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Kelly O'Brien

Trinity College, kobrien822@gmail.com

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AN EXAMINATION OF BACTERIAL COMMUNITIES
IN THE UPPER RESPIRATORY TRACTS
OF ASTHMATICS AND NON-ASTHMATICS

BY

KELLY A. O'BRIEN

A THESIS SUBMITTED TO
THE FACULTY OF THE DEPARTMENT OF BIOLOGY
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AN EXAMINATION OF BACTERIAL COMMUNITIES
IN THE UPPER RESPIRATORY TRACTS
OF ASTHMATICS AND NON-ASTHMATICS

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KELLY A. O'BRIEN

Honors Thesis Committee

Approved:

Lisa-Anne Foster, Advisor

Robert J. Fleming

Hebe M. Guardiola-Diaz

Date: _____

Abstract

The human body is colonized with commensal microbes that attach to the skin and mucosal membranes and function to protect the host as part of the innate immune response. These indigenous microbiota are able to prevent infections by blocking attachment sites and outcompeting invading pathogens for necessary nutrients. Disturbance of the host-microbe symbiotic balance may increase a host's susceptibility to disease. In addition, proper colonization of the normal flora has been implicated in playing a vital role in shaping the immune response. It has been hypothesized that reduced exposure to infectious microorganisms early in life can disrupt the development of normal immune regulation. This "hygiene hypothesis" states that the increased prevalence of allergic diseases in developed countries can be attributed to a more hygienic, westernized lifestyle that is characterized by a decrease in microbial challenges. Therefore, the allergic disease asthma, which is characterized by a heightened inflammatory response, could be the result of disturbed microbial colonization, hindrance of immune maturation, and subsequent dysregulation of the allergic response. It would be expected that asthmatic individuals would exhibit a less diverse and less abundant population of normal flora as compared to non-asthmatic individuals. This preliminary study investigated the bacterial communities found in the upper respiratory tracts of asthmatic and non-asthmatic subjects. Terminal restriction fragment length polymorphism (tRFLP) was used to examine the composition of bacteria in oropharyngeal samples collected from student volunteers at Trinity College. The tRFLP profiles of asthmatic and non-asthmatic subjects produced conflicting results, and it cannot be concluded if there is a difference in the diversity or abundance of bacterial communities in the upper respiratory tracts of both populations.

Introduction

Within the human body, a complex and symbiotic relationship exists between host cells and the indigenous microbiota known as normal flora. Shortly after birth, the skin and initially sterile mucosal membranes, such as the upper respiratory tract and the vaginal tract of the neonate are colonized with these commensal microbes. The greatest density of normal flora is found in the gastrointestinal tract, which contains approximately 10^{12} organisms per gram of intestinal content and around 1,000 different species (Macpherson and Harris, 2004). After approximately one week of life, colonization of the body is considered complete despite the fact that during the following three months there are enormous fluctuations in the abundance and number of species of bacteria (Björkstén, 2005). These differences are influenced by early life factors, which include type of birth and diet. For example, during birth, vaginally delivered babies are exposed to their mother's vaginal and fecal normal flora and will therefore acquire similar bacterial communities, such as *Lactobacillus* or *Prevotella*. Contrastingly, infants delivered by caesarean section will encounter maternal skin bacteria and will therefore acquire skin associated bacterial normal flora, such as *Staphylococcus* and *Corynebacterium* (Dominguez-Bello *et al.*, 2010). Diet can also influence colonization as studies have shown that intestinal flora differs between newborns who are breast-fed and those who are formula-fed (Harmsen *et al.*, 2000).

The normal flora of the body exists as a type of ecosystem that, once established, is surprisingly stable under normal conditions (Björkstén, 2005). Most importantly, these commensal microbes function as part of the innate (nonspecific) immune response to help defend the host from infection. By colonizing the epithelial cells of mucosal surfaces, the nonpathogenic normal flora act as an anatomical barrier and are able to outcompete invading

pathogens for attachment sites and the available necessary nutrients (Goldsby *et al.*, 2000). Normal flora can also produce compounds such as bacteriocins that inhibit or kill other bacteria. In addition to their role in the innate immune system, normal flora can also synthesize and excrete necessary vitamins and nutrients for the host. For example, *Eschericia coli* found naturally in the intestines is responsible for supplying the human body with Vitamin K (Todar, 2012).

Disturbance of the normal flora and any alteration of the existing bacterial symbiosis can result in disease by allowing pathogens to overcome protective factors. A common example of this, although a minor ailment, is digestive distress following the use of antibiotics. Broad-spectrum antibiotics, which act against a wide range of bacteria, can kill both invading pathogens as well as commensal microorganisms. The microbial ecosystem of the GI tract is disrupted, and as a result, food is improperly digested. In addition, many females experience yeast infections following antibiotic use because the vaginal tract flora is altered and the natural yeast is able to multiply and colonize in greater-than-normal amounts. While digestive distress and yeast infections are mostly just uncomfortable and fleeting, the disturbance of normal flora has also been implicated in the development of allergic disorders.

Over the past couple of decades, the prevalence of allergic and atopic diseases has been on the rise (Strachan, 1989), and this is especially true in industrialized countries with market economies (Burney, 1993). Atopic individuals have a genetic predisposition to a hypersensitive response and unusually high levels of IgE and allergy-related immune cells such as eosinophils and mast cells, in their bodies (Goldsby *et al.*, 2000). One of the earliest studies to recognize an increase in allergic disease among “westernized” individuals found that a white community in central Saskatchewan, Canada had a much higher prevalence of

asthma and eczema than the Metis (aboriginal) community in northern Saskatchewan. The Metis community was burdened with helminth infestation as well as other untreated viral and bacterial diseases. Gerrard *et al.* (1977) attributed this difference in allergic disease prevalence as the “price paid by some members of the white community for their relative freedom” from the bacterial diseases. Then in 1989, David P. Strachan found that the risk of allergic diseases was lower for individuals who had more siblings, i.e. an inverse relationship exists between household size and allergy prevalence (Strachan, 1989). Children who live with a large number of siblings are more often exposed to common infections early in life than children with a smaller family size. And as a result of these findings, Strachan developed the “hygiene hypothesis,” which states that the reduced microbial stimulation during infancy and early childhood, as a result of improved hygienic conditions, is associated with the increasing prevalence of allergic disorders (Strachan, 1989). Therefore, exposure to microbes is essential for the development of normal immune regulation.

Many studies have been conducted to investigate the hygiene hypothesis and whether or not early exposure to infectious microorganisms can have an effect on subsequent allergy development. In 1997, a Swedish study followed children from birth to 12 – 15 years of age and found that those born during the tree pollen season (spring) were less likely to develop sensitization to pollen and allergic rhinoconjunctivitis than those born between September and February (Nilsson *et al.*, 1997). In terms of pet ownership, early exposure to cats is associated with decreased cat sensitization later in life (Hesselmar *et al.*, 1999; Roost *et al.*, 1999) and dog exposure at birth is associated with decreased atopic dermatitis and wheezing at age 3 (Bufford *et al.*, 2008). Overall, exposure to household pets in the first years of life is associated with reduced allergic rhinitis, asthma, and decreased sensitization to indoor and

outdoor allergens in general in school children (Hesselmar *et al.*, 1999; Anyo *et al.*, 2002). Also, growing up on a farm, which is regarded as “less-hygienic” environment, is associated with reduced incidence of hayfever, asthma, and atopic sensitization in children (Kilpeläinen *et al.*, 2000; Riedler *et al.*, 2000). Finally, in addition to exposure to pollen and animals early in life, a study conducted in Italy found that respiratory allergy is less frequent in people who are heavily exposed to orofecal and foodborne microbes. These results supported the idea that a hygienic, westernized lifestyle in developed countries may contribute to the development of allergy.

