12-2020

Understanding the Microbial Population of Lactococcus lactis. subsp. Strains During Cheese Making and Initial Storage

Rhitika Poudel
Utah State University

UNDERSTANDING THE MICROBIAL POPULATION OF *Lactococcus lactis.* subsp.

STRAINS DURING CHEESEMAKING AND INITIAL STORAGE

By

Rhitika Poudel

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

_____	_____
Donald McMahon, Ph. D. Major Professor	Craig J. Oberg, Ph.D. Committee Member
_____	_____
Michael Lefevre, Ph.D. Committee Member	Randall Thunell, Ph.D. Committee Member

_____

D. Richard Cutler, Ph.D.
Interim Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2020

ABSTRACT

Understanding the Microbial Population of *Lactococcus lactis.* subsp. strains During Cheese Making and Initial Storage

by

Rhitika Poudel, Master of Science

Utah State University, 2020

Major Professor: Dr. Donald J. McMahon
Department: Nutrition, Dietetics and Food Sciences

Traditionally, starter cultures for cheddar cheese are combinations of *Lactococcus lactis* subsp. *lactis* and *cremoris*. Our goal was to compare their growth and survival during cheesemaking and after salting and pressing. Cheddar cheese was made in duplicate using two strains of *lactis* (600-M1, E36) and *cremoris* (B36, G61) using 520-kg of pasteurized milk and 31°C set and 38°C cook temperatures. Milled curd was salted with 2.0, 2.4, 2.8, 3.2 and 3.6% salt, pressed for 3 h and stored at 6°C and sampled after 6 d. Starter culture numbers were enumerated by plating on both M17 and Reddy's agar. Flow cytometry event numbers were divided into three groups based upon bacterial cells permeability to Sybr Green and propidium iodide: living-nonpermeable, living-semipermeable, and dead-permeable cells. Cheese make time (set-to-mill time) varied from 210 to 393 min depending on the strain. The two *cremoris* strains were different from each other in terms of acid production, increase in cell numbers during cheese manufacture and cheese make time. Acid production rates during cheesemaking (with cook temperature of 38°C) was more dependent on the individual strain than whether it was a *lactis* or *cremoris* strain. The cheese made with *lactis* strains contained ~4 times (~

0.5 log) more bacterial cells than those made using *cremoris*. None of the four strains tested were influenced by the amount of salt added to the curd (at least in the range of cheese S/M of 3.5 to 5.5%). Higher pH in cheeses with higher salting levels was attributed to the higher salt cheeses having up to 20 g/kg less moisture content. Based on flow cytometry, ~5% of the total starter culture cells in the cheese were dead after 6 d of storage. Another 5 to 15% of the cells were designated as being alive but live- semi permeable, with cremoris strains having the higher number of such cells. Live-nonpermeable cells were up to one log higher than plate count numbers depending on the strain. We concluded that using only plate counting does not fully describe the fate and activity of starter culture during cheese storage, and that flow cytometry provides additional information on microbiology of cheese that aids further understanding of the role of starter cultures in cheese aging and flavor development.

(91 pages)

PUBLIC ABSTRACT

Understanding the Microbial Population of *Lactococcus lactis.* subsp. strains During Cheese Making and Initial Storage

Rhitika Poudel

Cheddar cheese is manufactured by fermenting milk with lactic acid bacteria added as *Lactococcus lactis.* The two major subspecies used are *lactis* and *cremoris*. Starter culture for this study was prepared and donated by Vivolac Cultures Corporation, Greenfield, Indiana. Cheddar cheese was made in duplicate using two strains of *lactis* and *cremoris*. Milled curd was salted with 2.0, 2.4, 2.8, 3.2 and 3.6% salt, pressed for 3 h and stored at 6°C. The curd was sampled during cheese making and after 6d storage. Two different methods of enumeration were employed. One method involved plate counting which only enumerates those bacteria that can reproduce and form visible colonies. The other method was flow cytometry (FC) that counts the number of cells based upon their combination with fluorescent dyes, which could be divided into three groups: live, live-semipermeable, and dead cells.

None of the four strains tested were influenced by the amount of salt added to the curd. Acid production rates during cheesemaking (with cook temperature of 38°C) was more dependent on the individual strain than whether it was a *lactis* or *cremoris* strain. Based on flow cytometry, ~5% of the total starter culture cells in the cheese were dead after 6 d of storage. Another 5 to 15% of the cells were designated as being alive but live-semi permeable ,with cremoris strains having the higher percentage of such cells. Thus, it can be said that plate count does not fully describe the fate and activity of starter culture during cheese storage, and that flow cytometry provides additional information on

microbiology of cheese and can help track living cells during cheese storage and flavor development.

Dedicated to my family for their endless love, support and sacrifices. I wouldn't be where I am without you.

ACKNOWLEDGMENTS

First and foremost, I wish to express my sincere gratitude to my advisor, Professor Donald J. McMahon for giving me the opportunity to work and allowing me to grow as a research scientist. This journey would not have been possible without his support. I would like to thank him for the continuous motivation, patience and immense knowledge. His guidance has helped me in all the time of research and writing of this thesis.

I would like to thank my committee members Prof. Craig J. Oberg, Prof. Michael Lefevre, and Dr. Randall Thunell for their time, support and expertise. I would like to extend my thanks to Dr. Eric Bastian for his constant encouragement and insightful comments through BUILD dairy programs. Thanks to Tara B. Johnson and Kim Rasmussen for their kind support. Also, thanks to my fellow lab mates, Ireland Green, Brynli Tattersall and Sophie Overbeck for their cooperation. I would also like to thank Sapana Lohani and all my friends who in one way or another shared their support either morally or physically.

Special thanks to Kshitij Parajuli and Medha Khatiwada for their constant support and guidance and for all the love and care for the last 3 years. Special mention to darling Samaira.

Lastly, I would like to thank my family, my parents, Ram Chandra Poudel and Sudha Sharma, for always believing in me and giving me strength. I want to thank my brother Rhishav Poudel for always loving me, my sister Rhiju Poudel, for being my rock when I need it the most and always being supportive of my decision, my brother in law

Shiv Pandey, for his advice and making sure I never lost track of my dreams and my eight month old nephew Ahvaan for spreading light in our lives.

Rhitika Poudel

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

LAB = Lactic acid bacteria

SLAB = Starter Lactic acid bacteria

CFU/g = Colony forming units/gram

FC = Flow Cytometer

MANC = Metabolically Active Non Culturable

SG = SYBR Green

PI = Propidium Iodide

LSP = Live-semi permeable cells

INTRODUCTION

Cheddar cheese is manufactured by fermenting milk with lactic acid bacteria **(LAB)** generally using *Lactococcus lactis* starter cultures (Marilley and Casey, 2004). The two major subspecies of *Lactococcus lactis* used are *lactis* and *cremoris*. The *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains are traditionally differentiated based on arginine utilization or metabolism, temperature and pH sensitivity and salt stress (Kelly et al., 2010). *Lactococcus lactis* growth in milk is linked with the rapid production of lactic acid which promotes curd formation, prevents growth of pathogenic and spoilage bacteria and creates optimal biochemical compounds for ripening (Fernández et al, 2011). The starter LAB (**SLAB)** along with other bacteria present in the cheese, including nonstarter LAB (**NSLAB**) via their proteolytic and amino acid conversion pathways also contribute to the final texture and flavor of the cheese (Ruggirello et al., 2016).

Lactic acid bacteria are enumerated using both culture-dependent (cultivation on culture media) and culture-independent methods. Recent findings based on culture-independent methods, have shown the presence of live *L. lactis* cells in late ripened dairy products while findings from plate counting on culture media showed the absence of these microorganisms in late ripened stages (Dolci et al., 2008; Ruggirello et al., 2016).

This research aimed to study the growth of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* during cheese making, the influence of different levels of salting, and 6 d storage. Two different methods of enumeration were employed. One method involved plate counting which only enumerates those bacteria that can reproduce to form visible colonies. The other method was flow cytometry (**FC**) that counts the number of cells

based upon their combination with fluorescent dyes. This method can count cells that are dead as well as cells that are nonculturable but may still be metabolically active.

HYPOTHESIS AND OBJECTIVES

The hypothesis of this study*:*

*Lactococcus lactis s*tarter culture consisting of *L. lactis* subsp. *cremoris* subspecies grows to lower numbers during cheesemaking and undergoes more die-off after salting than does starter culture consisting of *L. lactis* subsp. *lactis* subspecies.

Objectives of this study are:

1. Evaluate fluorescent dyes that can be used to differentiate between living and dead cells of *Lactococcus lactis* using flow cytometry and develop a method that can be applied to cheese.
2. Manufacture cheddar cheese using single strains of *L. lactis* and compare extent of starter culture growth during cheesemaking and survival after salting by enumerating bacteria using:
    - Reddy's agar that differentiates between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*
    - Flow cytometry to differentiate between nonpermeable (living), semi-permeable (presumed to be living), and dead bacterial cells.

LITERATURE REVIEW

Cheddar Cheese Manufacture

Starter cultures, manufacturing stages, temperature, and ripening time defines the uniqueness of hundreds of cheeses that exist in the world. (Ryssel et al., 2010). Cheddar cheese is a rennet coagulated, semi-hard, cheese and the manufacturing process can be divided into two stages: (1) converting milk (already inoculated with SLAB) into curd using rennet, cutting coagulum, stirring and heating, whey draining, curd packing, cheddaring, salting, pressing and packaging; and (2) ripening of the curd which takes months to years (Singh et al., 2003). All the stages are equally important but one of the primary steps during cheese manufacture is acidification which involves the conversion of lactose to lactic acid by lactic acid bacteria. This event affects almost every stage of cheese manufacture and also the final product (cheese composition, texture, and flavor) (Singh et al., 2003).

Today, there are a number of various changes to the way in which Cheddar cheese is manufactured. The important factor in support of those changes remains the availability of reliable starter culture and mechanized system for cheese manufacture (Johnson, 2017).

Starter Culture

Starter cultures are inoculated directly into food material to produce desired changes in the finished product. Lactic acid bacteria play a pivotal role in this process and have a long record of application in various food products (Leroy and De Vuyst, 2004). By producing lactic acid during the fermentation process, LAB starter cultures also play

an essential part during cheese making as well as ripening aside from acid production (Ryssel et al., 2010).

The starter culture used widely for cheese production in the cheddar cheese industry are strains of *Lactococcus lactis* (Stuart et al., 1999). Lactococci are coccoid, homofermentative bacteria and occur singly, in pairs, or in chains. They are mesophilic bacteria that grow at 20 to 40°C (Teuber, 1995). The taxonomic position of *L. lactis* currently is phenotypically based (Schleifer et al., 1985), van Hylckama Vlieg et al., 2006; Rademaker et al., 2007) and includes two major subspecies, subsp. *lactis* and subsp. *cremoris*, both of which are used in making varieties of cheese and other fermented milk products. Acidifying rates and temperature dependence vary with both subspecies. Acid production from of *L. lactis* subsp. *lactis* is generally faster but *L. lactis* subsp. *cremoris* is considered more suitable for flavor development (Mills et al., 2011).

Five phenotypic characteristics that distinguish *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris* is the ability to grow at 40°C, in 4% NaCl and at pH 9.2, the ability to ferment maltose and capacity to deaminate arginine. *Lactococcus lactis* subsp. *cremoris* strains are reported to be negative for all these characteristics. (Fernández et al., 2011).

Flow Cytometry

Researchers constantly seek to develop techniques that can evaluate cell viability, and enumerate and monitor microbial populations, in various fields such as industrial microbiology, waste treatment plants, or clinical applications (Rose and Grimes, 2001). Traditional methods have relied on various technologies including, PCR, DNA extraction, plate counting, biochemical testing, and light or fluorescence microscopy to

examine growth, physiology, and morphology of microorganisms of interest (Franco-Duarte et al., 2019). However, while these technologies are widely recognized, most method don't measure or determine viability of cells. Moreover, these techniques are relatively slow and time consuming that take extensive lag time to generate a complete outline of data. One such technology that enables a better understanding of the viability of microbial population and generates data profile of microorganisms much faster is flow cytometry. This technique can replace viability counting on agar plates and give a more detailed result on not only live but dead and intermediate states (Berney et al., 2007).

Cytometry, as the name suggests, is a process for measuring the physical and chemical characteristics of cells or particles (Shapiro, 2005). In flow cytometry, the cells flow through a fluid stream and the measurements are made. It is an instrument that detects single cells or particles as they enter in front of a light beam and the signals from those illuminated cells are collated. It uses sheath fluid that focuses the cell suspension, causing cells to pass through a laser beam one cell at a time. Light scattered from the cells or particles is detected as they go through the laser beam. A detector in front of the light beam measures forward light scatter that gathers information about the relative size of the cell and several detectors to the side measure side light scatter that process information about cellular shape or granularity/ complexity (Nunez, 2001). Fluorescence detectors measure the fluorescence emitted from positively stained cells or particles. Data is collected from each stained cell and the extent of dye uptake of a particular stain allows the differentiation of cells into discrete sub-populations (Wilkinson, 2016). This information is displayed on a profile called a cytograph.

Flow cytometry (FC) is a unique technique that measures the chemical and physical characteristics of biological particles and cells, where data is provided for every particle detected. (Hickey et al., 2015). The advantages of FC are rapid multiparametric data acquisition (1 to 2 min), high numbers of cells examined per sample (>10,000), minimal sample volume (5 µl), high throughput, and less labor and space required compared with conventional plating techniques (Wilkinson, 2018).

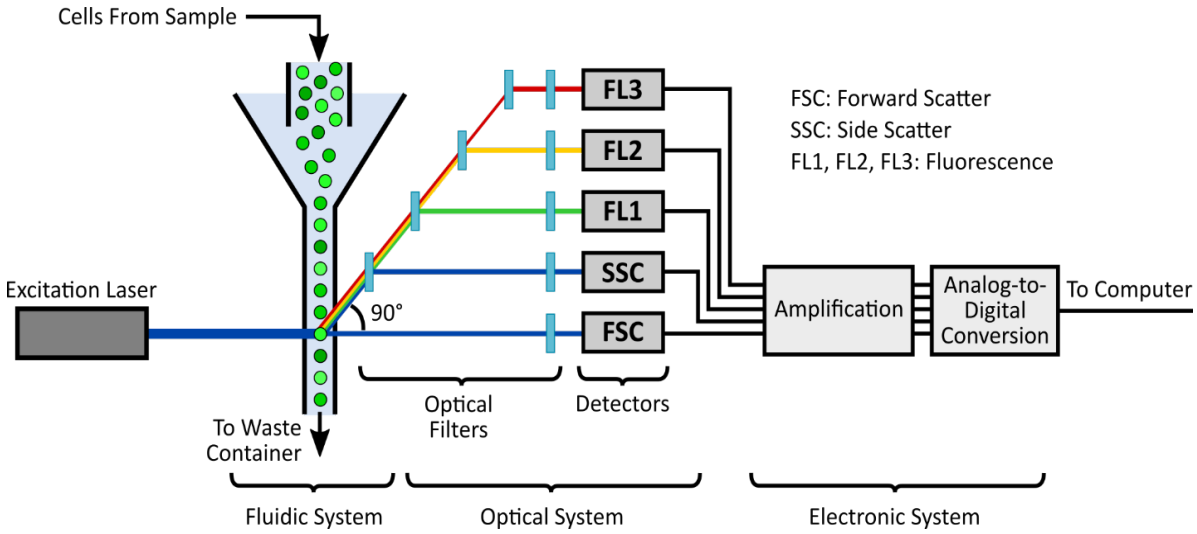


Figure 1. Schematic diagram of a typical flow cytometer showing the fluidic, optical, and electronic systems. Source: Castillo-Hair et al (2016)

The simple and quick assessment of the viability of a microorganism is an important aspect of **FC**. It has been used in metabolic studies of various microorganisms where one of the authors has used SYBR green as a fluorescent probe to determine the physiological state of *Staphylococcus epidermidis* (Cerca et al., 2011) while Jellet et al. (1996) assessed the metabolic activity of bacterioplankton communities in sea water. Researchers have also used FC for studying gene expression using reporter genes in bacteria and yeast (Álvarez-Barrientos et al., 2000).

Flow Cytometry has been applied to the study of changes in sub-populations of lactic acid bacteria (LAB) starters, and probiotic strains in fermented dairy products. It has provided insights into the degree of heterogeneity that develops in the microbial population during storage under various conditions (Wilkinson, 2018). Sheehan et al (2005) used FC to identify and enumerate intact and permeabilized cells in broth and in Cheddar cheese juice. These authors correctly addressed the importance of autolytic and permeabilization properties of LAB starter strains. Other studies on the fate of starter LAB include those of Ruggirello et al., (2016), Doolan et al, (2014) and Wilkinson (2012).

Live and Dead Staining

Flow cytometry in combination with fluorescent dyes is advancing as a technology for analysis of bacterial viability in food, industrial, and environmental samples. (Berney et al., 2007). Live/ Dead staining not only works with prokaryotic cells (bacteria) but also with eukaryotes such as yeast (Zhang and Fang, 2004; Boulos et al., 1999). This staining procedure enables differentiation between bacterial cells with intact or damaged membranes. Initially, it was assumed that membrane-compromised bacterial cells are dead while intact cells are metabolically active (Nebe-von-Caron et al., 2000). However, with the live/dead staining protocol, along with live and dead cells, flow cytometry has shown that intermediate cells are also observed (Berney, et al., 2007).

Several commercial single dyes and dye combinations are available allowing assessment of bacterial viability based on membrane integrity. In this research, green fluorescent SYBR Green (SG) and red fluorescent Propidium Iodide (PI), fluorescent dyes were used (Grégori et al., 2001). SYBR Green in combination with PI can be used

to assess bacterial cells in flow cytometer. SYBR Green is a membrane-permeant dye. Once inside the cell, it will preferentially bind to DNA, resulting in the formation of DNA: dye complex that, when excited at a wavelength of 488-nm blue laser light emits green and red light at wavelength λ_{max} = 520 nm that can be measured in FL1 and FL3 detectors, respectively (Habtewold et al., 2016). SYBR Green staining enables efficient analysis of the total bacterial cell concentration but on its own does not differentiate between intact and damaged cells (Berney et al., 2007). In contrast, PI is a membrane-impermeable dye (i.e., it is not actively taken up by intact cells), that binds to DNA in cells that have lost membrane integrity, i.e. dead cells/severely damaged cells and will fluorescence when excited at 535nm (Shapario, 2005) (Figure 2).

Double staining with these dyes, results in the fluorescence energy transfer from SG (donor molecule) to PI (accepter molecule) which reduces the SG fluorescence intensity while increasing PI emission intensity resulting in cells with damaged membranes exhibiting more fluorescence from PI than from SG depending on the extent of permeability of the cell membrane (Habtewold et al., 2016). Based on these different absorbance patterns, if a bacterial cell exhibits high SG fluorescence and low PI fluorescence then the cell membrane is assumed intact and the cell is as live and metabolically active. Conversely, a high PI fluorescence and low SG fluorescence implies that the cell membrane is highly permeable, so the cell is dead and therefore not metabolically active. Together, SG and PI can optimally discriminate bacteria with permeable (or disrupted) membranes versus intact membranes (Gatza et al., 2013).

It has been observed (Berney et al., 2007) that some bacterial cells examined using FC have an intermediate level of fluorescence when stained with both SG and PI.

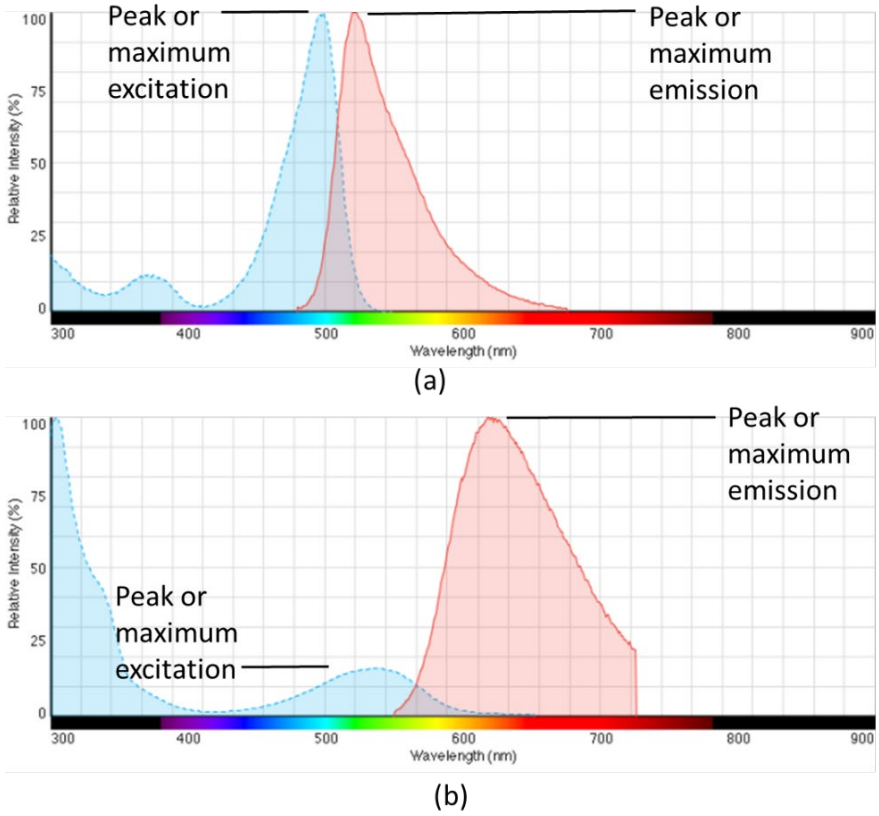


Figure 2. Spectral profiles. Light absorbance and light emission of (a) SYBR green I and (b) propidium iodide (PI). Source: Thermo Fischer Scientific

These cells are presumed to be metabolically active because of the uptake of SG, however, their cell membrane is still allowing some entrance to PI, so these cells have been designated as live- semi-permeable.

