ABSTRACT

Bacteroides fragilis Outer Membrane Vesicles are used for Secretion of a Discrete Subset of Bacterial RNAs that Stimulate an Immune Response in Colonic Epithelial Cells

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Alterations in the diversity and function of the gut microbiome are associated with changes in the host physiology, including inflammation. A critical component of the inflammatory response system are receptors capable of sensing foreign nucleic acids (e.g. small RNAs) that are carried as cargo in bacterial outer membrane vesicles (OMVs). The mechanisms by which human extracellular RNAs elicit immune responses have been well established, while the contribution of bacterial sRNA to host physiology remain unclear. We hypothesize that pathogenic and commensal microbes use OMV-associated small RNA species to differentially affect host inflammatory responses. First, we profiled the small RNA contents of purified OMVs from a commensal strain (NTBF) and a pathogenic strain (ETBF) of *Bacteroides fragilis.* To distinguish the differences in the sRNA profiles of both strains and their OMVs, we conducted small RNA-seq and identified enrichment of discrete sRNA species in OMVs that were also differentially expressed between the two strains. This evidence led us to investigate the differential effects of these OMVs upon intestinal epithelial cells. To understand the effects of OMVs on pattern recognition

receptors, we treated Toll-like receptor (TLR) reporter cells with NTBF and ETBF vesicles. We observed activation of TLR2 in a dose-dependent manner, and activation of TLRs 3 and 7 at high doses of OMVs. Using Caco-2 and HT29 cells exposed to OMVs from each strain, we ran qPCR to test several pro- and anti-inflammatory cytokines. We observed that both strains upregulate the expression of IL-1β and TGFβ, but NTBF stimulates a greater IL-8 response compared to ETBF. These results indicate that bacteria may preferentially load small RNAs into vesicles that target host cells, which differentially affect host immune responses through RNA-sensing pathways. Overall, our data suggest a key function of bacterial small RNAs and their OMV vehicles in controlling host immune system.

Bacteroides fragilis Outer Membrane Vesicles are used for Secretion of a Discrete Subset of Bacterial RNAs that Stimulate an Immune Response in Colonic Epithelial Cells

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A Dissertation

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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May 2023

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ACKNOWLEDGMENTS

I am truly grateful to everyone who have supported me through this project and dissertation. I have only succeeded because of the love and support of the village that has guided me over the course of my life.

I would like to begin by thanking my advisors Drs. Leigh Greathouse and Joseph Taube. The mentorship and support that you both have provided over the years has been critical to my development as a scientist, to the success of this dissertation, and to the pursuit of my career goals. I will be forever grateful that you both allowed me to join your labs for the OMV-TLR project; having the opportunity to work with you is one of the best decisions I have made in my life.

I would like to thank everyone from the Greathouse and Taube labs for the support they provided, whether it was critiques, methodology development, or assistance with experiments. I would like to especially thank Alysia Martinez and Michelle Pujol for their assistance in the analysis and qPCR validation of the RNA sequencing data. To Emily Lin for her assistance when I first joined the Taube lab in 2017. To Harry Ojeas for those early RNA isolation experiments that allowed us to get the OMV-associated RNA samples for northern blotting and qPCR. To Colin Scano for his work on the RNA synthesis, Julia Xu for her imaging of vesicle interactions, and to Ankan Choudury for his general critiques and assistance with bioinformatics. I would like to thank Dr. Bernd Zechmann for his assistance with TEM imaging, and also for the opportunity to work on a manuscript with him. Many thanks to Drs. Christie Sayes and Amanda Sevcik for their

guidance and collaboration with the nanoliposome synthesis. I am also thankful to my committee members for their guidance and support over the years: Drs. Bessie Kebaara, Erich Baker, and Chris Kearney. I would also like to thank Baylor Biology for being my home for the last 9 years.

My time at Baylor has allowed me to develop meaningful relationships with my peers, faculty, and staff that have supported my endeavor into science research. Dr. Kevin Jackson has supported my academic and personal growth since my freshman year, and Drs. Chris Rios, Beth Allison Barr, and Sara Dolan have been extremely supportive in the work that I have done with the GSA. The staff at the Baylor RoundUp and Baylor Photography, Julie Reed, Bruce Gietzen, Matthew Minard, Robbie Rogers, and Morty Ortega. All of these people gave me the opportunity to pursue my hobbies outside of academia and provided me with an outlet that prevented me from getting overwhelmed. To the staff of the BSB Starbucks and Which Wich for keeping me fed and caffeinated for 9 years and especially during the long hours spent in the lab and office. I am also thankful to Dr. Tamarah Adair for the opportunity to be apart of her SEA-PHAGES group in my freshman year.

I would like to also acknowledge everyone that have been by my side since the beginning. Without the love and support of these people I would not be where I am today. To my 7th grade science teacher Mr. Jason Li Ying and to Dr. Darcy Lidington for sparking an interest in research and love of science in me. To Saman Arfaie, Nalin Wijemuni, Amr Saleh, and Tanzim Hoque. To my friends at Baylor: Bryan King, Haleigh Parker, Charli Worth, Colin Scano, Madhur Wyatt, Julia Xu, Kayla Haberman. To my friends in GSA and to my clanmates in Othrys Sanctuary for the hours of stress relief in

Warframe. To my brothers at Delta Epsilon Psi past and present, especially Amaan Sheikh, Dustin and Jonathan Jaoude, Pranav Kapoor, and Shiva Dhiman. To Ryan Coleman and Kristen Bolte Coleman, Donna Daneshpajooh, Eric Soo and Jonathan Ross. To my first friend at Baylor, Pavithra Srinivasan. And most importantly to my parents and sister Abu Salmaan, Fouzia, and Hannah Sheikh.

DEDICATION

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

To my parents, sister, and the people that have raised and guided me. You have been instrumental in my development as a Muslim, a scholar, and a person.

ATTRIBUTIONS

Chapter Two:

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Chapter Three:

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Chapter Five:

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CHAPTER ONE

Introduction

Colorectal Cancer is a Major Public Health Concern

Colorectal cancer (CRC) is the third most-commonly diagnosed cancer in the United States and globally, and represents the second most common cause of cancer-related mortality [1,2]. The majority of CRC diagnoses and mortality are concentrated in developed nations [3] and result from factors such as a sedentary lifestyle, obesity, and a Western diet [4]. The risk of CRC development is increased in societies that consume higher portions of fats, processed red meats and grains, and low dietary fiber [5,6]. Socioeconomic progress in societies is accompanied with improvements in the citizens' quality of life, such as increased life expectancy and increased dietary options coinciding with a Westernized diet [5,7,8]. Such lifestyle changes increase the population of obese and overweight citizens which leads to an overall higher incidence of CRC diagnoses [9]. Abdominal adipose tissue accumulation is strongly associated with the CRC due to disruption of metabolic and inflammatory pathways in the intestines [10].

The higher life expectancy in developed nations is due to the high economic growth and productivity [11,12]. As per capita income increases, countries spend more on healthcare resources including physician accessibility, medical screening and research, and preventive medicine [11–15]. Advancements in CRC screening and treatments options have mitigated a portion of disease burden [7,16,17]. However, diagnoses and treatment costs present a huge economic burden on patients and the healthcare system, costing an estimated $14.1 billion

US dollars [18–20]. The issue is further exacerbated by socioeconomic and healthcare inequities, leading to increasing case numbers and a rising incidence in young adults, demonstrating the major risk that CRC poses to long-term public health and safety [16,17,21]. Current estimates project that CRC diagnoses to increase 210,000 cases and 80,000 death annually in 2040 [17]. The projected increases in CRC cases and its economic impact highlight the importance of understanding the contributors to and mechanistic evolution of colorectal carcinogenesis to develop better preventative and therapeutic options.

Chronic Inflammation Contributes to Colorectal Cancer

While the factors that contribute to CRC are complex, a major documented contributor to carcinogenesis is chronic intestinal inflammation [22,23]. Two major maladies that contribute to persistent colonic inflammation are Crohn's disease (CD) and ulcerative colitis (UC) and fall under the larger umbrella of Inflammatory Bowel Disease (IBD) [24]. The pathology of IBD is significantly associated with genetic and environmental factors that lead to dysregulation of the mucosa and epithelium [7,25]. These diseases can prime the intestinal tract for a subset of CRC known as colitis-associated CRC (CAC) [26–28]. A person suffering with IBD will have increased susceptibility to CAC with time and severity as sustained inflammation can induce intestinal barrier injury and promote epithelial mutagenesis [29–31]. The key characteristics of CAC include diagnoses at a younger age, and higher incidence of lesions and mutations [32,33].

While CAC shares many commonalities with CRC, it exhibits a distinct molecular pathogenesis pathway, including a distinct order of gene mutations in the colonic epithelia, leading to unique pathophysiological outcomes such as chemoresistance and poor prognosis [34]. Clinical diagnosis of CRC is dependent on endoscopic biopsy of intestinal

lesions and dysplasia [27,35–39]. However, while spontaneous CRC can rely on the histological appearance of "conventional" dysplasia, CAC-associated mutations tend to develop in histologically normal presenting tissue that go undetected prior to the appearance of cancerous lesions [27,35–39]. Early *TP53* mutations characterize the pathogenesis of CAC and act as the founding mutagenic event, while mutations in *KRAS* and *APC* occur much later than spontaneous CRC [39–41] (Figure 1.1). Additionally, CAC tends to develop in multiple locations at the same time due to the systemic nature of IBD and has higher genomic instability [42,43].

An important controlling component of intestinal inflammation is the microbiome and microbial community structure [44–46]. The numerous species of bacteria, viruses and fungi within the gut interact with host epithelial and immune cells in a symbiotic manner; disruptions, or dysbiosis to that balance can lead to chronic inflammation and pre-dispose patients to developing CAC [45,47,48].

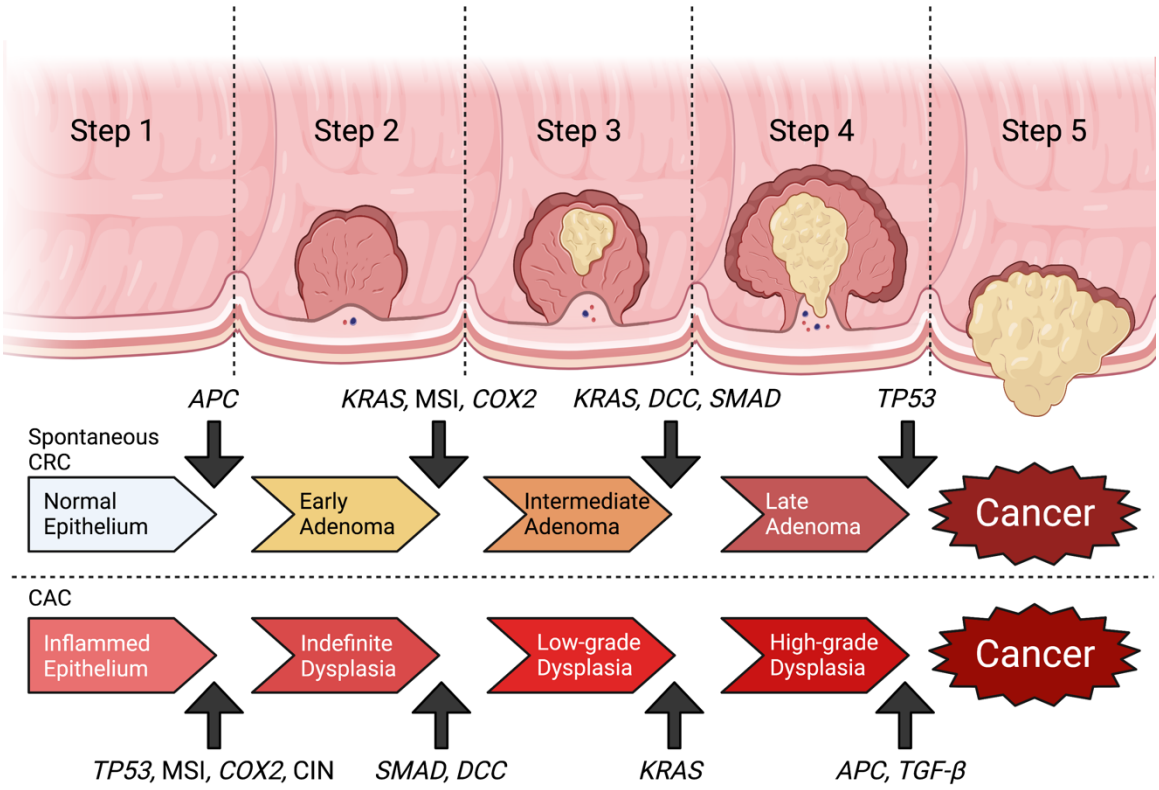


Figure 1.1: The differences in the molecular development of spontaneous CRC (top) and CAC (bottom). Intestinal inflammation is critical to the initiation of CAC and the temporal progression of associated mutations are different from spontaneous CRC. Mutations that occur early in spontaneous CRC, such as *APC* and *KRAS* happen much later in CAC. Meanwhile, mutations in *TP53, DCC,* and *SMAD* present earlier in CAC are a much later phenomenon in spontaneous CRC. CRC: Colorectal cancer, CAC: Colitis-associated cancer, *APC:* adenomatous polyposis coli, *DCC:* Deleted in Colorectal Cancer.

The Intestinal Microbiome Affects Colorectal Cancer Development

The gut microbiome is becoming increasingly recognized for its role in health and disease [49,50]. Three phyla make up the majority of human intestinal microbiome: *Firmicutes, Bacteroidetes*, and *Actinobacteria* [5,51] with most of the genera represented in community composition being obligate anaerobes such as *Bacteroides, Eubacteria, Bifidobacterium,* and *Fusobacterium* [5,51]. The initial colonization of an individual begins after birth through interactions with the maternal microbiome via delivery and milk feeding [52,53]. This primary microbiome mostly consists of anaerobic bacteria such as *Enterobacter, Enterococcus, Staphylococcus* and *Streptococcus* [52,53]. The microbiome experiences a shift

to a *Bifidobacterium* and *Lactobacillus* rich community through the introduction of solid foods [52,53]. Weaning from a milk-based diet to a purely solid food diet promotes the colonization of bacterial species belonging to the Firmicutes and Bacteroidetes phyla and the establishment of a community structure that remains stable for the rest of the individual's lifespan but can also adapt to environmental and intrinsic pressures [52,53].

The microbiome can provide many benefits to the host organisms as commensal microbes can facilitate a number of physiological pathways such as nutrient processing, immunotolerance and epithelial barrier maintenance [44,45,54–58]. Germ-free mice are an integral model for studying the role and effects of the microbiome on immune and disease development [59,60]. Studies using gnotobiotic animals demonstrate significant changes to the intestinal environment in the absence of a microbiome. Such alterations include poor development of the immune system and gut-associated lymphatic tissue, and poor development and renewal of the epithelial barrier [61–64]. Colonization of germ-free mice with commensal bacteria can lead to important systemic physiological shifts for the animal. These changes included restoration of the barrier integrity of the intestinal tract, and increased production of antibodies and migration of lymphocytes into the mucosal environment [63,65,66].

Perturbations of the microbiome, including changes in community structure and production of microbial products can have negative health effects on the host [45,47,48,50,67,68]. Factors such as disease, dietary and environmental changes can shift the radically change the proportions of the microbial community [44,45,50,68]. These changes in community structure give rise to adverse impacts on host health due to pathogens out-competing the symbiotic microbes, thus reducing the beneficial effects that they have on the hosts [67,69].

The dysregulation of microbial structure can give rise to many physiological changes and metabolic disorders in the host. Studies of individuals with obesity reveal an overall lower diversity of the microbiome compared to healthy individuals, and germ-free mice that receive the fecal microbiome of individuals with obesity gain weight in comparison to mice that receive a fecal matter transplant from healthy people, suggesting that the overall microbial community structure contributes to the health outcomes in the host [70,71]. The decrease in microbial diversity is also reflected in individuals with IBD and is associated with the development of CAC [50,72–76]. Carcinogenesis in the intestinal tract is associated with unique microbial community signatures. Early stages are associated with genera of *Fusobacterium, Parvimonas, Gemella* and *Leptotrichia*, lesions show enrichment of *Escherichia coli* and *Pseudomonas veronii,* and ~40-60% of carcinomas have high carriage of *Fusobacterium* (Figure 1.2) [77,78].

Variations in genes that code for immunity-related functions are associated with IBD and directly relate to interactions with microbiome. The most significant of these variations is in the nucleotide oligomerization domain 2 (*NOD2*) gene [46,79,80]. NOD2 is an intracellular pattern recognition receptor that interacts with bacterial peptidoglycan and is expressed in intestinal epithelial cells [81]. NOD2-dificient mice are shown to have an altered microbiome in modes of colitis when compared to wild-type mice [46,82,83]. People diagnosed with IBD that have NOD2 variations have a markedly different microbiome, containing a lower proportion of Firmicutes and enrichment of *Helicobacter, Mycobacteria* and *Escherichia* species [46,84]. General changes in the microbiome can lead to a pro-inflammatory environment, and cancer and can be associated with genetic variations. In addition to shifts in the microbial community structure, specific species of bacteria have

been found to directly initiate, or promote carcinogenesis, including *Helicobacter pylori, Fusobacterium nucleatum*, *E. coli* pks+ and enterotoxigenic *Bacteroides fragilis.*

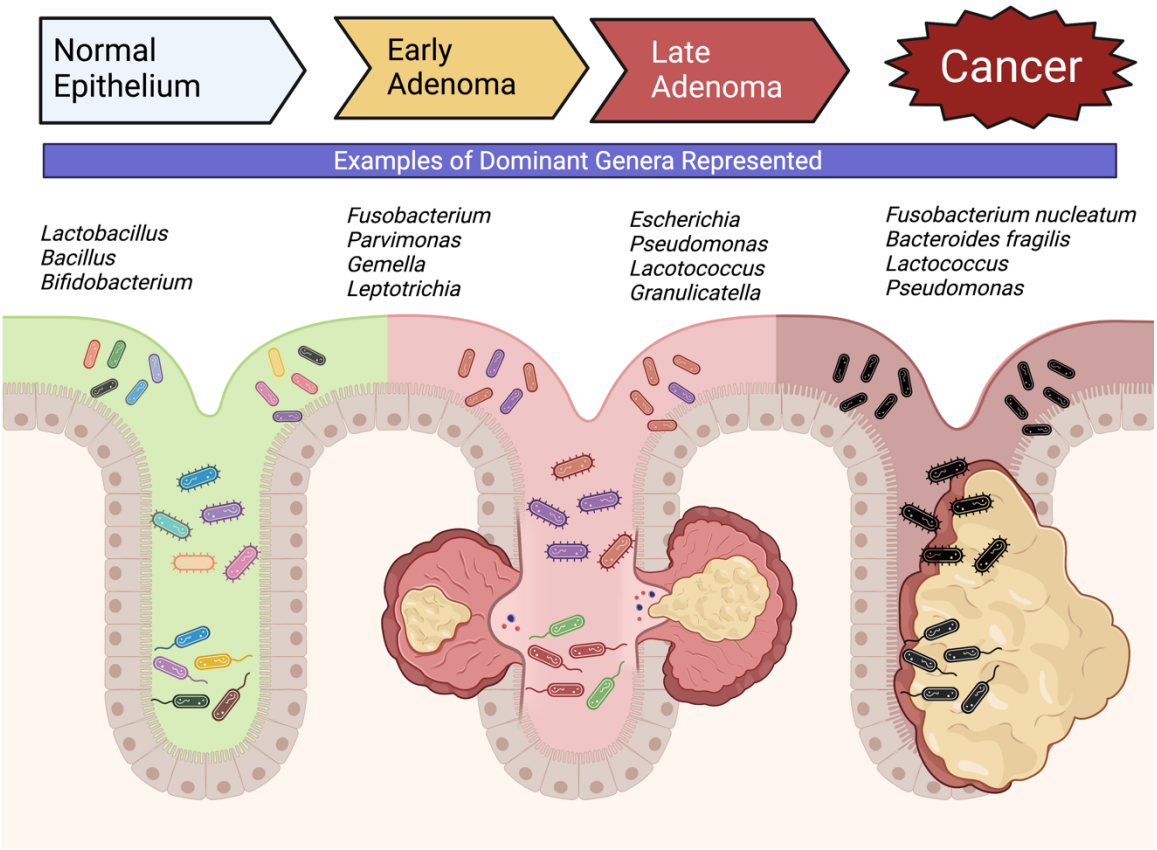


Figure 1.2: Colorectal cancer progression is associated with unique microbial signatures. Under normal, healthy conditions, the microbiome contains commensals belonging to the *Lactobacillus, Bacillus,* and *Bifidobacterium* genera. These proportions change with the development of precancerous lesions and adenomas. In early adenomas, enrichment of *Gemella, Leptotrichia, Parvimonas,* and *Fusobacterium* begin to be seen, followed by the emergence of *E. coli, P. veronii, Granulicatella,* and *Lactococcus* as cancer progression continues. As tumors develop and become established *F. nucleatum, B. fragilis, Pseudomonas,* and *Lactococcus* become prevalent in the microenvironment.

Bacteroides fragilis are Clinically Important Species in Colorectal Carcinogenesis

Among the microbes in the gastrointestinal tract, one of the most abundant gram-negative phyla is Bacteroidetes, accounting for about 50% of the species detected in sequencing data from patient samples [85,86]. *Bacteroides fragilis* is a gut commensal that constitutes ~1-2 % of cultured fecal samples [87], yet it has important roles in directly

influencing the host immune system and has been implicated in colitis and CAC [88–90]. An individual can be colonized *B. fragilis* early on in life and remain as a symbiotic microbe throughout the person's lifespan [91]. The strain of *B. fragilis* that acts as a commensal (NTBF) can promote the induction of $CD4^+$ and regulatory T cells through the secretion of polysaccharide A (PSA) [92,93], and experimental data demonstrate that NTBF can protect its host from colitis [89]. While NTBF provides immunomodulatory benefits to its host, *B. fragilis* can also exist as a pathogen (ETBF) that is associated with IBD and CAC (Figure 1.3) [94,95]. This pathogenic strain can be outcompeted by NTBF in a normal intestinal flora environment; NTBF can induce cell death in ETBF cells through a type VI secretion system [96,97]. While NTBF provides physiological benefits for the host organism, ETBF can promote the activation of inflammatory pathways through its toxins.

The *B. fragilis* genome exists in 3 distinct patterns. The ETBF genome is categorized as pattern I as it contain the 6-kb *B. fragilis* pathogenicity island (BfPAI) and 12-kb BfPAI flanking regions that encode for the *B. fragilis* toxin (BFT or fragilysin) [98–101]. The NTBF genome is split between pattern II and pattern III [100,102,103]. NTBF strains that fall under pattern II lack both the BfPAI and its flanking regions, while pattern III NTBF cells contain the flanking region with the BfPAI deleted from the genome [103]. Comparison of ETBF and pattern II NTBF genomes reveal conjugative transposons in the BfPAI flanking region of ETBF and homologous genetic elements in NTBF, suggesting that horizontal gene transfer of the BfPAI to NTBF gives rise to ETBF [102]. The appearance of the 12-kb BfPAI in pattern III NTBF cells suggests that recombination in ETBF cells deleted the BfPAI from the genome, resulting in a cell that cannot produce BFT [103]. Further comparison of the ETBF and NTBF genomes demonstrates a regulatory mechanism for the

expression of PSA via inversion of the PSA gene promoter [104,105]. Analysis of ETBF samples collected from intestinal biopsies of patients diagnosed with IBD revealed a high proportion of *bft*-positive cells with the PSA promoter in the "off" position, suggesting that a combination of BFT and low PSA expression in ETBF cells can exacerbate inflammatory responses in the colon [104,105].

BFT is a 21 kDa secreted zinc metalloprotease that is encoded by the BfPAI and can affect the intestinal epithelia by cleaving E-cadherin in tight junctions and promote cytoskeleton remodeling, resulting in diarrhea and chronic inflammation [98,106]. Despite BFT being a well characterized toxin, the method of secretion and delivery to host cells are not well understood. BFT does not appear to be secreted through known type I-VI secretion systems as direct secretion into the harsh extracellular environment of the intestine may result in the degradation of the protein [107,108]. While there is no association of BFT with the canonical bacterial secretion systems, there is increasing evidence demonstrating the packing and delivery of BFT through secreted outer membrane vesicles (OMVs) [108,109].

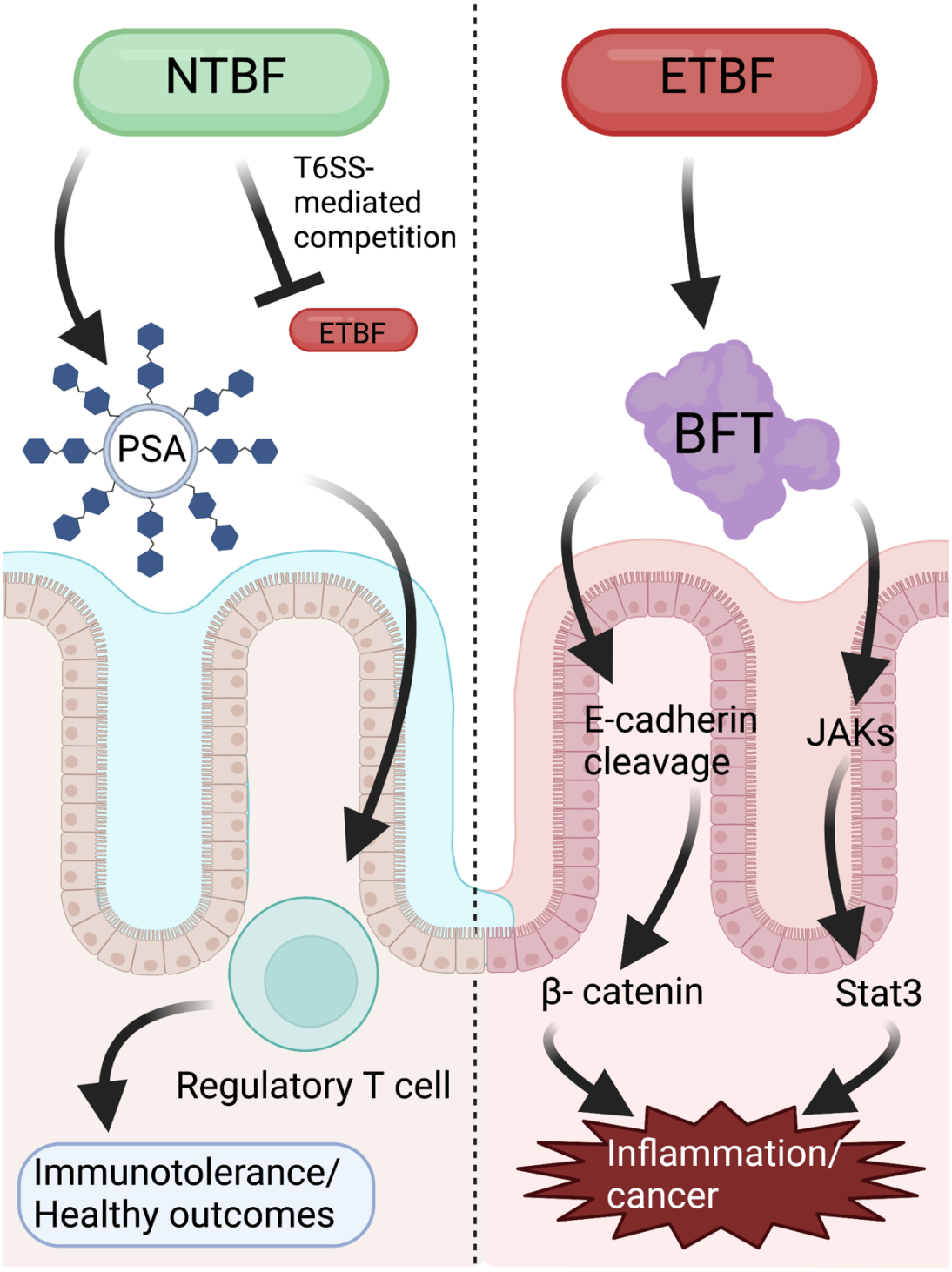


Figure 1.3: The different physiological outcomes with NTBF and ETBF. NTBF is a commensal that promotes immunotolerance in the host by inducing regulatory T cell proliferation via Polysaccharide A (PSA). It also competes with ETBF and promotes its cell death through a Type VI secretion system. ETBF produces the BFT protein which promotes cleavage of E-cadherin in the tight junctions. This promotes the movement of β-catenin into the nucleus and the transcription of pro-inflammatory cytokines. BFT also promotes inflammation through the activation of the JAK-STAT3 pathway.

Outer Membrane Vesicles are Used by Bacteria to Communicate with Host Cells

Cells in all domains of life produce extracellular vesicles (EVs) to facilitate cellular communication [110,111]. EVs enable cells to secrete sensitive biomolecules and factors that would otherwise degrade in the cellular microenvironment [110–114]. EVs serve to conserve the functions of these factors and have important roles in eukaryotic cell death and recycling, immune surveillance and activation, and cell physiology [110–114]. In the last decade there is growing evidence highlighting the importance of EVs in various pathologies such as cancer, Alzheimer's, diabetes mellitus and IBD [110,111].

