DISSERTATION

OCCUPATIONAL EXPOSURE TO BIOAEROSOLS AT COLORADO DAIRIES

Submitted by

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ABSTRACT

OCCUPATIONAL EXPOSURE TO BIOAEROSOLS AT COLORADO DAIRIES

The dairy industry is vital to the American economy and impacts both the general population and the workers immediately involved in dairy production. The United States is a significant contributor to the global industry producing approximately 14.6% of the global milk supply. To accomplish this, large herd dairy operations (>1000 head of cattle) operate 24 hours a day, 365 days a year. The long production hours and large herd size result in an increase in the number of injuries and illness in dairy workers. One type of illness diagnosed in dairy farmers is respiratory disease. Multiple researchers have shown that some workers in modern dairy operations have pulmonary function cross-shift declines and lower pulmonary function as well as increased rates of obstructive respiratory conditions such as chronic bronchitis, organic dust toxicity syndrome, occupational asthma, chronic obstructive pulmonary disease, and hypersensitivity pneumonitis (Reynolds, Lundqvist et al. 2013, Reynolds, Nonnenmann et al. 2013).

Respiratory disease is caused by exposure to bioaerosols that consist of bacteria, fungi (and the corresponding constituents), pollen, animal dander, feed, and manure. Although bioaerosol exposure can cause infection, the immunological response the body has to bioaerosols that result in decreased lung function is more prevalent in dairy workers. Although some researchers have examined culturable bacteria and fungi, the viable organisms only represent a small fraction of what is detected in the air at the dairies (Katja Radon and Jörg Hartung 2002). One method used to identify Gram-negative bacteria is the recombinant factor C (rFC) assay, a rapid diagnostic

assay to identify concentrations of endotoxins present in dairy environments. While endotoxins have explained a portion of the respiratory problems in dairy workers, they do not explain all of the respiratory diseases (May, Romberger et al. 2012). Little research has been performed to determine concentrations of fungi at dairies. Some work has been done using GC/MS to identify fungal markers, but the current research is the first study to use the rapid diagnostic (Glucatell) assay to quantify worker exposure to fungi at dairies.

The primary goal of this study was to better characterize dairy worker exposure to bioaerosols through two sample analysis techniques: next generation sequencing (NGS) and rapid diagnostic assays (rFC and Gluactell). The specific aims of this dissertation were to 1) identify similarities and differences in bacterial communities between button samplers and biosamplers co-located inside a cattle pen, 2) characterize worker exposure to the microbial community on dairy farms in comparison to environmental sources, and 3) characterize worker exposure to two bioaerosols constituents based on dairy worker task.

For Specific Aim 1, area air samples were taken for five consecutive days to compare the button and biosamplers co-located inside a fresh cow pen and then analyzed using NGS to determine the identity and quantity of bacteria. The current study was the first to compare the biosamplers and button samplers for NGS analysis at a dairy. The results from this study will help researchers make better decisions on the type of sampler that should be employed for collecting airborne bacteria.

The researchers found that the biosampler was more effective at collecting samples for NGS. The two samplers had significantly different microbial communities that were identified based on the Principle Coordinate Analysis (PCoA) plot. However, upon further analysis the alpha diversity plot showed relatively similar Shannon and Inverse Simpson indices suggesting

both samplers were sampling from the same core microbiome. Therefore, the difference between the samplers is likely due to the high variance in the samples and not actual differences in the microbial community. The alpha diversity plot also had a high operational taxonomic units (OTU) count indicating that the dairy microbiome has a high count of rare bacteria and a low count of dominant bacteria.

The biosampler had a higher relative abundance of bacteria across all five sampling days. The majority of the top identified bacteria were Gram-positive. Currently, little research has been done to assess the impact of Gram-positive bacteria on worker respiratory health. Based on these results, future research should focus on Gram-positive bacteria as they may substantially contribute to respiratory disease. Some of the identified bacterial genera have potentially pathogenic species, but data on the species level is needed to determine the potential for infection. Both viable and non-viable bacteria and their corresponding constituents can act as inflammagens, potentially causing cross-shift lung function decline and respiratory disease (May, Romberger et al. 2012). Both samplers collected bacterial communities that could be analyzed and used for NGS, but the biosampler was identified as the better sampler because of the higher OTU counts and greater bacterial diversity. However, depending on the type of sample information required, the button sampler may be advantageous because it can be used for personal samples and throughout the entire day.

For Specific Aim 2, personal and area air, hand swabs, and soil samples were collected at one dairy for five consecutive days and analyzed using NGS. The sample sets were then compared to identify differences and similarities between the sample type, identity of the bacteria, and potential for worker exposure.

The difference between sampler (button vs biosamplers) was significantly different. The sample type explained more than 50% of the differences seen in the microbial community. The biosampler compared to the button sampler had a lot of variation within their respective types which could explain some of the differences between the communities due to the differences in sampling length and time of day. The variation in the biosampler was mainly due to the second sample taken on each day. The area air samples had the highest relative abundance between the sample types. Soil was thought to have the highest relative abundance but because the number of samples were biased toward air samples (n=60 vs n=15) when the most prevalent top bacteria were chosen they were driven by the air samples. The majority of the bacteria were also found to be Gram-positive across all the samples. The most common source of the bacteria based on the genera information was soil which was expected based on the dusty nature of the dairy environment. Some genera identified have potential pathogenic species, but this dataset did not provide information on the species level. No conclusions can be made on the possibility of infection from the bacteria in these samples.

For Specific Aim 3, four dairies were recruited to assess airborne concentrations of Gram-negative bacteria, fungi and dust. Workers were binned into eight different tasks, and the task samples were compared to identify differences in exposure between the tasks. Differences in site and season were not statistically significant and were not included in subsequent analyses. The concentration of dust over a full work shift ranged from 0.95-5.6 mg/m^3 and were lower than expected. The highest dust concentration was below the Occupational Safety Health Administration Permissible Exposure Limit (OSHA PEL) of 10 mg/m^3 but was not below the suggested Occupational Exposure Limit (OEL) from the American Conference of Governmental Industrial Hygienists (ACGIH) of 2.4 mg/m^3 indicating that dust exposure may be a concern for

some of the tasks. Machine operators and milkers had the highest geometric mean dust concentrations with concentrations of 0.356 and 0.305 mg/m^3 respectively. The endotoxin concentrations ranged from 0.078-40 EU/m^3 which was lower than other research observing endotoxins concentrations at dairies and below the suggested OEL of 90 EU/m^3. Multi-task workers and milkers had the highest endotoxin concentrations (Donham 2000). The β-glucan concentrations ranged from 0.2-212 pg/m^3 with the highest task concentrations found in multi-task workers and machine operators. There is not a suggested OEL for β-glucans but concentrations measured in this study were higher than other studies in waste processing facilities (Douwes 2005). Ultimately, there was not one task that was consistently higher between the different exposure variables and there were no significant differences between any of the tasks. No conclusions or recommendations could be made on the task-based exposures at the dairies. However, even at low concentrations, exposure to agricultural dusts have been shown to induce responses from cytokines (Poole, Dooley et al. 2010). The genetic polymorphism TLR4 has also been demonstrated to cause workers to be more predisposed to sensitization to endotoxins at extremely low concentrations (Reynolds 2012).

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DEDICATION

To my dad. The man who always inspired me to pursue my dreams and taught me to never give up. Without his support, encouragement, and love I would never have dreamt of pursuing a PhD.

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LIST OF ACRONYMS

ACGIH American Conference of Governmental Industrial Hygienists

BIOM Biological Observation Matrix

BLS Bureau of Labor Statistics

CDC Centers for Disease Control and Prevention

COPD Chronic Obstructive Pulmonary Disease

CSU Colorado State University

CVMBS College of Veterinary Medicine and Biomedical Sciences

DNA Deoxyribonucleic Acid

EMP Earth Microbiome Project

EU Endotoxin Units

FEV_1 Forced Expiratory Volume in One Second

FVC Forced Vital Capacity

GOLD Global Initiative for Chronic Obstructive Pulmonary Disease

ISO International Organization for Standardization

NGS Next Generation Sequencing

NIOSH National Institute for Occupational Safety and Health

OEL Occupational Exposure Limit

OSHA Occupational Safety and Health Administration

OTU Operational Taxonomic Unit

PCoA Principal Coordinate of Analysis

PCR Polymerase Chain Reaction

PEL Permissible Exposure Limit

PVC Polyvinyl Chloride

PM Particulate Matter

rFC Recombinant Factor C

TLV Threshold Limit Value

TWA Time-Weighted Average

USDA United States Dairy Association

WHO World Health Organization

CHAPTER 1: INTRODUCTION

Dairy has an enormous impact on the global economy and daily lives of people throughout our nation. The United States produces approximately 14.6% of the annual global milk supply which equates to roughly 21 billion gallons of milk. This is accomplished through the endeavors of over 60,000 dairy farms across the country and 150,000 dairy farmers (Purdue Animal EducationDepartment 2008, Douphrate, Hagevoort et al. 2013, National Agricultural Statistics Service 2015). To meet the demand for production, most dairies are operated 24 hours a day, 365 days a year, milking cows up to three times a day. The long hours and 365 day-a-year production results in an increased risk of injury and illness among dairy workers. Over the past 50 years, dairy production has transformed from small family-owned operations to larger family-owned operations with much larger herd sizes (>1000 head of cattle). The increased job demand is met primarily by immigrant workers with no prior agricultural experience. Although job skills can be learned, an inexperienced immune system cannot always adapt, resulting in an increased risk of respiratory disease due to occupational exposure.

Bioaerosols are airborne particles of biological origin that include manure, feed, crops, bacteria, fungi, viruses, animal dander, both viable and non-viable as well as their corresponding constituents. Bioaerosols are common sources of airborne exposure for dairy workers that can result in various health problems such as respiratory disease, infections, and cancer (Douwes 2003, Walser, Gerstner et al. 2015). Exposure to bioaerosols can result in an immunological response that leads to decreased lung function (Lacey and Dutkiewicz 1994). Although most healthy people can adjust to some small exposures to bioaerosols in their natural environment, even a healthy adult cannot adapt to chronic exposure to high concentrations of dust, endotoxins,

fungi, and other bioaerosols. Many researchers have demonstrated a dose-response relationship between the concentration and length of exposure of air contaminants to the presence of reduced respiratory function such as shortness of breath and cough (Marescaux, Degano et al. 2016), (Radon, Danuser et al. 2001, Rask-Andersen 2011). One significant respiratory disease in farm workers globally is occupational asthma. Multiple researchers have identified a higher prevalence of asthma in people currently working and/or living on a farm (Reynolds, Nonnenmann et al. 2013, Vested, Basinas et al. 2015). Chronic obstructive pulmonary disease (COPD) is a significant problem for workers inside animal confinement operations. Researchers have demonstrated a relationship between time spent inside animal confinement buildings and shortness of breath (Radon, Danuser et al. 2001, Rask-Andersen 2011).

There is not a complete understanding of the resulting reduced pulmonary function and respiratory disease observed in agricultural workers across the world, but researchers have identified some evidence that links bacteria, fungi, and their constituents to respiratory disease. One problem associated with relating bacteria and fungi to respiratory disease is the lack of knowledge around true concentrations of the organisms and the impact their non-viable constituents have on human health.

Collecting viable bacteria and fungi is difficult. Typical personal air samplers can easily be hung on a worker, can sample the entire eight-hour work shift, have small pumps, and have samplers that do not require liquid. Air sampling equipment for collecting viable bacteria and fungi typically cannot run an entire eight-hour work shift, need agar plates or a liquid collection media as well as large sampling pumps that make sampling very difficult logistically. Even if samples could be collected for the viable organisms, the growing conditions must be ideal for the

organism to grow. The ultimate result is that the bacteria and fungi are not collected in concentrations that are equivalent to what exists in the natural environment.

There is also a large amount of bacterial and fungal constituents such as endotoxins, peptidoglycans, and β-glucans that persist in the environment after cell death that are contributing to decreased respiratory health of dairy workers. Endotoxins have been observed in a variety of different agricultural environments indicating that at least some of the reduced pulmonary function in dairy workers can be explained by endotoxins, but little work has been done to identify concentrations of β-glucans via rapid diagnostic assays. Although these markers represent a concentration of bacteria present in the sample, they do not identify the bacteria that are present or the abundance and concentration of such bacteria. The most recent technology, Next Generation Sequencing (NGS), is a way to both identify and quantify bacteria present in dairy environments. NGS can provide further information in the understanding of what bacteria are present and how those bacteria can directly impact human health as well as lead to potential areas of intervention.

There are no occupational health standards for concentrations of bacteria, fungi, or their corresponding constituents. Some researchers have proposed an endotoxin occupational limit of 90 EU/m^3, but the limit has not been adopted (Donham 2000, Reynolds, Nonnenmann et al. 2013). The most relevant occupational standard for dust concentrations at dairies is for 'particles not otherwise specified' which is set at 10 mg/m^3 by the Occupational Safety and Health Administration (OSHA) although there is a proposed occupational exposure limit (OEL) of 2.4 mg/m^3 from (Donham 2000) based on research in agricultural workers.

Bioaerosols, in particular bacteria and fungi, are thought to be causative agents in reduced pulmonary function in dairy workers around the world. A characterization of the dairy

microbiome and its impact on human health has not been done and little is known about the collection abilities of the different samplers available for air sampling in occupational environments. The Glucatell assay has never been employed in the agricultural setting to understand the concentration of airborne β-glucans and how that relates to worker exposure. The primary objective of this study was to better understand and characterize worker exposure to bacteria and fungi in dairy environments. This was accomplished through three specific aims:

Specific Aim 1: Identify similarities and differences in bacterial communities between button samplers and biosamplers co-located inside a cattle pen which will help understand differences in collection techniques of bioaerosols. Samples were collected using the button sampler and biosampler simultaneously inside a cattle pen. The DNA of the bacteria was sequenced and results were used to compare the most abundant bacteria found in both samplers.

Specific Aim 2: Characterize worker exposure to the microbial community on dairy farms in comparison to environmental sources to identify sources of exposure. The approach was to collect personal air and hand swabs from workers working at a cattle pen as well as area air samples and soil samples. All samples were analyzed using Next Generation Sequencing (NGS) to identify potential sources of bacteria in the dairy environment as well as identify any bacteria that are likely to have a direct impact on the health of the dairy worker.

Specific Aim 3: Characterize worker exposure to two bioaerosols constituents (endotoxins and β-glucans) based on task among dairy workers across four dairies to provide relevant information to identify potential interventions.

CHAPTER 2: LITERATURE REVIEW

Dairy Trends in the United States

Agriculture is one of the most prevalent job sectors in the world; the International Labor Organization estimates that there are 1.3 billion agricultural workers worldwide which accounts for close to half of the world's workforce. Agriculture also represents one of the most hazardous industries in the world accounting for approximately 170,000 of the 335,000 (50.7 %) occupational fatalities in the world (International Labour Organization 2016). Within the agricultural industry, the dairy industry is a unique sector because milk is produced 24 hours per day, 365 days of the year as opposed to the seasonal and light dependent work of other sectors such as crop production. Workers must meet the demand for continuous production of this valuable commodity, which in turn increases the potential for occupational illness and injury. Most large herd dairy operations (>1000 cattle) operate 24 hours a day, typically milking cows three times per day (Douphrate, Hagevoort et al. 2013). To meet the demands of continuous operation, work practices and pace have changed, increasing the number of workers needed for specific tasks. One worker doing the same task all day has much different exposures than a single person working on multiple tasks throughout the day. A worker doing one task all day has an increased risk of musculoskeletal injuries (due to the highly repetitive nature of the tasks) and respiratory disease (due to spending the entire day in an enclosed environment with high dust, endotoxin, fungi, and Gram-positive bacterial concentrations) (Douphrate, Hagevoort et al. 2013, Douphrate, Lunner Kolstrup et al. 2013, Reynolds 2013).

The United States has approximately 60,000 dairy farms around the country that produce roughly 21 billion gallons of milk every year that accounts for 14.6% of the supply of milk around the globe (Department 2008, Douphrate, Hagevoort et al. 2013, National Agricultural Statistics Service 2015). The demand for dairy products (including milk, cheese, yogurt, ice cream, and butter) in the United States continues to grow at a rate of approximately 0.4% a year (Horner 2004). For example, from 2004-2014, the average American increased consumption of dairy products from 580 to 614 pounds per year (USDA 2015). These demands are met by milking the cows as often as possible, generally up to three times per day, as well as increasing the number of cows at each dairy. As the number of cows increases, milking production speed also increases to ensure each cow can continue to be milked three times each day. With a larger herd size, more cows go through the milking parlor each day without changing the size of the milking parlor. An increase in pace must happen to accommodate a larger herd often without increasing the number of milkers inside the parlor. In addition to the increase in pace, a larger herd brings in more dirt, manure, and organic dust which increases the potential for respiratory disease in workers.

How dairies are owned and operated has changed drastically over the past 50 years. Previously, most dairies were small, family operated institutions with a small number of dairy cows. The majority of current dairy operations, while still family owned, have more than 2,000 head of cattle (Douphrate, Hagevoort et al. 2013). In 1990, there were approximately 195,000 milk cow operations in the United States while in 2022 the number of milk cow operations dropped to only 36,000 (Service 2011, National Agricultural Statistics Service 2015). Although the number of operations has decreased over the last two decades, the production of milk has

continued to increase from 147,721 million pounds of milk in 1990 to 226,462 million pounds of milk in 2022 (USDA 2015) further demonstrating the differences in dairy farm practices.

Dairy Workforce

The change in the size of the farm has also changed the workforce that supports this entity; a larger number of workers are needed to accommodate the larger herd size, approximately one worker is needed for every 80-100 dairy cows (Douphrate, Hagevoort et al. 2013). Therefore, approximately 20-25 workers are needed to operate a farm with 2,000 head of cattle. A single family that owns the dairy farm cannot fully support these large herd sizes and outside employees are required. Immigrant workers have met this demand for a larger workforce; approximately 70% of dairy workers are immigrant workers, the majority in the United States being Latino workers (Schenker and Gunderson 2013).

Most of the immigrant workforce has no previous work experience in the agricultural sector and therefore has a unique susceptibility to respiratory disease. Workers with no previous agricultural experience have naïve immune responses to organic dusts and other bioaerosols increasing their probability of developing a variety of different respiratory diseases such as chronic bronchitis, chronic obstructive pulmonary disease, and hypersensitivity pneumonitis (Reynolds, Nonnenmann et al. 2013). Rennie, Karunanayake et al. (2015) observed the effect of living and working on a farm and its relationship with asthma. The researchers found that workers without previous farm work or living experience have an increase in ever having asthma when they begin working or living on a farm (Rennie, Karunanayake et al. 2015). In addition to diseases that may result from exposure to organic dust, immigrant workers have higher rates of both fatal and non-fatal injuries than non-immigrant workers (Schenker 2010). In 2021, there were 1,130 Hispanic/Latino workers that suffered a fatal occupational injury; of those 1,130

workers, 727 (64%) of them were foreign-born (U.S. Bureau of Labor Statistics 2015). The differences in culture, lack of health care, and poverty associated with the immigrant workforce make this population vulnerable and more prone to injuries and illnesses. Many immigrant workers do not receive health insurance from their employer, cannot afford health care, and/or do not agree with medical practices in the United States. Poverty, lack of access to health care, and undocumented immigration status also results in underreporting of injuries and illnesses in immigrant workers suggesting that the actual rate of non-fatal injuries is much higher than is recorded by the U.S. BLS (Schenker 2010). Workers that are undocumented are less likely to report injuries, complain about unsafe work conditions, and more likely to take risk because of the fear of repercussion from law enforcement (Schenker and Gunderson 2013). The high rates of injuries and illnesses along with the underreporting in this population demonstrate the need for additional research in this area to help prevent future injuries and illnesses in this population.

Health and Safety in the Dairy Industry

A dairy farm is a very hazardous work environment with a myriad of safety hazards as well as exposures to a variety of different chemical and organic hazards. In 2021, there were 31 fatalities on dairy farms across the United States (U.S. Bureau of Labor Statistics 2015). Dairy farms also have a non-fatal injury incidence rate almost twice as high as the other industries in the United States with an incidence rate of 4.3 per 100 full-time workers in comparison to 2.9 per 100 full-time workers for the average of all industries likely with large amounts of underreporting due to the number of immigrant workers. Many injuries and fatalities in the dairy farm industry come from contact with animals, being struck by equipment, and transportation incidents (U.S. Bureau of Labor Statistics 2015).

Respiratory health is an important factor in the health and safety of dairy workers due to the full work shift exposure to a variety of different organic dusts that can lead to a variety of respiratory diseases. Researchers in Europe showed a dose-response relationship between the numbers of hours worked inside animal confinement buildings and shortness of breath, particularly in swine confinement operations (Radon, Danuser et al. 2001, Rask-Andersen 2011). Additional researchers have demonstrated a relationship between the concentration and length of exposure to air contaminants to the presence of reduced respiratory function (Marescaux, Degano et al. 2016). Multiple researchers have shown that workers in modern dairy operations have pulmonary function cross-shift declines and lower pulmonary function as well as an increased rate of obstructive respiratory conditions such as chronic bronchitis, organic dust toxicity syndrome, occupational asthma, chronic obstructive pulmonary disease, and hypersensitivity pneumonitis (Reynolds, Lundqvist et al. 2013, Reynolds, Nonnenmann et al. 2013). Many researchers have also examined the comparison between livestock and crop production farmers and differences in pulmonary function between the two groups. Eduard et al. (2009) found a higher prevalence of both chronic bronchitis (7.6%) and chronic obstructive pulmonary disease (14.0%) in livestock production farmers in comparison to crop production farmers.

Occupational asthma has proven to be a significant problem in farm workers across the world. Several researchers have demonstrated the increased prevalence of asthma in workers currently working and/or living on a farm (Reynolds, Nonnenmann et al. 2013, Rennie, Karunanayake et al. 2015). A study of California dairy workers showed that after adjusting for smoking status, dairy workers had an odds ratio of 2.73 in comparison to the control employees at a vegetable processing plant with a similar work population (race, age and sex) indicating that

the odds of developing asthma are 2.73 times more likely as a dairy worker than the vegetable processing plant control population (Sterk 2004, Reynolds, Nonnenmann et al. 2013). A longitudinal study by Rask-Andersen et. Al (2011) in Sweden assessed the prevalence of asthma among farmers with a 12 year follow-up period. During the 12 year follow-up period, the prevalence of asthma increased from 2% to 8.9%, a prevalence higher than that found in the general population during the same time period. Interviews of the farmers in the follow-up study showed a strong potential for work-related exposures leading to the higher prevalence of asthma; deterioration in lung function was reported to occur in conjunction with exposure to dust on their farms. Those farmers that had somehow reduced their dust levels or stopped working on farms reported an improvement in their lung function, however, it is difficult to determine a true cause and effect relationship without specific exposure data in this study (Rask-Andersen 2011). Eng, et al. (2010) examined occupational related asthma in New Zealand and found that 7.8% of 102 dairy farmers surveyed had asthma and 10.8% of those had adult-onset asthma which had an odds ratio of 1.4 with a confidence interval of 0.7-2.6 in comparison to the control population (Eng, 'T Mannetje et al. 2010).

Another significant problem found in animal confinement operations is the prevalence of chronic obstructive pulmonary disease (COPD). The Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) has created a standard to define different stages of COPD. The standard is based on two main measurements of pulmonary function which can be measured during a pulmonary function test. The forced expiratory volume in one second (FEV_1) measures the volume of air in liters during a one second forced exhalation. The forced vital capacity (FVC) is the total volume of air in liters exhaled. The GOLD standard defines normal lung function as a ratio of the FEV_1/FVC that is greater than 70%; once the ratio of FEV1/FVC falls

below the 70% cut-off, the severity of the COPD is categorized as mild, moderate, severe, and very severe (Sterk 2004).

