Growth and gas formation by *Lactobacillus wasatchensis,* a novel obligatory heterofermentative nonstarter lactic acid bacterium, in Cheddar-style cheese made using a *Streptococcus thermophilus* starter

Fatih Ortakci*, Jeffery R. Broadbent*[1], Craig J. Oberg†*, and Donald J. McMahon*[1]

*Western Dairy Center, Department of Nutrition, Dietetics, and Food Sciences, Utah State University, Logan, UT 84322-8700, †Microbiology Department, Weber State University, Ogden, UT 84408-2506

[1]Corresponding authors:

Donald J. McMahon

435-797 3644

Donald.McMahon@usu.edu

and

Jeffery R. Broadbent

Jeff.Broadbent@usu.edu

435-797-2113

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***Interpretive Summary*. Growth and gas formation by *Lactobacillus wasatchensis,* a novel obligatory heterofermentative nonstarter lactic acid bacterium in Cheddar-style cheese made using a *Streptococcus thermophilus* starter.** *Ortakci*.

Growth and gas formation in Cheddar-style cheese by *Lactobacillus wasatchensis* was studied when using a *Streptococcus thermophilus* starter. Since *St. thermophilus* does not utilize galactose during fermentation of lactose, galactose accumulates and serves as a carbohydrate source for nonstarter lactic acid bacteria that grow in cheese. *Lactobacillus wasatchensis* created large amounts of gas in cheese ripened at elevated temperature. No gas formation was observed cheeses stored at 6°C or control cheese stored at 12°C. Thus, *Lb. wasatchensis* contributes to gas formation in Cheddar-style cheese especially when *St. thermophilus* is included in the starter and elevated temperatures are used for storage.

ABSTRACT

A novel slow-growing, obligatory heterofermentative, nonstarter lactic acid bacterium (NSLAB) *Lactobacillus wasatchensis* WDC04 was studied for growth and gas production in Cheddar-style cheese made using *Streptococcus thermophilus* as the starter culture. Cheesemaking trials were conducted using *St. thermophilus* alone or in combination with *Lb. wasatchensis* deliberately added to cheese milk at a level of ~10^4 cfu/ml. Resulting cheeses were ripened at 6 or 12°C. At d 1, starter streptococcal numbers were similar in both cheeses (~10^9 cfu/g) and fast-growing NSLAB lactobacilli counts were below detectable levels (<10^2 cfu/g). As expected, *Lactobacillus wasatchensis* counts were 3 x 10^5 cfu/g in cheeses inoculated with this bacterium and below enumeration limits in the control cheese. Starter streptococci decreased over time at both storage temperatures but declined more rapidly at 12°C, especially in cheese also containing *Lb. wasatchensis*. Populations of fast-growing NSLAB and the slow-growing *Lb. wasatchensis* reached 5 x 10^7 and 2 x 10^8 cfu/g, respectively, after 16 wk of storage at 12°C. Growth of NSLAB coincided with a reduction in galactose concentration in the cheese from 0.6% to 0.1%. Levels of galactose at 6°C had similar decrease. Gas formation and textural defects were only observed in cheese with added *Lb. wasatchensis* ripened at 12°C. Use of *St. thermophilus* as starter culture resulted in galactose accumulation that *Lb. wasatchensis* can utilize to produce CO_2, which contributes to late gas blowing in Cheddar-style cheeses, especially when the cheese is ripened at elevated temperature.

KEY WORDS: Cheese, late blowing, nonstarter lactic acid bacteria, *Streptococcus thermophilus*, galactose.

INTRODUCTION

The manufacture of Cheddar cheese is characterized by use of mesophilic *Lactococcus lactis* starter strains and moderate cook temperatures (~39°C) (Michel and Martley, 2001). However, the "short-method" for Cheddar cheese manufacture (Bley et al., 1985) uses thermophilic *Streptococcus thermophilus* along with the regular mesophilic *Lc. lactis* subsp. *lactis/cremoris* starter culture so that rapid acid production continues even at cook temperatures of 42 to 43°C (Michel and Martley, 2001). Two advantages to using the "short-method" include reduced manufacturing costs and a 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/kg galactose in cheese (~0.6% (wt/wt)) and to unwanted CO_2 production by nonstarter lactic acid bacteria (**NSLAB**). This can lead to development of slits and fractures in the aging cheese (Tinson et al., 1982b; Michel and Martley, 2001). Galactose accumulates because only the glucose moiety of lactose is utilized by *St. thermophilus* so galactose is excreted back into the milk/cheese as part of a Lac S-mediated antiport system for lactose uptake (Tinson et al., 1982a; Hutkins and Ponne, 1991; Vaillancourt et al., 2004). Heterofermentative NSLAB are known to utilize residual galactose to produce CO_2, leading to gassy defect in Cheddar cheese (Radford and Hull, 1982; Tinson et al., 1982b) that results in economic losses to the cheese manufacturer (Golnazarian, 2001).

We recently reported that *Lactbacillus wasatchensis*[1] WDC04—a slow-growing, obligatory heterofermentative (OHF) NSLAB of cheese (Oberg et al., 2015)—can utilize galactose and produce gas in broth (Ortakci et al., 2015a) and in cheese (Ortakci et al., 2015b).

[1]In 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.

When this NSLAB is present in high numbers in aging cheese it promotes gas production via fermentation of galactose (or other hexoses) (Ortakci et al., 2015b). Based on these observations, we hypothesized that growth and gas production by *Lb. wasatchensis* would be promoted if *St. thermophilus* was included as part of a Cheddar cheese starter culture because the cheese would contain higher levels of residual galactose. We have also shown that *Lb. wasatchensis* readily grows on cell lysate material (presumably from the ribose that is released) so that starter culture lysis during cheese storage also promotes growth of *Lb. wasatchensis*. To confirm these findings, we inoculated milk with *Lb. wasatchensis* and then used *St. thermophilus* as a starter culture to make Cheddar-style cheese. Microbial populations, including starter culture, fast-growing NSLAB (which in our creamery is predominantly *Lactobacillus curvatus*) and the slow-growing NSLAB *Lb. wasatchensis* were then enumerated, along with measurements to determine the extent of gas formation, through 23 wk of cheese storage at 6 and 12°C.

MATERIALS AND METHODS

Bacterium and Growth

Working cultures of *Lb. wasatchensis* WDC04 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 (donated by Bioenergy Life Science Inc., Ham Lake, MN) (**MRS+R**). Cultures were incubated anaerobically using GasPak™ EZ at 23°C for 40 h. Cells for the cheesemaking experiments were propagated in 400 ml of MRS+R for 40 h at 23°C. Cells were harvested by centrifugation at 7,500 *g* for 10 min at 4°C, washed twice with sterile 0.1% (wt/vol) peptone water, and re-centrifuged after each wash. The cell suspensions were used in the cheesemaking trials. Cell suspension concentrations were determined by spread plate counts on MRS+R agar incubated anaerobically for 5 d at 23°C.

