5-2015

Contribution of a Novel Obligatory Heterofermentative Nonstarter Lactobacillus Species to Late Gassy Defect in Cheddar Cheese

Fatih Ortakci
Utah State University

CONTRIBUTION OF A NOVEL OBLIGATORY HETEROFERMENTATIVE NONSTARTER *LACTOBACILLUS* SPECIES TO LATE GASSY DEFECT IN CHEDDAR CHEESE

by

Fatih Ortakci

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

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

_____	_____
Dr. Donald J. McMahon Major Professor	Dr. Jeffery R. Broadbent Co-Major Professor
_____	_____
Dr. Craig J. Oberg Committee Member	Dr. Conly L. Hansen Committee Member
_____	_____
Dr. David W. Britt Committee Member	Dr. Mark R. McLellan Vice President of Research and Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2015

ABSTRACT

Contribution of a Novel Obligatory Heterofermentative Nonstarter *Lactobacillus* Species to Late Gassy Defect in Cheddar Cheese

by

Fatih Ortakci, Doctor of Philosophy

Utah State University 2015

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

This study sought to determine whether a recently isolated slow-growing nonstarter lactic acid bacterium, *Lactobacillus wasatchii* sp. nov., could be implicated in late gassy defect in Cheddar cheese. I demonstrated that *Lb. wasatchii* grows readily in the laboratory under cheese-like stress conditions of 5% salt and pH 5.2, and has the potential to survive pasteurization. *Lactobacillus wasatchii* can co-utilize ribose and galactose to maximize its growth. Due to being an obligatory heterofermentative, *Lb. wasatchii* produces CO_2 whenever it ferments a hexose such as galactose.

A second investigation extended these findings by examining the growth and gas forming characteristics of *Lb. wasatchii* in Cheddar cheese. The optimum growth of *Lb. wasatchii* and highest levels of gas production were observed in cheese supplemented with ribose plus galactose, and stored at 12°C rather than 6°C. *Lactobacillus wasatchii* also grew readily and produced gas in Cheddar cheese even without added ribose and

galactose, which corresponds with the ability of *Lb. wasatchii* to grow on starter cell lysate. A challenge still remains of how to easily enumerate *Lb. wasatchii* in cheese with a higher background population of other nonstarter lactic acid bacteria.

The third set of experiments explored the consequences on growth and gas production of *Lb. wasatchii* in Cheddar cheese made with *Streptococcus thermophilus*. Using *St. thermophilus* in cheesemaking results in galactose accumulation, which *Lb. wasatchii* then can utilize for growth, causing release of CO_2 with the end result of having blown Cheddar cheese. Results showed *Lb. wasatchii* or similar nonstarter lactic acid bacteria are likely to be particularly problematic in cheesemaking involving starter or adventitious *St. thermophilus*.

From these observations, it was concluded that *Lb. wasatchii* is a contributor to late gassy defect in Cheddar cheese and may be widely present as part of the nonstarter lactic bacteria population but has been undetected up until now. The late gassy defect is more pronounced at temperatures used for accelerated ripening of cheese and when there are substantial residual levels of galactose in the cheese. Thus, researchers and cheese manufacturers now must consider slow-growing obligatory heterofermentative nonstarter lactic acid bacteria when dealing with late gassy defect in cheese.

(199 pages)

PUBLIC ABSTRACT

Contribution of a Novel Obligatory Heterofermentative Nonstarter *Lactobacillus* Species to Late Gassy Defect in Cheddar Cheese

Fatih Ortakci

Cheddar cheese is usually aged for 3 to 24 months at temperatures ranging from 5 to 13°C. Ripening at elevated temperatures hastens the process, reducing manufacturing costs and enabling manufacturers to bring the product to market more quickly. However, cheeses ripened at elevated temperatures sometimes exhibit late gassy defect that may cause a textural defect, commonly referred to as slit defect. This results in crumbling and losses during cutting of as much as 50%, making slit defect a major economic issue in the cheese industry. Moreover, loose or blown cheese packages are unsuitable for sale in the supermarkets due to the consumer rejection.

Recently, a previously unnamed lactic acid bacterium was isolated from an aged blown USU Cheddar cheese. The bacterium is named *Lactobacillus wasatchii* sp. nov., after the Wasatch mountain range in Northern Utah. The current research demonstrates that *Lb. wasatchii*, whose presence was unsuspected before, is a likely cause of late gassy defect in Cheddar cheese. Three experiments were conducted to explore this problem.

The first experiment showed that *Lb. wasatchii* grows well on sugars available in cheese (ribose, galactose) under conditions that mimic cheese ripening, namely a high salt and low pH environment. It also survives high temperature short time pasteurization (72°C for 15 sec), which further implicates it in the late gassy defect in cheese.

A second experiment was conducted in which Cheddar cheese was manufactured with or without added *Lb. wasatchii* to cheese milk. Cheeses with added *Lb. wasatchii* had significantly higher gas formation compared to control cheeses, and the defect was greater at the elevated ripening temperature.

Finally, since cheesemakers often use a blend of starter cultures containing *Streptococcus thermophilus* to shorten the time required to make cheese, a third experiment was conducted to show that a gas-forming bacterium such as *Lb. wasatchii* can also produce gas in cheese made using *St. thermophilus* by utilizing accumulated galactose. Compared to control Cheddar cheese (no added *Lb. wasatchii*), cheese containing added *Lb. wasatchii* ripened at 12°C exhibited major textural defects and produced prodigious amounts of gas when *St. thermophilus* was included in the starter culture.

This research advances our understanding of late gassy defect in Cheddar cheese, implicating *Lb. wasatchii* as a likely contributor to late gas formation. Cheese manufacturers now must consider slow-growing, obligatory heterofermentative nonstarter lactic acid bacteria as a potential source of late gassy defect in Cheddar cheese.

DEDICATION

To my wife, who patiently stood by me through all of this, and to my children; there is no boundary and limit to what you can achieve.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my major advisor, Dr. Donald McMahon, for his expert guidance, caring, and patience throughout all of this. Without his support, I would never have made it through this experience. I would also like to thank my co-advisor, Dr. Jeff Broadbent, who allowed me to use his lab without any limitation and supported me with his brilliant ideas. It has been a great pleasure to receive direction from one of the best researchers in the field of lactic acid bacteria. To my dissertation committee members Drs. Craig Oberg, Conly Hansen, and David Britt for their support at every step of my program, I extend sincere thanks as well.

I want to thank my parents for teaching me the value of education and their continuous motivation and belief in me. Finally, I would like to thank my wife for her patience during whole my graduate school education. Without her support, this dissertation could never have been completed.

The research presented in this dissertation was funded by Western Dairy Center at Utah State University.

Fatih Ortakci

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

FHF	Facultative heterofermentative
G	Galactose
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
LBW	*Lactobacillus wasatchii*
MFGM	Milk fat globular membrane
MRS-R	de Man, Rogosa, and Sharp broth with ribose
MRS-V	de Man, Rogosa, and Sharp agar with vancomycin
MRS-R-V	de Man, Rogosa, and Sharp agar with ribose and vancomycin
NSLAB	Nonstarter lactic acid bacteria
OHF	Obligatory heterofermentative
PP	Pentose phosphate pathway
R	Ribose
St.	*Streptococcus thermophilus*

CHAPTER 1

GENERAL INTRODUCTION

In many cheese varieties, gas production (CO_2) by microorganisms may be desirable (e.g., eye production in Swiss and Dutch-type cheeses). In other types of cheeses such as Cheddar cheese any effects of gas formation such as holes, splits and cracks are undesirable. Unwanted gas production in cheese can fall into two categories: early blowing and late blowing. Early blowing, or gas production immediately after (or during) cheese manufacture is caused by bacteria such as coliforms that are present in milk either because of inadequate sanitation or use of unpasteurized milk (or otherwise heat-treated milk that kills such microorganisms). Late blowing refers to gas production that occurs later (2 months or more) during the aging process depending on storage temperature and other factors.

Coliforms are known to be major contributors to early gas production in raw milk cheeses. In aged Cheddar cheese, coliforms may be a cause of early gas production, particularly when there is a failure of the starter culture, such as by bacteriophage infection (Kleter et al., 1984; Mullan, 2000). High levels of coliforms can lead to defects in cheese during aging, which include early gas production, which leads to structural defects and negative impacts on cheese sensory quality. *Enterobacter aerogenes, Escherichia coli, Klebsiella aerogenes* has been shown to be problematic with respect to early gas production in cheese and found in raw milk (Alichanidis, 2007). Coliforms are acid sensitive and sensitivity is further increased with increased salt concentration and decreased water activity (Lawrence et al., 2004; Alichanidis, 2007).

The involvement of *Clostridium tyrobutyricum* in late gas production in brined cheeses such as Gouda has been well established (van den Berg et al., 2004). However, *Clostridium* would not be expected to cause problems in cheddar cheese of satisfactory salt, acid and moisture content (Hirsch and Grinsted, 1952; Kleter et al., 1984). Wild strains of urease-producing *Streptococcus thermophilus*-like bacteria can also produce gas in Cheddar cheese as they can grow in the regeneration section of pasteurizers such that relatively high levels may occasionally occur in pasteurized milk (Tinson et al., 1982; Mullan, 2000).

The focus of this research is on the late gas formation that occurs in Cheddar cheese on proper chemical composition (i.e., pH below 5.4 and salt-in-moisture content above 4.0%) as described by Lawrence et al. (2004) and Kleter et al. (1984) made from pasteurized milk, using properly sanitized equipment. Late gas formation in Cheddar cheese has been a recurring problem for over 100 years (Van Slyke and Hart, 1903). Occurrence of the defect tends to be sporadic and recurrent and has probably been experienced at most cheese making plants (Mullan, 2000). Late gas production has also been experienced in aged Cheddar cheese made in the Aggie Creamery at Utah State University. A new lactobacilli species was recently isolated from such cheese (unpublished data) that is suspected to be a cause for such gassy defect in ripening Cheddar cheese.

Cheddar Cheese Manufacture

Cheddar cheese is a semi-hard cheese variety with a smooth body, close texture and clean nutty flavor (Varnam and Sutherland, 1994). It is the second most popular

cheese in the United States, behind mozzarella, with an average annual consumption of 9.43 lb per capita (Anonymous, 2014a). The United States produced ~3.2 billion lb in 2013 (Anonymous, 2014b). Cheddar cheese production for the month of April 2014 was 282 million lb (Anonymous, 2014c). The average Cheddar cheese price received for the month of April was $2.24 per pound (Anonymous, 2014d). This relates to over seven billion dollars in sales of Cheddar cheese annually.

After the manufacture of Cheddar cheese, ripening is vital to change the rubbery, mildly tasty mass of fresh pressed curd into a homogenous mass with distinct flavor, aroma and body. For Cheddar cheese, aging for 3 to 12 month is common, during which operational costs and interest on capital cheese storage represent a significant portion of total cost (El Abboudi et al., 1991).

Any defects presented during ripening result in major economic losses for the cheese manufacturer. One such defect called “gassy” defect can be seen as openness of cheese texture or “blown wrappers” without texture change in cheeses aged longer than 3 months (Laleye et al., 1987). Gassy defect may create slits, cracks or voids in the cheese sometime during ripening but is not evident until the cheese is graded unless the cheese pack is loose. Even though this textural defect may not create a specific sensory defect, it may result in the inability of the producer to cut the blocks with uniformity and impacts the shredability of the cheese. If the defect is severe, the block will crumble upon cutting which can increase cutting losses from 10% (non-defective) up to 50% (Donnely et al., 2014). The time and money spent on the aging process is lost, therefore the cheese may

be sold at a lower price or used for processed cheese (Martley and Crow, 1996; Golnazarian, 2001).

Lactobacillus wasatchii

The organism isolated from Utah State University Cheddar cheese is tentatively called *Lactobacillus wasatchii* isolate WDC04. *Lactobacillus wasatchii* is a slow-growing organism and does not grow under conditions typically used for isolating and enumerating nonstarter lactic acid bacteria (**NSLAB**). Therefore, this organism and other obligatory heterofermentative (**OHF**) lactobacilli could have been overlooked because NSLAB enumeration and identification has been restricted to those lactic acid bacteria species that readily grow on MRS or Rogosa (sugar source is glucose) within 2 d at 30 or 37°C. Moreover, even with extended incubation times some lactobacilli species cannot form colonies in Rogosa agar (Anonymous, 2014e).

In studies on gassy defect in Cheddar cheese when coliforms and *Clostridium* were not detected, gassiness has been attributed to OHF lactobacilli species; *Lactobacillus brevis* and *Lactobacillus fermentum*, or described as a slow growing unusual bacterium (Elliott et al., 1981). However, organisms similar to the newly isolated oligotrophic species *Lb. wasatchii* would not grow under the time, temperature and media conditions used by previous researchers studying gassy defect in cheese.

The current research is focused on understanding the growth and gas-forming characteristics of *Lb. wasatchii* which is an OHF lactic acid bacterium meaning that when it utilizes hexoses for energy it produces equimolar amounts of CO_2, lactate, acetate/ ethanol through pentose phosphate pathway (Axelsson, 2004). The inability for this

organism to be detected (or enumerated) using standard methods used in the past, suggests that *Lb. wasatchii* or similar OHF lactic acid bacteria may be a widespread cause of late gas formation in Cheddar cheese.

Because past efforts for trying to identify the cause of late gas formation in Cheddar cheese have overlooked slow-growing OHF lactic acid bacteria, such as *Lb. wasatchii*, researchers have always had an incomplete understanding of microbiota of cheese and an incomplete list of potential causes of late blowing. This research will provide a major step forward in solving unwanted gas production in Cheddar (and similar) cheese. It will provide tools for identifying, enumerating and understanding OHF lactobacilli in cheese that have previously been overlooked, and whose presence was unsuspected. This will provide new insights for the cheese industry to solve problems of unwanted gas production in cheese. This would reduce the amount of cheese downgraded because it is unsuitable for slicing, and reduce the occurrence of puffy packs of shredded cheese that would be unsuitable for sale.

Hypothesis and Objectives

We hypothesized that growth of a slow-growing obligatory heterofermentative nonstarter lactic acid bacterium such as *Lactobacillus wasatchii* in Cheddar cheese is a principal cause of late gas formation.

Efforts to explore this hypothesis focused on the following objectives:

Objective 1. Determine the growth characteristics of *Lb. wasatchii* as a function of temperature, sugars, salt, pH, and presence of starter cell lysate.

Objective 2. Manufacture cheese with added *Lb. wasatchii* and determine the effect on gas production when the cheese is made using *Lactococcus lactis* subsp. *lactis*/*cremoris* starter culture and influence of added galactose and ribose.

Objective 3. Manufacture cheese with added *Lb. wasatchii* and determine the effect on gas production when the cheese is made using *Streptococcus thermophilus* starter culture.

REFERENCES

Alichanidis, E. 2007. Cheeses ripened in brine. Pages 332-333 in Cheese Problems Solved. P. L. H. McSweeney, ed. Woodhead Publishing Ltd, Cambridge, UK.

Anonymous 2014a. Accessed June 24, 2014.http://dairybusiness.com/seo/headline.php?title=2012-u-s-per-capita-cheese-consumption-a-new&table=headlines.

Anonymous 2014b. Accessed June 23, 2014. http://future.aae.wisc.edu/data/annual_values/by_area/154?tab=production.

Anonymous 2014c. Accessed June 23, 2014. http://usda.mannlib.cornell.edu/usda/ams/DYMCHEESE_PROD_CH.pdf.

Anonymous 2014d. Accessed June 23, 2014. http://future.aae.wisc.edu/data/monthly_values/by_area/14?tab=prices.

Anonymous 2014e. Accessed June 27, 2014. http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0627&org=82.

Axelsson, L. T. 2004. Lactic acid bacteria: classification and physiology. Pages 1-63 in Lactic Acid Bacteria. S. Salminen, and A. von Wright, ed. Dekker, New York.

Donnely, C., C. Golnazarian, and K. Boor. 2014. Accessed June 21, 2014. http://www.powershow.com/view/1e1e6- Page: 2.

El Abboudi, M., S. Pandian, G. Trepanier, R. E. Simard and B. H. Lee. 1991. Heat-shocked lactobacilli for acceleration of Cheddar cheese ripening. J. Food Sci. 56(4): 948–949.

Elliott, J. A., G. E. Millard, and R. A. Holley. 1981. Late gas defect in cheddar cheese caused by an unusual bacterium. J. Dairy Sci. 64(11): 2278-2283.

Golnazarian, C. 2001. Slit formation in Cheddar cheese: A comprehensive investigation of the microbiological parameters associated with this defect. PhD Thesis. University of Vermont., Burlington.

Hirsch, A., and E. Grinsted. 1954. Methods for the growth and enumeration of anaerobic spore formers from cheese with observations on the effect of nisin. J. Dairy Res. 21: 101.

Kleter, G., W. L. Lammers, and E. A. Vos. 1984. The influence of pH and concentration of lactic acid and NaCl on the growth of *Clostridium tyrobutyricum* in whey and cheese. 2. Experiments in cheese. Neth. Milk Dairy J. 38: 31-41.

Laleye, L. C., R. E., Simard, B. H Lee, R. A. Holley, and R. N. Giroux. 1987. Involvement of heterofermentative lactobacilli in development of open texture in cheeses. J. Food Prot. 50:1009-1012.

Lawrence, R. C., J. Gilles, L. K. Creamer, V. L. Crow, H. A. Heap, C. G. Honoré, K. A. Johnston and P. K. Samal. 2004. Cheddar Cheese and Related Dry-salted Cheese Varieties. Page 72 in Cheese: Chemical, Physics and Microbiology. Vol. 2. 3rd ed.

Patrick F. Fox, Timothy M. Cogan, and Timothy P. Guinee, ed. Elsevier Ltd, London, UK.

Martley, F. G., and V. L. Crow. 1996. Open texture in cheese: the contributions of gas production by microorganisms and cheese manufacturing practices. J. Dairy Res. 63: 489–507.

Mullan, W. M. A. 2000. Causes and control of early gas production in Cheddar cheese. Int. J. Dairy Tech. 53: 63–68.

Tinson, W., M. C. Broome, A. J. Hillier, and G. R. Jago. 1982. Metabolism of *Streptococcus thermophilus*. Australian J. Dairy Technol. 37:14-16.

van den Berg, G., W. C. Meijer, E. M. Düsterhöft, and G. Smit. 2004. Gouda and related cheeses. Page 107 in Cheese: Chemical, Physics and Microbiology. Vol. 2. 3rd ed. Patrick F Fox, Timothy M. Cogan, and Timothy P. Guinee, ed. Elsevier Ltd, London, UK.

Van Slyke, L. L., and E. B. Hart. 1903. The relation of Carbon Dioxide to proteolysis of ripening Cheddar cheese. New York (Geneva) Agr. Expt. Sta. Bull. 231, pp 20-41. (Obtained from Tretsven, 1945. Study of the gas evolution from ripening cheddar cheese. PhD Thesis. Graduate School of University of Wisconsin).

Varnam, A. H., and J. P. Sutherland. 1994. Milk and Milk Products Technology, Chemistry and Microbiology. 1st ed. Chapman & Hall, New York, NY.

CHAPTER 2

LITERATURE REVIEW

History of Late Gas Formation in Cheddar Cheese

Until 1903, perhaps it wasn't explicit whether the gases are evolving from a ripening Cheddar cheese is a biological charecteristic of the cheese. Van Slyke and Hart (1903) placed cheese in an enclosed system in order to control and collect evolved gas. They treated the first cheese with chloroform to inhibit microbial growth and the second cheese was not treated with chloroform. They reported that there were very little CO_2 evolved from chloroformed cheese during the first three weeks of ripening and then gas evolution ceased, however, control cheese evolved CO_2 continuously. They concluded that the factor responsible for gas formation and textural defects was of a biological character of the cheese.

In 1939, gas problem and therefore slit openness had also been experienced in New Zealand Cheddar cheese. Sherwood (1939) is the first researcher who attributed the slit openness defect to the presence of gas producing lactobacilli and described the problem as "most troublesome defect in Cheddar cheese." He had been established that defective cheeses evolve more CO_2 than non-defective cheese, defective cheese contained larger OHF lactobacilli than non-defective cheese and finally inoculation of gas forming lactobacilli to the cheese milk leads to the development of slit openness. He claimed the species to be responsible for the condition is mostly *Lb. brevis* and, to a lesser degree, *Lactobacillus plantarum*.

Dorn and Dahlberg (1942) reported that gas production by ripening Cheddar cheese was more uniform and minimized when cheese was made from pasteurized rather than raw milk. Gas liberated by partially ripened cheese consisted of 90 to 95% CO_2 and 5 to 10% hydrogen. Tittsler et al. (1947) inoculated milk with *Lb. brevis* and reported the development of objectionable flavors due to the rapid growth of organism in the cheese but they did not mention about the textural defects caused by *Lb. brevis*. Similar results were also reported by Dacre (1953) that *Lb. brevis* added to cheese milk caused an objectionable "yeasty" flavor in the final cheese.

In 1981, an asporogenous slow growing organism was isolated in large numbers (10^8) from a "blown" Cheddar cheese by Canadian researchers. Elliott et al. (1981) attributed the gas production in Cheddar cheese (usually from 9 to 12 mo of age) to this unusual bacterium which has similarities with *Lb. wasatchii* such as it was a slow-growing, anaerobic, gram positive, catalase negative, heat resistant against pasteurization but nonspore forming, tolerates 6.5% NaCI, does not produce gas in Durham tubes in 5 d in common broths at 32°C and does not produce gas from citrate. It grows but forms small pinpoint translucent colonies on Rogosa and MRS agars. The researchers inoculated cheese milk with the suspect organism and the resulting cheese exhibited gassiness after 6 mo at 10°C and uninoculated control cheese did not. However, they were not able to monitor the growth of the organism due to not having tools to enumerate it from Cheddar cheese during ripening in the presence of starter and NLAB. Although researchers invited microbiologists to facilitate future identification of this unusual type of late gassing in Cheddar in order that the full extent of the problem be known and so

that a solution might be found, there is no follow up in the literature perhaps due to the difficulty to develop a method for such a slow growing organism.

Laleye et al. (1987) reported that in swollen and fissured Cheddar cheese, lactobacilli were very numerous and included a significant portion of heterofermentative types which may be responsible for gassy defect in these cheeses. However, they were not able to enumerate the OHF lactobacilli from cheese rather gave the total lactic acid bacteria counts on MRS, MRS (pH 5.5), Rogosa, and APT mediums. As a future work, researchers suggested that a more detailed characterization of the lactobacillus species in non-defective versus gassy Cheddar cheese may assist in development of effective methods for control of the cheese ripening process.

Laleye et al. (1990) reported that *Lb. brevis* and *Lb. fermentum* contributed largely to the production of copious quantities of CO_2 and hence to the openness of the cheese with a one exception of one strain of *Lb. brevis* at 7°C. They attempted to solve the open texture problem by employing the strains of facultative heterofermentative lactobacilli as adjunct cultures. Researchers concluded that the addition of certain strains of facultative heterofermentative lactic acid bacteria to cheese milk reduced the frequency of gas production and hence openness encountered during ripening when this problem was caused by inoculated or adventitious OHF lactobacilli.

Finally, in 2001, Golnazarian attributed the slit defect in Cheddar cheese to lactobacilli species. She employed several strains of *Lb. paracasei*, *Lb. plantarum* and *Lb. curvatus* (all facultative heterofermentative LAB isolated from defective cheese) in cheese making and investigated the influence of those cultures on slit defect. From

defective cheese samples 55% of the isolates were identified as *Lb. curvatus*, 43% were *Lb. paracasei* and 2% were *Lb. plantarum*. From non-defective cheeses, the majority of the isolates were identified as *Lb. paracasei* (63%) and the remaining 37% were *Lb. curvatus* (although this was only based on API 50 CHL identification system and not genomic analysis).

In 2005, another OHF lactobacilli species, *Lb. danicus*, was isolated from Danish cheese after 5 d of incubation at 30 °C in MRS agar by Danish researchers Adamberg et al. (2005). Similar to the *Lb. wasatchii*, it rapidly ferments ribose preferantially over glucose at 24 °C, and *Lb. danicus* had a shorter lag-phase when grown on ribose than when grown on N-acetyl glucosamine . The researchers also reported that *Lb. danicus* fermented only Ribose at 30 °C in CRM (Bioscreen) although, in API-50CH tests performed at the same temperature, glucose, lactose, galactose, and NAG are also fermented. It was suggested that the temperature 24 °C is more favorable for the growth of *Lb. danicus* that is similar to our findings in *Lb. wasatchii* strain. However, the growth characteristics and gas formation of *Lb. danicus* strain in cheese has not been studied therefore the presence of that strain in a cheese cannot be correlated with gassy defect even though it is an OHF lactobacilli.

It is worth to mentioning here that some facultative hetereformentative (eg., *Lactobacillus casei*, *Lactococcus lactis subsp lactis biovar diacetylactis*) and obligative heterofermentative starter or adjunct LAB (*Leucocostoc dextranicus* and *Leuconostoc citrovorum*) can produce CO_2 from citrate and have been implicated in the blowing of film-wrapped cheese or open textures (Overcast and Albrecht 1952; Robertson, 1957;

Fryer et al., 1970) which is not the concern for the present study as they are deliberately added cultures.

REFERENCES

Adamberg, K., M. Antonsson, F. K. Vogensen, E. W. Nielsen, S. Kask, P. L. Møller, and Y. Ardö. 2005. Fermentation of carbohydrates from cheese sources by nonstarter lactic acid bacteria isolated from semi-hard Danish cheese. Int. Dairy J. 15:873-882.

Dacre, C. 1953. Cheddar cheese flavor and its relation to tyramine production by lactic acid bacteria. J. Dairy Res. 20:217.

Dorn, F. L., and A. C. Dahlberg. 1942. The gases evolved by Cheddar and Limburger Cheese. J. Dairy Sci. 25:727.

Elliott, J. A., G. E. Millard, and R. A. Holley. 1981. Late gas defect in cheddar cheese caused by an unusual bacterium. J. Dairy Sci. 64(11): 2278-2283.

Fryer, T. F, M. E. Sharpe, and B. Reiter. 1970. Utilization of milk citrate by lactic acid bacteria and 'blowing' of film wrapped cheese. J. Dairy Res. 37:17-29.

Golnazarian, C. 2001. Slit formation in Cheddar cheese: A comprehensive investigation of the microbiological parameters associated with this defect. PhD Thesis. University of Vermont., Burlington.

Laleye, L. C., R. E. Simard, B. H. Lee, R. A. Holley, and R. Giroux. 1987. Involvement of heterofermentative lactobacilli in development of open texture in cheese. J. Food Prot. 50:1009–1012.

Laleye, L. C., R. E., Simard, B. H Lee, R. A. Holley, and R. N. Giroux. 1990. Quality attributes of Cheddar cheese containing added lactobacilli. J. Food Sci. 55:114-118.

Overcast, W. W., and T. W. Albrecht. 1952. Gas production in Cheddar cheese caused by *Leuconostoc citrovorum*. J. Dairy Sci. 35(6): 554-558.

Robertson, P. S. 1957. The carbondioxide content of New Zealand Cheddar cheese. J. Dairy Res. 24:235.

Sherwood, I. R. 1939. The relation of certain lactic acid bacteria to open texture in Cheddar cheese. J. Dairy Res. 10:326-335.

Tittsler, R. P., G. P. Sanders, H. E. Walter, D. S. Geib, O. S. Sager, and H. R. Lochry. 1947. The effects of lactobacilli on the quality of Cheddar cheese made from pasteurized milk. J. Bacteriol. 54: 276.

Van Slyke, L. L., and E. B. Hart. 1903. The relation of Carbon Dioxide to proteolysis of ripening Cheddar cheese. New York (Geneva) Agr. Expt. Sta. Bull. 231, pp 20-41. (Obtained from Tretsven, 1945. Study of the gas evolution from ripening cheddar cheese. PhD Thesis. Graduate School of University of Wisconsin).

CHAPTER 3

GROWTH AND GAS PRODUCTION OF A NOVEL OBLIGATORY HETEROFERMENTATIVE CHEDDAR CHEESE NONSTARTER LACTOBACILLI SPECIES ON RIBOSE AND GALACTOSE

ABSTRACT

An obligatory heterofermentative lactic acid bacterium, *Lactobacillus wasatchii* sp. nov., isolated from gassy Cheddar cheese was studied for growth, gas formation, salt tolerance and survival against pasteurization treatments at 63°C and 72°C. Initially, *Lb. wasatchii* was thought to only use ribose as a sugar source and we were interested in whether it could utilize galactose. Experiments to determine rate and extent of growth and gas production in carbohydrate restricted (CR) de Man, Rogosa, and Sharpe (MRS) medium under anaerobic conditions with various combinations of ribose and galactose at 12, 23, and 37°C were conducted with 23°C being the more optimum growth temperature of *Lb. wasatchii*. When grown on ribose (0.1%, 0.5%, and 1%), maximum specific growth rates (μ_{max}) within each temperature were similar. When galactose was the only sugar, μ_{max} was 2 to 4 times lower than with ribose. At all temperatures, highest final cell densities (OD_{640}) of *Lb. wasatchii* were achieved in CR-MRS plus 1% ribose, 0.5% ribose and 0.5% galactose, or 1% ribose combined with 1% galactose. Similar μ_{max} values and final cell densities were achieved when 50% of ribose in CR-MRS was substituted with galactose. Such enhanced utilization of galactose in the presence of ribose to support bacterial growth has not previously been reported. It appears that *Lb. wasatchii* co-metabolizes ribose and galactose, utilizing ribose for energy and galactose

for other functions such as cell wall biosynthesis. Co-utilization of both sugars could be an adaptation mechanism of *Lb. wasatchii* to the cheese environment to efficiently ferment available sugars for maximizing metabolism and growth. As expected, gas formation by the heterofermenter was observed only when galactose was present in the media. Growth experiments with MRS plus 1.5% ribose at pH 5.2 or 6.5, with 0, 1, 2, 3, 4, or 5% NaCl revealed that *Lb. wasatchii* is able to grow under salt and pH conditions typical of Cheddar cheese (4 to 5% salt-in-moisture, ~pH 5.2). Finally, we found *Lb. wasatchii* cannot survive LTLT pasteurization but survives HTST lab pasteurization with 4.5 log reduction occurred. The ability of *Lb. wasatchii* to survive HTST pasteurization and grow under cheese ripening conditions implies that the presence of this nonstarter lactic acid bacteria can be a serious contributor to gas formation and textural defects in Cheddar cheese.

INTRODUCTION

Lactic acid bacteria (**LAB**) present in ripening cheese include deliberately added starter LAB and a variety of adventitious LAB referred to as nonstarter LAB (**NSLAB**). The NSLAB gain access to cheese through the milk or processing environment (Naylor and Sharpe, 1958; Peterson and Marshall, 1990; Martley and Crow, 1993; Somers et al., 2000).

The predominant NSLAB in Cheddar cheese are facultative heterofermentative (**FHF**) lactobacilli and, less frequently pediococci or obligatory heterofermentative (**OHF)** lactobacilli (Jordan and Cogan, 1993; Crow et al., 2001; Banks and Williams, 2004). Presence of OHF lactobacilli are a particular concern because these microbes may

promote the development of undesirable flavor and body defects including gas formation in Cheddar cheese (Dacre, 1953; Laleye et al., 1987; Khalid and Marth, 1990). Unwanted gas formation in Cheddar cheese is a recurrent and widespread problem in dairy industry that has probably affected most cheese plants (Mullan, 2000). Our group recently isolated a new *Lactobacillus* species from a "gassy" Cheddar cheese after incubation on de Man, Rogosa, and Sharpe (**MRS**) agar for 35 d at 6 °C. This bacterium was designated as *Lactobacillus wasatchii* sp. nov. (unpublished data).

Lactobacillus wasatchii is an OHF species and therefore uses the pentose phosphate pathway (**PP**) to generate energy from pentose and hexose sugars. Its preferred sugar is ribose, though hexoses such as galactose are also potential energy source in cheese. More importantly, hexose sugars can be fermented by OHF to lactate, acetate/ethanol plus CO_2, making *Lb. wasatchii* a potential contributor to gassy defect in Cheddar cheese.

This study explored growth characteristics of *Lb. wasatchii* with respect to ribose and galactose utilization, gas formation, tolerance to the salt and pH values found in Cheddar cheese, and its ability to survive pasteurization treatments. To our knowledge, this is the first report on growth and gas formation of a slow growing OHF lactobacilli species isolated as a NSLAB from a "gassy" Cheddar cheese.

MATERIALS AND METHODS

Materials

Lactobacilli MRS broth, proteose peptone, polypeptone, beef extract, yeast extract, GasPak™ EZ, and agar were purchased from Becton Dickinson Inc. (Sparks, MD); ribose was donated by Bioenergy Life Science Inc. (Ham Lake, MN), UHT milk was from Gossner Foods Inc. (Logan, UT), Tween-80 and bromcresol purple were from Sigma-Aldrich Inc. (St. Louis, MO), dipotassium phosphate was from Fisher Scientific Inc. (Fair Lawn, NJ), sodium acetate trihydrate and diammonium citrate were from MallincKrodt Baker Inc. (Paris, KY), galactose, and triamonium citrate were from Alfa Aesar Inc. (Ward Hill, MA), magnesium sulfate was from Alfa Aesar Inc. (Heysham, England).