In eukaryotes, EVs can be divided into two major groups (Figure 1.4). Shedding vesicles and exosomes [115,116]. Shedding vesicles arise directly from budding of the cell membrane. In order for budding to occur, the membrane undergoes certain molecular changes at the site of vesicular genesis including an increase of intracellular calcium and rearrangement of membrane lipids, and is generally triggered by movement of biomolecules to the area [117,118]. The influx of calcium into the cytoplasm activates the protease calpain to detach membrane proteins from the cytoskeleton and promote actin remodeling [117]. The budding and release of shedding vesicles occurs at lipid rafts on the plasma membrane. The increase in cytosolic calcium results in the activation of floppase to move lipids from the inner leaflet to the outer leaflet of the membrane and the deactivation of flippase to prevent lipid movement to the inner leaflet [118–120]. The activation of these enzymes result in the flipping of phospatidylserine to the outer leaflet of the phospholipid bilayer, resulting in vesicle formation [119].

Eukaryotic exosomes arise from intraluminal vesicles created within multivesicular bodies (MVB) [121–123]; these MVBs fuse with the plasma membrane and expel their contents

into the extracellular environment [123,124]. The formation of exosomes is dependent on the endosomal sorting complex required for transport (ESCRT) pathway [125–127]. The ESCRT pathway is dependent on the interactions of the ESCRT complex with endosomally ubiquinated proteins that facilitates their sorting into MVBs [127]. To transport a ubiquinated proteins from endosomes to an MVB, ESCRT-0 binds to ubiquitin and sequester the protein to the endosomal membrane. ESCRT-I, -II and -III will subsequently bind and promote budding of the membrane, flowed by Vpsp4-mediated cleavage [125,126,128]. The pathway regulating the transfer of MVBs to the plasma membrane is still poorly understood, however Rab GTPases including Rab5, Rab7, Rab27a/b, and Rab35 have been implicated in movement and fusion of MVBs to the cellular membrane [129–133].

In prokaryotic cells, the best described class of MVs are the outer membrane vesicles (OMVs) of Gram-negative bacteria [134–136]. OMVs have been implicated in roles like that of their eukaryotic counterparts: controlling cell population growth, the transfer of metabolites and plasmids, and contributing to maladies in the host organism [134,137]. These vesicles vary from 10 nm to 200 nm in diameter [135] and contain many of the same immunogenic biomolecules as the cells they are derived from including lipopolysaccharides (LPS), peptidoglycans (PGs), and nucleic acids [138–142]. In the intestinal tract, OMVs can be used by the bacteria to modulate host cell responses by interacting with pattern recognition receptors (PRRs) to activate downstream immune cascades to maintain immune tolerance of the microbiome, or to promote inflammation [48,143–148]. Analysis of OMVs demonstrate enrichment of specific biomolecules, though much is still unknown about the loading and export mechanisms that are involved in vesicle formation [107,108,139,140,149–151].

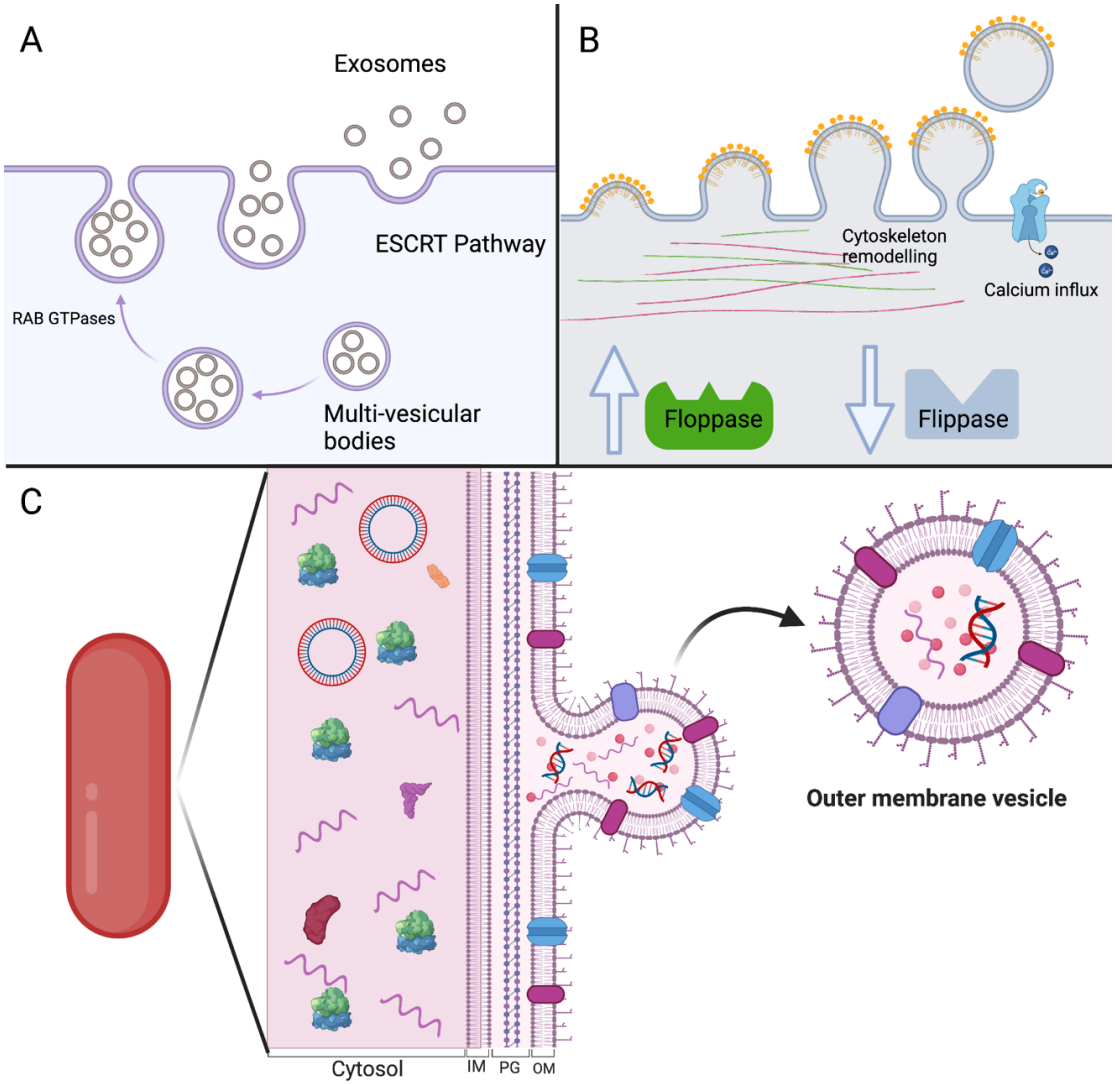


Figure 1.4: Comparison of vesicular formation mechanisms in eukaryotes and prokaryotes. Eukaryotes have two canonically described pathways for vesicle formation (A and B). Exosomes (A) arise in multivesicular bodies through and ESCRT pathway medicated mechanism and fuse with the cell membrane through a RAB GTPase mediated pathway, expelling the luminal vesicles into the extracellular space. Membrane/Shedding vesicles (B) form due to aggregation of lipid rafts and biomolecules near the cell membrane. An influx of calcium activates floppase and deactivate flippase to promote the transfer of phosphatidylserine from the inner leaflet to the outer leaflet of the cell membrane. This action promote cytoskeleton remodeling, leading to budding and the subsequent removal of vesicle from the cell membrane. Bacteria (C) produce outer membrane vesicle (OMVs), however the mechanisms for cargo loading and vesicle formation have yet to be fully elucidated.

Outer Membrane Vesicles are Enriched in RNA species that Contribute to Host Responses

Research to characterize the contents of OMVs has increased in the last decade, with most of the focus on the export of LPS, PGs, aminoglycosides, and virulence factors

[149,152–154]. Studies that characterize of the nucleic acid profiles, particularly RNA, of OMVs are not as numerous as their lipid and protein counterparts [155–157]. RNAs have important functions in cell physiology beyond acting as messengers in the central dogma [155].

In eukaryotes, non-coding RNAs can control transcription and translation, regulate immune responses, and modulate the cell cycle [158,159]. Cells can package RNAs into EVs and target distant sites to regulate physiology. The RNAs packaged into EVs are usually representative of the transcriptome and physiological state of their cell of origin, but differ in enrichment of certain RNA species [160]. EVs are taken up by recipient cells through several different mechanisms such as receptor mediated endocytosis, lipid raft interactions, clathrin and caveolin-dependent uptake and membrane fusion [161]. Seminal studies have demonstrated the importance of EV-derived RNA in cancer metastasis and the potential of these EVs as a biomarker for disease [162]. Colorectal cancer cells can enrich EVs with mutated *Rab13* mRNA and transfer them to recipient cells [163]. mRNAs packaged in EVs can also be translated by recipient cells and can affect in cell physiology processes [160,164]. Non-coding RNAs are also represented in EVs including lncRNAs, miRNAs, and tRNA fragments.

In bacteria, non-coding RNA fragments and sequences derived from intronic regions have been described in OMVs and can interact with host cells [155]. There is evidence of OMVs delivering RNAs to host cells to activate nucleic acid sensing PRRs like Toll-like receptors (TLRs) and retinoic acid inducible gene (RIG) receptors to affect immune responses [165,166]. Research to fully understand the effect of these RNA species on host cells and the mechanism by which they elicit their effects is underway and still in its infancy. OMVs can protect RNA species from RNases and enable efficient transfer of sequences

from the cell of origin to the recipient cell. There is also evidence that these RNAs act as eukaryotic miRNAs and can modulate host cell translation [157]. A methionine tRNA fragment from *P. aeruginosa* can interact with host MAPK mRNA and downregulate the expression of the pro-inflammatory cytokine IL-8 in human epithelial alveolar cells [157]. Despite recent advancements to understand the OMV transcriptomes, much is still unknown about the sorting and downstream effects of sequences that are enriched in the vesicles.

CHAPTER TWO

Contribution of the Microbiota and their Secretory Products to Inflammation and Colorectal Cancer Pathogenesis: The Role of Toll-like Receptors

This chapter is published as: Aadil Sheikh, Joseph Taube, K Leigh Greathouse, Contribution of the microbiota and their secretory products to inflammation and colorectal cancer pathogenesis: the role of toll-like receptors, *Carcinogenesis*, Volume 42, Issue 9, September 2021, Pages 1133–1142, https://doi.org/10.1093/carcin/bgab060

Abstract

Alterations in diversity and function of the gut microbiome are associated with concomitant changes in immune response, including chronic inflammation. Chronic inflammation is a major risk factor for colorectal cancer (CRC). An important component of the inflammatory response system are the toll-like receptors (TLRs). TLRs are capable of sensing microbial components, including nucleic acids, lipopolysaccharides, and peptidoglycans, as well as bacterial outer membrane vesicles (OMV). OMVs can be decorated with or carry as cargo these TLR activating factors. These microbial factors can either promote tolerance or activate signaling pathways leading to chronic inflammation. Herein we discuss the role of the microbiome and the OMVs that originate from intestinal bacteria in promoting chronic inflammation and the development of colitis-associated CRC. We also discuss the contribution of TLRs in mediating the microbiome-inflammation axis and subsequent cancer development. Understanding the role of the microbiome and its secretory factors in TLR response may lead to the development of better cancer therapeutics.

Chronic inflammation is a critical hallmark of cancer [23] as it increases the risk for most cancer types by nearly 20% and increases cancer-related mortality by more than 33% [167]. Chronic inflammation can be caused by several factors both exogenous and endogenous, including environmental exposures (e.g. tobacco smoke) and reactive oxygen species, respectively. Patients with inflammatory bowel disease (IBD) are at significantly higher risk for developing colorectal cancer (CRC) compared to the general population; epidemiological studies conclude that CRC accounts for 10-15% of all-cause mortality in patients diagnosed with IBD [26,30]. Repetitive exposure to tobacco smoke can promote the development of lung and colorectal cancers by triggering inflammation, and producing reactive oxygen species (ROS), which can lead to DNA damage [168,169]. Multiple studies demonstrate that genetic mutations (e.g. *APC, TP53, KRAS*) together with the production of pro-inflammatory cytokines (e.g. TNF-α, IL-6) can increase risk of CRC up to 70% [170,171]. In contrast to sporadic CRC, which occurs mainly due to hereditary mutations and/or environmental mutagens, inflammatory or colitis-associated cancer (CAC) is driven by several key players including signal transducer and activator of transcription 3 (STAT3), nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), and toll-like receptors (TLRs)[31]. A key component of immunoregulation comes from exposure to microbes residing in the gut and other organs. The microbial community is essential to educating the immune system, and promoting either tolerance or resistance, largely through the TLR pathway; a first line of defense [47]. Alterations in composition and function of the microbiome can induce inflammation in the colon, as well as induce cellular changes that initiate or promote carcinogenesis [47,48,51,68,75,172–174]. One mechanism that has been

understudied until recently is the activation of the TLR pathway via microbial outer membrane vesicles (OMVs). It is evident from observational and longitudinal studies that the immune system in combination with the microbiome is critical to controlling the inflammatory processes, and development of CRC and CAC [44,45,51,68], however, the factors triggering and sustaining this chronic inflammatory response have not been fully elucidated. In this review, we will highlight the microbiome-host interaction through the TLR pathway and discuss how OMVs engage this pathway to control chronic inflammation and carcinogenesis.

The microbiome is a factor in controlling inflammation and development of CRC

The best-characterized relationship between a single microbial species and cancer is the relationship between *Helicobacter pylori* and gastric cancer[175–177]. *H.* pylori is involved in early stages of gastric carcinogenesis through chronic gastritis[175,178] and harbors toxins including CagA and VacA that manipulate cell survival and chronic inflammation resulting in cancer[149,178]. Within the colon, several pathogens have been identified to be enriched in colorectal cancer, including enterotoxigenic *Bacteroides fragilis* (ETBF) and *Fusobacterium nucleatum*[95,101,179–181]. *F. nucleatum* selectively recruits tumor-infiltrating myeloid cells that up-regulate the expression of pro-inflammatory cytokines including IL-8, IL-1β and TNF-α to accelerate carcinogenesis [179]. Whereas, ETBF promotes intestinal epithelial cell (IEC) proliferation through E-cadherin cleavage, resulting in the release of pro-inflammatory cytokine IL-8 [101,182]. In addition to species-specific impacts on carcinogenesis, the colonization patterns of bacterial communities (distributed vs biofilm) also play an important role [54]. Biofilm analysis has

revealed enrichment of oral and colonic pathogens in CRC tissues, especially in the ascending colon [55]. Patients with familial adenomatous polyposis (FAP) tend to develop pre-cancerous lesions (polyps) early on in life leading to sproradic CRC [183]. Mucosal samples from patients diagnosed with FAP revealed patchy biofilms composed of *E. coli* and ETBF, and enrichment for oncotoxins including ETBF toxin and colibactin[183]. Due to the complexity of the microbiome-host interactions, it is likely that several factors within the microbial community trigger chronic inflammation and development of both CAC and sporadic CRC.

Viral and fungal infections can also elicit a pro-inflammatory reaction analogous to bacterial infections. Recent studies have demonstrated a viral contribution to inflammation and cancer development in the intestinal tract [184–186]. Human papillomavirus (HPV) is a well-documented initiator and virtually the sole cause of all cervical cancers [187–190]. Early HPV infection causes a combined inflammatory and immunosuppressive response that contributes to the development of pro-carcinogenic cervical lesions [191]. HPV has also been implicated in anal cancer [192], but its role in colorectal cancer is much more controversial [193]. The virus has been detected in CRC samples since the 1980s, however due to the numerous factors involved the etiology of CRC the role of the virus has yet to be determined [193–195]. Furthermore, metagenomic analysis of colon cancer patient fecal samples reveal differences between healthy and colorectal cancer viromes [184,186]. Viral communities belonging to *Siphoviridae* and *Myoviridae* taxa were found to be enriched in cancer samples [184] and viral taxon numbers drastically change in pre- and early cancer stages [186]. Together the findings suggest that viruses are another important key to microbial re-modelling in diseased states. Emerging research focusing on the impact of the

mycobiome on host health have also identified certain fungal taxa associated with cancer and IBD including species belonging to *Malassezia* and *Aspergillus* genera [196–198] . As the diverse taxa in the human microbiome interact with each other and mediate homeostasis with the host, deleterious alterations in the structure or function of the microbiome are likely the result of a multi-kingdom interaction, which leads to mucosal inflammation and contribute to colon carcinogenesis.

TLRs: a link between the microbiome and cancer

The mechanisms used by the immune system that promote tolerance are similar to the pathways used to control pathogen invasion [48,199–201]. In order for the host to recognize and respond to microbial antigens, the immune system has evolved a diverse recognition system with convergent signaling and cellular outcomes. At the forefront of the recognition system are pattern recognitions receptors (PRRs), including Toll-like Receptors (TLRs), that recognize specific microbial associated molecular patterns (MAMPs) unique to microbes. This system of receptors mobilizes the immune system to prevent "non-self" factors from harming the host and minimize damage to host cells. A well-known example of this system is the response to viral infection. Viruses can activate TLRs through the recognition of viral nucleic acids, which lack the same markers as host DNA/RNA. Likewise, bacteria produce microbial antigens including peptidoglycans and lipopolysaccharide (LPS) that can engage TLR signaling [202–210]. Microbial antigens engage with host PRRs to initiate immune responses including leukocyte recruitment and the production of cytokines [144,199,202] (**Fig. 2.1**). The numerous mechanisms that control immunotolerance in the gut are integral to maintaining homeostasis with the resident

microbiota, however abnormal shifts in the microbial community or pathogen invasion can overwhelm the immune system and promote chronic inflammation and disease.

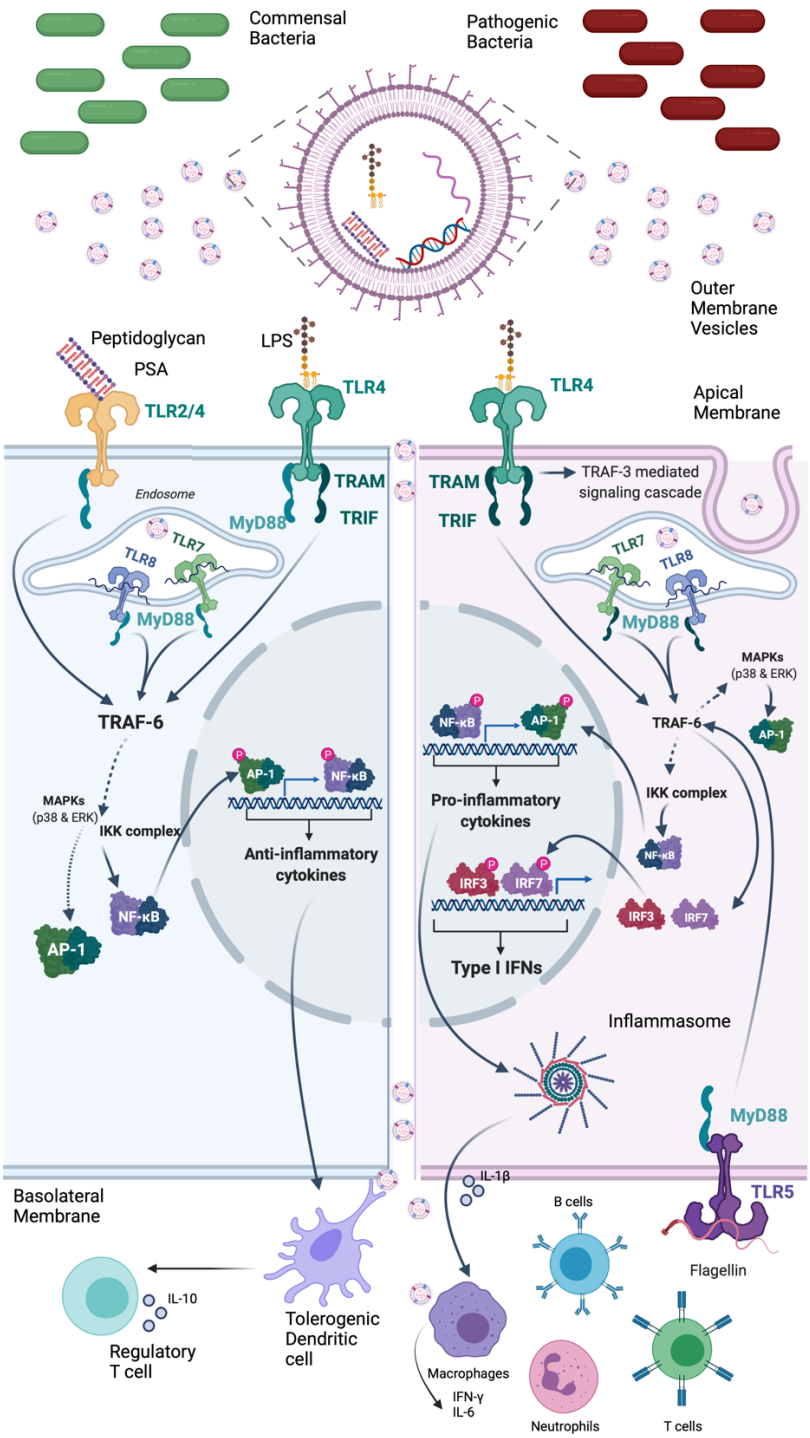


Figure 2.1: Bacteria release outer membrane vesicles that contain a variety of biomolecules including peptidoglycans, nucleic acids, proteins and lipopolysaccharides. These biomolecules can stimulate TLRs localized to the cell surface or subcellular compartments and activate downstream pathways that result in (A) pro-inflammatory or (B) anti-inflammatory signaling outcomes. Though TLRs share conserved signaling pathways, the inflammatory outcome (pro- or anti-inflammatory) resulting from OMV-TLR interaction will be influenced by factors such the localization of the TLR, the bacterial species of origin and the receptor ligand.

The GI immune system constantly monitors the microbial community and reacts against pathogenic species while maintaining tolerance towards commensals. TLRs play an integral role in the immunosurveillance of GI tract. These receptors have evolved to recognize a range of different MAMPs, and are expressed across many cell types to form one of the first lines of defense in the immune system. TLR4 was the first receptor described and recognizes LPS, a component of Gram-negative bacterial cell walls [211]. Since then, different classes of TLRs have been described to recognize a diverse range of MAMPs [205,207–210,212,213]. Upon ligand recognition, TLRs activate signaling cascades through either myeloid-differentiation factor 88 (MyD88), TIRAP, TRAM or TRIF to promote innate immune signals like NF-κB, p38, JNK, IFN and cytokine cascades [214]. The induction of the innate immune system through TLRs subsequently engages other immune components (i.e. the adaptive system) to target and remove pathogens from the local cellular environment. TLRs also prevent uncontrolled immune responses to ensure microbial tolerance, indicating an immunomodulatory role for these receptors.

The dynamic nature of the gut microbial community requires temporal and spatial regulation of TLR activation and expression to maintain homeostasis [215]. For example, TLR2 and TLR4 are expressed in low levels on the apical surface IECs to prevent over activation of the immune system due to the high levels of lipoprotein and LPS that are present in the gut lumen [216–219]. Whereas, TLR5 is predominantly located at the basolateral membrane and on DCs to prevent its access to luminal bacterial flagellin [220–222]. The

differences in the location and activation of TLRs ensures the homeostatic maintenance of the intestinal epithelial barrier through multiple downstream immune responses. Specifically, *Tlr4* expression in mice is located in the small intestinal crypt cells, specifically in those cells expressing the stem cell marker $Lgr5^{+}$. By deleting *Tlr4* in these cells, it was shown that *Tlr4* regulates their survival and proliferation [223]. Furthermore, *Tlr4* KO mice have lower incidence of CAC, indicating that location and expression of TLRs controls development of CAC [224].

In addition to responses to the microbiome as a whole, individual species within the microbiome are responsible for particular systemic effects through interactions with TLRs. Among specific commensals, *Lactobacillus crispatus* can modulate TLR2 and TLR4 expression in IECs through an ERK-dependent pathway by autoaggregation. This species can increase TLR2 expression and IL-10 production while also down regulating TLR4 and IL-6 expression [225]. *Bacteroides fragilis* (non-toxigenic; NTBF) has been shown to regulate inflammatory responses using the capsular component Polysaccharide A (PSA). PSA activates TLR2 to drive conversion of $CD4^{+}$ T cells into $Foxp3^{+}$ Treg cells that produce IL-10 during commensal colonization, effectively reducing the responsiveness of the gut to specific MAMPs [88]. The dynamic nature of the microbiome requires tolerance and surveillance by the host body to prevent pathogenic colonization while, at the same time, sparing commensal microbes. Any imbalances in the microbiome can subvert TLR signaling pathways leading to uncontrolled inflammation and contribute to diseases such as inflammatory bowel disease (IBD) which may eventually lead to the development of CAC [226–228].

TLR signaling during pathogen challenge results in an inflammatory response that aids the host immune system in clearing out the invading pathogen [202,214]. When a healthy host encounters a pathogenic challenge, TLRs experience a short-term change in expression to ensure pathogenetic clearance from the body. LPS from pathogens, *E. coli* and *P. gingivalis,* can drive TLR4-activated IL-8 responses and temporarily increase the expression of MYD88, IL-1β, NF-kB and TLR4 [218,229,230]. However, loss of microbial diversity in conjunction with chronic pathogen infection leads to uncontrolled chronic inflammation through constitutive activation of TLRs [231,232]. Enterobacteriaceae are present in low numbers in the gut, yet are the most commonly overgrown bacteria in chronic inflammatory conditions [233]. Pathogenic antigens interact with TLRs to increase the expression of pro-inflammatory genes[134], and the lack of commensal microbial factors to control pathogen growth exacerbate the inflammatory milieu. Activation of TLRs, in particular TLR3, has also been shown to induce apoptosis [234–237] and molecules that function within the apoptosis cascade can function as well to heighten inflammatory signaling. For example TLR3/4-TRIF signaling activates caspase-8 to produce mature IL-1β [238] which can be inhibited by IAP (inhibitor of apoptosis) [239]. Moreover, in epithelial cells, both apoptotic and inflammatory responses are curtailed by membrane-tethered mucins such as MUC1 which suppress TLR mediated pro-inflammatory responses by blocking association with TLR cytoplasmic tails [240]. Thus, the control of inflammatory and apoptotic responses are mediated by TLR pathways, and the persistent activation of these cascades can alter TLR expression creating a chronically inflamed state that eventually lead to disorders such as IBD and cancer [216,226,241–243].

TLRs across all cancer types demonstrate changes in their expression patterns that is dependent on the stage of cancer, suggesting a role for TLRs in cancer progression and highlighting a conserved phenomenon consistent with the inflammation-cancer axis [244] (**Fig. 2.2**). For example, reduced expression of TLR3, 5, and 7 and overexpression of TLR4 is a hallmark of CRC in comparison to normal colon or rectal tissues. As well, alterations in TLR expression in lung cancer can affect the treatment outcome; high expression of TLR5 associates with good prognosis, while elevated TLR7 expression is indicative of a poor outcome [245]. Analysis of CRC samples has also shown upregulation of TLR4, 7 and 9, with increased TLR4 and TLR7 expression being associated with poor prognosis [245–248]. One of the downstream targets of TLR4, through NF-kB, is NADPH Oxidase 1 (NOX1); an enzyme that contributes to production of reactive oxygen species (ROS). Upregulation of TLR4 leads to an increase in NOX1, increasing the potential of carcinogenic DNA mutations to occur[249]. Generally, the upregulation and hyperactivation of TLRs can signal downstream pathways that recruit white blood cells, such as macrophages and neutrophils, which have the ability to produce reactive oxygen species (ROS) that damage DNA, giving way to precancerous polyps [202]. Other pathways activated by TLR signaling include the STAT signals which promote hyperproliferation of epithelia and delay apoptosis, leading to the survival of neoplastic cells [214]. The microbial species that tend to associate with CRC are pathogenic in nature and can take advantage of the TLR pathways to promote CRC. A key example of this is *F. nucleatum*, a known CRC promoter. It can activate TLR4 through the p21-activated kinase 1 cascade to accumulate and stabilize β-catenin in cells resulting in decreased cell adhesion and increase cell division to promote tumorigenesis [250]. The microbial component of the TLR cascade in IBD and cancer highlight the important role

microbes play in affecting the balance of multiple pathways in the host that can lead to carcinogenesis. However, we are still uncovering new mechanism by which the microbiota can engage and control host TLR activation.

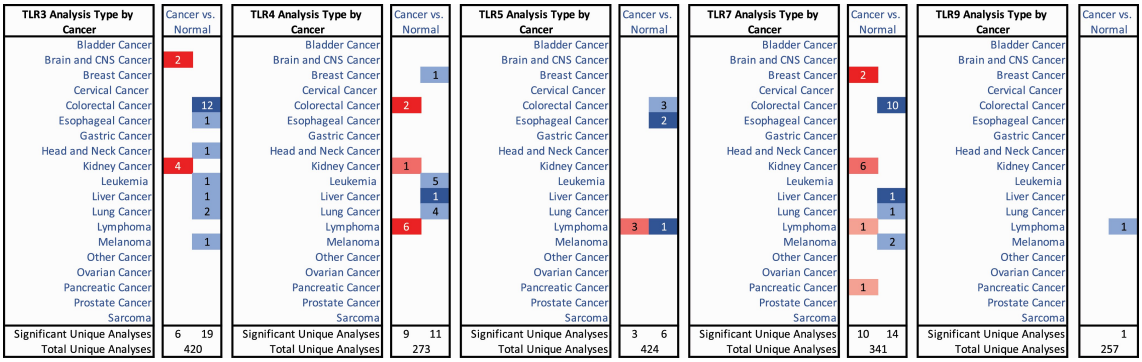


Figure 2.2: TLR3, 4, 5, 7 and 9 expression in TCGA Cancer or Normal Tissues from the Oncomine Database. Red indicates number of positive associations with that cancer type (overexpression in cancer), blue indicates number of negative associations with that cancer type (under expression in cancer).

