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MALDI-TOF: A Rapid Identification of Dairy Pathogens

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MALDI-TOF: A Rapid Identification of Dairy Pathogens

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Abstract

The proposed research study is a field validation study to benchmark against proven methods, a new methodology for the detection of microorganisms (Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry or MALDI-ToF) isolated from dairy farm and critical for safety and quality. The MALDI-TOF is a relatively new molecular technique extremely advantageous in terms of cost effectiveness, sample preparation easiness, turn-around time and result analysis accessibility. Although already successfully deployed in clinical diagnostic, it has not been evaluated for agricultural applications yet. In the dairy industry, Mastitis causes the most financial loss and a rapid diagnostic method as MALDI-TOF, will assist in the control and prevention program of mastitis, in addition to the sanitation and safety level of the dairy farms and processing facility. In the present study, we prospectively compared MALDI-TOF MS to the conventional 16S rRNA sequencing method for the identification of environmental mastitis isolates (481) and thermoduric isolates of pasteurized milk (248). Among the 481 environmental isolates, 454 (94.4%) were putatively identified to the genus level by MALDI-TOF MS and 426 (88.6%) were identified to the species level, but no reliable identification was obtained for 17 (3.5%), and 27 (5.6%) discordant results were identified. Future studies can help to overcome the limitation of MALDI database and additional sample preparation steps might help to reduce the number of discordance in identification. In conclusion, our results show that MALDI-TOF MS is a fast and reliable technique which has the potential to replace conventional identification methods for most dairy pathogens, routinely isolated from the milk and dairy products. Thus it's adoption will strengthen the capacity, quality, and possibly the scope of diagnostic services to support the dairy industry.

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Chapter I: Literature Review

Mastitis

Mastitis, a complex and multi-etiological infectious disease, is widespread in dairy cattle. It is defined by the inflammation of the mammary gland usually in response to injury by different agents. The inflammatory response is triggered to destroy or neutralize the source of the infection and to start the healing process of the udder (Harmon, 1994). The injury triggering the inflammation can have different sources such as physical trauma, chemical irritants, or microbes and their toxins. In dairy cattle, microorganisms, particularly bacteria, are the main cause of mastitis (Jones & Bailey, 2009). Pathogenic bacteria invade the udder, multiply in the milk-producing tissues, and produce toxins that are the immediate cause of tissue damage (Harmon, 1994).

Mastitis can be classified as clinical or subclinical. Clinical Mastitis is the presence of disease with visible signs that can be categorized as mild (e.g., flakes or clots in the milk, slight swelling of infected quarter) and severe (e.g., abnormal secretions, hot and swollen quarter or udder, fever, rapid pulse, loss of appetite, dehydration and depression). The most severe cases can be fatal (Erskine, Eberhart, Hutchinson, Spencer, & Campbell, 1988; Bradley, 2002). The clinical form of mastitis is the main cause of financial loss to dairy farmers through lowered milk production (Halasa, Huijps, Østerås, & Hogeveen, 2007). For every clinical case of mastitis, 15 to 40 subclinical cases will occur (Cremonesi, et al., 2009).

In the subclinical mastitis, there are no visible signs of the disease. However, the somatic cell count (SCC) of the milk will be above normal levels (above 300,000) indicating inflammation of the udder. If infectious, bacteriological culturing of milk will be generally positive for the presence of bacteria (Erskine, Eberhart, Hutchinson, Spencer, & Campbell,

1988). Concerning husbandry practices, the animals affected by sub-clinical mastitis can be source of infection for herd mates.

Mastitis Pathophysiology

Bovine mastitis, characterized as inflammation of the mammary gland, can have an infectious or non-infectious etiology (Bradley, 2002). The bovine mammary gland is composed of glandular tissue, gland cistern and branching network of ducts formed of epithelial cells ending in alveolar clusters that are the sites of milk secretion (McManaman & Neville, 2003) There is only one type of secretory epithelial cell that surround each alveolus within these clusters, forming a single layer over the cells (Linzell & Peaker, 1971). The apical junction complex that is composed of adherens- and tight-junctional elements connects all the secretory cells to each other (Linzell & Peaker, 1971; McManaman & Nevile, 2003). The function of the tight-junction is to inhibit any direct exchange of substances between vascular and milk compartments during lactation (Linzell & Peaker, 1971; McManaman & Nevile, 2003).

Mastitis occurs when potentially pathogenic microorganisms present in the environment enter the udder through the teat cistern colonizing it. The invading organism multiplies in the teat and mammary cisterns (Auldist and Hubble, 1998). As part of the host immune response, the intramammary infection is quickly followed by an influx of leucocytes, predominantly polymorphonuclear neutrophils (PMN) into the milk and elevated somatic cell counts of the milk (Auldist and Hubble, 1998; Bruckmaier & Blum, 2004). The tight junction permeability (Holdaway, 1990) across endothelial and epithelial layers increases due to the inflammatory reaction products including histamine, TNF, IFN-g and acute phase proteins (Nguyen, Beeman, & Neville, 1998; Pyorala, 2003). The increase in permeability of the tight junction allows immune components to reach the infection site (Nguyen, Beeman, & Neville, 1998).

Polymorphonuclear neutrophils (PMN) are the predominant leucocytes present in milk during the infection that are consequently responsible for the high somatic cell counts (SCC) (Auldist & Hubble, 1998; Pyorala, 2003; Bruckmaier & Blum, 2004). Considerable tissue damage of secretory cells is observed once the immune effector cells begin to combat the invading pathogens and their toxins. Furthermore, subsequent releases of enzymes like N-acetyl-b -D-glucosaminidase (NAG-ase) and Lactate dehydrogenase (LDH) are increased in the milk with the onset of mastitis (Burvenich, et al., 1994). Also necrotic mammary epithelial cells can be found with histological examination of mastitic glands (Nguyen, Beeman, & Neville, 1998).

Pathogens

There are several different bacteria that can be responsible for mastitis. These are generally present in the environment and categorized as infectious pathogens. The ability of these bacteria to colonize the outside surface and the internal locales of the mammary gland leads to spreading of the infection within a dairy cattle herd during milking. A series of recent surveys found that the most common contagious pathogens are *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp*. and *Corynebacterium bovis* (Carrillo-Casas & Miranda-Morales, 2012).

The environmental pathogens are those that are present in the environment of the animals such as moisture, mud and manure. They are the primary sources of exposure for environmental mastitis pathogens. The most frequently isolated environmental pathogens are environmental streptococci (usually *S. uberis* and *S. disgalactiae*) and gram-negative bacteria such as *Escherichia coli, Klebsiella spp.* and *Enterobacter spp,* coliforms etc (Harmon, 1994).

Threat to Animal and Human Health

Although mastitis is highly morbid and can cause much pain in the affected animals, it is rarely lethal. Two studies from France and Ireland, concerning the lethality of mastitis in dairy cattles, reported that mastitis had annual mortality rate of 0.22% (Faye & Pèrochon, 1995) and 0.19% (Menzies, Bryson, Mccallion, & Matthews, 1995). The cows infected with mastitis are at higher risk of being culled as the cost of treatment might be a burden for the dairy farmer and replacement of the sick cow would save the unwanted cost.

Because of the importance of milk and dairy products consumption in human diets, mastitis can be a health concern for human as well. In fact, diseases as tuberculosis, sore-throat, Q-fever, brucellosis, and leptospirosis are all caused by pathogens responsible for udder infections that can contaminate the milk rendering it unfit for human consumption (Sharif et al., 2009). As a result, mastitis is listed as a significant zoonosis in the World Organization for Animal Health Terrestrial Animal Health Code (World Organization for Animal Health, 2014). Some of the mastitis-causing bacteria are also responsible for human infection instances such as *Brucella, Campylobacter, Listeria, E.coli* etc. A lot of them cause intoxication of foods resulting in food poisoning such as the toxins produced by *S. aureus.* Although, pasteurization reduces the number of viable microorganisms but often does not destroy toxins produced by bacterial pathogens, hence it is very likely to get infected with bacterial toxins when raw milk is consumed or when pasteurization is faulty.

Moreover, some bacteria produce different heat stable toxins that can endure the boiling temperature, hence withstanding the pasteurization and sterilization processes. The transfer of heat-stable toxins produced by mastitis-causing pathogens in milk is a serious potential concern. Enterotoxins produced by enterotoxigenic strains of *S. aureus* have frequently been implicated in

cases of food poisoning. *Campylobacter, Salmonella* also found in the environment of the herd and also in bulk tank milk. Some strains of *E. coli* produces shiga toxin that can lead to severe conditions as bloody diarrhea and hemolytic uremic syndrome (Centers for Disease Control and Prevention, 2012). Despite the fact that it is brought about frequently by the consumption of ground beef, cases of contamination through raw milk consumption have been reported as well (Iowa Department of Public Health, 2014). The *E. coli O157:H7* is the most studied enteric pathogen among the Enterohemorrhagic *Escherichia coli* (EHEC). There are many outbreaks In the US caused by the Shiga toxin-producing E. coli, including the occurrence in 2012 that caused 29 outbreaks and 500 illnesses with 98 hospitalizations (CDC, 2012). Moreover in a Brazilian study, 5.8% of mastitic milk samples were contaminated with *E. coli* strains, and among them 64.5% belonged to the STEC group (Kobori, Rigobelo, Macedo, Marin, & Avila, 2004). The *E. coli* O157 and non-O157 STEC that causes human illness are considered highly infectious such that the encounter of these organisms are recommended to report to the Nationally Notifiable Diseases Surveillance System in the US (CDC, 2012).

In fact, milk from affected animals can be a threat to human health, especially if consumed by vulnerable people (children, pregnant, old people, people living with HIV-AIDS), and if it is consumed raw or not properly pasteurized. Antibiotic residue is a major public health concern, as people allergic to antibiotics, and development of antibiotic-resistant strains of bacteria (Pol & Ruegg, 2007). There is also concern for the safety of dried milk products used for infant formula, due to possible contamination with *Cronobacter* that can survive for longer period in such low water activity foods (Farakos & Frank, 2014). *Bacillus cereus*, a human pathogen found in both pasteurized and dried milk is a psychrotroph that can survive milk pasteurization (Notermans, et al., 1997; Lin, 1998). *B. cereus* has been frequently found in dried milk powders, however it

requires large number of growth (>10 5CFU/g) for causing an outbreak (Farakos & Frank, 2014). Again spores of *B. cereus* can survive for long periods, germinate and grow in foods that are not properly processed or under poor storage condition (Beuchat, et al., 2013).

Effect on Milk Composition

Mastitis changes the chemical and physical properties of the milk. The major milk protein casein, of high nutritional quality, declines in mastitic milk due to the invading organism. Such as, *E. coli* has a direct or indirect role in casein proteolysis that still needs to be determined (Moussaoui, et al., 2004). Also *S. aureus* produces proteases including serine protease, cysteine protease and metalloprotease that are involved in the casein proteolysis (Karlsson & Arvidson, 2002). Moreover, similar studies reported that *E. coli* and *S. aureus* are associated with increased levels of lactoferrin, protein content, proteose peptone, plasmin and lower levels of the casein/protein ratio, calcium, and phosphorus (Kawai, Hagiwara, Anri, & Nagahata, 1999; Coulona, et al., 2002; Hagiwara, Kawai, Anri, & Nagahata, 2003; Leitner, Krifucks, Merin, Lavi, & Silanikove, 2006). The whey proteins that derive from the blood mammary barrier disruption, has important implications for the manufacturing potential of the milk, particularly, but not exclusively, for cheese manufacture (Auldist & Hubble, 1998).

Also, Na^{+} and Cl^{-} increase in mastitic milk, while K^{+}, normally the predominant mineral in milk, declines (Auldist, Coats, Rogers, & Mcdowell, 1995). Because most calcium in milk is associated with casein, the disruption of casein synthesis contributes to lowered calcium in milk (Maréchal, Thiéry, Vautor, & Loir, 2011). Again serum proteins are found in the milk such as the immunoglobulins and serum albumin. The presence of immunoglobulins in milk induces the formation of agglutins, which can inhibit acid production in raw and pasteurized whole or skim milk, causing problems in the manufacture of cottage cheese (Salih & Sandine, 1984)

Effect on Dairy Products

To ensure the safety and quality of dairy products for human consumption, it is necessary for the industry to rapidly and accurately detect and identify milk-borne bacterial contaminants. In particular, the presence of bacterial contaminants (e.g. *Bacillus spp., Paenibacillus spp., Listeria spp., etc.*) in dairy foods is of significant concern to milk processors for several reasons including food quality (e.g. reduced shelf life, reduced cheese quality) as well as food safety. For example, *S. dysgalactiae* has such a huge impact on milk composition that no curd has been produced from infected milk in experimental cheese making. *S. dysgalactiae* infection results in reduced yields in both cheese and yogurt production. *S. dysgalactiae* directly generates (through its enzymatic activities) or activates the formation of short-chain peptides, which interfere with the coagulation process. Clotting time has also been shown to be significantly higher in *S. aureus* mastitic milk than in normal milk and curd firmness slightly decreased. Altogether these data show that most mastitis pathogens directly or indirectly affect milk coagulation by impacting either rennet or starter activity (Maréchal, Thiéry, Vautor, & Loir, 2011). The ability to rapidly screen milk, dry dairy powders and cheese for contamination without the use of culturing strategies would be highly beneficial to the dairy products industry.

