16S rRNA-BASED TAG PYROSEQUENCING OF COMPLEX FOOD AND WASTEWATER ENVIRONMENTS: MICROBIAL DIVERSITY AND DYNAMICS

A Thesis

by

KATHERINE GRACE MCELHANY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Food Science & Technology

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Microbial Diversity and Dynamics

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Approved by:

Chair of Committee,	Suresh D. Pillai
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ABSTRACT

16S rRNA-Based Tag Pyrosequencing of Complex Food and Wastewater Environments: Microbial Diversity and Dynamics. (December 2010)

Katherine Grace McElhany, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Suresh D. Pillai

Environmental microbiology has traditionally been performed using culture-based methods. However, in the last few decades, the emergence of molecular methods has changed the field considerably. The latest development in this area has been the introduction of next-generation sequencing, including pyrosequencing. These technologies allow the massively parallel sequencing of millions of DNA strands and represent a major development in sequencing technologies. The purpose of this study was to use both pyrosequencing and traditional culture-based techniques to investigate the diversity and dynamics of bacterial populations within milk and untreated sewage sludge samples.

Pasteurized and raw milk samples were collected from grocery stores and dairies within Texas. Milk samples were analyzed by plating, pyrosequencing, and an assay for the presence of cell-cell signaling molecules. Samples were processed, stored, and then evaluated again for spoilage microflora. The results of this study showed that raw milk had a considerably higher bacterial load, more diversity between samples, and a significantly higher concentration of pathogens than pasteurized milk. Additionally, this study provided evidence for varying spoilage microflora between raw and pasteurized milk, as well as evidence for the production of cell-cell signaling molecules by bacterial organisms involved in milk spoilage.

Four samplings of untreated sewage sludge were collected from wastewater treatment plants in seven different municipalities across the United States. Samples were subjected to quantification of selected bacterial organisms by culture and a pyrosequencing analysis was performed on extracted community DNA. The results of this study showed that untreated sewage sludge is inhabited by a huge diversity of microorganisms and that certain municipalities may have distinct bacterial populations that are conserved over time. Additionally, this study provided some evidence for seasonal differences in several of the major bacterial phyla. Lastly, this study emphasized the challenges of comparing results obtained by culture and pyrosequencing.

In conclusion, this study showed that both milk and sewage are highly diverse, dynamic environments that can contain organisms of public health concern. The use of both culture-based methods and pyrosequencing in this study proved a complementary approach, providing a more comprehensive picture of both microbial environments.

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NOMENCLATURE

CFU/mL	Colony Forming Units per Milliliter

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CHAPTER I
INTRODUCTION

Research of the past few decades has vastly increased our knowledge of microorganisms in the environment, both in terms of diversity and concentrations. Researchers have transitioned from thinking of bacterial species as discrete organisms to approaching environmental communities as continuous, dynamic entities in constant flux. Since the development of the field, culture-based methods have been the traditional method of environmental characterization. However, such methods have significant challenges, including a substantial bias towards easily-culturable organisms. The isolation of certain bacteria can be a long and tedious process and a huge portion of microorganisms cannot be cultured at all. The introduction of molecular methods has offered an alternate approach to microbial ecology and the development of specific technologies, such as next-generation sequencing, have propelled the field of environmental microbiology even further.

The purpose of the studies presented in this thesis were to perform bacterial diversity analysis on two different matrices critically important to human health. The first study examines raw and pasteurized milk, while the second is an analysis of untreated sewage sludge in wastewater treatment plants across the United States. Although fundamentally different, these two matrices share much in common. Both are environments that contain a wide variety of bacterial organisms. Both have been known to harbor pathogenic organisms that may cause outbreaks of illness. Both milk and sewage have historically been subjects of microbiological research and there is a substantial amount of scientific literature discussing the bacterial diversity of each. Both are environments in which

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This thesis follows the style of Applied and Environmental Microbiology.

microorganisms play a functional role—determining spoilage and sensory qualities for milk and involved in aerobic and anaerobic processes in sewage treatment. Lastly, the microbiological quality of both milk and municipal sewage impacts public health.

The overall objective of these studies was to understand the indigenous microflora of milk and sewage sludge samples using next-generation sequencing technologies and traditional culture-based techniques. The objective of the milk study was to compare the bacterial diversity of raw and pasteurized milk samples, understand microbial dynamics involved in spoilage and processing, and determine the levels of signaling molecules that are involved in bacterial cell-cell communication. It was hypothesized that such an analysis would reveal that bacterial populations within raw milk are fundamentally different than those present in pasteurized, store-bought milk. The objective of the wastewater study was to compare the bacterial diversity profiles of primary sludge samples between different municipalities and across seasons. A supporting objective of this study was to examine the correlation between results obtained by culture and by next-generation sequencing methods. It was hypothesized that the wastewater analysis would reveal significant differences between sludge sampled at different locations and at different times of the year.

CHAPTER II
LITERATURE REVIEW

Environmental Microbiology—Traditional and Metagenomic

History of Environmental Sampling

Since Anthony van Leeuwenhoek first looked through his handmade microscope at a drop of rainwater, microbiologists have been attempting to investigate, characterize, and classify the microbial world around them. The very first microbiological studies were environmental, but such work was soon abandoned in favor of clinical microbiology, which posed more urgent questions and more obvious benefits. However, as clinical microbiology expanded, scientists began to recognize that environmental exposure to microorganisms was a source of human illness and interest was renewed again (20). The types of environments that have been the subject of characterization studies are extremely diverse and include water, soil, air, and waste, as well as human-associated settings such as food processing centers and hospitals. Many environmental studies have focused on the search for pathogenic microorganisms, as the organisms that pose the most urgent threat to human health. Such environmental characterization studies have helped present more accurate viewpoints of the microbial world surrounding us and are even now constantly reshaping and reforming those perceptions as new methods are invented and new data is published.

Traditional vs. Molecular—Methods of Microbial Detection, Characterization, and Classification

Culture-based Methods

Environmental microbiology has traditionally relied on the culture-based characterization of bacteria, which has its own advantages and limitations. These types of studies generally rely on some variation of the following procedure: collection of an environmental sample, concentration or enrichment procedure, culture on a general or selective medium, isolation of discrete colonies, and are concluded by microscopic and metabolic characterization analyses (12). There are several advantages to these traditional methods. They produce live, viable bacteria that can be grown as needed and are easier to characterize functionally. Such organisms can also be stored as isolates for further study and use in experiments. However, for many bacteria, isolation and growth often requires specific media, special growth conditions, and an extended incubation period that can make the isolation process lengthy and complicated. Success of isolation can depend on many different variables: the specific protocol, incubation time and temperature, media, and the individual organism in question. Additionally, culture-based methods are burdened by extensive bias towards easily-culturable, proliferative, and adaptable organisms (33, 91). Therefore, there is no assurance that what grows on the media is representative of what exists in the sampled environment. Microscopy is the traditional alternative to culture-based methods, but many bacteria are indistinguishable by such methods (33).

Molecular Methods

Molecular techniques for bacterial identification have appeared over the last several decades, beginning with the development of the Polymerase Chain Reaction (PCR) and Sanger sequencing. The field advanced further with the realization of the potential of

16S rRNA genes, introduction of cloning techniques, advancement of multiplex and real-time PCR, and the evolution of array-based technologies. In environmental surveys, molecular based-methods provide a more accurate representation of bacterial diversity, but can give no assurance that the detected sequences were present in viable cells. Molecular methods are not necessarily a complete replacement for culture-based methods. Many molecular techniques have their own biases involved in DNA extraction, PCR, and cloning steps, although these are generally far less dramatic than those found in culture-based methods (12, 35, 77). Larger volumes of environmental samples can also be sampled with culture-based studies than molecular studies—allowing researchers to gain a more robust assessment of complex environments. However, the introduction of molecular methods has prompted new directions of research and new awareness of organisms in a variety of areas.

The molecular revolution is also important in that it has forced us to confront how we classify bacteria. Previously, bacteria were classified according to characteristics observed during culture, such as enzyme activity, substrate usage, colony characteristics, motility, etc. However, recognition of the potential of 16S rRNA genes for taxonomic classification changed much of the system established by culture-based characterization and is considered to be the best, most accurate method of bacterial identification and classification (55, 56, 67). 16S and 18S rRNA genes encode small subunit ribosomal RNAs that are highly conserved among bacteria and can be used to establish relationships between organisms (68). However, there are often discrepancies between traditional methods of classifying bacteria and molecular, DNA-based methods that are still being resolved (55). Many bacteria have had to be reclassified into new groups due to emerging 16S rRNA data.

Broad awareness of uncultivable bacteria was prompted by the introduction of molecular tools. Estimates vary, but it is generally believed that a mere 1-15% of bacteria are culturable using traditional methods (12, 68, 86). Of the 61 identified bacterial phyla,

only 30 have a representative organism that has been cultured in a laboratory (86). Studies of industrial wastewater have shown that microscopic counts of bacteria are 2-3 logs higher than culturable counts (33). New studies have provided additional evidence that environments previously considered sterile or low-bacteria, such as amniotic fluid, are colonized by bacteria that simply defy our most advance culture techniques (24). This "unculturability" is possible for several reasons. One is that certain bacteria require very precise growth conditions, such as specific nutrients and oxygen levels, that are difficult to replicate in a laboratory. Another is that certain bacteria can only grow as part of a consortium containing other supportive bacteria (86). Interactions between bacteria have been shown to play important roles in their responses and stimuli. Bacteria form biofilms with other microorganisms that allow them to withstand harsh environmental conditions and may facilitate communication (86). Bacteria also produce autoinducers that serve as communication molecules within and between bacterial species and can induce responses, such as the increased activity of virulence genes, in certain bacteria (63). Recent research has shown that the culture of many "unculturable" bacteria is likely possible with in-depth research and special techniques (12, 35). However, researchers must weigh the value of such studies against the immense expense, effort, and time that may be required.

Another area which further emphasizes the limitation of culture-based techniques is the growing knowledge of Viable-But-Not-Culturable (VBNC) cells in the environment. Generally believed to be a technique employed by stressed or injured cells to increase survival in adverse environmental conditions, VBNC bacteria are traditionally culturable organisms that are unable to grow on culture media, but still demonstrate some metabolic activity, such as the ability to transcribe genes and translate proteins (37). Classification of these cells falls into a kind of "limbo", as they are not capable of culture, but are neither technically dead. VNBC cells are of concern because some studies have shown them capable of returning to viability, and in some cases, virulence,

under the right conditions (37). Whether VBNC is an intermediary stage between viability and ultimate death of the cell needs further study.

Metagenomics

The purpose of microbial diversity studies is to discover new organisms, reveal the distribution of organisms within an environment, characterize microbial functions, and accurately classify organisms (12, 56). Metagenomics is generally defined as the culture-independent genomic analysis of whole microbial communities isolated directly from the environment (56, 62, 76). Metagenomics is an important emerging field because it purportedly provides a "less biased view" of bacterial distribution and molecular diversity while preserving community structure of the environment under analysis (76, 91). This field also has the advantage of permitting the inclusion of non-culturable bacteria into diversity studies, which has allowed researchers to more fully explore the prevalence of these organisms in the environment. Overall, metagenomics has paradigm-shifting potential in human health, soil, forensics, human genetics, microbial ecology, evolution, and paleobiology (68, 69).

Metagenomic Tools and Methods

The field of metagenomics has been driven by the invention of innovative molecular tools to study microbial diversity and ecology. Such developments are discussed in excellent reviews by Cardenas and Tiedje (2008), Petrosino *et. al.* (2009), and Shendure and Hanlee (2008) (12, 62, 74). These new methods have the potential to discover new genes, characterize existing genes, classify bacterial taxa, and help better define unculturable organisms (12). The first metagenomic studies were completed by 16S rRNA cloning, which requires the insertion of isolated 16S rRNA genes into plasmid vectors and host cells for sequencing (76, 84). This approach is commonly limited by the number of clones that can be created and sequenced—generally no more than 500 for

most studies (76). Other disadvantages include bias introduced by the PCR and cloning processes (76). In recent years, other tools have been introduced to improve metagenomic studies, including phylogenetic and functional gene microarrays that can characterize microbial ecosystems and microfluidics-based technologies that can sort and select organisms for more in-depth analysis (12, 84). However, this review focuses on what is generally considered the greatest technological advancement in metagenomics—next-generation sequencing.

Next-Generation Sequencing

Massively parallel sequencing, the newest tool in the molecular arsenal, further expands the potential of metagenomic analysis and has the potential to revolutionize the entire field. The first automated sequencing process to be developed was the Sanger sequencing, which produces 550-900 bp read lengths but is a tedious and expensive process capable of sequencing only 96 reads at a time (12, 68, 73). The development of next-generation technologies has permitted sequencing to become more affordable—allowing many smaller organizations and research groups access to immensely powerful sequencing tools for the first time (12, 74). Originally introduced by 454 Life Sciences (Branford, CT), pyrosequencing was the first deep sequencing tool to be marketed and available to researchers. However, other systems include the Solexa system (Illumina, San Diego, CA), the SOLiD system (Applied Biosystems, Carlsbad, CA), the HeliScope system (Helicos Biosciences, Cambridge, MA), and the Polonator system (Dover Systems, Salem, NH) (69, 74). All systems have high data output, but vary in read length capability (74). Next-generation sequencing technologies have the potential to evaluate bacterial and molecular diversity, complete functional analysis of bacterial communities, and perform a variety of other genomic analyses (12). However, many of these newer, deep-sequencing-based methods require in-depth and complicated bioinformatics databases and software for data processing, which is causing nearly as much of a technical renaissance as the sequencing tools themselves (69). Pyrosequencing, the

method of high-throughput sequencing used in this work, is discussed more fully in the next section.

Pyrosequencing

Pyrosequencing is an innovative next-generation sequencing system with an incredible potential for metagenomic analysis. Based on a "sequencing-by-synthesis" method, the platform utilizes specific enzymes and associated light reactions to record each nucleotide inserted into a complementary DNA strand. This allows the massively parallel sequencing of millions of DNA strands (69). Several preparatory steps are required before template DNA can be subjected to analysis by pyrosquencing. Immobilization of the template DNA is accomplished by attachment to magnetic beads, which then undergo emulsion PCR for amplification. The bead-attached templates are deposited on a specially designed plate that allows determination of the enzymatically-produced light reactions. Nucleotides and enzymes are added automatically at select intervals throughout the sequencing run by microfluidics.

The pyrosequencing principle itself is based on the concerted action of four separate enzymes: DNA polymerase, sulfurylase, luciferase, and apyrase. The polymerase enzyme moves along the template DNA strand, incorporating nucleotides into the growing complementary DNA strand. Each time a nucleotide is added, pyrophosphate molecules are released into the surrounding environment. These pyrophosphates act as substrates for the sulfurylase enzyme, which converts them into ATP molecules. The ATP then reacts with the luciferase enzyme to produce a light reaction. The machine is able to read the light reaction and determine which nucleotide was incorporated into the sequence. The sequencing results for each analyzed DNA fragment are recorded in the form of a pyrogram, in which each peak represents a nucleotide addition and provides information regarding the activity of the enzymes. For a more in-depth discussion of the technical aspects of pyrosequencing, please see the review by Rothberg *et. al.* (69).

After completion of the sequencing run, results are processed by a pipeline of customized bioinformatics software that checks the sequences for length and quality and subjects them to analysis via the database of choice. The pyrosequencing platform developed by 454 Life Sciences (Branford, CT) generates approximately 400,000 sequences or "reads" per sequencing set-up or "run". The sequencing depth, which is the number of sequences read per sample, is dependent on the number of samples (12, 74). The first pyroseqencing platform, the GS20, produced average sequencing reads of only 100 nucleotides. However, second-generation improvements, culminating in the GS FLX sequencer (454 Life Sciences, Branford, CT), produced sequence read lengths between 200-300 nucleotides. More recently, the introduction of the Titanium FLX Sequencer (454 Life Sciences, Brandford, CT) reagents has extended this capability to 350-500 nucleotides and beyond (62, 84).

Pyrosequencing represents a dramatic increase in sequencing capabilities. After preparation of the template DNA is complete, thousands of sequences can be generated from a single sample in a matter of hours. Additionally, all sequencing is accomplished simultaneously, which expedites the process immensely (12). However, pyrosequencing does have several issues that need attention by future research. Strings of consecutive identical bases, or homopolymers, can cause higher error rates in pyrosequencing because of the reliance on light signals (74). Error rates have decreased with the newest system, but no method currently exists to check for chimeras in systems producing such huge amounts of data (38). Additionally, although the average cost-per-sequence is lower than other methods, the cost required to maintain and run the sequencer platform can be prohibitive for single labs and smaller organizations. However, pyrosequencing and other deep-sequencing technologies have become much more affordable in only the last few years and are expected to become even more so (73). Two major types of studies are completed using pyrosequencing tools, both of which are discussed below.

High-Throughput 16S rRNA Sequencing—Microbial Diversity

Studies based on 16S rRNA evaluate the microbial diversity of an environment (84). In this type of analysis, universal bacterial primers are used to amplify hypervariable 16S rRNA regions and attach nucleotide barcodes before pyrosequencing (12). This approach is similar to the traditional cloning-and-sequencing method of early metagenomic studies, except on a much larger scale. The major advantages of pyrosequencing over the 16S rRNA cloning method is that it allows the sequencing of thousands of times the number of sequences and does not require the creation of a clone library (73). 16S rRNA genes encode small subunit ribosomal RNAs that are highly conserved among bacterial organisms, but contain highly variable genetic regions commonly used for taxonomic classification (68). These hypervariable regions are essentially fingerprints that, when compared against a 16S rRNA database, can identify an organism and help establish its evolutionary relationship to other microbes (12).

Sequence read lengths needed for 16S rRNA classification vary by publication. Some reports state that reads as short as 90 bp have been shown sufficient to assign taxa, while others indicate that reads of 200 bp and 400 bp can generally classify organisms to the family- and genus-level, respectively (12, 73). Others suggest that reads of 250-500 bp, covering several hypervariable regions, are required for taxonomic classification of characterized organisms, especially to the genus- and species-level (38, 62). Most second and third generation pyrosequencing platforms read across multiple hypervariable regions (62). The attachment of barcode tags during amplification allows samples to be mixed for the sequencing run and sorted during data analysis—further increasing sequencing capacity and allowing further reduction of cost (12, 84). The 16S rRNA pyrosequencing approach has been used to evaluate bacterial diversity in environments as diverse as wound biofilms, deep ocean environments, livestock gastrointestinal systems, various soil types, oral microflora, and the human gut (1, 3, 22, 25, 26, 44, 46, 78, 93).

High Throughput Metagenomic Sequencing—Molecular Diversity

If the 16S rRNA approach allows studies of "microbial diversity", then the metagenomic approach permits studies of "microbial ecology". Whereas the 16S method only sequences amplified ribosomal DNA, metagenomic sequencing or "whole-genome shotgun sequencing", sequences **all** DNA extracted from a community. The sequencing output of such an approach is a vast collection of random DNA fragments from the community metagenome—different parts of the genomes of different organisms. This method provides a vast array of genes for analysis instead of only one, allowing researchers to gather information about functional identifiers such as genes and metabolic pathways, as well as taxonomic structure, and provide a more comprehensive perspective of the ecosystem (76, 84). If enough coverage is achieved, assembly of whole genomes from metagenomic sequence data is possible. However, such achievements have only been demonstrated in low-diversity environments, as the computing and sequencing demands are currently too great for extremely complex environments (76). The increase of pyrosequencing read length capabilities will continue to improve accuracy of sequence classification for metagenomic studies (12). The first large-scale metagenomic project—the environmental whole-genome shotgun sequencing of seawater samples from the Sargasso Sea—is an example of a metagenomic study using Sanger sequencing (87). Metagenomic studies completed using pyrosequencing have examined marine viruses, honeybee colonies, deep mine environments, and ancient mammoth and Neanderthal genomes (4, 17, 28, 69).

Human Genome Project(54)

One study that has used both metagenomic and 16S rRNA deep sequencing approaches is the Human Microbiome Project. The Human Microbiome Project is a large project sponsored by the National Institutes of Health (NIH) that is using culture-independent methods to characterize human microbial diversity in locations such as skin, nasal and

oral cavities, gastrointestinal tract, and urogenital tract (61). The goal of this massive study is to gain a better understanding of the role that microorganisms inhabiting human bodies play in the health of their hosts. This project is remarkable in that it was one of the first major coordinated projects to make use of deep-sequencing technologies. The Human Microbiome Project is utilizing a multi-faceted, deep-sequencing approach that includes: 16S rRNA analysis to gain an overview of diversity and identify organisms of interest, metagenomic shotgun sequencing to further evaluate microbial ecology and functional capabilities, and whole-genome sequencing for select organisms (38, 61).

16S rRNA vs. Metagenomic: Strengths and Weaknesses

Of the next-generation techniques, pyrosequencing is generally preferred for goals such as genome sequencing of microbial organisms and metagenomic studies, mainly due to its longer sequencing read lengths (12). Both types of pyrosequencing approaches, 16S rRNA and metagenomic, have their strengths and weaknesses. 16S rRNA pyrosequencing performs an in-depth evaluation of bacterial diversity and is capable of detecting rare or minor populations in bacterial communities that may be missed by using other methods (62). However, 16S rRNA studies often have difficulty classifying novel or very divergent species, which requires sequencing of the entire 16S gene (around 1500 bp). For such applications, whole-genome metagenomic shotgun sequencing is more accurate (62, 68). Primer bias is also an issue for 16S rRNA studies—even standard 16S primers meant to target a broad range of microorganisms may miss environmental organisms that are not amplified by the chosen primers (38, 84). However, whole-community sequencing stretches the limits of current technologies in extremely diverse communities (76). Studies using pyrosquencing for complete genome recovery currently also have limitations in highly diverse environments. However, entire genomes may not always be required to gain valuable information about a microbial community (12).

Bacterial Diversity and Spoilage of Raw and Pasteurized Milk

Bovine milk is a basic food popular throughout the world. Milk is mainly composed of water, lactose, protein, and fat, but can vary according to different factors (54). Milk is well-suited for bacterial growth because of plentiful nutrients, high water content, and neutral pH (55). As a result, milk has a long history of microbiological research. However, the vast majority of research in this area has focused solely on organisms that influence the two major concerns of the industry—spoilage and safety.

Raw Milk Microflora and Spoilage

Some researchers maintain that fresh milk is sterile and that bacteria present in extracted milk originate from udder infections and environmental contamination during milking and processing (55). Others believe that low numbers of select organisms, mainly lactic acid bacteria, can colonize the udder of healthy cows (54, 82). Aseptically collected milk generally contains very few organisms, but bacterial load levels in fresh raw milk can range from a few to several thousand microorganisms, varying considerably from cow to cow (54, 82). Most bacteria in raw milk are the result of environmental contamination from sources such as the cow's teat and udder, animal feed, bedding, water, aerosols, human handling, and bulk tanks used for storage, as well as equipment used in milking, processing, and packaging (39, 45, 54, 82). Facility and equipment sanitation are important elements in reducing environmental contamination in milk (6).

Raw Milk Microflora

Reports vary somewhat on the dominant microflora found in raw milk, with variation shown dependent on season and geographical origin (29). Common raw milk microflora reported include lactic acid bacteria such as *Lactococcus* and *Lactobacillus*, as well as *Pseudomonas*, *Micrococcus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Listeria*,

Enterobacter, *Escherichia*, *Citrobacter*, *Klebsiella*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Aeromonas*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, and *Propionibacterium* (45, 54, 82). Several reports have indicated that raw milk tends to be dominated by gram-positive organisms (48, 54). Most published studies on milk have been culture-based, however a few have used molecular approaches and others have used 16S rRNA methods for isolate identification. One study using a cloning-based 16S rRNA analysis of raw milk showed that a majority of organisms were members of the Firmicutes phylum (Clostridiales and Lactobacillales), with lesser populations of Proteobacteria, Actinobacteria, and Bacteroidetes (21). However, analysis of isolates recovered from culture showed a higher prevalence of Gammaproteobacteria and *Staphylococcus*, highlighting the vast difference in results obtained by culture and by molecular methods. A molecular study of raw milk from four dairies in Israel found that each dairy had its own distinct microbial profile (39). Ercolini and colleagues screened and indentified 66 raw milk isolates using RAPD-PCR and 16S rRNA sequencing and found that *Pseudomonas* was the dominant genus, but that other frequently detected organisms included *Hafnia alvei*, *Serratia marcescens*, and *Citrobacter freundii* (29). Another study using cloning and sequencing to identify isolates found that *Lactococcus lactis* was a major organism in raw milk and that various mastitis-causing bacteria were prevalent throughout the samples (45). Interestingly, there seem to be conflicting viewpoints regarding the origin of lactic acid bacteria in raw milk—some regard them as indigenous flora of milk (21), whereas others consider them primarily environmental contaminants (65, 82).

Spoilage of Raw Milk

As a high-nutrient medium that can support substantial bacterial growth, spoilage of raw milk is a concern (82). Psychotrophic bacteria, or bacteria that are capable of growth at 7°C, are of interest in both raw and pasteurized milk because milk is generally kept at refrigerated temperatures to prevent spoilage (39, 82). Psychotrophic organisms may

produce enzymes, generally proteases and lipases, which cause milk spoilage (39). Refrigerated storage of raw milk prompts the medium to shift from being dominated by gram-positive organisms to being dominated by gram-negative and psychotrophic organisms (55). One study found that refrigerated raw milk was spoiled exclusively by gram-negative bacteria, mainly *Pseudomonas* spp. (83). The most common gram-negative psychotrophs reported in raw milk are members of the bacterial groups *Pseudomonas*, *Enterobacteriacae*, (mainly *Klebsiella*, *Serratia*, *Citrobacter*, *Hafnia*, and *Enterobacter* spp.), *Arthrobacter*, *Acinetobacter*, *Flavobacterium*, *Achromobacter*, *Aeromonas*, *Alcaligenes*, and *Chromobacterium*. Important gram-positive psychotrophs are mainly *Bacillus* spp, especially *B. cereus*, although *Listeria* species are occasionally found in raw milk and may grow at refrigerated temperatures (39, 45, 54, 55, 82).

Pathogenic Organisms in Raw Milk

Generally, pathogenic bacteria in raw milk come from one of two sources: mastitis, which is an infection of the cow's udder, or contamination as a result of environment or handling (54, 82). The presence of pathogenic microorganisms in raw milk is often attributed to fecal contamination during milking (6). Human pathogens found in raw milk include *Campylobacter jejuni*, *E. coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enterica*, *Brucella melentensis*, *Yersinia entercolitica*, and *Mycobacterium tuberculosis* (6, 40, 82). The most common diseases associated with raw milk consumption are salmonellosis and campylobacteriosis (40, 65). Most major pathogens of concern in raw milk cannot grow at refrigerated temperatures; however, milk's specific composition means that low-level contamination can be sufficient for infection. The fat present in milk protects pathogens in the stomach from inactivation by gastric acid and its fluidity allows for a short transit time through the gastrointestinal tract (65, 82).

Pasteurized Milk Microflora and Spoilage

Pasteurization, named for its inventor, Louis Pasteur, was introduced in the United States in the early 20th century to combat widespread disease outbreaks associated with the industrialization of milk production and transport arising in the late 1800s. The implementation of pasteurization reduced foodborne disease considerably in the United States (65). In the United States, the two main methods of pasteurization are batch pasteurization, which is defined as heating to 63°C for 30 minutes, or high-temperature, short-time (HTST) pasteurization, which is defined as heat treatment at 72°C for 15 seconds (52).

Pasteurized Milk Microflora

Bacteria in pasteurized milk may come from two sources: survival of the pasteurization process or post-pasteurization contamination (82). Pasteurization is effective against nearly all organisms, including pathogens and spoilage-associated microbes (54, 82). However, traditional pasteurization does not effectively sterilize the milk and a small percentage of organisms will survive—especially gram-positive spore-formers such as *Bacillus* and *Clostridium* (37, 82). However, few of these organisms are capable of growth at low temperatures or causing spoilage (82). Most bacteria found within pasteurized milk are actually post-pasteurization contaminants (54, 82, 90). Milk in the United States is not aseptically packaged, which allows for post-pasteurization contamination (90). Such contamination is common and often occurs through contact with aerosols and equipment associated with milk processing (82). Essentially, this means that the bacterial populations found in pasteurized milk do not represent what was originally present in milk before pasteurization. The most common post-processing bacterial contaminants are members of the *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Serratia*, *Enterobacter*, *Citrobacter*, and *Hafnia* genera, which enter the milk through pumps, pipes, valves, and filling equipment (54, 82).

Pasteurization, Processing, and Spoilage of Pasteurized Milk

The pasteurization process is generally considered to be responsible for the shift in microflora from the gram-positive, acid-producing bacteria of raw milk to the gram-negative, psychotrophic bacteria found in pasteurized milk (54). The use of specialized equipment has also increased the prevalence of organisms that can easily attach to equipment surfaces and resist cleaning, such as *Pseudomonas* and *Acinetobacter*, in milk (48, 54). One study noted that, although the raw milk tested by culture consisted mainly of gram-positive organisms, the samples collected from piping and tubing were predominantly gram-negative. This may explain the prevalence of gram-negative, psychotrophic organisms in pasteurized milk samples (48). The spoilage of refrigerated, pasteurized milk is generally the result of contamination with gram-negative, psychotrophic bacteria, mainly *Pseudomonas* spp. (54, 82). Organisms within the *Pseudomonas*, *Alcaligenes*, *Chromobacterium*, and *Flavobacterium* genera will generally out-compete other organisms at such low temperatures and are primarily responsible for spoilage (54, 82).

Bacterial Diversity of Untreated Municipal Sewage Sludge

Wastewater treatment plants are common fixtures throughout the United States and the world, generating valuable biosolids from human waste products. In municipal systems, sewers collect waste in the form of raw sewage from residential, commercial, and industrial locations and deliver it to wastewater treatment plants (85). Waste that enters a sewage treatment plant goes through a variety of processes to make it safe for disposal. Once treated, the biosolids produced are commonly used as fertilizer for agricultural activities—even with low levels of pathogens still present.

Wastewater Treatment and Research

Treatment processes vary significantly between plants, but generally utilize the same basic system: the sewage is dewatered through sedimentation to produce primary sludge, which then undergoes an aerobic or anaerobic biological treatment process in a holding tank. The sludge is held and mixed over a certain period of time, allowing the microorganisms in the waste to digest carbon sources present in the mixture. The main purpose of wastewater treatment is use bacterial organisms to break down organic compounds and nutrients that can cause putrification (73, 75). As raw sewage is known to contain pathogenic microorganisms, wastewater treatment processes that are engineered to generate biosolids must be validated to ensure sufficient pathogen reduction.

Given such applications, most studies in wastewater microbiology have explored bacterial concentrations and distributions in biosolids—mainly focusing on indicator or pathogen reduction due to treatment. There is also considerable research characterizing activated sludge, or waste currently undergoing the digestion process. These types of studies tend to focus on pathogenic organisms and bacteria important in the waste digestion process—either organisms that have desirable metabolic activities or that can be problematic (33). However, only a small percentage of studies have published information on raw sewage or sludge before the treatment process. Although part of the same process, populations present in activated sludge may vary significantly from those present in raw sewage and primary sludge, so studies on the former cannot be used to draw any conclusions. Very little information is available on the microbial composition of raw sewage and untreated sludges, especially addressing overall bacterial diversity. The studies that do exist are mainly culture-based and focus almost solely on pathogens or indicator organisms (7, 50, 57).

