Late blowing of Cheddar cheese induced by accelerated ripening and ribose and galactose supplementation in presence of a novel obligatory heterofermentative nonstarter *Lactobacillus wasatchensis*¹

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ABSTRACT

Lactobacillus wasatchensis 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, respectively. Milk was inoculated with (1) *Lactococcus lactis* starter culture, or (2) *Lc. lactis* starter culture plus *Lb. wasatchensis* (10^4 cfu/mL). In the control cheese with no added *Lb. wasatchensis*, starter numbers decreased from 10^7 initially to $\sim 10^4$ cfu/g over 23 wk of ripening at 6°C. When the cheese was ripened at 12°C, or if *Lb. wasatchensis* was added, the final starter counts were 1 log lower. In contrast, nonstarter lactic acid bacteria in the cheese increased from $<10^2$ cfu/g at press to 10^6 to 10^7 cfu/g after 23 wk, with higher numbers being observed with ripening at 12°C. In cheese with no added *Lb. wasatchensis*, levels of *Lb. wasatchensis* were initially below the enumeration threshold but counts of up to 10^3 cfu/g were detected after 23 wk. When the cheese was inoculated with *Lb. wasatchensis*, it could be enumerated throughout ripening, with final levels at 23 wk being dependent on whether ribose had been added to the cheese curd. With added ribose (with or without added galactose), *Lb. wasatchensis* grew to 10^7 to 10^8 cfu/g after 23 wk, whereas without added ribose it was 1 log lower. In all cheeses with added *Lb. wasatchensis*, greater gas formation was observed at 12°C, with most gas production occurring after ~ 16 wk. Very little gas

production was detected in cheese without added *Lb. wasatchensis* ripened at 12°C or in cheese with added *Lb. wasatchensis* ripened at 6°C. Adding a combination of ribose and galactose caused more gas formation, putatively because of the ability of *Lb. wasatchensis* to co-utilize both sugars and grow to high numbers, and then produce gas from galactose as ribose levels were depleted. Even without sugar supplementation, gas was observed in cheese with added *Lb. wasatchensis* after 16 wk. We also observed that *Lb. wasatchensis* could grow to high cell densities when grown in carbohydrate-restricted broth containing lactococcal cell lysate. This suggests that during cheese ripening, lysis of starter bacteria provides sufficient substrates (such as ribose) to allow growth of *Lb. wasatchensis* and, if fermentable hexose is available, the cheese will become gassy. We conclude that *Lb. wasatchensis* is a previously undetected contributor to late gas formation in Cheddar cheese and the defect is more pronounced when elevated ripening temperatures are used.

Key words: nonstarter, lactic acid bacteria, gas, cheese, starter autolysis

INTRODUCTION

Ripening cheese 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 yr and this defect continues to be one of the main concerns during accelerated ripening (Van Slyke and Hart, 1903; Mullan, 2000). This defect is manifest as openness of cheese texture (slits or cracks) or “blown wrappers” without any 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 usually evident until the cheese

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¹In a previous paper (Ortakci et al., 2015), this bacteria was called *Lactobacillus wasatchii*. The accepted name is *Lactobacillus wasatchensis* (Oberg et al., 2015), in homage to the Wasatch mountain range running between Weber State University and Utah State University, where this bacteria was first isolated and characterized.

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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 a cheese converter to cut the blocks with uniformity and also affects the slicing ability of the cheese. If the defect is severe, the block will crumble upon cutting, which can increase cutting losses from 10% (nondefective) up to 50% (Elliott et al., 1981; Donnelly et al., 2014). In addition, the time and money spent on the aging process is lost, as such cheese is downgraded and sold at a lower price (Martley and Crow, 1996; Golnazarian, 2001).

Gassy defect in cheese has been associated with poor sanitation (usually observed as early gas formation) and germination of *Clostridium* spores (late blowing; Fox et al., 1990; McSweeney and Fox, 2004). In properly manufactured Cheddar cheese with a pH <5.4 and salt-in-moisture (S/M) content >4.0% (Lawrence et al., 2004), gassiness has been attributed to obligate heterofermentative (OHF) nonstarter lactobacilli species such as *Lactobacillus brevis* and *Lactobacillus fermentum* (Sherwood, 1939; Laleye et al., 1987, 1990). It has often been difficult to identify the cause of gassiness, however, and Elliott et al. (1981) linked gassy defect in Cheddar cheese to an unidentified and slow-growing bacterium.

Our group has isolated a novel slow-growing OHF species, *Lactobacillus wasatchensis*, from a “gassy” Cheddar cheese manufactured at Utah State University (Oberg et al., 2015). We reported that *Lb. wasatchensis* produces gas from hexose sugars and can co-utilize ribose and galactose (Ortakci et al., 2015). These 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 that *Lb. wasatchensis* is able to grow under salt and pH conditions typical of Cheddar cheese (4 to 5% S/M, ~pH 5.2), and that there is potential for some survival of *Lb. wasatchensis* during HTST pasteurization (Ortakci et al., 2015).

