

**Identification of Novel Strains of *Pseudomonas lundensis*  
from Food Samples**

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## ABSTRACT

*Pseudomonas lundensis* is a psychrotrophic bacterium that is commonly implicated in the spoilage of different food products, especially milk and meat. Virtually all of the information that we have about this bacterium centers on its role in food spoilage; however, recent studies have provided evidence of its presence in the lungs of cystic fibrosis patients. Interestingly, this bacterium has never been directly cultured from a human specimen, underscoring the discrepancy between culture-dependent and culture-independent methods of identification. For this study, we set out to design a protocol for the isolation of *P. lundensis* from food samples and demonstrate its efficacy in inhibiting the growth of microorganisms commonly implicated in human infections. Additionally, we hoped to identify novel strains of *P. lundensis* from food samples for further characterization. Our experiments showed that the psychrotrophic nature of *P. lundensis* proved most useful in isolating it from other microorganisms, even when grown on a medium that generally supports the growth of a wide range of microorganisms. Upon application of the combination of low temperature incubation and a supportive growth medium to the isolation of *P. lundensis* from food samples, we were able to obtain four *P. lundensis* isolates from pasteurized 1% milk. Nearly all of the other isolates that we obtained were *Pseudomonas* species, with the exception of one *Paenibacillus* isolate and one *Stenotrophomonas* isolate. Our findings have demonstrated that the psychrotrophic nature of *P. lundensis* is effective at isolating it from other microorganisms, especially those that are commonly implicated in human infections, and may have the broader application of potentially culturing *P. lundensis* from human specimens in the future.

# INTRODUCTION

## I. *Pseudomonas*

*Pseudomonas* is a genus of aerobic bacteria that are members of the family *Pseudomonadaceae*, which consists of several hundred different species. Phenotypically, members of this genus present as small Gram negative bacilli (1, 2). Many members of this genus are polarly flagellated, allowing for motility (2). Some well-known species in the genus are *P. aeruginosa*, *P. syringae*, and *P. fluorescens*.

Ubiquitous in the environment, members of the genus *Pseudomonas* are widely distributed in soil and aquatic environments, both natural and man-made (1, 3). Some are also members of the normal flora of human body sites, including the oral cavity and the lungs (4). *Pseudomonas* species are commonly implicated as contaminants, including in meat and dairy products and pesticides and other chemicals, including organic acids (5).

The pathogenicity of *Pseudomonas* species ranges from species like the non-pathogenic rhizosphere bacterium *P. putida* to the pathogenic *P. syringae* and *P. aeruginosa*. *P. syringae* is phytopathogenic, meaning that it infects plants; conversely, *P. aeruginosa* is well-known as an opportunistic human pathogen (1). *P. aeruginosa* normally resides in soil and aquatic environments, but often contaminates surfaces in locations such as medical facilities. Patients with underlying illnesses, such as cystic fibrosis (CF), diabetes, or recent surgery, often acquire *P. aeruginosa*-related infections during hospitalization. Such infections often exhibit high morbidity and mortality, as antimicrobial resistant strains can develop quickly, making treatment difficult (6). The pathogenicity of many species in the genus is often overlooked or even not known, such as with members of the *P. fluorescens* species complex, as they are either not typically implicated in human infections or have little research looking into their potential effects on human health (4).

## II. Media and Bacterial Growth

### A. General Purpose Media

General purpose media contain components necessary for the growth of a wide range of microorganisms, which are generally non-fastidious. General purpose media contain some source of energy, carbon, nitrogen, phosphates, and metals, all of which are required for microbial growth (7). For the purposes of this study, this review focuses on three common general purpose media types: Trypticase Soy Agar (TSA), Lennox L agar (LB), and Nutrient Agar (NA).

TSA contains four main ingredients: pancreatic digest of casein, peptic digest of soybean meal, sodium chloride, and agar. Blood can be added for additional support of microbial growth. Although it isn't used to support the growth of any specific organism, it is suitable for the cultivation and isolation of a wide range of non-fastidious microorganisms (7, 8).

LB contains four main ingredients: peptone, yeast extract, sodium chloride, and agar. LB is primarily used to support the growth of *Escherichia coli* for a variety of purposes, including transformation, protein expression, and plasmid cloning and propagation, among others. However, LB can also be used to support the growth of other non-fastidious microorganisms (7, 9).

NA contains three main ingredients: peptone, beef extract, and agar. Like TSA, NA doesn't support the growth of specific microorganisms, but rather supports the growth of a wide range of non-fastidious microorganisms (7, 8).

## B. Selective Media

Selective media contain components that select for the growth of microorganisms of interest from a mixed population. These components can be specific carbon or energy sources that permit only a specific organism or class of organisms to grow, or they can be toxic substances that inhibit the growth of all microorganisms other than the desired organism or class of organisms (7). Many different kinds of selective media exist, but for the purposes of this study this review focuses on four: Cetrimide agar; Sabouraud Dextrose Agar (SDA); Violet Red Bile Agar (VBA); and de Man, Rogosa, and Sharpe agar (MRS).

Cetrimide agar contains five main ingredients: pancreatic digest of gelatin, magnesium chloride, potassium sulfate, cetrimide (tetradecyltrimethylammonium bromide), and agar. The primary use for Cetrimide agar is to selectively isolate *Pseudomonas aeruginosa* and other Gram-negative, non-fermentive bacteria (7, 8). On Cetrimide agar plates, *P. aeruginosa* appears as blue-green colonies due to the production of pyocyanin, which is characteristic of *Pseudomonas* species (10).

SDA contains four main ingredients: peptic digest of animal tissue, pancreatic digest of casein, dextrose, and agar. The primary use for SDA is to selectively isolate fungi, particularly dermatophytes; it can be used with or without antimicrobials to inhibit bacterial growth (7, 8).

VBA contains eight main ingredients: yeast extract, peptone, no. 3 bile salt, lactose, sodium chloride, neutral red, crystal violet, and agar. The primary use for VBA is to selectively isolate coliform organisms, especially from dairy products (7, 8).

MRS contains eleven main ingredients: dextrose, gelatin peptone, beef extract, sodium acetate, yeast extract, ammonium citrate, dipotassium phosphate, polysorbate 80, magnesium sulfate, manganese sulfate, and agar. The primary use for MRS is to selectively isolate Gram-positive cocci and members of the *Lactobacillus* genus, generally from clinical specimens (7, 8).

## C. Differential Media

Differential media contain components that allow for the identification of differential patterns of growth in different classes of microorganisms; for example, the production of acid from the metabolism of different carbohydrates or the decarboxylation of amino acids is exploited in order to identify the presence of certain microorganisms. Some differential media may contain pH indicators that visually detect these metabolic reactions, while others may contain chromogenic dyes that change color to indicate that a specific enzymatic reaction has taken place (7). The two types of differential media that are focused on in this study, MacConkey agar and *Pseudomonas*-isolation agar, can also be categorized as selective media.

MacConkey agar contains eight main ingredients: pancreatic digest of gelatin, peptones (meat and casein), lactose, bile salts, sodium chloride, neutral red, crystal violet, and agar. The primary use for MacConkey agar is the selective and differential isolation of enteric bacilli (7, 8). The presence of crystal violet and bile salts inhibit the growth of Gram-positive bacteria, allowing Gram-negative bacteria to thrive. On MacConkey agar plates, enteric bacilli that grow appear different colors based on whether or not they ferment lactose. Bacteria that ferment lactose appear as pink or red colonies on the agar, due to the presence of neutral red in the media; bacteria that do not ferment lactose appear as transparent or colorless colonies (11).

*Pseudomonas*-isolation agar contains five main ingredients: peptone, magnesium chloride, potassium sulfate, irtgasan (triclosan), and agar. An additional supplement, glycerol, must be added during the preparation of this media (8). The primary use for *Pseudomonas*-isolation agar is for the selective and differential isolation of *P. aeruginosa* and other

*Pseudomonas* species. The presence of irgasan, a broad-spectrum antimicrobial, inhibits the growth of microorganisms other than *Pseudomonas* species, as it is not active against members of the genus (7, 8). The medium's low phosphorus content, the presence of potassium sulfate, magnesium chloride, and the addition of a glycerol supplement all serve to promote the production of pyocyanin, a blue-green pigment characteristic of *Pseudomonas* species (7, 10).

### III. Bacterial Growth at Low Temperatures

Temperature is an extremely important factor when it comes to bacterial growth, and some bacteria prefer certain temperature ranges over others. Psychrophiles are microorganisms that grow best at a temperature around 15°C or colder, with a maximum around 20°C and a minimum around 0°C (12). Similarly, psychrotrophs are microorganisms that have the ability to grow well at cool temperatures, although their optimal growth temperature lies in a range of around 15-20°C. (13). Often, the terms psychrophile and psychrotroph are used interchangeably, as some confusion exists about the criteria for classification in these groups, as most bacteria do not have a temperature optima below 20°C (14). However, *Psychromonas ingrahamii* has demonstrated growth at -12°C, the lowest reported growth temperature for a bacterium (13). Many genera of bacteria have been isolated at low temperatures and classified as either psychrophilic or psychrotrophic, including *Micrococcus*, *Flavobacterium*, *Achromobacter*, *Bacillus*, *Clostridium*, *Vibrio*, *Acinetobacter*, and *Pseudomonas* (13). By comparison, mesophiles are microorganisms that have an optimal temperature range for growth around 25-40°C; typically, mesophiles will not grow well or at all at a temperature below 5°C (15).

Surprisingly, the large majority of Earth's available habitats are considered cold. Approximately 71% of the Earth's surface is part of a marine ecosystem, and 90% of the volume of the oceans exists at a temperature of 5°C or colder. The polar regions comprise around 14% of the Earth's surface, and that does not include all of the areas at high altitudes, which are also typically cold (13). Psychrophiles are found across all of these cold environments, from the depths of the oceans to arctic permafrost. An important distinction between psychrophiles and psychrotrophs is that psychrotrophs can also be found in cold environments that experience temperature fluctuations that extend outside of the psychrophilic growth temperature range, which occur due to seasonal changes in the intensity of solar radiation. Psychrotrophs generally have a wide growth range, compared to psychrophiles which can be thermally inactivated if exposed to higher temperatures (13).

Psychrophiles and psychrotrophs possess several physiological mechanisms that allow them to grow at temperatures at which true mesophiles and thermophiles cannot grow. One barrier to growth at low temperatures is membrane fluidity; at low temperatures, the cell membrane becomes more rigid, as cold temperatures cause the fatty acid chains in the membrane phospholipids to be more restricted. As the membrane becomes more rigid, the ability for nutrient and oxygen uptake is inhibited (16). Psychrophiles and psychrotrophs, however, are able to retain their membrane fluidity via the increased incorporation of unsaturated fatty acids into the membrane, a process called homeoviscous adaptation (13, 17, 18). This allows them to keep up the flow of nutrients and oxygen into the cell.

Ice crystallization is also a barrier to growth at low temperatures. Prolonged exposure to cold temperatures can result in the formation of ice crystals in the cytosol, which can perforate the membrane and lead to cell death (16). To combat this problem, psychrophiles and psychrotrophs employ the use of antifreeze proteins (AFPs), which can prevent ice formation

before and during freezing. Before freezing, AFPs act by lowering the freezing point of the cytosol in a non-colligative manner. When the cell begins freezing, AFPs control the size of the ice crystals that form, inhibiting the growth of large and potentially fatal ice crystals in favor of smaller, less harmful ones (19).

The production of exopolysaccharides has also been shown to play a role in alleviating the stress of growing at low temperatures; high concentrations of exopolysaccharides have been detected in Arctic sea ice where psychrophilic and psychrotrophic organisms have been isolated. When produced, exopolysaccharides appear to form thick gels that surround the cell, acting as a cryoprotectant (20). Together, these adaptations allow psychrophiles and psychrotrophs to grow at temperatures at which other organisms cannot.

#### **IV. The Microbiota of Milk and Meat**

The bacteria typically found in milk and meat, raw or otherwise, is generally referred to as spoilage bacteria. Raw milk, pasteurized milk, and ultra-high pasteurized milk all have the potential to harbor spoilage bacteria, depending on how the milk was handled, transported, and stored post-pasteurization (or lack thereof) (21). With meat, spoilage bacteria can be present in the animal prior to slaughter, or they can colonize the meat during processing, transport, or storage (22).

Milk, whether it be obtained from an animal source, such as cows or goats, or a human source, is a highly nutritious food that is primarily marketed for human consumption. Milk contains a variety of proteins, fats, carbohydrates, vitamins, minerals, and amino acids that not only provide a suitable source of nutrition for humans, but also for microbial populations (21). Raw milk, which is simply milk that hasn't been pasteurized or processed, generally contains a very diverse microbial community, as it hasn't been treated to remove such microorganisms (23, 24). Pasteurized milk ideally does not contain any pathogenic microorganisms, as the heat treatment of the milk at 161°F (72°C) for 15 seconds is designed for disinfection (23). However, this is not a fool-proof process. A newer technique, ultra-high pasteurization, heats the milk to at least 280°F (138°C) for 2 seconds, with the hope of destroying even more microorganisms. This process greatly extends the shelf life of the milk, making it safe to drink for 6-9 months prior to opening (23).

The microbiota of milk varies depending on the geographical location and the source, whether it be from a cow, a goat, or a human being. For the purposes of this study, this review focuses on cow's milk. Species from several genera are commonly found in raw cow's milk, including *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Acinetobacter*, *Corynebacterium*, *Enterococcus*, and *Pseudomonas* (21). With meat, the chemical composition favors the growth of a variety of microorganisms. Preservatives and different packaging methods are generally used to combat microbial growth. Several genera are typically found on freshly cut meat, including the psychrotrophs *Acinetobacter*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas*; *Staphylococcus*; *Moraxella*; lactic acid bacteria; and various genera in the *Enterobacteriaceae* family (22).

Temperature, specifically transport and storage at a cold temperature, has always been used to combat the growth of spoilage bacteria in food products; however, psychrotrophic bacteria has the potential to colonize these products (25). In both meat and milk in cold storage, the most common cause of spoilage is the presence of *Pseudomonas* species (21, 25, 26). Of the *Pseudomonas* genus, the most common species found in meat in cold storage are members of the

*P. fluorescens* group, *P. fragi*, *P. lundensis*, *P. migulae*, and *P. putida* (22). In milk in cold storage, the most common species are members of the *P. fluorescens* group, *P. fragi*, *P. lundensis*, *P. putida*, and *P. psychrophila* (26). Psychrotrophic bacteria are known to produce a variety of enzymes that, among other functions, contribute to the spoilage of milk and meat. These enzymes, which are active at low temperatures in psychrotrophs, include proteases and lipases. In milk, these enzymes are usually directed against casein, the dominant protein found in milk (27, 28, 29). In meat, these enzymes are usually directed against myofibrils and sarcoplasmic proteins. The metabolic byproducts of these bacteria also contribute to food spoilage (30).

## **V. *Pseudomonas lundensis* and the Clinical Laboratory**

*Pseudomonas lundensis* is a bacterium that was first isolated from refrigerated meat and proposed as a new species in 1986. *P. lundensis* is an aerobic, Gram-negative rod that, like many other members of the genus *Pseudomonas*, is motile by means of a polar flagellum. It is a psychrotrophic organism, with a temperature growth range of 0 to 33°C, although optimal growth occurs at a temperature of 25°C (28).

Although *P. lundensis* has primarily been implicated in milk and meat spoilage, recent research using culture-independent methods has demonstrated that it is present in diseased human lungs, particularly those of patients with cystic fibrosis (CF) (31). However, the role of *P. lundensis* in the lung microbiome and its potential role in respiratory disease is unknown. These findings are particularly significant as they underscore the major discrepancy between culture-dependent and culture-independent methods in the clinical laboratory. Growing the bacteria of interest out on agar after isolation from a diseased host is essential, as it is virtually impossible to uncover the functions of specific genes or the mechanism of different pathways through pure sequence data alone (32). We can detect the presence of a particular bacterium and identify the etiology of an infection using molecular techniques, but the ability to culture a bacterium allows us to observe products that it may produce and phenotypes that it may have, giving us insight into the potential roles it may play in things like host health and the planet's ecology. Culturing a specific organism can be difficult, however, as different organisms require different conditions for growth. When difficulties arise, it is not always obvious which growth conditions for a specific bacterium are not being adequately reproduced in the laboratory, creating even more challenges (32).

In this study, our aim was to determine the optimal culture conditions for the isolation of *Pseudomonas lundensis* from food samples, allowing us to identify novel strains of *P. lundensis* and potentially apply this protocol to culturing *P. lundensis* from clinical samples in the future. We began with a wide range of growth temperatures and media types that cater to the growth of many different classes of organisms in order to adequately capture the growth conditions best suited for isolating *P. lundensis*. Because *P. lundensis* is a psychrotrophic organism, we hypothesize that temperature, specifically growth at temperatures between 4-10°C, will be the growth condition with the most utility to our aim. As was aforementioned, research using culture-independent methods has shown that *P. lundensis* is present in diseased human lungs, specifically those of people with CF (31). Defining a protocol for the successful isolation of *P. lundensis* from other microorganisms commonly implicated in human infections would aid in our understanding of its potential roles in the lung microbiome and the disease process.



