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Assessment of microbial communities in a dairy farm from a food safety perspective

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ABSTRACT

Microbial communities associated with dairy farm operations have a significant influence on food safety, dairy product quality, and animal health. This study aimed to create a microbial mapping at a dairy farm to learn about their bacterial diversity, distribution, and potential dissemination pathways. The investigation included the detection of key zoonotic pathogens, enumeration of *Staphylococcus aureus* and *Escherichia coli* as indicators of typical bacterial loads in a dairy production environment, and a microbiome analysis using metagenomics. A total of 160 samples (environmental, udder swabs, feed, feces, raw milk, and water) were collected during winter ($N = 80$) and spring ($N = 80$). In winter, *Cronobacter* spp. were detected in four feed and two water samples; *L. monocytogenes* was identified in two samples, one from feces and one from a cattle mat; *E. coli* O157:H7 was found in two feed samples. On the other hand, during spring, *Cronobacter* spp. were present in four feed samples and one hallway drain, with only one feed sample testing positive for *E. coli* O157:H7, while *L. monocytogenes* was absent during the spring season. Regarding microbial counts, there was no significant difference between the two seasons ($p = 0.068$) for *S. aureus*; however, a significant difference ($p = 0.025$) was observed for *E. coli*. Environmental microbiome analysis showed the presence of Proteobacteria (46.0 %) and Firmicutes (27.2 %) as the dominant phyla during both seasons. Moraxellaceae (11.8 %) and Pseudomonadaceae (10.62 %) were notable during winter, while Lactobacillaceae (13.0 %) and Enterobacteriaceae (12.6 %) were prominent during spring. These findings offer valuable insights into microbial distribution within a dairy farm and potential risks to animal and human health through environmental cross-contamination.

1. Introduction

Exercising microbial control is a challenge the dairy industry faces constantly from farm to table. A wide diversity of microbial communities, whether resident or transient, can be found in any dairy farm operation, which, if left uncontrolled, may negatively affect human health by consuming dairy products (Osterhaus et al., 2020; Chen, 2022). The microbial ecosystem significantly influences the overall performance of any dairy operation, impacting animal health, milk production, quality, food safety, and workers' health. Within this context, the farm is required to foster production practices that ensure both public and animal health (Alonso et al., 2020). The microbial community in the dairy environment consists of ubiquitous bacteria, including microorganisms introduced and transferred by and between animals, workers, and fomites. This creates a microbial ecosystem that, if left unmanaged, can significantly compromise a dairy operation (Heredia and García, 2018) (Ouamba et al., 2022). Zoonotic bacteria frequently cause conditions that affect animal health, such as pneumonia, salmonellosis, listeriosis, and mastitis in dairy cattle (Nightingale et al., 2004; LeBlanc et al., 2006). These bacteria not only negatively impact animal welfare but also pose a public health risk (Rahman et al., 2020). They can be shed in cow feces or contaminate milk and spread in the environment, creating a transmission cycle.

The microbiota in raw milk is crucial in determining the overall safety and quality outcome of dairy products (Castellini et al., 2023). Raw milk can harbor pathogenic bacteria acquired from various sources and conditions, including intramammary infections, udder hygiene, general mudding conditions of the animal, and cleanliness at the milking parlors (Parente et al., 2020). The presence of these bacteria in dairy settings raises concerns about the safety of these products and underscores the intricate interplay between animal and human health within the dairy industry (LeBlanc et al., 2006, Garcia et al., 2019, CDC, 2022, Gebremedhin et al., 2022). While pasteurization effectively eliminates bacteria, toxin-producing and spore-forming organisms

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continue to challenge food safety and quality (Talukdar et al., 2017). Additionally, the increased interest in consuming raw milk products within the general population has raised concerns regarding potential health risks (Berge and Baars, 2020). These concerns are exacerbated by numerous foodborne outbreaks linked to raw milk, with pathogens such as *Staphylococcus aureus*, pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Cronobacter*, *Salmonella*, and *Campylobacter* identified as the primary causative agent.

Understanding the dynamics of pathogen transmission in the dairy farm environment and the frequency and concentration of key bacteria at the farm level is essential for developing efficient control strategies. These strategies are crucial to upholding the quality and safety of dairy products and enhancing livestock well-being (Li et al., 2018; Yuan et al., 2022). Exerting bacterial control and preventing propagation requires understanding current microbial dynamics and the populations residing in the dairy farm environment to implement targeted measures, including upholding stringent hygiene standards, employing effective animal disease management protocols, maintaining vigilant surveillance for signs of illness, and promoting workers' health and hygiene (Barkema et al., 2015, Quintana et al., 2020, Castro et al., 2022).

The general goal of this study is to assess and map the bacterial community found at a dairy farm operation, focusing primarily on foodborne bacterial pathogens, some of which can also cause animal infections. This study represents the microbial dynamics at the farm level and provides insights into the location points of pathogenic bacteria, microbial concentrations, and potential transmission routes. This assessment will serve as a foundational step in determining the necessity for enhanced environment microbial control to minimize the transmission of infectious diseases in animals and humans.

2. Materials and methods

2.1. Sample collection

This study explored bacterial communities by considering seasonal differences, sample type, and sample location. A dairy operation located in West Texas was selected to participate in this study, which is representative of a typical dairy farm in the United States with a milking process of about 4000 cows. A total of 160 samples were obtained during winter and spring (Table 1). In the case of feces, feed, and bedding, approximately 100 g per sample was collected. Feces from dairy cattle were obtained from freshly voided ground pats; animal feed was retrieved directly from the feeders; bedding material was manually collected from the animal pens.

Table 1
List of samples collected in winter and spring.

Zone	Sample	Number of samples/ season[1]	Total
Cattle pens	Bedding	6	12
	Feed	6	12
	Water	4	8
	Feces	6	12
Milking parlor	Milk	4	8
	Hallway drains (workers' area)	7	14
	Pit drains (cattle area)	7	14
	Milking cups	8	16
	Tubes and metal surfaces	8	16
	Towels[2]	5	10
	Worker's mat	7	14
	Cattle mat	7	14
	Udder	5	10
Total		80	160

[1] Equal number of samples obtained each time.
[2] Towels corresponded to those used to clean udders during the milking process, and only used towels were tested.

For fluid samples, approximately 100 mL were collected; water for animal consumption was obtained directly from the waterers, and raw milk was extracted from the bulk tanks. Environmental samples (drains, milking equipment, and mats) were collected using a sampling sponge (Whirl-Pak, Fort Atkinson, WI, USA) hydrated with HiCap™ Neutralizing Broth. Udders were swabbed for a few seconds immediately before farm personnel performed the routine teat disinfection without causing any discomfort to the animals. Udder swabs were obtained by the farm's overseeing veterinarian and provided to our research team. Fig. 1 depicts each type of sample collected and their location throughout the dairy farm. All samples were collected using disposable supplies to prevent contamination and transported under refrigeration to the microbiology laboratory at Texas Tech University's School of Veterinary Medicine in Amarillo, Texas, for same-day processing.

