MICROBIAL GLYCOSIDE HYDROLASES AND HOST CELL SIGNALING

MICROBIAL GLYCOSIDE HYDROLASES AND HOST CELL SIGNALING

By

CLAUDIA IXTEPAN TEJERO, B.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirement for the Degree

Master Science

Master of Science (2023)
McMaster University

(Biochemistry & Biomedical Sciences)
Hamilton, Ontario

TITLE: Glycoside Hydrolases and Host Cell Signaling.

AUTHOR: Claudia Ixtepan Tejero

B.Sc., Biomedical Science (Microbiology)

Benemerita Universidad Autonoma de Puebla, Puebla, Mexico.

SUPERVISOR: Dr. Michael G. Surette.

NUMBER OF PAGES: XIV, 101

LAY ABSTRACT

Glycans are sugars that cover cells of animals, plants, and microbes. These sugars on the external surface of the cell help them interact with and protect them from the environment. For our microbiomes – the microbes that live on or in us- glycans on both microbe and our cells are important for interactions between them and us. Many bacteria produce enzymes (such as glycoside hydrolases) that change or remove sugars from the cell surface to obtain nutrients and manipulate host proteins. These enzymes can be attached to the bacterial cell surface or released into the environment, where they can interact with glycans attached to mucosal surface and immune cells. The modification of cell glycans from the immune system can modulate inflammatory responses that could change the way gut cells react to certain kinds of food, such as legumes and wheat. My work focuses on understanding how human gut microbiome can use these tools to reshape glycans and their effects on our health.

ABSTRACT

Glycans, carbohydrate-based macromolecules, are present on the surfaces of all prokaryotes and eukaryotes cells. They play critical roles in cell-cell interactions, and host glycans also provide both binding sites and nutrition for commensal microbes. Many bacteria use glycoside hydrolases (GHs) on host-generated glycans and dietary glycans for nutrition and to modulate attachment sites and host protein function. The microbiota found in a single human gut sample has greater than 10,000 GH genes, and many of these gene products have specificity for host glycans. In eukaryotes, glycosylation of cell surface receptors is critical for proper localization and function, including receptors for innate immune signalling. The hypothesis of this project is that human-associated microbiota can express and secrete GHs that can alter the functioning of host immune signalling pathways. In this work, I measure GHs enzyme activity, use bacterial cell-free supernatant and purified enzymes to determine if they can modulate host cell signalling pathways, and examine glycans cell surface modifications by fluorescence microscopy using fluorescent-labelled lectins.

ACKNOWLEDGEMENTS

This thesis would not be possible without a large list of wonderful people I met before and after my studies at McMaster university. First, I want to thank my parents and sister, Juan C. Ixtepan, Liliana Tejero, and Karla Ixtepan, who, with love, inspired me day by day to be a better version of myself. During the past two years, we had a lot of inspirational talks that encouraged me to keep working for my dreams regardless of the difficulty of being away from home during the COVID-19 pandemic. Thank you for always believing in me.

To Dr. Michael G. Surette, who gave me the invaluable opportunity of being part of his laboratory at McMaster university. Mike, you are one of the kindest and most admirable mentors I have ever had. Thank you for all your advice, patience, and corrections in my work. Your constant feedback was an important pillar of my formation at McMaster university. I grew as a scientist under your supervision, and undoubtedly it was one of the most challenging and joyful moments of my life that allowed me to grow as a person.

To my committee meeting members, Dr. Lesly MacNeil and Dr. Sara Andres. Thank you for sharing your knowledge and for all the feedback and patience during the past two years of my project. I have learned and grown more than I could imagine; I will be forever grateful.

To my boyfriend, J. Miguel Capilla, who always supported me and encouraged me with kindness and love, giving me wise advice through all the random situations that happened to me during my studies at McMaster university.

To Laura Rossi, Blerina Kadiu, and Dr. Sheridan Baker, my first friends in Canada. Thank you for all the support and wise advice in and outside the laboratory. I am so glad I had the opportunity to meet wonderful people like you that worked with integrity and kindness.

To my lab mates: Dr. Evan Mann, Aarthi Pasupathi, Michelle Shah, Nancy ElChaar, Dominique Tertigas, Salah Uddin Khan, and Dr. Sharok Shekarriz from the Surette Laboratory for their friendship and constant feedback on my project. You made the time I spent in the laboratory fun and joyful.

Finally, I want to thank God for putting me on the right path to living this extraordinary adventure.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ConA	Concanavalin A
DAMPs	Damage associated molecular patterns
DSS	Dextran sulphate sodium
ER	Endoplasmic reticulum
GalNAc	N-Acetyl-D-galactosamine
GlcNAc	N-Acetyl-D-glucosamine
Man	D-Mannose
GHs	Glycoside Hydrolases
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
IBD	Inflammatory Bowel Disease
MWCO	Molecular weight cut off
PNA	Peanut agglutinin
PAMPs	Pathogen associated molecular pattern
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
SSN	Sequence similarity network
SBA	Glycine max agglutinin
WGA	Wheat germ agglutinin

DECLARATION OF ACADEMIC ACHIEVEMENT

The bioinformatic analysis of the human gut microbiome and identification of uncharacterized endo-β-N acetylglucosaminidase (GH18 and 85) GH33 and GH156 sialidases were performed by Evan Mann from the Surette Laboratory.

The Hek Blue mTLR4 assays were performed with the assistance of Arshpreet Bhatwa from the Schertzer Laboratory.

GH enzyme purification was performed by Aarthi Pasupathi from the Surette laboratory.

The author completed the rest of the work in this study.

CHAPTER ONE: Introduction

Human epithelial cell surfaces constantly interact with symbiotic microorganisms that play an important role in maintaining health homeostasis and disease(Belkaid & Hand, 2014; Wang et al., 2017).As with almost all eukaryotes, human cell surface and secreted macromolecules are covered with glycans. Many glycans can modulate or mediate a variety of events in cell-cell, cell-matrix, and cell-molecule interactions critical to complex multicellular organisms (Varki & Kornfeld, 2015; Whitfield et al., 2015). Extracellular glycans function as a layer that protects the cell surface from microbial contact and attachment such as in the case of mucins on the intestinal tract (Varki & Gagneux, 2015). Microbes that colonize mucosal surfaces can utilize and modify these extracellular glycans as a nutrient source (Tailford et al., 2015). Furthermore, microbial glycans such as LPS and LTA, can also be sensed by host cells and stimulate immune pathways (Comstock & Kasper, 2006). Our diets include both glycans and plant lectins (glycan binding proteins). Dietary glycans are important nutrients for both microbes and host, and lectins are potential immune modulators through their ability to recognize and bind to glycosylated cell surface receptors. Thus, glycans are at the interface of microbe-host-diet interactions and in this thesis, I am investigating the role of glycan degrading enzymes produced by the commensal microbiota in modulating microbe glycan/dietary lectin interactions with the host.

1.1Glycans

Glycans are carbohydrate-based macromolecules composed of single sugar molecules linked together by chemical bonds. They can be found as free polysaccharides or as glycoconjugates linked to other macromolecules such as proteins and lipids (Valverde et al., 2019). Protein-carbohydrate conjugates are most commonly N- and O-linked glycans define by the specific linkages (Figure 1). N-glycans begin with an N-acetyl galactosamine (GlcNAc) residue linked to the side chain amino group of asparagine in the peptide motif Asn-X-Ser/Thr (Stanley et al., 2022). In eukaryotes most N-linked glycans share a common core sequence of GlcNAc(β1–4)-GlcNAc(β1)–Asn. Moreover, the core structure of most N-glycan is composed of trimannosyl chitobiose unit ($Man_3GlcNAc_2$)(Meyer et al., 2021; Stanley et al., 2022).

There are three types of N-glycan structure: Oligomannose, composed of almost all mannose residues Man residues), Complex containing many different types of saccharides, and Hybrid which includes both extended mannose and mixed saccharide structures (Figure 2)(Stanley et al., 2022). These glycans have a crucial role in correct protein folding and conformation. They also mediate cell-cell adhesion by lectin-glycan interaction (Esmail & Manolson, 2021; Garbe & Collin, 2012).In addition, N-glycans are usually found on the surface of cells of the innate and adaptative system, such as neutrophils, monocytes, and macrophages, and also on immunoglobulins such as IgG (Radovani & Gudelj, 2022).

O-linked glycans result from the addition of a GalNAc residue on the hydroxyl side chain of serine or threonine residues (Garbe & Collin, 2012). Their structure has four subtypes depending on different linkage with GalNAc. Core1 (Gal addition in β1,3 linkage to GalNAc), Core 2 (GlcNAc addition in β1,6 linkage to core 1), Core 3 (GalNAc-α-O-Ser/Thr) and Core 4, derived from core 3 by the action of β1,6-N-acetylglucosaminyltransferase (Figure 2)(Bergstrom et al., 2015). Many O-glycans are less branched than N-glycans and are a significant component of mucins and immunoglobulins (González-Morelo et al., 2020; Varki et al., 1999).

One crucial component of mucus gut layer are complex glycosylated proteins called mucins. The majority of oligosaccharides on mucins are O-linked glycans that can serve as a nutrient source for many pathogens and commensal bacteria(Guzman-Aranguez & Argüeso, 2010). The mucins form a dense physical barrier between gut epithelium and microbiota preventing direct bacteria-host cell contact(Guzman-Aranguez & Argüeso, 2010). Moreover, gut microbiota can stimulate changes in host mucin glycosylation patterns resulting in the internalization of pathogens or dietary lectins, producing chronic inflammation such as in the case of inflammatory bowel disease (Reily et al., 2019).

Bacteria also produce glycosylated structures associated within the cell envelope and cell surfaces. Peptidoglycan forms a mesh-like network surrounding the cytoplasmic membrane of bacteria and is critical for bacterial cell structure. Gram-negative bacteria also produce an outer membrane containing lipopolysaccharides (LPS), while the Gram-positive bacteria peptidoglycan layer is covalently linked to

lipoteichoic acid (LTA). These glycolipids are important for bacterial survival and manage important interactions with elements of the host innate and adaptive immune system. A few serve as pathogen-associated molecular patterns (PAMPs) that are recognized via Toll-like receptor pathways(Cayuela, 2000; Varki & Kornfeld, 2015). Furthermore, most membrane protein receptors are glycosylated, which determine their activity (Al-Ghouleh et al., 2012).

1.2 Toll-like receptors (TLRs)

TLRs are a class of pattern-recognition receptors (PRRs) present in many host cells, including intestinal epithelial cells and gut-associated immune cells, which are involved in the inflammation process(Villena & Kitazawa, 2014). Currently in humans there are 10 members of TLR family (Kawai & Akira, 2006). TLR stimulation leads to the release of a wide variety of inflammatory cytokines and inflammatory modulators(El-Zayat et al., 2019). According to their function and location in host cells, they are classified into cell surface TLRs and intracellular TLRs. The focus of this project was cell surface TLRs, including TLR2 and TLR4, due to their interaction with the extracellular environment, microbiome, and host-derived diet metabolites(Kawasaki & Kawai, 2014). Cell surface TLR structure consists of an extracellular N-terminal domain, followed by a single helix transmembrane domain and a cytoplasm C-terminal domain(Sameer & Nissar, 2021). N-terminal domain includes ectodomains which include N-glycans glycosylated and serve as the ligand recognition site for PAMPs (Botos et al., 2011).

1.3 TLR2 and TLR4 ligands and signaling pathways

TLRs recognize ligands from different microorganisms such as protozoa, fungi, viruses and bacteria; and they also bind to diverse damage-associated molecular patterns (DAMPs) such as plasma membrane proteins and DNA (Sameer & Nissar, 2021). TLR2 ligands recognize different bacteria structures, such as lipopeptides of mycoplasma and gram-positive bacteria (Kawasaki & Kawai, 2014). Ligand activation of TLR2 occurs by dimerization with TLR1 or TLR6, conferring a horseshoe structure to the ectodomain forming an M shaped dimer with the ligand (de Oliviera Nascimento et al., 2012). The TLR4 ligand is mainly bacterial LPS with the lipid A moiety responsible for receptor activation(Schromm & Brandenburg, 2021). TLR4 stimulation occurs by the LPS-binding protein delivered by CD14, loading the LPS to complex TLR4/MD-2, which is anchored to the concave surface of TLR4(Botos et al., 2011). After PAMPs recognition, MyD88 pathways activate NF-κB and MAPKs inducing cytokine gene expression. TIRAP acts as an adaptor that recruits MyD88 to TLR2. Meanwhile, TLR4 activates MyD88- dependent and TRIF-dependent pathways (Kawasaki & Kawai, 2014).

1.4 TLR glycosylation

As with many other receptors on the cell surface, TLRs are glycosylated (Al-Ghouleh et al., 2012). N-linked glycosylation of TLR plays a key role in determining the resulting response (Cayuela, 2000). It has been reported the presence of 4 and 9 -N-linked glycosylation sites on TLR2 and TLR4 receptors; respectively (da Silva Correia & Ulevitch, 2002; Weber et al., 2004). TLR4 activity and stability depend on the formation of a complex TLR4/MD-2 in the ER. MD-2 is necessary for TLR4 glycosylation and translocation to cell surface (Amith et al., 2010) α-2-6- and α-2,3-sialylation of TLR4 has been shown to modulate activity and neuraminidase removal of sialyl residues promotes LPS activation of NF-Kb and cytokine secretion (Leifer & Medvedev, 2016). LPS binding activates the host enzyme neuraminidase 1 (Neu1), a sialidase that removes sialic acid from the surface glycans of the receptor, following MyD88/TLR4 activation of NF-κB in macrophage cells (Amith et al., 2010).

It has also been reported that lectins may act as TLRs agonists through binding of the N-glycans on the receptor, such as ArtinM (Mannose-binding) lectin from Jackfruit (El-Zayat et al., 2019). Nevertheless, there are only a few studies focusing on TLR's interactions with plant lectins(Batista et al., 2017; Gong et al., 2017; Mariano et al., 2014; Unitt & Hornigold, 2011).

1.5 Plant Lectins

Lectins are glycoproteins that bind to specific carbohydrate residues including those on the cell surfaces of bacteria and host cells (Mishra et al., 2019). Lectins are produced by all living organisms, and bind to specific carbohydrate moieties but have not enzymaic activity (Bellande et al., 2017). Plant lectins have a wide variety of structures and are involved in plant defence against pathogens (Lucius, 2020). Lectins are usually formed by two or four homo or heteromeric subunits which contain calcium and manganese ions required for saccharide binding (Sharon & Lis, 2007). Also, they can be found in almost all kinds of food containing legumes and grains. Most lectins are resistant to the digestive process in the gut due to their ability to remain stable in acidic environments (Barre et al., 2020; Boston & Ma, 2019). In addition, given the high degree of specificity of lectins for carbohydrate moieties, some dietary lectins have been shown to induce the NRPL3 inflammasome by binding on glycans present on cell surface receptors(Gong et al., 2017). This may indicate a contribution of lectins to inflammatory responses in food allergy, food intolerance, and inflammatory bowel diseases (Barre et al., 2020; Gong et al., 2017). The specificity of plant lectins for specific glycan moieties has also made them important tools for labelling cells and monitoring changes in cell surface glycans (Table 1). In this thesis, four different plant lectins have been used as tools for analyzing immune cell stimulation before and after treating them with GHs and other PAMPs.

1.6 Glycoside hydrolases (GHs)

Carbohydrate-active enzymes (CAZymes) include carbohydrate-degrading enzymes that include glycoside hydrolases (GHs), carbohydrate esterases and polysaccharide lyases (Wardman et al., 2022). GHs, the focus of this thesis, are enzymes that catalyze the hydrolysis of the glycosidic linkage of glycosides resulting in the formation of a sugar hemiacetal or hemiketal and the free aglycon (Davies & Henrissat, 1995; The CAZypedia Consortium, 2018). The enzymes are classified based on their site of action and their enzymatic mechanism (Figure 3) (Vuong & Wilson, 2010). In the first classification, exo- and endo- refers to the ability of glycoside hydrolase to cleave substrate at the end or within the middle of a polysaccharide chain, respectively (Gloster et al., 2008; McCarter & Stephen Withers, 1994). The second classification is based on the mechanisms of hydrolysis, which can be retaining or inverting. A typical retaining glycosidase needs a catalytic acid residue and a catalytic base residue, while an inverting glycosidase contains a general acid/base residue and a nucleophile(Vuong & Wilson, 2010). The organization of GHs into families was proposed based on amino acid sequence similarities and tertiary structure. Enzymes with different substrate specificities are sometimes found in the same family; on the other hand, enzymes that hydrolyze the same substrate are sometimes found in different families (Davies & Henrissat, 1995). There are currently 173 GH families identified in the Carbohydrate Active enZyme (CAZy) database (http://www.cazy.org/Home.html, accessed 12/29/2022), with new families

continually being discovered (Boston & Ma, 2019; Kaoutari et al., 2013). Each GH family represents proteins (or domains within composite proteins) that have the same structural fold and carry out the same enzymatic reaction with respect to the specific bond cleavage reaction. However, the actual macromolecular substrate for enzymes within a family can differ, and very few GHs enzymes have been characterized within most families.

Although GHs families have different protein folds, the mode of action and the specificity of the enzyme are dictated by the topology of the active site falling into three general classes, the pocket (endo-mode), the cleft, and the tunnel (exo-acting) (McCarter & Stephen Withers, 1994).

1.7 Microbial GHs

Glycoside hydrolases are widely distributed among commensal and pathogenic microbes. Microbes use these enzymes on gut mucosal glycans and dietary glycans for nutritional purposes; however, microbial degradation of mucins may contribute to inflammatory process in the gut as a result of the breakdown of this protective barrier. Also, microbes can modify host surface glycans to facilitate attachment and internalization (Koropatkin et al., 2008). Many pathogens possess GH for both nutrition and modulating glycan binding sites for colonization. For example, *S. pneumoniae* which encodes more than 40 known proteins that break glycosidic bonds including sialidase that is important for colonization of the airways (Hobbs et al., 2018). Mucin glycan degraders have different strategies to access

these glycans available in the gut, such as *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* that utilize a wide arrange of GHs such as exo-β-glucosaminidase (GH2), endo-β1-N-acetylglucosaminidase (GH18, GH85), sialidases (GH33, GH156), β-galactosidase (GH42), and α-galactosidase (GH110) (Koropatkin et al., 2008). Many of these GHs associated with the process of mucin degradation have been hypothesized to be extracellular (Belzer, 2022). Other species such as *Bifidobacterium bifidum, B. breve, B. longum* subsp. Infantis*, Lactobacillus spp., Clostridium spp.,* and *Escherichia coli* also possess the ability to degrade human milk oligosaccharides (Garrido et al., 2012).

In a preliminary analysis by Evan Mann in the Surette laboratory, ~10,000 GH genes were identified in individual human gut samples using culture-enriched metagenomics data. This includes a wide range of GH families. The GHs encoded within the microbiome have the potential to modulate the sites of microbiota-host interaction. The overarching focus of this research program was characterizing specific GH families that could act on N-glycans on the human cell surface and extracellular matrix glycome - that were predicted to be extracellular. Our particular interests are a new family of exo-sialidases (GH156) and endo-β-*N*-acetyl-glucosaminidases, which remove N-glycans (GH18 and GH85 families).

1.8 Family GH18 and GH85

Endo- β-N-acetylglycosaminidases (ENGases) are enzymes that cleave the chitobiose core (GlcNAc(β1–4)-GlcNAc(β1)–Asn) of N-glycans and are classified into two GHs families (GH18 and GH85) having a similar structure of eight α-helices and eight β-sheets (Fairbanks, 2017).

Glycoside hydrolase 18 family, better know as chitinases (Class II), currently contains more than 10,000 enzymes, that catalyze the hydrolysis of chitin oligosaccharides (β-1, 4 glycosidic bond) (Shrivastava, 2020). GH 18 family has been found in many organisms, such as bacteria, fungi, viruses, animals and plants, although it has not been reported that mammals synthesize chitin (Funkhouser & Aronson, 2007). GH18 activity in cells is associated with diverse physiological functions in bacteria, fungi and humans. A good example is *Saccharomyces cerevisiae* which requires GH18 for cell division during growth (Q.-S. Huang et al., 2012).

GH 85 family are also ENGases and it has been shown to cleave the GlcNAc-β1-4-GlcNAc in the conserved core of all human N-linked glycans (Fujita et al., 2001). Examples of bacterial ENGases with this activity are Endo D from *Streptococcus pneumoniae* which is able to hydrolyze fucosylated N-glycan core and Endo E from *Enterococcus faecalis* (Garrido et al., 2012). During infection, GH18 (Endo S) from *Streptococcus pyogenes*, and GH85 from *S. pneumoniae* use these enzymes on

host IgG gycans, reducing the affinity for the Fc receptor increasing their survival rate (Fan et al., 2012; Wardman et al., 2022).

1.9 Family GH 33 and GH156

Sialic acid is a carboxyl acid with 9 monosaccharides present in many kingdoms of life, such as bacteria and mammals, where the most abundant form of sialic acid is Neu5AC (Keil et al., 2022). Usually, sialic acid is found as a terminal modification in mucins (Bowles & Gloster, 2021). Sialidases (or neuroaminidases) are a large group of GHs enzymes that cleave terminal sialic acid from glycoproteins or glycolipids (Juge et al., 2016). They have been classified into five GHs family: 33,34,58,83 and 156. Gut bacteria sialidases are grouped in the GH 33 family includes bacterial and human extracellular sialidases that present a six-bladed β-propeller fold structure (Bule et al., 2019). GH156 family enzymes are exo-α-sialidase originally identified from a hot spring environment that hydrolyze α2,3- and α2,6-linked with a $(\beta\text{-}\alpha)_8$-hydrolase fold (Keil et al., 2022). The presence of GH156 in human gut microbiota was explored by (Mann et al., 2022), demonstrating that gut microbiota encode diverse GH156s exhibit sialidases activity. In humans, extracellular sialidases associated to membrane Neu3 act as structural modulators of membrane proteins playing an important role in receptor signalling pathways (Monti et al., 2000). Moreover, such as in the case of ENGases, bacterial pathogens use sialidases as a defence mechanism to promote mucosal infections, attachment and colonization. That is the case of *Streptococcus pneumoniae* that produce distinct neuraminidases such as NanA, and

Haemophilus influenzae on the respiratory tract that binds to the host cells by modifying cell surface glycans (Keil et al., 2022; Parker et al., 2009).

1.10 RATIONALE HYPOTHESIS AND AIMS

As with almost all eukaryotes, cell surface proteins in human mucosal epithelia and crucial molecules in innate and adaptative immunity are glycosylated. Appropriate glycosylation plays a central role in modulating cell-cell interactions and ligand-receptor interaction, determining the resulting response. Many pathogens exploit this process to interact with host cell membrane glycans as receptors for adhesion and internalization. Others, such as the human gut commensal microbes, encode a wide variety of extracellular GHs that can target the host glycome, potentially shaping physiology and innate immunity. The bacterial glycoside hydrolases have the potential to modulate receptor glycosylation by removing or exposing recognition sites for dietary lectins or PAMPS. However, despite the clear enzymatic specificity for specific glycoside bonds for each GH family, the specificity of different enzymes for different macromolecular substrates (such as specific glycoproteins, glycolipids, dietary fibres) is much less understood. Evan Mann from the Surette laboratory has carried out a bioinformatic analysis of the human gut microbiome and identified uncharacterized endo-β-N acetylglucosaminidase (GH18 and 85) and GH156 sialidases. In addition, he has generated sequence similarity networks that categorized distinct clusters of related proteins within each

GH family, and we hypothesize that each cluster represents a distinct macromolecular substrate (Figure 4). As part of this larger research program in the laboratory, I focused on different representatives from each of these clusters.

I hypothesize that GHs from the commensal gut microbiome reshape cell surface glycome modulating their responses to PAMPS and dietary lectins contributing to gut inflammatory diseases.

The core of this project is developing a screening test measuring GH enzyme activity from human gut samples from our lab collection focusing on three GHs families: GH156 (Sialidase), and GH18 and GH85 (endo-β-*N*-acetyl-glucosaminidases) sourced from human microbiome strains from our lab collection. In addition, we focus on understanding if GHs from human gut microbiota can modulate glycosylation on cell culture cell lines and immune cell receptors such as TLRs.

As a result, this thesis has three primary aims:

> **Aim 1** Develop a screening assay to measure glycosidase enzyme activity in bacterial-free cell supernatant and bacterial pellets from human microbiome isolates.
>
> **Aim 2** Use dietary plant lectins to stimulate Hek-Blue TLR2/TLR4 cells before and after treating them with LPS/LTA and GHs to answer the question: Do bacterial GHs modulate immune cell response to dietary lectins and PAMPs?

Aim 3 Monitor the effect of exogenous GHs on cell surface glycosylation of A549 cells using fluorescence microscopy with fluorescent-labelled lectins.

Table 1. Dietary lectins and their glycan specificity[1].

