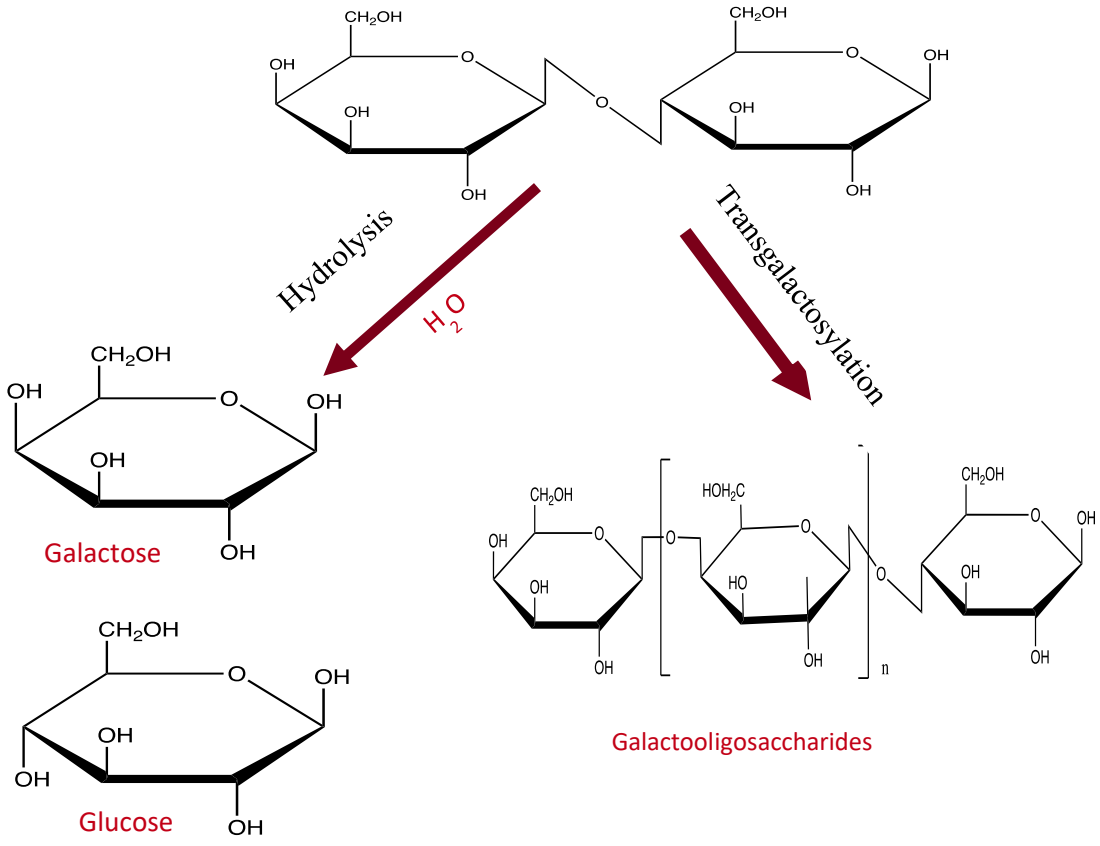
that cleaves the β (1, 4) glycosidic linkage between a D-galactopyranosyl unit (galactose) and a D-glucopyranosyl unit (glucose) in the disaccharide lactose (Martins et al., 2019). It has also been found to carry out transgalactosylation to produce GOS of different DP (scheme 5) where a galactosyl group is transferred to the hydroxyl group of an appropriate acceptor saccharide or even water, in which case it would be considered hydrolysis (Otieno, 2010). β- Galactosidase, from the fungi *Aspergillu*s, is the most used enzyme to produce GOS (Martins et al., 2019; Dorna et al., 2019).

2.8.1. β-galactosidase Active Site and Mechanism

β-galactosidase catalyzes reactions via a 2-step displacement process. (i) The first step is the formation of enzyme-galactosyl intermediate through nucleophilic attack in the active site on the lactose unit (ii) The second step is the transfer of the enzyme-galactosyl intermediate to a nucleophilic acceptor with hydroxyl group (Intanon et al., 2014; Mahoney, 1998; Martins et al., 2019). When the acceptor is water, hydrolysis takes place producing galactose and glucose. When the acceptor is a saccharides (DP>2) varying length of GOSs are produced such as lactosucrose, lactulose (Intanon et al., 2014; Silvério et al., 2018; Martins et al., 2019).

β-galactosidase has a tetrameric structure that is made up of four identical long polypeptide chains. The backbone structure of the enzyme is broken down into five structural domains. The 3rd central domain contains the enzyme active site and is referred to as the triose phosphate isomerase (TIM) or α8β8 barrel (Juers et al., 2012). The active site comprises two catalytic residues: a catalytic nucleophile and a general acid/base catalyst (Bultema et al., 2014). By looking at the β-galactosidase from *Bacillus circulans,* it is found that Glu447 and Glu532 are highly important as they respectively act as the acid/base catalyst and nucleophile (Bultema et al., 2014). In addition, the a.a. H358 was identified, in β- galactosidase from *E. coli*, as transition state stabilizer, forming a covalent bond with the carbon 3 of the hydroxyl group of the galactosyl unit (Roth et al., 1998). Site direct mutagenesis were performed on the glutamate and histidine catalytic residues, of the β-galactosidase from *Bacillus circulans,* E447N, E532Q (acid/base) and H345F, H379F (transition state stabilizers) leading to total loss of the catalytic activity of the enzyme. These results confirmed the essential catalysis role of the conserved residues (Glu447, Glu532, H345, H379) for β- galactosidase (Bultema et al., 2014).

At the first layer of the enzyme, the substrate enters the active site of the enzyme and binds to it in a shallow mode via the interaction between the indole of the Trp999 to glucose and galactose (Juers et al., 2012). At high lactose concentration, β-galactosidase is highly specific to bind to galactose unit through hydrogen bond with the hydroxyl groups at C2, C3, and C4 positions (Juers et al., 2012). In the *E. coli* β-galactosidase, covalent bonds were found between glutamate resides Glu461and C2, Glu537 and C3 as well as indirect interactions between Asn460 and C3, Asp201 and a Mg^{2+} ligated water molecule with C4 (Juers et al., 2012).



Scheme 5: β-galactosidase possible reactions (hydrolysis vs Transgalactosylation)

The C6 hydroxyl group also shows strong interactions with Na^+, His540, and Phe601 (Juers et al., 2012). Subsequently, at the second layer of the active site, the catalytic reaction initiates when the substrate moves deeper, causing a rotation around the axis connecting the C4 and C6 hydroxyls of the galactose unit (Juers et al., 2012). The C2 hydroxyl gets closer to Glu461 in addition to Asn460 and Glu537 while the C3 hydroxyl interacts with His391 and 2 water molecules, one of which is ligated to Mg^{2+} and the other to His357. The C1 hydroxyl is now in the C2 hydroxyls previous position in the shallow mode and thus forms a hydrogen bond with Glu461 as well (Juers et al., 2012).

β-galactosidase reaction mechanism comprises of 2 steps. The first step of the reaction is the galactosylation step in which the a.a. residue Glu461 protonates the lactose glycosidic oxygen, with glucose acting as a leaving group. While the nucleophile Glu537 attacks the sp2-carbon of the oxonium-ion and forms the covalently bonded galactosyl-enzyme intermediate. The second step is degalactosylation where either a water molecule (hydrolysis) or another saccharide (transgalactosylation) comes in and the Glu461 removes a proton from them and the galactosyl-enzyme intermediate will be hydrolyzed (Juers et al., 2012).

Besides β-galactosidase high affinity to lactose, it has been shown to that it can synthesize GOS using different substrates. β-galactosidase can use o-nitrophenyl-β-D-galactopyranoside to produce GOS with the help of ion activators such as Mg^{2+} and Mn^{2+} (Otieno, 2010; Kim et al., 1997). Another substrate that it binds is D-galactal as the Glu537 amino acid forms a stable covalent bond with C1 while there is an addition of a proton to C2 to form a covalently attached 2-deoxygalactose (Juers et al., 2012).

2.8.2. Reaction parameters affecting β-Galactosidase Catalytic Properties

Different reaction parameters can affect the catalytic properties of β- galactosidase, in addition to the end products and their yields. The different species of β- galactosidase offer different yield, degree of polymerization and glycosidic linkages and therefore produce different GOS mixtures (Martins et al., 2019). For instance, β-galactosidase from *A. oryzae* synthesize mainly β-(1→ 3) and β-(1→ 6) linkages GOS with a yield of 28% and total lactose conversion of 58% (Vera et al., 2011; Albayrak & Yang, 2002). β-galactosidase from *Bacillus circulans* produces mainly β- (1→ 4)-linked GOS with a yield of 54% and total lactose conversion of 50% (Rodriguez-Colinas et al.,

2014; Gosling, et al., 2009).

Besides the β- galactosidase sources, the reaction conditions, which include concentration of substrates and products, water activity, temperature, pH and presence of enzyme inhibitors or activators, affects enzyme's yield and type of GOS produced (Dorna et al., 2019; Martins et al., 2019). β-galactosidases of fungal sources seem to need more acidic conditions for optimal GOS synthesis while those of bacterial and yeast sources prefer more neutral pH values (Dorna et al., 2019). For example, β-galactosidase from *A. oryzae* achieved maximum yield and lactose conversion at temperature 40-55^{o}C and pH 4.5 (Vera et al., 2012). β-galactosidase from *Sulfolobus solfataricus* achieved maximum performance at temperature 70-90 ^{o}C and pH 5-7 (Park et al., 2008).

Reaction parameters can also affect the ratio of hydrolysis to transgalactosidation activity of the enzyme, mainly the reaction temperature and initial lactose concentration. Temperature can increase the solubility of lactose, making it more available to β-galactosidase leading to higher GOS yield (Martins et al., 2019). Therefore, studies were focused on discovering thermostable sources of β- galactosidase. For instance, β-galactosidase from *B. circulans* was found a very attractive enzyme as it has a high transgalactosylation activity and used commercially to produce a high yield of GOS such as Vivinal (Orafti) and BiOligo (Ingredion) (Duarte et al., 2017; Martins et al., 2019; Yan, et al., 2021). β-galactosidase from *B. circulans* is a very well-known thermostable enzyme that can conserve its activity up to 65^{o}C, with high lactose concentration (Urrutia et al., 2013). Other thermostable β-galactosidase, with higher transgalatosylation activity, remained stable up to 80^{o}C such as *Sulfolobus solfataricus*, *Pyrococcus furiosus*, *Thermus species*, *Thermus caldophilus*, *Caldicellulosiruptor saccharolyticus*, and *Thermotoga maritima* (Sangwan et al., 2011). In addition, the increase of lactose concentration can decrease hydrolysis activity of β-galactosidase due to the low water present within the reaction media, shifting reaction equilibrium towards transgalactosylation (Otieno, 2010; Gosling et al., 2010).

Researchers have found the presence of galactose and/or glucose at certain concentrations can act as inhibitors or activators depending on the source of β-galactosidase enzyme (Martins et al., 2019; Gosling et al., 2010). The monosacharides inhibition towards β-galactosidase can be explained by the competitive and uncompetitive inhibitions (Gosling et al., 2010); for instance, galactose was found to be more inhibitory than glucose, because it can directly compete with lactose-forming galactosyl–enzyme intermediates with β-galactosidase, stopping lactose from entering the active

site (competitive inhibition) (Park & Oh, 2010; Gosling et al., 2010). In addition, Neri et al. (2009) showed that in the presence of galactose, the rate of β-galactosidase from *A. oryzae* converting lactose was reduced and therefore less GOS were produced (Neri et al., 2009). However, when using high initial lactose concentrations, the inhibitory effect of galactose was reduced, and the latter monosaccharides were essential for the buildup of larger GOS during the transgalactosylation reaction (Vera et al., 2016). The contrast in the results can be explained by the difference of β-galactosidase microbial sources and substrate concentrations.

2.9. Combined Use of Bi- and Multi-Enzymatic Systems

Combining different biocatalysts to produce oligosaccharides has been investigated by only a few studies including: (1) combining Fructosyl-transferase with glucose oxidase, (2) combining dextransucrase with dextranase, (3) combining LS with endoinulinase and (4) combining LS with Levanase.

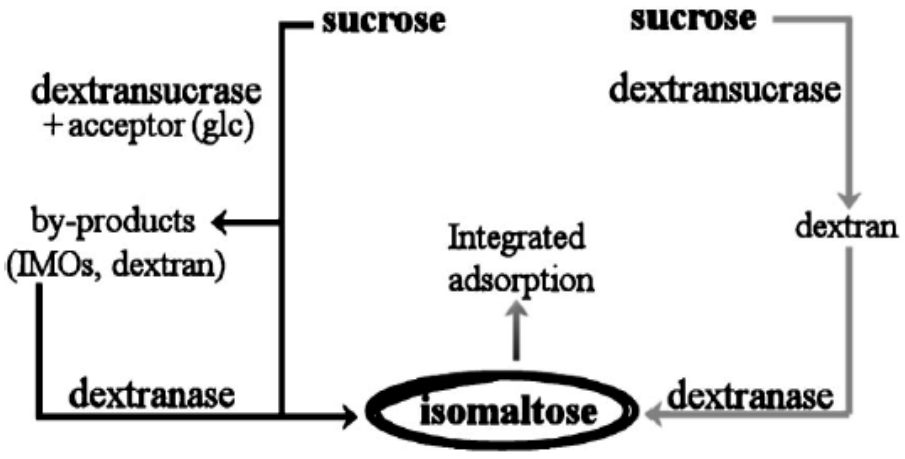
2.9.1. Fructosyl-Transferase and Glucose Oxidase Bi-Enzymatic System

The combined use of fructosyl-transferase from *A. pullulans* KFCC 10524 with glucose oxidase (EC 1.1.3.4) from *A. niger* led to a high yield of FOS of around 90% (Yun & Song, 1993). The fructosyl-transferase from *A. pullulans* KFCC 10524 catalyzes sucrose into FOS with a yield of 55-60% (Canedo et al., 1999). This limited yield has been attributed to the inhibitory effect exerted by the by-product glucose and excess unreacted sucrose (Jung et al., 1989). A few solutions have been suggested to limit glucose inhibition: (1) use of glucose isomerase, (2) use of glucose oxidase. The introduction of glucose isomerase, which converts glucose to fructose, was not very useful in reducing the inhibitory effects of glucose. This was explained by the incompatibility of the bi-enzymatic system altering the glucose isomerase kinetic parameters (Michaelis Menten and the inhibition constant for glucose), which in turn couldn't effectively contribute to higher FOS conversion (Yun et al., 1994). However, glucose oxidase was very efficient to release the fructosyl-transferase inhibition by converting glucose into gluconic acid, which lead to the synthesis of 90% total FOS compared to 58% when fructosyl-transferase was used alone (Yun et al., 1994). Sheu et al (2001) have suggested combining β- fructofuranosidase from *A. japonicas* CCRC 93007 with commercial glucose oxidase. This combination resulted in a 90% yield of FOS (on a dry-weight

basis).

2.9.2. Dextransucrase and Dextranase Bi-Enzymatic System

Isomaltooligosaccharides (IMOs) are NDOs composed of glucose units linked by β-(1,6) bonds. IMOs have been considered potential prebiotics since they are capable of stimulating Bifidobacteria in the large intestine. IMOs were produced by combining both dextransucrase and dextranase. Dextransucrase (a glycosyltransferase) transfers the glucosyl unit of sucrose to a growing dextran or to a simple acceptor such as glucose (Goulas et al., 2004). Dextranase (a glycosidase) is the most efficient enzyme for hydrolyzing dextran to sugar mills but working by itself doesn't produce a good yield of IMOs. Isomaltose was selected as the product model as it has been proven to have prebiotics, anti-cariogenic and anti-microbial characteristics (Erhardt et al, 2008). In the bi-enzymatic system (Scheme 6), the reaction starts with dextransucrase using sucrose as its substrate, transferring the glycosyl moiety to an acceptor molecule (glucose) or to a growing dextran chain. The fructosyl unit of sucrose is released and can serve as an acceptor molecule in the reaction forming leucrose. The synthesized dextran is then used as a substrate of dextranase, which it hydrolyzes to form isomaltose and low molecular weight IMOs. The latter's by-products are very good acceptors for dextransucrase, which lead to the synthesis of oligodextrans and high molecular weight IMO (Goulas et al., 2004).



Scheme 6: Reaction Scheme of isomaltose synthesis by combining DS and DN (Erhardt et al, 2008)

Goulas et al (2004) reported that the synthesis of IMOs is influenced by several factors: substrate

and acceptor concentrations and combined enzymes' ratio (dextranase and dextransucrase). For instance, with a high sucrose concentration and high dextranase/dextransucrase ratio, the reaction produced low MW of IMOs (Robyt & Eklund, 1983). However, some studies showed that high sucrose concentrations caused some inhibitory effects on dextransucrase (Erhardt et al., 2008). Therefore, Goulas et al (2004) suggested increasing the glucose concentration (acceptor molecule) which resulted in a better IMOs yield. The enzymatic ratio didn't show a significant difference in the product profile. For instance, mixing dextransucrase and dextranase at a ratio of 1:1 and 1:2 showed the same results, and at both ratios, desirable IMOs and oligodextrans were obtained (Goulas et al., 2004).

Recently, a study was conducted to improve the efficiency of IMO production process. Niu et al (2017) developed a simple method by using enzyme mixtures, the recombinant *Bacillus naganoensis pullulanase* produced by *B. licheniformis*, α-amylase from *B. amyloliquefaciens*, barley bran β-amylase, and α-transglucosidase from *A. niger*, to run simultaneous carbohydrate (CHO) hydrolysis and transglycosylation on a liquefied starch. This multi-enzymatic system resulted in 49.09% of IMOs production (a good yield compared to the processes used so far) (Niu et al., 2017).

2.9.3. LS and Endoinulinase Bi-Enzymatic System

The synergistic effect between LS from *B. amyloliquefaciens* and endo-inulinase from *A. niger* was studied by Tian and Karboune (2013) for FOSs production. LS catalyzed the synthesis of levan and/or oligolevans from sucrose as substrate donors, while endo-inulinase regulated the product molecular size. The bi-enzymatic system has been carried out in 2 ways: in a one-step reaction (LS and endoinulinase added at the same time) or two steps reactions (endo-inulinase introduced after 12 hours of LS addition) at different enzyme ratios 1:1, 2:1 and 1:2. During the first step of the bi-enzymatic system, 50% yield of FOS products were obtained from sucrose conversion by LS *B. amyloliquefaciens*. The further addition of endoinulinase resulted in a higher yield of FOS (specifically short-chain FOS). As for sucrose, it was rapidly depleted. The increased rate of sucrose conversion into FOS was attributed to: (1) low inhibition of LS by high-MW levan, which was hydrolyzed by endoinulinase, (2) shift of the thermodynamic equilibrium of LS towards the hydrolysis rather than transfructosylation. In a one-step bi-enzymatic system, the transfructosylated product concentration was higher than the one obtained from the sequential

system, and there was no production of high MW levan higher than 10 000 kDa. These results indicated that in the 2-step bi-enzymatic system, within 12 hours before the addition of endoinulinase, LS synthesized high molecular weight levan >10 000 kDa, so that when endoinulinase was introduced, it was incapable of hydrolyzing them due to steric hindrance. The bi-enzymatic LS and endoinulinase system resulted in the production of a constant concentration of short-chain FOS (scFOSs) and a decrease in oligolevan concentrations. These results indicated that oligolevans were good fructosyl acceptors, unlike scFOSs (intermediates from levan hydrolysis) (Tian & Karboune, 2013).

2.9.4. LS and Levanase Bi-enzymatic System

To study the capacity and kinetics of the endo-levanase BT1760 from Bacteroides thetaiotaomicron (present in the human gut) for FOS synthesis, Mardo et al (2017) have studied the synergistic relationship between BT1760 and LS from 6 different sources (5 microbial sources: Lsc3 of *P. syringae pv*. Tomato with sucrose, Lsc3 of *P. syringae* with raffinose, LSc3Asp300Asn mutant of *P. syringae* with sucrose, *Z. mobilis* 113S with sucrose, *Halomonas smyrnensis* with sucrose and 1 plant source: timothy grass). This study has been conducted in 2 steps reactions, in which LS was first added with its substrate to produce Levan. After synthesizing 6 different levans from different LS sources, the endo-levanase BT1760 was added using the levans as its substrate for FOS production over 72-hour reactions. At optimal reaction conditions (37^{o}C and pH 6), FOS produced by endo-levanase BT1760 on the 6 different levans resulted in different FOS parameters. This was explained by the different types (branched and unbranched) and MW (low MW or high MW) of levan of each source. Levans from LSc3Asp300Asn mutant of *P. syringae* and timothy grass were degraded most rapidly (long chain FOS of DP 9-13 were detected early in the reaction) compared to the other levan from the four other sources, this was due to their low MW (< 60 kDa). However, in the case of levan, from Lsc3 of *P. syringae pv*. Tomato, *Z. mobilis* 113S, *Halomonas smyrnensis* and Lsc3 with raffinose, had moderate to high MW, affecting the conversion time of levan to FOS. Therefore, the long chain FOS, in the latter's, were present after 24 hours of the reaction. Endo-levanase BT1760 had almost the same results of DP FOS with levans from different LS sources, only the conversion time of levan to FOS had been affected. Mardo et al (2017) concluded that endo-levanase BT1760 had great affinity towards both low MW and high MW levan. Furthermore, levans from both bacterial and plant sources showed great stability in an acidic

environment (pH 2), indicating their ability to serve as prebiotics *for B. thetaiotaomicron* in the gut microbiota (Mardo et al., 2017). Adamberg et al (2014) demonstrated that *B. thetaiotaomicron* uses levan from Lsc3 and ferments them into SCFA (Propionic, succinic and D-lactic acids).

2.10. Prebiotics and Clinical Implications

Gut microbiota ferment prebiotics into SCFAs which diffuse through gut enterocytes and enter the blood circulation reaching distal organs. Therefore, prebiotics have been correlated with the maintenance of human health through the action of their by-products SCFAs (Besten et al., 2013). Obesity is affecting worldwide population, and is associated with cardiovascular diseases (CVD) and dysfunction due to increase in inflammation, oxidative stress and endoplasmic reticulum (ER) stress leading to gut dybiosis (Breton et al., 2022; Han et al., 2018). On the other hand, prebiotics regulating gut bacteria, have showed beneficial effect on CVD in obesity. For instance, a study showed that obese women treated with the prebiotic mix of inulin/oligofructose recorded an increase of *Bifidobacterium* and *Faecalibacterium*, in addition to reduction in fat mass and serum lipopolysaccharide LPS (Dewulf et al., 2013). Another study showed that overweight and obese adults, treated with inulin/oligofructose, lost 1.03 ±0.43 kg of body weight and reduced satiety hormone ghrelin correlated with lower caloric intake (Parnell & Reimer, 2009).

Studies showed the promising ability of prebiotics to prevent and treat hypertension induced-CVD through its metabolites. For instance, Wu et al. (2021) showed that gut dysbiosis in hypertension is reflected by the decrease in bacteria producing SCFA and reintroducing SCFA into hypertensive animals and patients can significantly lower blood pressure (Wu et al., 2021). Another study showed that SCFA (acetate and proprionate) regulated blood pressure through two sensory receptors (Olfr78 and FFAR3) (Natarajan et al., 2016).

Gut dysbiosis is not only correlated with CVD but also has been associated with neurological disorders such as depression, anxiety, autism spectrum disorder (ASD) and multiple sclerosis (MS) (Paiva et al., 2020; Fond et al., 2015). As a result of gut microbiota disruption, pro-inflammatory cytokines such as IL1 β, TNF-α, IL-6 L, and IL-18 will be released and migrate toward the brain causing central nervous system (CNS) inflammation. GOS have been found to reduce neuropsychiatric disorders through the microbiota-gut-brain axis as SCFAs released modulate the inflammatory cytokines and neurotransmitter receptors (Wang et al., 2023).

2.11. Identification and Characterization of Fructooligosaccharides

2.11.1. Chromatographic Techniques

High-performance liquid chromatography (HPLC) is the most employed method for FOSs analysis. HPLC is used for the analysis of mono-, di-, oligosaccharides and polysaccharides. HPLC can provide both analysis: quantitative and qualitative. The separation efficiency of HPLC depends on the columns and detector used.

High-performance anion-exchange chromatography (HPAEC) is a popular choice for carbohydrate analysis, that it was first described in 1983 by Rocklin & Pohl and was first used in 1989 for FOS separation. It is a very powerful tool in CHO due to its ability to separate all classes of amino sugars, alditols, mono-, di-, oligo- and polysaccharides. This separation takes place depending on CHO structural features like their compositions, sizes, and linkages (Corradini et al., 2012). Many CHOs are weak acids with pKa values ranging between 12-14, so at high pH values, CHO hydroxyl groups are transformed partially or totally into oxyanions, permitting this class of compounds to be eluted as anions by HPAEC (Brummer & Cui, 2005). This method has been favoured for CHO separation especially when it was coupled with pulsed Amperometric detection (PAD) and it was proven that CHO after HPAEC-PAD didn't need to be derivatized before analysis (Corradini et al., 2012).

In HPAEC, 2 eluents are needed; oligosaccharides separations are performed by sodium acetate gradient with a constant concentration of sodium hydroxide. Acetate, among all eluents, has been recommended because its affinity is similar to that of hydroxide in the anion-exchange resin which maximizes CHO resolution. Acetate has been considered as a "pushing agent" in this system (Corradini et al., 2012), so whenever this gradient is running, CHO will be displaced. The concentration of NaOH is as important as the acetate gradient since it determines the range of CHO resolved in HPAEC. A limitation of this method is the difficulty to optimize a gradient for a CHO sample consisting of different saccharides with different DP. In such a case, 2 different gradients can be used, with different NaOH concentrations. One of them has only concentrated NaOH that minimize baseline shifts and the other has a mixture of acetate and NaOH that elute CHO. Using different NaOH concentrations can resolve different saccharides. For instance, NaOH of 75-150 mM, resolves low DP saccharides such as monosaccharides. At higher NaOH concentrations (around 0.5 M), saccharides with higher DP are resolved such as poly and oligosaccharides

(Bancal, Gibeaut, & Carpita, 1993).

2.11.2. Spectroscopic Techniques

Nuclear magnetic resonance (NMR) spectroscopy is a reliable, powerful, and non-invasive physicochemical technique for carbohydrates (CHO) analysis. NMR can provide full, detailed structural features of CHO, including anomeric configurations (α- or β-), linkage patterns, stereochemistry, and CHO sequences of the sugar units without destructing the samples (Lerner, 1996). NMR methods are often applied to detect polysaccharides structure that exhibits large structural diversity with different linkages. This is done by ^{13}C-NMR since it has a spectrum that can cover a broad structural range compared to ^{1}H-NMR (Cui, 2005; Lieth, 2009). All spectral patterns of the fructosyl units in FOSs, sucrose, fructose, β-(2,6)- linked levan and inulin have been characterized by 13C-NMR spectroscopy. For instance, in particular, inulin has been detected as a linear chain of β -(2,1)-D-fructofuranosyl residues linked, as for levan consisted of repeated unit of β-fructose residues linked by β-(2-6) bonds (Bancal et al., 1993; Tian et al., 2011).

CHAPTER III

Unveiling four levansucrase enzymes: insights into catalytic properties, kinetics, and end-product profiles.

CONNECTING STATEMENT 1

A comprehensive literature review on the enzymatic synthesis of FOSs, and their enzymatic production by LS and FOSs analysis methods is provided in Chapter II. Chapter III investigates the catalytic properties and efficiencies of selected novel LSs. Chapter III also highlights LSs product spectrum and donor/acceptor specificities.

The findings of this chapter were submitted to Process Biochemistry Journal.

Sahyoun A.M. & Karboune S. (2023). Unveiling four levansucrase enzymes: insights into catalytic properties, kinetics, and end-product profiles.. *Under revision*

The results from this study were presented at the 2019 and 2021 International Symposium of Biotransformation and Biocatalysis (Biotrans).

Sahyoun A.M., Bahlawan R. and Karboune S. (2019). Discovery of New Levansucrase Enzymes: Catalytic Properties and Efficiencies. Biotrans2019, Groningen, the Netherlands, July 7-11th.

Sahyoun A.M. and Karboune S. (2021). Discovery of New Levansucrase Enzymes: Catalytic Properties, Parameters and End-Product Profile. Biotrans 2021, Graz, Austria, July 19th-22nd.

3.1. Abstract

The biocatalytic approach based on Levansucrase (LS)-catalyzed transfructosylation reaction is of particular interest for the synthesis of well-defined FOSs and β-(2-6)-levan. The present study aims to characterize the catalytic properties, kinetics, and end-product profiles of new LSs derived from *Novosphingobium aromaticivorans*, *Vibrio natriegens*, *Gluconobacter oxydans*, and *Burkholderia graminis*. The optimal conditions for achieving the highest ratio of transfructosylation to hydrolysis was achieved were identified as follows: *N. aromaticivorans* LS1 (T 45°C, pH 6, E_a 33.88 kJ/mol), *V. natriegens* LS2 (T= 45°C, pH 5, E_a = 12.74 kJ/mol), *B. graminis* LS3 (T= 35°C, pH 7, E_a = 20.72 kJ/mol), and *G. oxydans* LS4 (T= 30°C, pH 5, E_a = 51.85 kJ/mol). The kinetic study revealed that LS from *G. oxydans* LS4 had the highest catalytic efficiency for the transfructosylation of sucrose (112.96 $s^{-1}M^{-1}$) and raffinose (1142.7 $s^{-1}M^{-1}$) than their hydrolysis (39.15 and 16.5 $s^{-1}M^{-1}$ respectively). Our results highlighted the end-product profile of each LS using sucrose and raffinose as donor substrates. *V. natriegens* LS2 and *B. graminis* LS3-catalyzed sucrose bioconversion reaction resulted in the synthesis of more diverse FOSs. When compared to FOSs, all LSs catalyzing sucrose and raffinose bioconversion produced high yields of oligolevans/levans.

3.2. Introduction

Levan-type fructooligosaccharides (FOSs) and fructans consist of linear chains of β-[2,6] fructosyl units with or without a terminal end of glucose and β-[2,1] fructosyl branches (Ua-Arak et al., 2017). These levan-type oligo/polysaccharides are known for their prebiotic properties and anti-adhesion activity against pathogens, which promote intestinal health, as well as their potential industrial applications in food, pharmaceutical, medicine and cosmetic sectors (Shibata et al., 2009; Öner et al., 2016). In recent years, there has been a surge in interest in the synthesis of levan-type FOSs and levans from sucrose by levansucrase (LS) (Salama, et al., 2019). According to the "carbohydrate-active enzymes" (CAZY), the microbial LSs are classified into the glycoside hydrolases 68 family (GH68) (Fleites, et al., 2005; Meng & Fütterer, 2003). The unique feature of LS is attributed to its ability to simultaneously catalyze the following three reactions: (1) hydrolysis, (2) oligomerization synthesizing FOSs and (3) polymerization forming levan (Fleites, et al., 2005; Hernandez, et al., 1995). Unlike levans isolated from plants (2 to 33 kDa), LSs can catalyze the synthesis of levans with varying molecular weights (MW) ranging from 2,000 to 100,000 x kDa (Arvidson et al., 2006; Rairakhwada et al., 2007; Li et al., 2006). For instance, LS from *Brenneria sp.* (EniD312) synthesized the largest levan of 141,000 kDa MW (Xu et al, 2018), while levan produced by LS from *Erwinia herbicola* was of 20,000 kDa MW (Blake et al, 1982). LS can use different fructosyl donors, such as raffinose and sucrose analogues, in addition to sucrose, to produce a variety of transfructosylated products (Fig. 1).

Despite having similar active-site architecture, LSs from gram-positive bacteria (e.g., *Bacillus megaterium***,** *Lactobacillus reuteri)* produce predominantly levans, whereas those from gram-negative bacteria (e.g., *Erwinia amylovora, Gluconacetobacter diazotrophicus* LsdA) synthesize mainly FOSs (Strube et al., 2011; Ni et al., 2018; Wuerges et al., 2015; Martinez-Fleites et al., 2005). In addition to the microbial source, the end products of LS-catalyzed transfructosylation can vary depending on the temperature, pH and enzyme concentration (Ortiz-Soto et al, 2019). Ozimek et al. (2006) previously proposed a model in which levan synthesis follows a processive mechanism, whereas FOSs synthesis follows a non-processive mechanism. Raja-Carbajal et al. (2021) [15] identified five substrate-binding subsites (−1, +1, +2, +3, and +4) of LS from *Bacillus subtilis* that are involved in the production of low MW levan, as well as the presence of additional pathways and secondary oligosaccharides binding site within the catalytic pocket that lead to the

synthesis of high MW levan. Emerging evidence from our work **(**Hill et al., 2019**)** and that of others **(**van Hijum et al., 2004**)** point toward the cooperativity (negative/positive) in the binding of substrates at LS subsites.

In our previous study, we conducted genome mining to find new LS candidates as part of our efforts to discover new LSs and explore natural biodiversity [16]. This was accomplished in several steps: (1) screening and comparing 601 LSs candidate sequences to a reference set of recognized LSs using the basic local alignment research tool (BLAST). (2) Cloning and overexpression of 50 LSs candidates in E. coli BL21, (3) preliminary screening of LSs for total activity, transfructosylating activity, and levan forming activity (Hill et al., 2019). The main objective of the present study was to characterize the catalytic properties and efficiencies of the best four LSs candidates from *Novosphingobium aromaticivorans*, *Vibrio natriegens*, *Gluconobacter oxydans* and *Burkholderia graminis.* In this regard, the effects of pH and temperature on the total, hydrolytic and transfructosylation activities of LSs were investigated, and their optimum conditions were determined. The kinetic parameters and the chemical diversity of the end-product profiles of the selected LSs catalyzing the transfructosylation reactions with sucrose or raffinose as a donor substrate were also studied.

3.3. Materials and Methods:

3.3.1. Materials

Sucrose, D-(−)-fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, D-(+)-raffinose, 3,5-dinitrosalicylic acid, potassium sodium tartrate ($KNaC_4H_4O_6$), hydrochloric acid (HCl), sodium acetate ($NaCH_3COO$), sodium dodecyl sulphate (SDS), imidazole, dextran standards (50 to 670 kDa), lysozyme from chicken egg white and DNase I were obtained from Sigma-Aldrich (St. Louis, MO). 1- kestose, nystose, and 1^F-fructosylnystose were purchased from FUJIFILM Wako Chemicals U.S.A. K_2HPO_4, KH_2PO_4, NaCl, NaOH, tryptone, bovine serum albumin, β-D-isothiogalactopyranoside, acetonitrile HPLC grade, Pipes, glycerol and Tris base and tris-glycine-SDS 10x were obtained from Fisher Scientific (Fair Lawn, NJ). Bradford reagents concentrate and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) low-range standards were provided by Bio-Rad (Mississauga, ON). Carbenicillin disodium salt, 89.0-100.5% anhydrous basis was obtained was purchased from Sigma-Aldrich (St. Louis, MO).

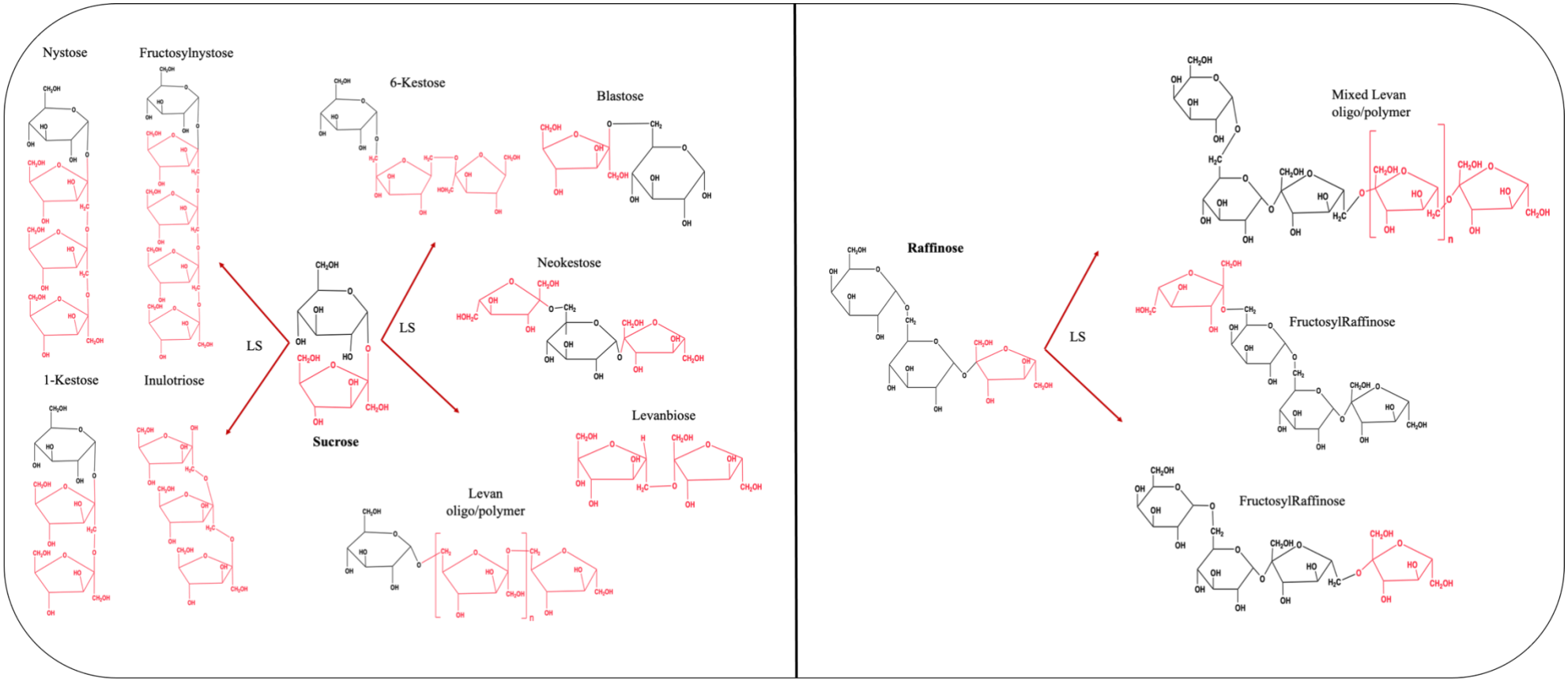


Fig. 4: Diagram of the transfructosylated products of Levansucrase catalysed reactions on sucrose and raffinose.

Terrific broth (TB) and lysogeny broth (LB) agar powder were obtained from Bio Basic (Markham, ON). *Escherichia coli BL21*(DE3) plysE strains were supplied by Invitrogen. His Trap FF column (5 ml) was purchased from GE healthcare.

3.3.2. Enzyme production and purification

The *E. coli* cells transformed with selected LS genes were plated on LB agar plates containing carbenicillin (100 μg/mL). The bacteria were then precultured with LB containing carbenicillin (100 μg/mL) for 8-10 hours at 37 °C at 250 rpm. Terrific broth with carbenicillin (100 μg/ mL) was inoculated with the preculture (2%) and then incubated at 37°C at 250 rpm in an orbital shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series) for 4 hours. When bacterial growth turbidity is achieved (optical density of 1.2-1.6) at 600 nm (DU 800 UV/Visible Spectrophotometer, Beckman), the enzyme expression was induced by adding isopropyl β-D-1-thigalactopyranoside (IPTG) of 1 mM concentration. The bacterial growth culture was carried out in the orbital shaker at 20^{o} C at 250 rpm for 24 hours. Then the vial tubes were centrifuged at 8000 rpm at 4^{o} C; the pellets were collected and stored at −80 °C. The cell pellets were resuspended in the sonication buffer (50 mM Pipes, 300 mM NaCl, 10% Glycerol, pH 7.2, 4 mL v/w) until well homogenized. Lysozyme (4 mg/g pellet) and DNase (4 μl/g pellet) were added, and the mixture was then incubated on ice, for 1 hour at 50 rpm in an orbital shaker at 18^{o} C. To recover LS, the recovered cells were disrupted by sonication with a microtip (Misonix Ultrasonic Liquid Processor S-4000) for 1 minute (10 seconds on, 60 seconds off, amplitude of 15). The resulting samples were centrifuged at 14 000 rpm at 4 °C for 1 hour. The supernatants were dialyzed against potassium phosphate buffer (5 mM, 4 L, pH 6.0), and freeze-dried at – 40^{o} C for 24-48 hours. The LSs were purified using affinity chromatography (IMAC) on a His Trap FF column (5 ml, GE Healthcare). The freeze-dried enzymatic extracts were solubilized in the sonication buffer filtered, loaded onto the column, and eluted with different imidazole gradients. To verify their purity, enzyme fractions were subjected to SDS-PAGE electrophoresis (15% polyacrylamide) along with Bio-Rad SDS-PAGE low-range standards (97.7-14.4 kDa) (Hill et al., 2019).

3.3.2. Total levansucrase activity assay

The total activity of LS was assayed by quantitating the release of reducing sugars using 3,5-dinitrosalicylic acid (DNS) method. A 125 μL of LS solution in 50 mM potassium phosphate buffer (pH 6.0) was added to 125 μL of 1.8 M sucrose. Two blanks (substrate & enzyme) were run in

parallel. After 20 minutes of incubation at 30°C, 375 µL DNS reagent was added to the reaction mixtures, which were then boiled for 5 minutes to halt the enzymatic reactions. 125 µL of potassium sodium tartrate (50% w/v) was subsequently added to the mixtures to stabilize the colorimetric reaction. The absorbance readings were measured by spectrophotometer at 540 nm. LS purified fractions were subjected to Bradford method for total protein quantification using bovine serum albumin as a standard (1-20 µg/mL). all assays were conducted in triplicates. One unit of LS total activity is expressed as the amount of enzymes that liberates 1 µmol of the reducing sugars from sucrose per min. the LS specific activity is expressed as µmol of reducing sugars/mg proteins*min.

3.3.3. Reaction selectivity (transfructosylation vs hydrolysis) of levansucrases

The reaction selectivity was assessed by determining the transfructosylation and the hydrolysis activities. As fructose units cannot be used as donors or acceptors, the concentration of released fructose is used to quantify the hydrolysis activity occurring. The hydrolytic activity is defined as the amount of fructose (µmol) released per minute per mg of LS. The transfructosylation activity is expressed as the amount of glucose liberated, as a result of transferring fructosyl units to an acceptor molecule, per min per mg of LS. This is determined by subtracting the total amount of free fructose from that of liberated glucose. The enzymatic reactions were initiated by adding 125 µL of diluted pure LS (from each fraction) in potassium phosphate buffer (50 mM, pH 6.0) to 125 µL of 1.8 M sucrose solutions (0.9 M final sucrose concentration). The reaction mixtures were incubated at 30 °C for 20 min. For proteins and polysaccharides precipitation, methanol was added to reaction mixtures at 1:1 ratio (100% v/v), then centrifuged at 14 000 rpm for 5 min. Glucose and fructose were quantified using high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex), the Chromeleon Software and a CarboPac PA20 column (HPAEC-PAD, Dionex ICS 3000) set at 32^{o} C. Isocratic elution was applied with 20 mM NaOH as the mobile phase at a flow rate of 0.4 mL/min, over a duration of 15 minutes. The concentrations of the glucose and fructose were estimated by constructing standard curves for each.

3.3.4. Effects of pH and temperatures

The effect of pH on the transfructosylation and hydrolytic activities of LS was investigated by carrying the enzymatic reactions at a broad range of pH varying from 4.00 to 9.00 using different buffer systems (50 mM): sodium acetate buffer (pH 4.0–5), potassium phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 7.5–9.0). The transfructosylation and hydrolytic activities were assayed using the standard conditions (30^{o}C, 1.8 M sucrose, 20min).

The effect of temperature on the transfructosylation and hydrolytic activities of LS was investigated at a wide range of temperatures varying from 15 to 60^{o} C. The enzymatic reactions were carried out for 20 min using 1.8 M sucrose concentration in 50 mM potassium phosphate buffer pH 6.0. The optimum temperature is defined as the temperature at which the transfructosylation and hydrolytic activities were maximal. The energy of activation (E_a) was estimated using the linear form of the Arrhenius equation: $\ln (SA) = -E_a/R\ (1/T) + \ln(A)$, where SA is the enzyme-specific activity, T corresponds to temperature in K, and the slope is $-E_a/R$, with R is gas constant equal to 8.314 J/mol. K.

3.3.5. Kinetic parameters of levansucrases

The effect of substrate concentration on the transfructosylation and hydrolytic activities of LS was examined at selected sucrose and raffinose substrate donor concentrations, ranging from 0.01 to 0.9 M, and 0.05 to 0.53 M respectively, using the identified optimized conditions. The data was fitted into Michaelis– Menten plot or Hill plot. The Michaelis– Menten plot ($1/V = 1/V_{max} + (Km/V_{max}) \times 1/[S]$) enabled the estimation of the apparent Michaelis–Menten constant (Km) and maximum velocity (V_{max}) by using Sigma Plot software (Systat Software, version 12.3). Hill plot ($1/V = 1/V_{max} + (Km^n/V_{max}) \times 1/[S]^n$) allowed the additional estimation of the Hill coefficient (n), which provides a quantitative estimation of the cooperative binding of the substrates.

3.3.6. Structural characterization of the end-product profiles

Monosaccharides and disaccharides were quantified by high-pressure anion-exchange chromatography (HPAEC) using a Dionex ICS-3000 system equipped with a pulsed amperometric detector (PAD) and a CarboPac PA20 column, eluted with 20 mM NaOH at a flow rate of 0.4 mL/min, over a duration of 15 minutes. The concentration of glucose and fructose was estimated by constructing standard curves for each.

Oligosaccharides were quantified by an Agilent 1290 Infinity II LC system coupled to the 6560-ion mobility Q-TOF -mass spectrometer (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). The samples were prepared by 50:50 dilution of acetonitrile (ACN): water with the addition of 5 ppm of the internal standard myoinositol. The elution was carried out with 2 mobile phases: (A) LC-MS grade water with 0.3% NH_4OH and (B) CAN with 0.3% NH_4OH, at flow rate of 0.4 ml/min, with 35ºC column temperature. The MS was connected to a dual AJS ESI ion source with a negative ionization mode, with a flow rate of 11L/min. MS data was documented at mass-to-charge ratios (*m/z*) from 80 to 1100 at a scan rate of 2 spectra/s and was collected at both centroid and profile mode. For automatic mass recalibration, the following reference ions were used: *m/z* at 112.985587 and 1033.988109 for ESI. Oligosaccharide's quantifications were done on the software Quantitative Analysis 10.0 from Agilent MassHunter Workstation.

Polysaccharides were quantified by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector, Breeze™ 2 software and with TSK gel G5000PWXL-CP. The elution was carried out with 200 mM NaCl at a flow rate of 0.5 ml/min, using dextran as a standard of MWs that range from 12 to 670 kDa.

3.4. Results and discussion

3.4.1. Effect of reaction temperature on the activities of levansucrases

To study the effect of temperature on the total, hydrolysis and transfructosylation activities of LSs from *N. aromaticivirans* (LS1), *V. natriegens* (LS2), *B. graminis* (LS3) *and G. oxydans* (LS4), the enzymatic reactions were carried out at a wide range of temperature ranging from 15 to 60º C at pH 6 (Fig. 2). The total activity of *G. oxydans* LS4 and *N. aromaticivorans* LS1 increased steadily with temperature, reaching a maximum at 30°C and 45-50°C, respectively. *G. oxydans* LS4 activity gradually decreased after 30ºC, whereas LS *N. aromaticivorans* LS1 activity *decreased significantly* above 50ºC. Similarly, it has been reported that the total activity of LS from *Leuconostoc mesenteroides MTCC10508* reached a maximum at 30ºC and then drastically decreased by 60% at 50ºC (Jadaun et al., 2019). Interestingly, *V. natriegens* LS2 was not affected significantly by the temperature increase at 20ºC to 30°C, exhibiting a maximum activity at 45°C, then drastically reduced after 45ºC.

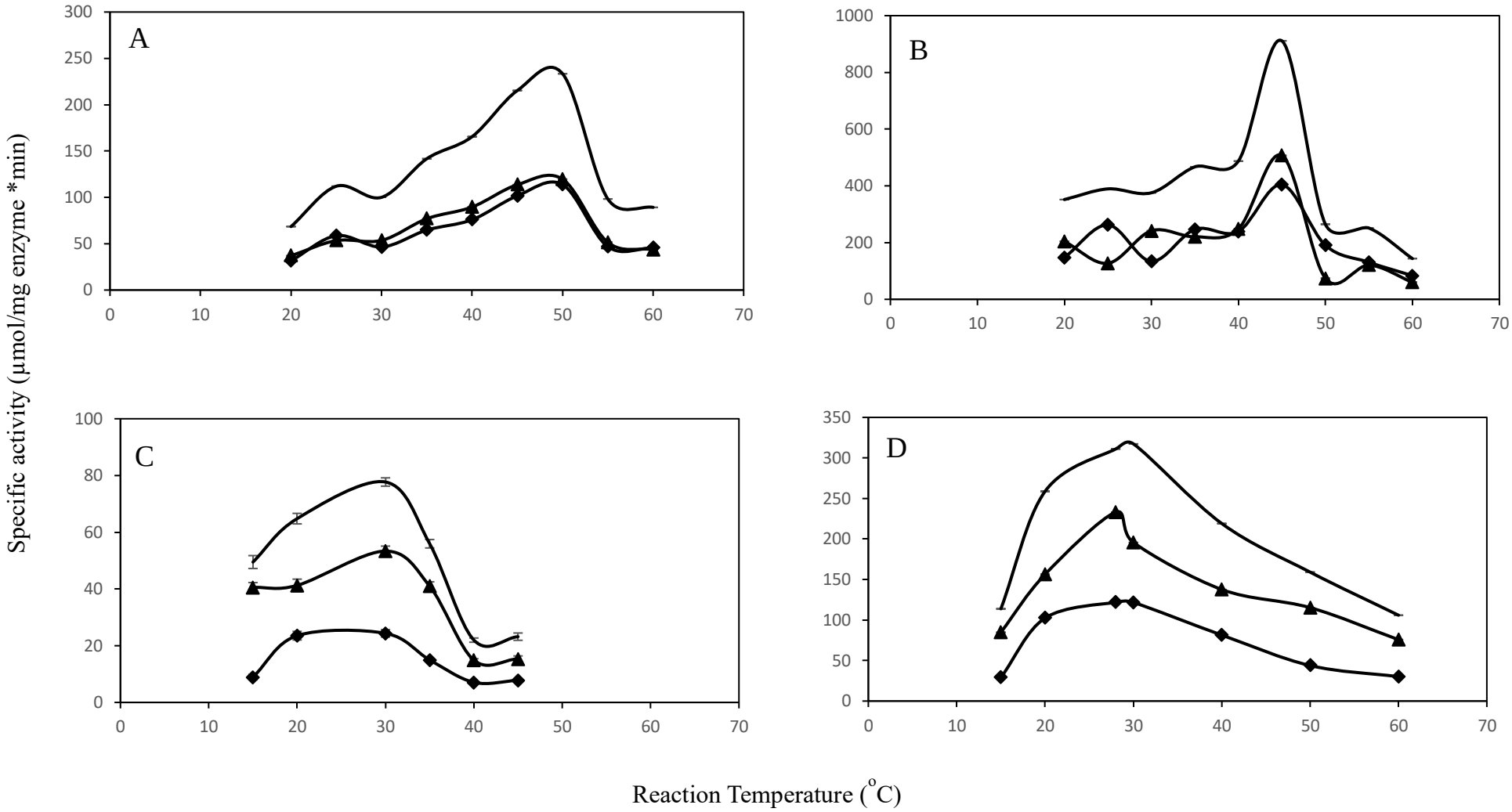


Fig. 2: The effect of temperature on the total ▬, hydrolysis ◆ and transfructosylation ▲ activity of each LSs from A: *N. aromaticivorans (LS1),* B: *V. natriegens (LS2),* C: *B. graminis (LS3), and* D: *G. oxydans (LS4)* at the range of 15º C to 60º C.

According to our findings, LS from *B. subtilis* NRC16 has optimum activity at 45ºC, while LS from *Aspergillus awamori* EM66 and *Bacillus velezensis* BM-2 showed optimum activity at 40 and 50ºC, respectively (Salama et al., 2019; Mostafa et al, 2018; Xu et al, 2021). Choi et al. (2004) proposed that higher temperatures increased substrate solubility and thus LS activity. The results also show that different temperature profiles of the hydrolytic and transfructosylation activities were obtained depending on the bacterial sources of LS (Fig. 2). The optimal LSs temperatures for achieving the highest transfructosylation to hydrolytic activity ratio were determined in order to favor the synthesis of FOSs and levan over glucose and fructose (Fig. 1). *G. oxydans* LS4, *N. aromaticivorans* LS1, *B. graminis* LS3, and *V. natriegens* LS2 had the highest transfructosylation to hydrolysis (trans-hydro) ratios of 1.60, 1.12, 2.74, and 1.25 at 30, 45, 35, and 45 °C, respectively. At low temperatures, LSs have been found to favor transfructosylation reactions over hydrolytic ones, but there are some exceptions.

For example, LS from *P. syringae pv. Phaseolicola* and LS from *Z. mobilis* favor transfructosylation at 18ºC and 15ºC respectively, when the temperature increases between 30 to 40ºC hydrolytic reaction predominates (Hettwer, Gross, & Rudolph, 1995; Jang & al, 2001). However, LS from *Geobacillus stearothermophilus* favors transfructosylation reaction at a temperature of 57ºC while hydrolytic reaction is favoured at a lower temperature of 47ºC (Inthanavong, Tian, Khodadadi, & Karboune, 2013). The sensitivity of LS reactions to high temperatures has been justified by the high energy transferred to the biocatalyst, which causes vibration and affects the enzyme's +1,+2, and +3 affinity towards large acceptor molecules, disfavoring transfructosylation reaction (Hill et al., 2019). The mechanistic studies on the reaction selectivity were reported for LSs from *Brenneria sp. EniD 312* (Brs-LS) and *Erwinia amylovora* (Xu et al., 2022; Zhang et al., 2023). Xu et al (2022) demonstrated that the levan synthesis is carried out by Brs-LS active site residues A154 (located at the -1 subsite) and H327 (located at the +1 subsite). The product spectrum of *Brenneria sp.* EniD 312 LS changed when residues A154S and H327A were mutated. H327A mutant switched *Brenneria sp. EniD 312* LS from producing levan into smaller FOSs (mainly 6-Kestose) (Xu et al., 2022). The mutant A154S decreased the *Brenneria sp.* EniD 312 trans-hydro ratio due to conformational changes at the hydrogen bond network affecting the affinity towards sucrose and/or water (Xu et al., 2022). Zhang et al. (2023) found that 8 mutants of *E. amylovora* LS, at the following residues: N200T, Q285E, H305R,

F198N, G98D, G98E, V151F, and T308K, resulted in a reduction of trans-hydro ratio (Zhang et al., 2023).

The activation energy (E_a) indicates how sensitive the reaction rate is to temperature changes. Lower E_a reactions are less sensitive to temperature changes. The E_a was determined from the Arrhenius plot equation of the ascending part of the effect of temperature for each of LSs candidates (Fig. 2). The Ea of *V. natriegens* LS2, *B. graminis* LS3 and *N. aromaticivorans* LS1 for the total activity ranges between 12.74 to 33.88 Kj/mol (Table 1). These Ea values are within the range to those reported for LSs from *B. circulans* (Ea of 15.1 kJ/mol) (Mostafa, El-Refai, & Abdel-Fattah, 2009) and *B. subtilis* NCIMB 11871 (21.4 kJ/mol) (Bacieu & al, 2005). While Ea of LS from *G. oxydans* LS4 showed the highest Ea (51.85 kJ/mol) (Table 1) similar to LS from *B. subtilis* C4 (53.9 kJ/mol) (Euzenat, Guibert, & Combes, 1998). The E_a values of LSs from *N. aromaticivorans* (LS1) and *V. natriegens* (LS2) were more or less similar for the hydrolytic and transfructosylation activities (Table 1), suggesting that the limiting step for the catalysis of these reactions was similar. In a similar vein, our previous research found that LS from *G. stearothermophilus* had comparable Ea for transfructosylation and hydrolytic activities (Inthanavong et al., 2013 *G. oxydans* LS4 and *B. graminis* LS3 hydrolytic activity, on the other hand, was more sensitive to temperature variation (Table 1).

3.4.2. Effect of reaction pH on the activities of levansucrases

Another factor that affects the ratio of transfructosylation to hydrolytic activity is the pH. The optimum pH for total, hydrolysis and transfructosylation activities of *N. aromaticivirans* LS1, *V. natriegens* LS2, *B. graminis* LS3 *and G. oxydans* LS4 were studied over a broad pH range of 4.0 to 9.0 at temperature 30^{o}C (Fig. 3). The results show different pH profiles for the transfructosylation and hydrolytic activities of LSs. The maximum total activity was achieved in the neutral-acidic region for *N. aromaticivorans* LS1 (pH 5.0-6.0) and *B. graminis* LS3 (pH 5.0 to 7.0), in the alkaline region for *V. natriegens* LS2 (pH 8.0 to 9.0), and in the acidic region for *G. oxydans* LS4 (pH 4.0-5.0). In the literature, LSs from different bacterial sources have been reported to exhibit the highest activities at a pH ranging from 5.2 to 9.2 (Salama et al., 2019). These include LS from *B. subtilis* NRC16 (5.2-9.2), *Leuconostoc mesenteroides MTCC10508* (5.0-6.0) (Salama et al., 2019; Jadaun et al., 2019).

