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GROWTH CHARACTERISTICS OF *LACTOBACILLUS WASATCHENSIS* AND ITS  
DETECTION AND ENUMERATION USING QUANTITATIVE POLYMERASE  
CHAIN REACTION

by

Isaac Brockbank Bowen

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

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

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UTAH STATE UNIVERSITY  
Logan, Utah

2018

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## ABSTRACT

Growth Characteristics of *Lactobacillus wasatchensis* and Its Detection and Enumeration Using Quantitative Polymerase Chain Reaction

by

Isaac Brockbank Bowen, Master of Science

Utah State University, 2018

Major Professor: Dr. Donald J. McMahon  
Department: Nutrition and Food Science

*Lactobacillus wasatchensis* can be part of the nonstarter lactic acid bacteria (**NSLAB**) microbiota of cheese and has been associated with unwanted gas production during storage. Experiments were performed to determine the upper limits of salt tolerance of *Lb. wasatchensis* (strain WDC04) at pH 5.2 and 6.5, what effect different levels of salt (0, 3.5, 4.5, and 5.5% w/v) have on growth at a cheese pH of 5.2 and 5.5, at what pH is optimal growth observed and what pH range supports growth. Further experimentation was done to observe growth on various carbohydrate sources typically found in aging cheese, and in cell lysate solutions prepared from common starter and adjunct LAB. Heat stability of *Lb. wasatchensis* at 157, 161, 165, and 169°F was tested using an industrial heat exchanger. In addition, a phenol-chloroform DNA extraction method was used to extract *Lb. wasatchensis* DNA from cheese samples. The purity of DNA was determined and quantitative polymerase chain reaction (qPCR) techniques were utilized to visualize the *Lb. wasatchensis* DNA present.

It was determined there is minimal growth of *Lb. wasatchensis* at pH 6.5 when grown in MRS broth containing 6.0% salt ( $\mu_{\max}$  of 0.012). Although growth was observed at all salt concentrations tested at pH 5.2, samples containing 6.0% salt still showed the slowest growth ( $\mu_{\max}$ , 0.035). Further, *Lb. wasatchensis* growth was shown to be slowest at salt concentrations of 5.5% at a cheese pH of 5.2 and 5.5, with increasing growth at lower salt percentages. *Lactobacillus wasatchensis* grows best at pH 8 followed closely by pH 6.0, 6.6, and 7.0. Growth was observed at pH 9 ( $\mu_{\max}$ , 0.361) while minimal growth was observed at pH 4.0 ( $\mu_{\max}$ , 0.015).

In various carbohydrate sources, growth was best in MRS broth containing ribose. However, growth was also observed on galactose, lactose, fructose, glucose, and N-acetylglucosamine. In cell lysate made from *Lactococcus lactis*, *Lactococcus cremoris*, *Streptococcus thermophilus*, and *Lactobacillus helveticus*, growth was seen in all four filtrates. The phenol-chloroform extraction method is effective and produces a relatively pure DNA product which can be visualized using qPCR, although the detection threshold is too high to be successful. Further experimentation is needed in this area before implementation to overcome challenges of primer dimers and non-amplification.

## PUBLIC ABSTRACT

Growth Characteristics of *Lactobacillus wasatchensis* and Its Detection and Enumeration Using Quantitative Polymerase Chain Reaction

Isaac B. Bowen

There are numerous challenges encountered during the manufacturing and storage of cheese by both the large-scale and artesian producers. One such challenge has been the formation of late gassy defect, which occurs when gas is produced by certain lactic acid bacteria found in the cheese block during storage and aging over a three month time period. Negative consequences of late gas production are slits and cracks in the cheese block and puffy cheese packaging, which cause significant financial losses for manufacturers along with poor consumer acceptance.

*Lactobacillus wasatchensis* is one such lactic acid bacterium shown to produce gas during cheese storage. This bacterium has now been found in cheese samples exhibiting late gas defect in the Midwest and Western states. The goal of this study was to further characterize and understand the growth attributes of *Lb. wasatchensis*, and thereby gain some understanding on how it enters the cheese vats and if there are possible ways to limit or inhibit its subsequent growth. An additional goal was to determine if we could effectively extract *Lb. wasatchensis* DNA from cheese samples and visualize using the qPCR molecular technique. If possible, this detection method would allow a faster and more sensitive approach to determining if *Lb. wasatchensis* is present in cheese blocks, which would help manufacturers know how long they should age their cheeses.

It was discovered that *Lb. wasatchensis* does not survive processing through an industrial heat exchanger and therefore must be entering the cheese vats by other means such as: cross-contamination, biofilm formation or aerosolizing. We also showed growth of *Lb. wasatchensis* is limited at an increased salt-in-moisture ratio in cheese. Additionally, we found that *Lb. wasatchensis* DNA can be extracted from cheese and visualized using qPCR, although further experimentation is needed to optimize this method.

## ACKNOWLEDGEMENTS

I want to express my appreciation to all those who helped, encouraged, and supported me over the past two years while in graduate school. While I could never name everyone who has aided me along the way, I do want to acknowledge several important people. My wife for being one of my greatest blessings, advocates, and laboratory assistants I could have asked for—whether it was helping count bacterial colonies at 2:00 am or making dilution blanks, you have become a wonderful microbiologist.

Also, a huge appreciation and thanks to Dr. Donald McMahon who was gracious enough to mentor me and allowed me an opportunity to work with him. I would be remiss if I did not thank as well Dr. Jeff Broadbent, Dr. Craig Oberg, and Dr. Michele Culumber who have not only become friends and role models over these past several years, but without whom I would not currently be writing this thesis. Along with Dr. McMahon, they have spent hours providing help, direction, and encouragement, as well as dealing with my incessant questions. Thank you!

I also want to thank the BUILD Dairy program (Building University-Industry Linkages through Learning and Discovery) at the Western Dairy Center and the support from Dairy West for helping fund my research.



*To my wife who continually deals with  
life's ever-changing plans*

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## LIST OF ABBREVIATIONS

- CFU – Colony forming unit
- CR-MRS – Carbohydrate restricted MRS broth
- DVS – Direct-to-vat set
- LAB – lactic acid bacteria
- MRS Broth – De Man, Rogosa, and Sharpe broth
- MRS-R – MRS broth supplemented with 1.5% (w/v) ribose
- NSLAB – Non-starter lactic acid bacteria
- qPCR- Quantitative polymerase chain reaction
- SE – Standard error
- $\mu_{\max}$  – Maximum specific growth rate
- M17-L Broth – M17 broth containing 1.5% (w/v) lactose
- OHF bacteria – Obligatory heterofermentative bacteria
- FHF bacteria – Facultative heterofermentative bacteria



## INTRODUCTION

Understanding the problems encountered during cheese manufacturing and storage has been a focus of the dairy community. There are a wide range of issues that both the large-scale manufacturing companies and novice cheesemaker deal with on a day to day and month to month basis. These challenges include achieving their proper moisture, fat, and salt targets along with developing proper flavor profiles or dealing with cheese “pinking.” However, one particular large challenging issue deals with unwanted gas formation during cheese ripening and storage—a persistent, widespread problem that has been around for over 100 years and affected most cheese plants (Van Slyke and Hart, 1903; Mullan, 2000).

During cheese making, there are both starter lactic acid bacteria (**LAB**) and non-starter lactic acid bacteria (**NSLAB**) present. Starter LAB are purposefully added as part of a bulk set or direct-to-vat set (**DVS**) to acidify the milk, whereas the NSLAB enter the cheese make process either through their inherent presence in the milk or due to contamination in the processing facility (Peterson and Marshall, 1990, McSweeney et al., 1993, Somers et al., 2001). Some NSLAB can have deleterious effects on both the body and flavor of ripening cheese (Khalid and Marth, 1990). A certain NSLAB, *Lactobacillus wasatchensis*, has been shown to produce gas in cheese during ripening, causing slits and cracks and “blowing” of packaging (Ortakci et al., 2015c, a). Typically, these slit and crack defects aren’t noticed until the cheese has been aged and graded, and can cause up to 50% cutting losses (Donnely et al., 2014). Both time and profit are lost due to the cheese being downgraded and sold at a lower cost.

It would be of great benefit to the cheese manufacturer to understand the growth characteristics of *Lb. wasatchensis*, and thereby, obtain further insight into how it enters the cheese making process, what conditions allow it to grow, and if any of its growth parameters could be utilized to reduce or eliminate its presence in the cheese. Also of interest to cheese manufacturers, would be to know whether or not *Lb. wasatchensis* is present in their final cheese blocks and at what numbers. Prior research has shown *Lb. wasatchensis* grows at pH 5.2 and 6.5, and up to 5% NaCl (Ortakci et al., 2015b). It also utilizes ribose and/or galactose as a carbohydrate source and grows well on lactococcal cell lysate (Ortakci et al., 2015c). Finally, in test tube experiments, *Lb. wasatchensis* exhibits some thermotolerant capabilities as no survival was noted at low-temperature, long-time pasteurization but some cells survived at high-temperature, short-time (HTST) pasteurization parameters (Ortakci et al., 2015b).

Current methods to detect whether or not *Lb. wasatchensis* is in cheese have been limited to plating the bacteria on de Man, Rogosa, and Sharpe (**MRS**) agar supplemented with 1.5% ribose (**MRS+R**), or extracting the bacterial DNA from a cheese sample in conjunction with an amplification step using polymerase chain reaction (**PCR**) with *Lb. wasatchensis* specific primers followed by agarose gel electrophoresis (Culumber et al., 2017). However, there are limitations with these two methods. When *Lb. wasatchensis* is plated, it is very slow growing and takes 5 days to appear on the agar plates. Also, data shows it has to be within  $\sim 1.5$  log colony forming units (**CFU**)/g of the other faster-growing NSLAB or it won't be detected (Culumber et al., 2017). When using PCR to amplify *Lb. wasatchensis* DNA followed by gel electrophoresis, it becomes time consuming and the detection limit is high, at least  $\sim 10^4$  log CFU/g.

One goal of this project is to expand upon previous research already conducted that explored some of its growth conditions. The upper limits of salt tolerance were measured at both pH 5.2 and 6.5 to determine where the growth of *Lb. wasatchensis* is inhibited. The growth at varying pH concentrations was observed with special attention given to milk pH (6.6) and cheese pH (5.2). In addition, a number of pH versus salt concentrations were used to observe growth at pH 5.2 and 5.5 at 0, 3.5, 4.5, and 5.5% salt—typical combinations used in cheddar cheese manufacturing. Growth of *Lb. wasatchensis* was also determined in presence of various carbohydrate sources.

Other goals are to determine how well *Lb. wasatchensis* can grow off cell lysates derived from common cheddar cheese starter lactic acid bacteria or adjunct cultures. To gain a more precise understanding of the thermotolerance of *Lb. wasatchensis*, a plant-scale pasteurizer was used and run at varying temperatures. We also determined if a phenol/chloroform DNA extraction method can be used to extract *Lb. wasatchensis* DNA directly from cheese samples, resulting in a pure enough product that can be amplified using quantitative real-time PCR (**qPCR**). Such a method would provide a less complicated and more sensitive approach to detect and enumerate *Lb. wasatchensis* in cheese—allowing cheese manufacturers to detect contamination early on in the cheese making process and decide whether or not they should age out their cheese.

## HYPOTHESIS AND OBJECTIVES

### Hypotheses:

1. *Lactobacillus wasatchensis* is present in the milk in cheese vats because of insufficient inactivation during HTST pasteurization.
2. Further characterization of the growth attributes of *Lb. wasatchensis* will help to understand how it is able to grow to high numbers in cheese and produce unwanted carbon dioxide.
3. The phenol-chloroform nucleic-acid extraction method will yield a clean product of the target *Lb. wasatchensis* gene when extracted from cheese samples to be used in quantitative real-time PCR.

### Objectives:

1. Determine the thermotolerance and percent survival of *Lb. wasatchensis* when inoculated into milk and pasteurized using an industrial HTST heat exchanger.
2. Measure the growth of *Lb. wasatchensis* at various pH concentrations with specific attention to milk (6.6) and cheese pH (5.2).
3. Determine the upper limits of salt tolerance for *Lb. wasatchensis* growth at pH 5.2 and 6.5.
4. Determine how well *Lb. wasatchensis* grows at a number of pH versus salt concentrations typical of cheddar cheese manufacturing and aging—including pH 5.2 and 5.5 with 0, 3.5, 4.5, and 5.5% salt

5. Observe growth of *Lb. wasatchensis* on various carbohydrate sources and on different LAB cell lysates characteristic of cell lysis that may occur during storage of cheese.
6. Determine the quantity of DNA extracted out from cheese samples with known amounts of *Lb. wasatchensis* using the phenol-chloroform extraction method.
7. Determine the efficacy of qPCR at amplifying *Lb. wasatchensis* DNA extracted from cheese samples.

