DISSERTATION

PATHOGENS, PULMONARY FUNCTION, AND THE NASAL MICROBIOME OF DAIRY WORKERS

Submitted by

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ABSTRACT

PATHOGENS, PULMONARY FUNCTION, AND THE NASAL MICROBIOME OF DAIRY WORKERS

Dairy workers are exposed to bioaerosols that are diverse in both size (0-100 µm in aerodynamic diameter) and inflammatory constituents (e.g. endotoxins, muramic acid, and β-glucans). Bioaerosol exposure at dairies is associated with a higher prevalence of chronic obstructive pulmonary disease (COPD), chronic bronchitis, asthma, respiratory pneumonitis, and asthma-like reductions in pulmonary function. More recently, opportunistic pathogens present at dairies such as the novel influenza D virus (IDV), influenza A (IAV), and livestock-associated Methicillin-resistant *Staphylococcus aureus* (MRSA) have also been a focus of research, as these pathogens can infect workers and pose a public health risk through community spread. Intrinsic factors such as genetics and childhood exposures likely play a major role in exposure response and respiratory disease pathology, but little research has been focused on the nasal microbiome's role in pathogen exposure and cross-shift changes of pulmonary function.

From a longitudinal (2-5 working shifts) cohort of dairy workers in the High Plains Region of the US, this research analyzed pathogens found in the nares of dairy workers via pre- and post-shift nasal lavages. The same nasal lavages underwent targeted 16S rRNA gene sequencing to quantify the bacterial communities that comprise the nasal microbiome. Spirometry was also performed on dairy workers pre- and post-shift to measure cross-shift changes in pulmonary function.

Overall, 32.1% (n=237) of nasal lavages tested positive for Methicillin-susceptible *Staphylococcus aureus* (MSSA), 11.4% tested positive for MRSA, 17.3% for IDV, 2.5% for

IAV, and 1.3% for influenzas C virus (ICV). Only 1 of the original 31 participants never tested positive for a pathogen during their workweek. Differences in nasal microbiome characteristics emerged based on pathogen positivity, and differential abundance analysis revealed significant differences in genera based on the positivity of both bacterial and viral pathogens.

The dairy workers in this study also experienced decreases in cross-shift pulmonary function. The average decrease in forced expiratory volume in one second (FEV1) over 108 working shift was -74.4 ml, and the average decrease of forced vital capacity (FVC) was -92.5 ml. Significant differences in microbiome characteristics did emerge based on post-shift and cross-shift spirometry performances, and taxonomic differences were noted in participants performing poorly on cross-shift FVC. The nasal microbiomes of workers also underwent community state typing, and participants in CST3 showed the most resilience to cross-shift changes in lung function.

This research also investigated the efficacy of a hypertonic saline nasal lavage in improving cross-shift changes in pulmonary function. From a cohort of 44 dairy workers, 22 workers received pre- and post-shift hypertonic saline nasal lavages with an osmotic concentration of 400 milliosmole (mOsm). The 22 participants in the control group received pre- and post-shift normotonic saline (308 mOsm) nasal lavages. Based on constructed mixed linear models, the treatment improved cross-shift outcomes of the forced expiratory flow at 25-75% of the vital capacity ($FEF_{25-75\%}$), but had little effect on FEV1 and FVC. The use of a pre- and post-shift lavage of any osmolarity, however, appeared to reduce the burden of cross-shift pulmonary function decline often experienced by dairy workers.

For the first time, this research showed that both viral and bacterial pathogens are present in the nares of US dairy workers. This work also identified the nasal microbiome characteristics

that may play a role in pathogen exposure and cross-shift lung function outcomes. The use of a saline nasal lavage as an intervention was also explored, and the intervention appeared to improve cross-shift pulmonary function outcomes.

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DEDICATION

Dedicated to my wife Suman and to my family. Thank you for the unconditional love and unwavering support. Thank you for always believing in me.

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CHAPTER 1: INTRODUCTION

Agricultural workers experience a large burden of respiratory disease likely caused by extensive and prolonged exposures to biological aerosols (bioaerosols).[1] Dairy operations generate bioaerosols that are diverse in size (<3-100um) and composition based on a variety of sources (animal feces, feed/bedding, human skin, etc.).[2–6] As such, dairy bioaerosols contain a taxonomically diverse set of bacterial species, inflammatory constituents, and potentially livestock-associated viruses such as influenza A and influenza D. These exposures have been previously associated with risk for inflammatory and obstructive lung diseases as well as decreased lung function, bronchial hyperresponsiveness, and respiratory symptoms such as coughing and wheezing.[1]

Recent research into the composition of bioaerosols collected from milking parlors at dairy operations suggests that larger particles between 10-100um in size are the most abundant particles by weight and subsequently contain the most inflammatory constituents.[6] Physiologically, these larger particles impact the nasopharyngeal region as their aerodynamic diameter physically restricts most of the particles in this range from passing through the larynx and into the ciliated airways.[7] While the pathological mechanisms of elicited inflammation and disease associated with bioaerosol exposure is not fully understood at this time, bacterial communities that make up the microbiome of the nasal passage may participate in the mediation of inflammatory responses occurring in this locus.[8]

Furthermore, the nasal microbiome of dairy workers may play a role in protecting them against opportunistic pathogens such as methicillin-resistant staphylococcus aureus (MRSA), influenza A (IAV), and the novel influenza D virus (IDV), all of which are emerging as biosafety

concerns at livestock operations.[4,6,9] Preliminary research suggests the increased taxonomic diversity observed in dairy worker's nasal microbiome may provide competition inhibiting staphylococci colonization.[10] As for viral exposures in dairy operations, the role of the nasal microbiome in protecting its host from viral colonization and infection is understudied. Interestingly, staphylococcus aureus in the airways protects against influenza infections by recruiting peripheral CCR2+ CD11b+ monocytes through promotion of TLR2 signaling.[8] Even less understood is the effect that host-microbial interactions have on lung function.

Despite decades of hazard recognition, little research into the development and application of successful controls has been conducted to protect agricultural workers from lung injury. Personal protective equipment (PPE) is the most studied control for this working population, but respiratory protective equipment is costly, bulky, and hierarchically the least effective control in reducing exposures. The research project and the subsequent aims are based on evaluation of a low-cost novel intervention that involves administering hypertonic saline nasal lavages to dairy workers before and after their shifts. Hypertonic saline has been shown to attenuate respiratory inflammation *in vitro* and it proves useful as a resuscitation fluid in trauma patients primarily because of its anti-inflammatory properties.[11,12] While hypertonic saline nasal lavages are expected to reduce upper respiratory inflammation, I was also interested in examining if the nasal lavages improve the dairy worker's cross-shift change in lung function.

Specific Aims

Specific Aim 1: Evaluate the nasal microbiome's potential role in pathogen exposures for dairy workers

Dairy workers (n=31) were recruited as participants from large-herd dairies in the High Plains region of the United States and administered nasal lavages before and after their shifts for

up to five consecutive days. Nasal lavages were analyzed via targeted PCR to determine if participants were carriers of livestock associated MRSA, influenza A, and/or influenza D. PCR-amplified 16S rRNA gene sequencing was also performed on the lavages to quantify the nasal microbiome of participants. I *hypothesized* that 1) *participants carrying MRSA and cattle-related MRSA would have lower alpha and beta diversity metrics in their nasal microbiome*, and 2) *certain taxonomic features would be lower in participants that carry influenza viruses*.

Specific Aim 2: Evaluate the relationship between the nasal microbiome and lung function in dairy workers

Thirty-one (31) participants also performed pulmonary function testing via spirometry before and after their shifts to determine cross-shift changes in lung function. For our purposes, the spirometry markers associated with lung function included forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), the subsequent ratio (FEV_1/FVC), and the forced expiratory flow at 25-75% of forced vital capacity (FEF_{25-75}). Literature on the airway microbiome's role in lung function is limited. A few identified studies have compared spirometry results to the microbiome with cohorts in various disease states.[13,14] One study that focused on aging and the microbiome found that microbial diversity and composition did not have a significant effect on lung function in healthy populations.[15]

Research examining the nasal microbiome of individuals diagnosed with the inflammatory disease chronic rhinosinusitis may provide insight into the nasal microbiota's role in protecting individuals from inflammation. A relationship between decreased upper respiratory tract (URT) microbiota diversity and increased URT inflammation has been established for individuals with chronic rhinosinusitis.[16,17] Furthermore, individuals with *Lactobacillus sakei* in their sinus microbiome also showed an increased resilience to infection from pathogenic

bacteria.[16] I *hypothesized* that, *in the context of occupational bioaerosol exposures, increased relative diversity in the nasal microbiome would protect from respiratory inflammation and therefore reduce the cross-shift change in lung function often experienced by dairy workers.*

Specific Aim 3: Explore the efficacy of a nasal lavage on dairy workers' cross-shift change in pulmonary function

For the intervention study, 44 dairy workers were enrolled as participants over 2-5 days. Half of the workers were randomly assigned into the treatment group and received a hypertonic saline nasal lavage while the other 22 workers were assigned into the control group and received a normotonic saline nasal lavage. Diurnal variation in non-occupational spirometry results point to a "morning-dip" phenomena where participants' worst performance in pulmonary function tests occurs in the morning and better results typically follow in the late afternoon.[18,19] Researchers investigating dairy and other agricultural workers often find deviations from the expected variations and observe decreases in spirometry markers in the evening following workers' shifts.[20–22] Observed decreases in markers associated with pulmonary obstruction, such as FEV_1, FEV_1/FVC, $Vmax_{50\%vc}$, and $Vmax_{25\%vc}$, suggests bioaerosol exposures in these working environments may increase pulmonary obstruction.

Based on a previous pilot project with similar methodology, I *hypothesized* that 1) *workers receiving normotonic and hypertonic saline nasal lavages would perform better in post-shift FEV_1, FVC, and $FEF_{25-75\%}$,*, and 2) *the treatment group would experience less of an inflammatory response to the dairy environment and should perform better on post-shift FEV_1, FVC, and $FEF_{25-75\%}$ measurements when compared to the control group.*

Together, my aims address important knowledge gaps in the role the microbiome plays in protecting livestock workers against opportunistic pathogens and respiratory inflammation. To

my knowledge, no other study has examined the relationship between the microbiome and lung function as it pertains to a cohort of bioaerosol-exposed workers. My third aim will not only help in evaluating a much-needed low-cost intervention for livestock workers but could also contribute to the ongoing discussion of the viability of biomarkers in predicting lung function.

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CHAPTER 2: REVIEW OF LITERATURE

2.1 Background

The United States dairy industry employed 157,140 workers in 2022.[1] An estimated 51-80% of these dairy workers are immigrant Latino workers, with many emigrating from remote regions of Mexico and Central America.[2–4] The increase in demand for immigrant workers over the last 20-30 years mirrors the organizational restructuring also occurring in this industry. Between 1990 and 2010, the number of milk cow operations in the United States shrunk from ~193,000 to ~62,000.[5] During that same period, the USDA reported increases in total milk production from 147,721 million pounds in 1990 to 199,871 million pounds in 2010.[6,7] Interestingly, this 35% increase in milk production was accomplished with a 9% decrease in total lactating cows. Technologic improvements including rotating milking parlors, robotic milking systems, advancements in cow health and nutrition, and improved artificial breeding techniques have played a major role in producers increasing milk production per cow.[6,8]

Increased efficiency at modern dairy operations affects not only the cows, but also the workers. At large herd dairies with >2,000 cows it is common to have 1 employee per 80-100 cows.[8] Furthermore, immigrant dairy workers report working 54 to 64 hours per week at their job.[2,4,9]Advancements in technology and production efficiency has contributed to a fast-paced, dynamic working environment where longstanding occupational health and safety concerns continue to impact workers. Dairy work is physically demanding, and the introduction of automated systems has led to greater task specializaton.[10] As a result, workers perform arduous movements repetitively – leading to an increased risk for musculoskeletal symptoms (MSS). In recent ergonomic assessments of large-herd US dairy workers, prevalence for at least 1 MSS in

the last 12 months was reported between 76% and 80%.[9,11] Over 55% of workers reported suffering from 2 or more MSS over that period. Approximately 85% of workers reported being kicked or stepped on by a cow.[9,11]

Of equal importance is the prolonged exposure to biologically-loaded dust (bioaerosols) that dairy workers experience every day. Dairy bioaerosols are a complex mixture of bacteria, viruses, fungi, associated inflammagens, and inorganic materials that are pervasive across all working tasks at the dairy. Historically, exposure to these bioaerosols has been associated with higher incidence of fixed airway disease, long-term decreases in pulmonary function, and increases in upper respiratory symptoms. More recently, public health concerns surrounding pathogenic viruses and antibiotic resistant bacteria present at dairies and other livestock operations have led to calls for increased surveillance of both workers and the environment.

The burden of poor respiratory outcomes is not felt equally across all dairy workers. As not all workers respond similarly to bioaerosol exposure, efforts to parse the intrinsic factors that modulate workers' exposure-response have become increasingly important.[12–15] Previous epidemiological studies have investigated the role of genetics and childhood exposure on occupational cohorts, but conflicting results has presented these intrinsic factors as providing both protection and increasing susceptibility.[16–20] Emerging as another potential factor is the nasal microbiome. The nose plays host to complex bacterial communities that include both commensal and pathobiont species.[21–23] The bacterial communities' role in host innate immunity is not well understood, but spending time at livestock operations does appear to significantly modify the nasal microbiome's composition.[24–27] Furthermore, the worker nasal microbiome has been proposed as a means of exposure of zoonotic pathogens to the community, and,

correspondingly, workers may bring community-associated pathogens and antibiotic-resistance bacteria that impact the animals.[28–32]

Ultimately, the exact pathology of respiratory disease as a result of bioaerosol exposure is not fully understood. This literature review outlines the current scientific knowledge surrounding bioaerosol composition, subsequent health outcomes, and potential interventions for protecting this workforce. A detailed discussion of acute changes in pulmonary function, intrinsic factors, and the dairy workers' nasal microbiome as it relates to exposure and respiratory disease is also provided. Globally, the dairy industry employes an estimated 240 million people.[33] The lessons and numbers discussed here are most relevant to areas of the world where modern agriculture practices have led to a proliferation of large herd commercial dairy operations. As countries like India, Brazil, and China continue to incorporate modern practices, their workers will be faced with the same challenges.[8] For all dairy workers, a detailed understanding of the hazards and implementable solutions will decrease the overall burden of respiratory disease.

2.2 Bioaerosols

Exposure to bioaerosols at dairy farms has repeatedly been associated with higher incidence of respiratory disease in dairy workers. [14,15,34] Dairy bioaerosols are a complex mixture of dust and biologically derived particulate matter and vary drastically in both size and composition. The sources that generate bioaerosols at dairy farms are also diverse, as Schaeffer et al. showed that bacterial aerosols at milking parlors were associated with animal feces, animal mucus, animal skin, birds, freshwater, humans, and soil.[35] The exact inflammatory constituents of bioaerosols that contribute most to respiratory disease is not known, so a detailed discussion of these aerosols is warranted.

2.2.1 Particle size distribution of dairy bioaerosols

In 2017, Schaeffer et al. deployed a 4-stage cascade impactor in the milking parlors of three large-herd dairies.[35] The impactor collected dust in four separate aerodynamic size fractions: >30, 10−30, 3−10, and <3 μm. Collected dust followed a bimodal size distribution, with masses being the highest in the largest (>30 μm) and smallest (<3 μm) size fractions.[35] While these ambient samples are not representative of individual worker exposure, their size distribution does inform the physiological deposition occurring during bioaerosol inhalation.

Previous investigations into the size distribution of dairy bioaerosols were limited by the use of personal cascade impactors with upper size cut-points of ~20 μm. Understanding the extent of particles that likely impact the initial portion of the upper respiratory system warrants deeper investigation into the host exposure-response occurring in the nose.

2.2.1.1 Health implications

Aerosol mixtures with an aerodynamic diameter cut-point of 4 μm are classified by the American Conference of Governmental Industrial Hygienists (ACGIH) as Respirable Particulate Matter. Theoretically, 50% of the particles in this size fraction are capable of penetrating deep into the airways of the respiratory system and may deposit in the gas-exchange region of the lung.[36,37] Larger particles, with aerodynamic diameters between 0-100 μm, are classified as Inhalable Particulate Matter. These particles may deposit anywhere in the respiratory system, but the larger particles in this size fraction are likely to deposit in the nasopharyngeal region.[35,36] Many constituents of bioaerosols, such as glucans, are also water-insoluble. Insoluble particles are less likely to be mucociliary cleared, and as a result many bioaerosol particles will penetrate past the mucous and into the epithelial cells.[38]

2.2.1.2 Exposure Limits

No formal exposure limits for inhalable or respirable dust at livestock operations have been established by any regulatory agency. Because of differences in individual susceptibility and the diverse composition of inflammatory constituents between any two bioaerosol samples, lawmakers and ACGIH have historically shied away from making bioaerosol exposure limits.[36] This issue is further complicated by a lack of consensus on the exact disease pathology following prolonged bioaerosol exposures.[14,15] ACGIH has set a threshold limit value-time-weighted average (TLV-TWA) for grain dust of 4 mg/m^3, but this standard is based on non-size-specific total-dust samples and therefore has little physiological relevance.[36] Additionally, the biological load of grain dust differs substantially from livestock operations, further reducing the utility of comparisons to this standard.[39] Following a two-year evaluation of pulmonary function in swine farm workers, Reynolds et al. suggested more stringent exposure limits of 2.5 mg/m^3 for total organic dust and 0.23 mg/m^3 for respirable dust over a standard 8-hour work shift.[40] A separate study conducted on poultry workers suggested exposure limits of 2.4 mg/m^3 and 0.16 mg/m^3 for total and respirable dust, respectively.[41] No proposed standards have been identified for exposure to dust in the inhalable fraction in livestock operations.

2.2.2 Endotoxin

2.2.2.1 Properties

Endotoxins are an inflammatory toxin and the primary outer membrane of gram-negative bacteria.[42] Endotoxin are comprised of proteins, lipids, and lipopolysaccharides (LPS), and the biochemical composition of these components differs between gram-negative bacterial species.[43] For example, Poole et al. found that dairy endotoxins more commonly consist of even numbered carbon chain length 3-hydroxy fatty acids (3-OHFA) compared to endotoxins in swine

facilities.[44] The dairy endotoxins also contained, on average, shorter-chain 3-OHFA (10 to 14 carbons in length) compared to the loner-chain 3-OHFA in swine facilities (C_{14}-C_{18}).[44] Upon stress or death, bacteria may shed their outer membrane components – thus aerosolizing endotoxins into the environment.

2.2.2.2 Health effects

Inhalation of endotoxin is associated with both respiratory inflammation and acute decrease in pulmonary function.[40,41,43–48] Following inhalation of dust containing endotoxin, LPS components bind to the lipopolysaccharide-binding protein (LBP) found in airway surface liquid (ASL).[43] LBP then transports macrophages and associated cell surface proteins, which begins an innate immune response.[43] For 4-24 hours following exposure, macrophages and neutrophils accumulate in the lung wall and are subsequently distributed among smaller airways.[45] Respiratory inflammation post-exposure can then be measured via nasopharyngeal swabs or nasal lavages.[49]

Exposure to endotoxin in livestock environments has also been associated with cross-shift decreases in pulmonary function. In cattle feedlot workers and dairy workers, Reynolds et al. reported the highest decreases in forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) across working shifts in the highest endotoxin exposure quartiles (Table 3).[12] In swine workers and poultry workers, significant decreases in spirometric markers were associated with exposures of 100 EU/m^3 and 614 EU/m^3, respectively. [40,41]

Historically, a disproportionate amount of research has focused on endotoxin as the primary inflammatory constituent of dairy bioaerosols.[49–53] While an important component of any dairy bioaerosol exposure assessment, decades of dairy research has largely concluded that endotoxins fail to account for all inflammatory responses.[14,15,34] This may be due to the chemical

structure of endotoxins present in the environment. The even-chained 3-OHFA endotoxins are believed to elicit a greater inflammatory response than the odd-chained 3-OHFA, but the shorter chains common in dairy environments (C_{10}-C_{14}) likely elicit less of an inflammatory response.[44,49,54]

2.2.2.3 Exposure levels

Endotoxin exposures are ubiquitous on dairy farms, but individual exposure concentrations may vary depending on job task, season of sampling, and region. In the last 10 years, exposure assessments focused on United States dairy farms have reported average concentrations of endotoxin between 2 and 1,368 endotoxin units per cubic meter (EU/m^3).[13,50,52,55–58] In exposure assessments conducted in Europe, average endotoxin concentrations between 128 and 360 EU/m^3 have been reported.[16,59,60] When looking at seasonal differences, Pfister et al. reported a geometric mean (GM) of 159 EU/m^3 in the winter and a GM of 104 EU/m^3 in the summer.[60] Basinas et al. reported a GM of 450 EU/m^3 in the winter and 290 EU/m^3 in the summer.[61] Pfister et al. also reported differences in endotoxin exposure based on task, and found that endotoxin exposure was greater on farms with automatic milkers.[60] This was in direct agreement with the findings of Basinas et al., that found the use of automatic milking systems and automatic manure handling methods were strongly associated with increased endotoxin exposure.[61] Particle size distribution of dairy aerosols, however, likely does not impact endotoxin concentration. Schaeffer et al. found that endotoxin was present in similar quantities across the entire inhalable size fraction (0-100 μm). This finding agrees with previous personal exposure assessments that found consistent endotoxin exposures in the respirable (Kullman et al.), thoracic (Basinas et al.), and inhalable size ranges (Garcia et al.).[16,52,62]

While no formal exposure limit for endotoxin exposure has been established by any regulatory agency, the Netherlands' Dutch Expert Committee on Occupational Safety (DECOS) has proposed an occupational exposure limit of 90 EU/m^3.[63] For livestock workers, Reynolds et al. and Donham et al. have proposed guidelines of 100 EU/m^3 and 61.4 EU/m^3, respectively.[64,65] The majority of studies identified here have found average exposures well over any of these proposed standards.

2.2.3 Gram-positive bacteria

2.2.3.1 Properties

Gram-positive bacteria are another major immunogenic constituent present in dairy farms.[44,50,66] Gram-positive bacteria possess a thicker peptidoglycan cell wall than Gram-negative bacteria and can further be differentiated based on shape.[67] Muramic acid is used as a biological marker of Gram-positive bacteria exposure, and similar to endotoxins, can be found in dairy bioaerosols across a range of size fractions. [44,50,66]

2.2.3.2 Health effects

An *in vitro* study in 1999 established an association between exposure to Gram-positive bacteria containing agricultural dust and subsequent excretion of the inflammatory cytokines IL-6 and IL-8.[68] After subjecting bronchial epithelial cells to Gram-positive bacteria obtained from dairy dust, Poole et al. reported that Gram-positive bacteria and endotoxin exposure may have a synergistic effect in eliciting respiratory inflammation.[44] Importantly, the research group blocked the TLR4 response of the epithelial cells to exclude possible inflammation from endotoxin in the dust. A more recent study conducted with workers in dairy parlors in Iowa; however, did not find a relationship between muramic acid exposure and pulmonary inflammation.[57] Inhalation of the Gram-positive bacteria *Saccharopolyspora rectivirgula* is suspected to be one of the main

causative agents of hypersensitivity pneumonitis (HP), and its presence in dairy barns has been established.[69]

2.2.3.3 Exposure levels

In dust collected from the floor of a dairy barn, Poole et al. reported a muramic acid concentration of ~6 ng/mg* (*quantity estimated from Figure 3a).[44] When performing microbial analyses on personal breathing zone (PBZ) samples taken from US dairy workers, Nonnemann et al. reported an average concentration of 3.61 ng/m^3 and Davidson et al. reported average an average concentration of 9.6 ng/m^3. Pfister et al. reported a GM of 960 colony forming units per meter cubed (CFU/m^3) when culturing gram-positive bacteria taken from PBZ samples of 42 French dairy workers.[60] Similar to endotoxin exposure, Pfister et al. also found Gram-positive bacteria concentrations were highest in the winter and positively associated with automatic manure handling practices.[60]

2.2.4 Other constituents

2.2.4.1 β-glucans

(1 → 3)-β-D-Glucans (β-glucans) are naturally occurring polysaccharides found in the cell wall of bacteria, fungi, yeast, and plants. [70] Inhalation of aerosolized β-glucan elicits an immune response in the airways, and occupational exposure has been associated with increases in both cytokine production and macrophage phagocytic activity.[45,70] While hazard recognition of β-glucans has long been established for grain workers, its contribution to bioaerosol mixtures and subsequent respiratory disease in livestock workers is not well understood.[71–74] PBZ samples taken from workers in a Dutch horse stable found GM concentration of β-glucan to be 9.5 µg/m^3.[75] When conducting personal sampling on 36 dairy workers over 137 working shifts, Martenies et al. found a GM concentration of 0.002 µg/m^3 and a maximum of 0.023 µg/m^3. [13]

Compared to the proposed occupational exposure limit of 0.150 µg/m^3, fully quantifying exposures to β-glucans for US dairy workers may not be a priority.[70]

2.2.4.2 Archaeal species

Archaeal species are prokaryotes that constitute the third domain of life.[76] Great scientific interest in the "extremophiles" of this domain has led to the identification of these microbes in ocean floors and rims of volcanoes, but their ubiquity in all environments and ability to aerosolize has also led to concerns regarding occupational exposures.[77,78] Nehme´et al. quantified archaeal species in inhalable bioaerosol samples collected from animal confinement environments, and found concentrations of 10^8 16S rRNA gene copies per cubic meter of air in swine facilities.[79] Archaeal concentrations in the bioaerosol samples were similar to bacterial concentrations, and much higher than levels previously recorded in schools, offices, and homes.[79,80] In a more recent investigation, Bønløkke et al. conducted personal sampling on 327 Danish livestock workers and found archaeal gene copies at $1.9x10^4/m^3$ in cattle workers – approximately two orders of magnitude lower than bacterial copies analyzed from the same samples.[81] Independent of their relative concentrations, the presence of certain archaeal species that have demonstrated immunomodulating and sensitizing properties may be more important in occupational settings.[82,83] In a murine model, Lecour's et al. exposed mice to the archaeal species M*ethanobrevibacter smithii* (MBS) and *Methanosphaera stadtmanae* (MSS), both of which have previously been observed in animal confinement operations.[79,83,84] MBS and MSS both exhibited immunogenic properties and triggered an immune response similar to the pathology observed in hypersensitivity pneumonitis (HP) and occupational asthma.[83]

2.2.4.3 Inorganic constituents

The inorganic fraction of dairy bioaerosols are likely an important piece of the exposure puzzle, but their composition has not been well quantified in the literature. Expensive analytical techniques, variability between types of farms, and differing soil compositions regionally present barriers to defining inorganic concentrations and compositions. In a 2000 review of inorganic dust exposure for agricultural workers, Schenker estimated that up to 20% of aerosolized dust particles at farms may be crystalline silica.[85] Molocznik reported total dust concentrations on Polish swine farms between 2.6 and 8.9 mg/m^3, with an estimated 2.2-7% of this dust comprised of crystalline silica.[86] Unfortunately, it is unclear how many samples were taken for this study, the scale of the pig farms, and if the samples were ambient or personal. Furthermore, crystalline silica in the Molocznik study was determined via colormetric spectrophotometry, a method the NIOSH Manual of Analytical Methods considers less precise than both x-ray diffraction (XRD) and infrared methods (IR). Even with imprecise methods and educated guesses, the presented data suggests possible overexposures based on the ACGIH TLV of 0.025 mg/m^3 and the updated OSHA permissible exposure limit (PEL) of 0.050 mg/m^3.[36,87] A comprehensive crystalline silica exposure assessment of livestock workers using NIOSH validated methods would contribute greatly to current knowledge.

Prolonged occupational exposure to crystalline silica is associated with silicosis, pulmonary fibrosis, and increased susceptibility to respiratory infections.[36] Autopsy studies conducted on agricultural workers' lungs obtained from the Fresno County, California Coroner's Office found a relationship between inorganic dust exposure and inflammation in the interstitial and epithelial tissue of respiratory bronchioles.[88,89] Additionally, cellular investigations show that crystalline silica in inorganic dusts collected from citrus and grape farms is a potent

immunomodulator that can stimulate the development of respiratory diseases such as pneumoconiosis and pulmonary fibrosis.[90] Cell injury from agricultural inorganic dust exposure has also been linked to reduced pulmonary function, persistent wheezing, chronic cough, and chronic bronchitis. [91,92]

2.3 Respiratory health outcomes in dairy workers

Decades of epidemiological research has established a strong link between occupational exposure to bioaerosols at dairy farms and negative respiratory health outcomes. A recent position paper published by Sigsgaard et al. in *Clinical and Translational Allergy* reviewed 73 livestock worker studies conducted between 2000 and 2018 and concluded that livestock workers are at higher risk for fixed airway diseases, reductions in pulmonary function, and persistent upper respiratory symptoms.[15] In 2000, Schenker et al. produced a comprehensive conference report titled "Respiratory Health Hazards in Agriculture" for the *American Thoracic Society*.[93] The report has over 900 references and details the link between bioaerosol exposure for agricultural workers and the development of numerous respiratory diseases including asthma, asthma-like reductions in pulmonary function, chronic airway disease, hypersensitivity pneumonitis, and interstitial fibrosis.[93] Published literature reviews conducted by Reynolds et al. and Seidel et al. focus solely on dairy workers, but their work agrees with latter; exposure to bioaerosol at dairy operations increases risk for development of respiratory disease and acute symptoms.[14,34]

2.3.1 Chronic disease

The development of chronic respiratory disease as a result of working at a dairy farm is a serious occupational health outcome. Working in dairy farm has statistically been associated with increased risk for developing chronic obstructive pulmonary disease, chronic bronchitis,

asthma, and hypersensitivity pneumonitis, all of which are discussed in further detail later.[14,34] Even following removal from exposure, workers who develop these diseases suffer greatly in their personal lives.

While the epidemiological studies discussed in this section provide a general understanding of the burden of these diseases among dairy workers, it is likely that the true burden of disease is underrepresented due to the healthy worker effect.[34,94] That is, a large number of workers will self-select out of agricultural work due to the development of disease before their disease can be captured in incidence rates. Any attempt to quantify the burden of occupational diseases with latency periods via cross-sectional methods naturally suffers from this bias. Even longitudinal studies may fail to capture workers who exit the workforce soon after hire. For example, grain workers often have a lower prevalence of asthma than control subjects because workers with pre-existing asthma will quickly self-select out of the industry.[93] The ramifications for overlooking these workers may be dire, as one study noted workers who left the grain industry due to asthma experienced greater declines in pulmonary function in subsequent years compared to asthmatic workers who continued to work.[95] In their final recommendations, Schenker et al. called for improved surveillance systems to monitor agricultural workers' respiratory disease.[93] Between the 25 years between of that publication and the writing of this review, no major improvements have been identified to this effect.

2.3.1.1 Chronic obstructive pulmonary disease (COPD)

2.3.1.1.1 Definition

Chronic obstructive pulmonary disorder (COPD) is characterized by irreversible airway obstruction that interferes with normal respiration. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has standardized spirometric criteria for diagnosing COPD so that a

pulmonary function test with a forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) ratio (FEV1/FVC) below 0.7 or an FEV1 below 80% of predicted indicates a strong enough obstructive pattern to diagnose COPD.[96] Before the development and publication of the current GOLD standards, agricultural health surveys deviated in interpretation of spirometry values based on regional and ideologic differences.