The ability of *Lb. wasatchensis* to readily utilize both ribose and galactose, to grow at cheese ripening temperatures, and to survive in the relatively harsh environment of cheese led us to hypothesize that *Lb. wasatchensis* is a contributor to late gas blowing and textural defects in Cheddar cheese (Ortakci et al., 2015). The aims of this work were to confirm that gas formation occurs in Cheddar cheese when *Lb. wasatchensis* is present, and to examine the effect of temperature (6 or 12°C) and substrate availability (ribose and galactose) on this phenomenon. Using these experimental parameters, we monitored the microbiota of the cheese for *Lb. wasatchensis*, starter lactococci, and NSLAB, as well as gas formation throughout 23 wk of ripening.

MATERIALS AND METHODS

Bacteria and Growth

Working cultures of *Lb. wasatchensis* WDC-04 (Oberg et al., 2015) 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% (wt/vol) ribose (R; donated by Bioenergy Life Science Inc., Ham Lake, MN), in which the cultures were incubated anaerobically using GasPak EZ (Becton Dickinson Inc.) at 23°C for 40 h. Cells for the cheese-making experiments were propagated in 400 mL of MRS+R for 40 h at 23°C. Cells were harvested by centrifugation at $6,000 \times g$ for 10 min at 4°C, washed twice with sterile 0.1% (wt/vol) peptone water, and then collected again by centrifugation. Concentration of cell suspensions was determined by anaerobic spread plate counts on 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* ssp. *lactis/cremoris* (DVS850, Chr. Hansen Inc., Milwaukee, WI) suspended in peptone water (optical density at 600 nm, OD₆₀₀, of ~0.9) was inoculated into 750 mL of M17 broth (Becton Dickinson Inc.) supplemented with 1% lactose (M17-L; Sigma-Aldrich Inc., St. Louis, MO) and incubated at 30°C for 24 h under aerobic conditions. The cells were then harvested by centrifugation ($7,500 \times g$; 10 min; 4°C), washed twice in 50 mL of sterile phosphate buffer and then suspended in 10 mL of sterile phosphate buffer. A 0.1-mL aliquot was diluted and plated on M17-L agar to enumerate starter numbers (Brannen 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 and pasteurized at 73°C for 15 s; then, 273 kg was transferred into each of 2 open stainless steel vats (which had previously been cleaned and heat sanitized for 30 min). Both batches of milk were warmed to 31°C, and then 0.2 g/kg of frozen pellets containing *Lc. lactis* ssp. *lactis/cremoris* starter culture DVS850 were added. To one of the vats, 10^4 cfu/mL of *Lb.*

wasatchensis was also added, and the milk was 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 single-strength annatto (DSM Food Specialties USA Inc., Eagleville, PA) and 0.16 mL/kg of double-strength (~ 650 international milk clotting units/mL) chymosin rennet (Maxiren; DSM Food Specialties USA Inc.) were added, and the milk was allowed to set undisturbed for 30 min. After cutting and heating, 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 and 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, whereas to the other portions was added (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 and then placed into plastic hoops and pressed overnight (140 kPa, ~ 18 h, $\sim 20^\circ\text{C}$). The cheese was recovered from the hoops and each block cut into 10 pieces of ~ 600 g each, and the pieces were individually vacuum packaged. Five pieces were stored at 6°C and 5 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, and 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. wasatchensis* does not grow quickly enough to be enumerated at 48 h. Like many NSLAB, *Lb. wasatchensis* is resistant to vancomycin but does not form colonies on MRS-V at 37°C within 48 h (data not shown).

The mean log numbers from the 3 replicates were plotted against storage time and a trend line fitted to

each set of data based on the highest R^2 obtained. The time (week) when the NSLAB37 trend line equaled and then surpassed the starter trend line was considered the crossover time (Oberg et al., 2011). All plate counts were performed in duplicate using the spread plate method.

Enumeration of *Lb. wasatchensis*. 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 of incubation at 23°C on MRS-R-V medium and marking all the visible colonies (~ 1.5 mm diameter), the plates were incubated anaerobically at 23°C for an additional 72 h. By this time, *Lb. wasatchensis* forms noticeable colonies (~ 1 mm diameter), which enables differential enumeration of this organism from other NSLAB23 colonies provided the number of *Lb. wasatchensis* colonies is within ~ 1.5 log of the NSLAB23 counts.