2.2. L. monocytogenes, Cronobacter spp., and E. coli O157:H7 prevalence

The detection of *L. monocytogenes, E. coli* O157:H7, and *Cronobacter* spp. was conducted via PCR using the GENE-UP® bioMérieux system (Marcy-l'Étoile, France) and the manufacturer's standardized procedures were followed. The details of the sample enrichment conditions are presented in Table 2. In brief, after the enrichment step, samples were homogenized for 2 min at 230 rpm and incubated according to the specific protocol for each microorganism as stipulated by the manufacturer. Upon incubation, a 20 μL aliquot was transferred to lysis tubes and lysed for 5 min at 2200 rpm in a centrifuge. The resulting lysed samples were transferred to new reaction tubes and subjected to PCR using the GENE-UP® thermocycler.

2.3. E. coli and S. aureus enumeration

E. coli and *S. aureus* were enumerated using the TEMPO® system (bioMérieux, Marcy-l'Étoile, France). Samples were weighted/measured following the process detailed in the previous section. Buffered peptone water (BPW, Remel, San Diego, CA, USA) was added to the samples and homogenized using a mixer operated at 230 rpm for 2 min. When necessary, 1 mL was drawn from each sample and utilized for serial dilutions, using BPW as the diluent. *E. coli* and *S. aureus* counts were determined by inoculating corresponding TEMPO® cards and incubated (37 ± 1 °C for 22–27 h for *E. coli* and at 37 ± 1 °C for 24-27 h for *S. aureus*). Bacterial counts were transformed to Log CFU/g or mL.

2.4. Bacterial isolation and confirmation

A separate step to attempt isolation was conducted from each sampling location where *S. aureus* was enumerated. Each sample was enriched using Brain Heart Infusion broth (BHI, BD Difco™, Franklin Lakes, NJ, USA) with 10 % NaCl and incubated at 37 °C for 24 h. Samples were streaked onto Mannitol Salt Agar (MSA, Remel, San Diego, CA, USA) and incubated overnight at 37 °C. Up to four presumptive *S. aureus* colonies were selected based on specific morphological characteristics from the MSA plate. Selected colonies were streaked onto Tryptic Soy Agar (TSA, Remel, San Diego, CA, USA) and incubated at 37 °C for 24 h. This process was repeated twice for improved isolation. Cryopreservation of isolates was carried out by transferring a colony to BHI and incubating at 37 °C for 18 h in an incubator shaker and further stored at −80 °C with 20 % glycerol.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) was utilized to confirm each isolate's identity. A 1 μL loopful was extracted from the frozen stock and introduced into Tryptic Soy Broth (TSB, Remel, San Diego, CA, USA), incubated at 37 °C for 18 h using a shaking incubator. Another 1 μL loopful was then streaked onto TSA and incubated at 37 °C for 24 h. The MALDI-TOF analysis employed two distinct methods; the first involved a direct smear, where a single colony was placed onto a plate spot, and 1 μL of

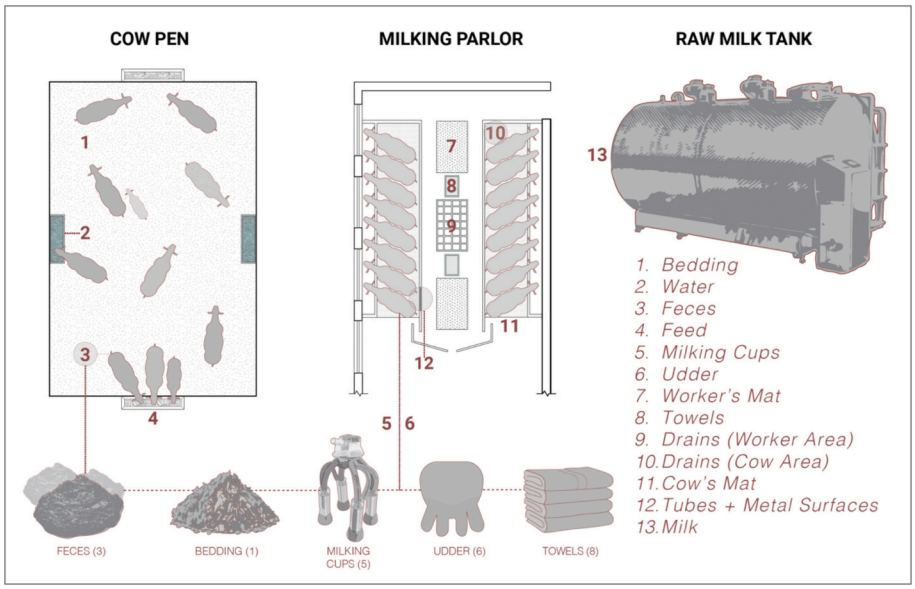

Fig. 1. Map of the dairy farm. Schematic representation of the sample collection locations.

Table 2
Enrichment media for detection of *L. monocytogenes*, *E. coli* O157:H7, and *Cronobacter* spp.

Sample[1]	Enrichment conditions[3]		
	L. monocytogenes	*E. coli* O157:H7[2]	*Cronobacter* spp
Feces, feed, water, and milk (10 or 25 g/mL)	25 g/mL of sample + 225 mL of LPT broth	25 g/mL of sample + 225 mL of BPW	10 g/mL of sample + 90 mL of BPW
Towels (10 or 25 mL)	25 mL of sample + 225 mL of LPT broth	25 mL of sample + 225 mL of BPW	10 mL of sample + 90 mL of BPW
Swabs - udder, drains, milking equipment, and mats (1 mL)	1 mL of sample + 10 mL of LPT broth	1 mL of sample + 10 mL of BPW	1 mL of sample + 10 mL of BPW

[1] Different enrichment conditions used following manufacturer's protocols, based on target organism microorganism and sample matrix.
[2] Milk samples were supplemented with acriflavine (10 mg/L).
[3] Enrichment media used for each sample type according to PCR kit insert protocol. BPW = Buffered Peptone Water; LPT = Listeria Phage Technology.

