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Understanding and Controlling the Effects of Dietary and Viral Factors on Intestinal Health and Allergy

Cassandra Suther

University of Massachusetts Amherst

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UNDERSTANDING AND CONTROLLING THE EFFECTS OF DIETARY AND
VIRAL FACTORS ON INTESTINAL HEALTH AND ALLERGY

A Dissertation Presented

by

CASSANDRA SUTHER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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The Department of Food Science
UNDERSTANDING AND CONTROLLING THE EFFECTS OF DIETARY AND VIRAL FACTORS ON INTESTINAL HEALTH AND ALLERGY

A Dissertation Presented

by

Cassandra Suther

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DEDICATION

To all those who’ve held a pipette and thought

“I’ve sure used a lot of plastic today”
ACKNOWLEDGEMENTS

Gandalf the Grey once told Frodo Baggins, “All we have to decide is what to do with the time that is given us”. The moral of this quote is something that has helped me through graduate school. Now that it has come to an end, my metaphorical finger has been bitten off and the ring has been thrown into Mount Doom. Just like Frodo, my journey was filled with memorable moments and my own fellowships (outside of the ones given academically). I want to take this moment to thank all of my family and friend’s who’ve helped me on my journey (and yes Elena, I did mention Lord of the Rings in my dissertation).

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Although not physically near me, I’d like to thank my family next. My best friend Lynsey, you are family after talking to me every day since 6th grade. Thank you for
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Thank you all for not being Gollum.
ABSTRACT

UNDERSTANDING AND CONTROLLING THE EFFECTS OF DIETARY AND VIRAL FACTORS ON INTESTINAL HEALTH AND ALLERGY

FEBRUARY 2023

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Directed by: Professor Matthew D. Moore

The rate at which the human diet has changed in recent centuries has surpassed that of our evolution. As a consequence, this could be deleterious to health. The gut microbiome is the collection of all microbiota and their genetic contents found within the gastrointestinal tract, and has been found to influence health in numerous ways. Its profound impact on the human body is essential for health and is heavily influenced by diet. As diet is a modifiable target, modulation of the gut microbiome through dietary intervention, such as through pre-probiotics, is of interest. Three different dietary ingredients (walnuts, frankincense, and *Bifidobacteria*) were evaluated for their pre-probiotic effects on gut microbiota composition, function and allergic disease. Models to study the effect of norovirus, a pathogen known to cause gastroenteritis, on food allergy and gut microbiota were also established.
Walnuts have been shown to positively affect gut health, cognitive function and cancer, yet their effect on host metabolism is not well defined. The work presented here suggests that walnuts to change the gut microbiota composition to induce more short chain fatty acid-producing bacteria. Along with this, metabolic changes in mice fed walnuts were observed, including a decrease in respiratory exchange ratio and increased activity. These changes could be due to the prebiotic effects of walnut composition, including fiber or polyphenol content. These findings suggest that walnuts may have prebiotic effects on the gut which leads to positive changes in metabolism.

*Boswellia serrata*, commonly known as frankincense, has been used for medicinal purposes for centuries, including treatment of asthma. However, the effect of consumption of a primary bioactive ingredient of it, 3-O-acetyl-11-keto-b-boswellic acid (AKBA), on the gut microbiome is not known. In our work, we investigate the effect AKBA consumption has on the gut microbiota and health of both healthy and allergic asthma-induced mice.

In healthy mice, AKBA significantly decreased gut bacterial richness in male mice but had no effect on female mice. *Akkermansia muciniphila*, previously reported to be associated with weight loss and anti-inflammation, was found to be significantly increased in both male and female mice, along with an increase in *Bifidobacterium* in female mice. These results show the potential benefits of dietary *Boswellia serrata* due to the modulation of gut microbiome composition, along with potential sex-based differences in its effects. Given its ability to shift the microbiota composition, as well as the fact our intestinal bacteria can significantly affect the development of allergies, the impact of *Boswellia serrata* acid on allergic asthma was explored.
Allergic asthma is the manifestation of an allergy in the lungs, which leads to coughing, wheezing and difficulty breathing. The gut microbiome composition has been shown to effect asthma development and exacerbation. In our study, we tested AKBA’s effect on development and severity of asthma and gut bacteria. AKBA treated mice showed significantly lower weight and airway inflammation. Asthma control mice showed a decrease in overall bacterial diversity while AKBA treated mice had increases in several bacterial genera associated with anti-inflammatory effects, including *Bifidobacteria*. The *Bifidobacteria* was further isolated from stool and identified via ITS sequencing as *B. pseudolongum* in all isolates. Further testing of *B. pseudolongum* via oral administration showed an alleviation in airway inflammation, suggesting *Boswellia serrata* has potential to serve as an anti-asthma agent via alteration of the microbiome and increases in *B. pseudolongum*.

Although the aforementioned dietary interventions appear to affect the gut microbiota and allergy, the effects of enteric viral infection on allergies have not directly been investigated. For this dissertation, two animal models, one targeting food allergy and the other norovirus, were developed to help study this proposed correlation. Further work should be continued on the extent norovirus has on allergic diseases and the microbiome.
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Chapter 1

INTRODUCTION

Trillions of microorganisms (bacteria, virus, fungi and protozoa) colonize our human body. They can be found throughout, including our skin, oral cavity, gut, respiratory and urinary tract. Of the microbes found, it is estimated that 70% flourish in the gut. Along with being the majority, our gut microorganisms are of interest due to their purposed effects on distant organs (like the lungs), protection against pathogens, and metabolism.

The gut microbiome in early life development is critical in the maintenance of proper immune balance as we age. However, certain bacteria, early life and throughout, are thought to have beneficial effects. Diet intervention is an easily controlled variable that can change gut composition and as a result, health. When considering diet and microbiota shifts, one of the most popularly studied is the effect of prebiotics.

Prebiotics were first defined as “selectively fermented ingredients that allow for specific changes, both in the composition of and/or activity in the gastrointestinal microflora that confer benefits upon hosts well-being and health.” The most recognized prebiotics are fiber. Specifically, soluble fiber (rather than non-soluble) is broken down and metabolized by our gut bacteria. However, it should be considered if dietary interventions which encourage positive change in composition in any way should be considered prebiotics, as other non-fiber substances have been recognized as having prebiotic effects.
Typically, prebiotics encourage probiotic population\textsuperscript{7}. Probiotics are bacteria which have associated health benefits. One of these health claims includes the increase of the bacterial metabolite short chain fatty acids (SCFA)\textsuperscript{7}. These metabolites are thought to increase energy and reduce inflammation by regulating immune responses. As such, increases in probiotic bacteria have been heavily associated with decreases in allergic diseases, including allergic asthma and food allergy\textsuperscript{3}.

Allergic asthma is the most common phenotype of asthma\textsuperscript{8}. The development of the disease is based on an overreaction to an allergen breathed into the lungs. Treatment for allergic asthma consists of a class of drugs called corticosteroids\textsuperscript{8}. Overuse of these drugs can trigger a rebound effect, causing reactions to worsen over time, along with other undesirable side effects\textsuperscript{9}. As such, less invasive treatments have been considered, including \textit{Boswellia serrata} resin\textsuperscript{4}.

\textit{Boswellia serrata} is a branching tree which produces a gum resin commonly known as frankincense\textsuperscript{10}. It contains active compounds thought to decrease allergy symptoms through the modulation of several inflammation pathways, including the 5-LOX pathway. However, it’s effect on the gut microbiome, and if this could be a contributing factor it it’s anti-inflammatory effect, is unknown.

It has been supported by research showing the protective nature of exposure to certain bacteria has against allergic disease, however, often overlooked is the effect viruses have on allergy\textsuperscript{11}. A common childhood disease, norovirus, has recently been shown to have potentially positive side effects following infection, including restored intestinal morphology and immune function in regular and gut microbiome deprived mice\textsuperscript{12}. However, it’s effect on allergy development is unknown.
To better understand the masterpiece behind the human microbiome, we investigated the effect different dietary ingredients, including walnuts, frankincense and *Bifidobacteria* had on gut microbiota composition, function and allergic disease. We also produced models to help study norovirus infection’s impact on allergy.

Our **long-term goal** is to develop diet-based strategies that provide alternative approaches to improve and study human gut health and allergic diseases. To reach that goal, the **overall objective** of this project is to study the interaction between gut microbiota, dietary ingredients and allergic disease. Our **central hypothesis** is that dietary components could modify gut microbiota composition and host function, which could benefit health. Moreover, we believe both walnuts and frankincense could change microflora to a more beneficial composition. We also believe frankincense, along with norovirus, could prevent allergic disease.

We will test our central hypothesis and achieve our objective by utilizing the following four **specific aims:**

1. **Determine walnut induced changes in gut flora, along with effects on host metabolism.** Two groups of mice will be fed a diet containing high levels of fat and sugar (a typical western diet). One group will be supplemented with 14% walnuts. Stool samples at different intervals will be taken and the 16S rRNA gene will be sequenced. Phyloseq will be used to analyze the microbiota data to observe the changes of microbiota diversity and compositions. The experiment will be repeated with mice given walnuts for 8-weeks to determine metabolic changes through a Comprehensive Lab Animal Monitoring System.
2. **Determine the effect a frankincense compound has on microbiota compositions and blood metabolites.** Healthy mice will be given an oral supplement of *Boswellia serrata*, every day, over the course of 2 weeks. Stool samples will be taken at the beginning and end of treatment to monitor a change. Phyloseq will be used to analyze the microbiota data to observe the changes of microbiota diversity and compositions. Blood will also be taken to observe differences in blood metabolites. Both male and female mice will be used to determine gender differences.

3. **Determine the effect frankincense and Bifidobacteria have on allergic asthma and the gut microbiome.** An OVA based murine allergic airway model will be used to study the effect an oral supplement of a frankincense compound has on both allergy symptoms and the gut microbiome. The compounds will be given every other day and then every day for 3 weeks. Stool samples will be taken before and after disease course. Phyloseq will be used to analyze the microbiota data to observe the changes of microbiota diversity and compositions. Lung fluid, tissue and blood will be taken to observe changes in immune reaction. The experiment will then be repeated with a *Bifidobacteria* supplement rather than frankincense.

4. **Develop a technique to study the effect norovirus has on food allergy development.** Two models will be developed. One to study food allergy and one for norovirus. For the food allergy, mice will be given OVA + cholera toxin orally over the course of 2-4 weeks. Following sensitization, an allergic reaction will be induced with a larger
dose of OVA. Allergy scoring, histamine, and IgE levels will be taken. To induce norovirus infection, mice will be given a single dose of MNV-1 to observe the course of infection. Stool samples will be taken for one week to detect viral shedding.
2.1 A Symbiotic Masterpiece: The Human Gut Microbiome

2.1.1 Gut Microbiome, noun. gət mi·cro·bi·ome

The human gut microbiome refers to the combined genetic material of all commensal bacteria, fungi, and viruses found within the human gut or gastrointestinal (GI) tract. Trillions of gut microbes from thousands of different species make up the highest density of microbes within the human body. It is typically dominated by five phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Tenericutes. Our gut microbiota has a broad impact on a range of medical attributes, including resistance to pathogens, intestinal epithelium upkeep, controlling immune function and digestion of dietary and pharmaceutical compounds. It has also been heavily linked to neurological function through a purposed gut-brain axis. In recent years, it has been used as a variable in the treatment of several diseases including allergy, autoimmune, metabolic disorders, cancers, gastrointestinal conditions and mental illness.

2.1.2 Diet and the Gut Microbiome

Excerpt taken from Appendix 3: The Gut Microbiome & The Big Eight

Diet shapes the configuration of the gut microbiome at early life. The gut microbiome of infants that were breastfed have a unique and beneficial composition that is not observed in those given formula when compared later in life. Gut microbiome dysbiosis in early life is thought to be related to development of allergies later in life. When children start a solid food diet (around 6 months) the gut microbiome shifts significantly for a second time. The adult gut microbiome generally can be classified into three distinct enterotypes that
are dominated by *Bacteroides, Prevotella*, or *Ruminococcus*, respectively\(^{19}\). The diet of the individual influences the enterotypes, with *Bacteroides* being associated with a Western-type diet high in proteins and fat, and *Prevotella* being associated with plant fiber consumption. As a modifiable target, modulation of the gut microbiome through dietary intervention (high fiber related diet), prebiotics or probiotics have seen an increase in both research interest and product development in recent years. However, the magnitude and duration of microbiome changes by dietary intervention have been largely inconsistent.

High levels of prebiotics can increase the production of short chain fatty acids (SCFA) from specific bacteria in Firmicutes, such as *Clostridia* (*Ruminococcus* enterotype) and *Bacilli*\(^{20}\). SCFAs are bacterial fermentation products and are profoundly affected by food intake. There are three common short chain fatty acids that tend to be produced by bacteria: acetate, propionate, and butyrate. Acetate is the acid produced in the highest quantity, but butyrate is thought to be the main energy source for colonocytes. These acids are absorbed into the portal vein during lipid digestion, and have been associated with major health benefits, including a reduced risk of inflammatory diseases, Treg function and increased energy levels\(^{21-22}\).

Other metabolites derived from commensal bacteria include long chain fatty acids (LCFA), glycolipid, histamines, vitamins B2/B9 byproducts and amino acids\(^{23,24}\). LCFA are major nutrients, including the clinically important \(\omega3\) and \(\omega6\) FAs, with \(\omega3\) FAs known to have anti-allergic and anti-inflammatory properties\(^{23}\). Pro-inflammatory LCFA metabolites, including stigma- and sitosterols and 8-hydroxyoctanoate, are associated with lower risk of allergy development\(^{23}\). These metabolites are thought to decrease IL-4 produced Th2 cells.
Western lifestyle and diet promote a change in bacteria which has not been associated with the human gut in evolution. These changes from millennia of human biology, even small, may lead to larger detrimental outcomes.

2.1.3 Walnut Consumption on the Gut Microbiome

Walnuts have gained “superfood” status because of their rich fat profile (omega-3 fatty acid, alpha-linolenic acid (ALA)), as well as phytochemicals, antioxidant polyphenols, and dietary fiber\(^{25}\). Walnuts have around double the amount of phenols compared to fruits and veggies, with its main polyphenol being pedunculagin, an ellagitannin\(^{26}\). After consumption, ellagitannins are hydrolyzed to release ellagic acid, which is converted by gut microflora to urolithin A and other derivatives such as urolithins B, C, and D\(^{26}\). Walnuts also contain around 7% fiber, a compound known to have profound effects on health\(^{27}\). However, it is uncertain which of these compounds (polyphenols, fiber, fats etc.) is responsible for the walnut associated health benefits, like decreased cancer, heart disease and increased brain health\(^{27}\). Several studies have shown the effect walnuts have on the microbiome.

Two studies, one conducted in rats and the other in humans, found an increase in \textit{Ruminococcaceae} following walnut interventions for ten or eight weeks, respectively\(^{27,28}\). However, in the rat study, an increase in \textit{Lactobacillus} and \textit{Roseburia} were also seen, along with a significant reduction in \textit{Bacteroides} and \textit{Anaerotruncus}\(^{27}\). The human study also reported an increase in \textit{Bifidobacteriaceae}. Both \textit{Ruminococcaceae} and \textit{Bifidobacteriaceae} are associated with health benefits because of their production of SCFA. Of interest, the human trial found a decrease of \textit{Lachnospiraceae}, whereas
*Lactobacillus* was found increased in mice, as stated above. The author suggests a difference in intervention for the discrepancies.

Colon cancer is thought to be beneficially effected by walnuts and microbiome studies have been conducted to look for its effects\(^{29}\). One study found a decrease in tumor size following a 7% walnut diet in male mice. Of interest, this effect was negated when walnut percentage was increased past 9%\(^{29}\). They found the walnut diet at 7% to increases in *Lactobacillus, Clostridiales, Clostridium, Lachnospiraceae*, and *Ruminococcaceae*. In groups past 9%, an increase in *Bacteriodes* was seen. A correlation analysis revealed Mice harboring the lowest number of tumors tended to have a reduced abundance of the *Bactroidetes* and *Lachnospiraceae* family and an increase in *Ruminococcaceae* and *Clostridium* XIVa genus\(^{29}\). The same group repeated their experiments and found three distinct gut microbiome enterotypes associated with cancer and walnut consumption. Mice with E2 induced by walnut consumption showed relative lower colon tumor burden, and included bacteria belong to the *Verrucomicrobiaceae* and *Lachnospiraceae* family, as well as *Porphymonadaceae, Lactobacillaceae, Ruminococcaceae*, and *Erysipelotrichaceae*\(^{30}\).

Decreased heart disease is linked to walnut nutritional composition. Studies have found enrichment of *Lachnospiraceae* following walnut and vegetable oils (in a fatty acid matched diet devoid of walnuts) were associated with improved cardiovascular risk factors\(^{31}\). It is suggested the ALA and linoleic acid found within the walnut affect the gut microbiome.

It is currently unknown what in walnuts causes the shift in microbial community towards more SCFA producing bacteria. It could be due to the 7% fiber content in the
walnuts, as traditionally seen prebiotics. However, non-dietary fiber source, such as polyphenols have been shown to have prebiotic effects.

2.1.4 Prebiotic Effects of Polyphenols

Polyphenols are bioactive substances from tea, fruits and veggies, cocoa and wine. They fight against oxidative stress in the body and prevent cancers, heart disease and inflammation. Around 90-95% of polyphenols reach the colon and are transformed into bioactive compounds by the gut microbiota. Several papers have shown polyphenols to increase *Akkermansia muciniphila* relative abundance, suggesting a prebiotic effect. *Akkermansia muciniphila* is a bacteria commonly associated with several health benefits, including weight loss and metabolic disease. Human consumption of red wine polyphenols significantly increased the relative abundance of *Bacteroides, Bifidobacterium, Enterococcus, Prevotella, Bacteroides uniformis, Eggerthellalenta*, and *Blautia coccoides-E. rectale* in the gut. Of interest, total cholesterol was significantly decreased, which was further correlated to changes in the *Bifidobacteria* number. Other humans’ studies have shown an increase in *Bifidobacteria* from consumption of a wild blue berry drink, Proanthocyanidin-rich extract from grape seeds and a resveratrol supplement. Other effects of polyphenols on gut bacteria include pathogenic inhibition. Tea polyphenols have been shown to modulate the gut community through the inhibition of pathogenic bacteria.

It is important to note the bile acid activity of polyphenols. Bile acids are important for the digestion and absorbance of lipids, along with the uptake of cholesterol and fat-soluble vitamins. Primary bile acids, chenodeoxycholic and cholic acid, are synthesized from cholesterol in the liver and moved to the colon, promoting absorption.
Deconjugation of bile acids, which makes them more readily absorbed, is catalyzed by bile salt hydrolase\textsuperscript{37}. Around 5-10\% of bile acid are not absorbed and metabolized by gut bacteria into secondary bile acids\textsuperscript{38}. Importantly, bile acids change the structure of the gut microbial community.

Numerous reports have described that polyphenols increases BA excretion, which corresponds to reductions in total and LDL cholesterol in animal and in-vitro studies\textsuperscript{37}. This could possibly be explained by an increase in bacteria which have bile salt hydrolase enzymes, as found in the three main phyla, Firmicutes, Bacteroidetes, and Actinobacteria\textsuperscript{37}. A study using a high fat diet on mice, along with catechin, curcumin, caffeic acid, rutin, ellagic acid, or quercetin, reduced the concentration of secondary BAs in the faeces\textsuperscript{39}. It is suggested polyphenols may have the ability to encourage the growth of bacteria which promote bile acid- deconjugation through bile salt hydrolase enzymes.

2.2 Two Sides of the Same Coin: Allergy and Asthma

2.2.1 Asthma and Food Allergy Pathology

\textit{Edited excerpt taken from Appendix 3: The Gut Microbiome & The Big Eight}

An allergen can be defined as abnormal immune response to the repeated exposure of certain antigens. Continuous exposure of the airways to inhalant antigens (asthma) or the GI to consumed antigens (food allergy), in sensitized individuals, may trigger an allergic reaction\textsuperscript{40}.

Allergic asthma is the most common phenotype of asthma. This is especially true in children. The disease is localized in the lungs, specifically the bronchial tubes, where they experience inflammation and remodeling. This leads to airway obstruction and over production of mucus. The clinical manifestations of asthma can be seen as repeated
episodes of wheezing, shortness of breath, and cough. Severe exacerbations (asthma attacks) may be life threatening and lead to the progressive loss of lung function. Common asthma antigens include pollen, molds, pests (dust mites, mice, cockroaches, dogs) and pets.

Food allergy has a similar pathology to asthma but occurs in the GI tract rather than the lung. It can manifest as minor gastrointestinal distress and skin rashes, to life-threatening anaphylactic shock. It is unknown why some individuals will develop an allergy to a specific antigen, while others will not. Food allergies have been rising at an alarming rate, nearing 6.5% (5% of adults and 8% of children) of the general population (of developed counties) affected to date. Of these food allergies, 90% are caused by “The Big Eight, a term referring to all major Food and Drug Administration (FDA)-regulated food products. These foods include cow milk, hen’s egg, fish, crustacean shellfish, tree nut, peanut, wheat, and soybean. As of January 1, 2023, the FDA will include sesame seed, for a total of nine major allergens.

For an allergic reaction to occur, the antigen must interact with the host immune system multiple times. One will not have an allergic reaction to an antigen the first time they come in contact. This process is defined as sensitization. Once the antigen has been introduced to the body, dendritic cells will interact with the antigen and proceed to T-cell locations to present the antigen to naive T cells, to form the classically seen Th2 cells. Th2 cells will produce certain cytokines, including IL-3, IL-4, IL-5, IL-9, and IL-13. From this, B cells will proliferate to produce IgEs, in place of other antibodies like IgG or IgM. These IgEs have a high affinity for FcεRI receptors found on mast cells and basophils and gear the cells up for further exposure, as sensitization is now complete. The processes of
sensitization can occur in several other locations in the human body, including the oral cavity, skin, and respiratory tract\textsuperscript{45}.

When there is reintroduction of the antigen, anaphylactic degranulation of the mast cells and basophils follows and there is a release of inflammatory mediators, including histamine, cytokines, and leukotrienes. At this point, the presence of the antigen in any body tissue (mouth, stomach, gut, lung) can induce an IgE mediated reaction that generally occurs a few minutes after exposure. Those with IgE mediated or mixed allergies can be identified based on the detection of food allergen specific IgE.

It has been hypothesized that the increase in allergies stems from the “hygiene hypothesis”, which states that early life exposure to microorganisms protect against allergic disease\textsuperscript{42}. The gut microbiome, termed a collection of microbiota and their genetic contents in the gastrointestinal tract, has been shown to play a part in the development of asthma, atopic dermatitis, and food allergies through mucosal tolerance and possible bacterial metabolites over the past decade.

2.2.2 Allergy and the Gut Microbiome

Both autoimmune and allergic diseases have increased in Western counties\textsuperscript{3}. Many believe this is a consequence of the “hygiene hypothesis”. This theory states that a lack of microorganisms (bacterial, viral, protozoa, and fungal) leads to reduced immune protection\textsuperscript{3}. This has been supported by research showing the protective nature of exposure to certain bacteria has against allergic disease. Current evidence suggest gut colonization with increased diversity promotes and maintains a balanced immune response in early development. The disruption of this process might have long-term health effects\textsuperscript{46}. There is evidence in both mice and humans of an early-life “critical
window” in which gut microbial dysbiosis’s effects are most prominent in immune development. This can be effected by a number of factors, including maternal and infant use of antibiotics and diet/breastfeeding. Disrupt in this critical window is thought to lead to increased asthma/allergy risk.

It is thought both the gut and lung microbiome are of importance when concerning the development and exacerbation of asthma. However, for the sake of this dissertation, only the gut microbiota will be disused. The connection between the lungs and gut has been repeatedly demonstrated in both human and mouse studies and has been termed the “gut-lung axis”. This connection was formed due to an observation of lung diseases being influenced by intestinal microbial changes, along with the gut microbiome of those with asthma differing from healthy counterparts.

Decreased abundances of genus *Lachnospira, Faecalibacteria, Roseburia, Lactobacillus rhamnosus* and *Bifidobacteria* have been associated with allergic asthma developed. An overabundance of pathogenic bacteria in the gut, including several species in the *Clostridium* genus, *Clostridium difficile, Clostridium neonatale* and *Clostridium coccoides*, along with *Bacteroides fragilis* have been correlated with asthma development.

Specific type of bacteria involved in the ecology of the gut is important. However, diversity and richness are thought to also contribute to decreased allergy. Like many diseases, decreased diversity has been linked to allergy occurrences.

For food allergy, alteration of the microbiota across “The Big 9” allergens (milk, egg, wheat, tree nut, peanut, finfish, shellfish, soy and now sesame seed) show a consistent, unique signature involving decreased allergy from the Firmicutes phylum.
Decreased diversity, antibiotic use and pathogenic bacteria have been associated as risk factors. Interaction of the microbiome and microbial metabolites (SCFA) with host immune response is likely the mechanisms by which the microbiome affects food allergy\(^3\).

There is currently not enough evidence to a recommendation the use of pre- or probiotics for treatment of food allergy or other allergic manifestations for humans. However, several animal studies have shown promise in alleviating both asthma and food allergy\(^3,52,53\).

There have been a few human trials studying the effect probiotics have when coupled with oral immunotherapy. The first randomized placebo-controlled trial on the effectiveness of a combination of *Lactobacillus rhamnosus* GG and peanut oral immunotherapy lead to 89.7% of those who received treatment to be desensitized, compared to 7.1% in the no bacterial control group\(^54\). *Lactobacillus rhamnosus* GG was used in a different peanut oral immunotherapy, with 67% of the children in the treatment group and 4% in the placebo desensitized following the end of the trial\(^55\). It has been proposed *Lactobacillus* promotes peanut tolerance by enhancing the tolerogenic effects of cells, including regulation of T cells.

### 2.2.3 Asthma and Frankincense

*Boswellia serrata* is a branching tree which produces a gum resin commonly known as frankincense\(^56\). The tree is native to the dry regions of India and the Middle East. Frankincense has a rich, centuries long history, being used in serval parts of the world for religious or medicinal proposes\(^56\). The active compounds of the resins are called the boswellic acids (BAs), a pentacyclic triterpenic\(^57\).
The BAs target a variety of cancers, inflammatory and bacterial diseases\textsuperscript{58–60}. This is possible due to its ability to function as a multitargeting agent, modulating several targets, including enzymes (5-LOX), growth factors (Vascular endothelial growth factor), kinases (I-κB kinases), transcription factors (STAT3), receptors (DR4), and others related to the survival and proliferation of cells (Myeloid leukemia 1)\textsuperscript{60}. The most potent and researched acid includes 3-O-acetyl-11-keto-b-boswellic acid (AKBA). Recently, AKBA was found to decrease neuroinflammation cause by lipopolysaccharide produced in the gut\textsuperscript{61}. However, the effects of AKBA ingestion on gut health has not been explored.

It has been well-established that AKBA has anti-bacterial and antifungal activity\textsuperscript{62}. However, the exact mechanism of antimicrobial action is not clearly established, as multiple studies have contradicting conclusions based on Gram stain, as inactivation does not seem to correlate with Gram status\textsuperscript{63–65}.

When AKBA has been used for asthma, it was found to decrease Th2 cytokines and OVA specific IgE production, along with airway hyperresponsiveness and inflammatory cell infiltration in mice\textsuperscript{10,58}. Of importance, a clinical trial found a daily treatment of 5-Loxin, an isolated AKBA supplement, to reduces pain scores and improves physical function\textsuperscript{66}.

