



AN ABSTRACT OF THE THESIS OF

Liam Robert Wustenberg for the degree of Master of Science in Food Science and Technology presented on September 20, 2011.

Title: Shelf Life and Microbial Content of Fluid Milk from Nine Processing Plants in Oregon

Abstract  
approved: \_\_\_\_\_

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High-temperature-short-time pasteurized fluid milk commonly has a shelf life around 16-19 days. The aim of this study was to determine the shelf life of fluid milk in Oregon and characterize the bacterial growth over 23 days. Samples of pasteurized 2% milk were collected at six time points from all nine fluid milk-processing plants within Oregon. Milk was collected at 23, 21, 19, 16, and 8 days pre-testing, allowing for aging to occur. Fresh samples were collected 24 hours pre-testing. Raw milk samples were collected twice at 23 days and within 24 hours of testing for enumeration of endospores. Samples from each time point were cultured for Total Plate Count (TPC), and gram-negative counts (GNC). Concurrently, shelf lives of the milk were determined by employing triangle tests

(N=25) where milk-drinking consumers were asked to distinguish between fresh and aged milk samples. Shelf lives varied greatly among the nine processing plants ranging from less than 8 to 21 days. Two accelerated milk shelf-life tests, the Pre-Incubation Count (PI) and the Moseley Keeping Quality (MKQ) test were also conducted. Both accelerated shelf life tests failed to accurately predict the shelf lives among plants. TPC's at 23 days of age were within one log value of  $10^6$ cfu/ml among all nine dairy plants. Endospore counts (ESC) ranged from  $10^3$ - $10^7$  at 23 days. ESC counts trailed the TPC and gram-negative counts, as high numbers of spores were not seen until after 16 days. GNC counts followed the TPC closely. The four dairies with the shortest shelf lives utilized coated paperboard carton packaging. Microbial content and packaging material showed no correlation. Pasteurization temperature did not correlate to the bacterial growth, although the five plants with the shortest shelf lives utilized the longest holding times. Bacterial species in raw and pasteurized milk samples were identified using Terminal Restriction Fragment Length Polymorphism (t-RFLP). *Paenibacillus* spp. were the most commonly found organism in the pasteurized samples. *Pseudomonas* spp. were only found in one pasteurized sample, however they were found in 56% of the raw milk samples. *Streptococcus* spp. were found in the pasteurized milk of the four plants with the shortest shelf lives and are a possible cause of reduced shelf life. This study demonstrates that although pasteurized milk processing is a well-established process, there is still significant variability in the milk quality and shelf life between dairy plants.

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SHELF LIFE AND MICROBIAL CONTENT OF FLUID MILK FROM NINE  
PROCESSING PLANTS IN OREGON

by  
Liam Robert Wustenberg

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I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Liam Robert Wustenberg, Author



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## **CHAPTER 1**

### **GENERAL INTRODUCTION**

#### **U.S. Milk Production**

High-temperature short-time (HTST) pasteurized fluid milk remains a competitor in U.S. beverage market. The U.S. is the leading cow's milk producer in the world supplying 193 billion pounds of milk in 2010. However, per-person consumption has decreased recently due to competition from extended shelf-life (ESL) products such as water and soda. Approximately 30 percent of the milk produced in the US is processed into fluid milk and cream, with the remainder being transformed into cheese, butter, powders and frozen products (NASS, 2011). Since the early 1900s, there have been numerous changes to the dairy production and processing industries in the U.S. The most prominent of which are a sharp reduction in total cow numbers, a near six-fold increase in average production per cow, substantially greater total annual milk production, a large decrease in the number of herds in the U.S., but a large increase in the number of cows per herd, a shift in cow numbers from the north and east to the west; and a decline in the per capita consumption of whole milk equivalent and fluid milk. Major advances in technology and dairy processing have drastically improved the safety of fluid milk (Chandan et al., 2008). Because of these advances in safety, there has been a shift in the focus of dairy research from the safety of fluid milk to general quality and acceptance of fluid milk. HTST fluid milk processors are still face challenges

extending the shelf life beyond 14 days. For HTST milk to continue to compete in the U.S. beverage market, there will need to be drastic increases in shelf life.

### **Oregon Dairy Industry**

There are nine different fluid milk processing plants in Oregon, along with several very small-scale processors. Eight of the nine processing plants produce HTST pasteurized milk, only one of the processing plants produces ultra-pasteurized milk as well. Oregon is different than most states in the U.S. in that it has many medium scale and small independent dairy processing plants. With increased density of fluid milk processors comes an increased likelihood of variability in fluid milk quality. The varying sizes of fluid milk processing plants may also cause variability in milk quality.

### **Fluid Milk Processing**

Current fluid milk processing practices can be divided into seven general steps, from the farm to the packaged product. These steps include:

1. Bulk milk handling and storage
2. Separation
3. Standardization
4. Homogenization
5. Pasteurization
6. Cooling
7. Packaging and storage

Figure 1 outlines processing steps one through six. Each one of these steps can be broken down into several stages. Bulk milk handling and storage starts on the farm where the cows are milked and the raw milk is cooled over several hours to below 7.2°C and stored in a raw milk bulk tank until pick-up. The milk is then transported in a tanker truck from the farm to processing plant. In some cases the farm is on the same grounds as the processing plant and no transportation is required. At the processing plant the milk is stored in silos and often mixed with raw milk from other dairy farms. Separation is the first step that takes place in the processing plant. During this step the milk is separated into a heavier skimmed milk fraction and a lighter cream fraction (Chandan et al., 2008). The separation step allows for standardization, which is the next step in the process. During standardization the processor can obtain a predetermined fat content. Blending the cream and skimmed milk fractions allows for the production of products with varying fat contents. Reduced fat milks are fortified with vitamins A and D. Vitamins A and D are both fat soluble so they are removed in the cream fraction and must be added back to the skimmed fraction. This usually takes place between the separation and homogenization step. If the vitamins are added before homogenization it allows for them to be dispersed properly. Homogenization can take place in either one or two stages and is always carried out at temperature of 37.2°C or higher. Often the cream is homogenized after separation and then added back into the skimmed milk during standardization. During the homogenization step the fat globules of the milk are reduced in size and the total surface area of exposed lipids is increased.

Homogenization prevents the milk fat from separating during storage. Once the milk is standardized it is heat-treated. Several pasteurization methods are commonly used for fluid milk processing, specifically HTST and ultra-high temperature (UHT) pasteurization. Low temperature long time (LTLT) pasteurization is not commonly used in fluid milk production since the rise of HTST and UHT pasteurization methods. HTST and UHT pasteurization utilize a regeneration step which makes them more efficient than LTLT pasteurization. Although UHT pasteurization produces a longer shelf life product when packaged aseptically, there still tends to be a preference for HTST milk by consumers (Blakely, 1995). There are off-flavors associated with the UHT pasteurization process, the most common of which is a cooked flavor (Blakely, 1995). Specifications for the pasteurization temperature and time combinations approved by the FDA can be found in Table 1.1.

Table 1.1. Temperature (°C) - time (min/sec.) relationships for pasteurization as specified by US FDA's Grade "A" Pasteurized milk ordinance.

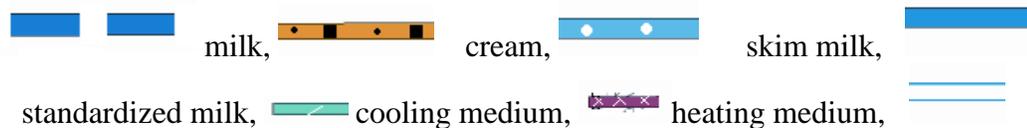
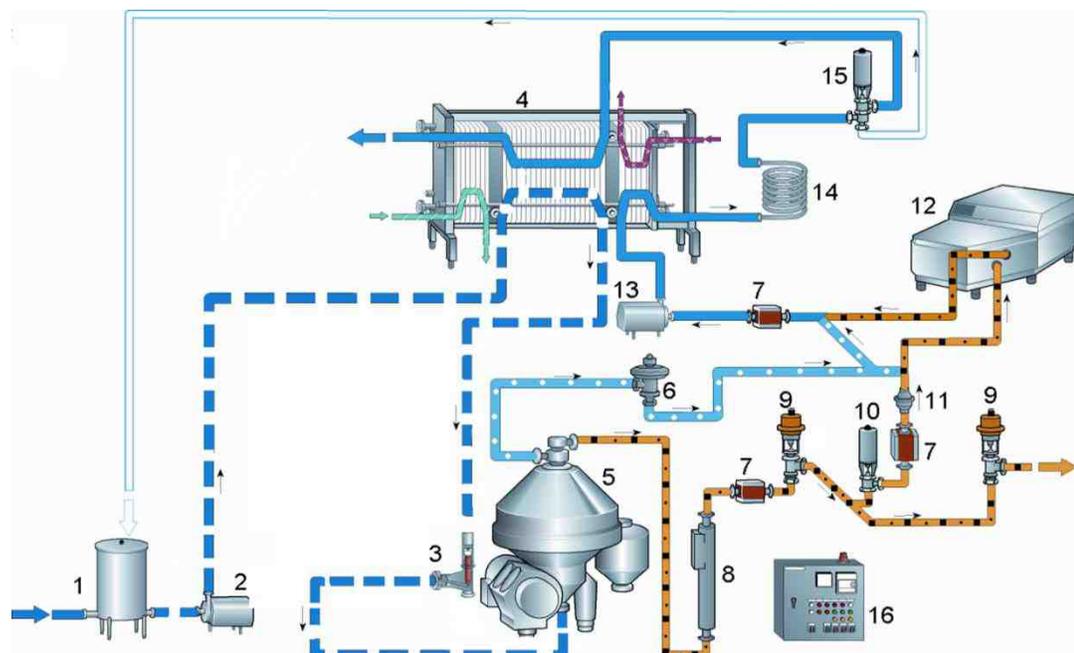
Temperature (°C)	Time (seconds)
<sup>a</sup> 63	30 min (LTLT)
<sup>a</sup> 72	15 (HTST)
89	1.0
90	0.5
94	0.1
96	0.05
100	0.01

<sup>a</sup> If the fat content of the milk product is 10% or more, or if it contains added sweeteners, the specified temperature shall be increased by 3°C.