## LITERATURE REVIEW

### Non-Starter Lactic Acid Bacteria

During cheesemaking, there are both starter LAB (added as either a bulk starter or DVS) and NSLAB present. The starter LAB are deliberately added during the cheese manufacture to with the main purpose of converting lactose to lactic acid. However, the NSLAB are not deliberately added but are present as part of the original milk microflora or enter as contaminants in the cheese manufacturing facility (Peterson and Marshall, 1990; McSweeney et al., 1993; Somers et al., 2001). These NSLAB can have advantageous effects (i.e., help with maturing and flavor development of cheese) or deleterious effects, such as producing gas during storage and aging causing late blowing defect (Crow et al., 2001; Settanni and Moschetti, 2010; Ortakci et al., 2015b).

NSLAB can be classified based on metabolic characteristics—either as facultative heterofermentative (**FHF**) or obligatory heterofermentative (**OHF**). The population of NSLAB is dominated mainly by FHF bacteria, consisting of *Lactobacillus curvatus*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and others. Less frequently present are the OHF lactobacilli (Williams and Banks, 1997; Crow et al., 2001). Both flavor and body defects, including the presence of gas as evident by slits and cracks, have been attributed to the presence of OHF lactobacilli in cheese (Laleye et al., 1987; Khalid and Marth, 1990; Ortakci et al., 2015b).

Initially, the NSLAB population is relatively small compared to the starter LAB added during cheese manufacturing. However, during aging the cheese environment becomes more advantageous for the growth of NSLAB. Eventually, the NSLAB,

including both FHF and OHF lactobacilli, usually surpass the starter lactococci numbers to become the dominant population in aging cheese (Williams and Banks, 1997; Banks and Williams, 2004; Culumber et al., 2017).

### ***Lactobacillus wasatchensis***

*Lactobacillus wasatchensis* WDC04 was originally isolated from a “gassy” cheddar cheese manufactured at the Utah State Creamery. Recently, it has been found to be present in cheeses with unwanted gas formation from multiple states from the Midwest and Western states. It was discovered on an MRS agar plate after incubation for 35 days at 6°C (Ortakci et al., 2015b, Culumber et al., 2017). This bacterium is a slow-growing OHF NSLAB. Typical NSLAB grow quickly (within 2 d) at 30 or 37°C with glucose as a carbohydrate source. *Lb. wasatchensis* is atypical taking 5 d to grow small (0.5 mm-1.0 mm), white, circular, smooth colonies. The optimal growth temperature is 23 to 25°C with very minimal growth at 37°C, and *Lb. wasatchensis* prefers ribose as an energy source. Galactose, a six carbon sugar, has also been shown to be co-utilized with ribose, but when utilized as an energy source, a carbon is cleaved off and released as CO<sub>2</sub> that causes gassiness and blowing packages in aging cheese (Ortakci et al., 2015b; Oberg et al., 2016; Culumber et al., 2017).

*Lactobacillus wasatchensis* has also been shown to grow well in an environment characteristic of the storage and aging of cheese. Growth was reported at up to 5.0% salt concentration. It has been shown to grow at storage temperature of 6 and 12°C and up to 30°C. *Lactobacillus wasatchensis* grows both in anaerobic conditions, hence its survival and growth in cheese and it is also aerotolerant (Oberg et al., 2016; Culumber et al.,

2017). Thus, *Lb. wasatchensis* is shown to not only survive the harsh conditions of the cheese environment but also thrive.

### **Salt Tolerance**

During the initial characterization of the growth attributes of *Lb. wasatchensis*, it was observed that it grows well at pH 5.2 and although its rate of growth slows down it can grow to high numbers with up to 5% salt (i.e., the normal salt-in-moisture levels for aged cheddar cheese). At pH 6.5, the results were similar (Ortakci et al., 2015b). Growth at 5% salt is not abnormal for NSLAB found in cheddar cheese and often up to 6% is required to inhibit growth (Lane et al., 1997).

### **Carbohydrate Metabolism**

*Lactobacillus wasatchensis* has shown to preferentially metabolize five carbon sugars, such as ribose, using the pentose phosphate pathway but can also metabolize six carbon sugars (hexoses) using the Leloir pathway. *Lactobacillus wasatchensis* can grow using galactose as the only sugar available, but only slowly and not to high levels. When a five-carbon sugar such as ribose is present, *Lb. wasatchensis* will grow at its maximum rate with an optimum temperature of ~23°C. Surprisingly, when ribose and galactose are both present, growth of *Lb. wasatchensis* also continues at its maximum rate without measurable gas production because galactose is not used to produce energy (Ortakci et al., 2015b). Based upon a genome analysis it was proposed that *Lb. wasatchensis* contains metabolic pathways that allows it to use ribose for its energy production while galactose is used for cell wall synthesis. Galactose, a six carbon sugar, can be utilized as an energy source but first a carbon is cleaved off which eventually goes to produce CO<sub>2</sub>



and cause gassiness and blowing packages in aging cheese, suggesting gas production occurs once growth slows or stops (Ortakci et al., 2015b).

### **Thermotolerance**

In the initial studies done to characterize *Lb. wasatchensis*, there was some indication that this bacterium may have some survival at pasteurization temperatures. Using a laboratory simulation of pasteurization in which  $6 \times 10^6$  CFU/mL of *Lb. wasatchensis* was added to test tubes of sterile milk at 63°C (145°F) and 72°C (161F) and held for 30 min and 15 s, respectively, and then cooled. For the 63°C treatment (equivalent to batch pasteurization), there was no recovery of any *Lb. wasatchensis*. However, for the 72°C treatment (equivalent to HTST pasteurization), the numbers of *Lb. wasatchensis* were reduced but only by 4.5 logs, which if correct, would be problematic for long operations of the pasteurizer and suggestive that *Lb. wasatchensis* cells may survive pasteurization and contribute to the NSLAB levels in the cheese vat (Ortakci et al., 2015b).

### **Cell Lysate Growth**

Based on the knowledge that *Lb. wasatchensis* metabolizes ribose as the primary energy source (Ortakci et al., 2015b), a study was performed to determine whether or not the bacterium could grow using lactococcal cell lysate (Ortakci et al., 2015c). During cheese ripening, autolysis of starter cells occurs releasing cellular components, including ribose and N-acetyl amino sugars. In particular, ribose is released in larger quantities than all the other sugars released by the starter LAB (Thomas, 1987, Rapposch et al., 1999). *Lactococcus lactis* ssp. *lactis/cremoris* bacterial cultures were grown, lysed, and

incubated with *Lb. wasatchensis* in absence of other carbohydrates. Growth of *Lb. wasatchensis* was considerably higher when grown with the lactococcal cell lysate than when solely supplemented with 1% ribose in MRS broth (Ortakci et al., 2015b). Growth reached 2.49 at OD<sub>600</sub>, therefore, lysis of the starter LAB *Lc. Lactis* ssp. *lactis/cremoris* provides an ample supply of exploitable energy that would allow *Lb. wasatchensis* to grow (Ortakci et al., 2015c)

### **DNA Extraction Methods**

In recent years, molecular and nucleic acid-based approaches have become common place to studying both starter LAB and NSLAB in both milk and cheese systems (Pega et al., 2016). Some of these approaches include: DNA microarrays, next generation sequencing and PCR based approaches (O Sullivan et al., 2013). Although each approach is different, they all rely on successful DNA extraction. In the case of a cheese system, there have been a variety of kits and extraction methods used, such as the Nucleospin Food Kit, DNAzol BD Reagent, Wizard SV Genomic DNA Purification System, Dneasy Tissue Kit, Puregene Yeast and Gram Positive Bacteria Kit, DNA Powersoil Kit, and the phenol-chloroform extraction method (Amagliani et al., 2007; Cezar et al., 2016; Pega et al., 2016).

The phenol-chloroform extraction method has been used extensively and shown to work well for cheese (Feligini et al., 2005; Monnet et al., 2006; Sambrook and Russell, 2006; O Sullivan et al., 2013; Dugat-Bony et al., 2015; Monnet et al., 2016). One of the greatest challenges of performing a DNA extraction is to produce a sufficiently high level of quality product. Cheese contains a high concentration of fat and protein which makes it difficult to get a clean product. The phenol-chloroform method helps mitigate the

problems encountered with extracting DNA from a cheese system by first disrupting the fat/protein matrix when a cheese sample is homogenized with salts (i.e., sodium citrate) and detergents (i.e., guanidine thiocyanate). Samples are also subjected to an enzyme treatment of proteinase K to lyse the bacterial cells and release their DNA. The DNA is purified with phenol-chloroform and precipitated with ethanol (Jany and Barbier, 2008; O Sullivan et al., 2013; Dugat-Bony et al., 2015).

### **Quantitative Real-Time Polymerase Chain Reaction**

Traditional methods to studying LAB involve plating the selected bacteria on synthetic media that contains certain nutrients for the growth of these bacteria. This method is time-consuming and has a number of limitations in its approach, especially when dealing with a cheese system where many bacteria are present (O Sullivan et al., 2013, Ferrario et al., 2017). In contrast to conventional methods, quantitative real-time polymerase chain reaction qPCR is a fast, accurate, and highly sensitive approach to studying and quantifying LAB individually or in aggregate (O Sullivan et al., 2013). qPCR also has the advantage of quantifying lysed and non-viable bacterial cells that would not be detected with standard spread plates (O Sullivan et al., 2013).

The basis behind PCR is to amplify a region of the genome within the target bacteria that is unique to said bacteria, thereby making copies of only the gene of interest and producing a pure product. It is common practice to use a part of the 16S rRNA gene as this is present in all bacteria and contains species-specific sequences which are small and can be utilized (Justé et al., 2008; Quigley et al., 2011). Amplification of the gene of interest is done by developing a forward and reverse primer that are specific to the

bacterium and anneal at highly conserved areas both before and after the target gene (Justé et al., 2008; O Sullivan et al., 2013).

Quantitative PCR differs from traditional PCR amplification by tagging the gene of interest with a fluorescent marker (typically SYBR Green or BOXTO) allowing the researcher to calculate the number of copies of the gene replicated in real-time and thereby determine the quantity of bacteria present. Traditional PCR is visualized on gel agarose as bands using electrophoresis and does not provide much information on the quantity of target DNA in the mix. The qPCR products can be considered absolute or relative. Absolute qPCR products are quantified based on the relation of the level of fluorescence compared to the calibration curve, which is made with known amounts of extracted DNA or PCR products. Relative qPCR products are quantified based on the presence of another housekeeping gene found within the bacterial genome (Monnet et al., 2006; O Sullivan et al., 2013).

## MATERIALS AND METHODS

### Materials

Lactobacilli MRS (**MRS**) broth came from Hardy Diagnostics (Santa Maria, CA); Oxyrase<sup>®</sup> for Broth, sodium chloride, glucose, fructose, 0.2 µm membrane filters, sodium citrate, potassium phosphate, acetate-EDTA, ethanol, and dipotassium phosphate were from Fisher Scientific Inc. (Fair Lawn, NJ); Tris-HCl and proteinase K were from Fisher Bioreagents (Pittsburgh, PA); bacteriological agar, proteose peptone, beef extract and yeast extract came from Becton Dickinson and Co. (Sparks, MD); ribose and GasPak EZ pouches were from VWR (Solon, OH); Tween-80, M-17 broth, guanidine thiocyanate, sodium dodecyl sulfate, phenol-chloroform-isoamyl alcohol, N-acetylmuramic acid, and N-acetylglucosamine were from Sigma-Aldrich Inc. (St. Louis, MO); Falcon 48 transparent micro-well plates with lids were manufactured by Corning Inc. (Corning, NY); sodium acetate and ammonium citrate were purchased from Mallinckrodt Baker Inc. (Paris, KY); magnesium sulfate was from Alfa Aesar Inc. (Heysham, UK); manganese sulfate, galactose, and lactose came from J.T. Baker Chemical Co. (Phillipsburg, NJ); milk was obtained from the Aggie Creamery at Utah State University (Logan, UT); 0.1-mm zirconium glass beads came from BioSpec Products (Bartlesville, OK); 2-mL microcentrifuge tubes were from VWR (Radnor, PA); and filtered stomacher bags were purchased from Whirl-Pak<sup>™</sup>, Nasco (Fort Atkinson, WI).

