Human Milk Oligosaccharides Combat Group B *Streptococcus* Infections Seamlessly from Test Tube to Mouse Model

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

May 13, 2022

Nashville, Tennessee

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Acknowledgements

Earning a Ph.D is not a solo venture for anyone; it takes an entire support system. Over my last four years at Vanderbilt, I have relied on my mentors, my colleagues, my friends, and family for their guidance throughout this journey. First, it goes without saying I need to thank my advisor, Professor Steve Townsend, for giving me the opportunity to join his lab. I will never forget the email I got from him in mid-March 2018 asking if I had chosen a school yet for my graduate studies. It was because of this personalized email that I knew not only that Vanderbilt was the school for me, but I wanted to work in his lab. Even after having troubles getting through his Structures and Mechanisms course, he took a chance on me. He believed I could make huge strides in the biology aspect of our lab, and this gave me the confidence I needed. I am thankful for his mentorship, not only in my scientific endeavors, but also in navigating being in grad school later in life. He truly has an open-door policy making it easy to stop in at any point to discuss whatever I needed.

I am very fortunate to have entered into Steve's lab as the collaboration with Professor Jennifer Gaddy was really taking off. I don't think I fully realized how amazing this collaboration would be when I joined the team. Jen is not only a close mentor, but also a friend. Her willingness to teach and extend her knowledge is unparalleled. No matter how busy she is, she is always willing to talk through the research. Her passion for science is infectious, and she makes coming into lab a joy. She has created a lab environment that is welcoming, fun, and collaborative. I am looking forward to continuing to learn from her as a postdoctoral researcher.

I have many of my colleagues to acknowledge for their mentorship that has shaped the scientist I have become. Dr. Kelly Craft was instrumental in passing on her knowledge of microbiology and navigating this joint mentorship under Steve and Jen. I am grateful that she helped paved the way

for bridging the chemistry-biology gap in our research. Dr. Schuyler Chambers has also played an important part in my training, as she took on learning the microbiology alongside me. Sabrina Spicer joined the lab in my third year, and she has become my go to person for advice in both science and as a friend. Her work ethic and positivity have driven me in so many ways. I am also thankful for Harrison Thomas who was an integral part of my Ph.D research, helping me design experiments and gather data. I also need to thank the remaining Townsend lab members, both past and present: Dr. Jamin Keith, Dr. Eric Huseman, Johny Nguyen, Lianyan Xu, Cleo Evans, Elizabeth Adams, and Alex Hughes. While my time was split between the Townsend and Gaddy labs, I always enjoyed our interactions, lab outings, and all of the "tough love" given during exam preparations. Additionally, the Gaddy lab members: Jacky Lu, Jamisha Francis, and Miriam Guevara have been instrumental in helping me understand the biology of our research. The Gaddy lab is a special group of scientists and humans I am lucky to work alongside.

Graduate school takes a toll on your mental health at times which is why I am grateful to have had a solid support system in my life to get me through these last four years. I met Lianyan and Bailey Bowser during orientation, and can say they have become my closest friends at Vanderbilt. From our late-night study sessions first semester, to our cooking nights and walks across campus, your friendship has meant so much to me. My two very best friends, Brooke Howe and Lauren Pippin have been so supportive of me, even living so far away. I have needed those phone calls, weekends away, and positivity in my life. And to Justin Medford, for coming into my life and being the support I needed during this challenging path. Thank you for letting me vent, helping me practice all my presentations, being excited for me about all my accomplishments, and of course all the fun times.

I sincerely need to thank my parents for being my biggest cheerleaders, and not even flinching when I told them at the age of 32, I wanted to get a Ph.D. They encouraged me and made sure I knew they would do anything to support me. They raised to me to follow my path and dreams, and because of that I have. A special thank you to my dad, who has read and proofread every single paper I have written (including this). I also couldn't have done this without those endless Southwest points you always seemed to have for whenever I needed a break. And to the rest of my family spread across the country, thank you for always being so proud of my accomplishments and rooting me on.

I finally need to thank Vanderbilt University for funding this opportunity, as well as my two other committee members: Professor David Cliffel and Professor Lars Plate. They have helped guide my research with their individual expertise. To my Masters advisor at San Jose State University (SJSU), Professor Laura Miller Conrad, without your push and encouragement I wouldn't be here.

My sincere appreciation goes out to all of you who have made this degree possible.

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3-FL	3-fucosyllactose
3’-SL	3’-sialyllactose
6’-SL	6'-sialyllactose
AAALAC	Association of Assessment and Accreditation of Laboratory Animal Care Act
AIP	autoinducing peptide
AMP	antimicrobial peptide
ANOVA	analysis of variance
ARA	arachidonic acid
ATCC	American Type Culture Collection
BibA	GBS immunogenic bacterial adhesin
BMI	body mass index
^{o}C	degrees Celsius
CAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
c-di-GMP	cyclic di-GMP
CIT	Vanderbilt Center for Innovative Technology
CFU	colony forming units
CPS	capsular polysaccharide
CS	Cesarean section
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid
DHPP	dihydropterin pyrophosphate
DHPS	dihydropteroate synthase

DI	deionized
DiHOME	dihydroxyoctadecanoic acid
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E	embryonic date
ELISA	enzyme-linked immunosorbent assay
EOD	early-onset disease
EPM	extraplacental gestational membrane
EpOME	epoxyoctadecanoic acid
EPS	extracellular polymeric substance
Fbs	fibrinogen-binding proteins
FBS	fetal bovine serum
FDA	Food and Drug Administration
FEG-SEM	field emission gun-scanning single electron microscopy
FIC	fractional inhibitory concentration
Fn	fibronectin
FOS	fructo-oligosaccharides
Fuc	fucose
g	gram
gal	galactose
GBS	Group B *Streptococcu*s, *Streptococcus agalactiae*
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor

GOS	galacto-oligosaccharides
H	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HILIC	hydrophilic interaction liquid chromatography
H_2O	water
HMO	human milk oligosaccharide
HvgA	GBS hypervirulent adhesin
IACUC	Institutional Animal Care and Use Committee
IAP	intrapartum antibiotic prophylaxis
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IRB	institutional review board
ISAPP	International Scientific Association for Probiotics and Prebiotics
KC	keratinocytes-derived chemokine
kEV	electronvolt
LA	linoleic acid
LDFT	lactodifucotetraose
Lmb	laminin-binding surface protein
LNFDI	lacto-*N*-difucohexaose I
LNFPI	lacto-*N*-fucopentaose I
LNFPII	lacto-*N*-fucopentaose II
LNnT	lacto-*N*-neotetraose
LNT	lacto-*N*-tetraose

LLOD	late late-onset disease
LOD	late-onset disease
MDR	multi-drug resistant
MET	macrophage extracellular trap
MIC	minimum inhibitory concentration
M	molar
mg	milligram
mL	milliliter
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MRSA	methicillin-resistant Staphylococcus aureus
MS	mass spectrometry
Neu5Ac	*N*-acetylneuraminic acid
NET	neutrophil extracellular trap
NF-κB	nuclear transcription factor
NH_3	ammonia
NH_4Cl	ammonium chloride
$(NH_4)_2CO_3$	ammonium carbonate
NH_4HCO_3	ammonium bicarbonate
NICU	the neonatal intensive care units
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
NO	nitric oxide
OD	optical density
OS	oligosaccharides

pABA	*para*-aminobenzoic acid
PBS	phosphate buffered saline
PE	phosphatidylethanolamine
PEN-STREP	penicillin G and streptomycin solution
PBP1a	surface-associate penicillin-binding protein
PbsP	plasminogen-binding surface protein
Pln	plasmin
PM	placental macrophage
PNAG	poly-*N*-acetylglucosamine
PPROM	preterm prelabor rupture of the membranes
PTB	preterm birth
qPCR	quantitative PCR
QS	quorum sensing
RPLC	reverse-phase liquid chromatography
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RU	repeating unit
SAR	structure-activity relationships
SCFA	short-chain fatty acids
ScpB	C5a peptidase
SEM	standard error of mean
SSURE	streptococcal surface repeat
ST	sequence type
TCS	two-component systems
THB	Todd-Hewitt broth

TMP	trimethoprim
TNF	tumor necrosis factor
tPA	tissue-type activators
TRIS	tris(hydroxymethyl)aminomethane
uPA	urokinase-type activators
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infections
VLBW	very low birth weight
VLOD	very late-onset disease
vol	volume
Vtn	vitronectin
W	watts
WHO	World Health Organization

Chapter 1

Group B *Streptococcus*: epidemiology, pathogenesis, prevention, and disease management

1.1. Abstract

Group B *Streptococcus* (GBS) is an opportunistic bacterium that typically colonizes the gastrointestinal and genital tracts. GBS infection during pregnancy puts the infant at risk for preterm premature rupture of the membranes (PPROM, i.e., the water breaking), severe invasive disease, or neonatal mortality. As maternal colonization is the primary route for transmission during labor and delivery, intrapartum antibiotic prophylaxis treatment (IAP) strategies are in place to reduce the risk for transmission. However, in addition to contributing to antibiotic resistance evolution, this strategy does not prevent ascending infection, or late-onset GBS infections. Here we describe the global burden of GBS infections; modes of transmission and their clinical manifestations; and current treatment and prevention tactics that are in place.

1.2 Clinical relevance of Streptococci

The *Streptococcus* genus encompasses a group of non-motile, gram-positive cocci that typically are arranged in pairs or chains. With over 50 known streptococcal species, many are normal inhabitants of the mucosal tissues in the respiratory, gastrointestinal, and genitourinary tracts.[2] However, several species have been associated with a wide range of human infections including soft tissue infections, meningitis, pneumonia, neonatal sepsis, and endocarditis.[3, 4] Streptococci are classified according to their Lancefield grouping which is based on the detection of carbohydrate antigens located on the surface of the cell wall.[5] Streptococci are also grouped by their hemolytic activity on blood agar plates. Beta (β) hemolytic species completely lyse the red blood cells, alpha (α) hemolytic species partially lyse them, and gamma (γ) hemolytic species

exhibit no hemolysis. Most *streptococcal* infections can be attributed to the β hemolytic Group A *Streptococcus* (GAS) or β hemolytic Group B *Streptococcus* (GBS). The most clinically relevant streptococcal pathogens responsible for causing severe human disease are GAS (i.e. *S. pyogenes)*, GBS (i.e. *S. agalactiae*), *S. equisimilis*, *S. pneumoniae*, and *S. viridans* (**Table 1.1**).[4]

Table 1.1. Clinically important Streptococci.

Species	Lancefield Classification	Hemolysis	Disease Outcomes
S. pyogenes	A	β	acute pharyngitis
S. agalactiae	B	β	neonatal sepsis and meningitis
S. equisimilis	C	β	endocarditis, bacteremia, pneumonia, meningitis
S. pneumoniae	none	α	pneumonia
S. viridans	none	α	endocarditis

1.3. Group B Streptococcus: an introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a β-hemolytic, gram-positive diplococcus (**Figure 1.1**). This bacterium is commonly isolated from the gastrointestinal and female reproductive tracts, with up to 40% of women carrying GBS during pregnancy.[6-9] While GBS is typically considered a member of the commensal bacterial flora, it is opportunistic in nature, causing serious invasive infections in those with weakened immune systems. Neonates are especially at risk, as GBS continues to be the leading cause of infant mortality and morbidity since it emerged in the 1970's.[10, 11]

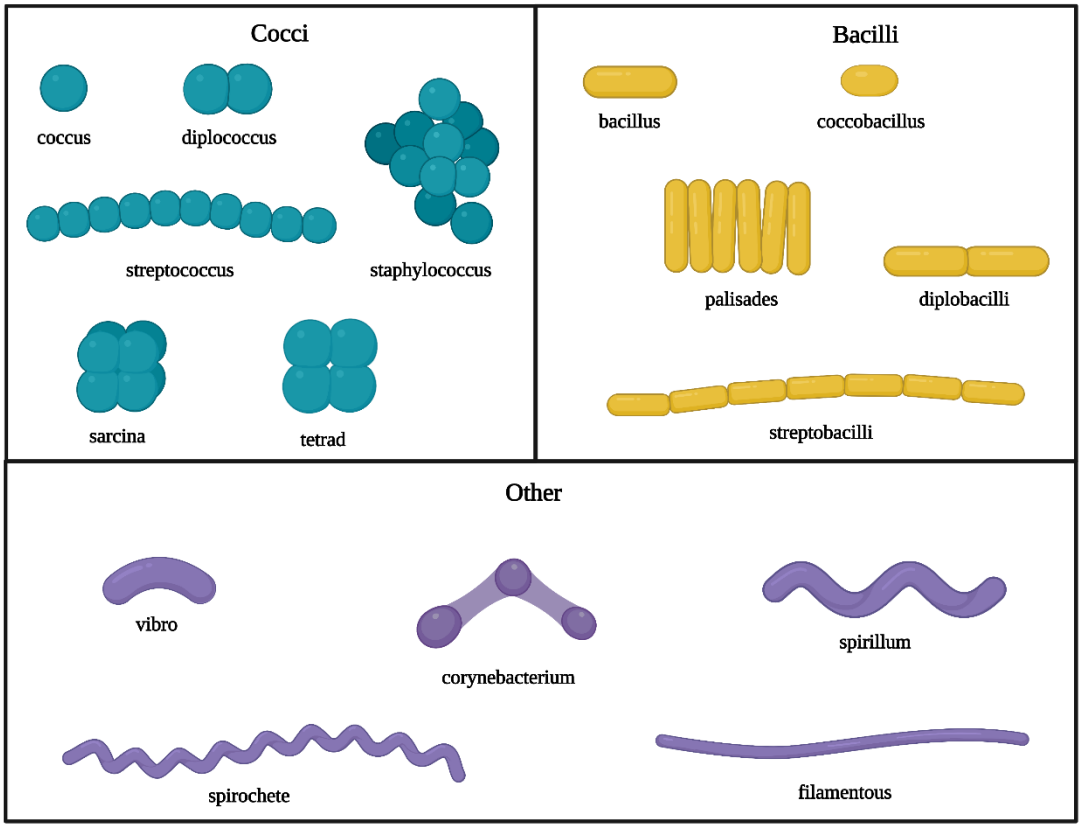

Figure 1.1. Most common types of bacteria. Group B *Streptococcus* is a diplococcus. Created with BioRender.com.

1.4. Modes of transmission

A number of maternal and neonatal risk factors have been identified for the colonization of GBS disease. Maternal risk factors include: heavy colonization ($\geq 10^6$ CFU/mL), mothers less than 20 years old, those of African or Latin American origins, and those having a history of GBS colonization in a previous pregnancy.[12-14] Primary infant risk factors include: babies delivered prematurely (less than 37 weeks gestation) and of a very low birth weight (less than 2500 g), intraamniotic infection, and prolonged rupture of the membranes.[12, 13, 15]

The most common route of GBS transmission occurs vertically either during pregnancy or parturition.[16, 17] Vertical transmission occurs when the infection is transferred from mother to neonate, while horizontal transmission is spread from environmental or nosocomial sources. The developing fetus is at risk of transmission *in utero* from a GBS colonized mother as this bacterium is known to ascend from the vagina into the placental membranes, eventually breaching through the amnionic cavity (**Figure 1.2**).[18] As a non-motile bacterium, it is not completely understood how GBS passages throughout the reproductive and gastrointestinal tracts. Additionally, nosocomial infections are often associated with infants in the neonatal intensive care units (NICU) through skin-to-skin contact from either the mother or hospital workers, or contaminated hospital equipment.[16]

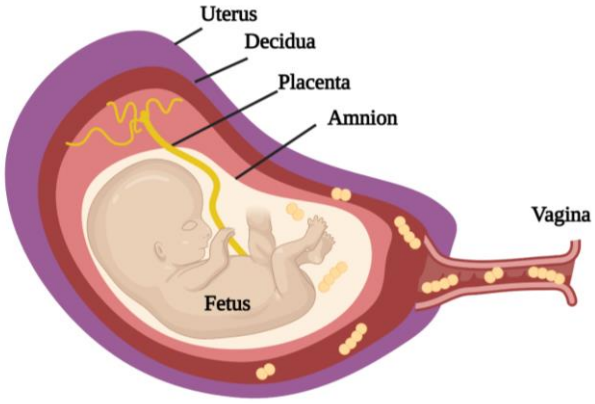

Figure 1.2. Conceptual diagram of GBS infection during pregnancy. Created with BioRender.com.

Controversially, breast milk is also a vehicle of transmission of GBS to the infant, with between 0.8 and 3.5% of mothers testing positive for GBS in their breast milk.[19-22] This is not surprising since, while it is well-known that breast milk provides essential nutritional, antimicrobial, and immunological components, it also contains over 200 bacterial species.[19, 23, 24] Indeed, breast milk from healthy, lactating women contains between10^3–10^5 CFU/mL of bacteria.[25] The most

commonly isolated species belong to the *Staphylococcus, Streptococcus, Lactobacillus, Pseudomonas, Bifidobacterium, Corynebacterium, Enterococcus, Acinetobacter, Rothia, Cutibacterium, Veillonella,* and *Bacteroides* genuses.[26, 27] There are two primary mechanisms for GBS transmission through the breast. One mechanism involves translocation from the maternal gastrointestinal tract to the mammary glands via the lymphatic system.[19] The other proposed mechanism is through retrograde flux from a colonized infant's oropharynx. The mammary ducts become infected through milk back flow, where the bacteria then multiply to higher concentrations.[19] This process can occur with or without mastitis.[19]

1.5. Clinical manifestations

Globally, over 319,000 newborns are affected by GBS every year, resulting in 57,000 stillbirths, and 90,000 infant deaths.[28] Additionally, maternal GBS colonization is largely associated with preterm birth, with up to 10% attributable to this bacterial infection.[18] Neonatal invasive GBS infections can cause meningitis, sepsis, pneumonia, and chorioamnionitis.[16, 18, 29] Complications can lead to long-term neurological and developmental impairments.

Universally, GBS colonization rates are constant amongst pregnant women; however, adverse pregnancy outcomes are much higher in low-income countries in sub-Saharan Africa and south Asia. While screening and the implementation of IAP is standard in higher income countries, this strategy is not readily available in developing countries. In an effort to decrease the global GBS burden, alternative prevention strategies must be explored including vaccine development and earlier recognition of signs and symptoms.

Table 1.2. GBS virulence factors involved colonization and infection

Virulence Factor	Mode of Action
Pore-Forming Toxins	
β-hemolysin	promotes cellular invasion and triggers host-cell lysis
cyclic adenosine monophosphate (CAMP) factor	forms pores in host-cell membrane
Host-Cell Adherence and Invasion	
fibrinogen-binding proteins (Fbs)	adheres to and enters host cells by binding to ECM fibrinogen
laminin-binding surface protein (Lmb)	adheres to host cells by binding to ECM laminin
GBS immunogenic bacterial adhesin (BibA)	adheres to host cells by binding complement regulatory protein C4bp
GBS hypervirulent adhesin (HvgA)	adheres to host cells and promotes colonization
C5a peptidase (ScpB)	mediates binding to fibronectin
plasminogen-binding surface protein (PbsP)	adheres to host cells by binding vitronectin, and plasminogen
pili	adheres to host cells
Resistance to AMPs	
surface-associate penicillin-binding protein (PBP1a)	promotes resistance
D-alanylation of lipoteichoic acid	decreases net negative charge on cell surface to repel AMPs
Other Virulence Factors	
capsular polysaccharide (CPS)	adheres to and invades host cells by binding to surface siglecs
CovR/S two-component system	Regulates hemolytic pigment expression and adherence to host cells

1.6. Virulence of GBS

As a pathogenic organism, GBS possesses a myriad of virulence factors which contribute to its ability to survive in the harsh host environment and cause invasive disease in humans. These factors are involved in host cell attachment; cell lysis; resistance to antimicrobial peptides (AMPs); evasion of host immunity; and adherence, invasion, and colonization of host cell surfaces (**Table 1.2**).

The pore-forming toxins β-hemolysin and cyclic adenosine monophosphate (CAMP) factor not only initiate cell lysis, but also facilitate intracellular invasion.[30] There are several surface adhesins that aid in host cell adherence through interactions with extracellular matrix components.[31] These include fibrinogen-binding proteins (Fbs), laminin-binding surface protein (Lmb), GBS immunogenic bacterial adhesin (BibA), GBS hypervirulent adhesin (HvgA), C5a peptidase (ScpB), plasminogen-binding surface protein (PbsP), and pili.[29, 32] Composed of three distinct proteins, the cell-wall anchored pili are crucial appendages necessary for adherence and GBS colonization. Pili additionally promote resistance to GBS AMPs through sequestration.[33] The surface-associate penicillin-binding protein (PBP1a) and reducing the membrane's net negative charge through a process of D-alanylation both contribute to AMP resistance.[33]

The sialic acid rich capsular polysaccharide (CPS) is a key virulence factor that has been well-studied and is characterized by its chemical composition and immunological response to antigens.[34, 35] To date, ten capsular serotypes have been identified, Ia (**1.4**), Ib (**1.5**), and II-IX (**1.1-1.3; 1.6-1.10**), with serotypes Ia, Ib, II, III, and V responsible for the majority of invasive GBS disease.[29, 30, 36] All CPS structures share a terminal side chain sialic acid residue that is a critical for their pathogenesis.[34] This structural feature mimics carbohydrate epitopes present on host cell surfaces which allows GBS to evade activation of the innate immune response.[37, 38]

Despite this common structural motif, CPS vary in their chain length, monosaccharide composition, and glycosidation patterns (**Figure 1.3**). All CPS structures are composed of β-D-glucose, β-D-galactose, and β-D-glucosamine, with serotype VIII uniquely containing β-L-rhamnose residues.[35, 39] Each repeating unit (RU) is four to seven monosaccharides in length; with ca. 50 to 300 RU per polymer; and connected by either β1→2, β1→3, β1→4, β1→6, α2→3, linkages.[34, 35]

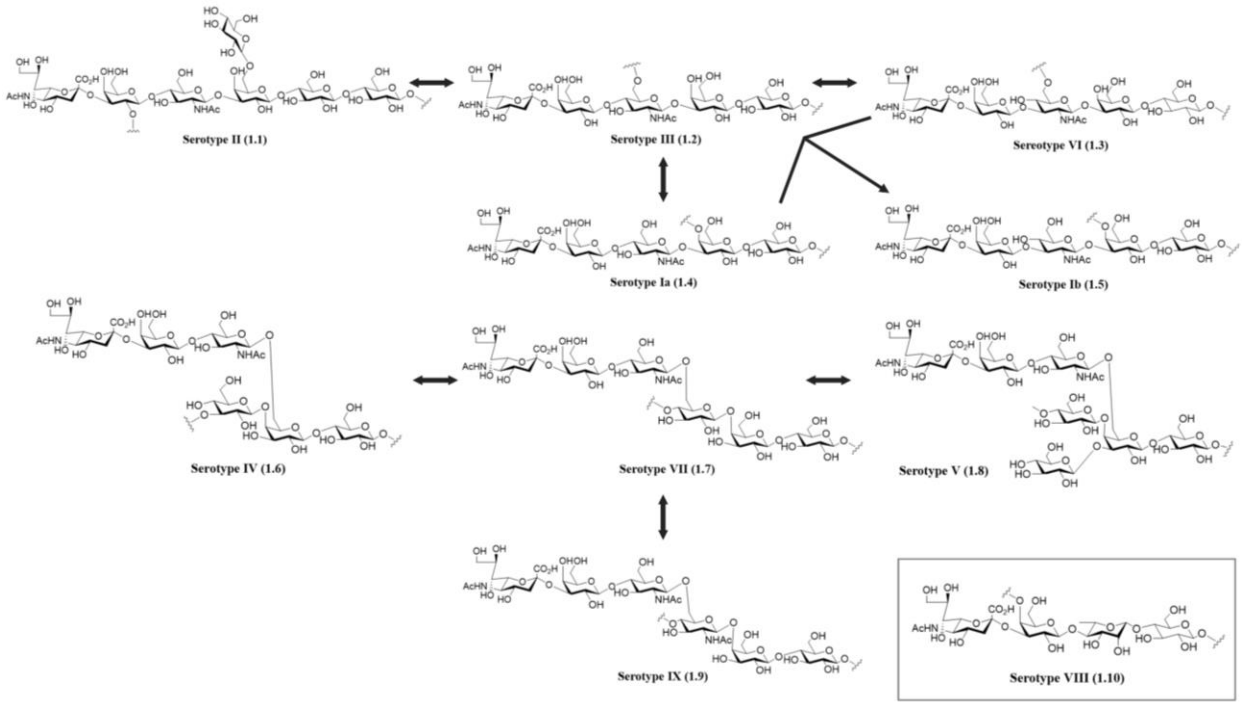

Figure 1.3. Chemical structures of the repeating units of Group B *Streptococcus* capsular polysaccharides. The repeating units are grouped according to structural and chemical similarities.[1]

Bacterial biofilms will be described in greater detail in Chapter 3, but a brief introduction to this well-known virulence factor is provided here. The ability of bacteria to colonize and persist within a host species is often dependent on the formation of biofilms. These three-dimensional structures are encapsulated by a self-produced extracellular matrix which allows them to adhere

to and invade host cells.[40-42] Bacterial biofilm infections are often implicated in morbidity and mortality due to their persistence in chronic infections.

In order for bacterial communities to survive in a wide range of environments, they must be able to sense and adapt to fluctuating conditions. This process is often carried out using two-component systems (TCS) which are known to coordinate virulence factor expression. TCSs consist of a membrane-bound sensor histidine kinase and a cytoplasmic response regulator. A variety of external signals are recognized including pH, osmolarity, availability of nutrients, antibiotic pressure, light, and temperature.[43-45] In response to the input signal, autophosphorylation occurs at a conserved active site histidine residue which is then transferred to the conserved aspartate residue on the response regulator (**Figure 1.4**).[45] With over 22 TCS identified in GBS to date, the CovR/S is one of the most well-studied with respect to its control of gene expression.[46] The CovR/S system has demonstrated to control several regulatory functions including cell envelope processes, cell membrane permeability, metabolism, and transportation of key molecules.[47] Due to its crucial role in the pathogenesis of GBS disease, the CovR/S system has been identified as a potential drug target.[48]

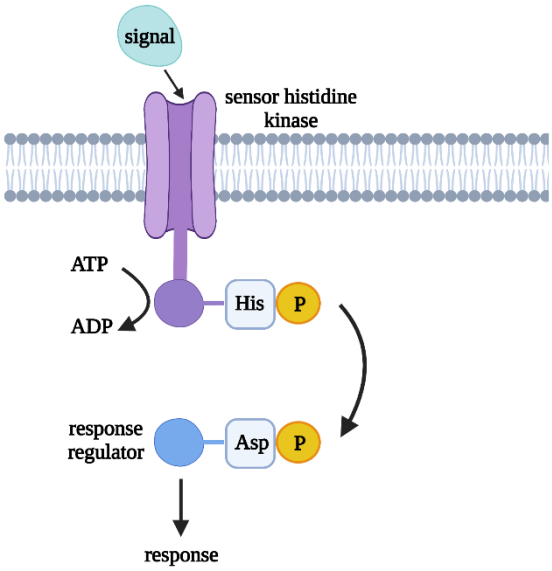

Figure 1.4. Model of a generalized two-component system signal transduction. Created with BioRender.com.

1.7 Types of GBS disease in infants

GBS infections in newborns are classified into three distinct clinical manifestations (**Table 1.3**). Early-onset disease (EOD) occurs within the first six days of life, late-onset disease (LOD) occurs between days 7 to 89 post parturition, and very late-onset disease (VLOD) occurs in babies over three months in age.

Table 1.3. Comparison of GBS infections

	Early-Onset Disease (EOD)	**Late-Onset Disease (LOD)**	**Late Late-Onset Disease (LLOD)**
Age	< 7 days old	7 days – 3 months	> 3 months
% of cases	60 – 70%	~30%	rare
Mode of Transmission	vertically from the mother during delivery through the birth canal	vertically, horizontally from hospital environment, or contaminated breast milk	horizontally from the environment or caretakers
Manifestations	Sepsis, pneumonia, meningitis	Meningitis, sepsis, bacteremia	Meningitis, sepsis, bacteremia
Mortality Rate	5 – 20%	~5%	low

1.7.1 Early onset disease (EOD)

EOD infections typically present within the first 24 hours as a result of vertical transmission from mother to infant during labor or following rupture of membranes.[13] Accounting for 60-70% of GBS disease in infants, serotypes 1a, III, and V predominate in these infections.[19, 49] The primary risks associated with EOD are pneumonia, bacteremia, sepsis, and meningitis, with mortality rates up to 20%.[50]

1.7.2 Late onset disease (LOD) and Very Late onset disease (VLOD)

While it is relatively well-known how EOD is transmitted to the infant, LOD infections can be acquired from a number of sources. Horizontal transmission from the mother or from caregivers, and exposure to infected surfaces during prolonged hospital stays are common routes of acquisition.[51, 52] Contaminated breast milk is also considered a source of early GBS transmission.[53, 54] Even though LOD infections are not as common as EOD, their manifestations can be just as deadly. Serotype III strains are most often implicated in LOD with bacterial meningitis being the primary risk factor presenting itself in up to 50% of these cases.[55] Serotype III is often found in neonatal invasive disease not only because these strains comprise ca. 20% of all isolates, but can be attributed to poor maternal CPS-specific immune response.[56, 57] Additional manifestations of LOD include bacteremia septicemia, cellulitis, osteomyelitis, and lymphadenitis.[52, 58] Babies born premature, at less than 37 weeks gestation are more susceptible for acquiring LOD infections.

VLOD, otherwise known as late-late-onset disease is more uncommon than both EOD and LOD. However, it still presents itself with similar manifestations and outcomes. Premature infants who have experienced prolonged hospital stays are most at risk for VLOD through horizontal transmission.

1.8 Prevention and treatment strategies

Vaccine administration to pregnant women could provide effective protection against GBS infection; however, research and design has proven challenging. The majority of GBS vaccines were developed to target the capsule, but due to serotype variability, capsular switching, and low

immunogenicity, these designs show limited efficacy.[18, 59] Currently, antibiotic treatment to GBS positive women is the only viable treatment option.

1.8.1 Intrapartum antibiotic prophylaxis during labor and delivery

Current guidelines for the prevention of EOD in newborns recommend pregnant women are vaginally/rectally screened for GBS colonization between 36 and 37 weeks gestation.[60] All women whose cultures are positive for GBS are encouraged to receive appropriate IAP at the onset of labor. The treatment course is dependent on antibiotic susceptibility of the specific strain as well as maternal allergies (**Figure 1.5**). β-lactams, specifically penicillin (**1.16**) and ampicillin (**1.17**), remain the preferred antibiotics. They have a narrow spectrum of antimicrobial activity against GBS leaving them less likely to acquire antimicrobial resistance.[10] However, with ca. 10% of women allergic to penicillin, alternative treatment options must be made available.[61] For penicillin allergic women who are low risk for anaphylaxis are recommended to take a first-generation cephalosporin, cefazolin (**1.18**).[62] For those who are high risk for anaphylaxis, clindamycin (**1.19**) can be given as an alternative if the GBS isolate has known susceptibility.[63] Erythromycin (**1.12**) is an excellent example of a failed antibiotic in the battle against GBS. In the early days of IAP, erythromycin was recommended as an alternative to β-lactam antibiotics. However, due to the evolution of resistance in 44% of strains, macrolides are no longer used as a treatment option.[50] Vancomycin (**1.20**) is given as a last-resort antibiotic to women who are at high risk for anaphylaxis and whose GBS isolate is clindamycin resistant.[50]

1.8.2 Traditional antimicrobial treatments and their pitfalls

Since the implementation of universal screening and IAP administration, incidences of GBS EOD have decreased significantly since the 1990's by about 80%.[64] During this same time period, incidences of GBS LOD have remained relatively constant, noting that IAP does not prevent these later infections.[64] IAP is also ineffective against GBS disease associated with preterm births including stillbirths and miscarriages.[65] For women who have PPROM, it is still possible to initiate IAP if she is swabbed for GBS culture immediately. If she is at low risk for anaphylaxis, and has a history of positive GBS colonization she can be given penicillin treatment while awaiting results.

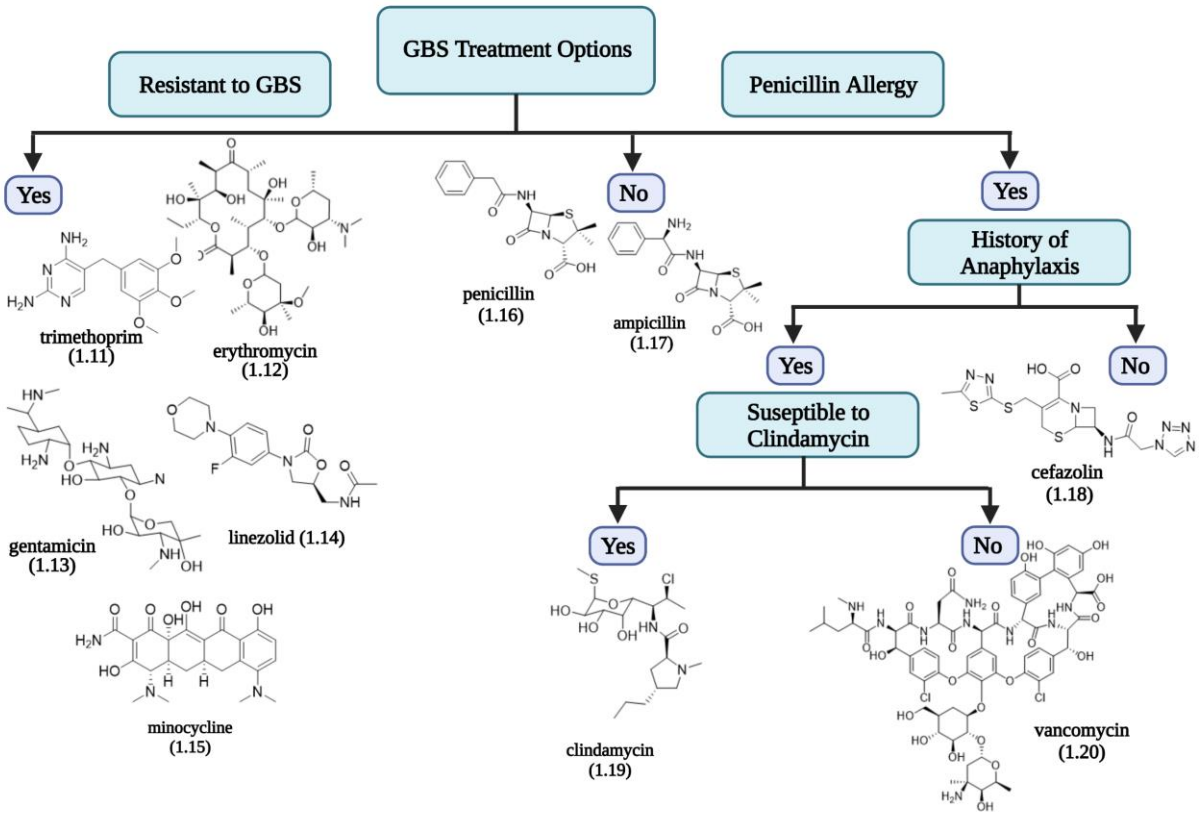

Figure 1.5. Guidelines for intrapartum antibiotic prophylaxis (IAP) for treatment of Group B *Streptococcus* (GBS) infections during labor and delivery.

With more than 30% of babies who are born vaginally exposed to IAP, there are a number of concerns that must be considered in addition to the risks of anaphylaxis mentioned above.[66] The emergence of antibiotic resistance among strains of GBS and other pathogens is heightened upon

increased exposure.[67] IAP has also been associated with adverse effects to the developing infant flora. Even with the downfalls of IAP administration, this course of treatment remains the optimal therapeutic for reducing the risk of infant morbidity and mortality related to GBS infection.

1.9 Purpose of dissertation

Group B *Streptococcus* (GBS) continues to threaten neonatal health as it is associated with preterm birth, sepsis, meningitis, and stillbirth. With the rise in antibiotic resistance and the accompanying disruption to the infant microbiome, alternative treatments are necessary for treating these GBS infections. Additionally, biofilms are an essential virulence factor linked to the pathogenesis of opportunistic bacteria, contributing to multidrug resistance. This dissertation focuses on how human milk oligosaccharides (HMOs) isolated from breast milk can be harnessed to combat pathogenic infections as a novel therapeutic option. As an antibiotic adjuvant, we utilized HMOs to potentiate the activity of select antibiotics against GBS. We not only focused on inhibiting growth and biofilm *in vitro*, but explore the antiadhesive properties of HMOs against *ex vivo* gestational tissues, and the prevention of ascending infection and adverse pregnancy outcomes in our *in vivo* mouse model. We concurrently took an interest in the prebiotic properties of HMOs and how these carbohydrates modulate the gut microbiota selectively promoting the growth of commensals over pathogens. We sought to gain understanding on how carbohydrate metabolism affects commensal-pathogen interactions in the developing microbiome. Together, these results will help us gain a better understanding of how breast milk can not only be used as a source of nutrition, but as an innovative treatment course against and prevention of GBS infections.

