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Identification and Description of Culturable Airborne Bacteria Suspended in Aerosols from the Milking Area in Two Dairy Farms in Puerto Rico by Using MALDI-TOF MS

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Abstract Due to its nutritional value and physicochemical characteristics, milk is susceptible to detrimental and pathogenic microorganisms. Therefore, the dairy industry needs to control the factors that pose risks to the safety and quality of the product. Thus, a microbiological analysis of the air was carried out in the milking area of two dairy farms in Puerto Rico. Triplicate air samples were collected from May to September 2021. The results showed that farm B had higher bacteria concentrations in most months. Sixty-three genera of bacteria were identified among all the samples collected from both farms using MALDI-TOF MS. Additionally, several important sanitary bacteria were detected in the samples, albeit at a low frequency. In conclusion, the results of this study demonstrate that the concentration of microorganisms in the air of milking areas can be influenced by different factors, such as the location of the farm, as well as temperature and relative humidity. Furthermore, MALDI-TOF MS proved to be a helpful and fast technique for identifying the isolated bacteria in the samples.

Keywords: *milk, food microbiology, environmental microbiology, milking area, environment*

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1. Introduction

Cows' milk is widely used product, because of its nutritional value. However, its physicochemical characteristics make it susceptible to detrimental and pathogenic microorganisms that may affect its quality [1]. In dairy farms, the microorganisms can vary by the facility's location, environmental conditions, management practices, storage, and the effectiveness of the cleaning processes [2]. Therefore, the dairy industry aims to control those factors that would put the safety and quality of the product at risk by implementing regulations and guidance for cleaning and disinfection, animal health, employee hygiene, and the farm's natural environment [3].

Identification of microorganisms in bioaerosols of farms have been proposed as a tool to identify possible detrimental and pathogenic microorganisms before the product reaches its destination. Currently, several microbiological methods are used to analyze the presence of microorganisms in the process environments that occur on livestock farms. Most of those methods sought to isolate, purify, classify, and identify the microorganisms found during environmental sampling [4,5,6]. However, many of these processes take long to obtain results, especially when identifying a microorganism. For this reason, instrumentation such as Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) is being proposed as an accurate and fast technique to identify microorganisms [7]. MALDI-TOF-MS uses protein analysis to identify bacteria, yeasts, viruses, and fungi, surpassing conventional identification methods that depend on a pure and fresh culture.

MALDI-TOF has been used successfully in several studies to identify microorganisms in livestock farms [8], including milk processing farms [9]. However, some/all these studies identified potentially pathogenic microorganisms such as *Aerococcus viridans*, *Corynebacterium striatum*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Enterococcus avium* [8]. Also, other bacteria that pose a risk of spoilage, such as *Staphylococcus* and *Bacillus* spp. [9].

Milk is a widely used human food product susceptible to deterioration caused by microbial contamination. Microorganisms in the environment surrounding livestock industries may affect the animals and the raw material (milk) obtained [10]. Therefore, the dairy industry needs to know the microbiological relationship between the environment and the process to ensure milk and animal health quality and safety. However, identifying this relationship is challenging in livestock farming because microbial identification is a slow and complex process [11]. Therefore, this study aimed to identify the bacteria suspended in aerosols from the milking area of two dairy farms in Lajas (farm A) and Hatillo (farm B) Puerto Rico by using MALDI-TOF MS.

2. Methodology

Microbiological sampling was conducted in the milking area of two dairy farms to isolate and identify the bacteria in bioaerosols using the MALDI-TOF MS technique. Samples were obtained monthly from May to September 2021 in three different areas of the milking process (entrance, middle, and exit).

2.1. Microbiological Sampling

The microbiological sampling was carried out according to Łukaszuk et al. [12] with some modifications. The bacteria suspended in the air were collected by using the SAS-Super 100/180 (International pbi Spa, Milan, Italy) air sampling system previously disinfected with 70% isopropyl alcohol. The equipment was programmed with a speed of 100L/min, and RODAC (replicate organism detection and counting) plate with Tryptic Soy Agar (TSA) / Lecithin was used for the isolation. The collected samples were covered with paraffin, placed in sterile bags, and transported at refrigeration temperatures of 4 - 6°C to the laboratory to be processed as soon as possible in a biological safety cabinet (Nuaire NU-440-400 Class II).

