Bacteria con leche: Bacterial populations and antibiotic resistance within conventional, USDA organic, and local milk

Kristen M. Wilson
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BACTERIA CON LECHE: BACTERIAL POPULATIONS AND ANTIBIOTIC RESISTANCE WITHIN CONVENTIONAL, USDA ORGANIC, AND LOCAL MILK

By
Kristen Majure Wilson

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2014

Approved by

Advisor: Professor Colin Jackson

Reader: Professor Lucile McCook

Reader: Professor John Samonds
ABSTRACT
KRISTEN MAJURE WILSON: Bacteria con leche: Bacterial populations and antibiotic resistance within conventional, USDA organic, and local milk
(Under the direction of Colin Jackson)

Concerns over food production and processing, along with increasing outbreaks of foodborne illnesses and antibiotic resistant infections have led many consumers to seek alternative food sources in the organic and local food markets. This study compared conventional, USDA certified organic, and local “farmer’s market” milk types to determine whether there is any noticeable benefit to purchasing organic or locally harvested milk in terms of their bacterial populations and level of antibiotic resistance.

Samples from various milk types were plated on Tryptic Soy Agar and Milk Agar plates to enumerate bacteria, and the cultivated bacteria tested for resistance to the antibiotics penicillin, erythromycin, tetracycline, and gentamicin. Bacterial cultures from the various milk samples were identified using 16S rRNA gene sequencing. Local farmer’s market milk had the most abundant and diverse bacterial composition, containing several potential pathogens, and a high general level of antibiotic resistance. Conventional milk samples also showed considerable bacterial counts and high levels of antibiotic resistance. On the other hand, USDA certified organic milk yielded no culturable bacteria on either TSA or MA plates. This study shows that those concerned with food safety should consume USDA organic milk to lessen their exposure to high numbers of antibiotic resistant and potentially pathogenic bacteria.
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Introduction

Concerns over food safety have been mounting in the minds of the American people because of increasing numbers of foodborne illness outbreaks. According to the Centers for Disease Control and Prevention, over 48 million Americans per year (roughly 15% of the US population) are reportedly affected by foodborne illnesses, with consequences ranging from sickness to hospitalization and death (CDC Estimates of Foodborne Illness in the United States 2011). There are about 30 known pathogens that cause foodborne illnesses, but an overwhelming majority of illnesses have unknown causes (CDC Estimates of Foodborne Illness in the United States 2011). This information, along with the concerns over food production and processing in the United States, has led consumers to seek alternatives in the local and organic food markets. In addition, the increased use of technologies such as genetically modified ingredients, antibiotics, pesticides, and hormones are of concern due to the lack of knowledge about the long-term consequences of consuming these additives.

One technology of particular concern is the use of antibiotics in healthy livestock to promote growth rather than to fight disease and the implications that this has for antibiotic resistant infections. This inappropriate usage, along with overuse of antibiotics in the medical community, has led to alarming levels of antibiotic resistance in bacteria and the diminished effectiveness of therapeutic antibiotics (Levy 1998). Over 40% of antibiotics produced in the United States are used in agriculture, either in animal feed or sprayed on produce (Levy 1998). Bacteria can transfer antibiotic resistance genes
through plasmids and transposons, potentially creating large populations of antibiotic resistant bacteria so that this agricultural use can have wide ranging effects. The impacts of antibiotic resistant bacteria in food products are not trivial; people placed on bacteria-free diets showed a 1000-fold decrease in the amount of antibiotic resistant bacteria in their feces (Levy 1998). This has serious implications for the human population as our healthcare system relies heavily on antibiotics for the treatment of primary infections as well as secondary infections arising from surgery, cancer, transplants, and other medical interventions (Smith and Coast 2013). The situation is now critical as the number of antibiotic resistant infections continues to rise, increasing the total burden of disease, and the development of new antibiotics and treatment strategies stalls (Spellberg et al. 2007, Ammerlaan et al. 2012). There is no real solution to the problem of antibiotic resistance; rather the human population must adapt alongside microbes and develop new antibiotics and strategies to keep pace with microbial adaptations (Spellberg et al. 2007). However, some things can be done to slow the spread of antibiotic resistance: eliminate the use of antibiotics for growth in healthy livestock, produce more pathogen-specific antibiotics, create other strategies for the use of existing antibiotics, reduce the use of antibiotics for viral and other non-bacterial infections, and limit the overall use of antibiotics in general (Levy 1998, Spellberg et al. 2007). These steps can reduce the selection pressure for microbes to take up antibiotic resistance genes and will slow the spread of antibiotic resistance among bacteria. Thereby, the medical community along with pharmaceutical companies can develop treatments and strategies to overcome the increasing number of multi-drug resistant infections.
Concerns about current food production and processing, along with increasing outbreaks of foodborne illnesses and antibiotic resistant infections, are leading many consumers to seek alternative food sources. Local and organic food markets still remain niche markets in the United States, but they continue to grow 20% annually, and consumers becoming more aware of food safety issues are turning to local and organic markets (Pino et al. 2012, Haas et al. 2013). There are two main motivations for consumers to purchase organic foods: food safety and ethical self-identity (Michaelidou and Hassan 2008, Pino et al. 2012). Regular consumers of organic foods are motivated primarily by an ethical obligation, while occasional consumers are motivated by food safety, especially during pregnancy or following a foodborne illness (Pino et al. 2012). Despite a lack of scientific data, most consumers perceive local and organic foods as healthier, safer alternatives to conventionally produced food (Haas et al. 2013). Studies have shown some health differences between animals and humans on organic diets versus conventional diets, but they have not produced sufficient evidence to conclude that organic food is healthier than conventional food (Huber et al. 2011). Regardless, consumers are becoming aware of the potential health risks associated with conventionally produced food and the technologies involved (i.e. genetically modified ingredients) with production and processing, and as a result, the public is searching for alternatives in the local and organic food market.