Organisms that affect quality and safety can come with the raw milk, or gain entry to pasteurized product in plant equipment during processing and packaging. Thermoduric organisms including mesophiles, psychrophiles, and especially gram-positive spore forming bacteria can survive milk pasteurization, causing early spoilage or lowered shelf life of the dairy products. Losses of fluid milk due to spoilage at the consumer level were estimated to be 18% in the US in 2008, which equates to approximately $4.2 billion worth of product (Buzby & Hyman, 2012). This loss can be due to the product reaching the shelf-life expiration date before being

consumed and then discarded (even if still consumable), or due to actual microbial spoilage because of the activity of psychrotrophic microorganisms or temperature abuse, resulting in spoilage. To further lengthen the shelf life of conventionally processed milk, reductions in spore forming organisms that survive pasteurization must be addressed.

Impact on Economy of Dairy Industry

Dairy is a vital part of the global food system and a universal agricultural production. The worlds total milk production was estimated at 748.7 million tonnes in the year of 2011, of which 620.7 million tonnes was cow's milk, produced by 260 million cows (IDF World Dairy Situation report, 2012). The data from the FAO showed that the gross production value of agriculture equals 3282 billion USD, where raw milk produced across the world equals 292 billion USD. The value of milk represented 8.9% of the value of all agricultural products in 2010, on a global scale (FAO, IFCN, 2010). The Dairy business plays a significant role to the agricultural economies of some particular countries where the milk production value accounts for more than 20% of the total agricultural value, whereas the average value represents between 8.5% and 10.5% depending on the year. Such countries include New Zealand (35%), Finland (26%), India (24%), Luxembourg (23%), Estonia (23%), Switzerland (21%) and Latvia (20%) (FAO, IFCN, 2010).

In the year of 2011, excluding trade within the European Union; world trade of dairy products (e.g. butter, skim milk powder, whole milk powder, condensed milk, yoghurt and cheese) summed up to 58.2 million tonnes in milk equivalents which represents 7.8% of world milk production (IDF World Dairy Situation report, 2012). FAO estimates, the trade of dairy products across the world to be at 64 billion USD, which is 5.9% of all of the agricultural products trade. Therefore, dairy industry plays a key role in the sustainability of the economy

across the world and financial loss in this sector reflects the forfeit in the entire economical system.

Mastitis Impact across the World

Domestic animal husbandry is a growing economic sector in most developing or underdeveloped countries. It is a noteworthy income source of the poor and particularly of women in developing countries. Bovine mastitis is one of the most significant production diseases of dairy animals, which directly or indirectly affect the economy of the dairy farmers and consequently affect the economy of the country. The major groups of mastitis causing organisms in Asia are *Staphylococcus aureus, Streptococci, E. coli, Corynebacterium spp*. and *Klebsiella spp* (Sharma, Pandey, & Sudhan, 2010).

In the year 2008 buffalo milk production in Asia represented 96.78% of the total volumes of world's buffalo milk production of 89.2 Million tons, where India and Pakistan from South and Southwest region principally contributed 93.17% (FAO, 2010). Therefore, buffaloes can be considered as the major sources of milk in this sub-region contributing as high as 68.35% of the total milk yield in Pakistan and 56.85% in total milk production in India (FAO, 2010). The prevalence of mastitis in Asia are shown below (Figure 1).

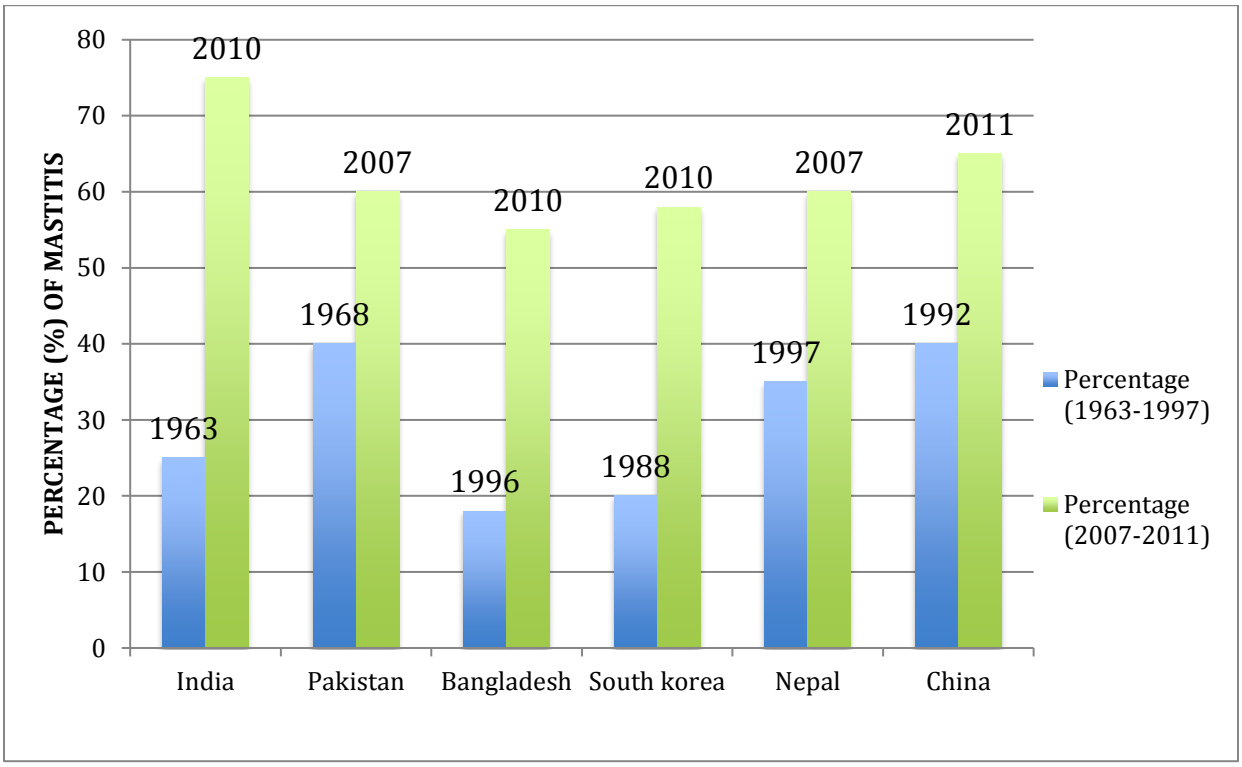

Figure 1: Prevalence of Mastitis in different countries of Asia including India, Pakistan, Bangladesh, South Korea, Nepal and China. The bar graph is showing the increase of the percentage of mastitis across Asia from the year 1963-1997 and 2007-2011, adapted from Neelesh et al., 2012.

Low productivity is proportional to poor animal health, particularly, mastitis which is the single largest issue with dairy animal in terms of economic losses. Mastitis is a monetarily critical disease of dairy cattle, representing 38% of the total direct expenses of the common production diseases (Kossaibati & Esslemont, 1997). The prevalence of bovine mastitis ranged from 29.34 to 78.54% (Ebrahimi, Lotfalian, & Karimi, 2007; Sharma & Maiti, 2010) in cows and 27.36 to 70.32% (Beheshti, Shaieghi, Eshratkhah, Ghalehkandi, & Maheri-Sis, 2010) in buffaloes.

In Pakistan, loss due to clinical mastitis was evaluated to INR (Indian National Rupee) 240 million per annum in Punjab just amid of 1978 (Chaudhry & Khan, 1978). Several reports

showed that the annual economic losses due to bovine mastitis was increased 114 folds in about 4 decades from 1962 (INR 529 million/annum) (Dhanda & Sethi, 1962) to 2001 (INR 60532 million/annum) (Dua & Banerji, 2001). This estimate might be substantially higher if losses due to sub-clinical mastitis had been incorporated, which are 15-40 times more prevalent than clinical form.

There are roughly 8,000 dairy farms and 472,000 cows in South Korea, where the yield is an average of 177,770,000 kg of raw milk per year. Another report revealed that around 68% of the total losses of milk resulted from drop in milk production in buffaloes in Nepal (Dhakal & Thapa, 2002). Again, bovine mastitis has essentially hindered the advancement of the dairy business in Bangladesh (Islam et al., 2011).

Economic losses caused by mastitis in Canada are about $300 million every year, reported by the dairy producers' (Dairy Farmers of Canada, 2012). Also the losses due to both clinical and subclinical mastitis vary between €17 and €198 per cow per year in the Netherlands (Groenestein, et al., 2011).

Mastitis Impact in the U.S.

Dairy industry is one of the most important agricultural sectors for the North American economy. In the United States, dairy farms produce almost 196 billion pounds of milk annually (USDA). Minnesota is the 6th largest dairy state, with approximately 4,000 producers and 469,000 dairy cows producing about 9 billion pounds of milk annually. Minnesota is home to several regional, national and international dairy food processors including Land O' Lakes Inc., Davisco Foods International Inc., Kemps, and Associated Milk Producers (AMPI, Inc.), among others. The dairy production and processing industry is the second largest agricultural business

in Minnesota, generating approximately $5.6 billion per year, with a total economic impact of about $11.5 billion, and supporting over 38,000 jobs in the state (Thiesse, 2012).

Mastitis costs the U.S. dairy industry about $1.7-2 billion annually or 11% of total U.S. milk production. The annual losses per cow from mastitis in the United States of America in 1976 were estimated to be US$ 117.35 per cow per year and a total loss of US$ 1.294 billion (Blosser, 1979); two decades later these losses had increased to US$ 185 to $ 200 per cow per year and the total loss increased to US$ 2 billion (Costello, 2004; Viguier, Arora, Gilmartin, Welbeck, & O'Kennedy, 2009). Despite decades of ongoing advancements, mastitis continues to be the most costly infectious disease on dairy farms (Erskine, Eberhart, Hutchinson, Spencer, & Campbell, 1988; Sargeant, Scott, Leslie, Ireland, & Bashiri, 1998; Erskine, Wagner, & Degraves, 2003; Riekerink, Barkema, Kelton, & Scholl, 2007; and Fetrow, 2000).

Much of the cost due to mastitis, is attributed to reduced milk production, discarded milk, and replacements, which are estimated at $102, $24, and $33 per cow per year as shown below (Table 1). The obvious costs for treatment medication, labor, and veterinary services are low, estimated to total $13 per cow (Costello, 2004).

Table 1**:** Different Category of Economic loss due to Mastitis, includes reduced milk production, discarded milk, replacements and treatment of the animal (Costello, 2004).

Category of loss due to Mastitis	**Cost US$ Per Cow**
Reduced Milk production	102
Discarded Milk	24
Replacement	33
Treatment	13
Total Cost	171

It must be recognized that mastitis cannot be completely eliminated from a herd, as most of the pathogens involved in causing mastitis are the natural inhabitants of the environmental flora of the cow barns. However, the total cost of mastitis in the average herd enrolled in DHI (Dairy Herd Improvement) is approximately $171 per cow. In general, it is assumed that milk had to be discarded for 6 days including 3 days treatment and 3 days withholding period due to the possible secretion of antibiotic residues in the milk. The treatment cost includes the veterinarian fees and the cost of drugs.

Importance of Rapid Diagnosis

Early diagnosis is of the utmost importance due to the high costs of mastitis to the dairy industry. Mastitis has several causative agents and the proper rapid detection of the pathogen is very important to address the control and prevention measures of Mastitis. A reliable rapid diagnostic test is a dire need for the wellbeing of the dairy industry, rapid turnaround time being the key factor because the storage of perishable foods for longer period is cost-effective and a big problem in such instances. Though the traditional milk culture techniques are useful for the

primary identification of important bacterial pathogens in mastitic milk samples, but most of them require longer time of incubation and adequate training to perform the tests. Also Biochemical tests can be nonspecific, slow, costly, and more importantly result interpretation can be critical in relation to the diagnosis of mastitis pathogens.

Biochemical Testing

Most of the biochemical tests are conducted on site to identify the infection of the cow through testing the milk and usually not helpful in detecting the presence of any pathogenic organisms. Some of the commonly used diagnostic techniques are California Mastitis Test (CMT), Culture test, Enzyme test, pH test, Portacheck, Fossomatic SCC, Electrical Conductivity test etc. (Viguier, Arora, Gilmartin, Welbeck, & O'Kennedy, 2009). Biochemical tests include catalase or coagulase tests where the enzyme catalase or coagulase is detected by adding specific reagent and API system (Analytical Profile Index), which is a biochemical panel containing chemically-defined dehydrated media for the manual identification and differentiation of bacteria to the species level.

California mastitis test (CMT) assay indirectly measures the somatic cell count (SCC) in milk samples. The CMT test applies a detergent that contains bromocresol-purple, which is used to break down the cell membrane of somatic cells, and the subsequent release and aggregation of nucleic acid forms a gel-like matrix with a viscosity that is proportional to the leukocyte number. The CMT assay is very cost effective where 350 tests costs about US$ 12 (Dingwell, Kelton, & Leslie, 2003). Also, it can be used 'on-site' or in the laboratory. It is a rapid and user-friendly assay, but it can be difficult to interpret and has low sensitivity and no information on the possible pathogens can be obtained through this rapid test.

Mastitis causes the increase of ionic particles in the milk and the Electrical conductivity (EC) test measures the increase in conductance in milk caused by the increase in levels of ions such as sodium, potassium, calcium, magnesium and chloride during inflammation (Norberg, et al., 2004). It can be used 'on-site' but non-mastitis-related variations in EC can present problems in diagnosis, as the test does not refer to any possible presence of pathogens or mastitis infection.

Different selective cultures are used to identify different microorganisms involved in causing mastitis. Some selective culture medium (e.g. Gram-negative or Gram-positive, Coliform specific) can identify specific pathogens causing mastitis. Although it can be used only in laboratory and the waiting time for results can be 24 to 48 hours. (Viguer, Arora, Gilmartin, Welbeck, & O'Kennedy, 2009)

The pH is a good indicator as well and can be used in mastitis detection. Milk pH rises due to mastitis and can be detected using bromothymol blue (Kitchen, 1981). It is easy to use, cost effective and rapid. On the other hand, it is not as sensitive as other tests. It is only an indicator of the inflammation and no information can be obtained about the possible pathogens.