The majority of fluid milk processing plants in Oregon utilize HTST pasteurization specifications (72°C, 15 seconds). After pasteurization the milk is cooled and readied for packaging. The packaging step has been shown to be critical in keeping microbial levels low (Gruetzmacher and Bradley, 1999). The milk can be packaged in several different containers such as high-density polyethylene (HDPE) bottles or coated paperboard cartons (CPC). Once packaged the milk is stored at approximately 4.4°C until delivery to the store shelf. All of these steps, from the farm to the packaged product, are critical in producing a high quality product.

This research focuses on determining the shelf lives as well as the microbial content of the fluid milk produced at nine different processing plants. One of the nine plants was shut down during the study so currently there are only eight major HTST processing plants in Oregon.

**Figure 1.1** Production line for pasteurized milk. Milk passes balance tank, regeneration section, separator, online standardization with homogenized cream, heating section, holding tube, regeneration section, and cooling section. diverted flow. . Balance tank (1), product feed pump (2), flow controller (3), plate heat exchanger (4), separator (5), constant pressure valve (6), flow transmitter (7), density transmitter (8), regulating valve (9), shut-off valve (10), check valve (11), homogenizer (12), booster pump (13), holding tube (14), flow diversion valve (15), process control (16)



Tetrapak (1995)

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **Shelf Life of Fluid Milk**

Shelf life is the age by which the quality of fluid milk changes from acceptable to unacceptable. The shelf life of fluid milk is impacted by several different factors. The use-by (UBD) and sell-by dates (SBD) are an estimation of shelf life. These dates may be used by the consumer to determine when milk will spoiled. By law, fluid milk must have a date printed on the container. However, it is the processor who decides how long the code date lasts. The law does not prohibit the sale of fluid milk that has surpassed its SBD or UBD. The total plate count is another benchmark for milk spoilage and has been used for decades. Pasteurized Grade A milk is required to have less than 20,000 cfu/ml total count and less than 10 cfu/ml for the coliform count (FDA, 2009). However, freshly pasteurized milk often contains less than 500 cfu/ml. Raw milk from a single producer must contain less than 100,000 cfu/ml total count and less than 300,000 cfu/ml total count if it is comingled (FDA, 2009). Although these regulations help to maintain good quality milk, bacterial growth is not the only cause of milk spoilage. Off-flavors in fluid milk can arise from many different root causes such as chemical reactions and absorption of off-flavors from the environment. Sensory evaluation is another tool for determining milk spoilage. With a combination of sensory methods one could determine when a milk samples has spoiled and define the off-flavors potentially giving clues as to what the cause of spoilage may have been. These methods range

from descriptive analysis panels, which can describe and characterize off-flavors in a product, to consumer acceptability testing, and discrimination testing. Currently, fluid milk processors taste the milk when the UBD or SBD is up to determine if they are meeting their benchmarks. This method does not prevent inferior quality milk from being sold. The sale of reduced quality milk has a negative effect on the consumer's perception of the product and the dairy industry as a whole.

### **Raw Milk Quality**

As with all food products, the quality of the raw milk directly affects the quality of the finished, pasteurized product (Cromie, 1992; Hayes, 2001; Nornberg, 2010). Contamination of raw milk can come from several sources including mastitic cows, dirty udders or teats, and poorly cleaned milking and storage equipment (Huck et al., 2007; Huck et al., 2008). Many different bacteria can be found in raw milk including both gram-negative bacteria (e.g. *Pseudomonas spp.*, *Aeromonas*, *Serratia spp.*, *Achromobacter spp.*, *Alcaligenes*, *Chromobacterium spp.*, and *Flavobacterium spp.*) and gram-positive bacteria (e.g. *Bacillus spp.*, *Clostridium spp.*, *Paenibacillus spp.*, *Streptococcus spp.*, *Staphylococcus spp.*) (Ternstrom et al., 1993; Surhaug and Stepaniak, 1997; Hayes et al., 2001; Nörnberg 2010). Psychrotrophic bacteria, which are able to propagate and metabolize at refrigeration temperatures (0-7°C) are major contaminants in raw milk and can be a common cause of fluid milk spoilage (Ternstrom et al., 1993; Moore, 2001). Psychrotrophic bacteria have become an increasing problem since the introduction of refrigerated raw milk-storage tanks on the dairy farm (Surhaug and Stepaniak,

1997). After the introduction of refrigerated raw milk storage, the rate of raw milk collection from the farm decreased to two or three collections per week. In some cases the milk is being further stored at the processing plant over weekends (Cromie, 1992). Despite the optimum growth temperature of psychrotrophic bacteria, which varies from 15-30°C (Morita, 1975), storage times of two days at 7°C or less, on the farm allows for these bacteria to grow and eventually dominate the raw milk flora (Cromie, 1992; Surhaug and Stepaniak, 1997). The law requires that the raw milk be stored for no longer than 48 hours on the farm (FDA, 2009).

Mastitis is of major concern when striving for great quality milk. Mastitis is characterized by a bacterial infection of the teat or udder of the cow. When a cow with mastitis is milked it can secrete viable bacterial cells in concentrations of up to  $10^7$  cfu/ml (National Mastitis Council, 2011). There are several bacteria that can cause a mastitis infection including, *Streptococcus*, *Staphylococcus*, *Enterococcus* and coliforms such as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* (National Mastitis Council, 2011). The other major problem associated with mastitis is an increased somatic cell count (SCC). Somatic cells produce heat stable proteases which can break down milk protein and cause off-flavors to develop in the pasteurized product (Ma et al., 2000). This increased proteolysis can also be detrimental to yields of other products such as cheese and yogurt (Politis, 1988). Because of this, the cheese industry offers premium prices for low SCC milk. The fluid milk industry has yet to offer such benefits. There is little research on the effects of increased SCC on fluid milk quality. Ma et al. (2000) conducted a study

on the effects of SCC on fluid milk quality. The research showed that milk with higher SCC counts had increased proteolytic and lipolytic activity in raw milk. They also found that off-flavors such as bitterness and rancidity in the pasteurized milk were associated with the high levels of proteolysis and lipolysis. Ma et al. (2000) found that milk with low SCC retained high organoleptic quality throughout a 21 day shelf life while milk with high SCC developed rancid and bitter off-flavors after 14 days of shelf life. Their study suggests that the onset of rancid and bitter off-flavors in the pasteurized milk is a result of the lipolytic and proteolytic activity in the raw milk. They also suggest that quality premium payment programs based on SCC be initiated to help improve fluid milk quality.

Psychrotrophs are capable of producing both proteolytic and lipolytic enzymes during growth at psychrotrophic temperatures, which may be heat-stable and remain active after pasteurization (Cromie, 1992; Ezzati et al., 2010; Nörnberg, 2010). Griffiths et al. (1988) found a correlation between storage time of raw milk at 2°C and 6°C and the initial psychrotrophic count of the pasteurized milk. Proteases produced by psychrotrophic bacteria are capable of spoiling milk after pasteurization. Nörnberg et al. (2010) found that the addition of proteases isolated from psychrotrophic bacteria found in raw milk, to UHT milk caused extensive coagulation after only 5 days. These proteases can also cause an increase concentration of peptides in milk, which can cause bitter flavors to predominate (Surhaug and Stepaniak, 1997; Nörnberg, 2010). Lipolytic enzymes that are produced by some *Pseudomonas* spp. can hydrolyze triglycerides, which are the

main lipid component of milk, creating short chain fatty acids or free fatty acids (FFA). An increase in FFA has been shown to cause strong, offensive defects such as rancidity and bitter off-flavors (Surhaug and Stepaniak, 1997; Boor et al., 1998; Nörnberg, 2010). Lipolysis due to microbial enzymes accounts for only a small portion of the lipolytic action in raw milk (Deeth and Fitz-Gerald, 1995). Much of the lipolytic break down that takes place before pasteurization is due the lipoprotein lipase (LPL), which is not produced by microorganisms but is inherent in the raw milk and not heat stable.

Non-microbial lipolysis due to LPL can be divided into two overlying categories: spontaneous lipolysis and induced lipolysis. Induced lipolysis is defined as lipolysis that is promoted by shearing of the milk fat globule by physical abuse of the milk, which exposes the lipid substrate to the LPL enzyme. Induced lipolysis can be triggered on the farm, during transportation or in the processing plant (Deeth and Fitz-Gerald, 1995). Spontaneous lipolysis can be defined as lipolysis that occurs when the milk has only been cooled after milking. Spontaneous lipolysis is affected by three main factors: the degree of lipase activity; the integrity of the milk fat globule membrane (MFGM); and the balance of lipolysis-activating and inhibiting factors (Cartier and Chilliard, 1990).