Cell-Free Extract and Growth

The cell suspensions containing $\sim 10^{10}$ cfu/mL of starter lactococci were divided into 0.5-mL aliquots and placed into 2-mL screw-cap tubes that contained 0.5 g of sterile 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 each, with 2-min intermittent cooling periods in an ice bath between bursts. After centrifugation (4°C, 15 min, $21,130 \times g$), the supernatant was collected and protein concentration determined with a modified Lowry Protein assay kit (Pierce/ThermoScientific, Rockford, IL) using 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 5-fold-concentrated carbohydrate-free MRS (Anonymous, 2015). This lysate solution was inoculated with 20 μL of *Lb. wasatchensis* (previously sub-cultured twice in MRS+R) and incubated at 23°C for 10 d under anaerobic conditions. Growth of *Lb. wasatchensis* was monitored by OD_{600} measurements taken at 0 and 10 d. A control was prepared by 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 vacuum-packaged 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 were packaged for each treatment with 5 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 d-0 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 manually pulled away from the cheese block (on the same side that was initially marked) as much as possible. A new 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 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 with 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 before opening the packs and sampling the cheese for microbial analysis. For each treatment, 4 packs were tested at 8 wk, 3 at 16 wk, and 2 at 23 wk of storage.

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) at 100% power with an endpoint setting of <0.4 mg of weight change over 2 s. 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 (Whatman International Ltd., Maidstone, UK), and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Corning, Medfield, MA). The 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 HPLC for lactose, galactose, lactic acid, acetic acid, citric acid, propionic acid, pyruvic acid, formic acid, and orotic acid at d 0 and after 16 wk of ripening as described by Phadungath (2011). For the sample preparation, about 5 g of cheese was manually homogenized for 90 s with 10 mL of 0.013 *N* sulfuric acid (Fisher Scientific, Fair Lawn, NJ) at 65°C using a high shear Omni mixer-homogenizer (model 17105, Omni International, Waterbury, CT). The extract was centrifuged (Jouan CR4-12 Centrifuges, Jouan Inc., Winchester, VA) at $7,000 \times g$ for 10 min at 4°C. 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 Whatman #4 filter paper. 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 Microcentrifuge) at $14,000 \times g$ 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 Karat Software, Beckman Coulter Inc.). The system was externally equipped with an intelligent refractive index detector (model RI-2031, Jasco Inc., Easton, MD), and a column heater (model 6301, Alltech Associates Inc., Deerfield, IL). The column used for separation of the analytes was a Rezex ROA-organic acid H^+ column (300 $\times$ 7 mm, 8 μ m, Phenomenex, Torrance, CA) held at 65°C, with a cation H^+ microguard cartridge (Bio-Rad Laboratories, Hercules, CA). The analysis was performed isocratically at a 0.6 mL/min flow rate using 0.013 *N* sulfuric acid as the mobile phase. Quantification of analytes was based on the external standard method described by Upreti et al. (2006b).

Experimental Design

The experiment was conducted as a randomized block with addition of *Lb. wasatchensis* as a whole plot factor, sugar addition as split plot factor, temperature as split-split plot factor, and ripening time as split-split-split plot factor. Statistical analysis of the effect of added sugar, storage time, temperature, and *Lb. wasatchensis* addition was performed using PROC GLIMMIX in SAS (version 9.1; SAS Institute Inc., Cary, NC) with Type III test of fixed effects. Differences between means were

Table 1. The effect of adding ribose or galactose, ripening temperature (6, 12°C), and time (0, 16 wk), and inoculated *Lactobacillus wasatchensis* (LBW) on the sugar and organic acid contents of Cheddar cheese

Source of variation	Sugar and organic acids									
	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 × sugar	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Temperature	NS	NS	**	NS	NS	**	NS	NS	NS	NS
LBW × temperature	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
Sugar × temperature	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Time	**	*	**	**	NS	**	**	**	**	**
LBW × time	NS	NS	*	NS	**	NS	*	NS	NS	NS
Sugar × time	**	NS	NS	NS	NS	NS	*	NS	NS	NS
Temperature × time	NS	NS	*	*	NS	**	NS	NS	NS	NS

* $P < 0.05$; ** $P < 0.01$; NS = $P > 0.05$.

determined using Tukey least squares means. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Initial Cheese Composition

Addition of *Lb. wasatchensis* to cheese milk or sugar supplementation of curd did not significantly ($P > 0.05$) affect the moisture, salt, S/M, pH, fat, lactose, or citrate levels of cheese (Table 1). Thus, those values were pooled across treatments. The pooled means of moisture and fat were $37.0 \pm 0.16\%$ and $32 \pm 0.1\%$ respectively, which are typical for Cheddar cheese. The pooled mean salt content was $2.14 \pm 0.03\%$ with S/M of $5.47 \pm 0.07\%$, which is higher compared with mean S/M levels in retail Cheddar cheese (4.3%) in the United States (Agarwal et al., 2011). However, S/M was in the range considered appropriate for good-quality aged cheese (4 to 6% S/M) as described by Lawrence et al. (1993). The pH (5.38 ± 0.03) was at the high end of expected range (5.0 to 5.4; Lawrence et al., 2004), but this was not unexpected as higher pH (i.e., >5.35) in Cheddar cheese typically occurs when salt concentrations are $>2.0\%$ or with S/M $\geq 5.7\%$ (Turner and Thomas, 1980; Thomas and Pearce, 1982; Lane et al., 1997). The higher retention of salt in our experiment may have resulted from 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). Mean initial lactose content was $0.42 \pm 0.024\%$ and citrate content was $0.13 \pm 0.01\%$ (Table 2). Initial lactic acid content was $\sim 1\%$, 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 correspond to cheese having high S/M levels and higher pH.

