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Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter lactobacilli species on ribose and galactose

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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 use only ribose as a sugar source and we were interested in whether it could also utilize galactose. We conducted experiments to determine the 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, with 23°C being the optimum growth temperature of *Lb. wasatchii* among the three temperatures studied. When *Lb. wasatchii* was 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, compared with ribose, μ_{max} was 2 to 4 times lower. At all temperatures, the highest final cell densities (optical density at 640 nm) of *Lb. wasatchii* were achieved in CR-MRS plus 1% ribose, 0.5% ribose and 0.5% galactose, or 1% ribose and 1% galactose. Similar μ_{max} values and final cell densities were achieved when 50% of the 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 medium. 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 that *Lb. wasatchii* cannot survive low-temperature, long-time pasteurization but survives high-temperature, short-time (HTST) laboratory pasteurization, under which a 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 bacterium can be a serious contributor to gas formation and textural defects in Cheddar cheese.

Key words: nonstarter lactic acid bacteria, late blowing, ribose, cofermentation

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., 2001).

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 the 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 *Lactobacillus wasatchii* sp. nov. (our unpublished

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data; GenBank accession number: AWTT00000000 as *Lactobacillus* spp. WDC04).

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, although hexoses such as galactose are also a potential energy source in cheese. More importantly, hexose sugars can be fermented by OHF to lactate, acetate or ethanol plus CO_2, making *Lb. wasatchii* a potential contributor to gassy defect in Cheddar cheese.

This study examined 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 lactobacillus species isolated as an NSLAB from a "gassy" Cheddar cheese.

MATERIALS AND METHODS

Materials

Lactobacilli MRS broth, proteose peptone, polypeptone, beef extract, yeast extract, GasPak EZ gas-generating pouches, and agar were purchased from Becton Dickinson and Co. (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 bromocresol 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 triammonium citrate were from Alfa Aesar Inc. (Ward Hill, MA), and magnesium sulfate was from Alfa Aesar Inc. (Heysham, UK).

A carbohydrate-restricted version of MRS (**CR-MRS**) was prepared by omitting glucose from the MRS broth formula. To 2 L of deionized water was added 20.0 g of proteose peptone No. 3, 20.0 g of beef extract, 10.0 g of yeast extract, 2.0 g of Tween-80, 4.0 g of ammonium citrate, 10.0 g of sodium acetate, 0.2 g of magnesium sulfate, 0.1 g of manganese sulfate, and 4.0 g of dipotassium phosphate. The CR-MRS was supplemented with different levels of ribose and galactose to study the 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 2 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 of CR-MRS broth acidified to pH 5.20 with HCl and supplemented with 0.1% galactose or ribose, 0.5% galactose or ribose, 1.0% galactose or 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 ($\mathbf{OD_{640}}$) 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 ($\boldsymbol{\mu_{max}}$) 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 to conduct 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 ~6×10^8 cfu/mL and the samples held at 63°C and 72°C for 30 min or 15 s, respectively. Samples were 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 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 were 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 1A, 2A, and 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 with galactose as the sole sugar (Table 1). In the presence of 1% ribose, μ_{max} of *Lb. wasatchii* were as follows: 23°C > 37°C = 12°C. When *Lb. wasatchii* was grown in the presence of 1.0% ribose at 12 and 23°C, exponential growth continued until final OD_{640} levels of ~1.3 to 1.4 were reached (Table 2), with lower OD_{640} achieved at lower sugar levels, indicating that available sugar 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 indicate 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 1B, 2B, and 3B), with μ_{max} of <0.01 at all temperatures (Table 1). Final cell densities were lower ($P < 0.05$) than when *Lb. wasatchii* was grown with ribose except for the lowest sugar level (0.1%) at 12 and 37°C (Table 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 (Biomerieux, Marcy l'Etoile, France) test even when held for longer than 48 h (our unpublished data). With slower growth occurring when galactose was the only sugar, the stationary phase in CR-MRS plus 0.5% galactose was only reached after 156 h at 23°C compared with 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 the galactose level was increased to 1% (Table 2).