α-Cyano-4-hydroxycinnamic acid (CHCA) matrix was added. The second method required the addition of up to three colonies to sterile microcentrifuge tubes, mixed with 20 μL of a 70 % formic acid solution in a 33:33:33 acetonitrile/water/methanol combination. After mixing vigorously for 1 min, 1 μL of the bacterial solution was applied to the target spot. Once the bacterial solution had almost dried, 1 μL of CHCA matrix was added and allowed to dry. The measurement process was automated, collecting spectra in linear TOF mode between 2000 and 20,000 Da. Spectra were matched to the microorganism using the SARAMIS database (v4.13.0 RUO database, Shimadzu Corporation, Kyoto, Japan). A bacterial test standard (Thermo Fisher Scientific™, MA, USA) containing *E. coli* DH5 was used for instrument calibration.

2.5. Microbiome analysis

For microbiome analysis, bedding material, cattle mats, cows' feces, feed, drains (pit and hallways), and worker mats were included, considering the availability and quality of DNA extracted that was suitable for this test. DNA extraction was conducted using a DNeasy PowerSoil Pro Kit (Qiagen, MD, USA) was used, following the manufacturer's protocol. Following individual extraction of each sample, DNA was standardized using nuclease-free water, with quality and quantity estimation carried out using the NanoDrop™ One (Thermo Fisher Scientific™, Waltham, MA, USA). When DNA concentrations met the quality standards, DNA from the same sample types was standardized and pooled, analyzing one sample type per season. With this process, a total of 14 genomic DNA samples (7 per season) were sent on dry ice to Novogene Corporation Inc. (Novogene Co, CA, USA) for amplicon metagenomics sequencing (Regions: V3-V4). At their research facilities, samples underwent additional quality control, library preparation, and Illumina sequencing (paired-end, 250 bp, NovaSeq6000- PE250), followed by data annotation. Novogene Co. performed the bioinformatic analysis and the generation of relative abundance plots utilizing R statistical software version 4.3.0.

2.6. Statistical analysis

Analysis of bacterial counts utilized R statistical software version 4.3.0. *E. coli* and *S. aureus* bacterial counts were log-transformed. When the data met normal distribution as verified by the Shapiro-Wilk test, an ANOVA was employed to evaluate zone, sample type, and season as primary factors. Subsequent post-hoc analysis was conducted using a pairwise *t*-test with Bonferroni adjustment. When normal distribution was not met, the Kruskal-Wallis test was applied. In cases of statistically significant outcomes, the Wilcoxon rank sum test served as an alternative post hoc method to the pairwise t-test. Statistical significance was determined at an alpha level of 0.05. Moreover, for microbiome analysis following the bioinformatics procedures, data were initially processed using Microsoft Excel 365 for Windows (2021, Microsoft Corp., Redmond, WA, USA). Subsequently, the data were imported into the R software computing environment, where various R packages (v4.3.1) were utilized for further analysis. The statistical significance of the results was determined using the Mann-Whitney (MW) non-parametric test, with a significance threshold set at $p < 0.05$ for all analyses. For alpha diversity assessment, we employed the Chao1 richness and Shannon diversity index. Chao1 estimated species richness, while Shannon considered richness and evenness. These metrics were illustrated using boxplots to visualize seasonal variations. To explore beta diversity, we utilized non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance. Bray-Curtis quantified compositional

dissimilarity between bacterial communities. The NMDS plot visually represented seasonal shifts in microbial communities.

3. Results

3.1. Pathogen prevalence

A subset of 100 samples ($n = 50$ from winter and n = 50 from spring), consisting of 62.5 % of the total samples collected for the enumeration of indicators, was selected to assess the presence of *L. monocytogenes*, *Cronobacter* spp., and *E. coli* O157:H7. These samples were randomly selected from the total of samples collected. During winter sampling, four feed and two water samples tested positive for *Cronobacter* spp.; two samples showed presumptive evidence of *L. monocytogenes*, one in feces and another in a cattle mat; and *E. coli* O157:H7 was detected in two feed samples. During spring, *Cronobacter* spp. was identified in four feed samples and one hallway drain, while *E. coli* O157:H7 was present in only one feed sample. *L. monocytogenes* was not found during the spring sampling. Overall, the prevalence of *Cronobacter* spp. 12.0 % (6/50) and 10.0 % (5/50) during the winter and spring, respectively. *E. coli* O157:H7 was detected in 4.0 % (2/50) of the samples during the winter and 2.0 % (1/50) in the spring. Lastly, *L. monocytogenes* exhibited a prevalence of 4.0 % (2/50) during the winter and absent (0/50) during the spring.

3.2. E. coli and S. aureus counts

E. coli and *S. aureus* were used as indicators of microbial loads in the farm environment. For better visualization of the farm layout, results were divided into two zones: cattle pens (bedding, feces, feed, and water) and milking parlor (towels, milk, pit drains, hallway drains, milking cups, tubes, and metal surfaces, worker's mat, cattle mat, and udder). *E. coli* counts showed a variation in microbial concentrations, and a statistical difference ($p = 0.025$) was found between winter and spring. The mean average *E. coli* count was higher during winter (4.39 Log CFU/g or mL) compared to the spring (3.96 Log CFU/g or mL) (Table 3). No statistical difference in *E. coli* counts was detected between the different zones ($p = 0.281$). Variations by sample type were observed, with *E. coli* counts displaying significant differences ($p < 0.001$). Among all the samples, the highest concentration of *E. coli* was observed in bedding, feces, cattle mats, hallway drains, and worker's mats (Fig. 2).

With respect to *S. aureus* counts, no statistically significant difference was observed when comparing the two seasons ($p = 0.068$). However, a trend towards higher *S. aureus* counts was noted during spring. Specifically, the mean *S. aureus* count was 2.19 Log CFU/g or mL during the winter, slightly increasing to 2.34 Log CFU/g or mL in the spring (Table 3). Furthermore, a significant difference was observed in zones and sample types ($p < 0.001$), indicating that *S. aureus* counts varied across these categories (Fig. 3). Additionally, no significant difference was found when analyzing the interaction between zone and season ($p = 0.301$), indicating that the levels of *S. aureus* within each location remained relatively consistent across different seasons. Bedding, feces, and feed were found to have the highest *S. aureus* concentrations (Table 3).

3.3. S. aureus confirmation

After microbial culturing, 237 isolates (bedding [$n = 12$], feces [$n = 15$], feed [$n = 6$], water [n = 6], towels [$n = 24$], milk [$n = 16$], pit drains [$n = 22$], hallway drains [$n = 18$], milking cups [$n = 20$], tubes and metal surfaces [n = 24], worker's mat [$n = 39$], cattle mat [$n = 17$], and udder [n = 18]) with typical morphology were recovered in both seasons, with 133 in the winter and 104 in the spring. All presumptive *S. aureus* isolates were subjected to confirmation using MALDI-TOF. A total of 182 out of the 237 isolates were confirmed to be *aureus* and non-*aureus* staphylococcal species and *Aerococcus viridans*. Among the various *Staphylococcus* species identified, *S. aureus* emerged as the predominant, being present in most of the samples, including those from cattle pens, the milking parlor, and raw milk collected from the bulk tank. In contrast, *S. epidermidis*, *S. equorum*, *S. hyicus*, and *S. xylosus* were the least dominant species within the samples, primarily observed in towels, pit drains, bedding, and feed (Table 4).