*Lactobacillus wasatchensis* (strain WDC04) cultures were obtained from -80°C freezer stocks from Weber State University (Ogden, UT). *Lc. lactis*, ssp. *lactis* and *cremoris* and *Streptococcus thermophilus* cultures came from Vivolac Cultures Corporation (Greenfield, IN). *Lactobacillus helveticus* culture was from Chr. Hansen

Inc. (Milwaukee, WI).

### **Growth and Storage of *Lactobacillus wasatchensis***

Working cultures of *Lb. wasatchensis* WDC04 were stored in MRS broth supplemented with 1.5% ribose (**MRS-R**) at ~25°C. Every 2 to 3 d fresh tubes of MRS-R were inoculated with *Lb. wasatchensis* from the 25°C working cultures to keep the bacterium in logarithmic growth. Every 3 to 4 wk new working cultures were started from the -80°C freezer stocks. Frozen cultures were stored at -80°C in 1-mL aliquots supplemented with 20% glycerine. Agar plates were made from MRS-R broth with the addition of 1.5% agar. Spread plates were stored at ~25°C in anaerobic incubation jars with GasPak EZ.

### **Micro-well Plate Preparation**

A fresh batch of *Lb. wasatchensis* was prepared by adding 4 mL of the working culture into 9 mL of MRS-R which was then allowed to incubate for 2 h. Following the incubation, the optical density was adjusted to ~0.900 at 600 nm (OD<sub>600</sub>). Into individual wells of the micro-well plates was added 930 µL of required MRS broth media such with ribose, varying pH, salt concentrations and or carbohydrate restricted (980 µL was used for controls that were not inoculated), 20 µL Oxyrase® (final concentration was 2% (vol/vol)), and 50 µL of *Lb. wasatchensis* broth. Experiments involving carbohydrate sensitivity, all *Lb. wasatchensis* cells were washed twice with phosphate buffer prior to use. All samples, including uninoculated controls, were prepared in triplicate wells on the plate. The lid was then placed on the plate and the plate inserted into an Infinite 200 Pro spectrophotometer (Tecan Production Corp., Chapel Hill, NC).

Spectrophotometer parameters were  $25 \pm 1^\circ\text{C}$ , orbital plate shaking, prior to reading, for 2 s at an amplitude of 3 mm and frequency of 218.3 rpm with a settle time of 5 s. Optical density at 600 nm was measured every 60 min for 48 h with 25 measurements per reading and 4 readings per well in a 2 x 2 pattern at 2.7 mm from the edge of the well. Maximum specific growth rate ( $\mu_{\text{max}}$ ) was calculated as the steepest linear portion of individual growth curves.

### **Thermotolerance**

A preliminary experiment was run to determine at what times samples should be collected from the HTST heat exchanger as the milk left the system. The pasteurizer was run with water until steady-state conditions were reached. The balance tank was allowed to drain and a 30-L batch of milk was added to the system, followed by water again. Samples were collected at the exit of the cooling section of the pasteurizer 10 s intervals starting at 60 s until the exit stream reverted back to water. Milk composition was determined at Dairy Herd Improvement (Logan, UT) by IR analysis

Determining the thermotolerance of *Lb. wasatchensis* was done when sterile MRS broth was prepared with 2% ribose and a 1% inoculum of *Lb. wasatchensis* was added to four separate 3-L bottles of broth. The bacteria was grown for 3 d at  $\sim 25^\circ\text{C}$  and then pelleted down in 250-mL bottles at  $4^\circ\text{C}$  at  $9150 \times g$  for 10 min. Bacterial cells were re-suspended in UHT milk and added to four separate 30-L batches of milk which had been previously pasteurized at  $62.8^\circ\text{C}$  ( $145^\circ\text{F}$ ) for 30 min. The *Lb. wasatchensis* bacteria were allowed an hour to acclimate to the milk environment at refrigeration temperatures before being run through the pasteurizer at either  $69.4$  ( $157^\circ\text{F}$ ),  $71.7$  ( $161^\circ\text{F}$ ),  $73.9$  ( $165^\circ\text{F}$ ), or  $76.1^\circ\text{C}$  ( $169^\circ\text{F}$ ). Triplicate samples were collected from the four batches of milk both pre

and post-pasteurization at 180, 200, and 220 s. A 1/10 dilution series was performed starting with no dilution up to  $10^{-7}$  and samples were plated on MRS-R agar and grown anaerobically at  $\sim 25^{\circ}\text{C}$  in jars containing GasPak EZ pouches. Colony counts were performed at 2, 5, and 10 d increments to determine the percent survival of *Lb. wasatchensis*.

### **Carbohydrate, salt, and pH**

***Carbohydrate restricted Media.*** Glucose, lactose, fructose, galactose, ribose, N-acetylglucosamine, and N-acetylmuramic acid each were prepared as 20% (wt/vol) stock solutions and filter sterilized with 0.2- $\mu\text{m}$  pore size membranes. Carbohydrate-restricted MRS (**CR-MRS**) broth was prepared by adding 10.0 g proteose peptone No. 3, 10.0 g beef extract, 5.0 g yeast extract, 1.0 g Tween-80, 2.0 g ammonium citrate, 5.0 g sodium acetate, 0.1 g magnesium sulfate, 0.5 g manganese sulfate, and 2.0 g dipotassium phosphate to 1 L of deionized water. The CR-MRS was lowered to a pH of 5.2 with HCl and 4.5% salt was added to simulate a typical cheese environment. From each carbohydrate stock solution, 7.5 mL was taken and added to 92.5 mL of CR-MRS for a final volume of 100 mL containing 1.5% (wt/vol) of each carbohydrate.

***Salt Tolerance.*** Based on previous work (unpublished data), a salt range from 5.4% to 6.0% with increments at every 0.1% NaCl was selected to determine growth characteristics of *Lb. wasatchensis* at the upper limits of salt tolerance. One-hundred milliliter quantities of MRS-R broth at pH 6.5 and pH 5.2 were made at each of these salt level (plus a batch with no added salt). In addition, MRS-R broth at pH and salt combinations typical for cheddar cheese were prepared: pH 5.2 and 5.5 at salt levels of



3.5, 4.5, and 5.5%. All samples were analyzed in triplicate for growth of *Lb.*

*wasatchensis* as described above.

**pH.** Aliquots of MRS-R broth covering a broad pH range (4, 5, 6, 7, 8, and 9) were prepared, as well as at pH levels characteristic of cheese and milk, i.e., pH 5.2 and pH 6.6, respectively. All samples were analyzed in triplicate for growth of *Lb.*

*wasatchensis* as described above

### **Bacterial Cell Lysate**

*Lactococcus lactis* ssp. *lactis* and *cremoris* and *St. thermophilus* were inoculated into M-17 broth containing lactose (**M17-L**). *Lactobacillus helveticus* DVS culture was inoculated into MRS broth. *Lc. lactis* and *Lc. cremoris* were grown at 30°C and *St. thermophilus* and *Lb. helveticus* were grown at 37°C. An overnight culture of each was taken and plated on M17-L or MRS-R agar plates to determine CFU/mL. One-mL aliquots were placed into 2-mL screw-cap tubes containing 0.25 g of sterilized glass beads. Samples were lysed using a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK) by alternating 6 bursts of 45 s each with 10 min of cooling in an ice bath. After disruption of the bacterial cells, samples were centrifuged for 15 min at 21,000 x g. A 0.1-mL sample of the cell lysate was plated on M17-L or MRS-R agar to determine the effectiveness of the cell lysis procedure. The supernatant was collected and filter sterilized using 0.2 µm pore size membrane filters. Some of the filtrate was mixed with CR-MRS broth at a 1:1 ratio. Additionally, the *Lc. lactis* filtrate was also combined with the CR-MRS broth at a 1/10 and 1/100 dilution. Both the filtrate alone and filtrate-broth mixture were loaded into a 48 micro-well plate and prepared following the procedure for micro-well plate preparation. In addition, both lysate solutions were inoculated with 50

$\mu\text{L}$  of *Lb. wasatchensis* and incubated anaerobically at 25°C for 10 d. Absorbance at 600 nm was taken at day 0, 5, and 10 d. Controls were run using sterilized ultrapure water in place of the cell lysate and all samples were run in triplicate.

### **Starter LAB in Cheese**

Old Juniper cheddar cheese was manufactured at Utah State Creamery and used to determine initial starter LAB (*Lc. lactis*) and adjunct (*Lb. helveticus*) bacterial counts. Samples of cheese were taken before salting of the curd, immediately following overnight pressing, and after 1 d of storage at 4.4°C. Eleven grams of cheese was taken from each sample group and homogenized in 99 mL of 2% sodium citrate in a stomacher at 230 rpm for 3 min. A dilution series was performed and 0.1-mL aliquots were used to make spread plates on both MRS and M17-L plates. The MRS plates were incubated at 37°C and the M17-L plates at 30°C for 24 h prior to colony counts being obtained. All samples were run in triplicate.

### **Phenol-Chloroform Nucleic-Acid Extraction**

Initially, to test the effectiveness of the phenol-chloroform extraction method in recovering DNA, 11 g of cheddar cheese were homogenized with 99 mL of sodium citrate at 260 rpm for 2 min in a stomacher. Prior to stomaching, *Lb. wasatchensis* bacterial cells were inoculated into the stomacher bag at 100  $\mu\text{L}$ , 500  $\mu\text{L}$ , 1.0 mL, and 10 mL quantities, and the extraction proceeded as normal. In conjunction with every DNA extraction performed, spread plates were performed on MRS-R agar to determine the starting quantity of bacteria present within each sample and compare this value with the amount of DNA extracted.

Once the 11 g of cheese was homogenized in 99 mL of sodium citrate, 1 mL of the homogenized cheese was collected into 2-mL microcentrifuge tubes and centrifuged at 12,000 x g for 5 min. The supernatant was discarded and another 1 mL of homogenized cheese was added and centrifuged again followed by discarding the supernatant. The pellet was then re-suspended with 400  $\mu$ L of 4 M guanidine thiocyanate in 0.1 M Tris-HCl and 50  $\mu$ L of 10% sodium dodecyl sulfate. The solution was transferred to a 2-mL tube with 350 mg of 0.1 mm sterile zirconium beads. Afterwards, 30  $\mu$ L of 20 mg/mL proteinase K solution was added to the tube and it was allowed to incubate on a heat block for 2 h at 55°C. Following the incubation, 150  $\mu$ L potassium phosphate buffer 0.2 M pH 8, 300  $\mu$ L 50 mM acetate-10 mM EDTA buffer pH 5, and 500  $\mu$ L of phenol-chloroform isoamyl alcohol (25:24:1 pH 8) were added and the tube was subjected to bead beating 3X for 45 s with 5 min of ice between cycles. Then, the samples were centrifuged at 10,000 x g for 2 min whereupon the aqueous phase was removed and added to a new microfuge tube. Another 300-500  $\mu$ L of phenol-chloroform was added and the sample was centrifuged at 10,000 x g for another 2 min. Approximately 500  $\mu$ L of the aqueous phase was removed and added to a fresh 2-mL microfuge tube with 800  $\mu$ L of 95% cold ethanol and 50  $\mu$ L of 3 M cold sodium acetate. The sample tubes were stored at -20°C overnight. Following the overnight incubation, the samples were centrifuged at 10,000 x g for 5 min, with the possibility of another 5 min if no pellet was observed. The ethanol was removed and the samples were washed with 75% cold ETOH by briefly vortexing the tube followed by centrifugation at 10,000 x g for 5 min. The ethanol was drained off and the samples is allowed to air dry. Any extracted product was stored in 10 mM Tris and put in the freezer. The amount of DNA

extracted from each sample (based on absorbance at 260 nm ( $A_{260}$ )) and respective purity (based on ratio of  $A_{260}$  and absorbance at 280nm ( $A_{280}$ )) were determined by using a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA).