1.10 References

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Chapter 2

Human Milk Oligosaccharides: combatting mechanisms of antibiotic resistance through adjuvant therapy against Group B *Streptococcus*

2.1 Abstract

Human milk oligosaccharides, a group of complex sugars only present in breast milk, possess potent antimicrobial activity and potentiate the activity of select antibiotics against GBS. Previously, we hypothesized HMOs permeabilize the cell membrane. This chapter describes the Townsend group's early efforts in HMO research and the basis for their utilization as antibiotic adjuvants alongside aminoglycosides, lincosamides, macrolides, and tetracyclines. We further discuss the powerful activity of HMOs in repurposing antifolate drugs, a class of drugs with known resistance against GBS. The culmination of this study was a validation of the hypothesis that HMOs permeabilize the bacterial cell membrane through untargeted metabolomic analyses. In this section of the study, we discovered that metabolites affiliated with cell membrane structure and function were perturbed by HMO treatment.

2.2 A brief review on infant feeding methods

While there are many factors that contribute to the decision on whether to breastfeed or formula feed a baby, most health experts including the American Academy of Pediatrics, recommend exclusive breast feeding for the first six months of life.[1] As solid foods are introduced, breast feeding should still be continued for at least the first year to provide the child with the best possible nutrition and protection against infection while training the child's immune system. According to the CDC, about 83% of babies will be breastfed immediately following birth, but at 3 months of age, that number decreases to 47%.[2] By 12 months of age, just 36% of babies are

still breastfeeding.[2] Most mothers start out breastfeeding because they are aware of the many benefits it provides to both mother and child. However, there are a number of factors that lead to formula supplementation. For some mothers, it is not possible to breastfeed due to low milk production, medications, or underlying health conditions.[3] In addition, some babies have breast milk allergies or refuse to latch onto the nipple.[3] While pumping is a feasible option, the time investment for women is another contributor for formula introduction as mothers go back to work and can no longer devote the time and energy required to breastfeed.[3] And while it is universally agreed that breast milk should still be the first choice to meet the nutritional needs of the baby through at least the first 6 months of life, formula manufacturers have advanced the formula contents to a level where it is a safe and healthy alternative when necessary.[1]

Breast feeding is strongly endorsed by health organizations, doctors, and even formula producing companies themselves. Even though most infants are being fed milk substitutes by the age of 6 months, breast milk remains a superior source of nourishment. Formula design is meant to duplicate the mother's milk. However, formula manufacturers are still unable to match the complexity of mother's breast milk.[4] Furthermore, breast milk is constantly changing along with the needs of the baby, and it provides protective antibiotics and bioactive components that cannot to be added to formula. Bovine milk and soy milk are the two most common bases for infant formula. The FDA regulates formula composition for precise quantities of proteins, fats, carbohydrates, vitamins, and minerals to ensure proper nutrition.[4] Additionally, iron fortification of formula is recommended for the prevention of anemia.

Human milk oligosaccharides (HMOs) are abundant in breast milk (see Section 2.4 and 2.5). While it is not synthetically or financially feasible to include all the known HMOs found in breast milk, many formula manufacturing companies supplement their formula with prebiotics to

mimic the beneficial properties the HMOs provide. With over 200 known structures, both chemical and enzymatic techniques have recently been investigated to synthesize HMOs in mass production, but many challenges still remain.[5, 6] Prebiotics in formula are designed to stimulate the growth of beneficial bacterial species including *Bifidobacterium* and *Lactobacillus*. The most commonly supplemented oligosaccharides (OS) are short-chain galacto-OS (GOS), long-chain fructo-OS (FOS) and polydextrose.[7, 8] However, it is important to recognize that none of these are actually found in breast milk.

2.3 Breast milk composition and its beneficial biomolecules

Colostrum is the first milk produced, developing during pregnancy and lasting until about 5 days after parturition.[9] This thick and yellowish liquid is nutrient rich in protective factors, antibodies, and proteins, and low in fats making it easier for a newborn to digest. Once the breast milk fully transitions into mature milk, the nutritional content remains relatively constant throughout lactation. As the sole source of hydration for the infant, ca. 87% of breast milk is water (**Figure 2.1A**).[4] The remaining macromolecular components in the average milk supply include 3-5% lipids, 1% proteins, and 7% carbohydrates (**Figure 2.1B**).[10]

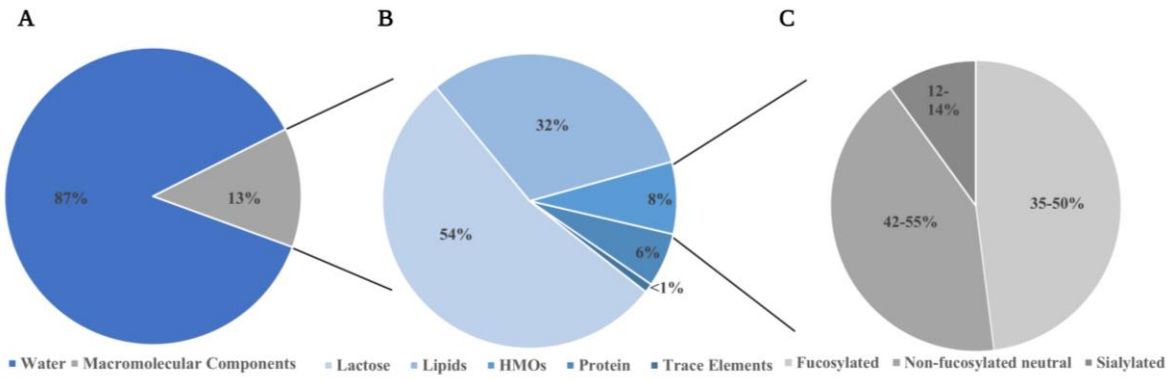

Figure 2.1. Average human breast milk composition displaying A) liquid and macromolecular components, B) the macromolecular component breakdown, and C) relative HMO composition.

Comprising up to 54% of the solid components found in human milk, the disaccharide lactose is the primary carbohydrate present.[11] Lactose can be broken down into the two monosaccharides glucose and galactose. Lactose provides an important source of energy for the baby, providing ca. 40% of the total calories.[4] Glucose provides the energy necessary for growth and development, while galactose is the major contributor for central nervous system development. Oligosaccharides are the third largest solid component of breast milk. These are virtually unique to humans and are only found in trace amounts in other mammals.

Human milk lipids are the largest source of calories for infants accounting for an average of 44% of the total energy requirements during the first six months of life.[12] In addition to providing energy requirements, lipids are a carrier of essential fat-soluble vitamins and the bioactive triacylglycerides, diacylglycerides, saturated and polyunsaturated fatty acids, and phospholipids.[13] Triglycerides are the main lipid component found in breast milk accounting for 98% of total milk fat and are used for the storage of energy.[12, 14] The remaining 2% of lipids that provide key functions are cholesterol, docosahexaenoic acid, arachidonic acid, and other complex lipids such as phospholipids, plasmalogens, and sphingolipids.[12, 14]

Fat content is the most variable macronutrient, not only across the duration of lactation, but also during each individual breastfeeding session. The first milk that is released from the breast, foremilk, is mostly water, and is lower in calories and fat content. As the breast empties, the fat content progressively increases until it reaches its peak in the hindmilk.

There over 400 proteins in human milk. These proteins can be divided into three categories: casein, whey, and mucin.[14] Mucins are heavily *O*-glycosylated, linear glycoproteins. They are one of the outer membrane components of the human milk fat globule, making up only a small percentage of the total protein content. Whey proteins are in liquid form and are easier to digest

than the larger, complex casein protein molecules. The whey to casein ratio in the early colostrum is as high as 90:10, the ratio drops in transition milk to 65:35, and mature milk stabilizes to about 60:40 throughout lactation.[15] The proteins found in the highest concentrations are casein, α-lactalbumin, lactoferrin, immunoglobulin IgA, lysozyme and serum albumin. Proteins in breast milk provide a wide array of functions including serving as an important source of amino acids necessary for growth and development; protection against bacterial infections; absorption of both micro and macronutrients; and shaping the immature microbiome.

The iron-binding glycoprotein lactoferrin is one of the most abundant proteins found in breast milk, comprising of 15 to 20% of total protein content.[16] The antimicrobial properties of lactoferrin against harmful pathogens arise from its ability to sequester iron thus rendering it unavailable for the bacteria to proliferate.[17] Lactoferrin also possesses anti-inflammatory and immunomodulatory properties necessary for the developing gastrointestinal tract of infants.[18, 19]

Another major whey protein encompassing 20 to 25% of the total protein content is α-lactalbumin which binds Ca2+ and Zn2+ ions driving the absorption of essential minerals.[17] In the mammary gland, α-lactalbumin is necessary for milk production as it forms a key intermediate lactose synthase complex. In addition to its bactericidal properties, it most importantly provides a rich source of essential amino acids including tryptophan, lysine, cysteine, leucine, isoleucine, and valine.

2.4 Discovery of human milk oligosaccharides and early studies

Since the late 18th century, it has been clear that babies who are breastfed have overall improved health and protection against infectious disease when compared to formula-fed babies. During the early 1900's mortality rates were as high as 30%; however, it was observed the rates were up

to seven times lower in breastfed babies.[20] Furthermore, there were lower incidences of infectious diarrhea. The initial discovery of HMOs was driven by the Austrian pediatrician and microbiologist, Theodor Escherich, who in 1886 published his research on the relationship between intestinal bacteria and the physiology of digestion in infants.[20, 21] Following this discovery, Ernst Moro, who studied under Escherich, and Henri Tissier, a graduate student at the Pasteur Institute in Paris, independently compared the microbial composition of the feces of breastfed to formula fed babies. Concurrently, the chemist Eschbach, was the first to discover that human milk contained a different kind of lactose than bovine milk and concluded that bovine lactose was more homogenous than human milk.[22, 23] Following this discovery, the chemist Deniges determined that while both bovine and human milk both contained the same type of lactose, human milk possesses an unidentified carbohydrate component.[20, 23] It wasn't until the early 1930's that Michel Polonowski and Albert Lespagnol, two French scientists, discovered a method to identify these unknown carbohydrates that they named "gynolactose".[24, 25] This "gynolactose" component which in addition to being insoluble in methanol was also not homogeneous with nitrogen and hexosamines essential components. Two decades later, Polonowski and Jean Montreui, an early pioneer studying carbohydrates and glycoconjugates, used two-dimensional chromatography to identify the first two HMOs, 2'-fucosyllactose (2'-FL) and 3-fucosyllactose (3-FL).[26]

In 1926, Herbert Schönfeld described a thermoresistant, growth-promoting factor for *Bifidobacterium bifidus* (originally classified as *Lactobaciullus bifidus*) which was termed the "bifidus factor" in the whey fraction of human milk. Schönfeld concluded this "bifidus factor" was a vitamin.[20, 27] However, the chemist Richard Kuhn and the pediatrician Paul György provided a connection between the intestinal bacteria research and "gynolactose". This

connection proved the "bifidus factor" was actually the human milk oligosaccharides.[23, 27, 28] Following this discovery, Montreuil and Kuhn identified and characterized several additional HMOs including the previously isolated 2'-FL and 3-FL.[20, 22]

In the last decade, researchers have examined the effects of HMOs on modulating the outcome of infectious diseases. In separate studies by the Le Doare and Bode groups, they both demonstrated the ability of HMOs to directly inhibit the growth of GBS both *in vitro* and *in vivo*.[29-31] In 2016, Le Doare and coworkers found a correlation between the mother's Lewis secretor status and GBS colonization in infants since HMO expression is related to Lewis blood type.[31] Interestingly, maternal Lewis secretor status has also been shown to shape infant gut microbiota; a result likely shaped by HMO presence or absence. The predominant HMO in secretor milk samples were 2'-FL and lacto-*N*-fucopentaose I (LNFPI), whereas non-secretor milk was characterized by lacto-*N*-fucopentaose II (LNFPII) and lacto-*N*-difucohexaose II (LNDFII). Differences in microbiota composition and quantity were found depending on secretor/non-secretor status. For example: *Lactobacillus spp*, *Enterococcus spp*, and *Streptococcus spp* were lower in non-secretor than secretor samples. *Bifidobacterium* were less prevalent in non-secretor samples compared to secretor samples. Despite no differences in diversity and richness, non-secretor samples had lower *Actinobacteria* and higher relative abundance of *Enterobacteriaceae*, *Lactobacillaceae*, and *Staphylococcaceae*.[32] There are four human milk groups defined: Lewis-positive Secretors and non-Secretors, and Lewis-negative Secretors and non-Secretors. Using ^{1}H nuclear magnetic resonance (NMR) spectroscopy methods, Le Doare and coworkers were able to assign a milk group based on the type of fucosylated HMO residues present in the milk sample.[31] Secretors were identified by those ^{1}H NMR spectra containing 2'-FL and other similar fucosylated HMOs. Lewis mothers were classified by ^{1}H NMR spectra comprised of peaks

equivalent to LNDFHI and LNDFHII. In their study they found that babies born to Lewis-positive mothers were less likely to acquire GBS infections as well as more likely to clear infection up to 90 days after birth when compared to infants born to Lewis-negative women. However, Le Doare et al. were not able to find a correlation between Secretor status and incidences of GBS colonization in either mother or baby. In addition to the *in vivo* work, they found that the presence of LNDFHI, a branched fucosylated HMO in the mother's milk sample was linked to an inhibition of GBS growth.[31]

In 2017, Bode and coworkers discovered that pooled samples of HMO extracts directly inhibited the growth of GBS in a dose-dependent manner.[30] This discovery was preceded by a study in which the Bode lab uncovered that HMOs provide protection to bladder epithelial cells through preventing the colonization of uropathogenic *Escherichia coli* (UPEC), the primary pathogen responsible for urinary tract infections.[33] In addition to pooled HMOs, Bode et al. also fractionated the HMOs into sialylated, acidic HMOs and non-sialylated, neutral HMOs using multidimensional chromatography. The Bode Group concluded that while the sialylated HMOs did not inhibit GBS growth, the neutral, non-sialylated HMO moieties provided narrow spectrum bacteriostatic activity against GBS growth. Specifically, lacto-*N*-tetraose (LNT) and the fucosylated LNFPI both inhibited GBS growth, while the isomer of LNT, lacto-*N*-neotetraose (LNnT) surprisingly did not. After expanding their studies to other species of bacteria including UPEC, *Streptococcus pyogenes* (Group A *Streptococcus*) *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, they found that HMOs did not inhibit the growth of these pathogens

2.5 HMO composition and biosynthesis

These structurally complex glycans are derived from only five basic monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and *N*-acetylneuraminic

acid (Neu5Ac) which range from 3 to 22 sugars in length (**Figure 2.2A**). HMO biosynthesis commences in the Golgi apparatus of mammary epithelial cells. At their reducing end, all HMOs contain a lactose core. Here, the glycosidic bond is catalyzed by the enzyme β-galactotransferase in association with α-lactalbumin.[5] Lactose can be elongated enzymatically by ß1-3 or ß1-6 linkages to Gal with either lacto-*N*-biose (Galβ1-3GlcNAc) or *N*-acetyllactosamine (Galβ1-4GlcNAc), respectively (**Figure 2.2B**). Lacto-*N*-biose typically terminates the chain, while *N*-acetyllactosamine further extends the chain by β1-3 and β1-6 linkages. Chain branching is introduced through β1-6 linkages and are labeled as *iso*-HMOs, while β1-3 linked linear chains are designated as *para*-HMOs. The HMO structure can subsequently undergo fucosylation with α1-2, α1-3 or α1-4 linkages or sialylation α2-3 or α2-6 linkages which is mediated by fucosyltransferases and sialyltransferases. The composition of HMOs varies significantly between mothers and over the course of lactation, however, typically, 35–50% are fucosylated, 12–14% are sialylated and 42–55% are non-fucosylated neutral HMOs (**Figure 2.1C**; **Figure 2.2C-E**).[34-36] To date, over 200 unique structures have been isolated and identified using liquid chromatography followed by mass spectrometry.[5]

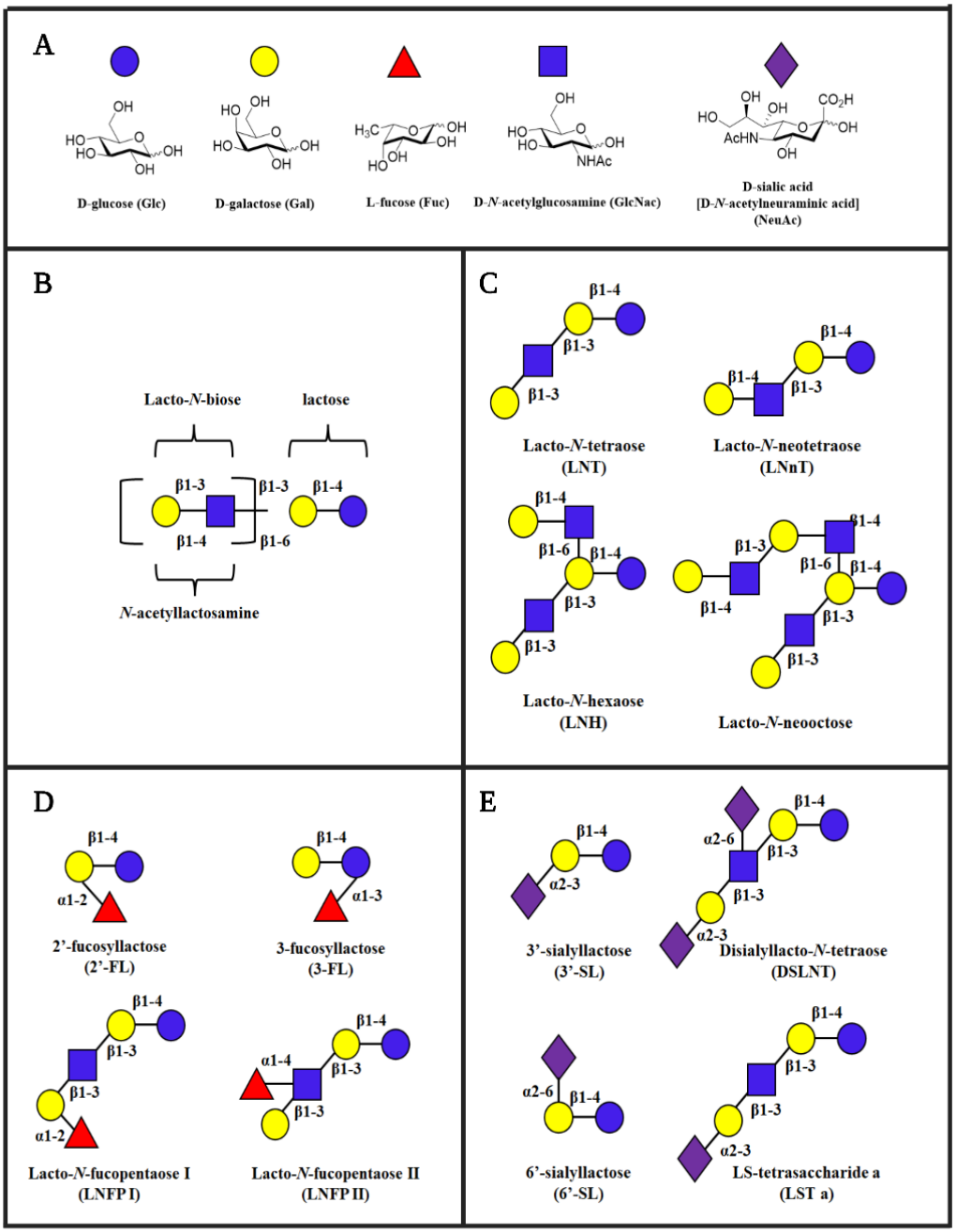

Figure 2.2. Basic HMO blueprint and representative HMOs. A) The five monosaccharides building blocks and their corresponding symbols. B) The structural blueprint for HMO biosynthesis. C) Selected non-fucosylated neutral HMOs. D) Selected fucosylated HMOs. E) Selected sialylated HMOs.

2.6 Biological activity of HMOs

2.6.1 HMOs as prebiotics

The definition of prebiotic has evolved over the last several decades as there is now a greater understanding of their mechanism of action and the health benefits they confer. Initially, prebiotics were defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health", with a focus on bifidobacteria and lactobacilli.[37] Most recently, the International Scientific Association for Probiotics and Prebiotics (ISAPP) updated the definition to "a substrate that is selectively utilized by host microorganisms conferring a health benefit."[38] The majority of prebiotics have a carbohydrate component and are found in high concentrations in foods such as bananas, legumes, beans, oats, garlic, and chicory root. In terms of development of the infant microflora, HMOs act as a powerful prebiotic. These non-digestible dietary fibers are able to resist gastric acidity and enzymatic hydrolysis, and gastrointestinal absorption. With only ca. 1% of HMOs absorbed into systemic circulation, the majority reach the distal small intestine and colon intact where they can be metabolized by the gut microbiota or excreted in the urine and feces.[22, 29, 39]

Compared to formula fed infants, bifidobacterial strains are of the highest abundance in breast fed infants. This is attributed to their ability to preferentially metabolize and feed on prebiotic HMOs.[38, 40] In addition to *B. bifidus*, *B. breve*, *B. longum*, *B. infantis*, and *B. pseudocatenulatum* are most commonly isolated from breast fed infants.[40] HMO degradation by *Bifidobacterium* spp. is mediated by either the adenosine triphosphate binding cassette transporter, carbohydrate binding proteins, or the cell-wall anchored glycosyl hydrolases.[41] *Bacteroides* spp. are also found in high concentrations as they are able to easily break down and metabolize several common

HMOs including 2'-FL, 3-FL, 3'-sialyllactose (3'-SL), 6'-siallylactose (6'-SL), and lactodifucotetraose (LDFT).[42] Mechanistically, *Bacteroides* spp. utilize extracellular enzymes to break down HMOs into simpler glycans which allows for transport into the cell where they can be further digested intracellularly.[43]

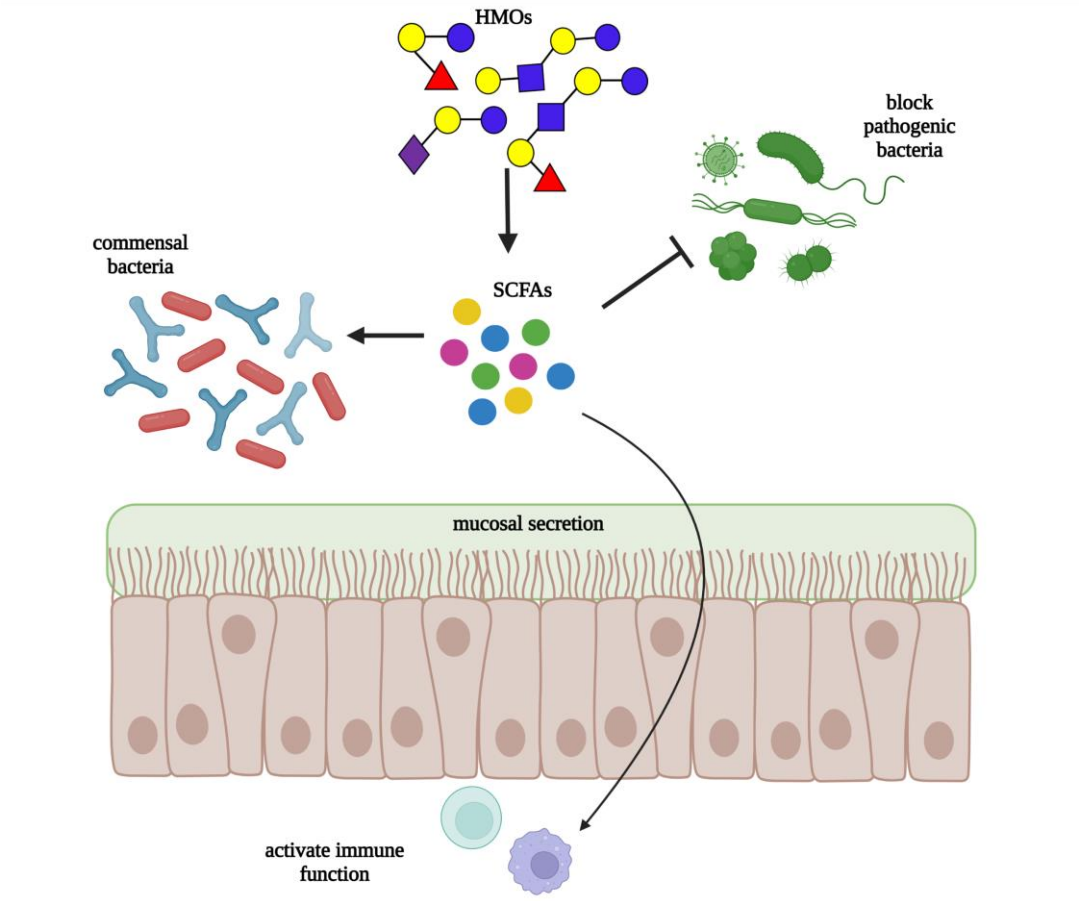

Figure 2.3. The effects of SCFA production as a result of HMO fermentation.

Short-chain fatty acids (SCFAs) are produced as the end product of bacterial fermentation of undigested HMOs. SCFAs stimulate mucus and antimicrobial peptide production, and lower the gastrointestinal pH which selectively allows for growth of beneficial commensals over pathogenic bacteria (**Figure 2.3**). SCFAs, notably acetic acid, butyric acid, and propionic acid, are an important energy source for intestinal epithelial cells which is necessary for establishing a

healthy gut microbiome. Additionally, SCFAs have been associated with and have been shown to play an important role in the activation of the immune and inflammatory responses.[44, 45]

2.6.2 HMOs as antiadhesives

The beneficial effects of HMOs go beyond the competitive advantage they provide to nonpathogenic commensals. They additionally have demonstrated they can prevent pathogenic adhesion to intestinal epithelial cells by acting as a soluble decoy receptor. Pathogenic invasion of bacteria, viruses, fungi, and parasites first begins with adherence to the epithelial surface, followed by colonization and proliferation of the infectious disease (**Figure 2.4A**). There are two mechanisms in which HMOs prevent pathogenic colonization. Microbes will either directly bind to HMOs that resemble cell surface glycans (**Figure 2.4B**) or HMOs will cause a conformational change in the receptor site through binding directly to gut epithelial cells (**Figure 2.4C**).[40, 46, 47]

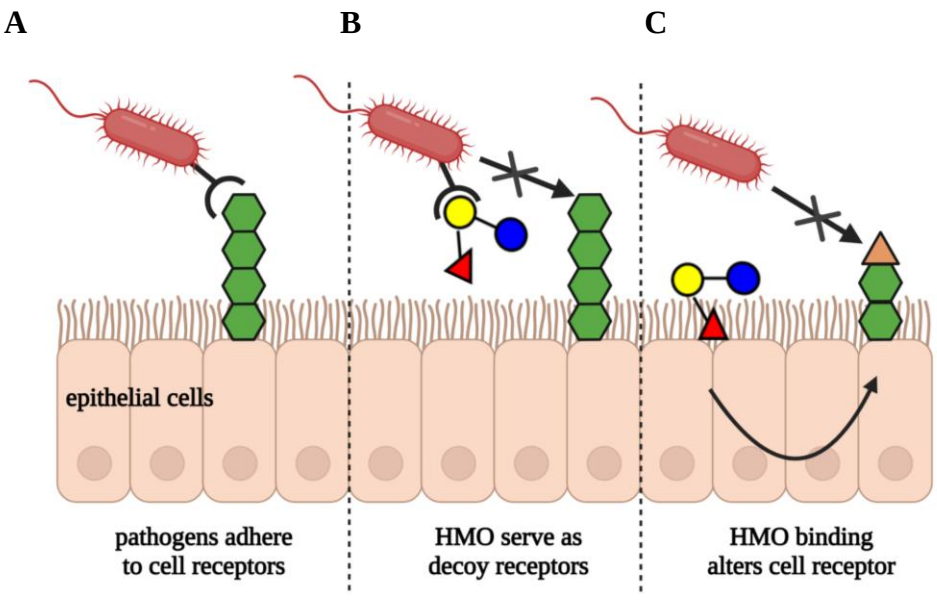

Figure 2.4. Mechanisms in which HMOs prevent adhesion of bacteria to epithelial cells surfaces. A) in the absence of HMOs, pathogens bind directly to cell surface glycan receptors. B) HMOs prevent adhesion of pathogens by resembling cell surface glycans. C) HMOs can bind directly to the epithelial cell surface, causing a conformational change in the receptor. Created with BioRender.com.

2.7 Antimicrobial properties of heterogeneous HMOs against GBS: previous studies

Following the results of the Le Doare and Bode groups, our interdisciplinary team focused on the synthesis and analysis of HMOs in infectious disease. Initial research initiated by Dr. Kelly Craft and Dr. Dorothy Ackerman was based on the hypothesis that HMOs possess both antimicrobial and antibiofilm activities against GBS. Bacterial growth was quantified employing a plate-based assay using spectrophotometric techniques measuring optical density at 600 nm (OD_{600}) over a period of 24 hours. In this assay, a heterogenous mixture of HMOs pooled from 14 donors was found to possess bacteriostatic activity against GBS, inhibiting growth up to 89% (**Table 2.1**).[48] The next steps following the elucidation of this impressive antimicrobial activity involved uncovering the most active single-entity HMOs present in the mixture. As expected, no single-entity HMOs were as potent as the pooled mixture with the understanding there are likely multiple mechanisms responsible for this inhibition.[49, 50]

Table 2.1. Pooled HMO inhibition across varying serotypes of GBS.

Strain	Serotype	MIC[a]	IC_{50} Values[a]	IC_{25} Values[a]
GB2	Ia	2.56	1.897	1.43
GB651	IB	5.12	2.616	2.33
GB653	II	5.12	2.741	2.64
GB590	III	5.12	2.417	2.30
GB83	IV	5.12	3.616	3.51
GB37	V	5.12	2.309	2.21
10/84	V	5.12	2.054	1.42

[a]all values in mg/mL

2.8 HMOs as an antibiotic adjuvant

Currently, antibiotics are the only recommended treatment for GBS infections in Western countries (see Section 1.8.1).[51] However, due to the rise in antimicrobial resistance and the adverse effects associated with the microbiome (see Section 1.8.2), novel therapeutic strategies are needed to combat these pitfalls. One promising approach involves combination treatments in

which either two or more antimicrobials are prescribed concurrently, or a non-antimicrobial agent is used alongside an antibiotic. Combination treatments often possess multiple mechanisms of action which can be used to synergistically potentiate and improve the utility of our current arsenal of antibiotics.

2.8.1 Previous studies

In a first-generation study of combination therapy of HMOs with select antibiotics, the HMOs were found to help potentiate the activity of a variety of antibiotics, including ribosomal targeting drugs and decrease the MICs of these drugs by up to 32-fold (**Table 2.2**).[52] To determine if there was strain specificity, the HMOs were assayed against three strains of GBS across various serotypes: GB2 (serotype Ia), GB590 (serotype III), and CNCTC 10/84 (serotype V). While a panel of antibiotics was chosen, interestingly β-lactams and glycopeptide antibiotics which inhibit cell-wall synthesis exhibited much more subtle effects compared to the ribosomal-targeting drugs in conditions of HMO supplementation. The ribosomal targeting antibiotics in this study were three antibiotics towards which GBS has evolved resistance. They were aminoglycosides, macrolides, and tetracyclines which were promising as this combination therapy could help to repurpose these antibiotics (**Table 2.2**). Based on the antibiotic/HMO combination studies, we further hypothesized that HMOs increase cell membrane permeability thereby increasing the efficacy of intracellular-targeting antibiotics. This was initially confirmed using a LIVE/DEAD Baclight assay which found that HMOs increase cell membrane permeability up to 30%.[52]

[53]**Table 2.2**. First-generation antibiotic/HMO combination study.

Antibiotic	GB2			GB590			CNCTC 10/84		
	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]
Penicillin	0.03	0.015	2	0.03	0.06	0	0.03	0.015	2
Ampicillin	0.125	0.0625	2	0.0625	0.0625	0	0.0625	0.0312	2
Cefazolin	0.125	0.0625	2	0.125	0.0625	2	0.125	0.0625	2
Clindamycin	0.0312	0.0078	4	0.0625	0.0156	4	0.0325	0.0156	2
Linezolid	2	1	2	2	1	2	2	1	2
Gentamicin	16	2	8	16	1	16	16	2	8
Erythromycin	0.0156	0.0001	16	0.0312	0.001	32	0.0156	0.0019	8
Minocycline	2	0.25	8	4	0.5	8	0.0625	0.0019	32

[a]MIC values in µg/mL [b]HMOs were dosed at 5 mg/mL [c]denotes fold change in MIC values

2.8.2 Expanded study

In an effort to validate the hypothesis that HMOs increase cell membrane permeability, the panel of antibiotics was expanded to include additional intracellular targeting antibiotics. We chose antibiotics that affect DNA and RNA synthesis, DNA replication, and folate biosynthesis (**Table 2.3**).[53] A similar combination assay was employed with this expanded study, and while we did identify additional antibiotic potentiation, it was not observed across all intracellular targets. The two nitrofuran-derived antibiotics, nitrofurantoin and furazolidone which impact DNA replication and protein production saw limited potentiation across the two strains of GBS. The two fluoroquinolones, ciprofloxacin and levofloxacin, which interfere with DNA gyrase and topoisomerase IV, the two enzymes that are necessary for DNA replication and ultimately cell viability also saw limited potentiation, only a 2-fold decrease in MIC. The rifamycin polyketides, rifaximin and rifampicin which act through inhibition of bacterial DNA-dependent RNA polymerase through suppression of elongation during transcription saw contrasting results. While rifaximin saw limited potentiation across both strains, rifampicin displayed an 8-fold decrease in MIC in GB2.

Table 2.3. Expanded second-generation antibiotic/HMO combination study.

Antibiotic	GB2			GB590			CNCTC 10/84		
	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]	MIC[a]	MIC[a,b] w/ HMOs	Fold Change[c]
Ciprofloxacin	2	1	2	2	1	2	2	1	2
Levofloxacin	1	0.5	2	1	0.5	2	1	0.5	2
Rifampicin	0.125	0.0156	8	0.0313	0.0313	0	0.0156	0.0078	2
Rifaximin	0.125	0.0625	2	0.125	0.0625	2	0.0313	0.0313	0
Trimethoprim	1024	2	512	>1024	32	>32	>1024	8	>128
Sulfisoxazole	>64	>64	0	>64	>64	0	>64	>64	0
Sulfadiazine	>64	>64	0	>64	>64	0	>64	>64	0
Nitrofurantoin	4	2	2	4	4	0	4	4	0
Furazolidone	64	32	2	64	32	2	64	32	2

[a]MIC values in µg/mL [b]HMOs were dosed at their IC_{25} values [c]denotes fold change in MIC values

2.9 Targeting folate biosynthesis with HMOs

2.9.1 HMOs synergistically potentiates trimethoprim activity in GBS

In this expanded study, we most notably uncovered the powerful activity of HMOs in combating antifolate antibiotic resistance in GBS. Trimethoprim (TMP; **1.11**) interferes with the folate biosynthesis pathway through inhibition of dihydrofolate reductase, the enzyme necessary to convert dihydrofolate (**2.5**) to tetrahydrofolate (**2.6**) (**Figure 2.5**). Sulfonamides (**2.3**), which are often used in combination with trimethoprim, also inhibit folic acid biosynthesis through an earlier step in the pathway. They are structural analogs of *para*-aminobenzoic acid (pABA; **2.2**); therefore, they are competitive inhibitors of the dihydropteroate synthase (DHPS), the enzyme which converts dihydropterin pyrophosphate (DHPP; **2.1**) and pABA to dihydropteric acid (**2.4**). The sulfonamides we tested in this study, sulfisoxazole and sulfadiazine, saw no potentiation of activity.