Initial galactose content of cheese was significantly different based upon sugar supplementation to the curd (Table 1). Ripening time and the interaction of time and sugar addition were also significant. In contrast, lactose content was only significantly influenced by ripening time (Table 1). It is usually assumed that lactose is consumed during ripening, but having relatively high S/M ($\sim 6\%$) can reduce the rate of lactose utilization by lactococcal starter cultures in cheese to almost zero (Turner and Thomas, 1980). Persistence of lactose in Cheddar cheese during storage was also shown by Fox et al. (1998), who reported the continued presence of lactose after 36 wk of ripening. Lactose content after 23 wk of ripening remained at $0.36 \pm 0.02\%$.

Galactose levels in cheese were not significantly influenced by addition of *Lb. wasatchensis* or by ripening temperature, and pooled mean values are presented in Table 3. Initial galactose content of the control cheese was 0.023%, with no significant change occurring during 16 wk of ripening. Adding ribose to the cheese curd caused no significant difference in initial galactose levels compared with the control cheese, but after 16 wk of storage, only 0.003% galactose remained ($P < 0.05$). This implies that a faster rate of galactose utilization occurs when more ribose than normal is present in cheese and agrees with our previous findings (Ortakci et al., 2015) that *Lb. wasatchensis* appears to concomitantly co-utilize ribose and galactose to maximize energy production and cell growth. Initial galactose levels for cheeses in which 0.5 and 0.25% galactose was added to the cheese curd were 0.238 and 0.125%, respectively. This suggests that half of the galactose added to the curd after salting was lost in the expelled whey during pressing. During ripening, the level of galactose in these cheeses decreased to levels similar to those of the control cheese

Table 2. Pooled means (%) for sugar and organic acids for Cheddar cheese at 16 wk of ripening

Item	Lactose	Lactic		Acetic		Propionic		Citric	Pyruvic	Formic	Uric	Orotic
		6°C	12°C	6°C	12°C	6°C	12°C					
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,b}Means with the same superscript letters were not significantly different from one another, $\alpha = 0.05$.

Starter Lactococci and NSLAB During Ripening

Starter lactococci numbers in cheese were influenced by ripening temperature, ripening time, and whether or not the milk had been inoculated with *Lb. wasatchensis* (Table 4). We detected no direct effect of adding *Lb. wasatchensis* on lactococci numbers but there was a significant interaction with time and temperature as the main effect was not significant. Adding ribose or galactose (separately or together) did not significantly affect lactococcal numbers and, therefore, pooled means across all sugar treatments were compared. Lactococcal numbers decreased when high numbers of *Lb. wasatchensis* were initially present and the rate and extent of decrease was dependent on ripening temperature (Figure 1).

The NSLAB numbers (meaning the fast-growing lactobacilli enumerated at 23°C and 37°C, not including *Lb. wasatchensis*) were only influenced by ripening time and temperature (Table 4). Adding ribose or galactose had no significant effect on NSLAB numbers, nor were there any significant interactions between sugar addition and ripening temperature or time. Adding *Lb. wasatchensis* was only significant as an interaction with ripening time and as a 3-way interaction with time and temperature. Therefore, NSLAB numbers were pooled over sugar and *Lb. wasatchensis* addition, and compared over ripening temperature and time as well as whether they were enumerated at 37°C on MRS-V agar or at 23°C on MRS-R-V agar (Figure 2). The influence of ripening temperature and time on both lactococci and NSLAB has been well demonstrated (Peterson and Marshall, 1990; Wilkinson et al., 1994; Fox et al., 1998; Oberg et al., 2011; McMahon et al., 2014).

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 was achieved around 4 and 8 wk of ripening at 12 and 6°C, respectively (Figure 1). This is sooner than previously reported NSLAB crossover times ≥ 4 mo of ripening of Cheddar cheese made with the same starter culture (McMahon et al., 2014). This could be due to the differences in S/M contents, as McMahon et al. (2014) reported levels 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 lactic acid bacteria, are sensitive to the salt concentration in cheese. Upreti et al. (2006a) reported that a S/M concentration in cheese above 4.5% results

Table 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)

Item	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, $n = 12$, $\alpha = 0.05$.