Cheesemaking

Fresh bovine milk was obtained from the George B. Caine Dairy Research and Teaching Center (Wellsville, UT) and transported to the Aggie Creamery at Utah State University (Logan, UT). The milk was standardized to a protein-to-fat ratio of 0.84, pasteurized at 73°C for 15 s, and 136 kg was added into an open stainless steel vat (each vat had previously been cleaned then heat sanitized for 30 min). All vats of milk were warmed to 31°C, and then 0.25 g/kg of frozen pellets containing *St. thermophilus* M6 starter culture (Chr. Hansen Inc., Milwaukee, WI) were added. For the experimental vats, ~10^4 cfu/ml of *Lb. wasatchensis* was also added and the milk allowed to ripen for 10 min. 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. Curd was stirred until a curd pH of 6.3 was reached with partial whey drainage. 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 and placed into open plastic containers. Curd was packed into plastic hoops and pressed overnight (140 kPa, ~18 h, ~20°C). The cheese was then de-hooped and each block cut into ten pieces of ~600 g, and each piece was vacuum packaged. Five pieces were stored at 6°C and five at 12°C. Control cheeses were made using the same procedure except that no *Lb. wasatchensis* was added. Cheesemaking 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% (wt/vol) sodium citrate (previously 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. 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.

St. thermophilus. *Streptococcus thermophilus* was enumerated as described by Tabasco et al. (2007) using M17 agar (Becton Dickinson Inc.) containing 1% (wt/vol) lactose (Sigma-Aldrich Inc., St. Louis, MO) incubated aerobically at 45°C for 24 h.

Fast-growing NSLAB. The method of Oberg et al. (2011) for enumerating NSLAB on MRS agar supplemented with 2 μg/ml vancomycin (**V**) incubated anaerobically at 37°C for 48 h was used to enumerate fast-growing NSLAB. These NSLAB counts were designated as **NSLAB37** and do not include *Lb. wasatchensis* since it requires ribose for growth, and even with ribose does not grow quickly enough to be enumerated in 48 h. *Lactobacillus wasatchensis* is resistant to vancomycin, as are most cheese NSLAB, but grow more slowly and will not form observable colonies on MRS-V within 48 h (Ortakci et al., 2015b).

A crossover time for when NSLAB37 numbers equaled and then surpassed starter numbers was determined for each cheese. 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 was considered the crossover time (Oberg et al., 2011). All plate counts were performed in duplicate using the spread plate method.

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 and marking all the colonies (~1.5 mm diameter), the plates were further incubated anaerobically at 23°C for an additional 72 h. By this time, *Lb. wasatchensis* formed observable colonies (~1 mm diameter), which enabled differential enumeration of this organism from the fast-growing NSLAB23 colonies (Ortakci et al., 2015b).

Relative Gas Measurements

Gas production during cheese storage was measured by determining the extent of loosening of the plastic bag around the cheese block (Ortakci et al., 2015b). 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 pulled tightly against the cheese (d-0 line). After 8, 16 and 23 wk, the cheeses were examined for gas production, which was manifest by a loosening of the package. If the plastic bag was no longer tightly held 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 two 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 a 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 package so that there was no longer any residual vacuum (compared to atmospheric

pressure), the seal line of the cheese package could be pulled the full 5 cm from the cheese. The distance the package 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, four cheese packages were tested for relative gas production at 8 wk, three at 16 wk, and two at 23 wk.

Chemical Analysis

Proximate composition of the cheeses was determined after approximately 3 d. Moisture content was measured by weight loss using ~4 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 weight change over 2 s. Fat content was measured by a modified Babcock method (Wehr and Frank, 2004). 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 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 HPLC for lactose, galactose, lactic acid, and propionic acid at d 1 and after 16 wk of storage as described by Phadungath (2011) and Ortakci et al. (2015b). About 5 g cheese were manually homogenized for 90 s with 10-ml of 0.013N sulfuric acid at 65 °C then centrifuged at 7,000 x *g* for 10 min. The samples were held at 4°C 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 65°C. 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. 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 was performed using PROC GLIMMIX in SAS (version 9.1; SAS Institute Inc., Cary, NC) with addition of *Lb. wasatchensis* as the main plot effect, storage temperature as a split plot effect and storage time as a split-split plot effect. Significance was declared at $P < 0.05$. Differences between means were determined using Tukey least squares means.

RESULTS AND DISCUSSION

Initial Composition

Significant differences were found in composition between control and *Lb. wasatchensis* supplemented cheeses ($P < 0.05$). Higher moisture (41.5%) and lower S/M (4.1%) levels were observed in cheese made with added *Lb. wasatchensis* ($P < 0.05$) (Table 1). However, salt, pH, and fat levels were the same in both cheeses ($P>0.05$). This effect of adding *Lb. wasatchensis* was unexpected. In previous work, when Cheddar cheese was made in a similar manner using *Lactococcus lactis* as the starter, adding *Lb. wasatchensis* had no effect on cheese moisture

content (Ortakci et al., 2015b). The set-to-salt time for the cheese was slightly longer (an additional 15 min) when *Lb. wasatchensis* was added but this would not cause an increase in moisture content. In a similar manner, cheese made with lactococcal starter and containing added *Lb. wasatchensis* took longer compared to making control cheese where no *Lb. wasatchensis* was added (Ortakci et al., 2015b).

Although S/M was lower in cheese with added *Lb. wasatchensis*, it was still within the S/M range 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 probably the result of the faster acidification rate (~195 min set-to-salt time) that occurred when using *St. thermophilus* as the starter culture rather than *Lc. lactis*.

Starter Streptococci and NSLAB during storage

Starter *St. thermophilus* numbers in the cheese were influenced by both storage time and temperature and there was a *Lb. wasatchensis* x storage time interaction ($P < 0.05$) (Table 2). Differences in streptococcal numbers based on addition of *Lb. wasatchensis* only occurred during storage at 12°C (Figure 1A). At 6°C starter numbers decreased ~1.5-log cfu/g after 23 wk for all cheeses (Figure 1B) but at 12°C there was a greater reductions in starter numbers ($P < 0.05$) in cheese with added *Lb. wasatchensis*. Similar results were also observed in previous work with Cheddar cheese made using lactococcal starters (Ortakci et al., 2015b). The basis for this effect is unclear since no obvious antimicrobial encoding gene(s) were found in the genome of *Lb. wasatchensis* (F. Ortakci, unpublished data)

Nonstarter lactic acid bacteria numbers increased from undetectable levels ($<10^2$ cfu/g) to 10^8 and ~10^7 cfu/g for NSLAB23 and NSLAB37, respectively after 23 wk storage (Figures 1, 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 storage, so the cheeses would be expected to have the same initial microbial background population.