COPD is typically a result of chronic irritation in the airways from some inhaled substance. In the dairy industry, this substance is thought to be exposure to organic dust throughout the dairy as well as a wide variety of different chemicals such as pesticides, fungicides, and cleaning products. Researchers in France determined that the the prevalence of COPD in dairy farmers was found in 12.0 ± 2.7% . Because smoking is the most common cause of COPD, whether the farmers smoked was also considered in the data analysis. Farmers that currently smoked or had smoked in the past had a higher prevalence of COPD than those that had never smoked, however, approximately 1/3 of the farmers with COPD have never smoked (Marescaux, Degano et al. 2016). Rinsky, (2015) determined that farmers with medium to large animal operations had an odds ratio 1.51 (95% CI: 1.21, 1.89) times higher than farmers that did not have animals suggesting that organic exposures generated by the animals was more likely to result in COPD Another group of researchers completed a cross-sectional study comparing non-farmer controls to farmers all aged between 40-75 years. Based on the GOLD criteria, there was a COPD prevalence of 5.1% observed in farmers while the COPD prevalence in non-farmers was found to be 2.9%. Once adjusted for age and smoking status, the prevalence was not found to be significantly different between farmers and non-farmers until further categorized by the type of farming. Farmers with cattle operations (specifically cattle breeding) were found to have significantly higher prevalence of COPD than non-farmers (Guillien, Puyraveau et al. 2016).

Bioaerosols

Bioaerosols can be defined as airborne particles that are derived from biological matter; including airborne bacteria (both viable and non-viable), fungi, viruses, animal dander, manure,

feed, plant matter, pollen, as well as many other constituents and metabolic products (Douwes 2003, Walser, Gerstner et al. 2015). In the past 30 years, bioaerosols have been recognized as a potential source for a variety of health problems including respiratory disease, infections, and cancer (Douwes 2003). Bioaerosols can be found in a wide variety of workplaces causing a myriad of respiratory diseases. Exposure to bioaerosols can result in an infection such as large quantities of *Aspergillus niger* growing in the lung, but more often result in decreased respiratory function due to the immunological response the body has to bioaerosol exposures (Lacey and Dutkiewicz 1994). Most healthy people can adjust to a small exposure to bioaerosols in their natural environment, but bioaerosols can represent a health risk for at-risk groups such as people with compromised immune systems or people suffering from allergies or respiratory disease. Approximately 1/3 of the world's population is found in these at-risk groups indicating the importance of the study of bioaerosols and their impact on the respiratory system and overall health (Walser, Gerstner et al. 2015). There is a substantial difference between general, everyday exposure and workplace exposure in areas with high dust concentrations such as those found in animal confinement operations. Although a healthy adult's body can adjust to small exposures to bioaerosols, when the exposure includes high concentrations of dust, endotoxins, fungi, and other organic dust components even a healthy adult cannot adapt completely.

Many researchers have found a wide range of dust concentrations on farming environments and have shown different concentrations based on different tasks on the farm. There currently is no standard in the United States that specifies an OEL for organic dust, endotoxins, or fungal matter. The most relevant OEL that can be used are for particles not otherwise specified (PNOS). The American Conference of Governmental Industrial Hygienists (ACGIH) recommends that airborne concentrations should be kept below 3 mg/m^3 for respirable particles

and below 10 mg/m^3 for inhalable particles (ACGIH 2015). ACGIH defines a respirable particle as "materials that are hazardous when deposited in the gas-exchange region" and an inhalable particle as "materials that are hazardous when deposited anywhere in the respiratory tract" (ACGIH 2015). The Occupational Safety and Health Administration (OSHA) requires that the concentration of particles not otherwise regulated should be kept below 15 mg/m^3 for total dust and below 5 mg/m^3 for respirable dust (OSHA 2016). Hinds et. al (1999) examined different sizes of particles or particulate matter (PM). Two types of PM are typically examined based on their deposition in the respiratory tract. $PM_{2.5}$ is fine particulate matter with an aerodynamic diameter cutoff size of 2.5 µm; PM_{10} is larger particulate matter with an aerodynamic diameter cutoff size of 10 µm. $PM_{2.5}$ is meant to mimic deposition in the respiratory tract because smaller particles are more likely to deposit in the respiratory tract while PM_{10} is meant to mimic deposition in the thoracic region (Hinds 1999). Donham et. al (2000) examined the exposure-response threshold and recommended an occupational exposure limit (OEL) of 2.4 mg/m^3 for total dust and 0.16 mg/m^3 for respirable dust, much lower than the OSHA and ACGIH limits. (Donham 2000).

Researchers studied organic dust, endotoxin, and 3-Hydroxy Fatty Acids (OHFA) on different farms types (feedlot, farms, dairy, and grain elevators) in Colorado and Nebraska. Across all facility types, workers were exposed to a geometric mean of 3.40 mg/m^3 of organic dust. However, when categorized by facility type, the dust concentration ranged from 2.37 to 5.09 mg/m^3 with dust concentrations at the dairy facilities averaging 2.37 mg/m^3 (Reynolds 2012). Other researchers examined the fine PM ($PM_{2.5}$) and inhalable PM (PM_{10}) concentrations on California dairies by task. The tasks included feeding, medical, milking, moving, and rebedding with mean $PM_{2.5}$ concentrations ranging from 33.55 µg/m^3 (0.0335 mg/m^3) (milking)

to 66.74 µg/m^3 (0.06674 mg/m^3) (feeding) and mean PM_{10} concentrations ranging from 930.4 µg/m^3 (0.9304 mg/m^3) (milking) to 1272 µg/m^3 (1.272 mg/m^3) (feeding). Across the five different tasks, the mean $PM_{2.5}$ concentration was 52.38 µg/m^3 (0.05238 mg/m^3) and the inhalable PM concentration was 1081 µg/m^3 (1.081 mg/m^3) (Garcia, Bennett et al. 2013).

Basinas, Sigsgaard et al. 2014 assessed organic dust and endotoxin concentrations across different seasons (summer and winter) in Denmark and found concentrations ranging from 0.9 to 1.1 mg/m^3 and an average across the seasons of 1.0 mg/m^3. In a review of dust and endotoxin concentrations, Basinas, Sigsgaard et al. (2015) summarized dust concentrations from a wide variety of studies as presented in the table below:

Table 2.1 Summary of dairy farm dust concentrations from Basinas, Sigsgaard et al. 2015

Reference	Fraction	Measure	N	Average (mg/m^3)	Range (mg/m^3)
Holness et al.	Total	GM	43	0.95	0.12-4.0
Louhelainen et al.	Total	AM	30	5.6	0.5-9.5
Virtanen et al	Total	AM	31	2.4	0.2-7.4
Kullman et al.	Inhalable	GM	159	1.78	0.007-53.6
Nieuwenhuijsen et al	Inhalable	GM	17	NS	0.3-0.62
Spaan et al.	Inhalable	GM	8	1.3	0.4-2.3
Spaan et al.	Inhalable	GM	4	1.5	0.7-2.7
Burch et al.	Inhalable	GM	15	2.4	NS
Basinas et al.	Inhalable	GM	124	1.0	<LOD-9.8
Samadi et al.	Inhalable	GM	62	0.89	<LOD-6.9
Garcia et al.	Inhalable	AM	225	0.99	NS

Researchers in both the United States and Europe characterized inhalation exposure to organic dust and have found concentrations to be extremely variable between facilities and tasks. A large number of the exposure characterizations reported organic dust concentrations that exceed the recommended occupational exposure limits of 2.4 mg/m^3 as recommended by researchers (Reynolds, Nonnenmann et al. 2013).

An important composition of bioaerosols are the bacterial and fungal constituents that continue to persist in the environment after the death of the microorganism. Although live bacteria and fungi can cause infections, , it is also important to understand the impact that viable and non-viable constituents have on respiratory health. Inhalation of these non-viable bacterial and fungal constituents can result in inflammation in the respiratory system, allergic, and immunotoxic effects (Hawley, Schaeffer et al. 2015). Constituents of bacteria and fungi can be categorized into two different areas based on the source of the constituent: structural constituents and proteolytic enzymes. Structural constituents are primary metabolites which include endotoxins, (1→3)-β-D-glucans, and peptidoglycans. In contrast, compounds that are excreted into the environment are secondary metabolites and include mycotoxins and proteolytic enzymes (Eduard 1997). Because these bacterial and fungal constituents are not viable, they cannot be cultured and other methods must be used to measure concentrations. Rapid diagnostic assays have been developed to measure the amount of those structural constituents such as endotoxins and (1→3)-β-D-glucans in samples.

Culture-Based Methods vs Rapid Diagnostic Assays

Culture-based methods to assess airborne bacterial contamination rely on the ability of microorganisms to grow on media at very specific conditions. Although this method has historically been used to determine concentrations of bacteria and fungi and can indicate some level of contamination, it is now known that this method does not provide a comprehensive assessment of the bacterial or fungal contamination present (Keer and Birch 2003). Culture-based methods also represent a biased method to determine which bacteria and fungi are present as the bacteria or fungi can only be grown after the ideal niche and specific culture conditions are found (Ward, Weller et al. 1990). Even from a culture of bacteria or fungi under ideal laboratory

conditions where there are ample nutrients and ideal temperature and humidity, it is impossible to get 100% bacteria or fungi to grow from a culture. Past research has estimated that approximately 1 in 1000 microbial cells are actually culturable although the true number varies between different species and growing conditions (Eduard 1997).

When the bacteria and fungi are cultured from the environment, there is substantial stress to the microorganisms resulting in even lower rates of culturable viability. In the dairy environment, many of the viable bacteria and fungi are killed using cleaning products across the dairy. Although the bacteria and fungi targeted by these cleaning chemicals are then no longer viable, the components of the bacteria and fungi such as endotoxins, peptidoglycans, and (1→3)-β-D-glucans are still present and can still lead to inflammation.

The sampling methods used to collect bioaerosol samples for analysis also pose a problem for collecting culturable samples. When a personal air sample is collected, it is typically collected over the entire shift, but during that time bacteria that have deposited on the filter become stressed and desiccated due to the high air flow that crosses the filter. Other samplers such as liquid impingers can be employed to collect bacterial and fungal samples in a less stressful way for the microorganisms but provide logistical problems when placing the sampler on a worker. Impingers are typically made of glass and contain liquid resulting in problems when a worker must bend over during their shift or move around. Because all the bacteria and fungi collected in a sample are not likely to grow properly in one medium, and because the bacterial and fungal components persist after cell death and continue to cause inflammation, other methods of analyzing concentrations of bacteria and fungi are necessary to understand true worker exposure.

Rapid diagnostic assays such as the rFC assay and the (1→3)-β-D-glucan assay provide a method for determining concentrations of bacterial constituents without having to culture the samples. Because a culture medium is not required, it is possible to get a more accurate sample concentrations to what workers are being exposed. Although cultures have been used for a long time to determine the concentration of bacteria and fungi, culture techniques only account for a small percentage of the bacteria that are actually present. Additionally, many researchers have shown that the components of bacteria and fungi can have the same or more significant impact on the respiratory system. By utilizing the rFC assay and the (1→3)-β-D-glucan assay, it is possible to measure a concentration of both viable and non-viable bioaerosol constituents.

Endotoxins

Endotoxins are found in the outer membrane of the cell wall of Gram-negative bacteria. Endotoxins have a pro-inflammatory reaction and exposure has been shown to cause airway inflammation, chronic obstructive pulmonary disease, chronic bronchitis, non-allergic asthma, reduced lung function, nose and throat irritation, and organic dust toxicity syndrome (Thorn 1998, Rylander 2006, Spaan 2008, Poole, Dooley et al. 2010). The endotoxin is a lipopolysaccharide that consists of an O-antigen, polysaccharide chains, and a toxic lipid A component (Willey 2008) (Figure 2.1). The lipid A component of the endotoxin is the biologically active component and consists of hydroxylated fatty acids of varying carbon chain lengths (Burch, Svendsen et al. 2009). The endotoxin is extremely important to the Gram-negative bacteria by aiding the bacteria in protection and contributes to the structural integrity of the cell membrane.

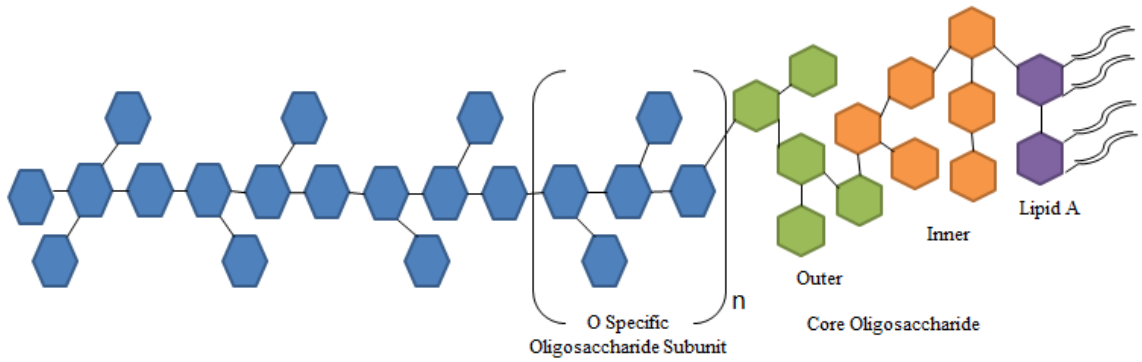

Figure 2.1 Endotoxin Structure (Laguri, Silipo et al. 2018)

The bacteria from which endotoxins come generally range from 0.3-60 µm in size; a single endotoxin is approximately 10 kDa but many form aggregates that weigh up to 1000 kDa (Sigma-Aldrich 2015). Many Gram-negative bacteria produce endotoxins and release large amounts of endotoxins at cell death and even some endotoxins during multiplication (Willey 2008). Endotoxins are found ubiquitously in the environment as Gram-negative bacteria are everywhere, however there are some environments such as agriculture that have a higher concentration of endotoxins than those found in "cleaner" environments such as a home or office. Kujundzic et. al (2006) assessed the concentration of endotoxins inside homes during both the winter and summer months and found that the average endotoxin concentration ranged from 0.56-2.6 endotoxin units (EU)/m^3 (Kujundzic 2006, Schierl, Heise et al. 2007). One EU is equivalent to 0.1 ng; the average range of endotoxins inside homes is 0.056-0.26 ng/m^3. (Donham 2000, Sigma-Aldrich 2017). This concentration of endotoxins is a relatively low concentration and would be representative of the concentration one would expect to find in a person's home or in most office environments. In contrast, research has been conducted in different agricultural environments that generally have higher concentrations of endotoxins. Poultry operations were found to have concentrations that ranged from 290-7700 EU/m^3, swine

operations had a range from 430-3700 EU/m^3 (Saito 2008), and dairy operations had average concentrations around 300 EU/m^3 with individual exposures exceeding 10,000 EU/m^3 (Reynolds, Nonnenmann et al. 2013). Endotoxins can cause a variety of health effects most of which affect the respiratory system.

It is not recent knowledge that there is a correlation between endotoxin exposure and decreased lung function. Castellan, Olenchock et al. (1987) reported a strong exposure-response correlation ($r=-0.85$, $p<0.0001$) between the concentration of endotoxins and a decrease in lung function even at low concentrations of endotoxins (Castellan, Olenchock et al. 1987). Researchers exposed humans to a single inhalation of 40 µg of endotoxins; after this single inhalation, participants had a decreased FEV_1 and an increase in reported symptoms such as chest tightness, airway irritation, fever, headache, joint and muscle pain, and nausea. In the alveolar lavage fluid of the patients, there was also an increase in the number of neutrophils and lymphocytes indicating an increase in inflammation (Thorn 1998).

Researchers examined occupational endotoxin exposures and its impact on respiratory function both in agriculture and specifically in dairies. Vogelzang, van der GULDEN et al. (1998) researched the long-term effects of exposure to endotoxins in pig farmers in the Netherlands. The researchers followed the pig farmers over three years and took a long-term average of worker exposure in conjunction with pulmonary function testing. Over the three years, the average organic dust exposure was 2.63 mg/m^3 and the average endotoxin exposure was 105 ng/m^3 while worker lung function decreased; the FEV_1 decreased by 73 mL/year and the FVC decrease by 55 mL/year (Vogelzang, van der GULDEN et al. 1998). Another research group assessed California dairy workers performing five different tasks (feeding, medical, milking, moving, and rebedding) and the subsequent endotoxin concentrations. Across the five

tasks, the geometric mean of endotoxin concentrations ranged from 163.3-368.9 EU/m^3 much higher than the suggested occupational exposure limit of 90 EU/m^3 (Garcia, Bennett et al. 2013). V.E. Arteaga et al. (2015) examined occupational exposure to endotoxins on dairies in comparison to a non-dairy control facility in California. The research team measured endotoxins using the Lonza rFC assay and compared those results to pulmonary function test results. Cross-shift decreases in lung function were found for both FEV_1 and FVC of -44.3 mL and -35.6 mL respectively with endotoxin concentrations ranging from 0.3-2061.3 EU/m^3 and a geometric mean of 331.5 EU/m^3 (Arteaga, Mitchell et al. 2015). Researchers assessed the prevalence of COPD in never smoking farmers in Europe working inside animal confinement operations. Exposure to total dust and endotoxin concentrations were measured for 105 farmers; of those 105 farmers, 18 (17%) were diagnosed with COPD. Total dust and endotoxin both showed a dose-response relationship with COPD with the highest COPD prevalence found in farmers with high dust and endotoxin concentrations (Monsó, Riu et al. 2004). Basinas, Sigsgaard et al. performed a literature review of endotoxin exposure studies in 2015. The results for dairies are summarized in Table 2.2 below.

Table 2.2 Summary of studies measuring dairy farm endotoxin concentrations from Basinas, Sigsgaard et al. 2015

Reference	Fraction	Measure	N	Average (EU/m^3)	Range (EU/m^3)
Kullman et al.	Inhalable	GM	159	647	25.4-34,800
Nieuwenhuijsen et al	Inhalable	GM	17	10.9	NS
Spaan et al.	Inhalable	GM	8	560	62-2230
Spaan et al.	Inhalable	GM	4	1570	444-3860
Smit et al.	Inhalable	GM	46	220	NS
Saito et al.	Inhalable	GM	17	752	NS
Basinas et al.	Inhalable	GM	124	358	<LOD-5890
Samadi et al.	Inhalable	GM	62	392	21-8292
Garcia et al.	Inhalable	AM	225	453	NS

Another method to analyze total endotoxin exposure is through the use of gas chromatography/mass spectrometry (GC/MS). GC/MS quantifies the 3-OHFA. The GC/MS method may provide a more reliable method for quantification of endotoxins because it is more sensitive than the rFC assay. Additionally, the rFC assay may have reactions with constituents of dust that interfere with the analysis (Burch, Svendsen et al. 2009).

Despite research dating back to 1987 that demonstrated a correlation between endotoxin exposure and a decrease in lung function, there is currently no standard in the United States or internationally for occupational endotoxin exposure. Based on research of the acute and chronic effects of occupational endotoxin exposure, the Dutch have proposed an occupational exposure limit of 90 EU/m^3 (Reynolds 2012) which is exceeded in many of the agricultural sectors. The prevalence of lifetime lung disease in farmers is much higher in comparison to the general non-farming population with a prevalence of 6-15% versus 2-3% respectively. Additionally, the "healthy worker effect" is thought to play a role where workers that are more susceptible to endotoxins exhibit more extreme symptoms and therefore choose not to work in environments where there is a relatively higher concentration in endotoxin exposure (Burch, Svendsen et al. 2009).

Respiratory disease is just the beginning of understanding the reaction the human body has from endotoxin exposure. Recent research indicates the potential other effects of endotoxin exposure such as psychological responses, increased blood pressure, and even a loss of bone density with continued occupational endotoxin exposure (Engler, Wegner et al. 2015, Espirito Santo, Ersek et al. 2015, Zhong, Urch et al. 2015).

Fungal Exposure and β-Glucans

Fungi have long been identified as causative agents of respiratory disease in humans; records as early as the middle ages report what is thought to be disease caused from mold exposure such as farmer's lung and silo unloader's syndrome (Perry, Iwata et al. 1998). In the 18th century, Bernardino Ramizzini recorded the first occupational illnesses after witnessing a grain with a powdery substance and consequent respiratory problems in farmers that handled that grain which was later found to be mold (Kuhn and Ghannoum 2003). In general, exposure to mold and fungi can impact humans through three different mechanisms: harmful immune response, direct infection by organism, and toxic irritants from mold by-products (Bush, Portnoy et al. 2006). Some conditions caused by exposure to fungal by-products and mold include hypersensitivity pneumonitis and allergies which are often observed by repeated exposure in an occupational setting. Farmer's lung is a disease that was first diagnosed in farmers with repeated exposure to antigens present on moldy hay or straw resulting in granulomas, sensitization, and hypersensitivity pneumonitis which was described by farmers as early as the 1850s (Grant, Blyth et al. 1972, Reboux, Piarroux et al. 2007). One historically relevant exposure to toxins from mold was the occurrence of St. Vitus' Dance, an epidemic of hallucinations, fainting, and a dancing mania. This is commonly believed to be a result of the toxins produced by the rye fungus *Claviceps purpurea* which produces lysergic and ergotamine (commonly known as ergot) (Midelfort 1999).

Researchers examined the effect of exposure to occupational respiratory disease such as asthma, COPD, increased bronchial responsiveness, and decreased lung function. Dosman, Lawson et al. (2004) discovered a higher prevalence of asthma and reduced pulmonary function in workers that had not been previously exposed to dust containing fungal components. These

authors examined four new workers at a swine facility that were previously healthy individuals but all developed acute onset of wheezing and coughing suggestive of asthma within weeks of beginning full-time employment. Although this is a small sample size, many other researchers have investigated new workers on different farm types and have seen very similar results; workers with a naïve immune system have a higher prevalence of developing occupational respiratory disease after beginning work on a farm.

Hypersensitivity pneumonitis is a disease that has common fungal sources such as *Aspergillus* and *Penicillium*. Both fungal genera are known allergens that are commonly found in occupational settings such as farms and composting facilities. Species of a variety of genera such as *Alternaria*, *Penicillium*, *Aspergillus*, and *Cladosporium* are known producers of type I allergens (allergens that bind IgE antibodies) (Douwes 2003).

Most researchers that study the impact of fungal exposure on worker health rely on culturable mold samples, but the culturable fungi are a small fraction of the fungal exposure in any occupational setting. In addition to the culturable mold exposure, there are large quantities of fungal constituents such as spores and β-glucans that can cause an allergic response. In addition to allergic responses caused by fungi, (1→3)-β-D-glucans are also known causative agents of non-allergic respiratory disease (Douwes 2003). (1→3)- β -D-glucan is a water-insoluble glucose polymer that is found in the cell wall of all fungi as well as some bacteria and plants. The (1→3)- β -D-glucans in the fungal cell wall are typically connected to lipids, proteins, and carbohydrates such as chitin, and mannan (Douwes 2005). (1→3)-β-D-glucan continues to persist after the death of the fungal species and continues to be toxic indicating that it can continue to impact respiratory health even if no culturable fungi can be found in the environment. Once inside the body, the (1→3)-β-D-glucans can have a wide range of biological

responses such as activating the neutrophils, macrophages, and eosinophils (Douwes 2005). The (1→3)-β-D-glucans are taken up by the macrophage and undergo a slow oxidative degradation that can take weeks or months to completely breakdown the entire (1→3)-β-D-glucan structure since humans do not possess a specific hydrolase to break down (1→3)-β-D-glucans (Rylander and Lin 2000).