DIETARY LECTIN	ABBREVIATION	BINDING SPECIFICITY
Arachis hypogaea (Peanut) agglutinin	PNA	GalNAc
Artocarpus heterophyllus (Jackfruit)	ArtinM	Man
Artocarpus integrifolia (Jacalin)	Jacalin	GalNAc
Amaranthus caudatus (Amacaa)	ACA	Galβ1-3GalNAc
Calystegia sepium (Heavenly trumpets)	Calsepa	Man
Concanavalin A (Canavalia ensiformis)	Con A	α-mannopyranosyl and α-glucopyranosyl
Chenopodium quinoa (Quinoa)	-	GlcNAc
Dolichus biflorus (Horse Gram)	DBA	α-D-GalNAc
Datura stramonium (Thorn apple)	DSA	Galβ1-4GlcNAc
Erythrina cristagalli lectins (Coral tree)	ECL	GalNAc
Glycine max (soybean) agglutinin	SBA	α- and β-GlcNac and galactopyranosyl residues
Maackia amurensis (Amur maackia)	MAH	Siaα2-3Galβ1-3
Lens culinaris agglutinin (Edible lentil)	LCA	Methyl-α-D-mannopyranoside
Oryza sativa L. (Asian rice)	OS	GlcNac
Phytolacca americana (Pokeweed)	PWM	(GlcNAc β1-4) n
Rcicinus communis (Castor bean)	RCA-1	B-D-GalNAc
Sambucus nigra lectin (Elderberry)	SNA	Sialic acid

Solanum tuberosum L. (Red potato)	STL	GalNAc
Triticum vulgaris agglutinin (Wheat germ)	WGA	NeuNac and GlcNAc
Urtica dioica (Perennial plant)	UDA	Mixture of Man 5 to Man9
Ulex europaeus (Common gorse)	UEA-I	Fuc α1-2Galβ1-4GlcNAc
Vicia villosa (Winter vetch)	VVA	α-linked terminal GalNAc
Vicia faba L. (Broad bean)	VFA	Man/Glc
Vigna unguiculata L. (Black-eye pea)	VU	GalNAc
Wisteria floribunda (Japanese wisteria)	WFA	GalNAc β1-4GlcNAc

[1], Adapted from ((Adamcová et al., 2021; Cappuccio et al., 2009; Inoue et al., 2013)).

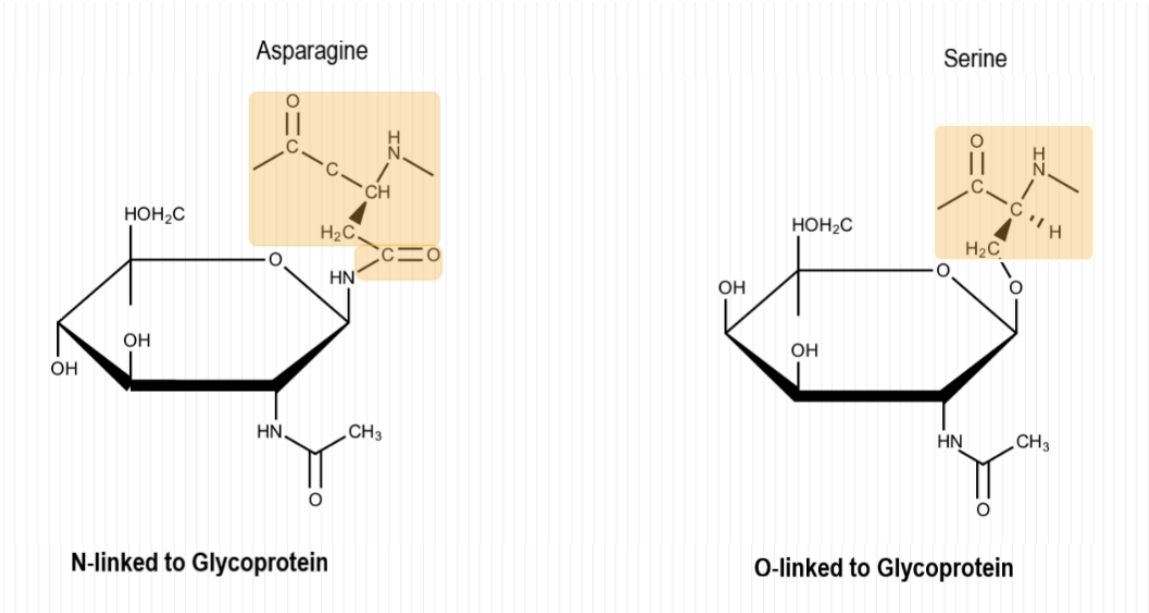


Figure 1. N- and O- linked glycosylation. The two most common forms of protein glycoconjugates, O-linked glycans can be link to either serine or threonine side chain hydroxyls. (Drawn with ChemDoodle).

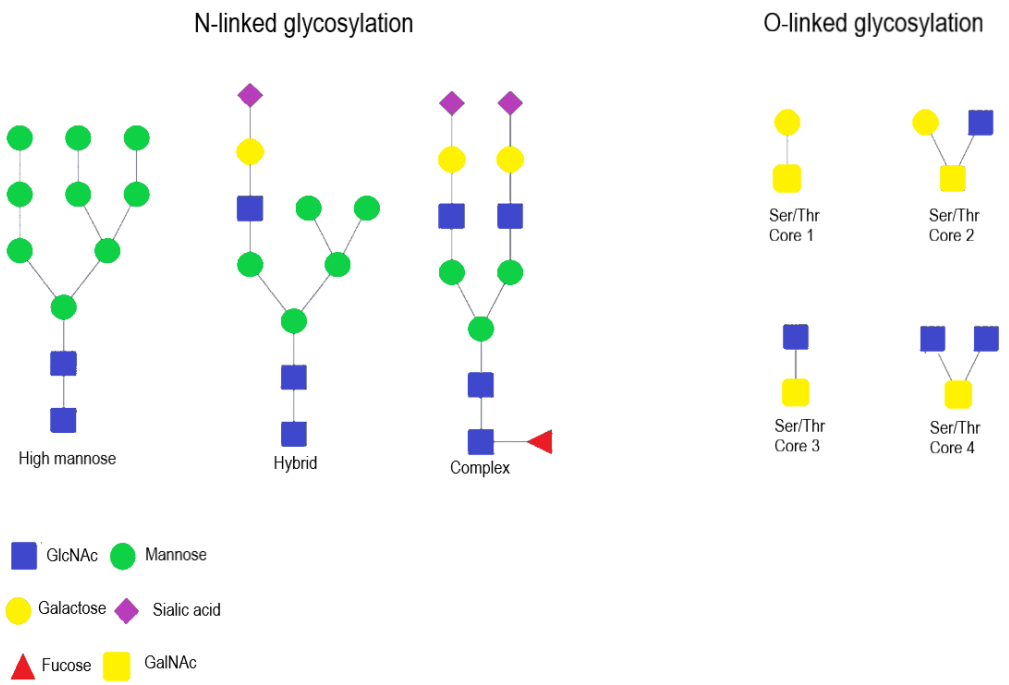


Figure 2. Types of N-glycans and O-glycans present in eukaryotes. N-Glycans are present in three general types of structures: High mannose (Oligomannose), Hybrid and complex. O-linked glycans are grouped by the core structure attached the Sr/Thr. Cores 1 to 4 are the most common core structures attached to Ser/Thr. (Adapted from(Sjögren & Collin, 2014).

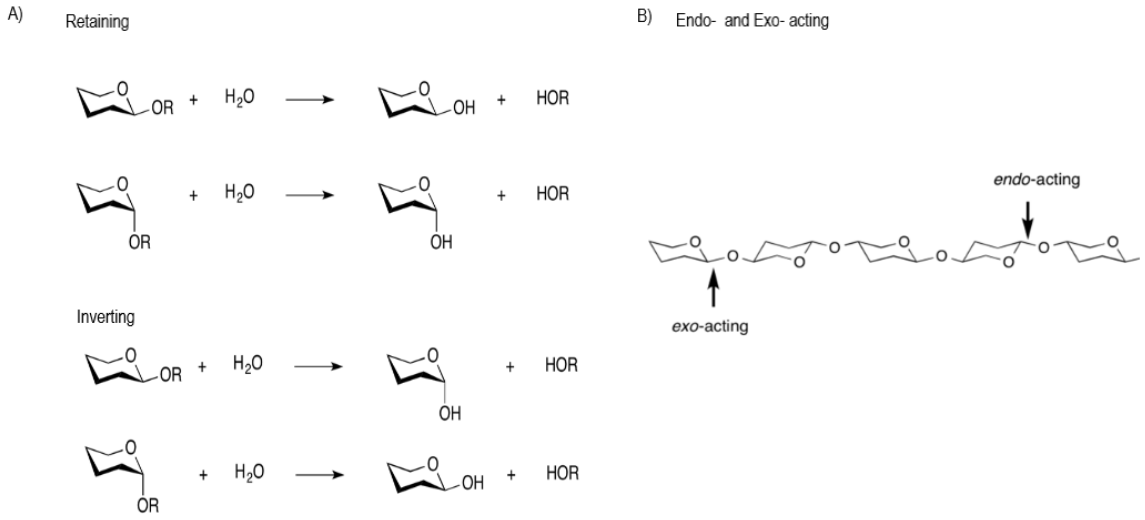


Figure 3. Glycoside hydrolase classification. A) Retaining and inverting mechanistic classification as first proposed by Koshland. In the retaining mechanism ihe resulting product has the same stereochemistry as the substrate. An inverting mechanism results in the product having the opposite stereochemistry to the substrate. B) Endo/exo acting classification, referring to the ability to cleave the substrate in the middle of a chain (Endo) or at the end of the chain (Exo). (Modified from CAZy database http://www.cazy.org/).

.

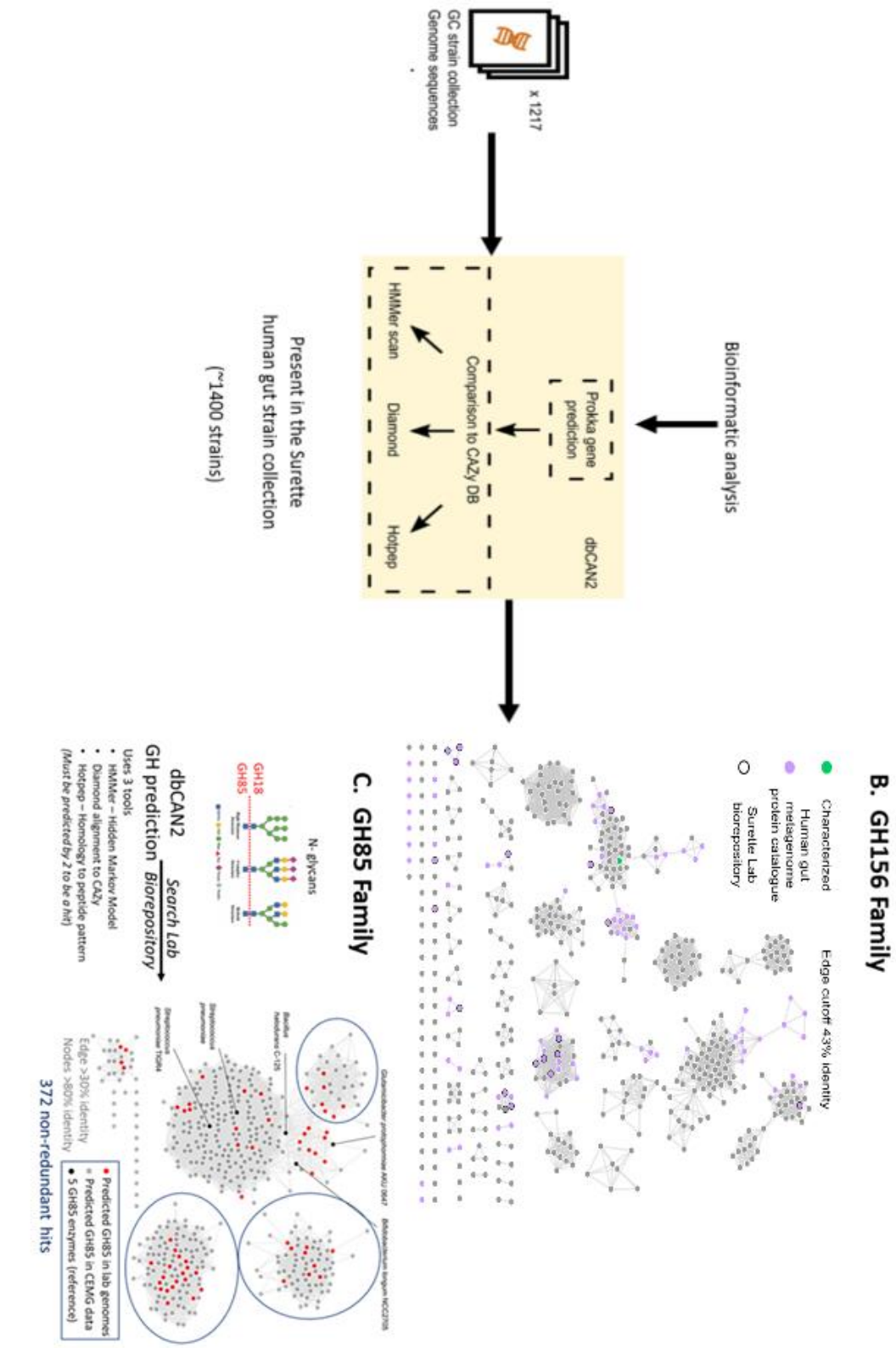

GC strain collection
Genome sequences
x 1217
Bioinformatic analysis
dbCAN2
Prokka gene prediction
Comparison to CAZy DB
HMMer scan
Diamond
Hotpep
Present in the Surette human gut strain collection
(~1400 strains)
B. GH156 Family
Edge cutoff 43% identity
Characterized
Human gut metagenome protein catalogue
Surette Lab biorepository
C. GH85 Family
N-glycans
GH18
GH85
dbCAN2 GH prediction
Search Lab Biorepository
Uses 3 tools
HMMer – Hidden Markov Model
Diamond alignment to CAZy
Hotpep – Homology to peptide pattern
(Must be predicted by 2 to be a hit)
Edge >30% identity
Nodes >80% identity
372 non-redundant hits
Predicted GH85 in lab genomes
Predicted GH85 in CEMG data
5 GH85 enzymes (reference)

Figure 4.General overview of the bioinformatic analysis for the identification of sequence similarity network clusters of GH18, GH185 and GH156 proteins in the human microbiome. A bioinformatic analysis were performed using dbCAN2 to compare gene sequences with CAZy data base. Each node represents a protein group (clustered with cdHit at 95% identity). Sequence similarity network (SSN) were used to categorize clusters of related proteins within each family. Proteins present within human microbiome databases are coloured mauve. Representatives from within the Surette lab strain collection are circled with black. B) GH156 SSN C) GH85 SSN. For more detailed methodology see Mann et al 2022).Figure was created by E. Mann.

CHAPTER TWO: Glycosidase enzyme activity in bacterial-free cell supernatant and bacterial pellets from human microbiome isolates.

2.1 BACKGROUND

Bacteria can encode a large number of GHs including those involved in primary metabolism and localized mostly into the cytoplasm. Other GHs are active outside the bacterial cells with some present on cell surface, while others are secreted to the surroundings (Berlemont & Martiny, 2016; Koropatkin et al., 2008). Gut microbes encode a diverse arsenal of surface-attached and released GHs involved in degradation of dietary and host derived glycans which in turn benefit the host (Hobbs et al., 2018). These enzymes also have a role in pathogenic bacteria, involved in nutrition, adherence to host cells and modulating the immune response through modification of glycoproteins (Sjögren & Collin, 2014). In this work, I established a screening assay to monitor the enzyme activity of GHs in cell-free supernatants and bacterial pellets. This was carried out with bacterial strains from our lab collection, prioritizing the human gut and upper respiratory tract strains that encode for predicted extracellular GH18, GH85, GH156, and GH33 enzymes belonging to different clusters in SSN, n=65 (Figure 5).

2.2 MATERIALS AND METHODS

2.2.1Bacterial strains.

65 bacterial strains from 22 different genera (Table 2) were selected, prioritizing strains containing predicted extracellular GH18, GH85, and GH156 enzymes belonging to different clusters using sequence similarity networks analysis (data not shown, provided by Dr. Evan Mann).

2.2.2 Cell-free supernatant and bacteria cell pellet recovery

Bacteria were grown from frozen stocks on BHI3 agar (Brain Heart Infusion agar supplemented with 1mg/L vitamin K, 10mg/L hemin, and 0.5g/L L-cysteine)(Lau et al., 2016) for 24 hrs for fast growers, 72 hrs for intermediate growers and 96 hrs for slow growers under anaerobic conditions at 37°C. Single colonies were subcultured in 2 ml of BHI3 broth or BHI3 broth diluted with H_2O (1:1 dilution) and grown at 37ºC in 1.5ml microfuge tubes (Fisher Scientific). Supernatants were recovered after centrifugation at 783×g for 20 minutes at 4ºC and stored at -20ºC. Pellets were resuspended in 500 µl of 0.85% NaCl solution and stored at 4ºC before testing. For bacteria that did not grow in BHI3 diluted broth medium, cell pellets were recovered from BHI3 agar by resuspending colonies in 500 µl of 0.85% NaCl solution (n=11).

2.2.3 Glycosidase enzyme activity measurement

A) α-GlcNAc, β-GlcNAc, and Sialic acid assays

Working solution mixes were prepared using 30 mM stocks of 4MU-α-D-N-acetylneuraminic acid, 4MU-N-acetyl-β-D-GlcNAc or 4MU-N-acetyl-α-D-GlcNAc, 250mM sodium acetate (pH 5.2) and H_2O for a final concentration of 2.6 mM. 96-well plates were used for the assays with a total volume of 100 µl. Each reaction contained 90µl dH_2O, 5 µl of sample (cell-free supernatant or bacterial pellets) and 5 µl of 4-MU-sugar working solution for a final concentration of 130 µM per well. Fluorescence (360nm excitation wavelength (EX) / 460nm emittance wavelength (EM) was measured in a Synergy H1 plate reader (BioTek) from 0 to 30 minutes at 30°C. The fluorescent units result for each assay was analyzed and expressed as fluorescence change per minute.

B) α-Fucose assays

Working solution mix was prepare using 10 mM stocks of 4MU-α-L-fucopyranoside, 250 mM sodium acetate (pH 5.2) and H_2O for a final concentration of 1.0 mM. 96-well plates were used for the assays with a total volume of 50 µl. Each reaction contained 40 µl of dH_2O, 5 µl of sample of interest, and 5 µl of 4-MU-sugar working solution for a final concentration of 100 µM per well. Fluorescence (360nm EX / 460nm EM) was measured in a BioTek Synergy H1 plate reader from 0 to 30 minutes at 30°C. The fluorescent units result on each assay was analyzed and expressed as fluorescence change per minute.

2.2.4 Modifications to reduce background in cell-free supernatant assays

Assays of bacterial supernatants grown in BHI3 broth had a high intrinsic fluorescence background. To reduce this background drop dialysis was performed using 0.025µm Mixed Cellulose Esters membranes (Millipore). 100 µl of cell-free supernatant was added on top of the membrane and left floating on 50 mM Tris-HCl buffer (pH 7.4). Buffer was changed after 1 hr before leaving it overnight at 4ºC. Alternatively, bacteria cells were grown in BHI3 media diluted 1:1. In order to avoid any background noise in the pellet samples, pellets were resuspended in 0.85% saline solution.

2.3 RESULTS

For the development of these assays, I used a modified method from a previously published protocol for sialidase activity measurement (Chen et al., 2014). 4-MU fluorophore with the substrates of interest α-D-N-acetylneuraminic acid, N-acetyl-β-D-GlcNAc, N-acetyl-α-D-GlcNAc and α-L-fucopyranoside substrate (Figure 6) were used to measured GHs enzyme activity. In this assay, enzyme activity was monitored over time *in vitro*. The objective was to create a screening tool to detect microbial GH activity without purifying the enzymes. By using bacterial pellets and cell-free supernatant, I addressed the question: Are GHs being released to the media or anchored to the membrane? Moreover, I showed a correlation between GH gene presence in our samples (predicted by bioinformatic analysis by Dr. Evan

Mann) and in vitro GH enzyme activity under rich media conditions (BHI3 supplemented media).

2.3.1 Cell-free supernatant measurement

For standardization of the assays, I measured the enzyme activity of β-GlcNAc, using *Enterococcus faecalis* (GC97) and *Rothia kristinae* (GC144) supernatant (Figure 7A). This assay showed a time-dependent enzyme activity for *E. faecalis* (GC97). Conversely, no activity was detected with *R. kristinae* (GC144) supernatant on this substrate. However, this assay was confounded by a high fluorescence background in our samples. BHI3 medium, supernatant samples on BHI3 media, and acetate buffer were measured without substrate and high background fluorescence was found to be dependent on the presence of BHI medium. In order to remove the background from supernatant samples, drop dialysis (Millipore 0.025µm Mixed Cellulose Esters membrane) was carried out. By using drop dialysis, I reduced the background from our samples; however, no GHs activity was recovered even from the previously 4MU-N-acetyl-β-D-GlcNAc positive *E. faecalis* (GC97) supernatant. As an alternative approach, bacteria were grown in BHI3 media diluted 1:1 in water (Figure 7B). The BHI3 medium 1:1 dilution showed a significant reduction in the background assays while conserving the enzyme activity of our samples. 4-MU-substrate assays were performed for BHI3 diluted broth with our glycans of interest (Figure 7B). Reduction of the background on our supernatant samples was achieved by using BHI3 medium diluted 1:1 on 4MU-N-acetyl-β-D-GlcNAc by 50% (Figure 8A).

Having established suitable reaction conditions using diluted BHI3 as a growth medium, GH activity was assayed for more strains, 46 samples were used to perform 4MU-N-acetyl-β-D-GlcNAc assays. 8 samples were used to perform 4MU-α-D-N-acetylneuraminic acid, 4MU-N-acetyl-α-D-GlcNAc and 4MU-α-L-fucopyranoside assays for standardizing the assays on these substrates. 3 (6.48%) of the supernatant samples showed activity for 4MU-N-acetyl-β-D-GlcNAc substrate. *Bacillus subtilis* (GC709)*, Enterococcus faecalis* (GC 97) and *Prevotella melaninogenica* (GC149) showed activity for this substrate (Figure 9A). 4 (50%) of the supernatant samples measured for sialic acid showed activity for 4MU-α-D-N-acetylneuraminic acid substrate. Active samples were from *Enterococcus durans* (GH27)*, Enterococcus faecalis* (GC97), *Bacteroides fragilis* (GC73) and *Lactobacillus plantarum* (GC4) (Figure 9B). 2 (25%) of the samples measured for α-GlcNAc showed activity for 4MU-N-acetyl-α-D-GlcNAc substrate (*Enterococcus faecalis* (GH97) and *Streptococcus* parasanguinis (GC897) (Figure 9C). Regarding the measurement of fucosylase activity, 7/8 (87.5%) of the samples showed activity for 4MU-α-L-fucopyranoside substrate. From this, 4 (50%) of the samples belonged to *Streptococcus* genera, as well as *Enterococcus faecalis* (GH97), *Enterococcus durans* (GH27) and *Bacteroides fragilis* (GH73) (Figure 9D).

2.3.2 Bacteria pellet measurement

65 samples were used to assess 4MU-substrate activity on bacterial pellet samples. 36/65 (55.3%) of the bacterial pellet samples showed activity for 4MU-N-acetyl-β-D-GlcNAc substrate (Figure 10). From this, 20 (30.7%) of the samples

belonged to *Bacteroides* genera*,* followed by 4 (6.15%) *Streptococcus*, 3 (*4.6%) Parabacteroides*, 1 (3.07%) as well as *Prevotella nanceiensis* (GC155), *Victivallis vandensis* (GC352), *Alistipes finegoldii* (GC1192), *Coprobacter fastidiosus* (GC1038), *Enterococcus faecalis* (GC97), *Gabonia massiliensis* (GC 960) and *Roseburia intestinalis* (GC447) (Figure 10).13/65 (20%) of the bacterial pellet samples showed activity for 4MU-α-D-N-acetylneuraminic acid substrate. 4 (6.15%) of the samples belonged to *Bacteroides,* followed by 2 *Prevotella melaninogenica* (GC149, GC329)*, Alistipes finegoldii* (GC124), *Barnesiella intestinihominis* (GC1181)*, Escherichia fergusonii* (GC707), *Monoglobus pectinilyticus* (GC559), *Parabacteroides merdae* (GC432)*, Paraprevotella clara* (GC1063) *a*nd *Streptococcus dentisani* (GC108) (Figure 11A). From these results, I concluded that endoglycosidases and sialidases in our samples are mainly anchored to the bacterial membrane.

