THESIS

COMPARATIVE ANALYSIS OF BACTERIAL AND FUNGAL COMMUNITIES IN TWO DAIRY PARLORS THROUGH THE USE OF PYROSEQUENCING, RIBOPRINTING, CULTURE TECHNIQUES, AND MICROSCOPIC ANALYSIS

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ABSTRACT

COMPARATIVE ANALYSIS OF BACTERIAL AND FUNGAL COMMUNITIES IN TWO DAIRY PARLORS THROUGH THE USE OF PYROSEQUENCING, RIBOPRINTING, CULTURE TECHNIQUES, AND MICROSCOPIC ANALYSIS

The purpose of this study was to compare three different analysis techniques used to characterize and identify bacteria and fungi. Pyrosequencing, culture techniques, and riboprinting were compared for all of the bacterial samples and pyrosequencing; culture techniques; and microscopic analysis was used to compare the fungal samples.

SKC BioSamplers were used to take area samples inside two modern dairy parlors from May 2012-January 2013. Four sampling sessions were completed at each dairy parlor. Four biosamplers ran side-by-side (two at a time) for 60 minutes at 12.5 l/min in addition to a lab and a field blank. A novel resuscitation buffer was used to collect and aid recovery of stressed bacteria and fungi. Three types of media were used to select for bacteria and fungi: tryptic soy agar (TSA) with a 5% sodium chloride addition for Gram-positive bacteria; Eosin methylene blue (EMB) for Gram-negative bacteria, and malt extract agar (MEA) with a chloramphenicol addition for fungi. Based on colony morphology, the five most commonly encountered bacteria from both TSA and EMB agar were subcultured and identified through riboprinting. Pyrosequencing was performed directly on the biosampler collection media.

The culturable bacterial concentrations and the pyrosequencing bacterial concentrations were within the same order of magnitude, which was unexpected. The culturable bacterial concentrations, with averages of 7500 CFU/m^3 and 500 CFU/m^3 for TSA and EMB plates respectively, were higher than the concentrations found in previous studies which could be a

result of the novel resuscitation buffer that was used as a collection media. Greater microbiome diversity was found through pyrosequencing analysis than the riboprinting analysis. The pyrosequencing data found many genera that include species that are pathogenic, but more work must be done to confirm if pathogenic species were found during sampling at these two dairy parlors. The riboprinting samples were identified on the species and strain level and found *Escherichia coli O157:H7* a known pathogen as well as *Pseudomonas aeruginosa*, an opportunistic pathogen.

The culturable fungi concentrations and the pyrosequencing concentrations were within the same order of magnitude, which was also unexpected. The pyrosequencing data had greater diversity than the microscopic analysis for the first two sets of samples that were sent for pyrosequencing. The second set of fungal samples that were sent for pyrosequencing came back as non-detect samples despite the growth of fungi on the agar. From the pyrosequencing data, there were many genera found that have pathogenic species, but more research needs to be conducted to determine the presence of the pathogenic species. There were no pathogenic fungal species found through the microscopic analysis.

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DEFINITION OF TERMS

bTEFAP	Bacterial Tag-Encoded FLX 454 Pyrosequencing
CFU	Colony Forming Units
CFU	Colony Forming Units
EMB	Eosin Methylene Blue
EU	Endotoxin Units
FEV	Forced Expiratory Volume
FVC	Forced Vital Capacity
GM	Geometric Mean
GSD	Geometric Standard Deviation
HICAHS	High Plains Intermountain Center for Agricultural Health and Safety
IL-6	Interleukin-6
LPS	Lipopolysaccharides
MEA	Malt Extract Agar
NAG	N-acetylglucosamine
NAM	N-acetylmuramic Acid
NIOSH	National Institute for Occupational Health and Safety
ODTS	Organic Dust Toxicity Syndrome
3-OHFA	3-Hydroxy Fatty Acids
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
RH	Relative Humidity
TNF-α	Tumor Necrosis Factor-α
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

CHAPTER 1: INTRODUCTION

The inhalation of dusts, specifically organic dusts has long been associated with respiratory problems particularly when looking at occupations in dusty environments. The agricultural industry has been recognized as a high risk occupation for respiratory problems as early as the 1500s when grain dust was recognized as a factor for decreased lung function in agricultural workers (Saiki 1998). Although more knowledge and awareness surrounds occupational respiratory problems, there are still many areas that need further research. As practices, techniques, and technology change, the risk for workers also continues to change. One such industry is the dairy industry. Previously, milk was collected by a large number of small farms; as technology changed, the dairy industry began to consolidate the farms creating a smaller number of farms with larger operations and a greater number of workers. Due to the need for an increased amount of labor, workers are more inexperienced and not acclimated to the animal confinement operation environment. Additionally, instead of completing different tasks throughout the farm, workers are spending entire 8 or 12 hour shifts in one spot such as the dairy parlor. Some of the respiratory symptoms associated with exposure to organic dusts include asthma, rhinitis, chronic obstructive airway disease, chronic bronchitis, and organic dust toxicity syndrome (Garcia, Bennett et al. 2013; Reynolds, Nonnenmann et al. 2013).

Organic dusts include any dust that is biological in origin such as fungi, bacteria, fecal matter, hay, feed, pollen, and animal dander. A bioaerosol is an aerosolized organic dust that contributes to the majority of respiratory disease in the agricultural industry (Douwes 2003). Dust, bacteria, fungi, and their corresponding constituents have all been linked to various respiratory diseases. Despite the link between bioaerosols and respiratory disease there continue to be no standards or guidelines in the United States and very few worldwide to help control and

reduce worker exposure. One of the important constituents of bacteria is bacterial endotoxins that are associated with Gram-negative bacteria. Previous studies have demonstrated the relationship between high endotoxin levels and decreased lung function (Reynolds, Clark et al. 2012). Based on the results from these studies, some abatement procedures have been developed to help reduce endotoxin levels.

Despite the reduction in endotoxin levels in many dairy farms, there are still a large number of workers with respiratory problems. Further research has indicated that one potential contributor to the decreased lung function in agricultural workers is Gram-positive bacteria which continue to be present despite the decreased endotoxin levels. Currently, research is being conducted to investigate the levels of muramic acid, a marker of Gram-positive bacteria, to determine concentrations of Gram-positive bacteria in different agricultural environments (Poole, Dooley et al. 2010). At present, little research exists regarding the characterization of bacteria and fungi, particularly on dairy farms. There are new methods for the analysis and identification of bacteria and fungi available that need further analysis to be used effectively. In order to help ensure a safe and healthy workplace for dairy workers it is vital to understand what bacteria and fungi are present to understand how to effectively control the exposures.

CHAPTER 2: LITERATURE REVIEW

Health Effects of Organic Dust Exposure

Organic dusts consist of any particulate matter of biological origin (animal, plant, and microbial). The study of organic dust is particularly important due to the large array of health effects associated with exposure to organic dust in many occupational environments. The routes of exposure includes direct contact, inhalation, or vector-born transmission. Organic dusts are known to cause respiratory problems, infectious diseases, cancer, and allergic reactions (Douwes 2003). High levels of dust present in the air can overload the clearance mechanisms of the respiratory system causing irritation (Donham, Cumro et al. 2000). Agricultural workers are of particular interest, due to the increased risk of respiratory mortality and morbidity in the agricultural industry (Reynolds, Clark et al. 2012). It is estimated that 22 million workers in the United States are involved in the production and processing of the country's food demonstrating the importance of research in this area (Carolina 2011).

Bioaerosols are organic dusts or biological particles suspended in the air. Bioaerosols are found over a variety of size ranges from <0.02 μm to >50 μm suggesting a wide variety of deposition in the different regions of the lungs. It is important to note that bioaerosols do not only contain live organisms but also contain dead organisms as well as their constituents that lead to an assortment of respiratory problems (Millner 2009).

Impact on the Dairy Industry

The dairy industry has changed in a unique way resulting in an increase in industrialized farms and a reduction in the number of small dairy farms. The Government Accountability Office estimated a 246% increase in large scale animal confinement operations from 1982 to 2002 raising important occupational health questions (Greger and Koneswaran 2010). As the

number and size of confinement operations increases, the number of workers required to maintain and operate these facilities also increases. With the increased need, more workers are hired that do not have a background in the dairy or agricultural industry increasing their susceptibility to organic dust exposure. Studies have shown that workers that have been previously exposed to organic dust had a decreased prevalence of asthma while workers not previously exposed had an increased susceptibility to asthma (Basinas 2011). Agricultural workers in general have increased respiratory morbidity not only in comparison to the general population but also to other occupations(Basinas 2011; Reynolds, Nonnenmann et al. 2013). Dairy farmers are more likely to have asthma, chronic bronchitis, rhinitis, and multiple other respiratory problems that are not seen in the general population (Mounchetrou, Monnet et al. 2012). Organic dusts found on dairy farms come from a variety of sources including but not limited to grain, manure, urine, animal dander, soil, and plants. Workers at dairy farms have the potential to be exposed to organic dusts and its harmful constituents during multiple operations such as milking, feed handling, manure handling, and bedding handling (Kullman, Thorne et al. 1998). While non-agricultural indoor concentrations of bacterial and fungal spores have been measured around 10^2-10^3 spores/m^3, concentrations on dairy farms have been found between 10^3-10^7 spores/m^3 indicating a higher risk for agricultural workers than nonagricultural workers (Lee, Adhikari et al. 2006).

In all agricultural work, the healthy worker effect plays an important role in how workers adapt to bioaerosols exposure. The healthy worker effect also complicates the understanding of the severity of respiratory problems associated with agricultural work. The healthy worker effect suggests that workers who are more susceptible to occupational hazards are more likely to leave the job quickly. Research has shown that workers who leave a job early have more severe

respiratory problems than workers who stay on the job for a longer period of time (Saiki 1998). The healthy worker effect in dairy farms particularly selects for workers without asthma. Multiple studies have indicated that workers with preexisting asthma, as well as those who quickly develop a sensitization to the organic dusts present on dairy farms are not likely to continue to work in the industry (Mounchetrou, Monnet et al. 2012).

Respiratory Diseases

Historically, respiratory disease in agricultural operations is one of the earliest noted occupational issues; respiratory symptoms as a result of the inhalation of grain dust were recognized as early as 1555 (Saiki 1998). Exposure to bioaerosols can result in allergic and non-allergic asthma, chronic obstructive airway disease, rhinitis, chronic bronchitis, hypersensitivity pneumonitis, and organic dust toxic syndrome (ODTS) (Garcia, Bennett et al. 2013). Multiple studies have shown a dose-response relationship between the number of working hours in animal confinement buildings and symptoms related to respiratory issues (Donham, Cumro et al. 2000; Iversen 2000). The respiratory issues associated with organic dust exposure are a result of both allergic and non-allergic responses. Allergic respiratory responses involves a type I inflammatory response induced by the immune system involving specific antibodies while the non-allergic response is non-immune specific (Douwes 2003). Both responses are caused by Gram-negative as well as Gram-positive bacteria and their constituents (Poole, Dooley et al. 2010).

Gram-Negative Bacteria

Endotoxins originate from Gram-negative bacterial cell walls and are comprised of lipopolysaccharides (LPS) that play an important role in the presence of respiratory problems in agricultural workers (Rylander 2006). The LPS is released from the bacteria when the cell dies

or lyses. The toxic portion of the LPS is lipid A which is thought to be the root cause of adverse health effects such as inflammation, shortness of breath, cough, and decreased lung function (Willey 2008). When endotoxins are inhaled, the result is an immune response causing increased inflammation in the airways as well as decreasing pulmonary function of the worker. Endotoxins contribute significantly to the increase in respiratory diseases among agricultural workers such as ODTS and chronic obstructive airway disease (Reynolds, Clark et al. 2012). Currently, there is no standard for the amount of endotoxin exposure in the workplace in the United States although an exposure limit based on work in the swine and poultry industry was recommended at 100 EU/m^3 (Donham et. al 2000). High endotoxin levels have previously been found in dairy farms and other agricultural settings; Reynolds et al. 2012 found a GM of 1166 endotoxin units (EU)/m^3 which exceed the recommended Dutch limits of 90 EU/m^3.

Gram-Positive Bacteria

Gram-positive bacteria as well as fungi are thought to be the cause of the high degrees of inflammation through exposure in-vitro using endotoxin depleted agricultural dusts in controlled cell studies. Gram-positive bacteria contain a large amount of peptidoglycan in the cell wall which has been shown to cause inflammatory responses in workers (Poole, Dooley et al. 2010). Peptidoglycan is a polymer composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The NAG and NAM sugars form a mesh-like structure used to create part of the cell wall in both Gram-negative and Gram-positive bacteria. In the Gram-positive bacteria the peptidoglycan layer is considerably larger than the layer found in Gram-negative bacteria (Willey 2008). The muramic acid in the NAM subunit has allowed for the use of gas chromatography-mass spectrometry to better characterize and identify the amount of peptidoglycan present in occupational settings (Poole, Dooley et al. 2010). This difference in

peptidoglycan in the Gram-positive bacteria has been demonstrated to result in inflammatory responses in workers. The inflammatory response is characterized by an influx of inflammatory cells and cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) (Larsson 1999).

Airborne Fungi

Fungal diseases have been reported from contact or inhalation of fungal spores while handling animal feces, soil, and grain in agricultural settings (Douwes 2003). Fungi play an important role in worker illness. Fungi often cause detrimental immune responses, irritation from toxic byproducts of the fungi, and infection (Bush 2006). Some genera of fungi that have been shown to cause respiratory problems include *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* (Saiki 1998). One important aspect of fungal inhalation is sensitization to fungi. Sensitization typically occurs when fungi are inhaled multiple times over a long period of time. A result of sensitization to certain genera of fungi such as *Alternaria* is the occurrence and/or increase in severity of asthma (Bush 2006). The presence of fungi in a work setting can also trigger allergic responses such as allergic rhinitis, allergic rhinosinusitis, and allergic asthma. No single genera of fungi results in these allergic responses; however the allergic response is higher with a higher concentration of fungi (Hamilos 2010).

Bioaerosol Collection Methods

SKC BioSamplers (SKC, Inc., Fullerton, CA) are a unique liquid impinger used to collect microorganisms in an effective manner. One advantage of using a liquid impinger is the flexibility for use in a variety of different tests. The collection liquid can be diluted and used over multiple media and analytical methods such as plating, pyrosequencing or polymerase chain reaction (PCR) (Lin, Reponen et al. 2000). Although there is still some sample loss, there is no

filter loss or need to extract from a filter. A traditional impinger brings in air through the inlet and the air flows straight into the liquid used for collection (Hinds 1999). On the other hand, the SKC BioSampler uses three nozzles that create a swirling motion in the liquid allowing for a gentle collection method with minimal bubbles (Figure 2.1). The small amount of bubbles helps reduce the amount of microorganisms that are resuspended or trapped in the air pockets created by the bubbles (Lin, Reponen et al. 2000). As the three nozzles pull in air, a swirling motion is created that raises the level of liquid in the collection vessel allowing the liquid to collect the microorganisms in the air.