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), resulting in longer crossover times. Having higher S/M levels in cheese impairs the fermentation of lactose to lactic acid by starter bacteria so there is less decrease in pH after pressing (Olson and Johnson, 1990), and an elevated 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 bacterial 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, it is not surprising that with the higher S/M content of our cheeses there was a shorter crossover time, with NSLAB numbers being higher than lactococci by ≤ 2 mo of ripening (Figure 1).

The total reductions in the numbers of starter lactococci during ripening at 12 or 6°C over 23 wk were similar, approximately 4.5 and 4 log, respectively. As stated above, we detected a significant interaction between

addition of *Lb. wasatchensis* and ripening time as well as a significant *Lb. wasatchensis* $\times$ time $\times$ temperature interaction ($P < 0.05$; Table 4). After 8 wk of ripening at 12°C, starter lactococci numbers were significantly lower in cheese containing added *Lb. wasatchensis* compared with the control cheese (i.e., cheese milk not inoculated with *Lb. wasatchensis*, $P < 0.05$; Figure 1). At 6°C, however, starter lactococci counts were the same ($P > 0.05$) in both cheeses although the trend lines also showed a slightly faster decrease in cheese with added *Lb. wasatchensis*.

As ripening progressed, starter lactococci numbers were similar in control and *Lb. wasatchensis*-treated cheese at 12°C, whereas at 6°C they were significantly higher in the control cheese (Figure 1). Having a greater reduction in lactococci numbers during ripening when *Lb. wasatchensis* had been added to the milk, corresponds with slower acid development and longer cheese-making times in these cheeses, even though the same amount of starter was used in all cheeses. We do not know the basis for this faster decrease in starter lactococci counts when high numbers of *Lb. wasatchensis* were present, as no genes encoding antimicrobial compounds have been found in the genome of *Lb. wasatchensis* (F. Ortakci and J. Broadbent, unpublished data).

Along with the faster decrease in lactococcal counts, NSLAB counts (both NSLAB23 and NSLAB37) in-

Table 4. The effect of adding ribose or galactose, ripening temperature (6 or 12°C), and time (0, 8, 16, 23 wk) on relative gas formation and on the numbers of starter lactococci, nonstarter lactic acid bacteria enumerated at 23°C (NSLAB23) or 37°C (NSLAB37), and added *Lactobacillus wasatchensis* (LBW) in Cheddar cheese ripening

Source of variation	Starter	NSLAB23	NSLAB37	LBW	Relative gas
LBW	NS	NS	NS	— ²	*
Sugar ¹	NS	NS	NS	**	*
LBW $\times$ sugar	NS	NS	NS	—	***
Temperature	**	**	**	**	**
LBW $\times$ temperature	NS	NS	NS	—	**
Sugar $\times$ temperature	NS	NS	NS	NS	NS
Time	**	**	**	**	**
LBW $\times$ time	*	NS	NS	—	**
Sugar $\times$ time	NS	NS	NS	**	NS
Temperature $\times$ time	**	**	**	**	**
LBW $\times$ temperature $\times$ time	*	NS	NS	NS	*

¹Cheese curd with no sugar, 0.5% ribose, 0.5% galactose, or 0.25% ribose plus 0.25% galactose.

²Cheese made without added *Lb. wasatchensis* was not included in the statistical analysis.

* $P < 0.05$; ** $P < 0.01$; *** $0.1 > P > 0.05$; NS = $P > 0.05$.

creased more rapidly ($P < 0.05$) and reached higher levels ($P < 0.05$) in cheese ripened at 12°C (Figure 1). There were no significant direct or interactive effects of sugar supplementation or *Lb. wasatchensis* addition on the numbers of NSLAB23 or NSLAB37 in cheese ($P > 0.05$). Initially, NSLAB numbers were $<10^2$ cfu/g and increased to $\geq 10^7$ and 10^5 to 10^6 cfu/g during 16 wk of 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 ($P > 0.05$), with significantly lower counts at 6°C ($P < 0.05$).

The increase in NSLAB counts in cheeses at higher ripening temperature (12°C) is in agreement with previous observations for ripening of full-fat Cheddar cheese (Cromie et al., 1987; Jordan and Cogan, 1993; Lane et al., 1997; Fenelon et al., 1999). It was interest-

Figure 1. Changes in microbiota during storage of Cheddar cheese at (A) 12°C and (B) 6°C showing decrease in starter lactococci without (○) and with (●) addition of *Lactobacillus wasatchensis* (pooled means over all sugar treatments) and increase in nonstarter lactic acid bacteria enumerated at 23°C (□, dashed line) and 37°C (■, solid line) (pooled over all treatments) with associated best-fit trend lines. Bars = SE ($n = 12$ for starter lactococci and $n = 24$ for nonstarter lactic acid bacteria).