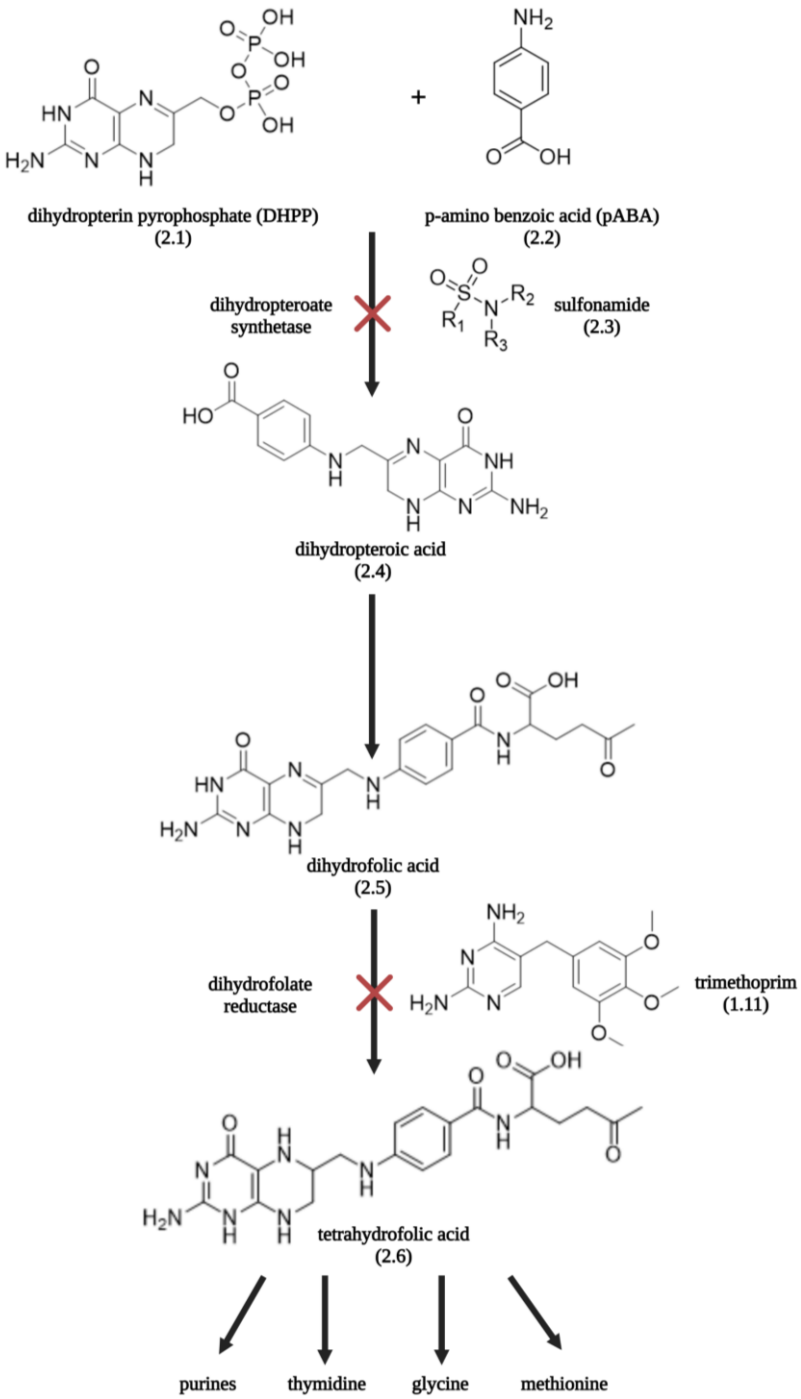

Figure 2.5. The folic acid synthesis pathway displaying the inhibition steps by sulfonamides and trimethoprim.

Results indicated that pooled HMOs could be co-dosed with TMP to synergistically increase GBS susceptibility to TMP. In addition to the three GBS strains assayed in the first-generation studies, strains GB651 (serotype Ib) and GB83 (serotype IV) were also included to span across the five strains that represent ca. 85% of all GBS isolates. In these combination trials, the HMOs were dosed at their IC_{25} in GB2 and CNCTC 10/84. For GB590, GB651, and GB83, the HMOs were dosed at the IC_{25} of a comparable GBS strain as the IC_{50} curves for these three strains did not supply high confidence limits (**Figure 2.6**). While GBS susceptibility was found to be strain specific, all representative GBS strains exhibited between 16 and 512-fold reduction in TMP MIC when dosed in coordination with HMOs (**Table 2.4**). The MIC of each strain was at least 512 µg/ml. The greatest reduction in MIC was found in GB2 with a 512-fold decrease from 1024 µg/ml to 2 µg/ml. CNCTC 10/84 was also significantly potentiated with at least a 256-fold decrease in MIC from at least 1024 µg/ml to 8 µg/ml. While the potentiation in GB590, GB651, and GB83 were not as great as the other two strains, they still saw MIC fold reductions of at least 64-fold, 16-fold, and at least 16-fold respectively in these GBS strains. These results were encouraging because GBS is intrinsically resistant to TMP and other folate-targeting antibiotics. Additionally, with HMOs used in combination with TMP as an adjuvant, we saw the greatest potentiation of an antibiotic across both the first- and second-generation studies.

Table 2.4. HMO potentiation of TMP across varying serotypes of GBS.

Strain	Serotype	MIC[a] of HMOs	MIC[b] of TMP	MIC[b,c] of TMP w/ HMOs	Fold Change[d]
GB2	Ia	2.56	1024	2	512
GB651	Ib	5.12	512	32	16
GB590	III	5.12	>1024	32	>64
GB83	IV	5.12	>1024	128	>16
10/84	V	5.12	>1024	8	>256

[a]MIC values in mg/mL [b]MIC values in µg/mL [c]HMOs were dosed at their IC_{25} values [d]denotes fold change in MIC values

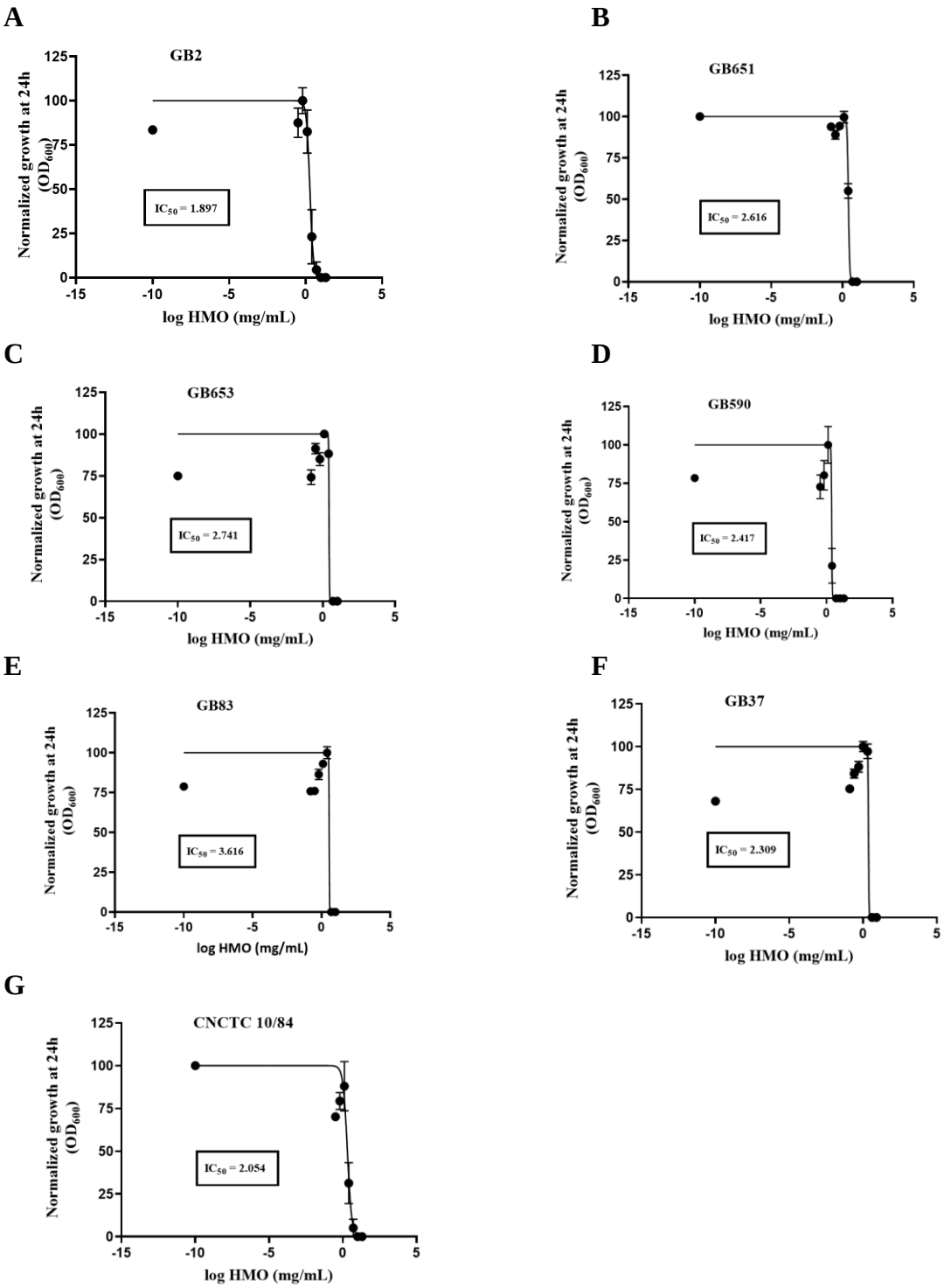

Figure 2.6. IC_{50} curves for the HMO cocktail against seven GBS strains in THB. Bacterial growth (OD_{600}) was recorded after 24 h of HMO treatment at 20.5; 10.25; 5.12; 2.56; 1.28; 0.64; 0.32; 0.16; and 0 mg/mL. A) HMO IC_{50} curve against GB2. B) HMO IC_{50} curve against GB651. C) HMO IC_{50} curve against GB653. D) HMO IC_{50} curve against GB590. E) HMO IC_{50} curve against GB83. F) HMO IC_{50} curve against GB37. G) HMO IC_{50} curve against CNCTC 10/84. Data displayed represent the mean normalized growth (OD_{600}) ± SEM of at least three independent experiments, each with 3 technical replicates. Mean normalized growth (OD_{600}) for each time point is indicated by the respective symbols.

Due to the significant potentiation of TMP against five strains of GBS, we sought to determine whether this HMO-TMP combination was synergistic in nature. Combination treatments can interact in ways to be synergistic, additive or antagonistic. If the two drugs together yield a greater inhibitory effect than the sum of each drug individually, it is considered synergistic. An additive effect generally is the sum of the drugs individual potencies, while antagonistic interactions occur when the drug combination is less effective than one or both of the drugs individually.

A checkerboard assay was employed to determine synergism of the HMO-TMP combination in which the MICs were measured for a range of concentration combinations. Synergy is determined by calculating the fractional inhibitory concentration (FIC) index value which is the sum of each FIC (ΣFIC) (**Figure 2.7**). The FIC is evaluated by dividing the MIC of each drug in combination by the MIC of each drug alone. The combination is considered synergistic when the ΣFIC is ≤0.5, additive when the ΣFIC is >0.5 to <4, and antagonistic when the ΣFIC is ≥4.

$$\Sigma FIC = FIC\ TMP + FIC\ HMOs$$

$$FIC\ TMP = \frac{MIC\ of\ TMP\ in\ combination}{MIC\ of\ TMP\ alone} \qquad FIC\ HMOs = \frac{MIC\ of\ HMOs\ in\ combination}{MIC\ of\ HMOs\ alone}$$

Figure 2.7. FIC index value calculation for determining synergy between TMP and HMOs.

In both GB2 and GB590, the HMO-TMP combination was found to be synergistic for a range of concentrations with ΣFIC values between 0.281 and 0.508. In GB2, when the HMOs were dosed from 0.64 and 1.28 mg/mL TMP was dosed from 4 to 32 µg/mL synergy was achieved (**Figure 2.8A**). In GB590, the synergistic combination was found when HMOs were dosed from 1.28 to 2.56 mg/mL and TMP was dosed from 8 and 128 µg/mL (**Figure 2.8B**). Knowledge of the synergistic combinations of HMOs and TMP is helpful in designing adjuvant therapy for the treatment of GBS and other bacterial infections.

A

Trimethoprim (µg/ml)	HMOs (mg/ml) 0	0.32	0.641	1.281	1.42	2.563	5.125	10.25
32				**0.281**	0.5312	0.5854	1.0312	2.0312	4.0311
16					0.5156	0.5697	1.0156	2.0155	4.0155
8					**0.508**	0.5619	1.0078	2.0077	4.0077
4					**0.504**	0.558	1.0039	2.0038	4.0038
2						0.5561	1.0019	2.0019	4.0018
1							1.0009	2.0009	4.0008
0							1	1.9999	3.9998

Growth
No Growth

B

Trimethoprim (µg/ml)	HMOs (mg/ml) 0	0.32	0.641	1.281	1.42	2.563	5.125	10.25
128					**0.375**	**0.402**	0.625	1.125	2.125
64					**0.313**	**0.34**	0.5625	1.0625	2.0625
32					**0.281**	**0.308**	0.5313	1.0313	2.0313
16							0.5156	1.0156	2.0156
8							**0.508**	1.0078	2.0078
4							**0.504**	1.0039	2.0039
0								1	2

Growth
No Growth

Figure 2.8. Checkerboard Assay in (A) GB2 and (B) GB590 showing the synergistic combinations (bolded) between trimethoprim and the HMO cocktail. The combination is considered synergistic when the ΣFIC is ≤0.5, additive when the ΣFIC is >0.5 to <4, and antagonistic when the ΣFIC is ≥4.

2.9.2 On target engagement of trimethoprim on the folate pathway

As previously mentioned, sulfonamides are often used in combination with trimethoprim because they provide broad spectrum antibacterial activity against both gram-positive and gram-negative pathogens. Both drugs inhibit enzymes in the folate synthesis pathway, working synergistically when taken together (**Figure 2.5**). The combination is most commonly taken to treat urinary tract infections, but also used to treat methicillin-resistant *Staphylococcus aureus* (MRSA), shigellosis, Pneumocystis pneumonia, bronchitis, travelers' diarrhea, and other bacterial infections.[54] Both sulfadiazine and sulfamethoxazole are used in combination with trimethoprim, typically given in a 5:1 ratio.[54, 55] We chose to look at the clinically relevant combination in both GB2 and GB590 to confirm the HMOs were not acting in place of the sulfonamides. The

sulfadiazine-TMP combination remained ineffective in both strains of GBS with an MIC of ≥512 µg/mL (**Table 2.5**). This result aligned with our hypothesis that HMOs do not target folate synthesis pathway, rather increase the activation of TMP through another mechanism not yet identified.

Table 2.5. TMP/Sulfadiazine combination assay in GBS.

Strain	MIC[a] of TMP	MIC[a] of Sulfadiazine	MIC[a,b] of TMP/Sulfadiazine Combination	Fold Change[c]
GB2	1024	>64	>512	0
GB590	>1024	>64	>512	0

[a]MIC values in µg/mL [b]1:4.85 ratio of TMP-sulfadiazine [c]denotes fold change in MIC values

Antifolate drugs are successful in the treatment of infectious disease as the folate end products in this pathway are essential to most living organisms. Folate is a necessary component in the synthesis of both nucleic and amino acids, as they are involved in one-carbon shuttling reactions which produce these biomolecules.[56] While antifolate drugs inhibit the *de novo* synthesis of nucleotides, additionally, bacterial cells can produce them via salvage reactions which convert free purine and pyrimidine bases such as thymidine into the corresponding nucleotide. Our next experiment explored whether the HMOs were assisting TMP in on-target engagement of the folate pathway. It was thought that thymidine supplementation would reverse the antimicrobial effects of the TMP/HMO combination treatment since thymidine is involved in the nucleotide salvage pathways. In the presence of thymidine at a concentration of 20 µg/ml, there an 8-fold increase in MIC in GB2 from 2 to 16 µg/ml, and a 4-fold increase in MIC in GB590 from 32 to 128 µg/ml (**Table 2.6**). This information tells us that GBS is able to utilize thymidine to reverse the effects of the combination therapy and partially salvage the folate pathway. We also

demonstrated that the MIC of the HMO cocktail was unaffected with thymidine supplementation, supporting our hypothesis that the folate pathway is not the target of the HMOs.

Table 2.6. HMO potentiation of TMP in the presence of thymidine.

Strain	THB Media			
	MIC[a] of HMOs	**MIC[b] of TMP**	**MIC[b,c] of TMP w/ HMOs**	**Fold Change[d]**
GB2	2.56	1024	2	512
GB590	5.12	>1024	32	>64
Strain	**THB Media +20 µg/mL thymidine**			
	MIC[a] of HMOs	**MIC[b] of TMP**	**MIC[b,c] of TMP w/ HMOs**	**Fold Change[d]**
GB2	2.56	1024	16	64
GB590	5.12	>1024	128	8

[a]MIC values in mg/mL [b]MIC values in µg/mL [c]HMOs were dosed at their IC_{25} values [d]denotes fold change in MIC

2.9.3 Uncovering the mechanism of action of HMOs using untargeted metabolomics

After demonstrating increased on-target activity of TMP through inhibition of folate biosynthesis, this led us to hypothesize that HMOs facilitate increased GBS cell permeability, restoring TMP cell penetrance and antibiotic activity within GBS strains that are initially TMP resistant. We wanted to further validate this hypothesis through global, untargeted metabolomic analyses to characterize the interactions between HMOs and GBS. This discovery-based approach allows for comparison of the complete metabolome. Untargeted metabolomic identifies metabolite perturbations across different biological conditions. This diagnostic tool has been useful in gaining insight into disease pathogenesis and microbial mechanism of action.

To evaluate the metabolic perturbations, we chose to study strain GB2 which was most susceptible to HMO treatment in all previous studies (**Table 2.1**). We compared our GBS control in THB medium alone to GBS treated with HMOs dosed at 1 mg/mL.[53] This concentration of

HMOs resulted in cellular death between 20 and 40%, allowing enough cellular mass to analyze for metabolic changes. We collaborated with the Vanderbilt Center for Innovative Technology (CIT) to isolate and characterize significant metabolites following a standard untargeted metabolomics technique.

Following sample preparation, metabolite extracts were first exposed to reverse-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC), followed by tandem mass spectrometry (MS/MS). Subsequent mass fragmentation and peak peaking allowed us compare identified significant metabolites between our GBS control to our HMO-treated sample. Our results revealed that HMOs significantly impacted several metabolic pathways in GBS (**Table 2.7**). The most statistically perturbed pathways ($p \leq 0.05$; fold change $\geq |2|$) were those of linoleic acid metabolism, sphingolipid metabolism, glycerophospholipid metabolism, pyrimidine metabolism, and pantothenate and CoA biosynthesis, with a focus on those metabolites critical cell membrane construction and structural integrity.

Table 2.7. Metabolic pathway analysis for untreated vs. HMO-treated GBS samples.

Metabolic Pathway	Total Metabolites	Number of Hits	Raw p-value	Impact Factor
linoleic acid metabolism	15	6	2.42E-05	0.771
sphingolipid metabolism	25	7	0.000595	0.215
glycerophospholipid metabolism	39	7	0.001334	0.271
pyrimidine metabolism	60	8	0.004362	0.238
pantothenate and CoA biosynthesis	27	3	0.1155	0.135

Linoleic acid (LA; **2.7**) metabolism is the most statistically impacted pathway upon HMO treatment. This aligns with our hypothesis, as linoleic acid metabolites are critical for cellular signaling, the host-immune response, and proper membrane construction.[57, 58] These polyunsaturated fatty acids are essential for humans in their diet since unlike plants they cannot be synthesized *de novo*.[59] In humans, LA metabolites play a vital role in brain function and

cardiovascular health.[60, 61] While GBS does not biosynthesize LA directly, it has the ability to uptake exogenous fatty acids from its' environment, in this instance from the THB media used in the growth assays. All identified linoleic acid precursors were accumulated in the HMO-treated population, with several metabolites having a 100-fold increase from the untreated controls (**Figure 2.9**). Two epoxyoctadecanoic acid metabolites were of particular interest, epoxyoctadecanoic acids (EpOMEs; **2.12**, **2.13**) and dihydroxyoctadecanoic acids (DiHOMEs; **2.10**, **2.14**). Accumulation of these metabolites is linked to changes in cell membrane fluidity and cell membrane construction.[62] These perturbations support that HMOs significantly alter cell membrane associated metabolites, likely causing increased GBS cell permeability.

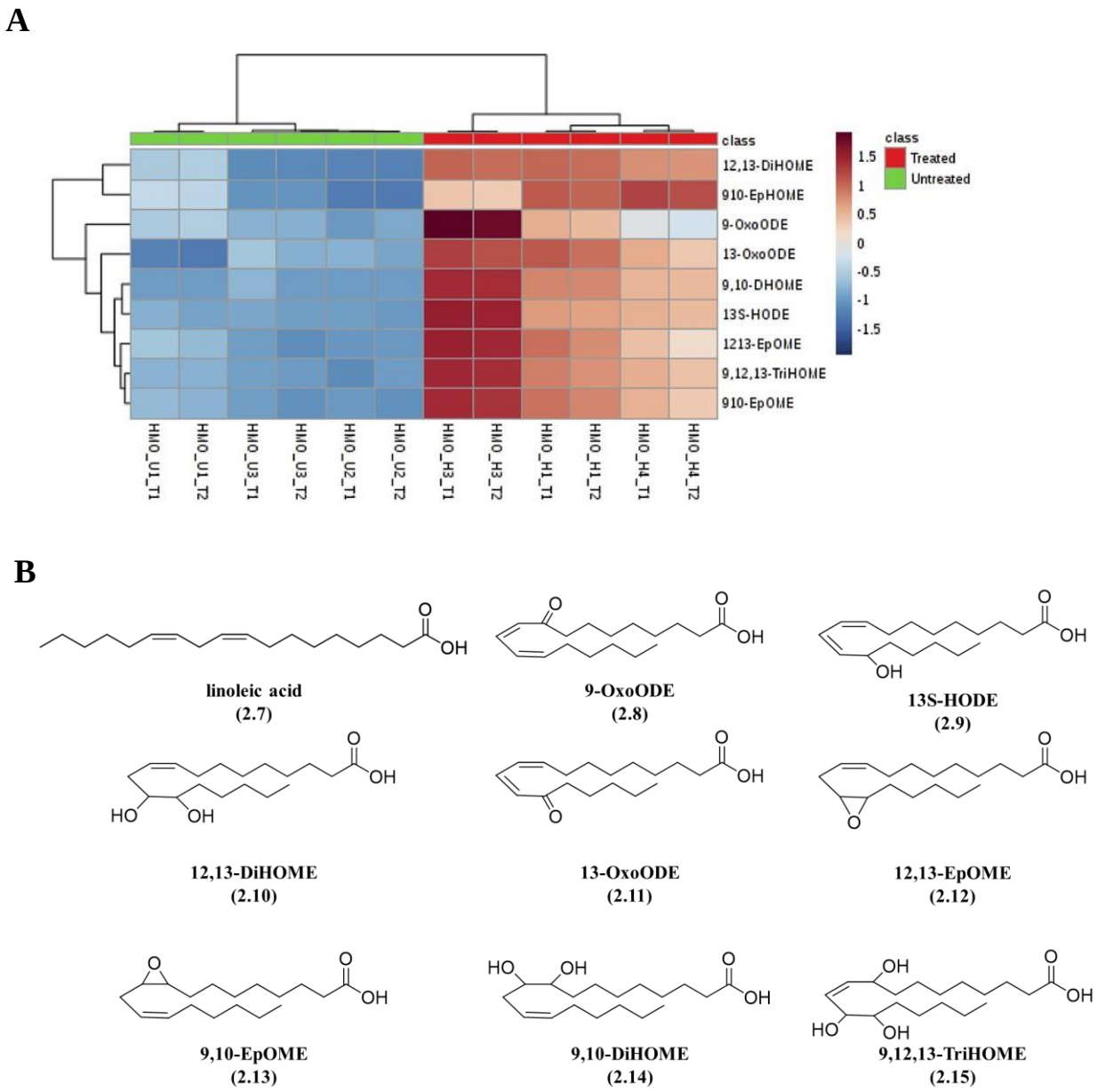

Figure 2.9. Significantly impacted linoleic acid metabolites upon HMO treatment. A) Heat map visualization of the significantly differently regulated linoleic acid metabolic pathway upon HMO treatment. Linoleic acid metabolism members shown here were detected by RPLC-positive LC-MS/MS analysis. Samples (columns) and metabolite compounds (rows) were processed using Euclidean average clustering via MetaboAnalyst 4.0. The heat map was generated for Pareto-scaled, log-transformed data, and colors are displayed by relative abundance, ranging from low (blue) to high (red), as shown in the legend. B) Structures of linoleic acid metabolites.

Similar to what we observed with linoleic acid metabolism, glycerophospholipid metabolism was also significantly impacted with up to a 50-fold increase in accumulation of these metabolites in the HMO-treated cultures compared to our untreated control. Comprised of two fatty acids esterified to a phosphate glycerol backbone and variable head group, glycerophospholipids are the most abundant components of cellular membranes (**Figure 2.10A&B**). These critical lipid constituents act as a natural barrier to protect cells against invasion and degradation by antimicrobials or in response to the host immune response.[63] Several metabolites associated with phosphatidylethanolamine (PE; **2.19**) catabolism such as PE(17:0/0:0) (**2.23**), PE(P-16:0/0:0) (**2.21**), and PE(19:1/12:0) (**2.20**) were found in higher abundances in the HMO-treated population (**Figure 2.10C&D**).[64] Additionally, choline (**2.16**), diethanolamine (**2.17**), and triethanolamine (**2.18**), three head groups that are found in the most common phosphoglycerides were increased upon HMO treatment.[65] This data provides evidence that the accumulation of these specific glycerophospholipid metabolites contributes to the collapse of membrane structure and integrity, allowing for passage of antibiotics when dosed with HMOs.

In addition to the two pathways described above that are directly related to membrane structure and function, HMOs also significantly perturbed other metabolic pathways. Unlike eukaryotic cells, bacteria do not produce sphingolipids directly, rather they are able to employ exogenous sphingolipids present in growth media or in host cells.[66] Sphingolipid metabolites, including sphingomyelin are important components of microbial cell membranes that must be incorporated for prolonged survival in the host.[66, 67] We also observed an increase in pyrimidine metabolites, the precursors required for DNA and RNA synthesis, and pantothenate and coa metabolites, the precursors involved in the biosynthesis of fatty acids.

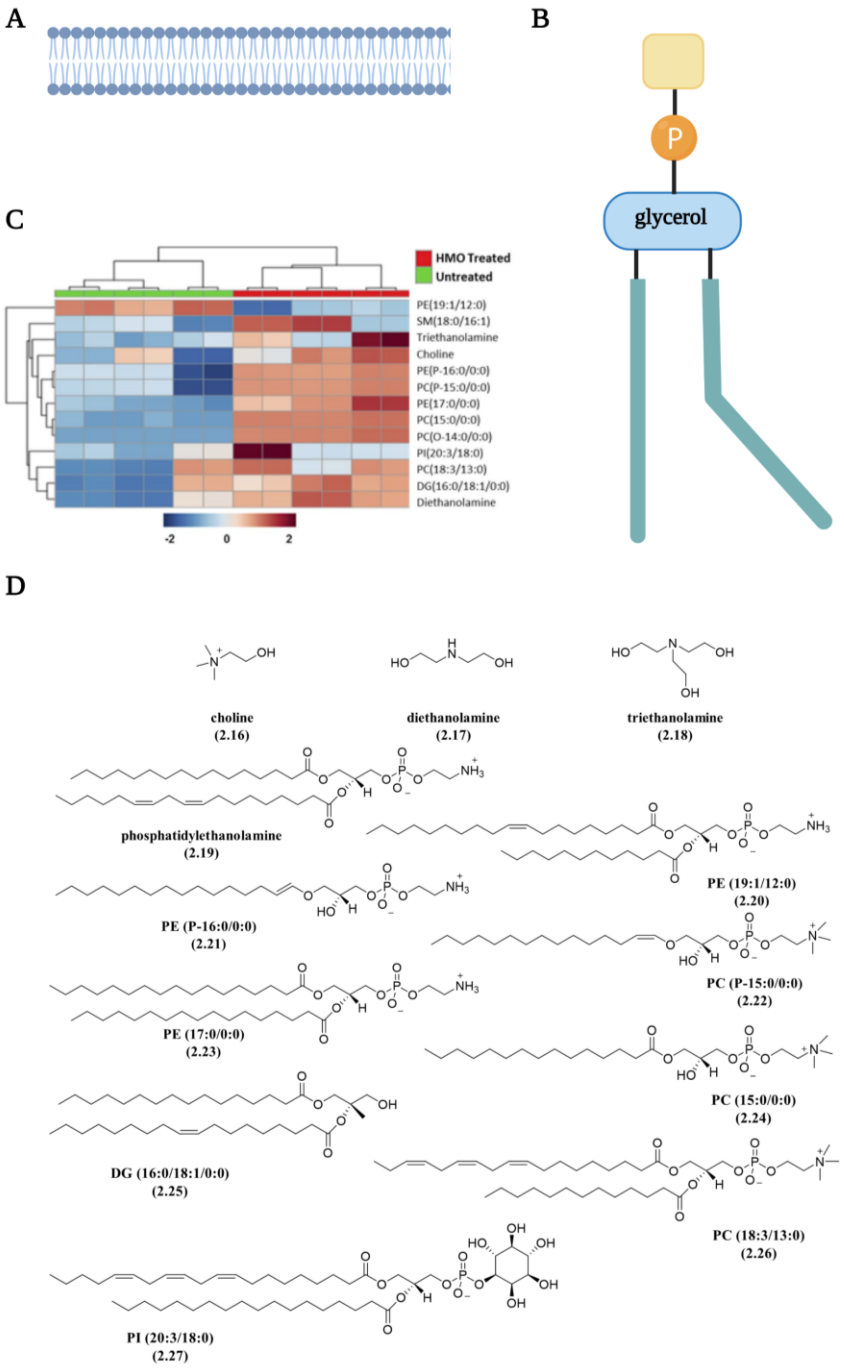

Figure 2.10. Representation of glycerophospholipid metabolites present in the cell membrane. A) The plasma membrane is composed primarily of glycerophospholipids. B) Glycerophospholipids are composed of a hydrophilic region (glycerol, phosphate, and a variable head group), and a hydrophobic region (two fatty acids). C) Heat map visualization of the significantly differently regulated glycerophospholipid metabolism pathway upon HMO treatment. Glycerophospholipid members shown here were detected by HILIC-positive LC-MS/MS analysis. Samples (columns) and metabolite compounds (rows) were processed using Euclidean average clustering via MetaboAnalyst 4.0. The heat map was generated for Pareto-scaled, log-transformed data, and colors are displayed by relative abundance, ranging from low (blue) to high (red), as shown in the legend. SM, sphingomyelin; PC, phosphocholine; PE, phosphoethanolamine; PI, phosphoinositol; DG, diglyceride; LysoPC, lysophosphatidylcholine. D) Structures of significant glycerophospholipid metabolites. Created with BioRender.com.

2.10 Conclusions and future outlook

In these studies on HMOs isolated from human breast milk, we discovered their ability to serve as adjuvants to antibiotics. We hypothesized heterogeneous HMO extracts function by increasing cell membrane permeability, therefore potentiating the action of intracellular targeting antibiotics such as aminoglycosides, lincosamides, macrolides, tetracyclines, rifamycins, and antifolates against GBS *in vitro*. We previously validated this hypothesis by means of a membrane permeability assay which exhibited an increase in membrane permeability in the HMO-treated samples compared to the untreated GBS control. We further probed this hypothesis by using untargeted metabolomic analyses where we revealed significant perturbations in cell membrane-associated metabolites.

Although these studies have shown the potent antimicrobial activity of HMOs and their effectiveness as an antibiotic adjuvant, we still have limited information on why only certain intracellular antibiotics were potentiated. The metabolomic analyses we employed only investigated the effects of HMO supplementation alone. Additional metabolomic analyses must be used to explore the various HMO-antibiotic combinations to further validate our proposed mechanism of action. Specifically, we need to understand why trimethoprim, a drug intrinsically resistant to GBS was so significantly affected upon HMO treatment.

Future work will also be expanded to include additional opportunistic pathogens implicated in adverse pregnancy outcomes including *A. baumannii*. As discussed in Section 2.7, the antimicrobial activity of a heterogeneous mixture of HMOs was assessed against *A. baumanni* indicating HMOs alone do not possess potent bactericidal activity.[48] Recently, Spicer et al. uncovered the potent antibiofilm activity of HMOs against 14 clinical strains of *A. baumannii* isolated from an array of anatomical sites, and spanning across antibiotic-resistant and

susceptible strains.[68] To follow up, we will focus on the utility of HMOs as an antibiotic adjuvant against *A. baumannii* infections. Since *A. baumannii* is a gram-negative bacterium, the panel of antibiotics we choose to explore will focus on enhancing the activity of gram-negative-targeting antibiotics including broad-spectrum cephalosporins, tetracyclines, carbapenems, beta-lactam/beta-lactamase inhibitor combinations, and polymyxins. These current antibiotic/HMO combination studies against GBS have shown how effective HMOs are in potentiating the activity of our current arsenal of antibiotics.

2.11 Experimental methods

Bacterial strains and culture conditions

Strain	Serotype	Source
S. agalactiae GB00002	Ia	clinical isolate, Shannon Manning, Michigan State
S. agalactiae GB00651	Ib	clinical isolate, Shannon Manning, Michigan State
S. agalactiae GB00653	II	clinical isolate, Shannon Manning, Michigan State
S. agalactiae GB00590	III	clinical isolate, Shannon Manning, Michigan State
S. agalactiae GB00083	IV	clinical isolate, Shannon Manning, Michigan State
S. agalactiae GB00037	V	clinical isolate, Shannon Manning, Michigan State
S. agalactiae CNCTC 10/84	V	ATCC

All strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37°C in ambient air overnight. All strains were subcultured from blood agar plates into

5 mL of Todd-Hewitt broth (THB) and incubated under shaking conditions at 180 rpm at 37°C overnight. Following overnight incubation, bacterial density was quantified through absorbance readings at 600 nm (OD_{600}) using a Promega GloMax-Multi detection system plate reader. Bacterial numbers were determined using the predetermined coefficient of an OD_{600} of 1, equal to 10^9 CFU/mL.

HMO isolation

Human milk was obtained from healthy, lactating women between 3 days and 3 months postpartum and stored between −80 and –20°C. Deidentified milk was provided by Jörn-Hendrik Weitkamp from the Vanderbilt Department of Pediatrics, under a collection protocol approved by the Vanderbilt University institutional review board (IRB no. 100897), or from Medolac. Milk samples were thawed and then centrifuged for 45 min. Following centrifugation, the resultant top lipid layer was removed. The proteins were then removed by diluting the remaining sample with roughly 1:1 (vol/vol) 180 or 200 proof ethanol, chilling the sample briefly, and centrifuging for 45 min, followed by removal of the resulting HMO-containing supernatant. Following concentration of the supernatant in vacuo, the HMO-containing extract was dissolved in 0.2 M phosphate buffer (pH 6.5) and heated to 37°C. β-Galactosidase from *Kluyveromyces lactis* was added, and the reaction mixture was stirred until lactose hydrolysis was complete. The reaction mixture was diluted with roughly 1:0.5 (vol/vol) 180 or 200 proof ethanol, chilled briefly, and then centrifuged for 30 min. The supernatant was removed and concentrated in vacuo, and the remaining salts, glucose, and galactose were separated from the oligosaccharides using size exclusion chromatography with P-2 gel (H_2O eluent). The oligosaccharides were then dried by lyophilization. Correspondingly, HMO isolates from donors were combined and solubilized in water to reach a final concentration of 102.6 mg/ml.

Broth microdilution minimum inhibitory concentration assay

All strains were grown overnight as described above and used to inoculate fresh THB or THB plus 20 μg/mL thymidine to achieve 5×10^5 CFU/mL. To 96-well tissue culture-treated, sterile polystyrene plates was added the inoculated medium in the presence of increasing concentrations of antibiotic or HMO cocktail to achieve a final volume of 100 μL per well. Bacteria grown in medium in the absence of any compounds served as the controls. The plates were incubated under static conditions at 37°C in ambient air for 24 h. Bacterial growth was quantified through absorbance readings (OD_{600}). The minimum inhibitory concentrations (MICs) were assigned at the lowest concentration of compound at which no bacterial growth was observed.