I also carried out assays for fucosylase activity. 14 (21.5%) of the bacterial pellet samples showed activity for 4MU-α-L-fucopyranoside. Of these, 7 10.7% were *Streptococcus,* with the remainder being *Alistipes indistinctus* (GC470)*, Barnesiella intestinihominis* (GC665)*, Bifidobacterium longum subsp. Infantis* (GC751)*, Escherichia fergusonii* (GC707) *Monoglobus pectinilyticus* (GC559)*,* and *Parabacteroides merdae* (GC432) (Figure 11C).

Only one of the 65 bacterial pellet samples assayed showed activity for 4MU-N-acetyl-α-D-GlcNAc substrate (*Barnesiella intestinihominis* (GC1181)) (Figure 11B), revealing a low enzymatic activity of bacterial pellets for this substrate.

Comparing predicted genes (Table 2) to the detected activity, all samples showing β-GlcNAc, α-Sialic acid, α-GlcNAc and α-fucose activity have were predicted in the bioinformatic analysis to have the appropriate glycoside hydrolases predicted as extracellular: GH18, GH85, GH33, GH89 and GH29 genes, respectively. On the other hand, 8/ 65 samples (*Alistipes finegoldii* (GC1992, GC124), *Bacteroides nordii* (GC 1072), *Bacteroides vulgatus* (GC561), *Cutibacterium acnes* (GH1045), *Paraclostridium benzoelyticum* (GC 263), *Prevotella clara* (GC 1063) and *Victivallis vandensis* (GC 1241)) with predicted genes did not show 4-MU activity for their predicted GH genes or any of the four substrates. (Table 2). Cell pellets from 5 samples displayed sialidase activity (GC1078, GC709,GC707, and GC982) and supernatant from GC 97, where no gene for GH33 or GH156 sialidase (predicted extracellular) was identified in the genome sequence analysis. Similarly, one strain (GC982) had fucosylase activity where no gene was predicted. The corresponding gene may be present in the organisms but missed because of the quality of the genome assembly or was not predicted to be extracellular. Alternatively, but less likely this activity could represent a new GH family and thus not detected with the dbCAN analysis.

2.4 DISCUSSION

In this study, I investigate GH activity in cell pellets and cell-free supernatants. Endoglycosidases of the GH18 and 85 famillies and sialidases in our samples were found to be cell associated rather than released into the medium. On the other hand, fucosidases were found to be released to the supernatant. Cell-free supernatant standardization of the glycosidase screening assays was challenging due to the high fluorescence background in our samples and the different growth rates across the species tested. Fluorescence measurement of BHI3 medium, supernatant samples on BHI3 media and acetate buffer as controls demonstrate high the background was due to the BHI medium and not from bacterial produced products (Figure 6B). GH enzyme molecular masses vary from 20 kDa to over 100 kDa (Stütz & Wrodnigg, 2011), while the membrane pore size used for drop dialysis is typically for a >100kDa molecule (Charcosset, 2011). This helped us reduce the background fluorescence of our samples. However, after treating our samples via drop dialysis and measuring glycosidase activity, I realized that our GH enzyme molecular masses were smaller than 100kDa, explaining why we lost β-GlcNAc activity on the samples after treatment. Reduction of the background by BHI3 media diluted 1:1 in water was selected as the best choice due to the simplicity of the method. Figure 5A reveals a reduction of 50% of background fluorescence using 4MU-substrate with bacterial supernatant on BHI3 media diluted compared with 4MU-substrate with bacterial supernatant on regular BHI3 broth as predicted.

Bioinformatic analysis revealed that up to 40% of all commensal bacteria encode GHs releasing monosaccharides from mucin O-linked glycans (Luijkx et al., 2020). In the 65 strains prioritized here, the number of GH families ranged from a minimum of 10 to a maximum of 62 with a mean of 38 GH families per genome. In general, *Bacteriodes* strains had the most GH families. I focused on GH18, GH85, GH33, GH89, and GH29 families and the predicted gene correlated closely with the measured substrate activity for each strain (Table 2). However, 8 isolates predicted to have GH18, GH85, GH33, GH89 and GH29 and do not show activity in any 4MU- substrate. These were intermediate or slow growing in BHI3 broth. The growth rate of these isolates could be one factor that interfere with the expression of those predicted genes(Klumpp & Hwa, 2014). Also, as samples were grown in BHI3 which is a rich medium also containing glucose, the expression of many genes could be repressed under these conditions(Tao et al., 1999). BHI3 was chosen as the medium of choice as it supports the growth of almost all human commensal bacteria, whereas more defined media are specific for different species and not known for most human commensals.

After comparing the four substrates on cell-free supernatant, I observed an increase in α-fucose activity measurement, indicating that fucosidases in our samples were being released to the supernatant, such as in the case of *S. pneumoniae* which is known to export many of their CAZy enzymes (Hobbs et al., 2019). Currently, gut microbiota α-fucosidases have been reported to play an important role in the degradation of intestinal mucin glycans (Wu et al., 2021). α-

fucosidases have been classified into the GH29 and GH95 families (Lombard et al., 2014), such as the GH29-B subfamily, found in *Bifidobacterium bifidum* and *Streptococcus pneumoniae* (Wu et al., 2021).

Bacteroides genomes encode a large number of CAZymes, including GHs. It has also been shown that EndoB displays activity against N-glycans, suggesting their participation in degrading these glycans on human gut surfaces (Trastoy et al., 2021). In the present study, β-D-GlcNAc and sialic acid measurements in cell-free supernatant show *Bacteroides* as the genus with the most activity for these substrates. This corresponds with previous sialidase activity with 4MU- N acetylneuraminic acid in *B. vulgatus* in mice during DSS-induced colitis (Y.-L. Huang et al., 2015). Meanwhile, *Barnesiella* and *Streptococcus* isolates showed GH enzyme activity in α-GlcNac and α-Fucose substrate corroborating the expression of α-Fucose in *Streptococcus pneumoniae* (Hobbs et al., 2019).

In relation to β-D-GlcNAc and sialic acid measurements in bacterial pellet screening assays, I concluded that endoglycosidases and sialidases in our samples are mainly anchored to the membrane since most of samples showing activity for those substrates were in bacterial pellets. Measurement of α-D-GlcNAc revealed low enzyme activity in bacterial pellets for this substrate. Also, samples showing β-GlcNAc, α-Sialic acid, α-GlcNAc and α-fucose activity are all predicted to have GH18, GH85, GH33, GH89, and GH29 genes, respectively (Table 2).

Bacteroides, Bacillus, Enterococcus, Lactobacillus, Prevotella, and *Streptococcus* were the main isolates expressing GH enzymes in bacterial supernatant differing from the genera present in bacteria pellets. The main isolates expressing GH enzymes on the cell membrane were *Alistipes*, *Bacteroides, Barnesiella, Bifidobacterium, Coprobacter, Enterococcus, Escherichia, Monoglobus, Streptococcus, Parabacteroides, Paraprevotella, Prevotella, Gabonia, Roseburia,* and *Victivallis.* The results presented here demonstrate that these activities can be measured directly in cell supernatants and cell pellets which provides a means for rapidly screening isolate collections. These assays could be adapted to more complex substrates. Although strains with specific activity had genes for corresponding GHs (and predicted to be extracellular), not all strains with predicted extracellular GHs displayed the corresponding activity. This is most likely due to low expression levels, and these could be examined under other growth conditions. A rich medium similar to BHI3 but with limiting glucose (which would support the growth of most human associated bacteria) would be a good medium to explore this further.

Table 2. Human gut strains from the Surette laboratory selected for extracellular GH assays.

		Predicted GH gene					Detected activity			
GC#	Isolate	GH 18	GH 85	GH 33	GH 89	GH 29	B-GlcNAc	Sialic acid	α-GlcNAc	α-Fucose
1992	*Alistipes finegoldii*	X		X		X				
124	*Alistipes finegoldii*			X	X	X				
470	*Alistipes indistinctus*			X		X				P
123	*Alistipes shahii*	X			X	X		P		
226	*Bacteroides caccae*			X	X	X	P			
209	*Bacteroides faecis*	X		X	X	X	P			P
1032	*Bacteroides faecis*	X		X	X	X	P			
73	*Bacteroides fragilis*	X		X		X	P	S/P		S
1059	*Bacteroides finegoldii*	X		X	X	X	P	P		P
603	*Bacteroides intestinalis*	X		X	X	X	P			
768	*Bacteroides nordii*	X		X	X	X	P			
1065	*Bacteroides nordii W*	X	X	X	X	X	P			
1072	*Bacteroides nordii W*	X	X	X	X	X				
189	*Bacteroides ovatus*	X		X	X	X		P		
722	*Bacteroides ovatus*	X		X	X	X	P			P
241	*Bacteroides thetaiotaomicron*	X		X	X	X	P	P		
756	*Bacteroides thetaiotaomicron*	X		X	X	X	P	P		P
939	*Bacteroides thetaiotaomicron*	X		X	X	X	P			
408	*Bacteroides uniformis*	X		X		X	P			P
504	*Bacteroides uniformis*	X		X		X		P		
615	*Bacteroides uniformis*	X		X		X				
651	*Bacteroides uniformis*	X		X		X	P			P
284	*Bacteroides vulgatus*	X		X	X	X		P		P
561	*Bacteroides vulgatus*	X		X	X	X				
619	*Bacteroides vulgatus*	X		X	X	X	P	P		

232	*Bacteroides xylanisolvens*	X		X	X	X	P	P		
343	*Bacteroides xylanisolvens*	X		X	X	X	P			P
1078	*Bacillus kyonggiensis*		X				S	P		
709	*Bacillus subtilis*						P	P		
602	*Bacillus velezensis*	X								P
665	*Barnesiella intestinihominis*		X	X	X	X		P		
1181	*Barnesiella intestinihominis*		X	X	X	X	P		P	
96	*Bifidobacterium breve*	X	X	X						P
751	*Bifidobacterium longum*		X				P			
466	*Butyricimonas paravirosa*	X	X			X	P	P		
962	*Coprobacter fastidiosus*	X	X	X		X		P		
1038	*Coprobacter fastidiosus*		X	X			P			P
1045	*Cutibacterium acnes*	X		X						
453	*Cutibacterium acnes*	X	X	X		X		P		
27	*Enterococcus durans*	X	X	X		X	S	S/P	S	S
97	*Enterococcus faecalis*	X					S/P	S		S
707	*Escherichia fergusonii*							P		
960	*Gabonia massiliensis*		X	X	X	X	P			P
559	*Monoglobus pectinilyticus*	X		X				P		
4	*Lactobacillus plantarum*		X				S			
982	*Lactococcus lactis*	X	X					P		P
432	*Parabacteroides distasonis*			X		X	P	P		
676	*Parabacteroides distasonis*		X			X	P			
1053	*Parabacteroides merdae*	X		X	X	X	P			
263	*Paraclostridium benzoelyticum*	X	X							
1063	*Paraprevotella clara*					X				
331	*Prevotella jejuni*	X	X	X		X	P	P		
149	*Prevotella melaninogenica*	X	X	X		X	S/P			

329	*Prevotella melaninogenica*	X	X	X		X		P		
155	*Prevotella nanceiensis*		X	X		X	P			
447b	*Roseburia intestinalis*	X	X			X	P			
144	*Rothia kristinae*	X	X					P		
84	*Streptococcus cristatus*		X			X	P			S
108	*Streptococcus dentisani*		X	X		X	P	P		S
109	*Streptococcus gordonii*		X			X	P			S/P
112	*Streptococcus intermedius*		X	X		X	P			S/P
897	*Streptococcus parasanguinis*		X			X	P		S	S
352	*Victivallis vandensis*			X		X	P			
1241	*Victivallis vadensis*			X		X				

X=Presence of GH gene. Green color: Fast grower, Yellow color: Intermediate grower. Red color: Slow grower. Samples in grey: Human gut strains. Samples in Blue: Upper respiratory strains. Detected activity: S (supernatant samples measurement) and P (bacteria pellet).

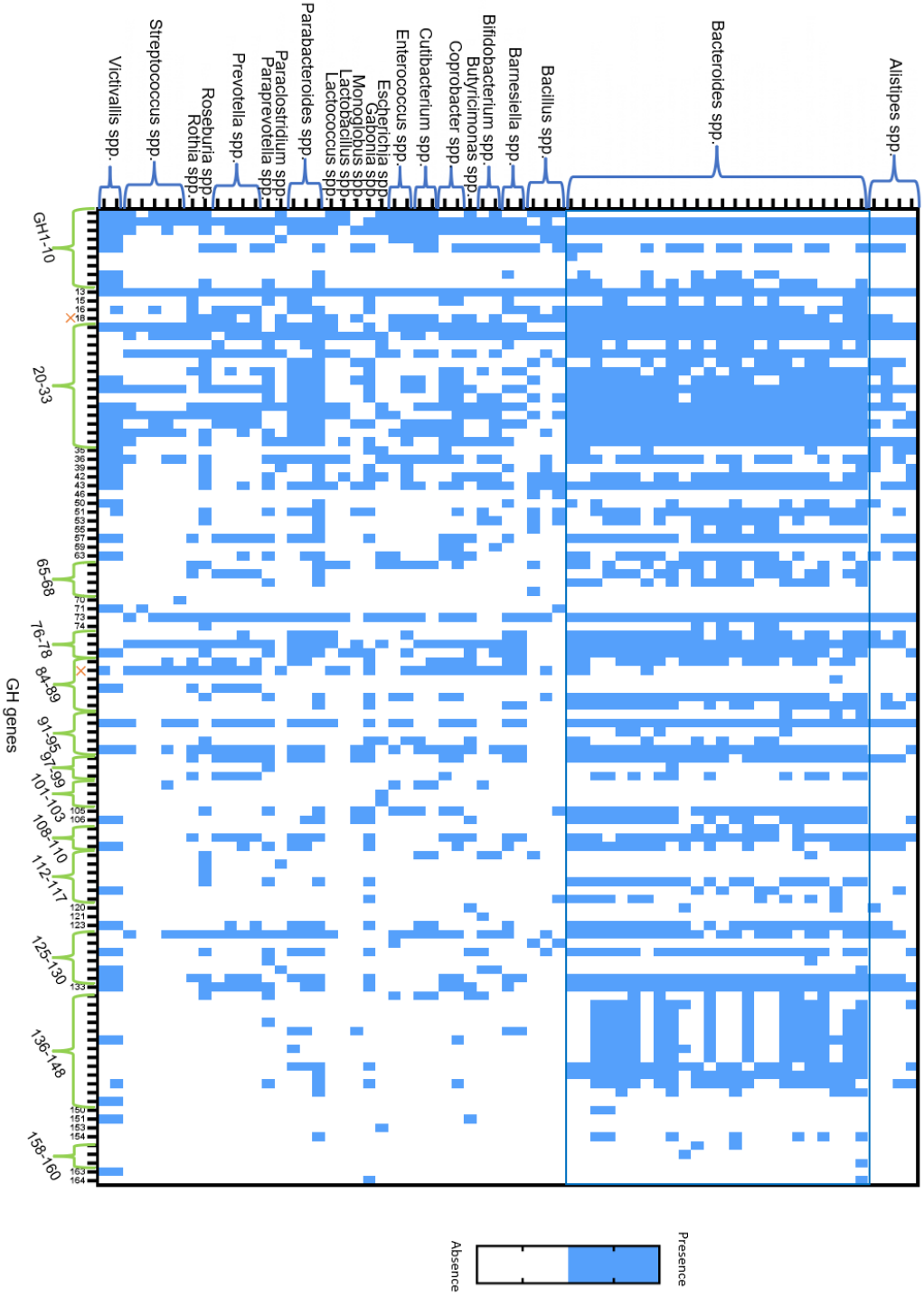


Figure 5.GH gene diversity representation of 65 strains from the Surette laboratory collection sorted by genera as predicted by dbCAN. These strains were the source of GH18 and GH85 (Orange X symbol) cloning and enzyme purification. The presence of at least one member of a GH family is represented by blue.

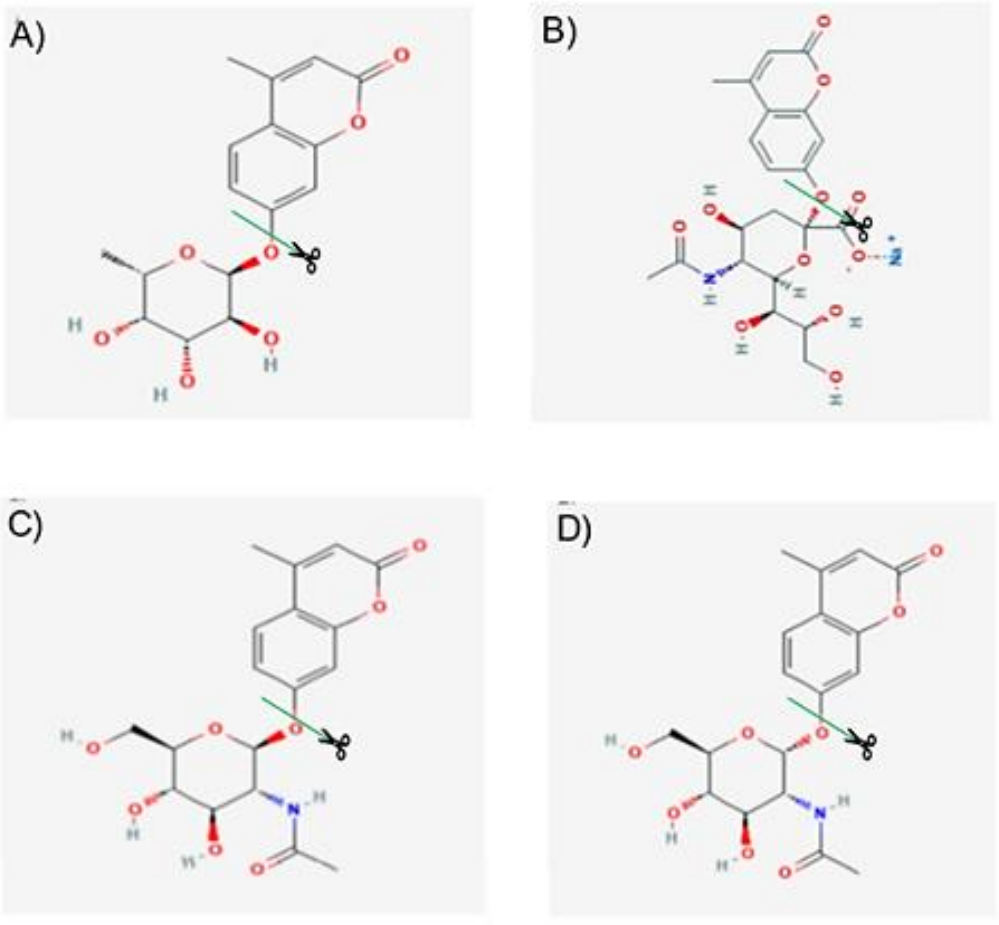


Figure 6. Structure of 4-MU-carbohydrate substrates used in this project. A) 4-Methylumbelliferyl-α-L fucopyranoside, B) 4-Methylumbelliferyl-α-D-N-acetylneuraminic acid, C) 4- Methyl umbelliferyl-N-acetyl-beta-D-glucosaminide, D) 4-Methylumbelliferyl N-acetyl-α-D-glucosaminide. Reproduced from the PubChem Compound Summary.

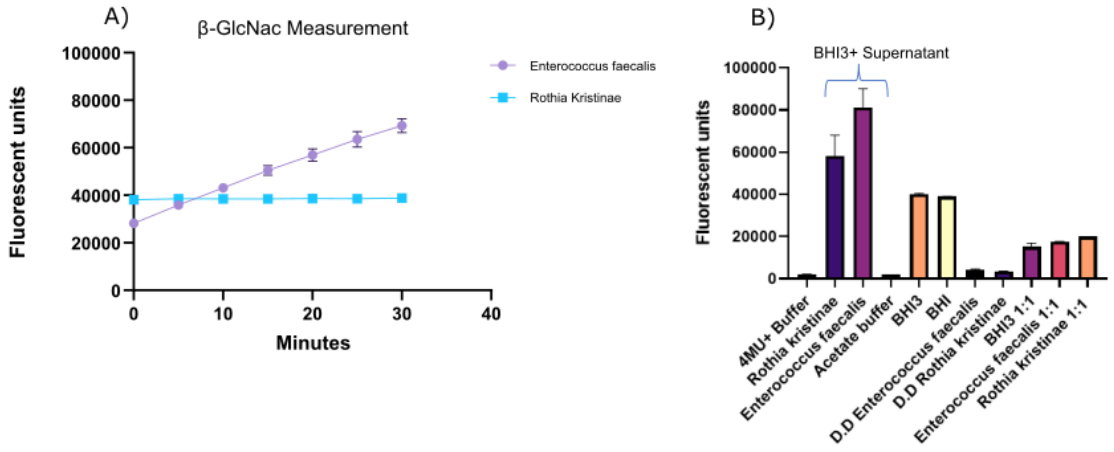


Figure 7. β-GlcNAc measurements in cell free supernatants and media controls. A) β-GlcNAc measurement of *Enterococcus faecalis* and *Rothia kristinae* cell free culture supernatants over time (30 minutes). The *E. faecalis* sample shows an linear increase in fluorescence over time corresponding to the cleavage of the glycosidic bond between 4MU and the substrate. No activity was detected with the *R.kristinae*. Not the high level of background fluorescence in both samples. B) Fluorescence measurement of experimental controls.4MU substrate with sodium acetate buffer, BHI3 supernatant of *E. faecalis* and *R. kristinae* samples, BHI3 and BHI broth without 4MU. Drop dialysis *E. faecalis* and *R. kristinae* samples, BHI3 1:1 dilution and BHI3 1:1 *E. faecalis* and *R. kristinae* dilution are shown on the right side of the graph.

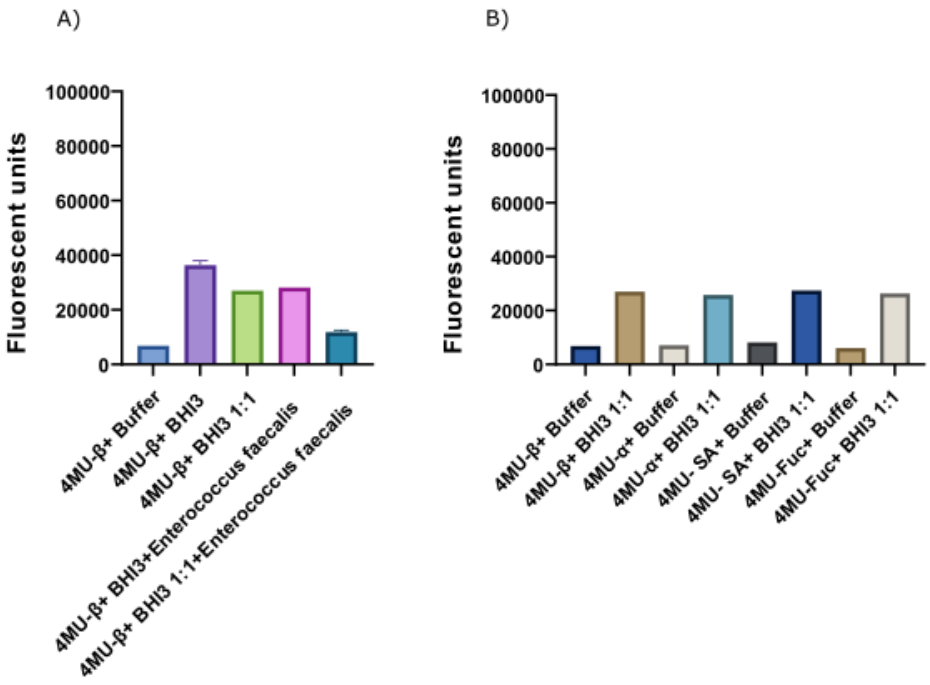
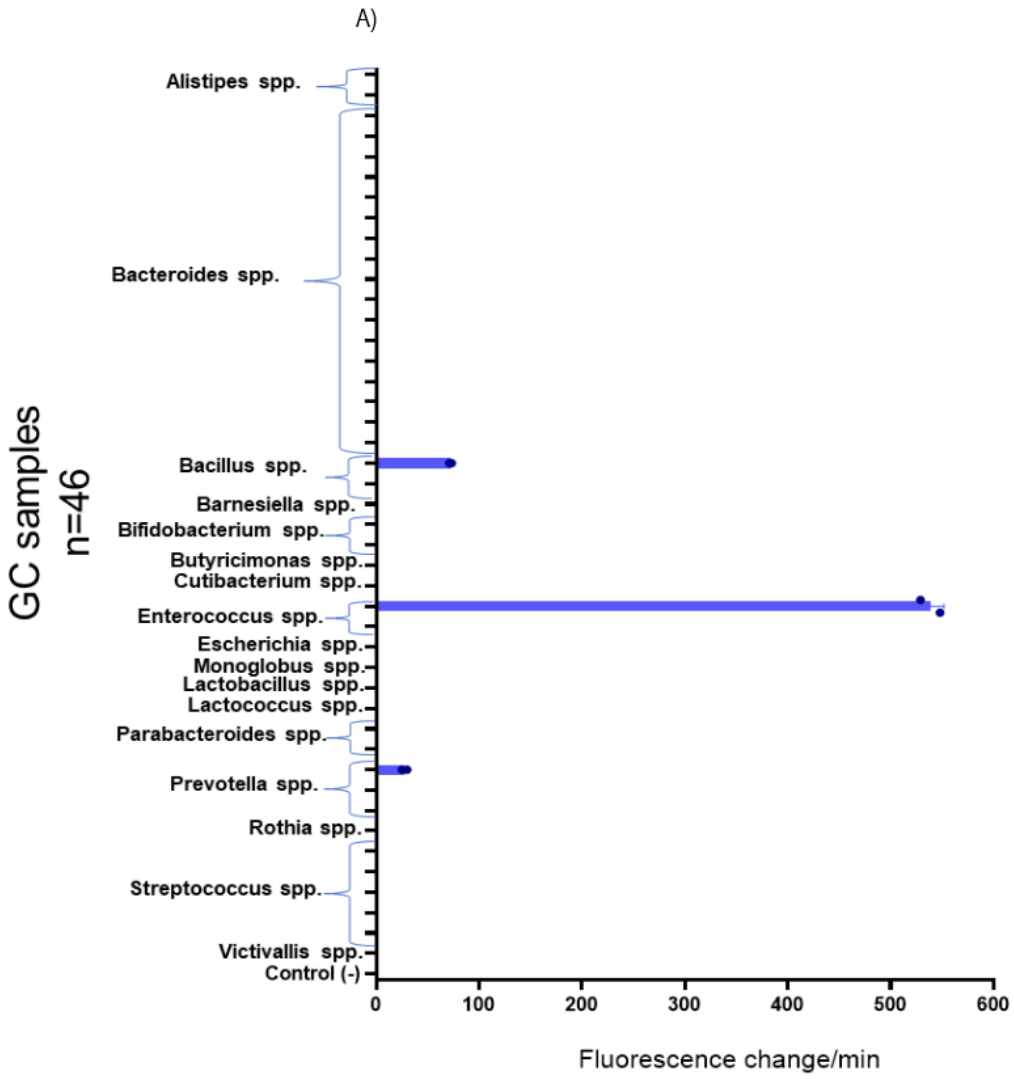


Figure 8. β-GlcNAc measurement with BHI3 broth and diluted BHI3 broth. A) Comparation of β-GlcNAc measurement with BHI3 broth and BHI3 broth 1:1 dilution with bacterial supernatant as controls on background elimination assays. 4MU with β-GlcNAc substrate with BHI3 and *E.s faecalis* and *R. kristinae* samples and 4MU with β-GlcNAc substrate with BHI3 1:1 and *E. faecalis* and *R. kristinae* samples are shown on the graph. B) 4MU substrate (α-D-N-acetylneuraminic acid, N-acetyl-β-D-GlcNAc, N-acetyl-α-D-GlcNAc and α-L-fucopyranoside) with BHI3 1:1 dilution measurement.


