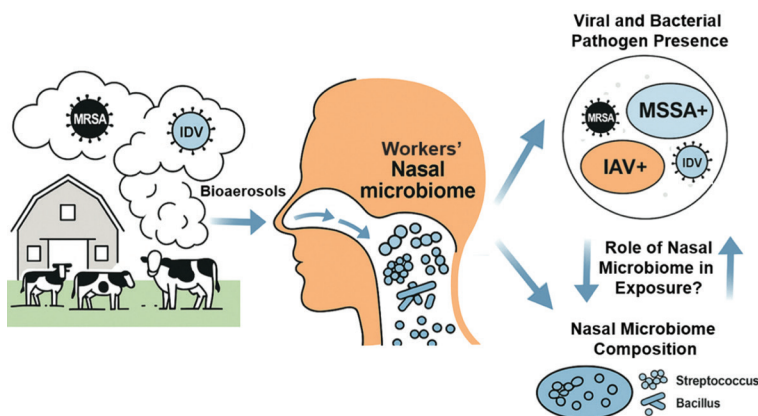




ORIGINAL ARTICLE

Potential Roles of the Nasal Microbiome in Pathogen Exposures in Dairy Workers

Graphical Abstract



Highlights

- Opportunistic pathogens were pervasive in the nostrils of dairy workers, with only 1 worker out of 32 never testing positive for at least one of our chosen pathogens (MRSA, MSSA, IAV, ICV, IDV).
- Few differences were observed in alpha and beta diversity metrics between lavages testing positive for pathogens, indicating that 'key player' genera may have a more modulating effect on pathogen positivity.
- Differential abundance analysis revealed significant differences in genera among lavages testing positive for MRSA, MSSA, IDV, and IAV.

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Brief Statement

Opportunistic pathogens including MRSA, MSSA, IAV, ICV, and IDV were present in nasal lavages taken from dairy workers before and after work. Following quantification of workers' nasal microbiome, lavages positive for IAV or MSSA clustered separately via robust Aitchison PCA. Differences in genera abundance (*Staphylococcus*, *Streptococcus*, and *Bacillus*) were also observed in lavages testing positive for MRSA, MSSA, IDV, and IAV.

Potential Roles of the Nasal Microbiome in Pathogen Exposures in Dairy Workers

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Abstract

Objective: Livestock workers are exposed to bioaerosols comprising pathogens including influenza D virus (IDV), influenza A (IAV), and methicillin-resistant *Staphylococcus aureus* (MRSA), all of which can colonize the upper airways (e.g., anterior nares) and pose public health risks. The role of the nasal microbiome in occupational exposure is not well understood.

Methods: Here, we characterized the nasal microbiomes of US dairy workers to investigate the microbiome's effects on pathogen carriage. We collected 237 lavages from 31 dairy workers to determine the presence of influenzas A, C, and D; methicillin-susceptible *Staphylococcus aureus* (MSSA); and MRSA. Nasal lavages were analyzed via PCR to quantify workers' nasal microbiomes, and differences in microbiome characteristics were analyzed according to pathogen presence.

Results: Overall, 32.1% of lavages tested positive for MSSA, 11.4% tested positive for MRSA, 17.3% tested positive for IDV, 2.5% tested positive for IAV, and 1.3% tested positive for ICV. Nasal samples positive for IAV and those positive for MSSA clustered separately with robust Aitchison PCA, but no significant differences in alpha diversity were observed. Differential abundance analysis revealed significant differences in genera among lavages testing positive for MRSA, MSSA, IDV, and IAV (e.g., *Staphylococcus*, *Streptococcus*, and *Bacillus*).

Conclusions: Future work is needed to ascertain whether the microbiome might protect against pathogen exposures among livestock workers.

Key words: Pathogens, influenza virus, MRSA, nasal microbiome, zoonoses, cattle, agricultural health, bioaerosols, nasal lavage

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INTRODUCTION

Agricultural workers experience a high burden of respiratory disease, probably because of repetitive and prolonged exposures to biological aerosols (i.e., bioaerosols) [1]. Dairy operations generate bioaerosols that vary substantially in size, spanning several orders of magnitude in aerodynamic diameter (i.e., <3–100 μm). The bioaerosols on dairy farms can originate from a variety of onsite sources,

including animals, humans, feces, and feed/bedding [2–6]. Bioaerosol exposures among dairy workers have been associated with risk of inflammatory and obstructive lung diseases; diminished 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 in 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 be divided into two broad groups, healthcare- and community-associated MRSA, which may pose potential threats to production animals (through zoonanthroponosis) [11]. Improved surveillance of exposures on dairy farms can help decrease the threat and burden of adverse outcomes among both people and animals [12]. For example, separate investigations have indicated that both IDV and the livestock-associated MRSA CC-398 variant can infect individuals with no livestock exposures [13,14]. Given existing evidence of community spillover of viral and bacterial pathogens originating from livestock operations, better understanding of these exposures and the subsequent host response is essential to protect workers and potentially mitigate future pandemics [14–17].