Rylander and Lin (2000) examined the impact of inhaling (1→3)-β-D-glucans and found that acute exposure to (1→3)-β-D-glucans can result in an inflammatory response where the neutrophils invade the lung tissue and airways and consequently excrete inflammatory cytokines (Rylander and Lin 2000). Other researchers investigated the correlation between (1→3)-β-D-glucans and self-reported respiratory symptoms. The result was a time-weighted average (TWA) of (1→3)-β-D-glucans of 14 µg/m^3 with no statistically significant correlation between (1→3)-β-D-glucans and self-reported symptoms (Eduard, Douwes et al. 2001). {Samadi et. al (2009) #8} investigated (1→3)-β-D-glucans in a horse stable.; they identified concentrations ranging from <LOD-631 µg/m^3 with significantly higher concentrations collected in the personal air samples.

Douwes et. al reviewed the (1→3)-β-D-glucan studies in 2005 looking at the different concentrations found in different types of locations as presented in Table 2.3 below.

Table 2.3 Summary of studies measuring dairy farm (1→3)-β-D-glucan concentrations from Douwes 2005 Microbial Communities and Sequencing

Reference	Environment	N	Assay	Range or Mean (ng/m^3)
Rylander et al.	Schools	46	LAL	0.2-0.55
Rylander et al.	Day Care	13	LAL	<0.1
Mandryk et al.	Sawmill	54	LAL	1.4
Mandryk et al.	Green mills	36	LAL	3
Thorn and Rylander	Waste Collectors	20	LAL	19.1
Douwes et al.	Compost	43	ELISA	0.54-4.85 µg/m^3
Wouters et al.	Waste Collectors	118	ELISA	1.3 µg/m^3
Heldal et al.	Waste Handlers	25	LAL	52
Gladding et al.	Waste Recycling	156	LAL	4.8-40.1

Eduard et al.	Farming	90	ELISA	0.82 µg/m^3

DNA Sequencing

The rapid diagnostic assays (such as the rFC and Glucatell) provide a quick and easy way to quantify markers of bacteria and fungi in any given sample. However, the assays do not provide information regarding the genus, species, or abundance of either. Information on the genus and species level can provide important information regarding possibility of infection, potential toxins produced, and source of the bacteria. All of this information makes it possible to make more informed decisions regarding worker exposure and interventions.

Bacteria are ubiquitous with concentrations averaging 10^4 to 10^6 cells/m^3 in typical environments. However, some environments such as dairy farms, composting facilities, and other occupational exposures far exceed these concentrations ultimately impacting the respiratory system of the workers in these environments (Bowers, Sullivan et al. 2011).

DNA and RNA sequencing is a field in which the methods are constantly changing to a newer, more advanced method that allow for deeper sequences. The first reliable method of DNA sequencing was the Sanger chain termination method that was developed in the late 1970s which gave way to the first automated capillary electrophoresis based sequencing techniques and finally the more recent massively parallel sequencing methods (illumina 2016). The most recent use of DNA sequencings is Next-Generation Sequencing (NGS). NGS uses the sequencing by synthesis method that allows reliable sequences of DNA to be synthesized and then read. Initially, the DNA template strand is fluorescently labeled. DNA polymerase catalyzes the reaction and incorporates a fluorescent label to the deoxyribonucleotide triphosphates (dNTPs). The DNA in the sample is broken into random fragments and an adapter is ligated onto the

fragments and then amplified through PCR and then gel purified (Figure 2.2) (illumina 2015, illumina 2016).

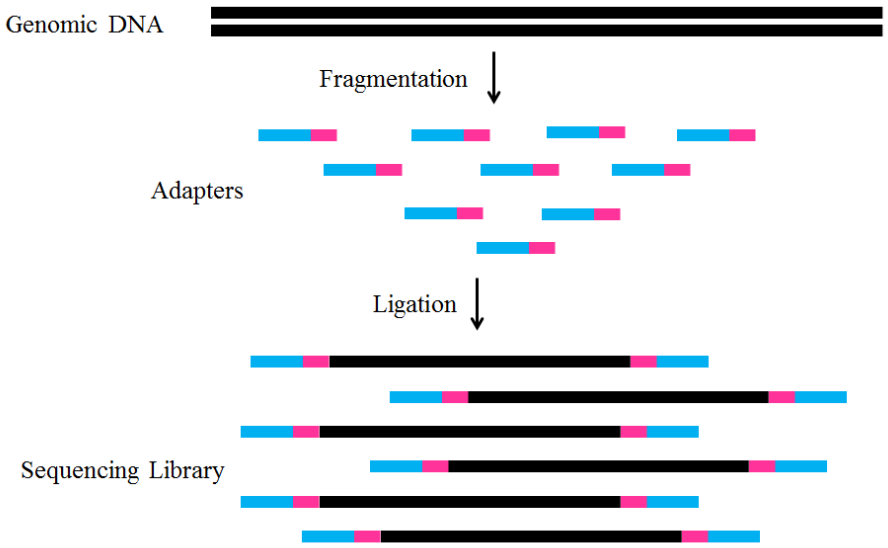

Figure 2.2 Steps of library preparation for NGS (Illumina 2016)

These fragments are then bound to a lawn of surface bound oligos that are complementary to the library adapters and amplified to create clonal clusters (Figure 2.2). After the clonal generation occurs, the sequencing process begins by adding, primers, DNA polymerase, and four labeled reversible terminators. The reversible terminators allow for the detection of single bases as the bases are incorporated into a single template strand of DNA. After the primers, terminators, and polymerase are added, laser excitation takes place; when a base is added, there is a fluorescent emission. The emission of fluorescence is recorded and the corresponding base addition is also recorded. This process is completed for all of the strands within each cluster simultaneously and is repeated for each base addition until all of the bases in each cluster are identified and sequenced (Mardis 2008, illumina 2015, illumina 2016).

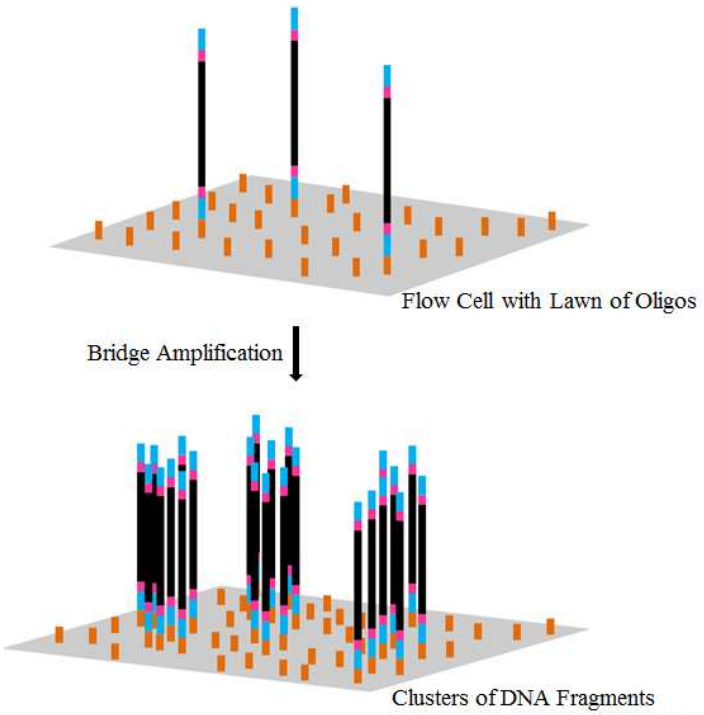

Figure 2.3 DNA fragment cluster generation and amplification (Illumina 2016)

The ability to sequence DNA samples with large sequences and high number of reads has been available for some time, but the ability to analyze the data output that is received from these large sequence reads continues to be problematic. The sequencing technology is outperforming the data analysis technology; however, bioinformaticists are becoming more common and increasing the knowledge and ability to understand the data that are received from these large sequence reads. The Earth Microbiome Project (EMP) is a prime example of the limitations and advances the technology has been making since it began in 2010. The main purpose of the EMP is to sequence and store the data from a wide array of different bacteria from a plethora of different samples including human, animal, plant, marine, freshwater, sediment, air, and many other media (Gilbert, Jansson et al. 2014). From these samples, the researchers involved in the EMP wanted to create a table with the abundance of all the different organisms, but the available resources could not handle the large amount of data that came from the operation taxonomic

units (OTUs). In order to keep up with these data, the researchers developed a new line of software called Biological Observation Matrix (BIOM) to analyze the data and QIIME to visualize the data. QIIME is now commonly used to analyze and visualize the sequencing data (Gilbert, Jansson et al. 2014).

Although significant work has been done to examine a variety of different samples such as different types of soil and water, little has been done to assess the impact this has on human health. The majority of researchers used other methods to assess the levels of bacteria and fungi that are present in agricultural settings such as culture techniques, end point assays, and PCR (Blais Lecours, Veillette et al. 2012). Blais Lecours, Veillette et al. (2012)examined the amount of archaeal and bacterial 16S rRNA genes through PCR to assess the concentration of archaea and bacteria in dairy barns in eastern Canada. This research team identified a wide range of bacterial and archaeal concentrations in different dairy barns, but overall found high concentrations of bacteria and archaea across all dairy barns average concentrations of 1.5×10^8 bacterial 16s rRNA genes per m^3 of air (Blais Lecours, Veillette et al. 2012). Although their research determined some of the genera that were found in the air samples, there is no quantity of each of the types of bacteria found and there is no specificity on the species level available from this data.

Although endotoxin analysis and health outcomes have previously been studied in the dairy industry, the use of the Glucatell assay to further characterize worker exposure to fungal markers in this environment has never been done. Additionally, little work has been completed to characterize the microbiome of worker air contaminant exposure at dairies and link potential sources of exposure. Based on the knowledge gaps, the primary objective of this study was to further characterize dairy worker exposure to bioaerosols. This was accomplished by 1)

comparing air sample collection techniques and samplers for NGS analyses; 2) characterizing the dairy microbiome to identify linkages between personal exposure and environmental sources; and 3) characterizing task-based exposures to bacteria and fungi based on novel rapid diagnostic assays to help drive interventions.

CHAPTER 3: COMPARISON OF SKC BUTTON AND BIOSAMPLER FOR MICROBIAL SEQUENCING IN ONE COLORADO DAIRY

SUMMARY

Airborne exposure to bioaerosols including bacteria and their corresponding constituents poses a threat to the respiratory health of dairy workers. To better understand the dairy microbiome and air sample collection techniques for high-throughput DNA sequencing, two samplers (SKC button sampler (n=10) and SKC biosampler (n=20)) were co-located inside a fresh cow pen for five consecutive days. Within the top genera of bacteria, there were more Gram-positive bacteria collected than Gram-negative bacteria, suggesting future studies should investigate different analysis techniques to develop a rapid diagnostic assay to identify the presence of Gram-positive bacteria.

The most prevalent genera of bacteria collected include *Staphylococcus*, *Clostridium*, *Pseudomonas*, and *Acinetobacter.* These generea have species linked to bacterial infections, particularly in people with compromised immune systems (Willey, Sherwood et al. 2011). Without species level information, it is impossible to know the explicit probability of infection. However, even without the species level information, the bacteria and their corresponding constituents have the ability to cause respiratory symptoms such as allergic asthma and COPD without causing a bacterial infection. Overall, the two samplers had significantly ($p<0.001$) different microbial communities based on the operational taxonomic units (OTUs). However, there was more variance within the bacterial samples from the biosamplers, which was potentially attributable to the shorter collection period and different worker tasks during collection time. The biosampler had a higher relative abundance of bacteria across all five days. There were no downward or upward trends in relative abundance during the week for the

bacterial genera in either sampler. Based on the analysis of richness, evenness, and dominance; the samplers were pulling from the same core microbiome as anticipated due to the uniform sampling environment. The microbiome in the fresh cow pen was driven mostly by many rare bacteria as opposed to a few dominant bacteria. The biosampler was better suited for collecting bacteria for high-throughput data analysis, but it posed a wide variety of logistical issues for personal sampling that the button sampler did not.

INTRODUCTION

Bacteria are found in every environment with concentrations averaging 10^4 to 10^6 cells/m^3 in most areas, but dairy workers are exposed to high concentrations of bacteria every day throughout their work shift. The high concentrations of bacteria and other organic dust are a result of cow manure, dust, feed, and slurries that ultimately result in concentrations that far exceed typical everyday activities. Exposure to these high bacterial concentrations result in a variety of respiratory problems such as cross-shift declines and lower pulmonary function as well as an increased rate of obstructive respiratory conditions such as chronic bronchitis, organic dust toxicity syndrome, occupational asthma, chronic obstructive pulmonary disease, and hypersensitivity pneumonitis (Reynolds, Lundqvist et al. 2013, Reynolds, Nonnenmann et al. 2013).

Researchers are trying to understand the source of dairy-worker bioaerosol exposure by determining concentrations of dust, endotoxins, Gram-positive bacteria, and fungi. Historically, culture techniques have been used to identify and quantify airborne bacterial concentrations, but typically have a much lower concentration than what is present in the air. Air sampling and collection techniques make it very difficult for bacteria to survive because the long sampling period often results in desiccation of the bacteria. Of the bacteria that do survive sample

collection, ideal culture conditions (e.g., temperature and growing media) must exist for each cell collected to grow. One set of culture conditions cannot accurately reflect all of the bacteria collected in an air sample, resulting in much lower concentrations of bacteria than in the natural environment.

Gram-negative bacteria can be quantified using a recombinant factor C (rFC) assay to determine endotoxin concentrations. Although this assay works for both viable and non-viable bacteria, it only quantifies the bacteria and does not identify which bacterial genera are present. Additionally, this assay only identifies Gram-negative bacteria which is only a small portion of the occupational bacterial exposure. In addition to quantification, next-generation sequencing (NGS) provides genus level information that can be used to identify the dairy microbiome. NGS has the benefit of providing information on both viable and non-viable samples while still providing information on Gram-negative and Gram-positive bacteria.

The Earth Microbiome Project has been focused on understanding the microbiome in many different environments such as soil, water, and the human gastrointestinal system, but little has been done to assess the occupational exposures in different industries. The dairy industry is a perfect candidate to assess bacterial exposure because it is an environment known to be rich in bacteria, but little work has been done to understand the composition of the microbiome and how it impacts human health in an occupational setting.

One limitation of collecting bacteria in an occupational setting is the types of samplers that are available for collection; most of the commonly used air samplers pose a harsh environment for bacterial survival while most of the samplers adequate for culture work are difficult to use on a worker. A commonly used sampler is the SKC button sampler (SKC Inc., Eighty Four, PA) which works well for inhalable personal samples but provides a harsh

environment for bacteria, often leading to bacterial desiccation. The button sampler is designed to collect dust with an aerodynamic diameter up to 100 µm (inhalable fraction). It is typically chosen for dirty environments, such as dairies, due to the presence of the screen to reduce the amount of large size deposits (such as manure splatter) that are often collected within other samplers. The SKC biosampler, a swirling impinger, provides a better environment for the bacteria due to its ability to be loaded with a liquid to help prevent desiccation which prevents the sampler from being placed on a worker. The biosampler requires the use of a high flow pump (12.5 L/min) which results in a shorter sample time due to the quick evaporation of the liquid and makes it impossible to place on a worker due to the size of the pump.

A gap exists in the scientific literature of the microbiome present in occupational settings, but to further assess the airborne concentrations, more information is needed regarding the types of samplers available for data collection. There are no studies that have used both the SKC button sampler and biosampler to determine the differences in bacterial collection for these two types of samplers for NGS in dairies. The historical data has included bacterial markers with no knowledge of which bacteria are present and the impact that the bacteria could have on worker health. This information is critical for developing future effective exposure control interventions. The purpose of this study was to compare the sampling results of the SKC button and biosampler and their ability to collect bacteria to further assess worker exposure.

METHODS

Sample Collection

One Northern Colorado large-herd dairy operation (i.e., greater than 1000 lactating cows) was recruited for this pilot project. Samples were collected for five consecutive days inside the fresh cow (recently calved) pen during July 2014. The samplers were placed side by side in the

middle of the pen on a box approximately three feet from the ground. Environmental data (temperature, relative humidity, carbon monoxide, and carbon dioxide) was measured using the TSI Q Trak. Only temperature and relative humidity were looked at as relevant data points. The SKC button samplers (SKC Inc. Eighty Four, PA) were sampled in duplicate for eight hours and were loaded with polyvinyl chloride (PVC) filters with a 0.5 µm pore size. Filters were pre- and post-weighed. The samplers were pre-calibrated to a flowrate of 4 L/min and post-calibrated to ensure the fluctuation in flowrate was within ±5%. A total of 10 samples were collected with the button samplers plus a laboratory and field blank for each sampling day. The field blanks were taken to the dairy and treated in the same manner as the samples but were not connected to a sampling pump. The laboratory blanks remained in the laboratory. Both blanks were weighed and analyzed in the same manner as the samples to ensure no cross-contamination occurred because of sample handling. The SKC Biosamplers (SKC Inc. Eighty Four, PA) sampled in duplicate in one hour increments for a total of two hours each day. The biosamplers were filled with 20 mL of resuscitation buffer (Andersson, Laukkanen et al. 1995) and calibrated to a flowrate of 12.5 L/min. The resuscitation buffer was a solution of polyethylene glycol, buffered peptone water, and tween that was autoclaved and only opened inside a biosafety cabinet to ensure sterility. The button samplers ran for 8 hours (the length of a work shift) while the biosamplers ran in two one-hour segments due to liquid evaporation and to help represent part of the work shift. After weighing the button filters and measuring the volume of resuscitation buffer from the biosamplers, the samples were transferred to 50 mL conical tubes and stored in a -80ºC freezer before being sent to Argonne National Laboratory for sequencing analysis.

Sample Analysis

Deoxyribonucleic Acid (DNA) from the microbial samples was extracted using a modified version of the PowerSoil DNA Isolation Kit (MO BIO). Genomic DNA was amplified using the Earth Microbiome Project (EMP) (Earth MicrobiomeProject 2017) (Van Bonn, LaPointe et al. 2015) barcoded primer set adapted for MiSeq by adding nine extra bases in the adapter region of the forward amplification primer that support paired-end sequencing (Caporaso, Lauber et al. 2012). The V4 region of the 16S rRNA gene (515F-806R) was amplified with region-specific primers that included the Illumina flowcell adapter sequences, and the forward amplification primer also contained a twelve-base barcode sequence (Apprill, McNally et al. 2015). Each 25 µL PCR reaction contained 12 µL of MO BIO PCR Water (Certified DNA-free), 10 µL of 5 Prime HotMasterMix (1×), 1 µL of Reverse Primer (5 µM concentration, 200 pM final), 1 µL Golay Barcode Tagged Forward Primer (5 µM concentration, 200 pM final), and 1 µL of genomic DNA. The PCR conditions were as follows: 94°C for 3 min to denature the DNA, with 35 cycles at 94°C for 45 seconds, 50°C for 60 seconds and 72°C for 90 seconds, with a final extension of 10 minutes at 72°C to ensure complete amplification. Following PCR, amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each of the products were pooled into a single tube so that each amplicon was represented equally. This pool was then cleaned using the UltraClean® PCR Clean-Up Kit (MO BIO) and quantified using Qubit (Invitrogen). After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 2 pM with a 30% PhiX spike for loading on the Illumina MiSeq sequencer.

Statistical Analysis

A principal coordinate analysis (PCoA) was used to visualize the similarities and dissimilarities between the button and biosampler Operational Taxonomic Units (OTUs). The OTUs are bins comprised of similar sequences of bacterial ribosomal Ribonucleic Acid (rRNA). These bins are selected by looking at the variable region of the 16S rRNA gene which is a highly conserved gene in bacteria. Using the Quantitative Insights into Microbial Ecology (QIIME) 1.9.1 toolkit, barcoded samples were de-multiplexed. Open-reference OTUs picking using the May 2013 Greengenes release was performed. The sequences were clustered at 97 % identity with the Greengenes database (Caporaso, Lauber et al. 2012).

Reads that did not match a reference sequence were then clustered de novo at 97 % identity. Representative sequences were aligned using PyNAST (Caporaso, Bittinger et al. 2010), and those that failed to align were discarded. These representative sequences were used to assign taxonomy to each OTU cluster using the RDP classifier (Wang, Garrity et al. 2007). A phylogenetic tree was built using FastTree 2.0, which was then used to calculate UniFrac distances (Barberán, Bates et al. 2014).

The samples were first analyzed using the phyloseq, vegan and deseq2 packages in RStudio. Alpha diversity was measured using Shannon index, Inverse Simpson index and the observed number of OTUs. From the Shannon and Simpson indices, an alpha diversity plot was generated to visualize the data. Beta-diversity clustering was analyzed using a permutational ANOVA (ADONIS) for categorical, using weighted unifrac distance matrix. Analysis of variance (ANOVA) was performed to assess significant differences in relative abundance of OTUs on different surface types.

The counts of the bacterial genera were combined for each sampling day within the sampler type. This was done for the four biosamplers each day and the two button samplers each day. The top 13 bacterial genera were then plotted by sampler type and date to better understand the variance across each day.

RESULTS

Figure 3.1 provides a visual representation of the similarities/differences within and between the button and biosampler area air samples taken inside the fresh cow pen. The button sampler data (represented in blue on the plot) have more bacterial community similarities than the biosampler data based on the tighter grouping of the data. The PCoA was used to determine how closely the sample sets from the button samplers and biosamplers were related based on similarities and differences in the OTUs.. . As shown in Figure 3.1, it was determined that the microbial communities between the two samplers were significantly different ($p < 0.01$) and that moderate correlation existed between the two samplers ($R^2 = 0.380$). However, the eigenvalues on the axes were relatively low indicating a high variance within the data sets. There was a large spread in the data from the biosamplers while the data from the button samplers were grouped more tightly. The data from the biosampler were anticipated to be grouped more tightly but separately from the button sampler.