# MATERIALS AND METHODS

## I. Bacterial Strains, Growth Media, and Temperature

### A. Determining Culture Conditions

Cultures were started from stocks of *Staphylococcus aureus* (strain 10), *Klebsiella pneumoniae* (strain I565), *Escherichia coli* (primary mouse gut isolate), *Pseudomonas fluorescens* (strain AU8050), *Pseudomonas aeruginosa* (strain PAO1), and *Pseudomonas lundensis* (strains AU1044, AU11122, AU11164, AU11235, and AU12644).

All bacterial strains were cultured in 20 mL LB broth (Lennox L Broth Base; Invitrogen), with the exception of *S. aureus*, which was cultured in TSB broth (Trypticase Soy Broth; Difco) at 37°C for 24 hours with shaking. The optical density (OD<sub>600</sub>) of each culture was measured to confirm sufficient growth. 1 mL of each culture was compared against a baseline established using LB broth; measurements were performed using an UltroSpec 500 pro spectrophotometer (Amersham Biosciences).

Each strain was serially diluted to 1:10 in sterile 1X PBS to 10<sup>-8</sup>. The serial dilutions were each plated in duplicate on LB (Invitrogen), NA (Nutrient Agar; Difco), TSA (Trypticase Soy Agar; Difco), SDA (Sabouraud Dextrose Agar; Difco), MacConkey (Difco), VBA (Violet Red Bile Agar; Difco), Cetrimide (Difco), *Pseudomonas*-isolation (Difco), and MRS (de Man, Rogosa, and Sharpe) agar plates. Each type of agar plate was incubated at 4°C, 10°C, room temperature (approximately 21°C), 30°C, and 37°C, for a total of 40 plates per strain.

### B. Isolation from Milk

Raw milk (farm sourced), 1% pasteurized milk (Meijer Brand), 2% pasteurized milk (Guernsey Farms), and ultra-high pasteurized 2% milk (Prairie Farms) were used for bacterial isolation. 20 mL of each type of milk was pipetted into a 125 mL Erlenmeyer flask and incubated at 4°C for the duration of each experiment.

Samples were plated at various intervals after initial incubation. When plating, a 200 µL aliquot of milk was serially diluted 1:10 in 1X PBS to 10<sup>-6</sup>. The serial dilutions were plated in duplicate on LB agar plates and incubated at 4°C and room temperature. An additional aliquot of the milk was diluted in sterile 1X PBS to 10<sup>-3</sup>, and 100 µL of the dilution was spread evenly with a sterile spreader on an LB agar plate, which was then incubated at 4°C. After sufficient growth (appearance of isolated colonies), all plates were wrapped in parafilm and stored at 4°C.

### C. Isolation from Meat

Raw, 10-day-old hamburger meat (purchase location/brand) was stored in a Ziploc bag at 4°C for the duration of the experiment. Samples were plated at various intervals after the initial incubation. A small (approximately 100 µg) sample of the meat was submerged in 2 mL of sterile water in a 5 mL round bottom tube and homogenized using a Tissue Tearor (BioSpec, model # 985370-395) on medium speed. When plating, a 200 µL aliquot of the homogenate was serially diluted 1:10 in sterile 1X PBS to 10<sup>-6</sup>. The serial dilution was plated in duplicate on LB agar plates and incubated at 4°C and room temperature. An additional aliquot of the homogenate was diluted in sterile 1X PBS to 10<sup>-3</sup>, and 100 µL of the dilution was spread evenly with a sterile spreader on an LB agar plate, which was then incubated at 4°C.

## D. Subculture of Milk Isolates

Milk sample isolates grown on LB agar at 4°C or 10°C that demonstrated at least 85% sequence homology with the *P. lundensis* strain AU1044 chromosomal sequence upon BLAST analysis were chosen for further characterization. A sterile loop was used to pick a colony from the agar plates that these isolates were derived and inoculate 20 mL of LB broth. Cultures were incubated at room temperature for 24 hours with shaking. The optical density of each culture was measured to confirm sufficient growth as previously described. The cultures were streaked onto LB agar plates and incubated at room temperature for 24 hours before being wrapped in parafilm and stored at 4°C.

## II. Quantification

Colonies were counted after an average of approximately 7 days (for plates incubated at 4°C), 5 days (for plates incubated at 10°C), and 24 hours (for plates incubated at room temperature, 30°C, and 37°C), although this slightly varied by agar type. The log CFU was calculated by taking the log of the average number of colonies in the lowest countable dilution multiplied by the dilution factor and divided by the volume plated (10 µL). For each bacterial strain, the log CFU for each plate type (agar, temperature) was plotted on a scatterplot using GraphPad Prism software. For the bacteria quantified from milk and meat, the log CFU for each type of milk and meat at each temperature was plotted on a bar graph using GraphPad Prism software.

## III. Gram Stains

Slides were prepared using colonies from the spread plates incubated at 4°C, broth cultures started from each of the selected milk sample isolates, and colonies from the streak plates made using those cultures. For colonies, a sterile toothpick was used to pick a representative of each distinct colony morphology (chosen on the basis of relative size, shape, and color), which was mixed with 10 µL of sterile water on the slide. For broth culture, a sterile loop was used to make a thin smear on the slide. Slides were allowed to air-dry. Cells were heat-fixed by passing the slide through a flame three times. Slides were Gram stained according to the manufacturer's instructions (Difco). Cells were visualized under a microscope at 100X with immersion oil. Photographs were taken using QCapture Suite PLUS software.

## IV. ExoU PCR

Colony PCR using primers specific to the *exoU* gene was performed on the 82 isolates from the 1% pasteurized milk and all of the selected milk sample isolates. A master mix was prepared using 20 pmol (0.4 µL) of the ExoU forward primer (5'-AGC-CGC-CCG-CCG-TTG-ACC-AG-3'), 20 pmol (0.4 µL) of the ExoU reverse primer (5'-GTG-ACC-GCG-CCG-CCC-TGC-TC-3'), and 24 µL of nuclease-free water per sample. 25 µL of master mix was added to each puReTaq Ready-To-Go PCR Bead tube (Illustra) and vortexed gently. A sterile toothpick was used to pick a colony of each distinct colony morphology and inoculate each reaction tube. PCR was run according to the following parameters:

Cycling Step	Temperature (°C)	Time	Number of Cycles
Initial Melt	95	10 min	1
Cycle Melt	94.3	20 sec	25
Annealing	64.6	20 sec	
Elongation	72	40 sec	
Final Elongation	72	1 min	1

## V. 16S rRNA PCR

16S rRNA gene-specific colony PCR was performed on all of the ExoU-positive isolates from the 1% pasteurized milk, all of the 2% pasteurized milk isolates and raw milk isolates, and eight of the nine selected milk sample isolates. A master mix was prepared using 20 pmol (0.4 µL) of D88 forward primer (GAGAGTTTGATYMTGGCTCAG), 20 pmol (0.4 µL) of E94 reverse primer (GAAGGAGGTGWTCCARCCGCA), and 24 µL of nuclease-free water per sample. 25 µL of master mix was added to each puReTaq Ready-To-Go PCR Bead tube (Illustra, cat. # 27955901) and vortexed gently. A sterile toothpick was used to pick a colony of each distinct colony morphology and inoculate each reaction tube. PCR was run according to the following parameters:

Cycling Step	Temperature (°C)	Time	Number of Cycles
Initial Melt	94°	2 min	1
Cycle Melt	94°	30 sec	29
Annealing	50°	45 sec	
Elongation	72°	2 min	
Final Elongation	72°	5 min	1
Soak	4°	Indefinitely	1

## VI. Gel Electrophoresis and DNA Extraction

Gel electrophoresis was used to verify PCR results and to extract DNA for sequencing. For 16S rRNA gene-specific colony PCR gels, 1 gram of agarose was mixed with 100 mL of 1X TAE. For *exoU* gene-specific PCR gels, 2 grams of agarose was mixed with 100 mL of 1X TAE. The mixture was then heated in the microwave for 90 seconds, with stirring at 30-second intervals. SYBR Safe was added to the heated gel, which was then poured into the gel box. Two twelve-well combs were put in place, and the gel was allowed to solidify for 20 minutes. A 1 Kb ladder was used for 16S rRNA gene-specific colony PCR gels and a 100 bp ladder was used for *exoU* gene-specific PCR gels. Gels were run for approximately one hour at 100 mV. For *exoU* gene-specific PCR gels, stock DNA from *P. aeruginosa* (strain PAO1) and *E. coli* (primary mouse gut isolate) and nuclease-free water were used as negative controls; stock DNA from *P. lundensis* (strain AU1044) was used as a positive control. Photographs of *exoU* gene-specific gels were taken using a ChemiDoc XRS+ Gel Imaging System with ImageLab software (Bio-Rad, cat. # 1708265). For 16S rRNA gene-specific colony PCR, gels were purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

## **VII. DNA Sequencing**

All 16S rRNA gene-specific colony PCR products were submitted for Sanger sequencing, along with 10  $\mu$ L of forward or reverse primer per sample. All DNA sequencing was conducted by the University of Michigan Medical School DNA Sequencing Core.

## **VIII. Sequence Analysis**

The sequence for each isolate was compared to the National Center for Biotechnology Information (NCBI) DNA sequence database and the *Pseudomonas lundensis* (strain AU1044) chromosomal sequence using BLAST analysis. Isolates grown on LB agar at 4°C or 10°C whose BLAST results returned matches for *Pseudomonas* species and whose sequences were at least 85% similar to the *P. lundensis* strain AU1044 chromosomal sequence were chosen for isolate banking and further characterization.

The isolates selected for further characterization were also sequenced and compared to the NCBI DNA sequence database and the *P. lundensis* strain AU1044 chromosomal sequence if it was not already one of the top five matches in order to confirm previous results.

## **X. Isolate Banking**

8 mL of each of the fresh cultures grown from the selected subset of milk sample isolates (101, 103, 105, 113, 206, R02, R03, and R04) was mixed with 2 mL of 10% glycerol (prepared from a 50% glycerol stock solution) and divided evenly among 5 cryogenic vials. All vials were stored at -80°C.

## RESULTS

### I. Determination of the optimal growth conditions for the isolation of *Pseudomonas lundensis*

In order to assess the growing conditions that would give us the best chance of isolating a novel strain of *Pseudomonas lundensis* from food samples, several different species of bacteria were plated on various agar types and incubated at different temperatures. We chose to vary two different growth conditions in our experiments, growth medium and temperature, both of which can be important in isolating a bacterium of interest. Figures 1A-J show the level of growth of each species of bacteria under each combination of growth conditions. A good level of growth was generally considered to be around 8-10 log CFU/mL, while a poor level of growth was generally considered to be  $\leq 5$  log CFU/mL. Inconsistent growth was considered to be growth that varied over a considerably large range across individual experiments.

#### A. Bacterial Growth at 4°C

At 4°C, only *P. fluorescens* strain AU8050 and *P. lundensis* strains AU1044, AU11122, AU11164, AU11235, and AU12644 grew well on all agar types, with the exception of VBA, Cetrimide, and MRS. On these agars, *P. fluorescens* strain AU8050 and the *P. lundensis* strains generally grew poorly or not at all. On all agar types, *E. coli* primary mouse gut isolate, *K. pneumoniae* strain I565, and *S. aureus* strain 10 exhibited no growth. *P. aeruginosa* strain PA01 also grew either very poorly or not at all on all agar types.

#### B. Bacterial Growth at 10°C

At 10°C, *S. aureus* strain 10 exhibited no growth on all agar types. *E. coli* primary mouse gut isolate did not grow on most agar types, but did exhibit good growth on LB, NA, and TSA. *P. aeruginosa* strain PA01 exhibited inconsistent growth on all agar types, ranging from approximately 5-10 log CFU. *K. pneumoniae* strain I565 grew well on all agar types, with the exception of *Pseudomonas*-isolation agar, on which it showed inconsistent growth ranging from approximately 5-10 log CFU. *P. fluorescens* strain AU8050 and the *P. lundensis* strains grew well on all agar types, with the exception of *Pseudomonas*-isolation agar, Cetrimide, and MRS, on which they exhibited inconsistent to no growth.

#### C. Bacterial Growth at Room Temperature

At room temperature (approximately 21°C), *S. aureus* strain 10 exhibited poor growth on all agar types, with the exception of LB, NA, SDA, and *Pseudomonas*-isolation agar, on which it grew well. *E. coli* primary mouse gut isolate grew well on all agar types, with the exception of MacConkey, VBA, Cetrimide, and *Pseudomonas*-isolation agar, on which it exhibited inconsistent growth. *K. pneumoniae* strain I565 also grew well on all agar types, with the exception of Cetrimide and *Pseudomonas*-isolation agar, on which it exhibited inconsistent growth. *P. aeruginosa* grew well on all agar types as well, although it did not exhibit any growth on MRS. *P. fluorescens* strain AU8050 and the *P. lundensis* strains grew well on all agar types, with a few exceptions. *P. fluorescens* strain AU8050 grew poorly on Cetrimide and not at all on MRS. The *P. lundensis* strains exhibited inconsistent growth on VBA, Cetrimide, and *Pseudomonas*-isolation agar, while not exhibiting any growth on MRS.

#### **D. Bacterial Growth at 30°C**

At 30°C, *S. aureus* strain 10 grew poorly on all agar types, with the exception of LB, NA, and TSA, on which it exhibited inconsistent growth. *E. coli* primary mouse gut isolate, *K. pneumoniae* strain I565, *P. aeruginosa* strain PA01, *P. fluorescens* strain AU8050, and the *P. lundensis* strains grew well on all agar types, again with a few exceptions for each species. *E. coli* primary mouse gut isolate and *K. pneumoniae* strain I565 exhibited inconsistent growth on Cetrimide and *Pseudomonas*-isolation agar. *P. aeruginosa* grew well on all agar types except for MRS, where it exhibited no growth. *P. fluorescens* strain AU8050 grew poorly or not at all on Cetrimide and exhibited no growth on MRS. The *P. lundensis* strains grew poorly or not at all on Cetrimide and *Pseudomonas*-isolation agar, while exhibiting no growth on MRS.

#### **E. Bacterial Growth at 37°C**

At 37°C, *S. aureus* strain 10 grew poorly on all agar types, with the exception of LB, NA, TSA, and *Pseudomonas*-isolation agar, on which it grew well. *E. coli* primary mouse gut isolate grew well on all agar types, with the exception of Cetrimide and *Pseudomonas*-isolation agar, on which it exhibited inconsistent growth. *K. pneumoniae* strain I565 grew well on all agar types, with the exception of MacConkey, Cetrimide, and *Pseudomonas*-isolation agar, on which it exhibited inconsistent growth. *P. aeruginosa* strain PA01 grew well on all agar types, with the exception of MRS, on which it exhibited no growth. *P. fluorescens* strain AU8050 did not exhibit growth on NA, VBA, or MRS, while exhibiting inconsistent growth on LB, MacConkey, SDA, TSA, Cetrimide, and *Pseudomonas*-isolation agar. Growth was generally better on LB, MacConkey, and *Pseudomonas*-isolation agar for *P. fluorescens* strain AU8050 than on SDA, TSA, and Cetrimide. The *P. lundensis* strains grew well on all agar types, with several exceptions. No growth was observed on NA, *Pseudomonas*-isolation agar, or MRS, while low to no growth was observed on VBA and Cetrimide.

Overall, no distinct differential growth pattern was observed based on agar type. However, based on these results, it is clear that 4°C is the optimal growth temperature for isolating strains of *P. lundensis* from other bacterial species, although *P. fluorescens* was observed to have very similar growth conditions to those of *P. lundensis*.

## **II. Assessment of the culturability of psychrotrophs and mesophiles from milk and meat**

Since the strains of *P. lundensis* that we tested grew at 4°C, unlike nearly all of the other species tested (with the exception of *P. fluorescens* strain AU8050), and grew well on LB agar at this temperature, we used this combination of growth conditions to attempt to isolate novel strains of *P. lundensis* from food samples. In order to monitor the levels of growth of psychrotrophic and mesophilic bacteria in raw milk, pasteurized 2% milk, ultra-pasteurized 2% milk, and raw hamburger meat (which was homogenized prior to plating), 1:10 serial dilutions of each food sample were plated on LB agar and incubated at 4°C and room temperature (approximately 21°C). The milk cultures and the raw hamburger meat were stored at 4°C for the duration of the experiment in order to preserve the viability of any psychrotrophs present, with plating occurring at various intervals over time periods ranging from roughly two weeks to one month, depending on the level of growth observed from each food sample. Food samples were

monitored for growth for at least 10 days before ceasing sampling. The ratios of the room temperature log CFU to the 4°C log CFU for each type of milk and meat were also calculated to determine if low-temperature incubation was excluding any organisms of interest.