2.2. Sample Analysis

Before working with the samples, the laboratory and the equipment were disinfected to avoid sample contamination. Next, the RODAC plates were incubated at 30 - 35°C for 48 - 72 hours. After the incubation period, the colonies were counted, and the concentration of microorganisms was calculated using the following formula:

$$CFU / ft^3 = \frac{Colonies, \Pr * 28.3}{Sample\ Volume(L)} \quad (1)$$

Where Colonies, Pr is the adjusted value of probable colony counts, 28.3 is used to express the CFU/m^3 value in CFU/ft^3, and sample volume was 100 L.

The colonies obtained from the microbiological sampling were subcultured on the TSA culture medium and incubated at 30 - 35°C for 24 - 48 hours. The most isolated colonies were selected, transferred with a sterile needle, and streaked on the TSA plate. In addition, Gram staining was performed with the pure cultures.

2.3. Bacterial Identification – MALDI TOF MS

Isolated and purified bacteria were identified with Bruker MALDI-TOF Biotyper (positive ion mode) by the direct method (colony placed with matrix solution of α-Cyano-4-hydroxycinnamic acid) and bacterial DNA extraction [13].

2.3.1. Direct Method – MALDI TOF MS

In the direct method, fresh (24 - 48 hours) and pure cultures of the microorganisms isolated and subcultured in TSA medium were used. Using a sterile needle, a small part of the colony was placed in the stainless-steel sample plate used by the MALDI-TOF-MS [14]. The colony was allowed to dry at room temperature in the previously disinfected biosafety cabinet for one minute. After drying, 1 µl of formic acid and 1 µl of the matrix were added. In addition, a BTS standard was used. This standard contains an extract of *Escherichia coli* DH5 alpha and worked as a positive control to warrant that the MALDI TOF worked properly and identified the characteristic profile of peptides and proteins [15]. The peptide mass fingerprinting (PMFs) was then passed to the Accugenix® portal database to identify the microorganisms. DNA extraction was performed for isolates that were not properly identified within the database.

2.3.2. DNA Extraction

In a 1.5 mL microtube, 300 µl of molecular-grade water and the isolated bacteria were added and mixed in a vortex for one minute. Next, 300 - 900 µl of ethyl alcohol was added and centrifuged (Benchmark MC-12 microcentrifuge) for 2 minutes at 13,000 rpm. The resulting supernatant was discarded, and the centrifugation step was repeated. Subsequently, the supernatant was discarded, and 50 µl of 70% formic acid was added and mixed on the shaker to dissolve the precipitate. After this step, 20-50 µl of acetonitrile was added, mixed, and centrifuged. Finally, 1 µl of the supernatant was placed in the cover plate of the MALDI-TOF-MS and allowed to dry at room temperature in the biosafety cabinet, after which 1 µl of the matrix was added. When the plate dried, it was placed in the MALDI-TOF MS for analysis following the same indications as in the direct method [15].

2.4. Statistical Analysis

A paired *t*-test at 0.05 significant level was used to determine if the concentration of airborne microorganisms was statistically different in each dairy farm during the sampling months.

3. Results

The results obtained in farms A and B during the sampling months are presented.

3.1. Bacterial Concentration

Farm B consistently exhibited higher levels of bacteria throughout the sampling months. The *t*-test analysis

conducted on the microorganism concentrations between both farms from June to September showed a significant difference, which is clearly displayed in Table 1.

Table 1. Statistical analysis results for the *t*-test analysis conducted on the microorganism concentrations (CFU/ft3) between both farms

Months	Farm	N	Mean	*P* value*
May	A	9	2463	0.093
	B	9	1630	
June	A	7	3883	0.005
	B	7	1424	
July	A	9	2484	0.018
	B	9	1242	
August	A	9	1364	0.001
	B	9	910	
September	A	9	1414	0.009
	B	9	1158	

* *P* values were determined by paired *t*-test at 0.05 significant level.

3.2. Predominant Identified Bacteria

A total of 200 isolated organisms were identified using MALDI-TOF MS, of which 17 families and 63 genera of bacteria were identified. The families found most frequently were *Bacillaceae*, *Staphylococcaceae*, *Moraxellaceae*, and *Planococcaceae* (Figure 1). Gram-positive microorganisms predominated with 58% occurrence, while 42% of the organisms isolated were Gram-negative.

