THESIS

EVALUATION OF SEASONAL VENTILATION CHANGES AND THEIR EFFECT ON AMBIENT DUST, ENDOTOXIN AND BIOAEROSOL CONCENTRATIONS IN A DAIRY PARLOR

Submitted by

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ABSTRACT

EVALUATION OF SEASONAL VENTILATION CHANGES AND THEIR EFFECT ON AMBIENT DUST, ENDOTOXIN AND BIOAEROSOL CONCENTRATIONS IN A DAIRY PARLOR

This pilot study measured the impact of seasonal ventilation changes on concentrations of organic dust, endotoxin and bacteria in one modern dairy milking parlor. Pyrosequencing, a new non-target specific molecular methodology was used to characterize airborne bioaerosols.

Area samples for inhalable dust, respirable dust, endotoxin, and bacteria were collected in one modern dairy parlor during both summer and winter seasons. Five sampling sessions were performed at approximately weekly intervals during each season. The summer season included an open facility with fresh mechanical air ventilation. The winter season consisted of a closed facility with no fresh air ventilation and forced heat. Aerosol size distributions, air velocity inside the parlor, humidity, temperature, and CO_2 were also sampled on each trip. Two-way ANOVA was performed to test statistically significant differences between variables.

No significant differences between mean concentrations of inhalable dust, respirable dust, or bacteria were seen by season. Endotoxin showed a near significant differen ce ($p=0.06$). CO_2 concentration doubled during the winter season as compared to

the summer ($p<0.001$) due to reduced ventilation. The aerosol size distribution did not vary between seasons (MMOD=12µm) indicating that seasonal ventilation changes do not affect the particle size distribution. The most common bacterial genera in both seasons were Clostridium (anaerobic), Oscillibacter and Staphylococcus – all Gram positive bacteria. Gram-positive bacterial genera occurred more frequently during the summer than winter seasons. Gram-negative bacteria cell counts increased in the winter season. Average concentrations of total bacteria in the facility during summer and winter were 2839 and 7008 counts/m^3, respectively.

This study was the first to apply Pyrosequencing to measure bioaerosols in a dairy environment. The diversity of bacteria and predominance of Gram-positive bacteria is consistent with studies in swine and poultry facilities. Concentrations of dust, endotoxin, and bacteria were low during both seasons in this milking parlor, most likely due to the new construction of the facility. These study results will inform the design of future comprehensive studies of aerosol exposure interventions in dairy operations. Understanding these aerosol exposures and potential interventions is important for reducing respiratory disease among workers in the dairy industry.

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DEDICATION

I want to dedicate this work to my loving family. To my husband Michael, thank you for all of your much needed support, love, and laughter. You made me smile throughout my frustration and you were there whenever I needed you. You have walked with me through this entire process and I couldn't have done it without you.

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LIST OF ACRONYMS

ACGIH	American Council for Governmental Industrial Hygienists
AOD	Aurora Organic Dairy
bTEFAP	Bacterial tag-encoded flexible amplicon pyrosequencing
bp	base pairs
CFU	Colony forming units
COPD	Chronic Obstructive Pulmonary Disease
CSU	Colorado State University
EU	endotoxin units
FEV_1	Forced Expiratory Volume in 1 second
FVC	Force vital capacity
GC/MS	Gas chromatography mass spectroscopy
HICAHS	High Plains Intermountain Council for Agricultural Health and Safety
3-OHA	3-hydroxy fatty acid
LOD	Limit of detection
LOQ	Limit of quantification
LAL	Limulus amebocyte assay
LPS	Lipopolysaccharides
MMOD	Mass mean optical diameter
MAPERC	Mountain and Plains Educational Research Center
NIOSH	National Institute for Occupational Safety and Health

OSHA	Occupational Safety and Health Administration
OEL	Occupational exposure guideline
ODTS	Organic Toxic Dust Syndrome
PNOR	Particles not otherwise regulated
PNOS	Particles not otherwise specified
ppm	parts per million
PPE	Personal protective equipment
rFC	Recombinant factor C assay
SCBA	Self-contained breathing apparatus
SOP	Standard operating procedure
σ	Standard Deviation
σ_g	Geometric Standard Deviation
TLV	Threshold Limit Value
TWA	Time weighted average

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CHAPTER 1

INTRODUCTION

Inhaling certain dusts or other particulates has been observed to produce breathing difficulties since Georgius Agricola in 1556 (Holt, 1987). Since that time, observations along with research have led to better understanding of respiratory illnesses in the work environment. Today, agriculture is ranked one of the most hazardous occupations in the United States (Kirkhorn, et al., 2000) (Rautiainen, et al., 2002). A segment of the agriculture industry in the United States is made up of animal confinement operations, with dairy farms falling into this category. The dairy industry has moved from small milking operations into much larger operations, with increased herd sizes (USDA, 2010) (GAO, 2001). Large animal confinement operations provide unique environments for many respiratory hazards to concentrate, increasing employee exposure to organic dusts, endotoxins, and bioaerosols (Rylander, et al., 1983) (Millner, 2009) (Barber, et al., 1992). Numerous correlations have been observed between working in animal confinement operations and respiratory symptoms, including chest tightness, wheezing, bronchitis, and cough along with other more severe respiratory conditions such as hypersensitivity pneumoconiosis, organic dust toxic syndrome (ODTS), as well as acute and chronic airway inflammation (Heederik, et al., 1991) (Donham, 1986) (Choudat, et al., 1994) (Reynolds, et al., 2009).

Organic dust, endotoxin, and airborne bacteria have all been found in animal confinement operations, including dairy operations (Kullman, et al., 1998) (Cormier, et al., 1991). These contaminants have the potential to cause various respiratory diseases (Schenker, et al., 1998). Organic dusts are complex mixtures of organic materials such as feed, hay, dander or other animal particles, urine, fecal material, chemicals, insect parts, bacteria, fungi, pollen grains, and endotoxins (Schenker, et al., 1998). Endotoxins are located on Gram-negative bacterial cell walls and are components of organic dust. Both Gram-negative and Gram-positive bacteria adhere to dust particles and are also a part of 'organic dust' (Schenker, et al., 1998) (DeMaria, et al., 1980).

Currently there are few guidelines pertaining to exposures that dairy workers may face. Most of the regulated occupational exposure levels regarding agricultural dust are set for grain dust (oat, barley, and wheat). A standard for organic dusts found in animal confinement operations does not currently exist (Burch, et al., 2010). Other standards set for dust by the Occupational Safety and Health Administration (OSHA) do not take into account the type of dust or the bioactive nature of dust found in animal facilities making adequate protection of the worker a concern (Donham, 1986). Characterization of exposures to endotoxin and bacteria have been researched, but not enough data exists to understand all of the health implications associated. Occupational limits for endotoxin exposure have been suggested, but a regulation has not yet been passed (DECOS, 2010) (Reynolds, et al., 1996) (Donham, et al., 1995). Few research studies exist on controlling airborne concentrations of organic dust, endotoxin, or airborne bacteria, while even less research exists regarding control specifically for the dairy environment. New methods of quantifying and identifying bacteria specific to animal confinement industries are

available, but need further testing. One aspect to providing a safe and healthy workplace for all employees in animal confinement operations will be to adequately understand the exposures associated with these contaminants along with methods of controlling the exposures for each specific work environment.

CHAPTER 2

LITERATURE REVIEW

History

Throughout history, many individuals have identified the relationship between dust inhalation that results in a subsequent disease outcome. Georgius Agricola noted in his work *De re Metallica* (1556) that certain kinds of dusts produced breathing difficulties (Agricola, et al., 1950). Later Bernardino Ramazzini (1713) made similar associations and observations, writing how the inhalation of certain materials produced a specific and identifiable disease outcome (Wright, 1940). In another observation, Ramazzini made an explicit reference regarding how farmers appeared to suffer from increased respiratory illness, which he believed resulted from dust exposure in their work environments (Donham, 1986). Today, agriculture is one of the most hazardous occupations in the United States (Kirkhorn, et al., 2000) (Rautiainen, et al., 2002). The nature of work in agriculture occupations provides common and almost unavoidable exposures to organic dusts and bioaerosols. Those employed specifically in agricultural animal operations, such as animal production (poultry, swine, beef, and dairy cattle), are exposed to many inhalable and potentially hazardous constituents, contributing to numerous respiratory diseases (Kirkhorn, et al., 2000) (Rautiainen, et al., 2002).

Animal Confinement Operations

Animal operations are known to be hazardous work environments (Rylander, et al., 1983) (Millner, 2009). The movement towards intensive livestock production has increased the confinement of the livestock, thereby increasing the concern for indoor air quality inside the animal housing (Barber, et al., 1992). Such a high density of animals located within enclosed buildings creates increased exposures to any dusts, bacteria, fungi, and toxins that may be released by the environment or animals (Millner, 2009). Airborne contaminants in buildings where animals are housed are of significant concern for the respiratory health of employees working inside these buildings. Numerous correlations have been observed between animal confinement operations and respiratory symptoms including chest tightness, wheezing, bronchitis, and cough along with other more severe respiratory conditions such as hypersensitivity pneumonitis, organic dust toxic syndrome (ODTS), as well as acute and chronic airway inflammation (Rylander, et al., 1983) (Rylander, et al., 2006) (Heederik, et al., 1991) (Choudat, et al., 1994). Much work has been done to quantify respiratory exposures in poultry and swine operations, whereas less is known about the airborne exposures in the dairy industry, specifically dairy parlors (Donham, 1986).

The Dairy Industry

In the last eight years, the dairy industry has condensed from many small operations into larger, regional facilities (USDA, 2010). Dairy operations with more than 500 head increased by 20% percent, from 2,795 operations in 2001, to 3,350 operations in 2009, with a decrease of 35% seen in smaller dairy farms (<500 head) (USDA, 2010)..

The increase in herd size produces higher levels of contaminants from cleaning, milking, and feeding, which can become concentrated in buildings where the animals and employees are located (Donham, 1986). Routine exposures to endotoxin in dairy parlors have been documented (Burch, et al., 2010) (Reynolds, et al., 2009). Dairy barns have also been confirmed to be rich with Gram-positive bacteria (Poole, et al., 2010). Dairy barns uniquely provide a humid environment for the growth of bacteria, which can then be aerosolized during milking and cleaning operations (Lange, et al., 1997). Inhalation of bioaerosols has potential to cause respiratory diseases such as ODTS, asthma, allergic rhinitis, hypersensitivity pneumonitis, and bronchitis (Donham, 1986) (Lange, et al., 1997). Employees in the dairy industry are exposed to the following airborne respiratory hazards: organic dusts, allergens, endotoxin, airborne microorganisms, noxious gases, fungi, and molds (Schenker, et al., 1998). In this thesis, specific hazards of organic dusts, endotoxin, and airborne bacteria and their control methods will be addressed.

Organic Dusts

Organic dusts of dairy or livestock facilities stem from organic materials and are mixtures of feed, hay, dander or other animal particles, urine, fecal material, chemicals, insect parts, bacteria, fungi, pollen grains, and endotoxins (Rask-Anderson, et al., 2006) (Schenker, et al., 1998). Organic dust can range in size from very small particles (<5µm) to greater than 30µm in diameter (Donham, 1986). Due to the large size distribution, organic dusts can penetrate and affect all regions of the lung, causing inflammation that may influence the occurrence of occupational respiratory diseases (Schenker, et al., 1998) (Rask-Anderson, et al., 2006). Symptoms associated to organic dust exposure that many research studies have documented are nose and throat irritation, cough, shortness of

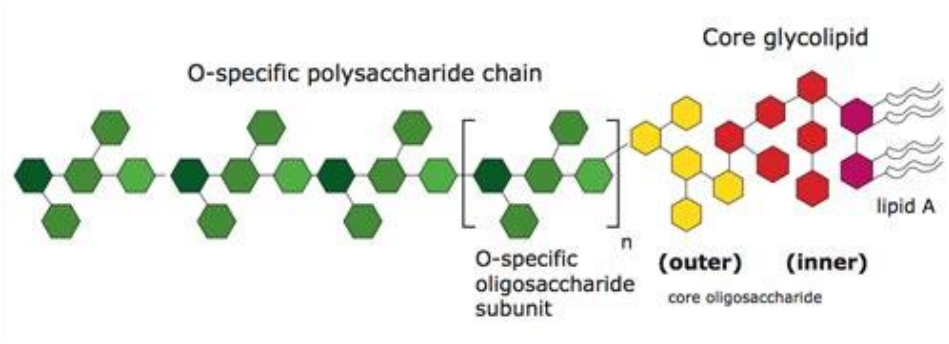
breath, and wheezing (Rylander, et al., 1989) (Rylander, et al., 2006) (Kullman, et al., 1998). Organic dusts in swine and dairy noted decreased lung function with decreases in forced expiratory volume in one second (FEV_1) as well as forced expiratory flow (FEF) (Cormier, et al., 1991) (Choudat, et al., 1994) (Donham, et al., 1995) (Donham, et al., 2000). Most of the guidelines regarding agricultural dust pertain to grain dust (oat, barley, and wheat) and a standard for organic dust found in animal confinement operations does not currently exist. The current TLV for organic dust falls under the term 'particulates not otherwise specified' (PNOS) due to the little data that exists. For PNOS to apply, the organic dust needs to be proven as insoluble or poorly soluble and have low toxicity (ACGIH, 2010). The TLV for PNOS suggest that airborne concentrations be kept under $3mg/m^3$ for respirable particulates and under $10mg/m^3$ for all inhalable particulates (ACGIH, 2010). The Occupational Health and Safety Administration (OSHA) have similar permissible exposure limits (PEL) regarding 'particulates not otherwise regulated'. According to OSHA, respirable particulates should be kept under $5mg/m^3$ and total particulate concentrations not to exceed $15mg/m^3$ (OSHA, 1989). Using these standards does not take into account the type of dust (i.e. organic, inorganic) or where the dust may have come from. A proposed occupational exposure guideline (OEL) of 2.4 mg/m^3 for total dust has been recommended to prevent adverse work-related health effects in swine and poultry production, but it has not yet been adopted. This proposed OEL is based on consistent findings using regression models that correlate dust exposure with associated decreases in forced vital capacity (FVC) and FEV_1. (Burch, et al., 2010) (Reynolds, et al., 2009) (Donham, et al., 2000). Research supports the conclusion that organic dusts have adverse effects on employee health and that the current safety

guidelines do not accurately protect the worker from such exposures (Schenker, et al., 1998) (Rylander, et al., 1989) (Christiani, 1996).