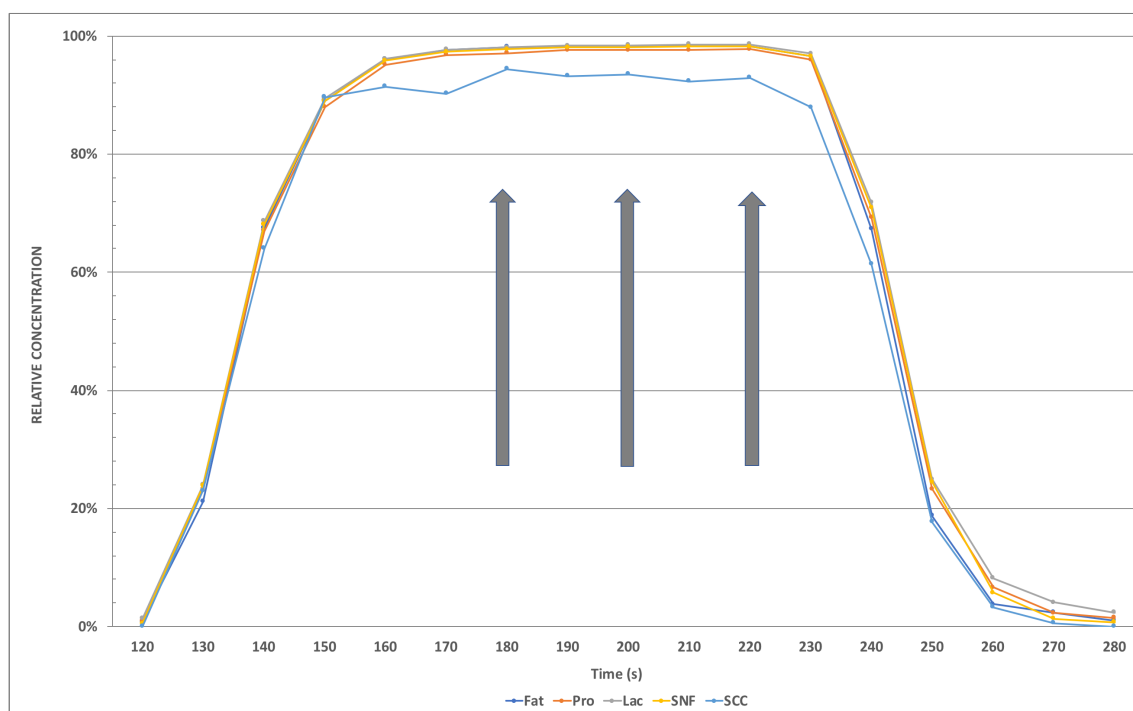
### **Quantitative PCR and Primers**

DNA samples were amplified using a QuantStudio quantitative real-time PCR using a SYBR Green fluorescent tag. The standard calibration curve was made using the 16S rRNA PCR product extracted from a pure culture of *Lb. wasatchensis* and amplified using the 27F and 1492R primers (Culumber et al., 2017). Cheese samples were run using 20  $\mu$ L per reaction, consisting of 2  $\mu$ L of DNA, 7  $\mu$ L sterile water, 10  $\mu$ L master mix, and 0.5  $\mu$ L of both the forward and reverse primers. The qPCR primers had been developed using the NCBI PrimerBlast tool by analyzing the 16S rRNA gene from *Lb. wasatchensis*. Based on product length, melting temperatures, self-complimentarity, and non-target matches, two primer pairs were selected. The primer pair used to produce the absolute qPCR product is 82F (5'-ATCATTCGCCCCATTCCAGG-3') and 256R (5'-GTTACAATGCCGCTGACGAC-3'). It is assumed the qPCR product appears only once per 16S rRNA gene sequence. This primer pair has been optimized and tested for specificity against 19 other common lactobacilli, one lactococci, and a leuconostoc, indicating it has a high specificity for only *Lb. wasatchensis* and will not amplify other bacterial DNA (unpublished data by Craig Oberg, Department of Microbiology, Weber State University, UT).

## RESULTS AND DISCUSSION

### PART 1. THERMOTOLERANCE

As shown in Figure 1, milk began to exit the pasteurizer after 120 s and continued until after 240 s. When a 30-L batch of milk is processed, the milk composition only reached ~98% of its original value indicating some slight mixing with the pre- and post-rinse water. Times for sampling milk during the subsequent thermotolerance test were selected at 180, 200, and 220 s. Since reduction in *Lb. wasatchensis* would be measured on a logarithmic scale, the slight dilution would be insignificant and all three sample times would have the same dilution.



**Figure 1.** Relative concentration of fat, protein (pro), lactose (lac), solids-not-fat (SNF) and somatic cell count (SCC) of the exit stream from the pasteurizer compared to composition of the 30 L of milk prior to pasteurizing. Arrows indicate sample times that were selected for triplicate sampling during the thermotolerance test.

**Table 1.** Destruction of *Lactobacillus wasatchensis* upon processing through a high-temperature-short-time plate heat exchanger with 15-s hold time, n = 3.

Set Temperature	Number of <i>Lactobacillus wasatchensis</i> <sup>1</sup>	
	Before Heating	After Heating
	------(CFU/mL)-----	
69.4°C (157°F)	6.0 x 10 <sup>8</sup>	≤10
71.7°C (161°F)	6.0 x 10 <sup>8</sup>	≤10
73.9°C (165°F)	4.5 x 10 <sup>8</sup>	≤10
76.1°C (169°F)	5.7 x 10 <sup>8</sup>	≤10

<sup>1</sup>Cell numbers counted through 10 d of anaerobic incubation at 23°C

Each 30-L batch of inoculated milk contained 10<sup>8</sup> CFU/mL of *Lb. wasatchensis* prior to pasteurization. After passing through the heat exchanger, there was no measurable survival of bacteria (≤10 CFU/mL) at any of the temperatures, including a sub-pasteurization treatment of 69.4°C for 15 s (Table 1). This represent >7 log reduction which is greater than a previous laboratory pasteurization test (Ortakci et al., 2015b) in which broth heated to 72°C was inoculated with *Lb. wasatchensis* and then cooled after 15 s. In that study, there was only a 4.5 log reduction.

*Lactobacillus wasatchensis* thus has a lower heat tolerance than initially thought, although the log reduction using a plant-scale heat exchanger, was similar to the reduction observed in milk held at 63°C for 30 min (Ortakci et al., 2015b). This can be explained by the greater, uniform heat exposure, turbulence, and mixing the *Lb. wasatchensis* cells receive in an industrial setting using a heat exchanger. Bacterial cells are exposed to heating as the milk passes through the warming regeneration section of the heat exchanger, then are held at the minimum of 72°C for 15 s, before being cooled down in the cooling section of the heat exchanger. In the laboratory method, the bacteria were

not added until after the milk reached 72°C, and cooling below 60°C occurred within a few seconds. Further, there was no mixing or turbulence with the test tube method. It therefore appears that the presence of *Lb. wasatchensis* as part of the NSLAB microbiota in cheese comes as an environmental post-pasteurization contaminant rather than as a result of its survival during pasteurization.

### **Summary**

Based off of our findings, the survival of *Lb. wasatchensis* cells is dependent on their exposure to a heat treatment rather than strictly an increase in temperature. When bacterial cells were exposed to HTST pasteurization conditions in a test tube at 72°C there was only a 4.5 log reduction. However, bacterial cells run through an industrial heat exchanger with an increased heat exposure due to the regeneration sections had a greater than 7 log reduction with no survival observed, even at sub-pasteurization temperatures of 69.4°C. It also appears the mixing and turbulence caused as the milk passes through a continuous HTST pasteurizer aids in the inactivation of the *Lb. wasatchensis* cells. Therefore, cheese manufacturers would be advised to look at possible causes of post-pasteurization contamination to determine how the bacteria are entering the cheese vats. Possible methods could be cross-contamination, biofilm formation, or through aerosolizing.

## PART 2. GROWTH CHARACTERISTICS

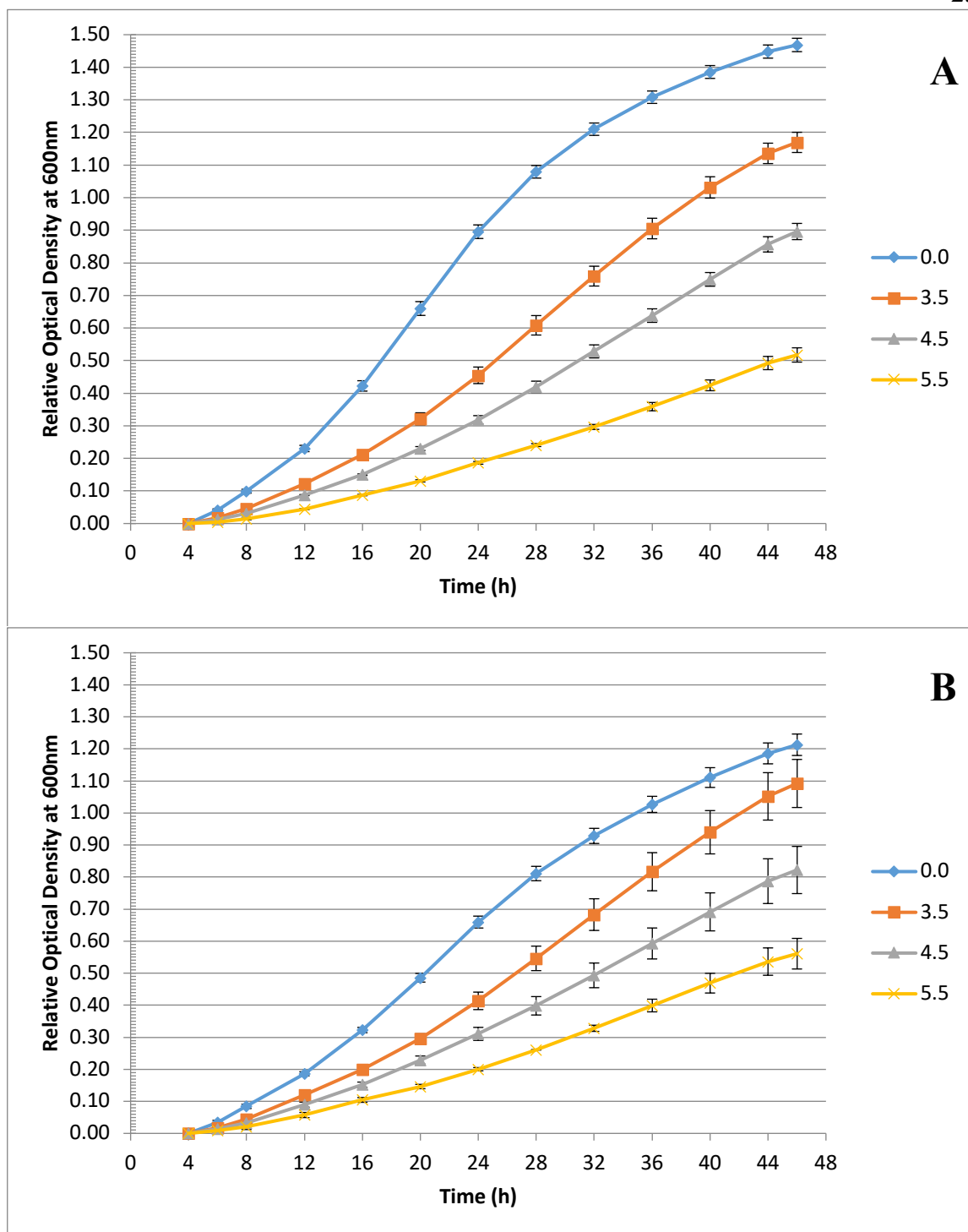
### Monitoring Growth of *Lb. wasatchensis* Using 48-well Plates

In preliminary studies (data not shown) it was observed that *Lb. wasatchensis* did not always grow well when it was inoculated into the wells of a 48-well plate. It was determined that the surface area of liquid in the wells compared to the volume was too large to produce an anaerobic environment within the liquid media (personal communication, Craig Oberg, Department of Microbiology, Weber State University, UT). Consequently, Oxyrase<sup>®</sup> for Broth was added to the wells to create a micro-anaerobic environment. As shown in Figure 2, adding Oxyrase<sup>®</sup> was effective in increasing the rate and extent of growth of *Lb. wasatchensis* in the micro-wells. There was also less variation among replicates as seen with smaller error bars. Therefore, it was determined Oxyrase<sup>®</sup> would be used in the subsequent experiments.

