Growth and survival characteristics of *Paucilactobacillus wasatchensis* WDC04

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ABSTRACT

Understanding characteristics that permit survival and growth of *Paucilactobacillus wasatchensis* as part of the nonstarter microbiota of cheese is important for minimizing unwanted gas formation in cheese that can cause downgrading because of slits and cracks. The ability of *Plb. wasatchensis* WDC04 to survive pasteurization was studied by inoculating raw milk with 10⁸ cfu/mL and measuring survival after processing through a high-temperature, short-time pasteurizer. Extent and rate of growth of *Plb. wasatchensis* WDC04 as a function of pH, salt concentration, and presence of various organic acids were studied using 48-well microplates in an automated spectrophotometer measuring optical density at 600 nm. Better growth in the 1-mL wells was obtained when a micro-anaerobic environment (similar to that which occurs in cheese) was created by enzymically removing the oxygen. Faster growth occurred around neutral pH (pH 6 to 8) than at pH 5 (cheese pH), whereas only marginal growth occurred at pH 4. Adding sodium chloride retarded growth of *Plb. wasatchensis* WDC04, but slow growth occurred even at salt concentrations up to 6%. At salt-in-moisture (S/M) concentrations found in cheese, the rate of growth at 3.5% S/M > 4.5% S/M > 5.5% S/M. Thus, low salt level in cheese is a risk factor for *Plb. wasatchensis* growth during cheese storage and unwanted slits and cracks. Some of the organic acids tested (propionic, formic, and citric) tended to suppress growth of *Plb. wasatchensis* WDC04 more than would be expected from their effect on pH. No survival of *Plb. wasatchensis* WDC04 after pasteurization was observed with the reduction in numbers being 8 logs or more. Even subpasteurization heating at 69°C for 15 s was sufficient to inactivate *Plb. wasatchensis* WDC04, so its presence as part of the nonstarter microbiota of cheese should be considered as a postpasteurization environmental contamination.

Key words: pasteurization, lactobacilli, nonstarter, salt

INTRODUCTION

Quality issues in cheese arising from the presence of obligatory heterofermentative (OHF) lactobacilli are problematic and a source of unwanted gas production leading to splits and downgrading of cheese. Cheese with slits, splits, or cracks is unsuitable for conversion into sliced cheese because modern slicing machines operate at up to 20 slices per second. One such OHF bacteria was isolated from a puffy bag of cheese in 2012 and given the name *Lactobacillus wasatchensis* (pronounced wa.satch.en'sis; Oberg et al., 2016) and has now been reclassified as a lactobacilli in the *Paucilactobacillus* (pronounced pow-kee.lac.to.bacil'lus) genera (Zheng et al., 2020). The species was initially presumptively designated as *Lactobacillus wasatchii* (Ortakci et al., 2015a). All of the previously designated members of the genus *Lactobacillus* can still be referred to as lactobacilli even though they have different genera names because they are still part of the *Lactobacillaceae* family (Zheng et al., 2020).

The *Paucilactobacillus* genus describes a group of lactobacilli that ferment few carbohydrates, and hence, their name is derived from the Latin *paucus* meaning “few.” They are gram-positive, rod-shaped, catalase negative, heterofermentative, and aerotolerant with growth observed between 20 and 37°C. Strains in this genus were predominantly isolated from fermented plant material including silage, pickles, and fruit mash- es, whereas *Plb. wasatchensis* was first isolated from cheese in Utah (United States) and named after the Wasatch mountains. These are lactobacilli that have adapted to hexose-depleted habitats as indicated by the lack of mannitol dehydrogenase in many strains of this genus. Among the heterofermentative lactobacilli, this property is shared only with *Secundilactobacillus* that is isolated from related habitats and found as secondary bacteria in fermentation processes. *Paucilactobacillus* species preferentially metabolize pentoses, and many

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strains, such as *Plb. wasatchensis*, do not ferment disaccharides (Zheng et al., 2020).

