DIETARY MODULATION OF
HOST-MICROBE INTERACTIONS

By

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AIEC – adherent invasive *E. coli*

IBD – Inflammatory Bowel Disease

BMDM – Bone marrow-derived macrophage

CD – Crohn’s Disease

DNA – deoxyribonucleic acid

iNos – inducible nitric oxide

LAMP – Lysosomal associated membrane protein

MDX – Maltodextrin

MOI – multiplicity of infection

n.d. – not detected

UC – Ulcerative Colitis

Rab – ras protein found in the brain

ROS – reactive oxygen species

SCV – *Salmonella* containing vesicle

*spp* – species
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*One time there was a protein was supposed to be tagged but wasn’t really tagged….

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Dietary Modulation of Host-Microbe Interactions

Abstract
by
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Inflammatory bowel disease (IBD) is a chronic, debilitating intestinal disease affecting an estimated 1.3 million individuals in the United States alone, with worldwide incidence rapidly rising. Crohn’s Disease (CD), one subtype of IBD, is believed to arise in response to environmental priming resulting in inappropriate immune responses against commensal bacteria in a genetically susceptible individual. While the scientific community has made great progress in understanding the genetics and altered immune responses observed in CD patients, little is known about the environmental factors important in shaping disease.

Sugar consumption and artificial sweeteners, as well as a “Western” diet, are environmental factors associated with onset of CD. We broadly hypothesized that diet was driving changes in bacterial adhesion promoting disease onset. **We identified a polymer of glucose, maltodextrin (MDX), that modulates both bacterial interaction with the environment as well as cellular response to intracellular bacteria.** MDX was identified as a phenotype-enhancing compound after panels of *Escherichia coli* species were subjected to an array of polysaccharides. Strains of adherent-invasive *E. coli* have been isolated from inflamed lesions of CD patients, while changes in spatial organization of the CD microbiome results in a bacterial biofilm adherent to the
intestine epithelium. *E. coli* species exhibit significant increases in bacterial adhesion after exposure to MDX and isolation of bacterial DNA from ileal CD patients had higher levels of the gene *MalX*, a bacterial gene require for metabolism of MDX. These data suggests the polysaccharide MDX can promote *E. coli* adhesion and may influence bacterial populations in CD patients.

Genetic studies have identified clusters of CD-associated genes in antibacterial pathways; therefore we looked at the effect of cellular MDX in the context of intracellular bacterial clearance. Exposure of cells to MDX impaired intracellular *Salmonella* clearance through alterations in trafficking resulting in enhanced *Salmonella* viability. Similar results were observed in mice infected with *Salmonella*, 100-fold more viable bacteria recovered from the cecum of MDX fed mice. Our work identifies a role for the polysaccharide MDX in shaping host-microbial interactions which may be important for development of chronic disease states like CD.
Chapter 1. Introduction

Chronic, debilitating inflammation of the intestine is one of the hallmarks of inflammatory bowel disease (IBD). IBD is an incurable, lifelong disease responsible for an estimated 700,000 physician visits, 100,000 hospitalizations, and extensive disability per year (CDC IBD Article, Accessed 10/01/13). There are an estimated 1.3 million cases of IBD in the United States alone\textsuperscript{1}. IBD is a diagnosis representing a spectrum of inflammation in the gastrointestinal tract. Location, pattern and extent of inflammation distinguish two of the major subtypes of IBD, ulcerative colitis (UC) and Crohn’s disease (CD). IBD is hypothesized to arise after an environmental trigger or priming results in an inappropriate immune response to bacteria in a genetically susceptible host (Figure 1.1). Currently, little is known about what factors are required for disease onset, especially the environmental variables priming the intestine. Our studies identified the dietary polysaccharide, maltodextrin (MDX), as an important environmental factor modulating bacterial adhesion and antibacterial responses in a manner which may contribute to CD pathogenesis.

1.1 Inflammatory Bowel Disease

Chronic, persistent inflammation of the intestinal tract is a shared characteristic of the two main classifications of IBD: UC and CD. UC presents with ulceration of the colonic epithelium, with inflammation initiating near the rectum and progressing continuously towards the small intestine. While UC is
Figure 1.1. The prevailing hypothesis leading to IBD onset requires environmental priming leading to inappropriate immune response to commensal bacterial in a genetically susceptible host. Select hypothesized etiologies are illustrated in the periphery.
limited to the colon, CD can occur anywhere throughout the intestinal tract with most cases presenting in the terminal ileum or colon. CD has different features, presenting with discontinuous transmural ulcers, also called skip lesions, that alter the appearance of the mucosa to appear in a cobblestone pattern while histologically, tissue with active disease contains abundant infiltrating polymorphonuclear cells, dysplasia, distortion of crypt/villus architecture, and noncaseating granulomas\textsuperscript{2}. As a result of this inflammation, the intestine can form areas with stricturing (abnormal narrowing of the intestine) or fistulas, where inappropriate connections occur between the intestine and other parts of the body. Additionally, extra-intestinal manifestations of CD can include uveitis, episcleritis, pyoderma gangrenosum, and erythema nodosum\textsuperscript{3}.

It has been observed that the incidence of CD has been increasing over the past fifty years, with the highest incidences occurring in Northern Europe, United Kingdom and North America\textsuperscript{1}. Formerly low-incidence regions with currently rising incidence include the remainder of Europe, Asia, Africa and Latin America indicating the world-wide impact of disease. Amongst the North American population, incidence of CD is estimated from 3.1 to 14.6 cases per 100,000 persons per year\textsuperscript{4}. Other studies list worldwide incidences range from 10.6 per 100,000 person-years in the UK to 20.2 per 100,000 person-years in Canada and 29.3 per 100,000 person-years in Australia\textsuperscript{5}. In 56\% of CD studies there has been a reported increase in incidence since 1980 without any studies reporting significant decreases in CD incidence in the same time period\textsuperscript{5}. 
1.2 Consequences of CD Diagnosis, Clinical Course and Outcomes.

There is no known cause for CD, and there is no cure. A diagnosis of CD requires lifelong care, with current treatment plans aimed at relieving symptoms and maintaining the disease in remission. Diagnosis can occur at any age with the most frequent ages diagnosed between 15-40 years of age\textsuperscript{4,6}. As disease follows periods of remission punctuated by active disease flares, repeated treatment therapies are used which lead to treatment failure and surgery for an estimated 75% of CD patients\textsuperscript{7}. A patient will usually present with abdominal pain, diarrhea, nausea, vomiting and weight loss\textsuperscript{3}. CD diagnosis is based on endoscopic procedure which allows the physician to determine locations of the intestine affected by inflammation as well as macroscopic observation of mucosal integrity. A diagnosis of CD requires microscopic evaluation of the tissue biopsy, with emphasis on the presence of granulomas, dysplasia and transmural ulceration.

After initial diagnosis, the treatment goal is to resolve the current symptoms, approached medically through the use of steroids. Several considerations are made when determining treatment including location and severity of disease leading to the use of three main classes of therapeutics: corticosteroids, immune modulators and biologics\textsuperscript{3}. The steroid prescription is intended to be short-term, to relieve the symptoms of the current disease flare. Disease generally alternates between periods of remission and flares of active disease; with a successful treatment regimen maintaining the disease in
remission. Smoking exacerbates disease symptoms and patients are advised to quit. There are currently no dietary recommendations for patients.

With increasing CD prevalence, diagnosis usually occurring at a young age and with no cure for disease, the direct and indirect costs of a lifelong disease are staggering. Inability to work, doctors’ visits, medication, procedures and surgical intervention contribute to the costs for affected patients, amounting to an estimated $19,000 per patient per year. These costs demonstrate a significant need to study variables contributing to disease onset. While progress has been made to understand and target biological pathways in patients, most treatments alleviate symptoms without targeting underlying cause of disease.

1.3 Inappropriate immune responses and genetic susceptibility:

Initially, it was observed that CD clusters in families, with about a 25-35% concordance of CD in monozygotic twins, compared to a 10% concordance in heterozygotic twins. Within the past fifteen years, genome-wide association studies (GWAS) have identified over 169 genes associated with CD susceptibility which cluster in functional pathways, such as IL-1R signaling and antibacterial detection and response. The first identified susceptibility gene is NOD2, nucleotide-oligomerization domain 2, which encodes a protein responsible for the recognition of a conserved fragment of the bacterial cell wall. Several other CD-susceptibility genes, such as ATG16L1, have been identified as important in the recognition, sequestration and clearance of
Figure 1.3.1. A sampling of cells and genes involved in CD pathogenesis. Several genes have roles in multiple cell types, and disease associated variants can result in functional consequences.
intracellular pathogens\textsuperscript{15-18} through anti-bacterial autophagy. The genes associated with CD cluster in pathways, with many related to innate and adaptive immune functions, specifically antibacterial responses\textsuperscript{11}. An overview of genes associated with disease and their function is shown in [Figure 1.3.1]. Based on functional analysis of susceptibility genes, impaired bacterial clearance repeatedly appears as a fundamental paradigm in disease onset.

1.4 Bacteria:

Multiple lines of evidence indicate a role for commensal bacteria in disease onset. In the intestine, commensal microbes colonize a mucosal layer overlaying the epithelium at an increasing density progressing from the small intestine to the large intestine, the regions most frequently involved in IBD (Figure 1.4.1). Several studies have identified that commensal bacteria are important in disease, and greatly exacerbate disease severity. Multiple experimental animal models implicate a role for bacteria in pathogenesis\textsuperscript{19}, including a requirement for the presence of luminal commensal
Figure 1.4.1 Schematic of commensal microbes present throughout the human body. Note the increasing density in commensals progressing from the stomach to the colon.
bacteria for inflammation\textsuperscript{20}, increased mucosal association and subsequent translocation of bacteria\textsuperscript{21}, and use of antibiotics to prevent or treat disease\textsuperscript{22}, and defective alterations in antibacterial signaling leads to chronic intestinal inflammation\textsuperscript{23-26}. Furthermore, most germ-free animals are frequently protected against intestinal inflammation or immune activation, but lose their protection after commensal colonization in relevant models\textsuperscript{19}.

Many observations in patients reaffirm the role of commensal bacteria in CD onset; for example disease traditionally occurs in the areas of the intestine with the highest bacterial loads (terminal ileum and colon). Fecal stream diversion will alleviate symptoms and treats CD, but removal of the diversion will return the patient to an inflamed status\textsuperscript{27}. Additionally, altered microbial populations and subsequent microbial metabolic capacity\textsuperscript{28,29}, association of adherent-invasive \textit{E. coli}\textsuperscript{30}, mucosal attachment\textsuperscript{21}, invasion and translocation\textsuperscript{31} with some short term success with antibiotic treatments\textsuperscript{22} reinforces that commensal populations have shifted from a healthy symbiotic relationship to an unhealthy community perpetuating a chronic inflammatory state.

\textbf{1.4.1 Specific bacteria species hypothesized to be associated with disease:} Although the GWAS studies suggest that intracellular bacterial clearance is likely playing an important role in CD onset, no single pathogen has been identified as responsible for disease. A number of intracellular pathogens have been hypothesized to have a role in CD onset, notably \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}\textsuperscript{32}, the causative agent of Johne’s disease in cows\textsuperscript{33}. Johne’s disease is a granulomatous colitis that presents histologically
similarly to CD\textsuperscript{33}. However, analysis of granulomas from CD patients did not yield amplification of \textit{M. avium paratuberculosis} DNA\textsuperscript{34} challenging its hypothesized role in human disease. In boxer and French bull dogs, adherent-invasive \textit{E. coli}(AIEC) species have been isolated from dogs with granulomatous colitis\textsuperscript{35,36}. AIEC has been repeatedly isolated from inflamed lesions of patients with disease, though it is less clear if the bacteria is present at time of diagnosis or rather observed due to its ability to persist in an inflamed region\textsuperscript{30}. AIEC strains have also been isolated from healthy (non-CD) patients suggesting that it may colonize under normal conditions but act opportunistically during periods of inflammation. This theme has been repeatedly observed, as in the case of \textit{Bacteroides fragilis}\textsuperscript{21,37}, another commensal bacteria that is found with an expanded population in CD patients. These observations have prompted the idea of a “pathobiont”, a commensal bacteria under normal, healthy conditions that can act opportunistically during inflammation to expand its population\textsuperscript{38,39}.

\textbf{1.4.2 Commensal reorganization}

In addition to the hypothesis that specific pathogens that may be associated with disease, there is a striking alteration in overall microbial populations and spatial organization of the commensal population in the intestine of CD patients\textsuperscript{19,21}. Under “healthy” circumstances, the human body hosts an extensive community of commensal microorganisms, allowing their colonization adjacent to the epithelium of our gastrointestinal tract and skin\textsuperscript{40,41}. This microbial consortium, collectively referred to as a microbiome, provides numerous beneficial effects including maintenance of a physical barrier to
prevent pathogen access to human tissue, degradation of toxic compounds and drugs, training for immune surveillance, and nutrient harvest by metabolism of food products that eukaryotic cells lack the ability to metabolize\textsuperscript{40,41}. In CD patients, changes in microbiome populations (termed dysbiosis) include increased bacterial burden with frequent incidences of \textit{Clostridium difficile} and Enterobacteriaceae species, and reduced numbers of \textit{Faecalibacterium prausnitzii}, Bifidobacteria and Lactobacilli\textsuperscript{42}. Microbiome dysbiosis is observed after diagnosis of disease so it is unclear if the population imbalances observed are required for disease onset or rather a product of the inflammatory state. One challenge to understanding IBD pathogenesis is the inability to predict disease onset. Studies of the microbiome does not tell us if the changes in bacteria are causative or correlative with disease state. Increased \textit{E. coli} species have been identified in patients at time of diagnosis which suggests that \textit{E. coli} may be important in disease onset\textsuperscript{43}.