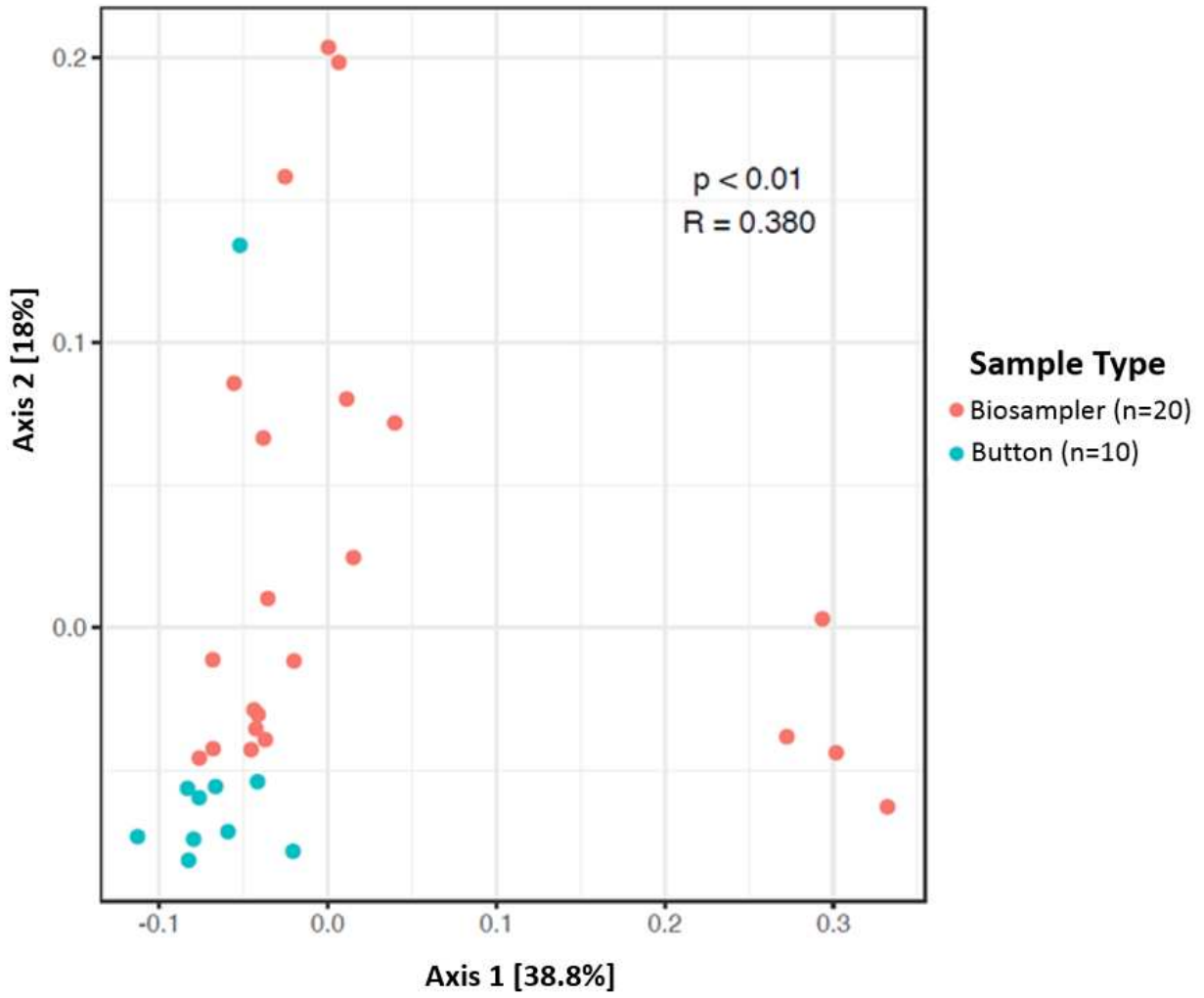

Figure 3.1. PCoA plot of SKC biosampler and SKC button sampler

The alpha diversity plot is a measure of evenness, richness, and dominance. As shown in Figure 3.2, the biosampler and the button sampler were relatively similar in these areas. Because both samplers collected air samples in the same environment, a similar core microbiome was expected for both samplers. Shannon measures richness and evenness; although relatively similar in richness and evenness, the button sampler had slightly higher results. The Simpson plot is a measure of evenness and dominance, and there was little difference observed between

the button and biosampler in dominance and evenness as expected. The large number of OTUs in the observed category suggests that the data were being driven by the rare bacteria; there were few bacteria that dominated the microbiome and an abundance of little represented bacteria.

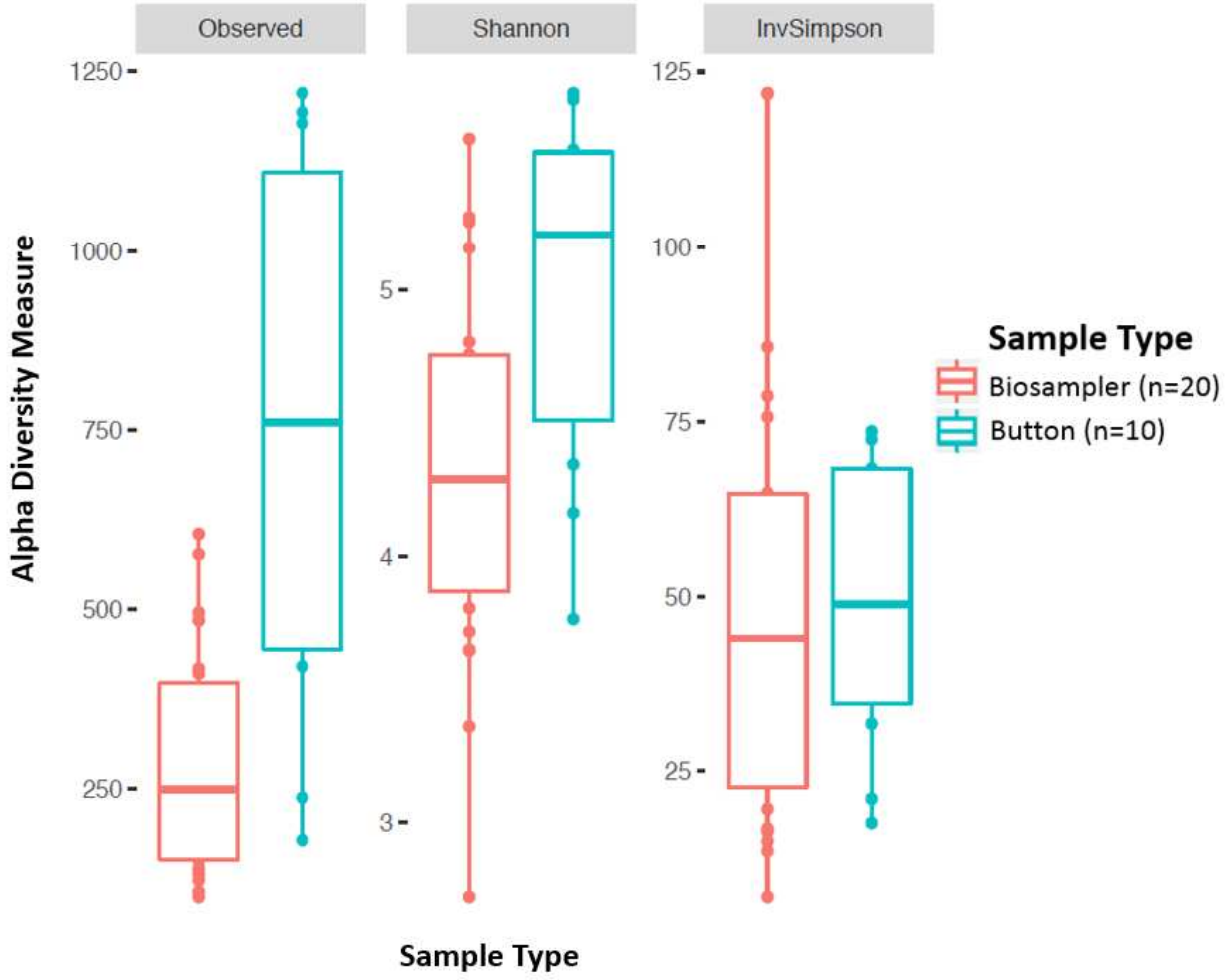

Figure 3.2 Alpha diversity plot comparison of the button and biosampler

As shown in Figure 3.3, the relative bacterial abundance was plotted against sampling date and sampler type. This categorization, provided an interesting perspective on the breakdown of the different bacterial genera. Some of the bacterial genera included in the top 10 had some potentially dangerous species such as *Acinetobactor*, *Staphylococcus*, *Pseudomonas*,

Corynebacterium, Clostridium, and *Methylobacterium*. Figure 3.3 provides a breakdown of the top 13 bacterial genera which had nine Gram-positive bacterial genera and four are Gram-negative bacterial genera. Table 3.1 provides additional information about each genera including the Gram staining result and potential sources of the bacteria.

Overall, there was a higher relative abundance of bacteria from samples collected using the biosampler in comparison to the button sampler. The button sampler was expected to have a higher relative abundance of bacteria due to the longer sampling time which corresponds to a higher air volume than the biosampler (1900 L vs 750 L) which was anticipated to result in a higher overall abundance of bacteria collected. Bacteria typically range from 0.5-5µm in diameter, suggesting that both samplers would be a suitable method for collecting bacteria.

Samples were collected for five consecutive days to identify if a temporal pattern existed for the overall bacterial trends or if there was a specific pattern for one bacterial genera. The weather during the five-day sampling period was relatively consistent; the temperature did not fluctuate more than 8ºF and rainstorms occurred each afternoon so weather should not have impacted the differences in bacteria. Overall, there was no significant trend for the temporal differences for the samplers. The button sampler had a relatively higher abundance for Days 2 and 5. Both samplers had a slight increase in relative abundance on Day 2 for the *Alicyclobacillus* and *Methylobacterium*. The biosampler had a slightly higher relative abundance on Day 3 in *Acinetobacter*, *Alicyclobacillus*, and *Methylobacterium*. The button sampler had a small increase on Day 5 in relative abundance of *Methylobacterium* and *Pseudomonas*. However, the relative abundance overall did not change very drastically between each day and no trend was visually identified.

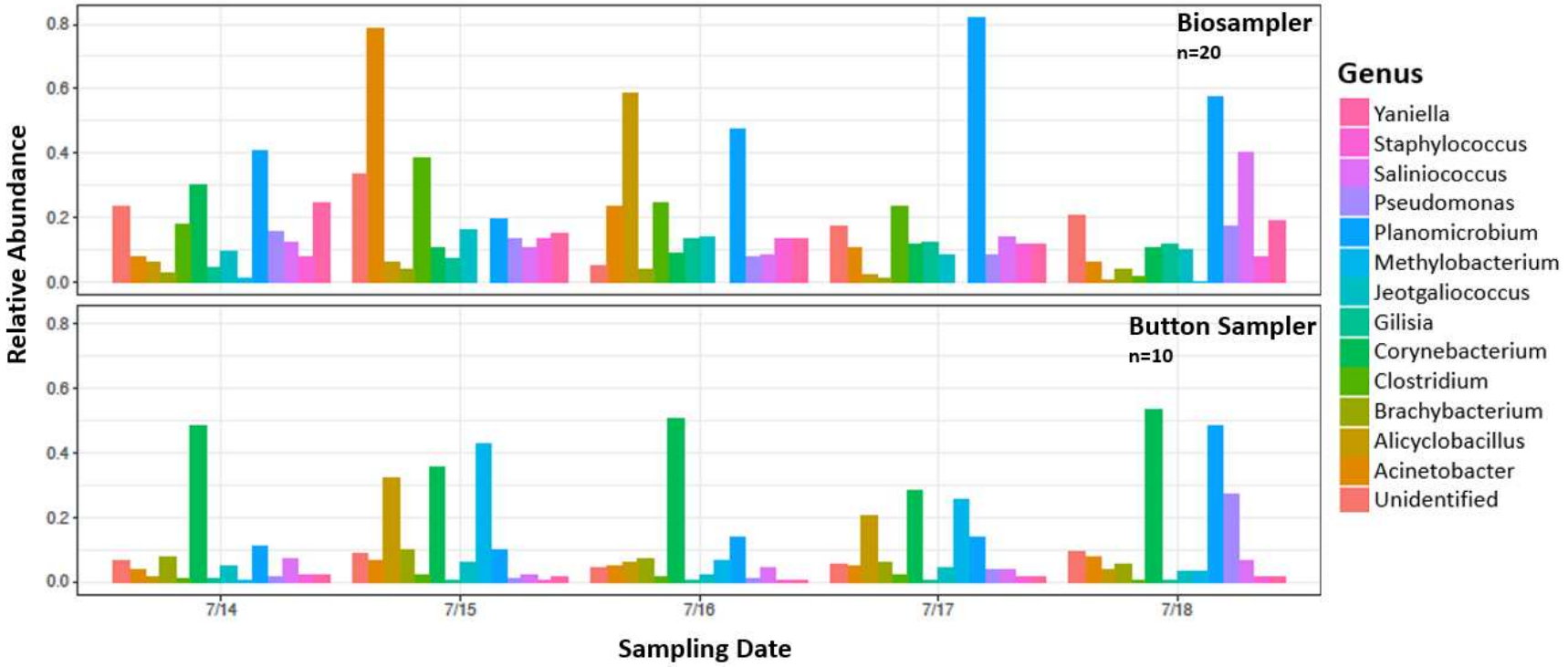

Figure 3.3 Relative abundance of bacterial genera for the biosampler and button sampler

Table 3.1 Breakdown of bacterial genera, source, and pathogenicity

Bacterial Genera	Gram Stain	Respiration	Potential Pathogenicity	Potential Source
Acinetobacter	Negative	Aerobic	Pathogenic Species	Soil
Gilisia	Negative	Aerobic	Non-Pathogenic	Water
Methylobacterium	Negative	Aerobic	Pathogenic Species	Ubiquitous
Pseudomonas	Negative	Aerobic	Pathogenic Species	Ubiquitous
Alicyclobacillus	Positive	Aerobic	Non-Pathogenic	Soil
Brachybacterium	Positive	Aerobic	Non-Pathogenic	Milk/Water
Clostridium	Positive	Anaerobic	Pathogenic Species	Soil
Corynebacterium	Positive	Aerobic/Anaerobic	Pathogenic Species	Soil/Water
Jeotgaliococcus	Positive	Facultative Anaerobic	Non-Pathogenic	Dust
Saliniococcus	Positive	Aerobic	Non-Pathogenic	Salt Water
Staphylococcus	Positive	Aerobic	Pathogenic Species	Ubiquitous
Yaniella	Positive	Aerobic	Non-Pathogenic	Soil

To further examine the difference in bacterial genera between the two samplers, the genera were examined more closely in Figure 3.4. The bacterial genera count for each sampler type were added together across the entire sampling period. The abundance of bacterial genera was then compared which showed a log fold change between the sampler types. In Figure 3.4, each data point represents a log-fold change in the different bacterial genera of the biosampler in comparison to the button sampler. For example, the biosampler had a relative abundance of *Methylobacterium* six times greater than that found in the button samplers. The different colors for the data points represent the five different bacterial phyla that are represented in this data set. The phylum with the greatest representation is *Proteobacteria*. The phylum *Proteobacteria* contains a wide range of Gram-negative bacteria that commonly reside in the soil and fecal matter such as *Salmonella*, *Vibrio*, and *Eschericia*. Although these bacteria reside in the phylum,

they were not found in the most abundant listing of bacteria. The relative abundance of bacteria in the biosampler ranged from 2-6 times greater than the relative abundance of bacteria in the button samplers. Not all bacterial genera identified in the top 13 bacteria in Figure 3.3 are represented in Figure 3.4 because the biosampler did not have a higher relative abundance for each bacterial genus represented.

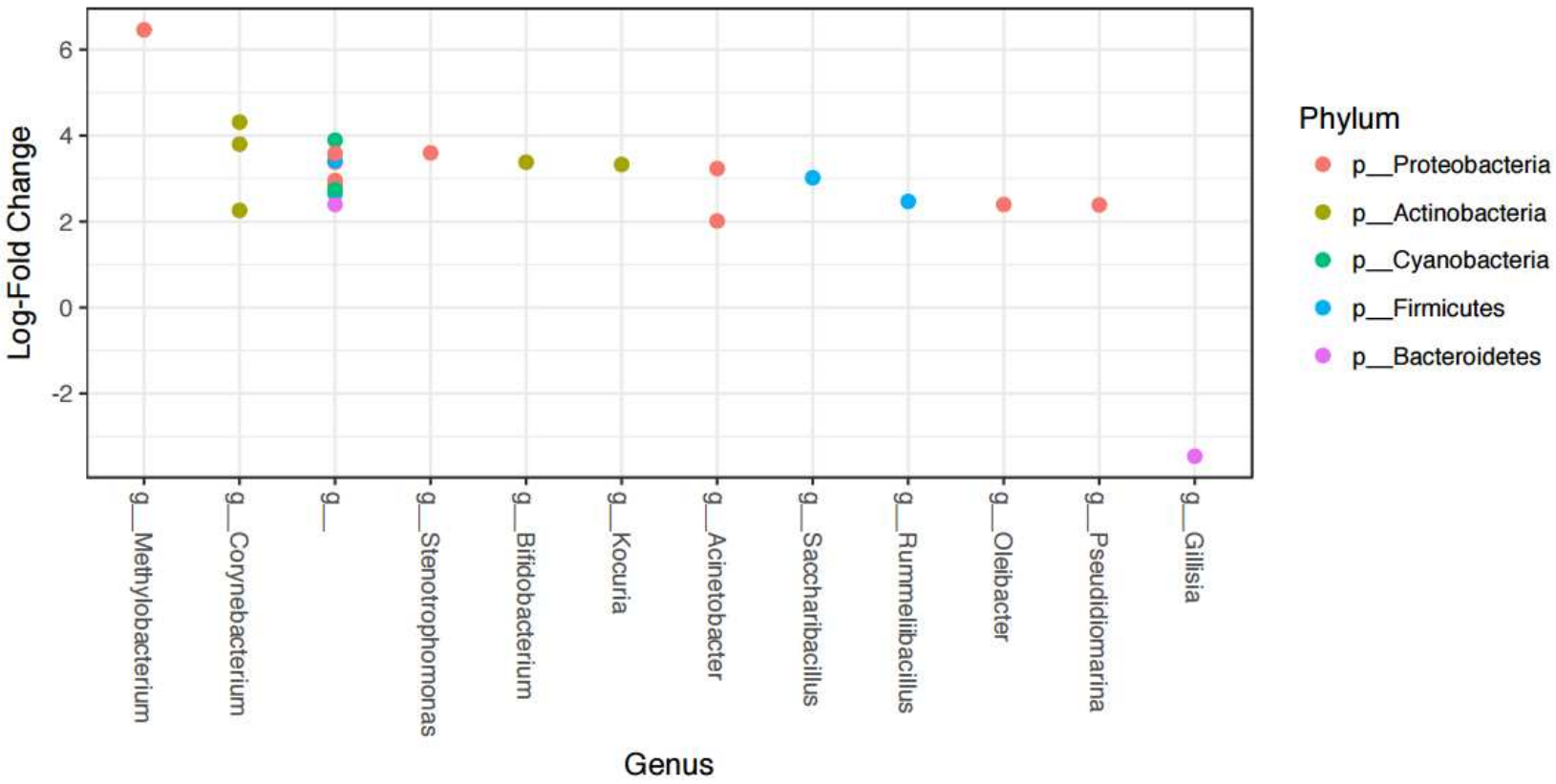

Figure 3.4 Bacterial genera with higher abundances in the biosampler vs the button sampler

DISCUSSION

Based on the results from Figure 3.1, there was little correlation between the OTUs in the two different samplers. The two samplers were anticipated to have different abundances, but were expected to have similar microbial communities since they were co-located inside a fresh cow pen. Ultimately, the samplers should have collected the same samples but at different flowrates and volumes. The larger opening in the biosampler could result in some conglomerated particles entering the biosampler that would not get past the screen on the button sampler. The changes in the environment throughout the day could have also resulted in slight differences in the aerosolized bacteria. Since the biosampler only collected samples for a short time period in the morning, it may not have captured the same air contaminants that the button sampler did. There were monsoons every afternoon during this week of sampling; before and during the monsoons there was an increase in wind and precipitation which may have resulted in different bacteria being aerosolized than the calmer morning conditions. It was not feasible during this pilot study to collect biosampler samples throughout the entire day, but if possible, future studies should operate biosamplers throughout the day and combine the samples to get a more accurate comparison.

The biosamplers had a larger variance than the button samplers which may be explained by the shorter sampling time or may be a result of the study design. The nature of repeated samples could be explaining the differences seen between the two samplers. The two button samplers were ran at the same time resulting in an artificially lower variance due to the lack of independence between the samples. Additional variability is introduced between the biosamplers because the samples are being run at different times of the day. Because the biosampler only sampled for a single hour, it likely captured different activities in the barn than the eight-hour

button samplers. During some of the biosampler collection time, other samples were being collected from the cattle in the same area. The additional sample collection could result in more dust and bacteria being disturbed during the first sampling hour and not the second resulting in differing concentrations. Future studies should limit sample collection time to either a known high or low cattle movement time to get a more uniform comparison within the biosampler data. The button sampler was run at the full 8-hour period to get a better representation of what was anticipated during a normal work shift.

Figure 3.2 was generated to better understand the richness, evenness, and the dominance in the microbial community in the dairy microbiome. The Shannon and Inverse Simpson plots showed few differences between the two samplers, suggesting that the samplers were pulling from the same core microbiome. The lack of difference between the richness, evenness, and dominance of the two samplers suggests that the differences in Figure 3.1 are likely due to the large variance of the biosampler. The alpha diversity plot also had a large observance of OTUs indicating the microbiome in the air had many rare bacteria and few dominant bacteria. Given the variety of sources at the dairy such as humans, cows, manure, soil, water, manure slurry, bedding, feed, etc. it is not unexpected that there was a wide variety of bacteria.

The most prevalent bacteria were from sources that were expected such as soil, water, and some that were ubiquitous (Table 3.1). Finding these bacterial genera in the environment does not signify that infection will occur or even that the pathogenic species are present in the environment. A deeper data analysis is required to identify the species level information to have certainty about the pathogenicity of the bacteria. Most of these bacterial genera are typical soil microbes; a possible explanation for the slight increase in these three bacteria is an increase in wind during specific times that could result in more soil being airborne in the direction of sample

collection. The knowledge that soil is the source for a large portion of the airborne bacteria can help identify methods of intervention such as dust suppression techniques. Most of the genera identified are aerobic (require oxygen) which could have been captured in a cultured sample (if the other culture conditions were correct). However, a few genera such as *Jeotgaliococcus*, *Clostridium*, and *Corynebacterium* are or can be anaerobic (require oxygen free environment) and would not have been captured in a typical culture further demonstrating the utility of the NGS.

Many of the genera found in the air samples have no pathogenic species and would therefore not result in infection. However, even though the bacteria may not cause infection the subsequent constituents such as endotoxins, peptidoglycans, and fungal mycotoxins can continue to result in reduced pulmonary function by causing allergic reactions and impacting the anatomy of the lungs.

Of the top 13 bacterial genera displayed in Figure 3.3, nine are Gram-positive while four are Gram-negative. Endotoxins (Gram-negative bacterial constituents) have been extensively studied in agriculture overall and specifically in the dairy industry. High endotoxin exposure has been linked to respiratory diseases and cross-shift pulmonary function decline in agricultural workers. Even though endotoxins can account for some of the respiratory disease, it does not explain the occurrence of all respiratory disease. Gram-positive bacteria and fungi are thought to also have an impact on the pulmonary function of workers (Douwes 2003). Unlike endotoxins, Gram-positive bacteria do not have a rapid diagnostic assay that can be used to measure the bacterial constituents and are therefore less understood than the Gram-negative bacteria in terms of occupational exposure.

There was no trend over the five-day sampling period observed within either of the samplers. There was no change in sampling conditions (e.g. weather, environment, activities) between the days therefore weather conditions were not anticipated to play a factor in the data.

The biosampler collected an overall higher abundance of bacteria. The biosampler was designed for bacterial collection; it has many features such as liquid collection media to prevent desiccation and a swirling motion for minimal bacterial damage that make it the perfect candidate for culturable samples. In contrast, the button sampler desiccates the bacteria while it sits on the filter especially over the long sample period (8 hours) often resulting in cell death. Although the biosampler is designed to better collect culturable bacteria, the bacteria analyzed using sequencing techniques are measured regardless of bacterial viability, so the higher air collection volume should have a bigger impact on the bacterial abundance. One explanation for this is the larger opening on the biosampler that could have collected larger particles or conglomerated particles such as dirt with bacteria attached which could get into the sampler. The biosamplers has a collection efficiency of 100% for particles larger than 1.0 µm but drops down to around 80% for particles less than 0.5 µm. The button sampler has the highest collection efficiency for particles less than 100 µm and is equipped with a 5.0 µm PVC filter (Inc. 2020).

Although the biosampler seems better suited for collecting bacterial samples, if the samplers are being used for personal air samples, the biosampler poses some logistical issues. The biosampler requires a large pump that can pull 12.5 L/min which is extremely heavy and cannot be worn by a worker. Additionally, the liquid collection medium makes it difficult for a worker to complete his/her tasks throughout the day because bending over can result in the liquid being spilled. The glass vial is also very fragile and if the worker drops the sampler, runs into a

wall, or is kicked by a cow is likely to break the sampler. The button sampler is designed for personal air sample collection, is small, relatively indestructible, and can be used with a pump that easily clips to a belt.