Table 2 shows the bacterial families and species occurrences during the sampling months in both farms. Thus, the *Bacillaceae* was the predominant family, with 15 species in all analyzed samples. The most frequent microorganisms of this family in the five sampling months were *Bacillus cereus* and *Bacillus altitudeinis/pumilus/safensis*, followed by *Exiguobacterium acetylicum* and *E. indicum* isolated during the months of July and August in both farms. Also, *in farm A was detected Bacillus subtilis* and *Exiguobacterium aurantiacum* from May to July.

The *Staphylococcaceae* was the second most frequently isolated group of microorganisms with 10 species. Of this family, the genus found the most in the five samplings was *Staphylococcus chromogenes* in both farms, followed by *Staphylococcus sciuri*, which was detected in May, July, and August in farm A. Something similar happened with *Staphylococcus gallinarum*, which was detected in farm B in May, June, and August.

The third most frequent group was the *Moraxellaceae* family, with seven species isolated and identified by the MALDI-TOF technique. All the bacteria identified were from the *Acinetobacter* genera. In this group, *A. indicus* was the most frequent bacteria, detected in May and September in both farms. The occurrence of the other species was random during the five sampling months in both farms.

Five species of bacteria were detected during the sampling months in farms A and B in the Enterobacteriaceae and Micrococcaceae families. For the *Planococcaceae* family, three species of *Kurthia* were identified, being *Kurthia massiliensis* the most frequent in both farms. Other families such as *Alcaligenaceae*, *Caulobacteraceae*, *Comamonadaceae*, *Corynebacteriaceae*, *Erwiniaceae*, *Flavobacteriaceae*, *Microibacteriaceae*, *Paenibacillaceae* and *Xanthomonadaceae*.

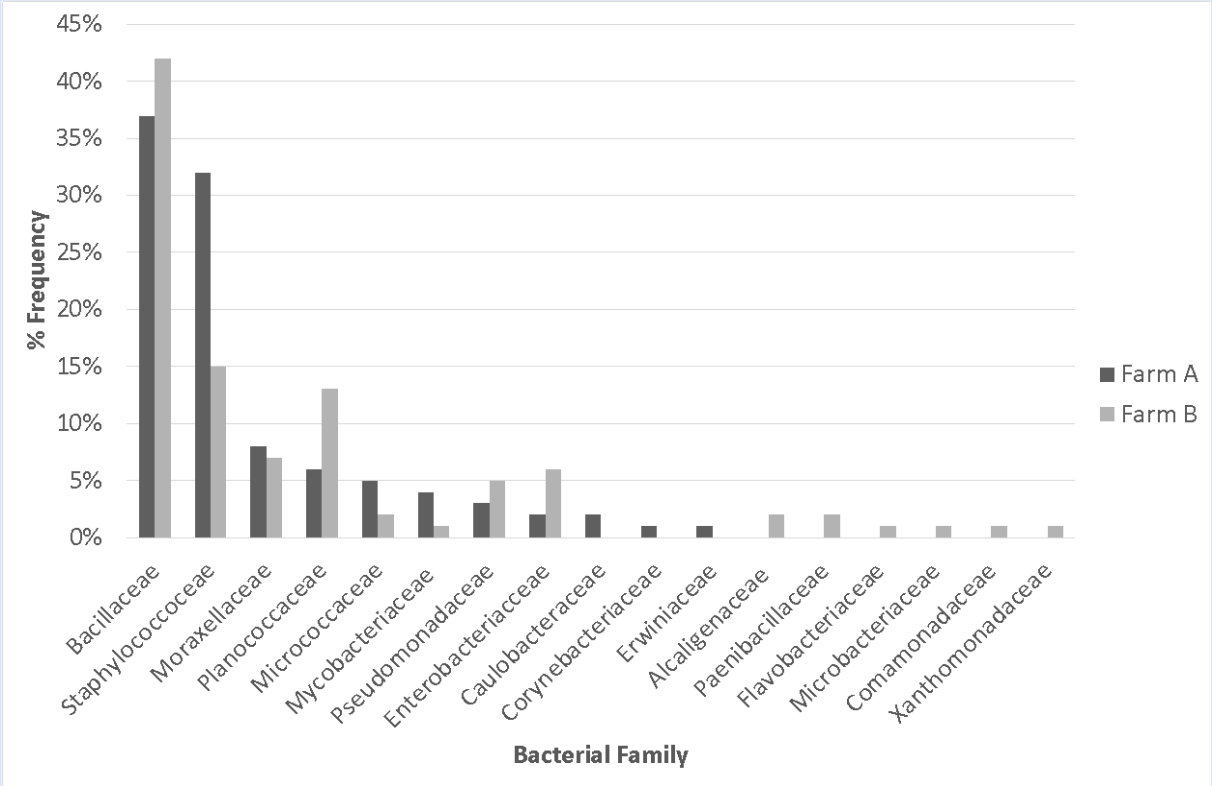

Figure 1. Frequency of bacterial families isolated in both farms (A & B). The frequencies were determined by the number of microorganisms identified in the family divided by the total number of all microorganisms identified