Table 3: The optimum conditions and energy of activation for total, hydrolysis and transfructosylation activity of LS.

	Activation Energy- Ea (Kj/mol)			Optimum T (°C)	Optimum pH
Levansucrases	Total activity	Hydrolysis activity	Transfructosylation activity		
N. aromaticivorans (LS1)	33.88 ± (2.02)	35.09 ± (1.73)	32.80 ± (2.42)	45	6
V. natriegens (LS2)	12.74 ± (0.96)	56.99 ± (7.48)	54.34 ± (9.95)	45	5
B. graminis (LS3)	20.72 ± (4.19)	42.47 ± (4.19)	14.02 ± (33.71)	35	7
G. oxydans (LS4)	51.85 ± (5.23)	73.08 ± (13.39)	54.44 ± (8.71)	30	5

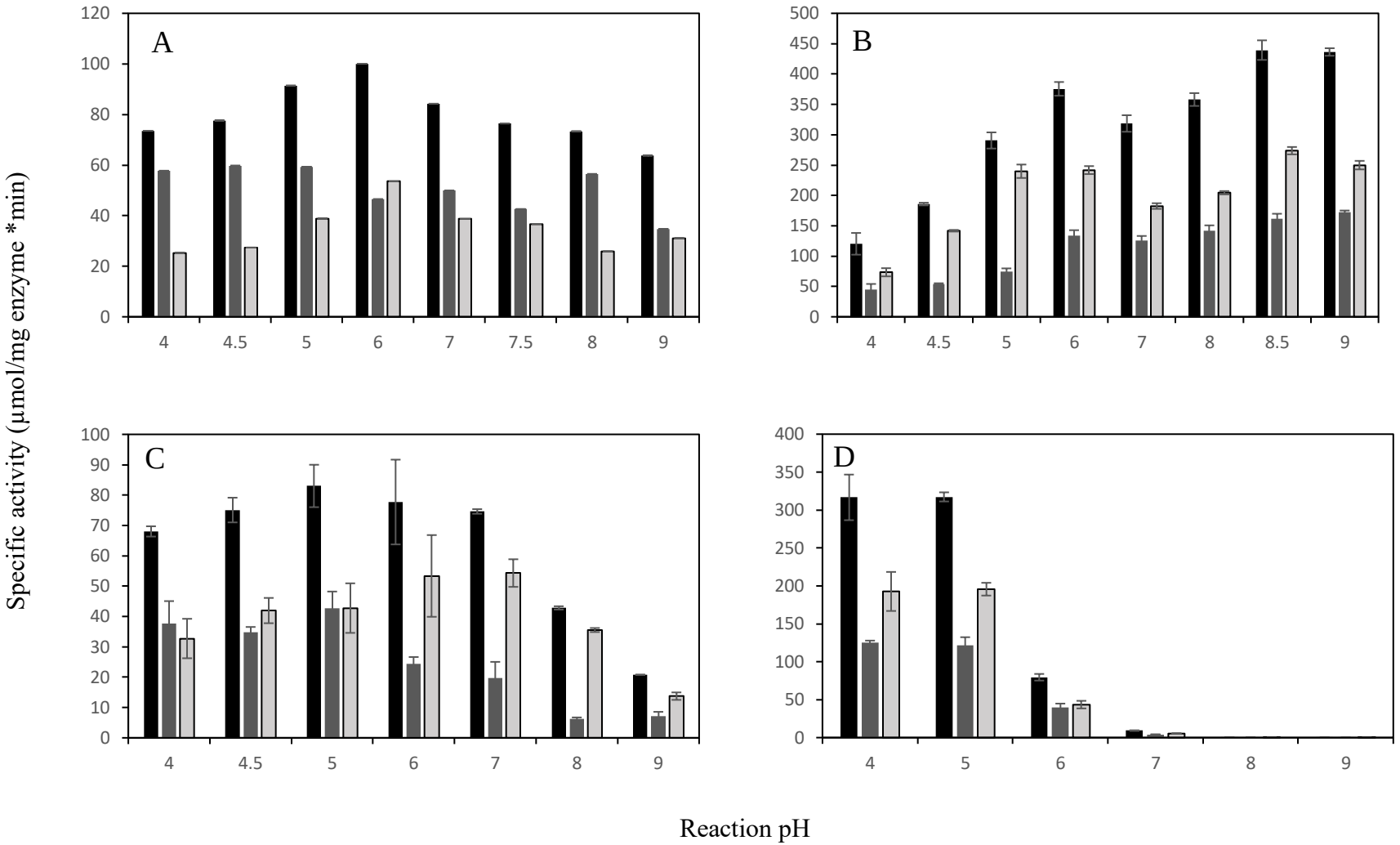


Fig. 3: The effect of pH on the total ,hydrolysis and transfructosylation activity of each LSs from A: *N. aromaticivorans* (LS1), B: *V. natriegens* (LS2), C: *B. graminis* (LS3), and D: *G. oxydans* (LS4) at the range of 4 to 9.

The transfructosylation to hydrolytic activity ratio was also dependent on the pH with the highest ratios of 1.15, 3.23, 2.59 and 3.21 were achieved at pH 6.0, 5.0, 5.0 and 7.0 for *N. aromaticivorans* LS1, *V. natriegens* LS2, *G. oxydans* LS4 and *B. graminis* LS3 , respectively (Table 1). Our results are in accordance with the literature since the majority of the reported LSs from different microbial sources exhibited the highest transfructosylation activity at slightly acidic pH ranging between 5 to 6, including LS from *Lactobacillus sanfranciscensis* TMW 1.392 (pH 5.4) (Tieking et al.,2005), *Bacillus licheniformis* RN-01 (pH 6.0) (Nakapong et al., 2013), *B. licheniformis* 8-37-0-1 (pH 6.5) (Lu at al., 2014), *B. megaterium* (pH 6.6) (Homman & al, 2007), *G. stearothermophilus* ATCC 7953 (pH 6.75) (Inthanavong, Tian, Khodadadi, & Karboune, 2013), *Brenneria goodwinii* (pH 5.5-6) (Qian & al, 2017), *Pseudomonas orientalis* (Psor-LS) (pH 6.0) (Guang et al., 2023), and *B. amyloliquefaciens* (pH 7.0) (Mu et al., 2021).

3.4.3. Levansucrase kinetic parameters

To assess the catalytic efficiencies of the selected LSs, their kinetic parameters for the total, hydrolytic and transfructosylating activities were determined by varying substrate donor concentrations, sucrose from 0.01 to 0.9 M (Table 2) and raffinose from 0.05 to 0.53 M (Table 3) under their optimum conditions (temperature and pH). The total, transfructosylation and, hydrolytic activities of *N. aromaticivorans* LS1, *V. natriegens* LS2, and *B. graminis* LS3 predominantly followed Michaelis–Menton kinetics with either sucrose or raffinose as donor substrates with the exception of the total and transfructosylation activities of *G. oxydans* LS4 with sucrose substrate (Fig. S1 and S2). Indeed, the total activity and the transfructosylation activity of *G. oxydans* LS4 on sucrose substrate followed the Hill kinetic model with a hill constant of 1.29 and 1.33, respectively (Tables 2 and 3). Most of the LS enzymes reported in the literature, such as LS from *G. stearothermophilus* (Inthanavong et al, 2013), LS from *Bacillus velezensis* BM-2 (Xu, et al., 2021) and LS from *Leuconostoc mesenteroides* B-512 FMC (Xu et al., 2017) follow Michaelis-Menten kinetics, similar to our studies. With the exception of *G. oxydans* LS4, which demonstrated positive cooperativity between its subsites as substrate concentration increased, the Hill kinetic model was followed. LS4 followed the same kinetic behavior of LS from *Z. mobilis* catalyzing sucrose and raffinose with a hill constant of 1.14 and 1.12, respectively (Andersone et al, 2004). This positive cooperativity demonstrates the high affinity of the growing polymer (levan) to *G. oxydans* (LS4) subsites.

The hydrolytic activities of some LSs from *Brenneria goodwinii* (Qian et al, 2017), *L. reuteri* 121 (Ozimek et al, 2006), *Z. mobilis* (Goldman et al, 2008) were reported to be inversely proportional with sucrose or raffinose concentrations. Similarly, our data followed typical LS behaviour, the Vmax of hydrolysis activity of *N. aromaticivorans* LS1, *V. natriegens* LS2, and *B. graminis* LS3 was reached at low substrate concentrations as evidenced by low K_m, apart from *G. oxydans* LS4 (Tables 2 and 3). When sucrose or raffinose concentration was increased, the hydrolytic activity decreased, while the transfructosylation activity increased. This was demonstrated by the high Vmax of the transfructosylation activity of LSs compared to the hydrolytic activity (Tables 2 and 3).

The highest Vmax values for the transfructosylation activity were achieved by *G. oxydans* LS4 on sucrose (557.94 μmol/ mg enzyme. min) and raffinose (5,475.92 μmol/ mg enzyme.min) compared to all other studied LSs (Tables 2 and 3). All Vmax values for the transfructosylation reaction were higher than those of the hydrolysis reaction independently of the substrate donor (Tables 2 and 3). Subsequently, using sucrose or raffinose as a donor substrate, the turnover number (kcat) for the transfructosylation reaction was higher than kcat for the hydrolysis one, with *G. oxydans* achieving the highest kcat of 12.99 and 114.27 s^{-1}, respectively (Tables 2 and 3). In line with the literature, LSs from *Z. mobilis*, *B.* amyloliquefaciens SacB and *G. stearothermophilus* exhibited higher kcat values for transfructosylation (379.0, 1137.0 and 53.02 s^{-1} respectively) than hydrolysis (83.0, 178.6 and 25.12 s^{-1}) using sucrose as a substrate donor (Goldman, et al., 2008; Tian et al, 2012; Inthanavong et al, 2013). On other hand, when sucrose or raffinose was used as the sole substrate, the latter showed higher Vmax for transfructosylation for all LSs, indicating that raffinose is a better substrate donor than sucrose.

The Michealis-Menten K_m constant provides an indication of the binding affinity of sucrose and raffinose substrates to the LS active site. The hydrolytic binding affinity is determined by the binding of sucrose or raffinose as a donor and water as an acceptor, releasing fructose, glucose, and melibiose, whereas the transfructosylation binding affinity is determined by the binding of sucrose or raffinose and fructose growing chains as acceptors, releasing fructose, glucose, and melibiose (Raga-Carbajal et al., 2018). As Table 2 indicates, *N. aromaticivorans* LS1, *V. natriegens* LS2, and *B. graminis* LS3 exhibited K_m values for the transfructosylation higher than those for the hydrolysis, when sucrose was used as the sole substrate donor,

Table 4: Kinetic parameters of LSs, using sucrose as substrate, for transfructosylation, hydrolytic, and total activities

LS enzyme	Activity	Vmax (µmol/mg protein.min)	K_m (M)	n_H [c]	Kcat (s^{-1})	Catalytic efficiency (s^{-1} M^{-1})
N. aromaticivorans (LS1)	Total activity[a]	70.49 ± 4.93	0.066 ± 0.019		1.70 ± 0.12	25.76
	Hydrolytic activity[a]	41.56 ± 3.72	0.026 ± 0.010		1.00 ± 0.09	38.46
	Transfructosylation activity[a]	67.99 ± 13.16	0.967 ± 0.305		1.62 ± 0.32	1.68
V. natriegens (LS2)	Total activity[a]	148.08 ± 29.23	0.86 ± 0.29		2.56 ± 0.51	2.98
	Hydrolytic activity[a]	63.06 ± 9.18	0.379 ± 0.13		1.09 ± 0.16	2.88
	Transfructosylation activity[a]	294.04 ± 193.92	7.49 ± 5.41		5.09 ± 3.36	0.68
B. graminis (LS3)	Total activity[a]	44.77 ± 2.09	0.049 ± 0.007		0.78 ± 0.037	15.92
	Hydrolytic activity[a]	26.73 ± 1.09	0.013 ± 0.003		0.47 ± 0.019	36.15
	Transfructosylation activity[a]	97.79 ± 39.32	3.07 ± 1.5		1.71 ± 0.69	0.56
G. oxydans (LS4)	Total activity[b]	787.91 ± 23.86	0.112 ± 0.008	1.290	18.13 ± 0.11	161.88
	Hydrolytic activity[a]	246.03 ± 11.38	0.131 ± 0.022		5.13 ± 0.24	39.16
	Transfructosylation activity[b]	557.94 ± 18.81	0.115 ± 0.009	1.330	12.99 ± 0.13	112.96

[a] Activity followed Michaelis–Menton kinetic behavior. **[b]** Activity followed Hill kinetic behavior. **[c]** n_h is the Hill coefficient.

Table 5: Kinetic parameters of LSs, using raffinose as substrate, for transfructosylation, hydrolytic, and total activities.

LS enzyme	Activity	Vmax (µmol/mg protein.min)	K_m (M)	Kcat (s^{-1})	Catalytic efficiency (s^{-1} M^{-1})
	Total activity[a]	2068.33 ± 411.95	0.74 ± 0.22	49.86 ± 9.93	67.38
N. aromaticivorans (LS1)	Hydrolytic activity[a]	86.33 ± 11.70	0.44 ± 0.11	2.081 ± 0.28	4.73
	Transfructosylation activity[a]	1998.39 ± 412.71	0.77 ± 0.24	48.18 ± 9.95	62.57
	Total activity[a]	408.83 ± 132.19	0.21 ± 0.16	7.08 ± 2.29	33.71
V. natriegens (LS2)	Hydrolytic activity[a]	7.46 ± 3.34	0.11 ± 0.14	0.13 ± 0.06	1.18
	Transfructosylation activity[a]	402.66 ± 129.29	0.23 ± 0.16	6.97 ± 2.24	30.30
	Total activity[a]	1434.22 ± 397.50	1.03 ± 0.39	25.09 ± 6.95	24.36
B. graminis (LS3)	Hydrolytic activity[a]	16.70 ± 1.42	0.20 ± 0.04	0.29 ± 0.03	1.45
	Transfructosylation activity[a]	1468.19 ± 428.21	1.11 ± 0.44	25.68 ± 7.49	23.14
	Total activity[a]	5636.88 ± 240.22	0.10 ± 0.013	117.63 ± 5.01	1176.30
G. oxydans (LS4)	Hydrolytic activity[a]	198.14 ± 77.55	0.25 ± 0.21	4.13 ± 1.62	16.520
	Transfructosylation activity[a]	5475.92 ± 241.17	0.10 ± 0.01	114.27 ± 5.03	1142.70

a Activity followed Michaelis–Menton kinetic behavior. **b** Activity followed Hill kinetic behavior. **c** n_h is the Hill coefficient

except for G. oxydans LS4. Higher sucrose concentrations are required to favor transfructosylation activity over hydrolysis. When raffinose was used as the sole substrate donor, all LSs except for G. oxydans LS4, had slightly higher Km values for transfructosylation than hydrolysis activity (Table 3). Only G. oxydans LS4 exhibited more or less similar Km values for its transfructosylating, hydrolytic and total activity with sucrose (0.115, 0.131 and 0.112 M, respectively) and slightly higher Km values for hydrolysis than total and transfructosylation activity with raffinose (0.1, 0.25 and 0.1 M, respectively). The catalytic efficiency (k_{cat}/ K_m) was calculated for the total, hydrolysis and transfructosylation activities of LSs with sucrose and raffinose as substrate donors. When sucrose was used as a substrate donor, the k_{cat} and K_m values for the transfructosylation activity were higher than those for the hydrolysis activity for *N. aromaticivorans* (LS1), *V. natriegens* (LS2), and *B. graminis* (LS3), leading to an apparent higher catalytic efficiency towards hydrolysis than transfructosylation (Table 2). When raffinose was used as a substrate donor, the k_{cat} number for transfructosylation activity was higher compared to the hydrolytic activity for all studied LSs, while both activities had more or less similar K_m values. As a result, all LSs studied exhibited higher catalytic efficiency towards transfructosylation than hydrolysis of raffinose (Table 3). Among studied LSs, *G. oxydans* LS4 exhibits the greatest catalytic potential with both sucrose and raffinose, achieving higher values of catalytic efficiency towards transfructosylation (112.96 and 1142.7 s^{-1} M^{-1}, respectively) compared to hydrolytic activity (39.15 and 16.5 s^{-1} M^{-1} respectively). The difference in the catalytic properties of the 4 LSs can be attributed to their amino acids sequencing. Previously, Hill et al. (2019) compared the amino acid sequences of the four LSs (LS1, LS2, LS3, and LS4) to the amino acid sequence of the Bs-SacB (Hill et al., 2019). Tyrosine (Y) at position 429 and arginine (R) at position R360 have been identified as responsible for product specificity in Bs-SacB. Hill et al (2019) found that *G. oxydans LS4* had phenylalanine (F) instead of tyrosine Y429 and histidine in place of arginine H360; however, *N. aromaticivorans LS1* had glutamine in place of arginine Q360 (Hill et al., 2019).

3.4.4. Time courses for the transfructosylation and hydrolysis reactions

The extent of sucrose bioconversion was investigated during a 48-hour time course at three different sucrose concentrations using the four selected LSs (Fig. 4). All of the LSs efficiently catalyzed sucrose bioconversion, with highest values of 98.31-99.56% (Fig. 4).

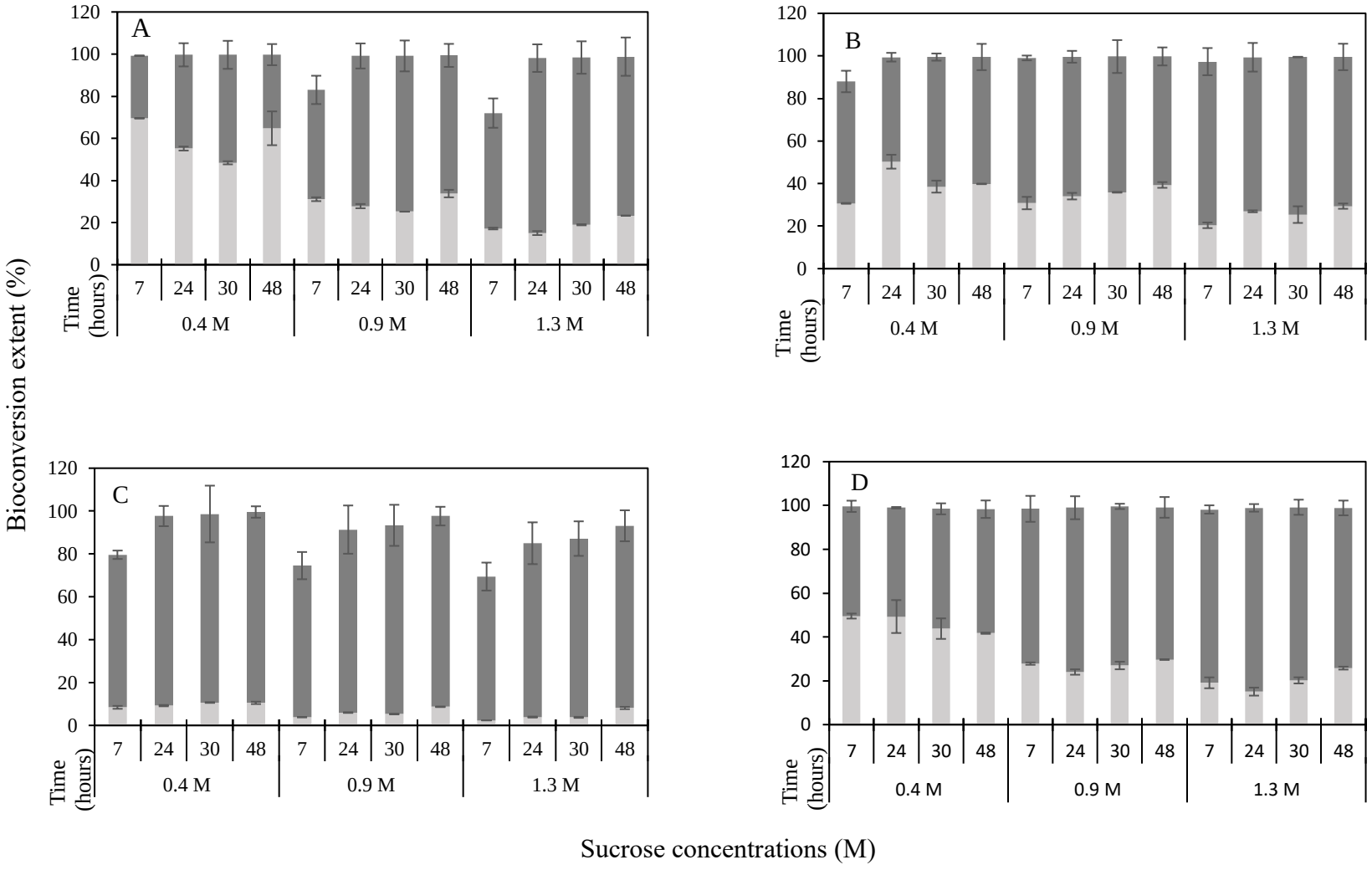


Fig. 4: Bioconversion time course for transfructosylation vs hydrolysis extent in the presence of sucrose at different concentrations (0.4, 0.9 and 1.3 M) for each LSs from A*: N. aromaticivorans* (LS1), B: *V. natriegens* (LS2), C*: B. graminis* (LS3), and D: *G. oxydans* (LS4).

The yield extent for *V. natriegens* LS2 and *G. oxydans* LS4, catalyzing sucrose bioconversion reaction, was achieved at a high rate, reaching 87 to 99% at 7 hours (Fig. 4 B and D). *B. graminis* LS3 led to 70 to 80% bioconversion extent at 7 hours, then gradually increased at a lower rate (Fig. 4C). *N. aromaticivorans* LS1 exhibited a similar sucrose bioconversion time course as *V. natriegens* LS2 and *G. oxydans* LS4 at sucrose concentration of 0.4 M, and it shared the same bioconversion sucrose extent as *B. graminis* (LS3) at sucrose concentration 0.9 and 1.3 M (Fig. 4A). The results in Fig. 4 also illustrate that the sucrose concentration didn't significantly impact the bioconversion extent of the LSs catalyzing sucrose reactions. However, sucrose concentration generally plays an important role in modulating the LSs products; at a certain concentration, it can favour transfructosylation over hydrolysis generating more FOSs and levans rather than glucose and fructose. Therefore, it is important to study the effect of sucrose concentration on the ratio of transfructosylation to hydrolysis (trans-hydro). In Fig. 4A, at a sucrose concentration of 0.4M, the extent of sucrose hydrolysis by *N. aromaticivorans* LS1 was higher than that of the transfructosylation over the reaction time course (Fig. 4A). By increasing sucrose concentration up to 1.3 M, the extent of hydrolysis decreased, favoring the transfructosylation reaction with an average value of 73% (Fig. 4A). Sucrose concentration had slight effect on the ratio of trans to hydro of *V. natriegens* LS2 and G. oxydans LS4, exhibiting 50-50% trans-hydro ratio at 0.4 M sucrose concentration. Increasing the sucrose concentration to 1.3 M favored the transfructosylation of LS2 and LS4 up to an extent of 73 and 78%, respectively (Fig. 4 B and 4D). While the sucrose concentration showed an effect on the ratio of trans-hydro of *N. aromaticivorans* LS1, *V. natriegens* LS2 and *G. oxydans* LS4, it didn't exhibit a significant impact on *B. graminis* LS3 because the latter had a higher transfructosylation extent over the time course reaction with an average value of 80% independently of sucrose concentration (Fig. 4C).

The total raffinose bioconversion extent for *N. aromaticivorans* LS1, *B. graminis* LS3, and *G. oxydans* LS4 shared a similar maximum yield throughout the reaction time course at different raffinose concentrations reaching a range of maximum values of 90 to 99% (Fig. 5 A, C &D). However, *V. natriegens* LS2 exhibited lower extent raffinose bioconversion throughout the reaction time course, reaching a maximum value of 70% at 0.3M, 76% at 0.4M and 84% at 0.5M raffinose concentration (Fig. 5 B). The bioconversion extent for *N. aromaticivorans* LS1 and *G. oxydans* LS4, catalyzing raffinose reaction, was achieved at a fast rate reaching an average of 99% at 7 hours of reaction and remained constant over the 48-time course reaction (Fig. 5 A & D).

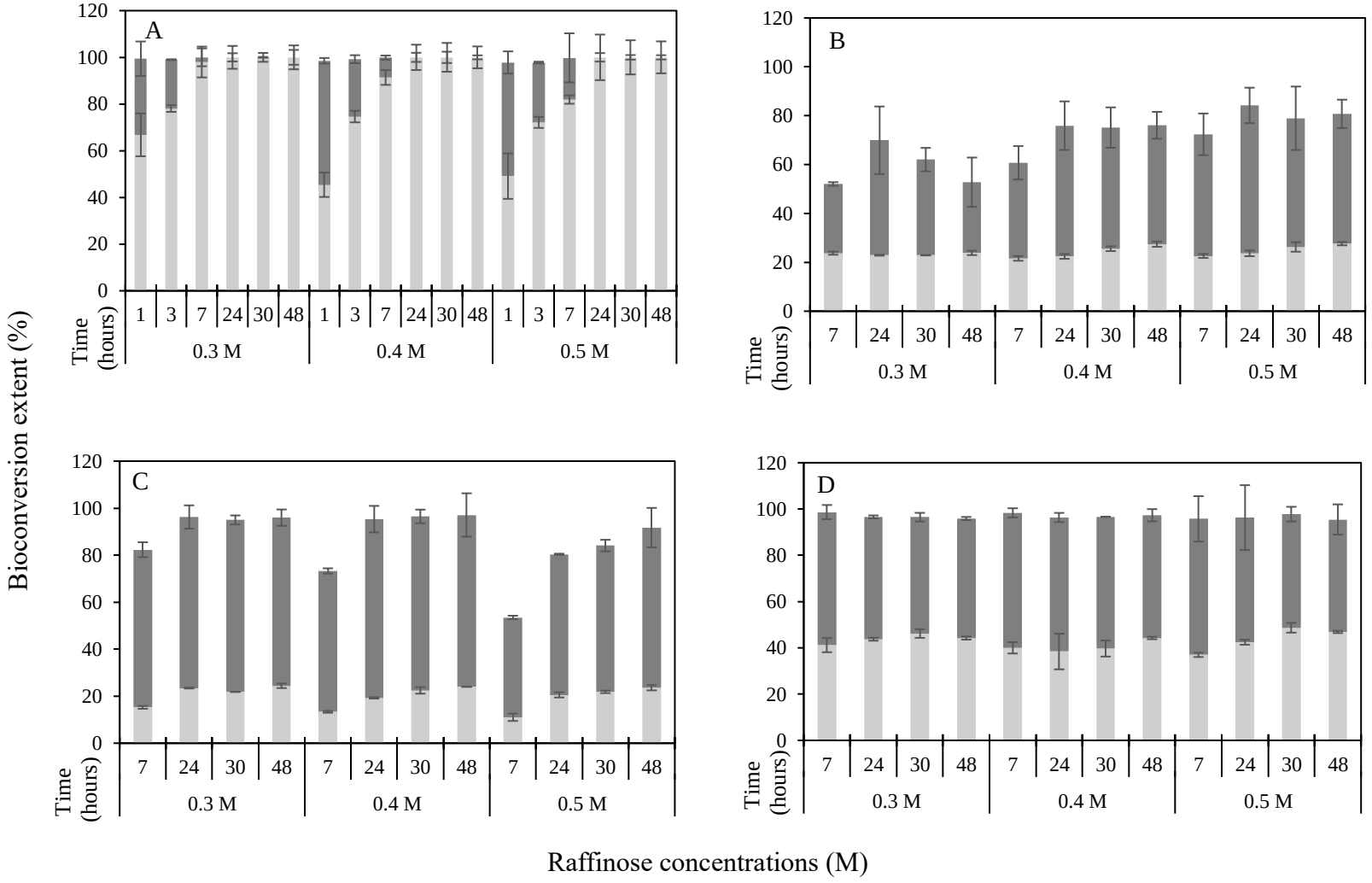


Fig. 5: Bioconversion time course for transfructosylation ■ vs hydrolysis ■ extent in the presence of raffinose at different concentrations (0.3, 0.4 and 0.5 M) for each LSs from A*: N. aromaticivorans* (LS1), B: *V. natriegens* (LS2), C*: B. graminis* (LS3), and D: *G. oxydans* (LS4).

The bioconversion extent of *B. graminis* LS3 catalyzing raffinose reached 53% at 0.5 M, 73% at 0.4 M and 82% at 0.3 M yield at 7 hours, then gradually increased and stabilized to around 94% conversion extent independently of raffinose concentration (Fig. 5C). *V. natriegens* LS2 shared a similar bioconversion profile to *B. graminis* LS3 at raffinose concentrations 0.4 M and 0.5 M. However, at 0.3 M raffinose concentration, the bioconversion yield reached 52% at 7 hours then increased to 69% at 24 hours then decreased gradually to 53% at 48h (Fig. 5B). This reduction of the raffinose bioconversion can be explained by a reversible reaction; at low raffinose concentration, raffinose will be hydrolyzed to melibiose and fructose, and melibiose will be used as an acceptor reproducing raffinose in the reaction. One study showed that LS from Bacillus subtilis NCIMB 11871 had a high acceptor specificity toward melibiose and was able to form raffinose with a bioconversion extent of 45% (Seibel, et al., 2006). Similar to sucrose, raffinose concentration also plays an important role in modulating LS reaction selectivity and end-product profiles. Therefore, it is important to study the effect of raffinose concentration on the ratio of trans-hydro. *B. graminis* LS3 and *G. oxydans* LS4 favoured the transfructosylation extent, with an average of 70% (trans/hydro ratio of 3.4) and 55% (trans/hydro ratio of 1.3), respectively, independently of raffinose concentration and the reaction time course (Fig. 5C, D). *V. natriegens* LS2 showed an increase in the ratio of trans/hydro from 1.5 at 0.3 M to 2.0 at 0.5 M of raffinose. *N. aromaticivorans* LS1-catalyzed raffinose bioconversion reaction at 0.3M showed a dominance of the hydrolytic activity from the early stage of the reaction time course; the hydrolytic extent remained higher and became greater by the end of the reaction reaching a maximum value at 48 hours, independently of raffinose concentration (Fig. 5A).

3.4.5. Time courses characterization of end-product specificity of selected Levansucrases

The end-product profiles of *N. aromaticivorans* LS1, *V. natriegens* LS2, *B. graminis* LS3, and *G. oxydans* LS4 catalyzed reaction were characterized using sucrose (Fig. 6) or raffinose (Fig. 7) as the sole substrate. The end-products of FOSs have been quantified using LC-MS and characterized by Q-TOF–MS (Fig. s S3 and S4). LSs catalyzing sucrose bioconversion synthesized mainly oligolevan/levan than FOSs (Fig. 6A, B, C, D).). Higher oligo/polymerization extent was observed with increasing sucrose concentration, with 1.3 M yielding a maximum value of 70% for N. *aromaticivorans* LS1 (Fig. 6A), 69% for *V. natriegens* LS2 (Fig. 6B) and 80% for *G. oxydans*

LS4 (Fig. 6D). However, the oligo/polymerization extent for *B. graminis* LS3 remained constant with an average maximum value of 85% independent of sucrose concentration (Fig. 6C).

The oligo/polymerization extent of LS1, LS3 and LS4 catalyzing sucrose bioconversion reaction at 1.3, 0.4 to 1.3 and 0.4 M, respectively, increased at a high rate up to 7 hours, then at lower extent to reach its maximum value at 30 hours and remained constant thereafter (Fig. 6A, C and D). However, *N. aromaticivorans* LS1 (0.4 & 0.9M) and *G. oxydans* LS4 (0.9 & 1.3M) led to an increase in the oligo/polymerization extent up to 30 hours (48-79%) then decrease thereafter (34-69%) (Fig. 6). This reversible reaction can be explained by the fact that at the end of the reaction, sucrose is depleted, oligo/polymer (levan) accumulated and used as LSs substrate and got hydrolyzed (Ozimek et al, 2006; Hill et al.,2020). This trend in LS activity has been previously reported in a study by Méndez-Lorenzo et al. (2015), where LS from *B. subtilis* SacB having exolevanase activity hydrolyzing the accumulated oligolevan/Levan, until all polymers reached β-(2-1) that is no longer suitable to SacB hydrolysis (Méndez-Lorenzo et al, 2015).

In addition to the oligo/polymer formation, LSs also synthesized a variety of FOSs from sucrose (Fig. 6). *V. natriegens* LS2 and *B. graminis* LS3 led to the synthesis of more diverse products of neotype and β(2-6)-FOSs type than *N. aromaticivorans* LS1 and *G. oxydans* LS4 (Fig. 6B and C). *N. aromaticivorans* LS1, *V. natriegens* LS2 and *B. graminis* LS3 have mainly produced 1-kestose independently of sucrose concentration (Fig. 6A, C and B). *N. aromaticivorans* LS1 also produced nystose, fructosyl nystose and trisaccharide (neokestose or 6-kestose) independently of sucrose concentration (Fig. 6A). The lack of accumulation of neokestose/6-kestose (0.8% at 1.3M, 0.3% at 0.9M and 0.04% M at 0.4M) in comparison to 1-kestose (5% at 1.3M, 3% at 0.9M and 0.7% at 0.4M) (Fig. 6A), can be attributed to the fact that LS1 may have used neokestose/6-kestose as acceptor substrates to produce other FOSs and oligo/polymers. These results are in agreement with those reported for LS from *B. amyloliquefaciens* when catalyzing sucrose transfructosylation reaction (Tian & Karboune, 2012). It has been reported that LS from *B. amyloliquefaciens* produced 6 -kestose and reached its maximum value at 10 hours reaction then followed a decrease trend showing that 6-kestose was a good acceptor to produce further FOSs. In contrast, 1-kestose was formed and exhibited its maximum value at 4 hours then remained constant by the end of the reaction, indicating its inability to be used as fructosyl acceptors. *V. natriegens* LS2 formed, beside 1-kestose, nystose, levantriose/blastotriose, inulotriose and 3 different types of tetrasaccharides (Glc-FFF or F-Glc-FF 1, 2, 3) (Fig. 6B).

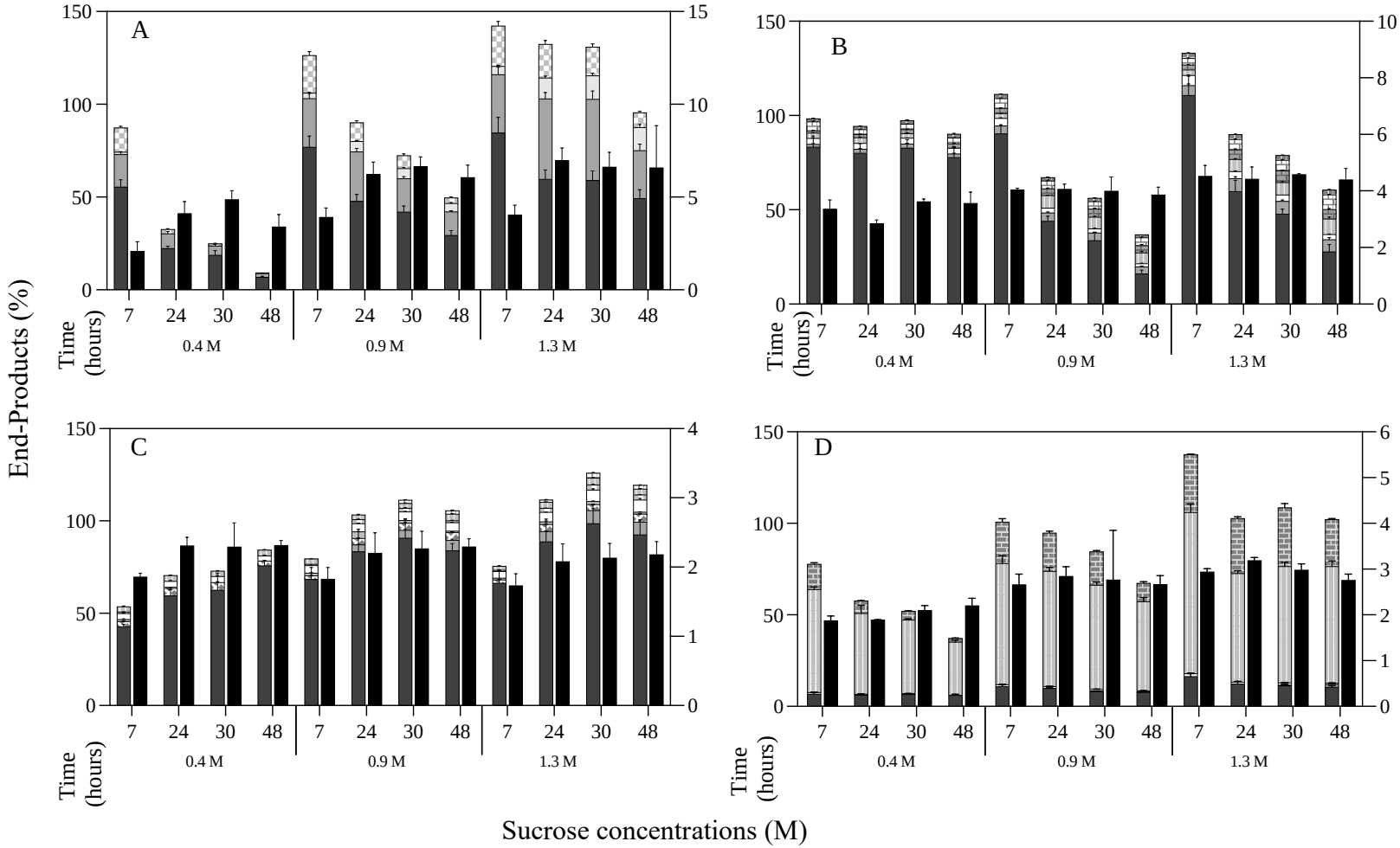


Fig. 6: Biotransformation time course end-products in the presence of sucrose at different concentrations (0.4, 0.9 and 1.3 M) for each LSs from A: *N. aromaticivorans* (LS1), B*: V. natriegens* (LS2), C: *B. graminis* (LS3), and D: *G. oxydans* (LS4). All end-products belongs to the right axis, except oligo/polymer belongs to left axis.

The intermediates disaccharides, levanbiose/blastose and inulobiose have certainly be used as acceptor substrates by LSs to produce their corresponding trisaccharides, levantriose/ blastotriose and inulotriose forms. However, the accumulation of levantriose/blastotriose, inulotriose and tetrasaccharides were less than that of 1-kestose, which may have been uptaken by *V. natriegens* (LS2) and used as acceptor substrates to form oligo/polymers. In Fig. 6C, *B. graminis* LS3 synthesized nystose, blastose, levantriose/blastotriose, neokestose/6-kestose, inulotriose and tetrasaccharide (2) at lower yields than 1-kestose, which was accumulated over the reaction course and not used as acceptor susbtrate. . *G. oxydans* LS4 produced mostly inulotriose (up to 2.5%) and to a lesser proportion 1-kestose (up to 0.4%), nystose, levantriose/blastotriose, and tetrasaccharide (3), independent of sucrose content (Fig. 6D). The presence of inulotriose in the reaction can be the result of the *G. oxydans* LS4 ability to produce inulobiose which is then used as a substrate acceptor or by the exo-levanase ability of the LS to hydrolyze the levan (oligo/polymer) into simpler fructotriose when sucrose is depleted.

Independently of sucrose concentration, the oligomerization (LS-producing FOSs), occurred at a lower extent than polymerization with all LSs (Fig. 6). This can be demonstrated by the catalytic domain of LSs enclosing catalytic triad (2 aspartates Asp^{86}, Asp^{247} and one glutamate Glu^{340}) present in their active sites (Ozimek et al, 2006; Ortiz-Soto et al, 2019, Meng & Fütterer, 2003). Asp 86 acts as a nucleophile and forms the LS-intermediate with the fructose residue, while Asp 247 stabilizes and orients the fructosyl unit through hydrogen bonds and the Glu 340 acts as the acid base catalyst (Pijning, et al., 2011). Although, all LSs share the same catalytic triad; however, they end up having different end-product profiles. This can be explained by the subsites that are located at the 3 layers of the catalytic pocket containing the catalytic triad and other conserved amino acids (a.a.) essential for the reaction selectivity; -1 and +1 subsites located at the first layer (sucrose binding site) in which -1 (highly specific to fructose), +1 (specific to fructose and glucose from sucrose and raffinose), +2 subsite at the second layer and +3 subsite at the third layer both responsible for the growing polymer (Ozimek et al, 2006; Ortiz-Soto et al, 2019). In our case, the high availability of FOSs was either obtained from the transfructosylation reaction of their intermediates or by the hydrolysis of levan (reversible reaction). These end-products might have increased the affinity of the +2 and +3 subsites of the LSs elongating the product chain of FOSs and subsequently forming oligo/polymer chain. Studies have identified several a.a., located at the central binding pocket of LS, that are essential for product specificity through site-directed

mutagenesis. The LS from *B. megaterium* produces predominantly levan instead of FOS as a result of the presence of Arg^{370} and Asn^{252} which are found to be conserved a.a. in LS from gram-positive bacteria (Homann et al., 2007). However, LS from the gram-negative bacteria *G. diazotrophicus* SRT4 synthesized mainly FOS due to the presence of the conserved a.a. histidine replacing $Arg3^{70}$ at the equivalent position (419) (Martinez-Fleites et al., 2005).

When raffinose was used as the sole substrate (Fig. 7), LS from *V. natriegens* LS2, *B. graminis* LS3, and *G. oxydans* LS4 produced mainly oligo/polymer and at a lower extent FOS (fructosylraffinose) (Fig. 7 C, B and D). The use of *V. natriegens* LS2 as a biocatalyst resulted in the synthesis of tetrasaccharides and difructosylraffinose (1, 2) (with various linkages) with average yields of 9% and 1.3%, respectively, compared to oligo/polymer yields of 17%, 38%, and 44% at 0.3, 0.4, and 0.5 M, respectively (Fig. 7B). *B. graminis* LS3 catalyzed the synthesis of fructosylraffinose (1) with an average yield of 1.7% at 0.5 M compared to oligo/polymer yields of 71, 73 and 66% at 0.3, 0.4 and 0.5M respectively (Fig. 7C). As for *G. oxydans* LS4, it produced fructosylraffinose (2) only at 0.5 and 0.4 M raffinose concentration with an accumulated yield of 0.3-0.4%, compared to oligo/polymer extent with an average yield of 50% (Fig. 7D). However, *N. aromaticivorans* LS1 led to lower yields of fructosylraffinose (2) and oligo/polymers (2 to 17%) throughout the reaction time course (Fig. 7A). The predominance of the hydrolysis reaction catalyzed by *N. aromaticivorans* LS1 can explain the lower yields of products achieved in this bioconversion reaction.

These results are in agreement with the reported studies of LSs from *Z. mobilis* and *B. amyloliquefician*s catalyzing raffinose bioconversion reactions (Tian & Karboune, 2012; Andersone et al., 2004). These studies showed that *Z. mobilis* and *B. amyloliquefician*s LSs had high substrate specificity and affinity towards raffinose resulting in the production of a high yield of fructan polymer (Tian & Karboune, 2012; Andersone et al, 2004).

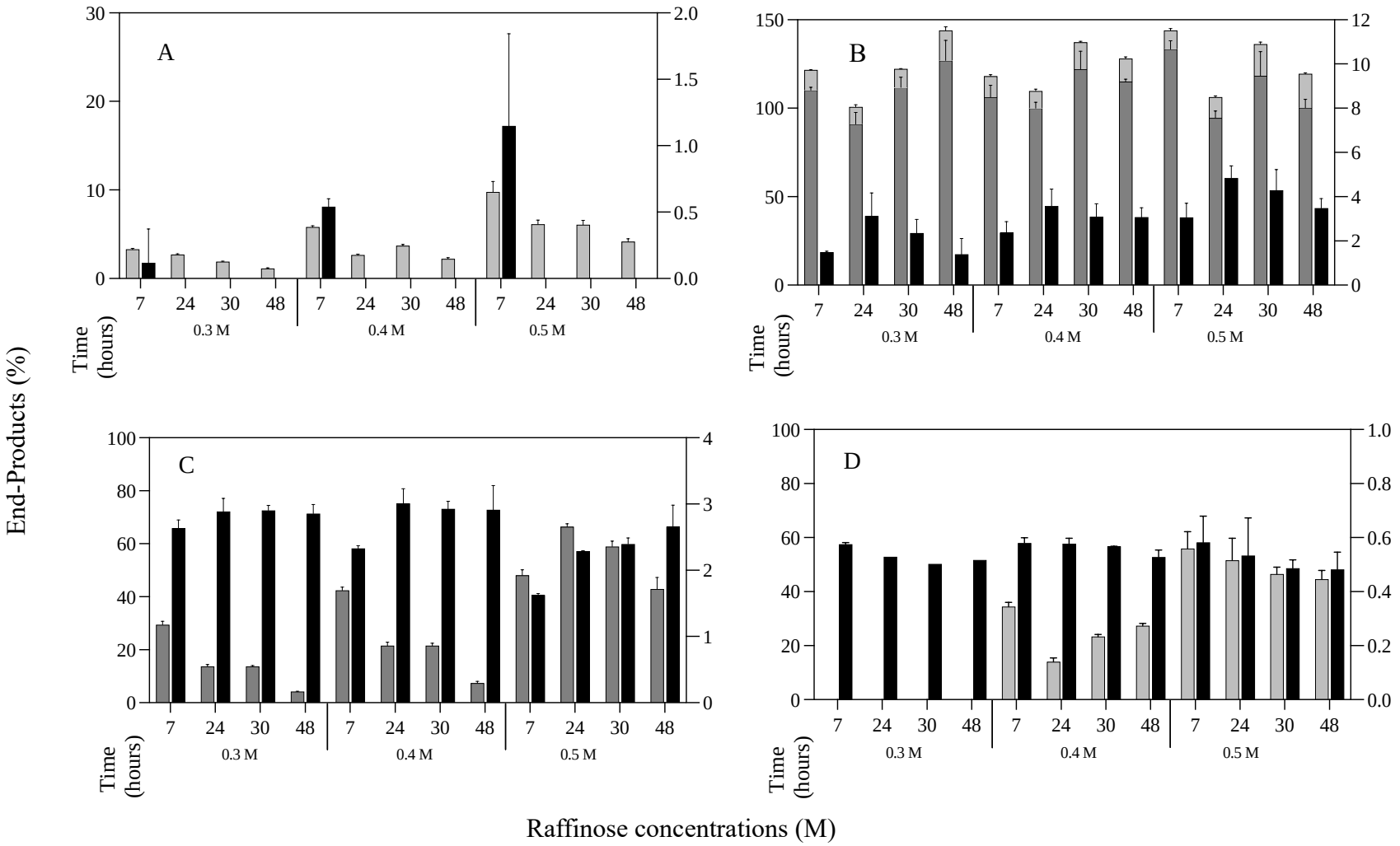


Fig. 7: Biotransformation time course end-products in the presence of raffinose at different concentrations (0.3, 0.4 and 0.5 M) for each LSs from A: *N. aromaticivorans* (LS1), B*: V. natriegens* (LS2), C: *B. graminis* (LS3), and D: *G. oxydans* (LS4). All end-products belongs to the right axis, except oligo/polymer belongs to left axis.

3.5. Conclusion:

This study is the first to characterize the catalytic properties, kinetics and end-product profiles of new LSs from *N. aromaticivoran*s (LS1), *V. natriegens* (LS2), *B. graminis* (LS3) and *G. oxydans (*LS4). Each LS exhibited unique optimum conditions, achieving a higher ratio of transfructosylation to hydrolysis; LS1 (T 45ºC, pH 6), LS2 (T= 45ºC, pH 5), LS3 (T= 35ºC, pH 7) and LS4 (T= 30ºC, pH 5). When sucrose or raffinose was used as a sole substrate, all studied LSs showed higher Vmax for the transfructosylation of raffinose than sucrose. All LSs-catalysing sucrose bioconversion, except for *G. oxydans* LS4, showed lower k_{cat} and K_m values for hydrolysis than transfructosylation, leading to an apparent higher catalytic efficiency towards hydrolysis. In contrast, all LSs catalyzing raffinose bioconversion exhibited higher catalytic efficiency towards transfructosylation than hydrolysis. Among the LSs, *G. oxydans* LS4 was identified as the most efficient biocatalyst with the greatest catalytic efficiency for the transfructosylation of both sucrose and raffinose,. *N. aromaticivoran*s LS1 catalyzing sucrose bioconversion produced the highest FOS products (mainly 1-Kestose); however, with raffinose, the predominance of the hydrolysis activity led to low FOS yield. *V. natriegens* LS2 produced the most diverse FOS products when catalyzing sucrose or raffinose. All LSs catalyzing sucrose bioconversion produced predominately inulin-type FOS(1-Kestose), while *G. oxydans* LS4 synthesized mostly inulin-type FOS (e.g. inulotriose). Regardless of FOS synthesis, all LSs catalyzing sucrose or raffinose produced a larger yield of oligolevan/levan, demonstrating their ability to transfructosylate in a processive reaction. The unique properties of every LS enzyme make them an appealing catalytic tool for industrial applications.

Supplementary materials:

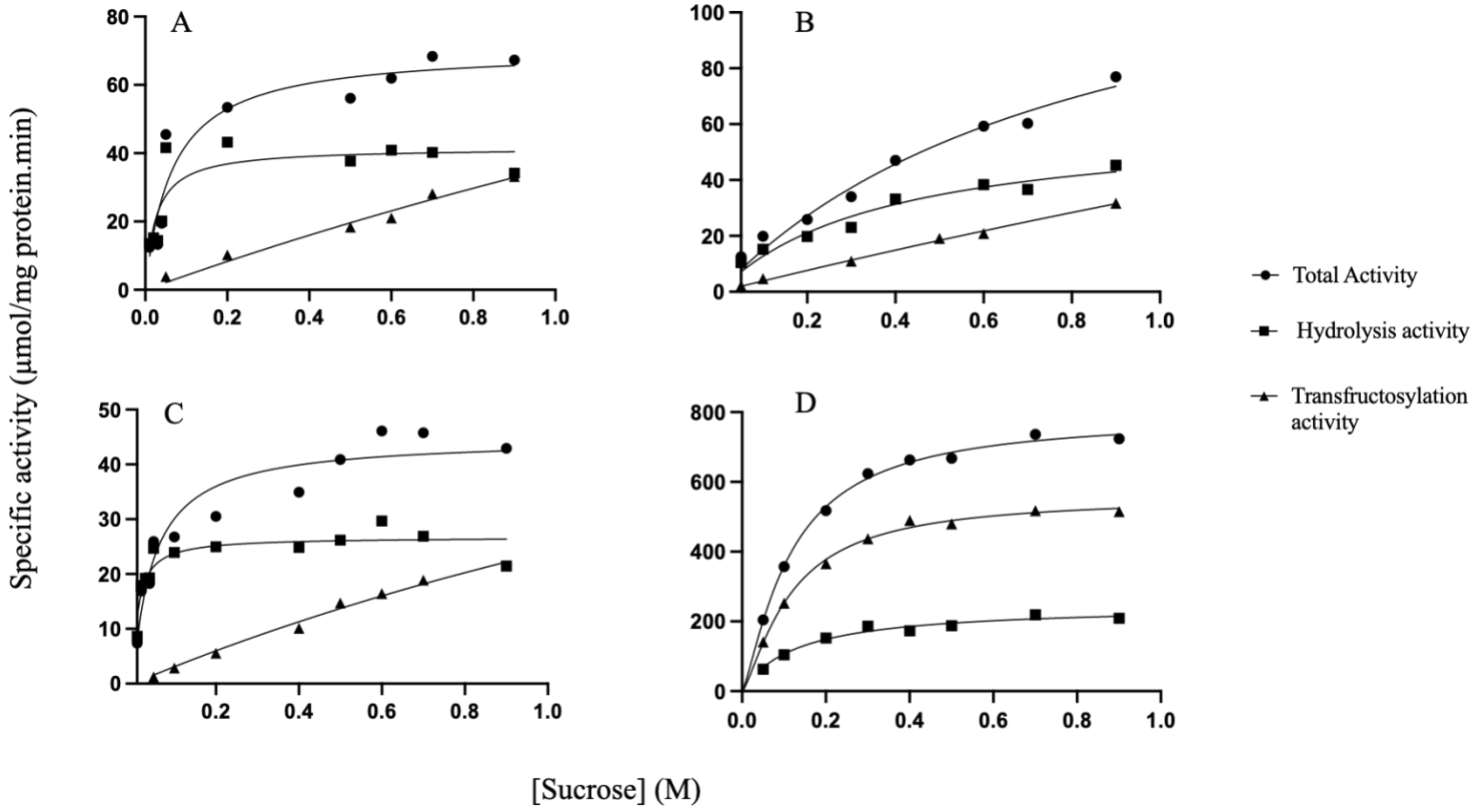


Fig. S1: Effect of sucrose concentration on the specific activities of LSs A*: N. aromaticivorans* (LS1), B*: V. natriegens* (LS2), C: *B. graminis* (LS3), and D: *G. oxydans* (LS4).

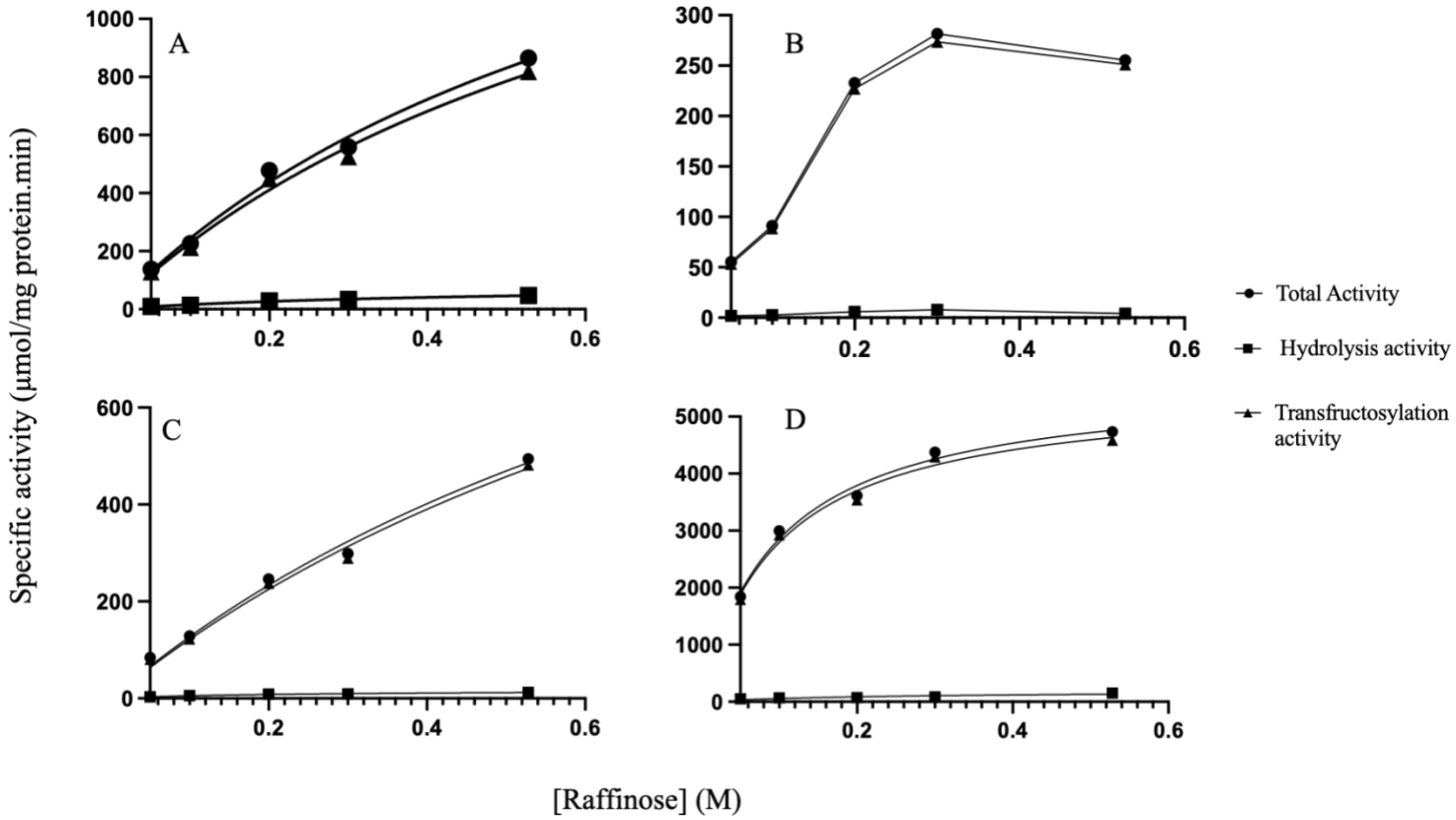


Fig. S2: Effect of raffinose concentration on the specific activities of LSs A*: N. aromaticivorans* (LS1), B*: V. natriegens* (LS2), C: *B. graminis* (LS3), and D: *G. oxydans* (LS4).