Allergy, or hypersensitivity, is an inappropriate and oftentimes heightened immune response that causes damage to the host. In a typical infection, however, the two main branches of the immune system known as the adaptive and the innate response will be able to recognize and eliminate invading pathogens without causing any self-harm. The innate response eliminates foreign microorganisms in a non-specific manner through multiple barriers, which include normal flora, mucosal membranes, and macrophages (cells that phagocytose and break down whole microorganisms). The immune cells of the adaptive response are able to recognize specific pathogens via a humoral or cell-mediated response. The B cells of the humoral response have membrane bound antibodies, which are able to recognize foreign antigens. Over time, as these cells are consistently exposed to a particular antigen, they can differentiate to plasma cells that secrete the specific antibody to the pathogen or to memory B cells that will be able to quickly recognize the antigen if it were to infect the host again. Therefore, the body is prepared to eliminate the antigen in the future. As an overview of the cell-mediated response, T cells recognize the antigen when it is presented by an infected, or altered, self-cell. As a result, T helper (T_H) cells produce

chemical messengers known as cytokines to stimulate other immune cells and cytotoxic T cells (T_C) respond by killing the altered self-cell. There are many areas of regulation in the immune response, but as a general overview, the immune cells are responsible for fighting off antigens with localized responses that do not cause any harm to the host, and in fact, can provide protection against future infections. In allergy, however, certain substances known as allergens trigger an inappropriate response that increases host sensitivity to the antigen, rather than protection. (Goldsby *et al.*, 2000)

Common allergens such as pollen, animal dander, and dust are antigens that will stimulate the humoral part of the adaptive immune response. Specifically, the majority of allergic reactions involve the actions of antibody (immunoglobulin) IgE. In a hypersensitive response, IgE antibodies, which are released by activated B cells and are membrane bound to histamine-releasing mast cells, recognize and bind to allergens. This cross-linking of allergen to mast cells induces the release of histamine and other molecules that will cause inflammation and irritation in the form of hives, eczema, or other local allergic responses. (Goldsby *et al.*, 2000)

Asthma is an example of an IgE mediated allergic disease with a hypersensitive response that occurs in the lower respiratory tract. Asthma attacks, which are a sudden worsening of asthma symptoms, such as wheezing, coughing, and shortness of breath, can be brought on by a number of triggers. These include typical allergens such as pollen and dust, and even allergen-independent factors such as exercise or cold weather. In an allergen-induced reaction, IgE triggers the degranulation of mast cells and later responses such as mucus secretion, vasodilation, bronchial hyperresponsiveness, and airway inflammation (Busse and Lemanske, 2001). Although bronchoconstriction is a major component of asthma

pathophysiology, the disease is typically regarded as an inflammatory disorder that involves recruitment of inflammatory immune cells, such as eosinophils and neutrophils, into the bronchial tissue (Goldsby *et al.*, 2000). In fact, a marked increase in blood eosinophil levels, known as eosinophilia, is associated with increased risk of asthma (Karakoc *et al.*, 2002). In addition, prevalence of asthma is very closely related to serum IgE level, and asthma related reactions are almost always associated with heightened IgE (Burrows *et al.*, 1989). High levels of IgE have also been found to precede allergic sensitization, indicating that a defect in immune system development can predispose an individual to allergic disease (Sherill *et al.*, 1999).

T lymphocytes (T cells) are white blood cells that also play a role in allergic asthma. In the immune system, the two major T helper (T_H) cell responses are known as T_H1 and T_H2 , which mediate cellular defense mechanisms and allergic inflammation, respectively. T_H2 cells produce the cytokines interleukin-4, 5, 6, 9, and 13 to recruit and communicate with other inflammatory cells (Busse and Lemanske, 2001). A balance exists between T_H1 and T_H2 immunity, as they inhibit each other to prevent exaggerated immune reactions. In atopic individuals as compared to nonatopics, however, there is a bias towards production of T_H2 cytokines (Romagnani, 1994). An imbalance between these helper lymphocytes has also been implicated in the development of asthma (Busse and Lemanske, 2001) as studies have revealed an overproduction of T_H2 cells as opposed to T_H1 cells in asthmatics (Holgate *et al.*, 2009). In addition, the amounts of IL-4, IL-5, and IL-13 cytokines are heightened in asthmatic airways (Neurath *et al.*, 2002). This dysregulation of immune cells can be linked to the hygiene hypothesis as less microbial pressure early in life could have delayed the postnatal maturation of the immune system. Therefore, the optimal balance between T_H1 and

T_H2 immunity was never able to develop, allowing the body to favor one immune response over the other.

It is possible that the overexaggerated allergic response exhibited in asthma and other inflammatory diseases can be related to normal flora. The link between the hygiene hypothesis, normal flora colonization, and subsequent development of allergy has been examined primarily in the gastrointestinal tract. The gastrointestinal tract is responsible for many bodily functions such as digestion, but it is also the body's largest organ of host defense. It drives development of the body's immune response, and colonization of balanced normal flora is important for proper maturation of immune effector cells (Björkstén, 2005). For example, the normal flora of the GI tract is the principal signal for postnatal maturation of T-cell function and the balance between T_H1 and T_H2 responses (Holt *et al.*, 1997). Therefore, disturbances in normal flora colonization could result in improper immune regulation and later development of allergic disease. Studies have shown that there is an association between these factors. Differences in neonatal gut microflora have been shown to precede atopic disorder (Kalliomäki *et al.*, 2001), reinforcing the idea that an individual can have a predisposition to allergic disease as a result of their infant normal flora colonization. A study conducted in 1999 revealed that allergic children in Estonia were less often colonized with commensal microbes of the genus *Lactobacilli* as compared to non-allergic children in Sweden (Björkstén, 1999). A later study found that infants who had non-allergic parents were also more frequently colonized with *Lactobacilli* compared to infants with allergic parents, and non-allergic infants acquired *Lactobacilli* more frequently than allergic infants. In addition, at one week of life, significantly more non-allergic children were colonized with the commensal microbe *B. bifidum* than allergic children (Johansson *et al.*,

2011). A more diverse gut microbiota early in life may help to prevent the development of allergy (Sjögren *et al.*, 2009).