Broth microdilution method for antibiotic combination

All strains were grown overnight as described above and the subcultures used to inoculate fresh THB or THB plus 20 μg/mL thymidine to achieve 5×10^5 CFU/ml. Freshly inoculated medium was then supplemented with HMOs at their IC_{25} (~1.24 mg/mL). To 96-well tissue culture-treated, sterile polystyrene plates was added the inoculated medium supplemented with HMOs in the presence of increasing concentrations of antibiotic. Bacteria grown in medium in the absence of any compounds served as one control. Bacteria grown in medium supplemented with HMOs in the absence of any antibiotic served as a second control. MICs were determined as previously described.

Synergy assay

Group B *Streptococcus* strains (GB2 and GB590) were grown overnight as described above and used to inoculate fresh THB to achieve 5×10^5 CFU/ml. To 96-well tissue culture-treated, sterile

polystyrene plates, 100µL of inoculated medium was added per well. Trimethoprim was two-fold serially diluted descending down the plate to achieve a final volume of 100 µl per well. The final row was left without any trimethoprim. The HMO cocktail was two-fold serially diluted going from right to left across the plate. The far-left column was left without any HMO cocktail. Bacteria grown in medium in the absence of either compound served as the controls. The plates were incubated under static conditions at 37°C in ambient air for 24 h. Bacterial growth was quantified through absorbance readings (OD_{600}). MICs were determined as previously described. The fractional inhibitory concentration (FIC) index was used to evaluate synergy. The calculation of the FIC index is as follows: ΣFIC = FIC A + FIC B = (MIC of drug A in the combination/MIC of drug A alone) + (MIC of drug B in the combination/MIC of drug B alone), where A is trimethoprim and B is the HMO cocktail. The combination is considered synergistic when the ΣFIC is ≤0.5, additive or indifferent when the ΣFIC is >0.5 to <4, and antagonistic when the ΣFIC is ≥4.

Statistical analysis

Statistical analyses were performed in GraphPad Prism Software v. 8.2.1. All data shown signify three biological experiments each with three technical replicates. Data are expressed as the mean ± SEM. The antimicrobial and combination assays additionally represent 3 technical replicates for each biological replicate.

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perturbations that result in potentiation of trimethoprim. mBio 11:e00076-20. https://doi.org/10.1128/mBio.00076-20

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Chapter 3

Controlling the virulence of infectious disease by targeting microbial biofilms

3.1 Abstract

In recent decades, the number of bacterial pathogens that are multi-drug resistant (MDR) have proliferated generating an urgent need to develop novel therapeutics with narrow-spectrum activity. Biofilms are an essential virulence factor linked to the pathogenesis of opportunistic bacteria, contributing to MDR and increased rates of morbidity and mortality because of persistent infections. We previously demonstrated that heterogeneous human milk oligosaccharide (HMO) extracts exhibit strong antibiofilm activity, but no single-entity HMO has been shown to decrease biofilm production in Group B *Streptococcus* (GBS). It was hypothesized that we could convert HMOs to antibiofilm compounds by incorporating a positive charge on the glycan with the knowledge that cationic molecules destabilize the extracellular matrix of biofilms. Four β-amino HMOs were synthesized and found to significantly inhibit biofilm production in both GBS and methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, we plan to elucidate the mechanism of action as to how the β-amino HMOs inhibit bacterial adherence and biofilm formation through proteomic analyses. This study provides necessary data for the development of additional compounds to effectively treat MDR bacterial infections, especially those impacted by the presence of robust biofilms.

3.2 The Role of biofilms in the pathogenesis of disease

Biofilms are often implicated in the pathogenesis of opportunistic bacteria, both gram-positive and gram-negative species. These structured colonies of microorganisms are found within a self-produced extracellular polymeric matrix which adheres to both biotic and abiotic surfaces (**Figure**

3.1).[1-3] Between 65% and 80% of chronic infections are associated with biofilm formation including cystic fibrosis, endocarditis, osteomyelitis, chronic wounds, periodontitis disease, recurrent urinary tract infections, and leptospirosis.[3, 4] All bacterial species, but especially biofilm-producing bacteria are difficult to eradicate due to the antimicrobial tolerance mechanisms they employ: efflux pumps, antibiotic-degrading enzymes, and target-site mutations.[1, 5, 6]

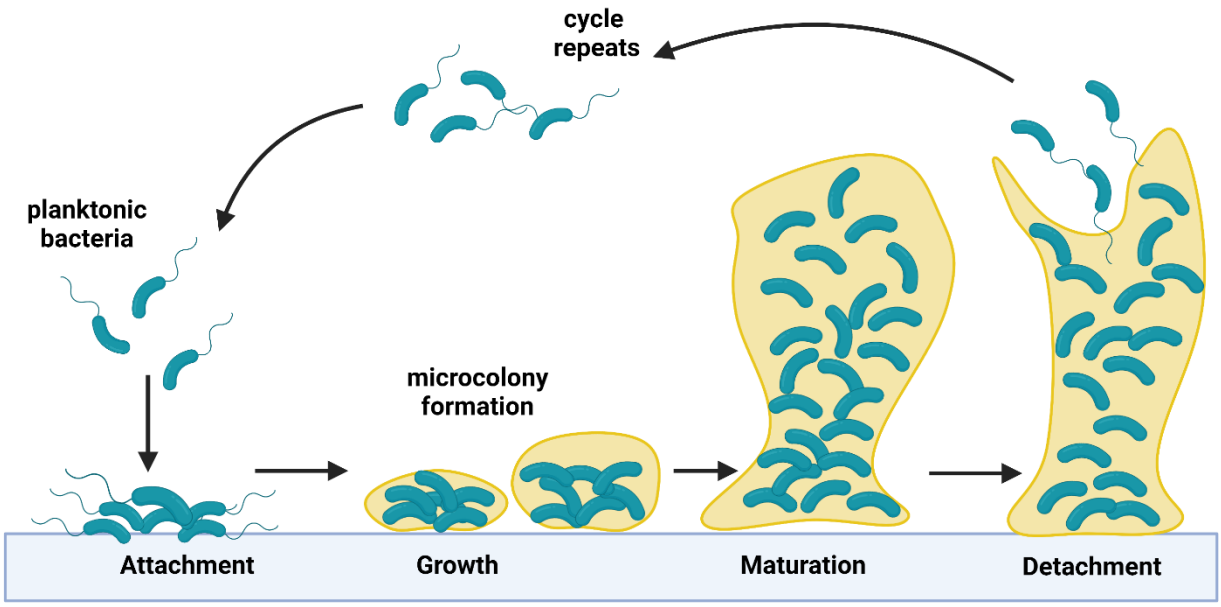

Figure 3.1. The biofilm life cycle. Stage 1: reversible attachment of planktonic bacteria to the surface. Stage 2: irreversible attachment of bacteria and proliferation through the formation of microcolonies. Stage 3: biofilm maturation. Stage 4: dispersion of the biofilms, releasing bacteria into the planktonic state. Created with BioRender.com.

3.2.1 The biofilm life cycle

The first step of biofilm formation is the initial contact and attachment to the surface. In this stage, it has been shown that the bacteria use their appendages such as pili, flagella, curli fibers, and fimbriae for surface attachment or rely on the physical interactions of van der Waals or electrostatic interactions.[7] After the initial attachment, the adherent cells are able to multiply through a process of cell division and recruitment resulting in the formation of microcolonies.

In the maturation phase, it has been shown that quorum sensing, a mechanism that regulates gene expression through cell-to-cell communication is a key feature for controlling virulence.[8, 9] In quorum sensing systems, single-celled bacteria generate small molecule signals, called autoinducers, (AI) in a low population environment (**Figure 3.2**). As AIs increase, they are recognized by the surrounding bacterial cells until they reach a threshold concentration. At high cell density, the signal molecules bind to receptor proteins, thereby leading to a coordinated response promoting changes in gene expression.

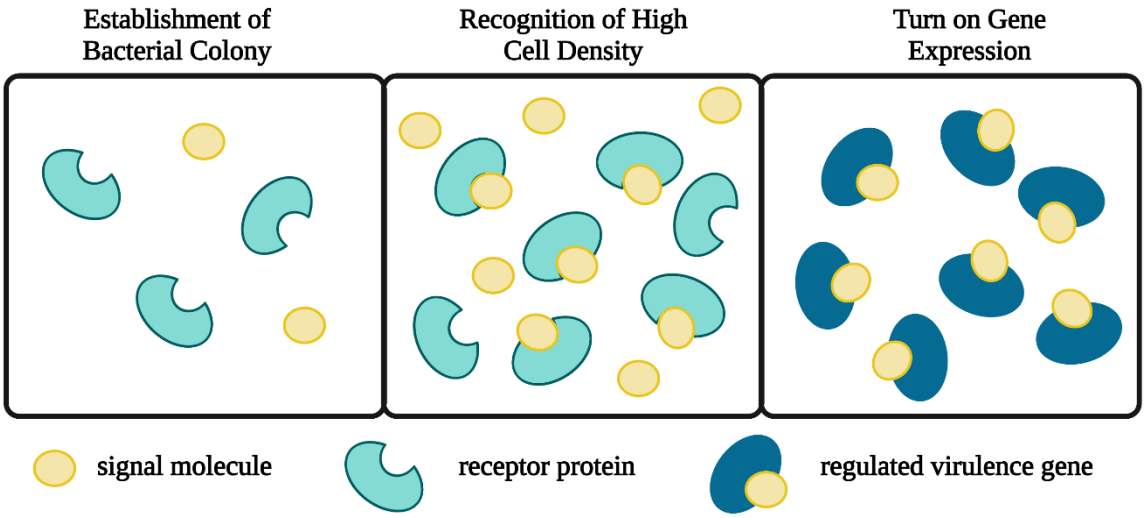

Figure 3.2. Overview of bacterial quorum sensing. Created with BioRender.com.

The final stage of biofilm development is the detachment and dispersal of cells from the biofilm matrix into the surrounding medium. Colonizing new environments is an essential process for survival of the bacteria and disease progression.

3.2.2 The extracellular matrix

The extracellular matrix is composed of microbial cells and the extracellular polymeric substance (EPS) consisting of polysaccharides, proteins, lipids, and extracellular DNA (**Figure 3.3**). The EPS is fully hydrated, accounting for up to 90% of the total mass which is held tightly together

through hydrogen bonding.[10, 11] The close proximity of the cells within the matrix increases the cell-to-cell communication amongst the community, resulting in enhanced virulence. The EPS is critical in immobilizing cells within the biofilm, not only providing structural integrity, but also providing protection from antimicrobials, host immune defenses, and harsh environmental conditions including ultraviolet radiation, extreme temperatures, osmotic stress, and pH.

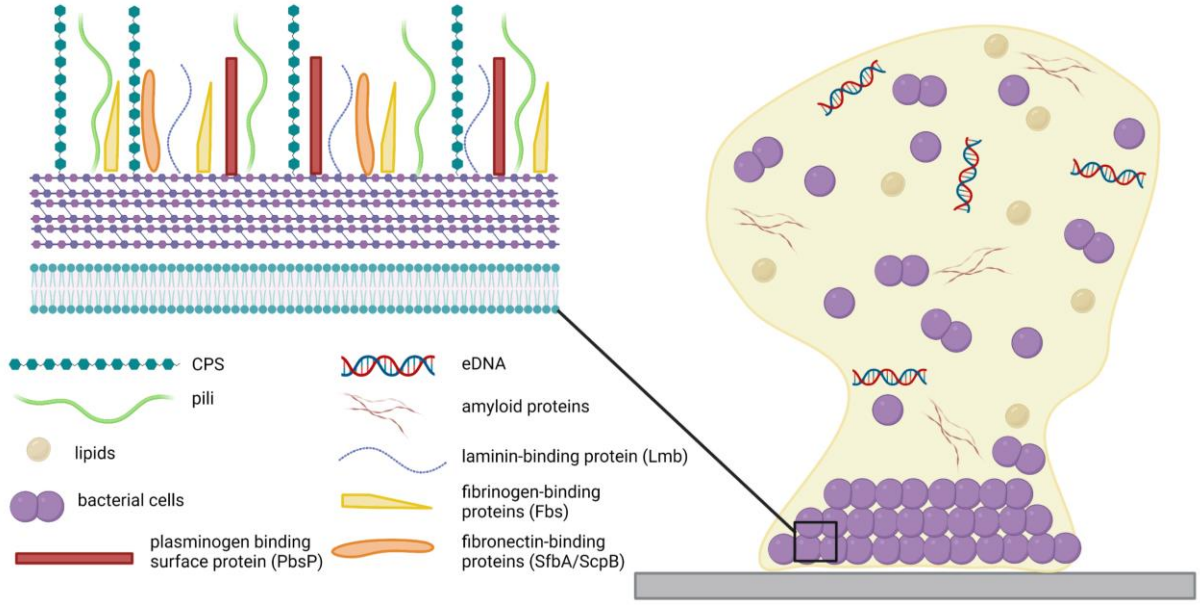

Figure 3.3. Extracellular polymeric substances (EPS) matrix. Created with BioRender.com.

Structurally, the biofilm matrix morphology is diverse, ranging from smooth and planar to mushroom-like macrocolonies.[12] Water-filled channels traverse the matrix, allowing for nutrient and oxygen distribution, and waste removal.[13] Polysaccharides and proteins are integral components of the matrix which can be categorized as either cell surface associated, or extracellularly secreted.[11] It is important to understand the structural and functional roles these biomolecules play in biofilm assembly, persistence, and proliferation.

Capsular polysaccharides (CPS) (see Section 1.6) are covalently linked to the cell surface of a broad range of pathogens, providing a protective barrier against environmental pressures and antimicrobial drugs. These highly hydrated molecules are structurally diverse due to the variable

monosaccharide constituents, glycosidic linkages, and branching of non-sugar moieties.[14] In gram-negative bacteria, the CPS chains are attached to either phospholipids or lipid-A molecules on the outer membrane, while in gram-positive bacteria they are usually anchored to the thick peptidoglycan layer of the cell wall.[14, 15] CPSs are often implicated in promoting biofilm formation by increasing motility and facilitating attachment of cells to each other and to surfaces.[16]

The cell-associated pili or fimbriae (see Section 1.6) are filamentous protein structures protruding from the cell envelope that aid in bacterial adhesion, motility, and host cell invasion and colonization.[17] Pili are composed of 0.3-2.0 μm long pilin subunits which arrange themselves in a cylindrical helices.[18, 19] Individually, the pilin covalently attach to the peptidoglycan layer of the cell wall through a specialized transpeptidase identified as a sortase enzyme.[20] Once the pili make contact with specific receptors on the host cell, cell-to-cell interactions between neighboring pili mediate biofilm formation. In GBS, there are three pilus types, 1, 2a, and 2b, with type 2a mostly associated with prolific biofilm formations.[21]

Fibrinogen, an essential protein required for blood coagulation, is also an ubiquitous constituent of the extracellular matrix.[22] The capability of GBS to bind to fibrinogen has been implicated in the colonization and invasion of host tissues, with respect to biofilm production and invasive disease. Along with the TCS CovR/S (see Section 1.6), the TCS RgfA/C has been shown to play a crucial role in fibrinogen binding.[23] The five best characterized proteins in the fibrinogen-binding protein (Fbs) family are FbsA, FbsB, FbsC or bacterial surface adhesin of GBS (BsaB), and the serine-rich repeat glycoproteins Srr1 and Srr2.[24] Specifically, FbsC has been identified an essential virulence factor in mediating biofilm formation of GBS. Structurally, FbsC is composed of two large immunoglobulin (IgG)-like tandem repeat domains and a C-terminal sortase-recognition motif (LPXTG) which is linked to the peptidoglycan layer of the cell wall.[25] It has been shown

that deletion of FbsC drastically reduced the ability of GBS to adhere to and invade human epithelial cells.[22]

The interaction between the cell surface lipoprotein, the laminin-binding protein (Lmb) and the ECM protein laminin is another crucial step required for GBS colonization and tissue invasion. Laminin is a basement membrane, heterotrimeric glycoprotein composed of α, β, and γ chains which provide structural integrity to the matrix and mediates binding between other ECM components.[26] Lmb was identified as a necessary adhesion protein through studies with the Δ*lmb* mutant which resulted reduced adherence to the epithelium compared to the wild type strain.[27]

Fibronectin (Fn) is another basement membrane glycoprotein found within the ECM that plays an essential role in mediating cell functions including growth, differentiation, migration, and most notably adhesion.[28] This large, secreted protein dimer consists of two protein chains, connected by disulfide bonds. Fibronectin is targeted by the GBS cell-surface anchored proteins C5a peptidase (ScpB) and streptococcal fibronectin binding protein A (SfbA). The dual-function ScpB is both a highly specific serine protease for the human C5a and modulates bacterial binding to fibronectin.[29] While SfbA is not directly involved in bacterial adherence, it plays a key role in invasion of epithelial cells, contributing to advanced GBS disease.[24] The binding abilities of these two proteins were confirmed in mutant studies with Δ*scpB* and Δ*sfbA*, which displayed reduced adherence and invasion.[30, 31]

The cell wall-anchored plasminogen binding surface protein (PbsP) contributes to microbial adhesion and invasion of host tissues. PbsP is a dual functional 521-aa protein consisting of two streptococcal surface repeat (SSURE) domains, a methionine and lysine-concentrated region, and a C-terminus LPXTG motif.[32, 33] As inferred by its name, PbsP interacts with plasminogen (Plg) on epithelial cell surfaces and in the extracellular matrix, as well as another ECM protein,

vitronectin (Vtn).[34] Plg is an inactive proenzyme, which upon binding with PbsP, converts to plasmin (Pln) by tissue-type activators (tPA) or urokinase-type activators (uPA), thus initiating colonization.[33] Vtn directly mediates bacterial adhesion through binding to the SSURE region of PbsP.[34]

3.2.3 Biofilms in GBS

Biofilm formation in GBS and other gram-positive bacteria is a key virulence factor promoting bacterial colonization and persistence in host tissues. The first indication that GBS was capable of forming biofilms came from a study by Marrie and Coserton in 1983.[35] They isolated GBS along with other pathogenic biofilm formers such as *S. aureus*, *E. coli*, *Staphylococcus epidermidis*, and *Candida albicans* on intrauterine devices removed from women suffering from pelvic inflammatory disease. This evidence was confirmed by SEM and TEM imaging. Numerous studies have since validated this initial study demonstrating the capacity of GBS to adhere to both abiotic and biotic surfaces.[36-38] Not surprisingly since GBS is a normal colonizer of the vaginal cavity, acidic pH has shown to stimulate biofilm formation compared to a neutral pH.[24, 39] Specifically, serotypes III and V have shown to display enhanced biofilm formation under acidic conditions.[40] In connection to GBS adherence, along with the capsule, several adhesions and surface-protruding proteins are responsible for increased biofilm. Prominent adhesions facilitating attachment to host surfaces include the Fbs, Lmb, ScpB, SfbA, BibA, and HvgA.[24] The type 2a pili are the fibrous protein structures are most often implicated in mediating attachment to host cells and promoting biofilm formation which makes them an attractive vaccine target.[37] While there are no currently

approved vaccines against GBS infections, targeting biofilms and their key components is a promising approach.

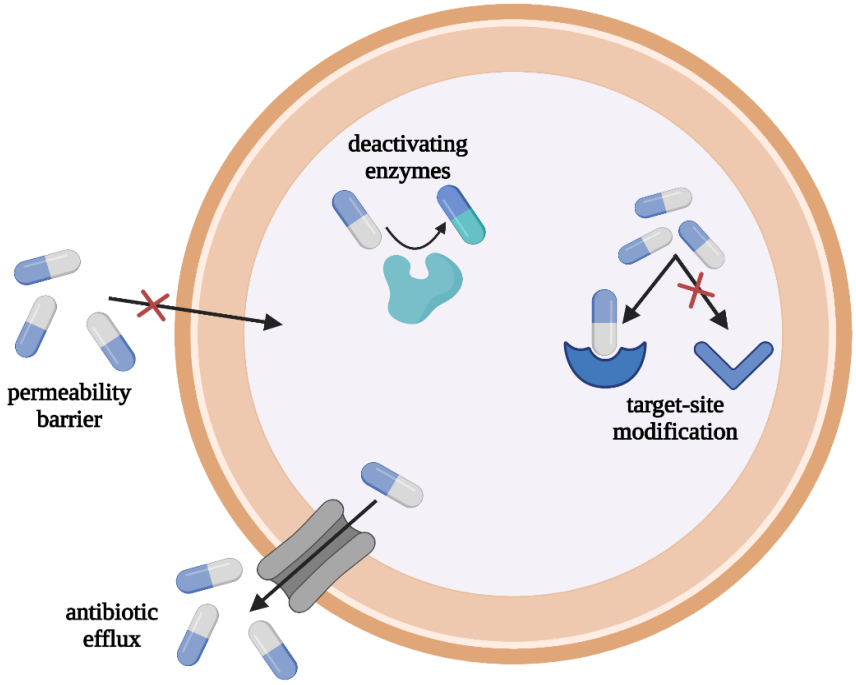

Figure 3.4. Mechanisms of resistance in gram-positive bacteria. Created with BioRender.com.

3.3 Current approaches to preventing and eradicating biofilms

The majority of antibiotics have been developed to generally target microbes in their planktonic state. As a result of this, most bacteria have developed a number of mechanisms in response to this treatment (**Figure 3.4**). Antimicrobial resistance falls into two categories: intrinsic and acquired. Intrinsic resistance mechanisms include limiting drug uptake through permeability barriers, pumping drugs out through non-specific efflux pumps, and inactivating antibiotics by bacterial enzymes. Acquired resistance genes are usually attained through horizontal gene transfer and their mechanisms include drug target site mutations, drug-specific efflux pumps, and modification of antibiotic-inactivating enzymes. However, bacteria within the biofilm are up to 1000 times more resistant to antibiotics compared to planktonic bacteria as a result of additional resistance mechanisms.[41-43]

While the EPS matrix of the biofilm does not provide an impenetrable barrier against antimicrobial dispersion, additional mechanisms contribute to increased antibiotic resistance and tolerance when compared to cells in the planktonic state. Reduced growth rates, harsh microenvironments, and drug interaction with EPS components all play a role in resistance development.[44] The cells located deep within the biofilm are metabolically dormant because they are deprived of nutrients and oxygen.[45, 46] This lifestyle limits the activity of essential cell functions including cell division, cell-wall synthesis, and translation.[45] The binding of constituents within the EPS matrix such as the negatively charged exopolysaccharides to positively-charged antibiotics also reduces antibiotic susceptibility. Due to the absence of selective antibiofilm drugs, most current treatments options against biofilm infections involve the long-term administration of high doses of one or more antibiotics.[47] Alternative antibiofilm strategies target prevention, dispersal, and disruption of biofilm communities.

Biofilm dispersal is an attractive approach to treat these infections. This mechanism releases bacteria back into its planktonic state in which these free-floating cells are more susceptible to antibiotic treatment. Dispersal mechanisms can be classified into two subtypes, active and passive. Active dispersion is initiated by the bacteria themselves through production of extracellular, degradative enzymes.[48] This is typically in response to environmental cues such as nutrient starvation, oxygen levels, nitric oxide, and antimicrobial pressure.[49] Passive dispersion involves direct removal of cells from the biofilm triggered by external forces including fluid shear, abrasion, and mechanical interventions.[49] Biofilm dispersal agents are most often used as an antibiotic adjuvant, as cells in the planktonic state are at more risk of spreading infection and colonizing new sites if left untreated.

Promising strategies aimed at biofilm disruption include the use of antimicrobial peptides (AMPs), nitric oxide-releasing antibiotics, enzymes, nanocarriers, and quorum sensing inhibitors (**Figure 3.5**). AMPs, often referred to as cationic antimicrobial peptides, are typically composed of between 12 and 50 amino acids and are rich in positively charged arginine and lysine residues.[47, 50] While most of the over 3000 natural peptides discovered to date have been studied for their antimicrobial activity, more recently they have been evaluated for antibiofilm activity.[51] The antibiofilm mechanisms of action of AMPs are independent from those antimicrobials used to treat planktonic cells. Mechanistically, AMPs are recognized to work by either membrane permeabilization or targeting intracellular components.[47] The human-derived cathelicidin LL-37 was the first identified AMP to both inhibit and eradicate biofilms in the gram-negative *Pseudomonas aeruginosa* and the gram-positive *Staphylococcus epidermidis* at sub-inhibitory concentrations.[52, 53] β-defensins are another class of AMPs that reduce the expression of biofilm production genes.[47] As a defense against AMP treatment, bacteria produce extracellular, peptide-degrading proteases or sequester AMPs with membrane-associated proteins.[54]

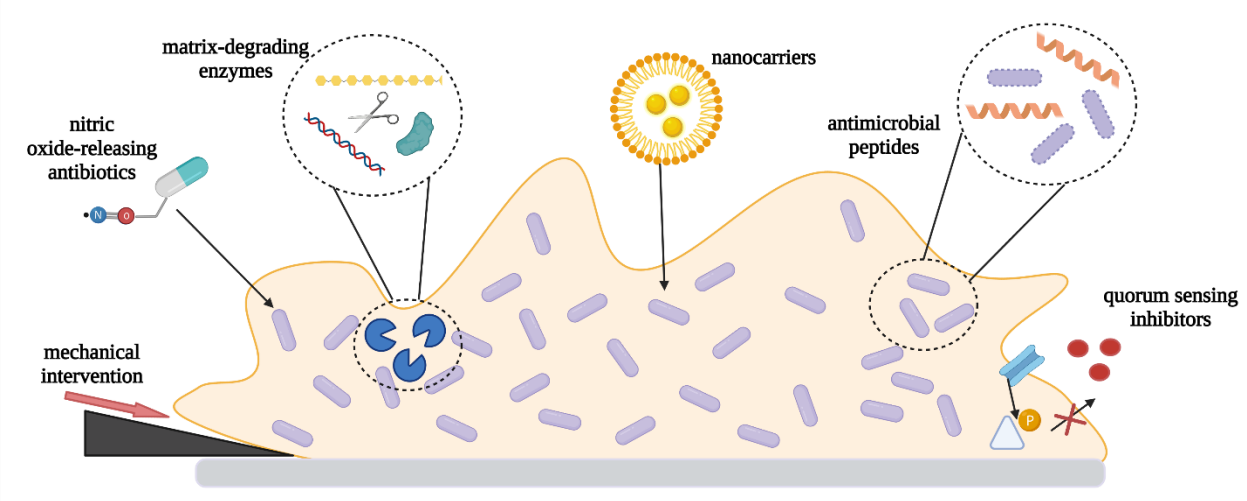

Figure 3.5. Biofilm dispersal strategies. Created with BioRender.com.

First discovered in *P. aeruginosa*, nitric oxide (NO) has shown to mediate biofilm dispersion at low concentrations against a myriad of microbial species.[55] NO treatment initiates production of phosphodiesterase enzymes, directly reducing the concentration of cyclic di-guanosine monophosphate (c-di-GMP) by hydrolytic cleavage of the phosphodiester bond .[56] C-di-GMP metabolism is highly correlated with bacterial pathogenicity through the establishment and colonization of biofilms. Due to the cytotoxic nature of nitric oxide, the gas is often delivered as a nitric oxide-functionalized antibiotic or prodrug, or as a synergistic combination with traditional antibiotics.[5, 42]

As mentioned above, bacteria produce extracellular enzymes that initiate biofilm dispersal by degrading matrix polymeric components such as proteins, extracellular DNA, exopolysaccharides, and lipids. These matrix-degrading enzymes can be categorized as glycosidases, proteases, and deoxyribonucleases. Dispersin B, a glycosyl hydrolase produced by the periodontal pathogen *Aggregatibacter actinomycetemcomitans*, is a well-known biofilm-dispersing agent. It has shown to display potent antibiofilm activity against a range of pathogens by degrading the matrix adhesive polysaccharide poly-*N*-acetylglucosamine (PNAG) through hydrolytic cleavage of the β-1,6 glycosidic linkages.[49] As a major component of the EPS, extracellular proteins are targeted for biofilm dissemination. Proteinase K treatment is known to initiate protein degradation and the release of nucleic acids.[44, 57] Extracellular DNA is an essential component of the EPS required for structural integrity and adhesion. Therefore, it is an attractive target for biofilm dispersal. DNase I has been identified as a treatment option for the prevention and removal of established biofilms by increasing matrix permeability, resulting in an increase of antibiotic efficacy.[44, 58, 59]

Nanoparticles have recently gained attention as a class of antibiofilm compounds due to their ability to improve the efficacy of antibiotic treatment through sustained drug release. These

particles are effective drug carriers because of their versatility, biocompatibility, low toxicity, and targeted delivery.[60] They act as a protectant to the antimicrobial agents against enzymatic destruction or electrostatic binding to the EPS. Since the discovery of the Doxil®, the first liposomal nanodrug approved by the Food and Drug Administration in 1995, several advances have been made in drug design and delivery.[61] Cationic lipid and polymer-based nanocarriers have been shown to be most effective in dispersing biofilms since both the EPS and bacterial cell wall are mostly anionic in nature.[62, 63] They are able to easily penetrate through the matrix and diffuse into the cell where they can release the drug to their intended target. Metallic nanoparticles composed of silver, gold, zinc oxide or copper oxide also have been demonstrated to be a promising strategy against biofilms, however, their high toxicity is of a significant concern.[64, 65]

Although it is not entirely clear how quorum sensing systems are involved in biofilm formation (see Section 3.2.1), targeting these pathways recently has been proposed as an antibiofilm strategy. Quorum sensing (QS) inhibitors are designed to inhibit the cell-to-cell communication amongst bacteria by preventing the signaling molecules from being detected, blocking the receptor proteins, or interrupting the signaling cascade. Gram positive bacteria use TCS (see Section 1.6) for the synthesis of these signaling molecules referred to as autoinducing peptides (AIPs). Enzymatic degradation or antibody neutralization of AIPs is the most common mechanism for quorum sensing inhibition.[66, 67] Some QS inhibition molecules competitively bind to the receptor site, therefore, preventing binding with the native AIPs.[66, 67] The mechanism of blocking the signal transduction cascade involves targeting the response regulators or regulatory factors.[66, 67]

3.4 Antibiofilm properties of heterogeneous HMOs against GBS

Early into our group's exploration into the antimicrobial properties of HMOs, we discovered they possess potent antimicrobial activity against several bacterial pathogens (see Section 2.6).[68] We

also investigated their ability to inhibit biofilm production against GBS, *A. baumannii*, and S. aureus (MRSA). Bacterial growth was evaluated at 24 hours by measuring optical density at 600 nm (OD_{600}). Optical density is used to estimate the concentration of bacterial cells in a culture per volume by measuring the amount of light scattered by the bacterial suspension. Following this biomass quantification, the growth medium was removed, leaving only the adherent bacterial cells. The wells were gently washed and stained with crystal violet. Biofilm was quantified by measuring optical density at 560 nm (OD_{560}). To account for any accompanying antimicrobial activity, biofilm production is expressed as a ratio of biofilm to biomass (OD_{560}/OD_{600}). In this assay, Townsend et al. found that a heterogenous mixture of HMOs pooled from 14 donors possessed antibiofilm activity against three strains of GBS and MRSA (**Table 3.1**).[68] Quantitatively, the pooled HMOs inhibited biofilm in GBS up to 93%, and in MRSA up to 60%. Unfortunately, we did not find any antibiofilm activity in the *A. baumannii strain* we tested.

Table 3.1. Antibiofilm activity of pooled HMOs against various bacterial pathogens.

Bacteria	Biofilm Inhibition
S. agalactiae (GBS)	93%
S. aureus (MRSA)	60%
A. baumannii	0%

3.5 Converting HMOs to antibiofilm compounds by way of the Kochetkov amination

In initial work, the Townsend group evaluated the antimicrobial and antibiofilm activity of a pooled mixture of HMOs. A pooled mixture is defined as combining milk samples from two or more people. We sought to explore the biological activity of single-entity HMOs with a goal of understanding which residues are responsible for specific functions vis-à-vis structure-activity relationships (SAR). While several individual HMOs did possess potent antimicrobial activity, most of these same compounds did not inhibit biofilm formation.[69, 70] We sought to discover new

antibiofilm compounds based on the HMO structure and elucidate how these compounds interact with biofilm disruption. We hypothesized that we could synthesize antibiofilm compounds by incorporating a positive charge on the anomeric carbon of single-entity HMOs.

In a pilot study initiated by Dr. Kelly Craft, 2′-fucosyllactose (2'-FL) was converted to the β-amino variant (βA-2′-FL, **3.1**) and evaluated for its antimicrobial and antibiofilm activity.[71] 2'-FL was initially chosen because it is the most abundant fucosylated oligosaccharide found in human milk and has been shown to play an important role in building up the infant gut microbiome.[72] 2'-FL serves as a prebiotic, promoting the growth of beneficial *Bifidobacterium* spp., while additionally protecting gut epithelium from pathogen adhesion by acting as a soluble decoy receptor for surface binding.[72-74] While neither compound possessed antimicrobial activity in GBS, the β-amino compound decreased biofilm production up to 46%. In this study, the mechanism of action remained unclear, but it was hypothesized that the positively charged glycan destabilized the biofilm matrix largely bound together by anionic molecules.[75]

3.6 Expanded β-amino HMO studies

We expanded this study to include the synthesis of three additional β-amino HMO compounds that spanned across fucosylated, acidic, and non-fucosylated neutral HMOs.[76] We initially questioned whether our results were exclusive to the structure of βA-2′-FL (**3.1**). We also proposed that we could expand our studies to different strains of gram-positive pathogens, including the ESKAPE pathogen MRSA. The three additional oligosaccharides were chosen based on the addition of a bulkier group near the reducing end (3-FL, **3.2**), a longer chain (LNT, **3.3**), and an acidic residue (6'-SL, **3.4**), allowing us to explore the implications of structure on biofilm formation.

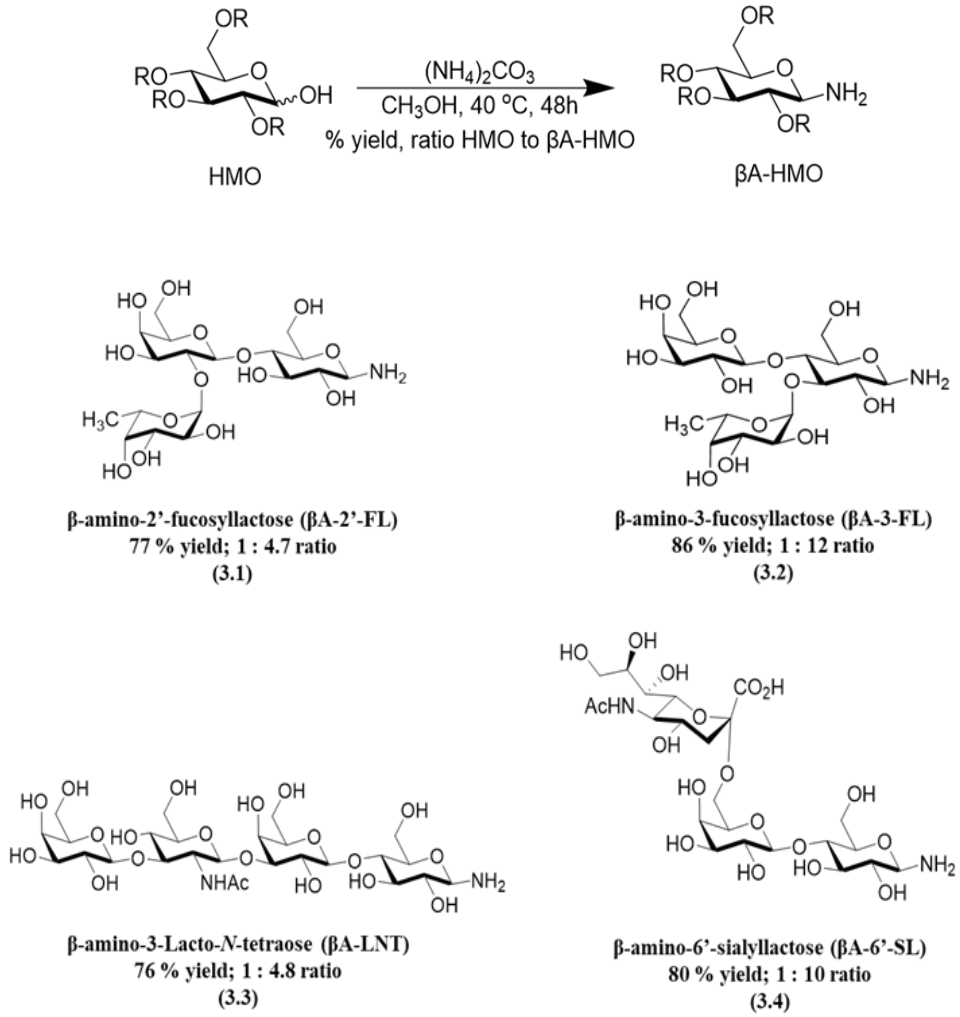

Figure 3.6. Synthesis of the four βA-HMOs used in the antibiofilm study under Kochetkov amination reaction conditions.