Also some Enzyme assays are used as well to detect enzymes involved in mastitis immune response, such as NAGase and LDH (Kitchen, 1976). However, such assays are rapid but might be laboratory-based only.

16S rRNA Sequencing

There are also a number of molecular techniques that are used for the identification of pathogens such as PCR, DNA sequencing or other DNA-based methods that are very sensitive and rapid. One such technique is the 16S rRNA sequencing (Figure 2) which is considered as the gold standard for bacterial identification purpose, as it provides very specific characterization in species level as well (Edwards, Rogall, Blöcker, Emde, & Böttger, 1989).

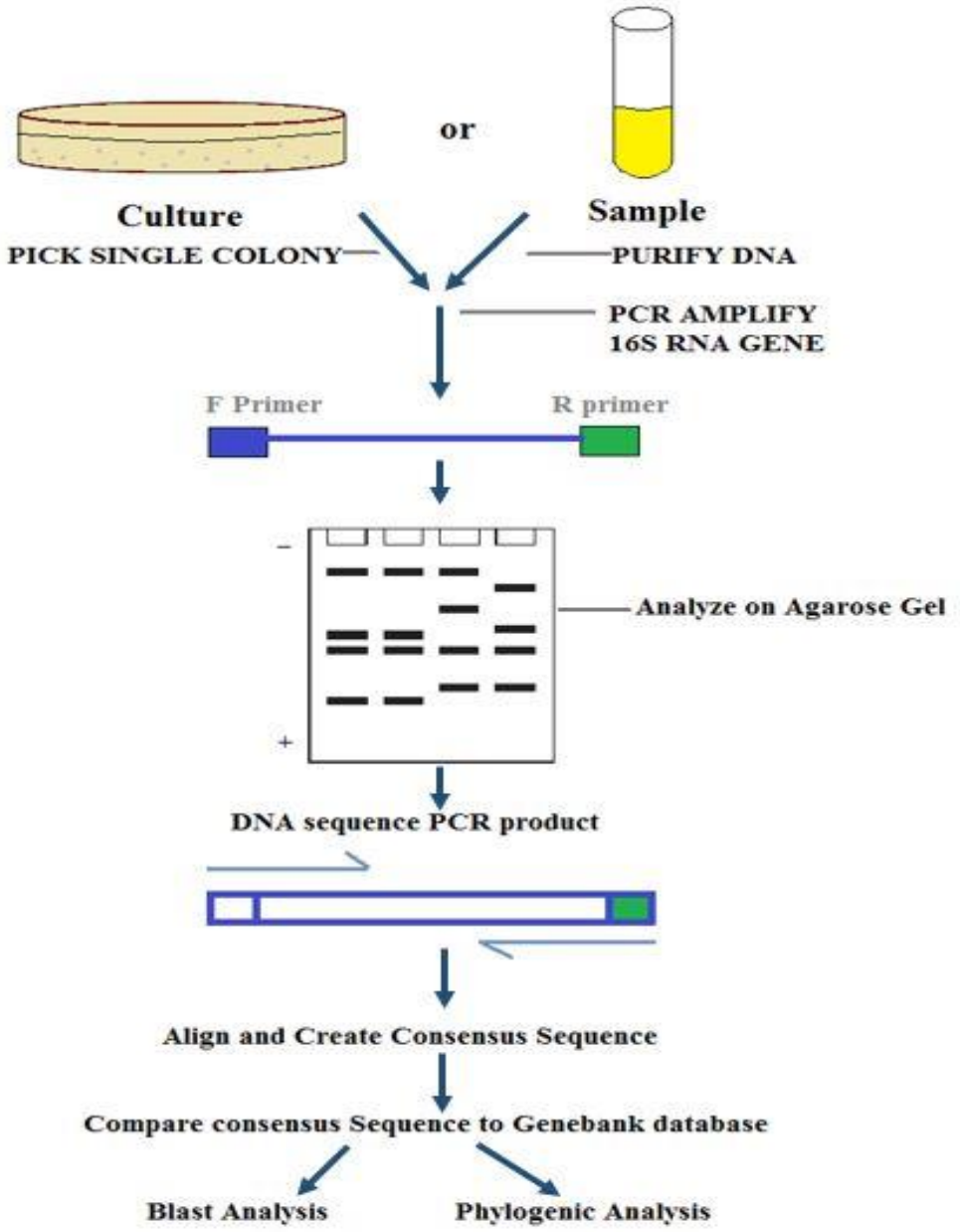

Figure 2. The 16S rRNA gene sequencing method showing gradual steps starting from the DNA extraction of pure bacterial colony, Amplification, Agarose gel electrophoresis, Sequencing and characterization through data analysis using 16S rRNA library.

It is important to have a rapid technique to evaluate products and sources of organisms along the product chain in an industrial setup (from farm through the processing plant), so that mitigation strategies can be developed. However, these techniques are relatively very time consuming and so are used almost exclusively for research but not for service samples (Zadoks, Middleton, McDougall, Katholm, & Schukken, 2014). Similarly, many of the systems employed for the isolation and identification of bacterial contaminants in dairy foods require expensive biochemical tests such as pathogen specific (e.g. *Salmonella* spp., *E. coli* 0157, *Listeria monocytogenes, Campylobacter* spp.) media or Petrifilm and/or DNA and RNA-based molecular techniques. These analyses are also time-consuming; increasing the holding time of the food products and thus impacting the production costs.

MALDI-TOF MS System

A new method of identification called MALDI-ToF, may overcome many of the aforementioned diagnostic limitations. MALDI-ToF, or matrix-assisted laser desorption ionization time-of-flight analysis, is a mass spectrometry-based method of identification. Simply, bacteria are shattered to protein fragments (peptides) with a laser beam and those peptides rise up an evacuated detection tube. The time taken by the peptides to reach the detection tube is called the “time of flight” or “ToF” and it is specific for the mass to charge ratio of the peptide. From this information, the peptide and protein composition can be presented as a mass spectrometry profile.

The comparison of the mass spectrometry profile with the MALDI-TOF database then allows for identification of bacterial genus and species based on the protein composition. The MALDI-TOF database is commercially available and can be updated by individual laboratory personnel or through purchasing updated version of the library. However, it is faster, less

expensive and potentially more universal (i.e. can identify more organisms) than biochemical or sequence-based identification of cultured isolates (Zadoks, Middleton, McDougall, Katholm, & Schukken, 2014). The great advantages of the technique includes rapid turnaround time, MALDI-TOF MS has been shown to identify organisms in <15 minutes whereas traditional methods take 5 to 48 hours depending on the method used (Cobo, 2013); a single MALDI-TOF MS system can be used for gram-positive bacteria, gram- negative bacteria, and yeast as well; the MALDI-TOF MS reference spectra database can be increased by editing commercially available software updates or by internal laboratory personnel; using MALDI-TOF MS in the clinical laboratory has been shown to lower costs for hospitals upwards of 53% annually (Seng, et al., 2009).

Also, while the current proposal focuses on detection of dairy microorganisms, because MALDI-ToF equipment can be used for detection of many kinds of pathogens. MALDI-TOF technology has already been successfully used in the field of virology where studies showed the comparison between the MALDI-TOF results and the other conventional methods (e.g. viral culture, PCR, nucleic acid based techniques). The concordance rate between MALDI-TOF and these established conventional techniques were high. Several approaches have been developed to detect different viruses in clinical specimen such as Human Herpes Viruses (HHVs), Poliovirus, Coxsackie virus A and B and Echo virus (Sjöholm, Dillner, & Carlson, 2007). Furthermore, MALDI-TOF could be useful for detecting drug resistance against some antivirals and antibiotic susceptibility testing (Zürcher, et al., 2012). Despite the fact that MALDI-ToF has already been validated and adopted for use by many human diagnostic laboratories (e.g. Rochester's Mayo Clinic), the library used by this method includes many human-derived pathogens that differ from

some animal-derived pathogens. As such, the method must be validated separately for use in veterinary diagnostic laboratories.

In the last few years, several European groups have investigated the use of MALDI-ToF for identification of a limited number of mastitis pathogens including several *Streptococcus, Enterococcus* and *Staphylococcus* species (Moser, Stephan, Ziegler, & Johler, 2013; Raemy, et al., 2013; and Werner, et al., 2012). However, before this instrument can be wholly accepted by the U.S. veterinary diagnostic laboratories, field validation of the MALDI-ToF must be completed using a broad range of North American-derived bacterial isolates of importance to animal health (e.g. bovine mastitis), food quality or food safety. During 2013 and early 2014, several veterinary diagnostic laboratories across western Europe, Canada and the U.S. have begun the process of evaluating MALDI-ToF for detection of mastitis, and other animal pathogens. The VDL is currently at the forefront of this process. The University of Minnesota VDL has acquired a MALDI-ToF instrument (Figure 3) and has been involved since 2013 in completing some preliminary internal validation studies evaluating the method's ability to detect common animal pathogens, including a large number of mastitis pathogens.

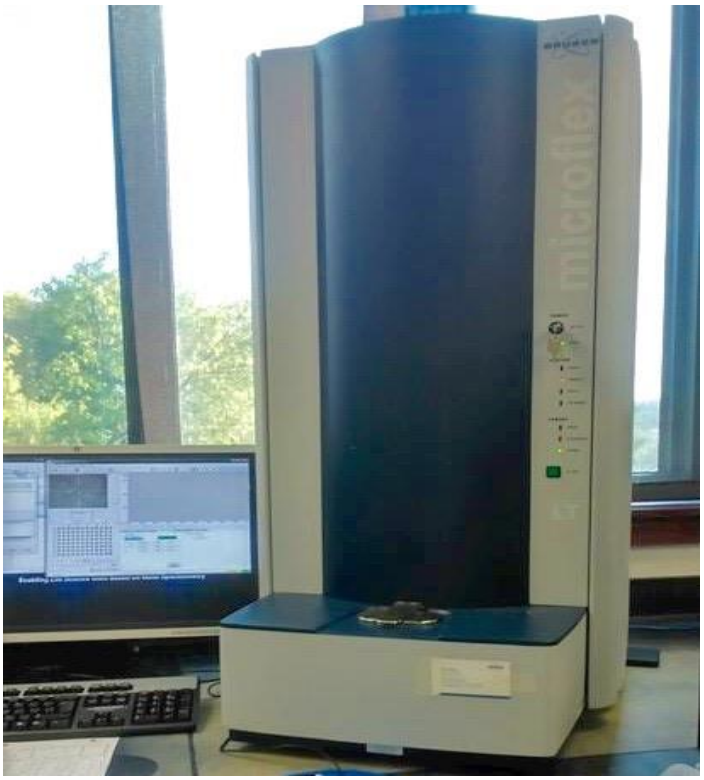

Figure 3. MALDI-TOF Mass Spectrometry machine at VDL.

With many advantages of using MALDI-TOF MS in the clinical microbiology laboratory, there are a few limitations that are noteworthy. A pure culture of the microorganism is currently still required and MALDI-TOF MS cannot be used with mixed cultures. In addition, traditional antimicrobial susceptibility testing is still required when a pathogenic organism is isolated and identified from culture. In that case, pure culture isolation is required which might be a problem in some instances. MALDI-TOF will fail to identify any new organism that is absent in the database and will show as unreliable identity. Moreover, there is also complexity in distinguishing some organisms that are closely related strains, due to the great sequence similarity of the microorganisms. These include *Streptococcus pneumoniae* and some of the *viridans Streptococci, or Shigella* species and *Escherichia coli* (Wieser, Schneider, Jung, &

Schubert, 2012). In such instances additional test needs to be conducted including, traditional biochemical tests, antigen detection, or molecular methods.

Objectives & Hypotheses

- ***Objective 1.*** Identification and characterization of all the isolates collected from Environment and Milk samples using 16S rRNA sequencing.
- ***Objective 2.*** Benchmark the MALDI-TOF identification against the 16S rRNA sequencing identification of the environmental isolates.
- ***Objective 3.*** Identification of Prominent Phyla in the Environment of Dairy Farm and Processing Facilities.

We hypothesize that we will be able to successfully characterize most of the pathogenic bacteria that causes mastitis and threat to the dairy products. We also hypothesize that the MALDI-ToF method will be accurate for detecting, at the level of genus and species, a large variety of important bacterial isolates derived from environmental samples of different dairy farms and processing facility. If our hypotheses are proven correct, the MALDI-ToF method can be adopted for use for service and research samples to detect dairy microorganisms affecting animal health, food quality or food safety. Furthermore, we may be able to add new species of interest to the in-house MALDI-ToF species library, thus creating a more universal list of species that can be identified. If our hypotheses are proven incorrect for one or more individual bacterial species studied, then it is still important to understand and report the limitations of this diagnostic technique so that alternate diagnostic techniques may be utilized when appropriate.

Chapter II: Materials and Methods

Sample Collection

The technicians in the UHL (Udder Health Laboratory) collected approximately 810 individual isolates that included Gram-positive and Gram-negative organisms. These were recovered by routine culture methods from milk samples from individual cows, already submitted by dairy producers to the UHL. No more than 10 bacterial isolates were derived from any one farm of origin. Selected isolates were frozen in a blood and/or glycerin suspension and retained at -80°C for later use in the project. Also participating food scientists collected samples of post-processed fluid milk or other dairy products. Samples were de-identified prior to culturing to maintain processor confidentiality. Processor milk samples or other dairy products were from unique lots. Samples were submitted, on ice, to the food science microbiology laboratory where they sampled, diluted and homogenized, using standard microbial laboratory practices (Wehr, 2004). 100 processor-sourced milk samples were collected for mesophilic and psychrophilic bacterial spore formers present that have survived pasteurization. Once recovered, isolates were grown on a non-selective agar medium and stored in glycerol at – 80 °C for later use. Between producer and processor sourced isolates, approximately 1,247 individual bacterial isolates were collected for use in the study.