2.2.4 Allergy, Viruses and the Gut Microbiome

\textit{Excerpt taken from Appendix 3: The Gut Microbiome & The Big Eight}

While not given the same amount of attention as the bacterial world in the gut, viruses and fungi may assist in the development and/or treatment of allergy. Asthma, hay fever, and peanut allergy were found inversely related to Hepatitis A, Herpes simplex virus 1, and \textit{Toxoplasma gondii} infections\textsuperscript{67}. Previous research has shown murine norovirus may
drive allergic disease, using egg ovalbumin, through changes in normal dendritic cell function. Interestingly, Kernbauer et al found murine norovirus infection of germ-free mice can replace the beneficial effects of commensal bacteria. Infection of murine norovirus 4 in non-obese diabetic mice was reported to led to positive changes in mucosal immunity, altering Tuft cell makers, cytokine secretion and mucosal antibodies. However, others have opposite conclusions, reporting norovirus and rotavirus infection may lead to increased protein absorption and sensitization in the gut. Diets of varying fiber and fat have been shown to change the virome, however, it is not well understood how these changes affect the human body. While dietary fungi have been known to cause allergic reactions, commensal fungi have not been studied for a possible cause or adjunct of disease. Allergy research must be conducted on these missing areas, as a major part of the human microbiome includes viral and fungal organisms.
CHAPTER 3

WALNUT-INDUCED CHANGES TO THE GUT MICROBIOME AND HOST METABOLISM

3.1 Introduction

*Juglans regia*, or Jupiter's nut, is a tree genus belonging to the walnut tree\(^2\). Walnuts are a globally popular tree nut with considerable economic importance to the food industry\(^2\). Along with popular use in traditional products, walnuts are emerging as a new plant-based protein alternative\(^3\). The popularity and “superfood” status of walnuts is largely due to their wide array of benefits on the digestive and neuropsychological systems\(^2,74\). In 2017, the FDA affirmed the body of walnut research and concluded that walnuts may reduce the risk of coronary heart disease\(^74\). Compared to other tree nuts, walnuts have the highest omega-3: omega-6 fatty acid profile (1:4.2) ratio. Walnuts also contain a high level of polyphenols, phytochemicals (phenolic acids, flavonoids, tannins, etc.) and fiber (6.4% soluble fructans)\(^25\).

Diet intervention is an easily controlled variable that can change gut composition and function. Although previous studies investigating the effects of walnut consumption on gut microbiota identified changes in different bacteria, a general trend towards higher levels of SCFA-producing bacteria from the Firmicute phylum was observed\(^27,28\). SCFA are thought to be primarily derived from microbial fermentation of insoluble fiber. However, polyphenols have been shown to also stimulate SCFA production, which are abundant in walnuts\(^37\). SCFAs act to regulate gene expression via their histone deacetylase activity and as well binding of GPCR receptors\(^75\). Cumulatively, these activities regulate immune function and act to control appetite. SCFAs also act as direct energy source,
providing 1.2–10% of the total energy obtained by an average western diet\textsuperscript{76}. Butyrate, the most abundant SCFA produced in the gut, has been shown to increase mitochondrial respiration and change substrate utilization\textsuperscript{77}. Polyphenols are also thought to regulate fat absorption and decrease cholesterol\textsuperscript{37}.

Here we investigate how a dietary intervention of a typical western diet in which walnuts substitute as primary source of calories from fats (saturated vs unsaturated, omega-3: omega-6 fatty acid) over a period of two months in mice. Our analysis focused on the effects of a western diet supplemented with 14% walnuts by total calorie on the composition of the gut microbiota as well as on basic metabolic parameters, such as respiration and energy utilization. Our early analysis has revealed increases in SCFA producing bacteria, increased fat utilization and changes in activity in mice. This may suggest walnuts serve as primary substrates that drive changes in gut microbial composition and function, likely by generating metabolites which increase energy efficiency.

3.2 Methods

3.2.1 Animal diet

C57BL/6 male mice, 8 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME, US) and housed in plastic cages with corncob bedding at the University of Connecticut Health (Farmington, CT). Animal rooms maintained a 12-h light/dark cycle and were pathogen free. Beddings from different cages was mixed every other day for a week for mice with the same gender to prevent microbiome cage effects and ensure a similar microbiome at the beginning of the study. Mice were then randomly assigned to either two groups, (1) 8 weeks of 14% walnuts or (2) control group that receives no
walnut (Fig 2.1). Whole raw walnut halves were provided by the California Walnut Commission (CA, USA) and placed into -20°C for storage. The whole raw walnuts were then finely ground and added to each diet. 14% walnut is consistent with a human dietary consumption of 56.6 g (2 oz) of walnuts, amounting to ~18% percent of total caloric intake based on 2000 calories per day. All control mice were maintained on a typical western diet (TWD). Contents of the fat sources were proportionally lowered to compensate for the addition of walnuts in walnut group.

3.2.2 Experimental timelines

Two different experiments were conducted using two different cohorts of mice. (1) Observing eight-weeks of walnut intervention on gut microbiota composition. The weight of two groups, walnut (n=5) and western control diet (n=5), were monitored weekly. Fecal pellets were collected before intervention (w0) and after intervention (w8). All fecal pellets were immediately frozen to -80°C until sequencing. (2) Observing the effect eight-weeks of walnuts on metabolic function. The weight of two groups, walnut (n=10) and western control diet (n=10), were monitored weekly. Before the being of the experiment, all groups were given the TWD without walnuts for two weeks. After, the walnut group was given the intervention for eight weeks. After this, all mice were given the control TWD again for an additional 4 weeks. Weight of the mice was taken every 2 weeks for 12 weeks.

3.2.3 Metabolic chamber

Respiratory exchange ratio, oxygen consumption, Co2 production, and spontaneous physical movement were measured simultaneously for each mouse in the second experiment, at week 8 and week 12, with a comprehensive laboratory animal
monitoring system (CLAMS) (Columbus Instruments, Columbus, OH) as described previously. All reported outcomes have been normalized to weight.

### 3.2.4 Processing of the 16S rRNA sequences

Raw 16S rRNA sequences (150-bp paired-end reads) were initially processed by BaseSpace software (Illumina, CA, US). For sample deconvolution, one mismatch was allowed in the primer and zero mismatches within barcodes. Deconvoluted sequences were then processed using the DADA2 data processing pipeline with default parameters to obtain amplicon sequence variants (ASVs). Final taxonomic assignment was conducted using RDP-classifier (v2.11) with a cut-off confidence value of 0.5. Reads with <0.5 confidence at a given taxonomical level were designated as “unclassified”.

### 3.2.5 Analysis of 16S rRNA sequence data

The relative abundance of ASVs agglomerated at the phylum and genus levels using the Phyloseq R package was visualized using a stacked bar plot. For simplicity, the relative abundance of only the 24 most abundant genera were displayed using a bar plot, with all others grouped into a 25th “other” category. All sample sequences were rarefied to 5,000 reads to account for uneven sampling depth. Alpha diversity was determined with the richness and Shannon diversity at the genus level. Wilcoxon sum rank test was performed to compare the alpha diversity of stool microbiomes between varying weeks. We utilized DESeq2 with significant values of adjusted p≤0.01 to identify specific taxa that were significantly enriched or depleted within specific samples. All plots were created with “ggplot2”, and all analyses were conducted in RStudio version 4.1.0.
3.3 Results

3.3.1 **Walnut supplementation does not alter bacterial diversity.**

Sequencing of all samples yielded 6,260,311 reads, with average of 26,303 reads per sample. Read clustering revealed 1924 unique ASVs which were then and rarefied to 5,000 reads per sample. No differences were found between baseline microbiomes between both groups and between individual cages, revealing that our approach of mixing bedding between cages to normalize the microbiome was effective (data not shown). Calculation of the Shannon diversity using the Shannon-Weiner species diversity index revealed increased microbial diversity after eight weeks of either the western diet (1.14± 0.18 vs 1.46±0.156, p=0.032) or the walnut diet (1.20±0.20 vs 1.80±0.12, p=0.0016) (Fig 3.1). However, richness remained unchanged after eight weeks of either diet.
Figure 3.1 (A-D) Diversity changes, shannon (A, C) and richness (B, D), for both walnut and western control diets between baseline and eight weeks. Phylum (E) and Top 25 genus (F) for both walnut and western control diets between baseline and eight weeks.

3.3.2 There was a clear composition variation between both groups following eight-week diet.

At the phylum level, both groups saw an increase in Actinobacteria following eight weeks of diet (DESeq2 p<0.01). The western control diet group also saw a decrease in Bacteroidetes and increase in Firmicutes (DESeq2 p<0.01). The
Firmicutes/Bacteroidetes ratio for the western control mice increased (2:8 to 3:7) following diet while the walnut mice’s ratio remained.

Composition at genus level varied between both groups at end point. However, both groups saw significant increases in similar bacteria, including Faecalibaculum, Bifidobacterium, and Lactococcus (DESeq2 p<0.01). Ileibacterium was also increased in both groups, however, the average relative abundance in the control groups (23.15±2.02) was vastly more than that found in the walnut diet (3.66±2.68).

Uniquely, the western control mice saw an increase in the Lachnospiraceae family. The walnut diet alone saw a significant increase in Bacteroidetes (DESeq2 p<0.01), making up a large portion of its relative abundance (26.2±12.64) (Fig 3.1). Anaerotruncus was also increased in the walnut group alone.

The western group saw significant decreases in Ruminococcus, Intestinimonas, Marvinbryantia and Muribaculaceae (DESeq2 p<0.01). Both groups decreased in Lachnospiraceae_NK4A136_group.

3.3.3  **Walnut supplementation changes energy production source with no effects on weight.**

The metabolic effects of walnut supplementation were conducted at week 8 and week 10. As was seen in the first experiment, both groups of mice steadily gained weight throughout the experiment (data not shown) and figure (Fig 3.2). No difference was seen in weight at any timepoint. Measurement of the respiratory exchange rate (RER) informs whether fat or carbohydrates serve as the primary energy source. As expected, the respiratory exchange rate (RER) was increased during active hours (night) for both groups at both eight and ten weeks (Fig 3.2). Although both groups of mice showed similar weight gains after eight weeks, mice consuming the walnut diet had a
significantly reduced RER for both day (0.77±0.2 vs 0.80±0.2, p=0.0047) and night (0.82±0.3 vs 0.88±0.2, p=0.0062) at week 8. Consistently, changing back to a western diet for four weeks after 8 weeks of walnuts had not effect weight. However, the RER at day (0.78±0.1 vs 0.78±0.1, p=0.8) and night (0.83±0.1 vs 0.83±0.1, p=0.65) no longer differed, indicating both groups were utilizing the same fuel source.

Figure 3.2 (A) Percent weight change between both walnut and western control following 14-week period. (B) average respiratory exchange ratio, (C) average heat, and (D) average ambulatory activity shown at both day and night for the first time point (eight weeks, walnuts being fed) and the second time point (ten weeks, walnut no longer in diet).

3.3.4 **Walnut supplementation of western diet increases activity without increasing energy expenditure and energy utilization efficiency.**

The average activity level was taken from a combination of x and y ambulatory movements as reported from the value of repeated broken beams due to movement. As
expected, both groups of mice saw an increase in activity at night at both week eight and ten, as mice are nocturnal. When mice were given walnuts for eight weeks, their activity was significantly higher than that of the western diet control group during the day (113.79±43.34 vs 54.78±22.35, p=0.01). At night, there was an average increase, although it was not significant (360.27±83.28 vs 279.44±104.21, p=0.83). Surprisingly, after switching back to a western diet for four weeks, the increased activity of the mice remained between groups at day (129.55±16.53 vs 100.07±22.33, p=0.015) and night (339.47±74.10 vs 251.10±71.61, p=0.83).

Heat production (kJ), representative of energy expenditure, was calculated from parameters obtained by indirect calorimetry. A linear regression was conducted against total activity. Heat was found to be directly related to activity in the walnut group at both time points (r = 0.38). However, no difference in heat was found between groups during both day and night at either of the time point (Fig 3.2). Mice on the western diet expended an average of 0.49±0.05kJ (8 weeks) and 0.46±0.05kJ (10 weeks) during the day and 0.59±0.05kJ (8 weeks) and 0.56±0.07kJ (10 weeks) during the night hours. Mice in the walnut group expended an average 0.50±0.03kJ (8 weeks) and 0.46±0.08kJ (10 weeks) during the day and 0.60±0.04kJ (8 weeks) and 0.57±0.09kJ (10 weeks) during night hours.

3.4 Discussion

Walnuts have been termed a “superfood” because of their high polyphenol and fiber content, and fat profile. We demonstrated an eight-week interventions of walnuts significantly changed the microbiome comparison of mice fed a higher fat, higher sugar or a “typical western” diet. Following walnut intervention, mice were shown to have
differed respiratory exchange ratio, and activity levels but similar energy expenditure than mice not fed walnuts. This suggests a walnut induced change in host metabolism.

Both groups of mice started out on a traditional chow designed to provide the required serving of all macronutrients needed for health development (18.6% protein, 6.2% fat, 44.2% carbohydrate). Mice were then switched over to a “typical western” diet, containing higher levels of fat and sugar (16.6% protein, 16.7% fat, 54.4% carbohydrate). Half the mice had 14% of their daily calories switched out for walnuts. When comparing both control western diet mice with the walnut mice, only the macronutrients type differed (based on walnut nutrition.) As such, its unsurprising that both groups of mice shared a similar increase and decrease in certain bacteria. This could be taken as a direct results of the increased fat and carbohydrates. Of interest, an increase in relative abundance of *Bifidobacteria* and *Ileibacterium* was seen in both groups.

*Bifidobacteria* is typically thought of as a beneficial probiotic bacteria, shown to have anti-inflammatory effects. It is curious to see a compositional increase of a bacteria often linked with health benefits increased by higher levels of fat and sugar. However, *Bifidobacteria* is known to metabolism simple sugars, such as sucrose. This addition of sugar may explain the increase in relative abundance across both groups.

*Ileibacterium* increased in both groups of mice, however, the western diet mice saw a much higher relative ambulance increase compared to the walnut group. Of importance, recent studies have shown that *Ileibacterium* responds strongly to a high-fat, high-sucrose diet, including increases up to 10-fold, comparable to our own study. *Ileibacterium* (gram-positive) is typically thought of as pathogenic bacteria, positively correlated with metabolic disorders and increased levels of LPS. However, their role
is still not well known. As the percent of *Ileibacterium* is much smaller in our walnut group, we hypothesis the addition of walnuts may have prevented the outgrowth of *Ileibacterium* brought on from the high fat, high sugar diet.

Walnuts may also encourage the growth of *Bacteroides*, which was found significantly increased in mice fed 14% walnuts, with a large relative abundance (26.2±12.64). Bacteroidetes (gram-negative) and Firmicutes (gram-positive) are the most abundant phyla in the human intestine\(^\text{20}\). Of interest, previously studies have shown a diet containing 9% or higher of walnuts to encourage the growth of *Bacteroides*\(^\text{29}\). Polyphenols, rich in walnuts, have also been shown to increase levels of *Bacteroides* in the gut\(^\text{34}\).

Members of the Bacteroidetes (*Bacteroides*) mainly producing acetate and propionate, while Firmicutes mostly produce butyrate\(^\text{20}\). Besides the conventional carbon sources, acetate is readily converted into acetyl-CoA within skeletal muscle. It has also been shown to increase resting energy expenditure and fasting fat oxidation\(^\text{83,84}\). In our study we saw our walnut mice have a shift in RER for higher fat oxidation and increased activity. As *Bacteroides* was so prevalent in the walnut mice, it is possible high levels of acetate were being produced following walnut inclusion, which in turn lead to changes in metabolism. However, both the walnut and western control groups had no difference in energy expenditure. This is of great interest, as the walnut mice had greater visible work output (activity levels) but used the same level of energy. Energy from the western control group might have been being utilized for something else inside the body. Other explanations for the difference between the metabolism of both groups could include
walnut included body composition changes and the difference in digestion of saturated vs polyunsaturated fats.

Changes in substrate utilization as presented by lower RER (fat utilization) in walnut mice could possibly be due to polyphenols purposed ability to inhibit key enzymes that are responsible for the digestion of dietary carbohydrates (α-amylase and α-glucosidase). Looking for carbohydrate levels in the stool of mice fed walnuts would be of interest.

Our study did have limitations due to a few items. (1) These findings were split into two separate studies. Our western diet/walnut inducted microbiome results are not the same mice found in our metabolism study. As such, we cannot confirm *Bacteroides* was found in the gut microflora of the metabolism tested mice. (2) In our gut microbial study, our baseline composition does not account for changes the higher fat, higher sugar diet had on the mice. However, our method did allow us to look at changes the higher fat, higher sugar diet had followed the controlled rodent chow.

Walnut’s effect on the gut microbiome should be furthered explored, including the persistence of walnuts induced microbiome changes and other metabolism related functions. As always, translation to humans is of great importance and should be considered.
Chapter 4

DIETARY BOSWELLIA SERRATA ALTERS THE GUT MICROBIOME AND BLOOD METABOLITES


4.1 Introduction

The gut microbiome, a collection of microbiota and their genetic contents from the gastrointestinal tract, has been shown to play an important part in health\textsuperscript{3,86}. Gut microbiome dysbiosis is thought to be linked to the development of disease and weight gain\textsuperscript{3,87}. Diet has a profound effect on the configuration of the gut microbiome. As a modifiable target, modulation of the gut microbiome through dietary intervention, including prebiotics or probiotics, have seen an increase in both research interest and product development in past years\textsuperscript{5}.

Gibson and Roberfroid first defined prebiotics as “selectively fermented ingredients that allow for specific changes, both in the composition of and/or activity in the gastrointestinal microflora that confer benefits upon hosts well-being and health”\textsuperscript{5}. Other criteria have been added to the definition of prebiotic, including being safe for ingestion and resistant to gastric acidity\textsuperscript{5}. Dietary fiber has been the most recognized prebiotic and include inulin, fructo-oligosaccharides, and other oligosaccharides\textsuperscript{5}. However, other non-fiber substances are increasingly being recognized as having prebiotic effects. Polyphenols are exhaustively metabolized by gut bacteria and are thought to produce beneficial by-products\textsuperscript{6}. Other studies have found rhubarb extract, a Chinese herb containing no fiber or polyphenols, to modulate the gut microbiome to increase Akkermansia muciniphila\textsuperscript{88}. 
*Boswellia serrata* is a branching tree that grows in the arid regions of India and the Middle East. These trees contain a gum resin commonly known as frankincense or olibanum. The use of *Boswellia serrata* varies, being utilized in cosmetics, materials, foods, and pharmaceutical products. For centuries, these gum resins have been used as a remedy for a variety of inflammatory and bacterial diseases. The active compounds are thought to be the boswellic acids (BAs) (pentacyclic triterpenic acids). BAs are commonly isolated and used in a variety of disease models and human research, including asthma, arthritis and several cancers. In recent years, BAs have been established as a multitargeting agent, modulating several targets, including enzymes 5-Lipoxygenase (5-LOX), cyclooxygenases 1 (COX-1), growth factors (Vascular endothelial growth factor), IkappaB kinase (I-κB kinases), transcription factors Signal Transducer and Activator Of Transcription 3 (STAT3), Death receptor 4 (DR4), and others related to the survival and proliferation of cells (Myeloid leukemia 1). Notably, BAs can inhibit 5-LOX and COX-1 activities by directly binding activity sites of these enzymes. The most potent and researched BA is 3-O-acetyl-11-keto-b-boswellic acid (AKBA). However, the effects of AKBA ingestion on gut health has not been explored.

It has been well-established that AKBA has anti-bacterial and antifungal activity. However, the exact mechanism of antimicrobial action is not clearly established, as multiple studies have contradicting conclusions based on Gram stain, as inactivation does not seem to correlate with Gram status.

In this study, we investigated the effect AKBA on the gut compositions of both health male and female mice. We also examined the antibacterial effect of AKBA through in vitro assays. Along with this, as changes in the gut microbiome are linked to changes in
circulating metabolites within the body, we also investigated differences in blood metabolites. Isolated AKBA itself contains no fiber or polyphenols and any changes to the gut microbiome would be due to a newly reported compound. To our knowledge, this is the first paper to report the effects of AKBA on the gut microbiome and the changes this causes in blood metabolites.

4.2 Materials and Methods

4.2.1 Animals

Eight-week-old BALB/cJ male and female mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US) and housed in plastic cages with corncob bedding at the University of Connecticut Health Center (Farmington, CT, US). Animal rooms maintained a 12-h light/dark cycle and were pathogen free. Mice were fed a 18% protein rodent diet (18.6% protein, 6.2% fat, 44.2% carbohydrate, 3.5% soluble fiber, 14.7% total fiber, 5.3% ash) purchased from Envigo, (Indianapolis, IN, US) and supplied autoclaved water. Bedding was mixed between all cages a week prior to start of experiment to homogenize the gut microbiomes within the same sex. Afterwards, mice within the same gender were randomized into AKBA groups (10 mice per group/5 mice a cage) and controls (5 mice per cage/5 mice per cage). For the AKBA group, mice were gavaged 200ul of 100mg/kg AKBA (pH 7) (AquaLOX, PLT Health Solutions, Morristown, NJ, US) once daily for 14 days (AKBA male n=10, AKBA female n=10). One cage from AKBA group was designated as AKBA1 and the other cage as AKBA2. For control groups, mice were gavaged with 200 ul PBS daily for 14 days (control males n=5, control female n=5). Because no previous studies have investigated effects of AKBA on the gut microbiome, 5-
10 mice /group were used, however, effect size and sample size could not be determined precisely.

Animal protocol 102063-0522 was approved by the University of Connecticut Health Center IACUC board.

4.2.2 Sample collection, DNA extraction and 16S rRNA amplification and sequencing

Fresh fecal pellets were collected upon defecation at Day 0 and Day 14, and stored in sterile RNA-/DNA-/RNAse-/DNAsefree microcentrifuge tubes at the beginning and end of experiment. Collected pellets were placed at −80 °C for storage following collection. DNA from pellets were extracted using PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany, Eur) according to the manufacturer’s protocol. Amplicon library preparation and sequencing were performed by the Microbial Analysis, Resources, and Services Facility at University of Connecticut, Storrs. Briefly, DNA extracts were quantified using the Quant-iT PicoGreen kit (Invitrogen, Waltham, MA, US). In brief, bacterial 16S rRNA gene was amplified using 30ng extracted DNA as template. The V4 region was amplified using 515F and 806R primers with Illumina adapters and dual indices (8 basepair)\textsuperscript{93}. Samples were amplified in triplicate 15ul reactions using Go-Taq DNA polymerase (Promega) with the addition of 3.3µg BSA (New England BioLabs). To overcome inhibition from host DNA, 0.1pmol primer without the indexes or adapters was added to the mastermix. The PCR reaction was incubated at 95°C for 3.5 minutes, then 30 cycles of 30 s at 95.0°C, 30 s at 50.0°C and 90 s at 72.0°C, followed by final extension of 72.0°C for 10 minutes. PCR products were pooled for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen). PCR products were normalized based on the concentration of DNA
from 250-400 bp then pooled using the epMotion 3075 liquid handling robot. The pooled PCR products were cleaned using Omega Bio-Tek Mag-Bind Beads according to the manufacturer’s protocol using 0.8x beads to PCR product. The cleaned pool was sequenced on the MiSeq using v2 2x250 base pair kit (Illumina, CA, US).

4.2.3 Processing of the 16S rRNA sequences

Raw 16S rRNA gene sequences were initially processed by BaseSpace software (Illumina, CA, US). One mismatch in primer and zero mismatch in barcodes were applied to sample deconvolution. Deconvoluted sequences were further processed using the DADA2 data processing pipeline with default parameters to obtain amplicon sequence variants (ASVs) 78. Final taxonomic assignment was conducted using RDP-classifier (v2·11) with 0.5 confidence value as cut-off. Reads with <0.5 confidence of classification was considered to be “unclassified” at a given taxonomical level.

4.2.4 Statistical Analysis of 16S rRNA gene data

ASV counts were converted to relative abundance and agglomerated at the phylum and genus levels using the Phyloseq R package to visualize the microbiome composition in a stacked bar plot. All 10 phyla were included in the barplot at phylum level, while the most abundant 24 genera were displayed in the barplot at genus level, including all additional genera grouped together in a 25th category as “other”. Sample reads were rarefied to 10,000 reads to account for uneven sampling depth.

Alpha diversity was determined with the Richness and Shannon diversity metrics. Wilcoxon sum rank test was performed to compare alpha diversity of stool microbiome between male and female mice from AKBA treated and control groups at day 0 and day 14. We also performed linear mixed regression with alpha diversity as response variable,
treatment, time points and their interaction term as fixed effect and individual mice as random effect.

Beta diversity was visualized by Principal Coordinates Analysis (PCoA) using the Bray-Curtis dissimilarity metrics. Microbiome community structure difference between two groups were determined by conducting PERMANOVA on Bray-Curtis dissimilarity and Jaccard indices using the Adonis function in the Vegan R package. Differential analysis of the relative abundance of various taxonomy in the microbial communities at the phylum and genus level was performed using LEfSe based on Linear discriminant analysis Effect Size (LDA)\(^4\). Effect size of differentially represented genera were represented by LDA scores. An adjusted p value <0.05 by false discovery rate (FDR) was considered statistical significance.

To identify relationships between baseline microbiome (\textit{Akkermansia} and \textit{Bifidobacterium}) at day 0 and the microbiome at day 14 in AKBA treated group, we performed a linear regression with cage as a covariate.

All plots were created with “ggplot2”, and all analyses were conducted in RStudio version 4.1.0.

4.2.5 Blood metabolome analysis with mass spectrometry

Mice were bled via cheek bleeding at the beginning and end of experiment. Blood was collected, allowed to clot at room temperature for an hour and spun down at 500g. The resulting serum was collected and stored at \(-80^\circ\text{C}\) until further analysis. For mass spectrometry preparation, serum samples were vortexed with 80% chilled aqueous methanol in a ratio of 1 (serum) :60 (methanol) (v/v). Protein was precipitated and centrifugated (14,000 g, 4 °C, 10 min). The supernatant was collected and dried under a
vacuum. The dried residues were reconstituted with 50% methanol and centrifuged (14,000 g, 4 °C, 15 min). Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) was performed at the University of Massachusetts Amherst (Amherst, MA, US) mass spectrometry facility to analyze the mouse serum for untargeted metabolomics. An equal volume of each serum sample was pooled to prepare the quality control (QC) sample. Five identical QC sample runs were conducted prior to running test samples, and one QC sample run was performed for every 8 sample runs throughout the experiment. Acquity UPLC HSS T3 column (2.1 mm×100 mm, 1.8 µm, Waters Co., MA, USA) was used to obtain chromatographic separation by injecting 5 µL aliquots of each sample. The column was maintained at 40 °C, with a flow rate of 0.5 mL/min. Solvent A contained 95% water with 5% acetonitrile and 0.1% formic acid and solvent B contained 100% ACN with 0.1% formic acid. The gradient started at 2% of solvent B and linearly increased to 95% at 8 min; once at 95%, solvent B was held for 2 min. Between the runs, the column was equilibrated at 2% of solvent B for 5 min. MS was conducted using the Thermo Fisher Orbitrap-Fusion in negative electrospray ionization mode at the detection range of 120-1000 m/z with 60000 full width at half maximum resolution. The following conditions were used for MS: spray voltage 3500 V, sheath gas flow rate 15 (arbitrary units), auxiliary gas flow rate 6 (arbitrary units), sweep gas flow rate 3 (arbitrary units), vaporizer temperature 275 °C, and ion transfer tube temperature 325 °C.

4.2.6 Identification of differently abundant metabolites and pathway activity

The raw data was analyzed by MetaboAnalyst (https://www.metaboanalyst.ca/). The metabolites of interest were identified by matching their profiles relating to accurate mass values, retention time, isotope peak matching, mass-to-charge ratio, and fragment
information against different databases, including ChemSpider (http://www.chemspider.com) and KEGG global metabolic network (https://www.genome.jp/kegg/). Pathway activity data was filtered by interquartile range, normalized by median, log transformed and set to an FDR cut off p-value of <0.05 using Fisher's exact test with pathways containing < 3 metabolites using Mummichog v2. Principal Coordinates Analysis (PCoA) were created with “ggplot2” in RStudio version 4.1.0. PERMANOVA was preformed between groups to a set p-value of <0.05.

4.2.7 Bacterial plating and load analysis

*Akermansia muciniphila* (ATCC BAA-835) and *Bifidobacterium pseudolongum* (isolated and identified in stools from mice treated with AKBA for 14 days) glycerol stocks were thawed and streaked onto a modified brain heart infusion (BHI) media that contains yeast extract, hemin, vitamin K1, l-cysteine and resazurin indicator. Following incubation for 24 hrs at 37°C in anaerobic conditions (7% H₂, 10% CO₂, N₂ balance), one colony was inoculated into 1 mL sterile BHI broth. Broth was incubated for 48 h at 37°C in anaerobic conditions. Subsequent 1:10000, 1:100000 and 1:1000000 dilutions were made and plated on to BHI plates containing either 0% wt/vol AKBA, 0.001% wt/vol AKBA, 0.01% wt/vol AKBA or 0.1% wt/vol AKBA. Agar plates were incubated for 48 h at 37°C in anaerobic conditions. Individual colonies were counted.