CONCLUSIONS

The PCoA indicated that the biosampler and button sampler had significant differences in the microbial community OTUs which was not anticipated based on the co-location of the samples. The biosamplers had a high variance between the individual samples while the button samples were grouped together which could be a result of activity in the barn during the biosampler collection period. However, both samplers represented a similar core microbiome (based on the Shannon and Inverse Simpson plots) which indicated that differences in OTUs were likely due to the variance in the samples. The large OTU count on the observance of the alpha diversity plot suggested the microbiome is being driven by a large number of rare bacteria as opposed to a few dominant bacteria. The wide variety of bacterial sources at the dairy suggest that bacterial diversity is expected in the samples. Gram-positive bacteria play a substantial role in this community and represent approximately 70% of the bacteria identified in the top 13 bacterial genera represented in both samplers. There were some genera that had potentially pathogenic species such as *Clostridium*, *Acinetobacter*, and *Staphylococcus* but conclusions could not be made on the pathogenicity without species level information. The bacterial sources were not surprising, as most of the bacteria came from soil, water, or were ubiquitous. There was no significant difference between the different sampling days. Overall, the biosampler had a higher relative abundance than the button sampler despite the shorter sampling time and smaller air volume. Based on the results from this pilot study and the larger abundance of bacteria, the

biosampler is better suited for both viable and non-viable bacterial collection but poses many logistical problems and cannot be used for personal sampling.

LIMITATIONS

One of the primary limitations of this study was the small sample size. With 20 samples representing the biosampler and 10 samples representing the button sampler, a total of 30 samples resulted in limited power, data analysis, and ability to make conclusions. A larger sample size may have provided more insight into the dominant bacteria in the dairy microbiome. Although the single sampling week provided an interesting perspective on the microbiome for one week, it only provides a relatively small snapshot of the dairy microbiome. Dust and endotoxin concentrations have previously been demonstrated to fluctuate based on the season; the same is anticipated for bacterial collection. A larger sample size that spans multiple weeks between seasons may provide greater understanding of the dairy microbiome. Differences in length of sampling time made it difficult to perform a direct comparison because the concentration of air contaminants can change throughout the day. Differences in wind direction, speed, humidity, precipitation, and activity in the barn can all change the amount of dust and bacteria in the air.

NGS does not provide information regarding the viability of the bacteria making it difficult to fully understand the health implications and potential infections as a result of exposure to the bacteria. Area air samples, although somewhat indicative of personal exposure, do not provide a true representation of personal exposure making it difficult to draw conclusions of personal exposure.

CHAPTER 4: COMPARISON OF BACTERIAL COMMUNITIES BETWEEN PERSONAL AIR SAMPLES AND ENVIRONMENTAL SAMPLES AT ONE COLORADO DAIRY

SUMMARY

Bacteria are ubiquitous and are an essential part of the earth's microbiome and the life of all living organisms. However, large concentrations of bacteria can have a negative impact on human health, particularly respiratory disease. The dairy industry is one example where bacterial concentrations are higher than average which has the potential to lead to reduced pulmonary function and respiratory disease such as allergic asthma, chronic bronchitis, and chronic obstructive pulmonary disease. Although bacterial markers, such as Gram-negative endotoxins, have been studied and have been demonstrated as an explanation for respiratory disease in dairy workers, it does not explain all of the respiratory disease. Gram-positive bacteria are thought to play a major role in respiratory disease, but have not been studied as extensively due to limitations in sample analysis. Personal and area air samples, soil, and hand swabs were collected at one Northern Colorado dairy to characterize the bacteria analyzed by Next Generation Sequencing (NGS). Area air samples that were upwind and downwind of the fresh cow pen had the highest abundance of bacteria with the majority of the bacteria identified in the top 20 as Gram-positive bacteria. Bacteria sequenced from the personal and fresh cow pen area air samples were highly correlated with the bacteria sequenced from the hand swabs. Bacterial genera in the top 20 bacteria that have potentially dangerous species included *Acinetobacter*, *Methylobacterium*, *Psychrobacter*, *Clostridium*, *Oerskovia*, and *Staphylococcus*. True understanding of the bacterial impact on health cannot be achieved without further analysis. Future studies should focus on Gram-positive bacteria since the researchers of the current study found that the majority of the bacteria in this dairy environment were Gram-positive.

INTRODUCTION

There are approximately 150,000 dairy farmers across the United States that supply dairy products globally. As technology has developed, U.S. dairy operations have adapted and changed, altering the way dairies are owned and operated. Milk production has changed from small herd operations to large herd operations (typically larger than 1000 head of cattle). The increase in herd size results in more milk production which requires more workers, longer shifts, more frequent milking, and 24 hour a day operations (Douphrate, Hagevoort et al. 2013). A larger workforce is needed to adapt to the larger herd size which has been met primarily by immigrant (e.g., Latino) workers with no previous agricultural experience (Schenker and Gunderson 2013).

Reduced pulmonary function and respiratory diseases such as COPD, chronic bronchitis, allergic asthma, hypersensitivity pneumonitis, and organic dust toxicity syndrome are more prevalent in agricultural workers (Reynolds, Nonnenmann et al. 2013). These illnesses are a result of chronic exposure to high concentrations of bioaerosols (airborne biological matter including bacteria, fungi, manure, feed, pollen, and corresponding constituents) (Douwes 2003, Walser, Gerstner et al. 2015). Endotoxins, peptidoglycans, and non-viable bacteria act as inflammagens that cause allergic reactions that transition to chronic bronchitis, hypersensitivity pneumonitis, and allergic asthma with chronic exposure (Hawley, Schaeffer et al. 2015).

Respiratory infections, caused by inhalation of viable bacteria can also have a major impact on the health of dairy workers. Culture-based methods have historically been used to assess the concentrations of airborne bacteria, but rely on the ability of the bacteria to grow in the pre-determined conditions. Although culture based methods can demonstrate the presence of bacteria in an environment, many of the bacteria do not survive sample collection and many that

do survive sample collection do not grow on the selected media or temperature. Eduard (1997) predicted that only 0.1% of the bacterial cells are culturable depending on the type of bacteria and culture conditions.

Rapid diagnostic assays to determine the concentration of bacterial endotoxins (a marker for Gram-negative bacteria) have been studied extensively in dairy environments, but endotoxins alone do not explain the prevalence of respiratory disease. Gram-positive bacteria are thought to play a large role in the bacterial microbiome in dairy environments as a causative factor in respiratory disease. Next Generation Sequencing (NGS) can be used to characterize the entire microbiome to better understand the bacterial microbiome and the exposure of dairy workers. NGS uses a reliable sequencing method to measure the abundance of bacteria and identify the genera present in the dairy microbiome.

Worker exposure is more than just air contamination, it extends to the sources of bacteria in their environment. NGS also allows a direct comparison of different bacterial sources to help better understand where the contamination is and how best to control it to protect worker health. The researchers of the current study examined personal and area air samples, soil, and hand swabs to characterize total worker exposure, explain sources, and identify methods to control worker exposure.

METHODS

Sample Collection

One large herd dairy (greater than 1000 lactating cows) in Northern Colorado was recruited for this pilot project. Samples were collected during five consecutive days in July 2014. Multiple sample types (personal air, area air, soil, and hand swabs) were collected to understand the relationship between the human exposure microbiome and the environmental

microbiome at the dairy operation. Dairy workers that worked in and around the fresh cow pen were recruited to allow for comparison of the area and personal samples. Hand swabs were collected from workers at the dairy as well as veterinarians that were collecting fecal samples during sample collection. This research was reviewed and approved by Colorado State University's Institutional Review Board.

Table 4.1 Number of Samples Collected by Type

Air Samples	
Button Personal Samples	16
Button Downwind	19
Button Upwind	20
Button Pen	10
Biosampler Pen	20
Total Personal	16
Total Area	69
Button Blanks	10
Biosampler Blanks	5
Hand Swabs	
Veterinarians	9
Dairy Workers	18
Total	27
Soil Samples	
Total	15

Sixty All samples were collected each day over the five-day sampling period. SKC button samplers (SKC Inc. Eighty Four, PA) were used to collect personal air samples from two dairy workers and a veterinarian.. Area air samples at three different locations (upwind, downwind, and inside the pen) were collected with the SKC button sampler. All SKC button samplers ran for an eight-hour sampling period with 0.5 µm pore size polyvinyl chloride (PVC) filters. Each sampler was pre- and post-calibrated to a flowrate of 4 L/min ±5%. The SKC Biosamplers (SKC Inc. Eighty Four, PA) were used to collect additional area air samples inside the fresh cow pen. The biosamplers sampled two subsequent one-hour sampling periods per day

filled with 20 mL of resuscitation buffer (a sterile solution made of polyethylene glycol, buffered peptone water, and tween) (Andersson, Laukkanen et al. 1995) and calibrated to a flowrate of 12.5 L/min ±5%. Hand swabs were collected daily at the beginning and end of each work shift for the dairy workers and after sample collection from the veterinarians using gauze pads wet just before wiping with resuscitation buffer and then placed in a sterile whirlpak bag. Soil samples were collected daily at three locations (upwind, downwind, and inside fresh cow pen) by scooping soil into a sterile 50 mL falcon tube in the same pre-decided location . The samples were then stored in a -80ºC freezer before being sent to Argonne National Laboratory for sequencing analysis.

Sample Analysis

The MO BIO Powersoil DNA Isolation kit was used to extract DNA from all samples types. Nine extra bases were added in the adapter region of the forward amplification primer to adapt the Earth Microbiome Project barcoded primer set for MiSeq to amplify the DNA (Caporaso, Lauber et al. 2012, Gilbert, Jansson et al. 2014, Project 2017). Region-specific primers that contained a twelve-base barcode sequence, the Illumina flowcell adapter sequences and the forward amplification primers, amplified the V4 region of the 16S rRNA gene (515F-806R) (Apprill, McNally et al. 2015). 10 µL of 5 Prime HotMasterMix (1×), 1 µL of Reverse Primer (5 µM concentration, 200 pM final), 1 µL Golay Barcode Tagged Forward Primer (5 µM concentration, 200 pM final) 12 µL of MO BIO PCR Water (Certified DNA-free), and 1 µL of genomic DNA was contained in each 25 µL PCR reaction. The PCR conditions are as follows: 94°C for 3 min to denature the DNA, with 35 cycles at 94°C for 45 seconds, 50°C for 60 seconds and 72°C for 90 seconds, with a final extension of 10 minutes at 72°C to ensure complete amplification. Quantification of the amplicons was completed using PicoGreen (Invitrogen) and

a plate reader. To represent the amplicons equally, different volumes of each product were pooled into a single tube. The UltraClean® PCR Clean-Up Kit (MO BIO) was used to clean the pool which was then quantified using Qubit (Invitrogen). Molarity of the pool was diluted to a final concentration of 2 pM with a 30% PhiX spike for loading on the Illumina MiSeq sequencer.

Statistical Analysis

Quantitative Insights into Microbial Ecology (QIIME) 1.9.1 toolkit was used to link the sequencing results with the sample identifier Open-reference OTU picking using the May 2013 Greengenes release was performed. This technique assigns sequences to the OTUs by clustering the sequences that have have similarities. The Greengenes database was used to cluster sequences at 97 % identity and sequences unmatched to a reference were clustered de novo at 97% identity (Caporaso, Lauber et al. 2012). PyNAST was used to align representative sequences. This system used a database to match the collected sample sequences to those pre-aligned in the database. Sequences that could not align were discarded (Caporaso, Bittinger et al. 2010). The RDP classifier used the representative sequences to assign taxonomy to each OTU cluster by comparing those sequences to the fasta database of pre-assigned reference sequences (Wang, Garrity et al. 2007). UniFrac distances were calculated using a phylogenetic tree built with FastTree 2.0 (Barberán, Bates et al. 2014).

The first step was an analysis with the Bioconductor package phyloseq in R Studio. Shannon and Inverse indices were used to measure the alpha diversity of the samples. The Shannon and Simpson indices provide a way to measure how many different bacteria one is likely to find in a given sample. The Shannon index provides a measure of richness (the number present) and the evenness (how relatively abundant the bacteria are). The Simpson index

provides a measure of evenness and dominance (dominance of one or a few species). A weighted unifrac distance matrix was used to analyze the Beta-diversity clustering. This analysis uses the species abundance information to determine the dissimilarities between the samples. Sourcetracker was computed using QIIME 1.9.1 using a Bayesian model. The SourceTracker was used to account for potential sample bias from soil samples by setting the soil samples as source samples, and samples belonging to either the workers or veterinarians were collapsed and treated as the two possible sources to the location sink community. Models were run following QIIME tutorial guidelines (Knights, Kuczynski et al. 2011).

RESULTS

The top 20 bacteria were identified and plotted to identify the relative abundance for each of the sample types. Separated by sample type, air samples had the highest relative abundance (button followed by biosampler). Further categorized by location within sample type, the upwind air samples had the highest relative abundance followed by the downwind air samples and the samples inside the pen . The soil samples inside the pen had the highest relative abundance. Of the 20 identified genera, 15 (75%) were Gram-positive while only 5 of the 20 (25%) were Gram-negative. Approximately 45% (9) of the bacterial genera identified in the top 20 bacteria have potential pathogenic species.

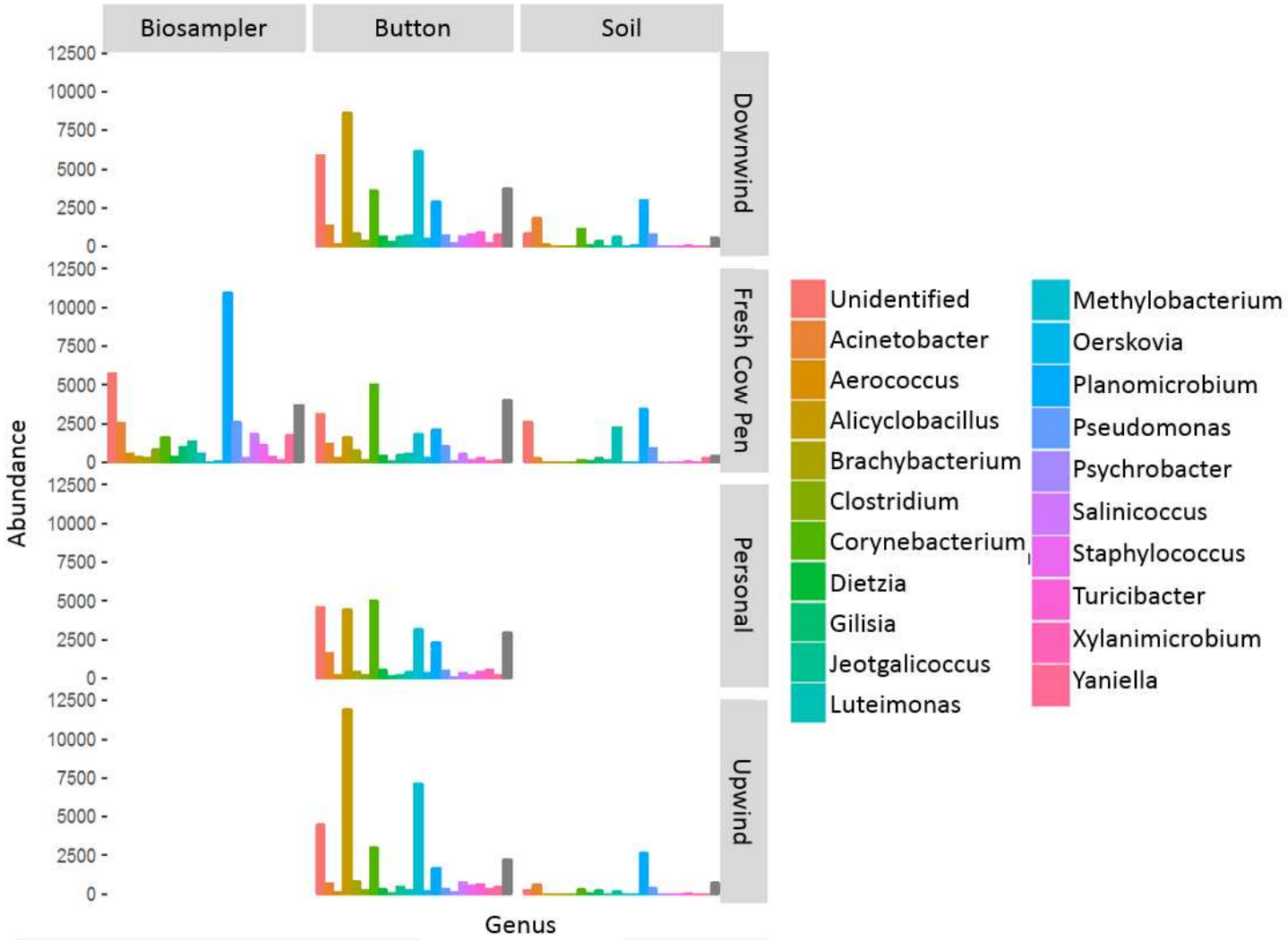

Figure 4.1 Relative abundance of top 50 bacteria for all sample types (n=137)

Overall, 75% of the bacteria were Gram-positive while 25% were Gram-negative, further substantiating the importance of further research and understanding of the health effects of Gram-positive bacteria (Table 4.1).

Table 4.2 Gram stain, respiration and pathogenicity of top bacterial genera

Bacterial Genus	Stain	Respiration	Potential Pathogenicity	Potential Source
Acinetobacter	Negative	Aerobic	Pathogenic Species	Soil
Gilisia	Negative	Aerobic	Non-Pathogenic	Water
Luteimonas	Negative	Aerobic	Non-Pathogenic	Animal Flora
Methylobacterium	Negative	Aerobic	Pathogenic Species	Ubiquitous
Pseudomonas	Negative	Aerobic	Pathogenic Species	Ubiquitous
Psychrobacter	Negative	Aerobic	Pathogenic Species	Soil
Aerococcus	Positive	Facultative Anaerobic	Pathogenic Species	Soil
Alicyclobacillus	Positive	Aerobic	Non-Pathogenic	Soil
Brachybacterium	Positive	Aerobic	Non-Pathogenic	Milk/Water
Clostridium	Positive	Anaerobic	Pathogenic Species	Soil
Corynebacterium	Positive	Aerobic/Anaerobic	Pathogenic Species	Soil/Water
Dietzia	Positive	Aerobic	Non-Pathogenic	Animal Flora
Jeotgalicoccus	Positive	Facultative Anaerobic	Non-Pathogenic	Dust
Oerskovia	Positive	Aerobic	Pathogenic Species	Soil/Skin
Planomicrobium	Positive	Aerobic	Non-Pathogenic	Soil
Salinococcus	Positive	Aerobic	Non-Pathogenic	Water
Staphylococcus	Positive	Aerobic	Pathogenic Species	Ubiquitous
Turicibacter	Positive	Anaerobic	Non-Pathogenic	Gastrointestinal
Xylanimicrobium	Positive	Facultative Anaerobic	Non-Pathogenic	Soil
Yaniella	Positive	Aerobic	Non-Pathogenic	Soil

The PCoA plot identifies the similarities and dissimilarities between the different sample types (hand swab, button, biosampler, and soil) to identify how much the microbial community

is driven by these differences. As illustrated in the PCoA in Figure 4.2, the four sample types were significantly different ($p<0.001$).

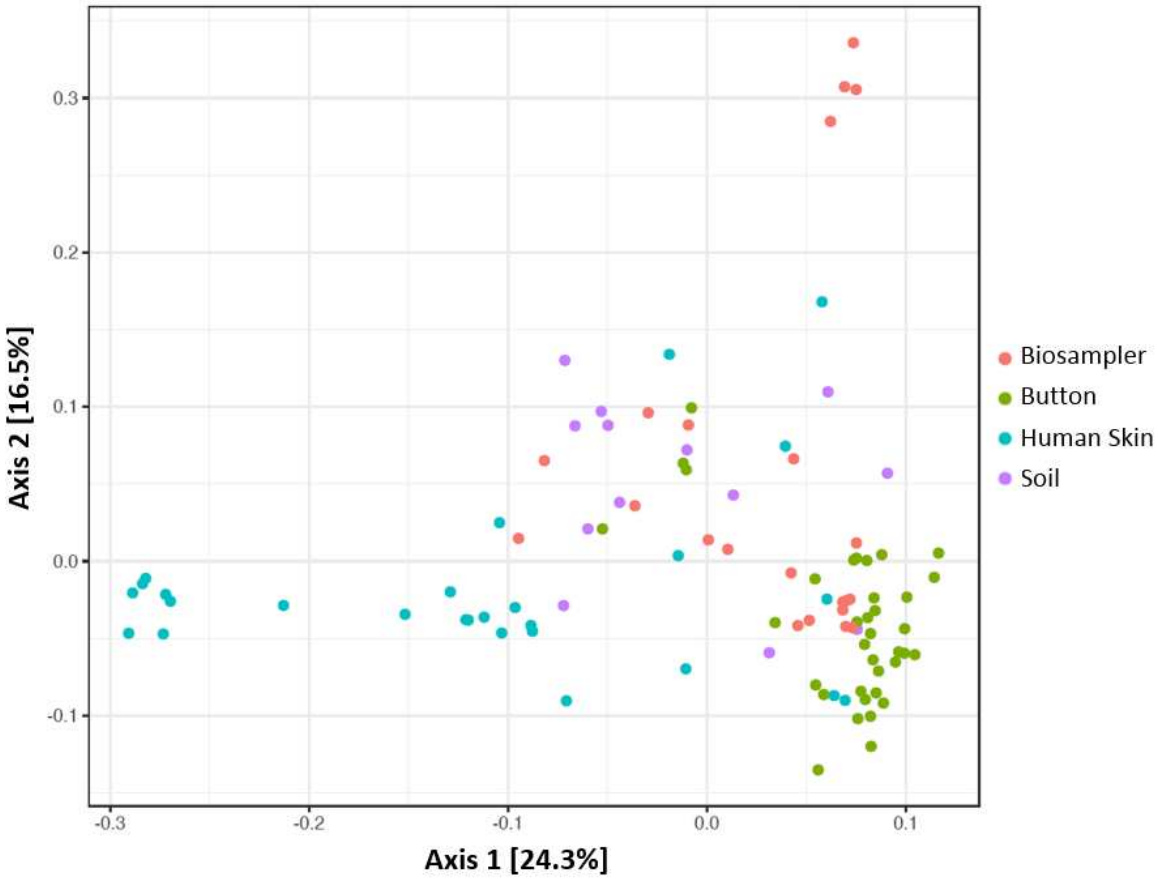

Figure 4.2. PCoA plot of air, human, and soil sample types

Samples were further categorized by individual samples in Figure 4.3. Most of the variance in the biosampler data came from a single biosampler (biosampler 2). The variance from the hand swabs came from the worker samples as opposed to the veterinarian samples.