3.4. Descriptive microbial analysis of the farm environment

Of the 13 sample types (as indicated in Table 1), only seven of them were included in the microbiome analysis (bedding, cattle mat, feces, feed, hallways drain, pit drain, and worker mat). This was due to the availability of usable DNA in terms of quantity and quality. Even though the protocol was followed equally for all sample types, not all of them had DNA in sufficient quantity and quality accepted for this test. Composite DNA samples were created per each sample type. A total of 14 samples, including seven from winter and seven from spring, were subjected to microbiome analysis. The results revealed that there was no variation in microbial communities between the two seasons, as

Table 3
Summary table of *S. aureus* and *E. coli* counts in winter and spring.

Zone	Sample type (n)/each season[1]	Microorganism			
		S. aureus (Log CFU/unit[2]±SE[3])		*E. coli* (Log CFU/unit[2]±SEM[3])	
		Winter	Spring	Winter	Spring
Cattle Pens	Bedding (6)	4.44 ± 0.09	4.54 ± 0.06	5.96 ± 0.55	4.88 ± 0.68
	Feces (6)	4.06 ± 0.16	4.28 ± 0.12	6.66 ± 0.24	5.74 ± 0.28
	Feed (6)	3.61 ± 0.13	4.18 ± 0.08	2.29 ± 0.39	2.83 ± 0.23
	Water (4)	0.70 ± 0.00	−0.30 ± 0.00	1.84 ± 0.10	0.78 ± 0.23
Milking Parlor	Towels (5)	0.56 ± 0.23	1.19 ± 0.83	4.69 ± 0.68	4.00 ± 0.13
	Milk (4)	1.26 ± 0.33	2.26 ± 0.06	1.01 ± 0.31	−0.30 ± 0.00
	Pit drains (7)	1.20 ± 0.23	1.15 ± 0.16	3.79 ± 0.61	4.04 ± 0.27
	Hallway drains (7)	2.57 ± 0.48	3.09 ± 0.74	5.25 ± 0.39	5.08 ± 0.10
	Milking cups (8)	0.86 ± 0.28	2.22 ± 0.21	2.42 ± 0.64	4.60 ± 0.10
	Tubes and metal surfaces (8)	1.73 ± 0.36	2.07 ± 0.29	3.87 ± 1.16	4.65 ± 0.46
	Worker's mat (7)	2.15 ± 0.31	1.75 ± 0.19	5.07 ± 0.10	4.73 ± 0.15
	Cattle mat (7)	3.22 ± 0.11	2.31 ± 0.20	6.62 ± 0.16	5.50 ± 0.25
	Udder (5)	1.29 ± 0.46	1.68 ± 0.32	3.83 ± 1.10	3.93 ± 0.23
Overall		2.19 ± 0.16	2.34 ± 0.17	4.39 ± 0.29	3.96 ± 0.20

[1] The number in parenthesis represents the samples collected each season, and equal numbers were obtained in winter and spring. The total of samples tested was 160; 80 per season.
[2] Unit = g or mL as presented in results, according to the type of sample.
[3] Standard error of the mean.

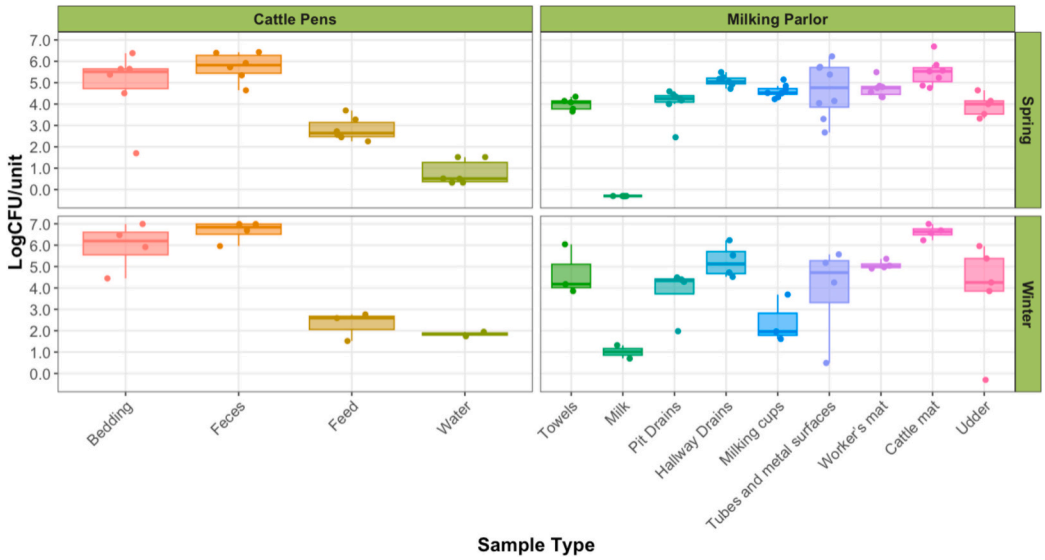

Fig. 2. *E. coli* counts during winter and spring in different samples collected in a dairy farm in West Texas. The horizontal line within the boxplot represents the median. The upper and lower limits of the box represent the interquartile range, while the bars extending from the box represent values up to 1.5 times the interquartile range. The plot depicts individual data points as dots.