Table 2. Bacterial families and species occurrence during the sampling months in farms A & B

		May		June		July		August		September	
Family	**Species**	**Farm A**	**Farm B**	**Farm A**	**Farm B**	**Farm A**	**Farm B**	**Farm A**	**Farm B**	**Farm A**	**Farm B**
Alcaligenaceae	*Alcaligenes faecalis*				x						
Bacillaceae	*Alkalihalobacillus gibsonii*							x			
	Bacillus amyloliquefaciens/velezensis						x		x		
	Bacillus altitudinis/pumilus/safensis	x	x	x			x	x	x	x	x
	Bacillus cereus	x	x	x	x	x	x	x			x
	Bacillus firmus/oceanisediminis	x									
	Bacillus indicus	x									
	Bacillus licheniformis	x	x	x							
	Bacillus marisflavi	x	x								
	Solibacillus silvestris or Bacillus (Solibacillus) isronensis							x			
	Bacillus subtilis	x		x		x					x
	Exiguobacterium acetylicum	x				x	x	x	x	x	
	Exiguobacterium aurantiacum	x		x		x					
	Exiguobacterium indicum	x				x	x	x	x		
	Exiguobacterium profundum					x		x		x	x
	Rossellomorea marisflavi			x		x					
Caulobacteraceae	*Brevundimonas aurantiaca / intermedia / mediterranea / nasdae / vesicularis*							x			
Comamonadaceae	*Delftia acidovorans/lacustris*				x						
Corynebacteriaceae	*Corynebacterium* sp.										x
	Corynebacterium efficiens				x						
Enterobacteriaceae	*Cronobacter dublinensis*								x		
	Enterobacter cloacae		x								
	Enterococcus casseliflavus					x					
	Klebsiella aerogenes		x								
	Klebsiella pneumoniae			x					x		
	Serratia marcescens				x						
Erwiniaceae	*Pantoea agglomerans*			x							
Flavobacteriaceae	*Myroides odoratus*				x						
Microbacteriaceae	*Leucobacter aridicollis*				x						
Micrococcaceae	*Arthrobacter gandavensis/koreensis*			x	x						
	Glutamicibacter mysorens	x									
	Rothia aerolata									x	
	Rothia sp.					x					
	Rothia endophytica						x				
Moraxellaceae	*Acinetobacter baumannii*					x					
	Acinetobacter indicus	x	x							x	x
	Acinetobacter refrigeratorensis	x									
	Acinetobacter tandoii									x	
	Acinetobacter schindleri									x	
	Acinetobacter towneri	x									
	Acinetobacter soli		x								
Microbacteriaceae	*Microbacterium arborescens*			x		x				x	x
Paenibacillaceae	*Paenibacillus hunanensis*								x		
	Paenibacillus lactis		x								
Planococcaceae	*Kurthia gibsonii*						x				
	Kurthia massiliensis	x	x			x		x	x		x
	Kurthia senegalensis		x								
Pseudomonadaceae	*Pseudomonas aeruginosa*	x	x								
	Pseudomonas composti/oleovorans				x						
	Pseudomonas koreensis/chlororaphis				x						
	Pseudomona putida group				x	x					
	Pseudomonas oryzihabitans	x									
Staphylococcaceae	*Macrococcus bovicus*			x						x	x
	Macrococcus canis/caseolyticus	x								x	
	Macrococcus carouselicus										x
	Macrococcus sp.	x									
	Staphylococcus arlettae										x
	Staphylococcus auricularis			x							
	Staphylococcus chromogenes	x	x	x	x			x	x	x	
	Staphylococcus gallinarum		x		x				x		
	Staphylococcus sciuri	x	x			x		x			
	Staphylococcus xylosus							x		x	
Xanthomonadaceae	*Stenotrophomonas maltophilia*										x