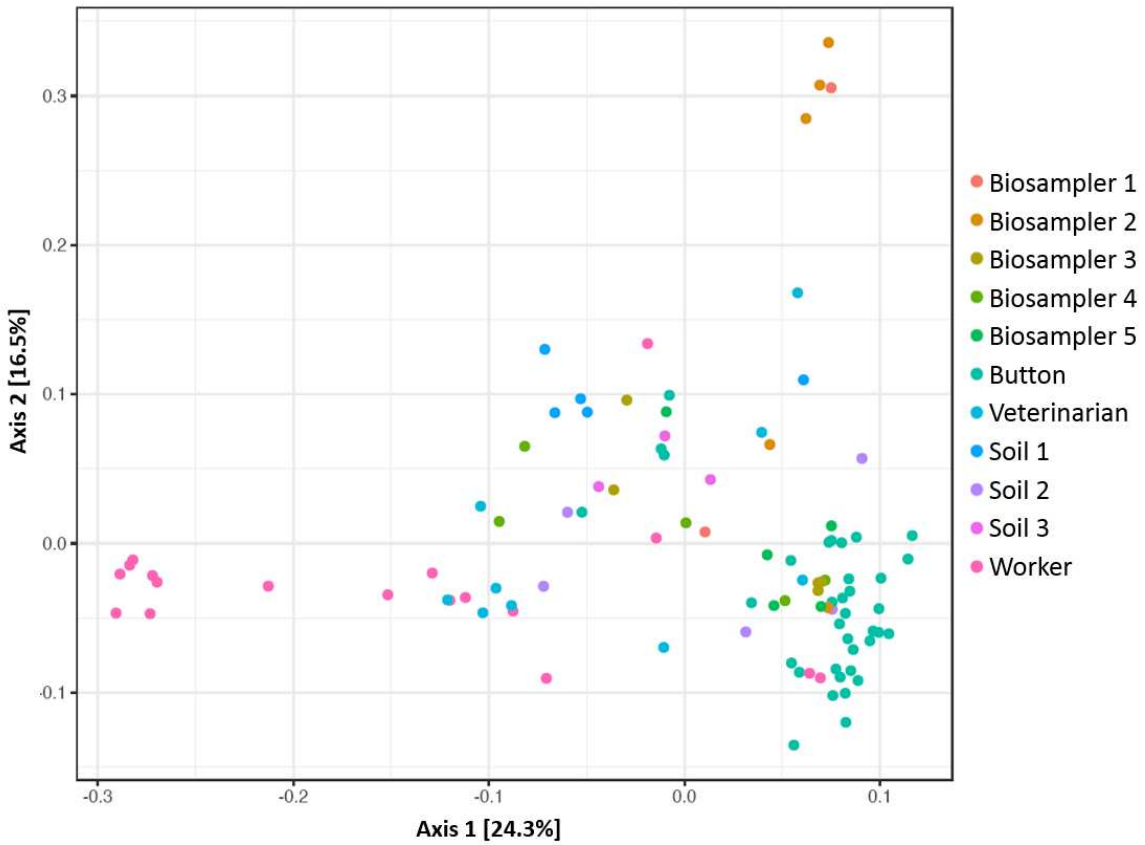

Figure 4.3 PCoA plot broken down by individual sample type

The alpha diversity plot which identifies evenness, richness, and dominance in Figure 4.4 suggests that the biosampler, button, and soil samples were relatively similar. The Shannon index explains richness and evenness, and all of the sample types overlapped suggesting similar richness. The Simpson index explains evenness and dominance which were all very close. These indices indicate that all of the sample types were driven by the same core microbiome which was anticipated because all samples were taken at one dairy in very close proximity to one another.

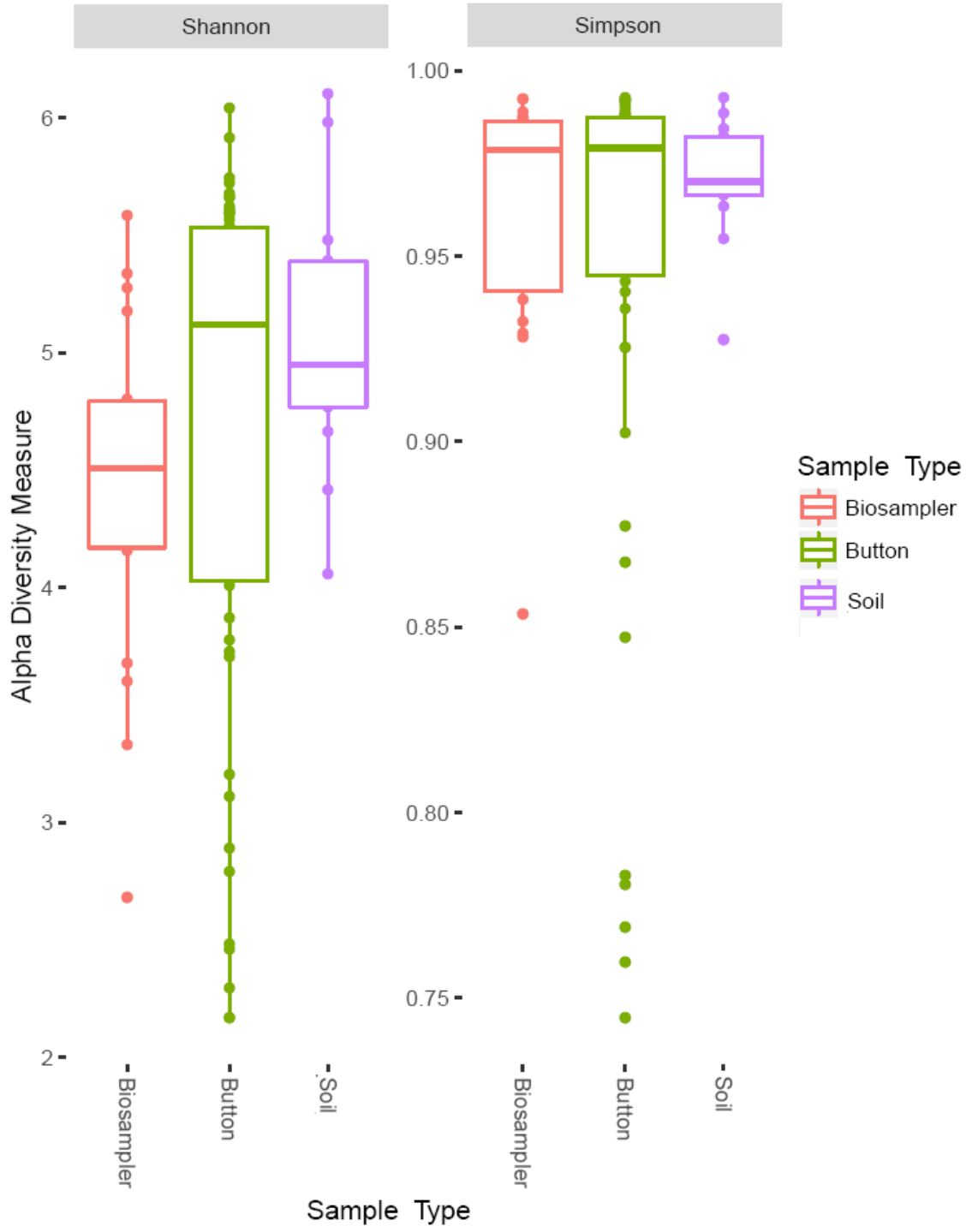

Figure 4.4. Alpha diversity plot by sample type

To better understand the human hand swabs and the source of human exposure, a SourceTracker with a mean frequency was used to predict the source of the hand swabs. The

SourceTracker estimates the average contribution of the individual sources to a designated sample using Bayesian modeling (Henry, Schang et al. 2016). Air samples inside the pen were the best predictors for the worker hand swabs. The soil samples also had a relatively high prediction frequency.

The veterinary samples correlated most closely to the samples inside the pen as anticipated because they were collected after the veterinarians took cow samples inside the pen. The veterinarians were only in this location for a couple of hours and did not have the same exposures outside of the pen as the workers and therefore did not correlate as closely.

Figure 4.5 SourceTracker prediction frequency for personal samples

DISCUSSION

The air samples had the highest relative abundance in the top genera of bacteria which was unexpected because air is typically considered to have less bacteria than soil. Within the air samples, the upwind had the highest relative abundance followed by the downwind samples. The upwind and downwind samples could have a higher relative abundance due to the airflow outside the fresh cow pen. Because the pen is partially enclosed, there is less wind that is aerosolizing dirt, fecal matter, and consequently bacteria that is being captured by the air samples. On the upwind side of the pen there was a road for vehicle and foot traffic that could have caused more dust and fecal matter to be stirred up. The upwind location was also close to the location where manure was flushed out of the pen daily with large amounts of water. The water ran directly in front of the samplers and could have aerosolized bacteria near the samplers resulting in higher abundances of bacteria that were not seen in the other samples.

The samples taken inside the fresh cow pen had the highest relative abundance between the three soil locations. This was anticipated because the soil sample was taken inside the pen where the cows laid, defecated, and urinated suggesting the sample would be substantially contaminated with bacteria. The most common bacterial genera found across all three soil locations was *Planomicrobium*, a Gram-positive, non-pathogenic soil microbe that was anticipated in the samples. *Jeotgaliococcus*, a Gram-positive, non-pathogenic bacterial genus that is commonly found in soil and skin cells was another predominant genus.

The personal samplers were anticipated to have a higher abundance of bacteria than the area air samples. Researchers anticipated that the personal samples would have the highest dust concentration due to the tasks done by workers that generate dust and consequently bacteria throughout the day. The majority of the personal samples had higher dust concentrations than

the area samples, therefore it was anticipated that the personal samples would also have a higher relative abundance of bacteria. However, because the area samples were closer to the ground, the samplers could have collected more bacteria than the samples in the workers' breathing zones. This was further substantiated by the fact that the majority of the bacteria were common soil microbes.

Overall, 75% of the bacteria were Gram-positive while 25% were Gram-negative, further substantiating the importance of further research and understanding of the health effects of Gram-positive bacteria (Table 4.1). Many of the genera identified are common bacteria found in soil which was expected due to the nature of the sampling environment. The dairy is a dusty environment where the dust often becomes aerosolized. Workers breathe in the dust along with the viable and non-viable bacteria associated with the dust that can ultimately result in reduced pulmonary function and respiratory disease.

Some of the bacterial genera have potential pathogenic species such as *Acinetobacter*, *Methylobacterium*, *Psychrobacter*, *Pseudomonas*, *Clostridium*, *Oerskovia*, and *Staphylococcus*. Although approximately 45% of the bacterial genera in the top 20 bacteria have potential pathogenic species, this does not guarantee that infection will occur in workers or that the pathogenic species are represented in this environment. The genus level provides useful information about the bacteria to help characterize the microbiome, but without more in-depth analyses that identify each bacterium at the species level, it is impossible to make conclusions on whether these bacteria could result in a direct bacterial infection. Based on previous studies, direct infection (from viable bacteria) is not the only health effect from exposure to bacteria. Non-viable bacteria contain inflammagens that result in allergic reactions that can lead to an

increased prevalence of respiratory disease (Thorn 1998, Rylander 2006, Spaan 2008, Poole, Dooley et al. 2010).

NGS still provided useful information that could not have been gathered using culture techniques. Although it is unknown how many of the bacteria were viable or non-viable, it can be assumed that most were non-viable. This is based on the knowledge that in a typical sample taken, it is estimated that only 0.1% can be cultured due to the specific conditions required for growth (Vartoukian, Palmer et al. 2010). Some of the bacteria were also anaerobic (cannot survive in an oxygen-rich environment) and therefore would not have grown in typical culture methods.

The similarities and differences between the different sample types (button, biosampler, human skin, and soil) are depicted in the PCoA plot in Figure 4.2. The four sample categories were found to be significantly different ($p<0.001$) which indicates that more than 50% of the differences in the microbial communities can be explained by the sample category. There was a large variance in the biosampler samples and human skin samples with samples stretching to the end of their corresponding axes. The large variances found in the biosampler data may have been due to the short sampling time and activities that took place during the one-hour sampling periods. Because the veterinarians were collecting samples simultaneously during some of the collection periods, but not all, the biosamplers may have been exposed to varying levels of activity inside the pen and therefore varying levels of bacterial aerosolization. The large spread in the human skin data may be attributable to the different microbiome found on the human skin which can change daily. The two sampled workers were also performing different tasks throughout the day and could therefore have different exposures. Both subjects were working in/around the fresh cow pen, but one worker spent most of his day working directly with the

cows while the other worker spent most of his time as a machine operator. The soil data had a moderate amount of variation within the samples. There was a relatively small amount of grouping within the samples which may be explained by the different sample locations. Based on observations during sample collection, the upwind and downwind soil sample locations had very similar soil compositions while the samples inside the pen contained fecal matter, urine, and more animal dander. The button sample data had very little variance between the samples suggesting they were sampling the same microbial community.

The sample types were further categorized based on the individual sample location or identifier. Three of the points of high variation in the biosampler data were from the second biosampler in the first duplicate of sample trials. It is possible the pump was not initially calibrated correctly due to a mechanical error, but unlikely since the pump was also used for "biosampler four" immediately after. The calibration tubing did not provide a perfect fit on each sampler and the glass in the samplers was imperfect which could have resulted in further deviations from calibration despite the calibrator reading the correct airflow.

The worker hand swabs and the veterinarian hand swabs were grouped separately from each other indicating that the two groups were likely receiving a different exposure. This would be expected since the subjects were completing different tasks. Prior to collecting the hand swab samples, the veterinarians were collecting fecal samples with exposure between 1-2 hours. The workers were completing various tasks across the dairy such as moving cows, moving manure, and feeding the cows. Soil sample one was taken inside the fresh cow pen and was grouped slightly away from the other two samples which would be expected due to the nature of the soil composition inside the pen.

Based on the alpha diversity plot in Figure 4.4, the microbial community from the air and soil samples was relatively similar. This could be explained by the fact that all samples were taken from the same dairy environment. However, more diversity would be expected in the soil samples than in the air samples.

The SourceTracker plot in Figure 4.5 provided a source match between one sample and a set of other samples to identify if the bacteria were contributing to the source and if the sample could be used as a good predictor. The best source for the hand swabs were the air samples collected inside the fresh cow pen. This was somewhat expected because each of the workers was chosen because they worked in and around the fresh cow pen as their main task throughout the day. Soil was a good source match to the worker hand swabs but not the veterinarian hand swabs. The workers spend their entire day at the dairy touching various surfaces where soil has been airborne and deposited on surfaces such as cows, pens, machinery, and other outside equipment. The exposures that the veterinarians received at this dairy represents a very small portion of their day. The entire time the veterinarians were at the dairy, they were collecting fecal samples with gloved handsand were not touching the surfaces where soil had deposited, therefore soil was not anticipated as a good predictor of hand samples from veterinarians. Personal air samples were expected to be the most significant predictor for the worker hand swabs because the both samples were collected from the same person and therefore the same source. However, what a worker touches with their hands can be extremely different from what they are breathing in.

CONCLUSIONS

The different sample types had similar relative abundances, but the area air samples located upwind and downwind of the fresh cow pen had the highest relative abundance. The

higher abundances could be a result of the increased vehicle/foot traffic in the area, the flush of the manure, or the increased wind that was present outside of the pen. Gram-positive bacteria were more prevalent (73%) in the top 20 bacterial genera of these samples implying that more investigation into the health effects of Gram-positive bacteria should be explored as well as rapid diagnostic assays that provide a quicker and more economical method for sample analysis. Some of the bacterial genera identified in the top 20 have species that are potentially pathogenic; without further investigation into the bacteria to the species level, it is impossible to know if the pathogenic species were present in this environment. However, even if no pathogenic species were present, the presence of the bacteria themselves in high concentrations could result in respiratory disease by acting as inflammagens. As demonstrated in the PCoA plot of the sample types, there was a significant difference between the sample types that can explain over 50% of the variance for the samples. Further, the air samples from the pen were found to be the best predictors for the worker hand swabs followed closely by the personal samples as illustrated in SourceTracker. This was not surprising; the workers were recruited because they worked in or around the pen for most of their tasks throughout the day. Since the air samples had the highest abundance of bacteria, the method to control worker exposure would be soil/dust suppression in the working environment. Logistically, soil/dust suppresion can have many problems particularly for workers that have varying tasks throughout the day. Workers should take time to pratice proper hygiene before eating and before returning home. There were a variety of different bacteria found in this environment which could result in worker illness particularly if ingested. Proper hygiene practices would help reduce the amount of bacteria the workers are potentially ingesting.

LIMITATIONS

The small sample size of 105 total samples that were categorized into many different sample types [60 air (10 personal, 30 button area, and 20 biosampler area), 15 soil, and 30 hand swabs (20 workers and 10 veterinarians)] made it difficult to draw conclusions. Within the air samples, there was a substantial variation between and within the different sample types making it challenging to identify trends and patterns within and between samples. With a larger sample size, the variation could be explained and any trends identified. Two workers are not representative of the entire staff at the dairy. Even within the workers selected, they each had different tasks throughout the day which often have very different exposures. Tasks, and consequently exposure, can change dramatically from day-to-day making it very difficult to understand true worker exposure especially with only 10 personal air samples. The seasonal variation can cause large fluctuations in concentrations of bacteria which were not captured in this small data set over a single week. The exposures captured in this study were only representative of one week in summer.

The personal air sampling data does provide some information, but it is difficult to relate the air sampling information to health effects with no health data. Collecting health data such as pulmonary function tests in conjunction with the personal air samples may have provided more insight into the true impact of the exposure data, but the small sample size restricts the information that could be gained.

NGS is a very useful tool that provides a large amount of data and further information that can be gleamed from the literature. The researchers of the current study were able to identify a variety of different bacterial genera, their sources, and potential pathogenicity. The lack of information on the species level makes it impossible to make determinations regarding

potential for infection and definite sources of the bacteria. Along with the species level information it would be helpful to know the bacterial viability to understand true infection potential. NGS does not provide information on whether the bacteria are viable or if the samples only consist of bacterial constituents. Knowing the viability of the bacteria could help in determining potential controls by identifying if bacteria need to be killed or if the non-viable bacteria are causing most of the respiratory health problems.

CHAPTER 5: EVALUATION OF TASK-BASED EXPOSURES TO AIRBORNE ENDOTOXINS AND B-GLUCANS AMONG COLORADO DAIRY WORKERS

SUMMARY

Agricultural bioaerosols have long been identified as causative agents of respiratory disease in dairy workers. Conditions such as chronic bronchitis, allergic asthma, and chronic obstructive pulmonary disease have been found in dairy workers where concentrations of dust and bacteria are high. Historically, culture work has been done to identify the concentrations of bacteria and fungi, but this collection method does not capture all of the airborne bacteria and fungi in agricultural settings. Rapid-diagnostic assays that capture bacteria or fungi and their constituents provide a better understanding of workers' true exposures. Personal air samples (n=110) for eight tasks at four Northern Colorado dairies were sampled across three seasons to identify the tasks with the highest exposures to gain a better understanding of the exposure and identify different control measures. Of the eight tasks, no single task was higher across the three different variables (dust, endotoxin, and β-glucan) and none of the tasks had statistically significant differences in concentration. Area air (n=297) samples were collected in three different locations (inside the milking parlor, upwind, and downwind).The parlor area samples had the highest airborne concentrations of dust, endotoxin, and β-glucan although only endotoxin concentration had a statistical significance. Based on these results, more work should be done to understand the differences between the tasks by following workers over a longer period. Intervention studies should focus on the parlor to help reduce worker exposure.

INTRODUCTION

The dairy industry in the United States supports more than 150,000 workers that contribute to the international dairy economy by supplying approximately 15% of the dairy products

(Douphrate, Hagevoort et al. 2013). Along with many production industries, the dairy industry has adapted to new technology over the past 50 years to increase production. This adaptation has resulted in an increase in herd size, workforce, and overall dairy operations. Immigrants have filled in the gaps in the workforce but often have no previous experience in agriculture, introducing ill-adapted immune systems for the work. With the increase in herd size, the concentrations of air contaminants such as bacteria and fungi (and their constituents), pollen, manure, and feed also increase. Previous researchers have indicated higher concentrations of dust and endotoxins in agricultural settings including dairies that exceeded the recommended occupational exposure limits (OELs) of 2.4 mg/m^3 and 90 EU/m^3 respectively (Donham 2000, Reynolds 2012, Basinas, Sigsgaard et al. 2014).

Bioaerosols as air contaminants and the corresponding health response have been studied for many years. As a result, many researchers have found a relationship between bioaerosol exposure and a higher prevalence of reduced pulmonary function, chronic obstructive pulmonary disease, occupational asthma, shortness of breath, chronic bronchitis, and pulmonary pneumonitis in agricultural workers (Marescaux, Degano et al. 2016); (Radon, Danuser et al. 2001, Rask-Andersen 2011); (Rinsky 2015) .

Endotoxins, markers of Gram-negative bacteria, have been identified as inflammagens that can result in airway inflammation, non-allergic asthma, and reduced pulmonary function (Thorn 1998, Rylander 2006, Spaan 2008, Poole, Dooley et al. 2010). Endotoxins are found in the outer cell wall of all Gram-negative bacteria and continue to persist and impact the respiratory system after cell death. Dairy operations have shown average endotoxin concentrations around 300 EU/m^3 with individual exposures exceeding 10,000 EU/m^3 (Reynolds, Nonnenmann et al. 2013). There is currently no occupational standard for endotoxin concentration although the Dutch have

a proposed standard of 90 EU/m^3 (Donham 2000, Douphrate, Hagevoort et al. 2013). Based on this Dutch standard, dairy exposures often far exceed the recommended upper limit of exposure. As research progresses in the understanding of the body's response to endotoxins, other effects of chronic exposure are being recognized including loss of bone density, increased blood pressure, and psychological response. (Engler, Wegner et al. 2015, Espirito Santo, Ersek et al. 2015, Zhong, Urch et al. 2015).

Dating back to the 18th century, fungi have been identified as sources of occupational exposure such as farmer's lung caused by *Aspergillus niger*. Exposure to fungi can result in many of the same conditions as endotoxins including Chronic Obstructive Pulmonary Disease (COPD), reduced pulmonary function, hypersensitivity pneumonitis, and allergic asthma (Grant, Blyth et al. 1972, Dosman, Lawson et al. 2004, Reboux, Piarroux et al. 2007) making it difficult to identify a single exposure source for health outcomes. Culturable samples have been used historically to identify fungal species and their concentrations in the air. However, culturable samples represent only a small fraction of the true airborne concentrations due to sample collection techniques and fungi growing requirements.

In addition to the culturable fungi exposure, both viable and non-viable fungi contain β-glucans that can cause an allergic response and can persist after cell death. The cell wall of all fungi contains (1→3)-β-D-glucan, a water-insoluble glucose polymer. After inhalation, the body quickly responds to the presence of β-glucans by activating the macrophages, eosinophils, and neutrophils (Douwes 2005). β-glucans have not historically been studied in agricultural settings; this study is the first to examine concentrations of β-glucans in any agricultural setting. Most of the research has been focused in waste processing facilities, therefore comparable concentrations for this assay are unknown. Further, there is no proposed occupational exposure limit.

The researchers of the current study hope to bridge the gap in the knowledge of fungal and bacterial constituents in the dairy environment by examining exposure to β-glucans, endotoxins, and dust during different tasks at four Northern Colorado dairies.

METHODS

Sample Collection

Four Northern Colorado dairies with large-herd dairy operations (>1000 lactating cattle) were recruited for participation in this study. Both personal (n=110) and area air (n=297) samples were collected over nine weeks from March-September 2015. The goal for each sampling week was to monitor seven workers performing different tasks during their full-shift; 38 total workers were sampled, many of whom were sampled multiple times, during the sampling period for a total of 110 personal samples. The tasks sampled included birthing, irrigation, machine operator, medical, milking, mixing feed, rebedding, and multi-task. A questionnaire was completed by each worker before and after the work shift to document the task(s) completed while wearing the sampling pump.

Area samples were also collected at each of the four dairies. Samplers were placed inside, upwind, and downwind of the milking parlor (n=297). Samples were collected for the same full work shift as the personal samples.

The SKC Button Sampler (SKC Inc. Eighty-Four, PA) was used to collect both area and personal samples with a polyvinyl chloride (PVC) filter with a 0.5 µm pore size. The samplers were pre-calibrated to a flowrate of 4 L/min and post-calibrated to ensure the flow-rate did not fluctuate more than ±5%. Each filter was pre- and post-weighed to determine the weight of dust collected; before weighing, the filters were desiccated for 24 hours. Dust concentrations were

calculated based on the differences between the pre- and post-weighed filter and the total volume of air sampled.

The Colorado State University Institutional Review Board reviewed and approved all human subjects study protocols related to this study.