Figure 2.1 SKC Biosampler Diagram

One important issue associated with the use of the biosampler is the quick evaporation of liquid in the collection vessel. To counteract this problem, either a non-evaporating liquid may be used or sampling must be completed over a shorter amount of time to ensure that the liquid does not evaporate completely (Lin, Reponen et al. 2000). In this particular project, a novel buffer was used to help increase the survival of the bacteria and fungi. A resuscitation buffer was developed by Andersson et al. (1995) that provides an environment to sustain the life of the

bacteria and fungi while also not promoting growth to keep concentrations consistent with the environmental concentration. The resuscitation buffer contains polyethylene glycol (PEG), peptone, and Tween 80; each component plays a key role in the survival of the bacteria and fungi. The peptone is a water-soluble protein that acts as a food source for the microorganisms. The PEG helps increase the osmotic pressure reducing the amount of water that will flow in the cells and maintain the membrane integrity. Tween 80 is a detergent which reduces the amount of agglomeration between particles and microorganisms. Through the use of the three components it is possible to provide an environment that promotes the survival of the bacteria and fungi through air sampling and sample handling.

Traditionally, culture techniques have been used to determine bacterial concentrations as well as determine some of the genera that are present in the environment. However, culture techniques are limited to bacteria or fungi that grow at the conditions set by those running the cultures. Many bacteria that are both pathogenic and allergenic do not grow under the commonly used conditions. Large numbers of bacteria exist in the environment that only grow under specific conditions such as high salt concentrations, extreme temperatures, or lack of oxygen that are still harmful to both people and animals. One way to solve this problem is to use molecular techniques such as pyrosequencing to determine the presence of such bacteria in the environment without having to provide the variety of different conditions that are necessary for the bacteria to grow.

Pyrosequencing

Pyrosequencing is an important method for the analysis and identification of bacteria and fungi. Pyrosequencing, also known as sequencing by synthesis, employs a series of enzymatic reactions to add nucleotides to a primed template (Willey 2008). As each nucleotide is added to

the strand of DNA, a pyrophosphate is released which is then converted to ATP providing the energy to emit light. The light emitted is directly proportional to the number of nucleotides added making it possible to determine how many nucleotides are added to the DNA strand. Because the identity of the nucleotide is known it is possible to obtain the sequence of the DNA as the nucleotides are added (Novais and Thorstenson 2011).

In order to identify microbial samples, the same technique is employed while focusing on the 16S rRNA gene. The 16S rRNA gene possesses a region that is highly conserved as well as a region that contains distinctive species sequences (Ahmadian 2006). By examining the 20-100 distinctive nucleotides present in the 16S rRNA gene, the genus can be identified as well as different strains of bacteria present (Ronaghi 2001). The same techniques are applied for fungi with the focus on the 18S rRNA gene. This gene contains a highly conserved region as well as a region with specific sequences for each different genus. The variable region consists of approximately 40 nucleotides and can be used to identify the genus and species of different fungi present in the sample (Gharizadeh, Norberg et al. 2004).

In order to receive a positive identification at a species level, the bacteria or fungi must match the database more than 97%. A match of 95-97% is required for a positive identification at a genus level (Research and Testing Laboratory 2012).

The use of pyrosequencing to analyze an agricultural sample was first done in a poultry barn in 2010. This study found a large number of non-viable bacteria and fungi that place workers at risk for a variety of different respiratory diseases (Nonnenmann, Bextine et al. 2010). Pyrosequencing was first applied in the dairy industry in 2011 which found higher concentrations of Gram-positive bacteria than Gram-negative bacteria suggesting that although endotoxins are extremely important in considering worker health, it is also vital to look at the

constituents of Gram-positive bacteria and further examine how both types of bacteria are altering worker health. Additionally, high concentrations of anaerobic bacteria were found (Funk 2011); although anaerobic bacteria does not grow under normal growing conditions, the bacteria still play a vital role in worker respiratory health.

Riboprinting

Riboprinting, also known as ribotyping, is a method of analysis to identify different bacteria found in a variety of samples. The general mechanism for ribotyping starts with cell lysis to obtain DNA (Clark 1997). The DNA is then isolated and fragmented through the use of a variety of restriction enzymes. The various fragments present after segmentation are then separated through gel electrophoresis (Bouchet, Huot et al. 2008). Based on the location and intensity of the bands on the gel it is possible to compare the bands to known species of bacteria to obtain the identity of the bacteria (Typing 2012). The Riboprinter® is a new system that automates the current method while also transferring the gel to a membrane and applies a chemiluminescent agent that allows the bands to be visualized. The instrument then takes a picture of the membrane and compares the band distance and intensity to a database that allows for the identification of the bacteria (DuPont 2005). Due to the automation and quick analysis of the Riboprinter® the instrument is able to analyze the 5S, 16S, and 23S rRNA genes to gain more specificity in the identity of different bacterial strains.

This study is the first to use riboprinting to analyze environmental air samples in the agricultural setting as well as the first study to use pyrosequencing to analyze air samples collected with a SKC BioSampler in the dairy environment. Additionally, this is the first use of the resuscitation buffer in conjunction with the biosamplers and pyrosequencing.

CHAPTER 3: PURPOSE AND SCOPE

The modernization of dairy parlors throughout the United States has altered the manner in which workers on dairy farms are utilized. Due to the increase in production and the consolidation of the milking process, employees are spending the entirety of the work shift inside the dairy parlor. Other studies have indicated that workers in animal confinement buildings have a higher rate of respiratory symptoms stemming from exposure to organic dust. An important component of the organic dust is bacteria and fungi that are being aerosolized and consequently inhaled by the workers along with the constituents of both the bacteria and fungi such as endotoxins and mycotoxins.

The purpose of this study was to characterize the types of bacteria and fungi present within the dairy parlor and to evaluate several different methods of analyzing the bacteria and fungi. Comparison of the different analytical methods, will increase the knowledge of how to effectively measure levels of bacteria and fungi not only on dairy farms but in other agricultural sectors as well. Characterization and identification of bacteria and fungi, will help to better understand how the different genera and species could be contributing to the decrease in worker health. The comparison of different analysis methods and the identification of different bacteria and fungi, will also help in developing possible exposure control methods to help maintain a safe and healthy workplace.

CHAPTER 4: MATERIALS AND METHODS

Facilities

Aerosol samples were collected at two different modern dairy facilities in the Northern Colorado Area. Four samples were collected at each of the two facilities. Samples were collected inside the milking parlor at each facility. Site 1 was a traditional milking parlor; samples were collected at the bottom of the stairs that lead up to the milking stations as seen in Figure 4.1. Site 1 had a herd size of 1300 cows with approximately 100 cows in the milking parlor when milking operations were running. During the spring and summer months, site 1 was open and had fans running at all times; during the winter months the milking parlor was completely enclosed. Site 2 was an organic dairy that used a rotary milking system; samples were collected in the southeast corner of the milking parlor as seen in Figure 4.2. Site 2 had a herd size of 1800 cows with approximately 50 cows in the milking parlor while milking operations were running. Site 2 was open during both the winter and summer months. Samples were collected over four seasons from May 2012-January 2013.

The SKC Biosamplers ran for 60 minutes for each sampling trip. Four samples were collected in parallel duplicates over two consecutive sessions. A total n = 16 samples were collected at each dairy location reaching a total n = 32 for both sampling locations. A field blank and a lab blank were collected for each session to assess if there was any contamination present in the handling of the materials.