A baseline mouse stool pellet was vortexed in 1mL of sterile PBS. From that, 200 uL of stool slurry was added to six tubes of 5mL modified BHI media (as mentioned above). Half the tubes received AKBA, for a final concertation of 0.1% wt/vol. Broth was incubated for 48 h at 37°C in anaerobic conditions. Following incubation, 250uL of broth was extracted using PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany, Eur)
according to the manufacturer’s protocol. qPCR was then performed using NEB Luna® Universal qPCR Kit (New England Biolabs, Ipswich, MA). A 16S rRNA gene primer targeting the V4 region was used. Amplification was then performed for 2 mins of 95°C for enzyme activation, then 30 cycles at 95°C for 30 s and 60°C for 15 s. At least two reactions were performed per sample with at least two separate reactions run. Averages were taken between groups.

4.3 Results

4.3.1 Baseline Microbiome Composition

A total number of 2,202,430 high-quality reads were obtained for an average of 36,707 reads per sample. Reads were clustered into 665 unique ASV. At the phylum, we found a higher relative abundance of Actinobacteria phyla in female mice compared to male mice at baseline (Figure 4.1A), with the linear discriminant analysis score of 4.80 (p<0.05). (Figure 4.1D). At the genus level, we showed the top 24 most abundant genera in AKBA treated and control mice (Figure 4.1B). Linear discriminant analysis with LEfSe identified Bifidobacterium and Turicibacter were significantly higher in female mice, comparing to male mice (Figure 4.1E). The microbiome difference by gender was further supported by PERMANOVA statistical tests which indicated significant differences between male and female mice at baseline using the Bray-Curtis (p=0.019) and Jaccard (p=0.001) distance metrics, as illustrated by a PCoA plot (Figure 4.1C).

Additional PERMANOVA testing was performed to determine any microbiome differences between control and AKBA treated groups, for male and female mice separately at baseline. No significant differences were found between AKBA groups and
control group with either the Bray-Curtis or Jaccard metrics for male (p=0.412, 0.423) and female (p=0.138, 0.267) mice at baseline.

Based on these findings, it was chosen to analyze the male and female mice separately to compare differences in the microbiomes following AKBA or control treatment.
Figure 4.1 Figure 1A-1E. Stool microbiome composition at Day 0 baseline. (A) Bar plots at phylum level for AKBA1 (cage 1 in AKBA treated mice), AKBA2 (cage 2 in AKBA treated mice) and control groups. There is a higher relative abundance of Actinobacteria in female mice at baseline. (B) Bar plots of top 24 genera for AKBA1, AKBA2 and control groups. (C) Principal Coordinates Analysis (PCoA) plot using Bray-Curtis dissimilarity displaying strong clustering of female and male baseline microbiome samples. PERMANOVA results indicate significant differences using Bray-Curtis and Jaccard metrics. (D-E). Significant phylum and genera between male and female identified by linear discriminant analysis.

4.3.2 Modulation of male microbial communities with AKBA supplementation

We first compared bacterial diversity difference between AKBA treated group and control group at either baseline or Day 14. Shannon diversity was not significantly at both time points (Figure 4.2A). Bacterial richness was significantly lower in male mice after 14 days of AKBA treatment, compared with control male mice at Day 14 (Wilcoxon, p=0.045) (Figure 4.2C). Linear mixed model with individual mice as random effect and treatment, time points and their interaction as fixed effect showed richness changed differently over time in AKBA group and control group in male mice (p=0.02), with richness significantly decreased from baseline to Day 14 in AKBA group (p=0.005). Principal Coordinates Analysis (PCoA) using Bray-Curtis dissimilarity displayed strong clustering by treatment group at Day 14. PERMANOVA testing indicated significant differences between treatment groups at Day 14 with Jaccard (p=0.003) distance metrics, and marginal significant difference with Bray-Curtis (p=0.094) dissimilarity (Figure 4.2E).

Further analysis to evaluate AKBA-specific taxonomy differences in the treatment group microbiomes was performed. Bar plots of relative abundance of top taxa at the phylum and genus level indicated an increase in Akkermansia (phylum Verrucomicrobia) in AKBA-treated mice (Figure 4.3A,4.3B). LEfSe testing for differential abundance at the phylum and genus level further verified this finding (Figure 4.4A, 4.4B). Notably, there
was a high level of variation in the increase of *Akkermansia* among one male mice, which had no *Akkermansia* at baseline but had a 53% abundance increase following treatment. In addition, a decrease in abundance of the genera *Megasphaera*, *Agathobacter*, and *Ruminococcus gnavus* group was observed with AKBA treatment, as these genera were significantly higher in abundance in the control male mice (LDA scores of 4.18, 4.08, and 4.04, respectively). Unlike female mice, male mice contain no detectable *Bifidobacterium* at baseline, with only 4 mice showing limited relative abundance after treatment (0.01-0.4%)

**Figure 4.** (A-D) Alpha diversity was determined for male and female mice using the Shannon and Richness metrics. Significant differences were found between male AKBA and control treatment groups in Richness at Day 14. (E-F) PCoA plots using Bray-Curtis dissimilarity and PERMANOVA test to show strong clustering and indicate significant difference between treatment groups for male and female mice at Day 14.
4.3.3 Modulation of female microbial communities with AKBA supplementation

There were no significant differences in alpha diversity between AKBA group and control group at the baseline time point, or post-AKBA supplementation (Figure 4.2B, 4.2D). Alpha diversity in both groups were not changed significantly from baseline to Day 14 in either group. PCoA using Bray-Curtis dissimilarity showed strong clustering by treatment group at Day 14, and PERMANOVA tests yielded significant results with Bray-Curtis dissimilarity ($p=0.005$) (Fig 4.2F) and Jaccard ($p=0.026$) distance metrics.

Bar plots of relative abundance appear to show an increase in the Actinobacteria phylum in AKBA-treated mice at Day 14 (Figure 4.3A). Further LEfSe analysis revealed a significantly higher presence of Verrucomicrobia and Actinobacteria phyla at Day 14, and Akkermansia and Bifidobacterium genera (LDA scores of 4.96 and 5.04, respectively), along with a significantly lower presence of Anaeroplasma (LDA score of 5.26) (Figure 4.4D).
Figure 4.3 (A-B) Bar plots showing relative abundance of phyla (A) and genera (B) in AKBA treated and control male and female mice.
Figure 4. A-D. LDA effect size showing differential microbiota at the phylum level for male (A) and female (C) mice, and at the genus level (B, D) following 14 days treatment in AKBA and control groups.

4.3.4 Baseline *Bifidobacterium* and its response to AKBA treatment

*Akkermansia* and *Bifidobacterium* were found significantly enriched in female mice following 14 days of AKBA treatment, however, only *Akkermansia* was found significantly enriched in male mice (Figure 4.4). To determine whether the abundances of these bacteria at the baseline were associated their response to AKBA treatment at Day 14, we conducted a linear regression analysis with the relative abundances of these bacteria at baseline and at Day 14 after controlling for cages. We did not find any association between the abundances of *Bifidobacterium* and *Akkermansia* at baseline and day 14 in female mice in AKBA group after controlling for cages. No association was found for male mice baseline and endpoint *Akkermansia* after AKBA treatment.

4.3.5 AKBA displays an antimicrobial effect on overall bacteria load and

*Akkermansia* but no effect on *Bifidobacterium* in vitro.

Due to the observation of increased relative abundances of *Akkermansia* and *Bifidobacterium* after AKBA treatment, we further tested whether AKBA has any direct effect on the growth of these bacteria *in vitro*. We plated *Akkermansia muciniphila* (gram-negative) and *Bifidobacterium pseudolongum* (gram-positive) in the presence of different concentrations of AKBA in BHI plates (0%, 0.001%, 0.01% and 0.1%). Interestingly, *Akkermansia* only grew in control plates containing 0% AKBA (108±4 at $10^{-5}$ dilution), showing strong inhibition at even low levels of AKBA. By contrast, *Bifidobacterium* growth was not affected by any AKBA concentrations.
Next, we wanted to check AKBA’s overall effect on bacterial load. After inoculating mouse stool in either BHI or BHI+AKBA, we found a significant decrease in Cq values following qPCR. The BHI control group had an average Cq value of 16.16±0.44 while the BHI+AKBA had a Cq value of 21.11±1.01. Extraction, broth, and qPCR controls were negative.

4.3.6 AKBA induces changes in circulating blood metabolites in both male and female mice

To understand metabolite changes before and after 14-day treatment with AKBA, we performed untargeted MS and identified 397 metabolomic features for males and 406 metabolomic features for females. PCoA plots of metabolomic communities show are shown (Figure 4.5). However, at the whole metabolome level, PERMANOVA analysis showed no significant difference between groups (p-value >0.05, PERMANOVA).

Feature level change was observed (adjusted p-value <0.05, Wilcoxon) for both male and female mice. Males saw 4 significant feature changes (2 increases and 2 decreases following treatment) and females saw 12 changes (10 increases and 2 decreases following treatment). One metabolite change with an m/z and retention time of 163.0594_184.36 (Possible formulas C₄H₁₀N₃O₄ or C₁₀H₁₂S) was found decreased between both groups.

All metabolite feature change possible identities are shown in Fig S1. Control mice from both sexes had no significant changes (data not shown).

Prediction of pathways activities directly from LC-HRMS peaks was conducted using Mummichog (v2.0) in MetaboAnalyst 3.0. Activity in 2 pathways was predicted for female mice at a cut off p-value of <0.05; specifically, steroid biosynthesis (compounds hits 1/41, Fisher's exact test, p=0.08) and primary bile acid biosynthesis (compound hits
1/46, Fisher's exact test, p=0.08). Calcitretol, which is known to stimulates intestinal calcium transport or 3beta,7alpha-Dihydroxy-5-cholestenoate, a derivative from a bile acid, were shown to change with the AKBA supplementation. No pathways were identified in male or control mice.

![Figure 4.5](image)

**Figure 4.5** Principal coordinate analysis of blood metabolites in AKBA treated mice at Day 0 and Day 14 for male (A) and female (B) mice.

### 4.4 Discussion

Acetyl-11-keto-beta-boswellic acid consumption altered the gut microbiome composition of both healthy male and female BALB/cJ mice. Interestingly, only *Akkermansia* was affected in male mice given AKBA, increasing throughout. However, *Bifidobacterium* were increased, along with *Akkermansia*, in female mice fed AKBA. The increase in *Bifidobacterium* in females, while not in males, could potentially be gender specific. Female mice are thought to naturally have higher counts of *Bifidobacterium* in the gut. However, it should be noted the higher abundance of *Bifidobacterium* in female mice at baseline vs male mice. It is also important to note the difference in relative abundance of *Bifidobacterium* between cages within female mice.
*Bifidobacterium* is a probiotic bacterium that is abundant in the guts of children during the first year of life, and has been detected in lower levels in adults.

*Bifidobacterium* is known to secrete beneficial short chain fatty acids and lower inflammation.\(^9^5\) *Turicibacter* (part of the Firmicutes phylum) is commonly found in the intestines of animals.\(^9^6\) *A. muciniphila* is a mucin degrading, gram-negative, oval-shaped, non-motile, and strictly anaerobic bacteria. It makes up around 0.5-5% of the total microbial composition of the human gut and its population has been correlated with reduces in obesity, type 2 diabetes and inflammatory bowel disease.\(^9^7,9^8\) *A. muciniphila* is thought to be a “next generation probiotic”, meaning it displays characteristics that are suspected to promote health, but is not part of the lactic acid group of probiotics that have been heavily studied.\(^9^9\) As this study was conducted in healthy, young mice, future work should investigate the potential benefits of AKBA in disease and aging models. Many diseases, which AKBA has been traditionally used to treat (allergies, cancers), are known to have dysbiosis of the microbiome. *A. muciniphila* and *Bifidobacterium* have been shown to be negatively correlated with asthma and are thought to help protect against different forms of cancer.\(^1^7,1^0^0–1^0^2\).

*Akkermansia* and *Bifidobacterium* can be increased through changes in diet, with increased dietary fiber or possibly increases in polyphenols. However, the supplement of AKBA administered in this study contains neither of these, suggesting a different mechanism of action for the increase in these potentially beneficial bacteria than previously reported. To further test AKBA’s promotional effect on *Akkermansia* and *Bifidobacterium*, the bacteria were plated on different concentrations of AKBA. Interestingly, AKBA completely inhibited the growth of *A. muciniphila* at all
concentrations tested but had no effect on Bifidobacterium. AKBA has been used as an antimicrobial in different applications (skin, nails, teeth)\textsuperscript{63–65}. However, the exact mechanism of antimicrobial action of AKBA is not well-characterized. Seeing as \textit{A. muciniphila} is gram-negative (inhibited) and \textit{Bifidobacterium} is gram-positive (not inhibited), one could suspect that Gram status may influence sensitivity to AKBA; however, previous studies have demonstrated the antimicrobial effect of AKBA on an array of gram-positive and gram-negative bacteria and fungi\textsuperscript{63–65}. In this study, we found the bacterial load of a stool culture to be significantly lowered with the addition of AKBA. Larger concentrates of AKBA may be needed for different bacteria as well. It should be noted that the conditions found \textit{in vivo} (GI tract) and \textit{in vitro} (BHI plates) are vastly different environments and may affect bacterial growth conductions.

Overall richness was not affected by AKBA consumption in female mice but was found to decrease significantly in males. A decrease in recorded ASV is not surprising, as AKBA displayed antimicrobial effects in this study and previous reports, however, more bacteria residing in the females may be resistant to AKBA’s activity. Differential sex dependent baseline microbiomes may affect these endpoint results and should be considered for gender specific nutrition.

Although AKBA inhibited \textit{A. muciniphila in vitro}, it is possible AKBA removed other bacteria in the gut, enabling \textit{A. muciniphila} to opportunistically grow. When less bacteria are present surrounding the mucin layer of the colon, more mucin is available for \textit{A. muciniphila} to thrive on, increasing the relative abundance\textsuperscript{97}. It is also possible the antimicrobial impact of AKBA has less effect on the bacteria \textit{in vivo} near the mucin layer.
of the gut compared to bacteria in the lumen, resulting in a relatively higher abundance of mucin residents such as *Bifidobacterium* and *Akkermansia*.

*Bifidobacterium* levels in the gut have been reported to be inversely correlated with inflammation in disease models. The bacteria itself is thought to exhibit anti-inflammatory properties. AKBA is well known for its own anti-inflammatory properties. From this, it begs the question if an increase of *Bifidobacterium* is a direct effect from the body’s responses to decrease in inflammation, or if the increase in *Bifidobacterium* is a mediator of AKBA’s anti-inflammatory properties. Further research should be conducted to further elucidate the role of AKBA and *Bifidobacterium* on the anti-inflammatory effects of AKBA consumption observed here.

The blood metabolomics in mice changed after 2 weeks of AKBA supplement, with only 4 metabolites changing in males and 12 metabolites changing in females. However, both did share one metabolite change with an m/z and retention time of 163.0594_184.36. Most of the feature-level compounds found from our untargeted MS analysis were unidentifiable, either due no results in the database used or there being too many potential candidates to confidently identify a compound. Mummichog analysis identified two possible compound changes involved in steroid biosynthesis and primary bile acid biosynthesis in females. Changes in the gut microbial community can lead to changes in bile acids, with reduced bile acid levels in the gut being associated with bacterial overgrowth and inflammation.

Despite interesting and innovative findings in our study, there are several limitations. First, our findings were based on an unbalanced design which led to less statistical power when compared to a balanced design. Second, the study is only
conducted using one mice strain, one AKBA dosage, one intervention course. It would be interesting to test effect of AKBA on the microbiome using different strains of mice, and test whether the microbiome effect is dosage and time dependent. Third, the study was a preclinical study. Given the microbiome are different between mice and humans, how AKBA changes the gut microbiome or microbial metabolites in human is of great interest.

*Boswellia serrata* is defined as “generally recognized as safe” by the United States Food and Drug Administration. As such, future research on the prebiotic effect of AKBA should be explored. There is already an established relationship between herbal medicine and an increase in *Akkermansia muciniphila*. Several Chinese medicines, including rhubarb, houttuynia, *Ganoderma lucidum* and *Pueraria lobata* have been shown to increase *Akkermansia muciniphila* 

With the prospects of more commercial food product use, AKBA’s antimicrobial properties should be further explored. The effect of bacterial load on the gut during long term AKBA consumption, as well as the extent and persistence of AKBA changes to the gut microbiome, would be of interest.

**4.5 Conclusions**

To our best knowledge, this is the first work to demonstrate effects of the *Boswellia serrata* active compound AKBA on the gut microbiome in healthy mice. We observed different effects of AKBA consumption on the gut microbiome for both male and female mice, as well as the effects of the AKBA on blood metabolites. As AKBA has been observed to suppress *Akkermansia* growth and have no effect on *Bifidobacterium*, further work should be conducted on its direct effect on different bacteria and the gut microbiome as well as their mechanisms of action.
CHAPTER 5

DIETARY INDIAN FRANKINCENSE (BOSWELLIA SERRATA) AMELIORATES MURINE ALLERGIC ASTHMA THROUGH MODULATION OF THE GUT MICROBIOME


5.1 Introduction

Allergy is defined as an abnormal immune reaction to the repeated exposure of certain antigens. Continuous exposure of the airways to inhalant antigens, in sensitized individuals, may trigger allergic asthma. Allergic asthma is the most common phenotype of asthma, especially in children. The disease is characterized by inflammation and remodeling of the bronchial tubes, causing airway obstruction and over production of mucus. This leads to the clinical manifestations of asthma, including repeated episodes of wheezing, shortness of breath, and cough. Severe exacerbations may be life threatening and are associated with progressive loss of lung function. As asthma is associated with significant morbidity and mortality, there is a need to expand therapeutic options.

The “gut–lung axis” is a theory which connects the gut microbiome to lung health. The gut microbiome in early life development, including a broad bacterial diversity, is critical in the maintenance of proper immune balance throughout life. Dysbiosis of the gut microbiome has been associated with allergies. Supplementation of probiotics (Lactobacillus and Bifidobacterium) have shown beneficial effects in allergic asthma in animal models. These probiotics are thought to modulate the host immune cells, including T helper Th2, Th17, regulatory T (Treg) cells and B cells, which are directly associated to allergic asthma. Commensal gut bacteria have been
associated with decreased airway inflammation in animal model of asthma as well, including *Prevotella, Faecalibacterium* and *Akkermansia*\(^47,106\).

The use of probiotics as a therapeutic strategy for asthma in humans is not conclusive\(^4\). However, dietary interventions which selectively increase the abundance of microbes that provide metabolic benefits, such as short chain fatty acid production, is currently being investigated and may provide benefits supplementation of probiotics can not\(^103\).

*Boswellia serrata* is a branching tree which produces a gum resin commonly known as Indian frankincense or olibanum\(^56\). The plant is native to the dry regions of India and the Middle East. The resin has a rich, centuries long history, being used in serval parts of the world for medicinal proposes. The active compounds of the resins are thought to be the boswellic acids (BAs) (pentacyclic triterpenic acids)\(^56\). There are six different types of BAs, with 3-O-acetyl-11-keto-b-boswellic acid (AKBA) being most potent\(^57\). The BAs target a variety of cancers, inflammatory and infectious diseases\(^58–61,66\). BA have been shown to improve asthma phenotypes in mice models\(^58,107–109\), and have also shown promising results in a small clinical trial\(^17\). However, it is unclear what specific types of BAs were tested in these studies. BAs have been established as a multitargeting agent, modulating several targets, including enzymes (5-LOX), growth factors (Vascular endothelial growth factor), kinases (I-κB kinases), transcription factors (STAT3), receptors (DR4), and others related to the survival and proliferation of cells (Myeloid leukemia 1)\(^60\). Whether the gut microbiome is involved in the effect of BAs on asthma is unknown.
We recently showed the most potent BA, AKBA, to have a prebiotic effect on the gut microbiome, specifically increasing *Akkermansia* and *Bifidobacterium* in healthy mice (manuscript accepted). In this study, we investigated whether an oral supplement of AKBA could attenuate allergic asthma using an OVA allergic airway model in BALB/cJ mice, and if this response is mediated by changes in the gut microbiome.

5.2 Materials and Methods Induction

5.2.1 Induction of allergic airway model and AKBA supplement

Eight-week-old BALB/cJ female mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US) and housed in plastic cages with corncob bedding at the University of Connecticut Health (Farmington, CT.). Animal rooms maintained a 12-h light/dark cycle and were pathogen free. Mice were fed a 18% protein rodent diet (18.6% protein, 6.2% fat, 44.2% carbohydrate, 3.5% soluble fiber, 14.7% total fiber, 5.3% ash) purchased from Envigo (Indianapolis, IN, US) and supplied autoclaved water.

Allergic airway phenotype was induced by using an ovalbumin (OVA, hen egg) sensitization and challenge model as previously described with modifications. Briefly (Fig. 1), two groups of mice (OVA control and OVA + AKBA) were sensitized with intraperitoneal ovalbumin plus alum on days 0 and 7, challenged for 3 days (day 14 to day 17) via nebulized OVA at 1% in PBS for 30 mins, and harvested 48hr after final challenge. Supplementation groups (OVA + AKBA) were given 100mg/kg of water soluble AKBA (pH 7) (PLT Health Solutions, Morristown, NJ, US) in autoclaved mouse water, via gavage, every other day for 10 days and then every day for the remainder of the experiment (9 days). OVA control and naïve mouse continued to receive normal autoclaved mouse water. Mice were weighted at the beginning and end of the experiment.
Experiments involving AKBA were conducted twice, with the first group containing 4 mice per group and the second containing 15 mice per group (4 per group for naïve/negative control). Experiment procedures were identical, and results were pooled for n=20 (n=4 for naïve/negative control) (Figure 5.1A). Animal protocol 102063-0522 was approved by the University of Connecticut Health IACUC board.

5.2.2 Repetition of experimental procedures with B. pseudolongum supplement

Experiments were repeated, as stated above, with oral gavages of B. pseudolongum culture in place of AKBA supplementation (Figure 1B). Groups were designated as OVA control (n=10), OVA + B. pseudolongum (n=10) and naïve (n=5). OVA + B. pseudolongum groups were given $10^8$ CFU B. pseudolongum in culture medium via gavage, every other day for 10 days and then every day for the remainder of the experiment (9 days).

**Figure 5.1** (A) Experimental designs to observe the effects of the *Boswellia serra* compound AKBA (A) and B. pseudolongum (B) on an allergic airway model.
5.2.3 BAL, blood, and lung tissue collection

Serum was obtained through blood collect from mice via cardiac puncture at the time of sacrifice (euthanized with 150mg/kg ketamine and 10mg/kg xylazine) and was allowed to clot for a minimum of 30 minutes. Clotted blood was spun down at 1500 x g for 15 min. Serum was isolated and stored at −80 °C until further analysis. To obtain broncho-alveolar lavage (BAL) fluid cells from the mice, lungs were lavaged with 2 mL of saline. BAL cells were pelleted at 500 x g for 5 min at 4 °C. Red lysis buffer was used to remove any residual red blood cells. Cell free fluid was saved separately and stored at −80 °C. Total cell counts were taken manually using a hemocytometer. Cytospin preparations were fixed in methanol and stained using May-Grunwald and counter stained with Giemsa. For histopathologic assessment, the left lungs were removed and fixed with 4% buffered formalin for 72 h and then placed in 70% ETOH.

5.2.4 Flow cytometry and IgE

Multiple inflammatory mediators were analyzed in the cell-free supernatant of BAL fluid using a multiplex flow-cytometry based assay according to manufacturer’s recommendations (BioLegend, San Diego, CA, US). Volume of cell free fluid was concentrated using a 50K centrifugal filter spun at 3000g for 10-15 minutes. Samples were run on a ZE5 Cell Analyzer (Bio-Rad, Hercules, CA, US) and sample processing was conducting using software provided from the kit’s manufacturer (BioLegend, San Diego, CA, US). Serum specific ovalbumin IgE levels was detected by using an ELISA kit with a sensitivity of 3.8 ng/mL per manufacturer’s protocol (Cayman Chemical Company, Ann Arbor, MI, US).
5.2.5  **Tissue histology and scoring**

Lung sectioning and staining was performed by the UConn Health Research Histology Core (Farmington, CT, US). Formalin-fixed, paraffin-embedded lungs were sectioned and stained with hematoxylin and eosin (for gross pathology) and periodic acid-Schiff with hematoxylin counterstain (for mucus production). Sections from the left lung were evaluated in their entirety and representative were images taken. Pathologic scoring was performed as described previously\textsuperscript{111}. Briefly, a blinded pulmonologist graded each stain using a light microscope. For inflammation scores, 0 corresponded to no detectable inflammation; 1 to mild peribronchiolar/perivascular cuffing with inflammatory cells; 2 to significant peribronchiolar/perivascular clustering; and 3 to significant clustering and airway remodeling (e.g., smooth muscle hypertrophy and hyperplasia). For mucus scores, 0 corresponded to no visible mucus; 1 to occasional and punctate mucus staining in the airways; 2 to presence of ring-like mucus structures in <10% of airways; and 3 to presence of ring-like mucus structures in >10% of airways. Half scores were permitted\textsuperscript{111}.

5.2.6  **Measurement of AHR**

Airway reactivity was assessed based on total respiratory system resistance (Rrs) response to increasing doses (0–100 mg/ml) of acetyl-β-methacholine chloride (Sigma-Aldrich, St. Louis, MO). Mice were euthanized (150mg/kg ketamine and 10mg/kg xylazine), underwent tracheostomies and were mechanically ventilated using the flexiVent system (SCIREQ, Montreal, CA). Airway reactivity was determined by assessing forced oscillatory mechanics every 10 s for 4 min following each methacholine challenge.
5.2.7 Analysis of immune responses

Means from the groups were analyzed for statistical significance by using the Mann-Whitney test, one-way analysis of variance or an unpaired t-tests. The significance level for tests included p < 0.05 and were preformed using Prism 7 (GraphPad Software, La Jolla, CA).

5.2.8 Sample collection, DNA extraction and 16S rRNA amplification and sequencing

Fresh fecal pellets were collected upon defecation and stored in sterile RNA-/DNA-/RNAse-/DNAsefree microcentrifuge tubes at the beginning and end of experiment (Fig 1). Collected pellets were placed at −80 °C for storage following collection. DNA from pellets were extracted using the PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Amplicon library preparation and V4 region of 16S rRNA gene sequencing were performed at Illumina HiSeq 4000 (Illumina, CA, US) platform.

5.2.9 Processing of the 16S rRNA sequences

Raw 16S rRNA sequences (150-bp paired-end reads) were initially processed by BaseSpace software (Illumina, CA, US). One mismatch in primer and zero mismatch in barcodes were applied to sample deconvolution. Deconvoluted sequences were further processed using the DADA2 data processing pipeline with default parameters to obtain amplicon sequence variants (ASVs) 78. Final taxonomic assignment was conducted using RDP-classifier (v2·11) with 0·5 confidence value as cut-off. Reads with <0·5 confidence of classification was “unclassified” at a given taxonomical level.
5.2.10 Analysis of 16S rRNA sequence data

ASV counts were converted to relative abundance and agglomerated at the phylum and genus levels using the Phyloseq R package to visualize the microbiome composition in a stacked bar plot. All 10 phyla were included in the bar plot at phylum level, while the most abundant 24 genera were displayed in the bar plot at genus level, with additional genera being grouped together in a 25th “other” category. Sample reads were rarefied to 10,000 reads to account for uneven sampling depth. Alpha diversity was determined with the richness and Shannon diversity metrics at the ASV level. Wilcoxon sum rank test was performed to compare alpha diversity of stool microbiome between asthma control and AKBA treated mice at day 0 and day 19. Beta diversity was visualized by principal component analysis (PCA). DESeq2, with an adjusted p value<0.05, was used for the differential taxa analysis. All plots were created with “ggplot2” and all analyses were conducted in RStudio version 4.1.0.

5.2.11 Bifidobacterium Isolation and Identification

Mouse feces stored at -80°C were thawed and mixed with 1 mL sterile peptone water. Subsequent 1:10 and 1:100 dilutions were made using the suspended fecal slurry and sterile peptone water. Approximately 150 uL was introduced to Bifidobacterium-specific medium (BSM) agar containing 55 g/L DeMann Rogosa-Sharpe agar (BD Difco, NJ, US), 15 g/L agar, 0.5% (wt/vol) L-cysteine hydrochloride (Sigma-Aldrich, MO, US), and 0.5% (wt/vol) mupirocin (AppliChem Panreac, IA, US) to select for Bifidobacteria. 112,113 Agar plates were incubated for 48 h at 37°C in anaerobic conditions (7% H₂, 10% CO₂, N₂ balance) (Coy Labs, MI, USA). Individual colonies were selected and grown on BSM plates under the same conditions. Upon confirmation of uniform
colony morphology, individual colonies were selected and grown on MRS plates supplemented with 0.5% (wt/vol) L-cysteine hydrochloride under the same conditions.