Microorganisms of the genus *Lactobacilli* have been implicated in potential probiotic use to stimulate a proper immune response in the gastrointestinal tract (Salminen and Deighton, 1992). Allergic subjects who were exposed to *Lactobacillus*-based probiotics exhibited a shift in T_H cell immunity from an exaggerated T_H2 response to the development of a T_H1 response with suppression of T_H2 cytokines (Ghadimi *et al.*, 2008). In addition, 6-month old infants who were fed daily *Lactobacillus*-based supplements exhibited promotion of a more diverse and evenly distributed gastrointestinal normal flora, indicating a healthier microbial ecosystem (Cox *et al.*, 2010). Depletion of commensal bacteria can result in GI inflammatory diseases, such as Crohn's disease and ulcerative colitis, which have been found to have significantly different microbiota as compared to healthy individuals (Frank *et al.*, 2007).

As many studies have revealed the relationship between normal flora and the proper maturation of the immune system, the importance of these results has been clearly shown in studies of germ free (GF) animals. Germ free rodents have never been exposed to any type of microorganism, pathogenic or non-pathogenic, while specific pathogen-free mice are colonized solely by normal flora. When challenged with ovalbumin, a typical protein used to stimulate an allergic reaction, germ free mice had significantly more production of IL-4, a T_H2 mediating cytokine, as well as a significantly reduced T_H1 response as compared to specific pathogen-free mice. When the neonatal germ free mice were recolonized with *Bifidobacterium infantis*, typically found in the GI normal flora, the exaggerated T_H2 response was mitigated (Sudo *et al.*, 1997). A more recent study found that after induction of

allergic airway inflammation, GF mice had elevated lymphocytes, eosinophils, basophils, T_H2 associated cytokines, and IgE production as compared to specific pathogen free mice (Herbst *et al.*, 2011). Overall, GF mice had an exaggerated allergic response and increased bronchial hyperresponsiveness, indicating that normal flora plays a pivotal role in modulating this immune reaction.

The gastrointestinal tract and the upper respiratory tract are both mucosal surfaces with normal flora lining the epithelial cells. Inflammatory GI diseases are attributable to a dysregulation in the immune response, and because differences have been found in the composition of normal flora in the GI tract of allergic and non-allergic individuals, it can be hypothesized that a similar relationship exists in the upper respiratory tract. Patients with moderate to severe chronic obstructive pulmonary disease (COPD) exhibit exaggerated airway inflammation, and the normal flora of COPD patients has been found to be remarkably less diverse than the flora of healthy patients (Erb-Downward *et al.*, 2010). Therefore, the allergic disease asthma, characterized by an inflammatory response, could also be the result of disturbed microbial colonization, hindrance of immune maturation, and subsequent dysregulation of the allergic response. It would be expected that asthmatic individuals would exhibit a less diverse and less abundant population of normal flora as compared to non-asthmatic individuals.

The purpose of this pilot study was to use molecular, rather than culture-based tools, to examine the bacterial flora colonizing the oropharynx in individuals with and without asthma. The benefits of molecular tools as compared to culture-based have been well documented in the literature. This study aims to process biological samples using Terminal Restriction Fragment Length Polymorphism (tRFLP). This technique exploits the base pair

differences found in the bacterial 16s rRNA gene. The 16s rRNA transcribed by this gene is a component of the small ribosomal subunit and plays a vital role in protein synthesis. Therefore, it is present in all bacterial species, and it is highly conserved evolutionarily.

Previous studies have targeted the 16s rRNA gene during Polymerase Chain Reaction (PCR) amplification by using fluorescently labeled primers that are universal to all bacterial species (Liu *et al.*, 1997). During amplification, the primers anneal to highly conserved regions of the 16s rRNA gene, producing an amplified fragment with labeled terminal ends. Then, the DNA is digested by restriction enzymes so that the slight polymorphisms between species will produce different fragmentation patterns. The resulting fluorescently labeled terminal fragments are unique to each species and can be used to generate a genetic “fingerprint.” By using this method of tRFLP, this study will examine the bacterial communities in the upper respiratory tracts of asthmatic and non-asthmatic individuals. Specifically, the relative amount of bacteria present in each group as well as the overall diversity of the bacterial species present in the upper respiratory tract will be analyzed. A greater understanding of the normal flora present in asthmatic versus non-asthmatic individuals may offer alternative treatments in the form of probiotics.

METHODS

Collection of Throat Swab Samples From Trinity College Students

In December 2010, the Trinity College Health Center collected throat swab samples from Trinity College students. The participants signed an informed consent and completed an anonymous questionnaire regarding current health status, tobacco use, alcohol use, recent antibiotic use, and chronic disease (Appendix). Then, Health Center employees obtained oropharyngeal throat swab samples from the participants. The samples and corresponding questionnaire were labeled with matching identification numbers and then separated. In this manner, student researchers remained “blind” to the data. One milliliter of dH₂O was added to the throat swab sample tubes, and then they were refrigerated until they were ready to be analyzed. Questionnaires remained locked in a drawer in the office of Dr. Lisa-Anne Foster until throat swab analysis was complete.

Preparation of PCR template from Throat Swab

Throat swabs were swirled around the 1mL of dH₂O to resuspend as many bacterial cells as possible. The suspended cells were centrifuged for 5 minutes at 13,000 rpm, and then the supernatant was transferred to a sterile 1.5 mL tube. An additional 1mL dH₂O was added to the tube containing the centrifuged pellet, and pipetted up and down to thoroughly resuspend the cells.

Amplification of the 16s rRNA gene: Polymerase Chain Reaction

Fluorescently labeled universal primers U1 Fam (5'-[6-fam]CCAGCAGCCGCGGTAATACG-3') and U2 Hex (5'-[5hex]ATCGGYTACCTTGTTACGACTTC-3') were selected to anneal to the 16s rRNA region of the bacterial genome during amplification. In a total reaction mixture of 50 μ L, reagents from the Qiagen PCR Core Kit (stored at -20°C) including, 5 μ L of 10x buffer, 10 μ L of Q buffer, 1 μ L of dNTP and 0.25 μ L of Taq polymerase, were added to 28.35 μ L of dH₂O and 5 μ L of DNA template. In addition, 0.2 μ L of each 50 μ M fluorescently labeled primer (U1 Fam and U2 Hex) was added for a final concentration of 0.2 μ M. An additional negative control was prepared with all necessary components, except 5 μ L of dH₂O in replacement of DNA template. The PCR tubes were placed in the thermal cycler to amplify the DNA via the 16s rRNA AMP cycling program.

The program began with a 5-minute initial denaturation at 94.0°C, followed by 30 cycles of a 1 min denaturation at 95.0°C, 1 min annealing at 50.0°C, 1 min extension at 72.0°C, 2 min final extension at 72.0°C. The program ended with a final hold at 4.0°C.

Gel Electrophoresis

In a gel electrophoresis chamber, 0.5x TBE buffer was poured over polymerized 1.2% agarose gel and then loaded with 2 μ L of 6x gel loading dye that had been combined with 10 μ L of amplified 16s rRNA products. The DNA in bacterial samples was not quantified. In addition to the samples and the negative control, a ladder containing 9 μ L dH₂O, 2 μ L 6x gel loading dye, and 1 μ L of 1 kilobase (kb) DNA ladder, was added as a measure of fragment size to detect the ~996 bp 16s rRNA gene. The gel was run at approximately 80 to 100 volts for 60 – 90 minutes. Upon completion, the gel was removed

from the electrophoresis chamber and bathed in a solution of 25 μ L of 10 mg/mL EtBr in 200 mL dH₂O for approximately 20 minutes to stain the bands.