### **A. Culturability from Raw Milk**

Figures 2A and 2B show the level of growth of psychrotrophic and mesophilic bacteria grown from raw milk. Distinct colonies appeared after one day for the plates incubated at room temperature and an average of 5.42 days for the plates incubated at 4°C. For both sets of plates, the log CFU/mL was relatively low on all plates up until around 7 days of incubation of the initial raw milk culture. There was only a very slow increase in bacterial growth observed over the next 16-17 days. The level of bacterial growth was very similar for both temperatures, with the highest level reached being approximately 9 log CFU/mL. Figure 2C shows the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated. The ratios ranged from 0.992 to 1.34 (average of 1.04), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.

### **B. Culturability from Pasteurized 2% milk**

Figures 3A and 3B show the level of growth of psychrotrophic and mesophilic bacteria grown from pasteurized 2% milk. Distinct colonies appeared after an average of 1.22 days post-plating for the plates incubated at room temperature and an average of 6.40 days post-plating for the plates incubated at 4°C. For both sets of plates, growth wasn't observed up until 13 days of incubation of the initial pasteurized 2% milk culture, but showed a general upward trend over the next 16-17 days. This upward trend in growth was much steeper with the 4°C plates, but the highest level of bacterial growth reached was very similar with both temperatures at approximately 9 log CFU/mL. Figure 3C shows the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated. The ratios ranged from 1 to 2.35 (average of 1.37), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.

### **C. Culturability from Ultra-Pasteurized 2% Milk**

Figures 4A and 4B show the level of growth of psychrotrophic and mesophilic bacteria grown from ultra-pasteurized 2% milk. No growth was detected on any of the plates over the entire time period that samples were plated and observed, suggesting that ultra pasteurization is highly effective at eliminating even psychrotrophic organisms from milk.

### **D. Culturability from Raw Hamburger Meat**

Figures 5A and 5B show the level of growth of psychrotrophic and mesophilic bacteria grown from the homogenized raw hamburger meat. Distinct colonies appeared one day post-plating for the plates incubated at room temperature and an average of 5.38 days post-plating for the plates incubated at 4°C. For both sets of plates, the level of bacterial growth was high initially and increased only slightly over the roughly two-week time period that samples were plated. The highest level of bacterial growth reached was about the same with both temperatures at around 9.50 log CFU/mL. The plates at both temperatures always appeared overgrown, so no isolates were obtained from the meat. Figure 5C shows the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated. The ratios ranged from 0.998 to

1.02 (average of 1.01), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.

The culturability of mesophiles compared to psychrotrophs from raw milk, pasteurized 2% milk, ultra-pasteurized 2% milk, and raw hamburger meat was approximately equal, indicating that the organisms grown at room temperature are the same as those grown at 4°C. Although no isolates could be obtained from the raw hamburger meat, a total of 17 isolates were obtained from the various types of milk, with eight of those isolates grown under the growth conditions identified as being optimal for the isolation of *P. lundensis*. Those eight isolates were chosen for further characterization, as we suspected that they were likely *P. lundensis* (at least 85% sequence homology to *P. lundensis*) or another pseudomonad.

### **III. Taxonomic identification of milk sample isolates**

#### **A. Gram stains**

Testing the isolates for their Gram stain reaction was the first step in identification; we were particularly hoping to see Gram negative bacteria, which could potentially lead us to *P. lundensis*. All isolates grown from the pasteurized 2% milk and raw milk and all of the ExoU-positive isolates from the pasteurized 1% milk were tested for their Gram stain reaction. Of the five isolates grown from the pasteurized 2% milk, three of them (isolates 207, 208, and 209) repeatedly stained very poorly or not at all, so a definitive reaction to the Gram stain could not be determined. Isolates 205 and 206 stained well; isolate 205 appeared as a Gram positive bacillus, while isolate 206 appeared as a Gram negative bacillus. All four isolates (isolates R01, R02, R03, and R04) grown from the raw milk and all of the ExoU-positive isolates (isolates 101, 102, 103, 105, 113, 122, 126, 139, 140, 141, 142, and 147) grown from the pasteurized 1% milk appeared as Gram negative bacilli (not shown).

#### **B. Colony PCR with ExoU gene-specific primers**

In order to rule out those isolates that were likely not *P. lundensis*, we performed colony PCR with *exoU* gene-specific primers on all 82 of the isolates from the pasteurized 1% milk. Of the 82 isolates grown from the pasteurized 1% milk, 12 of the isolates appeared positive, indicated by a bright band at 500 base pairs (the approximate size of the ExoU PCR product), after gel electrophoresis was performed on the ExoU PCR products (not shown).

#### **C. Colony PCR with 16S rRNA gene-specific primers, sequencing, and BLAST analysis**

In order to identify each of the milk sample isolates, colony PCR with 16S rRNA gene-specific primers was performed on all of the isolates grown from the pasteurized 2% milk and raw milk and all of the ExoU-positive isolates from the pasteurized 1% milk. All colony PCR products were run on an agarose gel to confirm the presence of a PCR product before being sent for sequencing.

Table 1 shows the BLAST analysis results of the sequences obtained for these isolates. All isolates had at least one match for a *Pseudomonas* species, with the exception of isolate 205, which only returned results for *Paenibacillus* species; it was eliminated from being chosen for further characterization and banking. Isolates 102, 103, 105, and 113 all had the *P. lundensis* AU1044 chromosomal sequence as one of their top five results with a high percentage of



identity, ranging from 96 to 98%; these isolates were selected for further characterization and banking, although isolate 102 did not end up growing when subcultured. Isolates 105 and 113 returned the *P. lundensis* AU1044 chromosomal sequence as the top result, both with a percentage of identity of 98%. Isolate 101 matched to two different strains of *P. lundensis* upon initial isolation, strains Z54a and W47a, but with a relatively low percentage of identity at 86%. The remaining isolates grown from the pasteurized 1% milk (122, 126, 139, 140, 141, 142, and 147) returned results for various other *Pseudomonas* species, including psychrotrophic species *P. psychrophila* and *P. fragi*, as well as *P. aeruginosa*, which is not commonly isolated from milk. Isolate 147 was most similar to the bacterium *Stenotrophomonas maltophila*, as nearly all of the results indicated a high similarity to *S. maltophila* strains.

The sequences for isolates 206, R02, R03, and R04 were all 95-98% identical to the *P. lundensis* AU1044 chromosomal sequence, but it was not listed as one of the top results; other results included matches for various *Pseudomonas* species, including *P. psychrophila*, *P. fragi*, *P. helleri*, and *P. weihenstephanensis*. These isolates were also selected for further characterization and banking.

Of the 17 total isolates that were obtained from the various milk types, eight of these were chosen for further analysis on the basis of which agar they were grown on, what temperature they grew at, and the percentage of sequence homology they showed with the *P. lundensis* strain AU1044 chromosomal sequence upon BLAST analysis. All eight isolates were grown on LB agar. Isolates 101, 103, 105, 206, R02, R03, and R04 were grown at 4°C, while isolate 113 was grown at 10°C. All of the isolates showed at least 85% sequence homology with the *P. lundensis* strain AU1044 chromosomal sequence upon BLAST analysis.

## **IV. Definitive taxonomic identification of selected subset of milk sample isolates**

### **A. Gram stains**

In order to more definitively identify them, we subcultured and banked a specific subset of milk sample isolates (101, 103, 105, 113, 206, R02, R03, and R04) that were previously identified by BLAST analysis as having a high similarity to the *P. lundensis* strain AU1044 chromosomal sequence (at least 85% sequence homology). First, Gram stains were performed on fresh colonies grown from the banked isolates, the results of which matched with what we saw in previous Gram stains of the original colonies of these isolates, which were all Gram-negative bacilli (Fig. 6).

### **B. Colony PCR with ExoU gene-specific primers**

Colony PCR using primers specific to the *exoU* gene was performed on fresh colonies grown from the banked isolates, followed by gel electrophoresis of the PCR products. Figure 7 shows the ExoU agarose gel of the eight isolates that were previously identified as having a high likelihood of being *P. lundensis* (or even another pseudomonad). As indicated by the bright band at approximately 500 base pairs (the approximate size of the ExoU PCR product), isolates 101, 103, 105, and 113 appeared positive for the ExoU PCR product, demonstrating the presence of the *exoU* gene in these isolates.

### C. Colony PCR with 16S rRNA gene-specific primers, sequencing, and BLAST analysis

16S rRNA gene-specific colony PCR, sequencing, and BLAST analysis was performed on fresh colonies grown from the banked isolates in order to confirm that we had pure isolates and to further prove their identities. Table 2 shows the results of the BLAST analysis. The sequences for isolates 101, 103, 105, and 113 were all highly similar to the *P. lundensis* strain AU1044 chromosomal sequence, with a percentage of identity ranging from 95-98%; the *P. lundensis* sequence was also the top BLAST result for these isolates. All four of these isolates were also demonstrated to be Gram-negative bacilli and positive for the *exoU* gene by *exoU* gene-specific PCR, confirming their identities as strains of *P. lundensis*.

The sequences for isolates 206 and R03 had 96% and 97% homology with a strain of *P. fragi* (strains DBC and PF04), respectively. *P. fragi* was the top BLAST result and appeared more than once in the top five BLAST results for these isolates. As these isolates were also demonstrated to be Gram-negative bacilli and negative for the *exoU* gene by *exoU* gene-specific PCR, we concluded that these isolates are both *P. fragi*.

The sequences for isolates R02 and R04 were more variable in their BLAST results, with high percentages of sequence homology (98% and 97%) with different strains of both *P. helleri* and *P. psychrophila*. Both of these isolates were also demonstrated to be Gram-negative bacilli and negative for the *exoU* gene by *exoU* gene-specific PCR. Because the percentage of sequence homology between the isolates and *P. helleri* and *P. psychrophila* are identical, we can only conclude that these isolates are a species of *Pseudomonas*, most likely either *P. helleri* or *P. psychrophila*.

Isolate	Source	Growth on LB at		Gram stain	16S rRNA identification	%ID	ExoU PCR
		4°C	10°C				
101	Pasteurized 1% milk	+	-	-	<i>Pseudomonas lundensis</i>	98%	+
103	Pasteurized 1% milk	+	-	-	<i>Pseudomonas lundensis</i> <i>Pseudomonas psychrophila</i>	97% 97%	+
105	Pasteurized 1% milk	+	-	-	<i>Pseudomonas lundensis</i> <i>Pseudomonas psychrophila</i>	97% 97%	+
113	Pasteurized 1% milk	-	+	-	<i>Pseudomonas lundensis</i>	95%	+
206	Pasteurized 2% milk	+	-	-	<i>Pseudomonas fragi</i> <i>Pseudomonas psychrophila</i>	96% 96%	-
R02	Raw milk	+	-	-	<i>Pseudomonas helleri</i> <i>Pseudomonas psychrophila</i>	98% 98%	-
R03	Raw milk	+	-	-	<i>Pseudomonas fragi</i>	97%	-
R04	Raw milk	+	-	-	<i>Pseudomonas helleri</i> <i>Pseudomonas psychrophila</i> <i>Pseudomonas lundensis</i>	97% 97% 97%	-

## DISCUSSION

This study was performed in order to identify the optimal conditions for selectively growing *Pseudomonas lundensis* and apply those growth conditions to isolating novel strains of *P. lundensis* from food samples. By observing the level of growth exhibited by a variety of bacterial species on different agars at different temperatures, it was determined that the psychrotrophic nature of *P. lundensis* would be the most valuable when attempting to isolate novel strains from food samples. Most of the bacteria we observed grew best at a temperature between 21°C and 37°C, other than *P. fluorescens* strain AU8050, which grew well at lower temperatures. There did not appear to be a pattern of differential growth based on agar type that could be exploited to isolate *P. lundensis* from food samples, which was why LB agar was chosen, as *P. lundensis* universally grew well on this agar.

Other studies have shown that *P. lundensis* can be isolated from milk and meat at low temperatures (21-23, 25-30), but have not focused on specifically demonstrating the poor growth of other species of bacteria under the same conditions at which *P. lundensis* grows well. Our experiments showed that organisms commonly implicated in human infections, such as *E. coli*, *K. pneumoniae*, *S. aureus*, and *P. aeruginosa*, grow very poorly or not at all at 4°C, the same temperature at which several strains of *P. lundensis* grow very well. Although LB agar is a general purpose medium that supports the growth of a wide range of bacteria, we showed that temperature is a significant enough selective pressure to inhibit the growth of microorganisms that would otherwise grow on this agar.

Using the combination of a low temperature (4°C) and a supportive medium (LB agar), we attempted to isolate *P. lundensis* from raw milk, pasteurized milk, ultra-pasteurized milk, and raw hamburger meat. In addition to the low-temperature incubation, we also monitored the growth of mesophiles in each of the food samples by plating dilutions at room temperature. For each food sample, the ratios of growth at room temperature to growth at 4°C were all very close to 1, so it appears that the organisms growing at room temperature are the same as those growing at 4°C. Additionally, the morphological appearance of the colonies on the plates at both temperatures was extremely similar, also pointing to the conclusion that the organisms growing at both temperatures are likely the same.

From the milk, we were able to obtain four isolates of *P. lundensis* (isolates 101, 103, 105, and 113). We were also able to obtain isolates of other pseudomonads (all other isolates, other than isolates 147 and 205, which were identified as *Stenotrophomonas maltophilia* and *Paenibacillus odorifer*, respectively) from milk using this same protocol. These isolates were identified on the basis of 16S rRNA gene sequence and gel electrophoresis of *exoU* gene-specific PCR products. Isolates 103 and 105 were identified as *P. lundensis*, but they both had 16S rRNA sequences that were highly similar to those of *P. lundensis* and *P. psychrophila*; however, the presence of PCR amplification of the *exoU* gene in these isolates definitively determined their identity as *P. lundensis*. Additionally, isolate R04's 16S rRNA sequence was highly similar to those of *P. helleri*, *P. psychrophila*, and *P. lundensis*; in this case, the lack of PCR amplification of the *exoU* gene ruled out *P. lundensis* as its identity, so we concluded that its identity is likely that of *P. helleri* or *P. psychrophila*. In the future, full sequencing of these isolates could be performed using sequencing technology such as the MinION device (Oxford Nanopore Technologies). Although we were able to isolate *P. lundensis* on several occasions, most of our isolates were identified as other pseudomonads, indicating that more selectivity may be required in order to specifically target *P. lundensis*. However, low-temperature incubation is not typically

used for bacterial culture in clinical laboratories (34-37), so it may be sufficient in trying to culture psychrotrophs like *P. lundensis* from clinical samples.

Our protocol was not effective in isolating any strains of *P. lundensis* from ultra-pasteurized milk; in fact, we were not able to culture any bacteria from it at all. This suggests that ultra pasteurization is either highly effective in eliminating bacteria, even psychrotrophs, from milk, or that our protocol was not catering to the favored growth conditions of any bacteria potentially present in the milk. Additionally, our protocol was not effective in isolating any strains of *P. lundensis* from hamburger meat, given time constraints and difficulty diluting the homogenized meat in order to obtain isolated colonies suitable for testing. The meat that we used had a very high bacterial load initially, even when significantly diluted; in future experiments, fresh meat should be obtained and more plates should be prepared with a range of dilutions of the meat homogenate in order to grow isolated colonies that can be used for identification tests. Despite this, the *P. lundensis* isolates obtained from milk demonstrates the efficacy of our protocol in specifically isolating *P. lundensis* from food samples.

In the clinical setting, *P. lundensis* is relatively unknown. Our group has recently published the sequence of the first human isolate of *P. lundensis*; other isolates of *P. lundensis* have been sequenced, but all of them have been from spoiled meat (31). Before our group's work, *P. lundensis* had not previously been isolated from human specimens. *P. lundensis* still has not been directly cultured on agar plates from human specimens, despite the aforementioned evidence of its presence in diseased human lungs. Culture-independent methods of identification, such as high-throughput sequencing, more frequently report the presence of a bacterium in human metagenomic DNA obtained from different body sites than do culture-dependent methods of identification (4, 33). This is true of many different species of bacteria, but is especially true of *P. lundensis*, as it has never been reported by standard clinical laboratory culture methods. Typically, respiratory tract specimens, where *P. lundensis* DNA has been detected, are inoculated on very specific media plates. Each specimen is usually streaked onto a sheep blood agar plate, a MacConkey agar plate, a phenylethyl alcohol plate, and a chocolate agar plate and incubated at a temperature between 33°C and 37°C in order to obtain the best results (34-37). Although a range of agars and temperatures are used, it is clear that a specific set of organisms is being targeted, which sets the stage for missing the presence of other bacteria that may be of importance.

There may be a variety of reasons why a particular bacterial species does not show up in routine clinical laboratory cultures. As *P. lundensis* is primarily implicated as a food spoilage microorganism and has been identified as a psychrotrophic organism, it is likely that standard clinical laboratory culture practices are simply missing the mark in creating an optimal growing environment for the bacterium. Although it does not appear that *P. lundensis* is causing acute illness in the patients where it has been detected, its clinical significance has yet to be explored. The features that make it a key player in food spoilage could very well have pathogenic effects in a human host, especially one already susceptible to opportunistic infection, such as a patient with cystic fibrosis.