Individual colonies were selected from MRS plates and grown in MRS broth supplemented with 0.5% (wt/vol) L-cysteine hydrochloride for 14 h under the same conditions. Liquid cultures were preserved as 25% (vol/vol) glycerol freezer stocks at -80°C. Isolated liquid cultures were extracted using the Quick-DNA Fungal/Bacterial Kits (Zymo, Irvine, CA, US).

Following extract, DNA was amplified using a qPCR Master Mix (New England Biolabs, Ipswich, MA) and ITS targeted primers (Forward – CAAGGCCATCAACTGTTCA and Reverse ACGTCGTGCTGCTCGAATGT\textsuperscript{114,115}) using cycling conditions of 95°C for 3 min, 25 cycles of 95°C for 30 secs, 55°C for 30 secs and 72°C for 1 min and then a final extension of 72°C for 5 min. Samples were cleaned using a PCR cleanup kit (Qiagen, Hilden, Germany) and sent to Genewiz (South Plainfield, NJ, US) for sanger sequencing. Species identification was then confirmed using sequence files in Basic Local Alignment Search Tool software (GenBank, NIH, Bethesda MD, US) against the NCBI Reference Sequence database.

5.3 Results

5.3.1 AKBA supplementation decreased weight and the severity of tissue inflammation in the lungs

Mice were given 100mg/kg of AKBA enterally as described (Fig. 5.2A) and experienced a significant decrease in weight when compared to both the OVA control group and naïve group (unpaired t.test, p=<0.0001) (Fig 5.2A). OVA + AKBA saw a total average loss of -2.32±4.19% of starting weight while the OVA control and naïve group saw a positive weight gain of 8.36±6.18% and 5.04±0.71% respectively.
OVA exposure led to evidence of peribranchial/perivascular and inflammation in the lungs (Fig. 5.2B), specifically clusters of inflammatory cells surrounding the airways, mucus buildup and airway smooth muscle. Blinded comparison of the inflammation and airway smooth muscle remodeling revealed that OVA+AKBA mice had slightly less severe histopathologic changes than OVA control mice (2.0±0.49 vs. 2.5±0.24, unpaired t.test, p=0.1823). However, the OVA+AKBA animals showed a significantly lower mucus production in mucus scoring (2.0±0.4 vs. 2.5±0.2, unpaired t.test, p=0.0278).

5.3.2 AKBA attenuated ovalbumin-dependent allergic airway inflammation with decreased airway hyper-reactivity

Exposure to OVA induced allergic airway disease caused airway hyper-reactivity and bronchoconstriction in response to the receptor agonist methacholine (Fig. 5.2C). No significant difference was seen between all three groups at 0mg/mL (ANOVA test, p=0.341). Experimental assessment of respiratory function using the FlexiVent system revealed that OVA+AKBA animals had significantly lower increases in total respiratory system resistance in response to methacholine challenge when compared to OVA control animals at 12.5mg (2.38±0.55 vs 4.65±0.94, unpaired t-test, p=0.02) and 25mg (3.56±1.25 vs 9.01±3.07, unpaired t-test, p=0.04). No significant difference was found between OVA control and OVA+AKBA at higher concentrations. However, trends are seen with OVA+AKBA having lower averages of lung resistance when compared to the OVA control group. Naïve mice were significantly less reactive than the OVA control group at concentrations of 12.5mg/mL (unpaired t-test, p=0.02), 25mg/mL (unpaired t-test, p=0.03) and 100mg/mL (unpaired t-test, p=0.03). Importantly, the OVA+AKBA groups was not significantly different from control groups at any concentration.
A

OVA Sensitization

OVA Sensitization

OVA Challenge

Sacrifice

Day 0

7

14 - 16

19

Stool Collection

Groups
OVA control (n= 20)
OVA + AKBA (n= 20)
Naïve (n= 4)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (g)</th>
<th>Endpoint (g)</th>
<th>Overall % change</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA control</td>
<td>19.93±1.02*</td>
<td>21.59±1.85*</td>
<td>+8.36±6.18%*</td>
</tr>
<tr>
<td>OVA + AKBA</td>
<td>19.83±1.23*</td>
<td>19.37±1.20*</td>
<td>-2.32±4.19%</td>
</tr>
<tr>
<td>Naïve</td>
<td>19.35±0.94*</td>
<td>20.33±1.12*</td>
<td>+5.04±0.71%*</td>
</tr>
</tbody>
</table>

B

OVA control (Hematoxylin and eosin)

OVA + AKBA (Hematoxylin and eosin)

Histochemistry score

OVA control (Periodic acid-Schiff)

OVA + AKBA (Periodic acid-Schiff)

Mucus score

C

Lung Resistance (cmH2O.s/mL)

- OVA control
- OVA + AKBA
- Naïve

Methacholine mg/mL

0.04

0.02

0.01

0.00
**Figure 5.2** (A) Induction of allergic airway model, AKBA supplementation course and average weight (g) of mice and percent change following the duration of experiment. Colored lines corresponding to AKBA/PBS supplementation. Values in each column which have different letters are significantly different (p<0.05). (n=15). (B) Representative pictures of both OVA control and OVA + AKBA lung histology and scoring (n=5 mice/group). (C) Total respiratory system resistance (Rrs) following increasing doses of aerosolized methacholine. Data represent mean ± SD. n = 4 all groups. *Significant differences are labeled for OVA control vs. OVA+AKBA mean.

### 5.3.3 AKBA attenuated ovalbumin-dependent allergic airway inflammation with decreased concentrations of inflammation markers

Our allergic airways model was able to produce elevated white blood cells in the BAL of all mice sensitized and challenged with OVA. The OVA control group of mice had 78.88% (Mann-Whitney test, p=0.0008) more leukocyte counts than the naïve group. Total leukocyte counts were significantly lower in those given 100mg/kg AKBA versus the OVA control group by 46.69% (Mann-Whitney test, p=0.0002)(Fig 5.3A). However, the AKBA group had higher counts than the naïve control, by 60.38% (Mann-Whitney test, p=0.0212).

Cell type in BAL also differed between the 3 groups. Both OVA sensitized groups of mice (OVA control and OVA + AKBA) showed a significant increase in airway eosinophilia when compared to naïve mice. Percent eosinophil was lower in OVA + AKBA groups, having 31.4% eosinophil percentage compared to the 48.9% concentration the OVA control group displayed (Figure 5.3B). As expected, no eosinophils were found in the naïve group. Absolute number of eosinophils/ml showed mice from the OVA control group had significantly higher counts (48934.39± 8321.83 eosinophil/mL) when compared to the AKBA group (17317.95 ± 5552.67 eosinophil/mL) (Figure 5.3C.)
Figure 5.3 (A) BAL fluid was analyzed for total cell number in three groups of mice as follows: mice that were sensitized and challenged with ova (column 1) (n= 20); mice that were sensitized and challenged with ova and treated with AKBA solution (column 2) (n= 19); naive mice (column 3) (n= 4); Values represent mean ± SD. (B) Cell differentiations were averaged and based on percentage (naïve n=4, OVA control and OVA + AKBA = 20). (C) Total eosinophil was calculated representing mean ± SD (OVA control and OVA + AKBA n=20). (D-G) BAL fluid was analyzed for cytokine makers in both experimental groups of mice (n=14-15). Values represent mean ± SD. *Indicates a significant difference between AKBA-treated mice vs OVA control (p= 0.05 (H) Serum was taken at sacrifice for assessment of OVA-specific IgE using anti-OVA ELISA. OVA control and OVA + AKBA (n = 10) values represent mean ± SD. Naïve animals (n = 4) are represented by the dotted line. Indications of significant difference include ** as p= 0.05, *** as p= 0.001 and **** as p= <0.0001.

Four cytokine markers were measured using flow cytometry of the BAL fluid in both OVA control and OVA + AKBA groups (Fig 5.3E-H). The OVA + AKBA groups showed significant lower levels of classic asthma markers IL-5 (62.53±43.62 ng/mL vs 31.39±17.71 ng/mL, Mann-Whitney test, p=0.0079) and IL-4 (18.72±14.72 ng/mL vs
9.30±6.67 ng/mL, Mann-Whitney test, p=0.0229) when compared to the OVA control group. No significant difference was found between the groups when observing IL-13 (6.81±6.15 ng/mL vs 4.15±3.15 ng/mL) or IL-10 (14.54±11.61 ng/mL vs 19.77±12.19 ng/mL). However, trends are seen, with a decrease in IL-13 and increase in IL-10 (anti-inflammatory) for OVA + AKBA groups.

Allergen-specific serum IgE is a hallmark of allergic asthma and allergic airway disease. As such, serum OVA-specific IgE appeared following OVA exposure, with non-detectable levels in naïve animals (Fig. 5.3H). However, no difference was found between OVA control and OVA + AKBA groups of mice (1120.71±433.94 ng/mL vs 1275.98±503.22 ng/mL Mann-Whitney test, p=0.3223).

5.3.4 AKBA supplement inhibits loss of microbiome diversity in OVA induced allergic airway model

A total number of 4,604,531 high-quality reads were obtained for an average of 68,724 reads per sample. Reads were clustered into 16,920 unique ASV. There was a significant decrease in the Richness measure of alpha diversity (p=0.0079) and Shannon diversity (p=0.0079) from OVA control mice when compared between baseline and endpoint (Figure 5.4A, 5.4B), suggesting the allergic asthma model is associated with dysbiosis of the gut microbiome. Twenty-four different genera were found significantly decreased in OVA control mice between baseline and end point (DESeq, p<0.05) (Supplementary Table 1). None of these bacteria were decreased in the OVA + AKBA group. As such, the OVA +AKBA group maintained its overall richness and Shannon diversity from baseline to the endpoint (Figure 5.4A, 5.4B). No significant difference was found between the groups at the same time point; However, OVA+AKBA group had a trend of higher diversity than OVA control group (p=0.095) at the endpoint (Figure 4C,
Both OVA and OVA + AKBA were distinguishable by analysis of PC1 and PC2 at endpoint (Figure 5.4E). No difference was found between baseline microbiomes for both groups for diversity and baseline bacterial composition.

**Figure 5.4** (A-D) Alpha diversity was determined for OVA control and OVA+AKBA mice using Shannon and Richness metrics. (E) PCA plots by analysis of PC1 and PC2 show distinguishable clustering for both OVA+AKBA and OVA control mice at endpoint. (F-G) Bar plots showing relative abundance changes between baseline, endpoint, OVA control and OVA+AKBA for phylum (F) and genus (G).
5.3.5 Dietary AKBA supplement significantly increases relative abundances of Bifidobacterium

A difference was observed between baseline and endpoint at both phylum and genus level for the OVA control group of mice (Fig 5.4F, 5.4G). At the phylum level, Proteobacteria was found significantly diminished, along with Verrucomicrobia and Actinobacteria in OVA control mice. However, Firmicutes and *Lactobacillus* were found significantly increased (DESeq, p<0.05). Interestingly, we found significantly higher relative abundances of Actinobacteria phyla and *Bifidobacterium* genus in OVA+AKBA vs OVA control mice (Fig 5.4F, 5.4G). *Bifidobacterium* increased by a 15.94±15.84-fold change in the OVA+AKBA while only increasing by a 0.75±1.27-fold change in the OVA control (Wilcoxon test p= 0.07). Other six bacteria, with relatively lower abundances than *Bifidobacterium*, were also significantly changed in AKBA treated mice between baseline and endpoint, including increases in *Lactiplantibacillus*, *Lacticaseibacillus* and *Turicibacter*, along with decreases in *Hyphomicrobium*, *Solirubrobacter* and *Steroidobacter* (DESeq, p<0.05).

5.3.6 *Bifidobacterium* ASV was identified as *Bifidobacterium pseudolongum*

Only one *Bifidobacterium* ASV was shown to increase in our experiments. To further identify the taxonomy of this *Bifidobacterium* ASV, we combined all 5 OVA + AKBA stool pellets at the endpoint and isolated 15 random *Bifidobacterium* colonies using *Bifidobacterium* selective media. All 15 bacterial samples were identical, and the ITS sequences are most closely related to *Bifidobacterium pseudolongum* strain UMB-MBP-01 in NCBI nucleotide database with accession number CP022544.1 (98.92% identity). To explore whether *B. pseudolongum* has mediated protection of asthma in AKBA
treated mice, the allergic airway model was repeated with oral gavages of $10^8$ CFU *B. pseudolongum* (Figure 5.5B).

![Diagram](image)

**Figure 5.5** (A) Induction of allergic airway model, *B. pseudolongum* supplementation course and average weight (g) of mice and percent change following the duration of experiment. Colored lines corresponding to $1 \times 10^8$ *B. pseudolongum* oral supplementation. Values in each column which have different letters are significantly different (p<0.05). (n=10 for experimental groups, n= 5 for naïve group). (B) Percent relative abundance of *B. pseudolongum* at both baseline and endpoints. Ova + *B. pseudolongum* were given $1 \times 10^8$ *B. pseudolongum* supplementation according to experimental design.

### 5.3.7 *B. pseudolongum* attenuated ovalbumin-dependent allergic airway inflammation with decreased concentrations of inflammation markers

Considering we found AKBA supplementation significantly decreased body weight in mice, we first examined whether *B. pseudolongum* treated mice captured a similar phenotype (Fig 5.5A). Indeed, mice given *B. pseudolongum* were found to gain weight significantly slower than both the OVA control and naïve group of mice (unpaired t-test, p=0.013, p=0.007). No weight change or percent change was seen between the OVA control and naïve group (unpaired t.test p=0.75).
Next, we measured airway inflammations in *B. pseudolongum* treated mice as we had done in the AKBA experiment. The OVA control group of mice had 92.2% (Mann-Whitney test, p= 0.0010) more leukocyte counts than the naïve group. The *B. pseudolongum* group also had significantly higher counts than the naïve control, by 87.0% (Mann-Whitney test, p=0.0016). However, total leukocyte counts were significantly lower in those given *B. pseudolongum* versus the OVA control group by 36.1% (Mann-Whitney test, p=0.036) (Fig 5.6A).

Cell type also differed between the 3 groups. OVA challenged mice showed a significant increase in airway eosinophilia when compared to naïve mice (75311.21±16642.62 cells/mL vs 0 cells/mL). Percent eosinophil was lower in OVA + *B. pseudolongum* groups (62381.80± 8869.84 cells/mL), having 44.6% eosinophil percentage compared to the 55.0% concentration the OVA control group displayed (Fig 6B, 6C). As expected, no eosinophils were found in the naïve group. *B. pseudolongum* did not affect ovalbumin-blood IgE levels (Supplementary Fig. 1B).
Figure 5.6 (A) BAL fluid was analyzed for total cell number in three groups of mice as follows: mice that were sensitized and challenged with ova (column 1) (n= 10); mice that were sensitized and challenged with ova and treated with B. pseudolongum (column 2) (n= 10); naive mice (column 3) (n= 5); Values represent mean ± SD. (B) Cell differentiation were counted and based on percentage. (C) Total eosinophil was calculated representing mean ± SD. (D-G) BAL fluid was analyzed for cytokine markers in both experimental groups of mice (OVA n=10, OVA + B. pseudolongum n=8). Values represent mean ± SD. Indications of significant difference include ** as p= 0.05, *** as p= 0.001 and **** as p= <0.0001.

Four cytokine markers were measured using flow cytometry of the BAL fluid in both OVA control and OVA + B. pseudolongum groups (Fig 5.6D-G). The OVA + B. pseudolongum group showed significant lower levels of IL-5 (69.50±16.67 ng/mL vs 34.03±25.24 ng/mL, Mann-Whitney test, p=0.0079) when compared to the OVA control group. No significant difference was found between the groups when observing IL-4
(40.08±9.70 ng/mL vs 24.40±18.24 ng/mL) IL-13 (14.05±4.30 ng/mL vs 11.88±8.00 ng/mL) or IL-10 (10.37±5.82 ng/mL vs 14.43±12.70 ng/mL). However, encouraging trends are seen, with a decrease in IL-4 and IL-13 and increases in IL-10 (anti-inflammatory) for OVA + B. pseudolongum.

5.3.8 Successful colonization of B. pseudolongum and its impact on other microbial compositions

To determine colonization status of B. pseudolongum, we profiled the gut microbiome before and after treatment in OVA + B. pseudolongum and OVA control groups. We found that the OVA control and the OVA + B. pseudolongum group had a significant decrease in Shannon diversity between baseline and endpoint (Wilcoxon, p=0.0029, p=0.0068. Supplementary Fig. 1C).

As expected, the top taxa at the phylum and genus showed a higher relative abundance of Actinobacteria phyla and Bifidobacterium genus in OVA+ B. pseudolongum vs OVA control mice at the end of the experiment (DESeq p<0.05). There was an average of 0.233± 0.56% and 1.393 ± 1.52% relative abundance in the starting OVA and OVA + B. pseudolongum mice, respectfully. At the end of the experiment, there was 1.29±1.737% and 5.65±4.820% relative abundance found within the gut (Figure 5.5B). This data suggests that Oral gavages of B. pseudolongum colonized successfully in the guts of the OVA + B. pseudolongum group of mice.

To test whether colonization of B. pseudolongum affected other bacterial compositions, we performed a comparison between baseline and endpoints compositions. Both groups shared changes in microbiota, including increases in the genus Lactobacillus, Eubacterium ventriosum and decreases in Oscillibacter (DESeq p>0.05). The Erysipelatoclostridium genus was found increased only in the OVA + B.
pseudolongum group (Supplementary Fig. 1C). No differences in bacterial abundance or diversity were found between OVA control and OVA + B. pseudolongum at baseline.

5.4 Discussion

A water-soluble dietary form of frankincense (AKBA) was shown to help alleviate and potentially protect against, allergic asthma in mice. As mentioned, AKBA is thought to improve inflammatory diseases through a variety of different mechanisms. Here, we suggest a new pathway involving the modulation of the gut microbiome, with increases in B. pseudolongum, to help decrease allergic asthma inflammation.

It is not uncommon to see a loss of diversity in both asthma animal models and asthmatic patients. Loss of gut microbiome diversity is thought to be related to, or the cause of, inflammatory diseases. However, dietary AKBA was shown to keep the supplemented animals from loss of gut microbiome diversity while increases probiotic-like bacteria such as Lactiplantibacillus, Lacticaseibacillus and anti-inflammatory related bacteria like Bifidobacterium. Bifidobacterium and Lactiplantibacillus levels in the gut have been reported to be inversely correlated with inflammation in disease models. Our findings suggest the consideration of AKBA as a prebiotics to facilitate asthma treatment.

Certain bacterial genera associated with anti-inflammatory effects (including Lactobacillus and Bifidobacterium) are thought to regulate Th2 inflammatory cells and prevent allergies by lowering IgE levels. Our data did not find a change in blood IgE levels following AKBA or B. pseudolongum supplementation. However, as asthma is a lung disease, differences in IgE levels may be observed in the lungs as opposed to systemically.
Bifidobacterium is a known probiotic bacteria and has been used in asthma studies before for its anti-allergy effects\textsuperscript{52,119}. Many of these studies look at specifically B. infantis, an important infant gut bacteria\textsuperscript{119}. In our study however, we saw an increase in B. pseudolongum. This bacteria is mainly found in the guts of animals with fur (calves, piglets, chickens, dogs, and others)\textsuperscript{120,121}. However, it is only found in small numbers in humans\textsuperscript{122}. To date, B. pseudolongum has not been widely discussed in human health. Interestingly, decreases in allergic disease are thought to be linked to animal ownership (cat, dog, farm animals)\textsuperscript{123–125}. The protective effect of animals may include an increase in microbial diversity. Very importantly, B. pseudolongum has been found increased in the gut of children who have continuous interaction with an animal\textsuperscript{126}. With our study, we show B. pseudolongum to have a protective effect on alleviating airway inflammation in asthma. More research should be conducted on the protective nature of B. pseudolongum and its linkage to animal exposure and asthma.

Following B. pseudolongum supplementation in the second set of experiments, only one genus, Erysipelatoclostridium, was found significantly different from the OVA control group. Erysipelatoclostridium is a Gram-positive anaerobic bacterium that is part of the normal human gut microbiota and is known to cause infections in different parts of the body\textsuperscript{127}. However, it has also been found to be a butyrate producing bacteria\textsuperscript{128}. Butyrate production in the gut has been associated with major health benefits, including a reduced risk of inflammatory diseases and Treg function\textsuperscript{3}. Previous studies have also associated human dairy intake with increases in Erysipelatoclostridium spp\textsuperscript{129}. Bifidobacterium is commonly used in dairy fermentation. The connection between the two bacteria should be further explored. It should be noticed, however, that the overall
microbiome composition following *B. pseudolongum* supplementation was not significantly different. This leads us to hypothesize that *B. pseudolongum*’s anti-asthma effect is specific to itself and not due to a change in microbiome composition.

An interesting question is what causes the alteration of the gut microbiome upon administration of AKBA in our allergic asthma model. We previously showed AKBA increased relative abundances of *Bifidobacterium* in healthy mice, and AKBA selectively inhibited certain gut microbiota including *Akkermansia* in vitro, but showed no direct feeding effect on *Bifidobacteria*⁴. Thus, one possibility is that the increased *Bifidobacterium* is a result of less competition for growth in the gut, as both *Bifidobacteria* and *Akkermansia* can colonize gut mucosa and use mucin as energy source. Other studies have shown *B. pseudolongum* increased in the gut of mice which survived lethal influenza infections. From this, it was believed that this bacteria may enhance the host influenza resistance when lethal infection occur¹³⁰. However, there is a need to understand the gut microbiomes response to decreases in inflammation and if a state of anti-inflammation can increase certain microbes.

Another interesting finding from our study is weight loss in mice given AKBA. It is possible AKBA may increase weight loss potential through microbiome changes. *Bifidobacterium* has been shown to contribute to weight loss in both mice and humans¹³¹,¹³². In our study, we also found *B. pseudolongum* supplementation retarded weight gain of the mice. Fasting blood glucose levels were seen decreased in patients given *B. longum*. *B. longum*’s potential to regulate host metabolisms or insulin resistance should be explored. It would be of interest to test the effect of AKBA or *B. pseudolongum* supplementation on obesity related asthma.
Despite our innovative findings, there are several limitations in this study. First, our study is only conducted using one AKBA dosage, and one intervention course. It is important to test the effect of AKBA during different time points and lower dosages to better translate to humans. Also, our findings between naive/negative control groups were based on an unbalanced design which led to less statistical power when compared to a balanced design. It should also be mentioned that while only one ASV of *Bifidobacteria* was found increased and all 15 isolated colonies were identified as *B. pseudolongum*, it is possible only *B. pseudolongum* was culturable. Also, this is a preclinical study. The gut microbiome between mice and humans are different and the effect of AKBA or *B. pseudolongum* may differ when given to humans. Because of this, AKBA gut microbiota changes in human is of great interest.

Of interest, *B. pseudolongum* should be investigated as a new human probiotic and fermentation starter. *Bifidobacterium animalis* subsp. *lactis* is commonly used in fermented food products for its flavor characteristics and health effects. As *B. pseudolongum* is animal derived, different characteristics may occur when used with animal dairy products. Additionally, the stability of AKBA in a variety of food products should be explored.

A small human trial, of a daily 500 mg of a combination of boswellic acids containing 6.25 mg, showed a decrease in inhalation therapy for asthmatic patients. A more recent clinical trial has shown 1:1 ratio of extracts of *Boswellia serrata* gum resin and *Aegle marmelos* fruit containing 30 mg AKBA to decrease overall asthma scores and serum IL-4, and increase IFN-γ compared to a placebo group. However, no microbiome study has been conducted.
AKBA has also been shown to help other diseases through the inhibition of the 5-LOX pathway, including cognitive dysfunction caused from lipopolysaccharide\textsuperscript{61,135}. As lipopolysaccharide are an important factor when discussing gut health, a microbiome study following such models would be of great interest.

We have used mice in this study as the smallest possible animal model to observe both gut microbiota and immunological changes due to allergic asthma. However, translation from animal study to humans can have problems. As AKBA has been shown to lessen asthma and arthritis in humans, it is a good candidate to investigate further into AKBA-human gut microbiome interactions\textsuperscript{66}. AKBA’s effect on the gut microbiome following different allergic diseases, along with the several other diseases it may positively impact (cancer, diabetes, COVID-19), should be investigated for similar results\textsuperscript{136–138}.

5.5 Conclusion

We showed oral AKBA attenuated airway inflammation and improved airway hyperresponsiveness. This effect, at least partially, may be mediated by increases of a specific bacterial species that is commonly found in pets, farm animals and other fur animals. The beneficial effects of AKBA on the gut are still largely undiscovered and may prove to be an important supplement to human health. Future aspects of AKBA and \textit{B. pseudolongum} as a functional ingredients should be explored.
CHAPTER 6

DEVELOPMENT OF NOROVIRUS AND FOOD ALLERGY MURINE MODELS

6.1 Introduction

Public awareness of the importance of hygiene has certainly intensified in the wake of the SARS-CoV-2 pandemic. A similar phenomenon was seen with bacteria in early development, where for many years the public increased hygiene practices to lower exposure to bacteria\textsuperscript{139}. However, this had unintended consequences, as predicted by the “hygiene hypothesis”. This theory states that a lack of microorganisms (bacterial, viral, protozoa, and fungal) leads to reduced immune protection. It has been supported by research showing the protective nature of exposure to certain bacteria has against allergic disease\textsuperscript{11}. Alternatively, the potential beneficial effects of exposure to viral infections have not been widely explored, especially early in development.

Food allergies are increasing at an alarming rate, with 10\% of the general U.S. population affected\textsuperscript{140}. A food allergy is defined as an immune reaction to the repeated exposure of certain foods and can be life threatening to both children and adults\textsuperscript{42}. It has been hypothesized that the increase in allergies could in part be due to the “hygiene hypothesis” and that negative changes in the gut microbiome lead to increases in allergy susceptibility and symptoms\textsuperscript{42}.

Norovirus, a common foodborne and environmental virus, is known to pose a significant public health and economic burden, as it is the leading cause of foodborne illness, fourth leading cause of foodborne death, and results in $2$ billion in medical costs yearly in the United States\textsuperscript{141–143}. However, recent studies have suggested potentially positive side effects of noroviral infection, including restored intestinal morphology and
immune function in regular and microbiome deprived mice\textsuperscript{12,144}. Norovirus has also been shown to have other positive effects on the gut microbiome and is thought to serve a protective role by decreasing the host inflammatory response to bacterial pathogens by replacing functions of commensal bacteria\textsuperscript{144}.

Thus, we propose investigating a significant and prevalent foodborne and environmental virus, norovirus, and its effect on food allergy development and modulation of the gut microbiome. For this dissertation, animal models for food allergy and norovirus were created for further evaluation of this proposed gap in research.

6.2 Methods

6.2.1 Mice

Eight-week-old C3H/HeJ female mice were purchased from the Jackson Laboratory (Bar Harbor, ME, US) and housed in plastic cages with corncob bedding at the University of Connecticut Health (Farmington, CT.). Animal rooms were maintained at a 12-h light/dark cycle and were pathogen free. Mice were fed a 18% protein rodent diet (18.6% protein, 6.2% fat, 44.2% carbohydrate, 3.5% soluble fiber, 14.7% total fiber, 5.3 % ash) purchased from Envigo (Indianapolis, IN, US) and supplied autoclaved water. Beddings from different cages was mixed every other day for a week for mice with the same gender to prevent microbiome cage effects and ensure a similar microbiome at the beginning of the study.

6.2.2 Food allergy model

An allergic phenotype was induced by using ovalbumin (OVA, hen egg) sensitization and challenge model as previously described with modifications\textsuperscript{145}. Briefly (Fig. 1A), one group of mice, OVA group, were sensitized via gavage with 5 mg
ovalbumin plus 10 μg cholera toxin either two times on days 0 and 7 or four times on day 0, 7, 14 and 21. On day 21 (two sensitization) or day 28 (four sensitizations), 10 mg of only OVA was gavaged into each mice, divided into 2 doses, at 30-minute intervals. Allergy symptoms were scored as follows: 0, no symptoms; 1, scratching around nose and head; 2, puffiness around eyes and mouth; 3, wheezing, labored respiration, cyanosis around mouth and tail; 4, no activity after prodding, or tremor and convulsion. 5, death. Mice were then euthanized, and blood was taken. Control group of mice were given only PBS for all sensitization and challenge.