Unique and distinct bacterial communities comprising those in the upper respiratory system, including the nasal microbiome, reside at this critical interface of exposure between workers and the environment. According to current understanding, the nasal microbiome is more homogeneous between individual than other areas of the body; is often colonized by *Staphylococcus*, *Corynebacterium*, *Alloicoccus*, *Haemophilus*, *Streptococcus*, *Granulicatella*, and *Moraxella*; and often shows dysbiosis, marked by a lack of diversity, in various respiratory disease states [18–20]. Substantial bioaerosol exposures are likely to affect the nasal microbiome. For example, the nasal microbiomes in dairy workers has not been extensively studied, but has been shown to have high diversity in species richness and novel taxa found primarily at dairy farms [21]. Dairy workers acquiring unique bacterial taxa from the dairies where they work is consistent with previous findings indicating that environmental factors substantially modulate the human microbiome [22,23]. Furthermore, the extended work shifts and increased task specialization common at modern dairies leave workers vulnerable to repetitive exposures over the course of the workweek.

The nasal microbiome in dairy workers may affect exposure to, and infection by, opportunistic pathogens including MRSA, IAV, and IDV, all of which are emerging biosafety concerns at livestock operations [4,6,17]. The high taxonomic diversity observed in dairy workers' nasal microbiomes has been suggested to provide competition that inhibits *Staphylococcus* colonization [21]. However, the role of the nasal microbiome in viral colonization and infection is understudied in the context of viral exposures in dairy operations. Interestingly, *Staphylococcus aureus* in the airways has been found to protect against influenza infection by recruiting peripheral CCR2+ CD11b+ monocytes through promotion of TLR2 signaling [24]. A recent epidemiological study has suggested that the bacterial community structure of the nasal microbiome can

protect against influenza infections; however, supporting data suggest that this structure is uncommon in adults [25].

Here, we used nasal lavages collected from a longitudinal intervention study to advance understanding of the nasal microbiome characteristics that might potentially affect occupational pathogenic exposures. The study design and associated nasal lavage specimens provided a unique opportunity to simultaneously quantify day-to-day pathogen presence within the upper respiratory system in dairy workers and to identify the bacterial communities that compose the nasal microbiome, both within and between workers. By identifying nasal lavages positive for IAV, ICV, IDV, methicillin-susceptible *Staphylococcus aureus* (MSSA), and/or MRSA, we were able to identify the nasal microbiome characteristics that differed between exposures.

METHODS

Participant recruitment and sample collection

This study was nested within a larger intervention study in which participants were recruited among workers at five large herd dairies (>2,000 cows) in the Southwestern United States between May 2019 and January 2020, through a snowball sampling approach. The exclusion criteria included 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 before the lavage. Workers in 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 the enrollment, the workers were asked to participate for five consecutive workdays; however, workers unable to participate for five consecutive days were not excluded. From the parent study, the participants were split evenly into treatment and control groups, such that every other participant received treatment. Because of the design of the intervention study, half the participants received a hypertonic saline nasal lavage with an osmotic concentration of 400 milliosmole (mOsm), and the other half received a normotonic saline lavage with an osmotic concentration of 308 mOsm. All participants provided written consent, in English or Spanish, to participate, and all study protocols were approved by the Colorado State University Institutional Review Board.

Full biological sampling procedures were as previously described [26]. Briefly, nasal lavages were collected before and after each shift, for a total of two lavages per participant per day. The participants therefore received as many as ten total lavages over the duration of the study. The participants were instructed to tilt their heads back while a trained researcher administered 5 ml lavage fluid into each nostril over a 10 second period. After the 10 seconds, the participants tilted their heads forward to expel

the lavage fluid into a sterile specimen cup. The lavages were pipetted into a conical vial, and 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 divided into aliquots for viral analysis, *Staphylococcus aureus* analysis, and polymerase chain reaction (PCR) amplification to identify bacterial communities. A 40% glycerol stock was added to each *Staphylococcus aureus* aliquot in a one-to-one ratio, to aid in deep freezing and storage, by limiting the formation of ice crystals that typically puncture cellular membranes and denature proteins. All aliquots were then immediately stored in -80°C freezers until analysis.