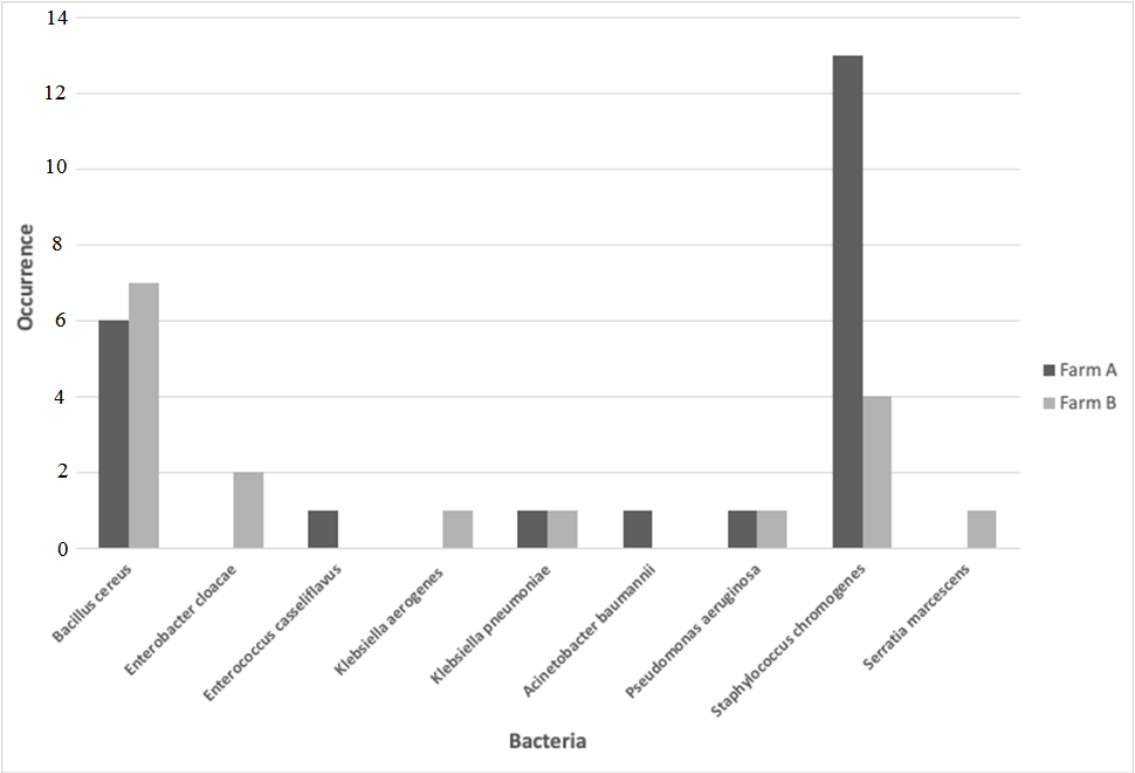

Figure 2. The occurrence of microorganisms of sanitary importance during all the sampling months

3.3. Microorganisms of Sanitary Importance

In this study, most of the sanitary-important bacteria detected in the sampling had a low occurrence (Figure 2), except for *Staphylococcus chromogenes* and *Bacillus cereus*. The other microorganisms detected in low frequency were *Enterobacter cloacae*, *Enterococcus casseliflavus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Serratia marcescens*.

4. Discussion

4.1. Bacterial Concentration

In this research, the number of bacteria suspended in the process area air may be influenced by some factors related to the conditions of this area. For example, temperature, relative humidity, and ventilation directly affect the quantity and distribution of bacteria in these process environments [16]. Also, in the case of milk-producing farms, the conditions of the milking place, the place available for the animals, and its design influence the concentration of microorganisms suspended in the air [17]. Additionally, other factors related to practices on the farm contribute to the concentration of air-suspended microorganisms, such as the frequency with which the facilities and equipment are cleaned before and after each milking process [11]. In this investigation, when comparing the number of cattle owned by both farms studied, farm B has a higher number than farm A. Also, farm A has a more rigorous cleaning system to perform the milking process. These practices may be responsible for farm B obtaining a higher number of bacteria suspended in the air in the milking area.

4.2. Predominant Bacteria Identified

4.2.1. *Bacillaceae*

The most frequent bacterial family in this study was the *Bacillaceae*. This family is commonly found in milking farm environments, contaminating dairy products and affecting their quality and shelf life with the presence of endospores produced by some genera belonging to this family [18]. Controlling this type of spore-producing microorganism in places of large-scale milk production is an important challenge for producers [19].