Environmental Isolates

A total of 810 environmental isolates were collected form VDL (Veterinary Diagnostic Lab). The bacterial isolates will be grown in the appropriate culture medium for further identification using the 16S rRNA sequencing. Blood Agar (with 5% sheep blood and Tryptic Soy Agar) was used for the isolation and cultivation (Figure 4), as it is an appropriate medium for the growth of a wide variety of fastidious microorganisms. Moreover, the morphology can be

observed clearly and hemolysis activity of the bacteria can be seen on blood agar, which serves as a way of partial identification and categorization of the pathogen (Faddin,1985).

Figure 4. Isolation of Mastitis pathogens (Bacteria) on 5% sheep blood agar.

Thermoduric Isolates

Milk samples were obtained from the DQCI (Dairy Quality Control Institute) for the isolation of mesophilic and psychrophilic bacterial cultures. The samples were prepared in three different ways as reported below (Table 2):

Table 2**.** The list of Milk samples received from DQCI.

No.	**Sample**	**Received**	**Treatment**
1	Raw Milk	87	Frozen raw milk
2	Heat Treated	87	Heated to 80°C for 12 minutes then frozen
3	Heat Treated	87	Heated to 80°C for 12 minutes and held at refrigeration temperature for 5 to 7 days before freezing.

For the isolation of thermoduric and spore forming organisms, only heat-treated milk samples were used. A total of 87 milk samples were collected from different farms by DQCI. Three replicates from each of the 87 milk samples were made, where the first set was kept as raw without any treatment. The other two sample sets were heated to 80°C for 12 minutes, and storage was done in two different ways for experimental purposes, where one of them were frozen right after heating and the other set was kept in the refrigerator for 5 to 7 days before freezing. The two different storage conditions were intended to examine the growth of mesopilic and psychrophilic spore forming organisms, respectively.

For the primary isolation of bacteria from the milk samples, three different agar media were used including NA (Nutrient agar), LB agar (Luria broth) and BHI agar (Brain heart infusion). Among them the BHI agar performed best with more bacterial growth comparatively, as the purpose was to grow most bacteria including fastidious ones (Figure 5). At first, 500 µl of each milk sample from both replicate sets were spread on the agar medium using sterile spreading rods. After plating, the plates were placed at 37°C for the growth of mesophilic spores and at 7°C for 10 days to grow psychrophilic spores. Depending on the amount of growth, the

samples were plated again with diluted milk samples for sub-culturing single colonies. To obtain pure colonies, the single colonies from the BHI agar plates were further sub-cultured into SBA (Sheep Blood agar) and incubated at two different temperatures as previously mentioned.

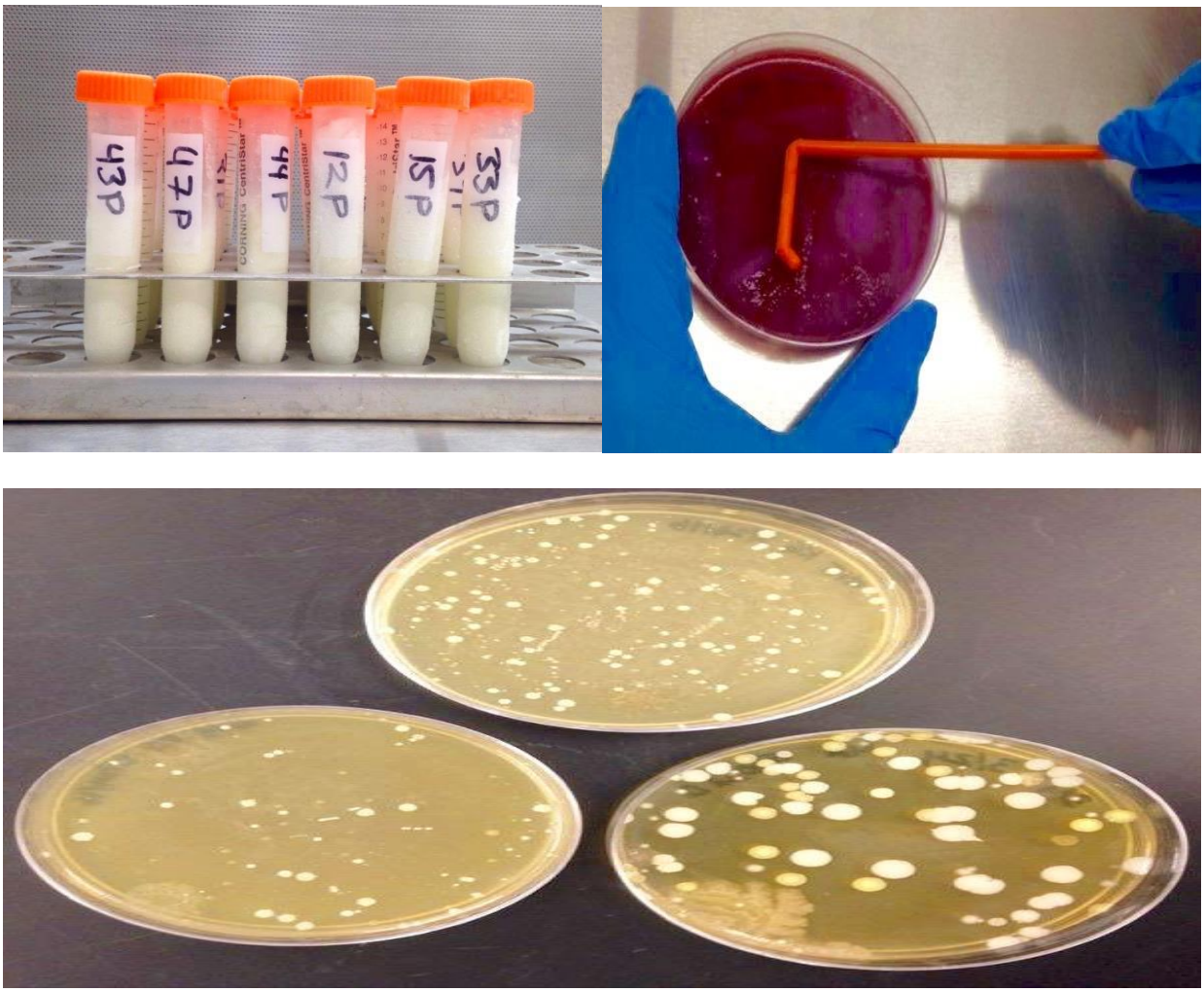

Figure 5. Isolation of Thermoduric organisms from heat-treated milk samples.

Stocking and Storage of Isolates

Glycerol stock of bacteria can serve the purpose for the long-term storage and future use of the bacterial isolates (Swift, 1920). After the isolation of bacteria on blood agar, they were further processed for making glycerol stocks. For each of the 1,247 isolates, two glycerol stocks were made. 20% glycerol was used for the stocking, where overnight grown bacterial culture in BHI broth were pipetted in 20% glycerol solution and vigorously vortex to generate uniform mixture. The glycerol stock vials were kept in -80° C for preservation and future use.

Preparation of BHI broth

Brain-heart infusion broth (BHI) is a general-purpose highly nutritious growth medium made with cow or porcine heart and brain (Table 3). It is used for culturing both fastidious and non-fastidious microorganisms (Faddin, 1985).

Table 3**.** The components and amount for BHI broth preparation.

Component	**Amount**
BHI powder	37 g
DI H_2O	1000 ml

At first the BHI powder was weighed to 37g and put in an Erlenmeyer flask, following Instructions from the manufacturer (Hardy Diagnostics, California, U.S.). Using a graduated cylinder, 1000 ml DI water was added slowly to the flask. Magnetic stir bar was inserted and the top of the flask was wrapped with aluminum foil. Then the flask was placed on a magnetic stirrer with hot plate. The heating plate was adjusted to boiling temperature and speed was set on medium for the stirring. The heat and stirrer were turned off when the broth started boiling. Then

the magnetic stir bar was removed cautiously and the broth was autoclaved at load cycle 3 (Liquid medium < 6L). After cooling down to room temperature, the broth was used to make bacterial culture and stock.

Preparation of 20% Glycerol solution

The addition of glycerol to the culture stabilizes the bacterial plasmid, protects from damage to the cell membranes and keeps the cells alive in freezing temperature (Gibson & Khoury, 1986). The glycerol stock of bacteria can be stored stably at -80°C for many years.

In a glass beaker 20 ml of Glycerol was added using a pipetter and 80 ml of DI water was added using graduated cylinder (Table 4). The solution was mixed thoroughly using the pipetter and autoclaved at Load Cycle 3 (Liquid medium < 6L) in the autoclave. After cooling down to room temperature the 20% Glycerol solution was used to prepare the bacterial stock.

Table 4. The components and amount for making 20% Glycerol solution.

Component	**Amount**
Glycerol	20 ml
DI H_2O	80 ml

Preparation of 20% Glycerol stock

After both the BHI broth and 20% glycerol was made and cooled down to room temperature, 5 ml of BHI broth were transferred to falcon tubes. Pure cultures of bacterial isolates were introduced into the broth using sterile loop and incubated overnight at 37°C. At first, 800 µl of 20% Glycerol solution was added to 1000 µl cryovials and then 200 µl of overnight pure

bacterial culture was added (Table 5). The turbidity must be observed for bacterial culture in the broth to ensure growth. Also the cryovials were labeled carefully according to the sample ID. Finally the mixture was vortexed vigorously and kept in the -80°C freezer in separate labeled boxes for later use.

Table 5**.** The components and amount for making 20% Glycerol Stock.

Component	**Amount**
20% Glycerol solution	800 µl
Overnight bacterial culture in BHI broth	200 µl

16S rRNA Sequencing

Isolates obtained from blood plates were initially classified by 16S rRNA sequencing, as described by Lane (1991). Each of the isolate has a unique number and other related information, that were recorded carefully. Four different universal primers were tested to select the best working primer set (Forward and Reverse primer) that works for most isolates (Greisen, Loeffelholz, Purohit, & Leong, 1994; Hou, Fink, Radtke, Sadowsky, & Diez-Gonzalez, 2013). The universal primers that were used in this study are as followed in the table below (Table 6):

Table 6**.** The list of primers for the amplification of the template.

Primer	**Sequence (5'--------3')**
1492R	TACGGYTACCTTGTTACGACTT
27F	AGAGTTTGATCMTGGCTCAG
8F	AGA GTT TGA TCC TGG CTC AG
63F-1389R	5'- CAGGCCTAACACATGCAAGTT-3'
BACT2F	5'-CAAACAGGATTAGATACCCTG-3'

PCR Amplification

Polymerase chain reaction, better known as PCR, is a technique used extensively in molecular biology. The technique uses a single piece of DNA and amplifies it to thousand to millions of copies. It requires two primers for forward and reverse reaction, DNA template to be amplified, DNA polymerase to elongate the strand, Deoxynucleoside triphosphates (dNTPs) to provide nucleotide to the growing strand and buffer solution providing suitable environment for the reaction (Lane, 1991). In this project, PCR amplification of the 16S rRNA sequence was done using 4 different forward primers and reverse primer for all samples (Figure 6). The forward primers are usually named according to the restriction site and the target site of the promoter where they will bind (Dieffenbach, Lowe, & Dveksler, 1993).

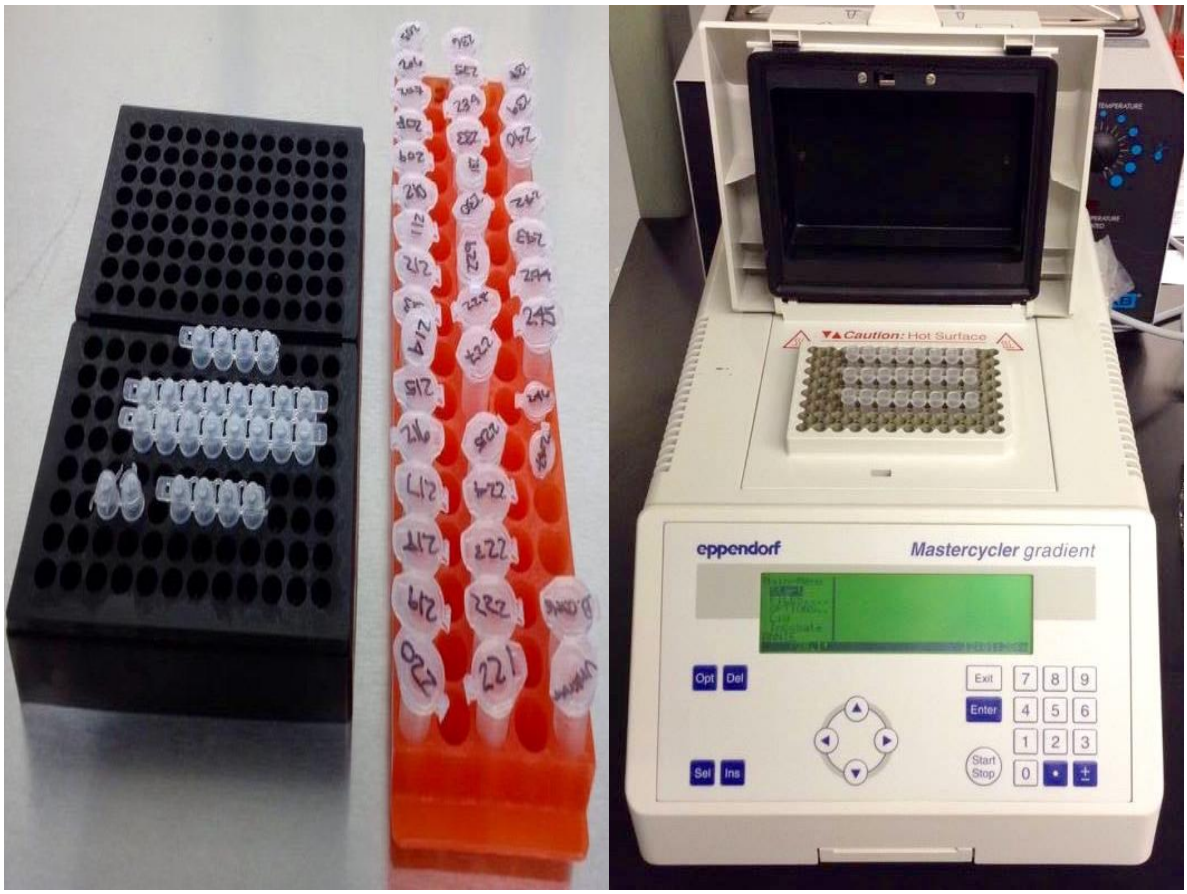

Figure 6: PCR reaction preparation and Thermal Cycler

Primer Working Stock Preparation

As mentioned above, four different universal primers were used to amplify the 16S rRNA sequence and only the 8F-1492R primer set performed best for all the samples. After receiving the lyophilized primer, it was spanned before opening to insure that the primer pellet is at the bottom of the tube. Following the manufacturer's instructions (Table 7), 100µM stock solutions of primers were prepared by adding DI water according to the nano mole of the oligo (IDT, Coralville, USA).