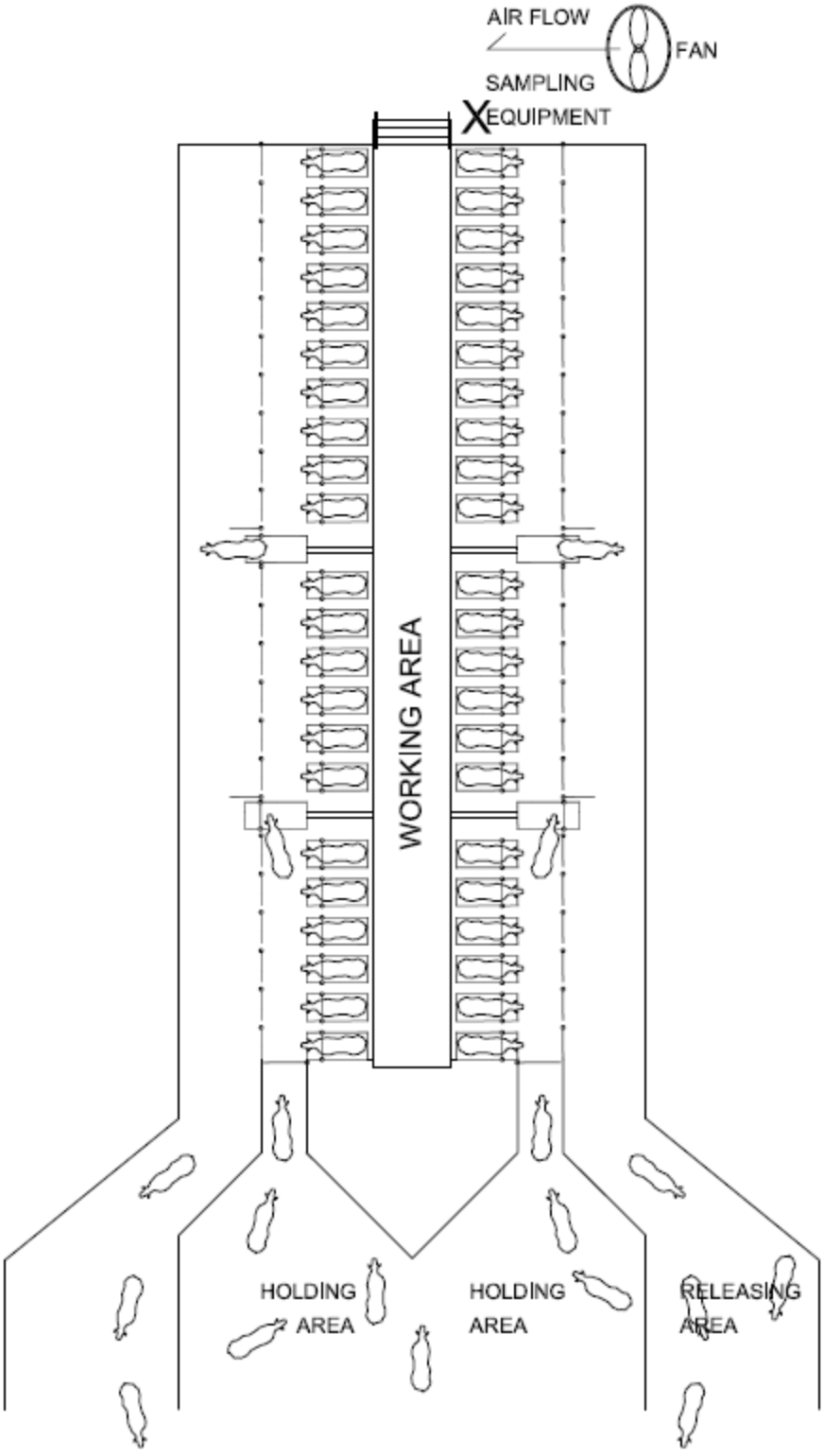

Figure 4.1 Schematic Diagram of Sampling Area at Site 1

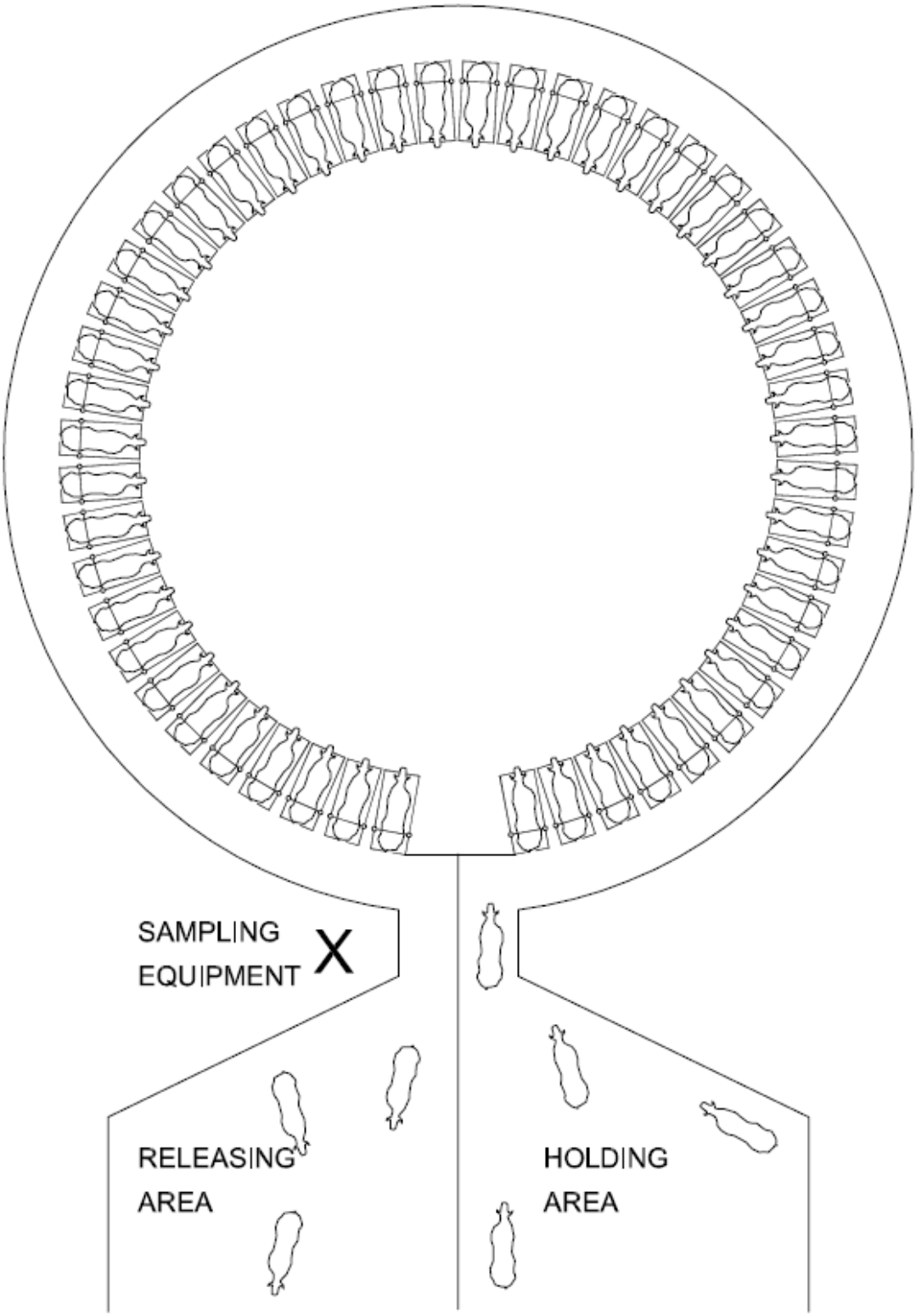

Figure 4.2 Schematic Diagram of Sampling Area at Site 2

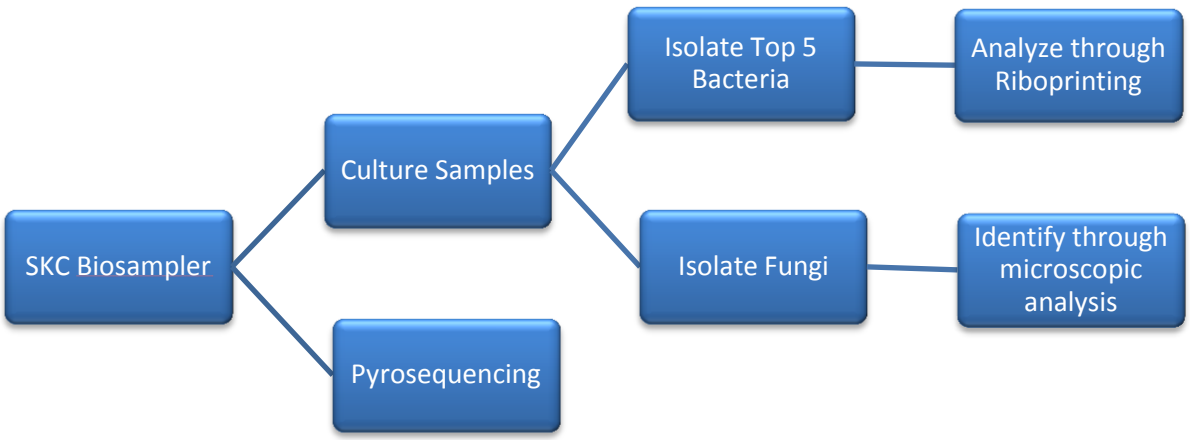

Figure 4.3 Overview of Sample Analysis

Biosampler Preparation

SKC BioSamplers (SKC, Inc., Fullerton, CA) were used to collect viable samples to generate cultures as well as for the pyrosequencing analysis of bacteria and fungi. Prior to use, the biosamplers were washed in an industrial strength detergent, allowed to dry, and then each piece was autoclaved in individual pouches. The day prior to sampling, 20 mL of the resuscitation buffer (Andersson, Laukkanen et al. 1995) (specific preparation is listed in Appendix A) was added to each collection vessel in a sterilized biosafety cabinet with sterile pipettes to prevent any contamination. The buffer consists of 10% polyethylene glycol (PEG), 0.1% peptone, and 0.05% Tween 80 solution. After the addition of the resuscitation buffer, the cap was placed on the collection vesicle and Parafilm® was placed around the seal to prevent any evaporation or contamination. The samples were stored in the fridge at 2-5°C overnight.

The SKC pumps were calibrated to a flow rate of 12.5 liters per minute (l/min) the night before with a biosampler that was not used for data collection to avoid any contamination from the air in the lab.

Sample Collection

The collection vesicles were transported to each site in a cooler on ice blocks. Once at the sampling site, the biosamplers were assembled immediately prior to attachment to the pump. The inlet portion and outlet portion were kept in their respective sterile autoclave bags until assembly. While sampling, the vesicle cap was placed in a sterile Whirl-Pak® bag to avoid contamination. Samples were collected for 60 minutes at a flow rate of 12.5 l/min. After sampling, the inlet and outlet sections of the sampler were removed and the cap was replaced along with Parafilm® around the seal. The collection vesicles were then placed back in the cooler and transported back to the lab on ice blocks. Once in lab, the remaining volume was measured, dilutions were made, samples were plated, and the remaining sample was frozen in a -80°C freezer. A 1:10 dilution of the collection media with sterile resuscitation buffer was made for the four samplers used to collect air samples; no dilution of the lab blank or field blank was made. All samples were plated in triplicate for each different media type for the undiluted sample as well as the 1:10 dilution.

Plating Methods

Three different types of media were used to identify the bacteria and fungi. Tryptic soy agar (TSA) with a 5% NaCl addition was used to select for Gram-positive bacteria, Eosin methylene blue agar (EMB) was used to select for Gram-negative bacteria, and malt extract agar (MEA) with chloramphenicol was used to select for the fungi (specific agar preparation methods are listed in Appendix A). To grow a countable sample, 0.1 mL of the dilution was added to

each plate and dispersed with a spreader that was dipped in a 70% ethanol solution and flame sterilized; all plates were done in triplicate. The TSA and EMB plates were incubated at 37°C ± 1°C while the MEA plates were incubated at 25°C ± 1°C to help promote growth of the desired organism type. The TSA plates were then counted after three days and the EMB and MEA plates were counted after five days. In addition to an overall count, a subculture count was also conducted to identify the colonies with the highest prevalence on each type of agar. Then, five to six of the most predominant colonies, based on morphological characteristics, on each agar type were selected for isolation and further analyzed using riboprinting for bacteria and microscopic analysis for fungal identification.

Fungal Identification

All fungi identification was done from isolated plates of the most abundant fungi present in each sample set. Fungal identification was completed using gross and microscopic morphology of the fungi. Lactophenol cotton blue was used to stain the fungal samples for easier microscopic identification. One drop of lactophenol cotton blue was added to a microscope slide and a piece of tape was then folded in half and used to pick up a sample of the fungi. The tape was then spread out over the drop of lactophenol cotton blue and examined under a microscope for further examination. Identification was based on the following mycology identification keys: *Medically Important Fungi* by David H. Larone; *The Medical Mycology Handbook* by Mary Campbell and Joyce Stewart; *Illustrated Genera of Imperfect Fungi* by H. Barnett and Barry Hunter; and *Mycology Online* by Adelaide University. Samples were identified by Stephen Reynolds and Amanda VanDyke with a quality control check by Douglas Rice.

Riboprinting

Once samples were isolated on EMB and TSA plates, the isolates were transferred to TSA plates that did not contain NaCl (a non-selective agar) for preparation of freezer stocks. From these isolated plates, a colony was selected using a sterile loop and transferred to a 10mL sterile tube of tryptic soy broth (TSB) and inverted to spread the colony throughout the media. The broth was then incubated with shaking for 18-24 hours at 37°C ± 1°C. Then, 500µL of 60% glycerol in TSB was added to a cryovial along with 500µL of the bacterial broth culture. The cap was then sealed and immediately placed in a -80°C freezer as a stock sample.

Prior to analysis, freezer stocks were used to make fresh plates of the bacteria. A minimum of two isolation streaks were completed on TSA plates and incubated at 37°C for 18-24 hours. For proper analysis the samples had to be fresh (less than 24 hours old) and gram stained. For Gram-positive bacteria two colonies were picked from a lawn on the TSA plate and transferred to 40 µL of sample buffer. For the Gram-negative bacteria, a single colony was picked from the lawn on the TSA plate and transferred to 200 µL of sample buffer. Next, 30 µL of each sample was transferred to another container and then heat treated. After the heat treatment, lysing agents A and B were added to each sample and the samples were then placed in the RiboPrinter® to complete the analysis.

Pyrosequencing

For each sampling trip, 3 mL of the resuscitation buffer from each sampling vessel was transferred to a 15 mL centrifuge tube and placed in the -80°C freezer until ready for shipment. Once ready for shipment the centrifuge tubes were placed in a Styrofoam centrifuge rack and placed on dry ice and shipped with two-day shipping to Research and Testing Laboratory in Lubbock, TX for pyrosequencing. Samples from sampling trips 1, 2, 5 and 7 (5/3/12, 5/16/12,

11/15/12, and 12/13/12 respectively) were sent for bacterial tag-encoded FLX 454 pyrosequencing (bTEFAP).

All samples sent for pyrosequencing were analyzed using the bTEFAP technique as previously described (Dowd 2008; Nonnenmann, Bextine et al. 2010; Ishak 2011). After samples were received by Research and Testing Laboratory, the samples were thawed and spun down at 14,000 rpm. Three hundred µL of the supernatant from the resuscitation buffer was then added to 500 µL of RLT buffer (Qiagen, Valencia, CA). Bacterial and fungal DNA was lysed from this 800 µL sample using 0.1 mm sterile glass beads in a Qiagen Tissue Lyser (Qiagen). The sample was then centrifuged and 100 µL of the supernatant was combined with 100 µL of ethanol. After being added to a DNA spin column, a Qiagen Stool Kit (Qiagen) was used to recover the DNA using standard protocol starting at step five. The steps in the Qiagen Stool Kit begin by adding one InhibitEX tablet and vortexing the sample until the tablet is suspended and allowing solution stand for one minute. Then, the sample was then centrifuged for three minutes followed by transferring 200 µL of the supernatant to a new centrifuge tube and adding 15 µL of proteinase K and 200 µL of buffer AL and vortexing the sample for approximately 15 seconds. The sample was then incubated for 10 minutes at 70°C followed by the addition of 200 µL of ethanol and vortexing. The lysate was placed in a QIAamp spin column and centrifuged for one minute. Then, 500 µL of Buffer AW1 and AW2 were added to the QIAamp spin column. The spin column was then placed in a centrifuge tube and 200 µL of Buffer AE was added to the tube and the sample was centrifuged to elute the DNA. A final concentration of 20 ng/µL was used.

bTEFAP was performed as previously described using the Titanium protocols and reagents (Dowd 2008; Nonnenmann, Bextine et al. 2010; Ishak 2011). The PCR primers for the FLX amplicon processing were Gray 28F primer (5'GAGTTTGATCNTGGCTCAG) and

Gray519r primer (5'GTNTTACNGCGGCKCTG) for the 16S gene. PCR was completed with a mixture of Hot Start and Hot Star high-fidelity Taq polymerases with a total of 30 cycles. After sequencing, all low quality sequence ends and tags, failed sequence reads, and primers were removed from the sequences. The black box chimera check software depleted sequences of non-bacterial ribosome sequences. The remaining sequences were used to identify the bacteria based on the operational taxonomic units (OTUs) using the BLASTN+ (KrakenBLAST www.krakenblast.com) algorithm. The BLASTN+ outputs and data reduction analysis was performed using a .NET and C# analysis. All sequences with >97% positive identification were identified at a species level, 95-97% at a genus level, and 90-95% at a family level.

Quality Control

To account for nonspecific differences between samples, controls and replicates were used for all samples. For each sampling session, a lab blank and field blank were collected. Both the lab and field blank were prepared at the same time and in the same manner as the other collection vessels. The blanks were placed in the refrigerator along with the other samples overnight, but the lab blank was left in the refrigerator during sampling collection. The field blank was transported in the same cooler and treated in the same manner as the other collection vessels in the field but was never attached to a sampling pump. Additionally, for each media type a control plate was placed in the incubator with all inoculated plates and incubated for the same time at the same temperature to check for contamination within media preparation procedures. Both undiluted, 1:10 dilutions, and blank samples were completed in triplicate for each collection vessel to assess variability between plates.

Environmental Conditions

Temperature, relative humidity, carbon dioxide, and carbon monoxide were measured at each site for both indoor and outdoor conditions. A Model 8554 Q-Trak (TSI INC., Shoreview, MN) was used for both measurements. The Q-Trak was set to log all measurements and record a measurement every five minutes. One Q-Trak was set on top of the IESL 3-stage cloud impactor which is where the biosamplers were also located for the indoor measurements. The outdoor measurements were completed with another Q-Trak that was hung on the antenna of the truck adjacent to the parlors during the entire sampling period.

Data Analysis

Each plate that was cultured was counted for the number of colonies. Based on those plate counts, the number of colony forming units (CFU) was calculated per mL of resuscitation buffer as well as the CFU for the total volume of resuscitation buffer. Based on the CFU calculations as well as the sampling time and flowrate it was then possible to calculate CFU/m^3 (specific calculations in Appendix B). For each calculation, a value was calculated for each individual plate and then averaged for the triplicate of the three plates. All of the above calculations were completed with Microsoft Excel. Bar graphs were created with SigmaPlot and then used to compare sampling sessions as well as identify trends within the data.

The pyrosequencing data was first separated by sampling date and sampler. Then, the top 30 identifications within the genus counts were used to compare and analyze. Stacked bar graphs were created with SigmaPlot to further visualize the breakdown of the top genera of both the bacteria and the fungi. The number of hits for each postiviely identified genus was then used to determine the number of CFU/m^3 present in the sample for each genus and then combined for

the total value of CFU/m^3 for the entire sample submitted for pyrosequencing in the same manner as the calculations done for the cultured bacteria demonstrated in Appendix B.

The riboprinting results were calculated by counting the number of morphologically similar colonies that occurred and were positively identified through the use of riboprinting. The counts were then placed in Microsoft Excel and the frequency in which the morphologically, positively identified colonies appeared was claculated. The pie chart was then greated using Microsoft Excel.

Prior to data analysis, a test of normality was done with MiniTab which found that the data was not normal but was log-normal. Therefore, natural log transformations were completed for all of the data and the GM was used to further analyze all of the data. SigmaPlot was used to create all figures.

In order to better understand the correlations present in the data, Spearman correlations were completed to compare Gram-positive bacteria, Gram-negative bacteria, fungi, temperature, relative humidity, wind speed, carbon dioxide concentration, and carbon monoxide concentrations. The Spearman correlations were completed using SigmaPlot and only p values < 0.05 were considered statistically significant.

CHAPTER 5: RESULTS AND DISCUSSION

Viable Bacteria Concentrations

The arithmetic and geometric means and standard deviations for the TSA and EMB agars can be found in Table 5.1 and Table 5.2. The geometric mean (GM) of the TSA and EMB plates ranged drastically from one sampling trip to the next. The TSA GM ranged from 1,320 to 18,000 CFU/m^3, a difference of 16,700 CFU/m^3. The EMB GM range was much lower in comparison to the TSA plates; it ranged from 65.8 to 1230 CFU/m^3, a difference of 1170 CFU/m^3. The geometric standard deviations (GSD) were relatively small indicating small intra-sampler variability (Table 5.1 and 5.2). The differences between samplers could be a result of washing down within the dairy parlor during sampling altering the bacterial concentrations found within the dairy parlor.