Host-microbe communication: Outer Membrane Vesicles and Inflammation

In order for the host to recognize commensals and pathogens, it requires a well-developed communication system to interact with these microbes and maintain symbiosis [251]. The extracellular environment contains factors including enzymes (i.e. proteases and nucleases) that can degrade cell secreted factors. One method that both prokaryotes and eukaryotes use to overcome this issue of extracellular degradation is by packaging cargo (e.g. protein, ribonucleotides, metabolites) in extracellular vesicles (EVs)[252]. EVs are a ubiquitous cellular vehicle that enables signal transduction and transfer of biomolecules[110]. EVs come with a diverse range of functions, cargo and composition, effectively segregating them into subclasses [253]. Specific eukaryotic cells, like B-cells and dendritic cells, use EVs for antigen presentation to initiate adaptive immune responses[254,255]. Others cell types like neurons use EVs to potentiate action potentials and stimulate targets[256],

while adipocytes secrete EVs in a paracrine manner to mediate insulin processing[257]. EVs also serve as a regulatory factor in the intercellular interactions of cancer[258]. Biomolecules, including nucleic acids, metabolites and proteins that exist within EVs from normal cells, can be also be used by tumor cells to produce EVs to "educate" non-cancerous cells to promote carcinogenesis [110,252,259,260]. These vesicles enable cancer cells to control the nature of local environment and cellular processes to promote inflammation, immune evasion, and advance disease progression[261,262].

In a similar manner, prokaryotic cells use EVs to facilitate inter- and intracellular signaling [135,263]. Within bacteria the most well-characterized class of EVs are outer membrane vesicles (OMVs) derived from the outer membrane of Gram-negative bacteria[135,151]. OMVs are composed of the same biomolecules that form the outer membrane including proteins (i.e. porins) and LPS and cytosolic components like nucleic acids[135]. They facilitate multiple functions; controlling population size, horizontal gene transfer of antibiotic resistant material and virulence factors. The metabolites in OMVs are highly immunogenic and interact with host PRRs. The major PRRs involved with microbial surveillance include retinoic acid-inducible gene 1[264] and melanoma differentiation associated protein 5 [265] (RNA detection), absent in melanoma 2 (microbial DNA detection) [266] and TLRs (detection of nucleic acids, peptidoglycans, and LPS) [205,207,208,210,212,213,267,268]. Since TLRs are expressed in almost all cells and present on the cell surface and within sub-cellular compartments, they are one of first components activated in an immune response. Their MAMP-TLR binding activates the innate immune system through NF-κB to generate inflammatory responses to combat pathogens[202,269,270]. TLR-mediated inflammatory responses are heavily implicated in IBD and CRC/CAC [171,216,271–275], suggesting that TLR

microbial surveillance of the GI tract is a key mediator of pathogenesis. Given that the TLR pathway is a key mechanism by which host cells recognize pathogens and is activated during cancer development, it is possible that this pathway also mediates the process of chronic inflammation-associated carcinogenesis by the microbiome through engagement with OMVs.

Much like their eukaryotic counterparts, OMVs are enriched with certain species of biomolecules, suggesting a selective export pathway involved in OMV biogenesis[135,137,142,155]. OMVs have garnered interest due to their potential to be used as novel biomarkers or as a therapeutic delivery system (e.g. vaccines) for certain diseases, and research shows that TLRs are one of the molecular pathways OMVs engage[134,263]. Many of the biomolecules that decorate or are packaged in OMVs interact with TLRs, including LPS (TLR4), peptidoglycan (TLR2) and nucleic acids (TLR3/7/8/9) [205,209,210,213,267,268]. Depending on the species of bacteria, the OMVs can engage with TLRs to maintain homeostasis or promote disease; however, the mechanisms by which OMVs control homeostasis is not well understood. *E. coli* C25 is a commensal strain that uses OMVs to interact with IECs. The OMV-IEC interaction upregulates the expression of TLR2/4/5 and promotes the expression of the proinflammatory cytokine IL-8, resulting in mild inflammation and prevents bacterial internalization into the epithelium [276]. Furthermore, compared to pathogenic strains of *E. coli*, OMVs derived from commensal *E. coli* cultures increase the expression of TLR 1/5/6/7/8/9 and the expression of IL-10, IL-12A, IL-1α, and IL-1β, in IECs to promote intestinal immunotolerance [277]. Likewise, other commensal microbes use OMVs to affect intestinal homeostasis by engaging with immune cells [134,278].

B. fragilis (NTBF) promotes intestinal immunotolerance by engaging with TLR2 on DCs using PSA [89,279]. Likewise, *B. fragilis* (NTBF) OMVs are enriched with PSA that activate TLR2-mediated upregulation the production of IL-10 in DCs and enhances the production Tregs to confer protection from experimental colitis[279]. The intestinal symbiont *Bacteroides vulgatus* secretes OMVs that promote anti-inflammatory responses by engaging with TLR2 and TLR4 on CD11c+ DCs [280]. The health status of the host can also affect how responsive host cells are to commensal OMVs. Specifically, OMVs from *Bacteroides thetaiotaomicron* (Bt) stimulate TLR-dependent IL-10 and IL-6 expression in DCs derived from healthy individuals, while cell cultures from patients diagnosed with IBD were non-responsive to Bt OMVs, suggesting an alteration in TLR expression or activation of components of the TLR pathway in IBD [281]. Together these results demonstrate that *commensal-derived* OMVs deliver immunomodulatory antigens that aid homeostatic regulation in the intestine. In contrast, the interactions between the *pathogen-derived* OMVs and host cells result in the integration of microbial antigens within host cells that affect intracellular pathways and exacerbate disease states[282–284].

OMVs from pathogens are well documented to elicit chronic inflammatory responses and can exacerbate IBD and cancer. Epithelial cells are key in immunosurveillance of pathogens and often initiate innate immune response. *P. gingivalis* OMVs induce strong TLR2 (peptidoglycan) and TLR4 (LPS) responses and activate TLR7/8/9 (nucleic acids) to a lesser extent in the epithelia [144]. The *E. coli* toxin cytolysin A is present on the OMV surface and can interact with epithelial TLR4 to increase intracellular concentrations of Ca^{2+} which upregulates the expression of IL-6 and IL-8 [229]. In gastric cancer, *H. pylori* OMVs deliver virulence factors (i.e. CagA) that activate TLR

and NF-kB pathways in gastric cells and B cells, which contribute to increases in pro-inflammatory cytokine expression and cell proliferation [145,285]. Since *H. pylori* is a well-documented oncogenic bacterium that uses OMVs to initiate carcinogenesis, it is likely that other intestinal microbiota use OMVs in a similar manner to affect carcinogenesis as well. Specifically, RNA within OMVs derived from *Aggregatibacter actinomycetemcomitans* promotes pro-inflammatory TNF-α through TLR8 and NF-kB signalling [138]. Additionally, vesicular delivery of DNA and RNA derived from *Staphylococcus aureus* can trigger potent IFN-β responses in macrophages through endosomal TLRs [286]. OMVs derived from the microbiome of a mouse model of colitis interact with TLR4 on IECs, which result in pro-inflammatory responses from macrophages, particularly the release of IL-8 [146]. The treatment of the macrophages with OMVs from individuals with IBD promotes macrophage polarization towards M1 and M2 types resulting in a pro-inflammatory response and potentiation of intestinal epithelia damage [146]. Peptidoglycan from OMVs can also be internalized by IECs via TLR2 for transport to the basal membrane. After the peptidoglycan is transferred across the IEC, it interacts with macrophages and triggers the secretion of IL-6 that exacerbate inflammation in CRC[287]. Therefore, OMVs, much like their eukaryotic counterparts, represent an important component of intercellular interactions between microbes and host cells by interfacing with TLRs.

It should be noted that aside from TLR interactions, the antigens and biomolecules in the OMVs can interact with host receptors to also produce an immune response. The host cell interacts with OMVs through several different surveillance mechanisms and is not just limited to TLR-OMV interaction to promote an immune/inflammatory response

[134,200]. The combined response resulting from the activation or repression of these pathways through OMVs allow the microbiome to promote inflammation and cancer [134,179]. In epithelial cells, receptors like nucleotide-binding oligomerization domain-containing protein 1 (NOD1) can be stimulated by OMVs from *H. pylori, P. aeruginosa,* and *Neisseria gonorrohoeae* to induce the production of pro-inflammatory cytokines and chemokines [154]. Additionally, OMVs from pathogens including *Treponema denticola* and *P. gingivalis* can disrupt the tight junction barriers of epithelia to enable further pathogen invasion to underlying tissue [288,289]. Depending on whether the microbe is a commensal or a pathogen, the OMVs they produce have to potential to activate different signaling pathways to promote either inflammatory or tolerant conditions in the GI tract (Table 2.1). However, more investigation is required to elucidate the molecular mechanisms governing these interactions and the selection of cargo they carry to the host, especially those that control carcinogenesis.

Table 2.1

A summary of all the effects of the microbiome on the GI tract

Bacteria	Effects	Reference
Lactobacillus crispatus	Upregulate TLR2 and IL-10 and downregulate TLR4 and IL-6 in IECs. Upregulates IL-10 production in DCs	[89,225,279,280]
Bacteroides fragilis	Converts $CD4^+$ T cells into $Foxp3^+$ Treg cells via TLR2	[88]
Escherichia coli	*E. coli* derived LPS result in TLR4 activation and chronic pro-inflammatory responses in IECs; Commensal *E. coli* can promote mild inflammation to promote immunotolerance	[229,276,277]
Fusobacterium nucleatum	Decreases cell adhesion and increases cell division through TLR4 activation and β-catenin stabilization	[250]
Bacteroides vulgatus	Engage with TLR2 and TLR4 on CD11c+ DCs to promote anti-inflammatory responses	[280]
Bacteroides thetaiotamicron	Stimulates TLR-dependent IL-10 and IL-6 expression in DCs	[281]
Aggregatibacter actinomycetemcomitans	Small RNAs in OMVs can promote TNF-α through TLR8 and NF-kB signaling	[138]
Pseudomonas aeruginosa	OMVs can activate NOD1 in epithelial cells	[154]

Conclusion

The microbiome can influence host physiology to tolerate commensals and prevent abnormal community changes or enable conditions that favor disease [44]. Receptors present on host cells, including TLRs, interact with microbial derived biomolecules and activate cellular pathways that mobilize the immune system and affect inflammatory status[45]. EVs, specifically prokaryotic OMVs, represent an important mode of MAMP delivery to host cells to produce an immunomodulatory effect on the host[134]. Together, EVs and TLRs are

essential components of microbe-host interactions and have implications in homeostasis and diseases including cancer. Additionally, TLRs and EVs are promising avenues of therapeutic development; the significant role TLRs play in immunity makes them potential drug targets and the immunogenic properties of EVs present an alternative to vaccines/medication. The agonists of TLRs 3/4/7/8/9 (synthetic compounds or microbial-derived biomolecules) are currently included on the National Cancer Institute's list of potential cancer therapeutic agents[290,291]. Currently, there are a handful of TLR agonists that have been approved for therapy or adjuvants in cancer treatments and more therapeutic agonists are in development [290,292]. While TLRs represent a potential therapeutic target in host cells, researchers are investigating microbial-derived EVs for vaccine and therapy development. Eukaryotic EVs have proven their potential as a future therapeutic platform that target TLRs; specific EVs can promote TLR3-mediated anti-tumor immunity in melanoma models[293]. Likewise, microbial OMVs are garnering more interest as stable vaccine platforms and as a form of therapeutic delivery[263]. Moreover, OMVs can be engineered to deliver targeted pharmacological molecules and antigens to recipient cells, including OMVs that specifically target cancer cells [263]. Additionally, the native molecules that make up OMVs interact with host receptors to provide an added benefit of immune system activation[294,295]. Measuring the efficacy of OMV-based cancer vaccines or therapeutics in pre-clinical trials will be critical, and much work needs to be done before we can consider moving towards clinical trials[263,294]. Foremost, we will need to address the selection of antigens to be loaded into OMVs, how the OMVs are delivered to the site of the tumor, and the impacts (immunosuppressive or immunostimulatory) of the OMVs on TLR signaling and other receptors in CRC pathogenesis. Such advances will allow us to

use OMVs as both biomarkers and as potential therapeutics for combatting CRC and other inflammatory-associated cancers.

CHAPTER THREE

Imaging and Characterization of *Bacteroides fragilis* Outer Membrane Vesicles through a Novel Staining Methodology

A portion of this chapter is in preparation as: Sheikh, A., Zechmann, B., Sayes, C., Taube, J., & Greathouse, K. L. (2023). A Preparation of Bacterial Outer Membrane with Osmium Tetroxide and Uranyl Acetate Co-stain Enables Improved Structural Determination by Transmission Electron Microscopy. *Micoscopy*.

Introduction

The human gut microbiome is an important mediator of host health [47,51,58,199,296]. Resident microbes contribute to host physiology by regulating the immune system [2,6–8], and epithelial cell signaling pathways. The establishment of interkingdom bacterial communication is important to maintain the balance of symbiosis between the host and the microbiome [44,56,76,141]. Resident microbes employ many different routes of signaling to communicate with host cells and other microbes. The major classes of cell-cell signaling in bacteria include quorum-sensing (QS), type I-VI secretion systems and extracellular vesicles (EVs) [143,145,155,156,297]. Quorum sensing and the secretion systems rely on the movement of cytoplasmic and periplasmic proteins into the extracellular space [298–303]. QS pathways control bacterial cell density population and the secretion systems contribute to the transfer of biomolecules in between cells and to virulence [298–303]. Since the extracellular environment contains factors that can degrade secreted molecules, EVs enable cells to transfer and protect molecular cargo in for effective cell-cell communication [304].

Extracellular vesicles are nanometer sized vesicles ubiquitous to both prokaryotic and eukaryotic cells and are released extracellularly [111,141,155,163,253,260,305]. The study of EVs is growing due to their critical and varied roles in paracrine signaling, intercellular interactions, and pathology. Bacteria secrete EVs that have similar biomolecular components as their cell of origin [146,147,306–309]. Gram-negative bacteria in particular release outer membrane vesicles (OMVs) that can elicit potent immune responses in the host cells [109,144–147,277,279,281,307].

Accurate imaging of OMVs aids in the determination of their physical characteristics, and mechanisms of action [148,150,310,311]. Transmission electron microscopy (TEM) enables high resolution imaging of OMVs, most notably through negative staining [110,114,312,313]. The most common negative stain used for sample preparation is uranyl acetate (UA). Alternate contrasting agents, however, such as osmium tetroxide enable simultaneous fixation and staining of vesicles for TEM imaging.

Uranyl acetate is a commonly utilized negative stain for TEM for over 60 years [314,315]. Uranyl acetate is an acetate salt of uranium oxide which binds to a wide range of biomolecules [316,317]. Though uranyl acetate can stain lipids with sialic acid groups like glycoproteins and gangliosides [317], OMVs are composed primarily of LPS and their lipids lacks the moieties necessary for interactions with the uranium oxide ions [318], leading to issues with imaging the OMVs post-UA staining. Osmium tetroxide; however, preserves the structure of cellular samples and membranes by reacting with the unsaturated double bonds of phospholipids [319,320]. The reaction of osmium tetroxide with unsaturated lipids provides an alternative for the negative staining of OMVs.

To further improve upon the current OMV imaging techniques, the study osmium tetroxide staining as an improved method for TEM imaging of OMVs. In this study, we compared the negative staining of OMVs from enterotoxigenic *Bacteroides fragilis* (ETBF) and non-toxic *Bacteroides fragilis* (NTBF) under two conditions: uranyl acetate staining alone versus co-staining osmium tetroxide with uranyl acetate. Additionally, we sought to characterize the protein and size distribution profiles of OMVs post-isolation.

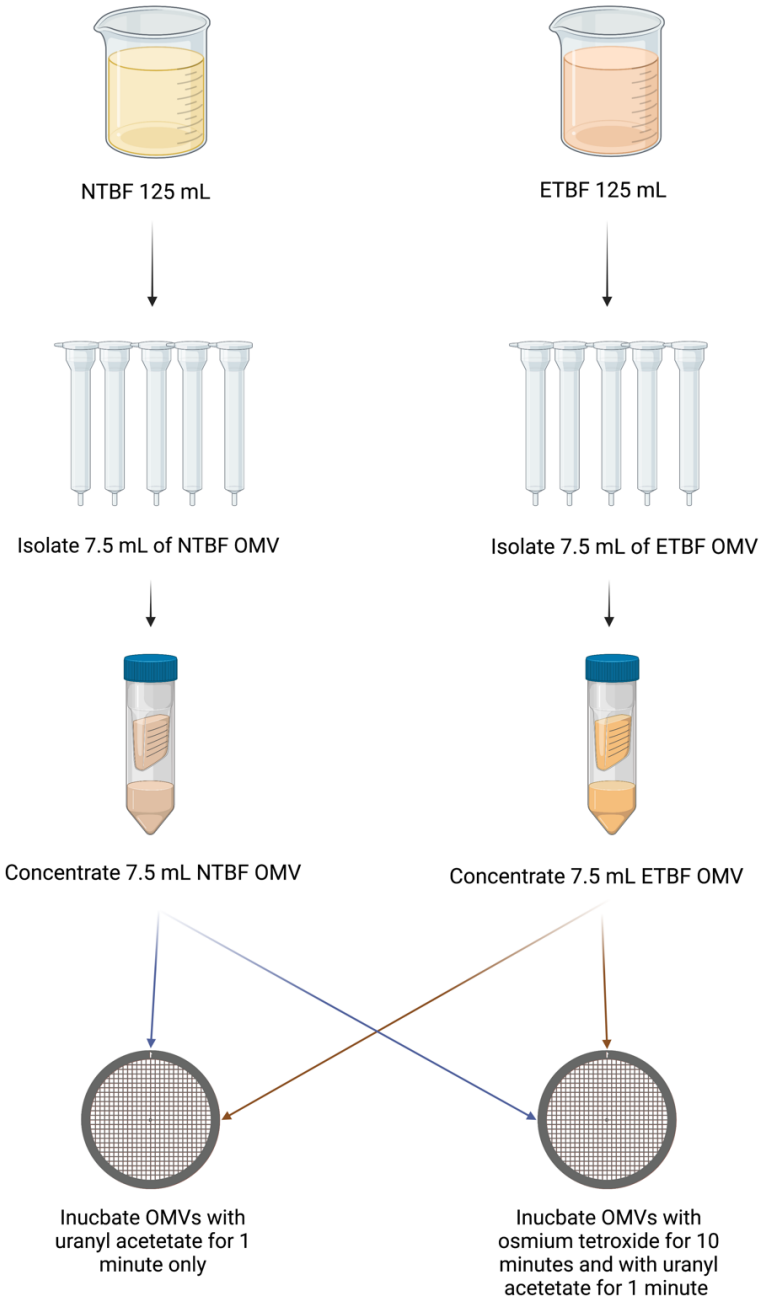


Figure 3.1: Graphical Overview of OMV Visualization Workflow.

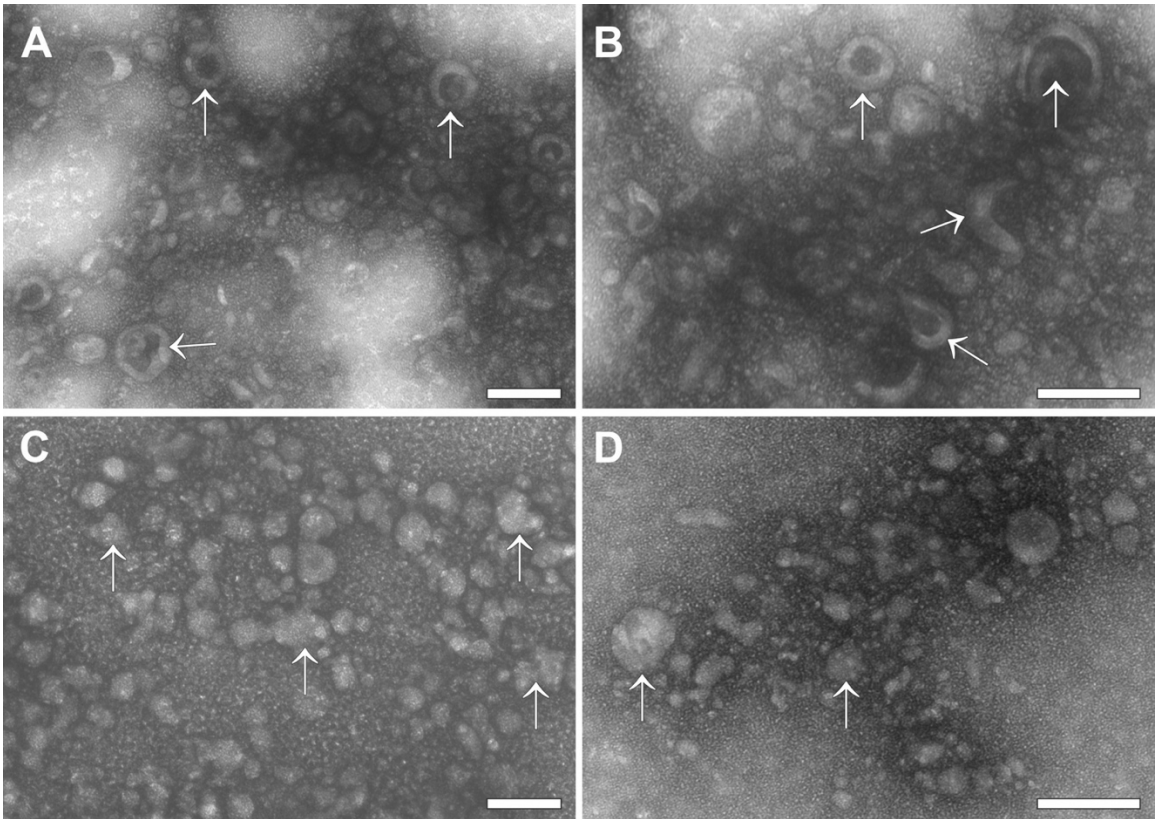


Figure 3.2: TEM micrographs of ETBF (A and B) and NTBF OMVs (C and D) stained with uranyl acetate. OMV samples appear to be aggregated in small areas of the grid. Some of the vesicles appear to be collapsed or experienced a loss of structure, as denoted by arrows Scale bars, 100 nm. X 50, 000 magnification

OMVs Stained with Uranyl Acetate have Poor Structure Preservation and Visualization Quality

To test the staining quality of uranyl acetate alone, OMV samples were incubated on copper grids for 5 minutes, followed by two wash steps and a one minute incubation with UA (Figure 3.1). Images were captured at 50, 000 magnification. Observations of the OMVs showed that the vesicles tended to form aggregate clusters on the grid (Fig. 2). Additionally, there is inconsistent staining on the samples, including areas of low contrast and overstaining on certain groups. Vesicles stained with UA appeared to be collapsed (Figure 3.2A and B) or broken apart (Figure 3.2C and D). All of these factors prevented

complete enumeration of ETBF and NTBF OMVs (Figure 3.4B and D) as individual vesicles could not be discerned from the noisy background.

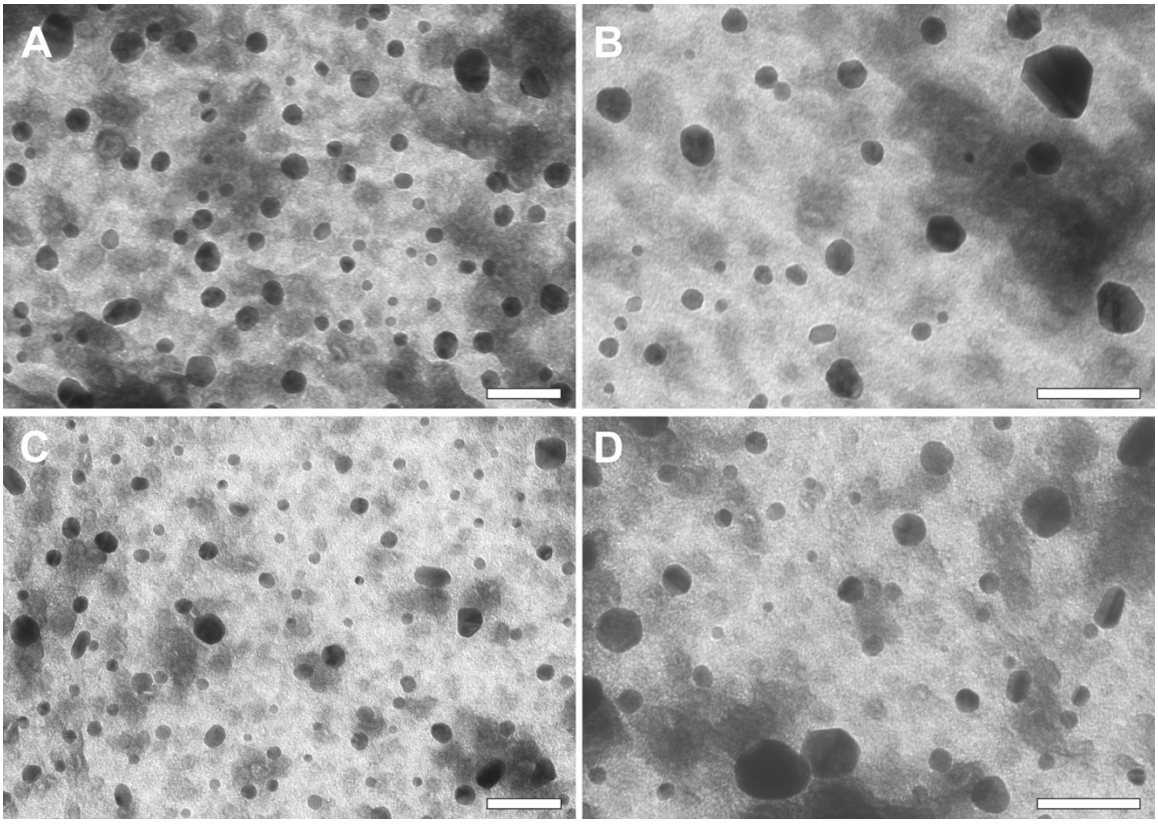


Figure 3.3: TEM micrographs of ETBF (A and B) and NTBF (C and D) OMVs stained with osmium tetroxide and uranyl acetate. Samples appear more dispersed on the grids and lack the collapsing characteristic seen in the UA samples. Scale bars, 100 nm. X 50, 000

OMVs Co-incubated with Osmium Tetroxide and Uranyl Acetate Demonstrate Better Fixation Quality Compared to Uranyl Acetate Alone

In order to address ways to improve OMV TEM imaging, we asked whether osmium tetroxide could be used in conjunction with UA to increase image quality. To this end we incubated an equivalent volume of OMVs with 4% osmium tetroxide for 10 minutes and followed up with a 1-minute UA stain. Imaging of the samples showed several improvements compared to UA alone (Figure 3.3). OMVs appeared darker in the images and vesicles were not clustering, allowing individual vesicles to be discerned more easily.

To compare the efficacy of the osmium tetroxide and UA co-staining of OMVs over just UA alone, we measured the diameter and roundness of vesicles in each condition (Figure 3.4). In co-stain condition a greater number of smaller vesicles closer to 20 nm were observed (Figure 3.4A and 3.4B), Additionally, these vesicles appeared to be rounder than UA alone (Figure 3.4C and 3.4D). Altogether, this data shows a higher efficacy of OMV staining when using jointly osmium tetroxide and UA compared to a solo UA stain.

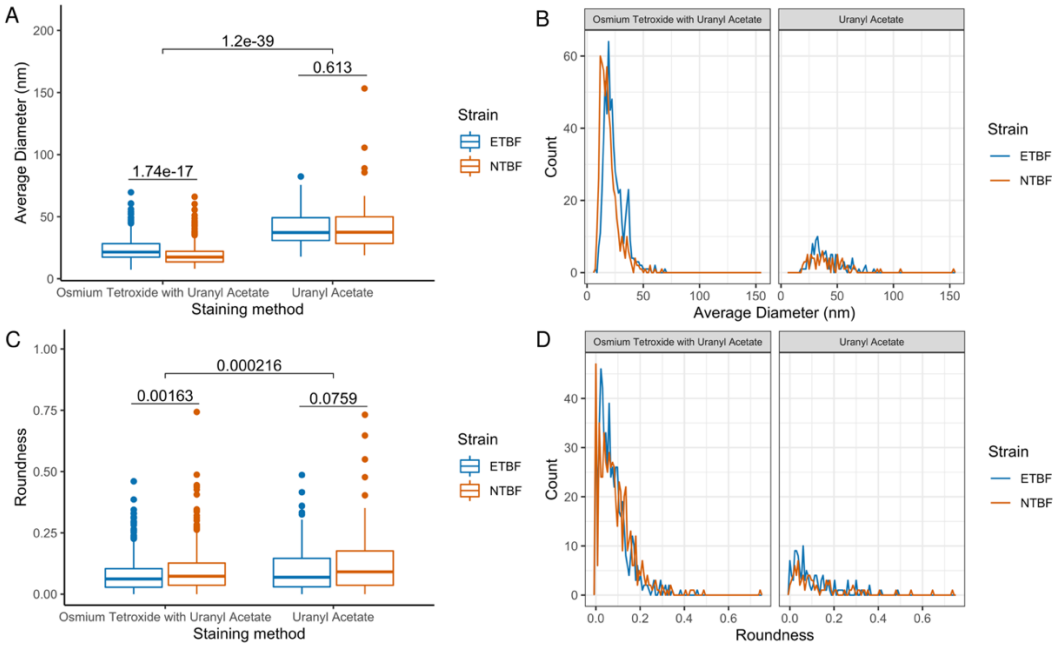


Figure 3.4: Quantitative analyses of OMV size (A and B) and roundness (C and D). Comparisons between different staining methods is shown as a box blot (A and C) and density plot (B and D). Statistics were calculated using Welch's t-test.