The search for organically grown food is not limited to meat and produce items, as organic beverages and dairy products make up 20% of all organic market sales (Haas et al. 2013). Organic milk has better fatty acid compositions than non-organic, including n-3 linoleic acid and conjugated linoleic acid (CLA), and children on organic diets may
have lower body weights, exhibit fewer allergies, and are at a lower risk for developing eczema (Huber et al. 2011). Studies have also shown that nursing women who consume organic milk have higher levels of CLA in their breast milk (Huber et al. 2011). These findings are still far from conclusive, but they show that there is some benefit to consuming organic milk over conventionally produced milk. Consumers, concerned with the antibiotics and hormones utilized in milk production, are switching to local, organic, and even raw food market sources. However, consumers must educate themselves on the risks as well as the benefits, especially in regards to raw milk. According to the CDC, over 3,000 illnesses and two deaths were due to the consumption of raw milk or raw milk products between 1998 and 2011 (Food Safety News 2014). Many potentially pathogenic bacteria, such as *Escherichia coli*, *Campylobacter*, and *Salmonella*, are known to contaminate milk during production, making ultra-pasteurized organic milk a safer choice than raw or local milk (Food Safety News 2014). Furthermore, organic milk is defined and regulated by law, whereas local food is less delineated or regulated (Haas et al. 2013). In addition, raw milk does not have the same level of federal regulation as organic milk but is instead restricted by state, with some states allowing raw milk sales on or off the farm, but rarely in grocery stores (Food Safety News 2014). All in all, consumers who are unsure about conventionally produced food and milk must remain educated and cognizant in their choices of consumption, and more studies are needed on the potential microbiological components present in all types of milk.

The objectives of this study were to investigate the microbiological quality of conventional, USDA organic, and local “farmer’s market” milk with a particular focus on the presence of antibiotic resistant bacteria. The initiative behind this study was to
determine whether there is any noticeable benefit to purchasing organic or locally harvested milk in terms of their microbial populations and level of antibiotic resistance. The hypothesis was that USDA organic milk would contain fewer bacteria with a reduced level of antibiotic resistance than conventional milk because it is ultra-pasteurized and produced without the use of antibiotics. Locally produced “farmer’s market” milk was hypothesized to contain more bacteria because of less stringent processing, but that these bacteria would show less antibiotic resistance because of an absence of antibiotics during its production.
Methods

Sample Collection

Samples of commercial whole milk were purchased from various stores in Oxford, MS in July 2013. Three types of milk were purchased: Regular, USDA Certified Organic, and Farmer’s Market Organic milk. Five brands were purchased and processed on July 15, 2013, and another five brands were obtained and processed on July 17, 2013. A summary of the samples is available in Table 1. Each sample was purchased on the day it was to be used. Expiration dates were taken into consideration when purchasing, with regular milk having an expiration date of at least one week beyond the date of purchase and organic milk for one month. Immediately after purchasing, the milk was brought to the laboratory and placed in the refrigerator, seal intact, until it was processed.