### 6.2.3 IgE and histamine levels

Serum specific ovalbumin IgE and histamine levels was detected by using an ELISA kit with a sensitivity of 3.8 ng/mL (IgE) and 0.03 ng/ml (histamine) per manufacturer’s protocol (Cayman Chemical Company, Ann Arbor, MI; Abcam Cambridge, United Kingdom).

### 6.2.4 Murine norovirus propagation

Isolated strains of murine novovirus-1 (MNV-1) were purchased from ATCC (Manassas, VA) and propagated in RAW 264.7 cells (ATCC, Manassas, VA) pre manufacturer instructions. Virus was titered using a plaque assay in RAW 264.7 cells and frozen in single-use aliquots at −80°C.

### 6.2.5 Murine norovirus model

Before infection, stool was collected. Murine norovirus infection was induced via oral gavage with a total 1x10⁴ plaque forming units (10⁶ pfu/mL) virus in 100μl PBS. Following infection, stool and weight were taken every 24hrs until 72hrs and then once a week for two weeks. (Figure 1B).
6.2.6 Murine norovirus identification

Murine norovirus was extracted from whole stool using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). All extracted RNA was aliquoted into minimal use volumes and stored at −80 °C until use. Following extraction, viral RNA was amplified using the Luna qPCR kit (New England Biolabs, Ipswich, MA) and MNV-1 targeted primers (Forward – CAC GCC ACC GAT CTG TTC TG and Reverse GCG CTG CTG CGC CAT CAC TC\textsuperscript{146}) using cycling conditions of 95°C for 3 min, 25 cycles of 95°C for 30 secs, 55°C for 30 secs and 72°C for 1 min and then a final extension of 72°C for 5 min.

6.3 Results

6.3.1 Oral gavage of food allergy requires four sensitization time points

No allergy was induced in mice when oral sensitization were administered two times (Fig. 6.1A) based on allergy score. All mice only sensitized twice had allergy scores of 0 following final challenge dose.

![Figure 6.1](image)

**Figure 6.1.** Experimental designs to test food allergy (A) and murine norovirus (B).

Alternatively, when mice were given four sensitization (Fig. 6.1A) doses instead, allergy was seen in a majority of the mice (7/8 mice) based on allergy score (p<0.0005)
(Fig 2A). Of the five mice tested from this group, there was a significant increase in blood IgE levels, 24.45 ng/mL vs 0.00 ng/mL, and although not significant, there was an observed average increase in histamine levels for the allergy vs control (Fig 6.2B & 6.2C).

![Figure 6.2](image)

**Figure 6.2.** (A) Average allergy score taken 30 minutes after last challenge (n=8, n=4). (B) Average histamine and (C) IgE levels using blood taken 30 minutes after last challenge. Two groups included allergy group (n=5), mice sensitized orally to OVA and naïve control (n=2).

### 6.3.2 Murine norovirus 1 infection is limited to 72 hours

There was significant weight change observed in mice given murine norovirus on the second day (48hrs). An average decrease in weight of 2.76% was seen when compared with the original weights (p=0.02) (Table 6.1). No other significant change in weight was seen.
Table 6.1. Mouse weight following MNV-1 infection (% change)

<table>
<thead>
<tr>
<th># NoV</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1</td>
<td>0.0</td>
<td>-2.1</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>-0.5</td>
<td>-3.6</td>
<td>-3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>0.0</td>
<td>-4.9</td>
<td>-1.2</td>
<td>-0.6</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>1.9</td>
<td>-0.5</td>
<td>2.9</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Change in mouse weight (g) following murine norovirus infection. Decrease in weight is indicated with red coloring. "*" indicates a p<0.05 when compared to original weight.

No MNV-1 was detected at baseline for any of the mice. However, viral Cq value increased 24hrs after the initial dose (Table 6.2.) On the third day, more than half (3/5) of the mice had no MNV-1 detected in their stool. No mouse had MNV-1 detected on day 7.

Table 6.2. Detection levels (Cq) of MNV-1 in mouse stool following infection.

<table>
<thead>
<tr>
<th># NoV</th>
<th>0hr</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1</td>
<td>0.00</td>
<td>27.94</td>
<td>36.21</td>
<td>38.4</td>
<td>0.00</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>0.00</td>
<td>30.40</td>
<td>38.40</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>0.00</td>
<td>30.65</td>
<td>38.48</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>0.00</td>
<td>30.95</td>
<td>37.31</td>
<td>38.4</td>
<td>0.00</td>
</tr>
<tr>
<td>Mouse 5</td>
<td>0.00</td>
<td>30.87</td>
<td>36.23</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

6.4 Discussion

With food allergies thought to be heavily linked to the dysbiosis of the gut microbiome and its immune effects, it is unknown if norovirus has a positive, negative, or non-consequential effect. It would be of great interest to investigate if norovirus influences the development and severity of food allergies and if the gut microbiome is a key factor. With these experiments, we developed the groundwork for testing this proposed influence.

We found that this food allergy model requires at least four sensitizations to elicit an immune response. Different varieties of food allergy models exist, including those relying on intraperitoneal injection for sensitization, rather than the gavage-based method used here\(^3\). Our own OVA allergic airway model includes only two intraperitoneal
injection of OVA with alum. As this mixture is going straight into the blood stream, a stronger, more consistent response is given. For the model described in this chapter, a gavage with cholera toxin was used. We used this method, rather than intraperitoneal injection, to better mimic a naturally occurring food allergy in the gut. Yet, as this is not as direct, it is not surprising more than two sensitization doses were needed. A higher immune response could be elicited with a longer course of sensitization. However, as the allergy score had an average of 2.0±0.5, it is important to keep mice under a score of 4.0, as this requires immediate euthanasia and may overtake any effect murine norovirus could have on the mouse.

MNV-1 is known to be a self-limiting infection in mice, and we showed its predisposition to linger for around 72hrs. Previous studies have shown MNV-1 shedding to stop after 48hrs as well\textsuperscript{147}. No other clinical signs were seen in the mice (watery stool, disrupted behavior) other than a decrease in weight following 48hrs. Weight loss is one of the main indicators for overall mouse health, as infection tends to cause anorexia and dehydration\textsuperscript{148}. Typically, euthanasia is required in mice following a 20% decrease at any time. However, MNV-1 is not typically reported to cause decrease in weights\textsuperscript{149}. However, future work should include testing of higher concentrations of MNV-1, as this study only looked at the effect of one dose/concentration.

Future work should include testing both models simultaneously to assess if infection with murine norovirus prevents allergy development, and if the gut microbiome has a connected impact. This approach will provide new insight on whether this common childhood infection influences the development and severity of food allergies, based on viral/host interactions and modulation of the gut microbiome and immune profile.
Regardless of positive, negative, or nonconsensual outcomes, this data will provide us with important information on how norovirus infection influences the microbiome and immune profile of the human enteric tract.
Chapter 7

CONCLUDING REMARKS

The impact our gut microbiota has on human biology is still yet to be fully understood. We understand its importance in digestion and energy utilization and see the strong connection it holds to immune and cognitive function; however, the extent and mechanisms to which this occurs is still not well understood. Diet is recognized as a major predictor and modulator of our gut microbiome and its effects should be considered an important variable in human nutrition, similar to that of essential nutrients like macro-micronutrients. In this dissertation, we demonstrated the impact diet can have on energy utilization and allergic diseases.

Our gut microbiota metabolize important compounds, like short chain fatty acids and digestible polyphenols, that promote energy and anti-inflammatory effects. We found that an eight-week intervention of 14% walnut consumption increased the relative abundance of *Bacteroides*. From this, we hypothesized its impact on host metabolism, seen through changes in RER, and activity level, are due to increases in certain *Bacteroides* produced SCFAs, like acetate. This is the first instant of walnuts being seen as a method to effect host metabolism.

We looked at the prebiotic effects of other novel compounds as well, like the herbal remedy resin found within the *Boswellia serrata* tree, more commonly thought of as frankincense. We specifically tested the isolated pentacyclic triterpene acetyl-11-keto-beta-boswellic acid. Of interest, this isolated triterpene contained no fiber or polyenoic compounds. However, it was still found to positively change the microbiome in our studies. In health mice, AKBA was found to increase next-generation probiotic bacteria.
Akkermansia, along with traditionally seen probiotics like Bifidobacteria in only females. This result was seen again in our asthma study using AKBA, where not only was a decrease in asthma symptoms seen, but an increase in Bifidobacteria in female mice as well.

With this dissertation, we investigated a novel species of Bifidobacteria isolated from our AKBA treated asthma mice, B. pseudolongum. This is the first reported instance of B. pseudolongum correlation with anti-allergy effects, along with the proposed idea this animal derived bacteria may plan a larger role in a theory related to pet ownership and lower allergy occurrences. In the future, we wish to study the effect norovirus infection, a common foodborne pathogen, has on allergic diseases. In this dissertation, we set the groundwork for both a food allergy and norovirus models in mice for future work.

Many new questions have arisen from this work that could be further investigated. Firstly, the translation of the data presented here into food items would be of interest. As AKBA showed promising use for the alleviation of asthma symptoms and modulation of the gut microbiome, it would be novel to look into the use of this water-soluble compound in drinks or smoothies. It would also be of interest to further identify B. pseudolongum’s characteristics (pH sensitivity, growth rate) for use in yogurts as a new probiotic species. The antimicrobial activity of AKBA should be better defined, as well as further correlating B. pseudolongum and pet ownership. Lastly, the completion of the work on the effect norovirus has on food allergy would be a promising project and give new insight into health and human wellness.

The gut microbiome is a very complex system with many different factors and potential mechanisms for affecting health, many of which are only beginning to be
partially understood. The interplay between diet or infection, the gut microbiome, and host factors will likely be the subject of decades of future investigation. This dissertation provided further knowledge on how these different potential factors influence each other and more globally may influence health.
APPENDIX A

QUANTIFICATION AND DISCOVERY OF PCR INHIBITORS FOUND IN FOOD MATRICES COMMONLY ASSOCIATED WITH FOODBORNE VIRUSES


1.1 Introduction

Norovirus is considered the leading cause of foodborne illness in the United States and globally $^{150,151}$. Human norovirus causes acute gastroenteritis, which may lead to severe vomiting, diarrhea and abdominal cramping. It has a low infectious dose, ranging from 18-2800 genomic equivalents, and is resistant to many common active ingredients in sanitizers and disinfectants, along with traditional food preservation and processing methods. $^{152,153}$ This makes sensitive and accurate detection critical for control of noroviral transmission. Detection and quantification of norovirus involves the use of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR); however, one challenge in its utilization is the presence of compounds in food that can inhibit detection and accurate quantification. Traditionally, nucleic acid extraction is used to avoid these substances, however, coextraction and purification of unwanted compounds is common $^{154}$.

Foodborne norovirus outbreaks occur year-round. The top two most implicated food categories in foodborne norovirus transmission are bivalve mollusks and produce, mainly being leafy greens and berries $^{155-157}$. Berries, and particularly citrus fruits, contain higher levels of pectin; for instance, raspberries are around 0.97% pectin $^{158}$. Pectinase treatment is sometimes implemented as a pretreatment in the detection of
viruses in berries\textsuperscript{159,160}. Demeke et al.\textsuperscript{161} reported that the addition of apple pectin at 500:1 (polysaccharide to DNA) or 6\% [w/v] pectin per reaction, did not display any inhibitory effect on PCR\textsuperscript{161}. In contrast, a subsequent report by Pandey et al.\textsuperscript{162} found that when using random amplification of polymorphic DNA (RAPD), 1500 ng of pectin to 1.5 ng of DNA, or 12\% [w/v] pectin per reaction, was needed to observe an inhibitory effect\textsuperscript{162}. Other work has found inhibition of traditional PCR with as low as 0.5\% [w/v] pectin\textsuperscript{163}. One step RT-qPCR was not used in these studies; thus, none of these have been able to sensitively quantify the effect of pectin on TaqMan-based one step RT-qPCR quantification. In addition to being generally more sensitive than traditional PCR, one step RT-qPCR has an additional enzyme (reverse transcriptase) that can potentially factor into detection inhibition.

Previous reports suggest that the glycogen content of oysters is around 4.6 to 17.1\%, depending on age and season of harvest\textsuperscript{164}. Several early reports suggest that glycogen may be a PCR inhibitor\textsuperscript{165,166}. When adding dilutions of oyster glycogen to PCR reactions containing poliovirus RNA, one study found glycogen at <3.13\% [wt/vol] did not inhibit amplification but higher concentrations did partially (6.25\% glycogen; decreasing band intensity) or completely (12.5\% and 25\% glycogen; no visible band) inhibit PCR in older traditional PCR chemistries. Numerous other reports observe unknown inhibitors present in oyster extracts\textsuperscript{167,168}. For example, Andersen et al.\textsuperscript{169} observed inhibition when serial dilutions of oyster meal were added to PCR reaction mixtures\textsuperscript{169}. This suggested that compounds other than glycogen could be the main inhibitory compounds present in oyster samples. Furthermore, the previously tested concentration of glycogen, 3.13\% (w/v), is an unlikely amount to get co-extracted with
RNA and end up in the final reaction. Kaufman et al. investigated if mantle fluid within oysters could be used to detect norovirus rather than meat and found that the fluid significantly raised the detection limit in a PCR-based assay. Although not explored further, the authors speculated that the oyster hemolymph could have been the source of inhibition observed. Hemocyanin is a blue respiratory protein found in mollusks’ hemolymph that carries oxygen in a similar fashion to hemoglobin to mammals. Hemocyanin is composed of several protein subunits, with each containing two copper atoms that together bind to a single oxygen. The hemocyanin structure differs between arthropods and mollusks, with mollusks having larger subunits. Hemoglobin has been reported to be a common PCR inhibitor found in blood, and hemocyanin mainly differs from hemoglobin by having a copper central ion instead of iron. Thus, the authors hypothesize that hemocyanin may have potential to inhibit RT-qPCR.

The purpose of this study is to observe and quantify the degree of inhibition that occurs from inhibitory compounds found in produce (pectin) and mollusks (hemocyanin, glycogen) by comparing Cq shifts. Each are thought to be inhibitory components found in produce and mollusks, respectively. Bacteriophage MS2, an ssRNA phage and common norovirus surrogate, was chosen as a model (+) ssRNA genome to test the potential inhibitory effects to one step RT-qPCR, as it is widely available and easily cultivable.

1.2 Materials and Methods

1.2.1 MS2 Propagation and RNA Extraction

The bacteriophage MS2 (15597-B1) was used as a model (+) ssRNA virus, as it has the advantage of being readily propagated to high levels in bacteria. Escherichia coli
strain C-3000 (15597) was obtained from ATCC (Manassas, VA) and a plaque assay was conducted as described by manufacturer’s instructions (ATCC 2018). MS2 stocks were filter sterilized and stored in -80°C until use. RNA was Trizol extracted according to manufacturer’s instructions (Trizol Reagent, Invitrogen, Carlsbad, CA). All extracted RNA was aliquoted into minimal use volumes and stored at − 80 °C until use.

Table A1.1. Real time RT-qPCR primer sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’→ 3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>632F</td>
<td>GTC GCG GTA ATT GGC GC</td>
<td>632</td>
</tr>
<tr>
<td>708R</td>
<td>GGC CAC GTG TTT TGA TCG A</td>
<td>708</td>
</tr>
<tr>
<td>b650P</td>
<td>AGG CGC TCC GCT ACC TTG CCC T</td>
<td>650</td>
</tr>
</tbody>
</table>

Primer and probe selection were based on previously published data targeting the assembly protein gene of MS2.175 aNucleotide corresponding to 5’ of primer of the Escherichia virus MS2 sequence (GenBank NC_001417). bProbe was modified to include FAM at the 5’ base and BHQ at the 3’ base.

1.2.2 Generation of a MS2 RNA Standard by RT-qPCR.

The amplifiable genomic copies of the MS2 extract was determined by using an RNA standard curve as previously described176. Serial dilutions of the extracted MS2 RNA was amplified using the 632F, 708R and 650P primer-probe set175 (Table 1) with NEB Luna® Universal One-Step RT-qPCR Kits (New England Biolabs, Ipswich, MA) and 3µl of template. A 10-minute reverse transcription cycle was conducted at 55°C, followed by enzyme inactivation at 95 °C for 10 minutes. Amplification was then performed for 45 cycles at 95 °C for 15 sec and 60 °C for 60 sec. At least two reactions were performed per sample with at least three separate reactions run.

1.2.3 Addition of inhibitory substance into RT-qPCR reactions.

Pectin (93854), glycogen (G8751) and hemocyanin (H7017) were purchased from Sigma-Aldrich (St. Louis, MO) and stored according to manufacturer’s instructions. Inhibitory compounds of interest were diluted into water solutions and added to RT-
qPCR reactions in desired concentrations (Table A1.2). Serial dilutions of extracted RNA were then added to each desired well. The samples were then amplified with 632F, 708R and 650P (Table 1) using NEB Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA). All reactions were performed in triplicate with no inhibitor and no template controls. Reactions used in gel electrophoresis analysis were cleaned using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and loaded on 2% agarose gels stained with 1× GelRed (Biotium) at 80 V for 1.5 hour run time.

**Table A1.2** Levels of PCR inhibitory compounds tested

<table>
<thead>
<tr>
<th>Pectin</th>
<th>Hemocyanin</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% (wt/v)</td>
<td>0.25% (wt/v)</td>
<td>10% (wt/v)</td>
</tr>
<tr>
<td>0.125% (wt/v)</td>
<td>0.125% (wt/v)</td>
<td>5.0% (wt/v)</td>
</tr>
<tr>
<td>0.0625% (wt/v)</td>
<td>0.0625% (wt/v)</td>
<td>2.5% (wt/v)</td>
</tr>
</tbody>
</table>

### 1.2.4 Data analysis

Statistical analysis was conducted in JMP using a connecting letters report. A p-value of less than 0.05 was considered significant. Negative reactions were considered results with a Cq value of 0.00. Negative reaction values (0.00) from replicates were not calculated into the average positive Cq values; Instead, replicates which displayed one negative reaction were indicated with a subscript “x”, two negative reactions with a subscript “y” and three negative reactions with a subscript “z”. The limit of detection (LOD) was calculated as the level for which no negative reactions were observed.

### 1.3 Results

The limit of detection for the MS2 RT-qPCR assay was found to be 10 PCR Units/reaction. Pectin was observed to cause significant inhibitory effects. Pectin at 0.25% was shown to cause the most severe effect, with complete inhibition (Figure 1). Samples containing 0.125% pectin showed an average 7.44±1.67 Cq difference when
compared to the control at the highest dilution, 10^6 copies/reaction, with negative reactions starting to occur at 10^4 (Table 3). No reactions at 0.125% reported a positive Cq after the 5th dilution (100 copies/reaction). There is a total average difference of 0.74±1.11 Cq between the 0% control and 0.0625% pectin samples and were not found significantly different from one another. The 0.125% and 0.25% pectin samples have a total average difference of 13.72±2.15 Cq and 30.44±0.75 Cq when compared down to the detection limit (10 PCR units), as multiple reactions were completely inhibited (Table A1.3).

![Figure A1.1](image)

**Figure A1.1.** Average Cq values of positive RT-qPCR reactions containing varying amounts of pectin. The percentage 0.25% is not shown, as the average Cq for all reactions was 0.

Data shown on the figure corresponds to the average of the positive values, reactions with no signal were excluded from the average calculations.

**Table A1.3.** Average difference between control Cq with samples contaminated with pectin

<table>
<thead>
<tr>
<th>PCR Units</th>
<th>0.25% (wt/v)</th>
<th>0.125% (wt/v)</th>
<th>0.0625% (wt/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000000</td>
<td>22.31±0.30^a</td>
<td>7.44±1.67^b</td>
<td>0.27±0.79</td>
</tr>
<tr>
<td>100000</td>
<td>25.52±0.68^a</td>
<td>8.57±1.36^b</td>
<td>0.26±1.13</td>
</tr>
</tbody>
</table>

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92
Glycogen was not found to cause any inhibitory effects up to 10% (wt/v) (Supplemental Materials). Because of this, hemocyanin was investigated as a possible inhibitor present in mollusks. Interestingly, hemocyanin was found to cause significant inhibition at low levels. The apparent LOD for 0.25% (w/v) was found to be $10^6$ copies/reaction (Table A1.5), after which there was complete inhibition at $10^5$ copies/reaction. The detection limit of 0.125% hemocyanin was found to be 1000 PCR Units/reaction (Table A1.5). This concentration also began to encounter negative reactions at 100 copies/reaction with an average 9.32±0.99 Cq increase when compared with the control at this dilution (Table A1.4). The 0.25% and 0.125% hemocyanin samples had significant differences from the 0% control, with average differences of 24.85±0.39 Cq and 11.29±0.47 Cq when compared down to the detection limit (10 PCR units), respectively. There is a total average difference of 1.65±0.28 Cq between the 0% control and 0.0625% hemocyanin samples down to 10 PCR units, which were not found significantly different from one another.

<table>
<thead>
<tr>
<th></th>
<th>0.72±0.85</th>
<th>10.09±4.14</th>
<th>0.72±0.85</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>29.00±0.55</td>
<td>10.09±4.14</td>
<td>0.72±0.85</td>
</tr>
<tr>
<td>1000</td>
<td>32.04±0.76</td>
<td>9.26±3.47</td>
<td>0.97±1.29</td>
</tr>
<tr>
<td>100</td>
<td>35.14±0.77</td>
<td>8.33±0.77</td>
<td>1.49±1.64</td>
</tr>
<tr>
<td>10</td>
<td>38.61±1.46</td>
<td>38.61±1.46</td>
<td>0.75±1.76</td>
</tr>
<tr>
<td>1</td>
<td>40.08±0.29</td>
<td>40.08±0.29</td>
<td>1.09±0.33</td>
</tr>
</tbody>
</table>

Levels connected by letters are significantly different (p<0.05) than 0% control. Those levels sharing the same letter are not significantly different for each column. Replicates with one negative reaction. Replicates with two negative reaction. Replicates with three negative reaction.
**Figure A1.2.** Average Cq values of positive RT-qPCR reactions containing varying amounts of hemocyanin. Data shown on the figure corresponds to the average of the positive values, reactions with no signal were excluded from the average calculations.

**Table A1.4.** Average difference between control Cq with samples with hemocyanin

<table>
<thead>
<tr>
<th>PCR Units</th>
<th>0.25% (wt/v)</th>
<th>0.125% (wt/v)</th>
<th>0.0625% (wt/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000000</td>
<td>13.44±0.40\textsuperscript{a}</td>
<td>3.25±0.62\textsuperscript{a}</td>
<td>0.65±0.09</td>
</tr>
<tr>
<td>10000</td>
<td>16.53±0.13\textsuperscript{a}</td>
<td>4.55±0.22\textsuperscript{a}</td>
<td>1.16±0.12</td>
</tr>
<tr>
<td>1000</td>
<td>14.77±0.14\textsuperscript{a}</td>
<td>5.09±0.32\textsuperscript{a}</td>
<td>1.30±0.10</td>
</tr>
<tr>
<td>100</td>
<td>31.80±0.15\textsuperscript{b}</td>
<td>7.52±0.15\textsuperscript{a}</td>
<td>1.88±0.26</td>
</tr>
<tr>
<td>10</td>
<td>34.51±0.99\textsuperscript{b}</td>
<td>9.32±0.99\textsuperscript{a}</td>
<td>2.71±0.90</td>
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<tr>
<td>1</td>
<td>38.04±0.53\textsuperscript{b}</td>
<td>38.04±0.53\textsuperscript{b}</td>
<td>2.17±0.20</td>
</tr>
</tbody>
</table>

\textsuperscript{ab}Levels connected by letters are significantly different (p<0.05) than 0\% control. Those levels sharing the same letter are not significantly different for each column. \textsuperscript{x}Replicates with one negative reaction. \textsuperscript{y}Replicates with two negative reaction. \textsuperscript{z}Replicates with three negative reaction.

Because previous research has shown hemoglobin to cause some form of fluorescence quenching, reactions testing all percentages of hemocyanin solutions with 100000 and 10 PCR units were analyzed using agarose gel electrophoresis. Results from the gels did not differ from those from the RT-qPCR results at 0.125\% (Supplemental Material). Unsurprisingly however, hemocyanin at 0.25\% did not show up on the gel at
100000 PCR units, as RT-PCR visualized via gel has lower sensitivity than probe-based RT-qPCR.

**Table A1. 5.** Limit of detection in PCR Units/reaction for samples contaminated with inhibitory substances

<table>
<thead>
<tr>
<th>Pectin (wt/v)</th>
<th>0.25%</th>
<th>0.125%</th>
<th>0.0625%</th>
<th>0.0%</th>
<th>0.25%</th>
<th>0.125%</th>
<th>0.0625%</th>
<th>0.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>100000</td>
<td>10</td>
<td>10</td>
<td>1000000</td>
<td>1000</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Limit of detection was calculated as the level for which no negative reactions were observed.

### 1.4 Discussion

In this study, pectin and hemocyanin were found to cause significant inhibition of one step RT-qPCR for bacteriophage MS2, a model (+)ssRNA virus. Pectin and hemocyanin had complete inhibition at 0.25% (w/v). Glycogen was not found to cause inhibition, even with levels as high as 10% (w/v) of reactions, despite having been previously implicated as being an inhibitor. All these molecules are present in foods commonly implicated in foodborne norovirus transmission; specifically produce and mollusks.

All hemocyanin reactions containing 1 PCR Units/reaction of RNA were found to be negative, minus a single reaction (the reason for a 0.00 standard deviation in Table 4) in the 0% control, which gave a Cq of 40.35 (data not shown). This MS2 assay was found to have a LOD of 10 PCR units/reaction, however sporadic detection is not uncommon for RT-qPCR reactions near their limit of detection. This can be seen throughout the paper, as many lower concentrations have replicates with negative results. Differences near the no inhibitor control limit of detection are not likely due to inhibition interactions, which can clearly be seen when comparing the 0% control and 0.0625% hemocyanin sample (Table 5).
Previous work has only reported pectin causing inhibition at higher concentrations than observed in this work. This could be due to multiple factors related to the previous reports, which used different PCR formulations, were not one-step real time RT-qPCR, and were not generally as analytically sensitive. It is possible that contemporary one-step RT-qPCR kits may be more prone to inhibition as the reverse transcriptase and real time probe are additional potential targets for inhibition. Future work identifying the degree to which the added reverse transcriptase step in the one-step assay renders the assay vulnerable to inhibition would be valuable. These results highlight the importance of pectinase in RNA extraction from produce, as little as 0.125% (w/v) can cause false negatives. Pectinase treatment has been found to work well, with reports of it aiding elimination of false-negative results in norovirus detection in produce\(^{159,160}\). However, the mechanism of pectin inhibition is still unknown. Previous reports have suggested that the acidic nature of pectin may cause the inhibition, as other acidic polysaccharides have also been observed to cause inhibition, including dextran sulphate and ghatti gum\(^{161}\).

PCR inhibitors cause false negatives and higher detection limits. Common practice to remove inhibitors from norovirus samples has traditionally been through added steps in RNA extraction, however, carryover contamination is still common. This is especially true for compounds used in extraction, including phenols and alcohols. GlycoBlue (Invitrogen, Carlsbad, CA) is a popular coprecipitant used in many RNA extraction methods. It consists of blue dye covalently linked to glycogen derived from mussels, comparable to the glycogen tested in this study. Invitrogen has stated in their manuals that the addition of GlycoBlue should not disrupt RT-PCR reactions. This study further supports this claim, as the GlycoBlue used in RNA extraction is added to PCR in
smaller amounts compared to the concentrations tested in this study. Further, the amount of glycogen found in oysters generally ranges from 4.6 to 17.1%, depending on age and season. Realistically, glycogen would not be found in PCR reactions higher than those tested in this study. In all, these results suggest that glycogen is not likely a major contributor to the PCR inhibition observed with mollusk samples.