OMVs Vary in Size and Contain Strain-specific RNA Profiles

To characterize the size distribution of the vesicles we conducted nanoparticle tracking analysis and found that vesicles in our concentrated samples ranged from 40 nm to 150 nm in diameter, with a majority of vesicles falling in between 50 to 70 nm (Figure 3.5). Silver staining of concentrated vesicles showed different protein banding profiles for

NTBF and ETBF OMVs (Figure 3.6), suggesting that OMV cargo differs amongst separate strains of bacterial species.

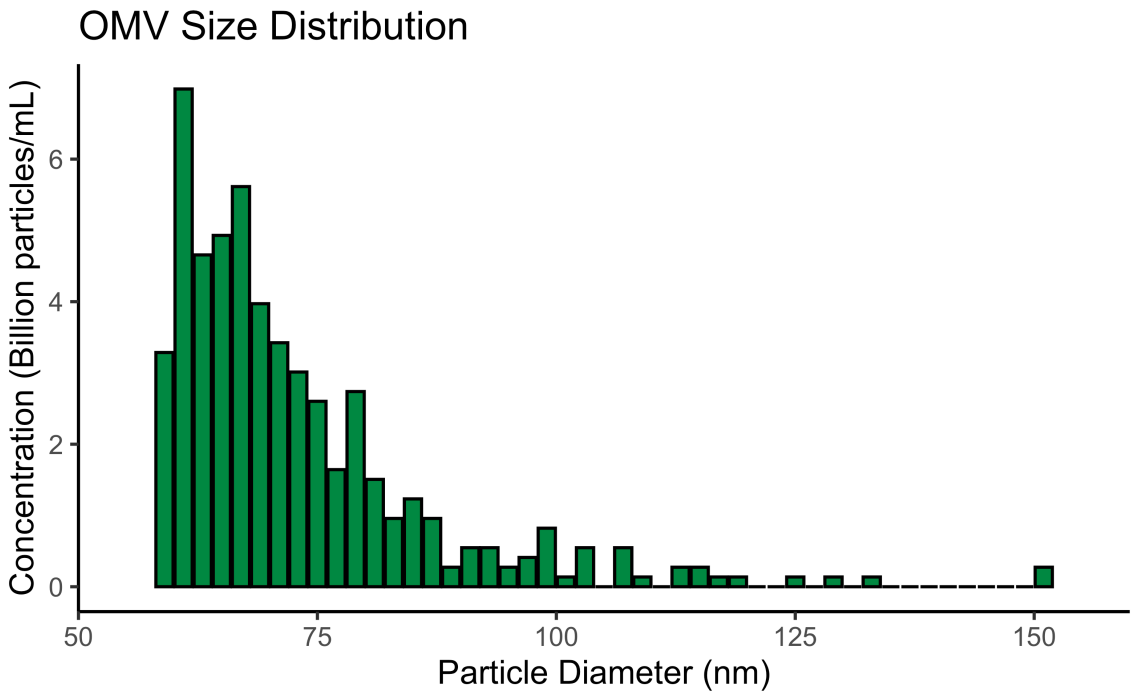


Figure 3.5 Nanoparticle tracking analysis (NTA) demonstrate a wide distribution of OMV sizes. NTA analysis reveals the concentration and size distribution of centrifuge-concentrated vesicles.

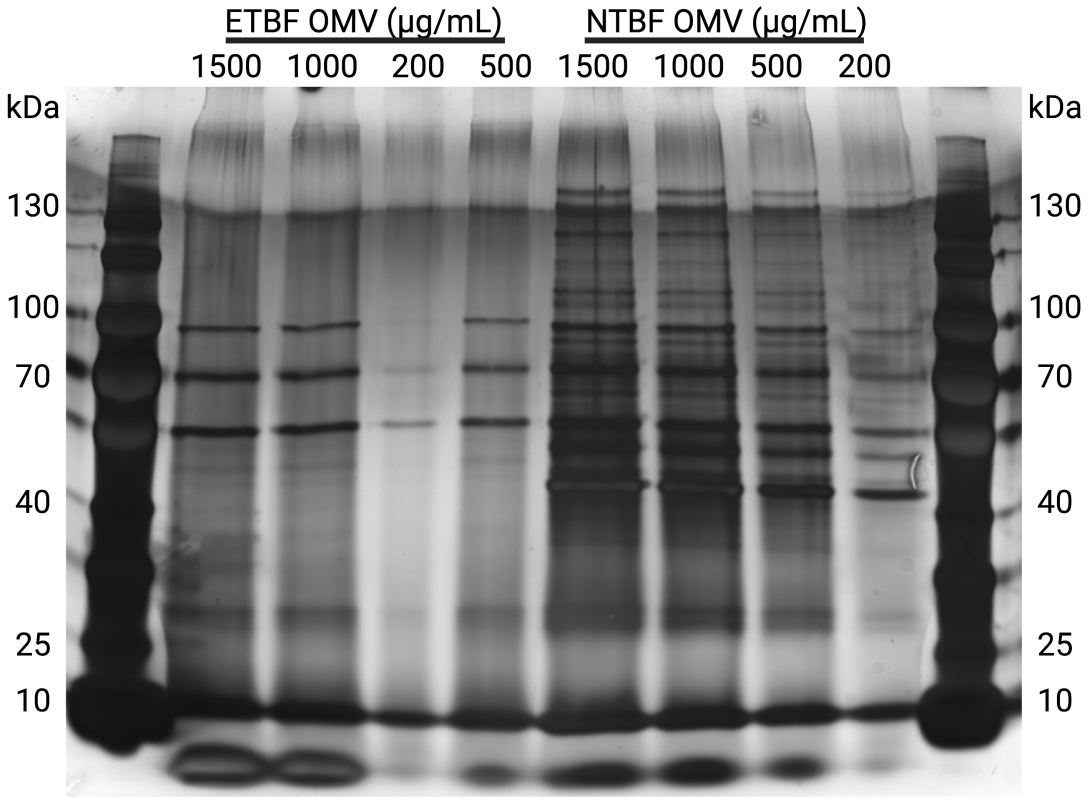


Figure 3.6: Silver staining of OMV samples of ETBF and NTBF show different protein profiles. ETBF and NTBF OMVs at different concentrations were run on a 10% SDS-PAGE gel followed by silver staining. Visualization of bands demonstrate different protein profiles of ETBF and NTBF vesicles.

Discussion

Electron microscopy techniques are important to visualize OMVs after isolation as they cannot be viewed through conventional light microscopic methods [113,321]. Preparation of OMVs for TEM imaging involves negative staining with UA. However, the lack of a fixation step results in the modification of the OMV structure as the stain dries, preventing collection and analysis of meaningful data from the samples. In Fig. 2 we stained OMVs from ETBF (A and B) and NTBF (C and D) with UA and observed collapsed vesicles that formed ‘donuts’. Additionally, we observed irregularly shaped vesicles and lipid fragments that may have formed from the destruction of OMVs. The low contrast of the images combined with the irregular, collapsed shape of the vesicles makes it difficult to discern

individual vesicles for quantitation. Such an issue prevents a thorough analysis of OMV samples.

Through this work, we sought to improve the imaging of OMVs by testing the addition of osmium tetroxide to a uranyl-acetate staining protocol. Uranyl acetate has been the primary stain choice for EM sample preparation for over 60 years [314,315]. It also has been described as a fixative, however samples require a different preparation method in order for those properties to take effect [322]. The reactive properties of UA allow it to act as a general stain without much specificity [314,315], however this can lead to issues with imaging sub-cellular structures as UA can form artifacts and uneven contrast throughout the grid [317]. Osmium tetroxide specifically reacts to unsaturated double bonds in phospholipids of membranes to give rise to osmate (VI) esters, allowing the osmium ion to integrate with the sample and fix them [320]. These fixative properties on lipids can preserve vesicular structure for further characterization and analysis [323]. We supplemented the UA with a pre-treatment of our samples in 4% osmium tetroxide for 10 minutes and then transferred the grid to the UA. We were able to clearly view images with high contrast (Fig. 3), the vesicles were observed as round, individual specimens with no irregular shaping.

Quantification of the images shows that the co-stain is able to visualize smaller, more round vesicles compared to UA alone (Fig. 4). In respect to the co-stain, the fixative properties of osmium on lipids allows it to stain vesicles to preserve the structure to a much greater degree than UA alone. The added contrast of UA in the co-stain allows us to visualize smaller vesicles much easier than the single stain condition, enabling a greater enumeration of OMVs for representative quantification. The low contrast of UA alone, in

combination with irregular vesicle structure, yields lower enumeration sample size and prevents accurate measurement of the OMVs.

The strong immunogenicity and structural stability of OMVs also make them a promising platform for therapeutic development [263,294]. Analysis of OMVs demonstrate enrichment of specific biomolecules, though much is still unknown about the loading and export mechanisms that are involved in vesicle formation [107,108,139,140,149]. Electron microscopy allows for the visualization of OMVs without disrupting sub-cellular structures and is essential for understanding the mechanisms of vesicle formation from bacterial cells and interactions with host cells. The development of a general stain protocol such as the one we described is an integral step in further understanding the structure and characteristics of OMVs from bacteria that lack the markers typically used in immunogold staining and other protein/ligand specific staining methods. Here we modified a protocol to prepare OMVs for TEM imaging while preserving their structure for quantitative analysis.

Materials and Methods

Bacterial Strain and Growth

Enterotoxigenic *Bacteroides fragilis* (ETBF) and non-toxigenic *Bacteroides fragilis* (NTBF) were provided as a gift by Dr. Cindy Sears at Johns Hopkins University. Bacteria were cultured in brain heart infusion (BHI) broth under anaerobic conditions overnight at 37˚C until an OD reading of 0.8 – 1 was achieved.

Outer Membrane Vesicle Isolation and Concentration

OMVs were isolated from ETBF and NTBF using the ExoBacteria OMV Isolation Kit (System Biosciences, EXOBAC100A-1) as per the manufacturer's instructions. Briefly 125 mL of ETBF and NTBF cultures were spun down at 8000 x g for 15 minutes at 4˚C and the resulting supernatant was filtered through 0.45 μm vacuum filter (Thermo Scientific, 168-0045). Filtered supernatant was run through the ExoBacteria OMV isolation column and each column yielded 1.5 mL of eluted OMVs. Five columns were used per culture resulting in 7.5 mL of each strain. The resulting solution of OMVs was concentrated using centrifugal concentration tubes (Thermo Scientific, 88532) at 3000 x g for 15 minutes.

Transmission Electron Microscopy

Concentrated OMVs were prepared for TEM imaging by staining uranyl acetate, osmium tetroxide separately, and co-staining with osmium tetroxide and uranyl acetate. For uranyl acetate, copper grids were incubated on 10 μL drops of OMVs for 5 minutes, followed by two washes on DI water drops for 2.5 minutes each and finally stained with 2% uranyl acetate for 1 minute. For the osmium tetroxide and uranyl acetate co-stain, 10 μL of OMVs mixed with an equivalent volume of 4% osmium tetroxide for 10 minutes on copper grids followed by staining with 2% uranyl acetate for 1 minute. All grids had excess liquid blotted off with filter paper and were allowed to dry overnight. The grids were then imaged with a TEM (JEM-1010, JEOL Inc., Tokyo, Japan), a minimum of 7 images per test condition were obtained.

Post Imaging Analysis

The diameter and size of OMVs were measured using the Olympus CellSens Dimensions Software (Olympus America Inc., Version 2.2). Prior to analysis a number generator was used to pick 5 random images per experiment to sample for counting. To determine the "roundness" of the OMVs, we used Equation 1 as previously described [324]. Briefly, the diameter of the OMVs in the x-direction was divided by the diameter from the y-direction and subtracted from 1, the absolute value of the resulting number was then recorded.

$$R_{OMV} = \left|1 - \frac{X\text{-}axis\ diamter\ OMV}{y\text{-}axis\ diamter\ OMV}\right| \text{ (Equation 1)}$$

OMV Protein Characterization and Size Profiling

OMV samples were diluted to concentrations of 1500 µg/mL, 1000 µg/mL, 500 µg/mL and 200 µg/mL. All samples were run on a 10% SDS-PAGE gel (Bio-Rad, 4568033) at 175V. The gel was subjected to silver staining using the Pierce Silver Stain Kit (Thermo Scientific, 24612) as per the manufacturer's instructions. NTA was conducted using the Exoid (Izon Science Limited) with default parameters; data was graphed using GraphPad Prism 9.

Data Analysis and Visualization

Data analysis and visualization was completed using R (version 4.2.1 – "Funny-Looking Kid') and RStudio (version 2022.07.2 – "Spotted Wakerobin").

Figure Preparation

Figures 3.1, 3.4, 3.5, and 3.6 were prepared using BioRender.com. Figures 3.2 and 3.3 were prepared in Adobe Illustrator.

CHAPTER FOUR

Identifying and Characterizing the RNA species of *Bacteroides fragilis* Outer Membrane Vesicles

Introduction

Research on nucleic acids demonstrates the versatility of RNAs beyond mediating the production of protein from DNA[155,325,326]. Regulatory non-coding RNAs (ncRNAs), their formation, and mechanisms of action have been best described in eukaryotic [158,159,327]. However, there is growing evidence of non-coding RNA from bacteria and their role in cell physiology and host-microbe interactions [141,155,326]. Given that RNA is ubiquitous among all cell types, host cells require sophisticated sensing mechanisms to distinguish self from non-self [204]. The various receptors and pathways that have evolved in eukaryotic cells to protect them from pathogen challenge highlight the importance of coding and non-coding bacterial RNA as pathogen-associated molecular patterns (PAMPs) [328,329].

Bacterial ncRNAs have been described in model organisms such as *E. coli*, *P. aeruginosa*, and *S. aureus* [329–331]. These RNAs, particularly small RNAs (sRNAs), have been implicated in many important cellular functions, including cell population control, combatting bacteriophage infection, biofilm formation, and protein expression [42,141,155,332]. Though bacteria do not have bona fide miRNAs, these sRNAs may act on a target mRNA through base pairing to alter translation or transcript stability, in a manner similar to eukaryotic miRNAs [326,328,333]. The pairing of the sRNAs to their corresponding target mRNA is facilitated by chaperone proteins such as Hfq, or by binding to RNA-binding proteins [328,332]. The formation mechanisms of sRNAs are complex and diverse; sources

include transcription of intergenic regions, targeted processing of larger RNA transcripts, degradation of mRNA, or total mRNAs that can also be translated into RNAs. The collective mechanisms of formation and action of bacterial sRNAs have important influences on cellular processes, and their presence in cultures and extracellular spaces suggests that bacteria actively secrete these RNA to modulate physiology on distant targets [155].

The presence of RNases in the extracellular space poses a great risk to secreted RNAs. One strategy that bacteria employ to prevent extracellular RNA degradation is the packaging of RNA sequences into outer membrane vesicles (OMVs) [155]. The OMV-mediated delivery of RNA fragments to target sites have a range of molecular outcomes including modulation of genetic and protein expression, and metabolic processes. Recent improvements in RNA sequencing have enabled researchers to characterize OMV-associated RNA fragments with high fidelity. Aside from delivery nucleic acid, OMVs contain a range of biomolecules including toxins such as the *Bacteroides fragilis* toxin (BFT/fragilysin) [108,109].

BFT is a 20 kDa metalloprotease toxin and is the sole known toxin encoded by the enterotoxigenic strain of *B. fragilis* (ETBF). The toxin is encoded through a 6-kb region (*bft*) unique to the ETBF genome, known as the *B. fragilis* pathogenicity island [101]. ETBF causes diarrhea and chronic inflammation by using OMV-delivered BFT to cleave E-cadherin in intestinal tight junctions and promote cytoskeleton remodeling [98,106]. Unlike ETBF, the non-toxic strain (NTBF) of *B. fragilis* uses polysaccharide A (PSA) to promote the induction of $CD4^+$ and regulatory T cells in the intestine [92,93]. Though several biomolecules have been demonstrated to be associated with the OMVs *B. fragilis*, the RNA

profiles of ETBF and NTBF have yet to be fully described. Here we investigate and validate the RNA sequences found in the OMVs of NTBF and ETBF. We hypothesize that the OMVs contain are an RNA profile that is unique from the original bacterial cell, and that these vesicles are enriched with specific RNA sequences.

Results

The RNA Profiles of Outer Membrane Vesicles are Distinct from Whole Bacterial Cells

To fully characterize the RNA content of OMVs, we isolated small RNA samples from ETBF and NTBF OMVs and whole cell cultures and performed Illumina sequencing to determine the types of RNA sequences represented in each sample by aligning all of them to both the ETBF and the NTBF genomes. The cross-alignment of samples to both genomes was conducted to perform downstream DESeq2 analysis to determine enrichment of specific RNA sequences. We first characterized the proportion of gene coding sequence types in each of the samples (Figure 4.1). In the OMV samples we found a significantly higher proportion of protein coding sequences when compared to the whole cell (WC) samples. Additionally, the OMV samples had significantly lower proportions of sequences that aligned to genes that coded for tRNAs, RNases, and pseudogenes when compared to WC samples across both genomes (Figure 4.1, Tables 4.1 and 4.2).

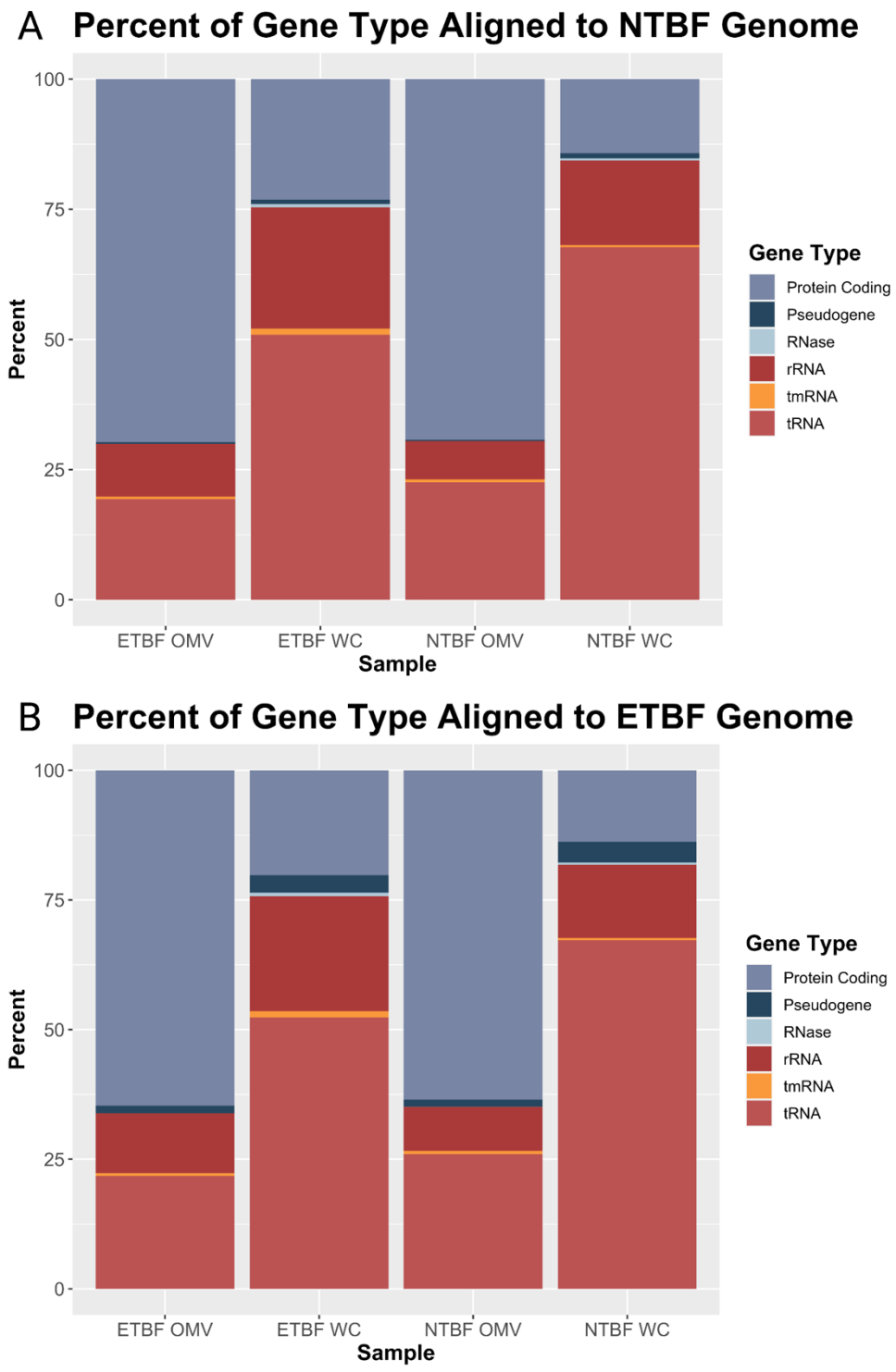


Figure 4.1: Protein coding RNAs are enriched in OMVs compared to whole cell (WC) samples. Illumina sequencing of *B. fragilis* OMV and WC samples and alignment to the NTBF (A) and ETBF (B) genomes reveal an enrichment of protein coding aligned RNAs in OMVs over WC samples. Additionally, there is a lower proportion of rRNA, tRNA and pseudogene aligned RNA sequences in OMVs in comparison to their WC counterparts. Statistical significance was determined using a paired t-test highlighted in Tables 4.1 and 4.2.

Table 4.1: Proportion of Gene Types Represented in Data Aligned to NTBF Genome

Gene Type	Percent of Reads Represented in Each Sample (Average of Triplicate)					
	ETBF OMV	ETBF WC	Statistical Significance Between ETBF OMV and ETBF WC (p)	NTBF OMV	NTBF WC	Statistical Significance Between NTBF OMV and NTBF WC (p)
Protein Coding	69.73	23.16	0.0006	69.26	14.25	0.0006
Pseudogene	0.32	0.85	0.008	0.28	0.99	0.0009
RNase	0.004	0.66	0.005	0.004	0.4	0.04
rRNA	10.16	23.30	0.05	7.38	16.21	ns
tmRNA	0.44	1.15	0.02	0.56	0.44	ns
tRNA	19.34	50.89	0.0	22.53	67.71	0.008

Table 4.2: Proportion of Gene Types Represented in Data Aligned to ETBF Genome

Gene Type	Percent of Reads Represented in Each Sample (Average of Triplicate)					
	ETBF OMV	ETBF WC	Statistical Significance Between ETBF OMV and ETBF WC (p)	NTBF OMV	NTBF WC	Statistical Significance Between NTBF OMV and NTBF WC (p)
Protein Coding	64.68	20.25	0.0006	63.48	13.79	0.001
Pseudogene	1.48	3.32	0.07	1.41	4.01	0.02
RNase	0.004	0.68	0.005	0.005	0.40	0.04
rRNA	11.55	22.20	ns	8.48	14.13	ns
tmRNA	0.50	1.19	0.02	0.64	0.44	0.02
tRNA	21.79	52.34	0.02	25.98	67.22	0.009

We next sought to identify to specific RNA sequences that are enriched in OMV samples and WC samples, by strain, for further downstream characterization. Accordingly, we performed DESeq2 analysis to determine enrichment of specific RNA sequences in the

OMVs when compared to the WC extracts. Analysis of RNA sequences that align to gene coding regions of the ETBF and NTBF genomes show enrichment of certain RNA sequences in OMV samples when compared to WC samples and vice versa (Figures 4.2 and 4.3). Due to the number of genes that were significantly upregulated in each condition, we set the Log_2 fold change cut-off value at 5 to limit our genes of interest to only the highest enriched RNA sequences and a p-value cut-off of 10^{-4}. Bacterial sRNAs may arise as fragments of tRNAs or gene-coding mRNAs, or as non-coding transcripts from intergenic regions of the bacterial genome. To determine the presence of intergenic transcripts, we cataloged and measured expression of intergenic RNA from OMV and WC samples. When we align all our samples, we observe enrichment of RNAs that arise from intergenic regions of the NTBF and ETBF genomes (Figure 4.4 and 4.5). Comparison of the strains revealed a subset of RNA sequences that align to coding regions and intergenic regions that are enriched in whole cell samples of ETBF over NTBF and vice-versa (Figure 4.2 and Figure 4.4). To determine the differences of the RNA profiles of OMVs between strains, we performed a DESeq2 analysis of NTBF against ETBF OMVs. We found very little differential enrichment in between ETBF and NTBF OMVs (Figure 4.6) using the criteria that we had established for our OMV and WC analyses ($Log_2FC = 5$, p-value = 10^{-4}). We observed enrichment of two NTBF-specific genes in ETBF OMVs that matched our filtering criteria (Figure 4.6A), while we observed three ETBF genes that were enriched in ETBF OMVs that fit within our significance threshold but did not exceed the Log_2FC criteria (Figure 4.6B).

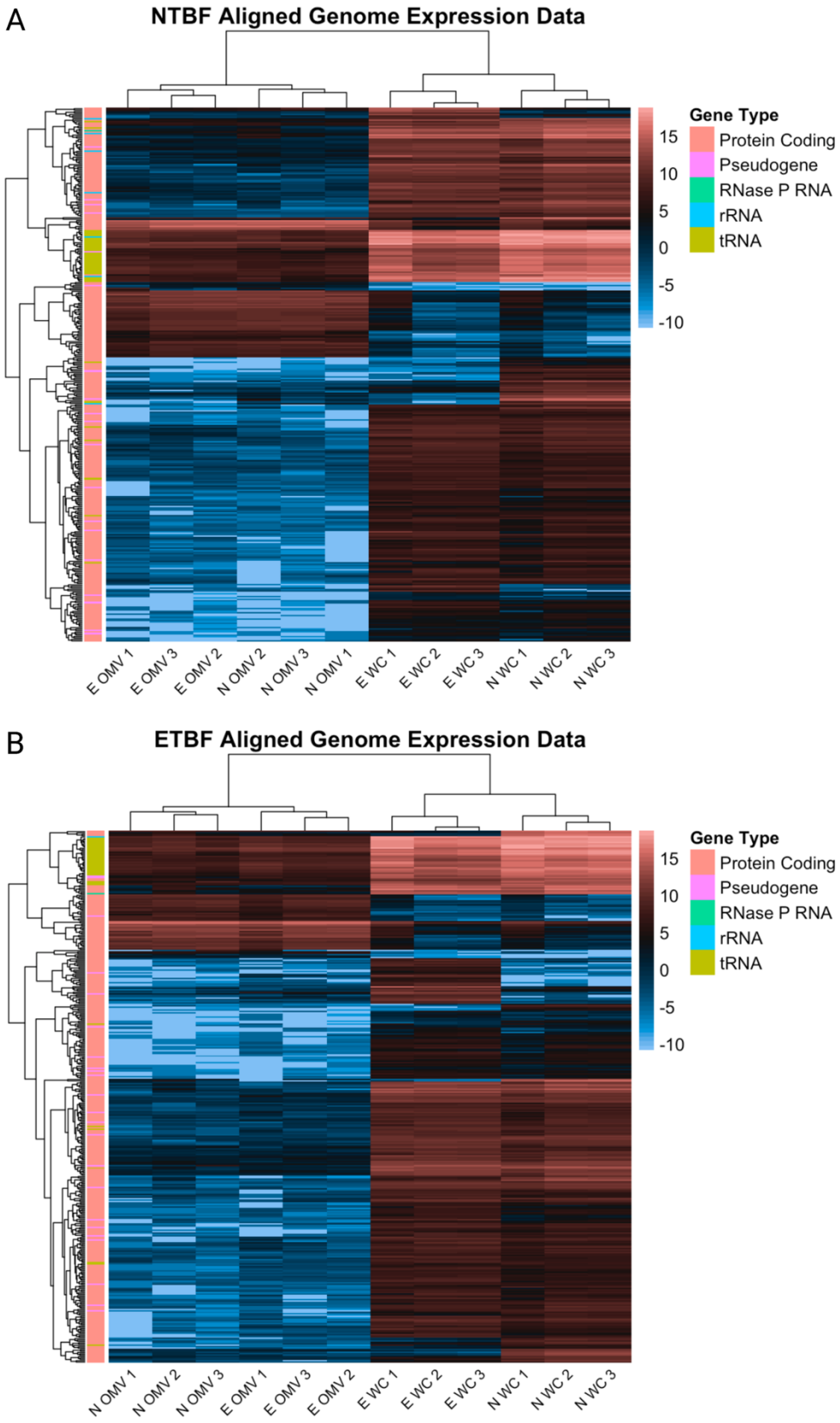

A
NTBF Aligned Genome Expression Data
Gene Type
Protein Coding
Pseudogene
RNase P RNA
rRNA
tRNA
15
10
5
0
-5
-10
E OMV 1
E OMV 3
E OMV 2
N OMV 2
N OMV 3
N OMV 1
E WC 1
E WC 2
E WC 3
N WC 1
N WC 2
N WC 3
B
ETBF Aligned Genome Expression Data
Gene Type
Protein Coding
Pseudogene
RNase P RNA
rRNA
tRNA
15
10
5
0
-5
-10
N OMV 1
N OMV 2
N OMV 3
E OMV 1
E OMV 3
E OMV 2
E WC 1
E WC 2
E WC 3
N WC 1
N WC 2
N WC 3

Figure 4.2: *B. fragilis* gene coding RNAs are enriched in OMV and whole cell (WC) samples. RNAseq analysis of gene coding regions using DESeq2 reveals enrichment of RNA sequences in OMVs and WC samples when they are aligned to the NTBF (A) and ETBF (B) genomes. OMV samples are represented in the columns on the left side of the heatmaps and WC samples are represented on the right sides. E = ETBF and N = NTBF. The numbers represent the biological replicates of each sample set.