2.3.1.1.2 Burden on dairy workers

A causal link between dairy work and increased risk for COPD has been established in the literature.[14,97] Eduard et al. defined COPD as 5% the lower limit of normal (LLN) of FEV1/FVC ratio and found dairy farmers had an odds ratio of 1.30 for developing COPD compared to crop farmers.[98] Monso et al. used GOLD standards for COPD diagnosis and concluded that animal confinement workers, including dairy workers, were at a greater risk for developing COPD.[99] A study led by World Health Organization (WHO) researchers in Macedonia also established a cause-effect relationship between dairy farming and COPD. Using the GOLD standard for diagnoses, the WHO group reported a significant (p=0.023) difference in COPD prevalence between a group of dairy workers (n=72) and office workers (n=52).[100] In their study, 10.7% of participating dairy workers had COPD compared to 2.7% of office workers (n=52).[100] They also reported that working in a dairy for over 20 years was a significant confounding factor in the development of COPD, supporting the dose-response theory of bioaerosol exposure and fixed airway disease. In a follow-up study of 83 dairy workers in Macedonia, Stoleski et al. reported 8.4% of dairy workers (n=83) met the diagnostic criteria for COPD, compared with 3.8% of office workers (n=80).[101] This difference was not significant. In a study of 575 male dairy farmers in the French Doubs region, Marescaux et al. found a prevalence of COPD of 12% using the GOLD criterion.[102] Smoking, age, and type of farm (traditional vs.

modern) were all found to modify COPD outcomes in this workgroup. Jouneau et al. found a surprisingly low prevalence of COPD in a separate cross-sectional analysis of French dairy workers in the Brittany region.[103] Out of the 1203 farmers participating in their study between 2012 and 2017, only 16 (1.3%) cases of COPD were identified.[103] The authors contributed the early adoption of modern farming practices in Breton as a potential reason for their findings.

2.3.1.2 Chronic bronchitis

2.3.1.2.1 Definition

The American Thoracic Society defines chronic bronchitis as a cough productive of sputum for at least three consecutive months per year occurring for two or more consecutive years.[104] Inflammation via tracheobronchial mucus production leads to airway obstruction which manifests symptomatically into a chronic cough in persons suffering from this subset disease of COPD.[105] Due to the self-reported nature of the diagnostic criteria, agricultural researchers can conveniently assess incidence of chronic bronchitis in farming populations without the assistance of physicians or spirometry.[98]

2.3.1.2.2 Burden on dairy workers

Dairy farmers in America and Europe have historically had higher incidence of chronic bronchitis and associated symptoms when compared to control populations. Babbott et al. measured a statistically significant difference in prevalence of chronic sputum production for Vermont dairy workers (25%) compared to Vermont workers employed in production, fabrication, and construction (17%).[106] A 1993 survey of 5,703 French dairy farmers found that 9.3% of respondents suffered from chronic bronchitis.[107] A separate survey conducted by Huchon et al. in 2002 found that the general French population had chronic bronchitis rates closer to 4.1%.[108] Choudat et al. also found that French dairy workers were at a much higher risk

for morning cough, diurnal cough, and morning phlegm than the general French population. [109] Similar results were presented by Chaudamanche et al.; their analysis of a 1994 French survey found 6.5% of dairy workers fit criteria for chronic bronchitis compared to 0.9% of nonfarming controls.[110] Thaon et al.'s more recent study confirms that dairy workers still suffer from chronic bronchitis, usual morning cough, and usual morning phlegm when compared to nondairy agricultural workers and nonagricultural worker populations.[111] An investigation studying Norwegian dairy workers confirmed similarly elevated rates of chronic bronchitis among dairy workers (6.4%) compared to crop workers (4.4%). [98]

2.3.1.3 Asthma

2.3.1.3.1 Definition

Asthma is another obstructive respiratory disease characterized by airway inflammation. Eosinophilic inflammation and inflammation of type 2 helper T lymphocytes in people with asthma manifests into symptoms such as airway obstruction, chest tightness, coughing, and intensification of mucous production.[112] Diagnosing asthma often involves administering a bronchodilator to patients following initial spirometry.[96] Unlike COPD sufferers, asthmatics' spirometric results following bronchodilator administration typically show an improvement in airway obstruction. Although once considered different diseases with different pathologies, asthma and COPD have seen a recent association in the medical community due to similarities in symptoms, environmental risks, and potential genetic disposition. [112,113]Physicians now consider diagnosing patients with three or more overlapping symptoms with asthma-COPD overlap syndrome (ACOS). [112]

Work-related asthma (WRA) is asthma that can be developed from exposure to sensitizers or irritants at work (occupational asthma) or asthma that is worsened by the working

environment (work-exacerbated asthma).[114] Recent estimates suggest that anywhere from 9 to 25% of adult asthma cases in Europe and the United States fit WRA criteria. [115,116]

2.3.1.3.2 Burden on dairy workers

Although farming and early childhood exposures to farm activities has been suggested as a protective measure from adult-onset asthma, adults entering the dairy workforce without childhood exposure are at an increased risk of developing asthma.[117] A study of New York dairy farmers found a significantly elevated risk (OR=1.54) for asthma in dairy farmers compared to rural nonfarming residents. [118]A large New Zealand study (n=2,903) examining asthma in various occupations found an OR of 1.4 (95% CI 0.7-2.6) for dairy farmers (n=102) developing adult-onset asthma. [119] Based on a study following Danish farming students, Omland et al. found an even higher OR of 2.5 (95% CI 1.1-5.3) for dairy workers to develop "new" asthma compared to rural nonfarmers.[120]A 12-year study of Swedish farmers found an increase in adult onset asthma compared to nonfarming populations; however, it is unclear how many farmers were dairy workers.[121] Mazurek et al. studied American farmers via a 2011 USDA National Agricultural Statistics Service Farm and Ranch Safety Survey and found that while farm operators had a lower overall prevalence of asthma than the general population (5.1% vs. 8.2%), farm workers with asthma were at a greater risk than the general working population for worker related asthma (15.4% vs 9.7%).[122] Dairy workers were not separated from other livestock operators in this survey. The same Farm and Ranch Safety Survey conducted in 2006 found that 24.8% of asthma cases in farmers were work related.[123] Combined with the 2011 Survey findings that found both small farm operators on less than 101 acres and farmers in the north were more likely to suffer from work related asthma, this data supports the hypothesis that reduced indoor

animal confinement and farm modernization practices may reduce the risk for workers to develop asthma. [122,123]

2.3.1.4 Hypersensitivity Pneumonitis (HP)

2.3.1.4.1 Definition

Hypersensitivity pneumonitis, also known as farmer's lung in the US and allergic alveolitis in Europe, is an allergic lung disease marked by mononuclear inflammation in the terminal bronchioles, interstitium, and alveoli found in the lung's parenchyma region.[14,124] Hypersensitivity pneumonitis develops during reoccurring exposures to organic and inorganic dusts causing sensitization and hypersensitivity to specific antigens found within the dusts.[14,124] Hypersensitivity pneumonitis is largely an occupational disease with different causative agents present in different occupational environments. Historically, *Micropolyspora faeni* has been identified as a common hypersensitivity pneumonitis antigen found in moldy compost, bedding, and feed that may be present at dairy barns.[124,125] More recently, *Absidia corymbifera*, *Erotium spp.*, *Wallemia sebi*, *Mesophilic streptomyces,* and *Thermophilic actinomycetes* have been identified as other hypersensitivity pneumonitis causative agents potentially present at agricultural operations.[125]

Chronic hypersensitivity pneumonitis may occur in certain individuals and typically follows one of three pathologies. The first two pathologies involve regression to interstitial lung fibrosis, with the difference that only one of the pathologies includes acute recognizable episodes. The third pathology manifests into chronic obstructive lung disease. The exact reasons for some individuals developing lung fibrosis and some individuals developing COPD are unknown, but suspected predictors include the offending antigen, cigarette smoking, and genetics.[126] Hypersensitivity pneumonitis must be diagnosed by a physician and typically

involves screening for occupational exposure to a known antigen, antibody testing, examination of chest radiographs, stethoscopic examination for crackles, and weight loss monitoring.[126,127]

Acute symptoms for hypersensitivity pneumonitis include dyspnea, fever, chills, chest tightness, and coughing. Symptoms typically subside following removal from the exposure.[124,127] Some acute symptoms, most notably dyspnea and chronic cough, may linger for weeks following initial symptom onset.[128]

2.3.1.4.2 Burden on dairy workers

A study of 5,703 dairy farmers in the Doubs region of France in the early 1990s found a prevalence of 1.4% of dairy farmers self-reporting clinical hypersensitivity pneumonitis.[107] The researchers in this study also found a strong positive correlation between participants reporting chronic obstructive pulmonary disease and hypersensitivity pneumonitis, an unsurprising result as hypersensitivity pneumonitis can pathologically lead to COPD. One interesting finding was that hypersensitivity pneumonitis prevalence had a linear increase with altitude. The researchers suggested that this increase was likely due to the higher altitudes in Doubs, France reporting larger rainfalls and therefore providing a more supportive environment for the growth of causative agents in bedding and silage.[107] A similar cross-sectional analysis involving participants enrolled in the Agricultural Health Study from 1993-1997 was conducted to determine the burden of hypersensitivity pneumonitis in American farm workers. Although not specific to dairy farms, 2.2% of the 21,393 respondents indicated they had received a clinical diagnosis of farmer's lung.[129] When adjusted for age, state, and smoking, dairy work and associated tasks including silage and grain handling increased participants' odds for clinical diagnosis of farmer's lung. From 1982 to 1994 researchers estimated the incidence of farmer's lung among Swedish farm workers, most of which dairy farmers, stayed at a consistent 2-

3/10,000.[121] Arya et al. observed a sharp decline in farmer's lung in Irish farmers from 1997-2002, which lead to them publishing of a paper titled "Farmer's lung is now in decline" in 2006.[130] While anecdotal in nature, Dr. Yvon Cormier echoed this sentiment when he published a commentary in the Canadian Respiratory Journal stating that his clinic in Quebec, Canada went from diagnosing 20 to 30 new cases of farmer's lung per year to just 1 case every 2-3 years.[131] Farm modernization practices, most notably hay and silage antimicrobial efforts, are attributed to reducing farmer's lung incidence since the turn of the century. More recent epidemiological data for hypersensitivity pneumonitis incidence in dairy workers is limited, and the current burden on dairy workers should be further investigated.

2.3.2 Acute respiratory health outcomes

Acute respiratory health outcomes include upper respiratory symptoms, short-term changes in pulmonary function, and respiratory system inflammation that can be measured immediately following a worker's shift. These measurements are important for two reasons: (i) they have an immediate effect on workers' quality of life during and after exposure, and (ii) acute health outcomes following bioaerosol-exposure may be important pieces of the disease pathology puzzle. For example, cross-shift changes in lung function following exposures to grain dust has been shown to be a useful indicator for longitudinal changes in lung function over a worker's career.[132]

2.3.2.1 Reductions in pulmonary function

Decreased lung function is a major clinical marker of many of the fixed airway diseases that burden dairy workers.[133] Exposure to bioaerosols in dairies can also cause decreased lung function in otherwise healthy workers.[134] Mechanistically, dust particles across the inhalable size fraction can settle or impact airway walls causing bronchoconstriction. The ability of dust to

cause bronchoconstriction is largely dose-dependent, i.e. the more dust a worker breathes the greater the lung function impairment.[41,135–137] Organic aerosolized components in the dust, including bacteria and associated constituents, also cause inflammation in monocyte and epithelial cells of the airways – further contributing to the inflammatory response.[44,68]

2.3.2.1.1 Measurement and definitions

Spirometry is the measuring of breath and the most common pulmonary function test (PFT) performed in field research.[133,138]Participants submitting to a spirometry PFT, under the instruction of a respiratory therapist or trained technician, inhale deeply and exhale into a mouthpiece as forcefully and quickly as they can.[139] A minimum of 3 and a maximum of 6-8 "acceptable efforts" are obtained from participants during normal spirometry testing.[139] Responsibility for determining acceptable efforts relies on the discretion of the spirometry administrator and general guidelines for acceptability determination are both subjective (time between inhale and exhale, participant effort, and participant form) and objective (limited variation of flow rates between efforts, minimum of 6 second effort, and plateau of 1 second where effort is unchanged).[139]

Spirometry tests result in the collection of several spirometric outcomes that inform clinicians and researchers on different aspects of a participant's lung health. Forced vital capacity (FVC) measures the amount of gas a participant exhales after full inspiration.[139] FVC is the primary maneuver performed during spirometry and provides clinicians with a quantitative volume of air expired by the participant in liters.[140] Forced expiratory volume (FEV1) is simply the volume of air (liters) a participant exhales during the first second of an FVC maneuver.[139] Generally, healthy participants will be able to exhale at least 70-80% percent of their total lung volume in the first second of spirometry.[96,139] This ratio of exhaled air in one second over total

volume exhaled is referred to as FEV1/FVC and may be presented in scientific literature as a fraction or as a percentage. These metrics are useful for understanding lung disease as individuals with obstructive diseases often perform poorly on FEV1/FVC, and individuals with restrictive diseases perform poorly on both FEV1 and FVC.

Peak expiratory flow rate (PEFR) or peak expiratory flow (PEF) is the maximum speed or flow rate a participant achieves during a PFT. Measured in units of liters per minute (L/m) or liters per second (L/s), PEFR can be readily obtained during an FVC maneuver. Due to high variability between efforts of the same participant, PEFR may have limited practicality in field research outside of monitoring occupational asthmatic populations.[133,139,140] Forced expiratory flow 25-75% (FEF25-75) is another spirometric value measuring maximum expiratory flow during the middle 50% of an FVC maneuver.[140] While PEFR gives insight into the function of larger airways, FEF25-75 may provide more detailed information on small and medium airway function.[133,140] FEF25-75 may provide clinicians with nuanced data and earlier detection of mild obstructive disorders in smaller airways, but all primary sources referenced in this review agree that FEF25-75 is highly variable, difficult to interpret, and may be of limited use to diagnostic practitioners.[133,139,140]

Historically, these spirometric markers were not only presented as volumes of air, but also as predicted percentages. Predicted percentages compare a participant's spirometry performance to expected values based on their height, weight, age, sex, and race/ethnicity. These expected values are calculated from large epidemiological studies where researchers perform spirometry tests on a wide range of the population. The largest and most common study referenced for these predicted percentages is the CDC's Third National Health and Nutrition Examination Survey (NHANES III), which collected health data on over 30,000 individuals

between 1988 and 1994.[138] In recent years, the use of these predicted values has become antiquated and controversial, as there is no physiological reasoning for predicting pulmonary function values based on race and ethnicity.[141,142] More current papers trend toward using mixed and general linear models that incorporate a variety of covariates to explain differences in pulmonary function.[134,143]

2.3.2.1.2 Spirometry strengths and weaknesses

Portability, ease of use, and short duration of PFTs makes spirometry a powerful tool for onsite assessment of lung function of dairy workers before and after shifts. Portable spirometers weigh less than 5 pounds and can easily be installed and calibrated in a matter of minutes. Interactive displays present vital information to technicians regarding the effort of the participant, acceptability of the efforts, and reproducibility of the efforts.[139] Spirometry PFTs take 15 minutes or less of a participant's time and quickly allows workers to attend their shift or go home.[133] Setup, calibration, and administration of the PFT is managed by a single technician allowing other researchers to collect supplementary data.

Results in the form of FVC, FEV1, FEV1/FVC, PEFR, and FEF25-75 can assist in determining incidence of obstructive diseases in dairy farmers.[140] Daily variability and cross-shift changes in PFTs from before and after a worker's shift can provide vital insight into the physiological response of working in the dairy environment.[134] Daily variability and cross-shift changes can also indicate the effectiveness of proposed interventions – such as the administration of a hypertonic saline nasal lavage to dairy workers.

Spirometry limitations stem largely from the daily variability of lung function and the overall effort participants exert during testing. Researchers performing spirometry on 15 healthy participants and 24 participants with chronic bronchitis found that a healthy person may have up

to 5% variation in FVC and FEV1 when performing spirometry on consecutive days.[144] Spirometry conducted on participants with chronic bronchitis indicated an even larger daily fluctuation of 15-17%.[144]

Most spirometric values rely heavily on obtaining the maximum effort from the participant for each maneuver performed. While spirograms and flow-volume curves can provide real-time feedback to technicians regarding participant effort, technicians may be limited on the amount of influence their feedback provides to participants. In the absence of maximum effort from a participant PEFR values should be discarded entirely.[140]

Additionally, spirometry maneuvers may be tiresome and uncomfortable for patients suffering from severe obstructive disease.[140] Dairy workers with chronic severe obstructive disease are unlikely to continue working and participating in studies; however, workers with undiagnosed treatable illnesses such as respiratory infections and pneumonia may still find spirometry challenging.

2.3.2.1.3 Normal diurnal variation in pulmonary function

Circadian rhythms impact pulmonary function in most individuals leading to a natural variability among test outcomes depending on the time of the day. Bagg & Hughes observed this diurnal variation among 40 asthmatic individuals over a 10-day period and coined the occurring phenomenon as a "morning dip."[145] In their study, 30 individuals showed a consistent morning dip in peak expiratory flow rate and subsequent higher peak expiratory flow rates during evening tests.[145] Medarov et al. performed a retrospective analysis on 4,756 individuals that performed pulmonary function testing during a 5-year period at the PFT Laboratory of Long Island Jewish Medical Center in New Hyde Park, NY.[146] Their study confirms the highest spirometric values of individuals is typically during the evening; however, they found the lowest pulmonary function

tests (PFTs) to be at noon rather than in the morning. Based on PFTs from asthmatic individuals, Medarov et al. hypothesized the morning dip likely effects asthmatic individuals more than healthy individuals.[146] Furthermore, the retrospective analysis found the largest diurnal variation occurring in FEV1 values. Teramoto et al. had similar findings following diurnal spirometry on 60 healthy young subjects, 60 healthy elderly subjects, and 30 subjects with COPD.[147] While they found no significant differences in diurnal variations of FVC, FEV1, or PEFR among the young and elderly subjects, a morning dip for participants with COPD was observed.[147] More recently, Rhee and Kim performed pulmonary function testing on 20 healthy individuals and found evidence of a morning dip in their participants (2015).[148] On average, the participants in their study showed increases of 4.2% and 8.7% for FVC and FEV1, respectively, between 9 am tests and 5 pm tests. [148]

2.3.2.1.4 Burden on dairy workers

Livestock workers exposed to bioaerosols during their shift suffer from larger diurnal variations in pulmonary function compared to non-exposed individuals. Commonly referred to as the "cross-shift change," noteworthy results have been observed in several studies that compare pre-shift spirometry tests to post-shifts results. Donham et al. observed an average cross-shift FEV1 reduction of 1.10% in percent predicted in poultry workers and only a 0.02% decrease in controls.[41] Significant reductions in cross-shift pulmonary function for the poultry workers was associated with dust exposures of 2.4 mg/m^3 for total dust and 0.16 mg/m^3 for respirable dust.[41]

A cross-sectional study of 210 workers at large-herd dairies in California provides the most comprehensive and relevant results regarding cross-shift changes in dairy workers' pulmonary function. On average, the dairy workers experienced a mean change of −65.2 mL (95% CI: -118.6 to -11.9) and −103.1 mL (95% CI: -168.2 to -38.0) for FEV1 and FVC,

respectively.[134] Compared to a control cohort of 46 vegetable workers, the dairy workers had lower baseline lung function and suffered from greater declines in cross-shift lung function. Thirty percent of the participating dairy workers experienced 3% or greater decreases in cross-shift FVC and FEV1 volumes. Only 12-15% of the control workers experienced a similar decline.[134] Self-reported asthma-like symptoms in dairy workers was noted as an indicator of cross-shift decline in pulmonary function. This finding may give larger credence to the Long Island Jewish Medical Center study that found asthmatics experience a larger "morning dip" in spirometry testing.[146] The asthma-like symptoms, which included "self-reported coughing, wheezing, feeling tightness in the chest, or feeling short of breath when exercising, working, or when exposed to cold air, animals, cows/livestock, dust, pollen, cigarette smoke, grain dust, or dairy chemicals" were noted in 40% of the participants.[134]

Another cross-sectional study of 62 workers at large-herd dairies in Iowa, Minnesota, Wisconsin, and South Dakota found a cross-shift change in percentage of predicted FEV1 values of -1.16%.[149] Similarly, 40.3% of the dairy workers in this study reported having at least one of asthma-like symptom. More recently, Martenies et al. did not find any statistically significant differences in cross-shift change in a study of 35 dairy workers in the High Plains region of the United States.[13] Exposure to dust, endotoxin, and 3-OHFA have also been suggested to negatively impact cross-shift pulmonary reductions in workers at grain elevators, corn farms, and cattle feedlots.[12]

2.3.2.2 Respiratory inflammation

Occupational exposure to bioaerosols is also associated with increased respiratory system inflammation. Inflammation occurs following exposure to various biological constituents of dairy dust including endotoxins (Gram-negative bacteria), peptidoglycans (Gram-positive

bacteria), and β-glucans (fungi). Sigsgaard et al. provide a simple summary (see Fig. 1) of the different innate and adaptive immune responses occurring in respiratory system macrophages following exposure to these three constituents: (i) endotoxin exposure triggers toll-like receptor (TLR) 4 response, (ii) peptidoglycan exposure signals TLR2, peptidoglycan recognition proteins (PGRPs), and nucleotide-binding oligomerization domain molecules (NODs), and (iii) exposure to β-glucans stimulates the expression of Dectin-1 on both macrophages and neutrophils.[15] Repeated innate immune reactions as a response to these exposures is the most likely pathogenesis for many respiratory disease associated with dairy farming including COPD and chronic bronchitis.[15] Allergen-specific adaptive immune responses that stimulate respiratory inflammation are likely responsible for the development and exacerbation of asthma, wheeze, and HP in workers.[15] For example, sensitization to bovine dander and hair in affected dairy workers causes an exaggerated IgE mediated immune response and increases risk for the development of occupational asthma and rhinoconjunctivitis.[150]

2.3.2.2.1 Measurement and definitions of inflammatory biomarkers

Collection of inflammatory biomarkers from dairy workers following exposures is integral to developing a greater understanding of the inflammation-mediated diseases that burden the workforce. The most effective measurements are similar to the cross-shift changes in pulmonary function: measure workers' before their shift and compare their baseline inflammatory expression to that occurring post-shift. While significant interest lies in the inflammation occurring in deeper portions of the respiratory system, bronchial lavages and bronchial brushing are invasive and painful procedures that often lie outside of a field researcher's scope of practice.[151,152] Research by McDouggall et al. and Hawley et al. posit that nasal epithelial cells in *in vitro* studies respond similarly to bronchial epithelial cells during

exposure challenges.[153,154] Of course, workers and their entire respiratory systems will not behave exactly like *in vitro* studies when exposed to bioaerosols, but in theory the collection of upper respiratory inflammation markers is still valuable to overall understanding. Inflammatory biomarkers are most often collected via nasal lavages or nasopharyngeal swab. Novel methods to measure inflammation include the collection of exhaled nitric oxide – a biomarker associated with airway inflammation and exhaled in greater volumes by persons with obstructive lung disease.[155]

2.3.2.2.2 Burden on dairy workers

Burch et al. collected nasal lavages post-shift from 174 agricultural workers in Nebraska and Colorado, 15 of whom were dairy workers. For the dairy workers, mean concentration of inflammatory markers were 744 polymorphonuclear neutrophils per ml (PMN/ml), 41 myeloperoxidase ng per ml (MPO ng/ml), 245 IL-8 pg/ml, 5,385 albumin ng/ml, and 1.07 eosinophilic cation protein ng per ml (ECP ng/ml).[49] For all measured biomarkers, mean concentrations were higher in dairy workers and feedlot workers (n=55) compared to grain elevator workers (n-46) – suggesting livestock-associated bioaerosols contain a more potent mixture of inflammatory constituents. Increases in 3-OHFA constituents of endotoxins in collected dusts were associated with 200-300% increases in mean PMN, MPO, albumin, and ECP expression across all workers.[49] As part of an intervention to test the efficacy of a hypertonic saline (400mOsm) nasal lavage to reduce inflammation, Erlandson et al. performed nasal lavages on 10 dairy workers before and after their shifts for 5 consecutive days.[156] In their pilot study, the nasal lavage was both the intervention and the sample, as the nasal lavage was subsequently collected from participants noses and analyzed for the pro-inflammatory cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p70, IL-13, interferon gamma, and tumor necrosis factor

(TNF)-α and the anti-inflammatory cytokine IL-10. Because of the intervention aspect of the study, eliciting conclusions from their results and applying it to dairy workers worldwide would be inappropriate; however, participants appearing to not respond to the intervention did express detectable levels of IL-1b, IL-2, IL-6, and IL-8 in their post-shift lavages.[156]

In a more recent study, Nonnenmann et al. compared cross-shift results in exhaled nitric oxide for 62 dairy parlor workers from dairies in the US Midwest.[57] Interestingly, workers experienced a statistically significant mean decrease of 1 ppb across the work shift.[57] Differences in exposure were not associated with differenced in cross-shift exhaled nitric oxide. The researchers also noted that the dairy workers did not present concentrations of exhaled nitric oxide outside of a normal, healthy population. They concluded that exposure to dairy bioaerosols does not produce enough lung inflammation to detect notable differences in exhaled nitric oxide.[57] It is also possible that their study suffered from small sample size and the natural diurnal variation of exhaled nitric oxide that both healthy and asthmatic individuals reportedly experience.[157]

2.3.2.3 Acute respiratory symptoms

Common upper respiratory tract (URT) and lower respiratory tract (LRT) symptoms experienced by dairy workers include cough, wheeze, dyspnea (shortness of breath), chest tightness, nasal congestion, increased sputum production, and sore throat.[14,15,93] Chronically occurring respiratory symptoms during and after exposures negatively impact workers' quality of life, and in serious cases likely contributes to workers self-selecting out of the industry.[93] Symptoms are also crucial in the diagnoses of the fixed airway diseases that affect workers, e.g., wheeze is common among asthmatic individuals and a chronic productive morning cough is a defining clinical factor of chronic bronchitis diagnosis. Field studies that include the assessment

of respiratory symptoms are almost always self-reported, with questionnaires typically being adopted from the American Thoracic Society (ATS) Division of Lung Disease questionnaire (*ATS-DLD-78A*) and previous exposure assessments.[13] Participants may underreport their symptoms for a variety of reasons, including distrust of researchers, fear of losing their job, or cultural differences. Therefore, the true burden of respiratory symptoms among dairy workers is likely underreported.

2.3.2.3.1 Burden on dairy workers

Between 1994 and 1997, 20,468 farmers and pesticide applicators in Iowa and North Carolina enrolled in the Agricultural Health Study. Hoppin et al. analyzed this cross-sectional survey and determined the number one risk factor for wheeze was working directly with livestock.[158] Based on the 1,131 participants that reported working in dairy operations, an OR of 1.26 (95% CI 1.08 to 1.48) was reported for having a wheeze.[158]

In their cross-shift assessment of 62 US dairy parlor workers, Nonnenmann et al. reported that 40% of workers had either a cough, phlegm, wheeze, or shortness of breath before their shift. Interestingly, a statistically lower (p=0.008) 23% of participants reported have one of these symptoms post-shift.[57] In a study comparing 48 French dairy workers to 78 referent workers, the dairy workers reported statistically higher prevalences of 'fits of coughing' and chest tightness.[109] Babbott et al. compared 198 Vermont dairy workers to 516 industry workers and found statistically higher prevalence of dyspnea (40% vs 29%) and chronic sputum production (25% vs 17%) in dairy workers.[106]

In a more recent cross-sectional survey comparing 226 California dairy workers to 49 vegetable processing-plant workers, no significant differences were observed in self-reported nasal irritation, throat irritation, cough, phlegm production, wheeze, or chest tightness.[159]

Martenies et al. also recently reported in their pulmonary survey of 36 Colorado dairy workers that respiratory symptoms were uncommon, but the results were not fully shown.[13] These reports of low respiratory symptoms may be attributed to the nature of dairy farming in Colorado and California, where cows and workers spend much of the year outside instead of in fully contained barns in regions like Vermont and the Midwestern states of the US.

2.4 Respiratory zoonoses

Present at the human-animal interface of dairy farms are zoonotic pathogens – a serious risk that serves both as an exposure and a health outcome for cows and humans alike.[160] Pathogens may jump between species via inhalation routes, with zoonotic viruses most often infecting respiratory tracts and bacterial pathogens colonizing in the nose of workers and animals.[160–162] These zoonotic pathogens pose a threat beyond individual animal and human infection, as their spread between different species is often accompanied by horizontal gene transfer and increased community fitness – creating viral and bacterial strains that are more contagious, resilient, and/or virulent. [34,163] As seen with the SARS-CoV-2 respiratory virus, these zoonotic pathogens can have potential public health implications far beyond the scope of workplaces and localized regions. One lesson learned from both the Spanish flu and COVID-19 pandemics is that even following the initial wave of infection and global spread, these pandemic pathogens may morph into different variants and impact humans long beyond initial spread. While predicting the next pandemic virus may not be possible, increased surveillance on dairy farms may aid in the early detection of future pandemic pathogens.