SYBR green also binds to extraneous material (inorganic particles, nonbacterial organic particle, or free DNA) in the sample that is recorded by FC as events (Gatza et al., 2013). These can be distinguished from actual bacterial cells based on their forward and side scatter properties and the extent of their fluorescent signal. However, having a large extraneous signal from such non-cellular fluorescing material reduces the number of relevant events recorded from bacterial cells because FC instruments stop counting

when a maximal number of events (1,000,000) is reached. Events from such debris should, therefore, be eliminated from a measurement so that more relevant events can be recorded. Hence, setting a threshold value (lowest signal intensity recorded for an event) is very important. Once, the particles have been identified, the threshold should be set in such a way that the noise doesn't interfere with the analysis while still capturing the entire cellular population. (Hall et al., 2011).

Metabolically Active Non-Culturable (MANC) Cells

Traditionally, bacterial cell viability is monitored by plating on bacteriological media with the appearance of visible colonies. If colonies are formed on the appropriate media, the number of bacteria can be calculated as the number of colony-forming units (**cfu**) per ml or grams plated. If colonies are not visible then the bacterium is often considered dead, although it may be that the particular bacterium is unable to grow (multiple) on that particular medium. Another possibility is that the bacterium may have lost the metabolic ability to divide and to reproduce. Such a non-culturable state has been observed when bacteria are present in some environments such as cheese (Ruggirello et al., 2014) and for many pathogenic bacteria (Pienaar et al., 2016)

In cheese, lactococcal starter culture numbers start out high (10^7 to 10^9 cfu/g) and based on plate counting decline over 3 to 6 mo to 10^4 cfu/g or less during the storage and aging of Cheddar cheese (Fenelon et al., 2000; Ganesan et al., 2007). These numbers are determined by plating on microbiological media and it is assumed that 99.9% or more of the starter culture cells in the cheese have died often releasing their cellular contents into the surrounding cheese environment (Oberg et al., 2011; McMahon et al., 2014).

However, based upon DNA extraction from cheese, lactococcal number (intact cells) levels remained high (10^7 cfu/g) throughout storage (Ganesan et al., 2014).

It has been observed that when carbohydrates become limited in a growth medium, lactococci can enter a non-culturable state as their metabolism changes to utilize other components such as amino acids as energy sources rather than sugars (Ganesan et al., 2007). Based on this study it was proposed that in conjunction with the genes responsible for sugar metabolism being repressed under such sugar starvation conditions, some genes responsibility for initiating cell replication and division are also repressed. So, while the cells remain viable, they are no longer culturable and are not enumerated using techniques that rely on bacterial growth (cell division) for the formation of colonies. Carbohydrate starvation conditions can occur after manufacture as the lactose (which was plentiful during cheesemaking) is consumed by the starter culture during the initial stage of cheese storage and aging (Oliver, 2005). Any conversion of the starter culture lactococci into a MANC state would result in those cells not being enumerated using standard plate counting techniques but they would be counted using flow cytometry.

MATERIALS AND METHODS

Starter Cultures

Lactococcus lactis subsp. *lactis* (strains 600-M1, E36) *and L. lactis* subsp. *cremoris* (strains B36, G61) starter cultures were obtained from Vivolac Cultures Corporation (Greenfield, IN). Cultures were grown at 31°C, using external pH-control in a dairy-based lactose limited production medium. The pH was controlled between 5.8 and 6.0 with the addition of ammonium hydroxide. Incubation was continued until substrate exhaustion occurred (when no further decrease in pH was detected) and then cooled to 4°C. Culture activity was measured by adding 0.3 mL of culture to 10 mL of ultra-high temperature processed milk (Gossner Foods, Logan, UT) in a test tube (in duplicate) and incubated in a water-bath at 32°C for 2.5 h (personnel communication, Dr. Randall Thunell, Vivolac Cultures Corporation). Culture activity was the difference in mean pH of the inoculated milk compared to pH of uninoculated tubes of milk. Subsequently, culture addition to milk in the cheese vat was set at 0.45% (wt/wt) when starter activity was 1.5. If culture activity was lower, then the amount to be added to the vat was increased to 0.50%. Conversely, if the activity was higher, e.g., 1.6 or 1.7, the percentage used for cheese making was reduced to 0.4% or 0.35%, respectively.

Cheese Manufacture

Fresh cow's milk was obtained from the George B. Caine Dairy Research and Teaching Center (Wellsville, UT) and transported to the Gary Haight Richardson Dairy Products Laboratory at Utah State University (Logan, UT). Milk was pasteurized (72°C

for 15 s) and 544 kg of pasteurized milk was pumped into a Tetra Scherping horizontal cheese vat (Tetra Pak Cheese and Powder Systems Inc., Winsted, MN).

The milk was warmed to 31°C, starter culture added according to its activity and after mixing for 5 min, 45 mL of double-strength chymosin rennet (Maxiren; DSM Food Specialties USA Inc., Eagleville, PA) was added and the milk allowed to set undisturbed. Thirty minutes after renneting the curd was cut. After cutting and healing, the curd/whey mixture was stirred for 10 min, heated to 38°C over 25 min, and then was stirred for another 20 min. The curd/whey mixture was pumped from the vat onto a drain table (Kusel Equipment Co., Watertown, WI) and stirred until a curd pH of 6.3 was reached (~25 min). The remaining whey was then drained, and the dry curd was stirred for approximately 10 min. The curd was allowed to mat together, cut into slabs, and cheddared for a target time of 180 min until the curd pH was reached 5.4. The standard make procedure is shown in Appendix A.

Curd was milled, separated into five 11-kg portions, and placed into open plastic containers. Curd was mixed with either 2.0, 2.4, 2.8, 3.2, or 3.6% (wt/wt) salt using 3 applications with 5 min between each application. Salted curd was allowed to stand for 5 min before being placed into plastic cheesecloth-lined stainless steel hoops and pressed in a horizontal press for 3 h (140 kPa, ~20°C). The cheese was then de-hooped, cut into ~1-kg pieces, individually vacuum packaged, and stored at 6°C.

Cheese Sampling

During cheesemaking, curd samples were collected after culture addition, after cutting, before draining, at curd pack, and before salting. The pH was measured using a glass combination pH electrode connected to a pH meter (Orion Star™ A211, Thermo

Fisher Scientific) and milk and curd samples immediately prepared for plate counting. Cheese was sampled after 6 d of storage at 6°C and evaluated using both plate count and flow cytometry.

Proximate Analysis

Chemical analysis of the cheeses was determined after approximately 5 d. Moisture content was measured by weight loss using approximately 3.1 g of grated cheese left overnight in a vacuum oven (Model 5831; National Appliance Company) at 100°C in triplicate. Salt was measured by homogenizing grated cheese with distilled water in a Stomacher 400 (Seward Ltd., Worthing, UK) for 4 min at 260 rpm. Whatman #1 filter paper was used to filter the slurry, and the filtrate was analyzed using a chloride analyzer (Model 926; Corning Diagnostic Corp., Medfield, MA) in triplicate. Salt-in-moisture concentration (**S**/M) was calculated as salt/(moisture + salt) and expressed as a percentage. The pH was measured by stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm then measured using a glass electrode.

Three cheeses from each starter culture strains that best fit target S/M levels of 3.5, 4.5, and 5.5% were selected and used for bacterial plate count and FC analysis.

Plate Counting

Eleven grams of milk, curd, or cheese were stomached with 99 mL of sterile 2% (wt/wt) sodium citrate buffer for 4 min at 260 rpm. One milliliter aliquots were then serially diluted with 9mL dilution blanks containing of 1% peptone. One hundred microliters of sample was plated on both M17 and Reddy's agar and aerobically

incubated at 32°C for 24 h. The *cremoris* strain was counted within a 30 to 40 h window while *lactis* strain was counted within 36 to 48h on Reddy's agar (Reddy et al., 1972).

Gram Staining and Chain Length

A smear of each bacterial sample was applied onto a slide, air dried and then heat fixed by passing it through a flame. First, five drops of crystal violet were added to the slide and washed briefly with water after a standing time of one minute. Then, five drops of iodine solution were added to the slide, made to stand for one minute and washed briefly with water After that, decolorizer was added to the slide until the purple color stopped running and the slide was washed briefly with water. Finally, five drops of safranin were added to the slide, washed with water and examined under the microscope at 100X oil immersion. The chain length was counted and divided by the number of chains to get an average chain length.

Flow Cytometry.

In cultured milk. Ultra-high temperature sterile milk was inoculated with a mixed strain lactococcal starter culture (DVS850, Chr. Hansen Laboratories) and incubated overnight at 37°C. A negative control containing only dead cells was obtained by heating the cultured milk at 85°C for 12 min. An aliquot of milk was then diluted 500-fold in PBS buffer, and then one of five nucleic acid dyes were added in combination with PI:

- Carboxy fluorescein diacetate (**cFDA**) in which 10 µL of 1mM cFDA was added to 990 µL of sample cells in combination with 5 µL of PI. Fluorescence from

cFDA only occurs upon intracellular enzymatic metabolism after its permeation into living cells. After heating the cultured milk, dead cells were also detected.

- SG (Invitrogen; SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO, cat No. S7563) in which 5 µL of SG was added to 1000 µL of sample cells in combination with 1 µL PI.
- SYTO24 (Invitrogen; SYTO™ 24 Green Fluorescent Nucleic Acid Stain - 5 mM Solution in DMSO, Eugene, OR, USA)/PI in which 10 µL was added to 990 µL of the sample in combination with 10 µL of 0.2mM PI.
- Thiazole Orange (Sigma-Aldrich, model no. 39006, MO, USA) in which 2 µL was added in 1000 µL of the sample in combination with 1 µL PI.
- Syto 9 (Invitrogen; SYTO™ 9 Green Fluorescent Nucleic Acid Stain, cat No. S34854) in which 1 µL was added to 1000 µL sample cells in combination with 20 µL PI.

In Cheese. *Flow* cytometry was used to evaluate the ratio of live, dead, or live-semi permeable cells in cheddar cheese after 6 d of storage. Eleven grams of cheese were obtained from within each block of cheese was stomached with 99 mL of sterile 2% (wt/wt) sodium citrate buffer for 4 min at 260 rpm. Buffered peptone (Oxoid CM0509, cat No. 2436693) and saline (PBS 10×, Gibco, cat No. 70011) was filtered through a 0.2-µm filter (Corning Incorporated; cat No. 431219). The sample was 10-fold diluted with buffered peptone and centrifuged (Beckman; Microfuge Lite; cat No. 365606) at 10,000 rpm for 15 min. The supernatant was discarded, and the pellet resuspended in 1,000 µl of sterile saline. The sample was again 10-fold diluted with saline. Five microliters of SG

and 1 µl of PI (Sigma- Aldrich; cat No. MKCC9922) were added to the sample and incubated for 15 mins at 37°C.

Measurements were obtained using a BD Accuri® C6 plus flow cytometer (BD Accuri® cytometer, Belgium). Primary dyes were excited by a 488 nm (20 mW) laser and collected through a 530/30 band pass filter (collected as FL1) and PI resultant fluorescence collected through a >670 nm band pass filter (collected as FL3). All parameters were collected as logarithmic signals using a threshold of 2,000 on FL1, below which no fluorescence intensity events were recorded.

Statistical analysis and data visualization

The experiment was done with cheese being made twice for each strain. The cheeses were made at 8 separate times and curd divided into batches, salted and pressed. Statistical analysis was made using MS Excel and Statistical Analysis System (SAS Inc., Cary, NC) analysis tool. Tukey's honestly significant difference at 5% level of significance was used to compare bacterial cell numbers.

RESULT AND DISCUSSION

Starters and Cheese Manufacturing

There were some individual differences in cheese manufacturing time between vats even though all cheeses were made using the same make procedure (Appendix A). Differences to reach target pH levels are shown in individual make records (Appendix B). For example, when using *Lactococcus lactis* subsp. *lactis* 600M1, replicate 2 took longer to reach the expected pH of cook temperature (pH 6.55). After that, the acid development and drop in pH were similar in both replicates. In replicate 1 of *Lactococcus lactis.* subsp. *lactis* E36, the drain table was accidentally maintained at too high a temperature therefore, acid development was initially slower after draining as compared to replicate 2. After adjusting the temperature of drain table and turning the curd every five minutes instead of ten minutes, the pH started to decrease but it still took replicate 1 more than 120 minutes to reach the milling pH. Both the replicates of *Lactococcus lactis.* subsp. *lactis* E36 were milled at pH ~ 5.6 which was higher than the target pH of 5.45. This was necessary due to time constraints to complete curd manufacturing and pressing of cheese within the allocated time.

In the case of *Lactococcus lactis* subsp. *cremoris* B36 *lactis*, replicate 2, took a longer time than replicate 1 to reach the pH of cook temperature (pH 6.55), pH of draining (pH 6.30) and pH of milling(5.45). However, after draining the whey, replicate 1 of *Lactococcus lactis* subsp. *cremoris* B36 did not drop in pH as targeted. When cheese was made using *Lactococcus lactis* subsp. *cremoris* G61, replicate 1 dropped in pH much faster than predetermined time according to the cheese make sheet (Appendix A). The

steps such as pumping over, stacking two-high during cheddaring, and milling the curd had to be cut short than expected in order to meet the pH targets. However, in replicate 2, these steps went according to the predetermined time and pH in the cheese make sheet.

Total cheese manufacturing time for *Lactococcus lactis* subsp. *lactis* (600M1, E36) *and Lactococcus lactis* subsp. *cremoris* (G61, B36) strains from starter addition to milling varied from 200 to 390 min depending on the strain (and replicate) rather than its subspecies designation (Table 1). The *Lactococcus lactis* subsp. *cremoris* B36 had the longest make time, followed by *Lactococcus lactis* subsp. *lactis* E36. *Lactococcus lactis* subsp. *cremoris* is more sensitive to temperature and is likely to have more physiological changes than *Lactococcus lactis* subsp. *cremoris lactis* (Kim et al., 1999).

The shortest manufacturing time was when using *Lactococcus lactis* subsp. *lactis* 600M1 followed by *Lactococcus lactis* subsp. *cremoris* G61. This occurred even though the starter culture was added according to its measured culture activity which was comparable within the subspecies (Table 2). It was observed that the activity of starter culture did not completely correlate with the cheese making time (Table 1 and 2). This could be because activity test is done at a static temperature whereas cheddar cheese manufacturing process involves several temperature ramps. Also, starter activity in milk at 32°C is not a reliable predictor of cheese make time without considering other factors which can affect acid production. The other factors can include number of copies of lac operon in a strain (Singh etal., 2006), transport mechanisms of lactose into the cell (Thompson, 1987) and sensitivity of cells to temperature.

Table 1. Make times for cheese made using single strains of *Lactococcus lactis*.

***Lactococcus lactis* strain**	**Subspecies**	**Replicate**	**Time From Adding Starter to Draining Whey**	**Time From Draining Whey to Milling Curd**	**Total Time From Adding Starter to Milling Curd**
			----------(min)----------		
600M1	*lactis*	1	110	100	210
600M1	*lactis*	2	139	115	254
E36	*lactis*	1	150	224	374
E36	*lactis*	2	176	176	352
B36	*cremoris*	1	178	186	364
B36	*cremoris*	2	205	188	393
G61	*cremoris*	1	109	107	216
G61	*cremoris*	2	139	130	269

Table 2. Starter culture activity and amount added to the milk and plate count numbers in milk after adding starter based on colony counted on Reddy's agar.

***Cheese made using L. lactis* subspecies strains**	**Replicate**	**Culture activity**	**Starter addition to milk**	**Milk in the vat**	**Culture added to milk**	**Lactococci numbers in milk after adding starter culture**
		----------(%)----------		----------(kg)----------		(log cfu/g)
600M1	1	1.6	0.45	605	2.7	6.6
600M1	2	1.7	0.4	533	2.1	7.1
E36	1	1.7	0.4	540	2.9	7.4
E36	2	1.7	0.4	538	2.2	7.6
B36	1	1.6	0.4	547	2.2	5.1
B36	2	1.4	0.55	549	3.0	6.1
G61	1	1.6	0.45	551	2.5	7.1
G61	2	1.5	0.45	544	2.4	7.4

Microbial numbers during cheese making

The starter culture amount was calculated according to its measured culture activity. The time allocated for mixing of the starter culture into the milk in the vat before the milk was sampled was only around 5 mins. This could have caused insufficient mixing of the starter into the milk resulting in a low after starter addition number (Table 2). Based on average chain length i,e, 2.8 for *Lactococcus lactis* subsp. *lactis* 600M1, 2 for *Lactococcus lactis* subsp. *lactis* E36, 16 for *Lactococcus lactis* subsp. *cremoris* B36, 15 for *Lactococcus lactis* subsp. *cremoris* G61 which was calculated from the micrograph shown in Figure 3, the starter culture number were within a half log difference or did not change from after cutting the curd to before salting the cheese. (Table 3). Thus, taking the concentration factor and chain length into account, interestingly, there was no variation in the cell count numbers during cheesemaking.

Plate counting was done on Reddy's agar, which is a differential agar. The ability of *Lactococcus lactis* subsp. *lactis* and the inability of *Lactococcus lactis* subsp. *cremoris* to hydrolyze arginine to create ammonia formed the basis of differentiation between these species. Ammonia liberated from arginine is detected by pH changes occurring in the medium. *L. lactis* subsp. *cremoris* strains should give yellow colonies surrounded by yellow zones whereas *lactis* strains should give white colonies devoid of zones. (Figure 4).

In this research, *Lactococcus lactis* subsp. *cremoris* B36 strain gave both white and yellow colonies for samples after draining, even though it was counted within 40-h window required for this media. Arginase activity can be a variable trait in some strains (R.Thunell, Personal Communication). Hence, Reddy's agar does not always distinguish

between *cremoris* or *lactis* strains making it impractical to use in a definitive way when cheese is made using a starter culture containing multiple strains. There is also the issue, that not all *cremoris* strains contain an active arginase gene and so would produce white colonies. Likewise, some *lactis* strains do exhibit arginase activity and would therefore produce yellow colonies on Reddy's agar.

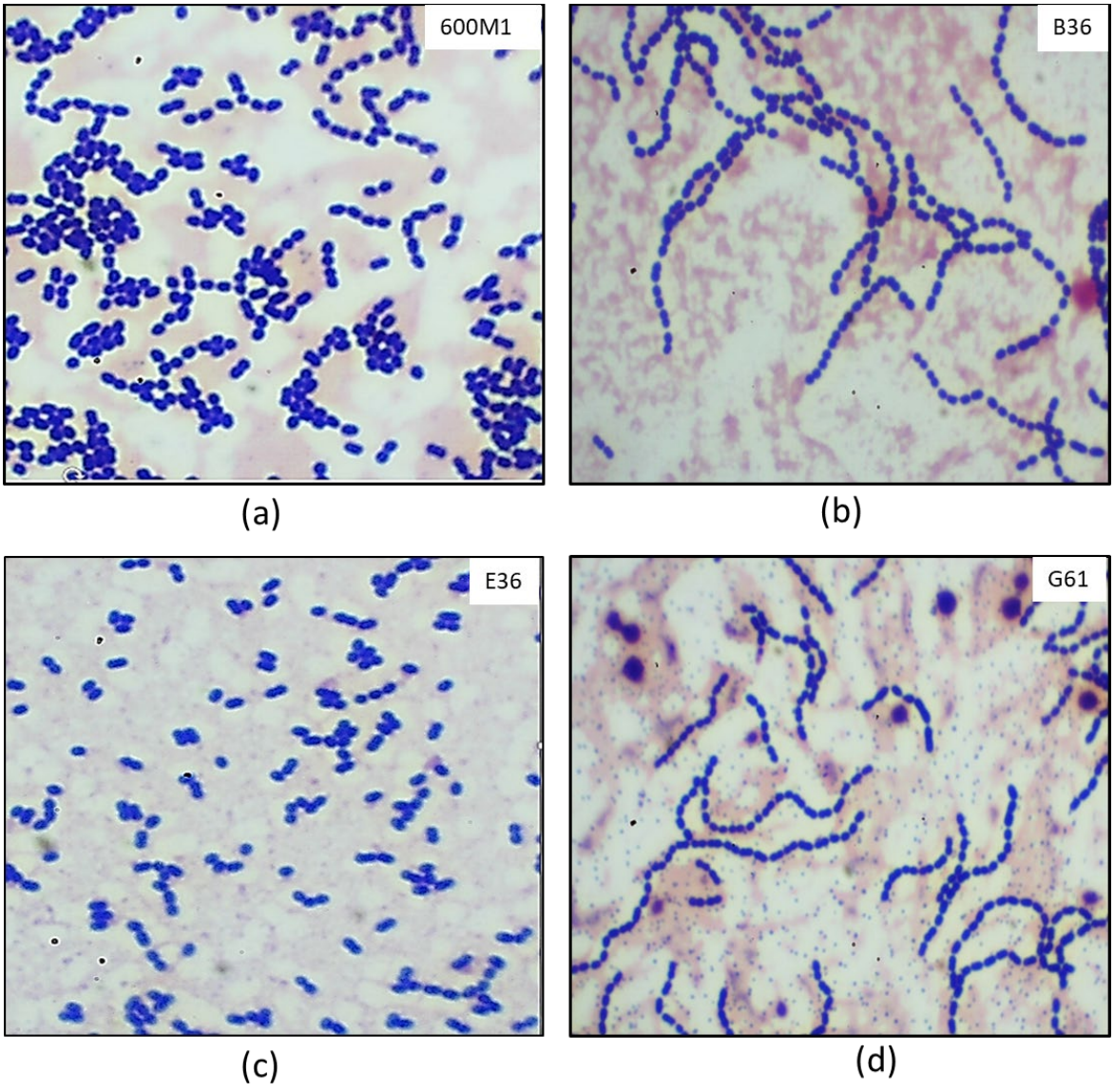
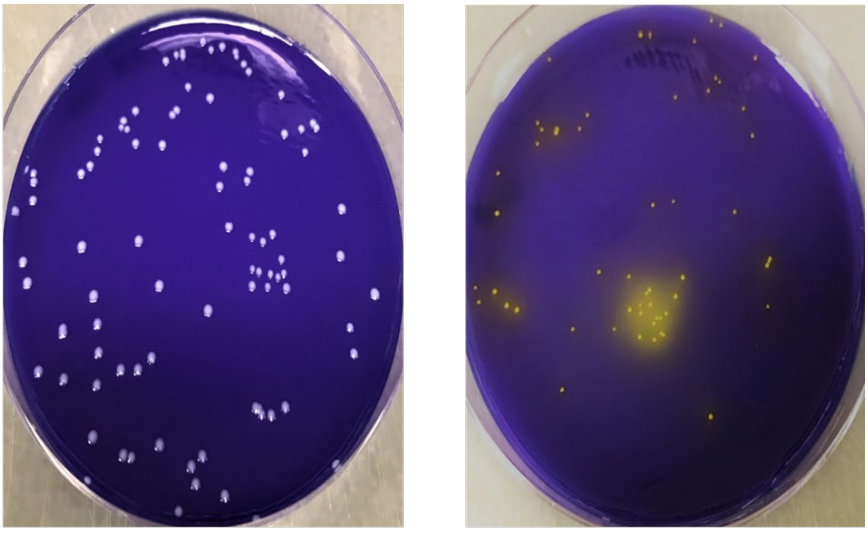


Figure 3. Gram stained images of *Lactococcus lactis* showing chains of cells for *lactis* subspecies strains (a) 600M1 and (c) E36, and *cremoris* subspecies strains (b) B36 and (d) G61 (Images courtesy of Dr. Randall Thunell, Vivolac).