The type species for the *Paucilactobacillus* genus is *Plb. vaccinostercus* (Zheng et al., 2020), which has been isolated from cow dung, fermented tea leaves, and fermented cereals. Other species include *Plb. hokkaidonensis* (isolated from grass silage), *Plb. kaifaensis* (isolated from fermented Chinese cabbage), *Plb. nenjiangensis* (isolated from pickles), *Plb. oligofermentans* (isolated from marinated poultry meat at the end of its shelf life, and from fermented olives), *Plb. suebicus* (isolated from fermented cherry mash, cider, and silage), and *Plb. wasatchensis* (isolated from cheese).

Many of the lactobacilli that have been associated with dairy foods also have reclassified genus names. The homofermentative *Lactobacillus delbrueckii* remaining as the type species for the *Lactobacillus* genus with *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, and *Lactobacillus kefiranofermentans* being its most common dairy members. The homofermentative casei group of lactobacilli is now designated as *Lactocaseibacillus* with *Lactocaseibacillus casei* as the type strain and also includes *Lactocaseibacillus paracasei* and *Lactocaseibacillus rhamnosus*.

The most common nonstarter lactic acid bacteria (NSLAB) found in cheese in North America is now designated as *Lactilactobacillus curvatus* and is part of a homofermentative genus whose members have a broad range of habitats (*latus* means wide), and the strains in the species lead a free-living lifestyle and are mesophilic (many strains are also psychrotrophic). *Lactilactobacillus sakei* is the type species. Another group of homofermentative lactobacilli is the *Ligilactobacillus* genus that have a host-associated lifestyle (from *ligare* meaning to unite). The type species is *Ligilactobacillus salivarius* and several of the species commonly occur in fermented foods and are used commercially as starter cultures or probiotic cultures. This genus also includes *Ligilactobacillus ruminis* that is found in the rumen of cows as well as in the gut of humans (and other mammalian species).

The homofermentative *plantarum* lactobacilli group is now designated as the *Lactiplantibacillus* genus based on their being found in both milk and plant-based fermented foods. *Lactiplantibacillus plantarum* is the type species. A group of lactobacilli with spoilage potential (*loigos* meaning destruction, ruin, or havoc) form the *Loigolactobacillus* genus with *Loigolactobacillus coryniformis* being the type species. *Loigolactobacillus bifermentans* has been isolated from Dutch-style cheese where it can cause small cracks due to fermentation of lactic acid into acetic acid, ethanol, traces of propionic acid, carbon dioxide, and hydrogen. Another member

of this genus is *Loigolactobacillus rennini* that was isolated from rennet and associated with cheese spoilage.

There are multiple genera of heterofermentative lactobacilli in addition to *Paucilactobacillus*. A group of lactobacilli that can produce exopolysaccharides from sucrose and produce a slimy texture to foods (*limosus* means slimy) make up the genus *Limosilactobacillus*. These bacterial species generally form stable associations with species in the *Lactobacillus* genus. The type species is *Limosilactobacillus fermentum* and also included in the group is *Limosilactobacillus reuteri*. The *Secundilactobacillus* genus is another group of bacteria that, like the *Paucilactobacillus*, have been isolated from hexose-depleted habitats such as silage, beer, liquor mash, and apple cider. They share a common trait with the *Paucilactobacillus* bacteria of not being able to reduce fructose to mannitol and are found as spoilage organisms in fermented foods after the primary fermenting bacteria has depleted the substrate of hexoses. A few species have also been found in silage. The type species is *Secundilactobacillus malefermentans*.

A group of lactobacilli known for their gas-producing ability is the *Levilactobacillus* genus (*levare* means to lift up or release) that have leavening ability as shown in their use in sourdough bread. The type species is *Levilactobacillus brevis*, which has also been associated with unwanted gas production in cheese.

Culumber et al. (2017) found that the incidence is not localized to just the type strain (WDC04) isolated in Utah, but extended across the United States with various strains being isolated from cheeses made in the western and midwestern states, suggesting that *Plb. wasatchensis* is probably ubiquitous in cheese. Further isolates of species closely related to *Plb. wasatchensis* WDC04 have been obtained from cheese produced in the eastern United States and a cheese produced in Ireland (unpublished data). Isolation of *Plb. wasatchensis* and other OHF NSLAB from cheese is hampered because of their slower growth rate than more prevalent NSLAB. In the case of *Plb. wasatchensis*, its optimum growth temperature of ~23°C results in very little growth at the 37°C incubation temperature normally used to enumerate NSLAB, such as *Ltlb. curvatus* or *Lcb. casei*, in cheese.