2.4.1 Viral

2.4.1.1 Influenza D Virus

Influenza D virus (IDV) has emerged as a relatively new pathogen of particular interest as outbreaks in cattle and dairy cows have been reported in Asia, Europe, and North America since 2011. [160,164,165] Hause et al first identified the novel influenza D virus obtained from an Oklahoman swine operation as having only 50% homology to human influenza C virus (ICV).[165] Additionally, the novel virus was not recognizable to human ICV antibodies. This led to the creation of a new genus within the Orthomyxoviridae Family coined as influenza D virus.[165]It was soon suggested that bovine and swine confinement operations were likely the most common reservoir for this virus.[164,165] Influenza D virus has since been identified in dairy and cattle herds in Texas, California, Kansas, Minnesota, Nebraska, Oklahoma, Mississippi, Florida, Argentina, Ireland, France, Italy, Turkey, Japan, and China .[165–175] Although IDV is generally mild in pathology, it can also trigger bovine respiratory disease (BRD) – an often fatal ailment for cattle.[173,176]

2.4.1.1.1 IDV in humans

The occupational and communal presence of IDV is currently understudied. Original estimates from the cohort that first sequenced IDV in Oklahoma suggested that 1.3% of the general human population had serum antibodies receptive to the virus.[177]

The first human subjects research to identify IDV in humans was performed with 35 cattle workers and 11 non-exposed control participants in north-central Florida.[167] Microneutralization assays performed on serum samples found IDV in 34 (97%) of the 35 workers exposed to cattle on at least a weekly basis for the past 6 months. Two (18%) of the eleven control participants also tested positive for IDV seroprevalence.[167] Haemagglutination

Inhibition (HI) assay was later performed on 1,281 human serum samples collected in Italy from 2005 to 2017 to determine the prevalence of IDV antibodies in the population.[178] Overall, a general increase from 2005 to 2017 was observed, and the total positive antibody samples for the entire time period was 335 (21.9%) out of 1,281.[178]

Most recently, IDV was observed in the nares of US dairy workers. Leibler et al. performed pre- and post-shift nasal lavages on 31 workers over 123 working shifts and found that 21 workers had at least one IDV-positive lavage during the study.[179] Over these 123 working shifts, seven personal breathing zone bioaerosol samples were also positive for IDV.[179] Exposure to IDV may also occur in public spaces, as ambient bioaerosol sampling conducted at Raleigh-Durham International Airport in North Carolina found IDV in 1 (4.1%) out of 24 samples.[180]

The exact pathology for human infection and the associated risks of IDV is poorly understood.[176] Su et al. hypothesize that the virus could only have to undergo a slight genetic change to infect the lower respiratory tract.[181] Su et al. also suggest that the virus appears to mutate relatively slowly and that IDV immunity may be persistent for a substantial time period following initial infection.[181] Almost all publications focusing on Influenza D virus agree that more research should be conducted regarding the occupational and communal health risks associated with the virus.[160,179]

2.4.1.2 Other viruses

Influenza D virus is not the only influenza that may be spread between animals and humans at dairies. In their investigation of US diary workers, Leibler et al. found evidence of influenza A virus (IAV) in 4% lavages.[179] Using Basic Local Alignment Search Tool (BLAST) taxonomic classification, one of the lavages was determined to be H9N2 – an avian flu strain

originally isolated from Chinese chickens.[182] The presence of H9N2 in dairies is concerning, as the virus is considered a 'pre-pandemic' virus due to its ability to horizontally integrate genetic traits from other viruses it encounters.[163] IAV likely travels to dairies via nesting birds that visit and live at the farm.[34] Leibler et al. also found evidence of the human and pig associated influenza C virus (ICV) and nonspecific pancoranaviruses in the nasal lavages of workers.[179]

2.4.2 Bacterial

Zoonotic bacterial pathogens at dairy farms and other livestock operations can also cause infection in workers and subsequently spread to community members. A study of Pennsylvanian women working at dairy farms found that 10.4% of workers reported contracting an illness from an infected animal, with many of these illnesses being the result of bacterial infection.[183] Dairy farms in the United States have also been suggested as potential reservoirs for dangerous *Listeria monocytogens* bacterial strains – a common foodborne illness that kills over 250 Americans every year.[184]

2.4.2.1 Methicillin resistant Staphylococcus aureus (MRSA)

In recent years methicillin resistant *Staphylococcus aureus* (MRSA) has received significant attention for its presence in dairy farms and swine operations. Specifically, the livestock-associated MRSA (LA-MRSA) clonal complex 398 (CC398) variant has seen increased surveillance, as many reported cases occur in non-exposed individuals.[185] Larsen et al. published a report in 2015 that found 34% of individuals hospitalized with MRSA CC398 infections in Denmark were never exposed to livestock.[186]

The burden of LA-MRSA on dairy workers is understudied, but preliminary results presented by Seidel et al. at the *2022 International Society of Exposure Science Conference* found MRSA positivity in 27 nasal lavages from 237 total lavages taken from US dairy workers.

The burden on US and European swine workers is better understood. In a longitudinal study of 21 swine workers in North Carolina, 16 workers were either intermittent or persistent nasal carriers of LA-MRSA.[29] Rinsky et al. performed a cross-sectional analysis of swine workers in North Carolina that included a nasal swab for *Staphylococcus aureus.*[32] LA-MRSA CC398 was observed in the nares of 14/69 (20%) participants, with 13 of these participants reportedly working at livestock operations that used antibiotics.[32] Subsequent whole genome sequencing conducted by Randad et al. at North Carolina pig farms showed evidence that MRSA transmission commonly occurred between workers and pigs.[162]

European researchers have found that the nares of livestock workers may also serve as a route of transmission for community exposure to LA-MRSA. Ingham et al. swabbed Danish pig truck drivers and found that 19 out of 47 tested posted for MRSA.[187] When following nine of these drivers for a follow-up study, two of their spouses tested positive for nasal carriage of LA-MRSA.[187] Zohorul Islam et al. also performed nasal swabs on Danish swine workers and found that most workers carried CC398 in their nasal passages, and many of them tested positive days after being removed from the environment.[27]

Conversely, the nares of dairy works also provide a means of exposure for community-associated bacterial strains to impact animals. While reverse zooanthroponosis is not well documented in dairy environments, there is evidence that workers introduce novel strains of multi-drug resistant tuberculosis to cows.[34,188,189] Increased surveillance of antibiotic resistant bacteria at dairies will therefore be necessary to protect workers, animals, and the community.

2.5 Factors modulating exposure and disease

The burden of respiratory health outcomes is not felt equally across all dairy workers. Recent investigations have focused on identifying the intrinsic factors that modulate individual

exposure responses, with a primary focus on uncovering spatiotemporal and genetic factors that appear to provide protection against bioaerosol exposure. For example, Stein et al. published a seminal paper in the *New England Journal of Medicine* examining differences in the innate immune response between Amish and Hutterite children.[190] Both groups have similar genetics and both groups experience bioaerosol exposure in youth; however, the Amish have more traditional farming and hygiene practices. As a result, Amish children experience more of an innate immune response to exposure as opposed to an allergic response. This is evidenced by decreases in serum IgE and eosinophils in white blood cells post exposure, and subsequent increases in tumor necrosis factor and interferon regulatory factor 7 signaling proteins.[190] These responses provide a greater protection against allergy and asthma development in Amish children compared to Hutterite children.

2.5.1 Intrinsic factors

2.5.1.1 Timing of exposure

Research over the last two decades has supported the theory that growing up on a farm protects against developing atopy and allergic asthma later in life.[117,191,192] Between 1994 and 1998 Omland et al. studied a group of Danish students and found that those growing up on a farm had a much lower risk for developing adult-onset asthma (OR 0.5, 95% CI: 0.3–0.98).[17] Conversely, they found those who entered into the dairy workforce at adulthood increased their risk for developing asthma (OR 2.47, 95% CI: 1.14–5.34).[17] The same research group later published that adult exposure to cattle may provide protection against allergic sensitization.[19]

Protection against atopy and asthma is valuable, but the adaptive immune response is likely only responsible for the development and exacerbation of asthma and upper respiratory symptoms. Early childhood exposures may not influence the innate immune response, as was

observed in the Amish and Hutterite Children study.[190] Again, the innate immune response is likely responsible for the development of COPD, chronic bronchitis, and decreases in lung function. In a 15-year longitudinal study of Danish dairy and swine farmers, Bolund et al. found that childhood farm exposure provided protection against decreases in FEV1 and FEV1/FVC.[18] Further research focused on the timing of exposure and its effect on the innate immune response should be considered.

2.5.1.2 Genetics

Genetic variations may also play a role in individual susceptibility to the effects of bioaerosol exposure. Genetic mutations in TLR4, specifically the missense polymorphisms Asp299Gly and Thr399Ile appear to have localized protective properties that promote a hyporesponsive reaction to endotoxin inhalation.[193] Known as the 'hyporesponsive phenotype,' individuals possessing this genetic mutation performed better in spirometry parameters following subjection to endotoxin than similarly exposed non-mutated individuals in a small (n=83) non-occupational study.[193,194] Prevalence of the hyporesponsive phenotype in Arbour et al. 's study was between 3.3% and 7.9%.[193]

Reynolds et al. found a prevalence of TLR4 mutations Asp299Gly and Thr399lle to be 6.7% & 8.8%, respectively, in a group of 134 agricultural workers from Colorado and Nebraska.[12] In this study spirometry was performed on participants before and after a typical shift at a cattle feedlot, dairy farm, grain elevator, or corn farm. Spirometry results were then grouped into quartiles based on inhalable dust exposures. Workers with the TLR4 mutations Asp229Gly and/or Thr399lle experienced a larger cross-shift reduction in both FEV1 and FVC.[12] This finding suggests organic dusts inundated with endotoxins and other inflammatory constituents from agricultural work environments may elicit a stronger systemic inflammatory

response among those possessing a 'hyporesponsive phenotype'. A study involving 434 Dutch agricultural workers, none of whom worked in animal confinement operations, did not find a relationship between endotoxin exposure, TLR4 mutation, and prevalence of a wheeze.[53]

CD14 is another endotoxin receptor in the human genome that has polymorphisms that may modulate host and exposure interactions.[195] Smit et al.'s study of Dutch farmers revealed three CD14 polymorphisms that increased the inflammatory response to endotoxin exposure.[196]Individuals with these polymorphisms performed worse when comparing FEV1 spirometric values following exposure to endotoxins. Furthermore, whole blood samples obtained from these workers expressed greater levels of proinflammatory cytokine IL-1B following ex vivo endotoxin exposure.[197]

Toll like receptor-2 (TLR2) is the transmembrane protein responsible for activation of the NF-κB pathway following exposure to gram-positive bacteria.[198] Poole et al. subjected 'wild-type' mice and TLR2-deficient mice to gram-positive bacteria-containing organic dust collected from a swine confinement feeding operationTLR2-deficient mice exhibited less bronchial inflammation and subsequently reduced cytokine expression following exposure to the organic dust.[44] Lung macrophages from the TLR2 deficient mice were then isolated and subjected to the organic dust. The TLR2-deficient lung macrophages expressed smaller amounts of the proinflammatory cytokines TNF-a, IL-6, and CXCL-1.[44]

2.5.2 Human nasal microbiome

2.5.2.1 Defining the human microbiome

The human body and its organ systems host thousands of unique species and distinct bacterial communities that vary in structure and diversity.[199–201] These communities are known as the human microbiome and their composition varies as greatly from person to person as it

does from organ system to organ system, i.e. one person's colon microbiome may be more similar to another's oral cavity.[202] Differences in microbiomes are often quantified via alpha and beta diversity metrics. Alpha diversity represents the richness or distribution of taxa within a single sample, while beta diversity measures the similarity of taxonomic metrics between two or more samples. Translational research over the last 20 years has pointed to the microbiome's role in human health – a possible modulating factor in various disease states where genetics, behavior, and environmental exposures fail to tell the entire story.[203]

2.5.2.2 Differences in livestock worker nasal microbiome

The human nasal microbiome and its role in occupational bioaerosol exposure blurs the boundary between intrinsic and extrinsic factors. As respiratory inflammation is a direct result of inhalable bioaerosols, the nasal microbiome may act as the first line of defense during exposure. Cross-sectional studies of livestock workers have shown that working in a livestock operation modifies the nasal microbiome, with livestock workers exhibiting greater alpha diversity in their nares than non-exposed controls.[24,25] This phenomenon is in part due to unique genera, such as Ruminococcaceae, which are associated with cows and other animals raised as livestock.[25]

Zohorul Islam et al. designed a study to measure the temporality of changes in the nasal microbiome post-exposure.[27] After recruiting pig farmers and non-exposed individuals, Zohorul Islam et al. measured their nasal microbiomes 2 hours before, immediately after, and 48 hours after exposure to a Danish pig farm. Surprisingly, the workers experienced larger changes after exposure and less of a shift back to pre-exposure baseline compared to the short-term visitors.[27] The researchers concluded that the impressionable nature of the livestock workers' nasal microbiome may provide a route of exposure for zoonotic pathogens to travel between environments.[27]

2.5.2.3 Protective characteristics of the nasal microbiome

Conversely, it is also theorized that increased alpha diversity in dairy workers nasal microbiomes may afford protection against bacterial and viral pathogens. Shukla et al. observed that dairy workers with a higher species richness were less likely to test positive for Staphylococci in their nostrils. The nasal microbiome's role in viral exposures is less understood, both occupationally and in the community. Bacillus strains may have antiviral properties, as one study identified a Bacillus spore nasal spray as a potential intervention for children suffering from respiratory syncytial virus (RSV).[204] Similarly, another study found that *Bacillus subtilis* inhibited the proliferation of IAV *in vitro* and protected against infection in mice.[205]

The gap in knowledge of the nasal microbiome's role in acute health outcomes following exposure is even less understood. In clinical studies, a relationship between decreased upper respiratory tract (URT) microbiota diversity and increased URT inflammation has been established for individuals with chronic rhinosinusitis.[206,207] Dysbiosis of Lactobacillaceae in various airway microbiomes may also impact lung function, as a decrease in this family have previously been associated with chronic rhinosinusitis, increased IL-6 and C reactive protein inflammation, and asthma.[208–210] From an occupational exposure perspective, no previous studies have been identified that attempt to parse the impact of the nasal microbiome on post-exposure inflammation or lung function.

2.6 Controls and interventions

Despite centuries of hazard recognition and decades of improved quantification of health outcomes in dairy workers, interventions to protect workers are sorely lagging. Successful development and implementation of controls provides a substantial challenge to occupational health professionals for many reasons. Unlike other livestock operations, large dairies operate on

24-hour production cycles with cows continuously entering milking parlors and receiving routine medical attention throughout the night. Long and strenuous working shifts, contact with large and unpredictable animals, and the operation of heavy machinery poses additional challenges that severely limit 'one-size-fits-all' approaches to reduce inhalation exposures.[4,9] Technological advancements in dairies have improved milk production and efficiency, but their translation to improved respiratory outcomes is minimal. For example, herringbone and rotary milking parlors are new inventions that greatly improve the amount of cows can be milked per hour; however, exposure assessments have determined neither parlor configuration substantially reduces the burden of inhalable bioaerosols.

A few identified studies have attempted to reduce either exposure levels or subsequent health outcomes in dairy workers. These studies and their strengths and weaknesses are briefly discussed in this section. The hypertonic saline nasal lavage intervention, which is a major focus of this research, is then introduced and discussed in more detail.

2.6.1 Previous interventions

2.6.1.1 Parlor washing

In 2012 Choudhry et al.'s group tested an administrative control that doubled the number of times a milking parlor in a modern dairy operation was cleaned by employees over an 8-hour shift.[211] While typically instructed to clean the parlor 4 times during a shift, workers were instructed to clean the parlor 8 times. Dairy workers used an automated system to clean the stalls, cow walkways, and other surfaces with reclaimed water from the onsite waste lagoon. Other than frequency, cleaning protocols were not changed for this study.

Dairy workers in both the control group (n=10) and intervention group (n=10) were fitted with personal air monitoring pumps to measure dust and endotoxin in both the inhalable and

respirable size fractions. Mean concentrations showed improvements for the intervention group across all criteria: inhalable dust decreased from 3.98 mg/m^3 to 2.25 mg/m^3, respirable dust from 0.25 mg/m^3 to 0.12 mg/m^3, inhalable endotoxin from 1108 EU/m^3 to 875 EU/m^3, and respirable endotoxin from 5.58 EU/m^3 to 3.96 EU/m^3.[211]

While the administrative intervention is relatively simple, increasing parlor washing has significant limitations that impact its adaptability across the industry. First, relative humidity under intervention conditions increased significantly, which may create an environment more favorable to microbial growth. Aerosolized endotoxin concentrations were lower in the intervention group, but over time increased microbial growth may lead to higher exposures of endotoxin and other inflammatory constituents. Parlor washing is also resource intensive in both water usage and man-hours, and many dairies may not have the capital to commit to this intervention. Finally, parlor washing as an intervention theoretically only improves exposures for employees working in the milking parlor. Due to increased task specialization in dairy operations, many workers are unlikely to spend any significant amount of their shift in the milking parlor.[212] Instead they may spend time in barns, corrals, grain storages, and maternity pens, where bioaerosol concentrations are similar to those found in milking parlors.[50,51,60]

2.6.1.2 Bedding material selection

Samadi et al. conducted an exposure assessment in Dutch dairy barns to determine which bedding material, if any, could best reduce dairy workers' bioaerosol exposure.[213]Personal and area sampling was conducted to compare the conditions in barns using different types of bedding (e.g. compost, sawdust, chopped straw, and combinations). When comparing personal air sampling results, sawdust bedding was associated with the lowest exposures. Workers in dairy barns with sawdust bedding had geometric means exposures of 0.40 mg/m^3 for inhalable dust

and 137 EU/m^3 for endotoxin. Workers in dairy barns with compost and combination bedding had concentrations between 1.45 – 1.63 mg/m^3 for inhalable dust and 574 - 1268 EU/m^3 for endotoxin.[213]

Bedding material selection may be important as one study of large-herd dairy works in California found that workers spending 75% or more of their time rebedding had the highest average endotoxin exposure.[51] One concern is that the crystalline silica content of sawdust bedding may simply mean swapping bioaerosol exposure for dangerous inorganic dusts.[14]

2.6.1.3 PPE – N95

NIOSH specifications require that certified N95 respirators filter 0.3 um and larger particles with an efficiency of at least 95%. Because agricultural dust particles and associated bioaerosols are often in the 0.3 – 1.0 um or larger mean size range, Lee et al.'s research group investigated the effectiveness of N95 respirators to reduce organic dust exposures in agricultural settings[214]. While generally protective against inorganic dust particles, the N95 respirators provided inadequate respiratory protection for more than 50% of the organic dust constituents measured. Notably, proinflammatory constituents including culturable bacteria, aspergillus/penicillium, and ascospores had raw mean values under the minimum recommended workplace protection factor (WPF) of 10.[214]

2.6.2 Hypertonic saline nasal lavage

In 2022 Erlandson et al. published a pilot study testing the efficacy of a low-cost, low-burden hypertonic saline (HTS) nasal lavage designed to reduce upper respiratory inflammation in dairy workers.[156] Because a significant portion of diary bioaerosols fall into the inhalable range, many of these particles deposit into the nasopharyngeal region.[35] A HTS nasal lavage intervention could therefore reduce inflammation and promote an anti-inflammatory response at

the primary location of dairy dust particle deposition. For their study, and the intervention study of this dissertation, the HTS nasal lavage had an osmotic concentration of 400 milliosmole (mOsm).

2.6.2.1 Justification

Hypertonic saline has precedence as an anti-inflammatory agent in medical applications. Early investigations into the viability of hypertonic saline as a resuscitation fluid found intravenous application of HTS positively modulated T-cell recovery from immunosuppression; which subsequently protected mice from sepsis – a gram-negative bacterial infection.[215] Interestingly, in vitro human peripheral blood mononuclear cells (PBMC) challenged with phytohemagglutinin (PHA) showed a higher response of IL-2 cytokine signaling when HTS was added post challenge.[215] The IL-2 proliferation finding suggests HTS directly effects inflammatory protein signaling pathways.

HTS has also been shown to attenuate inflammation in patients with cystic fibrosis. Reeves et al. collected bronchoalveolar lavage fluid samples from patients with cystic fibrosis and treated the samples with glycosaminoglycan lyases and HTS.[216] The samples treated with HTS showed decreases in IL-8 compared to control samples. The researchers then successfully treated patients *ex vivo* with a nebulized spray of HTS. Cystic fibrosis patients receiving this spray experienced significant decrease in IL-8 in their sputum when comparing pre- and post-treatment levels.[216] Elkins et al. also successfully treated cystic fibrosis patients with bronchodilators and nebulized sprays of HTS.[217] Patients receiving the HTS spray performed better on certain spirometric markers compared to control patients who only received a bronchodilator.[217]

Mitra et al. and Banerjee et al. have also shown HTS's ability to attenuate the expression of inflammatory markers *in vivo.* [218,219]

2.7 Conclusion

Decades of occupational health research in dairy workers has greatly advanced understanding of the respiratory exposures dairy workers experience, and the health outcomes associated with these exposures. Researchers have largely been successful at quantifying the particle size distribution, inflammatory constituents, and source profiles of dairy bioaerosols based on geography, task, and dairy parlor configuration. The respiratory diseases and health effects dairy workers experience as a result of these exposures has also been documented. Globally, dairy work is positively associated with COPD, chronic bronchitis, asthma, hypersensitivity pneumonitis, and asthma-like reductions in pulmonary function.

Organizational changes in dairies appear to be modifying the way dairy work is done. The emphasis on increasing milk production per cow has led to greater task specialization and differences in exposures between tasks. Furthermore, more recent epidemiological studies have reported mixed results in respiratory health outcome among dairy workers. Future epidemiological studies should enroll workers longitudinally and focus on enrolling workers from a variety of tasks.

Intrinsic factors modulating exposures and disease outcomes are also important research needs. Bioaerosols affect individuals differently, which is why regulatory agencies and professional associations are lagging on implementing occupational exposure limits for bioaerosol constituents. Current understanding of disease pathology suggests workers that have had previous childhood livestock exposures are more resilient to bioaerosols as adults. Less is

understood about the role of genetics, as mixed results have emerged from studies looking at polymorphisms of endotoxin receptors.

The role of the microbiome in bioaerosol exposure is also largely a mystery. Occupational exposure to livestock appears to modify the nasal microbiome, but studies linking these differences to health outcomes are limited. Differences in taxonomic composition, richness, and distribution of microbiota in the nares of dairy workers may provide protection against adverse health outcomes, but further research is needed. A better understanding of the intrinsic factors and microbiome differences among individuals may lead to the emergence of more suitable controls for this occupational group.

Recent research into the viral and bacterial pathogens present at dairies poses additional risks to workers and community members alike. Increased surveillance of potential pandemic pathogens at livestock operations, such as influenza A, will be necessary to avoid future pandemics. Improved consensus among researchers and public health officials will also be necessary to quantify exposures, infections, and outbreaks in this workforce. For example, there is little official guidance on the difference between presence, carriage, and infection for pathogens residing in the nares of livestock workers. Defining these terms will be crucial for future researchers.

Despite extensive research focus on respiratory health outcomes in dairy and other livestock workers, controls for bioaerosol exposures are understudied. The development of controls for dairy workers is restricted by unique challenges facing the industry: workers are often scheduled for long and unusual hours, workers interact with large and unpredictable animals, and workers may perform different tasks throughout their shift. Dairy work is also one of the oldest professions, and changes to work practice are slowly adopted throughout the

industry. Controls and interventions will only succeed if operators and workers agree to implement them.

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CHAPTER 3: THE NASAL MICROBIOME'S POTENTIAL ROLE IN PATHOGEN EXPOSURES FOR DAIRY WORKERS

Summary

Background: Livestock workers are exposed to polydisperse bioaerosols (0-100 µm in aerodynamic diameter) comprised of diverse bacterial and viral constituents. Consequently, opportunistic pathogens such as the novel influenza D virus (IDV), influenza A (IAV), and livestock-associated Methicillin-resistant *Staphylococcus aureus* (MRSA) may infect workers and pose a potential public health risk for community members. While our understanding of the nasal microbiome in dairy workers is increasing, its role in pathogen exposure and subsequent community spread is not well understood. Here, we characterized the nasal microbiome from a sample of US dairy workers to understand how microbiome composition may impact nasal carriage of zoonotic pathogens.

Results: From a cohort of 31 dairy workers, we collected 237 nasal lavages taken before and after shifts over a workweek to determine the presence of influenzas A, C, and D, Methicillin-susceptible *Staphylococcus aureus* (MSSA), and MRSA. The same nasal lavages were then analyzed via PCR to quantify the bacterial communities that comprised the workers' nasal microbiome, and differences in microbiome characteristics were analyzed based on the presence or absence of our targeted pathogens. Overall, 32.1% of nasal lavages tested positive for MSSA, 11.4% for MRSA, 17.3% for IDV, 2.5% for IAV, and 1.3% for ICV. Temporal analysis of positive lavages suggests MRSA presence in the nares is exposure related and transient in nature, while MSSA carriage is more persistent in dairy workers. Only 1 of the 31 dairy workers did not test positive any pathogens during their workweek. Nasal samples positive

for IAV and those positive for MSSA clustered separately from each other using Robust Aitchison Principle Coordinate Analysis (PCA), but no significant differences were observed in alpha diversity. Differential abundance analysis revealed significant differences in multiple genera for lavages testing positive for MRSA, MSSA, IDV, and IAV.

Conclusion: Key genera present in certain dairy workers' nares may therefore provide protection against pathogens. Future studies should implement metagenomic techniques to better characterize the modulating effect the nasal microbiome has on pathogen exposure.

Introduction

Agricultural workers experience a large burden of respiratory disease likely caused by repetitive and prolonged exposures to biological aerosols (i.e., bioaerosols).[1] Specifically, dairy operations generate bioaerosols that vary substantially in size – spanning several orders of magnitude in aerodynamic diameter (i.e., <3-100um). The composition of bioaerosols on dairy farms can be based on a variety of onsite sources, including animals, humans, feces, feed/bedding, etc.[2–6] Bioaerosol exposures among dairy workers have been associated with risk for inflammatory and obstructive lung diseases as well as decreased lung function, bronchial hyperresponsiveness, and respiratory symptoms such as coughing and wheezing.[1–8] More recently, interest in livestock exposures has shifted to opportunistic zoonotic pathogens and their implications for public health.

Dairy bioaerosols contain a taxonomically diverse set of bacterial species, inflammatory constituents (e.g., endotoxin), and livestock-associated pathogens such as influenza A (IAV), influenza C (ICV), the emerging influenza D virus (IDV), and Methicillin-resistant *Staphylococcus aureus* (MRSA).[6,9,10] MRSA strains can divided into two broad groups, healthcare- and community-associated MRSA, which may pose potential threats to production

animals (via zooanthroponosis).[11] Improved surveillance of exposures on dairy farms can help reduce the threat and burden of adverse outcomes among people and animals.[12] For example, separate investigations have identified evidence that both IDV and the livestock-associated MRSA CC-398 variant can infect individuals with no livestock exposures.[13,14] With clear evidence of community spillover of viral and bacterial pathogens originating from livestock operations, a better understanding of these exposures and the subsequent host response is vital to protecting workers and potentially mitigating future pandemics.[14–17 16]

Present at this critical interface of exposure between worker and environment reside unique and distinct bacterial communities that comprise the upper respiratory system, with specific attention to the nasal microbiome. Our current understanding is that the nasal microbiome is more homogenous between populations than other areas of the body, is often colonized by *Staphylococcus, Corynebacterium, Alloiococcus, Haemophilus, Streptococcus, Granulicatella, and Moraaxella,* and dysbiosis marked by a lack of diversity is commonly observed in various respiratory disease states.[18–20] Substantial bioaerosol exposures likely affects the nasal microbiome. For example, the nasal microbiome of dairy workers is less studied, but has been shown to be more diverse in species richness and novel taxa found primarily at dairy farms.[21] Dairy workers acquiring unique bacterial taxa from the dairies they work is congruent with previous findings that environmental factors have been shown to substantially modulate the human microbiome. [22,23] Furthermore, the extended work shifts and increased task specialization common at modern dairies leaves workers vulnerable to repetitive exposures over the course of their workweek.

The nasal microbiome in dairy workers may provide protection against opportunistic pathogens including MRSA, IAV, and IDV, all of which are emerging as biosafety concerns at

livestock operations. [4,6,17] Research suggests the increased taxonomic diversity observed in dairy worker's nasal microbiome may provide competition to inhibit staphylococci colonization.[21] As for viral exposures in dairy operations, the role of the nasal microbiome in protecting its host from viral colonization and infection is understudied. Interestingly, *Staphylococcus aureus* in the airways protects against influenza infections by recruiting peripheral CCR2+ CD11b+ monocytes through promotion of TLR2 signaling.[24] A recent epidemiological study suggests the bacterial community structure of the nasal microbiome can protect against influenza infections, but supporting data suggested this structure was uncommon in adults.[25]

Here, we used nasal lavages collected from a longitudinal intervention study to ascertain an improved understanding of the nasal microbiome characteristics that may provide protection against occupational pathogenic exposures. The nasal lavage specimens provide a unique opportunity to simultaneously quantify pathogen presence in the nostrils of dairy workers and the bacterial communities that comprise their nasal microbiome. By identifying nasal lavages that were positive for IAV, ICV, IDV, Methicillin-susceptible *Staphylococcus aureus* (MSSA), and/or MRSA, we were able to identify the nasal microbiome characteristics that may play a role in protection or susceptibility.

Methods

Participant recruitment and sample collection

This study was nested in a larger intervention study where participants were recruited from five large herd dairies (>2000 cows) in the Southwestern United States between May 2019 and January 2020 using a snowball sampling approach. Exclusionary criteria included the recent use of immunosuppressive, anti-autoimmune, or chemotherapy medications, recent surgery, chest injuries, or a history of stroke or heart disease. Participants were asked not to perform their

own nasal rinse before their work shift and to refrain from smoking tobacco and e-cigarettes within 15 minutes of the lavage. Workers from all roles at the dairies were invited to participate, including those performing tasks such as milking, feeding, caring for animals, maintenance, operations, and office work. As part of enrollment, workers were asked to participate for 5 consecutive workdays; workers unable to participate for five consecutive days were still included. From the parent study, participants were split evenly into treatment or control group where every other participant received treatment. Because of the design of the intervention study, half of the participants received a hypertonic saline nasal lavage with an osmotic concentration of 400 milliosmole (mOsm) and half the participants received a normotonic saline lavage with an osmotic concentration of 308 mOsm. All participants provided written consent in English or Spanish and all study protocols were approved by the Colorado State University Institutional Review Board.

Full biological sampling procedures have previously been described in Erlandson et al.[26] Briefly, nasal lavages were collected before and after each shift, for a total of 2 lavages per participant per day. Participants could therefore receive up to 10 total lavages over the duration of the study. Participants were instructed to tilt their head back while a trained researcher administered 5 ml of lavage fluid into each nostril over a 10 second period. Following the 10 seconds, participants tilted their head forward to expel the lavage fluid into a sterile specimen cup. Lavages were pipetted into a conical vial and then a protease inhibitor cocktail (PIC) was added onsite in a 1% volume to volume ratio. Lavages were transported on ice to a laboratory at Colorado State University where they were aliquoted for viral analysis, *Staphylococcus aureus* analysis, and PCR amplification for bacterial communities. Forty percent glycerol stock was added to the *Staphylococcus aureus* aliquot in a one-to-one ratio to aid in deep freezing and

storage. Glycerol stock limits the formation of ice crystals that typically puncture cellular membranes and denature proteins. All aliquots were then immediately stored in -80^{o}C freezers until analysis was performed.

Pathogen laboratory analysis

Methicillin-susceptible *Staphylococcus aureus* (MSSA) analysis of the nasal lavages was performed at Colorado State University via culture-based methods. Briefly, lavages were inoculated on to Tryptic Soy agar and Mueller Hinton agar plates using "lawn streaking" techniques. Following incubation, growth for selective and differential media was identified and, if present, positive colonies were transferred to BHI Broth.

To determine the presence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in lavages, antibiotic sensitivity testing (AST) was performed using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol outlined by the *American Society for Microbiology.*[27] For the scope of this paper, resistant samples were reported as positive and intermediate and susceptible samples were reported as negative.