The four β-amino HMO compounds were synthesized under Kochetkov amination conditions (**Figure 3.6**). The Kochetkov reaction is an effective route for the synthesis of glycosylamines via placement of an amine at the reducing end of monosaccharides.[77] This reaction is performed without the use of any protecting groups to stereoselectively convert the starting glycan to solely the β isomer. Traditionally, this reaction requires 40-50-fold excess of the aminating agent and long reactions (up to 5 days) under thermal conditions. Microwave-assisted synthesis has enabled a more efficient route, shortening reaction times significantly and reducing the amount of the ammonium salt by 10-fold.[78-80] However, since our syntheses involves starting materials composed

of either three or four monosaccharides, it was necessary to tease out the ideal conditions needed for these complex oligosaccharides. Using 2'-FL as our model substrate, we first screened four different ammonia sources: ammonium carbonate ($(NH_4)_2CO_3$), ammonium bicarbonate (NH_4HCO_3), ammonium chloride (NH_4Cl), and ammonia (NH_3) in methanol, identifying $(NH_4)_2CO_3$ as top aminating agent. Interestingly, microwave conditions produced low-yielding β-amine products; therefore, we chose to explore thermal reaction conditions. We found that using 5-fold excess of $(NH_4)_2CO_3$ in methanol at 40 ^oC for 48 hours yielded superior conversion ratios of starting material to product when compared to microwave conditions in all four HMOs (**Figure 3.6**).

3.7 β-amino HMOs inhibit microbial adhesion in GBS and MRSA

Both the antimicrobial and antibiofilm activity were assessed in GBS (GB00590) and MRSA (USA300). To assess antimicrobial activity, bacteria was grown in the presence of the ca. 5 mg/mL of β-amino HMOs or their parent compounds over a period of 24 hours. Bacteria grown in media alone and the parent HMOs served as controls. Biofilm was assessed at 24 hours and expressed as a ratio of biofilm to biomass to account for any accompanying antimicrobial activity. As expected, based on previous studies, no single compound evaluated displayed significant antimicrobial activity in either GBS (**Figure 3.7A**) or MRSA (**Figure 3.7B**). Following the same trends as in the previous study, while no parent HMOs displayed antibiofilm activity, all the β-amino HMOs significantly inhibited biofilm production in both strains (**Figure 3.8**). In GBS, biofilm production was decreased by an average of 62% and in MRSA by an average of 42%.

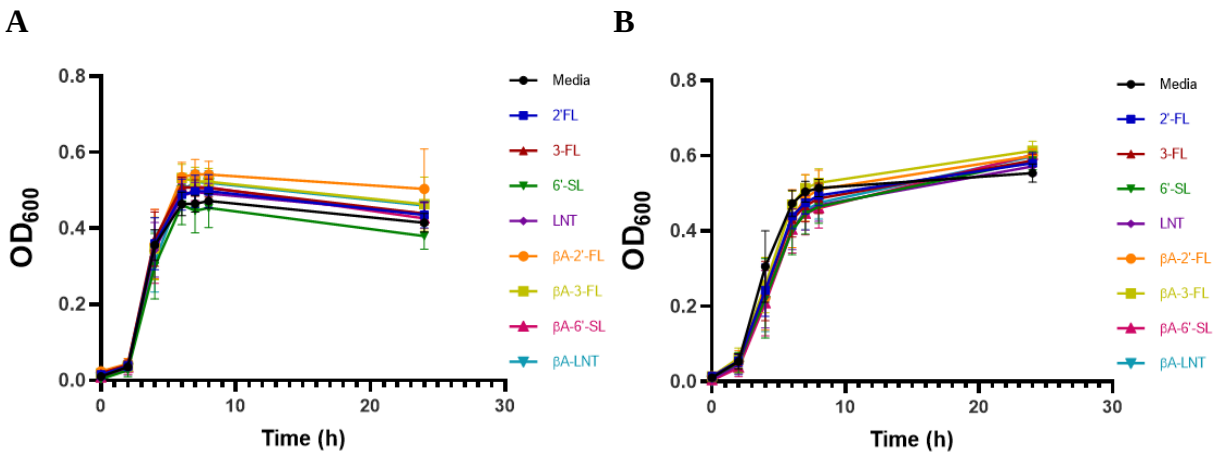

Figure 3.7. Evaluation of HMO and βA-HMO antibacterial activity at ca. 5 mg/mL on (A) GBS (GB00590) and (B) methicillin-resistant *S. aureus* (USA300). Growth was quantified via OD600 readings at 0, 2, 4, 6, 7, 8, and 24 h. Data displayed represent the relative mean growth ± SEM of three independent experiments, each with three technical replicates.

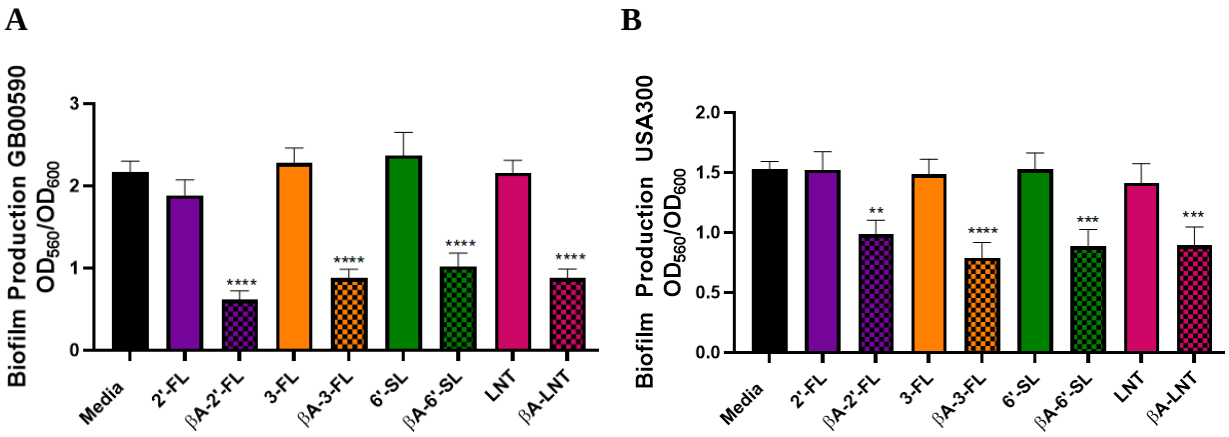

Figure 3.8. Evaluation of HMO and βA-HMO antibiofilm activity at ca. 5 mg/mL in (A) GBS (GB00590) and (B) methicillin-resistant *S. aureus* (USA300). Biofilm was quantified via OD_{560} readings at 24 h. Biofilm production is expressed as a ratio of biofilm/biomass (OD_{560}/OD_{600}). Data displayed represent the relative mean biofilm/biomass ratios ± SEM of three independent experiments, each with three technical replicates. In (A) and (B), **** represents $p < 0.0001$, *** represents $p = 0.0010$, and ** represents $p = 0.0081$ by one-way ANOVA with *post hoc* Dunnett's multiple comparison test comparing biofilm production in HMO-supplemented Todd-Hewitt Broth (THB) to biofilm production in HMO-free THB.

To further explore SAR of the βA-HMOs, we questioned whether any amino substituents at the C1-position at the reducing end would inhibit biofilm formation in gram-positive species. It is important to note that ammonia sources alone did not influence biofilm activity (**Figure A.1.1**). We chose two monosaccharides, glucose and galactose, and one disaccharide, lactose. Following

the same thermally activated Kochetkov amination conditions, the three carbohydrates were converted to their β-amino equivalents and assessed for antibiofilm activity. We were greatly encouraged as we observed with the βA-HMOs, all three βA-glycans significantly inhibited biofilm production in both GBS and MRSA when compared to the medium alone control (**Figure 3.9**). Additionally, while two of the parent sugars increased biofilm growth, glucose in GBS and MRSA, and lactose in MRSA, all three of the βA-sugars significantly decreased biofilm when compared to the parent molecule. Together, these two studies help validate our hypothesis that amino substituents at the C1-position inhibit biofilm formation; however further exploration into SAR of additional HMOs will be needed to confirm the above noted results.

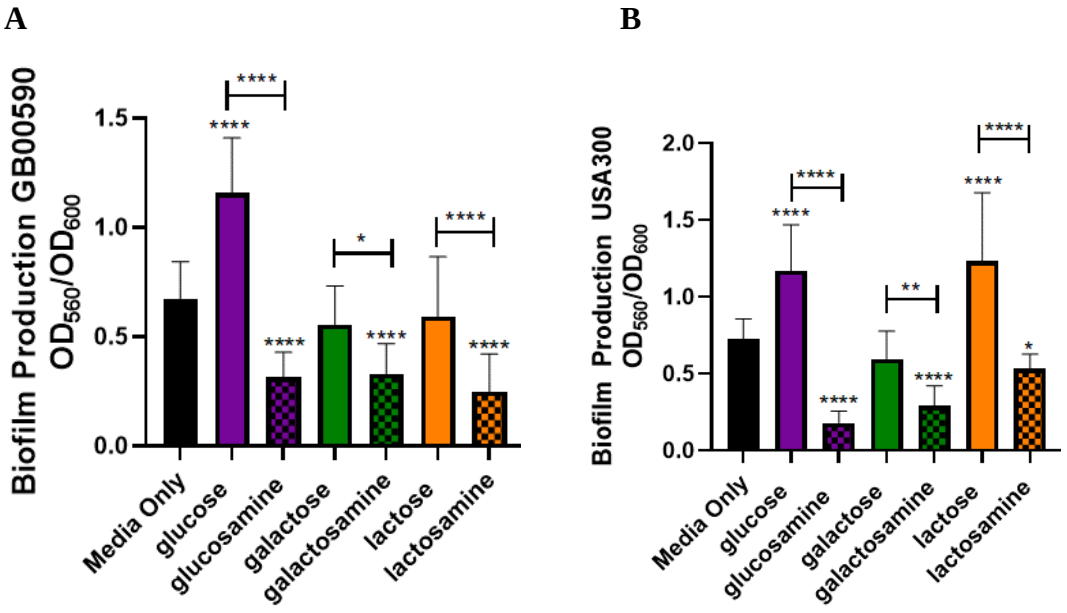

Figure 3.9. Evaluation of amino mono- and disaccharides on biofilm activity at ca. 5 mg/mL in (A) *S. agalactiae* (GB00590) and (B) methicillin-resistant *S. aureus* (USA300). Biofilm was quantified via OD_{560} readings at 24 h. Biofilm production is expressed as a ratio of biofilm/biomass (OD_{560}/OD_{600}). Data displayed represent the relative mean biofilm/biomass ratios ± SEM of three independent experiments, each with three technical replicates. In (A) and (B), **** represents $p < 0.0001$, ** represents $p = 0.0015$, and * represents $p = 0.0130$ by one-way ANOVA with *post hoc* Dunnett's multiple comparison test comparing biofilm production in HMO-supplemented Todd-Hewitt Broth (THB) to biofilm production in glycan-free THB.

3.8 Visualization of β-amino HMO supplementation on GBS and MRSA biofilms

To further assess biofilm production due to the presence of β-amino HMOs, field emission gun-scanning single electron microscopy (FEG-SEM) was employed to visualize changes in biofilm architecture (**Figure 3.10**). This technique allowed us to visually analyze structural differences between the biofilms upon supplementation with the β-amino HMOs when compared to the bacteria in media alone. *S. agalactiae* (GB00590) is classified as a gram-positive, diplococcus species that is typically found in pairs with the tendency to form chains. When GBS is grown in medium alone, the cells tend to form globular microcolonies with vertical cell stacking. Except for 3-FL, all compounds imaged as expected based on the crystal violet biofilm assay. Both 2'-FL and 6'-SL displayed similar biofilm architecture and adherence to the coverslip as to the media alone control. 3-FL adhered significantly less than the control, but still formed in smaller microcolonies. Upon supplementation with the βA-HMOs, both bacterial adherence and biofilm formation was limited to small chains, losing all globular structure.

Like GBS, *S. aureus* (USA300) is classified as a cocci-shaped gram-positive bacterium that tend to form in "grape-like" clusters. In media alone, *S. aureus* easily adhered to the coverslip, forming robust biofilms with numerous nutrient channels spanning the extracellular matrix. Both 2'-FL and 3-FL supplementation did not modify the architecture of the biofilms, but 6'-SL formed smaller colonies. All three βA-HMOs almost completely abolished biofilm formation with limited bacterial adherence.

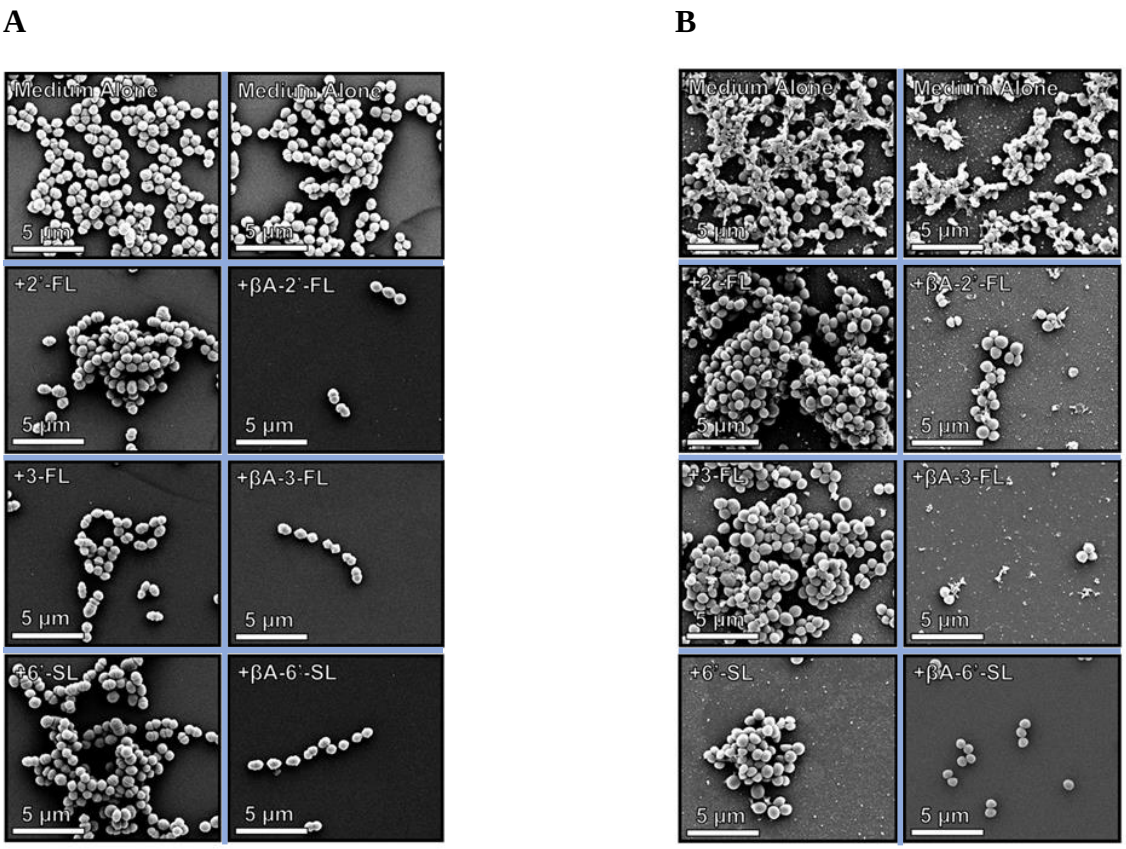

Figure 3.10. High-resolution FEG-SEM analyses of (A) GBS (GB00590) and (B) methicillin-resistant *S. aureus* (USA300) bacterial biofilm formation. FEG-SEM imaging of bacterial biofilms were performed on GBS and MRSA samples grown in medium alone (Medium Alone), or medium supplemented with 2′-FL (+2′-FL), βA-2′-FL (+βA-2′-FL), 3-FL (+3-FL), βA-3-FL (+βA-3-FL), 6′-SL (+6′-SL), or βA-6′-SL (+βA-6′-SL). Micrographs were collected at 20000× magnification, and magnification bars indicate 5 µm.

3.9 Conclusions and future outlook

In this study we synthesized four antibiofilm compounds through the conversion of single-entity HMOs to their β-amino derivatives utilizing thermal Kochetkov amination conditions. We evaluated these compounds for their ability to inhibit bacterial growth and biofilm production in two gram-positive species, Group B *Streptococcus* (GBS) and methicillin-resistant *Staphylococcus aureus* (MRSA). Using a colorimetric assay and visualizing using FEG-SEM, we observed that all four βA-HMOs significantly suppressed microbial adherence, therefore, restricting the ability of both pathogens to form biofilms.

Our initial studies on these β-amino antibiofilm compounds have focused on the gram-positive species GBS and MRSA. With the rise in antibiotic resistance, there is a critical need to find alternative treatments for the remaining ESKAPE pathogens, named for their ability to escape traditional antimicrobial treatment. It is not surprising that four of these six pathogens are gram-negative species as these strains are intrinsically harder to treat due to the presence of both an outer membrane in addition to the peptidoglycan cell wall and cytosolic membrane. The ability of these organisms to form robust biofilms is associated with the pathogenicity of these infections. In addition to inhibiting bacterial growth, it is also imperative to find treatments to prevent biofilm formation during the attachment stage. Our early focus will be on two of the gram-negative ESKAPE pathogens, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, and the ability of β-amino HMOs to inhibit biofilm formation. Preliminary data in *K. pneumoniae* suggests we will see similar trends to what we observed in gram-positive bacteria (**Figure A1.2**).

Our future work will also focus on evaluating the molecular response bacteria experience when they engage with β-amino HMOs. Proteomic techniques have become a powerful tool to promote a better understanding of the mechanisms of microbial pathogenesis and their response to environmental stressors. Proteomic analysis allows us to identify alterations in protein content, pathways, signaling cascades, and protein-protein interactions.[81] We have hypothesized that these positively-charged glycans are interacting with negatively-charged biofilm matrix components such as DNA and polysaccharides with the knowledge that cationic molecules destabilize the extracellular matrix of biofilms.[75, 82] Additionally, we have hypothesized the β-amino compounds are disrupting bacterial adherence, therefore, limiting biofilm-forming capacity. Proteomic analysis will help us gain insight into whether bacterial appendages such as pili or flagella, or two-component systems involved in biofilm production are affected by β-amino supplementation.

3.10 Experimental methods

Bacterial strains and culture conditions

Strain	Source
S. agalactiae GB00590	clinical isolate, Shannon Manning, Michigan State
S. aureus strain USA300	The *S. aureus* strain used was USA300 JE2,[83] a laboratory-adapted strain derived from the parental USA300 strain isolated from a skin and soft tissue infection.[84]
A. baumannii strain 19606	ATCC
K. pneumoniae 13883	ATCC

All strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37°C in ambient air overnight. All strains were subcultured from blood agar plates into 5 mL of Todd-Hewitt broth (THB) and incubated under shaking conditions at 180 rpm at 37°C overnight. Following overnight incubation, bacterial density was quantified through absorbance readings at 600 nm (OD_{600}) using a Promega GloMax-Multi detection system plate reader. Bacterial numbers were determined using the predetermined coefficient of an OD_{600} of 1, equal to 10^9 CFU/mL.

Bacterial growth assays

Bacterial strains were grown overnight as described above and used to inoculate fresh THB at a multiplicity of infection (MOI) of 10^6 colony forming units per 200 μL of growth medium in 96 well tissue culture treated, sterile polystyrene plates (Corning, Inc.). HMOs and βA-HMOs were dissolved in DI water to achieve a concentration of 80 mg/mL and filtered through a 0.2 μm syringe filter. HMOs or βA-HMOs were added to achieve a final carbohydrate concentration of ca. 5

mg/mL. Bacteria grown in THB in the absence of any HMOs served as the control. Cultures were grown under static conditions at 37 °C in ambient air for 24 h. Growth was quantified through spectrophotometric reading at OD_{600} with readings taken at 0, 2, 4, 6, 7, and 8 h then a final reading at 24 h.

Bacterial biofilm assays

Bacterial strains were grown overnight as described above and used to inoculate fresh THB at a multiplicity of infection (MOI) of 10^6 colony forming units per 200 μL of growth medium in 96 well tissue culture treated, sterile polystyrene plates (Corning, Inc.). HMOs and βA-HMOs were dissolved in DI water to achieve a concentration of 80 mg/mL and filtered through a 0.2 μm syringe filter. HMOs or βA-HMOs were added to achieve a final carbohydrate concentration of ca. 5 mg/mL. Bacteria grown in THB in the absence of any HMOs served as the control. Cultures were incubated under static conditions at 37 °C in ambient air for 24 h. Bacterial growth was quantified through absorbance readings at an optical density of 600 nm (OD_{600}). Following growth quantification, the culture medium was removed, and wells were washed gently with phosphate buffered saline (PBS, pH 7.4) to remove nonadherent cells. The remaining biofilms were stained with a 10% crystal violet solution for 10 min. Following staining, wells were washed with PBS and allowed to dry at room temperature for at least 30 min. The remaining crystal violet stain was solubilized with 200 μL of 80% ethanol/20% acetone solution. Biofilm formation was then quantified through absorbance readings at an optical density of 560 nm (OD_{560}). Results are expressed as biofilm/biomass ratios (OD_{560}/OD_{600}).

High-resolution field-emission gun scanning electron microscopy (FEG-SEM) analyses

Bacterial biofilms were analyzed via FEG-SEM as previously described. (32–34) Briefly, bacterial cells were cultured in biofilms adhering to glass coverslips coated with poly-l-lysine overnight in the culture conditions described above. HMOs and βA-HMOs were dissolved in DI water to achieve a concentration of 80 mg/mL and filtered through a 0.2 μm syringe filter. HMOs or βA-HMOs were added to achieve a final carbohydrate concentration of ca. 5 mg/mL. The following day, bacterial cells were fixed in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 0.05 M sodium cacodylate buffer pH 7.4. Samples were dehydrated with sequential washes of increasing concentrations of ethanol before being subjected to critical point drying, mounting on aluminum stubs, and sputter coating with 20 nm of gold–palladium. Samples were viewed using an FEI Quanta 250 field-emission gun scanning electron microscope at 5 kEV with a spot size of 2.5.

Statistical analyses

All data shown signify three independent experiments each with three technical replicates. Data are expressed as the mean ± SEM. Statistical analyses were performed in GraphPad Prism Software v. 8.2.1. Statistical significance for growth was determined using two-way ANOVA with post hoc Dunnett's multiple comparison test comparing growth in the presence of HMOs, βA-HMOs, or ammonia sources to growth in media alone. Statistical significance for biofilm production was determined using one-way ANOVA with post hoc Dunnett's multiple comparison test comparing biofilm production in the presence of HMOs or βA-HMOs to biofilm production in media alone.

Microwave-activated Kochetkov amination procedure

HMO (0.1 mmol) was dissolved in solvent (2 mL), ammonia source (5× mass of HMO) was added, and the reaction was irradiated for 1 h at 200 W and 50 °C. The reaction mixture was diluted to 45 mL with water in a 50 mL conical centrifuge tube, frozen with liquid nitrogen, and lyophilized repeatedly until a constant mass of white solid was obtained. The ratio of conversion was determined by integration of the C-1 anomeric protons of the starting material to that of the desired product (**Table A1.1**).

Thermally activated Kochetkov amination procedure

HMO (0.1 mmol) was dissolved in solvent (2 mL), ammonia source (5× mass of HMO) was added, and the reaction warmed for 48 h at 40 °C in an oil bath. The heating medium was silicone oil purchased from Sigma-Aldrich (St. Louis, MO, USA) and was carried out in a Pyrex crystallizing dish. The reaction mixture was diluted to 45 mL with water in a 50 mL conical centrifuge tube, frozen with liquid nitrogen, and lyophilized repeatedly until a constant mass of white solid was obtained. Ratio of conversion was determined by integration of the C-1 anomeric protons of the starting material to that of the desired product (**Table A1.1**).

This chapter was adapted from "Leveraging Stereoelectronic Effects in Biofilm Eradication: Synthetic β-Amino Human Milk Oligosaccharides Impede Microbial Adhesion as Observed by Scanning Electron Microscopy" published in The Journal of Organic Chemistry and has been reproduced with the permission of the publisher and my co-authors including: Kelly M. Craft, Lianyan L. Xu, Schuyler A. Chambers, Johny M. Nguyen, Keevan C. Marion, Jennifer A. Gaddy, and Steven D. Townsend. Moore RE, Craft KM, Xu LL, Chambers SA, Nguyen JM, Marion KC, Gaddy JA, Townsend SD. 2020 Leveraging Stereoelectronic Effects in Biofilm Eradication: Synthetic β-Amino Human Milk Oligosaccharides Impede Microbial Adhesion As Observed by Scanning Electron Microscopy. J. Org. Chem 85 (24), 16128-16135.
https://doi.org/10.1021/acs.joc.0c01958

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Appendix A1:
Data Relevant to Chapter 3

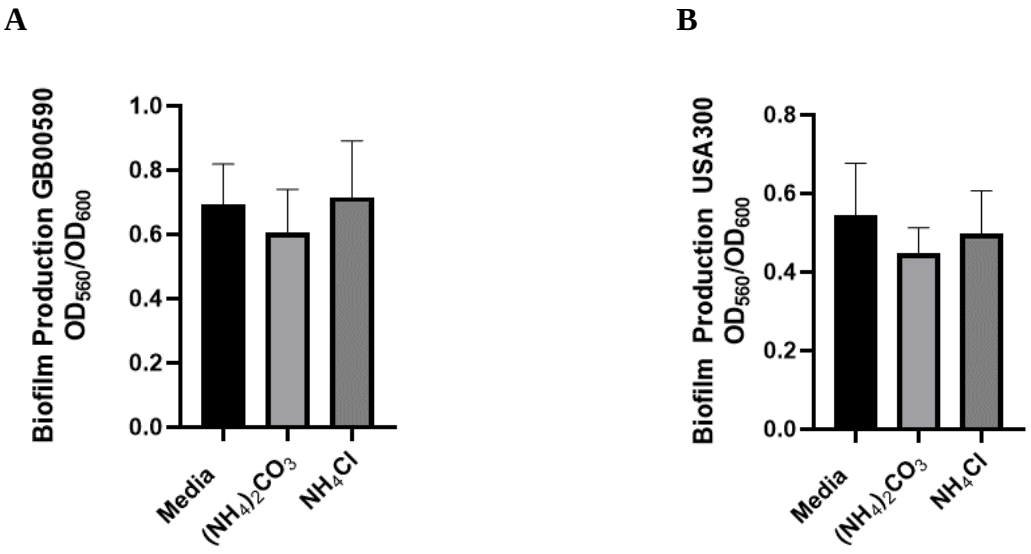

Figure A1.1. Evaluation of ammonia sources on biofilm production at ca. 5 mg/mL in (A) *S. agalactiae* (GB00590) and (B) methicillin-resistant *S. aureus* (USA300). Biofilm was quantified via OD_{560} readings at 24 h. Biofilm production is expressed as a ratio of biofilm/biomass (OD_{560}/OD_{600}). Data displayed represent the relative mean biofilm/biomass ratios ± SEM of three independent experiments, each with three technical

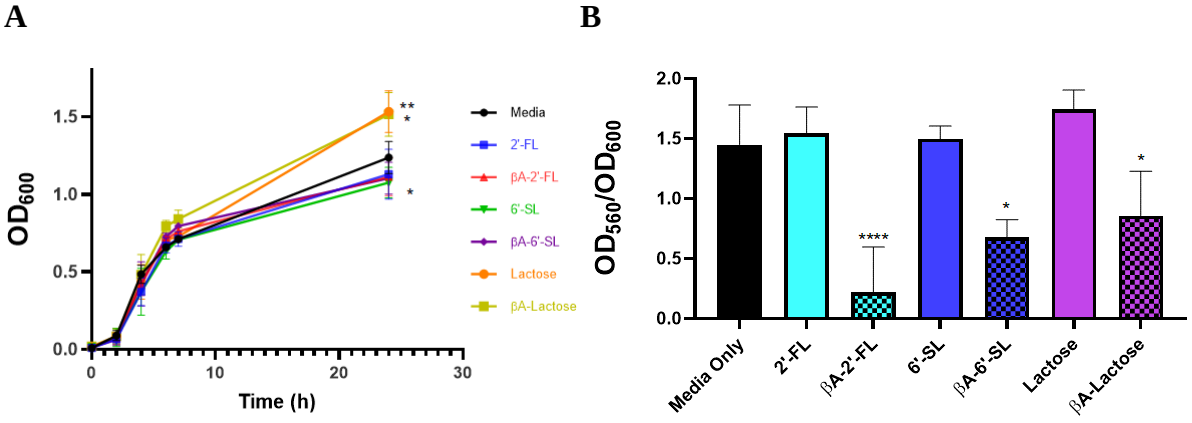

Figure A1.2. Evaluation of HMO and βA-HMO antibacterial activity at ca. 5 mg/mL *K. pneumoniae* (ATCC 13883). Growth was quantified via OD_{600} readings at 0, 2, 4, 6, 7, 8, and 24 h. Mean OD_{600} for each time point is indicated by the corresponding symbols. Biofilm was quantified via OD_{560} readings at 24 h. Biofilm production is expressed as a ratio of biofilm/biomass (OD_{560}/OD_{600}). (A) Growth of *K. pneumoniae* in the presence of parent HMOs and βA-HMOs. (B) Biofilm production of *K. pneumoniae* in the presence of parent HMOs and βA-HMOs. Data displayed represent the relative mean growth or biofilm/biomass ratios ± SEM of three independent experiments, each with three technical replicates. In (A) ** represents p = 0.0073, and * represents p = 0.0405 and p = 0.0139 by by two-way ANOVA with post hoc Dunnett's multiple comparison test comparing the growth of *K. pneumoniae* in each oligosaccharide supplementation condition to the growth of *K. pneumoniae* in medium alone. In (B) **** represents p < 0.0001, and * represents p = 0.0149 and p = 0.0302 by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing biofilm production in carbohydrate-supplemented Todd-Hewitt Broth (THB) to biofilm production in carbohydrate-free THB.

Table A1.1 Anomeric Proton Shifts to determine HMO: βA-HMO Ratio based on ^{1}H NMR

HMO	HMO shift (ppm)	βA - HMO	βA – HMO shift (ppm)
2'-FL	5.10	βA -2'-FL	3.97
3-FL	5.07	βA -3-FL	3.97
6'-SL	5.13	βA -6'-SL	4.01
LNT	5.10	βA -LNT	3.98

* 1H NMR data obtained in MeOD

Chapter 4

Governing bacterial competition in a two species microbiome through HMO supplementation

4.1 Abstract

The temporal development of the infant microbiome is driven by a variety of early life events with feeding choice of particular importance. Both breast milk and infant formula contain a prebiotic component- either naturally occurring, unique human milk oligosaccharides (HMOs) or supplemented plant-based oligosaccharides. These carbohydrates modulate the gut microbiota selectively promoting the growth of commensals over pathogens. To characterize how prebiotics govern these relationships, we have designed a two species microbiome in which pathogenic Group B *Streptococcus* (GBS) interacts with the commensal, *Streptococcus salivarius*. We discovered that, while GBS suppresses the growth of *S. salivarius* attributed to increased lactic acid production, galacto-oligosaccharides (GOS) supplementation circumnavigates this effect. This result contributes to our understanding on how carbohydrate metabolism affects commensal-pathogen interactions in the developing microbiome.

4.2 The infant gut microbiome

The development of the gut microbiome from *in utero*, through parturition, and into early childhood plays a critical role in modulating essential functions related to immune, endocrine, neural, and metabolic pathways. Dysbiosis of the microbial community, especially early in life, has been implicated in a number of diseases and conditions including type 1 diabetes, asthma, inflammatory bowel disease, food allergies, Crohn's disease, autoimmune diseases, inflammatory bowel disease, and necrotizing enterocolitis.[1-5] The primary factors influencing the

establishment of the infant gut microbiota are maternal diet and antibiotic use, mode of delivery, gestational age at birth, feeding method, and introduction to solid foods (**Figure 4.1**; **Table 4.1**).

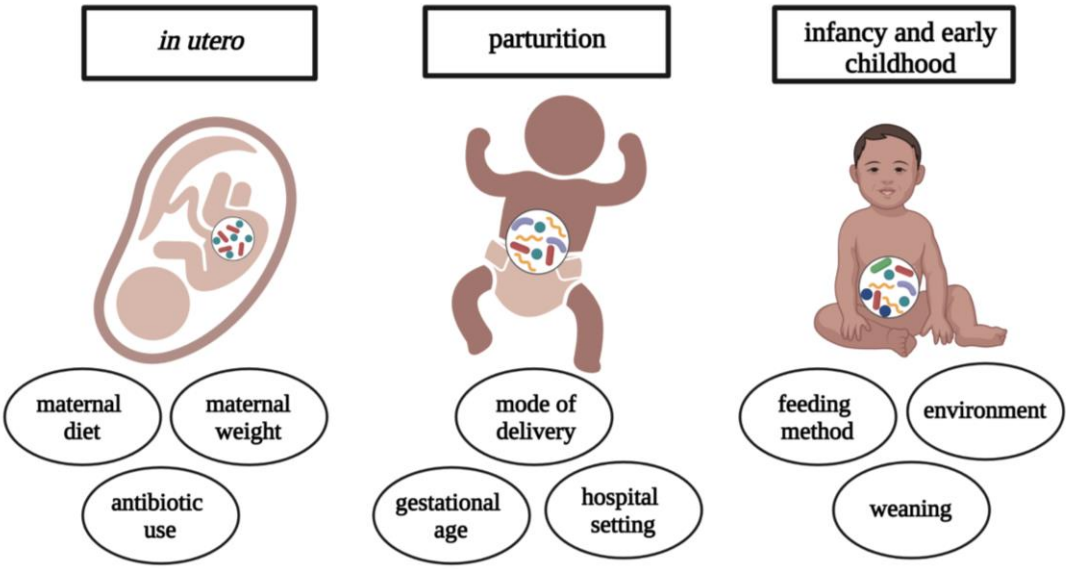

Figure 4.1. Factors contributing to gut microbial composition from gestation through early childhood. Created with BioRender.com.

4.2.1 Factors influencing the development of the microbial community

Traditionally, the developing fetal environment was thought to be sterile; however, as a result of recent studies challenging this hypothesis, this belief remains a highly debated subject.[6, 7] The current understanding with the "sterile womb paradigm" is that the human placenta remains free of any microbes until during and after birth.[8] This occurs vertically from the mother, or horizontally, either nosocomially or from the environment (see Section 1.4). The *in utero* colonization hypothesis states that microbial colonization begins prior to birth. Jimenez and et al., Collado et al. and Aagaard et al., were three of the first groups to use next-generation sequencing techniques such as 16S rRNA pyrosequencing, quantitative PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), and Illumina sequencing to detect microbial populations in the placenta, amniotic fluid, umbilical cord, and meconium from healthy pregnancies.[9-11]

However, this concept remains controversial since these techniques have inadequate detection limits to study "low density" bacterial populations.