A)
GC samples
n=46
Alistipes spp.
Bacteroides spp.
Bacillus spp.
Barnesiella spp.
Bifidobacterium spp.
Butyricimonas spp.
Cutibacterium spp.
Enterococcus spp.
Escherichia spp.
Monoglobus spp.
Lactobacillus spp.
Lactococcus spp.
Parabacteroides spp.
Prevotella spp.
Rothia spp.
Streptococcus spp.
Victivallis spp.
Control (-)
0
100
200
300
400
500
600
Fluorescence change/min

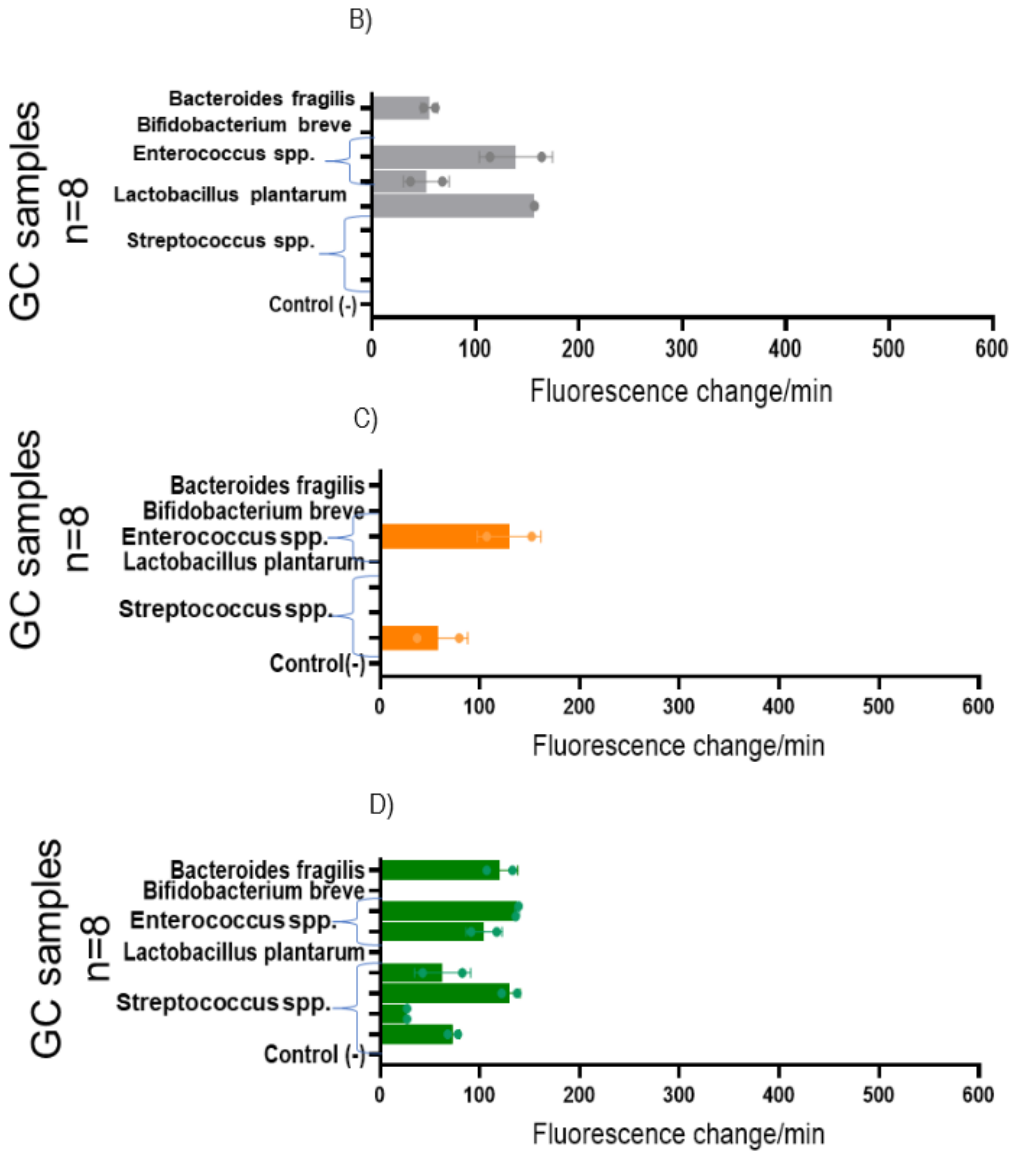


Figure 9. Glycosidase measurement on cell- free supernatant of 17 different bacteria genera (n = 65 isolates). A) β-GlcNAc measurement on cell-free supernatant (n=46). B) Sialic acid measurement cell-free supernatant (n=8). C) α-GlcNAc measurement cell-free supernatant (n=8). D) Fucose measurement cell-free supernatant (n=8). Activity is expressed in fluorescence change per minute.

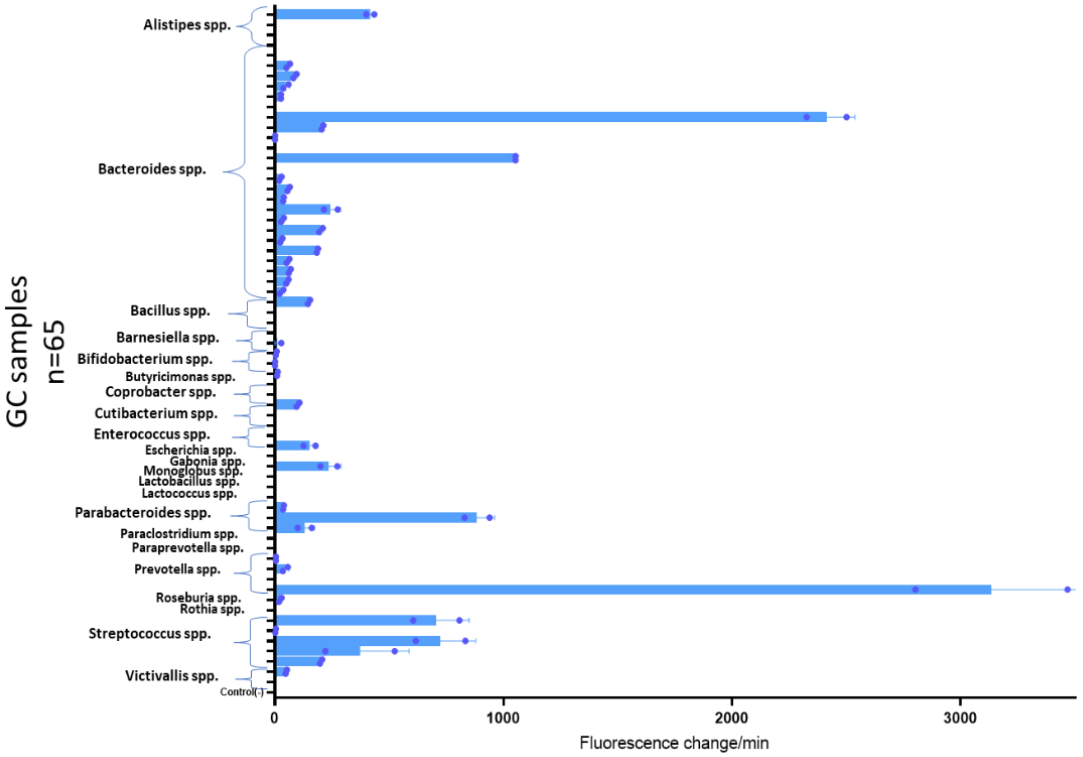
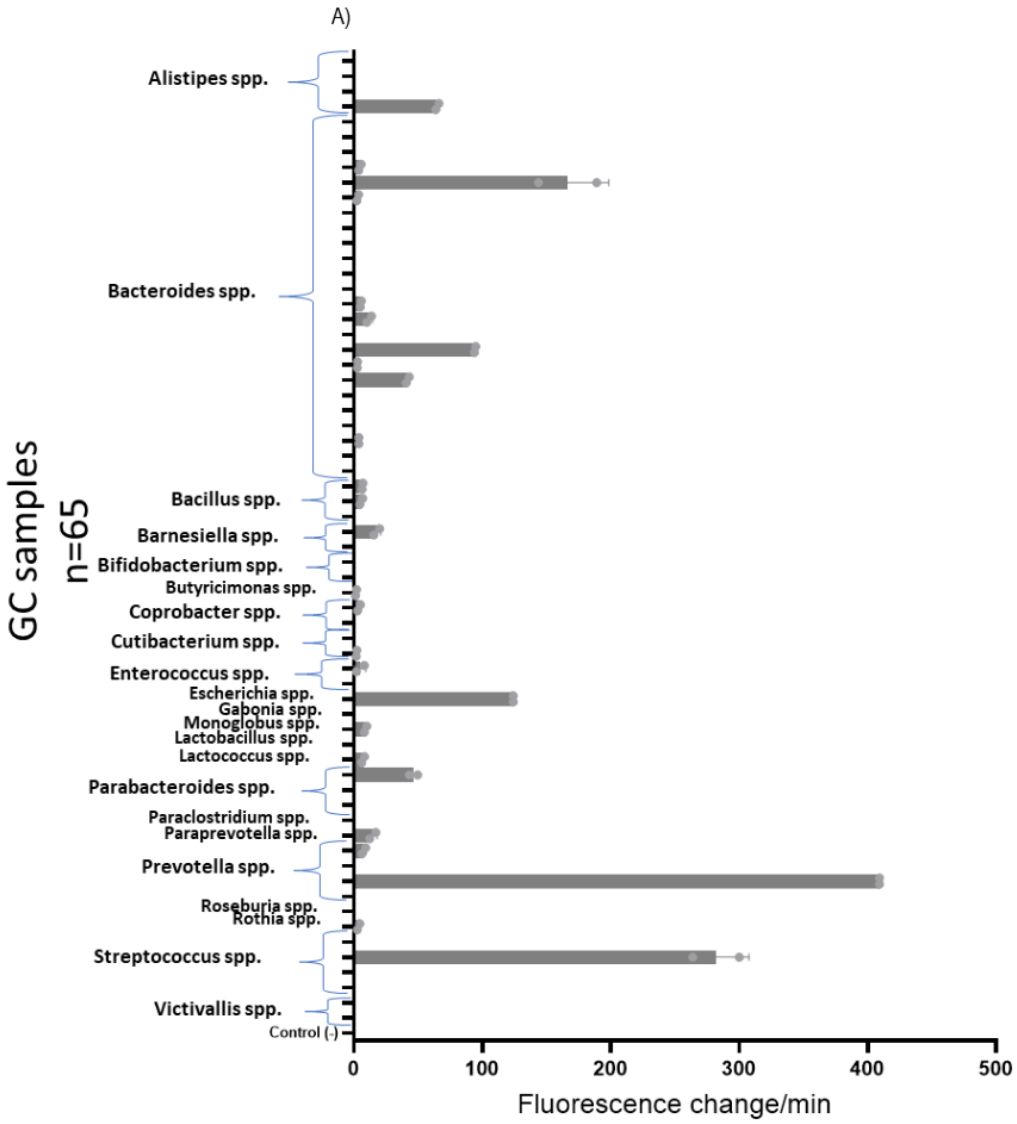
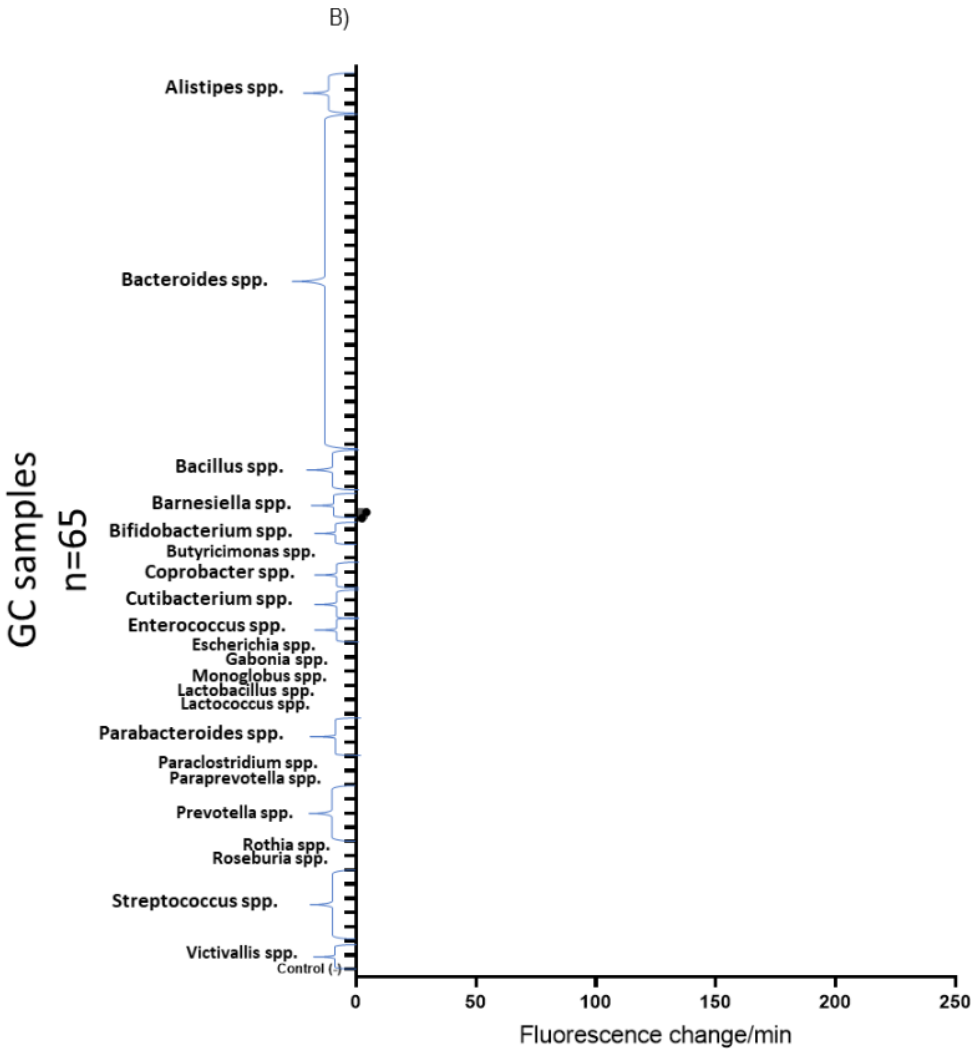


Figure 10. β-GlcNAc measurement on bacteria pellets of 17 different bacteria genera (n=65 isiolates). Activity is expressed in fluorescence change per minute.


A)
Alistipes spp.
Bacteroides spp.
Bacillus spp.
Barnesiella spp.
Bifidobacterium spp.
Butyricimonas spp.
Coprobacter spp.
Cutibacterium spp.
Enterococcus spp.
Escherichia spp.
Gabonia spp.
Monoglobus spp.
Lactobacillus spp.
Lactococcus spp.
Parabacteroides spp.
Paraclostridium spp.
Paraprevotella spp.
Prevotella spp.
Roseburia spp.
Rothia spp.
Streptococcus spp.
Victivallis spp.
Control (-)
GC samples
n=65
0
100
200
300
400
500
Fluorescence change/min


B)
GC samples
n=65
Alistipes spp.
Bacteroides spp.
Bacillus spp.
Barnesiella spp.
Bifidobacterium spp.
Butyricimonas spp.
Coprobacter spp.
Cutibacterium spp.
Enterococcus spp.
Escherichia spp.
Gabonia spp.
Monoglobus spp.
Lactobacillus spp.
Lactococcus spp.
Parabacteroides spp.
Paraclostridium spp.
Paraprevotella spp.
Prevotella spp.
Rothia spp.
Roseburia spp.
Streptococcus spp.
Victivallis spp.
Control (-)
0
50
100
150
200
250
Fluorescence change/min

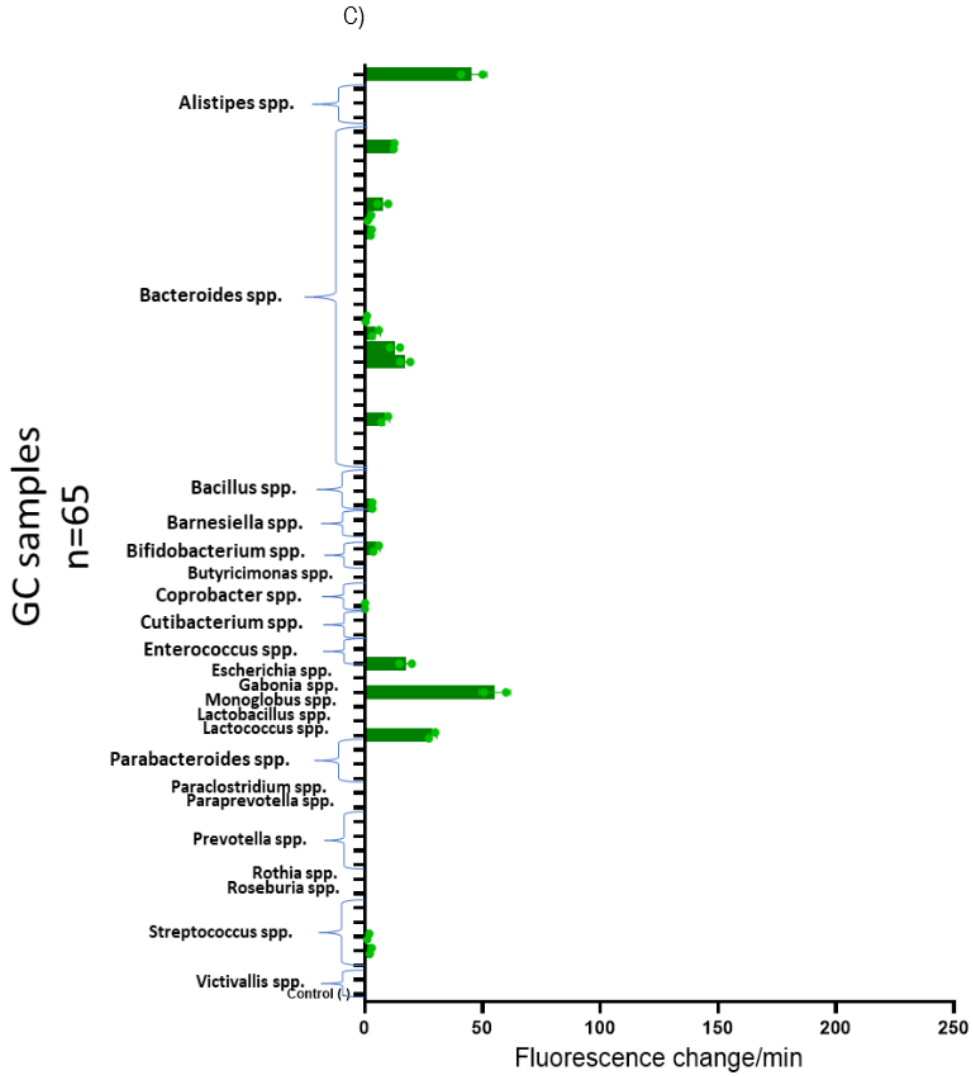


Figure 11. Glycosidase measurement on bacteria pellets of 17 different bacteria genera (n=65 isolates). A) Sialidase measurements. B). α-GlcNAc glycosidase measurements. C). Fucosidase measurements. Activity is expressed in fluorescence change per minute.

CHAPTER 3: Do bacterial GHs modulate immune cell response to dietary lectins and PAMPs?

3.1 BACKGROUND

Since the 1960s, lectins have been used as a research tool due to their role in a wide range of biological activities, such as cell recognition and inflammation (Souza et al., 2013). Plant lectins have the ability to interact with bacterial cell membranes and immune cells of the host. There are a few studies that exhibit the ability of plant lectins to modulate immune response through their interaction with surface glycans moieties present in immune cells (Mishra et al., 2019; Souza et al., 2013). In the case of inflammatory bowel disease, food intolerance, and food allergy, dietary lectins may be found in peripheral circulation as a consequence of increased intestinal permeability during inflammatory responses (Freed, 1999). Moreover, ConA lectin treatment has shown an increase in the expression of different toll-like receptors and proinflammatory cytokines in murine macrophages via NF-kB pathway (Mishra et al., 2019). TLRs recognition of PAMPS results in the activation of the NF-kB transcription factor. As TLRs ectodomain contains N-linked glycosylation sites, some lectins interact with TLRs, such as ArtinM plant lectin, which recognizes N-glycans of TLR2 and CD14 (Ricci-Azevedo et al., 2017). Although the exact role of glycans on TLRs is still not well understood,(Amith et al., 2010) demonstrate that Neu1 sialidase works with TLR2,3 and 4 on cell surface membrane of macrophage cells after activation of the receptor by its ligand (Amith et al., 2010).

In this chapter I investigate plant lectins, PAMPs (LPS or LTA), and microbial GHs for their ability to modulate host cell signalling pathways. As a first screening assay, I followed a protocol used (Gong et al., 2017) to measure plant lectins, stimulation in THP-1 cells through the activation of NLRP3 inflammasome. In this study Gong reported that plant lectins induce caspase-1 and IL-1β after activation of the inflammasome. As a simpler system, I used Blue-hTLR2 and HEK Blue-mTLR4 cells to evaluate my primary hypothesis - GHs can alter host immune signalling pathways by modifying glycans on cell surface receptors such as TLRs, increasing or decreasing the inflammatory response to dietary lectins or PAMPs.

As an alternative to the THP1 cells, HEK Blue-hTLR2 and HEK Blue-mTLR4 cells were used to evaluate the ability of lectins and bacterial GHs to modify host cell signaling. These two systems provided a simpler assay which report responses specific to the expressed TLR. HEK Blue- mTLR4 cells are generated by co-transfection of the murine TLR4, MD-2, and CD14 co-receptors genes and an inducible SEAP reporter gene into HEK293 cells (*HEK-Blue™ MTLR4*, 2016). HEK Blue- hTLR2 are obtained by co-transfection of the human TLR2 and SEAP genes (*HEK-Blue™ HTLR2*, 2016). SEAP (secreted form of embryonic alkaline phosphatase) is released into the cell culture supernatant when expressed in HEK293 cells and detected using a colorimetric phosphatase substrate (*SEAP Reporter Gene System*, 2016). Activation of NF-kB results in the expression of SEAP via the ELAM proximal promoter, which can be detected using HEK Blue Detection Media (InvivoGen).