A carbohydrate-restricted version of MRS (**CR-MRS**) was prepared by the omission of glucose from the MRS broth formula. To 2 L of deionized water was added 20.0 g proteose peptone No. 3, 20.0 g beef extract, 10.0 g yeast extract, 2.0 g Tween-80, 4.0 g ammonium citrate, 10.0 g sodium acetate, 0.2 g magnesium sulfate, 0.1 g manganese sulfate, and 4.0 g dipotassium phosphate. The CR-MRS was supplemented with different levels of ribose and galactose to study growth properties of *Lb. wasatchii*.

Bacterium and Growth

Stock cultures of *Lb. wasatchii* were maintained at -80°C in MRS broth supplemented with 1.5% Ribose (**MRS+R**) and 10% glycerol. Working cultures was prepared by two successive transfers into 10 ml of MRS+R broth, with anaerobic incubation using GasPak™ EZ at 23°C for 40 h after each transfer. Growth of *Lb.*

wasatchii was evaluated by inoculation of the working culture into 10 ml CR-MRS broth acidified to pH 5.20 with HCl and supplemented with 0.5 % galactose or ribose, 1.0% galactose, ribose, or a 0.50:0.50 combination) or 2.0% sugar (1% ribose plus 1% galactose). Optical density of the cell suspensions were followed at 640 nm after inoculation and every 12 h thereafter at 12, 23, or 37°C and during anaerobic incubation in jars containing GasPak™ EZ. Maximum specific growth rate (μ_{max}) was calculated as the slope of the steepest linear portion of the growth rate curves. Broth samples containing Durham tubes were similarly prepared, inoculated, and incubated to test for gas production. Working cultures were prepared in duplicate for conducting growth curves and gas formation experiments.

To test NaCl tolerance of *Lb. wasatchii* at pH 5.2 or 6.5, *Lb. wasatchii* working cultures were prepared in triplicate and inoculated into MRS+R broth containing 0, 1, 2, 3, 4, or 5% (wt/wt) NaCl. Growth at 23°C under anaerobic conditions was followed by spectrophotometrical (OD_{600}) measurements every 8 h until the stationary phase was reached.

Thermotolerance

The ability of *Lb. wasatchii* to withstand pasteurization treatment was assayed by heating 9.9 ml of UHT milk to 63°C and 72°C in sterile polypropylene tubes. Once the desired temperature was reached, each tube was inoculated with 0.1 ml of *Lb. wasatchii* working culture (prepared in triplicate) containing ~6 x 10^8 cfu/ml of and the samples held at 63°C and 72°C for 30 min or 15 s, respectively, then placed in a 31°C water bath (the set temperature commonly used for making Cheddar cheese) for 2 h. These

treatments were designed to mimic the high temperature short time (**HTST**) continuous pasteurization used in large-scale cheese operations and the low temperature long time (**LTLT**) batch pasteurization often used by small-scale artisan cheese makers. Samples were then plated on MRS+R agar in duplicate and incubated at 23°C anaerobically for 5 d.

Statistical Analysis

Statistical analysis of the effect of different temperature, sugar, pH, and NaCl treatments on μ_{max} and final cell density of *Lb. wasatchii* were performed using PROC GLM in SAS (version 9.1, SAS Institute, Cary, NC) and differences between means determined using REGWQ multiple range test and Tukey Least Squares Means.

RESULTS

Growth

Ribose. Growth curves for *Lb. wasatchii* at 23, 37, and 12 °C in CR-MRS with ribose at pH 5.20 are represented in Figures 3.1A, 3.2A, and 3.3A, respectively. Within each temperature, significantly higher μ_{max} values were observed when *Lb. wasatchii* was grown on CR-MRS plus ribose ($P<0.05$) compared to galactose as the sole sugar (Table 3.1). In the presence of 1% ribose, μ_{max} of *Lb. wasatchii* was 23°C > 37°C = 12°C. When grown in the presence of 1.0% ribose at 12 an 23°C, exponential growth continued until final OD_{640} levels of ~1.3 to 1.4 were reached (Table 3.2), with lower OD_{640} achieved at lower sugar levels, indicating available sugars was a limiting factor on extent of growth. Less cell growth occurred at 37°C with OD_{640} only reaching 0.75. Assuming that

exponential growth ends when the sugars are depleted, the lower final cell density at 37°C may be indicative that more of the energy obtained via fermentation is being used to maintain cell viability because of energy-intensive stress responses at the higher temperature.

Galactose. When galactose was the only sugar, growth of *Lb. wasatchii* was slow (Figures 3.1B, 3.2B and 3.3B) with similar μ_{max} of less than 0.01 at all temperatures (Table 3.1). Final cell densities were lower ($P < 0.05$) than when *Lb. wasatchii* was grown with ribose except when grown with the lowest sugar level (0.1%) at 12 and 37°C (Table 3.2). Slower utilization of galactose by *Lb. wasatchii* in the absence of ribose was expected, as we had previously seen that galactose did not provide a positive response on the API 50 CHL test even when held for longer than 48 h. With slower growth occurring with galactose as the only sugar, stationary phase in CR-MRS plus 0.5% galactose was only reached after 156 h at 23°C compared to 24 h in CR-MRS plus 0.5% ribose. At 37°C, the extent of bacterial growth remained low (final $OD_{640} \leq 0.22$) even when galactose level was increased to 1% (Table 3.2).

Combined Ribose and Galactose. When a 1:1 blend of galactose and ribose was used, μ_{max} rate was not significantly different than for ribose alone ($P<0.05$), with only a slight difference observed when grown at 23°C (Figures 3.1, 3.2, and 3.3 and Table 3.1). In general, final cell densities were similar when total sugar content was the same (Table 3.2). This indicates that galactose utilization by *Lb. wasatchii* is slower when there is no ribose present but provides almost the same rate of growth as ribose when both sugars are present.

Salt Tolerance

The growth characteristics of *Lb. wasatchii* grown in MRS+R with 0 to 5% NaCl at pH 6.5 and 5.2 are shown in Figures 3.4A and 3.4B, respectively. After 48 h, an OD_{600} of 2.0 was reached in all media except for 5% salt at pH 5.2 which had an OD_{600} of 1.75 and only reached OD_{600} of 2.0 after 60 h. At pH 6.5, there was a slight decrease in μ_{max} when grown with 4% NaCl although this was not observed with 5% NaCl (Table 3.3). At pH 5.2, there was also significantly lower μ_{max} at both 4 and 5% NaCl ($P<0.05$). Final cell densities were the same ($OD_{600} = 2.0$) except at 5% NaCl with had final OD_{600} of 1.96 ($P<0.05$). A combination of salt and lower pH causes a decrease in μ_{max}, but *Lb. wasatchii* can grow in the same environment that occurs during Cheddar cheese ripening (~pH 5.2, 4 to 5% salt-in-moisture). Such salt tolerance is expected for NSLAB isolated from Cheddar cheese, Jordan and Cogan (1993) observed growth of NSLAB such as *Lactobacillus casei*, *Lb. plantarum* and *Lb. curvatus* in 6% and some up to 8% (wt/wt) NaCl. Typically, at least 6% salt is needed to slow growth of NSLAB (Lane et al., 1997) and even then NSLAB populations in Cheddar cheese still reached about the same numbers at all salt levels (2.8 to 6.1%, salt-in-moisture) after 6 mo of storage. It is interesting to note that strains of *Lactobacillus danicus*, the NSLAB that is phylogenetically closest to *Lb. wasatchii*, was susceptible to salt and had negligible growth at 4% NaCl and none detected with 6.5% NaCl (Kask et al., 2003).

Gas Formation

Gas formation by *Lb. wasatchii* was only observed when galactose was present in the media. No gas formation was observed at 23°C when the sole sugar source was ribose

or when the total sugar concentration, both ribose and galactose, was below 0.5%. At 12°C, no gas formation was observed at sugar contents of <1.0%. This may be because of the higher solubility of CO_2 at lower temperatures (CRC, 2009). At 37°C, gas formation was only detected in CR-MRS containing 1% ribose plus 1% galactose.

Thermotolerance

Subjecting *Lb. wasatchii* to HTST heat treatment (72°C for 15 s) resulted in~ 4.5-log reduction, from 6 x 10^6 cfu/ml to 9.2x10^1 cfu/ml surviving after cooling to 31°C. In contrast, no detectable colonies of *Lb. wasatchii* (i.e., <10^1 cfu/ml) were found after the LTLT treatment (63°C for 30 min). Survival of lactobacilli after milk pasteurization has been previously reported and underscores the potential for lactobacilli in milk to be a source of NSLAB in cheese made from pasteurized milk (Turner et al., 1986; Golnazarian, 2001; Beresford et al., 2001). The finding that *Lb. wasatchii* can withstand HTST indicates the bacterium could gain access to cheese directly or produce biofilms in the cheese processing environment that provide a regular source of contamination.

DISCUSSION

Metabolic Capability

Lactobacillus wasatchii sp. nov. (unpublished data) is an OHF lactobacilli closely related to *Lb. suebicus* (isolated from apple and pear mashes), *Lb. vaccinostercus* (isolated from cow dung), *Lb. hokkaidonensis* (isolated from timothy grass silage), *Lb. oligofermentans* (isolated from poultry) and *Lb. danicus* (isolated from cheese). None of these species is regularly isolated from cheese, which could be due to the fact that

NSLAB isolation methods do not incorporate the long time, low temperature conditions used to isolate *Lb. wasatchii* and *Lb. danicus* (Kask et al., 2003; Oberg et al., 2011; Broadbent et al., 2013). Because its closest phylogenetic relatives are associated with plant materials and cow dung, we speculate that the origin of *Lb. wasatchii* was a dairy farm.

Lactobacillus wasatchii is an OHF lactobacilli possessing genes encoding phosphoketolase but lacking the genes encoding fructose-1,6-diphosphate aldolase. Thus, *Lb. wasatchii* ferments pentose and hexose sugars through the PP. Utilization of hexoses via OHF lactobacilli results in CO_2, lactate, and acetate/ethanol production, whereas pentose metabolism does not yield CO_2 (Axelsson, 2004). An OHF lifestyle corresponds with the finding that gas formation was only observed when *Lb. wasatchii* was grown in CR-MRS plus galactose or CR-MRS plus ribose and galactose.

As opposed to common cheese NSLAB that are FHF lactobacilli, *Lb. wasatchii* preferentially utilizes ribose over glucose and other sugars (unpublished data). Slow utilization of hexoses and active fermentation of pentoses was also reported for the OHF *Lb. vaccinostercus* KOZAKI and OKADA sp. nov. strains that were isolated from cow dung using a medium containing xylose as the sole carbon source (Okada et al., 1978). Another phylogenetic relative of *Lb. wasatchii*, *Lb. oligofermentans* sp. nov., also utilized glucose very weakly (Koort et al., 2005).

Ribose Fermentation

The heterolactic fermentation of ribose results in a slightly different end product pattern compared to galactose fermentation. No CO_2 is formed, and since no dehydrogenation steps are necessary to reach the intermediate xylulose-5-phosphate, the reduction of acetylphosphate to ethanol to regenerate NAD^+ becomes redundant. Instead, acetylphosphate can be converted by acetate kinase in a substrate-level phosphorylation step to acetate and ATP. Fermentation of ribose thus leads to production of equimolar amounts of lactic acid and acetic acid and net 2 mol ATP/mol ribose consumed (Axelsson, 2004).

Two amino sugars that are precursors to the peptidoglycan are N-acetylglucosamine and N-acetylmuramic acid. Both amino sugars are made from fructose-6-phosphate (**F6P**) that acts as the backbone molecule for cell wall synthesis (White, 2007). *Lactobacillus wasatchii* possesses a gene encoding transketolase that condenses two pentoses with F6P being one of the metabolic outputs with the remaining carbons eventually being converted into gylceraldehyde-6-phosphate. Based on this information, we speculate that when *Lb. wasatchii* is grown in CR-MRS plus ribose, ribose is utilized for both cell wall synthesis and ATP generation to support cell division as shown in Figure 3.5 (Pathway directions {1}, {2} and {3}).

At higher concentrations of ribose, generally, the μ_{max} of *Lb. wasatchii* is the same as at lower concentrations. Thus, PP is operating as fast as possible in generating energy when *Lb. wasatchii* was grown in CR-MRS plus either ribose concentrations. It is interesting the similar μ_{max} values are achieved when a ribose-galactose mixture is used

even at the low level of 0.05 % ribose plus 0.05% galactose (Table 3.1). The only notable change that is seen with increasing sugar concentration is that the time over which exponential growth occurs is lengthened and a higher final cell density is attained.

Galactose Fermentation

Lactobacillus wasatchii grows very slowly when galactose is the sole carbohydrate source of energy (μ_{max}= 0.005, 0.009, and 0.008 on 1% galactose at 12, 23, and 37°C, respectively). At 37°C, *Lb. wasatchii* showed only limited growth with a final OD_{640} of ~0.2 reached when galactose was the sole sugar (0.1% vs. 1%). It is interesting, that *Lb. wasatchii* reached significantly higher final cell densities when grown on ≥0.5% galactose at 12 and 23°C versus 37°C ($P<0.05$). Significantly lower final cell density at 37°C may be due to more of the ATP produced by fermentation being utilized to sustain cell viability because of energy-intensive stress responses at the higher temperature. Similar results were found by Adamberg et al. (2005) who reported slower growth of *Lb. danicus* with glucose or galactose at 30°C compared to 24°C. However, ribose utilization rates by *Lb. danicus* were the same at both temperatures. In comparison, utilization of hexose sugars by *Lb. casei*/*paracasei* was higher at 30°C compared to 24°C while ribose utilization did not change (Adamberg et al., 2005).

Analysis of the *Lb. wasatchii* genome suggests galactose enters the cell via a permease and then fermented into the Leloir pathway and converted to glucose-6-phosphate (**G6P**) as shown in Figure 3.5. The G6P then be utilized using PP via dehydrogenation to 6-phosphogluconate, followed by decarboxylation to ribulose-5-

phosphate (**R5P**) and CO_2 (pathway direction {4}, {5}, {1}, {2}). Both of these steps require reduction of NAD^+ to NADH.

The R5P can then be further metabolized in the PP to lactate and acetate/ethanol with potential of generating up to net 2 ATP. However, the need to re-oxidize NADH may direct the pathway from acetylphosphate towards ethanol production rather than acetate. Thus, galactose utilization through Leloir and PP would supply 1 mol each of lactic acid, ethanol, and CO_2, and net 1 mol ATP/mol of galactose (Axelsson, 2004).

There are two possible explanations for the much slower growth of *Lb. wasatchii* on galactose compared to ribose: (1) there is a rate limiting step in the pathways leading to conversion of galactose into R5P, or (2) the need to re-oxidize NADH requires conversion of acetylphospate into ethanol rather than acetate so that only 1 mole of ATP per mole of galactose is produced as reported by Axelsson (2004).

Co-metabolism of Galactose with Ribose

There have been a few instances in which growth of lactobacilli is increased in the presence of two sugars compared to either of the sugars alone. Gobetti et al. (1995) reported that a fructose negative strain of *Lactobacillus sanfrancisco* (another OHF species) grows faster when it co-ferments fructose in the presence of maltose; maltose is consumed for energy and fructose serves as an external electron acceptor for re-oxidation of NADH. This does not seem to be the case for *Lb. wasatchii* as neither galactose nor ribose is known to function as an external electron acceptor.

In general, FHF lactobacilli such as *Lb. plantarum* can utilize both pentoses and hexoses although Westby (1989) and Westby et al. (1993) reported a strain of *Lb.*

plantarum (NCIMB 8026) that was unable to utilize ribose in the absence of glucose. They offered two hypotheses to explain this observation: (1) *Lb. plantarum* NCIMB 8026 lacks the pathways to produce F6P from pentose sugars through transketolase or via fructose-1,6-bisphosphatase thus being unable to make C_6 units from C_5 sugars and needing an external source of C_6 units for biosynthesis of peptidoglycan and other cell building blocks; or (2) that phosphoenolpyruvate (**PEP**) production during pentose metabolism (compared to hexose fermentation via glycolysis) in *Lb. plantarum* NCIMB 8026 was insufficient to support the PEP-dependent uptake of ribose. According to Neidhardt et al. (1990) only one PEP molecule is produced per ribose molecule metabolized (versus two PEP molecules per glucose) leaving no PEP molecules for the other cellular functions such as peptidoglycan synthesis.

With *Lb. wasatchii*, transketolase is available to covert pentoses into F6P, thus producing the needed C_6 building blocks for peptidoglycan. Also, for *Lb. wasatchii* the uniqueness is improved utilization of a hexose in the presence of a pentose rather than the other way around. So, neither of these hypotheses explain the mechanism of galactose and ribose co-utilization by *Lb. wasatchii* (which appears highly adapted to ferment ribose). Ribose metabolism in *Lb. wasatchii* is more profitable than galactose (or other hexose) fermentation in terms of energy production. Fred et al. (1921) reported that certain groups of pentose-fermenting LAB commonly found in silage, sauerkraut, and related substances, showed high acid production from pentose sugars while, hexose sugars yielded low acid but high ethanol production. Once again, this observation is probably a reflection of the substrate energetics; with 2 ATP per pentose but only 1 ATP

from hexoses due to the need to re-oxidize NADH to NAD^+ using the ethanol branch of PP.

To explain growth attributes of *Lb. wasatchii* during co-utilization of ribose and galactose, it is necessary to consider the potential fates of each sugar with regard to energy yield and cellular building blocks. Since the similar μ_{max}, and final cell densities were observed when *Lb. wasatchii* is grown in the presence of ribose plus galactose or ribose alone, the rate of energy production and cell wall synthesis is likely the same. Given that *Lb. wasatchii* has the gene for G6P isomerase; it can convert G6P to F6P and utilizes galactose as a ready source of hexose for peptidoglycan synthesis (Figure 3.5, pathway direction {4}, {6}).

In a parallel manner, final cell densities of *Lb. wasatchii* is identical for cells grown in ribose or with 50% of the ribose replaced with galactose (except for 0.5% ribose vs. 0.25% ribose plus 0.25% galactose at 23°C). This further suggests that only ribose is being used for energy production and that an insignificant amount of ribose is being diverted for peptidoglycan synthesis by transketolase conversion of pentoses to F6P (Figure 3.5, pathway direction {1}, {2}). This hypothesis is supported by findings in *Bifidobacterium breve* where Degnan and McFarlane (1991) found cells grown in the presence of ^{14}C arabinose (a pentose) and glucose (a hexose) did not incorporate carbon from arabinose into cellular macromolecules.

We propose that when an OHF LAB such as *Lb. wasatchii* has both ribose and hexoses available for growth, that the ribose is primarily utilized for ATP production via the lower portion of the PP (Figure 3.5, pathway direction {1}, {2}), while the hexose is

utilized for synthesis of peptidoglycans and other cellular macromolecules (Figure 3.5, pathway direction {4}, {6}). This has the advantage of maximizing ATP production as the need to re-oxidize NADH is minimized when only ribose is fermented. The extent of ribose that is diverted from the PP for peptidoglycan synthesis would depend on the relative amounts of hexoses present. A consequence of such simultaneous co-metabolism is that acetate would be expected as the end product rather than ethanol from acetylphosphate. When ribose is depleted, then galactose would need to be fermented down the PP to provide energy to the cell. This corresponds with our observations that gas production occurred towards the end of exponential growth or early stationary phase (after 48 h at 23°C).

Our results clearly demonstrate that *Lb. wasatchii* can co-utilize ribose and galactose which are two potential substrates for NSLAB (Tinson et al., 1982; Thomas, 1987; Rapposch et al., 1999; Michel and Martley, 2001) in Cheddar cheese. We also have shown that *Lb. wasatchii* is quite tolerant to salt and pH conditions that usually exist in ripening Cheddar cheese. The ability to readily consume mixed putative cheese sugars, grow at cheese ripening temperatures as well as survival against harsh environment of cheese, support our hypothesis that *Lb. wasatchii* contributes late gas blowing and textural defects in Cheddar cheese. To better understand the adaptation of *Lb. wasatchii* to cheese microenvironment, it would be desirable to study whether other sugars in milk and cheese (e.g., lactose, N-acetylgalactosamine, N-acetyl neuraminic acid, mannose, fucose, N-acetylglucosamine) can also be co-utilized by *Lb. wasatchii* in the presence of ribose. When describing carbohydrate utilization abilities of bacteria, such co-utilization

should also be considered as our initial testing of *Lb. wasatchii* led us to believe that it was not capable of utilizing galactose.

CONCLUSIONS

A new obligatory heterofermentative nonstarter lactic acid bacterium, *Lactobacillus wasatchii* sp. nov. (isolated from a blown Cheddar cheese) was shown to require ribose for rapid growth unlike other cheese NSLAB that grow well on glucose. Due to its OHF nature, *Lb. wasatchii* utilizes six and five carbon sugars through the pentose phosphate pathway. Fermentation of hexoses such as galactose will produce CO_2, so OHF have been implicated in late blowing of Cheddar cheese. We speculate that when ribose and galactose are both available, *Lb. wasatchii* uses ribose to produce energy and galactose for peptidoglycan synthesis and growth. This capability is well suited to cheese ripening and we have shown that *Lb. wasatchii* can grow under cheese-like stress conditions of low pH (5.2), and at least up to 5% salt content. It also has the potential to survive the HTST pasteurization used in large scale dairy processing, which may explain how it gains entry to the milk processing environment.

REFERENCES

Adamberg, K., M. Antonsson, F.K. Vogensen, E.W. Nielsen, S. Kask, P.L. Møller, and Y. Ardö. 2005. Fermentation of carbohydrates from cheese sources by nonstarter lactic acid bacteria isolated from semi-hard Danish cheese. Int. Dairy J. 15:873-882.

Axelsson, L. T. 2004. Lactic acid bacteria: classification and physiology. Pages 1-63 in Lactic Acid Bacteria. S. Salminen and A. von Wright, ed. Dekker, New York.

Banks, J. M., and A. G. Williams. 2004. The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. Int. J. Dairy Tech. 57(2-3): 145-152.

Beresford, T. P., N. A. Fitzsimons, N. L. Brennan, and T. M. Cogan. 2001. Recent advances in cheese microbiology. Int. Dairy J. 11:259–274.

Broadbent, J. R., C. Brighton, D. J. McMahon, N. Farkye, M. Johnson and J. Steele. 2013. Microbiology of Cheddar cheese made with different fat contents using a *Lactococcus lactis* single-strain starter. J. Dairy Sci. 96:4212–4222.

CRC. 2009. CRC Handbook of Chemistry and Physics: A Ready-Reference Book of Chemical and Physical Data. 90th ed. CRC Press, Boca Raton, FL.

Crow, V. L., B. Curry, and M. Hayes. 2001. The ecology of nonstarter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. Int. Dairy J. 11:275–283.

Dacre, J. C. 1953. Cheddar cheese flavour and its relation to tyramine production by lactic acid bacteria. J. Dairy Res. 20: 217–223.

Degnan, B. A., and G. T. MacFarlane. 1991. Glucose dependent uptake of arabinose in *Bifidobacterium breve*. J. Applied Bacteriology. 71:xxviii-xxix.

Fred, B. Y., W. H. Peterson, and J. A. Anderson. 1921. The characteristics of certain pentose-destroying bacteria, especially as concerns their action on arabinose and xylose. J. Biological Chem. 48:385-411.

Gobbetti, M., A. Corsetti, and J. Rossi. 1995. Maltose-fructose co-fermentation by *Lactobacillus brevis* subsp. *lindneri* CB1 fructose-negative strain. Appl. Microbiol. Biotechnol. 42: 939–944.

Golnazarian, C. 2001. Slit formation in Cheddar cheese: A comprehensive investigation of the microbiological parameters associated with this defect. PhD Thesis. University of Vermont, Burlington.

Jordan, K. N., and T. M. Cogan. 1993. Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. Ir. J. Agric. Food Res. 32:47–55.

Kask, S., K. Adamberg, A. Orlowski, F. K. Vogensen, P. L. Moller, Y. Ardö, and T. Paalme. 2003. Physiological properties of *Lactobacillus paracasei*, *L. danicus*, and *L. curvatus* strains isolated from Estonian semi-hard cheese. Food Res. Int. 36:1037-1046.

Khalid, N. M., and E. H. Marth. 1990. Lactobacilli – their enzymes and role in ripening and spoilage of cheese: A review. J. Dairy Sci. 73: 2669–2684.

Koort, J., A. Murros, T. Coenye, S. Eerola, P. Vandamme, A. Sukura and J. Björkroth. 2005. *Lactobacillus oligofermentans* sp. nov., associated with Spoilage of Modified-Atmosphere-Packaged Poultry Products. Appl. Environ. Microbiol. 71(8): 4400-4406.

Laleye, L. C., R. E. Simard, B. H. Lee, R. A. Holley, and R. Giroux. 1987. Involvement of heterofermentative lactobacilli in development of open texture in cheese. J. Food Prot. 50:1009–1012.

Lane, C. N., P. F. Fox, E. M. Walsh, B. Folkertsma, and P. L. H. McSweeney. 1997. Effect of compositional and environmental factors on the growth of indigenous and non-starter lactic acid bacteria in Cheddar cheese. Lait 77: 561–573.

Martley, F. G., and V. L. Crow. 1993. Interaction between nonstarter microorganisms during cheese manufacture and ripening. Int. Dairy J. 3: 461–483.

Michel, V., and F. G. Martley. 2001. *Streptococcus thermophilus* in cheddar cheese--production and fate of galactose. J. Dairy Res. 68(2): 317-325.

Mullan, W. M. A. 2000. Causes and control of early gas production in Cheddar cheese. Int. J. Dairy Tech. 53: 63–68.

Naylor, J., and M. E. Sharpe. 1958. Lactobacilli in Cheddar cheese. III. The source of lactobacilli in cheese. J. Dairy Res. 25:431–438.

Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the Bacterial Cell: A Molecular Approach. Sinauer Associates. Sunderland, MA.

Oberg, C. J., L. V. Moyes, M. J. Domek, C. F. Brothersen, and D. J. McMahon. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. J. Dairy Sci. 94: 2220-2230.

Okada, S., Y. Suzuki, and M. Kozaki. 1978. A new heterofermentative lactobacillus species with Meso-diaminopimelic acid in peptidoglycan, *Lactobacillus vaccinostercus* KOZAKI and OKADA sp. nov. J. Gen. Appl. Microbiol. 25:215-221.

Peterson, S. D., and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: A review. J. Dairy Sci. 73: 1395-410.

Rapposch, S., F. Eliskases-Lechner, and W. Ginzinger. 1999. Growth of facultatively heterofermentative lactobacilli on starter cell suspensions. Appl. Environ. Microbiol. 65: 5597–5599.

Somers, E. B., M. E. Johnson, and A. C. L. Wong. 2000. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84:1926–1936.

Thomas, T. D. 1987. Cannibalism among bacteria found in cheese. NZ J. Dairy Sci. Technol. 22: 215–219.

Tinson, W., M. F. Ratcliff, A. J. Hillier, and G. R. Jago. 1982. Metabolism of *Streptococcus thermophilus*. 3. Influence on the level of bacterial metabolites in Cheddar cheese. Aust. J. Dairy Technol. 37:17–21.

Turner, K. W., R. C. Lawrence, and J. Lelievre. 1986. A microbiological specification for milk for aseptic cheese making. NZ J. Dairy Sci. Technol. 21:249–254.

Westby, A. 1989. *Lactobacillus plantarum* as a microbial antagonist. PhD. Thesis, University of Reading., Reading, UK.

Westby, A., L. Nuraida, J. D. Owens, and P. A. Gibbs. 1993. Inability of *Lactobacillus plantarum* and other lactic acid bacteria to grow on D-ribose as sole source of fermentable carbohydrate. J. Appl. Bacteriol. 75:168–175.

White, D. 2007. The Physiology and Biochemistry of Prokaryotes. 3rd ed. Oxford University Press, New York.

Table 3.1. Maximum specific growth rate (μ_{max}) of *Lactobacillus wasatchii* cells grown at 12, 23, or 37°C on ribose, galactose, or ribose plus galactose.

	μ_{max}		
Sugar[1]	**12°C**	**23°C**	**37°C**
	----------(OD$_{640}$/h) ----------		
0.1R	0.0085^{hij}	0.0235^{cd}	0.019^{de}
0.5R	0.0145^{efghi}	0.0365^{a}	0.0195^{de}
1R	0.0155^{efgh}	0.0355^{ab}	0.0215^{de}
0.1G	0.003^{j}	0.0095^{ghij}	0.008^{ij}
0.5G	0.006^{j}	0.0095^{ghij}	0.0075^{ij}
1G	0.005^{j}	0.009^{ghij}	0.008^{ij}
0.05R+0.05G	0.010^{fghij}	0.021^{de}	0.0185^{de}
0.25R+0.25G	0.016^{efg}	0.0285^{bc}	0.021^{e}
0.5R+0.5G	0.018^{de}	0.0385^{a}	0.0205^{de}
1R+1G	0.017^{def}	0.0375^{a}	0.0195^{de}

[1]Percent concentration (wt/vol) of ribose (R), galactose (G) or combination of ribose and galactose (R+G) in carbohydrate restricted MRS.

[a-j]Means values with the same letter are not significantly different from each other (α = 0.05).

Table 3.2. Final cell densities[1] (OD640) reached when *Lactobacillus wasatchii* cells were grown at 12, 23, or 37°C in ribose, galactose, or ribose plus galactose.

Sugar[1]	**12°C**	**23°C**	**37°C**
0.1R	0.3^{j}	0.35^{j}	0.225^{kl}
0.5R	0.795^{de}	0.8^{de}	0.705^{fg}
1R	1.44^{a}	1.37^{b}	0.75^{ef}
0.1G	0.298^{j}	0.214^{l}	0.205^{l}
0.5G	0.68^{gh}	0.69^{fgh}	0.215^{l}
1G	0.58^{i}	0.755^{ef}	0.195^{l}
0.05R+0.05G	0.335^{j}	0.335^{j}	0.300^{j}
0.25R+0.25G	0.83^{d}	0.83^{d}	0.62^{hi}
0.5R+0.5G	1.36^{b}	1.285^{c}	0.835^{d}
1R+1G	1.42^{ab}	1.4^{ab}	0.72^{fg}

[1]Final OD_{640} measured at 204 h for growth at 12°C and 72 h for growth at 23 and 37°C.
[2]Percent concentration (wt/vol) of ribose (R), galactose (G) or combination of ribose and galactose (R+G) in carbohydrate restricted MRS.
[a-l]Means values with the same letter are not significantly different from each other (α=0.05).

Table 3.3. Maximum specific growth rate (μ_{max}) of *Lactobacillus wasatchii* cells grown at 23°C on MRS plus 1.5% ribose containing 0 to 5% NaCl at pH 5.2 or 6.5

NaCl[1]	μ_{max}	
	pH 5.2	**pH 6.5**
	----------(OD_{600}/h) ----------	
0%	0.05^{cde}	0.061^{abcd}
1%	0.064^{abc}	0.076^{a}
2%	0.058^{bcde}	0.056^{bcde}
3%	0.057^{bcde}	0.068^{ab}
4%	0.044^{e}	0.048^{de}
5%	0.044^{e}	0.053^{bcde}

[1]Percent concentration (wt/wt) of NaCl (0 to 5%) in MRS supplemented with 1.5% ribose.

[a-e]Means values with the same letter are not significantly different from each other (α=0.05).