Endotoxin

Endotoxin exposure has been acknowledged to adversely effect lung function (DeMaria, et al., 1980) (DECOS, 2010) (Rylander, et al., 1998) (Zejda, et al., 1994). Influenza-like symptoms, shortness of breath, chest tightness, airway inflammation, cough, and decrease in FEV_1 and FEF have all been reported associated with endotoxin exposure (Kirkhorn, et al., 2000) (Rylander, 2002) (Smit, et al., 2008) (Donham, et al., 2000). Endotoxins are noninfectious, heat stable lipopolysaccharides (LPS) that are found on the outer cell wall of Gram-negative bacteria (Rylander, et al., 1982) (Rylander, 1985). Endotoxins are frequently found as part of organic dust in animal operations (Reynolds, et al., 2009). Inhalation of endotoxin containing material or the endotoxin itself induces lung inflammation by eliciting an immune response within the respiratory system at a cellular level (Poole, et al., 2010). Lipid A, the biologically active lipid part of the LPS, is hypothesized to be the cause of adverse health effects associated with endotoxin exposure (Kirkhorn, et al., 2000) (Burch, et al., 2010). See figure 2.1 for visual representation of endotoxin and lipid A structure.

Figure 2.1: Gram-negative bacterial endotoxin (lypopolysaccharide) structure (BIOMIN, Herzogenburg, Austria).



Lipid A is made up of hydroxylated fatty acids of varying lengths. Previous studies have used endotoxin's 3-hydroxy fatty acid (3-OHA) agents as chemical markers of endotoxin in environments (Burch, et al., 2010). The internalization of endotoxin (LPS) in macrophages and neutrophil cells results in local production of inflammatory cytokines in the lung that may migrate into the blood, possibly causing some of the suspected systemic reactions (DECOS, 2010) (Rylander, 2002). This migration of cytokines into the lung and possibly the blood may lead to the observed clinical effects of airway inflammation, bronchoconstriction, toxic pneumonitis, and ODTS (Rylander, 2002). Guidelines regarding total dust (TLV, PEL) leave room for error when applied to organic dusts containing endotoxin because the biological activity of the airborne particulates in the dust is not taken into account (Barber, et al., 1992). Guidelines specifically regarding endotoxin exposure in the workplace have been suggested, but a regulation has yet to be set. Recommendations made by the Health Council of the Netherlands suggests that endotoxin exposure not exceed 90 endotoxin units (EU)/m^3 as an 8 hour time weighted average (TWA) (DECOS, 2010). In swine and poultry facilities, recommended limits for total endotoxin exposure are 614 EU/m^3 and 900 EU/m^3,

respectively (Donham, et al., 1995) (Reynolds, et al., 1996). Currently there are no specific recommendations made for dairy facilities. Lack of data regarding endotoxin exposure and corresponding symptoms is problematic in creating a standard that will keep workers in animal operations safe (Reynolds, et al., 1996) (Rask-Anderson, et al., 1989).

Airborne Bacteria

Airborne bacteria are components of organic dusts. Little documentation about the concentration and variety of bacteria in organic dust exists for animal confinement industries (Rylander, et al., 2006) (Dutkiewicz, 1978) (Nonnenmann, et al., 2010) (Fallschissel, et al., 2010). Airborne bacterial concentrations may have an integral role to play in influencing some of the symptoms seen with organic dust exposure (Poole, et al., 2010). Until recently, understanding any kind of worker exposure to airborne bacteria required culture-based techniques (Nonnenmann, et al., 2010). A majority of bacteria found in the air cannot be grown in culture. Also, only living bacteria found in the sample will grow, underestimating the actual airborne bacterial concentrations (Nonnenmann, et al., 2010) (Fallschissel, et al., 2010) (Letourneau, et al., 2009). Due to the drawbacks associated with culture-based techniques, new studies have characterized the bacteria through the use of pyrosequencing and other molecular-biological techniques for DNA or RNA (Nonnenmann, et al., 2010) (Dowd, et al., 2008). Bacterial tag-encoded flexible amplicon pyrosequencing (bTEFAP) method has been utilized in bacterial identification and concentration of airborne bioaerosols in poultry facilities and has been used to characterize bacteria found in cattle feces (Nonnenmann, et al., 2010) (Dowd, et al., 2008). Pyrosequencing offers identification of bacteria in the sample with a very high

level of precision (≥95%) as well as being able to describe the genera and species of bacteria as percentages and counts (Nonnenmann, et al., 2010). Significant differences in concentrations observed between DNA/RNA methods and culture-based methods are credited to the possible limited detection capability of culture-based methods (Nonnenmann, et al., 2010). Currently guidelines have not been set regarding counts of bacteria (count/m^3). This study is the first to use the bTEFAP method to determine airborne bacterial concentrations in dairy parlors.

Research Studies

Symptoms of increased cough, wheezing, shortness of breath, as well as nose and throat irritation have all been reported among workers in swine, poultry, and dairy professions. A study on airway inflammation among workers in poultry houses reported that baseline FEV_1 values were significantly lower among poultry workers than controls (Rylander, et al., 2006). The study also reported a significant increase in the symptoms of dry cough, cough with phlegm, and shortness of breath among the poultry workers as compared to controls. Research regarding the respiratory health of swine producers reported an increase in symptoms of chronic bronchitis as well as increased asthmatic symptoms when compared to non-farming counterparts (Zejda, et al., 1993). Lower mean values of FEV_1 were also consistently observed among swine producers when compared to non-farming controls after controlling for age, height, and smoking. A five year prospective study on respiratory disease in swine confinement workers documented that 34% of workers reported episodes of ODTS when compared to blue-collar controls (Donham, et al., 1990).

Endotoxin exposure among swine farmers has been associated with decreased lung function and increased occurrence of chronic cough (Zejda, et al., 1994). Zejda *et al.* (1994) noted that respiratory response was related to the presence of endotoxin, not the dust levels, after looking at swine workers in low dust levels. A study looking at agricultural workers from various industries found a positive dose-response relationship between endotoxin exposure and adverse respiratory effects such as wheezing, shortness of breath, and cough (Smit, et al., 2008). Endotoxin moiety 3-OHA has been significantly associated with increased nasal airway inflammation (Burch *et al*. 2009). 3-OHA has also been seen to induce inflammatory cytokine responsiveness in human monocytes (Poole, et al., 2010). Donham *et al.* (1989) found a relationship between endotoxin exposure and subsequent decrease in FEV_1 in non-smoking swine confinement workers.

Average concentrations of endotoxin measured in various animal industry ranges between 220EU/m^3 and 11,443EU/m^3 (Kullman, et al., 1998) (Zejda, et al., 1994) (Smit, et al., 2008) (Saito, et al., 2009). Endotoxin concentrations calculated in dairy farms varied from 220EU/m^3 to 850EU/m^3 (Saito, et al., 2009) (Smit, et al., 2008) (Reynolds, et al., 2009). The presence and absence of symptoms observed with varying levels of endotoxin contributes to the difficulty of setting a standard regarding a safe exposure level (DECOS, 2010)

Endotoxin exposure in adulthood has been associated with many adverse health effects (Heederik, et al., 1991) (Smit, et al., 2008) (Zejda, et al., 1994). However, research supports the concept that endotoxin exposure in childhood may be preventative for development of allergies and atopy (Rylander, 2002) (von Mutius, et al., 2000) (Rask-Anderson, et al., 1989) (Ernst, et al., 2000) (Merchant, et al., 2005). A study performed

by von Mutius *et al.* (2000) found that populations of Swiss children living on farms who were exposed to endotoxin during childhood had decreased seasonal symptoms of hayfever and decreased development of atopic sensitization when compared to peers not raised on farms. Significant lower prevalence of asthma was found among children who were raised on farms as opposed to peers in the same rural area without farm contact (Ernst, et al., 2000). Rask-Anderson *et al.* (1989) found no correlation between endotoxin exposure and respiratory symptoms in adult Swedish dairy farmers. Subject choice may have influenced the lack of correlation if dairy farmers chosen were exposed to endotoxins from farm environments as children. The author did not look at childhood exposure. A study by Merchant *et al.* (2005) on animal farming operations in Iowa reported that allergies and atopy of children decreased with farming exposure, but not the prevalence of asthma. The author suggested that this most likely occurred from the different nature of Iowa farming to farming in Europe. European farms tend to be smaller and are in close proximity to living quarters. The farm in Iowa was an intensive livestock operation located away from the employee housing. Children were only exposed when brought to the farm on a visit or to help their parents. The brief and infrequent exposure to the farm environment likely contributed to the increased prevalence of asthma. Because endotoxin elicits an immune response upon inalation, it has been suggested that tolerance can be developed to endotoxin exposure and that early exposure can be beneficial in immune development (Rylander, 2002) (Rylander, 1981) (Burch, et al., 2010) (von Mutius, et al., 2000) (Ernst, et al., 2000). Today, fewer individuals are likely to grow up with exposure to endotoxin during childhood, due the decreasing number of family farms and increases in confinement operations among the animal industry.

Research performed on dairy workers in Colorado found that most of the workers did not have farming backgrounds (Roman-Muniz, et al., 2006). The movement away from small farming operations to large, intensive livestock operations limits childhood exposures and increases the likelihood that endotoxin exposure would cause the associated symptoms seen in workers of animal confinement operations (USDA, 2010) (Ernst, et al., 2000).

Another element of the complex organic dust mixture is both Gram-negative and Gram-positive airborne bacteria. Gram-negative bacteria are associated with occurrence of endotoxin, which is a part of the bacterial cell wall (DeMaria, et al., 1980). Normal air will usually contain from 10^3 to 10^4 bacteria/m^3 (Rylander, 1985) . The lung can defend against these normal environmental exposures, but adverse reactions to bacteria occur when the respiratory system is compromised or overloaded (Rylander, 1985). Histamine release and congregation of macrophages and neutrophils aid the inflammation process associated with bacterial inhalation (Rylander, 1981). Bacteria found in swine and poultry have been documented to induce precipitating antibodies and hypersensitivity pneumonitis among those exposed (Rylander, 1985) (Cormier, et al., 1990) (Cormier, et al., 1991) (Eduard, 1997). In a research study regarding biological agents, prevalence of COPD and chronic bronchitis were higher in livestock farmers than crop farmers and dairy farmers especially had significantly increased rates of COPD (Eduard, et al., 2009). Subjects ever exposed to biological dusts had an increased risk of chronic obstructive bronchitis (OR 3.19; 95% CI 1.27 to 7.97), emphysema (OR 3.18; 95% CI 1.41 to 7.13), and COPD (OR 2.70, 95% CI 1.39 to 5.23) (Matheson, et al., 2005).

Concentrations of airborne bacteria vary among the different animal confinement industries. An average of $3.3x10^5$ CFU/m^3 of culturable bacteria was found across five

types of swine housing (Chang, et al., 2001). In Milner *et al.* (2009) the average airborne bacterial exposure concentration for dairy farms was found to be $1.7x10^7$ CFU/m^3. A study looking at the swine barns reported culturable mesophilic bacteria in concentrations up to $2.29x10^6$ CFU/m^3 (Letourneau, et al., 2009). Letourneau *et. al* (2009) also used quantitative polymerase chain reaction (PCR) to identify fungi in the facility with measured success, a method similar to bTEFAP, but without the use of pyrosequencing. Use of the bTEFAP technique by Nonnenmann *et al.* (2010) yielded total bacterial counts of 7503 cells/m^3 in poultry houses. Specifically, poultry and swine production operations have been found to contain abundant levels of *Bacilllus, Corynebacterium, Enterococci, Escherichia coli, Staphylococcus,*and *Actinobacter,* with many other bacterial genera being found (Rylander, 1985) (Cormier, et al., 1990) (Nonnenmann, et al., 2010) (Eduard, 1997). Potential lung toxicity from airborne bacteria is correlated to not only the total number of bacteria but also the bacterial species involved (Cormier, et al., 1990). Data regarding counts and species of bacteria found in the dairy operations is at this time very limited.

Contaminant Control

Concern for the hazard associated with occupational exposure to dust and bioaerosols necessitates a method of control. Control of dust and other contaminants in animal confinement operations have been attempted with demonstrated successes, but prove yet to be effective for all agents involved or for the work environment. Using a self-contained breathing apparatus (SCBA) or supplied air respirator is limited in animal confinement operations because of heat and the uncomfortable nature of the device, hindering the employees working ability (Kirkhorn, et al., 2000). To use SCBAs there

must also be training and purchasing of equipment, making it costly in addition to cumbersome. The N-95 disposable respirator was found to reduce acute negative health effects when worn by workers not previously exposed in swine confinement barns (Dosman, et al., 2000). Engineering control methods should be assessed first and are usually a better option than the use of personal protective equipment (PPE) because they do not rely on employee compliance. Adequate ventilation of animal housing, decreasing the number of animals, and management of manure will generally minimize dust and endotoxin exposure, but a study performed by Kim *et al.*(2007) discovered that significant differences between total dust and airborne microorganisms was not seen when ventilation rates were changed (Millner, 2009) (Kim, et al., 2007). Kim *et al.* (2007) looked at three ventilation conditions in a pig growing/finishing facility and documented that as ventilation rates increased concentrations of total dust and airborne microorganisms did not see a statistically significant reduction. Respirable dust concentrations were controlled well by the increase in ventilation (Kim, et al., 2007).

A common dust control method tried in swine and poultry housing was the sprinkling of oil, either to the feed or air, to reduce dust generation (Barber, et al., 1992) (Ellen, et al., 2000) (Nonnenmann, et al., 2004) (Zhang, et al., 1996) (Senthilselvan, et al., 1997). Oil sprinkling has shown to be an effective method in reducing total dust and endotoxin levels, but not microbial concentrations. Barber *et al.* (1992) found that the addition of soybean oil to pig feed significantly reduced overall dust levels, but respirable dust fractions were not affected. This observation has led to hypothesis that oil will not affect the reduction of particle sizes smaller than 5.0μm (Barber, et al., 1992). There was also no significant difference in microorganism counts between the oil and no oil

treatments according to Barber *et al.* (1992). A 52% reduction in total dust levels was seen in piggeries when canola oil was sprayed at an amount of ~8g/pig/day (Nonnenmann, et al., 2004). Oil sprinkling in a swine confinement building effectively reduced dust concentrations from 37-89% depending on the oil application rate, according to Zhang *et al.* (1996). The more oil that was sprinkled, the more dust control was observed, but slippery walkways were observed with a daily dosage of $20mL/m^2$. The author suggests the application of $10mL/m^2$ of oil to effectively control the dust and minimize any safety hazards (Zhang, et al., 1996). Reductions in endotoxin and dust concentrations were seen in swine barns upon sprinkling of canola oil throughout the facility (Senthilselvan, et al., 1997). Application of oil sprinkling on the floor of the swine facility once a day decreased endotoxin levels (EU/m^3) from 3,983.5±498.3 to 452.3±65.8 (Senthilselvan, et al., 1997). Similar drops were seen in dust concentrations (mg/m^3) from 2.41±0.09 down to 0.15±0.02. Respirable dust concentrations were not measured in this study to see if oil sprinkling was effective (Senthilselvan, et al., 1997). Poultry houses also saw a reduction of 65% in the total dust after spraying with 10% oil and pure water mixture (Ellen, et al., 2000). Oil has shown to be very effective in controlling dust concentrations in animal confinement facilities.