This study has demonstrated the efficacy of a protocol for selectively growing *P. lundensis* and isolating it from food samples. Reiterating the importance of culture in characterizing a microorganism, there is much we could learn about *P. lundensis* and its place in the lung disease process from culturing it from clinical specimens. Sequence data is significant, but alone it is not enough to elucidate the mechanisms of specific genes and gene pathways or their role in host health. The broader implication of this study is the potential for the growth

conditions identified as being effective in isolating *P. lundensis* from food samples to be applied in the clinical setting for directly culturing *P. lundensis* from human specimens.

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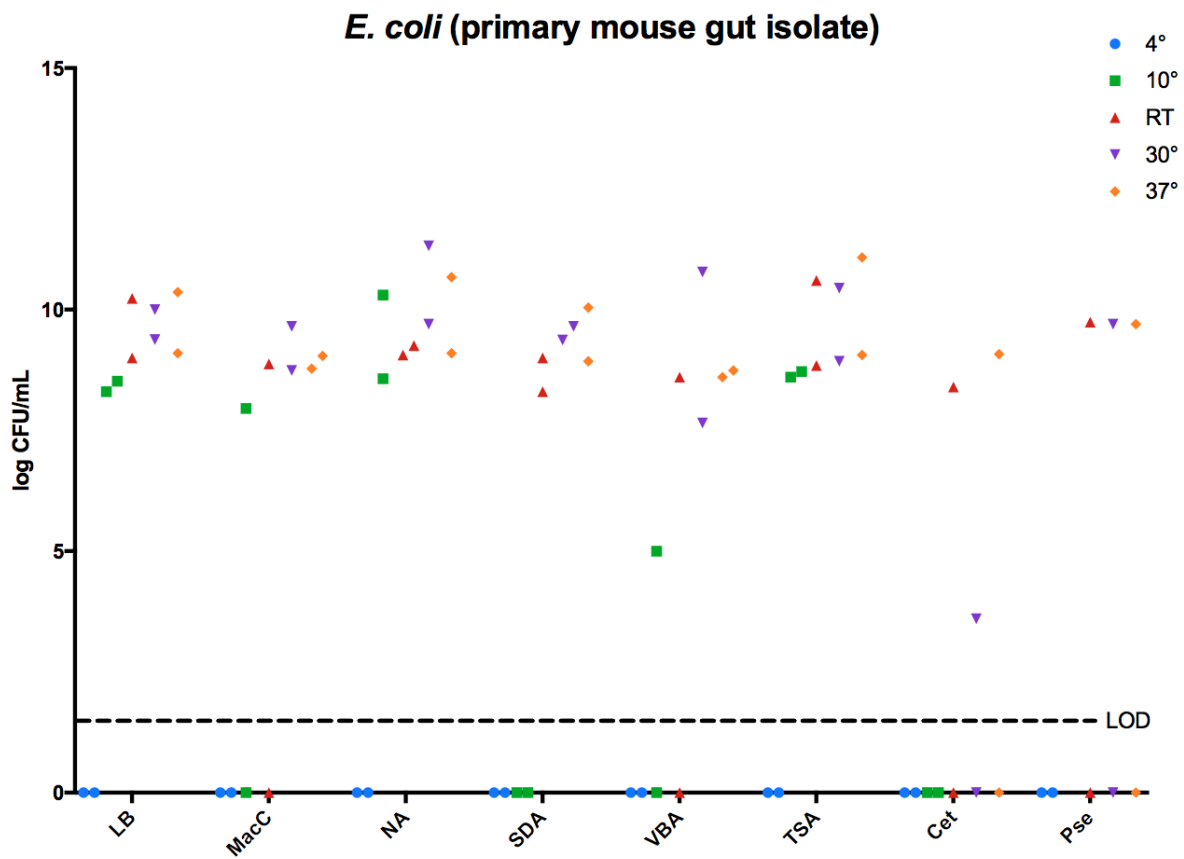
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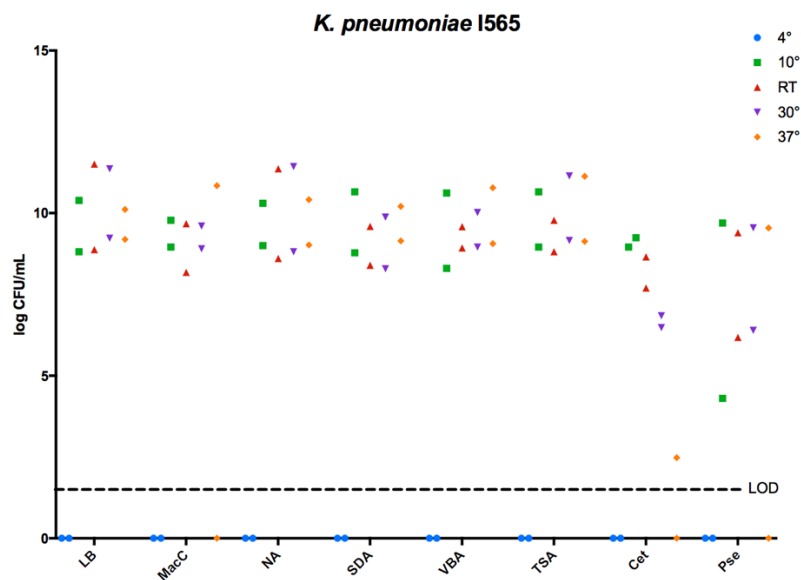


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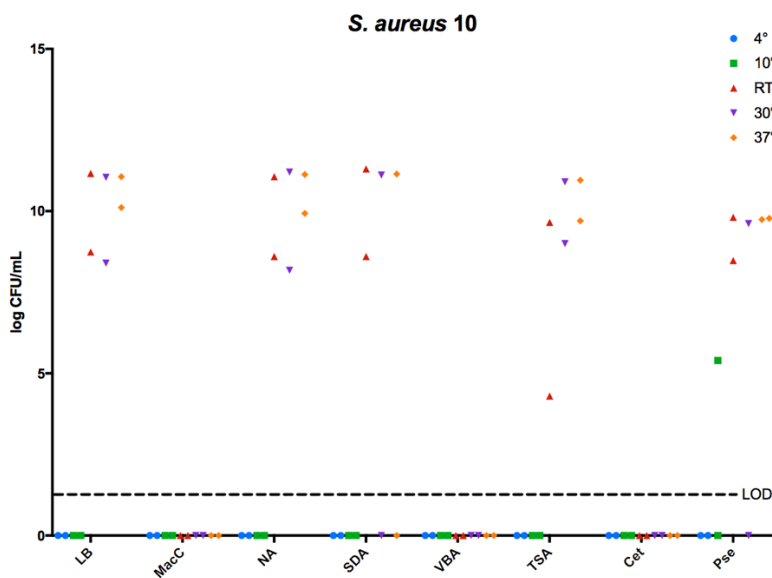
## FIGURES



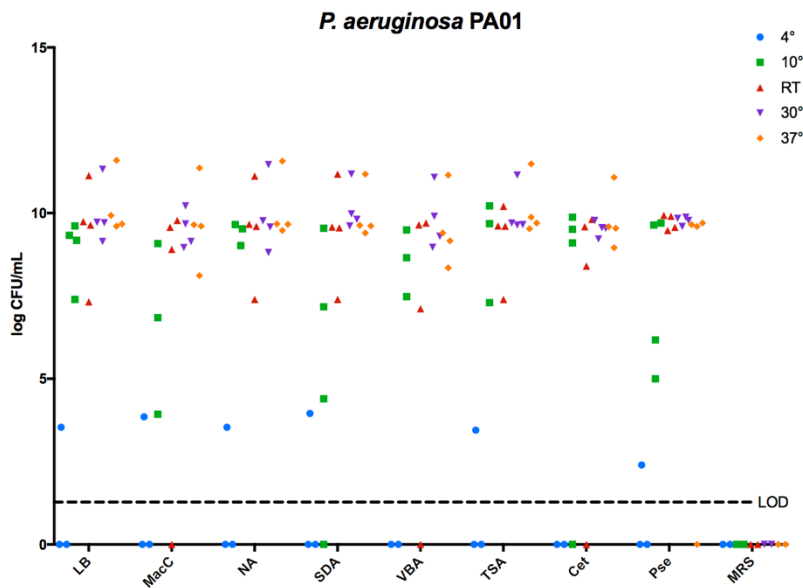
**Figure 1A. Differential growth of *Escherichia coli* (primary mouse gut isolate) at different temperatures on various agar types.** 1:10 serial dilutions of bacterial culture were plated on different agar types (Lennox L agar, MacConkey agar, Nutrient Agar, Sabouraud Dextrose Agar, Violet Red Bile Agar, Trypticase Soy Agar, Cetrinide agar, and *Pseudomonas*-isolation agar) and incubated at 4°C, 10°C, room temperature (approximately 21°C), 30°C, and 37°C until distinct colonies appeared. The average number of colonies were counted at the lowest dilution in which distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. Data points represent individual plates. *E. coli* primary mouse gut isolate was unable to grow at cool temperatures on all agars, only growing on a few agars at 10°C; poor growth was also demonstrated at all temperatures on Cetrinide agar and *Pseudomonas*-isolation agar.



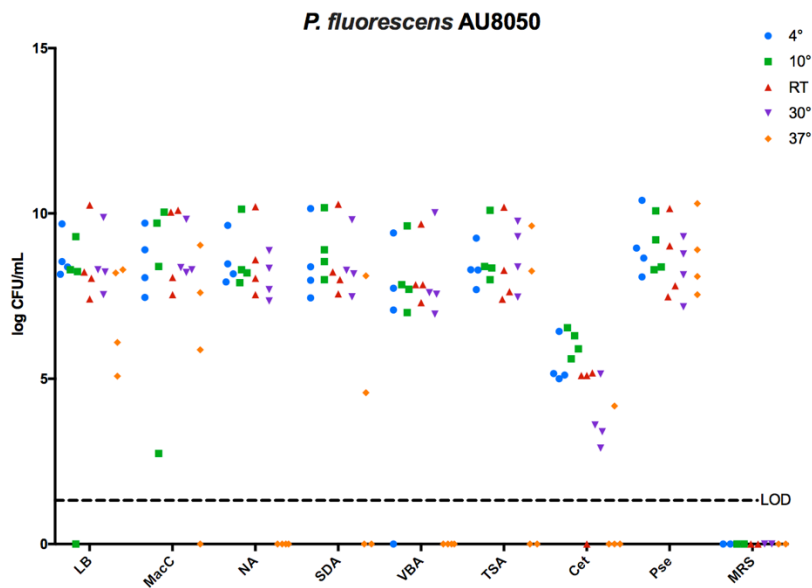
**Figure 1B. Differential growth of *Klebsiella pneumoniae* (strain I565) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *K. pneumoniae* strain I565 was unable to grow at 4°C on all agar types; poor growth was also demonstrated at all temperatures on Cetrimide agar and *Pseudomonas*-isolation agar.



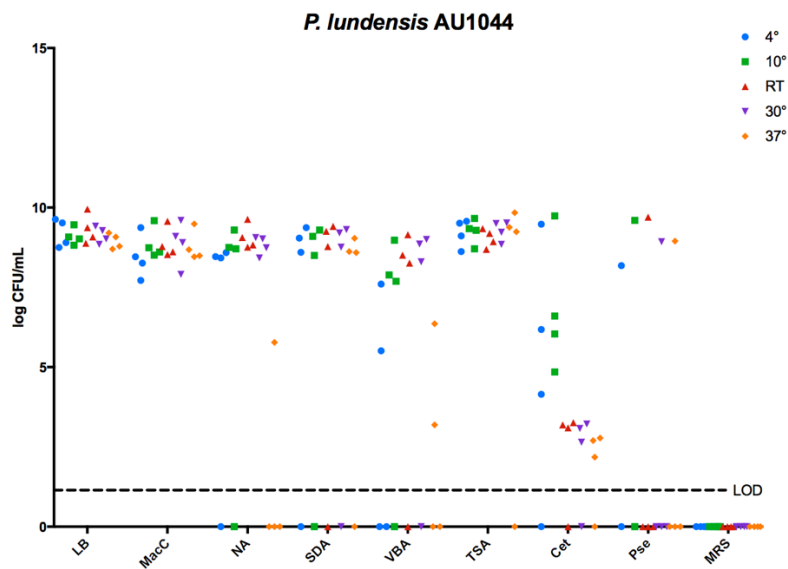
**Figure 1C. Differential growth of *Staphylococcus aureus* (strain 10) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *S. aureus* strain 10 was unable to grow at cool temperatures on all agars; growth was also absent at all temperatures on MacConkey agar, Violet Red Bile agar (VBA), and Cetrimide agar.



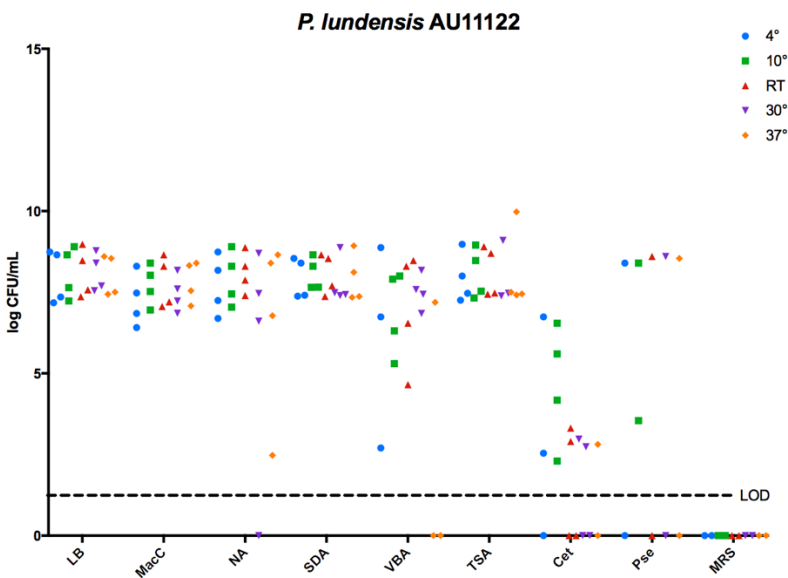
**Figure 1D. Differential growth of *Pseudomonas aeruginosa* (strain PA01) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *P. aeruginosa* strain PA01 demonstrated inconsistent growth at cool temperatures on all agars; little to no growth was observed at 4°C. Growth was also absent at all temperatures on MRS agar.



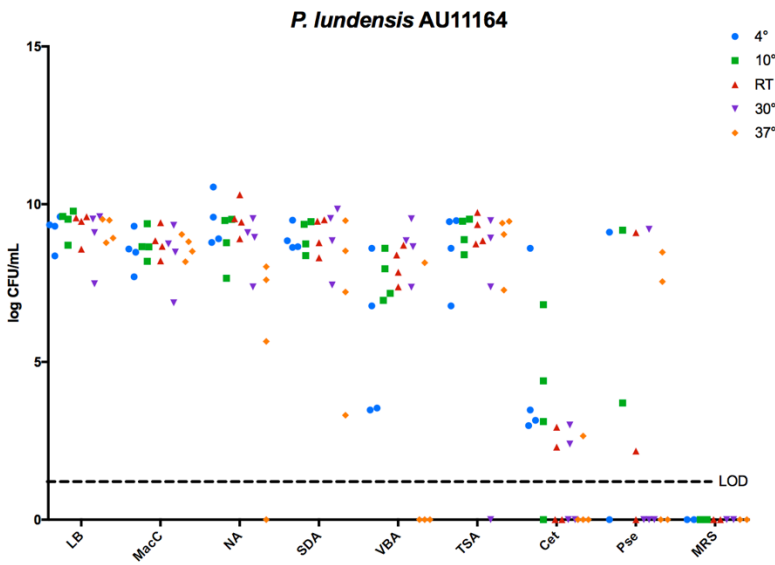
**Figure 1E. Differential growth of *Pseudomonas fluorescens* (strain AU8050) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *P. fluorescens* strain AU8050 demonstrated little to no growth at 37°C on most agars; poor growth was also demonstrated at all temperatures on Cetrimide agar and absent at all temperatures on MRS agar.