Paucilactobacillus wasatchensis preferentially metabolizes 5-carbon sugars, such as ribose, using the pentose phosphate pathway (Ortakci et al., 2015a). It can slowly grow by metabolizing 6-carbon sugars such as galactose using the Leloir pathway. When this occurs, a carbon is cleaved off that eventually goes to produce CO₂, which causes gassiness and blowing packages in aging cheese (Ortakci et al., 2015a). Some of the risk factors recently identified for having unwanted

gas production and formation of splits and cracks during storage of Cheddar cheese are (1) the cheese initially having high numbers of NSLAB that include *Plb. wasatchensis* or other OHF lactobacilli; (2) high levels of residual galactose in the cheese that can come from use of *Streptococcus thermophilus* as a component of the starter culture; (3) use of elevated temperature during cheese storage; and possibly (4) high numbers of starter culture in the cheese curd followed by cell lysis that makes ribose and other nutrients available to NSLAB in the cheese (Ortakci et al., 2015a,b,c; Oberg et al., 2016; Culumber et al., 2017).

To minimize the presence of *Plb. wasatchensis* in cheese it is necessary to understand its entry point into the cheese factory, its presence in cheese milk, and its growth characteristics and survival. The most likely origin of *Plb. wasatchensis* on the dairy farm is silage as closely related OHF lactobacilli have been isolated from fermented plant material (Oberg et al., 2016). This organism could then enter the cheese factory in the raw milk, and a previous study (Ortakci et al., 2015a) suggested that 0.003% of the bacterial cells of *Plb. wasatchensis* WDC04 could survive a pasteurization treatment of 15 s at 72°C. Such survival would allow entry of *Plb. wasatchensis* into the cheese processing facility where it could become part of biofilms that then seed NSLAB into milk, whey, and cheese curd. However, in that same study no viable *Plb. wasatchensis* WDC04 was recovered with a pasteurization heat treatment of 63°C for 30 min.

Because one strategy for preventing unwanted gas production in cheese is to keep the numbers of *Plb. wasatchensis* in the cheese low, it is important to know its growth characteristics and carbohydrate sources that can promote its growth in cheese. Ortakci et al. (2015a) and Oberg et al. (2016) have demonstrated the rapid growth of *Plb. wasatchensis* WDC04 on ribose with slower growth on galactose. These 2 sugars are present in cheese from lysis of starter (or adjunct) culture cells and incomplete fermentation of lactose by the starter cultures, respectively. Ribose makes up about 40% of RNA in bacterial cells (Zeibich et al., 2019). Two other materials that could be scavenged and utilized from lysed cells are *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). These are components and building blocks for the peptidoglycan of bacterial cell (glucosamine and NAG are among the most abundant sugars on the planet; Moye et al., 2014) and can provide biofilm bacteria with carbon, nitrogen, and energy.

Our aim was to further characterize the potential carbohydrate sources that would support growth of *Plb. wasatchensis* WDC04 in cheese, to confirm its salt

tolerance and whether it can survive HTST pasteurization. We were also interested in whether the presence of various organic acids in cheese influence *Plb. wasatchensis* WDC04 growth.

MATERIALS AND METHODS

Materials

Chemicals and Media. Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth was from Hardy Diagnostics (Santa Maria, CA) and Becton Dickinson and Co. (Sparks, MD); Oxrase for Broth, sodium chloride, glucose, fructose, 0.2- μ m membrane filters (for filter sterilization), sodium citrate, potassium phosphate, acetate-EDTA, ethanol, citric acid, and dipotassium phosphate were from Fisher Scientific Inc. (Fair Lawn, NJ). Tris-HCl was from Fisher Bioreagents (Pittsburgh, PA); bacteriological agar, proteose peptone no. 3, beef extract, and yeast extract were from Becton Dickinson and Co. Ribose and GasPak EZ pouches were from VWR (Solon, OH); and Tween-80, SDS, NAM, and NAG were from Sigma-Aldrich Inc. (St. Louis, MO). Falcon 48 transparent micro-well plates with lids were from Corning Inc. (Corning, NY). Sodium acetate and ammonium citrate were purchased from Mallinckrodt Baker Inc. (Paris, KY); formic acid was from Mallinckrodt Pharmaceuticals (St. Louis, MO); magnesium sulfate was from Alfa Aesar Inc. (Heysham, UK); manganese sulfate, galactose, and lactose came from J.T. Baker Chemical Co. (Phillipsburg, NJ); lactic, propionic, and acetic acids were from Avantor Performance Materials LLC (Center Valley, PA).