Table 3. Relative lactococcal numbers in curd during cheesemaking calculated as plate count numbers (cfu/g) multiplied by average chain length for each strain and divided by curd concentration factor to correct for shrinkage of curd as whey is expelled.

			Curd concentration factor			
			1.1	4.0	5.0	8.0
			Relative Bacterial Numbers			
***Lactococcus* strain**	**Replicate**	**Average Chain Length**	**After Cutting Curd**	**Before Draining Curd**	**After Curd Packed Together**	**Before Salting Curd**
			----------log(cfu/g)----------			
600M1	1	2.8	8.5	8.6	8.5	8.5
600M1	2	2.8	8.2	8.1	8.3	8.5
E36	1	2.0	8.5	8.5	8.6	8.5
E36	2	2.0	8.6	8.1	8.4	8.3
B36	1	16	8.4	7.9	7.7	8.8
B36	2	16	8.1	8.3	7.7	8.5
G61	1	15	9.0	8.8	8.7	8.6
G61	2	15	9.2	8.8	8.7	8.7



(a) (b)

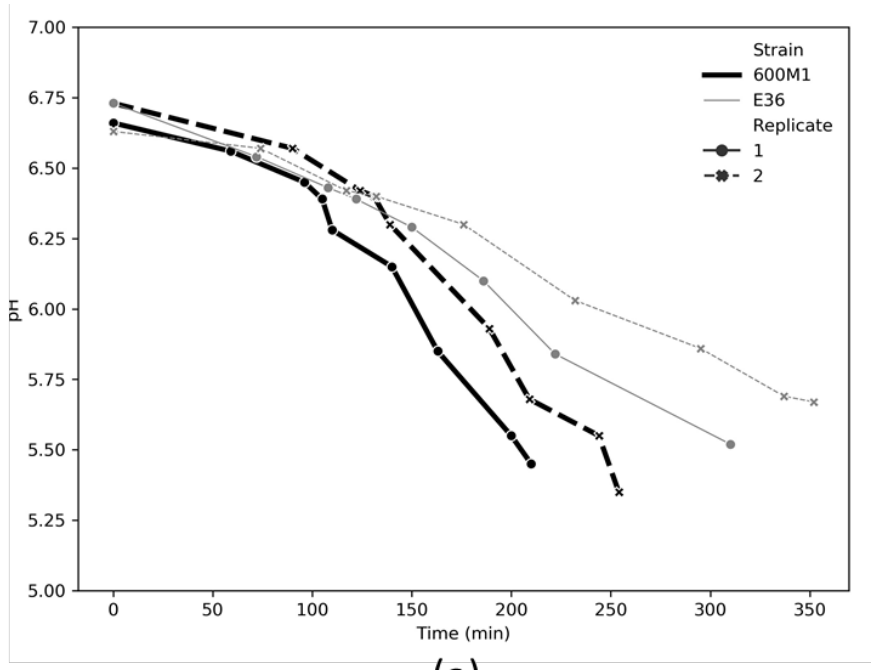
Figure 4. (a) Representing the colonies of *Lactococcus lactis* subsp. *lactis* E36 strain and (b) *Lactococcus lactis* subsp. *cremoris* G61 strain.

pH during Cheesemaking

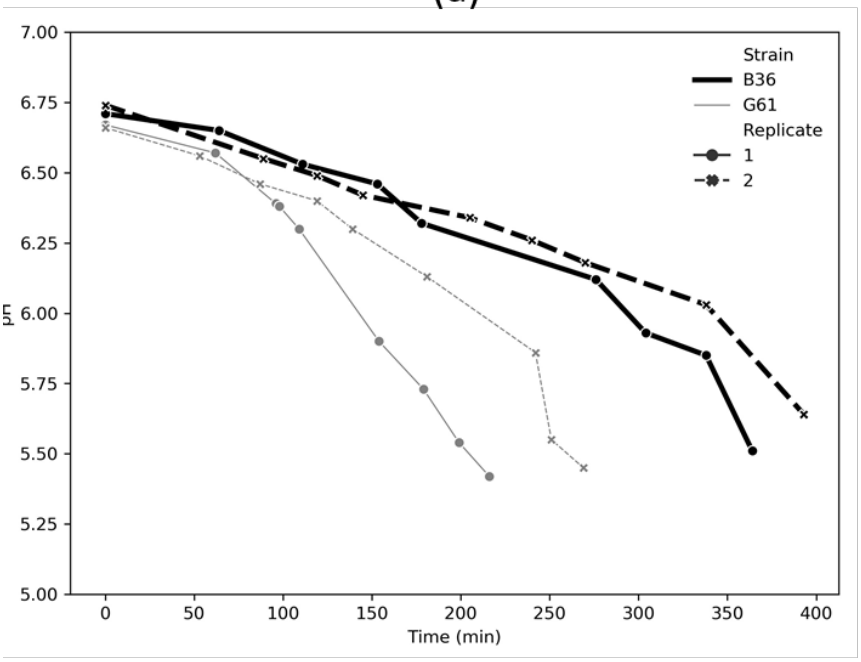
The rate at which pH dropped during cheesemaking (Figure 5) varied between strains (and between replicates of the same strain) as demonstrated by differences in cheese making time. The cheesemaking steps of whey draining and curd milling were performed based upon the curd reaching set pH values (pH 6.3 and pH 5.45, respectfully). The expectation was that with a standardized cook temperature of 38°C that the *lactis* strains would produce faster acidification of the curd than the *cremoris* strains based upon their tolerance of slightly higher temperatures. Figure 5a and 4b shows plot of pH change of each replicate of *Lactococcus lactis* subsp. *lactis* (600M1, E36) and *Lactococcus lactis* subsp. *cremoris* (B36, G61) during the cheese-making process. There were some differences in the pH change behavior between the different strains and within replicates.

A higher change in pH was observed for replicates of *L. lactis* subsp. *lactis* 600M1 and G61 followed by replicates of *L. lactis* subsp. *cremoris* E36 and B36. For some strains there were deviations, eg., above the temperature range during cooking for replicate 2 of *L. lactis* subsp. *cremoris* B36 and curd kept too warm during cheddaring for replicate 2 of *L. lactis* subsp. *lactis* E36. In our study, both replicates of *Lactococcus lactis* subsp. *cremoris* B36 were affected by temperature (~38C) and therefore slow in acid production- draining (~ 6.33) to milling (~5.6) which caused milling of the curd to occur at a higher pH than planned. In contrast, the pH value for the replicate 2 of *Lactococcus lactis* subsp. *lactis* 600M1 decreased rapidly from draining (~6.30) to milling (5.35).

It was observed that the replicate with the least amount of issues during cheese making had the shortest cheese make times. The replicate 1 of *Lactococcus lactis* E36,



(a)



(b)

Figure 5. (a) Acidification of curd while manufacturing cheese using *Lactococcus lactis* subsp. *lactis* strains 600M1, *Lactococcus lactis* subsp. *lactis* E36 (b) Acidification of curd while manufacturing cheese using *Lactococcus lactis* subsp. *cremoris* B36, and *Lactococcus lactis* subsp. *cremoris* G61 with replicate 1(solid lines) and replicate 2 (dotted lines). The change in pH is demonstrated during cheese making process (adding rennet to milling) at 8 separate occasion**s.**

600M1 *lactis* and both the replicates of G61 *cremoris* went according to the pre-determined pH listed in the cheese making sheet (Appendix A) with draining occurring at pH 6.30 to milling at pH 6.45. The pH change behavior of replicate 1 of *Lactococcus lactis* subsp. *lactis* 600M1 and *Lactococcus lactis* subsp. *cremoris* G61 were similar throughout the cheese making process (from adding starter to pressing) and had the same rate of acid production despite being a different subspecies (Figure 4). Both *Lactococcus lactis* subsp. *lactis* 600M1 and *Lactococcus lactis* subsp. *cremoris* G61 continued producing acid even after cooking the curd (38°C). Acid production by *Lactococcus lactis* subsp. *lactis* E36 was slowed down at 38°C and it took 60% longer for *Lactococcus lactis* subsp. *lactis* E36 to reach pH of 5.5 (310 compared to 190 minutes). Whereas *Lactococcus lactis* subsp. *cremoris* B36 required almost double the amount of time to reach pH 5.5 as compared to *Lactococcus lactis* subsp. *lactis* 600M1 and *Lactococcus lactis* subsp. *cremoris* G61 strains, and its acid production was severely curtailed at 38C and required the curd to cool down during cheddaring before acid production picked up again. Rate of pH decrease plays an essential role in dissolving the colloidal calcium phosphate from casein matrix, thus releasing great part of calcium to the whey during the cheese making process (Sameen et al., 2008)

Even though *Lactococcus lactis* subsp. *cremoris* strains are reported to have extremely reduced fermentation and enzyme activity profiles (Fernández et al., 2011), *Lactococcus lactis* subsp. *cremoris* G61 strain showed greater fermentation than *L. lactis* subsp. *lactis* E36 strain. From this result, that rate of acid production appears to depend upon specific strain, and not all *L. lactis* subsp. *cremoris* strains are slow acid producers and not all *L. lactis* subsp. *lactis* strains are fast acid producers.

Salt Effect on Cheese Moisture

Cheese moisture content decreased as more salt was added to the curd (Table 4). The cheese that received the low salt treatment was approximately 23 g/kg higher in moisture content than the other cheeses. More whey expulsion occurred with cheeses that received higher salt treatment as shown by Lu et al. (2015). This trend of higher moisture with lower salt addition to cheese has been previously shown (Sutherland, 1977; Guinee 2004; Fuca et al., 2012; McMahon et al., 2014). The initial expectation was that adding 2.8% (wt/wt) salt to the milled curd would produce a cheese with S/M levels of ~4.5% for a cheese with moisture of ~36%. In most of the cheese made during this experiment, this was achieved with S/M of 4.1, 4.6, 5.0, 4.2, 4.5, 4.5, 4.4, and 4.6% being obtained. Adding 2.0% (wt/wt) salt was expected to produce a cheese with S/M of ~3.5% which would be similar to that of a low-fat cheese or 33% reduced salt cheddar cheese. The S/M in the individual batches ranged from 3.1 to 3.8%. Adding 3.6% (wt/wt) salt to the curd was expected to represent the higher end of the salt level (~5.5%) shown to produce optimum cheese flavor in an aged cheddar cheese (Lawrence et al., 1984). In general, these S/M levels were achieved with batch variation.

Moisture levels (mean ±SE) for low, medium, and high salt cheeses were 36±1.9%, 35. ± 2.9%, and 34 ± 1.5%, respectively. Also, salt levels (mean ±SE) for the low, medium, and high salt level cheeses were 1.37 ± 0.3%, 1.66± 0.2%, and 1.91±0.4%, respectively.

pH at 6th day Storage

The fermentation of residual lactose in curd by starter culture continued even after pressing. At 6 d storage, the pH of all replicates was observed to be lower than the

milling pH. However, the amount of decrease was not the same for any replicates. In low salt cheeses, the decrease in pH was larger compared to pH of high salt cheeses (Table 5).

Table 4. Moisture, salt and salt-in-moisture (S/M) of cheese made using single strains of *Lactococcus lactis* as a function of salt added to the curd.

Strains	Subspecies	Replicate	Salt Addition to Curd	Cheese Moisture	Cheese Salt	Cheese S/M
			----------(%)----------			
600M1	*lactis*	1	2.0	36.7	1.20	3.1
			2.4	36.2	1.32	3.5
			2.8	35.2	1.53	4.2
			3.2	34.5	1.67	4.6
			3.6	34.1	1.89	5.3
600M1	*lactis*	2	2.0	36.6	1.27	3.3
			2.4	35.2	1.41	3.9
			2.8	34.7	1.67	4.6
			3.2	35.1	1.60	4.4
			3.6	34.4	1.87	5.1
E36	*lactis*	1	2.0	35.3	1.35	3.7
			2.4	34.9	1.40	3.9
			2.8	33.6	1.76	5.0
			3.2	33.4	1.84	5.2
			3.6	33.1	1.88	5.4
E36	*lactis*	2	2.0	36.7	1.41	3.7
			2.4	36.8	1.60	4.2
			2.8	36.2	1.56	4.1
			3.2	35.4	2.16	5.8
			3.6	34.4	2.17	5.9
B36	*cremoris*	1	2.0	35.8	1.32	3.6
			2.4	35.7	1.36	3.7
			2.8	34.5	1.63	4.5
			3.2	35.0	1.67	4.5
			3.6	33.6	1.81	5.1
B36	*cremoris*	2	2.0	36.6	1.45	3.8
			2.4	36.4	1.55	4.1
			2.8	35.7	1.68	4.5
			3.2	35.1	1.85	5.0
			3.6	34.3	2.00	5.5
G61	*cremoris*	1	2.0	36.3	1.41	3.8
			2.4	35.6	1.43	3.9
			2.8	35.7	1.63	4.4
			3.2	34.8	1.87	5.1
			3.6	34.1	1.92	5.3
G61	*cremoris*	2	2.0	36.9	1.45	3.8
			2.4	36.2	1.40	3.7
			2.8	34.8	1.67	4.6
			3.2	34.2	1.72	4.8
			3.6	34.0	2.03	5.6

Salt did not inhibit the acid production and the cheese continued to decrease in pH for 3 of the strains. The decrease in pH could be the function of moisture. Lower moisture cheeses have less residual lactose remaining in the cheese as less lactose lost in the whey. They have higher relative amount of protein and associated calcium phosphate which increases the buffering capacity of the cheese.

According to Yun et al. (1993), moisture content tends to be lower with lower milling pH because of the longer total time of making cheese. This was observed for some cheeses but not for all. It was observed that in general, low pH cheeses had higher moisture and lower cheese S/M as seen in Tables 4 and 5.

Relationship between pH and moisture

Cheeses with the higher salt level (3.6% added salt) had lower moisture because of increased whey syneresis. There was ~20g/kg decrease in moisture content between the cheeses with lowest salt levels compared to those with the highest.

Acid production rate, cheese making time, and target moisture content is of importance in cheese making process. If acid production is too fast, the curd moisture will be too high when the milling pH is reached and there will be more residual lactose in the cheese. This will result in cheese with a lower pH and acidic taste. In contrast, if acid development is too slow, the cheese making time increases. As a result, curd will lose more moisture during the process. The curd will need to be milled at a high pH due to time constraint, which can result in high pH cheeses that a have a flat flavor (which is a defect in cheddar cheese). Therefore, the more the cheese making process can be standardized, and the standard procedure followed, the more consistent the cheese will be in meeting target moisture and salt specifications.

Table 5. The pH of cheeses made using *L. lactis* subsp. *lactis (600M1, E36) and L. lactis* subsp. *cremoris strain (B36, G61)* at sixth day storage with different salt levels and weight of salt applied to 10.9-kg of cheese curd during cheese making.

***L. lactis* strains**	**Replicates**	**Milling pH**	**Salt Addition to Curd**	**Day6-pH**	**Decrease from milling pH**
			%		
600M1	1	5.45	2.0	5.04	0.41
			2.4	5.04	0.41
			2.8	5.10	0.35
			3.2	5.14	0.31
			3.6	5.27	0.18
600M1	2	5.35	2.0	4.94	0.41
			2.4	5.05	0.30
			2.8	4.99	0.36
			3.2	4.96	0.39
			3.6	5.00	0.35
E36	1	5.49	2.0	5.37	0.13
			2.4	5.33	0.16
			2.8	5.44	0.05
			3.2	5.36	0.13
			3.6	5.38	0.11
E36	2	5.67	2.0	5.36	0.31
			2.4	5.21	0.46
			2.8	5.47	0.20
			3.2	5.41	0.26
			3.6	5.49	0.18
B36	1	5.51	2.0	5.07	0.44
			2.4	5.05	0.46
			2.8	5.15	0.36
			3.2	5.10	0.41
			3.6	5.20	0.31
B36	2	5.64	2.0	5.33	0.31
			2.4	5.40	0.24
			2.8	5.43	0.21
			3.2	5.37	0.27
			3.6	5.42	0.22
G61	1	5.42	2.0	5.37	0.05
			2.4	5.23	0.19
			2.8	5.45	-0.03
			3.2	5.37	0.05
			3.6	5.46	-0.04
G61	2	5.45	2.0	5.32	0.13
			2.4	5.34	0.11
			2.8	5.37	0.08
			3.2	5.44	0.01
			3.6	5.45	0.00

Optimizing Flow Cytometry

When the minimum signal threshold for the FL1 photomultiplier (capturing fluorescence through the 530 nm band pass filter) was set to 10, most of the events recorded had a signal strength of <1,000 and only a small number of events were >1,000 (Figure 6a). The events with low fluorescence were considered to be from non-cellular material as SG (and other fluorescent nucleotide dyes) can bind to extraneous material such as inorganic particles, non-bacterial organic particle or free DNA (Gatze et al., 2013). Eliminating such non-cellular events as a way to reduce the signal-to-noise ratio is more critical when studying bacteria using FC compared to other uses such as studying mammalian cells that are much larger than bacterial cells (Bunthof and Abee, 2002). This becomes more important in a medium such as cheese which has a high fat and protein content. One strategy is to produce a cheese extract with low levels of protein and fat by using high hydraulic pressure and then physically separating the fat (Sheehan et al., 2005). However, in this study, solubilizing the cheese using citrate was performed as a hydraulic press was not available and more samples could be processed (hydraulic pressing takes ~4 h per cheese sample).

When the threshold was increased to 1,000, more of the measured events collected were considered to be from bacterial cells. The cytographs had a distinct population of events that had FL1 fluorescence of 2,000 to 60,000 units (Figure 6 b). Similar noise was observed when a blank sample was measured. The “noise” was following through from the cheese sample preparation (Figure 7). For counting of bacteria in cheese by FC, it was determined that the threshold could be raised to 2,000,

and this was subsequently used. The extent of this "noise" would decrease as more events related to bacterial cells are recorded as part of the total 1,000,000 events.

Sample Dilution

Since the FC instrument being used stops counting events after one million events, it is important to eliminate most of the noise by thresholding so that all the relevant events could be recorded. It is also important to have the optimum dilution of the sample. With too much dilution, there would not be enough events recorded, while if the sample is not diluted sufficiently, there is too much extraneous matter and the background noise is too high. With bacterial populations in cheese of about 10^8 to 10^9 cells/g, it was determined after many trials that a dilution factor of 10^3 was optimum for cheese samples being prepared by citrate solubilization and being analyzed in this flow cytometer.