Determination of Microbial Populations within Samples

Each sample of milk was serially diluted in a sterile dilute yeast extract solution to give dilutions of $10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$. Dilutions were plated onto Tryptic Soy Agar (TSA) and Milk Agar (MA) plates following aseptic technique. TSA consisted of 9 g Pancreatic Digest of Casein, 3 g Papaic Digest of Soybean, 3 g Sodium Chloride, 9 g Agar and 600 mL H$_2$O; MA consisted of 3 g Tryptone, 1.5 g Yeast Extract, 0.6 g Dextrose, 0.6 g Skim Milk, and 9 g Agar combined with 600 mL H$_2$O. Each brand was diluted and plated separately to avoid contamination. In addition, the dilutions were
Table 1: Information on milk samples used in this experiment.

<table>
<thead>
<tr>
<th>Brand</th>
<th>Cultivation Method</th>
<th>Processing</th>
<th>Store Acquired</th>
<th>Date Acquired</th>
<th>Packaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Value</td>
<td>Conventional</td>
<td>Pasteurized, Homogenized, “No Artificial Growth Hormones”</td>
<td>Walmart</td>
<td>7/15/2013</td>
<td>1.89 L in Plastic Carton</td>
</tr>
<tr>
<td>Great Value</td>
<td>USDA Organic</td>
<td>Ultra-Pasteurized, Homogenized</td>
<td>Walmart</td>
<td>7/15/2013</td>
<td>1.89 L in Cardboard Carton</td>
</tr>
<tr>
<td>Horizon</td>
<td>USDA Organic</td>
<td>Ultra-Pasteurized, Homogenized</td>
<td>Walmart</td>
<td>7/15/2013</td>
<td>1.89 L in Cardboard Carton</td>
</tr>
<tr>
<td>Turner’s</td>
<td>Conventional</td>
<td>Pasteurized, Homogenized, “No Artificial Growth Hormones”</td>
<td>Walmart</td>
<td>7/15/2013</td>
<td>473 mL in Plastic Carton</td>
</tr>
<tr>
<td>Simple Truth</td>
<td>USDA Organic</td>
<td>Ultra-Pasteurized, Homogenized</td>
<td>Kroger</td>
<td>7/17/2013</td>
<td>1.89 L in Cardboard Carton</td>
</tr>
<tr>
<td>Kroger</td>
<td>Conventional</td>
<td>Pasteurized, Homogenized</td>
<td>Kroger</td>
<td>7/17/2013</td>
<td>1.89 L in Plastic Carton</td>
</tr>
<tr>
<td>Best Choice</td>
<td>Conventional</td>
<td>Pasteurized, Homogenized</td>
<td>Cost Savers</td>
<td>7/17/2013</td>
<td>1.89 L in Plastic Carton</td>
</tr>
<tr>
<td>Stremick’s Heritage</td>
<td>USDA Organic</td>
<td>Ultra-Pasteurized, Homogenized; “No hormones, antibiotics, or pesticides used.”</td>
<td>Cost Savers</td>
<td>7/17/2013</td>
<td>1.89 L in Cardboard Carton</td>
</tr>
</tbody>
</table>
plated in ascending order, from $10^{-3}$ to $10^0$. Samples were pipetted using individually wrapped, pre-sterilized glass pipettes, then spread evenly on the plate using an ethanol sterilized glass spreader. On July 15, 2013, the following brands were plated and incubated for 48 hours at 37°C: Brown’s Dairy, Great Value Regular, Great Value Organic, Horizon, and Turner’s. The remaining five brands were plated and incubated for 48 hours at 37°C on July 17, 2013: Best Choice, Kroger, Simple Truth, Stremick’s, and Prairie Farms. The number of colonies appearing on plates after 48 hours was determined and the colonies on the plate assessed for bulk antibiotic resistance and preserved for DNA sequence identification.