Lipoprotein lipase is not heat stable, but the off-flavors caused by its activity in raw milk can be transmitted into the pasteurized product. Because of the heat-instability of LPL, occurrence of off-flavors in the pasteurized milk due to LPL action can be avoided by taking special care during the production,

transportation and pre-pasteurization processing steps. One major cause of LPL spoilage in pasteurized milk is the mixture of raw and pasteurized product during processing (Chandan, 2008). LPL action can also be promoted pre-pasteurization by different feed types, physical abuse, homogenization, and freezing and thawing of the raw milk. Off-flavors due to lipolytic activity can occur within 24 hours depending on temperature of milk and the degree of activity of the lipase (Clark et al., 2009). Rancidity due to LPL action is most common in un-homogenized milk but can occur in homogenized milk if pasteurization does not immediately follow (Clark et al., 2009). Homogenized raw milk has more surface area that is exposed to lipase and therefore is susceptible to hydrolytic rancidity (Deeth, 1986).

The other major spoilage mechanism that occurs pre-pasteurization is oxidation. Vitamin A, milk protein, and lipids are all prone to oxidation which can create off-flavors that carry through to the pasteurized product (Barrefors et al., 1995). The off-flavors that occur due to oxidation include cardboardy, metallic, tallowy, stale, oily, and fishy (Havemose, et al. 2006; Hedegaard et al., 2006). There are two different types of oxidation that occur in fluid milk: metal induced-oxidation; and light-induced oxidation. The metal-induced oxidation is primarily associated with unsaturated lipids and commonly occurs due to three common causes: direct contact with raw metals; introducing trace metals to the feed of milking cows; or, the presence of divalent cations (Cu, Fe, Mn) in the water supply used for cleaning milking equipment. Exposure to these metals catalyses an auto-oxidation of the lipids, forming a free radical, which produces aldehydes, ketones

and other compounds that can negatively affect the sensory profile of the raw milk. Light induced oxidation can be characterized by flavors such as wet-cardboard, medicinal, burnt protein, or chemical like (Clark et al., 2009) Exposure to ultra-violet or fluorescent light triggers two different types of reactions: a vitamin A oxidation; and a protein breakdown reaction. Exposure to sunlight for as little as 15 minutes minutes has been found to cause light induced off-flavors (Chapman, 2006). For the protein breakdown to occur riboflavin is required. Riboflavin is known to be naturally abundant in raw milk (Dunkley et al., 1962). Both metal-induced and light-induced oxidation can occur within 24 hours of exposure to causative agents (Clark et al., 2009).

### **Pasteurized Milk Quality**

Bacterial contamination of high temperature short time (HTST) pasteurized milk can be traced back to two main causes; contamination of the raw milk by psychrotrophic, gram-positive, endospore-forming (PGPE) bacteria, which survive pasteurization, or post-pasteurization contamination by both psychrotrophic gram-negative (PGN) bacteria or PGPE bacteria (Ternstrom et al., 1993; From and Boor, 2004; Huck et al., 2007; Huck et al., 2008).

HTST pasteurization kills 99.999% of pathogens (FDA, 2009). However, pasteurization is not a solution to bacterial spoilage of fluid milk. Endospores formed by PGPE bacteria, which are present at any given time during the bacterial growth cycle, can survive HTST pasteurization and spoil the milk during its shelf

life. It has been shown that pasteurization kills the vegetative form of the PGPE bacteria, but activates the spore to germinate (Curran and Evans, 1944; Keenan et al., 1964). PGPE bacteria such as *Paenibacillus* and *Bacillus* are especially difficult to control as they can be found on the dairy farm as well as in the processing plant (Huck et al., 2007; Huck et al., 2008). PGPE bacteria have the ability to form biofilms on stainless steel equipment and continually contaminate milk passing through pipelines and equipment (Teh et al., 2011; Burgess et al., 2010). These biofilms are able to form on chemically inert surfaces such as stainless steel and can go unnoticed on the farm, in the tanker trucks, and in the processing plant for extended periods of time (Carpentier and Cerf, 1993).

As well as being a problem in raw milk, PGN bacteria are often found as post pasteurization contaminants in the HTST pasteurized milk. PGN bacteria can be found in several common areas in the fluid milk processing plant. It has been shown that sources of contamination in the processing plant include the pasteurizer, the filling heads and the carton forming mandrels (Gruetzmacher and Bradley, 1999). The filling heads and the carton forming mandrels were found to be greater sources of contamination than the pasteurizer. The pasteurizer tends to be a source of post pasteurization contamination, particularly following nonproduction days such as weekends. Gruetzmacher and Bradley (1999) found that increased CIP cycling times and proper maintenance of filling heads and forming mandrels decreased the amount of contamination and increase the shelf life from nine to 20 days. The bacteria that contaminate the milk from these processing points can be

either PGPE that have survived pasteurization and now inhabit the dairy plant or PGN bacteria that have been introduced through outside sources (Fromm and Boor, 2004).

### **Accelerated Shelf life Tests**

Accelerated Shelf life tests, such as the Moseley Keeping Quality (MKQ) test and the Preliminary Incubation (PI) test, were developed for the processing plants to help maintain a quality benchmark for their fluid milk products. The PI and MKQ test use an elevated incubation temperature to accelerate the growth of bacteria in the milk in an attempt to predict the shelf life of the milk. The MKQ test uses an initial TPC, and a seven day incubation at 7.2°C, followed by another TPC. The PI test uses a modified TPC (incubated at 21°C for 48 hours) on a milk sample that has been incubated at 21°C for 18 hours. These methods are often used to predict shelf life by testing raw or pasteurized milk (Martin et al., 2011; White, 1993). Martin et al. (2011) showed that the use of the PI test on raw milk was not accurate in predicting the shelf life of pasteurized milk. The cause of this inaccuracy was attributed to post pasteurization contamination (Martin et al., 2011). White (1993) showed that the PI and Moseley tests were not reliable without the simultaneous utilization of rapid detection methods such as immunoassays, flow cytometry, or chemiluminescent labeling. However, these rapid detection methods tend to be very expensive (White, 1993).

## Use of Sensory Analysis in the Dairy Industry

Although microbial quality, shelf life, and shelf stability remain key methods for defining a high quality dairy product, flavor is another way to define quality (Drake, 2004). Consumer's acceptance of fluid milk is strongly determined by the aroma and taste characteristics of the product (Santos et. al., 2003). Previous research on fluid milk has lacked the most in sensory analysis, using only the traditional descriptive analysis method to describe the off-flavors in the milk (Hayes, 2002; Drake, 2004; Carey, 2005). This method is unique to the dairy industry when compared to the more common quantitative descriptive analysis method (QDA) (Claasen and Lawless, 1992). The traditional dairy industry method differs in that panelists who judge dairy flavor accurately, require years of experience. Also the terms used are already set and defined. Instead of the panel creating their own terms, as seen in the QDA method, the terms are already set and describe different spoilage phenomena rather than individual flavors in the milk.

Hayes et al. (2002) conducted a study investigating the aroma characteristics of milk that had been spoiled by several *Pseudomonas* spp. Milk samples were inoculated with three different strains of *Pseudomonas* spp. (*P. fragi*, *P. fluorescens*, and *P. putida*) and analyzed by a trained panel at specific time points during the milks incubation. The panel was able to differentiate milk spoiled by *Pseudomonas* strains. Certain aromas tended to predominate at different time points throughout the shelf life. During the first week fruity flavors were most commonly noted while during the second week the aroma was described as rotten

and cheesy. Barn aromas were perceived most during the third week. Species and strain had an effect on when spoilage characteristics were perceived. This study provides useful information about the aroma characteristics imparted by *Pseudomonas* spp. Similar to other studies using QDA methods this study provides very specific information about a single mechanism of spoilage. However, this single mechanism of spoilage, as important as it is, is a small part of the overall fluid milk shelf life problem.

K.J. Boor and others (2001, 2004) have investigated heat-resistant bacteria, the avenues in which they contaminate milk and, specifically, how they cause milk to spoil. The studies investigated bacterial content, sensory characteristics and included a chemical analysis of the free fatty acid content in the milk. Results from these studies suggest that the next hurdle in extending fluid milk shelf life is to eliminate contamination by Gram-positive psychrotrophic endospore-forming bacteria. These bacteria survive pasteurization and grow at refrigeration temperatures spoiling the milk (Fromm and Boor, 2004; Boor, 2001). Fromm and Boor (2004) found that the main cause of spoilage was the growth of heat-resistant psychrotrophic organisms such as *Paenibacillus*, *Bacillus* and *Microbacterium*. They demonstrated that although sanitation practices are improving, bacterial spoilage is still limiting shelf life. A quantitative descriptive analysis (QDA) panel was used to identify the off-flavors occurring in the milk and at the time of spoilage. Principle component analysis (PCA) revealed five major components. Component one was related to aromas such as hay/grain and sour/fermented,

flavors such as nutty and rancid along with a metallic aftertaste. Component two was defined by a flat taste and aftertastes of cardboard, drying, lingering and sour. The mean scores of components one and two increased over time. Component three showed buttery flavors and sweet tastes, which decreased over time. Higher scores for butter/sweet may be an indicator of lower bacterial numbers (Fromm and Boor, 2004). They used chemical analysis methods to evaluate the extent of lipolysis and proteolysis in the milk. There was no connection made between the principle components and any causative factor, but did show that fatty acid profiles increased over the 17 day period. Other studies have demonstrated that increased free fatty acid levels have been shown to cause rancid type off-flavors in milk (Santos et al., 2003). Fromm and Boor (2004) isolated and identified one organism that has been shown to demonstrate lipolytic action in milk. At the same time, these results also reveal important connections between consumer's perceptions of off-flavors and the spoilage actions that are causing the defects to arise. For example, QDA provides a benchmark for high quality milk which is made up of components relating mostly to favorable attributes such as butter and sweet flavors (Fromm and Boor, 2004). However, a finite shelf life was never defined for the milk samples tested. It seems that without defining a specific time when the milk was spoiled it would be difficult to draw accurate conclusions on what was contributing most to spoilage at the time when the shelf life ended.