In poultry houses, the use of electrostatic precipitators has been shown to reduce dust from house emissions with 40-60% efficiency (Takai, et al., 1998) (Chai, et al., 2009). Poultry facilities have seen some dust reduction with the use of electrostatic space charge systems (Millner, 2009). A reduction of *Salmonella enteritidis* from 89% to 39% was found on the feathers of chicks with the use of an electrostatic space charge system downstream (Gast, et al., 1999). Reduced levels of airborne dust particles were also

observed with the presence of the electric space charge system (Gast, et al., 1999). Wet scrubbing devices can be used to capture particles and have been shown to be efficient at removing dust from the air (Takai, et al., 1998). Unfortunately, both electrostatic precipitators and wet scrubbers can be impracticable when applied to this work environment. The size of the system that would be required to effectively handle the large volumes of air in modern livestock buildings would be costly and energy intensive (Takai, et al., 1998). Reducing dust particles as well as airborne microorganisms would help in decreasing the spread of disease between animals or between animals and humans.

Summary and Research Needs

Studies performed by Reynolds *et al*. (2009) and Burch *et al.* (2010) have reported respiratory illnesses among employees in the dairy industry. Studies of the dairy environment have observed high exposures to organic dust and bioaerosols, which have been shown to increase lung inflammation and decrease airway function in swine and poultry (Rask-Anderson, et al., 2006) (Heederik, et al., 1991) (Donham, 1986). Airborne bacteria are found inorganic dust and are either Gram-negative or Gram-positive in origin. Much data has been published regarding endotoxin from Gram-negative bacterial cell walls, but there is a lack of research regarding the true diversity of exposure to Gram-positive bacteria and other microbial constituents. Poole *et al*. (2010) published information confirming the importance Gram-positive bacteria play in pulmonary response. Because endotoxin and airborne bacteria (Gram-negative and positive) both are biological agents found in organic dust, research needs to be aimed at making a

distinction between the exposures, understanding how each may affect the lung (Eduard, et al., 2009).

In addition to a better understanding of the role endotoxin and airborne bacteria play in the pulmonary response, additional research needs to be done to look across the current testing methods and view possible inconsistencies. Research has been done comparing various testing methods for endotoxin, but little research has been done to compare airborne bacterial testing methods using RNA or DNA (Saito, et al., 2009) (Reynolds, et al., 2005) (Thorne, et al., 2010). There is a research need to identify and better understand the concentration and variety of the bacteria that make up organic dusts in various animal confinement industries. Studies using different testing methods for bacteria are difficult to compare, and the current culture based methods have limitations. New molecular biological methods using DNA/RNA offer significant advancement in the area of bacterial identification, but their applicability bioaerosol sampling needs refinement. The bTEFAP method for characterizing and quantifying bacteria appears to be a solution in airborne bacterial identification, but further testing using this method needs to occur to test its reliability and accuracy. The bTEFAP method should additionally be compared to other standard bioaerosol techniques such as Q-PCR, fluorescence, and culture-based.

Control of dust and bioaerosols in the swine and poultry environment have been studied in detail, with possible methods showing great potential, but such methods have not been evaluated in the dairy environment. The dairy facility has shown similar exposures to respiratory hazards that have been controlled in swine and poultry. Another research focus regarding occupational health exposure in the dairy environment should be

evaluating if the effective control methods in swine and poultry would be effective in the dairy industry as well.

CHAPTER 3

PURPOSE AND SCOPE

Industrialized dairy operations have made the milking process very streamlined and efficient, keeping operations running 24hrs per day, seven days a week. Cows are pushed through the milking process at high rates, requiring employees to stay in confined environments until their shift (8-10hrs) is finished. Research has demonstrated that workers in animal confinement operations suffer from increased respiratory health issues; however, it is not well known how ventilation changes in such facilities will affect the quantity, dispersion, or growth of organic dusts, endotoxins, and bacteria in the air. Understanding how changes in the ventilation will affect the concentrations of such exposures is essential in reducing exposures and preventing respiratory illness.

The purpose of this study was to evaluate how different seasonal changes in the ventilation of a dairy facility would affect the aerosol size distribution as well as the concentrations of organic dust, bacteria, and endotoxin in one dairy parlor. Results will facilitate design of further comprehensive research on reducing exposures to these contaminants. The project also proposed to evaluate the application of pyrosequencing to air sampling environments to characterize Gram-positive and Gram-negative airborne bacteria. This pilot study data will facilitate the design of more comprehensive studies

regarding respiratory disease among dairy workers and take the next step to begin identifying and evaluating effective interventions to reduce occupational respiratory disease.

CHAPTER 4

MATERIALS AND METHODS

Background

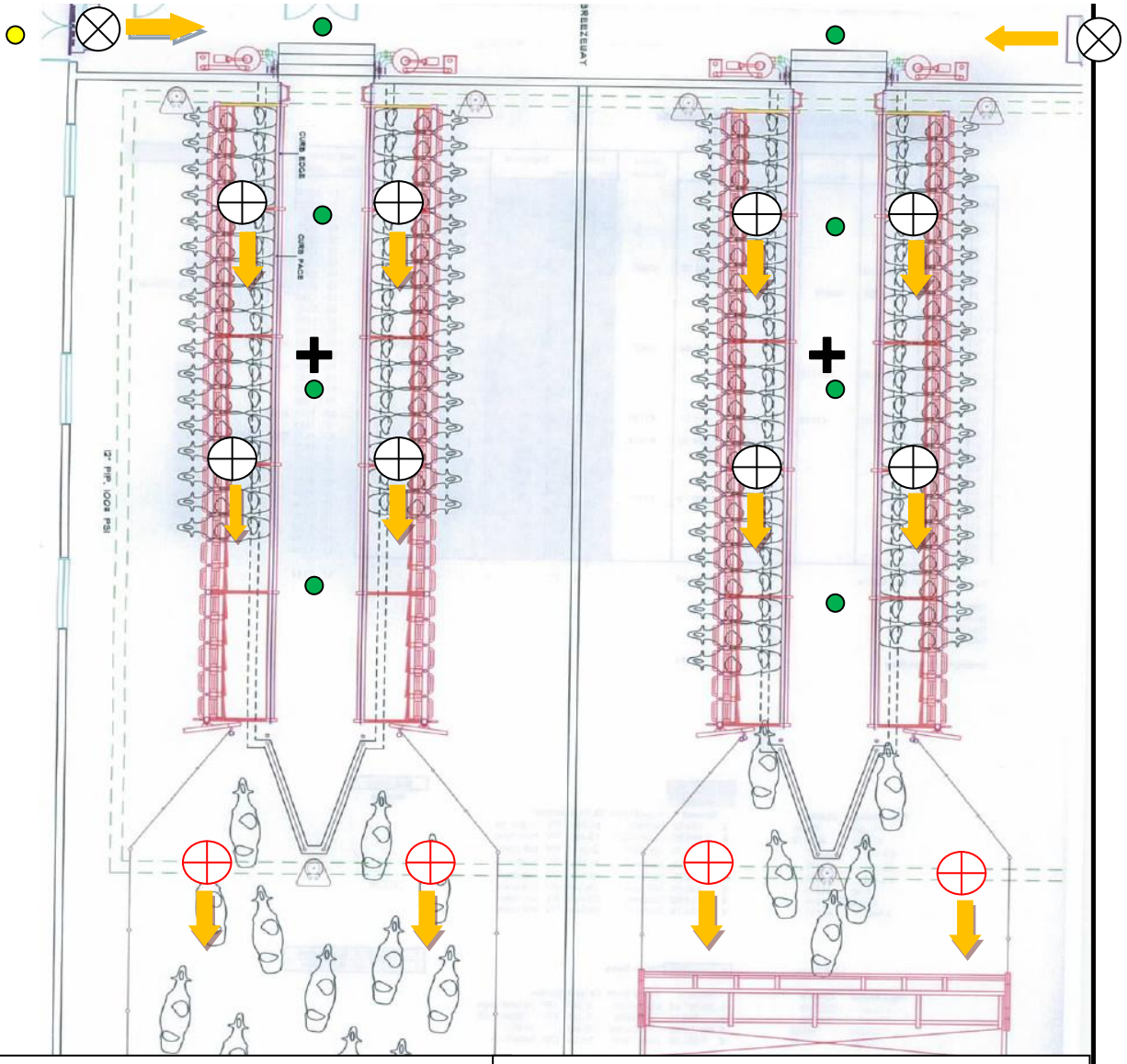
All data collection was performed at one modern milking parlor located in Colorado. Five sampling trips were performed approximately weekly during July 2010 to September 2010. These five sampling trips established baseline data for warm conditions, when the parlor has all fans running and curtains open to outside air movement. The second five sampling trips were performed biweekly in December 2010, and established baseline data for the colder season, which represented a worst case scenario. During the colder months, the facility is completely closed, fans are off, and heaters occasionally run. Comparisons were made between different seasons to understand how these seasonal ventilation changes effect the air movement and concentration of airborne endotoxin, microbial, and dust.

The study dairy is one of four commercial dairies operated by the same company. The study facility employs roughly 64 employees and cares for 4,900 head of cows. Cows are milked three times per day in three shifts during a 24 hour period. The milking parlor is two Double 24 Parallel Parlors side by side. The facility is enclosed and employees use automated milking equipment. The milking parlor area opens into a holding barn, which is fully covered except for an opening in the roof for fresh air, and

moveable curtains halfway up the exterior walls to allow air movement when necessary or to hold in heat. Heavy plastic curtains separate the milking alley from the holding pen. Mechanical ventilation in the facility consisted of eight stationary axial fans above the milking alley to move air in the facility (2 hp, 36 inches in diameter). Two large tunnel fans were located in the front walkway drawing air from outside into the facility (1 hp, 46 inches in diameter). In the eaves separating the milking parlor from the holding pen were four tunnel fans (1hp, 36 inches in diameter) pulling air from the milking parlor into the holding area. See Figure 4.1 for a blueprint of the milking parlor with fan locations as well as sampling locations.

All sampling was performed by the same individual to minimize potential sampling bias. Sampling was performed for four hours and conducted during 1st shift (7am-3pm), specifically from 8am-12pm. All sampling pumps were hung from tripods approximately four feet high and placed in the middle of the milking alley, approximately 30ft into the milking alley and 30ft from the back wall of the milking alley heading into the holding area. See Figure 4.1 for a visual diagram of sampling locations. For the rest of the discussion the label of 'worker zone' designates the milking alley way where the employees work.

Figure 4.1: Location of fans and sampling locations.



Key: Fan Locations at dairy parlor

= Axial Fan (2 hp, 36 inches in diameter)

= Tunnel Fan (1 hp, 46 inches in diameter)

= Axial Fan (1hp, 36 inches in diameter)

= Direction of fan air stream

Key: Approximate Sampling locations at dairy parlor

=Location where anemometer reading was taken

= Location where outside measurements of temperature, humidity, and CO_2 were taken

= Location where tripod was set up for all area samples and where PAS measurements were taken. Also where measurements of indoor temperature, humidity, and CO_2 were taken

All preparation for sampling visits was performed in the Industrial Hygiene Laboratory of Colorado State University, Fort Collins, Colorado. All pumps were pre and post-calibrated using a Bios Dry-Cal Lite Primary Flow Meter (Bios, Butler, NJ). Pumps were calibrated to 4.0L/min if used for an inhalable button sampler and to 2.5L/min for use with a cyclone sampler. Pump numbers and calibration flow rates, button sampler numbers, cyclone sampler numbers, microbial sample numbers, filter pre-weights and post weights, as well as all environmental conditions during the sampling visit were documented on a sampling data sheet (Appendix A).

Dust Collection

Inhalable dust samples were collected using 25mm SKC Button aerosol samplers (SKC Inc., Eighty Four, PA) attached to personal sampling pumps (SKC AirCheck XR5000, Eighty Four, PA) calibrated at 4.0 L/min. The PVC filters (5.0µm, SKC Inc., Eighty Four, PA) were weighed prior and post sampling to determine mass of dust collected. Pre and post-weighing occurred after allowing filters to dry in a dessicater for 24 hours prior to weighing and post sampling to remove moisture. All weighing was performed using a Mettler MX5 analytical microbalance (Mettler Toledo, Sercom, Columbus, OH). One sample was collected in each worker zone and a field blank was used for control. Field blanks were suspended in the same area as the sampling occurred. Dust concentrations were reported as mg/m^3.

Respirable Dust

Respirable dust samples were collected using 37mm aluminum cyclone samplers (SKC Inc., Eighty Four, PA). The PVC filters (SKC, 5.0µm) were handled identically to the Button sampler filters. Personal sampling pumps (MSA Escort ELF, Pittsburgh, PA) attached to cyclone samplers calibrated at 2.5 L/min were placed in each worker zone. A field blank was again used as a control. Respirable dust samples were reported as mg/m^3.

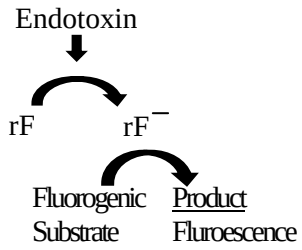
Aerosol Size Distribution

Particle size distributions were measured using a Grimm portable aerosol spectrometer (Grimm Industries, PAS 1:108, S/N 8F0020008, Douglasville, GA). The Grimm meter was placed on top of the tripod in each worker zone and allowed to run for an average of 15 minutes per sample. Two samples were taken in each worker zone during the four-hour sampling interval. A total of four measurements were made each trip. The meter could not be kept running for the full sampling period in the facility due to possible water damage to the instrument. Grimm Dust Monitor Software (#1177/01 v3.0) was used to obtain dust size mass distributions. Distributions were used to calculate the mass mean optical diameter (MMOD) and the geometric standard deviation (σ_g). The MMOD is the center of the dust size distribution where 50% of the particle size is above the calculated MMOD and 50% is below the MMOD value on a log scale. The σ_g identifies the variation of the particle size in the distribution. Formulas for calculating the MMOD and σ_g can be found in Appendix B. MMOD and σ_g for trips 01, 02, and 03 are not available due to malfunction of the instrument. MMOD values were reported in µm.

Endotoxin

Filters used to collect dust in both the cyclone and button samplers were placed in sterile 100mL tubes and frozen (0°F) after post weighing for later endotoxin analysis. Endotoxin analysis was performed using the Pyrogene Recombinant Factor C (rFC) Endotoxin Assay (Lonza Inc, Walkersville, MD). Factor C (rFC) is activated by endotoxin binding. The activated enzyme acts upon the fluorogenic substrate to produce product fluorescene. Figure 4.2 represents the pathway of endotoxin detection using the rFC method.

Figure 4.2: Visual representation of the rFC endotoxin detection method of the assay (Lonza Inc, Walkersville, MD).