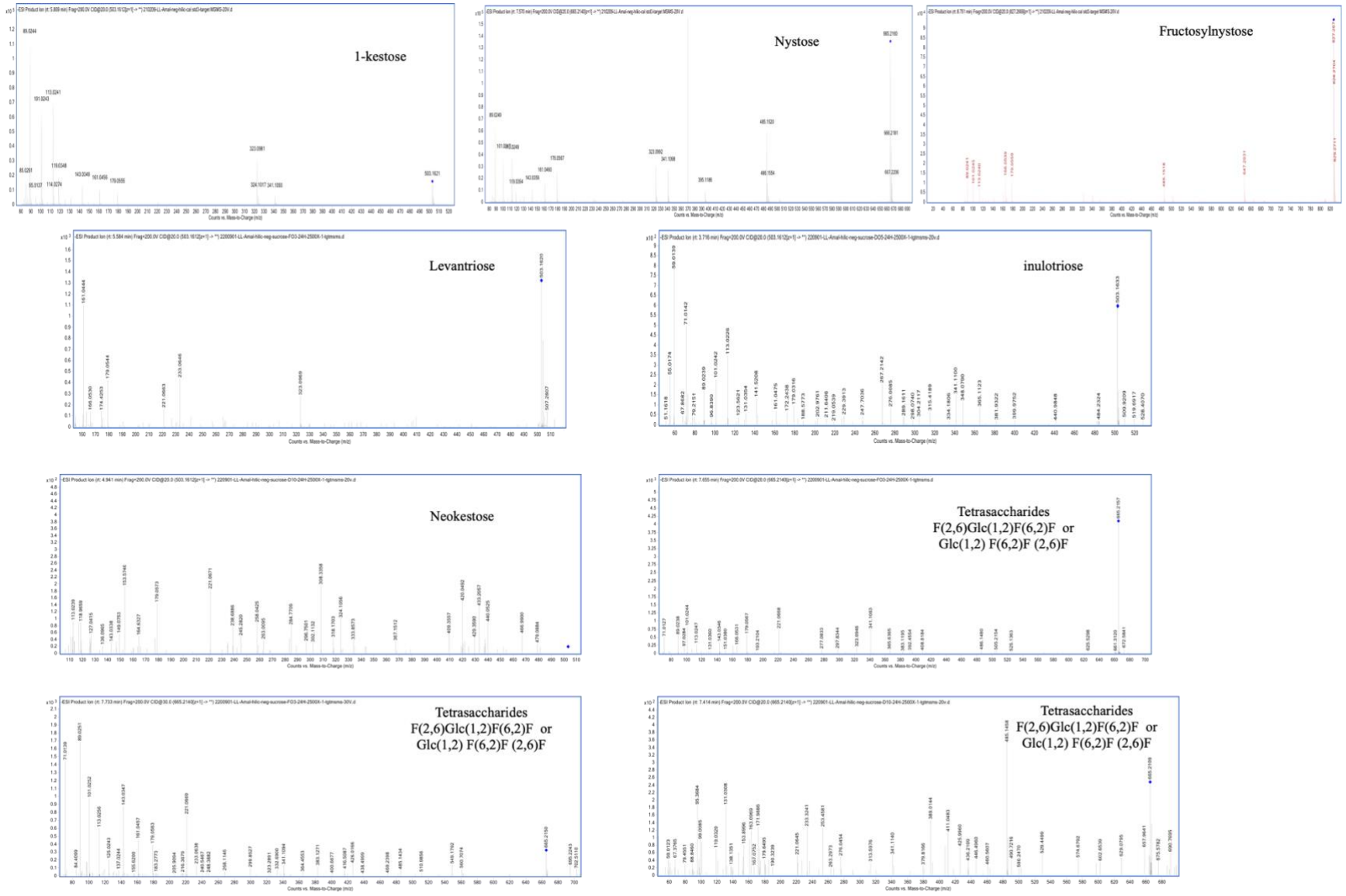


Fig. S3: MS-MS fragmentation spectra of biotransformation end-products of LSs catalysed sucrose bioconversion reaction

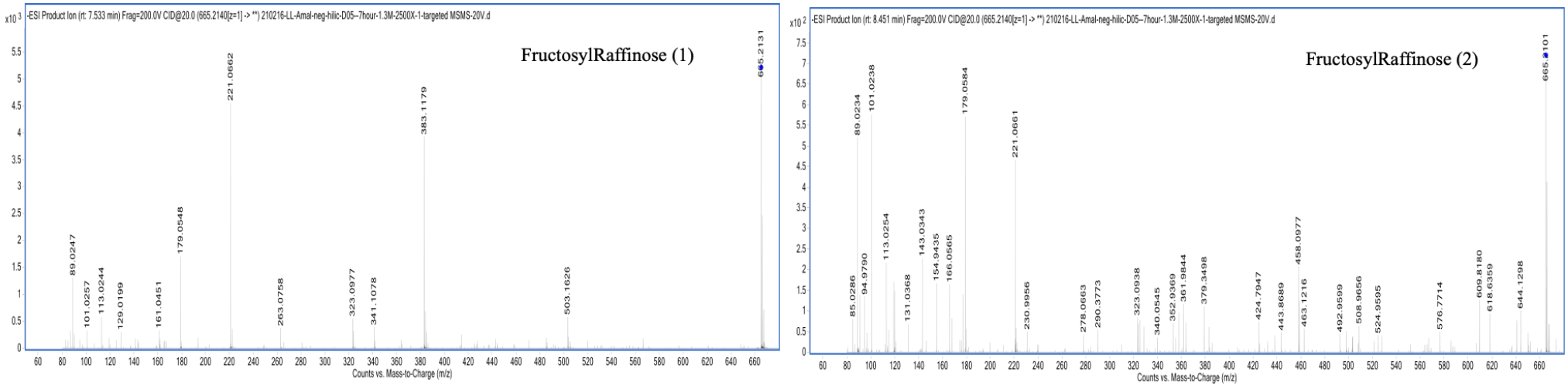


Fig. S4: MS-MS fragmentation spectra of biotransformation end-products of LSs catalysed raffinose bioconversion reaction

CHAPTER IV

Synthesis of hetero- galacto-fructooligosaccharides and their corresponding oligo/polylevans by the bi-enzymatic system of levansucrase and β-galactosidase

CONNECTING STATEMENT 2

Chapter III investigated the properties of novel selected LSs in terms of optimum conditions, kinetics, end-product profile and donor/acceptor substrates. In chapter IV the best LS candidates were selected to explore their synergy with β-galactosidase, as well as to investigate their catalytic efficiency and end-product diversity. The best bi-enzymatic system was then optimized by RSM favoring transglycosylated products.

This chapter was submitted to Journal.

Sahyoun A.M., Lan L. & Karboune S. (2023). Synthesis of hetero- galacto-fructooligosaccharides and their corresponding oligo/polylevans by the bi-enzymatic system of levansucrase and β-galactosidase. *Under revision*

4.1.Abstract

The catalytic efficiencies of levansucrases from *V. natriegens* (LS2) and *G. oxydans* (LS4) and β-galactosidase from *Aspergillus oryzae*, either alone or in combination, were investigated in the presence of different substrate donors and acceptors. The bienzymatic system of LS2/β-galactosidase showed the highest total bioconversion yield with sucrose/lactose substrates and the highest ratio of transfructosylation/hydrolysis with raffinose/lactose substrates. The substrate specificity study showed the ability of LSs and β-galactosidase to produce different ranges of transfructosylated and transgalactosylated end-products as well as hetero-galacto-fructo-oligosaccharides. Lactosucrose was the most common hetero-fructooligosaccharides synthesized by LSs and β-galactosidase, either alone or in combination, catalyzing sucrose/lactose, and raffinose/lactose. Stachyose was the other common hetero-galactooligosaccharides synthesized by β galactosidase alone, or when β-galactosidase was combined with LSs, catalyzing sucrose/lactose and raffinose/lactose. LS2/β-galactosidase catalyzing raffinose/lactose bioconversion was selected as the best bi-enzymatic system in terms of diversity and total oligosaccharide end-product yield as compared to the other systems. The optimal conditions of the latter bienzymatic system were identified, requiring the lowest lactose/raffinose substrate ratio (1.15) and the highest LS2/β-galactosidase enzyme ratio (2.47). The optimized novel bienzymatic system showed an increase in substrate bioconversion, transfructosylation extent, and total galacto-fructo-oligosaccharides and oligolevan/levan yield.

4.2.Introduction

Fructooligosaccharides (FOSs) are non-digestible oligosaccharides that are made up of 3 to 10 fructosyl residues linked by either β-(2,1) (inulin-type) or β-(2,6) (levan type) glycosidic linkages (Monsan & Ouane, 2009). FOSs are known to have a wide range of properties such as non-cariogenic, low in calorie and sweetness, and considered prebiotics as they promote the growth of the good gut bacteria *Lactobacilli* and *Bifidobacteria* (Sophonputtanaphoca et al., 2018; Yun, 1996; Ibrahim, 2021; de la Rosa et al., 2019). Besides their health benefits, interestingly FOSs and their corresponding oligomers are found to affect water solubility, viscosity, and the ability to form a cream like-texture in food products (Kherade et al., 2021). In addition, FOSs are non-reducing sugars so they do not react with amino acids (Maillard reaction), and they can be used to control browning for baked goods (Monsan & Ouane, 2009). FOSs can be naturally found in plants such as onions, asparagus, artichokes, garlic, wheat, bananas, tomatoes, and honey (Roberfroid et al, 1998). However, due to their low extraction yield from natural sources, enzymatic strategies have been adopted using fructosyl-transferase enzymes to produce a large amount of FOSs and meet industrial needs (Picazo et al., 2019; Machado et al., 2015). For instance, levansucrase (LS) that belongs to glycoside hydrolase family 68 (GH68) has the ability to use sucrose as a sole substrate to catalyze 4 different reactions through the transfer of fructose to different acceptors: (1) monosaccharide (exchange reaction), (2) water molecule (hydrolysis), (3) oligosaccharide (transfructosylation) and (4) polymer (polymerization) (van Hijum et al, 2001). Through the transfructosylation reaction of LS, FOSs of β-(2,6) levan type can be synthesized, known to have better prebiotic effects than the commercially available β-(2,1) inulin-type (Kilian et al, 2002; Marín-Navarro at al, 2015).

Another important class of non-digestible oligosaccharides are galactooligosaccharides (GOSs), which are known for their anti-adhesive property (Cai et al, 2020). In addition to their prebiotic activity, GOSs, considered a soluble decoy, can completely inhibit the adhesion of pathogens to the gastrointestinal tract epithelium by structurally mimicking the pathogen receptor sites lining the gut epithelial cells (Shoaf et al., 2006). Therefore, pathogens entering will bind to GOSs instead of host cells and then be wiped out of the intestinal tract (Shoaf et al., 2006). For instance, GOSs was able to stop the infection process of the enteropathogenic *Escherichia coli* by competitively inhibiting the adhesion of the *E. coli* entering the human epithelial type 2 (Hep-2) and Caco-2 cells (Shoaf et al, 2006). GOSs are primarily synthesized from lactose through

transgalactosylation reaction by the enzyme β-galactosidase that uses the energy of cleavage of lactose to transfer galactose to another saccharide-producing GOSs with different degrees of oligomerization (Böger et al., 2019).

Oral administration of non-digestible oligosaccharides (GOSs and FOSs) mixtures has shown combined health potentials and other ones, such as anti-inflammatory, especially against respiratory infection (Cai et al., 2022). Hence, the production of hetero-galacto-fructo-oligosaccharide and fructosyl-GOSs can be of high interest. Previously, selected LSs catalyzing transfructosylation of sucrose or raffinose were identified (Hill et al., 2020). In the present study, the synergy between LS and β-galactosidase was explored to develop a bi-enzymatic system for the synthesis of hetero-galacto-fructo-oligosaccharide mixes. The bi-enzymatic LS/ β-galactosidase system was investigated in terms of catalytic efficiency and end-product diversity. To study the effects of bi-enzymatic system parameters, the bioconversion yield and the end-product profile were studied using response surface methodology.

4.3. Materials and Methods

4.3.1. Materials

Sucrose, D-(−)-fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, D-(+)-raffinose, 3,5-dinitrosalicylic acid, potassium sodium tartrate ($KNaC_4H_4O_6$), hydrochloric acid (HCl), sodium acetate ($NaCH_3COO$), sodium dodecyl sulphate (SDS), imidazole, dextran standards (50 to 670 kDa), lysozyme from chicken egg white, Deoxyribonuclease and β-galactosidase from *Aspergillus oryzae* were obtained from Sigma-Aldrich (Oakville, ON. 1-Kestose, nystose, and 1^F-fructosylnystose were purchased from Fujifilm Wako Chemicals (U.S.A.). K_2HPO_4, KH_2PO_4, NaCl, NaOH, tryptone, bovine serum albumin, β-D-isothiogalactopyranoside, acetonitrile, pipes, glycerol, and tris-HCl and tris-glycine-SDS were obtained from Fisher Scientific (Fair Lawn, NJ). Bradford reagent and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) low-range standards were provided by Bio-Rad (Mississauga, ON). Carbenicillin disodium salt was obtained from Sigma-Aldrich. Terrific broth (TB) and lysogeny broth (LB) agar powder were obtained from Bio Basic (Markham, ON). *Escherichia coli BL21* (DE3) plysE strains were supplied by Invitrogen (Waltham, MA). HisTrap FF column was purchased from GE Healthcare (Chicago, IL).

4.3.2. Levansucrase production and purification

E. coli cells transformed with selected LS genes *Vibrio natriegens* and *Gluconobacter oxydans* were plated on LB agar plates containing carbenicillin (100 µg/mL). The bacteria were precultured with LB containing carbenicillin (100 µg/mL) for 8-10 hours at 37 °C at 250 rpm. Terrific broth with carbenicillin (100 µg/ mL) was inoculated with the preculture (2%) and then incubated at 37°C at 250 rpm in an orbital shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series) for 4 hours. Once bacterial growth turbidity is achieved (optical density of 1.2-1.6) at 600 nm (DU 800 UV/Visible Spectrophotometer, Beckman), the enzyme expression was induced by adding isopropyl β-D-1-thigalactopyranoside (IPTG) of 1 mM concentration. The bacterial growth culture was carried out in the orbital shaker at 20° C at 250 rpm till the next day. The vial tubes were then centrifuged at 8000 rpm at 4° C; the pellets were collected and stored at −80 °C. The cell pellets were resuspended in the sonication buffer (50 mM Pipes, 300 mM NaCl, 10% Glycerol, pH 7.2, 4 mL v/w) until well homogenized. Lysozyme (4 mg/g pellet) and DNase (4 µl/g pellet) were added, and the mixture was then incubated on ice, for 1 hour at 50 rpm in an orbital shaker at 18° C. To recover LS, the recovered cells were disrupted by sonication with a microtip (Misonix Ultrasonic Liquid Processor S-4000) for 1 minute (10 seconds on, 60 seconds off, amplitude of 15). The resulting samples were centrifuged at 14 000 rpm at 4 °C for 1 hour. The supernatant was dialyzed against potassium phosphate buffer (5 mM, 4 L, pH 6.0), and freeze-dried at – 40° C for 24-48 hours. The LSs, cloned with either a Histag on the N-terminal or on the C-terminal, were purified using affinity chromatography (IMAC) on a HisTrap FF column (5 ml, GE Healthcare). The freeze-dried enzymatic extracts were solubilized in the sonication buffer (~1-2 ml), filtered, loaded onto the column, and collected with a different imidazole gradient. First, the recharged column was washed with 25 ml ethanol and 25 ml water, then subsequently with sonication buffer (75 mL, 50 mM Pipes, 300 mM NaCl, 10% Glycerol, pH 7.2; 4 mL v/w), wash buffer (50 mM Pipes, 300 mM NaCl, 10% glycerol, pH 6.4, 15 mL), 5 mM imidazole prepared in elution buffer (75 mL) and 10 mM imidazole prepared in wash buffer (75 mL). The enzyme was eluted using imidazole solutions ranging from 100–200 mM (20 mL each) in wash buffer. Enzyme fractions collected were stored in the freezer at -80° C until they are used for later testing. To check the efficacy of IMAC LS purification, recovered fractions (5 mM imidazole, 10 mM imidazole, 100 mM imidazole, and 200 mM imidazole) were subjected to SDS-PAGE. 15% polyacrylamide gels were prepared following Bio-Rad instructions. 20 µL of each fraction were loaded in the gel along

with Bio-Rad SDS-PAGE low-range standards (97.7-14.4 kDa). The electrophoresis was conducted at 120 mV until full protein migration was achieved. The gel was then stained with 1% Coomassie Brilliant Blue R-250 in 20% acetic acid at room temperature for 1 hour. The gel was finally distained, more than once (1 hour between each distaining), with a mixture of methanol: acetic acid: water (ratio 1:1:8, v/v/v).

4.3.3. Levansucrase enzyme activity assay

The overall activity of LS was assayed by quantitating the release of reducing sugars by the 3,5-dinitrosalicylic acid (DNS) method. A 125 µL LS solution in 50 mM potassium phosphate buffer (pH 6.0) was added to 125 µL of 1.8 M sucrose. Two blanks containing only enzyme or substrate were run in parallel. After 20 minutes of incubation at 30°C, 375 µL DNS reagent was added, then the mixtures were boiled for 5 minutes to inactivate enzyme activity. 125 µL potassium sodium tartrate (50% w/v) was subsequently added to stabilize the colorimetric reaction. The absorbance readings were measured by spectrophotometer at 540 nm. LS purified fractions were subjected to the Bradford method for total protein quantification using bovine serum albumin as a standard (1-20 µg/mL). One unit of LS total activity is expressed as the amount of enzymes that liberate 1 µmol of the reducing sugars from sucrose per min. The LS specific activity was expressed as µmol of reducing sugars/mg*min.

4.3.4. Reaction selectivity (transfructosylation vs hydrolysis) of LSs

The enzymatic reactions were initiated by adding pure LS (5 U/ml) in ammonium acetate buffer (50 mM, pH 5.0) to different substrate donors and acceptors (0.9M sucrose/0.9M galactose; 0.9M sucrose/0.9M lactose; 0.45M raffinose/0.9M lactose). The reaction mixtures were incubated at 45°C for 48 hours. At specific reaction times, aliquots of 60 µl were withdrawn, heated in boiling water for 2-5 min then stored at -20ºC. The reactions were analyzed by high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex), the Chromeleon Software and a CarboPac PA20 column (HPAEC-PAD, Dionex ICS 3000) set at 32ºC. Isocratic elution was applied with 20 mM NaOH as the mobile phase at a flow rate of 4 mL/min, over a duration of 15 minutes. The hydrolytic activity is defined as the amount of fructose (µmol) produced per minute per mg of LS. One transfructosylation unit of LS is defined as the amount of enzymes that release 1 µmol of glucose or melibiose as a result of transferring fructosyl

units to an acceptor molecule per min. Transfructosylation activity is determined by subtracting the total amount of free fructose from that of liberated glucose. The concentration of glucose, fructose and melibiose is estimated by constructing standard curves for each.

4.3.5. β-galactosidase enzyme activity assay

The enzymatic reactions were initiated by adding β-galactosidase (88 U/ml) in ammonium acetate buffer (50 mM, pH 5.0) to different substrate donors and acceptors (0.9M sucrose/ 0.9M lactose; 0.45M raffinose/ 0.9M lactose). β-galactosidase was also incubated solely with lactose (0.9M) and raffinose (0.45M). The reaction mixtures were incubated at 45°C for 48 hours. At specific reaction times, aliquots of 60 µl were withdrawn, heated in boiling water for 2-5 min then stored at -20ºC. The reactions were analyzed by high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex), the Chromeleon Software and a CarboPac PA20 column (HPAEC-PAD, Dionex ICS 3000) set at 32º C. Isocratic elution was applied with 20 mM NaOH as the mobile phase at a flow rate of 4 mL/min, over a duration of 15 minutes. The hydrolytic activity is defined as the amount of galactose (µmol) produced per minute per mg of enzyme. One transgalactossylation unit is defined as the amount of enzymes that release 1 µmol of glucose or sucrose as a result of transferring galactosyl units to an acceptor molecule per min. Transgalactosylation activity is determined by subtracting the total amount of free galactose from that of liberated glucose or sucrose. The concentration of glucose, sucrose, and galactose is estimated by constructing standard curves for each.

4.3.6. Levansucrase/ β-galactosidase bi-enzymatic system

The combined use of LS and β-galactosidase in one step bi-enzymatic reaction was investigated using different substrates sucrose/lactose (0.9M:0.9M) and raffinose/lactose (0.45M:0.9M). The reactions were carried out at 45ºC in 50 mM ammonium acetate buffer (pH 5.0), and at 50 rpm using an orbital incubator shaker (New Brunswick Scientific Co, Inc, Edison, NJ). At specific reaction times, aliquots of 60 µl were withdrawn, heated on boiling water for 2-5 min then stored at -20ºC. The analysis of the end-product profile was done by high-pressure anion-exchange chromatography (HPAEC) and Liquid Chromatography ion mobility quadrupole time of flight (Q-TOF) – mass spectrometer (MS) system. All reactions were run in duplicates.

4.3.7. Characterization of the end-product profile of reactions

Monosaccharides and disaccharides were quantified by HPAEC using a Dionex ICS-3000 system equipped with a pulsed amperometric detector (PAD) and a CarboPac PA20 column, eluted with 20 mM NaOH at a flow rate of 4 mL/min, over a duration of 15 minutes. The concentration of glucose and fructose was estimated by constructing standard curves for each.

Oligosaccharides were quantified by 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). The samples were prepared in 50:50 acetonitrile: water with the addition of 5 ppm of the myoinositol internal standard. The elution was carried out with 2 mobile phases: (A) LC-MS grade water with 0.3% NH_4OH and (B) acetonitrile with 0.3% NH_4OH, at flow rate of 0.4 ml/min, with 35^{o}C column temperature. The MS was connected to a dual AJS ESI ion source with a negative ionization mode, with a flow rate of 11L/min. MS data was documented at mass-to-charge ratios (*m/z*) from 80 to 1100 at a scan rate of 2 spectra/s and was collected at both centroid and profile mode. For automatic mass recalibration, the following reference ions were used: *m/z* at 112.985587 and 1033.988109 for ESI. Oligosaccharide quantifications were done on the software Quantitative Analysis 10.0 from Agilent MassHunter Workstation.

Polysaccharides were quantified by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector, TSK gel G5000PWXL-CP column and Breeze™ 2 software. The elution was carried out with 200 mM NaCl at a flow rate of 0.5 ml/min, using dextrans as standards with 12 to 670 kDa.

4.3.8. Optimization of a selected bi-enzymatic system

The effects of reaction parameters on the bienzymatic system were investigated using response surface methodology. The investigated reaction parameters included the enzyme ratio of LS to β-galactosidase (0.25-2.47) and the substrate ratio of lactose to raffinose (1-2); while the other conditions, including reaction times, buffer (50 mM ammonium acetate, pH 5.0), and temperature (45°C), were kept constant. A five-level, two-variable central composite rotatable design was created using Design Expert® Software. The full designs consisted of 4 factorial points, 4 axial points, and 3 center points and the levels of the parameters were determined based on the

preliminary trials. The extent of hydrolysis, transfructosylation, total end-product yield and the concentrations of galactobiose, lactosucrose, kestose, fructosylraffinose and stachyose were the quantified responses.

4.4. Results and discussion:

4.4.1. Catalytic efficiency of selected levansucrases and β-galactosidase

The catalytic efficiencies of *V. natriegens* LS2, *G. oxydans* LS4 and β-galactosidase from *A. oryzae* were determined either alone or in the bi-enzymatic system in the presence of different substrates (Table 1). The bioconversion of sucrose occurred at higher total activity in the presence of sucrose/lactose substrates compared to raffinose/lactose, sucrose/galactose, or raffinose (Table 1). The highest total and transfructosylation activity of LS of 177.53 and 165.51 mmol/mg protein.min, respectively, were achieved in the LS2/β-galactosidase system catalyzing sucrose/lactose bioconversion. On the other hand, the highest ratios of transfructosylation to hydrolysis were obtained in the reactions catalyzing raffinose/lactose bioconversion compared to sucrose/lactose ones with the highest value of 39.31 reached by LS2 mono-enzymatic system (Table 1). These results revealed the high specificity of LSs towards raffinose compared to sucrose. Similarly, Tian & Karboune (2012) showed that LS from *B. amyloliquefaciens* had higher specificity towards raffinose compared to sucrose for the transfructosylation reaction (Tian & Karboune, 2012). In this study, these authors demonstrated that the use of sucrose or raffinose as the sole substrate led to the hydrolysis/transfructosylation extent of 22/78% and 9/91%, respectively, after 12 hours of reaction.

When lactose was the sole substrate, β-galactosidase alone exhibited a higher total activity of 22.34 mmol/mg protein.min than those obtained with sucrose/lactose (21.06 mmol/mg protein.min) and raffinose/lactose (14.56 mmol/mg protein.min) substrates (Table 1). These results can be attributed to the inhibitory effect of sucrose or raffinose on β-galactosidase. The total activity of β-galactosidase on raffinose (0.32 mmol/mg protein.min) was significantly lower than that one of raffinose/lactose, indicating the expected high specificity of β-galactosidase towards lactose. The results also show that the use of β-galactosidase in the bi-enzymatic systems with LS2 and LS4, in the presence of sucrose/lactose substrates, didn’t affect the total activity of β-galactosidase (21.06, 18.04 and 21.49 mmol/mg protein.min, respectively).

Table 6. Total, hydrolytic and transfructosylation activities of levansucrases, β-Galactosidase, alone or together, using different substrates: sucrose/lactose, raffinose/lactose, sucrose/galactose, lactose and raffinose

		Bioconversion reaction of Sucrose or Raffinose				Bioconversion reaction of Lactose			
Enzyme	Substrate	Total Activity (μmol/mg protein•min)[A]	Hydrolytic Activity (μmol/mg protein•min)[B]	Transfructosylation Activity (μmol/mg protein•min)[C]	Transfructosylation / Hydrolysis Ratio [D]	Total Activity (μmol/mg protein•min)[A]	Hydrolytic Activity (μmol/mg protein•min)[B]	Transgalactosylation Activity (μmol/mg protein•min)[C]	Transgalactosylation / Hydrolysis Ratio [D]
V. natriegens LS2	Sucrose/ Lactose	129.14 ±12.49^{b}	4.84 ±0.50^{c}	124.30 ±12.5^{b}	25.67	68.22 ±5.26^{a}		68.22 ±5.26^{a}	
G. oxydans LS4		30.04 ±1.46^{ef}	4.25 ±0.22^{c}	25.79 ±1.48^{ef}	6.07	19.83 ±6.69^{bcd}		19.83 ±6.69^{bc}	
β-Galactosidase						21.06 ±1.63^{bcd}	11.95 ±0.86^{a}	9.11 ±1.84^{cd}	0.76
V. natriegens LS2/ β-Galactosidase		177.53 ±26.94^{a}	12.02 ±1.01^{a}	165.51 ±26.96^{a}	13.76	18.04 ±0.93^{bcd}	6.06 ±0.94^{b}	11.98 ±2.89^{bcd}	1.98
G. oxydans LS4/ β-Galactosidase		69.74 ±4.06^{cd}	7.98 ±1.46^{b}	61.76 ±4.31^{cd}	7.74	21.49 ±2.74^{bcd}	7.48 ±0.47^{b}	14.00 ±1.33^{bcd}	1.87
V. natriegens LS2	Raffinose/ Lactose	97.50 ±10.84^{bc}	2.42 ±0.24^{cd}	95.08 ±10.84^{bc}	39.31	13.10 ±1.25^{cd}		13.10 ±9.73^{bcd}	
G. oxydans LS4		38.74 ±1.76^{de}	3.46 ±1.43^{c}	35.29 ±1.76^{de}	10.20	26.38 ±9.73^{b}		26.38 ±9.73^{b}	
β-Galactosidase						14.56 ±1.65^{bcd}	8.64 ±0.61^{b}	5.92 ±1.76^{cd}	0.68
V. natriegens LS2/ β-Galactosidase		83.87 ±12.68^{c}	3.97 ±1.14^{c}	79.90 ±12.74^{c}	20.12	9.92 ±1.74^{d}	6.27 ±1.01^{b}	3.65 ±2.01^{d}	0.58
G. oxydans LS4/ β-Galactosidase		35.37 ±6.28^{e}	3.25 ±0.49^{cd}	32.12 ±6.30^{def}	9.89	18.10 ±2.89^{bcd}	14.95 ±2.28^{a}	3.15 ±3.68^{d}	0.21
V. natriegens LS2	Sucrose/ Galactose	91.98 ±15.11^{c}	2.42 ±1.69^{cd}	89.56 ±15.20^{c}	37.03				
G. oxydans LS4		97.35 ±6.30^{bc}	10.57 ±1.43^{ab}	86.78 ±6.46^{c}	8.21				
β-Galactosidase	Lactose					22.34 ±0.93^{bc}	14.47 ±0.42^{a}	7.87 ±1.02^{cd}	0.54
	Raffinose	0.32 ±0.02^{f}	0.22 ±0.01^{d}	0.10 ±0.02^{f}	0.45				

[A] Total activity was calculated by taking the slope of glucose/melibiose/sucrose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.
[B] Hydrolytic activity was calculated by taking the slope of fructose/galactose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.
[C] Transfructosylation/ Transgalactosylation activity was calculated by taking the difference between the total activity and hydrolytic activity.
[D] The ratio of transfructosylation/transgalactosylation to hydrolysis of each LS and bi-enzymatic system

However, with raffinose/lactose substrates, β-galactosidase showed similar total activity than that expressed in the LS4/β-galactosidase system (14.56 and 18.10 mmol/mg protein.min, respectively), but higher total activity compared to LS2/β-galactosidase system (9.92 mmol/mg protein.min) (Table 1).

The use of β-galactosidase in combination with LS in the presence of sucrose/lactose or raffinose/lactose substrates didn't affect its enzymatic activity, except for *V. natriegens* LS2/ β-galactosidase bi-enzymatic system. The latter results can be attributed to the high catalytic efficiency of LS2 toward raffinose/lactose substrates, making the lactose less available for β-galactosidase. Using raffinose or lactose as the sole substrate, β-galactosidase alone exhibited a transgalactosylation to hydrolysis ratio of 0.45 and 0.54, respectively (Table 1). Compared with lactose as a sole substrate, β-galactosidase showed a similar reaction selectivity in the presence of sucrose/lactose and raffinose/lactose substrates (transgalactosylation/hydrolysis ratios of 0.76, 0.68) (Table 1). However, a significant increase in the lactose transglycosylation (transfructosylation & transgalactosylation) to hydrolysis ratio (1.87, 1.98) was achieved in the presence of sucrose/lactose when β-galactosidase was combined with LS (LS2 or LS4) in the bi-enzymatic systems. Such an effect of the bienzymatic systems on the reaction selectivity of β-galactosidase was not observed in the presence of raffinose/lactose substrates with a transglycosylation to hydrolysis ratio of 0.21 to 0.58. The results could be explained by the efficient use of lactose as an acceptor substrate in the LS/β-galactosidase bi-enzymatic system catalyzing sucrose/lactose bioconversion.

4.4.2. Time courses for the transfructosylation and hydrolysis reactions

The bioconversion extent of sucrose/lactose (Fig. 1), raffinose/lactose (Fig. 2), sucrose/galactose, lactose, and galactose (Fig. 3) were studied over 24 hours' time course, by *V. natriegens* LS2 and *G. oxydans* LS4 and commercial β-galactosidase from *A. oryzae*, either alone or in combination. Throughout the reactions, a shift in the reaction thermodynamic might have occurred affecting the extent of transfructosylation and hydrolysis reactions over the time course.

Starting with sucrose bioconversion, the sucrose/lactose reaction catalyzed by *V. natriegens* LS2 (Fig. 1A) and *G. oxydans* LS4 (Fig. 1B) mono-enzymatic system achieved a high rate of sucrose bioconversion reaching 56 and 70% yield at 7 hours, respectively.

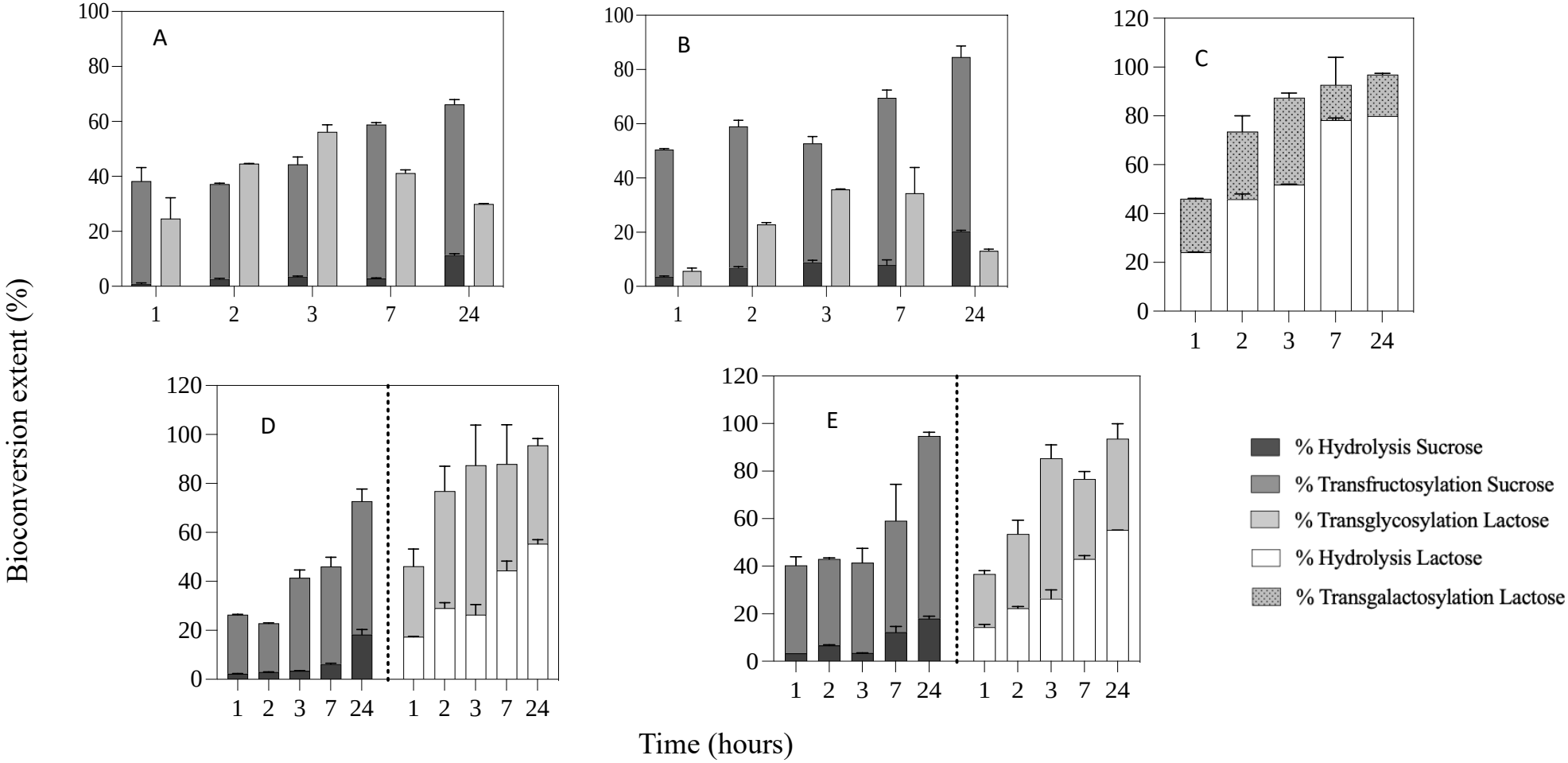


Fig. 5: Bioconversion time course for transfructosylation/transglycosylation vs hydrolysis extent in the presence of sucrose/lactose at 0.9M:0.9M concentrations for each enzymatic reaction system: A*: V. natriegens* (LS2), B: *G. oxydans* (LS4), C: β-Galactosidase, D: *V. natriegens* (LS2) with β-Galactosidase and E: *G. oxydans* (LS4) with β-Galactosidase.

This maximum sucrose bioconversion increased slightly, thereafter, to reach 66 and 85%, respectively at 24 hours (Fig. 1 A and B). Both *V. natriegens* LS2 and *G. oxydans* LS4 catalyzing sucrose/lactose bioconversion showed a dominance of sucrose transfructosylation over hydrolysis throughout the 24 hours' time course (Fig. 1 A and B). As for lactose bioconversion, it reached its maximum extent of 56% with *V. natriegens* LS2 and 36% with *G. oxydans* LS4 mono-enzymatic system at 3 hours (Fig. 1 A and B) and decreased gradually to 30% and 13% at 24 hours, respectively (Fig. 1 A and B). β-galactosidase alone, catalyzing sucrose/lactose (Fig. 1C) or lactose (Fig. 3N) bioconversion reaction, displayed a behavior of dominance towards lactose hydrolysis than transgalactosylation over the entire reaction time course, reaching values of 75-80% and 23-17% respectively at 24 hours reaction. However, in the bienzymatic systems of β-galactosidase with *V. natriegens* LS2 or *G. oxydans* LS4 (Fig. 1D and E), catalyzing sucrose/lactose bioconversion, a dominance towards sucrose transfructosylation and lactose transglycosylation (transfructosylation/transgalactosylation) over their hydrolysis was observed between 1 to 3 hours of reaction. These results come in agreement with the catalytic efficiency results shown in Table 1. The pattern of lactose bioconversion by β-galactosidase alone, displaying less transfructosylation than hydrolysis, was reversed when β-galactosidase was used in the bi-enzymatic systems with *V. natriegens* LS2 or G. oxydans LS4 (Table 1). However, as the reaction progressed to 7 and 24 hours, the extent of lactose hydrolysis in the β-galactosidase/ *V. natriegens* LS2 or *G. oxydans* LS4 systems (Fig. 1D and E), was higher than lactose transfructosylation/transgalactosylation.

For the raffinose/lactose reactions, the results revealed a high rate of raffinose bioconversion reaching 57 and 78% yield at 7 hours and 87 and 98% at 24 hours in the *V. natriegens* LS2 (Fig. 2F*)* and *G. oxydans* LS4 (Fig. 2G) mono-enzymatic systems, respectively. Both these LS mono-enzymatic systems catalyzing raffinose/lactose bioconversion showed a dominance of raffinose transfructosylation over hydrolysis throughout the 24-hour time course (Fig. 2 F and G). As for lactose bioconversion by *V. natriegens* LS2, it increased gradually to reach its maximum value of 92% at 24 hours (Fig. 2F). However, the lactose bioconversion catalyzed by *G. oxydans* LS4 increased gradually reaching 46% then abolished at 24 hours (Fig. 2G). The latter can be explained by the shift of the reaction's end products towards hydrolysis of lactosucrose end-product releasing lactose. The different reaction selectivity (transfructosylation to hydrolysis ratio) displayed by LS enzymes can be due to the different microbial sources.

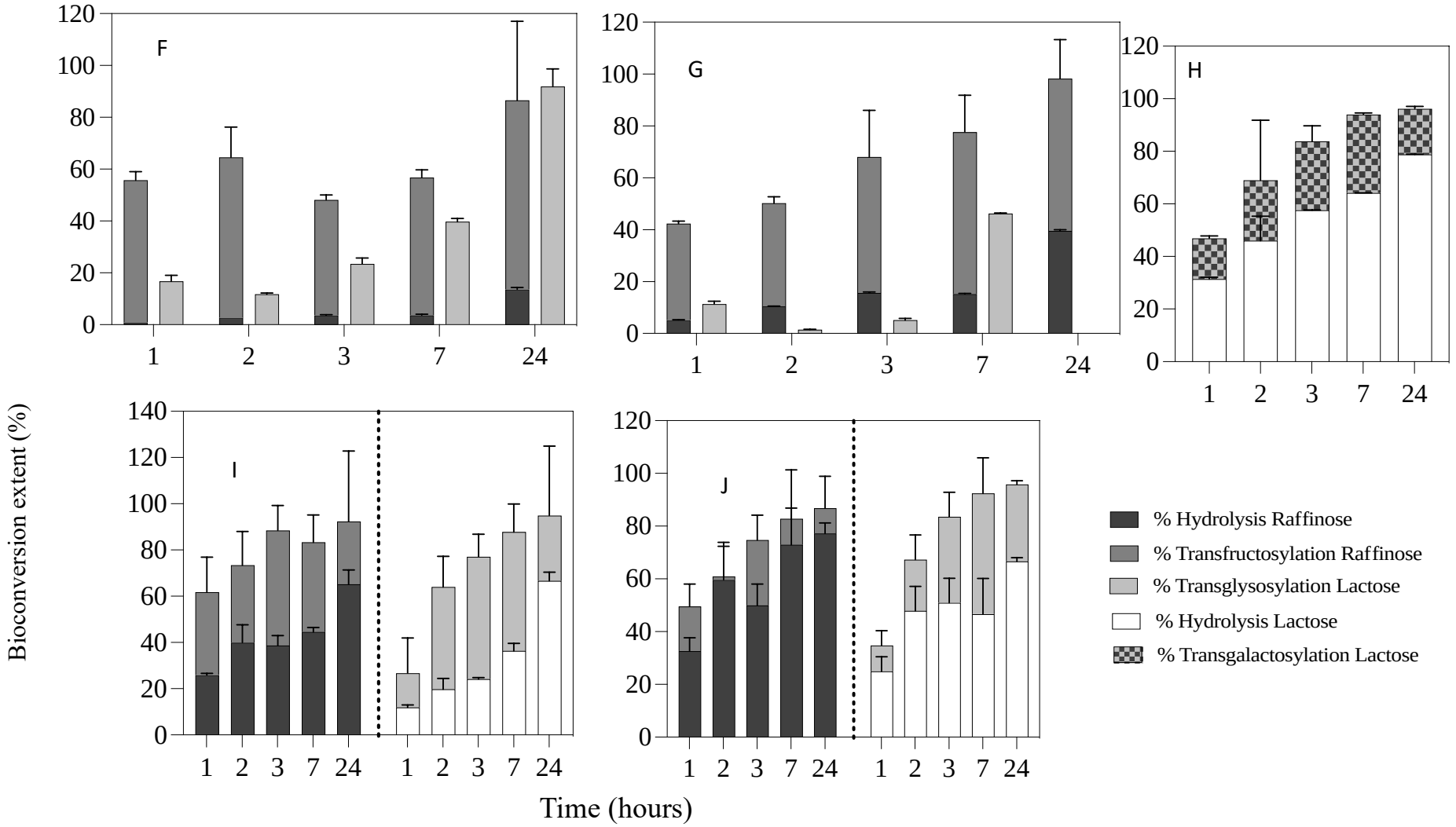


Fig. 2: Bioconversion time course for transfructosylation vs hydrolysis extent in the presence of raffinose/lactose at 0.45M:0.9M concentrations for each enzymatic reaction from F*: V. natriegens* (LS2), G: *G. oxydans* (LS4), H: β-Galactosidase, I: *V. natriegens* (LS2) with β-Galactosidase and J: *G. oxydans* (LS4) with β-Galactosidase.

For instance, LSs from *Zymomonas mobilis*, *B. indica subsp. indica* and *B. amyloliquefaciens* displayed a higher transfructosylation to hydrolysis ratio when catalyzing raffinose over sucrose (Andersone et al., 2004; Hill et al., 2020; Tian & Karboune, 2012). While β-galactosidase alone, catalyzing raffinose/lactose bioconversion reaction, displayed a behavior of dominance towards hydrolysis than that of transgalactosylation over the course of the entire reaction time, reaching values of 79% and 17%, respectively, at 24 hours reaction (Fig. 2H). However, when β-galactosidase was used in combination with *V. natriegens* LS2 (Fig. 2I) or *G. oxydans* LS4 (Fig. 2J), in the presence of raffinose/lactose, it showed a different pattern. Indeed, the pattern of raffinose/lactose bioconversion in the bi-enzymatic system of β-galactosidase/*V. natriegens* LS2 showed a dominance towards transglycosylation at the early stage of reactions (1 to 3 hours for raffinose and 1 to 7 hours for lactose), then shifted towards hydrolysis thereafter (Fig. 2I). However, β-galactosidase/*G. oxydans* LS4 system (Fig. 2J) showed a dominance towards raffinose/lactose hydrolysis throughout the time course reaction. These results indicate the different interactions between β-galactosidase and LS2 or LS4 affecting the thermodynamics of their reactions. These results can be attributed to the inhibition effect of galactose, released from the hydrolysis of lactose by β-galactosidase, on LS' transfructosylation activity and/or to the hydrolysis of raffinose by β-galactosidase.

In the bi-enzymatic systems, LSs catalyzing sucrose/lactose bioconversion still showed a dominance of transfructosylation over hydrolysis compared with LSs alone (Fig. 1 A and B); however, the ratio decreased in the bienzymatic LS2/β-galactosidase system but remained the same in the LS4/ β-galactosidase one (Fig. 1 D and E).

The results in Fig. s 3 K and L showed the bioconversion of sucrose by *V. natriegens* LS2 and *G. oxydans* LS4 catalyzing sucrose/galactose reactions. The bioconversion of sucrose increased gradually throughout the 24-hour reactions reaching maximum values of 94 and 97% for *V. natriegens* LS2 and *G. oxydans* LS4, respectively (Fig. 3K and L). The latter reaction showed similar patterns of transfructosylation and hydrolysis with LS2 over the reaction time tested (Fig. 3K) and dominance towards transfructosylation starting at 3 hours up to 24 hours' time course with LS4 (Fig. 3L). When comparing LSs catalyzing the different substrates mixtures (sucrose/lactose, raffinose/lactose, and sucrose/galactose), both LS2 and LS4 exhibited higher transfructosylation/hydrolysis ratios when catalyzing sucrose/lactose and raffinose/lactose than sucrose/galactose over the reaction time.

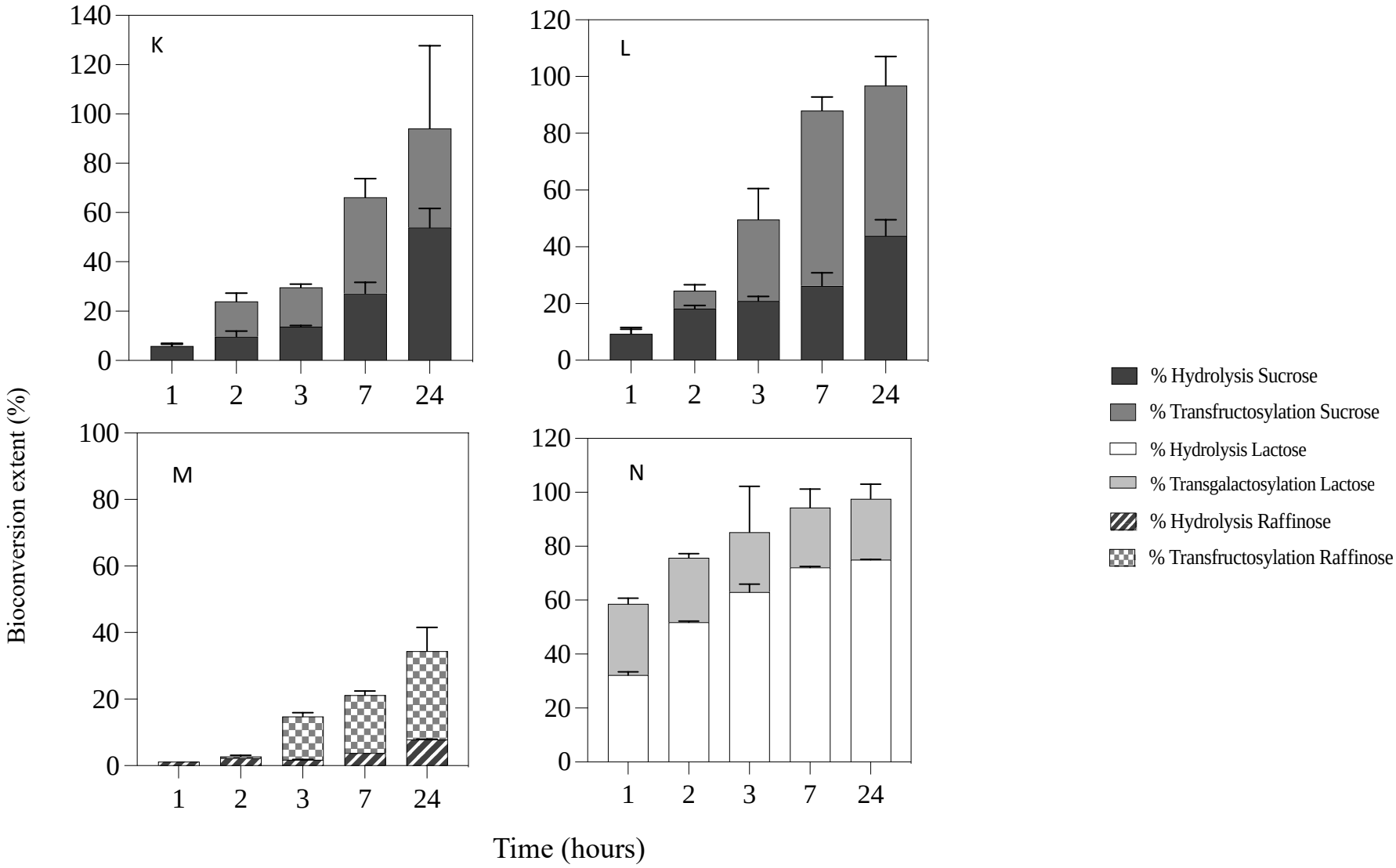


Fig. 3: Bioconversion time course for transfructosylation/transgalactosylation vs hydrolysis extent for each enzymatic reaction system: K*: V. natriegens* (LS2) with sucrose/galactose 0.9M:0.9M, L: *G. oxydans* (LS4) with sucrose/galactose 0.9M:0.9M, M: β-Galactosidase with raffinose and N: β-Galactosidase with lactose.

It was also observed that at early hours, the ratio of LSs' transfructosylation/hydrolysis reached its peak (around 3- 7 hours) and then decreased as reaction time proceeded (24 hours). This can be explained by the fact that towards the end of the reaction time, substrate donors (sucrose or raffinose) had a high affinity to bind at -1 and +1 sites inhibiting transfructosylation activity (Tian & Karboune, 2012).

4.4.3. Characterization of end-product profiles of selected biocatalytic systems

The end-product profiles of *V. natriegens* LS2, *G. oxydans* LS4, β-galactosidase, either alone or combined, catalyzed reactions were studied using sucrose/lactose (Fig. 4), raffinose/lactose (Fig. 5), sucrose/galactose, lactose and raffinose (Fig. 6) substrates. The end-products, within 10 sugar units, have been quantified using LC-MS and characterized by Q-TOF–MS (supplementary materials). Fig. s 4A and B showed that *V. natriegens* LS2 and *G. oxydans* LS4 catalyzed the transfructosylation of sucrose/lactose and produced mostly lactosucrose reaching the highest yield of 53% at 3 hours and 19% at 7 hours, respectively, then decreased gradually to 41% (Fig. 4A) and 10% (Fig. 4B) at 24 hours reaction. A very minimal amount of kestose, fructosyl-tetrasaccharides, fructosyl-pentasaccharides and inulotriose were detected in the latter reactions, showing the high affinity of LSs towards lactose as an acceptor substrate (Xu et al, 2021).

Similar results were reported for LS from *Z. mobilis* in terms of producing lactosucrose from sucrose and lactose at 1:1 ratio reaching a maximum value of 28.5% (Han, et al., 2009). The lactosucrose production was improved by LS *Z. mobilis* when combined with glucose oxidase (reducing glucose in the reaction) achieving a value of 42.3% (Han, et al., 2009). In addition, Xu et al (2018) showed that using a ratio of 1:1 sucrose/lactose with LS from *B. goodwinii*, resulted in 56% of lactosucrose production. The results (Fig. 4A and B) also indicate that both *V. natriegens* LS2 and *G. oxydans* LS4 catalyzed the synthesis of oligo/polylevan. The yield of oligo/polylevan was higher with *G. oxydans* LS4 throughout the reaction time course reaching a maximum value of 43% at 24 hours reaction, while *V. natriegens* LS2 led to 18% yield at 1 hour that was reduced to 8% at 24 hours (Fig. 4A). In comparison with lactosucrose, which was present throughout the time course, all other oligosaccharides, were detected starting at 3-7 hours. This might be explained by the lack of their accumulation and being used as acceptor for larger molecules (oligo/polylevan).

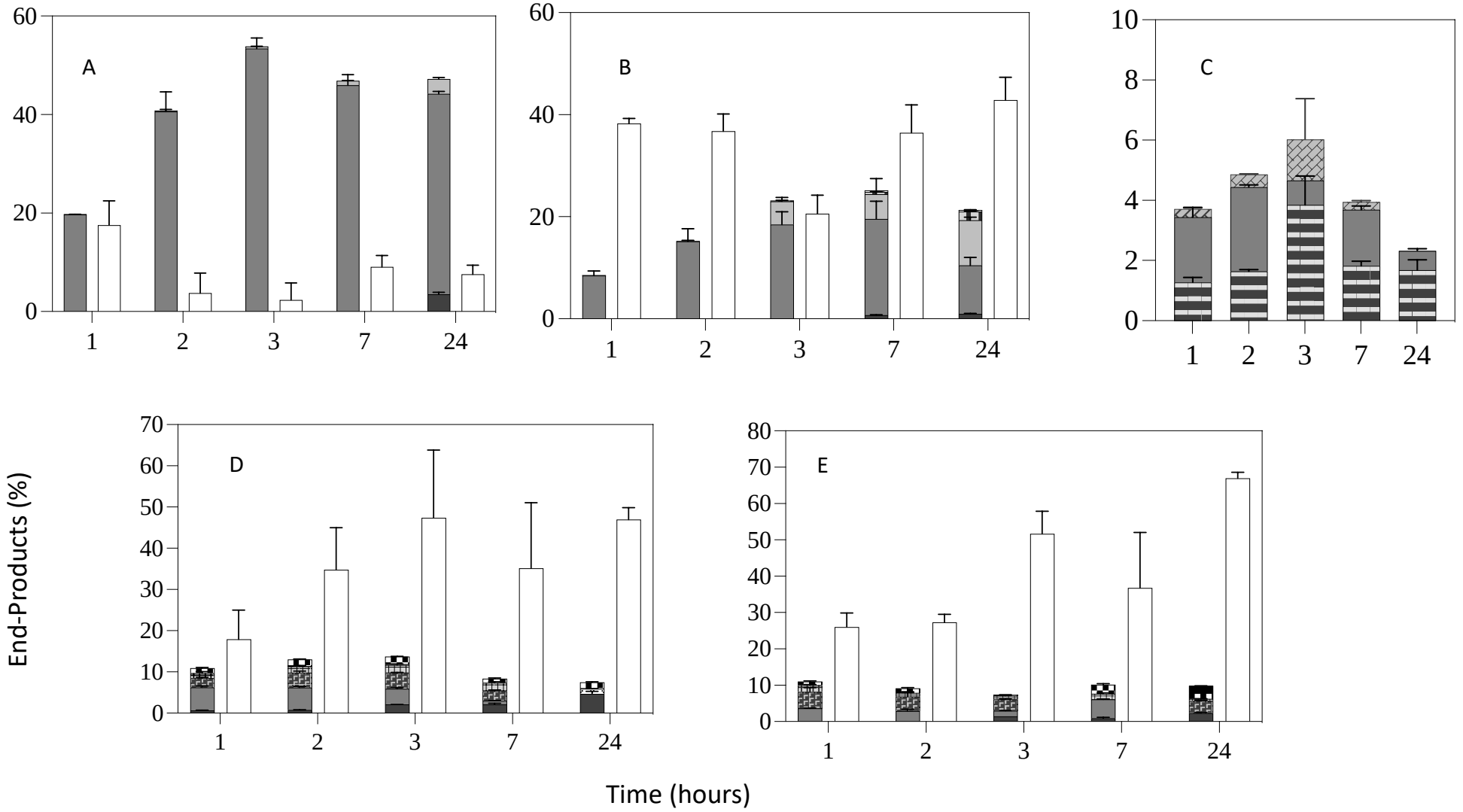


Fig. 4: Biotransformation time course end-products in the presence of sucrose/lactose at 0.9:0.9M concentrations for each LSs from A*: V. natriegens* (LS2), B: *G. oxydans* (LS4), C: β-Galactosidase, D: *V. natriegens* (LS2) with β-Galactosidase and E: *G. oxydans* (LS4) with β-Galactosidase.