The Gram-positive bacterial concentrations were higher in comparison to data from previous studies on dairy farms such as the work done by Bradley Lester in 2008. Lester found Gram-negative geometric mean bacterial concentrations around 300 CFU/m^3; this study found concentrations in the same order of magnitude at concentrations approximately 500 CFU/m^3. Lester's study found Gram-positive bacterial concentrations at approximately 400 CFU/m^3 which is much lower in comparison to the average concentration of 7500 CFU/m^3 found in this study (Lester 2008). A possible explanation for this increase in bacterial concentration is the novel resuscitation buffer that was used for a collection media which could provide a more habitable environment for both Gram-positive and Gram-negative bacteria.

Table 5.1 Arithmetic and geometric mean and standard deviation by sample date and site for TSA Agar (CFU/m^3)

Site	Date	Arithmetic Mean	Standard Deviation	Range	Geometric Mean	Geometric Standard Deviation
1	3-May-12	2630	1980	84.4-212000	1320	6.44
1	16-May-12	8830	5290	183-19900	7590	1.94
1	8-Nov-12	14100	8860	3370-24000	4830	2.14
1	13-Dec-12	19200	7770	2310-26100	18000	1.53
2	12-Jun-12	7650	2450	512-10000	7300	1.44
2	15-Nov-12	14610	4740	126-18000	13900	1.50
2	29-Nov-12	4350	1750	60.1-6860	4120	1.44
2	24-Jan-13	3510	1710	62.6-5380	3100	1.77

Table 5.2 Arithmetic and geometric mean and standard deviation by sample date and site for EMB Agar (CFU/m^3)

Site	Date	Arithmetic Mean	Standard Deviation	Range	Geometric Mean	Geometric Standard Deviation
1	3-May-12	529	584	72.0-8880	350	2.77
1	16-May-12	570	264	347-20800	530	1.54
1	8-Nov-12	1240	173	191-1410	1230	1.15
1	13-Dec-12	419	243	76.9-701	357	2.00
2	12-Jun-12	51.1	38.7	49.1-123	65.8	1.38
2	15-Nov-12	144	81.2	60.8-255	127	1.80
2	29-Nov-12	185	166	0.00-430	144	2.19
2	24-Jan-13	330	92.9	0.00-438	320	1.33

Based on the assumption that the TSA plates are only growing Gram-positive bacteria due to the NaCl addition, the Gram-positive bacteria are better able to withstand the harsh environmental sampling conditions. The average CFU/m^3 for TSA plates over all of the sampling sessions was 8370CFU/m^3 which is considerably higher than the EMB plate average of 391 CFU/m^3. There was significantly less growth on the EMB plates suggesting that the Gram-negative bacteria are less likely to withstand the environmental sampling conditions or are less

likely to grow in the sample buffer. It is unlikely that the sample buffer inhibited the growth for the Gram-negative bacteria because the PEG actually provides more nutrients for Gram-negative bacteria such as *Pseudomonas* species (Andersson, Laukkanen et al. 1995).

The bacterial concentrations were plotted in chronological order from spring to winter in Figure 5.1. As anticipated, the bacterial concentrations were higher in the winter and fall months than the spring and summer months. This difference in concentration can be explained by the increase in ventilation during the summer and spring months within the dairy parlors. During warmer weather, the majority of the doors are left open and a larger number of fans are used in the dairy parlors decreasing the concentration of airborne bacteria. Based on this pattern, the concentrations of bacteria were also plotted against temperature and a Spearman correlation was completed and found no statistically significant correlation between colony concentration and temperature inside the dairy parlor or outside.

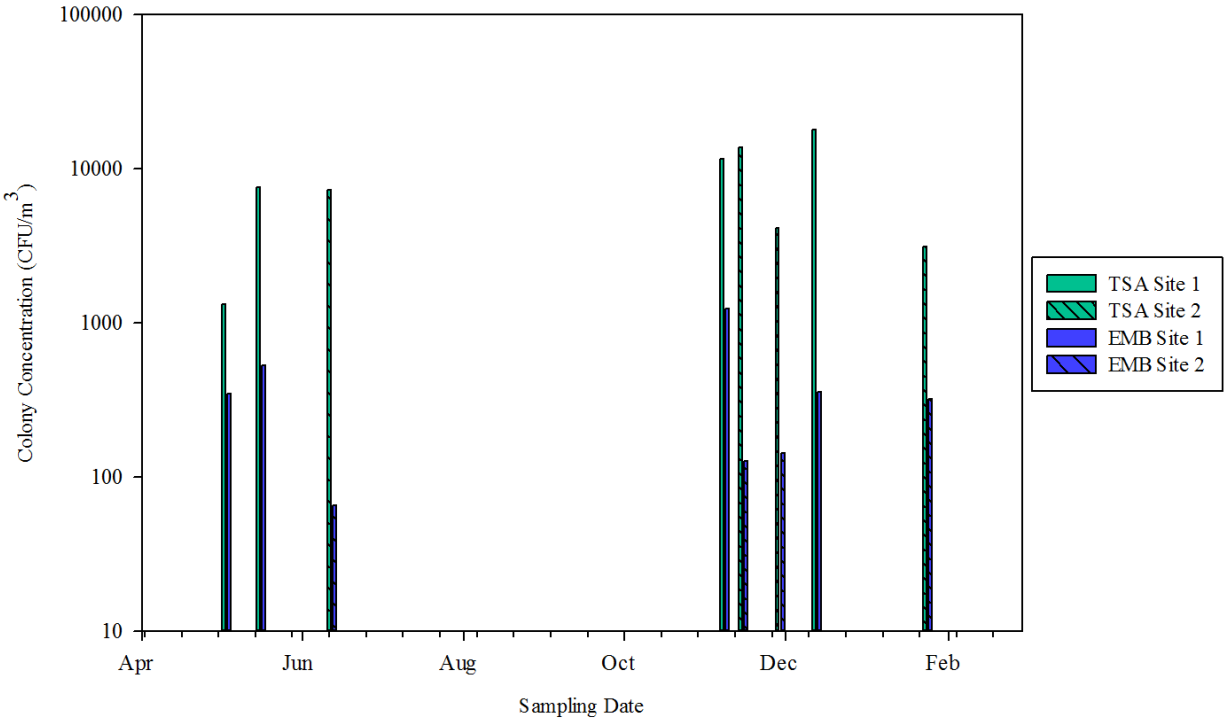

Figure 5.1 Geometric mean of bacterial concentration for all TSA and EMB Plates

Spearman correlations were completed for all of the different media types to analyze the correlation between the different types of bacteria as well as the fungi. There was no correlation between Gram-positive and Gram-negative bacteria or Gram-positive bacteria and fungi. However, there was a statistically significant correlation between Gram-negative bacteria and fungi (Figure 5.2) with a correlation coefficient of 0.905 at $p < 0.05$. Therefore, as the concentration of Gram-negative bacteria increased, the concentration of fungi also increased.

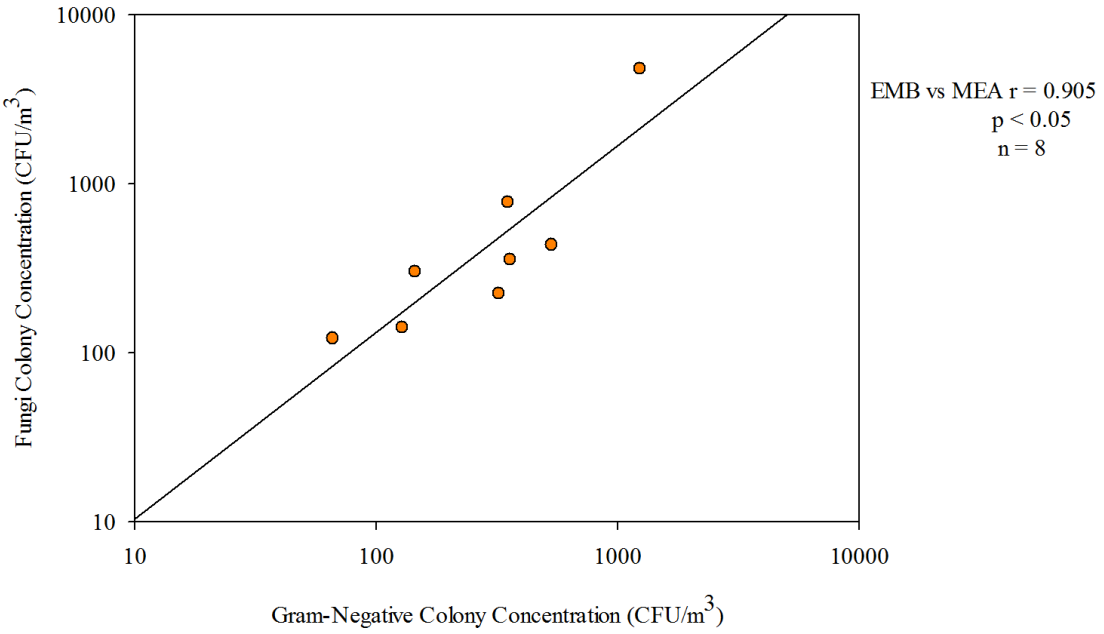

Figure 5.2 Spearman correlation of Gram-negative bacteria and fungi

Bacteria Pyrosequencing Results

The pyrosequencing bacterial concentrations from the resuscitation buffer were on the same order of magnitude as the culturable bacterial concentrations. The pyrosequencing concentrations were slightly higher in most cases. One reason for this difference is the fact that some of the bacteria that are found in the pyrosequencing data are no longer viable and therefore cannot be cultured and counted; only RNA is left to be examined which can be found in pyrosequencing. Additionally, the growth conditions for the bacteria are not optimal for all types

of bacteria. Some of the bacteria are anaerobic, requiring an environment that lacks oxygen. Additionally, the incubation temperature of 37°C is optimal for pathogens, but is not ideal for all types of bacteria, many bacteria prefer warmer or cooler temperatures. The TSA and EMB media select for Gram-positive and Gram-negative bacteria respectively, but are meant to be non-selective within their respective categories which do not necessarily provide the ideal growth conditions for certain bacteria. Unfortunately, due to the small number of pyrosequencing samples, a Spearman correlation was not completed to test the significance of the correlation.

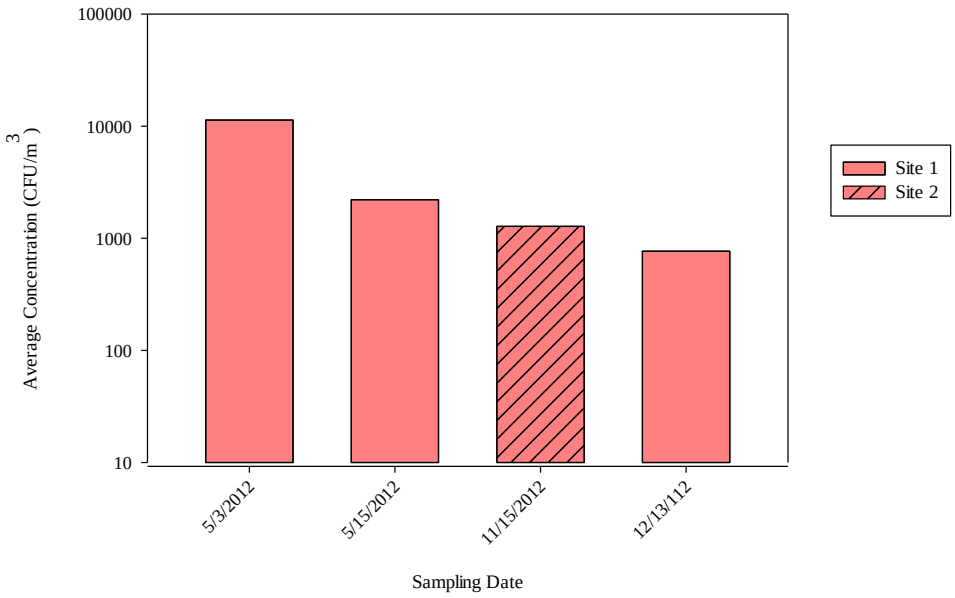

Figure 5.3 Geometric mean of bacterial pyrosequencing concentrations

There was large variability between sampling trips when analyzing the pyrosequencing data. The top 20 genera of bacteria and fungi were not comparable between sampling trips. Therefore, each set of pyrosequencing data was analyzed separately. The top five genera of bacteria found during the sampling trip on May 3, 2012 were *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Carnobacterium*, and *Bacillus* (Figure 5.4). The *Carnobacterium* and *Bacillus*

genera are both Gram-positive bacteria while the *Psychrobacter*, *Acinetobacter*, and *Pseudomonas* genera are Gram-negative. This particular sampling session had an extremely high abundance of the genus *Pseudomonas* in comparison to the other samples in this study. The high genus counts present in the lab blank is a result of sampling error; the lab blank was used to calibrate the pumps and consequently acquired a large number of bacteria in the process. However, the field blank, which was not used for any sampling, did not have bacteria present in the sample indicating that the resuscitation buffer used for collection as well as the practices used to prepare the samples was sterile.