In this study, the most frequent *Bacillaceae* isolated was *B. cereus*. This result is comparable with the study by [20], where the *Bacillus* genus was most abundant during sampling on a chicken farm. In another study presented by [21], the two bacteria with the highest frequency in the microbiological monitoring of seven dairy farms were *B. cereus* and *Clostridium perfringens*, with the microorganism *B. cereus* appearing first in 93% of the samples taken.

B. cereus is commonly found in the environment (soil, water, air). It is a bacterium of interest in food microbiology due to its ability to produce enterotoxins that affect people's health [22]. Also, *B. cereus* is resistant to food cooking and pasteurization processes [23]. Additionally, it can cause infections such as bacteremia, meningitis, and brain abscesses [24]. The other bacteria with high frequency in the *Bacillaceae* family were *Bacillus altitudeinis*/*pumilus*/*safensis*. These microorganisms contaminate food and pharmaceutical facilities, causing product quality problems [25]. In addition, *Bacillus*

pumilus has been implicated in food poisoning, human infections, and bacteremia in humans [26].

Exiguobacterium was another genus frequently isolated in farm sampling and belonging to the *Bacillaceae* family. This genus is versatile and present in various environments, temperature ranges, pH, heavy metals, and salinity [27]. *Exiguobacterium* can be an essential resource for natural alternatives to the environment and agricultural practices (for its ability to promote plant growth and improve productivity) [28]. However, this genus of bacteria has been isolated as a contaminant from colostrum samples on dairy farms in the Czech Republic [29]. The researchers characterized this microorganism as one of the environmental contaminants present in 75% of the samples analyzed.

4.2.2. *Staphylococcaceae*

During this research, the *Staphylococcaceae* family was the second most frequent group isolated in both farms. The members of this family are characterized by being bacteria with a coccus morphology, Gram-positive, catalase-positive, and non-motile. They are antibiotic-resistant organisms and do not produce spores. These organisms survive various non-physiological environmental conditions and can grow in oxygen-rich (aerobic) environments or oxygen-depleted (anaerobic) conditions. Some genera of *Staphylococcus* spp. have been isolated from bovine udders, raw milk, storage tanks, and cheese samples [30]. This bacterium is highly related to the development of mastitis in susceptible cattle [31].

Some species of the *Staphylococcaceae* family (i.e., *S. aureus*, *S. chromogenes*, *S. sciuri*, *S. xylosus)* inhabit the skin, oral cavity, respiratory system, and intestine also are opportunistic pathogens. These bacteria can cause suppurative lesions and septicemia in humans and animals [32]. Multiple studies have used MALDI-TOF to identify *Staphylococcus* spp. in raw milk and cheese [30,33,34]. These studies demonstrate that the *Staphylococcus* identification technique by MALDI is reliable for identifying this family.

In this research, the bacterial species found most frequently in the five samplings was *Staphylococcus chromogenes*, with a frequency of 80% for farm A and 40% for farm B. This bacterium is a coagulase-negative, Gram-positive coccus, which can be isolated from dairy cattle with mastitis. *S. chromogenes* was isolated in the study by [20] from air samplings over several months in different areas of a poultry plant. Furthermore, *S. chromogenes* was isolated in bovine milk samples from 76 farms in Austria and identified using MALDI-TOF MS [35].

4.2.3. *Moraxellaceae*

The family *Moraxellaceae* comprises a heterogeneous group of bacteria widely distributed in different natural habitats and have diverse ecological and clinical significance [36]. Additionally, this family has members that can colonize the mucosa and skin of humans and animals, causing infections. Among the bacteria genera in this family are *Moraxella* spp., *Acinetobacter* spp., and *Psychrobacter* spp. [37].

Studies abundantly detected microorganisms belonging to the *Moraxellaceae* family in milk from farms in Henan and Heilongjiang Province [10]. The authors suggest that these microorganisms in milk may be due to transfer from feces into milk. These results relate to those in this investigation, where several species of *Acinetobacter* were isolated from the milking area air. Species of this genus, such as *A. baumannii*, isolated from raw milk, have demonstrated the ability to resist multiple antibiotics [38]. This adaptation risks the health of dairy farm animals due to the bacteria's ability to survive, potentially infecting animals and spreading.