Carey et al. (2005) conducted a study on the shelf life of fluid milk products in New York State over a ten year period. They investigated total plate count,

coliform count and used a 6-8 person panel to determine acceptability for 23 dairy plants. It was not specified whether they used the same panelists throughout the study. The study found that overall acceptability of flavor for the 23 processors improved over the ten-year period (Carey et al., 2005). This study, unlike other similar investigations, used an acceptance method to determine the shelf life of the products and compared that to the microbial results. The acceptability scale ranged from 1-10, with 9 and 10 representing excellent scores and scores of <6 being unacceptable. The proportion of acceptable scores significantly increased from 1992 to 2000. The combination of microbial and sensory methods, as seen in this study, is capable of providing valuable information about the shelf life of the fluid milk as well as the spoilage mechanisms leading up to consumer rejection. However, the sensory method used to determine the shelf lives was an inadequate method for the prediction of acceptability. Generally, consumer acceptance testing is carried out with a large number of consumers (>50). Using a small panel for acceptance testing is subject to panelist influence effects and criterion effects.

### **Difference Testing: The Triangle Test**

Discrimination tests are often used in the food industry for quality control situations in which small differences in taste, flavor, and aroma must be realized. Difference testing, just as any other sensory test, is subject to a few problems such as order affect, position bias, and response bias, response bias being of the highest concern (O'Mahony, 1995). However, there are ways of eliminating these problems. Response bias can be eliminated through two methods. First is to use a

forced choice method such as the triangle test. Triangle testing is a sensitive discrimination method with minimized bias that is able to discriminate between two different food products. However, triangle tests must be given to the subject with very clear and careful instructions to eliminate the response bias. For this test a consumer subject is presented with three samples, two of them identical, and one odd sample. The subject is required to taste them and pick the odd sample (O'Mahony, 1995). The second way to eliminate response bias is to apply signal detection measures such as d-prime analysis to your data (O'Mahony, 1995). D-prime is an estimation of perceived sensory difference between two stimuli. In addition to calculating the significance, d-prime analysis also reveals the magnitude of the differences between samples. It assigns the subject's responses to a combination of sensitivities and biases while eliminating any differences in criteria that people might have (Claasson and Lawless, 1992). When  $d' \geq 1$  there is a significant difference between the samples.

Position bias and the effect of order of tasting are two potential problems with triangle testing. Although simply randomizing and counterbalancing the order in which the samples are presented can solve the position bias problem, the effect of serving order is slightly more difficult to avoid (O'Mahony, 1995). The serving order of the samples can seriously alter the ability of the subject to discriminate between the samples (O'Mahony, 1995).

The triangle test is a very sensitive and effective discrimination method when used by a knowledgeable person under the correct applications. There is no

literature cited that uses discrimination testing to define the shelf life of fluid milk. The determination of a finite shelf life is the first step in determining the root causes of spoilage. The triangle test is a sensitive and efficient discrimination test that can determine when consumers can detect a difference between the fresh and aged milk samples.

## CHAPTER 3

### **Determining the Shelf Life and Microbial Content of Fluid Milk from Nine Processing Plants in Oregon**

#### ABSTRACT

High-temperature-short-time pasteurized fluid milk commonly has a shelf life around 16-19 days. The aim of this study was to determine the shelf life of fluid milk in Oregon and characterize the bacterial growth over 23 days. Samples of pasteurized 2% milk were collected at six time points from all nine fluid milk-processing plants within Oregon. Milk was collected at 23, 21, 19, 16, and 8 days pre-testing, allowing for aging to occur. Fresh samples were collected 24 hours pre-testing. Raw milk samples were collected at 23 days and 24 hours pre-testing for the enumeration of endospores. Pasteurized samples from each time point were cultured for Total Plate Count (TPC), and gram-negative counts (GNC).

Concurrently, shelf lives of the milk were determined by employing triangle tests (N=25) where milk-drinking consumers were asked to distinguish between fresh and aged milk samples. Shelf lives varied among the nine processing plants from less than 8 to 21 days. Two accelerated milk shelf-life tests, the Pre-Incubation Count (PI) and the Moseley Keeping Quality (MKQ) test were also conducted. Both accelerated shelf life tests failed to accurately predict the shelf lives among plants. TPC's at 23 days of age were within one log value of  $10^6$ cfu/ml among all nine dairy plants. GNC counts followed the TPC closely. Endospore counts (ESC)

were ranged from  $10^3$ - $10^7$  at 23 days. ESC counts trailed the TPC and gram-negative counts, as high numbers of spores were not seen until after 16 days. The four dairies with the shortest shelf lives utilized coated paperboard carton packaging. TPC and packaging material showed no correlation. Pasteurization temperature did not correlate to the bacterial growth, although the five plants with the shortest shelf lives utilized the longest holding times. Bacterial species were identified using Terminal Restriction Fragment Length Polymorphism (t-RFLP). *Paenibacillus* spp. were the most commonly found organism in the pasteurized samples. *Pseudomonas* spp. were only found in one pasteurized sample, however they were found in 56% of the raw milk samples. *Streptococcus* spp. were found in the pasteurized milk of the four plants with the shortest shelf lives. This study demonstrates that although pasteurized milk processing is a well-established process, there is still significant variability in the milk quality and shelf life between dairy plants.

## INTRODUCTION

High temperature short time-pasteurized (HTST) fluid milk continues to compete in the U.S. beverage market with extended shelf life (ESL) products, which can remain on the shelves for an extended period of time. Fluid milk producers and processors still face challenges extending shelf life beyond 14 -18 days (Carey et al., 2005; Fromm and Boor, 2004) . Shelf life can be defined as the time between processing and when the milk becomes unacceptable for human consumption and is considered a quality indicator rather than a safety predictor. Generally, fluid milk quality and shelf life are assessed using a combination of microbial, chemical and sensory analysis (Carey et al., 2005). The main focus of fluid milk research continues to be characterizing bacterial contamination and spoilage (Boor, 2001; Fromm and Boor, 2004; Ranieri and Boor, 2009; Gruetzmacher and Bradley Jr, 1998; Huck et al., 2007; Huck et al., 2008).

Spoilage bacteria grow in milk producing undesirable flavor and aroma compounds significantly limiting the product's organoleptic quality. Enumerating the total number of bacteria is the most common way of assessing shelf life of fluid milk. Previous research has shown that organoleptic changes in milk become detectable at bacterial counts of  $10^6$  or greater (Bishop and White, 1986; Clark et al., 2009). Microbial spoilage is generally attributed to one of two distinct bacteria groups; spoilage due to psychrotolerant Gram-negatives (PGN), or spoilage due to psychrotolerant, gram-positive endospore-forming bacteria (PGPE) (Ternstrom, 1993; Fromm and Boor, 2004; Huck et al., 2007; Huck et al., 2008). Gram-

negative contamination is commonly attributed to post pasteurization contamination because they are typically destroyed by pasteurization (Meer, 1991). Due to the slow growing nature of PGPE bacteria, their presence as spoilage organisms have previously been masked by large numbers of PGN bacteria (Schroder, 1984; Fromm and Boor, 2004; Huck, 2008). Recent research has suggested that with advances in sanitation practices, post-pasteurization contamination is less prevalent and spoilage due to PGPE bacteria is the major factor limiting shelf life of pasteurized milk (Ranieri and Boor, 2009; Huck et al., 2007; Huck et al., 2008).

Fromm and Boor (2004) found *Paenibacillus*, *Bacillus* and *Microbacterium* to be the most frequently isolated organisms found in 2% HTST fluid milk. Their data show that PGPE bacteria, particularly *Paenibacillus*, are present in the raw milk supply and tanker trucks (Huck et al., 2007; Huck et al., 2008). Results from these studies suggest that the next hurdle in extending fluid milk shelf life is to eliminate contamination by PGPE bacteria. These organisms have been shown to occur on the dairy farm and in the processing plant (Huck et al., 2007; Huck et al., 2008). Trained sensory panels have shown that a number of off-flavors can be attributed to PGPE growth (Meer, 1991; Fromm and Boor, 2004). However, there are other causes of spoilage in fluid milk beyond bacterial growth such as chemical reactions and absorption of off-flavors from the environment.

Although microbial quality, shelf life, and shelf stability are necessary for defining a high quality dairy product, flavor is another way to define quality

(Drake, 2004). Milk flavor is typically determined using descriptive analysis methods, which utilize trained panelists (Drake, 2004). However, untrained consumers are the ultimate judge of milk flavor and product acceptability (Claassen and Lawless, 1992). The triangle test is a sensitive and efficient discrimination test that can determine when consumers can detect a difference between fresh and aged milk. To our knowledge, there is no literature cited that uses discrimination testing to define the shelf life of fluid milk products.