Sample Analysis

After collecting the post-weight, the filters were placed in a 50-mL falcon tube and placed in a -80ºC freezer until ready for further analysis. Each filter was extracted in 10 mL of 0.05% tween solution while shaking for one hour at 100 rpm at 25ºC.

After extraction, samples were vortexed for one minute to resuspend the sample and endotoxin analysis was performed according to the Lonza (Lonza Inc. Allendale, NJ) rFC assay standard protocol. Four assay standards (5.0 EU/mL, 0.5 EU/mL, 0.05 EU/mL, and 0.005 EU/mL) were prepared from a 20 EU/mL stock solution and LAL water with a one-minute vortex between each sample. The 96-well plate was loaded with 100 µL of sample and standard. Two samples were spiked on each plate by adding 10 µL of the 5 EU/mL standard to the samples to be spiked. The plate was placed on the preheated (37ºC) reader and incubated for 10 minutes while the working reagent was prepared with 5.5 mL of fluorogenic substrate, 4.4 mL of the rFC assay buffer reagent, and 1.1 mL of the rFC enzyme solution. After the incubation period, the plate was removed from the reader and 100 µL of the working reagent was added to each well. The plate was placed back in the pre-heated reader where the reaction incubated for one hour before being read.

The remaining extract was aliquoted into 5 mL tubes and stored in a -80ºC freezer until the (1→3) β-D-glucan assay was ready to be performed. The Cape Cod Glucatell assay (Associates of Cape Cod, Inc. Falmouth, MA) was performed according to the standard protocol

for the (1→3) β-D-glucan kinetic assay kit. Samples were thawed at room temperature and vortexed for one minute to resuspend the sample before proceeding with the assay. Six standards (100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, and 3.125 pg/mL) were made from the reagent water and the glucan standard vial. The 96-well plate was loaded with 25 µL of either sample or standard and the Glucatell agent was prepared. The Glucatell reagent was resuspended with 2.8 mL of the Glucatell reagent and 2.8 mL of the pyrosol buffer and swirled gently; 100 µL of the resuspended Glucatell reagent was added to each well. The 96-well plate was placed in the pre-heated reader where the plate was read every 10 seconds for 60 minutes.

Statistical Analysis

SAS version 9.4 (SAS institute, Cary, NC) was used to perform the statistical analysis. Descriptive statistics were used to identify geometric mean concentrations for the different exposure variables. Linear regression with a generalized estimating equation (GEE) was used to determine the statistical significance of the differences between the task-based exposure. GEE was chosen because of the repeated measures within the sampling data including the same worker sampled multiple times and the area samples taken in the same place over multiple days. The area samples were compared using a difference of least square means to identify differences between both location and site.

RESULTS

Data collection resulted in 407 total air samples with 110 personal samples from 38 different workers and 297 area samples. Samples were first categorized by season (spring, summer, and fall) and no significant difference was found for any of the analyzed variables ($p < 0.05$) between the seasons. Samples were then categorized by dairy and no significant

differences ($p < 0.05$) were found between the four different sites. Therefore, season and site were not included as factors in the following analyses.

The personal samples were categorized by work task; the worker questionnaires identified twelve different tasks. Due to small sample size some categories were combined where there were similar tasks. Truck driving was combined with machine operator, medical care and dry cows were combined with medical, and outside was combined with multi-task. The final categories included birthing (n=29), irrigation (n=8), machine operator (n=8), medical (n=20), milking (n=19), mixing feed (n=5), multi-task (n=12) and rebedding (n=4). Workers were categorized based on the task they spent the majority of their time performing.

Dust concentration was measured in mg/m^3 and ranged from 0.0041 mg/m^3 (rebedding) to 1.6 mg/m^3 (mixing feedThe highest dust concentration was below the American Conference of Governmental Industrial Hygienists Threshold Limit Value (ACGIH TLV) of 10 mg/m^3 and the suggested OEL of 2.4 mg/m^3. Overall, based on the geometric mean of the dust concentrations (Table 5.1), machine operator had the highest exposure (0.36 mg/m^3) followed by milking (0.31 mg/m^3) and birthing (0.24 mg/m^3) although none of the differences between the tasks were statistically significant. A graphical representation of the dust concentrations is found in Figure 5.1.

Table 5.1 Geometric mean of dust concentrations by task

Task	GM (mg/m^3)	GSD (mg/m^3)
Birthing (n=29)	0.242	2.81
Irrigation (n=8)	0.238	2.85
Machine Operator (n=8)	0.356	2.17
Medical (n=20)	0.132	2.94
Milking (n=19)	0.305	2.30
Mixing Feed (n=5)	0.177	6.16
Multi-task (n=12)	0.222	1.88
Rebedding (n=4)	0.045	5.25

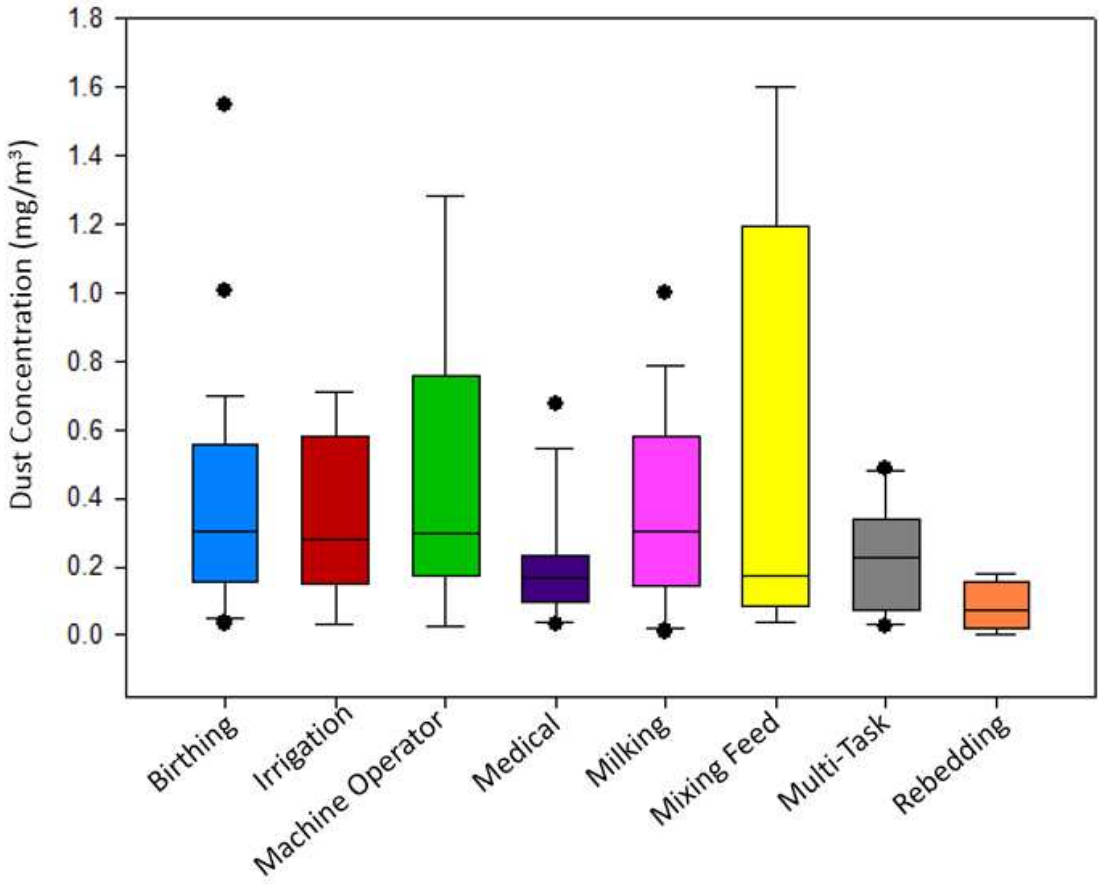

Figure 5.1 Personal exposure to dust concentration by task (n=110)

Each task was further categorized by participant ID to examine the variability within a personal sample between days (Figure 5.2). There was a large amount of variability for each individual participant suggesting that the dust concentration changed daily.

Endotoxin concentration was used to measure the concentration of Gram-negative bacterial constituents which was categorized by personal tasks. There were no significant differences ($p<0.05$) between the eight different tasks for endotoxin exposure. The endotoxin concentration ranged from 0.078 EU/m^3 (mixing feed) to 40 EU/m^3 (milking) with an overall geometric mean of 5.8 EU/m^3 (Table 5.3), all well below the proposed Dutch OEL of 90 EU/m^3. Multi-task had the highest geometric mean (10 EU/m^3) followed by milking (7.7 EU/m^3), and medical (7.4 EU/m^3).

Table 5.2 Geometric Mean of endotoxin concentration by task

Task	GM (EU/m^3)	GSD (EU/m^3)
Birthing (n=29)	5.4	4.3
Irrigation (n=8)	3.9	2.1
Machine Operator (n=8)	4.6	1.6
Medical (n=20)	7.4	5.2
Milking (n=19)	7.7	4.8
Mixing Feed (n=5)	1.2	2.1
Multi-task (n=12)	10	2.4
Rebedding (n=4)	3.62	8.5

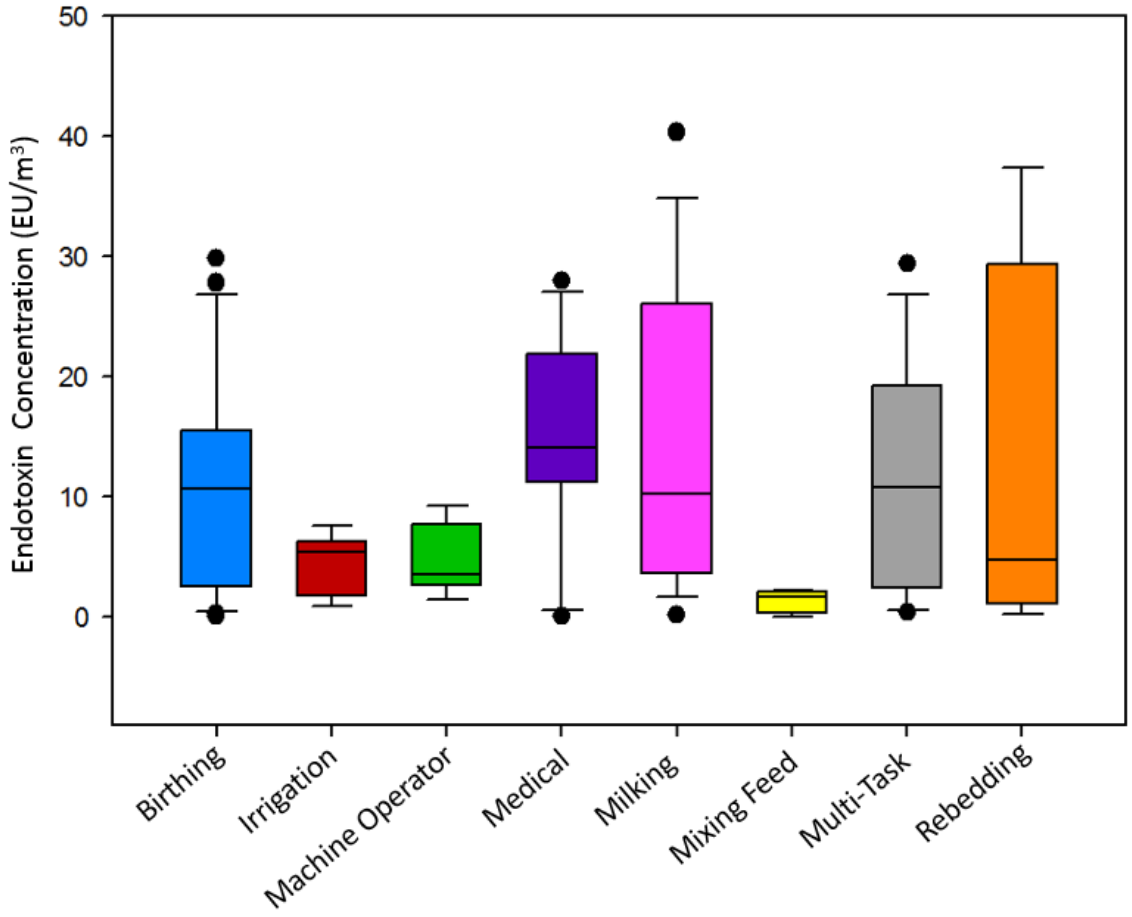

Figure 5.2 Task-based exposure to endotoxin concentrations

The endotoxin concentration for the individual tasks also had a lot of variation within each participant (Figure 5.4). There were some participants that had exposures that were similar from day-to-day, but most participants had endotoxin exposures that ranged widely from one day to the next. This study is the first to assess fungal concentrations in an agricultural environment through the use of the β-glucan assay. β-glucan concentration was used as a marker for fungal concentrations; the β-glucan concentration was categorized by task and ranged from 2.4 pg/m^3 (rebedding) to 430 pg/m^3 (machine operator) (Figure 5.5). The β-glucan concentration had a much larger range than any of the other markers of worker exposure, but appears to be typical of the β-glucan assay based on waste processing facilities.

Table 5.3 Geometric mean of β-glucan concentration by task

Task	GM (pg/m^3)	GSD (pg/m^3)
Birthing (n=29)	100	2.6
Irrigation (n=8)	80	2.4
Machine Operator (n=8)	140	2.6
Medical (n=20)	62	2.8
Milking (n=19)	89	2.3
Mixing Feed (n=5)	60	1.8
Multi-task (n=12)	150	1.7
Rebedding (n=4)	42	7.8

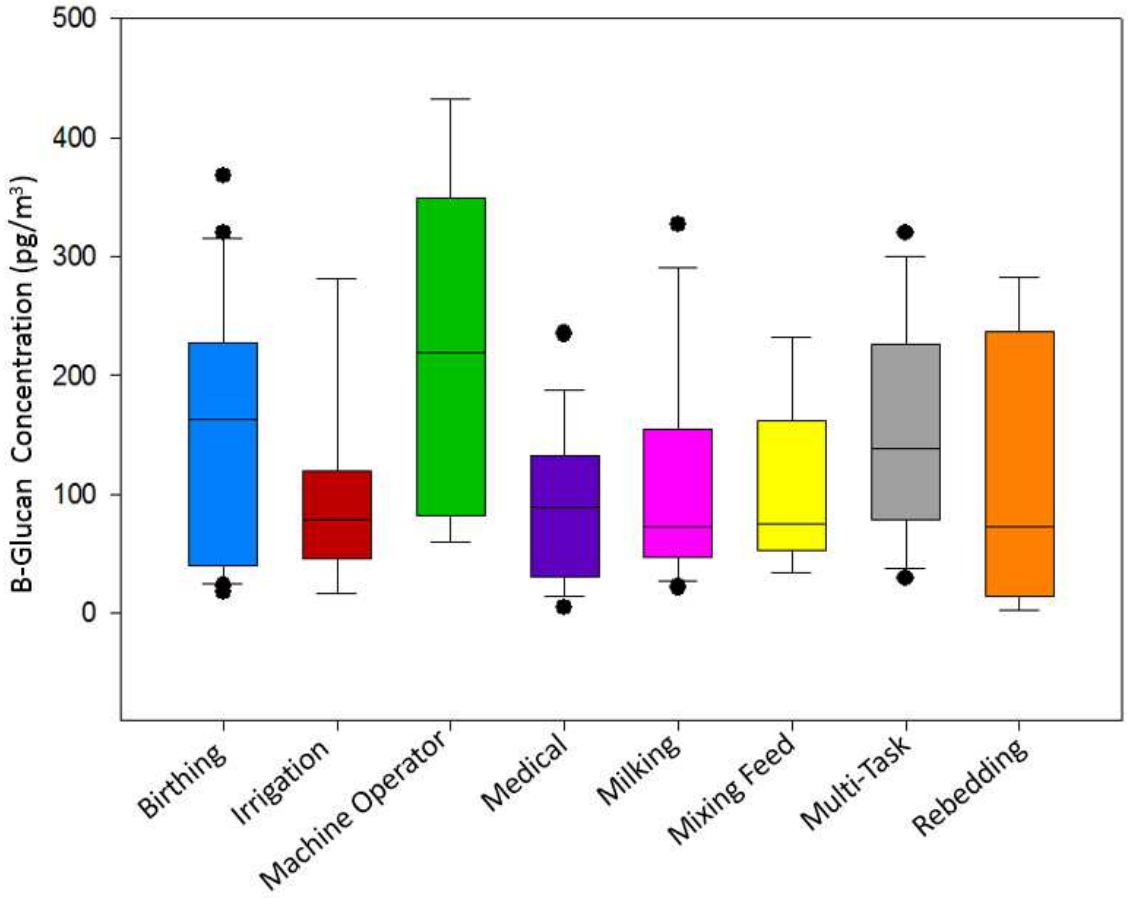

Figure 5.3 Task-based exposure to β-glucan concentrations

Each of the three markers identified for worker exposure (dust, endotoxin, and β-glucan) were also used to assess potential sources surrounding the milking parlor to identify opportunities for interventions. Samplers were placed inside, upwind, and downwind of the parlor. The difference in contaminant concentrations for the sites was not statistically significant for any of the variables. The milking parlor had the highest concentration across the three different variables, but only had a statistically significant difference than the other sample locations ($p<0.001$) for the endotoxin concentrations (Figure 5.8). The dust concentration and the β-glucan concentration were not statistically significantly different between the different locations (Figure 5.7 and Figure 5.9 respectively). The endotoxin concentration had a statistically significant difference between all three locations as shown in Table 5. and was lowest downwind. The estimates shown in Table 5.4 represented the difference between the locations.

Table 5.4 Difference of least square means for area endotoxin concentrations

Location	Location (Reference)	Estimate	Standard Error	P-value
Downwind	Parlor	-10.3	1.04	<0.001
Downwind	Upwind	-3.65	1.04	0.004
Parlor	Upwind	6.65	1.04	<0.001