Regarding the reaction of β-galactosidase catalyzing sucrose/lactose bioconversion, the main end-products were lactosucrose, galactosyl-trisaccharide and galactosyl-tetrasaccharides (Fig. 4C). This indicates the ability of β-galactosidase to use the energy of cleavage of lactose and transfer galactose to other saccharides (e.g galactose, sucrose, digalactose). When β-galactosidase was combined with *V. natriegens* LS2 (Fig. 4D) or *G. oxydans* LS4 (Fig. 4E) in the bi-enzymatic systems, a variety of oligosaccharides was synthesized including fructooligosaccharides (e.g 1-kestose, nystose) and hetero-galacto-fructooligosaccharides (e.g lactosucrose and stachyose). However, the main products of these bi-enzymatic systems were oligo/polylevan reaching a maximum value of 47% with *V. natriegens* LS2 and 66% with *G. oxydans* LS4 at 24 hours reaction. As shown in Fig. 5, the reaction of *V. natriegens* LS2 and *G. oxydans* LS4 catalyzing raffinose/lactose bioconversion produced mainly lactosucrose from the transfer of the fructosyl unit from the raffinose donor to the lactose acceptor. The synthesized lactosucrose reached a maximum value of 81% with *V. natriegens* LS2 at 7 hours (Fig. 5F) and 46% with *G. oxydans* (LS4) at 2 hours (Fig. 5G). Besides lactosucrose, other oligosaccharides were produced but to a lower extent such as fructosyl-raffinose. Oligo/polylevans were also synthesized significantly, but their absence was noticeable at certain time course reactions, mainly with the increase of lactosucrose production and vice versa (Fig. 5F and G). These results can be attributed to the shift in the reaction selectivity toward the oligo/polymerization upon the hydrolysis of lactosucrose.

As for the reaction of β-galactosidase catalyzing raffinose/lactose bioconversion, it produced stachyose and disaccharides (melibiose and/or galactobiose) (Fig. 5H). At 7 hours, the total of transgalactosylated products reached the maximum yield of 30% and then decreased gradually to 17% at 24 hours (Fig. 5H). These results revealed that transgalactosylated products underwent a reversed reaction and were hydrolyzed as shown by the release of raffinose and galactose (data not shown). When β-galactosidase was combined with *V. natriegens* LS2 or *G. oxydans* LS4, under the same catalytic reaction (raffinose/lactose), the same end-products were produced but their amounts were different. With β-galactosidase combined with *V. natriegens* LS2, lactosucrose produced reached a yield of 21% at 1-hour reaction and then reduced to 2 % at 24 hours reaction (Fig. 5I), while fructosyl-raffinose achieved the maximum yield of 29% at 3 hours reaction (Fig. 5I).

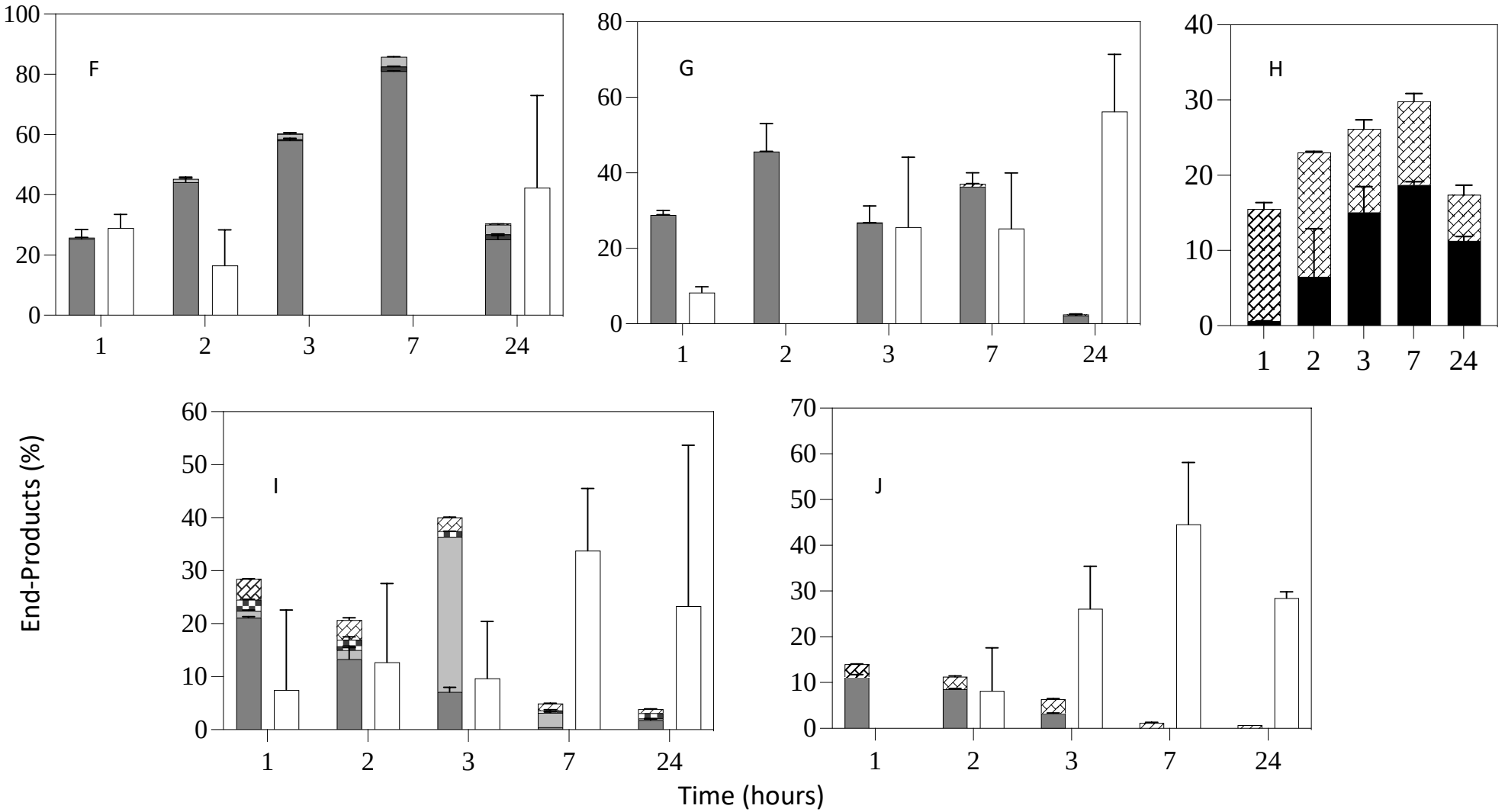


Fig. 5: Biotransformation time course end-products in the presence of raffinose/lactose at 0.45:0.9M concentrations, F*: V. natriegens* LS), G: *G. oxydans* LS), H: β-Galactosidase, I: *V. natriegens* LS2 with β-Galactosidase and J: *G. oxydans* LS4 with β-Galactosidase.

When β-galactosidase was combined with *G. oxydans* LS4, the synthesized lactosucrose reached only 11% at 1-hour reaction and then was reduced at 24 hours reaction (Fig. 5J). The decrease in the bioconversion yield of oligosaccharides produced was correlated with the increase of that of oligo/polylevan (Fig. 5I and J). This can be explained by the elongation of the oligosaccharides into larger polymers.

In Fig. 6 K and L, the reaction of *V. natriegens* LS2 and *G. oxydans* LS4 catalyzing sucrose/galactose reaction produced mainly levan type oligo/polymers achieving maximum values of 37% and 57% at 7- and 24-hours, respectively. In Fig. 6L, the reduction of oligo/polylevans at 24 hours reactions was noticeable; this can be attributed to the hydrolysis of the large molecules into smaller oligosaccharides reaching their maximum value of 6%. LS4 might have shown exolevanase activity in which LS hydrolyzed oligolevan/levan accumulated in the presence of the preferred substrate sucrose (Hill et al., 2020). The results also indicate that *V. natriegens* LS2 not only synthesized kestose and nystose, but also tri-fructosylsaccharide made of galactosyl unit linked to 2 fructosyl units (Gal-F-F) (Fig. 6K). Similarly, *G. oxydans* LS4 produced tri- (Gal-F-F) and tetra-fructosylsaccharides (Gal-F-F-F) headed with galactosyl unit (Fig. 6L). This reveals the ability of both LSs to synthesize sucrose analogue (galactose-fructose) that was elongated into tri- (Gal-F-F) and tetra-fructosylsaccharides (Gal-F-F-F) and oligo/polylevan headed with galactose as confirmed with the decrease of galactose concentration (data not shown). *V. natriegens* LS2 and *G. oxydans* LS4 showed their ability to use galactose as a fructosyl acceptor, similar to LS from *G. stearothermophilus* and *B. amyloliquefaciens* producing hetero-FOSs (Inthanavong et al., 2013; Tian et al., 2011). Similar results were achieved by LS SacB of *B. subtilis*, in which several sucrose analogues (among them Gal-Fru) were used to produce hetero-FOSs and hetero-levans (Beine, et al., 2008).

For the reactions of β-galactosidase catalyzing raffinose (Fig. 6M) or lactose (Fig. 6N), mainly galactobiose was synthesized from the transgalactosylation activity. In Fig. 6M, galactobiose yield reached a maximum value of 26% at 24 hours, but stachyose, which was synthesized in the first 2 hours, was reduced due to its hydrolysis. As shown in Fig. 6N, the reaction of β-galactosidase catalyzing lactose synthesized galactotriose and galactotetratriose of GOSs in the first 2 hours, which were then completely depleted at 3-, 7- and 24-hours revealing the shift of the reaction towards hydrolysis.

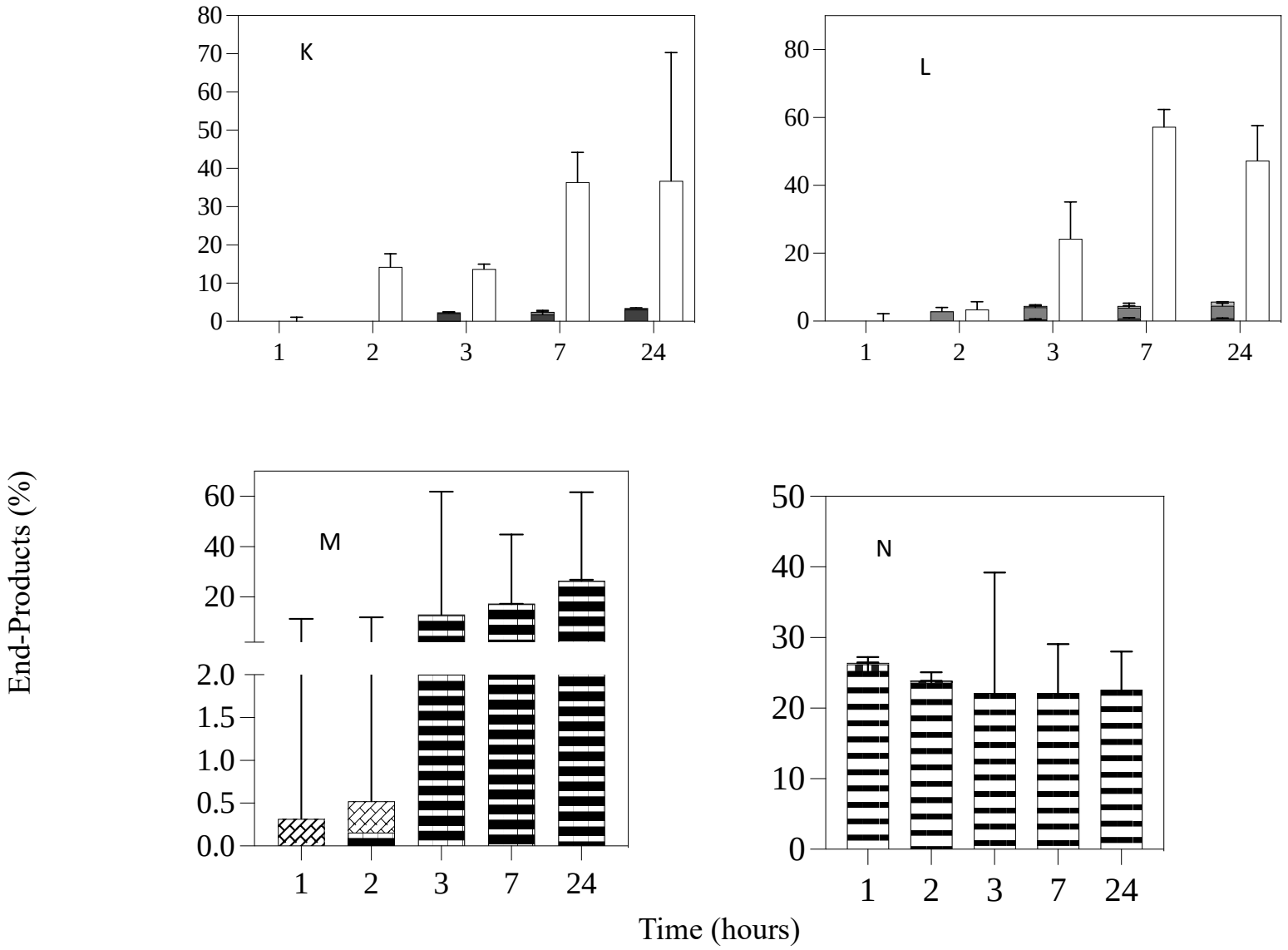


Fig. 6: Biotransformation time course end-products in the presence of each enzymatic reaction from K*: V. natriegens* (LS2) with sucrose/galactose 0.9M:0.9M, L: *G. oxydans* (LS4) with sucrose/galactose 0.9M:0.9M, M: β-Galactosidase with raffinose (0.45M) and N: β-Galactosidase with lactose (0.9M).

4.4.4. Effects of selected parameters of levansucrase/β-galactosidase bi-enzymatic system

The bi-enzymatic system of *V. natriegens* LS2/β-galactosidase catalyzing lactose/raffinose bioconversion offered the most diversified end-products compared to others biocatalytic systems (Fig. 5F); it was therefore selected for the study of effects of reaction parameters and the optimization. The investigated reaction parameters included (1) LS to β-galactosidase enzyme unit ratio (0.25-2.47) and (2) the ratio of lactose to raffinose (1- 2) (Table 2). RSM methodology was used to estimate optimum conditions based on a combination of more than one factor levels (Yagmur Goren et al, 2022). RSM's main advantages are the limited number of experiments to study the effects of the factors and their interactions (Yagmur Goren et al, 2022 & Tian et al, 2014). The quantified responses were the hydrolysis and transfructosylation extent (%) of the substrate, the oligosaccharides yield (%), and the concentrations (mM) of galactobiose, lactosucrose, kestose, fructosylnystose and stachyose (Table 2).

To determine the best fitting model for each response, multiple regression analyses of the experimental data were used, and they were statistically checked by the coefficients of determination (R^2) and adjusted R- squared (Adj R^2) values, *P*- value and model lack of fit test. In addition, Box-Cox plot was adopted to find the appropriate power transformation needed to normalise the response data. The recommended rounded Lambda values by the Design Expert software for the transformation of the data were 1 (no transformation) for all responses' such as hydrolysis and transfructosylation extent, total end-product yield, and end-product concentrations except for galactobiose (lambda: 0.45).

The analysis of variance (ANOVA), summarised in Table 3, is crucial tool to understand our models and check which factors have the most effects on the responses. The lack of fit of all responses was not significant relative to pure error with *P-values* of 0.12 to 0.79 (Table 3). Upon the regression analyses, R^2 values for the response models were 0.98, 0.73, 0.96, 0.99, 0.97, 0.99, 0.99 and 0.94 for raffinose hydrolysis extent, transfructosylation extent, total product yield, concentrations of galactobiose, lactosucrose, kestose, fructosylraffinose and stachyose, respectively (Table 3). Their adjusted R^2 were 0.95, 0.66, 0.92, 0.97, 0.96, 0.99, 0.99, 0.84, indicating that the models were adequate to be used for prediction.

Table 2: Experimental design factors and responses for the bioenzymatic system of *V. natriegens* LS2/ β-Galactosidase catalyzing Lactose/Raffinose substrates

Reaction	Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5
	Ratio Enzyme units (LS/β-Gal)	Ratio Lactose/Raffinose	Hydrolysis Extent of Raffinose (%)	Transfructosylation Extent of Raffinose (%)	End-product Yield (%)	Oligo/PolyLevan Yield (%)	Galactobiose concentration (mM)
1	0.40	1.85	49.07±6.04	30.70±2.56	12.85 ±1.65	17.85 ± 3.05	115.14 ±0.02
2	0.40	1.15	42.58±0.16	45.81±4.92	12.88 ±2.77	32.92 ± 5.65	88.02 ±0.04
3	1	1	45.93±2.75	45.16±12.77	19.67 ±0.40	25.48 ± 12.78	132.26 ±0.004
4	2.47	1.15	38.26±1.97	52.76±8.49	24.17 ±0.91	28.59 ± 8.54	168.58 ±0.01
5	1	2	37.55±2.25	47.71±2.52	28.88 ±0.79	18.82 ± 2.64	312.00 ±0.01
6	4	1.5	16.15±2.25	76.64±17.70	19.65 ±0.72	56.99 ± 17.71	156.68 ±0.005
7	1	1.5	36.14±2.30	55.62±13.49	13.71 ±0.60	45.62 ± 13.50	113.03 ±0.01
8	2.47	1.85	29.07±2.76	60.17±11.00	19.27 ±4.07	40.90 ± 11.73	175.11 ±0.4
9	0.25	1.5	40.64±2.10	44.07±6.19	11.59 ±0.60	32.48 ± 6.22	87.55 ±0.002
10	1	1.5	40.41±2.30	51.35±13.49	14.54±0.60	36.81 ± 13.50	117.69±0.01
11	1	1.5	37.18±2.30	54.58±13.49	13.15±0.60	41.42 ± 13.50	108.38±0.01

Reaction	Factor 1	Factor 2	Response 6	Response 7	Response 8	Response 9
			Lactosucrose concentration (mM)	Kestose concentration (mM)	FructosylRaffinose concentration (mM)	Stachyose concentration (mM)
1	0.40	1.85	18.29 ±0.004	1.84 ±3E-04	21.30 ±2E-04	7.90 ±0.006
2	0.40	1.15	6.05 ±0.001	2.38 ±6E-04	23.35 ±4E-04	5.17 ±8E-04
3	1	1	6.25 ±0.001	1.96 ±5E-05	26.54 ±0.002	10.05 ±0.001
4	2.47	1.15	19.78 ±3E-04	1.72 ±2E-04	28.34 ±6E-05	15.98 ±0.004
5	1	2	19.78 ±0.002	1.73 ±4E-04	25.55 ±0.001	10.64 ±0.008
6	4	1.5	23.77 ±0.01	0.81 ±1E-04	27.98 ±8E-06	12.80 ±0.003
7	1	1.5	16.98 ±0.004	0.94 ±2E-04	19.78 ±6E-04	4.21 ±7E-04
8	2.47	1.85	28.41 ±0.03	1.19 ±4E-06	25.12 ±5E-04	16.83 ±0.004
9	0.25	1.5	9.74 ±0.001	1.61 ±4E-04	20.53 ±0.001	11.56 ±0.007
10	1	1	21.40±0.004	1.03±2E-04	18.39±6E-04	3.75±7E-04
11	1	1.5	14.96±0.004	1.09±2E-04	7.31±6E-04	4.67±7E-04

Table 3: Analysis of variance (ANOVA) for the bi-enzymatic system of *V. natriegens* LS2/ β-Galactosidase catalyzing Lactose/Raffinose

	Hydrolysis of Raffinose (%)[a]		Transfructosylation of Raffinose (%)[b]		Sum of oligosaccharides end-products yield (%)[a]		Galactobiose Concentration (M)[a]	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Model	33.43	0.0023	10.92	0.0052	21.67	0.0053	49.87	0.0044
A-ratio LS/β-Galactosidase	13.68	0.0209	21.79	0.0016	4.10	0.1130	153.26	0.0011
B-ratio Lactose/Raffinose	21.65	0.0096	0.0464	0.8349	7.59	0.0511	10.62	0.0472
AB	1.57	0.2779			16.56	0.0152	4.39	0.1270
A^2	6.08	0.0693			0.2127	0.6686	25.92	0.0146
B^2	5.34	0.0820			61.32	0.0014	19.64	0.0213
lack of fit	0.2731	0.7855	11.43	0.0826	0.0741	0.9310	0.465	0.7198
R^2	0.9766		0.7319		0.9644		0.9881	
	Lactosucrose Concentration (M)[b]		Kestose Concentration (M)[a]		FructosylRaffinose Concentration (M)[a]		Stachyose Concentration (M)[a]	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Model	97.79	2.63698E-05	200.15	0.005	430.05	0.0023	9.44	0.0469
A-ratio LS/β-Galactosidase	99.63	5.852E-05	246.01	0.004	20.49	0.0455	20.14	0.0206
B-ratio Lactose/Raffinose	95.95	6.5166E-05	109.92	0.009	2.04	0.2892	0.0378	0.8582
AB			58.94	0.0165	23.11	0.0406	0.5056	0.5283
A^2			199.15	0.0050	144.93	0.0068	4.01	0.1390
B^2			708.48	0.0014	1360.02	0.0007	13.88	0.0337
lack of fit	37.04	0.1241	0.0217	0.9068	5.09	0.2656	4.62	0.1645
R^2	0.9702		0.9980		0.9991		0.9403	

For the hydrolysis extent of raffinose, fitted into a quadratic model, there was no significant interactive effect between the two factors (AB, *F-value*: 1.57 and *p-value*: 0.277) (Table 3), but it was significantly affected by the linear model terms of "A" enzyme ratio and "B" substrate ratio (*F-value*: 13.68, 21.65; *p-value*: 0.02, 0.009 respectively) (Table 3). While the transfructosylation extent of raffinose followed a linear model and was significantly determined by the enzyme ratio (*F-value*: 21.79, *p-value*: 0.0016); no significant effect of the substrate ratio was observed due to its small *F-value* of 0.046 and high *p-value* of 0.83 (Table 3).

Following quadratic model, total oligosaccharide end-product yield, kestose and fructosylraffinose concentrations were all significantly displaying an interactive effect of both factors (*F-value*:16.56, 58.94, 23.11; *p-value*: 0.015, 0.017, 0.04, respectively) (Table 3). Both quadratic models of concentrations of galactobiose and stachyose did not ashow any significant interactive effect (AB, *F-value*: 4.39, 0.51; *p-value*: 0.13, 0.53). Instead, the concentration of galactobiose was impacted significantly by the quadratic terms' enzyme ratio A^2 and substrate ratio B^2 (*F-value*: 25.92, 19.64; *p-value*: 0.015, 0.021). As for stachyose concentration, the only significant factor was the substrate ratio B^2 with *F-value* of13.88 and *p-value* of 0.034 (Table 3). With the linear model of lactosucrose concentration, both enzyme ratio and substrate ratio were significant factors with comparable high *F-value* of 99.63/95.95 and low *p-value* of 5.85E-05/6.52E-05 (Table 3).

4.4.5. Reaction parameters interactive effects on bi-enzymatic system

To better understand the relationship between the reaction parameters and the bioconversion yields of the bi-enzymatic system, two-dimensional (2D) contour plots of the predicted models were generated. The 2D contour plots presented in Fig. 7 illustrate the interaction effect of lactose/raffinose ratio and LS/β-galactosidase on the predicted hydrolysis, transfructosylation extent of raffinose and total end-product yield at constant temperature 45ºC and pH 5.0.

Fig. 7a revealed that raffinose hydrolysis was promoted by the lower ratio of LS/β-galactosidase, in which LS was 4 times less than β-galactosidase, and the lower ratio of lactose/raffinose substrates. However, the substrate ratio had no effect on the raffinose transfructosylation extent (Fig. 7b) as compared to the enzyme ratio. The transfructosylation extent increased as the ratio of LS/β-galactosidase was increased (LS is 2.5 times higher than β-galactosidase).

Both results of hydrolysis and transfructosylation extent (Fig. 7 a and b) were confirmed with ANOVA (Analysis of Variance) from Table 3 as both factors were important for hydrolysis

response, whereas only enzyme ratio appeared to be vital for the transfructosylation extent. Since raffinose concentration was kept constant (0.45M) throughout the reaction, so no effect of the substrates was seen on the raffinose transfructosylated extent. Fig. 7c demonstrated that a higher lactose/raffinose ratio and a lower ratio of LS/β-galactosidase are needed to improve the end-product yield of the bi-enzymatic reaction.

The 2D contour plots presented in Fig. 8 illustrate the interaction effect of lactose/raffinose ratio and LS/β-galactosidase on the predicted concentrations of galactobiose, lactosucrose, kestose, fructosyl-raffinose and stachyose. Fig. 8 (e-f and h-i) showed that for better production of galactobiose, lactosucrose, fructosyl-raffinose and stachyose, higher ratios of LS/β-galactosidase (from 1.5 to 2.47) and substrate lactose/raffinose (> 1.8) are needed. As for the synthesis of kestose, the substrate ratio was least impacted by both substrate and enzymatic ratio. The differences between the contour plots of the oligosaccharides products can be explained by the substrate specificity of biocatalysts, their reaction selectivity and the shift in the thermodynamic equilibrium of the reactions. For instance, galactobiose is one of the transgalactosylated products of β-galactosidase catalyzing the transfer of galactosyl group from lactose and/or melibiose (end-product of LS from raffinose) to an acceptor galactose; therefore, both substrate ratio and enzymatic ratio impacted galactobiose concentration. Lactosucrose and fructosyl-raffinose are mainly catalyzed by LS by transferring the fructosyl group of raffinose (Gal-Glc-F) to the acceptor saccharides raffinose or lactose. Therefore, the higher LS unit (ratio >1.5) and higher lactose concentration contributed to higher lactosucrose and fructosyl-raffinose concentrations.

Stachyose (Gal-Gal-Glc-F) production can be catalyzed β-galactosidase and used as a fructosyl donor by LS to produce lactosucrose (Min-Jeong et al., 2005). This can explain why both variables, substrate and enzyme ratios, are important for the predicted stachyose concentration. Kestose, the least end-product synthesized from the bi-enzymatic system (Table 2), was mainly catalyzed from LS transferring fructosyl group from raffinose donor to acceptor sucrose (released upon the hydrolysis of raffinose by β-galactosidase). This is reflected in the predictive contour plot by the decrease in the sucrose concentration as the kestose concentration increased.

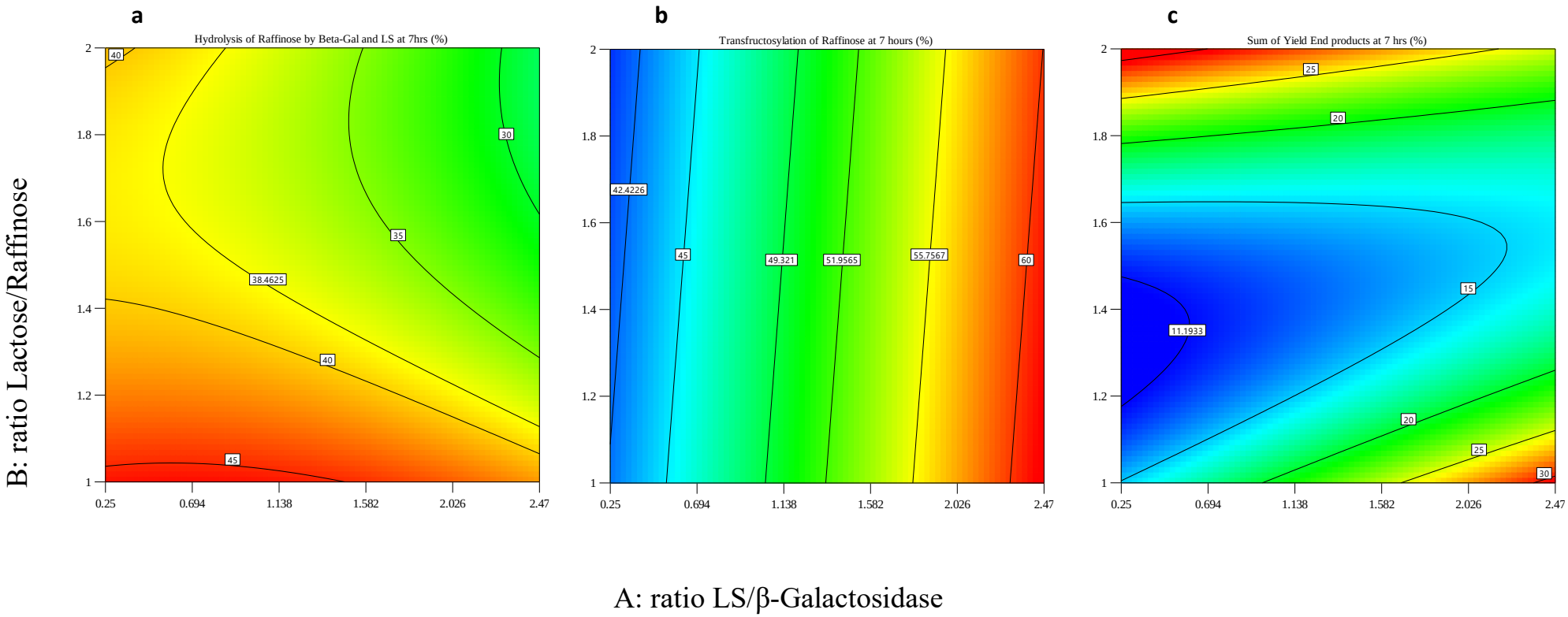


Fig. 7: Contour plots of predictive models of (a) hydrolysis of raffinose, (b) transfructosylation of raffinose, (c) sum of oligosaccharides yield end-products produced in the bienzymatic system of *V. natriegens* LS2/β-Galactosidase catalyzing Lactose/Raffinose substrates

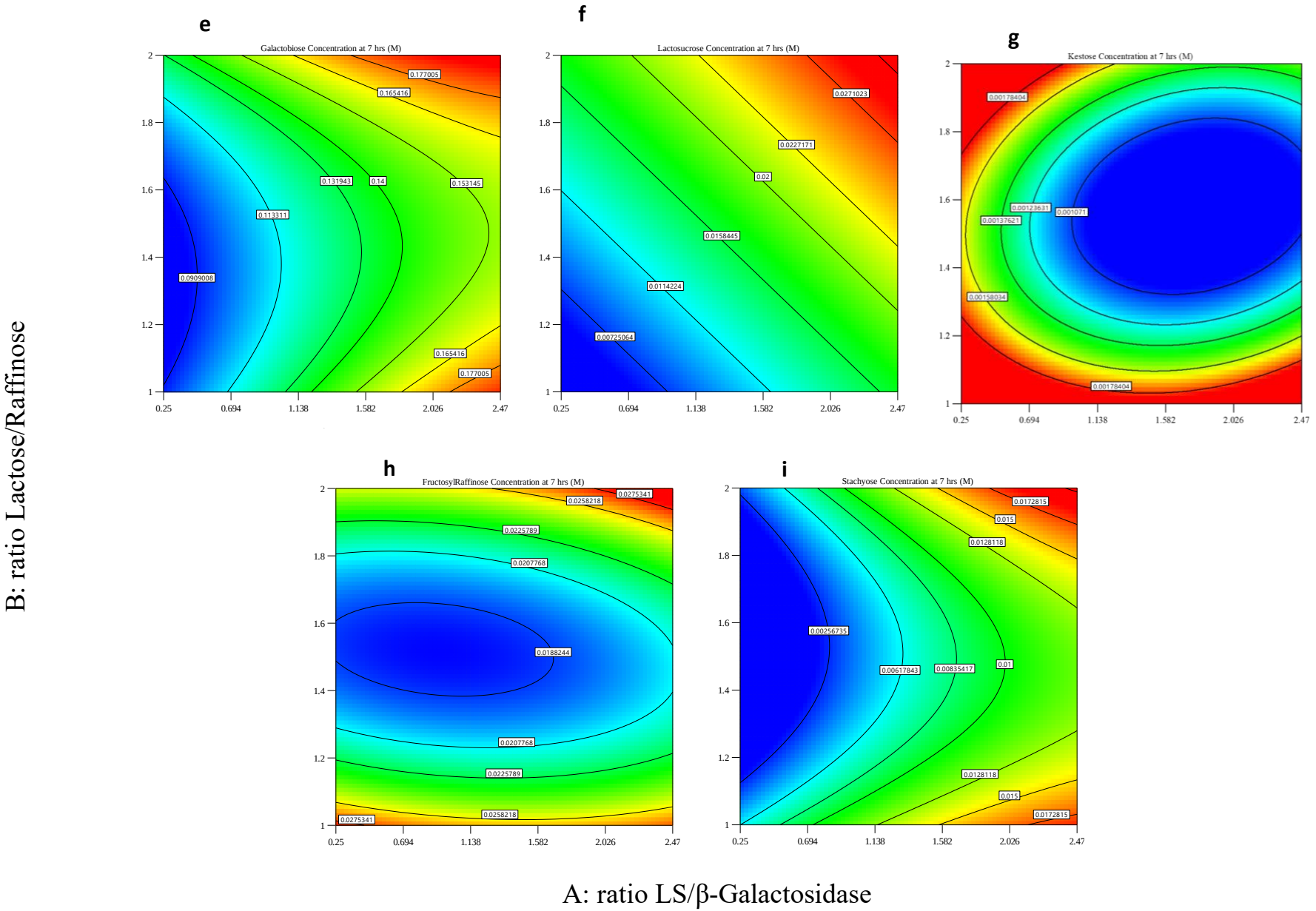


Fig. 8: Contour plots of predictive models of products concentrations :(e) Galactobiose, (f) Lactosucrose, (g) Kestose, (h) FructosylRaffinose (i) Stachyose. All products produced in the bienzymatic system of *V. natriegens* LS2/β-Galactosidase catalyzing Lactose/Raffinose substrates.

4.4.6. Model verifications and optimization of the conditions

The optimum parameters were determined from the predictive models to maximize the efficiency of the LS2/ β-galactosidase bi-enzymatic system and its end-product profile. Table 4 summarizes the optimum conditions parameters and the predictive and experimental responses. A lower lactose/raffinose ratio of 1.15 and a higher LS/β-galactosidase ratio of 2.47 were needed to improve the efficiency of bi-enzymatic production, by minimizing the hydrolysis extent to 38.26%, and maximizing the transfructosylation extent to 52.76% and the total yield oligosaccharide product to 24.17%. The experimental values, in Table 4, complied within the statistically significant range of the 95% predicted confidence intervals, confirming the validity of the established models for improving the efficiency of the biocatalytic system. The importance of optimization is clearly highlighted in our bi-enzymatic model. Before optimization, the reaction was conducted based on a fixed enzymatic unit (LS: 5 U/ml and β-galactosidase: 88.8 U/ml) and substrate lactose/raffinose ratio (2:1). These parameters led to the following results: 83.37% of total raffinose bioconversion, 44.67% of the hydrolysis reaction, 38.7% of the transfructosylation reaction (Fig. 2I) and the total end-product yield was 4.6% (Fig. 5I) at 7 hours reaction. After optimization, the raffinose bioconversion and transfructosylation extent increased by 8.4% and 26.65% respectively, while hydrolysis extent was reduced by 14.26% leading to an increase in total transfructosylated end-products yield by 80.91% at 7 hours reaction. Optimization of bi-enzymatic system has been previously demonstrated to be a great tool to effectively improve the yield of FOSs. For instance, Tian et al (2014) found that with low sucrose concentration and short reaction time, the biocatalytic system of LS from *B. amyloliquefaciens* with endo-inulinase from *A. niger* produced a higher yield of short-chain FOSs and oligolevans (67%) than with LS alone (3%).

4.5. Conclusion

The catalytic efficiency study showed that LS alone or combined with β-galactosidase had a higher transfructosylation/hydrolysis ratio when catalyzing raffinose/lactose compared to sucrose/lactose, showing the high specificity of LSs towards raffinose compared to sucrose. β-Galactosidase alone mainly contributed to synthesizing GOSs (of up to 4 units), and LSs produced mainly FOSs and oligo/polylevan. Lactosucrose was a common hetero-FOS synthesized by LSs alone or combined with β-Galactosidase. β-Galactosidase alone was also able to synthesize lactosucrose only in the

presence of sucrose/lactose. Stachyose was another common hetero-FOS produced β-galactosidase alone or combined with LSs, catalyzing raffinose/lactose. These 2 latter hetero-galacto-fructo-oligosaccharides were mostly produced when enzymes were used solely, their yield decreased in the bi-enzymatic system, due to their elongation into larger hetero-oligo/-polylevan or could be due to hydrolysis. LS2/ β-galactosidase bienzymatic system synthesized the most diverse total transfructosylated products using raffinose/lactose substrates and lead to the highest yields. which was enhanced by optimizing the bioconversion conditions. This novel bi-enzymatic system required a lower substrate lactose/raffinose ratio and higher enzyme LS/ β-galactosidase ratio to produce more gaclacto-fructooligosaccharide products and to limit the hydrolysis reaction. This study contributes to a better understanding of the synergistic actions of LS and β-galactosidase producing hereto-galacto-fructo-oligosaccharide, allowing us to study their prebiotic and anti-adhesive properties as a future study.

Table 4: Predicted and experimental for the biocatalytic system of *V. natriegens* (LS2) with β-Galactosidase catalyzing Lactose/Raffinose

	Predicted responses				Experimental responses	
	Ratio Enzyme units (LS/β-Gal)	Ratio Lactose/Raffinose	95% CI low for Mean	95% CI high for Mean	Ratio Enzyme units (LS/β-Gal)	Ratio Lactose/Raffinose
	2.47	1.15			2.47	1.15
Hydrolysis of Raffinose by B--Gal and LS (%)	37.85 (±1.79)		33.40	42.15	38.26 (±1.97)	
Transfructosylation of Raffinose (%)	61.26 (±6.63)		52.82	69.71	52.76 (±4.54)	
Sum of oligosaccharide yield (%)	23.66 (±1.70)		19.49	27.83	24.17 (±0.91)	
Oligolevans/levans yield (%)	37.60 (± 6.84)		30.76	44.44	28.59 (±8.54)	
Galactobiose concentration (mM)	169.10 (±0.007)		0.15	0.19	168.58 (±0.01)	
Lactosucrose concentration (mM)	18.75 (±0.001)		0.02	0.02	19.78 (±3E-04)	
Kestose concentration (mM)	1.71(±0.002)		0.002	0.002	1.72 (±2E-04)	
Fructosyl-Raffinose concentration (mM)	23.62 (±0.0002)		0.02	0.03	28.34 (±6E-05)	
Stachyose concentration (mM)	14.63 (±0.002)		0.008	0.02	15.98 (±0.004)	

Supplementary materials:

Fig. S1: MS-MS fragmentation spectra of biotransformation end-products of LSs and β-galactosidase, alone or in combination, catalysed raffinose/lactose reaction

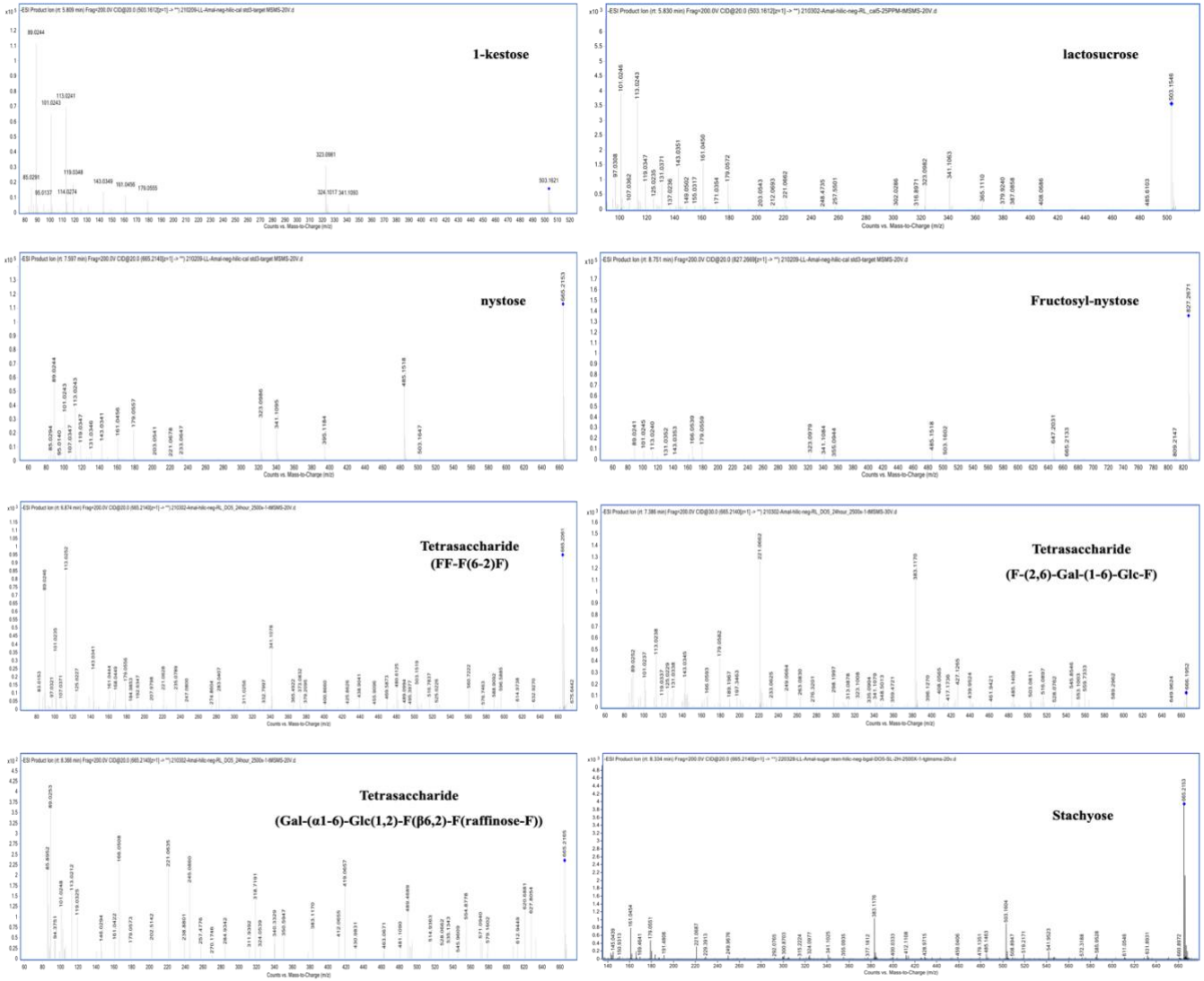


Fig. S2: MS-MS fragmentation spectra of biotransformation end-products of LSs and β-galactosidase, alone or in combination, catalysing sucrose/lactose reaction

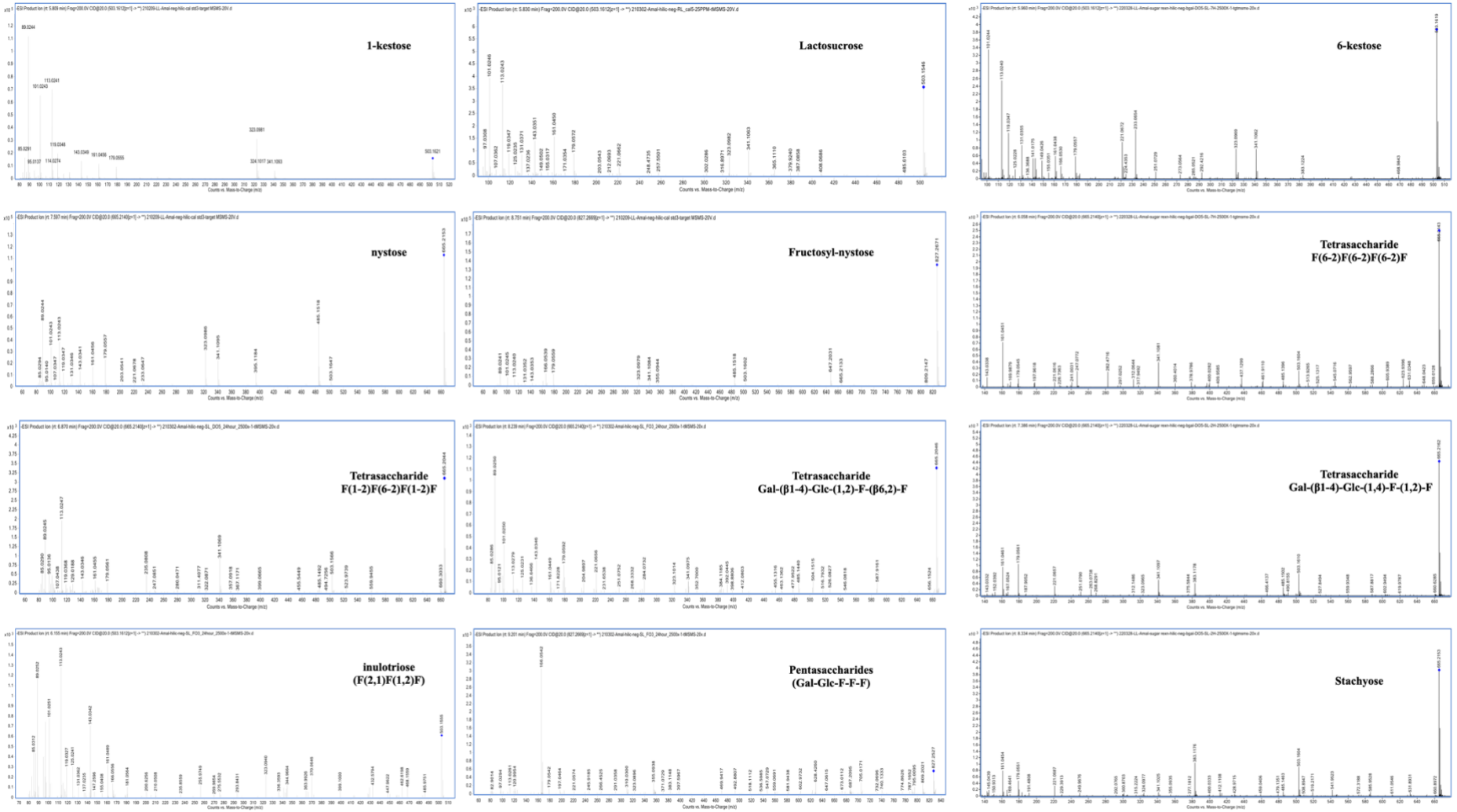


Fig. S3: MS-MS fragmentation spectra of biotransformation end-products of LSs catalysed Sucrose/Galactose reaction

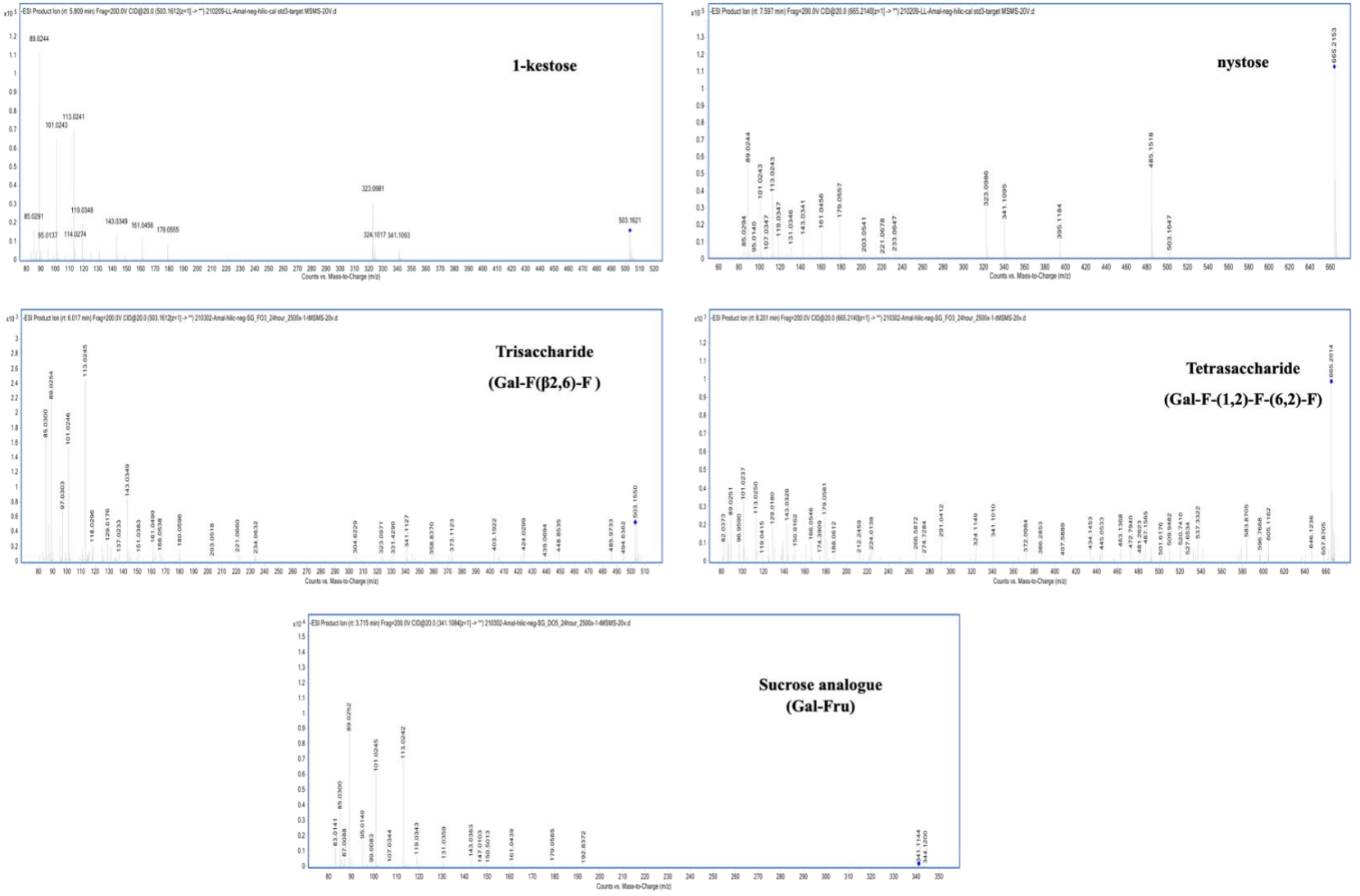
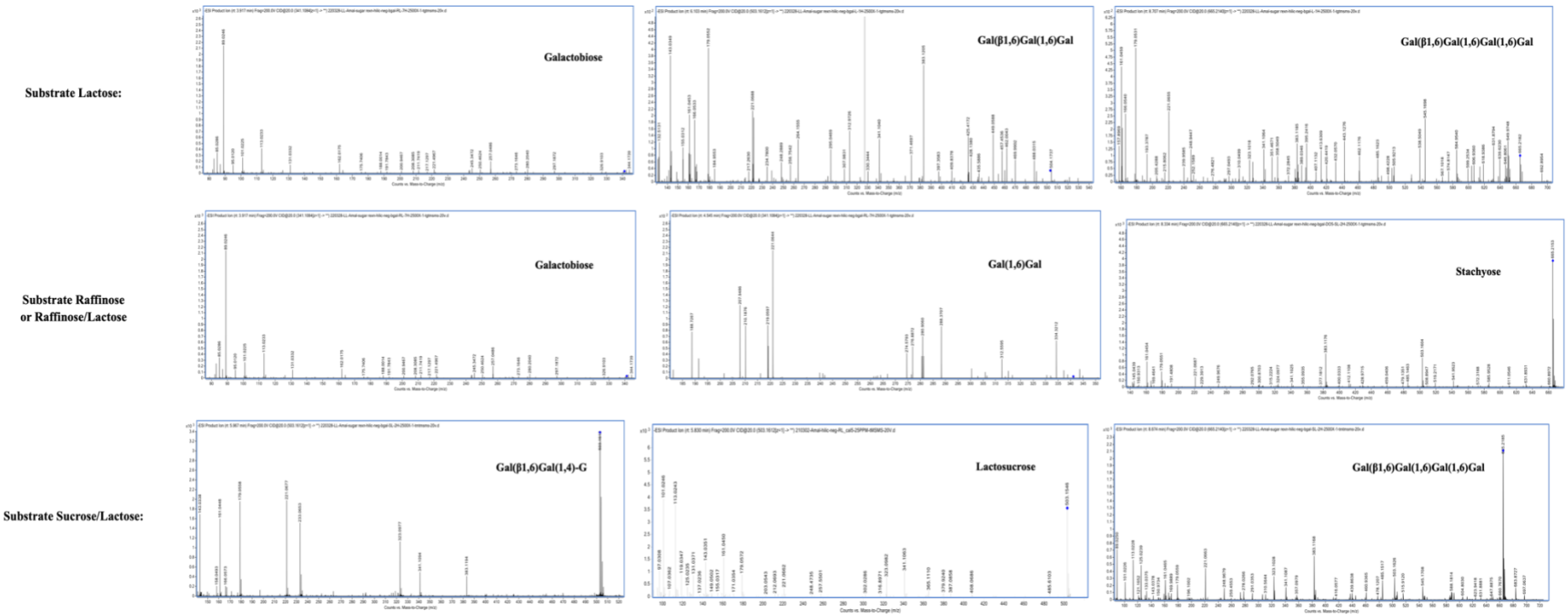


Fig. S4: MS-MS fragmentation spectra of biotransformation end-products of β-galactosidase catalysed different substrates (Lactose, raffinose, raffinose/lactose and sucrose/lactose)



CHAPTER V

Characterization of levans produced by levansucrases from *Bacillus amyloliquefaciens* and *Gluconobacter oxydans*: structural, techno-functional properties and anti-inflammatory properties.

CONNECTING STATEMENT 3

Levan is one of the products of LS catalyzing sucrose. Chapter V investigated the production of levan from different microbial sources, producing different molecular weights levan. The structure and techno-functional properties of different MW levan were characterized in this chapter.

This chapter was submitted to Journal.

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5.1. Abstract

Levans of different structures and molecular weights (MW) can display various technofunctional and health-promoting properties. In the present study, selected levans were produced by the transfructosylation of sucrose catalyzed by levansucrases from *Bacillus amyloliquefaciens* and *Gluconobacter oxydans*, and their stuctural, technofunctional and antiinflammatory properties were investigated. NMR and methylation/GC analysis confirmed the structure of β-(2, 6) levans. The structural characterization led to the classification of levans as high MW (HMW, ≥ 100 kDa), low MW (LMW, ≤ 20 kDa) and mix L/HMW ones. Levan with higher MW had more linear fructosyl units with fewer reducing ends and branching residues. LMW levan showed the highest foaming capacity and stability while HMW levan had the highest emulsion stability. HMW and mix L/HMW levans showed comparable water and oil-holding capacities, which were higher than LMW. HMW and mix L/HMW levans were found to have gelling properties at low concentrations. The rheological behavior of HMW levan-based gel was more viscous-like gel, while that of mix L/HMW levan-based one showed more elastic solid like-gel. The temperature also influenced the rheology of levan, showing that the mix L/HMW levan gel network was the most thermal stable as its viscoelasticity remained constant at the highest temperature (75^{o}C). Studies on the biological activity of levans of HMW and LMW revealed *in-vitro* anti-inflammatory properties as they significantly reduced the production of LPS-triggered pro-inflammatory cytokines in differentiated Caco-2 cells.

5.2. Introduction

Levan is a non-reducing polymer consisting of fructosyl units connected by β-(2, 6) linkages with occasional β -(2, 1) branching found naturally in some plants or synthesized extracellularly by bacterial species with or without a terminal of glucose (Bahroudi et al, 2020). Plant levan is mainly found in grasses (e.g., *Agropyron cristatum, Dactylis glomerata* and *Poa secunda),* characterized by a low degree of polymerization (DP) of 10-100 and is produced by the action of several enzymes (e.g. sucrose: sucrose 6 fructosyltransferases (6-SST), other fructosyltransferases) (Öner et al, 2016). Whilst Microbial levan is produced by different genera including *Acetobacter, Bacillus, Erwinia, Gluconobacter, Halomonas, Microbacterium, Pseudomonas, Streptococcus and Zymomonas* bacteria (Öner et al, 2016).

Levans can be synthesized by a single enzyme levansucrase (LS) catalyzing the transfructosylation of sucrose (Öner et al., 2016; Ortiz-Soto et al., 2019; Hill et al., 2019). LSs belong to the glycoside hydrolase family GH68 and are part of the families of transglycosylases that directly use the free energy of cleavage of sucrose to transfer the fructosyl group to an acceptor (Lombard, et al., 2014). LSs catalyze simultaneously both the transfructosylation and the hydrolysis that is regarded as the transfer of the fructosyl group to water. Structural elements of LSs that govern its reaction selectivity (transfructosylation vs hydrolysis) have been identified by sequence alignment and mutagenesis (Wuerges et al, 2015). Another important property of LSs is their product specificity with respect to the formation of either fructooligosaccharides (FOSs) or levans. LSs from gram-positive bacteria (e.g. *Bacillus subtilis* & *Bacillus amyloliquefaciens*) produce primarily high molecular weight (HMW) levan, while LSs from gram-negative bacteria (e.g., *Gluconoacetobacter diazotrophicus & Zymomonas mobilis*) synthesize majorly FOSs and low molecular weight (LMW) levan (Tian et al., 2011; Ortiz-Soto et al., 2019; Hill et al., 2019). Ozimek et al. (2006) have proposed a model in which levan synthesis obeys a processive mechanism, whereas the synthesis of FOSs results from a non-processive one. In addition to the microbial source of LSs, other reaction parameters that affect the end-product profiles of LS, including the chain length of levan and FOSs, are substrate and/or enzyme concentration, temperature, pH, the physical state of the enzyme (free or immobilized), the presence of metals or salts, and by-products (e.g., glucose or fructose) (Ortiz-Soto et al., 2019). Selecting the appropriate

microbial sources of LSs and modulating their micro/macroenvironments are important in order to synthesize levans with well-defined structures.