Microflora of Raw Sewage and Primary Sludge

An incomplete picture of raw sewage and primary sludge can be assembled from the limited culture-based studies available. Pathogenic organisms are well-known to exist in raw sewage and organisms such as *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., *E. coli* O157:H7, and protozoan pathogens such as *Cryptosporidium* and *Giardia lamblia* have all been detected in raw sewage sludge by culture and microscopy (33, 72). Indicator organisms are also present at high levels in raw sewage and primary sludge, including *E. coli*, total coliforms, fecal coliforms, fecal streptococci/enterococci, sulfite-reducing clostridia, enteric viruses, and a variety of phages (33, 50, 57). A study analyzing a mix of 2/3 raw sludge and 1/3 activated sludge also found high levels of viruses, total coliforms, fecal coliforms, and fecal streptococci (7). *Acinetobacter* has been a dominant genus detected in sewage using culture-based methods and organisms such as thermophilic campylobacters and *Arcobacter* have also been characterized in primary sludge (77, 80). However, many of these characterizations were performed as a prelude to a treatment study, not in an effort to characterize such environments, and should be considered poor representations of overall diversity.

Molecular Analysis of Activated Sludge

While traditionally culture-based, wastewater researchers have also used molecular techniques to investigate bacterial diversity in wastewater and pursue unculturable organisms (33, 66, 73). Molecular methods that have been used to explore wastewater environments include nucleic acid fingerprinting, fluorescent in situ hybridization (FISH), multiplex PCR, and 16S rRNA analysis (33, 73). Previous molecular studies have shown dominance of Proteobacteria, specifically the beta subclass, in activated sludge (43, 77). Genera such as *Arcobacter*, *Acinetobacter*, *Comamonas*, and *Aeromonas* were found in a study of activated sludge using a combination 16S-rRNA cloning and DNA probe hybridization approach (77). A study using culture-based methods and DNA

probes found that *Aeromonas*, *Acinetobacter*, *Pseudomonas*, and *Shewanella* were prominent in activated sludge, but that the prevalence of *Aeromonas* spp. were overestimated by using culture-based methods (43). Such molecular techniques are improving wastewater treatment by allowing the identification of organisms involved in the digestion process. Identification of a bacterium with good metabolic potential can lead to the development of techniques for selective enrichment and more efficient digestion (75). However, like the culture-based studies, these studies have been limited to activated sludge and biosolids.

Next-generation Sequencing of Wastewater and Biosolids

Two studies utilizing next-generation sequencing tools on wastewater products have been published. A metagenomic pyrosequencing study of activated sludge taken from a wastewater treatment plant in North Carolina showed considerable bacterial diversity that was dominated by members of the Proteobacteria phylum (~70%). The sequencing data showed poor assembly of genomes, which was unsurprising in such a diverse environment. Compared to other communities that have been studied using metagenomics, bacteria within the treatment plants appeared to express high levels of genes required for the breakdown of aromatic compounds (73). Additionally, an extremely high prevalence of tranposases was detected, indicating conservation within the sludge metagenome. Another recent study used 16S rRNA pyrosequencing to analyze bacterial diversity in biosolids and agricultural manure. This study was notable in that it used next-generation sequencing to identify pathogens in an environment (8). A relatively low prevalence of pathogens was detected, as would be expected in treated sludge. The pathogens that were detected were mainly opportunistic clostridia and mycobacteria. Bioinformatic analysis also revealed that waste products stabilized by different treatment processes had significant differences in bacterial community structure. However, waste products that had undergone similar stabilization processes had marked similarities in bacterial populations, even though treated in different plants.

Water Quality Indicators

A common practice in the water and wastewater industries is the use of indicators to represent water quality and safety of biosolids. Common bacterial indicators include enterococci, *Clostridium perfringens*, *Salmonella enterica*, fecal coliforms, and *Escherichia coli*. Indicators are organisms that are intended to represent water or wastewater quality, generally by indicating the presence of pathogens (33). There are obviously inherent difficulties in using one or two organisms to represent a diverse and dynamic environment of microbes. However, indicators are often necessary, as it is not technically or financially feasible to quantify a multitude of different pathogenic organisms before making a decision on water or soil quality. Effective indicator organisms must be found in waste, but not generally in the natural environment. Indicator organisms should parallel the concentration levels of known pathogens in water and waste and respond similarly to treatment processes. Additionally, the best indicator organisms are non-pathogenic and easily culturable or otherwise quantifiable (71). Bacterial organisms that meet such stringent specifications are difficult to find.

Study Summary and Purpose

Raw Milk and Pasteurized Milk: Diversity, Spoilage, and Pathogens

The purpose of this study was to examine bacterial communities present in raw and pasteurized milk samples using deep sequencing. Secondary goals included identifying population responses to processing conditions and bacterial organisms involved in spoilage. Five samples each of raw and pasteurized milk were purchased and evaluated by deep sequencing, plating on nonspecific media, and Autoinducer-2 (AI-2) analysis. This analysis was repeated with the milk samples after processing and refrigerated storage. Such information is needed for several reasons. There is very little information available on the bacterial communities present in raw and pasteurized milk—most

studies have focused almost entirely on pathogens and organisms responsible for spoilage. There have been very few metagenomic studies on milk and none using next-generation sequencing technologies. As a result of the bias inherent in the culture-based techniques used for previous characterization studies, it is likely that the available information on milk microflora is incomplete and perhaps even broadly incorrect. Community responses to processing were investigated because milk and other foods are often processed before sale and bacterial responses from a community perspective are largely unknown. Microbiological studies of milk spoilage are common, but generally only using culture-based techniques. Analysis of spoiled milk samples was included to compare these results to a less-biased community perspective obtained from deep sequencing.

A comparison of the microflora of raw and pasteurized milk is important because of the growing interest in raw milk consumption. Certain small segments of society have always consumed raw milk, especially farm families and personnel (41). However, a growing interest in raw or unpasteurized milk as a health food has been noted in the past few decades (10, 65). Raw milk is touted by advocates as having beneficial properties and a better taste (10, 65). Health claims associated with the consumption of raw milk include better nutrition, better tooth development and fewer cavities, improved immune system, enhanced fertility, and arthritis prevention/relief, as well as beneficial enzymes, hormones, and organisms such as lactobacilli (65). The Weston Price Foundation, which advocates for the legalized sale and healthful properties of raw milk, claims on its website that the process of pasteurization “destroys enzymes, diminishes vitamin content, denatures fragile milk proteins, destroys vitamins C, B12, and B6, kills beneficial bacteria, promotes pathogens and is associated with allergies, increased tooth decay, colic in infants, growth properties in children, osteoporosis, arthritis, heart disease, and cancer” (89). There is no peer-reviewed evidence to support any of these claims and no differences have been found between raw and pasteurized milk in nutritional studies (41, 65). A goal of this study was to evaluate the bacterial side of

these claims and provide data regarding the microbiological contents of raw milk. The unpasteurized nature of raw milk leaves it susceptible to harboring pathogenic organisms. Many clinical studies have been published describing infections and outbreaks associated with consumption of raw milk and cheese made from unpasteurized milk (13-15, 41). Illness associated with raw milk consumption is typically gastroenteritis, but serious complications can occur (41). A review of raw milk-associated outbreaks found that 46 raw milk-associated outbreaks were reported to the CDC between 1973 and 1992, however, it is likely that such outbreaks are underreported (10, 40). Pathogens in raw milk are especially dangerous to vulnerable populations such as children and the immunocompromised (10). Outbreaks have been reported in children after school field trips to dairy farms and, in one instance, after raw milk was provided at a school (41, 65). Therefore, an additional goal of this study was to evaluate and compare pathogen prevalence in raw and pasteurized milk samples.

Untreated Sewage Sludge from American Municipalities: Diversity and Seasonal Dynamics

Given the wide-spread usage of biosolids in commercial applications, this study sought to gain a comprehensive perspective of the bacterial communities entering the waste treatment process, using both next-generation sequencing analysis and traditional culture-based techniques. Samples were taken from seven representative wastewater treatment facilities in cities across the United States: Georgia, Wisconsin, Illinois, California, Ohio, Washington, D.C., and Texas. Such a study is important for several reasons. First of all, very few molecular studies have focused on surveying the microbial diversity of raw sewage or primary sludge and none have used next-generation sequencing tools. Use of such tools should provide a valuable and unbiased perspective of the diverse microbial ecosystem present in untreated waste, including whether or not bacterial diversity profiles in sewage sludge are similar or different across different

locations. This project is also unique because it serves as an opportunity to compare results obtained from metagenomic and culture-based analyses of the same samples.

This study is important from a pathogen-identification standpoint. Pathogens such as *Salmonella*, *E. coli*, and enteric viruses are well-known to exist in raw sludge and survive treatment to persist in biosolids at low levels. This study should provide another perspective on the prevalence of these pathogenic organisms through the use of deep sequencing technologies. However, we are aware of the presence of these pathogens in biosolids because we test for them routinely. This study may provide knowledge of other, less recognized, pathogens that are currently being reintroduced to the environment through the land application of biosolids (33).

This study also has the potential to help identify better indicator organisms for waste and wastewater treatment, which are urgently needed. Identification of better indicators will assist in the development of more effective waste treatment processes. If suitable indicators can be found for pathogenic organisms in sewage and other waste, operators will be able to swiftly judge if a sewage treatment method is effective at appropriately reducing pathogens loads. It will also assist public health officials in choosing appropriate organisms to serve as indicators of human fecal contamination in the environment. As treated sludge is often applied to agricultural fields as fertilizer, inadequately treated sludge could pose a health hazard and serve as a route of pathogen transmission to humans and crops. Other indicator organisms are needed because recent research has shown that indicators such as *Salmonella* spp. are capable of extended survival in surface water and that *E. coli* and enterococci may survive and even proliferate in some environments (70). These findings suggest that these organisms are not as well-suited for indicator purposes as may have been previously thought.

Lastly, it is important to study which organisms are present in untreated waste because the bacterial communities may provide valuable insights into the human populations

from which they were derived. The treatment plants sampled in this study received sewage input taken from suburban populations in seven major cities across the United States. Most gut pathogens are expelled at high levels in feces during symptomatic infection and studies have shown that various factors such as health, obesity, and antibiotic use may contribute to differences in gut microbiota (22, 36, 49). One recent study examined virus prevalence in raw sewage sampled across the United States, using the results to draw conclusions about the prevalence of specific enteric viruses in urban populations (9).

CHAPTER III
MATERIALS AND METHODS

Microbial Diversity in Milk Study

Collection of Samples

Five different samples of pasteurized, homogenized milk were purchased from grocery stores in the College Station, TX area in April 2009. Each sample consisted of whole milk from a different brand. For each sample, 2 gallons of milk was collected. All purchased milk was in sealed packaging and had not passed the expiration date. Raw milk in this study refers to milk that had undergone neither pasteurization nor homogenization before sale. Raw milk was purchased on-site from five different dairies within the state of Texas between June and July 2009. Four samples were collected from small family farms, while one was collected from a moderately-sized dairy operation that produced both pasteurized and raw milk. Two gallons of raw milk were purchased from each farm and all samples were stored on ice in coolers during transport to Texas A&M University. All collected samples were stored at 4°C until analysis. All milk samples were initially processed and stored within 24 hours of purchase from the grocery store, dairy, or farm.

Sample Processing

After collection, but before further processing, 150mL of each milk sample, raw and pasteurized, was used to plate, extract cell-free supernatant (CFS), and extract total community DNA. The protocols for each of these steps are given in the next section. Additionally, 250 mL of each of the milk samples was transferred aseptically using pipets into standing Whirl-Pak bags (Nasco, Fort Atkinson, WI), which were then

wrapped and tied closed. Filled bags were stored at 4°C for 60 days or until there were obvious signs of spoilage, such as major changes in coloration or composition.

Experimental Treatments

Store-bought milk samples were processed by electron beam irradiation and boiling. Raw milk samples were experimentally processed by simulated pasteurization, electron beam irradiation, and boiling. In the United States, batch or vat pasteurization is defined as heating milk for 30 minutes at 63°C (52). This was simulated in this study by using a water-bath to heat 25mL aliquots of the milk sample at 63°C for 30 minutes in sterile, 50mL glass test tubes. A control sample with thermometer was used to monitor the temperature and the 30-minute timer was started only after the control sample reached 63°C. Electron beam irradiation was performed at the National Center for Electron Beam Research at Texas A&M University. Samples were irradiated in 100mL packets at 1.0 kGy using a 10 MeV (Million Electron Volt), 18 Kilowatt Electron Beam Linear Accelerator (LINAC). For processing by boiling, 500mL of each milk sample was poured into a sterile 1L beaker. The milk was then boiled on a hotplate with stirring at low speed for 15 minutes. Time was started only when the physical boiling process began and the milk carefully observed to prevent overflow. After each experimental processing, 250mL of the processed milk was placed into Whirl-Pak bags for storage according to the previously described protocol. The remainder of the processed milk (150mL) was subjected to culture-based analysis, CFS extraction, and DNA extraction as described below.

Culture-Based Analysis

Aerobic Plating

For aerobic plating, 25mL of each milk sample was transferred aseptically to a 50mL conical tube (VWR, West Chester, PA). Dilutions were then made using 1mL of milk in 9mL of Butterfield's Phosphate Buffer. Undiluted milk (1 mL) was used to calculate bacterial load for store-bought, pasteurized milk and pasteurized milk samples after processing. For all raw milk samples and raw milk samples after irradiation and lab-pasteurization, 10^0 through 10^{-4} dilutions were plated. For all spoiled milk samples, 10^0 through 10^{-8} dilutions were plated. Undiluted milk (1 mL) was plated for all boiled samples, fresh and after storage. All designated dilutions were spread-plated onto Tryptic Soy Agar (BD, Franklin Lakes, NJ) and incubated for 5 days at 27°C. After incubation, plates were removed and all colonies counted and recorded.

Anaerobic Plating

The conical tube containing the milk sample was then transferred to a Bactron IV Anaerobic Chamber (Sheldon, Cornelius, OR) with an atmosphere of 90% Nitrogen, 5% Hydrogen, and 5% Carbon Dioxide through the airlock. All milk samples were diluted in pre-reduced anaerobically sterilized (PRAS) dilution blanks containing a mineral salts buffer (Anaerobe Systems, Morgan Hill, CA) using the same dilutions previously described. Aliquots were then spread-plated on PRAS Brucella Blood Agar (Anaerobe Systems, Morgan Hill, CA) and incubated within the anaerobic chamber at 27°C for 6-7 days. After incubation, plates were removed from the chamber and the total colonies counted. Indicators (Oxoid, Lenexa, KS) were used to ensure the non-presence of oxygen within the chamber throughout the plating and incubation period.

Cell Free Supernatant Extraction

To extract the cell-free supernatant (CFS) for autoinducer analysis, 5mL of milk was filtered through a 0.2µm filter (Millipore, Billeria, MA) using a 10mL syringe (BD, Franklin Lakes, NJ). Milk CFS was filtered into 1mL microcentrifuge tubes (VWR, West Chester, PA) and stored at -20°C until further use. CFS was extracted from all raw and pasteurized milk immediately after collection, all milk after processing, and all milk after storage.

DNA Extraction

Using aseptic technique, 125mL of milk was pipetted into sterile 250mL centrifuge bottles (VWR, West Chester, PA) and centrifuged for 15 minutes at 8000 x g. After centrifugation, the supernatant was discarded and the pellet washed and resuspended in 25mL of sterile Butterfield's phosphate buffer. The mixture was again centrifuged for 15 minutes at 8000 x g and the supernatant discarded. The pellet was again resuspended in 5 mL of sterile Butterfield's buffer. DNA was extracted in triplicate from 1mL of concentrated milk sample using the commercially available UltraClean DNA Extraction Kit (MoBio, Carlsbad, CA). Aliquots (1 mL) of each concentrated sample were pipetted into the initial bead-tube and the "High-Yield" manufacturer's protocol followed. Each extraction resulted in 50uL of DNA. The extraction for pyrosequencing analysis was chosen based on DNA quantification and qualification performed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Extracted community DNA was sent to the Research and Testing Laboratory in Lubbock, Texas for analysis by pyrosequencing. Further explanation and elaboration of the pyrosequencing process is provided later in this chapter.

AI-2 Analysis

The reporter strain V. *harveyi* BB170 was grown in an overnight culture in Autoinducer Bioassay (AB) media in a waterbath at 30°C with shaking. After incubation, 2uL of the late log culture was transferred to 10mL of fresh AB media (1:5000 dilution). Pre-formed AI-2 was synthesized to use as a positive control by growing an environmental isolate of *E. coli* in LB media with 0.5% glucose to a mid-log phase, centrifuging at 10,000 x g for 5 minute, and passing through a 0.2um filter. The AI-2 was then stored at -20°C until use. Stored cell-free supernatant (CFS) was thawed and vortexed to mix before use. The AI-2 assay was conducted in a white, flat-bottomed 96-well plate with cover (Whatman, Piscataway, NJ). Three wells were used for each sample. Each well received 90uL of diluted reported cells and 10uL of CFS from the sample of interest. Positive control, negative control, and inhibition control wells were also prepared in triplicate. Positive control wells consisted of 90uL of diluted reporter cells and 10uL of Preformed AI-2. Negative control wells consisted of 90uL of diluted reporter cells and 10uL fresh AB medium. Wells to measure inhibition activity were prepared by adding 90uL of diluted reporter cells, 5uL of a sample CFS, and 5uL of pre-formed AI-2. Three randomly chosen CFS samples were used for inhibition controls. Plates were then covered and incubated at 30°C with shaking (100 RPM) for 3-4 hours. After the incubation time, plates were periodically removed for luminescence readings at 30 minute intervals using a Wallac VICTOR2 plate reader (Perkin Elmer, Waltham, MA). Negative controls were monitored throughout the assay and the assay stopped when the average values began to increase. The values for luminescence taken from the previous reading were then selected and used to calculate relative AI-2 activity and inhibition.

Microbial Diversity in Municipal Sewage Study

Samples were collected from 7 different wastewater treatment plants across the continental United States. Untreated, primary sludge samples were taken from waste treatment plants in Washington D. C., Madison, Wisconsin, Cincinatti, Ohio, El Paso, TX, San Diego, CA, Chicago, Illinois, and Columbus, Georgia. Four sets of samples were collected from each location—two in the late summer/early fall period of 2009 and two in the late winter/early spring period of 2010. Sampling 1 took place between August 17th and September 1st, 2009. Sampling 2 took place between September 14th and September 28th, 2009. Sampling 3 was conducted between February 1st, 2010 and February 22, 2010 and Sampling 4 took place between March 1st and March 22nd, 2010. All samples were raw, primary sludge samples, that is, dewatered sludge with no treatment processing. No secondary sludge or digested sludge was incorporated as part of any of these samples. In all, 28 raw sludge samples were received and analyzed.

Samples were received at the Food & Environmental Microbiology lab at Texas A&M University the day after sampling. Each sampling collected 2500 mL of untreated sludge, which was shipped overnight on blue-ice. Dry weight and pH of each sludge sample was measured before microbiological analysis began. Other analyses, including *Legionella* spp., *Aeromonas* spp., virus, phage, and helminth ovum, were performed as a part of this project, but are not discussed in this document.

Anaerobic Heterotrophs

Aliquots (15mL) of raw sludge were transferred into a Bactron IV Anaerobic Chamber (Sheldon, OR) containing 90% Nitrogen, 5% Hydrogen, and 5% Carbon Dioxide through the airlock. Dilutions were made in blanks containing a pre-reduced, anaerobically sterilized (PRAS) mineral salt solution (Anaerobe Systems, Morgan Hill, CA). One hundred microliters (100uL) of the dilutions 10^{-4} through 10^{-7} were spread-

plated onto PRAS Brucella Blood Agar (Anaerobe Systems, Morgan Hill, CA), after which the plates were incubated within the anaerobic chamber at 27°C for 6-7 days. At the end of the incubation period, the plates were removed from the anaerobic chamber and the colonies counted.

Aerobic Spore-forming Bacteria

Fifteen milliliter (15mL) aliquots of the raw sludge samples were heated at 60°C in a water bath for 15 minutes in 50mL conical tubes (VWR, West Chester, PA). A control sample tube and thermometer were placed in the water bath to ensure that the sludge temperature reached 60°C prior to starting the 15 minute countdown. The heated sample was serially diluted (10^{-1} through 10^{-5}) in sterile water and 100uL was plated onto Tryptic Soy Agar (TSA) plates (BD, Franklin Lakes, NJ). The plates were incubated for 24 hours at 37°C, after which they were removed and enumerated.

Sulfite-reducing Clostridia/Presumptive *C. perfringens*

Fifteen milliliter (15 mL) aliquots of the raw sludge samples were heated at 60°C in a water bath for 15 minutes. A control sample tube and thermometer were placed in the water bath to ensure that the sludge temperature reached 60°C prior to starting the 15 minute countdown. The heated sample was serially diluted (10^{-1} through 10^{-5}) in 9mL sterile water blanks. One milliliter (1 mL) of each dilution was placed in the center of a empty, sterile petri plate, to which was added approximately 15mL of molten (~50°C), Perfringens Agar Base (Oxoid, Cambridge, UK) with added supplement containing D-cycloserine (Sigma-Aldrich, St. Louis, MO). The plates were gently swirled to mix and the medium was allowed to solidify. The plates were incubated anaerobically for 24 hours at 37°C using the GasPak EZ Container System (BD, Franklin Lakes, NJ). The plates were removed from the jar after incubation and large, black colonies were enumerated.

Total Coliforms/Fecal Coliforms/*E. coli*

Analysis of total and fecal coliforms in the sludge samples was performed using the EPA Method 1680. A 300mL portion of sludge was homogenized using a laboratory stomacher (Seward, Bohemia, NY) and the pH adjusted to 7.0-7.5 using a pH meter (Corning, Corning, NY) and 1M NaOH. Serial dilutions (10^{-1} through 10^{-7}) were made in flasks of 99mL phosphate buffer. For each dilution (10^{-3} through 10^{-7}), five test tubes of 10mL Lauryl Tryptose Broth (LTB) (BD, Franklin Lakes, NJ) with durham tubes were inoculated with 1.0mL of the diluted sewage sample. The inoculated LTB tubes were then incubated for 24 hours at 35°C. After incubation, the tubes were examined for turbidity and gas production. Each "positive" tube (showing gas production) was inoculated into a corresponding tube containing *Escherichia coli* (EC) broth and a durham tube. Inoculated EC tubes were incubated in a shaking waterbath for 24 hours at 44.5°C. Tubes were again examined for turbidity and gas production after incubation. Tubes exhibiting gas production were designated as "positive" and used in the MPN chart to calculate the MPN/mL of fecal coliforms.

The protocol used for enumerating total coliforms was taken from *Standard Methods for the Examination of Water and Wastewater* (34). The "positive" LTB tubes from the fecal coliform analysis were inoculated into corresponding 10mL Brilliant Green Lactose Bile Broth (BGLB) tubes (BD, Franklin Lakes, NJ) using an inoculating loop. The BGLB tubes were then incubated for 48 hours at 35°C. Tubes showing turbidity and gas production were scored as "positive" and were used as the basis of calculating the estimated MPN/mL using the MPN chart.

To calculate generic *E. coli* concentrations, a loopful of each positive EC tube from the fecal coliform analysis was streaked onto EC Medium with MUG plates (BD, Franklin Lakes, N. J.). The plates were then incubated for 24 hours at 35°C. After incubation, the plates were examined in a dark room using a hand-held UV lamp. Those plates with

fluorescing colonies were considered "positive" for generic *E. coli*. The fluorescent "positive" plates were used as the basis for calculating the MPN/mL of generic *E. coli* in the raw sewage samples.

Salmonella spp.

The EPA Method 1682 was used to evaluate the prevalence of *Salmonella* species in the raw sludge samples. Briefly, 300mL of the pH-adjusted samples were homogenized in a laboratory stomacher. Aliquots of the homogenized, pH-adjusted sample were then inoculated into 3 sets of 5 tubes of Trypticase Soy Broth (TSB) as follows: 20mL of raw homogenized sludge into 10mL 3X TSB, 10mL of raw homogenized sludge into 5mL 3X TSB, and 1.0mL of raw homogenized sludge into 10mL 1X TSB. The TSB tubes were incubated for 24 hours at 36°C. After incubation, six 30uL drops from each TSB tube were spotted onto corresponding plates of semisolid Rappaport-Vassiliadis (MSRV) media with added novobiocin (BD, Franklin Lakes, NJ). The MSRV plates were incubated for 16-18 hours at 42°C. Plates with "halo" spots after incubation, indicating motility, were stabbed using a sterile loop and streaked onto a Xylose Lysine Desoxycholate (XLD) plate (BD, Franklin Lakes, NJ). XLD plates were incubated for 18-24 hours at 36°C and examined for black or red colonies with black centers. Plates exhibiting such were marked as "positive" and positive plates were used to calculate initial concentration in MPN/mL using the MPN table provided in the EPA protocol.

Enterococci

1 mL aliquots of raw sewage were serially diluted (10^{-1} through 10^{-3} or 10^{-5}) in 99mL of sterile water. One Enterolert™ packet (IDEXX, Westbrooke, ME) was added to each dilution and the sample was thoroughly mixed. The entire volume was then transferred to a Quantitray 2000™ (IDEXX, Westbrooke, ME) and sealed as per the manufacturer's instructions using a Model 2X Quantitray Sealer (IDEXX, Westbrooke, ME). The sealed

Quantitrays™ were incubated for 24 hours at 41°C. After incubation, the fluorescent wells were counted using a hand-held UV light. The manufacturer-supplied MPN table was then used to estimate the MPN/mL of enterococci in each sample.

Shigella spp.

The raw sewage was serially diluted (10^{-1} through 10^{-3}) in phosphate buffer. One mL (1mL) aliquots of 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were each inoculated into a set of 5 test tubes containing *Shigella* broth with novobiocin. The tubes were incubated overnight with shaking at 37°C. Tubes showing turbidity were streaked onto Rainbow Agar (Biolog/FDA, unpublished protocol) and incubated for 24-48 hours at 35°C. The plates after incubation for examined for purplish, "mauve"-colored colonies.

DNA Extraction

DNA was extracted from approximately 0.275 g of wet sewage using the commercially available PowerSoil DNA Extraction Kit (MoBio, Solano, CA). The high-yield protocol was utilized, with a few modifications as described by Viau *et. al.* (2009) (88). Modifications included replacement of the initial bead-beating with heating at 70°C for 10 minutes, followed by bead-beating at 2500 rpm for 3 minutes. Additionally, the incubation time with buffers S2 and S3 was increased to 10 minutes at 4°C to improve removal of impurities. Each extraction resulted in 100uL of community DNA. Community DNA was extracted in triplicate from each received sample and then pooled into a composite sample with a volume of 300uL.

Pyrosequencing and Data Processing

The community DNA that was extracted from the raw sludge and milk samples as previously described was used for the deep sequencing-based microbial diversity analysis. Extracted community DNA from both studies, in 20µl aliquots, was sent to the Research and Testing Laboratory in Lubbock, Texas for analysis by 16S rRNA bacterial tag-encoded FLX amplicon pyrosequencing. The pyrosequencing procedure and subsequent bioinformatics processing were performed by Dr. Scot Dowd's laboratory at the Pathogen Research & Testing Laboratory in Lubbock, TX. A 50 µl PCR reaction was performed for each sample using 1µl of extracted DNA.

Massively Parallel bTEFAP and bTEFAP Titanium

Bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTETAP) was performed as described previously (5, 11, 26) at the Research and Testing Laboratory in Lubbock, TX. The new bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTETAP) approach is based upon similar principles to bTEFAP but utilizes Titanium reagents and Titanium procedures, a one-step PCR, a mixture of Hot Start and HotStar High Fidelity Taq Polymerases, and amplicons originating from the 27F region numbered in relation to *E. coli* rRNA. All bTETAP procedures were performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (www.researchandtesting.com).

Bacterial Diversity Data Analysis

Following sequencing, all failed sequence reads, low quality sequence ends, and tags were removed and sequences were depleted of any non-bacterial ribosome sequences and chimeras using custom software described previously (5, 11, 26) and the Black Box Chimera Check software B2C2 (described at

http://www.researchandtesting.com/B2C2.html). Sequences less than 150bp were removed for the original bTEFAP method and less than 300 bp for the bTEFAP titanium method. To determine the identity of bacteria in the remaining sequences, sequences were first queried using a distributed BLASTn.NET algorithm (27) against a database of high quality 16S bacterial sequences derived from NCBI. Database sequences were characterized as high quality based upon the criteria of RDP ver. 9 (16). Using a .NET and C# analysis pipeline, the resulting BLASTn outputs were compiled and validated using taxonomic distance methods, and data reduction analysis performed as described previously (5, 11, 26).

Bacterial Identification

Based upon the above BLASTn derived sequence identity (percent of total length query sequence which aligns with a given database sequence) and validated using taxonomic distance methods, the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 80% and 90% at the order level. After resolving based upon these parameters, the percentage of each bacterial ID was individually analyzed for each sample providing relative abundance information within and among the relative numbers of reads within a given sample. Evaluations presented at a given taxonomic level, except species level, represent all sequences resolved to their primary genera identification or their closest relative (where indicated).

CHAPTER IV

RESULTS OF MICROBIAL DIVERSITY IN MILK STUDY

Phylogenetic Profiles of Raw and Pasteurized Milk

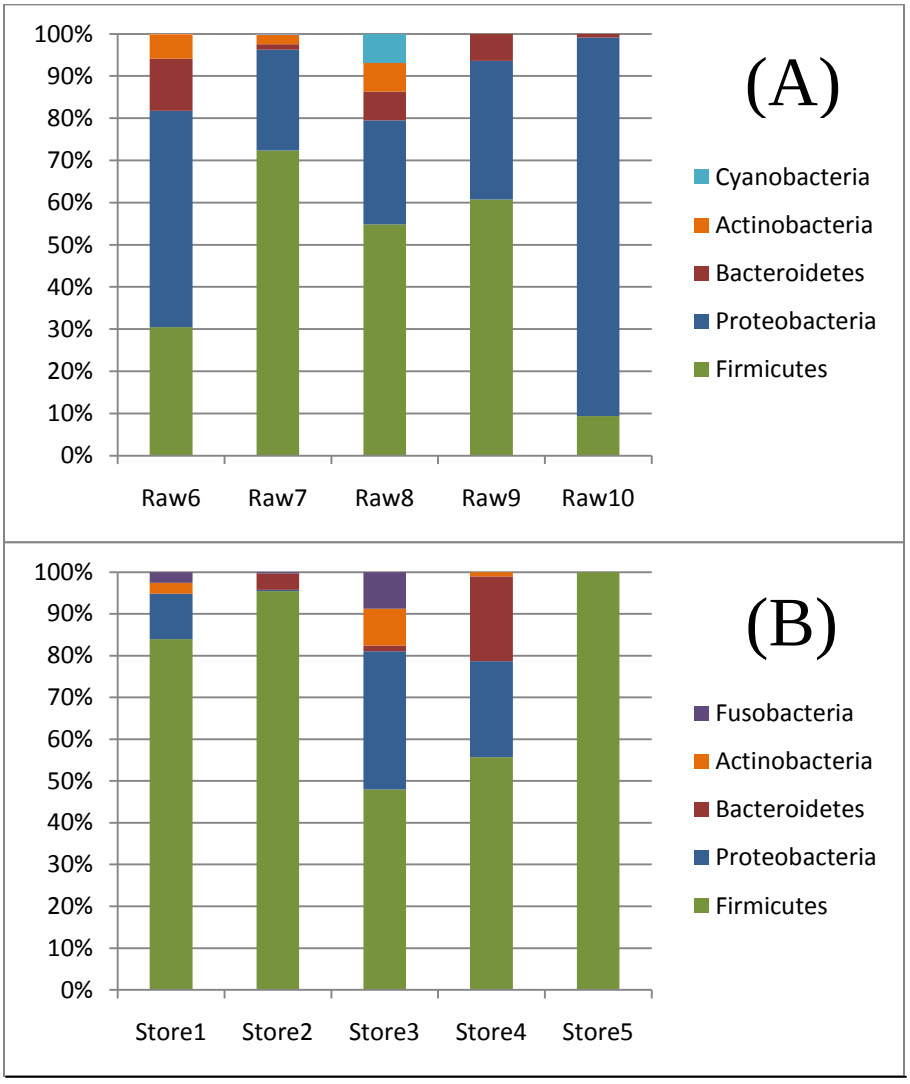


FIG. 1. Phylogenetic profiles observed in raw milk (A) and pasteurized milk (B) samples by pyrosequencing.