Combined Ribose and Galactose. The μ_{max} was not significantly different when a 1:1 blend of galactose and ribose was used compared with ribose alone ($P > 0.05$), with only a slight difference observed for growth at 23°C (Figures 1, 2, and 3; Table 1). In general, final cell densities were similar when the total sugar content was the same (Table 2). This indicates that galactose utilization by *Lb. wasatchii* is slower when there is no ribose present but that almost the same rate of growth as that with ribose is achieved when both sugars are present.

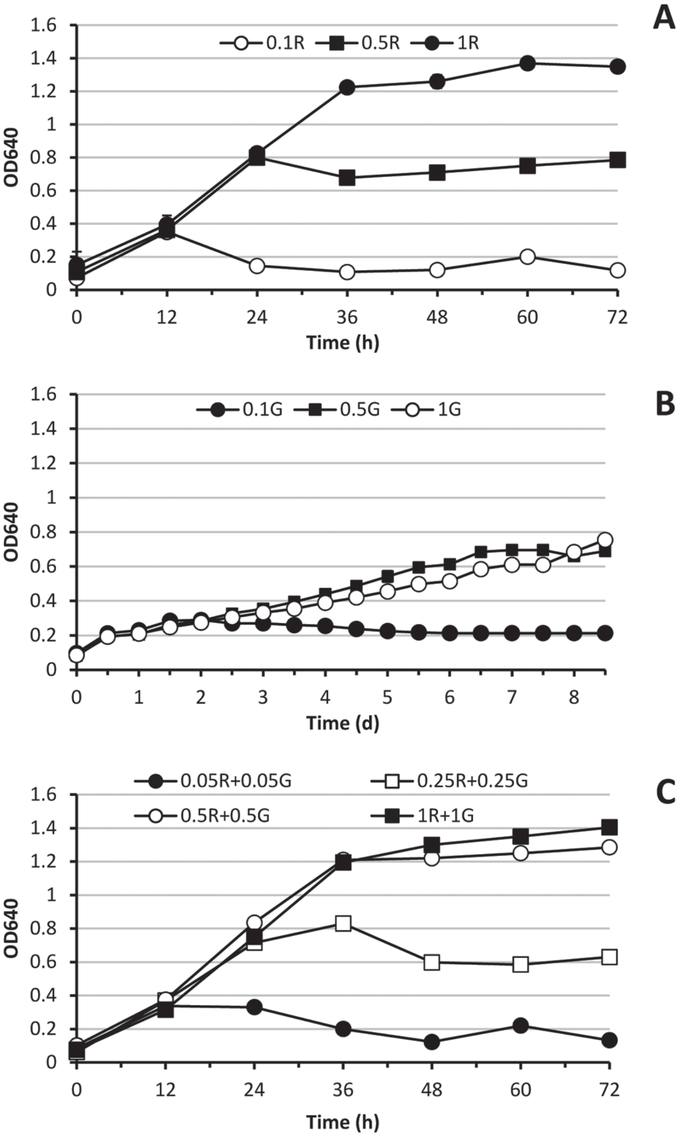

Figure 1. Growth of *Lactobacillus wasatchii* (optical density at 640 nm, OD_{640}) at 23°C in carbohydrate-restricted de Man, Rogosa, and Sharpe adjusted to pH 5.2 and supplemented with ribose (A), galactose (B), or a mixture of ribose and galactose (C). Numbers for each symbol represent the percentage concentration (wt/vol) of sugar (ribose, R, or galactose, G) added to the medium. Error bars (not visible) = SE (n = 2).

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 4A and 4B, respectively. After 48 h, an OD_{600} of 2.0 was reached in all cultures except for 5% salt at pH 5.2, which had an OD_{600} of 1.75 and only