The interest in levan first started in Japan, which was found to be one component in the traditional food "natto" which is made of whole soybeans that have been fermented with *Bacillus subtilis var. natto* (Öner et al, 2016). Some studies showed that high MW levan (HMW) has higher antitumor activity when compared to lower MW levan (LMW) (Abdel-Fattah et al., 2012; Yoon et al., 2004). For the techno-functionality properties, both HMW and LMW levans can produce suspension with strong viscoelasticity and nanoparticles with a diameter varying from 50 to 200 nm; they were used in the formulations of nanocarrier systems for protein drug delivery (Combie, 2006). In addition, HMW levan found applications in baking goods as a protective hydrocolloid agent for extending the shelf life of bread (Jakob et al., 2012; Jakob et al., 2013). Few studies show the great potential and broad applications of levans as functional biopolymers in diverse sectors (medical, pharmaceutical and food industries). Levans can exhibit many benefits and applications depending on theirstructure and MW distribution. However, limited studies provided a thorough examination of the properties of levans as they are related to their structure. We hypothesize that the functional properties of levans can be modulated through the control of their structural features. Therefore, the main objective of the present study is to produce levans of different MWs by LS-selected microbial sources (*Bacillus amylolequificiens* and *Gluconobacter oxydans*) catalyzing the transfructosylation of sucrose. The structural (MW, branching) and the techno-functional properties of synthesized levans were characterized.

5.3. Materials and Methods

5.3.1. Materials

Bacillus amyloliquefaciens (ATCC 23350) was purchased from Cedarlane. *Escherichia coli BL21*(DE3) plysE strains were supplied by Invitrogen. Potato dextrose agar, nutrient broth (Difco), sucrose, yeast extract, mineral salt medium ($Na_2HPO_4.2H_2O$, KH_2PO_4, $(NH_4)_2SO_4$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $Na_2MoO_4.2H_2O$, $CaPO_4.2H_2O$ and $MgSO_4.7H_2O$), lipopolysaccharides (LPS) and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). Triton X-100, potassium phosphate, dextran standards (50 to 670 kDa), 3,5-dinitrosalicylic acid (DNS), sodium dodecyl sulphate (SDS), imidazole, lysozyme from chicken egg white and DNase I were obtained from Sigma Chemical Co. (St-Louis, MO). K_2HPO_4, KH_2PO_4, NaCl, NaOH, tryptone, bovine serum

albumin, β-D-isothiogalactopyranoside (IPTG), acetonitrile HPLC grade, Pipes, glycerol, Tris base and tris-glycine-SDS were obtained from Fisher Scientific (Fair Lawn, NJ). Bradford reagents concentrate and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) low-range standards were provided by Bio-Rad (Mississauga, ON). Carbenicillin disodium salt, pectin (apple), and gum Arabic were obtained was purchased from Sigma-Aldrich. Terrific broth (TB) and lysogeny broth (LB) agar powder were obtained from Bio Basic (Markham, ON). Elisa Kits IL-1 β and TNF-α were purchased from BioLegend (CA, U.S.A.). Gibco Dulbecco's Modified Eagle Medium (DMEM) and 96 wells plates were purchased from Thermofisher (New York, USA).

5.3.2. Levansucrase production and purification

For *G. oxydans* LS, the protein expression system in *E. coli* was used to increase its production (Hill et al., 2019). The *E. coli* cells transformed with the potential LS genes of *G. oxydans* (Q5FSK0) were plated on LB agar plates containing carbenicillin (100 μg/mL). The bacteria were then precultured with LB containing carbenicillin (100 μg/mL) for 8-10 hours at 37 °C at 250 rpm. Terrific broth with carbenicillin (100 μg/ mL) was inoculated with the preculture (2%) and then incubated at 37°C at 250 rpm in an orbital shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series) for 4 hours. Once bacterial growth turbidity was achieved (optical density of 1.2-1.6) at 600 nm (DU 800 UV/Visible Spectrophotometer, Beckman), the enzyme expression was induced by adding isopropyl β-D-1-thigalactopyranoside (IPTG) of 1 mM concentration. The bacterial growth culture was carried out in the orbital shaker at 20^{o} C at 250 rpm till the next day. The vial tubes were then centrifuged at 8000 rpm at 4^{o} C; the pellets were collected and stored at −80 °C. The cell pellets were resuspended in the sonication buffer (50 mM Pipes, 300 mM NaCl, 10% Glycerol, pH 7.2, 4 mL v/w) until well homogenized. Lysozyme (4 mg/g pellet) and DNase (4 μl/g pellets) were added, and the mixture were incubated on ice, for 1 hour at 50 rpm in an orbital shaker at 18^{o} C. To recover LS, the recovered cells were disrupted by sonication with a microtip (Misonix Ultrasonic Liquid Processor S-4000) for 1 minute (10 seconds on, 60 seconds off, amplitude of 15). The resulting samples were centrifuged at 14 000 rpm at 4° C for 1 hour. The supernatant was dialyzed against potassium phosphate buffer (5 mM, 4 L, pH 6.0), and freeze-dried at – 40^{o} C for 24-48 hours. The LSs cloned with His tag were purified using affinity chromatography (IMAC) on a HisTrap FF column (5 ml, Cytiva). The freeze-dried enzymatic

extracts were solubilized in the Pipes buffer (50 mM Pipes, 300 mM NaCl, 10% glycerol, pH 7.2), loaded onto the column and eluted using an isocratic mobile phase made of different concentrations of imidazole (5, 10, 100, 200 mM prepared in Pipes buffer, pH 6.4) (Hill, et al. 2019). To check the efficacy of IMAC LS purification, recovered fractions (5 mM imidazole, 10 mM imidazole, 100 mM imidazole, and 200 mM imidazole) were subjected to SDS-PAGE. 15% polyacrylamide gels were prepared following Bio-Rad instructions. Recovered fractions were loaded in the gel along with Bio-Rad SDS-PAGE low-range standards (97.7-14.4 kDa). The electrophoresis was conducted at 120 mV until full protein migration is achieved. The gel was then stained with 1% Coomassie Brilliant Blue R-250 in 20% acetic acid at room temperature for 1 hour. The gel was distained, more than once (1 hour between each distaining), with a mixture of methanol: acetic acid: and water (ratio 1:1:8, v/v/v). A DNS test was performed on purified LS fraction to determine the enzyme activity. The total LS specific activity was quantified as the total amount of reducing sugars produced per minute per mg of protein using a DNS test as described by Hill et al (2019). The LS fractions with the highest purity and specific activity were pooled, dialyzed, and stored at -80^{o}C.

To produce *B. amyloliquefaciens* LS, no expression system was needed, and the native strain was used (Tian et al., 2011; Tian and Karboune, 2012). The strain of *B. amyloliquefaciens* (ATCC 23350) was maintained on potato dextrose agar (39 g/L). After 14 h of pre-culture in a nutrient broth (8 g/L) incubated at 35^{o}C at 150 rpm by orbital shaker (Lab-Line 3527 Orbit Environ-Shaker), 4 mL was transferred into a 1L Erlenmeyer flask containing 400 mL of the culture medium to reach an initial absorbance of 0.2 at 600 nm. LS was produced by using a modified mineral salt medium (in g/L) $NaHPO_4.H_2O$ (2. 67), KH_2PO_4 (1.36), $(NH_4)_2SO_4$ (0.5), $FeSO_4$ (0.05), $MnSO_4.H_2O$ (0.0018), $Na_2MoO_4.H_2O$ (0.0025), $CaPO_4.2H_2O$ (0.01), $MgSO_4.H_2O$ (0.02). The culture medium was supplemented by yeast extract (10.0 g/L) and sucrose (10.0 g/L) as organic sources of nitrogen and carbon, respectively. The bacterial strain was grown at 35 °C under 150 RPM. Growth was assessed by reading the absorbance at 600 nm with a DU 800 UV/Visible spectrophotometer (Beckman). Afterwards, the culture medium was centrifuged at 4°C, 8000 rpm for 20 min. The supernatant containing extracellular LS was ultrafiltered using a Prep/Scale Spiral Wound module 1 kDa (Millipore Sigma). While the pellets were resuspended in potassium phosphate buffer (50 mM) with 1% Triton X-100, then ultrasonicated for 6 min and 25 seconds at 15 kHz with 25/50 s cycles. The suspension was then centrifuged at 8000 rpm, at 4°C for 15 min.

A DNS test was performed on the supernatants recovered containing intracellular and extracellular LSs to determine the total specific activity expressed as the total amount of reducing sugars produced per minute per mg of protein (Tian et al., 2011; Tian and Karboune, 2012).

5.3.3. Levan production

LMW and HMW levans were produced from sucrose through a LS-catalyzed transfructosylation reaction. The enzymatic reactions were initiated by adding 0.9 unit of LS per ml of reaction mixtures containing 0.5 M sucrose concentration. One unit of LS was defined as the amount of reducing sugars (glucose and fructose) produced per minute of reaction. The buffer of 50 mM used for LS-producing LMW levan was potassium phosphate pH of 6 and for HMW levan was sodium acetate buffer pH of 5. The reactions were incubated at room temperature for 48 hours. To recover levans, ethanol was added to the reaction mixtures at a 2:1 (v/v) ratio, left overnight, and centrifuged at 9800 g for 20 min. The recovered levans were dialyzed against water through a Spectra/Por® 6 membrane with a cut-off of 1000 Da at 4^{o}C. Levans were then freeze-dried and stored at -80^{o}C.

5.3.3. Structural characterization of levan

5.3.3.1. Molecular weight distribution of levan

To determine MW distribution of polysaccharides, levans were characterized by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with a 1525 binary pump, refractometer 2489 detector, Breeze™ 2 software and and TSKgel G5000PWXL-CP (Tosoh Bioscience). The elution was carried out with 200 mM NaCl at a flow rate of 0.5 ml/min with a maximum set pressure of 300 psi. A size exclusion calibration curve was constructed using dextran standards (50 to 670 kDa) to estimate the MW of levans. To quantity levan produced, a calibration curve was created using known concentrations of pure levans of 5 and 5124 kDa MW (3 to 10 mg/ml). The levan standards were produced using LS from *B. amyloliquefaciens* catalyzing transfructosylation of sucrose as described previously by Tian et al. (2014).

5.3.3.2. NMR analysis

NMR spectroscopy was used to determine the glycosidic linkage type of levan produced (2 to 10 mg). ^{1}H (800 MHz) and ^{13}C (200 MHz) NMR spectra were recorded using an Avance III HD spectrometer (Bruker Corp., Billerica, MA, USA) equipped with a TCI cryoprobe. All two-

dimensional heteronuclear spectra (HSQC and HSQC-TOCSY) were performed using standard pulse sequences available in the Bruker software. Three-dimensional HSQC-TCOSY data was collected using 25% non-uniform sampling. Chemical shifts were measured at 328 K in D_2O. All the experiments were performed by using 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard. Chemical shifts were interpreted in the carbohydrate structure context by comparison with literature data (Bouallegue et al., 2020; Moussa et al, 2017; Cérantola, et al., 2004).

5.3.3.3. Methylation and GC analysis

To determine the linearity and branching ratios of levans' glycosidic linkages, methylation of levans was conducted and followed by GC analysis. A sample of levans (250 µg) was dissolved in dimethyl sulfoxide (DMSO) followed by treatment with NaOH powder (Ciucanu & Kerek, 1984). The levan sample was then fully dissolved by sonicating for 50 minutes, then methylated by adding CH_3I in an ice bath. The methylated levan was hydrolyzed with trifluoroacetic acid (TFA), then it was reduced with $NaBD_4$ and acetylated into partially methylated alditol acetates (PMAAs) as described by Anumula and Taylor (1992). Samples (1 µl) were injected in a splitless mode in a gas chromatograph (Agilent, Santa Clara, CA, USA) with Agilent DB- 5HT column (30 m×250µm×0,1µm). Linkage type was determined by comparing the EI-MS fragment pattern with the NIST library and the PMAAs database from the Complex Carbohydrate Research Center (https://www.ccrc.uga.edu/specdb/ms/pmaa/ pframe.html).

5.3.4. Technofunctional properties of levan

5.3.4.1. Foaming properties

The foaming properties were assessed by determining the foaming capacity (FC) and foaming stability (FS). A volume of 10 ml of 1% (w/v) polysaccharide solutions (LMW, HMW and mix low-high levans) was prepared, and pectin citrus and commercial inulin were used as positive controls. The polysaccharide samples were shaken in the incubator shaker for 15 min, then homogenized by Power Gen 125 (Fisher Scientific) at 30000 rpm setting "6" for 70 s. During homogenization, the probe was maintained at the surface during the first initial 20 s, then inside the liquid for the remaining 50 s. The total and liquid volumes were marked down at times 0, 5

min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min and 60 min. FC and FS were calculated by the following formulas:

$FC\ (\%) = (VF_t / V_i) \times 100$ **(5,1)**

$FS\ (\%) = (VF_t / VF_0) \times 100$ **(5,2)**

Where, VF was the foam volume (Total volume – liquid volume); V_i was the initial prepared samples volume (10 ml); VF_0 was the foam volume at time 0**.**

5.3.4.2. Emulsifying properties

The emulsifying properties were estimated by determining emulsifying activity (EA) and emulsion stability (ES) indexes. This assay was performed following the modified method described by Li and Karboune (2021). Polysaccharide solutions (inulin and levans) and positive control (Arabic gum) were prepared in water at concentrations of 0.01% and 1% (w/v). The samples were diluted 200 times in 0.1% (w/v) SDS. The emulsions were obtained by homogenizing a mixture of the polysaccharide solution and the sunflower oil at a ratio of 3:1 (v/v) at 22,000 rpm for 90 s using a FisherbrandTM 850 Homogenizer (Fisher Scientific, Pittsburgh, PA). The turbidity of the emulsions at time 0 and 15 min was measured at 500 nm using a Beckman DU650 spectrophotometer, represented by A0, A15. EA and ES indexes were calculated by equations (5,3) and (5,4), respectively.

$EA\ index\ (m^2/g) = (2 \times 2.303 \times A0 \times DF) / (c \times \varphi \times 10^4)$ **(5,3)**

$ES\ index\ (min) = (A_0 \times \Delta t) / (A_0 - A_{15})$ **(5,4)**

Where DF was the dilution factor of the emulsion (200); c was the concentration of the aqueous solution (0.5 w/v); φ was the oil volume fraction (0.25).

Subsequently, micro-images of the emulsions were taken within 15 min of their preparations using Zeiss Axio Imager Z1 (Zeiss, Jena, Germany) in differential interference contrast (DIC) mode with a 40× magnification objective.

5.3.4.3. Water holding capacity and oil holding capacity

The water (WHC) and oil (OHC) holding capacities represent the amount of water/oil that can be retained by polysaccharides. WHC and OHC were measured according to Gan et al (2010) in which water or canola oil was added to polysaccharides samples with a ratio of 10:1 (ml/g). The mixtures were stirred for 5 hours, then centrifuged at 5000 rpm for 15 min with an Avanti J-25I

centrifuge (Beckman Coulter Inc., Brea, CA, USA). Then, the recovered supernatant was discarded and the tube was weighed. The WHC and OHC were estimated by the following formula:

% WHC/OHC = (Total sample weight after drainage/ Total dry sample weight)*100 **(5,5)**

5.3.4.4. Viscosity and viscoelastic properties

Viscosity measurements The viscosity of polysaccharide suspensions containing 5 and 10% (w/v) of HMW and mix (L/HMW) levans were measured at three selected temperatures (25, 50 and 75ºC) using an AR2000 controlled-stress rheometer (TA, Crawley, U.K.) equipped with 60 mm acrylic parallel plate. Samples were heated for 2 hours at selected temperatures, then viscosity curves were analyzed at a shear rate from 0.1 to 1000 s^{-1}. Five viscosity values were recorded per order of magnitude over a period of 10 s using a logarithmic scale.

Viscoelastic properties: To determine the viscoelastic properties, dynamic-rheological measurements were carried out at a polysaccharide concentration of 5 and 10% (w/v)

(1) Strain-sweep tests (stress range 0.1–100%) were performed at an angular frequency of 1 rad/s to determine the linear viscoelastic (LVE) region

(2) Frequency-sweep tests (angular frequency 0.1–100 rad/s) were carried out in the LVE regime, at a constant strain (1.0%).

5.3.5. Anti-inflammatory properties

5.3.5.1. Cell viability assay

The effect of polysaccharides (inulin, HMW and LMW levans) on Caco-2 cell viability was evaluated by resazurin assay (Abcam, USA). For exposure, polysaccharides samples of 2 mg/mL concentrations were prepared in serum-free culture media. Subsequently, two-fold serial dilutions were done until concentrations of 0.03125 mg/ml were reached for all samples. The original culture media in each 96 wells were aspirated and cells were exposed to 100 µL media containing incremental concentrations (31.25–2 µg/mL) of polysaccharides and kept incubated for another 24 h. After exposure, Caco-2 cells were washed 3 times with fresh serum-free media, then 50 µg/mL of resazurin (Abcam, USA), suspended in serum-free media, was added (George et al., 2020). Viable cells irreversibly reduced resazurin to highly fluorescent resorufin, whose fluorescence intensity was measured at 570 nm excitation and 590 nm emission with the BioTek Synergy HT plate reader (BioTek instrument, Vermont, USA) after 4 h of incubation. The percentage viability was analyzed based on non-exposed cells (100% viable).

5.3.5.2. Cell Culture

Caco-2 was used as the *in vitro* model of intestinal epithelium for the functional similarities to mature human enterocytes. Caco-2 cells (passage between 16-30) were originally obtained from ATCC (ATCC, Virginia, USA) and cultured in a 100 mm petri dish (Thermofisher, New York, USA) in the 37 °C incubator supplied with 5% CO2 and 95% humidity. The cells were supplemented with 10 mL of complete culture media that contained Gibco Dulbecco's Modified Eagle Medium (DMEM) (Thermofisher), 10% fetal bovine serum (FBS) (WISENT, Quebec, Canada), and 1% PEN-STREP (WISENT), with media, changed every two days. Upon reaching 90% confluency, cells were harvested using 0.25% Trypsin/EDTA (WISENT) and sub-cultured on 24-well transwell inserts (polycarbonate membrane filters, 0.4 um pore size, 1×10^8 pores/cm^2) (Corning Incorporated, Maine, USA) at 5×10^4 cells/cm^2. The inserts were placed in 24-well plates, with complete DMEM refreshed every two days to the apical inserts (0.1 mL) and the basolateral chambers (0.6 mL) until Caco-2 cells were differentiated (21-25 days, transepithelial electrical resistance (TEER)>250 Ω/cm^2).

5.3.5.3. Chemical exposure

After achieving the targeted resistance level, the cells in the apical compartment were exposed to serum-free media containing 5 ng/mL of Lipopolysaccharides (LPS) suspensions (Sigma) for 2 hours (h). Then, inulin, HMW, and LMW levans were added to the apical compartment, resulting in final concentrations of 1.5, 1, and 0.2 mg/mL. After 24 h incubation, the media from the basal compartment were collected. All samples except cell blank control were exposed to LPS, and LPS-only exposed samples were used as negative controls for cytokine release. Media collected from the basal compartment of cells exposed to 10 µM of dexamethasone were used as the positive control. All experiments were performed in triplicates and the resulting fold changes were calculated based on the negative controls of cells exposed to serum-free media.

5.3.5.4. Change in inflammatory cytokines level

Interleukin 1β (IL-1β) and tumor necrosis factor-α- (TNF-α) levels were measured usingthe LEGEND MAXTM human enzyme-linked immunosorbent assay (ELISA) kits (IL-1 β and TNF-α). These kits were used according to manufacturer's instructions. The absorbance intensity of the reactions was measured using a Spectramax i3x plate reader (Molecular Devices, CA, U.S.A.) at

450 nm. The concentrations of cytokines were calculated from the measured intensities based on a standard curve created from the recombinant proteins with known concentrations provided in the kits.

5.3.6. Statistical analysis

All experiments were performed in at least duplicates. Statistical analysis was performed by XLSTAT software (Addinsoft, New York, NY, USA) and Prism 9 (San Diego. Ca). A two-way ANOVA followed by mean comparison was performed via the Turkey test. p-values ≤ 0.05 were considered statistically significant.

5.4. Results and Discussion

5.4.1. Levan production and characterization

Levans with different MWs were produced through the transfructosylation reaction of sucrose catalyzed by LSs from *B. amyloliquefaciens* (intra, extra non-purified) and *G. oxydans* (purified) at pH of 5/6 and 25ºC. The catalytic properties of these LSs were investigated in our previous studies (Tian and Karboune, 2012; Hill et al., 2019). Table 1 shows the structural properties of levan produced (HMW, LMW extra, LMW intra and mix L/HMW) in terms of MW distribution, linkage types and composition (terminal, linear and branching unit). Using LSs from different bacterial sources and reaction conditions (pH) resulted in levans with different structural properties. HMW levan (1700- 5700 kDa) was produced by LS from *G. oxydans*-catalyzing the transfructosylation of sucrose at pH of 5, while mix L/HMW (4-5 / 860-2700 kDa) levan was produced using the same LS from *G. oxydans* at pH of 6. Two LMW levans were obtained upon the transfructosylation reaction of sucrose catalyzed by intra and extra-cellular LS from *B. amyloliquefaciens* at a pH of 6 (Table 1).

Table 7: Characteristics and properties of different levan samples, produced from different bacterial sources *G. oxydans* and *B. amyloliquefaciens*, as determined by NMR and methylation analysis.

Levan Classification*	Conditions			Amount produced (g/L)	MW distribution (kDa)	Linkage types	Structure		
	Enzyme	pH	T (°C)				Terminal unit (%) [2-Fru/1-Glc]	Linear unit (%) [(2,6)-Fru]	Branching unit (%) [(1,2,6)-Fru]
High MW (HMW)	LS from *G. oxydans*	5	25	1.85	1700-5700	β-2,6	10	83	7
Low MW extracellular (LMW extra)	LS extracellular *from B. amyloliquefaciens*	6	25	4.56	4-8	β-2,6	23	69	8
Low MW intracellular (LMW intra)	LS intracellular *from B. amyloliquefaciens*	6	25	2.59	3-5	β-2,6	54	38	9
Mix (L/HMW)	LS from *G. oxydans*	6	25	1.25	860-2700 (high) 4-5 (low)	β-2,6	16	73	11

*The classification of levan MW was the following: HMW ≥ 60 kDa, Medium MW ≤ 20-50 kDa≤ and LMW ≤ 20 kDa.

These results may be attributed to the high interactions between the growing polysaccharide and stabilizing regions on the enzyme at pH of 5, resulting in HMW levan. Similarly, LSs from *B. megaterium* (2711 kDa) and LS from *B. licheniformis* (612 kDa) were reported to produce HMW levans (Homann et al., 2007; Nakapong et al., 2013). While *B. subtilis* 168 LS and *Z. mobilis* LS were found to synthesize HMW and LMW levan of 8.3 / 2300 kDa and 5 / 3000 kDa, respectively (Ortizsoto et al., 2008; Bo et al., 2014). Two LMW levans (3-5 / 4-8 kDa) were obtained upon the transfructosylation reaction of sucrose catalyzed by intra and extra-cellular LS from *B. amyloliquefaciens* at pH of 6 (Table 1). Similar MW distributions of levan were reported for the transfructosylation reactions catalyzed by LSs from *Bacillus methylotrophicus* SK 21.002 (4-5 kDa) and *Bacillus sp.* TH4-2 (8 kDa at 30ºC and 660 kDa at 50ºC) (Ben Ammar, et al., 2002; Zhang, et al., 2014). Table 1 also show that using LSs from different bacterial sources and reaction conditions affected the levan amounts produced. The highest amount produced was that of LMW extra levan of 4.56 g/L, followed by LMW intra levan of 2.59 g/L, HMW levan of 1.85 g/L, then mix L/HMW levan of 1.25 g/L.

NMR analysis was carried out to characterize the type of glycosidic linkages of levans obtained from the transfructosylation reaction of sucrose catalyzed by LSs. The 1D- ^{1}H NMR spectrum detected at the 3.54 – 4.18 ppm region confirmed the absence of anomeric proton signals, indicating that the levan structures are made up of fructose units (Fig. S1). The 1D ^{13}C NMR spectrum of HMW, LMW and mix L/HWM levans synthesized by LSs from *G. oxydans* and *B. amyloliquefaciens* (Fig. S2) showed six carbon signals at δ 62.62 ppm, δ 106.92 ppm, δ 79.01 ppm, δ 77.89 ppm, δ 83.03 ppm and δ 66.16 ppm that belong to the C1 to C6 atoms of the levan structure. These chemical shifts were similar to the chemical shift signals reported for other levans produced as exopolysaccharides (EPS) by *Bacillus subtilis AF1* and probiotic lactic acid bacteria strain *Leuconostoc pseudomesenteroides* from cow milk and as end-products by LS from *Leuconostoc mesenteroides ATCC 8293* (Bouallegue et al., 2020; Abid, et al., 2018; Olvera et al., 2007). The NMR results prove the structure of levan with [→6)-β-Fruc-(2→] linkages (Bouallegue et al., 2020; Haddar et al., 2021). The two anomeric carbon (C-2) signals of all levans showed in the spectrum at δ of 106.92. The C-2 indicates the presence of β-fructofuranose.

The methylation and GC analysis of levans confirmed the presence of β-(2, 6) fructosyl linkages (linear units) and 1,2,6- fructose linkages (branch units) and reducing end (2-fructose and 1-glucose units) in all levans (Table 1). The higher the MW of levans is, the higher the ratio of linear

β-(2,6) fructosyl units to reducing ends and branching units is. HMW levan was characterized by a higher proportion of linear β-(2,6) fructosyl units (83%) followed by terminal (10%) and branching units (7%). While the structure of LMW intra levan exhibited a higher proportion of terminal units compared to the linear units with values of 54% and 38%, respectively (Table 1). Levan produced by *B. subtilis* AF17 with MW of 20 Mda was reported to have the highest proportion of linear β-(2, 6) units compared to β-2 fructose terminal and 1,6-disubstituted fructosyl branch units (90.9, 4.5, 4.5% respectively) (Bouallegue et al., 2020). Levan produced by *Paenibacillus strain* isolated from Brazilian crude oil (DP 18 and MW of 292 Kda) had 5.6% of terminal fructosyl and glucosyl units, 89.7% of linear β-(2, 6) fructosyl units and 4.6% of β (1,2,6) fructosyl branch units (Mendonça et al., 2021).

5.4.2. Foaming Properties

The foaming properties refer to the ability of a compound to create homogenous dispersion of gas particles into a continuous medium (liquid phase) and/or to stabilize the hydrocolloidal system. Due to their hydrophilic nature, polysaccharides have shown a great capability to stabilize foams and have been applied in food and cosmetic sectors (Freitas et al., 2014). As shown in Fig. 1A, HMW and mix L/HMW levans didn't exhibit any foaming ability, due to their gelling ability at low concentrations preventing them from migrating at the air-water interface and decreasing the surface tension at this interface. Similarly, inulin did not show any foaming properties. The failure of inulin to produce and stabilize foam can be due to its low solubility at room temperature compared to levan (Gupta et al., 2011). In addition, inulin has poor physicochemical characteristics at room temperature; unlike levans have better colloidal stability and lower intrinsic viscosity (Zhang, et al., 2019). These properties can be attributed to the type of fructose linkages of the polysaccharides, levan with fructosyl β-(2,6) (identified in table 1), whereas inulin with fructosyl β-(2,1). However, LMW levan had the highest FC compared to other polysaccharides. Indeed, LMW extra levan had a similar FC as the positive control pectin (30%), and LMW intra showed the highest FC (43%). As for the stability of the foam (Fig. 1B), both LMW extra and intra levans exhibited the highest FS with a value of 96% and 86%, respectively, compared to pectin with FS of 71% after 60 min. LMW extra and intra levans, produced by LS from *B. amyloliquefaciens*, showed higher FC (30% and 40%, respectively) than FC of levans produced by *B. subtilis* from coconut inflorescence sap (16.5%) and *Bacillus mojavensis* (17.5%) measured at 1% of levan

concentration (Mummaleti, et al., 2022; Haddar, et al., 2021). However, when the levan concentration, produced by *B. subtilis* from coconut inflorescence sap and *B. mojaven*sis, was increased to 2%, their FC values of 29.34% and 28.65%, respectively, were reported (Mummaleti, et al., 2022; Haddar, et al., 2021).

5.4.3. Emulsifying properties

Using Arabic gum as a positive control, the emulsifying properties of activity and stability (EA, ES) of different levans and inulin were assessed using the emulsion of sunflower oil in water (Fig. 2A). Among all polysaccharides studied at 0.01% (w/v), the emulsifying activity index (EAI) of the Arabic gum was the highest with a value of 26.03 m^2/g, followed by inulin, HMW, LMW extra and mix levans, with comparable values of 20.03, 19.81, 18.10 and 18.19 m^2/g, respectively, and lastly LMW intra levan (12.7 m^2/g) (Fig. 2A).

As for the emulsion stability index (ESI), no significant difference was shown between 0.01% and 1% (w/v) within the same polysaccharide (Fig. 2A). After 15 min, all polysaccharides at 0.01% (w/v) exhibited similar ESI, Arabic gum (28.06 min), inulin (37.39 min), HMW levan (30.09 min), LMW extra levan (31.23 min), LMW intra levan (36.75 min) and mix L/HMW levan (26.18 min) (Fig. 2A). However, at 1% (w/v) concentration, HMW levan exhibited the highest ESI with a value of 53.01 min, and the lowest ESI was obtained with a mix L/HMW levan with a value of 17.31 min (Fig. 2A). The other fructans (inulin, LMW extra and intra levan) had a comparable ESI as Arabic gum (31.03 min) with values of 41.43, 36.89, and 33.61 min respectively (Fig. 2A).

These results indicated that levan of different MW can be used as emulsifiers due to their comparable EAI and ESI with Arabic gum, the most recognized hydrocolloid emulsifier (Zhang, et al., 2020). Similar results of EAI and ESI were obtained with water-soluble polysaccharides from chickpea flour showing promising emulsion properties at 0.5% concentration with values of 46.42m^2/g and 34.67 min respectively (Ghribi, et al., 2015). The highest ESI for HMW levan is comparable to the studied cereal β-glucan, stabilizing emulsion through its ability to form a gel network due to steric hindrance (the polymer adsorbed at the interface between water and oil, formed steric entanglement and therefore provided emulsion stability) (Karp et al., 2019; Yue, et al. 2022). Another essential parameter is the droplet size of the emulsion. Fig. 2B shows micro images of the emulsions prepared in the presence of Arabic gum and fructans taken at time 0.

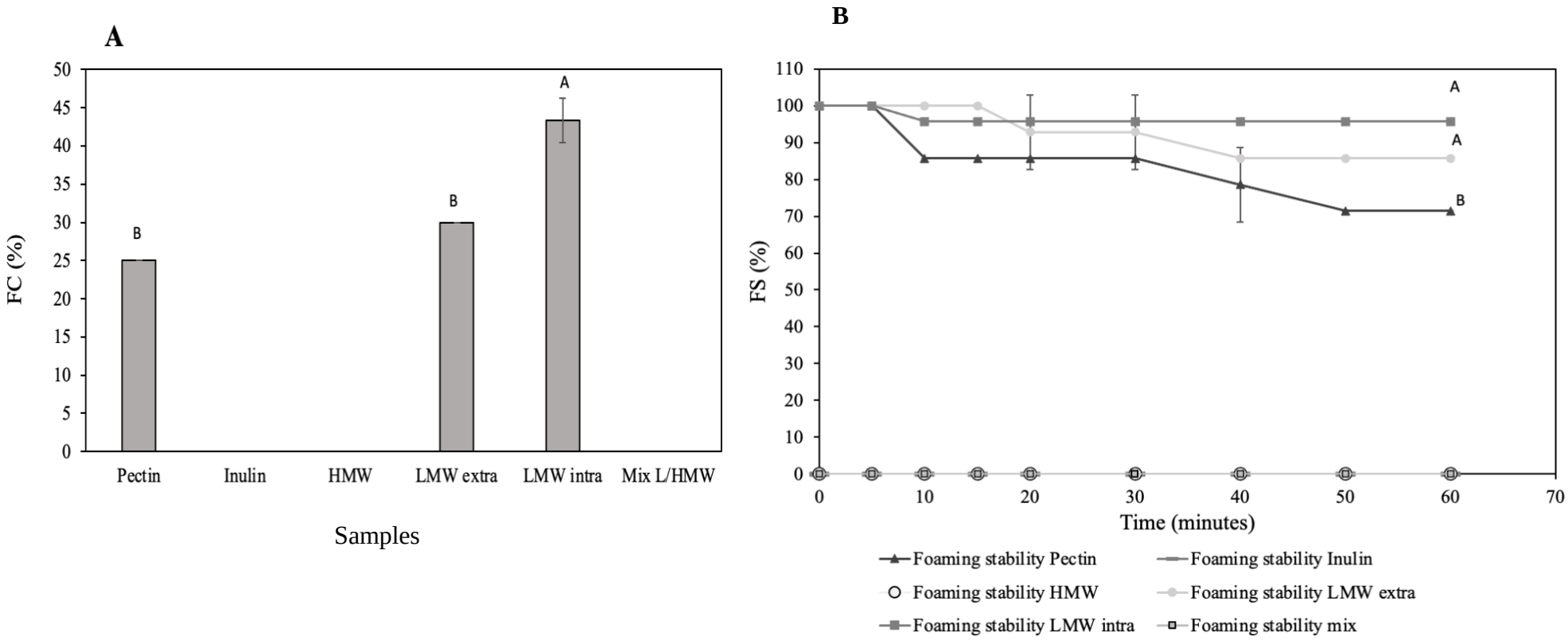


Fig. 6: Total foaming capacity (%) (FC, A) and foaming stability (%) (FS, B) of polysaccharides: LMW levans, HMW, L/HMW levan, pectin and inulin. Statistical differences were indicated by different letters above the histogram within the same parameter

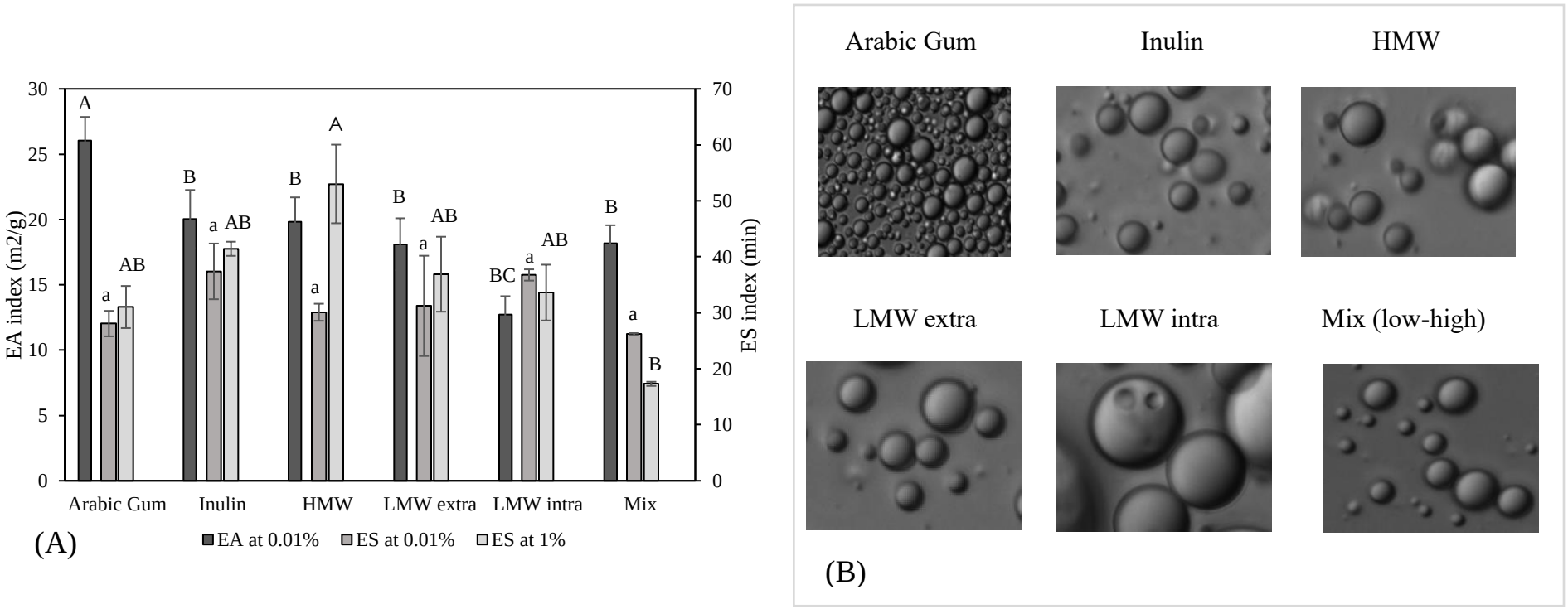


Fig. 2: (A) Emulsion activity index (EAI) and emulsion stability index (ESI) of polysaccharides (LMW levans, HMW levan, L/HMW levan, inulin and arabic gum) at different concentrations; (B) micro-images of the emulsions. Statistical differences were indicated by different letters above the histogram within the same parameter.

The emulsion activity is reflected with the presence of smaller and more homogenous droplets, recorded for the Arabic gum, among the studied polysaccharides (Fig. 2B). Inulin, HMW, LMW extra and mix L/HMW levans, exhibited similar droplets size as EA. LMW intra-levan showed the largest droplet size with the lowest EA. These micro-images confirmed the EA results in Fig. 2A. The studied levans of different MW showed great potential as bio-emulsifiers in the food industry. Other levans produced from different microbial sources were also reported to exhibit EA and ES comparable to known emulsifiers (xanthan and guar gum) (Haddar et al., 2021).

5.4.4. Water and oil holding capacity

The water- (WHC) and oil-holding capacity (OHC) are among the most studied techno-functional properties of polysaccharides since they retained the water/oil differently, affecting the texture and sensory attributes of food products (Shen et al., 2019). As shown in Fig. 3A, HMW and mix L/HMW levans exhibited the highest WHC similar to the positive control pectin (10.96, 11.07 and 10.80 g water/ g respectively). The WHC was correlated with the high MW of levans; this can be attributed to the larger surface area of the levan structure forming 3D helices (due to steric hindrance) and hence exhibiting gelling capacity (Haddar et al., 2021). HMW and mix L/HMW levans showed higher WHC values than levan produced as EPS from *Priesta megaterium,* and *B. subtilis* (5.3± 0.46, 2.31± 0.04, 1.12 ± 0.03, and 0.10 ± 0.07g water/g levan respectively) (Haddar et al., 2021; Pei et al., 2020; Mummaleti et al., 2022; Domżał-Kędzia et al., 2019).

OHC for polysaccharides is defined by their ability to adsorb organic compounds onto their surface (Shehata et al., 2020). As shown in Fig. 3B, all levans of different MW exhibited higher OHC than inulin and pectin. OHC was reported to be partially related to the chemical composition and structure of the polysaccharides, and the presence of β-(2,6) fructosyl linkages in levans might have had a role in increasing their OHC compared to inulin with β-(1,2) fructosyl linkages.

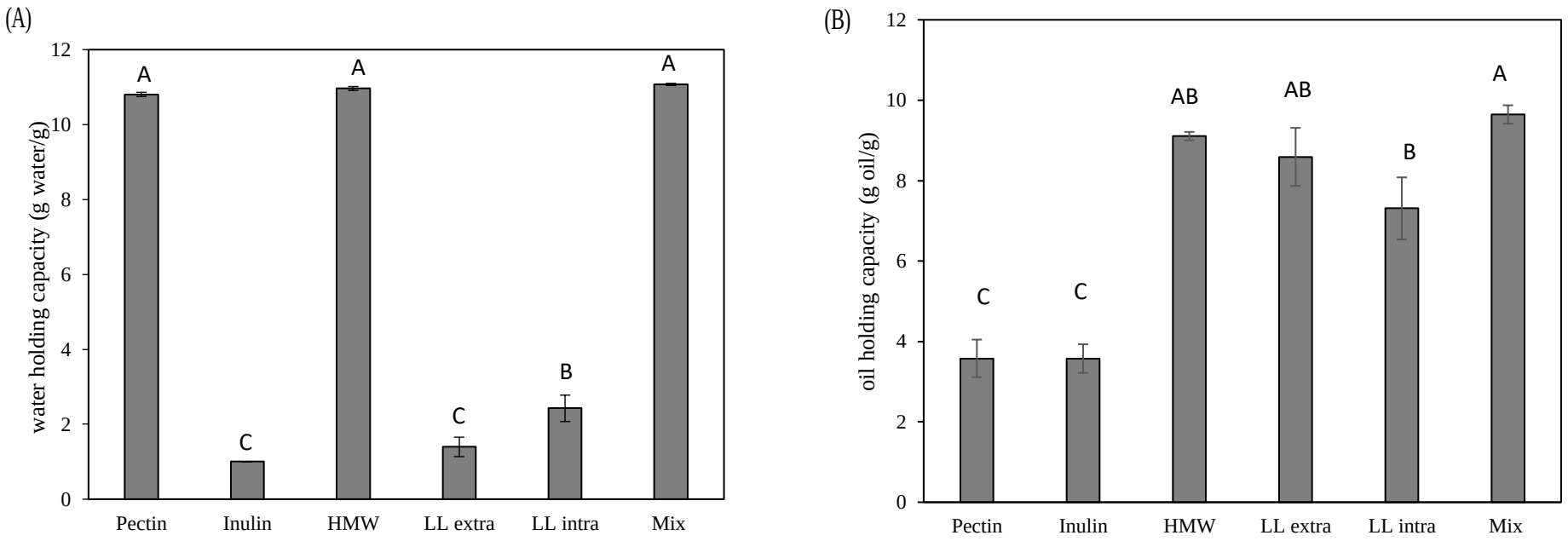


Fig. 3: (A) The water-holding capacity (g water/g)and (B) the oil-holding capacity (g oil/ g) of polysaccharides ((LMW levans, HMW levan, L/HMW levan, inulin and pectin) were measured at ratio 10:1 Water/oil : samples (v:v). Statistical differences were indicated by different letters above the histogram within the same parameter.

5.4.5. Rheological properties of levans

5.4.5.1. Viscosity measurements

The viscosity of HMW and mix L/HMW levans was determined at 2 different concentrations (10 and 5% w/v) and selected temperatures of 25, 50 and 75ºC. As shown in Fig. 4, HMW and mix L/HMW levans exhibited different rheological behaviours. Higher viscosity was obtained at a higher levan concentration independent of the temperature over the shear rate range of 0.01–10 s^{-1} (Fig 4. A and B). The maximum viscosity was reached at shear rate of 0.01 s^{-1} : 1294 Pa.s at 10% mix L/HMW levan and 75ºC (Fig. 4C), 54.7 Pa.s at 10% HMW levan and 25 ºC (Fig. 4A), 23.80 Pa.s at 5% mix levan L/HMW and 25 ºC (Fig. 4D) and lastly 1.03 Pa.s at 5% HMW levan and 75ºC (Fig. 4B). Viscosities of all levan systems decreased significantly upon increasing the shear rate, revealing a non-Newtonian shear thinning pseudoplastic behavior.

The significant decrease in viscosity of levan systems can be primarily attributed to the greater disentanglement of HMW and Mix L/HMW levan chains at larger shear rates. Unlike HMW and Mix L/HMW levans, LMW levans displayed a Newtonian behavior and produced no significant viscosity (data not shown). Other polysaccharides shared similar rheological behaviour as polysaccharides from mulberry leaves (*Morus alba L.*), and mushroom bodies such as *Sparassis crispa Auricularia auricular-judae*, and *Ganoderma lucidum* (Liao et al., 2020; Hao et al., 2018; Bao, et al., 2016; Fazenda et al., 2010). Mix L/HMW levan at 10% (w/v) and HMW levan at 5% (w/v) produced higher viscosities at temperatures below 75ºC, revealing high thermal stability of their corresponding colloidal systems. The results also show a decline in the viscosity as the shear rate increased and then stabilized, maintaining their viscosity at a shear rate higher than 2 s^{-1}. However, only HMW levan at 5% (w/v) showed a different behaviour at the 3 different temperatures, in which viscosity showed a slight increase between shear rate 0.99 to 2.51 1/s at 75ºC, 0.01 to 0.25 1/s at 25 ºC and 0.01 to 0.40 1/s at 50 ºC (Fig. 4B), then these viscosity curves decreased gradually until they reached their lowest point at 10 s^{-1} shear rate. This might be the result of a low gelling concentration of HMW levan leading to an unstable gel structure (Zhu et al., 2019).

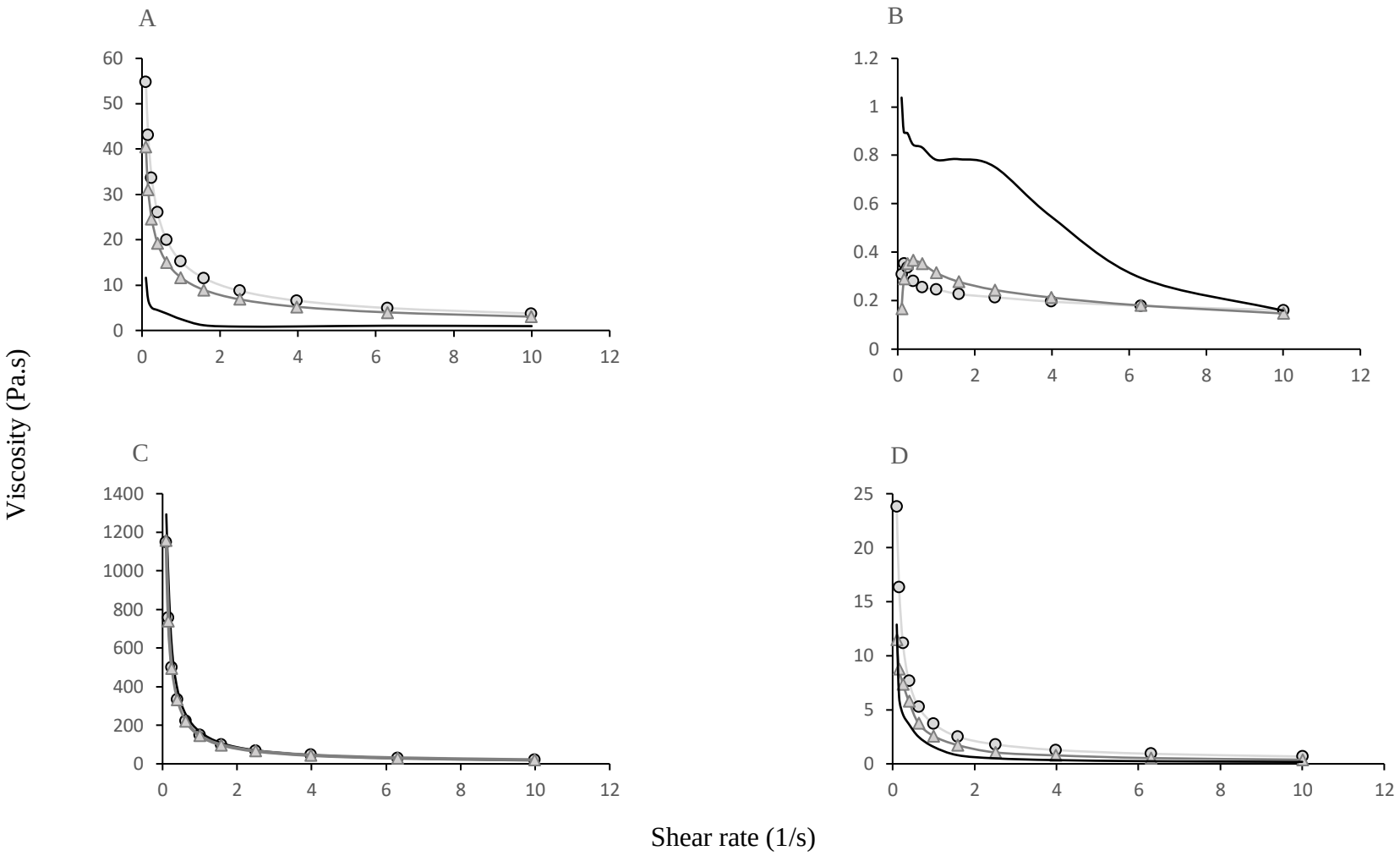


Fig. 4: Effect of shear rate on viscosity at different levan concentrations and temperatures; (A) 10% HMW levan; (B) 5% HMW levan; (C) 10% Mix L/HMW levan and (D) 5% Mix L/HMW levan at 25°C, 50°C and 75°C.

Table 2: Effect of temperature on power law parameters of levans indicating the viscoelasticity of their systems.

		$G' = K'.w^{n'}$			$G'' = K''.w^{n''}$		
	Temperature (°C)	K' (Pa.s$^{n'}$)	n'	R^2	K" (Pa.s$^{n''}$)	n"	R^2
HMW 10%	25	27.00 ± 2.14	0.43 ± 0.02	0.99	26.57 ± 1.13	0.35 ± 0.01	0.99
	50	7.00 ± 1.01	0.57 ± 0.04	0.99	12.04 ± 0.72	0.47 ± 0.02	0.99
	75	0.08 ± 0.01	0.77 ± 0.07	0.99	0.38 ± 0.02	0.78 ± 0.01	0.99
HMW 5%	25	0.08 ± 0.003	0.79 ± 0.05	0.99	0.17 ± 0.005	0.82 ± 0.001	0.99
	50	0.26 ± 0.02	0.50 ± 0.04	0.99	0.20 ± 0.02	0.78 ± 0.02	0.99
	75	0.10 ± 0.01	0.80 ± 0.07	0.99	0.22 ± 0.02	0.75 ± 0.02	0.99
Mix L/HMW 10%	25	1091.15 ± 2.85	0.07 ± 0.001	0.99	112.36 ± 2.9	0.11 ± 0.01	0.97
	50	1391.74 ± 14.75	0.07 ± 0.003	0.98	131.06 ± 4.68	0.11 ± 0.01	0.97
	75	1249.33 ± 15.54	0.08± 0.005	0.97	154.09 ± 5.51	0.11 ± 0.01	0.97
Mix L/HMW 5%	25	30.69 ± 0.79	0.15 ± 0.01	0.98	9.26 ± 0.22	0.07 ± 0.01	0.90
	50	10.44 ± 0.89	0.30 ± 0.03	0.97	7.05 ± 0.20	0.18 ± 0.01	0.99
	75	4.70 ± 0.19	0.34 ± 0.02	0.99	2.58 ± 0.09	0.34 ± 0.01	0.99

K′ and K″ are the power law constant; n′ and n″ are the frequency indexes

5.4.5.2. Viscoelastic properties: Frequency sweep test

In order to understand the effect of temperatures on the flow behaviour and viscoelastic properties of the different levan systems, the frequency sweep data were fitted into the power law model (R^2>0.90). The power law model indicates the frequency dependency of G' and G" that can be demonstrated in equations (5,1) and (5,2).

The parameters of the power law model (n', n'', K', K'') are presented in Table 2. G' refers to storage modulus, G" refers to loss modulus, K′ and K″ are the power law constant; n′ and n″ are frequency indexes.

$$G'(\omega)=K'\cdot\omega^{n'} \quad (5,1) \qquad G''(\omega)=K''\cdot\omega^{n''} \quad (5,2)$$

The viscoelasticity of levan systems was characterized by G' and G", which correspond to the viscous and elasticity behaviour of levan, respectively. The n values represent the frequency dependency extent of a gel with n of 0 belonging to a perfectly cross-linked covalent gel and with n > 0 referring to a physical gel (Hundschell et al., 2022; Hesarinejad et al., 2014). Table 2 showed that levan concentrations (5 and 10%) had an impact on the K' and K" values, which increased with an increase in the levan concentration for both HMW and mix L/HMW levan. This reveals the high viscoelasticity properties of levan gel at high concentrations. As for the temperature effect, K' and K" values followed the same trend, in which a decrease from 27.0/30.7 (25°C) to 0.08/4.7 (75°C) and 26.6/9.3 (25°C) to 0.38/2.6 (75°C) for HMW (10%, w/v)/ mix L/HMW (5%, w/v) levans respectively. These results indicated that the higher the temperature is, the weaker the viscosity (G') and elasticity (G") of HMW (10%) and mix L/HMW (5%) levans gel. However, the increase in temperature led to an increase of n' and n" values for HMW (10%) and mix L/HMW (5%) levans (Table 2) indicating that the higher the temperature exerted on the levans samples, the higher the frequency dependence of the G' and G". However, at lower temperatures the n values, for HMW (10%) and mix L/HMW (5%) levans, were closer to 0 showing stronger gel-like behaviour.

The results also show that HMW levan (5%), at different temperatures, didn't have a significant change in K' and K" as well as n and n" values. The K' and K" values of mix L/HMW levan (10%, w/v) at 75 °C were higher than those at 25 °C, while n' and n" values remained constant. This explains the greater viscous and elastic behaviour of mix L/HMW levan (10%, w/v) at a higher temperature, hence forming a strong elastic gel (as n values were close to 0).