Examination of Antibiotic Resistance within Select Samples

Samples were tested for antibiotic resistance to four common antibiotics: penicillin, gentamycin, tetracycline, and erythromycin. Based on the counts of microbial populations (see Results), only eleven of the twenty samples were tested, all using the $10^0$ dilution plates. The samples tested were: Brown’s TSA, Brown’s MA, Best Choice TSA, Best Choice MA, Kroger TSA, Turner TSA, Turner MA, Great Value Regular TSA, Great Value Regular MA, Prairie Farms TSA, and Prairie Farms MA. 1 mL of sterile yeast extract solution was added directly to the surface of the agar of those $10^0$ plates and a glass spreader used to scrape the bacteria off the agar and into suspension. 1 mL of the buffer/bacteria suspension was removed and pipetted into a sterile micro-centrifuge tube. This micro-centrifuge tube (the $10^0$ tube) was used to create a $10^{-1}$ dilution tube by adding 0.1 mL of the bacteria-buffer suspension to the 0.9 mL of buffer. 100 µL of each of the $10^0$ and $10^{-1}$ dilutions was plated on Mueller-Hinton (MH) Agar plates (1.2 g Beef
Extract, 10.5 g Acid Hydrolysate of Casein, 0.9 g Starch, 10.2 g Agar with 600 mL H2O) using sterile glass pipettes, and the liquid was spread evenly on the plate with a sterilized glass spreader. Four antibiotic disks, each containing one of penicillin, gentamycin, tetracycline, or erythromycin at 10 U, 10 µg, 30 µg, and 2 µg, respectively, were added to the plate, and the plates were incubated for 48 hours at 37°C. The remaining bacteria-buffer suspension in the 10^0 dilution tube was centrifuged at 12,000xg for 1 minute, the supernatant removed, and the pellet was frozen for DNA analysis.

DNA Extraction and Sequencing

The frozen pellet of each sample was allowed to thaw to room temperature and DNA extracted using a Mo Bio UltraClean Microbial DNA Isolation Kit, following the detailed protocol supplied by the manufacturer (Mo Bio Laboratories, Carlsbad, CA). The presence of DNA in each extraction was confirmed through agarose gel electrophoresis (Results, Figure 4). Bacterial tag-encoded FLX amplicon 454 pyrosequencing (bTEFAP) (Dowd et al. 2008) was conducted on the 16S rRNA gene from each sample, through a dedicated sequencing facility (MR DNA, Shallowater, TX). Bacterial specific 16S rRNA gene primers 939f and 1392r (Jackson et al. 2001, Baker et al. 2003) were used in the sequencing reaction. A single-step PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation,
Danvers, MA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following the manufacturer’s guidelines. A negative control amplification was used in the same 454 reaction and gave no valid reads. Raw pyrosequence data derived from the sequencing process was transferred into FASTA files for each sample, along with sequencing quality files. Files were accessed using the bioinformatics software Mothur (Schloss et al. 2009) where they were processed and analyzed following general procedures recommended by Schloss et al. (2011) and as described previously (Jackson et al., 2013). Final sequence types obtained were identified by BLAST searches through GenBank.
Results

Bacterial Counts

After incubation, the visible bacterial colonies were counted on the dilution plate that contained <300 colony-forming units (CFU). The following plates yielded visible colonies: Brown’s TSA, Brown’s MA, Great Value regular TSA, Great Value regular MA, Turner TSA, Turner MA, Kroger TSA, Prairie Farm TSA, Prairie Farm MA, Best Choice TSA, and Best Choice MA (e.g. Figure 1). Once the bacterial count was obtained for each agar plate, calculations were carried out to express the number of bacteria as per mL and as the number of bacteria in the 1.89 L bottle from which each sample was acquired (Table 2). In general, the ultra-pasteurized USDA organic milk did not produce any culturable bacteria on the TSA and MA plates, while almost all of the conventionally produced milk gave visible colonies. The farmer’s market (Brown’s Dairy) milk and the Best Choice brand of milk contained noticeably higher numbers of bacteria, compared to the other brands. There was no consistent pattern to whether the samples grew better on TSA or MA: three samples (Kroger, Great Value, Brown’s Dairy) grew better on TSA compared to MA; while three other samples (Turner’s, Best Choice, Prairie Farms) grew better on MA. These differences in growth were sometimes substantial; for example, the Great Value sample yielded 14 times more colonies on TSA than MA; while the Best Choice samples gave 4 times as many colonies on MA than TSA. The number of culturable bacteria in each milk sample was also expressed per 8 oz serving in order to assess the amount of bacteria to which typical consumers might be exposed (Figure 2).
Figure 1: Example of the variety of bacterial colonies found on TSA and MA plates after plating milk samples. A: Brown’s TSA, $10^0$ dilution. B: Turner’s TSA, $10^0$ dilution.
Table 2: CFU counts of each sample of milk per mL and per 1.89 L carton, as obtained using tryptic soy agar (TSA) and milk agar (MA) plates.