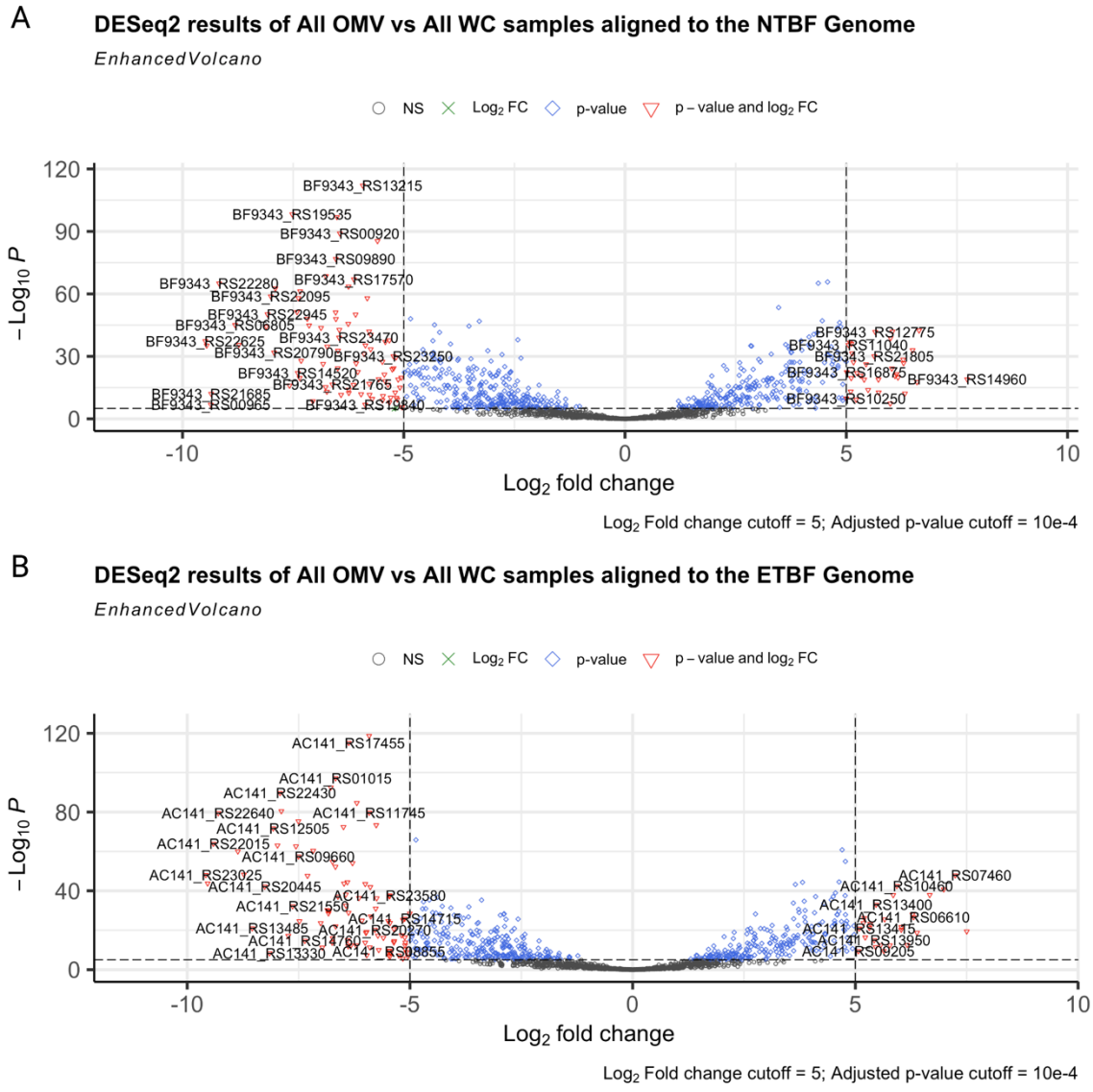
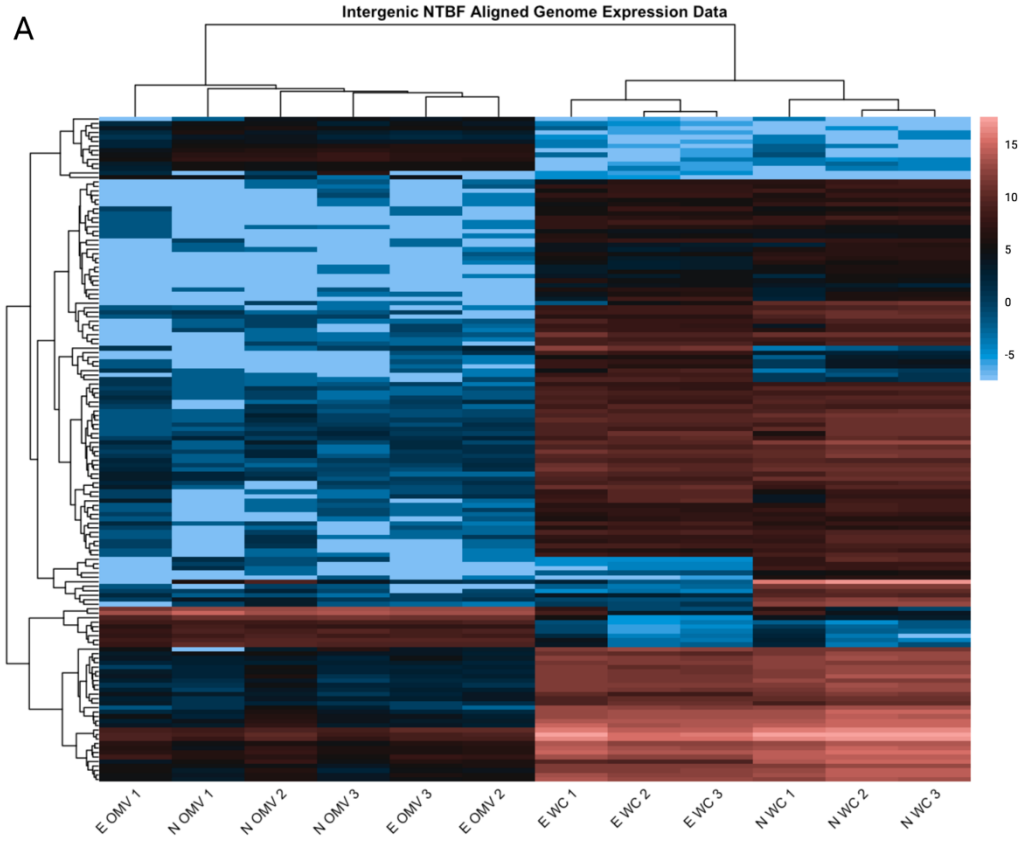


Figure 4.3: *B. fragilis* gene coding RNAs that are enriched in OMV and whole cell (WC) samples can be identified. RNAseq analysis of gene coding regions using DESeq2 reveals enrichment of RNA sequences in OMVs and WC samples when they are aligned to the NTBF (A) and ETBF (B) genomes. DESeq analysis was achieved by pooling the count data of all OMVs against all WC samples. To limit size of dataset for analysis, log_2FC was set at 5 and adjusted p value was set to 10^{-4}. The positive x direction denotes enrichment in OMVs, the negative x direction denotes enrichment in WC.

A


Intergenic NTBF Aligned Genome Expression Data
15
10
5
0
-5
E OMV 1
N OMV 1
N OMV 2
N OMV 3
E OMV 3
E OMV 2
E WC 1
E WC 2
E WC 3
N WC 1
N WC 2
N WC 3

B

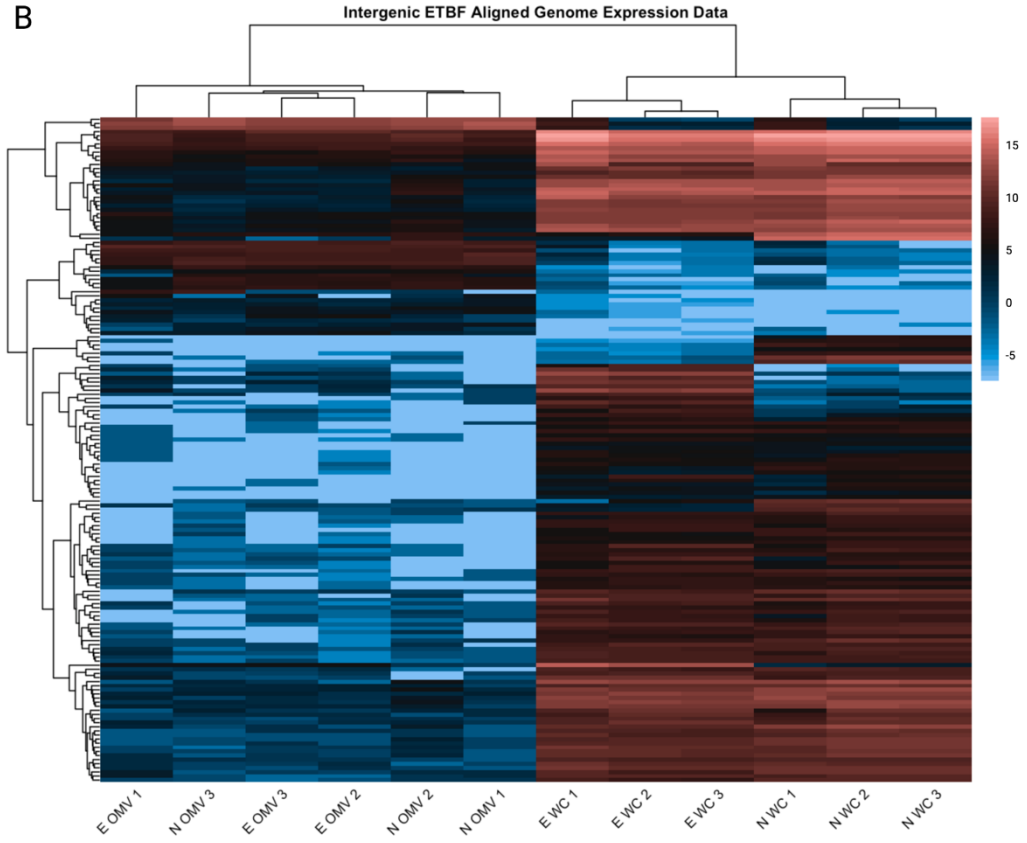

Intergenic ETBF Aligned Genome Expression Data
15
10
5
0
-5
E OMV 1
N OMV 3
E OMV 3
E OMV 2
N OMV 2
N OMV 1
E WC 1
E WC 2
E WC 3
N WC 1
N WC 2
N WC 3

Figure 4.4: RNA sequences from intergenic regions of the *B. fragilis* genome are enriched in OMV and whole cell (WC) samples. RNAseq analysis of intergenic coding regions using DESeq2 reveals enrichment of RNA sequences in OMVs and WC samples when they are aligned to the NTBF (A) and ETBF (B) genomes. OMV samples are represented in the columns on the left side of the heatmaps and WC samples are represented on the right sides. E = ETBF and N = NTBF. The numbers represent the biological replicates of each sample set.

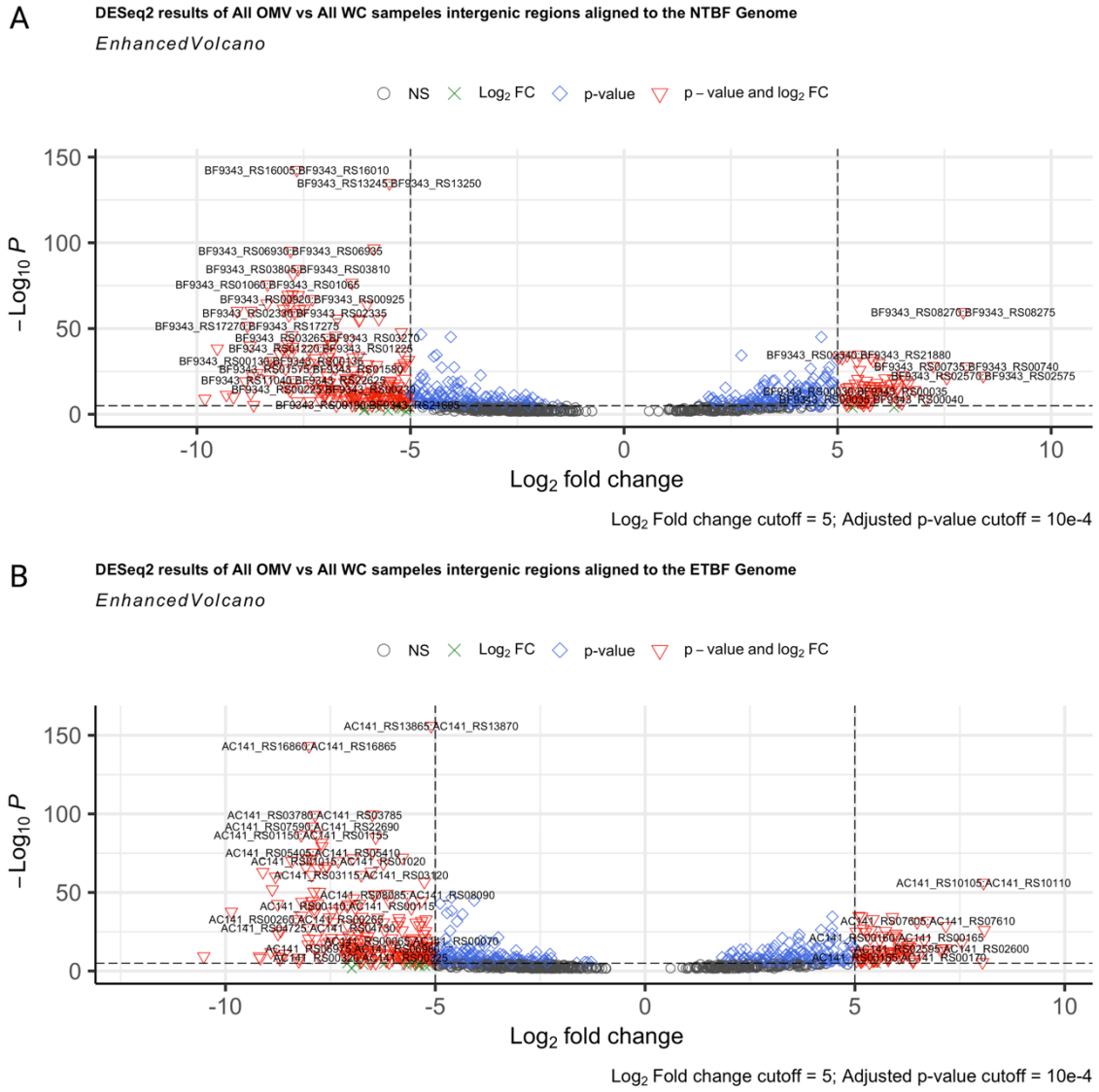


Figure 4.5: *B. fragilis* intergenic RNAs that are enriched in OMV and whole cell (WC) samples can be identified. RNAseq analysis of intergenic regions using DESeq2 reveals enrichment of RNA sequences in OMVs and WC samples when they are aligned to the NTBF (A) and ETBF (B) genomes. DESeq analysis was achieved by pooling the count data of all OMVs against all WC samples. To limit size of dataset for analysis, log_2FC was set at 5 and adjusted p value was set to 10^{-4}. The positive x direction denotes enrichment in OMVs, the negative x direction denotes enrichment in WC.

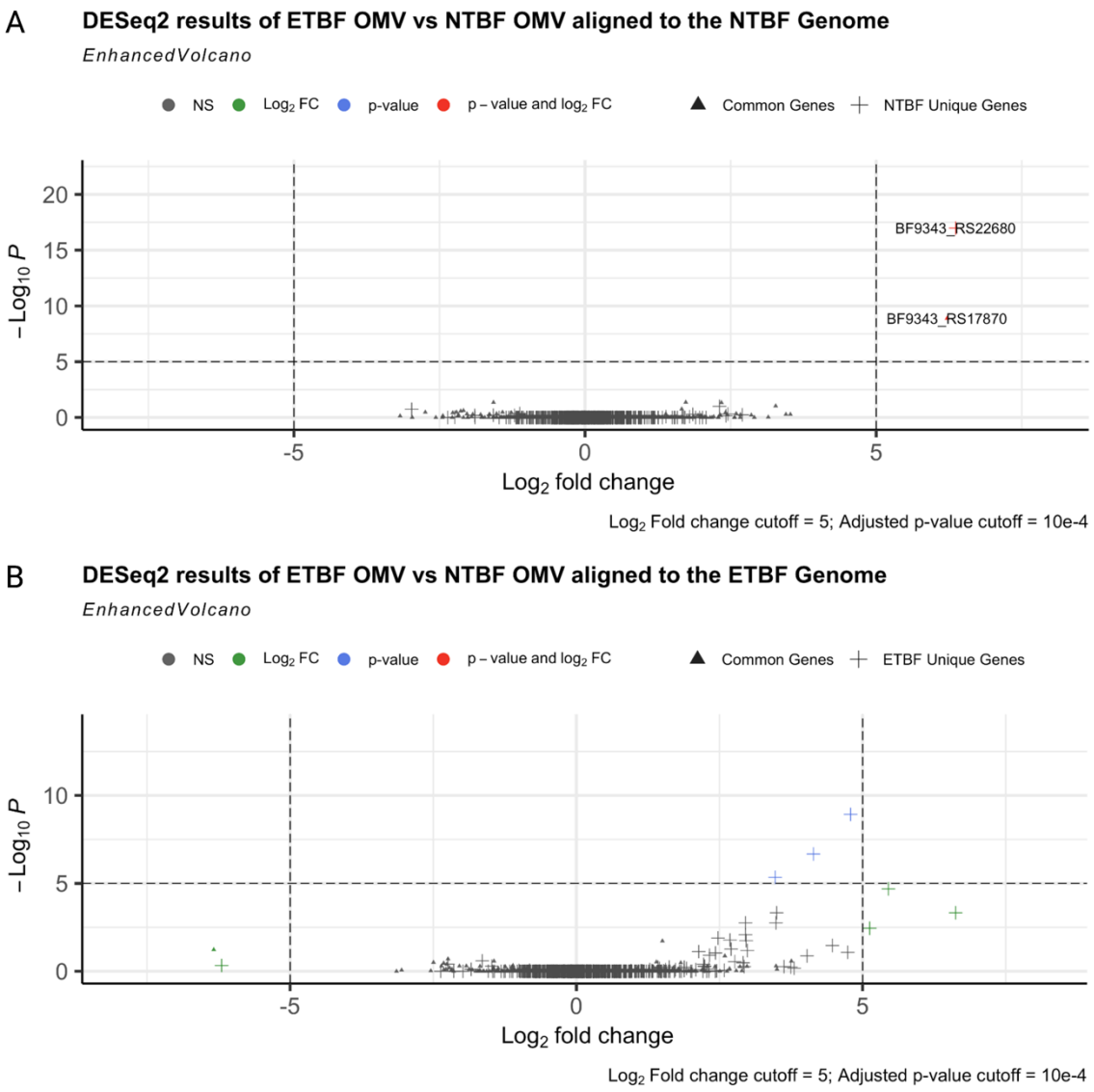


Figure 4.6: *B. fragilis* gene coding RNAs do not demonstrate high differential enrichment between ETBF and NTBF OMVs. RNAseq analysis of gene coding regions using DESeq2 between ETBF and NTBF OMVs demonstrates low differential enrichment of RNA sequences in OMVs and WC samples when they are aligned to the NTBF (A) and ETBF (B) genomes. Only 2 genes that are unique to NTBF are found to be upregulated in ETBF OMVs (A). DESeq analysis was achieved by pooling the count data of all OMVs against all WC samples. The positive x direction denotes enrichment in ETBF OMVs, the negative x direction denotes enrichment in NTBF OMVs.

OMV-Associated RNA Sequences are Distinct in Size and Abundance Between OMV and Whole Cell Samples

To validate and extend the RNA-seq results, we performed northern blotting analysis and qPCR on concentrated total RNA of OMV and WC samples. Using the dataset

generated from the DESeq2 analysis in conjunction with the count data, we identified several targets from ETBF and NTBF genome aligned data for further validation (22 sequences in total). We targeted genes that met our enrichment criteria ($Log_2FC = 5$, p-value = 10^{-4}) and had high count numbers (~2000 counts). We established six categories of putative targets: sequences that align with coding regions that are common in the ETBF and NTBF genomes that are enriched in OMVs, sequences that align with coding regions that are common in the ETBF and NTBF genomes that are enriched in WCs, sequences that align with intergenic regions of the NTBF genome that are enriched in OMVs, sequences that align with intergenic regions of the NTBF genome that are enriched in WCs, sequences that align with intergenic regions of the ETBF genome that are enriched in OMVs, and sequences that align with intergenic regions of the ETBF genome that are enriched in WCs. For each category we selected the top three-most differentially enriched RNA sequences from the DESeq2 analysis. The two NTBF genes that were shown to be enriched in ETBF OMVs over NTBF vesicles (Figure 4.6A) were also included in the dataset. To control for the expression of target sequences in our samples, we identified 2 genes that were not highly differentially expressed between OMV and WC samples, and that were present in high count numbers across all samples. One of the identified genes coded for a phenylalanine tRNA (tRNA-Phe) and the other gene coded for rubredoxin, which acted as the normalization gene for all of our qPCR data.

Table 4.3: PCR Primers

Sequence Name	Code Name	Strain Specificity and Enrichment	Sequence Length (nt)	Expected Product Size (nt)	Forward Primer (5'- 3')	Reverse Primer (5' – 3')
Cytochrome Ubiquinol Oxidase Subunit I	O-g2	Common gene/Enriched in all OMV samples	73	245	GCAGTACGTTGC TCTGTGGA	TAACCGTCGG TCCTTTCTTG
Redox-regulated Atpase Ychf	O-g3	Common gene/Enriched in all OMV samples	1096	212	CGAGAGCCGTA TCCAGAAAG	TTCGTCCACAT TGCAAACAT
OMV Intergene ETBF 1	O-iE1	ETBF genome-specific intergenic sequence enriched in OMV samples	305	210	AGAGGCGATAT AACGCAGGA	TTTGTTGCATC AGACGAACG
OMV Intergene ETBF 2	O-iE2	ETBF genome-specific intergenic sequence enriched in OMV samples	186	112	AAAAAGGTGGT ACTTCCTCTGA	CTTTCGCCAA ACCATCTCTC
OMV Intergene ETBF 3	O-iE3	ETBF genome-specific intergenic sequence enriched in OMV samples	152	150	TTGCTATGAACG AACAGTTGC	TTGGTTTTATC TGCATATTTA AGTGTA
OMV Intergene NTBF 1	O-iN1	NTBF genome-specific intergenic sequence enriched in OMV samples	189	1113	AAAAAGGTGGT ACTTCCTCTGA	TCTTTCGCCAA ACCATCTCT
OMV Intergene NTBF 2	O-iN2	NTBF genome-specific intergenic sequence enriched in OMV samples	264	206	GGTGCGAATCC GGTTATATG	CCCGGCTTCA ATGATTTTAC
OMV Intergene NTBF 3	O-iN3	NTBF genome-specific intergenic sequence enriched in OMV samples	159	115	CAGTTGCCTTGC CATTATTGT	AGTGTACCCC GAAACGACAG
YtxH domain-containing protein	W-g1	Common gene/Enriched in all WC samples	1443	202	ATGGGAAGTGG AAATGCAAA	CTACTTTGGC ACCAGCCTTC
Glutamate decarboxylase	W-g2	Common gene/Enriched in all WC samples	1443	172	GTGCATTGGCTA TCGGTTCT	TCAATCTGCC ACAACTGAGC
Type I deoxyribonuclease HsdR	W-g3	Common gene/Enriched in all WC samples	2046	240	GTTGAAGCCGG AGAACAGAG	CTGCCACCAT TACCGAAGAT
WC Intergene ETBF 1	W-iE1	ETBF genome-specific intergenic sequence enriched in WC samples	428	248	ACCGGTAAAGC ATGCAAAAA	TTTGAGCCTCT TGTCGGATT
WC Intergene ETBF 2	W-iE2	ETBF genome-specific intergenic sequence enriched in WC samples	428	181	CCGGCATTTTTC GTCTTAAA	CGAAGCTTGT CTCGGTTTTC
WC Intergene ETBF 3	W-iE3	ETBF genome-specific intergenic sequence enriched in WC samples	1017	155	TGCGAGATTTTG TGTTGGAA	ATGTAACCGC CCATTCGTAA
WC Intergene NTBF 1	W-iN1	NTBF genome-specific intergenic sequence enriched in WC samples	212	151	ATCGCAGCAAA GGTAGTTGG	GCCGAGATGC TTTTCCTTTT
WC Intergene NTBF 2	W-iN2	NTBF genome-specific intergenic sequence enriched in WC samples	428	181	CCGGCATTTTTC GTCTTAAA	CGAAGCTTGT CTCGGTTTTC
WC Intergene NTBF 3	W-iN3	NTBF genome-specific intergenic sequence enriched in WC samples	533	166	TTTTGTACCGTT GCAAATAGC	AAGTCGCTCACA CCTTAGGC
Rubredoxin	Rubredoxin	Common Gene/High count number in all OMV and WC samples	162	151	CATTTGCACCGT TTGTGATT	GCCATTATACGG TTCGAAATCT
Hypothetical Protein	O-EN1	NTBF-specific gene that is upregulated more in ETBF OMV than NTBF OMV	210	164	TCACCAAAACTT TCCGTTCC	CCTTTTGGGCACG TAGTGAT
DUF4373 Domain-containing Protein	O-EN2	NTBF-specific gene that is upregulated more in ETBF OMV than NTBF OMV	834	179	CCTGGTAGGACT TGGAATCG	CTACTTTGGCACC AGCCTTC

The primer sets for qPCR of the RNA sequences were created using the complete gene or intergenic region from the genome assembly using the Primer3 program (Table 4.3). We selected two RNA sequences for northern blot validation. The probes from northern blotting were developed by creating oligonucleotides that were complementary to the densest region of the RNAseq read alignments using the Integrative Genome Viewer. One of the coding RNA sequences that we found to be enriched in OMVs via DESeq2 aligned to an O-succinlybenzoic acid co-enzyme A ligase (Table 4.4). The Northern blot probing and qPCR yielded successful detection of the tRNA-Phe and CoA ligase sequences in our RNA samples (Table 4.5, Figure 4.7).

Table 4.4: Primer Pairs and Northern blot Sequences for tRNA-Phe and CoA Ligase

Sequence Name	Strain Specificity and Enrichment	Sequence Length (nt)	Read Length (nt)	Read Sequence	Forward Primer (5'- 3')	Reverse Primer (5' – 3')	Northern probe sequence (5'- 3')
tRNA – Phe	Common tRNA/High count number in OMV and WC samples	73	26	CACTACGTTCGGGACGTAGGGGTCGGGCGTTCGAGTCGCCTCATTCCG	GGTGCCATAGCTCAGTTGGT	GTGCCACCAGGAATCGAAC	AACACAGGGGCCAAGCTAAGGACCAC
O-succinyl benzoic acid-CoA ligase	Common gene/Enriched in all OMV samples	1096	27	TTTGGTAGAGAAGGGGGCAAATGCAGA	CTGCCGGACCGATATTCTTA	AGGCCGGTACAACATACTCG	AATCCTCTCCGCCAACACGAAAACCATCTCTTCCCCCGTTTACGTCTCAATGGCCTTTGTTTCTACTTCCTCGAAG

Table 4.5: DESeq2 Log_2FC for for tRNA-Phe/CoA Ligase and Detection via qPCR

Gene Name	Log_2FC When Aligned to NTBF Genome	Log_2FC When Aligned to ETBF Genome	Enriched in OMV or WC	Detected in NTBF WC	Detected in NTBF OMV	Detected in NTBF OMV + RNase	Detected in NTBF WC	Detected in NTBF OMV	Detected in NTBF OMV + RNase
tRNA – Phe	3.19	3.19	Enriched in OMVs, high count number in WC	✓	✓	✓	✓	✓	✓
O-succinyl benzoic acid-CoA ligase	-1.53	-0.96	Enriched in WC, high count number in OMVs	✓	✓	✓	✓	✓	✓

In order to confirm that the RNA sequences were contained within the OMVs and not coated onto the vesicle surface or were present in culture supernatant, we performed an RNase protection assay on the vesicles prior to RNA isolation and ran them with matched RNA samples of untreated OMVs (Figure 4.8). Analysis of RNA concentrations recovered from untreated vesicles compared to OMVs treated with RNase revealed significantly less RNA in RNase-treated vesicles (Figure 4.8). Running the RNA samples through a denaturing gel demonstrated that OMVs protect specific RNA sequences (Figure 4.9). PCR analysis of RNase treated vesicles with the primer set for the tRNA-Phe and CoA Ligase yielded successful detection of target sequences (Figure 4.10). Visualization of the northern blots for the tRNA-Phe and CoA Ligase showed that the probes bind to shorter OMV-associated RNAs lower in the blot compared to the WC samples, suggesting that the sequences contained in the OMVs are fragments of the larger RNA transcript (Figures 4.11 and 4.12). To further confirm that our identified RNA sequences of interest are protected

from RNase degradation by being packaged into vesicles, we ran qPCR on RNase-treated samples and detected our sequences (Figures 4.13, 4.14, 4.15, and 4.16; Table 4.5).