Purification

Protocol for purification with the QIAquick PCR Purification Kit was followed according to the manufacturer's (QIAGEN) instructions. Buffer PB was added to the amplified product at a ratio of 5:1. If the mixture was an orange or violet color, 10 μ L of sodium acetate was added to produce a color change to yellow. The entire mixture of Buffer PB, amplified product, and sodium acetate was centrifuged in a QIAquick column for 1 minute at 13,000 rpm, and the flow through was discarded. Then, 750 μ L Buffer PE was added and centrifuged in the same manner twice, with discarding of the flow through in between cycles. The cap of the QIAquick column, containing the QIAquick membrane, was transferred to a sterile 1.5mL centrifuge tube, and 35 μ L Buffer EB was added directly to the membrane. The columns were left to stand vertically for 1 minute, and then centrifuged again to collect the purified product. Purification of products was completed both after PCR and again after restriction digestion.

Restriction Digestion

In sterile 1.5mL centrifuge tubes, 15µL of purified product, 2µL of dH₂O, 2µL of 10x NEBuffer 4, and 1µL of restriction enzyme were combined. Possible restriction enzymes included *AluI*, *HaeIII*, and *MnII*. The reaction mixture was incubated at 37°C for 4 hours.

| Enzyme | Restriction Site |
|--------|--|
| AluI | 5'...AG↓CT...3' 3'...TC↑GA...5' |
| HaeIII | 5'...GG↓CC...3' 3'...CC↑GG...5' |
| MnII | 5'...CCTC(N) ₇ ↓...3' 3'...GGAG(N) ₆ ↑...5' |

Preparation of Purified Samples for Fragment Analysis by Capillary Electrophoresis

In a 96 well plate, 48 samples were prepared at two different dilutions. Purified throat swabs samples were combined with highly deionized formamide at both a 5µL:5µL and 7µL:3µL mixture, respectively. Purified known organisms were combined with highly deionized formamide at both a 1µL:9µL and 5µL:5µL mixture, respectively. The 96 well plates were packaged and mailed to the DNA Analysis Facility on Science Hill at Yale University for capillary electrophoresis on a 3730x1 96-Capillary Genetic Analyzer.

Software Analysis of DNA Fragments Using PeakScanner

Fluorescently labeled peaks on the electropherograms, representing U1 Fam and U2 Hex terminal fragments of the amplified region of the bacterial 16s rRNA genes, were labeled using the program *PeakScanner* (Applied Biosystems). Number of peaks, abundance, and size were recorded for each sample in Microsoft Excel. After all data for throat swab samples was collected and tabled, subject surveys were opened from their sealed envelope.

Then, to eliminate experimenter bias, the surveys were matched with electropherogram data. The average number of U1 Fam and U2 Hex peaks for every subject was calculated, and the means between asthmatic and non-asthmatic subjects were compared using a student's t-test (Microsoft Excel). In addition, the total abundance of U1 Fam and U2 Hex peaks for every subject was calculated, and the totals between asthmatic and non-asthmatic subjects were compared using a student's t-test (Microsoft Excel).

RESULTS

The goal of this study was to use Terminal Restriction Fragment Length Polymorphism (tRFLP) to quantify the number of bacterial species present and the abundance of bacterial communities in the upper respiratory tracts of asthmatic and non-asthmatic subjects. The fluorescently labeled universal primers, U1 Fam and U2 Hex, were used to anneal to the terminal ends of the 16s rRNA bacterial gene during PCR amplification of throat swab samples. DNA samples were purified and digested with the restriction enzyme Mnl1. The digested samples were purified again and sent to Yale University for fragment analysis by capillary electrophoresis. The Capillary Genetic Analyzer detected the terminal fragments of each amplified gene by the fluorescently labeled U1 Fam and U2 Hex primers.

Our lab received fragment analysis data from Yale University in the form of electropherograms. The Y-axis represented the relative fluorescence, and ultimately, the abundance of the fragment. The X-axis represented the size of the DNA fragment (in base pairs). The green peaks corresponded to the terminal fragments labeled with the U1 Fam primer while the blue peaks corresponded to the terminal fragments labeled with the U2 Hex primer.

This study was concerned with investigating the diversity and abundance of the bacterial communities found in the upper respiratory tract of asthmatic and non-asthmatic individuals. In an electropherogram, the pair of a U1 Fam peak and a U2 Hex peak corresponds to the two terminal fragments of a gene of a single bacterial species. Therefore, the total number of peaks in an electropherogram is representative of the bacterial diversity (the number of species) found in the analyzed throat swab sample. In addition, the relative

fluorescence (height) of the peaks indicates the abundance of the fragments. Therefore, the total height of all the peaks in an electropherogram can be used to determine the relative bacterial abundance in a throat swab sample.

In our investigation, sixty throat swab samples that were collected from Trinity College students were analyzed. Digestions were completed using the restriction enzyme *MnII*, while further analysis with addition enzymes is underway. Eleven students identified themselves as asthmatics while forty-nine students identified themselves as non-asthmatics. Non-asthmatics had significantly more U2 Hex peaks than asthmatics (Table 1, t-test, $p=0.0193$), indicating that non-asthmatics had more bacterial species, represented by the U2 Hex terminal fragments, present in their throat swab samples. However, there was no difference between the average number of U1 Fam peaks in the asthmatic and non-asthmatic samples (Table 1, t-test, $p=0.1231$). Because the terminal fragments are paired together for one species, there should be one U1 Fam peak for every U2 Hex peak in a given sample. Therefore, within an experimental group the average number of U1 Fam and U2 Hex peaks should be the same. This is seen in the asthmatic population, as the average number of both the U1 Fam peaks and the U2 Hex peaks is 2.05 (Table 1). Unfortunately, the non-asthmatic population deviates from this expected result as the average number of U1 Fam peaks is 2.84 and the average number of U2 Hex peaks is 3.60 (Table 1), indicating that there are more U2 Hex peaks than U1 Fam peaks. As a result, it cannot be determined if the number of bacterial species present in the upper respiratory tract is different between asthmatics and non-asthmatics. At this time, the data is conflicting as one primer (U1 Fam) indicates that there is the same number of species present, while the other (U2 Hex) indicates that non-asthmatics

have a greater number of bacterial species in their throat swab samples. Both primers need to be in agreement for a conclusion to be made.