Figure 2. Increase in *Lactobacillus wasatchensis* during storage of Cheddar cheese at (A) 12°C and (B) 6°C, control (no added sugars) cheese (○, solid line), and cheeses with 0.5% ribose added (□, solid line), 0.5% galactose added (●, dashed line), and 0.25% ribose and 0.25% galactose added (■, dashed line) with associated best-fit trend lines. Bars = SE ($n = 3$).

ing that there were significant differences ($P < 0.05$) in NSLAB numbers when enumerated on MRS-R-V at 23°C (i.e., NSLAB23) compared with being enumerated on MRS-V at 37°C (i.e., NSLAB37; Figure 1). Whether this is because there are other NSLAB that do not grow rapidly at 37°C or that require ribose for rapid growth is unclear. It does suggest that the NSLAB population in Cheddar cheese is not as uniform as previously thought and that the common use of MRS (or MRS-V) incubated at 37°C for enumerating NSLAB bacteria in cheese should be revisited.

As in previous cheese-making experiments (McMahon et al., 2014), we observed some vat-to-vat variations in NSLAB levels, with most NSLAB counts reaching 10^4 to 10^6 cfu/g within 8 wk, whereas in other replicates, the NSLAB counts remained low ($\leq 10^2$ cfu/g) for up to 16 wk. However, NSLAB numbers in all replicates reached 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). This can arise from differences in cleaning and sanitation of cheese vats as well as differences in the cheese-making history of the vats (i.e., what cheeses had been made and what cultures had previously been used in the vats). It may also be a reflection of there being multiple strains of indigenous NSLAB in the cheese microbiota during ripening (Beu-vier and Duboz, 2014).

Organic Acids

Pooled means for organic acids after 16 wk ripening showed that concentrations of lactic, acetic, propionic, pyruvic, formic, and orotic acids increased in all cheeses, whereas citric acid decreased ($P < 0.05$; Table 2). No significant differences could be attributed to adding ribose or galactose into cheese curd, and ripening temperature was the only main level significant factor influencing levels of lactic, acetic, and propionic acids (Table 1).

There was only a small increase in lactic acid during ripening, reaching 1.13% after 16 wk, whereas propionic acid levels significantly increased during 16 wk from an initial 0.08 to 0.21% and 0.42% at 6 or 12°C, respectively (Table 2). The increase at 6°C storage was similar to 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 at 12°C compared with 6°C were concomitant with higher numbers of NSLAB in cheese ripened at 12°C (see Figure 1). Such NSLAB activity has been reported to increase propionic acid levels in cheese during storage (St-Gelais et al., 1991; Bouzas et al., 1993). *Lactobacillus curvatus* has been identified as the dominant NSLAB in cheese made at Utah State University (Broadbent et al., 2013) and genomic data suggest it has the metabolic capability to produce propionic acid (J. Broadbent, unpublished data).

Acetic acid levels were significantly affected by time and by the temperature $\times$ time interaction with higher levels obtained at the elevated ripening temperature (Table 2). No significant increase was observed at 6°C ($P > 0.05$), whereas a 7-fold increase at 12°C occurred 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, whereas Upreti et al. (2006b) 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. Surprisingly, adding ribose to cheese curd did not have a significant effect on acetic acid levels in any of the cheeses even though

fermentation of pentoses by Group II and Group III LAB yields equimolar amounts of lactic acid and acetic acid, and a net 2 moles of ATP per mole of ribose consumed (Axelsson, 2004). Observing no differences in acetate levels in control versus ribose-supplemented cheeses could be due to the lysis of starter lactococci, which also releases ribose into the cheese microenvironment (Thomas, 1987).

Citric acid levels were significantly affected by time, *Lb. wasatchensis* $\times$ time, and sugar $\times$ time interactions (Table 2). Although statistically significant, there was only a minor decrease in citric acid during storage from initial 0.132 to 0.08% after 16 wk. Although citric acid levels were significantly affected by *Lb. wasatchensis* over time, no citrate permease or citrate lyase encoding gene(s) were found in the genome (J. Broadbent and F. Ortakci, unpublished data). Formic, uric, and orotic acids also significantly increased during ripening ($P < 0.05$).

Cheese with Added *Lb. wasatchensis*

As stated above, adding *Lb. wasatchensis* before cheesemaking had a significant effect on starter lactococci levels. However, NSLAB counts were not significantly affected by adding *Lb. wasatchensis* (Table 4). *Lactobacillus wasatchensis* numbers increased by 3 logs during ripening, and the extent of growth was influenced by both ripening temperature and sugar supplementation. Faster growth occurred at 12°C (Figure 2), which was expected because its optimum growth rate occurs at $\sim 23^\circ\text{C}$ (Ortakci et al., 2015). Most growth of *Lb. wasatchensis* occurred during the first 8 wk of ripening with higher overall 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 of ripening in cheese containing ribose or ribose plus galactose were the same ($P > 0.05$). Adding galactose on its own to the cheese curd did not ($P > 0.05$) increase growth of *Lb. wasatchensis* compared with cheese with no added sugars (Figure 2). Similar growth on cheese with ribose and ribose plus galactose is in accordance with previous observations that *Lb. wasatchensis* can co-utilize galactose when ribose is present (Ortakci et al., 2015) to maximize its growth in cheese.