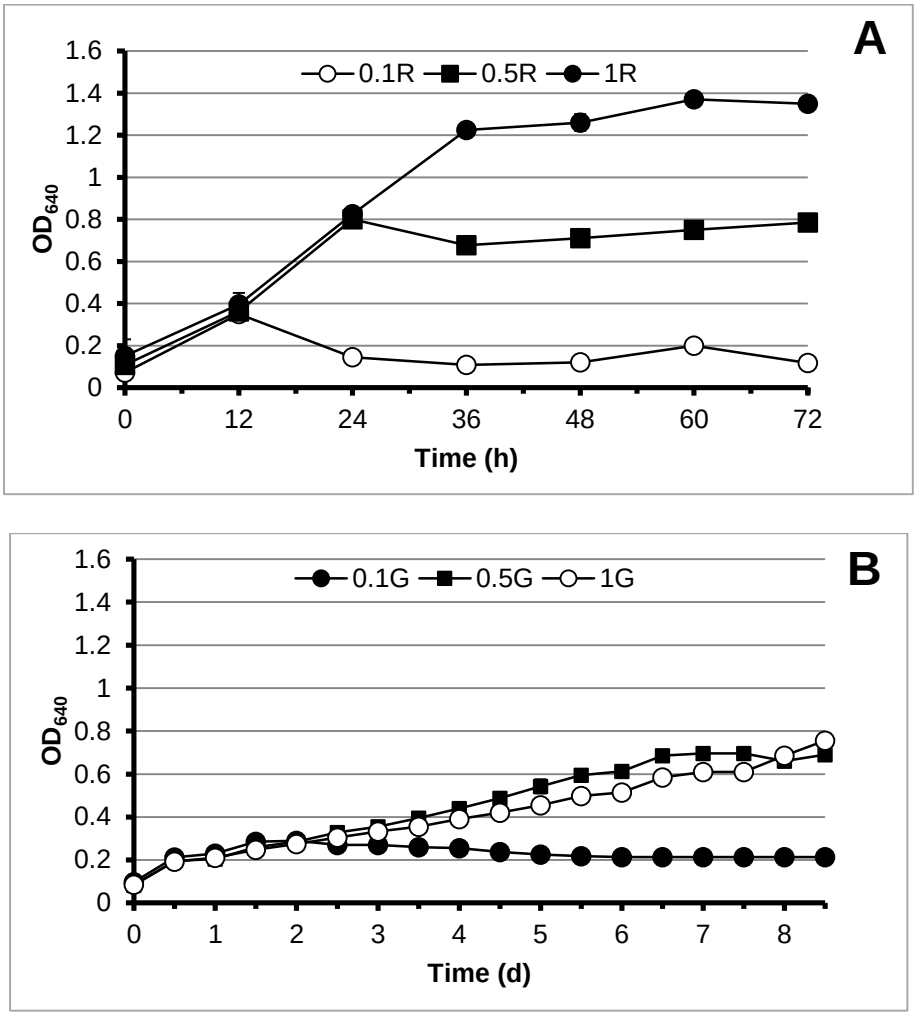

Figure 3.1. Growth of *Lactobacillus wasatchii* (OD640) at 23°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

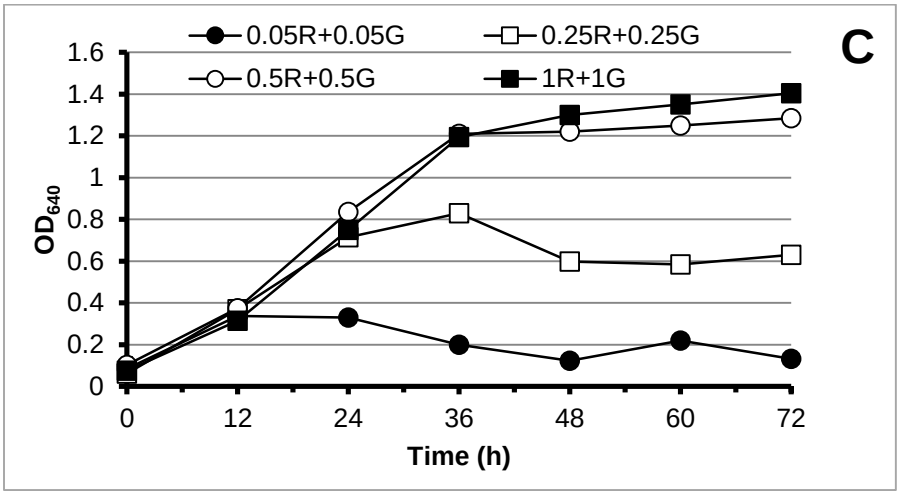

Figure 3.1. Continued.

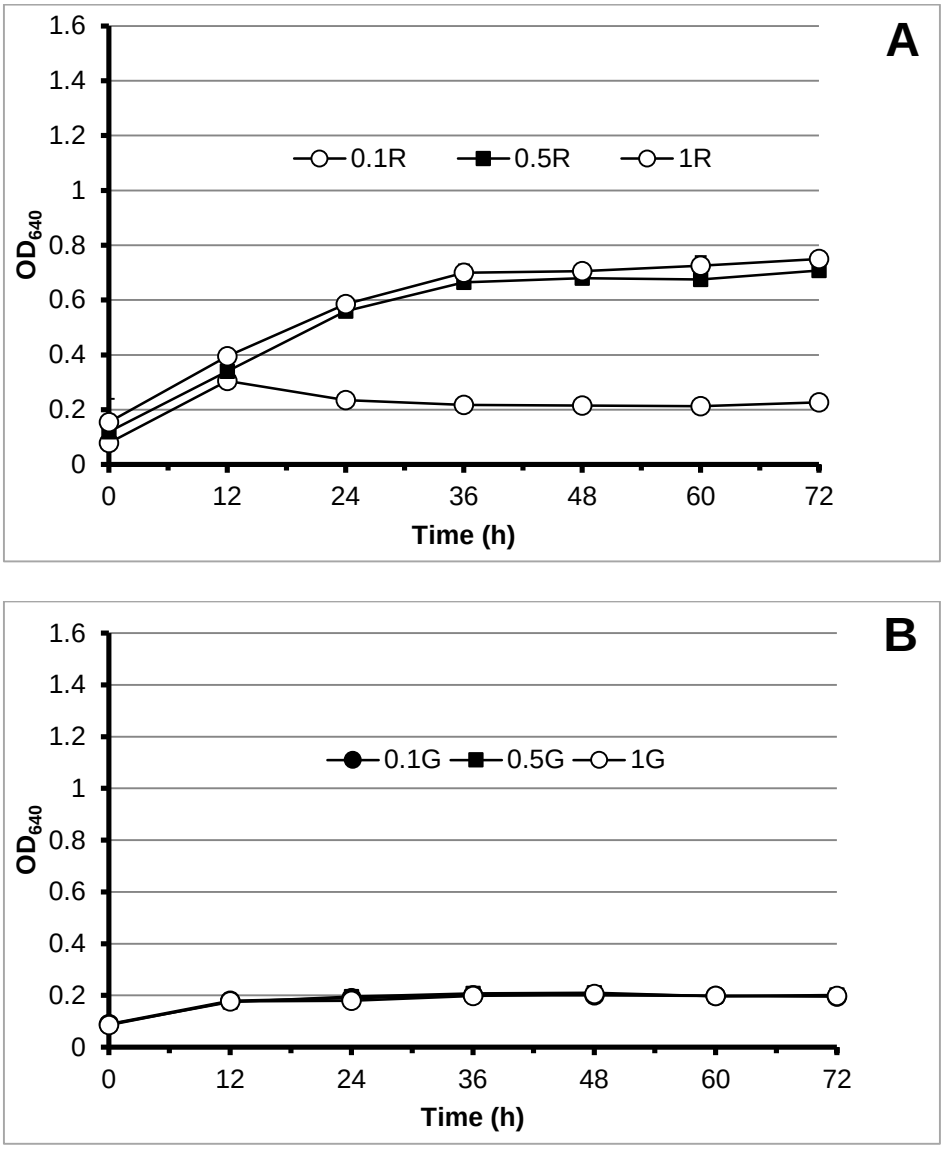

Figure 3.2. Growth of *Lactobacillus wasatchii* (OD_{640}) at 37°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

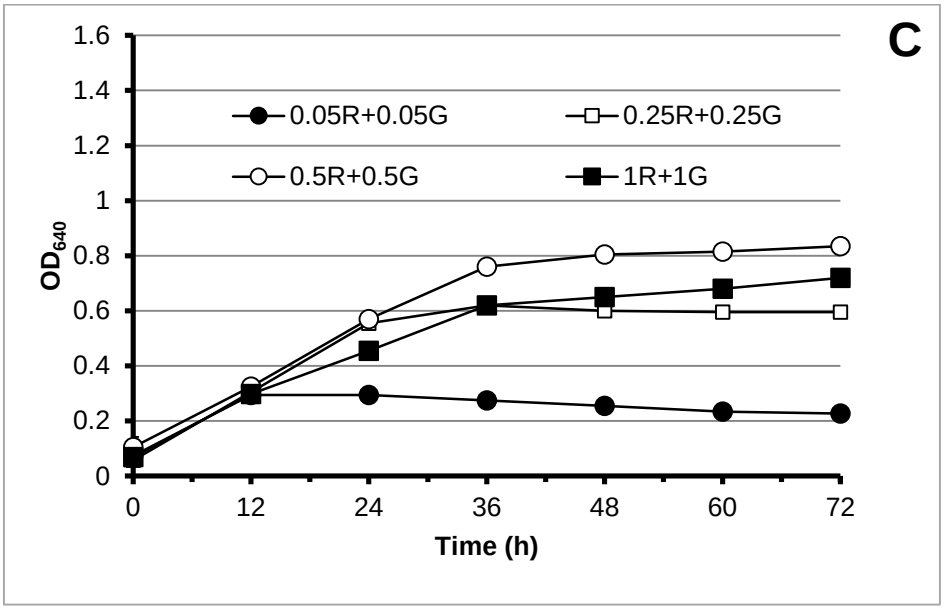

Figure 3.2. Continued.

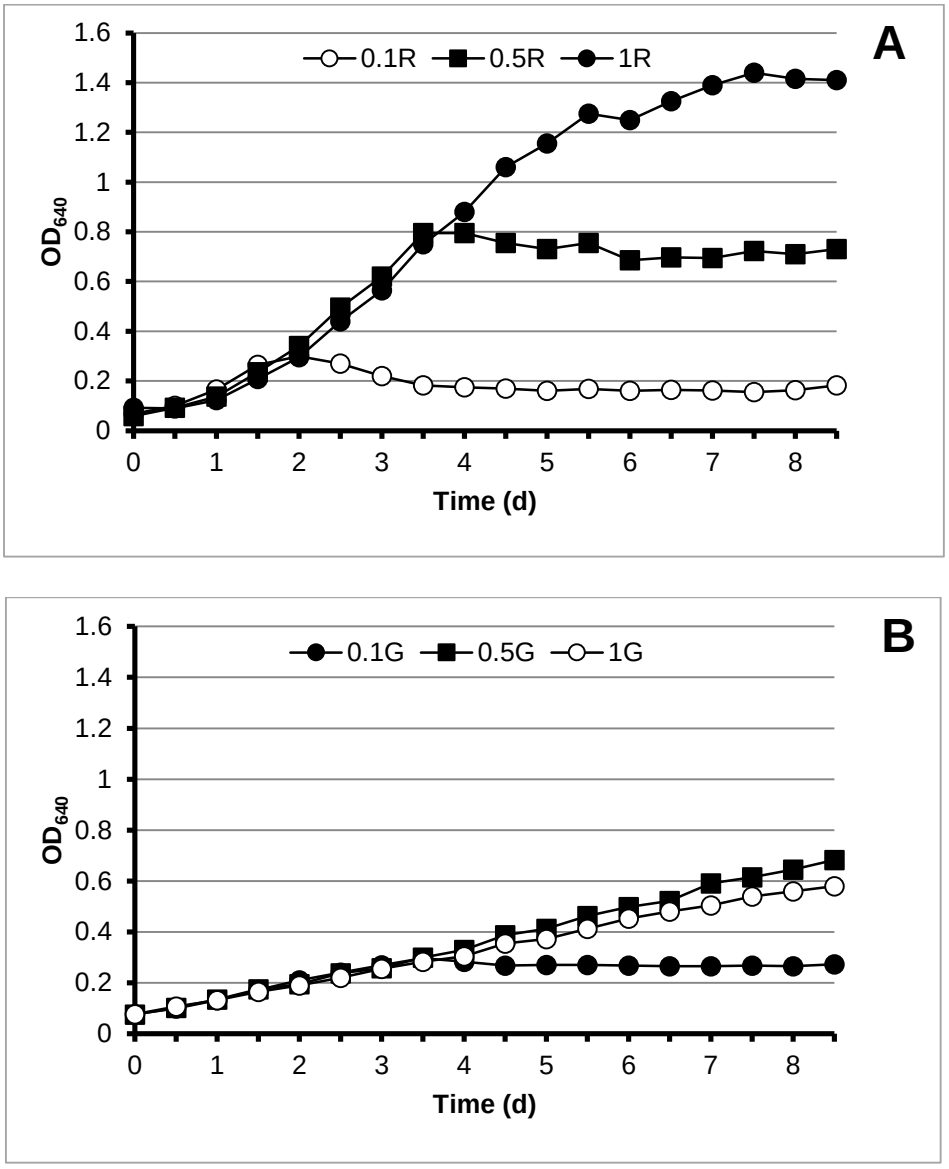

Figure 3.3. Growth of *Lactobacillus wasatchii* (OD_{640}) at 12°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

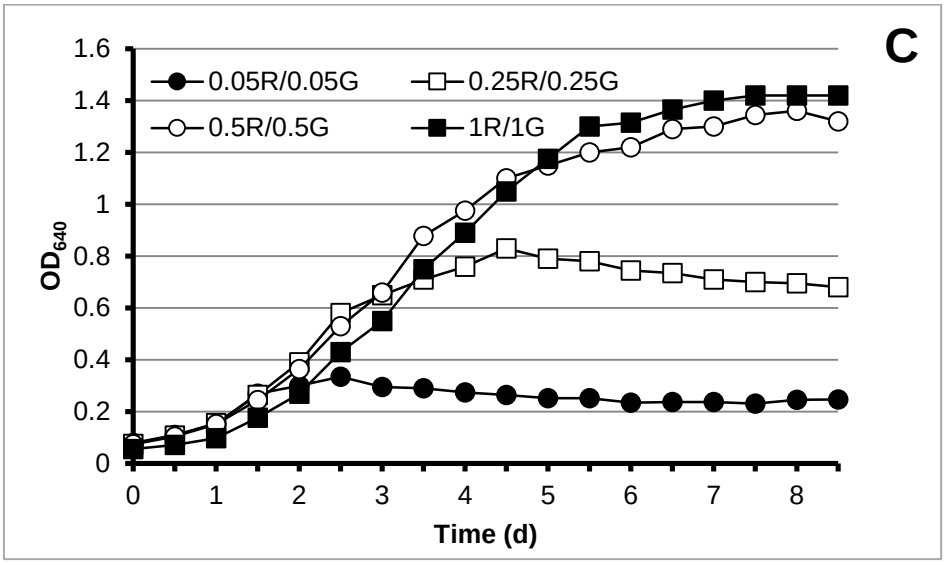

Figure 3.3. Continued.

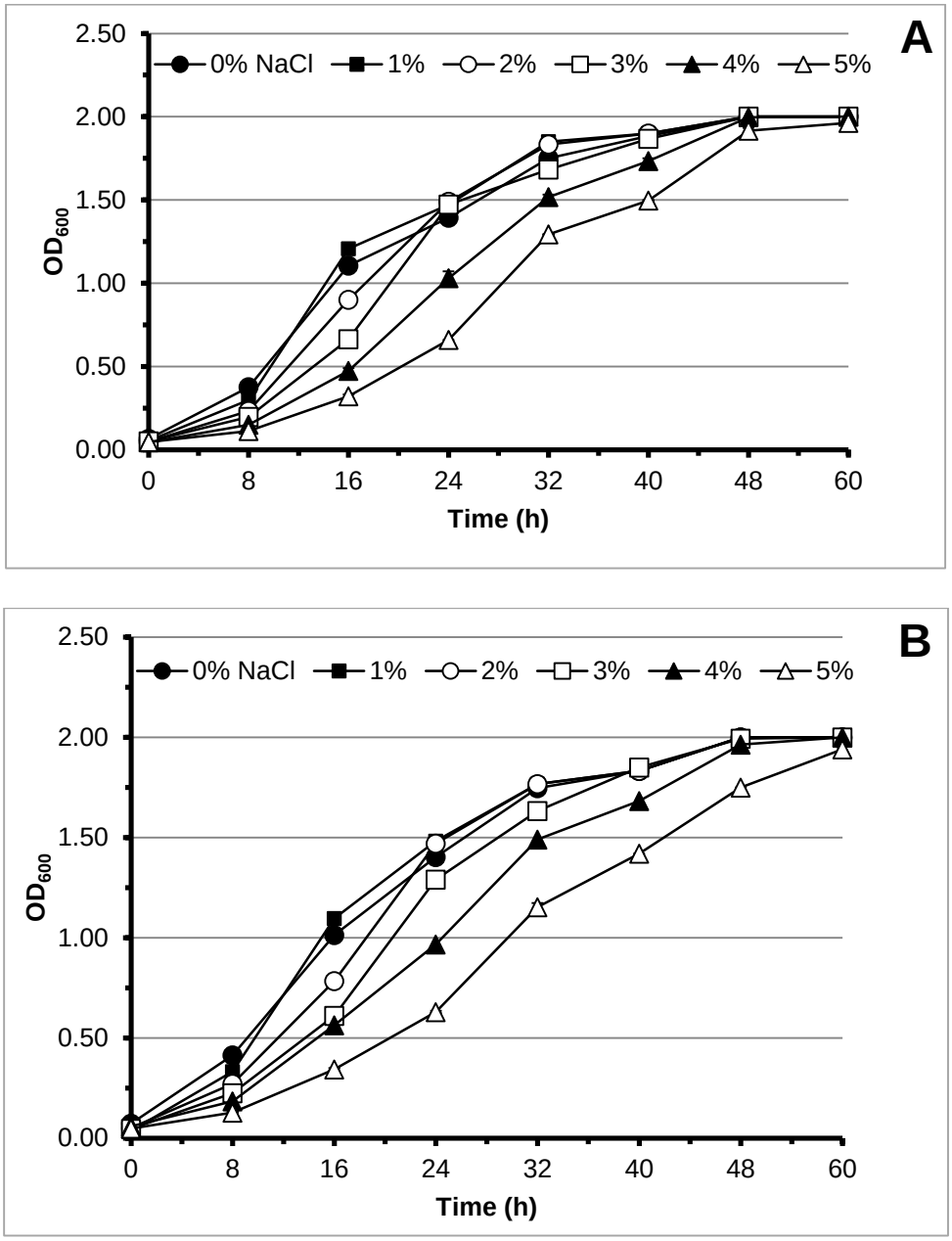

Figure 3.4. Growth of *Lactobacillus wasatchii* (OD_{600}) in regular MRS broth supplemented with 1.5% ribose (wt/vol) plus 0 to 5% NaCl and adjusted to pH 6.5 (panel A) or pH 5.2 (panel B). Error bars = SE (n=3).

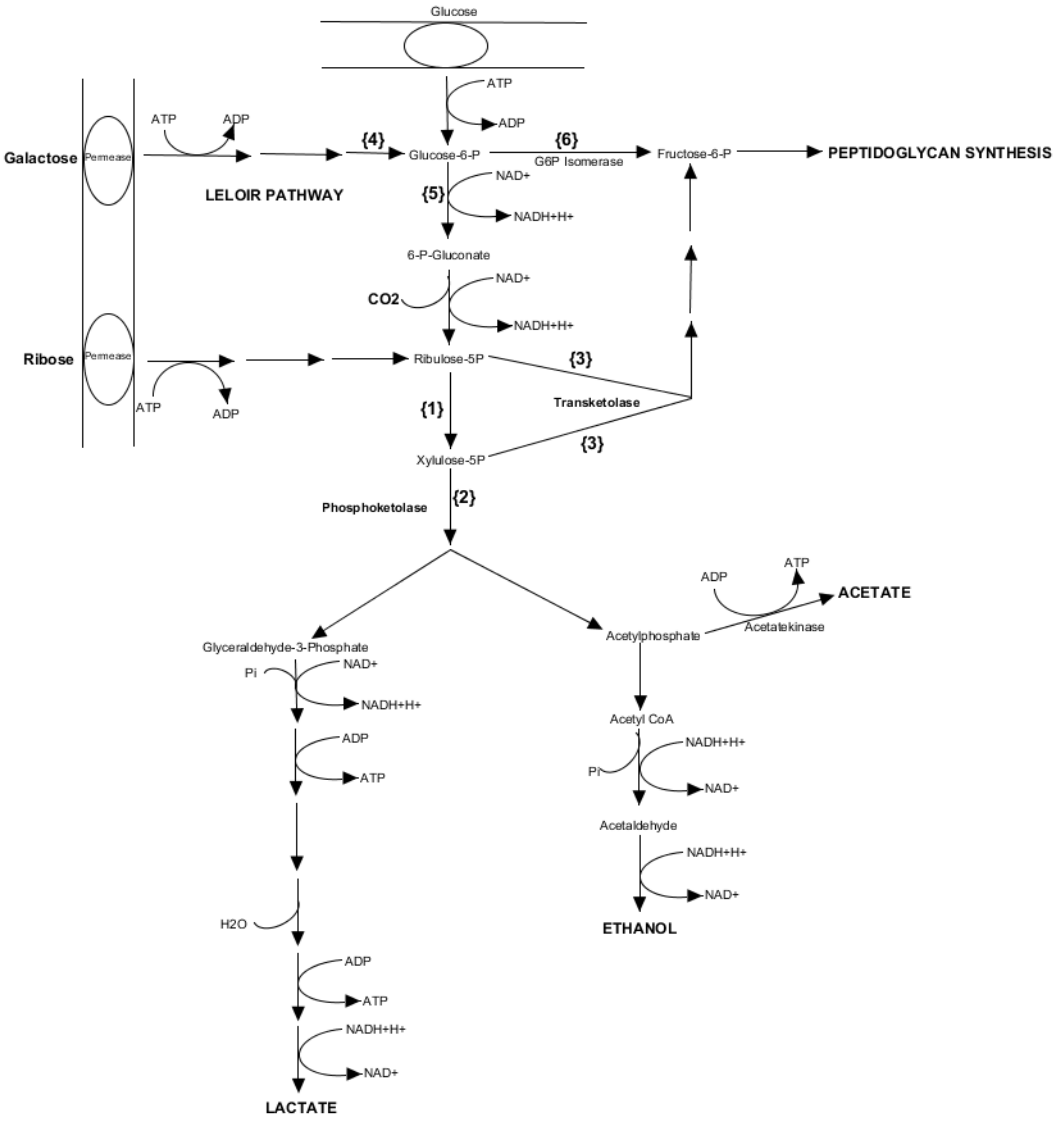

Figure 3.5. Proposed pathways for ribose and galactose utilization by *Lactobacillus wasatchii.*

CHAPTER 4

LATE BLOWING OF CHEDDAR CHEESE INDUCED BY ACCELERATED RIPENING AND RIBOSE AND GALACTOSE SUPPLEMENTATION IN PRESENCE OF A NOVEL OBLIGATORY HETEROFERMENTATIVE NONSTARTER LACTOBACILLI SPECIES

ABSTRACT

Lactobacillus wasatchii sp. nov. has been studied for growth and gas formation in a control Cheddar cheese and in cheese supplemented with 0.5% ribose, 0.5% galactose, or 0.25% ribose plus 0.25% galactose using regular and accelerated cheese ripening temperatures of 6 and 12°C. Cheese milk along with starter lactococci was inoculated with *Lb. wasatchii* at a level of 10^4 cfu/ml whereas a control vat was inoculated with starter lactococci only. Starter numbers in both cheeses decreased from 10^7 to ~10^3 cfu/g at 23 wk of ripening at both temperatures, except the control cheese at 6°C which had one log higher final cell counts. Unlike starter bacteria, nonstarter lactic acid bacteria started at <10^2 cfu/g in the cheese and reached 10^6 to 10^7 cfu/g with higher numbers observed at 12°C. *Lactobacillus wasatchii* grew to ≥10^8 cfu/g in cheese supplemented with ribose (alone or with galactose) at elevated temperature which was ~1-log higher compared to the control and galactose-supplemented cheeses. In all cheeses with adjunct *Lb. wasatchii*, highest growth and gas formation was observed at 12°C although most gas production occurred at ≥16 wk. Adding both ribose and galactose provided substantially higher growth and gas formation because of the ability of *Lb. wasatchii* to co-utilize both

sugars; producing gas from galactose as a result of the obligatory heterofermentative nature of the bacterium. Even without sugar supplementation, gas was observed in the presence of adjunct *Lb. wasatchii* after 16 wk. We have observed that *Lb. wasatchii* can grow to high cell densities when grown in carbohydrate-restricted broth containing lactococcal cell lysate. During cheese ripening, lysis of starter bacteria would provide sufficient substrate (such as ribose) to allow growth of *Lb. wasatchii* during cheese ripening and the presence of any hexoses in cheese would allow *Lb. wasatchii* to produce gas. We conclude that *Lb. wasatchii* is a previously undetected contributor to late gas formation in Cheddar cheese and the defect is more pronounced when elevated ripening temperatures are used.

INTRODUCTION

Cheese ripening at elevated temperatures is technically the simplest method to accelerate maturation and the lower refrigeration costs may provide overall savings to the producer (Wilkinson, 1993; Folkertsma et al., 1996). However, late gas formation ("gassy defect") in Cheddar cheese has been a recurring problem for over 100 years and this defect is one of the main concerns during accelerated ripening (Van Slyke and Hart, 1903; Mullan, 2000). Manifest as openness of cheese texture or "blown wrappers" without texture change in cheeses aged longer than 3 mo (Laleye et al., 1987). Gassy defect tends to be sporadic and recurrent and has probably been experienced at most cheese making plants (Mullan, 2000). The slits, cracks or voids caused by gassy defect in cheese are not evident until the cheese is graded unless the cheese pack is loose. Even though this textural defect may not create a specific sensory defect, it may result in the

inability of the producer to cut the blocks with uniformity and impacts the slicing ability of the cheese. If the defect is severe, the block will crumble upon cutting which can increase cutting losses from 10% (non-defective) up to 50% (Elliott et al., 1981; Donnely et al., 2014). The time and money spent on the aging process is lost, therefore the cheese may be sold at a lower price or used for processed cheese (Martley and Crow, 1996; Golnazarian, 2001).

Gassy defect in cheese can result from poor sanitaton (observed as early gas formation) and germinatin of *Clostridium* spores (late blowing) (Fox et al., 1990; McSweeney and Fox, 2004). In properly manufactured Cheddar cheese with pH below 5.4 and salt-in-moisture content above 4.0% (Lawrence et al., 2004) gassines has been attributed to obligative heterofermentative (**OHF**) nonstarter lactobacilli species such as *Lactobacillus brevis* and *Lactobacillus fermentum* (Sherwood, 1939; Laleye et al., 1987; Laleye et al., 1990). However, Elliott et al. (1981) linked gassy defect in Cheddar cheese to a slow growing unusual bacterium.

Our group isolated a novel slow-growing OHF species, *Lactobacillus wasatchii* spp. nov. from a "gassy" Cheddar cheese manufactured at Utah State University (see Chapter 3). We reported that *Lb. wasatchii* produces gas from the hexose and can co-utilize ribose and galactose (unpublished data) which are potential substrates for nonstarter lactic acid bacteria (**NSLAB**) in Cheddar cheese (Tinson et al., 1982; Thomas, 1987; Rapposch et al., 1999; Michel and Martley, 2001). We also showed *Lb. wasatchii* is able to grow under salt and pH conditions typical of Cheddar cheese (4 to 5% salt-in-

moisture, ~pH 5.2), and that there is potential for some survival of *Lb. wasatchii* during HTST lab pasteurization (see Chapter 3).

The ability to readily consume mixed putative cheese sugars, grow at cheese ripening temperatures as well as survival against harsh environment of cheese, led us to hypothesize that *Lb. wasatchii* contributes late gas blowing and textural defects in Cheddar cheese (see Chapter 3). Thus, the aim of present work was to confirm the gas formation in Cheddar cheese made from milk inoculated with *Lb. wasatchii*. The curd was also supplemented with ribose and galactose to promote growth of *Lb. wasatchii*, and the cheese was stored at 6°C or accelerated ripened at 12°C. We monitored the microbiota of the cheese for *Lb. wasatchii*, starter lactococci and nonstarter lactic acid bacteria as well as gas formation throughout 23 wk of ripening.

MATERIALS AND METHODS

Bacteria and Growth

Working cultures of *Lb. wasatchii* were prepared from frozen stocks stored at −80°C by sequential transfer twice into de Man Rogosa, and Sharpe (**MRS**) (Becton Dickinson Inc., Sparks, MD) broth containing 1.5% ribose (**R**) (donated by Bioenergy Life Science Inc., Ham Lake, MN), in which the cultures were incubated anaerobically using GasPak™ EZ at 23°C for 40 h. Cells for the cheese making experiments were propagated in 400 ml MRS+R for 40 h at 23 °C. Cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C and washed twice (7,000 rpm for 10 min at 4°C) with sterile 0.1% (wt/vol) peptone water. Concentration of cell suspensions was determined by anaerobic spread plate counts in MRS+R agar after 5 d at 23°C. The cell suspensions

were subsequently used in cheese making experiments after proper dilutions were made to reach desired numbers in cheese milk.

Frozen pellets of *Lactococcus lactis subsp. lactis/cremoris* (DVS850) suspended in peptone water (OD_{600} ~ 0.9) was inoculated into 750 ml of M17 broth (Becton Dickinson Inc., Sparks, MD) supplemented with 1% lactose (Sigma-Aldrich Inc., St. Louis, MO) (**M17-L**) and incubated at 30°C for 24 h under aerobic conditions. The cells were then harvested by centrifugation (8,000 rpm; 10 min; 4°C), washed twice in 50 ml phosphate buffer and suspended in 10 ml phosphate buffer and a 0.1 ml aliquot was plated on M17-L agar to enumerate starter numbers (Branen and Keenan, 1969; Thomas, 1987; Rapposch et al., 1999). The cell suspensions were stored at -80°C until used in cell free extract experiments.

Cheese Making

Fresh bovine 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). The milk was standardized to a protein-to-fat ratio of 0.84, pasteurized at 73°C for 15 s, and 273 kg was transferred into each of two open stainless steel vat (which had previously been cleaned then heat sanitized for 30 min). Both batches of milk were warmed to 31°C and 0.2 g/kg of frozen pellets containing *Lactococcus lactis ssp. lactis/cremoris* starter culture (DVS850; Chr. Hansen Inc., Milwaukee, WI) were added. To one of the vats, 10^4 cfu/ml *Lb. wasatchii* was also added and the milk allowed to ripen for 20 min in both vats. Then, 0.12 mL/kg of a 32% (wt/wt) $CaCl_2$ solution (Nelson-Jameson Inc., Marshfield, WI), 0.13 ml/kg of

anatto and 0.16 mL/kg of double-strength (~650 international milk clotting units/mL) chymosin rennet (Maxiren; DSM Food Specialties USA Inc., Eagleville, PA) were added, and the milk allowed to set undisturbed for 30 min. After cutting and healing, the curd/whey mixtures were stirred for 10 min, heated to 39°C over 35 min, and then stirred for another 10 min. The curd was stirred until a curd pH of 6.3 was reached with partial whey drainage. The remaining whey was then drained and curd was allowed to mat together, cut into slabs, and cheddared for approximately 160 min until the curd pH reached 5.4. Curd was milled, then salted (30 g/kg of curd) in 3 applications with 5 min between each application. Salted curd from each vat was separated into four 7-kg portions in open plastic containers. One portion served as a control with no sugar added, while to the other portions was added on a wt/wt basis: 0.5% ribose, 0.5% galactose (Alfa Aesar Inc., Ward Hill, MA) or 0.25% ribose plus 0.25% galactose. The curd was mixed for 5 min then placed into plastic hoops and pressed overnight (140 kPa, ~18 h, ~20°C). The cheese was recovered from the hoops and each block cut into 10 pieces of ~600 g each, and the pieces were vacuum packaged seperately. Five pieces were stored at 6°C and five at 12°C. Cheese making trials were conducted in triplicate.

Microbial Enumerations

At 0, 8, 16, and 23 wk, cheese samples (11 g) were collected from the interior of each cheese and homogenized in 99 ml of sterilized 2% sodium citrate (warmed to 45°C) using a Stomacher 400 Circulatory laboratory blender (Seward Laboratory Systems Inc., Bohemia, NY) set for 3 min at 230 rpm (Broadbent et al., 2013). Serial dilutions were prepared in 0.1% sterile peptone water, then lactococcal starter was enumerated on M17-

L agar after aerobic incubation at 30°C for 24 h. The method of Oberg et al. (2011) was used for enumerating NSLAB on MRS agar supplemented with 2 μg/ml vancomycin (**V**) incubated anaerobically at 37°C for 48 h. Such relatively fast-growing NSLAB counts were designated as **NSLAB37** and *Lb. wasatchii* does not grow quickly enough to be enumerated at 48 h. Like many NSLAB, *Lactobacillus wasatchii* is resistant to vancomycin but does not form colonies on MRS-V within 48 h (data not shown).

The mean log numbers from three reps were plotted against storage time and a trend line fitted to each set of data based on the highest R square obtained. The time (in weeks) when the NSLAB numbers equaled and then surpassed starter numbers were considered the crossover time (Oberg et al., 2011). All plate counts were performed with spread plate method in duplicate.

Enumeration of *Lb. wasatchii*. Nonstarter lactic acid bacteria were also enumerated on MRS-V agar supplemented with 1.5% ribose (**MRS-R-V**) after 48 h of anaerobic incubations at 23°C and these counts were designated as **NSLAB23**. After obtaining counts for NSLAB23 after 48 h incubation at 23°C on MRS-R-V media and marking all the colonies (~1.5 mm diameter), the plates were incubated anaerobically at 23°C for an additional 72 h. By this time, *Lb. wasatchii* forms noticeable colonies (~1 mm diameter), which enables differential enumeration of this organism from other NSLAB23 colonies.