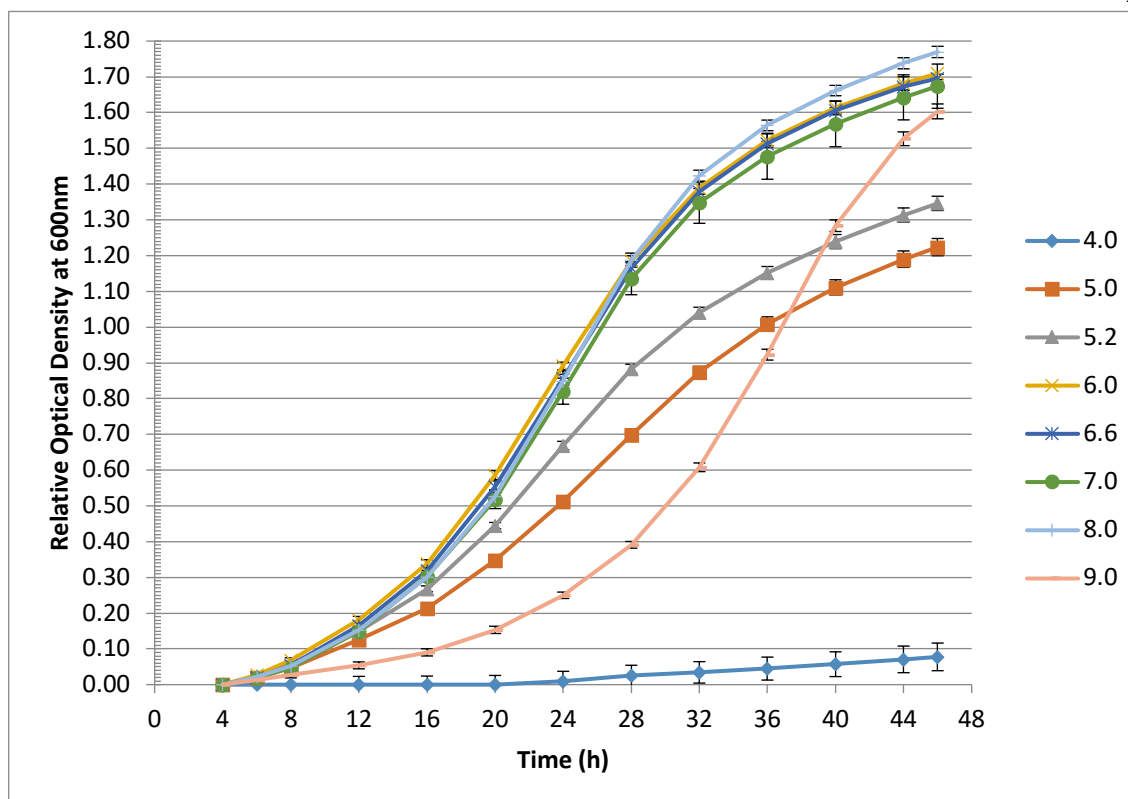
### pH Tolerance

It was observed that *Lb. wasatchensis* is well-suited for growing at a variety of pH levels. The highest optical density was seen at pH 8.0, followed closely by pH 6.0, 6.6, and 7.0. Growth was also observed at pH 5.0 and 5.2 and also up to pH 9.0 but minimal growth was seen at pH 4.0 (Figure 3). Although the initial growth of *Lb. wasatchensis* was slower at pH 9, indicative of a longer lag phase as the bacterial cells adjusted to the increased basic environment, it had the highest specific growth rate compared to the other pH levels (Table 2). The initial stress response undergone by the bacterial cells to survive in the basic environment may activate additional genes that appear to protect and help accelerate growth in the new environment after 24 h.





**Figure 2.** Growth of *Lactobacillus wasatchensis* at 25°C in MRS-Ribose broth at pH 5.2 supplemented with 3.5, 4.5, and 5.5% salt, based on optical density measured at 600 nm both with Oxyrase® enzyme (A) and without Oxyrase® (B), error bars = SE, n = 3.



**Figure 3.** Growth of *Lactobacillus wasatchensis* at 25°C in MRS broth supplemented with 1.5% ribose as a function of pH levels based on optical density measured at 600 nm, error bars = SE, n = 3.

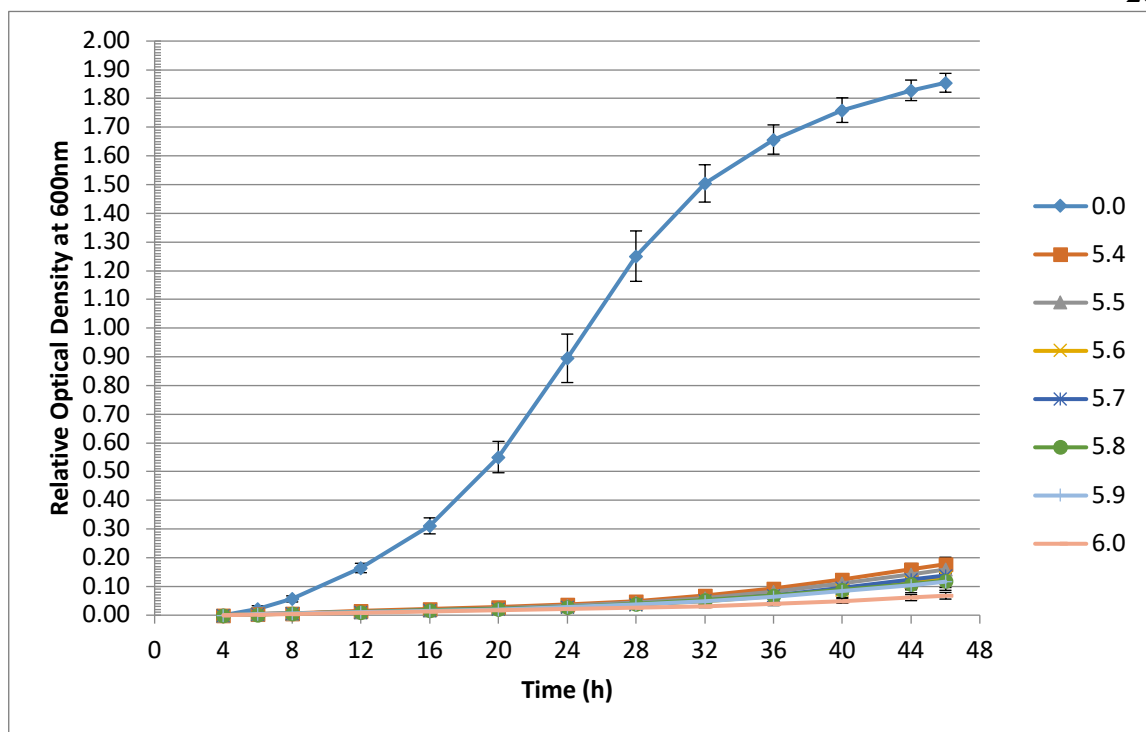
**Table 2.** Maximum specific growth rate ( $\mu_{\max}$ ) of *Lactobacillus wasatchensis* as a function of pH when grown at 25°C in MRS broth supplemented with 1.5% ribose based on optical density measured at 600 nm ( $OD_{600}$ ).

pH	$\mu_{\max}$ OD <sub>600</sub> /h
4.0	0.015
5.0	0.175
5.2	0.224
6.0	0.305
6.6	0.310
7.0	0.315
8.0	0.338
9.0	0.361

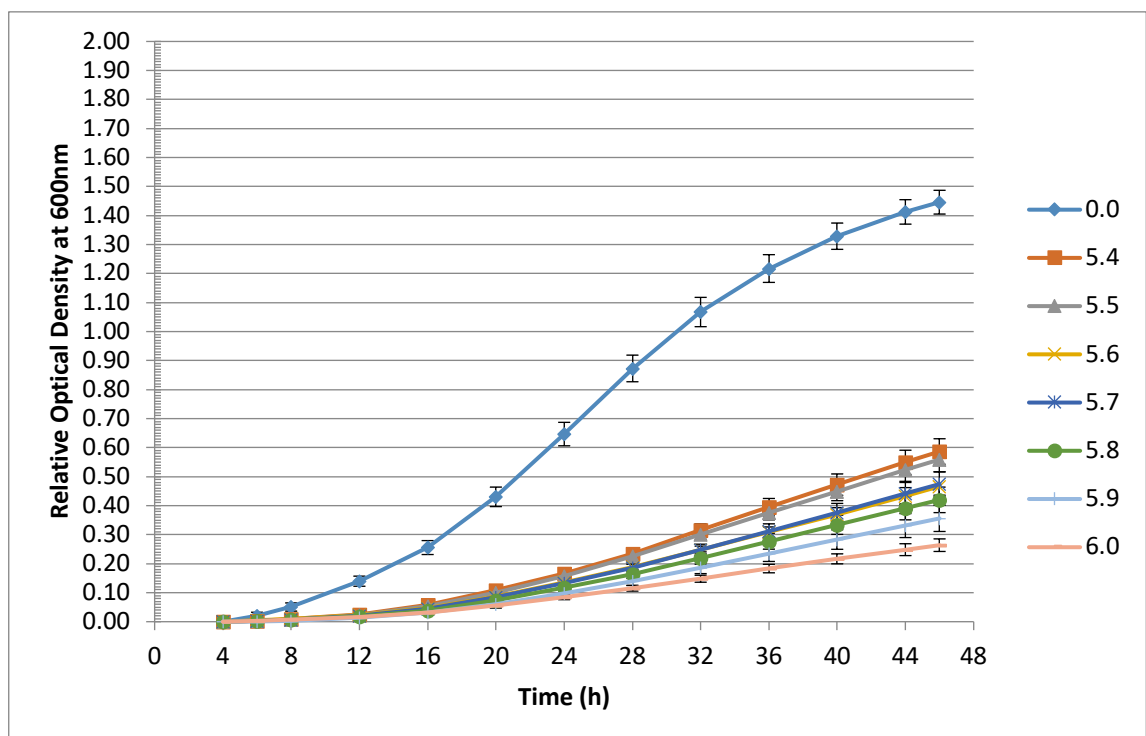
The growth observed at pH 9 could also indicate a low level of contamination with another bacteria, as lactobacilli are generally not recognized as growing at pH 9.

### **Salt Tolerance**

When *Lb. wasatchensis* was grown at pH 5.2, adding salt slowed down the rate of growth (Figure 2) but even with 5.5% salt it still reached OD<sub>600</sub> of 0.5 after 46 h incubation. However, the bacterium is more sensitive to salt when it is at pH 6.5, and less growth occurred. With a salt content of 5.4%, OD<sub>600</sub> was only ~0.2 after 46 hours (Figure 4). Increasing salt concentration further slowed growth. At pH 6.5 and with no salt addition, *Lb. wasatchensis* grew to an optical density of ~1.85. At pH 5.2, growth of *Lb. wasatchensis* with no salt addition grew to an optical density of ~1.44 (Figure 5). The  $\mu_{\max}$  at pH 5.2 was lower than at pH 6.5, going from 0.356 to a 0.226 when *Lb. wasatchensis* was grown without added salt. (Table 3). Both the growth curves and corresponding  $\mu_{\max}$  for the salt range 5.4 to 6.0% at pH 5.2 had a >50% increase when compared to their counterparts at pH 6.5. This suggests when *Lb. wasatchensis* is grown in the absence of salt, it grows to higher numbers at a pH closer to neutral (Figure 4 compared to Figure 5). However, when pH is lower (such as pH 5.2 which is typical of cheese) it can tolerate higher salt levels. As was mentioned in regards to *Lb. wasatchensis* growth at pH 9, when the stress response is activated, due to high or low pH levels (i.e., pH 5.2) or increased salt concentrations, it appears additional genes are turned on causing a bioprotective effect which help the bacteria adapt and facilitate better growth than when grown at a pH of 6.5 with the same salt concentrations.



**Figure 4.** Growth of *Lactobacillus wasatchensis* at 25°C in MRS-Ribose broth at pH 6.5 supplemented with 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, and 6% NaCl over 46 h, based on optical density measured at 600 nm, error bars = SE, n = 3.



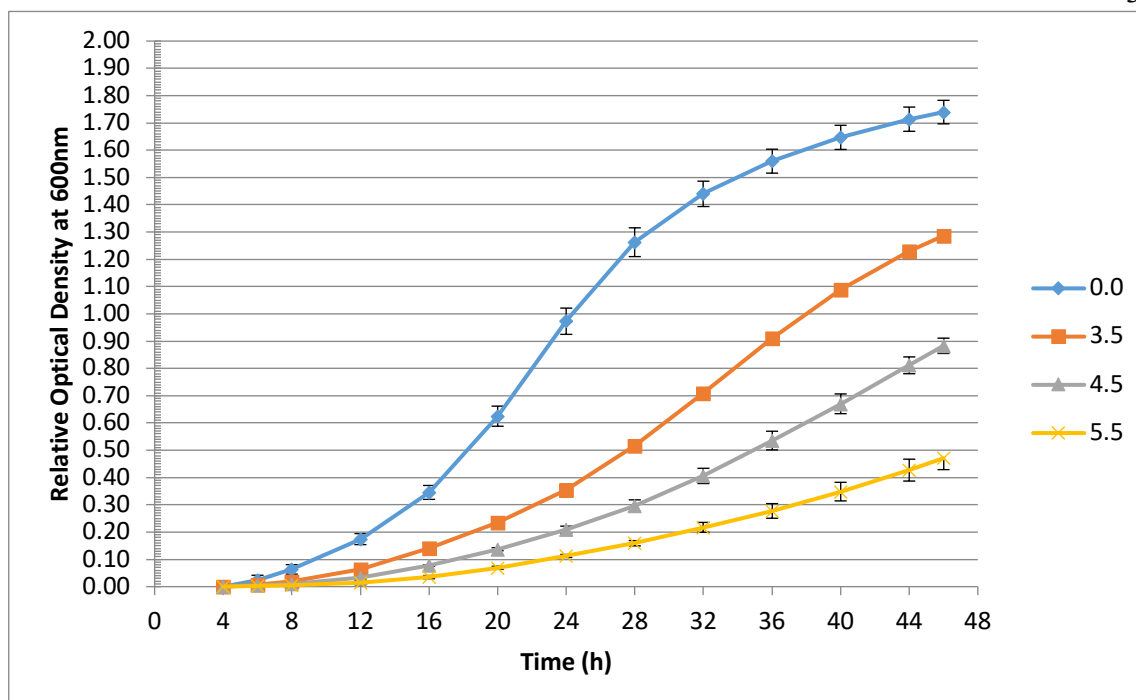
**Figure 5.** Growth of *Lactobacillus wasatchensis* at 25°C in MRS-Ribose broth at pH 5.2 supplemented with 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, and 6.0% NaCl over 46 h, based on optical density measured at 600 nm, error bars = SE, n = 3.