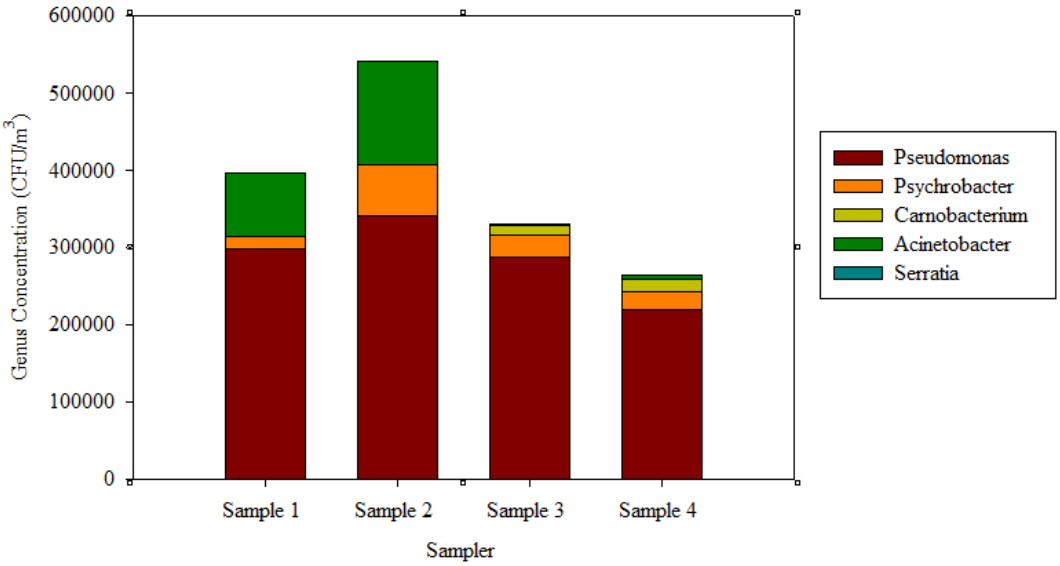

Figure 5.4 Pyrosequencing results for sampling trip at Site 1on May 3, 2012

The breakdown of the genera of bacteria for the sampling trip at Site 1 on May 16, 2012 looks very different in comparison to the sampling trip on May 3, 2012. The sampling session on May 16, 2012 has a much larger diversity of bacterial genera but the top five bacteria are still very similar (Figure 5.5). The five most common genera for the May 16th sampling session are *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Bacillus*, and *Clostridium.* Bacillus and Clostridium are the only genera in the top five bacteria that are Gram-positive. The genus

Clostridium is anaerobic and would therefore not be viable under normal growing conditions. There was also sampling error in the lab blank for this sampling session as it was used to pre-calibrate and post-calibrate the pumps. However, the field blank was treated in the same manner as the other pumps but was not used for any sort of air sampling. There was an extremely low count (0.1%) found in the field blank sample.

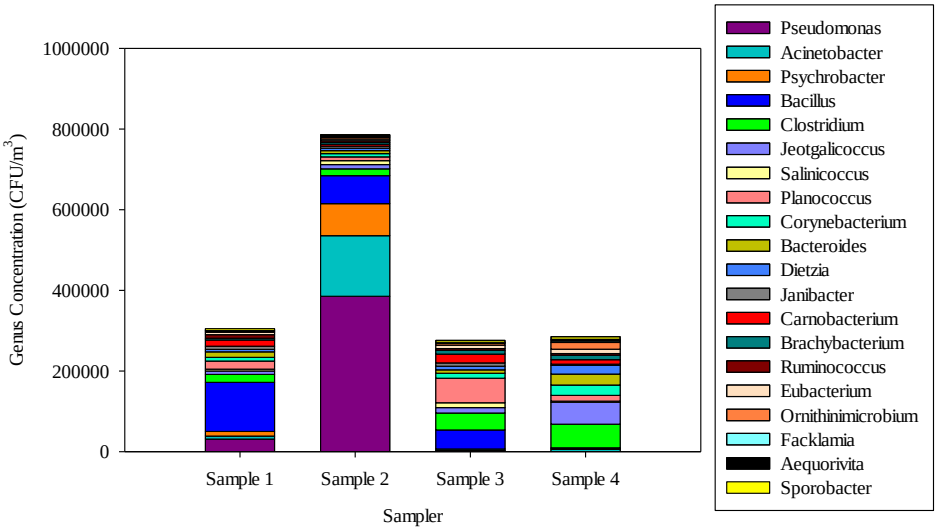

Figure 5.5 Pyrosequencing results for sampling trip at Site 1 on May 16, 2012

The top five bacterial genera in November and December did not resemble the top five bacterial genera found in May. One possible explanation for this is the seasonal and consequently temperature variability in the different sets of sampling sessions. The top five bacterial genera during this sampling session were *Serratia*, *Pseudomonas*, *Oscillibacter*, *Ruminococcus*, and *Clostridium*. From these top five genera, *Serratia*, *Pseudomonas*, and *Oscillibacter* are all Gram-negative while *Clostridium* and *Ruminococcus* are both Gram-positive. The sampling trip on November 15, 2012 was the only sampling session that has pyrosequencing data from Site 2.

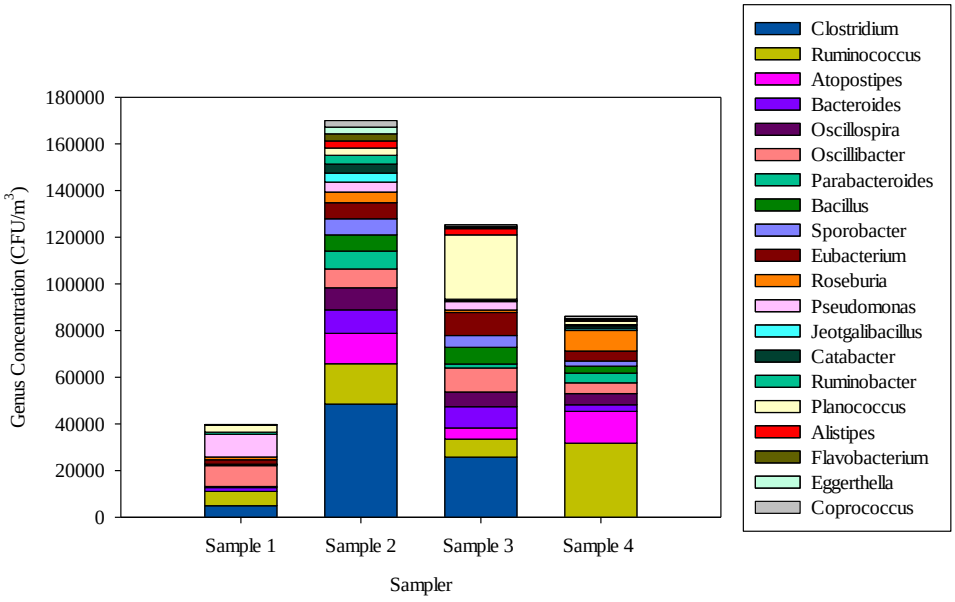

Figure 5.6 Pyrosequencing results for sampling trip at Site 2 on November 15, 2012

The sampling session on December 13, 2012 had a different set of top five bacterial genera that included: *Clostridium*, *Eubacterium*, *Bacteroides*, *Jeotgalibacillus*, and *Oscillospira*. From these top five bacteria, *Clostridium* and *Jeotgalibacillus* are Gram-positve; *Bacteroides* and *Oscillospira* are Gram-negative; and *Eubacterium* is Gram-variable.

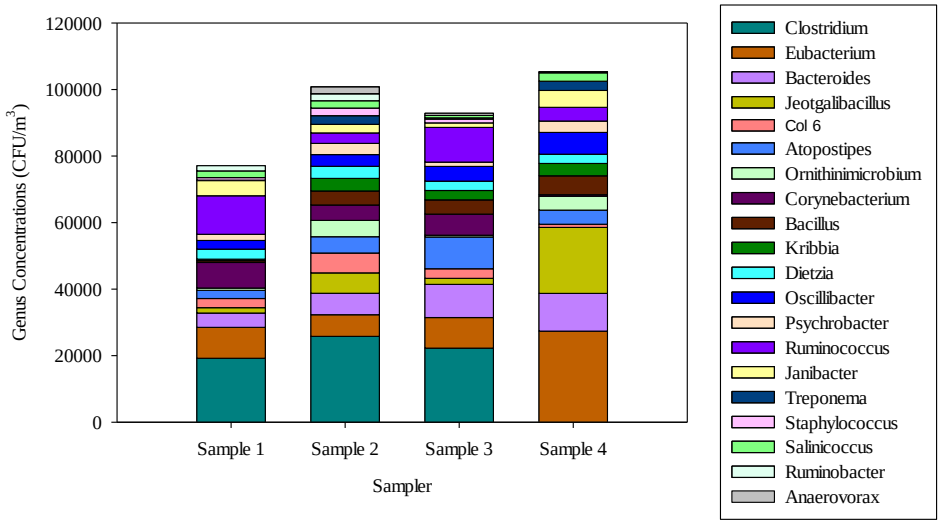

Figure 5.7 Pyrosequencing results for sampling trip at Site 2 on December 13, 2012

The list of the top 20 bacteria for the pyrosequencing data for each sampling site was compiled and more information was found to further analyze the potential pathogenicity of the bacteria as well as the source. Based on this information, there were a lot more Gram-negative bacteria (Table 5.3) found in the pyrosequencing data than Gram-positive bacteria (Table 5.4). Additionally, the Gram-negative bacteria had a lot more genera that had potential pathogenic species associated with them than the Gram-positive bacteria. Approximately 50% of the Gram-negative bacteria that were identified had potential pathogenic species while only 37% of the identified Gram-positive bacteria had pathogenic species. The potential origins of the bacteria are consistent with items that are expected in the dairy parlor such as soil, water, fecal matter, animal rumen, and milk and workers. Many of the pathogenic genera are commonly found in soil which could be used to further examine potential abatement procedures to reduce the exposure to these bacterial genera. Additionally, a lot of bacteria are commonly found in salt water and only grow in conditions with high salt concentrations. A potential explanation for this is the cleaning products that are used to clean the dairy parlor between shifts which often contain salts promoting the growth of the bacteria that thrive in high salt environments. There were many bacterial genera that were anaerobic which would not grow in the conditions used to grow these bacteria which could explain the lack of diversity found in the riboprinting data. All of the information regarding the bacterial genera was found through the use of *Bergey's Manual of Systematic Bacteriology Volumes 1-5* (2009).

Table 5.3 Characteristics of Gram-negative bacteria found through pyrosequencing

Genus	Respiration	Potential Pathogenicity	Potential Source
Acinetobacter	Aerobic	Pathogenic Species	Soil
Aequorivita	Aerobic	Non-Pathogenic	Salt Water
Alistipes	Anaerobic	Pathogenic Species	Fecal Matter
Aquimarina	Aerobic	Non-Pathogenic	Salt Water
Bacteroides	Anaerobic	Pathogenic Species	Human Flora
Brumimicrobium	Aerobic/Anaerobic	Non-Pathogenic	Salt Water
Butyrivibrio	Anaerobic	Non-Pathogenic	Animal Rumen
Desulfonatronum	Aerobic/Anaerobic	Non-Pathogenic	Sediment
Halomonas	Aerobic	Pathogenic Species	Salt Water
Leptospira	Aerobic	Pathogenic Species	Water
Marinobacter	Aerobic/Anaerobic	Non-Pathogenic	Water
Massilia	Aerobic	Pathogenic Species	Soil
Methylobacterium	Aerobic	Pathogenic Species	Ubiquitous
Paludibacter	Anaerobic	Non-Pathogenic	Water
Pantoea	Facultative Anaerobic	Pathogenic Species	Plants
Parabacteroides	Anaerobic	Pathogenic Species	Human Flora
Paracoccus	Aerobic	Non-Pathogenic	Soil
Phascolarctobacterium	Aerobic	Non-Pathogenic	Fecal Matter
Prevotella	Anaerobic	Pathogenic Species	Human Flora
Pseudobutyrivibrio	Facultative Anaerobic	Non-Pathogenic	Animal Rumen
Pseudomonas	Aerobic	Pathogenic Species	Ubiquitous
Psychrobacter	Aerobic	Pathogenic Species	Soil
Ruminobacter	Anaerobic	Non-Pathogenic	Animal Rumen
Serratia	Facultative Anaerobe	Pathogenic Species	Soil/Water
Sphingobium	Aerobic/Anaerobic	Pathogenic Species	Soil
Treponema	Anaerobic	Pathogenic Species	Bovine Intestinal Tract

Table 5.4 Characteristics of Gram-positive bacteria found through pyrosequencing

Genus	Respiration	Potential Pathogenicity	Potential Source
Aerococcus	Facultative Anaerobic	Pathogenic Species	Soil/Dust
Anaerovorax	Anaerobic	Non-Pathogenic	Water/Food
Atopostipes	Facultative Anaerobic	Pathogenic Species	Manure
Bacillus	Aerobic	Pathogenic Species	Ubiquitous
Brachybacterium	Aerobic	Non-Pathogenic	Milk/Water
Carnobacterium	Aerobic	Non-Pathogenic	Milk
Clostridium	Anaerobic	Pathogenic Species	Soil
Corynebacterium	Anaerobic/Aerobic	Pathogenic Species	Soil/Water
Desemzia	Microaerophillic	Non-Pathogenic	Insects
Dietzia	Aerobic	Non-Pathogenic	Human/Animal Flora
Janibacter	Aerobic	Non-Pathogenic	Soil/Water
Jeotgalicoccus	Facultative Anaerobic	Non-Pathogenic	Dust
Kribbia	Facultative Anaerobic	Non-Pathogenic	Oil
Oscillospira	Anaerobic	Non-Pathogenic	Animal Rumen
Planococcus	Aerobic	Non-Pathogenic	Salt Water
Ruminococcus	Anaerobic	Pathogenic Species	Animal Rumen/ Human Intestines
Salinicoccus	Aerobic	Non-Pathogenic	Salt Water
Staphylococcus	Aerobic	Pathogenic Species	Ubiquitous

Riboprinting Results

From the top most abundant bacteria based on colony morphology from the TSA and EMB plates it was possible to obtain good positive identifications. The riboprinter only reports a positive identification when the similarity is greater than 80%. Based on the positive identifications, the most common genus found was *Bacillus* followed by *Pseudomonas*, *Brevibacillus*, and *Eschericia* as seen in Figure 5.8. All of the *Bacillus* genera and species that were identified are not pathogenic, however *Pseudomonas aeruginosa* is an opportunistic pathogen and *Escherichia coli O157:H7* is a known pathogen. There were multiple problems

associated with the riboprinter that resulted in fewer identifications than was expected. Because a minimum of eight samples need to be run at a single time, freezer stocks were made of each isolate for future riboprinting. After an attempt at recovery was made there were a large number of samples that could not be regrown resulting in no identification. A possible explanation for this is that some of the samples that could not be regrown were yeast that appeared to be bacteria and the freezer stock solution is not meant to maintain yeast cultures. Additionally, the database for the riboprinter does not include all of the isolates that were identified. The riboprinter does however allow for the identification on the species and even strain level in many cases in a timely manner.