There were no significant differences in either NSLAB number (i.e., NSLAB23 or NSLAB37) observed in the cheese as a function of storage temperature or addition of *Lb. wasatchensis* ($P>0.05$). The absence of a temperature effect was unexpected, as prior studies usually report higher NSLAB counts at elevated storage temperatures (Peterson and Marshall, 1990; Fox et al., 1998; Ortakci et al., 2015b). Similar numbers of NSLAB at both storage 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*—the predominant NSLAB found in Cheddar cheese manufactured at the Utah State University creamery (Broadbent et al., 2013)—gain net two moles of ATP via the Embden-Meyerhof pathway per mole of galactose or other hexose consumed (Axelsson, 2004). Thus, free galactose could provide sufficient energy for NSLAB to reach high final cell densities following storage at both temperatures.

Similar to the previous work (Ortakci et al., 2015b), NSLAB23 counts were significantly higher than NSLAB37 counts during the 23 wk of cheese storage ($P < 0.05$) (Figures 1 and 2). The most likely explanation for this difference is that a temperature of 37°C inhibits growth of some NSLAB just as we observed when trying to enumerate *Lb. wasatchensis*. Even though NSLAB counts were not influenced by the storage temperature of the cheese, there were differences in the crossover times observed between storage at 6°C and 12°C. There were also differences in the crossover time depending on whether *Lb. wasatchensis* had been added to the milk (Figures 1 and 2). Crossover occurred earlier in cheese with added *Lb. wasatchensis* (Figure 1) as there was a more rapid decrease in *St. thermophilus* numbers. Crossover between starter

streptococci and NSLAB numbers occurred during the first 8 to 10 wk in cheeses with added *Lb. wasatchensis* stored at 12°C but not until after 23 wk for the control cheese (Figure 2). When stored at 6°C, the NSLAB numbers never reached the same level as the starter because of the longer retention of viable starter streptococci.

Sugar and Organic Acids

Sugar and organic acid profiles of the cheeses were similar in both control and *Lb. wasatchensis*-added cheeses stored at either 6 or 12°C. Lactose content of the cheese was 0.2% at d 1 and similar levels were observed after 16 wk of storage. Persistence of lactose in Cheddar cheese during storage has been previously shown by Fox et al. (1998) who reported the continued presence of lactose after 36 wk of storage. A substantial amount (0.6 to 0.7%) of galactose was observed in all cheeses at d 1. Accumulation of galactose occurred because *St. thermophilus* uses only the glucoe moiety of lactose to produce lactic acid and does not utilize the galactose moiety (Tinson et al., 1982a; Hutkins and Morris, 1987; Mora et al., 2002; Vaillancourt et al., 2004). Adding *Lb. wasatchensis* to the cheese milk did not have an impact on initial galactose concentrations because the *Lb. wasatchensis* utilizes galactose only very slowly in the absence of ribose (Ortakci et al., 2015a). Galactose levels fell to 0.1% to 0.2% after 16 wk of storage in all cheeses and this can be attributed to the growth of *Lb. wasatchensis* along with other NSLAB.

Initial lactic acid levels in the cheeses (~1.0%) was lower than previously reported by McSweeney and Fox (2004) and McMahon et al. (2014) who both found 1.4 to 1.5% lactic acid in the cheese at d 1. This may be related to using *St. thermophilus* as the starter culture whereas previous reports were for Cheddar cheese made using *Lc. lactis* starter cultures. Perhaps the relatively fast make time and the cheeses only having an initial pH in the range ~5.2 to 5.3 may

account for the lower lactic acid concentrations in the cheeses. Lactic acid levels increased to 1.7% after 16 wk in all cheeses at both storage temperatures. Assuming this was a function of NSLAB metabolism, this was not surprising as all cheeses had similar NSLAB numbers after 16 wk. The slightly higher lactic acid levels after 16-wk storage compared to our previous study (Ortakci et al., 2015b) could be because of the cheese's having lower S/M in this study. Production of lactic acid during storage is influenced by S/M (Upreti et al., 2006b).

Propionic acid also increased in all cheeses during cheese storage. Levels of propionic acid increased from an initial 0.02% to 0.2% after 16 wk of storage. This occurred concomitantly with NSLAB counts reaching levels of $\geq 10^6$ cfu/g and NSLAB 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), does have the metabolic capability to produce propionic acid (J. Broadbent, unpublished data).

***Growth of* Lb. wasatchensis.** In the control cheese, we were unable to detect *Lb. wasatchensis* because the enumeration method has a detection limit requiring *Lb. wasatchensis* numbers to be within 1.5 log cfu/g of the fast-growing NSLAB population (Ortakci et al., 2105b). Since *Lb. wasatchensis* is a part of the NSLAB microbiota in our cheesemaking facility, it was probably present at numbers too low to detect by enumerating using the MRS-R-V plate count method. This presents a challenge for determining the cause of sporadic late gas blowing in Cheddar cheese as slow growing NSLAB like *Lb. wasatchensis* can be difficult to detect and enumerate if the NSLAB populations are high.

When *Lb. wasatchensis* was added in sufficient numbers during cheese manufacture it can be easily enumerated and population growth observed during cheese storage as shown in

Figure 3. Temperature had a significant effect on growth of *Lb. wasatchensis* during storage ($P < 0.05$). The greatest increase in *Lb. wasatchensis* cell numbers occurred during the first 8 wk at either temperature. Higher counts were observed at the elevated storage temperature of 12°C compared to 6°C. This corresponds with the rapid reduction in numbers of *St. thermophilus* at 12°C (Figure 3).

Lactobacillus wasatchensis is capable of growing on carbohydrates released during the lysis of other bacteria (particularly starter culture bacteria), and can reach high cell densities (OD_{600} of 2.49) when it's grown on carbohydrate-restricted MRS broth containing lactococcal cell lysate (Ortakci et al., 2015b). There was a clear complimentary pattern in the trend lines of growth of *Lb. wasatchensis* matching a decrease in starter streptococci numbers (Figure 2). We postulate that free galactose remaining in cheese after lactose fermentation by *St. thermophilus* and ribose released via its subsequent lysis enables co-utilization of both sugars by *Lb. wasatchensis* in a manner that maximizes its rate and extent of growth (Ortakci et al., 2015a). Slower growth of *Lb. wasatchensis* during the first 8 wk of storage at 6°C supports our hypothesis because starter streptococci counts never decreased below 10^9 cfu/g during this period, thus providing little cell lysate material to provide the needed pentose to support growth of *Lb. wasatchensis*.