**Table 3.** Maximum specific growth rate ( $\mu_{\max}$ ) of *Lactobacillus wasatchensis* at 25°C as a function of varying salt concentrations in MRS broth containing 1.5% ribose at pH 5.2 and 6.5 supplemented with salt at 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, and 6.0% NaCl, based on optical density measured at at 600 nm ( $OD_{600}$ ).

% NaCl	$\mu_{\max}$	
	pH 5.2	pH 6.5
	$OD_{600}/h$	$OD_{600}/h$
0.0	0.226	0.356
5.4	0.080	0.035
5.5	0.075	0.032
5.6	0.064	0.027
5.7	0.065	0.029
5.8	0.058	0.021
5.9	0.048	0.022
6.0	0.035	0.012

### Growth at Cheese Conditions

Growth of *Lb. wasatchensis* at both pH 5.2 and 5.5 containing 3.5, 4.5, and 5.5% salt is shown in Figures 2A and 6, respectively. This represents, a medium and high pH for cheddar cheese, and a low, medium and high salt-in-moisture content. As already shown, *Lb. wasatchensis* grows quicker when no salt is added and reach  $OD_{600}$  of  $\sim 1.74$  at pH 5.5 and  $\sim 1.48$  at pH 5.2 after 46 h with  $\mu_{\max}$  of 0.348 and 0.238  $OD_{600}/h$ , respectively (Table 4). Then as salt was added, the growth rate slowed down with slightly slower growth occurring at pH 5.2 compared to pH 5.5. Although the growth curves at 3.5, 4.5 and 5.5% salt had not reached stationary phase after 46 h incubation, they likely would have eventually reached the same  $OD_{600}$  as broth with no added salt as shown by Ortakci et al. (2015a). Although growth of *Lb. wasatchensis* at 5.5% NaCl was the slowest, positive growth was still seen after 46 hours and optical density was  $>0.40$  at pH 5.2 and 5.5.



**Figure 6.** Growth of *Lb. wasatchensis* based on optical density measured at 600 nm at 25°C in MRS-Ribose broth at pH 5.5 supplemented with 3.5, 4.5, and 5.5% salt over 46 h, error bars = SE, n = 3.

**Table 4.** Maximum specific growth rate ( $\mu_{\max}$ ) of *Lactobacillus wasatchensis* at 25°C as a function of varying salt concentrations when grown in MRS broth containing 1.5% ribose at pH 5.2 and 5.5 supplemented with salt at 3.5, 4.5, and 5.5% NaCl, based on optical density measured at at 600 nm ( $OD_{600}$ ).

% NaCl	$\mu_{\max}$	
	pH 5.2	pH 5.5
	$OD_{600}/h$	$OD_{600}/h$
0.0	0.238	0.348
3.5	0.154	0.202
4.5	0.111	0.141
5.5	0.068	0.079

These and prior findings indicate *Lb. wasatchensis* is able to grow in cheddar cheese during storage and under ripening conditions. Not only does it grow at a normal cheese pH range of 5 to 6, but also is not completely inhibited by salt at concentrations up to 6% at pH 6.5 or 5.2. There is an increased risk of *Lb. wasatchensis* growth and subsequent gas production at a salt content  $\leq 3.5\%$ . As salt content is increased, the rate of growth of *Lb. wasatchensis* is retarded. Further, as previously noted in Figures 2A and 6, *Lb. wasatchensis* continues to show some positive growth in conditions typical of cheddar cheese at pH 5.2 and 5.5 in up to 5.5% salt. These observations indicate *Lb. wasatchensis* is robust in surviving the conditions characteristic of a cheese environment and would be able to grow in a variety of cheeses, in particular cheddar-type cheeses.

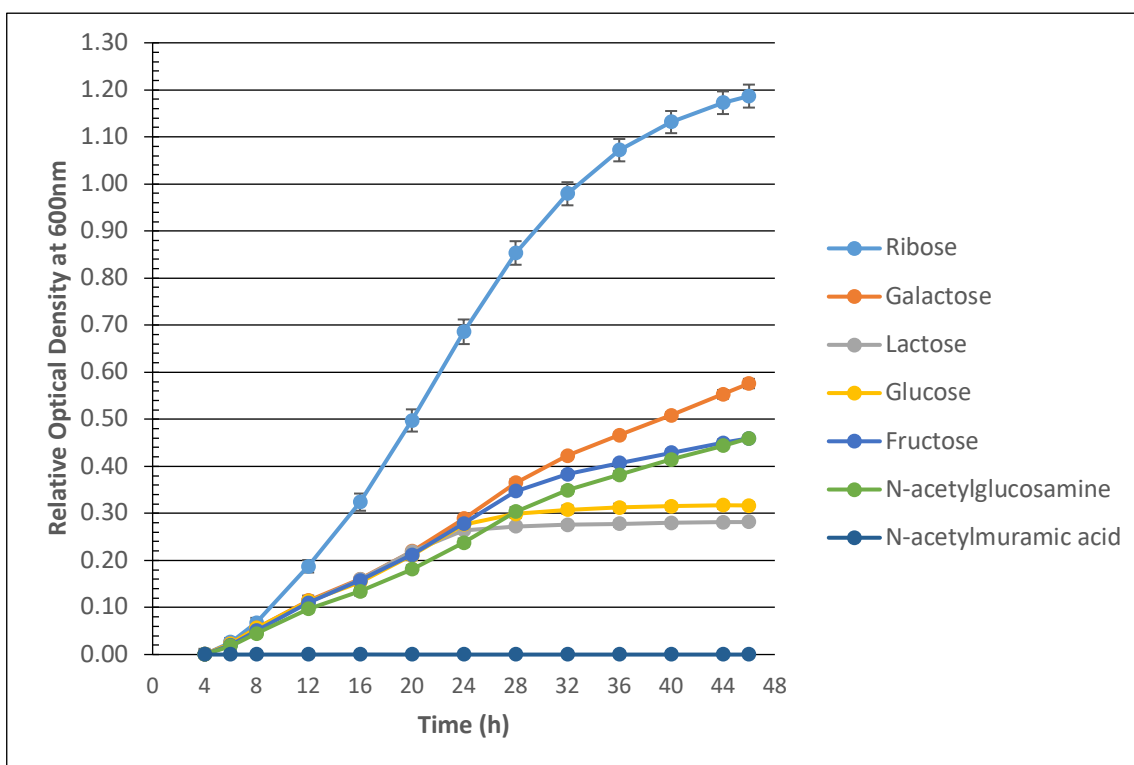
When there is a big variation in salt content during manufacture of cheese, any cheese at the low end of the range (i.e. Monterey cheese with 42% moisture and 1.4% salt) will have faster growth of *Lb. wasatchensis* and be more susceptible to unwanted gas production. To decrease the likelihood of growth of *Lb. wasatchensis* and subsequent unwanted gas production in cheese, manufacturers are advised to select cheese for aging that have salt-in-moisture content towards the upper target level. This coincides with historical information that the best aged cheddar cheese contain between 4.5 and 5.5% salt-in moisture (Lawrence et al., 1987). Maintaining this salt level would help slow growth of NSLAB populations in cheese.

### **Carbohydrate Utilization**

Growth of *Lb. wasatchensis* on various carbohydrate sources is shown in Figure 7. The fastest and highest level of growth occurred with ribose. No growth was observed on the N-acetylmuramic acid. The other sugars (galactose, lactose, glucose, fructose, and

N-acetylglucosamine) had similar  $\mu_{\max}$  (Table 5), however, they grew to different extents. (Figure 7). Since the *Lb. wasatchensis* cells had been washed twice with phosphate buffer prior to inoculation into the various carbohydrate-restricted broth, it is unlikely the differences were because of carryover of nutrients.

With galactose, *Lb. wasatchensis* was still in exponential grown phase after the 46 h of incubation, while growth ceased (or slowed down) after ~24 h for lactose and glucose, and after ~32 h for fructose. These observations suggest all available sugar had been depleted and therefore there was no energy to support further growth. Although initially glucose, lactose, and fructose followed the same growth rate of galactose, assuming energy costs for cell wall synthesis and cell division remain consistent, it is



**Figure 7.** Growth of *Lactobacillus wasatchensis* at 25°C in MRS without added carbohydrate at pH 5.2 with 4.5% NaCl containing various carbohydrate sources, based on optical density measured at 600 nm over 46 h, error bars = SE, n = 3.



**Table 5.** Maximum specific growth rate ( $\mu_{\max}$ ) *Lactobacillus wasatchensis* at 25°C as a function of varying carbohydrates of when grown in MRS broth containing 1.5% ribose at pH 5.2 supplemented with 4.5% NaCl, based on optical density measured at 600 nm ( $OD_{600}$ ).

<b>Carbohydrate source</b>	<b><math>\mu_{\max}</math></b>
	<b><math>OD_{600}/h</math></b>
<b>Ribose</b>	<b>0.174</b>
<b>Galactose</b>	<b>0.076</b>
<b>Lactose</b>	<b>0.059</b>
<b>Glucose</b>	<b>0.065</b>
<b>Fructose</b>	<b>0.069</b>
<b>N-acetylglucosamine</b>	<b>0.066</b>
<b>N-acetylmuramic acid</b>	<b>0.000</b>

hypothesized use of these sugars by *Lb. wasatchensis* has a higher energy cost and faster usage of resources.

Previous research showed how *Lb. wasatchensis* will co-metabolize galactose if present with ribose (Ortakci et al., 2015c). Although there are no conditions where these carbohydrates were tested with ribose, it is possible that *Lb. wasatchensis* could also co-metabolize lactose, glucose, fructose, and N-acetylglucosamine (Figure 7). Fructose is of less importance as it is not expected to be found within cheese.

### **Bacterial Cell Lysate**

When grown on *Lc. lactis*, *Lc. cremoris*, *St. thermophilus*, and *Lb. helveticus* cell lysate solutions, *Lb. wasatchensis* showed growth at d 5 of incubation on all four bacterial cultures. At d 10 of growth, there was an interesting decrease in growth observed (lower OD) on all cell lysate solutions with the exception of *Lb. helveticus* which had an increase in growth. Growth of *Lb. wasatchensis* was also observed in the control tubes at d 5 with a decrease in optical density at d 10.