Table 7**.** Preparation of 100 µM primer stock.

Oligo Amount (nmoles)	**Amount of DI H_2O**
8F- 20.8 nmole	208 µl
1492R- 25.4 nmole	254 µl

From the 100µM primer stock, 10 µM working stock was prepared by 1:10 dilution. In two separate sterile 1 ml tubes, 100 µl of Forward and Reverse primer were added following the addition of 900 µl of RNAase free water. The 100µM primer stocks were stored at -20°C for later use.

PCR Reaction and Sample Preparation

Fast Cycling PCR Kit from QIAGEN was used to amplify the templates. A 20µl reaction mixture was prepared following the manufacturer's instructions (Table 8). Amount of all the reagents used for PCR is given in table below.

Table 8. The list of reagents and amount used for a single PCR reaction.

Components	**Amount for 1 reaction**	**For *110 reaction**
Master Mix	10 µl	1100 µl
Q Solution	4 µl	440 µl
Forward & Reverse Primer	1 µl (0.5 each)	110 µl
RNAase free Water	3 µl	330 µl
Template DNA	2 µl	---
Total =	20 µl	Total= 1,980 µl

A 96 well PCR plate was used for mixing the PCR reaction and amplification in the thermal cycler. In the 96 well plate, 95 samples and one control were run for amplification. The PCR reaction mixture was made for a total of 110 reactions instead of 96, to avoid the shortage due to pipetting loss. In a sterile plastic vial 1100 µl of Fast cycling PCR Master Mix was added. Then 440 µl Q solution, 330 µl of RNAase free H_2O and 110 µl of Forward & Reverse primer solution were added to the vial. The mixture was vortexed briefly to evenly mix all the components.

For a single PCR run, 95 samples were prepared at a time. In a sterile 1.5ml plastic tube, 200 µl of DI water was added. Using the tip of the pipette tips, very little amount of culture was taken form the center of the pure single colony, to avoid contamination. The culture was mixed thoroughly using vortex. All the tubes were labeled carefully according to the sample ID.

At first, 18 µl of PCR reaction mixture was added to the entire well in the 96 plate and then using a multichannel pipetter 2 µl of sample was added to each well with vigorous mixing. The DNA templates (Sample) were added at last to reduce contamination. The PCR plate was covered tightly with domed shape strip caps and placed inside the thermal cycler for amplification run.

The thermal cycler program was set for 40 cycles with the denaturation temperature at 95° C, annealing temperature at 55°C and elongation temperature at 72°C (Table 9). Annealing temperature varied for different samples so adjustments were made, ranging from 50°C, 55°C, 65°C and 68°C (QIAGEN Fast Cycling Handbook, 2012).

Table 9**.** Thermal cycler program for PCR reaction.

Stage	**Cycle Step**	**Temperature (°C)**	**Time**
Hold	Initial denaturation	95	10 min
Cycle (25 to 40 cycles)	Denature	95	30 sec
	Anneal	55	30 sec
	Extend	72	60 sec
Hold	Final Extension	72	7 min
Hold	Final Hold	4	∞

Gel Electrophoresis

Gel electrophoresis is a standard tool in biological laboratories in purpose of separating DNA and RNA fragments according to their size. It uses a gel as medium containing the sample, which runs through an electrical field (Johansson, 1972). There are types of gel used for electrophoresis based on the size of sample. For this project Agarose gel was used for which is easy to cast and samples can easily be recovered from the gel (Figure 7).

Figure 7**:** Gel Electrophoresis Chamber.

Running Buffer Preparation

Tris-borate-EDTA (TBE) buffer was used for making Agarose gel and as running buffer. A beaker was filled with 800 ml of filtered DI (Deionized) water and put in a stir bar. All the components were weighed according to the amount shown in the chart below (Table 10). At first 54g of Tris base was added, then 2.9g of EDTA acid. Boric acid (27.5g) was added after the EDTA acid was fully dissolved. Another 200 ml of filtered DI water was added using graduated cylinder to reach the final concentration of 1000 ml or 1 Liter. After mixing for a couple of minutes, the mixture was poured into a sterilized 1 Liter glass bottle. Finally the glass bottle was autoclaved on cycle 3. To reach the working solution of 0.5X buffer, the 1 Liter buffer was diluted with 9 Liter DI water.

Table 10. The list of reagents used to make 5X TBE.

Components	**Amount**
Tris Base	54g
EDTA Acid	2.9 g
Boric Acid	27.5g
DI Water	1 Liter

1% Agarose Gel Preparation

In this project, after amplifying the 16S rRNA sequence of the templates, gel electrophoresis was used to detect the amplified desired product size of 1400 to 1500 bp (Base pair). For the gel electrophoresis 1% Agarose gel was used. Amount of each reagent in preparation of gel and sample loading is given in the table (Table 11).

Table 11. The list of reagents used to make 1% Agarose Gel.

Components	**Amount**
Agarose Powder	1 g
0.5X TBE Buffer	100 ml

1g Agarose gel powder was measured and taken in a sterile bottle and mixed with 0.5X TBE buffer. The top of the bottle was covered with plastic wrap and placed into a microwave for 3 minutes. The heating was observed carefully in case of overflow of liquid due to boiling. The mixture was placed into a water bath at 50°C and was not poured until it reached down to the temperature from boiling point. For gel with smaller pore size, 2% gel can be prepared when

needed. Amount of all the reagents are same as 1% Agarose, only instead of using 1g of gel powder, 2 g can be used.

Sample Preparation and Loading on Gel

After cooling down to 50°C, the liquid gel was poured on top of the Gel electrophoresis chamber and left for 30 minutes to set and solidify. After the gel was solidified, it was put on the electrophoresis tank. The wells in the gel were loaded with the amplified template and DNA ladder (Table 12).

Table 12**.** The list of reagents used to prepare sample and loading the Gel.

Components	**Amount**
DNA Ladder	6 µl
Loading Dye	2 µl
Template	10 µl

One well was loaded with 6 µl DNA ladder (GelPilot 100 bp plus-QIAGEN) mixed with 2 µl loading dye (EZ-VISION DNA dye), for measuring the size of the amplified sequences. Amplified samples of 10 µl were mixed with 2 µl of loading dye. All the wells were loaded with 12 µl sample. After loading the sample, gel was run at 70V (voltage) for 45 minutes. Images of the bands forming were visualized under UV light and picture was taken for recording the presence of desired bands.

PCR Product Purification (ExoSAP-IT)

When PCR amplification is complete, any unconsumed dNTPs and primers remaining in the PCR product mixture will interfere with these methods (Bell, 2008). ExoSAP-IT is a reagent that removes these contaminants. After the amplification of the PCR product and Gel Electrophoresis run, templates with desired product size or DNA band (1400-1500 bp) were identified under UV light observation and selected to send out for sequencing. PCR products were purified using ExoSAP-IT reagent by Affymetrix. The procedure was followed as manufacturer's instructions with adding 2 µl of the ExoSAP-IT clean up reagent to 5 µl PCR product. After mixing the mentioned amount, the mixture was incubated at 37°C for 15 minutes and heated at 80°C for another 15 minutes to inactivate ExoSAP-IT. The entire program including the incubation and heating temperature and time were set in the Thermal cycler machine for repeated use.

Data Analysis of 16S rDNA Sequencing

The amplified samples were sent for sequencing to GENEWIZ, which is a reknowned research organization providing different adavanced research services. Using the sanger sequencing method, forward and reverse primer sequences were obtained for the amplified samples. The forward and reverese primer sequences were assembeled together using the software Geneious version R10 (Kearse, et al., 2012). After retrieving the whole sequences for each sample, the taxonomic classification of 16S rRNA PCR products were assigned using NCBI Targeted Loci BLAST, comparing with the 16S rRNA library and further identify the unknown isolates. In the NCBI Targeted Loci BLAST 16S rRNA database, the result shows a combination of the highest alignment score (Max score), the total alignment scores (Total score), the percentage of query covered by alignment to the database sequence, the best (lowest) Expect

value (E-value) of all alignments, and the highest percent identity (Max identity) of all query-subject alignments from the sequence database (NCBI Resource Coordinators, 2016). Generally, the first hit shows the highest Max and total score, lowest E value, and highest Identity percentage. However, the percentage of query cover does not reflect the highest number all the time, it might be same or slightly lower than the highest score.

Data Analysis of MALDI-TOF

The result data from MALDI-TOF is analyzed by the commercial software and referrence library in the laboratory. It generates result very fast as a single run takes around 20 mins and it gives the specific name of the unknown bacteria by matching up with library. Also it provides a second prediction as well and no reading if there is not sufficient amount of cell debris of the bacteria or if there is no record in the referrence library. It is very easy to analyse the result of the MALDI-TOF as it provides the species name of the bacteria tested.

The MALDI-ToF method is run in duplicate for each isolate tested, with the 2 closest matches from the isolate library reported for each test, and a ‘diagnostic certainty score’ reported for each. The manufacturer (Bruker Corp., Germany) recommends a diagnostic certainty score of 2.0 or greater be attained to be confident in the accuracy of a diagnosis at the species level and a score between 1.8 and 2.0 is accurate to the genus level.

Therefore, the 16S rRNA sequencing was used to validate the MALDI-ToF performed by the Diagnostic lab at the University of Minnesota. All the isolates were analyzed and the comparison confirmed true genus and species identification for each organism tested. The results of the 16S rRNA sequence analysis were compared to the MALDI-ToF identification and the discrepancies were further analyzed in the result section.

Chapter III: Results

Thermoduric Isolate Identification

From the heat-treated milk samples, mesophilic and psychrophilic bacteria were isolated. Culturing the milk samples in the appropriate agar media collected a total of 447 isolates, where the numbers of psychrophilic isolates were 226 and mesophilic isolates were 221. For the identification purpose, unknown amplified samples were sent out for obtaining the complete 16S rRNA gene sequences. Due to reaction failure during the Sanger sequencing, only 248 sequences were retrieved combining 118 psychrophilic isolates and 130 mesophilic isolates. The table below (Table 13) shows all the Genus and Species obtained from the milk samples using the 16S sequencing identification method.

Table 13**:** List of Genus and Species of isolates collected from heat-treated milk samples.