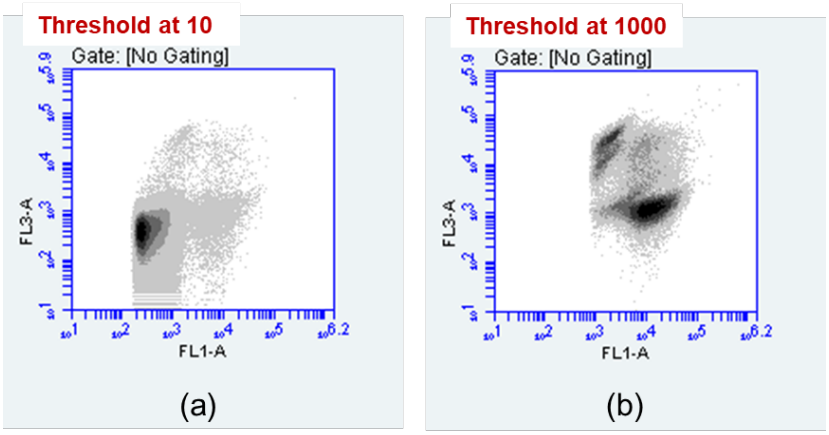


Figure 6. Cytographs of a cheese preparation stained with SYBR Green and propidium iodide showing the effect of threshold level on proportion of total events recorded that were considered non-cellular (FL1 < 1,000) and presumed bacterial cellular events (FL1 > 1,000) when the threshold was set at 10 (a) or 1,000 (b).

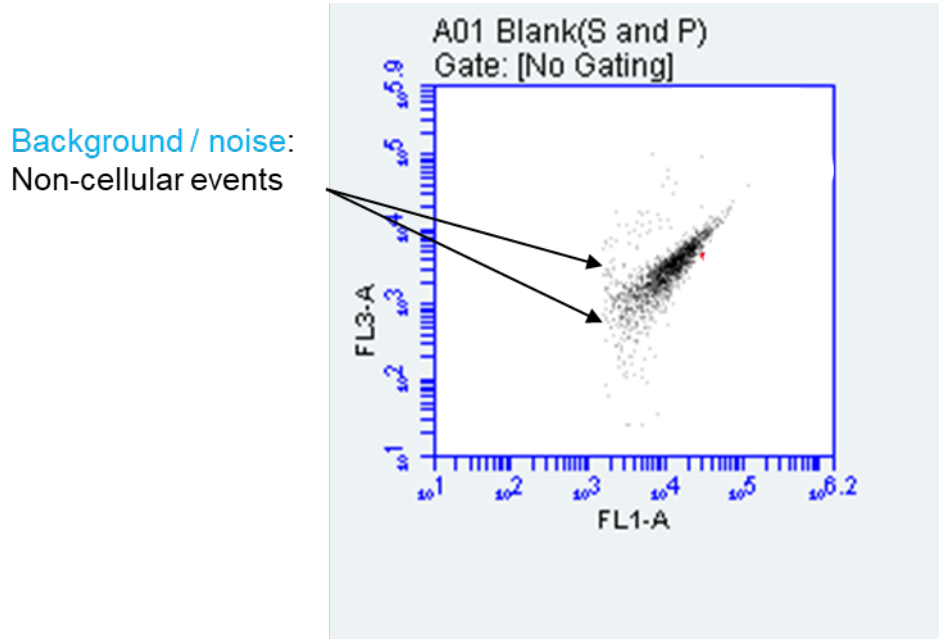


Figure 7. Cytograph of a blank (using sterile-saline instead of cheese) stained with SYBR Green and propidium iodide showing that even when using a threshold of 2,000 there are still some non-cellular events that are recorded as noise.

Differentiation of Live, Live- semi permeable and Dead cells

The staining of the samples with SG and PI enabled differentiation of three main populations: live, live – semi permeable (LSP), and dead cells in cheese (Figure 8). SG stained total (live and dead) bacterial population irrespective of membrane damage or non-culturable nature of bacterial cells (Figure 8a), whereas PI stained severely damaged and dead cells (Singh et al., 2019) (Figure 8b). The optical filters were set up such that red fluorescence (from PI) was measured above 630 nm (FL3) and green fluorescence (from SG) was measured at 520 nm (FL1). In figure 8a, when using only SG we can see that most fluorescence was detected by detector FL1 with some fluorescence picked up by detector FL3. It was observed that the fluorescence was at about 45 from the axis and

considered total bacterial cells including noise (non- cellular events). After simultaneously staining with SG and PI (Figure 8b), we could still see the same sample pattern, however two new populations of events could be observed with low FL1 and high FL3 fluorescence, , indicating not only that PI has entered dead or damaged cells but also that SG fluorescence has been lowered because of energy transfer to PI. The other population has similar FL1 signal as the normal live cells and also an increased FL3 signal that is presumed to be coming from some PI being able to permeate into these cells. Hence, they have been designated as live semipermeable (Sheehan et al., 2005).

It was observed that after the heat treatment at 85°C for 12 min, the concentration of dead cells increased, and the live cell population disappeared (Fig 9b). Thus, we were able to locate the dead cell population along with LSP and live cells (Fig 9a). Correctly setting the discriminator gates to select bacterial counts from background signals is a critical task and could be significant with food samples containing an unknown or variable microbiota. (Comas-Riu and Rius, 2009).

Selection of SYBR Green

Cytographs obtained using the five nucleotide fluorescent dyes (SG, Syto 9, Syto 24, carboxy fluorescein diacetate (cFDA), Thiazole orange) in combination with PI are shown in Figure 10. It was observed that only SG and Syto 24 dyes gave a distinct dead population while other dyes (cFDA, TO, Syto 9) were not able to differentiate live and dead population. In this study, SG was preferred over Syto 24 because of its consistent results and reproducibility.

Microbial number at 6 day storage.

Plant Counts. Based upon salt level added to the curd, there was no significant difference (P>0.572) in plate count numbers in replicates of any strains (Table 6 and 7). The salt tolerance

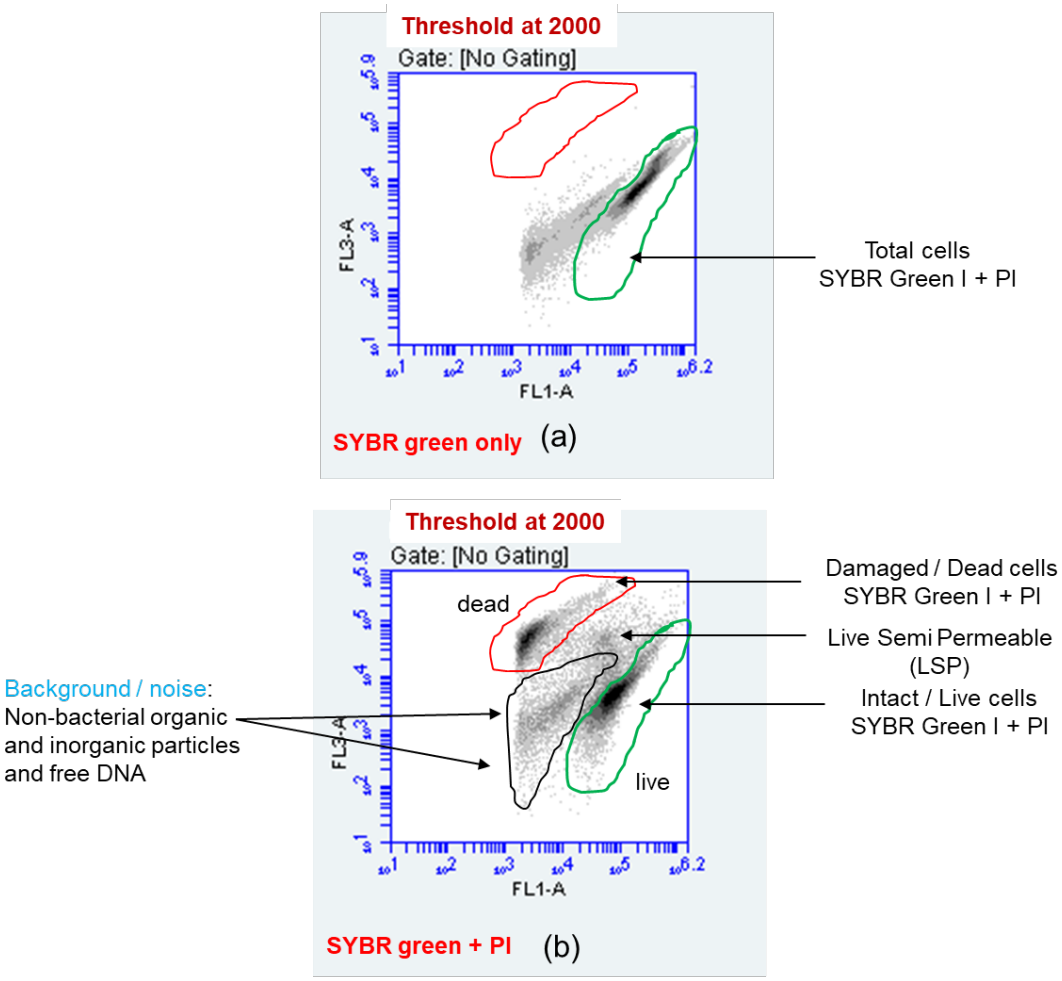


Figure 8. (a) Flow cytometric dot plot fluorescence of SYBR green only representing total cells irrespective of the status of viability. (b) Flow cytometric dot plot of *Lactococcus lactis* subsp. starter culture representing live, live-semi permeable and dead cells in standard salt cheese treated with SYBR green in combination with PI.

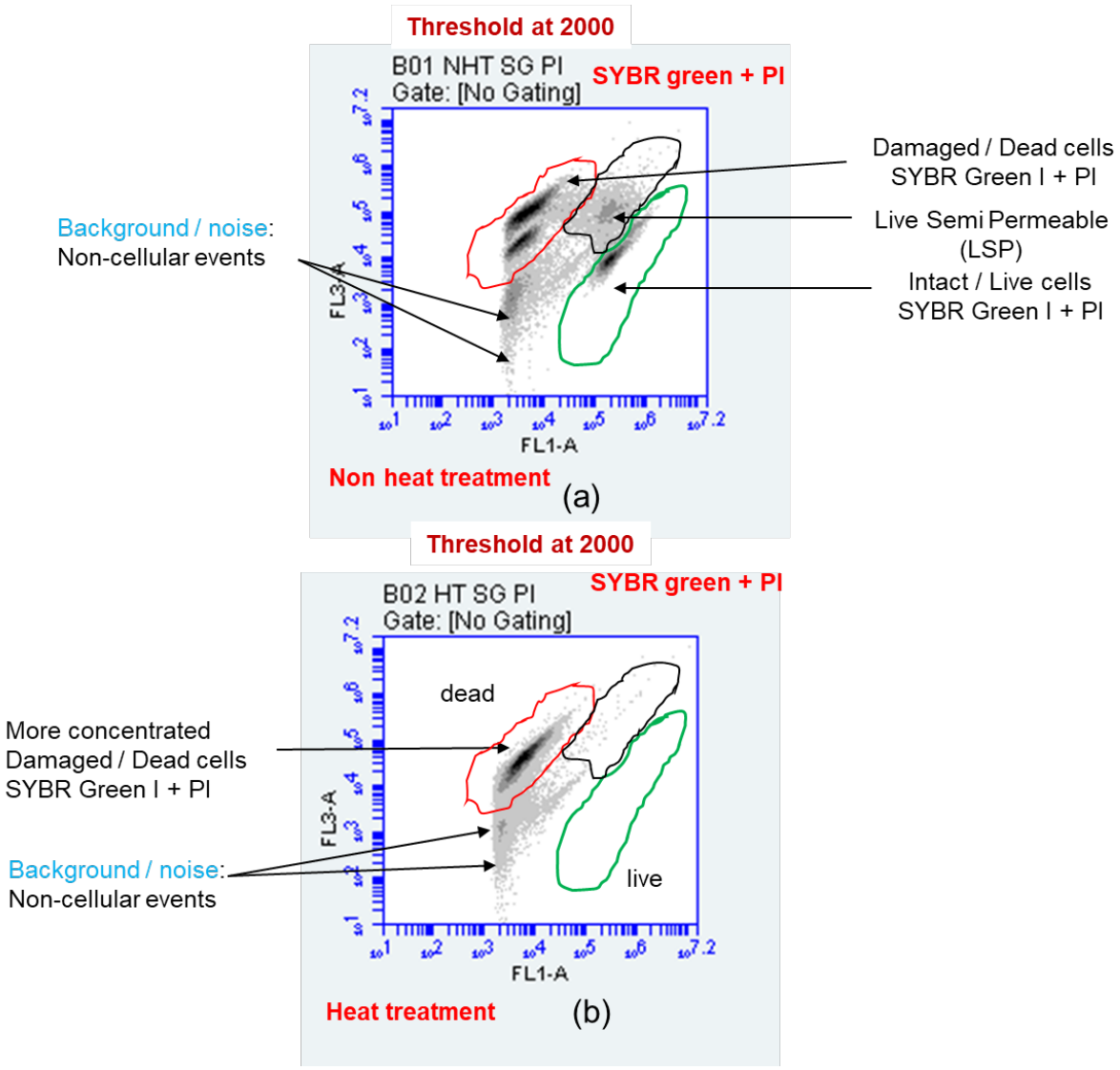


Figure 9. Flow cytometric dot plot fluorescence of SYBR green in combination with PI. Ultra-high temperature sterile milk was inoculated with a mixed strain lactococcal starter culture and incubated overnight at 37°C. (a) The clusters of cells representing live, live-semi permeable, and dead cells can be seen. (b) A negative control containing only dead cells was obtained by heating the cultured milk at 85°C for 12 min. FL1 represents the green fluorescence while FL3 represents red fluorescence.

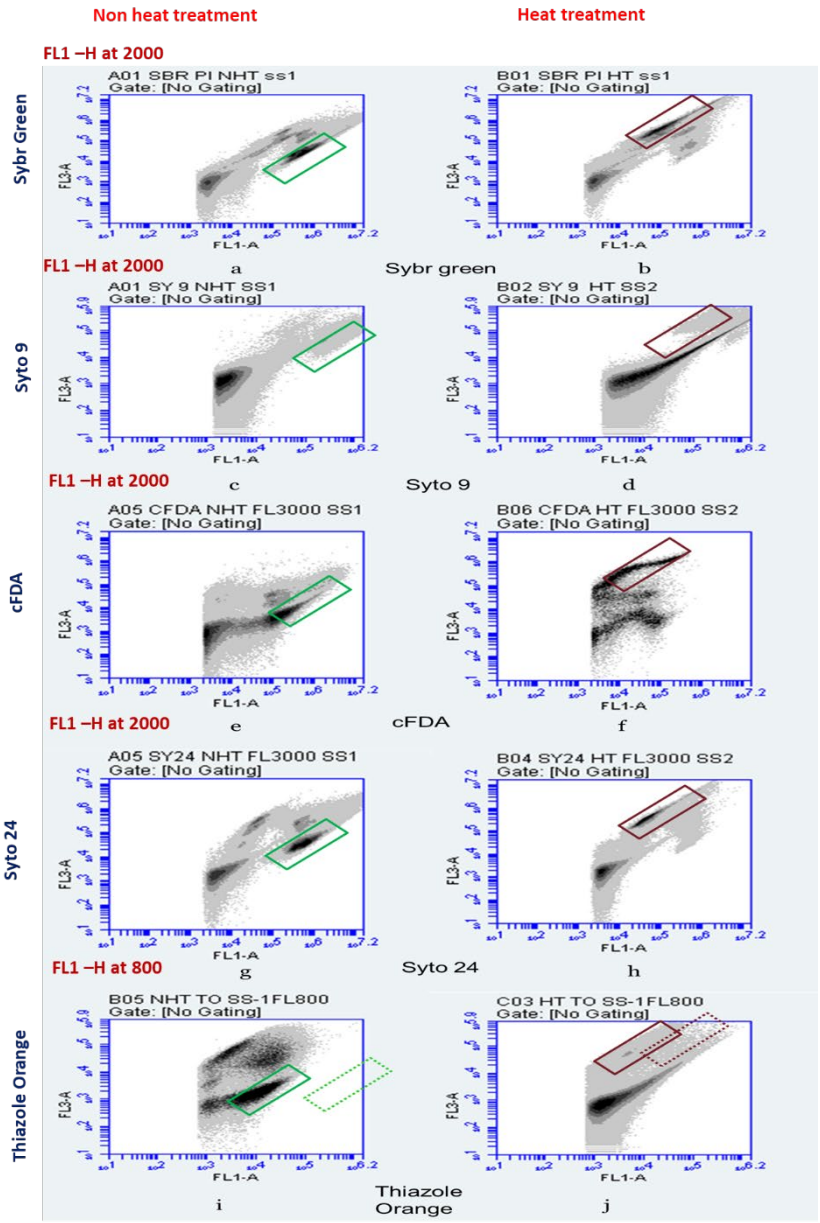


Figure 10. Comparison of flow cytometric dot plot fluorescence of five different dyes (Sybr Green, Syto 9, Syto 24, carboxy fluorescein diacetate (cFDA) or Thiazole Orange) in combination with PI. Ultra-high temperature sterile milk was inoculated with a mixed strain lactococcal starter culture and incubated overnight at 37°C. A negative control containing only dead cells was obtained by heating the cultured milk at 85°C for 12 min. FL1 represents the green fluorescence while FL3 represents red fluorescence. The clusters of cells representing the total cell counts can be seen in Figures II a, c, e, g, and i. The intact cells are assumed to be the cells gated with green box while the dead cells are assumed to be the cells gated with red box in Figures II b, d, f, and h. Red and green dotted box in Figure II i and j represent the position of dead and live cells that would occur with Sybr green and PI.

Table 6. Analysis of variance for plate count number at 6 d storage showing P-value**.**

Source	DF	Sum of Squares	Mean Square	Error DF	F-Value	Pr > F
S/M	2	0.058344	0.02917	18	0.58	0.572
Strains	3	7.867178	2.62239	18	51.8	<.0001
Residual	18	0.911179	0.05062			

Table 7. Analysis of variance for plate count number after multiplying with average chain length at 6 d storage showing P-value.

Source	DF	Sum of Squares	Mean Square	Error DF	F-Value	Pr > F
S/M	2	0.06	0.03	18	0.58	0.572
Strains	3	0.68	0.23	18	4.51	0.0159
Residual	18	0.91	0.05			

of modern starter culture strains used in the manufacture of cheese is greater compared to that reported before the advent of pH-controlled starter culture preparation (McMahon et al 2014).

There was significant difference in plate count number between individual *Lactococcus lactis* subsp. strains ($P < 0.05$) as shown in Table 6. Also, when cell numbers were multiplied by average chain length (~2 to 3 for *lactis*, and ~16 for *cremoris*), there was significant difference between the individual *Lactococcus lactis* subsp. ($P < 0.05$) (Tables 7). The biggest difference between replicates was for *L. lactis* subsp. *cremoris* B36 with SE = 0.16 (Table 8). This could be because replicate 2 of *Lactococcus lactis* subsp. *cremoris* B36 remained above the temperature range during cooking. After pooling over S/M levels the difference in cell numbers in cheese was

minimal (Table 8). In contrast, when compared based on CFU/g the cheese made *L. lactis* subsp. *cremoris* strains the starter culture was thought to be at log lower level than cheese made using *L. lactis* subsp. *lactis* strains was minimal.

Table 8. *Lactococcus lactis* cell numbers in 6-d cheese calculated as CFU/g multiplied by average chain length (see Table 3) as a function of subspecies strain and salt-in-moisure (S/M) level.

			Bacterial Numbers		
		Nominal		Pooled	
Subspecies	**Strains**	**S/M**		**Mean**	**SE**
		(%)	---------log10 cells/g-------		
		3.5	9.4		
lactis	600M1	4.5	9.2	9.30[a]	0.06
		5.5	9.3		
		3.5	9.4		
lactis	E36	4.5	9.3	9.37[a]	0.05
		5.5	9.4		
		3.5	8.9		
cremoris	B36	4.5	8.9	8.94[a]	0.16
		5.5	9.0		
		3.5	9.1		
cremoris	G61	4.5	9.1	9.12[a]	0.04
		5.5	9.1		

[a]Pooled means with the same superscript letter were not significantly different.

Flow Cytometry

Three main sub populations showing live, dead, and live- semi permeable cells can be differentiated by flow cytometry (Figure 10). When bacterial numbers were counted using flow cytometry, significant difference between individual *L. lactis* strains ($P < 0.0001$) could be observed but no significant differences were observed based upon salt content of the cheese for any cells (live, live-SP, and dead cells) (Table 9). Therefore, mean cell numbers were pooled over salt level (Table 10). This supports the

lack of salt sensitivity of these starter culture strains observed using tradiational plate counting. All of the cheese samples contained ~5% dead cells. Of the cells that were considered to be living based upon their high SG fluorescence, ~5% of total cells of the *lactis* strains were considered to be live- semi permeable (because of their increased PI fluorescence), and ~15% of the *cremoris* strains (Table 10). This may show an increased likelihood of cells of *cremoris* strains to become more permeable or it may just be an individual strain property. More strains would need to be studied to make a conclusion of it being general subspecies property.

Comparing the plate count number (CFU /g) and live FC number (events/g) after 6 d storage, it can be observed that the live cell count numbers are half log higher than plate count numbers for *L. lactis subsp*. *lactis* 600M1 and *L. lactis subsp*. *cremoris* G61 and one log higher for *L. lactis subsp*. *cremoris* B36 while both the numbers (PC and FC) are similar for *L. lactis subsp*. *lactis* E36. Flow cytometry numbers were higher than the plate count number for all strains at d6 storage. This could be because FC counts all the bacterial cells irrespective of membrane status of the cells while plate count numbers only counts cells that are intact (live cell population).