Research on pasteurized milk quality has been on-going for decades. Yet milk still spoils within less than three weeks. This is a significant barrier to efficient sales and distribution and has caused renewed focus on extended shelf-life (ESL) milks. Still consumers have expressed a preference for pasteurized milk over ESL milk and there is a need to continue the work to optimize shelf-life of pasteurized milk (Blake, 1995). Lately improved microbiological methods, such as terminal Restriction Fragment Length Polymorphism (t-RFLP) have become available which provide improved tools for identifying and controlling microbial contamination in milk.

The aim of this study was to determine the shelf life of fluid milk in Oregon as well as characterize the microbial content. For the purposes of this study, shelf life was defined as the age of milk when consumers could perceive a difference between fresh and aged milk samples.

## METHODS

### Sample Collection

All pasteurized samples were half-gallon containers (paper or plastic) of 2% fat fluid milk. Samples were collected from all HTST processing plants (nine plants) in Oregon. The first and last (23 d pre-testing (PT) and Fresh) samples were collected directly from the dairy plants. Samples collected on 21d, 19d, 16d, and 8 d PT were shipped overnight through Fed Ex or UPS in Styrofoam cooler boxes with ice. Ten pasteurized samples were collected each time for 23d, 21d, 19d, 16d, and 8d. Twelve fresh samples were collected the day before sensory testing.

Samples were stored at 6°C in the original packaging until testing.. Consumer triangle testing was carried out the day after the fresh sample collection. Standard plate counts were carried out two days after the fresh samples collection. Raw milk samples for spore enumeration were collected on the first and last collection days.

### Microbiological Analysis

Standard plate counts were carried out according to Standard Methods for the Examination of Dairy Products (Laird et al., 2004). Samples were diluted as needed using Hi-Veg peptone buffer solution. (VWR International, Radnor, PA).

Gram-negative counts (GNC) were carried out utilizing Velveteen Replica Plating Squares in conjunction with Scienceware® Replica Plating Tool. Copies were made from two SPC plates, with counts between 25 and 250, onto two Gram-

negative selective pseudomonas agar plates (VWR International, Radnor, PA). The plates were incubated for 48 hours at 32°C. Dilution factors from the SPC plates were carried through to the GNC. The Gram-negative nature was confirmed by Gram staining colonies from each testing day according to Current Protocols in Microbiology (Coico et al., 2007).

Two raw milk samples, corresponding to the pasteurized samples of 23d and fresh, were collected from each plant for enumeration of spores. All raw samples were heat treated in a water bath (80°C, 12 minutes) within 24 hours of collection (Frank and Yousef, 2004). Each sample was transferred into a sterile glass container in 200ml aliquots prior to heating. After heat treatment, the samples were poured into seven sterile tubes in 20ml volumes and stored at 6°C. Plating was carried out on standard plate count agar in duplicate on 0d, 8d, 16d, 19d, 21d, and 23d post heat treatment (PHT) (VWR International, Radnor, PA). Colonies from each sampling day were stained using a spore staining method according to Current Protocols in Microbiology (Coico, et al., 2007). Dilutions were performed as needed using a 0.1% solution of Hi-Veg peptone buffer (VWR International, Radnor, PA). Counts from the two raw milk heat treatments from each plant were averaged.

### **Accelerated Shelf-life Tests**

The Preliminary Incubation (PI) and the Moseley Keeping Quality (MKQ) tests were carried out alongside the microbial and sensory analysis using the pasteurized

samples picked up 23 days P.T. For the PI test, 10ml of milk was incubated at 21°C for 18 hours  $\pm$  10 minutes in a screw-capped culture tube (VWR International, Radnor, PA). The sample was then plated in duplicate on aerobic plate count Petrifilm™ (3M, St. Paul, MN) and incubated at 21°C for 48 hours (Laird et al., 2004). For the MKQ test, freshly processed samples were plated for SPC according to Standard Methods for the Examination of Dairy Products (Wehr and Frank, 2004), incubated at 7°C for five days and plated again for SPC. Simultaneously a 10ml of milk was aseptically poured into a sterile screw-capped glass bottle and incubated for the second SPC. Both PI and MKQ tests were carried out within 24 hours of the first milk collection according to the Guidelines for Maintaining and Testing Fluid Milk Shelf Life (Dairy Practice Council, 2004). A 0.1% solution of Hi-Veg peptone was used for all dilutions during the accelerated shelf life testing.

### **Sensory Analysis**

Triangle testing was carried out to determine when milk drinking consumers could tell a difference between an aged and a fresh milk sample. The triangle tests were carried out within 24 hours of the fresh sample collection. Twenty-five subjects were tested for each dairy. Subjects were required to be non-smoking, drink milk at least once a week, and to be between the ages of 18 and 50 years old. All subjects were recruited by email from the city of Corvallis, OR. Subjects were compensated with 15-dollar gift cards for Fred Meyer's. The Oregon State University Internal Review Board approved the sensory study.

Samples from the nine dairies were tested over three weeks. Four dairies were tested each week for the first two weeks. Plant #901 was tested separately on the third week. Samples from five time points for each dairy were compared with fresh milk samples. The milks were aged at 8d, 16d, 19d, 21d, and 23d. Each subject performed a triangle test at each time point for two dairies, totaling 10 triangle tests for each subject. Subjects were presented with 10 sets of three samples, five sets from two different dairies. Subjects took a mandatory 10-minute break after five triangle tests. Samples were coded with three-digit blinding codes and the serving orders were fully randomized across dairies and samples.

Samples of approximately 50 milliliters were poured into 2 oz. silo cups. Samples acclimated in cups at refrigeration temperature in cups for no more than three hours before being presented to the subjects. The panelists were given an ID number and were told to rinse with room temperature spring water at least once in between tasting. When tasting, the subjects were given as much time as needed to make their decision. Subjects were told to expectorate the sample into the container provided. Once done tasting a set of three samples, the subjects were asked to identify which of the three samples was different from the other two.

### **Terminal Restriction Fragment Length Polymorphism (t-RFLP)**

Following the initial experiment, nine pasteurized, 2% half-gallon milk samples were collected from the nine dairy plants for a second experiment. Samples were aged at 6°C until the testing date (8d, 16d and 21d of age) while raw

samples were treated within 24 hours of testing. At the time of testing three samples were mixed equally in a sterile Erlenmeyer flask. Bacterial DNA was isolated by centrifugation and frozen for further extraction. DNA extractions were performed on the pellets using a Mo Bio PowerFood™ DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). Polymerase chain reaction (PCR) was applied to the DNA extractions. Agarose gel (Invitrogen Corp., Carlsbad, CA) electrophoresis was utilized to confirm successful 16s DNA isolation. The PCR product was purified using a Qiagen® QIAquick PCR Purification Kit. Samples were digested using MspI and RsaI enzymes. Samples were sent out for t-RFLP analysis at the Oregon State University Center for Genome Research and Biocomputing. Pure PCR product was cloned using a TOPO TA Cloning® kit (Invitrogen Corp., Carlsbad, CA) and the resulting plasmids were transformed using One Shot® chemically competent *Escherichia coli* cells. The clone libraries were sent to Functional Biosciences, Inc. for 16s rRNA sequencing. 16s rRNA sequences were matched to peaks containing 10% or more of the total peak area on the t-RFLP profile. SPC were performed on all raw and pasteurized milk samples (Laird et al., 2004).

### **Statistical Analysis**

The number of correct discriminations was counted for each time point of each dairy and converted to  $d'$  values (Ennis, 1993), which indicate the magnitude of the differences between samples. The  $d'$  analysis (Bi et al., 1997) was further

performed in order to measure the significance. A significant difference was detected between aged and fresh milk when the  $d'$  values exceeded 1.03 (1-tailed test).

## RESULTS AND DISCUSSION

All dairy plants producing fluid milk in Oregon participated in this study. Thus it provides a complete picture of milk quality within one state.

D-prime analysis is a method that reveals whether the differences perceived by consumers are significant. In addition to calculating the significance, d-prime analysis also reveals the magnitude of the differences between samples and eliminates any differences in criteria that people might have (Claassen and Lawless, 1992). When  $d' \geq 1$  there is a significant difference between the two samples. D-prime values for the milks from the nine dairy plants are outlined in Table 3.1. The d-prime curves varied in consistency. For some plants, such as 629, 578, 901, 435 and 265 the  $d'$  values varied in magnitude throughout the time points, often rising and falling across the critical value line (Table 3.1). This is most likely due to day-to-day variations in milk quality from day to day. There are many variables on a day-to-day basis that affect milk quality including sanitation practices and the initial raw milk quality (Timmons et al., 2001). Other plants had more consistent increase in d-prime values throughout the shelf life of the milk (977, 401, 734 and 221) (Table 3.1). These plants likely have more consistent milk quality with little day-to-day variation.