Analysis of bacterial and viral pathogens

MSSA analysis of the nasal lavages was performed at Colorado State University via culture-based methods. Briefly, lavages were inoculated on tryptic soy agar and Mueller-Hinton agar plates with “lawn streaking” techniques. After incubation, growth on selective and differential media was identified. If growth was present, positive colonies were transferred to BHI broth.

To determine the presence of MRSA in lavages, we performed antibiotic sensitivity testing with the Kirby-Bauer disk diffusion susceptibility test protocol outlined by the *American Society for Microbiology* [27]. In the scope of this work, resistant samples are reported as positive and intermediate, and susceptible samples are reported as negative.

The presence of IAV, ICV, and the novel IDV was determined by Professor Gray’s team at Duke University in 2020–2021, through previously described real-time and conventional reverse transcription polymerase chain reaction methods (qRT-PCR and RT-PCR) [28]. Specific primers and probes for each virus of interest can be found in the supplementary materials.

Nasal microbiome analysis of nasal lavage fluid from participants

High-throughput 16S rRNA gene sequencing was performed at the University of Oklahoma Health Sciences Center, to quantify the bacterial communities composing the nasal microbiome of the dairy workers. Bacterial genomic DNA from the nasal lavages was isolated with Quick-DNA kits from Zymo Research (Irvine, CA). According to the methods 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 with 5 ng genomic DNA for PCR amplification of a ~460 bp product spanning the 16S rRNA V3 and V4 regions. Libraries were indexed to multiplex for sequencing on the Illumina MiSeq platform with a MiSeq 600 Cycle Reagent Kit v2 to collect 300 bp paired-end reads. The primers used for nasal microbiome quantification are available in the supplementary materials section. Quality control and quality assurance principles (including blank analysis and validation of

sequences) were implemented and maintained according to the procedures at the sequencing core at the University of Oklahoma Health Sciences Center and Illumina’s 16S library preparation protocol [29].

Data analysis and nasal microbiome processing were performed with the qiime2–2022.2 (q2) platform [30]. The q2 DADA2 plugin was used to denoise sequences and generate a feature table, which was then filtered for mitochondria and chloroplasts [31]. A taxonomic classifier was then trained with SILVA 138 SSURef NR99 full-length sequences and SILVA 138 SSURef NR99 full-length taxonomy (primers in S1 Table) [32]. Taxonomic classification of amplicon sequence variants was performed with this custom-trained classifier. To explore the evolutionary relationships among bacterial species, we generated a phylogenetic tree via the q2 sepp-fragment insertion plugin and SILVA’s 128 SEPP reference database. Sequences without a match were placed in the most representative branch point and denoted as unannotated sequences.

Statistical analysis

Each participant provided as many as ten repeated measures for analysis. We established individual positivity for each targeted pathogen by dividing the number of positive lavages by the total lavages collected. We determined the prevalence by dividing the number of participants testing positive for a pathogen during the study by the overall number of participants. Only one positive lavage throughout a participant’s workweek was required to be considered prevalent. An array of total lavages by participant and a heat map visually representing lavages at every time point was constructed. For both overall positivity and prevalence calculations, lavages were able to be positive for more than one pathogen.

To evaluate the relationships between bacterial pathogen positivity on day 1 of sampling and on subsequent sampling days, we performed separate McNemar’s Tests on samples for both MSSA and MRSA positivity. Contingency tables were then constructed by matching samples according to positivity and whether the samples were collected on day 1 of the study.

Diversity metrics and differential abundance analysis were then used to determine differences in nasal microbiome characteristics among samples testing positive for pathogens. First, alpha diversity was determined with the q2 breakaway package, which estimates species richness with frequency ratios [33]. Pairwise Kruskal-Wallis comparisons based on the presence and absence of pathogens were subsequently performed to identify significant differences in alpha diversity. Next, analysis of beta diversity between samples was performed with the q2 DEICODE package, which uses the robust Aitchison distance to depict and display beta diversity via biplots [34]. Pairwise PERMANOVA comparisons of beta diversity were also performed in DEICODE to infer significant differences in samples positive for pathogens.