Growth of *Lb. wasatchensis* in *Lc. lactis* cell lysate and its 1/10 and 1/100 fold

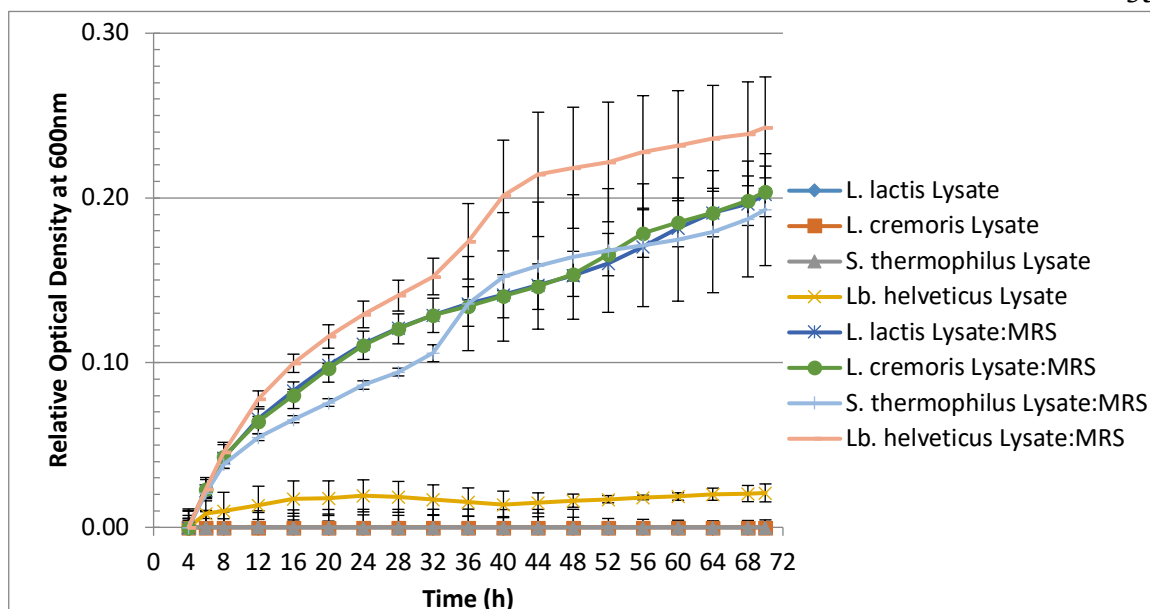
dilutions showed minimal growth at 5 d in the 1/10 and 1/100 samples. At d 10, there was minimal growth. Due to the contradictory data between the two experiment the results are not shown.

When grown in the 48 micro-well plate for 3 d in CR-MRS supplemented with *Lc. lactis*, *Lc. cremoris*, *St. thermophilus*, and *Lb. helveticus* cell lysate filtrate, *Lb. wasatchensis* grew on all four solutions. After 70 h, all four solutions showed positive growth, which would be expected to continue. The best growth was seen when *Lb. wasatchensis* was grown on the *Lb. helveticus* lysate combined with CR-MRS. In addition, growth was also observed on the *Lb. helveticus* lysate solution containing no MRS broth, although growth was relatively unchanging after 16 h and only reached an optical density of ~0.02 (Figure 8). Teusink and Molenaar (2017) showed LAB can use amino acids for growth and energy metabolism, which may explain how the *Lb. wasatchensis* cells were able to have limited growth utilizing solely lysate.

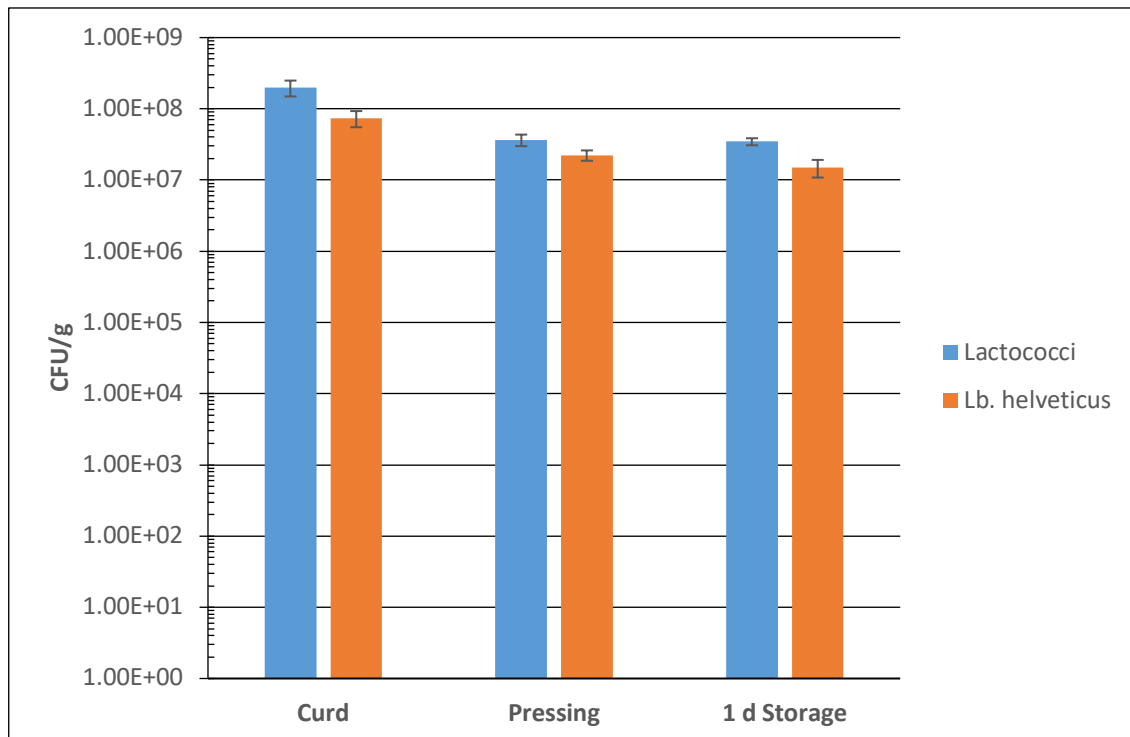
### **Starter LAB in Cheddar Cheese**

The highest number of CFU/g of cheese were observed for both starter lactococci and *Lb. helveticus* when sampled in the curd immediately before salting. Starter lactococci bacterial cells were in more abundance than the adjunct culture with counts being  $>10^8$  CFU/g. After overnight pressing of the cheese curd, there was a decrease in the number of both lactococci and lactobacilli cells with the most noticeable drop being in starter lactococci cells being almost a one log reduction. There was a minimal decrease in bacterial cells after a further day of storage.

Determining the lactococci and lactobacilli bacterial numbers in freshly made cheddar cheese showed the lactococci dominated the microflora initially (Figure 9).



**Figure 8.** Growth of *Lactobacillus wasatchensis* based on optical density measured at 600 nm when grown in cell lysate and in carbohydrate restricted MRS at pH 5.2 supplemented at a 1:1 ratio with starter LAB and adjunct culture cell lysate. Grown at 25°C with measurements taken at every hour for 70 h.



**Figure 9.** Colony Forming Units of lactococci and *Lactobacillus helveticus* per gram of cheese sample taken before salting, immediately following overnight pressing, and after 1 d of storage at 4.4°C.

After salting, both lactococci numbers and lacobaciili numbers dropped. Assuming most lysis was of the starter LAB (*Lc. lactis* subspecies *lactis* and *cremoris*) strains, this represents an 80% lysis of cells happening immediately after cheese manufacturing to support growth of *Lb. wasatchensis* and other NSLAB during cheese storage.

## Summary

*Lactobacillus wasatchensis* is suited to grow in conditions typical of cheese storage and ripening. It can grow in a wide range of pH concentrations from 5.2 up to 9.0 with growth observed in up to 6.0% salt at pH 5.2 and pH 6.5. At pH 9, after an extended lag phase, *Lb. wasatchensis* had the highest  $\mu_{\max}$ . In the absence of salt, faster growth was observed at pH 6.5 than at pH 5.2, but the reverse was observed in the presence of 3.5% salt or more. This suggests an enhancement of growth as a side effect of bioprotective mechanisms when *Lb. wasatchensis* cells are stressed. It may be there are additional genes activated when cells are challenged against harsh growth conditions which help protect and improve growth. In addition, although growth was still observed at cheese pH levels of 5.2 and 5.5 in 5.5% salt, there was a substantial decrease in growth when compared to the 3.5 and 4.5% salt concentrations. This suggests a decreased likelihood of growth and subsequent gas production of *Lb. wasatchensis* when the salt-in-moisture content is kept high. While *Lb. wasatchensis* grows best on ribose, galactose, lactose, glucose, fructose and N-acetylglucosamine can also be utilized by *Lb. wasatchensis*. This extends the list of carbohydrate sources available to support growth than previously shown. *Lactobacillus wasatchensis* also grows on the lysis of common starter and adjunct cultures. An 80% decrease in bacterial CFU/g was observed within the first 24 h after salting the curd.

### PART 3. DNA EXTRACTION

The cheddar cheese samples inoculated with *Lb. wasatchensis* had increasing CFU/g from  $1.9 \times 10^7$  in sample group A to  $1.1 \times 10^9$  in sample group D (Table 6). The recovery of extracted DNA from these cheeses ranged from 8.3 ng/ $\mu$ L to 39.5 ng/ $\mu$ L (Table 7). The  $A_{260}/A_{280}$  ratio was consistently between 1.81 or above 1.91, indicating relatively pure DNA extracts. The optimal is considered 1.90 for nucleic acids.

The phenol-chloroform DNA extraction method was thus effective at extracting DNA from cheese samples. However, other work at USU (Tyler Allen, unpublished data 2018) has shown that with direct extraction of DNA from cheese, such as the phenol-chloroform method, there is a problem with extraneous DNA (i.e., not from living bacterial cells) which can persist in milk and cheese. Based upon this finding, it would be better to use a method that first harvests the cells from the cheese, followed by a DNA extraction protocol.

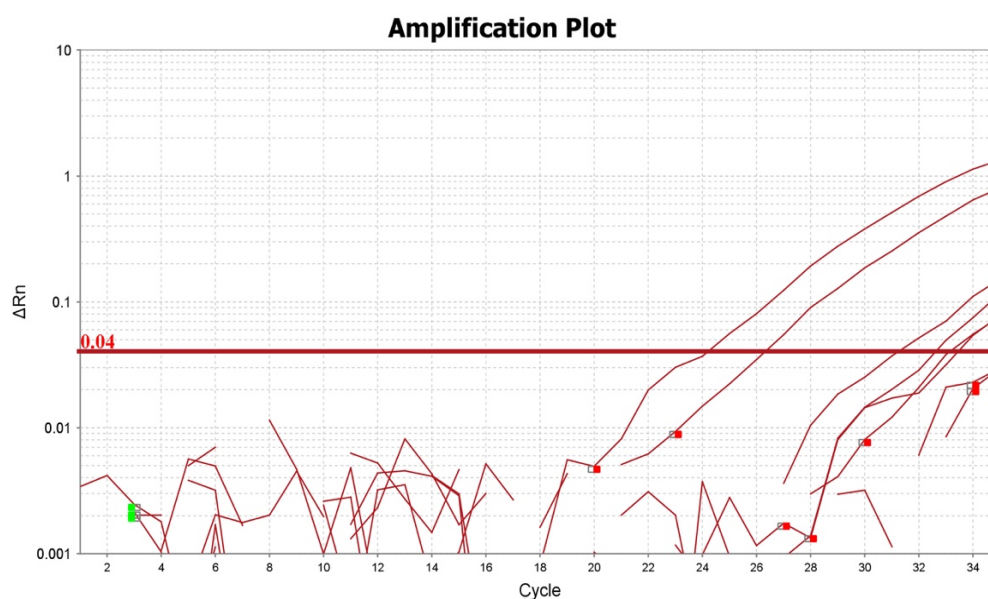
There was also a lot of variation in the amount of DNA extracted from the cheeses and quantity of DNA was not proportional to the number of *Lb. wasatchensis* cells (although count of the total number of bacteria in the cheese was not performed). Whether the variance occurred because of uneven spatial distribution of the bacteria community in the cheese or inherent in the extraction method, it was not determined. Interestingly, the extract from cheese B contained 39 ng/ $\mu$ L DNA whereas the extract from cheese D had only 18.4 ng/ $\mu$ L, even though it contained one log more of *Lb. wasatchensis*. Although cheese D had less total DNA extracted, when amplified, it contained the greater amount of *Lb. wasatchensis* DNA, as indicated by the lower CT value (Figure 10 and Table 8).

**Table 6.** Bacterial counts from four cheeses made from milk inoculated with 100  $\mu\text{L}$ , 500  $\mu\text{L}$ , 1 mL, and 10 mL of *Lactobacillus wasatchensis*. Counts were performed on MRS agar spread plates supplemented with 1.5% ribose after 5 d of anaerobic incubation at 25°C.

Cheese	Inoculation Volume	<i>Lb. wasatchensis</i>
		CFU/g
A	100 $\mu\text{L}$	$1.9 \times 10^7$
B	500 $\mu\text{L}$	$7.6 \times 10^7$
C	1 mL	$2.2 \times 10^8$
D	10 mL	$1.1 \times 10^9$

**Table 7.** Concentration and purity of DNA extracted from cheese inoculated with known amounts of *Lactobacillus wasatchensis* cells using the phenol-chloroform method. DNA purity of each sample group determined by the ratio of absorbance at 260 nm to absorbance at 280 nm ( $A_{260}/A_{280}$ ).