3.2 MATERIALS AND METHODS

3.2.1 THP-1 cell maintenance

THP-1 cells were obtained from the Bowdish Laboratory at McMaster University, Hamilton, Canada. Cells were maintained, and cultured as described by (Tsuchiya et al., 1980) using RPMI 1640 media with 10%FBS (Gibco, heat-inactivated) and 50µM 2-ME, replacing it every two days. Cultures were measured with a hemocytometer and Trypan Blue staining to measure cell number and viability. To differentiate into macrophages THP-1 cells were treated for 3hr with 100 nM phorbol 12-myristate 13-acetate (PMA) and then incubated overnight in Corning Falcon 6 well cell culture plates.

3.2.2 THP-1 cell stimulation

For induction of inflammasome, $6x10^5$ macrophages were plated overnight in Corning Falcon 6 well cell culture plates before priming them for 3hrs using Opti-MEM media (Reduced Serum Media) supplemented with 1% FBS (Gibco, heat-inactivated) and 100ng/ml of ultrapure LPS. Cells were stimulated with lectins (Con A 30 µg/ml, WGA 10 µg/ml, and PNA 10 µg/ml) for 6 hrs. IL-8 cytokine was measured in Optimed media (Reduced serum media) + LPS and lectins for 6 hrs. As controls, IL-8 was measured in RPMI media with cells, RPMI 1640 media with 10%FBS (Gibco, heat-inactivated) and 50 µM 2-ME (Culturing conditions), Optimed media with 10% FBS, and Optimed media with 100 ng/ml ultrapure LPS. The supernatant was recovered. In order to maintain the supernatant in good

condition, the Invitrogen Sample Prep Guide for Immunoassays protocol was followed (by centrifuging the medium at 19722 xg at 4ºC for 10 minutes in order to remove any cell or cellular debris before storage at -80ºC).

3.2.3 ELISA IL-8 measurement

Human IL-8 ELISA kit (Invitrogen) was used to analyze the supernatant recovered. Serial dilutions of the standard solution were made from Hu IL-8 10 ng/ml to generate a standard curve. 50 µl of standards and the samples were added to a precoated 96-well plate with HU IL-8 antibody. Two empty wells were used as a negative control. Biotin conjugate solution was added to the wells before incubating for 1hr and 30 minutes at room temperature. After that time, wells were washed 4 times with 1x Wash buffer before adding 1x Streptavidin-HRP solution incubation for 30 minutes. The wash step was repeated before adding the chromogen to the wells and incubating for 30 minutes. Finally, a stop solution was added before reading absorbance at 450 nm.

3.2.4 Hek Blue cell maintenance

HEK Blue-hTLR2 and HEK Blue- mTLR4 cells were obtained from the Bowdish Laboratory and the Schertzer Laboratory at McMaster University, Hamilton, Canada. Cells were maintained, and culture as described by Anhê *et al.* 2021 and Invitrogen Protocols (*HEK-Blue^{TM} HTLR2*, 2016; *HEK-Blue^{TM} MTLR4*, 2016; *SEAP Reporter Gene System*, 2016). Cells were cultured in DMEM media with 4.5 g/l glucose, 10% FBS (Gibco, heat-inactivated), 100 µg/ml Penicillin, 100 µg/ml

Streptomycin, 100 µg/ml Normocin and 2 mM L-Glutamine at 37ºC, 5% CO_2 (Growth medium). Media was replaced twice a week by washing the cells with PBS and incubating for 2 minutes at 37°C to detach cells from the surface. After the first passaging, cells were maintained on selection media (Growth medium with InvitroGen-HEK Blue Selection antibiotics) to ensure the maintenance of the transfected plasmid. Cells were cultured until they reached 70-80% confluency before transferring to a new flask or using for assays.

3.2.5 HEK Blue cell -SEAP assays

SEAP assays were used to assess the potential of lectins to activate TLR4 and TLR2 receptors on HEK cell lines. These assays were performed on Flat Bottom 96 well plates for tissue culture. For each cell line, plant lectins (Table 2) and a know TLR ligand, LPS (tlrll-3pelps, InvivoGen) or LTA (tlrl-lta, InvivoGen), were used to stimulate HEK Blue-hTLR2 and HEK Blue- mTLR4 cells (Figure 12).The following concentrations were used on each of the four lectins Con A (C0412-5MG, Sigma-Aldrich), WGA (L9640-25MG, Sigma-Aldrich), SBA (L1395-5MG, Sigma-Aldrich) and PNA (L0881-10MG, Sigma-Aldrich) : 5, 20, and 40 µg/ml**.** Each assay was performed with a standard curve of ultrapure *E. coli* LPS 0111: B4 from InvivoGen for TLR4 receptor and LTA from InvivoGen for TLR2 receptor. The following concentrations were used as a standard curve: *E. coli* LPS: 5, 10, 20, 50,100 ng/ml, LTA: 5, 10, 20, 50, 100 ng/ml.

Each assay (in duplicate) was carried out in 200 µl in 96 well plates with $1x10^4$ HEK reporter cells per well. 20 µl of plant lectins, LPS, LTS or ultrapure water (as a negative control) were added to final concentrations as indicated. The plate was read at 630 nm at 6 hours and 24 hours. To measure potential LPS contamination of the selected plant lectins (WGA and SBA), 20 µl of Polymyxin B (100 ng/ml) were added to the 96 well plates in duplicates before adding 160 µl of HEK reporter cells.

3.2.6 Hek Blue cell -SEAP assays with glycoside hydrolase treatments

Commercial GH enzymes (α2-3,6,8,9 Neuraminidase A (P0722S, New England BioLabs), Endo D (P0742S, New England BioLabs), and Endo S (P0741S, New England BioLabs) commercial enzymes and GH18 (GC123), GH18 (GC408), GH85 (GC124), GH156 (GC1282) purified enzymes (provided by Aarthi Pasupathi) were used to test their effect on TLR4 and TLR2 activity. Commercial enzymes (46 µg/ml) were added to duplicate assays. 180 µl of HEK mTLR4 cells were added into each well ($1x10^4$ cells per well). Alternatively, 20 µl (45 µg/ml in Hek Blue mTLR4 cells and 2.4 µg/ml in Hek Blue hTLR2 cells) of purified GH enzymes to duplicate assays *E. coli* LPS was added at 5,10, 20 and 50 ng/ml. The plate was read at 630 nm at 6 hours and 24 hours.

3.3 Results

3.3.1 Lectin modulation of NLRP3 inflammasome inTHP-1 cells

For this assay plant lectins, PAMPs (LPS or LTA), and microbial GHs were used to measure their ability to modulate host cell signalling pathways. A previously published protocol (Gong et al., 2017) was followed to measure plant lectins stimulation of THP-1 cells through the activation of NLRP3 inflammasome. In this previous study, specific lectins further activated LPS stimulate THP1 cells.

Cells were stimulated with lectins (Con A 30 µg/ml, WGA 10 µg/ml, PNA 10 µg/ml and SBA10 µg/ml) for 6 hrs on Optimed media. The supernatant was recovered and measured with an ELISA IL-8 kit. Cells stimulated only with Optimed media and FBS were used as negative control, meanwhile, cells stimulated with Optimed media and LPS were used as a positive control. Cytokine measurement in cell supernatant after treatment with Optimed media with LPS shows an increase in IL-8; meanwhile, cell supernatant after treatment with Optimed media with LPS and lectins shows a decrease on the IL-8 release on ConA, WGA and PNA. (Figure 13). This result was the opposite of the published report but reproduced in biological replicates.

3.3.2 Hek Blue-mTLR4 assays

As an alternative approach, Hek-Blue cells, which express a single TLR and report activation through expression of an NF-κB dependent secreted alkaline phosphatase, were used. Hek Blue-mTLR4 stimulation with ConA, SBA, WGA,

and PNA lectins reveals a dose-dependent increase of SEAP secretion after stimulation with WGA and SBA lectins, although lower than the activity obtained with LPS (Figure 14A). It is important to note that in these assays the lectins are added at ~1000 fold higher concentrations than the LPS. This lectin dependent activation of TLR4 is consistence with some published data, but it is possible that the active plant lectins contain low levels of LPS and are not directly activating TLR4. To evaluate this, Polymyxin B (100ng/ml) was co-administered with the SBA and WGA lectins in the assays. This antibiotic binds LPS and prevents LPS activation of TLR receptors. The results show a reduction in TLR4 stimulation by these lectins in the presence of Polymyxin B (Figure 14 B). This is consistent with low level LPS contamination in the SBA and WGA lectin preparations which confound the results, although other explanations are possible. Because the lectins are present at concentrations ~1000X higher than LPS controls, the level of contamination is quite low.

Hek Blue-mTLR4 assays with ConA alone did not show any activity indicating no LPS contamination. However, when ConA was combined with LPS at 10, 20, and 50 ng/ml, an increase of SEAP activity above LPS alone was observed at 24hrs (Figure 15). This is consistent with ConA interacting with TLR4 or a co-receptor to stimulate LPS binding and TLR4 signaling.

Hek Blue-mTLR4 assays with Neu A, and Endo S with LPS at 10 and 50 ng/ml after 24 hrs do not show any significant change in TLR4 stimulation. However, the addition of commercial Endo D enzyme at 45 µg/ml reduced the response to LPS.

(Hek Blue-mTLR4 treatment with GH18 (GC123, GC408) and GH85 (GC124) purified enzymes from our laboratory collection showed a large decrease in SEAP activity after 24 hrs. (Figure 17).

3.3.3 Hek Blue-hTLR2 assays

Hek Blue-hTLR2 cells stimulation with the four lectins shows SBA and WGA lectins caused an increase in SEAP secretion at 24 hrs. In these assays, our LTA (standard curve) at 24 hrs produces lower levels of SEAP secretion than lectin stimulation alone. Interestingly, these were the same lectins that stimulated the Hek Blue-hTLR4 cells (Figure 14). Polymyxin B (100 ng/ml) was used to treat the SBA and WGA lectins in the assays. Our results show a reduction in TLR2 stimulation after being treated with Polymyxin B (Figure 18 B). This does suggest that these samples have an TLR2 agonist that binds polymyxin. As LPS activation of TLR2 has been reported to require soluble CD14 cells which may not be present in our assay, this requires further investigation (Kirschning et al., 1998).

ConA alone did not activate the Hek Blue-hTLR2 cells; however, when ConA was added with LTA (at 10,20, and 50 ng/ml) increased SEAP activity relative to LTA alone was observed (Figure 19). Intriguingly, this reflects the combined synergy of ConA with LPS on Hek Blue-hTLR4 cells reported above.

The LTA dependent activation of Hek Blue- hTLR2 cells was also measured in the presence of glycan modifying enzymes. A modest decrease in SEAP activity was observed when the cells were treated with commercial enzymes (Neu A, Endo D

and Endo S) (Figure 20) but not with GH156 (GC1282) or GH18 (GC123) enzymes purified in our lab. (Figure 21). It should be noted that the LTA activity on the reporter cells was very low and not as expected. These assays should be carried out with a more active TLR2 agonist.

3.4 DISCUSSION

NLRP3 inflammasome is present in mucosal macrophages and dendritic cells; it is activated in bacterial, viral and fungal infections as well as on exposure to environmental irritants (Mao et al., 2018). Activation of NLRP3 inflammasome is a two-step process: priming and activation. Expression of caspase 1 and pro IL-1 β (priming step) can be induced after recognition of PAMPs by TLRs (Kelley et al., 2019). IL-1 β family has a crucial role in pro-inflammatory activity. Moreover, IL-1 β induces IL-8 synthesis by regulating ROS and NF-kB activation by BLT2-Nox1 pathway (Cullen et al., 2015; Kim et al., 2010). There is evidence that shows that some lectins can potentiate pro-inflammatory responses, and I sought to use this assay to investigate whether microbiota-encoded GHs could modulate this activity.

LPS stimulation of THP-1 cells led to the release of IL-8 and secretion of IL-1 β in lower amounts (Cullen et al.,2015). In a previous study (Gong et al., 2017) reported that plant lectins such as WGA further induce IL-1 β via NLRP3 inflammasome in THP-1 cells stimulated with LPS. I was unable to reproduce these results with THP-1 cells, and I consistently showed a decrease in IL-8 production with LPS combined with ConA, WGA or PNA lectin compared with cells stimulated with LPS

alone. This suggests that ConA, WGA and PNA lectin could be acting as antagonists reducing cell stimulation or that cell stimulation time should be extended for 24 hrs. The proposal that these lectins are acting as antagonists is not consistent with the results of previous work with THP-1 cells (Gong et al., 2017). Lectins such as ConA act as agonist of TLR2 and TLR6 in mesenchymal stem cells(M. Zhou et al., 2014). Some of the reasons that could be contributing to the struggles reproducing these data could be the difference between cell numbers on the assays or any source of contamination, such as LPS or other PAMPs in the lectins used.

Due to the difficulties reproducing the previous study, I switched to a more straightforward system, the Hek-Blue SEAP reporter cells, in collaboration with the Schertzer laboratory. In this system, addition of TLR2 or TLR4 agonists, leads to the activation of NF-kB, resulting in the expression and secretion of SEAP (Gregory et al., 1994; Jardin et al., 2008).

Although all assays were measured after 6 and 24 hrs, most of SEAP expression was detected on 24 hrs assays on Hek Blue-mTLR4 and Hek Blue-hTLR2 cell lines. Lectin cell stimulation assay on TLR4 and TLR2 shows an increase in SEAP expression as a result of receptor activation and NF-kB production. Showing that some lectins such as WGA and SBA can modulate how cells respond after being stimulated with a TLR ligand being consistence with some published data in HEK cells were SBA act as agonist of TLR4 meanwhile, ConA has been reported to stimulate TLR2 and TLR4 (Unitt & Hornigold, 2011). In order to corroborate this

response, Hek-Blue TLR4 and TLR2 cells were stimulated with lectins, and polymyxin B which blocks the effect of LPS through binding to lipid A. LPS-PMB effect is dose dependant and specific for LPS (Shatri & Tadi, 2022). As a result, I observed a reduction in TLR4 and TLR2 stimulation after treating them with PMB. Concluding that none of the lectins proved on the first assay directly activated TLR4 or TLR2 receptor and that the SEAP secretion was due to levels of LPS in WGA and SBA.

Regarding cell stimulation with ConA and LPS, our data show that ConA alone does not activate these receptors but acts synergistically with LPS and LTA on TLR2 and TLR4 receptors, respectively. These results demonstrate that ConA can modulate how cell response after stimulation with TLR ligand on Hek-Blue-mTLR4 but not in Hek-Blue-hTLR2 cells, being the opposite (Sodhi et al., 2007) reported that macrophages treated with ConA (10 µg/ml) enhanced expression of TLR2 and TLR6 after 12 hrs, and this is a possible explanation for the increased response of TLR4 ligand on Hek-Blue cells after being treated with ConA.

It has been previously reported that Neu 1 removal of sialic acid residues on glycans on TLR4 facilitates receptor dimerization and activation (Allendorf et al., 2020; Amith et al., 2010). This indicates that glycan modification of TLR4 can affect activity and motivated experiments to determine if bacterial GH enzymes can modulate cell response. TLR4 treatment with commercial enzymes NeuA, and EndoS had no effect, but Endo D decreased the response to LPS. Three bacterial GH enzymes purified in our lab also significantly decreased response to LPS. As

it has previously reported, N-linked glycans of TLR4 and MD-2 have an essential role in the activation of the receptor. These results suggest that removal of N-glycan modification on TLR4 or its co-receptors reduces activity(de Haas et al., 2020). However, this preliminary result need repeating. It is also possible that these purified enzymes have contaminants that are toxic to the cells and the decrease in activity just reflects a decrease in viable cells. This requires further investigation and assays to directly measure glycan removal.

Similar experiments with the TLR2 reporter cell line were less striking. Although the commercial enzymes appeared to cause a modest decrease in SEAP production in response to LTA, the baseline response of these cells to LTA was very low. This may reflect a problem with the LTA or the cell line.

The goal of this aim was to develop a host -cell assay for monitoring lectin and/or bacterial GH activity on host cell signaling, I was unable to recapitulate the co-activation of the NLRP inflammasome by LSP and lectins reported by (Gong et al., 2017). Switching to a simpler assay in cells expressing a single TLR and straightforward colorimetric reporter shows promise. I was able to show that both the SBA and WGA used I this work appear to have low level LPS (or similar TLR agonist). The results with ConA were more promising and this lectin stimulate response in both the TLR2 and TLR4 reporter cell lines. ConA has high affinity for high mannose and hybrid N-glycans which could explain the observed activity. The response should be block by active GH18 or GH85 enzymes and this was suggested by the results reported here. N-glycan modification on specific receptors

can be cell line dependent and is not known for the HEK cell line. Regardless these assays merits further investigation.

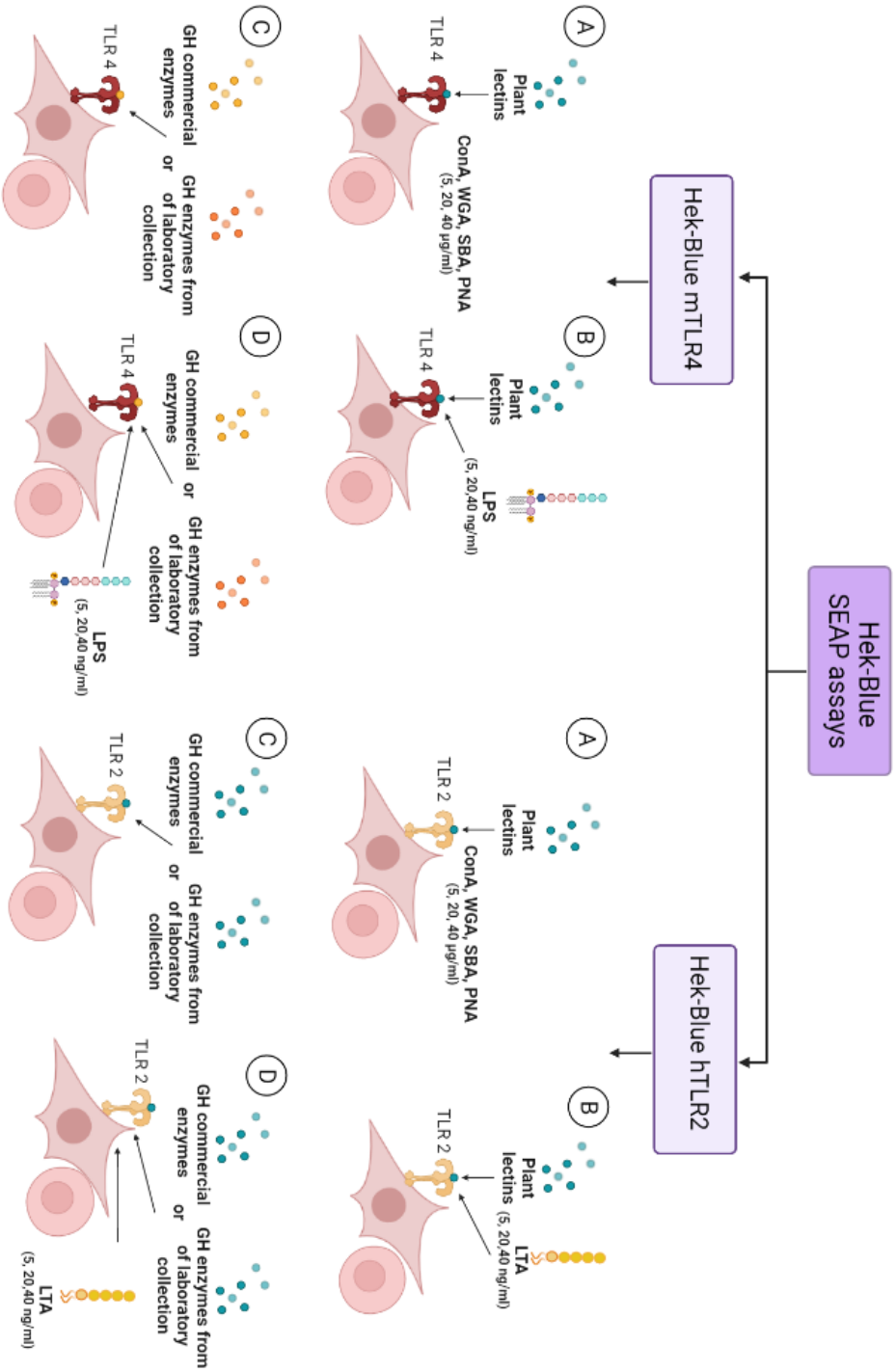


Figure 12. SEAP assays on Hek-Blue cells. These assays were performed using two Hek cell lines mTLR4 and hTLR2. A) Plant lectins ConA, WGA, SBS and PNA (5, 20,40 µg/ml) were used to stimulate Hek cells. B) Plant lectins ConA, WGA, SBS and PNA (5,20,40 µg/ml) and LPS for mTLR4 (LTA for hTLR2) were used to stimulate Hek cells. C) GH commercial enzymes NeuA, EndoD, EndoS and GH enzymes from our laboratory collection were used to stimulate mTLR4 and hTLR2 cells. D) GH commercial enzymes NeuA, EndoD, EndoS, GH enzymes from our laboratory collection with LPS (mTLR4) or LTA (hTLR2) were used to stimulate cells.

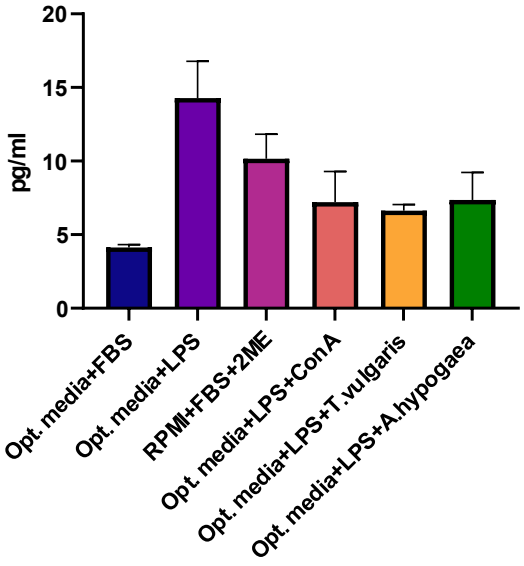


Figure 13. IL-8 measurement on THP-1 cells stimulated with LPS and dietary lectins. Optimed media with FBS, Optimed media with LPS, and RPMI with FBS, 2ME were used as a control. After cell stimulation with lectins ConA, WGA and PNA a decrease in IL-8 measurement was observed.

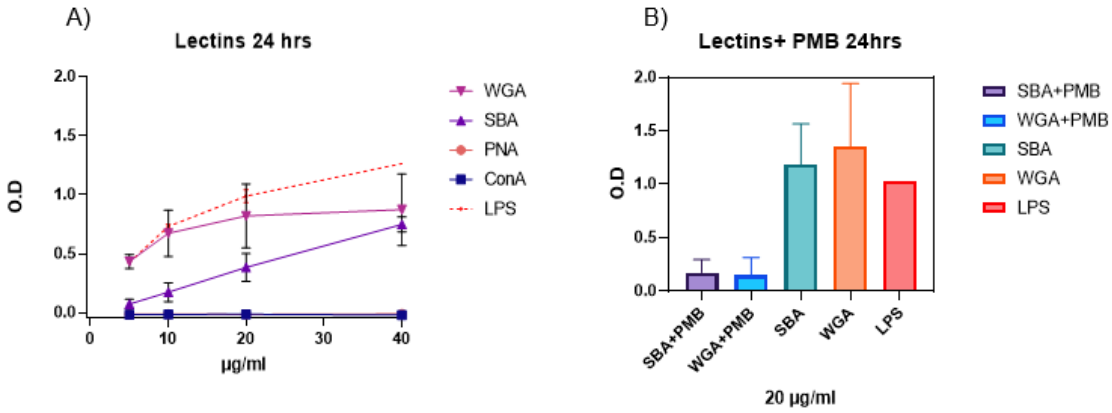


Figure 14. Hek Blue mTLR4 reporter cells to measure activity of LPS and dietary lectins. A) Hek Blue mTLR4 stimulation with lectins (ConA, WGA, SBA, and PNA) at 24 hrs. Cell stimulation with LPS curve 5,10,20,50 and 100 ng/ml is represented as a red line (Positive control). B) Hek Blue mTLR4 stimulation with lectin stimulation before and after treatment With Polymyxin B (100 ng/ml) at 24 hrs.