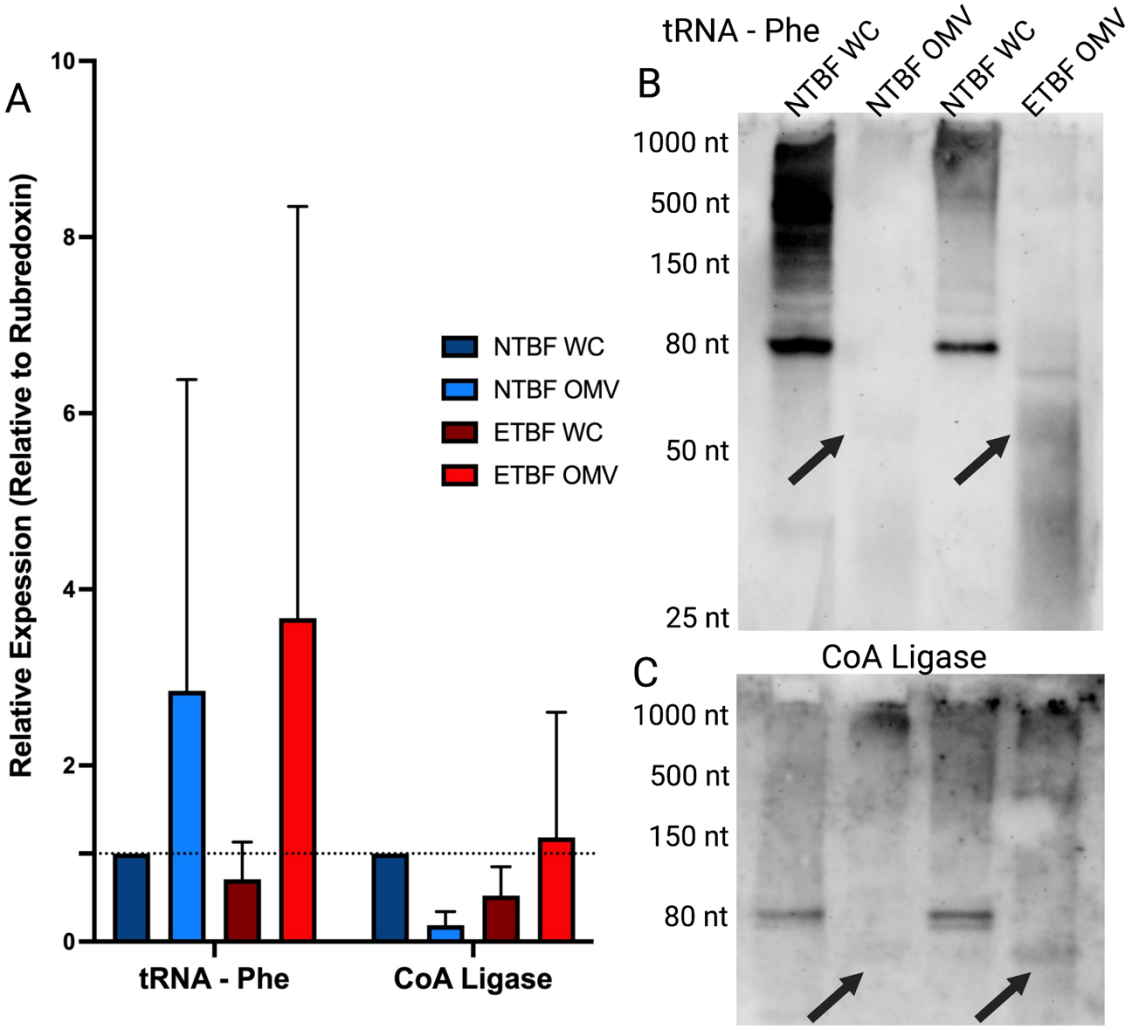


Figure 4.7: OMV-associated RNAs can be quantified through qPCR and northern blotting. Using gene sequence-derived primers, OMV target genes were detected through qPCR (4A) and normalized to a common gene found in both OMV and WC samples. To confirm the size of tRNA (4B) and CoA Ligase (4C) transcripts, northern blot probes complimentary to the RNAseq reads were used and successfully detected transcripts in OMVs (denoted by arrows).

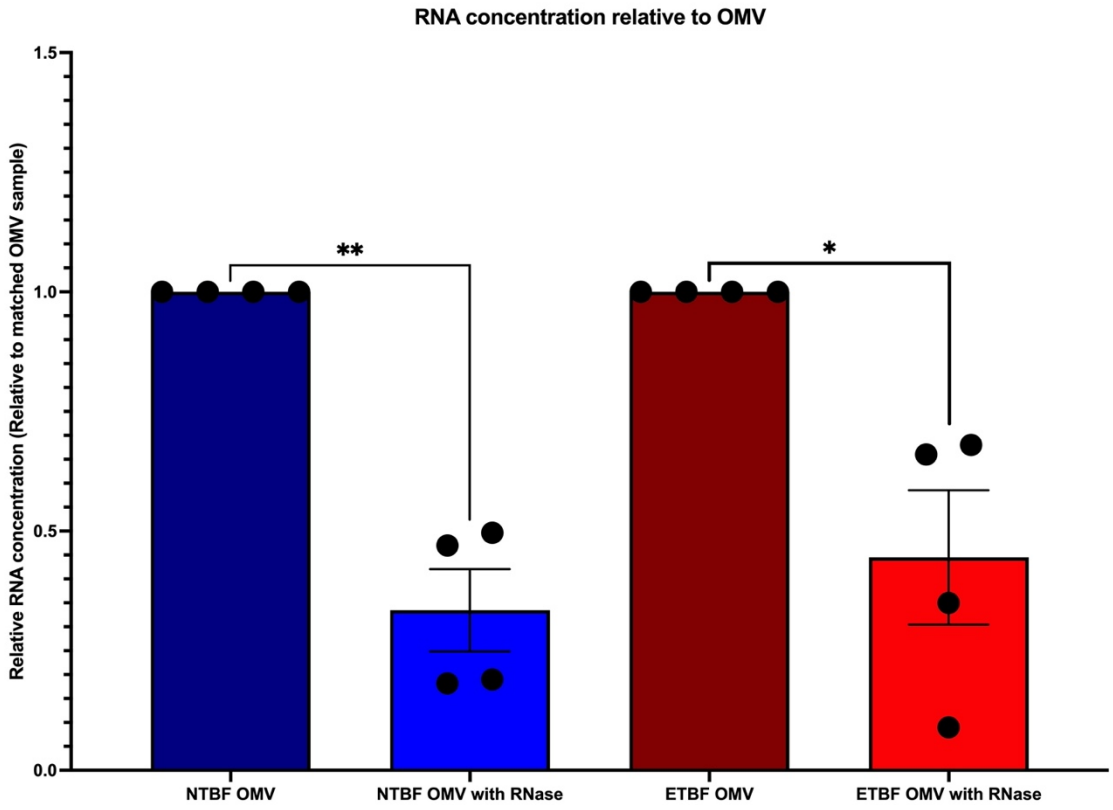


Figure 4.8: RNase degrades OMV-associated RNAs. OMV samples were treated with 10 μg/mL of RNase A for 30 minutes prior to RNA extraction. After RNase treatment there was a loss of RNA in the OMV samples in comparison to their matched untreated controls. Statistical significance was determined using a Welch's t-test. *$p < 0.05$, ** $p < 0.01$.

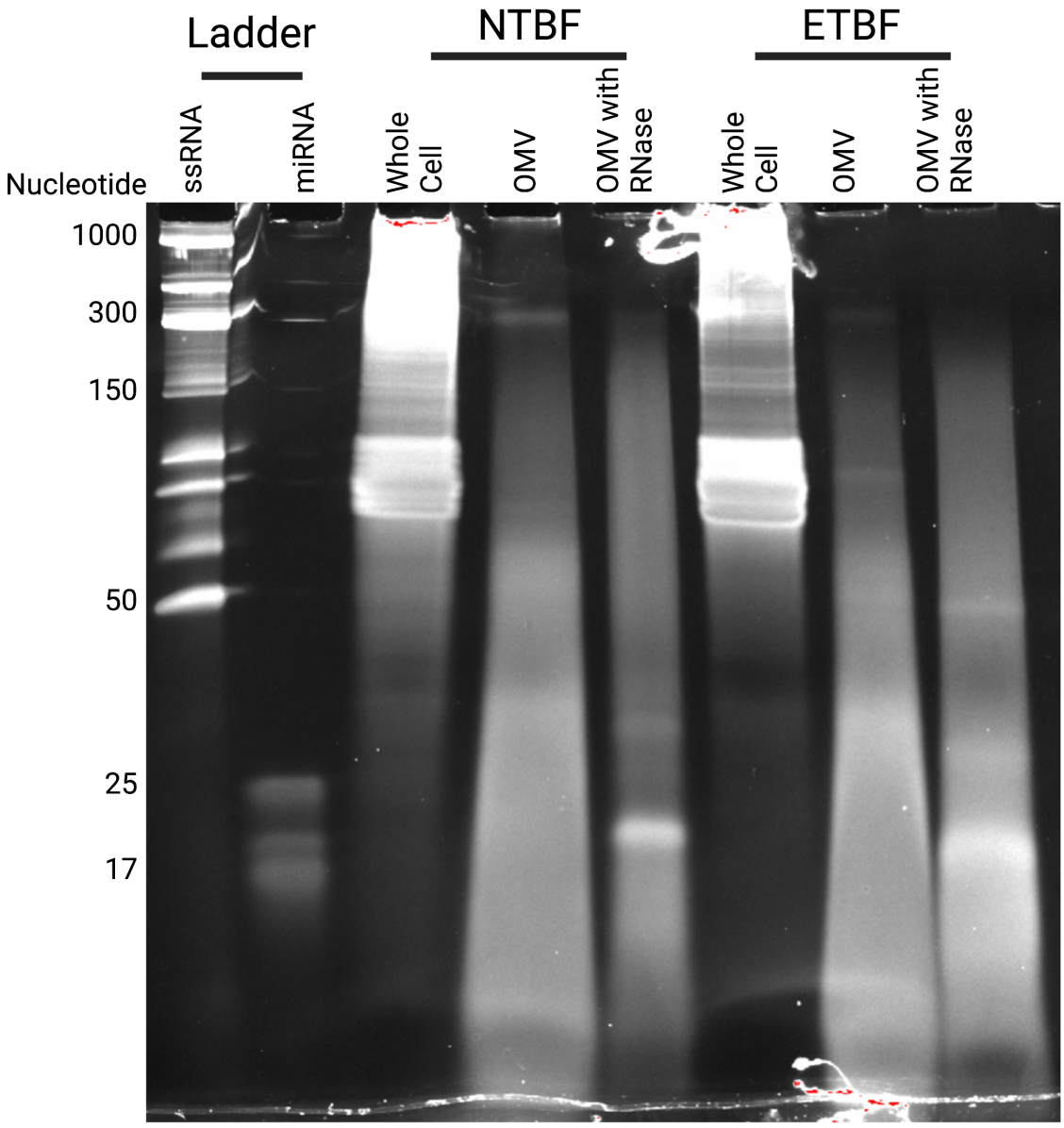


Figure 4.9: OMV-associated RNAs can be protected from RNase degradation. 10 μg of RNA samples were run through a denaturing gel and stained with GelRed. Comparison of OMV RNA with RNase treated vesicle RNA show a loss of streaking in the lane and specific bands becoming more prominent.

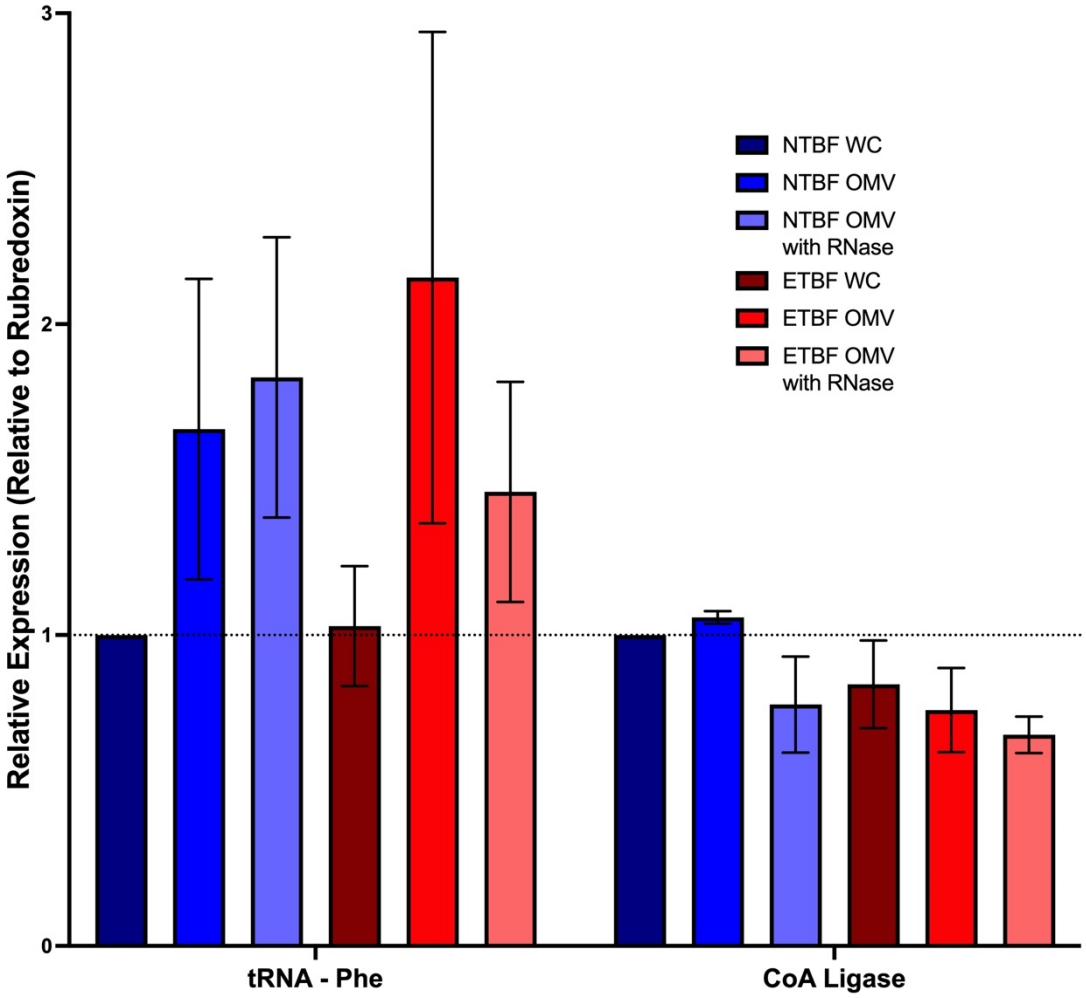


Figure 4.10: Specific OMV-associated RNAs are protected from RNase degradation and detected through qPCR. OMV samples were treated with 10 μg/mL of RNase A for 30 minutes prior to RNA extraction. PCR of RNase – treated vesicles demonstrate detectable amounts of target sequences. PCR data was normalized to Rubredoxin expression and referenced to the NTBF WC data. Testing with Kruskal-Wallis yielded statistical significance in the tRNA-Phe dataset ($p = 0.0463$). Dunn's multiple comparisons did not demonstrate any significance between samples for the target. The CoA Ligase dataset did not yield any significance.

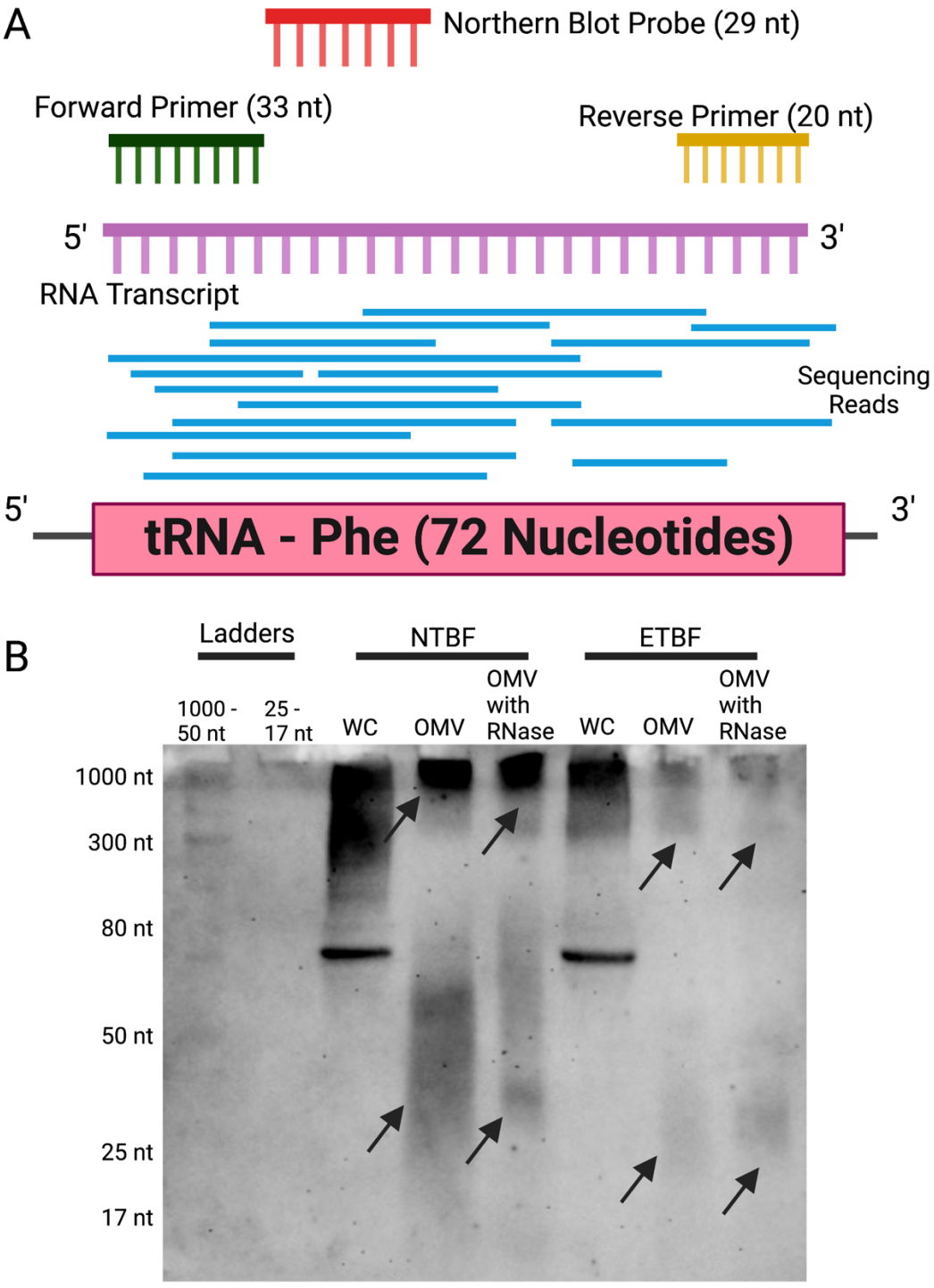


Figure 4.11: RNase protected tRNA-Phe RNA sequences can be detected through northern blotting. To confirm the size of tRNA-Phe, northern blot probes complimentary to the RNAseq reads were used and successfully detected transcripts in RNase-treated OMVs (denoted by arrows). Schematic diagrams for the tRNA (A) show the development of the qPCR primers and the northern blot probes. Read alignments from the RNAseq data (Blue) were spread throughout the tRNA gene. The primers (green and yellow) were developed based on the whole gene sequence (pink) while the northern blot probes (red) were developed based on the density of read alignments (orange) to the gene.

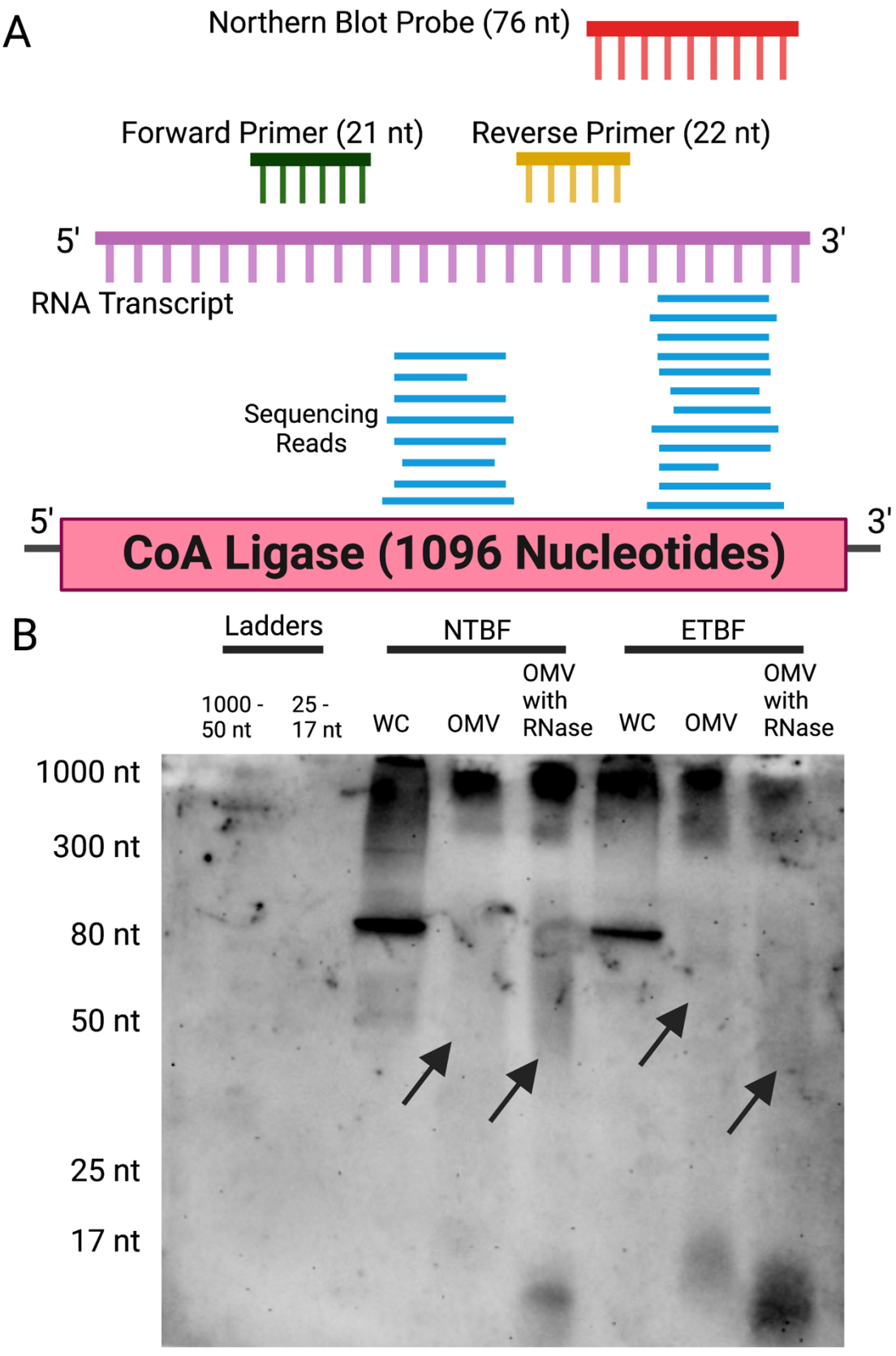

A
Northern Blot Probe (76 nt)
Forward Primer (21 nt)
Reverse Primer (22 nt)
5'
3'
RNA Transcript
Sequencing Reads
5'
3'
CoA Ligase (1096 Nucleotides)
B
Ladders
NTBF
ETBF
1000 - 50 nt
25 - 17 nt
WC
OMV
OMV with RNase
WC
OMV
OMV with RNase
1000 nt
300 nt
80 nt
50 nt
25 nt
17 nt

Figure 4.12: RNase protected CoA Ligase RNA sequences can be detected through northern blotting. To confirm the size of CoA Ligase, northern blot probes complimentary to the RNAseq reads were used and successfully detected transcripts in RNase-treated OMVs (denoted by arrows). Schematic diagrams for the CoA Ligase (A) show the development of the qPCR primers and the northern blot probes. Read alignments from the RNAseq data (Blue) were concentrated in 2 region of the CoA Ligase gene, one region in the center of the gene, and one region towards the 3' end of the gene. The primers (green and yellow) were developed based on the whole gene sequence (pink) while the northern blot probes (red) were developed based on the density of read alignments (orange) to the gene.