The goal of in-field viral detection of norovirus may be unobtainable using PCR technology. One of the many factors preventing this is the need to concentrate virus and perform RNA extraction to remove PCR inhibitors. However, some foods may have less inhibitory compounds compared to others and not require extensive purification. The levels of inhibitory compound used in this work range from those excess to what realistically would occur with direct use of food in reaction down to levels likely to be residual carryover after purification. For example, glycogen the highest concentration tested (10%), would be the equivalent of using direct oyster tissue as template in an oyster with 50% glycogen content—well beyond the amount of glycogen naturally found in oysters. Based on our observations, raspberries containing as little as 0.313% total pectin content or more could cause inhibition if directly used in reaction formulations as described. Raspberries have around 1% pectin, meaning that even crude sample extraction methods need an element to remove pectin to avoid inhibition. Further studies may be conducted on testing raw food samples vs only inhibitory compounds to observe the degree to which the compounds alone are responsible for inhibition.

Hemocyanin, a molecule found in the hemolymph of mollusks, was found to be inhibitory to RT-qPCR at very low concentrations. To the authors’ knowledge, little has been known about which specific compounds in mollusk matrices cause PCR inhibition.
This is important, as hemocyanin is the most abundant protein in mollusk hemolymph, and can be at levels as high as 85.6% of total hemolymph protein in in some crustaceans. In mollusks, hemocyanin functions similarly to hemoglobin—a well-documented PCR inhibitor in mammals. Unlike hemoglobin, a central copper group is found instead of an iron group. Free metal ions within a PCR solution are thought to cause reduction in the specificity of primers by replacement of magnesium. Hemoglobin was thought to release free iron ions and cause an inhibitory effect through this mechanism, but it was found that iron alone does not affect PCR mechanisms in the same way as hemoglobin. In fact, ionic iron and copper do not substantially inhibit PCR alone. However, more recently, it was found that when using FeCl$_3$ and hemoglobin in PCR reactions, almost ten times the amount FeCl$_3$ was needed to cause a similar inhibitory effect as hemoglobin, though the specific mechanism by which this occurs is not known. One hypothesis concludes that hemoglobin causes fluorescence quenching by binding dyes within the central cavity. Although fluorophores were used in this paper, it should be noted that fluorescence quenching was not observed as the mechanism for the inhibition for hemocyanin, suggesting that the hemocyanin protein itself may be directly disrupting the enzymatic activity in RT-qPCR reactions. Hemocyanin differs in structure, having no central cavity or porphyrin rings as hemoglobin does. This is likely why no fluorescence quenching was observed, as the same degree of inhibition was observed with RT-qPCR signal and was confirmed using gel electrophoresis. Further studies should be conducted on more efficient ways to remove this compound, as well as the degree to which it may carryover through RNA extraction. Solvent based extractions can frequently result in carryover of protein if phenol is not homogenized or removed properly. Column-based
extraction methods traditionally result in less protein contamination, however, columns overloaded with sample can result in carryover. Both norovirus and hemocyanin are similar in diameter, being ~40nm and 35nm respectively \cite{172,181}, and would thus both be selected for with filtration techniques. Similarly, norovirus and hemocyanin have similar isoelectric points (pI), both being around 5-6 \cite{181,182}, meaning both may be precipitated into solution at similar pH.

1.5 Conclusion

In sum, this work quantifies the degree of inhibition that occurs from three compounds present in foods associated with norovirus transmission, demonstrating that pectin and hemocyanin should be considered when testing produce and mollusks. This information helps inform sample preparation for PCR-based detection of foodborne pathogens from produce and mollusks, as well as identifies a hitherto unreported specific inhibitory compound found in mollusks.
APPENDIX B

RECENT DEVELOPMENTS IN ISOTHERMAL AMPLIFICATION METHODS FOR THE DETECTION OF FOODBORNE VIRUSES


2.1 Introduction

Viruses are the leading cause of foodborne illness in the United States, representing about 65% of known foodborne illnesses. A variety of viruses are known to cause foodborne disease, including human norovirus, enteroviruses, hepatitis A and E viruses, astroviruses, rotaviruses and adenoviruses, among others. These viruses are typically enteric, meaning they replicate in the gut and can be transmitted through the fecal-oral route. Numerous foods have been implicated in foodborne virus transmission, many of which are ready-to-eat foods that are commonly consumed without cooking. In many cases, viral contamination of food occurs from food handlers and/or fecally contaminated water. Some examples of foods commonly implicated in foodborne virus outbreaks include berries, spinach, oysters and other mollusks. While norovirus is the most prevalent of these viruses, it has been estimated that norovirus, rotavirus, hepatitis A and hepatitis E viruses collectively kill over 620,000 people worldwide each year, highlighting the importance of controlling their spread.

Unlike many bacterial and fungal foodborne pathogens, an enrichment step or growth of pathogen for higher detection, is not feasible for routine detection of foodborne viruses. Further, the high transmissibility and low infectious dose of many foodborne viruses means that portable, rapid, and sensitive detection of viruses is important for controlling their spread. Ligand-based detection methods, including enzyme-linked
immunosorbent assay (ELISA) and immunochromatography, can offer portability and relatively rapid results. However, these methods often lack the analytical sensitivity required for detection of such viruses in foods and the environment. Further, such methods can also have high rates of false positive or negative results depending upon the ligand used. The current gold standard for the detection and quantification of foodborne viruses is real time reverse transcriptase polymerase chain reaction (RT-qPCR). This technique often has a relatively favorable analytical sensitivity; however, traditional RT-qPCR can lack portability, is prone to food matrix-associated inhibition, and can take >1.5 hours.

Isothermal amplification methods have been the focus of research for several decades, as many isothermal amplification techniques offer the benefits of better portability, favorable analytical sensitivity, rapid time to result, potentially superior inhibitor tolerance, and potentially higher fidelity. Several isothermal techniques have shown promise for detection of foodborne and enteric viruses. Some examples of these include Recombinase Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), Nucleic Acid Sequence-Based Amplification (NASBA), Rolling Circle Amplification (RCA), Helicase-Dependent Amplification (HDA) and Cross Priming Amplification (CPA). In addition to portability and rapidity, these isothermal techniques have demonstrated sufficient analytical sensitivity for detecting the low viral levels at which food and environmental contamination often occurs. The purpose of this review is to highlight and discuss these techniques and progress in the development of isothermal amplification methods for foodborne viruses, with a particular focus on the performance of these methods in complex matrices.
2.2 Consistency is Key: Popular Isothermal Amplification Methods

Recombinase Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP) and Nucleic Acid Sequence-Based Amplification (NASBA) have been the most frequently reported isothermal methods developed for foodborne viruses. For the purposes of this review, only these methods will be covered.

Nucleic acid sequence-based amplification (NASBA) was first developed by Greene et al., and is designed to detect only RNA targets. Sometimes referred to as transcription mediated amplification (TMA), the assay is based on the reaction of two enzymes, avian myeloblastosis virus reverse transcriptase (RT), and RNase H/T7 DNA dependent RNA polymerase (DdRp), which work together to start a strand-dependent amplification reaction. Reactions run for around 60-90 minutes at a constant temperature ranging from 40-55°C.

- Loop-Mediated Isothermal Amplification (LAMP) is a technique that was developed about two decades ago, in 2000. Since its invention, many commercial kits have emerged. LAMP employs auto-cyclizing strand displacement of genomic material using a Bst DNA polymerase. This process includes a set of four primer pairs, which recognize six specific sequences on the target strand. This technique has been reported to be more sensitive than PCR in some cases when detecting different viruses. The high specificity is thought to be due to the use of eight specific primers. The process may take place at 65°C in a single tube. With the addition of a reverse transcriptase enzyme, RNA templates may also be targeted. Quantitative real time LAMP was introduced in 2004 for real time detection of genomic material with the introduction of fluorescent dyes. One of the main disadvantages of LAMP assays is the increased difficulty in primer design. Further, dimer formation can produce false positive results in many cases. A few
factors can affect the limits of detection and sensitivity in LAMP assays, including time, temperature and MgSO₄ concentration.

Recombinase polymerase amplification (RPA) has also been the focus of numerous viral detection reports. The procedure was developed in 2006 and is currently commercialized by TwistDx, a subsidiary of Abbott Laboratories. The process combines isothermal recombinase-driven primer targeting of template with strand-displacement DNA synthesis. Additionally, some work suggests it can better tolerate crude DNA/RNA extraction methods compared to PCR (Moore and Jaykus, 2017). Like LAMP, RPA can target RNA with the inclusion of a reverse transcriptase. Traditionally, primer design had been a challenge for viral targets because of the longer recommended length required for primers; however, TwistDx protocols demonstrate that PCR-length primers may be compatible with RPA, although the specific requirements for optimal primer design have not been completely elucidated. Temperatures for the RPA reaction may range from 22 - 45°C, and in most cases, RPA has been reported to have relatively high fidelity. RPA can tolerate some degree of target sequence mismatch, which may be an advantage allowing broader reactivity for viral targets with more genomic diversity. Although RPA has been suggested to be more tolerant than PCR in the presence of known inhibitors, it still can be inhibited by high genomic DNA concentrations and high levels of sample-associated inhibitors. RPA is more expensive than PCR for reagent costs currently, but the possibility to eliminate sample pretreatments could make up for such costs. Along with minimal sample pretreatment, RPA does not require an expensive, bulky thermocycler due to its low, constant reaction temperature. However, RPA has not yet been approved for clinical application by the FDA and may only be used for research purposes.
2.3 Two Bucket Syndrome: Caliciviridae Family: Norovirus

Human noroviruses are members of the *Norovirus* genus in the *Caliciviridae* family, and are the most common cause of known foodborne illness in the United States. Particles of norovirus are relatively small (around 37 nm in diameter), contain no lipid envelope, and have a 7.5-7.7 kilobase positive-sense single stranded RNA genome. The *Norovirus* genus has been divided into six genogroups (GI–GVI), with three causing disease in humans (GI, GII, and GIV). Noroviruses from genogroup II, genotype 4 (GII.4) cause most of the gastroenteritis cases across the globe. As of 2021, the CDC estimates that 900 deaths and 110,000 hospitalizations occur annually due to norovirus. Although the majority of cases stem from person-to-person transmission, which can occur in close (nosocomial) settings, a notable portion of transmission is associated with foods, often through food handler contamination. Norovirus gastroenteritis has been called the “two bucket disease” due to the severe vomiting and diarrhea that those infected often experience.

The first use of isothermal amplification for detection of norovirus was utilization of NASBA for Norwalk virus (GI.1), considered the lab type strain. This assay had a detection limit of $10^4$ PCR units/mL of stool filtrate. Using the same method, GI and GII strains were evaluated, finding positive results for 13/17 different strains tested under the one broadly reactive primer set. Subsequently, a real-time NASBA assay was formulated that utilized broadly reactive JJV2F and COG2R primers to target GII genotypes. This method reportedly displayed a superior limit of detection (a very low 0.01 PCR units) and specificity when compared to a previously established RT-qPCR assay. However, this real-time assay was only tested on GII and showcases a current
challenge in norovirus detection; the need to simultaneously detect all genotypes in the GI and GII genogroups of the *Norovirus* genus accurately, specifically, and quickly. As NASBA only requires a forward and reverse primer, the work may easily be translated to PCR or other isothermal techniques, like RPA. This translation may not work so easily for other isothermal techniques that require numerous primers like LAMP; however, one group does report successful adaptation of LAMP primers for a PCR norovirus assay.\(^{215}\)

The foundational report applying RT-LAMP for norovirus detection was published in 2006.\(^{216}\) Sensitivities were found to be $10^2$ and $10^3$ copies/reaction for GI and GII, respectively, when using endpoint analysis by gel electrophoresis and observation of turbidity. Interestingly, the limit of detection was determined to be similar for gel electrophoresis and observation of turbidity. LAMP reactions produce magnesium pyrophosphate during amplification, which can be observed as a clear white precipitate formed in the tube for endpoint detection. Similarly, nucleic acid intercalating dyes have been used for endpoint detection because they give an immediate visible readout that does not necessarily require complex equipment. However, this type of reaction does suffer in sensitivity and there is an increased possibility for false positives due to dye signal from nonspecific amplification. Common types of these intercalating dyes used include hydroxynaphthol blue dye (HBD) and SYBR green I. Khairuddin et al. added SYBR I to their reaction, which resulted in a visual detection limit of 22 copies/μL NoV plasmid surrogate.\(^{217}\) HBD has been used in LAMP as well, which resulted in a $10^3$ copy/reaction detection limit from extracted stool taken from an outbreak prevalent in China.\(^{218}\) As the use of endpoint dyes allows for fast results, Iturriza-Gómez et al. compared the performance of the aforementioned HBD assay and a commercial RNA
amplification kit. This specific kit, Loopamp RNA, has been used several times for the detection of norovirus. Both assays resulted in comparable detection limits (10^3 copies/reaction); however, the HBD endpoint method was found to produce signal faster by an average of 3 minutes. The use of HBD in LAMP is beneficial for quick detection with or without UV light.

As stated earlier, human norovirus outbreaks have been associated with consumption of contaminated filter feeding oysters; however, detection of noroviruses in oysters remains a challenge. Jeon et al. tested the use of RT-LAMP to detect norovirus in live Pacific oysters. An assay was created to observe inhibition or irregularities in extraction of GI and GII from live Pacific oysters for both traditional and one-step RT-LAMP. Hemocytes and the exsanguinated tissue from individual oysters were individually subjected to RNA extraction and amplification. Primary testing with an RNA standard suggested the sensitivity of both the GI and GII one-step RT-PCR assays to be 4.7 x 10^2 human norovirus GI copies/μL, while the real-time RT-LAMP assay had a lower detection limit of 4.7 x 10^1 genomic copies/μL. When using oyster RNA extract as the template, the detection limit for the RT-LAMP was found to be 2×10^2 copies per 1 g of digestive gland, suggesting inhibition occurred with a reduction in sensitivity.

Moore et al. reported the first application of RPA for the detection of noroviruses. Detection limits for GII.4 New Orleans were shown to be low in both kit-extracted and crudely-extracted (boiled stool) RNA in RT-RPA. A detection limit of 3.40 ± 0.20 log10 genomic copies per reaction for purified RNA was reported, which was higher than that of an established RT-qPCR method, but comparable to traditional RT-PCR assays for norovirus. More recently, Han et al. report an RT-RPA for all GII
genogroup noroviruses (including GII.4, GII.P16-GII.2 and GII.P17-GII.17) with a limit of detection of 166 copies/μL\(^2\). When testing real life samples, both the RT-RPA and RT-qPCR resulted in similar positive food samples (8/20) and stool samples (10/18). However, RT-qPCR did yield 1 more positive water sample (7/17 vs 6/17) than the RT-RPA assay. However, a larger number of samples in each of these matrices need to be evaluated to better understand any differences in sensitivity between the two assays. Ma et al. report broad detection of murine norovirus, including strains that contained seven-point mutations from the target RPA sequence\(^2\), suggesting that RPA does have some capacity to allow for even broader detection of multiple norovirus strains. More work needs to be done to understand the capacity of this technique to detect diverse norovirus strains more broadly, as well as withstand matrix-associated inhibitors present in foods. Additionally, colorimetric, or dye-based detection and validation has not been reported for RT-RPA of norovirus.

2.4 Small but Deadly: Picornaviridae Family

Enteroviruses are members of the Picornaviridae family and derive their name from the fact that they were initially isolated from the intestinal tract\(^2\). These positive-sense single-stranded RNA viruses include a broad range of viruses in the Enterovirus genus, and cause a variety of human diseases, including the common cold, acute flaccid paralysis, acute hemorrhagic conjunctivitis, aseptic meningitis, and myocarditis. Hand, foot, and mouth disease and other diseases in this genus can be transmitted through the fecal-oral route. There are five major groups of enteroviruses: polioviruses, group A and B coxsackieviruses, echoviruses and general enteroviruses. Enteroviruses have been
found to be able to persist on foods and environmental surfaces for notable periods of
time under normal household storage conditions 227.

One of the major routes through which these viruses contaminate foods is through
water. Thus, Zhao et al. reported a LAMP assay that was able to broadly detect human
enteroviruses, with a focus on human enterovirus A and human enterovirus B, from water
and stool samples 228. The detection limit was found to be 10 genomic copies/μL. The
assay detected enterovirus coxsackievirus A16 (HEV-A), enterovirus 71 (HEV-A),
coxsackievirus B3 (HEV-B), coxsackievirus B5 (HEV-B) and echovirus 30 (HEV-B), but
not a panel of other enteric viruses, showing its specificity for HEV-A and HEV-B.
Further, the developed assay showed promise when enteroviruses were inoculated into
drinking water and stool samples, as all water samples tested positive, with only one of
the stool samples coming back negative, possibly due to inhibitory substances in the stool
193. Fox et al. 229 utilized the commercial NASBA-based NucliSens® kit to detect
multiple enteroviruses after designing a broadly reactive primer set for detection of
human enterovirus A and B. All clinical samples tested were positive, including extracted
cerebrospinal fluid, respiratory and stool samples 229. Further, the limit of detection for
the assay was found to be <100 copies RNA per reaction with no cross reaction observed
against rhinoviruses (other members of the Picornaviridae family).

2.4.1 Enterovirus 71

Enterovirus 71 (EV71), along with coxsackievirus A16 (below), causes hand,
foot, and mouth disease, with clinical manifestations of these diseases being nearly
identical. However, each of these can result in different chronic health conditions, and
EV71 tends to be much more severe 230. While the infection is typically self-limiting, it
may cause brainstem encephalitis, aseptic meningitis, and acute flaccid paralysis. Laboratory testing for the virus involves isolation of EV71 from stool, throat-swab, or cerebrospinal fluid samples.

Multiple studies have been conducted using RT-LAMP to detect EV71 in a variety of clinical samples, including nasopharyngeal swabs, stool, throat swabs, and rectal swabs. A meta-analysis by Leu et al. discusses 10 studies, with a total of 907 samples, comparing RT-LAMP to real time RT-PCR methods for EV71 detection between 2011-2012 \(^{231}\). The analysis suggested comparable performance of RT-LAMP methods to real time RT-PCR, as the pooled sensitivity and specificity were found to be 0.99 (95% CI: 0.97 - 1.00) vs 0.97 (95% CI: 0.94-1.00), respectively. More recently, Wang et al. further evaluated this method, finding a limit of detection of 0.01 PFU per reaction. No cross-reaction of the assay was found with poliovirus 1, coxsackievirus A16, rotavirus, norovirus, sapovirus and astrovirus \(^{232}\). Despite the promise of this assay for EV71 detection, more evaluation in food and water samples is needed.

In trend with new technologies, Yin et al. \(^{233}\) recently reported an RT-RPA method for detection of EV71. The analytical sensitivity was found to be 3.767 log10 copies per reaction with the clinical sensitivity (95%) and specificity (100%) being comparable to real time RT-PCR. This is particularly important as it can be difficult for these assays to differentiate EV71 with coxsackievirus A16.

### 2.4.2 Coxsackievirus Group A

Along with causing mild hand, foot, and mouth disease, coxsackievirus infection is the most common cause of viral heart disease \(^{234}\). Infection is shown to be more problematic in children with myocarditis and in adults with pneumonitis \(^{235}\). There are
two groups of coxsackieviruses, group A and group B, and distinguishing between both is important for proper treatment. Because of the similarities between EV71 and coxsackievirus A16, both have commonly been studied simultaneously.

Multiplex endpoint RT-LAMP has been used for simultaneous detection of EV71 strain C4 (EV71-C4) and coxsackievirus A16 using HBD dye. Detection limits were around 0.33 and 1.58 of a 50% tissue culture infective dose (TCID50) per reaction of EV71-C4 or CVA16, respectively. The dye successfully detected the virus in extracted stool samples with 100% accuracy and no cross reactivity. However, as stated before, treatment for each disease varies and applying incorrect treatment may be detrimental to the patient; therefore, it is important to differentiate these viruses. As such, an assay exclusively targeting coxsackievirus A16 using RT-LAMP has been reported, with a detection limit of 81 copies per reaction after RNA extraction of clinical samples. EV71 viruses were used with zero cross reactivity between them, suggesting strong specificity; however, other subtypes of coxsackieviruses were not tested. A similar study combined RT-LAMP technology with a lateral flow device, which had a sensitivity of 0.55 TCID50 per reaction and 100% specificity in detecting coxsackievirus A16.

While coxsackievirus A16 is the most common cause of hand, foot, and mouth disease, A6 has become a major cause of outbreaks in the United States and is strongly associated with adult cases. Thus, there is a need to distinguish both for proper treatment. Wang et al. report an RT-RPA assay for coxsackievirus A6 with a detection limit of 400 copies per reaction, with 100% specificity when 234 clinical samples were tested. Further, the real-time RT-RPA assay showed no significant difference in sensitivity or specificity with established real-time RT-PCR assays. Reverse transcription recombinase-
aided amplification assay (RT-RAA), a new isothermal amplification technology similar to RT-RPA, was also developed for coxsackievirus A6 and found to have a detection limit of 38 copies per reaction, superior to that of the aforementioned RT-RPA assay 239.

2.4.3 Coxsackievirus Group B

Coxsackievirus group B viruses can cause spastic paralysis, gastroenteritis, herpangina, pleurodynia, pericarditis, meningoencephalitis, aseptic meningitis and colds, and account for more than 25-50% of viral myocarditis cases 240,241. Newborns are especially susceptible to difficult outcomes from these viruses 242. Jaianand et al. report an RT-LAMP detection assay for coxsackievirus group B, including B1-B5 234. This assay produced more positive signals than an established RT-PCR assay when tested against 31 positive stool samples, and showed no cross reaction with other enteroviruses, suggesting both favorable sensitivity and specificity. Of group B coxsackieviruses, B3 is the most common serotype, being responsible for fifty percent of viral myocarditis. Monazah et al. 240 report another RT-LAMP assay for coxsackievirus B3 with a detection limit of 0.1 pg RNA per reaction with no cross-reactivity with coxsackievirus A16, echovirus, and rhinovirus. Zeinoddini et al. reported RT-LAMP that exhibited similar analytical sensitivity for coxsackievirus B3 detection (0.1 pg RNA per reaction), as stated above, and was superior to both NASBA and RT-PCR, which both detected 10 pg RNA per reaction 243.

2.4.4 Poliovirus

Polioviruses were the first viruses to be categorized as foodborne. Immunization has made wild-type strains rare 184. However, detection is still important in assisting global efforts to eradicate the pathogen. Poliovirus (type 1) is still endemic in two
countries, Afghanistan and Pakistan. Poliovirus Sabin strain has been used for development of an RT-LAMP assay, with a reported limit of detection of 400 copies per reaction in 50 minutes. It should be noted that this assay’s LOD is significantly higher than other optimized RT-LAMP systems for other pathogens, though it was inferior to a traditional RT-qPCR assay. To the authors’ knowledge, this is the only isothermal technique described for detection of polioviruses.

2.4.5 Hepatovirus A

Hepatitis A virus (HAV) was first classified in the Enterovirus genus as enterovirus 72, but was subsequently given a distinct genus, Hepatovirus. These viruses are further subtyped based on sequence similarity of the genes that code for the VP1 and VP3 surface proteins. Further, these viruses are not as diverse as other enteric viruses, with all seven genotypes showing 85% genetic similarity. Being both an enteric and bloodborne pathogen, strains belonging to genotypes I and III are most predominant in humans. However, 80% of suspected cases belong to genotype I, with strain IA being the most prevalent globally.

RT-LAMP was first introduced for the detection of hepatitis A virus by Yoneyama et al., targeting the three strains, IA, IB and IIIB. The detection limit for all three strains was found to be 0.4–0.8 focus forming units (FFU) per reaction. Positive results could be seen via naked eye without need of a dye due to noticeable turbidity increase, similar to other LAMP reactions. Further, no cross reaction with other enteric viruses (polioviruses; norovirus genotypes I and II; sapovirus genotypes I, IV and V; and hepatitis E virus) was observed; however, the assay was not capable of distinguishing between different HAV strains.
NASBA was used for the detection of hepatitis A on a variety of spiked agricultural samples, including wastewater, lettuce and blueberries. It was found that when using buffered HAV target RNA, sensitivity was 0.4 ng of RNA/mL (compared to 4 ng/mL of RNA with RT-PCR) with dot blot hybridization used for visualization of the reaction. Discussion on the results of the non-extracted environmental samples can be found later in this review and in Table 2.

2.5 Do Not Wish Upon This Star: Astroviridae Family: Astrovirus

Astroviruses are non-enveloped, positive-sense, single-stranded RNA viruses from the Astroviridae family. The six points of the capsid shell give these viruses a “star-like” appearance and their name. These viruses have mostly been observed to cause self-limiting gastroenteritis in animals. At least 8 serotypes known to infect humans exist, which are all antigenically different from the strains reported to infect other animals. Astroviruses are relatively prevalent among children, causing an estimated 5%-10% of gastroenteritis cases in children. Foodborne outbreaks of the viruses are thought to be limited, with undercooked or raw seafood and water being the most commonly associated vehicles of foodborne transmission. Yang et al. developed an RT-LAMP assay with HBD endpoint detection for detection of astrovirus serotype I, observing a limit of detection for the assay at 36 copies/μL. When used on RNA extracted from sewage treatment plant water samples, viral RNA was found in 41.7% of samples, while an established PCR method only observed 33.3%, suggesting that this RT-LAMP assay may display better sensitivity, and/or better tolerate matrix-associated inhibitors present in sewage, than RT-PCR. Wei et al. found lower sensitivity for serotype 1 (stool samples),
when compared to the previously mentioned study, with a detection limit of \( \sim 100 \) RNA copies reaction \(^{250}\).

### 2.6 More than just the Common Cold: Adenoviridae Family: Adenoviruses

Adenoviruses are icosahedral, non-enveloped DNA viruses, with genomes of approximately 26–45 kb in length \(^{251}\). They were first isolated from civilians and army recruits who showed symptoms of other respiratory diseases \(^{252}\). Viral infection typically can cause pneumonia, cystitis, conjunctivitis, hepatitis, myocarditis, intussusception, encephalitis and is one of the causes of the common cold \(^{252}\). While many serotypes of the virus are thought to cause upper respiratory infection, serotypes 40 and 41 are known causes of gastroenteritis. As enteric adenoviruses, they spread not only through the fecal-oral route but also through respiratory droplets. This group of viruses is said to be the cause of 5-20% of worldwide cases of acute gastroenteritis among infants and young children \(^{253}\).

Ziros et al. report a LAMP assay utilizing SYBR Green for the endpoint detection of adenoviruses 40 and 41 in sewage samples with a process time of 60 minutes and 100% accuracy when compared to an established PCR method \(^{253}\). The sensitivity of this assay for viral DNA purified from sewage samples was found to be 50–100 copies per reaction. This procedure was found to be successful in detection from extracted clinical fecal samples as well, with no false positives observed. Similarly, a multiplex RPA assay has recently been developed for detection of adenoviruses in wastewater samples with a lateral flow strip. The detection limit for viral DNA extracted from water samples was found to be 50 copies per reaction with 100% specificity and sensitivity when testing 21
samples. While both methods have similar detection limits for use in water, RPA takes significantly less time and requires fewer primers.