Presence of influenza A virus (IAV), influenza C virus (ICV), and the novel influenza D virus (IDV) was performed by Professor Gray's team at Duke University in 2020-2021 using previously described real-time and conventional reverse transcription polymerase chain reaction methods (qRT-PCR and RT-PCR).[28]

Nasal microbiome laboratory analysis

High throughput 16S rRNA gene sequencing to quantify the bacterial communities that comprise the nasal microbiome of the dairy workers was performed at the University of Oklahoma Health Sciences Center. To isolate the bacterial genomic DNA from the nasal lavages, Quick-DNA kits from Zymo research were used (Irvine, CA). Following methodologies outlined

in Illumina's 16S Metagenomic Sequencing Library Preparation protocol, 16S rRNA gene libraries were constructed with IDT DNA Technology primers and KAPA reagents. Individual libraries were constructed using 5ng of genomic DNA for PCR amplification of a ~460bp product spanning the 16S rRNA V3 and V4 regions. Libraries were indexed in order to multiplex for sequencing on the Illumina MiSeq platform using a MiSeq 600 Cycle Reagent Kit v2 to collect 300bp paired end reads.

Data analysis and nasal microbiome processing was performed using the qiime2-2022.2 (q2) platform.[29] The q2 DADA2 plugin was used to denoise sequences and generate a feature table.[30] A taxonomic classifier was then trained using pre-formatted full-length reference sequences from SILVA and the primers listed in Table S3.1.[31] Taxonomic classification of amplicon sequence variants (ASV) was performed using this custom-trained classifier. To explore the evolutionary relationships between bacterial species, a phylogenetic tree was generated via the q2 sepp-fragment insertion plugin and SILVA's 128 SEPP reference database.[3132] Sequences without a match were placed in the most representative branch point and denoted as unannotated sequences.

Statistical analysis

Each participant was able to provide up to 10 repeated measures for analysis. Individual positivity for each targeted pathogen was established by dividing positive lavages by the total lavages collected. Prevalence was determined by dividing participants who tested positive for a pathogen during the study by overall participants. A participant only needed one positive lavage throughout their workweek to be considered prevalent. An array of total lavages by participant and a heat map visually representing lavages at every time point was then constructed. For both

overall positivity and prevalence calculations, lavages could be positive for more than one pathogen.

To evaluate the relationship bacterial pathogen positivity on day one of sampling with subsequent sampling days, separate McNemar's Tests were performed on samples for both MSSA and MRSA positivity. Contingency tables were then constructed by matching samples based on positivity and if the samples were taken on day 1 of the study.

Diversity metrics and differential abundance analysis was then performed to determine the nasal microbiome's role in pathogen exposures at dairies. First, alpha diversity was determined using the q2 breakaway package, which estimates species richness via frequency ratios.[32] Pairwise Kruskal-Wallis comparisons based on the presence and absence of pathogens was then performed to determine if there were significant differences in alpha diversity. Next, analysis of beta diversity between samples was performed using the q2 DEICODE package, which uses Robust Aitchison Distance to depict and display beta diversity via biplots.[33] Pairwise PERMANOVA comparisons of beta diversity were also performed in DEICODE to infer significant differences in samples positive for pathogens.

Taxonomy was then imported in R Studio (version 2022.07.2) and quantitative ranking of abundance was performed using the *phyloseq* pacakge.[34] After the top 30 genera were identified, Wilcoxon signed-rank tests were performed based on the presence or absence of our targeted pathogens. Significant results were identified as samples where a particular genus was more or less likely to occur based on a pathogen's positivity (p-value <0.05). These results were also visualized as boxplots using the *microbiomeutilities* package.[35] P values of <0.05 were used for all statistical analyses.

Results

Sampling results

Thirty-one (31) participants were recruited and sampled across a total of 119 working shifts. Participants were enrolled in the study for multiple days across a workweek, ranging from 2-5 days; of these, 65% of the participants enrolled for 4 or 5 days. Following pathogen analysis and nasal microbiome quantification, n=237 lavages were included in this study. Demographics of the workers are summarized in Table 3.1, and the working tasks participants reported during their shift are in Table 3.2.

Table 3.1: Worker Demographics (n=31)

	Yes
Working at the dairy one year or longer	71.0%
Reported milking	9.7%
Reported direct animal contact	54.8%
Reported feeding or maintenance in parlor and stalls	29.0%
Reported administrative work	9.7%
Male	77.4%
20-29 years old	32.3%
30-39 years old	48.4%
40-49 years old	9.7%
50+	9.7%
Smoker (current or former)	16.1%

Table 3.2: Working tasks reported during study period

Category	Task
Production	Milking
Maintenance	Maintenance
	Milking parlor maintenance
	Lagoon/waste maintenance
	Repairing pens and gates in corral
Animal care	Regular medical care
	Tending to sick or injured animals
	Hoof trimming
	Moving animals
	Mixing feed
	Feeding
	Rebedding and scraping stalls in corral
Reproduction	Birthing
	Calfing
	Breeding
	Husbandry
Administrative	Office

Pathogen Results

The lavages testing positive for pathogens are summarized in Table 3.3. A total of 76 (32.1%) nasal lavage samples tested positive for MSSA and 27 (11.4%) tested positive for MRSA. The viral results have previously been described by Leibler et al., 2023. Briefly, 6 (2.5%) lavages tested positive for IAV, 3 lavages for ICV (1.3%), and 41 (17.3%) lavages tested positive for IDV.

Table 3.3: Nasal lavages testing positive for viral and microbial pathogens (n=237)

Targeted pathogen	Percentage (number of positive lavages)
Influenza D virus (IDV)	17.3% (41)
Influenza A virus (IAV)	2.5% (6)
Influenza C virus (IAC)	1.3% (3)
Methicillin-resistant S*taphylococcus aureus* (MRSA)	11.4% (27)
Methicillin-susceptible S*taphylococcus aureus* (MSSA)	32.1% (76)

Overall prevalence of participants testing positive for lavages is summarized in Table 3.4. Over the course of a workweek, 14 participants (prevalence 45.2%) tested positive for MRSA and 8 of these participants had multiple lavages test positive throughout the week. Six out of 27 (22.2%) positive samples occurred on the first day of sampling. When compared to later sampling days, a McNemar's paired test found significantly more negative lavages on day one ($p=0.0001$). This increased likelihood of positive lavages occurring on days 2-5 suggests MRSA presence in dairy workers is exposure related. As most participants began our campaign following one or two days off from work, it is plausible that susceptible dairy workers 'clear' *Staphylococcus aureus* from their nares after an extended period away from the dairy. Upon return to work, these workers again test positive for MRSA. Fourteen of the MRSA positive lavages (51.9%) were taken pre-shift. Of the 14 MRSA positive lavages observed pre-shift, 6 had corresponding post-shift lavages test positive for MRSA.

Throughout the workweek 22 participants (prevalence 71%) had at least one nasal lavage test positive for MSSA, and 17 of these participants had multiple lavages test positive throughout the week. Twenty-five out of 76 (32.9%) positive samples occurred on the first day of sampling. When compared to all lavages taken on the first day, a McNemar's paired test did not find a significant difference in MSSA results on day one ($p=0.09$). Further, only thirty-four of the MSSA positive lavages (44.7%) were taken after the working shift. The lack of correlation

between time of exposure and MSSA positivity suggests these dairy workers are at an increased risk for more permanent nasal colonization of MSSA. A total of 9 participants (prevalence 29%) had 4 or more lavages test positive throughout the week, and 2 participants had 9 positive lavages. Comparatively, only 2 participants had 4 or more lavages test positive for MRSA throughout the week, suggesting persistent MSSA carriage is more common among dairy workers than persistent MRSA carriage. Together, 8 participants (prevalence 25.8%) had at least one nasal lavage that tested positive for both MSSA and MRSA.

Over the duration of the study, six workers had one nasal lavage positive for IAV (prevalence: 19.4%), three workers had one nasal lavage test positive for ICV (prevalence: 9.7%), and 21 workers had at least one nasal lavage positive for IDV (prevalence: 67.7%).

Each lavage and corresponding pathogen positivity is visualized in Figure 3.1. Of the 237 lavages taken, 29 (12.2%) tested positive for two or more pathogens. During the workweek we also identified six workers that tested positive for more than one influenza (prevalence: 19.4%).

Table 3.4: Prevalence of workers having at least one positive lavage during study (n=31)

Targeted pathogen	Percentage (workers with at least one positive lavage)
Influenza D virus (IDV)	67.7% (21)
Influenza A virus (IAV)	19.4% (6)
Influenza C virus (IAC)	9.7% (3)
Methicillin-resistant S*taphylococcus aureus* (MRSA)	45.2% (14)
Methicillin-susceptible S*taphylococcus aureus* (MSSA)	71% (22)

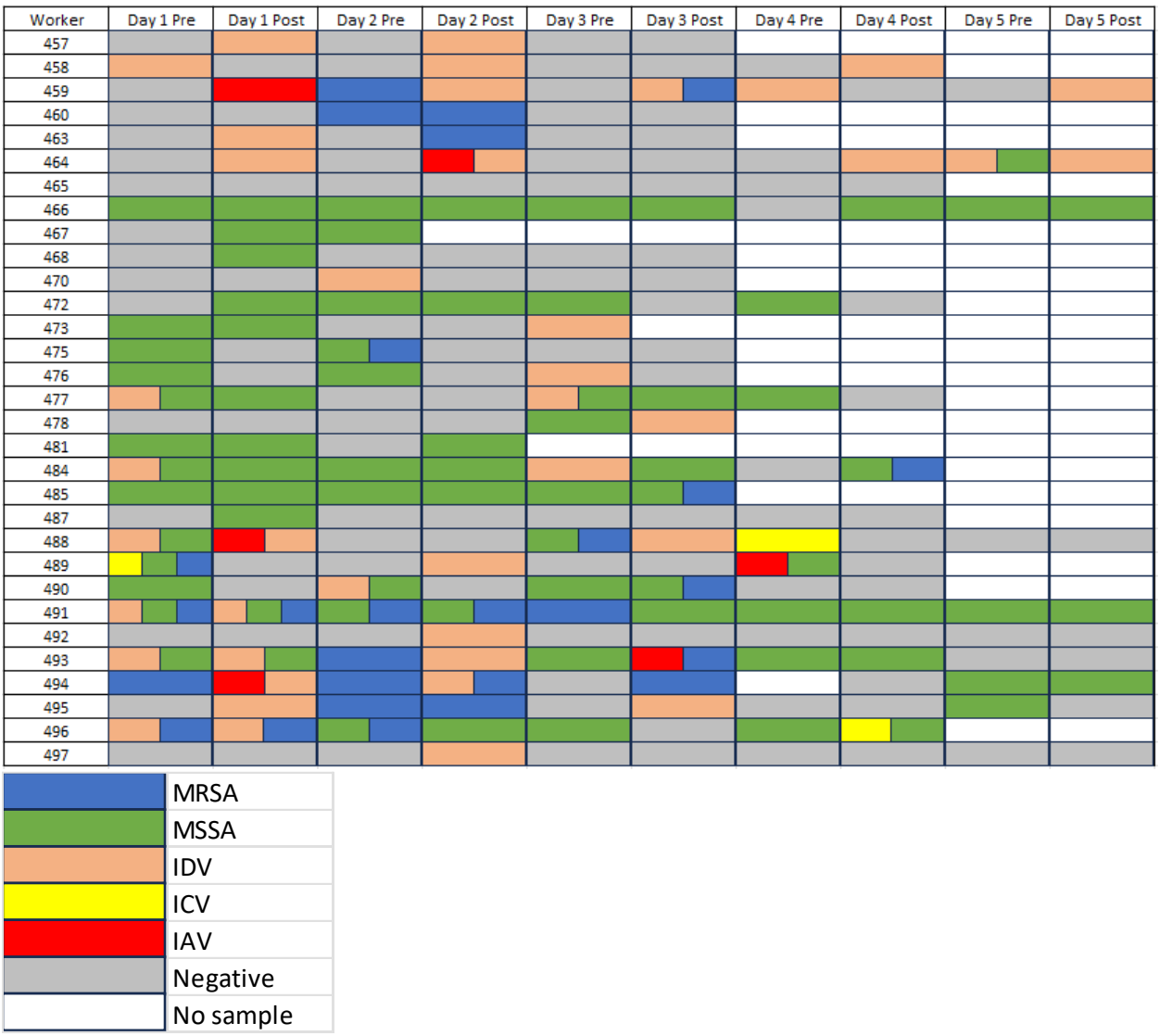

Figure 3.1: Heatmap of positive lavages for each participant. Multiple samples tested positive for 2 or 3 pathogens, which is indicated via multicolor boxes. Time points without a sample are indicated by white boxes and were due to either no participation or incomplete data.

Microbiome Results

Polymerase chain reaction (PCR) amplification of the 16S rRNA genes of our 237 nasal lavages generated a total of 2,967,539 reads with an average read of 12,154 reads per sample. Sequencing depth ranged from 1,244 to 89,085 reads. No significant differences in alpha or beta diversity metrics were observed based on the type of nasal lavage (hypertonic vs normotonic) participants received.

Based on Robust Aitchison Principle Components Analysis (PCA) generated by the DEICODE package, significant PERMANOVA pairwise differences in beta diversity were observed between participants (p=0.001), pre and post shift lavages (0.001), and lavages taken from participants working in the office (0.001). Kruskal-Wallis pairwise comparisons calculated by the 'breakaway' package revealed significant differences in alpha diversity between participants (p = 0.0003) and lavages taken before and after shifts (p = 0.007) (Figure 3.2).

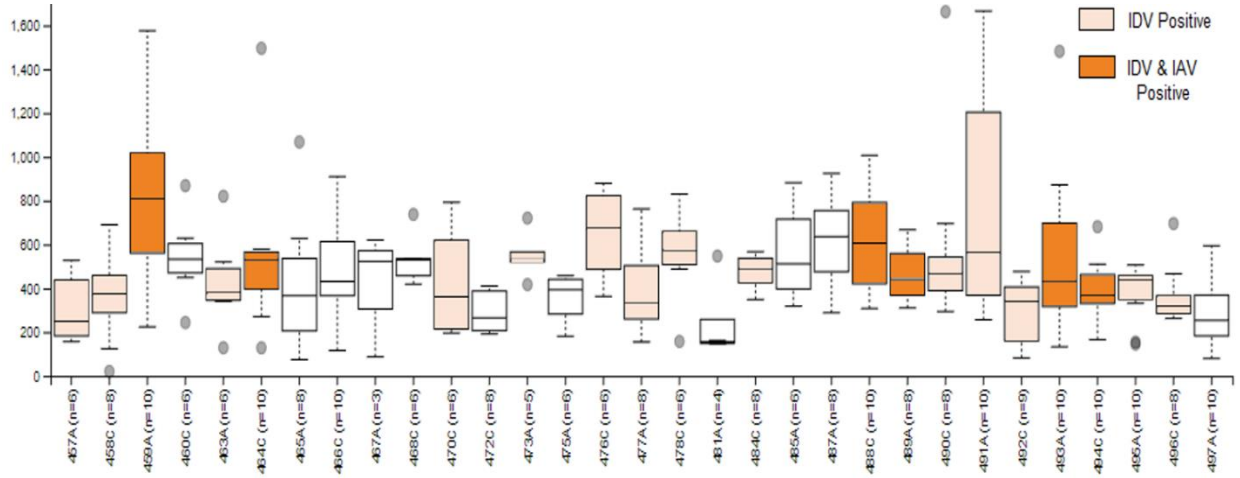

Figure 3.2: Alpha diversity comparisons of richness via the 'breakaway' package based on study participants (x axis). Colors represent participants that tested positive for a specific pathogen during the workweek (prevalence) and provide a visualization for the endemic nature of influenzas at dairy environments.

The top 5 phyla represented in the nasal lavages were *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Chloroflexi*. The top 30 genera ranked by pooled presence in the nasal lavages are summarized in Table 3.5. Significant differences in certain abundant genera were observed in lavages based on the presence of pathogens. These differences are denoted in the right column of Table 3.5, and their arrows indicate if the genus is more prevalent (upwards arrow) or less prevalent (downward arrow) based on a positive sample. For example, the genus

staphylococcus is, on average, significantly more abundant in nasal lavages that tested positive for MSSA compared to lavages that tested negative.

Table 3.5: Top 30 genera ranked by pooled presence in lavages

Genera	Significance differences in pooled presence based on pathogen positivity
Unknown	
Staphylococcus	MSSA ↑
Corynebacterium	
Streptococcus	MRSA ↑, MSSA↓
Moraxella	
Cutibacterium	MSSA ↑
Dolosigranulum	
Anaerococcus	MSSA ↑
Antarcticibacterium	
Psychrobacter	
Prevotella	MRSA ↑
Puia	
Peptoniphilus	MSSA ↑
Bacillus	IDV ↑
Turicibacter	
Sphingomonas	
Bacteroides	
Lawsonella	
Romboutsia	
UCG-005	
Enhydrobacter	
JG30-KF-CM45	
Acinetobacter	
Neisseria	
Methylobacterium-Methylorubrum	IAV ↑, ICV ↑
Atopostipes	
Porphyromonas	MRSA ↑
Clostridium_sensu_stricto_1	
Phyllobacterium	
Alloprevotella	MRSA ↑

Microbiome's role in resistance

There was a significant difference (p=0.04) in beta diversity between MSSA positive and MSSA negative lavages using the Robust Aitchison PCA (Figure 3.3). A significant difference

(p=0.04) in beta diversity between IAV positive and IAV negative lavages was also observed. No significant differences in alpha diversity were observed, although the largest differences in alpha diversity were between IAV positive and IAV negative samples. All pairwise results for alpha and beta diversity differences in positive and negative lavages are shown in Figure 3.4.

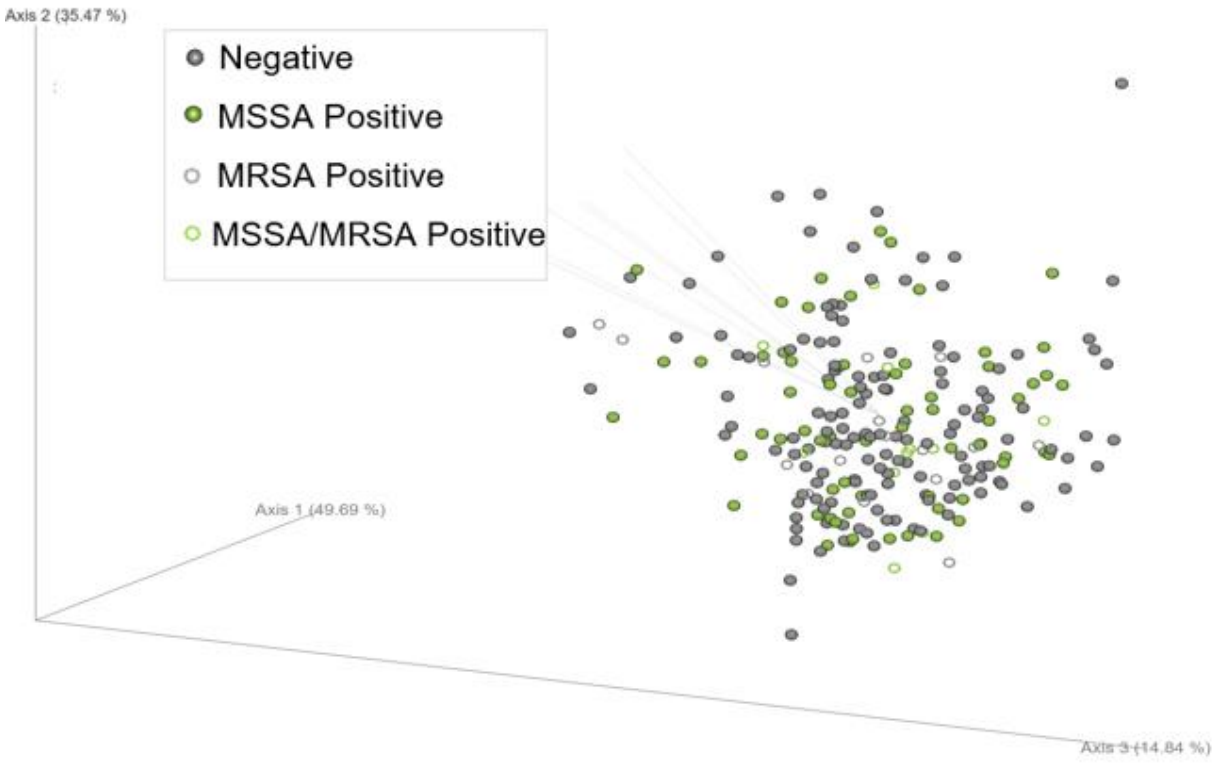

Figure 3.3: Biplot visualizing beta diversity of dairy workers' nasal microbiome using Robust Aitchison PCA

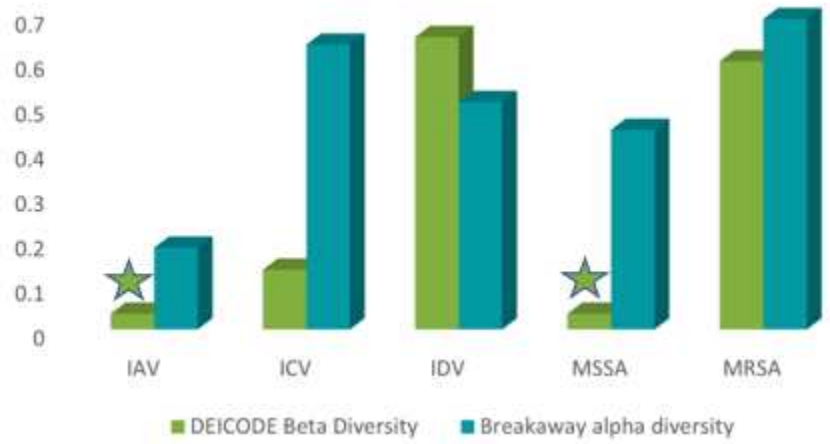

Figure 3.4: Alpha and beta diversity comparing lavages testing positive or negative for pathogens. Alpha diversity comparisons represent Kruskal-Wallis pairwise comparisons and beta diversity shows PERMANOVA results. Significant results indicated with a star.

At the genus taxonomic classification, *Streptococcus* (p=0.019), *Alloprevotella* (p=0.005), *Prevotella* (p=0.002), and *Porphyromonas* (p=.007) were significantly more abundant in nasal lavages that tested positive for MRSA than those nasal lavages that tested negative. In MSSA positive nasal lavages, *Anaerococcus* (p=0.0004), *Cutibacterium* (p=0.006), *Staphylococcus* (p=0.0001), and *Peptoniphilus* (p=0.005) were significantly more abundant, while Streptococcus (p=0.002) was significantly more abundant in MSSA negative lavages. Bacillus was significantly more abundant in nasal lavages testing positive for IDV (p=0.003). *Methylobacterium- Methylorubrum* was significantly more abundant in nasal lavages testing positive for IAV (p=0.035) and ICV (p=0.045). Differences in taxonomic abundance based on pathogen results are summarized in Table 3.5.

Discussion

Our work demonstrates that opportunistic pathogens are present in the nostrils of dairy workers. Timing of positive lavages and elevated prevalence suggests workers are likely exposed to MRSA at dairies and that their carriage of MRSA is transient in nature. These results agree

with other investigations that found swine workers were at a high risk for *S. aureus* exposures.[36–40] When compared to occupations traditionally considered "high-risk" for MRSA prevalence, such as nursing, our observed prevalence of 45.2% stands out. A recent systematic review of healthcare workers estimated prevalence to be between 1.8% and 36% depending on setting (i.e., nursing home vs emergency room) and occupation.[41] In a recent study of healthcare workers in a Vietnamese ICU, 29.1% tested positive for MRSA on their hands or in their nose.[42] Because individuals with MRSA carriage are at a higher risk for skin and bloodstream infections, increased surveillance for dairy workers is warranted to ensure their health and safety, which in turn, can help increase productivity and reduce costs associated with health-related absenteeism.

The timing of MSSA positive lavages and repeated positive lavages for many participants indicates MSSA colonization is occurring in this workforce. With an observed prevalence of 71%, workers in our study were more than twice as likely to test positive for MSSA compared to the general public.[43,44]

The presence of IDV and IAV, two viruses whose respective reservoirs are often livestock, provides further evidence for the transmission of viral pathogens between humans and animal hosts at livestock operations.[10,16,45,46] With only 3 nasal lavages testing positive for ICV, it is likely that transmission of this virus occurs infrequently at dairies. Interestingly, only 1 of the 31 participants (3.2%) never tested positive for a viral or bacterial pathogen during their workweek.

Colonization and transient carriage of *Staphylococcus aureus* has previously been associated with individual-to-individual infection incidents, which may have an important role in community spillover as workers bring home these pathogens and interact with family and community members.[47] Inversely, the well-established theory of transmission of microorganisms

between humans provides a direct route for the introduction of these pathogens to dairies, i.e. asymptomatic carriers may transmit pathogens to cows and surfaces during their work shift.[48,49] The implications of exposure to zoonotic IDV at work to community-level infection with this emerging pathogen remains unclear. Future studies should consider testing family members for IDV seroprevalence. As IDV infections are typically asymptomatic, hematologic community surveillance would provide greater insight into the transmission dynamics occurring beyond the dairy.

Our results comparing pathogen presence to the nasal microbiome builds on earlier findings from Shukla et al, elucidating a deeper understanding of the nasal microbiome characteristics that may play a role in pathogen susceptibility.[21] Significant differences in beta diversity were observed between lavages testing positive for MSSA and IAV and lavages testing negative for MSSA and IAV. No significant differences in alpha diversity were observed based on the presence or absence of targeted pathogens. One interpretation of our diversity metrics is that specific bacterial taxa in a worker's nasal microbiome may modulate pathogen exposures in the workplace. To test our hypothesis, we conducted compositional analysis and found several genera that were associated with pathogen susceptibility. Only one genus, *Streptococcus*, was positively associated with resistance for MSSA. Conversely, a positive association was found between *Streptococcus* and MRSA. A brief description of the significant genera based on pathogen presence and the current understanding of their role in the nasal microbiome follows.

Streptococcus abundance paradoxically increasing susceptibly for MRSA and providing protection against MSSA is likely due to its ubiquity in the nose and upper respiratory tract of most humans. [50–52] The genus may not play a role in exposures and differences may be attributed to individual factors and small sample sizes. *Alloprevotella* and *Prevotella*, which were

positively associated with MRSA susceptibility in our cohort, are often observed to be in dysbiosis in various disease states.[53] For example, patients with asthma have been shown to have higher *Prevotella* abundance compared to healthy controls.[54] In patients with early rheumatoid arthritis, *Prevotella* and *Alloprevotella* dominated the oral microbiome.[55] Increased abundance of these genus among our cohort is interesting, as dairy workers entering the workforce as adults are at a higher risk for allergic and inflammatory respiratory diseases. [7,56]

Porphyromonas was also positively associated with MRSA susceptibly among our participants. *Porphyromonas* is a common, but understudied genus, whose presence in the nasal microbiome is positively associated with sleep apnea.[51,57] Sleep disorders have been show to not only impact worker productivity, but also increase the risk of occupational injury twofold.[58–61] Considering the high physical demands of the work and previous findings estimating ~80% of dairy workers already suffer from musculoskeletal symptoms, future investigations may warrant collecting self-reported data on sleep.[62 64]

Anaerococcus, *Cutibacterium*, *Staphylococcus*, and *Peptoniphilus* were significantly more abundant in nasal lavages that tested positive for MSSA. The elevated presence of staphylococcus genus in samples that also cultured *Staphylococcus aureus* species is logical. While pathogenic species within the *Anaerococcus* and *Peptoniphilus* genera are known to cause infections in the bloodstream, a relative decrease in these genera in the nasal microbiome has also been observed in patients with chronic rhinosinusitis.[63–66] Possibly the most interesting result is the positive association of *Cutibacterium* with MSSA positive lavages, a finding in agreement with previous *in vitro* assessments that the most common porphyrin excreted by *Cutibacterium* species, Coproporphyrin III, promotes the growth of *Staphylococcus aureus* in the nose.[52,67]

The elevated abundance of *Bacillus* in nasal lavages testing positive for IDV is surprising, as many *Bacillus* strains have previously shown antiviral properties. In a randomized control study of children infected with respiratory syncytial virus (RSV), a nasal-spray of 5 billion *Bacillus* spores resulted in faster recovery times and alleviation in symptoms.[68] Another study found *B. subtilis* successfully inhibited IAV replication *in vitro* and protected mice against IAV infection.[69] Less is known about *Methylobacterium–Methylorubrum* and potential viral interactions, but one study found a lower relative abundance of *Methylobacterium–Methylorubrum* in the gut microbiome of HIV-infected men. [70] The differently enriched abundance of two potentially viral-resistant genera in our influenza-positive lavages points to the lack of understanding of the microbiome's role in virus susceptibly.[71]

Overall, the nasal microbiome of dairy workers in this study were compositionally similar to the US dairy workers enrolled in Shukla et al, with both groups sharing 4 of the same top 5 phylum and 4 of the same top 5 identifiable genera.[21] Many of the top genera observed in our participants were also observed in US livestock workers enrolled in the Kates et al. microbiome study, although differences in methodology do not allow for direct comparisons.[72] Islam et al conducted an investigation that grouped the nasal microbiome of Danish swine workers, short-term visitors, and pigs into separate community state types (CSTs). Again, direct comparisons to community state types are inappropriate, but the CSTs that swine workers most likely were grouped to were dominated by similar genera shown here.[38] Together, this work demonstrates livestock exposure modulates the human nasal microbiome and that larger studies with different livestock exposures are needed to identify a potential nasal microbiome phenotype associated with livestock work.

Significant differences in beta diversity and the taxonomic composition of lavages that tested positive for opportunistic pathogens was observed. The differentially enriched abundance of multiple genera in pathogen-positive lavages indicates that the presence of certain microbiota likely increases worker susceptibly for viral and bacterial pathogens common in the dairy environment. Making sense of which genera are key players and the exact mechanisms in which they act, however, is difficult. Overall, there seems to be a positive association between lavages testing positive for MSSA or MRSA and several important genera. For individuals with a predisposition to increased *Cutibacterium*, MSSA susceptibility was observed in both this study and previous work.[52,67] Nasal microbiome trends based on viral positivity were less evident. Future investigations should be aimed at understanding the characteristics of workers' nasal 'virome' and how host factors may modulate pathogenic exposures.

To our knowledge, our work is the first to study the microbiome composition of dairy workers within the context of multiple viral and bacterial pathogens present at dairies.[10 37–41] Additional investigations focused on seasonal differences and virus exposures in dairies are needed to support this theory. Diversity characteristics and taxonomic composition likely do not account for the 'full picture' in pathogenic exposures, and future studies with next generation sequencing that include accurate species level sequencing and metabolites is needed to parse the immunophysiology of exposures in dairies.

Limitations

Relatively low species abundance in the nose and the large amount of lavage fluid collected for the intervention led to a lower 16S rRNA gene sequencing depth than desired. Low sequencing depth can potentially impact downstream analysis and thus skew findings. However, given the similarities observed between the composition observed in our study and those in

Shukla et al. and others – it appears the nasal microbiomes represented in our samples are representative of dairy workers at large. Administering two lavages per day and up to ten lavages over the course of the week could also contribute to low sequencing depth, as some washout of bacterial communities is expected. Furthermore, significant differences in alpha and beta diversity were observed between pre and post shift lavages, but it is difficult to determine if differences were due to washout, exposures during work, or normal diurnal variation in the nasal microbiome. Future studies with multiple time points in the day should use nasal swabs for microbiome characterization.