The taxonomy was then imported into R Studio (version 2022.07.2), and quantitative ranking of abundance

was performed with the phyloseq package [35]. After the top 30 genera were identified, Wilcoxon signed-rank tests were performed according to the presence or absence of targeted pathogens. Significant results were identified for samples in which a particular genus was more or less likely to occur according to a pathogen's positivity (p -value < 0.05). These results were also visualized as boxplots with the microbiomeutilities package [36]. Significance thresholds of p values < 0.05 were used for all statistical analyses.

RESULTS

Sampling results

A total of 31 participants were recruited and sampled across a total of 119 working shifts. The participants were enrolled in the study for multiple days (range: 2–5 days) across the workweek. A total of 65% of participants enrolled for 4 or 5 days. After pathogen analysis and nasal microbiome quantification, $n = 237$ lavages were included in this study.

Most enrolled dairy workers were experienced: 71% of participants reported that they had worked at their current dairy for 1 year or longer. More than half the workers (54.8%) reported direct animal contact during work, 9.7% reported milking as a responsibility, and 29% reported feeding or performing maintenance in parlors and stalls as a responsibility. Most workers identified as male (77.4%), and 16.1% of workers were current or previous smokers. Workers throughout the study reported performing a variety of tasks, including milking, facility maintenance, medical care, tending to sick or injured animals, hoof trimming, moving animals, mixing feed, feeding, rebedding stalls, birthing, calving, breeding, and office work.

Bacterial and viral pathogens found in the nares of dairy workers

A total of 76 (32.1%) nasal lavage samples tested positive for MSSA, and 27 (11.4%) tested positive for MRSA. The viral results were described by Leibler et al. in 2023 [10]. Briefly, six (2.5%) lavages tested positive for IAV, three lavages tested positive for ICV (1.3%), and 41 (17.3%) lavages tested positive for IDV.

Examination of the prevalence of pathogen positivity over the course of the workweek indicated that 14 participants (prevalence 45.2%) tested positive for MRSA at least once, and eight participants had multiple positive lavages throughout the week. Moreover, 6 of 27 (22.2%) positive samples occurred on the first day of sampling. McNemar's paired test indicated significantly more negative lavages on day 1 than on later sampling days ($p = 0.0001$). The elevated likelihood of positive lavages occurring on days 2–5 suggested that MRSA presence in the dairy workers was exposure related. Because most participants started study participation after one or two days off from work, susceptible dairy workers might plausibly have cleared *Staphylococcus aureus* from their nares after an extended period away from the dairy, then again tested positive for MRSA after return to work. Fourteen of the MRSA

positive lavages (51.9%) were collected pre-shift. Of the 14 MRSA positive lavages observed pre-shift, six had corresponding post-shift lavages positive for MRSA.

Throughout the workweek, 22 participants (prevalence 71%) had at least one nasal lavage positive for MSSA, and 17 of these participants had multiple positive lavages throughout the week. A total of 25 of 76 (32.9%) positive samples occurred on the first day of sampling. McNemar's paired test did not indicate a significant difference in MSSA results between day 1 lavages and all lavages collected on the first day ($p = 0.09$). Furthermore, only 34 of the MSSA positive lavages (44.7%) were collected after the working shift. The lack of correlation between time of exposure and MSSA positivity suggested that these dairy workers had elevated risk of permanent nasal MSSA colonization. Nine participants (prevalence 29%) had four or more positive lavages throughout the week, and two participants had nine positive lavages. In comparison, only two participants had four or more lavages positive for MRSA throughout the week, thus suggesting that persistent MSSA carriage was more common than persistent MRSA carriage among the dairy workers. Eight participants (prevalence 25.8%) had at least one nasal lavage positive for both MSSA and MRSA.

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

Each lavage and the corresponding pathogen positivity are visualized in Fig 1. Of the 237 lavages collected, 29 (12.2%) tested positive for two or more pathogens. During the workweek, six workers tested positive for more than one influenza (prevalence: 19.4%).

The nasal microbiome differs in dairy workers according to pathogen presence

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

On the basis of robust Aitchison principal components analysis (PCA) with the DEICODE package, we observed significant PERMANOVA pairwise differences in beta diversity among participants ($p = 0.001$), between pre- and post-shift lavages (0.001), and among lavages collected from participants working in the office (0.001). Kruskal-Wallis pairwise comparisons calculated with the breakaway package revealed significant differences in alpha diversity among participants ($p = 0.0003$), and between lavages collected before and after shifts ($p = 0.007$).