In the control cheese with no added *Lb. wasatchensis*, there were still *Lb. wasatchensis* in the cheese, which in some replicates reached levels of 10^5 to 10^7 cfu/g after 16 to 23 wk, especially when the cheese was stored at 12°C. *Lactobacillus wasatchensis* is indigenous to the facility in which the cheese was manufactured but we were not able to enumerate it before 16 wk—the threshold for detection of *Lb. wasatchensis* requires its numbers to be within 1.5 log of the faster-growing

NSLAB that initially appear on the MRS-R-V agar. At population levels below this threshold, the plates contain too many fast-growing colonies to distinguish and count any slower-growing colonies that may appear after the initial 2-d incubation. We speculate that because *Lb. wasatchensis* grows slower than the predominant NSLAB in cheese, under normal cheese ripening conditions, it usually takes 4 to 6 mo to achieve similar levels and be enumerated.

In cheeses made from milk with added *Lb. wasatchensis*, we were able to count *Lb. wasatchensis* throughout the 23 wk of ripening because the initial level of *Lb. wasatchensis* was $\sim 10^5$ cfu/g and, therefore, above the threshold compared with other NSLAB. Even though slower growing compared with *Lb. curvatus*, *Lb. wasatchensis* grew to $>10^6$ and 10^7 cfu/g after 8 wk at 6 and 12°C, respectively.

Relative Gas

All treatments (sugar treatment, addition of *Lb. wasatchensis*, storage temperature, and time) significantly affected relative gas formation in cheese (Table 4). The interaction terms involving time, temperature, and *Lb. wasatchensis* addition were also significant. Gas formation was significantly higher ($P < 0.05$) in cheese containing added *Lb. wasatchensis* compared with control cheese at both ripening temperatures (Figure 3), whereas no gas formation was observed by 23 wk at 6°C in the control cheese. Similarly, Laleye et al. (1990) found that adding OHF species such as *Lb. brevis* or *Lb. fermentum* during cheese making resulted in open texture in cheese after 2 mo of ripening, whereas no openness was observed in a control cheese. It has also been recognized that gas formation can occur in cheese without any openness being observed because CO₂ does have some solubility in the aqueous phase of cheese (Laleye et al., 1987).

Interactions involving sugar addition were not significant except there was a tendency ($P = 0.073$) for the sugar $\times$ *Lb. wasatchensis* interaction to influence gas production (Table 4). When pooled over time and temperature, relative gas production based on sugar and *Lb. wasatchensis* addition was significantly higher when 0.25% ribose plus 0.25% galactose was added compared with adding either galactose or ribose alone, or the no-sugar-added control. The highest gas formation occurred in cheese containing added *Lb. wasatchensis* as well as ribose and galactose ($P < 0.05$; Figure 3). In these cheeses, the presence of ribose supported growth of *Lb. wasatchensis* to high numbers and presence of galactose enabled OHF fermentation with CO₂ release. When only ribose was added to the cheese, there was more growth of the *Lb. wasatchensis* but less gas forma-

tion ($P < 0.05$) because OHF bacteria do not produce CO₂ from pentoses (Axelsson, 2004).

Gas formation during storage was significantly higher at 12°C 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. wasatchensis*, with levels being about 0.5 log higher at 12°C. This increased growth at the elevated storage temperature could account for the higher measurements of relative gas formation, but solubility of CO₂ may also be a factor. At 6°C, there is higher solubility of CO₂ compared with 12°C (CRC Handbook of Chemistry and Physics, 2009). Thus, even with the same level of gas production, there would be more CO₂ present in the gaseous state at 12°C (evident as looser packs) and more probability of openness as well, occurring as slit and crack formation in the cheese. This was confirmed when a *Lb. wasatchensis*-treated cheese with no apparent gas production (i.e., a tight pack) was moved from 6°C to room temperature ($\sim 22^\circ\text{C}$) and after a while the package became loose (data not shown).