The lack of milkers recruited may underestimate the burden of pathogenic exposures in dairy work. Milkers spend their entire shift near cows, and the milking parlor is one of the most bioaerosol rich environments at the dairy.[6] Interestingly, the inclusion of office workers in this data set likely did not add to this underestimation, as 13/30 (~43%) of the lavages collected from office workers contained at least one targeted pathogen. Three of these lavages contained multiple pathogens. Furthermore, office workers in this study had similar exposures for total inhalable dust and endotoxins when compared to other tasks (results not shown). These findings are not surprising as all offices in this study were situated on or near dairy operations, and many shared buildings with milking parlors. Office workers at these operations are not strictly limited to their office environment, and they often assist with other tasks or speak to workers in areas that would increase their exposures.

Ultimately, it is difficult to draw conclusions regarding MRSA colonization in this workforce from culture results. While we did perform temporal analysis from our repeated measures, it is difficult to infer if there is clonal MRSA being spread around a workgroup or zoonotic MRSA passing between animals and humans. Another unlikely possibility is that the

lavage intervention increases susceptibility to MRSA carriage, and that is why MRSA-positive lavages were more common on days 2-5.

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CHAPTER 4: EVALUATION OF THE RELATIONSHIP BETWEEN THE NASAL MICROBIOME AND LUNG FUNCTION IN DAIRY WORKERS

Summary

Objective: The nasal microbiome's role in lung function is poorly understood. Dairy and other livestock workers were recently shown to have more diverse nasal microbiomes than non-exposed individuals, but they also suffer from greater decreases in cross-shift lung function. Here, we investigated the association between dairy worker nasal microbiome characteristics and resilience to cross-shift change in lung function.

Methods: As part of a larger intervention study, dairy workers were enrolled for 2-5 days and performed pre- and post-shift spirometry for each shift (n=108 working shifts). The intervention study included the collection of pre- and post-shift nasal lavage, and post-shift nasal lavages were analyzed to quantify the bacterial communities that comprise workers' nasal microbiome. Cross-shift spirometry results were compared to nasal microbiome characteristics, and workers were also grouped into different community state types (CSTs). A linear mixed model was also created to understand the impact of a hypertonic saline nasal lavage treatment on cross-shift changes in lung function compared to a normal saline nasal lavage.

Results: Dairy workers in this study experienced decreases in cross-shift spirometry outcomes. Significant differences in microbiome characteristics did emerge based on post-shift and cross-shift spirometry performances. Taxonomic differences were also noted for participants performing poorly on cross-shift FVC. Community state typing revealed 3 different CSTs, and samples falling into CST3 showed the most resilience on both cross-shift FVC and FEV1

outcomes. Significant differences in cross-shift outcomes were not associated with receiving a hypertonic saline nasal lavage.

Conclusion: The nasal microbiome of dairy workers may impact acute changes in lung function following work exposures. Based on differences in taxonomic abundance, the use of a *Lactobacillacea* probiotic spray may warrant investigation in this cohort. Future studies should continue grouping dairy workers into CSTs via their nasal microbiome, to better understand the intrinsic differences that serve to protect some workers against adverse changes in cross-shift pulmonary function.

Introduction

Dairy workers experience extensive and prolonged exposures to bioaerosols at work.[1–5] Bioaerosols are generated in dairies by a diverse set of sources including cows, humans, birds, and soil. Together, these sources generate a polydisperse mixture of bioaerosols spanning the entire inhalable size fraction (<3-100 µm) that are replete with diverse bacterial species and inflammatory constituents such as endotoxin, gram-positive bacteria, and the livestock-associated pathogens influenza D virus and methicillin-resistant *staphylococcus aureus* CC398.[2,6–12]

As a result of these exposures, dairy workers have historically had higher incidence of upper respiratory symptoms and fixed airway disease including chronic obstructive pulmonary disease (COPD), chronic bronchitis, asthma, and asthma-like reductions in pulmonary function.[13–15] In a 2016 cross-sectional study of 1,638 French dairy and cattle workers, Guillien et al. reported a significantly higher odds of prevalence of COPD in dairy workers compared to nonfarming controls.[16] Significantly higher rates of asthma were also reported in a 2005 cross-sectional study of 1,140 New York dairy farmers, for which the association between dairy work

and asthma was independent of smoking, BMI, and age.[17] In a twelve-year longitudinal study of French dairy farmers, Gainet *et al.* observed an accelerated decline in the ratio of forced expiratory volume in 1 second to forced vital capacity (FEV1/FVC) in dairy farmers compared to nonfarming controls and an even sharper decline in FVC in dairy farmers working on traditional farms.[18]

Acute changes in lung function for dairy workers may provide valuable insight into the exposure-response relationship between bioaerosol inhalation and respiratory health. By comparing pre- and post-shift pulmonary function tests, a cross-shift change in lung function provides a direct physiological response to the inhalation of dairy dust. While non-exposed individuals typically follow a circadian rhythm where their worst pulmonary function performance occurs in the morning, exposure to bioaerosols appear to negatively modulate these diurnal variations in lung function. [1920]

Importantly, not all dairy workers respond similarly to these bioaerosol exposures. Intrinsic factors such as the timing of initial exposure across the life course (childhood vs adult) and genetics have increasingly been associated with protection against respiratory disease, upper respiratory inflammation, and in some cases, even an improvement in cross-shift reductions in FEV1 and FVC.[19–24] Missing from these preliminary assessments of intrinsic factors, however, is the role of the nasal microbiome in workers' exposure response.

Spatially, the nasal microbiome sits at the interface between exposure and worker; potentially impacting the uptake of both pathobiont and benign microorganisms and modulating the host's response to immunogenic constituents.[25–27] Physiologically, the nasal microbiome is also at the interface between health and disease, serving as both a transitory marker of exposure in workers and as an understudied marker of effect. Shukla et al. recently demonstrated that the

nasal microbiome of dairy workers is more diverse than non-bioaerosol exposed controls, and that many of the unique taxa residing in the worker's nares is directly associated with dairy operations.[28] The role of the of the nasal microbiome in lung function for bioaerosol-exposed workers; however, has not been investigated.

Here, we analyzed nasal lavages collected from a longitudinal intervention study to ascertain the role the nasal microbiome may play in cross-shift pulmonary function for dairy workers. By performing spirometry on individuals before and after their shift and comparing these results to the nasal microbiome observed in post-shift nasal lavages, we can begin to understand the microbiome characteristics that may impact cross-shift decrements in lung function. We hypothesized that a greater microbial diversity in species within participants (i.e. higher alpha diversity) would improve cross-shift pulmonary function. We also hypothesized that dairy workers nasal microbiome would be grouped into distinct community state types (CST), and that participants falling within certain state types would be more resilient to cross-shift changes in pulmonary function.

Methods

Participant recruitment and sample collection

Dairy workers were recruited from large herd dairies (>2,000 lactating cows) in the High Plains region of the United States via long-term partnerships with participating dairies. We invited workers from all roles at the dairy to participate, and participants reported performing a variety of tasks including, maintenance, animal care, reproduction, milking, and administrative work. Additional workers were recruited via snowball sampling. Exclusionary criteria for this study were developed to consider the safety of performing nasal lavages and spirometry, and for the use of medication that may impact our interpretation of the results. Any potential participant

taking immune-suppressive, anti-autoimmune, or chemotherapy medications, or having a medical history of chest injuries, stroke or heart disease, or a recent chest or sinus surgeries was not invited to participate. All participants provided written consent in English or Spanish, and the study protocols were approved by the Colorado State University Institutional Review Board.

Participants of this study were recruited for a larger intervention study occurring over five consecutive working days. As part of the intervention, half of the workers received a hypertonic saline nasal lavage with an osmotic concentration of 400 milliosmole (mOsm) and half the participants received a normotonic saline lavage with an osmotic concentration of 308 mOsm. Lavages were administered to workers before and after each shift, and the study was designed to minimize differences in exposures between groups. While the efficacy of the intervention was not a primary focus of this investigation, we were interested in examining the treatment's effect on cross-shift change of lung function. Analyses were also stratified by treatment group to ensure the treatment was not significantly impacting the workers' nasal microbiome. Any worker who was unable to participate for five consecutive days was still invited to participate. To reduce interference with the lavage and spirometry, participants were asked not to smoke or vape within 15 minutes of data collection or to perform their own lavage before their shift.

To quantify the bacterial communities that comprise the nasal microbiome of the dairy workers, nasal lavages were collected after every working shift. In a seated position, participants tilted their head back with their neck outstretched while a trained researcher administered 5 mL of fluid into each nostril over a ten second period. Participants were instructed not to breathe, swallow, or move during the 10 second period. Following the tenth second, participants were instructed to tilt their head forward and allow the lavage to drain naturally into a sterile specimen

cup held by the participant. Protease inhibitor cocktail (PIC) was added immediately to the lavage onsite in a 1% volume to volume ratio based on the amount of lavage returned from each participant, placed on ice, and transported to Colorado State University to be frozen at -80°C until analysis.

As part of the intervention study, the treatment group (n=16) received a hypertonic saline nasal lavage with an osmotic concentration of 400 milliosmole (mOsm) and the control group (n=15) received a normotonic saline lavage with an osmotic concentration of 308 mOsm. Samples were analyzed to assess any potential differences in alpha or beta diversity in the nasal microbiome based on the type of nasal lavage received. Statistical analysis was also performed to assess differences between treatment and control groups for inhalable dust exposure, endotoxin exposure, and lung function results.

Personal air sampling was conducted on participants each day to quantify inhalable organic dust and endotoxin exposures. SKC Inc. AirChek XR5000 personal pumps fitted with SKC Button Aerosol Samplers ran for the entire duration of the shift at a flowrate of 4L/min (SKC Inc., Eighty Four, PA). Calibration of each pump was performed before and after every shift using a BIOS DryCal DC-Lite primary flowmeter (Mesa Labs, Lakewood, CO) to ensure post flowrates remained within ±0.5L of pre flowrates. Personal air sampling pumps were fitted with 25 mm SKC poly vinyl chloride (PVC) filters.

To measure lung function and the cross-shift change in lung function, pulmonary function tests (PFTs) via spirometry were conducted before and after every shift using Koko Legend II Portable Office Spirometers from nSpire Health (Longmont, CO). Trained researchers administered spirometry tests following guidelines from the National Health and Nutrition Examination Survey's (NHANES) 2008 *Respiratory Health Spirometry Procedures Manual,* as

previously described. Researchers had participants perform forced air maneuvers until three reproducible maneuvers or a maximum of six maneuvers were achieved. American Thoracic Society (ATS) defines a reproducible forced air maneuver as having 5% or less variability than the prior maneuver. Only expiratory pulmonary function tests were conducted; inspiratory tests were not performed in this study. Bronchodilators were not administered to any participants for this study. Following completion of the study, all pulmonary function tests were reviewed by a pulmonologist and a follow-up letter with result interpretations were mailed to participants.

Outcome analysis

Spirometry results were downloaded from the portable spirometer and stored on REDCap. The primary spirometric measurements obtained from PFTs were forced expiratory volume in 1 second in liters (FEV1), forced vital capacity in liters (FVC) and the subsequent ratio (FEV1/FVC). Cross-shift changes in all spirometric measurements were calculated by subtracting the pre-shift result from the post-shift result, i.e., participants who performed worse in spirometry following their shift would have negative cross-shift results. For nasal microbiome comparisons, post-shift spirometry results were also presented as NHANES III predicted values. These values include corrections for participants' age, sex, height, weight, and race/ethnicity.

Exposure analysis

Following each sampling campaign, personal air samples were desiccated for 12-24 hours to reduce moisture. Gravimetric analysis for inhalable organic dust was conducted using a Mettler Toledo (Columbus, OH) MX5 scale. Weights were calculated before and after each sampling period using anti-static weighing methods. Two repetitions for each filter were collected to ensure each weight had a variability of less than 0.05 mg. Inhalable dust levels were

then calculated by taking the difference between the post-weight mean and the pre-weight mean (mg) and dividing by the total volume of air collected by the pump (m^3).

Subsequently, samples were extracted in 10 ml of 0.05% Tween20 and shaking at room temperature for one hour as previously described.[1,6] Briefly, aliquots were analyzed for endotoxins using a PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay Kit (Lonza, Hayward, CA) and a fluorescence plate reader. Dilution factors for endotoxin analysis were selected post sampling based on visual inspection of the filter and preliminary assays. Personal exposures to endotoxins were calculated by dividing endotoxin units by the volume of air collected on the sample (EU/m^3).

Nasal microbiome processing

16S rRNA gene sequencing to quantify the nasal microbiome was performed at the University of Oklahoma Health Sciences Center. Bacterial genomic DNA was isolated from nasal lavages via Zymo Quick-DNA kits, and 16S libraries were constructed with IDT DNA Technology primers and KAPA reagents. PRC amplification of a ~460bp product spanning the 16s rRNA V3 and V4 regions led to the generation of individual libraries, which were then indexed for sequencing on the Illumina MiSeq platform.

Microbiome processing and initial data analysis was performed using the qiime2-2022.2 (q2) platform [29]. The q2 DADA2 plugin was used to denoise sequences and generate a feature table, and a taxonomic classifier was trained via full-length reference sequences from SILVA and our specific primers.[30,31] Taxonomic classification of amplicon sequence variants (ASV) was performed using this custom-trained classifier, and a phylogenetic tree was generated using the q2 sepp-fragment insertion plugin and SILVA's 128 SEPP reference database.[31,32]

Statistical analysis

To assess dairy workers' exposure to inhalable dust and endotoxin, descriptive statistics were performed via geometric means, geometric standard deviations, and 95% CI for both the treatment and control groups. Shapiro-Wilk tests for both inhalable dust and endotoxin exposures confirmed neither exposure was normally distributed for both treatment groups, therefore unpaired two-sample Wilcoxon tests were performed to test for any significant differences between the treatment and control group. Arithmetic means, standard deviations, and 95% CI were used to describe post-shift PFTs and the cross-shift changes in pulmonary function.

To estimate the association between the type of nasal lavage participants received (normotonic vs hypertonic), work-shift exposures (inhalable dust and endotoxin) and their effect on post-shift spirometry and cross-shift pulmonary function, mixed linear models were generated using the lme4 package in R Studio.[33] Models were controlled for gender, age, height, and weight. Mixed linear models were chosen to account for repeated measures as each participant recorded multiple spirometry tests for this study. P values for significant differences in spirometric measurements were generated in the lmerTest package via Satterthwaite's method.[34]

For nasal microbiome comparisons, post-shift spirometric markers were also categorized into "poor" or "acceptable" results based on their NHANES III predicted values. Any spirometric marker scoring lower than 80% of predicted value was considered "poor." Cross-shift results were then categorized as having "significant decreases" if decreases of -82 ml for FEV1 and -104 mL for FVC were observed post-shift. These reference points were selected based on a literature review of agricultural and other workers experiencing significant decreases in pulmonary function post shift (Table 4.1). Cross-shift decreases of -200 mL or more were

categorized as “clinical” decreases, as these values are commonly used by physicians to denote physiological relevance in short term changes in pulmonary function.[35]

Table 4.1: Reference points for cross-shift changes in pulmonary function

Source	Cross-shift change	Working population	Statistical significance
Eastman et al. 2013[36]	-65.2 ml in FEV1, -103.1 ml in FVC	210 dairy workers	Yes, significant difference between dairy workers and 47 control workers
Fell et al. 2011[37]	-37 ml in FEV1	95 cement workers in Norway	Significant decrease in FEV1
Zele et al . 2020[38]	-123 ml in FEV1, -129ml for FVC	306 textile workers in Ethiopia	Significant decrease compared to 156 control workers
Paudyal et al. 2015[39]	-74.25 ml in FEV1, -80.86 ml in FVC	384 textile workers in Nepal	Significant decrease in cross-shift for both
Du et al. 2020[40]	-110 ml in FEV1	80 underground miners	Significantly larger increase compared to 20 surface level miners
Average significant cross-shift decrease in spirometric markers	**-82 ml for FEV1 and -104 ml for FVC**		

Spirometry results were then compared to microbiome data to quantify any potential differences in the nasal microbiome based on pulmonary function outcomes. Alpha diversity was determined using the q2 breakaway package, which estimates species richness via frequency ratios.[41] Pairwise Kruskal-Wallis comparisons based on pulmonary function results was performed to determine significant differences in alpha diversity. Analysis of beta diversity between samples was then performed using the q2 DEICODE package, which uses Robust Aitchison Distance to depict and display beta diversity via biplots.[42] Pairwise PERMANOVA

comparisons of beta diversity were also performed in DEICODE to infer significant differences in samples based on pulmonary function testing.

Taxonomy was imported into R Studio (version 2023.09.1) and absolute ranking of abundance was performed using the *ANCOM-BC2* pacakge.[43,44]The ANCOM-BC2 package conservatively estimates differential abundance while correcting for both taxon-specific biases and the sampling fraction (i.e. accounting for library size to microbial load ratios).[45] The ANCOM-BC2 package is also appropriate for repeated measurements. Briefly, mixed linear models were created with the spirometric markers of interest and sampling day set as fixed effects, and participants set as a random effect. Multiple pairwise comparisons then generated false discovery rate (FDR) p-values to infer significant differences between samples.

Finally, community state typing (CST) was performed by clustering samples into groups using Dirichlet Multinomial Mixtures (DMM) analysis via the *bluster* Bioconductor package in R.[46] DMM allows for grouping of samples that feature different sequence sizes and rare taxa, both of which are expected to occur in our samples. First, we determined the appropriate number of clusters using Laplace approximation and visualizing the results via the *miaViz* package in R (Figure 4.1). [47] Once 3 clusters were selected as the appropriate grouping, samples were clustered and visualized as an Euclidian principal coordinates analysis (PCoA) plot with different clusters represented by color and cross-shift spirometric performance represented by shape.

For all statistical analysis, a p-value of <0.05 was considered significant.

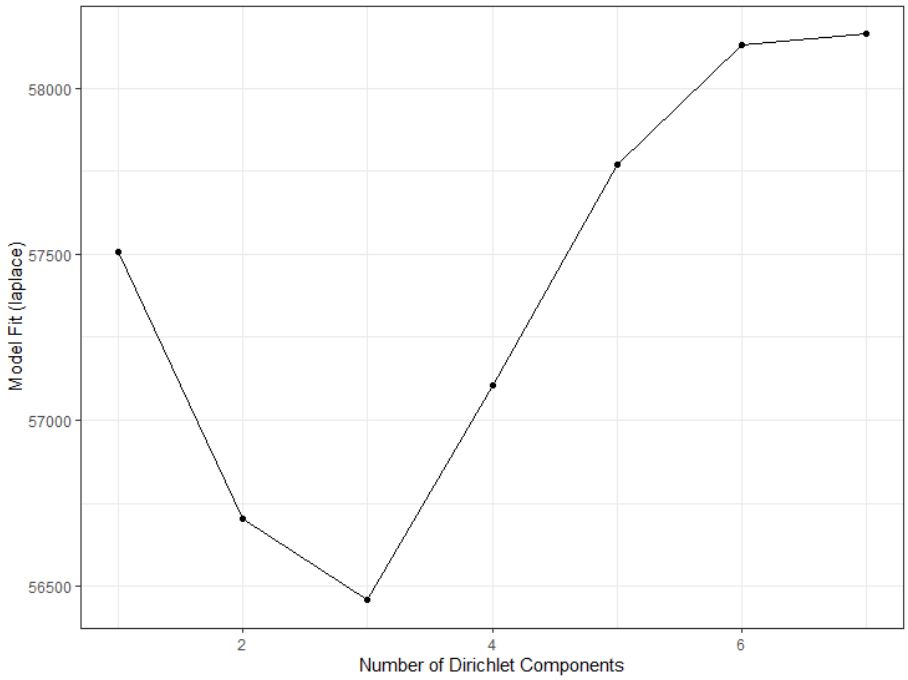

Figure 4.1: Laplace approximation for the best number of clusters in DMM analysis. The lowest value visualized via the *miaViz* package is our optimal k value. For the participants of this study, 3 CSTs were generated based on the Laplace approximation.

Results

A total of 31 participants were recruited and 108 working shifts were represented in this study. Based on participant recruitment and scheduling, 16 participants received a hypertonic saline nasal lavage (treatment) and 15 participants received a normotonic saline nasal lavage (control). All participants were enrolled for 2-5 consecutive working days, and ~48% of participants were enrolled in the study for 4 or 5 days. Worker demographics are summarized in Table 4.2.

Table 4.2: Worker Demographics

Worker variables	TOTAL (n=31)	TREATMENT (n=16)	CONTROL (n=15)
Male	77.4%	68.8%	86.7%
20-29 years old	32.3%	25%	40%
30-39 years old	48.4%	62.5%	33.3%
40-49 years old	9.7%	12.5%	6.7%
50+ years old	9.7%	0	20%
Work details			
Working at the dairy one year or longer	71%	62.5%	86.7%
Reported direct animal contact	71%	62.5%	80%
Reported milking	12.9%	12.5%	13.3%
Reported feeding or maintenance in parlor and stalls	35.5%%	25%	7 – 46.7%
Reported administrative work	9.7%	12.5%%	6.3%
Behavior			
Smoker (current or former)	12.9%	25%	0%

Personal air monitoring for inhalable dust and endotoxin was performed during the entirety of each working shift. Due to pump malfunction or calibration issues following sampling, 3 of the 108 working shifts do not have representative exposure data. The geometric mean exposure to dust during work at the dairies was 0.28 mg/m^3 and geometric mean exposure to endotoxin was 38.9 EU/m^3. Wilcoxon rank sum tests with continuity correction was performed on both exposures, and no significant differences were noted between treatment groups for inhalable dust (p-value = 0.22) or endotoxin (p = 0.92). Accordant exposure between treatment groups is important for our spirometry analyses, as acute pulmonary function has previously been shown to have an inverse dose-response relationship in livestock bioaerosol exposures.[48,49] Exposure data stratified by treatment group is presented in Table 4.3.

Table 4.3: Exposure Results (Geometric means and GSD)

Participants	Inhalable dust in mg/m^3 (95% CI)	Endotoxin in EU/m^3 (95% CI)
Treatment (n=50)	0.25 (0.18 – 0.33)	38.1 (21.7 – 66.8)
Control (n=55)	0.31 (0.24 - 0.40)	39.6 (23.4 – 67.0)
Total (n=105)	0.28 (0.23 – 0.34)	38.9 (26.6 – 56.7)

Spirometry Results

Pulmonary function tests via spirometry were performed before and after each of the 108 shifts in this study. Descriptives for post-shift results are presented in Tables 4.4 and 4.5, and descriptive statistics for the cross-shift change in pulmonary function are presented in Tables 4.6 and 7. The average post-shift FEV1 for our cohort was 3.27 liters, the average FVC was 4.09 liters, and the average FEV1/FVC ratio was 80%. The mean unadjusted cross-shift change in FEV1 and FVC was -74.4 mL and -92.5 mL, respectively. During the course of the study, 17/31 (54.8%) participants averaged decreases in cross-shift FEV1 and 24/31 (77.4%) participants averaged decreases in cross-shift FVC (Figures 4.2 & 4.3).

Table 4.4: Descriptives of post-shift results (n=108)

Spirometric parameter	Mean (95% CI)	Q1, Median, Q3	Range
FEV1 (liters)	3.27 (3.13 – 3.42)	2.72, 3.27, 3.855	1.47 – 4.78
FVC (liters)	4.09 (3.92 – 4.26)	3.38, 3.94, 4.76	2.13 – 5.89
FEV1/ FVC (%)	80 (78-81)	77, 82, 85	58 – 91

Table 4.5: Post-shift results based on treatment

Spirometric parameter	Treatment (n=52)	Control (n=56)
FEV1 mean in liters (95% CI)	3.34 (3.10 - 3.58)	3.2 (3.04 - 3.36)
FVC mean in liters (95% CI)	4.17 (3.91 - 4.43)	4.03 (3.83 - 4.23)
FEV1/ FVC % (95% CI)	80 (78-82)	80 (78-82)

Table 4.6: Descriptives of cross-shift change (n=108)

Spirometric parameter	Mean (95% CI)	Q1, Median, Q3	Range
FEV1 (ml)	-74.4 (-25.6 to -124.3)	-200, -65, 80	-1090 to 620
FVC (ml)	-92.5 (-31.2 to -154.8)	-270, -75, 70	-1070 to 1320

Table 4.7: Cross-shift change based on treatment

Spirometric parameter	Treatment (n=52)	Control (n=56)
FEV1 mean (95% ci)	-65.3 ml (-145.1 to 14.5)	-82.6 ml (-19.1 to -145.5)
FVC mean (95% ci)	-116.3 ml (-216.2 to -16.4)	-71.2 ml (-148.5 to 2.7)

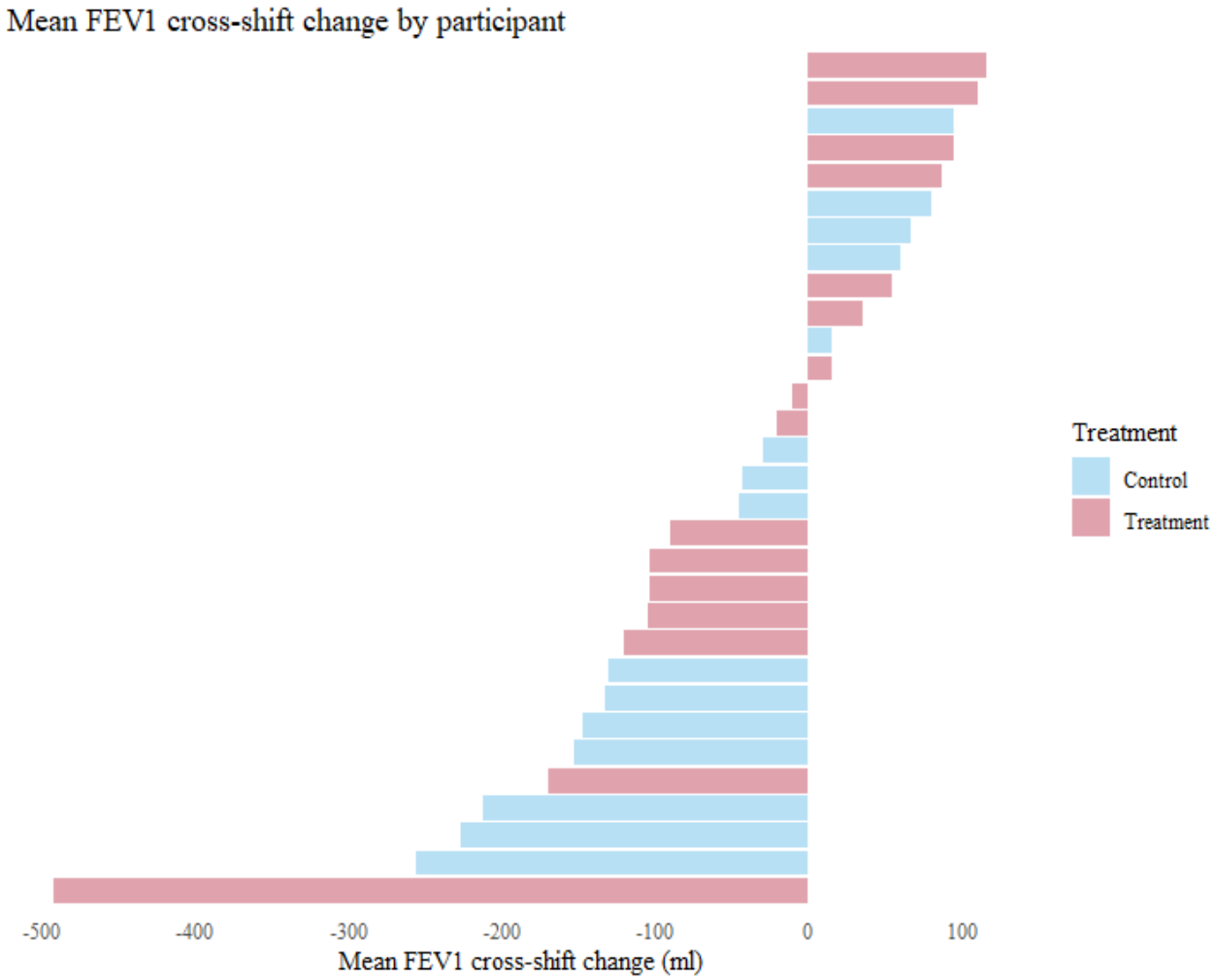

Figure 4.2: Unadjusted mean cross-shift change in forced expiratory volume in one second (FEV1) by participant. Participants performed spirometry pre- and post-shift for 2-5 working days, and their results were averaged for analysis of key spirometric markers. The colors indicate if participants received a hypertonic saline nasal lavage (treatment) or a normotonic saline nasal lavage (control). As expected, 19/31 (61.3%) of participants experienced cross-shift decreases in FEV1.

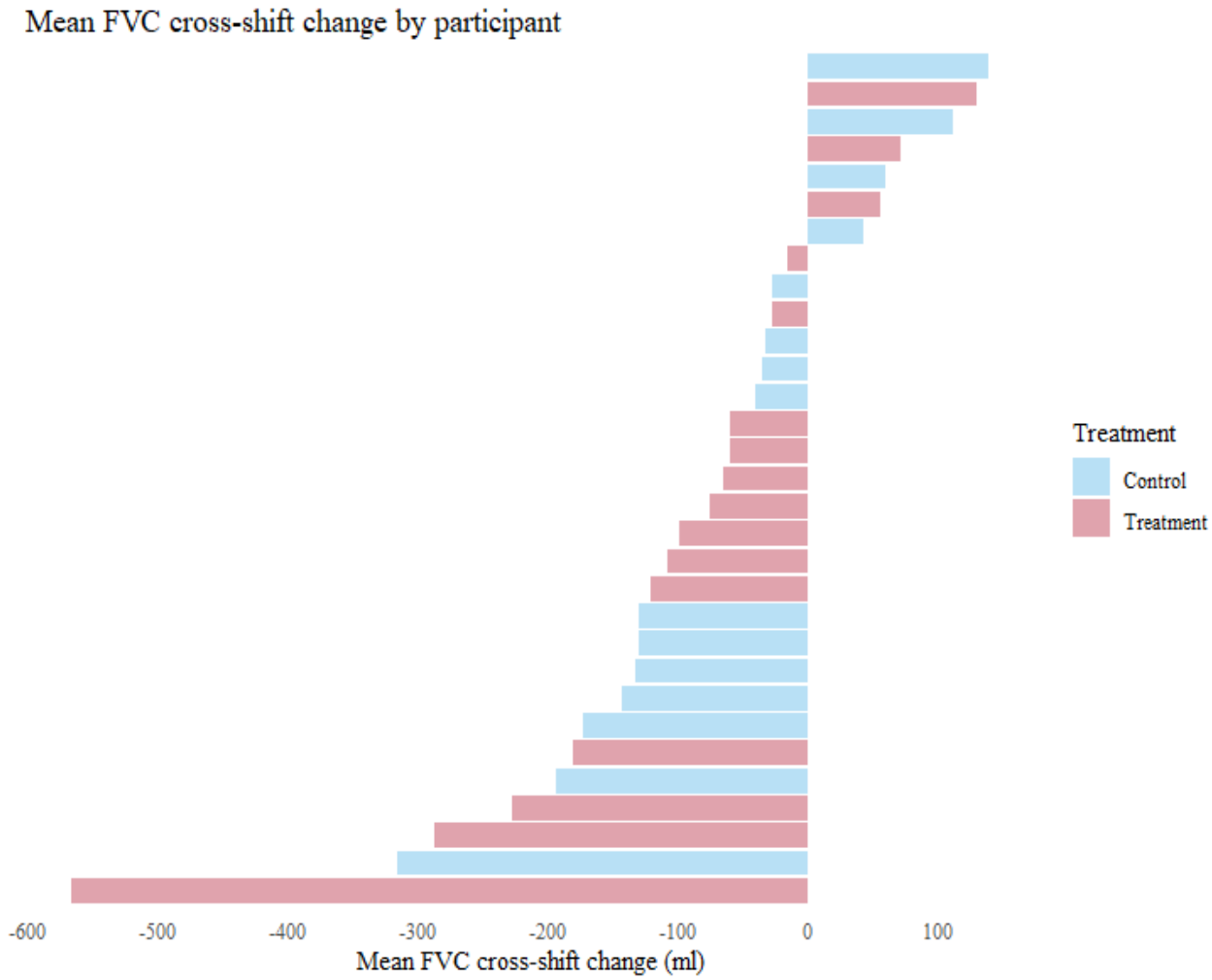

Figure 4.3: Unadjusted mean cross-shift change forced vital capacity (FVC) by participant. Participants performed spirometry pre- and post-shift for 2-5 working days, and their results were averaged for analysis of key spirometric markers. The colors indicate if participants received a hypertonic saline nasal lavage (treatment) or a normotonic saline nasal lavage (control). Interestingly, a larger percentage of participants experienced a decrease in FVC (77.4%) compared to a decrease in FEV1 (61.3%).