4.2.4. *Enterobacteriaceae*

The *Enterobacteriaceae* family are Gram-negative bacteria associated with warm-blooded animals' gastrointestinal tract. Several bacteria in this family are pathogenic microorganisms related to food spoilage and safety. Therefore, they are considered a high-risk factor in raw milk. This family includes genera such as *Escherichia*, which inhabits the feces of animals; *Enterobacter*, *Citrobacter*, *Serratia*, and *Klebsiella*, present in soils, water, and grains [14]. Mastitis in cattle is associated with Gram-negative bacteria, especially those of the *Enterobacteriaceae* family. These bacteria mainly affect cows after milking due to contact of the udder with contaminated water and farm areas.

However, phenotypic methods often misidentify *Enterobacteriaceae* at the genus and species level. Therefore, there is an interest in using more precise techniques for identifying enterobacteria, such as MALDI-TOF MS. This interest was demonstrated in the study by [14], where 183 members of *Enterobacteriaceae* were identified with MALDI-TOF MS in milk, cow feces, water samples, and from the process area in a dairy farm in Rio de Janeiro, Brazil. The authors' statements are related to the findings of our investigation, in which we isolated several genera of the *Enterobacteriaceae* family (including *Cronobacter* sp., *Enterobacter* sp., *Enterococcus* sp., and *Klebsiella* spp.) from air samples collected in milking areas.

4.2.5. *Micrococcaceae*

The *Micrococcaceae* family comprises a diverse group of microorganisms exhibiting various morphological and chemical-taxonomic properties that distinguish between genera and species [39]. These bacteria are found in the skin and mucous membranes of humans and animals and can also be in the environment. Some species in this family can cause opportunistic infections in hosts. A study by [40] detected *Micrococcaceae* in environments associated with a dairy farm, including the bedding area and air during summer and winter. Our study's findings align with Nguyen et al.'s results, as we isolated *Arthrobacter* spp., *Glutamicibacter* sp., and *Rothia* spp. from the air in the milking area.

4.2.6. *Pseudomonadaceae*

This bacterial family comprises Gram-negative rod-shaped bacteria, saprophytic microorganisms in soil and marine waters, and some pathogens of animals, humans, and plants [41]. In addition, members of this family, such as *Pseudomonas* spp., are classified as plant growth promoters through certain growth factors, nutrients,

and disease prevention [42]. Studies have shown the presence of *Pseudomonas* spp. in milk samples and at various sampling points in the analyzed milk dairies [43]. Its presence in milk was related to inadequate handling of milking processes, hygiene, and product storage. These results are associated with this study since one of the bacteria isolated from this family was *Pseudomonas aeruginosa* during May in both farms. Furthermore, this *P. aeruginosa* bacterium has been related, isolated, and identified with the MALDI-TOF technique in an outbreak of mastitis in a herd of dairy cows where it was determined that the outbreak occurred with a single strain of the microorganism using a cleaning solution that contained the bacteria [44].

4.2.7. *Planococcaceae*

The *Planococcaceae* family is taxonomically heterogeneous. According to [42], more than one hundred species of bacteria are grouped into fourteen genera. These are primarily bacilli and Gram variables; some can produce motile and aerobic spores. Also, the *Planococcaceae* family may be prevalent in the microbiota of cheese ripening. It was a family of organisms found in the sampling of dairy farms [46]. A study isolated and molecularly characterized microorganisms from the *Planococcaceae* family, which was possible in a sampling carried out in dairy farm storage tanks [47]. In this study, *Kurthia massiliensis* was the family member most prevalent during the samplings. One author described *K. massiliensis* as a Gram-positive, aerobic, encapsulated, motile rod shape; no evidence of pathogenicity exists for this microorganism [48].

4.3. Predominant Bacteria Identified

In both farms, a group of sanitary-importance microorganisms was found with variable occurrence. Although the capacity of these microorganisms to cause problems is moderate, they are agents that can cause diseases in humans and animals or represent a risk to the environment [49]. The sanitary-important microorganisms identified were *Enterobacter cloacae*, a nosocomial pathogenic bacterium responsible for bacteremia and respiratory, urinary, and intra-abdominal tract infections. Several strains of this microorganism have already been detected as resistant to antibiotics, which has caused a significant increase in infections [50]. In addition, this microorganism in dairy products can indicate unsanitary production or improper handling, which constitutes a safety problem for the consumer [51].