Raw whole milk was from the Caine Dairy Teaching and Research Center (Utah State University, Wellsville) and pasteurized (63°C for 30 min) by the Aggie Creamery (Utah State University). Ultra-high-temperature milk was obtained from Gossner Foods (Logan, UT). Whirl-Pak filtered stomacher bags were purchased from Nasco (Fort Atkinson, WI).

Bacterial Cultures. Working cultures of *Plb. wasatchensis* WDC04 were obtained from frozen stocks (Weber State University, Ogden, UT) and grown in MRS broth supplemented with 1.0 to 1.5% ribose (MRS-R) at 25°C. Every 2 to 3 d, fresh tubes of MRS-R were inoculated with the bacterium to keep the bacterium in logarithmic growth. Frozen cultures were stored at -80°C in 1-mL aliquots supplemented with 20% sterile glycerine. Agar plates were made from MRS-R broth with the addition of 1.5% agar. Spread plates were stored at ~25°C in anaerobic incubation jars with GasPak EZ. Test batches of *Plb. wasatchensis* WDC04 were prepared by adding 4 mL of working cul-

ture into 9 mL of MRS-R and incubated for 2 h, then adjusted to an optical density (OD) at 600 nm of 0.90 for pH, salt, and carbohydrate studies, and 0.25 for studying inhibitory effects of organic acids.

Carbohydrate-Restricted MRS. A stock solution of carbohydrate-restricted MRS (**CR-MRS**) broth was prepared by adding the ingredients listed in Table 1 to deionized water. To simulate potential for growth in a cheese environment, the solution was then adjusted to pH 5.2 with HCl and 4.5% salt added. Twenty percent (wt/vol) stock solutions of each carbohydrate (glucose, lactose, fructose, galactose, ribose, NAG, and NAM) were filter sterilized (0.2- μ m pore size) and then 7.5-mL aliquots were added to 92.5 mL of CR-MRS to produce individual broth solutions containing 1.5% (wt/vol) of a specified carbohydrate.

Thermotolerance

Thermotolerance of *Plb. wasatchensis* was studied using a HTST pasteurizing heat exchanger (model SP15, APV Crepaco, Tonawanda, NY) in the Gary Haight Richardson Dairy Products Laboratory (Utah State University) operating at 20 L/min with regeneration, heating, and cooling sections. It was previously determined that a 30-L aliquot of milk begins to exit the pasteurizer 2 min after its addition to the pasteurizer's constant level tank. Undiluted heat-processed milk could then be obtained between 3 min and 3 min 40 s (Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-18597>).

Four 3-L bottles of sterile MRS broth containing 2% ribose (wt/vol) were inoculated with 1% (vol/vol) *Plb. wasatchensis* culture and incubated for 3 d at 22°C. Then, it was transferred to 250-mL bottles and centrifuged at $9,150 \times g$ for 10 min at 22°C. The pellets from 8 tubes were collected and resuspended in UHT milk and then added to 30-L cold pasteurized milk and held for 1 h to allow *Plb. wasatchensis* bacteria to acclimatize before the milk was heat processed. One 30-L batch of inoculated milk was heat processed for 15 s at each of the following temperatures: 69, 72, 74, and 76°C.