Overall, participants in the treatment group had a larger cross-shift decrease in pulmonary function compared to participants in the control group (Tables 4.8 & 4.9). That is, receiving a hypertonic saline nasal lavage did not appear to improve cross-shift lung function compared to receiving a normotonic nasal lavage. As inhalable dust exposures increased, spirometric measurements decreased across all outcomes. An inverse association between endotoxin

exposure and FEV1 measurements was also observed, with a significant association between endotoxin exposure and cross-shift decreases in FEV1 being noted (p value = 0.041).

Table 4.8: Associations (95% CI) of post-shift lung function between treatment and control groups based on total inhalable dust and endotoxin exposures*+

Marker	Treatment	P value	Inhalable dust (mg/m^3)	P value	Endotoxin (EU/m^3)	P value
Post-shift FVC (liters)	0.011 (-0.442 to 0.464)	0.962	-0.228 (-0.652 to 0.197)	0.297	0.0001 (-0.0028 to 0.0030)	0.944
Post-shift FEV1 (liters)	0.069 (-0.345 to 0.483)	0.745	-0.162 (-0.460 to 0.134)	0.288	-0.00007 (-0.0206 to -0.0019)	0.949

*Coefficients reflect change based on treatment group, 1 mg/m^3 changes in inhalable dust, and 1 EU/m^3 changes in endotoxin.
+Pulmonary function tests were adjusted for height, weight, gender, and age

Table 4.9: Associations (95% CI) of cross-shift change in lung function between treatment and control groups based on total inhalable dust and endotoxin exposures*+

Marker	Treatment	P value	Inhalable dust (mg/m^3)	P value	Endotoxin (EU/m^3)	P value
Cross-shift change in FVC (ml)	-286.8 (-575.1 to 1.6)	0.056	-314.6 (-747.2 to 117.9)	0.158	0.136 (-5.68 to 0.748)	0.136
Cross-shift change in FEV1 (ml)	-177.2 (-402.0 to 47.5)	0.1271	-224.1 (-558.0 to 109.8)	0.192	-2.61 (-5.08 to -0.148)	**0.041**

*Coefficients reflect change based on treatment group, 1 mg/m^3 changes in inhalable dust, and 1 EU/m^3 changes in endotoxin.
+Pulmonary function tests were adjusted for height, weight, gender, and age

Microbiome results

Polymerase chain reaction (PCR) amplification of the 16s rRNA V3 and V4 regions of our 108 nasal lavages generated a total of 744,646 reads with an average of 6,895 reads per sample. We observed no significant differences in species richness within samples based on the type of lavage received, participant sex, if the participant worked in the office, or sampling date. Beta diversity metrics via Robust Aitchison Principal Components Analysis (PCA) estimated no

significant differences between samples based on participant sex, office work, or day of the study. Pairwise PERMANOVA comparisons did reveal a statistically significant higher beta diversity for participants receiving a hypertonic saline nasal lavage, compared to participants that received a normotonic saline lavage (p-value 0.048).

The nasal microbiome of dairy workers in this study contained a total of 20 unique phyla, with the top 5 abundant phyla being *Euryarchaeota, Firmicutes, Proteobacteria, Cyanobacteria*, and *Actinobacteriota*. At the family level, 173 unique family were identified, and the top 30 are presented in Table 4.10.

Table 4.10: Top 30 family ranked by absolute abundance in our nasal lavages

Rank	Family
1	*Methanobacteriaceae*
2	*Lactobacillaceae*
3	*Moraxellaceae*
4	*Rhodocyclaceae*
5	*Comamonadaceae*
6	*Burkholderiaceae*
7	*Oxalobacteraceae*
8	*Neisseriaceae*
9	*Planococcaceae*
10	*Alcaligenaceae*
11	*Carnobacteriaceae*
12	*Idiomarinaceae*
13	*Saccharospirillaceae*
14	*Cellvibrionaceae*
15	*Hahellaceae*
16	*Streptococcaceae*
17	*Enterobacteriaceae*
18	*Succinivibrionaceae*
19	*Peptostreptococcales-Tissierellales*
20	*Aerococcaceae*
21	*Gastranaerophilales*
22	*Acholeplasmataceae*
23	*Actinomycetaceae*
24	*Izemoplasmatales*
25	*Nitrosococcaceae*
26	*Arcobacteraceae*
27	*Campylobacteraceae*
28	*Sulfurovaceae*
29	*Sphingomonadaceae*
30	*Fodinicurvataceae*

The nasal microbiome and pulmonary function

We observed differences in the nasal microbiome based on the associated pulmonary function results collected at the same time. In pulmonary function tests where participants

performed poorly on the post-shift FEV1/FVC ratio (<80% predicted) or in cross-shift FVC (a decline of -104 mL or greater), pairwise PERMANOVA tests via Robust Aitchison PCA estimated significant differences in nasal microbiome beta diversity (p values of 0.020 and 0.046, respectively). Poor post-shift FEV1/FVC ratio was also associated with a significant difference in alpha diversity based on pairwise Kruskal Wallis comparisons. Paradoxically, for all significant differences in alpha diversity associated with lung function results, the poor performing lung function tests had higher diversities.

At the family taxonomic classification, a significant decrease in *Cellvibrionaceae* abundance in the nasal microbiome was observed in lung function tests performing poorly in post-shift FEV1 (<80% predicted; p value 0.02). A significant decrease in *Lactobacillaceae* was also associated with a poor cross-shift FVC results (p-value 0.01).

Community State Typing

A total of 3 CSTs were identified across the nasal microbiome of dairy workers participating in this study. Characterization of the abundance of family for each CST is in Figure 4.4. For all CSTs, *Moraxellaceae* was the most abundant family. Following *Moraxellaceae,* CST 1 was characterized by an even distribution between *Peptostreptococcales-Tissierellales, Prevotellaceae, Streptococcaceae,* and *Corynebacteriacea.* In CST 2, Sphingomonadaceae, *Corynebacteriaceae, Carnobacteriaceae,* and *Intrasporangiaceae* were the most abundant family after *Moraxellaceae*. CST 3 was dominated by *Moraxellaceae and Peptostreptococcales-Tissierellales,* with *Chitinophagaceae, Propionibacteriaceae,* and *Corynebacteriacea* following.

When compared to the associated cross-shift outcomes of FVC and FEV1, differences in bacterial community composition were observed (Figures 4.5 & 4.6). Pulmonary function tests paired with samples found in CST 3 performed better than CST 1 and CST 2 for both cross-shift

declines in FEV1 and FVC. For FEV1, 17/22 (77.3%) of the cross-shift PFTs in CST 3 were normal, compared to 27/54 (50%) for CST 1 and 16/32 (50%) for CST 2. When looking at FVC, 14/22 (63.6%) of the cross-shift PFTs in CST 3 were normal, compared to 30/54 (55.6%) for CST 1 and 15/32 (46.9%) for CST 2. The nasal lavages in CST 2 had the highest corresponding proportion of clinical differences in cross-shift FEV1 and FVC with 10/32 (31.3%) cross-shift FEV1 declines of -200 mL or more and 12/32 (37.5%) FVC declines of -200 mL or more occurring in this group.

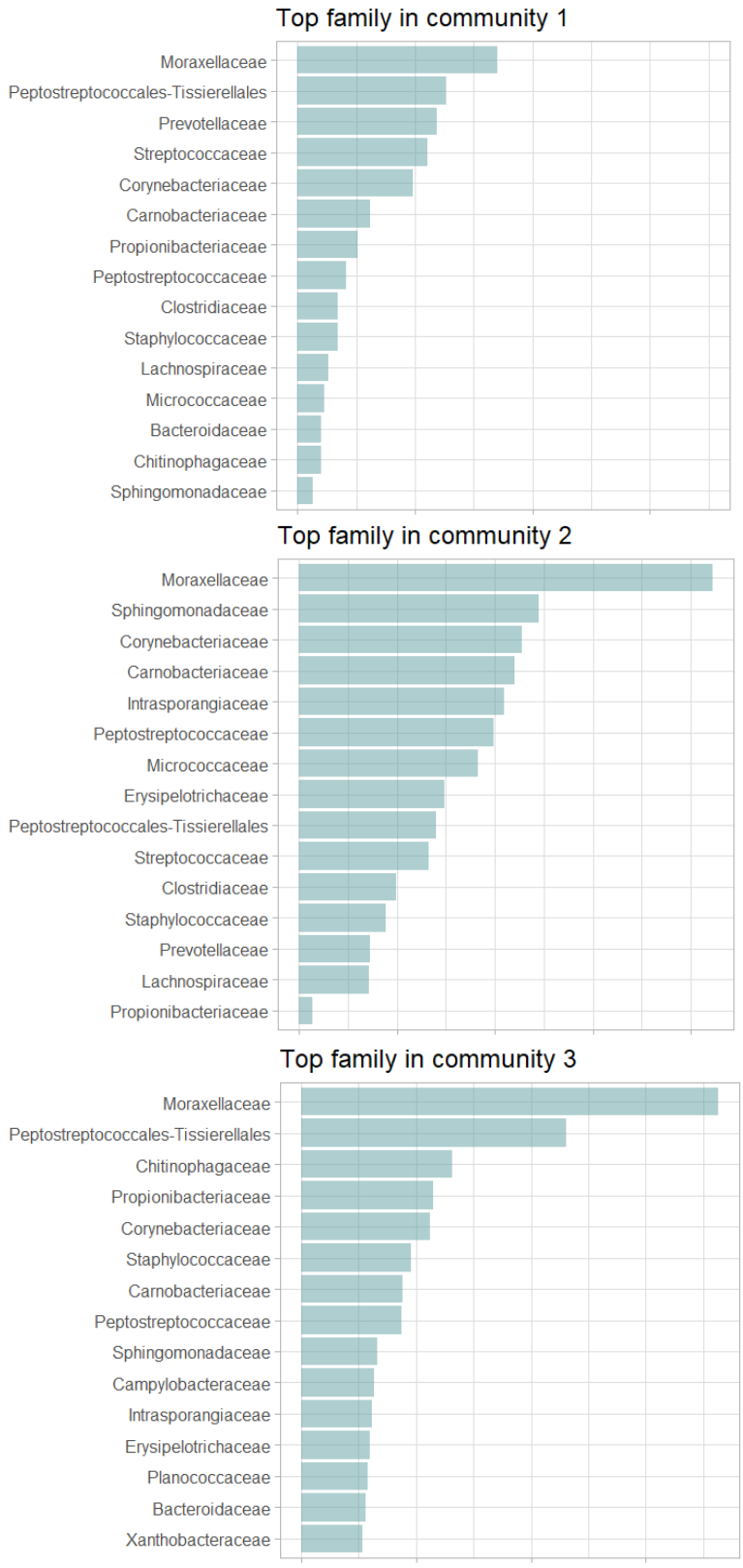

Figure 4.4: Abundance of microbiota taxa at the family level for each community state based on Dirichlet Multinomial Mixtures (DMM) analysis.

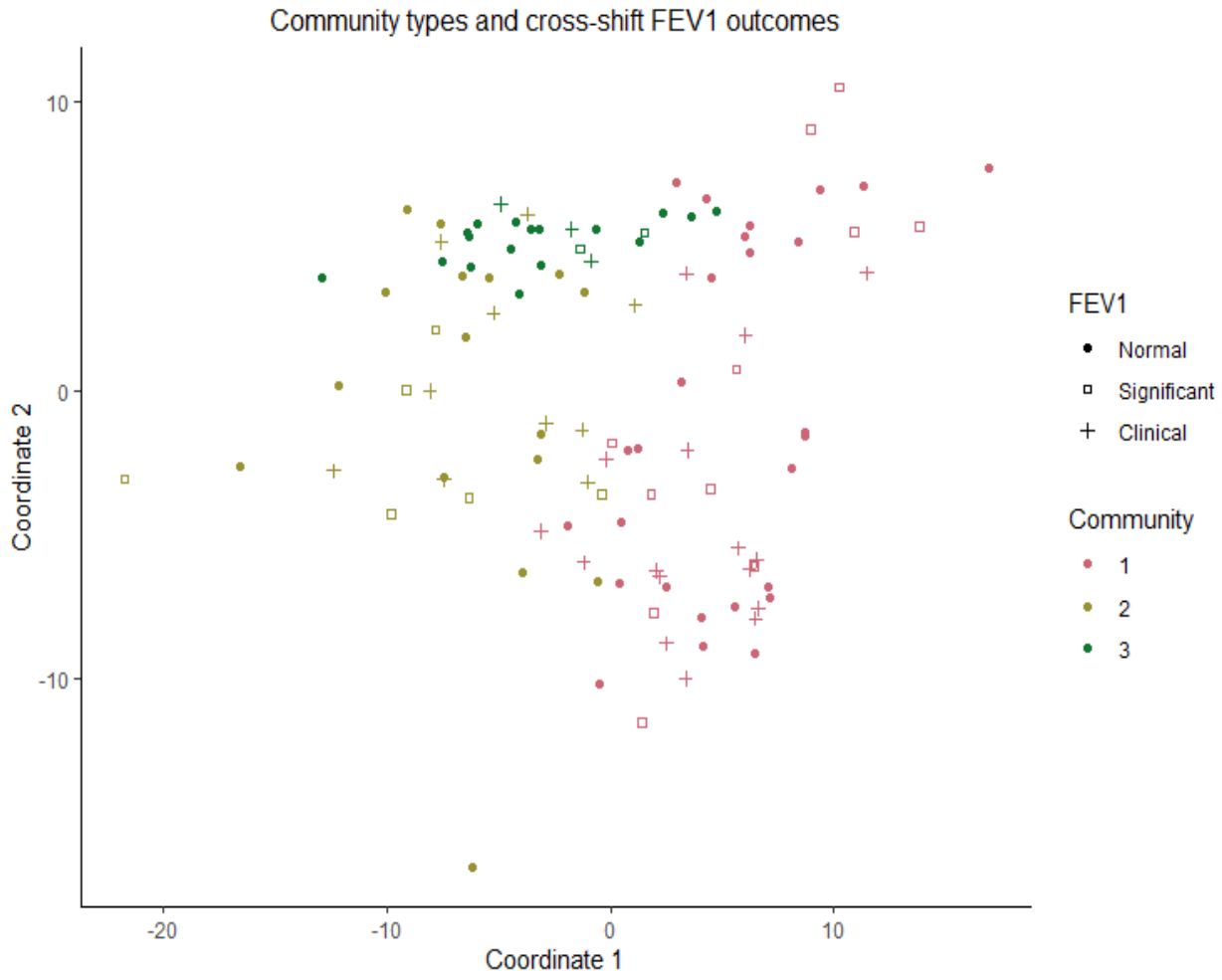

Figure 4.5: Euclidean PCoA plot visualizing the bacterial community composition in participants based on cross-shift FEV1 outcomes. Community state types are represented by different colors and cross-shift results are represented by shape. A cross-shift decline of > -82 mL for FEV1 was categorized as normal, -82 to -200 was categorized as significant, and any decline greater than -200 mL was categorized as clinical.

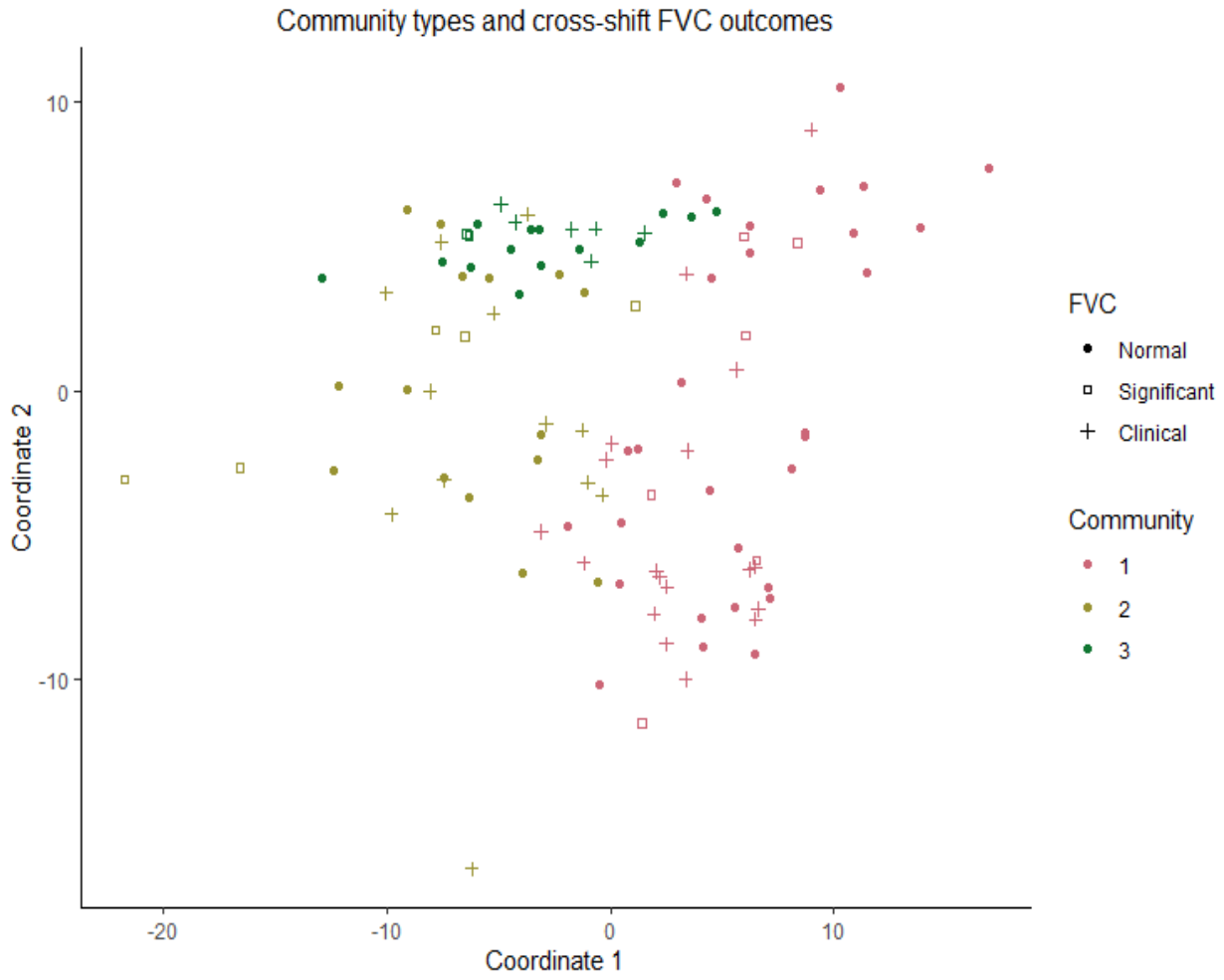

Figure 4.6: Euclidean PCoA plot visualizing the bacterial community composition in participants based on cross-shift FVC outcomes. Community state types are represented by different colors and cross-shift results are represented by shape. A cross-shift decline of > -104 mL for FEV1 was categorized as normal, -104 to -200 was categorized as significant, and any decline greater than -200 mL was categorized as clinical.

Discussion

Our cross-shift pulmonary function findings support the emerging theory that exposure to dairy bioaerosols can cause acute asthma-like reductions in pulmonary function. [24,36,50,51] Out of 108 total cross-shift pulmonary function tests represented in this study, 60 (55.6%) had post-shift declines of -82 mL or more for FEV1 and 59 (54.6%) had post-shift declines of-104 mL or more for FVC. Following a typical pattern of normal diurnal variation, where the worst performances

occur in the morning and improve throughout the day, a slight increase in both markers would be expected.[52–54] While these results only capture a few working days for a subset of dairy workers, previous research from Eastman et al. showed similar mean decreases of -65.2 mL and -103.1 mL in California dairy workers post shift for FEV1 and FVC, respectively.[36] Furthermore, these acute shifts in lung function may be predicative of more serious long-term changes. In their longitudinal studies of grain handlers, Tabona et al. noted that short-term decreases in FEV1, FVC, and FEF25-75% over the workweek were statistically correlated with long-term changes in these parameters after a six-year follow-up.[55] Based on previous longitudinal studies of French dairy workers that found associations between long-term dairy farming and lung disorders, participants experiencing these short-term changes may be at risk for more serious long-term changes in lung function. [18,56]

With geometric means of 0.28 mg/m^3 for inhalable dust and 38.9 EU/m^3 for endotoxin, and no significant differences in exposures between treatment groups, worker exposure to bioaerosols during our study supports other recent dairy exposure assessments. Generally, exposure to inhalable dust in modern dairy environments appears to be trending lower (typically < 1.0 mg/m^3), but inflammatory constituents such as endotoxin still pose respiratory challenges to susceptible individuals. [2,4,5,50,51,57]

Our overall goal was to determine if any microbiome characteristics contributed to improved post-shift pulmonary function or a reduction in the cross-shift decreases commonly observed in dairy workers. As this was part of a larger intervention study, we wanted to test if the hypertonic saline nasal lavage significantly modulated spirometry outcomes. To this effect, a linear mixed model that accounted for changes in treatment group (hypertonic vs normotonic lavage), exposure to inhalable dust, and exposure to endotoxin was created. For all post-shift and

cross-shift outcomes analyzed, the only significant effect of these three factors was a negative association between endotoxin exposure and cross-shift FEV1. This outcome is expected, as other exposure assessments have found associations between endotoxin exposure and acute lung obstruction in workers.[24,58–60] The treatment's effect on the nasal microbiome also appeared limited, as only a significant change in beta diversity was observed between the treatment and control groups. No significant differences in alpha diversity or taxonomy composition were noted between the two groups.

For our assessment, spirometry results were also categorized as significantly decreased or normal diurnal variation based on previous cross-shift assessments in the literature. Differences in microbiome characteristics were analyzed based on these groupings, and samples were clustered together based on microbiome similarity to see if patterns existed based on cross-shift results and post-shift PFT outcomes. Slight differences based on microbial diversity metrics and taxonomic abundance were noted for several spirometric outcomes. Furthermore, sample grouping via community state typing suggests certain microbial phenotypes may provide protection against cross-shift decrements of pulmonary function.

When comparing the nasal microbiome to our paired pulmonary function tests, significant increases in alpha and beta diversity were observed for spirometry tests with post-shift FEV1/FVC markers below 80% of their predicted values. Cross-shift FVCs that declined by -104 mL or more were also associated with a significant increase in alpha diversity. Furthermore, taxonomic analysis revealed differentially abundant taxa based on spirometric outcomes: a significant decrease in *Cellvibrionaceae* was associated with pulmonary function tests that had poor post-shift FEV1 (<80% predicted) and a significant decrease in *Lactobacillaceae* was associated with a decrease in cross-shift FVC of -104 ml or more.

Previous livestock-worker assessments that presented nasal microbiome community state typing showed temporal changes in the microbiome based on timing of exposures.[61,62] Here, we demonstrated that community state typing can also be useful in identifying microbial phenotypes that may contribute to host resilience during bioaerosol exposure. For both cross-shift spirometric outcomes tested, spirometry tests paired with nasal microbiomes in CST 3 outperformed the other two CSTs. CST 3 was dominated by the abundance *Moraxellaceae,* and *Peptostreptococcales-Tissierellales,* and was the only CST to have *Chitinophagaceae* and *Propionibacteriaceae* in its top 5 families (Figure 4.4).

Ultimately, our hypothesis that a higher microbial diversity would improve cross-shift pulmonary function was rejected. As increases in alpha diversity metrics were only associated with decreases in spirometric outcomes, a higher total number of species in the nasal microbiome may not afford protection against cross-shift changes in pulmonary function following bioaerosol exposure. Overall community composition may play a role, but the only significant difference in Robust Aitchison Distances between communities occurred in samples performing poorly in post-shift FEV1/FVC.

Literature on the upper respiratory microbiome's role in lung function is limited – and non-existent for occupational cohorts. A few identified studies have compared spirometry results to the microbiome with cohorts in various disease states, and one study focused on aging and the microbiome found that microbial diversity and composition did not have a significant effect on lung function in healthy populations.[63–65] Similar findings in patients with COPD were discussed by Segal et al., as they found pulmonary function was not a reliable predictor for lung microbiome phenotypes.[27] Research examining the nasal microbiome of individuals diagnosed with the inflammatory disease chronic rhinosinusitis, however, did establish a relationship

between decreased upper respiratory tract microbiota diversity and increased upper respiratory tract inflammation.[66,67] Future studies investigating cause and effect relationships between the nasal microbiome and pulmonary function should include a larger cohort and livestock workers from other animal confinement operations.

A lower relative abundance of *Lactobacillaceae* in the nasal microbiome of participants performing poorly in cross-shift FVC may have implications for future interventions targeting livestock workers. Many genera of *Lactobacillaceae* are normal residents of the nasal microbiome, and a decrease of these taxa has previously been in found in patients with chronic rhinosinusitis.[68] In one study quantifying the lower respiratory tract microbiome of smokers, a dysbiosis in *Lactobacillaceae* was associated with IL-6 and C reactive protein inflammation.[69] A decrease in *Lactobacillaceae* in the lung microbiome of asthmatic rats has also been observed.[70] Probiotic sprays containing *Lactobacillus* species has been proposed as a potential treatment for inflammatory upper respiratory diseases where airway dysbiosis is identified as a contributing factor. While these sprays are still in early development and their efficacy in an occupational setting is unknown, they may provide a low-risk solution for workers who are susceptible to cross-shift changes in pulmonary function.

The exact pathology of fixed airway disease in livestock workers is still unknown. Because bioaerosol exposure affects individuals differently, intrinsic factors such as genetics, allergy, and timing of first exposures have been proposed as potential modulators.[20–24,71] To our knowledge, this is the first time the composition of the nasal microbiome has been examined for its role in bioaerosol inhalation exposure-response. Our findings suggest the nasal microbiome has a small effect on cross-shift pulmonary function outcomes, which may have serious implications for livestock workers' long-term pulmonary health.

Limitations

Compared to previous livestock worker nasal microbiome studies, our sequencing depth was relatively low.[28,61] Low sequencing depth was likely a result of the nasal lavage intervention design, which consisted of two 10 mL lavages per participant day. Our previous work did identify significant microbiome differences between pre- and post-shift lavages, but washout, occupational exposures, and diurnal variations in the microbiome may contribute equally to these differences. To account for relatively low sequencing depth, all nasal microbiome analyses including alpha diversity, beta diversity, differential abundance, and community state typing were performed with packages designed for high levels of sparsity. Furthermore, taxonomic composition was presented at the family level. Future studies should continue using nasal swabs for microbiome characterization.

Spirometry is an effort-dependent measurement and therefore relies on full participant cooperation for meaningful results. Even with real-time feedback from modern portable spirometers, technicians may be limited on the amount of influence their feedback provides to participants.[72] Physical fatigue may also negatively impact post-shift pulmonary function tests, as workers finishing long and strenuous shifts may not be motivated to provide maximum effort during the test. To preserve the largest number of datapoints, some spirometry efforts that did not meet the ATS reproducibility standards were included in this study. Tests where the interpreting pulmonologist suspected effort issues were removed. Interestingly, patients with lung disorders such as chronic bronchitis have been shown to have less reproducible lung function tests.[73] While official diagnoses of chronic bronchitis were not conducted as part of this study, it is a disease that has a higher prevalence among dairy workers.[56,60,74,75]

The use of NHANES III predicted values is controversial as differences in pulmonary function based on ethnicity/race have limited physiological support.[76,77] Their inclusion in this study was not to provide diagnostic support or makes claims about this population's overall lung health. Instead, the reference values provided a categorical variable whereupon microbial differences based on lung function could be evaluated, i.e., post-shift spirometric markers scoring under 80% of predicted values could be summarized as "poor" and spirometric markers scoring greater than 80% could be summarized as "normal." Using this value as a baseline, we were able to understand differences in nasal microbiome characteristics based on pre-established metrics.

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CHAPTER 5: EXPLORING THE EFFICACY OF A NASAL LAVAGE ON DAIRY WORKERS' CROSS-SHIFT CHANGE IN PULMONARY FUNCTION

Summary

Objective: Dairy workers have repeatedly been shown to suffer from cross-shift reductions in pulmonary function. Our research group has developed a low-cost hypertonic saline nasal lavage that is designed to be used by dairy workers before and after their shifts. Based on the lavage's anti-inflammatory effects, the lavage may also reduce the burden of cross-shift changes in pulmonary function.

Methods: A total of 44 dairy workers from the High Plains Region of the United States were recruited for 2-5 consecutive working days (n=154). Half of the participants received a hypertonic saline nasal lavage pre- and post-shift (treatment group), and half of the participants received a normotonic saline nasal lavage pre- and post-shift (control group). Pre- and post-shift spirometry was also performed on participants, and the treatment's effect on cross-shift pulmonary function was assessed via mixed linear models.

Results: Participants receiving the hypertonic saline nasal lavage performed better on cross-shift forced expiratory flow at 25-75% of the vital capacity ($FEF_{25-75\%}$). There were no major differences between treatment groups in forced expiratory volume in one second (FEV1) and forced vital capacity (FVC). Compared to previous dairy worker investigations that reported unadjusted cross-shift pulmonary function values, the workers in our study performed better on both cross-shift FEV1 and cross-shift FVC.

Conclusion: The use of a hypertonic saline nasal lavage pre- and post-shift reduced the burden of cross-shift $FEF_{25-75\%}$ declines in dairy workers. Based on comparisons to previous

dairy workers investigations, the use of a lavage with any salinity may improve cross-shift reductions in pulmonary function. Our findings support the theory that regular saline has anti-inflammatory therapeutic effects. Saline lavages may improve respiratory outcomes in other bioaerosol-exposed working populations.