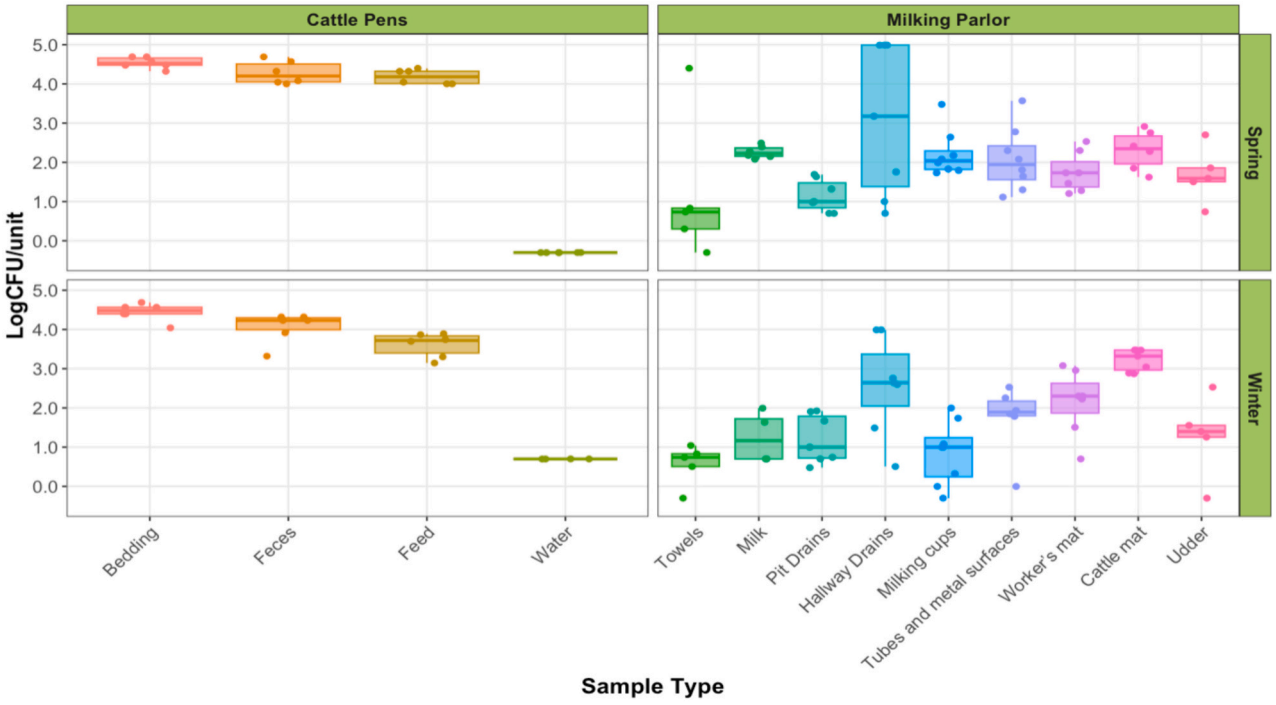

Fig. 3. *S. aureus* counts during winter and spring in different samples collected in a dairy farm in West Texas. The horizontal line within the boxplot represents the median. The upper and lower limits of the box represent the interquartile range, while the bars extending from the box represent values up to 1.5 times the interquartile range. The plot depicts individual data points as dots.

indicated by non-significant results for both the Chao1 and Shannon diversity indices ($p > 0.05$) (Fig. 4a). Furthermore, when considering the general structure of the microbiome communities, beta diversity did not differ between winter and spring, as illustrated by the NMDS analysis (Fig. 4b).

3.4.1. Seasonal differences

Proteobacteria and Firmicutes were the dominant phyla in both seasons. During the winter, Proteobacteria accounted for 46.0 % of the relative abundance, while Firmicutes comprised 27.2 %. Conversely, Firmicutes was slightly higher than Proteobacteria in the spring, with abundances of 35.8 % and 32.54 %, respectively. Among other prevailing phyla identified in both seasons, Actinobacteriota was consistently present in winter (7.9 %) and spring (11.4 %), while Bacteroidota was found during winter and spring at 7.6 % and 6.7 %, respectively (Fig. 5a). When comparing phyla between both seasons, no statistical difference was observed ($p > 0.05$) (Fig. 5b).

Moraxellaceae (11.8 %) and Pseudomonadaceae (10.62 %) stood out when examining the family-level distribution of microbial communities during winter, followed by Carnobacteriaceae (8.0 %), Aeromonadaceae

Table 4
Confirmed isolates from presumptive *S. aureus* positive samples by MALDI-TOF.

Microorganism	Isolates[1]/season							
	Winter (n)	%	Sample[2]	Spring (n)	%	Sample[2]	Total (n)	%
S. aureus	68	63.6	Feces, Towels, Milk, Hallway drains, Pit drains, Milking cups, Tubes and metal surfaces, Worker's mat, Udder	42	56.0	Water, Towels, Milk, Hallway drains, Milking cups, Tubes and metal surfaces, Worker's mat, Cattle mat, Udder,	110	60.4
S. simulans	11	10.3	Towels, Pit drains, Tubes and metal surfaces, Worker's mat, cattle mat, bedding,	12	16.0	Bedding, Hallway drains, Pit drains, Tubes and metal surfaces, Worker's mat	23	12.6
S. chromogenes	3	2.8	Pit drains, Cattle mat	6	8.0	Water, Hallway drains, Tubes and metal surfaces, Udder	9	4.9
S. sciuri	8	7.5	Bedding, Hallway drains, Worker's mat	2	2.7	Water, Worker's mat	10	5.5
S. haemolyticus	5	4.7	Pit drains, Towels, Tubes and metal surfaces	3	4.0	Tubes and metal surfaces, Bedding, Towels	8	4.4
S. saprophyticus	2	1.9	Worker's mat	3	4.0	Feed, Towels, Hallway drains	5	2.7
S. lentus	2	1.9	Water, Bedding	1	1.3	Pit drains	3	1.6
S. epidermidis	1	0.9	Towels	0	0.0	–	1	0.5
S. equorum	1	0.9	Pit drains	0	0.0	–	1	0.5
S. hyicus	1	0.9	Bedding	0	0.0	–	1	0.5
S. xylosus	0	0	–	1	1.3	Feed	1	0.5
A. viridans	5	4.7	Feces, Tubes and metal surfaces	5	6.7	Hallway drains, Pit drains, Bedding, Milking cups	10	5.5
Total	107	100	–	75	100	–	182	100

[1] Multiple isolates were obtained from the same sample.
[2] Sample types where *Staphylococcus* spp. and *A. viridans* were detected on the farm.