The level of fluorescence measured is proportional to the endotoxin concentration in the sample. The sample endotoxin level is calculated relative to a standard curve. All samples were extracted in 10 ml certified pyrogen-free (PF) water (Lonza, Inc.) containing 0.05% Tween 20 for 1 hr at 22°C with continuous shaking. Serial dilutions of endotoxin standards (*Escherichia coli* O55:B5; Lonza, Inc.) and sample extracts were prepared using sterile, PF water with Tween 20. Samples were added to a 96-well plate followed by 100 µl of a mixture of enzyme, buffer, and fluorogenic substrate (Thorne, et al., 2010). All samples, control and regular, were run in triplicate to ensure quality control. Cyclone and blank samples were run at full strength due to low dust

concentrations. Button samples were run on a 1:25 dilution. Plates were incubated at 37°C for 1 hr and read in a fluorescence microtiter plate reader (Biotek Instruments, Winooski, VT, USA; FLX800TBIE) at excitation/emission 380/440 nm (Saito, et al., 2009). Background fluorescence was subtracted. Four assay reagent blank wells served as control and reference for the pf status of the reagent water, centrifuge tubes, pipette tips, and microplates (Saito, et al., 2009) (Thorne, et al., 2010). Quality assurance spiking assays were run with samples to assess matrix interference or enhancement (Thorne, et al., 2010). Results were given in endotoxin units (EU). Triplicate results were further analyzed using Excel (Microsoft, 2010) to observe if all triplicate results were within three standard deviations of the mean. Blanks were subtracted from sample results and ending values were divided by the volume of air to report data in EU/m^3.

Airborne Bacteria

Sampling for bioaerosols was performed using Button samplers with a specific 3.0µm gelatin filter (SKC, Product # 225-9551, Eighty Four, PA). Gelatin filters arrived with 10 packages of five filters and were refrigerated (38°F). A new five pack of filters was used each sampling trip to minimize contamination. To inhibit possible contamination, Button samplers were washed in a 70% ethanol solution prior to filter application and sampling. Gelatin filters were suggested to be kept refrigerated (38°F) by the manufacturer until use and were transported on ice to and from sampling location. Counters were wiped down with a 70% ethanol solution before filters were applied to the cleaned button. Forceps used for application were wiped with a new ethanol wipe between samples. All filter loading for microbial sampling was performed the night before sampling occurred. Buttons with filters were then placed in personal Whirlpak

bags (Nasco, 4oz) to prevent contamination and placed in refrigerator (3°F) overnight. Immediately post sampling, buttons with the sampled filters were returned to their designated Whirlpak bag and transported on ice back to the laboratory. Counters were again cleaned using 70% ethanol before filters were removed from buttons. Filters were placed in sterile 100ml tubes and frozen (0°F) until shipping for analysis. Appendix E contains standard operating procedures (SOPs) used for airborne bacterial collection. Again, forceps were cleaned using a new ethanol wipe between each sample. To control for the possible contamination on the first five trips, bacterial concentrations found on the blanks were subtracted from the sample counts.

Due to the possible contamination identified in the first five sampling trips, a more stringent cleaning method was employed between sampling trips to ensure no contamination would occur in winter season samples. Button samplers were allowed to soak for 20 minutes in a bleach solution (Bleach-Rite Disinfectent, MarketLab, Caledonia, MI) before being autoclaved prior to sampling. Buttons were autoclaved in personal autoclave bags. The autoclave cycle consisted of 20 minute dry sterilization (121°C) with 20 minutes of drying. Filters were applied to autoclaved button samplers the night before sampling and all filter application and removal from the buttons was performed in a biohazard hood. Forceps were wiped with a new ethanol wipe between each sample. Samplers were again placed in personal Whirlpak bags during transport to prevent contamination. The same sterile care was taken post sampling when filters were removed from buttons and placed in sterile 100ml tubes. Appendix D contains the SOP used for winter season collection. Tubes were then placed in freezer (0°F) until ready to ship.

Pyrosequencing

Frozen airborne bacterial samples were overnighted on ice packs to the Research and Testing Laboratory in Lubbock, TX, for pyrosequencing. Samples were overnighted containing only the filter inside the sterile tube, no reagents were added. Only samples belonging to trips one and two were overnighted with 10ml of DNAse free water added before shipment, but due to leakage of one of the containers, the addition of water was discontinued prior to shipment of other samples. Samples were analyzed using the bacterial tag-encoded flexible (FLX) amplicon pyrosequencing (bTEFAP) technique as previously described (Dowd, et al., 2008) (Nonnenmann, et al., 2010). The genomic DNA collected by the gelatin filters was extracted in 500µl RLT buffer in a Qiagen TissueLyser (Qiagen, Valencia, CA),followed by a DNA spin column, and quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France) (Nonnenmann, et al., 2010). Massively Parallel Titanium Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as previously described, utilizing titanium reagents and a one-step PCR, mixture of Hot Start and HotStar (Qiagen, Valencia, CA) high fidelity taq polymerases, and amplicons originating from the 27F region numbered in relation to E.coli rRNA (Dowd, et al., 2008). Following sequencing, all failed sequence reads and low quality sequence ends and tags were removed, and sequences were depleted of any non-bacterial ribosomal DNA sequences and chimeras using custom software and Black Box Chimera Check software B2C2 (Research and Testing Laboratories, LLC, Lubbock, Texas). Sequences less than 300 base pairs (bp) were also removed. To determine the identity of bacteria in the samples, sequences were queried

using a distributed Basic Local Alignment Search Tool (BLASTn) .NET algorithm against a database of high quality 16s bacterial ribosomal DNA sequences derived from the National Center for Biotechnology Information (NCBI) database (Dowd, et al., 2008) (Nonnenmann, et al., 2010). Database sequences were characterized as high quality based on the criteria of the Ribosomal Database Project (version 9). Using a .NET and C# analysis pipeline (Dowd, et al., 2008), the resulting BLASTn outputs were compiled, validated using taxonomic distance methods and data reduction analysis as previously described (Dowd, et al., 2008) (Nonnenmann, et al., 2010). Results were obtained in both genera and species of bacteria. Of the genera reported, inhalable concentrations were calculated through dividing the cells counted by the volume of air sampled to report cell counts/m3. Individual species that accounted for the majority of a particular genus were also reported. Estimates of total bacteria were calculated by adding the total number of sequences identified for the bTEFAP procedure (Nonnenmann, et al., 2010).

After data was broken down into cell counts per sample by genera, data was further analyzed using Excel. Genera containing ≤ 3 cells were eliminated. Counts were then summed and divided by the quantity of air sampled per sample for counts/m^3. The 10 bacterial genera with the highest counts were reported per season. Gram-negative bacteria was calculated by identifying using the highest 20 genera in each sample, identifying whether Gram-negative or positive and then adding those 20 sample counts. Data is recorded for each sampling trip and worker zone located in parlor from where the sample was taken. Data is reported in bacterial cell counts/m^3.

Quality Control Pilot

Because of the possible contamination suspected in trip five of the first round of sampling, a control project was performed before sampling began on the winter conditions to test where possible contamination may have occurred. Samples of de-ionized water, ethanol, nuclease-free water, and LAL reagent water were placed in sterile, 100mL centrifuge tubes, shipped, and analyzed as described before for bTEFAP analysis. Gelatin filters from new and old packages were also placed in 100mL centrifuge tubes as well as filters from buttons on which were performed three different cleaning methods, which were also sent in for bTEFAP analysis. Cleaning methods included washing the button in 70% ethanol using various waters before applying a filter as well as autoclaving the button before filter application. Sample filters that were placed on differently cleaned buttons were left out at room temperature for four hours inside an individual Whirlpak bag to simulate handling conditions, but not allow airborne contamination from the laboratory. All work regarding filter loading and unloading was performed in a biohazard hood to control for room contamination. Forceps were wiped with a new ethanol wipe between samples. Samples were shipped and analyzed as stated above. Data received was also analyzed as stated above. A detailed chart with sample ID and corresponding sample can be found in Table 4.1.

Table 4.1: Quality control sample ID with corresponding sample sent in for bTEFAP analysis.

Sample ID	Sample type
01113010	DI water 10ml
02113010	Qiagen nuclease free water lot # 430135350
03113010	Lonza LAL reagent water lot # EL0795 exp:12Apr08
04113010	Fisher 100% reagent ethanol lot # 056671-36 exp: Jan. 2009
05113010	Fisher Absolute Ethanol 200 proof Lot # 107005
06113010	Autoclaved buttons before filter was added, let sit out at room temp for 4 hrs. Filters from new package SKC gelatin filters lot # 030912602090048
07113010	Autoclaved buttons before filter was added, let sit out at room temp for 4 hrs. Filters from new package SKC gelatin filters lot # 030912602090048
08113010	Filter from new package of SKC gelatin filters lot # 030912602090048
09113010	Filter from new package of SKC gelatin filters lot # 021012602090104
10113010	Filter from old opened package of SKC gelatin filters lot # 030912602090048
11113010	Washed button using Qiagen nuclease free water (lot# 430135350) and 200 proof ethanol (lot# 107005) (30%/70% mix). Used newer filters, new 5pack, lot # 021012602090104
12113010	Washed button using Qiagen nuclease free water (lot# 430135350) and 200 proof ethanol (lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104
13113010	Washed button using Lonza LAL reagent water (lot# HL0277 exp:12sep2010) and 200 proof ethanol (Lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104
14113010	Washed button using Lonza LAL reagent water (lot# HL0277 exp:12sep2010) and 200 proof ethanol (Lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104

Parlor Conditions

Measurements of the temperature, humidity, carbon dioxide (CO_2), and carbon monoxide (CO) concentrations were also taken during every sampling trip in each worker zone as well as outside the facility. Measurements were obtained using a Q-TRAK Indoor Air Quality meter (TSI, St. Paul, MN, Model# 8554, Serial# S10406, Last Factory Calibration Feb. 2010). These environmental factors were recorded once every hour in each worker zone and outside the facility during the four hour sampling period. Measurements were averaged per trip and in each worker zone. All temperature measurements were reported in °F, all humidity measurements were reported in % relative humidity, and all CO_2 and CO measurements were reported in parts per million (ppm).

Air Movement

Indoor air movement was measured using a TSI Alnor Velometer (TSI, St. Paul, MN, AVM440, Last factory calibration April 2010). Air speed (ft/min) was found for eight locations in the parlor to give an overview of air movement in the facility. Air speed measurements were averaged in each worker zone and in the breezeway with results presented in ft/min.

Statistical Analysis

Statistical analysis was performed using SAS (Enterprise Guide 4.2). Statistical inferences were based on a $P<0.05$ level of significance. Based upon plots of residuals versus predicted values, data where residuals appeared normal and independent were

analyzed using a two way analysis of variance (ANOVA) test. For data where the residuals did not appear normal or independent, a log10 transformation was then applied for correction before being analyzed by a two way ANOVA. Correlations programs in SAS were also used to determine if possible relationships existed between variables.

CHAPTER 5

RESULTS AND DISCUSSION

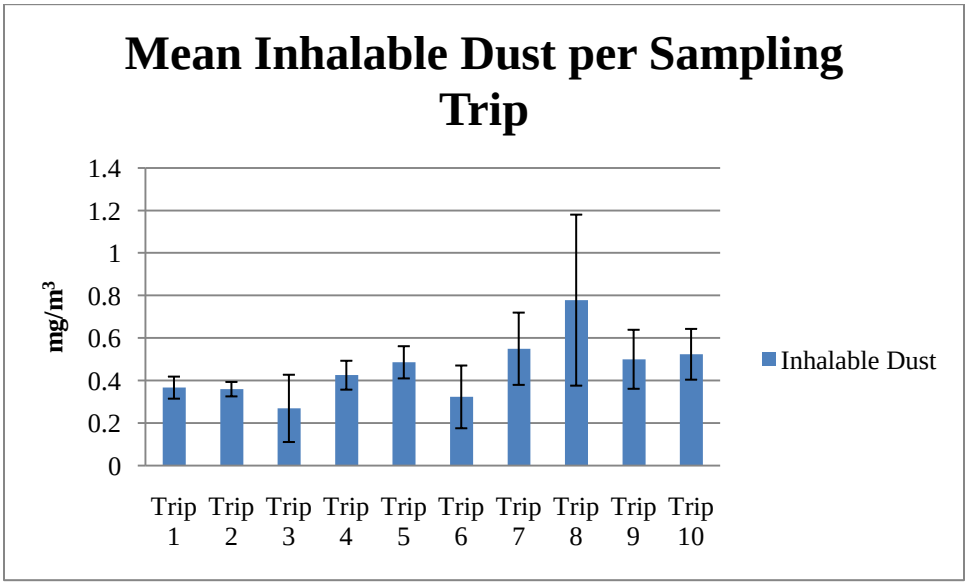
Overview

The intent of this research project was to collect data that would aid in understanding of how different changes in the seasonal ventilation of a dairy facility would impact the concentrations of organic dust, bacteria, and endotoxin in dairy parlor environments. Inhalable dust, respirable dust, and Grimm PAS meter readings were useful in determining dust concentrations and size distributions at the facility, which could be compared upon changing the ventilation in the building. Analysis for endotoxin concentrations in the facility helped to determine what the baseline exposure level was for employees working in dairy environment and how that may change depending on conditions in the facility. The application of pyrosequencing for bioaerosols is relatively new and offers a more extensive identification of the Gram-positive and negative bacteria that make up air samples, as well as increase the ease of determining concentrations of the bacteria. Currently not much is known about microbial concentrations in the air of facilities or how worker health could be affected, but knowledge regarding the type and concentration of bacteria can lead to development of occupational exposure guidelines for workers.

Inhalable Dust

Inhalable dust concentrations averaged over all sample locations for each trip can be found in Figure 5.1.The mean inhalable dust level and standard deviation (σ) for summer and winter was 0.38mg/m^3 and 0.53mg/m^3 with 0.10 and0.22 respectively. No statistically significant difference was found between seasons (p=0.096) or worker zones in the facility (p=0.498). Inhalable dust data did not require log transformation and plots of residuals did not improve upon log transformation. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the blanks. The LOD and LOQ were determined by finding the standard deviation of all blank samples and multiplying by three and ten, respectively. Only one sample was below the LOQ (0.30mg) while all samples were above the LOD (0.09mg).

Figure 5.1: Mean inhalable dust concentrations by sampling trip. Error bars indicate ± each sample standard deviation.



The current TLV for total dust is 4mg/m^3 (ACGIH, 2010). The PEL as stated by OSHA for PNOR as total dust is 15 mg/m^3 (OSHA, 1989). We sampled inhalable dust in this study, making it more difficult to compare our concentrations to a published guideline. Research using a button sampler in the dairy environment revealed that the sampler behaved more like a total dust sampler, which is most likely due to the inhalable range size distribution found (MMAD=7.7µm) (Reynolds, et al., 2009). This value differs from the distribution found in our dairy facility (MMOD=12µm). The inhalable dust values measured in our facility are well below any published guideline and are much lower than what has been found in poultry, swine, and other dairy facilities (Zejda, et al., 1994) (Ellen, et al., 2000) (Kullman, et al., 1998). Our average dust concentration for summer and winter were 0.38mg/m^3 and 0.53mg/m^3, respectively. Kullmen *et al*. (1998), found dust concentrations of 0.74mg/m^3 for total dust and 0.07mg.m^3 for respirable fractions. This is very similar to the concentrations for both inhalable and respirable dust found in this study. Previous studies have noted that total dust patterns are not influenced greatly by the change in ventilation rate due to gravity and particle size/weight (Kim, et al., 2007). Dust inside livestock buildings is not uniformly distributed and dust distribution depends on several factors. Dust sources, the airflow characteristics, animal activities and worker activities will all have an effect on particles in the air (Takai, et al., 1998). The dairy facility where the samples were collected is a newer facility, built in 2006. Care and planning was taken in the design and implementation of the building to provide the best possible environment for both animal and employee.