Relative Gas and Splits in Cheese

The largest amount of gas was produced in cheese with added *Lb. wasatchensis* and ripened at 12°C (Figure 4). All effects and interactions were significant at $P \leq 0.01$ (Table 2). At 6°C, cheese with added *Lb. wasatchensis* showed no sign of gas formation during 23 wk of storage. An association between elevated storage temperatures and gassy defect in cheese has been reported previously (Elliott et al., 1981; Laleye et al., 1987). Elliott et al. (1981) found that

gas formation occurred in cheese stored 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. wasatchensis*. Nonetheless, the observation that *Lb. wasatchensis* did not promote gassy defect at 6°C is different from our recent research with cheese made using *Lc. lactis* (Ortakci et al., 2015b) in which addition of *Lb. wasatchensis* did cause gas production at both 6 and 12°C. This difference may be an indication that lysis of starter culture cells provides *Lb. wasatchensis* with additional hexose sugars for gas production as well as the pentoses needed to promote its growth. Our current work using *St. thermophilus* is in accordance with starter cell lysis promoting growth and gas production. When cheese was ripened at 12°C there was a rapid drop in *St. thermophilus* numbers with ~99% of the cells being lysed within 2 wk (streptococcal numbers drop from 10^9 cfu/g to an estimated 10^7 cfu/g) while at 6°C only about one third of the cells appear to had been lysed within the first 2 wk of cheese storage (Figure 1).

While most of the growth of *Lb. wasatchensis* 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 storage temperature (Figure 4). This may be a result of needing to have most of the available ribose consumed before any extensive production of CO_2 occurs, or it may be related to the solubility of CO_2 in cheese at 6°C (Ortakci et al., 2015b). We know that galactose utilization by *Lb. wasatchensis* is considerably slower when there is no ribose present (Ortakci et al., 2015a) and, as also shown by Axelsson (2004), fermentation of a hexose, such as galactose, as an energy source is needed for CO_2 production by OHF lactic acid bacteria. In the presence of both ribose and galactose, however, *Lb. wasatchensis* can rapidly utilize ribose to generate ATP for the cell, while the biochemical pathways are available to simultaneously utilize galactose to provide peptidoglycan precursors for cell wall synthesis and cell growth. Then, when the supply

of ribose is exhausted, the cell switches to fermentation of galactose for energy so gas production is observed. This has been noticed when *Lb. wasatchensis* was grown in carbohydrate-restricted MRS broth supplemented with ribose and galactose and gas production occurred towards the end of the exponential growth phase and in cheese supplemented with both ribose and galactose (Ortakci et al., 2015a, 2015b).

We postulate that insufficient gas production to loosen packages at 6°C is probably a combination of having one log less *Lb. wasatchensis* than at 12°C, and the increased solubility of CO_2 at the lower storage temperature (CRC, 2009). Having slightly higher moisture (41.5%) in the cheeses in this study compared to 37% in a previous study (Ortakci et al., 2015b) may also be a factor since higher CO_2 levels would be required to saturate the water portion of the cheese before the CO_2 is released to loosen the package. Having no gas production in the control cheese stored at 12°C was unexpected as it had been observed previously (Ortakci et al., 2015b). Perhaps there was insufficient growth of the indigenous *Lb. wasatchensis* present in the cheese but these could not be enumerated against the high level of fast-growing NSLAB in the cheese.

Cheese having higher moisture levels in this current study probably accelerated the occurrence of late gas defect including the development of irregular shaped voids and round eyes in gassy cheese that had been supplemented with *Lb. wasatchensis* (Figure 5). Such defective cheese would not be suitable for sale in supermarkets, leading to consumer rejection and avoidance of purchase. In addition, cutting losses of up to 50% for cheese with slits and cracks is a major economic concern for cheese manufacturers (Golnazarian, 2001).

In large scale dairy processing, wild type *St. thermophilus* are often 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 usual 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 starter culture) used in later vats. However, both approaches 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 these conditions and, if an OHF NSLAB such as *Lb. wasatchensis* is present as part of the resident NSLAB population, this sugar could stimulate it's growth and lead to defects with late gas production. Deliberate use of *St. thermophilus* as a starter culture can increase the risk of this problem occurring, and this should be considered when using this strategy to shorten make times for Cheddar cheese. Similarly, using elevated temperatures to shorten the storage time for cheese ripening also increases the risk of having gassy defects develop in the cheese.

CONCLUSIONS

This study examined consequences of having an OHF bacteria as part of the background nonstarter microbiota of Cheddar cheese on late gas production when the starter culture contains *St. thermophilus* and when the cheese is ripened at elevated temperatures. Milk was deliberately inoculated with *Lb. wasatchensis*, a slow-growing OHF NSLAB species, Cheddar-style cheese was made using a *St. thermophilus* culture and gas formation was monitored during aging at regular and elevated storage temperatures. *Lactobacillus wasatchensis* was able to grow (~3 log) and produced gas after 8 wk at the elevated storage temperature (12°C). However, at the lower storage temperature (6°C) no gassiness was observed in the cheese containing added *Lb. wasatchensis*, probably because of lower growth (1-log lower counts) during storage. Also, the control cheese did not exhibit any sign of gas formation after 23 wk of storage at either

temperature. Utilization of *St. thermophilus* as part of a starter for Cheddar cheese when *Lb. wasatchensis* (or similar bacteria) is present as a NSLAB should be avoided, especially if a manufacturer is using higher than normal storage temperatures. We conclude that *Lb. wasatchensis* is a contributor to late gas production (late blowing) in Cheddar cheese, and has previously been undetected because of inherent difficulties in its enumeration because (1) it is slow growing, (2) it requires ribose to grow and form colonies, and (3) its optimum growth temperature is lower than that used in common methods to detect NSLAB in cheese.

ACKNOWLEDGMENT

The use of trade names in this publication does not imply endorsement by Utah State University of the products named or criticism of similar ones not mentioned. We thank Bioenergy Life Science Inc. for donation of ribose. This research was supported by the National Dairy Council as part of the cheese applications program at the Western Dairy Center and by the Utah Agricultural Experiment Station, Utah State University, and is approved as journal paper No. 8774.

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Table 1. Mean (±SE) composition of Cheddar-style cheese (at d 3) made using *Streptococcus thermophilus* with (LBW+) or without *Lactobacillus wasatchensis* (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 with same letter within each row were not significantly different, α=0.05.

Table 2. ANOV *P*-values for starter streptococcal numbers and gas production based on addition of *Lactobacillus wasatchensis* (LBW) during manufacture of Cheddar-style cheese and subsequent storage at 6 and 12°C for 23 wk.

		P-value	
Source of Variation	**DF**	**Starter**	**Gas**
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

LIST OF FIGURES

Figure 5 (print version). Textural differences after 16 wk of storage at 12°C in (A) control cheese or (B) cheese containing inoculated *Lactobacillus wasatchensis* added at 10^4 cfu/ml of milk.