**Table A2.1.** Comparison of limits of detection observed for different isothermal assays developed against foodborne and enteric viruses

<table>
<thead>
<tr>
<th>Isothermal Amplification Method</th>
<th>Vial Target</th>
<th>Limit of detection with reaction volume</th>
<th>aLimit of detection normalized to /μL</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caliciviridae Family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinase Polymerase Amplification (RPA)</td>
<td>Norovirus</td>
<td>0.8–10.0 LGC/50μL reaction</td>
<td>0.068 ± 0.004 LGC/μL</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>1.66 × 10^2 copies/μL using 50μL reaction</td>
<td>166 copies/μL</td>
<td>225</td>
</tr>
<tr>
<td>Loop-Mediated Isothermal Amplification (LAMP)</td>
<td>Norovirus</td>
<td>10^2 and 10^3 copies/25μL reaction</td>
<td>4 and 40 copies/μL</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>22 copies/μL using 10μL reaction</td>
<td>22 copies/μL</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>10^3 copies/25μL reaction</td>
<td>40 copies/μL</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>10^3 copy/20μL reaction</td>
<td>50 copies/μL</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>10^3 copy/20μL reaction</td>
<td>50 copies/μL</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>4.7 × 10^2 copies/μL using 25μL reaction</td>
<td>470 copies/μL</td>
<td>233</td>
</tr>
<tr>
<td><strong>Picornaviridae Family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic Acid Sequence-Based Amplification (NASBA)</td>
<td>Norovirus</td>
<td>10^4 PCR units/ml using 20μL reaction</td>
<td>10 PCR units/μL</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>0.01 PCR units using 20μL reaction</td>
<td>0.0006 PCR units/μL</td>
<td>214</td>
</tr>
</tbody>
</table>

**Enterovirus 71**

3.767 log10 copies/50 μL reaction

0.07594 log10 copies/μL
<table>
<thead>
<tr>
<th>Test System</th>
<th>Virus</th>
<th>Copies/RNA/Reaction</th>
<th>Copies/RNA/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recombinase Polymerase Amplification (RPA)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus A16</td>
<td>0.55 TCID50/25μL reaction</td>
<td>0.022 TCID50/μL</td>
<td>237</td>
</tr>
<tr>
<td>Coxsackievirus A6</td>
<td>400 copies/50 μL reaction</td>
<td>8 copies/μL</td>
<td>238</td>
</tr>
<tr>
<td><strong>Loop-Mediated Isothermal Amplification (LAMP)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human enterovirus A &amp; B</td>
<td>10 genomic copies/μL using 25μL reaction</td>
<td>10 genomic copies/μL</td>
<td>228</td>
</tr>
<tr>
<td>Enterovirus 71</td>
<td>0.01 PFU/25μL reaction</td>
<td>0.00004 PFU units/μL</td>
<td>232</td>
</tr>
<tr>
<td>Enterovirus 71</td>
<td>0.33 TCID50/reaction per 25μL reaction</td>
<td>0.0132 TCID50/μL</td>
<td>236</td>
</tr>
<tr>
<td>Coxsackievirus A16</td>
<td>1.58 TCID50/reaction per 25μL reaction</td>
<td>0.0632 TCID50/μL</td>
<td>236</td>
</tr>
<tr>
<td>Coxsackievirus A16</td>
<td>81 copies/reaction per 25μL reaction</td>
<td>3.24 copies/μL</td>
<td>235</td>
</tr>
<tr>
<td>Coxsackievirus B</td>
<td>0.1 pg RNA/12.5μL reaction</td>
<td>0.008 pg RNA/μL</td>
<td>240</td>
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<tr>
<td>Coxsackievirus B</td>
<td>0.1 pg RNA/12.5μL reaction</td>
<td>0.008 pg RNA/μL</td>
<td>243</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>400 copies/12.5μL reaction</td>
<td>32 copies/μL</td>
<td>245</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>0.4–0.8 FFU/12.5μL reaction</td>
<td>0.016–0.032 FFU/μL</td>
<td>247</td>
</tr>
<tr>
<td><strong>Nucleic Acid Sequence-Based Amplification (NASBA)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human enterovirus A &amp; B</td>
<td>&lt;100 copies RNA per reaction</td>
<td>No volume provided</td>
<td>229</td>
</tr>
<tr>
<td>Coxsackievirus B</td>
<td>10 pg RNA/20μL reaction</td>
<td>0.5 pg RNA/μL</td>
<td>243</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>0.4 ng of RNA/ml using 25μL reaction volumes</td>
<td>0.0004 ng RNA/μL</td>
<td>248</td>
</tr>
<tr>
<td><strong>Astroviridae Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loop-Mediated Isothermal Amplification</td>
<td>Astrovirus</td>
<td>3.6 copies/μL using 25μL reaction volumes</td>
<td>249</td>
</tr>
</tbody>
</table>
### Adenovirus Family

<table>
<thead>
<tr>
<th>Method</th>
<th>Adenovirus</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinase Polymerase Amplification (RPA)</td>
<td>50 copies/50µL reaction</td>
<td>1 copies/µL</td>
</tr>
<tr>
<td>Loop-Mediated Isothermal Amplification (LAMP)</td>
<td>50–100 copies/20µL reaction</td>
<td>2.5–5 copies/µL</td>
</tr>
</tbody>
</table>

*Limit of detection normalized to per µL for the purposes of comparison; however, volume associated dependence of reaction cannot be dismissed.

LGC = log$_{10}$ genomic copies

PFU = Plaque forming units

TCID$_{50}$ = Median Tissue Culture Infectious Dose

#### 2.7 Isothermal Amplification and Tolerance of Matrix-Associated Inhibitors

Inhibitory substances present in food and environmental samples pose a problem for detection of viruses via PCR and other molecular methods. Numerous components in clinical, environmental, and food samples have been characterized as inhibitors for PCR$^{255}$. Such inhibitors may include bile salts, complex polysaccharides, collagen, heme, humic acid, proteinases, and calcium ions$^{256,257}$. Although there are a variety of isothermal methods to use, most typically contain an RNA/DNA extraction step before amplification to prevent background noise and disruption of the amplification reaction from inhibitors$^{258}$. However, this requires more specialized equipment and can compromise the portability of any downstream detection assay at point of care. More recently, a few studies have observed the difference between using traditionally extracted and crudely extracted samples prior to amplification. These approaches typically involve crude extraction steps, like heating the sample to break down viral particles and release
genomic nucleic acid. Furthermore, research should be conducted on effects of inhibitors during both upstream and downstream processing of isothermal assays (Fig A2.1).

### 2.7.1 Clinical Samples

Stool is commonly contaminated with foodborne viral particles and rapid detection plays a critical role in prevention of outbreaks. Moore et al.\textsuperscript{224} investigated the direct boiling of 20\% and 2\% fecal suspensions of norovirus GII.4 New Orleans with RT-RPA and RT-qPCR assays. The RT-RPA assay displayed a higher positivity rate for 20\% stool (61\%) compared to RT-qPCR (18\%), and 61\% versus 58\% positivity for 2\% stool; taken together, this suggests that RT-RPA may exhibit a higher tolerance of inhibitors present in stool compared to real time RT-PCR.

In addition to stool, other reports suggest higher tolerance of inhibitors associated with different clinical samples for isothermal assays compared to PCR. Nie et al. tested 145 nasopharyngeal swabs using RT-PCR and RT-LAMP. The assay demonstrated a sensitivity and specificity of 90.3\% and 100\%, respectively, using RT-PCR and 86.83\% and 100\% when using RT-LAMP. Direct RT-LAMP of EV71 on nasopharyngeal swabs that were heat-treated displayed a detection limit of 0.8 TCID50/μL\textsuperscript{259}. As opposed to the reports for stool above, this suggests that real time RT-PCR may have better tolerance than RT-LAMP when using nasopharyngeal samples with potential inhibitors. RT-LAMP has also been used for HEV in crudely processed animal tissue culture samples\textsuperscript{245}. Echovirus 11 and EV71 strains were also directly detected from crudely processed animal tissue culture samples, with the assay displaying a sensitivity of 28,000 copies and 7,400-13,000 copies per 12.5μL, respectively\textsuperscript{245}. 
2.7.2 Water

As previously stated, water is a vector for several enteric viruses. Untreated metropolitan wastewater can contain a variety of contaminants, including bacteria, chemicals, human and agriculture biological waste, and pollution. Sewage samples processed without extraction (heated at 100 °C for 5 min) were tested by Ziros et al. for adenovirus 40 and 41 using LAMP. The assay was able to accurately detect 93.75% (15/16) of screened urban sewage samples. Jean et al. also tested crudely processed sewage samples for HAV strain HM-175 using NASBA. Spiking 1000 HAV PFU/μL (before treatment) into unprocessed raw wastewater, wastewater after aerobic digestion with activated sludge, and wastewater after aerobic digestion and UV treatment were tested using a NASBA assay with dot blot hybridization. Positive signals were observed in all samples; however, a weaker signal was obtained in the unprocessed raw wastewater, suggesting some level of matrix inhibition.

2.7.3 Food

Surprisingly, few reports exist analyzing and comparing the performance of isothermal assays for viral detection in crudely treated food or food concentrate samples. Currently, a few papers on the amplification of foodborne bacteria have been published. A smaller study was conducted on lettuce and blueberries inoculated with HAV strain HM-175 using NASBA, in which samples were spotted with $10^5$ HAV PFU/μL and eluate from the samples was heat treated for extraction. All eluates showed a recovery of 80% \(^{248}\). In all, it appears that many isothermal amplification methods show promise for use in crudely processed samples, but much more work is needed, especially in food and environmental samples.
### Table A2.2. Matrix inhibition without nucleic acid extraction when using isothermal amplification

<table>
<thead>
<tr>
<th>Isothermal Amplification Method</th>
<th>Sample Matrix</th>
<th>Viral Target</th>
<th>Limit of detection with reaction volume</th>
<th>Limit of detection normalized to /μL</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinase Polymerase Amplification (RPA)</td>
<td>20% heat treated human fecal suspensions</td>
<td>GII.4 New Orleans norovirus</td>
<td>Detected 61% of boiled stool samples</td>
<td>N/A</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>2% heat treated human fecal suspensions</td>
<td>Norovirus GII.4 New Orleans</td>
<td>Detected 58% of boiled stool samples</td>
<td>N/A</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Simple heat-treatment of nasopharyngeal swab specimens</td>
<td>EV71</td>
<td>1.6 TCID50/20μL reaction</td>
<td>0.8 TCID50/μL</td>
<td>259</td>
</tr>
<tr>
<td>Loop-Mediated Isothermal Amplification (LAMP)</td>
<td>Sewage samples</td>
<td>Adenoviruses 40 and 41</td>
<td>Detected 93.75% (15/16) of urban sewage samples</td>
<td>N/A</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>Raw wastewater</td>
<td>Hepatitis A virus</td>
<td>10⁶ PFU/mL per 25μL reaction</td>
<td>1000 PFU/μL</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Wastewater after aerobic digestion with activated sludge</td>
<td>Hepatitis A virus</td>
<td>10⁶ PFU/mL per 25μL reaction</td>
<td>1000 PFU/μL</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Wastewater after aerobic digestion and UV treatment</td>
<td>Hepatitis A virus</td>
<td>10⁶ PFU/mL per 25μL reaction</td>
<td>1000 PFU/μL</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>Hepatitis A virus</td>
<td>10⁸ PFU/mL per 25μL reaction</td>
<td>100000 PFU/μL</td>
<td>248</td>
</tr>
</tbody>
</table>
Blueberries  Hepatitis A virus  $10^8$ PFU/ mL per 25μL reaction  100000 PFU/μL

*Limit of detection normalized to per ul for the purposes of comparison; however, volume associated dependence of reaction cannot be dismissed.

LGC = $\log_{10}$ genomic copies

PFU = Plaque forming units

2.8 Discussion

Numerous isothermal amplification techniques have been reported for foodborne and enteric viruses. Of these, NASBA was the subject of much of the first series of investigation but has quickly been replaced by loop-mediated isothermal amplification with an increasing amount of focus. However, RPA has most recently been the subject of much focus for isothermal amplification of foodborne and enteric viruses, likely due in part to its shorter reaction time and reduced number of required primers. However, the use of LAMP’s several primers for specificity may be useful for viruses that are less conserved, like HAV. The use of broadly reactive and serotype-specific primers should be accurately designed in each instance and utilized based on situation. Multiple isothermal assays for a number of these viruses show promise, with comparable sensitivities to real time PCR-based methods, with less time to result and more portability in instrumentation. Further, some reports suggest that such isothermal methods may have potential to better tolerate sample matrix-associated inhibitory substances, meaning more crude nucleic acid extraction techniques may be able to be utilized to further realize the true portability of these downstream isothermal detection techniques.

However, more work needs to be conducted on the degree to which these assays can tolerate inhibitory substances from food and environmental samples, as well as the use of endpoint dyes in place of costly fluorescent probes. A fast, accurate, “suitcase”
diagnostic is within the realm of possibility for isothermal amplification. However, more work is needed for detection of these viruses from food and environmental samples—including upstream sample concentration steps—as the level of viruses in these samples is often low. As further work is conducted for improved, portable upstream sample concentration and purification techniques is conducted, the need for identification of downstream detection techniques that can maintain their sensitivity in the presence of residual food and environmentally associated inhibitors is needed. In all, the collected reports discussed here reveal the promise of isothermal amplification techniques for foodborne viruses given their rapidity, portability, sensitivity, and specificity; however, more research and further development of these techniques is needed to better realize their utilization for routine detection of these viruses in food and environmental samples.
3.1 Introduction

A food allergy is defined as an abnormal immune reaction to the repeated exposure of certain foods\(^{42}\). It can manifest as minor gastrointestinal distress and skin rashes, to life-threatening anaphylactic shock \(^{42}\). These adverse symptoms and the burdens associated with avoiding food products can disrupt the quality of life. It is unknown why some individuals will develop an allergy to a specific antigen, while others will not\(^{43}\).

Food allergies have been rising at an alarming rate, nearing 6.5% (5% of adults and 8% of children) of the general population (of developed counties) affected to date\(^{42}\). Of these food allergies, 90% are caused by “The Big Eight”, a term referring to all major Food and Drug Administration (FDA) regulated food products. These foods include cow’s milk, hen’s egg, fish, crustacean shellfish, tree nut, peanut, wheat, and soybean\(^{42}\). Genetic influences should not be attributed as the main contributing factor in the rise of allergic diseases. It has been hypothesized that the increase in allergies stems from the “hygiene hypothesis”, which states that early life exposure to microorganisms protect against allergic disease\(^{42}\). The gut microbiome, termed a collection of microbiota and their genetic contents in the gastrointestinal tract, has been shown to play a part in the development of asthma, atopic dermatitis, and food allergies through mucosal tolerance and possible bacterial metabolites over the past decade.

Despite being a very timely and important topic, no recent reviews exist addressing the gut microbiome and its relationship with allergens specific to each of the big eight
foods. This review will present the gut microbiological and dietetic factors associated with the development and treatment of food allergies. Food antigens will be explored individually for their connection to the gut microbiome.

Figure A3. 1. Summary of bacterial and dietary changes involved in food allergy

3.2 We Are What We Eat: Diet and the Microbiome

Trillions of gut microbes from thousands of different species make up the highest density of microbes within the human body\textsuperscript{265}. Diet shapes the configuration of the gut microbiome at early life. The gut microbiome of infants that were breastfed have a unique and beneficial composition that is not observed in those given formula when compared later in life\textsuperscript{19}. Gut microbiome dysbiosis in early life is thought to be related to development of allergies later in life. When children start a solid food diet (around 6 months) the gut microbiome shifts significantly for a second time. The adult gut microbiome generally can be classified into three distinct enterotypes that are dominated by \textit{Bacteroides}, \textit{Prevotella},
or *Ruminococcus*, respectively. The diet of the individual influences the enterotypes, with *Bacteroides* being associated with a Western-type diet high in proteins and fat, and *Prevotella* being associated with plant fiber consumption. As a modifiable target, modulation of the gut microbiome through dietary intervention (high fiber related diet), prebiotics or probiotics have seen an increase in both research interest and product development in recent years. However, the magnitude and duration of microbiome changes by dietary intervention have been largely inconsistent.

High levels of fiber can increase the production of short chain fatty acids (SCFA) from specific bacteria in Firmicutes, such as *Bacteroidetes*, Clostridia (*Ruminococcus* enterotype) and *Bacilli*. SCFAs are bacterial fermentation products and are profoundly affected by food intake. There are three common short chain fatty acids that tend to be produced by bacteria: acetate, propionate, and butyrate. Acetate is the acid produced in the highest quantity, but butyrate is thought to be the main energy source for colonocytes. These acids are absorbed into the portal vein during lipid digestion, and have been associated with major health benefits, including a reduced risk of inflammatory diseases and Treg function. A decrease in SCFA may lead to an increase in pathogenic bacteria due to a decrease in gut pH. Pathogenic bacteria can cause epithelial damage to the colon walls, and many believe food allergies are an epithelial barrier disease.

Other metabolites derived from commensal bacteria include long chain fatty acids (LCFA), glycolipid, histamines, vitamins B2/B9 byproducts and amino acids. LCFA are major nutrients, including the clinically important ω3 and ω6 FAs, with ω3 FAs known to have anti-allergic and anti-inflammatory properties. Pro-inflammatory LCFA metabolites, including stigma- and sitosterols and 8-hydroxyoctanoate, are associated with
lower risk of allergy development\textsuperscript{23}. These metabolites are thought to decrease IL-4 produced Th2 cells.

The current dietary interventions for those with food allergies include tolerance or simply avoidance. The enrichment of SCFA-producing bacteria in the body has been investigated as a potential treatment, as stated above, using the enrichment of the Firmicutes phylum. One may naturally increase these bacteria by introducing higher levels of fiber, or prebiotics, into their diet. High fiber diets used in mice were found to decrease allergic sensitization\textsuperscript{268}. Other options for dietary changes may include an increase in food derived probiotic bacteria found within fermented foods. Histamine can be derived from bacterial and dietary sources, including fermented foods. It should be noted that dietary and bacterial derived histamine’s role in food allergy is still currently unknown, however some data suggests the intestinal inflammation brought on from dietary histamine may enhance sensitization\textsuperscript{24}. Western lifestyle and diet promote a change in bacteria which has not been associated with the human gut in evolution. These changes from millennia of human biology, even small, may lead to larger detrimental outcomes.

3.3 Just One Bite, or Maybe Two: The Mechanisms of Food Allergy

Certain food may cause nonimmune responses in the body, including digestive enzyme disorders. However, these should not be confused with true food allergies. Food allergies are atopic disorders in which the body has a hypersensitive immune response to a normally harmless food derived protein\textsuperscript{43}.

The most common cause of food allergy is defined as an immediate IgE-mediated reaction. For an allergic reaction to occur, the antigen must interact with the intestinal mucosa multiple times. One will not have an allergic reaction to a food the first time they
ingest it. This process is defined as sensitization. For food sensitization to occur, the protein must get to the intestinal epithelium without denaturing. They are then transferred from the lumen into the mucosa through gut epithelial cells and other specialized cells\textsuperscript{45}. Once in the mucosa of the gut, mucosal dendritic cells interact with the antigen and proceed to T-cell locations to present the antigen to naive T cells, to form the classically seen Th2 cells. Th2 cells will produce certain cytokines, including IL-3, IL-4, IL-5, IL-9, and IL-13. From this, B cells will proliferate to produce IgEs, in place of other antibodies like IgG or IgM. These IgEs have a high affinity for Fc\varepsilon RI receptors found on mast cells and basophils and gear the cells up for further exposure, as sensitization is now complete\textsuperscript{43}. The processes of sensitization can occur in several other locations in the human body, including the oral cavity, skin, and respiratory tract\textsuperscript{45}.

When there is reintroduction of the antigen, anaphylactic degranulation of the mast cells and basophils follows and there is a release of inflammatory mediators, including histamine, cytokines, and leukotrienes. At this point, the presence of the food antigen in any body tissue (mouth, stomach, gut) can induce an IgE mediated reaction that generally occurs a few minutes after exposure. Those with IgE mediated or mixed food allergies can be identified based on the detection of food allergen specific IgE.

Other forms of food allergy include delayed non-IgE mediated reactions and pollen food syndrome (PFS), which are less common and often confused for IgE mediated food allergies\textsuperscript{43,269}. Delayed non-IgE mediated food allergies involves antigen contact with sensitized T-lymphocytes, as opposed to mast cells, which then damage the gut mucosa. This type of reaction is associated with disorders such as celiac disease and may occur hours later, involving vomiting and diarrhea\textsuperscript{43}. Interestingly, pollen food syndrome is still
an (IgE)-mediated disease, however, it is a consequence of an already established pollen allergy cross reacting with antigens, similarly structured to pollen, found within certain foods\textsuperscript{269}. This is commonly seen in nuts, fruits, and vegetables. However, both are not within the scope of this review.

3.4 “Good Source of Protein?”: Interactions of Food Matrices and Gut Permeability

Allergy type and dosage can affect immune response, along with adjuvants in the gut. The more food protein that makes it intact to the gut, the more chance there is of sensitization. While most are broken down by gastric acid and digestive enzymes, some intact proteins and peptides can move further into the large intestine. Certain factors can affect sensitization by allowing for a strong or weaker epithelial wall; including genetics, alcohol, anti-inflammatory drugs, pathogens, and stress\textsuperscript{43}. Gut bacterial populations and bacteria-derived proteases and peptidases may affect protein absorption into the bloodstream\textsuperscript{270}. Cooking promotes protein denaturation through various forms of heat processing (pasteurization, blanching, convection/conduction). However, peanuts and tree nuts are commonly roasted and many of their allergic proteins are not broken down in the process\textsuperscript{271}. It is also believed that the heating of peanuts and shrimp may enhance allergenicity, by aggregating protein to be more resistant during digestion\textsuperscript{272}.

Along with this, a few of the big eight food allergens can be consumed uncooked in some cases. Soybeans, finfish, and shellfish are commonly eaten raw. This uncooked or harder to digest factors, along with a higher protein content, may contribute to why these foods are considered to cause more sensation in individuals than other foods. Interestingly, many children may tolerate baked milk and egg if given in a wheat matrix, as other
ingredients eaten together are known to decrease the proteins availability to interact with the epithelial wall\textsuperscript{272}.

It is currently unclear why these food proteins cause a negative immune response in some. The gut-associated lymphoid tissues maintain tolerance by differentiating against self or non-self-antigens and through the recognition the pathogens\textsuperscript{273}. However, if this process breaks down, the body can no longer tell the different between friend or foe. Tolerance can be achieved to negate harmful effects. Oral tolerance to a food allergen can develop after frequent expose to the antigen due to possible changes and mechanisms involved in the dendritic cells, gut epithelial cells, and the gut microbiome. However, at this time, the exact mechanisms of desensitization is unknown\textsuperscript{45}.

3.5 The Gut, the Big Eight and the Correlation Between the Two

These is a clear difference between each food allergy, both in sensitization period and severity of reaction. Children tend to outgrow cow milk and hen egg allergies, but peanut and shellfish allergies can occur later in life and may be very severe. Table A3.1 displays the different food proteins that have been found to induce allergic reactions.

| Cow’s milk | Casein, whey, bovine serum albumin, immunoglobulin G heavy chain and a-lactalbumin\textsuperscript{274} |
| Hen’s egg | Whites: Ovomucoid, ovalbumin, ovotransferring, and lysosome\textsuperscript{275}  
Yolk: Livetin, vitellenin and apoprotein B\textsuperscript{275} |
| Peanut | Cupin(Ara h 1, 3), prolamin(Ara h 2, 6, 7, 9), profilin(Ara h 5), bet v-1-related proteins (Ara h 8), oleosin (Ara h 10,11) and defensin(Ara h 12, 13)\textsuperscript{276} |
| Tree nut | Vicilin, 2S albumin, nsLTP, legumin, bet v 1-like, oleosin, 60 s acidic ribosomal prot. P2, manganese superoxide dismutase and profilin\textsuperscript{277} |
Shellfish | Tropomyosin, arginine kinase, myosin light chain, and sarcoplasmic calcium-binding protein

Finfish | Parvalbumins, enolase, aldolase, tropomyosin, vitellogenin

Soy | Hull proteins, kunitz trypsin inhibitor, glycinin, α-subunit of β-con-glycinin, and 50-kd protein with homology to chlorophyll A-B binding protein and starvation-associated message

Wheat | α-Amylase/trypsin inhibitor, album, globulins, serine proteinase inhibitor, lipid protein transfer, thioredoxin, peroxidase, gliadin, thiol reductase, and thaumatin-like protein

### 3.5.1 Cow’s milk

Often confused with lactose intolerance, a cow milk allergy (CMA) can be a reaction to the main two proteins in milk, casein, and whey (Table 1). It affects 2-6% of children, making it the most common childhood food allergy. The typical onset age for milk allergy is 4.3 months into life, or when exposed to milk after breast feeding. Most children become tolerant by the age of three, however, some do not show resolution until teenage years. Milk allergies can leave children with a challenge of getting proper nutrition. Infant formula typically comprises of a blend of cow’s milk whey and casein, vegetable fat, lactose sources, vitamin/mineral mix, and probiotic bacteria. Because of the use of cow milk protein in these typical formulations, a substitute is required. Formula is used widely, as only 38% of newborns exclusively breastfed globally. In the United States, only 75% of infants are exclusively breastfed starting from birth, with 67% of all children relying on infant formula for some portion of their nutrition after three months. Only 13% of new mothers meet the recommendation of breastfeeding exclusively for six months. Exclusive breastfeeding has been linked to reduction of specifically cow milk sensitization, however, it should be noted that many different studies have been carried out with conflicting results between breastfeeding, formula feeding, and milk allergy.
The exact mechanism as to why certain children will develop CMA remains poorly understood\textsuperscript{288}. Increasing evidence suggests that a normal gut microbiome is critical to suppress CMA. Studies have shown germ-free animals to be at a higher risk for sensitization to cow milk protein\textsuperscript{289,290–292}. Maternal and infant use of antibiotics has been associated with an increased risk of the child developing a CMA\textsuperscript{293}. In addition, compositions of the early life gut microbiome differ between CMA resolution and continuing allergy in children, with enrichment of Clostridia and Firmicutes in the infant gut microbiome of those whose CMA resolved.\textsuperscript{294} In a study of 226 children, little over half (56\%) had the allergy resolved, with the gut microbiome composition between the two groups being significantly different between 3-6 months. This was not seen between other ages, supporting the importance of the early life gut microbiome in CMA resolution.

There are two different theories involving the Clostridia class from Firmicute phylum and its involvement in milk allergy. One theory states Firmicutes and those in the Clostridia class are beneficial, but others claim they may be harmful. Bacteria from Clostridia are major butyrate producers. Butyrate is known to regulate colonic regulatory T cells that are essential for immune tolerance\textsuperscript{295,296}. Butyrate also allows for regulation of the intestinal epithelial barrier which may decrease the intake of food antigens into the blood. Lower abundance of the Firmicutes phylum and the Clostridia class has been seen in children who’s allergy did not resolve\textsuperscript{294}. However, it is theorized that not all Firmicutes and products of the Clostridiales species lead to protection, but only specific species and even strains. In one study, treatment with extensively hydrolyzed casein formula combined with \textit{Lactobacillus rhamnosus} GG lead to an increase of Firmicutes, including \textit{Roseburia}, \textit{Blautia} and \textit{Coprococcus} (all from the Clostridiales order), in both allergic and healthy
groups\textsuperscript{297}. \textit{Oscillospira} was only seen in those whose allergies resolved. In their analysis of fecal butyrate levels, higher levels were found in those who became tolerant to milk allergies. Interestingly, there were strain-specific differences of \textit{Blautia} and \textit{Roseburia} between the tolerant and allergic patients. It should be noted that this study was conducted on stool after allergic symptoms start to appear, and that the importance of an increased abundance of Firmicutes may be during sensitization. The gut microbiome develops in early life and becomes mature at 2-3 years old\textsuperscript{19}. Longitudinal characterization of the microbial dynamics before and after sensitization and challenging will provide a holistic view of the evolving microbiome and development of CMA. Another Clostridia species, \textit{Anaerostipes caccae}, has been shown to reduce the risk of developing CMA\textsuperscript{298}. When using a fecal microbiota transplantation (FMT) from CMA children into mice, the mice became more susceptible to anaphylactic reactions to milk protein when compared to mice treated with healthy children’s stool, in which higher levels of Clostridiales and \textit{Anaerostipes caccae} were found. Gene expression analysis of intestinal epithelial cells of recipient mice identified genes involved in pyruvate metabolism, \textit{acot12} and \textit{me1}, were upregulated in the ileum of CMA-colonized mice, compared to the healthy-colonized mice. An integrated analysis identified a strong negative correlation of \textit{Anaerostipes caccae} with pyruvate metabolism. The latter was speculated to be one key intermediate during glycolysis, a metabolic pathway for colonocytes during gut microbiome dysbiosis. This study highlights that host-microbiome interaction is central to regulating tolerance to dietary antigens.

The counterargument for the beneficial effects suggests that the higher concentration of butyric acid produced by bacteria in the Firmicutes phylum can increase the permeability
of intestinal mucosa, thus possibly allowing higher amounts of cow milk protein to enter the bloodstream\textsuperscript{299,294}. A metagenome functional prediction conducted on the stool of allergic children found a decrease in fatty acid metabolism in children whose allergy resolved\textsuperscript{294}. It was suggested that cow milk lipids drive proinflammatory effects, and a decrease in fatty acid metabolism may be positively correlated with less inflammation. Other studies have found lower levels of branched-chain short fatty acids in healthy infants compared to allergic infants\textsuperscript{300}. This study also found high levels of \textit{Clostridium coccoides} in those with a cow milk allergy, along with an increase of butyric acid. Other studies have also found increased levels of \textit{Clostridium coccoides} in groups which did not develop tolerance to CMA\textsuperscript{301}. However, as stated above, it is important to remember that it is possible not all Firmicutes may lead to protection, even being as specific as species and strains.