Aersosol Size Distribution

The Grimm PAS data values were fairly consistent throughout the entire sampling period. Mean values for the MMOD and σ_g by season are presented in Table 5.1. There was no significant difference between the means by season (p=0.84) or worker zones (p=0.43). The summer season consisted of only two sampling trips due to equipment malfunction (trips four and five) but there is no evidence to support that comparison would be significant if sample sizes per group were equal. All results for Grimm dust meter have been included in Appendix C.

Table 5.1: Average MMOD and σ_g by season.

Season:	MMOD (µm)	σ_g
Summer	11.8±3.7	3.35±0.27
Winter	12.1±2.4	2.83±0.39

The distribution of dust in the facility (MMOD and σ_g) is similar to what has been found in other dairy facilities. The average MMOD value found in this study was 11.8µm for the summer and 12.1µm for the winter, with an overall average of 12µm. Reynolds *et al*. (2009) published values found in a dairy smaller than our distribution (MMOD=7.7µm). Kullman *et al.* (1998) found in dairy environments a mass mean aerodynamic diameter (MMAD) value of 13.5µm. MMAD values in poultry facilities were mostly larger, finding 12.6µm for cage-housed bird facilities and 16.6µm for floor-housed bird facilities (Kirychuk, et al., 2008). Barber *et al.* (1991) found an average MMAD value of 14µm in swine facilities (Barber, et al., 1991). Both the MMOD and

MMAD are the value at which 50% of the particles are smaller than the MMOD/MMAD and 50% of the particles are larger than the MMAD/MMOD allowing MMOD values and MMAD values to be directly compared. The difference is whether the measurement method used optical light scattering to count the particles or a gravimetric analysis of a filter. Our distribution suggests that the dairy facility has mostly large particles (>10µm), leading to better understanding of lung deposition. For the little respirable dust data that was gained, it appeared that respirable dust concentrations were very low, which is now validated by the MMOD value, indicating that particles around 12µm are more abundant.

Respirable Dust

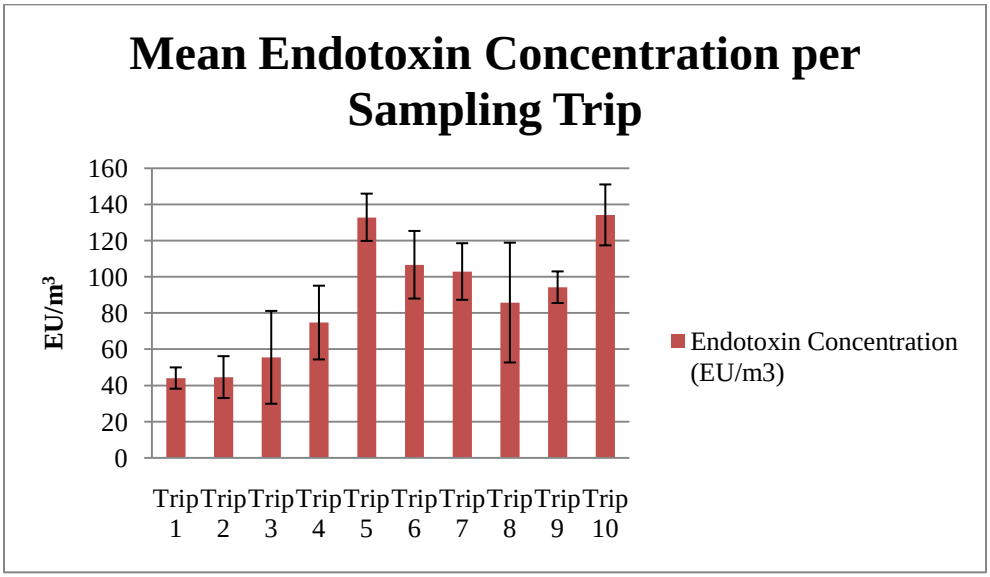
Respirable dust concentrations were found to be low and under the published guidelines by OSHA for dust exposure. The TLV for respirable dust exposure for an eight-hour workday is 3mg/m^3 (ACGIH, 2010). The OSHA permissible exposure limit for particulates not otherwise regulated (respirable fraction) for an eigth-hour workday is set at 5mg/m^3 (OSHA, 1989). All of the respirable dust concentrations found in this study were under both of these values. Respirable dust samples were very inconsistent in this study. In some cases, the 37mm PVC filters returning from sampling for respirable dust weighed less than before the sampling occurred. It is unknown what caused the drop in weight after sampling and the problem occurred for six of the 20 samples collected over the entire sampling period. Other sampling trips then had very large changes between sides of the facilities as well as between trips in the same season. Concentrations for respirable dust samples ranged from 0.008mg/m^3 to 1.2mg/^{3}, not including filters that saw a drop in weight post-sampling. The average concentration for the respirable dust samples for the summer and winter were 0.33mg/m^3 and 0.24mg/m^3 respectively (not

including samples found weighing negative values). Endotoxin analysis on respirable dust samples also yielded highly variable results (0.063-9.98 EU/m^3). Respirable dust and endotoxin appeared to have a highly variable relationship. The LOD for respirable dust samples was 0.23mg, higher than most of the actual samples. Due to the inconsistencies and extremely low values, the respirable dust samples, including respirable endotoxin, were excluded from the study and no statistical analysis was performed.

Endotoxin

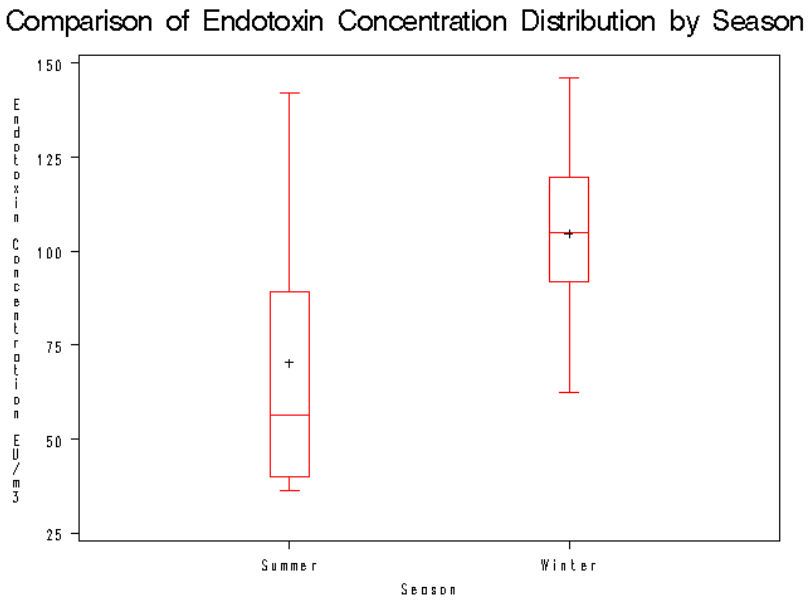
Endotoxin mean concentrations over all locations for each sampling trip can be found in Figure 5.2. When broken down by season, there was not a statistically significant difference between summer and winter values (p=0.1). There was a near significant difference found between the summer and winter (p=0.06) when data was log transformed (base 10). Plots of residuals did not improve considerably upon log transformation although the significance changed slightly with log transformation. There was not a significant difference between worker zone samples with or without log transformation (p=0.16, p=0.24). The mean endotoxin concentration for summer and winter were 70.3 EU/m^3 and 104.7 EU/m^3, respectively. All samples were above the LOD (4.3 EU/sample) and LOQ (14.4 EUsample).

Figure 5.2: Mean endotoxin concentration by sampling trip. Error bars indicate ± each sample standard deviation.



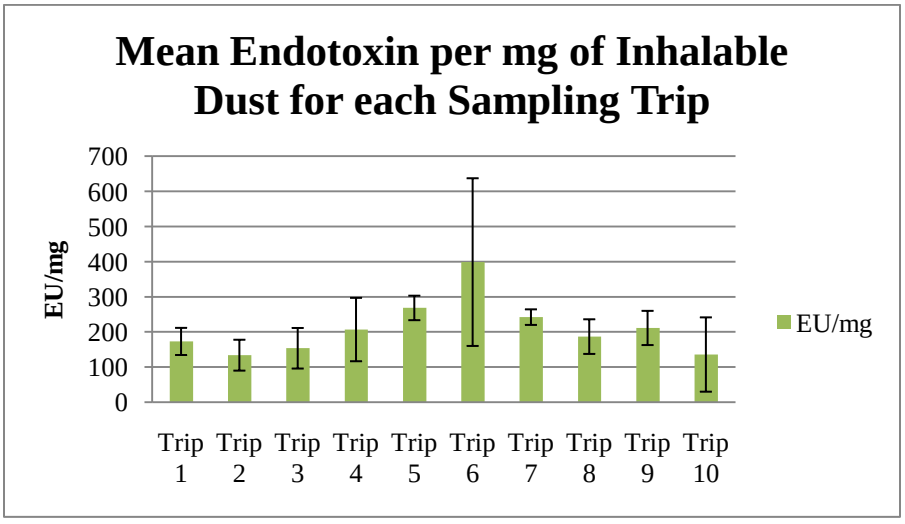
Endotoxin results were near significant when viewing summer and winter ($p=0.06$) after a log transformation. Overall endotoxin concentrations did increase during the winter months, but the larger distribution of values during the summer decreased the significance of the difference. See Figure 5.3 for the distribution of endotoxin during the different seasons. The box plot displays the range of the data (end caps) with the mean of the data as the plus sign. The median is the red line in the middle of the box and the edges of the box denote the upper quartile (25% of the values are above) and lower quartile (25% of values are below) with the box itself contain 50% of the data. All box plots in this study are formatted as stated above. Due to the facility being completely closed with less air movement, it makes sense that the overall endotoxin concentration would increase at that time, even if a statistically significant increase is not seen.

Figure 5.3: Endotoxin (EU/m^3) distribution by season.



When endotoxin is normalized by the mass of dust in the sample the increase in endotoxin suggested before is not seen. Instead, it appears that the occurrence of endotoxin follows the small increase in dust. Figure 5.4 provides visual representation of the change in EU/mg throughout sampling trips.

Figure 5.4: Changes in EU/mg throughout sampling trips. Error bars indicate ± each sample standard deviation.



The influence that the dust concentration plays on the endotoxin concentration can be better seen by comparing distributions of EU/mg and inhalable dust (mg). See figures 5.5 and 5.6 for the distributions of EU/mg and inhalable dust (mg) per season.

Figure 5.5: Seasonal distribution of endotoxin units (EU) per mg of dust

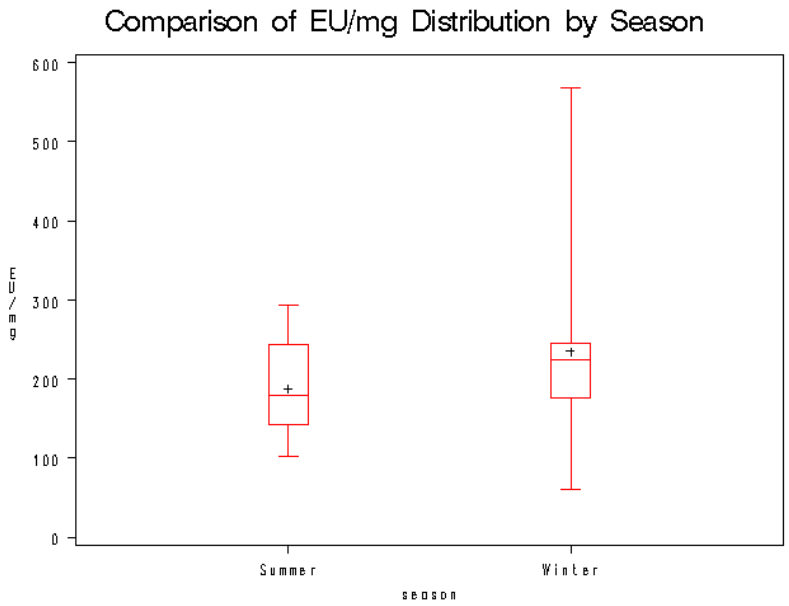
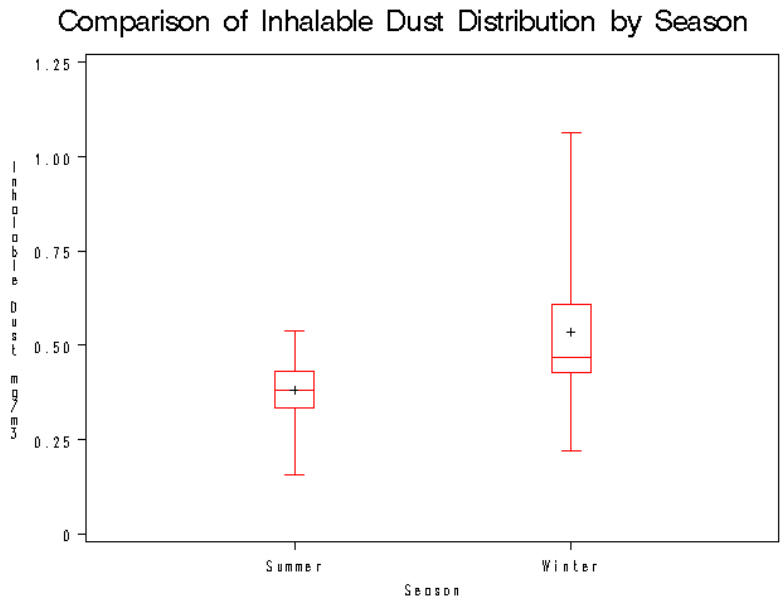


Figure 5.6: Seasonal distribution of inhalable dust



The box plot displays the range of the data (end caps) with the mean of the data as the plus sign. The median is the red line in the middle of the box and the edges of the box denote the upper quartile (25% of the values are above) and lower quartile (25% of values are below) with the box itself contain 50% of the data. These seasonal distributions are very similar to one another, indicating that dust is playing a role in endotoxin concentrations in this facility. Looking back at the seasonal distribution of EU/m^3 (Figure 5.3) and comparing to the seasonal distribution of EU/mg (Figure 5.5) indicates that the loadings of endotoxin seen in this facility do not necessarily increase into the winter, rather the dust concentration increases, allowing the concentration of endotoxin to also increase. Endotoxin adsorbs to the dust particle, and a previous study has suggested that binding preference is given to particles greater than 10µm as opposed to particles less than 10µm (Millner, 2009). If binding preference is given to particles larger than 10µm, , our aerosol size distribution (MMOD=12µm) appears to be effective for endotoxin binding, then explaining why the endotoxin concentration only seemed to increase when the dust concentration increased Other studies have found that endotoxin may not correlate to dust concentrations, but this study appears to suggest that they do (Millner, 2009) (Kirychuk, et al., 2010).