NO.	**Genus**	**Species**
1	*Acinetobacter*	*radioresistens*
2	*Arthrobacter*	*agilis*
3	*Aureimonas*	*phyllosphaerae*
4	*Bacillus*	*paralicheniformis*
5	*Bacillus*	*aryabhattai*
6	*Bacillus*	*safensis*
7	*Bacillus*	*kochii*
8	*Bacillus*	*siralis*
9	*Bacillus*	*pumilus*
10	*Bacillus*	*oleronius*

NO.	Genus	Species
11	*Bacillus*	*axarquiensis*
12	*Bacillus*	*circulans*
13	*Bacillus*	*aerius*
14	*Bacillus*	*ginsengi*
15	*Bacillus*	*subtilis*
16	*Bacillus*	*galliciensis*
17	*Bacillus*	*pseudomycoides*
18	*Bacillus*	*hisashii*
19	*Bacillus*	*amyloliquefaciens*
20	*Bacillus*	*licheniformis*
21	*Bacillus*	*clausii*
22	*Bacillus*	*sonorensis*
23	*Bacillus*	*galactosidilyticus*
24	*Bacillus*	*flexus*
25	*Bacillus*	*lonarensis*
26	*Bacillus*	*thermoamylovorans*
27	*Brachybacterium*	*muris*
28	*Brachybacterium*	*nesterenkovii*
29	*Brevibacterium*	*frigoritolerans*
30	*Brevundimonas*	*bacteroides*
31	*Clavibacter*	*michiganensis*
32	*Curtobacterium*	*oceanosedimentum*
33	*Enterococcus*	*faecalis*

NO.	Genus	Species
34	*Frigoribacterium*	*faeni*
35	*Hydrogenophaga*	*caeni*
36	*Janibacter*	*hoylei*
37	*Kocuria*	*varians*
38	*Lysinibacillus*	*halotolerans*
39	*Microbacterium*	*lacticum*
40	*Microbacterium*	*aurum*
41	*Microbacterium*	*lacus*
42	*Micrococcus*	*flavus*
43	*Micrococcus*	*yunnanensis*
44	*Micromonospora*	*aurantiaca*
45	*Oceanobacillus*	*sojae*
46	*Oceanobacillus*	*caeni*
47	*Paenibacillus*	*rhizosphaerae*
48	*Paenibacillus*	*xylanexedens*
49	*Paenibacillus*	*tundrae*
50	*Paenibacillus*	*borealis*
51	*Paenibacillus*	*barengoltzii*
52	*Paenibacillus*	*amylolyticus*
53	*Paenibacillus*	*lactis*
54	*Rothia*	*dentocariosa*
55	*Salmonella*	*enterica*
56	*Sphingomonas*	*trueperi*

NO.	Genus	Species
57	*Sporosarcina*	*psychrophila*
58	*Sporosarcina*	*siberiensis*
59	*Sporosarcina*	*contaminans*
60	*Staphylococcus*	*epidermidis*
61	*Staphylococcus*	*cohnii*
62	*Staphylococcus*	*chromogenes*
63	*Staphylococcus*	*hominis*
64	*Staphylococcus*	*wameri*
65	*Streptococcus*	*oralis*
66	*Streptococcus*	*mitis*
67	*Streptococcus*	*salivarius*
68	*Streptococcus*	*rubneri*
69	*Virgibacillus*	*halotolerans*
70	*Virgibacillus*	*proomii*

Prevalent Genera in Milk Samples

The characterization of the isolates showed frequent occurrence of some predominant genera. Many different species were found from the same genus with repeated occurrence. The table below (Table 14) shows the list of genera with the number of occurrences.

Table 14: List of Prevalent Genera from the heat-treated milk samples.

Genus	**Species Occurrence**	**Total Species Occurrence**
Bacillus	23	135
Paenibacillus	7	13
Staphylococcus	5	12
Streptococcus	4	7
Microbacterium	3	37
Sporosarcina	3	6
Micrococcus	2	5
Oceanobacillus	2	4
Other	21	29

A total of 71 different species were identified from the milk samples where the different prevalent genera included *Bacillus, Paenibacillus, Staphylococcus, Streptococcus, Microbacterium, Micrococcus and Oceanobacillus* (Figure 8). The most frequent genus was *Bacillus* with a total of 135 repeated occurrences and 23 different species. Some of the most frequent species of the genus *Bacillus* were *Bacillus licheniformis* with 38 occurrences, *Bacillus safensis* with 18 occurrences, *Bacillus paralicheniformis* with 16 occurrences, *Bacillus aerius* with 13 occurrences, *Bacillus circulans* with 9 occurrences and *Bacillus pumilus* with 8 occurrences. There were 7 different species of *Paenibacillus* including *Paenibacillus amylolyticus* with 4 occurrences, *Paenibacillus tundra* with 3 occurrences and *Paenibacillus borealis* with 2 occurrences. Then *Microbacterium* with 3 different species and a total 37 occurrences included the species *Microbacterium lacticum* with 33 occurrences, *Microbacterium*

aurum with 2 occurrences and *Microbacterium lacus* with 2 occurrences. Again the 5 different species of *Staphylococcus* included *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus cohnii, Staphylococcus hominis* and *Staphylococcus wameri.* Also 4 different *Streptococcus* species were found such as *Streptococcus mitis, Streptococcus oralis, Streptococcus salivaris* and *Streptococcus rubneri.* Some other comparatively less frequent genus were *Sporosarcina, Micrococcus, Oceanobacillus, Virgibacillus, lysinibacillus and Brachybacterium*.

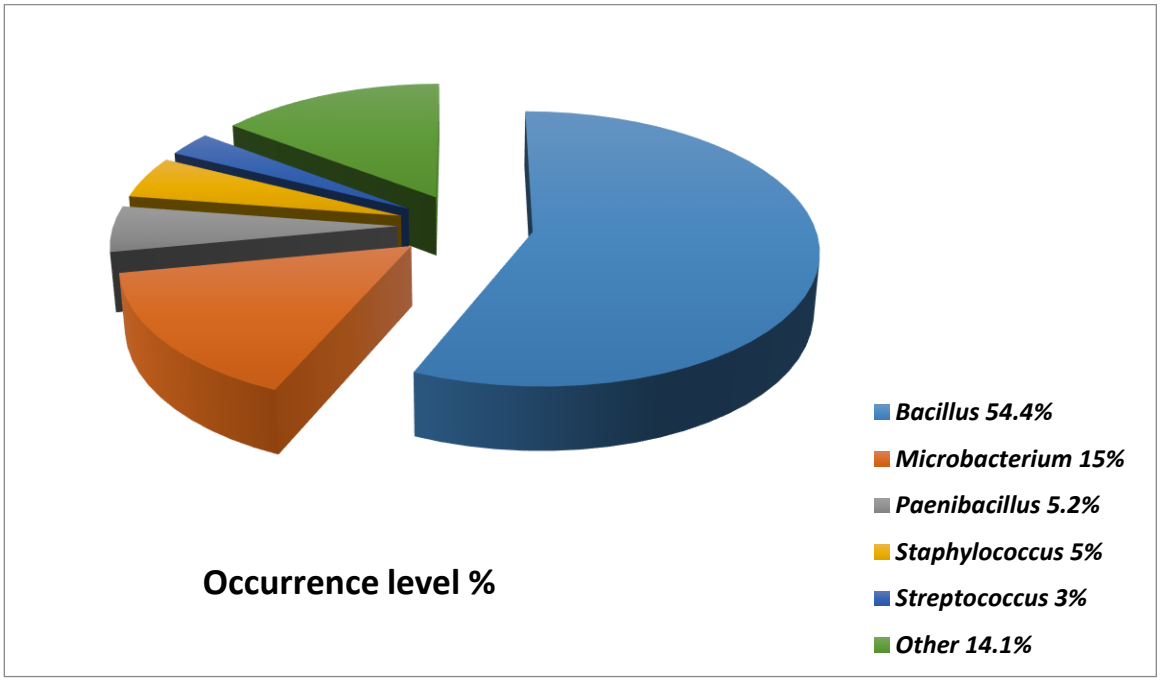

Figure 8: The pie chart in above figure is showing the occurrence rate (in percentage %) of the different prevalent Genera isolated from the milk samples. The *Bacillus* with 54.4%, *Microbacterium* 15%, *Paenibacillus* 5.2%, *Staphylococcus* 5%, *Streptococcus* 3% and other genera 14.1%.

The *Bacillus* genus represented 54.4% of the total bacterial isolates collected from the pasteurized milk samples. The occurrence level of *Microbacterium* was 15%, *Paenibacillus* 5.2%, *Staphylococcus* 5%, *Streptococcus* 3% and other genera constituted about 14.1% of the total bacterial population collected from the heat-treated milk samples. Some very less frequent genera that represented the other category included *Acinetobacter, Arthrobacter, Aureimonas, Clavibacter, Brevundimonas, Curtobacterium, Frigoribacterium, Hydrogenophaga, Janibacter, Kocuria, Micromonospora, Rothia, Sphingomonas, Enterococcus* and *Salmonella.*

Environmental Isolates

The Environmental samples were collected from different dairy farms across Minnesota. The total number of isolates received from the Veterinary Diagnostic Lab (VDL) were 810 and 664 isolates were amplified. After the Sanger sequencing 481 sequences were retrieved due to reaction failures. The table below (Table 15) shows all the Genus and Species identified from the environmental samples using 16S sequencing technique.

Table 15: List of Genus and Species of isolates collected from environmental samples.

NO.	Genus	Species
1	*Acinetobacter*	*gandensis*
2	*Aerococcus*	*viridans*
3	*Aerococcus*	*urinaeequi*
4	*Citrobacter*	*freundii*
5	*Corynebacterium*	*argentoratense*
6	*Enterobacter*	*cloacae*
7	*Enterobacter*	*xiangfangensis*

NO.	Genus	Species
8	*Enterobacter*	*ludwigii*
9	*Enterobacter*	*kobei*
10	*Enterobacter*	*asburiae*
11	*Enterococcus*	*faecium*
12	*Enterococcus*	*faecalis*
13	*Enterococcus*	*thailandicus*
14	*Enterococcus*	*hirae*
15	*Enterococcus*	*saccharolyticus*
16	*Enterococcus*	*casseliflavus*
17	*Escherichia*	*fergusonii*
18	*Escherichia*	*hermanni*
19	*Klebsiella*	*pneumoniae`*
20	*Klebsiella*	*oxytoca*
21	*Lactococcus*	*lactis*
22	*Lactococcus*	*formosensis*
23	*Lactococcus*	*garvieae*
24	*Listeria*	*innocua*
25	*Lysinibacillus*	*fusiformis*
26	*Macrococcus*	*caseolyticus*
27	*obesumbacterium*	*proteus*
28	*Ochrobactrum*	*tritici*
29	*Pantoea*	*agglomerans*
30	*Pantoea*	*brenneri*

NO.	Genus	Species
31	*Paracoccus*	*denitrificans*
32	*Psedomonas*	*guariconesis*
33	*Pseudomonas*	*putida*
34	*Pseudomonas*	*punonensis*
35	*Pseudomonas*	*knackmussi*
36	*Pseudomonas*	*indoloxydans*
37	*Pseudomonas*	*protegens*
38	*Pseudomonas*	*lundensis*
39	*Pseudomonas*	*guariconensis*
40	*Pseudomonas*	*pictorum*
41	*Pseudoxanthomonas*	*suwonensis*
42	*Psychrobacter*	*maritimus*
43	*Raoultella*	*terrigena*
44	*Riemerella*	*anatipestifer*
45	*Shigella*	*flexneri*
46	*Shigella*	*dysenteriae*
47	*Staphylococcus*	*aureus*
48	*Staphylococcus*	*chromogenes*
49	*Staphylococcus*	*sciuri*
50	*Staphylococcus*	*simulans*
51	*Staphylococcus*	*haemolyticus*
52	*Staphylococcus*	*saprophyticus*
53	*Staphylococcus*	*agnetis*

NO.	Genus	Species
54	*Staphylococcus*	*cohnii*
55	*Staphylococcus*	*devriesei*
56	*Staphylococcus*	*epidermidis*
57	*Staphylococcus*	*xylosus*
58	*Staphylococcus*	*hominis*
59	*Streptococcus*	*pyogenes*
60	*Streptococcus*	*agalactiae*
61	*Streptococcus*	*uberis*
62	*Streptococcus*	*dysgalactiae*
63	*Streptococcus*	*porcorum*
64	*Trueperella*	*abortisuis*
65	*Weissella*	*parameseneroides*

Prevalent Genera in Environmental Samples

After the identification of the isolates, it was seen that some genera were more frequent than the others. Again many different species were found to be from the same genus with repeated species occurrence. The table below (Table 16) shows the list of genera with the number of individual species occurrences.

Table 16: List of Prevalent Genera from the Environmental samples.

Bacterial Genera	**Species occurrence**	**Total Species occurrence**
Staphylococcus	11	157
Streptococcus	4	86
Escherichia	3	55
Klebsiella	2	53
Lactococcus	3	31
Enterococcus	6	29
Pseudomonas	9	11
Citrobacter	1	7
Aerococcus	2	6
Shigella	2	4
Other	22	42
Total	65	481

From the environmental samples, 64 different species were identified. The frequency of occurrence for some of the genus was more than the others (Figure 9), such as *Staphylococcus* was found to be the most prevalent genus for having 11 different species and a total occurrence number of 157 isolates. The different species of *Staphylococcus* were *Staphylococcus aureus, Staphylococcus chromogenes, Staphylococcus cohnii, Staphylococcus simulans, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus*

agnetis, Staphylococcus devriesei, Staphylococcus xylosus and *Staphylococcus hominis* etc. The second most prevalent genus was *Streptococcus* with a total occurrence of 86 isolates of 4 different species including *Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae.* Then *Escherichia* with 3 different species and 55 isolates included *Escherichia fergusonii, Escherichia coli* and *Escherichia hermanni.* The genus *Klebsiella* with 53 isolates and 2 different species included *Klebsiella oxytoca and Klebsiella pneumonia.* The *Lactococcus* genus had 31 isolates with 3 different species including *Lactococcus lactis, Lactococcus formensis and Lactococcus garviae.* The other prevalent genus *Enterococcus* with 6 different species included *Enterococcus faecalis, Enterococcus hirae, Enterococcus thailandicus, Enterococcus saccharolyticus, Enterococcus casseliflavus and Enterococcus faecium.* Again *Pseudomonas* had 9 different species, such as *Pseudomonas putida, Pseudomonas lundensis, Pseudomonas guariconesis, Pseudomonas punonensis, Pseudomonas knackmussi, Pseudomonas indoloxydans, Pseudomonas protegens, Pseudomonas parafulva* and *Pseudomonas pictorum.* Some other prevalent species were *Citrobacter freundii, Aerococcus viridans, Aerococcus urinaeequi, Shigella flexneri and Shigella dysenteriae.*