Based upon FC, when cheese was made using the *lactis* strains the initial bacterial load in the cheese was four times higher than when using *cremoris* strains, i.e., 2 x 10^9 cells compared to plate count numbers. When comparing statistical data for live cells, it was observed that *L. lactis subsp*. *lactis* 600M1 was significantly different from *L. lactis subsp*. *cremoris* B36 and *L. lactis subsp*. *cremoris* G61 (Table 11). For LSP cells, there was no significant difference between any of the strains except that *L. lactis subsp*. *lactis* 600M1 and *L. lactis subsp*. *lactis* E36 had significant difference. Moreover, for dead

cells, there was no significant difference between *L. lactis subsp*. *cremoris* B36 and *L. lactis subsp*. *lactis* E36.

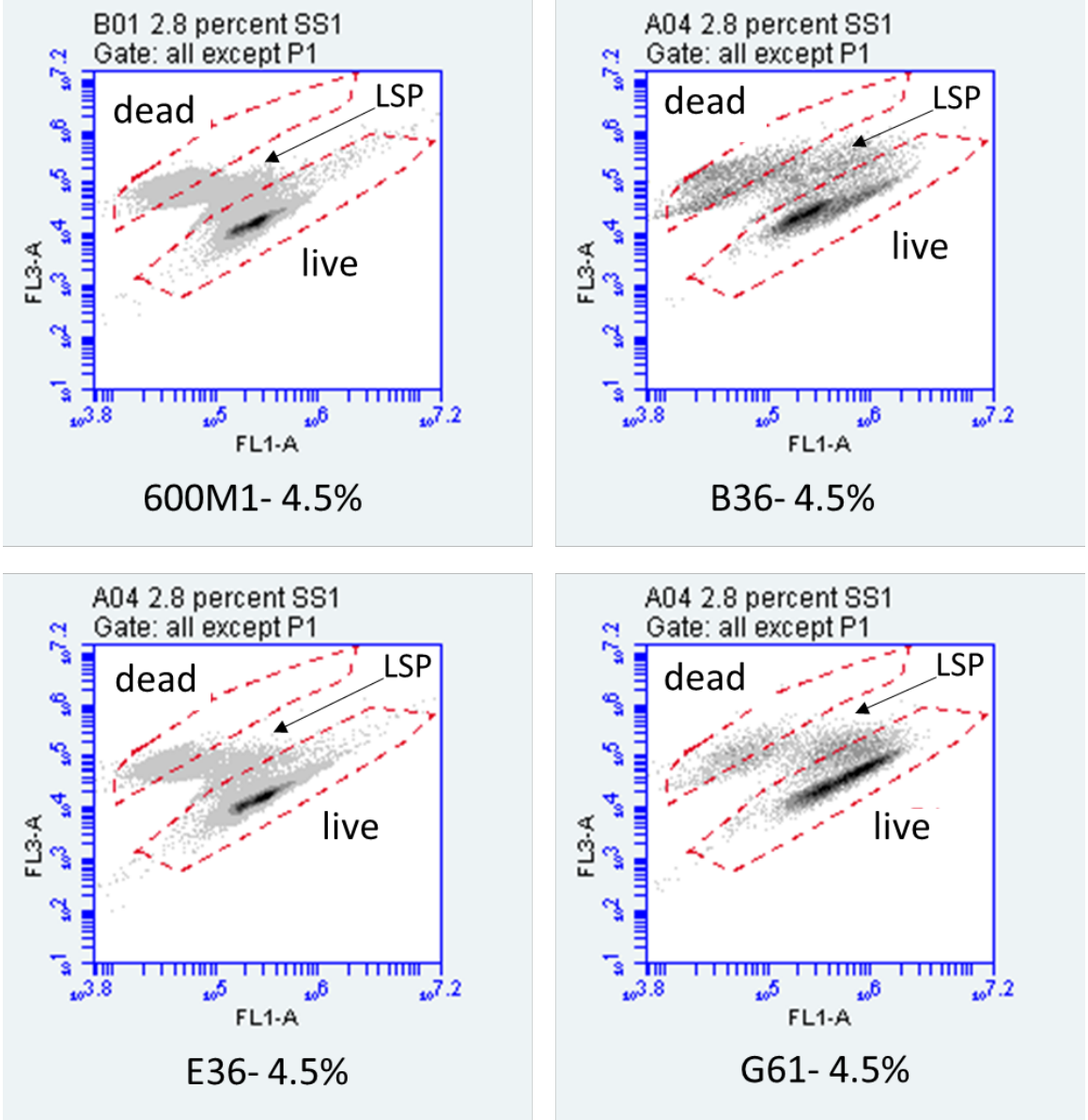


Figure 11. Flow cytometric dot plot of *Lactococcus lactis* subsp. *lactis* (600M1, E36) and *Lactococcus lactis* subsp. *cremoris* (B36, G61) at 4.5% S/M, representing live, live-semi permeable, and dead cells.

Table 9. Analysis of variance for live, live semi permeable and dead cell count number (FC) in cheese made using individual strain starter cultures and with different salt-in-moisture (S/M) levels after 6d storage at 6°C.

Cell Category	Source of Variation	DF	Sum of Squares	Mean Square	F-value	Pr>F
Live - normal cells	S/M	2	0.0233	0.0117	0.66	0.528
	Strains	3	2.5022	0.8341	47.23	<0.0001
	Residual	18	0.3178	0.0177		
Live- SP cells	S/M	2	0.2385	0.1193	2.09	0.1531
	Strains	3	0.5955	0.1985	3.47	0.0378
	Residual	18	1.0286	0.0572		
Dead cells	S/M	2	0.0598	0.0299	0.67	0.5245
	Strains	3	1.9529	0.6510	14.56	<0.0001
	Residual	18	0.8048	0.0441		

Table 10. Percentage of cells in cheese made using single *Lactococcus lactis* subsp. *lactis* strains (600M1 and E36) and *L. lactis* subsp. *cremoris* strains (E36 and G61) after 6 d of storage, measured using flow cytometry that were categorized as live-normal cells, live-semi permeable (Live-SP) cells and dead cells, pooled over salt levels.

Strain	Subspecies	Live-normal Cells	Live-SP Cells	Dead Cells
		----------%----------		
600M1	*lactis*	91	5	4
E36	*lactis*	96	3	3
B36	*cremoris*	78	16	6
G61	*cremoris*	87	13	4

Table 11. Mean cell count numbers in cheese made using single single *Lactococcus lactis* subsp. *lactis* strains (600M1 and E36) and *L. lactis* subsp. *cremoris* strains (E36 and G61) after 6 d storage at 6°C categorized using flow cytometry as live-normal, live - semi permeable (Live-SP) and dead cells.

	Bacterial Cell Numbers		
Strains	**Live-Normal cells**	**Live-SP cells**	**Dead cells**
	----------$\log_{10}$ cells/g----------		
600M1	9.35[a]	8.16[a]	8.08[a]
E36	9.24[a]	7.75[ab]	7.77[a]
B36	8.66[b]	8.06[ab]	7.53[ab]
G61	8.65[b]	7.90[b]	7.32[b]

[ab]Means within a column with the same superscript letter were not significantly different.

CONCLUSION

The two *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains were different from each other in terms of acid production, increase in cell numbers during cheese manufacture and cheese make time. During cheese manufacture, *L. lactis* subsp. *lactis* 600M1 and *L. lactis* subsp. *cremoris* G61 strains had the same rate of acid production and behaved similarly during entire cheese make time while *L. lactis* subsp. *cremoris* B36 and *L. lactis* subsp. *lactis* E36 had longer cheese make time. Therefore, acid production rates during cheesemaking (with cook temperature of 38°C) was more dependent on the individual strain than whether it was a *lactis* or *cremoris* strain.

When average chain length was taken into account (~2 to 3 for *L. lactis* subsp. *lactis* strains, and ~16 for *L. lactis* subsp. *cremoris* strains), the plate count numbers were comparable to the FC cell numbers. In both the cases, the variation in cell count numbers between the strains were minimal. Therefore, chain length should be considered when comparing starter culture numbers in cheese when using plate and not just infer differences based upon CFU numbers.

When making cheddar cheese with individual strains of lactococci, the cheese made with *L. lactis* subsp. *lactis* strains contained ~4 times (~ 0.5 log) more bacterial cells than those made using *L. lactis* subsp. *cremoris* strains as shown by both plate counting and flow cytometry. *Lactococcus lactis* subsp. *cremoris* was most influenced by salting with pH after salting only dropping about 0.1 pH units while a pH drop of 0.2 to 0.4 units was more typical of the other strains. The amount of salt added (at least in the range of cheese S/M of 3.5 to 5.5%) had little in any influence on continued fermentation of lactose by the

starter cultures during overnight pressing and 6 d of storage. Higher pH in cheeses with higher salting levels was attributed to the higher salt cheeses having up to 20 g/kg less moisture content (and hence contained less lactose and had higher buffering capacity) rather than any inhibitory effect of higher S/M levels on the starter cultures.

Plate counting only gives information about live bacterial population and does not fully describe the fate and activity of starter culture during storage. Flow cytometry on the other hand, provides additional information about different cell states including metabolically active but non-culturable cells, and further aids in understanding the role of starter cultures in cheese aging and flavor development.

Based on flow cytometry, ~5% of the total starter culture cells in the cheese were dead after 6 d of storage. Another 5 to 15% of the cells were designated as being alive but semi-permeable, with *L. lactis* subsp. *cremoris* strains having the higher number of such cells. Further work is needed to determine if the number of these live-semi permeable cells increases during storage of the cheese, and to determine their relevance to development of cheddar cheese flavor.

REFERENCES

Álvarez-Barrientos, A., J. Arroyo, R. Cantón, C. Nombela and M. Sánchez-Pérez. 2000. Applications of flow cytometry to clinical microbiology. Clinical Microbiol. Rev. 13:167-195. https//:doi.org/10.1128/CMR.13.2.167

Berney, M., F. Hammes, F. Bosshard, H.U. Weilenmann and T. Egli. 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl. Environ. Microbiol. 73:3283-3290. https//:doi.org/10.1128/AEM.02750-06

Bunthof, C. J. and T. Abee. 2002. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. Appl. Environ. Microbiol. 68:2934-2942. https//:doi.org/10.1128/AEM.68.6.2934-2942.2002

Boulos, L., M. Prevost, B. Barbeau, J. Coallier and R. Desjardins. 1999. LIVE/DEAD® BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37:77-86. https://doi.org/10.1016/S0167-7012(99)00048-2

Castillo-Hair, S. M., Sexton, J. T., Landry, B. P., Olson, E. J., Igoshin, O. A., & Tabor, J. J. 2016. FlowCal: a user-friendly, open source software tool for automatically converting flow cytometry data from arbitrary to calibrated units. ACS synthetic biology, 5:774-780. https://doi.org/10.1021/acssynbio.5b00284

Cerca, F., Trigo, G., Correia, A., Cerca, N., Azeredo, J., & Vilanova, M. 2011. SYBR green as a fluorescent probe to evaluate the biofilm physiological state of *Staphylococcus epidermidis*, using flow cytometry. Canadian journal of microbiology, 57:850-856. https://doi.org/10.1139/w11-078

Comas-Riu, J., & Rius, N. 2009. Flow cytometry applications in the food industry. Journal of industrial microbiology and biotechnology, 36:999-1011. https://doi.org/10.1007/s10295-009-0608-x

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., & Cocolin, L. 2008. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. International journal of food microbiology, 122:302-311. https://doi.org/10.1016/j.ijfoodmicro.2007.12.018

Doolan, I. A., Nongonierma, A. B., Kilcawley, K. N., & Wilkinson, M. G. 2014. Partitioning of starter bacteria and added exogenous enzyme activities between curd and whey during Cheddar cheese manufacture. International Dairy Journal, 34:159-166.

Fenelon, M. A., O'Connor, P., & Guinee, T. P. 2000. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. J. Dairy Sci. 83:2173–2183. https://doi.org/10.3168/JDS.S0022-0302(00)75100-9

Feng, J., Wang, T., Zhang, S., Shi, W., & Zhang, Y. 2014. An optimized SYBR Green I/PI assay for rapid viability assessment and antibiotic susceptibility testing for Borrelia burgdorferi. PloS one, 9:e111809. https://doi.org/10.1371/journal.pone.0111809

Fernández, E., Alegría, Á., Delgado, S., Martín, M. C., & Mayo, B. 2011. Comparative phenotypic and molecular genetic profiling of wild *Lactococcus lactis* subsp. *lactis* strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* genotypes, isolated from starter-free cheeses made of raw milk. Appl. Environ. Microbiol., 77:5324-5335. https://doi.org/10.1128/AEM.02991-10

Franco-Duarte, R., Černáková, L., Kadam, S., S Kaushik, K., Salehi, B., Bevilacqua, A., ... & Relison Tintino, S. 2019. Advances in Chemical and Biological Methods to Identify Microorganisms—From Past to Present. Microorganisms 7:130. https://doi.org/10.3390/microorganisms7050130

Fucà, N., McMahon, D. J., Caccamo, M., Tuminello, L., La Terra, S., Manenti, M., & Licitra, G. 2012. Effect of brine composition and brining temperature on cheese physical properties in Ragusano cheese. Journal of dairy science, 95:460-470. https://doi.org/10.3168/jds.2011-4438

Ganesan, B., Weimer, B. C., Pinzon, J., Dao Kong, N., Rompato, G., Brothersen, C., & McMahon, D. J. 2014. Probiotic bacteria survive in Cheddar cheese and modify populations of other lactic acid bacteria. Journal of Applied Microbiology, 116:1642–1656. https://doi.org/10.1111/jam.12482

Ganesan, B., Stuart, M. R., & Weimer, B. C. 2007. Carbohydrate starvation causes a metabolically active but nonculturable state in *Lactococcus lactis*. Appl. Environ. Microbiol., 73:2498-2512. https://doi.org/10.1128/AEM.01832-06

Grégori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S., & Denis, M. 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. Appl. Environ. Microbiol., 67:4662-4670. https://doi.org/10.1128/AEM.67.10.4662-4670.2001

Guinee, T. P. 2004. Salting and the role of salt in cheese. International Journal of Dairy Technology, 57:99-109. https://doi.org/10.1111/j.1471-0307.2004.00145.x

Hall, T., Biosciences, I., & Carlsbad, C. 2011. BioEdit: an important software for molecular biology. GERF Bull Biosci, 2:60-61.

Hickey, C. D., Auty, M. A. E., Wilkinson, M. G., & Sheehan, J. J. 2015. The influence of cheese manufacture parameters on cheese microstructure, microbial localisation and their interactions during ripening: A review. Trends in food science & technology, 41:135-148. https://doi.org/10.1016/j.tifs.2014.10.006

Hicks, C. L., & Ibrahim, S. A. 1992. Lactic bulk starter homogenization affects culture agglutination. Journal of food science, 57:1086-1092. https://doi.org/10.1111/j.1365-2621.1992.tb11270.x

Hicks, C. L., Morales, M. M., & Russell-Campbell, E. 1998. Use of agglutination-sensitive bulk starters in the manufacture of stirred curd cheese. Journal of dairy science, 81:621-630. https://doi.org/10.3168/jds.S0022-0302(98)75616-4

Jellett, J. F., Li, W. K., Dickie, P. M., Boraie, A., & Kepkay, P. E. 1996. Metabolic activity of bacterioplankton communities assessed by flow cytometry and single carbon substrate utilization. Marine Ecology Progress Series, 136:213-225. https://doi.org/10.3354/meps136213

Johnson, M. E. (2017). A 100-Year Review: Cheese production and quality. Journal of Dairy Science, 100:9952-9965. https://doi.org/10.3168/jds.2017-12979

Kelly, W. J., Ward, L. J. H., & Leahy, S. C. 2010. Chromosomal diversity in *Lactococcus lactis* and the origin of dairy starter cultures. Genome Biology and Evolution, 2:729–744. https://doi.org/10.1093/gbe/evq056

Kim, W. S., Ren, J., & Dunn, N. W. 1999. Differentiation of *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains by their adaptive response to

stresses. FEMS Microbiology Letters, 171:57-65. https://doi.org/10.1111/j.1574-6968.1999.tb13412.x

Leroy, F., & De Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology, 15:67-78. https://doi.org/10.1016/j.tifs.2003.09.004

Lu, Y., & McMahon, D. J. 2015. Effects of sodium chloride salting and substitution with potassium chloride on whey expulsion of Cheddar cheese. Journal of dairy science, 98:78-88. https://doi.org/10.3168/jds.2014-8600

McMahon, D. J., Oberg, C. J., Drake, M. A., Farkye, N., Moyes, L. V., Arnold, M. R., … Broadbent, J. R. 2014. Effect of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full-fat Cheddar cheese. Journal of Dairy Science, 97:4780–4798. https://doi.org/10.3168/JDS.2014-8071

Mills, S., Ross, R. P., & Coffey, A. 2011. Lactic acid bacteria *Lactococcus lactis*.

Nebe-von-Caron, G., Stephens, P. J., Hewitt, C. J., Powell, J. R., & Badley, R. A. 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. Journal of microbiological methods, 42:97-114. https://doi.org/10.1016/S0167-7012(00)00181-0

Nunez, R. 2001. Flow Cytometry: Principles and Instrumentation.

Oberg, C. J., Moyes, L. V., Domek, M. J., Brothersen, C., & McMahon, D. J. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. Journal of Dairy Science, 94(5), 2220–2230. https://doi.org/10.3168/JDS.2010-3934

Oberg, C. J., Moyes, L. V., Domek, M. J., Brothersen, C., & McMahon, D. J. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. Journal of dairy science, 94:2220-2230. https://doi.org/10.3168/jds.2010-3934

Oliver, J. D. 2005. The viable but nonculturable state in bacteria. The journal of microbiology, 43:93-100.

Pienaar, J. A., Singh, A., & Barnard, T. G. 2016. The viable but non-culturable state in pathogenic Escherichia coli: A general review. African journal of laboratory medicine, 5:1-9. http://dx.doi.org/10.4102/ajlm.v5i1.368

Rademaker, J. L. W., Herbet, H., Starrenburg, M. J. C., Naser, S. M., Gevers, D., Kelly, W. J., … van Hylckama Vlieg, J. E. T. 2007. Diversity analysis of dairy and nondairy *Lactococcus lactis* isolates, using a novel multilocus sequence analysis scheme and (GTG)5-PCR fingerprinting. Applied and Environmental Microbiology, 73:7128–7137. https://doi.org/10.1128/AEM.01017-07

Reddy, M. S., Vedamuthu, E. R., Washam, C. J., & Reinbold, G. W. 1972. Agar medium for differential enumeration of lactic streptococci. Applied Microbiology, 24:947-952.

Rose, J. B., & Grimes, D. J. 2001. Reevaluation of microbial water quality: powerful new tools for detection and risk assessment. American Society for Microbiology Washington DC.

Ruggirello, M., Cocolin, L., & Dolci, P. 2016. Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models. Food microbiology, 59:112-118. https://doi.org/10.1016/j.fm.2016.05.001

Ruggirello, M., Dolci, P., & Cocolin, L. 2014. Detection and viability of *Lactococcus lactis* throughout cheese ripening. PLoS One, 9:e114280. https://doi.org/10.1371/journal.pone.0114280

Ryssel, M. 2010. Investigation of the physiology of genetically modified strains of *Lactococcus lactis*, and their potential for accelerated ripening of cheese. Department of Food Science, University of Copenhagen.

Schleifer, K. H., Kraus, J., Dvorak, C., Kilpper-Bälz, R., Collins, M. D., & Fischer, W. 1985. Transfer of *Streptococcus lactis* and Related Streptococci to the Genus Lactococcus gen. nov. Systematic and Applied Microbiology, 6:183–195. https://doi.org/10.1016/S0723-2020(85)80052-7

Singh, T. K., Drake, M. A., & Cadwallader, K. R. 2003. Flavor of Cheddar Cheese: A Chemical and Sensory Perspective. Comprehensive Reviews in Food Science and Food Safety, 2:166–189. https://doi.org/10.1111/j.1541-4337.2003.tb00021.x

Singh, S. K., Ahmed, S. U., & Pandey, A. 2006. Metabolic engineering approaches for lactic acid production. Process Biochemistry, 41:991-1000. https://doi.org/10.1016/j.procbio.2005.12.004

Shapiro, H. M. 2005. Practical flow cytometry. John Wiley & Sons.

Sheehan, A., O'loughlin, C., O'Cuinn, G., FitzGerald, R. J., & Wilkinson, M. G. 2005. Cheddar cheese cooking temperature induces differential lactococcal cell permeabilization and autolytic responses as detected by flow cytometry: implications for intracellular enzyme accessibility. Journal of Applied Microbiology, 99:1007-1018. https://doi.org/10.1111/j.1365-2672.2005.02718.x

Stuart, M. R., Chou, L. S., & Weimer, B. C. 1999. Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *lactococcus lactis* subsp. *lactis*. Applied and Environmental Microbiology, 65:665–673. https://doi.org/10.1128/AEM.65.2.665-673.1999

Sutherland, B. J. 1977. Some observations on salt and moisture concentration gradients in Cheddar cheese. Australian Journal of Dairy Technology (Australia). 32:17-18 http://hdl.handle.net/102.100.100/302546?index=1

Teuber M. Geis A. Neve H. 1992. The genus Lactococcus. In: The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (Balows A., Ed.), 2nd edn., pp. 1482–1501. Springer-Verlag, New York.

Teuber, M. 1995. The genus Lactococcus. In The genera of lactic acid bacteria (pp. 173-234). Springer, Boston, MA.

Thomas, T. D., & Pearce, K. N. 1981. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. New Zealand Journal of Dairy Science and Technology.