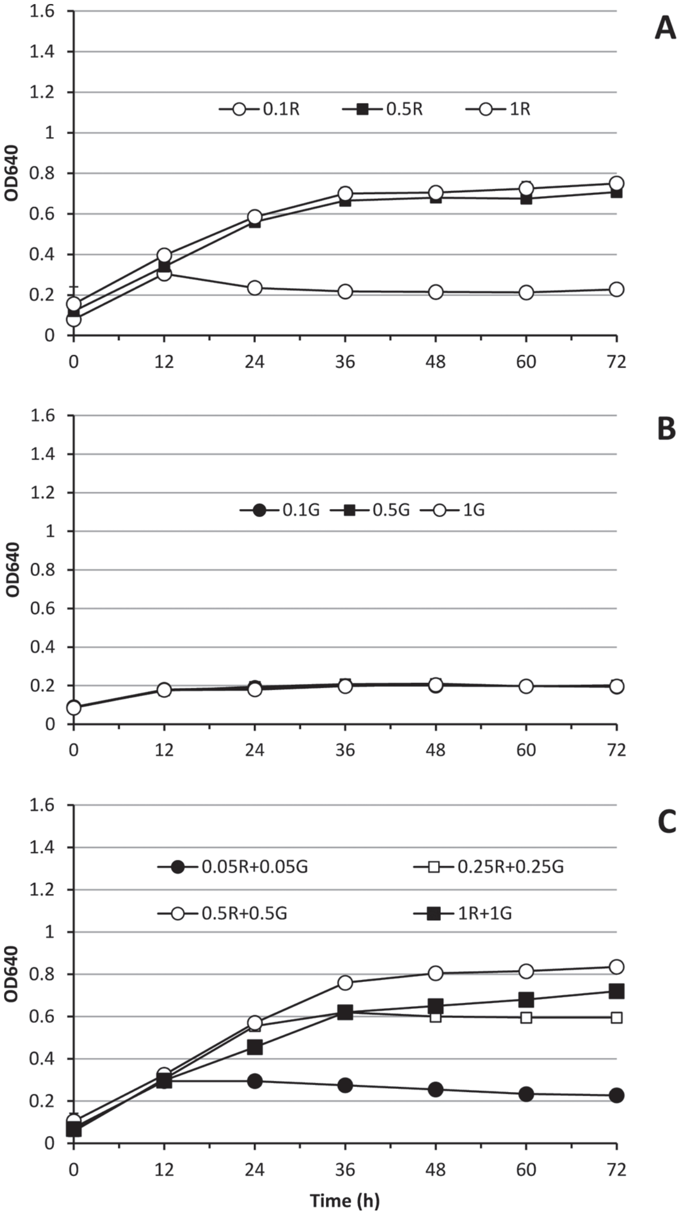

Figure 2. Growth of *Lactobacillus wasatchii* (optical density at 640 nm, OD_{640}) at 37°C in carbohydrate-restricted de Man, Rogosa, and Sharpe adjusted to pH 5.2 and supplemented with ribose (A), galactose (B), or a mixture of ribose and galactose (C). Numbers for each symbol represent the percentage concentration (wt/vol) of sugar (ribose, R, or galactose, G) added to the medium. Error bars (not visible) = SE (n = 2).