The pasteurizer was run with cold water until steady-state conditions were reached, then the constant level tank was allowed to drain, and then a 30-L batch of cold milk was added to the system, followed by cold water when the milk had drained. A sample of unprocessed milk was retained, and triplicate samples of cooled heat-processed milk were collected as the milk exited the heat exchanger 180, 200, and 220 s after the milk was added to the constant level tank. Dilution series for the milk was performed and samples were plated on MRS-R agar and grown anaerobically at 25°C in jars containing GasPak EZ pouches. Colony counts

Table 1. Carbohydrate-restricted *Lactobacillus* de Man, Rogosa, and Sharpe (MRS) broth based on de Man et al. (1960)

Material ¹	Quantity (g/L)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Sodium acetate	5.0
Ammonium citrate	2.0
Dipotassium phosphate	2.0
Tween-80	1.0
Magnesium sulfate	0.1
Manganese sulfate	0.05

¹Proteose peptone no. 3, beef extract, and yeast extract (Becton Dickinson and Co., Sparks, MD); sodium acetate and ammonium citrate (Mallinckrodt Baker Inc., Paris, KY); dipotassium phosphate (Fisher Scientific Inc., Fair Lawn, NJ); Tween-80 (Sigma-Aldrich Inc., St. Louis, MO); magnesium sulfate (Alfa Aesar Inc., Heysham, UK); manganese sulfate (J.T. Baker Chemical Co., Phillipsburg, NJ).

were performed after 2, 5, and 10 d and the percentage survival of *Plb. wasatchensis* was calculated.

Growth Studies

Growth of *Plb. wasatchensis* WDC04 was studied utilizing an Infinite 200 Pro spectrophotometer (Tecan Production Corp., Chapel Hill, NC) using 48-well plates with lids. For control samples (uninoculated blank), 980 μ L of the appropriate broth was added into a well. For test samples, 930 μ L of broth was added followed by 50 μ L of *Plb. wasatchensis* broth. To help establish anaerobic-like conditions, 20 μ L of Oxyrase solution was added to reduce oxygen levels in the medium (or 20 μ L of sterile deionized water as a comparison). Samples were inoculated in triplicate, along with triplicate uninoculated wells. A lid was placed on the plate, which was then inserted in the spectrophotometer.

The orbital plate shaking was set at 2 s with an amplitude of 3 mm and a frequency of 218 rpm with a settle time of 5 s. Every 60 min during 48 h of incubation, OD was measured using 25 measurements per reading, 4 readings per well in a 2×2 pattern at 2.7 mm from the edge of the well. A 3-h running average was calculated at each time point for each well to smooth the data. Optical density at 4 h was considered as the starting level and subsequent average change in OD during incubation over 48 h was determined after correcting for the average change in OD of the control uninoculated wells. The difference in absorbance for each 2-h period was calculated and the largest value was designated as maximum specific growth rate ($\mu_{\max}$) and expressed as OD/h.

Effect of salt concentration and pH was studied using MRS-R broth containing up to 6% (wt/vol) sodium chloride and adjusted to various pH levels. For carbohydrate utilization, CR-MRS containing 1.5% (wt/vol)

Table 2. Concentrations of organic acids found in aged Cheddar cheese (McMahon et al., 2014)

Acid	Concentration (mM)		
	Low	Mid	High
Lactic	450	505	560
Formic	26	63	100
Propionic	6	38	70
Citric	12	13.5	15
Acetic	2.5	9.8	17

of an individual carbohydrate was used. For organic acids (lactic, formic, propionic, citric, and acetic), 10-fold concentration solutions were prepared at low, mid, and high concentrations (Table 2) based on their measured levels in aged Cheddar cheese (McMahon et al., 2014). In place of some of the MRS-R broth, 100 μ L of the organic acid solution was added using a 0.2- μ m sterile filter.

RESULTS AND DISCUSSION

Pasteurization

No viable *Plb. wasatchensis* were recovered from milk pasteurized at 72, 74, or 76°C with a 15-s hold time, or from milk that was subpasteurization heat treated at 69°C. No colonies were observed on any of the plates (including the 10⁻¹ dilution plate) even when the plates were incubated for 10 d to allow for recovery of any sublethally injured cells. This represents a greater than 8 log reduction of *Plb. wasatchensis* when milk is heated in a commercial-style HTST pasteurizer. Our prior study (Ortakci et al., 2015a) had predicted a 4.5 log reduction, and this difference suggests that the amount of heat exposure in the test-tube experiment was less than what occurs in an industrial setting. In the laboratory model, the *Plb. wasatchensis* cells were added to medium already heated to 72°C and then rapidly cooled after 15 s. In contrast, in the HTST system the cells are exposed to a longer period of heating as the milk passes through the warming regeneration section of the heat exchanger, then are held at the set temperature for 15 s, before being cooled down in the cooling section of the heat exchanger. This time discrepancy was less of an issue when using a 63°C for 30 min pasteurization treatment in which heating and cooling in a test tube also showed no survival after pasteurization in broth containing $\sim 6 \times 10^6$ cfu/mL of *Plb. wasatchensis* WDC04 (Ortakci et al., 2015a). Because *Plb. wasatchensis* does not survive pasteurization, its presence as part of the NSLAB microbiota of cheese appears to be through environmental contamination postpasteurization.