Both sets of samples consisted mainly of organisms from the Firmicutes and Proteobacteria phyla (Figure 1). The majority of the pasteurized milk samples were dominated by Firmicutes, however the overall prevalence of this phylum varied quite widely from sample to sample. Significant proportions of Proteobacteria and Actinobacteria appeared in Pasteurized Samples #1, #3, and #4. Populations of Fusobacteria were present in Samples #1, #2, and #3 and significant populations of Bacteroidetes were found in Samples #2, #3, and #4. Sample #5 was entirely made up of Firmicutes. Similarly, the raw milk samples were also dominated by members of the Firmicutes and Proteobacteria phyla. Raw Samples #6, #7, #8, and #9 had fairly similar profiles at the phylum-level, consisting mainly of Firmicutes, Proteobacteria, and Bacteroidetes. Actinobacteria also appeared in Samples #6, #7, and #8, while Sample #8 had a significant population of Cyanobacteria. Raw Sample 10 differed significantly from the other raw milk samples, being almost completely dominated by Proteobacteria.

Comparison of the Microflora of Raw and Pasteurized Milk

The aerobic and anaerobic plate count data, as well as sequence numbers detected in samples by pyrosequencing, are shown for both pasteurized and raw milk samples (Table 1). The number of sequences detected in the pasteurized milk samples were extremely low, between 50-311 sequences; indicating that the bacterial density in these samples was not very high. Plate counts of pasteurized milk were also low, ranging from 1 to 79 CFU/mL in aerobic plating and 0 to 1 CFU/mL in anaerobic plating. The culture-based plating results support the conclusion that the microorganisms isolated from pasteurized milk were mainly obligate aerobes. Between 146 and 12656 sequences were detected in the raw milk samples. Plate counts were also considerably higher, ranging from $2.6x10^2$ to $2.3x10^5$ CFU/mL in aerobic plating and 73.0 to $9.9x10^5$ CFU/mL in anaerobic plating. Using the Mann-Whitney Rank Sum test, aerobic and anaerobic plate counts of raw milk were shown to be significantly higher than those of pasteurized milk ($p=0.008$ for both). However, the number of sequences detected in pasteurized and raw

milk samples was not found to be significantly different (p=0.091). Raw Sample #6 appeared to contain mainly obligate aerobic organisms, even though the aerobic and anaerobic plate counts for the other samples were fairly similar. Additionally, Raw Sample #8 appeared to differ from the other raw milk samples collected, having low plate counts and comparatively many fewer sequences detected.

TABLE 1. Bacterial load detected in collected milk samples

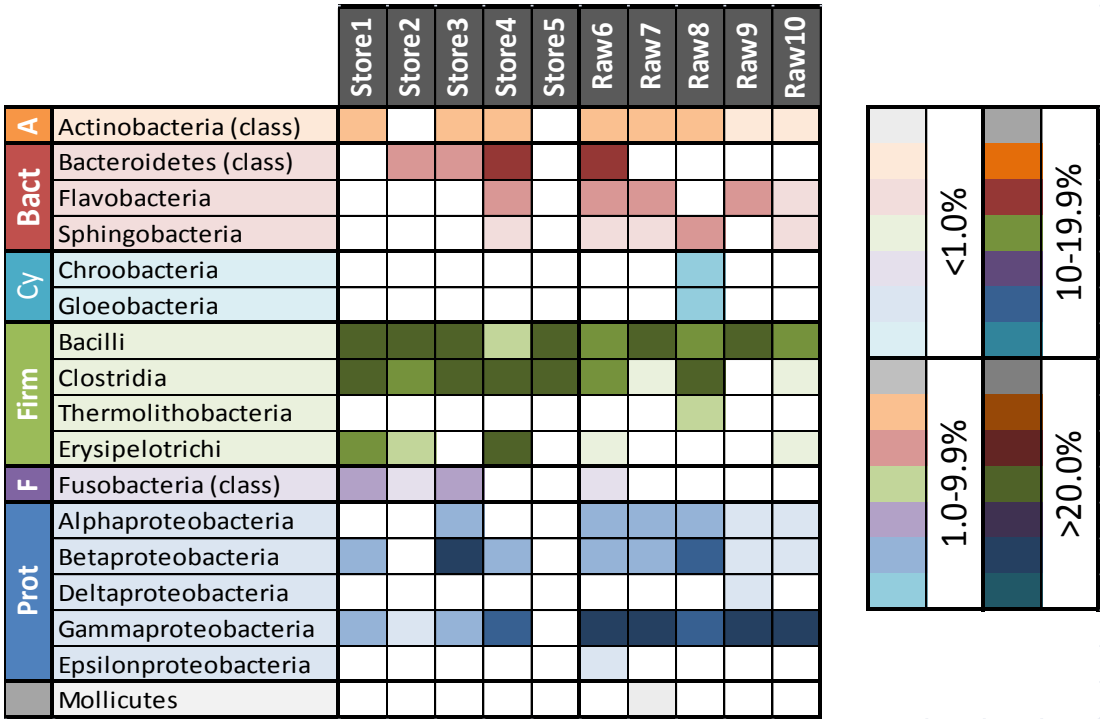
Pasteurized	**Store 1**	**Store 2**	**Store 3**	**Store 4**	**Store 5**
Aerobic Load[a]	3.0	5.0	22.0	79.0	1.0
Anaerobic Load	0.0	1.0	1.0	0.0	0.0
Sequences[b]	311	283	148	300	50
Raw	**Raw 6**	**Raw 7**	**Raw 8**	**Raw 9**	**Raw 10**
Aerobic Load	$8.9x10^2$	$2.1x10^3$	$2.6x10^2$	$2.3x10^5$	$2.2x10^5$
Anaerobic Load	73.0	$1.4x10^3$	$3.8x10^2$	$3.6x10^5$	$9.9x10^5$
Sequences	1330	3655	146	12045	12656

[a] Bacterial load in CFU/mL
[b] Represents number of sequences detected in each sample by pyrosequencing

As previously mentioned, the dominant bacterial phyla detected in both the pasteurized and raw milk samples were Firmicutes and Proteobacteria, with smaller proportions of other phyla. Both raw and pasteurized milk samples were similar in that each milk sample appeared to have its own distinct bacterial profile. This was reflected in a visual representation of the bacterial diversity in the pasteurized and raw milk samples at the

class-level (Table 2). The purpose of this table was to provide an impression of dominant groups of organisms and how the different milk samples related to each other. There were few patterns or conserved genera that could be detected across samples for either milk type.

TABLE 2. Visualization of bacterial diversity present in pasteurized and raw milk samples at the class-level[a]



[a] Different colors represent different phyla, namely Actinobacteria (A), Bacteroidetes (Bact), Cyanobacteria (Cy), Firmicutes (Firm), Fusobacteria (F), and Proteobacteria (Prot), while the intensity of the color represents the prevalence of the class of organisms, ranging from less than 1.0% to greater than 20%. Bacterial phyla that were not assigned a color are presented in gray-scale.

In all, 38 different genera were detected in the five pasteurized milk samples. Firmicutes was the dominant phylum detected, with organisms split between the Clostridia, Bacilli, and Erysipelotrichi classes. Clostridia were mainly *Clostridium* spp. Many of these were identified as *C. lituseburense*, which was the only bacterial species detected in all 5 samples. Bacilli were commonly *Staphylococcus* and *Streptococcus* spp., while the detected Erysipelotrichi were almost entirely *Turicibacter* spp. Surprisingly, *Pseudomonas*, widely believed to be one of the dominant bacterial genera in milk, was detected in only 3 out of the 5 samples overall (29, 79).

Comparatively, 130 different bacterial genera were detected in the raw milk samples. Raw milk samples were also dominated by Firmicutes and Proteobacteria. The majority of the Firmicutes sequences were members of the Bacilli class, with significant populations of Clostridia showing up in Raw Samples #6 and #8. Common Bacilli included *Staphylcoccus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp., and *Bacillus* spp., while Clostridia were mainly *Clostridium* spp. Clostridia in raw milk are of interest because of their potential to survive pasteurization. Proteobacterial organisms were dominated by Gammaproteobacteria, in which genera such as *Acinetobacter*, *Pseudomonas*, and *Serratia* were common. *Pseudomonas* appeared more common in the raw milk than in the pasteurized samples and was the dominate genus in two of the raw milk samples. Many of the organisms considered to be “classic” raw milk microflora, as described in the literature review, were detected within the samples.

Statistical Comparison of Populations

Statistical comparisons of raw and pasteurized milk samples were performed using the Mann-Whitney Rank Sum test at the phylum, class, order, family, and genus-level to determine whether bacterial populations within samples of each type of milk (raw or pasteurized) were significantly different at these levels (Table 3). There were no statistically significant differences between any of the milk samples at the class- or

phylum-levels. Three of the pasteurized milk comparisons had significant differences at the genus- and/or family-levels. The comparisons that showed statistical significance involved Store Sample #2. All of the raw milk samples were significantly different from one another at the genus-level. Additionally, all of the raw milk comparisons that did not involve Raw Sample #6 were significantly different at the family-level and a select number of comparisons were also significantly different at the order-level.

TABLE 3. Statistical comparisons of milk populations[a]

	Pasteurized Milk									
	1 vs. 2	1 vs. 3	1 vs. 4	1 vs. 5	2 vs. 3	2 vs. 4	2 vs. 5	3 vs. 4	3 vs. 5	4 vs. 5
Genus	p=0.009				p=0.009		p<0.001			
Family	p=0.033						p=0.006			
Order										
	Raw Milk									
	6 vs. 7	6 vs. 8	6 vs. 9	6 vs. 10	7 vs. 8	7 vs. 9	7 vs. 10	8 vs. 9	8 vs. 10	9 vs. 10
Genus	p=0.005	p=0.030	p=0.002	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Family					p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
Order					p=0.036	p=0.035		p=0.018		

[a] A green-colored box represents that the difference between the two samples was significantly different (p-value<0.05). P-value are listed for each comparison that was statistically significant.

Prevalence of Lactic Acid Bacteria in Raw and Pasteurized Milk Samples

The prevalence of four lactic acid bacteria genera within the raw and pasteurized milk samples, namely, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* was evaulated. These genera are considered "friendly" lactic acid bacteria important for food production and, occasionally, in probiotic supplements.

TABLE 4. Lactic acid bacteria species and overall prevalence in raw and pasteurized milk samples

	Store 1	Store 2	Store 3	Store 4	Store 5	Species
Lactobacillus		0.71				*L. mucosae*
Lactococcus		12.01				*L. lactis*
Leuconostoc						*N/A*
Streptococcus	6.75	48.8	19.6	1.7	26	*S. dysgalactiae, S. thermophilus, S. iniae, S. gallolyticus, S. equi, S. suis*
TOTAL	6.8%	61.5%	19.6%	1.7%	26.0%	
	Raw 6	**Raw 7**	**Raw 8**	**Raw 9**	**Raw 10**	**Species**
Lactobacillus	2.26		13.7		3.43	*L. casei, L. graminis, L. brevis, L. curvatus, L. plantarum*
Lactococcus		19.89		0.16	3.1	*L. lactis, L. garvieae*
Leuconostoc		0.25				*L. mesenteroides*
Streptococcus	0.9			<0.01	<0.01	*S. equi, S. iniae, S. mitis*
TOTAL	3.2%	20.1%	13.7%	0.2%	6.5%	

A comparison of the prevalence of lactic acid bacteria in the pasteurized milk samples showed that most samples had significant portions of lactic acid bacteria, with many

different species detected (Table 4). However, these sequences were mainly streptococci of various species. The overall prevalence of lactic acid bacteria in raw milk was lower than pasteurized milk, but raw milk samples appeared to have higher proportions of organisms such as *Lactobacillus* and *Lactococcus* spp. However, for both pasteurized and raw milk, the concentrations of these organisms were highly variable between samples. In pasteurized milk, prevalence of these lactic acid bacteria ranged from 1.7% in one sample to 61.5% in another, while prevalence in raw milk ranged from 0.2% to 20.1%. A comparison of raw and pasteurized milk using the Mann-Whitney Rank Sum test found no significant difference between LAB populations (as defined by these genera) in raw and pasteurized milk samples ($p = 0.310$).

Prevalence of Pathogenic Organisms in Raw and Unpasteurized Milk Samples

Analysis of the milk microbiota showed the presence of organisms that are classical indicators of fecal contamination. Fecal indicators such as *Escherichia* spp., *Bacteroides* spp., and *Enterococcus* spp. were detected in several of the samples, both raw and pasteurized. Organisms commonly defined as "pathogens" were also detected in the milk samples (Table 5). Evidence for the designation of the selected bacteria as pathogenic organisms is provided in the appendices (Table A-1). Pathogenic organisms detected in pasteurized milk were solely *Staphylococcus aureus*, which was found in only one sample and which totaled less than 10 sequences. However, one sequence each of common foodborne pathogens *Salmonella enterica, Shigella boydii*, and *Campylobacter jejuni* was detected in separate milk samples (Raw #1, Raw #4, and Raw #5). Other organisms of public health importance that were detected in the raw milk samples included *Coxiella burnetti* and *Clostridium perfringens*. While these pathogens constituted very minor portions of the microflora, large numbers of opportunistic pathogens such as *Aeromonas hydrophila, Acinetobacter baumannii, Pseudomonas aeruginosa, Serratia marcescans,* and *Staphylcococcus aureus* were detected in many of the raw milk samples. *Staphylococcus aureus* was detected in high numbers in Raw

Sample #7, while *Acinetobacter baumannii* and *Aeromonas hydrophila* were shown to make up a significant portion of Raw Sample #9. Additionally, *Pseudomonas aeruginosa* and *Serratia marcescans* made up more than 70% of all sequences detected in Raw Sample #10. None of the pathogens detected were found in all five raw milk samples, but *Acinetobacter baumannii*, *Aeromonas hydrophila*, and *Klebsiella pneumoniae* were detected in four of the five samples—indicating that these organisms may be common in raw milk. A comparison of the two sample sets using the Mann-Whitney Rank Sum test found that prevalence of the selected pathogenic organisms was significantly higher in the raw milk samples than in the pasteurized milk samples (p=0.008).

TABLE 5. Pathogenic organisms detected in raw and pasteurized milk samples[a]

	Store 1	Store 2	Store 3	Store 4	Store 5	Raw 6	Raw 7	Raw 8	Raw 9	Raw10
Acinetobacter baumannii						21	2		700	17
Aeromonas hydrophila						19	6		430	1
Campylobacter jejuni [a]						1				
Clostridium perfringens							1			
Coxiella burnetii						8				
Enterobacter cloacae							7		395	271
Enterococcus faecalis						21	153		44	
Enterococcus faecium									8	
Klebsiella pneumoniae						3	58		44	88
Pseudomonas aeruginosa							9	15		6527
Salmonella enterica									1	
Serratia marcescens									32	2570
Shigella boydii										1
Staphylococcus aureus				8			1708		214	50

[a] Major pathogens of interest are shown in bold for emphasis. The number within each box represents the number of sequences identified in each sample. The squares highlighted in yellow represent organisms confirmed to greater than 97% of sequence similarity.

Spoilage Microflora of Unprocessed and Processed Milk Samples

One of the secondary goals of this study was to evaluate the microbial ecology of milk spoilage, both with and without processing. For the purposes of this study, spoilage was defined as the presence of a bacterial load exceeding $2.0x10^4$ CFU/mL after the refrigeration period, which is the legal limit for processed milk samples (31).

TABLE 6. Bacterial load detected in unprocessed milk samples after refrigerated storage

Pasteurized	Spoil 1	Spoil 2	Spoil 3	Spoil 4	Spoil 5
Aerobic Load[a]	$1.0x10^8$	$5.2x10^8$	$1.8x10^8$	$7.0x10^6$	$3.6x10^6$
Anaerobic Load	$3.5x10^6$	$1.1x10^7$	$3.8x10^6$	$4.1x10^6$	$5.6x10^6$
Sequences[b]	16877	10164	16196	7437	11264
Raw	Spoil 6	Spoil 7	Spoil 8	Spoil 9	Spoil 10
Aerobic Load	$1.4x10^8$	$9.2x10^7$	$1.1x10^8$	$2.0x10^8$	$2.5x10^8$
Anaerobic Load	$1.0x10^8$	$1.6x10^8$	$1.3x10^7$	$1.2x10^8$	$1.9x10^8$
Sequences	13555	14219	9843	8441	7674

[a] Bacterial load in CFU/mL
[b] Represents number of sequences detected in each sample by pyrosequencing

For most samples, spoilage was characterized by a dramatic change in overall bacterial composition, an increase in the number of organisms, and, often, the dominance of a handful of bacterial genera. In the majority of samples, refrigerated storage caused the

bacterial load to increase dramatically, as measured by both plate counts and detectable sequence numbers (Table 6). A comparison of raw and pasteurized milk samples after spoilage using the Mann-Whitney Rank Sum test found that aerobic plate counts and number of sequences detected were not significantly different ($p=0.548$ for both). However, there were significantly higher anaerobic plate counts in the spoiled raw milk samples ($p=0.008$).

Spoilage Microflora of Pasteurized Milk Samples

Bacterial diversity associated with spoilage of the unprocessed and processed pasteurized milk samples differed between samples and treatment (Table 7). Despite the microbial diversity of the original samples, the pasteurized milk samples were dominated by only three genera after spoilage: *Janthinobacterium* (Store Samples #1), *Pseudomonas* (Store Samples #2 & #3), and *Paenibacillus (Store Sample #5).* *Paenibacillus* spp. also appeared as minor populations in two of the other samples (Store Samples #1 and #2). Interestingly, those samples that were dominated by *Janthinobacterium* spp. and *Pseudomonas* spp. also exhibited anaerobic plate counts approximately a log or two lower than their aerobic plate counts. This is in contrast to the samples that were dominated by *Paenibacillus* spp., in which the aerobic and anaerobic plate counts were quite similar (Table 6). It should be noted that the genera listed as dominating the spoiled milk samples were often comprised of two or more bacterial species, indicating that one specific species did not always dominate.

TABLE 7. Visualization of bacterial diversity present in pasteurized milk samples after refrigerated storage at the class-level[a]

	Class	Milk1F	Milk2F	Milk3F	Milk4F	Milk5F	Irr-1F	Irr-2F	Irr-3F	Irr-4F	Irr-5F
A	Actinobacteria						N/A	N/A		N/A	N/A
F	Bacilli						N/A	N/A		N/A	N/A
Bact	Bacteroidetes						N/A	N/A		N/A	N/A
	Flavobacteria						N/A	N/A		N/A	N/A
Prot	Alphaproteobacteria						N/A	N/A		N/A	N/A
	Betaproteobacteria						N/A	N/A		N/A	N/A
	Deltaproteobacteria						N/A	N/A		N/A	N/A
	Epsilonproteobacteria						N/A	N/A		N/A	N/A
	Gammaproteobacteria						N/A	N/A		N/A	N/A

[a] Samples that had plate counts less than the spoilage standard are represented by a "N/A", denoting that no spoilage was present, even though sequences may have been detected. The milk samples processed by boiling were not included in the results table as none of the boiled pasteurized milk samples showed any significant culture growth after the storage period.

Pasteurized milk samples were also allowed to spoil after treatment with Electron beam irradiation and boiling. The plate counts and sequences numbers for these samples are given in Appendix B. Only one processed sample met the requirements for spoilage after refrigerated storage, suggesting that processing generally eliminated those organisms capable of causing spoilage. The one spoiled sample (Irr #3) was dominated by *Pseudomonas* spp.—the same genus found in the untreated sample after spoilage. Spoilage populations of samples Store #3 and Irradiated #3 were compared using the Mann-Whitney Rank Sum test. However, no statistical significance was found (p=1.00 at genus-level).

Spoilage Microflora of Raw Milk Samples

Bacterial diversity associated with spoilage of the unprocessed and processed raw milk samples also differed between samples and treatment (Table 8). Like the pasteurized milk samples, spoilage of the raw milk samples was also characterized by the detection of organisms such as *Pseudomonas* spp. and *Janthinobacterium* spp. *Pseudomonas* spp. were the major organisms detected in four out of the five raw milk samples (Raw Samples #6-9) that had undergone spoilage. The remaining sample (Raw Sample #10) was dominated by *Serratia* spp. As was found in the pasteurized milk samples, these dominant genera were often made up of more than one species. The raw milk samples showed a wide diversity of *Pseudomonas* species after spoilage, including *P. gessardi*, *P. panacis*, *P. trivialis*, *P. cedrina*, *P. chlororaphis*, *P. fluorescens*, and *P. putida*. Other minor populations detected within the spoiled raw milk samples included *Leuconostoc* spp., *Janthinobacterium* spp., *Pectobacterium* spp., and *Enterococcus* spp.

Raw samples were also allowed to spoil after treatment with Electron beam irradiation, a lab-pasteurization process, or boiling. The plate counts and sequences numbers for these samples are shown in Appendix B. After storage, all of the irradiated raw milk samples and three of the lab-pasteurized raw milk samples (Past #6, #9, and #10) met the requirements for spoilage. The three lab-pasteurized samples tended to be dominated by the same genera responsible for spoilage in the unprocessed samples, although there was an absence of the minor populations detected. However, the irradiated raw milk samples tended to harbor a greater diversity of organisms after spoilage, including *Enterococcus* spp., *Acinetobacter* spp., and a variety of lactic acid bacteria. Spoilage populations of raw and processed milk samples were compared using the Mann-Whitney Rank Sum test, but none of the comparisons were statistically significant.

TABLE 8. Visualization of bacterial diversity present in raw milk samples after refrigerated storage at the class-level[a]

	Class	Raw-6F	Raw-7F	Raw-8F	Raw-9F	Raw-10F	Past-6F	Past-7F	Past-8F	Past-9F	Past-10F	irr-6F	Irr-7F	Irr-8F	Irr-9F	Irr-10F
A	Actinobacteria							N/A	N/A							
F	Bacilli							N/A	N/A							
F	Clostridia							N/A	N/A							
	Opitutae							N/A	N/A							
Bact	Bacteroidetes							N/A	N/A							
Bact	Flavobacteria							N/A	N/A							
Bact	Sphingobacteria							N/A	N/A							
Prot	Alphaproteobacteria							N/A	N/A							
Prot	Betaproteobacteria							N/A	N/A							
Prot	Deltaproteobacteria							N/A	N/A							
Prot	Epsilonproteobacteria							N/A	N/A							
Prot	Gammaproteobacteria							N/A	N/A							
	Spirochaetes							N/A	N/A							

[a] Samples that had plate counts less than the spoilage standard are represented by a "N/A", denoting that no spoilage was present, even though sequences may have been detected. The milk samples processed by boiling were not included in the results table as none of the boiled raw milk samples showed any significant culture growth after the storage period.

Other Spoilage Results

It is important to note that DNA sequences assigned to various organisms were detected even in the samples in which "spoilage" (as defined by this study) did not take place. As the viability of the detected organisms could not be validated, they were not included in the microflora results and are represented by a "N/A". The sequences detected in these samples tended to be similar to the genera detected in the original samples and most likely represent fragmented DNA that survived the various processing methods. Hundreds of sequences were detected in some of these "non-spoiled" samples, although sequences numbers tended to be much lower than those detected in "spoiled" samples. Considerable numbers of sequences, ranging from 0 to 7099, were even detected in boiled samples after the refrigerated storage (Table B-3). Additionally, some of the same pathogenic organisms detected in the original samples were still detectable after the storage period, although generally at much lower levels than in the initial samples. However, several of the irradiated raw milk samples showed high levels of certain opportunistic pathogens after spoilage, including *Staphyloccoccus aureus* and *Enterococcus faecium*, suggesting that populations of these organisms increased during storage. Interestingly, 935 and 28 *Rickettsia rickettsii* sequences were detected in boiled Raw Samples #6 and #7, respectively, after storage. This organism was not detected in any of the other samples—original, processed, or spoiled.

Analysis of Autoinducer-2-like Activity in Raw, Pasteurized, and Spoiled Milk Samples

Out of all the raw, pasteurized, and spoiled milk samples, only four samples showed evidence of Autoinducer-2-like (AI-2-like) activity using the *V. harveyi* BB170 reporter strain assay (Table 9). Samples were defined as exhibiting AI-2-like activity if they showed a 10-fold or greater increase over negative controls. All samples positive for AI-2-like activity were portions of Raw Samples #9 and #10 that had undergone

spoilage. In order to establish the inhibitory capabilities of the milk matrix on the reporter strain assay, milk aliquots were mixed with a high concentration of pure AI-2-like molecules and analyzed. These inhibition studies revealed that different milk samples inhibited between 68.16% and 99.10% of spiked AI-2. Therefore, given these results, it is likely that AI-2-like molecules may have been present in lower levels in the other milk samples but were not detected because of the inhibitory activity of the milk.

TABLE 9. Milk samples showing Autoinducer-2-like activity

Sample	Fold-Increase[a]
Raw 9 Spoiled	12.31
Raw 10 Spoiled	109.39
Pasteurized 10 Spoiled	180.39
Irradiated 10 Spoiled	143.19

[a] "Fold-increase" represents increase in fluorescence over negative controls.

Comparing these results to the microflora data generated by pyrosequencing shows that all of the samples showing AI-2-like activity had a significant population of *Serratia* spp. The AI-2-like activity seems to coincide with the prevalence of *Serratia proteamaculans* in these samples. In each sample in which AI-2-like activity was detected, *S. proteamaculans* made up between 20% and nearly 100% of the total microflora. Additionally, this organism does not appear in such high levels in any of the other milk samples.

CHAPTER V
RESULTS OF MICROBIAL DIVERSITY IN MUNICIPAL SEWAGE STUDY

Primary sewage sludge samples were collected from municipal wastewater treatment plants in Chicago, IL, Cincinatti, OH, El Paso, TX, Columbus, GA, San Diego, CA, Madison, WI, and Washington D.C. Chemical analysis showed that the sludge samples were slightly acidic, ranging in pH from 5.3 to 6.54. The solids content of the sludge samples were variable, ranging from 0.11% to 5.22%. Solids content and pH for each sample are provided in Appendix C.

Phylogenetic Profiles of Bacterial Communities within Sewage Sludge

All of the sewage sludge samples were similar in that they were made up mainly of bacteria from the Proteobacteria, Bacteroidetes, Firmicutes, and Fusobacteria phyla (Figure 2). Except for two of the El Paso samples, Proteobacteria was the dominant phylum in all samples, followed by Bacteroidetes, Firmicutes, and Fusobacteria in prevalence. Samples collected from the El Paso treatment plant did not match the patterns seen in the other samples, having much higher levels of Firmicutes and tending to be dominated by Bacteroidetes instead of Proteobacteria. Firmicutes appeared to be a minor population in the other samples. However, other sludge samples also showed more subtle unique characteristics. For example, the Madison samples consistently showed a higher proportion of organisms from the Fusobacteria phylum, whereas Chicago samples seemed to have a higher incidence of Verrucomicrobia.