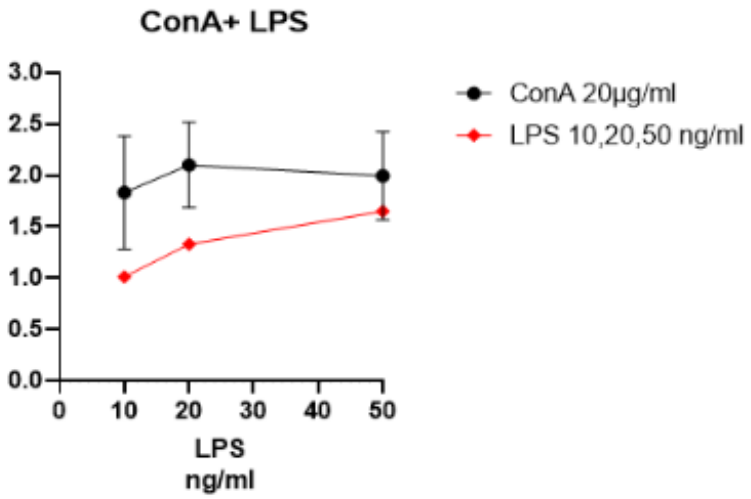


Figure 15. Hek Blue mTLR4 stimulation with ConA lectin and LPS at different concentrations. Cell stimulation with LPS (10, 20 and 50 ng/ml). is represented as a red line (Positive control). ConA lectin (20 µg/ml) shows an increase in TLR4 stimulation by LPS.

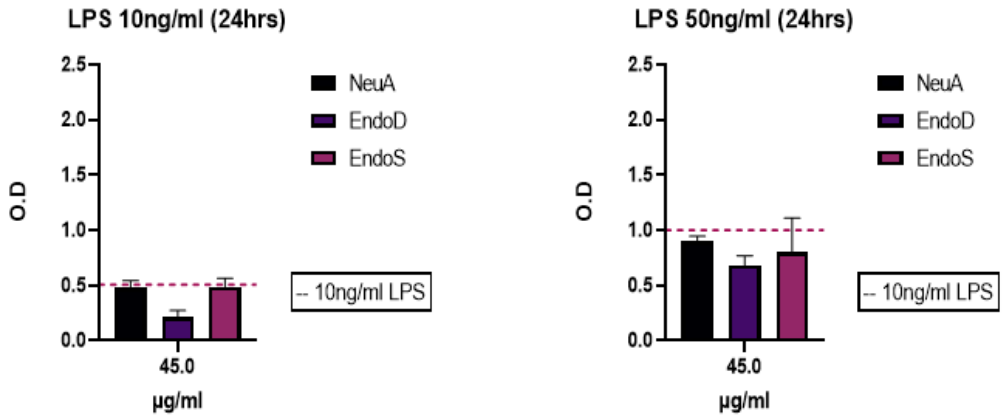


Figure 16. Hek Blue hTLR2 treatment with commercial enzymes. Hek Blue mTLR4 were treated with commercial enzymes NeuA, EndoD, and EndoS (45 µg/ml) and LPS (10-50 ng/m)l. Cell stimulation with LPS 10-50 ng/ml is represented as a red line (Positive control).

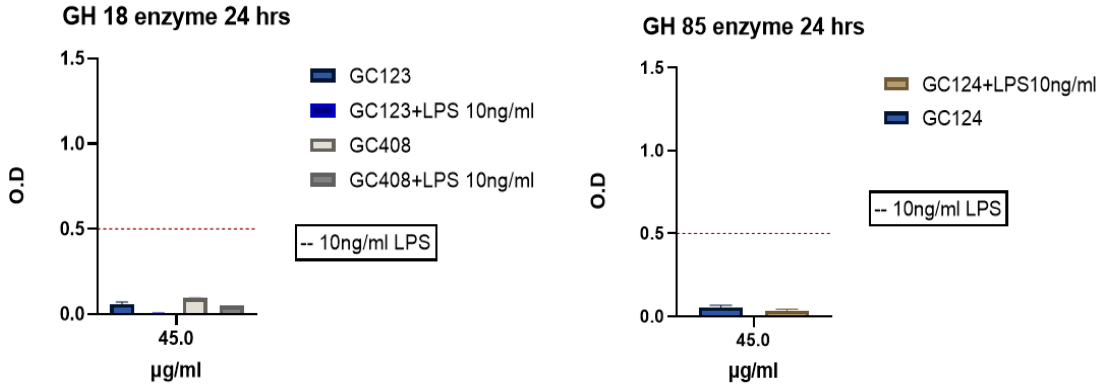


Figure 17. Hek Blue hTLR2 treatment with GH18 and GH85 enzymes. Hek Blue mTLR4 were treated with GH18 and GH85 enzymes from our laboratory collection. Cell stimulation with LPS 10-50 ng/ml is represented as a red line (Positive control).

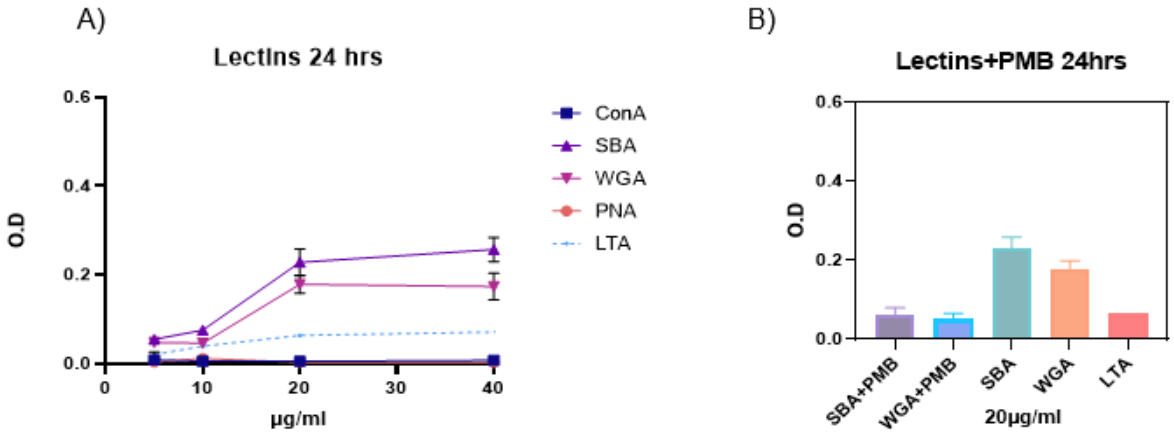


Figure 18. Hek Blue hTLR2 reporter cell activity in the presence of LTS or dietary lectins. A) Hek Blue hTLR2 stimulation with lectins (ConA, WGA, SBA, and PNA) at 24 hrs. Cell stimulation with LTA is represented as a red line (Positive control). B) Hek Blue hTLR2 stimulation with lectin stimulation before and after treatment With Polymyxin B (100 ng/ml) at 24 hrs.

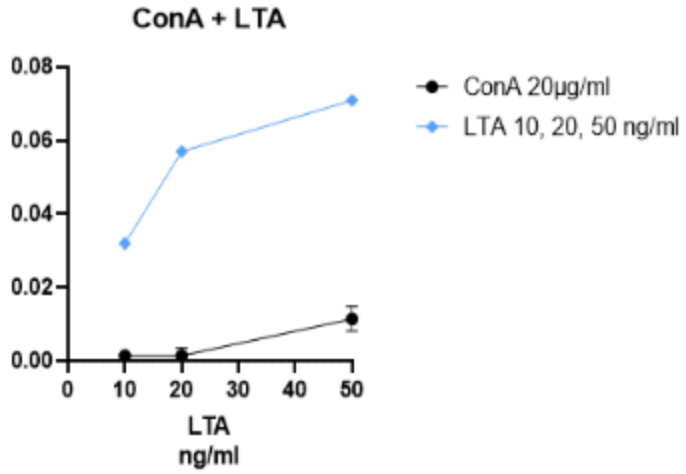


Figure 19. Hek Blue hTLR2 stimulation with ConA lectin and LPS. Hek Blue hTLR2 stimulation with ConA lectin 20µg/ml and LPS at different concentrations (10, 20 and 50 ng/ml). Cell stimulation with LTA is represented as a blue line (Positive control). ConA lectin shows an increase in TLR2 stimulation.

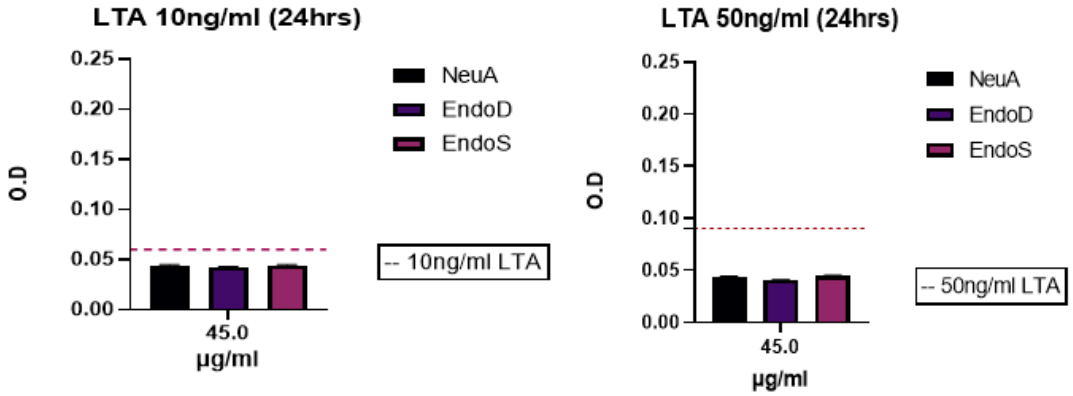


Figure 20. Hek Blue hTLR2 stimulation with commercial enzymes and LPS. Hek Blue hTLR2 stimulation (24 hrs) with commercial enzymes Neu A, Endo D and Endo S 45 µg/ml and LTA at 10-50 ng/ml. Cell stimulation with LTA 10-50 ng/ml is represented as a red line (Positive control).

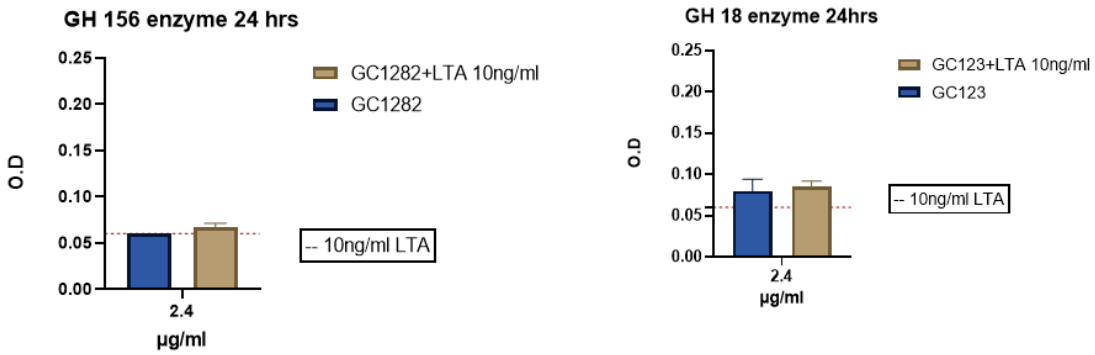


Figure 21. Hek Blue hTLR2 stimulation (24 hrs) with GH 156 and 18 enzymes from our laboratory collection. Cell stimulation with LPS 10-50 ng/ml is represented with a red line (Positive control).

CHAPTER 4: Visualization of glycosylation of A549 cells using fluorescent-labelled lectins and the effects of glycoside hydrolases

4.1 BACKGROUND

As a result of the carbohydrate affinity of lectins, fluorescent-labelled lectins have been widely used to characterize surfaces of eukaryotic cells and polysaccharides due to their low cost, specificity, stability, and ease of generation (Johnsen et al., 2000). Lectins are classified according to their affinity to monosaccharides. In this work, three different binding specificities were selected. Concanavalin A (ConA) is an α-mannose-binding lectin that usually binds to high mannose and hybrid N-glycans but fails to bind highly branched complex N-glycans (Cummings & Etzler, 2009). On the other hand, Wheat germ agglutinin (WGA) is one of the most used lectins in research, and it binds specifically to N- acety-D-glucosamine (GlcNAc) and N-acetyl neuraminic acid (sialic acid) (Ryva et al., 2019). Finally, Arachis hypogaea (PNA) binds to galactose/ N- acetyl-galactose (GalNAc) (Natchiar et al., 2007).

A reduction in the expression of sialidases promotes a sustained sialylation on glycoconjugates that facilitates cell immune escape and tumor proliferation (X. Zhou et al., 2020). A549 cells is a cell line derived from human alveolar adenocarcinoma. This cell line is derived from hypotriploid alveolar basal epithelial cells and is one of the most used models to study Type II alveolar epithelium (Okumiya et al., 2006). Moreover, they are easy to grow and an ideal model for

microscopy bases assays due to their ability to grow adherently in monolayer (Korrodi-Gregório et al., 2016).

In this work, I used A549 cells as a model for the epithelial cell layer and optimization of lectin staining protocols. I further explored the effect of bacterial cell extracts on glycosylation by monitoring changes in lectin staining. Four bacterial strains were used in this work: *Enterococcus faecalis* (GC97)*, Rothia kristinae* (GC144)*, Prevotella melaninogenica (*GC 149*),* and *Cutibacterium acnes* (GC 453*)*. The commercial enzyme α2-3,6,8,9 Neuraminidase A was used as a positive control for sialidase activity.

4.2 MATERIALS AND METHODS

4.2.1 Cell culturing and maintenance

A549 cells were obtained from the Bowdish Laboratory at McMaster University, Hamilton, Canada. Cells were maintained, and culture as described by (Lau et al., 2016). Cells were cultured in T75 flasks with DMEM 10 media supplemented with 1% L-glutamine, 10% FBS (Gibco, heat-inactivated), and 100 µg/ml Penicillin/Streptomycin at 37ºC, 5% CO_2. Media was replaced twice a week by washing the cells twice with PBS followed by trypsinizing with 0.25% of Trypsin/EDTA to detach cells from the surface. Cells were transferred to a 50 ml conical tube containing 20 ml of warm DMEM medium and centrifuged for 5 minutes at 440 xg. Supernatant was discarded, and pellet were resuspended in 5 ml of warm DMEM media before transferring to a T75 flask with 10 ml of media.

Cell concentration and viability were monitored with a hemocytometer and Trypan Blue, respectively. Bacterial contamination was measured by plating cell culture medium on BHI3 and TSY agar plates once per week during the cell maintenance period.

4.2.2 Bacteria supernatant recovering

Four bacterial strains that expressed GH activity (Chapter 2) were used in this: *Enterococcus faecalis* (GC97), *Rothia kristinae* (GC144), *Prevotella melaninogenica (*GC149*),* and *Cutibacterium acnes* (GC453*)*. Samples were grown from frozen stocks on BHI3 agar (Brain Heart Infusion broth supplemented with 1 mg/L vitamin K, 10 mg/L hemin, and 0.5 g/L L-cysteine) (Lau et al., 2016) for 24 hrs for *E. faecalis*, 72 hrs hours for *R. kristinae* and 96 hrs for *P. melaninogenica* and *C. acnes*, under anaerobic conditions at 37ºC (Table 2). Single colonies were subculture in 5 ml of BHI3 broth for 24hrs at 37ºC in conical tubes. Supernatants were recovered after centrifugation at 783 xg for 20 minutes at 4ºC and stored at -20ºC.

4.2.3 A549 Cell Double Fluorescent staining assays

A) Intracellular glycan staining

Cells were cultured on T75 flasks with DMEM 10 media supplemented with 1% L-glutamine, 10% FBS (Gibco, heat-inactivated), and 100 µg/ml Penicillin/Streptomycin at 37ºC, 5% CO_2, until they reached a concentration of $6x10^5$ cells/ml before transferring them to a 4-well chamber slide system (Thermo

Scientific Nunc) allowing them to attach to the surface for 24hrs before each assay. Cells were treated with 5 units of commercial enzyme α2-3,6,8,9 Neuraminidase A as a positive control and with 5 µl of *E. faecalis* (GC97)*, R. kristinae* (GC144)*, Prevotella melaninogenica (*GC149*),* and *Cutibacterium acnes* (GC453*)* supernatants for 30 minutes before staining. Fluorescent-labelled lectins were used to stain the glycans present in A549 cells following the protocol reported by (André Dias et al., 2018; Gong et al., 2017) . Cells were fixed with 4% paraformaldehyde for 20 minutes before permeabilizing using 0.1% Triton 100x for 15 minutes and blocked with 10% FBS (Gibco, heat-inactivated) in PBST for 45 minutes. A549 cells were washed with 1X PBST (NaCl: 137mM, KCl: 2.7Mm, Na_2HPO_4: 10Mm and Tween 20 detergent: 0.1%) and incubated for 1hr with both fluorescent-labelled lectins, Con A, Alexa Fluor 350 conjugate (W11262, Invitrogen) 30 µg/ml, PNA (FLK-2100, Vector Laboratories) 10µg/ml, and WGA, Alexa Fluor 594 conjugate (W11262, Invitrogen) 10 µg/ml in PBS at 37°C. Cells were washed once with PBS and dried before visualizing on the microscope at 20x. Slides were visualized on a LEICA DM4000B fluorescence microscope paired with a Spot RT/SE Slider camera. This camera was set to an exposure of 1 second for WGA and Con A staining, gain 1.0x, and camera gamma of 0.54 before each experiment. Each 4 chambered slide included a positive control (cells treated with NeuA) and a negative control (Untreated cells). Con A with WGA staining and ConA with PNA staining images were merge using image J software.

B) Cell surface glycan staining

Cells were grown and treated with neuraminidase and cell supernatants as described above. Fluorescent-labelled lectins staining was done without the fixing step. Cells were blocked with DMEM with 0.5% BSA and 1% L-Glut for 2 hrs. A549 cells were washed with 1X PBST (NaCl: 137mM, KCl: 2.7Mm, Na_2HPO_4: 10Mm and Tween 20 detergent: 0.1%) and incubated for 10 minutes with both fluorescent-labelled lectins, Con A 30 μg/ml and WGA 10 μg/ml in PBS at 37°c. Cells were washed once with PBS and dry before seeing in the microscope at 40x. Slides were visualized on a LEICA DM4000B fluorescence microscope paired with a Spot RT/SE Slider camera. This camera was set to an exposure of 150ms for WGA and 107ms for Con A staining, gain 1.0x, and camera gamma of 0.54 before each experiment. Due to different stages of the cells present on our slides, this protocol stained attached and cells in suspension.

4.3 RESULTS

Fluorescent labelled lectins are useful probes for monitoring global glycosylation patterns in cells. Here I applied protocols for lectin staining of A549 cells and investigated whether treatment of these cells with bacterial supernatants with know GH activity would alter the staining patterns. *Enterococcus faecalis* (GC97)*, Rothia kristinae* (GC144)*, Prevotella melaninogenica (*GC149*),* and *Cutibacterium acnes* (GC453) were used to treated cells before staining with fluorescent-labelled lectins.

Commercial enzyme α2-3,6,8,9 Neuraminidase A was used as a positive control for the cleavage of terminal sialic acid residues on host cell glycans.

For standardizing the method, I followed the protocol from (André Dias et al., 2018; Gong et al., 2017) for fluorescent-labelled lectins. After fixing, permeabilizing, blocking, washing, and staining the cells. I obtained a series of merge microscope images at 20x on a Leica Fluorescence Microscope (ConA with WGA, and ConA with PNA lectins). Differences in ConA, WGA and PNA lectin staining were observed due to cell morphology (surface attached cells vs cells in suspension). Figure 22 shows A549 cells attached cells, stained intracellular with ConA and WGA. Both lectins provide low level uniform staining of cells. ConA staining was to be high on the nuclear membrane in A549 cells. On the other hand, WGA staining on these cells focused on cell nucleus. This staining is consistent with the expected labelling of permeabilized cells.

Pre-treatment with bacterial culture supernatants altered the staining pattern on fixed cells. To compare the staining patterns more closely, fluorescent microscopy images recovered at 20x were merge using image J in order to observe ConA and WGA staining together (Figure 23). A decrease in ConA staining was observed after treatment with *Enterococcus faecalis* (GC97) and *Rothia kristinae* (GC144) supernatant on attached cells. Meanwhile, there was an increase in WGA staining on suspension cells after. *Rothia kristinae* (GC144), and *Cutibacterium acnes* (GC453) supernatant treatment. In general, A549 cells treated with bacterial supernatants had reduced background cytoplasmic staining, Cells treated with

NeuA 10, and 5 units showing and increase on fluorescent intensity on both staining on attached and suspension cells.

Regarding PNA and ConA staining (Figure 24), there was an increase in both staining on A549 cells with Neuraminidase A 5 units. There was and increase of PNA staining with *Enterococcus faecalis* (GC 97) and *Cutibacterium acnes* (GC 453) supernatant. Moreover, there was a greater increase of PNA staining after treatment with *Rothia kristinae* (GC144) supernatant.

Modifications to the staining protocol, such as reduction of exposure time, removal of the fixing and permeabilization steps before staining with lectins , were done to focus on cell surface glycans (Figure 25-26). WGA staining reveals an increase in the fluorescence intensity on suspension cells and no change on attached cells after treating them with Neu A commercial enzyme compared to the negative control. On the other hand, cells treated with *Prevotella melaninogenica (*GC 149*),* and *Cutibacterium acnes* (GC 453) supernatant shows a decrease in WGA on attached cells, meanwhile cells in suspension treated with the same bacterial supernatant shows an increase on WGA staining. (Figure 23).

Con A staining reveals an increase in the fluorescence of suspension cells after treating them with *Rothia kristinae* and *Prevotella melaninogenica* compared with untreated attached cells (Figure 26).

4.4 DISCUSSION

Microscope images of ConA and WGA staining (Figure 22), revealed N-glycans on A549 cells membrane without bacteria supernatant treatment. According to Con A specificity, this lectin will be binding to mannose residues on N-glycans or highly mannose structures present on cell membrane. On the other hand, as part of the fixing and permeabilizing step, WGA staining reveals the presence of N-and O-linked glycans on cell nucleus corresponds with the binding of WGA to GlcNAc of β-1,4-GlcNAc-linked residue (Goldstein et al., 2020; Takahashi & Fukusato, 2017). These lectins have been widely used for staining permeabilized cells and the results are consistent with the know staining patters.

Merge microscope images after bacterial supernatant treatment (Figure 23), shows a decrease of fluorescent intensity on ConA. This reduction could be the result of GHs present on the bacterial supernatant, more over, *Enterococcus faecalis, Cutibacterium acnes* and *Rothia kristinae,* have been predicted to express GH 18 and 85 ENGases that could be hydrolysing GlcNAc residues from native N-linked glycan expressed by A549 cells removing mannose residues on cells(Fairbanks, 2017; Funkhouser & Aronson, 2007). On the other hand, the increase of WGA staining on suspension cells *Rothia kristinae* (GC 144)*,* and *Cutibacterium acnes* (GC 453) supernatant treatment is a result of the N- and O glycans physical exposure observed in round suspension cells.

The increase on ConA and WGA intracellular lectin staining after treatment with NeuA suggests that terminal sialic acid residues are being removed from glycoproteins, glycopeptides, and oligosaccharides present A549 cells. This action increases the accessibility of mannose residues to the Con A lectin on N-linked glycans (Angeloni, 2004).

PNA lectin staining on A549 cells increase after NeuA suggests increased exposure of galactose residues after removing sialic acid, as PNA has a specificity for N-acetylgalactosamine (Figure 24) (Mustac et al., 1996). The increase of PNA staining after *Rothia kristinae* (GC144) supernatant treatment could be a result of GH18 or 85 acting on GlcNAc residues on O-glycans structure, this will expose galactose residues increasing PNA binding(Fairbanks, 2017).

In figure 25, WGA staining analysis reveals an increase in the fluorescence of cells on suspension after treating them with Neu A commercial enzyme, being the result of the N- and O glycans physical exposure observed in round suspension cells. The treatment of this enzyme on A549 cell removes one of the two binding sites of WGA (NeuNac and GlcNAc), explaining the reduction of WGA staining. Moreover, an increase of WGA /ConA extracellular staining, might be dependent on cell line morphology, changes on staining on suspension cells are related to an increase of a homogeneous exposure to the staining, apparently increasing WGA and ConA staining. Also, cells treated with bacterial supernatant from *Cutibacterium acnes* (GC 453) and *Prevotella melaninogenica* (GC 149) show a decrease in WGA staining on attached cells. According to the previous bioinformatic work,

Cutibacterium acnes possess GH18 and GH85 families, respectively. Meanwhile, *Prevotella melaninogenica* possesses gene for GH85 enzymes. Both families of enzymes exhibit the ability to cleave the β-1,4 glycosidic bond found in the conserved core of all human N-linked glycans and O-linked glycans present in cell membrane (Garbe & Collin, 2012; Varki et al., 1999). This enzyme can be secreted to the media, reducing the amount of GlcNAc residues where WGA can bind on cells.

After treating cells with *Rothia kristinae* (GC 144) and *Prevotella melaninogenica* (GC 149) Con A staining was increased, these might be a result of the change in cell morphology present in a few sections on the slide. Changing from attached cells to suspension cells might expose cells homogeneously to our lectin staining.