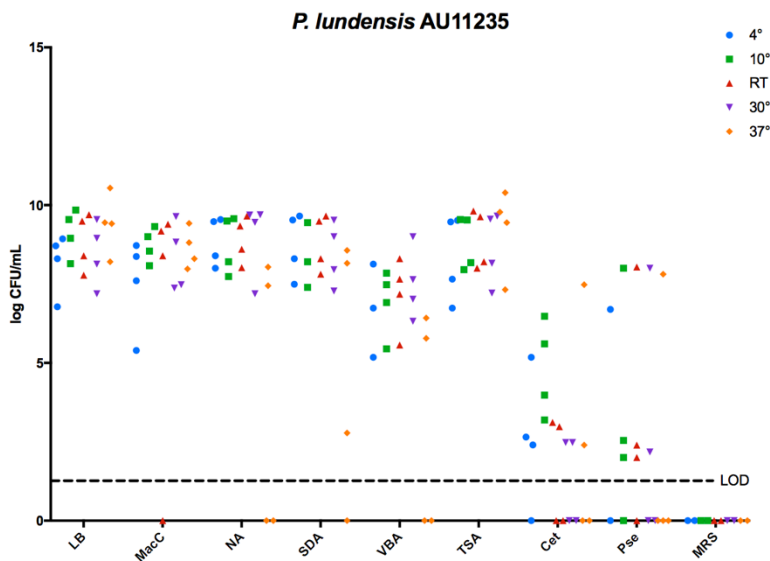
**Figure 1F. Differential growth of *Pseudomonas lundensis* (strain AU1044) at different temperatures on various agar types.** 1 See Fig. 1A for methodology. Data points represent individual plates. *P. lundensis* strain AU1044 demonstrated poor growth at all temperatures on Cetrimide agar and *Pseudomonas*-isolation agar; growth was also absent at all temperatures on MRS agar.



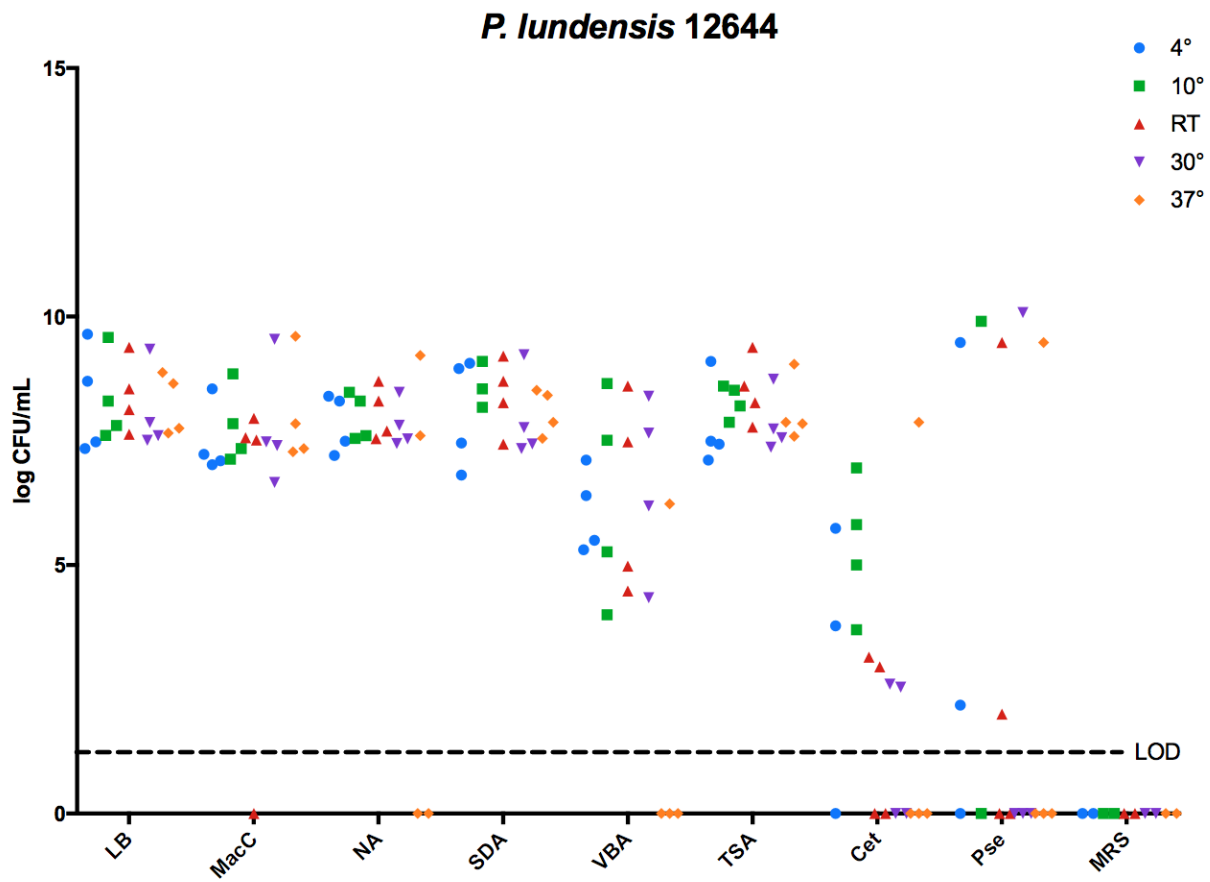
**Figure 1G. Differential growth of *Pseudomonas lundensis* (strain AU11122) at different temperatures on various agar types.** 1 See Fig. 1A for methodology. Data points represent individual plates. *P. lundensis* strain AU11122 demonstrated poor growth at all temperatures on Cetrimide agar and *Pseudomonas*-isolation agar; growth was also absent at all temperatures on MRS agar.



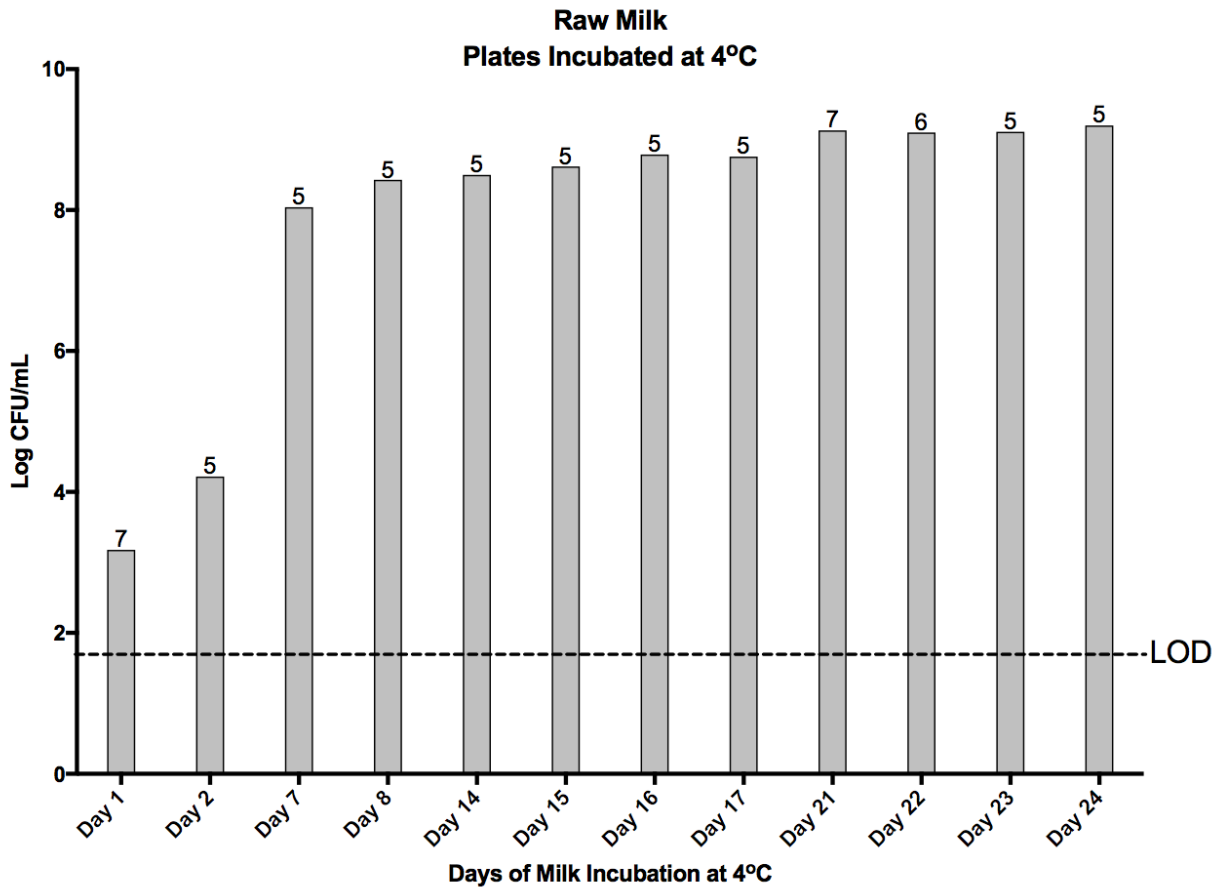
**Figure 1H. Differential growth of *Pseudomonas lundensis* (strain AU11164) at different temperatures on various agar types.** 1 See Fig. 1A for methodology. Data points represent individual plates. *P. lundensis* strain AU11164 demonstrated poor growth at all temperatures on Cetrimide agar and *Pseudomonas*-isolation agar; growth was also absent at all temperatures on MRS agar.



**Figure 1I. Differential growth of *Pseudomonas lundensis* (strain AU11235) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *P. lundensis* strain AU11235 demonstrated poor growth at all temperatures on VBA agar, Cetrimide agar, and *Pseudomonas*-isolation agar; growth was also absent at all temperatures on MRS agar.

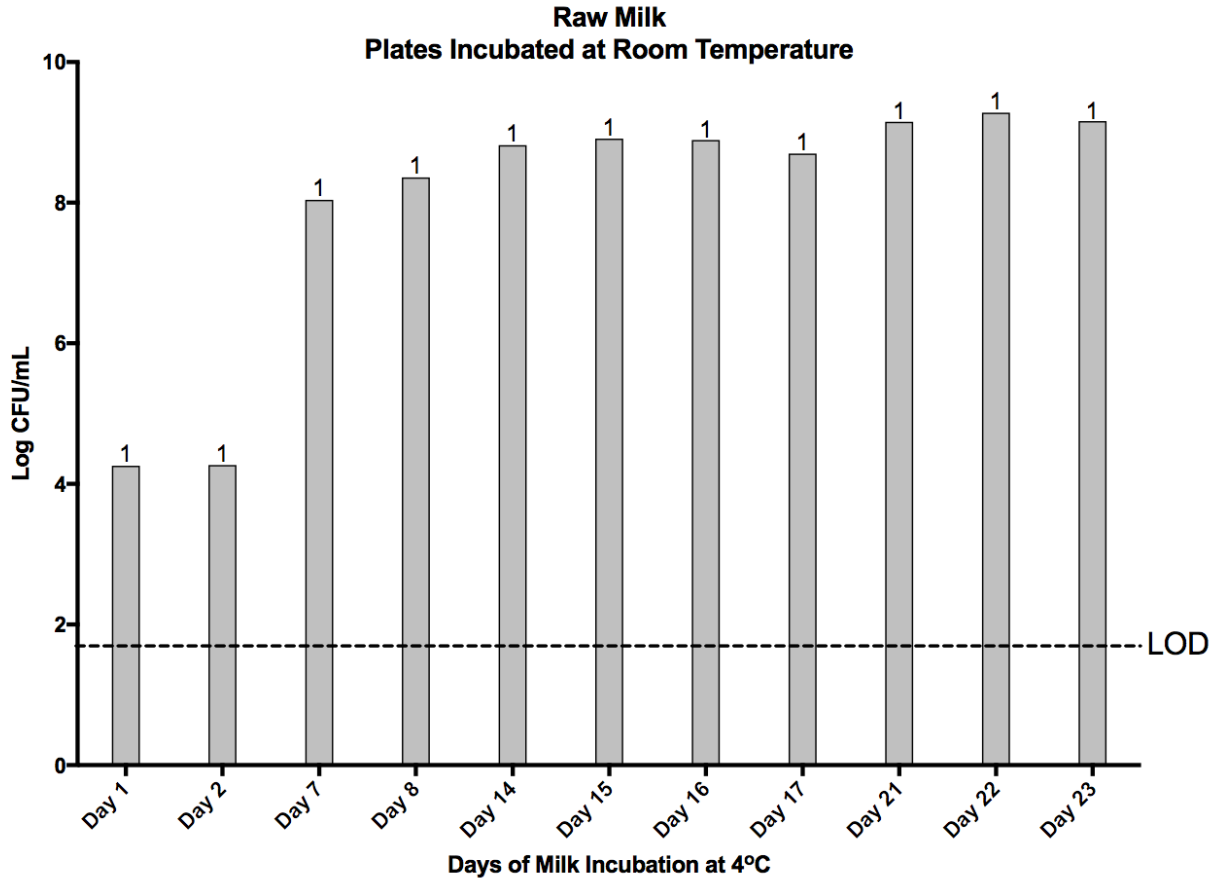


**Figure 1J. Differential growth of *Pseudomonas lundensis* (strain AU11164) at different temperatures on various agar types.** See Fig. 1A for methodology. Data points represent individual plates. *P. lundensis* strain AU11164 demonstrated poor growth at all temperatures on VBA agar, Cetrimide agar, and *Pseudomonas*-isolation agar; growth was also absent at all temperatures on MRS agar.

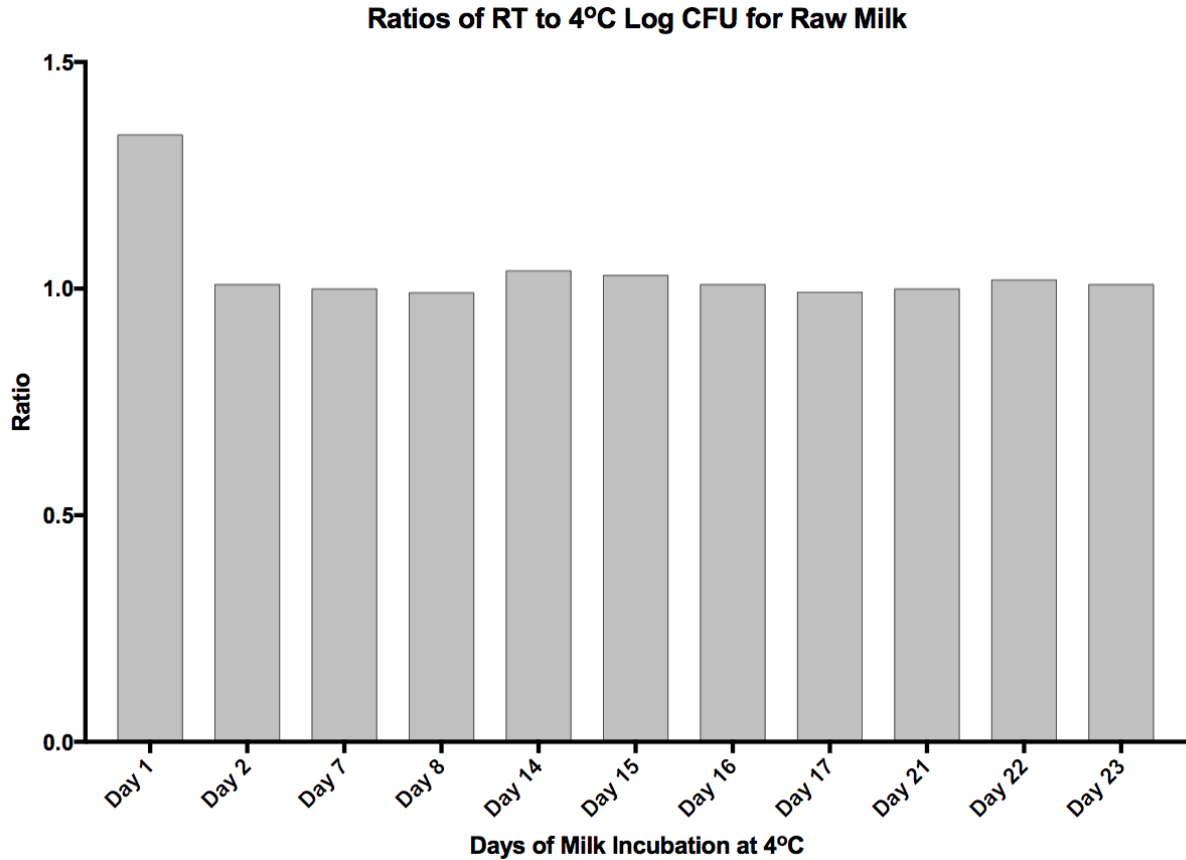


**Figure 2A. Culturability of psychrotrophic bacteria from raw milk at 4°C.** Raw milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were periodically plated in duplicate on LB agar plates and incubated at room temperature. The number on top of each column represents the number of days post-plating at 4°C that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of psychrotrophic bacteria from raw milk reaches a relatively stable level at around seven days of incubation of the initial milk culture. On average, growth was observed 5.42 days post-plating.