Cell Free Extract and Growth

The cell suspensions containing ~10^{10} cfu/ml of starter lactococci were divided into 0.5 ml aliquots placed into 2 ml sized screw-cab tubes that contained 0.5 g of 100

µm diameter glass beads (BioSpec Products, Bartlesville, OK). Cells were disrupted in a Mini-Beadbeater (Biospec Products) at a speed setting of homogenize, in 12 bursts of 30 s, with 2 min intermittent cooling periods in ice bath. After centrifugation (4°C, 15 min, 15,000 rpm), the supernatant was collected and protein concentration determined with a Modified Lowry Protein assay kit (Pierce, ThermoScientific) using bovine serum albumin (BSA) (2 mg/ml stock solution; Pierce) as the standard. To determine the efficacy of lysis, 0.1 ml of the lysate was plated on M17-L agar and incubated at 30°C for 24 h. The remaining supernatant was filter sterilized (0.2 µm pore size membranes) and 1 ml of the filtrate was mixed with 0.2 ml of 5X carbohydrate free MRS (Anonymous, 2015). The lysate-carbohydrate free MRS solution was inoculated with 20 µl of *Lb. wasatchii* (sub-cultured twice in MRS+R) and incubated at 23°C for 10 d under anaerobic conditions. Growth of *Lb. wasatchii* was monitored by OD_{600} measurements taken at 0 and 10 d. A control was prepared with adding 1 ml of sterile ultrapure water instead of lysate. Growth studies on starter lysate were performed in triplicate.

Cheese Gas Measurements

Gas production during storage of cheese was measured by the extent of loosening of the plastic bag around the cheese. After pressing, similar sized (~600 g) blocks of cheese were inserted into plastic bags (QME355 3.5 mil; Vilutis and Co. Inc., Frankfurt, IL) and the bag vacuum-sealed ~5 cm distance from the cheese block. Ten blocks for packaged for each treatment with five stored at each temperature (6 and 12°C). The vacuum packaged cheese was then visually examined and on the side of the pack that was just sealed, a line was drawn along the pack at the position where it was tightly pulled

against the cheese. After 8, 16 and 23 wk, the cheese packs were examined for gas production and loosening of the package. If the pack was no longer tightly held against the cheese, the pack was pulled away from the cheese block (on the same side which was initially marked) as much as possible. A line was then drawn on the bag at the point at which the 2 layers of the bag were still held together by any residual vacuum inside the bag. The distance between that line and the d 0 line was then measured and used as an expression of relative gas formation. The more gas that was produced the further the plastic bag could be pulled away from the cheese block. When sufficient gas production had occurred inside the cheese pack so that there was no longer any residual vacuum (compared to atmospheric pressure) the cheese pack could be pulled the full 5 cm from the cheese to the seal. The distance the pack could be pulled was calculated in relation to this maximum distance and expressed as relative gas production. All available packs were tested for relative gas production prior to opening the packs and sampling the cheese for microbial analysis. So that for each treatment, 4 packs were tested after 8 wk, 3 after 16 wk, and 2 after 23 wk.

Chemical Analysis

Proximate composition of the cheeses was determined after approximately 3 d. Moisture content was measured by weight loss using ~3.7 g of grated cheese in a microwave moisture analyzer (Model SMART System 5; CEM Corporation, Matthews, NC) using program CHEESELF. Fat content was measured by a modified Babcock method (Richardson, 1985). Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher. The slurry was filtered through a

Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (Model 926, Corning, Medfield, MA). Salt-in-moisture (**S/M**) content was calculated as salt/(moisture + salt) and expressed as percentage.

Sugar and Organic Acid Analysis

Cheese samples from each treatment were analyzed by an HPLC for lactose, galactose, lactic acid, acetic acid, citric acid, propionic acid, pyruvic acid, formic acid, and orotic acid at time 0 and after 16 wk of ripening as described by Phadungath (2011). For the sample preparation, about 5g cheese were manually homogenized for 90 s with 10-mL of 0.013N sulfuric acid (at 65°C) using a high shear Omni mixer-homogenizer (model 17105, Omni International, Waterbury, CT). The extract was centrifuged at (Jouan CR4-12 Centrifuges, Jouan, Inc., Winchester, VA) at 7,000 x *g* for 10 min. The samples were held at 4°C for 20 min to solidify the fat layer, which was removed with a spatula, and the supernatant was filtered through filter paper (Whatman #4; Whatman International Ltd., Maidstone, England). A 0.5 mL aliquot of filtered supernatant was poured into a 0.5-mL Microcon® (Millipore Corporation, Bedford, MA) centrifugal filter device with a molecular weight cut-off of 3,000 Da and micro-centrifuged (Jouan A14 Microcentrifuges, Jouan, Inc., Winchester, VA) at 14,000 x *g* for 20 min to remove soluble peptides. The filtrate collected from the micro-centrifuge was directly injected into the HPLC system.

The HPLC system (Beckman Coulter Inc., Fullerton, CA) was equipped with a photodiode array detector set at 210 nm, and data processing software (System Gold® HPLC, 32 KaratTM Software, Beckman Coulter Inc., Fullerton, CA). The system was

externally equipped with an intelligent refractive index detector (JASCO Model RI-2031, Jasco, Inc., Easton, MD), and a column heater (Alltech® Model 6301, Alltech Associates Inc., Deerfield, IL). The column used 172 for separation of the analytes was a Rezex ROA-organic acid H+ column (300x7mm, 8µm, Phenomenex) held at 65°C, with a cation H+ microguard cartridge (Bio-Rad Laboratories, Hercules, CA). The analysis was performed isocratically at 0.6 mL/ min flow rate using 0.013 N sulfuric acid (Fisher Scientific, Fair Lawn, NJ) as the mobile phase. Quantification of analytes was based on the external standard method described by Upreti et al. (2006a).

Experimental Design

The experiment was conducted as a randomized block with split-split-split plot design. Statistical analysis of the effect of added sugar, storage time, temperature and *Lb. wasatchii* addition was performed using PROC GLIMMIX in SAS (version 9.1; SAS Institute Inc., Cary, NC) and differences between means determined using Tukey least squares means. Significance was declared at $P<0.05$.

RESULTS AND DISCUSSION

Initial Cheese Composition

Addition of *Lb. wasatchii* to cheese milk or sugar supplementation of curd did not significantly impact ($P>0.05$) the initial composition of cheese. Therefore, moisture, salt, S/M, pH, fat, lactose and citrate values across those two treatments were pooled (Table 4.1). The average S/M levels of the cheeses in the current study were higher compared to levels in typical retail Cheddar cheese (4.3%) in the USA (Agarwal et al., 2011).

Internationally, however, S/M was in the range for good quality aged cheese (4 to 6% S/M) as described by Lawrence et al. (1993). The pH was at high end of expected range (5.0 to 5.4) (Lawrence et al., 2004) which perhaps relates to higher S/M and slower activity of the starter culture. Higher pH (i.e., >5.35) in Cheddar cheese typically occurs with salt concentrations are greater than ~ 2.0% with S/M of 5.7% or higher (Turner and Thomas, 1980; Thomas and Pearce, 1982; Lane et al., 1997). The high retention of salt in our experiment may have occurred because of the longer time allowed after salting and before pressing while the sugars were being added. In previous experiments, the same make procedure had produced cheeses with 1.4% to 1.8% salt (Oberg et al., 2011; Broadbent et al., 2013: McMahon et al., 2014).

Lactose content was the same in all cheeses while galactose content varied based upon sugar supplementation to the curd (Table 4.2, Table 4.3). Storage time was the only factor that had an impact on lactose content with a decrease ($P<0.05$) to 0.36 at 16 wk. Since the S/M levels in the cheeses were relatively high, the continuing presence of lactose during storage was not unexpected. Turner and Thomas (1980) found that in Cheddar cheese manufactured with *Lactococcus lactis*/*cremoris* stored at 12°C with S/M values near 6%, the rate of lactose utilization declined to near zero by 16 d leaving high levels of residual lactose. Persistence of lactose in Cheddar cheese during storage was also shown by Fox et al. (1998) who reported continued presence of lactose after 36 wk of ripening.

Initial galactose content was significantly different ($P<0.05$) based on sugar supplementation. Cheeses made with 0.5% galactose or 0.25% ribose plus 0.25%

galactose added to the curd contained 0.24% and 0.13% galactose, respectively, compared to <0.03% in the control cheese (Table 4.3). Losses of galactose in expelled whey during pressing probably occurred (as with salt) and there may have been some utilization of galactose by starter lactococci during overnight pressing of the salted curd at room temperature. Further reductions ($P < 0.05$) in galactose occurred during storage. Galactose was completely consumed during the first 16 wk of storage in the control or ribose-supplemented cheeses and had decreased to 0.08% in which galactose had been added (alone or with ribose).

Starter lactococci and NSLAB during ripening

Supplementation of curd with ribose or galactose had no significant impact on numbers of starter lactococci or NSLAB in any of the cheeses (Figure 4.1, Table 4.5) nor were significant interactions with ripening temperature or time ($P>0.05$). Inoculation of *Lb. wasatchii* to cheese milk was only significant as an interaction with ripening time, and as a three-way interaction with time and temperature. Therefore, pooled means across all sugar treatments for lactococci and sugar plus *Lb. wasatchii* inoculation for NSLAB counts were evaluated against ripening temperature and time. Not surprisingly, ripening temperature and time and their interactions significantly affected both starter lactococci and NSLAB numbers (Peterson and Marshall, 1990; Wilkinson et al., 1994; Fox et al., 1998). It was interesting to note that starter lactococci numbers decreased when high numbers of *Lb. wasatchii* were initially present and the rate and extent of decrease was dependent on ripening temperature (Figure 4.1).

The best fit of the data was most often obtained using power or log trend lines, especially during the first few weeks of aging when the greatest growth of the NSLAB and greatest decrease in starter culture counts occurred. The crossover time when NSLAB numbers became greater than lactococci numbers occurred in all cheeses during the first 8 wk of ripening at either temperature. Based on the fitted trend lines, it appears that crossover time is achieved after around 4 and 8 wk of ripening at 12 and 6°C, respectively (Figure 4.1). This is sooner that McMahon et al. (2014) previously reported NSLAB crossover occurred only after 4 mo of ripening from the cheese made with the same starter culture. This could be due to the differences in S/M contents as McMahon et al. (2014) reported around 4.7% S/M, whereas the S/M levels in the present study were 5.5%.

Lactococcus lactis starter cultures, along with many other LAB, are sensitive to the salt concentration in cheese. Upreti et al. (2006b) reported that S/M concentration in cheese above 4.5% results in a more rapid decrease in starter lactococci during initial ripening. Conversely, starter bacteria numbers can remain at a higher concentration in the aging cheese if the S/M is low (<4.5%) (Lane et al., 1997). Higher S/M levels impaired the fermentation of lactose to lactic acid by starter bacteria that also contributes stabilizing the cheese pH (Olson and Johnson, 1990) and the salt concentration can continue to affect the growth of bacteria during cheese storage. Although the typical S/M concentration in Cheddar cheese (4.0 to 5.5%) is not sufficient to prevent all microbial growth, in combination with a low pH and refrigerated ripening temperature, it prevents growth of pathogens and influences LAB populations (McMahon et al., 2014). A high

salt concentration also decreases lactose metabolism by starter lactococci, leaving more residual lactose available to be used by NSLAB during ripening (Turner and Thomas, 1980). Thus, salt concentration affects the population dynamics of NSLAB by determining how quickly NSLAB will grow in the ripening cheese as well as the final number of NSLAB.

The total reduction in the numbers of starter lactococci during ripening at 12 or 6°C over 23 wk were similar and around 4.5 and 4 log, respectively. As stated above, there was a significant impact of added *Lb. wasatchii* x time as well as *Lb. wasatchii* x time x temperature interaction on the numbers of starter lactococci in cheese ($P<0.05$) (Table 4.5). After 8 wk of ripening at 12°C, starter lactococci numbers were significantly lower in cheese containing added *Lb. wasatchii* compared to control cheese (cheese milk not inoculated with *Lb. wasatchii*) ($P<0.05$) (Figure 4.1). At 6°C, however, starter lactococci counts were the same ($P>0.05$) in both cheese although the trend line also showed a faster decrease in cheese with added *Lb. wasatchii*. As ripening progressed, starter lactococci numbers were similar in control and *Lb. wasatchii* treated cheese at 12°C whereas at 6°C counts were significantly higher in the control cheese (Figure 4.1). Higher reductions in the numbers of starter lactococci in cheese in the presence of added *Lb. wasatchii* corresponds with our observations in cheese making that slower acid development (longer cheese-making time) occurred even though the same amount of starter used in ripening when the milk was inoculated with *Lb. wasatchii* compared to the control vat. However, the basis for faster reduction in starter lactococci counts in cheese

ripening is unknown since no antimicrobial compound encoding genes were found in the genome of *Lb. wasatchii*.

Along with the faster decrease in lactococcal counts, NSLAB counts (both NSLAB23 and NSLAB37) increased more rapidly ($P<0.05$) and reached higher levels ($P<0.05$) in cheese ripened at 12°C (Figure 4.1). There was no significant impact of sugar supplementation or *Lb. wasatchii* inoculation on the numbers of NSLAB23 or NSLAB37 in cheese ($P>0.05$). NSLAB numbers increased in cheese from an initial nondetected level ($<10^2$ cfu/g) to $\geq 10^7$ and 10^5-10^6 cfu/g during 16 wk ripening at 12 and 6°C, respectively. The highest growth occurred within the first 8 wk of ripening at both 6 and 12°C. Within each temperature, NSLAB numbers were the same between 8 and 16 wk of ripening ($P>0.05$) and significantly lower counts achieved at 6˚C ($P<0.05$). The increase in NSLAB counts in cheeses as ripening temperature increased from 6 to 12° C is in agreement with previous observations for full fat Cheddar cheese (Cromie et al., 1987; Jordan and Cogan, 1993; Lane et al., 1997; Fenelon et al., 1999). After 23 wk, however, NSLAB numbers were the same and significantly lower ($P<0.05$) compared to 8 and 16 wk in cheese ripened at either temperature (Figure 4.1).

Interestingly, there were statistically significant differences ($P<0.05$) observed in the numbers of NSLAB23 and NSLAB37 with higher numbers obtained at 23˚C ($P<0.05$) incubation on MRS-R-V (Figure 4.1). This could be due to the differences in sugar and temperature conditions used during incubations as 1.5% ribose was extra in MRS-R-V plates incubated at 23°C. Moreover, an incubation temperature of 23°C compared to 37°C could be more favorable for the growth of NSLAB in Cheddar cheese.

We observed some variations in NSLAB counts that in one replicate, NSLAB23 and NSLAB37 numbers were $\leq 10^2$ cfu/g up to 16 wk of ripening at 6°C compared to the other replicates in which NSLAB counts reached 10^4 to 10^6 cfu/g in 8 wk. However, NSLAB numbers in all replicates reached to 10^4 to 10^7 cfu/g after 23 wk of ripening. Such differences in cheese biota (especially in indigenous NSLAB populations) within an individual cheese manufacturing facility have been reported previously (Broadbent et al., 2003; Oberg et al., 2011). These can arise from differences in cleaning and sanitation of cheese vats as well as differences in cheesemaking history of the vats (i.e., what cheeses had been made, and what cultures had previously been used in the vats) and could be due to the differences of salt tolerance between NSLAB strains exist in cheese during ripening.

Organic Acids

Lactic, acetic, citric, propionic, pyruvic, formic, and orotic acids were detected in all cheese samples after 16 wk of ripening (Table 4.4). No significant differences caused by adding ribose and galactose into cheese curd were observed, thus pooled means for organic acids are shown in Table 4.4. The overall concentration of organic acids in all cheeses increased (except for citric acid) significantly during storage ($P<0.05$). Ripening temperature had a significant impact on lactic and propionic acids with higher concentrations obtained at elevated temperature ($P<0.05$). Initial lactic acid levels were ~1% which is lower than previously reported by McSweeney and Fox (2004) and McMahon et al. (2014) who found 1.4 to 1.5% lactic acid after the pressing of Cheddar cheese. Lower lactic acid levels in the present study likely relates to higher S/M levels

causing slower activity of the starter lactococci, and resulting in higher pH of the cheese (5.38) at pressing.

Propionic acid levels also significantly increased from 0.08% to 0.21% or 0.42% after 16 wk of storage at 6 or 12°C, respectively. The increase at 6°C storage was similar with the findings of McMahon et al. (2014) who reported a 3-fold increase after 3 mo storage of Cheddar cheese. The 2-fold higher propionic acid levels achieved at 12°C compared to 6°C after 16 wk relates to higher numbers of NSLAB in cheese ripened at 12°C (Figure 4.1) since NSLAB activity has been reported to increase propionic acid concentration in cheese during storage (St-Gelais et al., 1991; Bouzas et al., 1993). At least one common NSLAB in Utah State University Cheddar cheeses, *Lactobacillus curvatus* (Broadbent et al., 2013), appears to have the metabolic capability to produce propionic acid (J. R. Broadbent and C. J. Oberg, unpublished data).

Acetic acid levels were significantly impacted by time and by the temperature x time interaction with higher levels obtained at elevated temperature (Table 4.2, Table 4.4). No significant increase was observed at 6°C ($P>0.05$), whereas a 7-fold increase at 12°C noticed after 16 wk of ripening ($P<0.05$). Lues and Bekker (2002) reported that acetic acid concentration in Cheddar cheese initially decreased rapidly and then increased after 3 wk of storage. Upreti et al. (2006a) also reported a decrease in acetic acid content during the first few months of storage from an initial concentration of 0.15 to 0.06 g/kg. McMahon et al. (2014) reported some increase in acetic acid concentration over the ripening period, but also a decrease in acetic acid concentration from 6 to 9 mo.

Surprisingly, adding ribose to cheese curd did not have a significant impact on acetic acid levels in any of the cheeses although fermentation of pentose by Group II and Group III LAB yields equimolar amounts of lactic acid and acetic acid and net 2 mol ATP/mol ribose consumed (Axelsson, 2004). No differences in acetate in control versus ribose supplemented cheeses could be due to the lysis of starter lactococci which releases ribose into the cheese microenvironment (Thomas, 1987).

Citric acid levels were significantly affected by time, *Lb. wasatchii* x time, and sugar x time interactions ($P<0.05$). There were significant but minor decreases occurred during storage, as citrate levels dropped from 0.132 to 0.08%. Although citric acid levels were significantly impacted by *Lb. wasatchii* over time, no citrate permease or citrate lyase encoding gene(s) were found in the genome. Formic, uric, and orotic acids were also significantly increased by ripening time ($P<0.05$).

Cheese with added *Lb. wasatchii*

As stated above, inoculating milk with *Lb. wasatchii* prior to cheesemaking had a significant effect on starter lactococci. However, NSLAB counts were not significantly affected by added *Lb. wasatchii* (Table 4.5). *Lactobacillus wasatchii* numbers increased over 3-log during ripening, and the extent of growth was influenced by ripening temperature and sugar supplementation of curd. Faster growth occurred at elevated temperature as expected from previous work (see Chapter 3) (Figure 4.2). Most growth of *Lb. wasatchii* occurred during the first 8 wk of ripening with higher counts ($P < 0.05$) achieved at 12°C and when ribose (alone or with galactose) was added to the cheese curd. The final numbers reached after 23 wk ripening in cheese containing ribose or ribose plus

galactose were the same (P>0.05) and highest between all sugar treatments. Adding galactose on its own to the cheese curd did not (P > 0.05) increase growth of *Lb. wasatchii* compared to the cheese with no added sugars (Figure 4.2). Similar growth on cheese with ribose and ribose plus galactose is in accordance with the previous work that *Lb. wasatchii* reached similar final cell densities on MRS plus 0.5% ribose versus MRS plus 0.25% ribose + 0.25% galactose (see Chapter 3). Thus, *Lb. wasatchii* can benefit from this co-utilization by maximizing its cell densities in the stressful environment of Cheddar cheese (4-6% S/M, pH<5.4) where minimal nutrients exist.

In the control cheese with no *Lb. wasatchii* deliberately inoculated into the cheese milk, there were still indigenous *Lb. wasatchii* detected in some replicates at a level of 10^5 to 10^7 cfu/g after 16 to 23 wk, especially when the cheese was stored at 12°C. *Lactobacillus wasatchii* levels were not able to determined in the control cheese prior to this time as the threshold for detection of *Lb. wasatchii* required *Lb. wasatchii* numbers to be within about 1.5 log of the fast growing NSLAB that are enumerated using the same plate. At population levels below this threshold the plates contain too many fast growing NSLAB to distinguish and count any new colonies that may appear after the initial 2 d incubation. We speculate that because *Lb. wasatchii* grows slower than predominant NSLABs in Cheddar cheese, it takes 4 to 6 mo to achieve similar levels (i.e, with 1.5 log). Since *Lb. wasatchii* was isolated from cheese made in our pilot plant, it is likely a part of resident microbiota in the plant, and contaminates the cheese as opportunity provides.

In the cheeses that were made from milk inoculated with *Lb. wasatchii*, we were able to count *Lb. wasatchii* throughout the 23 wk ripening because the initial levels of *Lb. wasatchii* was ~10^5 cfu/g and therefore above the threshold compared to other NSLAB. Even though slower compared to other predominant NSLAB, *Lb. wasatchii* grew to above 10^6 and 10^7 after 8 wk at 6 and 12°C, respectively. Thus, the numbers of *Lb. wasatchii* was either the similar or higher than the fast growing NSLAB throughout ripening at either temperature.

Relative Gas

All treatments (sugar treatment, addition of *Lb. wasatchii*, storage temperature and time) significantly affected the relative gas formation in cheese (Table 4.6). The interaction terms involving time, temperature and *Lb. wasatchii* addition were also significant. Interactions involving sugar addition were not significant except there was a tendency (P=0.0727) for the sugar x *Lb. wasatchii* interaction to influence gas production (Table 4.6). When pooled over time and temperature, relative gas production based on sugar and *Lb. wasatchii* addition was significantly higher when 0.25% ribose plus 0.25% galactose was added compared to adding galactose or ribose alone, or the no-sugar added control. The highest gas formation with *Lb. wasatchii* in cheese supplemented with ribose plus galactose sugars (P<0.05) correlates with the faster growth of *Lb. wasatchii* in the same cheese (Figure 4.2). Lower gas formation in cheese supplemented with ribose only (P<0.05) was expected as CO_2 is not formed from pentose sugars (Axelsson, 2014).

Gas formation was significantly higher (P<0.05) in cheese containing added *Lb. wasatchii* compared to un-inoculated control cheese at both temperatures (Figure 4.3). No

gas formation was observed at 6°C in un-inoculated control cheese. Laleye et al. (1990) found that inoculation of cheese milk with OHF species such as *Lb. brevis* or *Lb. fermentum* resulted in open texture in cheese after 2 mo of ripening whereas no openness was observed in a control cheese although gas formation can occur without any openness being observed (Laleye et al., 1987).

Gas formation during storage at 12°C was significantly higher than at 6°C ($P<0.05$). This was expected as elevated ripening temperatures can increase the occurrence of openness in cheese (Elliott et al., 1981; Laleye et al., 1987). At both 6 and 12°C, there was growth of *Lb. wasatchii* with levels being about 0.5 log higher at 12°C. This increased growth at the higher storage temperature could account for the higher measurements of relative gas formation, but solubility of CO_2 may also be a factor. At 6°C, there is higher solubility of CO_2 compared to 12°C (CRC, 2009) and even with the same level of gas production there would be more CO_2 present in the gaseous state at 12°C (evident as looser packs) and more probability of openness as well as slits and crack formation in the cheese. This was confirmed when a *Lb. wasatchii* treated cheese that had been stored at 6°C and was observed to have a tight pack was moved to room temperature (~22°C) and after a while the package became loose (data not shown).

The possibility that slow growing bacteria may contribute late blowing in Cheddar cheese has been previously suggested (Elliott et al., 1981). In that study, the bacterium was not identified (except for being a slow growing, salt tolerant, non-sporeforming, heat tolerant, Gram positive, rod), but its addition to cheese milk at 10^1 and 10^4 cfu/ml yielded gas formation during storage at 10°C but not at 4.5°C.

Even though the most rapid growth of *Lb. wasatchii* occurs during the first 8 wk of ripening, observations of later blowing usually occur after this time. This may be a consequence of needing to have most of the available pentose consumed before any extensive production of CO_2 occurs, or it may be related to solubility of CO_2 in cheese and the need for the cheese water phase to become saturated in CO_2 before any loosening of the pack is observed.

Fermentation of citrate also results in CO_2 production by NSLAB which could contribute to gas formation in cheese (Overcast and Albrecht, 1952). However, relatively low amounts of gas would be expected since only 0.13% of citrate was detected in cheese after press in the present study. In a parallel manner, Hoglung et al. (1972) reported that utilization of citrate was not essential for the development of openness indicating other sources of CO_2 were involved.

Growth of *Lb. wasatchii* on Starter Cell Lysate

After bead beating of ~10^{10} cfu/ml of starter lactococci, the numbers in the supernatant of the cell lysates were less than 5 x 10^4 cfu/ml. This indicates the efficacy of lysing to be ~99.999%, which yielded around ~4000 µg/ml protein in the supernatant. When *Lb. wasatchii* was grown in carbohydrate free MRS containing supernatant of lysed starter lactococci, final cell densities reached up to 2.49 (OD_{600}) from an initial cell density of ~0.157 (OD_{600}) after 10 d of incubations. On the other hand, final cell densities only reached to ~0.06 from 0.001 (OD_{600}) when grown in control treatment where lysate was replaced with water (Figure 4.4).

Although growth of *Lb. wasatchii* in cell lysate was slower compared to MRS plus 1% ribose (see Chapter 3), final cell densities reached were considerably higher in the lysate. This likely explains the 2-log increase in *Lb. wasatchii* counts in cheese without added sugar over 23 wk at both 6 and 12°C since starter counts dropped from 10^7 to ~10^4 cfu/ml within the first 8 wk of ripening (Figure 4.1, Figure 4.2). Along with *Lb. wasatchii,* other fast growing NSLAB increased concomitantly with this drop in starter lactococci counts (Figure 4.1, Figure 4.2) possibly by utilizing the sugars released from the starter cells on autolysis (Fox et al., 1998). It has been suggested that both ribose and n-acetyl amino sugars release from starter cells on autolysis (Thomas, 1987). Lysates of starter lactic acid bacteria cells considered source of nutrients for NSLAB and of the different sugars present in the starter cell lysates, ribose is the most readily released sugar from starter lactococci (Thomas, 1987; Rapposch et al., 1999). Thomas (1987) found that *Lb. brevis,* OHF species, reached the highest cell densities on lactococcal cell lysate among the different NSLAB tested Thomas (1987). Rapposch et al. (1999) found that *Lb. paracasei* reached to 10^7 from an initial level of 10^2 cfu/ml in the lysate of *Lactobacillus helveticus*. Thus, the breakdown products of starter cells can clearly supply the carbon sources needed for growth of *Lb. wasatchii* and other NSLAB. However, there would also be nutrients available from MFGM material that supports the growth of NSLAB in cheese (Moe et al., 2013).

CONCLUSIONS

This study explored the consequences of inoculation of cheese milk with a novel slow-growing OHF *Lb. wasatchii* sp. nov. on late gas formation in Cheddar cheese. We

recently reported that *Lb. wasatchii* utilizes galactose very slowly in the absence of ribose, and that fermentation of galactose (or other hexose) as an energy source is necessary for CO_2 production (see Chapter 3). We confirmed in the present study that *Lb. wasatchii* is a contributor to late gas blowing in Cheddar cheese. Likelihood of gas formation increased at accelerated ripening temperature and when ribose and galactose was added to cheese curd. *Lactobacillus wasatchii* grew best in the presence of added ribose or ribose plus galactose. Co-utilization of galactose with ribose by *Lb. wasatchii* appeared to increase the occurrence of late gas formation in cheese. However, *Lb. wasatchii* also grew ~2-log in cheese when the curd was not supplemented with ribose. Since *Lb. wasatchii* is able to grow in the cell free extracts of starter lactococci, increased numbers of *Lb. wasatchii* could be attributed to substrates released by starter lactococci on autolysis during ripening. To reduce the occurrence of the gas problem by *Lb. wasatchii* or similar slow-growing OHF species, it would be desirable to study whether adjunct facultative heterofermentative lactic acid bacteria could be utilized in cheese making.

REFERENCES

Agarwal, S., D. McCoy, W. Graves, P. D. Gerard, and S. Clark. 2011. Sodium content in retail Cheddar, Mozzarella, and process cheeses varies considerably in the United States. J. Dairy Sci. 94:1605–1615.

Anonymous 2015. Accessed February 12, 2015. https://www.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/288110.pdf.

Axelsson, L. T. 2004. Lactic acid bacteria: classification and physiology. Pages 1-63 in Lactic Acid Bacteria, S. Salminen, S. and A. von Wright, ed. Dekker, New York.

Bouzas, J., C. A. Kantt, F. Bodyfelt, and J. A. Torres. 1993. Time and temperature influence on chemical aging indicators for a commercial Cheddar cheese. J. Food Sci. 58:1307–1313.

Branen, A. L., and T. W. Keenan. 1969. Growth Stimulation of *Lactobacillus* Species by Lactic Streptococci. Appl. Microbiol. 17(2):280–285.

Broadbent, J. R., C. Brighton, D. J. McMahon, N. Farkye, M. Johnson and J. Steele. 2013. Microbiology of Cheddar cheese made with different fat contents using a *Lactococcus lactis* single-strain starter. J. Dairy Sci. 96:4212–4222.

Broadbent, J. R., K. Houck, M. E. Johnson, and C. J. Oberg. 2003. Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. J. Dairy Sci. 86:2773–2782.

CRC. 2009. CRC Handbook of Chemistry and Physics: A Ready-Reference Book of Chemical and Physical Data. 90th ed. CRC Press, Boca Raton, FL.

Cromie, S. J., J. E. Giles, and J. R. Dulley. 1987. Effect of elevated ripening temperatures on the microflora of Cheddar cheese. J. Dairy Res. 54:69–76.

Donnely, C., C. Golnazarian, and K. Boor. 2014. Accessed June 21, 2014. http://www.powershow.com/view/1e1e6- Page: 2.

Elliott, J. A., G. E. Millard, and R. A. Holley. 1981. Late gas defect in Cheddar cheese caused by an unusual bacterium. J. Dairy Sci. 64(11):2278-2283.

Fenelon, M. A., M. P. Ryan, M. C. Rea, T. P. Guinee, R. P. Ross, C. Hill, and D. Harrington. 1999. Elevated temperature ripening of reduced fat Cheddar made with or without lacticin 3147 starter culture. J. Dairy Sci. 82:10–22.

Folkertsma, B., P. F. Fox, and P. L. H. McSweeney. 1996. Accelerated ripening of Cheddar cheese at elevated temperatures. Int. Dairy J. 6: 1117- 1134.

Fox, P. F., J. A. Lucey, and T. M. Cogan. 1990. Glycolysis and related reactions during cheese manufacture and ripening. CRC Critical Reviews in Food Sci. Nutr. 29:237–253.

Fox, P. F., P. L. H. McSweeney, and C. M. Lynch. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust. J. Dairy Technol. 53: 83–89.

Golnazarian, C. 2001. Slit formation in Cheddar cheese: A comprehensive investigation of the microbiological parameters associated with this defect. PhD Thesis. University of Vermont, Burlington.

Hoglund, G. F., T. F. Fryer, and J. Gilles. 1972. The influence of lactobacilli on open texture in Cheddar cheese. NZ J. Dairy Sci. Technol. 7:159-160.

Jordan, K. N., and T. M. Cogan. 1993. Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. Ir. J. Agric. Food Res. 32:47–55.

Laleye, L. C., R. E., Simard, B. H. Lee, R. A. Holley, and R. N. Giroux. 1987. Involvement of heterofermentative lactobacilli in development of open texture in cheeses. J. Food Prot. 50:1009-1012.

Laleye, L. C., R. E., Simard, B. H. Lee, R. A. Holley, and R. N. Giroux. 1990. Quality attributes of Cheddar cheese containing added lactobacilli. J. Food Sci. 55:114-118.

Lane, C. N., P. F. Fox, E. M. Walsh, B. Folhersma, and P. L. H. McSweeney. 1997. Effect of compositional and environmental factors on the growth of indigenous non-starter lactic acid bacteria in Cheddar cheese. Lait. 77:561–573.

Lawrence, R. C., J. Gilles, and L. K. Creamer. 1993. Cheddar cheese and related dry-salted cheese varieties. Pages 1-38 in Cheese: Chemistry, Physics and Microbiology, Vol. 2. 2nd ed. P. F. Fox. Major Cheese Groups. Chapman & Hall, London, UK.

Lawrence, R. C., J. Gilles, L. K. Creamer, V. L. Crow, H. A. Heap, C. G. Honoré, K. A. Johnston, and P. K. Samal. 2004. Cheddar cheese and related dry-salted cheese varieties. Page 72 in Cheese: Chemical, Physics and Microbiology. Vol. 2. 3rd ed. Patrick F. Fox, Timothy M. Cogan, and Timothy P. Guinee, ed. Elsevier Ltd, London, UK.

Lues, J. F. R., and A. C. M. Bekker. 2002. Mathematical expressions for organic acids in early ripening of a Cheddar cheese. J. Food Comp. Anal. 15:11–17.

Martley, F. G., and Crow, V. L. 1996. Open texture in cheese: the contributions of gas production by microorganisms and cheese manufacturing practices. J. Dairy Res. 63: 489–507.

McMahon, D. J., C. J. Oberg, M. A. Drake, N. Farkye, L. V. Moyes, M. R. Arnold, B. Ganesan, J. Steele, and J. R. Broadbent. 2014. Impact of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full fat Cheddar cheese. J. Dairy Sci. 97(8): 4780-4798.