Figure 5 (online version). Textural differences after 16 wk of storage at 12°C in (A) control cheese or (B) cheese containing inoculated *Lactobacillus wasatchensis* added at 10^4 cfu/ml of milk.

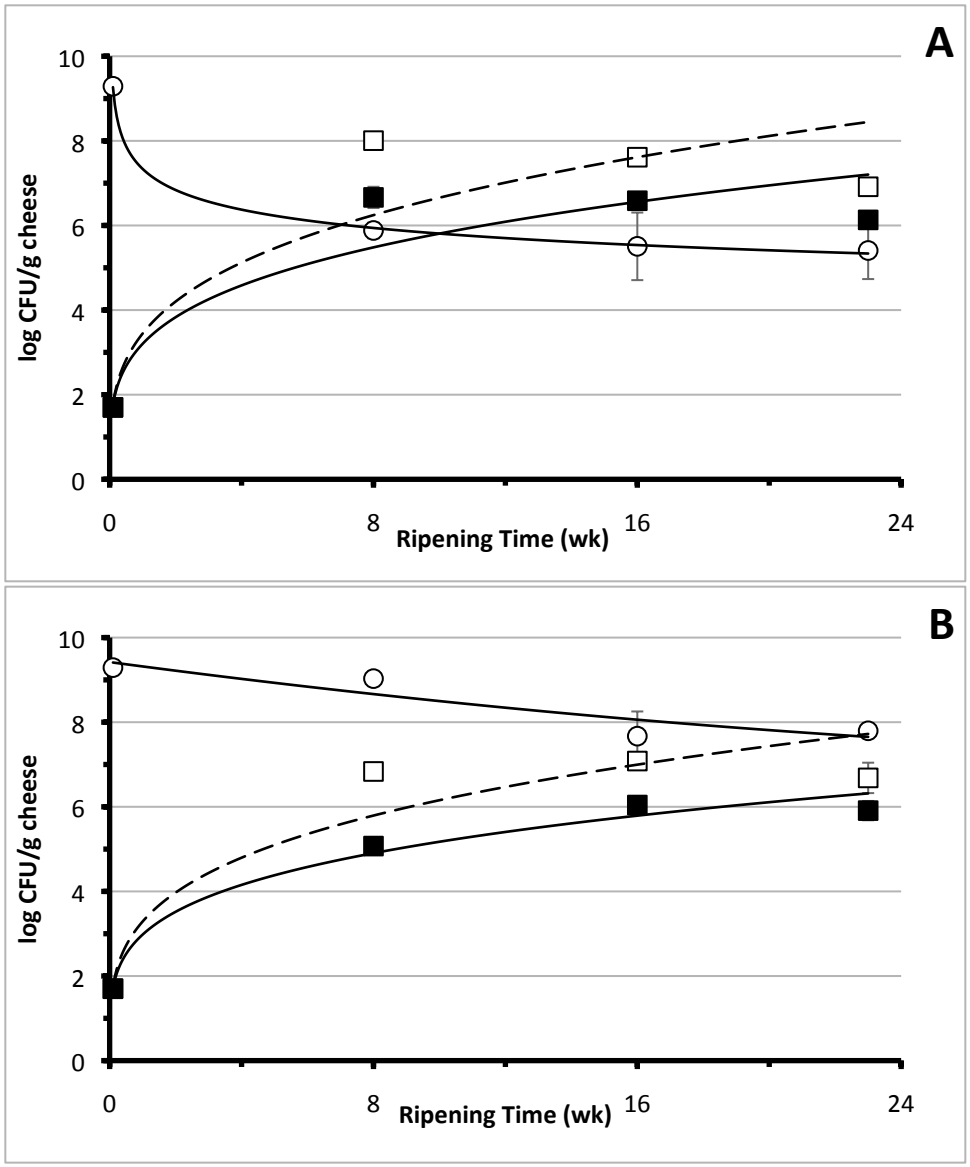

A
10
8
6
4
2
0
log CFU/g cheese
0
8
16
24
Ripening Time (wk)
B
10
8
6
4
2
0
log CFU/g cheese
0
8
16
24
Ripening Time (wk)