Phylogenetic profiles of individual locations appeared fairly consistent over time, but there were some possible trends across samplings. The prevalence of Proteobacteria in

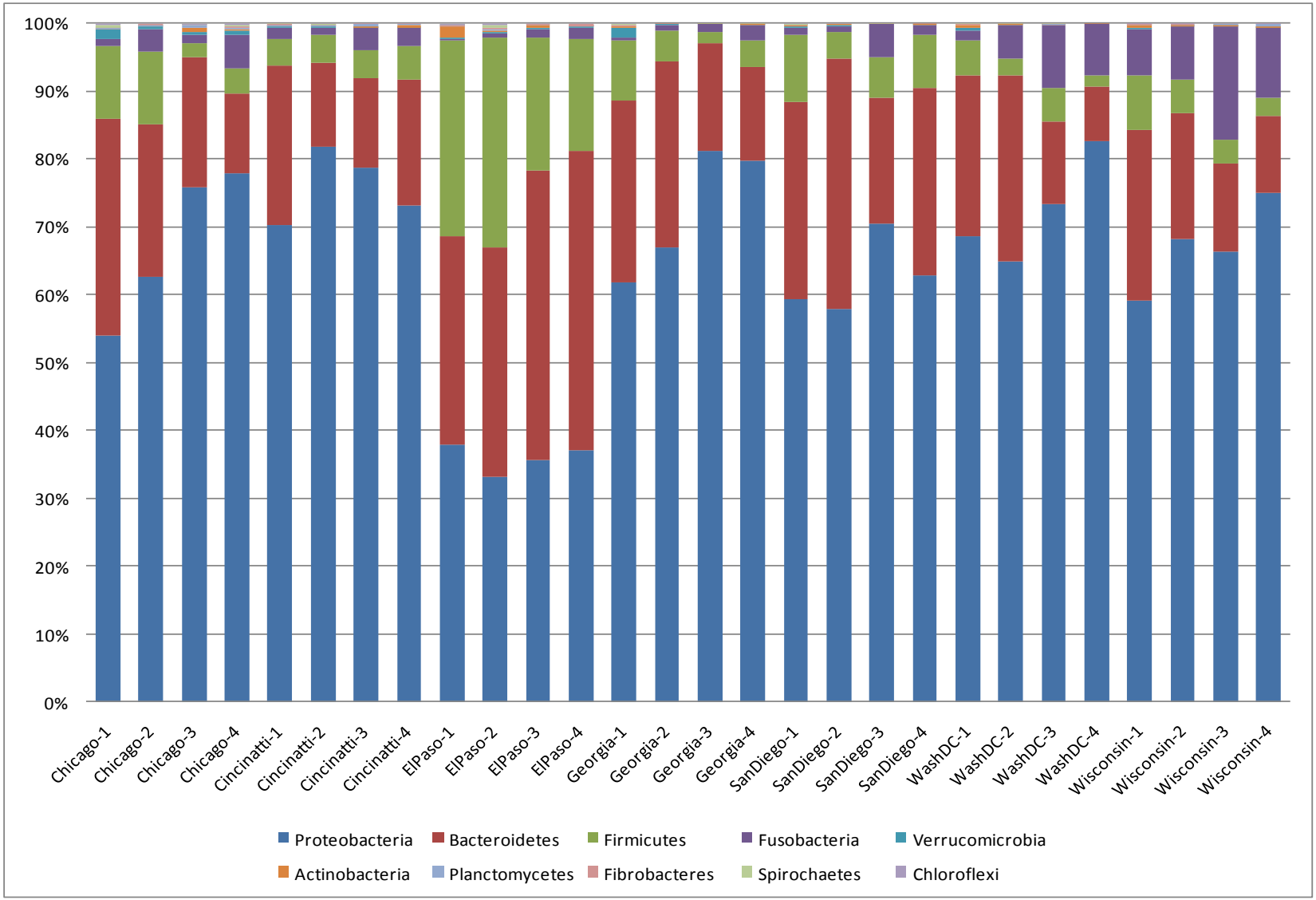


FIG. 2. Distributions of major bacterial phyla within sewage sludge samples grouped by location.

the samples increased as the sampling progressed at the expense of the other phyla, making up an average of 58.56%, 62.16%, 68.78%, and 69.09% of total sequences in Samplings 1-4, respectively. The deep sequencing results indicated that the prevalence of Bacteroidetes decreased from the first to the last sampling, making up an average of 27.13%, 25.58%, 19.17%, and 19.32% of total sequences. The Firmicutes also showed a similar trend, with an average proportion of 10.76%, 8.81%, 6.00%, and 5.94% across samplings. Fusobacteria exhibited an average prevalence of 1.77%, 2.77%, 5.40%, and 4.30% across the four samplings. However, the El Paso samples did not follow the

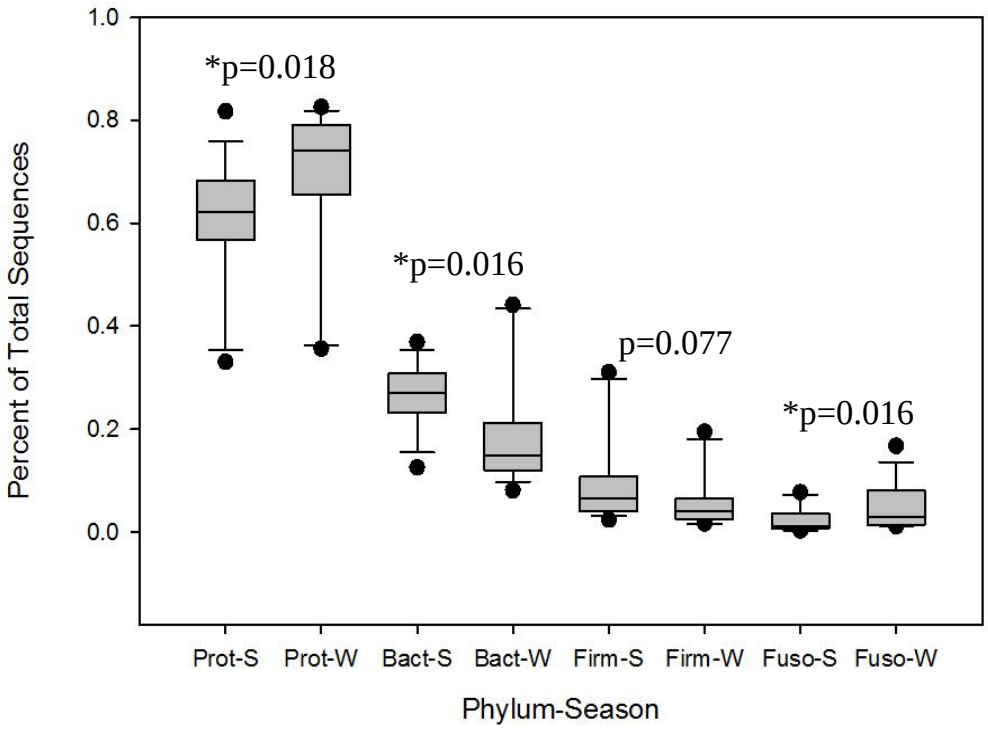


FIG. 3. Seasonality of major phyla in sewage sludge**.** Boxplots representing prevalence data (in percent of total sequences) from each season, summer/fall (S) and winter/spring (W), are shown for four phyla: Proteobacteria (Prot), Bacteroidetes (Bact), Firmicutes (Firm), and Fusobacteria (Fuso). P-values represent the comparison of seasonal values using the Mann-Whitney Rank Sum test, and the presence of an asterisk represents that the difference between the two groups was statistically significant.

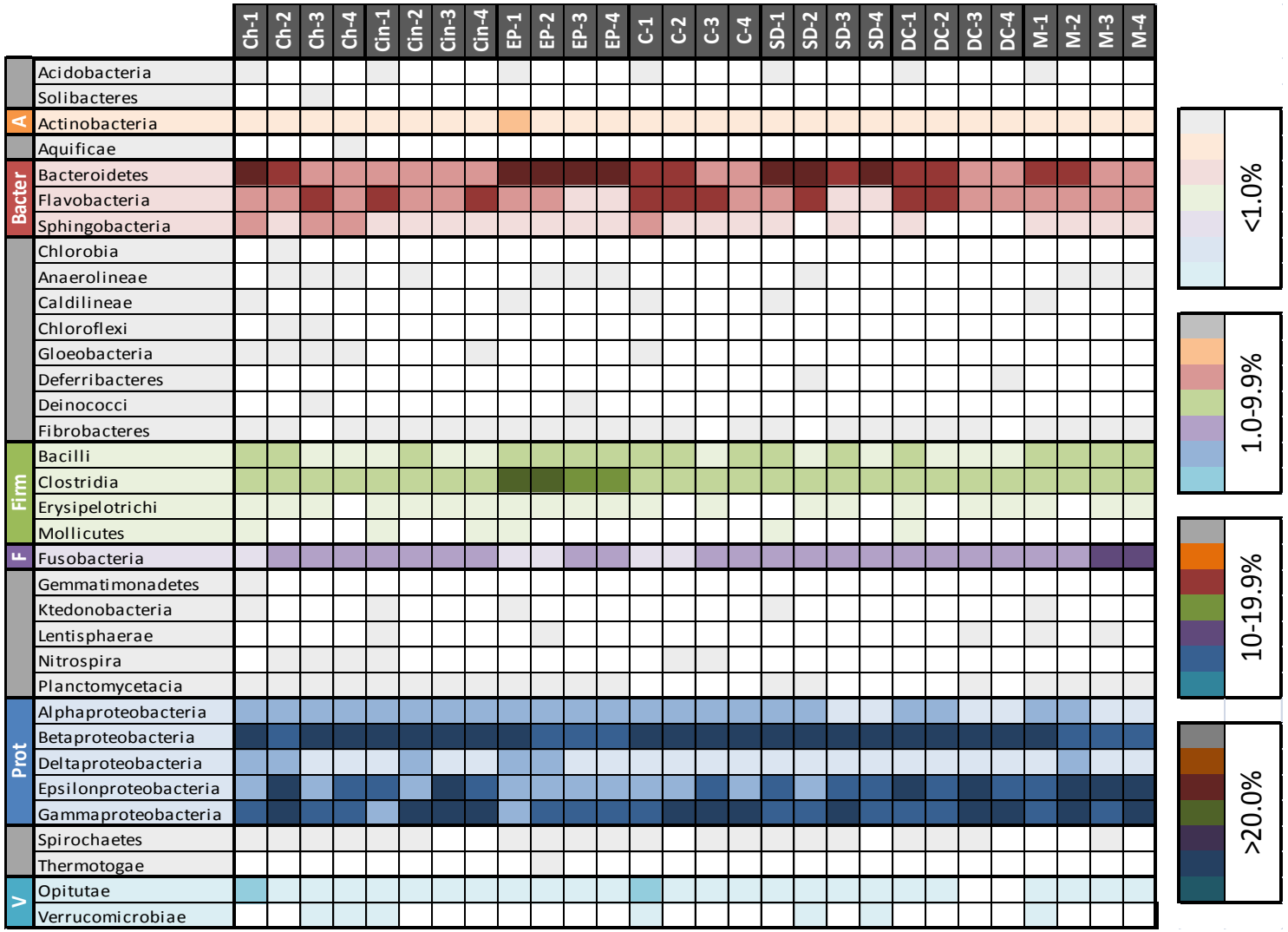
patterns exhibited by the other samples, as the percentage of Bacteroidetes increased from the summer/fall to the winter/spring samplings.

Statistical comparisons of the prevalence of these four major phyla by season were performed using the Mann-Whitney Rank Sum test and the results are presented along with boxplot summaries of the data (Figure 3). This analysis showed that populations of Proteobacteria ($p=0.018$) and Fusobacteria ($p=0.016$) were significantly higher in the winter/spring samplings, while populations of Bacteroidetes ($p=0.016$) were significantly higher in the summer/fall samplings. There was no statistical significance between populations of Firmicutes across seasons ($p=0.077$).

Comparison of Microflora of Sewage Sludge Samples

A vast diversity of bacterial organisms were found in the untreated sludge, with more than 350 different genera detected in all samples. This diversity is reflected in a visual representation of the sewage sludge microflora at the class-level (Table 10). This table provides an impression of the dominant groups of organisms and how the sludge samples related to each other. In the table, classes of organisms are grouped by phylum for comparison. The sludge samples are notable for the large number of bacterial classes represented at low levels. These classes were present sporadically throughout the samples and generally comprised less than 1% of total sequences. As previously mentioned, the dominant bacterial phyla detected in the sewage sludge samples were Proteobacteria, Bacteroidetes, Firmicutes, and Fusobacteria. Most Bacteroidetes detected belonged to the classes Bacteroidetes and Flavobacteria. Major genera present within the Bacteroidetes class were *Bacteroides*, *Parabacteroides*, *Petrimonas*, *Paludibacter*, and *Prevotella*. Within the Flavobacteria class, *Riemerella* spp., *Flavobacterium* spp., and *Chryseobacterium* spp. were common. Firmicutes detected within the sewage sludge samples were mainly Clostridia and Bacilli. Common genera within Clostridia included *Clostridium*, *Butyrivibrio*, *Phascolarctobacterium*, and *Sporobacterium*. Bacilli were

TABLE 10. Visualization of bacterial diversity present in sewage sludge samples at the class level[a]



[a] Different colors represent different phyla, while the intensity of the color represents the prevalence of the class in each sample, ranging from less than 1.0% to greater than 20%. Bacterial phyla that were not assigned a color are presented in gray-scale. Samples are organized by location: Chicago (Ch), Cincinatti (Cin), El Paso (EP), Columbus (C), San Diego (SD), Washington, D.C. (DC), and Madison (M).

commonly *Streptococcus* spp., with some *Staphylococcus* spp. Common genera within the Fusobacteria class included *Leptotrichia*, *Propionigenium*, and *Sebaldella*.

Proteobacteria made up a large portion of the organisms detected in the sewage sludge samples and were predominantly Betaproteobacteria, Epsilonproteobacteria, and Gammproteobacteria. Common Betaproteobacteria included *Achromobacter* spp., *Acidovorax* spp., *Comamonas* spp., *Dechloromonas* spp., *Hydrogenophaga* spp., *Kingella* spp., *Propionivibrio* spp., *Thauera* spp., *Uruburella* spp., and *Zooglea* spp. *Acidovorax* was a very prevalent genus detected across samples, making up more than 10% of all sequences in many samples. Epsilonproteobacteria were almost exclusively *Arcobacter* spp., which was the most predominant genus detected across the sewage sludge samples. *Arcobacter* species made up greater than 10% of all sequences in a majority of the samples, greater than 20% of sequences in several samples, and comprised up to 31.1% of a single sample (Madison-3). Gammaproteobacteria were primarily genera such as *Acinetobacter*, *Aeromonas*, *Enhydrobacter*, *Pseudomonas*, *Shewanella*, *Tolumonas*, and *Xylophilus*. *Acinetobacter* and *Aeromonas* spp. were also especially prevalent throughout the sludge samples.

TABLE 11. Genera detected in all sewage sludge samples by pyrosequencing

Conserved Genera (Average %)		
Arcobacter (12.54%)	*Propionivibrio* (2.48%)	*Uruburuella* (1.13%)
Acidovorax (9.49%)	*Paludibacter* (1.96%)	*Sulfurospirillum* (0.67%)
Bacteroides (5.90%)	*Clostridium* (1.85%)	*Sebaldella* (0.51%)
Parabacteroides (4.00%)	*Dechloromonas* (1.81%)	*Enterobacter* (0.41%)
Chryseobacterium (2.90%)	*Leptotrichia* (1.81%)	*Desulfobulbus* (0.35%)
Zoogloea (2.74%)	*Comamonas* (1.56%)	*Dysgonomonas* (0.22%)
Prevotella (2.72%)	*Enhydrobacter* (1.44%)	*Rhodobacter* (0.20%)
	Thauera (1.25%)	

In all, 22 different genera were detected in every sewage sludge sample, ranging in average prevalence from 0.20% (*Rhodobacter*) to 12.54% (*Arcobacter*) across all collected samples (Table 11). This represents, from the hundreds of bacterial genera that were detected, a snapshot of the conserved organisms found in all samples.

Statistical Comparisons of Sewage Sludge Samples

Statistical comparisons of sewage sludge samples were performed using the Mann-Whitney Rank Sum test at the phylum, class, order, family, and genus-level to determine whether bacterial populations within paired samples were significantly different at these levels (Table 12). There were no statistically significant differences between any of the sewage sludge samples at the class- and phylum-levels. All of the samples were significantly different at the genus-level, most with p-values less than 0.001. However, significant differences were seen between only some samples at the family- and order-levels, indicating that these samples were composed of distinct bacterial populations. Additionally, some of these differences appeared to be conserved across samplings. Comparisons of samples Columbus-San Diego, Columbus-Washington, D.C., and Columbus-Madison were significantly different in all four samplings, while El Paso-Columbus, San Diego-Washington, D.C., and Washington, D.C.-Madison were significantly different in three out of the four samplings.

TABLE 12. Statistical comparisons of sewage sludge populations[a]

	Chicago vs. Cincinatti	Chicago vs. El Paso	Chicacgo vs. Columbus	Chicago vs. San Diego	Chicago vs. WashDC	Chicago vs. Madison	Cincinatti vs. El Paso	Cincinatti vs. Columbus	Cincinatti vs. San Diego	Cincinatti vs. WashDC	Cincinatti vs. Madison	El Paso vs. Columbus	El Paso vs. San Diego	El Paso vs. WashDC	El Paso vs. Madison	Columbus vs. San Diego	Columbus vs. WashDC	Columbus vs. Madison	San Diego vs. WashDC	San Diego vs. Madison	WashDC vs. Madison
	Set 1																				
Genus	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Family							0.038	0.01	0.015	0.05	0.042	0.042	0.043			0.025	0.034	0.047	0.022		
Order																					
	Set 2																				
Genus	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Family												0.02	0.014	0.029	0.014	0.012	0.003	0.013	0.019		0.021
Order																					
	Set 3																				
Genus	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Family								<0.001		<0.001	<0.001					<0.001		<0.001	<0.001	<0.001	<0.001
Order																	0.047				
	Set 4																				
Genus	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Family												0.039				0.002	0.002	<0.001		<0.001	<0.001
Order																					

[a]A green-colored box represents that the difference between the two samples was significantly different (p-value<0.05). P-values are listed for each comparison that was statistically significant.

Detection of Pathogenic Organisms within Sewage Sludge Samples

Various bacterial organisms commonly regarded or referred to as "pathogens" were detected within the untreated sludge samples (Table 13). Evidence for the designation of these organisms as pathogenic bacteria is provided in the appendices (Table A-1). Nearly all samples showed considerable numbers of *Acinetobacter baumannii* and *Aeromonas hydrophila*. Other pathogens detected in a majority of samples included *Klebsiella pneumoniae*, *Salmonella enterica*, and *Serratia marcescans*. Other organisms such as *Brucella melitensis*, *Clostridium botulinum*, *Coxiella burnetti*, *Legionella pnuemophila*, *Rickettsia* spp., *Shigella* spp., *Vibrio cholerae*, and *Yersinia enterocolitica* appeared sporadically in low levels throughout the samples.

Total pathogen concentrations of selected organisms within sampling sets were compared using the Mann-Whitney Rank Sum test. Sampling 1 was not included in this analysis because of the lack of organisms identified to the species-level. Between the samplings, only Sampling Sets 2 and 4 were statistically significant ($p=0.038$), with pathogen concentrations in Sampling 4 significantly higher than pathogen concentrations in Sampling 2. The relatively low prevalence of pathogens in the first sampling set was likely the result of the pyrosequencing platform's increasing read length capabilities and improved identification over the course of the study, as the data set produced from analysis of the first sampling had far fewer organisms identified to the species-level than the data sets produced from the last three samplings. However, the large number of organisms indentified as *Acinetobacter* spp., *Aeromonas* spp., and *Serratia* spp. in this first sampling suggests that the levels of these species might have been similar.

TABLE 13. Pathogenic organisms detected in sewage sludge samples[a]

	Chicago-1	Chicago-2	Chicago-3	Chicago-4	Cincinatti-1	Cincinatti-2	Cincinatti-3	Cincinatti-4	El Paso-1	El Paso-2	El Paso-3	El Paso-4	Columbus-1	Columbus-2	Columbus-3	Columbus-4	San Diego-1	San Diego-2	San Diego-3	San Diego-4	Wash DC-1	Wash DC-2	Wash DC-3	Wash DC-4	Madison-1	Madison-2	Madison-3	Madison-4
Acinetobacter baumannii		24		24		80	33	49		24	77	155		75	88	63		34	112	51		53	22	23		25	45	21
Aeromonas hydrophila		59	26	33		87	360	160		1	19	49		23	48	63		70	454	117		63	351	589		128	253	283
Brucella melitensis		4	17	1		1		3														1				2		
Clostridium botulinum				1				2																				
Coxiella burnetii			8															1										
Klebsiella pneumoniae	4	19	9			6	4	3	2	6	21	26		9	29	27	1	31	17	9	2	13	15	28		8	3	5
Legionella pneumophila			3	1			1																			2		
Pseudomonas aeruginosa						4				2				1				4										1
Rickettsia rickettsii			5	1																								
Salmonella enterica	4	3		2	2	3		3				1	1	1		2		4				2	3	6	3	1	3	3
Serratia marcescens		3	3	1		1	6	1			1	7		5	37	23		4	2	4		3	8	8				4
Shigella sp	2																4				1							
Shigella boydii												1																
Shigella sonnei											1	5							1	5		1		1				
Staphylococcus aureus		1								61				3														
Streptococcus pyogenes		27																								1		
Vibrio cholerae																										1		
Yersinia enterocolitica																			3									4

[a] Major pathogens of interest are shown in bold for emphasis. The number in each box represents the number of sequences detected for each organism. The squares highlighted in yellow represent sequences confirmed to greater than 97% of sequence similarity.

Comparison of Pyrosequencing and Culture-based Analyses of Sewage Sludge Samples

The organisms quantified by culture-based assays included anaerobic heterotrophs, aerobic spores, sulfite-reducing Clostridia, enterococci, *Shigella* spp., total coliforms, fecal coliforms, *Escherichia coli*, and *Salmonella* spp. The detection limits for each of these assays are provided in Appendix C. To compare pyrosequencing data to bacterial groups such as aerobic spores and fecal coliforms, certain assumptions were made. Anaerobic heterotrophs were defined as any organism that was either a facultative or obligate anaerobe—which was the vast majority of organisms detected in the samples. Sulfite-reducing clostridia were defined as *C. perfringens* and aerobic spores were defined as organisms belonging to the genus, *Bacillus.* Total coliforms were defined as organisms within the Enterobacteriacae family and fecal coliforms were defined as organisms belonging to the *Escherichia, Enterobacter, Klebsiella,* or *Citrobacter* genera.

The selected bacterial organisms were isolated from most of the samples by culture, while fewer were detected by pyrosequencing (Table 14). The three groups of organisms that were detected in every sample by both culture and pyrosequencing were anaerobic heterotrophs, total coliforms, and fecal coliforms. The levels of these organisms ranged from 10^6 to 10^{11} CFU/dry gram. Organisms detected consistently by culture, but sporadically by pyrosequencing included aerobic spores (detected by pyrosequencing in approximately ~29% of samples), enterococci (~54%), *Shigella* spp. (~32%), *E. coli* (~42%), and *Salmonella* spp. (~64%). In three of the samples, *Shigella* spp. were detected by pyrosequencing even though no organisms were isolated by culture. Interestingly, sequences identified as *E. coli* were only detected in the two summer/fall samplings. Isolation of *Salmonella* spp. using the MPN method suggested that fairly low levels of the pathogen existed in the sewage sludge. However, the organisms were

TABLE 14. Detection of selected organisms by culture-based methods and pyrosequencing[a]

	log$_{10}$ (CFU/dry g)								
	Anaerobic Heterotrophs	Aerobic Spores	Sulfite-reducing Clostridia	Enterococci	*Shigella* spp.$^{\alpha}$	Total Coliforms	Fecal Coliforms	Generic E. coli	*Salmonella* spp.
Ch-1	10.74	6.39	6.47	7.04	BD	8.70	7.98	7.98	1.11
Ch-2	10.94	7.34	7.10	6.63	2.66	9.02	8.15	8.15	1.72
Ch-3	9.48	5.60	6.42	6.37	BD	7.90	7.52	7.52	1.03
Ch-4	9.97	8.43	6.41	6.59	1.64	8.31	7.03	6.85	2.30
Cin-1	10.46	6.03	6.25	6.39	1.63	8.60	7.51	6.94	0.59
Cin-2	9.51	6.95	5.83	6.54	2.05	8.00	6.98	6.00	-0.02
Cin-3	9.94	5.72	5.95	6.39	1.19	8.57*	7.74	6.99	-0.51
Cin-4	9.71	6.80	6.23	5.99	1.57	7.74	6.61	6.19	-0.67
EP-1	10.19	6.47	6.38	6.45	1.38	7.36	6.81	6.70	1.67*
EP-2	10.07	6.23	6.44	6.42	1.25	7.50	6.62	6.62	0.45
EP-3	10.45	6.93	6.70	6.55	1.04	7.87	6.87	6.60	BD
EP-4	11.01	5.73	6.15	6.52	BD	8.02	6.98	6.98	1.49*
C-1	10.90	8.80	5.89	6.43	2.97	8.34	7.86	7.86	1.12
C-2	10.70	6.74	6.52	7.08	2.19	8.70	7.86	7.66	0.30
C-3	10.35	6.83	6.42	6.09	2.19	8.84	6.78	6.26	BD
C-4	10.81	5.73	6.30	5.22	1.28	8.62	7.07	6.52	0.84
SD-1	9.69	6.54	5.91	6.21	1.33	8.43	7.16	7.16	0.90
SD-2	10.01	6.50	6.02	6.70	2.30	7.91	7.61	7.61	1.67
SD-3	10.39	6.34	6.35	4.69	2.70	8.35	7.50	7.50	1.60*
SD-4	10.08	5.94	6.15	4.13	0.55	8.02	7.67	7.67	1.49*
DC-2	10.39	8.56	6.13	4.29	1.86	8.87	8.03	7.58	1.92
DC-2	10.36	7.05	6.35	4.27	1.33	8.43	6.12	6.12	0.83
DC-3	10.44	5.78	6.01	5.58	0.71	8.00	7.15	6.97	1.66*
DC-4	10.16	5.85	6.04	4.61	BD	8.59*	7.28	6.62	1.59*
M-1	10.78	7.70	5.95	6.71	1.85	8.62*	7.11	7.11	1.62
M-2	10.05	7.17	5.80	6.27	1.40	8.09	7.25	7.25	1.56*
M-3	10.02	7.01	5.62	5.69	1.35	8.35	7.19	7.05	-0.12
M-4	9.99	7.07	5.60	5.87	1.41	8.18	6.96	6.83	0.01

* = Maximum Level Detectable, $^{\alpha}$ = Presumptive, BD = Below Detection Limits

[a] Bacterial loads determined by culture-based methods are shown in each box. Boxes that are highlighted in orange represent detection of the organism within the sample by pyrosequencing, while boxes that are not highlighted represent no detection of the organism.

detected fairly consistently (~64%) using pyrosequencing. Sulfite-reducing clostridia, or presumptive *Clostridium perfringens*, were detected in considerable levels by culture, ranging from 10^5 to 10^6 spores per dry gram, but were not detected in any of the samples analyzed by deep-sequencing.

CHAPTER VI
DISCUSSION

Raw Milk and Pasteurized Milk: Diversity, Spoilage, and Pathogens

Bacterial Load and Diversity of Raw and Pasteurized Milk

The aerobic and anaerobic plate counts performed in this study demonstrated that the raw milk samples had a considerably higher bacterial load than the pasteurized milk samples. In fact, two of the raw milk samples collected in this study exceeded the microbiological standards required for raw milk intended for pasteurization to be labeled "Grade A Pasteurized Milk" (54). However, the exception to this generalization was Raw Sample #8, which showed much lower sequence numbers, bacterial loads, and pathogen prevalence as compared to the other raw milk samples. This may be due to the fact that this sample was collected from a larger dairy operation that sold both raw and pasteurized milk, whereas the other four samples were acquired from small family farms. Such a difference could be due to different hygienic practices and different conditions during milking, processing, and storage (45).

Each raw and pasteurized milk sample contained a unique phylogenetic profile and distribution of bacterial organisms, suggesting that the organisms present in each sample were influenced by environmental contamination or handling. It is likely that the detected microflora were representative of each milking and processing environment, which emphasizes the potential impact of environmental conditions on bacterial load. However, according to these results, the notion that raw milk is dominated by gram-positive bacteria and pasteurized milk by gram-negative bacteria seems to be false (48, 54). Both the raw and pasteurized milk samples in this study were dominated by gram-positive organisms. The pasteurized milk samples actually contained a higher proportion

of gram positive organisms than the raw milk samples. Additionally, the statistical comparisons of the milk populations showed that the raw milk samples had more significantly different samples at the genus, family, and order-levels than the pasteurized milk samples, indicating that raw milk samples are inhabited by more diverse and distinct bacterial populations than pasteurized milk samples.

The results of this study showed that there was no significant difference between the prevalence of selected lactic acid bacteria (*Streptococcus* spp., *Lactococcus* spp., *Lactobacillus* spp., and *Leuconostoc* spp.) in raw and pasteurized milk. Additionally, there is little evidence to support the idea that lactic acid bacteria are consistently major populations in raw milk (45, 54). Raw milk samples did have a higher prevalence of bacteria such as *Lactobacillus* spp. and *Lactococcus* spp., but the levels of such bacteria in the raw milk samples varied widely— ranging from greater than 20% in one sample to less than 1% in another. This gives support to the idea that lactic acid bacteria in raw milk are mainly the result of environmental contamination, as normal flora would be expected to be present in more consistent levels. Lactic acid bacteria were not generally major populations within spoiled milk samples, except for those that had been irradiated—indicating that such organisms had difficulty competing with other bacteria under normal circumstances. It is possible that past studies of raw milk have overestimated the populations of lactic acid bacteria as a result of their relative ease of culture.

Indicator and Pathogenic Organisms Within Raw and Pasteurized Milk Samples

The detection of some organisms classically considered to be indicators of fecal contamination in both raw and pasteurized milk samples, such as coliforms and enterococci, raises certain questions. However, other studies have provided evidence to suggest that bovine feces is not a primary source of indicators in raw milk and that there are likely other environmental sources of contamination (32, 42).

Raw milk was shown to contain significantly more of the selected pathogenic organisms than pasteurized milk, in which there was almost total absence of any known "pathogen". Raw milk had a high prevalence of certain opportunistic pathogens and emerging infectious organisms, such as *Acinetobacter baumannii*, *Aeromonas hydrophila*, *Serratia marcescans*, and *Staphylococcus aureus*. Some of these organisms made up large portions of all sequences detected in a sample. The low levels of *Salmonella*, *Campylobacter*, and *Shigella* spp. sequences detected indicates that these pathogens were a minority of the overall raw milk microflora, although such small sequence numbers could translate to hundreds of organisms when converted into equivalent milk volume. Additionally, the infectious doses for some of these organisms can be extremely low (41, 81). The detection of *Salmonella* spp. is of concern because *Salmonella* grows very well in milk and can survive in cultured milk products (65). Some of the same pathogens were detected in the spoiled milk samples, but generally at much lower levels—supporting the idea that many pathogenic organisms are not capable of competition with other flora at low temperatures (82). It is unknown why the organism *Rickettsia rickettsii* was found in significant numbers only in raw milk samples that had been boiled and stored. Overall, the detection of pathogens in raw milk was not very surprising, as one survey of raw bulk tank milk from 248 different producers found that 13% contained at least one bacterial pathogen (41). The results of this study suggest that raw milk can be a source of pathogen exposure for consumers. This exposure may pose a serious health risk, especially to individuals that are immunocompromised.

Spoilage Microflora of Processed and Unprocessed Milk

In this study, refrigerated storage prompted dramatic changes in bacterial flora, which has also been shown in previous studies (45). Spoilage of the milk samples was generally characterized by higher plate counts and the dominance of a small number of genera. The most common organisms found after spoilage in the pasteurized milk samples were *Paenibacillus spp.*, which are gram-positive bacteria. These results suggest

that gram-positive organisms may play a bigger role in the spoilage of pasteurized milk than has previously been suspected (54, 82, 90). The fact that no common bacterial genus or species was found in all of the spoiled milk samples, raw or pasteurized, indicates that spoilage is likely dependant on initial conditions and handling. This study provided evidence that raw milk appears to have a higher predominance of anaerobic organisms after spoilage than pasteurized milk. Additionally, several of the pasteurized milk samples were dominated by *Janthinobacterium* spp. and *Paenibacillus* spp. during spoilage, which are not organisms generally associated with milk spoilage in the literature (39, 45, 54, 55, 82). However, many of the *Pseudomonas* species detected in this study have been commonly associated with spoilage of raw and pasteurized milk (45, 58).

The raw irradiated milk samples seemed to have a different selection, distribution, and number of spoilage organisms than the other spoiled samples. This was an intriguing result, suggesting that the irradiation process somehow altered the bacterial competition within the milk during spoilage. However, this difference was not statistically significant, most likely because bacterial diversity in the spoiled samples was too low to achieve statistical power. It is possible that the organisms common to these irradiated samples, such as *Enterococcus* spp. and lactic acid bacteria, demonstrated some resistance to the irradiation process and provided these organisms with a competitive advantage during spoilage. The detection of microbial DNA in samples after an essentially sterilizing treatment emphasizes the resiliency of DNA and the limitation of molecular methods, in that the presence of DNA does not necessarily represent viable bacteria.

Quorum sensing is the coordination of gene expression in bacterial communities through the production and response to specific low-molecular-weight signaling molecules known as autoinducers. Autoinducer-2 (AI-2) has been shown to influence gene expression in both gram-positive and gram-negative organisms. It is thought that AI-2

may serve as a kind of universal signal for interspecies and intraspecies communications among bacteria (51). The detection of AI-2-like activity in several of the spoiled samples indicated that organisms participating in the raw milk spoilage were producing AI-2-like cell-cell signaling molecules. These results coincide with precious studies that also found autoinducer activity in milk and mik-based products (2, 51, 64). Autoinducer activity has also been associated with milk spoilage by *Serratia proteomaculans*, which was a major organism detected in the spoiled milk samples exhibiting AI-2-like activity in this study (2).