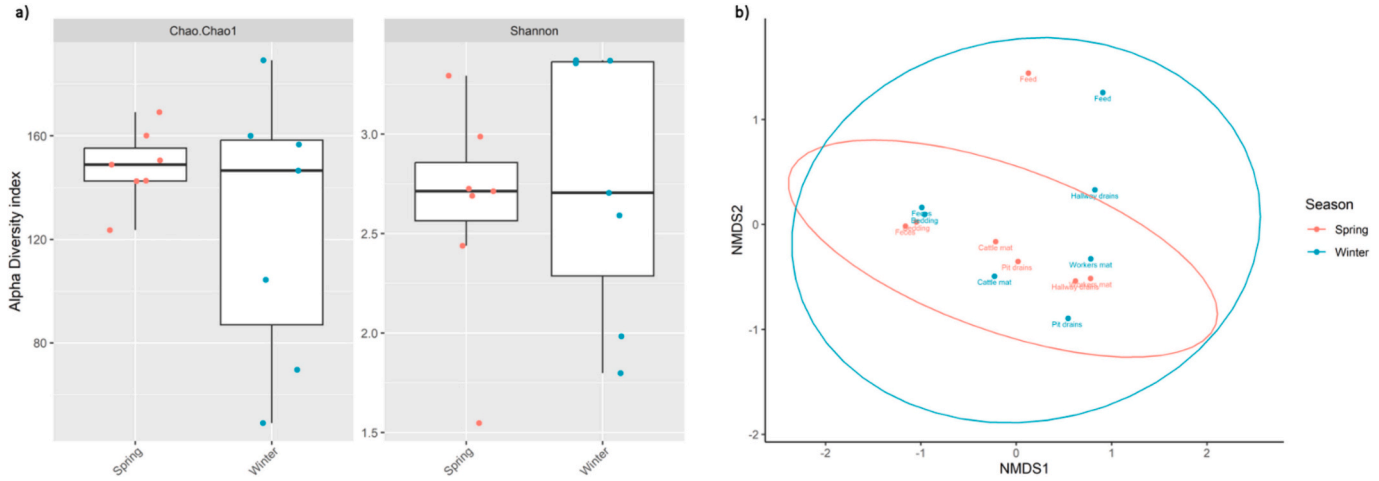

Fig. 4. a) Environmental microbiota of a dairy farm across seasons. Boxplots of alpha diversity as measured by Chao1 richness and Shannon diversity index. **b)** Non-metric multidimensional scaling (NMDS) plot of the Bray-Curtis distance for bacterial communities between seasons.

(7.6 %), and Enterobacteriaceae (7.4 %). On the other hand, during spring, Lactobacillaceae (13.0 %), Enterobacteriaceae (12.6 %), Moraxellaceae (9.2 %), Streptococcaceae (6.5 %), and Micrococcaceae (5.5 %) exhibited the highest abundances (Fig. 6a). Moreover, there was no statistical distinction ($p > 0.05$) when comparing families between the two seasons (Fig. 6b).

3.4.2. Sample type differences

When analyzing the distribution of phylum across different sample types, Proteobacteria was the dominant group in hallway drains (69.1 %), pit drains (53.0 %), cattle mats (47.7 %), and worker's mats (44.7 %). In contrast, Firmicutes were prevalent in feces (50.1 %), bedding (39.7 %), and feed (38.3 %) (Fig. 7a).

The microbial composition exhibited distinct patterns in various sample types at the family level. Lactobacillaceae dominated in feed (56.3 %), Corynebacteriaceae in bedding (22.9 %), Enterobacteriaceae in pit drains (21.4 %) and cattle mats (17.0 %), Moraxellaceae in hallway drains (17.0 %), Streptococcaceae in worker's mats (12.0 %), and Oscillospiraceae in feces (10.0 %). It is worth noting that the Enterobacteriaceae family exhibited a consistent presence in feces, pit drains, cattle mats, hallway drains, and worker's mats. In addition, the Staphylococcaceae family was also among the primary families detected in bedding (Fig. 7b).

4. Discussion

This investigation highlighted the presence of *Cronobacter*, *L. monocytogenes*, and *E. coli* O157:H7 at various locations in the dairy farm setting. *Cronobacter* is a major health hazard that has been link to infant formula outbreaks. The presence of this organism in feed samples aligns with a study conducted in Australian dairies that examined samples such as soil, feces, feed, and trough water, which similarly emphasized the occurrence of *Cronobacter* in animal feed (McAuley et al., 2014). Molloy et al. (2009) studied *Cronobacter* in food animals and their environment to identify if animal production was a risk factor for transmission of the foodborne pathogen; consistently with our study, the authors found the organism in dried animal feed (Molloy et al., 2009). However, neither of those two studies found *Cronobacter* in trough water, which contrasts with the findings in the present investigation, where two water samples displayed the presence of *Cronobacter*.

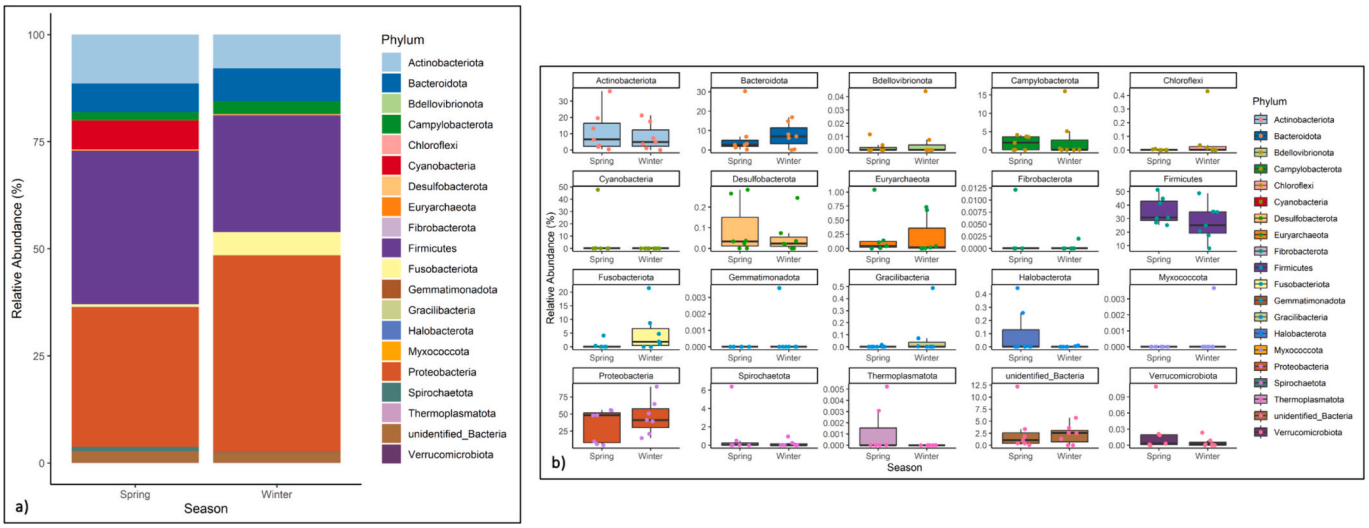

Fig. 5. a) Descriptive analysis of phylum-level relative abundance in winter and spring. **b)** Comparative analysis of dominant phyla in winter and spring.