Table 1: Average number of U1 Fam and U2 Hex peaks for asthmatic and non-asthmatic throat swab samples digested with Mnl1 (means reported \pm standard deviation).

| | U1 Fam | U2 Hex |
|----------------------|-----------------|-----------------|
| Asthmatic (n=11) | 2.05 \pm 1.31 | 2.05 \pm 1.62 |
| Non-Asthmatic (n=49) | 2.84 \pm 2.07 | 3.60 \pm 2.69 |

For all sixty samples, the total peak heights were calculated to determine total bacterial abundance. Non-asthmatics had significantly more abundant U1 Fam terminal fragments than asthmatics (Table 2, t-test, $p=0.0343$). However, there was no difference in the abundance of U2 Hex terminal fragments between the two groups (Table 2, t-test, $p=0.3131$). Unfortunately, in a similar manner to the data for the average number of peaks, there is a primer discrepancy between the U1 Fam and the U2 Hex terminal fragments. For any given species, the terminal fragments may be different sizes (in terms of base pairs), but they should be equally abundant. For the asthmatic population, the total abundance of U2 Hex fragments appears to be much larger than the total abundance of U1 Fam fragments, however, with the large variation indicated by the standard deviations, the difference is not significant (Table 2, t-test, $p=0.0606$). Within the non-asthmatic population, however, the U2 Hex abundance is significantly greater than the U1 Fam abundance (t-test, $p=0.0032$). Similarly to the previously stated results, this imbalance indicates that there are more U2 Hex

fragments than U1 Fam fragments in a given sample. Therefore, it cannot be determined if there is a difference in total bacterial abundance between asthmatics and non-asthmatics. While one primer (U1) suggests a greater bacterial load in non-asthmatic subjects, the other primer (U2) suggests that the bacterial abundance is the same in both populations.

Table 2: Average height of U1 Fam and U2 Hex peaks for asthmatic and non-asthmatic throat swab samples digested with Mnl1 (means reported \pm standard deviation).

| | U1 Fam Height | U2 Hex Height |
|----------------------|---------------------|---------------------|
| Asthmatic (n=11) | 198.36 \pm 163.43 | 621.41 \pm 652.62 |
| Non-Asthmatic (n=49) | 403.82 \pm 565.33 | 864.89 \pm 901.33 |

Although the data cannot confirm a significant difference in the number of peaks between asthmatics and non-asthmatics, it is interesting to note the differences in peak distribution. All asthmatic throat swab samples had between 0 and 6 average peaks present on their electropherograms, with zero percent of the population above 5 total peaks (Figures 1 & 2). Non-asthmatics, on the other hand, had a wider distribution of peaks. For example, 8.16% and 2.04% of the non-asthmatic population had between 6 – 7.99 and 10 – 11.99 average U1 Fam peaks, respectively (Figure 1) In addition, 10.20% of the non-asthmatic population had between 6 – 7.99 average U2 Hex peaks, with 6.12% between 8 – 9.99 and 4.08% between 10 – 11.99 (Figure 2). The general trend for both experimental groups appears to be a decrease in percentage of the population as the average number of peaks increases (Figures 1 & 2).

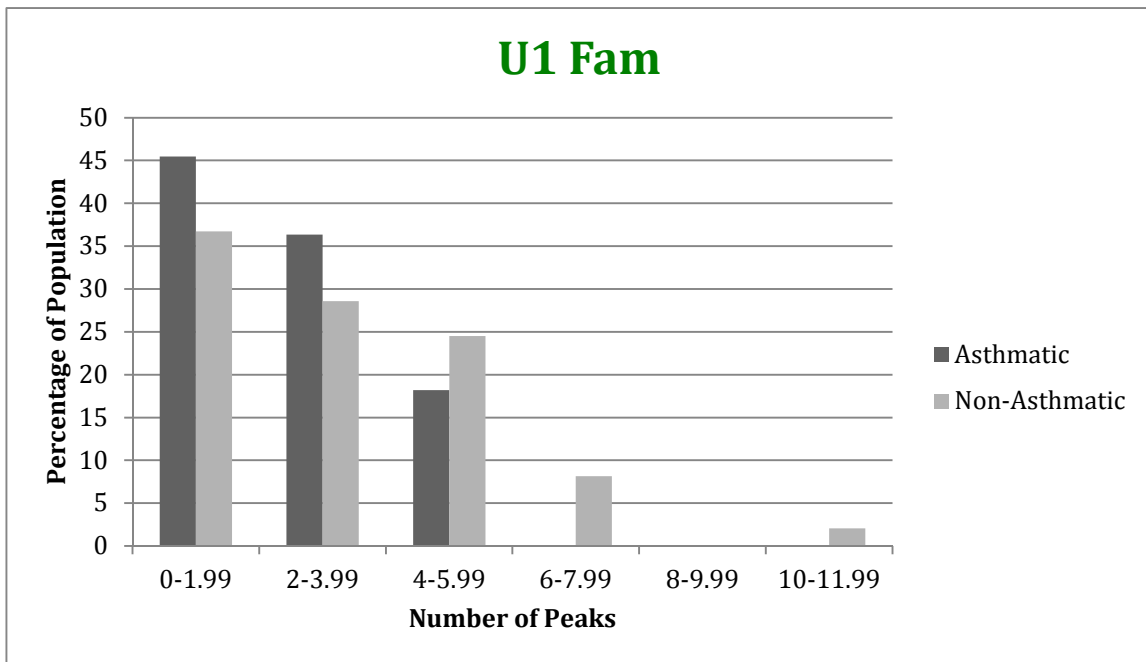


Figure 1: U1 Fam peak distribution.

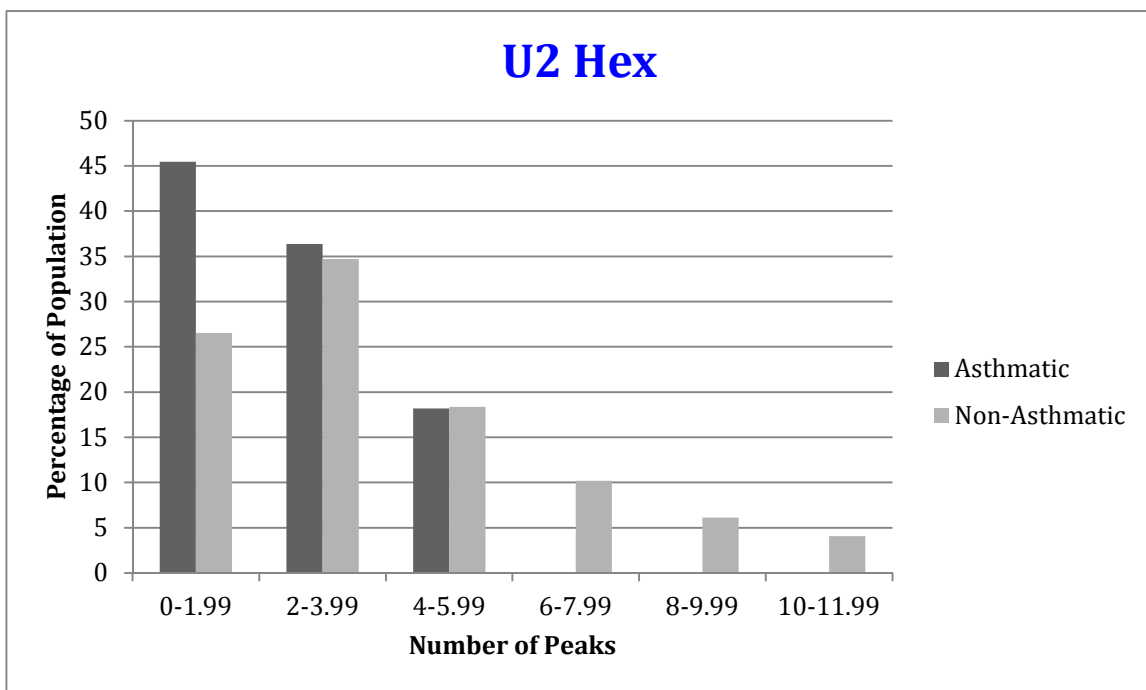


Figure 2: U2 Hex peak distribution.