Discussion

Overall, there did appear to be significant differences between the raw and pasteurized milk samples collected in this study. This difference could be the result of the different environments in which milking took place and the hygienic standards with which the milk was subsequently handled. The exposure of milk to the environment and, thereby, possible microbial contaminants could depend upon a huge number of variables, including handling, equipment, hygienic practices, number of employees, size of operation, and climate. This study highlights the importance of not generalizing raw milk microbial populations, as each raw milk sample was found to possess a very unique microbiological profile. Additionally, this study provided evidence against some of the more popular assumptions in milk microbiology, such as the fact that pasteurized milk is dominated by gram-negative organisms and that refrigerated spoilage is almost always caused by gram-negative organisms (48, 54, 55, 82). This study also demonstrated how culture-based methods can complement molecular techniques, proving that organisms detected at the end of the storage period were viable in large numbers. Lastly, this study of milk microflora suggested that public health concerns regarding pathogens in raw milk are well-founded.

However, this study did have several limitations. This study simulated batch-pasteurization using a water bath. However, milk is also pasteurized by the high-temperature, short-time (HTST) method (54), which is a challenge to simulate in a research laboratory and hence was not performed in this study. This study provided some evidence for a difference in spoilage patterns between unprocessed and processed milk samples, although this difference was not statistically significant. More in-depth analysis is needed of how processing, especially irradiation, affects milk spoilage. Future milk spoilage studies using metagenomics could also provide a valuable perspective by taking aliquots of a sample at smaller time intervals in order to reveal more subtle, intermediate fluctuations in bacterial populations. Additionally, one of the raw milk samples had a much lower bacterial load than the others, which coincidentally was collected from a larger dairy operation instead of a small family farm. This indicates that the relationship between dairy size and bacterial load should be investigated further.

Overall, this study presented a novel view of the bacterial populations present in raw and pasteurized milk. Such data can be used to establish raw milk regulations and policy founded on empirical scientific evidence. However, this study is also important in that it emphasizes the vast bacterial diversity present in a commonly consumed food. Humans consume a huge amount and variety of foods during their lifetimes and there is a vast resource of literature that establishes the presence of significant populations of microbes within many of these foods. Much effort goes into preventing pathogens in food, but we consume vast numbers of organisms that are often ignored. Metagenomic analysis has been performed on human microflora, soil, and water samples, but food is considered a challenge because of the comparatively lower bacterial load and the presence of inhibitors such as fats and proteins that can interfere with molecular analysis. This study was important because it was the first performing an in-depth metagenomic analysis of a single food.

Untreated Sludge from American Municipalities: Diversity and Seasonal Dynamics

Bacterial Diversity of Untreated Sewage Sludge

Given the fecal make-up of sewage sludges, it is intriguing that the major phyla detected in the sewage sludge samples were Proteobacteria and Bacteroides, when the dominant phyla in the human gut are Bacteroides and Firmicutes (49). However, there is some evidence that the competitive fitness of human fecal bacteria is less than other, unidentified bacteria present in the treatment plant environment (73). It is unknown what factors were responsible for the differences in phylogenetic profiles between samples, especially in the El Paso samples, but possibilities include climate, input, and infrastructure of the sewage system and treatment plant. This study also presented evidence for seasonal differences in populations of Proteobacteria, Bacteroidetes, and Fusobacteria. It seems possible that temperature and climate may be a determining factor in the concentrations of Bacteroidetes in the samples. The cities of El Paso and San Diego have consistently warm climates and also showed comparatively higher and more prevalent year-round populations of *Bacteroides* spp. and *Parabacteroides* spp., which were more prevalent overall in the summer/fall samplings.

Bacterial populations of all sludge samples were significantly different at the genus-level, indicating immense diversity in all samples. Meaningful differences between sludge samples appeared in the comparisons at the family- and order-levels, suggesting that there are significantly different bacterial populations between municipalities. Additionally, some evidence of conserved differences between sludge samples across sampling sets indicates that these differences may be inherent to the location. Detailed analysis of the dominant genera found in the untreated sludge revealed that certain organisms were common across many of the samples. The high prevalence of genera such as *Arcobacter* spp. and *Aeromonas* spp. is of concern, given the status of these organisms as emerging foodborne pathogens (19, 47). In total, 22 different genera were

conserved across all sludge samples, indicating that they may comprise a "core" wastewater microbiota that could serve as a potential source of indicator organisms. Some of these genera, including *Clostridium* spp. and *Enterobacter* spp., are currently used as indicator organisms. However, the potential for other organisms conserved within these sludge samples should be further evaluated, as new indicator organisms are currently needed by the wastewater industry.

Pathogenic Organisms Present in Untreated Sewage Sludge Samples

The variety of pathogens present in the sewage sludge was surprising, including the etiological agents of cholera, brucellosis, Q fever, and Legionnaire's disease. Additionally, high levels of various opportunistic pathogens and emerging infectious pathogens were also detected. A relatively high incidence of *Salmonella* spp. has been found in past studies of raw sewage, which was generally confirmed by this study (72). *Salmonella* spp. were detected consistently by both culture-based and pyrosequencing methods in a majority of the sewage sludge samples. The results of this study also suggest that there is some evidence for different levels of pathogenic organisms between samplings/seasons. Untreated sludge would likely pose a health threat, given the number of pathogens detected. These results reiterate the need to dispose of such waste properly and prevent runoff into recreational areas and other areas with human activity. It is also important to evaluate the ability of these pathogens to survive wastewater treatment and improve risk assessment for land application of biosolids.

Comparison of Bacterial Detection by Pyrosequencing and Culture

This study was unique in that it compared results obtained by pyrosequencing to a culture-based analysis of bacterial indicators. There are many challenges with comparing the results of two methods as vastly different as culture and pyrosequencing. However, it is clear that the two methods are not equal and have distinct patterns of detection. The

high levels of total and fecal coliforms in both culture and pyrosequencing results were unsurprising given the fecal input. Complete lack of detection of *C. perfringens* by pyrosequencing suggests that many of the organisms detected in the TSC pour-plate method may have been other, less recognized, sulfite-reducing clostridia. A wide variety of other *Clostridium* species were detected in each of the raw sludge samples, lending support to this theory.

There does not appear to be any correlation between the detection of certain organisms based on culture and the incidence of detection by pyrosequencing. *Salmonella* spp. were detected fairly consistently by pyrosequencing at low concentrations, whereas other, more prevalent, organisms were not. However, pyrosequencing did detect *Shigella* spp. in several samples that were below detection limits by culture. It is also unknown why no sequences identified as *E. coli* were found in the two winter/spring samplings when high levels were detected by the culture-based assay. These discrepancies could be a result of the tiny amount (1uL) of DNA used for the pyrosequencing analysis, as compared to the rather large amount of sludge used for culture-based methods. Additionally, issues with amplification bias and database classification remain a possibility when working with next-generation sequencing technologies.

Discussion

The results of this study indicated that there were distinct differences in the microflora of sewage sludge sampled from different locations and provided some evidence for seasonal variations. It also captured a snapshot of the tremendous diversity of organisms present in untreated sludge. Several decisions were made for simplification purposes of such a vast amount of data, including not performing an in-depth examination of the different genera and species present in the samples. This could be corrected by future work and more extensive analysis. One important result of this study was that it demonstrated the vast, complicated environment that is commonly represented by a

handful of indicators such as enterococci and *Salmonella* spp.—organisms which make up only a minute portion of the actual microbial environment.

All in all, this study takes the first step towards a more in-depth microbiological understanding of sewage sludge. Previous studies have shown that microbes found in wastewater treatment are poorly characterized and underrepresented in databases (73). Molecular techniques are improving wastewater treatment by allowing the identification of organisms involved in the process. Identification of bacterial organisms with good metabolic potential can lead to the development of techniques for more efficient digestion and better knowledge of sewage ecology will allow for improved processes such as nitrification and phosphate removal (33, 75). Additionally, much of how the wastewater digestion process actually works is still unknown (75). This study helps address that knowledge gap by providing a better understanding of the bacterial communities feeding into the process. Future studies need to address existing communities in the digestor and how these communities mesh within the treatment plant.

One of the key questions prompted by this study is whether or not the information obtained from analyzing raw sludge reveals any valuable information about the original population. As a staggering number of factors could contribute, such as climate, treatment plant, and infrastructure environment, as well as commercial and industrial inputs, it is rather a stretch to make any assumptions at this point in time. However, while each of the raw sludge samples had many similar characteristics in terms of bacterial diversity, each sampling location appeared to have a distinct variation on the general profile and a different variety of dominant organisms, suggesting that certain unknown influencing factors remained constant for each location across time. These results suggest that this area deserves further exploration.

CHAPTER VII
CONCLUSIONS

These two projects were significantly different, but founded on exploration of deep-sequencing based technologies for characterization of specific environments important to human health. Much of the research in both of these fields tends to concentrate on "relevant" organisms—organisms responsible for disease, spoilage, or functional properties. However, this study provided a less-biased overview of the entire community of both environments and presented some intriguing results. Interestingly, the sequences detected in the milk samples, in general, appeared to be more easily classified into lower-level classifications such as 'genus' and 'species' than the sequences detected in the sewage samples, suggesting that bacteria in milk are generally better characterized than bacteria in sewage. Organisms detected in these samples were defined as 'pathogens' based on a review of scientific literature and available data from the Center for Disease Control. However, the term "pathogen" is subjective and there are many other organisms that could have potentially been included on this list.

One of the strengths of this study was the successful combination of molecular and culture-based approaches to build a comprehensive picture of the milk and sludge environments. One of the main drawbacks to such a molecular approach is that such methods only detect the DNA of bacteria in an environment—not viable, culturable cells. This is a legitimate concern and supports that notion that results found in such an approach should be explored further using other methods. Additionally, pyrosequencing analyses only 1µL of every sample—meaning that only a tiny fraction of each sample is actually characterized at a time. However, the mere existence of nonculturable organisms is a strong argument to why alternatives to strictly culture-based studies are integral to microbiology. Metagenomic and other molecular-based methods should be thought of as complementary to, not a replacement for, culture-based methods.

Building on these projects, an interesting next step would be a metagenomic study examining gene transcription and metabolic capabilities for these environments. Additionally, characterization is needed for many of the genera indentified in this study, as there is minimal information available regarding a large portion of them. Finally, this study only used deep sequencing to explore bacterial members of the milk and sewage microflora, but the microbial world is made up of a staggering diversity of viruses, phages, fungi, and protozoa. Such studies are needed to provide a comprehensive view of microbial ecology and fill in the gaps generated by current research.

Challenges for Metagenomics

Metagenomic and deep-sequencing technologies provide massive output, large coverage of target, speed, and ease-of-use, but the field has not been without growing pains. The terminology used for these assays needs to become more well-defined. Conserved protocols for procedures such as DNA extraction and data processing are essential to ensure that information can be compared across data sets and across platforms. Other needs include reproducibility, indicators for data quality that can be reported, and the ability to combine data obtained from different sequencing technologies (74).

Additionally, metagenomic analyses are only as good as the databases and computing tools on which they depend. Metagenomic studies and other next-generation-based research are filling databases with unclassified data from sequencing projects faster than researchers can process it (38, 73). Database creation and curation, as well as the archiving of data generated by new sequencing projects, needs to be a top priority (74). Computational technologies need to be developed at the same rate as metagenomic technologies to be able to analyze the massive quantities of data generated from high-throughput systems and extract useful information. Also needed are training programs that teach bioinformatic tools and allow researchers to make sense of the data that they are producing.

Final Thoughts

Results obtained from pyrosequencing and other metagenomic approaches should be thought of as an impetus, the jumping-off point for more detailed research. At this time, metagenomics can be compared to a picture taken of the earth from a satellite. Such a picture provides a broad view and may generate too much information to be processed fully. However, it can identify areas of curiosity and interest to be further investigated by a more in-depth exploration. As such satellite photos can reveal and explore areas inaccessible to exploration teams, so can metagenomic and molecular-based approaches explore areas currently inaccessible to traditional microbiology. It is clear that metagenomics is changing microbiology—marrying the field to genomics, computer science, and ecology and forcing researchers to develop expertise, albeit rudimentary, in all of these fields. Such approaches are forcing microbiologists to confront preconceived notions regarding bacterial virulence, classification, and distribution. Ultimately, the partnership of scientific knowledge and technology has reaching a turning point in which the amount of information that it would have previously taken a scientist their entire career to collect can now be obtained in a few days. The full implications of this potential on the field on environmental microbiology remain to be seen.

Despite the shortcomings of next-generation sequencing, a look back at the vast amount of knowledge gained from exploring this technology brings some perspective. Next-generation sequencing accomplished the sequencing of a human genome in two months and using less than a million dollars (69). Ambitious projects such as the Sargasso Sea study and Human Microbiome Project have provided invaluable knowledge about these respective environments. Project scopes cannot continue to expand without the utilization of such sequencing methods (74). As computing ability increases, whole-genome sequencing is most probably the future of this technology, although there will likely be need for 16S rRNA surveys in specific applications. Metabolomics approaches using next generation technologies, such as metaproteomics and metatranscriptomics,

have the potential to illuminate metabolic activity and functional capabilities of complex bacterial communities. Such studies will allow researchers to assign function to taxonomy and explore how bacterial communities respond to environmental changes (91). We are really only beginning to grasp the incredible microbial diversity present in the environment and this fact makes effective and efficient DNA sequencing one of the essential molecular technologies required for the future.

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APPENDIX A

TABLE A-1. Organisms defined as pathogens in this study

Organism	Significance
Acinetobacter baumannii	Multi-drug resistant strains estimated to account for 1.3% of nosocomial bloodstream infections (92)
Aeromonas hydrophila	Can cause gastroenteritis; some information suggesting foodborne pathogen (19, 59)
Brucella melitensis	Agent of brucellosis; Category B Bioterrorism Agent; Rare in the U.S, but estimated over half of a million cases worldwide annually (30)
Campylobacter jejuni	Agent of campylobacteriosis; common food-borne pathogen; estimated to affect over 2 million people in the U.S. every year (59)
Clostridium botulinum	Agent of botulism; generally less than 30 cases of foodborne illness and less than 100 cases of infant botulism reported annually (59)
Clostridium perfringens	Can cause soft-tissue infections and gastroenteritis (59)
Coxiella burnetti	Agent of Q fever; Category B Bioterrorism Agent; approximately 51 cases reported per year (53)
Enterobacter spp.	Responsible for estimated 3.9% of hospital-acquired bloodstream infections (92)
Enterococcus faecalis/faecium	Responsible for estimated 9.4% of hospital-acquired bloodstream infections; common in patients with neutropenia (92)
Klebsiella pneumoniae	Can cause community-acquired pneumonia, especially in the immunocompromised; can also cause wound and urinary tract infections (59)
Legionella pneumonphila	Agent of Legionnaire's disease; one of the three most common causes of severe pneumonia; 23,076 cases reported to the CDC from 1990 to 2005 (23, 60)
Pseudomonas aeruginosa	Responsible for estimated 4.3% of hospital-acquired bloodstream infections (92)
Rickettsia rickettsii	Agent of Rocky Mountain Spotted Fever; highly virulent tick-borne illness; 2092 cases reported in U.S. in 2006 (18)
Salmonella enterica	Agent of salmonellosis; common food-borne pathogen; estimated to cause more than 1.4 million infections and 600 deaths in the U.S. annually (59)
Serratia marcescens	Responsible for estimated 1.7% of hospital-acquired

	bloodstream infections (92)
Shigella spp.	Agent of shigellosis; common food- and water-borne pathogen; estimated that nearly 450,000 cases occur in the U.S. every year (59)
Staphylococcus aureus	Responsible for estimated 20.2% of hospital-acquired bloodstream infections (92)
Streptococcus pyogenes	Common agent of pharyngitis, but can also cause bacteremia, streptococcal toxic shock syndrome, and necrotizing fasciitis; approximately 4500 cases of invasive disease were reported in the U.S. in 2004 (59)
Vibrio cholera	Agent of cholera; common in countries and areas with poor sanitation (59)
Yersinia enterocolitica	Primarily enteric pathogen; one infection reported yearly per 100,000 people in the U.S. (59)

APPENDIX B

TABLE B-1. Bacterial load detected in irradiated milk samples after refrigerated storage

Pasteurized	Irr 1	Irr 2	Irr 3	Irr 4	Irr 5
Aerobic Load[a]	2.0	2.0	$2.7x10^8$	0.0	0.0
Anaerobic Load	0.0	0.0	16.0	0.0	0.0
Sequences[b]	343	354	14653	392	1918
Raw	**Irr 6**	**Irr 7**	**Irr 8**	**Irr 9**	**Irr 10**
Aerobic Load	$1.0x10^6$	$2.9x10^6$	$3.0x10^5$	$4.9x10^6$	$3.5x10^8$
Anaerobic Load	$8.3x10^4$	0.0	$1.9x10^5$	$4.1x10^6$	$8.8x10^7$
Sequences	14174	5278	6715	6797	4535

[a] Bacterial load in CFU/mL
[b] Represents number of sequences detected in each sample by pyrosequencing

TABLE B-2. Bacterial load detected in lab-pasteurized milk samples after refrigerated storage

Raw	Past 6	Past 7	Past 8	Past 9	Past 10
Aerobic Load[a]	$1.4x10^8$	6.0	2.0	$7.2x10^7$	$3.3x10^8$
Anaerobic Load	$3.7x10^6$	0.0	0.0	$4.9x10^6$	$7.0x10^8$
Sequences[b]	14151	1449	383	11116	8217

[a] Bacterial load in CFU/mL
[b] Represents number of sequences detected in each sample by pyrosequencing

TABLE B-3. Bacterial load detected in boiled milk samples after refrigerated storage

Pasteurized	**Boil 1**	**Boil 2**	**Boil 3**	**Boil 4**	**Boil 5**
Aerobic Load[a]	2.0	0.0	1.0	0.0	0.0
Anaerobic Load	0.0	0.0	0.0	0.0	0.0
Sequences[b]	0	0	431	212	0
Raw	**Boil 6**	**Boil 7**	**Boil 8**	**Boil 9**	**Boil 10**
Aerobic Load	0.0	0.0	0.0	0.0	0.0
Anaerobic Load	0.0	0.0	0.0	0.0	0.0
Sequences	3872	291	186	7099	3187

[a] Bacterial load in CFU/mL

[b] Represents number of sequences detected in each sample by pyrosequencing

APPENDIX C

TABLE C-1. Measured pH of sewage sludge samples

	Set 1	**Set 2**	**Set 3**	**Set 4**
Chicago, IL	6.30	5.65	6.12	6.51
Cincinatti, OH	5.90	5.75	5.99	6.05
Columbus, GA	6.54	5.43	5.80	5.91
El Paso, TX	5.50	6.50	5.30	5.75
Madison, WI	6.12	5.52	6.05	6.15
San Diego, CA	5.86	5.35	5.86	5.60
Washington D.C.	6.49	5.75	6.12	6.05

TABLE C-2. Measured percent solids of sewage sludge samples

	Set 1	**Set 2**	**Set 3**	**Set 4**
Chicago, IL	1.82%	0.23%	0.99%	0.46%
Cincinatti, OH	4.01%	3.44%	4.37%	3.17%
Columbus, GA	0.11%	1.09%	1.33%	3.89%
El Paso, TX	3.42%	4.12%	3.26%	5.22%
Madison, WI	3.83%	4.42%	4.11%	3.59%
San Diego, CA	3.42%	3.45%	4.07%	5.13%
Washington D.C.	0.74%	3.42%	3.51%	4.11%

TABLE C-3. Detection limits of culture-based assays

	Highest % Solids[a]	**Lowest % Solids[a]**
Anaerobic Heterotrophs	<2.28	<3.96
Aerobic Spores	<2.28	<3.96
Sulfite-reducing clostridia	<1.28	<2.96
Enterococci	<1.28	<2.96
***Shigella* spp.**	<0.54	<2.21
Total Coliforms	<0.54	<2.21
Fecal Coliforms	<0.54	<2.21
Generic *E. coli*	<0.54	<2.21
***Salmonella* spp.**	<(-0.91)	<0.77

[a] Detection limits in $\log_{10}$CFU/dry g of sewage sludge

APPENDIX D

TABLE D-1. Genera detected in sewage sampling 1

SEWAGE SAMPLING 1							
NAME	Chi-1	Cin-1	EP-1	Col-1	SD-1	WDC-1	Mad-1
Abiotrophia	9	0	0	0	34	0	0
Acetanaerobacterium	0	0	0	0	0	0	1
Acetivibrio	9	7	43	2	1	0	3
Acetobacterium	0	2	4	0	0	0	0
Acetonema	0	1	0	0	0	0	0
Achromobacter	98	715	94	147	137	66	165
Acidaminobacter	2	0	0	0	0	0	0
Acidaminococcus	0	2	73	3	15	0	8
Acidimicrobium	0	1	2	1	0	0	0
Acidiphilium	1	1	0	0	0	0	0
Acidisphaera	1	0	0	0	0	0	0
Acidovorax	78	745	198	280	258	187	222
Acinetobacter	0	3	0	0	0	0	0
Acrocarpospora	0	0	0	0	0	0	2
Actinobacillus	0	1	23	0	25	0	3
Actinobaculum	0	1	0	0	2	0	0
Actinomyces	0	0	0	0	0	0	1
Aeromonas	146	49	35	111	198	116	180
Aggregatibacter	0	0	2	0	3	0	0
Akkermansia	0	0	0	3	0	0	0
Alcanivorax	1	0	0	0	0	0	0
Alicycliphilus	6	10	0	7	11	1	3
Alishewanella	0	0	0	0	0	1	0
Alistipes	4	2	21	4	5	4	0
Alkaliflexus	11	15	5	2	8	2	26
Alkaliphilus	0	0	0	0	0	1	0
Alkanindiges	0	0	0	0	0	1	1
Allobaculum	1	0	0	1	0	0	0
Aminobacterium	2	0	5	5	3	0	3
Aminomonas	0	1	12	2	2	0	6
Anabaena	0	0	0	1	0	0	0
Anaerobacter	0	0	0	1	0	0	0
Anaerofilum	1	1	8	0	0	0	0
Anaerofustis	2	2	0	0	0	0	0
Anaeromyxobacter	0	0	0	1	0	0	0
Anaerophaga	0	1	0	3	0	0	3
Anaeroplasma	0	0	0	0	0	1	0

Anaerosinus	2	0	4	4	1	1	2
Anaerospora	0	0	1	0	0	0	0
Anaerostipes	0	0	0	0	0	0	1
Anaerotruncus	2	0	4	0	1	0	1
Anaerovorax	9	4	4	1	1	0	3
Antarctic	0	3	0	0	0	0	0
Aquabacterium	6	3	0	20	2	1	4
Aquaspirillum	0	0	0	2	0	0	0
Aquimonas	2	0	0	0	1	0	0
Aquitalea	3	5	2	4	5	1	0
Aranicola	1	0	0	0	0	0	0
Arcobacter	198	519	60	168	395	416	611
Arthrobacter	0	2	2	1	0	0	0
Asteroleplasma	1	1	12	0	5	0	0
Azoarcus	2	1	1	2	3	0	3
Azonexus	0	4	8	18	14	7	3
Azospira	1	0	1	3	7	0	0
Azospirillum	0	0	0	1	1	0	0
Azovibrio	0	0	1	0	0	0	1
Bacillus	6	1	0	12	0	1	1
Bacteriovorax	2	0	0	5	0	0	0
Bacteroides	334	116	166	78	314	93	291
Beggiatoa	3	0	0	0	0	0	0
Beijerinckia	1	0	0	0	0	0	0
Bosea	0	0	0	0	0	1	0
Brachybacterium	1	0	0	0	0	0	0
Brachymonas	2	3	6	5	1	5	2
Bradyrhizobium	0	0	0	1	1	0	1
Brevundimonas	0	0	1	0	0	0	2
Brochothrix	0	1	0	0	0	0	0
Brooklawnia	1	0	0	0	0	0	0
Bulleidia	1	1	2	0	0	0	0
Burkholderia	0	19	6	0	50	66	0
Buttiauxella	1	0	1	0	0	1	0
Butyrivibrio	2	6	177	0	38	0	2
Byssovorax	9	0	0	1	0	0	0
Caldilinea	6	0	3	1	1	0	0
Cand. Amoebinatus	0	0	0	0	0	0	1
Cand. Odyssella	0	2	0	0	0	8	0
Carnobacterium	0	1	0	0	0	0	0
Caryophanon	0	0	0	1	0	0	0
Catabacter	0	0	0	0	0	0	1
Catenibacterium	0	0	1	0	0	0	0
Cerasibacillus	0	0	0	2	0	0	0
Cetobacterium	2	1	0	0	1	0	4
Chelatococcus	0	0	1	0	0	0	0

Chitinibacter	0	0	0	0	0	0	1
Chitinimonas	2	0	0	1	0	0	0
Chitinophaga	0	0	0	0	3	0	0
Chromobacterium	1	0	0	2	0	0	0
Chryseobacterium	70	502	169	472	229	241	158
Citrobacter	4	2	2	5	6	4	9
Clostridium	62	25	13	125	7	14	17
Comamonas	7	74	68	23	146	19	61
Coprococcus	2	1	14	1	3	0	0
Coxiella	1	0	0	0	0	0	1
Cupriavidus	2	0	3	0	1	0	1
Curtobacterium	0	0	39	0	0	2	0
Curvibacter	10	0	14	2	3	1	4
Cytophaga	24	10	3	31	1	1	2
Dechloromonas	71	45	14	93	22	23	24
Delftia	0	3	0	0	0	0	0
Denitratisoma	4	0	0	0	0	0	0
Derxia	3	0	1	0	0	0	0
Desulfobacter	7	0	1	0	3	0	2
Desulfobulbus	21	13	23	17	13	12	12
Desulfomicrobium	0	0	2	0	0	1	0
Desulfonema	1	0	0	0	0	0	0
Desulforhopalus	0	1	0	0	0	0	0
Desulfovibrio	2	0	5	3	4	1	1
Dialister	0	0	2	1	0	1	0
Diaphorobacter	3	1	0	0	1	1	1
Dokdonella	1	0	3	0	1	0	3
Dorea	6	0	4	8	2	0	3
Dysgonomonas	1	2	1	2	11	5	4
Elizabethkingia	0	1	0	0	0	0	0
Empedobacter	0	1	0	0	0	0	4
Enhydrobacter	7	236	2	77	4	31	58
Enterobacter	14	5	3	33	9	10	7
Enterococcus	5	0	0	1	0	0	3
Erysipelothrix	9	2	1	1	0	1	4
Escherichia	4	0	2	1	8	0	3
Ethanoligenens	0	0	6	0	2	0	1
Eubacterium	8	2	10	2	8	2	5
Faecalibacterium	0	2	26	3	5	3	2
Ferribacterium	22	1	0	2	1	0	0
Fibrobacter	3	9	3	13	3	3	2
Finegoldia	0	1	0	0	0	0	0
Flavobacterium	18	87	3	24	4	3	16
Fluviicola	0	0	0	1	0	0	0
Formosa	0	0	0	1	0	0	0
Frigovirgula	4	2	1	0	3	2	19

Fusibacter	12	9	1	3	0	1	6
Fusobacterium	3	2	2	0	9	0	1
Garciella	0	0	0	0	0	1	0
Gemella	11	0	0	0	40	0	0
Gemmata	1	0	0	0	0	0	0
Gemmatimonas	1	0	0	0	0	0	0
Geobacter	1	0	0	1	0	0	4
Geothrix	7	5	1	12	1	2	0
Ginsengisolibacter	0	0	0	0	0	1	0
Gracilibacter	3	1	0	3	0	1	1
Granulicatella	0	0	1	0	0	0	0
Haemophilus	0	0	1	0	6	0	4
Haliangium	1	0	0	0	0	0	0
Haliscomenobacter	16	0	4	0	1	0	0
Halomonas	0	0	0	0	0	2	0
Halothiobacillus	0	0	2	0	0	0	0
Helicobacter	8	16	1	0	0	0	0
Herbaspirillum	2	0	1	0	0	0	1
Hespellia	0	0	1	0	0	0	0
Holophaga	4	0	0	2	1	0	0
Hydrocarboniphaga	2	1	0	0	0	0	0
Hydrogenophaga	13	43	4	12	3	1	3
Hyphomicrobium	0	5	1	1	1	0	1
Ilyobacter	4	1	0	0	0	0	11
Inquilinus	1	0	3	0	2	0	0
Isosphaera	1	0	0	0	0	0	0
Kaistella	1	7	0	0	0	0	4
Kingella	0	0	84	0	141	0	0
Klebsiella	15	4	22	6	13	10	4
Kluyvera	1	0	0	1	1	1	0
Labrys	0	2	0	0	0	0	1
Lachnobacterium	2	1	0	1	1	0	0
Lachnospira	0	1	0	0	0	0	1
Lactobacillus	9	0	2	1	4	1	2
Lactococcus	5	0	0	0	0	0	7
Legionella	1	0	0	0	0	1	0
Leptolinea	1	0	0	0	0	0	0
Leptothrix	3	22	1	56	0	0	1
Leptotrichia	9	64	1	9	9	25	163
Leucobacter	0	0	1	0	2	0	0
Levilinea	4	0	3	0	0	0	4
Luteococcus	1	0	0	0	0	0	0
Lysobacter	11	3	11	0	3	0	4
Magnetospirillum	0	3	0	0	1	1	1
Marinilabilia	2	1	0	3	0	5	3
Marinospirillum	0	2	0	0	0	0	0

Massilia	1	0	0	0	0	0	0
Megasphaera	0	0	0	0	1	0	0
Methylibium	6	0	0	0	0	0	0
Methylobacter	1	0	2	0	1	0	1
Methylobacterium	0	1	0	1	1	0	0
Methylocaldum	2	1	1	1	1	0	0
Methylococcus	0	0	3	1	0	0	0
Methylocystis	0	0	0	0	1	0	0
Methylophilus	0	0	0	2	0	0	1
Methylosarcina	0	1	0	0	0	0	0
Methylovorus	0	0	0	0	0	0	1
Microbulbifer	1	0	1	0	0	0	0
Microlunatus	0	0	0	0	0	1	0
Microscilla	0	0	0	0	1	0	0
Microvirgula	0	3	0	0	0	0	0
Mitsuaria	0	1	0	0	0	0	0
Mitsuokella	2	1	0	1	2	0	0
Mogibacterium	0	1	0	1	0	2	0
Moraxella	1	0	11	0	7	0	3
Moryella	0	0	1	0	0	0	0
Muricauda	3	0	0	1	0	0	0
Myceligenerans	0	0	1	0	0	0	0
Mycobacterium	0	0	0	0	0	2	0
Myroides	0	2	0	0	0	0	1
Neisseria	1	2	64	4	89	13	11
Niastella	0	0	0	0	0	0	1
Nitrobacter	0	1	0	0	0	1	0
Nitrosococcus	0	0	2	0	1	0	0
Nitrosomonas	3	0	1	1	0	0	0
Nitrospira	0	1	0	0	0	0	0
Nocardiopsis	0	0	0	0	0	5	0
Nonomuraea	0	0	0	0	0	0	1
Novosphingobium	1	0	1	0	0	0	2
Ochrobactrum	0	1	0	0	0	0	2
Oligotropha	0	0	1	0	0	0	0
Olsenella	0	0	0	0	1	0	0
Opitutus	47	8	8	45	4	11	8
Oribacterium	3	0	0	0	1	0	0
Ornithinicoccus	0	0	0	0	0	0	4
Oscillibacter	0	0	0	0	1	0	0
Oxalobacter	0	0	1	0	2	0	0
Paludibacter	236	57	57	167	21	34	80
Pantoea	6	3	2	5	8	12	3
Papillibacter	9	17	34	1	14	2	3
Parabacteroides	171	104	230	67	279	112	123
Paracoccus	0	2	0	4	0	1	3