Table 4.6: DESeq2 Log_2FC for Identified RNA Sequences and Detection via qPCR

Gene Code	Log_2FC When Aligned to NTBF Genome	Log_2FC When Aligned to ETBF Genome	Enriched in OMV or WC	Detected in NTBF WC	Detected in NTBF OMV	Detected in NTBF OMV + RNase	Detected in ETBF WC	Detected in ETBF OMV	Detected in ETBF OMV + RNase
O-g2	5.07	5.22	OMV	✓	✓	✓	✓	✓	✓
O-g3	4.81	4.92	OMV	✓	✓	✓	✓	✓	✓
O-iE1	5.26	N/A	OMV	✓	✓	✓	✓	✓	✓
O-iE2	5.99	N/A	OMV	✓	✓	✓	✓	✓	✓
O-iE3	6.67	N/A	OMV	✓	✓	✓	✓	✓	✓
O-iN1	N/A	5.42	OMV	✓	✓	✓	✓	✓	✓
O-iN2	N/A	6.40	OMV	✓	✓	✓	✓	✓	✓
O-iN3	N/A	6.81	OMV	✓	✓	✓	✓	✓	✓
W-g1	-8.26	-8.11	WC	✓	✓	✓	✓	✓	✓
W-g2	-6.14	-5.89	WC	✓	✓	✓	✓	✓	✓
W-g3	-5.92	-5.93	WC	✓	✓	✓	X	X	X
W-iE1	-7.91	N/A	WC	✓	✓	✓	✓	✓	✓
W-iE2	-7.64	N/A	WC	✓	✓	✓	✓	✓	✓
W-iE3	-6.44	N/A	WC	✓	✓	✓	✓	✓	✓
W-iN1	N/A	-7.87	WC	✓	✓	✓	✓	✓	✓
W-iN2	N/A	-7.59	WC	✓	✓	✓	✓	✓	✓
W-iN3	N/A	-8.67	WC	✓	✓	✓	X	X	X
O-EN1	N/A	6.37	OMV	✓	✓	✓	X	X	X
O-EN2	N/A	6.22	OMV	✓	✓	✓	✓	✓	✓

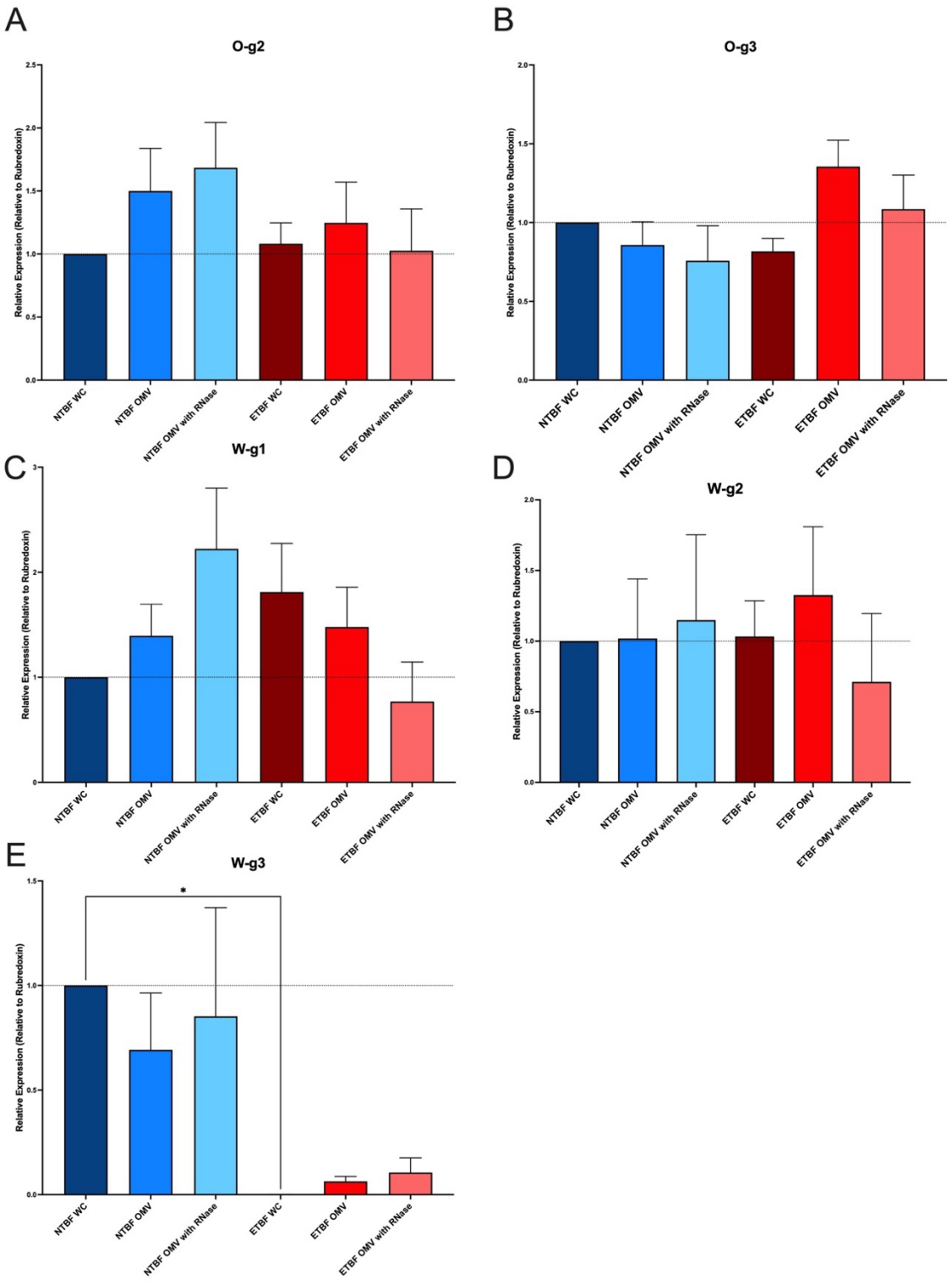


Figure 4.13: Gene coding RNA sequences are protected from RNase degradation in OMVs and can be detected through qPCR in OMVs and WC samples. Using gene sequence-derived primers, target sequences that aligned with coding region of the NTBF and ETBF genomes that were enrich in OMVs (A,B) and WC (C-E) samples were detected through qPCR. Statistical significance was determined using a Dunn's multiple comparisons test. *$p < 0.05$

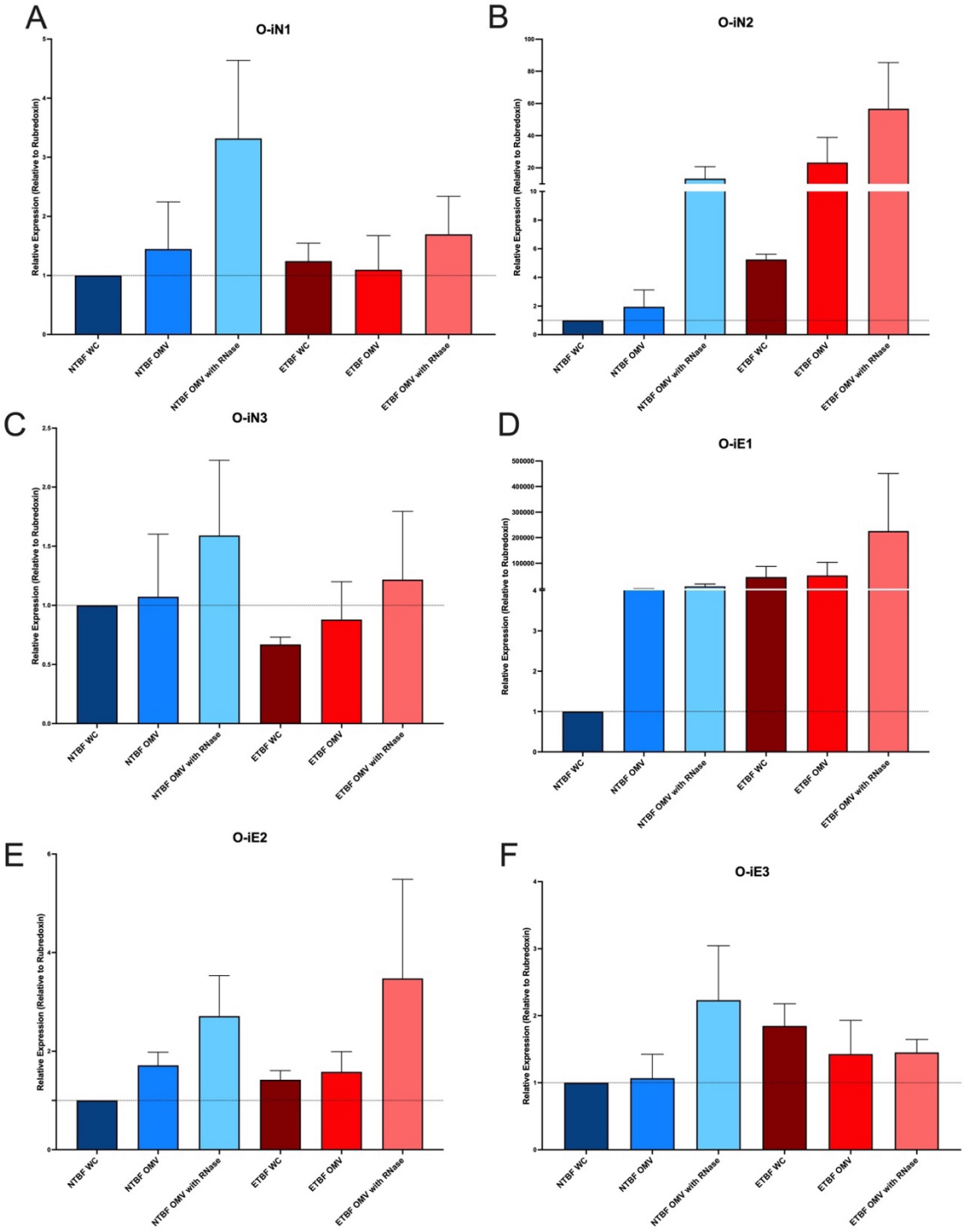


Figure 4.14: Bacterial OMV-associated intergenic sequences are detected through qPCR. Using gene sequence-derived primers, OMV intergenic target sequences were detected through qPCR. Statistical testing using ANOVA and Dunn's multiple comparisons test yielded no statistical significance.

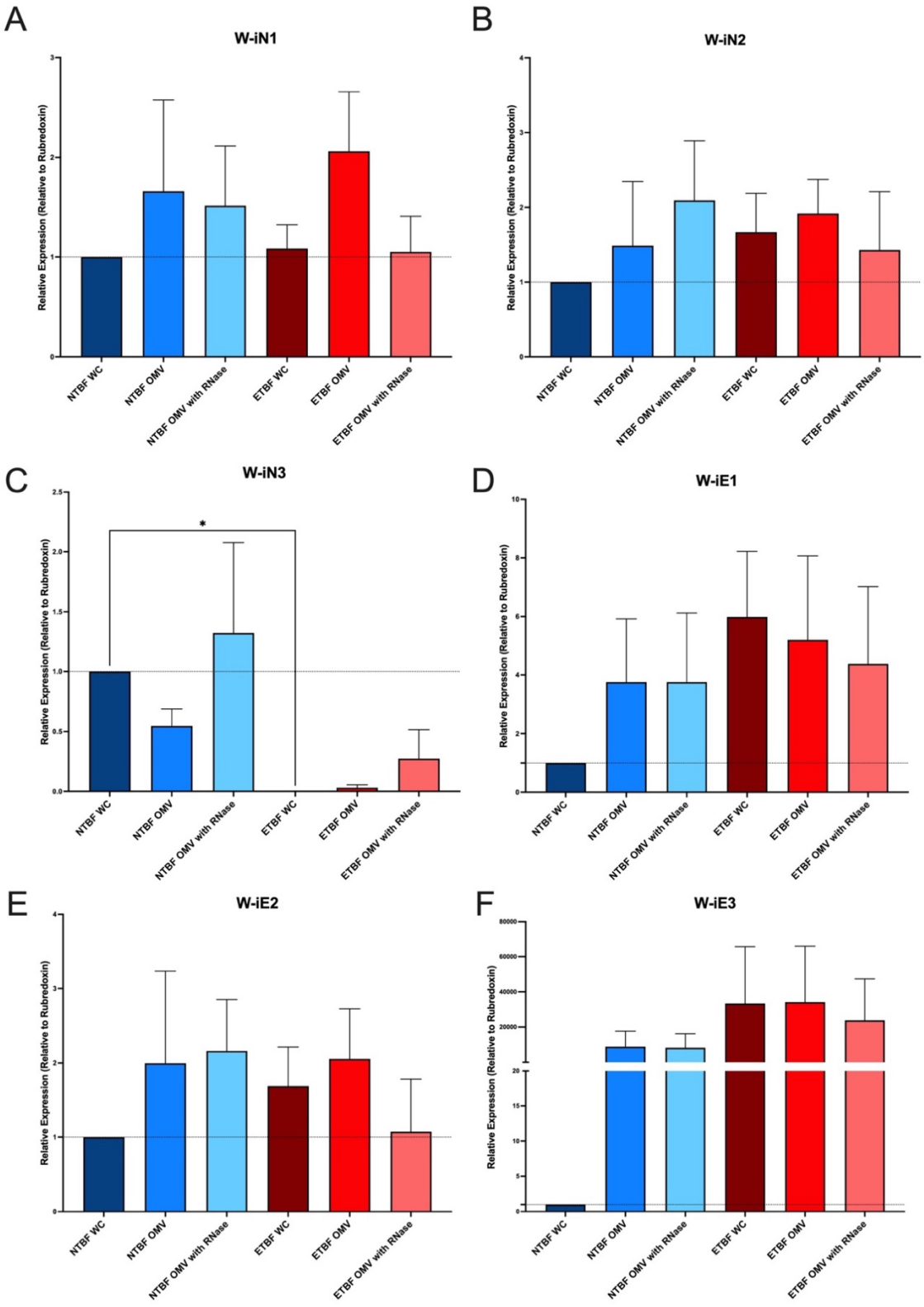


Figure 4.15: Bacterial whole cell-associated intergenic sequences are detected through qPCR. Using gene sequence-derived primers, whole cell intergenic target sequences were detected through qPCR. Statistical significance was determined using a Dunn's multiple comparisons test. *$p < 0.05$

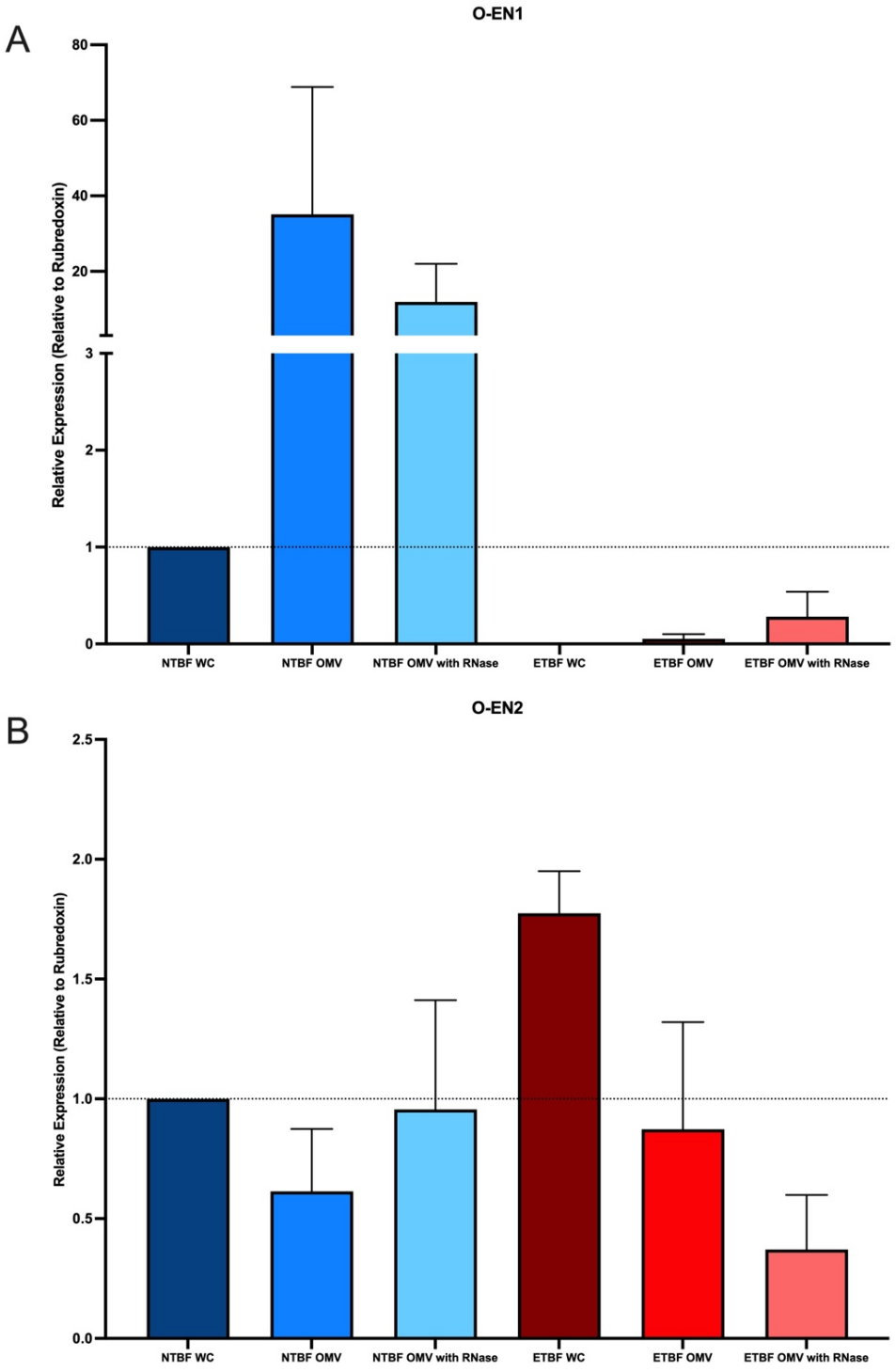


Figure 4.16: Bacterial OMV-associated sequences are detected through qPCR. The RNAseq comparison analysis yielded two NTBF-specific gene that were upregulated in ETBF OMVs over NTBF OMVs. Using gene sequence-derived primers, OMV target genes were detected through qPCR. Statistical testing using ANOVA and Dunn's multiple comparisons test yielded no statistical significance.

Discussion

There has been a growing interest in recent years in understanding the effects of bacteria-derived OMVs on host intestinal physiology and health [108,134,135,143,276]. The immunogenic effects of OMVs on host cells and their ability to be easily transformed to present specific antigens warrants further investigation as a platform for therapeutic development and vaccine development [160,263,295]. The effects of major biomolecular components of OMVs such as LPS, peptidoglycans, and secreted proteins (i.e: toxins) have been the subject of a majority of the research surrounding the characterization and downstream effects of OMVs on host cells [108,149,153,154,287]. Compared to these biomolecules, the role of nucleic acids (DNA and RNA) in OMVs, has only been recently begun to be characterized due to improvements in the isolation and high-throughput sequencing of OMV-derived nucleic acids [155]. The role of OMV-derived RNA in host physiology and disease has been described in common bacterial species that are clinically relevant such as *E. coli, P. aeruginosa, V. cholerae,* and *P. gingivalis* [138,138,157,325,334]. As work continues to further understand the role of the microbiome and of specific bacterial species on host health, it is imperative to understand the RNA profiles of their derived OMVs as they can have important modulatory effects on downstream host pathways.

We sought to profile and characterize the RNA species contained within OMVs from two strain of *B. fragilis*, a commensal (NTBF) and a pathogenic (ETBF) strain due to their distinct clinical effects in patients in cases of chronic inflammation and colorectal cancer [89,92,94,108]. Using Illumina sequencing on RNA samples that we isolated from OMV samples, we were able to align the reads to both genomes of interest. Profiling the OMV-derived RNA reads to the general classes of gene coding regions of both genomes revealed

an enrichment for protein coding-aligned RNA reads and a depletion of reads that aligned to tRNAs, and rRNAs. The presence of these general categories of reads in proportions that are different in OMVs from their cells of origin suggests that bacteria have a molecular mechanism that directs more fragments of protein coding RNA reads into OMVs compared to other genic-aligned classes.

To further understand the differences in the RNA read profiles of OMV and WC samples, we performed DESeq2 analysis to identify specific genes and intergenic regions of interest that are enriched in each sample condition and in between strains. Using this dataset in conjunction with the read alignment count numbers, we are able to identify several sequences of interest for further validation. Other researchers have demonstrated the presence of specific OMV-associated RNA sequences using qPCR for genes that are known to be enriched in the vesicles of their species of interest [157,334]. The RNA profiles of *B. fragilis* is not as well-characterized as other model bacteria species (i.e. *E. coli*), requiring a *de novo* approach to validate the presence of the sequences and genes identified *in-silico*.

We were able to successfully detect our genes of interest through qPCR in OMV and WC samples using primers generated from the genome-assembly derived gene sequences. The detection of these genes prompted us to add an extra level of certainty in our findings with northern blotting. The presence of RNA bands WC samples confirmed our detection of the identified sequences of interest. The binding of the probe to the OMV samples occurred in lower areas of the blot compared to WC samples. These data suggest that the OMVs do not contain the full-length transcript of the target sequences, but rather

various smaller fragments of the sequences are loaded in the vesicles for extracellular transport as evidenced by the smears in the respective lanes on the blot.

To ensure that the detection of our target sequences is due to internalization of transcripts within vesicles and not from external presentation on the outer membrane or secretion into the culture broth, we treated our vesicle samples with RNase. We found that there was a modest decrease in the concentration of RNA isolated from RNase-treated vesicles compared to their untreated counterpart, suggesting that a portion of the RNA we initially isolated is not contained within the vesicles. The appearance of probe-binding on the northern blot in these samples demonstrate that the sequences of interest are loaded into the OMVs to protect them from degradation as vesicular delivery to the target site occurs. These findings correspond with other studies investigating RNA fragments in OMVs [157]. *P. aeruginosa* has been described to load a tRNA fragment into its OMVs for delivery to human epithelial cells where the fragment can act in a similar manner to miRNA and down regulate IL-8 expression in the host cells [157]. The presence of RNA fragments in our NTBF and ETBF OMV samples may suggest a similar function of these sequences as the *P. aeruginosa* tRNA fragment and warrants further investigation.

Overall, our data demonstrate that NTBF and ETBF OMVs are enriched in specific RNA sequences that are primarily aligned with coding regions of the *B. fragilis* genome. Through this work, we demonstrate a novel methodology to detect and validate the presence of uncharacterized RNA sequences using qPCR and northern blotting that can be applied to other model organisms for novel RNA sequence discovery and validation. RNase treatment of the vesicles demonstrate that our sequences of interest and their fragments are contained inside the OMVs for transport to target sites. Future work to

understand the interactions of these RNA fragments with host cells, and their interactions with host receptors and downstream molecular machinery can promote an improved understanding of the contribution of OMV-derived RNAs in immune and inflammatory responses in clinical cases such as IBD and colorectal cancer.

Materials and Methods

Bacteria Strain and Culture Preparation

Enterotoxigenic *Bacteroides fragilis* (ETBF; 86-5443-2-2) and non-toxigenic *Bacteroides fragilis* (NTBF; NCTC 9343) were provided as a gift by Dr. Cindy Sears at Johns Hopkins University. Bacteria were cultured in brain heart infusion (BHI) broth under anaerobic conditions overnight at 37˚C until an OD_{600} reading of 0.8 – 1 was achieved.

Ultracentrifugation Isolation of Outer Membrane Vesicles

One liter of NTBF and ETBF cultures were spun down at 8000 x g for 15 minutes at 4˚C and the resulting supernatant was filtered through 0.45 μm vacuum filter (Thermo Scientific, 168-0045). The filtered supernatant was ultracentrifuged at 100, 000 x g for 2 hours at 4˚C (Beckman Coulter 70 Ti) and pelleted vesicles were resuspended in PBS.

Column Isolation and Concentration of Outer Membrane Vesicles

OMVs were isolated from NTBF and ETBF using the ExoBacteria OMV Isolation Kit (System Biosciences, EXOBAC100A-1) as per the manufacturer's instructions. Briefly 250 mL of ETBF and NTBF cultures were spun down at 8000 x g for 15 minutes at 4˚C and the resulting supernatant was filtered through 0.45 μm vacuum filter (Thermo Scientific, 168-0045). Filtered supernatant was run through the ExoBacteria OMV

isolation column and each column yielded 1.5 mL of eluted OMVs. 5 columns were used per culture resulting in 15 mL of each strain. The resulting solution of OMVs was concentrated using centrifugal concentration tubes (Thermo Scientific, 88532) at 5500 x g for 15 minutes.

Small RNA Isolation

Matched samples of RNA isolated from whole bacteria and corresponding OMVs were isolated in three individual preparations. RNA samples were isolated using the GeneJET RNA Purification Kit (Thermo Scientific, K0732) as per the manufacturer's instructions for bacteria RNA isolation. RNA quality was assessed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA concentrations were determined using the Qubit3 RNA HS Assay Kit (Invitrogen, Q32852).

RNase Protection Assay and Total RNA Isolation

Concentrated OMVs were incubated with 10 μg/mL of RNase A at 37°C for 1 hour. The solution was then treated with RNA*secure* RNA Inactivation Reagent (Invitrogen; AM7006) for 10 minutes. Three matched preparations of whole cell pellets, RNase-treated OMVs and untreated OMVs were subjected to hot phenol RNA extraction. Whole cell pellets were treated in 1.5 mL of 0.5 mg/mL of lysozyme in 10 mM of Tris-HCl solution and incubated at 37°C for 5 minutes. For each 1.5 mL of whole cell and OMV samples, add 150 μL of 10 % SDS and 30 μL of 7.5 M ammonium acetate. Add equal amounts of phenol:chloroform:isoamyl alcohol (25:24:1; pH ~5.2) and vortex until the solution is emulsified. Incubate at 65°C for 15 minutes with a brief vortex every 5 minutes and then chill on ice for 5 minutes. Centrifuge the samples at 15, 000 x g for 10 minutes at 4°C,

collect the aqueous layer and add equal amount of chloroform and vortex the solution. Centrifuge again at 15, 000 x g for minutes at 4°C and remove the aqueous layer. Add 100 μL of 7.5 M ammonium acetate and 2X chilled 95% ethanol to the mixture, vortex, and centrifuge at 15, 000 x g for 20 minutes at room temperature. Decant the supernatant, add 80% ethanol to wash the RNA pellet and centrifuge at 15, 000 x g for 20 minutes for 4°C. Decant the supernatant and air dry the pellet briefly and resuspend the pellet in 100 μL of ultrapure water.

RNA Sequencing and Analysis of Bacteroides Fragilis Small RNAs

Small RNA extractions were quantified by Quant-iT RiboGreen RNA assay (Life Technologies, Thermo-Fisher) and assessed for purity using a Thermo Scientific Nanodrop 2000 spectrophotometer. To create indexed sequencing libraries, the Takara Biosciences (formerly Clontech) SMARTer smRNA-Seq kit for Illumina (catalog 635029) was used, which is suitable for sequencing small RNAs from low-yield samples. Illumina adapter and index sequences were incorporated without ligation to ensure diverse small RNAs are represented. 50 ng of starting material was used for each library.

Resulting libraries were quantified using Biotium Accuclear 7 DNA standards High Sensitivity assay (fluorometric quantitation) and the size distribution determined by Agilent Bioanalyzer using the High Sensitivity DNA assay. Fragment molarity and samples were normalized to 1.0 nM before pooling for sequencing at loading concentration of 1.8 pM on the Illumina NextSeq 500 using a High output 75 cycle reagent kit v2. Sequence reads were then demultiplexed using bcl2fastq2 before subsequent bioinformatic analysis.

Reads from all samples were trimmed and aligned to both *B. fragilis* genomes (ETBF: 86-5443-2-2, NTBF: NCTC 9343) using the STAR aligner with default parameters. RNA enrichment analysis and visualization was completed using R (version 4.2.1 – "Funny-Looking Kid') and RStudio (version 2022.07.2 – "Spotted Wakerobin"). Data analysis and visualization was completed using tidyverse (version 1.3.2), DESeq2 package (version 1.4.2), ggplot2 (version 3.3.6), EnhancedVolcano (version 1.14.0), and pheatmap (version 1.0.12).

Read Coverage Analysis

RNA coverage from sequencing data was visualized with *Integrative Genome Viewer* (Version 2.8.12). Northern blot probes were created by taking the complementary sequence of the densest read regions of the identified genes and intergenic regions of interest.

qRT-PCR of RNAs

Total RNA (1 μg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) as per the manufacturer's instructions. The qPCR was performed with diluted cDNA using primer sets for genes identified through DESeq2 analysis. PCR amplification of RNA was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems, 4367659). All qPCR experiments were run in technical quadruplicates and biological triplicates and a mean value was used for the determination of RNA levels. Images were generated using GraphPad Prism 9.

Northern Blotting of Bacterial RNAs

Northern blots of total RNA samples were conducted as follows. Northern blot probes were created by taking the complementary sequence to the densest read mapping to genes of interest according to *IGV*. Probes were labeled with DIG using the 2nd generation DIG oligonucleotide 3'-end labeling kit (Roche, 03353575910) according to the manufacturer's instructions and diluted to a concentration of 1 ng/mL in 10 mL of ULTRAhyb ultrasensitive hybridization buffer (Invitrogen, AM8670).

10 μg of RNA samples were loaded onto 15% Mini-PROTEAN Tris-borate EDTA (TBE)-Urea gel (Bio-Rad, 4566053) and run for 2 hours at 90 V in 1X TBE buffer. Transfer of RNA was completed in 1X TBE buffer onto a positively charged nylon membrane (Invitrogen, AM10102) for 3 hours at 60V in cold conditions. Blots were UV crosslinked and incubated in hybridization buffer for 1 hour before being probed overnight at 42°C. Probes were poured off and blots were washed twice in 2X SSC with 0.1% SDS buffer for 15 minutes, twice in 0.1X SSC with 0.1% SDS buffer with 5 minutes and 1X SSC buffer for 10 minutes. Blots were processed using the DIG wash and block buffer set (Roche, 11585762001) as per the manufacturer's instructions and imaged using ChemiDoc MP Imaging System (Bio-Rad, 12003154) for 20 minutes.

Figure Preparation

All figures were prepared using BioRender.com

CHAPTER FIVE

The Effects of Outer Membrane Vesicles on the Cytokine Responses of Colonic Epithelial Cells

Introduction

The influence of the intestinal microbiome on its host organism has many important consequences in terms of metabolism and homeostasis [44–46]. Significant alterations to the microbial community structure can result in pathologies that can lead to adverse health outcomes such as IBD and CRC in the context of germ-line or somatic mutations [38]. The gastrointestinal tract interacts with the microbiomes primarily through the use of PRRs expressed on epithelial and immune cells [50]. Under normal conditions, microbial biomolecules can engage with PRRs to "train" the immune system and promote immunotolerance in the intestine [61,89,93,200]. In diseased states, overabundance of certain microbial metabolites can result in altered activity of the pathways that are downstream of the PRRs. The OMVs produced by the microbiome represent one of the various delivery mechanisms for these bacterial biomolecules. A class of PRRs that have important implications in inflammation and CRC are TLRs [50,93,273].

TLRs have evolved to recognize a diverse array of biomolecules including lipoprotein, PGs, LPS, and nucleic acids [209,210,213,335]. As such, these receptors are expressed across many cell types as one of the first lines of defense in the immune system [202]. Upon ligand interaction, TLRs activate signaling pathways through either TRIF or MyD88, which engage with proteins and enzymes such as MAPK and IKK complexes to promote innate immune signals like NF-κB and cytokine cascades [214]. TLR-mediated immune

activation eventually leads to the engagement the adaptive immune system to remove pathogens from the site of infection/challenge. The primary method of adaptive immune cell recruitment to the site of TLR activation is through the production of cytokines.

Cytokines are important mediators of cellular communication during immune responses and are produced by a wide variety of cells [336]. Chemokines are a subgroup of cytokines that induce the chemotaxis of leukocytes to the site of production to mount an appropriate immune response [337]; one chemokine with particular relevance to CRC is IL-8 which is coded by the (C-X-C motif) ligand 8 (*CXCL8*) gene [338]. IL-8 is canonically described as a neutrophil chemotaxis factor [339]. However, studies suggest that IL-8 can act on other cell types to promote cell migration, proliferation and angiogenesis, resulting in important clinical implications in diseases such as cancer [338,340,341]. In the intestines, overproduction of IL-8 correlates with increasing severity of inflammation in people diagnosed with IBD [342]. Additionally, sustained IL-8 production can increase metastatic potential of CRC and can affect prognostic outcomes in patients [343,344]. Despite recent evidence demonstrating the OMV-mediated control of IL-8 responses in human airway epithelial cells [157], the interplay of OMVs and IL-8 responses in CRC have yet to be fully elucidated.