Endotoxin concentrations (EU/m^3) measured in this facility were extremely low when compared to what has been found in swine and poultry facilities and slightly lower than other dairy facilities (Reynolds, et al., 2009). The average winter concentration found for in this dairy facility was 104.7 EU/m^3, whereas Smit *et al.* (2008) presents an average value of 220 EU/m^3. Values greater than 800 EU/m^3 have been documented in dairy barns in Colorado and Nebraska (Reynolds, et al., 2009). Again, the lower

endotoxin concentrations seen in this study could be attributed to the newer construction of the facility. We assume there would be a small degree of correlation between endotoxin and dust concentrations due to endotoxins adhering to dust particles in the air. Airborne bacteria and endotoxin have shown a preference for adhering to particles >10µm, which explains the very low endotoxin results found in our respirable dust samples (range 0.06-10 EU/m^3) (Kim, et al., 2007). A study by Kirychuk *et al.* (2009) also supports this hypothesis that endotoxin binds better to larger particles. Data on EU/mg observed in this study seem to agree with Kirychuk's study. Although larger endotoxin loading is usually found in larger particle size distributions, endotoxin is still found in sometimes high amounts in respirable dust fractions (Kirychuk, et al., 2010). The endotoxin loading in respirable dust will be more dangerous due the increased deposition into the deeper areas of the lung (Kirychuk, et al., 2010). Even though endotoxin may preferentially bind to larger particles in the non-respirable fraction, endotoxin concentrations in the respirable fraction pose a much larger threat to pulmonary inflammation in workers.

Environmental Conditions

Log transformation was not required on any environmental conditions after viewing plots of residuals. Statistical analysis of the environmental conditions revealed significant seasonal differences in temperature (decrease), humidity (increase) and air movement (decrease) ($p=<0.0001$, $p=0.0012$, and $p=0.019$, respectively). This is as expected with changing the seasons and ventilation of the building. See Figures 5.7 and 5.8 for representation of changes seen across the sampling trips. Average summer temperatures and σ were found to be 75.3°F in the milking parlor and 76.5°F outdoors.

Average winter temperatures were 60.6°F inside milking parlor and 37.2°F outdoors. Average humidity inside the milking parlor for the summer and winter was 59% and 84.8%, respectively, with the average humidity outside the facility for summer and winter being 41.9% and 36.1%, respectively. Air movement varied the least between the seasons with an average for summer and winter measuring 52.3ft/min and 35.9ft/min, respectively. The increase in CO_2 concentrations was statistically significant from summer to winter ($p=<0.001$). Average values for the summer and winter were 921ppm and 2489ppm, respectively. Measurements for CO_2 were also taken outside to allow for background levels. Levels outside for the summer were 370ppm and winter 411ppm. Measurements for CO were taken but levels were consistently zero.

Figure 5.7: Changes in temperature, humidity, and air movement by sampling trip.

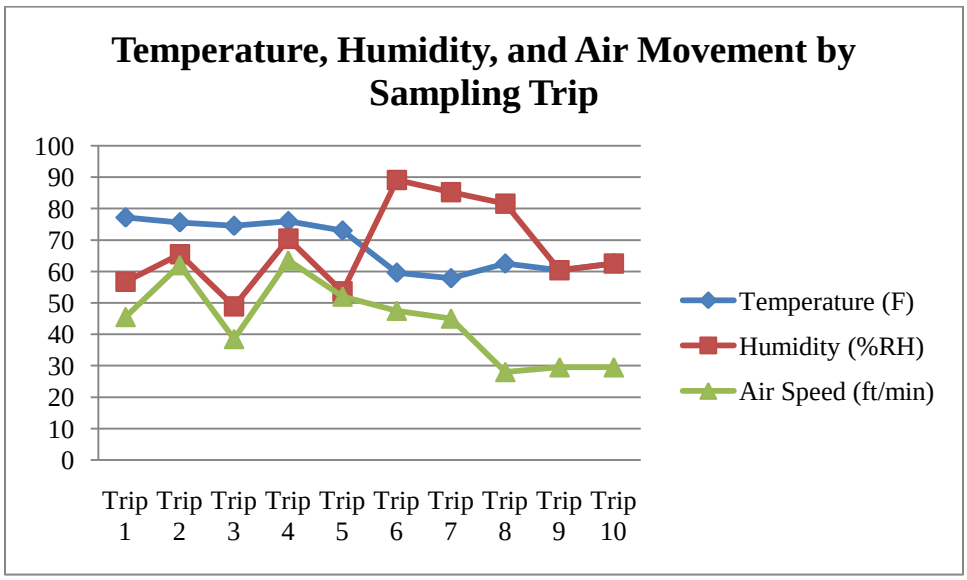


Figure 5.8: Mean CO_2 concentrations in ppm by sampling trip. Error bars indicate ± each sample standard deviation.

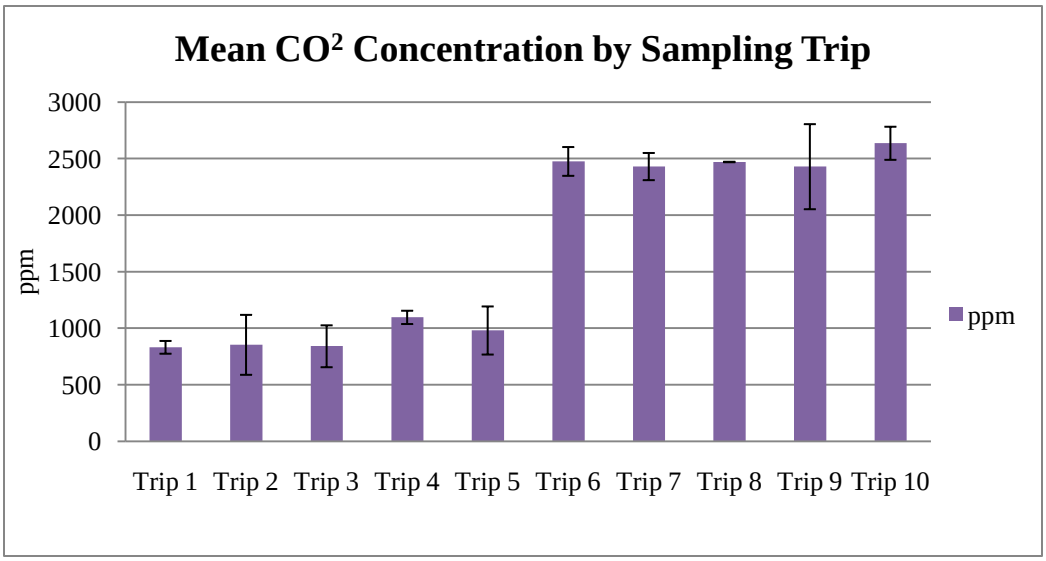


Table 5.2: Mean values and σ of environmental conditions by season.

Season:	Temperature (°F)	Humidity (%RH)	CO_2 (ppm)	Air Movement (ft/min)
Summer	75.3±1.6	59±8.8	921±115	52.3±11.5
Winter	60.6±2.0	84.8±13.3	2489±86	35.9±11.7
Summer outside	76.5±1.8	41.9±13.3	370±37	
Winter outside	37.2±7.8	36.1±4.2	411±32	

Environmental conditions of the facility (air speed, temperature, humidity, CO_2,) help to give background and understanding to what may be occurring in a facility. All

conditions were significantly different from one another for the seasonal differences. Specifically, CO_2 can be an index of the ventilation in a building (NIOSH, 2010). With CO_2 concentrations more than doubling in the winter months, it can be assumed that the airflow in the facility was poor and fresh air was not supplied as needed. A negative correlation was observed between air speed observed in the facility and CO_2 concentrations ($p=0.0032$, $r^2=-0.63$) but moderate positive correlations were observed for both endotoxin and airborne bacteria with CO_2 ($p=0.034$, $r^2=0.48$ and $p=0.05$, $r^2=0.45$). Again, if CO_2 is used as an index of the extent of the ventilation in the facility, it could be assumed that as levels of CO_2 would increase from decreased ventilation, that levels of endotoxin and airborne bacteria would also increase. The TLV and PEL for CO_2 is 5000ppm (ACGIH, 2011). The highest level of CO_2 observed during sampling was recorded to be 3186ppm for this facility. An average concentration of CO2 in a swine facility observed 2632ppm, lower than what was found in the dairy facility (Zejda, et al., 1994).

Airborne Bacteria

Bacterial concentrations required log base 10 transformation after observing plots of residuals. Differences in mean total bacterial counts were not statistically significant between seasons ($p=0.2$). There was no significant difference between worker zones ($p=0.45$) regarding total counts/m^3, but the different sides of the facility as well as different trips did observe widely varying concentrations as well as genera. Bacterial counts during the 10 sampling trips ranged from 373-14,638 counts/m^3 with an average of 4924±4196 counts/m^3. Table 5.3 contains bacterial concentrations per trip. Figure 5.9

depicts distribution of bacterial counts by trip and Figure 5.10 represents the five most reported genera overall.

Table 5.3: Total bacterial counts broken down by trip and by side of facility, right side worker zone=RS and left side worker zone=LS

Total Bacterial Cell Counts/m3		
Trip #	RS	LS
1	5978	9134
2	642	842
3	1962	552
4	1930	1460
5	2308	3583
6	1052	373
7	14638	11079
8	4238	6306
9	9665	5447
10	7540	9744

Figure 5.9: Mean airborne total bacteria counts observed by sampling trip. Error bars indicate ± each sample standard deviation.

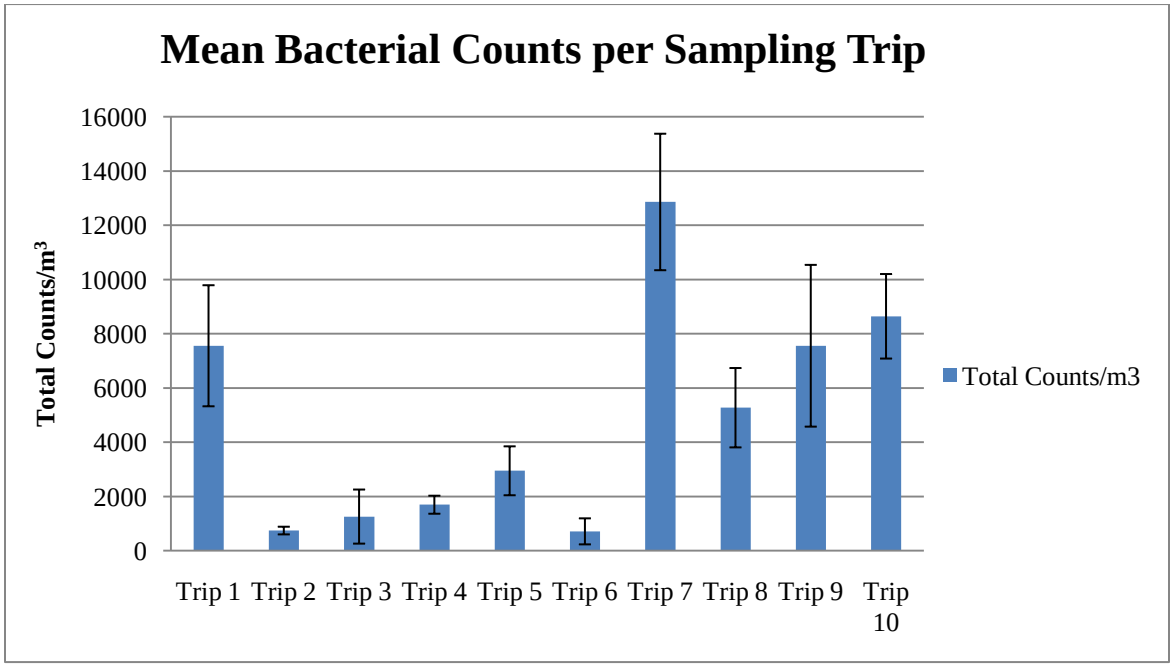
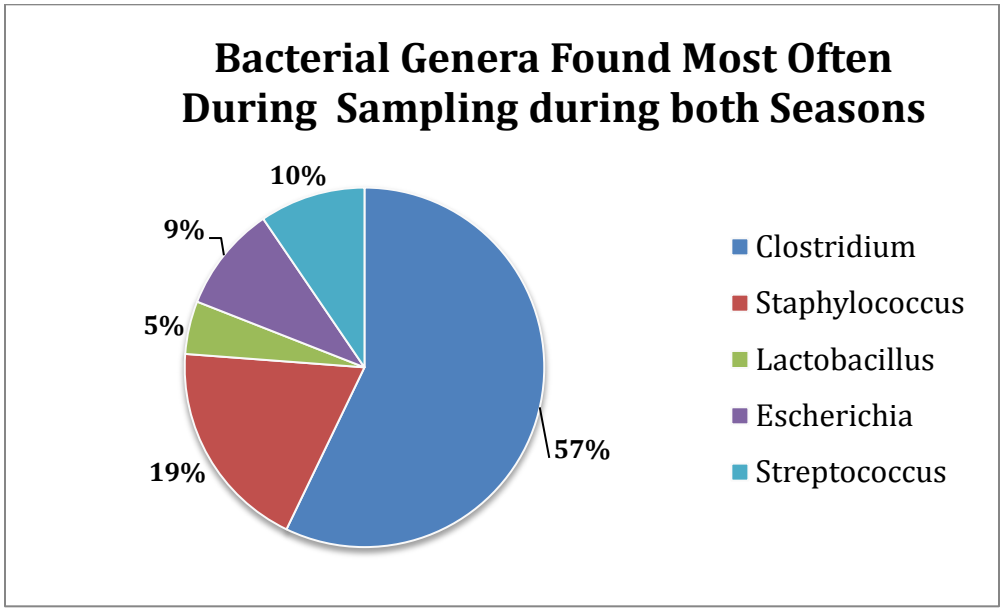


Figure 5.10: Breakdown of five most common Genera of bacteria seen across all seasons and sampling trips.



Airborne bacteria levels were highly variable and largely inconsistent between worker zones and sampling trips. The bTEFAP method is new and not much testing for its accuracy has been performed when applied to air sampling. Its ease of use and large quantity of results obtained would make it a useful method in bacterial identification and concentration. During our sampling, contamination problems on blanks were observed and it is uncertain where the contamination was stemming from. Results from the control pilot reveal that contamination was present in all control samples. Bleaching and autoclaving the Button samplers was assumed to be an adequate method for controlling bacterial contamination, but counts found in control pilot samples revealed that there was still bacterial DNA. Field blanks were also sent into the lab with the samples and counts found on field blanks were subtracted from the samples. Lab blank contamination from

facility sampling did not necessarily match field blank contamination, also indicating that contamination is occurring at a different source or step in the sampling process. The percent of contamination per sample ranged between 0%-84% and averaged 34%. Appendix E contains the percent contamination found in each sample with the method of calculation.