Parapedobacter	0	0	0	0	0	0	1
Pasteurella	0	0	0	0	1	0	0
Paucisalibacillus	2	1	6	0	4	0	3
Pedobacter	0	1	0	15	0	3	0
Pedomicrobium	1	0	2	0	1	0	0
Pelobacter	0	0	0	0	1	0	2
Peptococcus	1	3	2	0	0	0	1
Peptoniphilus	0	0	0	0	0	0	1
Peptostreptococcus	0	0	1	0	1	0	1
Petrimonas	0	0	1	0	0	0	0
Phenylobacterium	0	1	0	0	1	2	0
Pirellula	2	0	2	0	0	0	3
Planctomyces	2	3	1	0	1	0	0
Planococcus	0	0	0	1	0	0	0
Pleomorphomonas	2	1	3	1	1	2	0
Plesiomonas	0	1	0	0	0	0	0
Polyangium	3	0	0	0	0	0	0
Polynucleobacter	6	0	0	3	2	3	3
Porphyromonas	0	4	10	0	0	0	27
Prevotella	73	153	172	27	123	8	45
Prolixibacter	1	0	0	2	1	0	0
Propionicicella	0	0	0	0	0	0	1
Propionicimonas	0	0	1	0	0	0	0
Propionigenium	11	6	1	1	13	4	24
Propionivibrio	151	87	43	127	85	95	70
Proteiniphilum	1	0	0	0	0	0	0
Proteus	0	0	0	0	0	1	0
Providencia	0	0	0	0	0	0	1
Pseudobutyrivibrio	1	3	2	0	2	0	0
Pseudochrobactrum	0	0	1	0	0	0	1
Pseudoclavibacter	0	0	0	0	0	0	1
Pseudomonas	0	1	2	0	0	0	0
Pseudoramibacter	1	0	3	0	0	0	0
Pseudoxanthomonas	5	1	2	1	4	1	3
Psychrobacter	13	11	0	0	1	0	3
Psychroserpens	2	2	0	2	0	1	0
Ralstonia	1	7	6	11	1	3	3
Ramlibacter	1	0	1	0	0	0	2
Raoultella	1	0	0	0	0	0	0
Rathayibacter	0	1	0	0	0	0	0
Rhabdochromatium	1	0	0	0	0	0	0
Rheinheimera	1	0	0	0	0	0	0
Rhizobium	2	1	4	11	3	3	0
Rhodobacter	3	4	3	3	6	3	3
Rhodocyclus	1	0	0	0	0	0	0
Rhodoferax	8	4	0	0	0	0	0

Rhodomicrobium	0	0	1	0	0	0	0
Rhodoplanes	1	0	0	0	0	0	0
Rhodopseudomonas	0	0	1	1	0	0	0
Rhodovibrio	0	0	0	0	1	0	0
Rikenella	49	17	15	13	16	3	9
Roseburia	14	16	10	1	6	5	14
Roseomonas	3	0	0	1	0	0	0
Rubrivivax	4	0	1	0	0	0	1
Ruminobacter	0	0	0	0	1	0	0
Ruminococcus	10	13	71	7	27	2	14
Saccharomonospora	0	0	0	1	0	0	0
Salmonella	4	2	0	1	0	0	3
Sarcina	1	0	0	0	0	0	0
Sebaldella	1	4	4	2	5	1	14
Sediminibacterium	1	0	0	0	0	0	0
Segetibacter	0	1	0	0	0	0	0
Sejongia	0	1	0	0	0	0	0
Selenomonas	0	0	0	0	1	0	0
Serratia	1	0	0	1	0	0	0
Shigella	2	0	0	0	4	1	0
Shinella	1	0	0	1	2	0	2
Simplicispira	3	11	5	2	7	1	2
Sinorhizobium	58	58	33	16	46	33	65
Smithella	0	0	0	0	0	0	1
Soehngenia	0	0	1	2	2	0	0
Sorangium	1	0	0	2	0	0	0
Sphingobacterium	7	1	0	2	0	4	2
Sphingomonas	1	1	0	24	4	0	0
Sphingopyxis	1	6	2	1	1	0	0
Sphingosinicella	0	1	0	0	0	0	0
Spirochaeta	7	0	2	3	0	0	0
Sporacetigenium	1	1	8	0	7	0	4
Sporanaerobacter	0	0	0	5	0	11	0
Sporobacter	1	3	6	2	17	1	2
Sporobacterium	9	4	154	13	29	2	3
Sporocytophaga	7	3	0	0	4	0	0
Sporotalea	0	0	0	0	0	0	1
Stella	0	0	3	0	0	0	0
Stenotrophomonas	2	4	3	5	3	3	5
Sterolibacterium	12	0	0	0	2	1	0
Stigonema	1	0	0	0	0	0	0
Streptococcus	71	37	93	75	47	55	97
Succiniclasticum	0	1	21	0	6	0	0
Succinivibrio	0	0	0	0	0	0	1
Sulfuricurvum	20	11	4	17	3	10	2
Sulfurospirillum	32	22	20	24	25	42	15

Sulfurovum	0	0	0	0	0	0	1
Sutterella	0	0	0	4	1	0	0
Syntrophococcus	0	1	0	0	0	0	0
Syntrophus	1	0	0	0	0	0	0
Tannerella	2	0	42	1	2	0	0
Terriglobus	0	0	0	0	0	0	1
Terrimonas	3	2	0	0	0	0	0
Tetrasphaera	0	0	0	1	0	0	0
Thauera	92	172	36	241	71	71	33
Thermanaerovibrio	1	2	2	3	4	0	1
Thermomonas	4	1	3	2	8	0	1
Thioalkalispira	0	1	1	0	1	0	0
Thiobacillus	0	3	0	3	3	1	1
Thiobacter	0	1	1	0	1	0	0
Thiohalocapsa	0	0	0	0	1	0	0
Thiomonas	0	0	2	0	1	1	0
Thiothrix	1	5	10	15	1	2	2
Tissierella	15	8	2	0	0	1	0
Tolumonas	244	108	73	142	40	90	71
Treponema	1	5	0	2	7	1	0
Trichlorobacter	1	0	0	0	1	1	1
Trichococcus	9	0	0	1	0	0	8
Turicibacter	0	0	0	2	0	0	0
Uruburuella	7	29	18	16	57	16	86
Veillonella	17	0	16	4	8	1	8
Verrucomicrobium	0	1	0	0	0	0	1
Victivallis	0	1	0	0	0	0	1
Virgibacillus	0	0	0	1	0	0	0
Vitreoscilla	2	39	0	3	12	18	21
Vogesella	1	0	0	2	0	0	0
Wautersiella	0	18	0	0	0	0	0
Wolinella	0	0	0	0	1	0	0
Yaniella	0	0	0	1	0	0	0
Yersinia	1	0	0	0	0	0	0
Zoogloea	222	164	25	310	69	72	48
TOTAL	**3266**	**4756**	**2954**	**3443**	**3529**	**2190**	**3205**

TABLE D-2. Genera detected in sewage sampling 2

SEWAGE SAMPLING 2							
name	Chi-2	Cin-2	EP-2	Col-2	SD-2	WDC-2	Mad-2
Acetanaerobacterium	1	0	13	1	0	0	3
Acetivibrio	1	0	0	0	0	0	0
Acetobacterium	0	0	2	0	0	0	1
Achromobacter	0	0	0	0	1	1	2
Acidaminobacter	1	0	0	0	0	0	0
Acidaminococcus	1	0	7	0	1	0	0
Acidiphilium	2	0	0	0	0	0	0
Acidisphaera	2	0	0	0	1	0	0
Acidithiobacillus	0	0	1	0	0	0	0
Acidovorax	126	945	116	495	538	917	365
Acinetobacter	138	1416	88	413	71	232	455
Actinobacillus	0	0	8	0	14	0	3
Aeromonas	452	274	70	199	518	433	400
Afipia	10	1	3	1	1	0	0
Aggregatibacter	0	0	1	0	4	0	0
Akkermansia	0	0	0	0	2	0	0
Algibacter	0	0	0	0	1	0	0
Alicycliphilus	0	5	0	1	3	12	7
Alishewanella	0	4	0	0	0	0	0
Alistipes	3	2	29	0	2	0	2
Alkaliflexus	5	1	0	4	0	2	2
Amaricoccus	4	0	2	0	1	0	1
Aminobacter	0	0	0	0	1	0	0
Aminobacterium	0	1	18	0	2	0	1
Anaeroarcus	3	1	0	0	1	2	0
Anaerococcus	0	1	0	34	8	1	2
Anaerofilum	2	3	18	0	5	5	7
Anaerolinea	1	1	7	0	2	0	3
Anaerosinus	3	0	2	4	17	13	3
Anaerostipes	1	0	0	0	0	0	0
Anaerotruncus	0	1	13	0	0	2	0
Anaerovorax	20	9	0	5	0	10	12
Ancylobacter	1	0	0	0	0	0	0
Angulomicrobium	0	0	0	0	0	0	1
Aquabacterium	0	6	0	43	0	0	1
Aquimonas	0	1	1	0	0	0	0
Aquitalea	3	0	0	1	0	0	0
Arcobacter	1237	474	106	149	112	602	1253
Asanoa	0	0	0	0	0	0	1
Asticcacaulis	1	0	0	0	0	0	0
Azoarcus	9	4	7	30	3	6	1
Azohydromonas	0	0	0	1	0	0	0

Azonexus	7	2	1	30	3	7	4
Azospira	1	0	0	0	0	0	0
Azospirillum	1	0	0	0	0	0	0
Azovibrio	4	2	1	3	0	1	2
Bacillus	0	0	1	0	1	0	0
Bacteriovorax	0	0	0	1	0	0	0
Bacteroides	515	122	216	102	880	381	344
Beijerinckia	1	1	2	2	0	2	0
Bergeriella	0	0	7	0	57	3	0
Bergeyella	0	0	0	0	0	0	2
Bibersteinia	0	0	1	0	2	1	5
Bifidobacterium	0	2	0	0	0	0	0
Bordetella	0	2	0	2	0	0	0
Bosea	1	5	0	1	4	1	2
Brachymonas	9	7	2	7	18	21	15
Bradyrhizobium	0	0	1	0	0	0	0
Brenneria	1	0	0	0	0	0	0
Brevundimonas	3	9	2	1	5	2	9
Brucella	4	1	0	0	0	1	2
Bulleidia	1	0	1	0	1	0	0
Butyrivibrio	0	0	18	1	2	1	0
Caloramator	2	0	0	0	0	0	0
Campylobacter	7	2	2	17	0	5	2
Cand. Alysiosphaera	0	0	1	0	0	0	0
Cand. Amoebinatus	0	0	0	2	0	3	4
Cand. Aquirestis	2	0	0	0	0	0	0
Cand. Blochmannia	6	1	1	1	5	6	5
Cand. Kuenenia	2	2	1	0	0	0	4
Cand. Monilibacter	4	2	8	4	3	0	6
Cand. Nitrotoga	2	0	0	0	0	0	0
Cand. Odyssella	0	0	4	1	0	0	0
Cand. Symbiothrix	1	0	0	0	0	0	0
Catenibacterium	2	0	0	0	1	0	0
Caulobacter	3	0	0	0	0	0	1
Cellulomonas	1	0	0	0	0	0	0
Cellvibrio	2	0	1	0	0	0	1
Cetobacterium	7	4	0	0	1	6	16
Chelatococcus	1	1	0	0	0	0	0
Chitinibacter	0	0	0	0	0	1	0
Chitinimonas	0	0	0	2	0	0	0
Chitinophaga	0	1	2	0	0	0	0
Chlorobium	1	0	0	0	0	0	0
Chromobacterium	3	1	0	2	0	0	0
Chryseobacterium	48	81	21	241	94	229	66
Citrobacter	10	7	0	3	15	10	4
Clostridium	67	75	355	15	25	20	40
Collinsella	0	0	1	0	0	0	0

Comamonas	13	79	20	26	278	125	79
Conchiformibius	0	0	21	1	221	2	0
Conexibacter	1	0	0	0	0	0	0
Corynebacterium	0	0	0	0	1	0	0
Coxiella	0	0	0	0	1	0	0
Cupriavidus	9	3	11	19	2	9	4
Curvibacter	18	6	3	7	6	4	3
Cytophaga	0	1	0	1	0	0	0
Dechloromonas	84	63	5	106	26	78	43
Delftia	1	5	1	3	1	2	2
Denitratisoma	1	2	0	0	2	0	1
Denitrovibrio	0	0	0	0	2	0	0
Desulfobacter	5	6	1	0	6	1	1
Desulfobulbus	41	16	42	7	13	29	21
Desulfococcus	0	0	1	0	0	0	0
Desulfomicrobium	2	2	1	1	2	0	1
Desulforegula	8	3	0	2	0	1	2
Desulfovibrio	6	2	4	3	4	6	1
Desulfurivibrio	0	4	0	0	0	0	0
Devosia	0	0	0	0	1	0	0
Dialister	0	0	0	0	1	0	2
Diaphorobacter	2	25	3	19	14	21	14
Dickeya	0	0	0	0	1	0	0
Dinoroseobacter	0	0	0	0	0	1	0
Dokdonella	10	0	1	1	1	0	2
Dysgonomonas	2	2	1	10	16	10	3
Elizabethkingia	1	5	0	0	1	0	0
Enhydrobacter	26	186	4	152	5	137	132
Ensifer	0	3	1	1	0	0	2
Enterobacter	21	5	7	20	57	33	6
Enterococcus	9	0	0	9	13	0	2
Epilithonimonas	0	0	0	0	0	0	1
Erysipelothrix	0	1	0	0	1	0	0
Escherichia	13	1	2	2	24	4	4
Ethanoligenens	1	0	7	0	0	1	1
Eubacterium	4	7	6	3	2	6	14
Faecalibacterium	5	7	9	1	16	10	20
Fervidobacterium	0	0	1	0	0	0	0
Fibrobacter	6	9	8	1	0	1	8
Finegoldia	0	0	1	43	0	0	7
Flavobacterium	26	79	2	35	0	15	30
Fluviicola	1	1	1	1	0	0	0
Formivibrio	6	17	0	27	1	6	5
Formosa	0	0	0	3	0	0	0
Friedmanniella	0	0	0	0	0	1	0
Fusibacter	11	6	0	0	0	2	5
Fusobacterium	3	1	0	0	2	1	94

Gallionella	0	1	0	0	0	0	0
Geobacter	12	0	0	0	3	0	3
Giesbergeria	0	0	0	1	0	0	1
Gracilibacter	0	0	0	1	0	0	1
Granulicatella	0	0	0	0	0	0	1
Haematobacter	1	2	1	7	9	4	0
Haemophilus	0	0	5	0	24	0	0
Hahella	1	0	0	0	0	1	0
Haliscomenobacter	3	0	0	0	0	0	0
Halothiobacillus	0	0	2	0	1	0	0
Herbaspirillum	2	3	2	1	5	6	7
Hoeflea	0	0	0	0	0	0	1
Hydrogenophaga	22	21	0	5	0	8	6
Hyphomicrobium	7	2	1	0	3	0	1
Ideonella	0	0	1	1	0	0	1
Ignatzschineria	0	0	1	0	0	0	0
Ilyobacter	72	5	0	0	0	0	8
Inquilinus	2	0	0	0	0	0	0
Iodobacter	0	0	0	1	0	0	0
Janthinobacterium	0	1	0	0	0	0	0
Kaistia	0	0	0	0	1	0	0
Kaistina	0	0	1	1	2	5	0
Kineococcus	0	0	0	1	0	0	0
Kingella	0	1	0	0	0	0	1
Klebsiella	25	8	6	11	34	17	12
Kluyvera	0	0	0	1	3	3	0
Kozakia	0	0	0	2	0	0	0
Labrys	0	2	0	0	0	0	0
Lachnobacterium	1	0	3	0	0	0	0
Lachnospira	0	0	1	0	0	0	0
Lactobacillus	2	0	0	0	0	0	0
Lactococcus	40	0	0	2	0	0	7
Lamprocystis	0	0	0	0	1	0	0
Laribacter	6	3	3	4	53	31	26
Leadbetterella	0	0	0	0	0	0	1
Legionella	0	0	0	0	0	0	3
Leisingera	0	0	0	0	1	0	0
Leptonema	0	0	1	0	0	0	0
Leptothrix	0	2	0	9	0	1	0
Leptotrichia	75	42	4	29	13	256	247
Leucobacter	0	1	0	0	0	0	0
Leuconostoc	0	1	0	0	0	0	1
Luteimonas	0	3	0	0	0	1	0
Luteococcus	0	1	0	0	0	0	0
Lysobacter	0	0	0	0	1	0	0
Magnetospirillum	2	0	0	0	0	0	1
Mahella	0	1	3	0	0	0	0

Malikia	7	8	1	11	6	9	3
Mannheimia	0	0	2	0	16	0	0
Maricaulis	0	1	2	1	0	0	0
Marinilabilia	2	2	0	3	0	5	7
Marinobacter	0	1	0	0	0	0	0
Massilia	2	1	0	2	1	0	0
Megasphaera	0	1	3	0	0	1	0
Merismopedia	0	0	0	0	0	0	1
Mesorhizobium	0	1	0	1	0	0	0
Methylibium	5	4	1	2	0	2	6
Methylobacillus	1	4	0	1	0	0	0
Methylobacterium	0	1	1	0	0	0	0
Methylocaldum	0	2	1	0	4	3	1
Methylocapsa	0	0	1	0	0	0	0
Methylococcus	0	0	0	3	0	0	0
Methylomicrobium	3	0	8	2	1	0	0
Methylomonas	1	0	0	0	1	0	0
Methylophilus	0	1	0	2	0	0	0
Methylopila	0	0	1	0	0	0	0
Microbacterium	0	1	0	0	0	0	0
Microbispora	1	0	0	0	0	0	0
Microvirga	0	0	3	0	3	0	1
Microvirgula	12	13	0	22	1	4	2
Mitsuaria	0	3	0	0	0	1	0
Mitsuokella	2	0	0	0	1	1	1
Moraxella	0	1	16	0	16	0	0
Morganella	1	1	0	4	13	7	1
Muricauda	7	0	0	3	0	0	2
Myceligenerans	0	0	2	0	1	3	3
Mycobacterium	1	1	1	0	0	4	0
Nakamurella	0	1	0	0	0	0	0
Naxibacter	0	2	0	0	0	0	0
Neisseria	6	21	6	5	60	57	21
Nitrosomonas	2	3	0	9	0	1	1
Nitrosospira	3	0	0	0	0	0	0
Nitrospira	7	0	0	1	0	0	0
Nostocoida type II	1	0	1	0	1	0	0
Novosphingobium	2	6	3	2	3	4	1
Ochrobactrum	0	4	5	2	1	0	2
Opitutus	17	8	7	10	4	6	7
Oxalobacter	1	0	0	0	0	0	0
Paludibacter	193	86	63	149	9	52	117
Pandoraea	0	0	0	1	0	0	0
Pannonibacter	0	1	3	0	3	1	0
Papillibacter	0	0	69	0	0	0	0
Parabacteroides	259	90	175	122	286	364	221
Paracoccus	3	52	3	17	43	26	33

Parasporobacterium	0	1	0	0	0	0	0
Parvibaculum	0	1	0	1	0	0	0
Pasteurella	0	0	2	0	4	0	0
Pectinatus	0	0	0	0	0	1	0
Pectobacterium	0	2	1	1	1	1	1
Pedobacter	1	0	1	2	0	0	0
Pedomicrobium	0	0	1	0	0	0	0
Pelobacter	5	0	0	1	0	1	5
Pelomonas	2	2	0	4	0	1	0
Peptoniphilus	0	0	0	0	0	0	1
Peredibacter	0	1	0	0	0	0	0
Petrimonas	4	0	85	1	0	0	0
Petrobacter	3	0	0	0	0	2	0
Phascolarctobacterium	1	2	8	2	2	4	4
Phenylobacterium	3	1	3	2	5	1	3
Phyllobacterium	0	2	0	2	0	0	1
Pirellula	8	0	1	0	1	0	0
Planctomyces	1	0	1	0	1	0	0
Pleomorphomonas	2	3	5	3	2	7	4
Plesiomonas	0	1	0	0	1	1	0
Polynucleobacter	0	0	0	0	0	1	0
Porphyromonas	0	0	0	1	0	0	1
Pragia	1	0	1	1	0	2	0
Prevotella	73	37	268	22	69	56	60
Propionigenium	23	3	2	0	0	0	13
Propionispira	0	0	0	0	0	3	0
Propionispora	1	0	0	0	0	0	0
Propionivibrio	123	83	41	105	99	179	143
Proteiniphilum	4	0	2	4	1	1	3
Providencia	0	1	0	1	0	0	0
Pseudaminobacter	0	1	2	0	0	0	0
Pseudochrobactrum	0	0	0	0	0	1	0
Pseudomonas	30	137	30	36	41	69	81
Pseudoxanthomonas	9	7	5	3	7	6	4
Psychrobacter	0	0	1	0	0	0	4
Ralstonia	0	6	0	9	0	18	2
Ramlibacter	0	1	0	2	2	0	3
Raoultella	5	2	0	2	3	10	1
Rathayibacter	0	1	0	0	0	0	0
Rhizobium	5	28	1	10	7	16	5
Rhodobacter	5	7	8	5	5	6	6
Rhodocyclus	0	1	0	1	3	3	5
Rhodoferax	2	1	0	0	0	2	2
Rhodoplanes	1	0	1	0	0	0	0
Rhodopseudomonas	8	2	7	2	1	2	1
Rhodospirillum	1	0	0	0	0	0	0
Rhodovibrio	0	0	0	0	0	0	1

Rhodovulum	1	0	0	0	0	0	0
Riemerella	93	171	38	386	402	485	126
Roseateles	0	0	0	1	1	0	0
Roseburia	3	1	9	0	4	2	7
Roseiflexus	3	0	0	0	0	0	0
Roseococcus	0	0	0	1	0	0	0
Roseomonas	2	2	1	0	1	0	3
Roseospira	1	0	0	0	0	0	0
Rubrivivax	0	1	1	1	0	0	0
Ruminococcus	4	4	31	4	3	2	11
Runella	4	0	0	0	0	0	0
Salmonella	3	3	0	1	4	2	1
Samsonia	8	1	0	1	3	2	0
Sanguibacter	1	0	0	0	0	0	0
Sarcina	1	0	0	0	0	0	0
Schlegelella	0	0	1	0	0	0	0
Sebaldella	5	7	8	4	32	25	39
Sedimentibacter	4	0	1	0	0	1	0
Segetibacter	4	0	0	0	0	0	0
Selenomonas	6	0	1	1	8	1	0
Serratia	3	1	0	5	4	3	1
Shewanella	137	44	0	63	1	31	37
Shigella	0	0	0	0	0	1	0
Shinella	1	5	1	1	0	4	1
Shuttleworthia	1	0	0	0	0	0	0
Simplicispira	0	1	1	0	0	0	1
Smithella	1	0	0	0	0	0	0
Soehngenia	0	0	2	0	0	0	0
Sorangium	4	1	0	1	0	0	0
Sphingobium	2	2	1	1	0	0	0
Sphingomonas	1	1	3	0	1	2	2
Sphingopyxis	1	3	3	2	1	1	0
Sphingosinicella	1	0	0	0	0	0	0
Spirochaeta	4	9	0	0	0	2	0
Sporichthya	0	0	1	0	2	0	1
Sporobacter	2	6	16	10	2	4	10
Sporomusa	0	4	0	0	0	0	1
Staphylococcus	1	72	61	7	3	18	0
Stenotrophomonas	3	13	4	0	3	3	7
Stigonema	1	0	0	0	0	0	0
Streptococcus	312	16	108	32	28	25	95
Succiniclasticum	0	1	24	1	1	0	0
Succinispira	0	0	0	0	0	1	1
Succinivibrio	0	0	0	0	2	1	0
Sulfurimonas	6	0	0	0	1	0	0
Sulfurospirillum	120	6	18	18	12	89	21
Sulfurovum	0	0	0	0	0	1	0

Syntrophobacter	1	0	0	0	0	0	0
Syntrophorhabdus	5	61	0	10	1	3	22
Syntrophus	0	0	1	0	0	0	0
Tannerella	1	0	0	0	0	0	0
Tatlockia	0	1	0	0	1	0	0
Teichococcus	0	0	0	0	1	0	0
Terrimonas	9	0	3	0	0	0	1
Thauera	25	56	26	97	51	47	38
Thermanaerovibrio	0	0	4	0	0	0	0
Thermomonas	3	5	12	3	10	5	5
Thermovirga	1	0	14	0	1	0	0
Thiobacillus	1	0	0	0	0	1	0
Thiobacter	4	1	0	2	0	0	2
Thiomonas	0	0	1	0	0	1	0
Thiothrix	10	15	3	15	1	8	5
Tissierella	0	0	0	0	0	0	2
Tistrella	1	0	0	0	0	0	0
Tolumonas	232	77	6	98	7	57	26
Treponema	0	0	8	0	2	0	0
Trichococcus	2	1	0	1	0	0	11
Turicibacter	1	0	1	0	0	0	0
Uruburuella	7	39	14	18	66	61	117
Variovorax	0	0	0	0	0	1	1
Veillonella	87	0	1	4	34	2	3
Vibrio	0	0	0	0	0	0	1
Victivallis	0	0	1	0	0	0	0
Vitreoscilla	2	22	0	1	11	30	53
Vogesella	0	0	1	0	0	0	0
Volucribacter	0	0	4	0	9	0	0
Wautersiella	0	17	0	0	0	0	3
Xanthobacter	0	1	0	1	0	0	0
Xanthomonas	2	0	5	0	0	0	0
Xenophilus	0	1	1	4	0	0	0
Xylella	1	0	0	0	0	0	0
Xylophilus	3	3	6	1	4	1	1
Yeosuana	0	2	0	0	0	0	0
Yersinia	0	0	0	0	0	0	2
Zoogloea	287	131	18	189	19	170	68
TOTAL	**5644**	**5590**	**2680**	**3983**	**4767**	**5816**	**5410**

TABLE D-3. Genera detected in sewage sampling 3

SEWAGE SAMPLING 3							
name	Chi-3	Cin-3	EP-3	Col-3	SD-3	WDC-3	Mad-3
Acetanaerobacterium	0	0	1	1	0	0	0
Acetobacterium	0	1	3	0	1	1	1
Achromobacter	0	0	0	1	0	0	0
Acidaminococcus	0	0	1	0	0	0	3
Acidisphaera	13	0	0	0	0	0	0
Acidovorax	519	1060	280	496	1067	944	411
Acinetobacter	27	951	234	482	275	374	445
Actinomyces	0	0	0	0	1	0	0
Adhaeribacter	23	0	0	1	0	0	0
Aeromonas	114	572	150	388	891	751	618
Afipia	26	1	0	1	0	0	2
Aggregatibacter	0	0	0	0	1	0	0
Agrococcus	1	0	0	0	0	0	1
Agromyces	2	0	1	0	0	0	0
Algoriphagus	3	0	0	0	0	0	0
Alicycliphilus	2	20	5	1	6	2	1
Alishewanella	0	5	0	0	0	0	0
Alistipes	7	5	21	0	6	14	6
Alkaliflexus	4	9	2	6	2	1	8
Alkaliphilus	0	2	0	0	1	0	9
Alkanindiges	0	1	0	0	0	0	0
Allisonella	2	0	0	0	4	0	0
Amaricoccus	11	0	2	1	0	0	0
Aminobacter	1	0	1	0	0	0	0
Aminobacterium	2	0	1	0	6	0	19
Anaeroarcus	0	0	0	1	1	0	0
Anaerofilum	0	0	6	0	6	2	0
Anaerolinea	12	0	1	0	0	0	2
Anaeromyxobacter	2	0	0	0	0	0	0
Anaerosinus	0	2	8	0	21	0	3
Anaerotruncus	1	0	4	2	0	1	2
Anaerovibrio	0	0	0	0	2	0	0
Anaerovorax	0	3	0	2	2	1	5
Aquabacterium	221	5	0	13	1	3	0
Aquamicrobium	0	0	0	0	0	0	2
Aquicella	1	0	0	0	0	0	0
Aquiflexum	10	0	0	0	0	0	0
Aquimonas	46	0	14	0	0	0	0
Aquitalea	4	0	0	8	3	0	0
Arcobacter	361	1804	202	574	1073	1328	2047
Arthrobacter	0	5	0	0	0	0	0
Asticcacaulis	1	0	0	0	0	0	0

Atopostipes	0	0	0	0	1	0	0
Azoarcus	4	0	4	8	1	1	2
Azonexus	3	1	1	3	1	0	0
Azospira	0	0	0	0	1	0	3
Azospirillum	2	0	2	0	0	0	0
Azovibrio	11	0	5	5	1	5	3
Bacillus	1	0	0	0	0	0	0
Bacteriovorax	1	0	0	0	0	0	1
Bacteroides	44	98	735	20	738	150	183
Balneimonas	0	0	0	0	0	0	2
Bartonella	1	0	0	0	0	0	0
Bdellovibrio	9	0	0	0	0	0	0
Beijerinckia	1	0	1	0	1	0	1
Bergeriella	0	1	2	0	8	0	0
Bergeyella	0	0	0	0	0	0	1
Bibersteinia	0	0	3	0	3	0	3
Bordetella	0	0	1	0	0	0	0
Bosea	1	0	1	0	0	0	0
Brachymonas	7	3	15	3	24	6	4
Brevundimonas	3	1	1	1	5	1	1
Brucella	17	0	0	0	0	0	0
Bulleidia	1	0	0	0	1	0	0
Burkholderia	2	1	0	0	1	0	0
Butyrivibrio	0	0	1	1	5	3	0
Caloramator	0	1	0	0	0	0	0
Caminicella	0	4	0	0	4	1	5
Campylobacter	0	0	0	0	0	0	1
Cand. Alysiosphaera	2	0	0	0	0	0	0
Cand. Amoebinatus	2	7	0	0	2	7	7
Cand. Aquirestis	49	0	1	0	0	0	0
Cand. Blochmannia	0	0	2	0	3	1	0
Cand. Kuenenia	22	33	0	0	0	8	12
Cand. Midichloria	4	0	5	0	0	0	0
Cand. Monilibacter	26	0	12	0	1	1	1
Cand. Nitrotoga	22	1	0	0	0	0	0
Cand. Odyssella	2	0	8	0	0	0	0
Cand. Symbiothrix	0	0	0	0	0	1	0
Cand. Vestibaculum	0	0	1	0	0	0	0
Catenibacterium	1	1	1	1	0	1	0
Caulobacter	4	0	0	0	0	0	0
Cellulomonas	0	0	1	0	0	0	0
Cellulosimicrobium	1	0	0	0	0	0	0
Cellvibrio	8	0	0	0	0	0	1
Cetobacterium	9	104	3	0	9	13	75
Chelatococcus	0	1	0	0	0	1	0
Chitinibacter	1	0	0	3	1	5	6
Chitinimonas	1	0	1	1	3	0	1