Analysis of the spatial organization of the intestinal microbiome demonstrated striking differences in the organization of the microbiota in CD patients relative to non-IBD controls. In a healthy small intestine, goblet cells secrete mucins which is responsible for a protective layer which overlays the intestinal epithelium\textsuperscript{44}. The inner layer, immediately adjacent to the epithelium, is constructed of tightly packed mucins that become digested and more loosely packed as you approach the lumen of the intestine\textsuperscript{44}. This loosely packed mucin
**Figure 1.4.2.** Schematic illustrating the commensal colonization strategy in a healthy intestine (top) and the changes that occur in a CD patient (bottom).
matrix creates a framework for microbial adhesion and bacterial colonization\textsuperscript{44}. In CD patients, Swidsinski et al. observed the formation of a biofilm of microorganisms directly adjacent to the epithelial surface\textsuperscript{21}, demonstrating a loss of the protective mucosa and an increased density of microbial antigens constantly barraging the epithelium (Figure 1.4.2). Furthermore, contained within the bacterial biofilms of CD patients were expansive communities of \textit{B. fragilis}, \textit{Eubacterium rectale} and \textit{E. coli}\textsuperscript{21,45,46}, both emphasizing and supporting the idea of opportunistic pathobionts in CD pathogenesis.

Microbiome analysis of patients with CD has shown altered bacterial populations, demonstrating decreased bacterial diversity and abundance of opportunistic pathogens, indicating potential areas of study needed to understand the change in microbiome dynamics from healthy symbiotic relationship to one that is instrumental in promoting chronic inflammation.

\textbf{1.5 Host-Microbe Interactions}

\textbf{1.5.1 Cell types}

The human body has multiple cell types capable of responding to invasive pathogens and the intestine structure is designed to protect from infection, but also to facilitate rapid response to offending pathogens. In the intestine, a single layer of epithelial cells lines the intestinal tract. Interspersed within the epithelial cells are specialized goblet cells that secret mucins to protect the epithelium, and M-cells, whose flat topology (due to the lack of microvilli) is
intended to entice pathogens to infect as well as allow sampling of the luminal microbes and microbial antigens. Dendritic cells extend pseudopods into the intestinal lumen to monitor the environment while macrophage cells are recruited to sites of infection. Adaptive immune cells are localized in regions called Peyer’s patches and isolated lymphoid follicles, where an activated T-cell can interact with a B-cell. One hallmark of CD is the formation of granulomas, or macrophages that have been recruited to clear an infection but have failed and remain clustered within the lamina propria. Both epithelial cells and macrophages are frequently exposed to infection by a number of pathogens.

1.5.2 Crohn’s disease associated adherent-invasive E. coli LF82.

One bacterium associated with CD is adherent-invasive E. coli, with its prototypic strain identified as “LF82”. It was initially isolated from an inflamed lesion of a patient with ileal CD. Similar strains with parallel adherent-invasive pathologies have since been isolated from ileal lesions of other CD patients. LF82 expresses several adhesins, notably long polar fimbriae or type-1-pili, which can bind surface molecules CEACAM6 and gp96. While AIEC has been isolated from several patients with IBD, it has also been isolated from healthy individuals thus indicating that it may act as a pathobiont; capable of capitalizing on inflammatory conditions instead of initiating inflammation. Several aspects of AIEC infection are reminiscent of CD pathologies. For example, AIEC replication in macrophages is dependent on macrophage-secretion of TNFα. Furthermore, in vitro AIEC infection of epithelial cells represses autophagy.
Figure 1.5.3. Schematic of Salmonella trafficking patterns observed in macrophage and epithelial cells.
through microRNA expression\textsuperscript{55-57}, demonstrating bacterial-driven impaired autophagy. As impaired autophagy is observed in patients with certain CD-associated risk genotypes, recapitulating the phenotype through bacterial virulence gene expression may demonstrate an alternate route to a common disease phenotype.

1.5.3 \textit{Salmonella typhimurium}

While not a CD-associated gastrointestinal pathogen, \textit{Salmonella} occurs as a repeating theme in autophagy research and subsequently CD-research and has been associated with infectious gastritis\textsuperscript{58,59}, hypothesized to contribute to IBD onset. Approximately 30% of intracellular \textit{Salmonella} is targeted by autophagy for clearance\textsuperscript{60}. Infection of macrophage or epithelial cells follows a predictable pattern (Figure 1.5.3) where \textit{Salmonella} facilitates entry of the epithelial cell (or is phagocytosed by the macrophage) and is trafficked to an early endosome marked by Rab5\textsuperscript{+} protein\textsuperscript{61}. Maturation of the early endosome is indicated by the acquisition of Rab7\textsuperscript{+}, a late endosome marker\textsuperscript{61}. At this point, virulence gene expression dictates if \textit{Salmonella} will damage the vesicle and escape to the cytoplasm, where it will be rapidly ubiquitinated, permitting binding of several adapter proteins which then facilitate the formation of an autophagosome about the bacterium\textsuperscript{62}. A damaged vesicle will also be targeted for autophagy; or alternatively the \textit{Salmonella} can remain within the late endosome, acquire markers of a lysosome but through virulence gene expression prevent the acidification of the vesicle rendering an environment permissive for replication called the \textit{Salmonella} containing vesicle (SCV). In
epithelial cells, *Salmonella* expression of *sif* genes will lead to long finger-like projections extending off of the SCV. Uncontrolled replication of *Salmonella* within the SCV leads to inflammasome activation and pyroptosis.\textsuperscript{63}

1.5.4 **Avoidance of infection and strategies for intracellular pathogen clearance by macrophages and epithelial cells.**

To prevent pathogen infection, the presence of a dense mucin layer and commensal population physically blocks any rogue pathogen from accessing the epithelial layer. Secreted antimicrobial peptides, immunoglobulins, compliment and other compounds act as guards present within the mucosal layer to immediately target bacteria that breach the mucosa.\textsuperscript{64} The first goal of an epithelial cell is to avoid infection, however, sometimes that strategy fails. Epithelial and macrophage cells respond to bacterial presence through recognition of bacterial ligands including (but not limited to) lipopolysaccharide, peptidoglycan fragments, and flagellin through activation of Toll-Like receptors and Nod-Like receptors that initiate signaling cascades to facilitate inflammatory responses, alert other cells of pathogen detection and finally to facilitate clearance of the intracellular bacterium itself. Several mechanisms are utilized for bacterial clearance including autophagy induction in which a vesicle containing a bacterium or the bacterium itself is targeted leading to formation of an autophagosome vesicle around the bacterium. This vesicle can then fuse with a lysosome to degrade the contents, killing the bacteria. Normal maturation of vesicles results in lysosomal fusion and bacterial killing and a complementary
system leads to generation of radicals (such as superoxide as a product of the NADPH-oxidase system) which effectively kill the pathogen.

1.6 Environmental priming:

In addition to the striking clustering of CD in families, there is a geographical association with disease. “Westernized” societies have higher incidence of disease; a phenomenon that trends with increased Westernization of the modern world\textsuperscript{65}. “Westernization” includes increased food preservation techniques, increased hygiene, sterilization and changes in dietary practice. Notably, as CD clusters in families, it is important to note that families also share environment—including transmissible commensal microorganisms and environmental exposures. Immigration is also a strong predictor of disease susceptibility. Should one immigrate from an area with a low incidence of CD to an area with a high risk of CD, they adopt the risk of the new area\textsuperscript{65}. The opposite is also true, further emphasizing the importance of environmental variables on disease onset\textsuperscript{65}.

Smoking is the most strongly associated environmental variable with divergent effects for UC and CD. First identified due to its negative correlation with UC in 1982, smoking was shown to have a protective effect in epidemiological studies reflecting lower rate of relapse or need for colectomy\textsuperscript{66}. Despite the bizarre beneficial effect for UC, smoking is a risk factor for CD; increasing the risk for disease development 2-fold, the risk of disease flares, surgical intervention and post-operative disease reoccurrence\textsuperscript{66}.
In addition to smoking, one important environmental variable contributing to disease onset is diet; however diet is much more challenging to study. Issues with prospective studies (such as long length of study easily spanning 30+ years, cost, inability to predict if participants will develop IBD), or retrospective studies (such as recall bias or subconscious changes in dietary habits) have resulted in very little progress in identifying common dietary risk factors. There has been limited success in identifying an association with a diet low in fiber, high in refined sugar, red meat, carbohydrates and fat to be correlated with CD\textsuperscript{67}. Our current understanding of CD onset involves environmental, genetic and immune variables, yet our understanding of how each component contributes to disease initiation is limited. Furthermore, our appreciation for environmental variables contributing to disease is limited to such a degree that there are currently no clinical recommendations for patients aside from avoiding smoking or consumption of foods that trigger symptoms. This dearth of knowledge demonstrates an immediate necessity for further research into the role of diet and environmental factors in CD.

1.7 Diet-Microbe interactions.

1.7.1 Interplay between diet and microbiome.

The microbiome is dynamic, responding to changes in diet and lifestyle. While originally believed to be a relatively static commensal community, recent studies have demonstrated that microbial diversity can change rapidly depending on diet. The relationship between diet and microbiome has been shown through
a number of elegant studies\textsuperscript{68-75} demonstrated how dietary components can shift bacterial populations and how disease phenotypes acquired by in commensal microorganisms. The mounting evidence demonstrates a role for diet microbiome interactions in disease onset. One clear example of this is the transfer of a diet-dependent obese phenotype through microbiome transplants\textsuperscript{75}. In these, mice were fed either a high fat diet or a control diet to induce an obese phenotype. The microbiome was harvested and transplanted into previously germ-free animals. The mice receiving the obese microbiome gained weight rapidly recapitulating the obese phenotype despite being maintained on the normal diet. It is surprising to note that maintenance on the normal diet was not enough to revert phenotype; the ability of the microbiome to contain the obese phenotype demonstrates that there is remarkable potential contained within our commensal communities.

1.7.2 Robust alteration are observed in the CD microbiome As mentioned briefly, there are consistent changes in speciation of the microbiome in CD patients in studies worldwide, suggesting that microbiome alterations may be important in driving disease pathogenesis. Specifically, loss of species diversity, including disappearance of \textit{Faecalibacterium} and \textit{Roseburia} (and an overall decrease in Firmicutes) with blooms of \textit{Ruminococcus gnavus} and Enterobacteriaceae\textsuperscript{76} (including AIEC) lead to overall increases in bacterial density, particularly in the case of ileal CD. Furthermore, decreases in \textit{Faecalibacterium prausnitzii} correlate strongly with reoccurrence of post-
operative CD and the bacterium is suggested to be anti-inflammatory in mouse models\textsuperscript{76}.

### 1.7.3 Diet effects on microbial populations.

Several modern diseases have significant microbial contributions to disease onset, including obesity and atherosclerosis. In both CD and obesity there are increases in the numbers of Enterobacteriaceae, specifically \textit{E. coli}; however in contrast to CD, there are decreases in \textit{Bacteroides}, increases in \textit{Firmicutes}\textsuperscript{77} in obese individuals. Combining a high fat/high sugar diet in mice expressing human CEACAM adhesion molecules resulted in decreased thickness of the mucus layer and increased AIEC adhesion\textsuperscript{13}. Metabolic capacity of the commensal microbiome reflects dietary consumption; and \textit{E. coli} spp are capable of metabolism of more compounds than more favorable small intestinal bacteria.

### 1.7.4 Food additives and bacterially-driven GI disease.

Recent studies have implicated dietary food additives in a number of bacterially-driven GI illnesses. Like MDX, these polysaccharides are generally recognized as safe by the Food and Drug Administration and include additives such as carboxymethyl cellulose (CMC), modified starches, carrageenan, pectin, and xanthan gum. However, a growing number of studies link these polysaccharides to intestinal disorders in both animals and humans\textsuperscript{78-83}. Most recently reported is the association of necrotizing enterocolitis with ingestion of xanthan gum by preterm infants\textsuperscript{80}. Many of these studies also demonstrate that polysaccharide additives alter the intestinal microbiota, both in the types and amounts of bacteria present. Incorporation of CMC into the diet of a genetically susceptible (IL-10 knockout)
mouse lead to severe bacterial overgrowth in the small bowel\textsuperscript{78}. CMC is added to foods as a thickener, texture enhancer and food stabilizer, similar to another frequently utilized food additive, MDX. Studies in pre-term pigs fed MDX-based forumula observed significantly more necrotizing enterocolitis, blunting of the intestinal villus and weight loss\textsuperscript{79}. As these dietary additives are frequently integrated into foods, a more careful examination of their effect on gastrointestinal pathologies is necessary.

1.8 Maltodextrin

MDX is a polymer of glucose ranging from 2-20 glucose units in length linked by $\alpha$1-4 or $\alpha$1-6 linkages. Dietary MDX has previously been recognized in bacterial driven gastrointestinal pathologies, specifically when integrated into the diet of preterm piglets causes severe small bowel pathology and necrotizing enterocolitis\textsuperscript{79}. MDX is produced though chemical or enzymatic digestion of starch or endogenously via degradation of glycogen in muscle or liver tissue. Dietary MDX is hydrolyzed in the small intestine\textsuperscript{84}. Based on its natural occurrence and endogenous availability, MDX is considered “generally recognized as safe” by the FDA (Code of Federal Regulations Title 21 Volume 3 Revised as of April 1, 2013 21CFR184.1444). Availability of MDX as a food additive began in roughly the 1950s\textsuperscript{85} and its availability correlates with CD-incidence (Figure 1.8.1). Due to its unique characteristics as a texture enhancer or binding agent, MDX is utilized in a number of food products, medications, hand lotions and makeup. Based on preliminary analysis of grocery store products, MDX is estimated to be in about 50% of food products.
Figure 1.8.1. Incidence of CD between 1945 and 1985 correlates with per capita availability of MDX.
1.9 Hypothesis

Critical review of the literature demonstrates increased prevalence of CD concurrent with Westernization of diet and lifestyle, complimenting basic science research observing changes in microbiome in patients with CD and a relationship between diet, environment and microbiome facilitated our hypothesis proposing the environmental factor, MDX, driving changes in host-microbial interactions, leading to altered microbial phenotypes and impaired microbial clearance. The multivariable complexity of CD has made identification of these variables challenging. Furthermore, the microbiome is dynamic, responding to changes in diet and environment.