The top five 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, were unknown genus, *Staphylococcus*,

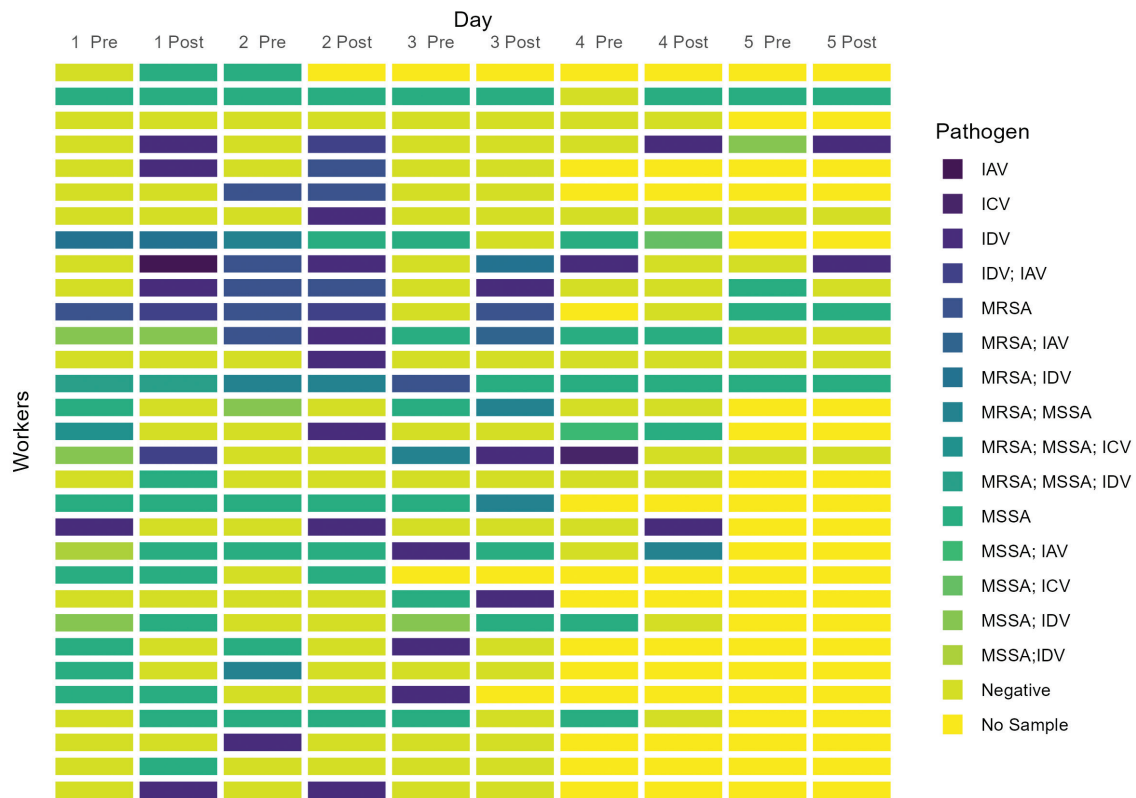


FIGURE 1 | Heatmap of positive lavages for each participant. Multiple samples tested positive for 2 or 3 pathogens. Time points without a sample were due to either no participation or incomplete data.

Corynebacterium, *Streptococcus*, *Moraxella*, *Cutibacterium*, *Dolosigranulum*, *Anaerococcus*, *Antarcticibacterium*, *Psychrobacter*, *Prevotella*, *Paia*, *Peptoniphilus*, *Bacillus*, *Turicibacter*, *Sphingomonas*, *Bacteroides*, *Lawsonella*, *Romboutsia*, *UCG-005*, *Enhydrobacter*, *JG30-KF-CM45*, *Acinetobacter*, *Neisseria*, *Methylobacterium-Methylorubrum*, *Atopostipes*, *Porphyromonas*, *Clostridium_sensu_stricto_1*, *Phyllobacterium*, and *Alloprevotella*.

Role of the microbiome in resistance

We observed a significant difference ($p = 0.04$) in beta diversity between MSSA positive and MSSA negative lavages with robust Aitchison PCA. A significant difference ($p = 0.04$) in beta diversity between IAV positive and IAV negative lavages was also found. 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 Fig 2.