Introduction

Dairy workers are continuously exposed to airborne dust originating from biological sources (bioaerosols) during normal working tasks.[1–5] Dairy bioaerosols are a complicated mixture of particles generated by sources ubiquitous at dairy farms, including cows, humans, birds, feed, and soil.[6] These bioaerosols are comprised of immunological constituents (endotoxin, β-glucans, peptidoglycans), pathogenic bacteria (Methicillin-resistant Staphylococcus aureus, Listeria monocytogenes), and zoonotic viruses (Influenza A, Influenza D, and coronaviruses), and inhaling them for extended periods has been linked to increased respiratory inflammation.[1,7–14] While the exact pathology between post-exposure inflammation and health outcomes is not completely understood, working at dairy farms has long been associated with respiratory disease, decreased lung function, and upper respiratory symptoms.[9,15–21] Recent review papers from both US and European-led research groups have outlined over 40 years of academic research linking dairy work to respiratory disease.[22–24]

Unfortunately, hazard recognition has far outpaced solutions for dairy workers. Unique challenges burdening the workforce include a 24-hour production cycle, greater task specialization, extended working shifts with odd hours, and contact with large, unpredictable animals.[24–27] The increasing reliance on immigrant workers across dairies in developing countries also presents social challenges that likely further complicates health outcomes.[28–30] Immigrant workers are often immunologically naïve, as they rarely enter the workforce with

prior livestock experience and are unlikely to have grown up on livestock farms.[19,27,29] The changing structural organization of dairies has also increased the physical demand of the work; as dairies continue to agglomerate and increase in average herd size, the ratio of workers to cows has steadily decreased.[27] Earlier proposed interventions, including increased parlor washing and cumbersome PPE, have failed to address these challenges in a way that meaningfully improves respiratory health outcomes.[31,32] To achieve widespread implementation, the dairy industry needs a low-cost, quickly implementable solution that does not restrict worker movement.

A hypertonic saline (HTS) nasal lavage offers a low-cost and minimally invasive procedure that can be self-administered quickly before and after each shift. HTS's anti-inflammatory properties have previously been recognized in medicine, where its use as resuscitation fluid helps stem systemic respiratory inflammation following physical trauma.[33] Multiple *in vitro* studies have also demonstrated HTS's ability to attenuate respiratory inflammation in both endothelial and epithelial cells, the latter of which is a focal point for the cascade of cytokine signaling that occurs post-bioaerosol exposure.[23,34,35] In an occupational setting, use of HTS is largely unprecedented. Previously, our research group pilot tested the HTS nasal lavage on a small sample of dairy workers over the course of a 5-day workweek. Workers receiving the HTS saw an increase in anti-inflammatory cytokine production compared to a similarly exposed control group receiving a normotonic saline lavage.[14] The study yielded mixed results; however, as statistical increases in two pro-inflammatory cytokines in the treatment group were also observed. Based on the intervention's ability to modulate upper airway inflammatory responses, we suspected it may also have a therapeutic effect on cross-shift pulmonary function in this cohort. The association between upper respiratory inflammation and

acute changes in pulmonary function are not well understood, but there appears to be a negative association in bioaerosol-exposed occupational groups.

The mixed results of the pilot study and the promise of a feasible low cost and low burden intervention warrant further investigation into the HTS's capacity to reduce inflammatory signaling. Given the anti-inflammatory prospects in the upper airways, we sought to evaluate the potential for the HTS lavage to improve the cross-shift decreases in lung function often experienced by dairy workers. Occupational bioaerosol exposure appears to disrupt the normal diurnal variation of lung function.[20,24,36,37] While healthy and non-exposed individuals are expected to perform worse on pulmonary function tests (PFTs) in the morning and subsequently improve throughout the day, many dairy workers perform better on pre-shift morning PFTs compared to post-shift PFTs in the evening.[7,20]

Cross-shift pulmonary function is a unique measurement, in that it serves as both a biomarker and a health outcome. For example, previous research in grain workers found that acute changes in pulmonary function were predictive of permanent long-term decreases in lung function beyond what is anticipated from normal aging.[38] Similar observations have been made in both swine workers and cotton textile workers.[39,40] An intervention that reduces cross-shift change in pulmonary function may find applicability outside of the dairy industry too. Documented occupational cohorts experiencing cross-shift changes in pulmonary function over the last twenty years include workers in other animal confinement operations, underground mines, textile factories, cement production, and fire services.[36,37,41–45]

Because upper respiratory inflammation is often indicative of lower respiratory inflammation, we hypothesized that workers receiving the HTS lavage would perform better on cross-shift forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and the

forced expiratory flow at 25-75% of the vital capacity (FEF25-75%). Traditionally, occupational exposure to dust is associated with bronchial hyperreactivity and therefore the HTS lavage's ability to attenuate negative FEV1 outcomes was of particular interest.

Methods

Participant recruitment

The goal of the intervention study was to recruit dairy workers for five consecutive working days. Dairy workers that could only participate 2-4 days during the sampling week were still invited to participate. Workers were recruited from large herd dairies (500-2000+ cows) in the High Plains region of the United States. Dairies were selected based on size, location, and their previous participation in High Plains Intermountain Center for Agricultural Health and Safety (HICAHS) led studies. Potential participants were recruited during on-site visits by members of the research group, and through snowball sampling for workers unable to attend on-site recruitment events. All dairy workers over the age of 18 who spoke English or Spanish were invited to participate, regardless of their role on the dairy farm. Exclusionary criteria were designed to protect against adverse reactions to both the nasal lavage and spirometry testing. Additionally, potential participants using anti-inflammatory medication, steroidal or non-steroidal nasal sprays, or drugs commonly used to treat auto-immune disease were excluded from this study. Before beginning the intervention study, all participants consented in English or Spanish. All study protocols and materials were approved by Colorado State University's Institutional Review Board (IRB).

Nasal lavage intervention

Based on their order of enrollment, participants were randomly placed into the treatment or control group. Both groups were administered a 10 mL nasal lavage before and after each shift

they were enrolled in the study. Participants were instructed to tilt their head back in a seated position while a trained researcher administered 5 mL of the lavage fluid into each nostril. After a 10 second period had passed, participants drained the lavage fluid into a sterile specimen cup.

For both pre- and post-shift lavages, the treatment group received a hypertonic saline (HTS) lavage with an osmotic concentration of 400 milliosmole (mOsm). The control group received a lavage with an osmotic concentration of 308 mOsm before and after their shift. All other methods described hereinafter were the same for both treatment and control groups.

Exposure assessment

To assess exposures to inhalable dust, personal samples were collected in the breathing zone of participants each day. Samples were collected using SKC Inhalable Button Samplers fitted with 25 mm SKC poly vinyl chloride filters (PVC) filters (SKC Inc., Eighty Four, PA). Button samplers were connected to SKC Airchek XR5000 personal pumps running at a flowrate of 4 L/min. Pump calibration was performed before and after every shift using a BIOS DryCal DC-Lite primary flowmeter (Mesa Labs, Lakewood, CO) to ensure post flowrates remained within ±5% of pre flowrates.

Inhalable dust was quantified gravimetrically using a Mettler Toledo MX5 Scale (Mettler Toledo, Columbus, OH). Desiccated PVC filters were weighed in the gravimetric laboratory in the Department of Environmental and Radiological Health Sciences at Colorado State University before being placed in the Button samplers. Following sampling, the filters were again desiccated for 24-48 hours before being weighed. Both pre- and post-shift filter weights were performed in duplicate, and weights were considered reproducible if they were within 10 µg of each other. The average of the pre-weight was then subtracted by the average of the post-weight and divided by volume of air collected to calculate personal dust exposures in mg/m^3.

Filters were then extracted by placing them in 10 mL of 0.05% Tween20 solution and shaking at 25°C for one hour. Following extraction, the samples were analyzed for endotoxin via a PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay Kit (Lonza, Hayward, CA). Briefly, extracted samples, blanks, and calibration standards were pipetted onto a sterile 96-well plate. Two to three samples on each plate were also spiked with 10 µL of endotoxin standard as a positive control to assess matrix effects and other interferences. After incubating the 96-well plate for 10 minutes at 37°C, the assay kit's working reagent was added to each well. The plate was then placed into a BioTek Syngergy HTX Multimode fluorescence plate reader and results were displayed using BioTek Gen5 software (BioTek, Winooski, Vermont). Endotoxin exposures were then divided by the volume of air and results presented in endotoxin units per meter cubed (EU/m^3)

Lung function measurement

Pulmonary function tests (PFTs) were conducted before and after every shift using KoKo Legend II Portable Office Spirometers (nSpire Health, Longmont, CO). A trained researcher administered spirometry to participants in accordance with the National Health and Nutrition Examination Survey (NHANES) *Spirometry Procedures Manual.*[46] Participants were instructed to perform forced air maneuvers until three reproducible maneuvers or a total of six maneuvers were achieved. Reproducible maneuvers were based on American Thoracic Society guidelines.[47] Bronchodilators were not administered to participants and inspiratory flows were not collected. All PFTs were then reviewed by a pulmonologist to verify validity and a letter with the pulmonologist's interpretation was mailed to participants. Spirometry results that were questioned by the pulmonologist were not included in the final analysis. Additionally, cross-shift changes in FEV1 or FVC that were greater than 1.0 L in either direction were not included, as

either the pre- or post-shift test that day was likely impacted by poor participant effort. Any participant days that did not include a pre- or post-shift lavage or PFT were also excluded from final analysis.

All spirometry results were stored on REDCap for analysis. The spirometric variables of interest were forced expiratory volume in 1 second in liters (FEV1) and forced vital capacity in liters (FVC). To assess the impact of the nasal lavage on acute changes in small airway lung function, the forced expiratory flow at 25-75% of the vital capacity ($FEF_{25-75\%}$) was also examined. Cross-shift changes in all spirometric markers were calculated by subtracting pre-shift values from post-shift values, therefore any participants performing worse on post-shift spirometry would have negative cross-shift markers.

Statistical analysis

Geometric means (GM) and geometric standard deviations (GSD) were calculated for both personal dust and endotoxin exposures. For data visualization, 95% confidence intervals of the GMs were also calculated using the ci.gm function in the survJamda package in R Studio (Posit, Boston, MA).[48] To explore the relationship between treatment groups and exposure, a mixed linear model was created using the lme4 package and p values were generated in the lmerTest package using Satterthwaite's method.[49,50] In the mixed linear model, the treatment group was set as a fixed effect. Participants and days (1-5) were included as random effects to account for repeat measures. Confidence intervals (95%) were also created via the Wald method from the generated model.

Arithmetic means and standard deviations (SDs) were calculated for worker demographics, FEV1, FVC, and $FEF_{25-75\%}$. Cross-shift spirometric markers were not compared to their predicted percent values, as most current reference values are either outdated, suffer from

small sample size, or inappropriately include race as a modifier.[51,52] Instead, the success of the treatment on cross-shift pulmonary function was determined by creating mixed linear models for all spirometric markers. Models were again generated in the lme4 package and P values were calculated using Satterthwaite's method. Participation in the treatment or control group was set as a fixed variable. To account for the demographic variables that impact pulmonary function, age, gender, height, weight, and smoking (current vs former/never) were also included as random variables. During analysis exposures to inhalable dust and endotoxin were found to be consistent between groups, therefore neither were included in the model. Participants and days (1-5) were included as random variables to account for repeated measures.

All analyses were conducted in R (R version 4.3.2) and p-values <0.05 were considered statistically significant. All figures were made in R Studio using the ggplot2 and ggthemes packages.[53,54]

Results

Between 2019 and 2022, 44 participants enrolled in the study from 5 dairies in the High Plains Region of the US. Participants were enrolled for 2-5 working shifts over a 5-day workweek, and a total of 154 working shifts had both pre- and post-shift pulmonary function tests that met the inclusion criteria for this study. Half of the participants (n=22) were in the treatment group and received an HTS nasal lavage pre- and post-shift, and half of the participants (n=22) received pre- and post-shift normotonic saline nasal lavages as a control. Pump malfunction and laboratory error impacted some personal exposure samples; 151 of these working shifts have representative dust results and 149 of the shifts have representative endotoxin results. Participant demographics are summarized in Tables 5.1 and 5.2.

Table 5.1: Quantitative participant demographics from the 44 dairy workers enrolled in this study

Variable	Total (n=44) Mean (sd), Range	Treatment (n=22) Mean (sd), Range	Control (n=22) Mean (sd), Range
Age	32.7 (7.9), 23-55	31.8 (5.4), 24-43	32.5 (9.3), 23-55
Height (in.)	66.8 (3.4), 60-75	67.2 (3.1), 61-71	66.3 (3.5), 60-73
Weight (lb)	175.6 (34.3), 110-250	173.3 (32.9), 110-250	179.4 (35.1), 119-242

Table 5.2: Dichotomous participant demographics

Variable	Total (n=44)	Treatment (n=22)	Control (n=22)
Sex			
Female	10 (22.7%)	7 (31.8%)	3 (13.6%)
Male	34 (77.3%)	15 (68.1%)	19 (86.4%)
Smoker*			
Current	6 (13.6%)	5 (22.7%)	1 (4.5%)
Former/Never	38 (86.3%)	17 (77.3%)	21 (95.4%)

*Smoker was defined as answering yes to the question: "Do you currently use any tobacco products (including e-cigarettes) on a daily basis?"

The geometric mean (GM) concentration and geometric standard deviation (GSD) for all personal inhalable dust (n=151) exposures was 0.41 mg/m^3 and 3.02, respectively. For personal endotoxin exposures, the GM concentration and GSD was 58.3 EU/m^3 and 7.15. The treatment group experienced a GM concentration of 0.45 mg/m^3 (GSD=3.55) for inhalable dust exposures and 62.4 EU/m^3 (GSD = 8.27) for endotoxin exposure. The control group experienced a GM concentration of 0.37 mg/m^3 (GSD = 2.57). for dust and 55.0 EU/m^3 (GSD = 6.32) for endotoxin. Geometric mean exposures stratified by treatment group and control are presented in Figures 5.1 and 5.2.

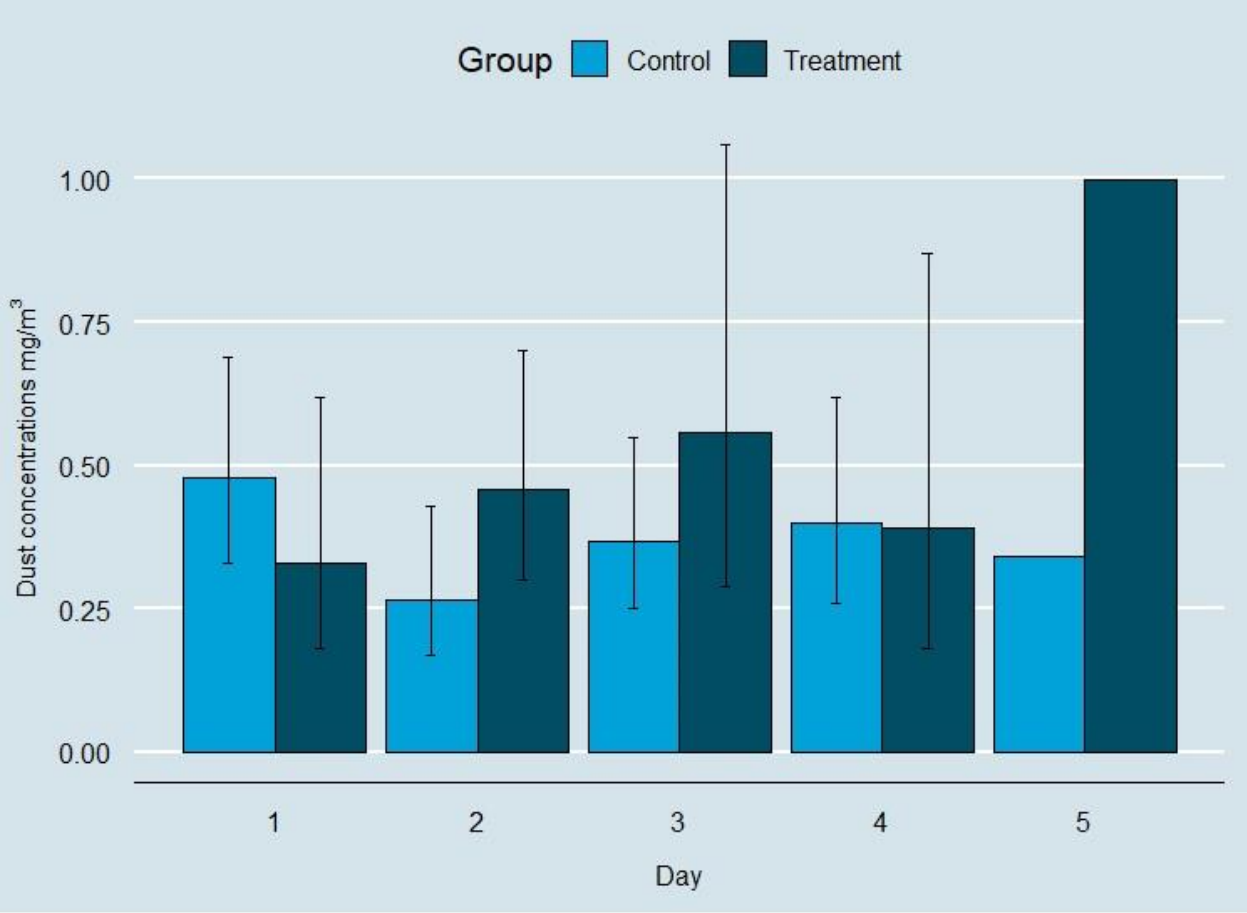

Figure 5.1: GM inhalable dust concentrations in mg/m^3 stratified by day and treatment group. The bars represent 95% CI based on the GM values using the ci.gm function in the survJamda package. The CIs for Day 5 were removed for scaling purposes: Day 5 Control (0.15-0.78); Day 5 Treatment (0.26-3.77)

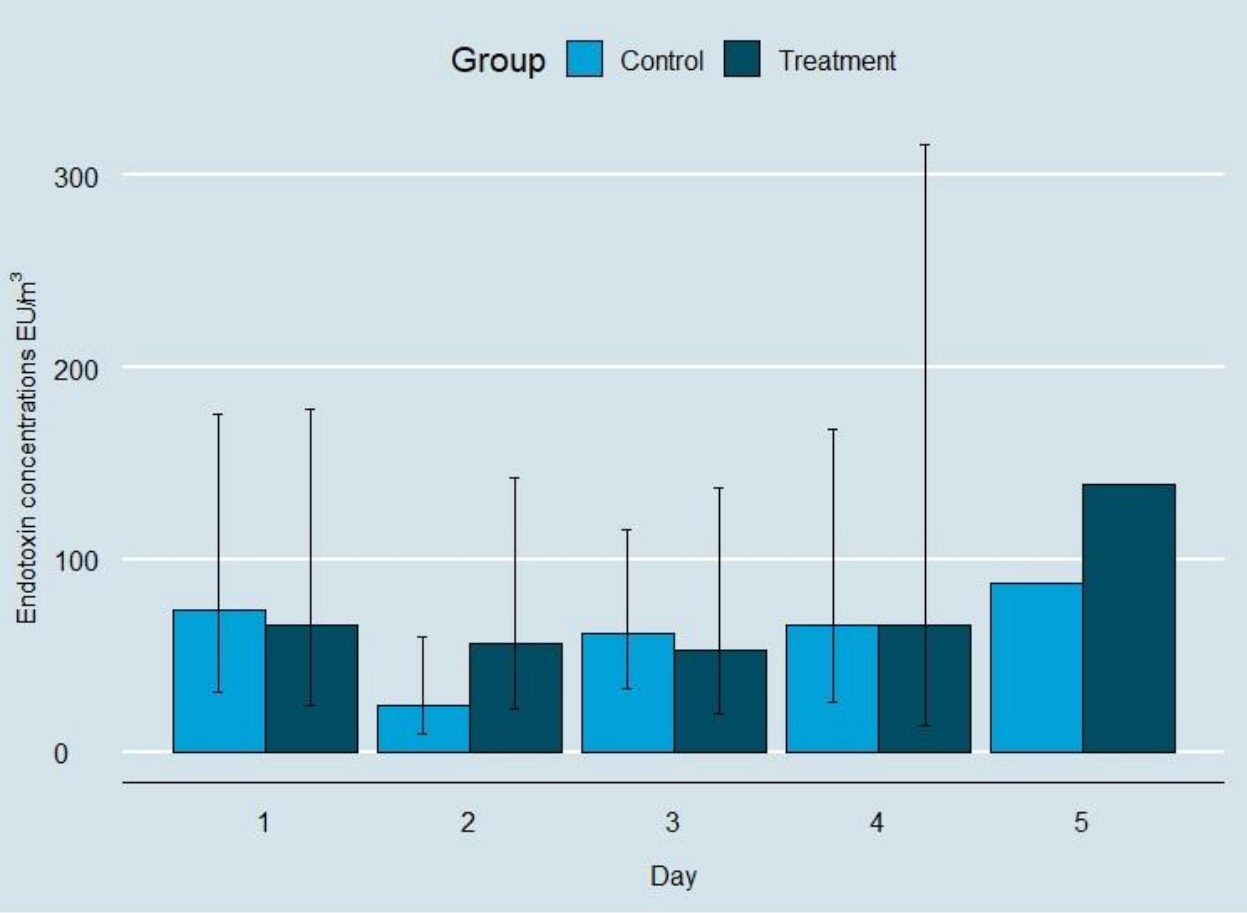

Figure 5.2: GM endotoxin concentrations in EU/m^3 stratified by day and treatment group. The bars represent 95% CI based on the GM values using the ci.gm function in the survJamda package. The CIs for Day 5 were removed for scaling purposes: Day 5 Control (28.3-273.1); Day 5 Treatment (31.4-623.72)

The mixed linear models for exposures found no significant differences between treatment and control groups. For inhalable dust, the regression coefficient (β) showed a small increase of 0.50 mg/m^3 from the control group to the treatment group. (p = .187, 95% CI[-.23 to 1.21]). When comparing endotoxin exposures, the regression coefficient (β) was 80.62 EU/m^3 between control and treatment groups (p = 0.310, 95% CI[-72.9 to 234.13]).

Out of 44 participants, 16 (36%) averaged a cross-shift increase in FEV1 during their enrollment. Nine of the 16 participants with positives FEV1s were from the treatment group, and 7 were in the control group. Only 10 (23%) participants experienced an average cross-shift

increase in FVC, 4 of whom were treatment participants. Half of the participants (22/44) averaged an increase in cross-shift $FEF_{25-75\%}$, and 13 of these participants were in the treatment group. Spirometric results from the 154 individual shifts showed 59 (38%) shifts had a cross-shift increase in FEV1, with 28 of those shifts representing treatment participants and 31 representing control participants. Independent of FEV1 results, 56 (36%) shifts had increases in FVC. Positive cross-shift FVC results were primarily experienced by control participants; 35 control shifts showed increases in FVC compared to only 21 treatment shifts. A total of 73 (47.5%) shifts had increases in cross-shift $FEF_{25-75\%}$, and 37 of these shifts were from participants in the treatment group.

On average, the 44 dairy workers in our study experienced decreases in cross-shift FEV1 (M =- 47.8 mL, SD = 228.8 mL), FVC (M = -86.4 mL, SD = 249.0 mL), and $FEF_{25-75\%}$ (M = -30.8 mL, SD=637). Daily FEV1 and FVC cross-shift results for each participant are displayed in Figures 3 & 4. When comparing participants based on treatment, the treatment group performed better on cross-shift FEV1 (M = -30.28 mL, SD = 228.6 mL) compared to the control group (M = -62.7 mL, SD = 229.2 mL). Conversely, the control group performed better on cross-shift FVC (M = -62.0, SD=267.1) compared to the treatment group (M = -114.9 mL, SD = 267.1 mL). The treatment group on average improved on cross-shift $FEF_{25-75\%}$ during their shifts (M = 81.3 mL/s, SD = 618.2), while the control group showed declines (M = -126.6 mL/s, SD = 640.9). Mean cross-shift changes stratified by treatment group and spirometric markers are shown in Figures 5.5 & 5.6.

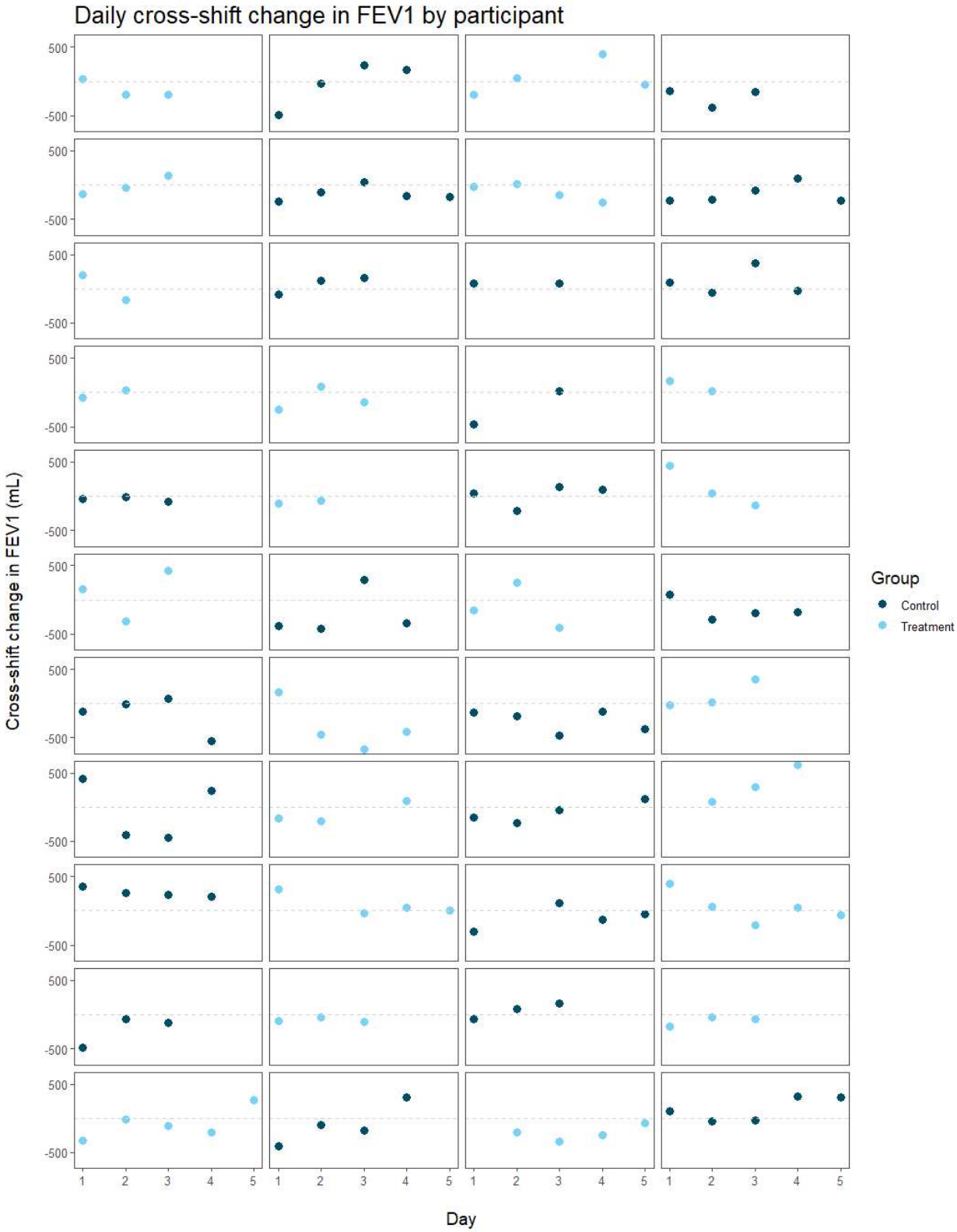

Figure 5.3: Daily cross-shift change in FEV1 (mL) by participant. Each multiple represents a single participant's cross-shift change in FEV1 during their enrollment in study. Treatment and control groups are differentiated by color.

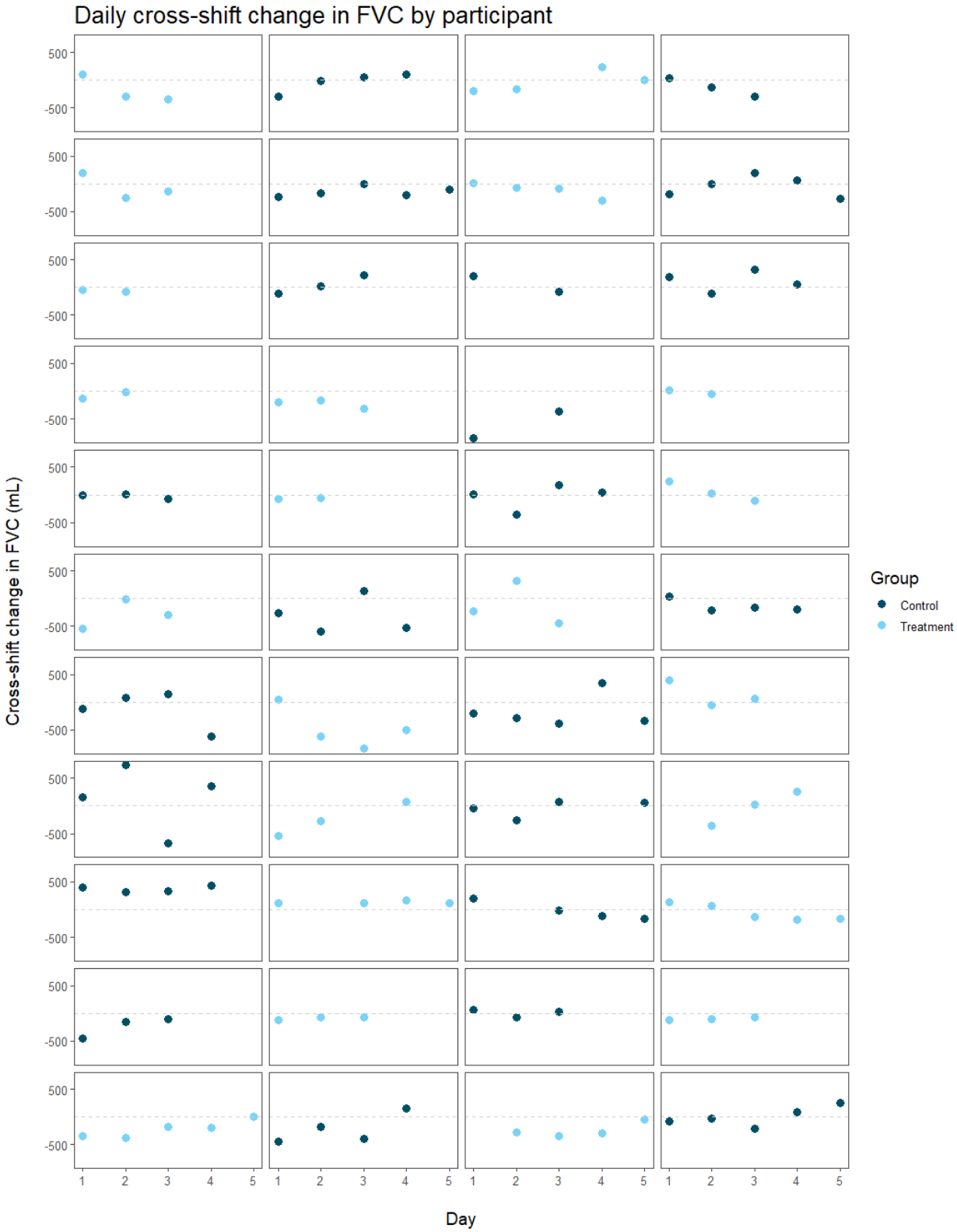

Figure 5.4: Daily cross-shift change in FVC (mL) by participant. Each multiple represents a single participant's cross-shift change in FVC during their enrollment in study. Treatment and control groups are differentiated by color.