These observations facilitated my hypothesis that dietary factors drive changes in host-microbial interactions important in CD pathogenesis. To test the hypothesis, I set out to identify dietary polysaccharides that alter microbial phenotypes with intent of mimicking patient-observed phenotypes in a controlled in vitro environment. As CD is a complex and multivariable disease, my first goal was to isolate variables that could be modeled in vitro but capture a clinically relevant phenotype. Given the relationship between diet and disease, I hypothesized that diet was selecting for changes in bacterial populations and promoting a biofilm-formation phenotype in CD-associated AIEC. To test this hypothesis, I modified bacterial growth media to assess a panel of polysaccharides and filler compounds; leading us to identification of MDX. In the first chapter I show that MDX, a ubiquitous dietary polysaccharide, dramatically enhances adhesion of the CD-associated AIEC LF82. Furthermore, a gene
required for microbial metabolism of MDX appears more frequently in adherent ileal bacteria from CD-patients suggesting a clinically relevant relationship between diet and bacterial colonization. Diet directly affects the commensal population and it is also important for mucosal health. The consortium of human cells present in the GI tract interacts with food products, largely to absorb any additional nutrients and to respond to food antigens. In chapter 3, I demonstrate how exposure of a number of different cell types to this dietary polysaccharide impairs clearance of an enteric pathogen through deregulated bacterial trafficking. Furthermore, we show that inclusion of MDX in a mouse diet exacerbates *Salmonella* infection. Together, these data demonstrate an important identification of an environmental factor capable of mediating alterations host-microbial interactions which may be important in CD disease pathology.

Chapter 2:

Crohn’s Disease-Associated Adherent-Invasive Escherichia coli Adhesion is Enhanced by Exposure to the Ubiquitous Dietary Polysaccharide Maltodextrin

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2.0 Abstract

Crohn’s disease (CD) is associated with intestinal dysbiosis evidenced by an altered microbiome forming thick biofilms on the epithelium. Additionally, adherent-invasive *E. coli* (AIEC) strains are frequently isolated from ileal lesions of CD patients indicating a potential role for these strains in disease pathogenesis. The composition and characteristics of the host microbiome are influenced by environmental factors, particularly diet. Polysaccharides added to food as emulsifiers, stabilizers or bulking agents have been linked to bacteria-associated intestinal disorders. The escalating consumption of polysaccharides in Western diets parallels an increased incidence of CD during the latter 20th century. In this study, the effect of a polysaccharide panel on adhesiveness of the CD-associated AIEC strain LF82 was analyzed to determine if these food additives promote disease-associated bacterial phenotypes. Maltodextrin (MDX), a polysaccharide derived from starch hydrolysis, markedly enhanced LF82 specific biofilm formation. Biofilm formation of multiple other *E. coli* strains was also promoted by MDX. MDX-induced *E. coli* biofilm formation was independent of polysaccharide chain length indicating a requirement for MDX metabolism. MDX exposure induced type I pili expression, which was required for MDX-enhanced biofilm formation. MDX also increased bacterial adhesion to human intestinal epithelial cell monolayers in a mechanism dependent on type 1 pili and independent of the cellular receptor CEACAM6, suggesting a novel mechanism of epithelial cell adhesion. Analysis of mucosa-associated bacteria from individuals with and without CD showed increased prevalence of *malX*, a gene
essential for MDX metabolism, uniquely in the ileum of CD patients. These findings demonstrate that the ubiquitous dietary component MDX enhances *E. coli* adhesion and suggests a mechanism by which Western diets rich in specific polysaccharides may promote dysbiosis of gut microbes and contribute to disease susceptibility.
2.1 Introduction

Crohn’s disease (CD) is a recurrent, debilitating, chronic inflammatory bowel disease with genetic, bacterial, and environmental factors contributing to disease pathogenesis. Extensive genetic studies have expanded our knowledge of predisposing factors, but indicate other risk factors are also important for CD development. Multiple studies indicate that bacteria are central to the onset and perpetuation of CD, as well as demonstrate alterations in the gut bacteria of individuals with CD. However, growing evidence indicates that environmental factors, such as smoking, diet and geography, may play key roles in disease development. Current disease models hypothesize that genetically susceptible individuals develop abnormal immune responses to bacteria in response to environmental stimuli, resulting in inflammatory bowel disease. Currently, it is unclear how environmental factors contribute to the development of disease.

Analyses of the bacterial communities associated with the intestinal mucosa or present in fecal samples of CD patients have identified specific alterations of the microbiome. These alterations include a decrease in microbial diversity, reduced representation of Firmicutes and increased levels of Proteobacteria. In particular, a lower amount of Faecalibacterium prausnitzii and a higher prevalence of Escherichia coli have been observed in the ileum of CD patients. These disease-associated E. coli have unique properties and are termed “adherent and invasive E. coli” (AIEC) for their ability to adhere and invade epithelial cells, as well as survive within macrophages. Interestingly,
these strains do not possess any of the classical virulence factor clusters common to other pathogenic *E. coli* strains\textsuperscript{94}.

Another striking change in the microbiota of CD patients is in the spatial organization of the microbiome. In healthy individuals, the microbiota is separated from direct contact with the intestinal epithelium, while in CD patients gut bacteria form a dense biofilm structure in intimate proximity with the epithelium\textsuperscript{21,46,95}. The factors which induce this dysbiosis are unclear, but genetics, lifestyle and a “Western” diet (a diet high in fats, sugar and protein but low in fiber) are all proposed to play a role\textsuperscript{96}.

Epidemiological studies show a striking increase in the incidence of CD since the 1950s in the United States\textsuperscript{1,97}. The reason for this explosion is unidentified, but data strongly support an environmental cause. During this time, the availability and popularity of pre-packaged foods escalated in the American diet\textsuperscript{98}. To improve the palatability and shelf life of packaged foods, polysaccharides are commonly added as emulsifiers, stabilizers, coating materials or bulking agents. These polysaccharides are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and include additives such as carboxymethyl cellulose (CMC), modified starches, carrageenan, pectin, and xanthan gum. However, a growing number of studies link these polysaccharides to intestinal disorders in both animals and humans\textsuperscript{46,79-83}. Most recently reported is the association of necrotizing enterocolitis with ingestion of xanthan gum by preterm infants\textsuperscript{80}. Many of these studies also demonstrate that
polysaccharide additives alter the intestinal microbiota, both in the types and amounts of bacteria present.

In light of evidence demonstrating that the composition of the gastrointestinal microbiota is responsive to changes in diet and the strong link between microbial dysbiosis and CD, we investigated whether a polysaccharide dietary additive, such as xanthan gum, CMC, mannitol, or modified starch, could promote CD-associated alterations in *E. coli* adhesion. The results of these experiments demonstrated that the modified starch, maltodextrin (MDX), markedly enhanced *E. coli* biofilm formation and epithelial cell adhesion. MDX is a polysaccharide comprised of α(1→4) and α(1→6) linked chains of 3-20 glucose units produced through the chemical and enzymatic hydrolysis of starch. Our findings demonstrate that MDX alters bacterial adhesion and support a model by which diet influences microbial phenotypes and may contribute to disease development.
2.2 Materials and Methods

2.2.1 Ethics Statement

The protocol (IRB#12-383) used to collect the human tissue samples used in this study was reviewed and approved by the Cleveland Clinic Institutional Review Board. This board determined that the collection and anonymous analysis of these de-identified, redundant surgical specimens was exempt from the requirement to obtain informed consent.

2.2.2 Patient Samples and Quantitative Real Time PCR

De-identified gastrointestinal tissue was collected from ileal and colonic resections by the Cleveland Clinic Tissue Procurement Service (IRB#12-383; Table S1). DNA was isolated from unblotted tissue using the Roche High Pure PCR Template Prep Kit for genomic DNA isolation. Real-time quantitative PCR (qPCR) was performed using 10ng DNA and primers for malX (5’ACGCGTTTTCCTTTGCAA3’/5’ACAGAACTGGCGCTACGA3’), E. coli 16S (5’CATGCGCGGTGTATGAAGAA3’/5’CGGGTAACGTCAATGAGCAAA3’)\(^{102}\) or Eubacteria (5’TCTTACGGGAGGCAGCAGT3’/5’GGACTACCAGGGGTATCTAATCCTGT3’)\(^{103}\) in iTaq SYBR Green Supermix with ROX (Biorad) on an ABI prism 7900HT using SDS2.4 software. Samples were run in triplicate with E. coli 16S and malX values normalized to Eubacteria levels using the equation: \(2^{\Delta C} = (C_{T_{malX}} - C_{T_{Eub}})\).
2.2.4 Biofilm formation assays

Specific biofilm formation assays were adapted from a microtiter plate protocol\(^\text{104}\). Briefly, M9 minimal medium (M9 Salts (BD Difco), 2mM MgSO\(_4\) (Acros), 100μM CaCl\(_2\) (Sigma)) supplemented with 0.4% sugars was prepared fresh the week of use. Glucose (Acros), MDX (Spectrum chemicals), fractionated MDXs, maltose, sucrose, D-mannitol, xanthan gum, cellulose and sucralose were purchased from Sigma. Aspartame was purchased from Supelco. “Measures like sugar” formulations of Splenda® and Equal®, as well as liquid Sweetleaf® stevia were purchased from a grocery store in Cleveland, Ohio. The final concentration of supplemental sugar (0.4% w/v) added to the minimal media was based on the estimated volume of the stomach (1L) and the serving size (0.5g) of the commercial products, Splenda® and Equal®. Overnight cultures of bacteria were grown in LB broth at 37°C without shaking. M9 medium was inoculated 1:100 and plated in triplicate in 96 well plates then incubated at 30°C for 24h. The OD\(_{600}\) of each well was measured, then wells were washed with phosphate buffered saline (PBS) and air dried for >20min. Wells were stained for 5min with 1% crystal violet, washed 4x dH\(_2\)O and air-dried overnight in the dark. Plates imaged using an Olympus 1X71 microscope with a 40x LucPlanFLN RC3 objective with complementary RC3 filter and a RTKE Diagnostic SPOT camera. Wells were then de-stained in 95% ethanol, OD\(_{540}\) measured and specific biofilm formation was calculated using the following equation: \[(\text{OD}_{540} - \text{medium OD}_{540})/\text{OD}_{600}\]. The exopolysaccharide matrix of 4% paraformaldehyde/PBS fixed
biofilms was stained with saturated Congo red overnight and bacteria counterstained with carbol fuscin. Growth curves were determined by measuring the OD$_{600}$ every 15min.

2.2.5 Scanning Electron Microscopy

Biofilms were grown on Thermanox® plastic coverslips (Nunc) for 24h at 30°C. Samples were washed with PBS and fixed in 4% paraformaldehyde (PF)/2.5% glutaraldehyde/PBS overnight at 4°C. After washing in PBS, samples were placed in 1% osmium tetroxide for 1h. Samples were washed with dH2O for 5 minutes, followed by dehydration in a graded series of ethanol (50%, 70%, 95% EtOH) with 3x10 minutes in absolute EtOH. Samples were dried 2x10 min in 100% EtOH:hexamethyldisilazane, 1:1 and 10 min in 100% hexamethyldisilazane x3 at RT. Samples were gold coated and examined with a JEOL Scanning Microscope (JSM 5310).

2.2.6 Type 1 Pili Expression Analysis

For analysis of the fim operon, biofilms were lysed using Roche High Pure PCR Template Preparation Kit for isolation of genomic DNA. Analysis of the fim operon was performed by PCR amplification of the fimA/E invertible element as described$^{105}$. 
2.2.7 Cell culture

HT29 cells (American Type Culture Collection (ATCC); gift of Carol de la Motte, Cleveland Clinic) were maintained in RPMI with 10% fetal bovine serum (FBS; Invitrogen). Caco2 cells (ATCC; gift of Lopamura Das, Case Western Reserve University) were maintained in DMEM with 20%FBS. Raw264.7 cells (ATCC; gift of Gabriel Nuñez, University of Michigan) were grown in DMEM with 10%FBS. Caco2:shCeacam6 and Caco2:shControl stable knockdown cell lines were generated by infection with MISSION shRNA lentiviruses (SHC002 and NM_002483.3-222s1c1, Sigma) followed by puromycin selection.

2.2.8 Cell adhesion assays

Infections of epithelial cell monolayers (multiplicity of infection (MOI)=10) were performed with 15-21d cultures of Caco2 cell lines, 5d cultures of HT29 cells or Raw264.7 cells plated the previous day. E. coli cultures were diluted in culture media for infection. For epithelial cell infections, wells were washed 2x PBS after 3h, and cells were harvested or media replaced for an additional 3h. For Raw264.7 cell infections, cells were washed 2x PBS and harvested 1h or 2h post-infection. At harvest, wells were washed 2x PBS, lysed in 0.1% Triton X-100/PBS (epithelial cells) or 1% Triton X-100/PBS (Raw264.7) and plated on LB plates in duplicate for calculation of colony forming units/well.
2.2.9 Cell invasion assays

HT29 monolayers and Raw264.7 cells were infected at an MOI of 10. After 2.5h and 5.5h infection of HT29 monolayers or 0.5h and 1.5h post-infection of Raw264.7 cells, cells were washed 2x PBS and media containing 100μg/mL gentamycin was added to the wells. Cells were incubated for an additional 30min, then cells were lysed in 0.1% Triton X-100/PBS (HT29) or 1% Triton X-100/PBS (Raw264.7) and plated on LB agar in duplicate for calculation of colony forming units/well.