Up to 25% of women are administered antibiotics during her pregnancy, with antibiotics accounting for 80% of all medications prescribed to pregnant women.[12-14] Antibiotics are necessary for the treatment of the urinary tract infections (UTIs), bacterial vaginosis, upper respiratory tract infections, and sexually transmitted infections.[12, 13] Additionally, ca. 50% of women in Western countries will receive antibiotics during labor and delivery (see Section 1.8.1).[15] Antibiotic use is standard practice for women delivering by Cesarean section (CS) to reduce the incidences of endometritis, UTIs, and wound infections.[16] As described in Section 1.7.1, IAP treatment is encouraged for those women planning a vaginal delivery who test positive for GBS cultures prior to the onset of labor. Prenatal antibiotic administration has been correlated with early disruptions in the microbial colonization and composition. Both CS-delivered infants and vaginally-delivered infants, whose mothers received IAP, exhibit overall lower microbial diversity when compared to infants whose mothers did not receive antibiotic treatment during labor and delivery. In general, these infants display lower levels of *Actinobacteria*, *Bacteroidetes*, *Bifidobacterium*, and *Firmicutes* spp., with increased abundance of *Proteobacteria* and *Clostridium* spp.[17-19]

Maternal diet during pregnancy has been shown to influence the developing fetal microbiome. Maternal obesity and high fat diets have been examined extensively on the impact this has for the infant's gut and immune function development. Besides the obvious health benefits to the mother associated with a low fat, high fiber diet, this régime is also positively correlated with increased microbial diversity in the fetus.[20] Collado et al. specifically uncovered that when compared to normal weight mothers, infants born to high BMI mothers were associated with elevated levels

of *Bacteroides* and *Staphylococcus* spp.[21] Additionally, diets high in vegetable and fruit consumption have been associated with the beneficial *Cutibacterium, Parabacteroides,* and *Lactococcus* spp., where low vegetable and fruit consumption is correlated with relative higher abundance of *Prevotella, Isobaculum, Hungatella*, *Lachnoclostridium, Flavonifractor*, *Erysipelatoclostridium, Phascolarctobacterium, Megamonas,* and *Sutterella* spp.[22, 23]

Mode of delivery (vaginal or CS) is one of the prominent contributors to fluctuations in the infant gut microbiome. Vaginal delivery usually is the ideal scenario; however, birth by CS is often a necessary procedure when the health or safety of the mother and/or baby is at risk. During a normal vaginal birth, the infant is exposed to maternal vaginal, fecal, and skin microbes through contact with the mother's vaginal and gut microflora. Babies born via CS have overall lower bacterial diversity with the majority of their microbiota made up of bacteria from the environment and their mother's skin. The gut microbes of vaginally delivered babies are dominated by *Bacteroidetes, Bifidobacterium, Lactobacillus, Prevotella,* or *Sneathia* spp., while CS-delivered babies are dominated by *Clostridium difficile,* and common skin microbes including *Staphylococcus, Streptococcus,* and *Propionibacterium* spp.[1, 24-26]

Infant feeding methods, primarily breastfeeding and formula feeding, have a large impact on how the gut microbiome develops, especially early in life. Breast milk remains the superior source of nutrition for infants during the first six months of life, however, breast milk is not always an option (see Section 2.2). Of all the beneficial components present in breast milk, the bifidogenic effect that HMOs exert on the infant's microflora is most significant (see Section 2.5.1). These abundant oligosaccharides selectively stimulate the growth of specific beneficial bacterial species establishing an environment rich in *Bifidobacterium* spp., accounting for 70% of all strains.[27] *B. bifidus, B. longum, B. infantis, B. breve, B dentium,* and *B. pseudocatenulatum*

are the dominant strains detected in the stools of breastfed babies.[28, 29] In addition to *Bifidobacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and the lactic acid-producing *Lactobacillus* spp., are present in high concentrations.[17, 24, 30] Advances in formula design have recently been made at an attempt to mimic the composition of human breast milk, however, the gut microbiota of formula fed babies remains distinct from their breast fed counterparts. Formula fed babies exhibit overall high bacterial diversity with higher abundances of *Clostridium*, *Staphylococcus*, *Bacteroides*, *Lactobacillu*s, and *Enterococcus* spp., and reduced presence of *Bifidobacterium* spp.[24, 26, 31, 32]

The period in which there is a switch from exclusively being fed by breastfeeding or formula feeding to an introduction to solid foods initiates a major shift in the structural and functional diversity of the microbial composition to a more adult-like state. Prior to this weaning period which begins at approximately four to six months, the gut favors the proliferation of *Bifidobacterium* spp. They are able to ferment HMOs and lactose: two of the largest components present in human breast milk. Following the cessation of breastfeeding (or formula feeding), a more complex microbial community is necessary to allow the growth of bacteria that are capable of utilizing complex carbohydrates, fats, proteins, and fiber. Following this transition persiod, the gut is dominated by *Lachnospiraceae*, *Bacteroides*, *Prevotella*, *Ruminococcus*, *Clostridium*, *Firmicutes*, and *Veillonella* spp., with decreased abundances of *Bifidobacterium*, *Staphylococcus* *Lactobacillu*s, *Enterococcus*, and *Enterobacter* spp.[24, 27, 33-35]

Other than the apparent determinants discussed above, environmental factors encountered in life play a pivotal role in gut microbial composition and variability. Pet and animal exposure, geographical location, and hospital environment all contribute to the developing microbiota. Somewhat surprisingly, the presence of indoor pets has been associated with a lower risk of

allergy development which has been linked to the altered microbial home environment of these households.[26] The accepted theory is that these pets introduce a vast variety of microbes into the home, sensitizing the infant's immune system. In homes with pets, babies are more likely to be colonized with *Peptostreptococcaceae,* and a lower abundance of *Bifidobacteriaceae*.[36, 37] Specific animal-derived *Bifidobacterium* species including *B. pseudolongum*, *B. thermophilum*, and *B. longum* have been detected in higher proportions in infants with pet exposure.[38]

Every year, approximately 15 million babies are born prematurely (less than 37 weeks gestation) worldwide due to complications during pregnancy, leading to increased rates of neonatal morbidity and mortality.[39] Preterm infants often need specialized medical care in a neonatal intensive care unit (NICU) because of the traumatic nature of their birth. Preterm babies are more prone to hospital-related infections due to the compromised immune systems. The NICU environment is colonized with a myriad of bacterial species including *Geobacillus*, *Halomona*s, *Shewanella*, *Acinetobacter* and *Gemella* spp.[40, 41] NICU babies are colonized with higher abundances of *Clostridia* species (specifically *C. perfringens*, *C. butyricum*, *C. difficile* and *C. paraputrificum*) when compared to term babies.[42-44] Additionally, very low birth weight (VLBW) NICU infants display higher prevalence of *Klebsiella*, *Enterobacter*, and *Enterococcus* spp., while normal-birthweight NICU infants have *Escherichia* is found in higher abundances.[41, 43, 45, 46]

Table 4.1. Factors influencing the infant microbiota.

Early Life Exposure	Impact on Microbiota
Antibiotic use	↑ *Proteobacteria* and *Clostridium* spp. ↓ *Actinobacteria, Bacteroidetes*, *Bifidobacterium,* and *Firmicutes* spp.*,*
Maternal diet	↑ High fat/low fiber: *Bacteroides* and *Staphylococcus* spp.; High vegetable/fruit: *Cutibacterium, Parabacteroides,* and *Lactococcus* spp. Low vegetable/fruit: *Prevotella*, *Isobaculum, Hungatella, Lachnoclostridium, Flavonifractor*, *Erysipelatoclostridium*, *Phascolarctobacterium, Megamonas*, and *Sutterella* spp.
Mode of delivery	↑ Vaginally delivered: *Bacteroidetes*, *Bifidobacterium*, *Lactobacillus, Prevotella*, or *Sneathia* spp. CS-delivered: *C. difficile*, *Staphylococcus*, *Streptococcus*, and *Propionibacterium* spp.
Feeding method	↑ Breastfed: *Bifidobacterium, Staphylococcus, Streptococcus, Propionibacterium*, and *Lactobacillus* spp. Formula-fed: *Clostridium, Staphylococcus, Bacteroides, Lactobacillu*s, and *Enterococcus* spp. ↓ Formula-fed: *Bifidobacterium* spp.
Introduction of solid foods	↑ *Lachnospiraceae*, *Bacteroides*, *Prevotella*, *Ruminococcus*, *Clostridium, Firmicutes*, and *Veillonella* spp. ↓ *Bifidobacterium, Staphylococcus Lactobacillu*s, *Enterococcus,* and *Enterobacter* spp.
Pet and animal exposure	↑ *Peptostreptococcaceae, B. pseudolongum, B. thermophilum,* and *B. longum* ↓ *Bifidobacteriaceae*
Hospital setting	↑ VLBW NICU: *Clostridia*, *Klebsiella*, *Enterobacter*, and *Enterococcus* spp. Normal birthweight NICU: *Escherichia* spp.

4.2.2 Probiotics: an introduction to the "good" bacteria

The infant's gut microbiota develops into a densely populated microbial community consisting of bacteria, viruses, and fungi. Bacteria are typically associated with harmful microorganisms; however, there are certain species designated as probiotics which are considered "friendly"

bacteria. Among the trillions of microbes, certain commensal species have evolved to modulate a mutualistic symbiotic relationship with the host. These live microorganisms are a combination of bacteria and yeasts that are naturally found in the digestive, urinary, and genital systems. They are additionally found in yogurt and other fermented foods such as sauerkraut, miso, and kimchi, as well as in the form of a dietary supplement. Commensal gut microbes play a critical role in host resistance against pathogenic organisms, while providing a nutrient-rich environment for the proliferation of symbiotes over pathogens. In this complex ecosystem, there lies a balance in which to regulate gut homeostasis and developing the gut's mucosal immune system. The most prominent microbes identified in healthy infants are *Firmicutes* (*Clostridium, Enterococcus, Lactobacillus, Streptococcus,* and *Ruminococcus*), *Bacteroidetes* (*Bacteroides* and *Prevotella*), and *Proteobacteria* and *Acinetobacteria*.[47-50]

4.2.3 Discovery of probiotics and early research

Long before the nutritional properties of probiotics could be identified, fermented food products were consumed for their therapeutic health benefits. While the history of probiotics can be traced backed to the ancient Greeks and Romans, it is the Russian scientist and Nobel Prize winner, Ilya Metchnikoff of the Pasteur Institute of Paris who was credited with this discovery in the late 19th century.[51] Metchnikoff focused his studies on a rural population in Bulgaria, whose diet was rich in soured milk and other fermented dairy products, and observed they lived exceptionally long lives despite their harsh living conditions. He concluded that milk fermented with lactic acid (traditional yogurt) helps suppress the growth of proteolytic bacteria due to the low pH environment. The initial probiotic he discovered in the yogurt, *Lactobacillus bulgaricus,* is the link he found between improved overall health and long life.[51, 52]

During this same period at the Pasteur Institute, Henri Tissier was the first to isolate *Bifidobacterium* spp. specifically in the infant gut of breastfed babies and found these species were useful in the treatment of diarrhea.[53] One of the first commercially available probiotics was a yogurt drink called Yakult, developed by the Japanese microbiologist, Minoru Shirota.[54] He observed that *Lactobacillus casei* Shirota, a type of lactic acid containing bacteria, could pass through the stomach intact and colonize the intestines. This bacterium became the base of his yogurt drink when he realized that consumption led to a decrease in pathogenic strains of bacteria.

For the next several decades, research focused on the isolation and discovery of probiotic strains that contributed to balance of the gut microflora. In 1953, the German bacteriologist Werner Kollath was the first to coin the term "probiotic", derived from Greek and Latin roots meaning "for life".[55] In the late 1980's, Roy Fuller expanded on this definition stating that probiotics are "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance."[56] In the mid 1990's probiotic research took off as scientists took interest in how the gut microbiome is shaped through the various microbial strains.

Probiotics have shown to be effective in the treatment of several diseases and disorders; however, it is important to note that the benefits are typically strain specific. They are known to prevent or reduce the symptoms of digestive disorders including: acute and antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel disease, necrotizing enterocolitis, respiratory infections, and infant colic.[57] In addition, probiotics are currently being researched for their clinical applications in adjuvant therapy for the treatment of type 2 diabetes, nonalcoholic fatty liver disease, metabolic syndrome, and colon and bladder cancer.[58]

4.2.4 Commensal species prevent colonization of pathogenic organisms

Commensal microbes have developed several defense mechanisms to either directly or indirectly impede invading pathogens (**Figure 4.2**). One mechanism by which commensal species prevent colonization of pathogens is by more efficiently competing for essential nutrients in the same ecological niche (**Figure 4.2A**). Not only do commensals starve competing pathogens for resources, but they also secrete toxins and antimicrobial bacteriocins that directly inhibit adhesion to epithelial surfaces (**Figure 4.2B**). Additionally, commensals induce the production of metabolites which stimulate a robust host immune response (**Figure 4.2C**). Upon encountering a pathogen, specific immune defenses are initiated, leading to secretion of proinflammatory cytokines, regulatory T cells, and specialized macrophages.[59] The interactions between commensals and pathogens are influenced by several early life exposures and experiences (see Section 4.2.2).

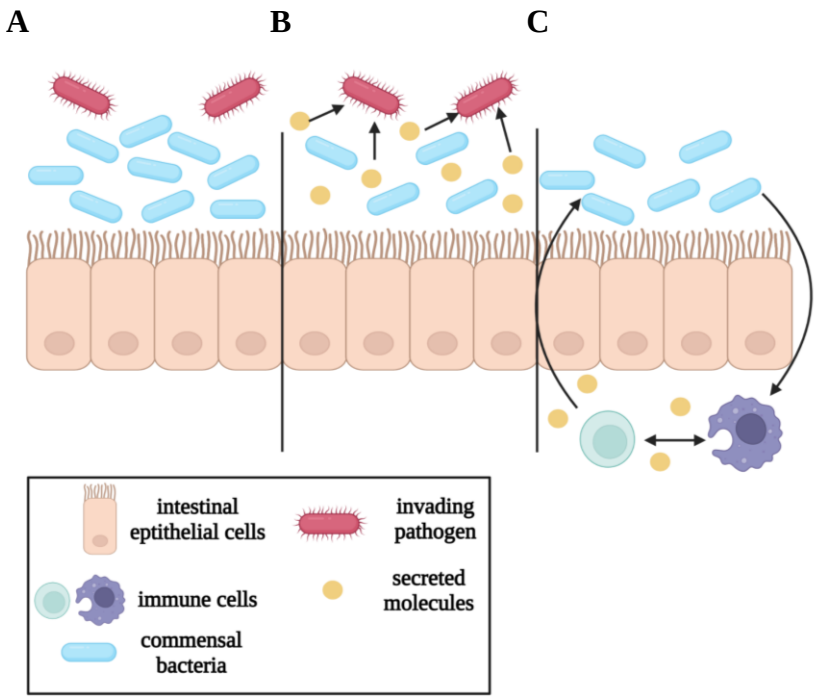

Figure 4.2. Mechanisms utilized by commensal bacteria to combat against invading pathogens. A) Commensal species outcompete pathogens for nutrients and energy sources. B) Commensal species secondary metabolites and antimicrobials that directly inhibit intestinal binding. C) Commensal bacteria initiate the immune response through the production of cytokines and immune cells. Created with BioRender.com.

4.3 The attempt to mimic human breast milk

The innumerable benefits that breastfeeding provides for the baby are well-established. Breast milk contains all the necessary nutritional, protective, and immunological components needed to build up the infant's immune system and actively support growth and development, especially early in life (see Section 2.3). And while most health organizations including the World Health Organization (WHO) and the American Academy of Pediatrics, endorse breastfeeding as the sole source of nutrition for the first six months of life, formula supplementation is a suitable alternative for those mothers unable to breastfeed for a variety of reasons (see Section 2.2).[60] Formula manufacturers strive to mimic the nutritional composition of human breast milk as closely as possible. This is a difficult task as the composition of breast milk is dynamic and complex, constantly responding to the infant's current needs.

4.3.1 Common infant formula additives

Infant formula is highly regulated by the Food and Drug Administration (FDA) and the WHO to ensure proper nutrition and safety for the baby. Available in three forms: powder, liquid, and ready-to-feed, cow's milk and soymilk are the primary bases for these formulas. For babies with sensitivities and allergies to soy or cow milk, there are specialized hypoallergenic and amino acid-based formulas available in these rare cases.[61] The specific composition of the major components of infant formula: proteins, lipids, and carbohydrates, varies amongst different manufacturers, but must fall within the set of established guidelines. Formulas are typically distinguished by their protein components: casein and whey; soy; hydrolyzed casein and whey in hypoallergenic formula, and non-allergenic amino acids. While casein and whey are also present in human breast milk, the proportions of these two substances are significantly different. In human milk, the casein/whey ratio varies between 20/80 early in lactation to 50/50 in the later

stages.[62] Infant formulas are typically higher in casein content, which is much harder for the infant to digest as these proteins curdle. The bioactive proteins α-lactalbumin and lactoferrin, together comprising up to 45% of total protein content in breast milk, have recently been supplemented into some infant formulas due to their known antimicrobial and immunomodulatory properties (see Section 2.3).[63, 64]

The lipid component of formula is the major energy source in the infant diet, accounting for 45-55% of the total energy consumed.[65] Composed of three fatty acids esterified to a glycerol backbone, triglycerides are the primary lipids found in both human breast milk and infant formula, comprising more than 95% of the total fat content.[66] Infant formulas are additionally enriched with the milk fat globule membrane, which has shown to increase the concentrations of phospholipids, sphingolipids, glycolipids, and glycoproteins. Polyunsaturated fatty acids such as DHA (docosahexaenoic acid) and ARA (arachidonic acid) are also included in many formulas to improve cognitive function.

The base of most infant formulas, lactose, is also the principal carbohydrate found in human breast milk, followed by HMOs which are composed of a lactose core (see Section 2.3). Manufacturers have strived to improve the quality of infant formula through oligosaccharide supplementation. While two native HMOs, 2'-FL (**3.1**) and LNnT (**4.1**) have recently been added to formula, more commonly, plant oligosaccharides are supplemented in an attempt mimic the prebiotic properties of HMOs.[67] Polydextrose (**4.2**), long-chain fructo-oligosaccharides (FOS, **4.3**), and short-chain galacto-oligosaccharides (GOS, **4.4**) are the most common plant fibers added to formula (**Figure 4.3**).[68, 69] These oligosaccharides have shown to stimulate the growth of beneficial *Bifidobacterium* spp., while suppressing the growth of pathogenic species such as *E. coli* and *Enterococcus* spp.[69-71] These prebiotic polymers are now considered standard formula

supplements; however, the mechanism by which these fibers affect the interactions within the microbial community is still unclear.

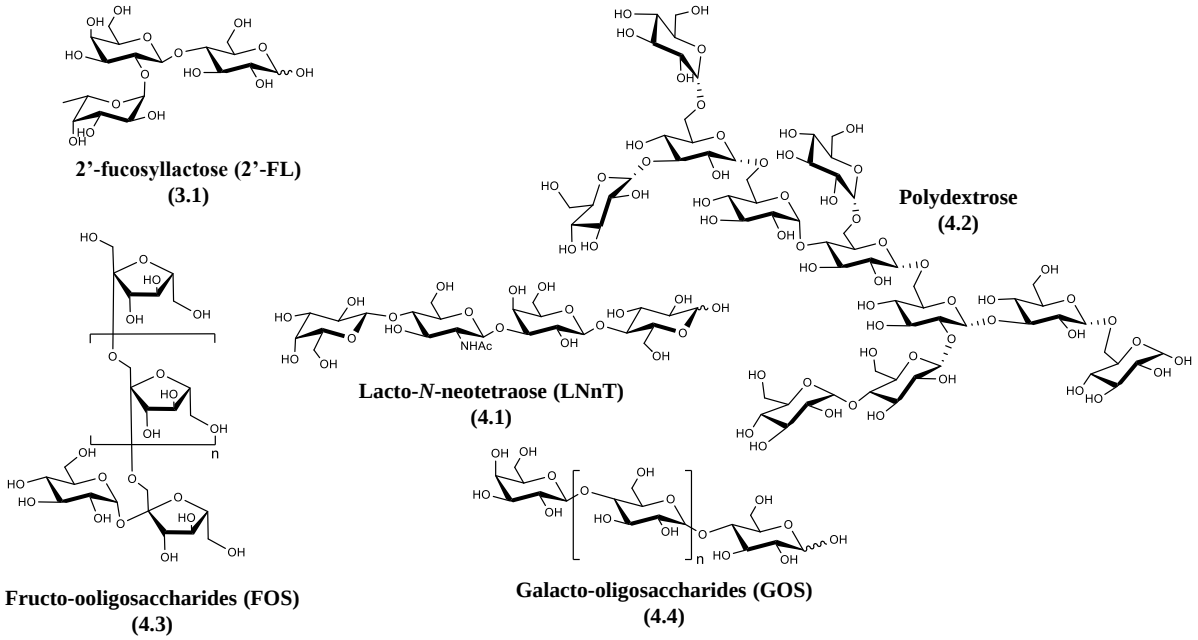

Figure 4.3. Structures of common oligosaccharide formula supplements.

4.4 Streptococcus salivarius as a prominent commensal species in the oral and gut microbiomes

Streptococcal species are amongst the most invasive group of pathogens, colonizing the mucosal surfaces of the intestines, mouth, and upper respiratory tract. They can also exist as commensal organisms, normal inhabitants of the oral cavity and gastrointestinal tract. *S. salivarius* is one earliest colonizers of the oral and gut microbiomes, remaining a predominant commensal species of the healthy, adult microflora.[72-74] Just hours after birth, *S. salivarius* is acquired by infants from their mothers through breastfeeding, taking up residence in the gut.[75] K12 and M18 are two of the most well-studied *S. salivarius* strains administered for probiotic or prophylactic treatment.[76] In addition to inhibiting *S. pyogenes*, the bacteria that causes strep throat, *S. salivarius* has been shown to regulate the host immune response through downregulation of

nuclear transcription factor (NF-κB) and the secretion of the proinflammatory cytokine interleukin (IL)-8 in human intestinal cells.[77, 78]

4.5 The fight for space in the mini microbiome of GBS and S. salivarius

With the knowledge that commensal species have a competitive advantage over pathogens for resources (see Section 4.2.4), we chose to explore a minimal two-species microbiome to characterize how prebiotics regulate these interactions. While we do recognize that the infant microflora is more complex than only two species, we were interested in the mechanisms behind how *S. salivarius* and GBS were affected by carbohydrate metabolism. We hypothesized that coculturing a commensal with a pathogen in addition to prebiotic HMOs and plant polymers would allow us to study their growth dynamics.

4.5.1 The effects of oligosaccharide supplementation on bacterial growth

We designed an experiment to screen five single-entity oligosaccharides (**Figure 4.4**), as well as a heterogeneous cocktail of HMOs for their antimicrobial activity in both *S. salivarius* and GBS. We chose *S. agalactiae* strain GB00002, a serotype 1a strain belonging to multilocus sequence type (ST)-23 as these strains are amongst the most common isolates associated with GBS disease, specifically EOD in newborns. *S. salivarius* was selected as of the first colonizers of the infant gut. Four of these molecules were native HMOs: 2'-fucosyllactose (2'-FL, **3.1**), 3-fucosyllactose (3-FL, **3.2**), 6'-siallyactose (6'-SL, **3.4**), and 3'-siallylactose (3'-SL, **4.5**). We also studied galacto-oligosaccharides (GOS, **4.4**), the most common formula supplement, and a heterogeneous cocktail of HMOs extracted from the breast milk of seven healthy donors. Antimicrobial activity was assessed in GBS (strain GB00002) and *S. salivarius* (strain ATCC 19258) by examining growth and viability over a period of 24 hours using a plate-based assay

(**Figure 4.5**). Growth was quantified usingOD_{600} absorbance readings, and cellular viability was assessed through serial dilution and plating onto blood agar plates, followed by enumeration of colony forming units (CFUs). GBS and *S. salivarius* were grown in THB medium alone or THB supplemented with ca. 5 mg/mL oligosaccharides. This concentration was selected because HMOs typically range between 5 and 25 mg/mL in human breast milk, depending on the stage of lactation.[79] Due to the limited supply of breast milk, we tested at the low end of this physiological relevant concentration.

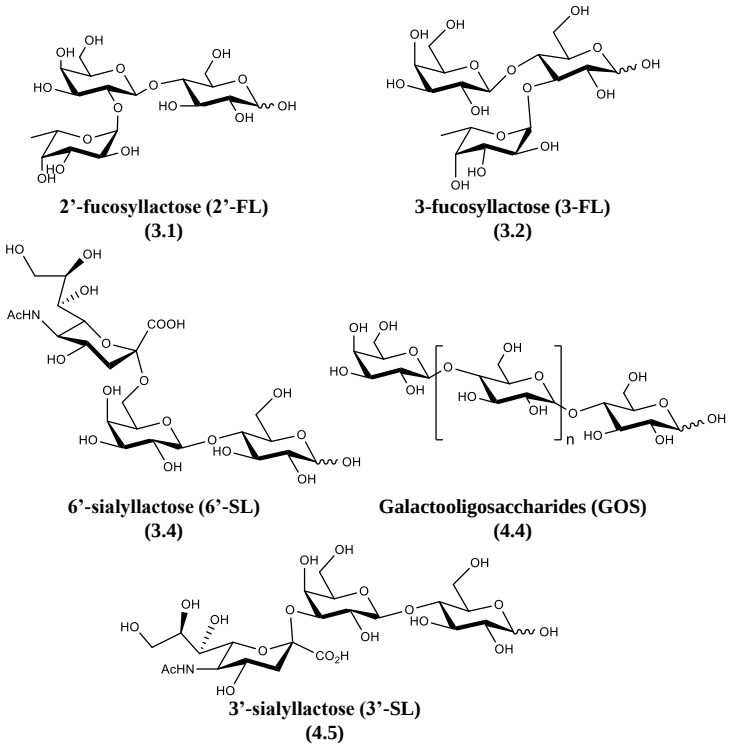

Figure 4.4. Structures of oligosaccharide prebiotics used in this study: 2'-fucosyllactose (2'-FL, 3.1), 3-fucosyllactose (3-FL, 3.2), 6'-sialyllactose (6'-SL, 3.4), galacto-oligosaccharides (GOS, 4.4), and 3'-sialyllactose (3'-SL, 4.5).

In *S. salivarius*, GOS increases growth starting at hour 6, with an increase of 64% at 24 hours.[80] We observed contrasting results with HMO cocktail supplementation, completely inhibiting growth of *S. salivarius* over the entirety of the 24-hour period. The four naturally occurring

HMOs did not have a significant effect on *S. salivarius* growth. Not surprisingly, in GBS, the HMO cocktail impressively suppressed growth entirely over the 24-hour period. However, in contrast to what we observed in *S. salivarius*, GOS reduced bacterial growth starting at hour 4, with a 24% decrease at 24 hours. 3-FL, 3'-SL, and 6'-SL additionally decreased growth significantly at 24 hours, by 11%, 14%, and 12%, respectively. We observed comparable trends for bacterial viability in both strains. As was seen with *S. salivarius* growth, at 6 hours, cellular viability began to increase when compared to our medium alone control, with an increase of 18% at 24 hours. Additionally, the HMO cocktail significantly inhibited viability over the entire 24-hour period in both *S. salivarius* and GBS.

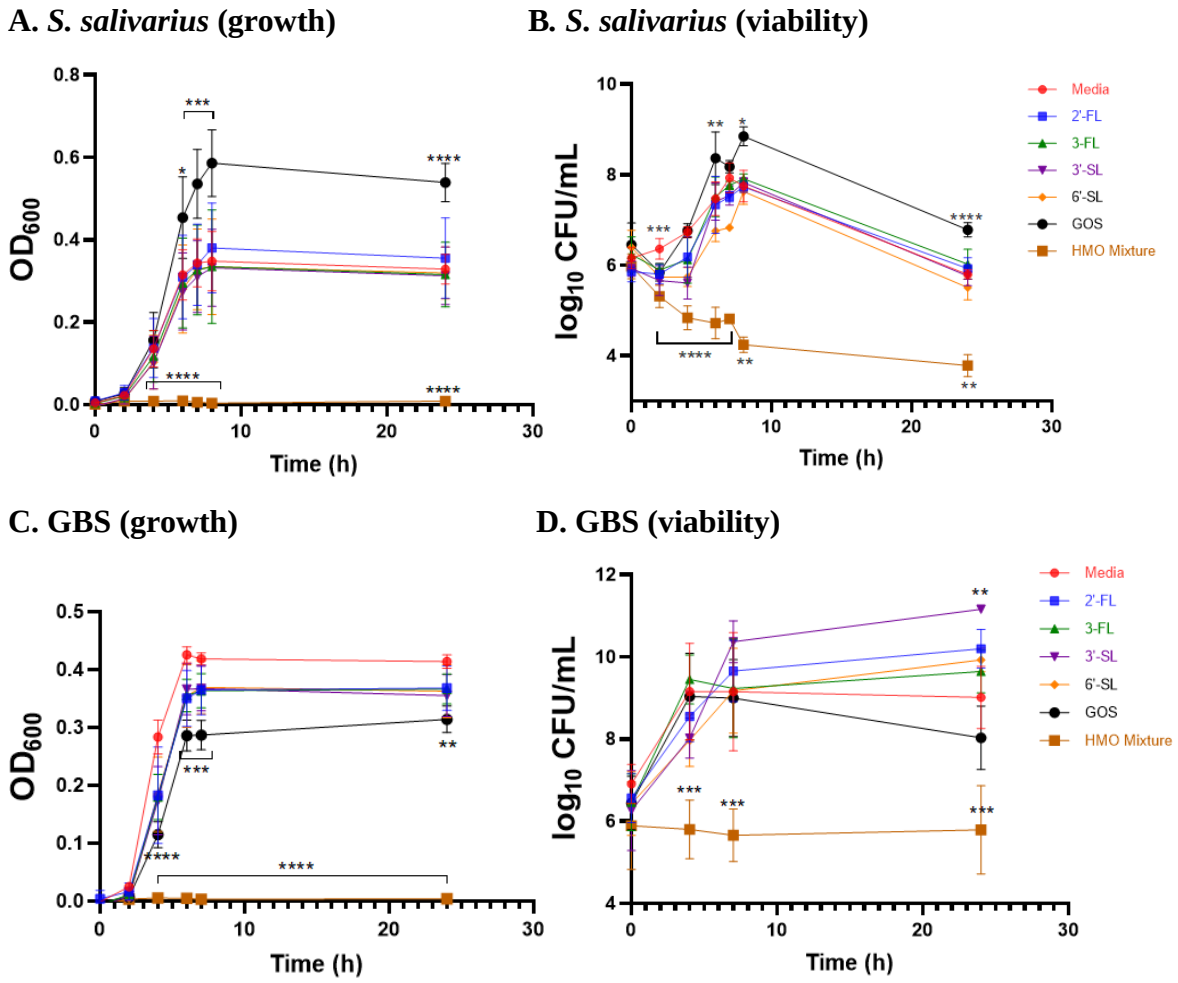

Figure 4.5. Effects of single-entity oligosaccharides and the HMO cocktail at ca. 5 mg/mL on growth and viability of *S. salivarius* (ATCC 19258) and *S. agalactiae* (GB00002). Growth was quantified via OD600 readings at 0, 2, 4, 6, 7, 8, and 24 h. Mean OD_{600} for each time point is indicated by the corresponding symbols. Viability was assessed by enumeration of CFU/mL performed at 0, 2, 4, 6, 7, 8, and 24 h for *S. salivarius* and 0, 4, 7, and 24 h for *S. agalactiae*. Log_{10} CFU/mL for each HMO and time point is designated by the corresponding symbols. (A) Growth of *S. salivarius* (OD_{600}) in the presence of single-entity oligosaccharides and the HMO cocktail. (B) Viability of *S. salivarius* (CFU/mL) corresponding to the OD values graphed in Figure 2A. (C) Growth of *S. agalactiae* (OD_{600}) in the presence of single-entity oligosaccharides and the HMO cocktail. (D) Viability of *S. agalactiae* (CFU/mL) corresponding to the OD values graphed in Figure 2C. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. In (A) **** represents $p < 0.0001$, *** represents $p = 0.0009$ and $p = 0.0003$, and * represents $p = 0.0132$ by two-way ANOVA with post hoc Dunnett's multiple comparison test comparing the growth of *S. salivarius* in each oligosaccharide supplementation condition to the growth of *S. salivarius* in medium alone. In (B) **** represents $p < 0.0001$, *** represents $p = 0.0005$, ** represents $p = 0.0085$, $p = 0.0062$, and $p = 0.0022$, and * represents $p = 0.0484$ by two-way ANOVA with post hoc Dunnett's multiple comparison test comparing the growth of *S. salivarius* in each oligosaccharide supplementation condition to the growth of *S. salivarius* in medium alone. In (C) **** represents $p < 0.0001$, *** represents $p = 0.0009$ and $p = 0.0007$, and ** represents $p = 0.0015$ by two-way ANOVA with post hoc Dunnett's multiple comparison test comparing the growth of *S. agalactiae* in each HMO supplementation condition to the growth *of S. agalactiae* in medium alone. In (D) *** represents $p = 0.0010$, $p = 0.0005$, and $p = 0.0002$, and ** represents $p = 0.0037$ by two-way ANOVA with post hoc Dunnett's multiple comparison test comparing the growth of S. agalactiae in each oligosaccharide supplementation condition to the growth of *S. agalactiae* in medium alone.

4.5.2 GBS suppresses the growth of S. salivarius in coculture

The contrasting results we observed with GOS across both strains spurred our desire to conduct further analyses. To our knowledge, this is the first study evaluating how the metabolism of GOS is modulated by the commensal-pathogen relationship. In our minimal, model infant microbiome design, we used the Transwell® plate system for coculturing, allowing us to characterize contact-independent microbial interactions (**Figure 4.6**). This method physically separates the two microorganisms, only permitting the diffusion of primary and secondary metabolites. The Transwell® system is useful for studying the growth dynamics of cocultured species as a result of these secreted molecules.

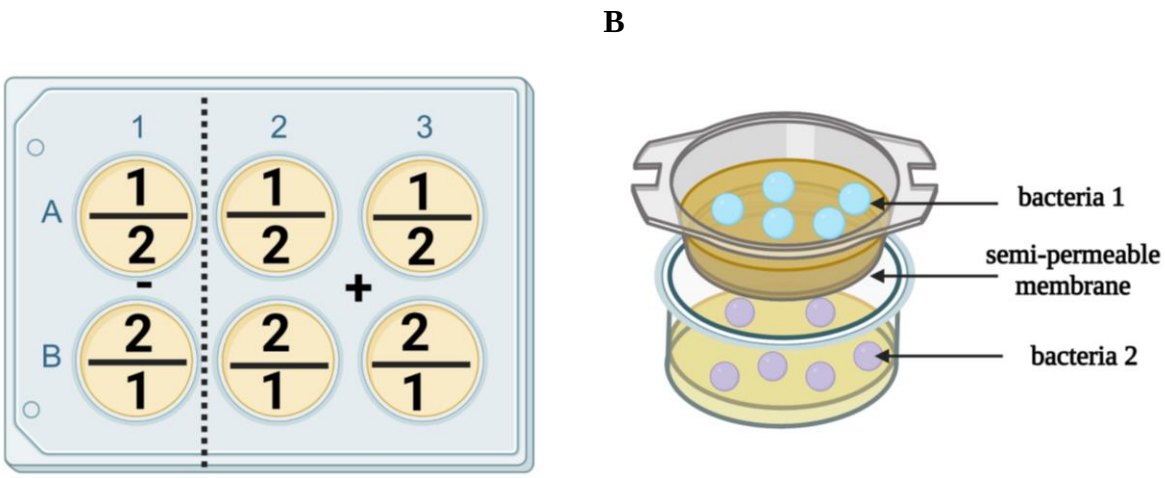

Figure 4.6. The Transwell® plate system for coculturing two strains of bacteria: *S. salivarius* (1) and GBS (2). A) The 6-well Transwell® plate is set up as shown. The wells are supplemented with (+) or without (-) galacto-oligosaccharides at ca. 5 mg/mL. B) One strain of bacteria is grown on top, one strain of bacteria on the bottom, and they are physically separated by semi-permeable membrane. Created with BioRender.com.

As a control, we cultured both strains in their own wells, with or without GOS supplementation. In terms of growth, we observed a 25% difference between the two strains in medium alone (**Figure 4.7**). As expected, GOS supplementation increased *S. salivarius* biomass by 67%. To our surprise, in coculture we observed a 286% difference in growth between the two strains. This

was a 40% decrease of *S. salivarius*, and a 40% increase of GBS when compared to the solo cultures. GOS supplementation in coculture rebounded growth in both *S. salivarius* and GBS to comparable growth of the solo culture.

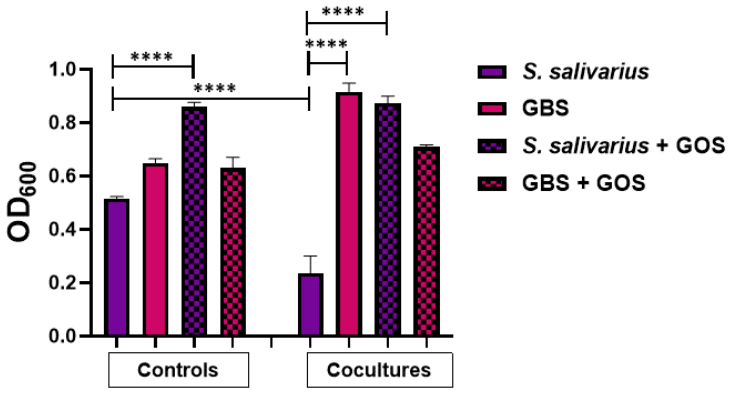

Figure 4.7. GBS suppresses growth of *S. salivarius* in coculture; GOS supplementation reverses this suppression. The first four bars represent controls in which *S. salivarius* (ATCC 19258) and GBS (GB00002) were grown separately, either with or without GOS supplementation at ca. 5 mg/ml. The last four bars represent the two strains grown in coculture either with or without GOS supplementation at ca. 5 mg/ml. Growth was quantified via OD_{600} readings at 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. **** represents $p < 0.0001$ by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing the mean growth of each condition with the mean of every other condition.