Though possible contamination was seen, analysis of bacterial data was still performed for research purposes. The analysis of bacterial data revealed that in the summer Clostridium (anaerobic), Oscillibacter, and Staphylococcus, all Gram-positive bacteria, were the most common genera seen. The sampling during the winter season yielded different bacterial results of Lactobacillales, Clostridium (anaerobic), and Turicibacter, all Gram-positive bacteria, as the highest genera of bacteria. View Figures 5.11 and 5.12 for overall percentage of bacteria during each season. Graphs are based on the four highest genera found for each trip, and then summed to find overall 10 highest genera per season. Up to 200 different genera of bacteria could be observed in some of the sampling trip results.

Figure 5.11: Summer season genera percentages. Bacteria in red denote Gram-negative bacteria and bacteria in black denote Gram-positive.

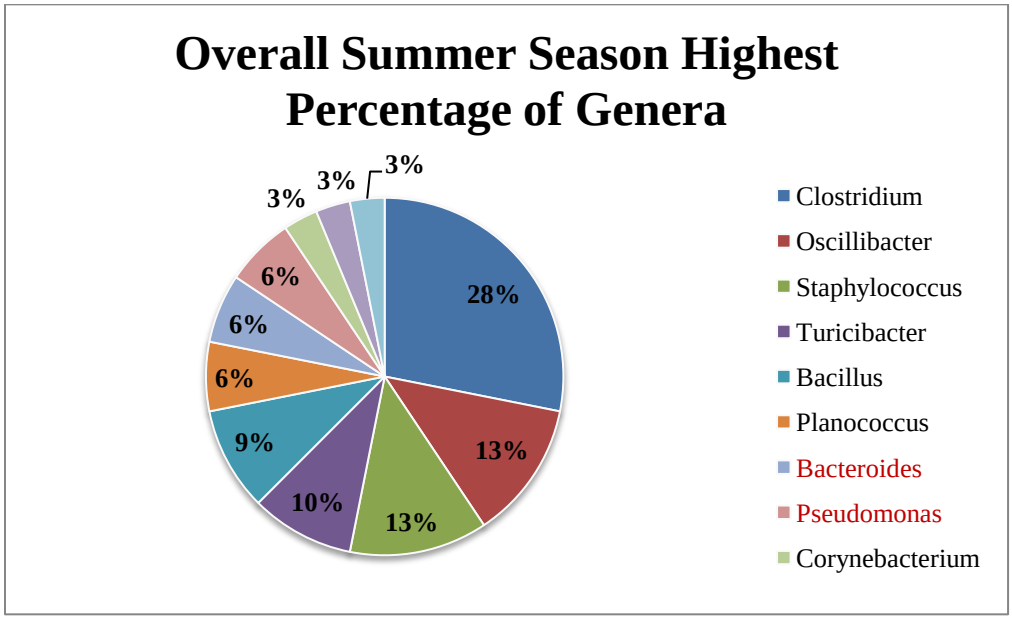
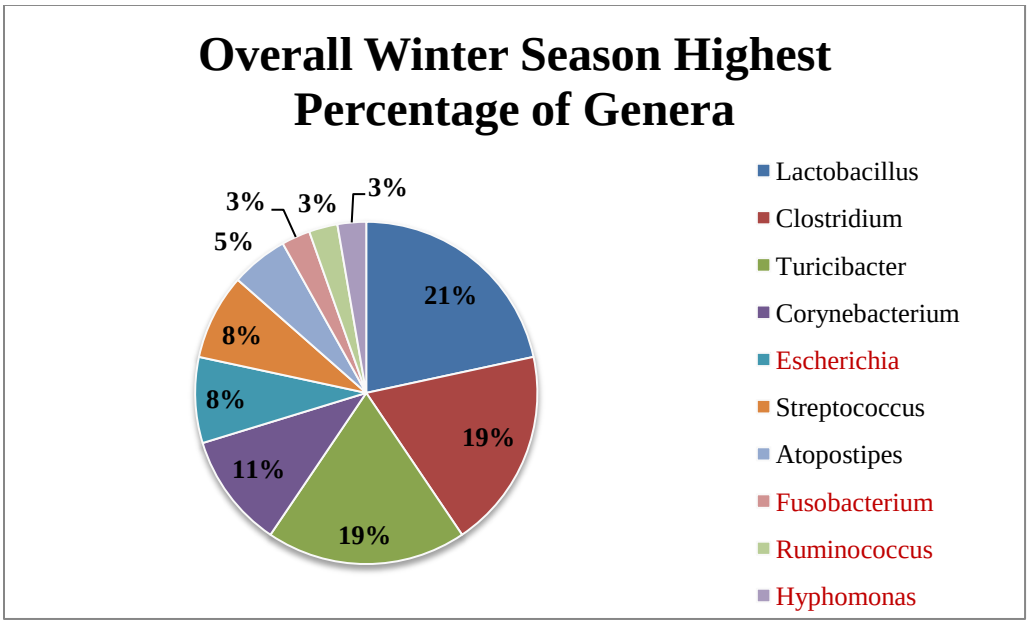
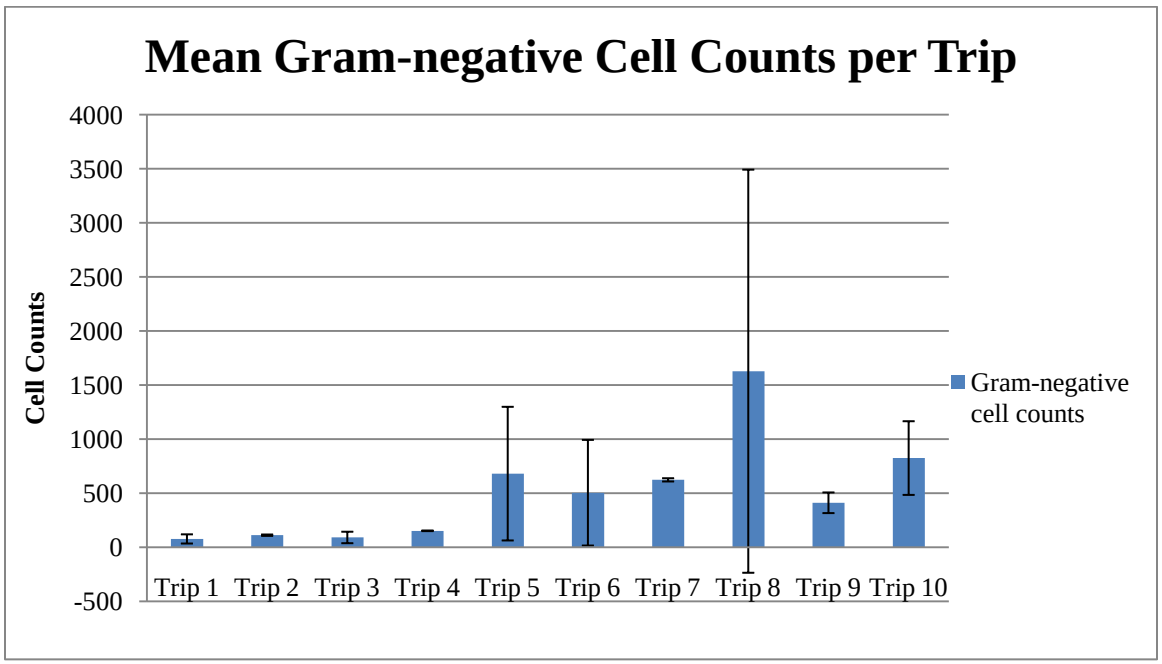


Figure 5.12: Winter season genera percentages. Bacteria in red denote Gram-negative bacteria and bacteria in black denote Gram-positive.



Count of bacteria for Gram-negative bacteria were viewed for possible association with endotoxin. Mean cell counts of Gram-negative bacteria found per sampling trip can be viewed in Figure 5.13.

Figure 5.13: Gram-negative mean cell counts each sampling trip. Error bars indicate ± each sample standard deviation.



Moving into the winter season (trips 6-10), an increase in Gram-negative bacterial cell counts can be seen when compared to the summer season (trips 1-5), but the variability of the samples is large. Mean cell counts of Gram-negative bacteria for the summer and winter season were 221 counts and 798 counts, respectively. Gram-negative bacteria made up anywhere between 1% and 83% of the total cell counts in each sample.

Both Gram-positive and Gram-negative bacteria were present in the dairy environment. Gram-postive organisms were found in this study more often than Gram-negative bacteria which may influence the decreased overall levels of endotoxin seen in

this facility when compared to other studies and animal industries. Endotoxin is a part of the Gram-negative bacterial cell wall, leading to the hypothesis that increased Gram-negative bacteria would be associated with increased occurrence of endotoxin. Gram-positive organisms have different chemical markers on their cell wall. Recent studies have indicated that exposure to peptidoglycan, a derivative of Gram-positive microbes, without the presence of endotoxin (Gram-negative bacteria) maintained increased inflammatory markers in human bronchial epithelial cells, demonstrating that Gram-positive bacteria, or agents other than Gram-negative bacteria, play an integral part of inflammation observed with organic dust exposure (Poole, et al., 2010). Gram-negative bacteria were seen more often during the winter season when compared to summer (Figure 5.11), possibly contributing to the near significant increase ($p=0.06$) in endotoxin concentrations from summer to winter. Knowledge that the overall amount of endotoxin is limited by the dust suggests that control methods be directed to controlling the dust, then control of endotoxin will also be achieved.

Only the top 20 bacterial genera were assessed for Gram-negative or Gram-positive status for this research due to the incredibly large numbers of genera gained though the bTEFAP method and large sample number(>200 genera, 20 samples). Using the bTEFAP technique in poultry housing yielded cell counts of bacteria and genera somewhat similar to results found in this study. Nonnenmann *et al.* (2010) found bacterial genera of Staphylococcus, Salinicoccus, and Lactobacillus with the most cell counts. Clostridium was found in Nonnenmann *et al.* (2010) but in much lower quantities than what was discovered in this study (247 counts/m^3 vs. 4170 counts/m^3). Differences between our study and others in the bacteria seen, as well as the concentrations are most

likely due to the different animals in the facilities. Overall, statistically significant differences were not seen between seasons, but view of Figure 5.6 reveals a general trend of larger bacterial counts/m^3 during the winter sampling season. Average concentrations of total bacteria in the facility during the winter and summer were 2839 and 7008 counts/m^3, respectively. Nonnenmann *et al.* (2010) found an overall concentration of 7503 counts/m^3 in poultry facilities. Nonnenmann *et al.* (2010) did not break down the total cell counts into amounts of Gram-negative and positive bacteria, allowing no comparison for our mean Gram-negative cell counts. Despite facing possible contamination issues, it appears are results are not too dissimilar to what has been found in other animal confinement operations. Very little data is available for comparison using the bTEFAP method.

Concern for contamination was present in virtually all samples. Results of the control project revealed contamination in every sample sent. Samples of new filters, never before opened yielded counts of bacteria per sample of 1233 and 2565, suggesting that the filter media is contaminated either upon packaging, or during laboratory analysis. Control filter samples were taken out of new sealed packages and placed in sterile 100ml tubes, then shipped on ice for analysis. All work was performed in a biohazard hood with forceps being cleaned between each sample. Table 5.4 contains the counts of bacteria for each quality control sample sent in for bTEFAP analysis. The * denotes that washing occurred by dipping the button into the solution, with the solution completely covering the button and agitating for one minute. The SOP for this Quality Control Pilot can be found in Appendix D under Winter Season SOP (reagents differ slightly but procedure is the same).

Table 5.4: Bacterial counts observed on quality control samples.

Sample ID	Sample type	Counts per sample
01113010	DI water 10ml	2871
02113010	Qiagen nuclease free water lot # 430135350	1429
03113010	Lonza LAL reagent water lot # EL0795 exp:12Apr08	2079
04113010	Fisher 100% reagent ethanol lot # 056671-36 exp: Jan. 2009	1131
05113010	Fisher Absolute Ethanol 200 proof Lot # 107005	3960
06113010	Autoclaved buttons before filter was added, let sit out at room temp for 4 hrs. Filters from new package SKC gelatin filters lot # 030912602090048	Missing
07113010	Autoclaved buttons before filter was added, let sit out at room temp for 4 hrs. Filters from new package SKC gelatin filters lot # 030912602090048	2728
08113010	Filter from new package of SKC gelatin filters lot # 030912602090048	1233
09113010	Filter from new package of SKC gelatin filters lot # 021012602090104	2565
10113010	Filter from old opened package of SKC gelatin filters lot # 030912602090048	1967
11113010	Washed* button using Qiagen nuclease free water (lot# 430135350) and 200 proof ethanol (lot# 107005) (30%/70% mix). Used newer filters, new 5pack, lot # 021012602090104	4775
12113010	Washed* button using Qiagen nuclease free water (lot# 430135350) and 200 proof ethanol (lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104	3163
13113010	Washed* button using Lonza LAL reagent water (lot# HL0277 exp:12sep2010) and 200 proof ethanol (Lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104	2954
14113010	Washed* button using Lonza LAL reagent water (lot# HL0277 exp:12sep2010) and 200 proof ethanol (Lot# 107005) (30%/70% mix)Used newer filters, new 5pack, lot # 021012602090104	1207

Speaking with the manufacturer (SKC) of the gelatin filters yielded new information regarding the filter use for pyrosequencing. The filters are manufactured from pig skin and irradiated for sterility. If the filter wasn't thoroughly irradiated to denature the DNA from the bacteria on the pig skin, pyrosequencing would amplify any non-viable bacterial DNA. This could have influenced the background contamination seen in our samples and Quality Control Pilot. Further research is needed to look into appropriate filters for air sampling to be used with pyrosequencing.

Correlations

Correlations between variables were evaluated with strong, moderate, or weak designations given to assess the strength of the correlation.