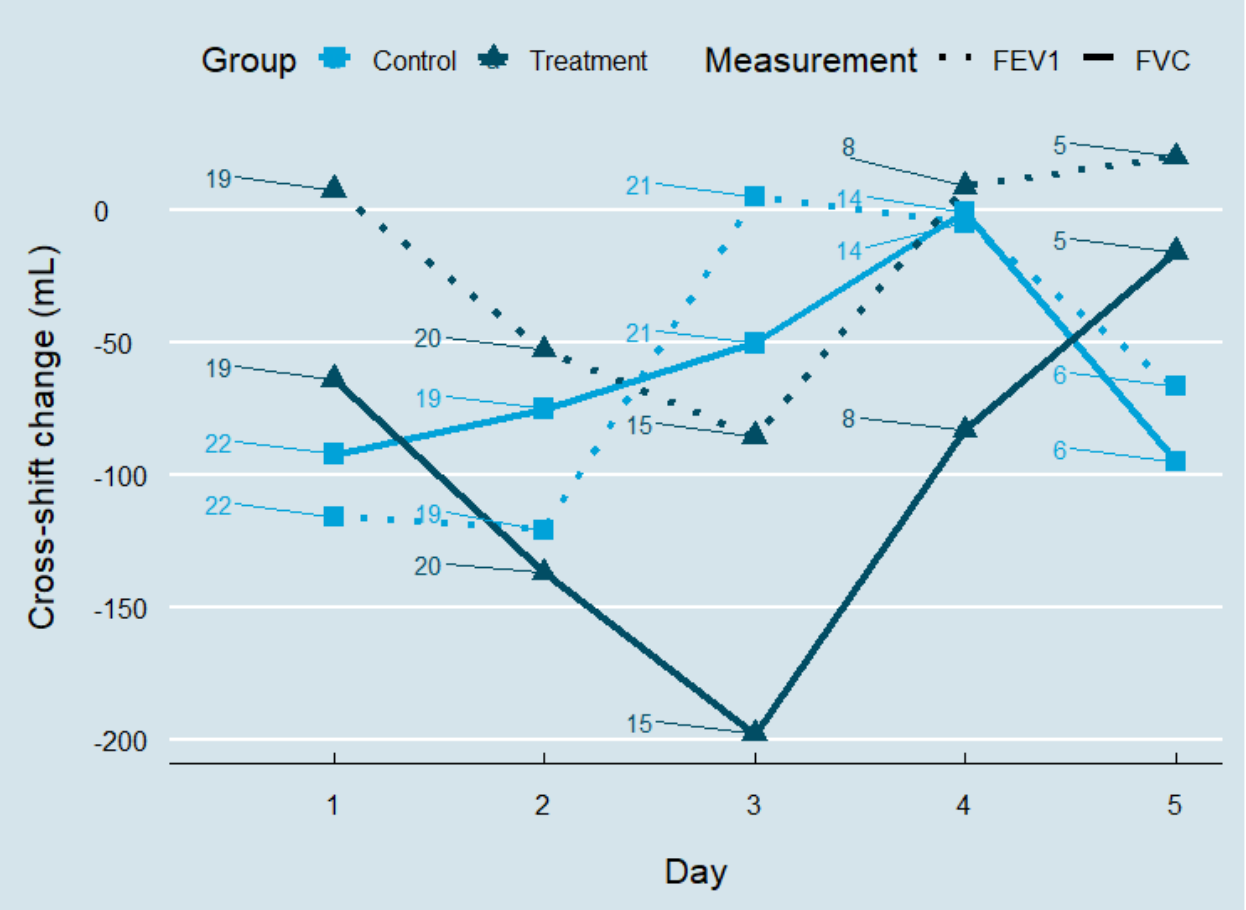

Figure 5.5: Average cross-shift change in FEV1 and FVC over the course of the study. Each spirometric marker is indicated by line type. Treatment and control groups are separated by color and shape. The number of shifts (n) for each data point are indicated with numbers.

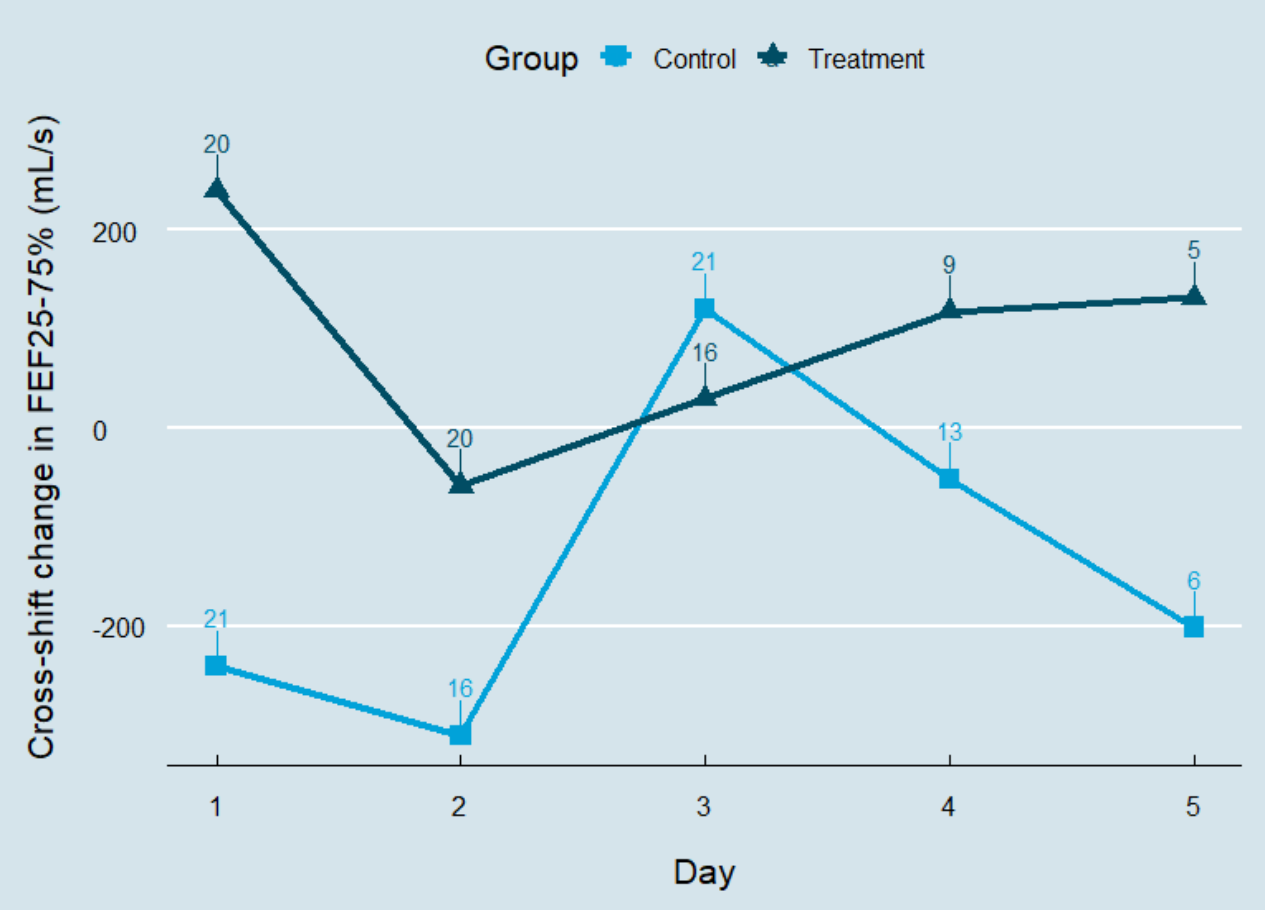

Figure 5.6: Average cross-shift change in $FEF_{25-75\%}$ separated by treatment and control group. The number of shifts (n) for each data point are indicated with numbers.

Separate cross-shift mixed linear models were generated for all spirometry outcomes of interest. Because the exposure models found no significant differences in inhalable dust or endotoxin exposures between groups, neither variable were included as fixed effects in the cross-shift models. For the dairy workers in our study, receiving a hypertonic saline nasal lavage appeared to have a negligible effect on the cross-shift change in FEV1 and FVC compared to receiving a normotonic lavage. The mixed linear models indicate that the treatment group, on average, performed slightly worse on cross-shift changes in FEV1 (β = -1.8 mL; p = 0.970) and FVC (β = -67.7; p =0.238) compared to the control group. The treatment did have a positive effect on cross-shift $FEF_{25-75\%}$ performance (β = 204 mL/s; p = 0.078). The 95% CIs for the

treatment's effect on FEV1 (-95.1 to 91.5), FVC (-178.3 to 42.8), and $FEF_{25-75\%}$ (-20.9 to 429.1) were wide. When looking at random effects, the day did not affect FEV1 and FVC outcomes (sd = 0), but it did impact cross-shift changes in $FEF_{25-75\%}$ (sd = 58.8). Given physiological differences and the intrinsic factors that are known to impact individual exposure responses, it was unsurprising that a variation between participants was observed for both FEV1 (sd = 80.2) and FVC (sd=112.6). Full results of the mixed linear models fixed effects are presented in Tables 5.3, 5.4, & 5.5.

Table 3: Linear mixed effect model for the treatment's effect on FEV1

Parameter	Regression Estimate β (mL)	Lower 95% CI	Upper 95% CI
HTS Lavage (Treatment)	-1.8	-95.1	91.5
Height	11.5	-7.5	30.6
Weight	-0.4	-2.0	1.2
Age	5.0	-0.7	10.7
Gender (male)	-74.4	-217.7	68.9
Current smoker	96.2	-35.7	228.3

Table 4: Linear mixed effect model for the treatment's effect on FVC

Parameter	Regression Estimate β (mL)	Lower 95% CI	Upper 95% CI
HTS Lavage (Treatment)	-67.7	-178.3	42.8
Height	8.3	-14.3	30.9
Weight	-0.2	-2.1	1.7
Age	4.1	-2.6	10.9
Gender (male)	-44.73	-214.4	125.0
Current smoker	56.2	-98	210.4

Table 5: Linear mixed effect model for the treatment's effect on FEF_{25-75}

Parameter	Regression Estimate β (mL/s)	Lower 95% CI	Upper 95% CI
HTS Lavage (Treatment)	204.1	-20.9	429.06
Height	-0.5	-746.2	45.2
Weight	0.5	-3.3	4.3
Age	9.8	-4.1	23.6
Gender (male)	-12.8	-359.1	333.4
Current smoker	93.5	-232.8	419.9

Discussion

The efficacy of a hypertonic saline (HTS) nasal lavage to reduce dairy worker's cross-shift changes in pulmonary function was investigated over 2-5 consecutive working days. Overall, dairy workers in our study experienced decreases in pulmonary function during their work shift based on the three primary spirometric outcomes: (i) FEV1, (ii) FVC, and (iii) $FEF_{25-75\%}$. Averages in cross-shift outcomes between our two participant groups found that workers receiving the HTS nasal lavage experienced a smaller decrease in FEV1 but a greater decrease in FVC compared to workers receiving a normotonic lavage. The HTS lavage did improve workers' unadjusted cross-shift $FEF_{25-75\%}$. When the cross-shift outcomes were compared in a mixed linear model that also incorporated participant characteristics and repeated measures factors, the hypertonic saline lavage appeared to have a therapeutic effect on cross-shift $FEF_{25-75\%}$, but little effect on cross-shift FEV1 or FVC. These numbers may not tell the full story of the lavages' effect on dairy workers' acute changes in FEV1 and FVC. Considering the potential for a therapeutic effect of normal saline in improving cross-shift pulmonary function outcomes in dairy workers, we wanted to also examine our entire study population within the context of receiving an intervention.

Investigating our cross-shift results under the assumption that both groups received a treatment leads to interesting comparisons. In 2013, Eastman and her research group from University of California, Davis published a comprehensive cross-shift respiratory health survey of 210 Californian dairy workers.[20] Their group discussed a previously proposed 3% decrement in unadjusted levels of FEV1 and FVC as a predictor for long term health outcomes. Out of their 210 representative shifts, 30% of dairy workers experienced a 3% or more decrease in unadjusted FEV1 and 29% experienced a 3% or more decrease in unadjusted FVC.[20] In our study

of 154 working shifts, 25% experienced a similar decrease in unadjusted FEV1 and only 17% experienced a similar decrease in unadjusted FVC.

While comparisons to previous occupational health surveys were not a major focus of our intervention study, the similarity between the California study and our study warranted further evaluation. Both studies had a working cohort that was over 90% Latino, and the mixed indoor and outdoor dairy configurations in California lead to similar exposures for High Plains workers.[20] To this point, the UC Davis research group published the exposure results of their evaluation under Garcia et al. in 2013.[55] Geometric mean endotoxin levels for the California dairies were higher than the results here (453 EU/m^3 vs. 58.3 EU/m^3), but inhalable dust levels were similarly low (0.99 mg/m^3 vs. 0.45 mg/m^3).[55] The use of unadjusted FEV1 and FVC cross-shift spirometry results in both studies allows for direct comparison, with participants' pre-shift PFTs serving as their own control for post-shift measurements. Unadjusted values also provide more physiological relevance when comparing prevalence of negative cross-shift health outcomes, i.e. the typical participant characteristics controlled for in pulmonary function studies (age, sex, height, and weight) have no biological basis on impacting diurnal variation of lung function.

The use of any nasal lavage, regardless of osmolarity, may therefore provide protection against cross-shift decreases of 3% or more in both FEV1 and FVC. While the differences between the Eastman et al. results and the data presented here are small, minor improvements in acute respiratory health are still worth investigating.

These findings point towards a potential lack of understanding of the anti-inflammatory effects of normal saline. As previously stated, hypertonic saline has outperformed normotonic saline in attenuating inflammation in several *in vitro* studies involving respiratory cells.[34,35]

Many *in vivo* studies, however, have highlighted improved physiological responses to normal saline as an anti-inflammatory treatment. For example, Ghosh et al. observed that RNS60, a 0.9% saline containing charged oxygenated nanobubbles, improved tidal volume and bronchoalveolar lavage (BAL) levels of several pro-inflammatory cytokines when inhaled by asthmatic rats.[56] In a systematic review of hip osteoarthritis clinical studies, Gazendam et al. concluded that intra-articular hip saline injections were just as effective in improving pain and hip function compared to corticosteroids, hyaluronic acid, and platelet-rich plasma injections.[57]

Remarkably, a similar problem to ours appeared in knee osteoarthritis clinical trials that used saline injections as placebo controls. For years, researchers suspected that a therapeutic anti-inflammatory effect from saline injections was interfering with trials also using experimental injections. Saltzman et al. published a meta-analysis in 2016 of 13 placebo-controlled trials using saline injections as a control between 2006 and 2016.[58] They concluded that saline injections improved patient-reported outcomes statistically and clinically for both pain and physical function. Their findings further support the theory that normal saline modulates inflammatory responses.

The treatment's effect on improving cross-shift changes in $FEF_{25-75\%}$ by 204 mL/s should not be dismissed. Patients with asthma have previously shown that decreases in $FEF_{25-75\%}$ are associated with increases in asthma symptoms, bronchial hyperreactivity, and increased respiratory inflammation via exhaled nitric oxide and serum eosinophil levels.[59] Qin et al. have also recently suggested that $FEF_{25-75\%}$ is a better predictor than FEV1 for airway responsiveness and respiratory inflammation in asthmatic patients.[60] The use of an HTS lavage before their shift therefore, may serve to both protect dairy workers against small airway inflammation and bronchial hyperreactivity.

$FEF_{25-75\%}$ is not always reported in other cross-shift investigations of workers, but in a study of 69 Norwegian bar workers exposed to cigarette smoke, Skogstad et al. reported an average decrease of -199 mL/s.[61] A similar average decrease of -170 mL/s was observed in a cohort 96 Norwegian cement workers.[41] In agricultural workers, Viet et al. reported an average decrease of -0.61% of predicted values in Coloradoan wheat harvesters, and Eastman et al. reported a decrease of -60 mL/s in baseline adjusted least square means in their dairy worker investigation.[20,62] In their investigation of 137 Coloradoan dairy worker shifts, Martenies et al reported a modest cross-shift increase of 0.69% of predicted values in $FEF_{25-75\%}$.[9] As our treatment group also experienced increases in unadjusted $FEF_{25-75\%}$, the HTS lavage may find applicability in other industries where workers suffer from acute changes in small airway function.

Ultimately, the inclusion of a control group that also received a normotonic saline lavage before and after their shift likely skewed our results. A lavage was selected for the control group so that inflammatory cytokine collection was consistent between both groups. Study design principles also necessitate a consistent delivery of both treatment and placebo between groups, as secluding participants that worked at the same dairy was not feasible or in the best interest of the dairy's fundamental goal of milk production. Future intervention studies involving any anti-inflammatory efficacy should therefore consider the potential therapeutic effects of saline during the study design phase.

We found that the hypertonic saline nasal lavage only outperformed a normotonic saline in improving dairy workers cross-shift $FEF_{25-75\%}$. Interestingly, the use of a nasal lavage with any osmolarity in our study points towards a reduction in negative cross-shift pulmonary outcomes of physiological relevance. Based on these mixed results, future studies may want to investigate the

use of a saline nasal lavage for occupations traditionally associated with decreased lung function. While a saline nasal lavage is not likely to be a simple cure-all, it may be a piece to the puzzle in reducing respiratory disease in dairy workers.

Strengths and Limitations

Our study did suffer from the omission of a true non-exposed control group. As part of our study design, office workers were originally intended to serve as non-exposed controls. During the collection of data, it was revealed that office workers faced similar exposure risks due to office locations on the dairies and the mixed tasks often performed by office workers. Downstream analysis confirmed this suspicion, as over 40% of the lavages taken from office workers in this cohort tested positive for at least one bacterial or viral pathogen.[12,63] The three workers in this cohort that did report performing office duties were therefore included in the final analysis as dairy workers.

Spirometry is effort dependent, and any study reporting spirometry results suffers from this limitation. Because dairy work is physically demanding, the potential for influence of fatigue on post-shift tests should also be noted. Future studies should incorporate non-effort dependent measures of pulmonary function such as oscillometry.

The inclusion of FEV1 and FVC is one strength of this study. FEV1 and FVC are the most used spirometric outcomes in both epidemiological studies and in clinical settings. The inclusion of $FEF_{25-75\%}$ is slightly more experimental. Historically, small fluctuations in this marker are difficult to interpret, and its diurnal variability as it relates to bioaerosol exposure is poorly understood.[64,65]

Our work here supports the use of cross-shift pulmonary function as a convenient, low-cost, and minimally invasive biomarker that can be used in intervention studies focused on

respiratory health. Unlike the measurement of cytokines, which may take minutes to hours to upregulate and is therefore difficult to correctly time collection, pulmonary function changes occur actively throughout a working shift and can be measured with participants in less than 5 minutes. Further decreases in pulmonary function are unlikely once the worker is removed from the exposure, except in rare occurrences of acute hypersensitivity pneumonitis. Transferring data from modern spirometers and oscillometers is also a relatively simple process, allowing for researchers to analyze data immediately and bypassing the need for meticulous laboratory preparation and analysis. Pulmonary function is also an important health outcome for workers, as participants are more likely to perceive changes in lung function compared to changes in cytokine inflammation signaling.

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CHAPTER 6: CONCLUSION

Future directions

The future of dairy worker research is at a critical junction for workers, scientists, and stakeholders. Decades of hazard identification, exposure assessments, and occupational epidemiology have greatly advanced our understanding of the exposures workers face and the negative health outcomes associated with the occupation. Missing from this pedigree of research, however, are implementable solutions to address concerns of respiratory disease in livestock workers. Sigsgaard and a taskforce of European researchers recently published a comprehensive overview on respiratory disease and allergy affecting livestock workers in *Clinical and Translational Allergy.* After a detailed review of the most up-to-date epidemiology and suspected disease pathology, the taskforce ended with a "Research needs" subsection that identified three specific areas with significant knowledge gaps: (i) follow-up studies, (ii) mechanisms and diagnosis, and (iii) prevention and intervention.

Their last identified need points to the elephant in the room of livestock worker health research: hazard recognition has far outpaced advancements in disease prevention and intervention. As we pointed out in our review "Lessons from Dairy Farmers for Occupational Allergy and Respiratory Disease" published in *Current Allergy and Asthma*, controlling bioaerosols and developing interventions for this workforce poses unique challenges. The dairy industry runs on a 24-h production cycle, and workers are scheduled for long shifts with odd hours. Combined with interactions with large, unpredictable animals, the use of bulky PPE such as respirators will not find widespread application. Choudry et al. proposed increasing the frequency of parlor washing, but the increased use of water is neither sustainable or

economically feasible for most dairy operators. Toward this effort, intervention studies must factor cost, burden, and adoptability in their design phases.

As part of this dissertation, I examined the efficacy of a hypertonic saline (HTS) nasal lavage intervention in reducing the burden of cross-shift changes in pulmonary function. In parallel, Erlandson et al. tested the HTS nasal lavage's ability to modulate the expression of both anti-inflammatory and pro-inflammatory cytokines in the upper respiratory system. Both works found that while the use of pre- and post-shift nasal lavages leads to slight improvement in selected health outcomes, the lavage will likely need to be a piece in a larger puzzle that addresses exposures. Thus, the development and testing of new interventions to reduce exposure and/or improve respiratory health outcomes is needed for both workers and stakeholders.

Astute observers will notice the other two sections of this dissertation focused on pathogens and the nasal microbiome of dairy workers. Dairy workers are a fascinating cohort in respect to their nasal microbiome. Compared to non-bioaerosol exposed individuals, dairy workers have a more diverse nasal microbiome that's shown to be more dynamic in microbial composition and taxonomic abundance. Improving our understanding of the effects the dairy environment have on workers' nasal microbiomes will improve our understanding of how much the human microbiome can be influenced by exposures and environments. The increased application of next-generation sequencing (NGS) will provide even greater scientific knowledge on the microbial molecular functions that impact host-microbe relationships.

Future research into the nasal microbiome of dairy workers will be part of a larger effort to better understand the intrinsic factors that affect respiratory disease pathology. Major questions still need answered regarding the role of host genetics and timing of exposures on dairy worker health, and future longitudinal studies following youth into the workforce would

nicely complement future microbial investigations. These studies may conclude that certain individuals are less fit to work on dairy farms compared to other individuals. Proposed interventions may even recommend excluding workers without previous childhood farm exposure or with a susceptible genotype.

From a pathogenic perspective, the dairy environment is increasingly seen as a petri dish for zoonotic diseases that can potentially spill over to non-exposed individuals. With the recent occurrence of the devasting COVID-19 pandemic, increased surveillance for viral and bacterial pathogens on dairy farms will be crucial to the early detection of virulent infectious diseases. The dairy environment and other livestock operations are also well-suited for investigations focused on the transfer of genetic material between microbes. Microbes naturally evolve and adapt to improve community fitness, but the misuse of antibiotics in animal populations appears to have accelerated the rate in which antibiotic-resistant genes (ARG) get passed between microbiota. Increased surveillance of this phenomena at dairy environments provides valuable insight into the public health implications of antibiotic-resistant bacteria. Future investigations may also include the enrollment of family members of dairy workers, to better quantify the spread of ARG and pathogens to non-exposed individuals.

In their Strategic Plan Intermediate Goals, NIOSH specifically addresses both fixed airway diseases and infectious disease transmission in the Agriculture, Forestry, and Fishing (AgFF) sectors. With strong federal funding support and a network of 12 CDC/NIOSH-supported Centers for Agricultural Safety and Health, US researchers are poised to continue leading the effort of developing interventions to improve dairy worker health worldwide.

As part of their focus on AgFF workers, NIOSH also highlights “Precarious Employment Arrangements” and “Work organization, Fatigue and Mental Health” as specific areas in need of

improved research. Current livestock worker health research tends to undervalue the role of the social determinants of health in our cohorts. Specifically, we are failing to incorporate political and socioeconomic factors into our hazard recognition models. These factors are major drivers of dairy organizational structures, and their overall impact on dairy worker health is seriously understudied. In 2013 Schenker and Gunderson published the paper "Occupational Health in the Dairy Industry Needs to Focus on Immigrant Workers, the New Normal" in the *Journal of Agromedicine.* Over 10 years later, our primary focus on immigrant workers has been enrolling them as participants in our studies.

The future of dairy worker health research should address the most pressing need: developing interventions to reduce respiratory disease in this workforce. Future investigations may also focus on pathogens, ARG, and the human microbiome, as these topics have public health implications beyond the farm. Proposed interventions will have to focus on immigrant workers and the unique challenges they face to find success with implementation. If newly developed interventions do not consider the social determinants affecting immigrant workers, the interventions will never succeed.

Conclusions

The original research presented here aimed to build on decades of previous respiratory health research on dairy workers. Specifically, the research addressed knowledge gaps in the nasal microbiome and how it may impact pathogen prevalence and cross-shift lung function in dairy workers. Furthermore, this research examined the efficacy of a novel hypertonic saline (HTS) nasal lavage in reducing the impact of dairy bioaerosol exposure on cross-shift changes in lung function.

This research sought to answer the following questions:

1. *Do certain nasal microbiome characteristics correlate to viral or bacterial pathogen susceptibility in dairy workers?*
2. *Is there a relationship between the nasal microbiome and lung function in dairy workers?*
3. *Does the administration of a hypertonic or normotonic saline nasal lavage before and after a working shift improve the cross-shift spirometric markers associated with pulmonary obstruction for dairy workers?*

For all research aims, dairy workers from the High Plains region of the United States were enrolled in an intervention study for up to 5 consecutive working days. Half of the workers received pre-and post-shift HTS nasal lavages (400 milliosmole (mOsm)) as a treatment, and half of the workers received normotonic nasal lavages (300 mOsm) as a control. Lavages were collected and then analyzed for the presence of bacterial and viral pathogens in workers' nares. To quantify the bacterial communities that comprise the nasal microbiome of these workers, all lavages also underwent targeted 16S rRNA gene sequencing. To understand the cross-shift change in pulmonary function experienced by workers, spirometry was performed pre- and post-shift with workers.

For the first time in the identified literature, this work demonstrated that opportunistic viral and bacterial pathogens reside in the nares of dairy workers at alarming levels. Of the first 31 participants enrolled in this study, only 1 (3.2%) participant never tested positive for a viral or bacterial pathogen. Out of a total of 237 nasal lavages collected, 41 (17.3%) tested positive for influenza D virus (IDV), a virus most commonly found in cattle and swine facilities. Over 32% of the lavages tested positive for Methicillin-susceptible *Staphylococcus aureus* (MSSA) and over 11% tested positive for Methicillin-resistant S*taphylococcus aureus* (MRSA).

The taxonomic composition of the nasal microbiome of these dairy workers was similar to previous investigations of livestock workers. Significant differences in beta diversity were observed between lavage testing positive or negative for IAV and MSSA, but no significant differences in alpha diversity were observed based on the presence of our targeted pathogens. Combined with the observed differences in taxonomic abundance based on bacterial pathogen presence, it is possible that certain 'key-player' taxa may increase worker susceptibility to bacterial pathogens. The role of the nasal microbiome in virus exposure was not elucidated.

When comparing the nasal microbiome of dairy workers to cross-shift changes in pulmonary function, a lower relative abundance of *Lactobacillaceae* was found in the nasal microbiome of participants performing poorly on cross-shift changes in forced vital capacity (FVC). *Lactobacillaceae* dysbiosis has also been observed in patients with chronic rhinosinusitis and rats with asthma, and one study found that reductions in *Lactobacillaceae* in smoker lung microbiomes was associated with increases in IL-6 and C reactive protein inflammation. Probiotic sprays using *Lactobacillus* species have previously been proposed as a treatment for inflammatory upper respiratory disease, and these findings suggest probiotic sprays may have some applicability in bioaerosol-exposed workers. Taxonomic community state typing (CST) via Dirichlet Multinomial Mixtures (DMM) was also performed at the family level in our study, and 3 distinct CSTs emerged from the cohort. Spirometry samples paired to the CSTs performed at different levels in cross-shift changes in both FVC and forced expiratory volume in one second (FEV1). CST3 was the smallest group, but also the most resilient to decreases in lung function during their shift. Participants with nasal microbiomes falling into CST2 performed the worst, with over 30% of the spirometry samples associated with CST2 experiencing decreases of -200 ml or more for either FEV1 or FVC.

Finally, this work confirmed recent findings that bioaerosol exposure negatively impacts cross-shift pulmonary function. On average, workers performed poorly in both cross-shift changes in FEV1 and FVC. While the hypothesis that a HTS nasal lavage would improve cross-shift changes in pulmonary function compared to a normotonic lavage was rejected, the use of a nasal lavage with any osmolarity may help with this burden. To assess this outcome, the unadjusted cross-shift values in this study were compared to the unadjusted cross-shift values in Eastman et al's 2013 assessment of dairy workers. Overall, the dairy workers receiving a saline rinse in this study performed better on cross-shift pulmonary function than the dairy workers in Eastman et al. These findings underlie the scientific community's lack of consensus on the anti-inflammatory properties of normotonic saline. In knee osteoarthritic clinical trials using saline injections as a placebo control, researchers have long suspected that normal saline injections are not appropriate as placebos. A 2016 meta-analysis of 14 saline placebo-controlled trials confirmed this suspicion.

This research built on the current understanding of dairy worker respiratory health. For the first time, cause and effect relationships between the nasal microbiome of dairy workers and specific health outcomes were investigated. Specific characteristics of the nasal microbiome were found to confer protection against bacterial pathogens, and a dysbiosis in *Lactobacillaceae* was found to decrease cross-shift FVC outcomes. Dairy workers' nasal microbiomes were also sorted into community state types for the first time, and participants falling into CST3 were the most resilient to cross-shift changes in pulmonary function. The efficacy of a saline nasal lavage on impacting cross-shift pulmonary function was examined, and workers receiving any osmolarity lavage pre- and post-shift appeared to perform better than workers not receiving a lavage. Furthermore, the answers found in this research went beyond the questions originally

asked. The presence of viral and bacterial pathogens in the nares of dairy workers was alarmingly high, and increased pathogen surveillance at dairies and other livestock operations is warranted. This work also contributes to the current understanding of the therapeutic effects of normal saline, as the use of normotonic saline as placebos in medical studies may not be appropriate.