According to the demographic data for both sample populations, all of the asthmatic participants and 77.55% of the non-asthmatic participants use alcohol. Most of the students do not smoke cigarettes, although 18.18% of asthmatics and 22.45% of non-asthmatics reported that they do use tobacco (Table 3).

Table 3: Demographic data for self-identified asthmatic and non-asthmatic Trinity College students. Values reported as percentages of population.

| | | Asthmatic (n=11) | Non-asthmatic (n=49) |
|-------------|-------------|------------------|----------------------|
| Gender | Male | 36.36 | 63.27 |
| | Female | 54.55 | 28.57 |
| | No Response | 9.09 | 8.16 |
| Alcohol Use | None | 0 | 22.45 |
| | Infrequent | 54.55 | 42.86 |
| | Habitual | 9.09 | 20.41 |
| | Binge | 36.36 | 14.29 |
| Tobacco Use | None | 81.82 | 77.55 |
| | Social | 9.09 | 20.41 |
| | Habitual | 9.09 | 2.04 |

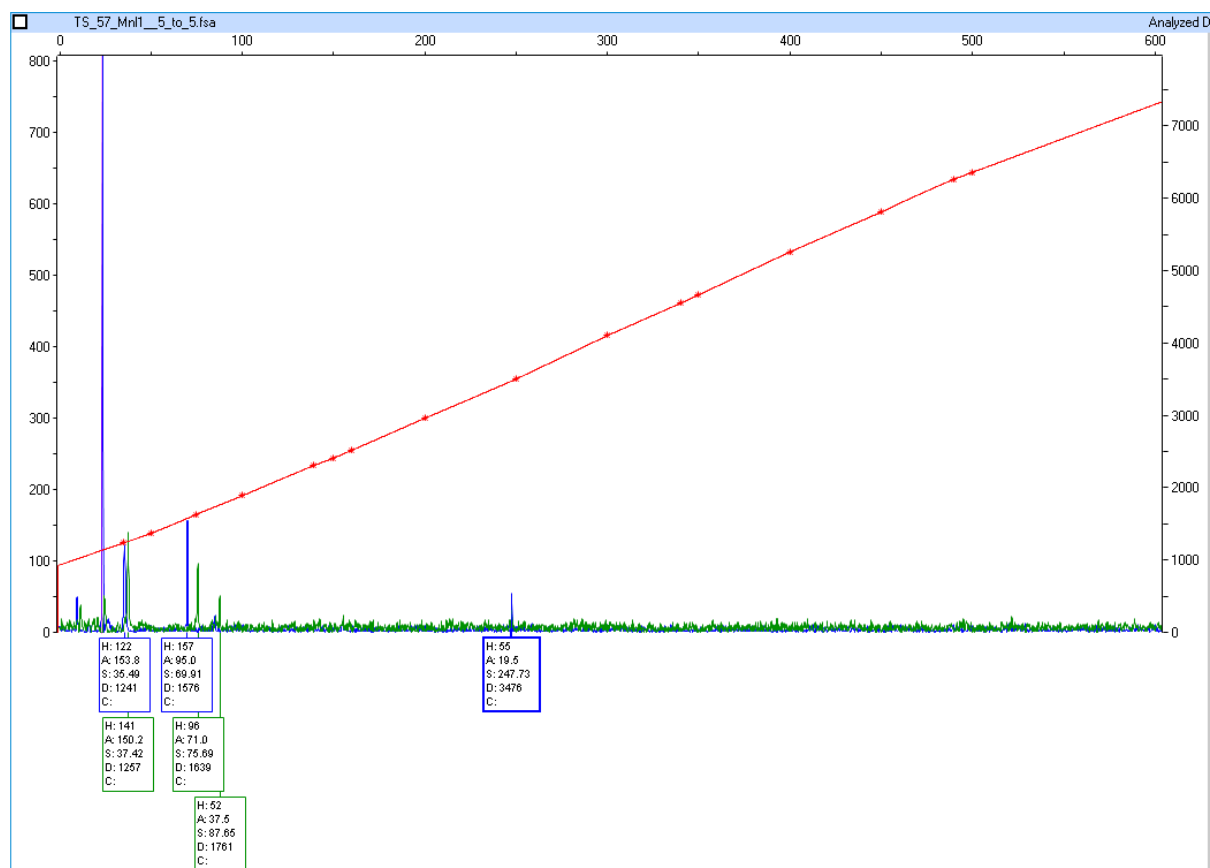


Figure 1: Chromatogram data representative of a self-identified asthmatic student at Trinity College (Sample 57 digested with restriction enzyme Mnl1).