McSweeney P. L. H., and P. F. Fox. 2004. Metabolism of residual lactose and of lactate and citrate. Pages 361-372 in Cheese: Chemistry, Physics and Microbiology, Vol 1: General Aspects, 3rd ed. P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, ed. Elsevier, London, UK.

Michel, V., and F. G. Martley. 2001. *Streptococcus thermophilus* in Cheddar cheese--production and fate of galactose. J. Dairy Res. 68(2):317-25.

Mullan, W. M. A. 2000. Causes and control of early gas production in Cheddar cheese. Int. J. Dairy Tech. 53: 63–68.

Moe, K. M., D. Porcellato, and S. Skeie. 2013. Metabolism of milk fat globule membrane components by nonstarter lactic acid bacteria isolated from cheese. J. Dairy Sci. 96:727–739.

Oberg, C. J., L. V. Moyes, M. J. Domek, C. F. Brothersen, and D. J. McMahon. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. J. Dairy Sci. 94:2220-2230.

Olson, N. F., and M. E. Johnson. 1990. Light cheese products: Characteristics and economics. Food Technol. 44:93‑96.

Overcast, W. W., and T. W. Albrecht. 1952. Gas Production in Cheddar Cheese Caused by *Leuconostoc citrovorum*. J. Dairy Sci. 35(6):554-558.

Peterson, S. D. and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: A review. J. Dairy Sci. 73: 1395–1410.

Phadungath, C. 2011. The efficacy of sodium gluconate as a calcium lactate crystal inhibitor in cheddar cheese. PhD Thesis. South Dakota State University, Brookings.

Rapposch, S., F. Eliskases-Lechner, and W. Ginzinger. 1999. Growth of facultatively heterofermentative lactobacilli on starter cell suspensions. Appl. Environ. Microbiol. 65:5597–5599.

Richardson, G. H., ed. 1985. Page 351 in Standard Methods for the Examination of Dairy Products. 15th ed. Am. Publ. Health Assoc., Washington, DC.

Sherwood, I. R. 1939. The relation of certain lactic acid bacteria to open texture in Cheddar cheese. J. Dairy Res. 10:326-335.

St-Gelais, D., G. Doyon, J. R. Rolland, and J. Goulet. 1991. Sugar and organic acid concentrations during ripening of Cheddar cheese-like products. Milchwissenschaft 46:288–291.

Thomas, T. D. 1987. Cannibalism among bacteria found in cheese. NZ J. Dairy Sci. Technol. 22:215–219.

Thomas, T. D., and K. N. Pearce. 1982. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. NZ J. Dairy Sci. Technol. 16: 253–259.

Tinson, W., M. F. Ratcliff, A. J. Hillier, and G. R. Jago. 1982. Metabolism of *Streptococcus thermophilus*. 3. Influence on the level of bacterial metabolites in Cheddar cheese. Australian J. Dairy Technol. 37:17–21.

Turner K. W., and T. D. Thomas. 1980. Lactose fermentation in Cheddar cheese and the effect of salt. NZ J. Dairy Sci. Technol. 15: 265-276.

Upreti, P., L. L. Mckay, and L. E. Metzger. 2006a. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: changes in residual sugars and water-soluble organic acids during ripening. J. Dairy Sci. 89:429–443.

Upreti, P., L. E. Metzger, and K. D. Hayes. 2006b. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Proteolysis during ripening. J. Dairy Sci. 89:444–453.

Van Slyke, L. L., and E. B. Hart. 1903. The relation of carbon dioxide to proteolysis of ripening Cheddar cheese. New York (Geneva) Agr. Expt. Sta. –Bull. 231, pp 20-41. (Obtained from Tretsven, 1945. Study of the gas evolution from ripening cheddar cheese. PhD Thesis. Graduate School of University of Wisconsin).

Wilkinson, M. 1993. Acceleration of cheese ripening. Pages 523-555 in Cheese: Chemistry, Physics and Microbiology, Vol. 1. 2nd ed. P. F. Fox, ed. Chapman and Hall Publishers, London, UK.

Wilkinson, M. G., T. P. Guinee, D. M. O'Callaghan, and P. F. Fox. 1994. Autolysis and proteolysis in different strains of starter bacteria during Cheddar cheese ripening. J. Dairy Res. 61: 249–262.

Table 4.1. Pooled means and SE for initial composition of cheese, (n=24).

	Moisture	**Salt**	**S/M[2]**	**pH**	**Fat**	**Lactose**	**Citrate**
Mean	37.03	2.14	5.47	5.38	32	0.419	0.132
SE	0.159	0.025	0.069	0.03	0.12	0.0236	0.0089

[1]All values except pH represent percentage.
[2]Salt-in-moisture.

Table 4.2. The effect of adding ribose or galactose, ripening temperature (6, 12°C), and time (0, 16 wk), and inoculated *Lactobacillus wasatchii* (LBW) on the sugar and organic acid content of Cheddar cheese.

	Sugar and Organic Acids									
Source of Variation	**Galactose**	**Lactose**	**Lactic**	**Acetic**	**Pyruvic**	**Propionic**	**Citric**	**Formic**	**Uric**	**Orotic**
LBW	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sugar	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
LBW x Sugar	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Temperature	NS	NS	**	NS	NS	**	NS	NS	NS	NS
LBW x Temperature	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
Sugar x Temperature	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Time	**	*	**	**	NS	**	**	**	**	**
LBW x Time	NS	NS	*	NS	**	NS	*	NS	NS	NS
Sugar x Time	**	NS	NS	NS	NS	NS	*	NS	NS	NS
Temperature x Time	NS	NS	*	*	NS	**	NS	NS	NS	NS

*$P<0.05$

**$P<0.01$

NS: Not significant, $P>0.05$.

Table 4.3. Pooled means of galactose concentrations (%) at 0 and 16 wk in cheese supplemented with no sugar (Control), 0.5% ribose (0.5R), 0.5% galactose (0.5G) or 0.25% ribose plus 0.25% galactose (0.25R+0.25G). Bars= SE (n=12).

	------Control------		**-------0.5R-------**		**-------0.5G-------**		**-0.25R+0.25G--**	
	0 wk	16 wk	0 wk	16 wk	0 wk	16 wk	0 wk	16 wk
Mean	0.023^{cd}	0.048^{cd}	0.061^{bc}	0.003^{d}	0.238^{a}	0.076^{bc}	0.125^{b}	0.078^{bc}
SE	0.0221	0.0246	0.0185	0.0199	0.0221	0.0205	0.0277	0.0204

[a-d]Means with the same superscript letters were not significantly different from one another. α=0.05.

Table 4.4. Pooled means for sugar and organic acids for Cheddar cheese at 16 wk of ripening.

		Lactic		Acetic		Propionic						
	Lactose	**6°C**	**12°C**	**6°C**	**12°C**	**6°C**	**12°C**	**Citric**	**Pyruvic**	**Formic**	**Uric**	**Orotic**
Mean	0.3642	1.127^{b}	1.4163^{a}	0.0082^{b}	0.022^{a}	0.214^{b}	0.423^{a}	0.0819	0.0137	0.02	0.0019	0.0011
SE	0.02248	0.0746	0.0745	0.0036	0.0035	0.0241	0.024	0.0087	0.0025	0.003	0.0002	0.0001

$^{a-d}$Means with the same superscript letters were not significantly different from one another. α=0.05.

Table 4.5. The effect of adding ribose or galactose, ripening temperature (6 or 12°C), and time (0, 8, 16, 23 wk) on the numbers of starter lactococci, nonstarter lactic acid bacteria enumerated at 23˚C (NSLAB23) or 37˚C (NSLAB37), and added *Lactobacillus wasatchii* (LBW) in Cheddar cheese ripening.

	Bacterial Counts			
Source of Variation	**Starter**	**NSLAB23**	**NSLAB37**	**LBW**
LBW	NS	NS	NS	-[2]
Sugar	NS	NS	NS	**
LBW x Sugar	NS	NS	NS	-
Temperature	**	**	**	**
LBW x Temperature	NS	NS	NS	-
Sugar x Temperature	NS	NS	NS	NS
Time	**	**	**	**
LBW x Time	*	NS	NS	-
Sugar x Time	NS	NS	NS	**
Temperature x Time	**	**	**	**
LBW x Temperature x Time	*	NS	NS	NS

**P<0.01

NS: Not significant, P>0.05

[1]Cheese curd added with; no sugar, 0.5% ribose, 0.5% galactose, or 0.25% ribose plus 0.25% galactose.

[2] Cheese made without added *Lb. wasatchii* is not included in the statistical analysis.

Table 4.6. The effect of adding ribose or galactose, ripening temperature (6 or 12°C), and time (0, 8, 16, 23 wk) on relative gas formation in Cheddar cheese.

Source of Variation	Relative Gas
LBW	*
Sugar[1]	*
LBW x Sugar	***
Temperature	**
LBW x Temperature	**
Sugar x Temperature	NS
Time	**
LBW x Time	**
Sugar x Time	NS
Temperature x Time	**
LBW x Temperature x Time	*

*P<0.05
** P<0.01
***0.1>P>0.05
NS: Not significant, P>0.05
[1]Cheese curd added with; no sugar, 0.5% ribose, 0.5% galactose, or 0.25% ribose plus 0.25% galactose.

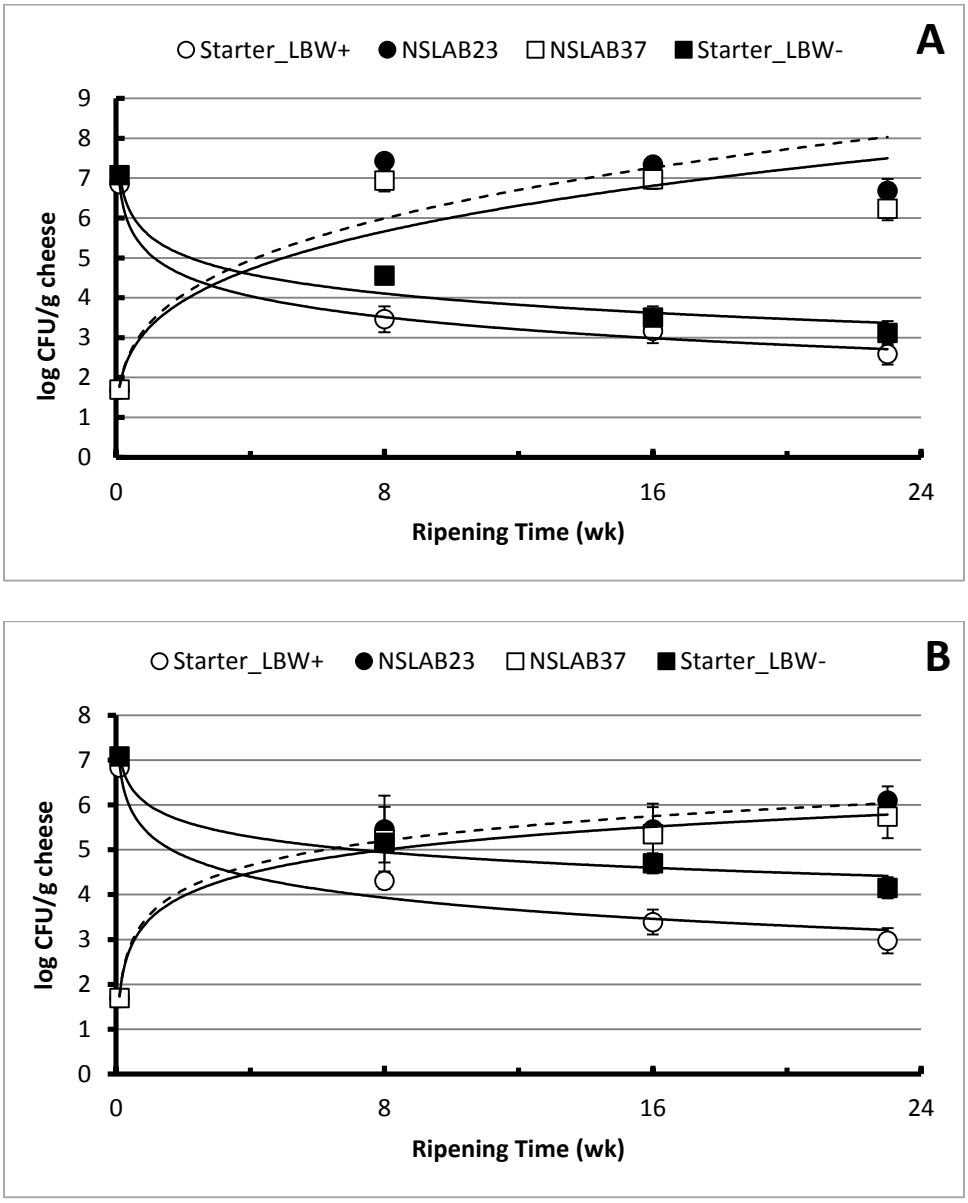

Figure 4.1. Changes in pooled mean numbers of nonstarter lactic acid bacteria enumerated at 23°C (NSLAB23) or at 37°C (NSLAB37), starter lactococci in cheese with (LBW+) and without added *Lactobacillus wasatchii* (LBW-), at 12°C (**A**) and 6°C (**B**). Bars = SE (n=12) for starter lactococci and n=24 for NSLAB23 and NSLAB37.

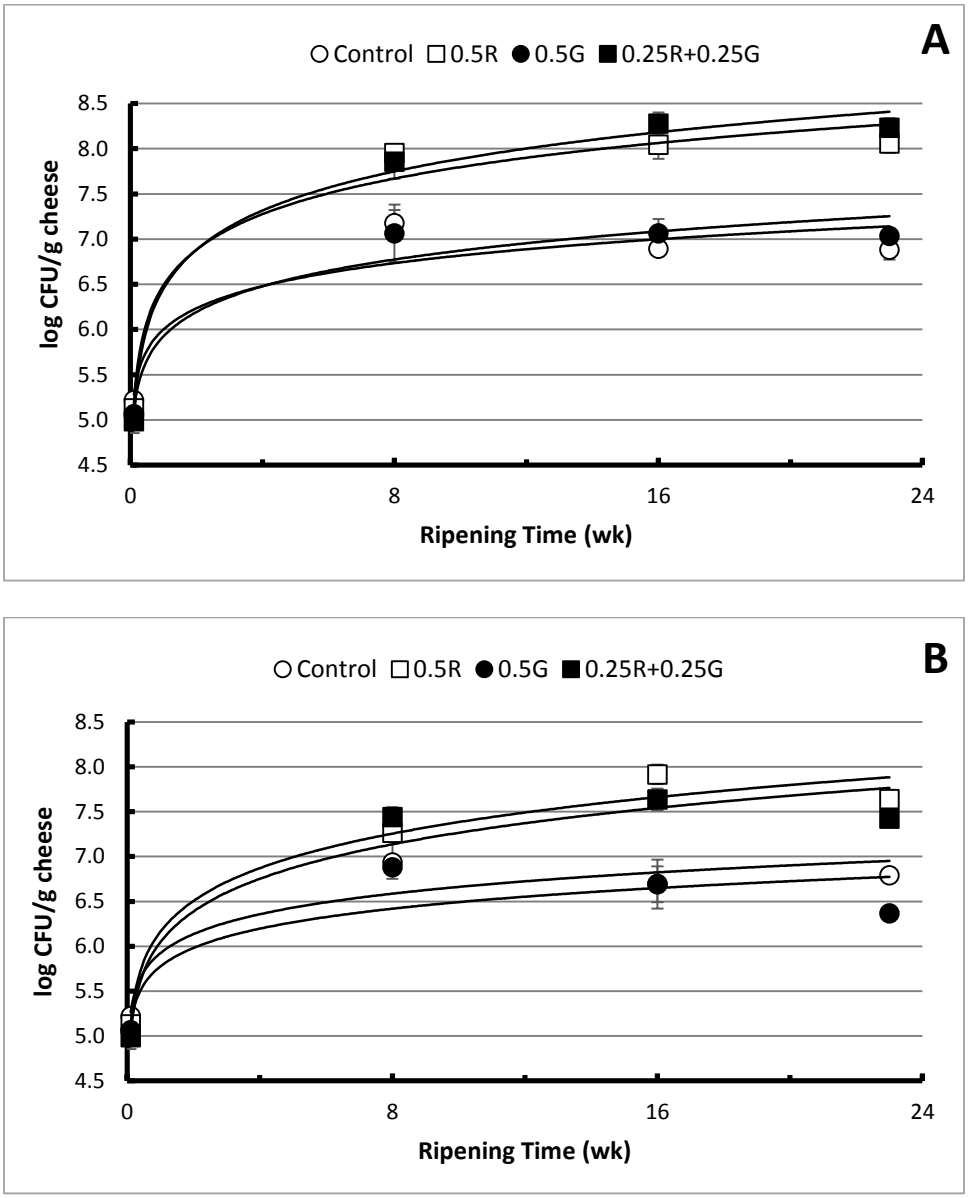

Figure 4.2. Changes in the mean numbers of *Lactobacillus wasatchii* as a function of time, and ripening temperatures of 12°C (A) or 6°C (B) in Cheddar cheese in which the curds were added with; no sugar (Control), 0.5% ribose (0.5R), 0.5% galactose (0.5G), 0.25% ribose plus 0.25% galactose (0.25R+0.25G), bars = SE (n=3).

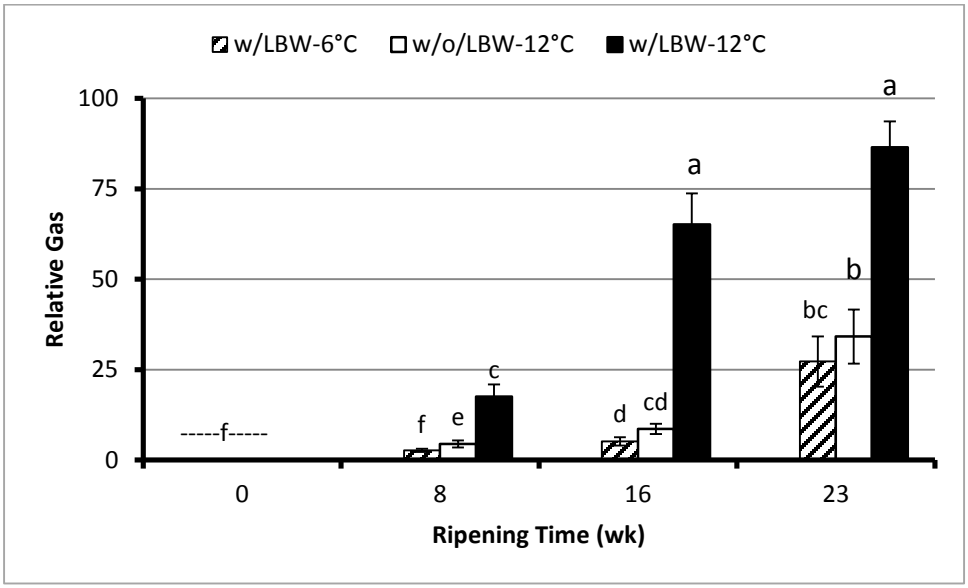

Figure 4.3. Pooled means of relative gas formation in *Lactobacillus wasatchii* inoculated cheese (w/LBW) or un-inoculated control cheese (w/o/LBW) during 23 wk storage at 6°C or 12°C. Bars=SE (n=12). [a-d]Means with the same superscript letters were not significantly different from one another. α=0.05.

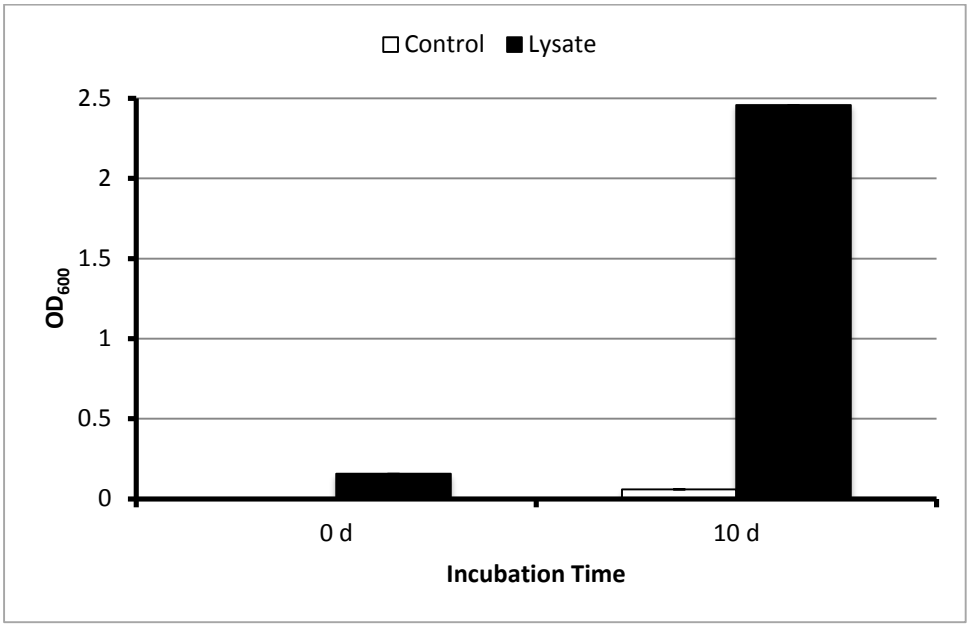

Figure 4.4. Growth of *Lactobacillus wasatchii* as measured by OD_{600} in carbohydrate free MRS containing starter cell lysate (■) or sterile water as control (□) during anaerobic incubation at 23°C at 0 and 10 d. Bars= SE (n=3).

CHAPTER 5

GROWTH AND GAS FORMATION BY A NOVEL OBLIGATORY HETEROFERMENTATIVE NONSTARTER LACTIC ACID BACTERIUM IN CHEESE MADE USING A *STREPTOCOCCUS THERMOPHILUS* STARTER

ABSTRACT

A novel slow-growing, obligatory heterofermentative, nonstarter lactic acid bacterium, *Lactobacillus wasatchii* sp. nov, was studied for growth and gas production in Cheddar cheese made using a *Streptococcus thermophilus* starter. Cheesemaking trials were conducted using starter *St. thermophilus* alone or in combination with *Lb. wasatchii* deliberately added to cheese milk at a level of ~10^4 cfu/ml. Then, cheeses were ripened at 6 or 12°C. At day 1, starter streptococcal numbers were similar in both cheeses (~10^9 cfu/g) and nonstarter lactic acid bacteria (NSLAB) counts were below detectable levels (<10^2 cfu/g). As expected, *Lactobacillus wasatchii* counts were 3 x 10^5 cfu/g in cheeses inoculated with this bacterium. Starter streptococci decreased over time at both ripening temperatures but fell more rapidly at 12°C, especially in cheese with *Lb. wasatchii*. Populations of NSLAB and *Lb. wasatchii* reached 5 x 10^7 and 2 x 10^8 cfu/g, respectively after 16 wk of ripening at 12°C, and their emergence was coincided with reduction in galactose concentration in the cheese from 0.6% to 0.1%. Levels of galactose at 6°C had similar decrease after 16 wk storage. Gas formation and textural defects were only observed in cheese with added *Lb. wasatchii* ripened at 12°C. Using *St. thermophilus* as starter resulted in galactose accumulation that, *Lb. wasatchii* can utilize, producing CO_2

which contributes to late gas blowing in Cheddar cheese, especially when the cheese is ripened at elevated temperature.

INTRODUCTION

The manufacture of Cheddar cheese is characterized by the use of mesophilic *Lactococcus lactis/cremoris* starter strains and use of moderate cook temperatures (~39°C) (Michel and Martley, 2001). However, the "short-method" for Cheddar manufacture (Bley et al., 1985) requires very rapid acid production rates which are obtained by including *Streptococcus thermophilus* with the regular mesophilic *Lc. lactis* subsp. *cremoris* starter culture and a slight increase in cook temperature (42-43°C) (Michel and Martley, 2001). Two advantages to using the short method include reduced manufacturing costs, and lower risk of bacteriophage infection (Cogan, 2011).

Unfortunately, this type of manufacturing process has also been linked to accumulation in the cheese of up to 33 mmol of galactose/kg (0.6% (wt/wt)) as well as carbon dioxide which is likely a result of nonstarter lactic acid bacteria (**NSLAB**) and leads development of slits, and fractures in the cheese (Tinson et al., 1982a; Michel and Martley, 2001). Galactose accumulates because the only the glucose moiety of lactose is utilized by *St. thermophilus* and galactose is excreted back into the milk/cheese as part of a Lac S-mediated antiport system for lactose uptake (Tinson et al., 1982b; Hutkins and Morris, 1987; Hutkins and Ponne, 1991; De Vos and Vaughan, 1994; Mora et al., 2002; Vaillancourt et al., 2004). Heterofermentative NSLAB can utilize this residual galactose; produce carbon dioxide, leading to gassy defect in Cheddar cheese (Radford and Hull,

1982; Tinson et al., 1982a), which is a major economic concern for the cheese industry (Golnazarian, 2001).

We recently reported that *Lb. wasatchii* is able to utilize galactose and produce gas in broth at the pH levels found in Cheddar cheese (see Chapter 3) and that when this NSLAB is present in high numbers in Cheddar cheese it promotes gas production (see Chapter 3). It was our hypothesis that growth of *Lb. wasatchii* would be promoted in Cheddar cheese if *St. thermophilus* was included as part of the starter culture. To test this hypothesis, cheese was made using *St. thermophilus* and microbial populations (starter culture, NSLAB, and *Lb. wasatchii*) as well as gas formation in cheese was monitored throughout ripening at 6 or 12°C. This is the first report in the literature on the effect of an obligatory heterofermentative (**OHF**) NSLAB species on late gas formation when the Cheddar cheese is made using a *St. thermophilus*.

MATERIALS AND METHODS

Bacterium and Growth

Working cultures of *Lb. wasatchii* were prepared from frozen stocks stored at −80°C by sequential transfer twice into de Man, Rogosa and Sharpe (**MRS**) (Becton Dickinson Inc., Sparks, MD) broth containing 1.5% ribose (**R**) (donated by Bioenergy Life Science Inc., Ham Lake, MN), in which the cultures were incubated anaerobically using GasPak™ EZ at 23°C for 40 h. Cells for the cheese making experiments were propagated in 400 ml MRS+R for 40 h at 23°C. Cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C and washed twice (7,000 rpm for 10 min at 4°C) with sterile 0.1% (wt/vol) peptone water. Concentration of cell suspensions was determined by

anaerobic spread plate counts in MRS+R agar after 5 d at 23°C. The cell suspensions were subsequently used in cheese making experiments after proper dilutions were made to reach desired numbers in cheese milk.

Cheese Making

Fresh bovine 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). The milk was standardized to a protein-to-fat ratio of 0.84, pasteurized at 73°C for 15 s, and 136 kg into each of 6 open stainless steel vats (each vat had previously been cleaned then heat sanitized for 30 min). All batches of milk were warmed to 31°C and 0.25 g/kg of frozen pellets containing *Streptococcus thermophilus* M6 starter culture (Chr. Hansen Inc., Milwaukee, WI) were added. To 3 of the vats, ~10^4 cfu/ml *Lb. wasatchii* (working culture) was also added and the milk allowed to ripen for 10 min in all vats. Then, 0.12 mL/kg of a 32% (wt/wt) $CaCl_2$ solution (Nelson-Jameson Inc., Marshfield, WI), 0.13 ml/kg of anatto and 0.16 mL/kg of double-strength (~650 international milk clotting units/mL) chymosin rennet (Maxiren; DSM Food Specialties USA Inc., Eagleville, PA) were added, and the milk allowed to set undisturbed for 20 min. After cutting and healing, the curd/whey mixtures were stirred for 10 min, heated to 39°C over 35 min, and then stirred for another 10 min. The curd was stirred until a curd pH of 6.3 was reached with partial whey drainage. The remaining whey was then drained and curd was allowed to mat together, cut into slabs, and cheddared until the curd pH reached 5.25. Curd was milled, salted (30 g/kg of curd) in 3 applications with 5 min between each application. Salted curd from each vat was

separated into two 7-kg portions, placed into open plastic containers. Curd was placed into plastic hoops and pressed overnight (140 kPa, ~18 h, ~20°C). The cheese was then dehooped, and each block cut into 10 pieces of ~600 g each, and each piece was vacuum packaged. Five pieces were stored at 6°C and 5 at 12°C. Cheese making was conducted in triplicate.

Microbial Enumerations

At 0, 8, 16, and 23 wk, cheese samples (11 g) were collected from the interior of each cheese and homogenized in 99 ml of sterilized 2% sodium citrate (warmed to 45°C) using a Stomacher 400 Circulatory laboratory blender (Seward Laboratory Systems Inc., Bohemia, NY) set for 3 min at 230 rpm (Broadbent et al., 2013). Serial dilutions were prepared in 0.1% sterile peptone water. *Streptococcus thermophilus* starter was enumerated as described by Tabasco et al. (2007) using M17 agar (Becton Dickinson Inc., Sparks, MD) containing 1% lactose (Sigma-Aldrich Inc., St. Louis, MO) incubated aerobically at 45ºC for 24 h. The method of Oberg et al. (2011) for enumerating NSLAB on de Man Rogosa, and Sharpe (**MRS**) (Becton Dickinson Inc., Sparks, MD) agar supplemented with 2 μg/ml vancomycin (**V**) incubated anaerobically at 37ºC for 48 h was used. Such relatively fast-growing NSLAB counts were designated as **NSLAB37** and does not include *Lb. wasatchii* as it does not grow quickly enough to be enumerated at 48 h. *Lactobacillus wasatchii* is resistant to vancomycin like most other NSLAB in cheese and grows very slowly at 37°C (see Chapter 3) and does not form colonies on MRS-V within 48 h (data not shown).

A “crossover time” for the time point when NSLAB numbers equaled and then surpassed starter numbers were determined. The mean log numbers from three reps were plotted against storage time and a trend line fitted to each set of data based on the highest R square obtained. The time (in weeks) when the lines intersect were considered the crossover time (Oberg et al., 2011). All plate counts were performed with spread plate method in duplicate.

In this experiment, the lowest dilution used for bacterial enumeration was 10^{-2}. For calculating mean numbers and when making plots of microbial numbers, a value of 5×10^1 cfu/g was used for samples with counts $<10^2$ cfu/g.

Enumeration of *Lb. wasatchii*. Nonstarter lactic acid bacteria were also enumerated on MRS-V agar supplemented with 1.5% ribose (**MRS-R-V**) after 48 h of anaerobic incubations at 23°C and these counts were designated as **NSLAB23**. After obtaining counts for NSLAB23 after 48 h incubation at 23°C on MRS-R-V media and marking all the colonies (~1.5 mm diameter), the plates were incubated anaerobically at 23°C for an additional 72 h. By this time, *Lb. wasatchii* forms noticeable colonies (~1 mm diameter), which enables differential enumeration of this organism from other NSLAB23 colonies.

Relative Gas Measurements

Gas production during storage of cheese was measured by the extent of loosening of the plastic bag around the cheese block (see Chapter 4). After pressing, similar sized (~600 g) blocks of cheese were inserted into plastic bags (QME355 3.5 mil; Vilutis and Co. Inc., Frankfurt, IL) and the bag vacuum-sealed ~5 cm distance from the cheese block.

Vacuum packaged cheese was then visually examined and on the side of the package that had just been sealed, a line was drawn along the plastic bag at the position where it was tightly pulled against the cheese (d 1 line). After 8, 16 and 23 wk, the cheeses were examined for gas production manifest by loosening of the package. If the plastic bag was no longer tightly pressed against the cheese, the plastic bag was pulled away from the cheese block (on the same side where it was initially marked) as much as possible. A line was then drawn on the plastic bag at the point at which the 2 layers of the bag were still held together by any residual vacuum inside the bag. The distance between that line and the d 1 line was then measured and used as relative measure of gas formation. The more gas produced, the further the plastic bag could be pulled away from the cheese block. When sufficient gas production had occurred inside the cheese pack so that there was no longer any residual vacuum (compared to atmospheric pressure), the cheese package could be pulled the full 5 cm from the cheese to the seal. The distance the pack could be pulled was calculated in relation to this maximum distance and expressed as relative gas production. All cheeses were tested for relative gas production prior to opening the packs and sampling the cheese for microbial analysis. Therefore for each treatment, 4 cheeses were tested at 8 wk, 3 at 16 wk, and 2 at 23 wk.

Chemical Analysis

Proximate composition of the cheeses was determined after approximately 3 d. Moisture content was measured by weight loss using ~3.7 g of grated cheese in a microwave moisture analyzer (Model SMART System 5; CEM Corporation, Matthews, NC) using program CHEESELF. Fat content was measured by a modified Babcock

method (Richardson, 1985). Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher. The resulting slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (Model 926, Corning, Medfield, MA). Salt-in-moisture (**S/M**) content was calculated as salt/(moisture + salt) and expressed as a percentage.