We hypothesized GBS was synthesizing a metabolite as a defense mechanism against a competitor, *S. salivarius*. To test this hypothesis, we examined the inhibitory effects of cell-free supernatants from overnight cultures in **Figure 4.7**. Interestingly, the suppressive phenotype employed by GBS was still observed upon treatment with cell-free supernatants (**Figure A2.1**). To identify whether secreted proteins, lipids, carbohydrates, or DNA are responsible for restricting *S. salivarius* growth we incubated the cell-free supernatants with proteinase K, lipase, α-amylase, or DNAase I for 24 hours (**Figure 4.8**). Somewhat surprisingly, we no longer observed *S. salivarius* growth inhibition by GBS from both the solo and cocultures in this study. We hypothesized adding 1 mM TRIS buffer to the cell-free supernatants would produce this

same result due to the highly buffered nature of each enzyme. Validating this hypothesis, we did not observe *S. salivarius* growth inhibition upon incubation with TRIS buffer. Therefore, we concluded suppression of *S. salivarius* is only observed under acidic conditions (**Table A2.1**).

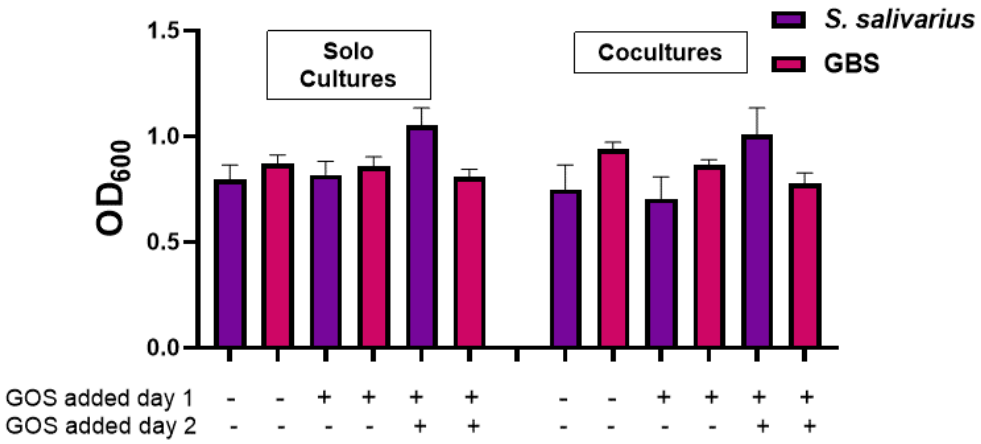

Figure 4.8. Suppression of *S. salivarius* by GBS is combatted when the supernatants from overnight cultures are treated with DNAase I, lipase, α-amylase, proteinase K, or 1 mM TRIS buffer. Supernatants from overnight solo cultures and cocultures are treated with enzyme or TRIS buffer to determine if the suppression of *S. salivarius* by GBS is reversed. Cultures with GOS supplementation were added at ca. 5 mg/ml. Growth was quantified via OD_{600} readings at 24 h. Data displayed is a combined from treatments with all four enzymes and TRIS buffer. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates.

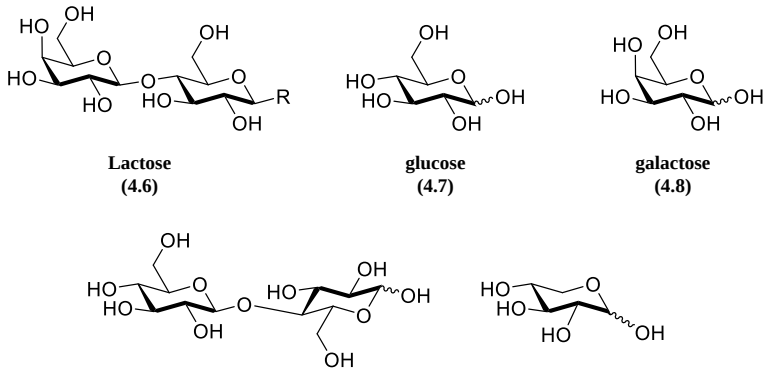

Figure 4.9. Structures of additional carbohydrates used in study examining effects against *S. salivarius* growth: lactose (4.6), glucose (4.7), galactose (4.8), cellobiose (4.9), and xylose (4.10).

4.5.3 Carbohydrate specificity on the effects of bacterial growth

We questioned whether the rebound of *S. salivarius* growth in coculture with GBS was specific to GOS or if other carbohydrates would produce this same phenotype. We selected to screen five additional carbohydrate molecules (**Figure 4.9**) in *S. salivarius* to determine if they provoke the same response as GOS (**Figure 4.10**). Lactose, glucose, and galactose all increased growth by 96%, 62%, and 48%, respectively. This was not surprising as lactose is a disaccharide composed of glucose and galactose monomers. We set up cocultures with supplementation of these three carbohydrates and compared them to GOS (**Figure 4.11**). Interestingly, only galactose triggered *S. salivarius* growth to fully rebound from the suppression caused by GBS, similar to what we observed with GOS. Since GOS is a polymer composed of between two and eight monomeric units of galactose, we do believe this reversal of *S. salivarius* inhibition is specific to galactose. In future studies, we plan on continuing exploration the of SAR in relation to this pathogen-commensal interaction.

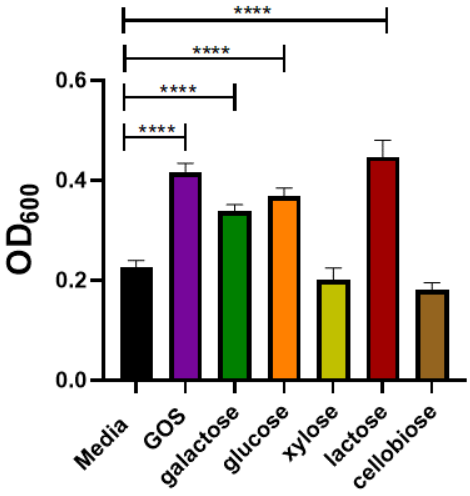

Figure 4.10. Effects of mono-, di-, and oligosaccharides at ca. 5 mg/mL on growth of *S. salivarius* (ATCC 19258). Growth was quantified via OD_{600} readings at 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. **** represents $p < 0.0001$ by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing growth in carbohydrate-supplemented Todd-Hewitt Broth (THB) to growth in carbohydrate-free THB.

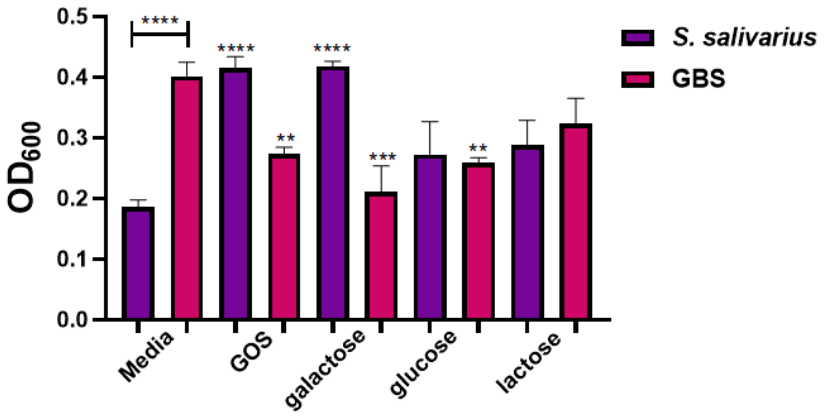

Figure 4.11. In coculture, GOS and galactose supplementation assist in circumnavigating the suppression of *S. salivarius* by GBS. Growth of *S. salivarius* (ATCC 19258) and GBS (GB00002) supplemented with ca. 5 mg/ml of GOS, galactose, glucose, and lactose were compared to growth of *S. salivarius* and GBS grown in THB medium alone. Growth was quantified via OD600 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. **** represents $p < 0.0001$, *** represents $p = 0.0006$, ** represents $p = 0.0099$ and $p = 0.0085$ by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing growth in carbohydrate-supplemented Todd-Hewitt Broth (THB) to growth in carbohydrate-free THB.

4.6 The effects of acidic environment on bacterial growth

GBS produces a myriad of virulence factors contributing to its ability to persist in the harsh host environment, contributing to its pathogenesis (see Section 1.6). Lactic acid has recently been implicated as a potential virulence factor of GBS.[81, 82] GBS produces lactic acid as an end product of anaerobic carbohydrate fermentation.[82] We hypothesized that lactic acid production was contributing to the suppression of *S. salivarius* growth. A simple lactic acid production assay was employed to measure the concentration of lactic acid present in all of the samples in **Figure 4.7**. Utilizing the lactic acid standard curve (**Figure A2.2**), the lactic acid concentration was calculated (**Figure 4.12**). In the solo cultures, GBS produced ca. 49.2 ng/µL lactic acid. The remaining cultures produced an average of 34.5 ng/µL lactic acid, 30% less than GBS in medium alone. We concluded that this significant increase in lactic acid production was likely contributing to GBS modulation of *S. salivarius*.

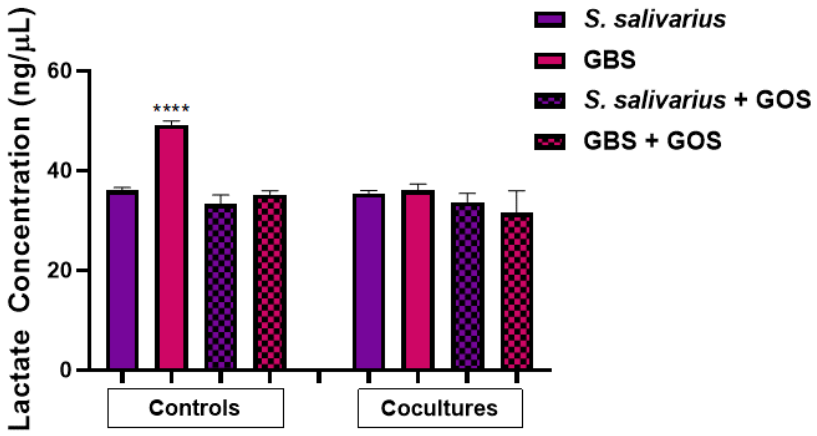

Figure 4.12. GBS in medium alone produces significantly more lactic acid compared to all other conditions tested. The first four bars represent controls in which *S. salivarius* (ATCC 19258) and GBS (GB00002) were grown separately, either with or without GOS supplementation at ca. 5 mg/ml. The last four bars represent the two strains grown in coculture either with or without GOS supplementation at ca. 5 mg/ml. Lactic acid concentration was quantified via OD_{450} readings at 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. **** represents $p < 0.0001$ by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing growth in S. agalactiae in medium alone to growth in all other conditions.

4.7 Conclusions and future outlook

In this study, we have provided convincing evidence that lactic acid production plays a significant role in the virulence of GBS against *S. salivarius*. We discovered that galactose/GOS supplementation reverses this suppression of *S. salivarius* growth. This work is an important step toward understanding how commensal-pathogen interactions are affected by carbohydrate metabolism. This two species minimal microbiome allowed us to characterize how microbes engage each other and the mechanisms they employ to survive in harsh environments.

Future studies will focus on continuing to characterize how prebiotic oligosaccharides modulate interactions between GBS and other prominent commensal species in the infant gut. The majority of commensals originate from *Bifidobacterium* and *Lactobacillus* species. The majority of the over 200 species of *Lactobacillus* identified to date have been isolated from the

gastrointestinal and vaginal tracts of humans and animals.[83] Named for their ability to convert glucose to lactic acid, they can be classified as either homofermentive (producing only lactic acid) or heterofermentive (producing either lactic acid, acetic acid, or alcohol and carbon dioxide). *Bifidobacterium* also produces lactic acid as the primary end product of carbohydrate metabolism along with short-chain fatty acids.[84]

Out next coculture design will focus on *Lactobacillus rhamnosus* GG, one of the most widely used probiotic strains to treat bacterial vaginosis, diarrhea in children, and acute gastroenteritis. The GG is derived from Sherwood Gorbach and Barry Goldin, the two scientists who discovered the strain in the feces of a healthy, human adult in 1983.[85] We are especially interested in how the HMO cocktail modulates the interactions between GBS and *L. rhamnosus* GG. In our preliminary data, as expected, the HMO cocktail suppressed growth of both strains when cultured separately. Surprisingly, in coculture, we no longer observed this growth inhibition upon supplementation with the HMO cocktail (**Figure A2.3**). Future experiments will aim to characterize the mechanisms at play causing this phenotype.

4.8 Experimental methods

HMO isolation

Human milk was obtained from 7 healthy, lactating women between 3 days and 3 months postpartum and stored between −80°C and –20°C. Deidentified milk was provided by Jörn-Hendrik Weitkamp from the Vanderbilt Department of Pediatrics. Milk samples were thawed and then centrifuged at 3750 rpm for 45 min. Following centrifugation, the resultant top lipid layer was removed. The proteins were then removed by diluting the remaining sample with roughly 1:1 (vol/vol) 180 or 200 proof ethanol, chilling the sample briefly, and centrifuging for

45 min at 3750 rpm, followed by removal of the resulting HMO-containing supernatant. Following concentration of the supernatant *in vacuo*, the HMO-containing extract was dissolved in 0.2 M phosphate buffer (pH 6.5) and heated to 37°C. 1 mL of β-Galactosidase from *Kluyveromyces lactis* was added, and the reaction mixture was stirred until lactose hydrolysis was complete. The reaction mixture was diluted with roughly 1:0.5 (vol/vol) 180 or 200 proof ethanol, chilled briefly, and then centrifuged at 3750 rpm for 30 min. The supernatant was removed and concentrated in vacuo, and the remaining salts, glucose, and galactose were separated from the oligosaccharides using size exclusion chromatography with P-2 gel (H_2O eluent). The oligosaccharides were then dried by lyophilization. Correspondingly, HMO isolates from donors were combined and solubilized in water to reach a final concentration of 102.6 mg/ml.

Bacterial strains and culture conditions

S. agalactiae strain GB00002 was previously recovered from a vaginal/rectal swab taken from a pregnant mother prior to childbirth [86]; it was previously classified as a serotype Ia strain belonging to multilocus sequence type (ST)-23 [87]. *S. salivarius* strain (ATCC 19258) is a type strain. *L. rhamnosus* (ATCC 7469) is a type strain.All strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37 °C in ambient air overnight. Strains were subcultured from blood agar plates into 5 mL of Todd-Hewitt broth (THB) and incubated under shaking conditions at 180 rpm at 37 °C in ambient air overnight. Following overnight incubation, bacterial density was quantified through absorbance readings at 600 nm (OD_{600}) using a Promega GloMax-Multi Detection System plate reader. Bacterial numbers were determined using the predetermined coefficient of 1 OD_{600} = 10^9 CFU/mL.

Bacterial growth assays

Bacterial strains were grown overnight as described above and used to inoculate fresh THB at a concentration of 10^6 colony forming units per 200 μL of growth media in 96-well tissue culture treated, sterile polystyrene plates (Corning, Inc.). Compounds were dissolved in DI water to achieve a concentration of 80 mg/mL and filtered through a 0.2 μm syringe filter. Compounds were added to achieve final carbohydrate concentrations of ca. 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL. Bacteria grown in THB in the absence of any compounds served as the control. Cultures were grown under static conditions at 37 °C in ambient air for 24 h. Growth was quantified through spectrophotometric reading at OD_{600} with readings taken at 0, 2, 4, 6, 7, and 8 h then a final reading at 24 h. Viability was assessed through serial dilution and plating onto blood agar plates followed by quantification of viable CFU/mL with readings taken at 0, 2, 4, 6, 7, 8, and 24 h for *S. salivarius* and 0, 4, 7, and 24 h for *S. agalactiae*.

Coculture model system

Bacterial strains were grown overnight as described above and used to inoculate fresh THB to achieve 5×10^5 CFU/ml. To 12-well tissue culture-treated, sterile polystyrene plates was added the inoculated media in the presence of HMO or carbohydrate to achieve a final volume of 3 ml per well. Bacteria grown in medium in the absence of any compounds served as the controls. To a 6-well culture treated, sterile, polystyrene transwell plate was added 3 ml of THB media below and above the membrane. Bacterial strains were grown overnight as described above and used to inoculate the fresh THB on each side of the membrane to achieve 5×10^5 CFU/ml (*S. agalactiae* on bottom and *S. salivarius* on top). Compounds were added to each side of the membrane to achieve a final carbohydrate concentration of ca. 5 mg/mL. Bacteria grown in THB in the absence of any compounds served as the control. Cultures were grown under static conditions at

37 °C in ambient air or in a CO_2 incubator for 24 h. Growth was quantified through spectrophotometric reading at OD_{600}.

Supernatant treated cultures

Cocultures were set up as described above. The media and cells from overnight growth plates were removed from each side of the transwell and transferred to 15 ml conical centrifuge tubes. The samples were centrifuged at 5000 rpm for 15 min to generate a bacterial pellet. The supernatant was removed and filtered through a 0.2 μm syringe filter. To a 6-well culture treated, sterile, polystyrene transwell plate was added 3 ml of THB media below the membrane. The filtered supernatant was added to the top of the membrane. Bacterial strains were grown overnight as described above and used to inoculate the fresh THB on the bottom of the membrane to achieve 5×10^5 CFU/ml (*S. agalactiae* if *S. salivarius* supernatant on top, *S. salivarius* if *S. agalactiae* supernatant on top). Compounds were added to each side of the membrane to achieve a final carbohydrate concentration of ca. 5 mg/mL. Bacteria grown in THB in the absence of any compounds served as the control. Cultures were grown under static conditions at 37 °C in ambient air for 24 h. For enzyme and buffer treated supernatants, 15 μl of either DNAase I, proteinase K, lipase, α-amylase or 1 mM TRIS buffer was added to supernatants and incubated for 1 hour at 37 °C before adding to the transwell plates. Cultures were then grown under static conditions at 37 °C in ambient air for 24 h. Growth was quantified through spectrophotometric reading at OD_{600}.

Lactic acid production assay

Cocultures were set up as described above. Lactate standards for colorimetric detection were prepared as described using the Sigma-Aldrich Lactate Assay Kit II. Media and cells were

removed and centrifuged the samples at 5000 rpm for 10 minutes to remove insoluble material. 50 µl of the soluble fraction was added to each well of a 96-well tissue culture treated, sterile polystyrene plates (Corning, Inc.). 50 µl of the appropriate Reaction Mix (as prepared from the Sigma-Aldrich Lactate Assay Kit II) was added to each well. The plates were mixed using a horizontal shaker for 30 minutes at room temperature while protected from light. The absorbance was read at OD_{450}. The values obtained from the lactate standards were used to plot a standard curve. The amount of lactate in each sample was determined from the standard curve.

Statistical analysis

All data shown signify three independent experiments each with three technical replicates. Data are expressed as the mean ± SEM. Statistical analyses were performed in GraphPad Prism Software v. 8.2.1. Statistical significance was determined using one-way analysis of variance (ANOVA) with *post hoc* Dunnett's multiple-comparison test comparing growth in the presence of ca. 5 mg/ml HMOs or carbohydrates to growth in medium alone.

This chapter was adapted from "Galacto-Oligosaccharide Supplementation Modulates Pathogen-Commensal Competition between *Streptococcus agalactiae* and *Streptococcus salivarius*" published in ChemBioChem and has been reproduced with the permission of the publisher and my co-authors including: Harrison C. Thomas, Shannon D. Manning, Jennifer A. Gaddy and Steven D. Townsend. Moore RE, Thomas HC, Manning SD, Gaddy JA, Townsend SD. 2021 Galacto-Oligosaccharide Supplementation Modulates Pathogen-Commensal Competition between *Streptococcus agalactiae* and *Streptococcus salivarius*. ChemBioChem. 23(3) e202100559. https://doi.org/10.1002/cbic.202100559

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Appendix A2:

Data Relevant to Chapter 4

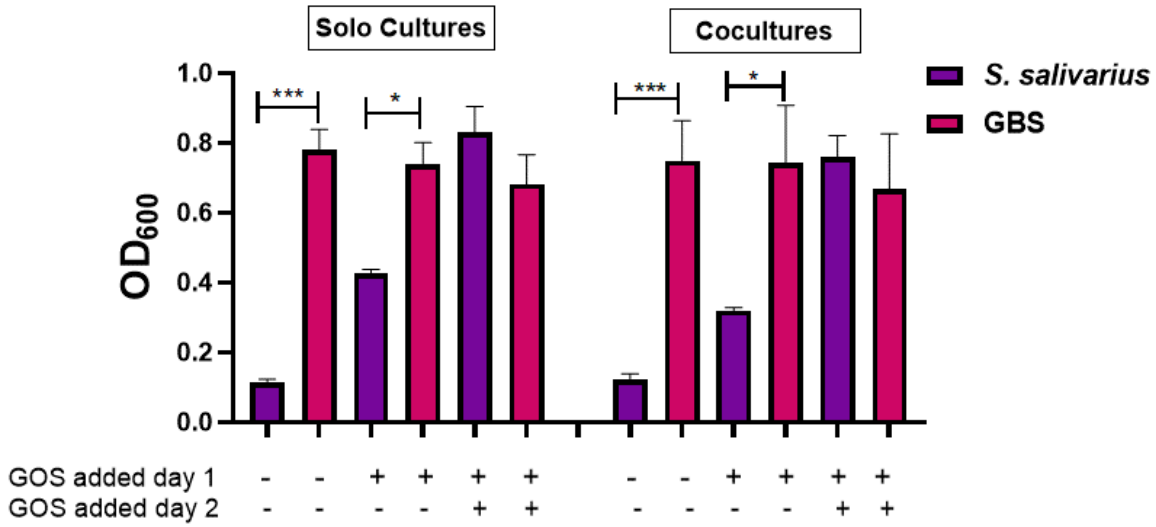

Figure A2.1. Overnight cultures treated with cell-free supernatants. Suppression of *S. salivarius* by GBS is still observed when the supernatants from overnight cultures are cocultured with whole cells. Cultures with GOS supplementation were added at ca. 5 mg/ml. Growth was quantified via OD_{600} readings at 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. *** represents p = 0.0002 or p = 0.0004 and * represents p = 0.0127 by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing the mean growth of each condition with the mean of every other condition.

Table A2.1. Recorded pH of data collected in Figure 4.7.

Condition	Time (h)					
	0	**2**	**4**	**6**	**8**	**24**
GBS	7	7	6	6	6	5
S. salivarius	7	7	7	7	7	6
GBS + GOS	7	7	6	6	6	6
***S. salivarius* + GOS**	7	7	7	6	6	6
Coculture - GOS	7	7	6	6	6	5
Coculture + GOS	7	7	7	6	6	6

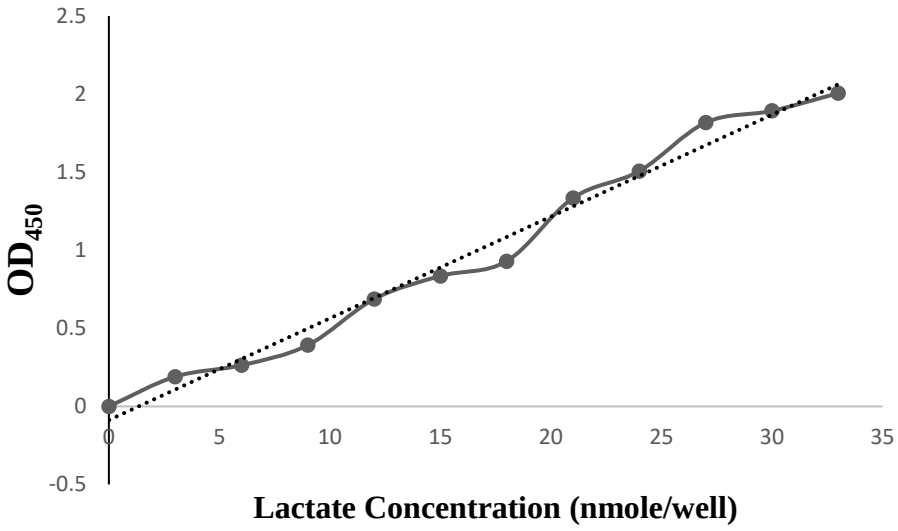

Figure A2.2. The amount of lactic acid present in each sample is calculated from the lactic acid production standard curve created fresh during each new assay run. The concentration (C) calculated using the equation $S_a/S_v = C$ in which $S_a = x$ (calculated from standard curve) and S_v = sample volume added to each well. The concentration is converted to ng/µL using the lactic acid molar mass (89.07 ng/nmole). The equation $y = 0.0651x – 0.0868$ was used to calculate lactic acid concentration.

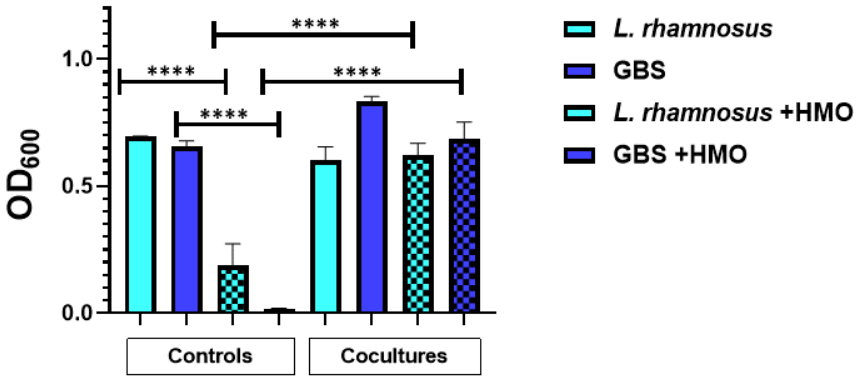

Figure A2.3. The HMO cocktail suppresses growth of L. rhanmonus and GBS when grown in medium alone; in coculture we no longer observe this growth inhibition upon supplementation with the HMO cocktail. The first four bars represent controls in which *L. rhamnosus* (ATCC 7469) and GBS (GB00002) were grown separately, either with or without HMO cocktail supplementation at ca. 5 mg/ml. The last four bars represent the two strains grown in coculture either with or without HMO cocktail supplementation at ca. 5 mg/ml. Growth was quantified via OD_{600} readings at 24 h. Data displayed represent the relative mean growth ratios ± SEM of three independent experiments, each with three technical replicates. **** represents $p < 0.0001$ by one-way ANOVA with post hoc Dunnett's multiple comparison test comparing the mean growth of each condition with the mean of every other condition.

Chapter 5

The powerful influence of HMOs at the host-pathogen interface

5.1 Abstract

GBS colonization of the rectovaginal mucosa is the primary risk factor for adverse pregnancy outcomes and neonatal invasive disease. Ascending infection of the reproductive tract may lead to maternal sepsis, chorioamnionitis, PPROM, preterm birth, stillbirth, and early-onset disease (EOD). We have previously demonstrated human milk oligosaccharides (HMOs), which are prominent, multi-functional glycans present in breast milk, display potent antimicrobial and antibiofilm activity against GBS *in vitro*.[1-3] We have also shown that GBS can adhere to and produce robust biofilms on gestational membrane tissues, as well as colonization and invasion of the fetal membranes in our *in vivo* mouse model.[4-6] In our work, we determined that HMOs inhibit GBS adherence and biofilm formation on EPMs collected from healthy, term, non-laboring C-section placenta, and to EpiVaginal™ human organoid tissue. HMOs also significantly reduced ascending infection in the mouse model. These results have significant implications on reducing the incidences GBS colonization and disease progression.

5.2 GBS adherence to and biofilm formation on gestational membranes

5.2.1 The role of the fetal membrane on adverse pregnancy outcomes

Excessive inflammation because of chorioamnionitis is often associated with ascending infection of the gravid reproductive tract (see Section 1.4). Once GBS colonizes the lower genital region, this leads to passage into the intrauterine cavity where the bacteria can then cross the gestational membranes surrounding the developing fetus. Invasive GBS infections are a major risk factor for triggering PPROM, preterm labor, preterm birth, stillbirth, and neonatal sepsis.[7-9] While it not

completely understood how a non-motile bacterium traverses from the vagina through the extraplacental membrane barrier, the relationship with adverse pregnancy outcomes is well-established.

We have previously shown that HMOs possess potent antimicrobial and antibiofilm properties against GBS *in vitro* (see Sections 2.7, 3.4 & 3.6). We sought to explore whether HMOs could disrupt GBS adherence to and biofilm formation on human gestational tissues. In our lab, we have developed an *ex vivo* model of GBS tissue infection within the human extraplacental gestational membrane (EPM).[4, 5] We have previously published data showing that GBS adheres to and promotes biofilm formation on these *ex vivo* human fetal tissues while eliciting a proinflammatory response to bacterial infection.[6]

5.2.2 HMOs inhibit GBS adherence and biofilm formation on gestational membranes

De-identified placental tissues from healthy, term, non-laboring C-sections were collected, and 12-mm sections were cultured *ex vivo*. To the maternal choriodecidual face of the EPM, GBS was cocultured in THB medium alone or THB supplemented with ca. 5 mg/mL of HMOs. The tissues were incubated for 24 hours, and then fixed and processed for microscopy analysis. FEG-SEM imaging revealed that GBS readily adheres to the gestational tissues, forming globular microcolonies (**Figure 5.1A**). Treatment with HMOs significantly reduced GBS adherence and biofilm formation to the EPM (**Figure 5.1B**). These results indicate that inhibiting GBS colonization could positively influence adverse pregnancy outcomes associated with placental inflammation.

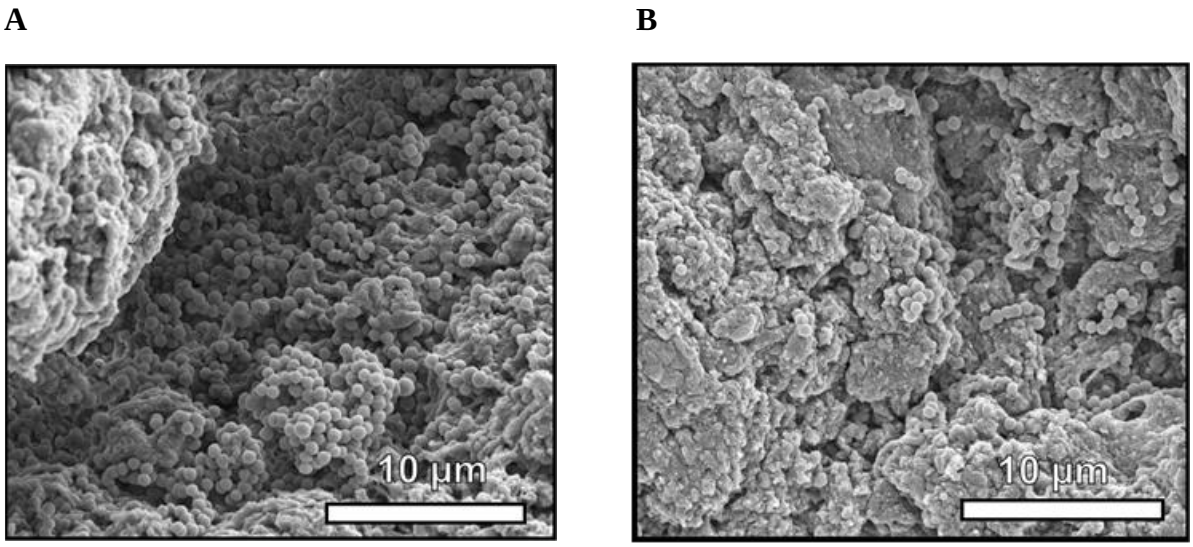

Figure 5.1. Human milk oligosaccharides prevent GBS adherence to and biofilm formation on gestational membranes. High-resolution field-emission gun scanning electron microscopy analyses of GBS strain GB00590 adherence to gestational tissues. FEG-SEM imaging of GBS adherence was performed on GB00590 samples grown in medium alone (A), or medium supplemented with ca. 5 mg/mL of HMOs (B). The addition of HMOs significantly inhibits GBS adherence. Micrographs were collected at 10,000× magnification and magnification bars indicate 10 µm.

5.3 GBS adherence to and biofilm formation on EpiVaginal™ tissues

5.3.1 The importance of vaginal colonization in the establishment of GBS disease

GBS is a common inhabitant of the genitourinary and gastrointestinal tracts, acting as the primary reservoir for vaginal colonization (see Section 1.3). Heavy rectal colonization is highly correlated with vaginal colonization as transfer is accepted to occur through passage from the rectum.[10] Vaginal carriage, especially late in pregnancy is of particular concern due to the risk of vertical transmission either by ascending transmission *in utero*, during passage through the birth canal, or through aspiration of infected amniotic fluid (see Section 1.4).[11] Since vaginal colonization is an important step in the pathogenesis of GBS, we sought to explore mechanisms in which we could prevent GBS adherence to vaginal tissues.

5.3.2 HMOs prevent GBS adherence to and biofilm formation on EpiVaginal™ tissues

The EpiVaginal™ 3D tissue model from MatTek Life Sciences was used to interrogate GBS interactions with the vaginal epithelium. Based on our studies with human fetal tissues, we hypothesized HMOs would inhibit GBS colonization of the luminal tissue surface. The reconstructed EpiVaginal™ organoid tissue model is a useful *in vitro* tool that closely resembles microbial infections of the vaginal mucosa. The tissues were cocultured with GBS in THB medium alone or THB supplemented with ca. 5 mg/mL of HMOs. Following incubation for 24 hours, the tissues were then fixed and processed for FEG-SEM imaging. Just as we observed with the EPMs, treatment with HMOs significantly inhibited GBS adherence and altered the biofilm morphology (**Figure 5.2**). Preventing GBS colonization of the vaginal epithelium is critical to blocking the progression of GBS disease.

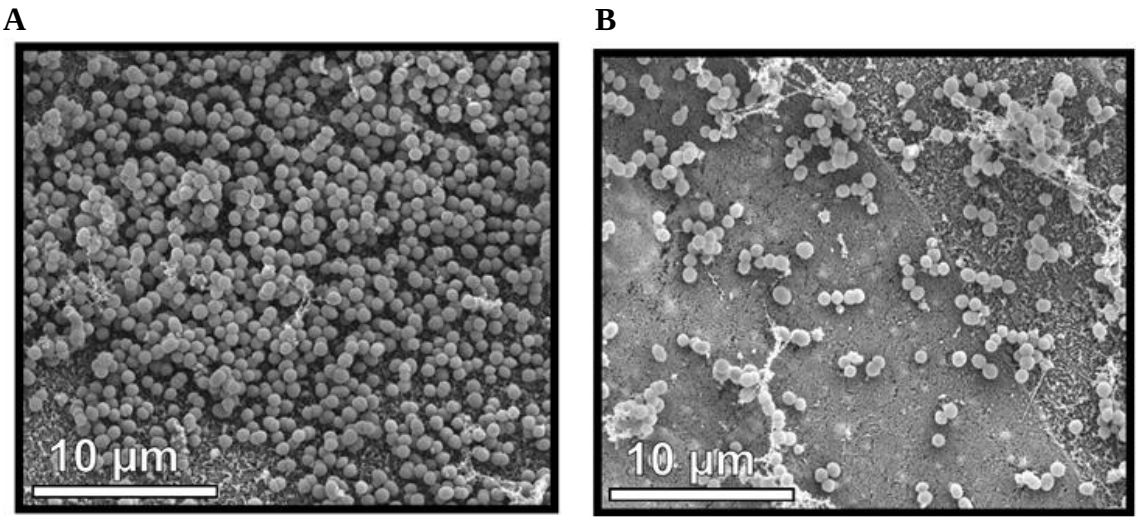

Figure 5.2. HMOs prevent GBS adherence to and biofilm formation on EpiVaginal™ tissues. High-resolution field-emission gun scanning electron microscopy (FEG-SEM) analyses of GBS strain GB00590 adherence to vaginal tissues. FEG-SEM imaging of GBS adherence was performed on GB00590 samples grown in medium alone (A), or medium supplemented with ca. 5 mg/mL of HMOs (B). The addition of HMOs significantly inhibits GBS adherence. Micrographs were collected at 10,000× magnification and magnification bars indicate 10 µm.