Chitinophaga	1	0	0	0	0	0	0
Chryseobacterium	4	18	7	96	28	64	12
Citrobacter	9	5	8	9	10	24	12
Clostridium	26	147	436	11	45	63	52
Coenonia	1	0	0	1	0	0	0
Collimonas	0	0	0	0	0	1	0
Comamonas	8	83	68	53	276	103	53
Conchiformibius	1	0	0	1	8	1	0
Conexibacter	1	1	0	0	0	0	0
Coprococcus	0	0	1	0	0	0	2
Coprothermobacter	0	0	0	0	3	0	0
Coxiella	8	0	0	0	0	0	0
Cupriavidus	46	3	1	2	7	6	0
Curvibacter	229	20	31	47	21	9	8
Cystobacter	4	0	0	0	0	0	0
Cytophaga	2	0	0	1	0	0	2
Dechloromonas	420	49	11	85	36	50	20
Defluvibacter	1	1	0	0	0	0	0
Deinococcus	0	0	2	0	0	0	0
Delftia	0	5	2	1	2	2	1
Denitratisoma	49	0	1	1	0	0	2
Desulfobacter	0	4	1	0	5	0	0
Desulfobulbus	2	3	17	1	22	17	19
Desulfomicrobium	0	0	1	0	1	1	1
Desulforegula	0	0	1	1	3	2	0
Desulfotomaculum	2	0	8	0	0	0	1
Desulfovibrio	0	0	5	0	1	0	0
Dialister	0	0	2	1	2	9	1
Dinoroseobacter	0	1	0	0	1	0	1
Dokdonella	24	0	7	0	1	0	0
Dorea	0	0	0	0	4	3	0
Duganella	0	0	0	2	0	0	0
Dyella	14	0	3	0	0	0	0
Dysgonomonas	1	12	20	4	42	13	18
Eggerthella	0	0	0	1	0	0	0
Ehrlichia	0	0	2	0	0	0	0
Elizabethkingia	0	1	0	4	1	0	0
Emticicia	1	0	0	0	0	0	0
Endoriftia	3	0	0	0	0	0	0
Enhydrobacter	2	22	18	221	23	182	10
Ensifer	0	3	0	3	0	4	0
Enterobacter	28	7	35	38	28	21	6
Enterococcus	0	1	1	1	0	1	4
Epilithonimonas	0	5	0	0	0	3	2
Erwinia	0	0	2	1	1	0	0
Erysipelothrix	0	0	0	0	1	0	1
Ethanoligenens	0	1	1	0	0	0	0

Eubacterium	3	22	62	13	8	37	11
Faecalibacterium	6	12	30	2	42	79	17
Fibrobacter	0	2	12	0	4	1	1
Finegoldia	0	0	1	0	0	0	0
Flavobacterium	654	622	7	492	12	160	166
Flexibacter	1	0	0	2	0	0	0
Flexithrix	1	0	0	0	0	0	0
Fluviicola	2	4	0	2	0	0	3
Formivibrio	0	0	3	3	2	3	3
Fusibacter	0	10	0	0	0	2	8
Fusobacterium	0	9	6	5	88	30	51
Gallionella	0	0	0	0	1	0	0
Gelidibacter	0	1	0	0	0	0	0
Geobacter	0	1	0	0	6	0	1
Giesbergeria	46	4	0	3	0	0	3
Gillisia	0	0	0	0	0	0	2
Gordonia	0	0	3	0	0	0	0
Gracilibacter	1	0	1	1	0	0	2
Haematobacter	30	1	2	3	3	2	3
Haemophilus	0	0	0	0	4	1	0
Hafnia	0	0	0	2	0	0	0
Hahella	0	0	0	0	5	0	0
Haliangium	18	0	0	0	0	0	0
Haliscomenobacter	35	0	0	0	0	0	0
Halomonas	1	0	0	0	0	0	0
Helicobacter	0	0	1	0	0	0	0
Heliobacterium	0	0	1	0	0	0	0
Herbaspirillum	11	0	2	126	4	7	0
Holdemania	0	0	0	0	1	0	0
Hydrogenophaga	127	381	1	109	0	22	1
Hydrogenophilus	0	4	0	37	0	17	0
Hymenobacter	5	0	0	0	0	0	0
Hyphomicrobium	10	1	1	0	0	0	0
Ideonella	36	1	3	0	0	0	0
Ignatzschineria	0	0	0	0	0	0	7
Ilyobacter	19	18	1	1	7	16	99
Inquilinus	1	1	0	0	0	0	0
Iodobacter	0	0	0	1	0	0	2
Janthinobacterium	2	4	0	31	0	10	0
Kaistella	0	0	2	0	1	0	3
Kaistia	2	0	0	0	1	0	0
Kaistina	6	0	3	0	0	1	0
Klebsiella	10	5	21	29	18	20	3
Kluyvera	9	2	1	4	13	0	0
Kocuria	1	0	0	0	0	0	0
Krokinobacter	4	0	0	0	0	0	0
Kytococcus	0	1	0	0	0	0	0

Lachnobacterium	0	0	138	0	5	0	0
Lachnospira	0	1	0	0	2	0	0
Lactobacillus	1	0	0	0	0	2	0
Lactococcus	19	23	1	14	0	22	34
Laribacter	0	0	28	0	22	2	3
Larkinella	0	0	1	0	0	0	0
Leadbetterella	2	0	0	0	0	0	0
Lechevalieria	2	0	0	0	0	0	0
Legionella	7	1	0	0	1	0	0
Leptospira	1	0	0	0	0	0	0
Leptothrix	14	1	2	2	1	0	0
Leptotrichia	10	91	22	26	96	426	397
Leucobacter	0	0	0	0	1	1	0
Lochheadia	3	0	0	0	0	0	0
Loktanella	1	0	0	0	0	0	0
Lutibacter	0	0	0	0	0	0	7
Lysobacter	2	0	2	1	0	0	0
Malikia	42	71	4	68	5	11	5
Maribacter	0	0	3	0	1	1	0
Maricaulis	0	0	3	0	0	0	0
Marinilabilia	1	4	1	1	3	18	20
Marinobacter	7	0	0	0	0	0	0
Massilia	1	4	0	41	0	23	0
Megasphaera	0	2	2	0	1	1	0
Meiothermus	1	0	0	0	0	0	0
Methylibium	102	21	1	29	2	12	3
Methylobacillus	230	1	0	1	0	0	0
Methylobacterium	1	0	2	0	0	0	0
Methylocaldum	1	0	2	0	1	0	0
Methylocapsa	3	0	0	0	0	0	0
Methylomicrobium	27	0	7	0	2	0	0
Methylosarcina	1	0	0	0	0	0	0
Microbacterium	2	2	3	0	0	0	5
Microcella	0	1	0	0	0	0	0
Micropruina	3	0	0	0	0	0	0
Microvirgula	0	2	2	11	5	4	0
Mitsuaria	10	0	0	5	1	0	0
Mitsuokella	0	1	0	1	4	0	0
Mogibacterium	0	0	1	0	0	0	0
Moraxella	0	0	1	3	10	0	0
Morganella	0	0	3	1	4	1	0
Muricauda	0	9	0	5	0	3	7
Myceligenerans	0	4	0	0	0	0	0
Mycobacterium	1	0	5	0	0	0	0
Mycoplana	0	1	0	0	0	0	0
Myxococcus	2	0	0	0	0	0	0
Nakamurella	0	0	0	0	0	0	1

Neisseria	0	0	42	1	181	3	8
Niastella	7	0	0	0	0	0	0
Nitratifractor	0	0	10	4	1	1	0
Nitratiruptor	0	0	0	1	0	0	0
Nitrobacter	3	0	0	0	0	0	0
Nitrosococcus	1	0	0	0	1	0	0
Nitrosomonas	1	0	1	5	2	0	1
Nitrosospira	14	4	2	0	0	0	0
Nitrospira	18	0	0	1	0	0	0
Nostocoida type II	4	0	2	0	1	0	0
Novosphingobium	48	1	10	4	0	0	0
Oceanimonas	0	0	0	0	1	1	0
Ochrobactrum	11	3	3	1	0	1	2
Opitutus	23	1	14	1	2	0	3
Ornithinicoccus	1	0	0	0	0	0	0
Ottowia	0	3	1	0	0	0	0
Oxalobacter	0	0	4	0	0	0	0
Paludibacter	81	120	56	44	15	123	132
Pandoraea	1	2	0	0	0	0	0
Pannonibacter	1	0	1	0	0	0	0
Pantoea	1	0	0	0	1	0	0
Papillibacter	0	1	4	0	0	3	0
Parabacteroides	40	108	518	24	380	146	212
Paracoccus	51	39	8	0	7	15	11
Paracraurococcus	2	0	0	0	0	0	0
Parvibaculum	1	0	0	0	0	0	0
Parvularcula	3	0	1	0	0	0	0
Pectobacterium	0	0	0	1	0	0	0
Pedobacter	14	5	0	1	0	0	1
Pedomicrobium	1	0	2	0	0	0	0
Pelobacter	0	0	0	0	3	2	9
Pelomonas	9	2	0	1	0	0	0
Peptoniphilus	0	1	0	0	0	0	0
Peredibacter	0	0	0	1	0	0	0
Petrimonas	6	9	24	0	6	2	9
Phascolarctobacterium	10	22	145	1	11	33	2
Phenylobacterium	9	0	0	0	1	1	2
Phyllobacterium	20	2	4	0	0	0	0
Pirellula	0	0	1	0	0	0	0
Planctomyces	0	0	1	0	0	0	0
Pleomorphomonas	13	0	10	0	3	1	2
Polaribacter	19	0	0	0	0	0	0
Polaromonas	17	7	0	2	0	0	0
Pragia	1	0	0	0	0	0	0
Prevotella	10	50	847	5	55	76	15
Procabacter	2	0	0	0	0	0	0
Prochlorococcus	1	0	0	0	0	0	0

Propionicimonas	1	1	0	0	0	0	0
Propionigenium	30	36	15	11	32	18	327
Propionivibrio	194	123	69	118	182	160	141
Prosthecobacter	7	0	0	0	0	0	0
Proteiniphilum	1	0	5	0	2	0	2
Pseudochrobactrum	0	0	3	2	0	0	0
Pseudomonas	79	417	31	85	73	126	85
Pseudoxanthomonas	245	7	22	1	4	1	3
Psychrobacter	0	0	0	0	3	0	28
Psychromonas	0	0	0	0	0	0	1
Psychroserpens	3	0	0	0	0	0	0
Ramlibacter	65	3	6	1	5	2	0
Raoultella	5	17	5	15	3	30	3
Rhabdochromatium	0	1	0	0	0	0	0
Rhizobium	20	36	4	20	4	13	2
Rhodobacter	140	13	1	7	2	6	5
Rhodocista	0	0	1	0	0	0	0
Rhodocyclus	0	1	0	0	1	0	1
Rhodoferax	399	46	1	26	0	11	2
Rhodopseudomonas	2	0	2	0	2	0	6
Rhodospirillum	1	0	1	0	0	0	0
Rickettsia	8	0	0	0	1	0	0
Rikenella	0	0	1	0	1	0	1
Robiginitalea	17	0	0	0	0	0	0
Roseburia	0	3	18	0	2	4	2
Roseiflexus	3	0	0	0	0	0	0
Roseomonas	11	0	0	0	0	0	0
Roseovarius	5	0	0	0	0	0	0
Rubrivivax	11	0	0	0	0	0	0
Ruminobacter	0	0	0	0	1	0	0
Ruminococcus	5	10	23	9	9	18	2
Runella	41	0	0	1	0	0	0
Salmonella	0	0	0	0	0	3	3
Samsonia	0	0	0	0	0	1	0
Sandarakinorhabdus	1	0	0	0	0	0	0
Sanguibacter	13	0	1	0	0	0	2
Schlegelella	11	0	3	0	0	0	0
Sebaldella	5	19	18	11	113	100	147
Sedimentibacter	2	1	0	0	0	0	0
Sejongia	1	11	0	7	0	1	0
Selenomonas	0	0	7	0	1	1	0
Serratia	3	6	1	37	4	8	2
Shewanella	18	18	15	149	22	88	15
Shigella	0	0	1	0	1	0	0
Shinella	0	2	0	1	1	0	0
Shuttleworthia	2	3	10	0	3	11	1
Silanimonas	1	0	0	0	1	0	0

Simplicispira	6	59	0	0	3	0	24
Smithella	3	0	0	0	5	0	0
Solibacter	1	0	0	0	0	0	0
Sorangium	12	0	0	3	0	0	0
Sphingobacterium	0	0	0	1	1	0	0
Sphingobium	3	0	2	0	0	5	0
Sphingomonas	6	1	14	2	8	0	0
Sphingopyxis	8	2	3	2	9	0	1
Sphingosinicella	91	6	0	1	1	0	0
Spirochaeta	3	0	0	1	0	3	0
Spirosoma	2	0	0	0	0	0	0
Sporichthya	1	0	1	0	0	0	0
Sporocytophaga	11	0	0	1	0	0	0
Sporomusa	6	18	1	2	2	7	11
Stella	4	0	2	0	0	0	0
Stenothermobacter	1	0	0	0	0	0	0
Stenotrophomonas	7	6	6	1	3	6	0
Sterolibacterium	1	0	0	0	0	0	0
Streptobacillus	1	0	0	1	0	4	3
Streptococcus	7	3	52	7	202	5	15
Succiniclasticum	0	0	20	0	1	0	0
Succinispira	0	1	5	1	2	2	0
Succinivibrio	0	2	0	0	0	0	0
Sulfurimonas	1	11	0	1	1	0	6
Sulfurospirillum	14	17	56	24	41	45	55
Sutterella	0	1	0	0	6	0	0
Syntrophomonas	0	0	0	0	1	0	0
Syntrophorhabdus	1	17	5	4	16	9	12
Syntrophus	2	0	0	0	0	0	0
Tannerella	1	0	2	0	1	0	1
Tatlockia	7	1	0	0	0	0	2
Tenacibaculum	5	0	0	0	0	0	0
Terrabacter	5	0	1	0	0	0	0
Terrimonas	133	0	4	6	0	0	2
Tetrasphaera	1	0	1	0	0	0	0
Thalassobius	3	0	0	0	0	0	0
Thalassolituus	1	0	0	0	0	0	0
Thauera	19	31	28	37	32	27	6
Thermanaerovibrio	0	0	4	0	3	2	0
Thermomonas	34	2	18	2	2	0	0
Thermovirga	0	0	0	0	0	0	1
Thiobacillus	0	0	2	2	0	0	0
Thiobacter	2	0	0	0	0	1	0
Thiomonas	0	0	1	0	0	0	0
Thiorhodospira	0	1	1	0	0	0	0
Thiothrix	5	0	1	0	3	0	0
Tissierella	0	0	1	0	0	0	0

Tolumonas	0	0	0	1	0	2	0
Treponema	0	0	1	0	1	0	3
Trichococcus	34	56	0	0	1	7	14
Tsukamurella	0	0	1	0	0	0	0
Turicibacter	0	0	0	1	0	2	4
Uruburuella	4	19	151	11	244	37	111
Vagococcus	0	0	0	0	0	0	3
Variovorax	5	0	0	2	0	0	0
Veillonella	2	0	30	2	13	1	1
Vibrio	1	0	0	0	0	0	0
Victivallis	0	0	0	0	0	2	1
Vitreoscilla	6	19	16	11	98	66	67
Vogesella	0	0	0	2	0	0	1
Volucribacter	0	0	0	0	2	0	0
Wautersiella	0	3	4	0	0	4	24
Xanthobacter	0	0	7	0	0	0	0
Xanthomonas	20	1	0	0	0	1	0
Xenophilus	1	0	0	0	0	0	1
Xylella	1	0	0	0	0	0	0
Xylophilus	35	389	23	5	27	24	2
Yersinia	2	3	0	0	3	6	11
Zoogloea	111	142	25	153	33	79	8
TOTAL	**6527**	**8406**	**5298**	**4571**	**7052**	**6498**	**6574**

TABLE D-4. Genera detected in sewage sampling 4

SEWAGE SAMPLING 4							
name	Chi-4	Cin-4	EP-4	Col-4	SD-4	WDC-4	Mad-4
Acetanaerobacterium	0	0	10	0	0	0	0
Acetobacterium	0	0	1	0	0	0	0
Acholeplasma	0	1	0	0	0	0	0
Achromobacter	0	0	1	0	0	1	0
Acidaminobacter	0	0	0	0	0	1	0
Acidaminococcus	0	0	1	0	0	0	0
Acidisphaera	4	1	0	1	0	0	0
Acidovorax	80	1026	340	522	310	899	374
Acinetobacter	210	883	285	322	101	277	404
Actinobaculum	0	0	0	0	2	0	0
Actinomyces	0	2	0	1	0	0	0
Adhaeribacter	4	0	0	0	0	0	0
Aeromicrobium	0	1	0	0	0	0	0
Aeromonas	93	270	390	398	297	1279	637
Afipia	3	2	1	0	0	0	0
Agrococcus	0	2	0	0	0	0	0
Ahrensia	1	0	0	0	0	0	0
Akkermansia	1	0	0	0	2	0	0
Alicycliphilus	0	20	9	2	12	4	0
Alishewanella	2	24	0	0	0	0	0
Alistipes	1	12	26	0	4	1	2
Alkaliflexus	4	14	3	15	0	2	3
Alkaliphilus	7	3	0	0	1	0	3
Alkanindiges	1	18	0	0	0	0	0
Allisonella	0	3	1	2	2	0	0
Aminobacter	0	1	0	0	0	0	0
Aminobacterium	0	2	0	0	4	0	4
Anaeroarcus	0	0	1	0	0	0	0
Anaerofilum	0	0	9	0	2	0	2
Anaerolinea	12	0	3	0	0	0	2
Anaeromusa	0	0	5	0	0	0	0
Anaeromyxobacter	1	0	0	0	0	0	0
Anaerosinus	0	2	12	3	7	3	0
Anaerotruncus	0	1	12	0	1	0	0
Anaerovorax	0	10	0	0	1	1	3
Aquabacterium	24	8	0	4	0	0	0
Aquiflexum	1	1	0	0	0	0	0
Aquimonas	5	3	2	0	0	0	0
Aquitalea	0	0	1	1	0	0	0
Arcobacter	506	796	362	347	434	877	1201
Arthrobacter	0	0	0	0	0	1	0
Asticcacaulis	0	0	1	0	0	0	1

Azoarcus	2	1	2	1	0	1	0
Azonexus	5	0	4	0	0	1	1
Azospira	0	0	1	1	0	0	0
Azospirillum	0	1	0	0	0	1	0
Azovibrio	9	1	8	1	1	3	5
Bacteriovorax	0	1	0	0	0	0	0
Bacteroides	31	53	1461	49	366	70	70
Bdellovibrio	3	0	0	0	0	0	0
Beijerinckia	1	2	2	1	0	0	0
Bergeriella	0	0	1	0	8	0	3
Bibersteinia	0	0	2	0	2	0	3
Bosea	0	2	0	0	0	0	0
Brachymonas	0	2	20	5	3	3	2
Brevundimonas	0	13	6	3	1	2	0
Brucella	1	3	0	0	0	0	0
Budvicia	1	0	0	0	0	0	2
Bulleidia	0	1	0	0	0	0	0
Burkholderia	0	0	3	0	0	0	0
Buttiauxella	0	0	1	0	0	1	1
Butyrivibrio	0	0	199	0	20	0	0
Caminicella	0	1	0	0	1	1	4
Cand. Amoebinatus	0	13	0	0	0	2	5
Cand. Aquirestis	12	3	0	0	0	0	0
Cand. Blochmannia	0	2	2	0	0	1	0
Cand. Kuenenia	8	14	1	0	0	0	16
Cand. Monilibacter	4	8	4	0	1	0	0
Cand. Nitrotoga	25	0	0	0	0	0	0
Cand. Odyssella	1	1	0	0	0	0	2
Cand. Symbiothrix	0	1	0	0	0	0	0
Catenibacterium	0	0	1	0	0	0	0
Cellvibrio	2	1	0	0	0	1	0
Cetobacterium	21	90	2	1	0	5	42
Chelatococcus	1	0	2	0	0	0	0
Chitinibacter	1	0	0	2	0	3	9
Chitinimonas	0	0	1	3	0	0	0
Chondromyces	0	0	0	1	0	0	0
Chryseobacterium	2	10	22	162	18	55	3
Citrobacter	1	2	27	4	5	30	2
Clostridium	61	207	426	46	45	21	13
Collimonas	0	1	0	0	0	0	0
Collinsella	0	1	0	1	0	0	0
Comamonas	5	51	98	54	99	101	41
Conchiformibius	0	0	6	0	4	0	0
Coprococcus	0	0	33	0	3	1	0
Cupriavidus	10	9	3	0	5	4	0
Curtobacterium	0	1	0	0	0	0	0
Curvibacter	56	44	24	15	11	6	8

Cystobacter	1	0	0	0	0	0	0
Cytophaga	3	1	0	0	0	0	0
Dechloromonas	687	97	35	80	16	39	16
Delftia	0	9	1	0	3	5	1
Denitratisoma	4	2	0	0	0	0	0
Denitrovibrio	0	0	0	0	0	1	0
Desulfobacter	1	2	2	0	0	1	0
Desulfobulbus	3	4	16	3	12	4	5
Desulfocapsa	0	0	1	0	0	0	0
Desulfomicrobium	0	0	0	0	2	0	0
Desulforegula	0	0	0	0	0	1	2
Desulfotomaculum	0	0	4	1	0	0	0
Desulfovibrio	2	0	4	2	6	0	0
Desulfurivibrio	0	1	0	1	0	0	0
Dialister	1	0	0	1	3	0	0
Dinoroseobacter	0	2	0	0	0	0	0
Dokdonella	10	3	3	0	1	0	2
Donghaeana	1	1	0	0	0	0	0
Dorea	0	0	0	0	0	0	3
Dyella	1	4	0	0	0	0	0
Dysgonomonas	1	8	51	10	21	20	27
Elizabethkingia	0	0	1	1	1	0	0
Emticicia	0	2	0	0	0	0	0
Endoriftia	5	0	0	0	0	0	0
Enhydrobacter	3	8	16	208	11	104	8
Ensifer	0	1	0	1	0	3	0
Enterobacter	9	4	44	48	6	30	7
Enterococcus	2	0	0	4	0	0	7
Epilithonimonas	0	2	0	0	0	0	0
Erwinia	0	0	0	3	2	0	0
Erysipelothrix	0	3	0	0	0	0	1
Erythrobacter	0	1	0	0	0	0	0
Ethanoligenens	0	0	0	0	0	0	2
Eubacterium	0	35	63	5	7	10	4
Faecalibacterium	5	8	29	1	39	12	2
Fibrobacter	9	4	22	1	3	0	3
Flavobacterium	160	987	14	127	3	33	83
Flexibacter	2	1	0	0	0	0	0
Fluviicola	0	1	0	1	0	0	1
Formivibrio	0	1	1	11	3	5	4
Formosa	2	0	0	1	0	0	0
Fusibacter	7	11	0	2	0	0	2
Fusobacterium	1	9	10	8	6	13	27
Geobacter	0	0	0	1	0	0	1
Giesbergeria	14	18	0	0	1	4	2
Gluconacetobacter	0	0	1	0	0	0	0
Gracilibacter	1	0	0	0	0	0	0

Haematobacter	3	4	1	2	3	0	0
Haemophilus	0	0	1	0	1	0	0
Hahella	1	0	0	0	0	0	0
Haliangium	2	0	0	0	0	0	0
Haliscomenobacter	13	4	0	0	0	0	0
Hallella	0	0	11	0	0	0	0
Halothiobacillus	0	0	1	0	0	0	0
Helicobacter	0	0	0	0	0	1	0
Herbaspirillum	3	1	11	50	4	5	0
Hirschia	0	0	1	0	0	0	0
Hydrocoleum	1	0	0	0	0	0	0
Hydrogenophaga	89	308	2	54	0	21	6
Hydrogenophilus	0	6	0	17	0	7	0
Hymenobacter	0	2	0	0	0	0	0
Hyphomicrobium	3	0	8	0	0	0	0
Ideonella	9	5	14	0	0	0	0
Ignatzschineria	0	0	0	0	0	0	3
Ilyobacter	56	12	2	2	0	20	33
Inquilinus	0	0	1	0	0	0	0
Iodobacter	0	0	0	1	0	0	2
Janthinobacterium	1	17	0	20	0	6	1
Kaistella	0	0	1	0	0	1	0
Kaistia	0	3	0	0	0	0	0
Kaistina	0	1	2	0	0	0	0
Kingella	0	0	0	0	1	0	0
Klebsiella	0	3	28	36	9	35	5
Kluyvera	1	1	1	8	0	5	2
Labrenzia	0	1	0	0	0	0	0
Lachnobacterium	0	5	50	0	14	0	0
Lactobacillus	0	0	3	0	0	0	0
Lactococcus	10	8	0	39	2	9	34
Lamprocystis	0	2	0	0	0	0	0
Laribacter	0	1	24	2	27	5	3
Legionella	3	1	0	0	0	0	1
Leptospira	3	0	0	0	0	0	0
Leptothrix	10	17	2	2	1	0	0
Leptotrichia	17	69	56	56	21	308	197
Lutibacter	2	0	0	0	0	0	11
Lysobacter	0	1	0	0	0	0	0
Magnetospirillum	1	1	0	0	0	0	0
Malikia	17	102	5	26	1	17	16
Maribacter	0	0	2	2	0	0	0
Maricaulis	0	2	1	0	0	0	0
Marinilabilia	1	2	5	1	0	2	25
Marinobacter	1	0	0	0	0	0	0
Marinomonas	1	0	0	0	0	0	0
Massilia	2	26	0	28	0	4	0

Megasphaera	0	1	1	3	0	1	0
Mesorhizobium	0	1	0	0	1	0	0
Methylibium	20	56	2	14	0	5	8
Methylobacillus	29	11	1	1	1	0	0
Methylobacter	1	0	0	0	0	0	0
Methylocaldum	3	0	1	0	0	0	1
Methylocapsa	0	4	0	0	0	0	0
Methylococcus	0	0	0	0	1	0	0
Methylomicrobium	3	0	0	2	0	0	0
Methylophaga	0	0	1	0	0	0	0
Methylophilus	0	0	5	0	0	0	0
Methylosarcina	0	6	0	0	0	0	0
Microbacterium	0	6	1	4	0	0	1
Microvirgula	1	3	0	5	1	4	3
Mitsuaria	0	1	0	3	0	0	0
Mitsuokella	0	0	1	1	2	1	1
Mogibacterium	0	0	2	0	0	0	0
Moraxella	1	1	10	0	1	0	0
Morganella	0	0	0	3	0	0	0
Muricauda	1	7	0	0	0	5	1
Myceligenerans	0	10	0	0	0	1	1
Mycobacterium	0	1	2	0	0	0	0
Myroides	1	2	0	0	0	0	1
Myxococcus	5	0	0	0	0	0	0
Neisseria	3	2	184	0	48	7	7
Nitratifractor	1	0	1	0	0	0	0
Nitratiruptor	2	0	0	0	0	0	0
Nitrosomonas	2	1	0	1	0	0	0
Nitrosospira	14	25	0	1	0	3	0
Nitrospira	4	0	0	0	0	0	0
Nocardioides	0	1	1	0	0	0	0
Nodularia	2	1	0	0	0	0	0
Nostocoida type II	3	0	0	3	0	0	0
Novosphingobium	21	6	5	0	0	0	0
Oceanimonas	0	0	0	1	0	1	0
Ochrobactrum	3	3	0	3	0	0	1
Opitutus	11	3	15	1	1	0	1
Ornithinicoccus	2	0	0	0	0	0	0
Ottowia	0	2	0	0	1	1	0
Oxalobacter	0	0	0	1	0	0	0
Paludibacter	114	152	31	80	7	133	86
Pandoraea	4	0	0	1	0	1	0
Pantoea	0	0	4	1	0	0	2
Papillibacter	0	0	41	0	0	0	0
Parabacteroides	43	72	378	45	258	105	176
Paracoccus	8	76	6	9	7	15	14
Parasporobacterium	0	1	0	0	0	0	0

Pectinatus	0	0	0	1	0	0	0
Pectobacterium	1	1	0	0	0	2	1
Pedobacter	8	5	0	0	0	0	1
Pedomicrobium	0	2	0	0	0	0	0
Pelobacter	0	0	0	1	0	0	3
Pelomonas	0	2	0	0	0	0	0
Peptostreptococcus	0	1	0	0	0	0	0
Petrimonas	2	0	10	0	2	0	5
Phascolarctobacterium	12	42	75	7	16	11	1
Phenylobacterium	1	4	11	0	0	0	0
Phyllobacterium	1	1	0	0	0	0	0
Pigmentiphaga	0	0	0	0	1	0	0
Pirellula	0	0	1	0	0	0	0
Pleomorphomonas	0	4	16	4	0	0	0
Plesiocystis	1	0	0	0	0	0	0
Polaribacter	2	2	0	0	0	0	0
Polaromonas	15	13	0	0	0	0	1
Porphyromonas	0	0	0	1	0	0	0
Pragia	0	0	2	0	0	3	0
Prevotella	5	69	1322	42	107	27	4
Propionigenium	90	28	16	6	2	9	83
Propionispira	2	0	0	0	0	0	0
Propionispora	2	1	0	0	0	1	0
Propionivibrio	132	116	113	134	71	169	114
Prosthecobacter	12	0	0	0	0	0	0
Proteiniphilum	0	0	0	0	3	0	2
Providencia	0	0	1	0	0	2	0
Pseudobutyrivibrio	0	0	5	0	2	0	0
Pseudochrobactrum	0	0	0	0	0	0	1
Pseudomonas	97	437	73	67	30	143	101
Pseudoxanthomonas	16	10	23	1	1	0	1
Psychrobacter	38	0	2	0	0	2	53
Psychromonas	5	0	0	0	0	0	0
Psychroserpens	0	0	2	0	0	0	0
Ramlibacter	18	7	3	1	0	3	0
Raoultella	1	4	5	12	1	27	4
Rhizobium	6	37	2	18	3	3	3
Rhodobacter	16	41	5	5	1	4	4
Rhodoblastus	0	0	1	0	0	0	0
Rhodocyclus	1	1	1	0	0	1	2
Rhodoferax	56	176	0	19	0	23	5
Rhodoplanes	0	3	2	0	0	0	0
Rhodopseudomonas	0	4	12	2	0	0	0
Rickettsia	4	0	0	0	0	0	0
Riemerella	0	0	0	1	0	0	0
Robiginitalea	1	2	0	0	0	0	0
Roseburia	0	6	18	1	0	2	0