Thompson, J. 1987. Regulation of sugar transport and metabolism in lactic acid bacteria. FEMS Microbiology Reviews, 3:221-231. https://doi.org/10.1111/j.1574-6968.1987.tb02462.x

Van Hylckama Vlieg, J. E., Rademaker, J. L., Bachmann, H., Molenaar, D., Kelly, W. J., & Siezen, R. J. 2006. Natural diversity and adaptive responses of *Lactococcus*

lactis. Current Opinion in Biotechnology, 17:183–190. https://doi.org/10.1016/J.COPBIO.2006.02.007

Wilkinson, M. G. 2018. Flow cytometry as a potential method of measuring bacterial viability in probiotic products: a review. http://hdl.handle.net/10344/6870

Yun, J. J., Barbano, D. M., & Kindstedt, P. S. 1993. Mozzarella cheese: impact of milling pH on chemical composition and proteolysis. Journal of dairy science, 76:3629-3638. https://doi.org/10.3168/jds.S0022-0302(93)77704-8

Zhang, T., & Fang, H. H. 2004. Quantification of Saccharomyces cerevisiae viability using BacLight. Biotechnology letters, 26:989-992. https://doi.org/10.1023/B:BILE.0000030045.16713.19

APPENDICES

APPENDIX A. CHEESE MAKE RECORDS

General Cheese Make Sheet

Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65			
Actual								

Cultures Used

Strain	Type	Activity	Date Made
A62	L. lactis ssp. cremoris		
600M-1	L. lactis ssp. lactis		

Rennet Used

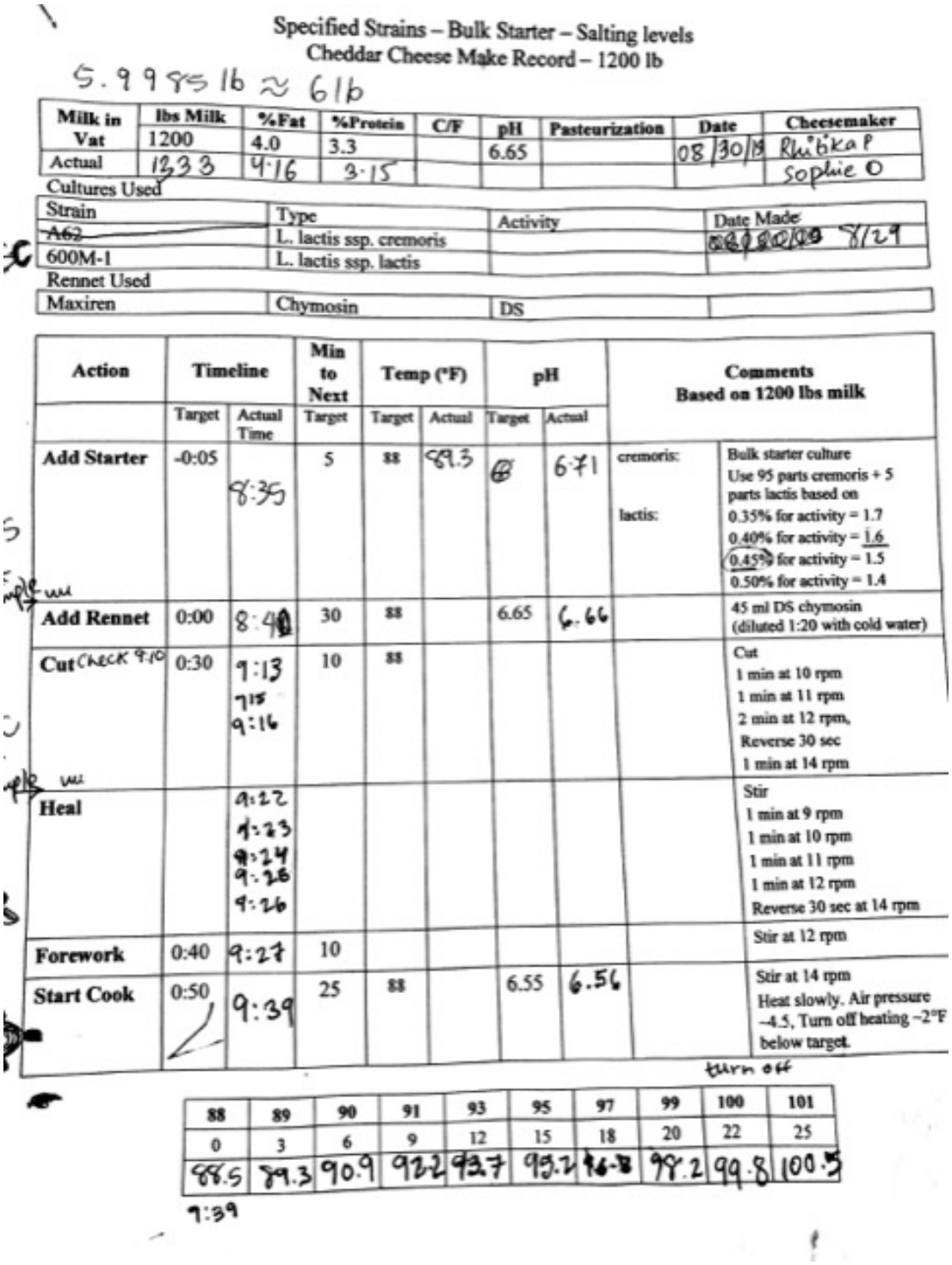
Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (ºF)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05		5	88				cremoris: lactis:	Bulk starter culture 0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00		30	88		6.65			45 ml DS chymosin (diluted 1:20 with cold water)
Cut	0:30		10	88					Cut 1 min at 10 rpm 1 min at 11 rpm 2 min at 12 rpm, Reverse 30 sec 1 min at 14 rpm
Heal									Stir 1 min at 9 rpm 1 min at 10 rpm 1 min at 11 rpm 1 min at 12 rpm Reverse 30 sec at 14 rpm
Forework	0:40		10						Stir at 12 rpm
Start Cook	0:50		25	88		6.55			Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25

End Cook	1:15		20	101		6.45			Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35		10			6.40			Warm drain table before pumpover
Start Draining	1:45		5	101		6.30			
Form curd into Pack	1:50		10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00		30	99		6.15			Cut into slabs ~12" wide x 12 long. Turn every 10 minutes
Stack 2 High	2:40		30	96		5.85			Turn every 10 minutes
Stack 3 High	3:10		30	94		5.55			Turn every 10 minutes
Mill	3:40		5	91		5.45			Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, on third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g
Salt 2	4:00		15	88					Add 2.4% salt, 272 g
Salt 3	4:00		15	88					Add 2.8% salt, 318 g
Salt 4	4:00		15	88					Add 3.2% salt, 363 g
Salt 5	4:00		15	88					Add 3.6% salt, 409 g
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15		180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese bloc
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum.package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box an store in back cooler Rm 117 a 42°F.

Lactococcus. lactis subsp. *lactis* Replicate 1.



Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

5.9985 lb ≈ 6lb

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		08/30/18	Rhibka P
Actual	1333	4.16	3.15					Sophie O

Cultures Used

Strain	Type	Activity	Date Made
~~A62~~	L. lactis ssp. cremoris		8/29
600M-1	L. lactis ssp. lactis		

Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (°F)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05	8:35	5	88	89.3	@	6.71	cremoris: lactis:	Bulk starter culture Use 95 parts cremoris + 5 parts lactis based on 0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00	8:40	30	88		6.65	6.66		45 ml DS chymosin (diluted 1:20 with cold water)
Cut CHECK 9:10	0:30	9:13 9:15 9:16	10	88					Cut 1 min at 10 rpm 1 min at 11 rpm 2 min at 12 rpm, Reverse 30 sec 1 min at 14 rpm
Heal		9:22 9:23 9:24 9:25 9:26							Stir 1 min at 9 rpm 1 min at 10 rpm 1 min at 11 rpm 1 min at 12 rpm Reverse 30 sec at 14 rpm
Forework	0:40	9:27	10						Stir at 12 rpm
Start Cook	0:50	9:39	25	88		6.55	6.56		Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

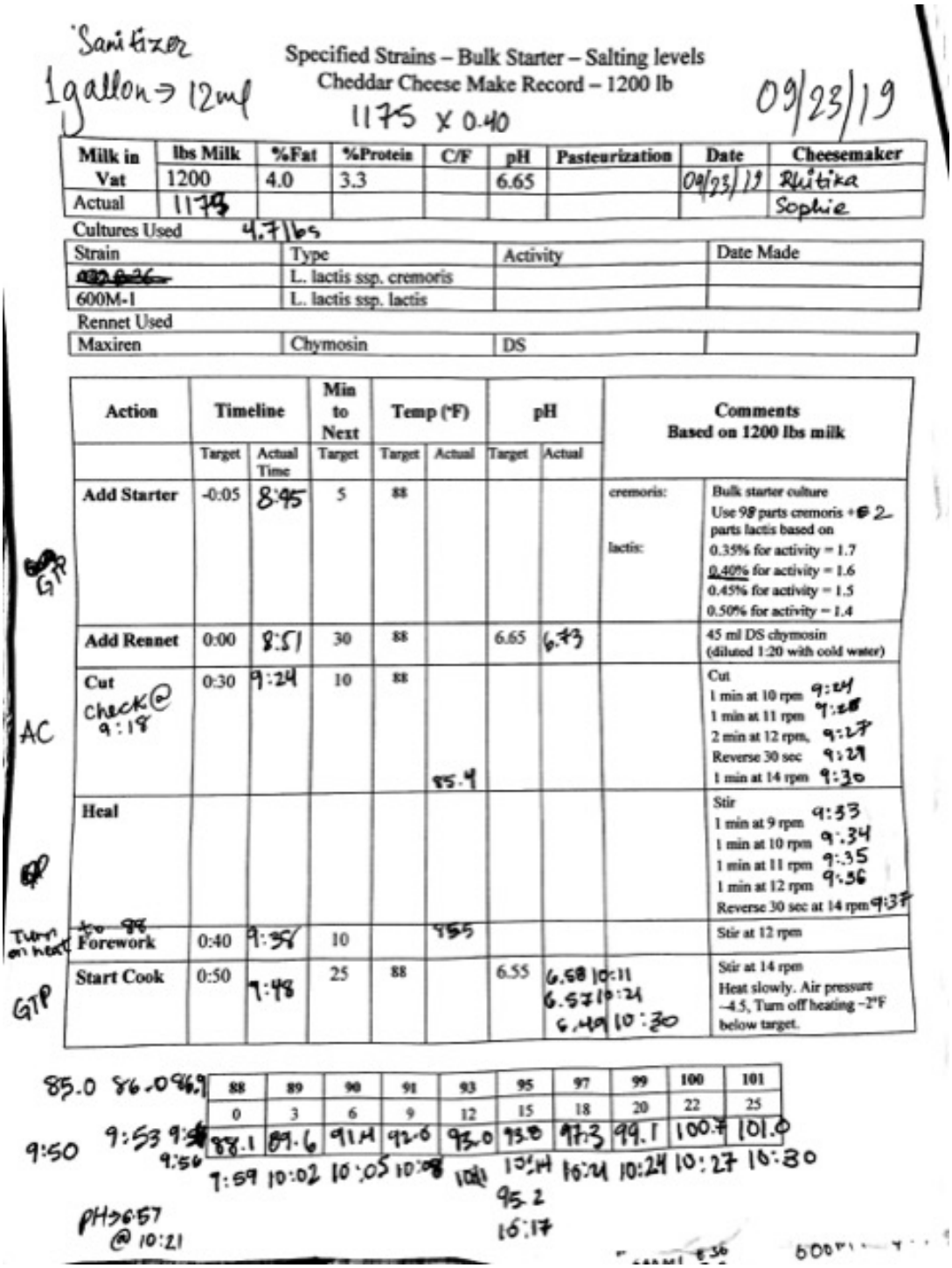
turn off

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
88.5	89.3	90.9	92.2	93.7	95.2	96.8	98.2	99.8	100.5

9:39

End Cook	1:15	10:16	20	101		6.45	6.45		Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35		10			6.40	6.39		Warm drain table before pumpover
Start Draining	1:45		5	101		6.30	6.28		
Form curd into Pack	1:50	10:55	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	11:00	30	99		6.15	6.15		Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	11:23	30	96		5.85	5.86		Turn every 10 minutes
Stack 3 High	3:10		30	94		5.55			Turn every 10 minutes
Mill	3:40		5	91		5.45			Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g
Salt 2	4:00		15	88					Add 2.4% salt, 272 g
Salt 3	4:00		15	88					Add 2.8% salt, 318 g
Salt 4	4:00		15	88					Add 3.2% salt, 363 g
Salt 5	4:00		15	88					Add 3.6% salt, 409 g
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15	4:00	180						Press for 3 hours
Dehoop	8:15	4:00							One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum.package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus. lactis subsp. *lactis* Replicate 2.



Sanitizer
1 gallon → 12ml

Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

1175 X 0.40

09/23/19

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		09/23/19	Rhitika
Actual	1175							Sophie

Cultures Used 4.7 lbs

Strain	Type	Activity	Date Made
~~[illegible]~~	L. lactis ssp. cremoris		
600M-1	L. lactis ssp. lactis		

Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline Target	Timeline Actual Time	Min to Next Target	Temp (°F) Target	Temp (°F) Actual	pH Target	pH Actual	Comments Based on 1200 lbs milk	
Add Starter	-0:05	8:45	5	88				cremoris: lactis:	Bulk starter culture Use 98 parts cremoris + 2 parts lactis based on 0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00	8:51	30	88		6.65	6.73		45 ml DS chymosin (diluted 1:20 with cold water)
Cut check @ 9:18	0:30	9:24	10	88	85.4				Cut 1 min at 10 rpm 9:24 1 min at 11 rpm 9:25 2 min at 12 rpm, 9:27 Reverse 30 sec 9:29 1 min at 14 rpm 9:30
Heal									Stir 1 min at 9 rpm 9:33 1 min at 10 rpm 9:34 1 min at 11 rpm 9:35 1 min at 12 rpm 9:36 Reverse 30 sec at 14 rpm 9:37
Turn on heat ~~to 98~~ **Forework**	0:40	9:58	10		85.5				Stir at 12 rpm
Start Cook	0:50	9:48	25	88		6.55	6.58 10:11 6.57 10:24 6.49 10:30		Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

85.0 86.0 86.9

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
88.1	89.6	91.1	92.0	93.0	93.8	97.3	99.1	100.7	101.0

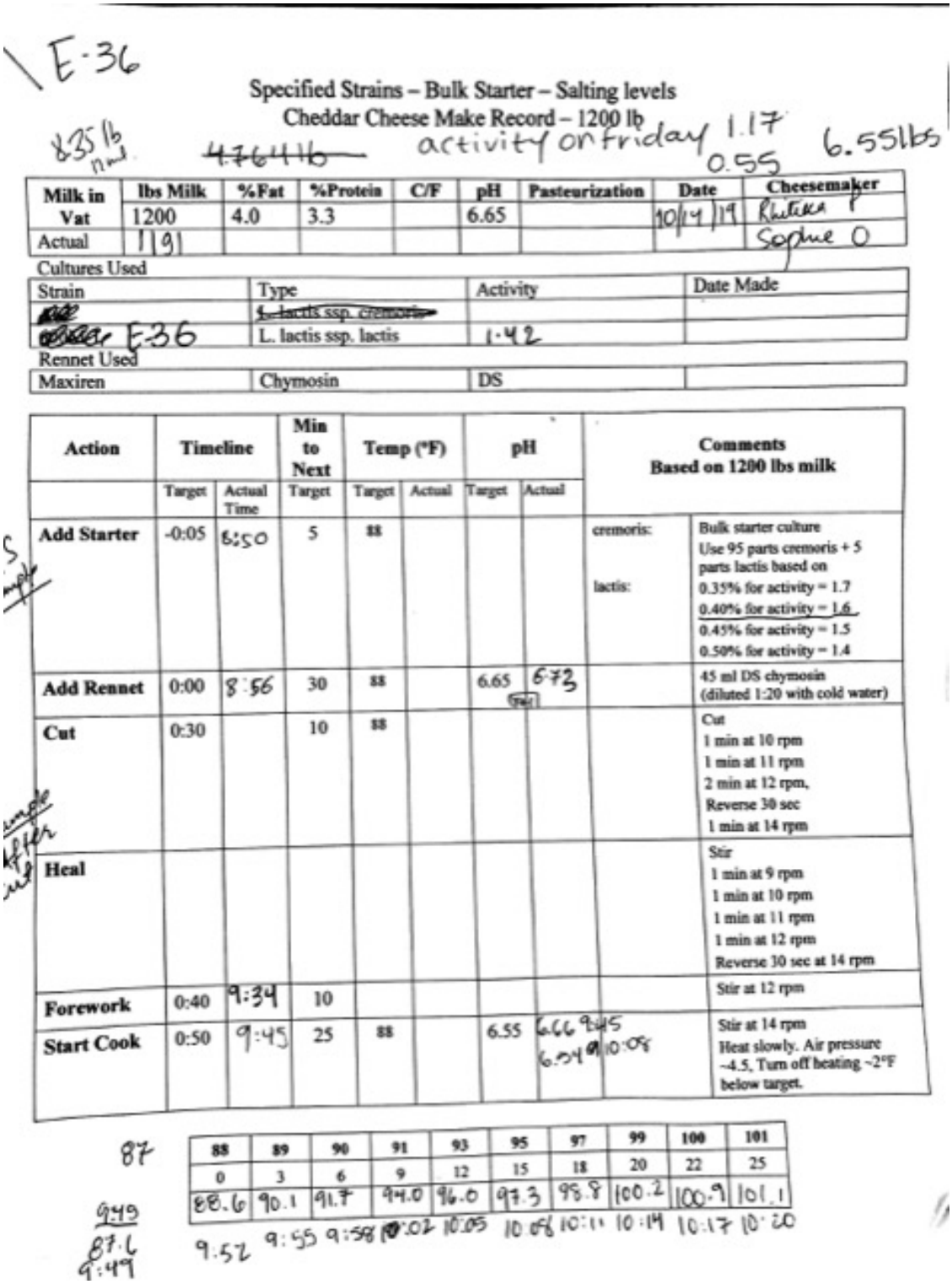
9:50 9:53 9:56 9:59 10:02 10:05 10:08 10:11 10:14 10:17 10:21 10:24 10:27 10:30

95.2

pH 6.57 @ 10:21

End Cook	1:15	1032	20	101		6.45	6.49 6.47 6.48	10:33 10:42 10:55	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35	11:02	10			6.40	6.40		Warm drain table before pumpover
Start Draining	1:45	11:10	5	101		6.30	6.30		
Form curd into Pack	1:50	11:27	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	11:40	30	99		6.15	5.95 5.93	12:50 1:00	Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40		30	96		5.85	5.77 5.68	12:10 12:20	5.62 12:30 Turn every 10 minutes
Stack 3 High	3:10	12:45	30	94		5.55	5.55		Turn every 10 minutes
Mill	3:40		5	91		5.45	5.35	1:05	Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g 218
Salt 2	4:00		15	88					Add 2.4% salt, 272 g 261
Salt 3	4:00		15	88					Add 2.8% salt, 318 g 305
Salt 4	4:00		15	88					Add 3.2% salt, 363 g 348
Salt 5	4:00		15	88					Add 3.6% salt, 409 g 392
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15	3:00	180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *lactis* E36- Replicate 1



E-36

Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

835 lb nmk 4764 lb activity on friday 1.17 0.55 6.55lbs

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		10/14/19	Rhutika
Actual	1191							Sophie O

Cultures Used

Strain	Type	Activity	Date Made
[redacted]	~~L. lactis ssp. cremoris~~		
[redacted] E-36	L. lactis ssp. lactis	1.42	

Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (°F)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05	8:50	5	88				cremoris:	Bulk starter culture Use 95 parts cremoris + 5 parts lactis based on
								lactis:	0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00	8:56	30	88		6.65	6.73		45 ml DS chymosin (diluted 1:20 with cold water)
Cut	0:30		10	88					Cut 1 min at 10 rpm 1 min at 11 rpm 2 min at 12 rpm, Reverse 30 sec 1 min at 14 rpm
Heal									Stir 1 min at 9 rpm 1 min at 10 rpm 1 min at 11 rpm 1 min at 12 rpm Reverse 30 sec at 14 rpm
Forework	0:40	9:34	10						Stir at 12 rpm
Start Cook	0:50	9:45	25	88		6.55	6.66 9:45 6.54 10:08		Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