<table>
<thead>
<tr>
<th>Sample:</th>
<th>Bacterial Count:</th>
<th>CFU/mL</th>
<th>CFU/1.89 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown’s TSA</td>
<td>49 colonies on $10^{-2}$</td>
<td>49,000</td>
<td>92.6 million</td>
</tr>
<tr>
<td>Brown’s MA</td>
<td>42 colonies on $10^{-2}$</td>
<td>42,000</td>
<td>79.4 million</td>
</tr>
<tr>
<td>GV Reg TSA</td>
<td>14 colonies on $10^0$</td>
<td>14</td>
<td>26,460</td>
</tr>
<tr>
<td>GV Reg MA</td>
<td>1 large colony on $10^0$</td>
<td>1</td>
<td>1,890</td>
</tr>
<tr>
<td>GV Org TSA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GV Org MA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Horizon TSA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Horizon MA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Turner TSA</td>
<td>4 large colonies on $10^0$</td>
<td>4</td>
<td>1,892</td>
</tr>
<tr>
<td>Turner MA</td>
<td>6 large colonies on $10^0$</td>
<td>6</td>
<td>2,838</td>
</tr>
<tr>
<td>Simple Truth TSA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Simple Truth MA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kroger TSA</td>
<td>2 large colonies on $10^0$</td>
<td>2</td>
<td>3,780</td>
</tr>
<tr>
<td>Kroger MA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Best Choice TSA</td>
<td>25 colonies on $10^{-2}$</td>
<td>25,000</td>
<td>47.25 million</td>
</tr>
<tr>
<td>Best Choice MA</td>
<td>104 colonies on $10^{-2}$</td>
<td>104,000</td>
<td>196.6 million</td>
</tr>
<tr>
<td>Stremick’s TSA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stremick’s MA</td>
<td>0 colonies</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prairie Farm TSA</td>
<td>6 colonies on $10^0$</td>
<td>6</td>
<td>11,340</td>
</tr>
<tr>
<td>Prairie Farm MA</td>
<td>34 colonies on $10^0$</td>
<td>34</td>
<td>64,260</td>
</tr>
</tbody>
</table>
Figure 2: Bacterial counts from various milk samples shown in CFU/8 oz glass of milk. Counts were obtained using tryptic soy agar (TSA) and milk agar (MA) plates.
The typical number of bacteria found in each sample was approximately 1000-2000 per 8 oz, although some samples contained upwards of 10 million bacteria in a single serving.

Analysis of Antibiotic Resistance Testing

Following incubation, the 11 plates that were tested for antibiotic resistance were examined. If the zone of inhibition (the gap between the disk and bacterial growth) was large (5-10 mm), the bacteria were regarded as being susceptible to that antibiotic. If the zone of inhibition was small (1-2 mm), the bacteria were regarded as being partially resistant to that antibiotic. If there was no visible zone of inhibition, the bacteria were regarded as being fully resistant to that antibiotic. Figure 3 shows the variety of resistance of each sample to the four antibiotics: gentamycin, tetracycline, erythromycin, and penicillin. In general, every sample tested was susceptible to gentamycin, and all of the samples, with the exception of one, showed resistance to penicillin. The sample with the least amount of antibiotic resistance was the Kroger brand, while Brown’s Dairy and Best Choice had the most antibiotic resistance (Table 3).

Sample Identification

The presence of DNA in each extraction was confirmed through DNA gel electrophoresis (Figure 4), and a portion of the 16S rRNA gene in each sample was subsequently sequenced. The most common sequence detected across all samples was identified as *Lactococcus lactis, lactis IL1403 strain*, and this sequence type accounted for at least 40% of the sequences obtained from all samples, with the exception of Best Choice MA. *Lactococcus lactis* was the only bacterium identified on the following plates: Turner
Figure 3: Examples of the variety of antibiotic resistance seen during testing of mixed cultures of bacteria obtained from milk samples using tryptic soy agar (TSA) and milk agar (MA) plates. Brown’s Dairy TSA (top left) and Best Choice TSA (bottom left) showed the highest resistance, while Kroger TSA showed the least resistance (top right). Other samples such as Turner’s MA (bottom right) were intermediate.
Table 3: Antibiotic resistance results for 11 samples of mixed cultures obtained from milk (Brown’s TSA and MA, Great Value TSA and MA, Turner TSA and MA, Kroger TSA, Prairie Farms TSA and MA, Best Choice TSA and MA). The samples showing resistance to an antibiotic are symbolized with ++++. The samples showing partial resistance to an antibiotic are symbolized with ++. The samples showing susceptibility to an antibiotic are symbolized with +.