Sugar and Organic Acid Analysis

All cheese samples were analyzed by an HPLC for lactose, galactose, lactic acid, and propionic acid at d 1 and after 16 wk of ripening as described by Phadungath (2011). About 5 g cheese was manually homogenized for 90 s with 10-mL of 0.013N sulfuric acid at 65°C then centrifuged at 7,000 g for 10 min. The samples were held at 4ÅãC for 20 min to solidify the fat layer and the supernatant was filtered then poured into a 0.5-mL Microcon (Millipore Corporation, Bedford, MA) centrifugal filter device with a molecular weight cut-off of 3,000 Da and micro-centrifuged at 14,000 x g for 20 min to remove soluble peptides. The filtrate was injected into the HPLC system containing a cation H+ microguard cartridge (Bio-Rad Laboratories, Hercules, CA) and Rezex ROA-organic acid H+ column (300 x 7 mm, 8 µm, Phenomenex) held at 65ÅãC. Separation was performed isocratically using 0.013 N sulfuric acid as the mobile phase with quantification of analytes based on the external standard method described by Upreti et al. (2006a).

Experimental Design

The experiment was conducted as a randomized block with split-split plot design. The cheese was made on 2 separate occasions. Each pressed block was cut into ~600 g

pieces, individually vacuum packaged, and randomly assigned to be stored at either 6°C or 12°C for various time periods. Statistical analysis of the effect of adding different starter cultures, storage time, storage temperature and *Lb. wasatchii* addition was performed using PROC GLIMMIX in SAS (version 9.1; SAS Institute Inc., Cary, NC) and differences between means determined using Tukey least squares means. Significance was declared at $P<0.05$.

RESULTS AND DISCUSSION

Initial Composition

Significant differences were found in the initial composition of control and *Lb. wasatchii* added cheese ($P<0.05$). Higher moisture (41.5%) and lower S/M (4.1%) levels were observed in cheese made with added *Lb. wasatchii* ($P<0.05$) (Table 5.1). However, salt, pH, and fat levels were the same in both cheeses ($P>0.05$). This effect of adding the *Lb. wasatchii* was unexpected. In previous work (see Chapter 4) when Cheddar cheese was made in a similar manner using starter lactococci, adding *Lb. wasatchii* had no effect on cheese moisture content. The set-to-salt time for the cheese was slightly different with a longer time required (15 min more) when *Lb. wasatchii* was added. In a similar manner, making cheese with lactococcal starter containing added *Lb. wasatchii* took longer compared to making control cheese where no *Lb. wasatchii* was added (see Chapter 4).

Although S/M was lower in cheese with added *Lb. wasatchii*, it was still within S/M levels reported by Agarwal et al. (2011) for Cheddar cheese made in the Unites States. The moisture content of the cheeses being at or above the legal maximum for

Cheddar cheese was perhaps a result of the fast acidification rate (~195 min set-to-salt time) when using *St. thermophilus* as the starter culture rather than *Lactococcus.*

Starter Streptococci and NSLAB during ripening

Starter *St. thermophilus* numbers in the cheese were influenced by storage time, temperature as well as *Lb. wasatchii* x time interaction ($P<0.05$) (Table 5.2). Differences in streptococci numbers based on addition of *Lb. wasatchii* only occurred during storage at 12°C. At 6°C starter numbers decreased ~1.5-log cfu/g after 23 wk for both cheeses (Figures 5.1, 5.2) but at 12°C there was a greater reductions in starter numbers ($P<0.05$) in cheese with added *Lb. wasatchii* (Figure 5.3). Similar results were also observed in the previous work with cheese made using lactococcal starters (see Chapter 4). The basis for this effect is unclear, since no antimicrobial encoding gene(s) were found in the genome of *Lb. wasatchii* (see Chapter 4)

Nonstarter lactic acid bacteria numbers increased from undetectable levels ($\leq 10^2$ cfu/g) to 10^8 and ~10^7 cfu/g for NSLAB23 and NSLAB37, respectively (Figures 5.1, 5.2). Within each replicate, levels and patterns of growth of NSLAB were similar, which was expected, as the salted curd from each replicate was divided into separate portions for ripening, so the cheeses would be expected to have the same starting microbial background population.

No significant differences existed in NSLAB (both NSLAB23 and NSLAB37) numbers observed in the cheese as a function of ripening temperature or addition of *Lb. wasatchii* ($P>0.05$). The absence of a temperature effect was unexpected, as prior studies often reported a higher NSLAB counts at elevated temperatures (Peterson and Marshall,

1990; Fox et al., 1998; see Chapter 4). Similar numbers of NSLAB at both temperatures in this study could be due to the availability of galactose as a readily available energy source. Facultative heterofermentative NSLAB such as *Lactobacillus curvatus*, predominant NSLAB found in Cheddar cheese (Broadbent et al., 2013), gain net two ATP via Embden-Meyerhof pathway per galactose or another hexose consumed (Axelsson, 2004). Thus, free galactose could provide sufficient energy for NSLAB to reach high final cell densities throughout ripening at both temperatures.

Similar to the previous work (see Chapter 4), NSLAB23 counts were significantly higher than NSLAB37 during ripening ($P<0.05$). The most likely explanation for this difference is that 37°C inhibits growth of some NSLAB just as we observed for *Lb. wasatchii*. Even though NSLAB counts were not influenced by temperature, there were differences in the crossover times between 12°C and 6°C as well as between cheese with and without added *Lb. wasatchii* (Figures 5.1, 5.2). Crossover occurred more quickly in cheese with added *Lb. wasatchii* (Figure 5.1) because the *St. thermophilus* numbers decreased more rapidly. Crossover between starter streptococci and NSLAB numbers occurred during the first 8 to 10 wk at 12°C in cheeses with added *Lb. wasatchii* and not till after 23 wk for the control cheese (Figure 5.2). When stored at 6°C, the NSLAB numbers never reached the same level as the starter because of the higher retention of viable starter streptococci numbers at 6°C.

Sugar and Organic Acids

Sugar and organic acid profile of the cheeses were similar in both control and *Lb. wasatchii* added cheese at 6 and 12°C. Lactose content of the cheese was 0.2% at d 1 and similar levels were observed after 16 wk of ripening. Such persistence of lactose in Cheddar cheese during storage has been previously shown by Fox et al. (1998) who reported continued presence of lactose after 36 wk of ripening. This perhaps relates to impaired fermentation of lactose to lactic acid by starter lactic acid bacteria at higher S/M levels (Olson and Johnson, 1990).

A substantial amount (0.6 to 0.7%) of galactose was observed in cheese at d 1. Accumulation of galactose was not surprising because *St. thermophilus* does not utilize the galactose moiety of lactose (Tinson et al., 1982b; Hutkins and Morris, 1987; Mora et al., 2002; Vaillancourt et al., 2004). Adding *Lb. wasatchii* to cheese milk did not have an appreciative impact on initial galactose concentrations, likely because *Lb. wasatchii* utilizes galactose very slowly and itself is a slow-growing species (see Chapter 3). Galactose levels fell to 0.1 to 0.2% after 16 wk of storage in all cheeses likely due to the growth of *Lb. wasatchii* and other NSLAB.

Initial lactic acid levels observed (~1%) in the current study was lower than previously reported by McSweeney and Fox (2004) and McMahon et al. (2014) who found 1.4 to 1.5% lactic acid in the cheese at d 1. Perhaps this was because of the relatively fast make time and the cheeses were not very acid with initial pH in the range ~5.2 to 5.3. Lactic acid levels increased up to 1.7% after 16 wk in control and *Lb. wasatchii* added cheese at both temperatures. This corresponds with similar NSLAB

numbers achieved in cheese stored at 6 or 12°C (P>0.05) that perhaps further relates to substantial reductions in free galactose content of cheese. The higher lactic acid levels could also be attributed to having lower S/M in the current study as production of lactic acid is influenced by S/M as reported by (Upreti et al., 2006b).

Propionic acid also increased during ripening in control and *Lb. wasatchii* added cheeses as previously shown (McMahon et al., 2014). Levels of propionic acid were up to 0.2% (from 0.02% at d 1) after 16 wk of ripening when NSLAB counts reached $\geq 10^6$ cfu/g. Nonstarter lactic acid bacteria activity has been reported to increase propionic acid concentration in cheese during storage (St-Gelais et al., 1991; Bouzas et al., 1993; McMahon et al., 2014). *Lactobacillus curvatus,* which is the predominant NSLAB found in our cheese (Broadbent et al., 2013), has the metabolic capability to produce propionic acid (unpublished data).

Growth of *Lb. wasatchii.* No adventitious *Lb. wasatchii* was detected in the control cheese during ripening at either 6 or 12°C which as previously shown (see Chapter 4) does not mean it was not present just that it was not with 1.5 log of the NSLAB counts. This makes it difficult to relate sporadic late gas blowing in Cheddar cheese when it may only be present in low numbers on most occasions.

Growth of deliberately added *Lb. wasatchii* is shown in Figure 5.3. Temperature had a significant effect on the increase in numbers during ripening ($P<0.05$). Higher counts were observed at the elevated ripening temperature of 12°C compared to 6°C with the greatest increase in cell numbers occurring during the first 8 wk at both temperatures.

This corresponds with the rapid reduction in the numbers of *St. thermophilus* in cheese with added *Lb. wasatchii* at 12°C (Figure 5.3).

We have previously shown that *Lb. wasatchii* is capable of growing on carbohydrates released during lysis of other bacteria, and can reach high cell densities (OD_{600} of 2.49) when it is grown on carbohydrate-restricted MRS broth containing lactococcal cell lysate (see Chapter 4). In addition, since there is a clear pattern in the trend lines for growth of *Lb. wasatchii* and decrease in the numbers of starter streptococci (Figure 5.3), we postulate that free galactose remaining from lactose fermentation by *St. thermophilus* and ribose released via its subsequent lysis enables co-utilization of both sugars by *Lb. wasatchii* in manner that maximizes its rate and extent of growth (see Chapter 3). Slower growth of *Lb. wasatchii* during the first 8 wk of ripening at 6°C supports our hypothesis because starter streptococci counts never fell below 10^9 cfu/g during this period.

Relative Gas and Splits in Cheese

A large amount of gas was produced in cheese with added *Lb. wasatchii* and ripened at 12°C. All effects and interactions were significant at $P \leq 0.01$ (Table 5.2). At 6°C, even cheese with added *Lb. wasatchii* showed no sign of gas formation during 23 wk of storage. An association between elevated ripening temperatures and gassy defect in cheese has been reported previously (Elliott et al., 1981; Laleye et al., 1987). Elliott et al. (1981) reported that gas formation was observed during storage at 10°C but not at 4.5°C and within 6 mo in cheese inoculated with a slow-growing gas forming bacterium that may be similar to *Lb. wasatchii*. Nonetheless, the observation that *Lb. wasatchii* did not

promote gassy defect at 6°C is different than our recent work in cheese made with *Lc. lactis* (see Chapter 4). Results from the latter study suggest *Lb. wasatchii* might derive hexose sugars for gas from lysed starter culture cells. Our current work using *St. thermophilus* confirms this, in that as stated above when the cheese was ripened at 12°C there was a rapid drop in *St. thermophilus* numbers from an initial 10^9 cfu/g to 10^6 cfu/g at 8 wk (Figure 1). In comparison, the *St. thermophilus* numbers stayed much higher when the cheese was stored at 6°C and were still at ~10^7 cfu/g at the end of 23 wk storage (Figure 5.1).

Corresponding differences in *Lb. wasatchii* numbers were observed with the cheese stored at 12°C having one log higher *Lb. wasatchii* counts than cheese stored at 6°C (Figure 5.3). At 12°C, faster lysis of *St. thermophilus* starter culture cells and increased numbers of *Lb. wasatchii* were similar to results found with cheese made with starter lactococci (see Chapter 4). Previous studies have shown that the growth of *Lb. wasatchii* is higher in the presence of cell lysate material since it uses the released carbohydrates, such as ribose and glycosylated proteins, for energy to support metabolism and growth (see Chapter 4).

While most of growth of *Lb. wasatchii* and the die off of starter bacteria occurred during the first 8 wk of storage, blowing of packs was not observed until after this time (and only at the elevated ripening temperature) (Figure 5.4). This may be a result of needing to have most of the available ribose (from lysing starter bacteria) consumed before any extensive production of CO_2 occurs, or it may be related to the solubility of CO_2 in cheese and the need for the cheese water phase to become saturated in CO_2 before

any loosening of the pack is observed (see Chapter 4). We recently reported that galactose utilization by *Lb. wasatchii* is considerably slower when there is no ribose present in the medium (see Chapter 3). As Axelsson (2004) reported, fermentation of a hexose such as galactose as an energy source is needed for CO_2 production in OHF lactic acid bacteria. In the presence of both ribose and galactose, however, *Lb. wasatchii* can rapidly utilize ribose to generate ATP for the cell, and the biochemical pathways are available to simultaneously utilize galactose to provide peptidoglycan precursors for cell wall synthesis and cell growth. Then, when supply of ribose is exhausted, the cell switches to fermentation of galactose for energy and gas production is observed. This has been noticed when *Lb. wasatchii* is grown in MRS broth at pH 5.2 (see Chapter 4) and the similar effect observed in our previous experiment with the highest gas production occurring in the cheese to which both ribose and galactose had been added. Even though the most rapid growth of *Lb. wasatchii* occurs during the first 8 weeks of ripening, observations of later blowing usually occurred after this time (see Chapter 4).

We postulate that lack of enough gas production to loosen packs at 6°C is probably a combination of having one log less *Lb. wasatchii* than at 12°C, and having an increased solubility of carbon dioxide (CRC, 2009). Having slightly higher moisture of 41.5% in these cheeses compared to 37% when the cheese made using starter lactococci (see Chapter 4) is another factor that higher CO_2 would be needed to saturate out the water portion of the cheese before it releases and loosens the package. Having no gas production in control cheese at 12°C is different than what was observed in cheese made with starter lactococci (loose packs after 16 wk). This corresponds with that adventitious

Lb. wasatchii numbers never reaching detectable levels (within 1.5 log of NSLAB) even after 23 wk of ripening in control cheese in the current study.

Having higher moisture levels in the current study allowed us to show the occurrence of the defect more explicitly in that irregular shaped void and round eyes were observed in gassy cheese (Figure 5.5). Such defective Cheddar cheese would not be suitable for sale in supermarkets, leading to consumer rejection and avoidance of purchase. As well, cutting losses of up to 50% for defective cheese is a major economic concern for cheese manufacturers (Golnazarian, 2001).

In large scale dairy processing, wild type *St. thermophilus* are found in pasteurized cheese milk (Hup and Stadhouders, 1979; Bouman et al., 1982; Martley and Crow, 1993). As Martley and Michel (2001) reported, if *St. thermophilus* is present in pasteurized milk at levels sufficient to increase the acid production rate during Cheddar cheesemaking without the cheesemaker's knowledge, the natural response to slow the acid production rate would be to increase the cook temperature or to reduce the amount of starter lactococci (i.e. mesophilic) being used in later vats. However, both approaches would unknowingly increase the growth and acid production by *St. thermophilus* over that of starter lactococci (Michel and Martley, 2001). Thus, galactose accumulation would be enhanced under such conditions and if an OHF NSLAB such as *Lb. wasatchii* is present as part of the resident NSLAB population, the sugar can stimulate the growth of such OHF NSLAB and lead to problems with gassy defect. Deliberate use of *St. thermophilus* starter culture obviously brings similar risks, and this should be

acknowledged in attempts to shorten the made time for Cheddar cheese, or shorten the ripening time by using elevated temperatures.

CONCLUSIONS

This study explored consequences of having an obligatory heterofermentative bacteria as part of the background nonstarter microflora of Cheddar cheese on late blowing when the starter culture contains *St. thermophilus* and when the cheese is ripened at elevated temperatures. We deliberately inoculated milk with *Lb. wasatchii*, a slow-growing OHF species, and made cheese using a *St. thermophilus* culture and studied gas formation during ripening at regular and elevated ripening temperatures. *Lactobacillus wasatchii* was able to grow (~3 log) during storage and produced gas after 8 wk of ripening at elevated temperature. However, no gassiness was observed in the cheese containing added *Lb. wasatchii* at 6°C, either because of having 1-log lower counts of *Lb. wasatchii* in the cheese during ripening or the higher solubility of CO_2 at colder temperature. Also, control cheese did not exhibit any sign of gas formation after 23 wk of ripening at either temperature. Utilization of *St. thermophilus* as a starter for Cheddar cheese or presence of *Lb. wasatchii* or similar organism as a NSLAB should be taken into consideration, especially if a manufacturer is using higher than normal ripening temperatures. We conclude that *Lb. wasatchii* is a contributor to late blowing in Cheddar cheese, and that this property is enhanced when the cheese is made using *St. thermophilus* starter culture.

REFERENCES

Agarwal, S., D. McCoy, W. Graves, P. D. Gerard, and S. Clark. 2011. Sodium content in retail Cheddar, Mozzarella, and process cheeses varies considerably in the United States. J. Dairy Sci. 94:1605-1615.

Axelsson, L. T. 2004. Lactic acid bacteria: Classification and physiology. Pages 1-63 in Lactic Acid Bacteria, S. Salminen, S. and A. von Wright, ed. Dekker, New York.

Bley, M. E., M. E. Johnson, and N. F. Olson. 1985. Factors affecting nonenzymatic browning of process cheese. J. Dairy Sci. 68:555-561.

Bouzas, J., C. A. Kantt, F. Bodyfelt, and J. A. Torres. 1993. Time and temperature influence on chemical aging indicators for a commercial Cheddar cheese. J. Food Sci. 58:1307-313.

Bouman, S., D. B. Lund, F. M. Driessen, and D. G. Schmidt. 1982. Growth of thermoresistant Streptococci and deposition of milk constituents on plates of heat-exchangers during long operating times. J. Food Prot. 45(9):806-12.

Broadbent, J. R., C. Brighton, D. J. McMahon, N. Farkye, M. Johnson and J. Steele. 2013. Microbiology of Cheddar cheese made with different fat contents using a *Lactococcus lactis* single-strain starter. J. Dairy Sci. 96:4212-4222.

Cogan, T. 2011. Microbiology of Cheddar cheese. Page 625 *in* Encyclopedia of Dairy Sciences. Vol. 1. 2nd ed. J. W. Fuquay, P. F. Fox, and P. L. H. McSweeney, ed. GBR: Academic Press, London, UK.

CRC. 2009. CRC Handbook of Chemistry and Physics: A Ready-Reference Book of Chemical and Physical Data. 90th ed. CRC Press, Boca Raton, FL.

De Vos, W. M., and E. E. Vaughan. 1994. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol. Rev. 15:217-237.

Elliott, J. A., G. E. Millard, and R. A. Holley. 1981. Late gas defect in Cheddar cheese caused by an unusual bacterium. J. Dairy Sci. 64:2278-2283.

Fox, P. F., and P. L. H. McSweeney, and C. M. Lynch. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust. J. Dairy Technol. 53:83-89.

Golnazarian, C. 2001. Slit formation in Cheddar cheese: A comprehensive investigation of the microbiological parameters associated with this defect. PhD Thesis. University of Vermont., Burlington.

Hup, G., and J. Stadhouders. 1979. Growth of thermoresistant streptococci in cheese milk pasteurizers. 3. Specific bacterial flora and its effect on cheese quality. Zuivelzicht. 71: (51/52): 1141-1143.

Hutkins, R. W., and C. Ponne. 1991. Lactose uptake driven by galactose efflux in *Streptococcus thermophilus*: evidence for a galactose-lactose antiporter. Appl. Environ. Microbiol. 57:941-944.

Hutkins, R. W., and H. A. Morris. 1987. Carbohydrate metabolism by *Streptococcus thermophilus*: A review. J. Food Prot. 50:876-884.

Laleye, L. C., R. E. Simard, B. H. Lee, R. A. Holley, and R. N. Giroux. 1987. Involvement of heterofermentative lactobacilli in development of open texture in cheeses. J. Food Prot. 50:1009-1012.

Martley, F. G., and V. L. Crow. 1993. Interactions between non-starter microorganisms during cheese manufacture and ripening. Int. Dairy J. 3:461-483.

Martley, F. G., and V. Michel. 2001. Pinkish colouration in Cheddar cheese-description and factors contributing to its formation. J. Dairy Res. 68:327-332.

McMahon, D. J., C. J. Oberg, M. A. Drake, N. Farkye, L. V. Moyes, M. R. Arnold, B. Ganesan, J. Steele, and J. R. Broadbent. 2014. Impact of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full fat Cheddar cheese. J. Dairy Sci. 97:4780-4798.

McSweeney, P. L. H., and P. F. Fox. 2004. Metabolism of residual lactose and of lactate and citrate. Pages 361-372 *in* Cheese: Chemistry, Physics and Microbiology, Vol 1: General Aspects, 3rd ed. P. F. Fox, P. L. H. McSweeney, T. M., Cogan and T. P. Guinee, ed. Elsevier, London.

Michel, V., and F. G. Martley. 2001. *Streptococcus thermophilus* in cheddar cheese--production and fate of galactose. J. Dairy Res. 68:317-25.

Mora, D., M. G. Fortina, C. Parini, G. Ricci, G. Giraffa, and P. L. Manachini. 2002. Genetic diversity and technological properties of *Streptococcus thermophilus* strains isolated from dairy products. J. Appl. Microbiol. 93:278-287.

Oberg, C. J., L. V. Moyes, M. J. Domek, C. F. Brothersen, and D. J. McMahon. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. J. Dairy Sci. 94:2220-2230.

Olson, N. F., and M. E. Johnson. 1990. Light cheese products: Characteristics and economics. Food Technol. 44:93‑96.

Peterson, S. D. and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: A review. J. Dairy Sci. 73:1395-1410.

Phadungath, C. 2011. The efficacy of sodium gluconate as a calcium lactate crystal inhibitor in cheddar cheese. PhD Thesis. South Dakota State University, Brookings.

Radford, D. R., and R. R. Hull. 1982. Utilization of carbohydrate in "short method" Cheddar cheese by mesophilic starters. Aust. J. Dairy Technol. 37:104.

St-Gelais, D., G. Doyon, J. R. Rolland, and J. Goulet. 1991. Sugar and organic acid concentrations during ripening of Cheddar cheese-like products. Milchwissenschaft. 46: 288-291.

Tabasco, R., T. Paarup, C. Janer, C. Peláez, and T. Requena. 2007. Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. Int. Dairy J. 17:1107-1114.

Tinson, W., M. F. Ratcliff, A. J. Hillier, and G. R. Jago. 1982a. Metabolism of *Streptococcus thermophilus*. 3. Influence on the level of bacterial metabolites in Cheddar cheese. Aust. J. Dairy Technol. 37:17-21.

Tinson, W., A. J. Hillier, and G. R. Jago. 1982b. Metabolism of *Streptococcus thermophilus*. 1. Utilization of lactose, glucose and galactose. Aust. J. Dairy Technol. 37:8-13.

Upreti, P., L. L. McKay, and L. E. Metzger. 2006a. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. J. Dairy Sci. 89:429-443.

Upreti, P., L. E. Metzger, and K. D. Hayes. 2006b. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Proteolysis during ripening. J. Dairy Sci. 89:444-453.

Vaillancourt, K., J. D. LeMay, M. Lamoureux, M. Frenette, S. Moineau, and C. Vadeboncoeur. 2004. Characterization of a galactokinase-positive recombinant strain of *Streptococcus thermophilus*. Appl. Environ. Microbiol. 70:4596-4603.

Table 5.1. Mean (±SE) composition of cheese (at d 3) made using *Streptococcus thermophilus* with (LBW+) or without *Lactobacillus wasatchii* (Control) added at 10^4 cfu/ml in milk.

	Control	**LBW$^+$**
Moisture	38.8^a (0.26)	41.5^b (0.16)
Salt	1.93^a (0.08)	1.78^a (0.03)
S/M[1]	4.73^a (0.18)	4.11^b (0.08)
pH	5.27^a (0.003)	5.31^a (0.01)
Fat	30.7^a (0.17)	30.5^a (0.00)

[1]Salt-in-moisture

[a,b]Means same letter within each row are not significantly different from each other. (α=0.05).

Table 5.2. The effect of *Lactobacillus wasatchii* inoculation (LBW), temperature, time and their interactions on the numbers of *Streptococcus thermophilus* (Starter) and relative gas formation in Cheddar cheese.

Source of Variation	DF	Starter (Pr > F)	Gas (Pr > F)
LBW	1	0.3256	0.0146
Temperature	1	0.0239	0.0012
LBW x Temperature	1	0.0383	0.0012
Time	3	<.0001	<.0001
LBW x Time	3	0.5184	<.0001
Temperature x Time	3	0.0964	<.0001
LBW x Temperature x Time	3	>0.05	<.0001

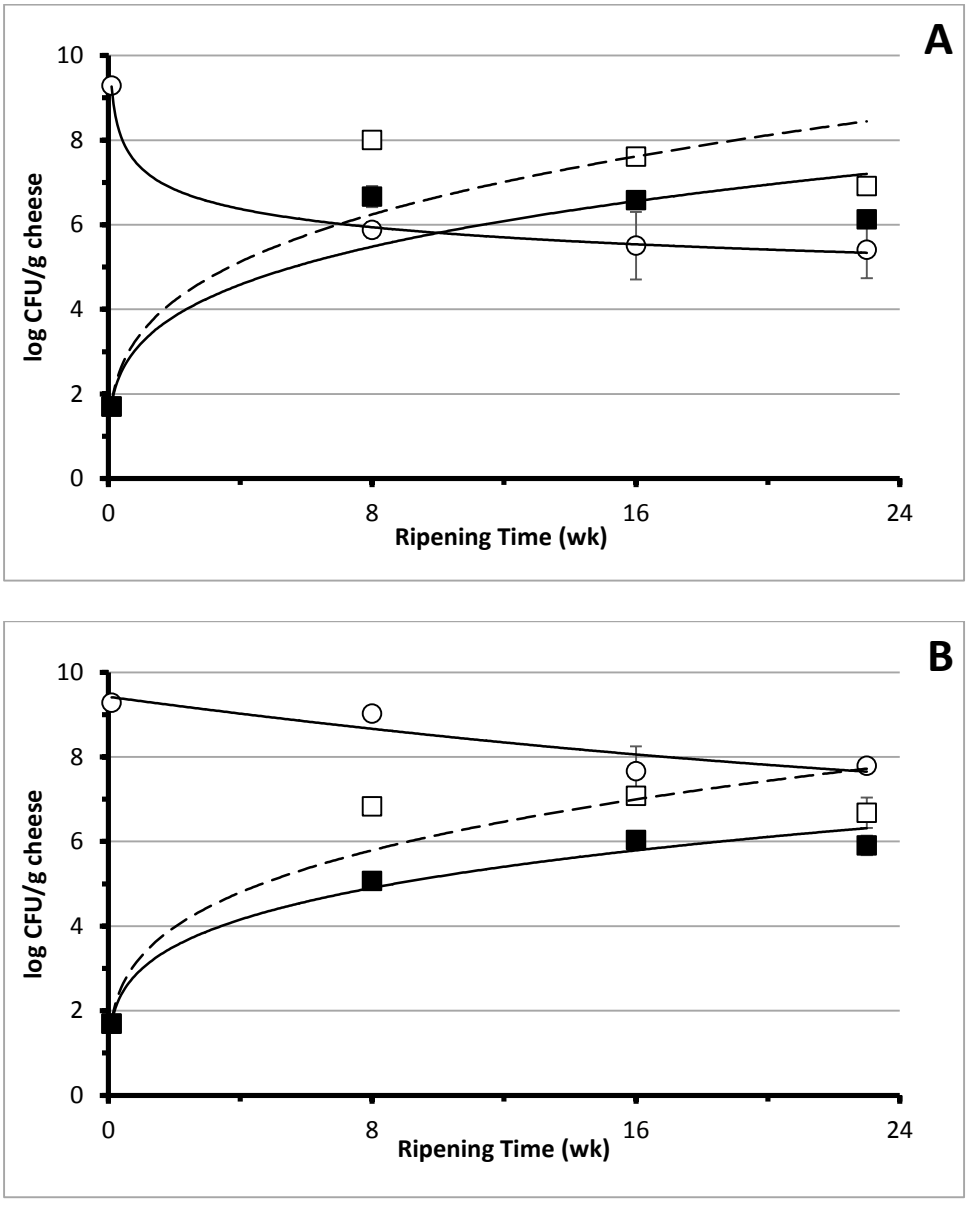

Figure 5.1. Changes in mean numbers of *Streptococcus thermophilus* (○), nonstarter lactic acid bacteria enumerated at 23°C (□, dashed line) or nonstarter lactic acid bacteria enumerated at 37°C (■) in cheese containing *Lactobacillus wasatchii* stored at 12°C (A) or 6°C (B). Bars=SE (n=3).

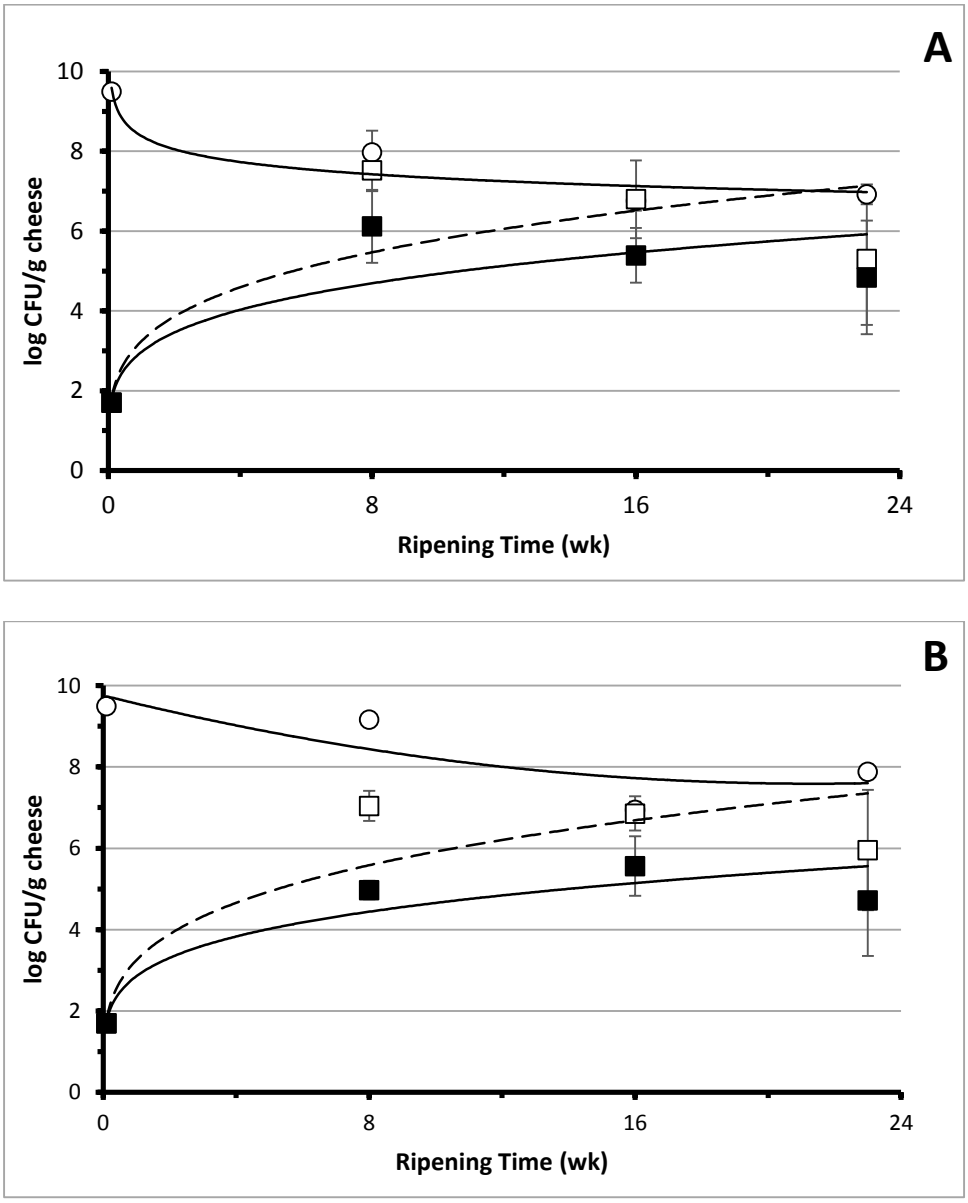

Figure 5.2. Changes in mean numbers of *Streptococcus thermophilus* (○), nonstarter lactic acid bacteria enumerated at 23°C (□, dashed line) or nonstarter lactic acid bacteria enumerated at 37°C (■) in control cheese stored at 12°C (A) or 6°C (B). Bars=SE (n=3).