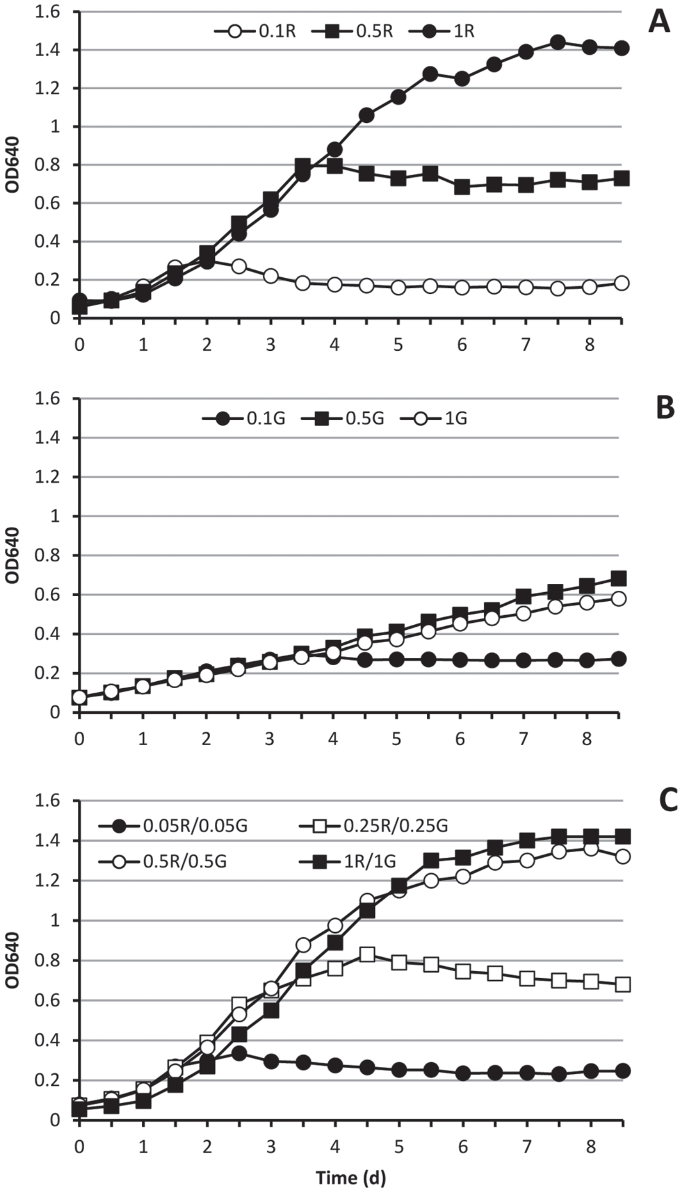

Figure 3. Growth of *Lactobacillus wasatchii* (optical density at 640 nm, OD_{640}) at 12°C in carbohydrate-restricted de Man, Rogosa, and Sharpe adjusted to pH 5.2 and supplemented with ribose (A), galactose (B), or a mixture of ribose and galactose (C). Numbers for each symbol represent the percentage concentration (wt/vol) of sugar (ribose, R, or galactose, G) added to the medium. Error bars (not visible) = SE (n = 2).

reached OD_{600} of 2.0 after 60 h. At pH 6.5, we observed a slight decrease in μ_{max} when *Lb. wasatchii* was grown with 4% NaCl, although this was not observed with 5% NaCl (Table 3). At pH 5.2, μ_{max} was significantly lower at both 4 and 5% NaCl ($P < 0.05$). Final cell densities were the same ($OD_{600} = 2.0$) except for 5% NaCl, which had a 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

Table 1. Maximum specific growth rate (μ_{max}, measured as optical density at 640 nm per hour, OD_{640}/h) of *Lactobacillus wasatchii* at 12, 23, or 37°C when grown in carbohydrate-restricted de Man, Rogosa, and Sharpe broth with various levels of ribose and galactose

Sugar (% wt/vol)		μ_{max} (OD_{640}/h)		
Ribose	Galactose	12°C	23°C	37°C
0.1	0	0.0085^{hij}	0.0235^{cd}	0.019^{de}
0.5	0	0.0145^{efghi}	0.0365^{a}	0.0195^{de}
1.0	0	0.0155^{efgh}	0.0355^{ab}	0.0215^{de}
0	0.1	0.003^{j}	0.0095^{ghij}	0.008^{ij}
0	0.5	0.006^{j}	0.0095^{ghij}	0.0075^{ij}
0	1.0	0.005^{j}	0.009^{ghij}	0.008^{ij}
0.05	0.05	0.010^{fghij}	0.021^{de}	0.0185^{de}
0.25	0.25	0.016^{efg}	0.0285^{bc}	0.021^{e}
0.5	0.5	0.018^{de}	0.0385^{a}	0.0205^{de}
1.0	1.0	0.017^{def}	0.0375^{a}	0.0195^{de}

[a–j] Mean values with the same letter are not significantly different from each other ($\alpha = 0.05$).

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; it had negligible growth at 4% NaCl and did not grow at 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 treatment (72°C for 15 s) resulted in a ~4.5-log reduction, from 6×10^6 cfu/mL to 9.2×10^1 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 that the bacterium could gain access to cheese directly or produce biofilms in the cheese-processing environment that provide a regular source of contamination.