- Strong correlation $r^2>0.75$
- Moderate correlation $r^2=0.4\text{-}0.74$
- Weak correlation $r^2<0.39$

Only statistically significant correlations are presented here. Correlations were not viewed heavily in this research. The strongest correlation was seen between CO_2 and humidity ($p=<0.001$, $r^2=0.89$). There was a moderate negative correlation between endotoxin and temperature ($p=0.0265$, $r^2=-0.50$). As the temperature decreased in the winter, ventilation decreased, CO_2 increased, and humidity increased, all probably influencing the endotoxin. Possibly a shift in bacterial distribution (greater concentration of Gram-negative bacteria in the winter) could have occurred to influence the increase endotoxin, but no statistically significant correlation between Gram-negative cell counts and endotoxin was observed. Decreased ventilation may have an increase on the type of

bacteria that propagate. Humidity and temperature had a strong negative correlation ($P<0.001$, $r^2=-0.80$). Inhalable dust and temperature had a moderate negative correlation ($p=0.05$, $r^2=-0.44$). Temperature and air speed had a moderate correlation ($p=0.01$, $r^2=0.55$). Endotoxin and CO_2 had a moderate correlation ($p=0.05$, $r^2=0.45$) and airborne bacteria and CO_2 had a moderate correlation ($p=0.034$, $r^2=0.48$). Appendix F contains all Pearson Correlation Coefficients.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

This study was the first to apply pyrosequencing to measure bioaerosols in a dairy environment. The diversity of bacteria and predominance of Gram positive bacteria is consistent with studies in swine and poultry facilities. It is difficult to evaluate potential occupational exposure from the airborne concentrations of bacteria found in this study due to the lack of exposure guidelines, but compared to studies in other enclosed livestock environments, high concentrations of bacteria are sometimes seen in the dairy facility (>14,000 counts/m^3). Gram-positive bacteria appear to be found in higher percentages in this dairy parlor than Gram-negative bacteria overall, but data showed an increase in Gram-negative bacteria during the winter season. Results from the quality control project yield information directing research to where testing and assessment should take place to aid in the development of a pyrosequencing method that will accurately sample airborne bioaerosols.

Concentrations of inhalable dust, respirable dust, and endotoxin were low during both seasons in this milking parlor. But the new construction of the facility may have influenced the differences seen in this facility compared to other dairy facilities.

The size distribution in the facility (MMOD 12µm) demonstrates that larger particles (>10µm) are more abundant and do not change with changing seasonal ventilation conditions. Endotoxin concentrations showed a near significant increase from summer to winter, which may be have been influenced by the increased occurrence of Gram-negative bacterial genera. Normalization of endotoxin with the mass of dust indicated that controlling dust might control the endotoxin concentrations in the facility.

These study results will inform the design of future comprehensive studies of aerosol exposure interventions in dairy operations. Understanding these aerosol exposures and potential interventions is important for reducing respiratory disease among workers in the dairy industry.

Recommendations

Research regarding the accuracy of the bTEFAP method applied to air sampling needs to be addressed before further testing takes place. Results of this project indicate possible contamination of the gelatin filter, which is used in sampling. Another possibility for the contamination source is the centrifuge tubes used for this study, which were not assessed. Once contamination issues are resolved, research can move forward with the use of this method to identify and quantify bacterial exposures in any industry, not just animal confinement operations.

In addition to understanding the application of the bTEFAP method to bioaerosol sampling, research needs to be done to compare the various airborne bacterial testing methods using RNA or DNA. This will help in comparing concentrations seen in facilities and understanding the role that will play in dose-response relationships between airborne bacteria exposure and disease.

Gram-positive bacterial exposure needs to be further studied, and a more thorough understanding of the relationship between organic dust and the percentage containing Gram-positive and Gram-negative organisms must be addressed. Understanding why Gram-positive or Gram-negative bacteria are seen and in what relation will aid in more accurate development of occupational exposure guidelines.

Control of dust and bioaerosols needs to be addressed specifically in the dairy environment. Useful and effective methods of dust control have been seen in swine and poultry industries, but have not been applied to the dairy industry. Further research should focus on controlling bioaerosols associated with animal confinement operations with control methods assessed in the dairy environment specifically. The milking parlor in this study appeared to have effective control of contaminants, but evaluation needs to be performed on the wider range of parlor designs as well as older facilities to apply lessons learned from this facility.

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APPENDICES

Appendix A:

Sampling Data Sheet

Area Sampling Sheet Dairy Study			
Collection Date:		Staff Initials:	
Left Side of Facility:			
Endotoxin Button # _____ Pump # _____ Sample ID:			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			
Gel Button # _____ Pump # _____ Sample ID			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			
Cyclone # _____ Pump # _____Sample ID:			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			
Right Side of Facility			
Endotoxin Button # _____ Pump # _____ Sample ID:			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			
Gel Button # _____ Pump # _____ Sample ID:			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			
Cyclone # _____ Pump # _____Sample ID:			
Pump Start Time:		Pump End Time:	
Pre-weight:		Pre-calibration:	
Post-weight:		Post-calibration:	
Comments:			

Blank Samples:

Endotoxin #	**Pre-weight**	**Post-weight**	**Sample ID:**
Gel #	**Lab Blank**	**Field Blank**	
Cyclone #	**Pre-weight**	**Post-weight**	

Comments:

Q-Trak Model # _____

Time:	Temp(°F)/Humidity(%):	Location:	Reading (ppm):

Grimm PAS Sampling

Location:	Time:	How long sampled:

Appendix B:

Formula for MMOD calculations

1. Mass in each size range was imported into an Excel spreadsheet.
2. The mass per each channel was then calculated by subtracting the mean in by each preceding mean.
3. The cumulative mass was calculated by adding up the mass in each channel for a cumulative mass per channel.
4. The cumulative percent was calculated by dividing the cumulative mass by the total mass, which is the 1st value in the mean column under the 0.23um channel
5. After Excel calculated the cumulative percent, the percents were plotted by hand onto a log probability plot.
6. The MMOD was found by looking at where the plot crossed the 50% axis.
7. The σ_g was found by dividing the MMOD by the value found crossing the 15.9% axis.
8. This same process was performed on all samples. See below for illustrations of the Excel spreadsheet.

Date	8/30/10: 8:11am			
Channel	Mean	Mass per channel	Cumulative mass	Cum. %
>0.23um	548.56	1.1	1.1	0.20%
>0.30um	547.46	1.24	2.34	0.43%
>0.40um	546.22	1.08	3.42	0.62%
>0.50um	545.14	1.05	4.47	0.81%
>0.65um	544.09	0.8	5.27	0.96%
>0.80um	543.29	1.24	6.51	1.19%
>0.90um	542.05	3.09	9.6	1.75%
>1.0um	538.96	11.21	20.81	3.79%
>2.0um	527.75	23.51	44.32	8.08%
>3.0um	504.24	23.94	68.26	12.44%
>4.0um	480.3	25.91	94.17	17.17%
>5.0um	454.39	62.12	156.29	28.49%
>7.5um	392.27	54.8	211.09	38.48%
>10.0um	337.47	102.51	313.6	57.17%
>15.0um	234.96	72.3	385.9	70.35%
>20.0um	162.66	162.66	548.56	100.00%
Total		548.56		

Appendix C:

All Grimm PAS Measurements

Season	Trip	Side	MMOD	σ_g
summer	4	RS	9	3.2
summer	4	LS	18	3.6
summer	4	RS	10	3
summer	4	LS	17	3.8
summer	5	RS	8.5	3.5
summer	5	LS	10	3.3
summer	5	RS	12	3.3
summer	5	LS	9.8	3.1
winter	6	LS	13	3
winter	6	RS	14	3.2
winter	6	LS	12	2.7
winter	6	RS	15.5	2.9
winter	7	RS	10	2.8
winter	7	LS	11	2.8
winter	7	RS	13	3.3
winter	7	LS	13	2.8
winter	8	LS	12	3.1
winter	8	RS	11	2.8
winter	8	RS	11	2.6
winter	8	LS	13	2.7
winter	9	RS	13	3.3
winter	9	LS	6.5	3
winter	9	RS	15	3.6
winter	9	LS	8	2.6
winter	10	RS	14	2.6
winter	10	LS	10	1.9
winter	10	RS	11	2.1
winter	10	LS	16	2.8

Appendix D:

Standard Operating Procedures (SOP) for Airborne Bacterial Collection

List of Materials:

- 25mm Inhalable Button Samplers (SKC Inc., Eighty Four, PA)
- 25mm Gelatin Filters (SKC, Product # 225-9551, Eighty Four, PA)
- Bleach Rite Disinfecting Spray (Product # ML3004, MarketLab, Caledonia, MI)
- 70% Ethanol Solution (30% Filtered water from sink, 70% Fisher 100% reagent ethanol)
- Ethanol wipes (Triad, Product # 10-3001, Brookfield, WI)
- 100mL centrifuge tubes (Falcon, Product # 352074, Franklin Lakes, NJ)
- Forceps (Aven, Product #18-499)
- Whirlpak bags (Nasco, Product # B00736WA)
- Biohazard Hood
- Autoclave
- Personal autoclave bags (Propper, Product # 024008, NY)

Summer Season SOP: To be done the night before the sampling trip

1. Wash buttons in 70% ethanol solution and let dry. (May want to perform this step earlier in the day.
    a. Make solution in beaker and let button samplers sit in there, gently agitating the beaker for approximately one minute. Make sure solution covers the whole button.
    b. Remove button using forceps and let air dry on counter wiped with a 70% ethanol solution in a spray bottle (this is already kept on hand in the lab).
2. Clean the counter space with 70% ethanol solution spray
3. Gelatin filters are kept in the refrigerator; take out only when ready to apply to samplers.
4. Set out the required number of buttons, write button numbers or sample designations on whirlpak bags
5. Spray gloves with ethanol solution and wipe forceps with a new ethanol wipe. Let forceps air dry for a couple seconds before grabbing filter, filter will dissolve if anything wet touches it.
6. Open a new package of gelatin filters, apply one filter to a button sampler
    a. Close sampler and place in designated whirlpak bag
7. Spray gloves again and wipe forceps with new ethanol wipe
8. Repeat steps 5—7 until number of samples is reached.

9. Place Whirlpak bags containing button samplers in refrigerator (38°F) until sampling next morning.
10. Transport samples on ice to and from sampling.
11. Post sampling, return buttons to designated bags.
12. Wash counter with the 70% ethanol solution before removing filters from buttons.
13. Spray gloves with ethanol solution and wipe forceps with a new ethanol wipe. Again, remember to let forceps air dry for a couple seconds before grabbing filter.
14. Place filter in labeled 100mL centrifuge tube.
15. Repeat steps 13 and 14 until samples are all in tubes.
16. Freeze (0°F) the centrifuge tubes containing samples until shipping for analysis.
17. Ship overnight on ice.

Winter Season SOP:

Perform the day before sampling or earlier:

1. Spray button samplers with Bleach-Rite disinfecting spray and let sit for 20min. Rinse with filtered water and let dry.
2. Place dry buttons in personal autoclave bags and autoclave (20min dry sterilization (121°C) with 20min of dry time).
3. Keep autoclaved buttons in bags until use.

Perform this section the night before sampling

4. Work in the biohazard hood for all of the filter application process.
5. Spray gloves with the 70% ethanol solution and wipe forceps with new ethanol wipe.
    a. Remember to let the forceps dry for a couple seconds (you can just hold them in the hood) so you don't dissolve the filter.
6. Open one autoclaved button and assemble the bottom half.
7. Open a new package of gelatin filters and place one on autoclaved button.
8. Place assembled button in personal Whirlpak bag (label and designate bags beforehand)
9. Repeat steps 4-8 until all button samplers are filled.
10. Place assembled buttons in Whirlpak bags in refrigerator and refrigerate (38°F) overnight.
11. Transport samples on ice to and from sampling.
12. At sampling location, use clean gloves to gently expose lab blank to air, then close Whirlpak bag and keep lab blank on ice.
    a. Might want to designate lab blank the night before on the bag.
13. Post-sampling, replace on microbial button samples to their personal Whirlpak bags and transport on ice to the lab.

14. Repeat steps 4-8 except instead of adding filters, remove them and place in 100mL centrifuge tubes.
    a. Filters may be kind of wet and dissolving from sampling, use care to pry them off of the button screen.
15. Place 100mL tubes with filters in freezer and freeze (0°F) until shipping for analysis.
16. Ship overnight on ice.

Appendix E:

Percent of Contamination per Sample and Method of Calculation

Method of Calculation:

1. Total Counts were summed for each sample and field blank
2. Field blank counts were divided by the total count in each sample
3. Percentage was rounded to the nearest decimal place

Season	Trip	Side	% Contamination of Sample
Summer	1	RS	6%
Summer	1	LS	4%
Summer	2	RS	84%
Summer	2	LS	72%
Summer	3	RS	0%
Summer	3	LS	0%
Summer	4	RS	56%
Summer	4	LS	63%
Summer	5	RS	50%
Summer	5	LS	35%
Winter	6	RS	17%
Winter	6	LS	44%
Winter	7	RS	8%
Winter	7	LS	10%
Winter	8	RS	60%
Winter	8	LS	62%
Winter	9	RS	23%
Winter	9	LS	35%
Winter	10	RS	25%
Winter	10	LS	20%

Appendix F:

Pearson Correlation Coefficients

Pearson Correlation Coefficients, N = 20 Prob > |r| under H0: Rho=0									
	Temperature	**Humidity**	**CO2**	**Dust**	**Endotoxin**	**Air**	**Bacteria**	**Endotoxinmg**	**Gramneg**
Temperature	1.00000	-0.80067	-0.91573	-0.44315	-0.49495	0.55396	-0.34148	-0.40195	-0.50077
		<.0001	<.0001	0.0504	0.0265	0.0113	0.1406	0.0790	0.0245
Humidity	-0.80067	1.00000	0.89185	0.19544	0.18811	-0.35550	0.39505	0.17886	0.23733
	<.0001		<.0001	0.4089	0.4271	0.1240	0.0847	0.4506	0.3137
CO2	-0.91573	0.89185	1.00000	0.36388	0.45136	-0.62504	0.47631	0.28426	0.36285
	<.0001	<.0001		0.1148	0.0458	0.0032	0.0337	0.2245	0.1159
Dust	-0.44315	0.19544	0.36388	1.00000	0.23385	-0.23801	0.26228	0.00285	0.83047
	0.0504	0.4089	0.1148		0.3210	0.3123	0.2639	0.9905	<.0001
Endotoxin	-0.49495	0.18811	0.45136	0.23385	1.00000	-0.37204	0.26912	0.40674	0.17336
	0.0265	0.4271	0.0458	0.3210		0.1063	0.2512	0.0751	0.4648
Air	0.55396	-0.35550	-0.62504	-0.23801	-0.37204	1.00000	-0.43535	-0.09609	-0.09742
	0.0113	0.1240	0.0032	0.3123	0.1063		0.0550	0.6870	0.6828
Bacteria	-0.34148	0.39505	0.47631	0.26228	0.26912	-0.43535	1.00000	-0.11576	0.15448
	0.1406	0.0847	0.0337	0.2639	0.2512	0.0550		0.6270	0.5155
Endotoxinmg	-0.40195	0.17886	0.28426	0.00285	0.40674	-0.09609	-0.11576	1.00000	0.08501
	0.0790	0.4506	0.2245	0.9905	0.0751	0.6870	0.6270		0.7216
Gramneg	-0.50077	0.23733	0.36285	0.83047	0.17336	-0.09742	0.15448	0.08501	1.00000
	0.0245	0.3137	0.1159	<.0001	0.4648	0.6828	0.5155	0.7216	