2.2.10 Immunofluorescence

Cells infected with LF82 on glass coverslips were fixed in 4%PF/PBS (Electron Microscopy Services) then permeabilized with 0.4% Triton X-100/PBS, blocked in 2%FBS/PBS and stained with anti-*E.coli* antibody (ab20640, Abcam). Coverslips were incubated with goat anti-rabbit-Alexa488 (Invitrogen) in 2%FBS/PBS and mounted on slides with Vectashield+DAPI (Vector Labs). For visualization of intracellular LF82, cell cytoplasm was visualized by addition of Cell Tracker CMFDA (1μM final concentration, Molecular Probes) during infection. Cells were infected for 45min, then washed 2x PBS and then incubated for an additional 30min in media containing 100μg/mL gentamycin. Coverslips were stained for *E. coli* as described above. Confocal microscopy performed using a Leica TCS-SP spectral laser scanning confocal microscope equipped with a Q-Imaging Retiga EXi cooled CCD camera and Image ProPlus Capture and Analysis software.
(Media Cybernetics). The z-stacks (0.5μM step, line average of 4) were imported into Volocity (version 6.0.1) and analyzed using ‘isosurface’ with 1-4% black and 1-2x brightness.

2.2.11 Statistical Analyses

Figures are representative outcomes of a minimum of three independent experiments. Experiments were performed in triplicate and significance determined by ANOVA with post-hoc analysis using unpaired t-tests with equal variance. qPCR data was analyzed by non-parametric Wilcoxon test to accommodate the non-Gaussian distribution of the data set.
2.3 Results

2.3.1 Maltodextrin enhances biofilm formation of CD-associated *E. coli* in vitro. We investigated whether specific polysaccharides used as emulsifiers, thickeners, coating agents or stabilizers in processed foods influenced growth or adhesive characteristics of an *E. coli* strain associated with Crohn’s disease, AIEC LF82. The growth of LF82 in minimal medium supplemented with CMC, xanthan gum, MDX, or mannitol was assessed in comparison to sucrose or glucose over 24 hours. LF82 grew in all media formulations with the exception of media supplemented with CMC or sucrose (Figure 2.3.1).

As microbial communities in the intestine form biofilm structures with characteristics distinct from actively growing planktonic cultures, the effect of the polysaccharides on LF82 specific biofilm formation on plastic was measured after 24 hours. Specific biofilm formation was strikingly enhanced in medium containing MDX relative to glucose-supplemented medium and more modestly increased in mannitol-containing medium (Figure 2.3.1). This is in contrast to LF82 growth in medium supplemented with CMC or xanthan gum which did not form detectable biofilms. Media with sucrose did not significantly alter LF82 biofilm formation in comparison to glucose-supplemented medium biofilms. Biofilm formation was confirmed by microscopy in wells stained with Congo red to visualize exopolysaccharide matrix production, a hallmark of biofilm formation (Figure 2.3.1). These results indicate that MDX, a polysaccharide derived from starch hydrolysis, markedly promotes biofilm formation of the CD-associated LF82 *E. coli* strain.
Figure 2.3.1. MDX strongly enhances *E. coli* biofilm formation. (A) Growth curves of LF82 grown in medium supplemented with the indicated polysaccharide or sugar. (B) Specific biofilm formation of LF82. Average +/-SD shown. **p<0.001, n.d.=none detected. (C) Micrographs of LF82 biofilms from B stained with Congo red to detect exopolysaccharide formation (pink) with bacteria counterstained with carbol fusion (blue).
MDX is included as a bulking agent in the no-calorie sweeteners Equal® (aspartame) and Splenda® (sucralose). Using these commercial sources of MDX, the growth and biofilm formation of LF82 was assessed. LF82 grew robustly in media supplemented with Equal® or Splenda® (Figure 2.3.2) and specific biofilm formation was strikingly enhanced in medium containing Equal® or Splenda® relative to glucose-supplemented medium (Figure 2.3.2). The effect of replacing MDX with glucose as a filler component for aspartame or sucralose was also tested in specific biofilm formation assays. No increases in biofilm formation were observed in glucose-containing medium supplemented with aspartame or sucralose (Figure 2.3.2). Likewise, addition of the artificial sweeteners to MDX-containing medium did not further increase biofilm formation over the levels observed in medium supplemented only with MDX. These findings indicate that MDX found in commercial sources can stimulate LF82 biofilm formation.

2.3.2 MDX promotes biofilm formation of multiple *E. coli* strains in a process dependent on MDX metabolism. MDX is utilized by bacteria through a conserved bacterial maltose/MDX metabolic system suggesting that MDX enhancement of biofilm formation may be a general characteristic of *E. coli*. A panel of *E. coli* strains including laboratory reference strains, additional AIEC strains, clinical isolates from individuals without inflammatory bowel disease and the probiotic *E. coli* Nissle 1917 (Table 2.1) were evaluated for growth and biofilm formation in medium supplemented with glucose or MDX. A majority of the strains tested (75%) showed a significant increase in MDX-stimulated specific biofilm formation relative to Table 2.1. to glucose-supplemented medium (Figure
2.3.3). These findings suggest that MDX affects a wide variety of *E. coli* strains and that this is not a unique feature of disease-associated strains.

**Table 2.1: Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>K-12 derivative, weak biofilm former</td>
<td>ATCC</td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12 derivative, strong biofilm former</td>
<td>ATCC</td>
</tr>
<tr>
<td>ECG023</td>
<td>Non-IBD control isolate, Serotype ONT:H-, Phylotype At, <em>iucD, fimH, fimAvMT78</em>, weak biofilm former</td>
<td>50</td>
</tr>
<tr>
<td>ECG043</td>
<td>Non-IBD control isolate, Serotype O83:H1, Phylotype B2, <em>ibeA, fimH, fimAvMT78</em>, strong biofilm former</td>
<td>50</td>
</tr>
<tr>
<td>AIEC07</td>
<td>Non-IBD AIEC isolate, Serotype O22:H7, Phylotype B1, <em>papC, iucD, fimH</em>, strong biofilm former</td>
<td>50</td>
</tr>
<tr>
<td>LF82</td>
<td>AIEC isolated from an ileal lesion of a CD patient, Serotype O83:H1</td>
<td>30</td>
</tr>
<tr>
<td>LF82Δ<em>fliC</em></td>
<td>Isogenic LF82 with <em>fliC</em> deletion, KanR</td>
<td>107</td>
</tr>
<tr>
<td>LF82Δ<em>fimH</em></td>
<td>Isogenic LF82 with <em>fimH</em> deletion, KanR</td>
<td>108</td>
</tr>
<tr>
<td>Nissle 1917</td>
<td>Fecal isolate, MutafloR® probiotic</td>
<td>Ardeypharm</td>
</tr>
</tbody>
</table>
Figure 2.3.2. MDX included as a bulking agent in non-calorie sweeteners enhances biofilm formation. (A) Growth of LF82 in medium supplemented with the indicated sweetener. (B) Specific biofilm formation of LF82. Average +/-SD shown. *p<0.05, **p<0.01. (C) Micrographs of biofilms from B stained with either crystal violet to detect adhered bacteria or Congo red to visualize the exopolysaccharide matrix. (D) Effect of aspartame or sucralose on biofilm formation of LF82. Average +/-SD shown. **p<0.01 € Micrographs of crystal violet stained biofilms from D.
MDX is a glucose polymer between 3-20 glucose units. The length of these polymers is defined by dextrose equivalent (DE), with longer chains having lower DE values\textsuperscript{101}. In studies of other carbohydrates, it has been demonstrated that chain length dramatically alters the biological effect of the carbohydrate polymers\textsuperscript{109}. Since the MDX tested is a heterogeneous mix of chain lengths, the effectiveness of different size MDXs to stimulate biofilm formation was investigated to determine whether a specific size range of MDX was required for this effect. All MDX-supplemented media, regardless of chain length, were sufficient to increase LF82 biofilm formation relative to glucose-supplemented medium in specific biofilm formation assays and confirmed by staining of biofilms with crystal violet or Congo red (Figure 2.3.3 and Figure 2.3.4). While all MDX-supplemented media promoted biofilm formation, longer MDX chains were more effective, suggesting that metabolism of MDX may be important for biofilm enhancement.

Addition of long polymers to solutions can alter osmolarity. Biofilm formation and LF82 adhesion to epithelial cells have been demonstrated to be influenced by changes in osmolarity\textsuperscript{110,111}. To evaluate whether MDX supplementation altered the osmotic strength of the medium, we measured the osmolarity of the media used in our assays by freezing-point osmometry. Minor changes in osmolarity (less than 20mOsm/kg) were observed in medium supplemented with either the heterogeneous mix of DEs (Mixed) or 4-7 DE MDX, while no change in osmolarity was detected in medium containing either 13-17 DE or 16.5-19.5 DE MDXs (data not shown). However, all MDX-containing media promoted biofilm
Figure 2.3.3. MDX promotes biofilm formation of multiple *E. coli* strains in a process dependent on MDX metabolism.  (A) Specific biofilm formation of a panel of *E. coli* strains.  (B) Micrographs of crystal violet stained biofilms from A.  (C) Specific biofilm formation of LF82 in medium supplemented with MDX of different chain lengths.  (D) Micrographs of crystal violet stained biofilms from C.  Average +/- SD shown.  *p<0.05, **p<0.01, n.d.=none detected.
Figure 2.3.4. Effect of different MDX chain lengths on LF82 growth and biofilm formation. (A) Growth curves of LF82 in M9 medium supplemented with the indicated sugar. (B) Micrographs of Congo red stained LF82 biofilms formed in medium supplemented with the indicated sugar for 24h.
formation, indicating that osmolarity changes are not required for MDX to induce biofilm formation of *E. coli*.

**2.3.3 MDX promotes type 1 pili expression which is required for enhanced biofilm formation.** To identify candidate bacterial adhesins responsible for MDX-mediated biofilm formation, LF82 biofilms were visualized by scanning electron microscopy (SEM). At low magnification, we observed enhanced adherence of LF82 in MDX-containing medium to the coverslip, as well as to other bacteria (Figure 2.3.5). At higher magnification, an increase in short, thin, hair-like projections ranging between 0.5μm to 1.5μm in length could be seen protruding from the surface of LF82 grown in medium supplemented with MDX. One adhesin with these characteristics is type 1 pili, which has been described as a major adhesive structure of LF82\textsuperscript{52,93}. Expression of type 1 pili is regulated by an invertible DNA element in the *fim* operon of the bacterial genome. To confirm the expression of type 1 pili by MDX, we assessed the position of the *fim* operon of LF82 in 24h biofilms by PCR (Figure 2.3.5). The adherent bacteria in glucose-containing medium were found to be a mixed population of type 1 pili expressors and non-expressors, similar to what was observed by SEM. In contrast, the bacteria in MDX supplemented medium were more homogenous with the *fim* invertible element in the “on” position, suggesting that type 1 pili are adhesins upregulated by LF82 when grown in MDX-containing medium. Next, we examined whether type 1 pili were functionally required for MDX-enhanced LF82 biofilm formation.
Figure 2.3.5. MDX increases biofilm formation via type 1 pili. (A) Scanning electron micrographs of LF82 biofilms. Arrowheads indicate bacteria-plastic (white) or inter-bacterial (black) adhesins. (B) Assessment of the LF82 fim operon by PCR to determine type 1 pili expression.
**Figure 2.3.6.** MDX increases biofilm formation via type 1 pili (A) Specific biofilm formation of LF82 in the presence or absence of 2% mannose. (B) Micrographs of crystal violet stained biofilms from C. (C) Specific biofilm formation of LF82 and isogenic mutant strains. (D) Micrographs of crystal violet stained biofilms from E. Average ±SD shown. 
*p<0.05, **p<0.01
Type 1 pili attach to cells and surfaces in a mannose-sensitive manner dependent on the tip protein FimH\textsuperscript{52,93}. Competition with mannose abrogated specific biofilm formation by LF82 in either glucose or MDX supplemented medium (Figure 2.3.6), indicating that MDX-enhanced biofilm formation is due to an upregulation of a mannose-sensitive adhesin. We confirmed this adhesin to be type 1 pili through the evaluation of isogenic mutant strains of LF82. A type 1 pili adhesin \textit{fimH} knockout strain (\textit{ΔfimH}) formed biofilms that were unaffected by the addition of MDX, whereas a flagellin knockout strain (\textit{ΔfliC}) increased biofilm formation in MDX-containing medium (Figure 2.3.6). Taken together, this data demonstrates that MDX increases biofilm formation through the upregulation of type 1 pili expression.

\textbf{2.3.4 MDX enhances LF82 adhesion to intestinal epithelial cells and macrophages, but does not promote invasion.} LF82 has been shown to adhere and invade intestinal epithelial cells\textsuperscript{52,93}. We assessed whether growth in MDX promoted adhesion and invasion of LF82 to human intestinal epithelial cell lines (Caco2 and HT29) and a macrophage cell line (Raw264.7). LF82 were grown overnight in medium supplemented with either glucose or MDX. These bacteria were brought into early log phase of growth in fresh medium for infection. To assess adhesion, the total number of adherent bacteria was quantified after 3 or 6 hours by colony counts and visualized by confocal
**Figure 2.3.7.** MDX selectively enhances LF82 adhesion to intestinal epithelial cell lines.  
(A) Adhesion of LF82 to Caco2 monolayers.  
(B) Immunofluorescent confocal micrographs of LF82 adhered to Caco2 monolayers. Green=LF82, blue=nuclei  
(C) Adhesion of LF82 to HT29 monolayers.  
(D) Immunofluorescent confocal micrographs of LF82 adhered to HT29 monolayers. Green=LF82, blue=nuclei  
(E) Intracellular LF82 recovered from HT29 monolayers.  
(F) Immunofluorescent confocal micrographs demonstrating the localization of LF82 (red) on the surface of HT29 cells (green). Nuclei = blue. Average +/-SD shown. *p<0.05, **p<0.01 relative to glucose.
microscopy. Growth in MDX supplemented media increased the number of LF82 adhered to intestinal epithelial cell monolayers at both timepoints in comparison to LF82 from glucose-containing medium (Figure 2.3.7). Similar results were observed in infections of Raw264.7 macrophages, with both quantitative and visual measures of adherence demonstrating increased LF82 adhesion when the bacteria were grown in MDX (Figure 2.3.7).