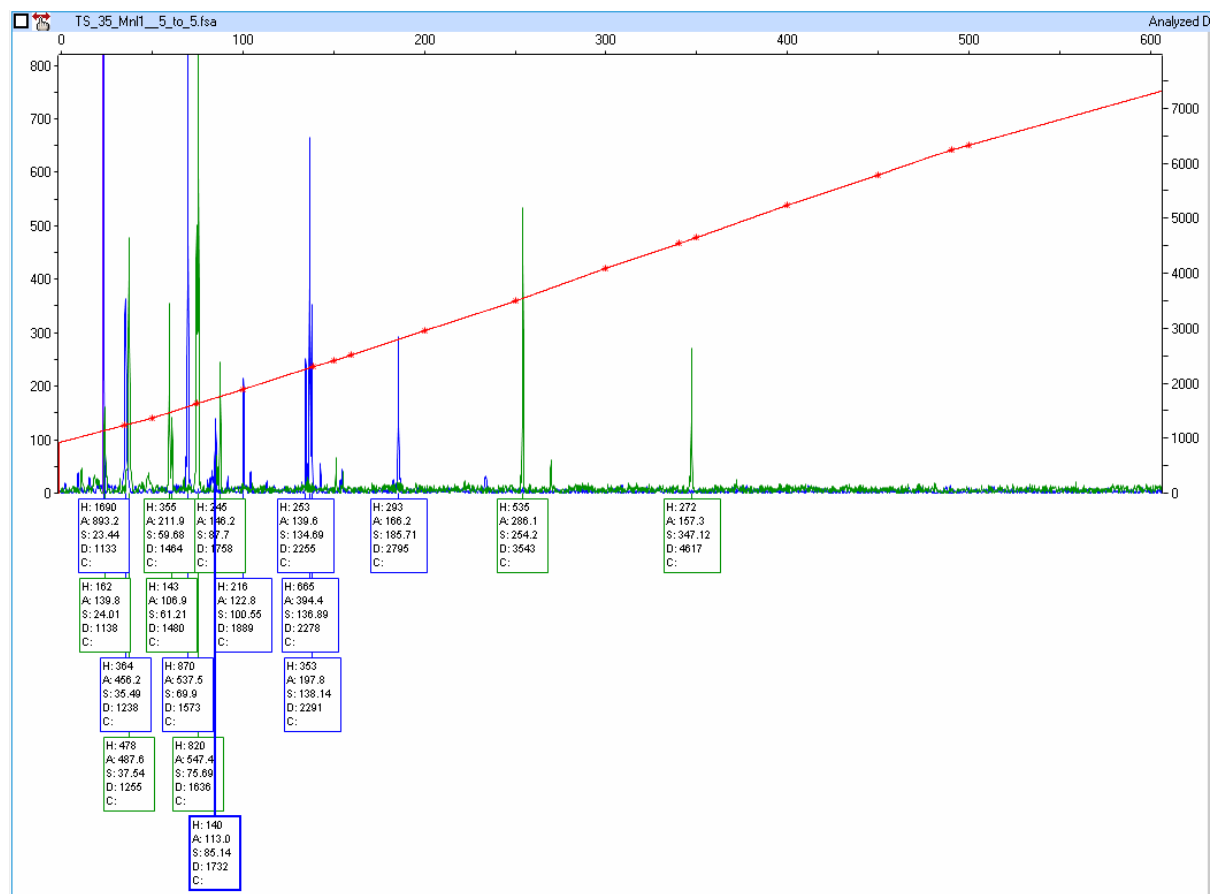
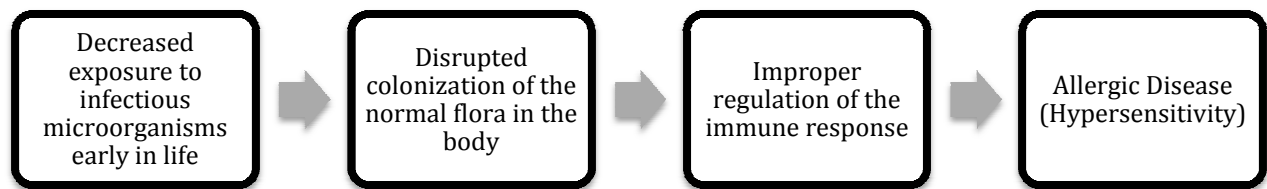


Figure 2: Chromatogram data representative of a self-identified non-asthmatic student at Trinity College (Sample 35 digested with restriction enzyme Mnl1).

DISCUSSION

In the past couple of decades, the prevalence of asthma has been steadily increasing, and in the United States currently there are 18.7 million adults and 7 million children living with asthma (Schiller *et al.*, 2010). There is no real answer to the question, “What causes asthma?” but it is known that the disease has a genetic origin (Cookson, 1999), and symptoms are triggered by a number of factors such as weather, respiratory infections, exercise, irritants in the air, and common allergens such as pet dander or dust (Anonymous, 2011). The frequency of symptom exacerbation, also known as asthma attacks, varies from person to person and depends on the severity of the disease in addition to how well it is controlled. Minor cases of the disease may cause slight breathing difficulties, while severe cases can result in death from a grossly over exaggerated inflammatory response. In fact, the inflammatory component of asthma was initially revealed upon examination of cadavers who had fatal asthma. These patients exhibited, among other symptoms characteristic of inflammation, lung infiltration by neutrophils and eosinophils, degranulated mast cells, and hyperplasia and hypertrophy of bronchial smooth muscle (Busse and Lemanske, 2001).

The increased prevalence of asthma and other allergic diseases has recently become quite a concern. The “hygiene hypothesis” has attributed the increased sensitivity to allergic disorders in the westernized world to a decreased exposure to infectious microorganisms early in life. Many studies have since supported this theory and additionally have found that the reduced microbial challenges in the first years of life can disrupt the proper colonization of normal flora in the body. As normal flora have been shown to regulate the development of the immune response, additional evidence has mounted for the hygiene hypothesis:



Asthma, specifically, is an IgE mediated allergic disease with a hypersensitive response that occurs in the lower respiratory tract. In an allergic reaction, the bronchioles restrict and become inflamed, resulting in breathing difficulty. In the airways, the presence of commensal bacteria has been demonstrated to be crucial for modulating the T_H2 allergic inflammatory response (Herbst *et al.*, 2011). Because the pivotal role of normal flora in immune regulation has been documented in the upper respiratory tract, this study set out to investigate whether or not bacterial diversity and abundance in the upper respiratory tract was different between asthmatics and non-asthmatics. If a disturbance in normal flora was responsible for the development of asthma, it would be expected that the asthmatic throat swab samples would exhibit less diverse and less abundant bacterial ecosystems. In this respect, the pathogens would be able to outcompete the normal flora in the asthmatic bacterial community.

The results of this study of eleven self-identified asthmatics and forty-nine self-identified non-asthmatics from Trinity College are inconclusive due to discrepancies in the primer data. Non-asthmatics had significantly more U2 Hex peaks than asthmatics, suggesting that a greater number of bacterial species were present in their throat swab samples. However, both groups had the same number of U1 Fam peaks, suggesting that the number of bacterial species present in their samples was the same. A similar issue with the primer data arose upon calculations of total bacterial abundance, represented by peak height.

Non-asthmatics had significantly more U1 Fam terminal fragments than asthmatics, suggesting a greater bacterial abundance in non-asthmatic throat swab samples. However, there was no difference in the abundance of U2 Hex terminal fragments between the two groups, suggesting that the bacterial abundance is the same in both populations. For a conclusion to be made, both primers should be in agreement in terms of significance.

In terms of sample preparation, there are limited areas that could contribute to the differences between the primers. In a PCR reaction, amplification of the 16s rRNA gene would not occur unless both primers bind to the terminal ends. In addition, the primers should have equal binding capacity, with the same sensitivity to degradation, and the fluorescent tags should not be able to detach from the primers. The Capillary Genetic Analyzer used by Yale University to separate the DNA fragments by capillary electrophoresis is programmed to have the same sensitivity to both fluorescent primers. Therefore, during the fragment analysis, the primers should be detected in equal amounts.

The probable explanations for why the two primers appear in conflicting amounts involve sequence variation between species, incomplete digestion, and the subjective nature of our analysis. It is likely that the sequence variations between species could result in some matching restriction sites, and therefore produce a common sized U1 terminal fragment but differing U2 terminal fragments. For example, if two species have a shared restriction site in equal distance from the U1 primer, their U1 terminal fragments will be the same size (in base pairs) and appear as a singular peak on the electropherogram. However, if the species have unique restriction sites at unequal distances from the U2 primer, they will produce terminal fragments of different sizes (in base pairs). On an electropherogram, this data would appear as a singular U1 peak with two distinct U2 peaks.

Another possible explanation for the variability in the primer data is that the purified DNA fragments were not digested completely. For example, if the 16s rRNA gene of a species had three MnlI restriction sites, four fragments would be produced, but only two would be the terminal fragments labeled with fluorescent primers. In an incomplete digestion, the fragment may only be cut at two out of the three sites, resulting in one “true” terminal fragment, and an additional fluorescently labeled fragment that resembles a terminal fragment in an electropherogram, but is larger than normal because it should have been digested. As a result, more than two terminal fragments, at altered abundances, may appear on the electropherogram for an individual species in a throat swab sample.