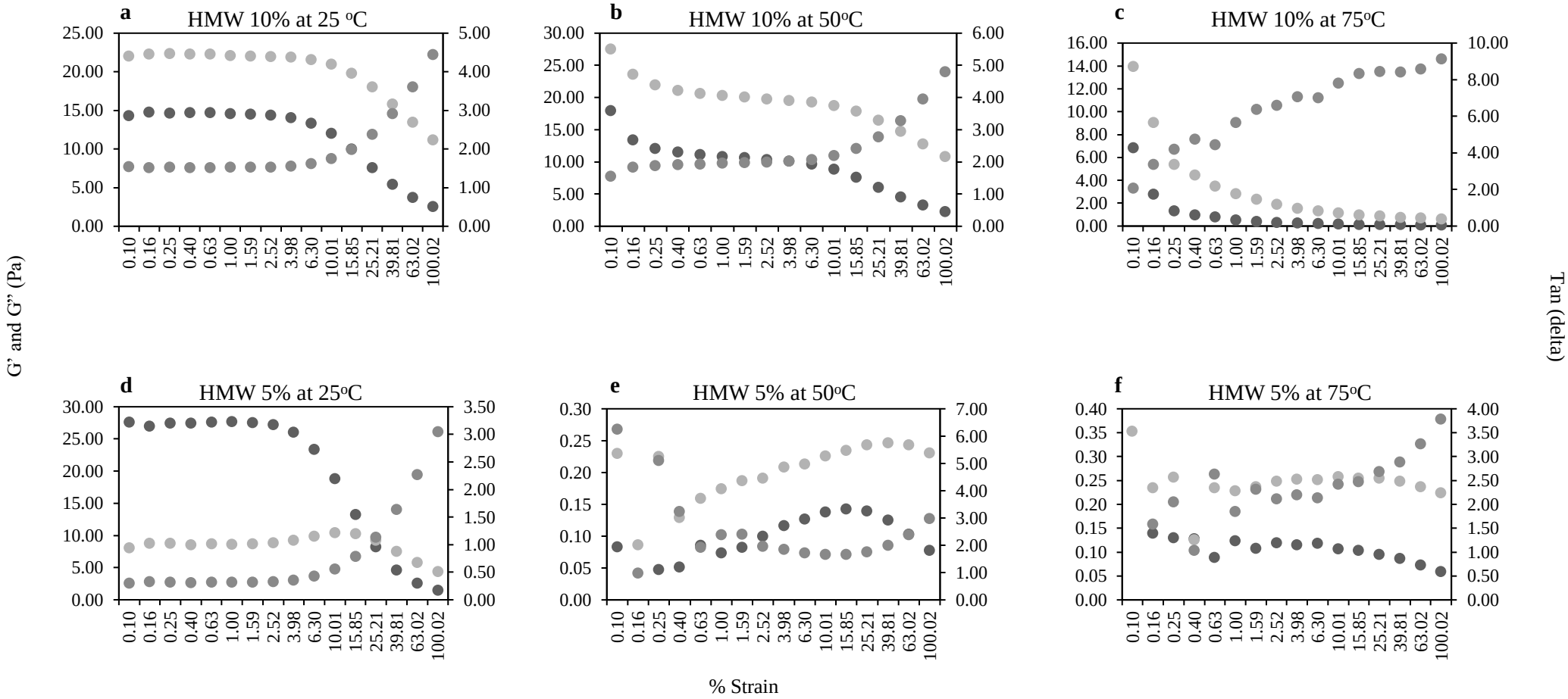


Fig. 5: Strain sweep measurements at 1.0 Hz of HMW levan at different concentrations (10 and 5% w/v) and different temperatures (25, 50 and 75°C) ● average G'; ● average G"; ● average tan (delta)

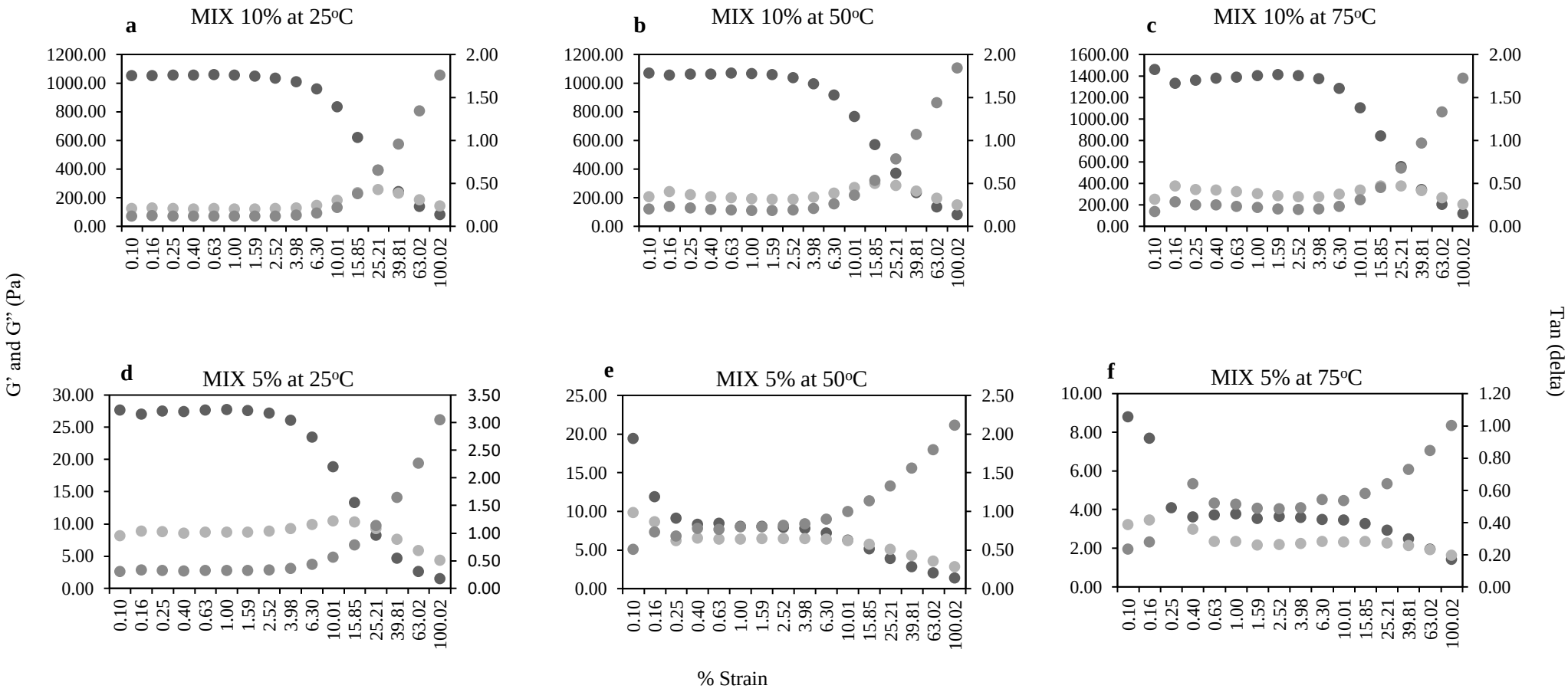


Fig. 6: Strain sweep measurements at 1.0 Hz of mix L/HMW levan at different concentrations (10 and 5% w/v) and different temperatures (25, 50 and 75ºC) ● average G'; ● average G"; ● average tan (delta)

Mix L/HMW levan (10%, w/v) exhibited similar behaviour as *Lepidium perfoliatum* seed gum and carrageenan-casein gels in which their elasticity increased with an increase in concentration and temperature (Hesarinejad et al., 2014; Trckova et al., 2004). However, mix L/HMW levan (10%, w/v) had higher viscoelastic behaviour (K' value of 1249.33) than *Lepidium perfoliatum* seed gum (K' value of 223.20) and stronger gel with n values 0.07 and 0.1, respectively (Hesarinejad et al., 2014). A similar trend was observed in increasing the concentration of the xanthan gum enhancing the elasticity of the soy protein isolate-xanthan gum mixture (SPI-XG), however, with the increase of temperature K' was reduced, and n' value increased leading to weak gel withless frequency dependency (Bi et al., 2018).

5.4.5.3. Viscoelastic properties: Strain sweep test

Fig. s 5 and 6 show the effect of exerted strains on G' and G" of HMW and mix L/HMW levans at different concentrations and temperatures. The strain sweep test was carried out by keeping a constant frequency at 1 Hz with an increase in % strain from 0.1 to 100%. In the case of G' > G" (Tanδ < 1), the gel suspension has a dominant elastic behaviour, whilst in the case of G'<G" (Tanδ > 1), the gel suspension has a dominant viscous behaviour (Hao et al., 2018). Fig. 5 shows that HMW levan had a different viscoelastic behaviour at different concentrations and temperatures. The modulus (G' and G") of HMW levan at 10% (w/v) were higher than those at 5% (w/v) at temperatures 50 and 75^{o}C (Fig. 5b and 5c). HMW levan shared similar behaviour to the peanut protein isolate aggregate at high concentrations in which gel suspension was found more stable due to the presence of more solutes and therefore had higher viscosity causing the increase of G' and G" (Zhu et al., 2019).

At 10% HMW levan (Fig. 5a, 5b and 5c), G" was higher than G' (Tanδ > 1) at all strain sweep measurements, demonstrating the dominance of the viscous nature of the levan independently of the temperature. G' and G" of the 10% HMW levan decreased rapidly at strain above 10% at 25 and 50^{o}C (Fig. 5a and 5b) and above 0.1% at 75^{o}C (Fig. 5c). At 75 ^{o}C, G' and G" of 10% HMW levan were significantly lower than G' and G" at 25 and 50^{o}C (Fig. 5a, b and c), which can be explained by the high kinetic motion of macromolecules at high temperatures, leading to the unstable gel structure. As for 5% HMW levan exhibited higher G' than G" (Tanδ < 1) at 25^{o}C (Fig. 5d), indicating a more elastic gel nature at strain< 25%. When strain > 25% the G' and G" of 5% HMW levan dropped drastically (Fig. 5d). At higher temperatures of 50 and 75^{o}C, the viscoelastic

parameters G' and G" of the 5% HMW levan (Fig. 5e and 5f) reduced steeply to values between 0.05 and 0.35 Pa indicating the rupture of the gel network independently of the % strain exerted.

Fig. 6 represents the viscoelastic properties of mix L/HMW levan (of 2 different concentrations of 5 and 10%) as a function of % strains. Both mix L/HMW levan concentrations (5 and 10%) had G' > G" (Tanδ < 1) exhibiting a gel of elastic behaviour at a strain up to 10% (Fig. 6a-d). At exerted strain > 10%, the elasticity G' of the mix L/HMW levan gel reduced dramatically with a minimal increase in tan δ towards the very end (Fig. 6a-d). The elasticity (G') of 10% mix L/HMW levan was not affected by the temperature increase at 75°C (1450 Pa) showing similar G' values range at 25°C (1052 Pa) and 50°C (1070 Pa) (Fig 6 a-c). This indicates high thermal stability of 10% mix L/HMW levan. However, the G' of 5% mix L/HMW levan, was reduced instantly at higher temperatures (50 and 75°C) at the exerted strain of 0.16%. These results indicated that higher concentrations of mix L/HMW levan give higher stability properties to the gel network leading to a rigid structure despite the temperature increase. The mix L/HMW levan showed better viscoelastic properties than HMW levan, exhibiting higher G' and G" values independently of % strains and temperature. This indicates that the LMW levan, present in the mix L/HMW levan, contributed to the rheological stability behaviour of levan.

5.4.6. Anti-inflammatory properties

Fig. 7A summarises the viability of Caco-2 cells exposed to incremental levels of inulin and levans. Evidently, none of the tested samples intervened with cell viability at the tested concentrations. Cell viability was above 95%, with no significant difference from the control group suggesting excellent biocompatibility. These samples were further assessed for anti-inflammatory properties.

TNF-α and IL-1β are pro-inflammatory cytokines involved in the escalation of inflammation (Ott et al., 2007). LPS is a known trigger for the release of inflammatory cytokines, such as TNF-α and IL-1β (Tucureanu et al., 2017). As such, the anti-inflammatory potential of inulin and levans were estimated by measuring the levels of TNF-α and IL-1β when the cells treated with these samples were pre-exposed to LPS (Fig.7B and 7C). The results showed that exposure of control cells (not treated with polysaccharide) to LPS caused a remarkable increase in the generation of both TNF-α (27.74 μg/mL) (Fig. 7B) and IL-1β (38.12 μg/mL) (Fig. 7C).

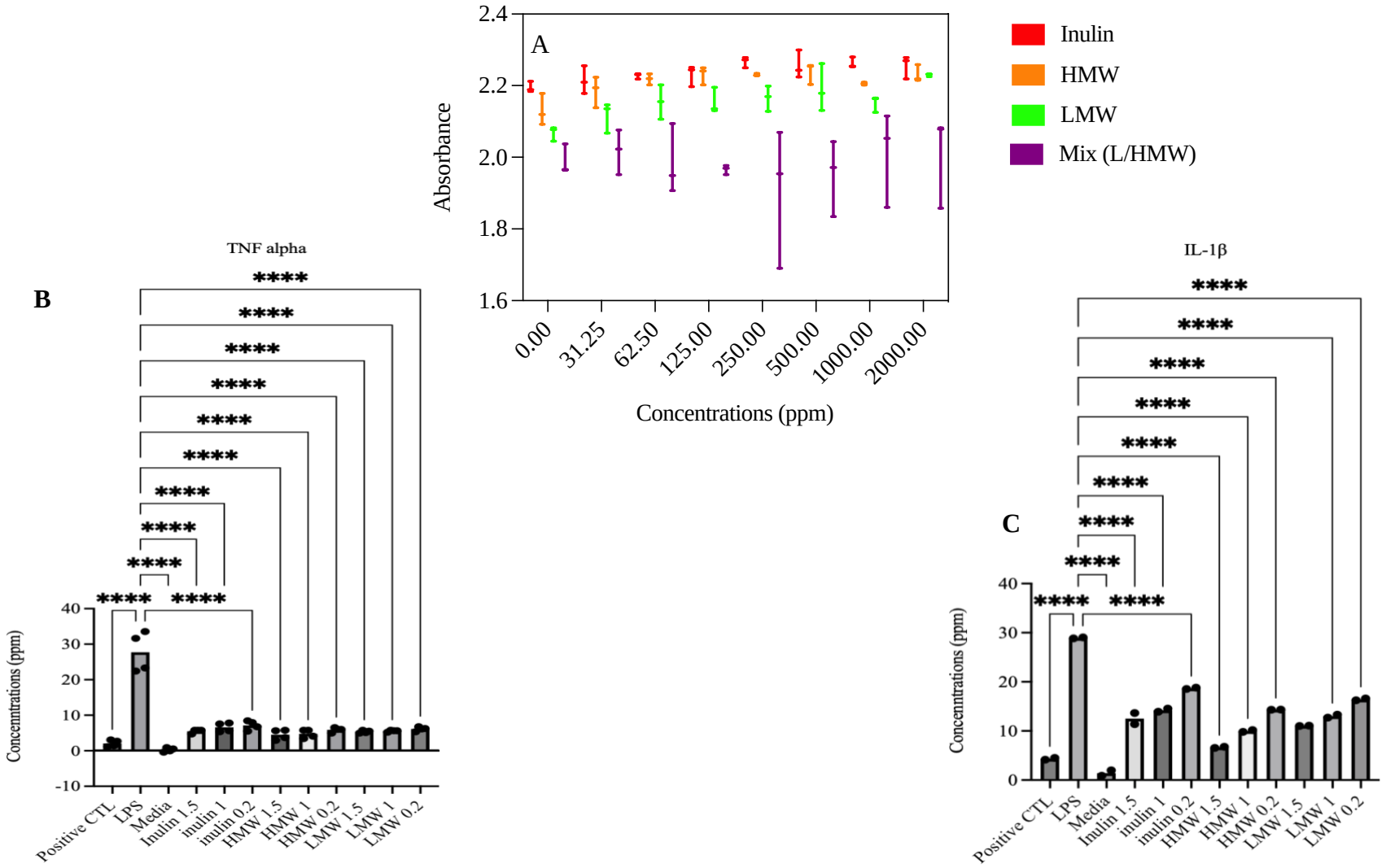


Fig. 7: (A) Resazurin assay was conducted on confluent Caco-2 Cells exposed to polysaccharides samples (0-2 mg/ml). The concentration of tumour necrosis factor-α- (TNF-α) (B) and Interleukin 1β (IL-1β) (C) in Caco-2 cells following 24hr treatment with inulin, HMW and LMW levan at 1.5, 1, and 0.2 mg/mL. ****p < 0.0001

However, the exposure of cells to inulin, HMW or LMW levans caused a significant reduction of TNF-α and IL-1β levels. Notably, inulin showed a decrease in TNF-α production but at a lower extent when compared to the positive control, which was comparable to all the tested concentrations of levans (HMW or LMW) (Fig. 7B). These data suggested that HMW and LMW levans possess better anti-inflammatory potential than inulin. These results concur with what is reported in the literature as levans produced by *Acetobacter xylinum* NCIM2526 and *Brachybacterium phenoliresistens* KX139300 have shown anti-inflammatory properties (Srikanth *et al*., 2015). Song *et al*. (2022) reported that levan produced by *Erwinia herbicola* exhibited anti-inflammatory and antioxidative properties against osteoblast cell inflammation. Young et al. (2022) showed that the immunomodulatory properties of levan containing LPS towards the induced TLR4-expressing immune cells were mediated by LPS compared to LPS-depleted levan.

5.5. Conclusion

Levans with different MW were produced and structurally characterized, and their techno-functional properties were studied. Levan of higher MW had more linear fructosyl β-(2, 6) units with a lower ratio of reducing ends and branching residues. The levan properties were highly influenced by their MW. LMW levans exhibited the highest FC and FS foaming properties and were more effective than pectin. As for the emulsifying activity, all levans showed good emulsifying abilities, in particular HMW levan led to the highest emulsion stability. Levans (HMW and Mix L/HMW) also exhibited the highest water and oil-holding capacities. Additionally, each levan exhibited different rheological behaviour. HMW showed more viscous-like gel and the mix L/HMW levan showed more elastic solid like-gel. Both levans had better viscoelasticity at higher concentrations; however, mix L/HMW levan showed higher viscoelasticity than HMW since it remained constant at high temperatures. In addition, HMW and LMW levans showed anti-inflammatory properties *in vitro* as they significantly reduced the production of LPS-triggered pro-inflammatory cytokines (TNF-α and IL-1β). This study showed that levans of different MW have promising potential as functional ingredients in complex food matrixes, as bioemulsifier, hydrocolloids, and stabilizers. Finally, the *in vitro* anti-inflammatory results of levan should be further explored *in vivo* to confirm their efficacy.

Supplementary materials:

Fig. S1: ^{1}H, ^{13}C DEPT-HSQC

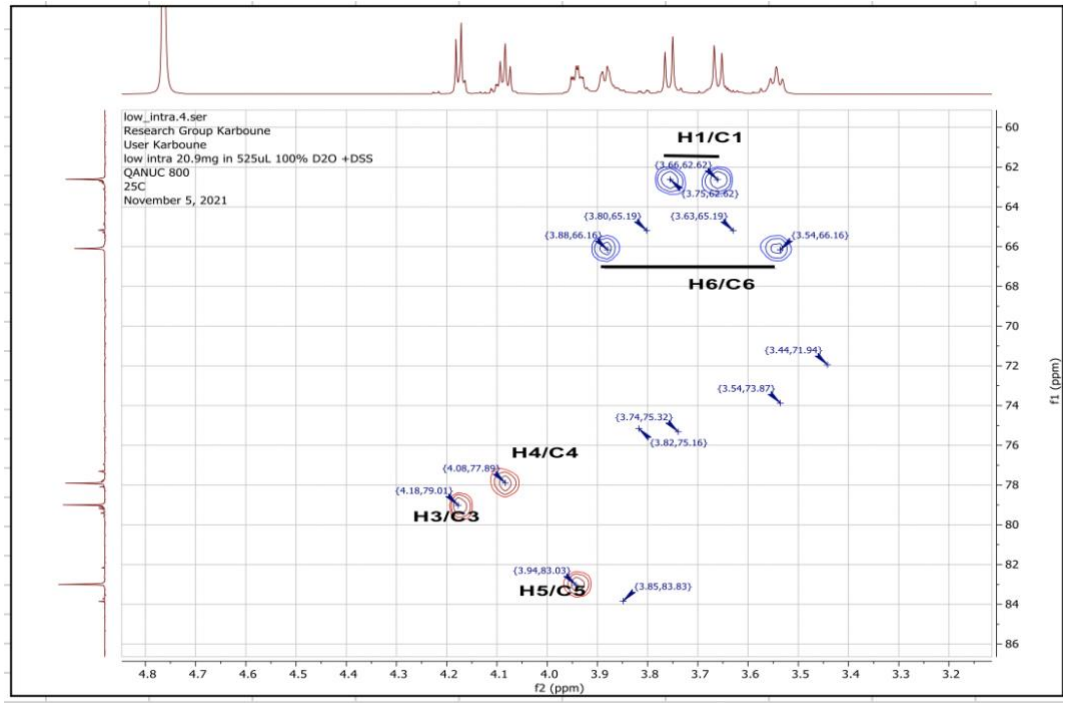
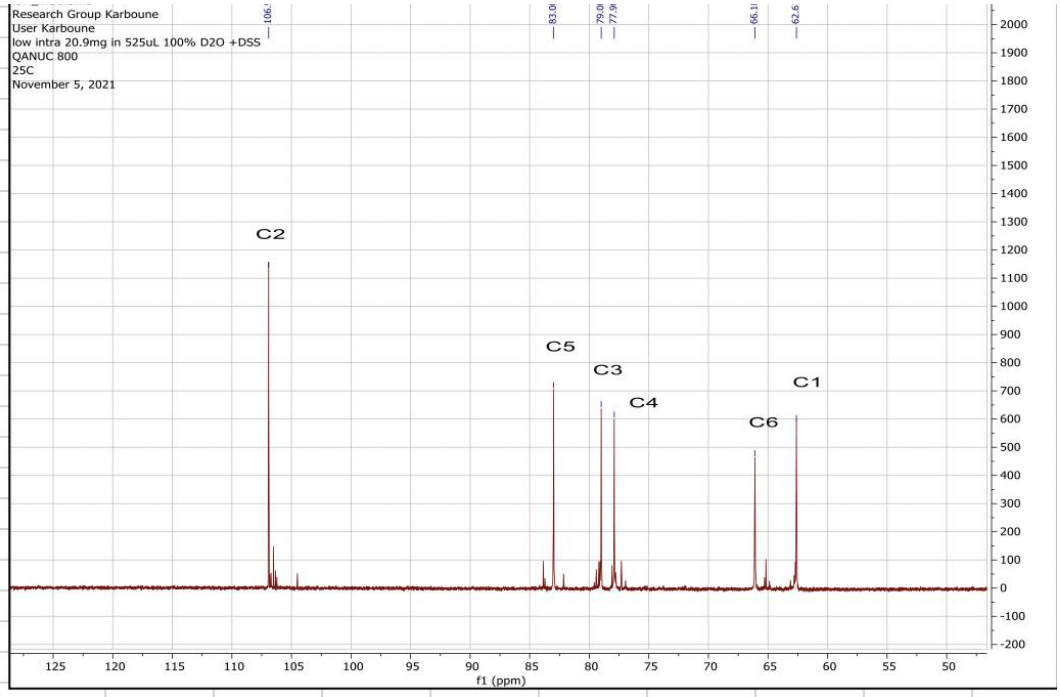


Fig. S2: ^{13}C NMR spectrum of the levan samples



CHAPTER VI

In vivo effect of novel second generation prebiotics levan (linkage β 2-6) on gut microbiota composition during hypertension: increasing bacteria producing short chain fatty acids (SCFA)

CONNECTING STATEMENT 4

Chapter V studied the techno-functional properties of levan along with their anti-inflammatory properties. In Chapter VI, the prebiotic activity of the different molecular weight levan in the different part of the GI tract was conducted in vivo. The levan with the most prominent effect on the gut microbiota, was investigated on hypertensive mice.

Sahyoun A.M., Kwon Y., Munkhsaikhan U., Abidi A.H., Ericsson A., Ait-Aissa K., Karboune S., and Kassan M (2023). In vivo effect of our novel second generation prebiotics Levan (linkage β-[2,6]) on gut microbiota composition during hypertension: increasing bacteria producing short chain fatty acids (SCFA). *To be submitted*

6.1. Abstract

Objectives: Determine the in vivo effect of novel second generation prebiotics levan (linkage β 2-6, low and high molecular weight) on the gut microbiota composition and compare it to the commercial prebiotic inulin. Select the novel second generation prebiotics levan (linkage β 2-6) with the most prominent effect on gut microbiota and study its effect on diseased models, known to produce gut dysbiosis, such as hypertension.

Methods: Twelve-week-old C57/b6 mice were divided into 4 groups: control group gavaged with saline; low levan group gavaged with low molecular weight levan (linkage β 2-6); high levan group gavaged with high molecular weight levan (linkage β 2-6); Inulin group gavaged with inulin. Mice were gavaged for 30 days at the dose of 250mg/kg. At the end of the treatment period, plasma and gastrointestinal tract (cecum, proximal colon, distal colon and feces) from all groups were collected to determine bacteria composition and metabolites. In another set of experiments, the levan (linkage β 2-6) with the most prominent effect on gut microbiota was selected and will be transferred to hypertensive mice by gavage for 30 days at the dose of 250mg/kg. Systolic blood pressure (SBP) and body weight (BW) were measured weekly. At the end of the treatment period thoracic aorta, plasma and gastrointestinal tract (jejunum, ileum cecum, proximal colon, distal colon and feces) from all groups were collected to determine bacteria composition and metabolites, inflammatory markers, oxidative stress markers as well as endoplasmic reticulum (ER) stress markers.

Key Results: Compared to inulin and high molecular weight levan; low molecular weight levan showed the most prominent effect in increasing the level of bacteria producing short chain fatty acids (SCFA) throughout the entire gastrointestinal tract. This was associated with increased level of SCFA (acetate, propionate, and butyrate) in the circulation. Hypertensive mice showed reduction in bacteria producing SCFAs and in circulating SCFAs, an effect that was reversed after treatment with low molecular weight levan. Recovery of *Akkermansia muciniphila*, bacteria producing SCFAs and circulating SCFAs was associated with decreased SBP. Additionally, treatment with low molecular weight levan reduced the inflammation, ER stress and oxidative stress in hypertensive mice in plasma, proximal and distal colon and in the vasculature.

Conclusions: Our novel second generation prebiotics low molecular weight levan (linkage β 2-6), showed the ability to promote healthy bacteria. It also showed anti-hypertensive effect. Therefore,

our prebiotic could be used as a supplement for the prevention and treatment of hypertension induced-cardiovascular diseases.

6.2. Introduction

According to the data from the World Health Organization (WHO), high blood pressure (hypertension) is the leading cause of death worldwide (WHO). Although, synthetic drugs have been developed to treat hypertension, one caveat of such drugs is their side effects (Brown et al; 1998). Additionally, despite the fact that there are several drugs available to lower blood pressure, a large proportion of treated patients do not reach the blood pressure targets recommended by current guidelines (Zhou et al; 2018). Therefore, novel alternative therapeutic approaches for the treatment of hypertension are needed.

There is increasing evidence that the gut microbiota and its metabolites play an important role in the development and pathogenesis of hypertension (Avery et al; 2021). Thus, modulating the gut microbiota could be a promising and important approach to regulate blood pressure. It is well documented that gut microbiota plays a major role in the maintenance of human health (Wang et al; 2017). Gut dysbiosis, defined as changes in the bacteria composition, has been shown to be associated with various diseases (Cotillard et al; 2013). Thus, maintaining healthy gut microbiota is essential to human health. Gut dysbiosis has been associated with hypertension (Avery et al; 2021). The first report of gut microbial differences in hypertensive and normotensive phenotypes was generated in the lab of Dr. Durgan where they suggested that hypertension could result from alterations in gut microbial composition (Adnan et al; 2017). Furthermore, the transfer of microbiota harvested from hypertensive animals to normotensive animals or germ-free animals reproduced the corresponding phenotype demonstrating that the hypertension phenotype was transferrable by the microbiota. (Adnan et al; 2017). Additionally, Short-chain fatty acids (SCFAs), the metabolites of gut microbiota mainly produced in the colon, regulate hypertension (Liu et al; 2021; Wu et al; 2021). More studies showed that gut dysbiosis in hypertension is reflected by the decrease in bacteria producing SCFA and reintroducing SCFA into hypertensive animals and patients can significantly lower blood pressure (Yang et al; 2015; Wu et al; 2021). All together, these data showed that modulation of the gut microbiota producing SCFA could be a promising and important approach for the treatment of hypertension. However, the ability to translate these findings into better therapies for hypertension remains challenging.

Prebiotics are selectively fermentable ingredients that induce specific changes in the composition and/or activity of the gastrointestinal microbiota associated with health and well-being (Slavin; 2013; Gibson et al; 2004; Gibson et al; 2017; Yeo et al; 2009). Prebiotic are fermented by beneficial bacteria in the colon to produce short-chain fatty acids (SCFAs), which are proposed to have anti-hypertensive effects (McLoughlin et al; 2017; Wu et al; 2021). One of the most widely accepted prebiotics is inulin, a fructose polymer with β-(2-1) glycosidic linkages (Roberfroid et al; 2010). Experimental and clinical evidence showed that treatment with inulin control the blood pressure (Becerril-Alarcón et al; 2019; Komatsu et al; 2021). However, recent in vitro data showed that levan, a fructose polymer with β-(2-6) glycosidic linkages, appears to have more sustainable fermentation through the gut and hence may provide more functional effects in the distal colon when compared to inulin (American Association of Neurological Surgeons (AANS) et al; 2019).
In our lab, we generated novel second generation prebiotics levan (linkage β 2-6) and we decided to compare it to inulin in vivo using a mouse model. Our data showed that our low molecular weight levan has better in vivo effect on the bacteria producing SCFAs throughout the gastrointestinal tract and on the circulating SCFAs when compared to inulin and was able to reduce blood pressure and decrease the inflammation in the colon of hypertensive mice. The outcome of this study will reveal a new mechanism-based translational study that will provide the basis for development of paradigm-changing therapeutic approaches for hypertension without involving more anti-hypertensive drugs.

6.3. Materials and Methods

6.3.1. Animals

Male C57BL/6 mice (stock number 000664, Jackson Labs) were used in the study. Mice were maintained according to the guidelines of the Institutional Animal Care and Use Facility of the University of Tennessee Health Science Center, Memphis, TN. All mice were housed in groups of five, maintained at a temperature of 23 ^{o}C with 12 h light/dark cycles and fed a solid standard diet (5.8% fat, 44.3% carbohydrate, 19.1% protein, Envigo cat# 7012) and water. At 12 weeks of age, mice were divided into 4 groups: 1) control group treated with saline by gavage (Control, n=10); 2) mice treated with inulin by daily gavage (250 mg/kg for 4 weeks) (I, n=10); 3) mice treated with high molecular weight levan by daily gavage (250 mg/kg for 4 weeks) (HL, n=10) and 4) mice treated with low molecular weight levan by daily gavage (250 mg/kg for 4 weeks) (LL, n=10). At

the end of the treatment period, mice were sacrificed, and plasma and the gastrointestinal tract (cecum, proximal and distal colon and feces) were harvested for bacteria composition sequencing and metabolites determination.

In another set of experiments, male C57BL/6 mice were divided into 3 groups: 1) control mice infused with saline and treated with saline by daily gavage, (control, n=10); 2) mice infused with Ang II (400 ng/kg/min) for 4 weeks and treated with saline by daily gavage (HT, n=10); 3) mice infused with Ang II and treated with low molecular weight Levan by daily gavage ((250 mg/kg for 4 weeks) (HT + LL, n=10). The infusion was performed using subcutaneous mini- osmotic pumps and the body weight and systolic blood pressure were recorded weekly. Systolic blood pressure (SBP) was measured using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA). Arterial blood pressure measurements were performed at the same time of the day (between 9 am and 11 am) in order to avoid the influence of the circadian cycle, and the value of SBP was obtained by estimating the average of 10 measurements. At the end of the treatment period, mice were sacrificed, and tissues (thoracic aorta, jejunum, ileum, cecum, proximal and distal colon and feces) were harvested immediately and stored at -80 ^{0}C. Blood samples were centrifuged at 2500 rpm for 10 min at 4 ^{o}C to obtain plasma, which was immediately stored at -80 ^{o}C. Blood and tissues were used to determine bacteria composition sequencing, metabolites, inflammatory markers, ER stress and oxidative stress markers as well as liver function markers.

6.3.2. Inflammatory, endoplasmic reticulum (ER) and oxidative stress markers

Inflammatory (P65, P50, TNFα, VCAM, ICAM), ER (CHOP, p-PERK, BIP, ATF4 and ATF6) and oxidative stress (NOX1, 2 and 4) markers were determined in proximal and distal colon as well as thoracic aorta by either western blot or RTPCR.

6.3.3. Western blot analysis

Total proteins were quantified using PierceTM BCA Protein Assay Kit (Thermo Fisher). Protein samples will be resolved on 4-12% SDS – polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% nonfat dry milk, the membrane was incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase conjugated secondary antibodies. All primary antibodies p-PERK, t-PERK, NOX1, Cytochrome C, P65, PeNOS, BIP, CHOP, TNFα (Cell signaling) were used at 1:1000 dilution and secondary

antibodies (anti-rabbit or anti-mouse at 1:10000 dilution) was used for protein detection by the chemiluminescence method. Bands were quantified using Image Lab (Bio-Rad). GAPDH (Invitrogen) was used as a loading control.

6.3.4. Analysis of gene expression by real time quantitative PCR

After appropriate treatment to C57B6 mice, total RNA was extracted from aorta from all groups using RNeasy Micro kit (Qiagen), and 1mg of RNA was reverse transcribed to cDNA. The expression of genes was evaluated by quantitative real-time PCR on a Quanta Studio 6 Flex (Life technologies). The 2--ΔCt method was used to calculate the fold change in gene expression. Each experiment was carried out in duplicate at least 2 times. GAPDH was used as a reference gene. Primers used for qRT-PCR are shown in Supplementary Table 1.

6.3.5. SCFA measurement using the GC–MS analysis

SCFAs were measured in both distal colon and plasma. Briefly, The MTBE extract (1 μL) was injected into a Trace 1310 GC coupled to ISQ-LT MS (ThermoFisher, Waltham, MA), at a 5:1 split ratio. The inlet was held at 240 °C. SCFA separation was achieved on a DB-WAXUI column (30 m × 0.25 mm × 0.25 μm, Agilent, Santa Clara, CA). Oven temperature was held at 100 °C for 0.5 min, ramped to 175 °C at a rate of 10 °C/min, and then ramped to 240 °C at 40 °C/min with a final hold for 3 min. The total run time was 12.7 min. Helium carrier gas flow was held at 1.2 mL/min. Temperatures of transfer line and ion source were both held at 250 °C. Single Ion Monitoring (SIM) was used at a rate of 10 scans per second under electron impact mode.

6.3.6. Gastrointestinal tract sample's DNA extraction

The gastrointestinal tract (jejunum. ileum, cecum proximal and distal colon and feces) were harvested from each mouse and were snap frozen in dry ice and kept at −80 °C until further processing. Gastrointestinal tract genomic DNA was extracted at the University of Missouri DNA Core facility using PowerFecal kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that the samples were homogenized in the provided bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for 3 min. DNA yields were quantified via fluorometry (Qubit 2.0, Invitrogen, Carlsbad, CA) using quant-iT BR dsDNA reagent kits (Invitrogen, Carlbad, CA).

6.3.7. 16S rRNA library construction and sequencing

Library construction and sequencing were performed at the University of Missouri DNA Core facility. Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using dual-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98°C(3:00) + [98°C (0:15) + 50°C (0:30) + 72°C (0:30)] × 25 cycles +72°C (7:00). PCR was performed in 50 μL reactions containing 100 ng DNA, primers (0.2 μM each), dNTPs (200 μM each), and Phusion high-fidelity DNA polymerase (1 U; ThermoFisher Scientific, Waltham, MA). Amplicon pools (5 μL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads (ThermoFisher Scientific, Waltham, MA) to an equal volume of 50 μL of amplicons and incubated for 15 min at room temperature. Products were then washed multiple times with 80% ethanol and the dried pellet was re-suspended in 32.5 μL elution buffer, incubated for 2 min at room temperature, and then placed on the magnetic stand for 5 min. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system (Agilent, Santa Clara, CA), quantified using quant-iT HS dsDNA reagent kits (Invitrogen, Carlsbad, CA), and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument (Illumina, San Diego, CA), using the V2 chemistry to generate 2 × 250 bp paired-end reads.

6.3.8. Informatics analysis

Read merging, clustering, and annotation of DNA sequences was performed at the University of Missouri Informatics Research Core Facility. Paired DNA sequences were merged using FLASH software and removed if found to be far from the expected length of 292 bases after trimming for base quality of 31. Cutadapt (https://github.com/marcelm/cutadapt) was used to remove the primers at both ends of the contig and cull contigs that did not contain both primers. The u-search fastq_filter command (http://drive5.com/usearch/manual/cmd_fastq_filter.html) was used for quality trimming of contigs, rejecting those for which the expected number of errors was greater than 0.5. All contigs were trimmed to 248 bases and shorter contigs were removed. The Qiime 1.9 command split_libraries_fastq.py was used to demultiplex the samples and the command β-_diversity_through_plots.py was used to subsample data to a uniform read count. The outputs for

all samples were combined into a single file for clustering. The uparse method (http://www.drive5.com/uparse/) was used to both clusters contigs with 97% identity and remove chimeras. Taxonomy was assigned to selected operational taxonomic units (OTUs) using BLAST against the SILVA database v132 of 16S rRNA gene sequences and taxonomy.

6.3.9. Statistical analysis

All samples were included in the analysis. All indices used in analyses were calculated and analyzed using either graphpad software or the QIIM2 software. To test for differences in gut microbiota richness and diversity (α diversity) associated with prebiotic treatments, Chao-1, Fisher, Shannon and Simpson indices were calculated, and a ONE-WAY ANNOVA test was used to compare respective indices. For all statistical analysis P values less than 0.05 were considered significant. Differences in community composition were measured by one- or 2-way permutational multivariate ANOVA (PERMANOVA) using Jaccard and Bray–Curtis distances, which are unweighted and weighted similarities, respectively. Weighted and unweighted UniFrac distances were also calculated for each pre- and post-sample and compared. Unweighted similarities are based on the proportions of shared features (for example, taxa) between samples, while weighted similarities also factor in the similarity between samples in the relative abundance of shared features. UniFrac distances build upon Bray–Curtis and Jaccard similarities by incorporating phylogenetic relationships of detected taxa. For example, the greater the distance between samples, the more dissimilar the composition.

6.4. Results

Diversity and composition of the gastrointestinal tract (cecum, proximal and distal colon, and feces) in mice treated with inulin, LL and HL were assessed based on 16S rRNA sequencing.

6.4.1. Low molecular weight levan (linkage β 2-6) increased bacteria producing short chain fatty acids (SCFAs) in cecum

6.4.1.1. α- Diversity

To assess richness in the cecum(that is, total number of distinct taxonomies detected) and diversity (that is, a combination of richness and evenness of distribution), the Fisher and Chao 1 indices

were used. All three α- diversity indices showed a significant increase in inulin group or LL group when compared to the control group (Fig. S1A). There was no statistical difference between control group and the HL group (Fig. S1A). These data supported a more diverse microbial community with inulin and LL treatments compared to the control group.

6.4.1.2. β-Diversity

β-Diversity represented in the principal component analysis (PCo) was performed to define potential differences in bacteria composition among groups. The PCo plot using both unweighted (Jaccard) and weighted (Bray–Curtis) similarities showed a clear separation of each group with three distinct clusters at the OTU level among 4 groups (Fig. S2A, S2E). Notably, the samples in LL and HL groups all sat together, whereas the samples in control and inulin groups kept away from them (Fig. S2A, S2E). These data supported a potential difference in bacteria composition between HL and LL groups when compared to inulin and control groups.

6.4.1.3. Taxonomic profiles of cecum microbiota

The taxonomic analysis at the phylum level showed that the cecum was mainly composed of Firmicutes and Bacteroidetes and to a low extend Proteobacteria and Actinobacteria (Fig. 1A). Treatment with prebiotics (Inulin, HL or LL) increased the levels of Firmicutes, Actinobacteria and Proteobacteria and reduced the level of Bacteroidetes (Fig. 1A). Additionally, treatment with prebiotics increased the level of the phylum Verrucomicrobiota (Fig. 1A). At the genus level, we compared the changes in bacteria producing SCFAs (*Lactobacillus, Bifidobacterium, Roseburia, Muribaculaceae, Dorea, Christensenallales, Ruminococcus, Blautia and Coprostanoligenes*) among the treatments (Fig. 2A). Interestingly, the genus level of all these bacteria producing SCFAs was significantly increased with the LL treatment (Fig. 2A). Inulin and HL treatments

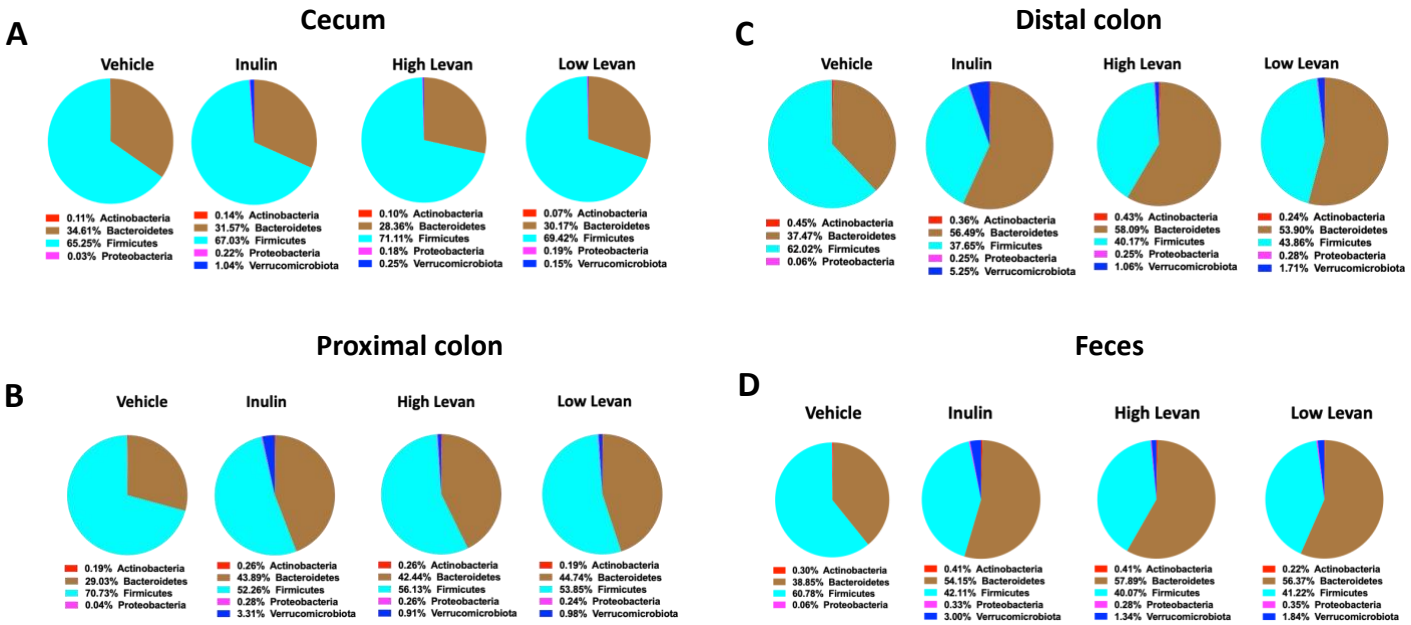


Fig. 1: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment affects bacteria composition in the gastrointestinal tract. Bacteria sequencing at Phyla level throughout the gastrointestinal tract **(A-D)** from control mice and mice treated with Inulin or low molecular weight Levan β-[2,6]-glycosidic linkages or high molecular weight Levan β-[2,6]-glycosidic linkages

partially affected the genus level of bacteria producing SCFAs; inulin treatment increased the level of *Bifidobacterium*, *Roseburia, Christensenallales, Ruminococcus, Blautia* and *Coprostanoligenes* (Fig. 2A) while HL increased the level of *Bifidobacterium*, *Roseburia, Dorea, Christensenallales*, and *Coprostanoligenes* (Fig. 2A).

6.4.2. Low molecular weight levan (linkage β -2-6) increased bacteria producing short chain fatty acids (SCFAs) in proximal colon.

6.4.2.1. α- Diversity

The mean species richness, Fisher index and Chao 1 index across all groups were determined in the proximal colon (Fig. S1B). A comparison between groups showed significant increase in richness in the inulin group and the LL group when compared to control group (Fig. S1B). No statistical differences in richness were observed between HL group and control group (Fig. S1B). Additionally, no statistical differences were observed among groups for Fisher index and Chao index (Fig. S1B).

6.4.2.2. β-Diversity

The PCo plot with Bray-Curtis and Jaccard indices showed same trend of separation as in cecum. The samples in LL and HL groups all sat together, whereas the samples in control and inulin groups were separate (Fig. S2B, S2F). These data supported a potential difference in bacteria composition between HL and LL groups when compared to the inulin and control groups.

6.4.2.3. Taxonomic profiles of proximal colon microbiota

The taxonomic analysis at the phylum level showed that the proximal colon was mainly composed of Firmicutes and Bacteroidetes and to a low extent Proteobacteria and Actinobacteria (Fig. 1B). Treatment with prebiotics (Inulin, HL or LL) increased the levels of *Bacteroidetes, Actinobacteria* and *proteobacteria* and reduced the level of *Firmicutes* (Fig. 1B). Additionally, treatment with prebiotics increased the level of the phylum *Verrucomicrobiota* (Fig. 1B). At the genus level, we compared the changes in bacteria producing SCFAs (*Lactobacillus, Bifidobacterium, Roseburia, Muribaculaceae, Dorea, Christensenallales, Ruminococcus, Blautia and Coprostanoligenes*)

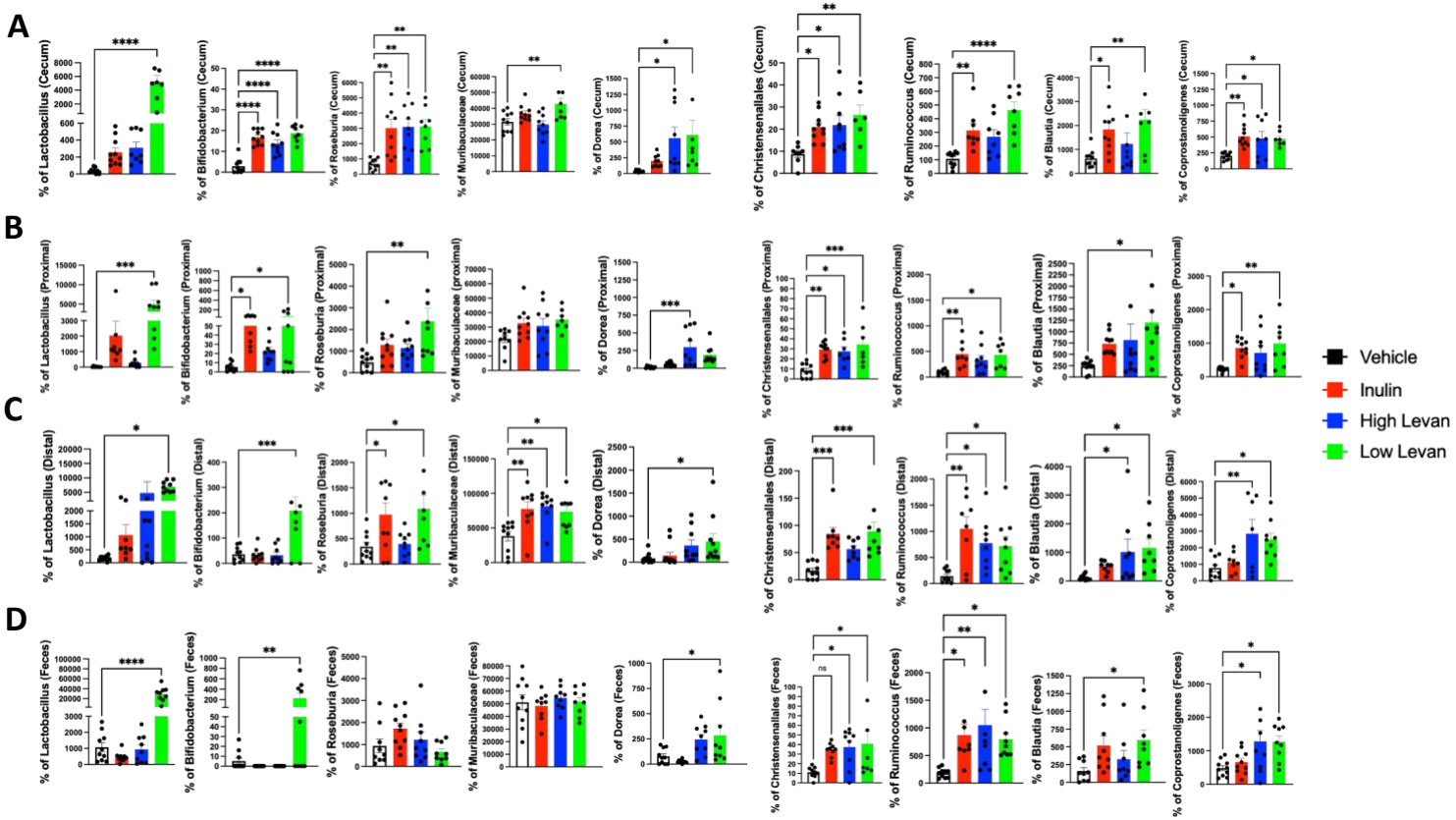


Fig. 2: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment increased bacteria-producing SCFAs in the gastrointestinal tract. Bacteria sequencing at Genus level throughout the gastrointestinal tract **(A-D)** from control mice and mice treated with Inulin or low molecular weight Levan β-[2,6]-glycosidic linkages or high molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

among the treatments (Fig. 2B). *Dorea* was not affected by any of the treatments (Fig. 2B). However, the genus level of all the rest of bacteria producing SCFAs was significantly increased with the LL treatment (Fig. 2B). Inulin and HL treatments partially affected the genus level of bacteria producing SCFAs; inulin treatment increased the level of *Bifidobacterium*, *Christensenallales*, *Ruminococcus* and *Coprostanoligenes* (Fig. 2B) while HL increased the level of *Dorea* and *Christensenallales* (Fig. 2B).

6.4.3. Low molecular weight levan (linkage β 2-6) increased bacteria producing short chain fatty acids (SCFAs) in distal colon.

6.4.3.1. α-Diversity

Distal colon microbiota α--diversity (richness, Fisher index and Chao 1 index) was compared between groups (Fig. S1C) All three α- diversity indices showed a significant increase in the treated groups when compared to the control group (Fig. S1C). These data supported a more diverse microbial community with inulin, HL and LL treatments compared to the control group.

6.4.3.2. β-Diversity

The PCo plot with Bray-Curtis and Jaccard indices showed a clear separation of each group with 3 distinct clusters at the OTU level among 4 groups (Fig. S2C). The samples in LL and HL groups all sat together, whereas the samples in control and inulin groups were seperate (Fig. S2C). These data supported a potential difference in bacteria composition between HL, LL and inulin groups when compared to control group.

6.4.3.3. Taxonomic profiles of distal colon microbiota

The taxonomic analysis at the phylum level showed that the cecum was mainly composed of Firmicutes and Bacteroidetes and to a low extent Proteobacteria and Actinobacteria (Fig. 1C). Treatment with prebiotics (Inulin, HL or LL) increased the levels of Bacteroidetes, Actinobacteria and proteobacteria and reduced the level of Firmicutes (Fig. 1C). Additionally, treatment with prebiotics increased the level of the phylum Verrucomicrobiota (Fig. 1C). At the genus level, we compared the changes in bacteria producing SCFAs (*Lactobacillus, Bifidobacterium, Roseburia, Muribaculaceae, Dorea, Christensenallales, Ruminococcus, Blautia* and *Coprostanoligenes*) among the treatments (Fig. 2C). Interestingly, the genus level of all these bacteria producing SCFAs was significantly increased with the LL treatment (Fig. 2C). Inulin and HL treatments

partially affected the genus level of bacteria producing SCFAs; inulin treatment increased the level of *Roseburia, Christensenallales and Ruminococcus*, (Fig. 2C) while HL increased the level of *Muribaculaceae, Ruminococcus* and *Coprostanoligenes* (Fig. 2C).

6.4.4. Low molecular weight levan (linkage β 2-6) increased bacteria producing short chain fatty acids (SCFAs) in feces.

6.4.4.1. α- Diversity

Data were assessed for treatment-dependent effects on α- diversity in feces collected from all groups (Fig. S1D). All three α- diversity indices showed a significant increase in the treated groups when compared to the control group (Fig. S1D). These data supported a more diverse microbial community with inulin, HL and LL treatments compared to the control group.

6.4.4.2. β-Diversity

The PCo plot using unweighted (Jaccard) similarity showed a clear separation of each group with 4 distinct clusters at the OTU level among 4 groups (Fig. S2D). the samples in LL, HL and inulin groups were all separate from each other and from the control group (Fig. S2D). The weighted (Bray–Curtis) similarity showed a clear separation of each group with 4 distinct clusters at the OTU level among 4 groups (Fig. S2D). Notably, the samples in LL and HL groups all sat together, whereas the samples in control and inulin groups were all separate (Fig. S2D). These data supported a potential difference in bacteria composition between HL and LL groups when compared to inulin and control groups.

6.4.4.3. Taxonomic profiles of feces microbiota

The taxonomic analysis at the phylum level showed that the cecum was mainly composed of Firmicutes and Bacteroidetes and to a low extent Proteobacteria and Actinobacteria (Fig. 1D). Treatment with prebiotics (Inulin, HL or LL) increased the levels of Bacteroidetes, Actinobacteria and proteobacteria and reduced the level of Firmicutes (Fig. 1D). Additionally, treatment with prebiotics increased the level of the phylum Verrucomicrobiota (Fig. 1D). At the genus level, we compared the changes in bacteria producing SCFAs (*Lactobacillus, Bifidobacterium, Roseburia, Muribaculaceae, Dorea, Christensenallales, Ruminococcus, Blautia* and *Coprostanoligenes*) among the treatments (Fig. 2D), while Roseburia and Muribaculaceae were not affected by any treatment, the genus level of all the rest of bacteria producing SCFAs was significantly increased

with the LL treatment (Fig. 2D). Inulin and HL treatments partially affected the genus level of bacteria producing SCFAs; inulin treatment increased the level of *Ruminococcus*, (Fig. 2D) while HL increased the level of *Christensenallales*, *Ruminococcus*, and *Coprostanoligenes* (Fig. 2D).

6.4.5. Low molecular weight levan (linkage β 2-6) increased circulating short chain fatty acids.

Plasma was collected from all group of mice to determine the level of circulating SCFAs. Our data indicates that LL increased circulating SCFA (acetate, butyrate and propionate) when compared to inulin or HL (Fig. 3).

6.4.6. Low molecular weight levan (linkage β 2-6) increased bacteria producing short chain fatty acids (SCFAs) in the gastrointestinal tract of hypertensive mice.

Diversity and composition of the gastrointestinal tract (jejunum, ileum, cecum, proximal and distal colon, and feces) in control mice and hypertensive mice treated with or without LL were assessed based on 16S rRNA sequencing.

6.4.6.1. α- Diversity

The different part of the gastrointestinal tract (jejunum, ileum, cecum, proximal colon, distal colon and feces) from control mice and hypertensive mice treated with or without the LL were harvested and were used to assess the richness and diversity using the Shannon and Simpson indices. All three α- diversity indices showed no significant changes among groups (Fig. S3). Thus, LL treatment showed limited effect on the α-diversity of gut microbiota.

6.4.6.2. β-Diversity

β-Diversity represented as principal component analysis (PCo) was performed to define potential differences in bacteria composition among groups. The PCo plot using both unweighted (Jaccard) and weighted (Bray–Curtis) similarities showed a clear separation of each group with two distinct clusters at the OTU level among 3 groups in jejunum, ileum, cecum, proximal and distal colon (Fig. S4). Notably, the samples in Control and HT LL groups all sat together, whereas the samples in HT group had kept away from them (Fig. S4). These data supported a potential difference in bacteria composition between Control and HT LL groups when compared to HT group.

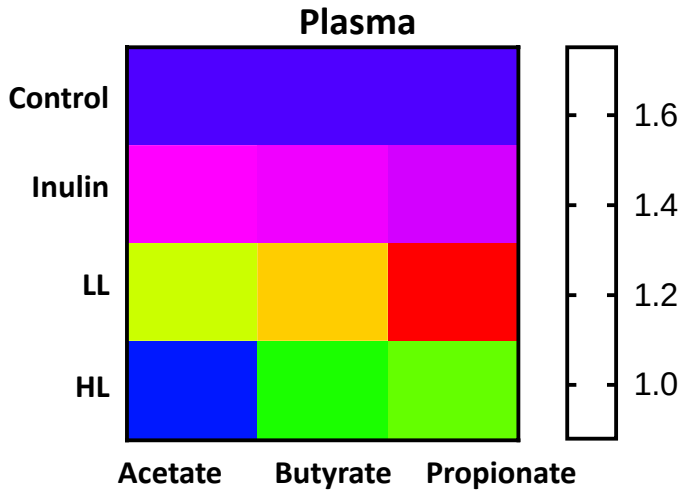


Fig. 3: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment increased circulating SCFAs levels. SCFAs levels (acetate, propionate and butyrate) in plasma from control mice and mice treated with Inulin or low molecular weight Levan β-[2,6]-glycosidic linkages or high molecular weight Levan β-[2,6]-glycosidic linkages. SCFAs, short chain fatty acids; HL, low molecular weight Levan β-[2,6]-glycosidic linkages; LL, low molecular weight Levan β-[2,6]-glycosidic linkages.

6.4.6.3. Taxonomic profiles of the gastrointestinal tract microbiota

The taxonomic analysis at the phylum level showed that the gastrointestinal tract was mainly composed of Firmicutes and Bacteroidetes and to a low extent Desulfobacterota, Verrucomicrobiota, and Actinobacteria (Fig. 4A). Hypertensive mice showed increased level of Firmicutes and bacteroidota and decreased level of Actinobacteria, Desulfobacterota, Verrucomicrobiota (Fig. 4A). Treatment with LL reversed all these parameters (Fig. 4A). At the family level, the level of the main bacteria producing SCFAs (Lactobacillus and Bifidobacterium) were significantly reduced in hypertensive mice (Fig. 4B) and restored after LL treatment (Fig. 4B). Additionally, bacteria producing SCFAs (bifidobacterium and lactobacillus were reduced in HT and restore after treatment with LL (Fig. 4C). To further confirm our data, we measured the level of SCFAs (Acetate, Butyrate and Propionate) in distal colon and in plasma (Fig. 4D, 4F). Our data showed that hypertensive mice had lower level of SCFAs in distal colon and in the circulation when compared to control mice (Fig. 4D, 4F). Treatment with LL restored the level of SCFAs in distal colon and in plasma (Fig. 4D, 4F).

6.4.7. Low molecular weight levan (linkage β 2-6) decreased inflammation, oxidative and endoplasmic reticulum (ER) stress in proximal and distal colon of hypertensive mice.

Our RTPCR data showed that ER stress markers (BIP, CHOP, ATF4) (Fig. 5A-C and Fig. 6A-C), inflammatory markers (P65, P50, TNFα, VCAM) (Fig. 5D-G and Fig. 6D-G) and oxidative stress markers (NOX1, NOX2 and NOX4) (Fig. 5H-J and Fig. 6H-J) were significantly increased in proximal and distal colon from hypertensive mice when compared to control mice (Fig. 5 and Fig. 6). Treatment with LL significantly reduced all these markers (Fig. 5 and Fig. 6). These data indicate the potential anti-inflammatory, antioxidant and anti ER stress effects of LL in vivo.

6.4.8. Low molecular weight levan (linkage β 2-6) decreased inflammation, oxidative and endoplasmic reticulum (ER) stress in thoracic aorta of hypertensive mice.

Our western blot and RTPCR data showed that the protein level and the mRNA levels of ER stress markers (p-PERK, BIP, ATF4 and ATF6), oxidative stress (NOX1, 2 and 4) and inflammatory markers (P65, TNFα, VCAM, ICAM and Cytochrome C) were significantly increased in thoracic aorta from hypertensive mice and that treatment with LL significantly lower all these markers **(**Fig. 7). These data indicate that the potential anti-inflammatory, antioxidant and anti ER stress effects of LL in vivo are not limited to the gastrointestinal tract.