In addition to LF82 adhesion, invasion of both HT29 and Raw264.7 cells was assessed by gentamycin protection assay and confocal microscopy. The total number of intracellular LF82 in HT29 epithelial cells was unaffected by MDX (Figure 2.3.7). In contrast, we observed increased numbers of intracellular MDX grown LF82 in Raw264.7 macrophages (Figures 2.3.8). The amount of intracellular LF82 was proportional to the number of LF82 adhered to the macrophages. Therefore, we postulate that the increased intracellular load of MDX grown LF82 in the macrophages is likely due to phagocytosis of a higher number of bacteria adhered to the surface of the cells. These findings indicate that MDX has a significant effect on LF82 adhesion to cells but does not increase the invasiveness of this strain.

2.3.5 LF82 adhesion to intestinal epithelial cells is enhanced by MDX in a type 1 pili dependent manner. LF82 has been shown to adhere to intestinal epithelial cells in a type 1 pili-dependent manner. To determine the role of type 1 pili in the enhanced adhesion of LF82 by MDX, binding of LF82 to cells
Figure 2.3.8. MDX selectively enhances LF82 adhesion to Raw264.7 macrophages. (A) Total amount of Raw264.7 cell-associated LF82. (B) Intracellular LF82 recovered from Raw264.7 cells. (C) Immunofluorescent confocal micrographs of LF82 (red) infected Raw264.7 cells (green). Nuclei = blue. Average +/-SD shown. **p<0.01, ***p<0.001 relative to glucose.
was competed by addition of mannose during infection, suggesting that LF82 adhesion may be mediated by type 1 pili (Figure 2.3.9). The requirement of type 1 pili for MDX enhancement of LF82 cell adhesion was confirmed by infection studies with the ΔfliC and ΔfimH LF82 mutant strains. In agreement with previous studies, overall adhesion to epithelial cells of both the ΔfliC and ΔfimH strains was significantly reduced (~2 logs) relative to the wild-type strain (Figure 2.3.9). However, while adhesion of the ΔfliC mutant strain was enhanced by exposure to MDX, there was no effect of MDX on the adhesion of the ΔfimH strain. Together this data demonstrates that the increased type 1 pili expression by LF82 grown in MDX not only enhances biofilm formation, but also increases adhesion to human intestinal epithelial cell monolayers.

2.3.6 MDX-enhanced LF82 adhesion to intestinal epithelial cells is independent of CEACAM6. Previous studies demonstrate that LF82 adhesion to ileal enterocytes from CD patients and colonization of the mouse intestine requires the binding of type 1 pili to the cellular receptor CEACAM6. Surprisingly, when we evaluated the expression levels of CEACAM6 by immunoblot of the intestinal cell lines used in our experiments, the HT29 cell line had no detectible CEACAM6 expression (Figure 2.3.10). This suggested that MDX-enhanced adhesion of LF82 was independent of CEACAM6. To confirm this finding, we generated Caco2 cell lines with the endogenous CEACAM6 expression stably knocked down by RNAi (Figure 2.3.10). In infection assays, loss of CEACAM6 expression did not alter the number of either glucose- or MDX-
Figure 2.3.9. MDX enhances epithelial cell adhesion in a type 1 pili-dependent manner. (A) Adhesion of LF82 to HT29 monolayers pre-incubated with 2% mannose. (B) Adhesion of LF82 isogenic mutants to HT29 monolayers. Average ± SD shown. *p<0.05, **p<0.01 relative to glucose.
Figure 2.3.10. MDX-enhanced LF82 adhesion to epithelial cells occurs via a mechanism independent of CEACAM6. (A) Immunoblots of CEACAM6 expression. (B) Adhesion of LF82 to Caco2 cell lines stably expressing shRNAs. Average +/- SD shown. **p<0.01, ***p<0.001 relative to glucose. (C) Immunoblots of CEACAM6 expression. (D) Immunofluorescent confocal micrographs of LF82 adhered to Caco2 cells used in B. Green=LF82, blue=nuclei.
exposed LF82 adhering to Caco2 monolayers, as quantitated by colony counts or visualized by confocal microscopy (Figure 2.3.10). These findings indicate that MDX promotes adhesion to intestinal epithelial cells via a mechanism independent of CEACAM6.

2.3.7 Bacteria with genes for MDX metabolism are more prevalent in the ileal mucosa of CD patients. AIEC strains have been isolated from CD patients with ileal disease and are implicated in CD pathogenesis\textsuperscript{30,49,89}. A recent study observed that these AIEC strains are present at the time of disease diagnosis and these strains were associated with carriage of a virulence factor, \textit{malX}\textsuperscript{43}. \textit{MalX} is a MDX-binding component of the maltose/MDX metabolism system\textsuperscript{113}. As MDX-grown LF82 form robust biofilms, we hypothesized that MDX metabolism may be beneficial for colonization of \textit{E. coli} in the terminal ileum of CD patients (patient characteristics listed in table 2.2). We designed real-time quantitative PCR (qPCR) primers for amplification of \textit{malX} to determine if bacteria with MDX metabolism genes were more prevalent in CD patient mucosal samples (Figure 2.3.11). DNA samples from intestinal mucosa were analyzed by qPCR for the presence of \textit{malX} and \textit{E. coli} 16S DNA normalized to total levels of \textit{Eubacteria} DNA. The normalization of these genes relative to total \textit{Eubacteria} levels allowed us to determine if the changes we observed were due to population shifts and not to overall increases in \textit{Eubacteria} levels that have been reported in CD patients\textsuperscript{45}. Specifically in ileal samples, we observed increased levels and prevalence of the \textit{malX} gene in CD patients as compared to controls.
(18% positive in ileal controls vs. 71% positive in ileal CD) (Figure 2.3.12). The prevalence of *E. coli* 16S DNA was not increased in these samples, indicating that *malX* is not a marker for *E. coli* (Figure 2.3.12). These findings suggest a link between MDX utilization and microbial changes in ileal CD.

**Table 2.2: Characteristics of Tissue Donors**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tissue</th>
<th>Median Age (Range)</th>
<th>Gender</th>
<th>Diagnoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Ileum</td>
<td>50 (24-78)</td>
<td>6/10 F 4/10 M</td>
<td>Colon cancer, familial figadenomatous polyposis, tumor, diverticular disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Ileum</td>
<td>34.9 (17-76)</td>
<td>9/18 F 9/18 M</td>
<td>Crohn's Disease</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>32 (22-67)</td>
<td>8/16 F 8/16 M</td>
<td>Crohn's Disease</td>
</tr>
</tbody>
</table>
Identification of *malX* Bacterial Strains for Analysis

Search of NCBI Gene database using “malX” as search term

133 *malX* entries found

Eliminated incomplete sequences & pseudogenes

63 *malX* genes from proteobacteria species

ClustlW2 alignment of enterobacteria *malX* genes

Eliminated sequences with limited identity & non-human pathogens (i.e. fish or plant pathogens)

Identification of 36 enterobacteria strains with *malX* genes

**Enterobacteria *malX* gene sequence species distribution**

- E. coli spp
- Shigella spp
- Y. enterocolitica
- Salmonella spp
- Klebsiella spp
- C. rodentium
Figure 2.3.11. Identification strategy for *malX*. bacterial strains to be analyzed by quantitative PCR in human mucosal samples. NCBI lists 63 proteobacteria species with gene sequences specific for *malX*. Excluding discontinued sequences and partial sequences, the remaining sequences were aligned using ClustlW2. Further sequences were eliminated if they lacked significant identity or were sequences from non-human pathogens for a final strain count of 36 gene sequences. The aligned sequence was used to generate a consensuses sequence which was then entered into the BiBiServ GeneFisher2 software. Parameters for primer design were length between 15 to 18bp, GC content of 45-65%, melting temperature between 57-63°C and a product size between 50 and 200bp. Candidate primer sets were also evaluated for possible amplification of the human genome. The primers selected were 5’ACGCGTTTCTTTTCGCAA3’ and 5’ACAGAACTGGCGCTACGA3’.
Figure 2.3.12. Bacteria with the malX gene are more prevalent in the mucosa of ileal CD. (A) Prevalence of the malX gene normalized to total Eubacterial DNA (Eub) amplified from mucosal samples by qPCR. (B) Prevalence of E. coli 16S DNA (E. coli) as measured in A. Mean indicated with bar. *p<0.0175
2.4 Discussion

The pivotal contribution of the intestinal microbiome to both health and disease is becoming increasingly appreciated through studies defining this “organ” and how it is altered in disease states\textsuperscript{73}. The large microbial community residing in the intestine provides protection against pathogens, aids in digestion and absorption of nutrients, production of micronutrients, neutralization of toxins, and the shaping of the immune system. Alterations in the composition or function of this microbial ecosystem has been demonstrated in diseases such as athlerosclerosis, obesity, metabolic syndrome, allergy, diabetes and inflammatory bowel disease\textsuperscript{114}. Therefore, elucidating factors which modulate the microbiota are of importance in understanding disease pathogenesis.

In the case of CD, pathogenesis is linked to intestinal dysbiosis characterized by an increase in total numbers of bacteria, a reduced diversity of bacterial species, and alterations in the spatial organization of the microbiome\textsuperscript{49}. Specifically, the concentration of bacteria in biofilms adhered to the intestinal epithelium of CD patients are 2 logs higher than found in controls\textsuperscript{21}. Additionally, ileal CD is associated with increased prevalence of \textit{E. coli} strains, including AIEC strains such as LF82\textsuperscript{30}. These AIEC strains have been identified in individuals newly diagnosed with CD\textsuperscript{43}, but because of their low prevalence (~6\%) in healthy individuals\textsuperscript{30} are not thought to be pathogens but rather pathobionts - opportunistic bacteria which under certain circumstances can promote disease\textsuperscript{115}. CD is a complex and multi-factorial disease with genetic, bacterial, and environmental factors contributing to disease pathogenesis\textsuperscript{1}. The factors that
contribute to changes in the microbiome of CD patients are not well understood. Our findings demonstrating increased adhesiveness and biofilm formation of *E. coli* grown in MDX, but not invasion, would suggest that exposure to MDX may be one factor which promotes the colonization of pathobionts, such as AIEC LF82.

One factor which clearly influences the composition and characteristics of the microbiota is diet. Dietary studies in both mouse models and humans demonstrate large shifts in the composition of the microbiota dependent on diet\(^68,70,96,99\). Comparisons of 16S rRNA gene profiles between mice harboring a humanized microbiota and fed a high-fat/high-sugar diet ("Western diet") versus those maintained on a low-fat/high polysaccharide diet revealed shifts in *Bacteroidetes* and an expansion of *Bacilli* and *Erysipelotrichi*\(^68\). Likewise, human studies comparing obese and lean twin pairs demonstrated changes in *Bacteroidetes* prevalence and a decrease in microbial diversity in obese individuals\(^68\).

Diet also causes alterations in the types of genes present in microbial communities and influences their metabolism. Consumption of a Western diet enriches nutrient processing genes, such as ATP-binding cassette (ABC) transporters and phosphotransferase systems (PTS)\(^68\). Interestingly, genes involved in MDX metabolism are in both these categories\(^116\). Notably, we show *malX* (a PTS gene of the maltose/MDX system) is more prevalent in the ileal mucosa-associated bacteria of CD patients. These findings suggest that MDX
metabolism may be associated with the dysbiosis observed in CD and other diseases linked to Western diet consumption.

MDX is a d-glucose polymer under 20DE linked primarily by α(1→4) bonds produced through the enzymatic and chemical degradation of corn, potato or rice starch. Since its introduction in the 1950s, it has been added to a variety of foods and health care products to improve texture as a thickener, filler or binding agent. These MDX-containing products are diverse and include items such as non-calorie sweeteners (such as Splenda® and Equal®), snack foods, breakfast cereals, salad dressings, fiber supplements, hand lotions and medications. MDX is generally recognized as safe by the FDA (Code of Federal Regulations, Title 21, Volume 3, Part 184, Subpart B, Section 184.1444) and consumption of MDX is unlikely to be sufficient to cause disease in the absence of other risk factors. However, reports demonstrate that consumption of MDX or other polysaccharide additives under certain circumstances may result in intestinal disease. Specifically, studies associate induction of necrotizing enterocolitis in preterm piglets fed MDX-supplemented formula and the production of diarrheal enterotoxin in infant milk-based formula made with MDX by *Bacillus cereus*. Additionally, the ubiquitous inclusion of MDX into foods of the American diet parallels a substantial increase in incidence of CD. Our findings demonstrate that MDX enhances bacterial adhesion and suggests a mechanism by which consumption of this ubiquitous dietary additive may promote disease in susceptible individuals.
MDX is metabolized in the small intestine by specific enzymes\textsuperscript{118,119}. Interestingly, one of these enzymes (maltase-glucoamylase) is inhibited by high levels of MDX, suggesting that a MDX-rich diet could result in increased MDX levels in the small intestine and enrichment of MDX-utilizing bacteria in this location. In our studies, we observed MDX-enhanced biofilm formation by multiple strains of \textit{E. coli} suggesting that MDX metabolism may be a dietary switch promoting colonization of these strains in new regions of the intestine (i.e. the ileum instead of the colon). Additionally, our data indicates that MDX-enhanced adhesion of LF82 is dependent on type 1 pili, but independent of the cellular receptor CEACAM6. This contrasts with studies defining CEACAM6 as a major cellular receptor for LF82 adhesion on CD patient enterocytes\textsuperscript{52}. However, ileal epithelial expression of CEACAM6 is increased by LF82 infection, as well as pro-inflammatory cytokines. RNAi-mediated knockdown of CEACAM6 studies by Barnich et al., 2007 were performed with either stimulated Caco2 cells or CD patient-derived enterocytes and demonstrated a significant decrease in LF82 adhesion\textsuperscript{52}. In our experiments, CEACAM6 knockdown did not affect LF82 adhesion to unstimulated Caco2 cells and this agrees with the authors’ findings where no correlation between the basal levels of CEACAM6 expression in various cell lines and LF82 adhesion levels was observed. Of current discussion is whether the high levels of CEACAM6 expression seen in CD patients are present before disease onset (promoting disease susceptibility) or a consequence of disease pathogenesis (promoting disease progression)\textsuperscript{52}. Our findings suggest a dietary mechanism by which LF82 could initially adhere to the
epithelium and then induce the expression of CEACAM6 as a secondary event to maintain its colonization of the ileum and promote disease.