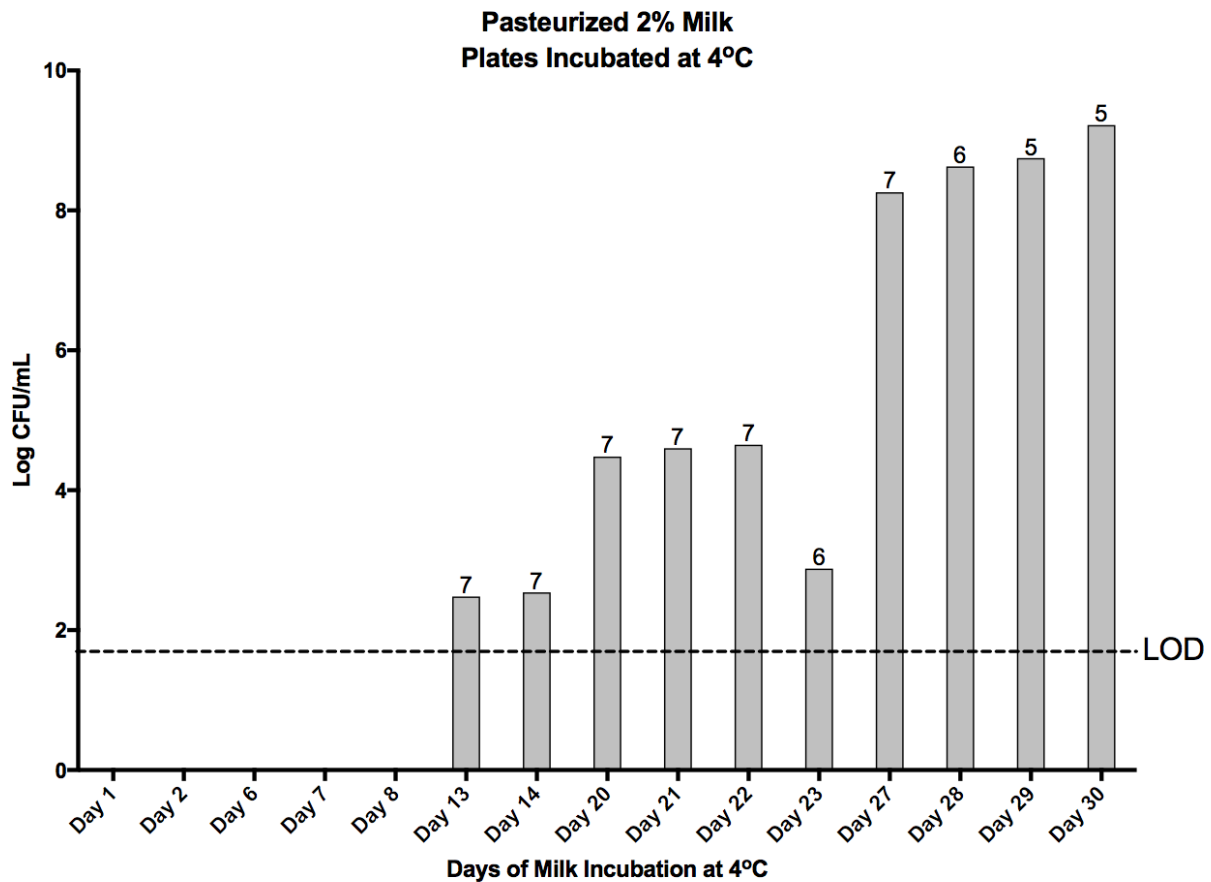




**Figure 2B. Culturability of mesophilic bacteria from raw milk at room temperature.** Raw milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were periodically plated in duplicate on LB agar plates and incubated at room temperature. The number on top of each column represents the number of days post-plating at room temperature that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of mesophilic bacteria from raw milk reaches a relatively stable level at around seven days of incubation of the initial milk culture. Growth was always observed one day post-plating.

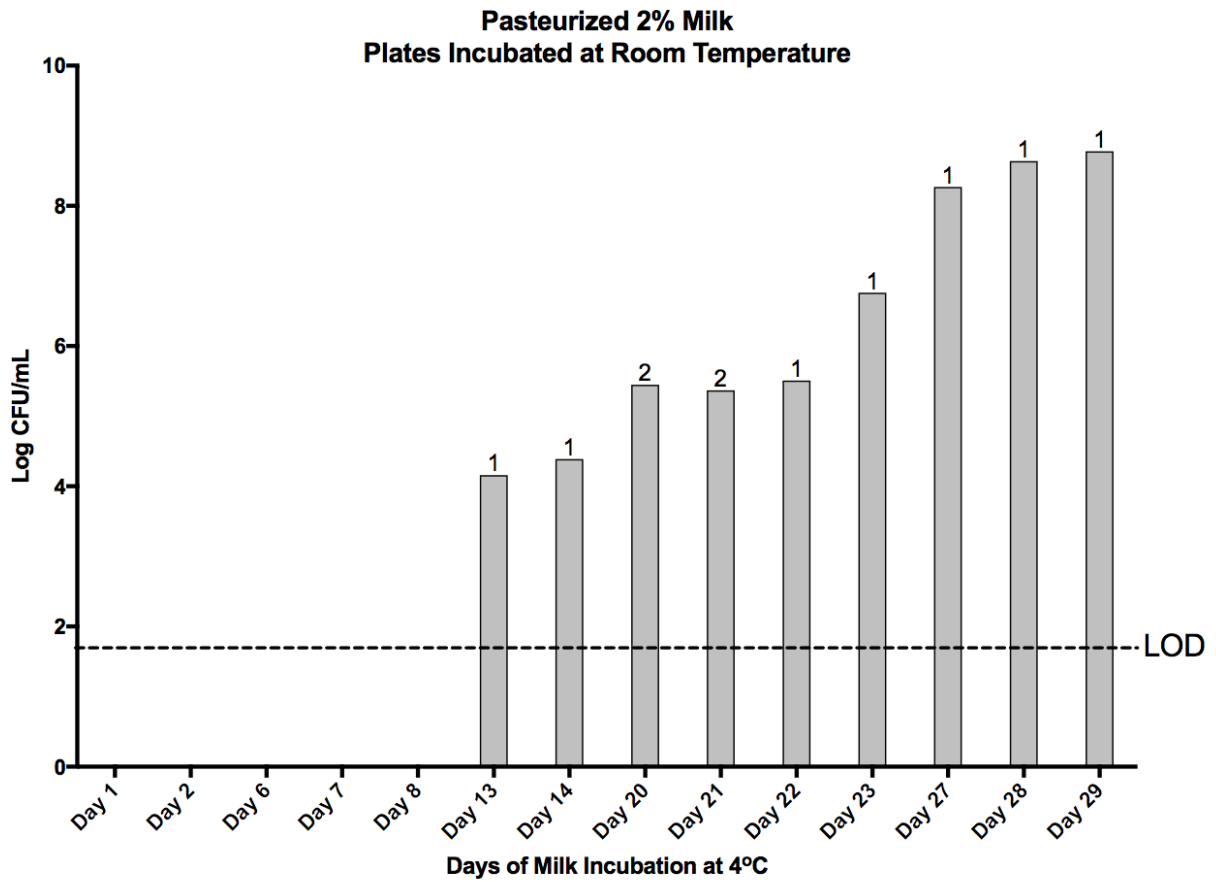


**Figure 2C. Comparison of culturability of mesophiles and psychrotrophs from raw milk.** In order to determine whether or not low-temperature incubation was excluding any organisms of interest, the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated were calculated. The ratios ranged from 0.992 to 1.34 (average of 1.04), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.

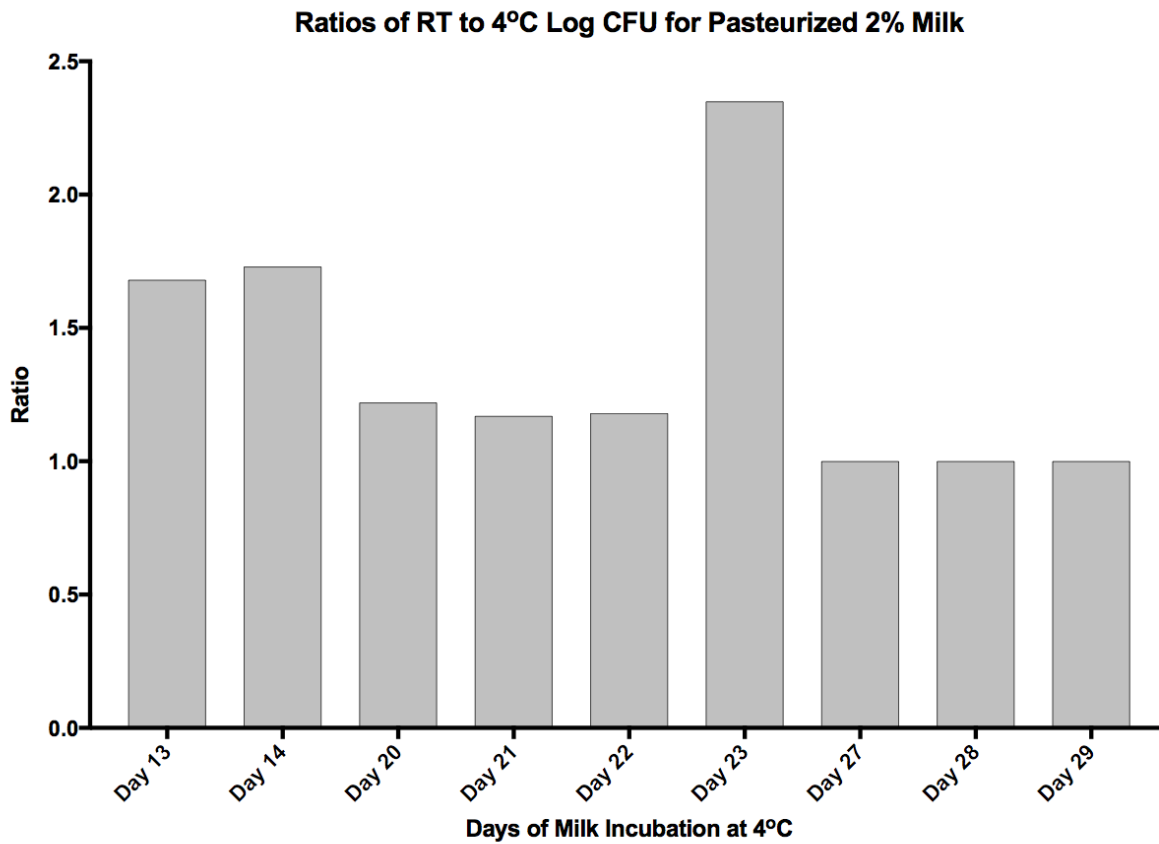


**Figure 3A. Culturability of psychrotrophic bacteria from pasteurized 2% milk at 4°C.**

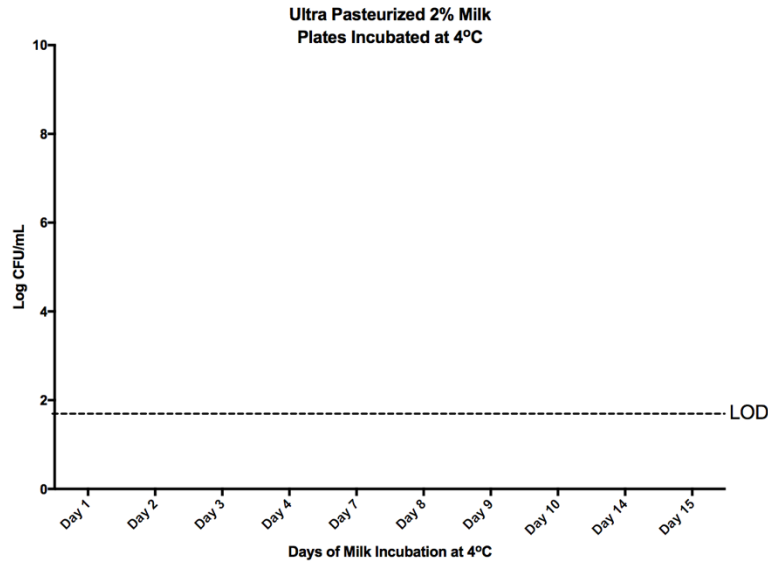
Pasteurized 2% milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were plated periodically in duplicate on LB agar plates and incubated at 4°C to quantify the growth of psychrotrophic bacteria. The number on top of each column represents the number of days post-plating 4°C that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of psychrotrophic bacteria from pasteurized 2% milk begins to be detectable at around 13 days of incubation of the initial milk culture; the level of bacterial growth remained relatively low until 27 days post-plating, when it nearly doubled. On average, growth was observed 6.40 days post-plating.



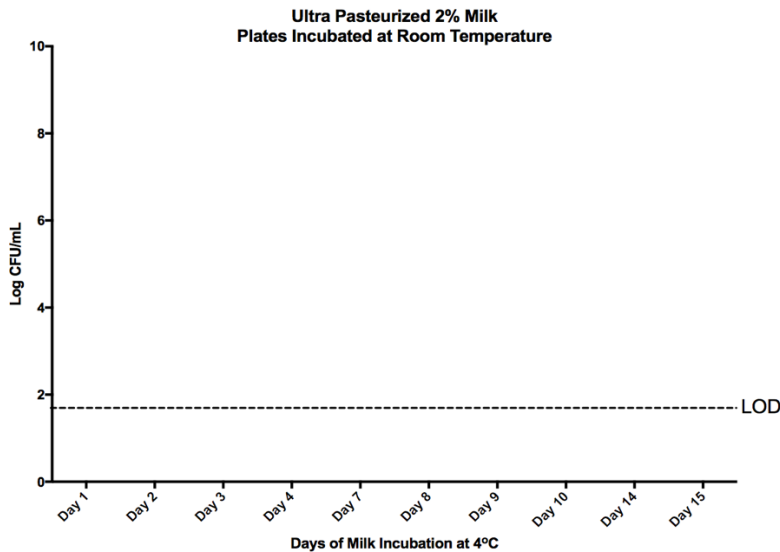
**Figure 3B. Culturability of mesophilic bacteria from pasteurized 2% milk at room temperature.** Pasteurized 2% milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were plated periodically in duplicate on LB agar plates and incubated at room temperature to quantify the growth of mesophilic bacteria. The number above each column represents the number of days post-plating at room temperature that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of mesophilic bacteria from pasteurized 2% milk began to be detectable after 13 days of incubation of the initial milk culture and increased steadily thereafter. On average, growth was observed 1.22 days post-plating.



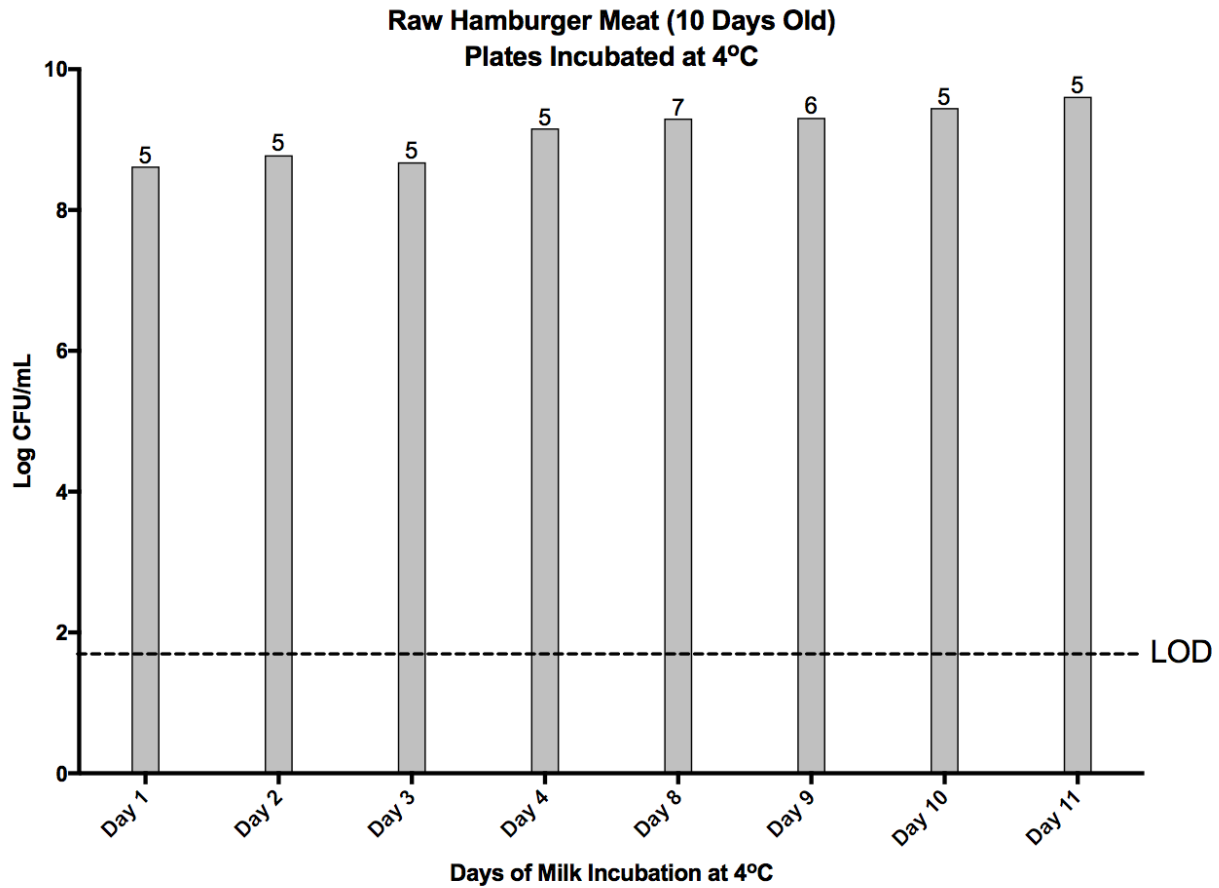
**Figure 3C. Comparison of culturability of mesophiles and psychrotrophs from pasteurized 2% milk.** In order to determine whether or not low-temperature incubation was excluding any organisms of interest, the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated were calculated. The ratios ranged from 1 to 2.35 (average of 1.37), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.