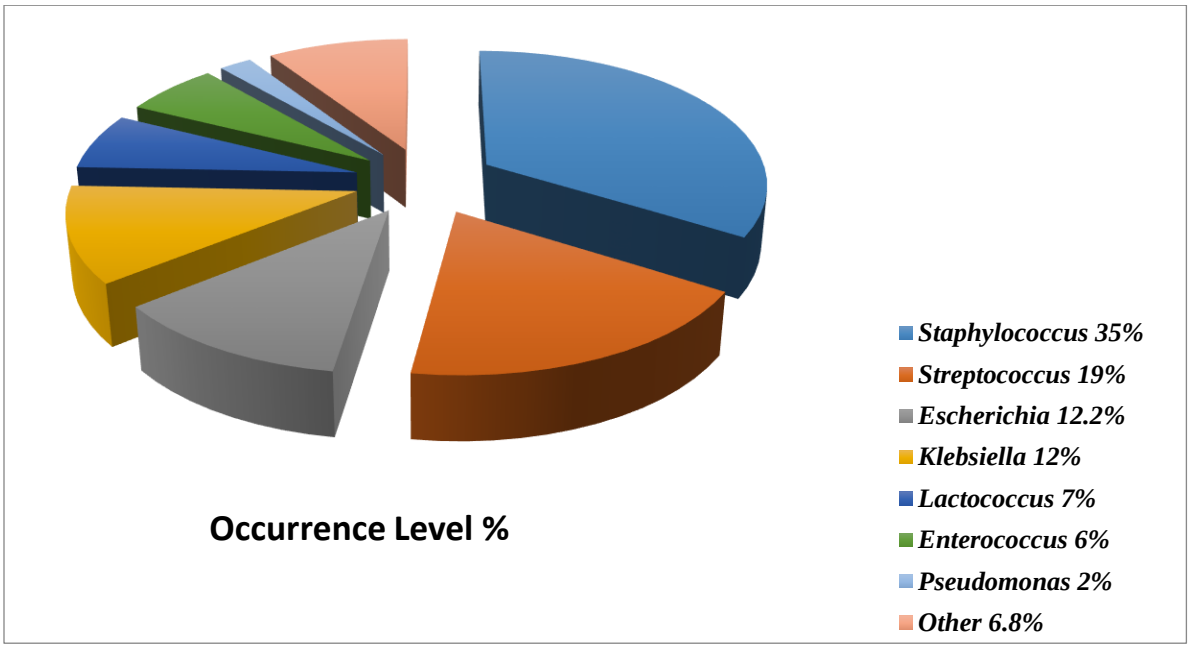

Figure 9: The pie chart in above figure is showing the occurrence rate (in percentage %) of the different prevalent Genera isolated from the Environmental samples. The *Staphylococcus* occupied 35%, *Streptococcus* occupied 19%, *Escherichia* occupied 12.2%, *Klebsiella* occupied 12%, *Lactococcus* occupied 7%, *Enterococcus* occupied 6%, *Pseudomonas* 2% and other genera occupied 6.8%.

The most prevalent genus *Staphylococcus* represented 35% of the total bacterial population of the environmental isolates. The other frequent genera *Streptococcus* occupied 19%, *Escherichia* occupied 12.2%, *Klebsiella* occupied 12%, *Lactococcus* occupied 7%, *Enterococcus* occupied 6%, *Pseudomonas* 2% and other genera occupied 6.8%. Some less frequent genera that occurred only once or twice over the entire bacterial population in the environmental samples, were categorized as the other genera and included *Acinetobacter, Enterobacter, Corynebacterium, Enterobacter, Listeria, Lysinibacillus, Macrococcus, Obesumbacterium, Ochrobactrum, Pantoea, Paracoccus, Pseudoxanthomonas, Psychrobacter, Raoultella, Riemerella, Trueperella* and *Weissella.*

Comparison of 16S rRNA Sequencing and MALDI-TOF Identification

The MALDI-TOF identification was compared with the 16S sequencing results to verify the accuracy of the test method. The MALDI-TOF identification result was obtained only for the environmental isolates. Hence, only environmental (481) isolates 16S identification was compared against the MALDI-TOF identification at both genus and species level. The table below (Table 17) is showing the outcome of the comparison.

Table 17: Comparison of 16S rRNA Sequencing and MALDI-TOF identification of the Environmental isolates.

16S sequencing and MALDI-TOF	**Number of isolates**
Total number of Isolates retrieved	481
Number of Genus level match	454
Number of Species level match	426
Number of complete mismatch	27
Number of mismatch at only Species level	26
Number of unreliable identification	17

The comparison of the identification of the environmental isolates included both genus and species level match, where all the 481 isolates 16S identification was compared against the MALDI-TOF identification (Figure 10). At the genus level, 454 isolates matched out of the total 481 isolates. The number of species level match was 426 out of 481 isolates, which makes the species level mismatch for 26 isolates out of the total. The number of complete mismatch where both genus and species did not match for 16S sequencing and MALDI-TOF identification was

low as 27 out of 481 isolates. Also there was some unreliable identification by MALDI-TOF, where the genus and species of a given isolate was not obtained. Such unreliable identity was for 17 isolates out of the 481 total.

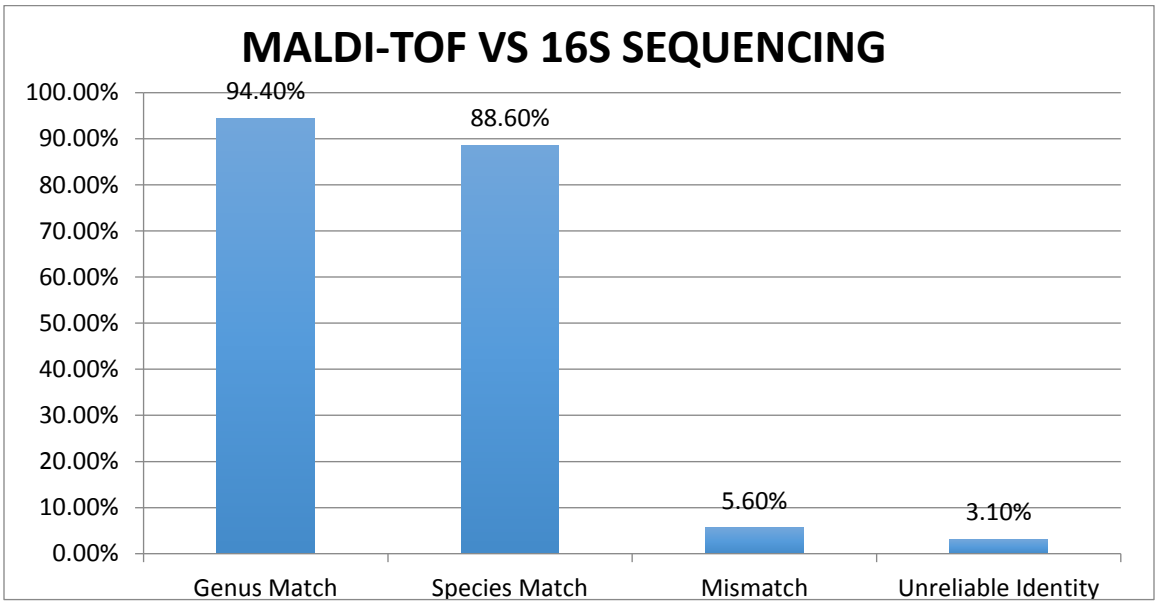

Figure 10: The comparison of MALDI-TOF and 16S sequencing identification of environmental isolates bar graph shows the percentage of Genus level match at 94.4%, Species level match at 88.6%, complete mismatch of both genus and species level at 5.6% and Unreliable identification of isolate by MALDI-TOF at 3.1%.

The environmental isolates characterized by MALDI-TOF had a genus level match of 94.4% with the 16S rRNA sequencing identification. The complete match at both genus and species level was 88.6% and complete mismatch was 5.6% where both genus and species were different than that of identified by the gold standard 16S rRNA sequencing method. The unrecognized identity by MALDI-TOF identification system was only 3.1% of the total number of isolates characterized.

Complete and Partial Misidentification by MALDI-TOF

The misidentification of the environmental bacterial isolates by MALDI-TOF was either complete mismatch or partial mismatch. The complete mismatch were those, where both genus and species were not the same as the 16S identification. Whereas the partial mismatch had only the genus level match with the 16S characterization but the species were different. The table below (Table 18) shows the complete misidentification by the MALDI-TOF mass spectrometry system.

Table 18: Complete mismatch of genus and species of isolates by MALDI-TOF identification.

16S rRNA identification	**MALDI-ToF identification**
Shigella flexneri	*Escherichia coli*
Pantoea agglomerans	*Enterobacter cloacae*
Citrobacter gillenii	*Klebsiella oxytoca*

The complete mismatch shows that all the *Shigella flexneri* that were characterized by 16S rRNA sequencing were *identified* as *Escherichia coli* by the MALDI-TOF. Also *Pantoea agglomerans* was identified as *Enterobacter cloacae* by the newer technique MALDI-TOF. All the *Citrobacter gillenii* were characterized as *Klebsiella oxytoca* by MALDI-TOF identification system.

The table below (Table 19) shows the misidentification of species from the same genus by the MALDI-TOF characterization method.

Table 19: Species mismatch of Environmental isolates by MALDI-TOF identification.

16S Sequencing Identification	**MALDI-ToF Identification**
Escherichia fegusonii	*Escherichia coli*
Escherichia hermannii	*Escherichia coli*
Lactococcus formensis	*Lactococcus garvieae*
Aerococcus urinaeequi	*Aerococcus viridans*
Klebsiella terrigena	*Klebsiella oxytoca*
Citrobacter freundii	*Citrobacter braakii*

While comparing the 16S and MALDI-TOF identification results of the environmental isolates, only species level mismatch was found for some isolates. Such as all the Escherichia *fegusonii* and *Escherichia hermannii* were identified as *Escherichia coli.* All the *Lactococcus formensis* were characterized as the species *Lactococcus garvieae.* The *Aerococcus urinaeequi* were characterized as *Aerococcus viridans* by the MALDI-TOF method. Again the *Klebsiella terrigena* and *Citrobacter freundii* were recognized as *Klebsiella oxytoca* and *Citrobacter braakii* respectively.

Unreliable Identification by MALDI-TOF

Also there was some unreliable identification where the genus and species of the environmental isolates were not identified by the MALDI-TOF method. The unreliable identity includes both Gram-positive and Gram-negative organisms.

- **Gram Positive**

- *Staphylococcus cohnii*
- *Streptococcus porcorum*

- *Macrococcus caseolyticus*
- *Acinetobacter gandensi*
- *Weissella paramesenerοides*
- *Enterococcus hirae*

❑ **Gram Negative**

- *Pseudomonas knackmussi*
- *Pseudomonas guariconesis*
- *Riemerella anatipestifer*
- *Psychrobacter maritimus*

Chapter IV: Discussion

Most of the isolates we identified from environmental samples belonged to the genera *Staphylococcus, Streptococcus, Escherichia, Klebsiella,* and *Pseudomonas*; all reported as common environmental mastitis pathogens in many studies (Harmon, 1994). We also identified many *Lactococcus* and *Enterococcus* species (31 and 29 respectively). According to Plumed-Ferrer, et.al, *Lactococcus* spp. are being identified from clinical mastitis more often recently than in the past, possibly because this genus was misidentified either as *Streptococcus* or *Enterococcus* by traditional culture techniques. Also further genotyping of the *Lactococcus lactis,* and *Lactococcus garvieae* in the particular study showed that the strains were different which implies the environmental transmission rather than contagious transmission of mastitis (Plumed-Ferrer, Uusikylä, Korhonen, & Wright, 2013). A study was conducted at the Wisconsin Veterinary Diagnostic Lab for the identification of mastitis pathogens from the environmental samples that were taken from dirt, manure, bedding, and milking machines. The different species that were found to be common with our findings included *Citrobacter sp., Aerococcus sp., Shigella sp., Corynebacterium sp., Acinetobacter sp., Enterobacter sp., Listeria sp., Pantoea sp.,* and *Trueperella sp.* According to the study, all these pathogens are reported as common Environmental mastitis pathogens (Wisconsin VDL, 2016).

In the pasteurized milk samples, we identified mainly *Bacillus, Paenibacillus, Microbacterium, Streptococcus, Staphylococcus, Micrococcus, Sporosarcina,* and *Oceanobacillus.* The *Bacillus spp*. constituted more than half of the total bacterial species identified from the heat-treated frozen milk samples. *Bacillus spp.* are Gram-positive spore-forming bacteria that can survive the usual pasteurization temperature and have been very commonly associated with milk and dairy product spoilage (Caceres, Castillo, & Pizarro, 1997;

Johnson, 2000; Rukure, & Bester, 2001). In agreement with other similar studies, the most frequent species of the genus *Bacillus* were *Bacillus licheniformis, Bacillus safensis Bacillus paralicheniformis*, *Bacillus aerius*, *Bacillus circulans*, and *Bacillus pumilus*, (SAMARŽIJA, Zamberlin, & Pogačić ,2012). Some species of *Bacillus* such as *B. cereus*, *licheniformis*,, and *subtilis* isolated from the milk samples, can produce different types of toxins implicated in food borne diseases (Griffiths, 1990; Svensson, et al., 2006). Also, many aerobic spore-formers were identified including *Paenibacillus*, *Sporosarcina*, and *Oceanobacillus*, which can be associated with environmental, bedding, and feeding factors within dairy farms (De Jonghe, Shaheen, Andersson, Salkinoja-Salonen, & Christiansson, 2006). Different extracellular enzymes including proteases and lipases, plays an important role in the spoilage of dairy products. Being resistant to heat, the extracellular enzymes can help the organisms to survive pasteurization (72°C for 15 s) and even ultrahigh temperature processing (UHT; 138°C for 2 s or 149°C for 10 s) (Cousin, 1982; Koka & Weimer, 2001). *Microbacterium spp.*, and *Streptococcus spp.* that were frequently isolated from the milk are very common dairy spoilage organisms, secreting such extracellular enzymes causing the spoilage of milk, and dairy products (Adams, Barach, & Speck, 1975).