Dairy Plant	d'-8 days	d'-16 days	d'-19days	d'-21 days	d'-23 days
401	0	0.93	0	0.65	(N/A)
901	0	0	1.36*	0	2.48*
977	0	0	0.51	2.01*	5.0*
734	0.55	(N/A)	1.36*	(N/A)	(N/A)
265	0.55	0	3.06*	1.91*	0.55
578	0	1.03*	3.85*	2.25*	3.85*
629	0	1.56*	2.13*	5.11*	3.9*
435	0	4.44*	1.51*	4.05*	(N/A)
221	1.26*	1.03*	2.25*	2.25*	3.85*

**3.1.** D-prime values for consumer sensory tests of fresh and aged fluid milk samples from nine dairy processing plants. Samples were aged at 6°C for up to 23 days and compared to fresh samples using triangle tests. For some plants, D-prime values increased consistently throughout the shelf life.

\* d' value was greater than 1.03 indicating a significant difference between the aged sample and a fresh sample.

(N/A)-The milk was separated and therefore subjects did not taste samples from that day. Separated samples were considered spoiled.

Shelf life was determined as the time point prior to the d-prime value exceeding 1.03 on two consecutive testing days. Shelf lives ranged from <8 days to 21 days (Table 3.1). However, the majority of milk samples reached 16 days or more without consumers perceiving a difference when compared with a fresh sample. For one company the consumers were able to distinguish between fresh milk and 8 day samples. Two companies of the nine had milk that was undistinguished from fresh milk at 21 days. Out of the nine plants tested, six did not reach their sell by date (SBD) or use by date (UBD) printed on container. This

suggests that the majority of the milk processors in this study are not meeting their own standards and need to improve the quality of their product. Two plants could extend their UBD or SBD. However it is worth noting that the definition of shelf-life utilized in this study is very stringent. In this study milk was considered spoiled as soon as it tasted different to fresh milk for two consecutive storage durations. Companies may decide that they are willing to allow a low level of flavor deterioration before considering the quality as unacceptable, i.e. the end of shelf-life.

Table 3.2: Shelf life (determined by consumer triangle testing), Sell by/Use by Date, P.I. and Moseley Predictions, Packing Type and Pasteurization Specifications for Nine Dairy Plants

Dairy Plant	Shelf Life (days)	Sell By (SBD) or Use By Date (UBD)	Pasteurization Temperature (°F / seconds)	Moseley Prediction & % Increase in TPC	P.I. Prediction	Packaging
Plant # 401*	21	SBD 18	N/A	Prob. (752%)	>14	Plastic
Plant # 901*	21	SBD 21	175.5, 18	No Pr. (28%)	10-14	Plastic
Plant # 977	19	SBD 18	178, 19.5	Prob. (250%)	10-14	Plastic
Plant # 734**	19	SBD 18	169, 15	No Pr. (44%)	>14	Plastic
Plant # 265	16	UBD 21	177, 23.5	No Pr. (-13%)	10-14	Plastic
Plant # 578	8	SBD 18	167.5, 26	No Pr. (59%)	10-14	Paper
Plant # 629	8	UBD 19	178, 27	No Pr. (-10%)	>14	Paper
Plant # 435*	8	SBD 20	171, 32	No Pr. (-1%)	10-14	Paper
Plant # 221	< 8	SBD 21	176, 27	No Pr. (-8%)	>14	Paper

\*, \*\*: Due to the large number of samples, the experiment was conducted over 3 trials. During the first trial consumers objected to tasting some of the older milk samples. Therefore milk samples were pre-tested by the researchers during the subsequent trials. Some samples that had strong off-flavors or had separated in the containers were not included for consumer testing.

\* consumers didn't test day 23 samples

\*\* consumers didn't test days 16, 21, 23 samples

All, but one company, reported pasteurization specifications. All plants pasteurized above the minimum FDA requirements of at least 161°F for a minimum of 15s (FDA, 2009). Pasteurization temperatures ranged from 167°F to 178°F for the nine plants, with most utilizing temperatures around 177°F (table 3.2). Pasteurization times varied from 15s to 32s. The majority of plants utilized pasteurization temperatures at least 7°F over the minimum required temperature and held the product for at least eight seconds longer. Elevated pasteurization temperatures of 161 and 168.8°F, over a 15 second period, have been shown to cause an increase in activation of bacillus spores over milk treated at 145.4°F for 30

min (Hanson et al., 2005). Dairy plants 977, 629, and 435 had  $\leq 2.0$  log cfu/ml endospores at 14 days (figure 3.1) and two of these plants utilized the highest pasteurization temperatures (178°F) among the nine plants. In contrast plants 578, 221, and 401 had the highest endospore counts at 14 days, but had pasteurization temperatures ranging from 169 to 177°F. Therefore our results do not support the hypothesis that increased pasteurization temperature leads to increased activation and outgrowth of endospores. Differences in endospore counts over 23 days were most likely not due to differences in pasteurization temperatures. It is interesting to note that the five dairy plants with the shortest shelf lives utilized the longest holding times (all above 20 sec.). Thus the variations among different holding times may be more important than the differences in temperature. It is also possible that the differences seen in endospore counts could be due to sanitation practices on the dairy or in the dairy plants, as PGPE bacteria have been found to be both pre- and post-pasteurization contaminants in fluid milk (Huck et al., 2008).

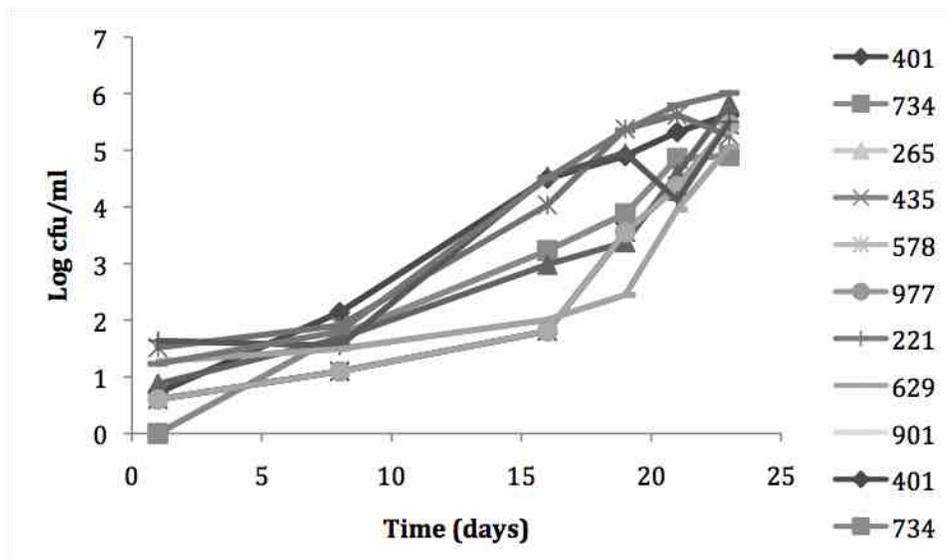


Figure 3.1: Endospore forming bacteria growth trends over 23 days for all nine dairy plants.

Moseley and PI tests did not accurately predict shelf life for the nine processing plants (Table 3.1). The PI test produced both false positive and false negative results, as it predicted that the dairy with the shortest shelf life (221) would last more than 14 days and that a plant with a 21-day shelf life (901) would last between 10 and 14 days. The MKQ test couldn't differentiate between milks from the nine dairy plants and predicted "no problem" for seven of the nine dairy plants. The test only predicted reduced shelf life for two of the three plants with the longest shelf lives. The Martin et al. (2010) tested the reliability of the MKQ and PI test when predicting shelf life by testing raw milk samples. They found that these tests could not predict pasteurized milk shelf life when applied to raw milk samples. Other studies have shown that MKQ and PI tests have low correlations to pasteurized product shelf life compared to other rapid methods (Kahn et al., 1987;

White, 1993). White (1993) suggests that the PI test is less effective by itself but can be useful when used in conjunction with a rapid detection method such as immunoassays, flow cytometry, or electronic counting. The results from this study indicate these tests are unable to predict pasteurized milk shelf life when applied to a freshly pasteurized sample. A major short coming of these two methods utilized to predict shelf-life is that they assume milk quality is only impacted by microbiological action. Flavor deterioration by chemical reactions such as rancidity and light or metal catalyzed oxidation are not detected by PI or MKQ.

The four plants with the shortest shelf lives used coated paperboard carton (CPC) packaging while the five plants with 16 day or greater shelf life used opaque plastic containers (Table 3.1). CPC packaging has previously been thought to be mostly impermeable to oxygen. However, off-flavors are still known to occur in milk packaged in CPC (Rysstad, 1998). Leong et al. (1992) found that CPC packaging can cause off-flavors in milk after only one day of storage. Fillers have been found to be important sources of contamination in the processing plant (Gruetzmacher and Bradley, 1999). Specifically, the carton-forming mandrels and filling heads were major sources of contamination within the filler. Therefore it is possible that the differences in shelf lives are not linked to the packaging materials but rather to the types of fillers and sanitation practices. This study did not detect differences in microbial count between milk packaged in CPC and high-density polyethylene (HDPE) packages. Other researchers have also failed to demonstrate

connections between packaging type and the microbial count (Moysiadi et al., 2003; Vassila et al., 2002).

Although shelf lives varied considerably, the total plate counts were similar at the end of their shelf lives among all nine dairies (figure 3.2). Seven of the dairy plants had counts between 6 and 7 log values at 23 days. Similar results were documented in several other studies (Cromie, 1991; Fromm and Boor, 2004; Hayes et al., 2002; Huck et al., 2007; Huck et al., 2008; Ranieri and Boor, 2009).