Roseomonas	7	1	2	0	0	0	0
Roseovarius	1	0	0	0	0	0	0
Rubrivivax	0	0	2	0	0	0	0
Ruminococcus	0	1	110	13	14	1	3
Runella	9	4	0	0	0	0	0
Salmonella	2	3	1	2	0	6	3
Samsonia	0	0	0	0	0	1	0
Sanguibacter	1	0	0	0	0	2	3
Sarcina	1	0	0	1	0	0	0
Schlegelella	0	0	1	0	0	0	0
Sebaldella	2	5	41	11	13	74	86
Sedimentibacter	1	0	0	0	0	0	0
Sejongia	1	10	0	11	0	0	0
Selenomonas	0	0	1	4	4	0	0
Serratia	1	1	7	23	4	8	7
Shewanella	6	5	31	168	8	162	21
Shigella	0	0	6	0	5	1	0
Shinella	0	2	0	1	0	0	0
Shuttleworthia	0	7	15	7	2	2	0
Silanimonas	1	0	0	0	0	0	0
Simplicispira	2	47	0	0	1	4	16
Smithella	0	0	0	0	0	0	1
Sorangium	5	5	0	0	0	0	0
Sphingobium	0	2	0	0	0	1	0
Sphingomonas	0	0	4	0	0	0	0
Sphingopyxis	1	10	6	0	0	0	0
Sphingosinicella	4	8	0	0	0	0	0
Spirochaeta	5	0	2	0	0	0	0
Sporanaerobacter	0	1	1	0	0	0	0
Sporobacter	0	0	1	0	0	0	0
Sporocytophaga	3	0	0	1	0	0	0
Sporomusa	10	4	1	2	2	5	5
Stenotrophomonas	0	10	14	1	1	1	3
Streptobacillus	0	0	0	0	0	2	0
Streptococcus	3	0	91	7	22	5	10
Succiniclasticum	0	0	2	0	5	0	0
Succinispira	2	0	16	1	0	0	1
Sulfurihydrogenibium	1	0	0	0	0	0	0
Sulfurimonas	2	0	2	0	0	0	3
Sulfurospirillum	12	4	38	26	22	20	26
Sutterella	0	5	0	0	0	3	0
Syntrophobacter	0	0	1	0	0	0	0
Syntrophococcus	0	0	1	0	0	0	0
Syntrophorhabdus	4	17	5	6	6	9	15
Syntrophus	3	0	0	0	0	0	0
Tannerella	0	1	2	0	0	0	0
Tatlockia	0	5	0	0	0	0	0

Tenacibaculum	1	0	0	0	0	0	0
Terrimonas	18	11	6	4	0	0	1
Tessaracoccus	0	2	0	0	0	0	0
Tetrasphaera	0	0	3	0	0	0	0
Thauera	8	29	35	44	23	18	21
Thermanaerovibrio	0	0	2	0	0	0	0
Thermomonas	0	6	10	6	4	2	2
Thermovirga	0	0	0	0	0	0	1
Thioalkalivibrio	0	0	1	0	0	0	0
Thiobacillus	1	2	1	3	0	0	0
Thiobacter	0	0	0	0	0	2	0
Thiorhodospira	0	0	1	0	0	0	0
Thiothrix	10	1	0	0	1	0	0
Tistrella	0	0	2	0	0	0	0
Tolumonas	0	0	2	1	0	1	0
Treponema	0	0	0	2	0	0	0
Trichococcus	15	25	0	2	0	3	14
Turicibacter	0	2	0	0	0	1	0
Ulvibacter	1	0	0	0	0	0	0
Uruburuella	1	33	201	17	105	30	68
Variovorax	0	2	0	0	0	0	0
Veillonella	7	1	9	5	6	1	0
Vitreoscilla	2	15	17	16	35	20	42
Volucribacter	0	0	0	0	1	0	0
Wautersiella	0	0	0	0	0	1	8
Xanthobacter	1	1	0	0	0	0	0
Xanthomonas	2	0	0	0	1	0	0
Xenophilus	0	1	2	0	0	0	0
Xylophilus	34	369	55	5	6	41	4
Yeosuana	0	5	0	0	0	0	0
Yersinia	0	2	0	1	0	4	23
Zoogloea	421	247	34	249	13	112	11
Zymomonas	1	0	0	0	0	1	0
TOTAL	**3868**	**7867**	**7588**	**3981**	**2876**	**5649**	**4508**

APPENDIX E

TABLE E-1. Genera detected in pasteurized milk samples

PASTEURIZED MILK SAMPLES					
name	Store 1	Store 2	Store 3	Store 4	Store 5
Anoxybacillus	102	0	0	0	0
Bacteroides	0	0	2	52	0
Brevibacterium	0	0	10	0	0
Clostridium	88	33	16	38	19
Corynebacterium	2	0	2	0	0
Escherichia	4	0	0	0	0
Eubacterium	0	7	21	0	0
Flavobacterium	0	0	0	7	0
Fusobacterium	8	1	0	0	0
Geobacillus	4	0	0	0	0
Helcococcus	0	13	0	19	0
Janibacter	1	0	0	2	0
Janthinobacterium	0	0	33	9	0
Jeotgalicoccus	0	39	0	0	0
Kluyvera	1	0	0	0	0
Lactobacillus	0	2	0	0	0
Lactococcus	0	34	0	0	0
Macrococcus	0	0	0	4	0
Ornithinimicrobium	1	0	0	0	0
Phascolarctobacterium	0	0	1	0	0
Planifilum	0	0	0	3	0
Pleomorphomonas	0	0	13	0	0
Prevotella	0	11	0	0	0
Propionibacterium	4	0	1	0	0
Pseudomonas	9	0	1	29	0
Ralstonia	20	0	0	1	0
Roseburia	0	0	1	3	0
Ruminococcus	0	0	0	2	0
Serratia	0	1	0	1	0
Shewanella	0	0	2	0	0
Sneathia	0	0	13	0	0
Sphingobacterium	0	0	0	2	0
Sporobacter	0	0	1	17	0
Staphylococcus	6	0	2	9	18
Stenotrophomonas	0	0	0	29	0
Streptococcus	21	138	29	5	13
Turicibacter	39	4	0	67	0

Vagococcus	1	0	0	0	0
Yaniella	0	0	0	1	0
TOTAL	**311**	**283**	**148**	**300**	**50**

TABLE E-2. Genera detected in raw milk samples

RAW MILK SAMPLES					
name	Raw 6	Raw 7	Raw 8	Raw 9	Raw 10
Acetanaerobacterium	15	0	0	0	0
Acetobacter	4	0	0	0	0
Acholeplasma	0	9	0	0	0
Achromobacter	0	0	0	0	2
Acidovorax	0	5	0	0	0
Acinetobacter	21	27	0	2683	204
Actinotalea	3	0	0	0	0
Aeromonas	28	8	0	580	1
Alishewanella	0	0	0	16	0
Alistipes	12	0	0	0	0
Anabaena	0	0	1	0	0
Anaerotruncus	2	0	20	0	0
Anoxybacillus	5	0	0	3	0
Aquabacterium	0	0	0	0	1
Azoarcus	0	1	0	0	0
Bacillus	100	0	5	3	0
Bacteroides	118	0	0	0	0
Beijerinckia	2	0	0	0	0
Brachybacterium	0	0	0	1	0
Brevundimonas	23	41	0	1	0
Buttiauxella	0	0	0	0	1
Campylobacter	1	0	0	0	0
Cand. Amoebinatus	0	5	0	0	43
Cand. Blochmannia	0	0	0	1	0
Cand. Monilibacter	4	0	0	0	0
Cellvibrio	0	0	0	0	3
Chryseobacterium	0	6	0	717	44
Citrobacter	3	5	0	4	0
Clostridium	73	10	26	0	3
Comamonas	19	0	0	37	81
Corynebacterium	0	81	2	0	0
Coxiella	8	0	0	0	0
Cytophaga	0	0	0	0	2
Delftia	0	0	1	1	3

Derxia	11	0	0	0	0
Dietzia	18	0	0	0	0
Duganella	0	2	0	0	0
Elizabethkingia	5	0	0	0	0
Empedobacter	11	0	0	0	3
Enhydrobacter	0	5	0	3	0
Enterobacter	0	7	0	395	274
Enterococcus	21	153	0	64	222
Eubacterium	59	0	5	0	0
Exiguobacterium	5	0	0	948	0
Flavobacterium	0	27	0	0	0
Friedmanniella	0	0	7	0	0
Georgenia	3	0	0	0	0
Gloeotrichia	0	0	1	0	0
Halomicronema	0	0	5	0	0
Halomonas	0	0	2	0	0
Helcococcus	0	0	0	0	1
Herbaspirillum	90	0	0	0	0
Hoeflea	0	0	0	0	1
Ideonella	0	9	0	0	0
Inquilinus	0	0	0	0	1
Janibacter	0	0	1	0	0
Janthinobacterium	0	61	0	0	0
Klebsiella	3	64	0	49	105
Kocuria	22	1	0	1	0
Kurthia	1	0	0	2	0
Lactobacillus	30	0	20	0	434
Lactococcus	0	727	0	19	387
Leuconostoc	0	9	0	0	0
Lysinibacillus	19	0	0	0	0
Macrococcus	0	0	0	6059	1
Megamonas	0	0	1	0	0
Merismopedia	0	0	2	0	0
Mesorhizobium	5	0	0	0	0
Methylocaldum	0	0	0	0	2
Microbacterium	0	0	0	0	5
Mogibacterium	11	0	0	0	0
Morganella	2	0	0	6	4
Nocardioides	2	0	0	0	0
Nostocoida type II	2	0	0	0	0
Novosphingobium	4	0	0	0	0
Ochrobactrum	10	3	0	0	46
Ornithinimicrobium	6	0	0	0	0
Ottowia	0	1	0	0	0
Paludibacter	1	0	0	0	0
Pannonibacter	3	0	0	0	0
Pantoea	0	0	0	0	1

Paracoccus	0	0	0	0	3
Paracraurococcus	9	0	0	0	0
Patulibacter	1	0	0	0	0
Pectobacterium	0	0	0	0	10
Pedobacter	0	3	0	0	0
Pelomonas	0	0	15	1	1
Petrimonas	3	0	0	0	0
Petrobacter	1	0	0	0	0
Planktothrix	0	0	1	0	0
Planococcus	0	0	0	0	2
Pragia	0	0	0	8	0
Prevotella	12	0	0	0	0
Pseudaminobacter	1	0	0	0	0
Pseudomonas	373	621	16	91	6698
Pseudoxanthomonas	1	13	0	0	15
Psychrobacter	0	0	0	1	0
Raoultella	0	0	0	0	82
Rhizobium	0	0	0	2	11
Rhodococcus	18	0	0	0	0
Rhodospirillum	0	0	2	0	0
Riemerella	0	0	0	0	1
Roseburia	19	0	0	0	0
Roseospira	1	0	0	0	0
Rothia	0	0	0	3	0
Ruminococcus	3	0	0	0	0
Salmonella	0	0	0	1	0
Samsonia	0	0	0	4	11
Sanguibacter	1	0	0	0	0
Sedimentibacter	0	18	0	0	0
Segetibacter	0	0	10	0	0
Serratia	0	0	0	32	3358
Shewanella	0	0	0	29	0
Shigella	0	0	0	0	1
Sneathia	1	0	0	0	0
Sphingobacterium	3	2	0	0	10
Sphingobium	0	0	0	0	8
Sphingomonas	0	0	0	0	1
Sporobacter	26	0	0	0	0
Staphylococcus	3	1727	0	216	135
Stenotrophomonas	24	0	0	3	422
Streptococcus	12	0	0	1	3
Syntrophorhabdus	0	0	0	10	0
Tetrasphaera	1	0	0	0	0
Thermolithobacter	0	0	3	0	0
Turicibacter	1	0	0	0	2
Vogesella	0	0	0	4	0
Wautersiella	0	4	0	46	0

Xanthomonas	0	0	0	0	4
Xylella	31	0	0	0	3
TOTAL	**1330**	**3655**	**146**	**12045**	**12656**

TABLE E-3. Genera detected in unprocessed pasteurized milk samples after storage

UNPROCESSED PASTEURIZED MILK SAMPLES AFTER STORAGE					
name	Milk-1F	Milk-2F	Milk-3F	Milk-4F	Milk-5F
Citrobacter	0	0	0	1	0
Geobacter	1	0	0	0	0
Hydrogenophaga	0	0	0	1	0
Janthinobacterium	16513	0	0	0	0
Paenibacillus	358	153	158	7431	11261
Pseudomonas	0	10010	16038	4	0
Serratia	5	0	0	0	0
Staphylococcus	0	1	0	0	3
TOTAL	**16877**	**10164**	**16196**	**7437**	**11264**

TABLE E-4. Genera detected in unprocessed raw milk samples after storage

UNPROCESSED RAW MILK SAMPLES AFTER STORAGE					
name	Raw-6F	Raw-7F	Raw-8F	Raw-9F	Raw-10F
Acinetobacter	2	0	1	4	2
Aeromonas	0	0	0	2	0
Arcobacter	1	1	0	0	1
Bacteroides	0	2	0	0	2
Brevundimonas	0	0	3	0	1
Buttiauxella	0	7	0	0	1
Carnobacterium	0	0	0	20	0
Comamonas	0	0	1	0	0
Delftia	0	0	0	1	0
Dickeya	0	1	0	0	0
Enterobacter	1	0	0	0	0

Enterococcus	3	0	88	867	2
Exiguobacterium	0	0	0	1	0
Flavobacterium	0	1	0	0	0
Herminiimonas	0	0	1	0	0
Janthinobacterium	0	1516	0	0	0
Klebsiella	0	0	0	28	2
Lactobacillus	0	0	0	0	31
Lactococcus	0	0	0	0	2
Leuconostoc	956	0	2	0	0
Macrococcus	0	0	0	18	0
Massilia	0	1	0	0	0
Microbacterium	0	0	2	0	0
Morganella	1	0	0	0	0
Pantoea	0	1	0	0	0
Pectobacterium	2	12	0	1145	0
Porphyrobacter	0	0	1	0	0
Prevotella	0	1	0	0	0
Pseudomonas	12585	12292	9733	4546	4
Raoultella	0	0	0	0	2
Rhodococcus	0	0	7	0	0
Serratia	4	384	0	1796	7615
Sphingomonas	0	0	3	0	0
Sphingopyxis	0	0	1	0	1
Streptococcus	0	0	0	0	8
Yersinia	0	0	0	13	0
TOTAL	**13555**	**14219**	**9843**	**8441**	**7674**

TABLE E-5. Genera detected in irradiated pasteurized milk samples after storage

IRRADIATED PASTEURIZED MILK SAMPLES AFTER STORAGE					
name	Irr-1F	Irr-2F	Irr-3F	Irr-4F	Irr-5F
Acidovorax	0	0	0	0	1
Acinetobacter	0	9	0	0	7
Anaerococcus	0	0	0	0	1
Anoxybacillus	34	0	0	0	0
Aquabacterium	0	0	0	0	3
Arcanobacterium	6	1	0	0	0
Arcobacter	0	64	0	0	0
Arthrobacter	0	2	0	0	0
Atopostipes	1	0	0	0	0

Bacillus	0	0	0	0	93
Bacteroides	0	4	0	0	40
Bifidobacterium	0	0	0	0	1
Brachybacterium	0	0	0	0	3
Bradyrhizobium	0	0	0	29	0
Brevibacillus	0	0	0	6	0
Brucella	0	0	0	0	3
Clostridium	9	25	0	20	256
Clostridium; Syntrophococcus	2	0	0	0	0
Corynebacterium	0	1	0	0	1
Coxiella	0	0	0	0	15
Diaphorobacter	0	0	0	0	100
Dietzia	1	0	0	0	0
Enterobacter	1	0	0	4	0
Enterococcus	0	0	0	0	11
Escherichia	2	7	0	5	0
Eubacterium	0	2	0	0	1
Eubacterium; Roseburia	0	2	0	0	0
Eubacterium; Sporobacter	0	1	0	0	0
Flavobacterium	0	0	0	11	0
Formosa	5	0	0	0	0
Fusobacterium	1	0	0	0	0
Gemmatimonas	0	0	0	0	3
Geobacillus	0	0	0	0	26
Helcococcus	23	5	0	0	0
Janibacter	25	18	0	0	0
Janthinobacterium	0	29	0	14	0
Klebsiella	0	0	0	2	0
Kytococcus	76	0	0	0	0
Laceyella	0	0	0	0	14
Lachnospira	0	0	0	0	4
Legionella	0	0	0	0	12
Leptotrichia	0	23	0	0	0
Lysinibacillus	0	0	0	0	1
Lysobacter	0	0	0	0	34
Methylococcus	0	0	0	0	5
Methylococcus; Methylomicrobium	0	0	0	0	10
Methylomicrobium	0	0	0	0	3
Micrococcus	1	1	0	0	6
Morganella	0	0	0	2	0
Mycobacterium	0	0	0	4	1
Mycoplasma	0	0	0	0	31
Nocardia	0	0	0	0	4
Nocardioides	0	0	0	0	6
Oceanobacillus	0	0	0	0	18
Ochrobactrum	0	0	0	0	1
Ornithinicoccus	0	0	0	0	3

Ornithinimicrobium	0	0	0	0	2
Paenibacillus	0	0	0	0	18
Pasteurella	0	4	0	0	1
Paucisalibacillus	0	0	0	0	3
Pectobacterium	0	0	0	3	0
Peptoniphilus	0	0	0	0	1
Phascolarctobacterium	0	2	0	0	0
Planococcus	0	0	0	0	28
Planococcus; Planomicrobium	0	0	0	0	1
Planomicrobium	0	0	0	0	5
Polaromonas	0	0	0	34	0
Promicromonospora	0	0	0	0	2
Propionibacterium	25	10	0	10	1
Pseudochrobactrum	0	0	0	0	1
Pseudomonas	10	8	14652	78	91
Psychrobacter	0	4	0	0	0
Ralstonia	0	0	0	1	0
Rhizobium	0	0	0	8	24
Riemerella	1	0	0	0	1
Roseburia	0	11	0	0	0
Ruminobacter	0	2	0	0	0
Ruminococcus	0	0	0	1	0
Salinicoccus	0	0	0	2	0
Serratia	0	0	0	7	0
Shewanella	0	0	0	0	9
Sphingomonas	0	0	0	2	0
Sporobacter	0	9	0	14	0
Staphylococcus	7	7	1	37	523
Stella	0	0	0	0	12
Stenotrophomonas	0	1	0	2	6
Streptococcus	46	90	0	50	312
Succinivibrio	0	0	0	0	12
Trichococcus	0	0	0	0	9
Turicibacter	67	12	0	46	129
Virgibacillus	0	0	0	0	5
Yaniella	0	0	0	0	4
TOTAL	**343**	**354**	**14653**	**392**	**1918**

TABLE E-6. Genera detected in irradiated raw milk samples after storage

IRRADIATED RAW MILK SAMPLES AFTER STORAGE					
name	Irr-6F	Irr-7F	Irr-8F	Irr-9F	Irr-10F
Acetobacter	18	0	0	0	0
Achromobacter	0	4	0	0	0
Acidovorax	283	120	166	0	0
Acinetobacter	390	35	666	244	3
Afipia	36	0	4	0	0
Algoriphagus	0	15	0	0	0
Alishewanella	0	2	0	0	0
Amaricoccus	0	25	0	0	0
Aquabacterium	4	1	13	0	0
Arthrobacter	0	160	546	0	0
Bacillus	9	0	0	0	0
Bergeyella	1	0	0	0	0
Blastobacter	0	3	0	0	0
Bosea	9	0	8	0	0
Brevibacillus	0	0	7	0	0
Brevibacterium	0	23	0	0	0
Brevundimonas	76	95	42	0	0
Buttiauxella	0	0	0	0	2
Cand. Amoebinatus	0	0	2	0	0
Capnocytophaga	0	1	0	0	0
Carnobacterium	0	0	0	75	0
Caulobacter	0	0	33	0	0
Chryseobacterium	2	9	8	0	0
Citricoccus	4	0	0	0	0
Citrobacter	0	8	0	0	0
Clostridium	2	1	42	0	0
Comamonas	30	0	0	0	0
Corynebacterium	0	16	26	0	0
Coxiella	6	0	0	0	0
Cryobacterium	0	0	0	0	1
Cupriavidus	6	0	0	0	0
Curtobacterium	0	5	0	0	0
Dechloromonas	10	0	0	0	0
Delftia	0	0	9	0	0
Enhydrobacter	0	17	0	0	0
Enterobacter	0	27	0	0	0
Enterococcus	10868	208	4929	1129	1
Erythrobacter	11	0	0	0	0
Eubacterium	0	1	0	0	0
Exiguobacterium	0	0	0	3	0
Flavobacterium	0	1	0	0	0
Friedmanniella	1	0	0	0	0

Georgenia	0	0	7	0	0
Haematobacter	0	10	0	0	0
Herbaspirillum	15	0	3	0	0
Herminiimonas	52	0	10	0	0
Hydrogenophaga	1	0	0	0	0
Janthinobacterium	3	0	0	0	0
Kineosporia	0	0	1	0	0
Kocuria	19	17	4	0	0
Lactobacillus	0	0	0	0	105
Lactococcus	0	1427	0	4507	11
Leuconostoc	327	0	0	0	0
Loktanella	0	1	0	0	0
Lysobacter	0	21	0	0	0
Macrococcus	0	0	0	824	0
Malikia	0	19	2	0	0
Microbacterium	206	25	0	0	0
Microlunatus	1	0	0	0	0
Mitsuaria	3	0	9	0	0
Morganella	0	2	0	0	0
Mycobacterium	1	0	0	0	0
Opitutus	0	28	0	0	0
Pannonibacter	1	0	0	0	0
Paracoccus	1	17	0	0	0
Pelomonas	1	7	33	0	0
Plantibacter	0	109	0	0	0
Polaromonas	1	0	0	0	0
Porphyrobacter	19	0	0	0	0
Prevotella	0	4	0	0	0
Propionivibrio	20	0	0	0	0
Pseudomonas	2	65	11	0	0
Psychrobacter	0	0	19	0	0
Raoultella	0	1	0	0	0
Rhizobium	0	1	0	0	0
Rhodobacter	9	0	1	0	0
Rhodococcus	1337	1	0	0	0
Sanguibacter	0	0	29	1	0
Selenomonas	0	8	0	0	0
Serratia	0	0	2	7	4252
Shewanella	0	0	1	0	0
Sphingobacterium	11	0	0	0	0
Sphingobium	5	0	0	0	0
Sphingomonas	24	309	0	0	0
Sphingopyxis	313	13	19	0	0
Staphylococcus	0	2333	34	0	0
Stenotrophomonas	4	0	25	0	1
Streptococcus	21	9	0	0	159
Streptomyces	0	0	1	0	0

Syntrophorhabdus	0	0	0	7	0
Terrimonas	0	13	3	0	0
Treponema	0	3	0	0	0
Variovorax	0	50	0	0	0
Veillonella	0	8	0	0	0
Xylella	11	0	0	0	0
TOTAL	**14174**	**5278**	**6715**	**6797**	**4535**

TABLE E-7. Genera detected in lab-pasteurized raw milk samples after storage

LAB-PASTEURIZED RAW MILK SAMPLES AFTER STORAGE					
name	Past-6F	Past-7F	Past-8F	Past-9F	Past-10F
Acidovorax	0	0	35	0	0
Acinetobacter	0	21	89	0	0
Anabaena	0	0	1	0	0
Anaerococcus	0	332	0	0	0
Bacteroides	0	0	2	0	0
Belnapia	0	1	0	0	0
Bradyrhizobium	0	5	0	0	0
Buttiauxella	0	0	0	0	3
Catonella	0	14	0	0	0
Caulobacter	0	2	0	0	0
Chitinophaga	0	21	0	0	0
Chryseobacterium	0	1	1	0	0
Citrobacter	0	1	0	0	0
Clostridium	0	0	1	0	0
Corynebacterium	0	43	0	0	0
Cryptosporangium	0	0	2	0	0
Cupriavidus	0	1	0	0	0
Devosia	0	0	6	0	0
Enhydrobacter	0	2	0	0	0
Enterobacter	0	14	0	0	0
Enterococcus	0	18	0	6	0
Eubacterium	0	3	0	0	0
Exiguobacterium	0	0	0	4	0
Finegoldia	0	24	0	0	0
Flavobacterium	0	4	0	0	0
Frankia	0	0	59	0	0
Gordonia	0	1	0	0	0
Haemophilus	0	4	0	0	0
Janthinobacterium	0	33	0	0	0
Lactococcus	0	45	0	0	0

Macrococcus	1	6	0	11	0
Microbacterium	0	0	6	0	0
Microcella	0	0	5	0	0
Mitsuaria	0	3	0	0	0
Nocardioides	0	0	9	0	0
Ochrobactrum	0	26	0	0	0
Prevotella	0	3	2	0	0
Propionibacterium	0	6	0	0	0
Propionivibrio	0	0	17	0	0
Pseudomonas	14150	98	77	11094	0
Rhodobacter	0	0	2	0	0
Rhodopseudomonas	0	1	0	0	0
Roseomonas	0	27	0	0	0
Ruminococcus	0	0	1	0	0
Serratia	0	6	0	0	8214
Staphylococcus	0	660	11	0	0
Stenotrophomonas	0	5	3	0	0
Streptococcus	0	18	0	0	0
Streptomyces	0	0	50	0	0
Succinivibrio	0	0	1	0	0
Syntrophorhabdus	0	0	1	1	0
Thermomonas	0	0	1	0	0
Xylophilus	0	0	1	0	0
TOTAL	**14151**	**1449**	**383**	**11116**	**8217**

TABLE E-8. Genera detected in boiled pasteurized milk samples after storage

BOILED PASTEURIZED MILK SAMPLES AFTER STORAGE					
name	Boil-1F	Boil-2F	Boil-3F	Boil-4F	Boil-5F
Achromobacter	0	0	0	1	0
Acinetobacter	0	0	0	77	0
Alcaligenes	0	0	10	0	0
Alistipes	0	0	3	0	0
Aquabacterium	0	0	0	3	0
Bacillus	0	0	12	0	0
Bacteroides	0	0	1	0	0
Clostridium	0	0	3	0	0
Corynebacterium	0	0	1	0	0
Cryobacterium	0	0	0	1	0
Desemzia	0	0	1	0	0
Enterococcus	0	0	0	89	0
Escherichia	0	0	1	0	0

Eubacterium	0	0	1	0	0
Gordonia	0	0	1	0	0
Hymenobacter	0	0	5	0	0
Janibacter	0	0	3	1	0
Listeria	0	0	1	0	0
Macrococcus	0	0	3	0	0
Micrococcus	0	0	1	0	0
Mitsuaria	0	0	0	1	0
Pelomonas	0	0	0	2	0
Petrobacter	0	0	0	1	0
Prevotella	0	0	6	0	0
Propionibacterium	0	0	86	7	0
Pseudomonas	0	0	55	4	0
Sneathia	0	0	185	0	0
Sneathia ; Streptobacillus	0	0	2	0	0
Staphylococcus	0	0	0	23	0
Stenotrophomonas	0	0	1	0	0
Streptococcus	0	0	3	0	0
Streptomyces	0	0	46	0	0
Thermicanus	0	0	0	2	0
Thermolithobacter	0	0	0	1	0
TOTAL	**0**	**0**	**431**	**213**	**0**

TABLE E-9. Genera detected in boiled raw milk samples after storage

BOILED RAW MILK SAMPLES AFTER STORAGE					
name	Boil-6F	Boil-7F	Boil-8F	Boil-9F	Boil-10F
Acetanaerobacterium	1	0	0	0	0
Achromobacter	0	0	0	0	3
Acidovorax	128	0	2	152	166
Acinetobacter	483	26	68	2746	579
Aeromonas	0	0	0	116	0
Afipia	1	0	0	7	28
Algoriphagus	5	0	0	0	0
Anaerococcus	65	0	0	0	0
Anaerotruncus	1	0	0	0	0
Aphanizomenon	1	0	0	0	0
Aquabacterium	21	22	0	19	12
Asticcacaulis	0	0	1	0	0
Atopostipes	32	0	0	0	0
Azoarcus	0	0	0	5	0
Azospirillum	41	0	0	0	0

Bacillus	27	0	0	0	0
Bacteroides	16	0	0	0	0
Bosea	0	0	0	1	0
Brevibacterium	121	0	0	0	0
Brevundimonas	19	2	0	48	97
Cand. Amoebinatus	0	0	0	0	1
Caryophanon	2	0	0	0	0
Caulobacter	0	0	1	5	20
Chitinophaga	1	0	0	0	0
Chryseobacterium	2	1	1	2313	13
Clostridium	41	0	0	40	65
Comamonas	0	0	0	32	30
Corynebacterium	49	0	0	0	10
Cryobacterium	67	0	0	0	0
Cupriavidus	2	0	1	0	0
Dechloromonas	25	0	0	1	20
Delftia	1	0	0	0	0
Eggerthella	0	0	0	1	0
Elizabethkingia	1	0	1	0	0
Enhydrobacter	1	0	7	27	0
Enterobacter	0	0	0	31	33
Enterococcus	70	0	0	16	26
Exiguobacterium	2	0	0	186	0
Faecalibacterium	30	0	0	31	0
Finegoldia	308	0	0	0	0
Flavobacterium	7	0	0	0	1
Gallionella	10	0	0	0	0
Giesbergeria	0	0	0	0	1
Herbaspirillum	0	0	16	19	5
Herminiimonas	0	0	0	40	0
Hydrogenophaga	0	2	0	0	0
Janibacter	0	0	6	9	0
Janthinobacterium	10	0	0	11	0
Kineosporia	1	0	0	0	0
Klebsiella	0	0	0	0	7
Kocuria	0	0	0	1	0
Lactobacillus	44	0	0	0	39
Lactococcus	0	12	0	3	133
Leifsonia	2	0	0	0	0
Leptothrix	0	1	0	0	0
Leptotrichia	0	0	16	0	0
Macrococcus	0	0	0	820	0
Massilia	31	0	1	1	63
Microbacterium	0	4	11	0	0
Microbispora	11	0	0	0	0
Micromonospora	5	0	0	0	0
Mitsuaria	2	5	2	8	5

Mycobacterium	61	0	0	0	0
Niastella	2	0	0	0	0
Olsenella	0	0	0	9	0
Ornithinimicrobium	0	0	0	0	5
Paracoccus	0	11	0	1	36
Pelomonas	0	0	2	1	0
Peptoniphilus	56	0	0	0	0
Planococcus	2	0	0	0	0
Prevotella	0	0	4	0	1
Propionivibrio	0	0	0	7	0
Pseudomonas	131	130	0	238	570
Pseudoxanthomonas	0	0	0	0	2
Raoultella	0	0	0	0	9
Rhodoferax	36	0	0	0	1
Rhodopseudomonas	0	0	29	0	0
Rickettsia	937	28	0	0	0
Roseburia	13	0	0	0	0
Rothia	0	0	0	7	0
Sanguibacter	45	0	0	0	0
Schlegelella	25	0	0	1	0
Serratia	26	1	0	0	913
Shewanella	0	0	0	16	0
Sphingobacterium	0	0	0	0	2
Sphingobium	0	0	0	0	15
Sphingomonas	23	0	0	0	23
Sphingopyxis	60	0	0	76	68
Staphylococcus	605	43	0	33	35
Stenotrophomonas	17	3	6	12	146
Streptococcus	94	0	10	0	4
Syntrophorhabdus	0	0	0	5	0
Terrimonas	34	0	0	0	0
Thermomonas	0	0	0	4	0
Tsukamurella	16	0	0	0	0
Xylella	2	0	0	0	0
Zoogloea	0	0	1	0	0
TOTAL	**3872**	**291**	**186**	**7099**	**3187**

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