<table>
<thead>
<tr>
<th>Milk Sample</th>
<th>Penicillin</th>
<th>Erythromycin</th>
<th>Gentamycin</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown’s TSA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Brown’s MA</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>GV Reg TSA</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>GV Reg MA</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Turner TSA</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Turner MA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Kroger TSA</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Prairie Farm TSA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Prairie Farm MA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>BC TSA</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BC MA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
Figure 4: Results of DNA extraction from 11 samples of milk cultured on either tryptic soy agar (TSA) or milk agar (MA) plates as confirmed via gel electrophoresis. DNA was extracted from Great Value regular TSA, Great Value regular MA, Prairie Farms TSA, and Prairie Farms MA (upper figure), as well as, Brown’s Dairy TSA, Brown’s Dairy MA, Turner’s TSA, Turner’s MA, Best Choice TSA, Best Choice MA, and Kroger TSA (lower figure).
TSA, Turner MA, Great Value MA, Prairie Farms TSA, Kroger TSA, and Best Choice TSA (Figure 5). The other plates examined (Great Value TSA, Prairie Farms MA, Best Choice MA, Brown’s Dairy TSA and Brown’s MA) each contained more than one identifiable bacterium based on sequence analysis (Figure 6). The TSA and MA plates obtained from Brown’s Dairy milk showed the most diverse set of bacteria, each giving five different sequences, although *Lactococcus lactis* was still the most common sequence type detected on those plates (Figure 7).
Figure 5: Bacteria from milk samples grown on Tryptic Soy Agar (TSA) and Milk Agar (MA) were sequenced and identified. This figure shows the microbial makeup (% sequences obtained) of Turner TSA, Turner MA, Great Value TSA, Great Value MA, and Kroger TSA. Each sample contained only *Lactococcus lactis*.  

* Lactococcus lactis, lactis IL1403 strain

Turner TSA

Turner MA

Great Value TSA

Great Value MA

Kroger TSA
Figure 6: Bacteria from milk samples grown on Tryptic Soy Agar (TSA) and Milk Agar (MA) were sequenced and identified. This figure shows the microbial makeup (% sequences obtained) of Best Choice TSA, Best Choice MA, Prairie Farms TSA, and Prairie Farms MA. Each color represents a different strain of bacteria, using the following legend.

<table>
<thead>
<tr>
<th>Color</th>
<th>Legend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td><em>Lactococcus lactis, lactis IL1403 strain</em></td>
</tr>
<tr>
<td>Red</td>
<td><em>Lactobacillus buchneri, NRRL B-30929 strain</em></td>
</tr>
</tbody>
</table>

Best Choice TSA

Best Choice MA

Prairie Farms TSA

Prairie Farms MA
Figure 7: Bacteria from milk samples grown on Tryptic Soy Agar (TSA) and Milk Agar (MA) were sequenced and identified. This figure shows the microbial makeup (% sequences obtained) of Brown’s Dairy TSA and Brown’s Dairy MA. Each color represents a different strain of bacteria, using the following legend.

- **Lactococcus lactis, lactis IL1403 strain**
- **Paenibacillus glycanilyticus strain DS-1**
- **Chryseobacterium bovis DSM 19482 strain H9**
- **Streptococcus agalactiae 2603V/R strain**
- **Sphingobacterium alimentarium strain WCC 4521**
- **Lactobacillus rhamnosus GG strain (ATCC 53103)**

### Brown's Dairy TSA

![Pie chart showing bacterial composition for Brown's Dairy TSA](chart)

### Brown's Dairy MA

![Pie chart showing bacterial composition for Brown's Dairy MA](chart)
Discussion