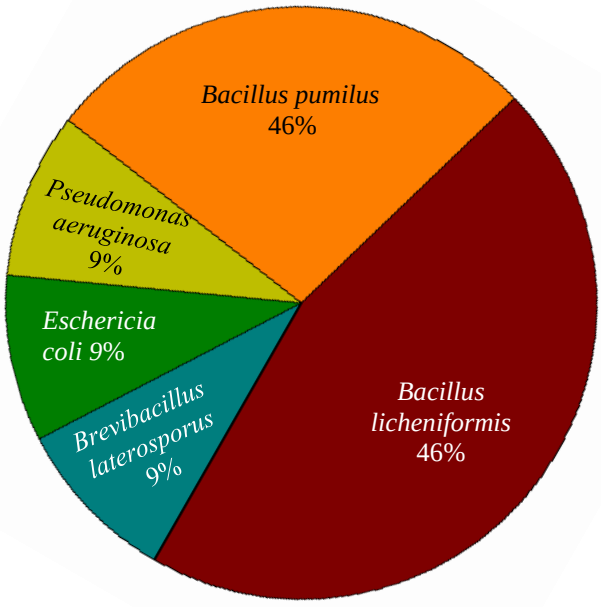

Figure 5.8 Bacterial riboprinting results

Culturable Fungi Concentrations

The fungal concentrations were lower than the Gram-positive bacterial concentrations, but generally higher than the Gram-negative concentrations. One possible explanation for less growth on the MEA plates is the way in which the colonies form. When the fungi grew on the

MEA plates, the colony generally took over the majority of the plate reducing the amount of other fungi that are able to grow on that plate due to a limited supply of space and nutrients. The GM of the MEA plates ranged from 122 to 4830 CFU/m^3, a difference of 4710 CFU/m^3 (Table 5.5). Lester's previous study (2008) found a concentration of approximately 250 CFU/m^3 which is much lower than the concentration of 800 CFU/m^3 that was found in this study a potential result of the resuscitation buffer.

Table 5.5 Arithmetic and geometric mean and standard deviation by sample date and site for MEA agar (CFU/m^3)

Site	Date	Arithmetic Mean	Standard Deviation	Range	Geometric Mean	Geometric Standard Deviation
1	3-May-12	1090	897	200-26300	781	2.70
1	16-May-12	484	272	285-2720	438	1.64
1	8-Nov-12	5730	3460	114-9630	4830	2.05
1	13-Dec-12	1140	753	76.9-2170	357	1.95
2	12-Jun-12	133	53.5	58.2-140	122	1.66
2	15-Nov-12	94.8	150	63.1-315	142	3.09
2	29-Nov-12	341.63	167.95	0.00-518.02	304	1.81
2	24-Jan-13	170	118	0.00-262	225	1.18

Concentrations of fungi were much more consistent among sampling trips in comparison to the bacterial concentrations. However, one sampling trip (November 8, 2012) was exceptionally high in comparison to the other sampling trips as seen in Figure 5.9. Without that sampling trip, the geometric means have a difference of 659 CFU/m^3, much smaller than the previous 4710 CFU/m^3. The sampling trip on November 8, 2012 had higher yeast counts which grow in smaller colonies and therefore result in higher concentrations. One possible explanation is that this sampling trip had a much higher relative humidity of 63.1% which was almost 10% higher than the next closest relative humidity of 53.8%.

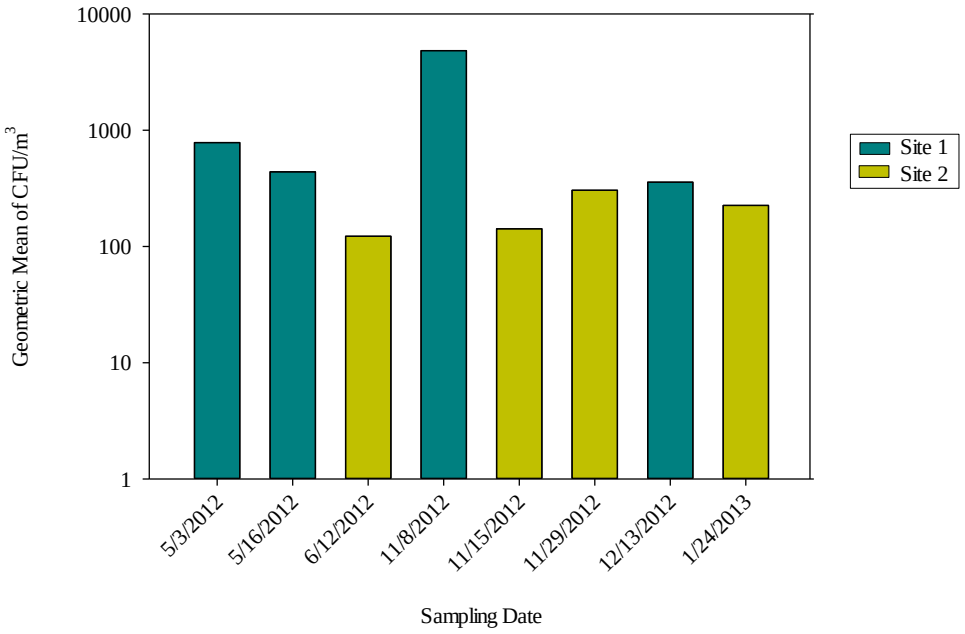

Figure 5.9 Geometric mean of fungi concentration for MEA plates

The geometric mean of the fungal concentrations from pyrosequencing were also plotted and compared to the culturable fungal concentrations. It was found that the concentrations were also consistent and within the same order of magnitude as seen in Figure 5.10. Due to the small number of pyrosequencing samples (n = 2) it was not possible to run a Spearman correlation to test the significance of the pyrosequencing and culturable samples.

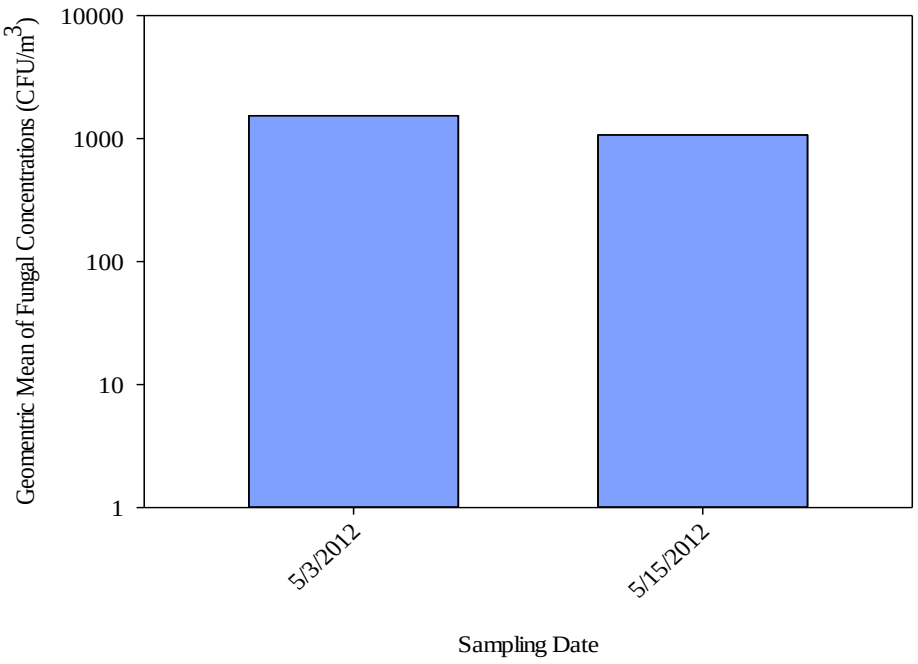

Figure 5.10 Geometric mean of fungal concentration for pyrosequencing data

All of the fungi that grew on the MEA agar were identified based on their microscopic and macroscopic morphology through the use of multiple fungal identification guides. The frequency of each fungal genus was plotted in Figure 5.11. *Cladosporium* was the genus that was found most frequently at 40%. *Aspergillus* was the second most common genus found in the fungi samples with a frequency of 26%. Some further analysis into species identification was done on some samples but it was difficult to determine for many of the samples collected. There were no pathogenic forms of *Aspergillus* identified in these samples. However, *Cladosporium* and *Alternaria* are known allergens and could potentially lead to respiratory symptoms which is concerning for the dairy workers. Refer to Appendix C for pictures of the samples isolated and identified.

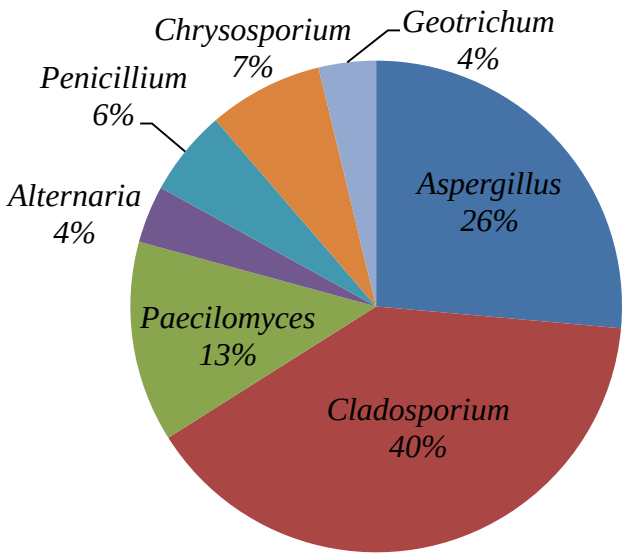

Figure 5.11 Percentage of fungal genera identification

Fungal Pyrosequencing Data

The pyrosequencing counts identified a wider variety of fungi than the culture techniques. A greater diversity of fungal genera and higher counts were found in pyrosequencing due to the specificity of the media for various fungi as well as differing incubation temperatures for the environmental fungi. Additionally, many of the fungi cannot survive the harsh sampling conditions and therefore cannot be cultured and grown on media. However, it is possible for there to be remnants present in the sample collection which can be identified through pyrosequencing.

The top five genera of fungi found during the sampling session on May 3, 2012 were *Pichia*, *Cryptococcus*, *Trichosporon*, *Cryptococcus (Filobasidiales)*, and *Phoma*. Of the top five genera found during this sampling session, all but the genus *Phoma* are considered yeast (Figure 5.12) .There was a large amount of *Cryptococcus* found in the lab blank for this particular sampling run. The most predominant yeasts found on the MEA plates were analyzed using an

API 20 C AUX (Biomerieux) test. All of the yeast isolated and tested were found to be in the genus *Cryptococcus*.

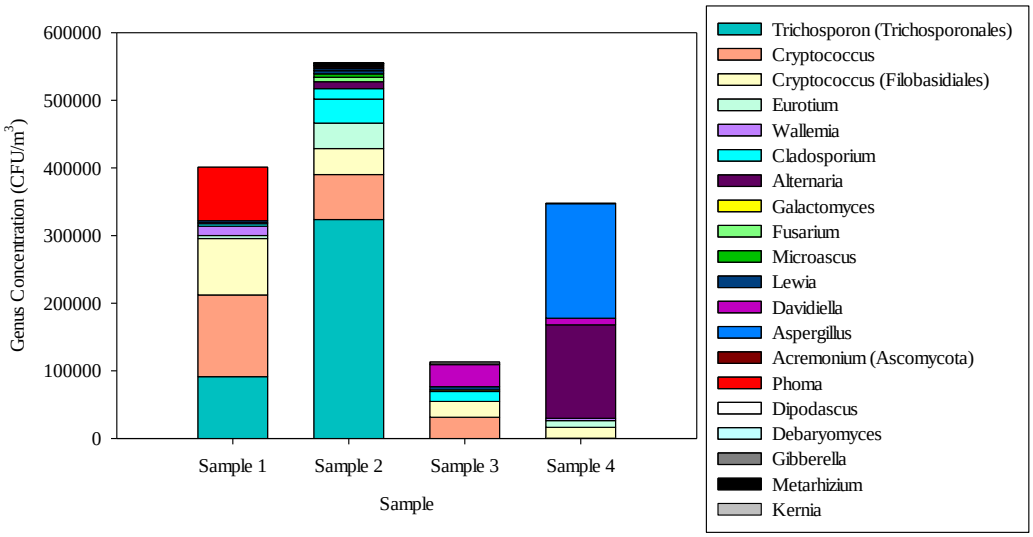

Figure 5.12 Fungal pyrosequencing counts for May 3, 2012 sampling session inside the dairy parlor at Site 1

The top five fungal genera found during the sampling trip at Site 1 inside the dairy parlor on May 16, 2012 are *Knufia*, *Alternaria*, *Cladosporium*, *Cryptococcus*, and *Davidiella*. Out of these top five genera, all of the isolates were filamentous fungi except for the genus *Cryptococcus* which was yeast.

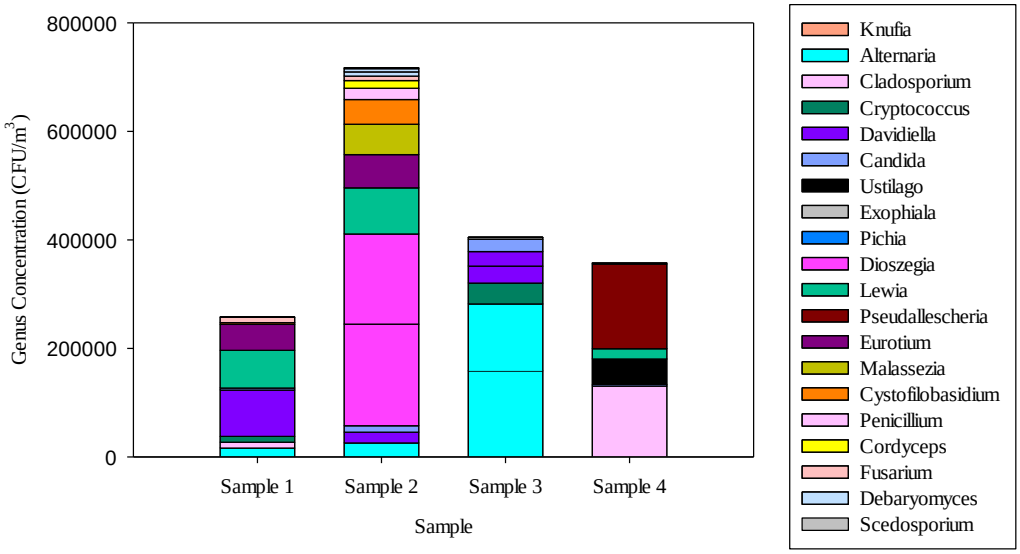

Figure 5.13 Fungal pyrosequencing counts for May 16, 2012 sampling session inside the dairy parlor at Site 1

Samples were sent for analysis for sampling sessions on November 15, 2012 and December 13, 2012 but there were no fungi found in the samples. It is unclear why this occurred, due to the high sensitivity of the pyrosequencing and its ability to identify nonviable samples. For both sampling sessions there was fungal growth on the MEA plates, although it was lower for the November 15, 2012 sampling session in comparison to the other sampling sessions. However, there was still growth indicating the presence of fungi in the samples. The lab blank and field blank agar plates were blank so there was no contamination in the media or resuscitation buffer. John Delton-Hanson made the recommendation of amplifying the samples further to observe some sort of identification but in order to keep the sample handling consistent, no additional amplification was used to increase the sensitivity of the pyrosequencing.