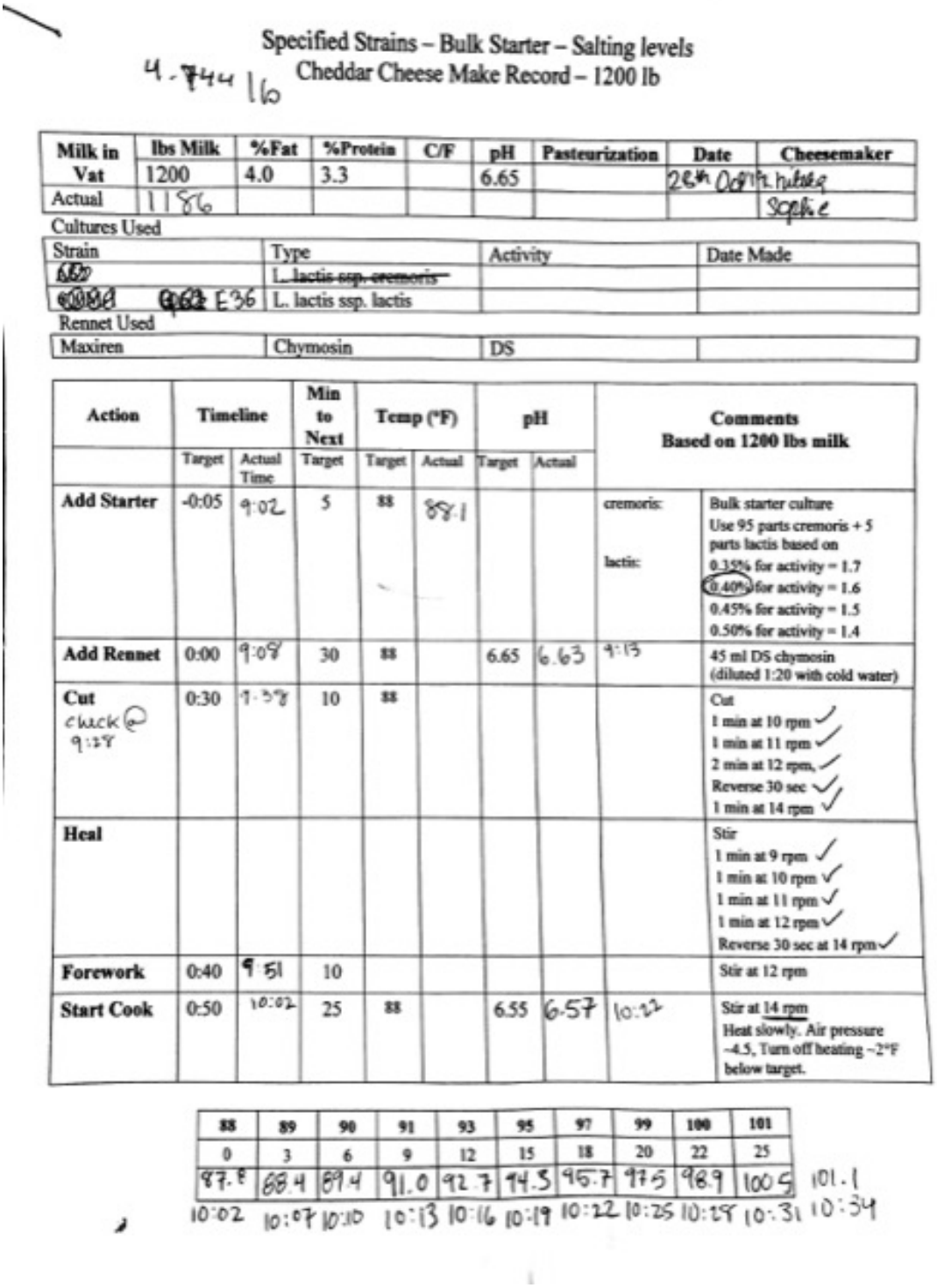
87

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
88.6	90.1	91.7	94.0	96.0	97.3	98.8	100.2	100.9	101.1
9:52	9:55	9:58	10:02	10:05	10:08	10:11	10:14	10:17	10:20

9:49
87.6
9:49

2nd Cook	1:15	10:28	20	101		6.45	6.50 6.43	10:28 10:44	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35	10:58	10			6.40	6.39 6.36	10:58 11:14	Warm drain table before pumpover
Start Draining	1:45	11:26	5	101		6.30	6.29	11:26	
Form curd into Pack	1:50	11:40	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn turn every 5 b/c cold	2:00	turn 11:54	30	99		6.15	6.10 6.02	12:02 12:19	Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	12:38	30	96		5.85	5.84 5.81	12:38 1:03	Turn every 10 minutes
Stack 3 High	3:10	1:24 2:06	30	94		5.55	5.48 5.52	1:06 2:06	Turn every 10 minutes
Mill	3:40		5	91		5.45			Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g
Salt 2	4:00		15	88					Add 2.4% salt, 272 g
Salt 3	4:00		15	88					Add 2.8% salt, 318 g
Salt 4	4:00		15	88					Add 3.2% salt, 363 g
Salt 5	4:00		15	88					Add 3.6% salt, 409 g
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15	3:10	180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *lactis* E36- Replicate 2



Specified Strains – Bulk Starter – Salting levels
4.744 16 Cheddar Cheese Make Record – 1200 lb

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		28th Oct	Mateo
Actual	1186							Sophie

Cultures Used

Strain	Type	Activity	Date Made
~~[illegible]~~	~~L. lactis ssp. cremoris~~		
~~[illegible]~~ E36	L. lactis ssp. lactis		

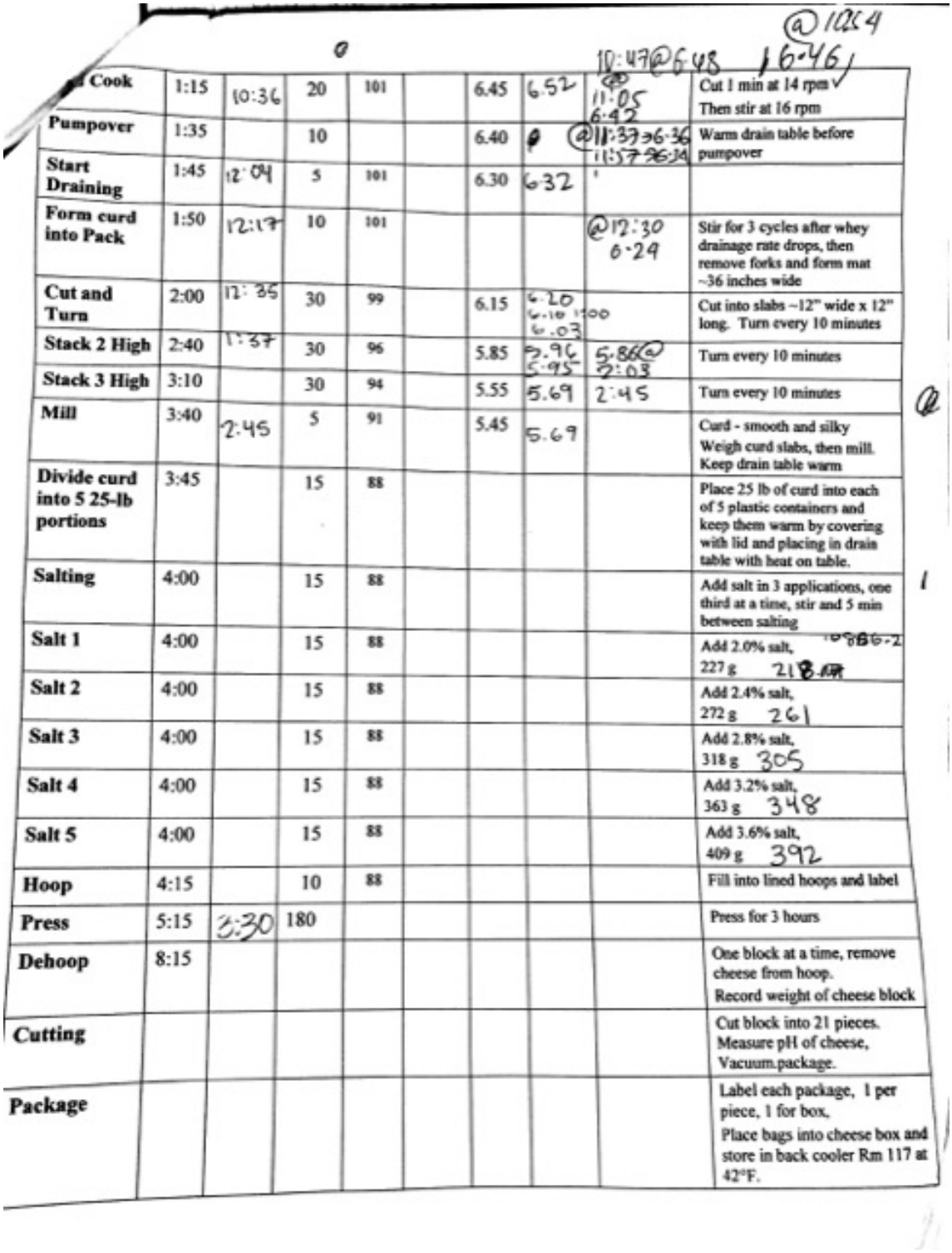
Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (°F)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05	9:02	5	88	88.1			cremoris:	Bulk starter culture
									Use 95 parts cremoris + 5 parts lactis based on
								lactis:	0.35% for activity = 1.7
									0.40% for activity = 1.6
									0.45% for activity = 1.5
									0.50% for activity = 1.4
Add Rennet	0:00	9:08	30	88		6.65	6.63	9:13	45 ml DS chymosin (diluted 1:20 with cold water)
Cut check @ 9:28	0:30	9:38	10	88					Cut 1 min at 10 rpm ✓ 1 min at 11 rpm ✓ 2 min at 12 rpm, ✓ Reverse 30 sec ✓ 1 min at 14 rpm ✓
Heal									Stir 1 min at 9 rpm ✓ 1 min at 10 rpm ✓ 1 min at 11 rpm ✓ 1 min at 12 rpm ✓ Reverse 30 sec at 14 rpm ✓
Forework	0:40	9:51	10						Stir at 12 rpm
Start Cook	0:50	10:02	25	88		6.55	6.57	10:12	Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

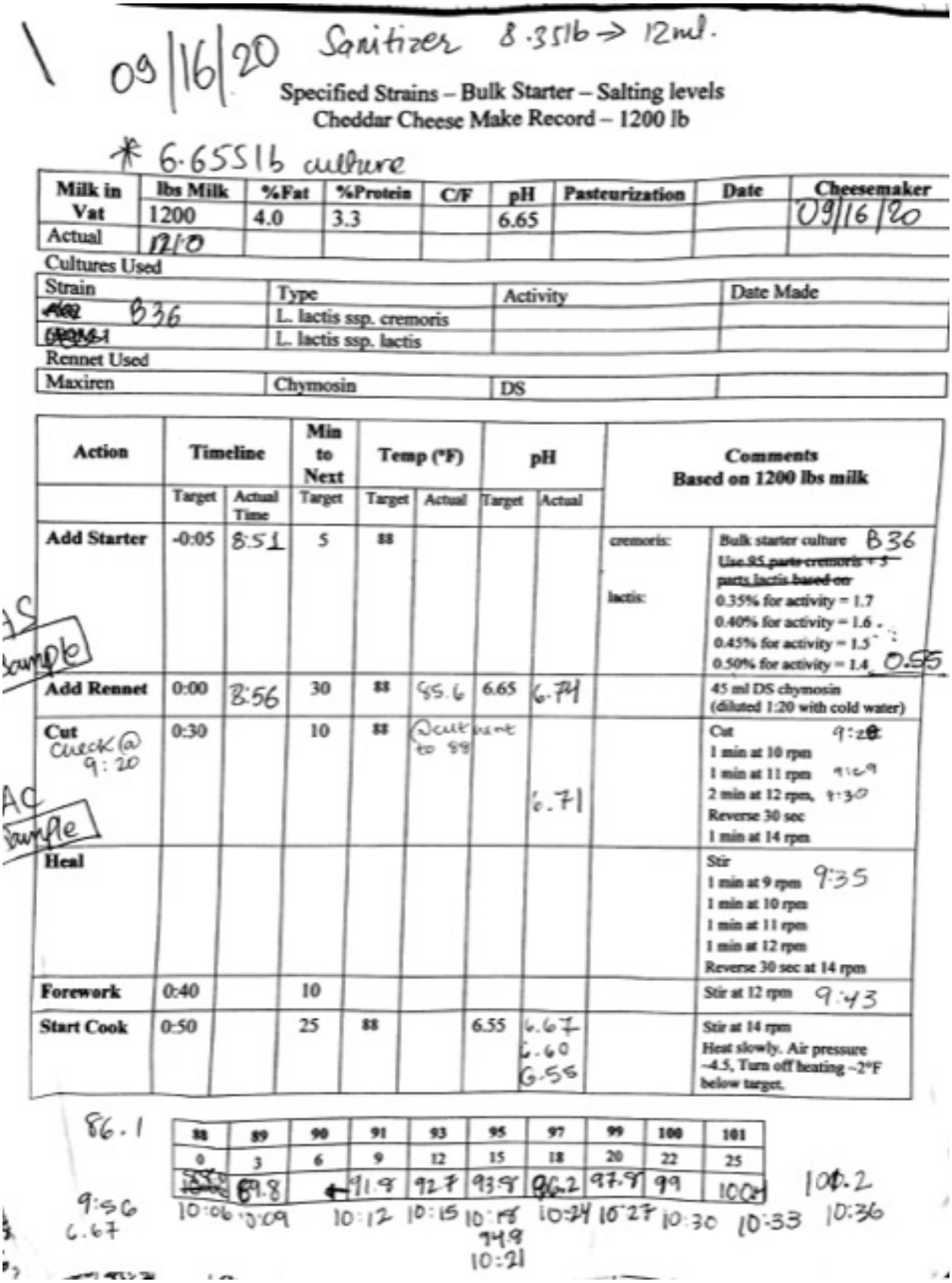
88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
87.8	88.4	89.4	91.0	92.7	94.5	96.7	97.5	98.9	100.5
10:02	10:07	10:10	10:13	10:16	10:19	10:22	10:25	10:28	10:31

101.1
10:34



Cook	1:15	10:36	20	101		6.45	6.52	10:47@6.48 / 6.46 / @11:05 6:42	Cut 1 min at 14 rpm ✓ Then stir at 16 rpm
Pumpover	1:35		10			6.40		@11:37 6.36 11:57 6.34	Warm drain table before pumpover
Start Draining	1:45	12:04	5	101		6.30	6.32		
Form curd into Pack	1:50	12:17	10	101				@12:30 6.29	Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	12:35	30	99		6.15	6.20 6.10 6.03		Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	1:37	30	96		5.85	5.96 5.95	5.86@ 2:03	Turn every 10 minutes
Stack 3 High	3:10		30	94		5.55	5.69	2:45	Turn every 10 minutes
Mill	3:40	2:45	5	91		5.45	5.69		Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g 218
Salt 2	4:00		15	88					Add 2.4% salt, 272 g 261
Salt 3	4:00		15	88					Add 2.8% salt, 318 g 305
Salt 4	4:00		15	88					Add 3.2% salt, 363 g 348
Salt 5	4:00		15	88					Add 3.6% salt, 409 g 392
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15	3:30	180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box. Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *cremoris* B36- Replicate 1



09/16/20 Sanitizer 8.351b → 12ml.

Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

* 6.6551b culture

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
Actual	1200	4.0	3.3		6.65			09/16/20

Cultures Used

Strain	Type	Activity	Date Made
~~402~~ B36	L. lactis ssp. cremoris		
~~B2356-1~~	L. lactis ssp. lactis		

Rennet Used

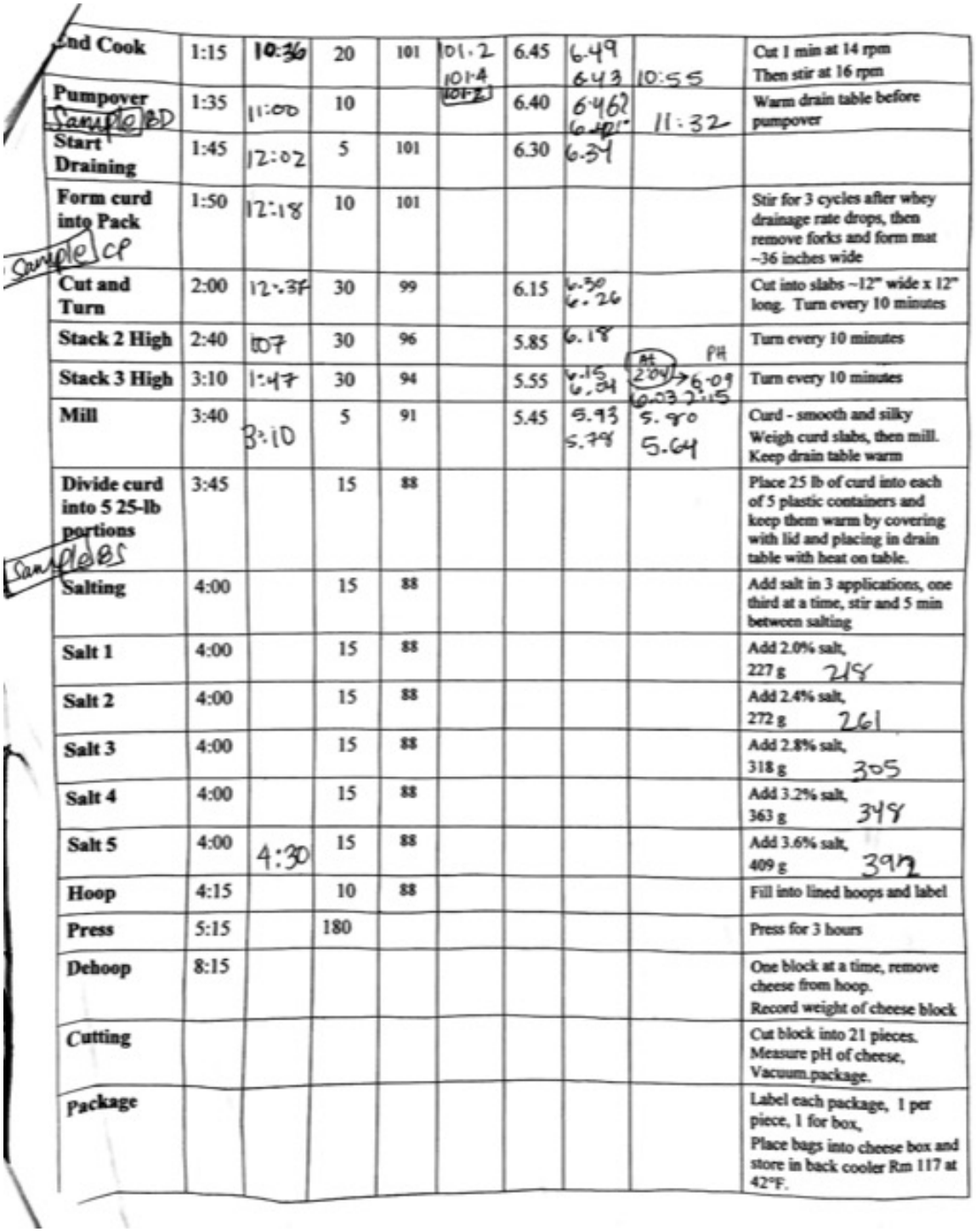
Maxiren	Chymosin	DS	

Action	Timeline Target	Timeline Actual Time	Min to Next Target	Temp (°F) Target	Temp (°F) Actual	pH Target	pH Actual	Comments Based on 1200 lbs milk	
Add Starter	-0:05	8:51	5	88				cremoris:	Bulk starter culture B36 ~~Use 95 parts cremoris + 5 parts lactis based on~~
								lactis:	0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4 0.55
Add Rennet	0:00	8:56	30	88	85.6	6.65	6.71		45 ml DS chymosin (diluted 1:20 with cold water)
Cut Check @ 9:20	0:30		10	88	Cut ment to 88		6.71		Cut 9:28 1 min at 10 rpm 1 min at 11 rpm 9:29 2 min at 12 rpm, 9:30 Reverse 30 sec 1 min at 14 rpm
Heal									Stir 1 min at 9 rpm 9:35 1 min at 10 rpm 1 min at 11 rpm 1 min at 12 rpm Reverse 30 sec at 14 rpm
Forework	0:40		10						Stir at 12 rpm 9:43
Start Cook	0:50		25	88		6.55	6.67 6.60 6.56		Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

86.1

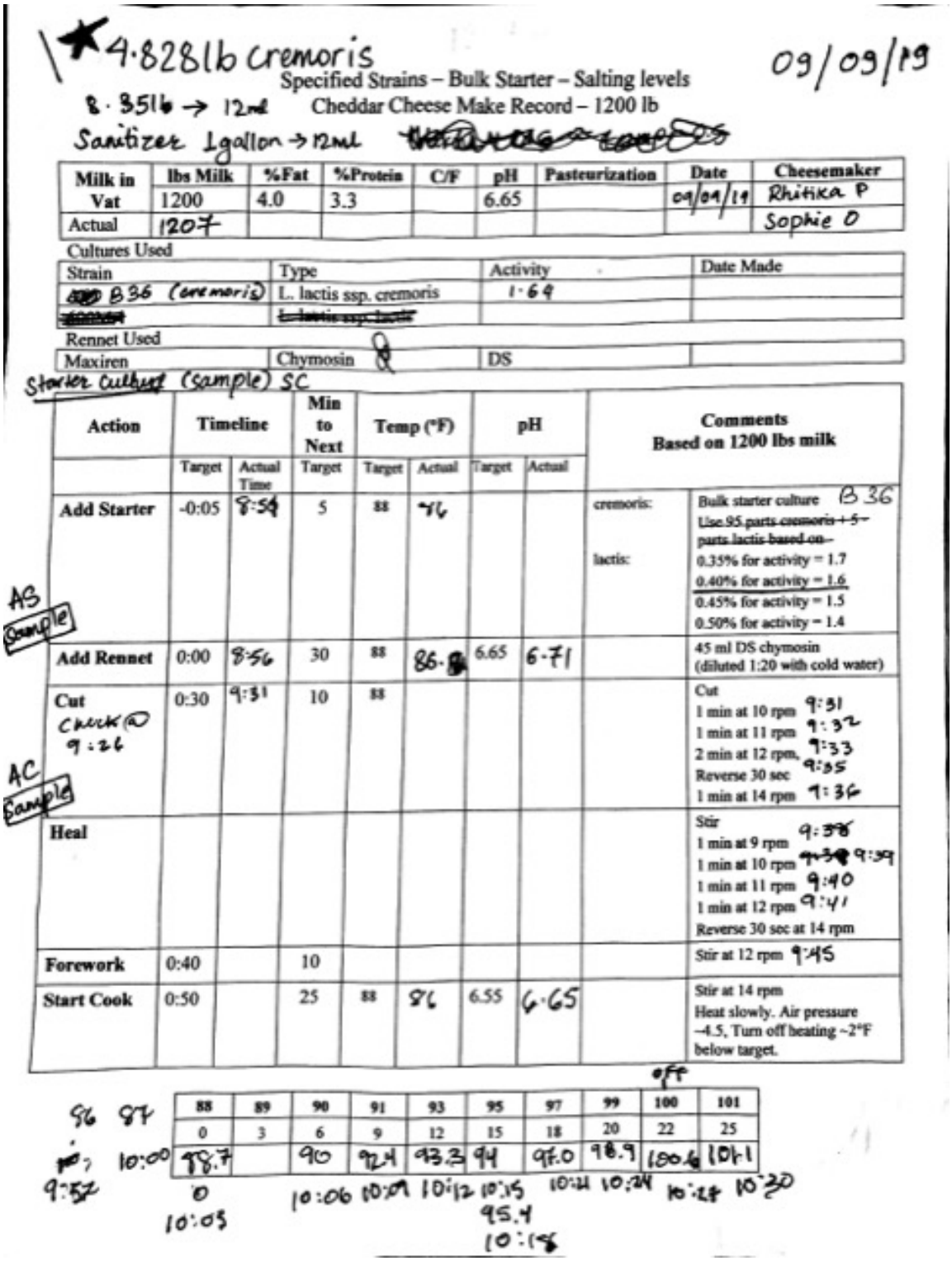
88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
~~85.9~~	89.8	←	91.8	92.7	93.9	96.2	97.8	99	100.1