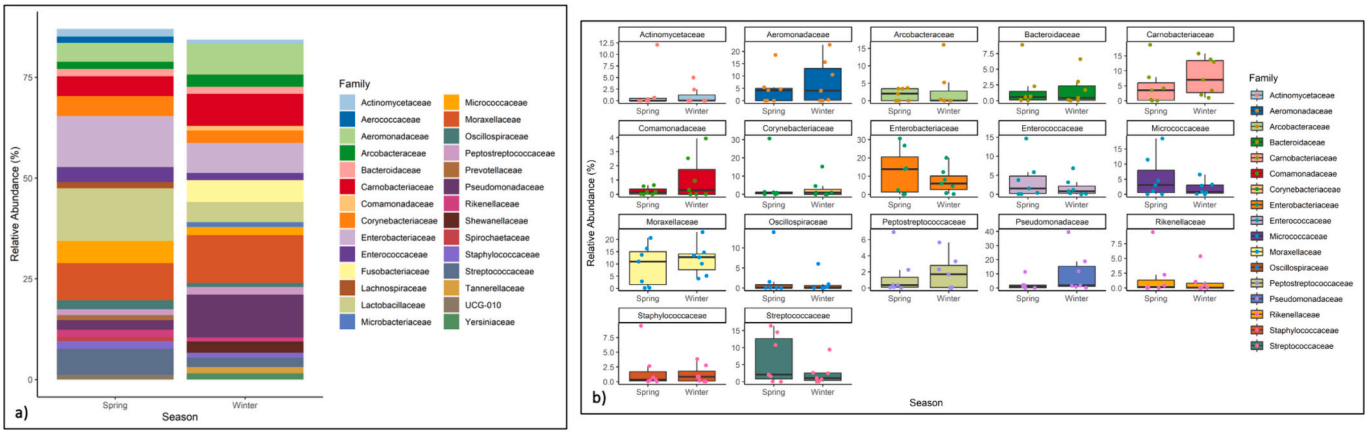

Fig. 6. a) Descriptive analysis of relative abundance at family level in winter and spring. **b)** Comparative analysis of dominant families in winter and spring.

Furthermore, detecting *Cronobacter* in a hallway drain sample from the farm could potentially be attributed to contamination from water. These findings are not surprising, as *Cronobacter* is commonly found in natural environments such as plants and organic materials, food from animal and plant-origin, and water (Beuchat et al., 2009, Kalyantanda et al., 2015, CDC, 2023). It is important to note that the presence of *Cronobacter* in the dairy farm environment poses a risk of transferring the organism to dairy manufacturing facilities. Raw milk trucks and personnel moving between the farm and processing facilities could carry the microorganism and transfer it to the dairy manufacturing environment.

E. coli O157:H7 was also present in the feed samples. Research has previously demonstrated the occurrence of this pathogen in a range of feed ingredients, including dried forage, grains, hay, and silage, suggesting their potential as sources of *E. coli* O157:H7 (Nazareth et al., 2021). While this microorganism is commonly associated with ruminant/cattle feces, *E. coli* O157:H7 could enter the animal's gastrointestinal tract through contaminated feed and establish itself within the animal, eventually being shed (Bach et al., 2002; Callaway et al., 2009; Atnafie et al., 2017).

On the other hand, evidence of *L. monocytogenes* in feces and cattle mats within the milking parlor, specifically during the winter season, concurs with prior studies that have reported *L. monocytogenes* in fecal samples (Nightingale et al., 2004; Ho et al., 2007). Additional research has highlighted an increased prevalence of *L. monocytogenes* shed by dairy cattle during the winter, especially from cows undergoing second lactation (Schoder et al., 2022). This seasonal trend raises an interesting aspect of transmission dynamics. The possible transfer of *L. monocytogenes* from the pen to the milking parlor could occur as cows transport the bacteria on their skin, possibly alongside traces of feces. This potentially designates cows as pathogen carriers, utilizing their movements across the farm as a mechanism for pathogen transmission (Nightingale et al., 2004).

Furthermore, a statistical difference in *E. coli* counts emerged between the winter and spring, with higher counts observed during winter. This difference, while statistically significant, was relatively modest. Other investigations have reported more substantial variations in *E. coli* counts between warmer months and colder months, revealing a difference of approximately 2.5 Log CFU/unit between seasons (Petersen and Hubbart, 2020). Data reported by the National Oceanic and Atmospheric Administration (NOAA) during sampling days of the present study shows an average of 11 °C ten days before each sampling event around the geographical location of the farm (NOAA, 2024). Research demonstrates that temperature's influence extends beyond thermal conditions. Factors like rainfall, air temperature, sunlight, and relative humidity contribute to bacterial growth (Litt et al., 2021).

The widespread presence of *E. coli* throughout the farm is not unusual; this organism is not only found in the gastrointestinal tract of cattle but is also considered environmental (Lambertini et al., 2015). *S. aureus* found across diverse samples collected during this study