To further analyze and characterize the fungal samples found through pyrosequencing, more information was found regarding the fungal genera in the top 20 through the use of *Mycology Online* through Adelaide University (Table 5.6). The majority of the fungi samples

identified were filamentous fungi and most of the fungi are also commonly found in soil. Approximately 20% of the fungi identified through pyrosequencing contain pathogenic species.

Table 5.6 Fungal characteristics of fungi identified through pyrosequencing

Genus	Filamentous/ Yeast	Potential Pathogenicity	Source
Acremonium	Filamentous	Opportunistic Pathogen	Soil
Alternaria	Filamentous	Pathogenic Species	Soil
Aspergillus	Filamentous	Pathogenic Species	Soil
Candida	Yeast	Pathogenic Species	Soil/Water/ Animal Feces
Cladosporium	Filamentous	Pathogenic Species	Air
Cordyceps	Filamentous	Non-pathogenic	Plants
Cryptococcus	Yeast	Pathogenic Species	Human Flora
Cystofilobasidium	Yeast	Non-pathogenic	Plants
Davidiella	Filamentous	Non-pathogenic	Plants
Debaryomyces	Yeast	Non-pathogenic	Salt Water
Dioszegia	Yeast	Non-pathogenic	Soil
Dipodascus	Yeast	Non-pathogenic	Water/Milk
Eurotium	Filamentous	Pathogenic Species	Soil
Exophiala	Filamentous	Pathogenic Species	Soil/Wood
Fusarium	Filamentous	Pathogenic Species	Soil
Galactomyces	Yeast	Opportunistic Pathogen	Plants
Gibberella	Filamentous	Non-pathogenic	Plants
Knufia	Filamentous	Non-pathogenic	Plants
Lewia	Filamentous	Non-pathogenic	Grain
Malassezia	Yeast	Pathogenic Species	Human Flora
Metarhizium	Filamentous	Non-pathogenic	Plants
Microascus	Filamentous	Pathogenic Species	Soil
Penicillium	Filamentous	Opportunistic Pathogen	Soil
Phoma	Filamentous	Non-pathogenic	Ubiquitous
Pichia	Yeast	Non-pathogenic	Milk
Pseudallescheria	Filamentous	Pathogenic Species	Soil/Water
Scedosporium	Filamentous	Pathogenic Species	Soil
Trichosporon	Filamentous	Opportunistic Pathogen	Human Flora
Ustilago	Filamentous	Non-pathogenic	Plants
Wallemia	Filamentous	Non-pathogenic	Soil

Pyrosequencing/Riboprinting Comparison

In order to accept a positive identification, pyrosequencing requires a match >97% while riboprinting requires a match of >85% both on a species level. Pyrosequencing will also provide a positive identification on the genus level when the match is between 95-97% (Research and Testing Laboratory 2012). Riboprinting does not provide positive identifications on the genus level; the riboprinter only identifies a positive match of bacteria on a genus and species level and will not identify only the genus. The pyrosequencing database has approximately 4,500 different bacterial species and 620 fungal species (NCBI 2013). The riboprinting database contains approximately 1,200 different bacterial species and no fungal species (DuPont 2005). Although neither of these databases encompasses the large number of bacteria and fungi that have been positively identified, the pyrosequencing database provides a larger number of bacteria that can be positively identified.

Pyrosequencing data is based not only on viable and culturable bacteria and fungi but also the fragments of the bacteria and fungi. Riboprinting requires that the bacteria are culturable and whether the bacteria are Gram-negative or Gram-positive. Because riboprinting requires the use of viable bacteria, it greatly limits the amount of bacteria that can be positively identified because the bacteria cannot be grown with all of the different possible variables that affect the growth of the bacteria. Additionally, viable bacteria are not the only health concern for the workers; the constituents of the bacteria and fungi are equally important to worker respiratory health. Pyrosequencing requires a long time for sample processing and results analysis in order to see the positive identifications. It can take a couple of weeks to receive pyrosequencing results while riboprinting takes only eight hours to report a positive identification.

For the purpose of this study, pyrosequencing was a better way to positively identify the bacteria and fungi. Pyrosequencing provides a larger database that covers both bacteria and fungi samples that are viable as well as those that are no longer viable. Riboprinting, although faster does not provide the specificity or large database that pyrosequencing does. However, riboprinting would be useful in a different research project such as the positive identification of a known species when looking for something such as contamination.

Environmental Conditions

The temperature inside the parlor was higher in the spring and summer months (ranging from 75.6-77.4°F) in comparison to the fall and winter months (ranging from 58.3-63.2°F) as seen in Figure 5.14. There was no statistically significant correlation between dairy parlor temperature and bacterial or fungal concentration. A Spearman correlation was also completed with outside temperature. There was no significant correlation between bacterial or fungal concentration and temperature.

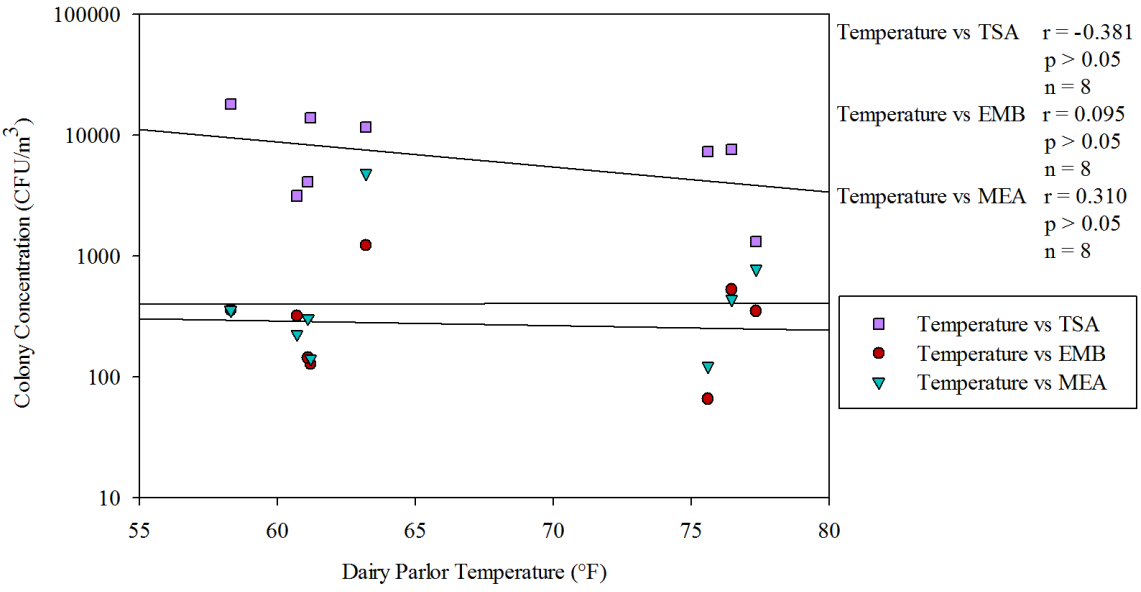

Figure 5.14 Spearman correlation of bacterial concentration and dairy parlor temperature

The relative humidity was generally lower in the spring and summer months (ranging from 34.9-48.3%) than the fall and winter months (ranging from 45.5 to 63.1%) although there was a small amount of overlap. There was a statistically significant negative correlation ($p < 0.05$) between Gram-positive bacteria and outside relative humidity (Figure 5.15). Therefore, as the outside relative humidity increased, the concentration of Gram-negative bacteria decreased. There was no statistically significant correlation between Gram-negative bacteria or fungi and outside relative humidity. Additionally, there was no correlation between colony concentration and relative humidity inside the parlor.

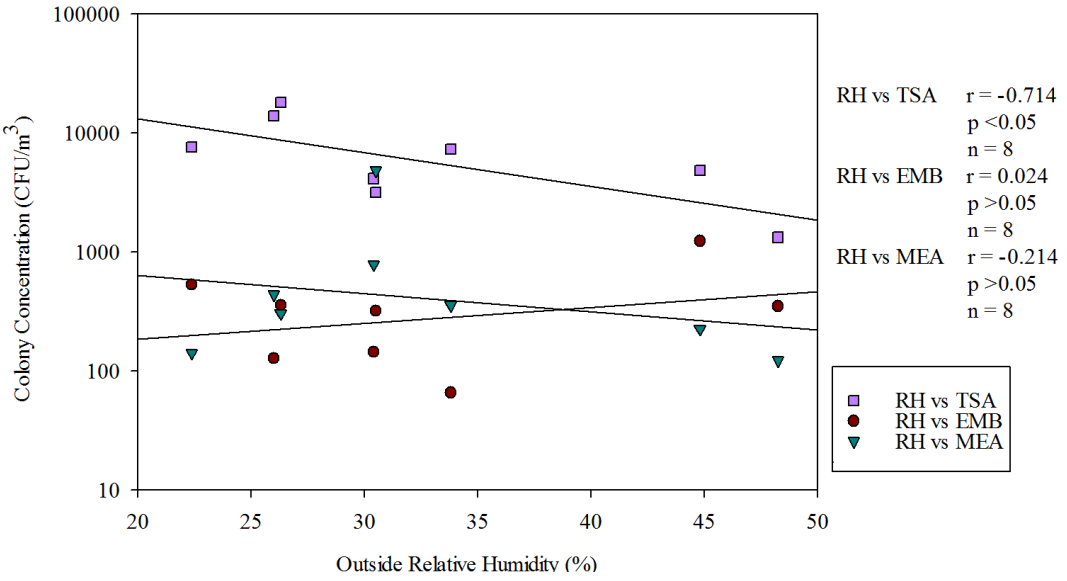

Figure 5.15 Spearman correlations of colony concentration and outdoor relative humidity

Based on Figure 5.16, there is no significant correlation for the concentration of CO_2 and Gram-positive bacteria or fungi. However, there is a positive correlation between CO_2 concentration and Gram-negative bacteria with a correlation coefficient of 0.810 with a $p<0.05$. Therefore, as the concentration of CO_2 increased, the concentration of Gram-positive bacteria also increased. The concentration of CO_2 ranges from 643-1752 ppm over all of the data. The spring and summer months range from 705-1752 ppm while the fall and winter months range

from 643-1354 ppm demonstrating a large amount of overlap between the two seasons. However, when the sampling sessions are separated by sampling site, there is a larger concentration of CO_2 inside the dairy parlor at Site 1 in comparison to the concentrations found at Site 2. Site 1 has CO_2 concentrations that range from 1341-1752 ppm while the CO_2 concentrations at Site 2 range from 643-898 ppm a difference of 445 ppm. There is no apparent correlation between carbon dioxide concentration and bacterial or fungal growth. The carbon dioxide concentration is higher than the levels measured in previous studies.

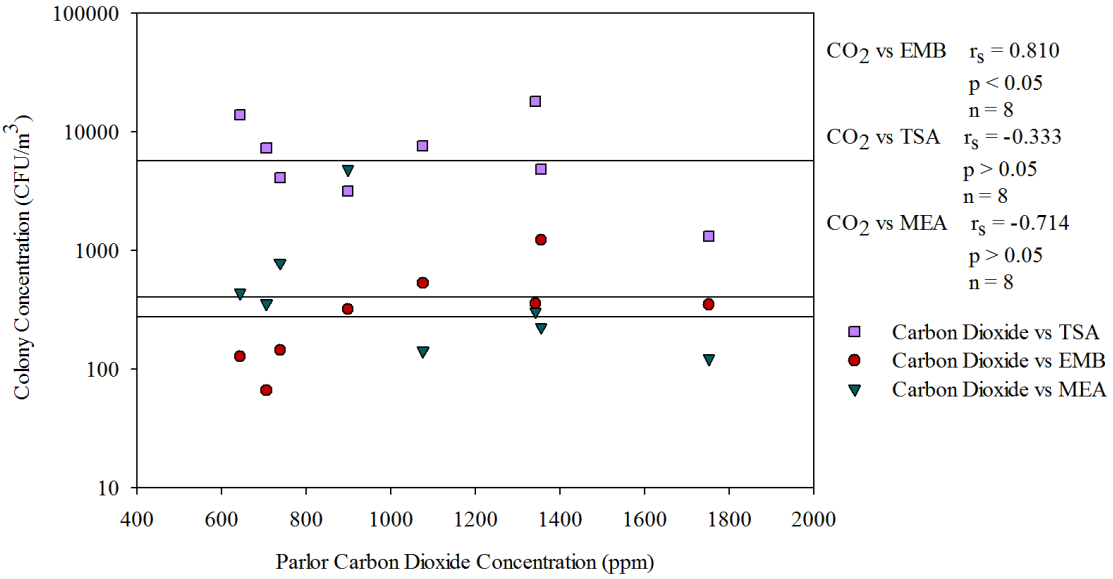

Figure 5.16 Spearman correlation of carbon dioxide concentration and colony concentration inside parlor

Based on the Spearman correlation, there was a statistically significant negative correlation between Gram-positive bacteria and carbon dioxide concentration. Therefore, as the concentration of carbon dioxide decreased outside there was an increase in the Gram-positive bacterial concentration (Figure 5.17). Despite the outliers present in the current data selection, when removed, there was still a statistically significant correlation between Gram-positive bacteria and outdoor carbon dioxide concentration. There was no statistically significant

correlation found between Gram-negative bacteria or fungi concentration and the carbon dioxide concentration.