Various microbial populations were found in milk samples from conventional, USDA organic, and local “farmer’s market” milk. Samples were plated onto Tryptic Soy Agar (TSA) or Milk Agar (MA) plates. TSA was used in this study because it is a generalized agar that allows many different bacteria to grow, while MA was utilized because of its specificity to the bacteria in milk. USDA organic milk samples, being ultra-pasteurized, yielded few or no colonies on the TSA or MA plates, while the conventional and “farmer’s market” milk samples gave substantial numbers of bacterial colonies. While some bacterial colonies covered the plate as individuals (as in Brown’s Dairy samples), others intermixed, forming a single large colony on the plate (as in the Turner milk sample). The latter situation resulted in lower bacterial counts, so that the numbers reported here are likely underestimates. The amount of bacteria found in a single 8oz glass of milk was calculated, which gives an estimate of the number of bacteria ingested by a typical consumer when, for example, drinking a glass of milk. Even allowing for this underestimate, the plates with few large colonies still averaged at least 1000-2000 bacteria per glass of milk. The plates with separate colonies spread over the plate yielded much higher numbers of bacteria per glass, upwards of 10 million. These bacterial counts indicate a consistent exposure to bacteria from milk, and potentially to antibiotic resistant bacteria, which has implications for the medical field. Consumers should be aware of these bacteria when ingesting different types of milk, and weigh the advantages as well as the disadvantages of ingesting these microorganisms. It
was not possible to determine the benefit (or lack thereof) of ingesting fewer bacteria in ultra-pasteurized organic milk versus ingesting more, possibly beneficial or harmless bacteria in the conventional or “farmer’s market” milk. It’s quite possible that by ingesting ultra-pasteurized USDA organic milk the consumer would be protected from potential pathogens or antibiotic resistant bacteria found in local or raw milk types.

Results from the antibiotic resistance tests showed an alarming number of antibiotic resistance bacteria in both conventional and farmer’s market milk samples (USDA organic milk samples were not tested for antibiotic resistance because no bacteria were cultured on either the TSA or MA plates). Bacteria grown on the plates from the conventional and farmer’s market milk samples were collected and tested for overall antibiotic resistance. Since the sample was not a pure culture, some of the plates may have contained more than one strain of bacteria that varied in susceptibility or resistance to a particular antibiotic. However, this was not of concern, because the overall resistance or susceptibility of each milk sample was in question. Overall, all of the samples tested showed susceptibility to gentamycin and considerable resistance to penicillin. Of all the samples, only two conventional milk samples were susceptible to erythromycin, and none of the samples were susceptible to tetracycline. Thus, there was a substantial amount of antibiotic resistance in both conventional and farmer’s market milk samples, particularly towards the commonly used antibiotics penicillin and tetracycline, and also to erythromycin. It was originally hypothesized that the farmer’s market milk sample would show less antibiotic resistance, but this was not shown to be the case. However, the farmer’s market milk contained more than one identifiable species, so that the increased overall resistance of this sample may actually reflect
differential resistance of multiple bacterial species, rather than one multi-resistant bacterium. On the other hand, all of the conventional milk samples contained only one or two identifiable bacteria, but they showed considerable antibiotic resistance. This suggests that those bacteria have antibiotic resistance genes to penicillin, tetracycline, and erythromycin.

*Lactococcus lactis* accounted for over 40% of 16S rRNA gene sequences found in each sample, showing that it was clearly the most prevalent bacterium in milk. *L. lactis* was the only bacterium cultured on TSA from any of the conventional milk samples, while, in contrast, the farmer’s market milk sample contained at least four other bacteria. *L. lactis* is a Gram-positive, nonpathogenic bacterium that is commonly found in milk and milk products, because of its ability to carry out lactic acid fermentation (Bolotin 2001). *L. lactis* is potentially beneficial and has been tested extensively for therapeutic applications as delivery vehicles for vaccines and in the treatment or prevention of inflammatory bowel disease (such as Chrohn’s Disease), allergies, and viral infections (Braat 2006, Wells, 2008). In addition to *L. lactis*, two conventional milk samples (Best Choice and Prairie Farms) grown on MA plates also contained *Lactobacillus buchneri*, a bacterium that was first isolated from commercial ethanol plants and is commonly added to corn silages to prevent spoilage (Liu 2011). The presence of *L. buchneri* in these samples might suggest contamination during milk production, since it is found on dairy farms, yet not commonly found in milk.