Enterococcus casseliflavus is a bacterium commonly found in human and animal intestinal tracts. It is one of the species of enterococci widely found in dairy cows [52]. This organism has been found in bulk tanks and milking equipment even after they have been disinfected. A study found the bacteria *E. casseliflavus* in a few of the 2,000 samples of cows suspected of mastitis in Poland [53]. In addition, cases of human infections have been reported where *E. casseliflavus* has shown antibiotic resistance [54].

Klebsiella pneumoniae is found on mucosal surfaces in mammals and the environment (water and soil). In humans, it is located in the gastrointestinal tract and nasopharynx. However, in immunocompromised people, it can cause pneumonia [55]. *K. pneumoniae*, an opportunistic pathogen, infects the mucosal epithelium and spreads to deep tissues and the bloodstream. It causes severe infections such as meningitis, ophthalmitis, bacteremia, and liver abscesses. *K. pneumoniae* is clinically challenging as it is resistant to certain antibiotics [56]. This bacterium, also associated with mastitis, was accurately identified by MALDI-TOF in isolates from the Mastitis Pathogen Culture Collection of the Canadian Bovine Mastitis and Milk Quality Research Network [57].

Acinetobacter baumannii is an opportunistic pathogenic bacterium causing nosocomial infections, such as pneumonia, skin infections, and meningitis. *A. baumannii* is one of the pathogens most resistant to antibiotics [58]. In addition, it causes mastitis, pneumonia, and sepsis in animals for consumption [59]. Therefore, a study recommends analyzing bulk tank milk for foodborne pathogens and *Acinetobacter* spp. due to their potential risk to milk quality and public health [60].

Pseudomonas aeruginosa is an opportunistic pathogenic bacterium, ubiquitous and persistent in the environment [61] Found in water, soil, and various physical media. *P. aeruginosa* is the main microorganism and the fifth worldwide cause of nosocomial infections. It is the second and fourth cause of urinary and surgical infections, respectively [61]. In addition, it is one of the pathogenic agents that cause mastitis [62]. One study described an outbreak of mastitis in 20 herds of dairy cows caused by *P. aeruginosa* related to poor cleaning practices. The investigation showed that the microorganism's transmission occurred with a contaminated cleaning solution used during the milking process [44].

Serratia marcescens is an opportunistic pathogenic bacterium belonging to the *Enterobacteriaceae* family and causing various lung and urinary tract infections, meningitis, and sepsis. One study described their results from two herds of dairy cows, where positive samples of *S. marcescens* were obtained in milk with mastitis [63]. It was concluded that this microorganism and its transmission are due to the mishandling of equipment and chemical compounds used during cleaning.

Bacillus cereus and *Staphylococcus chromogenes* were the sanitary-important microorganisms with a frequency of over 60% in the samplings on both farms. *B. cereus* is a bacterium commonly found in the environment and capable of producing toxins related to foodborne illnesses. Consuming these toxins can cause symptoms such as vomiting and diarrhea. In addition, these bacteria can contaminate animal feed [23]. As a spore-former, *B. cereus* resists pasteurization and adverse environmental conditions. This bacterium has been linked (although not as frequently) to mastitis in cows used in the dairy industry [64]. *S. chromogenes* is a bacterium classified as a pathogen for humans, resistant to antibiotics, and associated with nosocomial infections [65]. It is a pathogenic microorganism that can coagulate plasma and has been isolated from dairy cow mastitis [66]. Several of these microorganisms have already been isolated in previous research. For example, in the study by [67], conventional dairy farms in Canada were microbiologically sampled, reporting pathogens such as *Bacillus* spp.,

Streptococcus spp., *Staphylococcus aureus*, and *Escherichia coli* most related to mastitis disease in cows.

5. Conclusions

The results of this study show that the concentration of microorganisms in the air of milking areas can be influenced by different factors, such as the location of the farm, as well as temperature and relative humidity. The study also demonstrates the effectiveness of using MALDI-TOF MS to identify microorganisms quickly and accurately in dairy farms. This study successfully isolated and identified numerous bacteria that are commonly associated with animal production environments. Some of the identified bacteria are known to be part of the normal environmental flora, while others have been linked to livestock diseases. Additionally, MALDI-TOF MS accurately identified bacteria classified as opportunistic pathogens, capable of affecting the health of both animals and humans. These findings contribute to the understanding of the significance of comprehending and identifying the microbial flora present in dairy farms, thereby facilitating the development of new protocols to prevent and reduce microbial contamination.

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