Growth of *Plb. wasatchensis* Using 48-Well Plates

Paucilactobacillus wasatchensis, like other NSLAB found in cheese, is a facultative anaerobic bacteria and, hence, will only grow when oxygen is absent or at very low levels. Because the surface area to volume ratio of 1 mL of liquid in the wells of 48-well plates is relatively high compared with bottles and test tubes, the dissolved oxygen in the medium slows its growth. Using Oxyrase to create a micro-anaerobic environment by chemically removing oxygen increased both the rate and extent of growth of *Plb. wasatchensis* (Figure 1) under these experimental conditions. After 48 h of incubation, the OD increased from 1.2 to 1.5 when Oxyrase had been added to the medium. Adding Oxyrase also decreased the variation among replicate trials especially when the growth rate was slower (data not shown). After 48 h of incubation, standard error of means were as high as 0.075 when Oxyrase was not used and were only 0.02 to 0.03 with Oxyrase.

During the first few hours of incubation in the 48-well plates, sometimes the OD increased or decreased, especially when additives such as the organic acids were added to the medium. There is only marginal growth of *Plb. wasatchensis* at the beginning of incubation (not enough to affect OD readings), so the OD at 4 h was considered as the baseline for comparing with later OD readings. Any changes in OD before 4 h were considered to be the test medium and added components coming to equilibrium.

Figure 1. Growth of *Paucilactobacillus wasatchensis* at 25°C with (solid line) and without (dashed line) Oxyrase (Fisher Scientific Inc., Fair Lawn, NJ) added to de Man, Rogosa, and Sharpe (MRS)-ribose broth, pH 5.2, in 48-well plates based on optical density measured at 600 nm (OD₆₀₀), error bars = SE, n = 3.

pH

Growth of *Plb. wasatchensis* was similar in the range of pH 6 to pH 7 with mean $\mu_{\max} = 0.16$ OD/h. Lowering the pH to that typical of Cheddar cheese (pH 5.2 to 5.0) slowed down its growth with mean $\mu_{\max}$ dropping to 0.11 and 0.085 OD/h, respectively (Figure 2). After 48 h of incubation at 25°C, this slowing of growth was such that OD only reached 1.35 and 1.22, respectively, compared with 1.67 at pH 6.0. When the pH of the MRS-R broth was further lowered, there was acid inhibition of *Plb. wasatchensis* with only marginal growth occurring at pH 4 (Figure 2). Because fermentation of ribose by *Plb. wasatchensis* results in production of lactic acid and a lowering of pH, it may be that when it is grown in MRS-R broth adjusted to pH 5.2 or 5.0 some acid inhibition may occur during continued incubation. In this experiment, incubation was only conducted for 48 h, and the stationary phase, which occurs when ribose is depleted (Ortakci et al., 2015a), had not been reached. It is possible that if the medium was more strongly buffered to prevent acid inhibition then the same final OD would be reached as when it is grown at neutral pH but takes a longer time.

When the medium was adjusted to pH 8.0, growth was slightly faster ($\mu_{\max} = 0.17$ OD/h) and to a greater extent (final OD = 1.77 after 48 h) compared with 1.67 at pH 7.0 (Figure 3). This is probably a function of the pH of the medium remaining slightly higher during incubation. Increasing pH to 9.0 resulted in an extension of the lag phase in which no appreciable increase in OD was observed from 4 h to almost 24 h. However, during the next 24 h, the growth in *Plb. wasatchensis* was the fastest ($\mu_{\max} = 0.18$ OD/h) and it reached a similar OD level after 48 h. At pH 9 the bacterial cells would be under alkaline stress and the longer lag time may reflect an induced change in cellular metabolism to maintain a neutral intracellular pH. Also, during the initial slow growth of *Plb. wasatchensis*, enough lactic acid may have been produced to lower the extracellular pH to a level where normal growth could occur.