9:56 6.67 10:06 10:09 10:12 10:15 10:18 10:21 10:24 10:27 10:30 10:33 10:36 100.2



End Cook	1:15	10:36	20	101	101.2 101.4 101.2	6.45	6.49 6.43	10:55	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover Sample BD	1:35	11:00	10			6.40	6.46 6.48	11:32	Warm drain table before pumpover
Start Draining	1:45	12:02	5	101		6.30	6.34		
Form curd into Pack Sample CP	1:50	12:18	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	12:37	30	99		6.15	6.30 6.26		Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	107	30	96		5.85	6.18		Turn every 10 minutes
Stack 3 High	3:10	1:47	30	94		5.55	6.15 6.34	pH at 2:04 → 6.09 6.03 2:15	Turn every 10 minutes
Mill	3:40	3:10	5	91		5.45	5.93 5.78	5.90 5.64	Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions Samples BS	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g 218
Salt 2	4:00		15	88					Add 2.4% salt, 272 g 261
Salt 3	4:00		15	88					Add 2.8% salt, 318 g 305
Salt 4	4:00		15	88					Add 3.2% salt, 363 g 348
Salt 5	4:00	4:30	15	88					Add 3.6% salt, 409 g 392
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15		180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *cremoris* B36- Replicate 2



4.828lb cremoris 09/09/19

Specified Strains – Bulk Starter – Salting levels

8.351b → 12ml Cheddar Cheese Make Record – 1200 lb

Sanitizer 1gallon → 12ml

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		09/09/19	Rhitika P
Actual	1207							Sophie O

Cultures Used

Strain	Type	Activity	Date Made
B36 (cremoris)	L. lactis ssp. cremoris	1.69	

Rennet Used

Maxiren	Chymosin	DS	

Starter Culture (sample) SC

Action	Timeline Target	Timeline Actual Time	Min to Next Target	Temp (°F) Target	Temp (°F) Actual	pH Target	pH Actual	Comments Based on 1200 lbs milk	
Add Starter	-0:05	8:54	5	88	96			cremoris:	Bulk starter culture B36
								lactis:	Use 95 parts cremoris + 5 parts lactis based on: 0.35% for activity = 1.7; 0.40% for activity = 1.6; 0.45% for activity = 1.5; 0.50% for activity = 1.4
Add Rennet	0:00	8:56	30	88	86.8	6.65	6.71		45 ml DS chymosin (diluted 1:20 with cold water)
Cut	0:30	9:31	10	88					Cut 1 min at 10 rpm 9:31 1 min at 11 rpm 9:32 2 min at 12 rpm, 9:33 Reverse 30 sec 9:35 1 min at 14 rpm 9:36
Heal									Stir 1 min at 9 rpm 9:38 1 min at 10 rpm 9:39 1 min at 11 rpm 9:40 1 min at 12 rpm 9:41 Reverse 30 sec at 14 rpm
Forework	0:40		10						Stir at 12 rpm 9:45
Start Cook	0:50		25	88	86	6.55	6.65		Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

AB Sample

AC Sample

Check @ 9:26

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
98.7		90	92.4	93.3	94	97.0	98.9	100.6	101.1

96 87 off

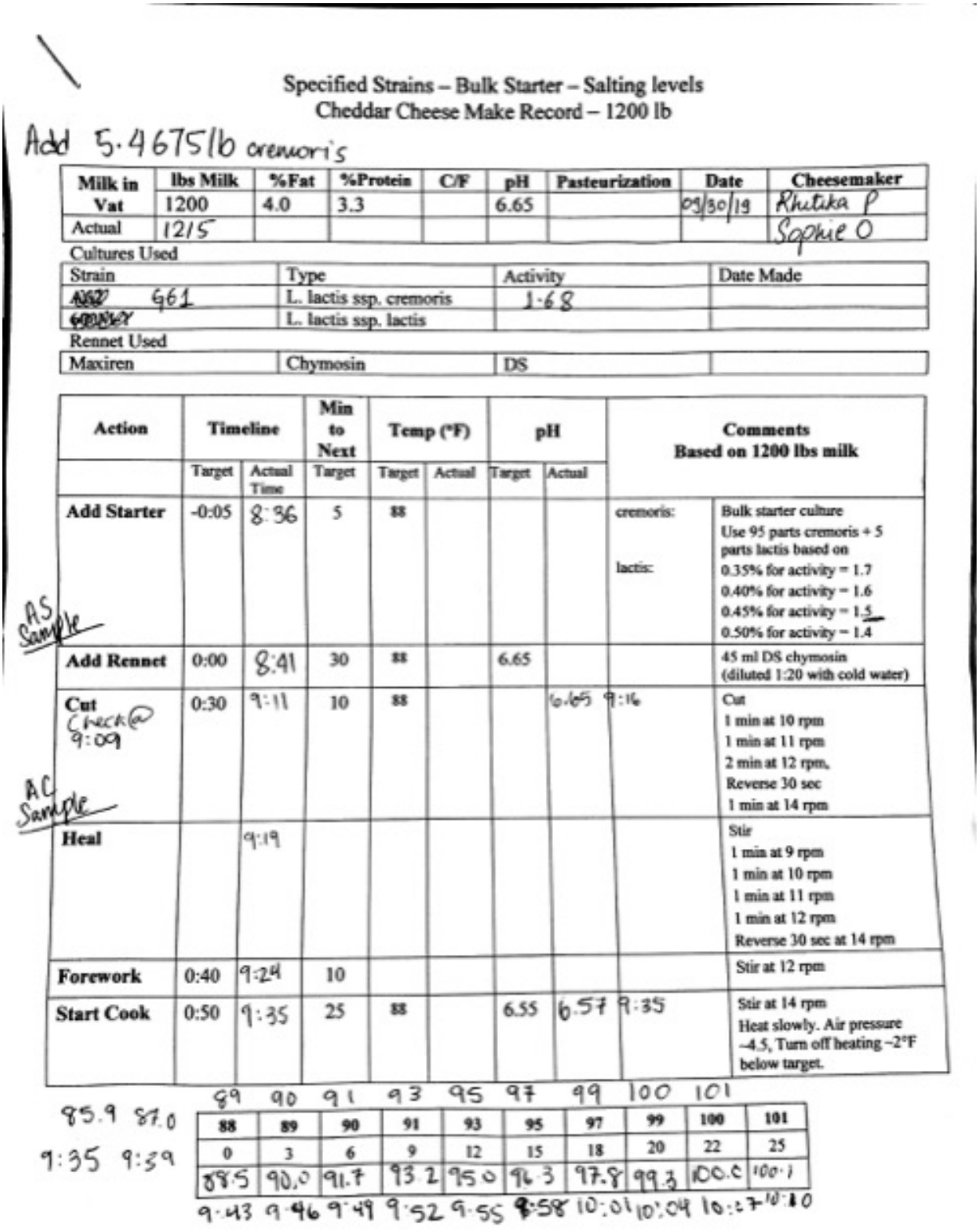
10:00 9:57

0 10:03

10:06 10:09 10:12 10:15 95.4 10:18 10:21 10:24 10:27 10:30

End Cook	1:15	10:30	20	101		6.45	6.57 6.53	10:36 10:47	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35	1057	10			6.40	6.50 6.46	11:29	Warm drain table before pumpover
Start Draining	1:45	12:16	15	101		6.30 SD	6.39 6.32	11:54	
Form curd into Pack	1:50		10	101	At 1:35 6.12			1:30	Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	1.14	30	99		6.15	6.20		Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	2:00	30	96		5.85	5.11 5.93		Turn every 10 minutes
Stack 3 High	3:10		30	94		5.55	5.85	2:34	Turn every 10 minutes
Mill	3:40	3:00	5	91		5.45	5.51		Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g 218
Salt 2	4:00		15	88					Add 2.4% salt, 272 g 261
Salt 3	4:00		15	88					Add 2.8% salt, 318 g 305
Salt 4	4:00		15	88					Add 3.2% salt, 363 g 348
Salt 5	4:00		15	88					Add 3.6% salt, 409 g 392
Hoop	4:15	4:20	10	88					Fill into lined hoops and label
Press	5:15	4:20	180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *cremoris* G61 Replicate 1



Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

Add 5.4675lb cremoris

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
	1200	4.0	3.3		6.65		09/30/19	Rhutika P
Actual	1215							Sophie O

Cultures Used

Strain	Type	Activity	Date Made
G61	L. lactis ssp. cremoris	1.68	
	L. lactis ssp. lactis		

Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (°F)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05	8:36	5	88				cremoris:	Bulk starter culture Use 95 parts cremoris + 5 parts lactis based on
AS Sample								lactis:	0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00	8:41	30	88		6.65			45 ml DS chymosin (diluted 1:20 with cold water)
Cut Check @ 9:09	0:30	9:11	10	88			6.65	9:16	Cut 1 min at 10 rpm 1 min at 11 rpm 2 min at 12 rpm, Reverse 30 sec 1 min at 14 rpm
AC Sample									
Heal		9:19							Stir 1 min at 9 rpm 1 min at 10 rpm 1 min at 11 rpm 1 min at 12 rpm Reverse 30 sec at 14 rpm
Forework	0:40	9:24	10						Stir at 12 rpm
Start Cook	0:50	9:35	25	88		6.55	6.57	9:35	Stir at 14 rpm Heat slowly. Air pressure –4.5, Turn off heating –2°F below target.

89 90 91 93 95 97 99 100 101

85.9 87.0

9:35 9:39

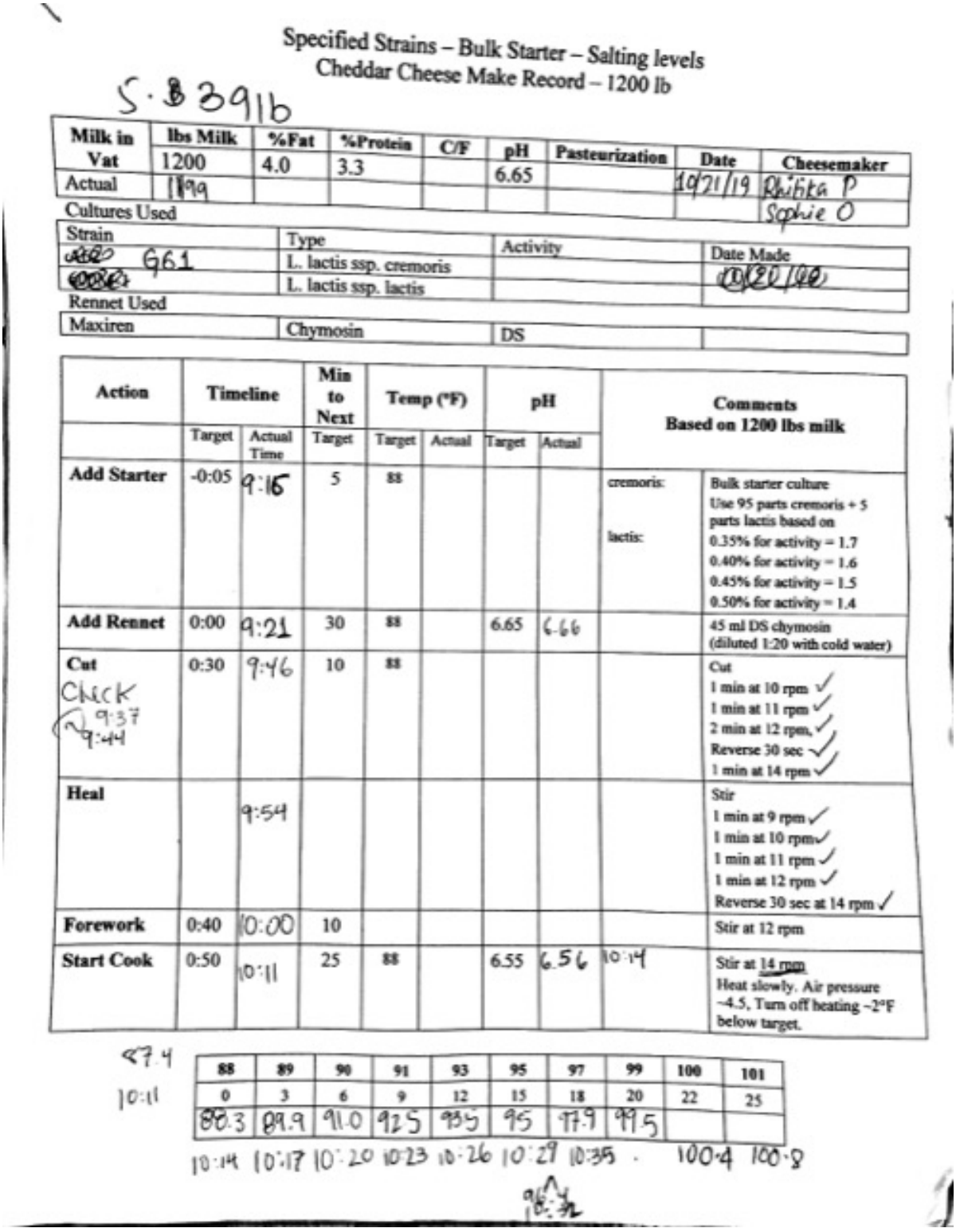
88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
88.5	90.0	91.7	93.2	95.0	96.3	97.8	99.3	100.0	100.1

9:43 9:46 9:49 9:52 9:55 9:58 10:01 10:04 10:07 10:10

Had to Pumpover early ; pH was dropping rapidly.

nd Cook	1:15	10:17	20	101	101.0	6.45	6.39	10:17	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35	10:A	10			6.40			Warm drain table before pumpover
Start Draining	1:45	10:30	5	101		6.30	6.30		
Form curd into Pack	1:50	10:50	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	11:05	30	99		6.15	6.15 5.90	11:15	Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	11:30	30	96		5.85	5.85 5.73	11:30 5.65 11:40	11:50 Turn every 10 minutes
Stack 3 High	3:10	12:00	30	94		5.55	5.54		Turn every 10 minutes
Mill	3:40	12:17	5	91		5.45	~~5.36~~	5.42	Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88					Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g
Salt 2	4:00		15	88					Add 2.4% salt, 272 g
Salt 3	4:00		15	88					Add 2.8% salt, 318 g
Salt 4	4:00		15	88					Add 3.2% salt, 363 g
Salt 5	4:00		15	88					Add 3.6% salt, 409 g
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15	1:20	180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Lactococcus lactis subsp. *cremoris* G61- Replicate 2



Specified Strains – Bulk Starter – Salting levels
Cheddar Cheese Make Record – 1200 lb

5·B 391b

Milk in Vat	lbs Milk	%Fat	%Protein	C/F	pH	Pasteurization	Date	Cheesemaker
Actual	1199				6.65		10/21/19	Rhitika P
								Sophie O

Cultures Used

Strain	Type	Activity	Date Made
G61	L. lactis ssp. cremoris		10/20/19
	L. lactis ssp. lactis		

Rennet Used

Maxiren	Chymosin	DS	

Action	Timeline		Min to Next	Temp (°F)		pH		Comments Based on 1200 lbs milk	
	Target	Actual Time	Target	Target	Actual	Target	Actual		
Add Starter	-0:05	9:16	5	88				cremoris: lactis:	Bulk starter culture Use 95 parts cremoris + 5 parts lactis based on 0.35% for activity = 1.7 0.40% for activity = 1.6 0.45% for activity = 1.5 0.50% for activity = 1.4
Add Rennet	0:00	9:21	30	88		6.65	6.66		45 ml DS chymosin (diluted 1:20 with cold water)
Cut Check 9:37 9:44	0:30	9:46	10	88					Cut 1 min at 10 rpm ✓ 1 min at 11 rpm ✓ 2 min at 12 rpm ✓ Reverse 30 sec ✓ 1 min at 14 rpm ✓
Heal		9:54							Stir 1 min at 9 rpm ✓ 1 min at 10 rpm ✓ 1 min at 11 rpm ✓ 1 min at 12 rpm ✓ Reverse 30 sec at 14 rpm ✓
Forework	0:40	10:00	10						Stir at 12 rpm
Start Cook	0:50	10:11	25	88		6.55	6.56	10:14	Stir at 14 rpm Heat slowly. Air pressure ~4.5, Turn off heating ~2°F below target.

87.4
10:11

88	89	90	91	93	95	97	99	100	101
0	3	6	9	12	15	18	20	22	25
88.3	89.9	91.0	92.5	93.5	95	97.9	99.5		

10:14 10:17 10:20 10:23 10:26 10:29 10:35 . 100.4 100.8

96.4
10:32

End Cook	1:15	10:50	20	101		6.45	6.46	10:48	Cut 1 min at 14 rpm Then stir at 16 rpm
Pumpover	1:35	11:04	10			6.40			Warm drain table before pumpover
Start Draining	1:45	11:20	5	101		6.30	6.30		
Form curd into Pack	1:50	11:40	10	101					Stir for 3 cycles after whey drainage rate drops, then remove forks and form mat ~36 inches wide
Cut and Turn	2:00	11:52	30	99		6.15	6.13 6.04 12:02	5.97 12:42	Cut into slabs ~12" wide x 12" long. Turn every 10 minutes
Stack 2 High	2:40	12:22	30	96		5.85	5.96	12:32 12:42	Turn every 10 minutes
Stack 3 High	3:10		30	94		5.55	555	1:06 1:13	Turn every 10 minutes
Mill	3:40		5	91		5.45	545	1:23 1:32	Curd - smooth and silky Weigh curd slabs, then mill. Keep drain table warm
Divide curd into 5 25-lb portions	3:45		15	88					Place 25 lb of curd into each of 5 plastic containers and keep them warm by covering with lid and placing in drain table with heat on table.
Salting	4:00		15	88				1085662	Add salt in 3 applications, one third at a time, stir and 5 min between salting
Salt 1	4:00		15	88					Add 2.0% salt, 227 g → 217.7
Salt 2	4:00		15	88					Add 2.4% salt, 272 g → 261.27
Salt 3	4:00		15	88					Add 2.8% salt, 318 g → 304.81
Salt 4	4:00		15	88					Add 3.2% salt, 363 g → 348.36
Salt 5	4:00		15	88					Add 3.6% salt, 409 g → 391.9
Hoop	4:15		10	88					Fill into lined hoops and label
Press	5:15		180						Press for 3 hours
Dehoop	8:15								One block at a time, remove cheese from hoop. Record weight of cheese block
Cutting									Cut block into 21 pieces. Measure pH of cheese, Vacuum package.
Package									Label each package, 1 per piece, 1 for box, Place bags into cheese box and store in back cooler Rm 117 at 42°F.

Take Sample!

APPENDIX B.

Raw data of Microbial numbers during Cheese making

Table 12. The cell count numbers of each replicate of lactis (600M1, E36) and cremoris strain (B36,G61) collected after culture addition or after starter (d0), after cutting (d0), before draining (d0), at curd pack (d0), before salting (d 0) and starter culture on Reddy's agar. The expected generation time for all the cultures was 1 hour.

L. Lactis ssp.	Replicates	Starter culture only	After Starter	After Cut	Before Draining	Curd Pack	Before Salting
600M1	1	9.8	6.6	8.1	8.7	8.7	9.0
600M1	2	10.0	7.1	7.8	8.2	8.5	8.9
E36	1	10.3	7.4	8.3	8.8	9.0	9.1
E36	2	10.5	7.6	8.3	8.4	8.8	8.9
B36	1	8.7	5.1	7.3	7.3	7.2	8.5
B36	2	8.7	6.1	7.0	7.7	7.2	8.0
G61	1	9.5	7.1	7.9	8.3	8.2	8.3
G61	2	9.7	7.4	8.0	8.2	8.2	8.5