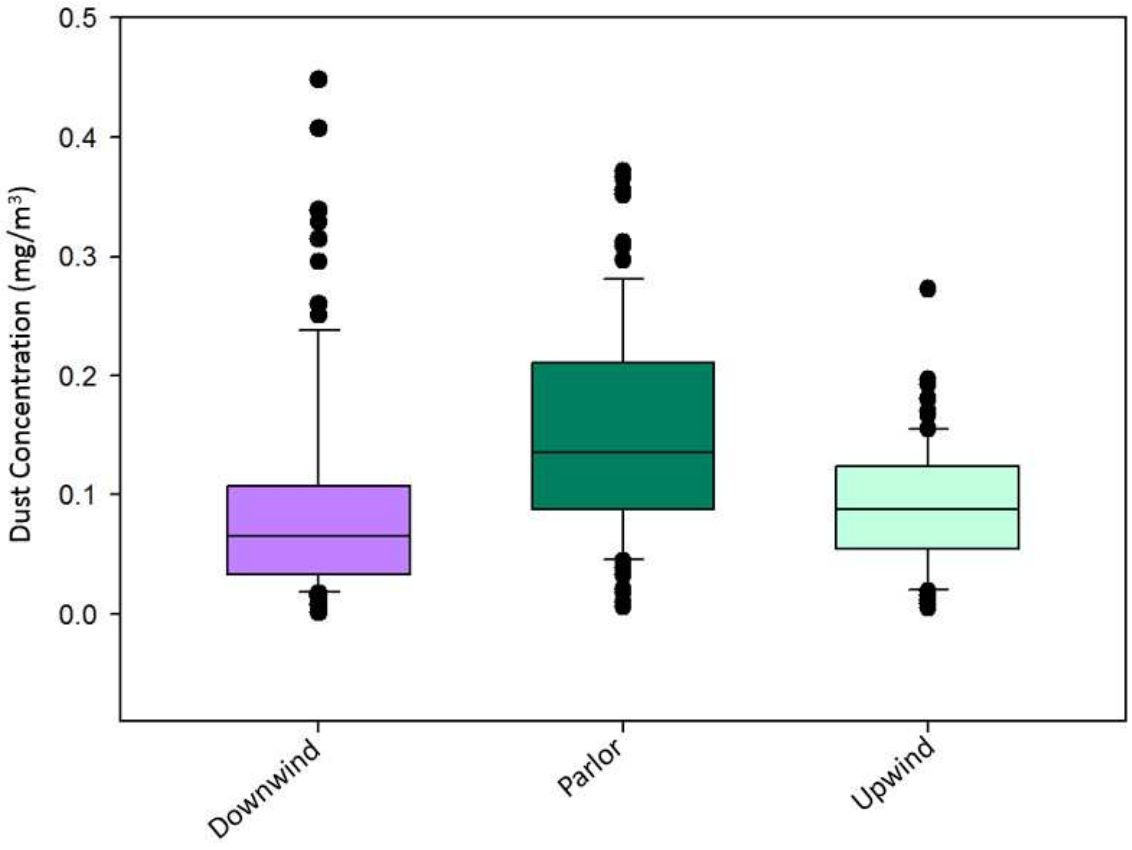

Figure 5.4 Area dust concentration by location

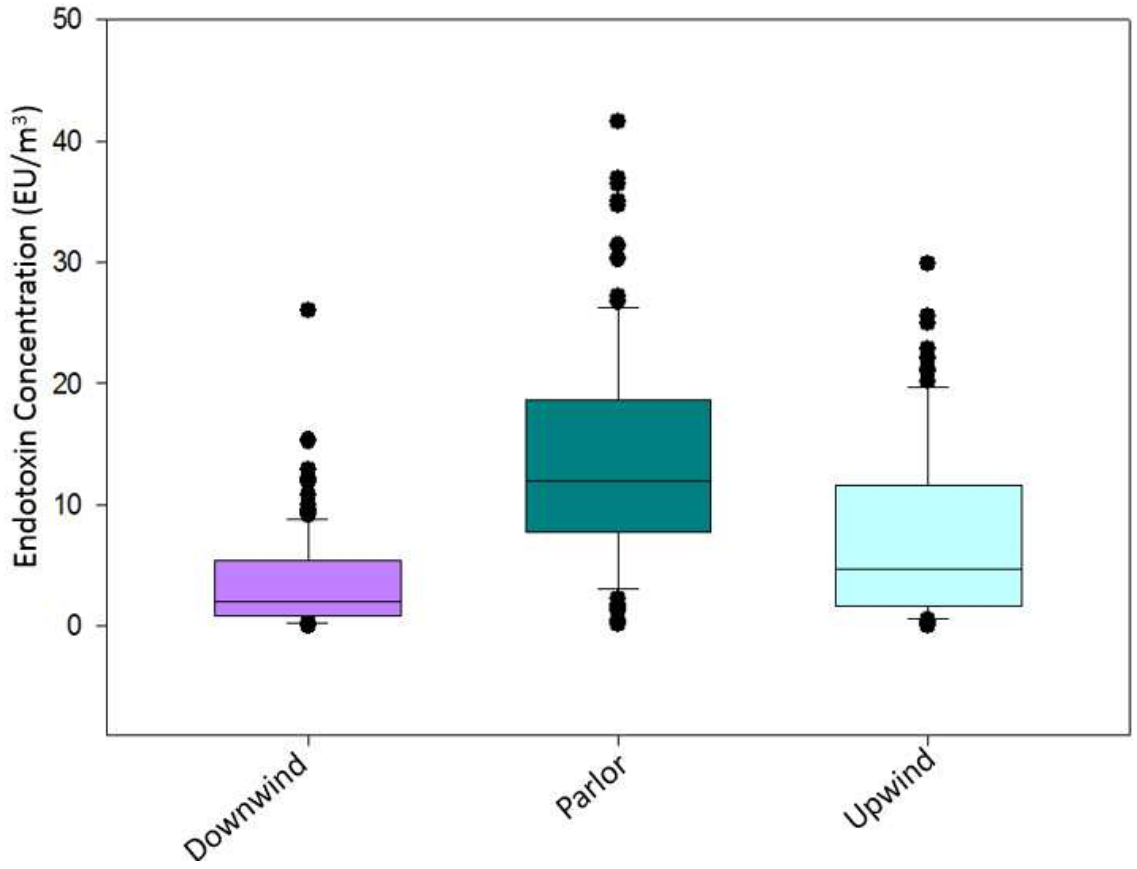

Figure 5.5 Area endotoxin concentration by location

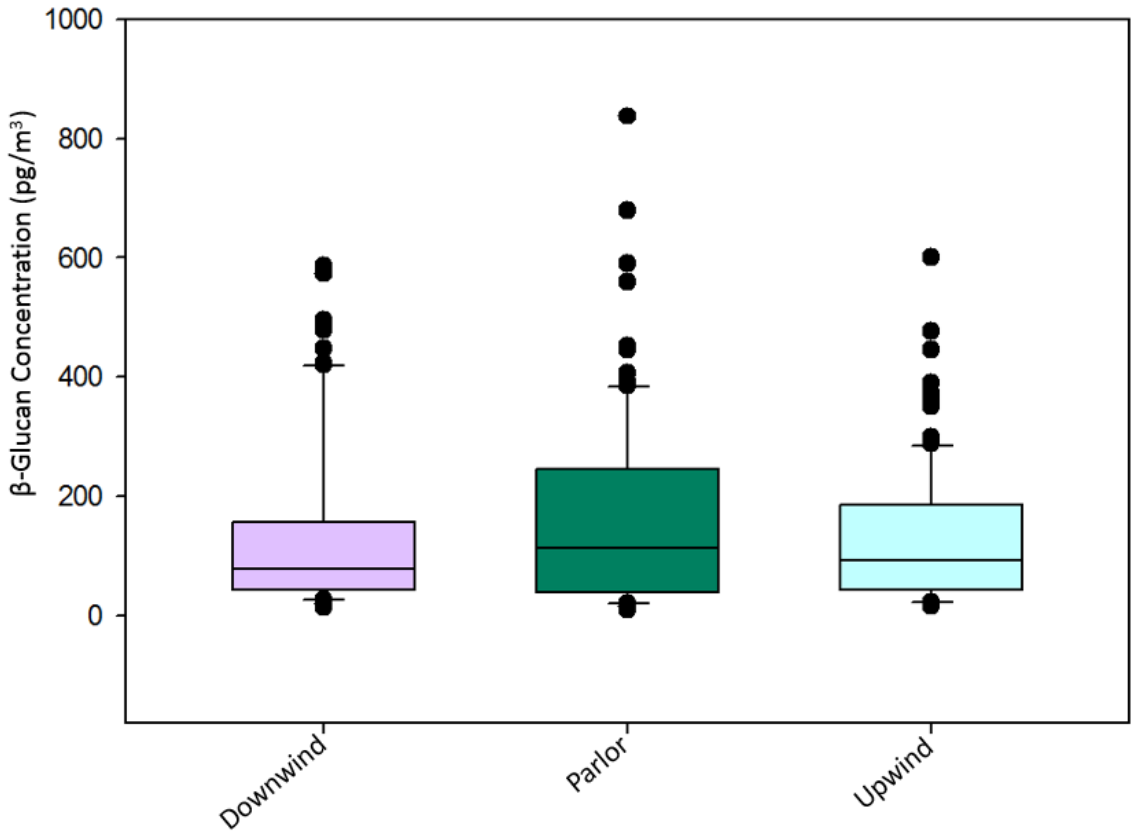

Figure 5.6 Area β-glucan concentration by location

Every area sample location was taken in triplicate which were categorized by sampling event (one day) in Figures 5.10, 5.11, and 5.12. In comparison to the personal samples, the area samples are more tightly grouped for each sampling event and are grouped more tightly from one day to the next although there are some outliers. Little variance was expected within a triplicate grouping for each because the samplers were co-located under the same sampling conditions.

DISCUSSION

Figure 5.1 depicts the breakdown of the dust exposure across the eight different tasks at four dairies in Northern Colorado. Machine operators had the highest geometric mean of exposure which was not expected. Machine operators spend the day inside an enclosed cab suggesting the filtered air conditioning should filter out most of the air contaminants. They may be moving large piles of dirt, manure, or feed while inside the machine and may not have the windows rolled up, may not have filtered air conditioning, or the filters in the equipment is not adequate to filter small particles increasing their potential for exposure. Future researchers should ask more questions surrounding machine operators such as the use of air conditioning, the efficiency of the air filter, windows, and the task being performed while using the machinery. Milking had the second highest geometric mean dust exposure which was anticipated based on previous research. Workers in the milking parlor are typically inside an enclosed parlor all day with little ventilation and high volumes of activity that generate dust such as the movement of cows. Mixing feed was expected to have the highest dust concentration due to the dusty nature of the task; plumes of dust are generated within the workers' breathing zones when mixing feed but was not included in the top activities that resulted in high dust exposures (Table 5.2). This range in dust concentration was lower than a variety of other studies reviewed (Basinas, Sigsgaard et al. 2014) in similar environments that ranged from 0.95-5.6 mg/m^3 suggesting the exposure was less than what was anticipated. One important follow-up to the dust concentrations found at these dairies is the health impact this exposure has on dairy workers that are chronically exposed as well as the constituents that are included in this dust.

Samples were categorized by participant ID to identify trends within a participant's exposure in Figure 5.2. Except for a few workers, there was a large within-worker variance suggesting different daily exposures. For example, participant 409 (birthing) and participant 423 (mixing feed) both had exposures that spanned both ends of the spectrum in terms of dust concentrations. Although workers may be binned into the same category during each day of the sampling period, workers were likely to have different tasks they were assigned daily that could have resulted in different exposures.

Workers were exposed to a geometric mean concentration of 5.8 EU/m^3 that ranged from 0.078-40 EU/m^3. In comparison to other studies where endotoxin concentrations were found to range between 4-225 EU/m^3 (Basinas, Sigsgaard et al. 2014), the concentrations measured in the current study were relatively low. The upper range of the endotoxin concentration was also well below the proposed Dutch occupational standard of 90 EU/m^3 suggesting that endotoxin concentrations may not be a concern in these Northern Colorado dairies. However, even at relatively low concentrations of exposure, researchers have demonstrated a cross-shift lung function decline in workers with genetic polymorphisms (TLR4) suggesting that the proposed OEL may not be low enough to prevent respiratory disease in more susceptible workers (Reynolds 2012).

Workers that performed multiple tasks across the dairy had the highest exposure followed by milking and medical. Multi-task was not predicted to have the highest endotoxin concentration and it is difficult to speculate what tasks were included within the multi-task category to identify why this group had the highest exposure. Milking was expected to have the highest endotoxin exposure based on previous studies that showed high dust and endotoxin concentrations inside the milking parlor due to the high activity and enclosed parlor. The

ranking in exposure of tasks was expected to follow more closely with the dust concentrations because an increase in dust is expected to have a corresponding increase in bacteria.

The current study was the first use of the β-glucan assay to identify concentrations of bacteria in dairies. Little work has been done with the Glucatell assay to assess occupational exposure to fungi. Researchers at waste processing facilities have done some investigation to identify fungal concentrations that resulted in concentrations of β-glucans that ranged from 0.2-212 pg/m^3 (Douwes 2005).

The β-glucan concentrations in the current study ranged from 2.4-430 pg/m^3 for the different tasks at the dairies which were substantially higher than those found in the waste processing facility studies (Douwes, 2005). Higher concentrations at the dairies were expected due to the dust-generating activities of workers on the dairies. The dairy environment has large amount of feed, manure (and processing), animal dander, soil, and milk which can all have high concentrations of fungi that can easily become aerosolized. Tasks at the dairy such as moving manure, mixing and moving feed, or rebedding are all expected to have high concentrations of fungi. The highest geometric mean β-glucan concentration was the multi-task category (150 pg/m^3) followed by machine operator (140 pg/m^3) and birthing (100 pg/m^3). It is difficult to make conclusions based on the multi-task category without further information on the individual participants and their corresponding tasks/exposures. Machine operators were not expected to have the relatively high β-glucan exposures because it was assumed they were in the cab with the windows rolled up and air being filtered by the air conditioning system. However, if the machine operators did not have the windows rolled up and did not have adequate filtration systems while they were moving large amounts of manure or bedding materials, a large amount of dust with fungi could be disturbed and aerosolized into the workers' breathing zone. In

addition, the birthing tasks were not expected to result in such relatively high concentrations of β-glucans. Workers in the birthing area were often on the ground with the birthing mothers and moving new calves which could have resulted in exposure to the fungi found in soil.

Although the exposure concentrations were relatively low in the current study, many other researchers suggest that low concentrations of bioaerosols can still result in cross-shift pulmonary function decline. A genetic polymorphism, TLR4, may result in predisposing workers to relatively low endotoxin sensitization, making them more susceptible to respiratory disease and reduced pulmonary function. Researchers have examined a variety of cytokines in response to agricultural dust exposure. Dusts from agriculture were found to induce monocyte tumor necrosis factor (TNF) alpha, interleukin (IL)-8, IL-6, and epithelial cells (Poole, Dooley et al. 2010). These cytokines are all markers of inflammation and demonstrate that the body is trying to repair and protect itself after exposure to agricultural dusts that contain endotoxins, β-glucans, and other bacterial constituents that can ultimately lead to reduced respiratory function in relatively low exposure concentrations.

Area air samples were collected simultaneously in three locations (upwind, downwind, and inside the parlor) at each dairy. The samples were first categorized by dairy site and it was found that there was no statistically significant differences for any of the three exposure measures (dust, β-glucan, and endotoxin); therefore site was ignored for subsequent analyses. Samples collected inside the milking parlor had the highest concentration of the threemeasured exposures and downwind samples had the lowest concentrations. Dust and β-glucan concentrations were not statistically significant for the difference in location, but endotoxins were significant ($p<0.05$). The parlor was anticipated to have the highest concentration of contaminants because it was an enclosed building. Concentrations of bioaerosols inside the

milking parlor were expected to increase because there was little wind to push the air contaminants out of the building. Additionally, during milking operations, there was always activity inside the parlor with the movement of cows, milking, and applying teat dips to each cow that could stir up dust, bacteria, fungi and their constituents. Samples were taken in triplicate at three locations, and it was found that the samples had very little variance within the triplicate grouping.

CONCLUSIONS

Three measured variables (dust, β-glucan, and endotoxin) were used to estimate respiratory exposure of dairy workers at four Northern Colorado dairies. The highest dust concentrations (4.6 mg/m^3) for the personal exposures were well below the ACGIH TLV of 10 mg/m^3 but not below the recommended OEL of 2.4 mg/m^3. Feed mixers, machine operators, and milkers had the highest dust exposures. Feed mixing workers were expected to have relatively high dust exposure concentrations due to the nature of the task and the plumes of dust that are typically generated with this task. The highest concentration of endotoxins for feed mixing was 40 EU/m^3 which is well below the proposed Dutch standard of 90 EU/m^3. Milking, medical, and rebedding workers had the highest endotoxin exposure concentrations which was expected based on previous studies. This was the first study to assess β-glucan concentrations on dairies; concentrations were higher than anticipated (as compared to results from waste processing facilities), but no work has been done using the β-glucan assay for exposures at dairies. Of the eight tasks evaluated, β-glucan concentrations were highest for the machine operators, multi-task workers, and birthing workers. Machine operators had relatively high exposures for all three variables (dust, β-glucan, and endotoxin) which was not anticipated. More information would be helpful to understand the high exposures such as use of in-cab air conditioning (and filter

efficiency), the machine operator tasks, and if the windows are kept rolled up. The three measured exposures had a relatively high amount of variability within each participant from day-to-day. Each participant was only sampled for three consecutive days which did not provide enough information to draw conclusions based on task due to the high day-to-day variability. Even though the exposure was relatively low for both dust and endotoxin concentrations, previous exposure studies indicate that the body still reacts to the bioaerosols based on the presence of inflammatory markers such as TNF alpha, IL-6, and IL-8. Additionally, the genetic polymorphism, TLR4, can lead to sensitization and cross-shift pulmonary function decline at relatively low concentrations of bioaerosols.

The area samples were taken in three different locations at each site: upwind, downwind, and inside the parlor. The measured air contaminants were not significantly different between site locations. Inside the parlor had the highest concentration for all three contaminants, but the difference in location was only statistically significant for the endotoxin concentrations. Downwind and upwind samples also had statistically significant different means for the endotoxin concentration but not for dust and β-glucan concentrations. Based on the area sampling results, future interventions to reduce worker exposure should focus on the milking parlor. The area samples that were taken in triplicate were typically grouped closely, but there was still a lot of variation between the days for all of the sample types. Based on the results of the current study, dairy workers are exposed to levels of dust, β-glucan, and endotoxin that can increase risk of pulmonary disease. In addition, the β-glucan assay proved to be useful in demonstrating the relatively high concentrations that could explain the increased risk of pulmonary disease.

LIMITATIONS

The relatively small sample size, especially within the tasks makes it difficult to draw conclusions based on worker exposure. The small sample size within tasks resulted in large variances between the samples and the inability to identify trends, differences, or similarities within the data set. There was large variance within each participant which may be explained in a larger data set that would result from monitoring workers for longer sampling periods.

Participant attrition was a problem during this study; multiple pumps were hung on the workers which resulted in the use of bulky sampling vests that were cumbersome for the workers to wear during their work shift causing workers to decline to participate. Some tasks such as milking were not as represented because of the bulkiness of the vest reducing arm movement and limiting the range of motion for their milking tasks.

CHAPTER 6: SUMMARY

MAJOR RESEARCH FINDINGS

This research was conducted to characterize dairy worker exposure to bioaerosols, guide future studies to develop interventions and ultimately reduce worker exposure. Dairy workers around the world are impacted by relatively high concentrations of bioaerosols that lead to reduced pulmonary function and a variety of respiratory diseases greatly impacting their quality of life. Characterization of the microbial community needs to be accomplished to understand the specific contaminants to which workers are exposed, but little work has been done using NGS for worker exposure. Rapid diagnostic assays have been used to relate exposure to Gram-negative bacteria to worker health outcomes, but no work has been published for fungal markers. This study helped accomplish the bioaerosol identification of dairy worker exposure by 1) comparing two air sampling devices for analysis by NGS, 2) characterizing the dairy microbiome to further assess occupational exposure, and 3) using rapid diagnostic assays to assess worker exposure by task. This was the first study to compare the SKC button sampler and SKC biosampler using NGS in dairies. It was also the first study to use the Glucatell assay to assess worker exposure to fungi on dairies.

SPECIFIC AIM 1

The objective of Specific Aim 1 was to examine the differences and similarities in sample collection techniques between the SKC button sampler and SKC biosampler using NGS to characterize bacterial communities. The two samplers were co-located inside a fresh cow pen for five consecutive days and bacterial DNA was sequenced for each sample. The bacterial

communities were analyzed to identify differences in bacterial collection between the samplers, trends between the sampling days, and information about the identified genera.

This was the first study to use NGS to compare the SKC button sampler and SKC biosamplers in a dairy environment. Overall, the biosampler was determined to be the better sampler for collection of bioaerosols in a dairy environment. The two samplers had significantly different OTUs, but the biosampler had a relatively larger variance. This could be associated with the changes of activity from hour one to hour two in the biosampler collection period while the button samples were collected over eight-hour periods averaged over the day. Because of the high variance illustrated in the PCoA plot and the similarities between the samplers in the alpha diversity plots, the difference between the samplers is likely a result of the high variance of the biosampler and not due to difference in the sample type. The alpha diversity plot had very similar Shannon and Inverse Simpson indices indicating that the samples between the two samplers had very similar core microbiomes. The OTU count for both samplers was very high, suggesting that the microbiome in these air samples was driven by a high number of rare bacteria and a low number of dominant bacteria.

The relative abundance for the top 13 bacteria found in both samplers was identified. The biosampler had a higher relative abundance of bacteria than the button sampler for all five sampling days. The button sampler had a higher sampling volume over the eight-hour sampling period, therefore it was expected to have the higher abundance. The button sampler is better suited for personal sampling to quantify true worker exposure.

Gram-positive bacteria represented 70% of the top identified bacteria. Little research has been done focusing on Gram-positive bacteria and their impact on the respiratory health of dairy workers due to limitations in the available sample analysis methods. Because so many Gram-

positive bacteria were identified in the current study, it is important to consider their impact during future health studies of dairy workers. Soil was the most common source for bacteria in these samples. This was likely due to the dusty nature of the environment where soil is being aerosolized and collected on the sample filters.

A few of the top identified bacterial genera such as *Staphylococcus*, *Clostridium*, *Pseudomonas*, and *Acinetobacter* have potentially pathogenic species. If the pathogenic species are present in relatively high concentrations, workers could become infected with these bacteria. However, species level information was not possible in the current study and conclusions could not be made on the presence of the pathogenic species or the potential health impacts associated with them. Even without the species level information, the presence of these bacteria could continue to result in reduced pulmonary function and respiratory disease such as allergic asthma, chronic bronchitis, and COPD due to the inflammatory reaction caused by bacterial inhalation.

Ultimately, the biosampler was identified as the better sampler in comparison to the button sampler for assessing airborne bacteria in dairy environments due to the larger abundance of bacteria collected during this study. Although the biosampler was better for collecting the area air samples, it was impossible to use it for collection of personal samples. Although the biosampler seems better suited for collecting bacterial samples, if the samplers are being used for personal air samples, the biosampler poses some logistical issues. The biosampler requires a large pump that can pull 12.5 L/min which is extremely heavy and cannot be worn by a worker. Additionally, the liquid collection medium makes it difficult for a worker to complete his/her tasks throughout the day. The button sampler is designed for personal air sample collection, is small, relatively indestructible, and can be used with a pump that easily clips to a belt.

Both samplers worked to collect bacteria for NGS analysis, therefore the final decision on which sampler to use relies on the type of air sample needed.

SPECIFIC AIM 2

The objective of Specific Aim 2 was to characterize worker exposure to the microbial community on dairy farms and identify potential environmental sources using NGS. One Northern Colorado dairy was recruited for this pilot study. Personal air, area air, hand swabs, and soil were collected for five consecutive days. The samples were analyzed using NGS to determine the relative abundance of bacterial genera, temporal patterns, and similarities/differences between the sample types.

Air samples had the highest relative abundance of bacteria. Upwind samples had the highest relative abundance followed by the downwind samples. The air samples were the only outside samples collected in areas where there was relatively more wind and movement of animals and people; which could be explained why there was a higher abundance in these samples. The soil samples revealed the lowest relative abundance of all the samples probably due to the low sample number. Soil samples from inside the cow pen had the highest relative abundance within the different soil samples likely due to the presence of manure, urine, and cattle.

The sample types had significantly different microbial communities. Sample type explained more than 50% of the differences found in the microbial communities. The biosamplers and hand swabs had large variances within their data sets. The hand swabs could be explained by natural differences between the microbiome on the skin. The biosampler variance is likely due to a problem with the sampler due to the consistent nature of the single sample with the large variance.

The majority of the top identified bacterial genera were Gram-positive. Some of the genera identified have potential pathogenic species. *Acinetobacter*, *Methylobacterium*, *Psychrobacter*, *Pseudomonas*, *Clostridium*, *Oerskovia*, and *Staphylococcus* all have strains of bacteria within their respective genera that could lead to infection in the dairy workers. Species level information was not available for this study; true potential for infection could not be inferred from the available data. In addition to potential infection, workers exposed to both viable and non-viable bacteria, could experience an inflammatory reaction that leads to respiratory disease and reduced pulmonary function. Soil was the most common source for the bacteria from all sample types. The dairy environment is typically very dusty with substantial cattle movement, wind, and vehicles—all aerosolizing soil and the bacteria in it.

The air samples were the best predictors for the bacteria found in the hand swabs. All of the sample types were relatively good predictors of the bacteria identified in the hand swabs. Good hygiene practices are the best control for preventing ingestion of potentially pathogenic bacteria.

SPECIFIC AIM 3

The objective of Specific Aim 3 was to characterize worker exposure to three bioaerosols constituents (dust, endotoxins and β-glucans) based on dairy worker task to provide relevant information to identify potential interventions. Personal and area air samples were collected at four dairies and samples were analyzed to determine the concentration of dust, endotoxins, and β-glucans in each of the samples. Workers provided their primary task throughout the day which culminated to eight different tasks. Data were then analyzed to determine differences between site, season, task, and location.

Differences in site and season were found to not be statistically significant across both the personal and area air samples and were not included as variables in any of the subsequent analyses. Task differences were analyzed for each of the three measures of exposure (dust, endotoxins, β-glucans), however there was not one task that was in the top three highest exposures for all three variables. Machine operators and milkers had the highest dust exposure across the dairies. In other research studies, milkers had the highest exposure because of the enclosed nature of the milking parlor that had less air movement and higher dust concentrations. The highest dust exposure was well below the OSHA PEL of 10 mg/m^3 but was not below the ACGIH OEL of 2.4 mg/m^3 suggesting the dust concentrations may be a health concern for some of the tasks. Multi-task and milking workers had the highest exposure to concentrations of endotoxins. Conclusions could not be made about the multi-task worker because the tasks between each of the multi-task workers varies so greatly. The highest endotoxin exposure was well below the proposed OEL of 90 EU/m^3. Multi-task and machine operators had the highest β-glucan concentrations. It was unexpected to measure such high β-glucan concentrations for machine operators. More information needs to be gathered to understand the reason behind the high levels of exposure in the machine operators, for example if the cab windows were rolled up and what tasks the operators were performing while operating the machinery. Even if the air contaminant concentrations are relatively low, it is still likely that workers could develop respiratory disease. Workers with the genetic mutation in TLR4 have been shown to have sensitization at very low endotoxin concentrations. Additionally, markers of inflammation have been induced at very low concentrations to organic dust.

Each individual worker had a relatively large day-to-day exposure variability for the β-glucan measurements, suggesting that the exposure changed depending on specific tasks between

days. Following workers for multiple weeks to accurately characterize their task exposures would provide very useful information.

Based on the results from the area samples, the milking parlor samples demonstrated the highest average contaminant concentrations across all three exposure variables, but only endotoxins were statistically different based on location ($p<0.001$). The parlor was anticipated to have the highest contaminant exposures due to the lack of ventilation and the number of cows bringing in dirt, bacteria, and fungi; leading to higher worker exposure. Since the milking parlor samples resulted in the highest contaminant concentrations, the parlor should be a focal point for designing future interventions. Because milking in the parlor is a relatively stationary task, there areample opportunities for engineering or administrative controls that can reduce worker exposure and improve the respiratory health of the parlor workers.

Overall, the researchers of the current study found varying contaminant concentrations both between tasks and within workers from day-to-day. Additional sampling should be performed, focusing on fewer tasks over longer periods to understand the temporal and task differences associated with dairy worker exposure. There was no significantly different contaminant concentration based on task to focus intervention studies, but the area air sampling results indicated that the parlor should be an area of focus. This was the first study to use the Glucatell assay to identify concentrations of β-glucans at dairies; future studies should continue to explore this method of assessing worker exposure.

CONCLUSIONS

Although there were many limitations in this study particularly with the small sample sizes across all three aims, this research provides useful information to further understanding dairy worker exposure. The researchers found that both the biosampler and button sampler can

collect airborne bacteria to be analyzed by NGS. The biosampler is the better sampler for airborne bacteria but it cannot be used for personal air monitoring. Soil was the source for the bacteria found in the airborne and personal samples suggesting that dust suppression techniques would be a viable intervention to reduce worker exposure. Novel information was collected as this was the first study to quantify dairy worker exposure to β-glucans. Worker exposure to dust and endotoxins also provided useful information on further understanding typical exposures across dairy environments. All of the information resulting from this study can be used to guide future work to design interventions, understand total worker exposure, reduce exposure, and create a healthier environment for dairy workers.

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