Table 2. Final cell density of *Lactobacillus wasatchii* measured as optical density at 640 nm (OD_{640}) when grown at 12, 23, or 37°C in carbohydrate-restricted de Man, Rogosa, and Sharpe broth

Sugar (% wt/vol)		Final cell density[1] (OD_{640})		
Ribose	Galactose	12°C	23°C	37°C
0.1	0	0.3^{j}	0.35^{j}	0.225^{kl}
0.5	0	0.795^{de}	0.8^{de}	0.705^{fg}
1.0	0	1.44^{a}	1.37^{b}	0.75^{ef}
0	0.1	0.298^{j}	0.214^{l}	0.205^{l}
0	0.5	0.68^{gh}	0.69^{fgh}	0.215^{l}
0	1.0	0.58^{i}	0.755^{ef}	0.195^{l}
0.05	0.05	0.335^{j}	0.335^{j}	0.300^{j}
0.25	0.25	0.83^{d}	0.83^{d}	0.62^{hi}
0.5	0.5	1.36^{b}	1.285^{c}	0.835^{d}
1.0	1.0	1.42^{ab}	1.4^{ab}	0.72^{fg}

[a–l] Mean values with the same letter are not significantly different from each other ($\alpha = 0.05$).

[1] Measured as OD_{640} after incubation for 72 h for growth at 23 and 37°C for medium containing ribose, and after 204 h for all samples incubated at 12°C and those with only galactose at 23 and 37°C.

Table 3. Maximum specific growth rate (μ_{max}, measured as optical density at 600 nm per hour, OD_{600}/h) of *Lactobacillus wasatchii* cells grown at 23°C in de Man, Rogosa, and Sharpe broth supplemented with 1.5% ribose as a function of salt and pH

NaCl (% wt/wt)	μ_{max} (OD_{600}/h)	
	pH 5.2	pH 6.5
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}

[a–e]Mean values with the same letter are not significantly different from each other ($\alpha = 0.05$).

DISCUSSION

Metabolic Capability

Lactobacillus wasatchii sp. nov. 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 because NSLAB isolation methods do not incorporate the low-temperature, long-time 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 lactobacillus 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 by OHF lactobacilli results in CO_2, lactate, and acetate or 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.

Compared with common cheese NSLAB that are FHF lactobacilli, *Lb. wasatchii* preferentially utilizes ribose over glucose and other sugars. 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).

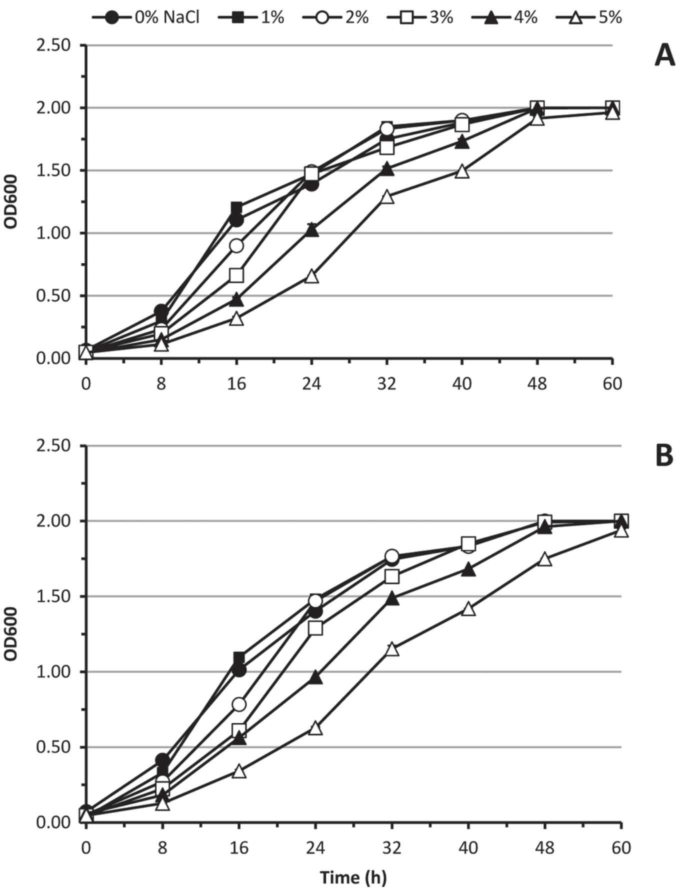

Figure 4. Growth of *Lactobacillus wasatchii* (optical density at 600 nm, OD_{600}) in regular de Man, Rogosa, and Sharpe broth supplemented with 1.5% ribose (wt/vol) plus 0 to 5% NaCl and adjusted to pH 6.5 (A) or pH 5.2 (B). Error bars (not visible) = SE (n = 3).