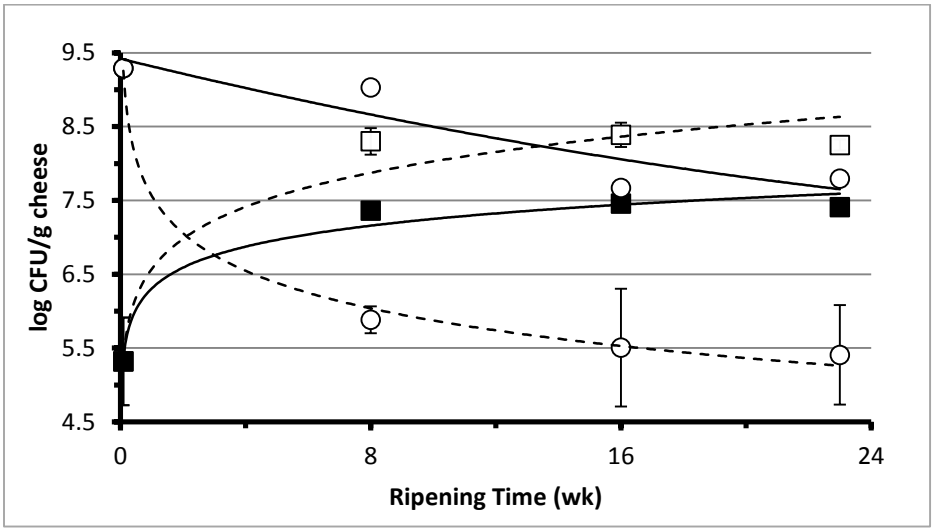

Figure 5.3. Changes in the mean numbers of *Lactobacillus wasatchii* (squares) and *Streptococcus thermophilus* (circles) in cheese ripened at 12°C (open symbols and dashed lines) or 6°C (solid symbols and lines). Bars=SE (n=3).

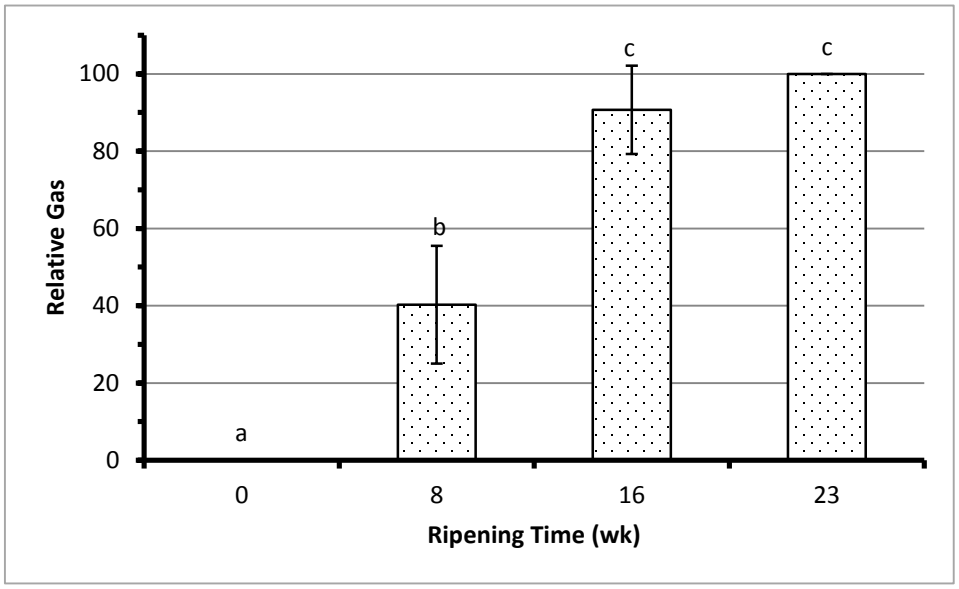

Figure 5.4. Relative gas formation in Cheddar cheese made using *Streptococcus thermophilus* inoculated with *Lactobacillus wasatchii* added at 10^4 cfu/ml of milk. Bars= SE (n=3).

[a-c] Means same letters are not significantly different from each other. (α=0.05).

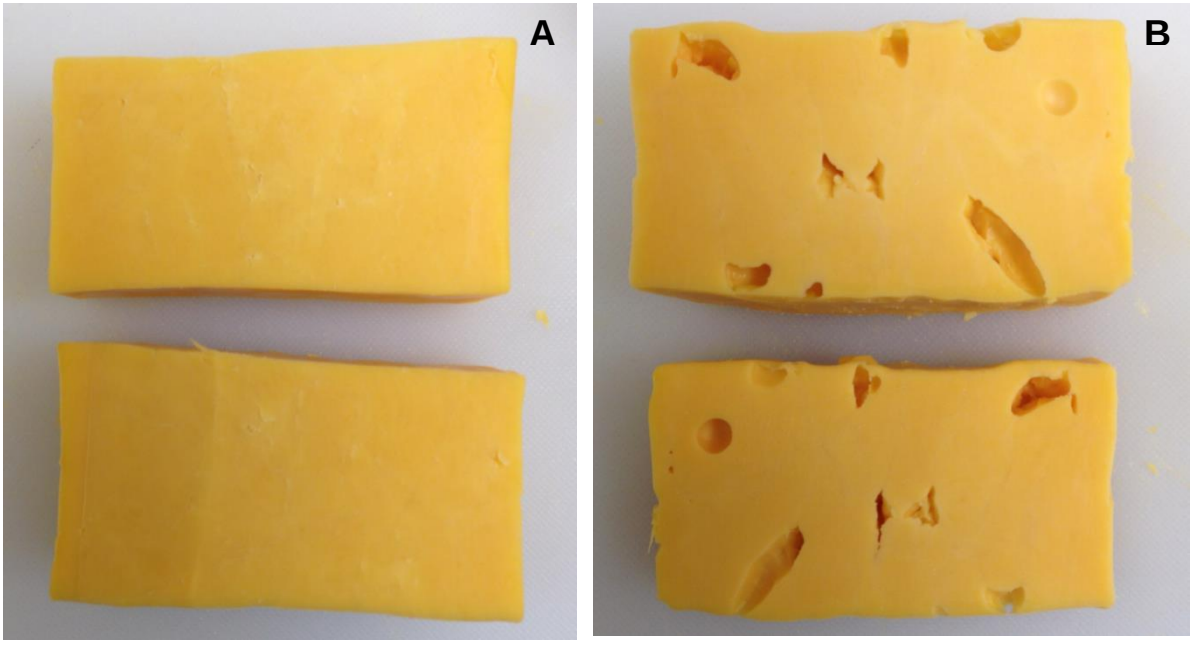

Figure 5.5. Textural differences after 16 wk of ripening at 12°C in (A) control cheese or (B) cheese containing inoculated *Lactobacillus wasatchii* added at 10^4 cfu/ml of milk.

CHAPTER 6

GENERAL SUMMARY AND CONCLUSIONS

This study sought to determine whether a recently isolated slow-growing obligatory heterofermentative nonstarter lactic acid bacterium, *Lactobacillus wasatchii* sp. nov., could be implicated in late gassy defect in Cheddar cheese. The study involved 3 interrelated investigations discussed in Chapter 3 through Chapter 5.

Investigation 1 explored the growth characteristics and gas formation of *Lb. wasatchii* in ribose and galactose, its NaCl and pH tolerance, and its thermotolerance. The results of this investigation demonstrated that;

1. *Lb. wasatchii* grew more readily on ribose compared to galactose as indicated by higher maximum specific growth rates ($P<0.05$) and higher final cell densities ($P<0.05$). Gas production was only observed in the presence of galactose (with or without ribose) that corresponds with the OHF lifestyle of *Lb. wasatchii*.
2. *Lb. wasatchii* can co-utilize galactose with ribose. Cells grown on carbohydrate restricted MRS broth containing 0.25% ribose and 0.25% galactose exhibited identical μ_{max} and final cell densities compared with cells grown on 0.5% ribose.
3. *Lb. wasatchii* tolerated at least 5% NaCl and cheese pH of 5.2 as indicated by similar μ_{max} and final cell densities achieved with control (no NaCl at pH 5.2 or 6.5). *Lb. wasatchii* survived HTST lab pasteurization with ~4.5-log

reduction whereas no colonies were detected following LTLT treatment (<10^1 cfu/ml).

Lactobacillus wasatchii is able to grow under cheese-like stress conditions of high salt and low pH. It can also survive HTST pasteurization typical of industry scale dairy processing. It appears that *Lb. wasatchii* is well suited to grow in aging Cheddar cheese and maximizes its growth by co-utilizing ribose and galactose, which are the potential sugars in cheese. Due to being an OHF, *Lb. wasatchii* produces CO_2 only in the presence of a hexose such as galactose, thus it can be implicated in late gas formation of Cheddar cheese.

Investigation 2 explored the growth of *Lb. wasatchii* in Cheddar cheese supplemented with ribose and/or galactose to promote gas formation at regular or accelerated ripening temperatures. Compared to control cheese, *Lb. wasatchii* reached higher cell counts (above 10^8 cfu/g) in cheese supplemented with ribose plus galactose at elevated temperature. On the other hand, cell counts were significantly lower in cheese supplemented with only galactose (10^7 cfu/g) were comparable to cell counts of control cheese ($P>0.05$). The greatest gas production in the cheese was observed in the presence of deliberately inoculated *Lb. wasatchii* at both temperatures with earlier and higher levels achieved at 12°C ($P<0.05$). The fastest gas production occurred in the cheese supplemented with ribose plus galactose ($P<0.05$) while cheese supplemented with either no sugar (control), or ribose, or galactose only, had significantly lower gas formation after 8 wk at 12°C. However, all the cheeses made with added *Lb. wasatchii* had the same amount of gas production during the rest of ripening at 12°C.

Although, no gas formation was observed in cheese without deliberately inoculated *Lb. wasatchii* at 6°C, some replicates exhibited gassiness after 16 wk at 12°C that adventitious *Lb. wasatchii* numbers exceeded the detectable limit (within ~1.5 log below NSLAB). We speculate that *Lb. wasatchii* is likely part of the resident microbiota in the USU dairy plant and contaminates cheese as opportunity provides.

Lactobacillus wasatchii is able to grow and produce gas in Cheddar cheese even without added ribose and galactose. This suggests that *Lb. wasatchii* is able to derive energy from the autolyzed starter culture as demonstrated in this experiment in which *Lb. wasatchii* cells reached an OD_{600} of 2.39 (higher than with 1% ribose) when grown on lactococcal starter cell lysate.

The presence of slow-growing OHF NSLAB was unsuspected in previous efforts that attempted to identify the cause of late gas production in aged cheese. This investigation further implicates *Lb. wasatchii* in late gas blowing problem in Cheddar cheese. The effect is more pronounced at elevated temperature which is common practice for cheese manufacturers to accelerate ripening.

Investigation 3 explored the consequences of making Cheddar cheese using only *Streptococcus thermophilus* with or without inoculated *Lb. wasatchii* during ripening at 6 or 12°C. Cheesemakers often use starter cultures containing *St. thermophilus* in order to accelerate the cheese making process. The downside of using *St. thermophilus* is galactose accumulation in cheese due to the inability of this bacterium to utilize galactose moiety of lactose. We hypothesized that accumulated galactose would be a substrate for

Lb. wasatchii to grow and produce CO_2 due to being an OHF NSLAB, resulting in late gassy defect in cheese.

In cheese containing added *Lb. wasatchii,* growth was observed at both ripening temperatures with ~1-log higher counts of *Lb. wasatchii* at 12°C (~10^8 cfu/g), however, gas formation was only observed at 12°C. On the other hand, in control cheeses *Lb. wasatchii* counts did not reach the detectable limit (within ~1.5-log of fast growing NSLAB) with no sign of gas formation observed at either temperature. Galactose accumulation was 0.6% at day 1 and 0.1% at wk 16 at in all cheeses ripened at both temperatures.

Using *St. thermophilus* in cheesemaking results in galactose accumulation, which *Lb. wasatchii* can utilize for growth with CO_2 released as a fermentation by product resulting in gassy Cheddar cheese. The presence of *Lb. wasatchii* is likely to be particularly problematic in cheesemaking involving utilizing *St. thermophilus* starter or containing adventitious *St. thermophilus*.

APPENDICES

APPENDIX A. STATISTICS FOR CHAPTER 3

Maximum Specific Growth Rates (μ_{max}) when grown on Ribose and Galactose

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	0.00577800	0.00019924	62.92	<.0001
Error	30	0.00009500	0.00000317		
Corrected Total	59	0.00587300			

R-Square	Coeff Var	Root MSE	umax Mean
0.983824	10.16865	0.001780	0.017500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	29	0.00577800	0.00019924	62.92	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	29	0.00577800	0.00019924	62.92	<.0001

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	3.167E-6
Critical Value of Studentized Range	5.83345
Minimum Significant Difference	0.0073

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	treatment
A	0.038500	2	123
A			
A	0.037500	2	1023

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	treatment
	A				
	A		0.036500	2	523
	A				
B	A		0.035500	2	423
B					
B	C		0.028500	2	223
	C				
D	C		0.023500	2	623
D	C				
D	C	E	0.021500	2	437
D		E			
D		E	0.021000	2	323
D		E			
D		E	0.021000	2	237
D		E			
D		E	0.020500	2	137
D		E			
D		E	0.019500	2	537
D		E			
D		E	0.019500	2	1037
D		E			
D		E	0.019000	2	637
D		E			
D		E	0.018500	2	337
D		E			
D		E	0.018000	2	112

Means with the same letter are not significantly different.

Tukey Grouping				Mean	N	treatment
	D		E			
	D	F	E	0.017000	2	1012
		F	E			
	G	F	E	0.016000	2	212
	G	F	E			
	G	H	F	E	0.015500	2	412
	G	H	F	E			
I	G	H	F	E	0.014500	2	512
I	G	H	F				
I	G	H	F	J	0.010000	2	312
I	G	H		J			
I	G	H		J	0.009500	2	823
I	G	H		J			
I	G	H		J	0.009500	2	923
I	G	H		J			
I	G	H		J	0.009000	2	723
I		H		J			
I		H		J	0.008500	2	612
I				J			
I				J	0.008000	2	937
I				J			
I				J	0.008000	2	737
I				J			
I				J	0.007500	2	837
				J			
				J	0.006000	2	812

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**treatment**
J			
J	0.004500	2	712
J			
J	0.003000	2	912

Final OD_{640} when grown on ribose and galactose

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	10.23429208	0.35290662	1243.72	<.0001
Error	30	0.00851250	0.00028375		
Corrected Total	59	10.24280458			

R-Square	Coeff Var	Root MSE	FinalOD Mean
0.999169	2.506990	0.016845	0.671917

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	29	10.23429208	0.35290662	1243.72	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	29	10.23429208	0.35290662	1243.72	<.0001

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**treatment**
A	1.44000	2	412
A			
B A	1.42000	2	1012

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	treatment
B	A			
B	A	1.40500	2	1023
B				
B		1.37000	2	423
B				
B		1.36000	2	112
	C	1.28500	2	123
	D	0.83500	2	137
	D			
	D	0.83000	2	212
	D			
	D	0.83000	2	223
	D			
E	D	0.80000	2	523
E	D			
E	D	0.79500	2	512
E				
E	F	0.75000	2	437
	F			
G	F	0.72000	2	1037
G	F			
G	F	0.70500	2	537
G				
G	H	0.68000	2	812

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	treatment
	H			
I	H	0.62000	2	237
I				
I		0.58000	2	712
	J	0.35500	2	823
	J			
	J	0.35000	2	623
	J			
	J	0.33500	2	323
	J			
	J	0.33500	2	312
	J			
	J	0.33000	2	723
	J			
	J	0.30000	2	337
	J			
	J	0.30000	2	612
	J			
	J	0.29750	2	912
	J			
K	J	0.29000	2	923
K				
K	L	0.22500	2	637
	L			
	L	0.21500	2	837

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	treatment
L			
L	0.20500	2	937
L			
L	0.19500	2	737

µmax during growth in various concentrations of NaCl

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.00295134	0.00036892	12.99	<.0001
Error	27	0.00076675	0.00002840		
Corrected Total	35	0.00371809			

R-Square	Coeff Var	Root MSE	umax Mean
0.793778	9.458215	0.005329	0.056343

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	5	0.00237677	0.00047535	16.74	<.0001
rep	2	0.00009301	0.00004650	1.64	0.2132
pH	1	0.00048156	0.00048156	16.96	0.0003

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	5	0.00237677	0.00047535	16.74	<.0001
rep	2	0.00009301	0.00004650	1.64	0.2132
pH	1	0.00048156	0.00048156	16.96	0.0003

Means with the same letter are not significantly different.

REGWQ Grouping			Mean	N	pH/NaCl
	A		0.90833	3	6.5-1%
	A				
B	A		0.81000	3	6.5-3%
B	A				
B	A	C	0.76500	3	5.2-1%
B		C			
B	D	C	0.73000	3	6.5-0%
B	D	C			
B	D	C	0.70167	3	5.2-2%
B	D	C			
B E	D	C	0.68000	3	5.2-3%
B E	D	C			
B E	D	C	0.66833	3	6.5-2%
E	D	C			
E	D	C	0.63333	3	6.5-5%
E	D	C			
E	D	C	0.60000	3	5.2-0%
E	D				
E	D		0.57000	3	6.5-4%
E					
E			0.52333	3	5.2-4%
E					
E			0.52333	3	5.2-5%

Final OD_{600} when grown on various concentrations of NaCl

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.01249722	0.00113611	7.87	<.0001
Error	24	0.00346667	0.00014444		
Corrected Total	35	0.01596389			

R-Square	Coeff Var	Root MSE	FinalODsalt Mean
0.782843	0.603355	0.012019	1.991944

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	11	0.01249722	0.00113611	7.87	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	11	0.01249722	0.00113611	7.87	<.0001

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	pH/NaCl
A	2.000000	3	5.2-0%
A			
A	2.000000	3	6.5-0%
A			
A	2.000000	3	5.2-1%
A			
A	2.000000	3	6.5-1%
A			
A	2.000000	3	5.2-2%
A			
A	2.000000	3	6.5-2%

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	pH/NaCl
A			
A	2.000000	3	5.2-3%
A			
A	2.000000	3	6.5-3%
A			
A	2.000000	3	5.2-4%
A			
A	2.000000	3	6.5-4%
B	1.963333	3	6.5-5%
B			
B	1.940000	3	5.2-5%

APPENDIX B. STATISTICS FOR CHAPTER 4

Initial Cheese Composition

Moisture

Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0.06195	0.1739
[1]Culture	Rep	0.06166	0.1822
Culture*[2]Cheese	Rep	0.1989	0.1868
Residual		0.2587	.

[1]Culture=with or without added Lb. wasatchii
[2]Cheese=No sugar, ribose, galactose or ribose plus galactose treatments

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.69	0.4941
Cheese	3	12	2.78	0.0869
Culture*Cheese	3	12	0.29	0.8344

Salt

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0.001284	0.006007
Culture	Rep	0.005279	0.007152
Culture*Cheese	Rep	0.000395	0.002995
Residual		0.006941	.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	2.34	0.2657
Cheese	3	12	1.86	0.1899

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture*Cheese	3	12	1.05	0.4063

Salt-in-Moisture

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0.02106	0.05535
Culture	Rep	0.03900	0.05468
Culture*Cheese	Rep	0.001662	0.02503
Residual		0.05964	.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	2.33	0.2664
Cheese	3	12	0.25	0.8572
Culture*Cheese	3	12	0.87	0.4846

pH

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0.01142	0.01750
Culture	Rep	0.01035	0.01054
Culture*Cheese	Rep	1.86E-23	.
Residual		0.000758	0.000310

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.44	0.5739

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Cheese	3	12	2.51	0.1082
Culture*Cheese	3	12	2.42	0.1164

Fat

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0	.
Culture	Rep	0.005252	0.04261
Culture*Cheese	Rep	0.03113	0.08047
Residual		0.1608	.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	1.95	0.2977
Cheese	3	11	3.12	0.0701
Culture*Cheese	3	11	1.29	0.3249

Starter Lactococci

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0	.
Culture	Rep	0.2740	0.2040
Culture*Cheese	Rep	0.005568	0.02992
Culture*Cheese*Temp	Rep	0.02138	0.03861
Residual		0.3317	0.04578

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	2.71	0.2413
Cheese	3	12	1.95	0.1762
Culture*Cheese	3	12	0.55	0.6566
Temp	1	16	33.02	<.0001
Culture*Temp	1	16	3.52	0.0792
Cheese*Temp	3	16	1.20	0.3423
Culture*Cheese*Temp	3	16	0.13	0.9429
Time	3	105	404.63	<.0001
Culture*Time	3	105	3.90	0.0110
Cheese*Time	9	105	0.69	0.7140
Culture*Cheese*Time	9	105	0.89	0.5400
Temp*Time	3	105	4.63	0.0044
Culture*Temp*Time	3	105	2.78	0.0445
Cheese*Temp*Time	9	105	0.50	0.8688

Culture*Temp*Time Least Squares Means

Culture	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
w	6	0.1	6.8358	0.3481	105	19.63	<.0001
w	6	8	4.3071	0.3481	105	12.37	<.0001
w	6	16	3.3908	0.3481	105	9.74	<.0001
w	6	23	2.9721	0.3481	105	8.54	<.0001
w	12	0.1	6.8479	0.3481	105	19.67	<.0001
w	12	8	3.4613	0.3481	105	9.94	<.0001
w	12	16	3.1638	0.3481	105	9.09	<.0001
w	12	23	2.5892	0.3481	105	7.44	<.0001
wo	6	0.1	7.0863	0.3481	105	20.35	<.0001

Culture*Temp*Time Least Squares Means

Culture	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
wo	6	8	5.1579	0.3481	105	14.82	<.0001
wo	6	16	4.6992	0.3481	105	13.50	<.0001
wo	6	23	4.1508	0.3481	105	11.92	<.0001
wo	12	0.1	7.0754	0.3481	105	20.32	<.0001
wo	12	8	4.5521	0.3481	105	13.08	<.0001
wo	12	16	3.5071	0.3481	105	10.07	<.0001
wo	12	23	3.1175	0.3481	105	8.95	<.0001

Conservative T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate		
wo	6	0.1	7.0863		A
					A
wo	12	0.1	7.0754		A
					A
w	12	0.1	6.8479		A
					A
w	6	0.1	6.8358		A
wo	6	8	5.1579		B
					B
wo	6	16	4.6992		B
					B
wo	12	8	4.5521	C	B

Conservative T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate			
				C	B	
w	6	8	4.3071	C	B	D
				C		D
wo	6	23	4.1508	C	E	D
					E	D
wo	12	16	3.5071	F	E	D
				F	E	
w	12	8	3.4613	F	E	
				F	E	
w	6	16	3.3908	F	E	
				F		
w	12	16	3.1638	F		
				F		
wo	12	23	3.1175	F		
				F		
w	6	23	2.9721	F		
				F		
w	12	23	2.5892	F		

The LINES display does not reflect all significant comparisons. The following additional pairs are significantly different: (wo 6 8,wo 12 8), (wo 6 23,wo 12 16), (w 12 8,w 6 23), (w 12 8,w 12 23), (w 6 16,w 12 23), (w 12 16,w 12 23).

Lactobacillus wasatchii

Covariance Parameter Estimates			
Cov Parm	**Subject**	**Estimate**	**Standard Error**
Intercept	**Rep**	0.01328	0.01572
Culture	**Rep**	0.000033	.
Culture*Cheese	**Rep**	0.004264	0.005501
Culture*Cheese*Temp	**Rep**	0	.
Residual		0.04092	0.007734

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	0	.	.	.
Cheese	3	6	50.12	0.0001
Culture*Cheese	0	.	.	.
Temp	1	8	54.15	<.0001
Culture*Temp	0	.	.	.
Cheese*Temp	3	8	2.72	0.1148
Culture*Cheese*Temp	0	.	.	.
Time	3	48	738.34	<.0001
Culture*Time	0	.	.	.
Cheese*Time	9	48	15.90	<.0001
Culture*Cheese*Time	0	.	.	.
Temp*Time	3	48	6.68	0.0007
Culture*Temp*Time	0	.	.	.
Cheese*Temp*Time	9	48	1.57	0.1520

Cheese*Temp*Time Least Squares Means							
Cheese	**Temp**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
Cont	**6**	**0.1**	5.2200	0.1396	48	37.38	<.0001

Cheese*Temp*Time Least Squares Means

Cheese	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Cont	6	8	6.9350	0.1396	48	49.66	<.0001
Cont	6	16	6.6900	0.1396	48	47.91	<.0001
Cont	6	23	6.7917	0.1396	48	48.64	<.0001
Cont	12	0.1	5.2200	0.1396	48	37.38	<.0001
Cont	12	8	7.1767	0.1396	48	51.39	<.0001
Cont	12	16	6.8933	0.1396	48	49.36	<.0001
Cont	12	23	6.8817	0.1396	48	49.28	<.0001
Gal	6	0.1	5.0650	0.1396	48	36.27	<.0001
Gal	6	8	6.8800	0.1396	48	49.27	<.0001
Gal	6	16	6.6933	0.1396	48	47.93	<.0001
Gal	6	23	6.3650	0.1396	48	45.58	<.0001
Gal	12	0.1	5.0650	0.1396	48	36.27	<.0001
Gal	12	8	7.0633	0.1396	48	50.58	<.0001
Gal	12	16	7.0617	0.1396	48	50.57	<.0001
Gal	12	23	7.0350	0.1396	48	50.38	<.0001
Rib	6	0.1	5.1283	0.1396	48	36.72	<.0001
Rib	6	8	7.2650	0.1396	48	52.02	<.0001
Rib	6	16	7.9150	0.1396	48	56.68	<.0001
Rib	6	23	7.6400	0.1396	48	54.71	<.0001
Rib	12	0.1	5.1283	0.1396	48	36.72	<.0001
Rib	12	8	7.9533	0.1396	48	56.95	<.0001
Rib	12	16	8.0450	0.1396	48	57.61	<.0001
Rib	12	23	8.0583	0.1396	48	57.71	<.0001
Rib_Gal	6	0.1	4.9850	0.1396	48	35.70	<.0001
Rib_Gal	6	8	7.4383	0.1396	48	53.27	<.0001
Rib_Gal	6	16	7.6367	0.1396	48	54.69	<.0001

Cheese*Temp*Time Least Squares Means

Cheese	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Rib_Gal	6	23	7.4233	0.1396	48	53.16	<.0001
Rib_Gal	12	0.1	4.9850	0.1396	48	35.70	<.0001
Rib_Gal	12	8	7.8567	0.1396	48	56.26	<.0001
Rib_Gal	12	16	8.2783	0.1396	48	59.28	<.0001
Rib_Gal	12	23	8.2317	0.1396	48	58.95	<.0001

Conservative T Grouping for Cheese*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Temp	Time	Estimate				
Rib_Gal	12	16	8.2783			A	
						A	
Rib_Gal	12	23	8.2317	B		A	
				B		A	
Rib	12	23	8.0583	B		A	C
				B		A	C
Rib	12	16	8.0450	B		A	C
				B		A	C
Rib	12	8	7.9533	B	D	A	C
				B	D		C
Rib	6	16	7.9150	B	D		C
					D		C
Rib_Gal	12	8	7.8567		D		C
					D		
Rib	6	23	7.6400		D	E	
					D	E	

Conservative T Grouping for Cheese*Temp*Time
Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Temp	Time	Estimate				
Rib_Gal	**6**	**16**	7.6367		D	E	
						E	
Rib_Gal	**6**	**8**	7.4383	F		E	
				F		E	
Rib_Gal	**6**	**23**	7.4233	F		E	
				F			
Rib	**6**	**8**	7.2650	F		G	
				F		G	
Cont	**12**	**8**	7.1767	F		G	H
						G	H
Gal	**12**	**8**	7.0633	I		G	H
				I		G	H
Gal	**12**	**16**	7.0617	I		G	H
				I		G	H
Gal	**12**	**23**	7.0350	I	J	G	H
				I	J	G	H
Cont	**6**	**8**	6.9350	I	J	G	H
				I	J		H
Cont	**12**	**16**	6.8933	I	J		H
				I	J		H
Cont	**12**	**23**	6.8817	I	J		H
				I	J		H
Gal	**6**	**8**	6.8800	I	J		H
				I	J		
Cont	**6**	**23**	6.7917	I	J		

Conservative T Grouping for Cheese*Temp*Time
Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Temp	Time	Estimate		
				J	
Gal	**6**	**16**	6.6933	J	K
				J	K
Cont	**6**	**16**	6.6900	J	K
					K
Gal	**6**	**23**	6.3650		K
Cont	**12**	**0.1**	5.2200		L
					L
Cont	**6**	**0.1**	5.2200		L
					L
Rib	**12**	**0.1**	5.1283		L
					L
Rib	**6**	**0.1**	5.1283		L
					L
Gal	**12**	**0.1**	5.0650		L
					L
Gal	**6**	**0.1**	5.0650		L
					L
Rib_Gal	**6**	**0.1**	4.9850		L
					L
Rib_Gal	**12**	**0.1**	4.9850		L

Nonstarter Lactic Acid Bacteria at 23°C (NSLAB23)

Covariance Parameter Estimates			
Cov Parm	**Subject**	**Estimate**	**Standard Error**
Intercept	**Rep**	0.5710	0.6113
Culture	**Rep**	0.04552	0.07819
Culture*Cheese	**Rep**	0	.
Culture*Cheese*Temp	**Rep**	2.94E-19	.
Residual		1.0162	0.1256

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	2.08	0.2862
Cheese	3	12	0.20	0.8971
Culture*Cheese	3	12	1.19	0.3554
Temp	1	16	35.06	<.0001
Culture*Temp	1	16	0.86	0.3664
Cheese*Temp	3	16	0.67	0.5852
Culture*Cheese*Temp	3	16	0.24	0.8643
Time	3	103	278.17	<.0001
Culture*Time	3	103	0.86	0.4623
Cheese*Time	9	103	0.27	0.9803
Culture*Cheese*Time	9	103	1.16	0.3296
Temp*Time	3	103	6.59	0.0004
Culture*Temp*Time	3	103	1.17	0.3244
Cheese*Temp*Time	9	103	1.29	0.2508

Temp*Time Least Squares Means

Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
6	0.1	1.7000	0.4902	103	3.47	0.0008
6	8	5.7331	0.4927	103	11.64	<.0001
6	16	6.0337	0.4902	103	12.31	<.0001
6	23	6.1785	0.4902	103	12.61	<.0001
12	0.1	1.6991	0.4902	103	3.47	0.0008
12	8	7.3943	0.4927	103	15.01	<.0001
12	16	7.3410	0.4902	103	14.98	<.0001
12	23	6.6821	0.4902	103	13.63	<.0001

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	Time	Estimate		
12	8	7.3943		A
				A
12	16	7.3410		A
12	23	6.6821		B
				B
6	23	6.1785	C	B
			C	
6	16	6.0337	C	
			C	
6	8	5.7331	C	
6	0.1	1.7000		D

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)			
LS-means with the same letter are not significantly different.			
Temp	**Time**	**Estimate**	
			D
12	**0.1**	1.6991	D

Nonstarter Lactic Acid Bacteria at 37°C (NSLAB37)

Covariance Parameter Estimates			
Cov Parm	**Subject**	**Estimate**	**Standard Error**
Intercept	**Rep**	0.9577	0.9836
Culture	**Rep**	0	.
Culture*Cheese	**Rep**	0.02871	0.08317
Culture*Cheese*Temp	**Rep**	0	.
Residual		1.3815	0.1804

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	0.02	0.8925
Cheese	3	12	0.32	0.8075
Culture*Cheese	3	12	0.93	0.4575
Temp	1	16	24.70	0.0001
Culture*Temp	1	16	0.86	0.3670
Cheese*Temp	3	16	0.12	0.9487
Culture*Cheese*Temp	3	16	0.33	0.8062
Time	3	102	163.78	<.0001
Culture*Time	3	102	0.74	0.5328

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Cheese*Time	9	102	0.48	0.8873
Culture*Cheese*Time	9	102	1.06	0.3968
Temp*Time	3	102	5.83	0.0010
Culture*Temp*Time	3	102	0.32	0.8123
Cheese*Temp*Time	9	102	0.71	0.6962

Temp*Time Least Squares Means

Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
6	0.1	1.7583	0.6176	102	2.85	0.0053
6	8	5.2147	0.6176	102	8.44	<.0001
6	16	5.5806	0.6148	102	9.08	<.0001
6	23	5.8510	0.6148	102	9.52	<.0001
12	0.1	1.6991	0.6148	102	2.76	0.0068
12	8	6.9356	0.6148	102	11.28	<.0001
12	16	6.9451	0.6176	102	11.24	<.0001
12	23	6.2344	0.6148	102	10.14	<.0001

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	Time	Estimate	
12	16	6.9451	A
			A
12	8	6.9356	A
12	23	6.2344	B

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	Time	Estimate		
				B
6	23	5.8510	C	B
			C	B
6	16	5.5806	C	B
			C	
6	8	5.2147	C	
6	0.1	1.7583		D
				D
12	0.1	1.6991		D

Relative Gas

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	66.81	0.0146
Cheese	3	12	4.09	0.0326
Culture*Cheese	3	12	3.00	0.0727
Temp	1	16	219.15	<.0001
Culture*Temp	1	16	2.90	0.1082
Cheese*Temp	3	16	0.42	0.7378
Culture*Cheese*Temp	3	16	0.81	0.5080
Time	3	87	179.68	<.0001

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture*Time	3	87	27.50	<.0001
Cheese*Time	9	87	0.74	0.6728
Culture*Cheese*Time	9	87	0.59	0.8059
Temp*Time	3	87	42.87	<.0001
Culture*Temp*Time	3	87	4.30	0.0071
Cheese*Temp*Time	9	87	0.23	0.9887