Salt Tolerance

When *Plb. wasatchensis* was grown in MRS-R at pH 6.5, addition of 5.4% salt strongly inhibited growth such that OD after 46 h of incubation at 25°C was only about 0.18. In comparison, when no salt was added growth was rapid and OD reached a level of 1.85. Adding more salt further suppressed growth so that at 5.5% salt the OD only reached 0.16 and with 6.0% salt it only reached 0.07. This is different than previously observed by Ortakci et al. (2015a) in which adding salt up to 5% slowed growth of *Plb. wasatchensis* WDC04 but

Figure 2. Growth of *Paucilactobacillus wasatchensis* at 25°C in de Man, Rogosa, and Sharpe (MRS)-ribose broth at pH 6.0, 5.2, 5.0, and 4.0 in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, n = 3.

all samples reached the same OD after 60 h of incubation. This may reflect differences in the environment within the medium when using test tubes containing 10 mL of medium compared with 1 mL in microwells. It may be that even with the addition of Oxyrase, the microwells are still more hostile for supporting growth of *Plb. wasatchensis* because of trace amounts of dissolved oxygen remaining.

Figure 3. Growth of *Paucilactobacillus wasatchensis* at 25°C in de Man, Rogosa, and Sharpe (MRS)-ribose broth at pH 7.0, 8.0, and 9.0 in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, n = 3.

Acidifying the MRS-R broth to make it more similar to cheese (pH 5.2 to 5.5) slows down the growth rate of *Plb. wasatchensis* (Figure 2), but the adaption made to cope with this acid stress apparently increases the ability of cells to deal with salt-induced stresses. The production of pH-stress proteins would provide cross protection against other stresses, which could explain increased growth being observed at cheese pH conditions when salt was added compared with growth at neutral pH.

In cheese, the salt-in-moisture (S/M) concentration varies considerably between blocks of cheese with standard deviations for salt content often being 0.15 percentage units. That is, even with a cheesemaking process with a target salt content of 1.7%, only 68% of the cheese blocks would be expected to be in the salt range of 1.55% to 1.95% salt. Assuming a normal distribution, 13% of the cheese blocks would have salt contents between 1.40% and 1.55% and 2% would only have from 1.25% to 1.40% salt. If the target moisture of the cheese was 38%, then this corresponds to cheeses with S/M levels of about 4.5% (target cheese), 3.9%, and 2.6%. Given that less whey is expelled when the salt content is low, the cheeses at the bottom end of the salt range would be even lower in S/M concentration.

When S/M levels of 3.5, 4.5, and 5.5% salt were tested as representing cheeses that were low, medium, and high in salt, respectively, it was clear that cheeses that are low in salt will promote more growth of NSLAB such as *Plb. wasatchensis* (Figure 4). Adding 3.5% salt in MRS-R broth at pH 5.2 resulted in little suppression of growth, with the OD reaching 1.2 after 46 h at 25°C compared with an OD of 1.5 for the control. With 4.5% salt, growth of *Plb. wasatchensis* was further repressed such that OD reached 0.9 and with 5.5% salt the OD after 46 h was only 0.5. Increasing the pH to 5.5 reduced the amount of acid stress and the growth rate of *Plb. wasatchensis* was slightly higher (Table 3). At higher salt levels, growth was further retarded, but even at a 6.0% salt concentration, complete inhibition of *Plb. wasatchensis* did not occur (Figure 5). Thus, although having lower salt levels in cheese is a risk factor for growth of *Plb. wasatchensis* and consequent potential unwanted gas production with formation of slits and cracks in the cheese, having high levels of salt will not prevent *Plb. wasatchensis* from growing during extended storage of Cheddar cheese.