The farmer’s market milk sample (Brown’s Dairy) had the most diverse set of bacteria that grew on the TSA and MA plates. The sequences in the sample from TSA contained *Lactococcus lactis, Paenibacillus glycanilyticus, Chryseobacterium bovis,*
Streptococcus agalactiae, and Lactobacillus rhamnosus. The sequences in the MA sample yielded two strains of Lactococcus lactis, Paenibacillus glycanilyticus, Streptococcus agalactiae, and Sphingobacterium alimentarium. Strangely, *P. glycanilyticus* is a polysaccharide-degrading soil bacterium (Dasman 2002); however, many organisms in the species *Paenibacillus* are shelf-life limiting organisms, and they have been implicated in the spoilage of pasteurized milk (Ranieri 2012). It is possible that it was not specifically the soil dwelling *P. glycanilyticus*, but a closely related milk-inhabiting organism. Regardless, while the genus *Paenibacillus* contains mostly environmental microorganisms, pathogenic *Paenibacillus* bacteria can cause infections in humans (Ouyang 2008) so that the presence of this genus in milk is potentially of interest. Another bacterium found in the Brown’s Dairy TSA sample was *C. bovis*, a Gram-negative bacterium that has been previously isolated from raw cow’s milk, that can cause defects in many food products, including milk (Hantsis-Zacharov 2008). As with *Paenibacillus*, the genus *Chryseobacterium* includes some potential human pathogens, including *C. indologenes*, which causes bloodstream infections (Chen 2013), and *C. meningosepticum*, which causes meningitis in premature infants and immuno-compromised patients (Ceyhan 2011).

*Streptococcus agalactiae* is another potential human and animal pathogen that was found in Brown’s Dairy TSA and MA samples. This bacterium can cause infections in both bovines and humans, although there are differences between the two strains (Shome 2012). Bovine *S. agalactiae* is a major pathogen in dairy cows that can lead to decreased milk quality (Shome 2012). The human variant of *S. agalactiae* is commonly found in the gastrointestinal, urinary, and genital tract of healthy females but can lead to
postpartum infections and sepsis in pregnant women and infants, as well as, bloodstream infections and meningitis in non-pregnant adults, who normally had an underlying medical condition (Chaiwarith 2011). Other bacteria obtained from the farmer’s market milk sample included *Lactobacillus rhamnosus* (found only in the Brown’s Dairy TSA sample), which is commonly found in milk and dairy products as a culture starter, as well as, being marketed as a probiotic (Douillard 2013) and *Sphingobacterium alimentarium*, a Gram-negative bacterium that has been previously isolated from raw cow’s milk (Schmidt 2012). Clearly, the farmer’s market milk sample contained a diverse set of potentially pathogenic, beneficial, and harmless bacteria. These bacteria could have come from contamination during production (e.g. *P. glycanilyticus*), from an infection in the dairy cow (e.g. *S. agalactiae*), or the bacteria could be native microorganisms to raw milk that would usually be removed during the processing of conventionally produced milk (e.g. *C. bovis, L. rhamnosus, S. alimentarium*).

These results show the inherent risk of ingesting local or conventional milk types over USDA organic. In a single 8oz glass of milk, the consumer would consume between 1000-2000 bacteria in the conventional milk and upwards of 10 million bacteria in the local milk; while consumers drinking an 8oz glass of USDA organic milk, having been ultra-pasteurized, would not ingest any culturable bacteria. In addition, by drinking either local or conventional milk types, the consumer would be exposed to a considerable amount of potentially pathogenic, antibiotic resistant bacteria. This has major implications for the consumer’s health and the medical field has a whole. Ingesting the antibiotic resistant bacteria found in local and conventional milk types potentially aids the spread of antibiotic resistant genes and puts consumers at risk of developing an antibiotic
resistant infection. Although raw milk was not tested specifically in this study, the
dangers of drinking raw milk have been persistently reported by the Centers for Disease
Control and Prevention. If the pasteurized farmer’s market milk contained upwards of 10
million bacteria per 8 oz glass of milk, then by extrapolation the raw milk would contain
even higher bacterial counts, with potentially more pathogens. While organic milk has
not been declared healthier than other milk types, there is a definite benefit in consuming
USDA certified organic milk that has been acknowledged in this study. By consuming
USDA organic milk, the consumer is not exposed to the thousands to millions of
potentially pathogenic, antibiotic resistant bacteria found in other milk types. These
results present the need for consumer awareness in milk selection. Consumers searching
for alternatives to conventionally produced milk should weigh the benefits alongside the
risk of ingesting raw and local milk. Although it will take time to reduce antibiotic use
both in the medical field and food production, consumers can avoid exposure to antibiotic
resistant bacteria by choosing to purchase and ingest organic, ultra-pasteurized milk. In
conclusion, more research is warranted to find new strategies to combat antibiotic
resistance and to treat antibiotic resistant infections; however, in the meantime consumers
can choose to consume organic foods to lessen their exposure to antibiotic resistant
bacteria and thereby reduce the risk of developing an antibiotic resistant infection.
LIST OF REFERENCES