Introduction of bacteria that are suppressive of CMA has long been proposed to both preventing sensitization or aiding immunotherapy. \textit{Lower counts of probiotic bacteria, Bifidobacteria and Lactobacilli}, have been reported in the guts of children with CMA\textsuperscript{283}. The clinical outcome by reintroduction of these bacteria is mixed. \textit{Lactobacillus casei} CRL431 and \textit{Bifidobacterium lactis} Bb-12 were given to infants diagnosed with CMA for 12 months\textsuperscript{284}. However, it was found these bacteria did not have any effect on the acceleration of milk allergy tolerance. Comparatively, a similar study was conducted, which looked at the most common probiotic on the market, \textit{Lactobacillus rhamnosus} GG and its ability to accelerates tolerance acquisition in infants with cow’s milk allergy\textsuperscript{302}, as \textit{L. rhamnosus} GG has been shown to promote respiratory and gut immunity\textsuperscript{303,304}. After 6 months of \textit{Lactobacillus rhamnosus} GG supplementation, 60\% of the infants with CMA
had resolved symptoms, compared to the 22% in the control group. It was hypothesized that the resolution of CMA could be related to the immunoregulatory role of the *Lactobacillus rhamnosus* GG. *Lactobacillus rhamnosus* GG is found to balance generation of Th2 related cytokines. With a more in-depth analysis of *Lactobacillus rhamnosus GG*, the probiotic was found to increase specific butyric acid producing bacteria in the gut of children whose allergies resolved after treatment.

*Bifidobacterium* is another common probiotic used outside of the *Lactobacillaceae* family and is commonly found in the gut of infants. An increase in these bacteria have been seen to improve the characteristics in other IgE or Th2 allergies. *Bifidobacterium breve* M-16V and a supplement containing a mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides, was introduced into the mice’s diet and the anaphylactic reaction to milk protein was measured. Combination of both were found to reduce ear swelling upon cow milk protein introduction in the mice, with less effectiveness when used separately. The protective effect of *Bifidobacterium* in the mouse model of food allergy is also evident in another study where serum IgE levels were lower after administration of *B. infantis* CGMCC313-2, compared to that in controls. A well-characterized healthy infant microbiota, containing high levels of *Bifidobacterium* and *Bacteroides*, were transplanted it into germ-free mice, which lead to an improved protection of milk allergy.

Unpasteurized milk is thought to protect from asthma and food allergy, however its benefits are controversial. Unpasteurized milk is known to contain many bacteria, including probiotics, which are otherwise inactivated in pasteurized milk. This includes lactic acid bacteria such, as *Lactobacillus, Streptococcus, Enterococcus, Lactococcus,*...
Leuconostoc, Weisella and Pediococcus. However, possible contamination with numerous pathogenic bacteria, including Staphylococcus aureus, Salmonella enterica, and Escherichia coli, may lead to further gut damage. Individuals should look for safer sources of probiotics, including other dairy products such as yogurts and cheese.

3.5.2 Hen’s egg

Egg allergies affect 0.5% to 2.5% of young children, starting around infancy. However, this is as high as 8.9% of infants in certain populations, making it the second most common food allergen in children. In addition to causing dietary restrictions, egg allergies have significant health consequences for children, as they cannot receive certain vaccines generated using eggs. Ovomucoid is considered the main allergen in egg because of its immunoreactivity and heat resistance (Table 1). While ovalbumin (OVA) is the most abundant potential allergen in eggs, it is easily denatured during thermal processing. Although most proteins are found within the egg whites, allergic children should avoid eggs altogether, as proteins can bleed into the yolk, along with the yolk having small amounts of allergic proteins themselves. Tolerance may be accelerated similarly to milk, with studies finding some children able to tolerate eggs faster after a diet including baked eggs.

Early life gut microbiome composition of those with egg allergies has been shown to play an important role in development of the disease. However, like the aforementioned milk studies, no association has been found between the gut microbiota and egg allergy resolution later in life (by the age of 8 years). Higher microbial diversity during this early stages of life has been found to correlate with egg allergy, sharing similar results to previous studies on other allergic diseases. At the phylum level, Firmicutes and
Verrucomicrobia are higher in infants with egg allergy, with *Ruminococcus* and *Lactococcus* associated on a genus level, when compared to health patients. A difference in purine metabolism was also observed, with less in those with egg allergy. A depletion of uric acid inhibits the activity of xanthine oxidase in purine metabolism, significantly changing the inflammatory responses of mice. This has been seen in asthma exacerbation, a mechanism proposed to be involved in sensitization.

Bacterial interventions are a common research topic to alleviate egg allergy. Oral administration of *Bifidobacterium bifidum*, *Lactobacillus casei*, and *Escherichia coli* were found to lower IgE, or overall immune response in OVA with cholera toxin mice; however, *E. coli* caused an unhealthy lack of weight gain. The differences in OVA specific fecal IgA levels and OVA specific serum IgG1 levels among the experimental bacteria suggests they are likely to inhibit allergy responses through different mechanisms. *Bifidobacterium longum*, but not *Enterococcus faecalis*, extracellular vesicle–derived extracellular vesicle–derived protein were found to suppress egg allergies by induced apoptosis to bonded mast cells in mice.

The effect of other bacteria, that are not lactic acid bacteria, has also been investigated in the egg allergy mouse model to explore possible benefits. Therapy with Clostridiales, *Subdoligranulum variabile* and a Bacteroidales consortium has been found to suppress allergy. In this study, a MyD88/ROR-γt pathway in nascent Treg cells was deficient in food allergy infants and mice. Inhibition of this pathway in Tregs abrogated protection. Oral supplements with 17 Clostridia strains, previously isolated and invested for positive effects, led to a reduction of OVA-specific IgE after sensitization and reduced diarrhea scores after challenge in mice.
OVA-sensitized mice with a mutation in the IL-4 receptor α chain (Il4raF709) harbor a distinct gut microbiome, with over-represented populations of *Lachnospiraceae, Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae*\(^{21}\). Fecal transplantation of the microbiome in OVA-sensitized *Il4raF709* mice promoted OVA-specific IgE responses and anaphylaxis. Interestingly, treatment with OVA-specific Treg cells led to a suppression of allergic response, accompanied by the suppression of allergy associated microbes. Taken together, these reports suggest that specific gut microbiota and their associated immune effects are at least partially responsible for OVA-mediated food allergy, providing additional rationale for microbiome-based intervention in food allergy.

3.5.3 Peanut

Peanut allergies are one of the most predominate food allergies to carry into adult life\(^ {320}\). At least 11 peanut antigens have been described (Table 1). The quality of life for a child with a peanut allergy has been perceived to be worse than a diabetic child\(^ {321}\). Peanuts can be easily hidden in foods, including their use as bulking agents in nonrelated items. Accidental exposure is common in children with peanut allergies, with annual incidence of 12-15% in these children\(^ {322}\). There has been an increase in peanut allergies, with a 21% increase since 2010, with 2.5% of children in the U.S. having a peanut allergy\(^ {323}\). Because of this and the severity of the reaction, peanut oral immunotherapy interventions have been developed\(^ {324}\). More recently in 2020, the FDA has approved the first immunotherapy treatment, termed “*Palforzia*” (125696)\(^ {325}\). Oral immunotherapy can be described as an introduction of peanuts to children at a young age that show signs of peanut sensitization in hopes of increasing the threshold that induces a reaction.
While *Palforzia* does not contain a bacterial component, many studies have included bacteria as an adjunct for the therapy. The first randomized placebo-controlled trial on the effectiveness of a combination of *Lactobacillus rhamnosus* GG and peanut oral immunotherapy led to 89.7% of those who received treatment to be desensitized, compared to 7.1% in the no bacterial control group\(^{54}\). *Lactobacillus rhamnosus* GG was used in a different peanut oral immunotherapy, with 67% of the children in the treatment group and 4% in the placebo desensitized following the end of the trial\(^{55}\). It has been proposed *Lactobacillus* promotes peanut tolerance by enhancing the tolerogenic effects of cells, including regulation of T cells. Other treatments that have been investigated include FMT and a rationally defined bacterial consortium supplement. A Boston Children’s Hospital treatment in which children are given oral encapsulated frozen fecal microbiota transplantations (NCT02960074)\(^{326}\), is set for completion in late 2020. Vedanta Biosciences has recently enrolled their first peanut allergy patient to receive VE416, an orally administered live biotherapeutic product containing a defined bacterial consortium. This is thought to have similar, if not more benefits than a FMT, without the need for inconsistencies in a live donor (NCT03936998)\(^{327}\). However, the potential impact of these new treatments remains to be seen.

Dietary interventions have also been reported to have beneficial effects for peanut allergy. The introduction of a high fiber diet, with vitamin A, in mice improved oral tolerance and protected against peanut allergy\(^{268}\). With only fiber and vitamin A, and not an added bacterial component, the gut microbial ecology of the mice was reshaped to have more diversity and an increase in Firmicutes, specifically Bacilli. In turn, this caused an increase in levels of acetate and butyrate. The direct feeding of acetate and butyrate in
drinking water was also found to protect mice from peanut sensitization. This diet lead to an increase in tolerogenic CD103+ dendritic cells, which are required for oral tolerance to antigens, and higher IgA serum levels. Specifically, this IgA increase is thought to be associated with Clostridia. Mice treated with antibiotics or germ-free mice have been reported to be more susceptible to peanut sensitization. Reintroduction of Clostridia bacteria, but not Bacteroides bacteria, blocks sensitization to peanut allergen in germ free mice. The presence of Clostridia was thought to be associated with an adaptive expansion of Tregs found in the intestine and induce immunoglobulin class switching from IgE to IgA. It is believed that IgA contributes to the immune response by reducing allergen uptake. Elevated levels of uric acid have been found in mice which have undergone sensitization to peanuts, as well as with children confirmed to have a peanut allergy. Uric acid is a by-product of purine metabolism, and the depletion of uric acid during sensitization of mice is known to prevent the development of IgE and IgG1. Changes in total antibody composition may help prevent sensitization.

3.5.4 Tree nuts

Tree nuts are a broad term used to describe any nut that grows on a tree, including almonds, brazil nuts, cashew nuts, hazelnuts, macadamia nuts, pecans, pistachios, and walnuts. They differ from peanuts, which are classified as legumes along with peas and soybeans. Consumption of walnuts and other tree nuts has been seen to increase healthy probiotic and butyric acid producing bacteria in the gut. About 4.9% of the general population has a tree nut allergy. The lack of this food source can have potential negative effects on health, including a lack of vegetable protein, dietary fiber, magnesium, potassium, copper, and vitamins E and K. Peanut and tree nut allergies overlap within
people commonly, as the structure between the antigenic proteins in both are similar. Unsurprisingly, a meta-analysis has found a stronger correlation between peanut and tree nut allergies. Along with this, many nut allergies are due to an allergic reaction to pollen and not to the nut protein itself. This phenomenon is referenced to as pollen food allergy syndrome. There are several different tree nut proteins that can cause allergy, most being found within the metabolic and storage protein family of the nut (Table A3.1).

For those with tree nut allergies, a decrease in Clostridiales and higher Bacteroidales has been observed. With peanuts and tree nuts taken together, a positive association can be seen of allergy with *Bacteroides*, *Bacteroides fragilis* and Bacteroidales, and a negative association with Clostridiales, *Prevotella* and *Ruminococcaceae*. There is currently no microbiome associated treatments for tree nut allergies. However, like peanuts, there are serval immunotherapy options which have been studied. As peanut and tree nut allergies are shown to have similar structure between allergic proteins, positive results from bacterial tree nut immunotherapies may occur as it has with peanuts. Similarly, future work on utilizing certain bacteria as adjuncts to treatment for tree nut allergies should be investigated.

### 3.5.5 Crustacean and molluscan shellfish

Crustaceans are a subsection of the phylum Arthropoda and include crab, rock lobster, prawn, and shrimp, among others. Mollusks generally consumed include gastropods (snail), bivalves (clam, oyster, scallop and mussel), and cephalopods (squid and octopus), and belong to the phylum Mollusca. Tropomyosin is the predominant allergic protein in crustacean, while mollusks contain less well-understood allergens, along with different tropomyosin (Table 1). Mollusk allergies are known to cross react with other
shellfish allergies regardless of these differences; however, crustacean and molluscan allergens do not cross-react with fish allergens. Recent studies have shown tropomyosins from house dust mites to also cause allergies in those allergic to shellfish tropomyosins. However, vertebrate tropomyosins are not known to cause disease. As of 2019, crustacean and molluscan allergy is the most common food allergy affecting adults in the United States. Importantly, while most of the big eight allergies develop in childhood, shellfish allergies can develop at any point in life, much like peanuts.

*Bifidobacterium* has been investigated to have a role in the protection of shellfish-related food allergy. *Bifidobacterium infantis* and *Bifidobacterium lactis*, bacteria commonly found in nursing mothers and the guts of infants, was found to reduce shellfish specific IgE in mice. *Bifidobacterium infantis* was found to revert bacterial proportion imbalance caused from the allergen, by increasing *Dorea* and decreasing *Ralstonia*. It was proposed this proportion of *Dorea/Ralstonia* is involved in Treg cell differentiation and could help balance the Th2/Treg ratio. *Bifidobacterium lactis* was found to increase the ratio of Treg and Th17 cells in a mouse model. Other studies have found *Bifidobacterium longum* and *Bacillus coagulans* to regulate gut dysbiosis and mitigate overactive Th2 response in tropomyosin-induced allergic mice. *Bifidobacterium longum* and *Bacillus coagulans* have also found to regulate gut arginine metabolism pathways, leading to the conclusion that metabolites of aspartate and arginine may be critical for prevention of food allergy. However, it should be noted these studies mostly focused on *Bifidobacterium*, and the effects of other bacteria has not been as well investigated. Future work should focus on investigating the role of other commensal bacteria in mitigating shellfish allergies.
3.5.6 Wheat

Gluten sensitivity is not a true wheat allergy. However, the insoluble gluten proteins, gliadins and glutenins are reported to be the most responsible for inducing IgE related sensitization, especially omega-5-gliadin (Table 1)\textsuperscript{281}. Celiac disease is a common non-IgE mediated reaction involving the intolerance to gluten. The prevalence of wheat allergies varies between different regions of the world, ranging from 0.2% to 1%\textsuperscript{281}. Like cow’s milk and egg allergies, wheat allergy is commonly outgrown by adulthood\textsuperscript{320}. Those with wheat allergies miss out on major fiber sources, like whole grains and bran\textsuperscript{341}. Many of these fibers are considered “prebiotic” and promote the growth of health gut bacteria\textsuperscript{341}. Not surprisingly, it has been seen that the loss in fiber significantly reduces the abundance of fiber-degrading bacteria, resulting in a reduction of SCFA levels\textsuperscript{342}. Luckily, within recent years, there has been an increase in wheat free, or gluten free, products in the food industry\textsuperscript{343}. While these products have been made for gluten intolerance in mind, those with an IgE medicated wheat allergy or gluten sensitivity may also benefit. There is currently no data on the interactions of the microbiome with wheat allergies. This may be of great interest, due to the large fiber components of many wheat products.

3.5.7 Soy

Soy is a complete protein, with sensitization normally occurring from the soybean hull proteins (Table 1)\textsuperscript{280}. 0.4% of children in the U.S. have a soy allergy, and 50% of those tend to outgrow it by adulthood\textsuperscript{344}. Interestingly, one report found eighty-eight percent of those with soy allergies were found to also have peanut allergies\textsuperscript{344}. Soy lecithin is a very common additive included in many food items. For instance, it is used as an emulsifier in chocolate and dairy products and filler in baked goods to reduce fat content. Unfortunately,
not much work has been completed on the effect of avoiding soy consumption on the gut microbiome, nor has there been any research on the potential role of the gut microbiome in soy allergy.

3.5.8 Finfish

The consumption of fish is beneficial, as they are high in omega-3 and omega-6 fatty acids. Omega-3 is well known to have many health benefits and has been seen to help with other IgE mediated conditions \(^{345}\). Fish oil tablets may be taken, but many of these capsules are heavily oxidized before reaching the consumer. Finfish allergens, as with shellfish, may not appear until adulthood. However, this may be due to a lack of fish in childhood diets. The main proteins that cause allergy are parvalbumins, with different variations found as the main protein in different fish (Table 1) \(^{279}\). Interestingly, previous studies have found that antacids encourage sensitization to parvalbumin in mouse models \(^{346}\). This is likely due to the raising of the pH in the stomach, allowing the intact protein to move along into the gut. As with wheat and soy, there is no previous research on the interactions of finish allergy and the microbiome.

3.5.9 Common or Unique - Summary of Work

Shown below in Table A3.2 is a summary of the work presented in this review. There are currently no studies of the role or interactions of the gut microbiome with wheat, soy, or fish allergies. It is possible that some of the microbiome changes and allergy mechanisms may overlap between these allergies and those described above. It is not uncommon for individuals to have multiple food allergies. It is also interesting to note the possible change multiple allergies may have on microbiome dysbiosis vs a singular allergy;
however, this has not been investigated. Therapies and supplements (Table A3.2) may be utilized as functional food additives

Table A3. 2 Summary of trends thought to promote/reduce risk or treat allergies for each of the big eight.

<table>
<thead>
<tr>
<th></th>
<th>Promote risk</th>
<th>Reduce risk</th>
<th>Therapies and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cow’s milk</strong></td>
<td></td>
<td></td>
<td><strong>Promote Risk</strong></td>
</tr>
<tr>
<td>General antibiotic use before and during pregnancy</td>
<td><strong>Reduce Risk</strong></td>
<td><strong>Therapies and supplements</strong></td>
<td></td>
</tr>
<tr>
<td>Short-Chain Fatty Acid m3</td>
<td>Increase in diversity h294</td>
<td><strong>Promote Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium coccoideus</strong> h300</td>
<td>Breastfeeding h287</td>
<td><strong>Reduce Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Atopobium</strong> h300</td>
<td>Increase in diversity h294</td>
<td><strong>Therapies and supplements</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lachnospiraceae</strong> h297</td>
<td>Firmicutes h294,297,298</td>
<td><strong>Promote Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ruminococcaceae</strong> h297</td>
<td><strong>Clostridia</strong> h,294,297,298</td>
<td><strong>Reduce Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacilli</strong> h4</td>
<td><strong>Oscillospira</strong> h297</td>
<td><strong>Therapies and supplements</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Anaerostipes cacaee</strong> h,298</td>
<td><strong>Roseburia</strong> h297</td>
<td><strong>Promote Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Bifidobacterium</strong> h,283,291</td>
<td><strong>Blautia</strong> h297</td>
<td><strong>Reduce Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacteria</strong> h4</td>
<td><strong>Bacteroides</strong> h,m291</td>
<td><strong>Therapies and supplements</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Hen’s egg</strong></td>
<td></td>
<td></td>
<td><strong>Promote Risk</strong></td>
</tr>
<tr>
<td>Increase in diversity h314</td>
<td><strong>Leuconostocaceae</strong> h314</td>
<td><strong>Reduce Risk</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lachnospiraceae</strong> h21,314</td>
<td><strong>Promote Risk</strong></td>
<td><strong>Therapies and supplements</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcaceae</strong> h314</td>
<td><strong>Reduce Risk</strong></td>
<td><strong>Promote Risk</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Therapies and supplements**

- Bifidobacterium infantis CGMCC313-2, 5 × 10^{10} CFU/mL for 6 days. m119
- Lactobacillus rhamnosus GG, 4.5 × 10^{7}–8.5 × 10^{7} CFU/g of formula for 6 months h42
- Lactobacillus rhamnosus GG, 1.4 × 10^{7} CFU/100 mL of formula for 6-12 months h17
- Bifidobacterium breve M-16V, 2 × 10^{9} CFU/g for 7 weeks h307
- Bifidobacterium longum, 5×10^{9} CFU daily for 36 days m317
- Clostridiales consortium, 5 × 10^{7} CFU twice weekly for five weeks m318
- Subdoligranulum variabile, 2.4 × 10^{6}
Lactobacillaceae\textsuperscript{m21}

Rikenellaceae\textsuperscript{m21}

Porphyromonadaceae\textsuperscript{m21}

Proteobacteria consortium\textsuperscript{m318}

Ruminococcaceae\textsuperscript{h314}

Promote Risk | Reduce Risk
---|---
Peanut | Therapies and supplements
\textsuperscript{c}Lactobacillus rhamnosus & peanut oral immunotherapy, 2 × 10\textsuperscript{10} CFU once daily with peanut protein for 18 months\textsuperscript{h54}
\textsuperscript{c}Lactobacillus rhamnosus GG & peanut oral immunotherapy, 2 × 10\textsuperscript{10} CFU of \textit{L} rhamnosus CGMCC 1.3724 and 2 g of peanut protein once daily for 18 months\textsuperscript{h55}
High fiber diet & vitamin A for 2 weeks\textsuperscript{m268}
Direct feeding of acetate and butyrate for 3 weeks\textsuperscript{m268}
aClostridia via 2 oral gavages, once a week\textsuperscript{m328}

<table>
<thead>
<tr>
<th>Promote Risk</th>
<th>Reduce Risk</th>
<th>Therapies and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textsuperscript{c}Bacteroidales\textsuperscript{h50}</td>
<td>\textsuperscript{c}Clostridiales\textsuperscript{h50}</td>
<td></td>
</tr>
<tr>
<td>\textsuperscript{c}Bacteroides fragilis\textsuperscript{h50}</td>
<td>\textsuperscript{c}Prevotella\textsuperscript{h50}</td>
<td></td>
</tr>
<tr>
<td>\textsuperscript{c}Bacteroidales\textsuperscript{h50}</td>
<td>\textsuperscript{c}Ruminococcaceae\textsuperscript{h50}</td>
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</table>

<table>
<thead>
<tr>
<th>Promote Risk</th>
<th>Reduce Risk</th>
<th>Therapies and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textsuperscript{e}Ralstonia\textsuperscript{m338}</td>
<td>\textsuperscript{e}Dorea\textsuperscript{m338}</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Promote Risk</th>
<th>Reduce Risk</th>
<th>Therapies and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finfish</td>
<td>No data present</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>Soy</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Prevention of sensitization
\textsuperscript{b}Therapeutic during allergic reaction
\textsuperscript{c}Increased oral tolerance
\textsuperscript{d}Worsened Sensitization
\textsuperscript{e}Bacteria found in the gut of either FA or health individuals

\textsuperscript{m}Mouse study
\textsuperscript{h}Human study
Many of the same mechanisms were proposed for each of The Big Eight, with some contradicting each other (Table A3.3). Many of the theories revolve around Clostridia and its abilities to regulate T cells. Due to its importance as the most prevalent childhood allergen, cow milk has been the most studied food allergen in term of microbiome association. Mechanistic differences between other allergens may occur, as currently there are not enough data to defer the theory. Importantly, there are currently no data available showing the effect that the gut microbiome has on wheat, soy, and finfish allergies.

Table A3. 3 Summary of proposed mechanistic actions of the effect the gut microbiome has on food allergies.

<table>
<thead>
<tr>
<th>Proposed mechanisms</th>
<th>Allergens Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in diversity strengthens and establishes immune system.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td>Exclusive breastfeeding prevents the introduction of cow’s milk required for sensitization.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td>Firmicutes, those in the Clostridia order and those which increased butyrate production, which regulate colonic regulatory T cells.</td>
<td>Cow’s milk, Hen’s egg</td>
</tr>
<tr>
<td>Those in the Clostridia order regulate innate lymphoid cell function to alter epithelial permeability and reduce allergen uptake into the systemic circulation.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td>Pyruvate metabolism from those in the Clostridia order and Leuconostocaceae family depletes uric acid, which inhibits the activity of xanthine oxidase.</td>
<td>Cow’s milk, Hen’s egg</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> and <em>Bacteroides</em> invoke foxp3 gene activation, which is responsible for the development and function of regulatory T cells.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td><em>B. infantis</em> increased the abundance of butyrate producing bacteria, which in-turn suppress the inflammatory responses triggered by Th2 cytokines.</td>
<td>Shellfish</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus GG promotes growth of SFCA producing bacteria.</td>
<td>Cow’s milk, Peanut</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> induces apoptosis to bonded mast cells.</td>
<td>Hen’s egg</td>
</tr>
<tr>
<td>Antibiotics decrease level of beneficial bacteria.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td>Firmicutes and those in the Clostridia order increased butyrate production, which in-turn increases the permeability of the intestinal mucosa.</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> and <em>Bacillus coagulans</em> regulate gut arginine metabolism pathways and the metabolites of aspartate and arginine may be critical for prevention of FA.</td>
<td>Shellfish</td>
</tr>
<tr>
<td>Mouse study</td>
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<td>Human study</td>
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h Human study; mh Mouse study
3.6 There's More Than Meets the Gut: Gaps and Future Perspectives

3.6.1 Viral and Fungal Interactions

While not given the same amount of attention as the bacterial world in the gut, viruses and fungi may assist in the development and/or treatment of food allergy. Asthma, hay fever, and peanut allergy were found inversely related to Hepatitis A, Herpes simplex virus 1, and *Toxoplasma gondii* infections. Previous research has shown murine norovirus may drive allergic disease, using egg ovalbumin, through changes in normal dendritic cell function. Interestingly, Kernbauer et al found murine norovirus infection of germ-free mice can replace the beneficial effects of commensal bacteria. Infection of murine norovirus in non-obese diabetic mice was reported to led to positive changes in mucosal immunity, altering Tuft cell makers, cytokine secretion and mucosal antibodies. However, others have opposite conclusions, reporting norovirus and rotavirus infection may lead to increased protein absorption and sensitization in the gut. Diets of varying fiber and fat have been shown to change the virome, however, it is not well understood how these changes affect the human body. While dietary fungi have been known to cause allergic reactions, commensal fungi have not been studied for a possible cause or adjunct of disease. Allergy research must be conducted on these missing areas, as a major parts of the human microbiome include viral and fungal organisms.

3.6.2 Clostridiales Cocktail

Next-generation probiotics is a term used to describe the use of commensal bacteria, rather than lactic acid food associated bacteria, as probiotics. There is a clear trend with the involvement of the Firmicutes phylum, and more so with the Clostridiales order, involved in the sensitization and treatment of food allergies. Clostridiales use as a therapy...
and a next-generation probiotic should be further investigated. Throughout this review, Clostridiales has been mentioned several times in multiple food allergens to be a beneficial bacteria. A strain specific difference of beneficial effects in *Roseburia* and *Blautia* give further evidence to the theory that not all Firmicutes, or SCFA producing bacteria, have the same effect on the body. *Dorea* is discussed through multiple studies for possible beneficial effects. For example, one study characterized 17 different Clostridia strains to have positive health effects on food allergy. Further investigation of the effects of Clostridiales on food allergies and use as a treatment option would be valuable, especially regarding specific species, dosage, and timing of administration.

### 3.6.3 Experimental Design and Analysis

Most microbiome studies utilize the 16S ribosomal RNA (rRNA) sequencing technique. However, only certain level of taxonomy can be reached, with many only going down to genus level. Virus and fungal communities are completely missed. As stated above, species and strain level differences likely contribute a larger part of allergy disease than previously discovered. Rarely functional characteristics of bacteria are further exploring as well, with many reports simply stating the taxonomical results from sequencing. With most gut microbiome studies, and those discussed in this review, only the large intestinal microbiome is analyzed, as stool is used for sampling. However, with food being digested in the small intestinal, it would be important to observe microbial and metabolic changes in this region as well. These important research details are required to further understand the complexity of the immune system and the entire microbiome.
3.7 Conclusions

Food allergies have an enormous impact on the quality of life. The rates of those with food allergies has notably increased in the past decade, suggesting that environmental factors are driving most of the increase\textsuperscript{42}. Numerous animal and human studies have supported this theory with the connection of the gut microbiome (Figure 1). Alteration of the microbiota across the big 8 allergies shows a consistent unique signature involving the Firmicutes phylum. Interaction of the microbiome and microbial metabolites (SCFA) with host immune response is likely the mechanisms by which the microbiome affects food allergy. The addition of probiotics, both traditional (\textit{Lactobacillus} and \textit{Bifidobacterium}) and next generation (Clostridia) along with other dietary interventions have shown significances in helping prevent and treat disease. This knowledge may be used to develop products utilizing these as functional additives. Further research must be done to identify potential differences in mechanisms, prevention, and treatment between different antigens.
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