The environmental samples and pasteurized milk samples had different predominant genera, which can be justified by the different sources of the organisms. In fact, the environmental isolates were collected from skin swabs of the cow where *Staphylococcus* and *Streptococcus* species can be found predominantly. Also, some samples included swabs from the cow bedding and feeding area where *Escherichia*, *Pseudomonas, Klebsiella*, *Enterobacter*, and other coliform bacteria can be commonly isolated. On the other hand, in the milk samples, bacteria can be introduced in the milk while in the processing facility after collection. Spore

forming bacteria can survive pasteurization and the heat shock of the high temperature activates surviving spores so that they are primed to germinate at a favorable growth temperature (Cromie, Schmidt, & Dommett, 1989). Also, *Staphylococcus* spp. and *Streptococcus* spp. were seen as predominant genera in both milk and environmental samples. However, there was a significant difference in the species level, such as *Streptococcus mitis, Streptococcus oralis, Streptococcus salivaris,* and *Streptococcus rubneri* were found in the pasteurized milk samples, which are common flora of human oral cavity, and upper respiratory tract (Davis, 1996), thus confirming the contamination in the milk through the processing facility. The environmental samples had more *Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, and Streptococcus uberis,* which are commonly reported environmental *Streptococcus* spp. that causes mastitis (Petersson-Wolfe & Currin, 2012). Likewise, *Staphylococcus epidermidis, Staphylococcus cohnii, Staphylococcus chromogenes and Staphylococcus hominis* were found in the milk samples but all the other species of *Staphylococcus* including *Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus agnetis, and Staphylococcus saprophyticus* were found in the environmental samples.

As the 16S sequencing identification is generally considered more reliable, and the database more updated than the MALDI-TOF diagnostic methodology, MALDI-TOF diagnoses were benchmarked against the 16S sequencing. The comparison of the two molecular identification methods result shows overall higher percentage of similarity in case of identification of the dairy pathogens collected form environmental samples. The genus level match was shown to be pretty high as 94.4% and species level match was moderately high as 86.6%. The species level match bears more importance as it shows both genus, and species identification of the organisms. However, only genus identification might help in determining the

level of sanitation, and hygiene of a dairy farm, as well as processing facility. Again, MALDI-TOF's complete mismatch, and unreliable identity was very low as 5.6%, and 3.1% respectively. This can be attributed to the database limitation of MALDI-TOF library, and fortunately this limitation can be overcome with more similar studies, and expansion of the library of MALDI-TOF.

Misidentification can occur due to an inaccurate taxonomic assignment of a given spectra in the MALDI-TOF MS database or a recent change in the taxonomy of a given species. Also, an error in the initial conventional identification system can be a possibility as well. In a study by Bizzini and Durussel, the repetition of conventional method of misidentified isolates showed that the initial identity obtained by the conventional method was incorrect and that the identity obtained by MALDI-TOF MS was correct. Some MALDI-TOF misidentifications were consistent by both genus and species level. For example, *Shigella flexneri*, *Pantoea agglomerans*, and *Citrobacter gillenii* were erroneously identified as *Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella oxytoca* respectively. This misdiagnosis indicates that the MALDI-TOF identification of closely related Gram-negative taxa might present some challenge. Furthermore, in a related study on the evaluation of MALDI-TOF performance, *Shigella* isolates were misidentified as *E. coli* and the discordances were associated with the limit of resolution of the MALDI-TOF MS method (Bizzini, Durussel, Bille, Greub, & Prodhom, 2010). Again, being closely related species with great genomic level similarity, *Shigella* spp. and *E. coli* are expected to have similar proteomes that might prevent their differentiation by MALDI-TOF MS (Johnson et al., 2000).

Again, MALDI-TOF could partially identify some organisms, where only the genus was identified correctly not species. For example, MALDI-TOF could not distinguish among closely

related strains of the genus *Escherichia*, where *Escherichia fegusonii*, and *Escherichia hermannii* were identified as *Escherichia coli*. Similarly, *Lactococcus formensis* was identified as *Lactococcus garvieae, Aerococcus urinaeequi* was identified as *Aerococcus viridans, Klebsiella terrigena* was identified as *Klebsiella oxytoca, Citrobacter freundii* was identified as *Citrobacter braakii.* For these partially identified strains, where misidentifications were consistent by only species level, the16S rRNA identification showed same query cover percentage (%) but slightly different Max score, where mainly the highest max score, total score, Max identity percentage, and lowest E-value were taken under consideration for species identification.

Among these partially identified organisms, some are Gram-positive cocci and some are Gram-negative rods. This misdiagnosis indicates the MALDI-TOF MS identification systems limitation in identifying closely related species of both Gram-positive cocci and Gram-negative rods. The MALDI-TOF MS successfully distinguished between Gram-positive and Gram-negative organisms. Otherwise, it would be very problematic and questionable for the fundamentals of the detection system, as the cell wall composition of Gram-positive and Gram-negative bacteria are significantly different from each other, where Gram-positive cell wall is high in peptidoglycan and Gram-negative cell wall has more lipid content. So it can be hypothesized that Gram-positive bacteria, might require an additional extraction step more frequently for their cell wall structure to yield a valid MALDI score (Bizzini, Durussel, Bille, Greub, & Prodhom, 2010).

MALDI-TOF technique was unable to identify some of the species where it showed the result as unreliable identity. Gram-positive organisms that were unidentified by MALDI-TOF included *Staphylococcus cohnii, Streptococcus porcorum, Macrococcus caseolyticus, Acinetobacter gandensi, Weissella paramesene*roides, and *Enterococcus hirae.* Unidentified

Gram-negative organisms were *Pseudomonas knackmussi*, *Pseudomonas guariconesis*, *Riemerella anatipestifer*, *and Psychrobacter maritimus.* The species that were unrecognized are very less frequent, mostly unrepeated, and occurred only once or twice over the entire bacterial isolates that were collected from the milk samples. So, it can be implied that these less frequent species were not updated in the MALDI-TOF library, so that they can be identified. As previously mentioned, this problem can be solved, as the database of MALDI-TOF can be updated easily by an internal lab personnel or by simply purchasing the most updated database software (Seng, et al., 2010).

Additionally, MALDI-TOF sample preparation is simple yet crucial, and requires to be performed carefully, as it was observed that too much bacterial load could reduce the quality of the results. Also, due to the small inter-spot distance on the MALDI plate, liquid smear between spots may result in cross contamination, inducing the unreliable identification. The extraction technique yields better result than the direct deposition technique. In addition, the quality of the spectra obtained by this technique is superior to the quality of the spectra obtained by the direct deposition technique. Therefore, better MALDI scores can be obtained. In a similar study, it was shown that protein extraction increased the overall yield of valid results by 25% (Bizzini, Durussel, Bille, Greub, & Prodhom, 2010). The rapid turnaround time is the biggest advantage of MALDI-TOF MS method, starting from the sample preparation to result analysis, it can be done by individuals with minimal training that can save the labor cost of the dairy industry greatly. Also, food samples need to be stored the entire time of the testing, until the results, and data analysis is done, and ensured that no repeat test is required. In this case, the rapidity of the test method will save the dairy industry big time by reducing the cost of preservation or storage process.

Both MALDI-TOF, and PCR requires pure bacterial culture, from there the steps of sample preparation for MALDI-TOF is only adding formic acid on the culture to kill the bacteria, and obtain cell debris; whereas PCR requires multiple steps of sample preparation including DNA extraction (if needed), PCR reaction preparation, amplification, gel run preparation, and detection of amplicons etc.

The result reading of MALDI-TOF is very easy, as it directly comes up with suspect of genus, and species. On the other hand, most of the times amplified PCR reaction needs to be sent out for sequencing to commercial gene sequencing labs, as most labs or institutions would not have the expensive equipment or technical support for this purpose. Again, after the sequences are obtained, bioinformatics tools need to be applied for retrieving the species identification, which requires advanced level of trainings on the respective area.

Some limitations, and drawbacks will always be present in a research study. Such as the sample size can be considered one of the study limitations, as bigger sample size always gives a better, and statistically significant result. Initially the sample size was 1,247 (Environmental isolate 800 & Milk isolate 447), but due to reaction failures in both PCR, and sequencing events, the final number got down to 729 (Environmental isolate 481 & Milk isolate 248). So, if the results from all the samples could be retrieved, it would surely contribute to more accurate findings.

All the environmental samples were collected from different dairy farms of Minnesota, including samples from skin and/or udder swab of Mastitis infected or normal looking cows, surroundings of the animal e.g. mud, manure, moisture, and bedding etc., and the heat-treated milk samples were collected from different farms, and dairy processing facility across Minnesota by the DQCI (Dairy Quality Control Institution). So, the sample collection site can be a limiting

factor as well, because all the samples of this study are collected from similar ecological environment, and climate, which resulted in less variation of bacterial population obtained. Consequently, it is adding to the limitation of database of MALDI-TOF, as newer species addition to the library can be possible only by conducting more studies with bigger sample size, and different geographical sites with varying environmental conditions.

There were some obvious limitations of the test method itself. MALDI-TOF result analysis shows some unreliable identities where no peaks were found for those unrecognized species. So it might require additional sample preparation other than just adding formic acid. For example, some organisms such as *Mycoplasma* spp. requires DNA extraction step prior to the exposure to laser beam (Pereyre, et al., 2013). Also, further test of catalase, coagulase or antibiotic susceptibility testing helps to identify some unrecognized identity. Therefore, the test method still needs some improvements in sample preparation or some alternate test method to avoid such kind of occurrences of unreliability.

Another big limitation of the study was part of the data collection, and result analysis. MALDI-TOF MS system is an expensive one-time setup, and was available only at the VDL (Veterinary Diagnostic Lab) of the University of Minnesota, thus all the MALDI-TOF testing was conducted there. However, MALDI-TOF result was obtained only for the environmental isolates, the pasteurized milk sample isolate test is still under progress, and the rest of the results will be provided once they are tested.

Some other potential study can be done including bigger sample size with geographical variety, as studies showed that the different season and weather condition had great impact on the predominance of different species of bacteria in the environment (Hantsis-Zacharov & Halpern, 2007). A comparison of predominant environmental flora of mastitis among different

farms, and across different states of the U.S. can be done. Also, even larger studies can include different regions or countries of the world that has bigger dairy industry. It would be very interesting to find the endemic pathogens of mastitis that are specific to ecological or environmental conditions. Moreover, MALDI-TOF MS system has the potential to change the way of functioning of microbiology laboratories. Hence, further prospective studies can be conducted to assess its cost-effectiveness and time to results in comparison to those for conventional techniques.

In the food industry, it is of high importance to detect foodborne pathogens as early as possible in order to prevent outbreaks of foodborne diseases, and the spread of foodborne pathogens. The conventional culture based methods can be very specific, and selective for microorganisms, but they are mostly time consuming, and laborious as well. Again, rapid detection methods are also more sensitive, specific, time-efficient, laborsaving, and reliable compared to conventional methods. Hence various rapid methods have been developed to overcome the limitations of conventional detection techniques.

One such method is the MALDI-TOF MS system, and it is full of possibility for the rapid detection of microorganisms based on their mass spectrometry profile. From the results of the current study, it can be concluded that MALDI-TOF MS has the potential to replace conventional identification techniques for the majority of routine isolates in the milk and dairy products. MALDI-TOF can detect the genus of the bacteria with high efficiency, and species level detection is very possible with the same efficiency by expanding the library, and added sample preparation steps. More similar studies can contribute to the availability of databases specifically designed for the identification of clinically significant strains of Mastitis, which will greatly serve the dairy industry. Moreover, disease or food category specific studies might help

to develop alternate techniques, and added sample preparation steps. Furthermore, laboratories with limited microbiological expertise will benefit by using this newer, and faster detection method.

The outcome of the current study includes successful characterization of bacterial isolates derived from environmental samples and pasteurized milk samples, using 16S sequencing method. The MALDI-TOF identification was benchmarked against conventional 16S sequencing identification for a set of environmental isolates derived from dairy farms and processing facility, where the comparison showed MALDI-TOF's high accuracy of genus level identification and a slightly lower species level identification. The study results were similar to the other relevant studies (Bizzini, Durussel, Bille, Greub, & Prodhom, 2010), confirming certain limitation of the MALDI-TOF MS system including database limitation and inadequate sample preparation. A few misidentifications of the bacterial species were observed in our study for some particular closely related strains of bacteria that were reported in other similar studies, thus confirming the relevance of data across the board for environmental dairy pathogens (Bizzini, Durussel, Bille, Greub, & Prodhom, 2010; Pereyre, et al., 2013). Also, we found many environmental bacterial isolates that were characterized by 16S sequencing but remained unrecognized by the MALDI-TOF system, those bacterial isolates can be added as new species of interest to the MALDI-ToF species library. Finally, the proposed study will surely contribute to better understand the advantages and limitations of the MALDI-TOF as a primary rapid identification system, let alone providing potential species addition to the dairy pathogens list of MALDI-TOF database.

In conclusion, it bears great significance to develop a rapid, and sensitive method for detection, and identification of dairy pathogens, particularly for commercial dairy industries all over the world. The MALDI-TOF MS technique itself is simple to use, fast, and reliable. The

rapid detection of pathogen in the milk, and in the dairy farm processing facility will help to address the sanitation, and hygiene practices. Also, the early detection of mastitis pathogens from subclinically infected animals, will help to control the mastitis situation in the farm, and assist in adopting prevention measures as well.

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