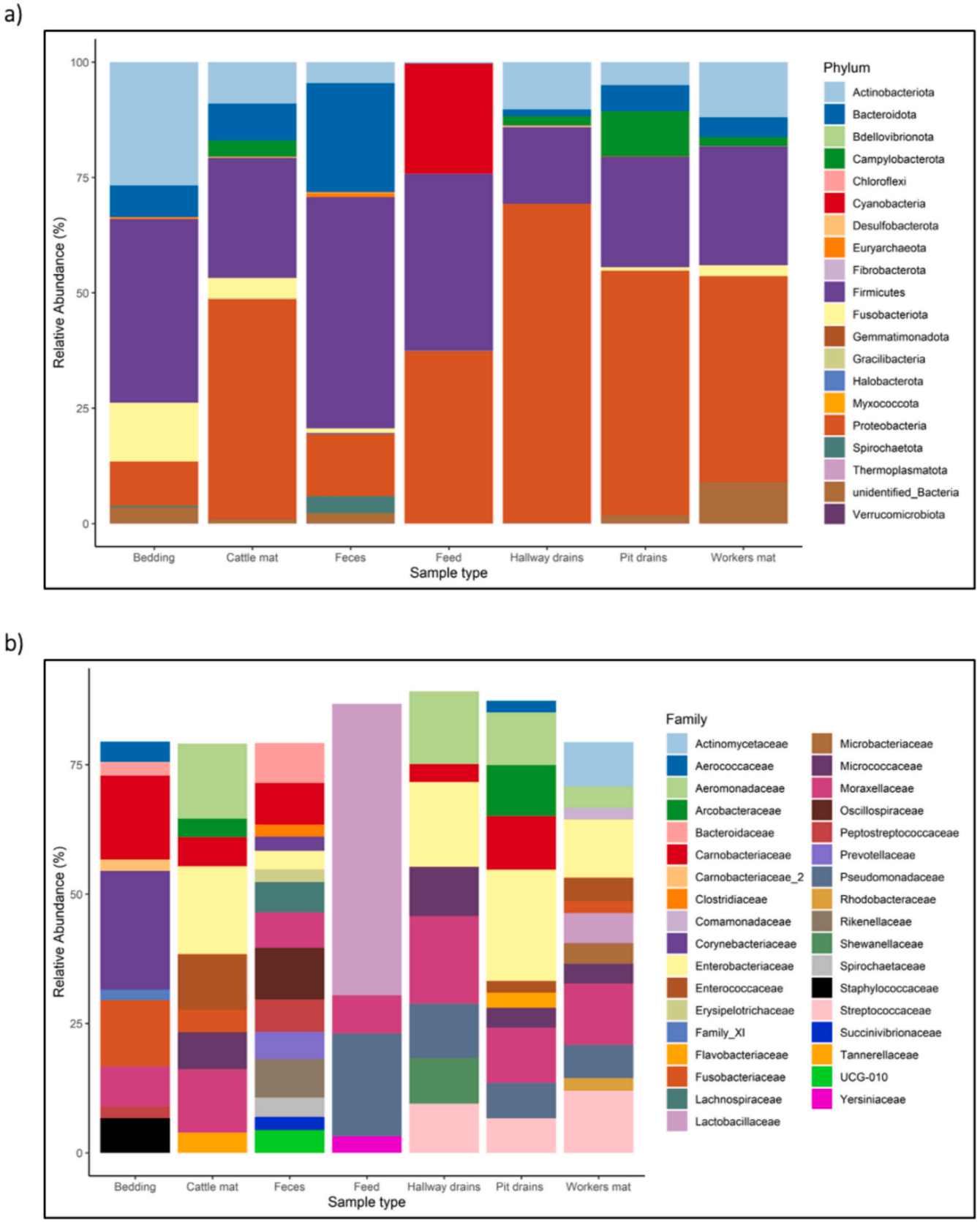

Fig. 7. **a)** Phylum and **b)** family-level relative abundance distribution distributed by sample type.

exhibited higher concentrations in cattle pens. Several research studies have reported the presence of *S. aureus* surrounding the cow's direct environment with variations between herds (Capurro et al., 2010; Rowbotham and Ruegg, 2016), and these findings are well documented. Consistent with observation from this investigation, previous studies have also highlighted the existence of *S. aureus* in the bulk tank and the milking production area. *Staphylococcus aureus's* ubiquity throughout the farm is notable, as it was isolated from various sources, including cows (teat skin and udder), humans, processing equipment, and the surrounding environment (Capurro et al., 2010; Haran et al., 2012; Silva et al., 2022). Furthermore, this investigation also showed the prevalence of non-aureus staphylococci. Compared to previous studies, specific microorganisms such as *S. haemolyticus*, *S. sciuri*, *S. equorum*, *S. chromogenes*, and *S. simulans* have also been identified within the dairy farm environment (De Visscher et al., 2014; Roberts et al., 2018).

Upon analyzing the overall farm microbiota, a consistent prevalence was seen across both seasons at the phylum level. Proteobacteria and Firmicutes emerged as the most abundant phyla in various sample types for both seasons. Firmicutes and Proteobacteria taxa have been reported as dominant in the post-gastric intestines, including both the small and large intestines (Muñoz-Vargas et al., 2018; Mu et al., 2019; Lin et al., 2023). Proteobacteria represent one of the largest prokaryotic divisions, encompassing the majority of known Gram-negative bacteria, including families like Enterobacteriaceae and Moraxellaceae. Conversely, the phylum Firmicutes comprises families such as Streptococcaceae, Staphylococcaceae, Oscillospiraceae, and Lactobacillaceae.

The abundant presence of Staphylococcaceae, Moraxellaceae, Corynebacteriaceae, Lactobacillaceae, and Streptococcaceae on bedding and airborne dust microbiota has been documented (Liu et al., 2019; Nguyen et al., 2020; Mtshali et al., 2022; Hoskisson, 2018). Rich organic material within the farm harbors the highest microbial diversity, facilitating the dispersal of microorganisms throughout the farm, which was consistent with *S. aureus* enumeration results in the present study. It has been reported that the status and type of bedding influence the growth of various bacterial taxa during use by cows (Manyi-Loh et al., 2016; Pandey et al., 2018; Alegbeleye and Sant'Ana, 2020).

Members of the Enterobacteriaceae family are known pathogens affecting both human and animal health. This family includes bacteria such as *Salmonella*, pathogenic *E. coli*, and *Cronobacter* spp., which have been associated with food poisoning (Bintsis, 2017). Furthermore, *E. coli* has been shown to cause clinical mastitis in cattle, impacting animal health (Goulart and Mellata, 2022). Bacteria within the Staphylococcaceae and Streptococcaceae families have been linked to mastitis infections in dairy cattle, as well as a range of human diseases, including food poisoning and throat infections (Argudín et al., 2010, Pumipuntu et al., 2019, Kanwal and Vaitla, 2022). Members of the Moraxellaceae family have been associated with infectious bovine keratoconjunctivitis (IBK), an ocular disease affecting cattle, while in humans, Moraxellaceae is recognized as an opportunistic pathogen responsible for causing a wide range of infections (Tan and Grewal, 2001; Loy and Brodersen, 2014).

5. Conclusion remarks

A diverse array of samples was selected to explore the microbial ecology and distribution in a dairy farm focusing on foodborne organisms. The selection was guided by the expectation that those from the cattle pen, such as bedding, feed, and feces, would harbor a significant concentration of microbes, as they are central components of the cattle environment (Ray et al., 2022). These findings provide valuable insights into the microbial landscape of the dairy farm.

The study demonstrated that some of the most important foodborne pathogens that have historically caused foodborne diseases and outbreaks linked to dairy products are highly prevalent in the primary production environment. This highlights the need for interventions at the farm level to effectively reduce the risk of these pathogens being transferred to dairy processing facilities, particularly more strict sanitation routines. Additionally, the widespread presence and their high bacterial loads of organisms such as *E. coli* and *S. aureus*, recognized as causative agents of mastitis, suggests that the environment plays an important role in disseminating these bacteria, increasing the chances of animal disease.

While the presence of a wide variety of microorganisms is expected, understanding their prevalence, distribution, concentration, and abundance on the farm provides significant information for dairy facilities to design and implement targeted control measures. Creating protocols that specifically target the reduction of microbial loads in the environment is recommended. This includes rigorous hygiene practices around the milking parlor and specific interventions to control microorganisms in the bedding material, which could serve as an aid to minimize bacterial transfer from the environment to animals, therefore contributing to enhancing both animal and public health.

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CRediT authorship contribution statement

Angela Perdomo: Writing – original draft, Methodology, Investigation, Formal analysis. **Alexandra Calle:** Writing – review & editing, Supervision, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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