Finally, an additional explanation for the disparity in primer data is the subjective nature of deciding what will be labeled as a “true peak” on the electropherogram. Our lab works diligently as a group to determine what peaks are representative of bacterial DNA fragments, as opposed to the series of very small peak fragments at the bottom that can be considered as “noise.” However, in this respect, our decisions have led to unequal labeling of peaks within a number of samples. For example, if an electropherogram appeared to have six true green peaks but only five true blue peaks, the labeling remained that way. It would be incorrect to label a sixth blue peak, that doesn’t actually appear to be a peak, just to make the numbers even.

To eliminate these issues, the characteristic terminal fragments of individual species should be identified. This can be achieved by running *in silico* digests of the 16s rRNA region. The sequence of the 16s rRNA gene is available for thousands of bacterial species on the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI). The sequence data can then be used to identify where the U1 and U2

primers will bind, as well as where the restriction enzymes, such as AluI, HaeIII, and MnlI, will cut the DNA. As a result, the “true” terminal fragments can be determined for given species when it is cut with a specific enzyme. For example, this technique would allow researchers to say that when a certain species is cut with the enzyme MnlI, it will produce a 26 bp U1 fragment and a 50 bp U2 fragment (hypothetically). These known fragment sizes can be used to recognize when multiple species have similar restriction sites, and they can also be used to identify a species by its terminal fragments in an electropherogram. In addition, the known fragment sizes can reveal incomplete digests. For example, a species that produces a terminal fragment of 30 bp and a non-terminal fragment of 60 bp can be considered incompletely digested if a 90 bp peak appears on the electropherogram. Finally, other studies that use tRFLP have access to software programs that can automatically determine a baseline threshold for identification of true peaks over noise, thus eliminating the subjective nature of analysis.

If the data had supported the hypothesis that non-asthmatics had significantly more bacterial diversity and abundance than asthmatics, the results could have been interpreted in a number of ways. Greater diversity of bacterial species in an environment, such as the upper respiratory tract, typically represents a healthy ecosystem. However, an increase in diversity and abundance can correspond to either an increased population of pathogenic microorganisms, an increased population of commensal organisms, or increased populations of both. The present species would have to be identified to determine the relationship between bacterial abundance and presence of allergic disease.

The normal flora varies throughout the body, and many bacterial species are specific to different anatomical sites. In the upper respiratory tract, for example, the typical

commensal organisms are of the genera *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Neisseria*, and *Haemophilus*. The normal flora of the gastrointestinal tract, on the other hand, includes the genera *Lactobacillus* and *Bifidobacterium*, among many others (Madigan *et al.*, 2005). Part of normal colonization of the commensal microbes involves these organisms localizing to the correct locations, and this process can be disturbed early in life. For example, as it has been shown that vaginally delivered babies acquire their mother's vaginal and fecal normal flora, such as *Lactobacillus* and *Prevotella*, across all body habitats (oral, nasopharyngeal, and gut), and infants who are delivered by caesarean section will acquire their mother's skin normal flora such as *Staphylococcus* and *Corynebacterium* across all body habitats. For both vaginally delivered and caesarean section delivered neonates. As vaginal birth is the natural route, the vaginal microbiota serves an early defensive role that the caesarean section delivered babies do not acquire. Instead, C-section babies acquire an abundance of surface skin microbiota in their bodies, and they are more susceptible to certain pathogens. It is documented that 64 to 82% of reported methicillin-resistant *Staphylococcus aureus* (MRSA) skin infections in newborns occur in C-section babies (Dominguez-Bello *et al.*, 2010). As both vaginally delivered and caesarean delivered babies may have abundant and diverse normal flora upon birth, it is essential that the bacterial communities colonize the proper mucosal surfaces.

Therefore, in analysis of bacterial abundance and diversity, it is important to take into account the commensal microbes that are normally present in the investigated area of interest. A reexamination of bacterial communities in the upper respiratory tracts of asthmatics and non-asthmatics should focus on identifying the species present. If oropharyngeal swabs of asthmatics revealed a decreased abundance of normal flora, such as

Streptococcus, *Staphylococcus*, *Corynebacterium*, *Neisseria*, or *Haemophilus*, as compared to non-asthmatic subjects, a possible link could be drawn between the improper colonization of commensal microbes and the development of allergic disease. In that case, the disturbance of normal flora in asthmatics could potentially be responsible for the exaggerated immune response.

This pilot study aimed to examine the bacterial communities in the upper respiratory tracts of asthmatics and non-asthmatics. A future study will be conducted in the laboratory of Dr. Lisa-Anne Foster that eliminates many of the confounding variables in this investigation, such as self-identification of asthma, antibiotic use, alcohol use, and tobacco use. Specifically, physicians at the Connecticut Children's Medical Center (CCMC) will obtain throat swab samples from children of certain eligibility requirements. The asthmatic children will be clinically diagnosed with asthma, as opposed to the self-identified participants in this study, and the non-asthmatic children will be in good health. The children will be between the ages of 5 and 18, and none of the participants will be tobacco users. In this study, many of the subjects were frequent users of both alcohol and tobacco. In addition, the clinical samples obtained from CCMC should have greater representative sample sizes from each experimental group. Future data may reveal if there is or is not a significant difference between the bacterial communities in the upper respiratory tracts of asthmatic and non-asthmatic individuals. Then, conclusions can be drawn on what effects the normal flora colonization has on development of the allergic disease asthma.

Appendix

After signing the informed consent page, please tear off this sheet and answer the following questions completely and truthfully.

Date: _____

Graduating Class: 20____

Sex: **M** or **F** (circle one)

Age: _____

Have you been sick (i.e. cough or cold) in the last 30 days? **Yes** or **No** (circle one)

What, if any, over-the-counter medications did you take? _____

Did you seek medical attention? **Yes** or **No** (circle one)

What, if any, medications were you *prescribed*? _____

Was this prescription for an antibiotic? **Yes** or **No** (circle one)

Did you finish the prescription? **Yes** or **No** (circle one)

If not, when did you stop? _____

Have you been diagnosed with a chronic health condition? **Yes** or **No** (circle one)

If **yes**, check all that apply:

☐ Immune system disorder, specify: _____

☐ Asthma

☐ COPD

☐ Other, specify: _____

When were you diagnosed? _____

What, if any, medications were you prescribed for this condition? _____

Do you continue to take this medication on a regular basis? **Yes** or **No** (circle one)

Do you smoke tobacco?

☐ I do not smoke tobacco

☐ I am a social smoker (5 or fewer cigarettes per week)

☐ I am a habitual smoker (1 pack or greater per week)

☐ Other, specify: _____

Alcohol consumption:

☐ I do not consume alcohol.

☐ I am an infrequent drinker (one drink per week)

☐ I am a habitual drinker (1-2 drinks per day)

☐ I frequently consume 4-5 drinks over a short period of time for the purpose of becoming intoxicated

Thank you for your time in filling out the questionnaire and providing a throat swab sample!

For Official Use Only

Sample Code: _____

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