Ribose Fermentation

The heterolactic fermentation of ribose results in a slightly different end-product pattern compared with galactose fermentation. No CO_2 is formed and, because 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 2 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 5 (pathway directions {1}, {2}, and {3}).

The $\mu_{\max}$ of *Lb. wasatchii* is generally the same at higher concentrations of ribose as at lower concentrations. Thus, PP was operating as fast as possible in generating energy when *Lb. wasatchii* was grown in CR-MRS with either ribose concentration. It is interesting that similar $\mu_{\max}$ values were achieved when a ribose-galactose mixture was used, even at the low level of 0.05% ribose plus 0.05% galactose (Table 1). The only notable change that was seen with the increasing sugar concentration was that the time over which exponential growth occurred was lengthened and a higher final cell density was attained.

Galactose Fermentation

Lactobacillus wasatchii grew very slowly when galactose was the sole carbohydrate source of energy ($\mu_{\max}$ = 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

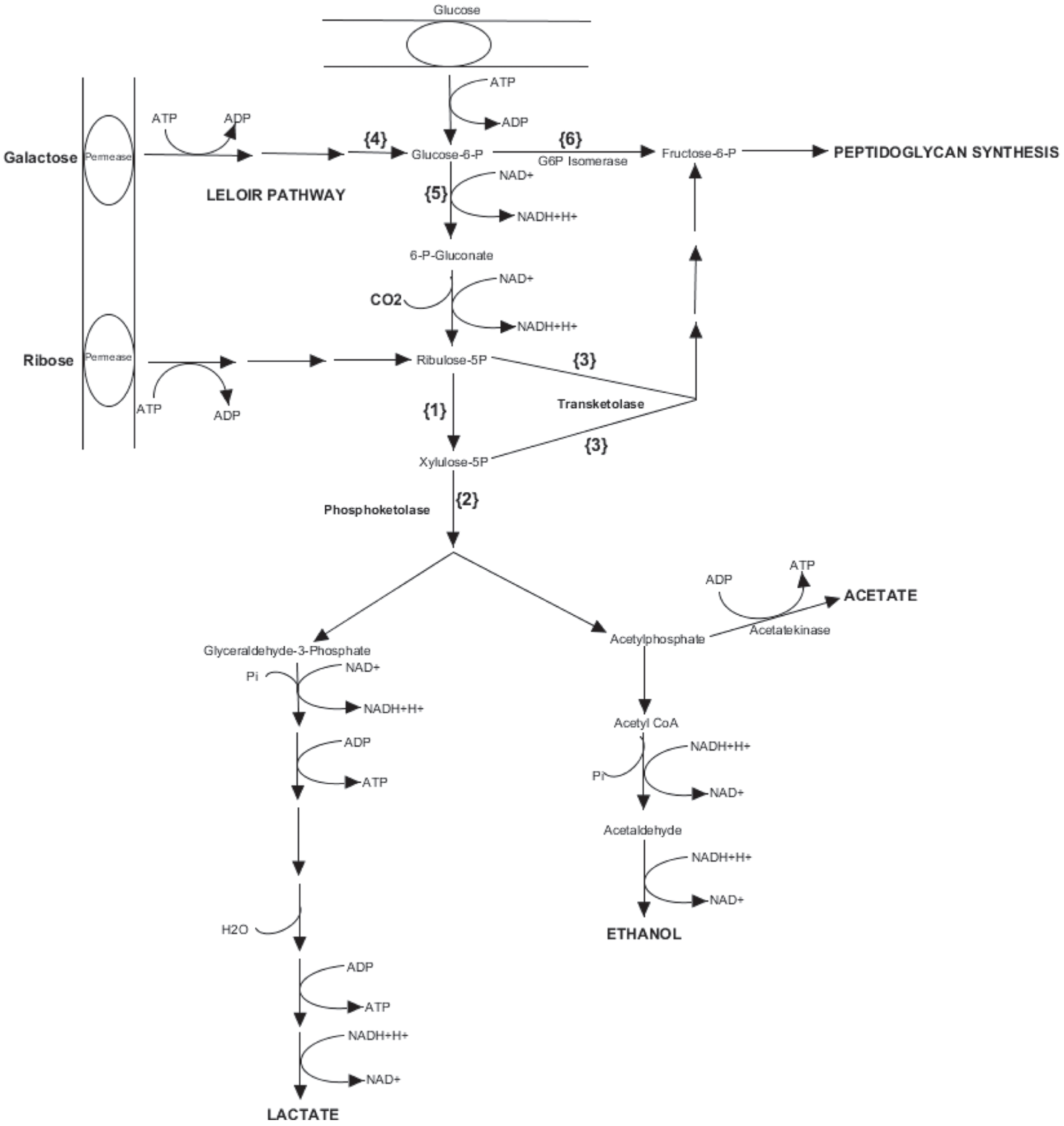

Figure 5. Proposed pathways for ribose and galactose utilization by *Lactobacillus wasatchii.* P = phosphate group. Steps {1} through {6} are described in text.

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 ($P < 0.05$). The 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 with 24°C. However, ribose utilization rates by *Lb. danicus* were the same at both temperatures. In comparison, utilization of hexose sugars by *Lb. casei* or *paracasei* was higher at 30°C compared with 24°C, whereas ribose utilization did not change (Adamberg et al., 2005).

Analysis of the *Lb. wasatchii* genome suggests that galactose enters the cell via a permease and is then fermented into the Leloir pathway and converted to glucose-6-phosphate (**G6P**), as shown in Figure 5. The G6P is then utilized using PP via dehydrogenation to 6-phosphogluconate, followed by decarboxylation to ribulose-5-phosphate (**R5P**) and CO_2 (pathway directions {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 or ethanol with the potential to generate up to net 2 ATP. However, the need to reoxidize NADH may direct the pathway from acetylphosphate toward ethanol production rather than acetate. Thus, galactose utilization through the Leloir and PP pathways would supply 1 mol each of lactic acid, ethanol, and CO_2, and net 1 mol ATP/mol of galactose (Axelsson, 2004).