At the genus taxonomic classification, *Streptococcus* ($p = 0.019$), *Alloprevotella* ($p = 0.005$), *Prevotella* ($p = 0.002$), and *Porphyromonas* ($p = 0.007$) were significantly more abundant in nasal lavages testing positive, rather than negative, for MRSA. *Anaerococcus* ($p = 0.0004$), *Cutibacterium* ($p = 0.006$), *Staphylococcus* ($p = 0.0001$), and *Peptoniphilus* ($p = 0.005$) were significantly more abundant in MSSA positive nasal lavages, whereas *Streptococcus* ($p = 0.002$) was significantly more abundant in MSSA negative lavages.

Bacillus was significantly more abundant in nasal lavages testing positive rather than negative for IDV ($p = 0.003$). *Methylobacterium-Methylorubrum* was significantly more abundant in nasal lavages testing positive rather than negative for IAV ($p = 0.035$) and ICV ($p = 0.045$).

DISCUSSION

Our work demonstrated the presence of opportunistic pathogens in the nostrils of dairy workers. The timing of positive lavages and elevated prevalence suggested that workers are likely to be exposed to MRSA at dairies and that their carriage of MRSA is transient. These results are consistent with those of other investigations indicating that swine workers have high risk of *S. aureus* exposure [37–41]. Compared with occupations traditionally considered to pose high risk of MRSA prevalence, such as nursing, the 45.2% prevalence observed herein is notable. A recent systematic review of healthcare workers has estimated a prevalence between 1.8% and 36%, depending on the setting (i.e., nursing home vs emergency department) and occupation [42]. In a recent study of healthcare workers in an intensive care unit in Vietnam, 29.1% tested positive for MRSA on the hands or in the nose [43]. Because individuals with MRSA carriage have elevated risk of skin and bloodstream infections, increased surveillance for dairy workers is warranted to ensure their health and safety, and consequently to increase

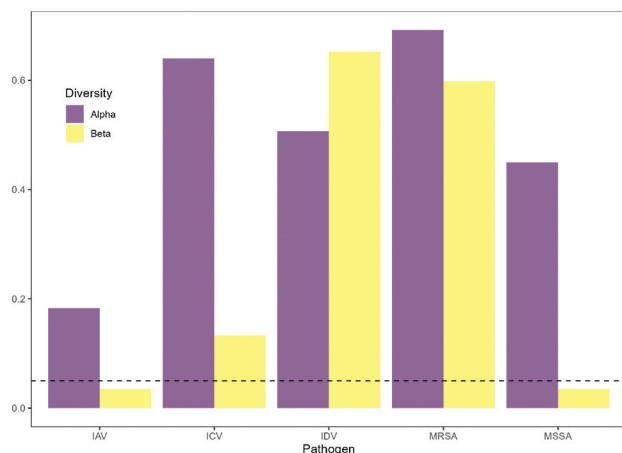


FIGURE 2 | Alpha and beta diversity comparing lavages testing positive or negative for pathogens. Alpha diversity comparisons represent Kruskal-Wallis pairwise comparisons and beta diversity shows PERMANOVA results. Significant results are below the dotted line.

productivity and decrease costs associated with health-associated absenteeism.

The timing of MSSA positive lavages and repeated positive lavages for many participants indicated MSSA colonization in this workforce. The workers in our study were more than twice as likely to test positive for MSSA (observed prevalence of 71%) than the general public [44,45]. The presence of IDV and IAV, two viruses whose reservoirs are often livestock, provided further evidence of the transmission of viral pathogens between humans and animal hosts at livestock operations [10,16,46,47]. Given that only three nasal lavages tested positive for ICV, transmission of this virus is likely to be infrequent 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* have been associated with individual-to-individual infection incidents, which might have an important role in community spillover, as workers carry these pathogens into their homes and communities [48]. In contrast, 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 the work shift [49,50]. The implications of exposure to zoonotic IDV in workplaces on community-level infection with this emerging pathogen remain unclear. Future studies should consider testing family members of dairy workers for IDV seroprevalence. Because 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 build on earlier findings from Shukla et al., elucidating a deeper understanding of the nasal microbiome characteristics that might have roles 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 according to the presence or absence of targeted pathogens. One interpretation of our diversity metrics is that specific bacterial taxa in a worker's nasal microbiome might modulate pathogen exposures in the workplace. To test our hypothesis, we conducted compositional analysis and identified several genera associated with pathogen susceptibility. Only one genus, *Streptococcus*, was associated with reduced MSSA prevalence in participants. In contrast, a positive association was found between *Streptococcus* and MRSA. A brief description of the significantly abundant genera according to pathogen presence and the current understanding of their roles in the nasal microbiome is provided below.