Figure 1

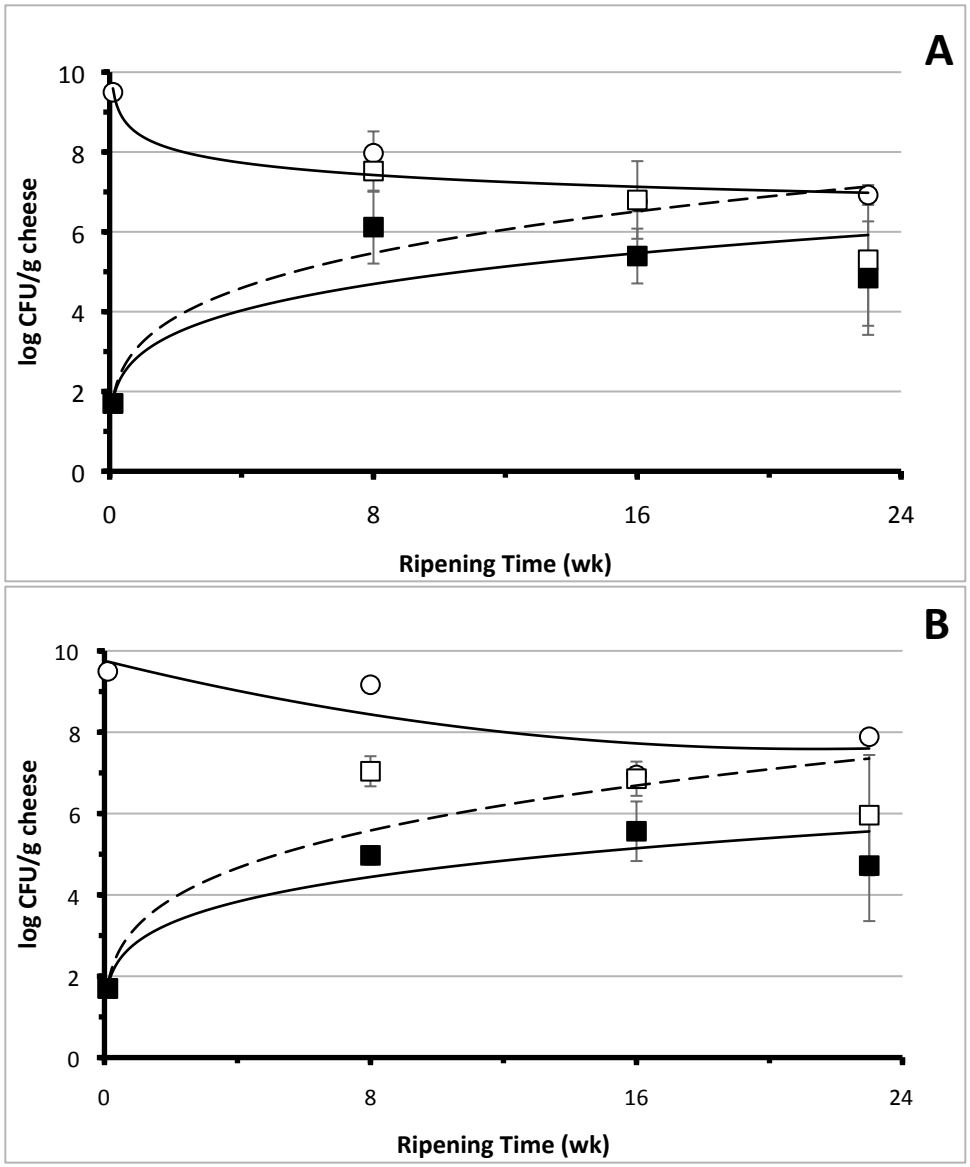
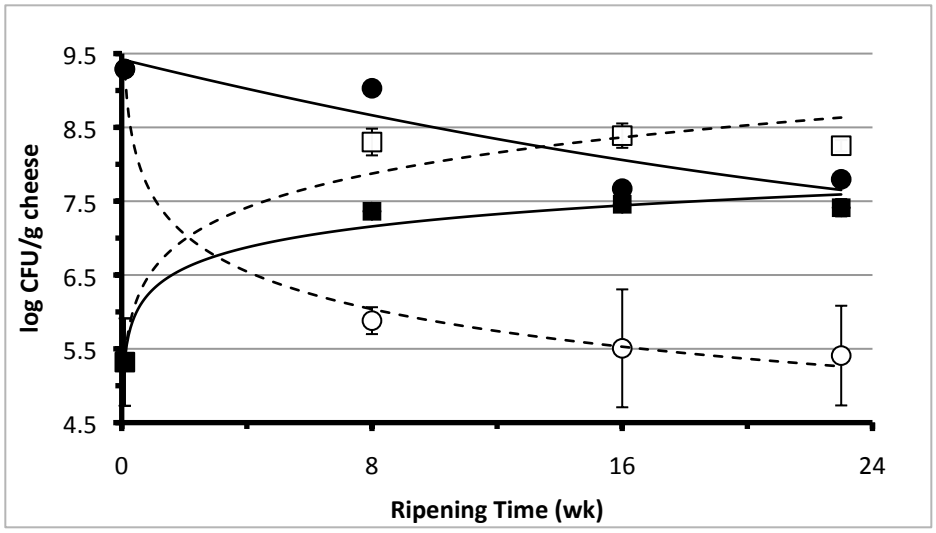

A
10
8
6
4
2
0
log CFU/g cheese
0
8
16
24
Ripening Time (wk)
B
10
8
6
4
2
0
log CFU/g cheese
0
8
16
24
Ripening Time (wk)

Figure 2.



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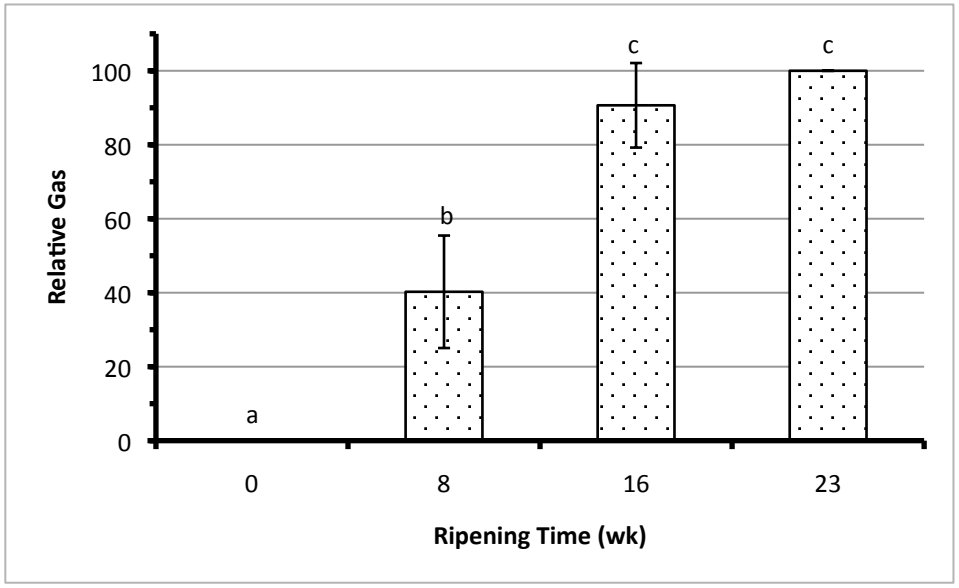

Figure 4

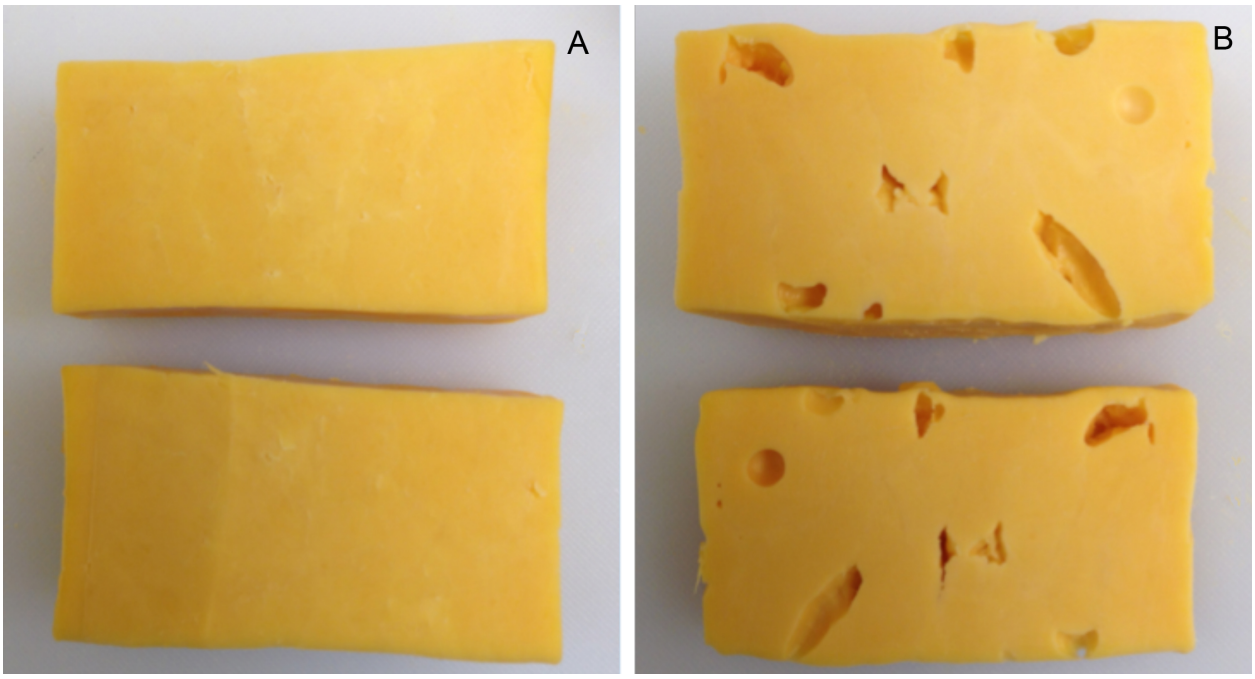

Figure 5.