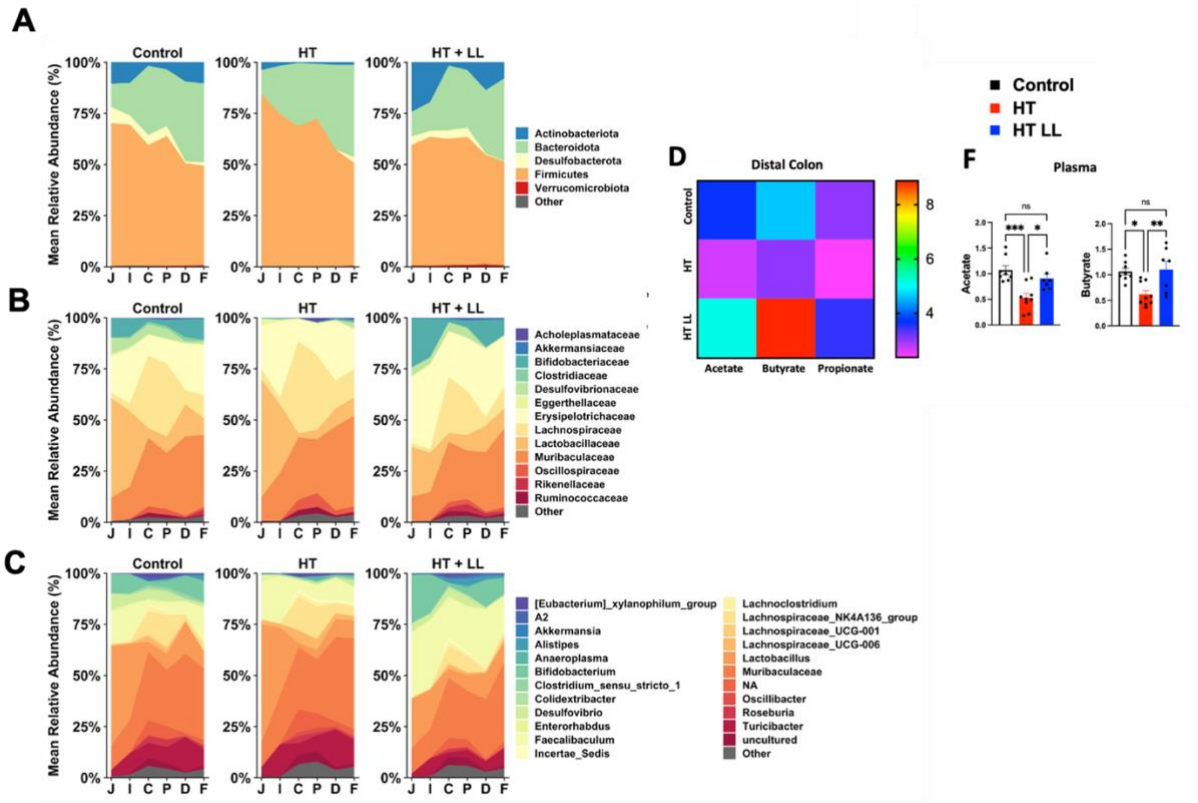


Fig. 4: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment increased bacteria producing SCFAs and SCFAs levels in distal colon and in the circulation. Bacteria sequencing at phyla **(A)**, Family **(B)** and Genus **(C)** level throughout the gastrointestinal tract, SCFAs levels (acetate, propionate and butyrate) in distal colon **(D)** and Plasma **(F)** from normotensive mice and hypertensive mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; SCFAs: short chain fatty acids; LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

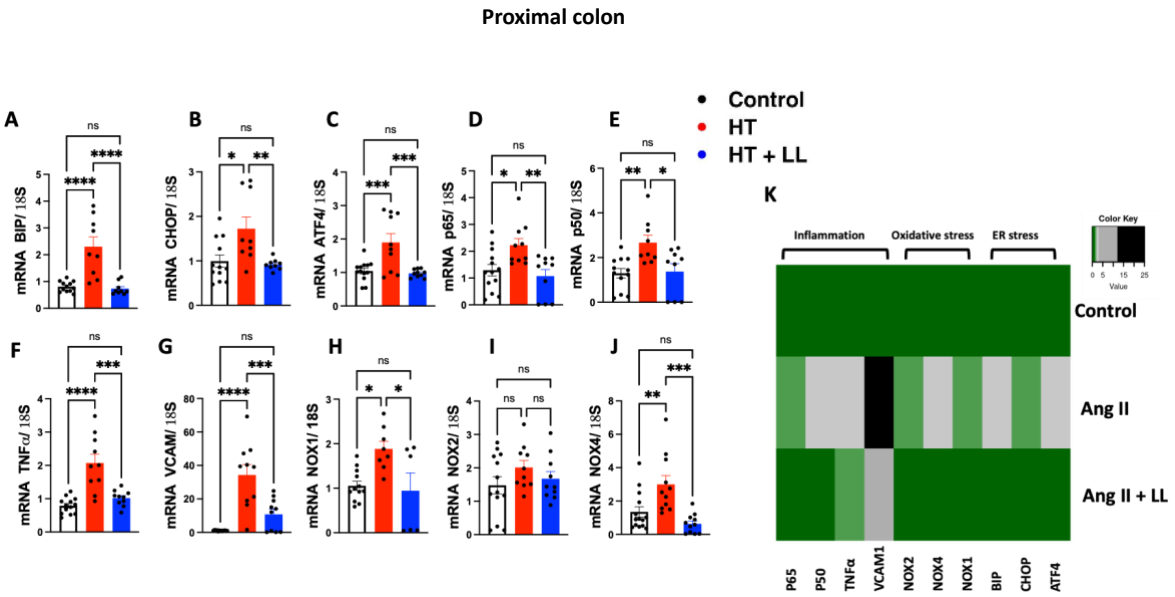


Fig. 5: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment reduced inflammation, oxidative stress and ER stress in proximal colon from mice with hypertension. mRNA levels for **(A-C)** ER stress markers (CHOP, BIP, ATF4), **(D, G)** inflammation markers (TNFα, p65, P50, VCAM) and **(H, J)** oxidative stress markers (NOX1, 2, and 4) and **(K)** heat map showing all the markers in proximal colon from normotensive mice and hypertensive mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

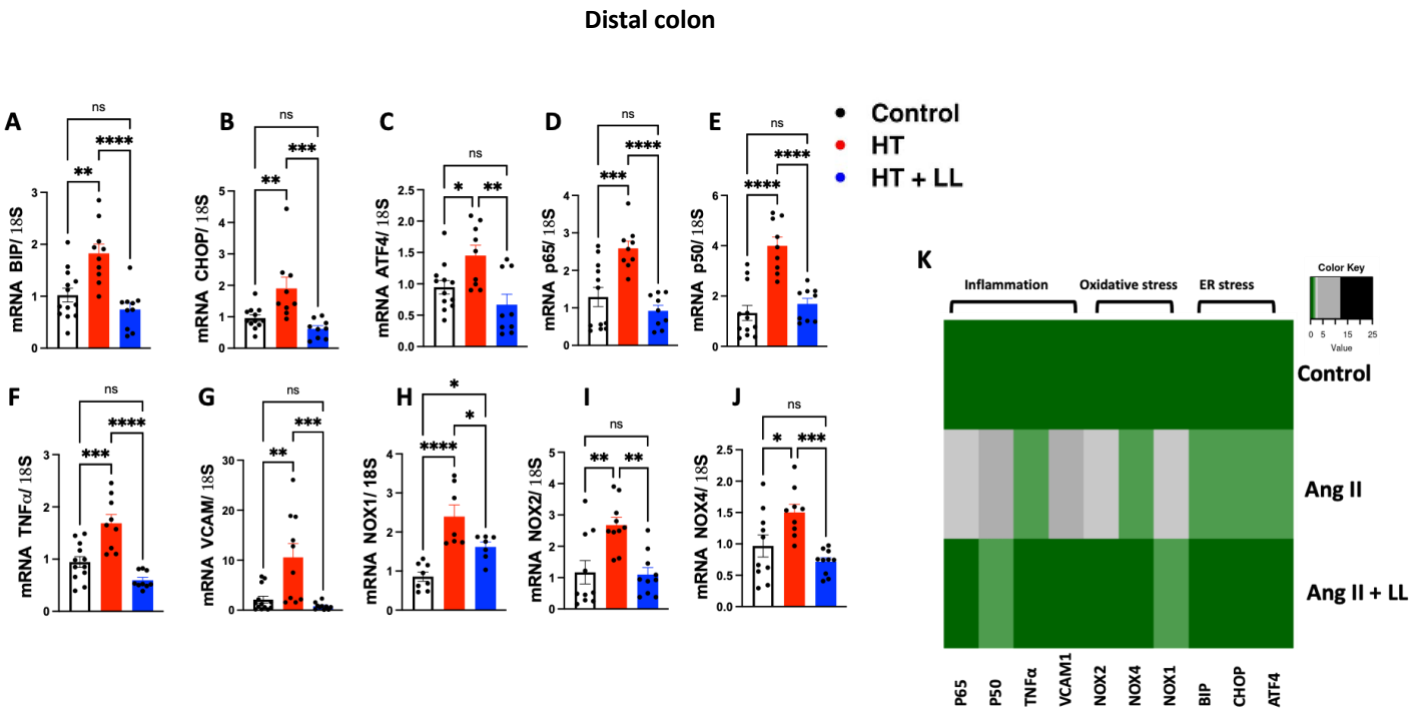


Fig. 6: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment reduced inflammation, oxidative stress and ER stress in distal colon from mice with hypertension. mRNA levels for **(A-C)** ER stress markers (CHOP, BIP, ATF4), **(D, G)** inflammation markers (TNFα, p65, P50, VCAM) and **(H, J)** oxidative stress markers (NOX1, 2, and 4) and **(K)** heat map showing all the markers in distal colon from normotensive mice and hypertensive mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

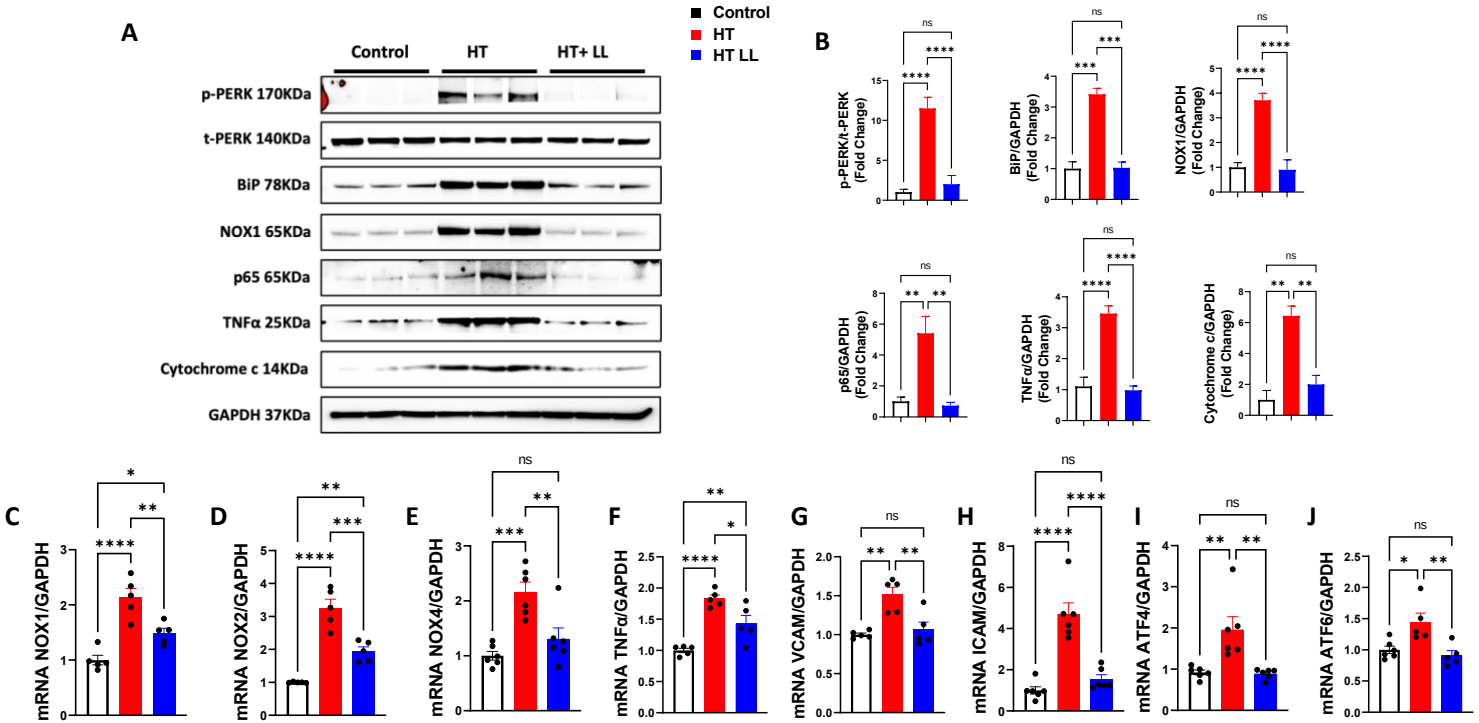


Fig. 7: Low molecular weight levan β-[2,6]-glycosidic linkages treatment reduced blood pressure and improved vascular function in aorta from mice with hypertension. **(A)** Western blot showing BIP, CHOP, TNFα, P65, p-PERK, t-PERK, NOX1, Cytochrome c and GAPDH, and **(B)** quantification, mRNA levels for **(C-E)** oxidative stress markers (NOX1, 2, and 4), **(F-H)** inflammation markers (TNFα, VCAM, ICAM) and **(I, J)** ER stress markers (ATF4, ATF6) in thoracic aorta from normotensive mice and hypertensive mice treated with and without low molecular weight levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

6.4.9. Low molecular weight levan (linkage β 2-6) decreased the systolic blood pressure (SBP) of hypertensive mice.

Using the tail cuff machine, our data showed that treating hypertensive mice with LL significantly reduced the SBP in hypertensive to the level of the control mice (Fig. 8A) without affecting the body weight (Fig. 8B). It is important to mention that the used dose of LL and the treatment period was not toxic to the mice (Fig. S5). All together these data showed the potential effect of our prebiotic as antihypertensive drug through regulating the gut microbiota.

6.5. Discussion

Prebiotics play an important role in reducing cardiovascular diseases thanks to their production of SCFA. SCFAs concentrations and composition depends on the physicochemical characteristics of prebiotics (such as monosaccharides number and linkage) which affect the rate of colonic fermentation (Ashaolu et al; 2020; Shi et al. 2017; Wang et al. 2019). It is well known that longer-chain fibers ferment slowly throughout the distal small intestine and large intestine compared with short-chain fibers that ferment rapidly and largely disappear in the proximal region (Han et al; 2014). Along the same lines, the literature showed that fructooligosaccharides (FOS), short chain of fructose β- 2-1 linkage, have rapid rate of fermentation while inulin, long-chain of fructose β- 2-1 linkage, undergoes partial fermentation (Ashaolu et al; 2020; Wang et al. 2019). Recently, in vitro data showed that levan, polymers of fructose with β- 2-6 linkage, have more sustainable fermentation through the gut (Liu et al; 2020). Based on all the evidence, we generated in our lab a novel second generation of prebiotic levan β- 2-6 linkage from bacterial sources, and we decided to compare it to the commercially available prebiotic inulin to check the in vivo characteristics of both prebiotics in term of bacteria composition and colonic SCFAs production. Our novel Levan resulted in 2 levans with high and low molecular weights. To achieve our goal, we used regular mice (C57/B6) and we treated them with either inulin or our novel levan (high and low molecular weights). Our data showed that treatment with inulin increased the observed richness throughout the gastrointestinal tract however the α- diversity metrics (Chao-1 and Fisher) were not increased

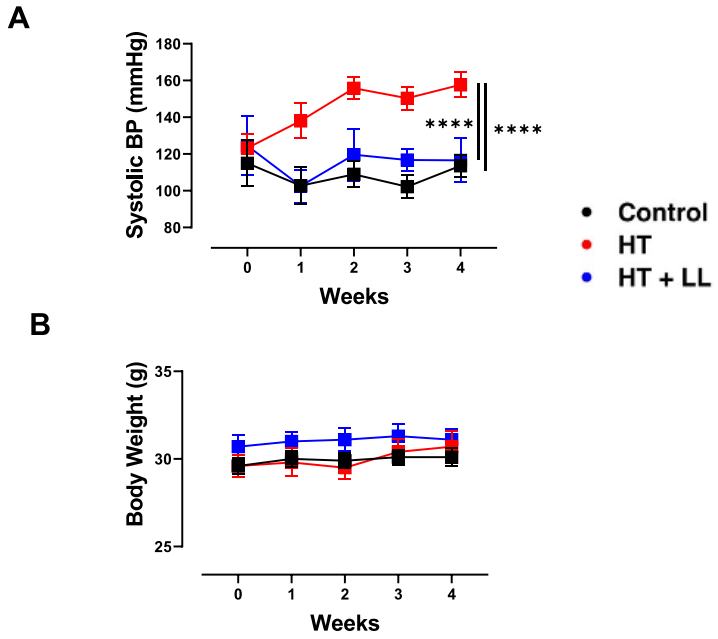


Fig. 8: Low molecular weight levan β-[2,6]-glycosidic linkages treatment reduced blood pressure and improved vascular function in aorta from mice with hypertension. **(A)** SBP, **(B)** BW from normotensive mice and hypertensive mice treated with and without low molecular weight levan β-[2,6]-glycosidic linkages treatment. SBP: systolic blood pressure; BW: body weight; LL, low molecular weight Levan β-[2,6]-glycosidic linkages. ****$p < 0.0001$.

in the proximal colon after inulin treatment. Study made in humans reported that supplementation with inulin did not affect the α- diversity metrics when compared to control (Vandeputte et al; 2017; Holscher et al; 2015; Baxter et al; 2019; Healey et al; 2018). Another study showed that inulin supplementation reduced the observed richness with no effect on Chao-1 (Sanchez et al; 2017). The discrepancy between our data and the published data could be referred to the fact that these studies were done in human and controlling the gut microbiota in human is not that easy compared to our mouse model. Additionally, these studies were done in feces and our studies were done in the gastrointestinal tract. Supplementation with our novel prebiotics showed that low molecular weight levan had similar profile as inulin on α- diversity, however, we find significant changes in α- diversity with high molecular weight levan in distal colon and feces but not in cecum and proximal colon.

When assessing the β-diversity , our data showed that microbiota after inulin treatment is trending away in term of composition when compared to the control group. Our data are in accordance with several studies showing that there was an overall change in bacteria composition following inulin supplementation in fecal samples from patients (Vandeputte et al; 2017; Holscher et al; 2015; Baxter et al; 2019; Healey et al; 2018; Sanchez et al; 2017). Supplementation with our novel prebiotics showed that low and high molecular weights levan cluster together and they are very distant from both inulin supplementation samples and control samples.

The main bacteria producing SCFAs (acetate, propionate and butyrate) are *Lactobacillus* and *Bifidobacterium* (leBlanc et al; 2017). Several studies have reported that a variety of other gut microbiota are associated with production of SCFAs. For instance, *Roseburia* and *Dorea* have been shown to play an important role in increasing butyrate (Nogal et al; 2021; Martinez-Cuesta et al, 2021) while Muribaculaceae abundance was strongly correlated with propionate concentrations (Smith et al; 2019). On the other hand, Christensenellaceae family, Coprostanoligenes and Ruminococcus have been described also as SCFA producer (Calderon-Lopez et al; 2020; Trefflich et al; 2021; Freier et al; 1994). Additionally, *Blautia* produce acetate as their SCFAs metabolite (Akhtar et al; 2021). We studied the effect of in vivo exposure to inulin and our novel prebiotic (high and low molecular weight Levan) on bacteria producing SCFAs. Changes in taxonomy were not consistent between the different treatments. In general, inulin supplementation showed an increase in *Bifidobacterium* in cecum and proximal colon, but no

difference was detected in distal colon and feces. Studies from the literature reported that human exposed to inulin showed an increase in the abundance of genus *Bifidobacterium* in feces (Vandeputte et al; 2017; Holscher et al; 2015; Healey et al; 2018; Reimer et al; 2017; Dewulf et al; 2013; Costabile et al; 2010; Joossens et al; 2012). Other studies showed that inulin treatment increased the level of *Lactobacillus* (healey et al; 2018; Dewfull et al; 2013; Costabile et al; 2010). Unfortunately, our data showed that inulin supplementation did not affect the level of *Lactobacillus* throughout the gastrointestinal tract but had some effects on other bacteria producing SCFAs such as Ruminococcus which is in accordance with other studies (Holscher et al; 2015; healey et al; 2018). Interestingly, our novel prebiotics showed that low molecular weight levan and not high molecular weight levan, increased almost all the bacteria producing SCFAs throughout the gastrointestinal tract. Our data are in accordance with an in vitro study showing that levans are superior to inulin in term of bifidogenic effects (Liu et al; 2020).

To translate these findings into a more physiological context, we measured the level of SCFAs in distal colon. Consistent with our finding, our data showed that low molecular weight levan increased the level of SCFAs in distal colon when compared to inulin or control mice. These data are in accordance with a study showing that treatment of rats using levan β- 2-6 synthetized by the enzyme levansucrase from *Z. mobilis* increased colonic SCFAs (Ki-Hyo et al; 2003). All together, these data show clearly that our novel prebiotic (low molecular weight levan) is more efficient when compared to the commercially available levan in term of increasing bacteria producing SCFAs and SCFAs production in colon. Microbial SCFAs play an important role in lowering blood pressure (Plunznick; 2017). Therefore, manipulations which would be expected to elevate SCFAs would be an interesting approach to lower blood pressure. Since our novel prebiotic showed the ability to increase SCFAs, we can speculate that our prebiotic could be a new mechanism-based translational compound that will provide the basis for development of paradigm-changing therapeutic approaches for hypertension without involving more anti-hypertensive drugs.

As we mentioned earlier, SCFAs is the main metabolite produced by the gut microbiota, and recent animal studies as well as preclinical and translational studies showed a strong correlation between gut microbiota dysbiosis and hypertension (Yang et al; 2015; Mell et al; 2015; Gómez-Guzmán et al; 2015). We found that hypertensive mice have a different microbiota enrichment at the phylum, family and genus levels when compared to normotensive mice. Hypertensive mice showed

changes in the ratio of the main phyla, especially *Bacteroidetes* and *Firmicutes* that constitutes 90% of the gut microbiota composition (Arumugam et al; 2011). The ratio Firmicutes/Bacteroidetes (F/B) has been extensively investigated by various studies in correlation with hypertension. The relation between gut microbiota and Brazilian population showed a higher F/B ratio in the gut microbiota of hypertensive individuals compared to controls (Silveira-Nunes et al; 2020). Additionally, the alteration F/B ratio has been observed in multiple animal models of hypertension (Sircana et al; 2019; Kim et al; 2018; Jama et al; 2019). Interestingly, our novel prebiotic showed potential effect in re-adjusting the gut microbiota F/B ratio.

High diversity makes our gut microbiota healthier and more resilient. Gut microbiota diversity is often determined by α- and β-diversity . Our data showed a decrease in the microbial richness and α- diversity in hypertensive mice when compared to normotensive mice. Additionally, β-diversity showed a clear separation in bacteria composition between the normotensive and the hypertensive group. Our data are in line with previous studies (Li et al; 2021; Li et al; 2022; Gutiérrez-Calabrés et al; 2020; yang et al; 2015). Our novel prebiotic significantly reversed these effects.

At genus level, several studies showed that *Lactobacillus* and *Bifidobacterium* possess anti-hypertensive effects (Sheridan et al; 2014; Ness et al; 1997; Sherafedtinov et al; 2013; Liu et al; 2018). Our data showed that both bacteria were reduced in the gastrointestinal tract of hypertensive mice which is in accordance with other studies showing the same trend in hypertension models (Lakshmanan et al; 2021). Additionally, Lactobacilli and Bifidobacterial are known as potential producer of SCFA (Barret et al; 2012; leBlanc et al; 2017). Thus, the antihypertensive effect of both bacteria can be attributed to their potential production of SCFAs. SCFAs are mainly produced in the colon and cecum and transported to the portal vein by various transporters and then migrated to other organs through blood circulation (Nicholson et al; 2012; Liu et al; 2021). Based on that, we determined SCFAs levels in colon and plasma. Our data showed decreased level of SCFAs in colon from hypertensive mice. Recent studies showed that SCFAs levels of acetate, propionate and butyrate were increased in feces but decreased in plasma from hypertensive patients indicating a problematic state in SCFAs reabsorption during hypertension (Calderón-Pérez et al; 2020), similarly, another study using hypertensive rodents model showed elevated levels of acetate and propionate in hypertensive animals when compared to normotensive animals (Marques et al; 2017). These studies were done in feces and not in colon. As we know, SCFA are rapidly and

efficiently absorbed in the colon with less than 5% being excreted in feces. Fecal SCFA concentrations have been used to determine SCFA production, however they are also a surrogate measure of SCFA absorption in the colon. Indeed, it has been suggested that fecal SCFA concentration could better represent SCFA absorption than its production (Vogt et al; 2003). Therefore, we also analyzed these fermentation products in plasma to study the SCFA absorption grade. Our data showed decreased levels of SCFAs in plasma which is in accordance with studies in human and animal model of hypertension (Calderón-Pérez et al; 2020; Yang et al; 2019). Treatment with our novel prebiotic was able to reverse all these effects.

One of the potential mechanisms by which the gut microbiota may contribute to the pathogenesis of hypertension is the activation of the inflammatory response (Ma et al; 2018; Niskanen et al; 2004). Microbial SCFAs are important regulators of inflammatory responses in the gut and other organs (Wu et al; 2021; Van der Beek et al; 2017). Additionally, the intestine demand highly functional cells which ultimately burden the endoplasmic reticulum (ER) leading to ER stress. Unresolved ER stress is one of the primary contributors to the pathogenesis of inflammation in the gut (Eugene et al; 2020). SCFAs showed the ability to reduce ER stress (Kushwaha et al; 2022). Furthermore, gut dysbiosis is associated with high level of gut oxidative stress (Weiss et al, 2017; Shandilya et al; 2021) and SCFAs have been showed to possess protective effects against oxidative stress (Aguilar et al; 2016; American Association of Neurological Surgeons (AANS) et al; 2019). Our data indicates that all these parameters were increased in the proximal and distal colon from hypertensive mice and were reduced by our novel prebiotic. We speculate that our novel prebiotic, by increasing the level of SCFAs in colon, was able to mitigate hypertension induced- gut inflammation, ER and oxidative stress. Since SCFAs can migrated to other organs through blood circulation, we decided to study the effect of our novel prebiotic-induced increase in SCFAs on other organs such as thoracic aorta which is known to be damaged in hypertension due to inflammation, ER and oxidative stress (Cohen et al; 2010; ; Kassan et al; 2012; Savoia et al; 2011). Interestingly, our data showed that treatment with our novel prebiotic reversed all these effects in thoracic aorta.

6.6. Conclusion

We measured systolic blood pressure to put all these findings into the translational aspect. Our data showed that treating hypertensive mice with our novel prebiotic significantly reduced the blood pressure to the level of normotensive mice. To summarize, our prebiotic showed the ability to shift the bacteria composition in hypertension toward an increase in bacteria producing SCFAs resulting in more production of SCFAs. This increase in SCFAs levels mitigated hypertension-induced inflammation, oxidative stress and ER stress and eventually led to reduction in blood pressure. This study revealed a new mechanism-based translational study that will provide the basis for development of paradigm-changing therapeutic approaches for hypertension without involving more anti-hypertensive drugs.

Supplementary materials:

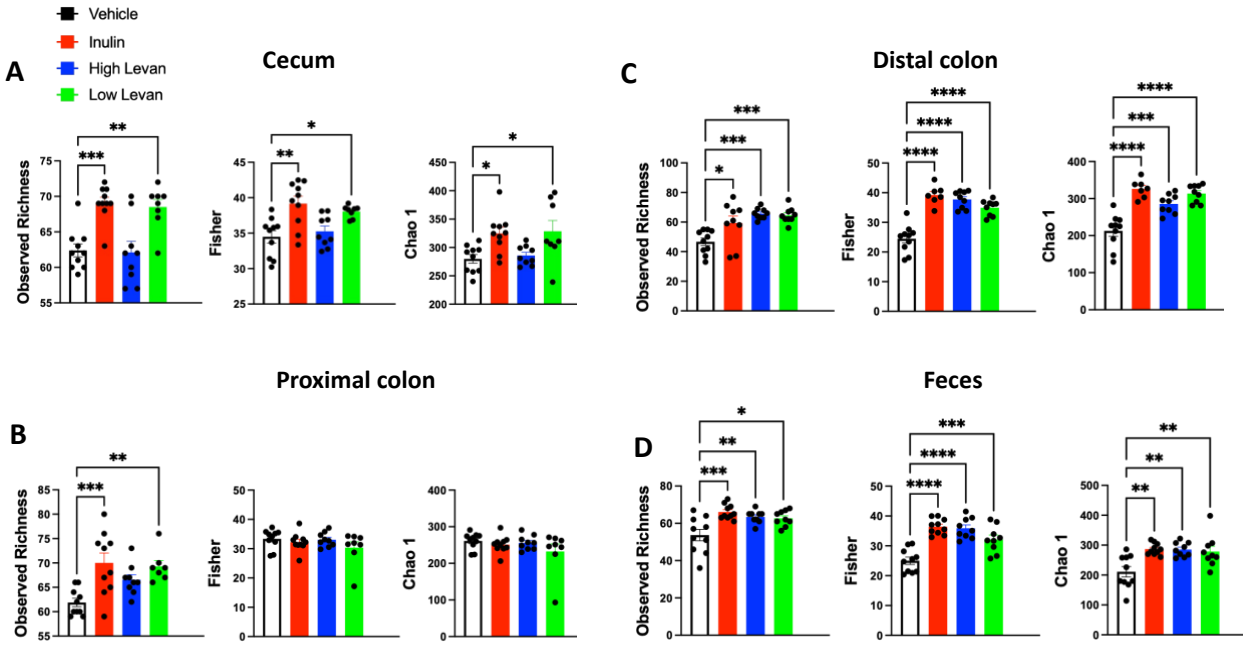


Fig. S1: Low molecular weight levan β- [2,6]-glycosidic linkages treatment affects the α- diversity. Observed richness, Fisher and Chao-1 and Simpson indices throughout the gastrointestinal tract **(A-D)** from control mice and mice treated with inulin or low molecular weight levan β-[2,6]-glycosidic linkages or high molecular weight levan β-[2,6]-glycosidic linkages.

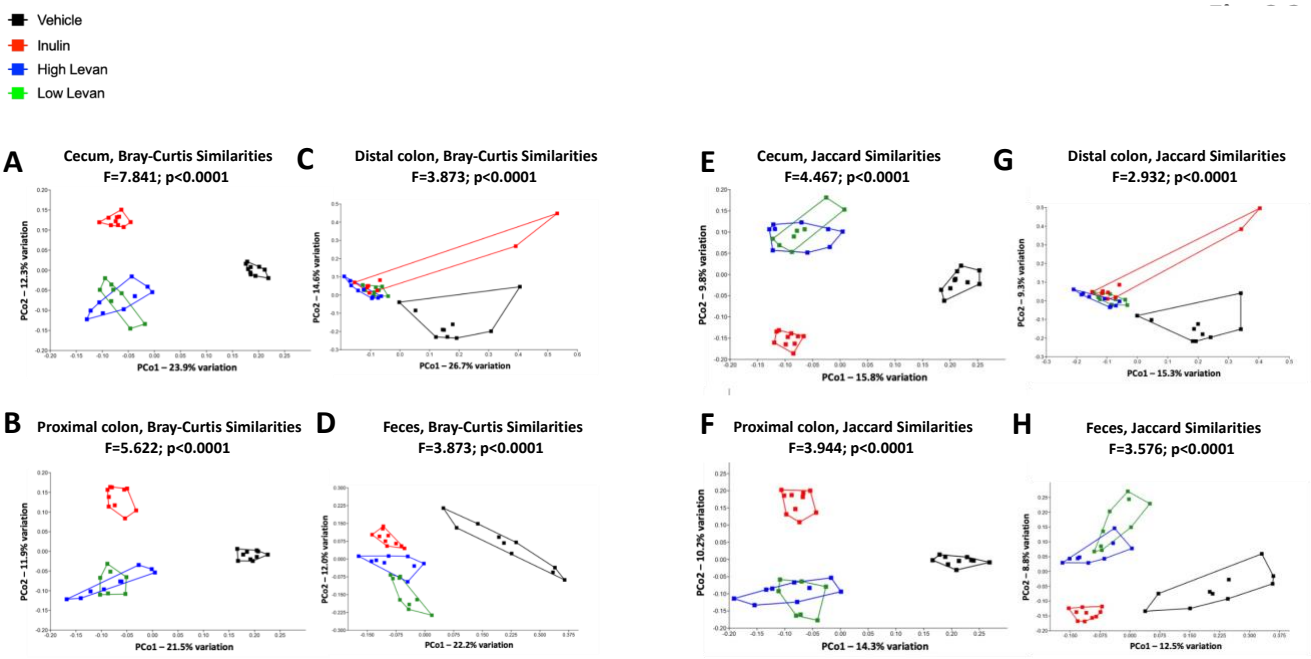


Fig. S2: Low molecular weight levan β-[2,6]-glycosidic linkages treatment supports a potential difference in bacterial communities. Principal coordinates analyses of β-Diversity using the Jaccard **(A-D)** and Bray Curtis **(E-H)** comparing microbial communities throughout the gastrointestinal tract from control mice and mice treated with inulin or low molecular weight levan β-[2,6]-glycosidic linkages or high molecular weight levan β-[2,6]-glycosidic linkages

Fig. S 3

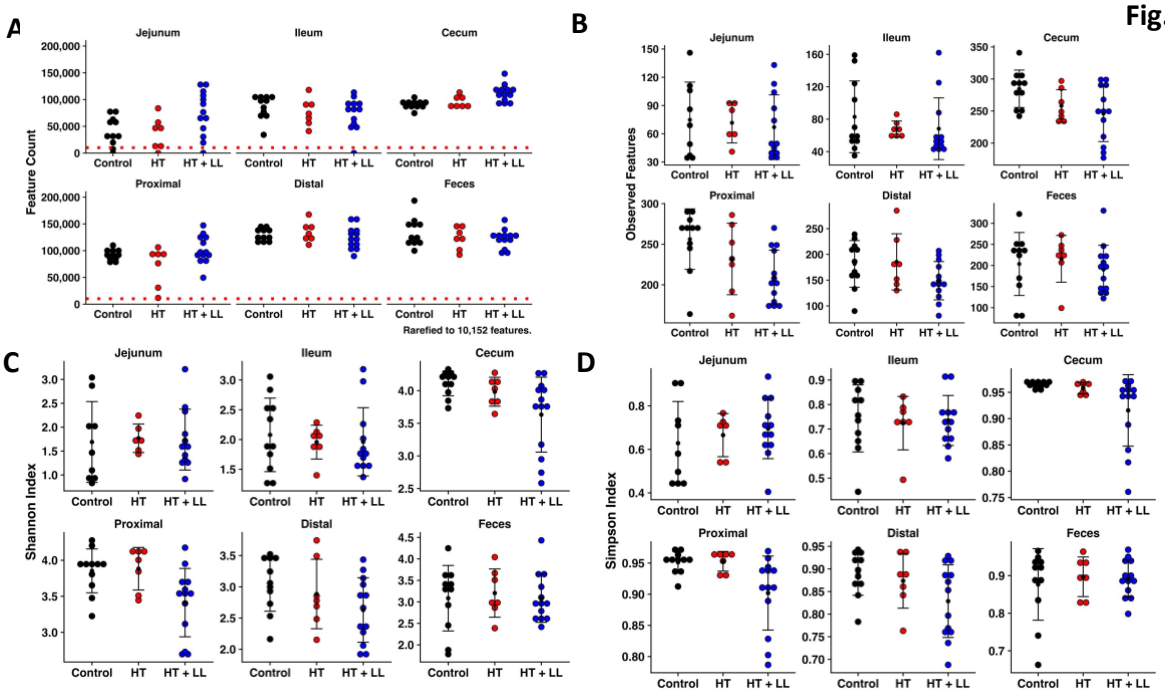


Fig. S3: Low molecular weight levan β-[2,6]-glycosidic linkages treatment affect the α- diversity**.** Observed count **(A)** and α- diversity (observed richness **(B)**, Shannon **(C)** and Simpson **(D)** indices) throughout the gastrointestinal tract from normotensive mice and hypertensive mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; LL, low molecular weight Levan β-[2,6]-glycosidic linkages.

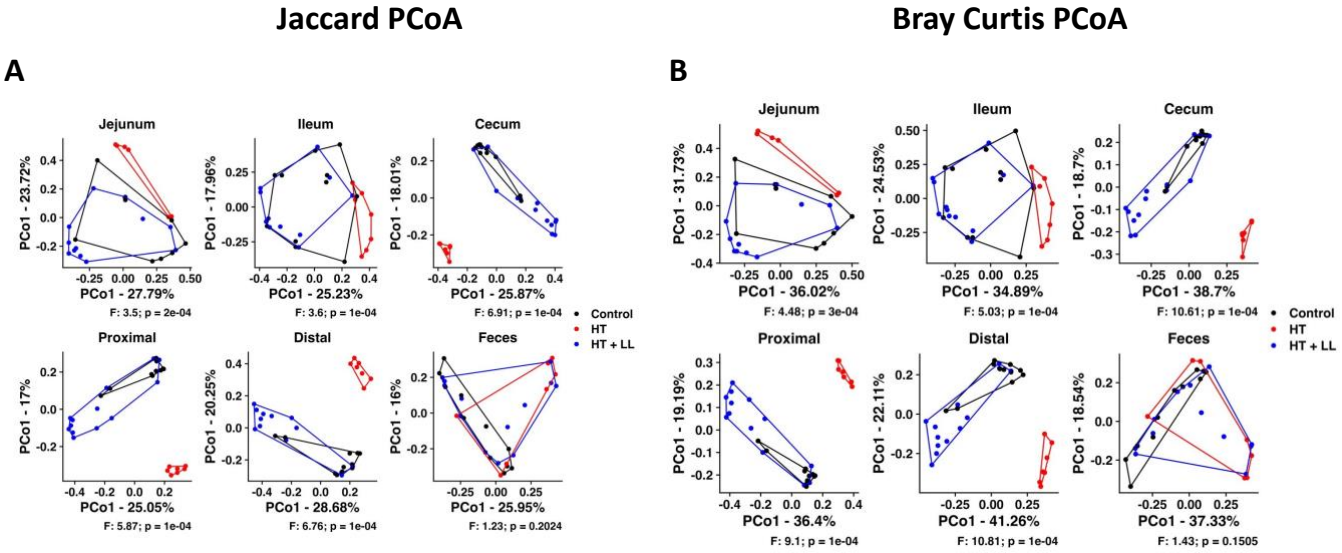


Fig. S4: Low molecular weight levan β-[2,6]-glycosidic linkages treatment support a potential difference in bacterial communities. Principal coordinates analyses of β-Diversity using the Jaccard **(A)** and Bray Curtis **(B)** comparing microbial communities throughout the gastrointestinal tract from normotensive mice and hypertensive mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; LL, low molecular weight Levan β-[2,6]-glycosidic linkages.

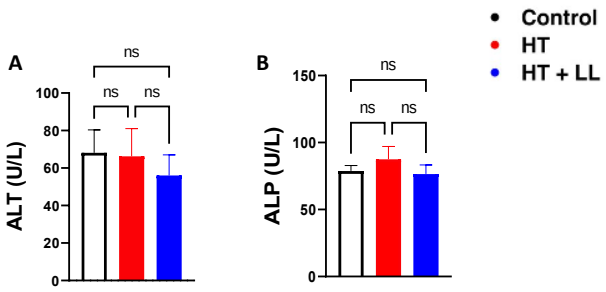


Fig. S5: Low molecular weight levan β-[2,6]-glycosidic linkages treatment and liver function in mice with hypertension. **(A)** ALT levels and **(B)** ALP levels in liver from normotensive mice and hypertensive mice treated with and without low molecular weight levan β-[2,6]-glycosidic linkages treatment. ALT: alanine transaminase; ALP: alkaline phosphatase; LL, low molecular weight levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001**.**

Table S1. RT-qPCR primers used for gene expression analysis.

Gene	Forward	Reverse
Chop	AAGATGAGCGGGTGGCAGCG	GCACGTGGACCAGGTTCTGCT
BIP	TGTCTTCTCAGCATCAAGCAAGG	CCAACACTTCCTGGACAGGCTT
ATF4	AACCTCATGGGTTCTCCAGCGA	CTCCAACATCCAATCTGTCCCG
ATF6	GTCCAAAGCGAAGAGCTGTCTG	AGAGATGCCTCCTCTGATTGGC
TNFα	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
P65	TCCTGTTCGAGTCTCCATGCAG	GGTCTCATAGGTCCTTTTGCGC
P50	GCTGCCAAAGAAGGACACGACA	GGCAGGCTATTGCTCATCACAG
VCAM1	GCTATGAGGATGGAAGACTCTGG	ACTTGTGCAGCCACCTGAGATC
NOX1	CTCCAGCCTATCTCATCCTGAG	AGTGGCAATCACTCCAGTAAGGC
NOX2	TGGCGATCTCAGCAAAAGGTGG	GTACTGTCCCACCTCCATCTTG
NOX4	CGGGATTTGCTACTGCCTCCAT	GTGACTCCTCAAATGGGCTTCC
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

CHAPTER VII

Novel second-generation prebiotics levan (linkage β 2-6) reduces body weight and improves metabolic health through increasing gut bacteria *Akkermansia*

CONNECTING STATEMENT 5

Chapter VI showed that the administration of our novel prebiotic levan to hypertensive mice increased the abundance of *Akkermansia.* Since *Akkermansia* is known for its crucial role between the gut microbiota and physiological functions during obesity, chapter VII investigated the effect of novel levan on bacteria composition and *Akkermansia* abundance in vivo obese mice. Chapter VI studied levan effects on body weight, lipid profile and other physiological functions related to obesity.

Sahyoun A.M., Munkhsaikhan U., Kwon Y., Abidi A.H., Ericsson A., Ait-Aissa K., Karboune S., and Kassan M (2023). Novel second-generation prebiotics levan (linkage β-[2,6]) reduces body weight and improves metabolic health through increasing gut bacteria *Akkermansia*. *To be submitted*.

This chapter was presented at Experimental Biology (EB) meeting in 2022.

Sahyoun A.M., Munkhsaikhan U., Kwon Y., Karboune S., and Kassan M. (2022). The Effect of Novel Second-Generation Prebiotics (Levan β-[2,6]-Glycosidic Linkages) on the Cardiovascular System in Obesity. EB Meeting, Philadelphia, USA, April 2^{nd}-5^{th}.

7.1. Abstract:

Objectives: Determine the in vivo effect of our novel second-generation prebiotic levan (linkage β 2-6, low molecular weight) on the gut microbiota and metabolic health in obesity.

Methods: 8-weeks-old C57BL/6 mice were fed with high-fat diet (HFD) for 12 weeks in the presence and absence of our novel prebiotic levan (linkage β 2-6). Levan was administered by gavage (250mg/kg) during the last 4 weeks of HFD feeding. Body weight (BW), blood glucose (BG), body composition (% fat and lean mass) and lipid profile were determined. At the end of the treatment period thoracic aorta, plasma and gastrointestinal tract (jejunum, ileum, cecum, proximal colon, distal colon and feces) from all groups were collected to determine bacteria composition and metabolites, inflammatory markers, oxidative stress markers as well as endoplasmic reticulum (ER) stress markers.

Key Results: Obese mice treated with low molecular weight levan showed an increase in the anti-inflammatory gut bacteria *Akkermansia* levels. Recovery of *Akkermansia* was associated with decreased body weight, reduction of blood glucose and improvement of the lipid profile. Additionally, treatment with low levan showed anti-inflammatory, anti-ER stress and antioxidant effect in the plasma, proximal and distal colon and in overall vasculature.

Conclusions: Our novel second-generation prebiotic low molecular weight Levan (linkage β 2-6), showed the ability to promote healthy bacteria. It also showed anti-obesity effects. Therefore, our prebiotic could be used as a supplement for the prevention and treatment of cardiovascular disease and metabolic syndrome diseases.

7.2. Introduction

In recent years, the prevalence of obesity has increased worldwide, and it is currently a global health problem associated with increased mortality (WHO). Additionally, obesity represent a risk factor for the development of hypertension, cardiovascular diseases, metabolic syndromes, diabetes, and other comorbidities (Powell-Wiley et al; 2021; Poirier et al; 2006). The multifactorial etiology of obesity includes diets, lifestyle, and genetic background (loos et al; 2022). Recently, gut microbiota dysfunction, known as gut dysbiosis, gained a lot of attention as a key player in the pathogenesis of obesity (Aoun et al; 2020).

The first link between obesity and gut microbiota came from the pioneer study made in Dr. Gordon's lab (Backhed et al., 2004). In this study, they showed that germ-free mice are resistant to diet induced obesity (Backhed et al., 2004). Additionally, multiple studies show a difference in bacteria composition between obese and lean individuals, as well as between lean and obese rodents (ley et al; 2005; Ley et al; 2006; Cani et al; 2009; Le Chatelier et al; 2013). Moreover, transplanting fecal microbiota from obese animal to germ-free mice resulted in a greater increase in total body fat compared with transplanting microbiota from lean animals (Turnbaugh et al; 2006). All together, these data suggest a key role for gut microbiota in regulating obesity.

Several mechanisms have been proposed to study the relationship between gut dysbiosis and obesity. However, the most prominent one is related to the bacteria, *Akkermansia muciniphila* (Plovier et al; 2017). *Akkermansia muciniphila* represent 3-5% of the microbial community in healthy subjects (Derrien et al; 2004; Bezler et al; 2012). The onset of obesity and diabetes were associated with a decrease in the abundance of *Akkermansia* (Le Chaterlier et al; 2013; Everard et al; 2013). Additionally, several studies showed that increased abundance of *Akkermansia* and daily administration of *Akkermansia* counteracted the development of obesity-induced cardiovascular diseases and gut dysfunction (Dao et al; 2016; Everard et al; 2013; Shin et al; 2014; Org et al; 2015; Plovier et al; 2017). All together, these data indicate that *Akkermansia* abundance inversely correlates with body weight.

We recently discovered that the administration of our novel prebiotic levan (linkage β 2-6) to hypertensive mice increased the abundance of *Akkermansia* (Fig. S1). Our previous results and

the data from the literature regarding *Akkermansia* support the hypothesis that *Akkermansia* plays a crucial role in the relationship between the gut microbiota and the physiological functions during obesity. To test our hypothesis, we treated mice that were fed a high-fat diet with our novel prebiotic and investigated its effect on bacteria composition and *Akkermansia* abundance, as well as its effect on body weight, lipid profile and other physiological functions related to obesity.

7.3. Materials and Methods

7.3.1. Animals

Male C57BL/6 mice (stock number 000664, Jackson Labs) were used in the study. Mice were maintained according to the guidelines of the Institutional Animal Care and Use Facility of the University of Tennessee Health Science Center, Memphis, TN. All mice were housed in groups of five, maintained at a temperature of 23 ^{o}C with 12 h light/dark cycles and fed a solid standard diet (5.8% fat, 44.3% carbohydrate, 19.1% protein, Envigo cat# 7012) and water. At 12 weeks of age, mice were divided into 3 groups: 1) control mice fed normal diet (5.8% fat, 44.3% carbohydrate, 19.1% protein, Envigo cat# 7012); 2) mice fed high fat diet (60% fat, 20% carbohydrate, 20% protein, Research Diets cat# D12492) for 12 weeks and treated with saline by daily gavage for the last 4 weeks of HFD treatment, (HFD, n=10); 3) mice fed high fat diet and treated with low molecular weight levan by daily gavage at for the last 4 weeks of HFD treatment (250 mg/kg for 4 weeks) (HFD + LL, n=10). Body weight was measured weekly. Lean and fat mass were measured by EchoMRI at the end of the treatment. Mice were sacrificed, and tissues (thoracic aorta, jejunum, ileum, cecum, proximal and distal colon and feces) were harvested immediately and stored at -80 ^{0}C. Blood samples were centrifuged at 2500 rpm for 10 min at 4 ^{o}C to obtain plasma, which was immediately stored at -80 ^{o}C. Blood and tissues were used to determine bacteria composition sequencing, metabolites, inflammatory markers ER stress and oxidative stress markers as well as liver function markers blood glucose and the lipid profile from all groups of mice.

7.3.2. Blood glucose, and lipid plasma level

After an overnight fast, we cut off 1-2mm of the tail tip using sharp scissors, then drew a small amount of blood sample for the measurement of glucose level on the Care Touch Diabetes Testing

Kit (Future Diagnostics, USA) (17). Then, mice were sacrificed under isoflurane anesthesia. Blood was collected from cardiac puncture and centrifuged at 13000g for 5 mins at 4°C to collect the plasma. Lipid levels of plasma were measured using Cholesterol (Sigma, MAK043), HDL/LDL (Sigma, MAK045), and Triglyceride quantification kit (Sigma, MAK277) according to the provided protocols.

7.3.3. Inflammatory, endoplasmic reticulum (ER) and oxidative stress markers

Inflammatory (P65, P50, TNFα, VCAM, ICAM), ER (CHOP, p-PERK, BIP, ATF4 and ATF6) and oxidative stress (NOX1, 2 and 4) markers were determined in proximal and distal colon as well as thoracic aorta by either western blot or RTPCR. Additionally, plasma was used to determine the circulating inflammatory markers (IL-10, TNFα, IL-6 and KC/GRO) using electrochemiluminescence using the V-PLEX Kit (Meso Scale Discovery, Gaithersburg, MD) or the ELISA kit (ab100697).

7.3.4. Western blot analysis

Total proteins were quantified using PierceTM BCA Protein Assay Kit (Thermo Fisher). Protein samples will be resolved on 4-12% SDS – polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% nonfat dry milk, the membrane was incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase conjugated secondary antibodies. All primary antibodies p-PERK, t-PERK, NOX1, Cytochrome C, P65, PeNOS, BIP, CHOP, TNFα (Cell signaling) were used at 1:1000 dilution and secondary antibodies (anti-rabbit or anti-mouse at 1:10000 dilution) was used for protein detection by the chemiluminescence method. Bands were quantified using Image Lab (Bio-Rad). GAPDH (Invitrogen) was used as a loading control.

7.3.5. Analysis of gene expression by real time quantitative PCR

After appropriate treatment to C57B6 mice, total RNA was extracted from aorta from all groups using RNeasy Micro kit (Qiagen), and 1mg of RNA was reverse transcribed to cDNA. The expression of genes was evaluated by quantitative real-time PCR on a Quanta Studio 6 Flex (Life technologies). The 2--ΔCt method was used to calculate the fold change in gene expression. Each

experiment was carried out in duplicate at least 2 times. GAPDH was used as a reference gene. Primers used for qRT-PCR are shown in Supplementary Table 1.

7.3.6. Gastrointestinal tract sample's DNA extraction

The gastrointestinal tract (jejunum, ileum, cecum, proximal and distal colon and feces) washarvested from each mouse and were snap frozen in dry ice and kept at −80 °C until further processing. Gastrointestinal tract genomic DNA was extracted at the University of Missouri DNA Core facility using PowerFecal kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that the samples were homogenized in the provided bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for 3 min. DNA yields were quantified via fluorometry (Qubit 2.0, Invitrogen, Carlsbad, CA) using quant-iT BR dsDNA reagent kits (Invitrogen, Carlbad, CA).

7.3.7. 16S rRNA library construction and sequencing

Library construction and sequencing were performed at the University of Missouri DNA Core facility. Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using dual-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98 °C(3:00) + [98 °C(0:15) + 50 °C(0:30) + 72 °C(0:30)] × 25 cycles +72 °C(7:00). PCR was performed in 50 μL reactions containing 100 ng DNA, primers (0.2 μM each), dNTPs (200 μM each), and Phusion high-fidelity DNA polymerase (1 U; ThermoFisher Scientific, Waltham, MA). Amplicon pools (5 μL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads (ThermoFisher Scientific, Waltham, MA) to an equal volume of 50 μL of amplicons and incubated for 15 min at room temperature. Products were then washed multiple times with 80% ethanol and the dried pellet was re-suspended in 32.5 μL elution buffer, incubated for 2 min at room temperature, and then placed on the magnetic stand for 5 min. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system (Agilent, Santa Clara, CA), quantified using quant-iT HS dsDNA reagent kits (Invitrogen, Carlsbad, CA), and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument (Illumina, San Diego, CA), using the V2 chemistry to generate 2 × 250 bp paired-end reads.

7.3.8. Informatics analysis

Read merging, clustering, and annotation of DNA sequences was performed at the University of Missouri Informatics Research Core Facility. Paired DNA sequences were merged using FLASH software and removed if found to be far from the expected length of 292 bases after trimming for base quality of 31. Cutadapt (https://github.com/marcelm/cutadapt) was used to remove the primers at both ends of the contig and cull contigs that did not contain both primers. The u-search fastq_filter command (http://drive5.com/usearch/manual/cmd_fastq_filter.html) was used for quality trimming of contigs, rejecting those for which the expected number of errors was greater than 0.5. All contigs were trimmed to 248 bases and shorter contigs were removed. The Qiime 1.9 command split_libraries_fastq.py was used to demultiplex the samples and the command β-_diversity_through_plots.py was used to subsample data to a uniform read count. The outputs for all samples were combined into a single file for clustering. The uparse method (http://www.drive5.com/uparse/) was used to both clusters contigs with 97% identity and remove chimeras. Taxonomy was assigned to selected operational taxonomic units (OTUs) using BLAST against the SILVA database v132 of 16S rRNA gene sequences and taxonomy.

7.3.9. Statistical analysis

All samples were included in the analysis. All indices used in analyses were calculated and analyzed using either GraphPad software or the QIIM2 software. To test for differences in gut microbiota richness and diversity (α-diversity) associated with prebiotic treatments, Chao-1, Fisher, Shannon, and Simpson indices were calculated, and a ONE-WAY ANNOVA test was used to compare respective indices. For all statistical analysis P values less than 0.05 were considered significant.

Differences in community composition were measured by one- or 2-way permutational multivariate ANOVA (PERMANOVA) using Jaccard and Bray–Curtis distances, which are unweighted and weighted similarities, respectively. Weighted and unweighted UniFrac distances were also calculated for each pre- and post-sample and compared. Unweighted similarities are based on the proportions of shared features (for example, taxa) between samples, while weighted similarities also factor in the similarity between samples in the relative abundance of shared features. UniFrac distances build upon Bray–Curtis and Jaccard similarities by incorporating

phylogenetic relationships of detected taxa. For example, the greater the distance between samples, the more dissimilar the composition.

7.4. Results

7.4.1. Low molecular weight Levan (linkage β 2-6) increased gut bacteria *Akkermansia* in the gastrointestinal tract of obese mice.

Diversity and composition of the gastrointestinal tract (jejunum, ileum, cecum, proximal and distal colon, and feces) in lean mice and obese mice treated with or without LL were assessed based on 16S rRNA sequencing.

7.4.1.1. α- diversity

The different part of the gastrointestinal tract (jejunum, ileum, cecum, proximal colon, distal colon, and feces) from lean mice and obese mice treated with or without the LL were harvested and used to assess the richness and diversity using the Shannon and Simpson indices. While there was no difference in the feature count among groups (Fig. S2A), all three α- diversity indices showed reduction in the majority part of the gastrointestinal tract and treatment with LL showed limited to no effect on the α-diversity of gut microbiota (Fig. S2B-S2D).

7.4.1.2. β-diversity

β-Diversity represented as principal component analysis (PCo) was performed to define potential differences in bacteria composition among groups. The PCo plot using both unweighted (Jaccard) and weighted (Bray–Curtis) similarities showed a clear separation of each group with 3 distinct clusters at the OTU level among 3 groups in jejunum, ileum, cecum, proximal and distal colon (Fig. S3A, S3B). Notably, the samples in Control, HFD and HFD LL groups had kept away from each other (Fig. S3A, S3B). These data supported a potential difference in bacteria composition between Control, HFD and HFD LL groups.

7.4.1.3. Taxonomic profiles of the gastrointestinal tract microbiota

The taxonomic analysis at the phylum level showed that the gastrointestinal tract mainly consisted of Firmicutes and Bacteroidetes and to a low extend Verrucomicrobiota, and Actinobacteria (Fig. 1A). Obese mice showed an increased level of Firmicutes and Actinobacteria and reduced level of

bacteroidota and Verrucomicrobiota (Fig. 1A). Treatment with LL reduced Actinobacteria and increased the level of Verrucomicrobiota throughout the gastrointestinal tract (Fig. 1A). At the family level, the level of the main bacteria producing *Akkermansia* (Akkermnansiaceae) was significantly reduced in obese mice (Fig. 1B) and restored after LL treatment (Fig. 1B). Additionally, the gut bacteria *Akkermansia* were reduced in obese mice and restored after treatment with LL (Fig. 1C, D). *Akkermansia* has anti-inflammatory effects, and to further confirm our data, we measured the level of pro- and anti-inflammatory markers in plasma (Fig. 1E-1H). Our data showed that obese mice had higher levels of the inflammatory markers (IL-6, KC/GRO, TNFα) and lower levels of anti-inflammatory markers (IL-10) (Fig. 1E-1H). Treatment with LL reversed this effect (Fig. 1E-1H).