The possibility that slow-growing bacteria may contribute to late blowing in Cheddar cheese has been previously hypothesized (Elliott et al., 1981). In that study, the bacterium was not identified (except for being a slow-growing, salt-tolerant, non-spore-forming, heat-tolerant, gram-positive rod), but its addition to cheese milk at 10^1 and 10^4 cfu/mL resulted in gas formation during storage at 10°C but not at 4.5°C. With *Lb. wasatchensis*, most of the growth occurs during the first 8 wk of ripening, whereas observations of gas production in cheese usually occur after this time. This suggests that growth is being supported by pentoses available in the cheese and production of CO₂ occurs

Figure 3. Pooled means of relative gas formation in *Lactobacillus wasatchensis* inoculated cheese (w/LBW) or un-inoculated control cheese (w/o/LBW) during 23 wk storage at 6°C or 12°C. Bars with the same letter (a to f) were not significantly different ($\alpha = 0.05$); error bars = SE ($n = 12$).

after the pentoses are consumed but while there are fermentable hexoses still present. There also needs to be sufficient CO₂ produced to exceed its solubility in cheese and then loosening of the pack may be observed.

Fermentation of citric acid can also result in CO₂ production in cheese by NSLAB (Overcast and Albrecht, 1952) although in our study there was only a relatively low level, with 0.13% present after pressing, decreasing to 0.08% during storage, with no differences occurring because of sugar addition or ripening temperature. Hoglund et al. (1972) also reported that substrates other than citrate could cause CO₂-related openness in cheese.

Growth of *Lb. wasatchensis* on Starter Cell Lysate

Production of a cell lysate using bead beating was successful in producing a supernatant containing ~4 mg/mL protein with a reduction in starter lactococci from ~10¹⁰ to <5 × 10⁴ cfu/mL. After centrifuging to produce a cell-free lysate and adding it to carbohydrate-free MRS, the growth of *Lb. wasatchensis* reached high cell densities (with an OD₆₀₀ up to 2.49) after 10 d incubation (initial OD₆₀₀ ~0.16). In contrast, very little growth occurred (only 0.06 increase in OD₆₀₀) in the control when no lysate was added (Figure 4).

Although growth of *Lb. wasatchensis* in cell lysate was slower compared with that on MRS plus 1% ribose (Ortakci et al., 2015), final cell densities reached were considerably higher in the lysate. This likely explains the 2-log increase in *Lb. wasatchensis* counts in cheese without any added sugars during the time when starter counts decreased from 10⁷ to ~10⁴ cfu/g (see Figures 1 and 2). Along with *Lb. wasatchensis*, the other faster-growing NSLAB increased concomitantly with this decrease in starter lactococci counts (see Figure 2) and probably utilized the same sugars (such as ribose and *N*-acetyl amino sugars) being released during autolysis of starter cells (Thomas, 1987; Fox et al., 1998). Of these released sugars, ribose is considered the most readily released from starter lactococci (Thomas, 1987; Rapposch et al., 1999). *Lactobacillus brevis*, another OHF species, has been shown to reach higher cell densities on lactococcal cell lysate than many other NSLAB (Thomas, 1987). Similarly, *Lb. paracasei* has been observed to grow to 10⁷ cfu/mL from an initial level of 10² cfu/mL in lysate of *Lactobacillus helveticus* (Rapposch et al., 1999). Another source of nutrients to support growth of NSLAB in cheese is that associated with the milk fat globule membrane material (Moe et al., 2013). Thus, there is clearly an adequate supply of carbohydrate material from autolysis-released products of starter cells or the components themselves to

Figure 4. Growth of *Lactobacillus wasatchensis* as measured by optical density at 600 nm (OD₆₀₀) in carbohydrate-free de Man, Rogosa, and Sharpe (MRS) broth containing starter cell lysate (■) or sterile water as control (□) during anaerobic incubation at 23°C at 0 and 10 d. Bars = SE (n = 3).

support growth of *Lb. wasatchensis* and other NSLAB during cheese ripening.

CONCLUSIONS

This study explored the growth in cheese of a novel, slow-growing OHF NSLAB, *Lb. wasatchensis* sp. nov., on late gas formation. This NSLAB uses galactose very slowly in the absence of ribose, and fermentation of galactose (or other fermentable hexose) as an energy source causes CO₂ production. Adding ribose to cheese curd stimulated the growth of *Lb. wasatchensis* during cheese ripening over 23 wk with most of the growth occurring during the first 8 wk when there is a large decrease in starter lactococci numbers. *Lactobacillus wasatchensis* grows well using cell lysate as its carbon source, presumably because of release of ribose and other sugars. The likelihood of gas formation in cheese increases when a temperature such as 12°C is used to accelerated ripening. Having a combination of ribose and galactose in the cheese also increased gas production. Co-utilization of galactose with ribose by *Lb. wasatchensis* appears to increase the occurrence and extent of late gas formation in cheese. Initially, ribose is consumed to promote growth with co-utilization of galactose, and then when ribose is depleted, the remaining galactose is used to produce energy, with CO₂ being generated. Even without ribose supplementation, there was a 2-log growth in *Lb. wasatchensis* indicating that starter cell autolysis provides adequate pentoses to support growth. Enumerating *Lb. wasatchensis* in cheese is still problematic as it needs to be present at numbers within 1.5 log of the faster growing NSLAB to be detectable on MRS-R-V agar.

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