Treatment of the A549 cells with bacterial supernatants resulted in altered lectin binding patterns, both in fixed/permeabilized cells and unfixed cells. As suggested above this may be the result of GH activity detected in these bacterial supernatants. However, the altered staining pattern may result from the cell response to PAMPs present in these bacterial supernatants. This confounds the interpretation and in the future the use of purified GH enzymes from these bacteria, free of PAMPs (which can be evaluated with the Hek-Blue cell lines in Chapter 3), can be used to explore specific effects of GH activity on surface exposed glycans.

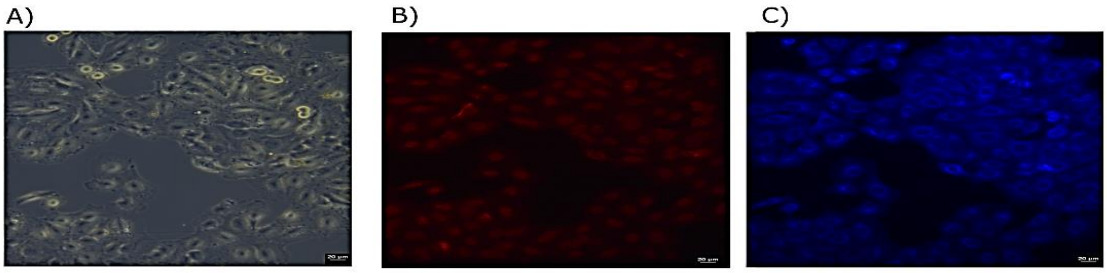


Figure 22. Fluorescence microscopy at 20x of intracellular glycans staining on fixed and permeabilize A549 cells. A) Cells untreated under phase contrast. B) Cells stained in red correspond to WGA lectin (10µg/ml). C) Cells stained in blue correspond to ConA lectin (30µg/ml).

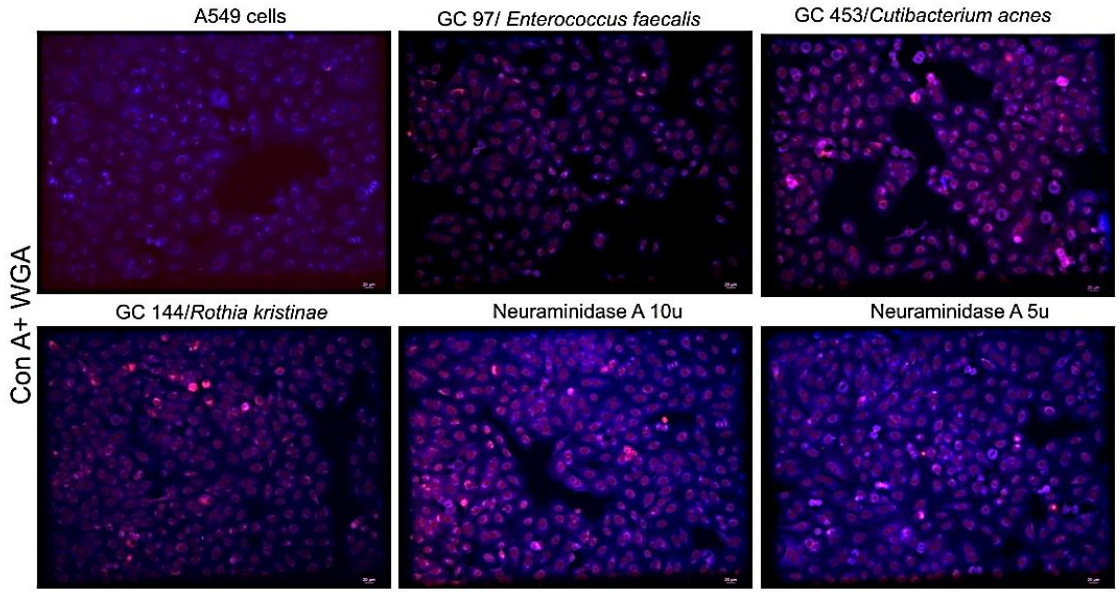


Figure *23*. Fluorescence microscopy at 20x of intracellular glycans staining on fixed and permeabilize A549 cells pretreated with bacterial culture supernatants, Merged fluorescence microscopy of ConA lectin (30µg/ml) and WGA lectin (10µg/ml). *S*taining at 20x of intracellular glycans on treated A549 cells monolayer with *Enterococcus faecalis*, *Cutibacterium acnes*, *Rothia kristinae*, Neuraminidase A 10 units (185 µg/ml) and Neuraminidase A 5 units (92.8 µg/ml).

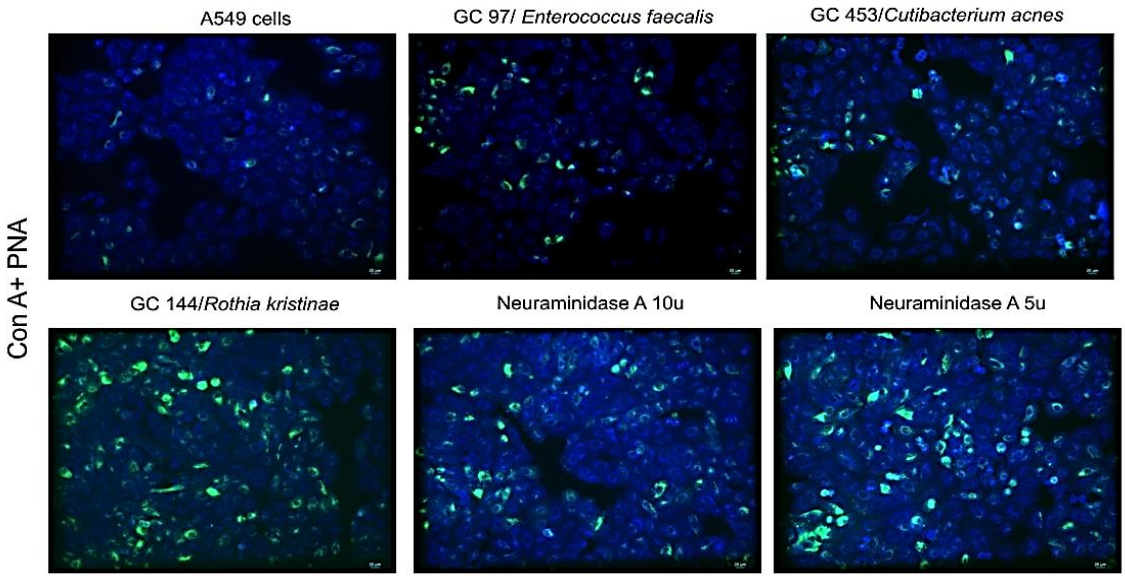


Figure 24. Fluorescence microscopy at 20x of intracellular glycans staining on fixed and permeabilize A549 cells pretreated with bacterial culture supernatants, Merge fluorescence microscopy of ConA lectin (30µg/ml) and PNA lectin (10µg/ml). Staining at 20x of intracellular glycans on treated A549 cells monolayer with *Enterococcus faecalis, Cutibacterium acnes, Rothia kristinae*, Neuraminidase A 10 units (185 µg/ml) and Neuraminidase A 5 units (92.8 µg/ml).

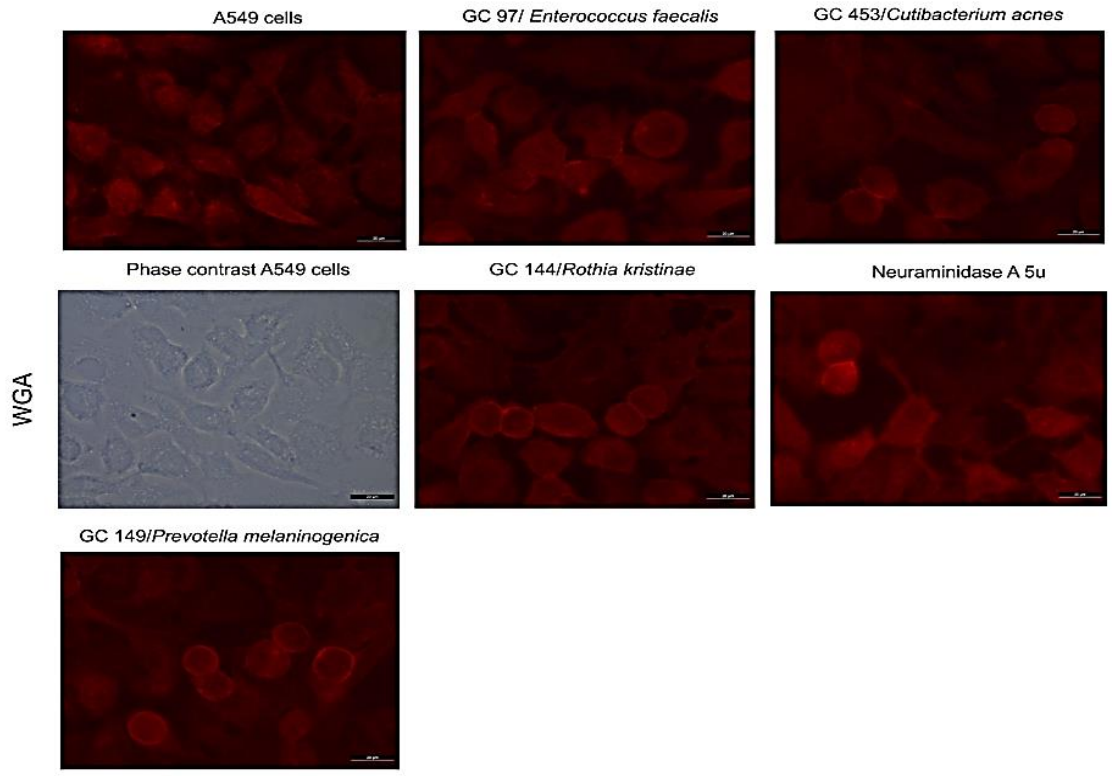


Figure 25. Fluorescence microscopy images at 100x of cell surface glycans on A549cells monolayer, stained with WGA lectin (10µg/ml). Controls: Untreated A549 cells strained with WGA lectin and phase contrast cells. Cells were treated with: Enterococcus faecalis, Cutibacterium acnes, Rothia kristinae, Prevotella melaninogenica and Neuraminidase A 5 units.

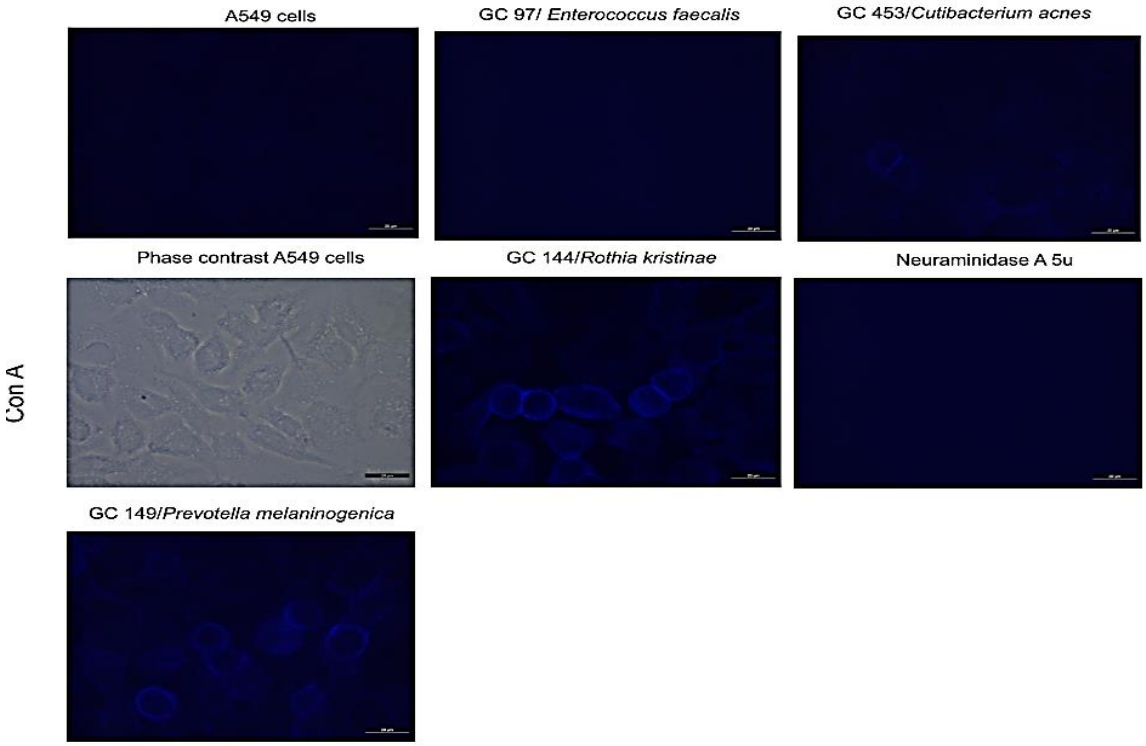


Figure 26. Fluorescence microscopy images at 100x of cell surface glycans on A549cells monolayer, stained with ConA lectin (30µg/ml). Controls: Untreated A549 cells strained with WGA lectin and phase contrast cells. Cells were treated with: Enterococcus faecalis, Cutibacterium acnes, Rothia kristinae, Prevotella melaninogenica and Neuraminidase A 5 units.

Chapter 5: CONCLUSION

Human gut commensal and pathogenic microbes encode a variety of glycoside hydrolases that can target extracellular host cell glycans and shape innate immune responses. For that reason, we can say that glycans are an interface of microbiome-host interaction. Moreover, bacterial glycoside hydrolases have the potential to modulate receptor glycosylation by removing or exposing recognition sites for plant lectins and other PAMPs. Although the detection of GH family enzymes from genome sequence is possible, the prediction of GH enzyme specificity is not. In addition, only a small fraction of known GH enzymes have been functionally characterized. There is a need to develop efficient methods to functionally characterize GH enzymes and of interest in this thesis are those found within the human microbiome.

Cell-free supernatant measurement of GH activity against the four glycans of interest shows that fucosidases in our samples appear to be primarily released into the supernatant. Also, in the present study, β-D-GlcNAc and sialic acid measurements in cell-free supernatant show *Bacteroides* as the genus with the most GH enzyme activity for these substrates. Meanwhile, *Barnesiella* and *Streptococcus* genera showed GH enzyme activity in α-GlcNac and α-Fucose substrate. It is important to note that for cell-free supernatant measurement for sialic acid, fucose and α-GlcNAc were only performed for 8 samples, it is probable

that if we expand the number of samples, we will find more variety of genus presenting activity for our substrates of interest.

Bacterial pellet measurement shows that endoglycosidases and sialidases in our samples are mainly anchored to the membrane due to the number of samples showing activity for those substrates in bacterial pellets. Finally, on these assays I concluded that *Bacteroides, Bacillus, Enterococcus, Lactobacillus, Prevotella,* and *Streptococcus* were the main genera expressing GH enzymes in bacterial supernatant differing from the genera present in bacteria pellets. The main genera expressing GH enzymes on the cell membrane were *Alistipes*, *Bacteroides, Barnesiella, Bifidobacterium, Coprobacter, Enterococcus, Escherichia, Monoglobus, Streptococcus, Parabacteroides, Paraprevotella, Prevotella, Gabonia, Roseburia,* and *Victivallis.*

The ability to measure enzymatic activity directly on cell pellets would allow for rapid screening of bacterial isolates. While the use of a fluorescent substrate was convenient, there was a high background from the preferred culture medium (BHI3) and this reduced the sensitivity of the assay of bacterial culture supernatants,

Plant based lectins are known to activate some host signalling pathways. As these recognize and bind to glycans on specific receptors, modulation of these immunostimulatory activities by changing glycan structure on these receptors is expected. I hypothesize that this is one mechanism by which commensal micrbiota can modulate diet -host interactions. To this end I set out to validate a published

activity of dietary lectins on the NLRP3 inflammasome. In that study (Gong et al., 2017) two lectins (ConA and WGA) stimulated LPS-dependent activation of the inflammasome in THP-1 cells. In the work reported here, I reproducibly observed that IL-8 measurement on THP-1 cells after ConA, WGA and PNA lectin stimulation decreased, compared with cells stimulated with LPS alone (positive control). Consequently, I moved to a simpler host cell system: Hek2-Blue cells which report the activation of a single TLR receptor per cell line. Lectin cell stimulation assay on TLR4 and TLR2 showed an increase in SEAP expression as a result of receptor activation and NF-kB production by WGA and SBA. However, I was able to demonstrate that this activity was likely due to low level LPS contamination Adding polymyxin B to these lectins abolished the TLR4 and TLR2 stimulation.

Cell stimulation with ConA and LPS, our data show that this lectin activates TLR2 and TLR4 receptors, increasing SEAP activity. This indicates that ConA can modulate how cell response after stimulation with TLR ligands on Hek-Blue-mTLR4 and Hek-Blue-hTLR2 cells. There was some reduction in this activity with one commercial GH enzyme and with partially purified GH18 and GH85 enzymes from human microbiota. These enzymes would be predicted to remove N-glycans to which ConA binds. Although more experiments are required to demonstrate this conclusively, these results suggest that ConA activation of Hek-Blue-mTLR4 and Hek-Blue-hTLR2 cells could be an efficient assay to screen for ENGAses active on the TLRs.

A549 cells membrane without bacteria supernatant treatment reveals the presence of N-glycans. ConA binding to mannose residues on N-glycans or highly mannose structures present on cell membrane. On the other hand, as part of the fixing and permeabilizing step, WGA staining reveals the presence of N-and O- linked glycans on cell nucleus corresponds with the binding of WGA to GlcNAc of β-1,4-GlcNAc-linked residue.

An increase of WGA /ConA extracellular staining, might be dependent on cell line morphology, changes on staining on suspension cells are related to an increase of a homogeneous exposure to the staining, apparently increasing WGA and ConA staining.

Treatment of the A549 cells with bacterial supernatants resulted in altered lectin binding patterns, both in fixed/permeabilized cells and unfixed cells.

Altered staining pattern may result from the cell response to PAMPs present in these bacterial supernatants.

5.1 Future directions

Future directions for the first aim of this study include scaling of the 4-MU screening assays with more samples in order to detect the presence of predicted GHs on our laboratory collection.

For my second aim, the future direction includes testing a higher amount of purified GHs from our laboratory collection on Hek-Blue cells and improving the purification steps in order to avoid unspecific activation of TLRs with reminding PAMPs.

Also, corroborate the LTA stimulation on Hek-Blue hTLR2 cells to solve the low stimulation showed on the assays.

Finally, for my third aim the future directions focus on quantified staining variations on the assay and use confocal microscopy for improvement optical resolution on the result image.

REFERENCES

Adamcová, A., Laursen, K., & Ballin, N. (2021). Lectin Activity in Commonly Consumed Plant-Based Foods: Calling for Method Harmonization and Risk Assessment. *Foods*, *10*, 2796. https://doi.org/10.3390/foods10112796

Al-Ghouleh, A., Johal, R., Sharquie, I. K., Emara, M., Harrington, H., Shakib, F., & Ghaemmaghami, A. M. (2012). The Glycosylation Pattern of Common Allergens: The Recognition and Uptake of Der p 1 by Epithelial and Dendritic Cells Is Carbohydrate Dependent. *PLoS ONE*, *7*(3), e33929. https://doi.org/10.1371/journal.pone.0033929

Allendorf, D. H., Franssen, E. H., & Brown, G. C. (2020). Lipopolysaccharide activates microglia via neuraminidase 1 desialylation of Toll-like Receptor 4. *Journal of Neurochemistry*, *155*(4), 403–416. https://doi.org/10.1111/jnc.15024

Amith, S. R., Jayanth, P., Franchuk, S., Finlay, T., Seyrantepe, V., Beyaert, R., Pshezhetsky, A. V., & Szewczuk, M. R. (2010). Neu1 desialylation of sialyl alpha-2,3-linked beta-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling. *Cellular Signalling*, *22*(2), 314–324. https://doi.org/10.1016/j.cellsig.2009.09.038

André Dias, S., Planus, E., Angely, C., Lotteau, L., Tissier, R., Filoche, M., Louis, B., Pelle, G., & Isabey, D. (2018). Perfluorocarbon induces alveolar epithelial cell response through structural and mechanical remodeling. *Biomechanics and Modeling in Mechanobiology*, *17*(4), 961–973. https://doi.org/10.1007/s10237-018-1005-z

Angeloni, S. (2004). Glycoprofiling with micro-arrays of glycoconjugates and lectins. *Glycobiology*, *15*(1), 31–41. https://doi.org/10.1093/glycob/cwh143

Barre, A., Damme, E. J. M. V., Simplicien, M., Benoist, H., & Rougé, P. (2020). Are Dietary Lectins Relevant Allergens in Plant Food Allergy? *Foods*, *9*(12), 1724. https://doi.org/10.3390/foods9121724

Batista, J., Ralph, M., Vaz, R., Souza, P., Silva, A., Nascimento, D., Souza, L., Ramos, M., Mastroeni, P., & Lima-Filho, J. (2017). Plant lectins ConBr and CFL modulate expression toll-like receptors, pro-inflammatory cytokines and reduce the bacterial burden in macrophages infected with Salmonella enterica serovar Typhimurium. *Phytomedicine*, *25*, 52–60. https://doi.org/10.1016/j.phymed.2016.12.005

Belkaid, Y., & Hand, T. (2014). Role of the Microbiota in Immunity and inflammation. *Cell*, *157*(1), 121–141. https://doi.org/10.1016/j.cell.2014.03.011

Bellande, K., Bono, J.-J., Savelli, B., Jamet, E., & Canut, H. (2017). Plant Lectins and Lectin Receptor-Like Kinases: How Do They Sense the Outside? *International Journal of Molecular Sciences*, *18*(6), Article 6. https://doi.org/10.3390/ijms18061164

Belzer, C. (2022). Nutritional strategies for mucosal health: The interplay between microbes and mucin glycans. *Trends in Microbiology*, *30*(1), 13–21. https://doi.org/10.1016/j.tim.2021.06.003

Bergstrom, K., Fu, J., & Xia, L. (2015). Biological Functions of C1GalT1 and Mucin-Type O-Glycans. In N. Taniguchi, T. Endo, G. W. Hart, P. H. Seeberger, & C.-H. Wong (Eds.), *Glycoscience: Biology and Medicine* (pp. 1073–1080). Springer Japan. https://doi.org/10.1007/978-4-431-54841-6_65

Berlemont, R., & Martiny, A. C. (2016). Glycoside Hydrolases across Environmental Microbial Communities. *PLOS Computational Biology*, *12*(12), e1005300. https://doi.org/10.1371/journal.pcbi.1005300

Boston, 677 Huntington Avenue, & Ma 02115 +1495-1000. (2019, January 24). *Lectins*. The Nutrition Source. https://www.hsph.harvard.edu/nutritionsource/anti-nutrients/lectins/

Botos, I., Segal, D. M., & Davies, D. R. (2011). The Structural Biology of Toll-Like Receptors. *Structure (London, England : 1993)*, *19*(4), 447–459. https://doi.org/10.1016/j.str.2011.02.004

Bowles, W. H. D., & Gloster, T. M. (2021). Sialidase and Sialyltransferase Inhibitors: Targeting Pathogenicity and Disease. *Frontiers in Molecular Biosciences*, *8*. https://www.frontiersin.org/articles/10.3389/fmolb.2021.705133

Bule, P., Chuzel, L., Blagova, E., Wu, L., Gray, M. A., Henrissat, B., Rapp, E., Bertozzi, C. R., Taron, C. H., & Davies, G. J. (2019). Inverting family GH156 sialidases define an unusual catalytic motif for glycosidase action. *Nature Communications*, *10*(1), 4816. https://doi.org/10.1038/s41467-019-12684-7

Cappuccio, J., Quiroga, M. A., Moredo, F., Fernández Canigia, L., Machuca, M., Capponi, O., Bianchini, A., Zielinski, G., Sarradell, J., Ibar, M., Vigo, G., Giacoboni, G., & Perfumo, C. (2009). Neonatal piglets mesocolon edema and colitis due to Clostridium difficile infection. Prevalence, clinical disease and pathological studies. *Braz J Vet Pathol Brazilian Journal of Veterinary Pathology*, *2*, 35–40.

Cayuela, M. F., Chantal. (2000). Microbial Modulation of Host Intestinal Glycosylation Patterns. *Microbial Ecology in Health and Disease*, *12*(2), 165–178. https://doi.org/10.1080/089106000750060422

Charcosset, C. (2011). 2.43—Membrane Systems and Technology. In M. Moo-Young (Ed.), *Comprehensive Biotechnology (Second Edition)* (pp. 603–618). Academic Press. https://doi.org/10.1016/B978-0-08-088504-9.00131-8

Chen, G.-Y., Brown, N. K., Wu, W., Khedri, Z., Yu, H., Chen, X., van de Vlekkert, D., D'Azzo, A., Zheng, P., & Liu, Y. (2014). Broad and direct interaction between TLR and Siglec families of pattern recognition receptors and its regulation by Neu1. *ELife*, *3*, e04066. https://doi.org/10.7554/eLife.04066

Comstock, L. E., & Kasper, D. L. (2006). Bacterial Glycans: Key Mediators of Diverse Host Immune Responses. *Cell*, *126*(5), 847–850. https://doi.org/10.1016/j.cell.2006.08.021

Cullen, S. P., Kearney, C. J., Clancy, D. M., & Martin, S. J. (n.d.). *Diverse Activators of the NLRP3 Inflammasome Promote IL-1b Secretion by Triggering Necrosis*.