Paradoxically increased *Streptococcus* abundance in MRSA-positive samples and increased abundance in MSSA-negative samples is likely to be due to the ubiquity of this genus in the nose and upper respiratory tract in most humans [51–53]. This genus might not have a role in exposures, and observed differences might be attributable 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 [54]. For example, patients with asthma have been shown to have higher *Prevotella* abundance than healthy controls [55]. In patients with early rheumatoid arthritis, *Prevotella* and *Alloprevotella* have been found to dominate the oral microbiome [56]. The elevated abundance of these genera among our cohort was interesting, given that dairy workers entering the workforce as adults have elevated risk of allergic and inflammatory respiratory diseases [7,57].

Porphyromonas was also positively associated with MRSA susceptibility among our participants. The presence of this common but understudied genus in the nasal microbiome is positively associated with sleep apnea [52,58]. Sleep disorders have been shown not only to influence worker productivity but also to increase the risk of occupational injury twofold [59–62]. Given the high physical demands of the work and previous estimates that ~80% of dairy workers already have musculoskeletal symptoms, future investigations collecting self-reported data on sleep may be warranted [63].

Anaerococcus, *Cutibacterium*, *Staphylococcus*, and *Peptoniphilus* were significantly more abundant in nasal lavages that tested positive rather than negative for MSSA. The elevated presence of the *Staphylococcus* genus in samples that also cultured *Staphylococcus aureus* species is logical. Whereas 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 [64–67]. Possibly the most interesting result was the positive association of *Cutibacterium* with

MSSA positive lavages, in agreement with previous *in vitro* assessments indicating that the most common porphyrin excreted by *Cutibacterium* species, coproporphyrin III, promotes *Staphylococcus aureus* growth in the nose [53,68].

The elevated abundance of *Bacillus* in nasal lavages testing positive for IDV was unexpected, because many *Bacillus* strains have been shown to have antiviral properties. In a randomized controlled study of children infected with respiratory syncytial virus, a nasal spray of 5 billion *Bacillus* spores resulted in faster recovery times and symptom alleviation [69]. In another study, *B. subtilis* successfully inhibited IAV replication *in vitro* and protected mice against IAV infection [70]. Although relatively less is known regarding *Methylobacterium-Methylobacterium* and potential viral interactions, one study has reported diminished relative abundance of *Methylobacterium-Methylobacterium* in the gut microbiome in HIV-infected men [71]. The differentially enriched abundance of two potentially viral-resistant genera in our influenza-positive lavages underscored the lack of understanding of the microbiome's role in virus susceptibility [72].

Overall, the nasal microbiomes of dairy workers in this study were compositionally similar to those in the US dairy workers in Shukla et al.: both study cohorts shared four of the same top five phyla and four of the same top five identifiable genera [21]. Many of the top genera observed in our participants have also been observed in US livestock workers enrolled in a microbiome study by Kates et al., although differences in methods between studies do not allow for direct comparisons [73]. Islam et al. conducted an investigation that grouped the nasal microbiomes of Danish swine workers, short-term visitors, and pigs into separate community state types [39]. Again, although direct comparisons to community state types are inappropriate, the swine workers' community state types were most likely to be dominated by similar genera to those observed in our study. Thus, our findings suggested that livestock exposure modulates the human nasal microbiome, and 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 were observed. The differentially enriched abundance of multiple genera in pathogen-positive lavages indicated that the presence of certain microbiota was likely to have increased worker susceptibility to viral and bacterial pathogens common in the dairy environment. However, determining which genera are key players and the exact mechanisms through which they act is difficult. Overall, a positive association was observed between lavages testing positive for MSSA or MRSA and several important genera. For individuals with a predisposition to elevated *Cutibacterium*, MSSA susceptibility was observed in both this study and previous work [53,68]. Nasal microbiome trends based on viral positivity were less evident. Future investigations should

be aimed at understanding the characteristics of workers' nasal viromes and how host factors might modulate pathogenic exposures.