Culture*Temp*Time Least Squares Means

Culture	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
w	6	0.1	-4.5630	0.2275	87	-20.06	<.0001
w	6	8	-3.5127	0.2275	87	-15.44	<.0001
w	6	16	-2.3143	0.2275	87	-10.17	<.0001
w	6	23	-1.2443	0.2598	87	-4.79	<.0001
w	12	0.1	-4.5630	0.2275	87	-20.06	<.0001
w	12	8	-1.6258	0.2275	87	-7.15	<.0001
w	12	16	0.04433	0.2602	87	0.17	0.8651
w	12	23	0.3367	0.5350	87	0.63	0.5308
wo	6	0.1	-4.5951	0.2275	87	-20.20	<.0001
wo	6	8	-4.5951	0.2275	87	-20.20	<.0001
wo	6	16	-4.5951	0.2275	87	-20.20	<.0001
wo	6	23	-4.1530	0.2275	87	-18.26	<.0001
wo	12	0.1	-4.5630	0.2275	87	-20.06	<.0001
wo	12	8	-3.3672	0.2275	87	-14.80	<.0001
wo	12	16	-1.6759	0.2275	87	-7.37	<.0001
wo	12	23	-1.0088	0.2353	87	-4.29	<.0001

T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate			
w	12	23	0.3367	A		
				A		
w	12	16	0.04433	A		
wo	12	23	-1.0088		B	
					B	
w	6	23	-1.2443	C	B	
				C		
w	12	8	-1.6258	C		
				C		
wo	12	16	-1.6759	C		
w	6	16	-2.3143			D
wo	12	8	-3.3672			E
					E	
w	6	8	-3.5127			E
wo	6	23	-4.1530			F
						F
wo	12	0.1	-4.5630			F
						F
w	6	0.1	-4.5630			F
						F

T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate	
w	12	0.1	-4.5630	F
				F
wo	6	16	-4.5951	F
				F
wo	6	8	-4.5951	F
				F
wo	6	0.1	-4.5951	F

Cheese Least Squares Means

Cheese	Estimate	Standard Error	DF	t Value	Pr > |t|
Cont	-3.1142	0.1662	12	-18.74	<.0001
Gal	-2.8701	0.1790	12	-16.03	<.0001
Rib	-2.9248	0.1681	12	-17.40	<.0001
Rib_Gal	-2.5897	0.1815	12	-14.26	<.0001

T Grouping for Cheese Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Estimate		
Rib_Gal	-2.5897		A
			A
Gal	-2.8701	B	A
		B	
Rib	-2.9248	B	

T Grouping for Cheese
Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Estimate	
		B
Cont	-3.1142	B

T Grouping for Culture*Cheese
Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Cheese	Estimate	
w	Rib_Gal	-1.6226	A
w	Gal	-2.2328	B
			B
w	Rib	-2.3673	B
			B
w	Cont	-2.4984	B
wo	Rib	-3.4823	C
			C
wo	Gal	-3.5075	C
			C
wo	Rib_Gal	-3.5567	C
			C
wo	Cont	-3.7301	C

Sugar and Organic Acids

Lactose

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	1.92	0.2997
Cheese	3	12	2.00	0.1672
Culture*Cheese	3	12	0.67	0.5838
Temp	1	16	0.60	0.4511
Culture*Temp	1	16	0.44	0.5180
Cheese*Temp	3	16	0.69	0.5725
Culture*Cheese*Temp	3	16	0.11	0.9518
Time	1	40	4.68	0.0366
Culture*Time	1	40	1.41	0.2413
Cheese*Time	3	40	0.71	0.5526
Culture*Cheese*Time	3	40	0.29	0.8313
Temp*Time	1	40	0.87	0.3573
Culture*Temp*Time	1	40	0.67	0.4176
Cheese*Temp*Time	3	40	0.71	0.5546

Time Least Squares Means					
Time	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
1	0.4187	0.02364	40	17.71	<.0001
16	0.3642	0.02248	40	16.20	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)	
LS-means with the same letter are not significantly different.	
Time	**Estimate**
1	0.4187 A
16	0.3642 B

Galactose

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	6.11	0.1320
Cheese	3	12	13.55	0.0004
Culture*Cheese	3	12	1.74	0.2123
Temp	1	16	0.12	0.7310
Culture*Temp	1	16	1.18	0.2928
Cheese*Temp	3	16	0.20	0.8982
Culture*Cheese*Temp	3	16	0.15	0.9261
Time	1	40	17.83	0.0001
Culture*Time	1	40	1.89	0.1774
Cheese*Time	3	40	7.06	0.0006
Culture*Cheese*Time	3	40	1.03	0.3895
Temp*Time	1	40	0.04	0.8498
Culture*Temp*Time	1	40	0.94	0.3392
Cheese*Temp*Time	3	40	0.17	0.9127

Cheese*Time Least Squares Means

Cheese	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Cont	1	0.02273	0.02209	40	1.03	0.3097
Cont	16	0.04763	0.02462	40	1.93	0.0602
Gal	1	0.2383	0.02207	40	10.80	<.0001
Gal	16	0.07614	0.02053	40	3.71	0.0006
Rib	1	0.06050	0.01845	40	3.28	0.0022
Rib	16	0.003423	0.01985	40	0.17	0.8639
Rib_Gal	1	0.1246	0.02769	40	4.50	<.0001
Rib_Gal	16	0.07783	0.02036	40	3.82	0.0005

T Grouping for Cheese*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Cheese	Time	Estimate		
Gal	1	0.2383		A
Rib_Gal	1	0.1246		B
				B
Rib_Gal	16	0.07783	C	B
			C	B
Gal	16	0.07614	C	B
			C	B
Rib	1	0.06050	C	B
			C	
Cont	16	0.04763	C	D
			C	D
Cont	1	0.02273	C	D
				D

T Grouping for Cheese*Time Least Squares Means (Alpha=0.05)			
LS-means with the same letter are not significantly different.			
Cheese	Time	Estimate	
Rib	16	0.003423	D

Acetic Acid

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.01	0.9203
Cheese	3	12	0.85	0.4942
Culture*Cheese	3	12	1.13	0.3766
Temp	1	16	3.72	0.0718
Culture*Temp	1	16	0.60	0.4513
Cheese*Temp	3	16	2.01	0.1530
Culture*Cheese*Temp	3	16	0.67	0.5812
Time	1	40	21.78	<.0001
Culture*Time	1	40	1.48	0.2302
Cheese*Time	3	40	1.52	0.2247
Culture*Cheese*Time	3	40	2.34	0.0877
Temp*Time	1	40	4.24	0.0461
Culture*Temp*Time	1	40	0.77	0.3846
Cheese*Temp*Time	3	40	2.23	0.0994

Temp*Time Least Squares Means						
Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
12	1	0.002987	0.003601	40	0.83	0.4117
12	16	0.02199	0.003531	40	6.23	<.0001

Temp*Time Least Squares Means						
Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
6	1	0.000705	0.003600	40	0.20	0.8458
6	16	0.008165	0.003612	40	2.26	0.0293

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)			
LS-means with the same letter are not significantly different.			
Temp	Time	Estimate	
12	16	0.02199	A
6	16	0.008165	B
			B
12	1	0.002987	B
			B
6	1	0.000705	B

Lactic Acid

Covariance Parameter Estimates			
Cov Parm	Subject	Estimate	Standard Error
Intercept	Rep	0	.
Culture	Rep	0.01662	0.01565
Culture*Cheese	Rep	0.006766	0.01021
Culture*Cheese*Temp	Rep	0	.
Residual		0.04813	0.009260

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.09	0.7972
Cheese	3	12	1.23	0.3403
Culture*Cheese	3	12	0.20	0.8961
Temp	1	16	10.30	0.0055
Culture*Temp	1	16	1.03	0.3263
Cheese*Temp	3	16	0.22	0.8816
Culture*Cheese*Temp	3	16	1.06	0.3923
Time	1	40	16.96	0.0002
Culture*Time	1	40	4.62	0.0378
Cheese*Time	3	40	0.19	0.9045
Culture*Cheese*Time	3	40	0.59	0.6221
Temp*Time	1	40	6.66	0.0136
Culture*Temp*Time	1	40	0.28	0.5983
Cheese*Temp*Time	3	40	0.28	0.8379

Temp*Time Least Squares Means

Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
12	1	1.0671	0.07582	40	14.07	<.0001
12	16	1.4163	0.07447	40	19.02	<.0001
6	1	1.0397	0.07591	40	13.70	<.0001
6	16	1.1270	0.07457	40	15.11	<.0001

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)			
LS-means with the same letter are not significantly different.			
Temp	**Time**	**Estimate**	
12	**16**	1.4163	A
6	**16**	1.1270	B
			B
12	**1**	1.0671	B
			B
6	**1**	1.0397	B

Propionic Acid

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	0.26	0.6625
Cheese	3	12	1.13	0.3751
Culture*Cheese	3	12	1.21	0.3472
Temp	1	16	30.07	<.0001
Culture*Temp	1	16	0.05	0.8184
Cheese*Temp	3	16	0.40	0.7560
Culture*Cheese*Temp	3	16	0.12	0.9467
Time	1	40	129.92	<.0001
Culture*Time	1	40	0.75	0.3909
Cheese*Time	3	40	0.16	0.9231
Culture*Cheese*Time	3	40	0.72	0.5445

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Temp*Time	1	40	27.88	<.0001
Culture*Temp*Time	1	40	0.02	0.9018
Cheese*Temp*Time	3	40	0.31	0.8213

Temp*Time Least Squares Means

Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
12	**1**	0.08506	0.02468	40	3.45	0.0013
12	**16**	0.4233	0.02406	40	17.59	<.0001
6	**1**	0.08402	0.02471	40	3.40	0.0015
6	**16**	0.2140	0.02406	40	8.90	<.0001

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	Time	Estimate	
12	**16**	0.4233	A
6	**16**	0.2140	B
12	**1**	0.08506	C
			C
6	**1**	0.08402	C

Citric Acid

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.17	0.7186
Cheese	3	12	1.33	0.3117
Culture*Cheese	3	12	1.46	0.2759
Temp	1	19	0.36	0.5542
Culture*Temp	1	19	0.03	0.8601
Cheese*Temp	3	19	0.37	0.7721
Time	1	47	27.05	<.0001
Culture*Time	1	47	4.15	0.0472
Cheese*Time	3	47	2.91	0.0444
Temp*Time	1	47	1.08	0.3049

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
1	0.1315	0.008897	47	14.78	<.0001
16	0.08187	0.008664	47	9.45	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
1	0.1315	A
16	0.08187	B

Orotic Acid

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.15	0.7352
Cheese	3	12	2.27	0.1323
Culture*Cheese	3	12	1.66	0.2289
Temp	1	19	1.61	0.2197
Culture*Temp	1	19	0.09	0.7624
Cheese*Temp	3	19	0.11	0.9547
Time	1	47	12.84	0.0008
Culture*Time	1	47	2.13	0.1510
Cheese*Time	3	47	0.72	0.5447
Temp*Time	1	47	1.73	0.1945

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
1	0.001470	0.000093	47	15.83	<.0001
16	0.001130	0.000090	47	12.55	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
1	0.001470	A
16	0.001130	B

Formic Acid

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.00	0.9594
Cheese	3	12	0.76	0.5404
Culture*Cheese	3	12	1.18	0.3581
Temp	1	19	0.00	0.9449
Culture*Temp	1	19	5.57	0.0292
Cheese*Temp	3	19	0.42	0.7399
Time	1	47	22.82	<.0001
Culture*Time	1	47	0.14	0.7123
Cheese*Time	3	47	1.66	0.1888
Temp*Time	1	47	0.01	0.9417

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
1	0.002312	0.003317	47	0.70	0.4891
16	0.01993	0.003180	47	6.27	<.0001

Differences of Time Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Time	Time	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
1	**16**	-0.01761	0.003687	47	-4.78	<.0001	<.0001

Tukey-Kramer Grouping for Time Least Squares Means (Alpha=0.05)	
LS-means with the same letter are not significantly different.	
Time	**Estimate**
16	0.01993 A
1	0.002312 B

Pyruvic Acid

Covariance Parameter Estimates			
Cov Parm	**Subject**	**Estimate**	**Standard Error**
Intercept	**Rep**	5.647E-6	0.000020
Culture	**Rep**	9.764E-6	0.000022
Culture*Cheese	**Rep**	0.000033	0.000024
Culture*Cheese*Temp	**Rep**	0	.
Residual		0.000044	8.851E-6

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	0.94	0.4337
Cheese	3	12	2.00	0.1684
Culture*Cheese	3	12	1.24	0.3385
Temp	1	16	0.28	0.6033
Culture*Temp	1	16	1.52	0.2354
Cheese*Temp	3	16	0.98	0.4287
Culture*Cheese*Temp	3	16	0.31	0.8147

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Time	1	40	0.83	0.3677
Culture*Time	1	40	12.38	0.0011
Cheese*Time	3	40	0.91	0.4428
Culture*Cheese*Time	3	40	7.23	0.0006
Temp*Time	1	40	0.51	0.4782
Culture*Temp*Time	1	40	0.92	0.3441
Cheese*Temp*Time	3	40	1.24	0.3070

Time Least Squares Means					
Time	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
1	0.01217	0.002521	40	4.83	<.0001
16	0.01370	0.002466	40	5.55	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	**Estimate**	
16	0.01370	A
		A
1	0.01217	A

Uric Acid

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	0.18	0.7094

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Cheese	3	12	1.20	0.3515
Culture*Cheese	3	12	0.45	0.7247
Temp	1	19	0.47	0.5022
Culture*Temp	1	19	0.69	0.4168
Cheese*Temp	3	19	0.40	0.7543
Time	1	47	12.69	0.0009
Culture*Time	1	47	2.80	0.1006
Cheese*Time	3	47	2.21	0.0989
Temp*Time	1	47	0.96	0.3334

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
1	0.000258	0.000156	47	1.65	0.1053
16	0.000919	0.000152	47	6.05	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
16	0.000919	A
1	0.000258	B

APPENDIX C. STATISTICS FOR CHAPTER 5

Initial Cheese Composition

Moisture

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	118.53	0.0083

Culture Least Squares Means

Culture	Estimate	Standard Error	DF	t Value	Pr > |t|
w	41.5267	0.2152	2	192.94	<.0001
wo	38.7600	0.2152	2	180.09	<.0001

T Grouping for Culture Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Estimate	
w	41.5267	A
wo	38.7600	B

Salt

Culture Least Squares Means

Culture	Estimate	Standard Error	DF	t Value	Pr > |t|
w	1.7813	0.05828	2	30.57	0.0011
wo	1.9267	0.05828	2	33.06	0.0009

T Grouping for Culture Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Estimate	
wo	1.9267	A
		A
w	1.7813	A

Salt-in-Moisture

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	23.93	0.0393

Culture Least Squares Means

Culture	Estimate	Standard Error	DF	t Value	Pr > |t|
w	4.1136	0.1403	2	29.32	0.0012
wo	4.7349	0.1403	2	33.75	0.0009

T Grouping for Culture Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Estimate	
wo	4.7349	A
w	4.1136	B

pH

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	9.31	0.0927

Culture Least Squares Means					
Culture	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
w	5.3100	0.008498	2	624.83	<.0001
wo	5.2733	0.008498	2	620.51	<.0001

T Grouping for Culture Least Squares Means (Alpha=0.05)	
LS-means with the same letter are not significantly different.	
Culture	**Estimate**
w	5.3100 A
	A
wo	5.2733 A

Fat

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	1.00	0.4226

Culture Least Squares Means					
Culture	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
w	30.5000	0.1179	2	258.80	<.0001
wo	30.6667	0.1179	2	260.22	<.0001

T Grouping for Culture Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Culture	**Estimate**	
wo	30.6667	A
		A
w	30.5000	A

Starter St. thermophilus

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	1.67	0.3256
Temp	1	4	12.58	0.0239
Culture*Temp	1	4	9.26	0.0383
Time	3	30	20.36	<.0001
Culture*Time	3	30	0.77	0.5184
Temp*Time	3	30	2.31	0.0964

Culture*Temp Least Squares Means						
Culture	**Temp**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
w	**6**	8.4467	0.3193	4	26.45	<.0001
w	**12**	6.5217	0.3193	4	20.42	<.0001
wo	**6**	7.9350	0.2996	4	26.48	<.0001
wo	**12**	7.7879	0.3193	4	24.39	<.0001

T Grouping for Culture*Temp Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Estimate	
w	6	8.4467	A
			A
wo	6	7.9350	A
			A
wo	12	7.7879	A
w	12	6.5217	B

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
0.1	9.3933	0.3193	30	29.42	<.0001
8	8.0121	0.3193	30	25.09	<.0001
16	6.7217	0.3193	30	21.05	<.0001
23	6.5642	0.2996	30	21.91	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
0.1	9.3933	A
8	8.0121	B

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
16	6.7217	C
		C
23	6.5642	C

Temp Least Squares Means

Temp	Estimate	Standard Error	DF	t Value	Pr > |t|
6	8.1909	0.2339	4	35.01	<.0001
12	7.1548	0.2404	4	29.77	<.0001

T Grouping for Temp Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	Estimate	
6	8.1909	A
12	7.1548	B

Lb. wasatchii

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Temp	1	2	17.99	0.0513
Time	3	12	63.49	<.0001
Temp*Time	3	12	2.02	0.1651

T Grouping for Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Temp	**Time**	**Estimate**	
12	**16**	8.3883	A
			A
12	**8**	8.3017	A
			A
12	**23**	8.2517	A
6	**16**	7.4583	B
			B
6	**23**	7.4117	B
			B
6	**8**	7.3633	B
6	**0.1**	5.3200	C
			C
12	**0.1**	5.3200	C

Nonstarter lactic acid bacteria at 23°C (NSLAB23)

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	0.93	0.4374
Temp	1	4	0.62	0.4745
Culture*Temp	1	4	1.01	0.3711
Time	3	27	102.54	<.0001
Culture*Time	3	27	1.01	0.4038
Temp*Time	3	27	0.74	0.5356

Time Least Squares Means

Time	Estimate	Standard Error	DF	t Value	Pr > |t|
0.1	1.7000	0.3858	27	4.41	0.0001
8	7.3508	0.3858	27	19.05	<.0001
16	7.0875	0.3858	27	18.37	<.0001
23	6.2146	0.3858	27	16.11	<.0001

T Grouping for Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Time	Estimate	
8	7.3508	A
		A
16	7.0875	A
23	6.2146	B
0.1	1.7000	C

Nonstarter lactic acid bacteria at 37°C (NSLAB37)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	1.60	0.3328
Temp	1	4	1.08	0.3584
Culture*Temp	1	4	0.21	0.6722
Time	3	22	114.34	<.0001
Culture*Time	3	22	1.32	0.2941
Temp*Time	3	22	2.89	0.0586
Culture*Temp*Time	2	22	0.24	0.7914

Time Least Squares Means					
Time	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
0.1	1.7000	0.2607	22	6.52	<.0001
8	5.7063	0.2607	22	21.89	<.0001
16	5.8963	0.2607	22	22.62	<.0001
23	Non-est	.	.	.	.

NSLAB23 versus NSLAB37

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Culture	1	2	1.01	0.4209
Temp	1	4	0.94	0.3862
Culture*Temp	1	4	0.85	0.4079
Time	3	56	79.42	<.0001
Culture*Time	3	56	0.74	0.5315
Temp*Time	3	56	1.20	0.3171
Culture*Temp*Time	3	56	0.12	0.9455

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Bacteria	1	56	176.60	<.0001
Culture*Bacteria	1	56	0.14	0.7104
Temp*Bacteria	1	56	0.92	0.3403
Culture*Temp*Bacteri	1	56	0.02	0.8819
Time*Bacteria	3	56	38.79	<.0001
Culture*Time*Bacteri	3	56	0.87	0.4628
Temp*Time*Bacteria	3	56	1.47	0.2312

Time*Bacteria Least Squares Means

Bacteria	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
NSLAB23	0.1	1.7000	0.3650	56	4.66	<.0001
NSLAB37	0.1	1.7000	0.3566	56	4.77	<.0001
NSLAB23	8	7.3508	0.3650	56	20.14	<.0001
NSLAB37	8	5.7063	0.3566	56	16.00	<.0001
NSLAB23	16	7.0875	0.3650	56	19.42	<.0001
NSLAB37	16	5.8962	0.3566	56	16.53	<.0001
NSLAB23	23	6.2146	0.3650	56	17.03	<.0001
NSLAB37	23	5.5882	0.3797	56	14.72	<.0001

Tukey-Kramer Grouping for Time*Bacteria Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Bacteria	Time	Estimate	
NSLAB23	8	7.3508	A
			A
NSLAB23	16	7.0875	A
			A

Tukey-Kramer Grouping for Time*Bacteria Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Bacteria	Time	Estimate		
NSLAB23	23	6.2146	B	A
			B	
NSLAB37	16	5.8962	B	C
			B	C
NSLAB37	8	5.7063	B	C
				C
NSLAB37	23	5.5882		C
NSLAB23	0.1	1.7000		D
				D
NSLAB37	0.1	1.7000		D

Relative Gas

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Culture	1	2	66.86	0.0146
Temp	1	4	66.86	0.0012
Culture*Temp	1	4	66.86	0.0012
Time	3	24	23.98	<.0001
Culture*Time	3	24	23.98	<.0001
Temp*Time	3	24	23.98	<.0001
Culture*Temp*Time	3	24	23.98	<.0001

Culture*Temp*Time Least Squares Means

Culture	Temp	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
w	6	0.1	-416E-17	5.4250	24	-0.00	1.0000
w	6	8	3.92E-15	5.4250	24	0.00	1.0000
w	6	16	-186E-16	5.4250	24	-0.00	1.0000
w	6	23	-181E-15	5.4250	24	-0.00	1.0000
w	12	0.1	-979E-16	5.4250	24	-0.00	1.0000
w	12	8	40.2500	5.4250	24	7.42	<.0001
w	12	16	90.6667	5.4250	24	16.71	<.0001
w	12	23	100.00	5.4250	24	18.43	<.0001
wo	6	0.1	-61E-15	5.4250	24	-0.00	1.0000
wo	6	8	-387E-16	5.4250	24	-0.00	1.0000
wo	6	16	3.29E-14	5.4250	24	0.00	1.0000
wo	6	23	3.3E-13	5.4250	24	0.00	1.0000
wo	12	0.1	2.01E-13	5.4250	24	0.00	1.0000
wo	12	8	1.24E-13	5.4250	24	0.00	1.0000
wo	12	16	-669E-16	5.4250	24	-0.00	1.0000
wo	12	23	-737E-15	5.4250	24	-0.00	1.0000

T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate	
w	12	23	100.00	A
				A
w	12	16	90.6667	A
w	12	8	40.2500	B

T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate	
wo	6	23	3.3E-13	C
				C
wo	12	0.1	2.01E-13	C
				C
wo	12	8	1.24E-13	C
				C
wo	6	16	3.29E-14	C
				C
w	6	8	3.92E-15	C
				C
w	6	0.1	-416E-17	C
				C
w	6	16	-186E-16	C
				C
wo	6	8	-387E-16	C
				C
wo	6	0.1	-61E-15	C
				C
wo	12	16	-669E-16	C
				C
w	12	0.1	-979E-16	C
				C
w	6	23	-181E-15	C

T Grouping for Culture*Temp*Time Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Culture	Temp	Time	Estimate	
				C
wo	**12**	**23**	-737E-15	C

CURRICULUM VITAE

Fatih Ortakci
Dept. of Nutrition, Dietetics, and Food Sciences
Utah State University
Logan, UT, 84322-8700
Phone: (435) 713-5370
E-mail: fatih.ortakci@usu.edu

EDUCATION

Ph.D., **Food Science** April, 2015
Utah State University GPA 4.00

- Thesis: "Contribution of a Novel Obligatory Heterofermentative Nonstarter *Lactobacillus* Species to Late Gassy Defect in Cheddar Cheese"
- Advisor: Dr. Donald J. McMahon
- Co-advisor: Dr. Jeffery R. Broadbent

M.S., Food Engineering August, 2010
Ataturk University GPA 91.18%

- Thesis: "Improving the Health Benefits of Probiotic Bacteria by Microencapsulation"
- Advisor: Dr. Selahattin Sert

B.S., Food Engineering June, 2008
Ataturk University GPA 3.57

RESEARCH INTERESTS

Dairy Microbiology
Genetics of Lactic Acid Bacteria
Microencapsulation
Probiotics
Dairy Processing and Technology

PROFESSIONAL PRACTICE

R&D and Technical Services Intern August-October, 2014
Saputo Inc., Green Bay, WI, USA

- Hands on experience in industrial scale manufacturing processes of a variety of cheeses such as; Mozzarella, Ricotta, Parmesan, Blue cheese, Cheddar cheese, Feta cheese, Gorgonzola, Monterey Jack, Pepper Jack, Romano, and Asiago.
- Assisted to the Tech Services Coordinator on developing a new culture media for cheese starter bacteria

Quality Control Manager August-December, 2008
Dogan Organic Dairy Products Inc., Gumushane, TR

- Responsible for the assurance of the microbiological quality of organic milks in the milking station of the farm.
- No milk spoilage had occurred during the working period.

AWARDS and HONORS

- 'Graduate Student Researcher of the Year 2014' in the College of Agriculture and Applied Sciences at Utah State University.
- 'Graduate Student Researcher of the Year 2014' in the Department of Nutrition and Food Sciences at Utah State University.
- Nominated for the 'Utah State University Graduate Student Researcher of the Year 2015' award.
- 'Best Poster Presentation Award' in the Institute of Food Technologists Bonneville Section Poster Competition. Salt Lake City, Utah, USA. April, 2014.
- John Seymour Memorial Scholarship for 2014-2015 Academic Year by Utah State University, College of Agriculture and Applied Sciences (USU-CAAS).
- John Seymour Memorial Scholarship for 2013-2014 Academic Year by USU-CAAS.
- Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Graduate Scholarship for 2012-2013 Academic Year by USU-CAAS.
- Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Graduate Scholarship for 2011-2012 Academic Year by USU-CAAS.
- Scholarship from the Council of Turkish Higher Education (YOK) for conducting research in the USA. March-June, 2010.

EXTRACURRICULAR ACTIVITY

- Founder and President of Turkish Student Association at Utah State University 2012-2013
- Vice President of TSA at Utah State University 2013-2014

WORKSHOPS

- Online Microbial Genetics classes from University of California, Berkley 2014
- Bioinformatics Tutoring 2013
- Grant Writing Workshop 2012
- Quality Control Workshop (GMP) 2012
- HACCP Workshop 2012
- Employee "Train-the-Trainer" Food Safety Workshop 2012
- Statistical Process Control Workshop 2012
- Safe Quality Foods Workshop 2012

SERVICE

- Editorial Board Member of Journal of Advances in Dairy Research 2013-present
- Reviewer of Food Research International 2011-present
- Reviewer of Journal of Food Quality 2011-present
- Reviewer of Journal of Food Science and Engineering 2011-present
- Reviewer of Journal of Food Processing and Preservation 2011-present

COMPUTER SKILLS

SPSS	SAS	MS Word, Excel, PowerPoint
RAST	MEGA6	NCBI
DNA OligoAnalyzer	Primer3	

PUBLICATIONS AND MANUSCRIPTS IN SCI/SCI EXPANDED JOURNALS

1. **F. Ortakci**, J. R. Broadbent*, C. J. Oberg, D. J. McMahon*. 2014. Growth and gas production of a novel obligatory heterofermentative cheddar cheese nonstarter lactobacilli species on ribose and galactose (Accepted for publication in JDS on 02/08/2015.)
2. **F. Ortakci,** J. R. Broadbent, W. R. McManus and D. J. McMahon*. 2012. Survival of Microencapsulated Probiotic *Lactobacillus paracasei* LBC-1e during manufacture of Mozzarella cheese and simulated gastric digestion. J. Dairy Sci. 95 (11): 6274-6281 (**Sixteen citations**).
3. **F. Ortakci*** and S. Sert. 2012. Stability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in yogurt and in an artificial human gastric digestion system. J. Dairy Sci. 95 (12): 6918-6925 (**Seven citations)**.
4. F. Ertugay, M. Baslar*, and **F. Ortakci.** 2013**.** Effect of Pulsed Electric Field (PEF) treatment on polyphenol oxidase, total phenolic compounds and microbial growth of apple juice. Tur J. Agric and For. Vol. 37:772-780.

Manuscripts submitted or in preparation for submission

5. C. J. Oberg*, M. D. Culumber, T. S. Oberg, **F. Ortakci**, J. R. Broadbent, and D. J. McMahon. 2015. *Lactobacillus wasatchii* sp. nov. a novel species associated with late gas production in Cheddar cheese (Submitted to Int. J. Systematic and Evolutionary Microbiology on 02/03/2015).
6. **F. Ortakci**, J. R. Broadbent, C. J. Oberg, D. J. McMahon*. 2015. Late blowing of Cheddar cheese induced by accelerated ripening and ribose and galactose supplementation in presence of obligatory heterofermentative nonstarter lactobacilli species (Submitted to J. Dairy Science on 02/13/2015).
7. **F. Ortakci,** J. R. Broadbent*, C. J. Oberg, D. J. McMahon*. 2015. Growth and gas formation by a novel obligatory heterofermetative nonstarter lactobacilli species to late gas formation in Cheddar cheese manufactured using *S. thermophilus* starter (Submitted to J. Dairy Science on 03/11/2015).

8. D. J. McMahon, **F. Ortakci**, L. Montierth, M. Culumber, J. R. Broadbent, and C. J. Oberg. 2015. A survey of slow growing nonstarter lactic acid bacteria in Cheddar cheese (Manuscript in preparation).

PUBLICATION IN REFEREED JOURNALS

F. Ortakci, M. Gurses*, S. Sert. 2010. Microbiological Quality of Turkish Safranbolu Delight. Journal of Agricultural Faculty of Ataturk University, 41: (2), 145-147.

ORAL PRESENTATIONS IN INTERNATIONAL CONFERENCES

1. **F. Ortakci***, J. R. Broadbent, C. J. Oberg, D. J. McMahon. 2015. Late blowing of Cheddar cheese induced by accelerated ripening and ribose and galactose supplementation in presence of a novel obligatory heterofermentative nonstarter lactobacilli species. (ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, Orlando, FL, Accepted).
2. **F. Ortakci*,** J. R. Broadbent, C. J. Oberg, D. J. McMahon. 2015. Growth and gas formation by a novel obligatory heterofermetative nonstarter lactobacilli species in Cheddar cheese manufactured using *S. thermophilus* starter (ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, Orlando, FL, Accepted).
3. **F. Ortakci.** 2013**.** Gas Forming Bacteria in Cheese. Global Cheese Technology Forum. Reno, NV (**Invited Speaker**).
4. **F. Ortakci*,** J. R. Broadbent, W. R. McManus and D. J. McMahon. 2012. Survival of microencapsulated probiotic *Lactobacillus paracasei* LBC-1e during manufacture of mozzarella cheese and simulated gastric digestion. ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, July 15-19, Phoenix, Arizona.

POSTER PRESENTATIONS IN INTERNATIONAL CONFERENCES

1. J. R. Broadbent*, C. J. Oberg, M. D. Culumber, T. S. Oberg, **F. Ortakci**, and D. J. McMahon. Characterization of *Lactobacillus wasatchii* WDC04, a novel species associated with late gas production in cheddar cheese. 11th International Symposium on Lactic Acid Bacteria. August 31-September 4, 2014. Netherlands.
2. **F. Ortakci***, C. J. Oberg, J. R. Broadbent, D. J. McMahon. 2014. The salt, pH, and thermotolerance of a novel nonstarter lactic acid bacterium that might be associated with slit defect in ripened Cheddar cheese. ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, Kansas City, Missouri, July 20-24**.**
3. C. J. Oberg*, M. D. Culumber, T. S. Oberg, **F. Ortakci**, J. R. Broadbent, and D. J. McMahon. 2013. Genomic Analysis of *Lactobacillus* WDC04, A Novel Species Associated With Late Gas Production In Cheese**.** ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, July 8-12, Indianapolis, IN, USA.
4. **F. Ortakci***, C. J. Oberg, J. R. Broadbent, T. S Oberg, D. J. McMahon. 2013. Gas formation and growth characteristics of an oligotrophic *lactobacillus* species isolated from cheddar cheese. ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, July 8-12, 2013, Indianapolis, IN, USA.

5. **F. Ortakci***, S. Sert., 2012. Viability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in Yogurt and Artificial Human Gastric Digestion System**.** ADSA-AMPA-ASAS-CSAS-WSASAS Joint Annual Meeting, July 15-19, 2012, Phoenix, AZ, USA.
6. **F. Ortakci*,** and S. Sert, 2010. Main components used for microencapsulation of probiotics.1st International Congress on Food Technology. November 3-6. Belek/Antalya Turkey.
7. H. Yildiz, A. Kavaz, **F. Ortakci** and S. Sert, 2010. A Traditional Food Product: Tarhana. Traditional Foods from Adriatic to Caucasus. 15-17 April Tekirdag/Turkey.
8. **F. Ortakci***, B. Cetin, and S. Sert. 2009. The use of microencapsulated probiotic bacteria in several foods. 6th Food Engineering Congress, Antalya/Turkey.

OTHER CONFERENCES

- IDF Cheese Ripening & Technology Symposium. 21-24 May, 2012. Madison, WI, USA.
- 10th Food Congress. May 21-23, 2008. Erzurum/Turkey.