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 x Sugar	NS	NS	NS	-	***
Temperature	**	**	**	**	**
LBW x Temperature	NS	NS	NS	-	**
Sugar x Temperature	NS	NS	NS	NS	NS
Time	**	**	**	**	**
LBW x Time	*	NS	NS	-	**
Sugar x Time	NS	NS	NS	**	NS
Temperature x Time	**	**	**	**	**
LBW x Temperature x Time	*	NS	NS	NS	*

*$P < 0.05$
** $P < 0.01$
***$0.1 > P > 0.05$
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.
[2] Cheese made without added *Lb. wasatchensis* is not included in the statistical analysis.

LIST OF FIGURES

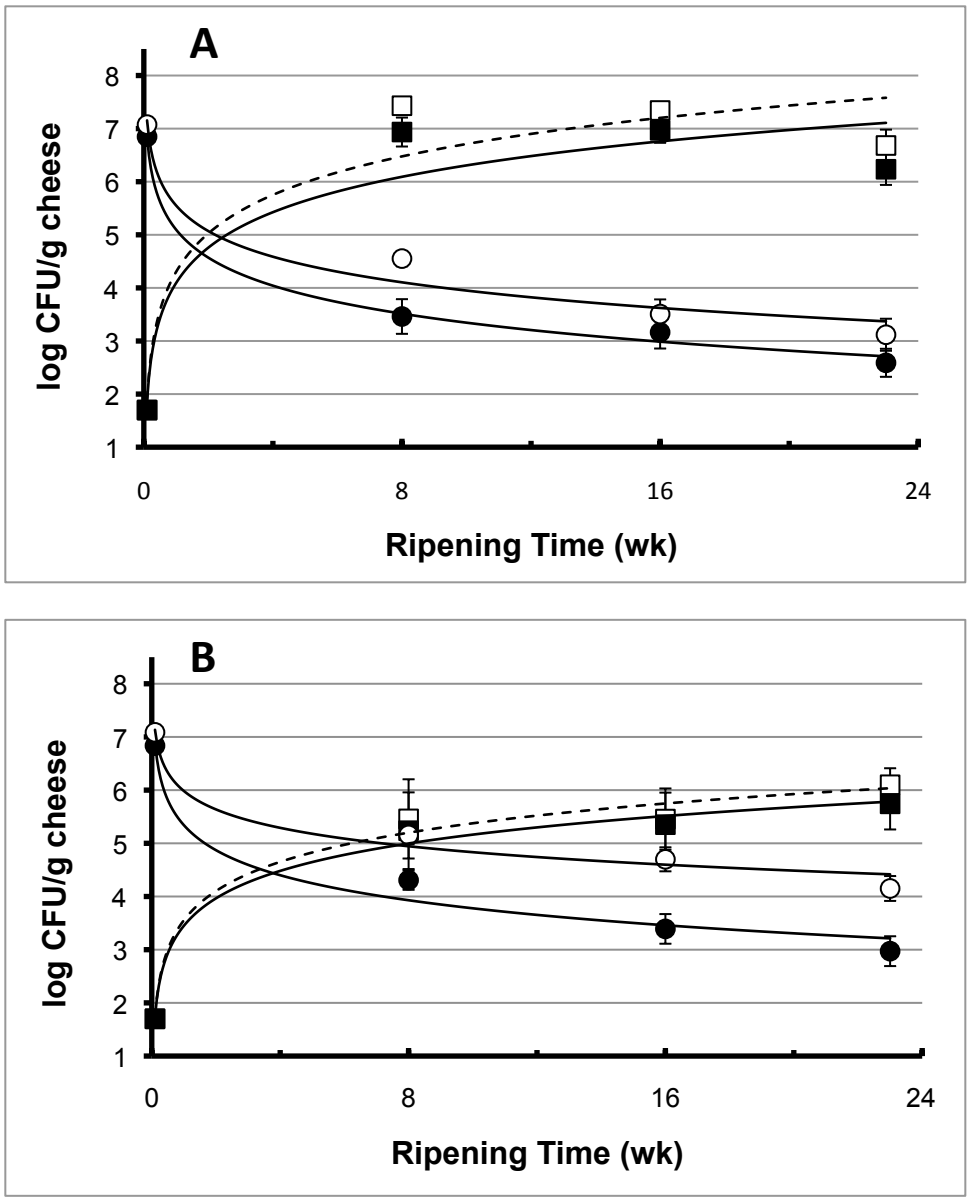
A
log CFU/g cheese
8
7
6
5
4
3
2
1
0
8
16
24
Ripening Time (wk)
B
log CFU/g cheese
8
7
6
5
4
3
2
1
0
8
16
24
Ripening Time (wk)