In this study, we sought to understand the interactions of NTBF and ETBF OMVs with TLRs and subsequent IL-8 responses. Given the diverse types of biomolecules found in OMVs, we hypothesized that the vesicles of *B. fragilis* can activate multiple sub-classes of TLRs. Additionally, we hypothesized that the OMVs of NTBF and ETBF can stimulate the transcription of IL-8 in a strain-specific manner. We discovered that TLRs that recognize peptidoglycans and RNAs are activated in the presence of OMVs,

and that IL-8 is produced in vesicle-treated CRC cell lines in a dose and strain-dependent pattern.

Results

OMVs Activate Innate Immune Receptors

Through our work to characterize the RNA profiles NTBF and ETBF OMVs, we observed enrichment of specific RNA sequence fragments in the vesicles. As such we worked to determine the effects of these vesicles on pathogen-recognition receptors and downstream molecular signals in colorectal cancer cells. To this end we sought understand the role that the vesicles of different strains of *B. fragilis* in contributing to immunomodulation in a clinically relevant sample.

To understand the interactions of host cell PRRs in the presence of NTBF and ETBF OMVs, we treated TLR-SEAP reporter cells with varying doses of column-isolated NTBF and ETBF vesicles (Figure 5.1). We used four different classes of TLRs that recognize bacterial biomolecules: TLR2 for peptidoglycans, TLR3/7 for dsRNA and ssRNAs respectively, and TLR4 for lipopolysaccharides. For the TLR2 reporter cells (Figure 5.2A) we observed significant activation of the TLR pathway in a dose-dependent manner for both *B. fragilis* strains with the activation peaks at 500 μg/mL. TLR3 (Figure 5.2B) and TLR7 (Figure 5.2D) had similar responses to both strains' OMV treatments with significant activation at 2000 μg/mL and the response falling off at lower doses of vesicles in both TLR classes. On the other hand, the TLR4 cells showed no evidence of significant activation in the presence of OMVs (Figure 5.1C).

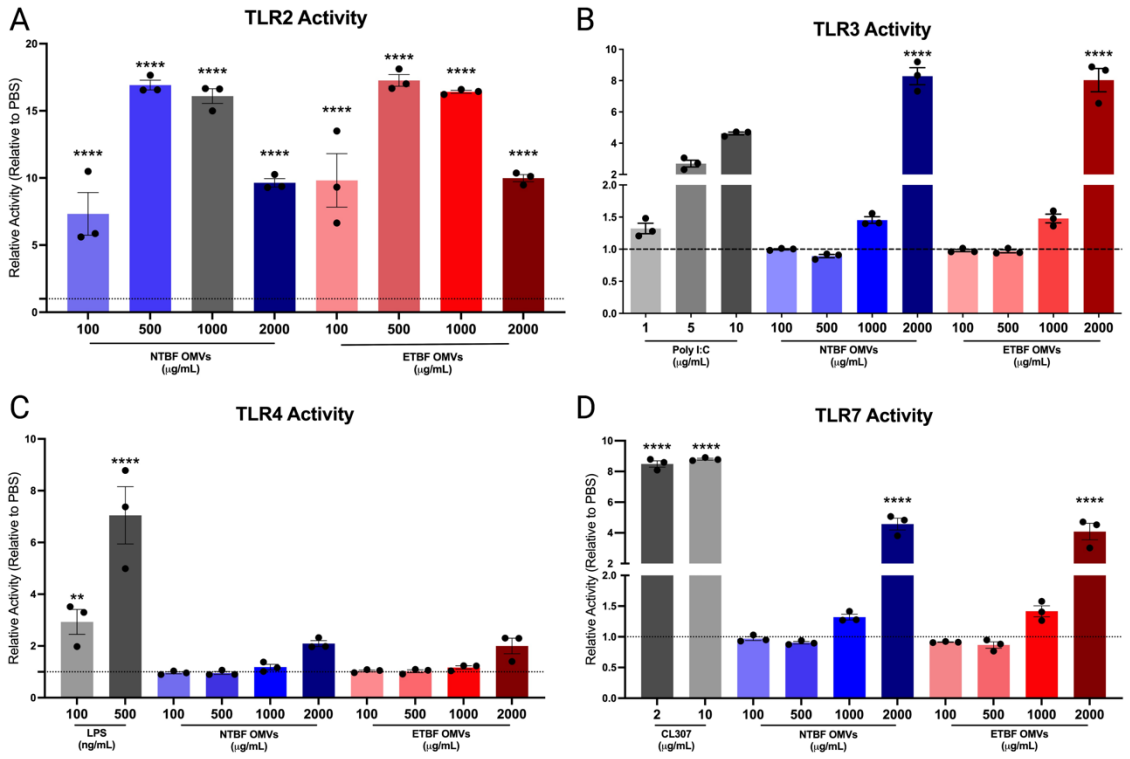


Figure 5.1: Administration of column-isolated NTBF and ETBF vesicles to TLR-SEAP reporter cells demonstrates activation of various TLRs. (A) TLR2, (B) TLR3, (C) TLR4 and (D) TLR7. TLR activity was calculated by comparing vesicle and positive control treatments (gray columns) to the corresponding TLR cells treated with PBS (PBS treatment activity is represented by the dashed lines at y = 1). Dots represent biological replicates and data is presented as mean ± SEM. Statistical significance was found using Dunnett's multiple comparison test. ** $p < 0.01$, **** $p < 0.0001$.

Outer Membrane Vesicles Induce Cytokine RNA Expression in Colon Cancer Cells

The activation of different TLR subtypes in presence of NTBF and ETBF OMVs prompted us to investigate the role that the vesicles of different strains of *B. fragilis* have in contributing to immune signaling in colon cancer. For these experiments, we used two different colorectal adenocarcinoma cell lines: Caco-2 and HT29-MTX (Figure 5.2). Caco-2 cells closely mimic the tight junction barrier of the colon, allowing the cell line to be used as a model to observe the interactions of the vesicles with host cells [345]. HT29-MTX cells are a stable sub-population of the HT29 cell line that are differentiated into a goblet cell phenotype and can produce mucosal proteins that are found in the gastrointestinal tract [346].

The different phenotypes of these cell lines represent different cell population of the colon and allow us to investigate the differential effects of vesicles on different cell types.

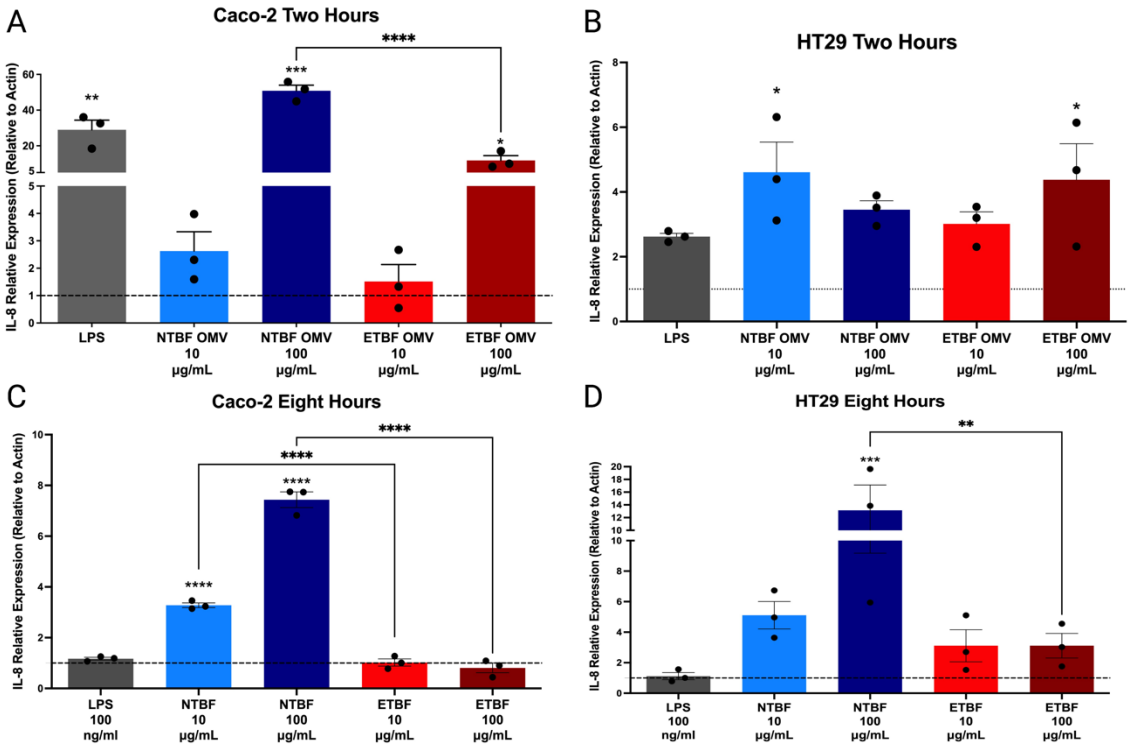


Figure 5.2: Outer membrane vesicles from NTBF and ETBF can elicit strong IL-8 response in colorectal adenocarcinoma cells. Treatment of Caco-2 and HT29-MTX adenocarcinoma with column-isolated ETBF and NTBF vesicles increase expression of IL-8 transcripts in cells relative to cells treated with PBS. (A) Treatment of Caco-2 cells with OMVs for 2 hours. (B) Treatment of HT29 cells with OMVs for 2 hours. (C) Treatment of Caco-2 cells with OMVs for 9 hours, (D) Treatment of HT29 cells with OMVs for 8 hours. Dots represent biological replicates and data is presented as mean ± SEM. Statistical significance was found using Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

We treated the cells for 2 hours (A and B) and 8 hours (C and D), with a low dose (10 μg/mL) and a high dose (100 μg/mL) of column-isolated vesicles. At 2 hours we observed a significantly higher induction of IL-8 expression in Caco-2 (5.2A) cells in response to 100 μg/mL treatments of NTBF and ETBF vesicles. At 10 μg/mL, we observed a general increase in IL-8 expression with only the NTBF OMVs while ETBF vesicle 10 μg/mL treatment remained close to baseline expression (5.2A). In HT29-MTX cells, we

observed a significant increase of IL-8 with treatment of 10 μg/mL of NTBF vesicles and 100 μg/mL of ETBF OMVs (5.2B) while NTBF at 100 μg/mL and ETBF at 10 μg/mL resulted in a general increase of IL-8 transcription (5.2B). Caco-2 cells do not express IL-8 in quantities that are detectable over the PBS controls for ETBF vesicles at 8 hours post-treatment (5.2C), while treatment with NTBF vesicles maintains a significant induction of IL-8 transcription in a dose-dependent manner (5.2C). HT29-MTX cells treated with ETBF OMVs for 8 hours show a general increase of IL-8 at both doses (5.2D). For NTBF OMVs we observed a significant increase of IL-8 expression at the 8-hour mark for concentrations of 100 μg/mL while treatments of 10 μg/mL yielded generally higher expression of IL-8 over the control treatment. Analysis of OMV treatments demonstrated higher expression of IL-8 with treatments of NTBF over ETBF at 100 μg/mL in Caco-2 cells at 2 hours (5.2A), Caco-2 cells at 8 hours (5.2C), and HT29 cells at 8 hours (5.2D). Treatments of Caco-2 cells with 10 μg/mL of NTBF vesicles yielded higher IL-8 transcription over matched treatments of ETBF OMVs (5.2C).

Discussion

The intestinal microbiome maintains a symbiotic relationship with the host organism and disruptions to that symbiosis can lead to chronic conditions and disease like IBD and CAC [5,45,47]. There is growing evidence that increased enrichment of certain species and strains of bacteria can lead to microbial dysbiosis and contribute to the poor health and prognostic outcomes in the host [106,179,277,347]. The microbiome interacts with the mucosal and epithelial layers through various mechanisms including OMVs [134,135]. There is growing evidence of the utility of these vesicles as an important regulator of inter-kingdom communication and host physiology through horizontal gene transfer, modulating

immune responses, and controlling host cell protein expression [88,134,142,157]. Due to the multi-faceted use of OMVs in a natural setting, there is much interest in their potential as a tool for therapeutic development. However, it is imperative that we understand the impacts of vesicles on human cells due to the various bacterial biomolecules that can elicit strong immune responses in the cells [134,263].

Through the work presented in this paper, we sought to determine the different types of TLRs that NTBF and ETBF OMVs have may potentially interact with to activate downstream immune mechanisms and to determine impact that these vesicles have on colorectal cancer cells. We used two strains of *B. fragilis* as our model organism due to their clinical relevance. As a commensal (NTBF) the bacteria is known to promote immunotolerance by inducing the proliferation of regulatory T cells in the intestine [88,143], while the pathogenic strain (ETBF) can produce a toxin that cleaves E-cadherin in tight junction to disrupt the epithelial barrier and contribute to chronic inflammation [101,106,182].

For our work, we investigated the role of TLR activation in the presence of OMVs. TLRs are innate immune receptors that recognize a wide range of foreign 'non-self' biomolecules and can modulate the host immune system through their activity [50,93,202]. We used TLR reporter cells that expressed four classes of TLRs that recognize different bacterial biomolecules that compose OMVs and their molecular cargos: TLR2 for peptidoglycan, TLR4 for LPS, TLR3 for dsRNA, and TLR7 for ssRNA [50,202,205,219]. We demonstrate that TLR2 activates in the presence of the OMVs of both strains in a dose-dependent manner with activation peaking at 500 μg/mL. This data suggests that the vesicle samples contain peptidoglycans, and that there is an ideal concentration of OMVs the achieves maximum activation of TLR2 and its downstream pathways. At lower

concentrations, there may not be enough activation of TLR2, and at higher concentrations the biomolecules in the vesicles maybe promoting cell death resulting in a decreased TLR2 response. TLR3 and TLR7 cells activated with treatments of OMVs at 2000 μg/mL. The high concentration of OMVs required to activate these cells suggests that the RNAs contained in the vesicles may not be causing immune responses through TLRs, but rather through other nucleic acid-sensing receptors like cGAS or RLRs. Though TLR4 does not significantly activate in the presence of OMVs, this data does not suggest the LPS is not found in our samples, rather that there may be other mechanisms of action (i.e caspase-4/11) that LPS is exerting its effects on cells [348].

The activation of different classes of TLRs in response to OMV treatment prompted us to investigate the effects on vesicles in a clinically relevant model. We treated monocultures of Caco-2 and HT29-MTX cells with NTBF and ETBF OMVs and found that the vesicles of both strains induce expression of the pro-inflammatory cytokine IL-8. We found that NTBF induces a much stronger IL-8 response than its pathogenic counterpart. At 2 hours, the Caco-2 cells responded to OMV treatment in a dose-dependent manner while HT29-MTX showed significant IL-8 expression with 10 μg/mL of NTBF OMVs and 100 μg/mL of ETBF OMVs. At 8 hours post-treatment we found that the response to ETBF OMVs in both cell lines fell to the expression found in PBS samples, while NTBF OMVs maintained the IL-8 response. Caco-2 and HT29-MTX cells expressed IL-8 in a dose-response manner to NTBF vesicles. The differences of cell lines' responses to NTBF OMVs maybe due to their different phenotypes and origins, as HT29-MTX is a goblet-cell phenotype subpopulation of HT29 that derives from intestinal crypts, while Caco-2 has its origins in villus enterocytes [143,346,349]. Other researchers have demonstrated

that OMVs from commensal *E. coli* strains can induce IL-8 expression in Caco-2 and HT29 in a dose-dependent manner [143]. These findings suggest a possible beneficial effect by priming the intestine to better respond to challenges from pathogen colonization as IL-8 is a chemokine that attracts neutrophils and macrophages to affected sites [143,339].

Altogether, our data demonstrate that ETBF and NTBF vesicles can produce strong inflammatory responses in epithelial colorectal cancer cells. OMVs activate certain classes of TLRs, suggesting a role for these receptors in host-vesicle interactions. Future work will require further investigation to solidify the role of TLRs in the host-microbe-OMV axis and to investigate the activity of other PRRs such as RLRs in the presence of OMVs. We will seek to understand the role of OMV-associated RNA fragments in the host microbe relationship and investigate other roles for cell physiology modulation other than interactions with PRRs. Creating synthetic oligonucleotides based on our sequences of interest and loading them into nanoliposomes will allow us to see the effects of a single sequence of RNA on the proteome of the host cell [157]. Additional work will be required to understand the utility of high IL-8 production in NTBF over ETBF. Overall, our research builds a foundation for understanding the role of OMVs on host immune responses through RNAs.

Materials and Methods

Bacteria Strain and Culture Preparation

Enterotoxigenic *Bacteroides fragilis* (ETBF; Strain 86-5443-2-2) and non-toxigenic *Bacteroides fragilis* (NTBF; NCTC 9343) were provided as a gift by Dr. Cindy Sears at Johns Hopkins University. Bacteria were cultured in brain heart infusion (BHI)

broth under anaerobic conditions overnight at 37˚C until an OD_{600} reading of 0.8 – 1 was achieved.

Column Isolation of Outer Membrane Vesicles

OMVs were isolated from NTBF and ETBF using the ExoBacteria OMV Isolation Kit (System Biosciences, EXOBAC100A-1) as per the manufacturer's instructions. Briefly 250 mL of ETBF and NTBF cultures were spun down at 8000 x g for 15 minutes at 4˚C and the resulting supernatant was filtered through 0.45 μm vacuum filter (Thermo Scientific, 168-0045). Filtered supernatant was run through the ExoBacteria OMV isolation column and each column yielded 1.5 mL of eluted OMVs. Vesicular sample concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, 23227).

Cell Lines

Caco-2 and HT29-MTX cells were received as a generous gift from Dr. Christie Sayes and HEK-Blue TLR cells were obtained from InvivoGen. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning Inc.; Kennebuck, ME, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio Inc.; Kerrville, Texas, USA) and 1X antibiotics (Penicillin/Streptomycin, Lonza; Basel, Switzerland). HEK-Blue TLR cells were additionally supplemented with 100 μg/mL of Normocin (Invivogen, ant-nr-1), 1X HEK-Blue Selection (Invivogen, hb-sel), 100 μg/mL of Zeocin (Invivogen, ant-zn-05), and 30 μg/mL of blasticidin (Invivogen, ant-bl-05). Cell lines were tested for mycoplasma every two weeks. Incubation occurred at 37°C with 5% CO_2.

TLR Reporter Assays

HEK-Blue TLR reporter assays (Invivogen) were carried out as per the manufacturer's specifications for specific TLRs. Cells were treated with 100 μg/mL and 10 μg/mL of ETBF and NTBF vesicles.

RNA Extraction and qRT-PCR

Table 5.1: Primer sets for Actin and IL-8

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
IL-8	ATGACTTCCAAGCTGGCCGT	TCCTTGGCAAAACTGCACCT

Caco-2 and HT29 cells were treated with 100 μg/mL and 10 μg/mL of NTBF and ETBF vesicles for 2 hours and 8 hours. Cells were lysed in the presence of Trizol Reagent. (Invitrogen, 15596026) and RNA was extracted following the manufacturer's instructions. RNA (250 ng) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) as per the manufacturer's instructions. The qPCR was performed with diluted cDNA using primer sets for cytokine genes (Table 5.1). PCR amplification of RNA was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems, 4367659). All qPCR experiments were run in technical quadruplicates and biological triplicates and a mean value was used for the determination of RNA levels.

Figure Preparation

All figures were prepared using BioRender.com

CHAPTER SIX

Conclusion

Research Objectives

The mechanisms by which the microbiome interacts with host cells are complex and multi-faceted [44,45]. Outer membrane vesicles (OMVs) represent one method of inter-kingdom communication [135] as the biomolecules associated with OMVs have context-dependent interactions with host cells, resulting in varying physiological outcomes [134]. The goal of this research is to determine the role of OMV-associated RNA sequences in modulating immune responses in colorectal cancer (CRC) cells. This work characterizes the RNA profiles of non-toxic and enterotoxigenic *Bacteroides fragilis* (NTBF and ETBF, respectively) OMVs and their impacts on RNA-sensing mechanisms and cytokine responses in CRC cells.

Our work began with improving upon existing transmission electron microscopy (TEM) imaging techniques and characterizing the physical properties of OMVs to ensure successful isolation of vesicles prior to RNA extraction. Current TEM protocols of vesicles rely on either negative staining with uranyl acetate or cryo-EM. Each method comes with unique pitfalls: uranyl acetate does not fix the vesicles, leading to sample modifications during imaging, and cryo-EM is expensive and requires significant amount of time to prepare samples [322,350]. Our work focused on co-incubation samples with osmium tetroxide and uranyl acetate to simultaneously fix and stain vesicles. Imaging of a higher quantity of sub-50 nm OMVs in our co-stain demonstrates an improved method of visualizing vesicles

for downstream analysis. Characterization of concentrated OMVs through silver staining of concentrated vesicles yielded unique protein profiles for NTBF and ETBF, demonstrating that OMVs of different bacterial strains can contribute to the differential physiological outcomes that the bacteria confer to their host. While our work corroborates previously established research that characterized the size profiles of vesicles and specific OMV-protein associations in *B. fragilis*, further research is required to expand the identification and characterization of the protein cargo of NTBF and ETBF OMVs.

The RNA cargo of OMVs have important implications in host-microbe interactions and physiology due to their contributions to phenomenon such as biofilm formation, pathogenicity, and immunomodulation [138,141,157]. Characterization of export mechanisms and of individual sequences are not as understood as alternative biomolecules such as LPS, peptidoglycans, and lipoproteins [153,154,351]. To this end we sought to investigate the RNA cargo of NTBF and ETBF OMVs. We performed Illumina sequencing on small RNA sequences isolated from matched samples of OMV and whole cell (WC) cultures of both strains. During analysis we conducted reciprocal alignment of all samples to both genomes so that we could conducted differential enrichment analysis to identify potential sequences of interest. Through our analysis we were able to identify general trends in the types of RNAs contained in OMVs, and enrichment of specific reads in the vesicular samples.

To validate the RNA-seq data and to determine localization of OMV-associated RNAs, we treated OMVs with RNase, and developed primer pairs for our sequences of interest and northern blot probes based on the read density data. Using Northern blots we observed probe binding in OMVs and RNase-treated OMV RNAs at nucleotide lengths below the expected transcript size suggesting that RNA fragments, rather than full-length

transcripts, are imported into OMVs as evidenced by the diffuse staining found in the lanes for the vesicular samples. On the other hand, qPCR of these samples resulted in successful detection of our genes and intergenic sequences of interest. Together with the enrichment data, we find evidence that bacteria can selectively load specific fragments of RNA sequences into the interior of OMVs for transport to target sites.

The enrichment of RNA sequences in the OMVs of *B. fragilis* prompted us to investigate the effects of the vesicles on immune signaling in CRC cells, particularly the contribution of the OMV-associated RNAs in the responses. To this end, we treated TLR reporter cells with OMVs and observed robust dose-dependent responses in TLR2, which recognizes peptidoglycans. On the other hand, we observed modest activation of the RNA-sensing TLRs, TLR3 and TLR7, which recognize double-stranded and single-stranded RNA respectively. Moving to a more clinically relevant model, we incubated Caco-2 and HT29-MTX cells with OMVs and observed strong *CXCL8* responses. Interestingly, NTBF induced a higher expression of *CXCL8* compared to ETBF. Such a phenomenon may be a result of alternate mechanism by which ETBF may exert its pathogenic effects (i.e: cleaving E-cadherin), or it may be due to an effort by NTBF to elicit mild inflammatory responses to promote tolerance to the microbiome. These sets of data all together demonstrate the importance of OMV delivery of RNA fragments in cell signaling and downstream responses.

Contributions

Outer membrane vesicles are a ubiquitous characteristic of Gram-negative bacteria, however their contributions to host physiology and disease are limited to model organisms such as *E. coli, P. aeruginosa,* and *S. typhimurium* [148,150,157,307,318]. OMVs contain different

types of biomolecules that elicit different responses depending on which receptor ligand binding occurs [50,143,144,156]. In this work we demonstrate the importance of *Bacteroides fragilis* OMVs in host immune responses, highlighting the critical role they play in modulating intestinal physiology.

Research on other model organisms have shed light on important mechanisms in OMV-host cell interactions; vesicles produced by commensals assist in maturation of the immune system, while pathogenic OMVs contribute to biofilm formation and pathology [50,143,144,156,303] Due to the heterogeneity of the cell populations found in the intestinal lining, current research focuses on the vesicles' ability to induce pro-inflammatory responses. OMVs of various pathogenic species such as *H. pylori, P. aeruginosa,* and *A. baumannii* induce potent cytokine responses (i.e.: IL-8, IL-1β and IL-6) in epithelial cells provide mechanistic evidence of the immunostimulatory capabilities of OMVs [145,147,149,347]. The various bacterial biomolecules contained in the vesicles interact with several classes of PRRs to lead to different physiological outcomes. The most characterized PRR to interact with vesicles is TLR4; OMVs contain LPS that can drive TLR4-mediated cytokine response [224,225,352]. Evidence from various species demonstrate upregulation of TLR4 and proteins involved in the response pathways such as MyD88 upon treatment with vesicles, along with induction of pro-inflammatory cytokines like IL-8, and IL-1β in a dose-dependent manner [50,134,224,225,352]. The effects of OMVs on the epithelial cells can lead to disruption in epithelial barrier integrity and can promote bacterial invasion into the submucosa, leading to chronic inflammation [106,108,229].

In addition to interactions with epithelial cells, OMVs can elicit response in immune cells and in certain cases can train immune system to promote tolerance to the

microbiome [63,89,143,225,279]. The interactions of OMVs with neutrophils, macrophages, and dendritic cells are dependent on the species of origin and their bimolecular cargo [353–355]. Generally, vesicles can stimulate these cells and drive the production of pro-inflammatory cytokines such as TNF, IL-1β, IL-8 and IL-6 [134,353–355]. Interactions with OMVs can also stimulate the upregulation of MHCII and promote the proliferation of T cells and B cells[356]. In contrast, certain cases such as with the OMV of *Neisseria meningitidis* and *H. pylori,* apoptosis and anti-inflammatory pathway activation will occur to facilitate bacterial colonization [149,285,357].

Our work contributes to the field of OMV biology by discerning the contributions of *Bacteroides fragilis*-derived RNA in immune response. We visualized the vesicles by developing a novel staining procedure that preserves the structure of the OMVs without relying on conventional fixation methods. Our RNAseq analysis with reciprocal alignments to the genomes of both strains provided us with previously uncharacterized sequences of interest contained within vesicles. We validated these sequences of interest through the use of northern blotting and qPCR. Through this methodology of analysis and validation we demonstrate a set of benchmarks that can be used for the discovery of novel RNA sequences in OMVs or whole cell bacteria. Our data from the TLR reporters and cell cultures demonstrate the contribution of microbial RNA in the intestinal immune response and highlight a mechanism by which bacteria can affect inflammatory pathways.

Applications and Future Directions

The research we conducted have important implications in clinical sciences. The role of the microbiome in chronic conditions and disease has become more appreciated in the last decade. Understanding the communication mechanisms between microbe and host

can aid in therapeutic development. Several diseases such as cancer, Alzheimer's, and obesity have been identified to have unique RNA and miRNA profiles as markers of diagnosis, prognosis, and treatment [55,158,358–360]. The addition of microbial OMV-associated extracellular RNA (exRNA) will assist scientists in improving the library of known diagnostic and prognostic markers for disease, and aid in the development of more accurate and targeted therapies. The strong immunogenicity of OMVs makes them promising platforms for vaccine and drug development, with one OMV-based vaccine already in circulation (Bexsero) [263,361]. However, it is imperative to fully understand the molecular cargo of OMVs and the pathways they effect on host cells to ensure proper quality control prior to undertaking such development.

In addition to providing mechanistic insight and therapeutic options in humans, the applications of this research can extend to alternative organisms. The impacts of the microbiome on host health can lead to it use as a population control measures in pest species [243,362,363]. Mosquito genera such *Ades* and *Culex* are known vectors for a variety of diseases such as malaria, West Nile and dengue [364–366]. Certain genera of bacteria such as *Wolbachia* can infect mosquitoes, promote viral resistance in populations, and render them sterile, leading to colony collapse [367–370]. Understanding the interactions of *Wolbachia* with their hosts and the contribution of their OMVs and exRNA can allow us to harness such interactions to reduce the disease burden in vulnerable areas.

The scope of this work has contributed to our understanding of the underlying mechanism of host-microbe interactions. Further investigation is required to ensure a thorough understanding of the influence of OMV-delivered RNA in host responses. Research focusing on other RNA sensing mechanism such as cGAS, RIG-1 and NLRs can

shed light on the full range of RNA-host interactions. Investigating the capacity of OMV RNA fragments to act as eukaryotic miRNA can deepen our understanding of immune responses to OMV treatment. To this end, we will investigate such interactions by loading synthetic RNAs that have sequences identical to the RNA reads identified in Chapter 4, load them into nanoliposomes for subsequent administration to colorectal epithelial cells and test for changes in immune responses via qPCR and proteomic analysis. In line with these investigations, conducting experiments that help our understanding of the specific mechanisms that contribute to host responses to the presence of vesicles, such as generation of TLR KO models, or using inhibitors of endocytosis. While the epithelium represents the first line of interaction with OMVs, it is important to understand the effects of vesicles on other cell types such as B cells, T cells, neutrophils, and dendritic cells. As such investigation of the effect of OMVs and their RNA cargo can add to the appreciation of the vesicular-host interface. Finally, to apply the mechanistic understanding to a biologically relevant system, animal model studies will help in seeing the effects of OMVs and OMV RNA in a complex environment.

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