Finally, although there is a strong link between AIEC strains and CD pathogenesis, no one bacterial strain has been causatively linked to CD\textsuperscript{49}. Other candidates include \textit{Mycobacterium avium} subspecies \textit{paratuberculosis}, \textit{Yersinia enterocolitica}, \textit{Listeria monocytogenes}, \textit{Salmonella} spp., \textit{Clostridium difficile}, \textit{Enterococcus faecalis}, and \textit{Campylobacter} spp\textsuperscript{120}. Interestingly, several of these disease-associated strains possess the \textit{malX} gene or have homologous systems for MDX utilization (Figure 2.3.11). This observation suggests that MDX metabolism may promote colonization of multiple CD-associated bacteria and is supported by our findings that \textit{malX} prevalence is significantly increased in ileal CD patient mucosa in the absence of increased \textit{E. coli} colonization. This leads us to postulate that consumption of MDX could increase bacterial loads in the ileum and prime these individuals to have a greater translocation of bacteria after intestinal injury. If these individuals carry other risk factors for CD (genetic variants of anti-bacterial response genes such as \textit{ATG16L1} or \textit{NOD2}, for example), this may result in the development of disease in these susceptible individuals. These findings describe a potential disease mechanism linking the ubiquitous dietary additive MDX to microbial changes in the intestine of CD patients and suggest a novel therapeutic area for the prevention and treatment of inflammatory bowel disease.
2.5 Acknowledgments

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Chapter 3:

The dietary polysaccharide maltodextrin promotes the intracellular survival of the enteric pathogen Salmonella

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3.0 Abstract

In the latter half of the 20th century, societal and technological changes have led to a shift in the composition of the American diet to include a greater proportion of processed, pre-packaged foods high in fat and carbohydrates with low dietary fiber (a “Western diet”). Over the same time period, there have been parallel increases in *Salmonella* gastroenteritis cases and a broad range of chronic inflammatory diseases. Several polysaccharide food additives have been linked to bacterially-driven intestinal inflammation and may contribute to the pathogenic effects of a Western diet. Therefore, we examined the effect of a ubiquitous polysaccharide food additive, maltodextrin (MDX), on clearance of the enteric pathogen *Salmonella* using both *in vitro* and *in vivo* infection models. When examined *in vitro*, macrophages exposed to MDX had defects in reactive oxygen species generation and altered vesicular trafficking that resulted in persistence of *Salmonella* in enlarged Rab7+ late endosomal vesicles. Enrichment of large Rab7+ vesicles was also observed in lamina propria cells of MDX-supplemented mice infected with *Salmonella*. Furthermore, these mice had higher cecal *Salmonella* loads and increased systemic inflammation. These findings suggest a link between consumption of processed foods containing the polysaccharide MDX with an increased risk for enteric infection.
3.1 Introduction

The composition of the American diet shifted dramatically in the latter half of the 20th century to include a greater proportion of processed, pre-packaged foods high in fat and carbohydrates with low dietary fiber (a "Western diet"). Over the same time period, a steady increase in non-typhoidal salmonellosis has been reported and *Salmonella* spp. are now the most common cause of foodborne bacterial disease outbreaks in the United States 121. Concurrently, there have been increases in a broad range of chronic inflammatory diseases, such as inflammatory bowel disease, diabetes, asthma, and atherosclerosis 3. Epidemiologic studies correlate Western diet consumption with an increased risk of death due to cardiovascular disease and cancer 2,122. Taken together, these findings suggest a relationship between diet and multiple diseases.

In addition to alterations in the relative proportions of fat, sugar and fiber, a Western diet also incorporates processed food constituents to promote longer shelf-life, improved texture or flavor, which studies postulate may have detrimental effects on health. Supporting this, recent case reports link outbreaks of necrotizing enterocolitis with consumption of a xanthan gum-derived food thickener by preterm infants 6,80. Additional studies have linked other thickening or emulsifying agents, such as carob-bean gum, modified starch, carboxymethyl cellulose, carrageenan, pectin and cellulose to bacterially-driven intestinal diseases 7,8,46,79,81-83. These findings suggest that food additives may alter antibacterial response mechanisms important in maintaining intestinal homeostasis.
Maltodextrin (MDX) is a polysaccharide commonly added to processed foods, cosmetics and medications as a filler, thickener, texturizer, or coating agent. MDX is produced through chemical and enzymatic processing of a variety of starches to result in chains of up to 20 glucose molecules linked by $\alpha(1-4)$ and $\alpha(1-6)$ glycosidic bonds $^{101}$. It is generally recognized as safe (GRAS) by the Federal Drug Administration (Code of Federal Regulations, Title 21, Volume 3, Part 184, Subpart B, Section 184.1444); however, there are links between MDX and alterations in intestinal microbiota, as well as increased necrotizing enterocolitis in animal models $^{9,79,117}$. To examine the contribution of this ubiquitous dietary additive to bacterially-driven disease, we investigated the effects of MDX on clearance of the enteric pathogen Salmonella using both in vivo and in vitro infection models.
3.2 Results & Discussion

3.2.1 Dietary MDX increases mucosal *Salmonella* colonization and systemic inflammation. The effect of dietary MDX on the clearance of an enteric pathogen was assessed using the streptomycin pre-treated mouse model of *Salmonella* colitis. The drinking water of 6 week old female mice was supplemented with 5% MDX for 2 weeks. Although daily water consumption was higher in the MDX-supplemented group (14.5+/−3.2mL/cage vs. 25.7+/−4.1mL/cage; p<0.00001), this did not translate into a difference in weight after 2 weeks (18.3+/−0.8g vs. 18.8+/−2.4g; p=0.6). MDX-supplemented mice had enhanced cecal colonization relative to control mice, as demonstrated by a 2-log increase in viable *Salmonella* recovered from cecal homogenates and confirmed by immunofluorescent staining of cecal tissue (Figure 3.2.1). This increased cecal bacterial colonization did not translate into measurable differences in tissue pathology between treatment groups as assessed by histologic analysis of neutrophil accumulation, goblet cell depletion, edema and epithelial erosion using an established scoring system (Figure 3.2.1). Additionally, no differences were observed in colonization of systemic sites (MLN, spleen or liver) (Figure 3.2.1). However, MDX-supplemented mice had higher serum amyloid A (SAA) levels post-infection indicating a greater induction of systemic inflammation in these mice (Figure 3.2.1). These findings are similar to the enhanced cecal *Salmonella* colonization described in rats fed a “Western style diet” supplemented with dietary fructo-oligosaccharides or inulin, suggesting that MDX consumption
Figure 3.2.1. Dietary MDX increases mucosal *Salmonella* colonization and systemic inflammation. (A) Confocal micrographs visualizing *Salmonella* colonization (green) in cecums from control or MDX-supplemented mice. Nuclei stained with DAPI (blue). Scale bars=100μm. (B) Quantitation of *Salmonella* recovered from the cecum, MLN, spleen and liver homogenates. (C) Average pathology scores of hematoxylin and eosin stained cecal tissue from infected mice. Pathology scores integrate analyses of epithelial integrity (Epithelial Injury), goblet cell hyperplasia (Goblet Cells), polymorphonuclear cell infiltration (PMN) and submucosal edema (Edema). (D) SAA levels from infected mice. Unpaired 1-tailed t-test, *p<0.05.
Figure 3.2.2. MDX exposure impairs *Salmonella* clearance. (A) Recovery of intracellular *Salmonella* from the indicated cell types cultured in glucose or MDX containing media. (B) Confocal micrographs of infected BMDM stained for *Salmonella* (green). Nuclei stained with DAPI (blue). Scale bars=10μm. (C) Recovery of intracellular *Salmonella* from the indicated cell types cultured in unsupplemented (No Sugar), glucose or MDX supplemented media. Unpaired 1- tailed t-test, *p<0.05, **p<0.01, *** p<0.001.
also promotes mucosal bacterial colonization and systemic inflammation in response to *Salmonella* infection.

### 3.2.2 MDX exposure impairs *Salmonella* clearance from intestinal epithelial cells and macrophages

Consumption of a Western diet has been shown to not only affect the intestinal microbiota, but also cellular anti-microbial defense mechanisms \(^{13-15}\). To determine whether MDX affects bacterial clearance in cell types critical to intestinal defense, cells were cultured in glucose-free media reconstituted with MDX or glucose for 24 h, infected with *Salmonella* and intracellular bacterial survival determined by gentamycin protection assay 90 min post-infection. MDX exposure enhanced intracellular *Salmonella* survival in human intestinal epithelial cell lines, primary human monocytes-derived macrophages and mouse bone marrow-derived macrophages (BMDM) (Figure 3.2.2). These results were confirmed by visualization of bacterial loads in BMDM by confocal microscopy (Figure 3.2.2). Furthermore, the observed suppression of *Salmonella* clearance after MDX exposure was not a result of glucose starvation, as cells cultured in glucose-free media had similar intracellular bacterial loads as glucose-supplemented cells (Figure 3.2.2). These results indicate that MDX exposure promotes intracellular *Salmonella* persistence in multiple cell types involved in gut defense.

### 3.2.3 *Salmonella* entry and trafficking to early endosomes are unaffected by MDX

Increased intracellular bacterial loads could be a result of defective
Figure 3.2.3. *Salmonella* entry and trafficking to early endosomes are unaffected by MDX. (A) Quantitation of *Salmonella*/cell 30 min post-infection in confocal micrographs. (B) Recovery of intracellular *Salmonella* 30 min post-infection. (C) Confocal micrographs of BMDM 15 min post-infection stained for *Salmonella* (red) and Rab5 (green). Nuclei stained with DAPI (blue). Scale bars=10μm. (D) Quantitation of *Salmonella* co-localizing with Rab5 vesicles/cell 15 min post-infection in confocal micrographs. Values are not significant by unpaired 1-tailed t-test.
bacterial clearance or enhanced bacterial entry. To discriminate between these two possibilities, the initial entry of *Salmonella* was assessed by confocal microscopy and gentamycin protection assays 30 min post-infection in BMDM. No discernible differences in intracellular *Salmonella* numbers were found between BMDM cultured in glucose or MDX containing media by quantification of confocal micrographs or recovery of viable intracellular bacteria (Figure 3.2.3). Further analyses of confocal micrographs showed that *Salmonella* is immediately trafficked to a Rab5+ early endosome in both media conditions (Figure 3.2.3). As early as 15 min post-infection, equivalent numbers of *Salmonella* co-localize with Rab5+ vesicles in both control and MDX-exposed cells (Figure 3.2.3). These findings indicate that MDX does not affect *Salmonella* entry or appropriate trafficking to early endosomes.

### 3.2.4 MDX promotes maturation of *Salmonella*-containing vesicles.

*Salmonella* is a facultative intracellular pathogen that replicates within membrane-bound “spacious” vesicles. Formation of spacious, *Salmonella*-containing vesicles (SCV) is important for bacterial survival within macrophages. These SCV acquire markers of late endosomes (Rab7) and selective lysosome-associated membrane proteins (Lamps) without accumulation of lysosomal hydrolytic enzymes through the action of bacterial effector proteins (Meresse et al., 1999). The ability to form and maintain these spacious SCV has been correlated with both the pathogenicity of *Salmonella* strains, as well as host susceptibility to infection, indicating both bacterial and cellular factors contribute
Figure 3.2.4. MDX promotes formation of spacious, Rab7+ *Salmonella*-containing vesicles. (A) Confocal micrographs of BMDM 90 min post-infection stained for Rab7 (green). *Salmonella* and nuclei stained with DAPI (blue). (B, C) Quantitation and size analyses of Rab7+ vesicles/cell 90 min post-infection from confocal micrographs. (D) Quantitation of *Salmonella*/cell 90 min post-infection from confocal micrographs (103 cells/data point). (E) Confocal
micrographs of BMDM 90 min post-infection and immunofluorescently stained for Rab7 (green). Salmonella and nuclei stained with DAPI (blue). Histogram profiles were generated along a line connecting the arrows in the merged insets. (F) Quantitation of *Salmonella* co-localizing with Rab7+ vesicles/cell 90 min post-infection by analysis of confocal micrographs. Scale bars=10μm. Unpaired 1-tailed t-test, *p<0.05, **p<0.001.
Figure 3.2.5. MDX does not alter association of Lamp2 with SCV but suppresses ROS generation. (A) Confocal micrographs of BMDM 90 min postinfection and immunofluorescently stained for Lamp2 (green) and *Salmonella* (red). Nuclei stained with DAPI (blue). Scale bars=10μm. (B) Quantitation of *Salmonella* co-localizing with Lamp2+ vesicles/cell 90 min post-infection from immunofluorescent confocal micrographs. (C) BMDM ROS generation 10 min post H2O2 stimulation. Unpaired 1-tailed t-test, *p<0.05, **p<0.01.
Figure 3.2.6. MDX consumption enhances the number of cells with large Rab7+ vesicles in intestinal tissue of *Salmonella* infected mice. (A) Confocal micrographs visualizing Rab7+ vesicles (green) in cecal tissue from Fig. 1. Nuclei stained with DAPI (blue). Scale bars=100μm. (B) Quantitation of the number of cells with Rab7 vesicles in 6 fields/group. Unpaired 1-tailed t-test, *p<0.05.
to this process\textsuperscript{123}. The co-localization of \textit{Salmonella} with Rab7\textsuperscript{+} vesicles was assessed in BMDM cultured in glucose or MDX supplemented media. At 90 min post-infection, dramatic differences in the total number and size of Rab7\textsuperscript{+} vesicles was apparent (~2 fold increases; Figure 3.2.4). In addition to a greater intracellular \textit{Salmonella} load, MDX exposed BMDM had more large (>0.5μm), Rab7\textsuperscript{+} vesicles as compared to control cells (Fig. 4b-d). Quantitation of confocal micrographs demonstrated an enrichment of \textit{Salmonella} within large, Rab7\textsuperscript{+} vesicles in MDX cultured cells (Figure 3.2.4). Rab7 is essential for the recruitment and transfer of Lamps to the SCV and Lamps transport cytosolic nutrients into the SCV\textsuperscript{16}. Surprisingly, although MDX enhanced Rab7 accumulation on SCVs, the co-localization of Lamp2 with \textit{Salmonella} was not different between glucose or MDX exposed cells (Figure 3.2.5).