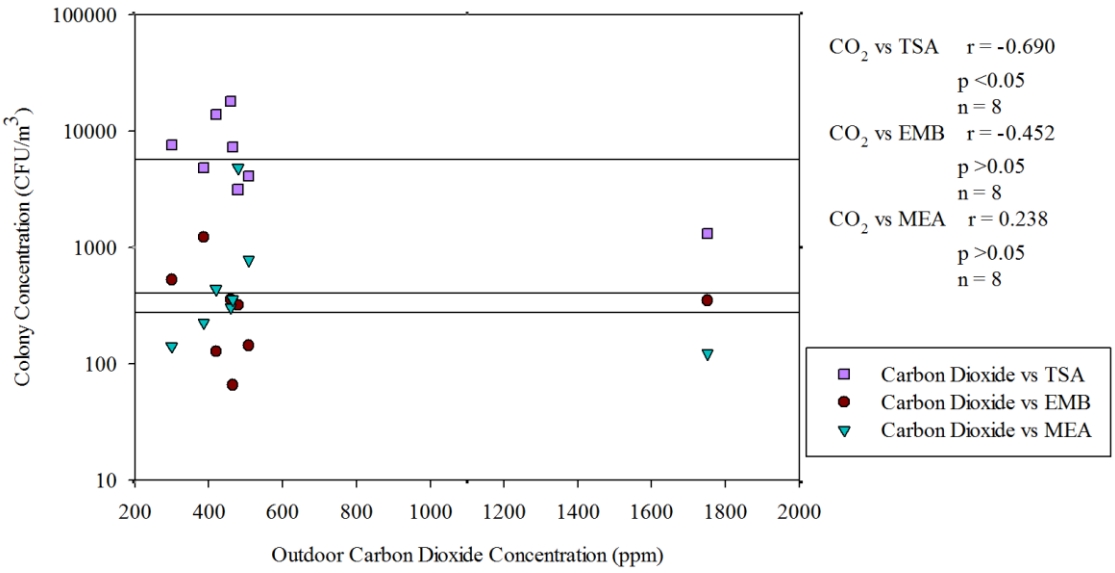

Figure 5.17 Spearman correlation of colony concentration and outdoor carbon dioxide concentration

There was no statistically significant correlation found between carbon monoxide concentration and colony concentration.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

This is the first study to research the use of pyrosequencing in conjunction with the biosamplers at a dairy parlor as well as the first study to use riboprinting for the analysis of environmental air samples collected in the agricultural industry. Bacterial concentrations from culture techniques varied based on the season and temperature likely due to the amount of ventilation and dilution air present when the weather was warmer which consequently decreased the concentration. The culturable concentrations found using the SKC Biosampler were much higher than those found in previous studies that used the SKC Biosampler in dairy environments. It was also found that as the outside relative humidity increased, the concentration of Gram-positive bacteria decreased and as the concentration of CO_2 increased, the concentration of Gram-negative bacteria increased. Additionally, as the concentration of Gram-negative bacteria increased, the concentration of fungi also increased.

Overall, the concentrations for the pyrosequencing data and the culturable bacteria were within the same order of magnitude which was not expected based on results from previous studies. Due to the small sample size, it was not possible to run correlation tests to determine if there was a statistically significant correlation between the different tests. However, the culturable concentrations were higher than the concentrations found in previous studies. The higher concentrations found in this study could be a result of the use of the resuscitation buffer that has not yet been used with the biosamplers or for air sampling. The pyrosequencing results had greater genus diversity than the culturable bacteria based on colony morphology which was expected based on the ability of pyrosequencing to analyze non-viable bacteria and fungi. The pyrosequencing results also revealed a number of bacteria that have potentially pathogenic

species. The potential pathogenic species present in the dairy farm could lead to more than just respiratory problems present in the facilities. With the positive identification of *E. coli O:157* there is also the possibility of contamination of food and the outbreak of gastrointestinal problems such as those cited at a correctional facility dairy in 2010 (CDC 2010).

The fungal pyrosequencing data also had more diversity than the culturable fungi and demonstrated a larger number of yeast than were found through the culture techniques. However, the second round of fungal samples that were sent from pyrosequencing were blank despite growth on the agar. There were no pathogenic forms of the fungi identified in the culturable and microscopic analysis, but multiple genera were positively identified through pyrosequencing that have pathogenic species.

Overall, pyrosequencing was the better analysis method for this study. In the comparison between pyrosequencing and riboprinting, the pyrosequencing data had a much greater number of positive identifications. Pyrosequencing has a larger database as well as the ability to analyze bacteria and fungi that are both viable and non-viable. The riboprinter was however able to give more information on the species and strain level that helped identify the pathogenicity of the bacteria. In contrast, riboprinting has no ability to identify fungi, has a smaller database, and requires bacterial growth in order to identify the bacteria. Based on these results, it will be possible to further understand the bacteria and fungi that are present on the dairy farm that could result in decreased respiratory function as well as future methods to abate the presence of such bacteria and fungi.

Limitations

Due to the nature of the biosamplers, samples were only collected for one hour. The use of the liquid media in the biosamplers results in a large amount of evaporation of the collection

media. To avoid evaporation, SKC recommends using ViaTrap oil that allows for an 8-hour sampling period but does not allow the liquid to be used for cultured analysis or for use in pyrosequencing. Therefore, the experiment is limited to one-hour sampling periods which may not be representative of the entire work shift. There is a possibility that the concentration of bacteria and fungi changes based on the number and length of washes during the work shift, when the cows are moving, and exactly what the workers are doing which changes throughout the work shift and may not be captured by the one hour samples.

Due to the high expense and length of time required to receive results, only four sets of samples were sent for pyrosequencing. This small number of pyrosequencing samples did not allow for a complete in-depth statistical analysis resulting in some gaps of knowledge. There is therefore no information on the seasonal variability of the pyrosequencing samples. Additionally, due to the small sample size it was not possible to compare the cultured samples to the pyrosequencing data for all of the samples.

Both analysis types, riboprinting and pyrosequencing, have a limited number of samples available in the database. Riboprinting has approximately 1,200 different bacterial species in its database; pyrosequencing has approximately 4,500 bacterial species and 620 different fungal species. Although the number of bacterial species is drastically different, these numbers still do not cover the entire list of bacterial species that could be collected in the dairy parlors. Therefore, the number of bacterial and fungal positive identifications is limited based on the number of samples in the database.

Samples were also limited to work within two dairies located in Colorado. Throughout the recruiting process, there was difficulty receiving consent to sample at the dairies so no other dairies were included in this study.

Future Work

Further research should be completed to assess the resuscitation buffer that was used as a collection media for this research project. Although it was recommended and a large amount of growth was present for all of the plates, it was not assessed prior to the commencement of this project. Therefore, it should be analyzed to determine if it is an effective collection media for all bacteria and fungi to ensure there is no bias present.

Additional investigation should examine the bacteria and fungi on a species level to determine if the microorganisms present are contributing to the health effects of workers. Further research should be done to gain a better understanding as to why some of the fungal pyrosequencing samples came back as blank while there continued to be growth on the agar.

An increase in the number of samples that undergo pyrosequencing will help increase the understanding of the bacteria and fungi that are generally present within the dairy parlors. In addition to further characterizing bacteria and fungi, it would help to analyze the difference between pyrosequencing and cultured samples to better understand the best methods for different desired results. Until recently, researchers have not had a feasible method to analyze the pyrosequencing data. However, there are now systems to analyze the large amount of data that accompanies the work done in pyrosequencing. Therefore, further analysis of the pyrosequencing data to improve comprehension of the presence of the bacteria and fungi as well as the role the microorganisms play in the health of the worker.

As previously mentioned, many of the bacteria and fungi survive in this environment due to their ability to thrive in a high salt environment and consequently withstand the cleaning process that takes place so often inside the dairy parlor. However, little is known about the role the amount of washing inside the parlor and specific cleaning agents play on the concentration of the bacteria and fungi in the air. Based on this knowledge, it is recommended that further work should be completed to gain a better understanding of the role that washing plays on the concentration of bacteria and fungi.

Due to the limitations set by the short sampling period, further work should also be completed to enhance the comprehension of the bacterial and fungal concentrations throughout the entire work shift. More information should also be completed to analyze the wind speed and direction both inside and outside the parlor to determine if the wind speed plays a significant role in the concentration of the bacteria and fungi. Background sample outside the dairy parlor would also be important to gain a better understanding of where the bacteria and fungi are coming from as well as examine the different exposures for workers across different tasks on the dairy.

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APPENDIX A:

Resuscitation Buffer Preparation

The resuscitation buffer consists of a 10% PEG, 0.1% peptone 0.05% Tween 80 solution. The resuscitation buffer was prepared by first combining 50g of PEG 8000 weight per volume into 1g of buffered peptone water. Then, 250 mL of Tween 80 was added and swirled to dissolve. Once dissolved, 500 mL of distilled water was added. The solution was then autoclaved for 15 minutes at 121°C. After autoclaving, the solution was cooled to approximately 50°C in a water bath. After cooling, the buffer was divided into sterile storage containers and stored in the refrigerator at 2-5°C until ready for use.

Agar Preparation

Tryptic Soy Agar with 5% NaCl

Start by adding 40.0g of TSA and 45.0g of the NaCl to a 2L flask. Then, add enough distilled water to bring the total volume to 1L and place the flask on a hotplate and heat with agitation until the solution boils for approximately one minute. Autoclave the solution at 121°C for 15 minutes. Before pouring plates, make sure the temperature has reached 55°C and swirl the flask to suspend any particles that may have settled.

Eosin Methylene Blue

Suspend 36.0g of EMB powder in 1L of distilled water. Heat with agitation until the solution boils for approximately one minute. Autoclave solution for 15 minutes at 121°C. Prior to pouring plates, reduce the temperature of the agar to 55°C and swirl the flask to suspend any particles that have settled.

Malt Extract Agar with chloramphenicol

Add 33.6g of MEA to a 2L flask and then add enough distilled water to bring the total volume to 1L and place the flask on a hotplate and heat with agitation until the solution boils for approximately one minute. Autoclave the solution at 121°C for 15 minutes. Once the solution has cooled to 55°C add 2mL of the chloramphenicol stock solution and agitate until the solution has dissolved.

Chloramphenicol stock solution

For a 10mL stock solution, dissolve 0.125g of chloramphenicol in a 70% ethanol solution in a sterile bottle. Agitate the solution until the entire solid has dissolved. Wrap the bottle in foil or store in a dark bottle in a 2-5°C refrigerator up to one month.

APPENDIX B:

Calculations

CFU/mL:

$$\frac{Colonies}{volume\ added\ to\ plate} = \frac{2}{0.1} = 20$$

CFU/mL with 1:10 dilution:

$$\frac{Colonies}{volume\ added\ to\ plate} \cdot dilution\ factor = \frac{2}{0.1} \cdot 10 = 200$$

CFU for Total Volume:

$$\frac{CFU}{ml} \cdot total\ volume = 20 * 13 = 260$$

CFU/m^3:

$$\frac{CFU\ for\ total\ volume}{m^3} = \frac{260}{0.749} = 347$$

APPENDIX C:

Fungal Identification Pictures

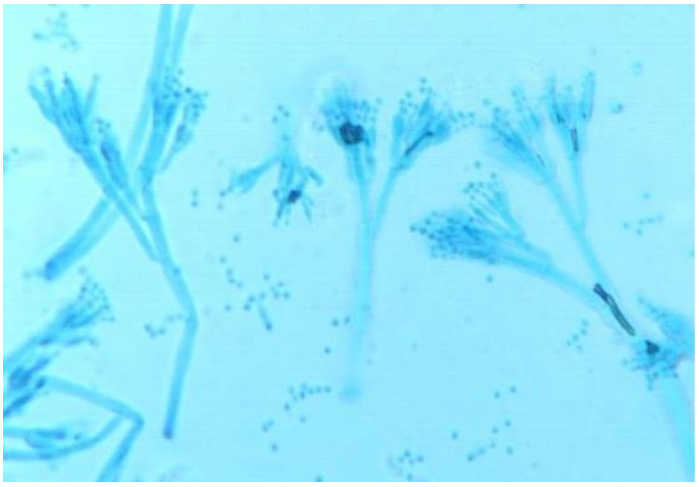

Figure C.1 Microscopic picture of fungal genus *Paecillomyces* at 400x magnification

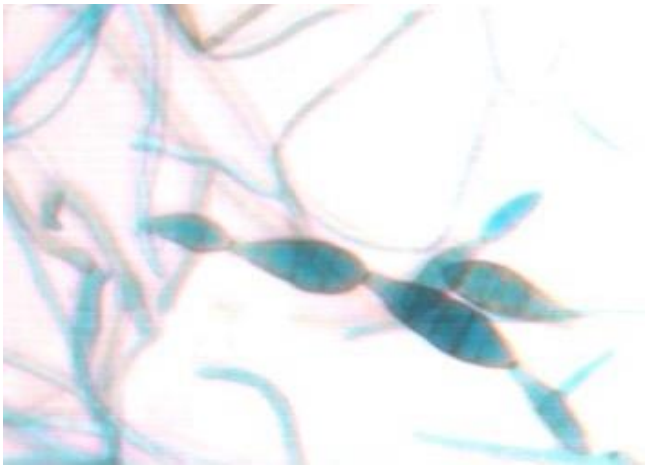

Figure C.2 Microscopic picture of fungal genus *Alternaria* at 400x magnification

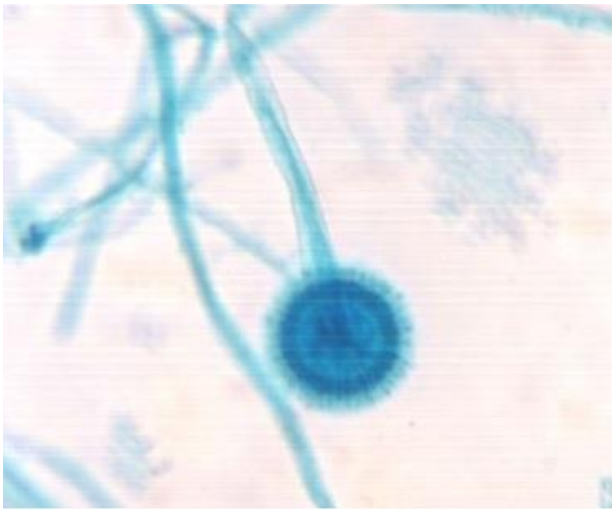

Figure C.3 Microscopic picture of fungal genus *Aspergillus* at 400x magnification

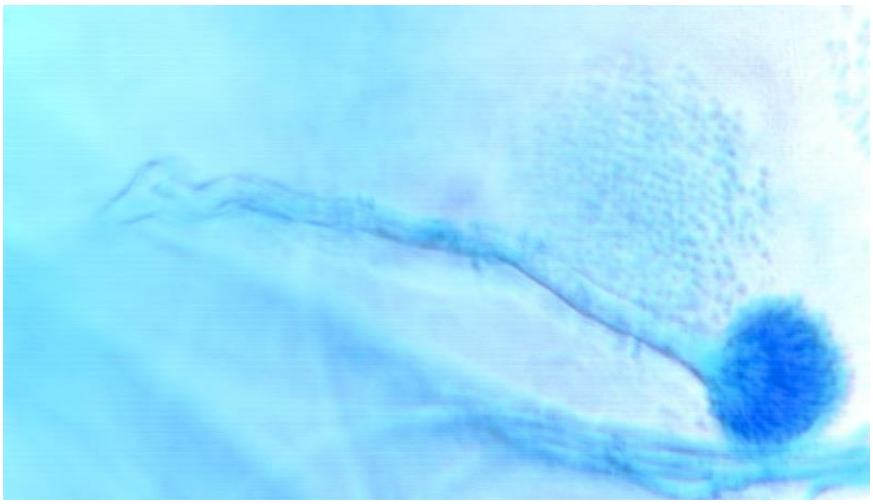

Figure C.4 Microscopic picture of fungal genus *Aspergillus* at 400x magnification