To our knowledge, this work is the first to study the microbiome composition of dairy workers in the context of multiple viral and bacterial pathogens present at dairies [10]. We observed differences in microbiome diversity; however, the consequences and implications are unclear. Further investigation is needed to improve understanding of the role of the nasal microbiome in health and disease among livestock workers. Additional investigations focused on seasonal differences and virus exposures in dairies are necessary to support this hypothesis. Diversity characteristics and taxonomic composition are unlikely to fully account for the full picture of pathogenic exposures, and future studies using next-generation sequencing, including accurate species-level sequencing and metabolites, are needed to parse the immunophysiology of exposures in dairies.

LIMITATIONS

The relatively low species abundance in the nose and the large amount of lavage fluid collected in the intervention led to a lower 16S rRNA gene sequencing depth than desired. Low sequencing depth can potentially influence downstream analysis and consequently skew the findings. However, given the similarities in composition between our study vs Shukla et al. [21] and others, the nasal microbiomes represented in our samples appear to be representative of dairy workers at large. Administering two lavages per day and as many as ten lavages over the course of the week might also have contributed to the low sequencing depth, because some washout of bacterial communities is expected. Furthermore, significant differences in alpha and beta diversity were observed between pre- and post-shift lavages, but whether the differences were due to washout, exposures during work, or normal diurnal variation in the nasal microbiome is difficult to determine. Future studies should include multiple time points in the day and should use nasal swabs for microbiome characterization.

Lavage blanks were not collected in the field to monitor background contamination; however, we used an optimal sampling strategy to ensure that the samples were collected with fidelity to aseptic practices (minimizing background contamination). Furthermore, the pre-shift lavage from each participant served as a baseline/control.

The lack of recruited milkers might have led to underestimation of the burden of pathogenic exposures in dairy work. Milkens spend their entire shifts near cows, and the milking parlor is one of the most bioaerosol-rich environments at dairies [6]. Interestingly, the inclusion of office workers in the data set probably did not add to this underestimation, because 13/30 (~43%) of the lavages collected from office workers contained at least one targeted pathogen, and three of these lavages contained multiple

pathogens. Furthermore, office workers in this study had similar total inhalable dust and endotoxin exposures to workers performing other tasks (results not shown). These findings were not unexpected, given that all offices in this study were situated in 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 other areas, thus increasing their exposures.

Ultimately, conclusions regarding MRSA colonization in this workforce are difficult to draw from culture results. Although we did perform temporal analysis from our repeated measures, whether clonal MRSA was spread around a workgroup or zoonotic MRSA passed between animals and humans is difficult to infer. Another unlikely possibility is that the lavage intervention might have increased susceptibility to MRSA carriage, thus resulting in more common MRSA-positive lavages on days 2–5.

In summary, we found meaningful presence of pathogens of public health concern in the upper airways in dairy workers, with respect to published data for occupational groups with traditionally high levels of pathogen presence. Livestock workers might therefore act as vectors and potentially introduce pathogens into communities or the farm environment. This study cannot indicate the directionality of the source–receptor relationship, but it highlights the concerns regarding pathogen transmission and the need for optimized biosecurity control measures.

Nasal microbiome composition also appeared to differ among participants exposed to pathogens. Participants with nasal lavage samples positive for IAV and those positive for MSSA clustered separately via robust Aitchison PCA, thus indicating a difference in beta diversity according to pathogen positivity. Differential abundance analysis also indicated differences in genera according to the positivity of selected pathogens. We observed no significant differences in alpha diversity according to pathogen positivity when estimating species richness via frequency ratios. Although our study indicated a relationship between the nasal microbiome and pathogen exposure in livestock facilities, further work is needed to identify the role of the nasal microbiome in modulating pathogen exposure.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article (and its supplementary information files). Raw sequencing data are available on the NCBI Sequence Read Archive (SRA) under accession No. PRJNA1280724.

ETHICS STATEMENT

All participants provided written consent in English or Spanish, and all study protocols were approved by the Colorado State University Institutional Review Board (IRB approval #3015).

AUTHORS CONTRIBUTIONS

JS, GE, SM, and JWS conceptualized the study. ZA provided support on nasal microbiome data analysis and interpretation. JHL provided guidance on study design and analysis of viral pathogen data. JN assisted with sample collection and supervised MRSA/MSSA laboratory analysis. GCG supervised viral laboratory analysis and data reporting. JS conducted data analysis and drafted the manuscript, to which all other authors contributed.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://zoones-journal.org/wp-content/uploads/2025/09/zoones20250012_Suppl.pdf.

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