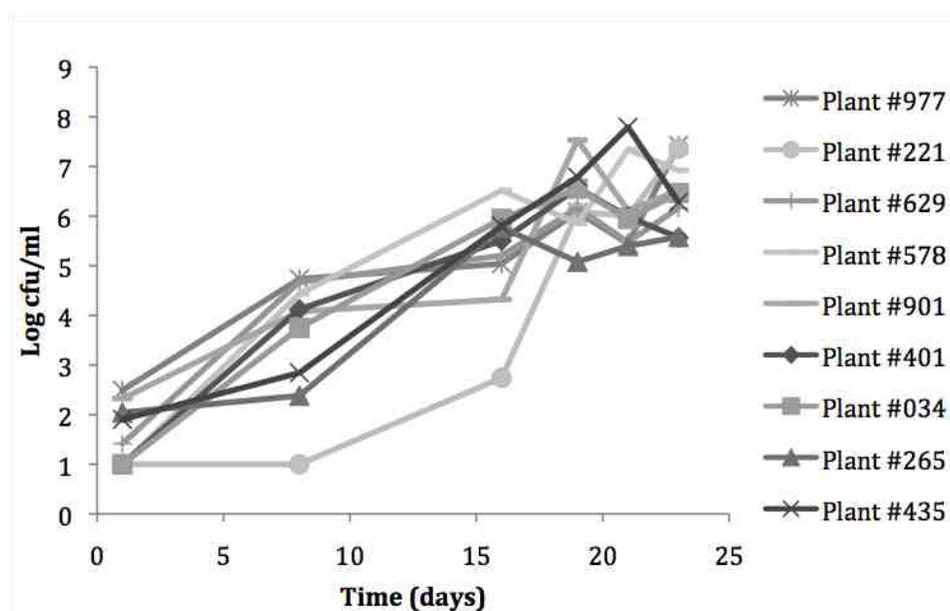
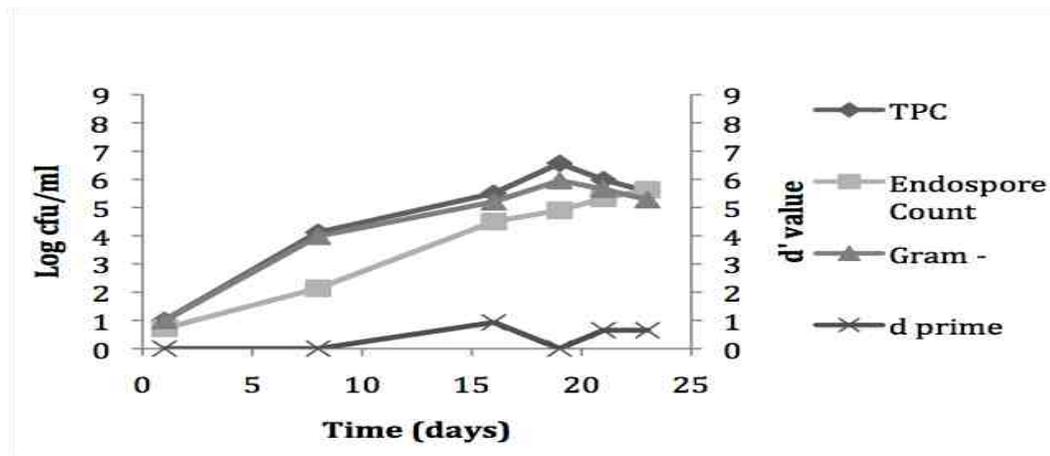


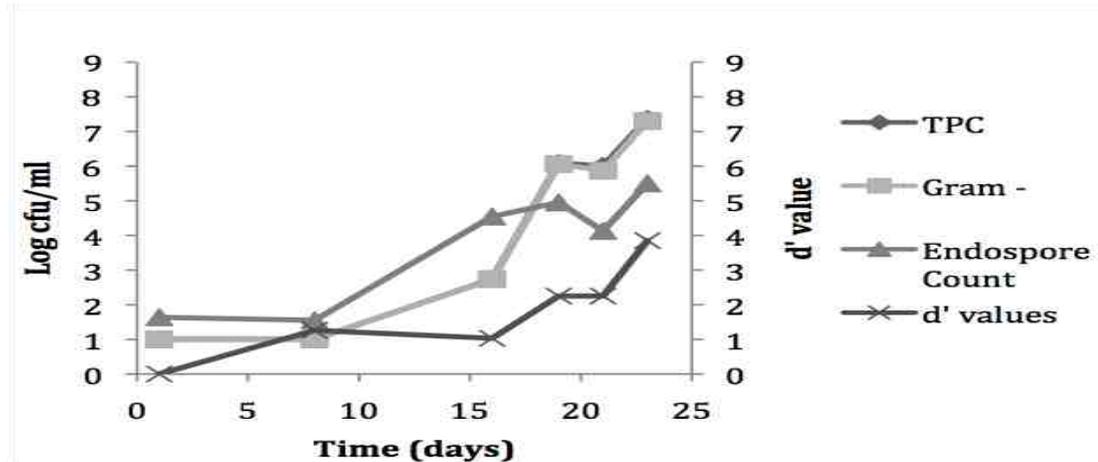
Figure 3.2. Total plate count trends for 2% milk, from nine dairy plants, stored at 6°C for over 23 days of storage.

For each dairy plant, TPC, GNC, and ESC were determined over the 23 days storage. Although the microbial plates were incubated at mesophilic growth temperatures and therefore ought to be indicative of mesophilic bacteria, the growth trends in overall numbers reflected psychrotrophic growth because the milk

samples were incubated at 6°C for up to 23 days. Milks from eight of the dairy plant exhibited similar growth patterns during storage. Thus results from only one of these plants are shown here (Figure 3.3A). The GNC counts were similar to the TPC, staying within one log value throughout the six time points for all dairy plants, suggesting that most of the TPC growth was gram-negative bacteria. The ESC counts trailed the TPC and GNC as they, generally, did not exceed four log values until 16 days or later. Ranieri and Boor (2009) found similar growth trends in a 2009 study on bacterial ecology of pasteurized fluid milk. Growth patterns in milk from dairy plant #221 were different (Figure 3.3B) for dairy plant 221, which had a shelf life of less than eight days, the TPC did not exceed two log values for more than 10 days. D' values are shown in Table 1 illustrate when the critical value of 1.03 is surpassed and consumers could discriminate between the aged sample and the fresh. There were no correlations between d' values and microbial counts for any of the companies. With mostly similar bacterial growth trends and differing shelf lives it was evident that more than just the bacterial loads contributed to the spoilage of milk from the nine processing plants. If it was not the TPC, GNC, or ESC, that impacted shelf-lives, then it was likely the specific species of bacteria that impacted milk spoilage. Thus a second experiment was initiated to characterize specific bacterial populations from each plant.



A



B

Figure 3.3: (A) Total plate counts, endospore counts, gram negative counts and d-prime trends for dairy 401 were typical. (B) Total plate counts, endospore counts, gram negative counts and d-prime trends for dairy 221 were atypical.

Previous studies, attempting to identify specific bacterial strains in milk, have utilized culture-based methods for identifying the bacteria present in the milk (Mayo, 2008). The disadvantage of this approach is that only bacteria that are easily cultured are identified while some are missed. Culture-independent analysis

of bacterial populations is very useful when not all organisms in a sample are easily cultured. The composition of fluid milk makes it a perfect growth medium for a variety of bacteria, which may not all be easily cultured on a growth medium (Cromie, 1991; Hantsis-Zacharov and Halpern, 2007; Ranieri and Boor, 2009). Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis is a widely used method for studying uncultured bacterial populations over time (Nakano et al., 2010). However, to our knowledge, there has been no published use of the t-RFLP method to characterize bacterial populations in fluid milk.

The aged milk samples analyzed in the second experiment were aged at 6°C for the duration of the shelf-life identified for each plant in the initial experiment; i.e. ranging from eight to 21 days. The second experiment was carried out six months after the first. TPC's for experiment two were mostly similar to TPC's in experiment one except for plant 435. Plant 435 had approximately a three log decrease in TPC at eight days, from the first experiment to the second. The decrease in TPC was most likely due to the reconfiguration of plant 435's sanitation program that took place in between experiments. The t-RFLP analysis of fluid milk samples revealed varying diversity and concentration of bacterial populations between plants and, pre- and post-pasteurization samples (Table 3.2). Approximately 65% of the organisms that were identified in the raw milk samples across the nine dairy plants were gram positive. After pasteurization and aging the gram-positive bacteria made up 90% of the organisms identified. Bacterial 16s RNA is destroyed when held at 47°C for 30 min and leakage of genetic material

absorbing at 260nm occurs (Miller and Ordal, 1973). Therefore, the RNA used in identifying the organisms in the corresponding raw milk samples was not extractable from the pasteurized samples.

Table 3.2: Bacterial species represented by a peak that was 10% or more of the total peak area were identified for the raw and aged samples. Percentages of total peak area and corresponding total plate counts for each peak are shown.