**Figure 4A. Absence of culturability of psychrotrophic bacteria from ultra-pasteurized 2% milk at 4°C.** Ultra-pasteurized 2% milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were plated periodically in duplicate on LB agar plates and incubated at 4°C to quantify the growth of psychrotrophic bacteria. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. No growth was detected on any of the plates over a 15-day period.

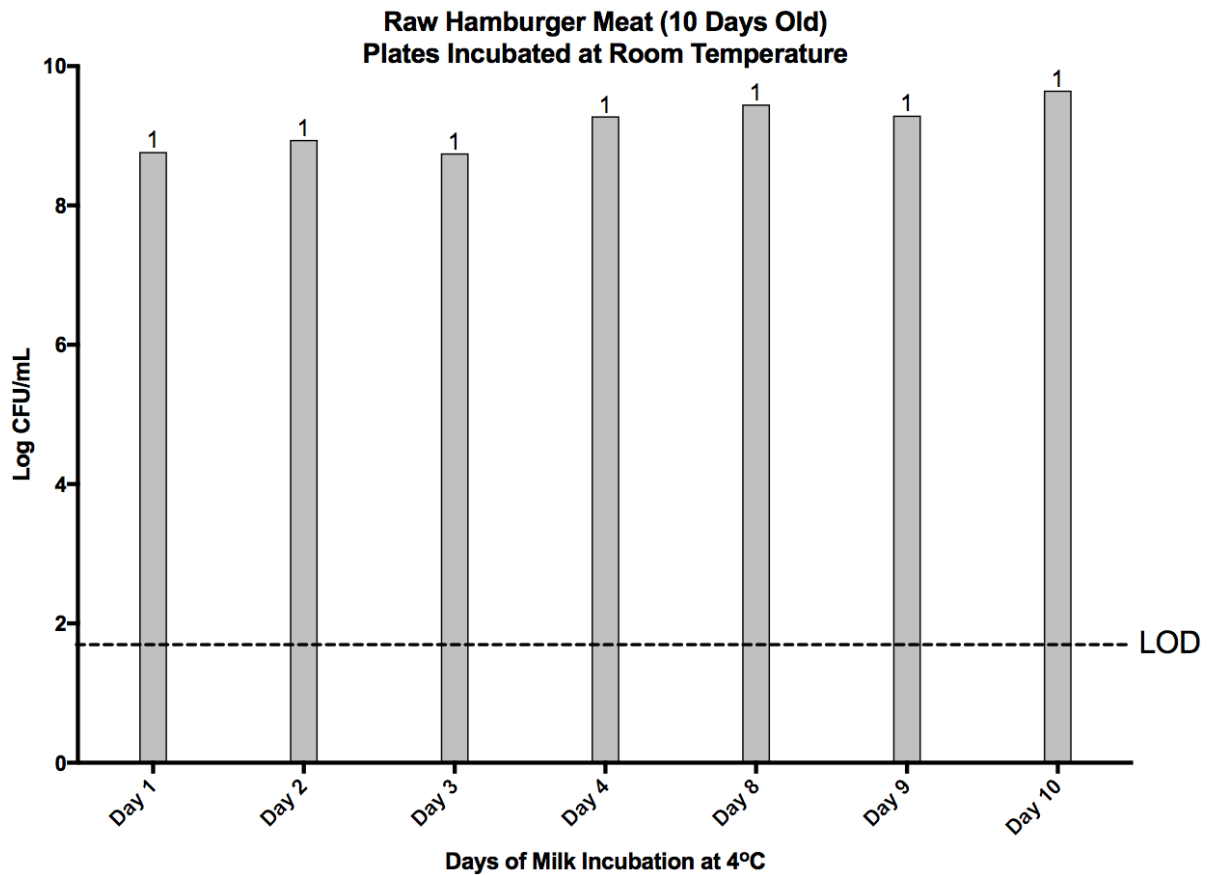


**Figure 4B. Absence of culturability of mesophilic bacteria from ultra-pasteurized 2% milk at room temperature.** Ultra-pasteurized 2% milk was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of the milk were plated periodically in duplicate on LB agar plates and incubated at room temperature to quantify the growth of mesophilic bacteria. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. No growth was detected on any of the plates over a 15-day period.



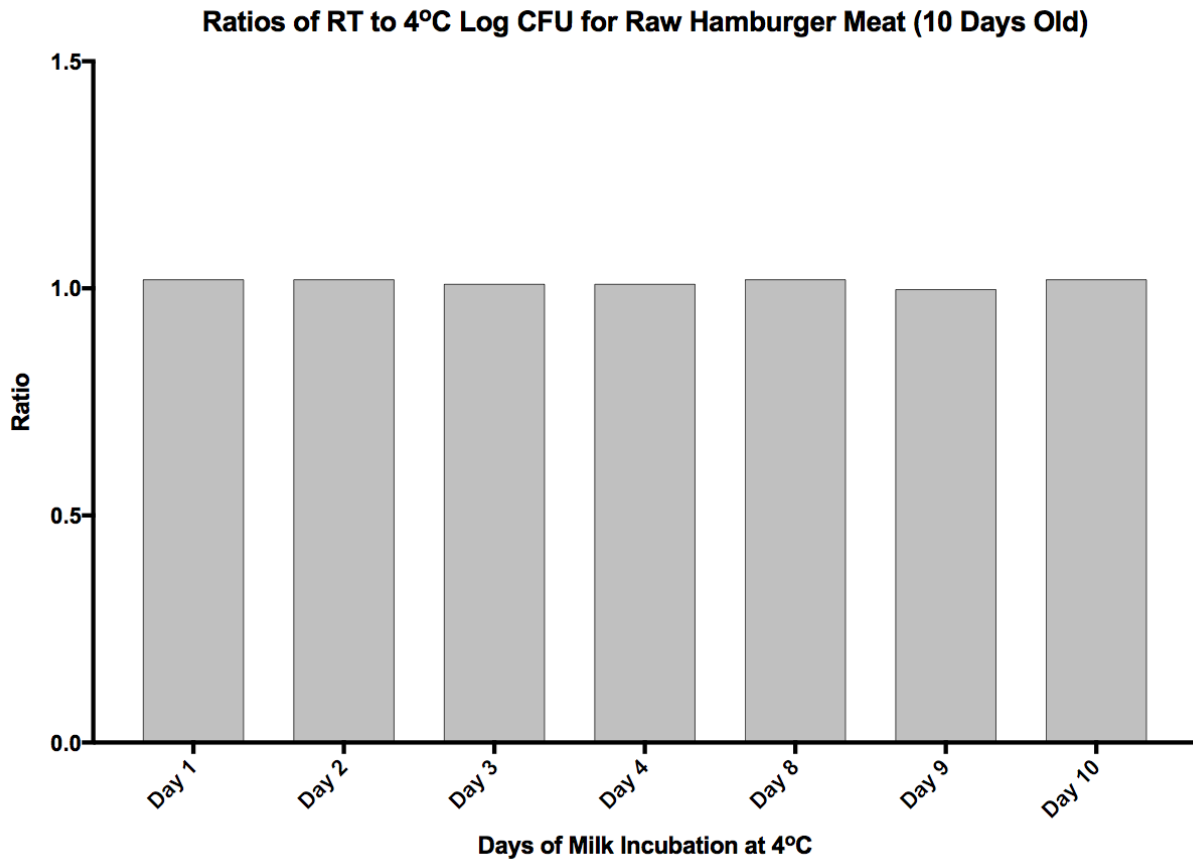
**Figure 5A. Culturability of psychrotrophic bacteria from raw hamburger meat at 4°C.**

Raw hamburger meat was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of homogenized raw hamburger meat were plated periodically in duplicate on LB agar plates and incubated at 4°C to quantify the growth of psychrotrophic bacteria. The number above each column represents the number of days post-plating at 4°C that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of psychrotrophic bacteria from raw hamburger meat is relatively stable over an 11-day period. On average, growth was observed 5.38 days post-plating.

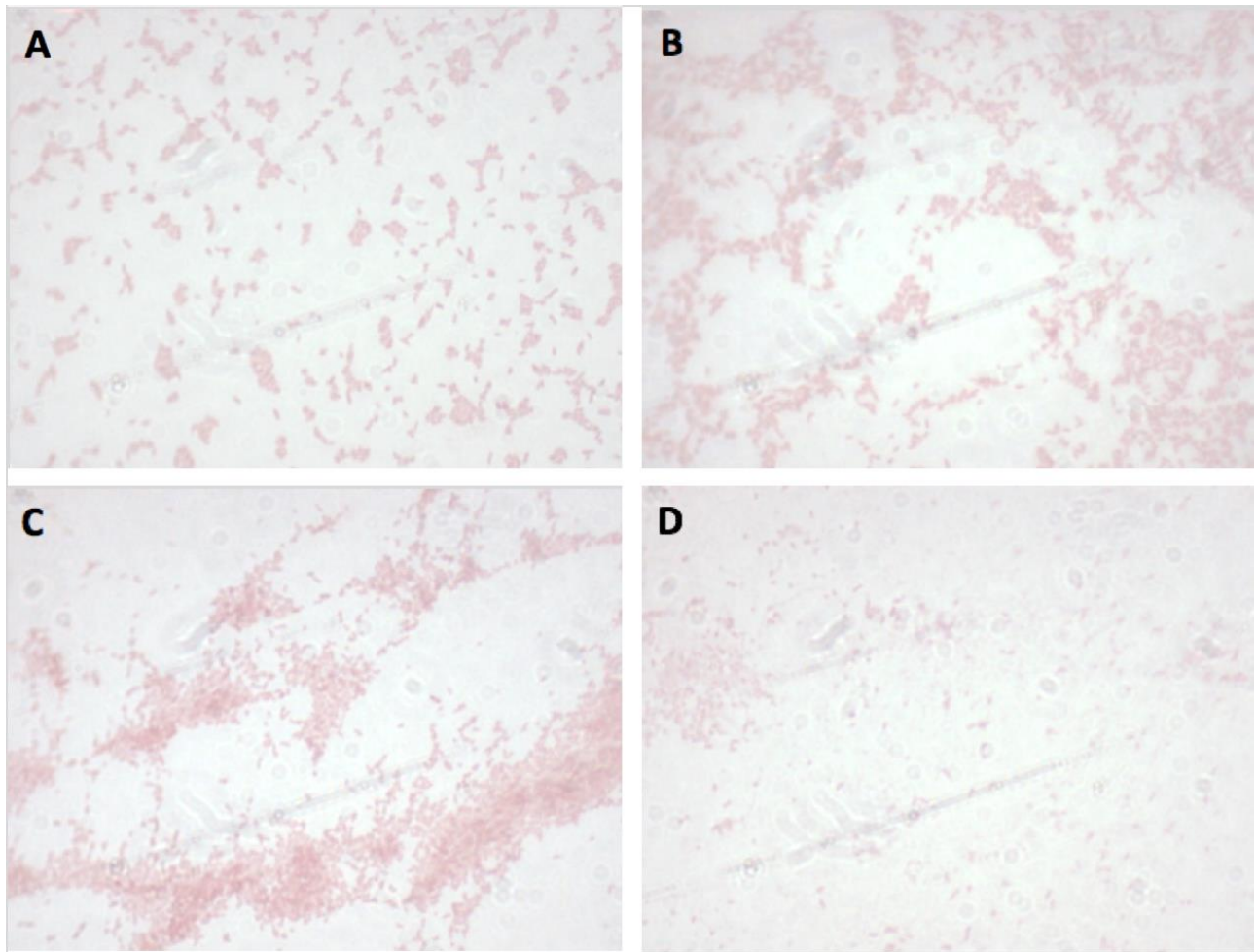


**Figure 5B. Culturability of mesophilic bacteria from raw hamburger meat at room temperature.** Raw hamburger meat was stored at 4°C for the duration of the experiment. 1:10 serial dilutions of homogenized raw hamburger meat were plated periodically in duplicate on LB agar plates and incubated at room temperature to quantify the growth of mesophilic bacteria. The number above each column represents the number of days post-plating at room temperature that passed before distinct colonies appeared. The limit of detection (LOD) is equal to 0.5 colonies in 10  $\mu$ L. The growth of mesophilic bacteria from raw hamburger meat is relatively stable over a 10-day period. Growth was always observed one day post-plating.

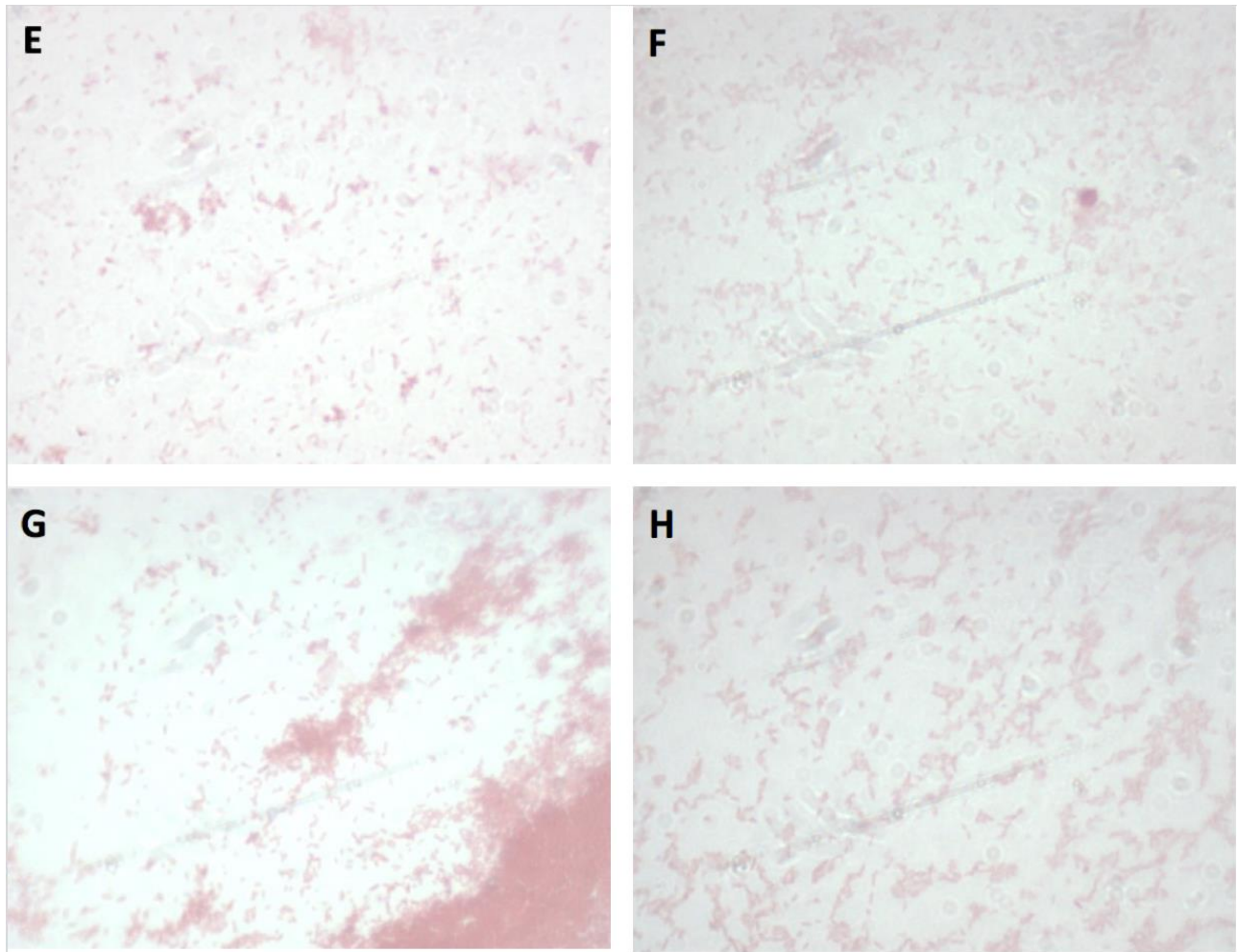




**Figure 5C. Comparison of culturability of mesophiles and psychrotrophs from raw hamburger meat.** In order to determine whether or not low-temperature incubation was excluding any organisms of interest, the ratios of the room temperature log CFU to the 4°C log CFU for each day that a sample was plated were calculated. The ratios ranged from 0.998 to 1.02 (average of 1.01), indicating that the organisms grown at room temperature are likely the same as those grown at 4°C.