5.4 GBS adherence to placental macrophages and induction of extracellular trap production

5.4.1 The implications of macrophage extracellular trap production on preterm birth

Arguably the most important organ for the growing fetus, the placenta is responsible for carrying out vital functions including gas exchange, delivery of nutrients, and removal of waste.[12, 13] The placenta first develops within the uterine wall in the first trimester and continues to grow throughout pregnancy. Connected by the umbilical cord, the placental tissues are composed of the fetally-derived amnion, the chorion, and the maternally-derived decidua (**Figure 5.3**). The placenta additionally serves as a barrier between the mother and fetus. Macrophages are a specialized, tissue-resident immune cells that play an essential role in the innate immune response to pathogens, homeostasis, and tissue remodeling and repair.[14] Found within the chorionic villi of the placenta, Hofbauer cells are fetally-derived macrophages present in high abundance throughout pregnancy, representing 20-30% of all leukocytes.[15] They are essential in mediating placental development through tissue remodeling, managing inflammation, and modulating immune function.[16, 17]

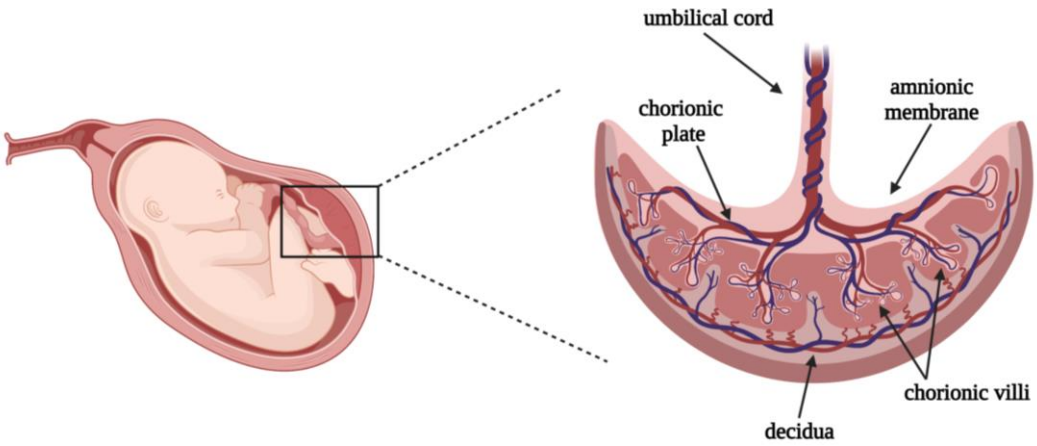

Figure 5.3. Schematic diagram of the developed human placenta. Created with BioRender.com.

These cells employ multiple mechanisms as part of the host response against microbial infections including phagocytosis; production of reactive oxygen and nitrogen species; generation of antimicrobial compounds; and macrophage extracellular trap (MET) formation.[18, 19] METs are web-like structures comprised of DNA fibers, histones, antimicrobial proteins, and cell-specific proteases (**Figure 5.4**).[20] ETs were first reported in 2004 when describing the process in which neutrophils immobilize and abolish pathogenic microorganisms, termed "ETosis".[21] In response to bacterial infection, placental METs have shown to secrete matrix metalloproteinases (MMPs) in high abundance.[22] This class of collagen-cleaving enzymes has been implicated in PPROM and preterm birth as they are known to hydrolyze ECM components, destroy tissue integrity, and weaken the fetal membranes.[23, 24]

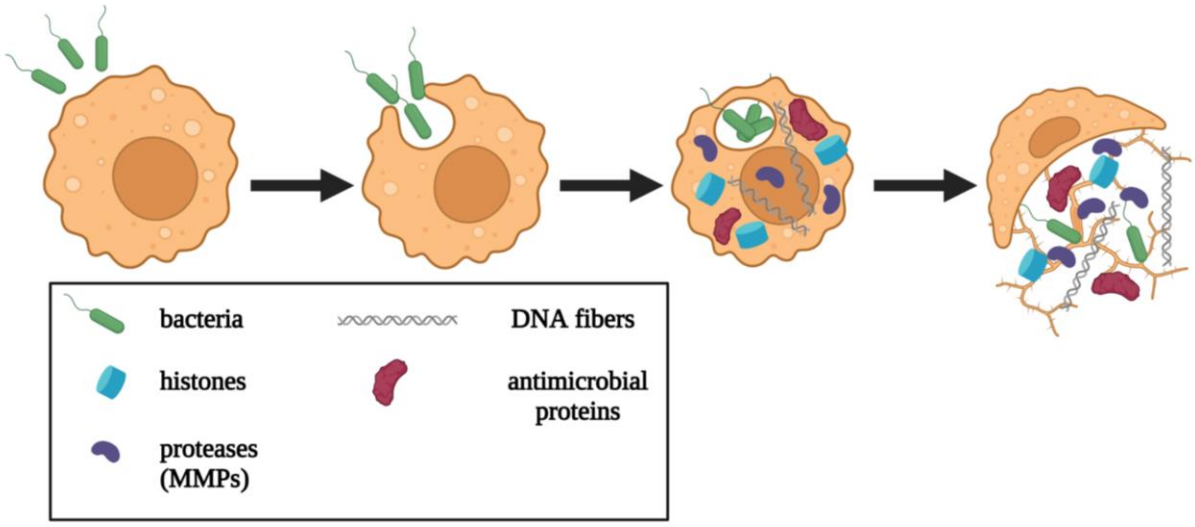

Figure 5.4. Schematic illustration on the process of macrophage extracellular trap (MET) formation. Upon contact, the macrophage engulfs the bacteria and initiates the production of histones, proteases (MMPs), DNA fibers, and antimicrobial proteins, leading to MET release. Created with BioRender.com.

5.4.2 HMOs inhibit GBS induction of macrophage extracellular traps

Gaddy et al. has previously published work demonstrating that GBS can adhere to placental macrophages and induce them to produce extracellular traps.[5, 25, 26] We have also shown there is

an accumulation of macrophages within the gestational tissues of our chorioamnionitis-induced, gravid mouse model.[27] We hypothesized HMOs would prevent GBS interactions with placental macrophages, therefore, inhibiting MET release. We sought to identify placental macrophage responses to HMO-treated GBS cocultures. *Ex vivo* isolation of placental macrophages was carried out, and these cells were cocultured with GBS in THB medium alone or THB supplemented with ca. 5 mg/mL of HMOs for 24 hours. The cells were fixed, and we examined these interactions using FEG-SEM microscopy. FEG-SEM analysis revealed enhanced MET formation in response to GBS when compared to the HMO-treated GBS cultures (**Figure 5.5**). These results could have massive implications in PPROM and preterm birth through inhibition of METs, laden with MMPs.

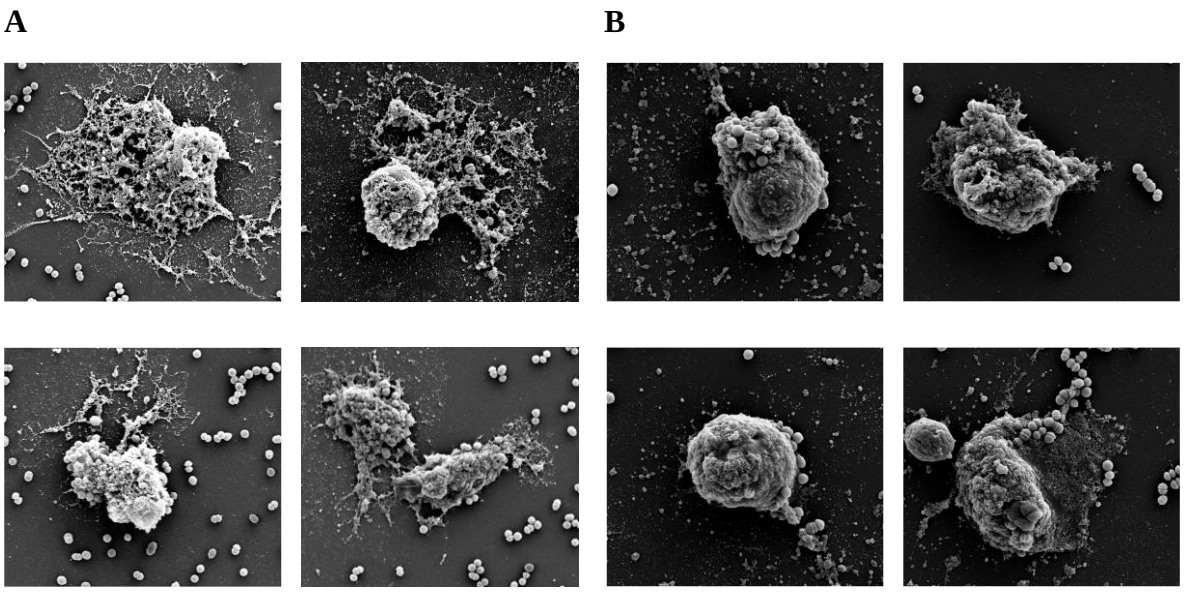

Figure 5.5. HMOs prevent GBS interactions with placental macrophages and inhibit GBS-induced MET release. High-resolution field-emission gun scanning electron microscopy (FEG-SEM) analyses of GBS strain GB00590 interactions with placental macrophages and MET formation. FEG-SEM imaging of GBS adherence was performed on GB00590 samples grown in medium alone A), or medium supplemented with ca. 5 mg/mL of HMOs (B). The addition of HMOs significantly prevents MET release. Micrographs were collected at 10,000× magnification.

5.5 HMOs prevent ascending infection during pregnancy in the mouse model

Animal models of vaginal colonization and ascending infection represent a useful tool for studying GBS disease during pregnancy. The murine mouse model mimics GBS infection of the maternal reproductive tract. We have previously demonstrated that GBS colonizes the vaginal mucosa and infiltrates the reproductive tissues of the mouse during pregnancy.[26, 28] Based on our *ex vivo* studies, we hypothesized HMOs would prevent or significantly reduce GBS colonization, ascending infection, and adverse pregnancy outcomes associated with disease progression.

To test this hypothesis, we vaginally inoculated pregnant mice with ca. 5 mg/mL of a pooled mixture of HMOs on embryonic day 14.5 (E14.5) (**Figure 5.6A**). As previously described, on E15.5 we infected the pregnant dams with GBS at a dose of $5x10^3$ to $1x10^4$ CFU allowing us to monitor disease outcomes including PPROM, preterm birth, and maternal death (**Figure 5.6C-D**).[26, 27, 29] Uninfected and untreated controls were also maintained. PPROM was identified by the presence of blood surrounding the vagina, and preterm birth was detected by the presence of pups, both occurring before E.21.5. We observed a significant increase in PPROM, preterm birth, and maternal mortality in our untreated GBS population which was recognized by the Mantel-Cox log-rank test ($p = 0.0163$, $p = 0.079$) and Gehan-Breslow-Wilcoxon test ($p = 0.018$, $p = 0.0803$). Excitingly, while we did observe one preterm birth in our uninfected HMO control, the HMO-treated, GBS infected population experienced no instances of PPROM, preterm birth, or maternal death.

In our mouse model of ascending infection during pregnancy, we set out to identify the capability of HMOs to prevent GBS invasion of reproductive tissues (**Figure 5.6B**). To study this model, pregnant mice were treated with ca. 5 mg/mL HMOs on E14.5. This was followed by vaginal inoculation of GBS strain GB590 on E15.5 at an infectious dose of $5x10^2$ to $1x10^3$ CFU.

Uninfected and untreated controls were also maintained. Two days post-infection, mice were sacrificed, and necropsy was performed on reproductive tissues (vagina, uterus, decidua, placenta, amnion, fetus) and analyzed for bacterial burden (**Figure 5.6E**). Although we did not observe a difference in colonization of the vagina, HMOs significantly reduced bacterial burden in the five additional tissues when compared to the untreated control mice. We observed a 2-log decrease in burden of the uterus, decidua, placenta, and amnion, and 1-log decrease in burden of the fetus in the HMO-treated mice compared to the untreated animals.

5.6 Conclusions and future outlook

Previously, we have shown HMOs possess potent antimicrobial and antibiofilm activity and potentiate the utility of select antibiotics against treating GBS infections *in vitro*.[1-3, 30] While preliminary studies revealed that HMOs perturb cell membrane permeability, we sought to better understand host-pathogen interactions. Our current work has demonstrated that a heterogeneous cocktail of HMOs inhibited GBS adherence and proliferation to an EpiVaginal™ human organoid tissue model, as well as to *ex vivo* human gestational tissues. These results were corroborated using high resolution imaging techniques. We additionally reported that in response to GBS interactions, HMOs prevent placental macrophage extracellular trap release which is implicated in inflammation of the fetal tissues leading to chorioamnionitis and preterm birth. Using an *in vivo* mouse model of ascending infection during pregnancy, HMOs also reduced bacterial burden of the reproductive tissues. Our results have shown that HMOs could be utilized as a potential therapeutic treatment for GBS infections and provide major contributions in reducing the pathogenesis of GBS disease during pregnancy.

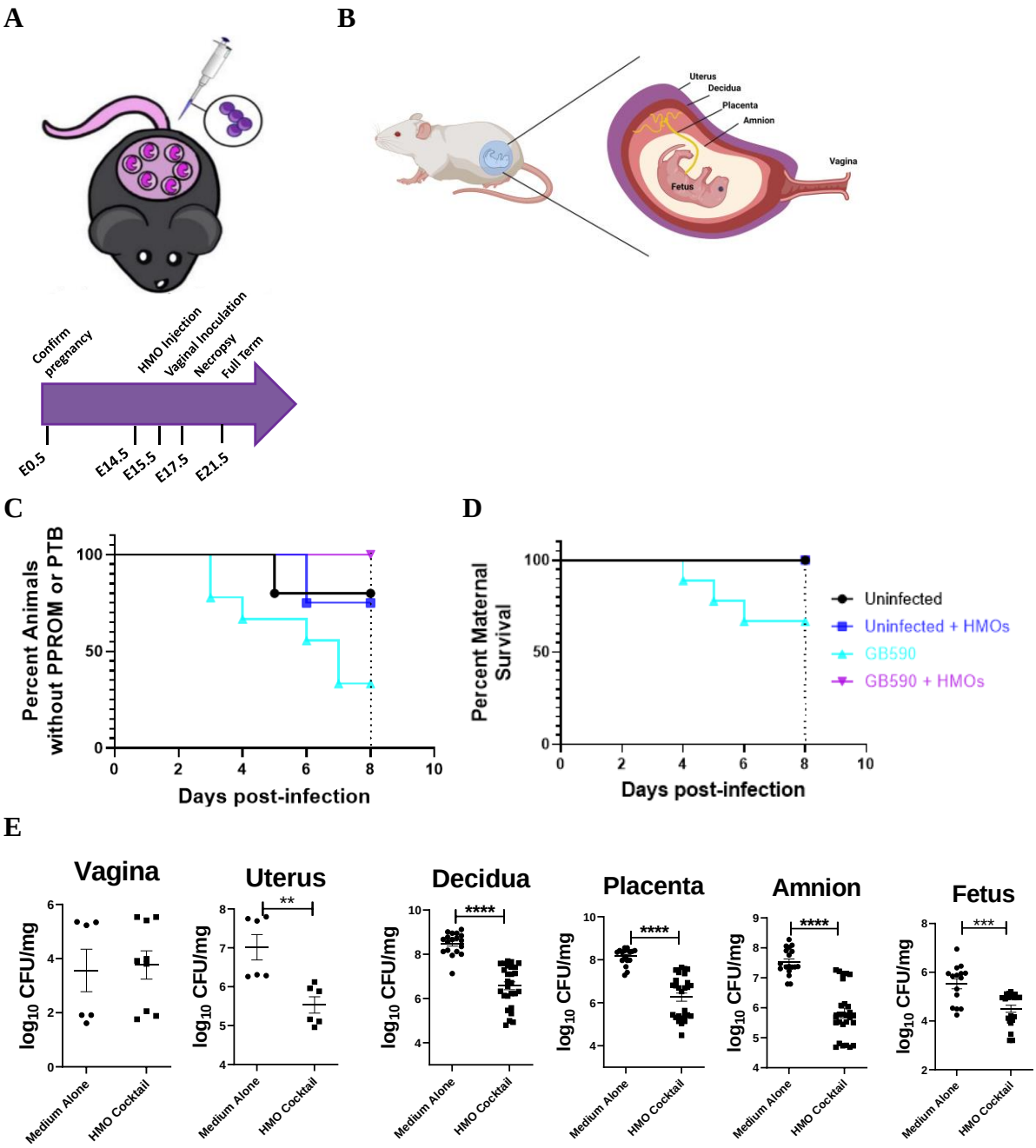

Figure 5.6. HMOs reduce cognate disease consequences associated with infection. A) Conceptual diagram of methods used in these studies. Pregnant mice were treated with HMOs on embryonic day 14.5 (E.14.5) and infected with GBS on E15.5. Mice were either sacrificed two days post-infection and reproductive tissues were collected for analyses or monitored until E21.5 for adverse disease outcomes. B) Conceptual model of reproductive tissues collected and analyzed for GBS burden. Analyses of percent animals C) without PPROM or preterm birth (PTB) or D) maternal survival. Dotted line indicates term for the average gestation in the C57BL6/J mouse model used in this study. p = 0.0163 Mantel-Cox log-rank test, n = 4-9 animals per group. E) Bacterial burden within reproductive tissues was evaluated by quantitative culture and the HMO cocktail promotes significant inhibition with respect to burden within the uterus, decidua, placenta, fetal membranes, and fetus. Bars indicate mean +/- SEM, **P = 0.0033, ***P = 0.0002, ****P < 0.0001 by Student's t test comparing the addition of the HMO cocktail to medium alone controls. Created with BioRender.com.

The first several years of our program studying HMOs for their antimicrobial and antibiofilm activities has been limited to *in vitro* studies. In the last year, we have adjusted out attention to *in vivo* and *ex vivo* studies, both on primary human tissues and in a murine animal model. Several questions remain following the initial studies we have completed.

With a concentration on our mouse model, we first desire to see if the HMOs can be administered directly into the stomach via the oral-gastric gavage method. We have only investigated how HMOs respond by vaginal inoculation which is not the ideal route for drug delivery. We know that HMOs are able to resist gastric acidity and enzymatic hydrolysis, reaching the small intestine intact where they can be metabolized by the gut microbiota.[31-33] With the knowledge that HMOs positively affect the infant microflora (See Section 2.6), we hypothesize that there is a correlation with gut health and reproductive health. Oral administration will allow us to investigate the ability of HMOs to prevent GBS infections of the reproductive tract by way of controlling microbial imbalances of the gut. It is also important for us to evaluate a range of HMO concentrations using this method. In our current studies, we have only utilized a concentration of 5 mg/mL, which is at the low end of physiological relevant concentrations of HMOs present in breast milk.[34-36]

While most of our work has focused on preventing GBS infections, we also want to determine whether HMOs can also clear infections. In our mouse model of ascending infection, we previously demonstrated that vaginal HMO treatment prior to bacterial inoculation is more effective than HMO treatment following establishment of infection (data not shown). In our oral administration of HMOs, we will assess both treatment before and after bacterial inoculation to determine if HMOs can clear infection using this method.

Future work will also focus on changes in host inflammation and proinflammatory cytokine production in response to HMO treatment of GBS infections. Histopathological examination will be used to identify architectural changes to gravid reproductive tissues. Increased abundances of polymorphonuclear immune cells in infected tissues are an indicative feature of inflammation. Previously, we have shown that in GBS-infected human EPM tissues *ex vivo* and *in vivo* fetal-placental mouse tissues there was an infiltration of polymorphonuclear cells when compared to uninfected controls.[26, 28]

Using multiplexed cytokine arrays and enzyme-linked immunosorbent assays (ELISAs), we will be able analyze immunological changes correlated with inflammation. We have previously demonstrated that GBS infection induces production of proinflammatory cytokines such as GM-CSF, IL-2, IL-6, IL-1β, IFN-γ, KC, MIP-1α, MIP-1β, MIP-2, and TNF-α.[4, 37] Upregulation of these cytokines has been to key to activating pathways related to inflammation, PPROM, and preterm birth.[38-40] We hypothesize HMO treatment will significantly reduce inflammation and proinflammatory cytokine production compared to untreated GBS infections.

5.7 Experimental methods

Bacterial Strains and Culture Conditions

The GBS strain used in this study was GB00590 (GB590) which represents the wild-type or parental strain. This strain was isolated from a vaginal rectal screen of a patient who had recently given birth and was previously classified as sequence type (ST)-19 using multilocus sequence typing. Bacterial strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar) plates at 37˚C in ambient air overnight. Bacteria were sub-cultured from blood agar plates into Todd-Hewitt broth (THB) and incubated at 37°C in ambient air overnight.

The following day, bacterial density was measured spectrophotometrically at an optical density of 600 nm (OD_{600}), and bacterial numbers were determined with a coefficient of 1 $OD_{600} = 10^9$ CFU/mL.

Human Placental Macrophage Isolation

De-identified placental tissue was collected from non-laboring women who delivered healthy, full-term infants by Caesarian section. Placental macrophages (PM) were isolated according to our previously published methods.[41] Briefly, a 30–60 g sample of tissue was excised from the placenta and washed three times in PBS, mechanically disrupted and enzymatically digested to a single cell suspension with DNase, collagenase, and hyaluronidase (Sigma-Aldrich). Cells were filtered and centrifuged, and CD14+ cells were isolated using the magnetic MACS Cell Separation system with CD14 microbeads (Miltenyi Biotec). Cells were incubated in RPMI 1640 medium (ThermoFisher) with 10% charcoal stripped fetal bovine serum (ThermoFisher) and 1% antibiotic/antimycotic solution (ThermoFisher) overnight at 37°C in 5% carbon dioxide. The following day, PMs were suspended in RPMI 1640 medium without antibiotic/antimycotic and distributed into polystyrene plates. Cells were seeded at a density of 200,000 cells per well in a polystyrene, 24-well culture plate in RPMI with 1 % antibiotic/antimycotic solution and 10 % charcoal dextran FBS (RPMI +/+), and then incubated for 24 h in a humidified atmosphere at 37 °C and 5 % CO_2.

Gestational Membrane Coculture

Gestational membranes were excised from placental tissues from females who delivered healthy infants at full term by Cesarean section without labor. Deidentified tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.

Gestational membranes were processed into 12-mm punch biopsy sections, and sections were placed with the amnion side down, in a 12-well dish containing Dulbecco's modified Eagle's medium (DMEM), high-glucose, HEPES, no-phenol-red cell culture medium (Gibco, Carlsbad, California) supplemented with 1% fetal bovine serum and PEN-STREP antibiotic/antimycotic mixture (Gibco). Sections were incubated overnight at 37°C in ambient air containing 5% CO_2; washed 3 times with prewarmed, sterile phosphate buffered saline (pH 7.4); and placed again in DMEM, high-glucose, HEPES, no-phenol-red cell culture medium (lacking the PEN-STREP antibiotic/antimycotic mixture). Bacterial cells were added to the choriodecidual surface of the gestational membranes at a multiplicity of infection of 1×10^6 cells per 12-mm diameter membrane, using a predetermined coefficient of bacterial density of 1 $OD_{600} = 10^9$ CFU/mL. HMOs were added to achieve a final carbohydrate concentration of ca. 5 mg/mL. Concomitantly, uninfected gestational membrane samples were also maintained. Cocultures were incubated at 37°C in ambient air containing 5% CO_2 for 24 hours and cells were fixed with 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) for at least 12 h prior to processing for microscopy.

Epi Vaginal Coculture

EpiVaginal™ (VEC-100™) tissues were purchased from MatTek Life Sciences. These tissues are cultured from normal, primary human-derived vaginal epithelial cells, and are similar to studying *in vivo* tissue. The tissues were processed into 8-mm diameter sections, and sections were placed in Nunc™ single well tissue culture plate inserts containing DMEM medium, F12 medium, phenol-red cell culture medium, supplemented with 5 µg/ml gentamicin (10% of normal gentamicin level) and 0.25 µg/ml amphotericin B. Sections were thawed and incubated overnight at 37°C in ambient air containing 5% CO_2; washed 3 times with prewarmed, sterile

phosphate buffered saline (pH 7.4); and placed in VEC-100-MM™ (MatTek Life Sciences) maintenance medium. Bacterial cells were added to the top face of the tissue at a multiplicity of infection of 1×10^6 cells per 8-mm diameter membrane, using a predetermined coefficient of bacterial density of 1 $OD_{600} = 10^9$ CFU/mL. HMOs were added to achieve a final carbohydrate concentration of ca. 5 mg/mL. Concomitantly, uninfected vaginal tissue samples were also maintained. Cocultures were incubated at 37°C in ambient air containing 5% CO_2 for 24 hours and cells were fixed with 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) for at least 12 hours prior to processing for microscopy.

Mouse Model of Ascending Vaginal GBS infection During Pregnancy

GBS infection of pregnant mice and subsequent analyses were performed as previously described.[26, 42] Briefly, C57BL6/J mice were purchased from Jackson laboratories and mated in harem breeding strategies (1 male to 3-4 females) overnight. The following day, pregnancy was confirmed by the presence of a vaginal mucus plug establishing the embryonic date (E0.5). On embryonic day 14.5 and 16.5 (E14.5 and E16.5) pregnant dams were anesthetized via inhalation of isoflurane and vaginally injected with an HMO cocktail at a concentration of ca. 5 mg/mL. On embryonic day 15.5 (E15.5) pregnant dams were anesthetized via inhalation of isoflurane and vaginally infected with $5x10^2$ to 10^3 colony forming units (CFU) in 0.05 mL of THB plus 10% gelatin. Uninfected controls were also maintained. On embryonic day 17.5 (E17.5) animals were euthanized by carbon dioxide asphyxiation and necropsy was performed to harvest reproductive tissues including vagina, uterus, placenta, decidua, fetal membranes, and fetus.

Quantifying Bacterial Burden in Host Tissues

To determine bacterial burden in reproductive tissues quantitative culture methods were employed as previously described.[26] Briefly, reproductive tissues were weighed and placed in sterile THB. Tissues were homogenized and subjected to serial dilution and plating onto blood agar to enumerate bacteria (CFU/mg) in host tissue.

PPROM, Preterm Birth, and Survival Analyses

GBS infection of pregnant mice and subsequent analyses were performed as previously described.[26, 42] Briefly, C57BL6/J mice were purchased from Jackson laboratories and mated in harem breeding strategies (1 male to 3-4 females) overnight. The following day, pregnancy was confirmed by the presence of a vaginal mucus plug establishing the embryonic date (E0.5). On embryonic day 14.5 (E12.5) pregnant dams were anesthetized via inhalation of isoflurane and vaginally injected with an HMO cocktail at a concentration of ca. 5 mg/mL. On embryonic day 15.5 (E15.5) pregnant dams were anesthetized via inhalation of isoflurane and vaginally infected with $5x10^3$ to $1x10^4$ colony forming units (CFU) in 0.05 mL of THB plus 10% gelatin. Uninfected, and infected (untreated) controls were also maintained. Animals were monitored daily for PPROM, preterm birth, and maternal survival. On embryonic day 21.5 (E21.5) animals were euthanized by carbon dioxide asphyxiation.

High-Resolution Field-Emission Gun Scanning Electron Microscopy (FEG-SEM) Analyses

Bacterial adherence was analyzed via FEG-SEM as previously described.[30, 43, 44] Briefly, bacterial cells were cultured in biofilms adhering to glass coverslips coated with poly-l-lysine overnight in the culture conditions described above. HMOs and βA-HMOs were dissolved in DI water to achieve a concentration of 80 mg/mL and filtered through a 0.2 μm syringe filter. HMOs or βA-HMOs were added to achieve a final carbohydrate concentration of ca. 5 mg/mL.

The following day, bacterial cells were fixed in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 0.05 M sodium cacodylate buffer pH 7.4. Samples were dehydrated with sequential washes of increasing concentrations of ethanol before being subjected to critical point drying, mounting on aluminum stubs, and sputter coating with 20 nm of gold–palladium. Samples were viewed using an FEI Quanta 250 field-emission gun scanning electron microscope at 5 kEV with a spot size of 2.5.

Immunohistochemical Analyses

Tissues were fixed in 4% neutral buffered formaldehyde overnight before being embedded into paraffin blocks. Samples were cut into 5-µm sections, and multiple sections were placed on each slide for analysis. Samples were deparaffinized with xylene, and heat-induced antigen retrieval was performed on the Bond Max automated IHC stainer (Leica Biosystems) using Epitope Retrieval 2 solution for 5 to 20 min. Slides were incubated with a rabbit polyclonal anti-GBS antibody (ab78846; Abcam) for 1 hour. The Bond Polymer Refine detection system (Leica Biosystems) was used for visualization. Slides were counter stained with eosin, dehydrated and cleared, and coverslips were added before light microscopy analysis was performed.

Cytokine Analyses

Mouse reproductive tissues, maternal sera, and amniotic fluid were analyzed by multiplex cytokine assays. Mouse tissues were placed in 1 mL of sterile PBS or THB + 10 mg/mL penicillin, then homogenized and passed through a 0.22 µm filter. Samples were frozen at -80˚C or on dry ice until analyses were performed. Samples were analyzed by Eve Technologies via multiplex cytokine array (Eve Technologies, Alberta, Canada) as previously described.[41]

Validation of host targets for specific cytokines (IL-1β, IL-6, KC, and TNF-α) were performed by sandwich ELISA (AbCam) as previously described.[4]

Histopathological Analyses

Reproductive tissues were subjected to a primary fixation in 4% formalin (neutral buffered) overnight. The following day, tissues were embedded in paraffin and sectioned into 5 µm thick sections for staining and microscopical analyses. Sections were stained with hematoxylin and eosin for histopathological examination and imaged with an OMAX M83ES compound light microscope with ToupView software package.

Statistical Analyses

Statistical analysis of parametric data with more than two groups was performed using one-way ANOVA with either Tukey's or Dunnet's post hoc correction for multiple comparisons; all reported P values were adjusted to account for multiple comparisons. For parametric data with two groups, a Student's t test or one-way ANOVA were used. P values of ≤ 0.05 were considered significant. Non-parametric data (such as log-transformed CFU data) were analyzed by Mann-Whitney U or Kruskal-Wallis tests. All data analyzed in this work were derived from at least three biological replicates (representing different placental samples). Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc.).

Ethics Statement

This study was carried out in accordance with the recommendations of the Vanderbilt University Medical Center Institutional Review Board. This protocol was approved by the Institutional Review Board (IRB #181998 and #00005756). All animal experiments were performed in accordance with the Animal Welfare Act, U.S. federal law, and NIH guidelines. All experiments

were carried out under a protocol approved by Vanderbilt University Institutional Animal Care and Use Committee (IACUC: M/14/034 and M/17/012), a body that has been accredited by the Association of Assessment and Accreditation of Laboratory Animal Care Act (AAALAC).

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About the Author

"To give anything less than your best, is to sacrifice the gift"

-Steve Prefontaine

Whenever someone asks where I am from, I always hesitate because I've "grown up" several times in different places across the United States. I was born December 27th, 1984 in Homestead, Florida. Sadly, the hospital I was born in was demolished by Hurricane Andrew in 1992. My parents, Alice and Mike, were both in the military, the Navy and Coast Guard, respectively, so shortly after I was born, we moved up the coast to Severna Park, Maryland. Oh, I should mention I'm the youngest of three children. I'm joined by my sister Laura (1978), and my brother Chris (1981). My parents both retired from the military in Severna Park, and still live there today, so I usually claim Maryland as my home. "Crabcakes and football — that's what Maryland does!" This pretty much sums it up! No, not really, but I do love Old Bay, blue crabs, and football. Severna Park was a great place to grow up. It's right on the Chesapeake Bay, we have all the seasons (ironically, I hate the cold now), you can drive to 5 states within an hour, and education was a priority.

Since I was young, I've always been competitive and determined to make my own money. I convinced my parents to let me play soccer and join the swim team when I was only 4 years old. I've never been a natural athlete, but always put in extra hours and effort to excel. Two of my greatest achievements were winning first place in the back stroke when I was 12 years old in the county meet, and scoring three goals in the championship game of a soccer tournament when I was in middle school. It was moments like this that drove me to always try and be a leader, and motivate others to tap into their potential.

My first job was when I was 10 years old as “mother’s helper”, and after realizing how good it felt to make my own money, I always had some source of income. My jobs were random: I had a paper route (remember when everyone read the paper?), sold cameras (remember getting your film developed?), and of course I worked in the service industry, preparing food and making cocktails.

I definitely wasn’t someone who always knew they wanted to be a scientist, but I did know that I would eventually pursue a career in STEM. In the 8th grade, I had a phenomenal algebra teacher named Mrs. White. I absolutely loved her class and she truly knew how to make math fun. All it took was her to joke to me one day that she was using my quizzes and exams as the answer key for me to use that as motivation to get 100% on every assignment. I still am proud that I received the “mathematician of the year award” that year; the button still hangs proudly in my childhood bedroom.

I don’t think I spent much of the next few years thinking much about what I wanted to do with my future, but once again during my junior year in high school I realized how much I enjoyed math. I serendipitously ended up taking an advanced calculus class by way of switching up my schedule to be in the same lunch period with all my friends. Turns out this class was such a success; it was then I decided I wanted to pursue a degree in engineering. At the same time, I was really enjoying my chemistry class, even tutoring some of my friends.

I applied to schools all over, really having no direction except that I wanted to enroll in a chemical engineering program. The University of Alabama offered me a nice scholarship, and the southern hospitality they showed me during my visitation is what sold me. I made the trek down to Tuscaloosa in 2003, and it was one of the best decisions I ever made. I joined the crew team my freshman year, and during my junior year we became an official division I varsity sport.

My favorite perk was getting to work out in the same gym as the football team! I also joined the engineering fraternity my freshman year, and had no clue how instrumental this group would continue to be throughout my college experience and beyond. As the first female regent (president) of the fraternity, this was a critical period I learned how to navigate being a leader in group dominated by men.

This is probably when I should say I graduated and when I knew I wanted to pursue my Ph.D. Well, I did graduate cum laude with a degree in chemical and biological engineering, but instead of venturing off into the real world, I made the decision to pursue a dream to travel the world and work on a cruise ship. I have to hand it to my parents; they didn't even try and stop me. I think they always knew I would find my way back to the sciences, and this was a necessary time in my life to just live a little. Ironically, working for Norwegian Cruise Lines over a number years was probably the hardest I have ever worked (and probably ever will work). It truly was an experience of a lifetime, island hopping and meeting people from all over the world. However, I worked 12-16 hours a day, 7 days a week, without a day off for 6 to 9 months at a time. It was the epitome of "work hard, play hard". It was during these years I learned about work ethic and how to be efficient with my time.

Following my cruise days, I was ready and motivated to make my way back to using my degree. I knew in order to be successful in my field, I would need to get back into school to refresh my skills and knowledge. In 2015, I enrolled in a Masters program at San Jose State University pursuing a degree in Chemistry. It was challenging being back in school almost 8 years later, but grateful for my P.I., Dr. Laura Miller Conrad for her guidance and support. Besides my college organic chemistry course, I had never run a chemistry reaction. My thesis project focused on synthesizing an inhibitor that targeted the production of pyocyanin, a blue-green pigmented

virulence factor produced by the opportunistic pathogen, *Pseudomonas aeruginosa*. During my time at SJSU, I worked on small molecule synthesis, photoaffinity labeling techniques, affinity chromatography, mass spectrometric analyses, and biological assays. It was about a year into my research project that I realized how rewarding my work was, and I desired to continue my education and pursue a Ph.D in chemistry.

I applied to 16 graduate schools because I was determined to get my Ph.D. at this point. Looking back, that was a little excessive, but I've always been someone who once I set my mind on something, I am going to complete it. Vanderbilt was my first choice for a number of reasons: I wanted to move closer to family and friends, the weather is pretty moderate, Nashville is a fun city, Vanderbilt has a great reputation, and most importantly Steve's research drew me in. I was accepted to several schools, but by mid-March I still had not heard from Vanderbilt. I will never forget the day I got the email from Steve asking if I was still interested in attending Vanderbilt. It was the day after I gave my final seminar for my master's thesis program at SJSU. I was elated, and before even visiting Vanderbilt, I made the decision to go there.

I started the program thinking I wanted to be an organic chemist with a slight interest in microbiology. After a rough first semester in the organic chemistry structure and mechanisms course, I knew I wanted to switch directions. I am grateful Steve took me on and trusted my skills enough to take on a larger aspect of the biology studies in our lab. For the last four years, I have been co-mentored by Steve and Dr. Jennifer Gaddy. While my path changed significantly from when I started at Vanderbilt, I am more passionate than ever about my research. I love talking about my research because its relatable and really does make an impact on the scientific community. I have taken an interest in women's health, specifically on how to prevent adverse pregnancy outcomes. I am looking forward to the next year working with Jen, and continuing to

soak up knowledge from her. In the future I would love to work in a clinical microbiology lab in a research hospital.