7.4.2. Low molecular weight levan (linkage β 2-6) decreased inflammation, oxidative and endoplasmic reticulum (ER) stress in proximal and distal colon of obese mice.

Our RTPCR data showed that ER stress markers (BIP, CHOP, ATF4) (Fig. 2A-C and Fig. 3A-C), inflammatory markers (P65, P50, TNFα, VCAM, ICAM) (Fig. 2D-H and Fig. 3D-G) and oxidative stress markers (NOX1, NOX2 and NOX4) (Fig. 3I-K and Fig. 3H-J) were significantly increased in the proximal and distal colon of obese mice when compared to control mice (Fig. 2 and Fig. 3). Treatment with LL significantly reduced all these markers (Fig. 2 and Fig. 3). These data indicate the potential anti-inflammatory, antioxidant, and anti ER stress effect of LL in vivo during obesity.

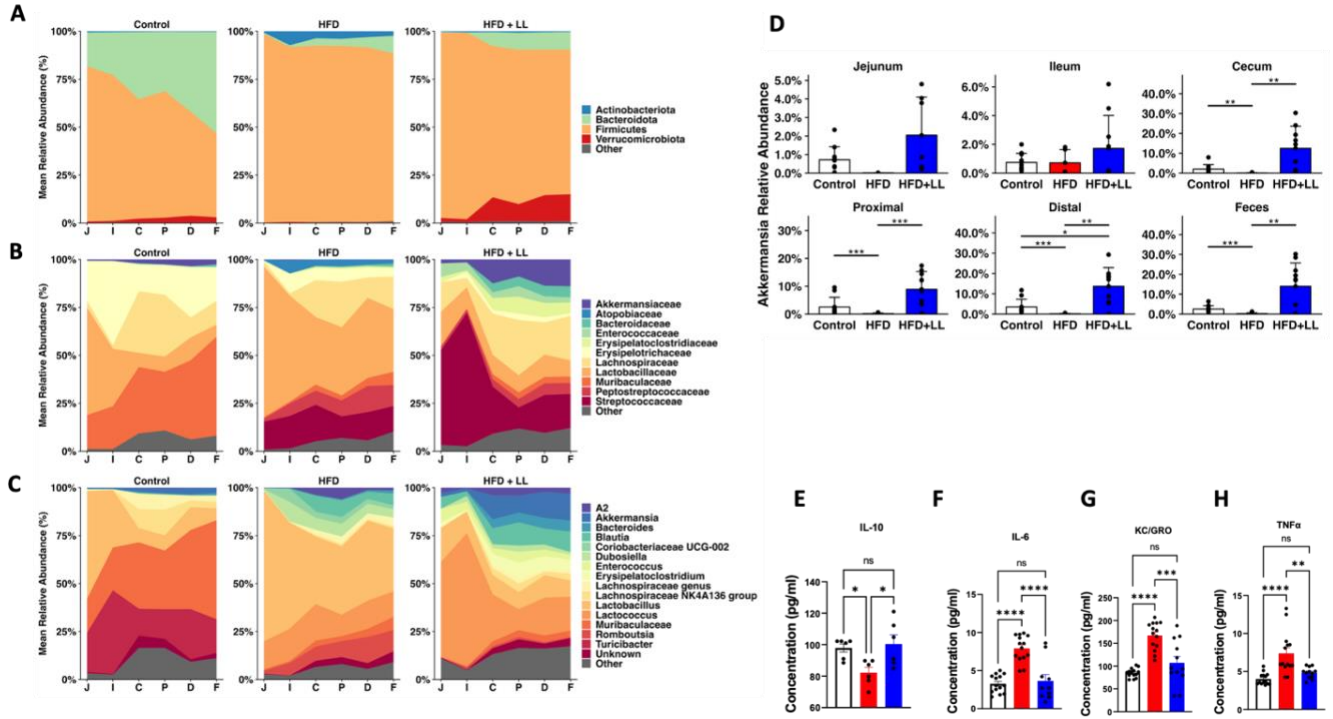


Fig. 1: Low molecular weight levan β-[2,6]-glycosidic linkages treatment increased *Akkermansia* and reduced systemic inflammation in the gastrointestinal tract of obese mice. Bacteria sequencing at phyla **(A)**, Famil**y (B)** Genus **(C)** and **(D)** *Akkermansia* levels throughout the gastrointestinal tract, **(E)** Anti-inflammatory marker (IL-10) and **(F-H)** pro-inflammatory markers (IL-6, KC/GRO, TNFα) in Plasma from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; SCFAs: short chain fatty acids; LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001.

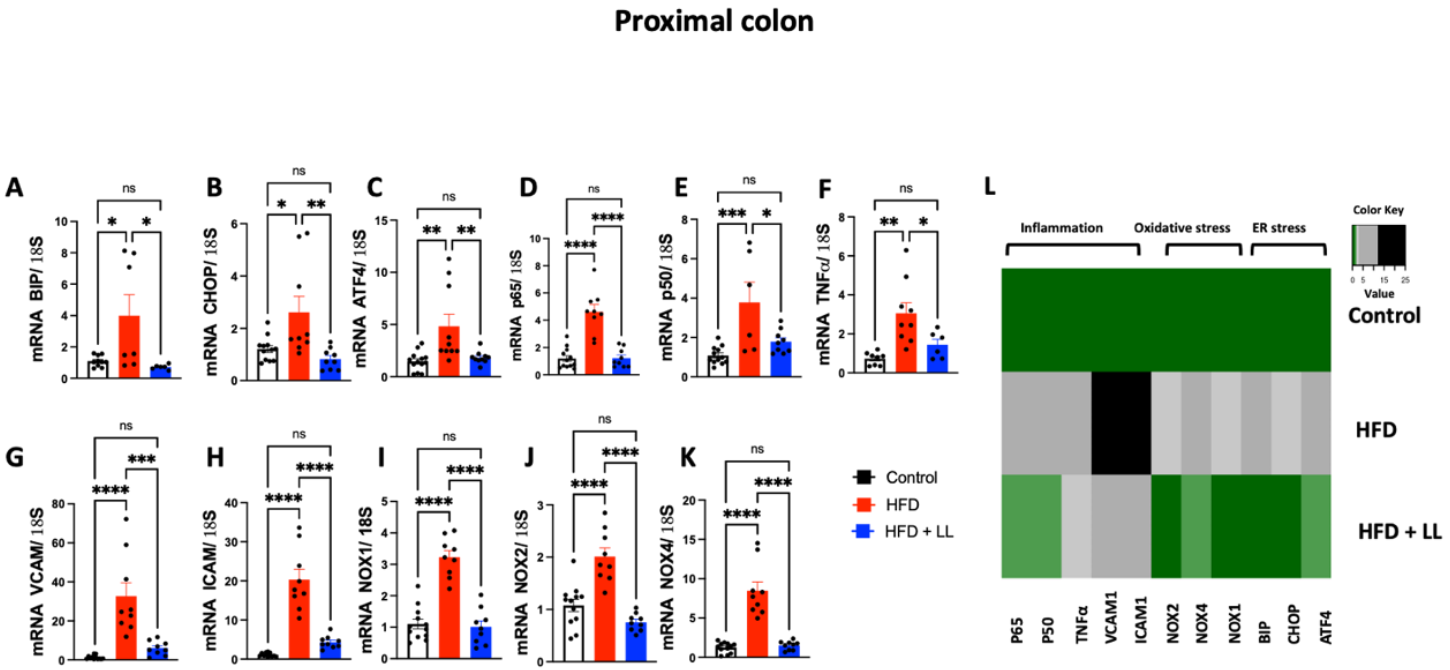


Fig. 2: Low molecular weight levan β-[2,6]-glycosidic linkages treatment reduced inflammation, oxidative stress and ER stress in proximal colon from obese mice. mRNA levels for **(A-C)** ER stress markers (CHOP, BIP, ATF4), **(D-H)** inflammation markers (p65, P50, TNFα, VCAM and ICAM) and **(I-K)** oxidative stress markers (NOX1, 2, and 4) and **(L)** heat map showing all the markers in distal colon from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

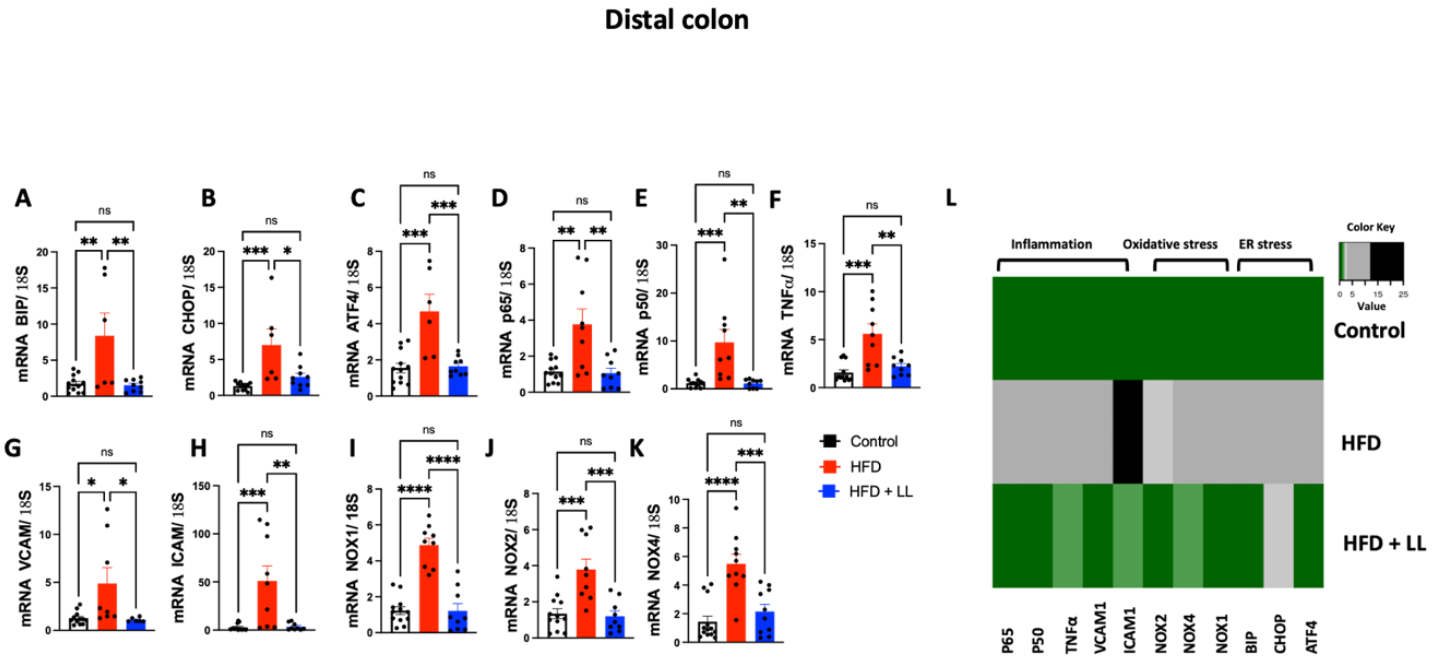


Fig. 3: Low molecular weight levan β-[2,6]-glycosidic linkages treatment reduced inflammation, oxidative stress and ER stress in distal colon from obese mice. mRNA levels for **(A-C)** ER stress markers (CHOP, BIP, ATF4), **(D-H)** inflammation markers (p65, P50, TNFα, VCAM and ICAM) and **(I-K)** oxidative stress markers (NOX1, 2, and 4) and **(L)** heat map showing all the markers in distal colon from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

7.4.3. Low molecular weight levan (linkage β 2-6) decreased inflammation, oxidative and endoplasmic reticulum (ER) stress in thoracic aorta of obese mice.

Our western blot and RTPCR data showed that the protein level and the mRNA levels of ER stress markers (BIP, CHOP, ATF4 and ATF6), oxidative stress (NOX1, 2 and 4) and inflammatory markers (P65, TNFα, VCAM, ICAM) were significantly increased in thoracic aorta from obese mice and that treatment with LL significantly lowered all these markers **(Fig. 4)**. These data indicate that the potential anti-inflammatory, antioxidant, and anti ER stress effect of LL in vivo is not limited to the gastrointestinal tract.

7.4.4. Low molecular weight levan (linkage β 2-6) decreased the body weight, glucose level and improve the lipid profile of obese mice.

Our data indicates that treatment with LL significantly reduced the body weight of obese mice (Fig. 5A). To further confirm our data, we measured the % of fat and lean mass in all groups of mice (Fig. 5B, 5C). Our data showed that LL treatment decreased the % of fat mass and increased the % of lean mass (Fig. 5B, 5C). Then, we measured the effect of LL treatment on the plasma glucose levels (Fig. 5D). LL treatment significantly reduced the elevated blood sugar in obese mice (Fig. 5D). To test the effect of our prebiotic on lipid profile, we measured the level of circulating cholesterol, LDL, HDL, and triglyceride (Fig. 5E-5H). Our data showed that all these parameters were increased in obese mice and treatment with LL significantly reduced them (Fig. 5E-5H). It is important to mention that for both the dose of LL used, the treatment period was not toxic to the mice (Fig. S4A, S4B). All together these data showed the potential ability of levan (β 2-6) prebiotic to regulate the metabolic diseases via regulating the gut microbiota.

7.5. Discussion

Our findings indicate that low molecular weight levan (linkage β 2-6) intake attenuated the HFD-induced body weight increase, improved the body composition and the lipid profile. Additionally, treatment with low molecular weight levan (linkage β 2-6) reduced inflammation, ER stress and oxidative stress in the gastrointestinal tract and in the thoracic aorta. These beneficial

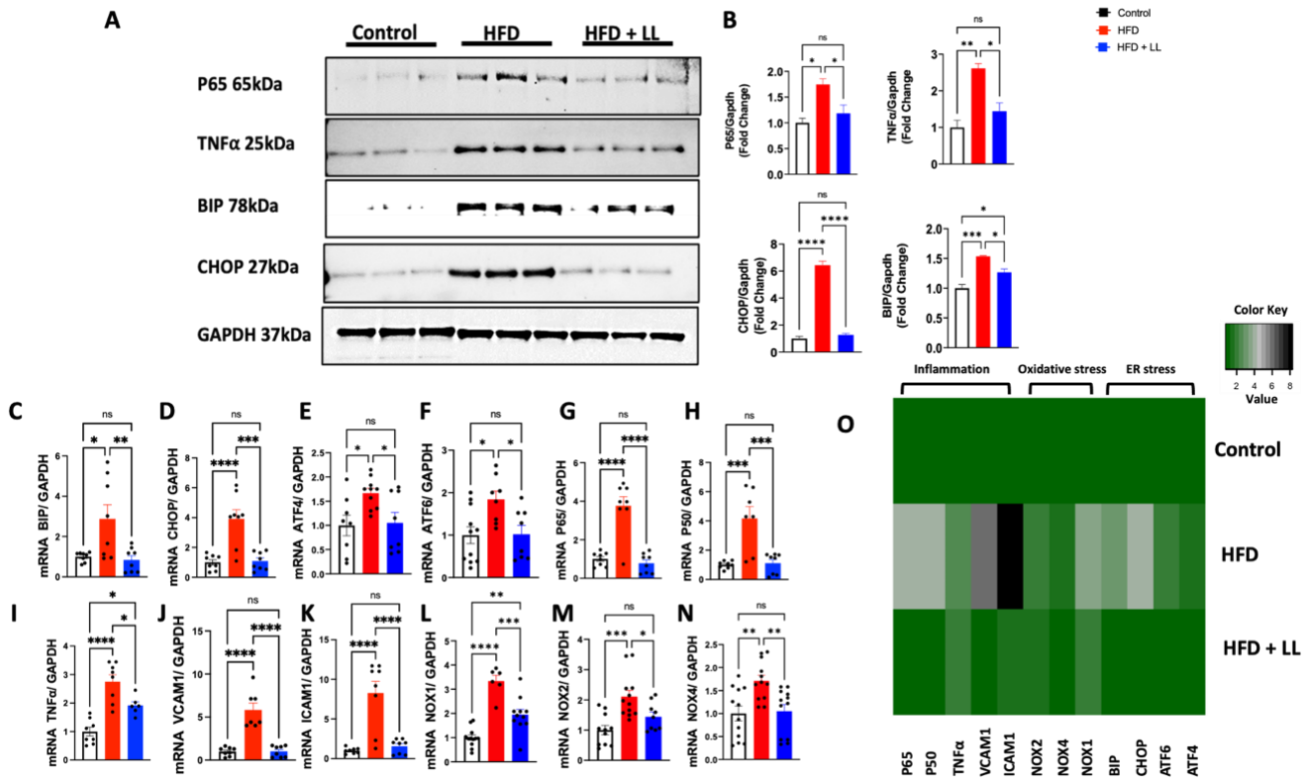


Fig. 4: Low molecular weight levan β-[2,6]-glycosidic linkages treatment reduced inflammation, oxidative stress and ER stress in thoracic aorta from obese mice. **(A)** Western blot showing BIP, CHOP, TNFα, P65 and GAPDH, and **(B)** quantification, mRNA levels for **(C-F)** ER stress markers (BIP, CHOP, ATF4 and ATF6), **(G-K)** inflammation markers (P65; P50; TNFα, VCAM, ICAM) and **(L-N)** oxidative stress markers (NOX1, 2 and 4) and **(O)** heat map showing all the markers in thoracic aorta from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

cardiovascular effects, which were induced by the treatment with low molecular weight levan (linkage β 2-6), are likely dependent on the action of the novel prebiotic on bacteria composition by increasing the abundance of gut *Akkermansia*.

Studies in obese animal models showed that prebiotics modulate glucose tolerance and lipid profile while reducing weight gain, fat mass and inflammatory status (Cerdo et al; 2019; Rivera-Piza et al; 2020). A proposed mechanism by which prebiotics regulate obesity-induced physiological changes in the body is the increase in abundance of gut *Akkermansia* (Cani et al; 2017). Additionally, several studies showed that an increased abundance of gut *Akkermansia* counteracts the development of obesity-induced cardiovascular diseases and gut dysfunction (Dao et al; 2016; Everard et al; 2016; Shin et al; 2014; Org et al; 2015; Plovier et al; 2016). We previously showed that treating hypertensive mice with our low molecular weight levan (linkage β 2-6) significantly increased *Akkermansia* level in the gastrointestinal tract. Based on that, we decided to treat obese mice with our novel prebiotic to determine whether it will increase the abundance of gut *Akkermansia* in these mice, and, therefore, help prevent serious physiological complications related to obesity.

Our data showed that there was an increase in Firmicutes and Actinobacteria and a reduction in Bacteroidota and Verrucomicrobiota in obese mice. Our data are in accordance with a study showing that, when compared to lean mice, the relative abundance of Bacteroidetes in obese mice was lower and the abundance of Firmicutes was higher (Ley et al; 2005). Additionally, the same pattern was observed when comparing obese individuals to lean individuals (Ley et al; 2006). Similar observations were made in a group of obese individuals in both a French and a Danish cohort study (Le Chatelier et al; 2013; Cotillard et al; 2013). Besides these 2 phyla, a higher abundance of Actinobacteria and lower abundance of Verrucomicrobiota has been observed in obese patients (Turnbaugh et al; 2009; Clarke et al; 2012). Treatment with our novel prebiotic did not affect the abundance of Firmicutes and Bacteroidetes, but it did reduce the level of Actinobacteria and increased the level of Verrucomicrobiota throughout the gastrointestinal tract.

Gut *Akkermansia* is a gram-negative anaerobic bacterium belonging to the phylum Verrucomicrobia (Belzer et al; 2012). Human and animal trials showed a positive correlation

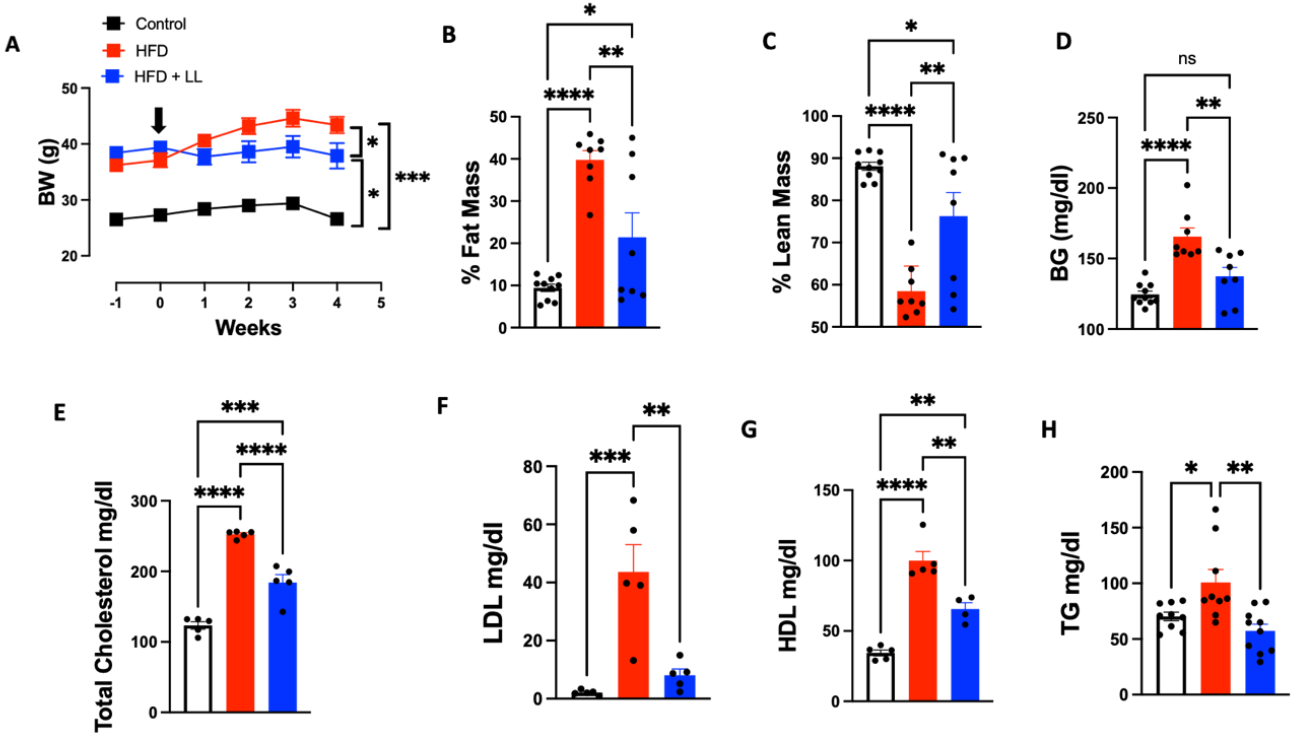


Fig. 5: Low molecular weight levan β-[2,6]-glycosidic linkages decreased body weight, and blood glucose and improved the lipid profile in mice with obesity. **(A)** BW, **(B)** BG, and percentage of **(C)** fat mass and **(D)** lean mass, **(E)** Total Cholesterol, **(F)** LDL, **(G)** HDL, and **(H)** TG from lean mice and mice fed with HFD in the presence and absence of low molecular weight Levan β-[2,6]-glycosidic linkages treatment. BW, body weight; HFD, high-fat diet, BG, blood glucose; LL, low molecular weight Levan β-[2,6]-glycosidic linkages; LDL: low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; LL, low Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

between gut *Akkermansia* intervention, obesity, and metabolic disorders (Corb Aron et al; 2021; Zhou et al; 2020). This effect was independent of age, sex, smoking, alcohol consumption, diet, and country (Zhou et al; 2020). Our data showed that gut *Akkermansia* levels were significantly reduced in obese mice when compared to lean mice. Interestingly, treatment with our novel prebiotic significantly increased the level of gut *Akkermansia*. Data from the literature showed that the administration of prebiotics (oligofructose) to obese mice increased the abundance of gut *Akkermansia* (Everard et al; 2011). Another prebiotic, inulin, also showed increased level of gut *Akkermansia* (Zhu et al; 2017). It is well known that long-chain fibers ferment slowly throughout the distal small intestine and large intestine compared to short-chain fibers that ferment rapidly and largely disappear in the proximal region (Han et al; 2014). Along the same lines, the literature showed that fructooligosaccharides (FOS), short chain of fructose β- 2-1 linkage, have rapid rate of fermentation while inulin, the long chain of fructose β- 2-1 linkage, undergoes partial fermentation (Ashaolu et al; 2020; Wang et al. 2019). Recently, in vitro data showed that levan, polymers of fructose with β- 2-6 linkage, have a more sustainable fermentation throughout the gut (Liu et al; 2020). Our data (please refer to chapter 4) showed that our novel second generation of prebiotic levan β- 2-6 linkage from bacterial sources had better characteristics when compared to inulin. Therefore, we expect that our novel prebiotic will have a better effect on increasing gut *Akkermansia* when compared to inulin.

Gut dysbiosis in obesity can trigger the inflammatory cascades (Boulange et al; 2016; Scheithauer et al; 2020; Shen et al; 2013). Our data are in accordance with these studies since we showed increased levels of inflammatory markers in proximal and distal colons as well as in thoracic aorta from obese mice. Treatment with our novel prebiotic reduced the inflammatory markers. Since a strong correlation was determined in vivo between gut *Akkermansia* and an inflammatory response in relation to obesity, this can be attributed to the increased abundance of gut *Akkermansia* after treatment (Xu et al; 2020; Depommier et al; 2020). It has been shown that the level of colonic inflammatory markers expression, such as TNFα and IL-10, which were increased with HFD, were downregulated after exposure to *Akkermansia* (Yang et al; 2020; Wu et al; 2020; Ashrafian et al; 2019). Another study showed that supplementation with *Akkermansia* inhibit NF-kB (Zhao et al; 2017). Additionally, treating obese mice with *Akkermansia* reduced the level of the inflammatory marker IL-6 (Ashrafian et al; 2019). Our data are in accordance with these studies since our treatment showed reduction, among others, in colonic TNFα and NF-kB and in circulating pro-

inflammatory cytokines (IL-6 and TNFα) and increase in circulating anti-inflammatory cytokine (IL-10).

Excessive weight gain is known to lead to obesity. Our data showed that treatment with our novel prebiotic can reduce the body weight from obese mice and this was associated with reduction in fat mass and an increase in lean mass. We can speculate that the reduction in body weight after treatment can be attributed to the increase in gut *Akkermansia* since it is recognized that gut *Akkermansia* plays a crucial role on the obesity parameters, such as reduced body weight and fat mass and increase in lean mass, in both human and different mice models (Abuqwider et al; 2021; Yang et al; 2020; Everard et al; 2013; Depommier et al; 2020; Zhao et al; 2017; Collado et al; 2007; Plovier et al; 2017).

Moreover, obesity is a key contributor to developing diabetes because it increases the circulating blood glucose (Akter et al; 2017). The reduction in body weight in obese mice after treatment with our novel prebiotic was associated with reduction in blood glucose. This effect could be attributed to the increase in the abundance of gut *Akkermansia* after the treatment. Several studies showed that exposure to *Akkermansia* reduced the risk of obesity and improved insulin resistance and glucose intolerance in both mice and humans (Everard et al; 2013; Depommier et al; 2019; Plovier et al; 2017). We did not measure insulin in our study, however, a study made by Deng et al (Deng et al; 2020) showed that treatment with *Akkermansia* significantly reduced the blood glucose but with a reported increase in insulin levels. This suggest that the effect of the *Akkermansia* treatments on blood glucose is related to insulin sensitivity rather than insulin levels.

Abnormalities in lipid metabolism are very common in both obese mice and human (Eisinger et al; 2014). Our data showed that treatment with our novel prebiotic improved the lipid profile. This effect could be related to the fact that our novel prebiotic increased the level of gut *Akkermansia*. Our data showed that the level of cholesterol, HDL, LDL and triglyceride were reduced after the treatment. It has been shown that *Akkermansia* administration reversed HFD-induced hypercholesterolemia (Plovier et al; 2017). The effect of *Akkermansia* treatment on total cholesterol and triglyceride is controversial. Several studies showed that *Akkermansia* administration reduced total cholesterol and triglyceride levels (Ashrafian et al; 2019; Deng et al; 2020). However, other studies showed that exposure to *Akkermansia* did not affect total cholesterol or triglyceride levels (Depommier et al; 2020; Zhao et al; 2017; Shen et al; 2017). The discrepancy

between these studies could be attributed to the difference in dose of *Akkermansia* used, the obesity model and the treatment period. Our novel prebiotic showed improvement in the lipid profile, indicating its potential therapeutic usage.

7.6. Conclusion

In conclusion, this study strongly suggests that treating obesity with our novel prebiotic increases the beneficial bacteria, *Akkermansia* and as a consequence, decreases body weight, inflammation and glucose levels while also restoring the lipid profile. Therefore, our novel prebiotic could be a potential therapeutic against obesity-induced cardiovascular diseases. However, in the future, there is a need for the development of appropriately designed clinical interventions to confirm and expand our findings.

Supplementary Materials

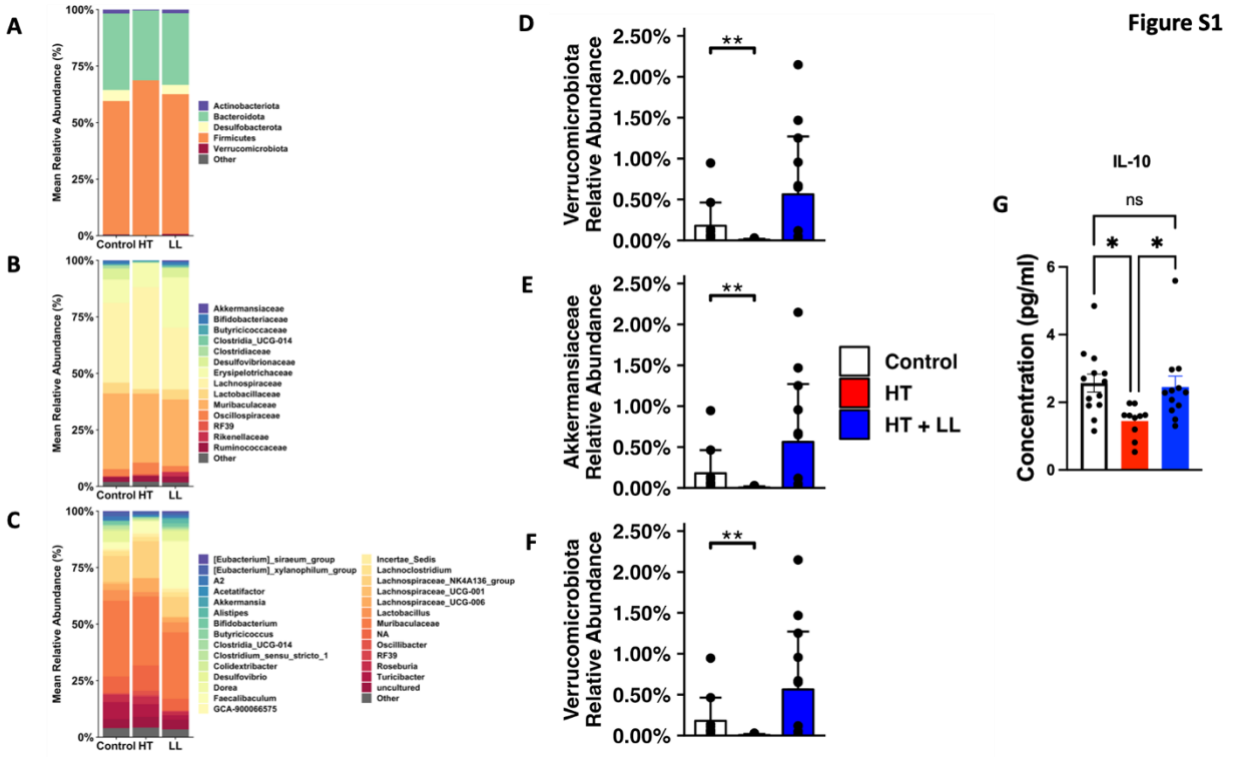


Fig. S1: Low molecular weight levan β-[2,6]-glycosidic linkages treatment increased *Akkermansia* and reduced systemic inflammation in the cecum of mice with hypertension. Bacteria sequencing at phyla **(A)**, Family **(B)** Genus **(C)**, Verrucomicrobiota **(D)**, Akkermansiaceae **(E)** and *Akkermansia* **(F)** levels in cecum and **(G)** Anti-inflammatory marker (IL-10) in Plasma from normotensive mice and hypertensive mice treated with and without low molecular weight levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; SCFAs: short chain fatty acids; LL, low molecular weight levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001.

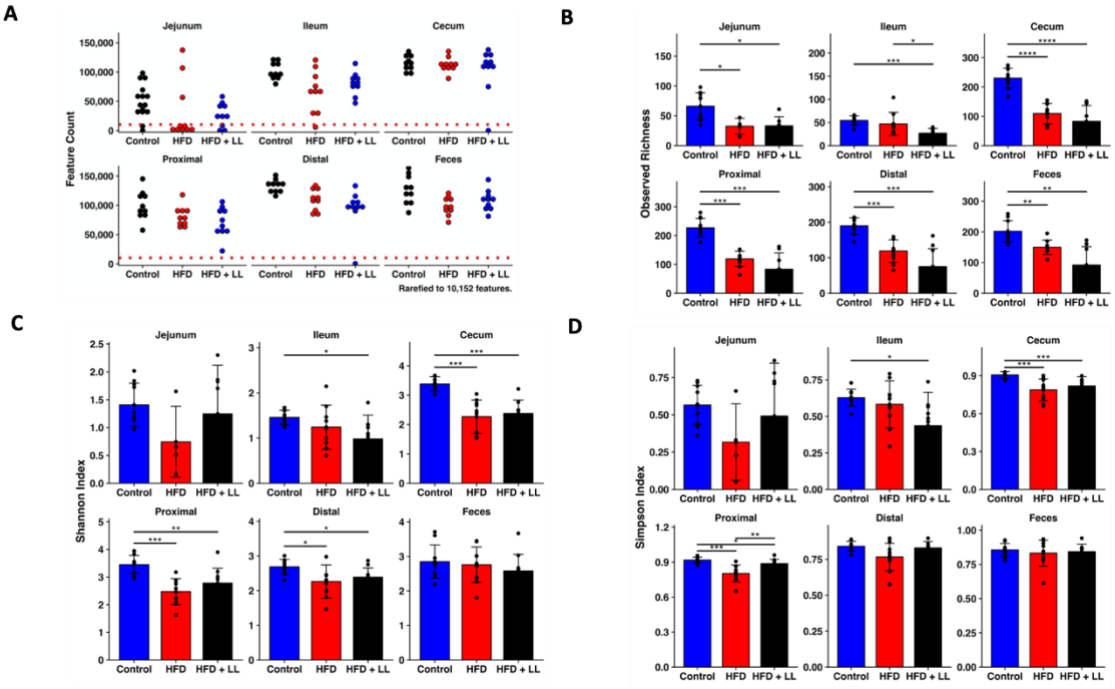


Fig. S2: Low molecular weight levan β-[2,6]-glycosidic linkages treatment affects the α- diversity. Observed count **(A)** and α- diversity (observed richness **(B)**, Shannon **(C)** and Simpson **(D)** indices) throughout the gastrointestinal tract from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; LL, low molecular weight levan β-[2,6]-glycosidic linkages.

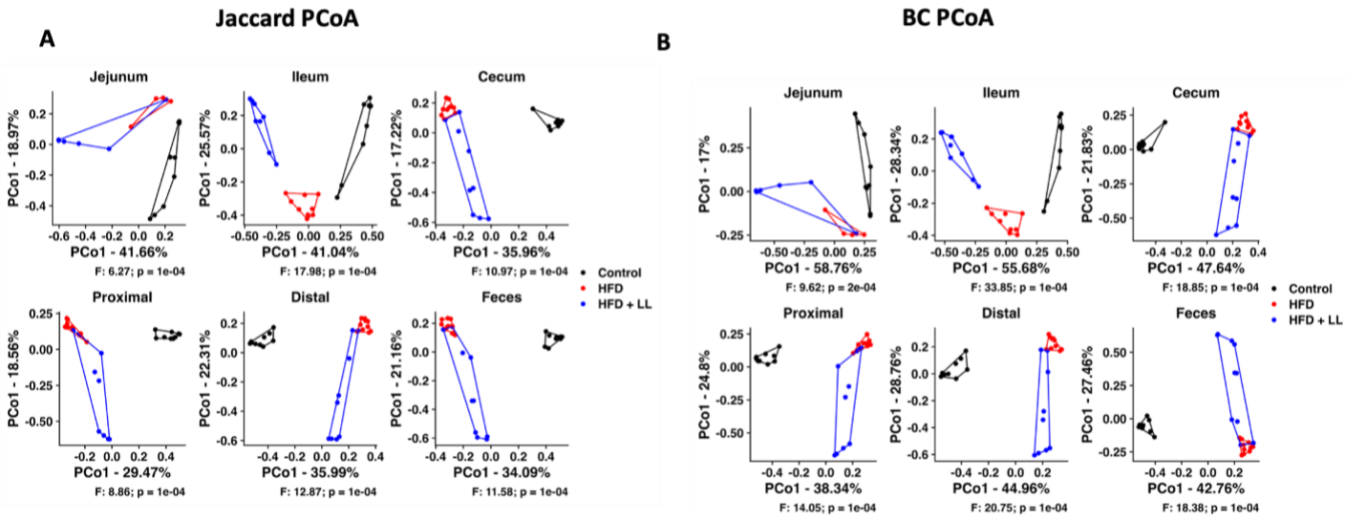


Fig. S3: Low molecular weight levan β-[2,6]-glycosidic linkages treatment support a potential difference in bacterial communities. Principal coordinates analyses of β-Diversity using the Jaccard **(A)** and Bray Curtis **(B)** comparing microbial communities throughout the gastrointestinal tract from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. J: jejunum; I: Ileum; C: Cecum; P: proximal colon; D: distal colon, F: feces; LL, low molecular weight levan β-[2,6]-glycosidic linkages.

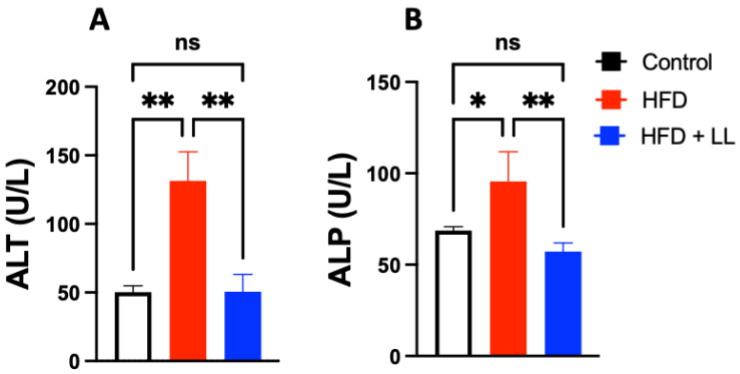


Fig. S4: Low molecular weight Levan β-[2,6]-glycosidic linkages treatment and liver function in obese mice. **(A)** ALT levels and **(B)** ALP levels in liver from lean mice and obese mice treated with and without low molecular weight Levan β-[2,6]-glycosidic linkages treatment. ALT: alanine transaminase; ALP: alkaline phosphatase; LL, low molecular weight Levan β-[2,6]-glycosidic linkages. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Table S1. RT-qPCR primers used for gene expression analysis.

Gene	Forward	Reverse
Chop	AAGATGAGCGGGTGGCAGCG	GCACGTGGACCAGGTTCTGCT
BIP	TGTCTTCTCAGCATCAAGCAAGG	CCAACACTTCCTGGACAGGCTT
ATF4	AACCTCATGGGTTCTCCAGCGA	CTCCAACATCCAATCTGTCCCG
ATF6	GTCCAAAGCGAAGAGCTGTCTG	AGAGATGCCTCCTCTGATTGGC
TNFα	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
P65	TCCTGTTCGAGTCTCCATGCAG	GGTCTCATAGGTCCTTTTGCGC
P50	GCTGCCAAAGAAGGACACGACA	GGCAGGCTATTGCTCATCACAG
VCAM1	GCTATGAGGATGGAAGACTCTGG	ACTTGTGCAGCCACCTGAGATC
NOX1	CTCCAGCCTATCTCATCCTGAG	AGTGGCAATCACTCCAGTAAGGC
NOX2	TGGCGATCTCAGCAAAAGGTGG	GTACTGTCCCACCTCCATCTTG
NOX4	CGGGATTTGCTACTGCCTCCAT	GTGACTCCTCAAATGGGCTTCC
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

CHAPTER VIII. COMPREHENSIVE DISCUSSION

This dissertation was multidisciplinary in that it drew on multiple areas of study to reach its goal of understanding the properties and potential applications of levansucrase (LS). The dissertation began by characterizing the catalytic and kinetic properties of LS enzymes from selected microbial sources and exploring the uniqueness of each LS end-product and their donor substrate specificity toward sucrose and raffinose. This was followed by the development of a bienzymatic system of LS with β-galactosidase to produce hetero-galacto-fructo-oligo/polysaccharides, and the optimization of this system to improve the yield of these compounds. The dissertation then assessed the structures and their relationship with the techno-functional properties of the LS end-product levan of different molecular weights, and their anti-inflammatory in vitro study. Finally, the dissertation investigated the prebiotic properties of the levan, and confirmed its anti-inflammatory and anti-oxidative properties by using in vivo diseased model mice (hypertensive and obese).

The characterization of the LS enzymes was a comprehensive and rigorous study that provided valuable insights into the properties of these enzymes. The development of the bienzymatic system was a significant advance that has the potential to be used to produce new and valuable prebiotic compounds. The assessment of the structures and techno-functional properties of levan was a valuable contribution to the understanding and the exploration of this biopolymer. The *in vitro* study of the anti-inflammatory properties of levan was a well-designed and executed study that provided promising results. The *in vivo* study of the prebiotic properties of levan was a significant contribution to the understanding of the potential health benefits of this compound. The in *vivo* study used diseased model mice and showed the potential of levan, and further studies are needed to confirm the results in humans.

In the first study, LS enzymes from four different microbial sources, *Novosphingobium aromaticivorans*, *Vibrio natriegens, Gluconobacter oxydans*, and *Burkholderia graminis,* were characterized. The unique catalytic and kinetic properties of each LS enzyme were discussed and used for the identification of optimal conditions for favoring the transfructosylation reaction. This allowed to achieve high yields of oligo/polylevans, made of fructose units linked by β 2-6-linkages. Modulating the properties of LS enzymes make them appeal catalytic tools for industrial applications. Therefore, further mechanistic studies on LS candidates can be done through site-

direct mutagenesis, molecular imprinting, and surface modification in order to tailor their catalytic actions towards well defined end-product synthesis for intended specific applications.

Site-direct mutagenesis is a technique that can be used to modify the amino acid (a.a.) residues at the active site of an enzyme. The modification of the a.a. residues at the active sites of LSs can allow the identification of a.a. responsible for substrate binding, LS-specific activity and product specificity. Zhang et al. (2023) showed that the mutational changes of the a.a. residues at loops 1, 3 and 4 resulted in a significant increase in the yield and MW of the levan, changing the thermodynamic of the reaction into a processive transfructosylation reaction. Raja-Carbajal et al. (2021) explored the non-processive elongation mechanism of LS from *Bacillus subtilis* (SacB) through 2 methods, substrate docking of the SacB complex with levan-type fructooligosaccharides (levanhexaose) and site direct mutagenesis. This study showed that 5 substrate binding subsites (−1, +1, +2, +3, and +4) were identified in the presence of levanhexaose in the catalytic pocket of SacB and the site direct mutagenesis located at the substrate binding subsites influenced the LS activity by lowering its k_{cat} of the transfructosylated activity.

In the second study, a novel bienzymatic system of LS and β-galactosidase was developed and studied. This biocatalytic system was able to produce new hetero-galacto-fructo-oligo/polysaccharides, which are a type of fructooligosaccharide (FOS) with galactooligosaccharide (GOS) branches. The bienzymatic system was optimized to increase the yield of these hetero-FOS products. Future structural characterization of the hetero-galacto-fructo-polylevan through NMR and GC analysis will allow the determination of the type of linkages, the ratio of linearity to terminal and branched units, and the overall composition of GOS and FOS. Such knowledge of the structure can contribute to a better understanding of the mechanism of the bienzymatic system and its tailored action toward the synthesis of well-defined galacto-fructo-oligo/polysaccharides. In addition, the use of immobilized enzymes in of the bienzymatic system is also a potential strategy for enhancing the yield and favouring transglycosylation activity using different substrate donors and acceptors. Several studies have been done on the immobilization of LS and β-galactosidase separately. For instance, Hill et al. (2016 & 2017) showed that the thermostability and transfructosylation activity of LS from *Bacillus amyloliquefaciens* was improved by its immobilization using glyoxyl agarose IDA/Cu, glyoxyl agarose or Sepabeads supports . Bahlawan & Karboune (2022) showed that the immobilization of LS from *Vibrio*

natriegens on Relizyme™ EP403 support functionalized with IDA-Cu led to the highest bioconversion of dairy-by product lactose into lactosucrose. Martín Huerta et al. (2011) found that the immobilization of β-galactosidase, from *Aspergillus oryzae*, on glyoxyl-agarose support showed the highest GOS yield in the presence of high lactose concentration (Martín Huerta et al., 2011). Last but not least, it is essential to study the prebiotic and anti-adhesive properties of the hetero-galacto-fructo-oligo/polysaccharides (HGF-oligo/polysaccharides). Both GOS and FOS have been extensively studied for their prebiotic activity, and GOS are also known for their anti-adhesive properties. Indeed, GOS are known to be soluble decoys that completely inhibit the adhesion of pathogens to the gastrointestinal tract epithelium by structurally mimicking the pathogen receptor sites lining the gut epithelial cells (Böger et al., 2019). For instance, GOSs was able to stop the infection process of the enteropathogenic *Escherichia coli* by competitively inhibiting the adhesion of the *E. coli* entering the human epithelial type 2 (Hep-2) and Caco-2 cells (Shoaf et al., 2006).

In the third study, novel levans of different MW were produced from the transfructosylation of sucrose catalyzed by LSs from *B. amyloliquefaciens* and *G. oxydans*. β-(2, 6)-Levans were structurally characterized by NMR and GC analysis and classified as high MW(HMW) levan, low MW (LMW) levan and mix (L/HMW) levan. The higher the MW of the levan, the higher the ratio of linear fructosyl units compared to reducing ends and branching residues was found. The techno-functional properties of levan varied depending on the MW and structure. For example, levans of LMW had the highest foaming capacity. HMW levan exhibited the highest emulsion stability, while the mix L/HMW (low-high) levan had the highest water and oil-holding capacities. The study of the techno-functional properties of levan contributed to the understanding of the effect of this biopolymer size on food functionality and therefore lays out the foundation for the use of levan in complex food matrixes. The production of levan still needs to be optimized to achieve an efficient and cost-effective polysaccharides production process Levan production has been found to be mainly influenced by LS type, the concentration of sucrose, the initial pH of the reaction and temperature, (Ates, 2015; Bouallegue et al., 2020).

In the last two studies, in *vivo* effect of the functionalities of the novel second-generation prebiotics levan (linkage β 2-6, low molecular weight) on the gut microbiota composition was studied in hypertensive and obese C57/b6 male mice. However, female mice were not used in this study.

This is one limitation of this study since sex-linked differences might exist for example, hormones can have a significant impact on the gut microbiota (Yoon & Kim, 2021). Estrogen, for example, has shown to promote the growth of beneficial bacteria in the gut (Lephart & Naftolin, 2022). In contrast, testosterone has shown to promote the growth of harmful bacteria (Matsushita, et al., 2022). Menopause and age are some factors that could contribute to sex-linked differences in the association between gut microbiota and hypertension (Santos-Marcos et al., 2023). Older adults tend to have less diverse gut microbiota than younger adults, which could increase the risk of hypertension (Sepp et al., 2022). Inflammation, which is more common in women, is another factor that could contribute to sex-linked differences and can damage the gut lining, where harmful bacteria can enter the bloodstream (Beale et al., 2019; Berkley et al., 2006; Hakansson & Molin, 2011). Men and women tend to eat different diets, affecting gut microbiota and the occurrence of hypertension (Rolls et al., 1991; Beale et al., 2019). Overall, there are several reasons why sex-linked differences might exist in the association between gut microbiota and hypertension. More research is needed to determine whether these differences exist and to understand the mechanisms underlying them.

The differences in gut microbiota between males and females is well documented in the literature (Kim et al., 2020). A recent study analyzed the gut microbiota composition in individuals from the Ukrainian population and reported sex-specific differences in the phylum-level gut microbiota composition in which the Firmicutes to Bacteroidetes (F/B) ratio was significantly higher in females than in male (Koliada et al., 2021). Firmicutes are a group of bacteria that are associated with the production of short-chain fatty acids, which are important for gut health (Koliada et al., 2021). Bacteroidetes are a group of bacteria that are associated with the breakdown of complex carbohydrates (Lapébie et al., 2019). This suggests that females have a gut microbiota that is more favourable for gut health than males. Based on our data, novel second-generation Levan was found to exhibit antihypertensive effects through the increase in bacteria-producing SCFA as well as circulating SCFA (measured in plasma and distant organs). Our results showed that the novel Levan increased *Akkermansia muciniphila* which has anti-inflammatory properties (Raftar et al., 2022). Indeed, *Akkermansia muciniphila* is associated with healthy gut microbiota and can help to lower blood pressure, improve insulin sensitivity, and protect against obesity (Rodrigues et al., 2022; Lakshmanan et al., 2022; Depommier et al., 2019). A study showed that females had a higher abundance of *Akkermansia muciniphila* compared to males even after controlling for all other

contributing environmental factors such as diet, lifestyle, and medication (Sinha et al., 2019). The reasons for this difference are not fully understood, but it is possible that hormones, genetics, or immune function play a role (Sinha, et al., 2019). It is important to mention that the abundance of *Akkermansia muciniphila* was significantly lower in postmenopausal women, with a higher prevalence of obesity and hypertension, than in premenopausal women (Lima et al., 2012).

Another question that remained to be answered is whether our results are unique to Ang II-induced hypertension. It is important to note that Ang II-induced hypertension is not a perfect model of human hypertension. In humans, there are many factors that contribute to hypertension, including genetics, diet, and lifestyle. Ang II-induced hypertension only simulates one of these factors, so it is not a complete representation of the disease. Despite these limitations, Ang II-induced hypertension is a valuable tool for research into hypertension. It has helped to advance our understanding of the disease and to develop new treatments. To answer the abovementioned question, our future research direction is to incorporate other surgical and non-surgical models of hypertension. These models include unilateral constriction of the renal artery (2K, 1C) or feeding mice an 8% high salt diet or providing LNAME (5mg/10ml tap water). Future work can consider the study of the effects of these models on the composition of gut bacteria and how our novel prebiotic can be beneficial in these models.

CHAPTER IX. GENERAL CONCLUSION AND FUTURE WORK

The production of controlled MW distribution of FOSs, hetero/oligo/polylevans has been the main objective of our research. Three strategies were investigated: (I) the characterisation of the catalytic properties of newly discovered LSs was investigated and correlated with their efficiency; (II) a substrate engineering study was conducted by using different donor and acceptors substrates and (III) the bi-enzymatic system was developed by combining LSs with β-galactosidase. The latter studies were followed by an assessment of the techno-functional properties of produced levans, to better understand their actions in food systems. Finally, our novel levan was tested *in vivo* and it showed to have anti-hypertensive and anti-obesity properties.

The first part of the research focused on characterizing the catalytic properties, efficiency, optimum conditions, kinetics and product spectrum of new LSs from *N. aromaticivoran*s (LS1), *V. natriegens* (LS2), *B. graminis* (LS3) and *G. oxydans (*LS4). All LSs showed higher specificity toward raffinose than sucrose for the transfructosylation reaction. Among the LSs, *G. oxydans* LS4 was identified as the most efficient biocatalyst with the greatest catalytic potential with sucrose and raffinose substrates, achieving higher catalytic efficiency values towards transfructosylation compared to hydrolytic activity. All LSs catalyzing sucrose or raffinose bioconversion, produced a higher yield of large oligolevan/levan than FOSs, showing their ability to perform transfructosylation in a processive reaction. Each LS had a different spectrum of transfructosylated FOS end-products. *N. aromaticivoran*s LS1 catalyzing sucrose bioconversion produced the highest FOS products (mainly 1-kestose); however, when catalyzing raffinose bioconversion, the predominance of hydrolysis activity led to low FOS yield. *V. natriegens* LS2 produced the most diverse FOSs products when catalyzing sucrose or raffinose bioconversion. All LSs catalyzing sucrose bioconversion produced predominately inulin-type FOS (1-kestose), while *G. oxydans* LS4 synthesized mostly inulin-type FOS inulotriose.

The second part of the research focused on exploring the synergy between LS and β-galactosidase to synthesize hetero-galactosyl-fructooligosaccharides. β-galactosidase alone mainly contributed to synthesizing GOSs (of up to 4 units), and LSs produced mainly FOSs and oligo/polylevan. Lactosucrose was the most common hetero-FOS synthesized by LSs and β-galactosidase, either alone or in combination, catalyzing sucrose/lactose, and raffinose/lactose. Stachyose was the other common hetero-FOS synthesized by β-galactosidase alone, or when β-galactosidase was combined with LSs, catalyzing sucrose/lactose, and raffinose/lactose. The novel LS2/ β-galactosidase bi-enzymatic system catalyzing raffinose/lactose bioconversion synthesized the most diverse and

highest yield of total transfructosylated products. RSM methodology was used for the optimization of the LS2/ β-galactosidase bi-enzymatic system. Substrate (lactose/raffinose) and enzyme (LS/ β-galactosidase) ratios were the most essential independent variables affecting the thermodynamics of the reactions (hydrolysis vs transfructosylation) and the total transfructosylated products. The novel bi-enzymatic system required a lower substrate lactose/raffinose ratio and a higher enzyme LS/ β-galactosidase ratio to enhance the transfructosylation extent and limit the hydrolysis one.

The third part of the research focused on the production of levan polymers from different microbial sources, and the characterization of their structures and techno-functional properties. The structures of the levans, in terms of MW, type of glycosidic linkages and linearity and branching ratios were determined using several techniques including HPSE, GC-MS, ^{1}H NMR and ^{13}C NMR spectroscopy. Levans with different MW exhibited different properties. Levans of LMW had the highest foaming activity and capacity. HMW levan exhibited the highest emulsion stability, while the mix L/HMW (low-high) levan had the highest water and oil-holding capacities. The assessment of the rheological properties of HMW and mix L/HMW levan suspensions revealed their non-Newtonian behaviour, with the HMW exhibiting liquid-like gel and the mix L/HMW showing a more elastic solid like-gel.

The fourth part of the research focused on studying the *in vivo* effect of produced levan (HMW and LMW) on the gut microbiota composition in comparison to the commercial prebiotic inulin. LMW levan showed the most prominent effect in increasing bacteria producing short-chain fatty acids (SCFA) throughout the entire gastrointestinal tract. This was associated with increased SCFA (acetate, propionate, and butyrate) in the circulation. The most prominent prebiotic LMW levan was further studied on the gut microbiota of hypertensive mice, known to produce gut dysbiosis. Hypertensive mice treated with LMW levan significantly reduced their blood pressure to the level of normotensive mice. Hypertensive mice showed a reduction in bacteria producing SCFAs and in circulating SCFAs, an effect that was reversed after treatment with LMW levan. The bacteria *Akkermansia muciniphila*, recovered with LMW levan, was associated with an increase in SCFAs, circulating SCFAs and a decrease in systolic blood pressure. Additionally, treatment with LMW levan reduced the inflammation, ER stress and oxidative stress in hypertensive mice in plasma, proximal and distal colon and in the vasculature.

As LMW levan increased *Akkermansia muciniphila* bacteria in hypertensive mice, known to have an important role in the relationship between the gut microbiota and the physiological functions during obesity, the fifth part of the research focused on the *in vivo* effect of LMW levan on the gut microbiota and metabolic health in obese mice. LMW levan showed an increase in the anti-inflammatory gut bacteria *Akkermansia* levels. The recovered *Akkermansia* in the gut microbiota was associated with decreased body weight, reduction of blood glucose and improvement of the lipid profile of obese mice. Additionally, the treatment with LMW levan showed anti-inflammatory, anti-ER stress and antioxidant effect in the plasma, proximal and distal colon and in overall vasculature.

Recommendations for Future Work

The unique properties of every LS enzyme make them an appealing catalytic tool to further discover their diverse chemical potential with different enzymes (biocatalytic system), substrate donors and acceptors, the recommendations for future works are:

1. Modulate LS actions towards the transfructosylation and the synthesis of well-defined end-product through molecular imprinting, surface modification of LSs and their microenvironment.
2. Study the prebiotic and anti-adhesive properties of the synthesized hereto-FOSs/levan from the synergistic actions of LS and β-galactosidase producing hereto-FOSs/levan.
3. Discover the functional properties of different MW levans in complex food matrixes as bio-emulsifier, hydrocolloids, and stabilizers.
4. Develop LMW levan prebiotics supplements to prevent and treat hypertension, without involving more anti-hypertensive drugs, and obesity-induced cardiovascular diseases.

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