The two possible explanations for the much slower growth of *Lb. wasatchii* on galactose compared with ribose are (1) that there is a rate-limiting step in the pathways leading to conversion of galactose into R5P, or (2) that the need to reoxidize NADH requires conversion of acetylphospate into ethanol rather than acetate so that only 1 mol 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 the growth of lactobacilli is increased in the presence of 2 sugars compared with 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 reoxidation of NADH. This does not seem to be the case for *Lb. wasatchii* because 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 2 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, and thus is unable to make C_6 units from C_5 sugars and needs 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 with hexose fermentation via glycolysis) in *Lb. plantarum* NCIMB 8026 is 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 (vs. 2 PEP molecules per glucose), leaving no PEP molecules for the other cellular functions such as peptidoglycan synthesis.

With *Lb. wasatchii,* transketolase is available to convert pentoses into F6P, thus producing the needed C_6 building blocks for peptidoglycan. In addition, for *Lb. wasatchii*, the uniqueness is improved utilization of a hexose in the presence of a pentose rather than the other way around. Therefore, neither of these hypotheses explains 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, whereas 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 reoxidize 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. Because similar μ_{max} and final cell densities were observed when *Lb. wasatchii* was grown in the presence of ribose plus galactose or ribose alone, the rates of energy production and cell wall synthesis are 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 5, pathway directions {4}, {6}).

In a parallel manner, final cell densities of *Lb. wasatchii* were 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 5, pathway directions {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, the ribose is primarily utilized for ATP production via the lower portion of the PP (Figure 5, pathway directions {1}, {2}), whereas the hexose is utilized for synthesis of peptidoglycans and other cellular macromolecules (Figure 5, pathway directions {4}, {6}). This has the advantage of maximizing ATP production, as the need to reoxidize 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 produce 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 toward 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 2 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 the 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, and survive in the 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 the cheese microenvironment, it would be desirable to study whether other sugars in milk and cheese (e.g., lactose, *N*-acetylgalactosamine, *N*-acetylneuraminic 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 because 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 6- and 5-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.

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