Dairy Plant	Age of sample	Bacterial sp. in Raw Milk (% of total t-RFLP peak area)	TPC Raw (cfu/ml)	Bacterial sp. in Aged Milk (% of total t-RFLP peak area)	TPC Aged (cfu/ml)
401	23	<i>P. IHB-B</i> (61.8)	$3.1 \times 10^3$	<i>P. telluris</i> (95.3)	$2.3 \times 10^5$
901	23	<i>Ps. fluorescens</i> (78.5) <i>P. IHB-B</i> (10)	$2.1 \times 10^4$	<i>P. telluris</i> (91.9)	$8.7 \times 10^6$
977	16	<i>Ps. brenneri</i> (40.4) <i>Ps. DD1</i> (30.3)	$1.0 \times 10^4$	<i>P. polymyxa</i> (44.7) <i>B. simplex</i> (34.3)	$6.8 \times 10^4$
734	16	<i>P. IHB-B</i> (59.5)	$1.7 \times 10^4$	<i>Ps. fluorescens</i> (91.2)	$8.5 \times 10^6$
265	16	<i>Staph. Aureus</i> (74.2)	$5.9 \times 10^3$	<i>B. C-21</i> (45.5)	$5.4 \times 10^3$
578	8	<i>Ps. fluorescens</i> (37.3) <i>P. telluris</i> (17.3) <i>P. IHB-B</i> (12.5)	$2.7 \times 10^4$	<i>P. telluris</i> (89.7)	$7.2 \times 10^4$
629	8	<i>Pr. Vulgaris</i> (36.5) <i>E. Pei061</i> (10.0)	$5.8 \times 10^3$	<i>S. thermophilus</i> (23.3)	$3.0 \times 10^2$
435	8	<i>S. Dysgalactiae</i> (36.0) <i>Ps. Poea</i> (28.3)	$8.3 \times 10^3$	<i>S. Uberis</i> (51.4)	$2.6 \times 10^2$
221	8	<i>S. thermophilus</i> (40.0) <i>M. caseolyticus</i> (37.0) <i>Ps. fluorescens</i> (12.0)	$2.9 \times 10^4$	<i>S. thermophilus</i> (98.9)	$3.4 \times 10^2$

Genus Abbreviations: *Ps.*=*Pseudomonas*; *P.*=*Paenibacillus*; *E.*=*Eubacterium*; *Pr.*=*Proteus*; *Staph.*=*Staphylococcus*; *S.*=*Streptococcus*; *B.*=*Bacillus*

Note: Samples were tested at the age closest to the time of spoilage determined in experiment one.

Raw milk usually contains a more diverse population of bacteria (Ternstrom et al., 1993; Desmaures et al., 1996; Ercolini et al., 2008). This study found less diversity in the raw milk samples. This is most likely because our cut off for peak identification was 10% of the total population and with increased diversity most peaks made up less than 10% of the total population.

With the exception of plant 734, the dairy plants with the longest shelf lives (401, 901, 977 and 265) contained primarily *Paenibacillus* and *Bacillus* spp. in the aged samples. Huck et al. (2007 and 2008) concluded that as PGN bacteria become scarcer; the PGPE bacteria will be the next major factor limiting the shelf life of fluid milk to 14d. Our data agrees that as PGNs are controlled in the processing plant the majority of the bacterial growth is due to PGPE bacteria such as *Paenibacillus* and *Bacillus*. But our data does not agree with the prediction that PGPE bacteria will limit shelf-life to within two weeks. Rather these products, with primarily PGPE contamination, are capable of reaching 21 days without consumers being able to discriminate between 21d old and fresh milk.

Besides the *Paenibacillus* spp., *Pseudomonas* was the most commonly identified organism found in both raw and pasteurized milk samples. Ternstrom et al. (1993) observed similar results. Although *Pseudomonas* spp. were only found in one pasteurized sample at greater than 10% of the total population (plant 734), it was found in low levels in pasteurized samples from dairy plant 578. Plant 578 also had a large population of *Pseudomonas fluorescens* (37.3% of total population) in the raw milk sample. *Pseudomonas fluorescens* does not survive HTST pasteurization. This suggests that re-contamination of *Ps. fluorescens* is occurring after pasteurization. Ternstrom et al. (1993) found results demonstrating similar recontamination by *Ps. fluorescens* in pasteurized milk.

*Staphylococcus aureus*, a common skin pathogen, was identified as the major organism the raw milk for plant 265, making up 74.2% of the population. *S.*

*aureus* is a common causative organism for mastitis (NMC, 2008). The organism is also frequently associated with the skin of cows and humans. The presence of this organism in the raw milk could be explained by several likely phenomena. It is highly likely that a mastitic animal was milked and the bacteria entered the raw milk during milking as infected cows can shed high numbers of bacteria into the milk supply. Another possibility would be contamination from the skin of the cow through improper sanitation. Because *S. aureus* is killed by pasteurization the occurrence of this common pathogen is of little concern to human health. However, it does demonstrate the importance of herd health.

*Streptococcus* spp. made up 20% or greater of the bacterial population in pasteurized milk samples for three of the five dairy plants with an 8 day shelf life. *Streptococcus* is ubiquitous on the dairy farm and is a common cause of both clinical and sub-clinical mastitis in cows (Bramley, 1982). Cows with clinical mastitis can shed up to  $10^7$  cfu/ml into the milk supply (Leigh, 1999). However, these cows are usually diagnosed quickly and are separated from the herd. Cows with sub-clinical mastitis do not show symptoms of mastitis and can go unnoticed and shed high numbers of bacterial and somatic cells into the milk supply jeopardizing the quality of the pasteurized product (National Mastitis Council, 2008). Mastitis can also cause an increase in somatic cell count (SCC) (Leigh, 1999). Increased SCC can cause increase lypolysis and proteolysis due to enzymes produced by the somatic cells, resulting in the formation of off-flavors in the milk (Ma, 1999). *Streptococcus* spp. are killed by HTST pasteurization. The presence of

*Streptococcus* spp. including *S. uberis* and *S. dysgalactiae* in both the raw and pasteurized milk suggests that the pasteurized milk is being re-contaminated after pasteurization. It also suggests that herd management and raw milk quality might be a limiting factor for the processors with short shelf life.

### CONCLUSION

This study demonstrated great variability between shelf life of milks produced within one state. Milks with a shelf life of 16 days or greater were characterized by endospore-forming bacteria and HDPE packaging while short shelf life milk was characterized by CPC packaging. Milks with short shelf lives were also characterized by *Streptococcus* spp. in both the raw and pasteurized. It is advisable that the dairy plants that produced milk with a short shelf life and contained *Streptococcus* spp. must advise their milk suppliers to focus on mastitis prevention. Furthermore these plants must improve their cleaning and sanitation practices. The plants with longer shelf life lives must focus on controlling PGPE bacteria in the milk supply and in the dairy plant.

## CHAPTER 4

### GENERAL CONCLUSIONS

Although HTST fluid milk still competes in the U.S. beverage market with ESL products such as juices, water and sodas, its shelf life remains around 16-19 days. For HTST fluid milk, which counts for a large part of the Oregon dairy industry, to remain competitive in the U.S. beverage market the shelf life must be extended. The goal of this study was to determine the shelf life of fluid milk in Oregon and characterized the microbial content.

This study demonstrated great variability between shelf life of milks produced within one state. Milk with longer shelf life was packaged in HDPE containers while short shelf life milk was packaged in CPC's. Milks with short shelf lives were also characterized by *Streptococcus* spp. in both the raw and pasteurized. It is advisable that the dairy plants that produced milk with a short shelf life and contained *Streptococcus* spp. must advise their milk suppliers to focus on mastitis prevention. Furthermore these plants must improve their cleaning and sanitation practices. The plants with longer shelf life lives must focus on controlling PGPE bacteria in the milk supply and in the dairy plant.

The impacts of this study have already started to become realized by two of the dairy plants involved. One processor, plant 435, showed significant improvement in quality after reviewing the initial results from this study. They changed their CIP schedule sanitation practices. As a result the TPC of their eight

day milk was reduced by approximately three log figures from experiment one to experiment two. Plant 221 report problems with oxidation in their 2% milk. They were advised to stop mixing their organic milk, which was left over in the holding tank with the soon to be pasteurized non-organic milk

The great variability in HTST fluid milk quality in Oregon is a hurdle that must be overcome if fluid milk is to compete with ESL products. The results from this study suggest that two major hurdles for extending fluid milk shelf life in Oregon are the improvement of raw milk quality and controlling post pasteurization contamination in the processing plant.

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## APPENDIX

### Recruitment Email: Consumer Sensory Test

Hello Fellow Students,

My name is Liam Wustenberg and I'm working for a Master's Degree with Dr. Lisbeth Goddik. This term I am conducting some sensory evaluations on milk. The testing will take place on July 15th. I am looking for students to take place in this study as subjects.

Being a subject includes tasting pasteurized 2% milk and picking the sample that is different from the other two (triangle test). Subjects will spit out the samples once they have tasted. The milk you will be tasting is no different that the milk you would buy at the store.

Your participation in the study will last for approximately 30 minutes. For your participation in this study, should you choose to be a subject, you will receive a \$15 gift card to Fred Meyers.

NOTE: This study is totally voluntary and you may leave or quit at anytime if you feel you cannot finish.

The study will be taking place on **Thursday July 15th**

If you are interested in participating reply to [liam.wustenberg@oregonstate.edu](mailto:liam.wustenberg@oregonstate.edu), with the following information...

- How often do you drink milk?
  - a) 1 glass/week b) 3 glasses/week c) >3 glasses/week
- What is your age?
- When are you free between 10am and 2pm on Thursday July 15th?

I appreciate your help!!!

Liam Wustenberg