\textit{Salmonella} also target antimicrobial effectors, such as NADPH oxidase and inducible nitric oxide synthase (iNOS) to help create this protective niche for replication \textsuperscript{17}. Studies examining the effect of diet-induced obesity or high fat diet consumption on macrophage function demonstrate reduced iNOS induction and impaired generation of reactive oxygen species (ROS), respectively \textsuperscript{14,15}. BMDM cultured in MDX-containing media did not have altered iNOS expression (data not shown), but had ~50% reduction of ROS production in response to hydrogen peroxide (Figure 3.2.5). Taken together, these results suggest that MDX promotes \textit{Salmonella} survival through the induction of a replicative niche in spacious Rab7\textsuperscript{+} vesicles.
3.2.5 MDX consumption results in increased numbers of cells with large Rab7+ vesicles in intestinal tissue of *Salmonella* infected mice. To determine whether expansion of this protective niche also occurs *in vivo* after dietary exposure to MDX, cecal tissue sections from *Salmonella* infected mice fed MDX-supplemented water for 2 weeks were stained for Rab7 and compared to infected, control mouse sections. In both sets of mice, cells with vesicular Rab7 staining were identified in the lamina propria (Figure 3.2.6). Quantitation of Rab7+ cells per field demonstrated increased numbers of cells with Rab7+ vesicles in the MDX supplemented mouse tissue relative to control mice (~2 fold; Figure 3.2.6).

Our findings indicate that MDX exposure promotes the formation of a replicative niche for *Salmonella*, which enhances intracellular survival and mucosal colonization. These results suggest a link between consumption of processed foods containing the polysaccharide MDX with an increased risk for enteric infection. With the association of enteric infection and development of chronic inflammatory diseases, such as inflammatory bowel disease\(^{18}\), we propose that a careful examination of the contribution of food additives to chronic disease induction is warranted.
3.3 Materials and Methods

3.3.1 Salmonella infection in vivo. All animal studies were approved by the Cleveland Clinic Institutional Animal Care and Use Committee (IACUC protocol 2013-0955) and performed in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Female 6 week old C57BL/6J mice from Jackson Laboratories (stock number 000664) were fed Harlan Teklad Global Irradiated Rodent Diet 2918 and water supplemented with or without 5% (w/v) MDX (DE 4-7; Spectrum Chemicals) for 2 weeks prior to infection. Water consumption was monitored daily and animal weight measured every other day. Infection studies were performed as described in\(^\text{10}\). One day prior to infection, food and water were withdrawn for 4h, then mice were orally gavaged with 100\(\mu\)L streptomycin (20mg) in water. Twenty hours post-streptomycin treatment, food and water were withdrawn for 4h and mice infected with \(10^8\) cfu *Salmonella enterica* serovar Typhimurium strain SL1344 (200\(\mu\)L suspension of log-phase cultures in PBS) by gavage. Food and water were returned 2h after infection and mice monitored for 48h. Mice were sacrificed by CO\(_2\) asphyxiation and blood collected by cardiac puncture into microtainer EDTA tubes (Fisher). Mesenteric lymph nodes (MLN), spleen and liver were harvested aseptically, weighed and homogenized in cold, sterile PBS for bacterial enumeration. Cecal content was removed and tissue was divided in half, weighed and either fixed in Histochoice (AMRESCO) or homogenized in cold, sterile PBS. Tissue *Salmonella* loads were quantified by plating serial dilutions of tissue homogenate on MacConkey agar plates supplemented with
streptomycin (50μg/mL). Hematoxylin and eosin-stained cecal sections (5μm thick) were evaluated for inflammation and pathology by a gastrointestinal pathologist blinded to treatment group using the scale detailed in\textsuperscript{10}. Levels of serum amyloid A were determined by ELISA (Life Technologies).

\textbf{3.3.2 Cells and cell lines.} All media and media supplements were obtained from Life Technologies, except where indicated. The HCT116 cell line (gift of Gabriel Nuñez, University of Michigan) was maintained in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose supplemented with 10\% fetal bovine serum (FBS). The HT29 cell line (gift of Carol de la Motte) was maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10\% FBS. Primary human peripheral blood monocytes were isolated from healthy donors by counterflow centrifugal elutriation by the Cleveland Clinic Clinical and Translational Sciences Collaborative using a protocol approved by the Cleveland Clinic Institutional Review Board and differentiated into macrophages by culturing in RPMI supplemented with 10\% FBS and 50ng/mL macrophage colony stimulating factor for 7 days. Murine bone marrow-derived macrophages (BMDM) were isolated as previously described\textsuperscript{124}. Briefly, bone marrow was flushed from femurs and tibias of C57BL/6J (Jackson Laboratories) or AKR mice (gift of Fabio Cominelli, Case Western Reserve University; used in Figure 2c analyses only) and plated on Petri dishes in DMEM supplemented with 10\% FBS, 30\% L929 conditioned media, non-essential amino acids and penicillin/streptomycin. After 5 days, BMDM were replated for analysis in experimental media for 24h.
Experimental media consisted of DMEM without glucose supplemented with 10% FBS and glucose (4.5g/L; Sigma) or MDX (4.5g/L; Spectrum Chemicals) and was made fresh every 2 weeks.

3.3.3 In vitro Salmonella infections. Cells were infected in triplicate with log phase Salmonella enterica serovar Typhimurium strain SL1344 at an MOI of 10 for 30 minutes followed by two washes in PBS and replacement of media containing 50μg/mL gentamycin (Sigma). Cells were harvested in 0.1% Triton X-100/PBS after 1 hour post-gentamycin addition and serial dilutions in LB broth plated on LB agar to determine viable intracellular Salmonella colony forming units (cfu).

3.3.4 Immunofluorescent staining and confocal microscopy. BMDM were plated on glass coverslips in experimental media 24 hours prior to infection. At indicated harvest times, cells were fixed in 4% paraformaldehyde/PBS (Electron Microscopy Services), followed by permeabilization in 0.4% Triton X-100/PBS. Cells were subsequently blocked in 0.2% FBS/0.4% Triton X-100/PBS followed by addition of antibodies in 0.2% FBS/PBS. Antibodies used are as follows: polyclonal rabbit anti-Rab5 (Cell Signaling, #21435), polyclonal rabbit anti-Rab7 (Cell Signaling #20942), monoclonal mouse anti-Lamp2 (Abcam G12A7), monoclonal mouse anti-Salmonella (Serotech), polyclonal rabbit anti-Salmonella (Thermo Scientific PA1-7244), goat anti-rabbit-Alexa488, goat anti-rabbit-Alexa568, goat anti-mouse-Alexa488, goat anti-mouse-Alexa568, goat anti-rat-
Alexa488, goat anti-mouse-Alexa633, goat anti-rat-Alexa633 (Life Technologies). After staining, coverslips were mounted on glass slide with Vectashield containing DAPI (Vector Laboratories) and sealed with nailpolish. Imaging was conducted using a Leica TCS-SP spectral laser scanning confocal microscope equipped with a Q-Imaging Retiga EXi cooled CCD camera and Image ProPlus Capture and Analysis software (Media Cybernetics). Image z-stacks were collected every 0.49μm spanning the full thickness of cells and exported for image quantitation using Image ProPlus Capture and Analysis software (Media Cybernetics). Vesicle size, number and co-localization of staining were calculated using a custom macro. Co-localization was verified by manual scoring of image stacks. A minimum of 2 fields were analyzed for each experimental condition and experiments were repeated at least three times. 3D Reconstructions were prepared using Volocity software. Image stacks were imported and visualized using 3D projections and uniform percent-black and percent-brightness for all images analyzed and presented.

3.3.5 Reactive Oxygen Species Measurement. BMDM were plated at 2.5×10^5 cells/mL in a 96 well plate 24 hours prior to analysis. Cells were washed in Hanks’ Balanced Salt Solution and incubated with 10mM Carboxy-H₂DCFDA (Invitrogen, C6827) for 30 minutes in the dark. After washing, cells were stimulated with 100mM H₂O₂ and fluorescence measured using a Spectromax plate reader recording excitation/emission 495/529nm every 22 seconds at 37°C. Assay performed in triplicate in 4 independent experiments.
3.3.6 **Statistical analysis.** Statistical analyses were performed using Prism software (Graph Pad) and one-tailed, unpaired t-tests were applied to each experiment to determine significance. P values 0.05 or less were considered significant. *In vitro* experiments were performed in triplicate in a minimum of three independent experiments. *In vivo* experiments were conducted with a total of 6 control mice and 7 experimental group mice. Data is presented as averages with standard error unless noted in the figure legend.
3.4 Acknowledgements

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Chapter 4. Discussion and Findings

4.1 Discussion and Findings

Environmental variables significantly shape and promote onset of Crohn’s disease; however, studies to identify specific environmental factors relevant to disease are challenging to perform. In the context of CD, a prospective study would be the optimal strategy to identify dietary factors contributing to disease onset. However, this study is largely impractical as it is impossible to control other environmental variables (such as exposures and lifestyle) and to predict disease onset; the size of the cohort required to make meaningful conclusions would be extensive and collection of data would span decades. We applied an alternate approach, utilizing available data regarding disease incidence and changes in dietary consumption to identify polysaccharides that may be important in disease onset. **This body of work describes identification of a dietary polysaccharide that associates chronologically with CD onset and has important properties mediating both bacterial adhesion and host response to invasive pathogens.**

In the second chapter, I identify the polysaccharide MDX as a commonly consumed dietary additive, whose increasing public availability is correlated with CD incidence. For the identification of this polysaccharide, we designed a high-throughput assay to test the effect of MDX containing food products on biofilm formation, a bacterial phenotype important in CD. This allowed us to isolate two specific variables associated with CD in an **in vitro** assay. First, MDX, by itself or
present in combination with artificial sweeteners, enhances bacterial biofilm formation. Second, this enhanced adhesion promotes more bacterial adherence to epithelial cells without changing the invasive properties of the bacteria. Together, these data demonstrates a mechanism by which a polysaccharide can enhance bacterial attachment to cells. MDX is frequently integrated into food products as a texture enhancer and binding agent. In a profile of grocery store food products, roughly 50% of surveyed products contained MDX. Based on MDX availability, it is reasonable to assume that most Americans are consuming some amount of MDX on a daily basis. It is important to understand the effect of dietary additives on gastrointestinal health.

To emphasize clinical relevance of this phenotype, I looked at surgical resections from both healthy donors and CD patients. From the acquired tissue, I isolated total DNA from the adherent bacterial population and host genomic DNA. I then utilized a quantitative-real time PCR approach to assess a gene required for bacterial MDX metabolism. The gene, malX, is also reported to be a virulence gene, likely due to its repeated observation in uropathogenic E. coli strains. MalX codes for a component of the phosphotransferase II system, responsible for phosphorylation of the sugar after passing the inner membrane into the bacterium. We hypothesize that the MalX gene may confer additional metabolic capability to a number of enteric pathogens allowing it to better survive in an environment like the gut, or to survive intracellularly in vesicles where glucose or MDX might be present. In the context of adherent bacteria from CD or control patients, significantly more copies of the MalX gene, relative to total
bacteria, were amplified from patients with CD in their ileum as opposed to healthy controls or colonic CD patients. This work is significant because it identifies a polysaccharide that correlates with disease and demonstrates its ability to modulate microbial phenotypes while correlating with bacterial populations found in patients with ileal CD.

The complimentary second story looks at MDX from the host perspective, observing how epithelial and macrophage cells cells handle the exposure to MDX with the challenge of bacterial infection. MDX is found endogenously as a degradation product of glycogen, often seen in liver or muscle cells. This suggests that the gut luminal MDX may illicit an inappropriate or unexpected response in epithelial or macrophage cells as they respond to a polysaccharide not normally seen by these cells at this dose or frequency. As MDX is consumed with increasing frequency by individuals consuming a low-sugar diet or athletes, it is important to understand the effects of dietary polysaccharide on human health. Our data demonstrates that both in vivo and in vitro exposure to MDX increases Salmonella bacterial loads, suggesting that a diet rich in MDX may explain why some individuals have severe cases of food poisoning or develop chronic intestinal disease.

In our murine model, six week old wild-type C57bl/6 mice were provided with drinking water supplemented with 5% MDX for two weeks. C57bl/6 mice are naturally susceptible to Salmonella infection due to a coding variant in the gene Nramp1. The Nramp1 coding mutation in C57bl/6 leads to rapid degradation of the Nramp1 protein resulting in severe Salmonella susceptibility as Nramp1 is
responsible for IL-1β secretion, reactive oxygen species generation, antigen presentation and other functions in macrophages\textsuperscript{126}. In our study, we utilized streptomycin pretreatment to clear commensal bacteria from the GI tract prior to infection with *Salmonella*. It is also important to note that *Salmonella* prepared for infection was grown to log phase, a point at which expression of SPI-1 virulence genes are prominent. Expression of these genes preferentially promotes a bacterial infection at the mucosa, as opposed to a late-log phase *Salmonella* expressing a different set of virulence gene that promote a systemic infection. Combining a susceptible mouse strain with antibiotic treatment and water supplemented with MDX allowed us to look that the effect of dietary supplementation on mucosal enteric pathogen infection and show diet-dependent increased *Salmonella* burden from mice fed MDX. Our data also demonstrates an increase in a systemic marker of inflammation as evaluated through plasma levels of serum amyloid A, suggesting that there is overall mouse stress though consequences of the increased bacterial burden are not yet appreciated. I hypothesize that the increased bacterial burden could lead to premature mouse demise (should the mouse be unable to control the infection) or a chronic infection (should the mouse be unable to clear all bacteria). It is interesting to note that the effect of MDX was localized to bacterial burden in the mucosa, and when hypothesizing the importance for human health, may indicate a preference towards enhanced enteric infection in individuals consuming a diet rich in MDX.