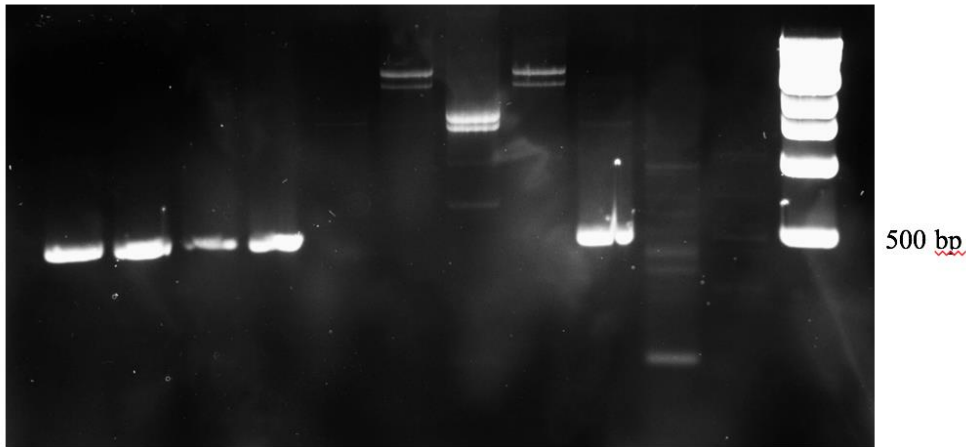


**Figure 6A. Gram stains of milk sample isolates 101, 103, 105, and 113.** All isolates from the milk samples were tested for their Gram stain reaction. Shown here are the Gram stains of the selected subset of isolates (those grown on LB agar at 4°C or 10°C and shown to have at least 85% sequence homology to the *P. lundensis* AU1044 chromosomal sequence). All isolates in the selected subset appeared as Gram negative bacilli. **(A)** Isolate 101 (pasteurized 1% milk). **(B)** Isolate 103 (pasteurized 1% milk). **(C)** Isolate 105 (pasteurized 1% milk). **(D)** Isolate 113 (pasteurized 1% milk).



**Figure 6B. Gram stains of milk sample isolates 206, R02, R03, and R04** All isolates from the milk samples were tested for their Gram stain reaction. Shown here are the Gram stains of the selected subset of isolates (those grown on LB agar at 4°C or 10°C and shown to have at least 85% sequence homology to the *P. lundensis* AU1044 chromosomal sequence). All isolates in the selected subset appeared as Gram negative bacilli. **(E)** Isolate 206 (pasteurized 2% milk). **(F)** Isolate R02 (raw milk). **(G)** Isolate R03 (raw milk). **(H)** Isolate R04 (raw milk).

Isolate: 101 103 105 113 206 R02 R03 R04 (+) (-) (-)



**Figure 7. Gel electrophoresis of *exoU* gene-specific PCR products for subset of milk sample isolates.** All isolates from the milk samples were run on a gel using primers specific for a region of the *P. lundensis* AU11122 *exoU* gene to aid in their identification. Shown here is the ExoU agarose gel of the selected subset of milk sample isolates (those grown on LB agar at 4°C or 10°C and shown to have at least 85% sequence homology to the *P. lundensis* AU1044 chromosomal sequence). The first eight lanes each represent an individual isolate. The lane designated with a (+) represents the positive control, *P. lundensis* AU1044 DNA. The lanes designated with a (-) represent the negative controls, *P. aeruginosa* PAO1 DNA and *E. coli* primary mouse gut isolate, respectively. The isolates were run alongside a 1 kb ladder. Isolates 101, 103, 105, and 113 were positive for the *exoU* gene, indicated by a bright band at approximately 500 bp on the gel (the ExoU PCR product is 494 bp).

Isolate	Description	Query Cover	E Value	Ident
101	<i>Pseudomonas</i> sp. M13(2015) 16S rRNA gene, partial	87%	0	87%
	<i>Pseudomonas</i> sp. strain S10 16S rRNA gene, partial	90%	0	86%
	<i>Pseudomonas</i> sp. strain V1/1/2 16S rRNA gene, partial	90%	0	86%
	<i>Pseudomonas lundensis</i> strain Z54a 16S rRNA gene, partial	90%	0	86%
	<i>Pseudomonas lundensis</i> strain W47a 16S rRNA gene, partial	90%	0	86%
	<i>Pseudomonas lundensis</i> AU1044 chromosome, complete genome	96%	0	86%
102	Uncultured bacterium clone WF16S_121 16S rRNA gene, partial	99%	0	97%
	<i>Pseudomonas</i> sp. KB44 16S rRNA gene, partial	97%	0	98%
	<i>Pseudomonas fluorescens</i> strain FLM05-1 16S rRNA gene, partial	97%	0	98%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	97%	0	98%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	97%	0	98%
103	<i>Pseudomonas</i> sp. KB144 16S rRNA gene, partial	93%	0	96%
	Uncultured bacterium clone F21 16S rRNA gene, partial	93%	0	96%
	<i>Pseudomonas fluorescens</i> strain FLM05-1 16S rRNA gene, partial	93%	0	96%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	93%	0	96%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	93%	0	96%
105	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	97%	0	98%
	Uncultured bacterium clone SN206 16S rRNA gene, partial	96%	0	98%
	Uncultured <i>Pseudomonas</i> sp. clone HLB-25 16S rRNA gene, partial	96%	0	98%
	<i>Pseudomonas</i> sp. R-37260 partial 16S rRNA gene, isolate R-37260	96%	0	98%
	<i>Pseudomonas</i> sp. KK-21-4 partial rRNA gene, isolate KK-21-4	96%	0	98%
113	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	94%	0	98%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	94%	0	98%
	Uncultured bacterium clone SN206 16S rRNA gene, partial	94%	0	98%
	Uncultured <i>Pseudomonas</i> sp. clone HLB-25 16S rRNA gene, partial	94%	0	98%
	<i>Pseudomonas</i> sp. R-37260 partial 16S rRNA gene, isolate R-37260	94%	0	98%
122	Uncultured bacterium clone 153T3 16S rRNA gene, partial	97%	0	93%
	<i>Pseudomonas fluorescens</i> strain 8F12 16S rRNA gene, partial	95%	0	94%
	<i>Pseudomonas psychrophila</i> strain 4G7 16S rRNA gene, partial	95%	0	94%
	<i>Pseudomonas psychrophila</i> strain 6A5 16S rRNA gene, partial	95%	0	94%
	<i>Pseudomonas psychrophila</i> strain 7B8 16S rRNA gene, partial	95%	0	94%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	95%	0	94%
126	<i>Pseudomonas</i> sp. GR4 16S rRNA gene, partial	94%	0	97%
	<i>Pseudomonas aeruginosa</i> isolate RW109 genome assembly, chromosome	98%	0	96%
	<i>Pseudomonas</i> sp. AK6U chromosome, complete genome	98%	0	96%
	<i>Pseudomonas aeruginosa</i> strain PB350 chromosome, complete genome	98%	0	96%
	<i>Pseudomonas aeruginosa</i> strain PB367 chromosome, complete genome	98%	0	96%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	92%
139	<i>Pseudomonas aeruginosa</i> strain N1418 16S rRNA gene, partial	95%	0	97%
	Uncultured bacterium partial 16S rRNA gene, clone 16Sps16-2h11.p1k	95%	0	97%
	Uncultured bacterium clone P5D23-408 16S rRNA gene, partial	95%	0	97%
	Uncultured bacterium clone P5D23-654 16S rRNA gene, partial	95%	0	97%
	Uncultured bacterium clone P5D23-665 16S rRNA gene, partial	95%	0	97%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	94%	0	93%
140	Uncultured bacterium clone 16S-24 16S rRNA gene, partial	66%	3e-93	77%
	Uncultured bacterium clone Luq_GN490_008 16S rRNA gene, partial	66%	2e-90	77%
	Uncultured <i>Pseudomonas</i> sp. clone LT12 16S rRNA gene, partial	66%	7e-90	77%
	<i>Pseudomonas</i> sp. BWDY-18 16S rRNA gene, partial	66%	7e-90	77%
	Uncultured bacterium clone Foos8B_81p 16S rRNA gene, partial	66%	3e-88	77%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	66%	4e-96	77%
141	<i>Pseudomonas aeruginosa</i> strain PBS29 16S rRNA gene, partial	98%	0	95%
	<i>Pseudomonas aeruginosa</i> strain AQGSSB12 16S rRNA gene, partial	98%	0	95%
	<i>Pseudomonas</i> sp. strain DASA1 16S rRNA gene, partial	98%	0	95%
	<i>Pseudomonas aeruginosa</i> strain KAVK01 16S rRNA gene, partial	98%	0	95%
	<i>Serratia marcescens</i> strain ABL16KA15 16S rRNA gene, partial	98%	0	95%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	91%

142	<i>Pseudomonas aeruginosa</i> strain N141B 16S rRNA gene, partial	98%	0	91%
	<i>Pseudomonas aeruginosa</i> strain E136a 16S rRNA gene, partial	98%	0	91%
	<i>Pseudomonas</i> sp. strain FQR8 16S rRNA gene, partial	98%	0	91%
	<i>Pseudomonas aeruginosa</i> strain SLI03 16S rRNA gene, partial	98%	0	91%
	<i>Pseudomonas aeruginosa</i> partial 16S rRNA gene, isolate DR1	98%	0	91%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	91%
147	<i>Pseudomonas hibiscola</i> strain PB19 16S rRNA gene, partial	98%	0	90%
	<i>Stenotrophomonas maltophila</i> strain PB12 16S rRNA gene, partial	98%	0	93%
	<i>Stenotrophomonas maltophila</i> strain PB2 16S rRNA gene, partial	98%	0	93%
	<i>Stenotrophomonas maltophila</i> strain ZFJ-10 16S rRNA gene, partial	98%	0	93%
	<i>Stenotrophomonas maltophila</i> strain FDAARGOS_325, complete genome	98%	0	93%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	91%	0	84%
205	<i>Paenibacillus</i> sp. strain 11 16S rRNA gene, partial	88%	0	97%
	<i>Paenibacillus</i> sp. P22V 16S rRNA gene, partial	88%	0	97%
	<i>Paenibacillus</i> sp. 56 16S rRNA gene, partial	90%	0	96%
	<i>Paenibacillus odorifer</i> strain DSM 15391, complete genome	95%	0	96%
	<i>Paenibacillus</i> sp. 7B-637 16S rRNA gene, partial	95%	0	96%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	80%	0	84%
206	<i>Pseudomonas</i> sp. sgn 179 partial 16S rRNA gene, isolate sgn 179	95%	0	98%
	<i>Pseudomonas</i> sp. MY1420 16S rRNA gene, partial	95%	0	98%
	<i>Pseudomonas</i> sp. Lz4W chromosome, complete genome	95%	0	98%
	<i>Pseudomonas weihenstephanensis</i> strain DSM 29166 16S rRNA, partial	95%	0	98%
	<i>Pseudomonas helleri</i> strain DSM 29165 16S rRNA, partial	95%	0	98%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	95%	0	97%
R02	<i>Pseudomonas psychrophila</i> strain PP02 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas</i> sp. R2SpM1P1C10 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas</i> sp. sgn14 partial 16S rRNA gene, isolate sgn 14	98%	0	98%
	<i>Pseudomonas helleri</i> strain DSM29165 16S rRNA, partial	98%	0	98%
	<i>Pseudomonas psychrophila</i> strain BS3667 genome assembly	98%	0	98%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	97%
R03	<i>Pseudomonas</i> sp. WR7#2 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas fragi</i> strain PF04 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas fragi</i> strain 4Lv.1 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas</i> sp. strain 8A7 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas</i> sp. strain 1F11 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	95%
R04	<i>Pseudomonas</i> sp. R2SpM1_1C10 16S rRNA gene, partial	89%	0	99%
	<i>Pseudomonas psychrophila</i> strain BS3667 genome assembly	89%	0	99%
	<i>Pseudomonas psychrophila</i> strain PP02 16S rRNA gene, partial	89%	0	99%
	<i>Pseudomonas</i> sp. sgn 14 partial 16S rRNA gene, isolate sgn 14	89%	0	99%
	<i>Pseudomonas</i> sp. Lz4W chromosome, complete genome	89%	0	99%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	89%	0	98%

**Table 1. BLAST results of milk sample isolates upon initial isolation.** Shown are the BLAST analysis results for all isolates grown from pasteurized 2% milk, raw milk, and all ExoU-positive isolates grown from pasteurized 1% milk. The top five BLAST results are shown for each isolate, as well as the alignment to the *P. lundensis* AU1044 chromosomal sequence if it was absent from the top five results. The query coverage column represents the percentage of the query sequence that was aligned. The E value column represents the level of random background noise in the search. The ident column represents the percentage of sequence homology between the query and each result. The isolates that were both grown on LB agar at 4°C and showed at least 85% sequence homology with the *P. lundensis* AU1044 chromosomal sequence were selected for further analysis.

Isolate	Description	Query Cover	E Value	Ident
101	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	94%	0	98%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	94%	0	98%
	Uncultured <i>Pseudomonas</i> sp. clone HLB-25 16S rRNA gene, partial	94%	0	98%
	<i>Pseudomonas</i> sp. R-37260 partial 16S rRNA gene, isolate R-37260	94%	0	98%
	Uncultured bacterium clone SN206 16S rRNA gene, partial	94%	0	98%
103	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	89%	0	97%
	<i>Pseudomonas psychrophila</i> strain P270 16S rRNA gene, partial	89%	0	97%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	89%	0	97%
	<i>Pseudomonas</i> sp. KB44 16S rRNA gene, partial	89%	0	97%
	Uncultured <i>Pseudomonas</i> sp. clone HLB-25 16S rRNA gene, partial	89%	0	97%
105	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	93%	0	97%
	<i>Pseudomonas psychrophila</i> strain P270 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	93%	0	97%
	Uncultured bacterium clone SN206 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas</i> sp. KB44 16S rRNA gene, partial	93%	0	97%
113	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	96%	0	95%
	<i>Pseudomonas</i> sp. KK-21-4 partial 16S rRNA gene, isolate KK-21-4	96%	0	95%
	Uncultured bacterium clone SN206 16S rRNA gene, partial	96%	0	95%
	Uncultured <i>Pseudomonas</i> sp. clone HLB-25 16S rRNA gene, partial	96%	0	95%
	<i>Pseudomonas</i> sp. R-37260 partial 16S rRNA gene, isolate R-37260	96%	0	95%
206	<i>Pseudomonas fragi</i> strain DBC genome	98%	0	96%
	<i>Pseudomonas psychrophila</i> strain BS3667 genome assembly	98%	0	96%
	<i>Pseudomonas fragi</i> strain NRRL B-727 genome assembly	98%	0	96%
	Uncultured bacterium partial 16S rRNA gene, clone AF-35726	98%	0	96%
	Uncultured bacterium clone BJ201110-38 16S rRNA gene, partial	98%	0	96%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	95%
R02	<i>Pseudomonas helleri</i> strain DSM 29165 16S rRNA, partial	98%	0	98%
	<i>Pseudomonas helleri</i> strain DSM 29165 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas psychrophila</i> strain AW2 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas psychrophila</i> strain Amm-01 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas psychrophila</i> strain Car-14 16S rRNA gene, partial	98%	0	98%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	98%	0	97%
R03	<i>Pseudomonas fragi</i> strain PF04 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas fragi</i> strain 4Lv.1 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas</i> sp. strain 1D2 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas</i> sp. strain 8A7 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas</i> sp. strain 1F11 16S rRNA gene, partial	93%	0	97%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	93%	0	96%
R04	<i>Pseudomonas</i> sp. R-37265 partial 16S rRNA gene, isolate R-37265	91%	0	97%
	<i>Pseudomonas helleri</i> strain DSM 29165 16S rRNA, partial	91%	0	97%
	<i>Pseudomonas helleri</i> strain DSM 29165 16S rRNA gene, partial	91%	0	97%
	<i>Pseudomonas psychrophila</i> strain AW2 16S rRNA gene, partial	91%	0	97%
	<i>Pseudomonas psychrophila</i> strain Amm-01 16S rRNA gene, partial	91%	0	97%
	<i>Pseudomonas lundensis</i> strain AU1044 chromosome, complete genome	89%	0	97%

**Table 2. BLAST results of subset of milk sample isolates upon single colony subculture.**

Shown are the BLAST analysis results of those isolates that were grown on LB agar at 4°C or 10°C and were previously shown to have at least 85% sequence homology with the *P. lundensis* strain AU1044 chromosomal sequence. The top five BLAST results are shown for each isolate, as well as the alignment to the *P. lundensis* AU1044 chromosomal sequence if it was absent from the top five results. The query coverage column represents the percentage of the query sequence that was aligned. The E value column represents the level of random background noise in the search. The ident column represents the percentage of sequence homology between the query and each result. Isolates 101, 103, 105, and 113 all appear to be *P. lundensis*; all other isolates appear to be other *Pseudomonas* species.