Cummings, R. D., & Etzler, M. E. (2009). Antibodies and Lectins in Glycan Analysis. In *Essentials of Glycobiology. 2nd edition*. Cold Spring Harbor Laboratory Press. https://www.ncbi.nlm.nih.gov/books/NBK1919/

da Silva Correia, J., & Ulevitch, R. J. (2002). MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *The Journal of Biological Chemistry*, *277*(3), 1845–1854. https://doi.org/10.1074/jbc.M109910200

Davies, G., & Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure*, *3*(9), 853–859. https://doi.org/10.1016/S0969-2126(01)00220-9

de Haas, P., Hendriks, W. J. A. J., Lefeber, D. J., & Cambi, A. (2020). Biological and Technical Challenges in Unraveling the Role of N-Glycans in Immune Receptor Regulation. *Frontiers in Chemistry*, *8*, 55. https://doi.org/10.3389/fchem.2020.00055

de Oliviera Nascimento, L., Massari, P., & Wetzler, L. (2012). The Role of TLR2 in Infection and Immunity. *Frontiers in Immunology*, *3*. https://www.frontiersin.org/articles/10.3389/fimmu.2012.00079

El-Zayat, S. R., Sibaii, H., & Mannaa, F. A. (2019). Toll-like receptors activation, signaling, and targeting: An overview. *Bulletin of the National Research Centre*, *43*(1), 187. https://doi.org/10.1186/s42269-019-0227-2

Esmail, S., & Manolson, M. F. (2021). Advances in understanding N-glycosylation structure, function, and regulation in health and disease. *European Journal of Cell Biology*, *100*(7), 151186. https://doi.org/10.1016/j.ejcb.2021.151186

Fairbanks, A. J. (2017). The ENGases: Versatile biocatalysts for the production of homogeneous N-linked glycopeptides and glycoproteins. *Chemical Society Reviews*, *46*(16), 5128–5146. https://doi.org/10.1039/C6CS00897F

Fan, S.-Q., Huang, W., & Wang, L.-X. (2012). Remarkable Transglycosylation Activity of Glycosynthase Mutants of Endo-D, an Endo-β-N-acetylglucosaminidase from Streptococcus pneumoniae*. *Journal of Biological Chemistry*, *287*(14), 11272–11281. https://doi.org/10.1074/jbc.M112.340497

Freed, D. L. J. (1999). Do dietary lectins cause disease? *BMJ : British Medical Journal*, *318*(7190), 1023–1024.

Fujita, M., Shoda, S., Haneda, K., Inazu, T., Takegawa, K., & Yamamoto, K. (2001). A novel disaccharide substrate having 1,2-oxazoline moiety for detection of transglycosylating activity of endoglycosidases. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1528*(1), 9–14. https://doi.org/10.1016/S0304-4165(01)00164-7

Funkhouser, J. D., & Aronson, N. N. (2007). Chitinase family GH18: Evolutionary insights from the genomic history of a diverse protein family. *BMC Evolutionary Biology*, *7*(1), 96. https://doi.org/10.1186/1471-2148-7-96

Garbe, J., & Collin, M. (2012). Bacterial Hydrolysis of Host Glycoproteins – Powerful Protein Modification and Efficient Nutrient Acquisition. *Journal of Innate Immunity*, *4*(2), 121–131. https://doi.org/10.1159/000334775

Garrido, D., Nwosu, C., Ruiz-Moyano, S., Aldredge, D., German, J. B., Lebrilla, C. B., & Mills, D. A. (2012). Endo-β-N-acetylglucosaminidases from Infant Gut-associated Bifidobacteria Release Complex N-glycans from Human Milk Glycoproteins*. *Molecular & Cellular Proteomics*, *11*(9), 775–785. https://doi.org/10.1074/mcp.M112.018119

Gloster, T. M., Turkenburg, J. P., Potts, J. R., Henrissat, B., & Davies, G. J. (2008). Divergence of Catalytic Mechanism within a Glycosidase Family Provides Insight into Evolution of Carbohydrate Metabolism by Human Gut Flora. *Chemistry & Biology*, *15*(10), 1058–1067. https://doi.org/10.1016/j.chembiol.2008.09.005

Goldstein, C. M., Murray, E. J., Beard, J., Schnoes, A. M., & Wang, M. L. (2020). Science Communication in the Age of Misinformation. *Annals of Behavioral Medicine*, *54*(12), 985–990. https://doi.org/10.1093/abm/kaaa088

Gong, T., Wang, X., Yang, Y., Yan, Y., Yu, C., Zhou, R., & Jiang, W. (2017). Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders. *The Journal of Immunology*, *198*(5), 2082–2092. https://doi.org/10.4049/jimmunol.1600145

González-Morelo, K. J., Vega-Sagardía, M., & Garrido, D. (2020). Molecular Insights Into O-Linked Glycan Utilization by Gut Microbes. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.591568

Gregory, B., Savino, R., & Ciliberto, G. (1994). A fast and sensitive colorimetric assay for IL-6 in hepatoma cells based on the production of a secreted form of alkaline phosphatase

(SEAP). *Journal of Immunological Methods*, *170*(1), 47–56. https://doi.org/10.1016/0022-1759(94)90244-5

Guzman-Aranguez, A., & Argüeso, P. (2010). Structure and Biological Roles of Mucin-type O-glycans at the Ocular Surface. *The Ocular Surface*, *8*(1), 8–17.

Hassan, S., Donia, A., Sial, U., Zhang, X., & Bokhari, H. (2020). Glycoprotein- and Lectin-Based Approaches for Detection of Pathogens. *Pathogens*, *9*(9), Article 9. https://doi.org/10.3390/pathogens9090694

HEK-Blue™ hTLR2. (2016, November 25). InvivoGen. https://www.invivogen.com/hek-blue-htlr2

HEK-Blue™ mTLR4. (2016, November 25). InvivoGen. https://www.invivogen.com/hek-blue-mtlr4

Hobbs, J. K., Pluvinage, B., & Boraston, A. B. (2018). Glycan-metabolizing enzymes in microbe–host interactions: The *Streptococcus pneumoniae* paradigm. *FEBS Letters*, *592*(23), 3865–3897. https://doi.org/10.1002/1873-3468.13045

Hobbs, J. K., Pluvinage, B., Robb, M., Smith, S. P., & Boraston, A. B. (2019). Two complementary α-fucosidases from Streptococcus pneumoniae promote complete degradation of host-derived carbohydrate antigens. *The Journal of Biological Chemistry*, *294*(34), 12670–12682. https://doi.org/10.1074/jbc.RA119.009368

Huang, Q.-S., Xie, X.-L., Liang, G., Gong, F., Wang, Y., Wei, X.-Q., Wang, Q., Ji, Z.-L., & Chen, Q.-X. (2012). The GH18 family of chitinases: Their domain architectures, functions and evolutions. *Glycobiology*, *22*(1), 23–34. https://doi.org/10.1093/glycob/cwr092

Huang, Y.-L., Chassard, C., Hausmann, M., von Itzstein, M., & Hennet, T. (2015). Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. *Nature Communications*, *6*(1), Article 1. https://doi.org/10.1038/ncomms9141

Inoue, K., Wada, J., Eguchi, J., Nakatsuka, A., Teshigawara, S., Murakami, K., Ogawa, D., Terami, T., Katayama, A., Tone, A., Iseda, I., Hida, K., Yamada, M., Ogawa, T., & Makino, H. (2013). Urinary Fetuin-A Is a Novel Marker for Diabetic Nephropathy in Type 2 Diabetes Identified by Lectin Microarray. *PloS One*, *8*, e77118. https://doi.org/10.1371/journal.pone.0077118

Jardin, B. A., Zhao, Y., Selvaraj, M., Montes, J., Tran, R., Prakash, S., & Elias, C. B. (2008). Expression of SEAP (secreted alkaline phosphatase) by baculovirus mediated transduction of HEK 293 cells in a hollow fiber bioreactor system. *Journal of Biotechnology*, *135*(3), 272–280. https://doi.org/10.1016/j.jbiotec.2008.04.006

Johnsen, A. R., Hausner, M., Schnell, A., & Wuertz, S. (2000). Evaluation of Fluorescently Labeled Lectins for Noninvasive Localization of Extracellular Polymeric Substances in Sphingomonas Biofilms. *Applied and Environmental Microbiology*, *66*(8), 3487–3491.

Juge, N., Tailford, L., & Owen, C. D. (2016). Sialidases from gut bacteria: A mini-review. *Biochemical Society Transactions*, *44*(1), 166–175. https://doi.org/10.1042/BST20150226

Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D., & Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nature Reviews Microbiology*, *11*(7), 497–504. https://doi.org/10.1038/nrmicro3050

Kawai, T., & Akira, S. (2006). TLR signaling. *Cell Death & Differentiation*, *13*(5), Article 5. https://doi.org/10.1038/sj.cdd.4401850

Kawasaki, T., & Kawai, T. (2014). Toll-Like Receptor Signaling Pathways. *Frontiers in Immunology*, *5*. https://www.frontiersin.org/articles/10.3389/fimmu.2014.00461

Keil, J. M., Rafn, G. R., Turan, I. M., Aljohani, M. A., Sahebjam-Atabaki, R., & Sun, X.-L. (2022). Sialidase Inhibitors with Different Mechanisms. *Journal of Medicinal Chemistry*, *65*(20), 13574–13593. https://doi.org/10.1021/acs.jmedchem.2c01258

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International Journal of Molecular Sciences*, *20*(13), 3328. https://doi.org/10.3390/ijms20133328

Kim, G.-Y., Lee, J.-W., Ryu, H.-C., Wei, J.-D., Seong, C.-M., & Kim, J.-H. (2010). Proinflammatory Cytokine IL-1β Stimulates IL-8 Synthesis in Mast Cells via a Leukotriene B4 Receptor 2-Linked Pathway, Contributing to Angiogenesis. *The Journal of Immunology*, *184*(7), 3946–3954. https://doi.org/10.4049/jimmunol.0901735

Kirschning, C. J., Wesche, H., Merrill Ayres, T., & Rothe, M. (1998). Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *The Journal of Experimental Medicine*, *188*(11), 2091–2097. https://doi.org/10.1084/jem.188.11.2091

Klumpp, S., & Hwa, T. (2014). Bacterial growth: Global effects on gene expression, growth feedback and proteome partition. *Current Opinion in Biotechnology*, *0*, 96–102. https://doi.org/10.1016/j.copbio.2014.01.001

Koropatkin, N. M., Martens, E. C., Gordon, J. I., & Smith, T. J. (2008). Starch catabolism by a prominent human gut symbiont is directed by the recognition of amylose helices. *Structure (London, England : 1993)*, *16*(7), 1105–1115. https://doi.org/10.1016/j.str.2008.03.017

Korrodi-Gregório, L., Soto-Cerrato, V., Vitorino, R., Fardilha, M., & Pérez-Tomás, R. (2016). From Proteomic Analysis to Potential Therapeutic Targets: Functional Profile of Two Lung

Cancer Cell Lines, A549 and SW900, Widely Studied in Pre-Clinical Research. *PLOS ONE*, *11*(11), e0165973. https://doi.org/10.1371/journal.pone.0165973

Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P., & Surette, M. G. (2016). Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Medicine*, *8*(1), 72. https://doi.org/10.1186/s13073-016-0327-7

Leifer, C. A., & Medvedev, A. E. (2016). Molecular mechanisms of regulation of Toll-like receptor signaling. *Journal of Leukocyte Biology*, *100*(5), 927–941. https://doi.org/10.1189/jlb.2MR0316-117RR

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., & Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research*, *42*(D1), D490–D495. https://doi.org/10.1093/nar/gkt1178

Lucius, K. (2020). Dietary Lectins: Gastrointestinal and Immune Effects. *Alternative and Complementary Therapies*, *26*(4), 168–174. https://doi.org/10.1089/act.2020.29286.klu

Luijkx, Y. M. C. A., Bleumink, N. M. C., Jiang, J., Overkleeft, H. S., Wösten, M. M. S. M., Strijbis, K., & Wennekes, T. (2020). Bacteroides fragilis fucosidases facilitate growth and invasion of Campylobacter jejuni in the presence of mucins. *Cellular Microbiology*, *22*(12), e13252. https://doi.org/10.1111/cmi.13252

Mann, E., Shekarriz, S., & Surette, M. G. (2022). Human Gut Metagenomes Encode Diverse GH156 Sialidases. *Applied and Environmental Microbiology*, *0*(0), e01755-22. https://doi.org/10.1128/aem.01755-22

Mao, L., Kitani, A., Strober, W., & Fuss, I. J. (2018). The Role of NLRP3 and IL-1β in the Pathogenesis of Inflammatory Bowel Disease. *Frontiers in Immunology*, *9*. https://www.frontiersin.org/articles/10.3389/fimmu.2018.02566

Mariano, V. S., Zorzetto-Fernandes, A. L., Silva, T. A. da, Ruas, L. P., Nohara, L. L., Almeida, I. C. de, & Roque-Barreira, M. C. (2014). Recognition of TLR2 N-Glycans: Critical Role in ArtinM Immunomodulatory Activity. *PLOS ONE*, *9*(6), e98512. https://doi.org/10.1371/journal.pone.0098512

McCarter, J. D., & Stephen Withers, G. (1994). Mechanisms of enzymatic glycoside hydrolysis. *Current Opinion in Structural Biology*, *4*(6), 885–892. https://doi.org/10.1016/0959-440X(94)90271-2

Meyer, B. H., Wagstaff, B. A., Adam, P. S., Albers, S.-V., & Dorfmueller, H. C. (2021). *N-glycan chitobiose core biosynthesis by Agl24 strengthens the hypothesis of an archaeal origin of the eukaryal N-glycosylation* (p. 2021.01.19.427365). bioRxiv. https://doi.org/10.1101/2021.01.19.427365

Mishra, A., Behura, A., Mawatwal, S., Kumar, A., Naik, L., Mohanty, S. S., Manna, D., Dokania, P., Mishra, A., Patra, S. K., & Dhiman, R. (2019). Structure-function and application of plant lectins in disease biology and immunity. *Food and Chemical Toxicology*, *134*, 110827. https://doi.org/10.1016/j.fct.2019.110827

Monti, E., Bassi, M. T., Papini, N., Riboni, M., Manzoni, M., Venerando, B., Croci, G., Preti, A., Ballabio, A., Tettamanti, G., & Borsani, G. (2000). Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochemical Journal*, *349*(Pt 1), 343–351.

Mustac, E., Melato, M., Sasso, F., Valkovic, T., Bottin, C., & Jonjic, N. (1996). The lectin-binding sites for peanut agglutinin in invasive breast ductal carcinomas and their role as a prognostic factor. *Journal of Cancer Research and Clinical Oncology*, *122*(11), 693–697. https://doi.org/10.1007/BF01209034

Natchiar, S. K., Suguna, K., Surolia, A., & Vijayan, M. (2007). Peanut agglutinin, a lectin with an unusual quaternary structure and interesting ligand binding properties. *Crystallography Reviews*, *13*(1), 3–28. https://doi.org/10.1080/08893110701382087

Okumiya, T., Keulemans, J. L. M., Kroos, M. A., Van der Beek, N. M. E., Boer, M. A., Takeuchi, H., Van Diggelen, O. P., & Reuser, A. J. J. (2006). A new diagnostic assay for glycogen storage disease type II in mixed leukocytes. *Molecular Genetics and Metabolism*, *88*(1), 22–28. https://doi.org/10.1016/j.ymgme.2005.10.016

Parker, D., Soong, G., Planet, P., Brower, J., Ratner, A. J., & Prince, A. (2009). The NanA Neuraminidase of Streptococcus pneumoniae Is Involved in Biofilm Formation. *Infection and Immunity*, *77*(9), 3722–3730. https://doi.org/10.1128/IAI.00228-09

Radovani, B., & Gudelj, I. (2022). N-Glycosylation and Inflammation; the Not-So-Sweet Relation. *Frontiers in Immunology*, *13*, 893365. https://doi.org/10.3389/fimmu.2022.893365

Reily, C., Stewart, T. J., Renfrow, M. B., & Novak, J. (2019). Glycosylation in health and disease. *Nature Reviews Nephrology*, *15*(6), 346–366. https://doi.org/10.1038/s41581-019-0129-4

Ricci-Azevedo, R., Roque-Barreira, M.-C., & Gay, N. J. (2017). Targeting and Recognition of Toll-Like Receptors by Plant and Pathogen Lectins. *Frontiers in Immunology*, *8*, 1820. https://doi.org/10.3389/fimmu.2017.01820

Ryva, B., Zhang, K., Asthana, A., Wong, D., Vicioso, Y., & Parameswaran, R. (2019). Wheat Germ Agglutinin as a Potential Therapeutic Agent for Leukemia. *Frontiers in Oncology*, *9*. https://www.frontiersin.org/articles/10.3389/fonc.2019.00100

Sameer, A. S., & Nissar, S. (2021). Toll-Like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility. *BioMed Research International*, *2021*, 1157023. https://doi.org/10.1155/2021/1157023

Schromm, A. B., & Brandenburg, K. (2021). TLR4 Ligands: Single Molecules and Aggregates. In C. Rossetti & F. Peri (Eds.), *The Role of Toll-Like Receptor 4 in Infectious and Non Infectious Inflammation* (pp. 39–56). Springer International Publishing. https://doi.org/10.1007/978-3-030-56319-6_3

SEAP Reporter Gene System. (2016, November 25). InvivoGen. https://www.invivogen.com/seap-reporter-gene-system

Sharon, N., & Lis, H. (Eds.). (2007). Specificity And Affinity. In *Lectins* (pp. 63–103). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6953-6_4

Shatri, G., & Tadi, P. (2022). Polymyxin. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK557540/

Shrivastava, S. (2020). Introduction to Glycoside Hydrolases: Classification, Identification and Occurrence. In S. Shrivastava (Ed.), *Industrial Applications of Glycoside Hydrolases* (pp. 3–84). Springer. https://doi.org/10.1007/978-981-15-4767-6_1

Sjögren, J., & Collin, M. (2014). Bacterial glycosidases in pathogenesis and glycoengineering. *Future Microbiology*, *9*(9), 1039–1051. https://doi.org/10.2217/fmb.14.71

Sodhi, A., Tarang, S., & Kesherwani, V. (2007). Concanavalin A induced expression of Toll-like receptors in murine peritoneal macrophages in vitro. *International Immunopharmacology*, *7*(4), 454–463. https://doi.org/10.1016/j.intimp.2006.11.014

Souza, M. A., Carvalho, F. C., Ruas, L. P., Ricci-Azevedo, R., & Roque-Barreira, M. C. (2013). The immunomodulatory effect of plant lectins: A review with emphasis on ArtinM

properties. *Glycoconjugate Journal*, *30*(7), 641–657. https://doi.org/10.1007/s10719-012-9464-4

Stanley, P., Moremen, K. W., Lewis, N. E., Taniguchi, N., & Aebi, M. (2022). N-Glycans. In *Essentials of Glycobiology [Internet]. 4th edition*. Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/glycobiology.4e.9

Stütz, A. E., & Wrodnigg, T. M. (2011). Chapter 4 - Imino sugars and glycosyl hydrolases: Historical context, current aspects, emerging trends. In D. Horton (Ed.), *Advances in Carbohydrate Chemistry and Biochemistry* (Vol. 66, pp. 187–298). Academic Press. https://doi.org/10.1016/B978-0-12-385518-3.00004-3

Tailford, L. E., Crost, E. H., Kavanaugh, D., & Juge, N. (2015). Mucin glycan foraging in the human gut microbiome. *Frontiers in Genetics*, *6*, 81. https://doi.org/10.3389/fgene.2015.00081

Takahashi, Y., & Fukusato, T. (2017). Chapter 13—Animal Models of Liver Diseases. In P. M. Conn (Ed.), *Animal Models for the Study of Human Disease (Second Edition)* (pp. 313–339). Academic Press. https://doi.org/10.1016/B978-0-12-809468-6.00013-9

Tao, H., Bausch, C., Richmond, C., Blattner, F. R., & Conway, T. (1999). Functional genomics: Expression analysis of Escherichia coli growing on minimal and rich media. *Journal of Bacteriology*, *181*(20), 6425–6440. https://doi.org/10.1128/JB.181.20.6425-6440.1999

The CAZypedia Consortium. (2018). Ten years of CAZypedia: A living encyclopedia of carbohydrate-active enzymes. *Glycobiology*, *28*(1), 3–8. https://doi.org/10.1093/glycob/cwx089

Trastoy, B., Du, J. J., Li, C., García-Alija, M., Klontz, E. H., Roberts, B. R., Donahue, T. C., Wang, L.-X., Sundberg, E. J., & Guerin, M. E. (2021). GH18 endo-β-N-acetylglucosaminidases use

distinct mechanisms to process hybrid-type N-linked glycans. *Journal of Biological Chemistry*, *297*(2), 101011. https://doi.org/10.1016/j.jbc.2021.101011

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International Journal of Cancer*, *26*(2), 171–176. https://doi.org/10.1002/ijc.2910260208

Unitt, J., & Hornigold, D. (2011). Plant Lectins are Novel Toll-Like Receptor Agonists. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2011.03.010

Valverde, P., Ardá, A., Reichardt, N.-C., Jiménez-Barbero, J., & Gimeno, A. (2019). Glycans in drug discovery. *MedChemComm*, *10*(10), 1678–1691. https://doi.org/10.1039/C9MD00292H

Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., & Marth, J. (1999). O-Glycans. In *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. https://www.ncbi.nlm.nih.gov/books/NBK20721/

Varki, A., & Gagneux, P. (2015). Biological Functions of Glycans. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Eds.), *Essentials of Glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press. http://www.ncbi.nlm.nih.gov/books/NBK453034/

Varki, A., & Kornfeld, S. (2015). Historical Background and Overview. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Eds.), *Essentials of Glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press. http://www.ncbi.nlm.nih.gov/books/NBK316258/

Villena, J., & Kitazawa, H. (2014). Modulation of Intestinal TLR4-Inflammatory Signaling Pathways by Probiotic Microorganisms: Lessons Learned from Lactobacillus jensenii TL2937.

Frontiers in Immunology, *4*. https://www.frontiersin.org/article/10.3389/fimmu.2013.00512

Vuong, T. V., & Wilson, D. B. (2010). Glycoside hydrolases: Catalytic base/nucleophile diversity. *Biotechnology and Bioengineering*, *107*(2), 195–205. https://doi.org/10.1002/bit.22838

Wang, B., Yao, M., Lv, L., Ling, Z., & Li, L. (2017). The Human Microbiota in Health and Disease. *Engineering*, *3*(1), 71–82. https://doi.org/10.1016/J.ENG.2017.01.008

Wardman, J. F., Bains, R. K., Rahfeld, P., & Withers, S. G. (2022). Carbohydrate-active enzymes (CAZymes) in the gut microbiome. *Nature Reviews Microbiology*, *20*(9), 542–556. https://doi.org/10.1038/s41579-022-00712-1

Weber, A. N. R., Morse, M. A., & Gay, N. J. (2004). Four N-linked Glycosylation Sites in Human Toll-like Receptor 2 Cooperate to Direct Efficient Biosynthesis and Secretion *. *Journal of Biological Chemistry*, *279*(33), 34589–34594. https://doi.org/10.1074/jbc.M403830200

Whitfield, C., Szymanski, C. M., & Aebi, M. (2015). Eubacteria. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Eds.), *Essentials of Glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press. http://www.ncbi.nlm.nih.gov/books/NBK453062/

Wu, H., Rebello, O., Crost, E. H., Owen, C. D., Walpole, S., Bennati-Granier, C., Ndeh, D., Monaco, S., Hicks, T., Colvile, A., Urbanowicz, P. A., Walsh, M. A., Angulo, J., Spencer, D. I. R., & Juge, N. (2021). Fucosidases from the human gut symbiont Ruminococcus gnavus. *Cellular and Molecular Life Sciences*, *78*(2), 675–693. https://doi.org/10.1007/s00018-020-03514-x

Zhou, M., Zhu, X., Ye, S., & Zhou, B. (2014). Blocking TLR2 in vivo attenuates experimental hepatitis induced by concanavalin A in mice. *International Immunopharmacology*, *21*(1), 241–246. https://doi.org/10.1016/j.intimp.2014.04.027

Zhou, X., Yang, G., & Guan, F. (2020). Biological Functions and Analytical Strategies of Sialic Acids in Tumor. *Cells*, *9*(2), Article 2. https://doi.org/10.3390/cells9020273