To identify a mechanism by which MDX mediates *Salmonella* survival, I used *in vitro* analysis of epithelial cells and macrophage cells to demonstrate
impaired *Salmonella* clearance after exposure to MDX. Both macrophages and epithelial cells possess several strategies for eliminating *Salmonella*, including targeting the bacterium or damage *Salmonella*-containing-vesicle by autophagy or fusion of late endosomes with lysosomes to degrade contents. As increased *Salmonella* numbers were observed after 90m of infection, a time point at which *Salmonella* clearance mechanisms should be activated, we hypothesized that impaired trafficking may be responsible for enhanced *Salmonella* viability. This hypothesis was supported by analysis of late endosomes labeled by Rab7 protein. In MDX-fed bone marrow derived macrophage (BMDM) cells we found an increase in overall Rab7+ late endosomes, and that a percentage of these vesicles were much larger than in control fed cells for this time point. Colocalization analysis demonstrated that more *Salmonella* was contained in late endosomes, suggesting a protective niche for bacterial replication. Strikingly, Lamp2+ vesicles had also formed at this time point, but failed to colocalize with *Salmonella* suggesting an inability of the cell to efficiently coordinate vesicular trafficking and fusion of the late endosome to the lysosome.

**Overall this work describes identification of the polysaccharide MDX as a potential risk factor for CD. In vitro and in vivo analysis of MDX-treatment shows changes in bacterial phenotypes and host-clearance capabilities, demonstrating a dietary component capable of modulating both microbe-host and host-microbe interactions.**
4.2 Future Directions

Perhaps the most exciting result from this work is the extensive opportunities for the project moving forward. We have identified a polysaccharide important in host-microbe interactions and have only begun to understand the implications of this relationship and its significance in disease states. In this section, I have included experiments that would expand on work completed as well as experiments that would further help us understand MDX as an environmental risk factor for CD.

4.2.1 Mouse-MDX Experiments.

One very important step would be to expand on our understanding of how MDX enhances *Salmonella* infection in mice. In the data presented in chapter 3, we use a specifically defined system to address mucosal *Salmonella* infection. By preferentially utilizing *Salmonella* that infects the mucosal surface we do not address the effect of MDX on systemic infection. I would like to compare both a systemic and local infection to understand the global effect of MDX. The results from these studies would clarify if the effect of MDX is localized to the intestine or promotes impaired systemic antibacterial clearance. Furthermore we chose to terminate our experiment after 48h of *Salmonella* infection. At this time point we did not see significant differences in weight loss or distress of the animals but we do recover significantly more bacteria from the cecum of the animal, as well as observe higher levels of serum amyloid a from the blood plasma. It is possible that a longer course of infection would allow us to understand how the increased
bacterial burden is handled. A complimentary study would follow fecal *Salmonella* after infection, testing the hypothesis that MDX promotes a chronic infection of the bacteria. We would anticipate that the control fed would clear the infection while the MDX would promote persistence. These studies would allow us to understand the role that MDX plays in enteric infection and potentially give us insight into chronic infection.

Additionally, IBD has a strong genetic requirement for disease onset. The mice we have utilized harbor a coding mutation in a *Salmonella* clearance gene called *Nramp1*. It would be interesting to test infection susceptibility after MDX exposure in a mouse without *Salmonella* susceptibility. Furthermore, exposing mice with CD-associated genetic variants in *Atg16l1* or *Nod2* would allow us to explore the relationship between environment and genotype after infection. In preliminary work looking at CD-related genotypes reconstituted in epithelial cells, we observe a combinatorial effect of MDX with susceptible genotype. This suggests that MDX could exacerbate a CD-genotype which may be important in disease onset.

Part of our hypothesis is that MDX alters commensal bacterial populations and promotes biofilm formation. Bacteria in a biofilm state are slow-growing and therefore less susceptible to antibiotics, which suggest that MDX may promote antibiotic resistance. In our mouse *Salmonella* infection model, we pre-treat our mice with the broad-spectrum antibiotic streptomycin to clear commensal microorganisms. It would be important to consider the effect of MDX on commensals to evaluate antibiotic efficacy in clearing the adherent population. If
our hypothesis is supported, fewer commensal microbes would be cleared from the intestine potential inhibiting effective Salmonella infection. As our in vivo data demonstrates increased Salmonella colonization of the cecum, it would be interesting to examine if MDX is restricting antibiotic efficacy and how this inhibition is mediating Salmonella infection potential. Finally, results from these studies would aid in our understanding of variables mediating antibiotic efficacy and the effect of MDX on commensal microorganisms.

**Defining MDX-mediated intestinal dysbiosis and altered homeostasis in mice.** We hypothesize that consumption of MDX will alter commensal bacterial populations and increase expression of bacterial adhesion proteins changing the host-microbe architecture of the small intestine. Support for this hypothesis extends from dynamic, diet-dependent shifts captured in fecal bacterial populations in human and mice; while evidence of MDX metabolism has been observed in adherent bacterial populations from patients diagnosed with Crohn’s disease (CD) ileitis. Despite a strong relationship between diet and commensal microbiome composition, diet remains an unexplored variable likely contributing to development of CD. Fundamentally, defining how MDX alters intestinal homeostasis will demonstrate how this commonly consumed polysaccharides contribute to ileitis susceptibility while simultaneously clarifying its hypothesized role as a risk factor for CD.

**How does MDX shift the commensal microbiome composition and alter the intestinal architecture?** To establish a baseline for how MDX alters commensal populations; MDX (DE 4-7, 5% w/v, Sigma) will be supplemented
into the drinking water of 6-8 week old WT C57bl/6 mice (n=10 per treatment group) for two weeks. It is anticipated that water consumption rates will be increased in the MDX-supplemented group, therefore animals will be observed for water consumption, weight gain, and for evidence of general malaise via grooming habits, activity levels and response to human interaction. Should excessive consumption (>10mL per mouse per 24h period) of the supplemented water occur, an alternate strategy for MDX delivery may be utilized or supplementation of the control water with glucose will assist in consumption normalization. To monitor changes in adherent bacterial populations, ileal and colonic biopsy samples will be taken every three days using a novel microendoscope (Colview Technology). Isolation of 16s ribosomal RNA (rRNA) will be analyzed using 454 pyrosequencing to generate a comprehensive view of changes in bacterial populations. Our expected results would reflect decreased diversity of bacterial populations (figure 4.2.1). Ideally, MDX will drive decreased bacterial diversity and the resulting populations will mimic what is observed in human IBD; anticipating a loss of Faecalbacterium and Roseburia, diminished Firmicutes and Faecalibacterium pausnitzii and increased Enterobacteriaceae (and g-proteobacteria) and Rumnococcus gnavus. From the information obtained regarding bacterial populations, further bioinformatics analysis will look at metabolic capacity of the commensal populations anticipating enrichment of carbohydrate metabolism genes. Taken together, these analyses will define a role for MDX in population shifts of commensal microbes in the intestine of the murine gastrointestinal tract.
While we anticipate changes in bacterial populations, we also hypothesize alteration of bacterial phenotypes evidenced by increased bacterial density, adhesion molecule expression and altered spatial organization. An overview of hypothesized outcomes is provided in figure 4.2.1. Using biopsy material collected as described, samples will be fixed in a non-aqueous solution (Carnoy’s Fixative, prepared fresh, in house) to preserve mucosal structure and commensal organization. Analysis of these mucosal-preserved tissue sections will focus on phenotypic alterations in bacterial adhesion; specifically to evaluate type 1 pili adhesin expression and mucosal layer thickness. These studies will utilize immunofluorescence staining for visualization of type 1 pili coupled with fluorescently conjugated DNA probes recognizing broad spectrum eubacteria enabling visualization of bacterial translocation, distribution of the commensal populations and changes in adhesion protein expression. Period acid-Schiff staining will enable visualization of the mucosal layer and goblet cells with quantitation using Image Pro Plus software to quantitate mucosal layer thickness and goblet cells quantity. At the conclusion of the experiment, mice will be terminated with intestinal tissue preserved for histology and evaluated for epithelial and villus architecture, overall intestinal health and bacterial translocation.

In a wild-type mouse strain, such as the C57bl/6 mouse, we would expect to see changes in bacterial populations dependent on MDX consumption; however it is less likely that we will observe phenotypes associated with IBD such as loss of mucosal barrier and goblet cell depletion. As both environmental
variables and susceptible genetic background are required for onset of human CD, repeating these studies in mice harboring genetic variants associated with CD will allow for assessment of the interplay between both variables. Follow up studies will replicate aforementioned analyses in mice with the ATG16L1\textsuperscript{T300A} variant and the Nod2\textsubscript{Leu1007fsinsC}. Complimentary to described analysis, mice will be monitored for signs of gastritis including loose or bloody stool. Ideal results would demonstrate that a combination of dietary MDX and risk genotype results in ileitis similar to human disease, as well as, alterations in microbiome composition and spatial organization as described above.
Figure 4.2.1: Hypothesized results from MDX-feeding studies. On the left panel, a schematic of the intestinal architecture shows the distribution of cells in the epithelium and associated commensal microbes. The central and right panels outline anticipated results after MDX feeding in a WT mouse (center panel) or a CD-associated risk genotype (right panel).

**WT Genotype**
6. decreased commensal diversity
7. increased bacterial translocation
8. loss of goblet cells
9. increased bacterial adhesion gene expression

**CD Risk Genotype**
10. decreased commensal diversity, increased bacterial density
11. loss of goblet cells
12. crypt branching, dysplasia
13. loss of paneth cells
14. loss of mucus barrier
15. increased bacterial translocation
16. increased bacterial adhesion gene expression
4.2.3 Salmonella-Infection Experiments. In chapter 2, our biofilm manuscript, we look solely at stains of *E. coli* in our biofilm formation model. It would be quite interesting to also look at additional enteric pathogens and other commensals to determine effect of polysaccharide on adhesion or competition between species. We had initially tried to integrate *Salmonella* into our biofilm assays; however we were unable to promote growth of the bacterium in our minimally defined media. *E. coli* has extensive metabolic capabilities, allowing growth under minimal circumstances. To circumvent the less-adaptable nature of *Salmonella* or other species, supplementation with amino acids, vitamins or cofactors may be sufficient to promote growth. These modifications to our minimal media formula would allow us to test additional strains and determine if MDX promotes similar adherent phenotypes in other bacterial strains. Understanding the full potential of dietary MDX on bacterial metabolism would allow us to appreciate the selective potential of dietary MDX and how it may be driving changes in bacterial populations in patients with CD.

Also demonstrated in chapter 2, I observed MDX-promoted increased adhesiveness of LF82 through upregulation of type-1-pili expression. Type 1 pili has been reported to stimulate expression of matrilysin\(^{127}\). Matrilysin is reported to activate pro-\(\alpha\)-defensins in mice and is important in mucosal epithelial repair and immunity. CD patients have decreased levels of \(\alpha\)-defensins compared to healthy controls\(^{128}\). I would hypothesize that increased bacterial adhesion through type-1-pili expression could hyperactive matrilysin would could
negatively affect levels of α-defensins and lead to further degradation of the protective mucin layer.

One minor peculiarity of our studies is that we’ve evaluated both *E. coli* spp. and *Salmonella* in MDX-dependent scenarios, although we had been testing differing hypotheses. It would be most interesting to ask if both the bacteria and the cells are exposed simultaneously to MDX how the outcome would be altered. After consumption of dietary MDX, both commensals and host cells are simultaneously exposed to MDX demonstrating a need for integration of variables when designing future experiments.

**4.2.4 Human Studies:**

Our central hypothesis is that MDX is an environmental risk factor for CD, driving impaired bacterial response by host cells and altering the commensal microbiome. While our *MalX* gene analysis demonstrates an increased incidence of this gene in patients with ileal CD, it does not address changes in overall microbiome composition. Moreover as no single bacterial pathogen has been shown responsible for disease onset, population studies demonstrating a diet-dependent shift may recapitulate population shifts in patients with active disease. To perform these studies, volunteers would be fed a controlled diet with supplemented MDX and provide either ileal biopsy or fecal samples, prior to intervention and throughout. Samples will be analyzed for bacterial 16s ribosomal RNA sequencing to identify populations of bacteria present after dietary intervention. Ideally, populations expanding after MDX exposure would
reflect MDX-metabolizing species. This proof-of-principal design would demonstrate if MDX directly alters microbiome populations in the small intestine. However, as we believe that MDX may be negatively affecting intestinal health, such a study would be unethical. An alternative strategy might be to restrict MDX from the diet of CD patients and monitor for improvement in overall health.

One idea to capitalize on the bacterial metabolism of maltodextrin would be to use MDX-conjugated antibiotics. Using an antibiotic targeting bacterial protein synthesis would support killing of adherent-phase bacteria, while conjugating it to MDX would promotes specificity of antibiotics against harmful bacteria (figure 2.3.11 details bacterial strains possessing the MalX gene). This strategy may also target Lactobacilli, a known probiotic and beneficial bacteria. Supplementation of the diet with Lactobacilli containing foods may circumvent any negative effects of an MDX-conjugated therapeutic. As a noted issue in CD is the inability to promote mucosal healing, this strategy may eliminate the bacterial biofilm and allow reformation of the mucosal barrier leading to potentially effective long term healing of the intestine.

Alternatively, utilizing antibodies targeting bacterial proteins in the MDX metabolic pathway would demonstrate a strategy where clearance of the bacteria is facilitated without the use of antibiotics. Other proteins involved in the MDX metabolism pathway are expressed on the outer membrane of the bacteria and therefore could be considered as better therapeutic targets than the intracellular MalX protein.
Identification of MDX demonstrates a specific, avoidable dietary factor that mediates host-microbe interactions. Fundamentally there are no clinical recommendations for dietary modifications and while our data lays the foundation for the idea that MDX interferes with normal antibacterial response in the intestine and may alter our commensal microbiome, these studies will help fully elucidate the role of dietary MDX in disease progression, and may lead to meaningful dietary modifications or the development of therapies to aid in the treatment of this devastating disease.
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