Figure 1

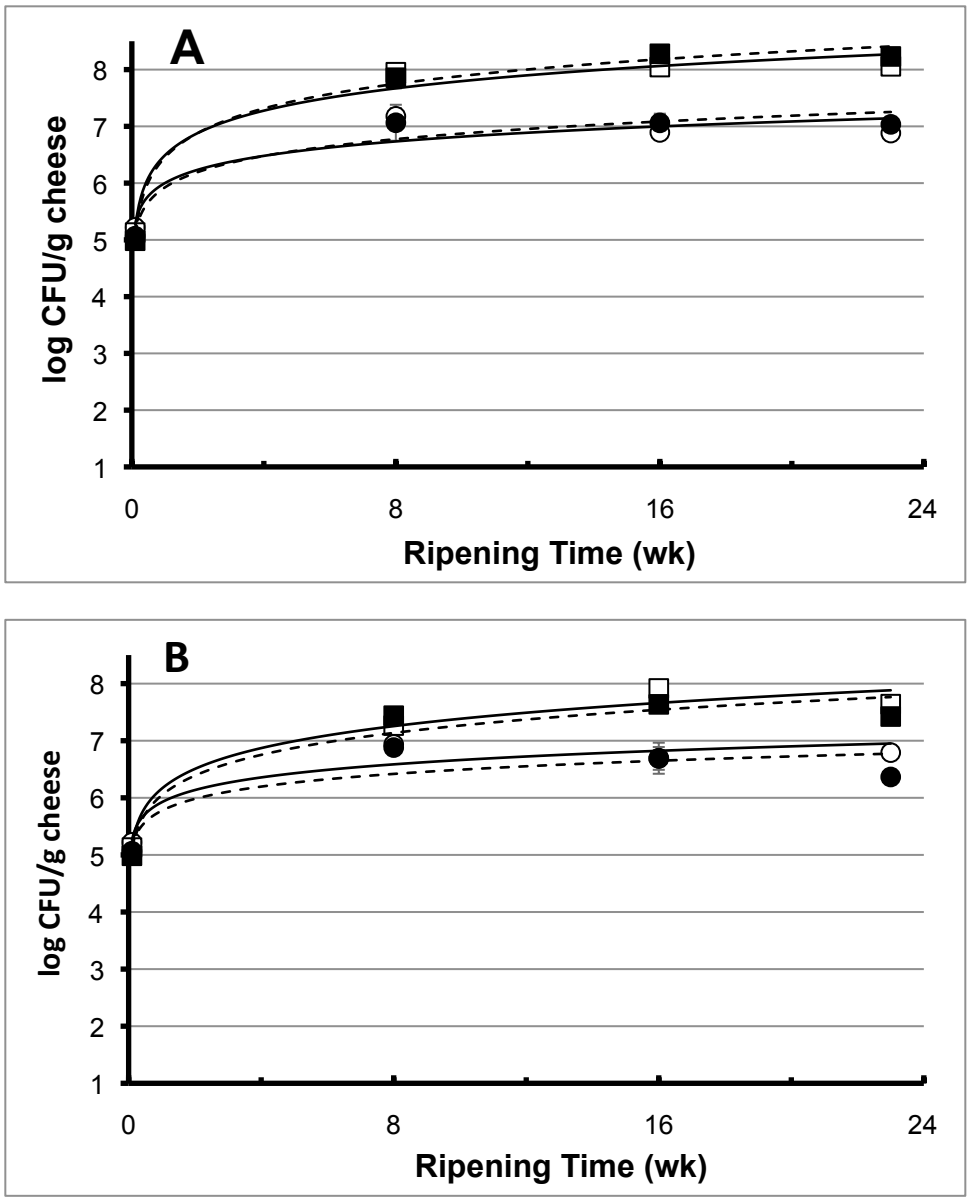
A
log CFU/g cheese
8
7
6
5
4
3
2
1
0
8
16
24
Ripening Time (wk)
B
log CFU/g cheese
8
7
6
5
4
3
2
1
0
8
16
24
Ripening Time (wk)

Figure 2

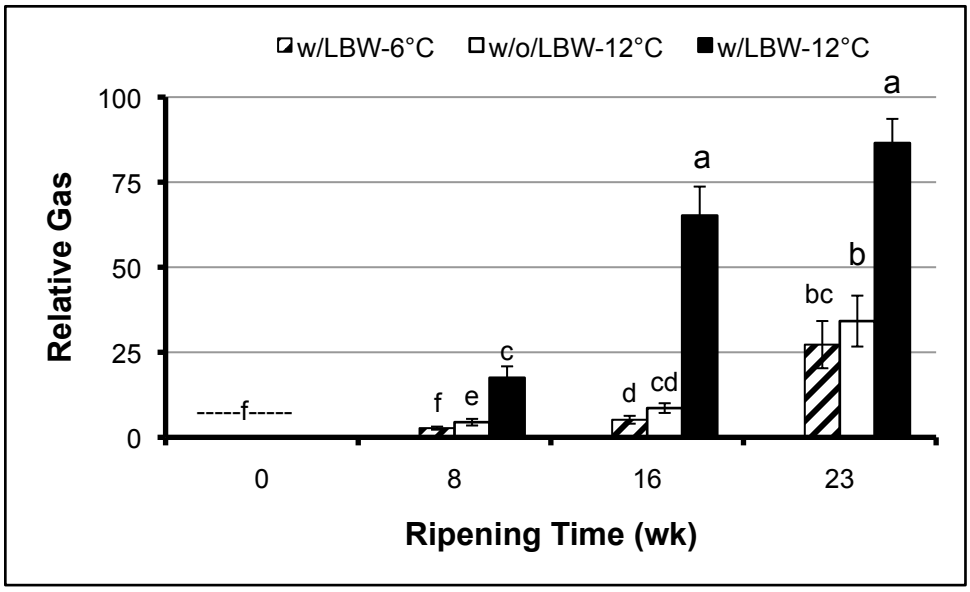

Figure 3 Ortacki et al Relative Gas

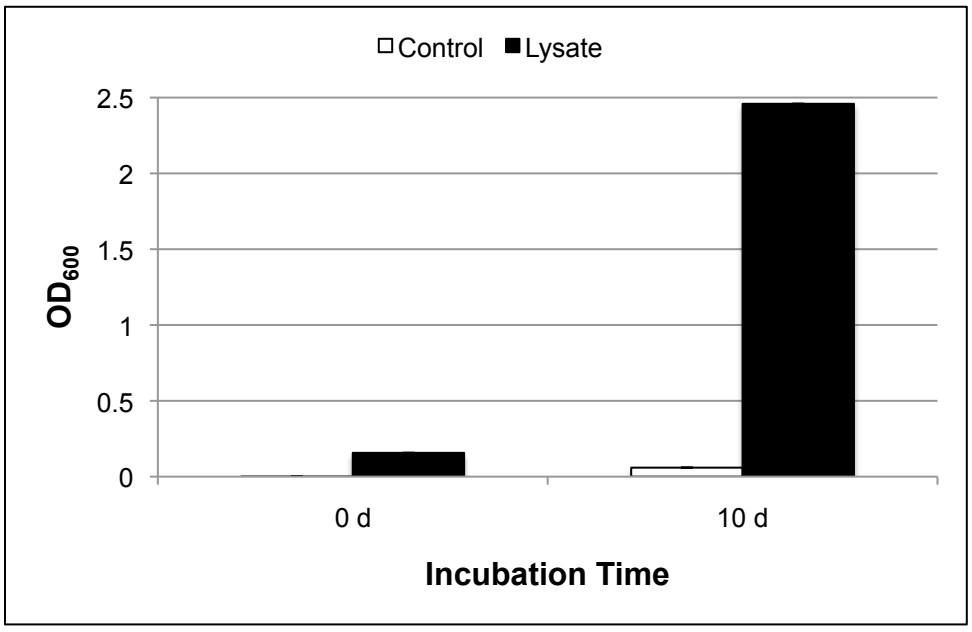

Figure 4 Ortakci et al Lysate growth