Cheese	Inoculation Volume	DNA Concentration	DNA Purity
		ng/ $\mu\text{L}$	260nm/280nm
A	100 $\mu\text{L}$	8.3	1.90
B	500 $\mu\text{L}$	39.5	1.83
C	1 mL	10.6	1.91
D	10 mL	18.4	1.91



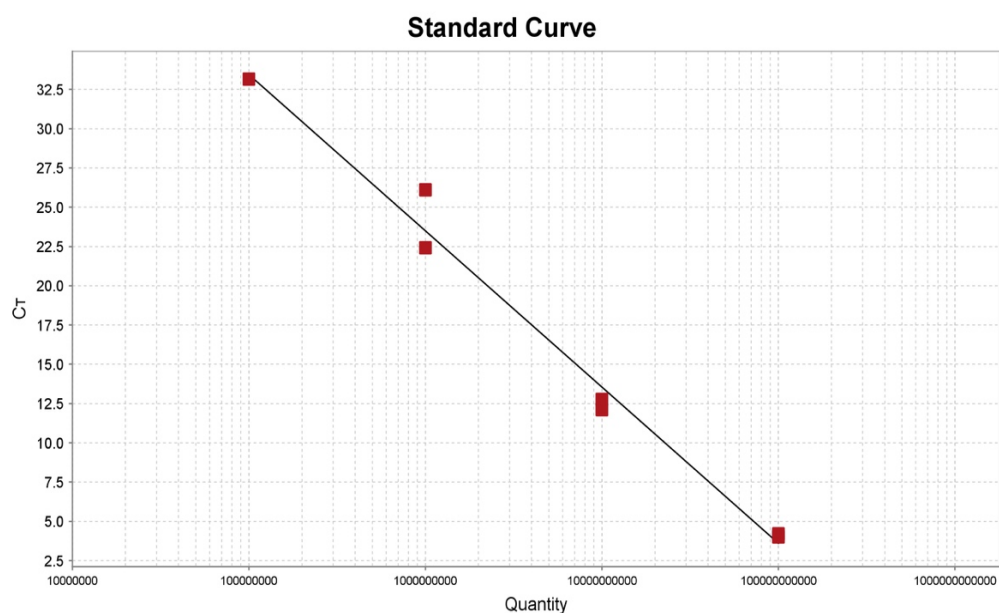
**Figure 10.** Quantitative PCR amplification of cheese samples inoculated with 100  $\mu\text{L}$ , 500  $\mu\text{L}$ , 1 mL, and 10 mL of *Lactobacillus wasatchensis*.

**Table 8.** CT values obtained from the amplification of *Lactobacillus wasatchensis* DNA extracted from cheese samples containing 100  $\mu$ L, 500  $\mu$ L, 1 mL, and 10 mL *Lactobacillus wasatchensis*, n = 2.

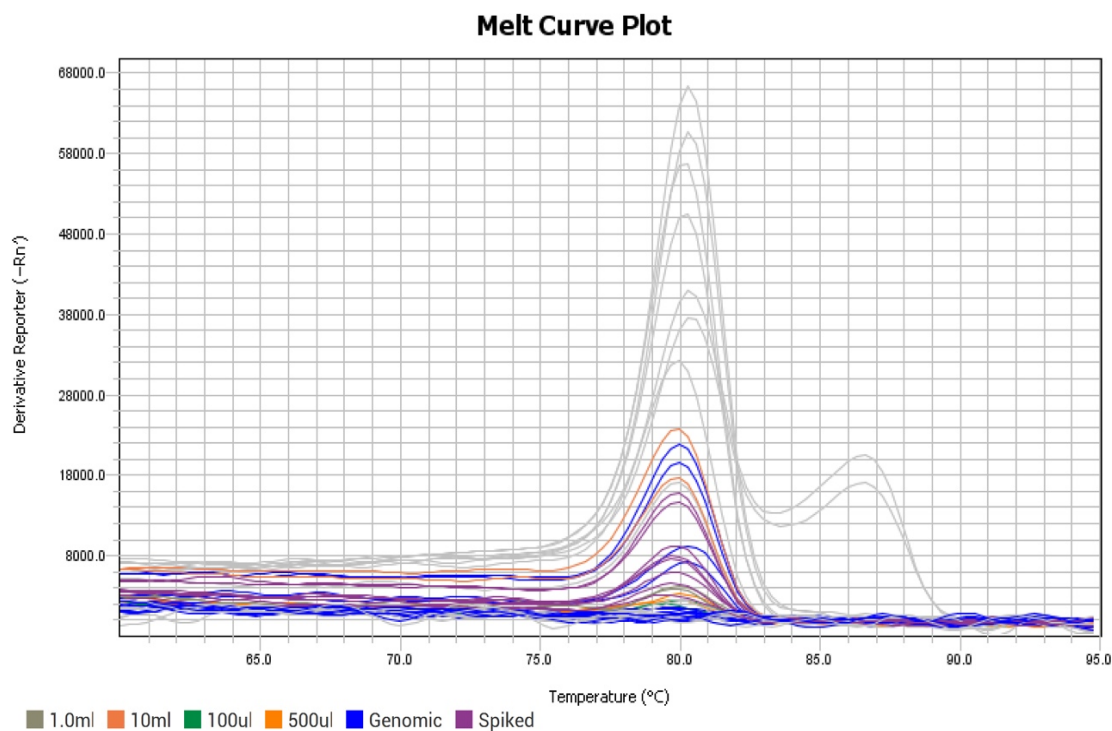
<b>Cheese</b>	<b>Inoculation Volume</b>	<b>CT value</b>
<b>A</b>	<b>100 <math>\mu</math>L</b>	<b>0.0</b>
<b>A</b>	<b>100 <math>\mu</math>L</b>	<b>0.0</b>
<b>B</b>	<b>500 <math>\mu</math>L</b>	<b>33.4</b>
<b>B</b>	<b>500 <math>\mu</math>L</b>	<b>33.1</b>
<b>C</b>	<b>1.0 mL</b>	<b>31.4</b>
<b>C</b>	<b>1.0 mL</b>	<b>32.6</b>
<b>D</b>	<b>10 mL</b>	<b>24.1</b>
<b>D</b>	<b>10 mL</b>	<b>26.3</b>

The amount of *Lb. wasatchensis* specific DNA was not proportional to the total DNA extracted.

There were a number of complications in performing the qPCR analysis with samples not amplifying or having more than one melting point peak (Figures 11 and 12). Even when making the calibration curve using serial dilutions, some of the samples did not amplify. Only one replicate of the most dilute sample amplified (Figure 11). A lack of amplification of cheese extracts may be because of PCR inhibitors being found in the cheese (Al-Soud and Rådström, 1998, Monnet et al., 2006), or whether or not the original cheese sample contains enough *Lb. wasatchensis*, and whether the phenol-chloroform extraction is able to recover that DNA from the cheese. Using qPCR, the detection threshold of  $\sim 8 \times 10^7$  CFU/g is higher than the  $10^5$  CFU/g that can be observed using standard PCR and gel electrophoresis (Tyler Allen, Utah State University, unpublished data 2018). The CT for cheeses B, C, and D of 33.3, 32.0, and 25.2 respectively corresponded to cell numbers of  $7 \times 10^7$ ,  $1 \times 10^8$ , and  $7 \times 10^8$  which were similar to the  $7.6 \times 10^7$ ,  $2.2 \times 10^8$ , and  $1.1 \times 10^9$  CFU/g determined by plate counting (Table 6).



**Figure 11.** Quantitative PCR standard curve made by diluting the amplified 16S rRNA PCR product of *Lactobacillus wasatchensis* grown in MRS broth and then serially diluted.



**Figure 12.** Melting peaks of 100  $\mu$ L, 500  $\mu$ L, 1.0 mL, and 10 mL *Lactobacillus wasatchensis* DNA sample groups monitored during qPCR amplification.



There was also occasionally two melting point peaks observed. The primer pair used was thought to be specific to *Lb. wasatchensis*, however, since it also occurred with some of the standard curve samples prepared from broth culture it may indicate non-specific primer binding and amplification (Monnet et al., 2006).

### **Summary**

The phenol-chloroform DNA extraction method from cheese produced a relatively pure DNA product. The amount of DNA harvested was not proportional to the amount of *Lb. wasatchensis* in the cheese. The threshold for detection using qPCR was  $\sim 8 \times 10^7$  CFU/g of cheese. The CT for extracts from cheese with higher levels of *Lb. wasatchensis* corresponded to CT values determined for the calibration curve. Further optimization and experimentation needed to cover the detection threshold for this method to be effective at measuring low levels of *Lb. wasatchensis* concentrations in cheese.

## CONCLUSION

We assumed *Lb. wasatchensis* was present in the cheese vats and final cheese product due to insufficient inactivation during HTST pasteurization. However, our hypothesis was proven incorrect. *Lactobacillus wasatchensis* was shown to be heat sensitive with low thermotolerant capabilities. Heating in a HTST pasteurizer heat exchanger caused a greater than 7 log inactivation of *Lb. wasatchensis* cells with no signs of survival, even at sub-pasteurization temperatures of 69.4°C for 15s.

By further characterizing the growth attributes of *Lb. wasatchensis*, we have come to understand better how it is able to grow to high numbers in cheese and produce unwanted carbon dioxide. *Lactobacillus wasatchensis* grows at milk pH (6.6) and cheese pH (5.2). After a longer lag period, the fastest growth rate was at pH 9. The upper limit of salt tolerance at pH 6.5 was >6.0% with only slight growth at 5.4 to 6.0%. At pH 5.2 there was >50% increase in growth rate at each salt concentration compared to pH 6.5. Perhaps in slightly acidic or alkaline conditions, there is a stress response that activates bioprotective genes that support growth.

At pH 5.2 and 5.5 *Lb. wasatchensis* grew at salt concentrations up to 5.5%, although at a slower rate than at 3.5 to 4.5% salt. Among the ever-growing list of risk factors causing late gassy defect, low salt concentrations is another. There was increased *Lb. wasatchensis* growth as salt concentrations dropped from 5.5 to 4.5% (the ideal range for aged cheddar cheeses) and even further growth at 3.5%. At low salt levels, the risk for poor quality cheese increases along with *Lb. wasatchensis* growth and subsequent unwanted gas production.

Observing growth on various carbohydrate sources indicated *Lb. wasatchensis* is

able to utilize additional carbohydrates than solely ribose and galactose as previously suggested based on API CH50 tests. Growth occurred with lactose, glucose, fructose, and N-acetylglucosamine being present as the only carbohydrate source. No growth occurred with N-acetylmuramic acid.

Using the phenol-chloroform DNA extraction method, DNA was obtained from cheese samples inoculated with known amounts of *Lb. wasatchensis* cells. However, there was too much variation in the concentration of DNA extracted from each sample although the extracts were acceptable. The work involving the qPCR method was not successful.

## FUTURE RESEARCH

Based off the experiments performed a number of questions arose which would be interesting to explore. Could it be *Lb. wasatchensis* is surviving in the HTST pasteurizer through biofilm formation and thus getting into the cheese vats? This port of entry would appear to be the most probable. Therefore, it might be of interest to run the pasteurizer for 8 to 12 h with milk inoculated with *Lb. wasatchensis* and take samples every 30 min to see if some cells begin to survive after long runs. It would also be advised to cheese manufacturers to look at additional post-pasteurization entry points of *Lb. wasatchensis* contamination (i.e., cross-contamination or aerosols).

It was proposed that *Lb. wasatchensis* utilizes different energy pathways to metabolize lactose and glucose. After 24 h of incubation *Lb. wasatchensis* ceases to grow on these carbohydrates suggesting there is no more sugar available for energy and growth. It would be interesting to use molecular techniques to explore whether or not there are different pathways utilized when metabolizing these sugars. It would also be beneficial to test if *Lb. wasatchensis* can co-metabolize lactose, glucose, fructose, and N-acetylglucosamine when present with ribose.

Further, it was suggested there may be a bioprotective effect elicited when *Lb. wasatchensis* cells are put into a stressful environment (i.e., pH 9 or pH 5.2 with salt). It appears there may be additional genes activated to help the bacteria survive the harsh conditions and grow better than their “more” neutral counterparts. Is what we suggest actually happening or what helps these cells grow better? It would be curious to look at gene activation of bacteria in stressful environments compared to those at neutral pH.

It may be beneficial to re-do the bacterial cell lysate experiments involving

growth over 10 d. There was contradictory data produced from the two experiments involving the controls and decreased growth at 10 d versus 5 d.

Finally, to improve the concentration of DNA extracted from cheese samples, rather than using proteinase K as the enzyme treatment during incubation try using a lysozyme/mutanolysin treatment.

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