Carbohydrate Utilization

As shown in previous studies (Ortakci et al., 2015a) the most rapid growth of *Plb. wasatchensis* occurs when ribose is available as an energy source. This was appar-

Figure 4. Inhibition of growth of *Paucilactobacillus wasatchensis* at salt levels typical of Cheddar cheese (3.5, 4.5, and 5.5% salt-in-moisture) at 25°C in de Man, Rogosa, and Sharpe (MRS)-ribose broth at pH 5.2, in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, $n = 3$.

ent when *Plb. wasatchensis* was grown in CR-MRS adjusted to cheese-like conditions (pH 5.2 and 4.0% salt) with ribose, galactose, or lactose as the carbohydrate (Figure 6). With ribose, $\mu_{\max}$ was 0.087 OD/h and the OD reached 1.2 in 46 h. In comparison, growth with galactose was slower ($\mu_{\max} = 0.038$ OD/h) and after 46 h only reached an OD of 0.06. This slower growth has been attributed to the need of the cells to re-oxidize NADH formed when galactose is converted to a 5-carbon sugar phosphate via the Leloir pathway so it can be fermented for the production of ATP in the pentose phosphate pathway (Ortakci et al., 2015a).

When lactose was the only carbohydrate available its growth rate was similar to that with galactose (Figure 6) but growth of *Plb. wasatchensis* stopped when the OD reached 0.3. This implies that the cells ran out of energy sources after 24 h. Similar behavior had been previously observed (Ortakci et al., 2015a) when *Plb.*

Table 3. Maximum specific growth rate ($\mu_{\max}$) for growth of *Paucilactobacillus wasatchensis* at 25°C in de Man, Rogosa, and Sharpe (MRS) broth containing 1.5% ribose at pH 5.2 and 5.5 based on salt concentration¹

Salt concentration (%)	$\mu_{\max}$ (OD ₆₀₀ /h)	
	pH 5.2	pH 5.5
0	0.119	0.174
3.0	0.077	0.101
4.5	0.056	0.071
5.5	0.034	0.040

¹OD₆₀₀ = optical density measured at 600 nm.

Figure 5. Growth of *Paucilactobacillus wasatchensis* at high salt levels from 5.5% to 6.0% at 25°C in de Man, Rogosa, and Sharpe (MRS)-ribose broth at pH 5.2, in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, $n = 3$.

wasatchensis was grown in different concentrations of ribose or galactose. When grown on CR-MRS with 0.1, 0.5 or 1.0% ribose, the growth rates were all the same and the difference was that exponential growth phase finished earlier at the lower ribose concentrations. This suggests that *Plb. wasatchensis* cells cannot use more than 0.1% ribose (more likely less than that) at a time so higher ribose concentrations just support growth for a longer time. This limitation of *Plb. wasatchensis* to

Figure 6. Growth of *Paucilactobacillus wasatchensis* at 25°C in carbohydrate-restricted de Man, Rogosa, and Sharpe (MRS) broth (pH 5.2, 4.0% salt) containing 1.5% ribose, galactose, or lactose in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, $n = 3$.

grow rapidly is evident when grown on MRS-R agar because other NSLAB form large colonies (~2 mm) within 48 h, whereas colonies of *Plb. wasatchensis* are barely visible at 24 h and require an additional 2 to 3 d of incubation to form countable colonies.

Growth of *Plb. wasatchensis* on CR-MRS containing only glucose (Figure 7) was similar for growth on lactose. It had a similar rate of growth ($\mu_{\max} = 0.033$ OD/h) and also stopped growing after 24 h when the OD reached ~0.3. Why less growth would occur on glucose compared with galactose was unexpected and followed the same pattern as growth on lactose (Figure 6). Utilization of galactose for energy or cell wall production was assumed to proceed with the initial conversion of galactose to 6-P-glucose. Why this should be limited when glucose is available (including through hydrolysis of lactose) is unclear. In contrast, growth of *Plb. wasatchensis* with fructose as the carbohydrate source (Figure 7) continued through the 46 h of incubation but slowed down after 24 h and only reached an OD of 0.45.

Another sugar that will support growth of *Plb. wasatchensis* is NAG, which along with NAM and associated peptides, are components of the bacterial cell wall. These 2 sugars are β -linked in an alternating pattern to form the mesh-like polymeric structure of peptidoglycan. When *Plb. wasatchensis* was grown in 1.5% NAG added to CR-MRS, its growth pattern was similar to that of fructose with $\mu_{\max}$ of 0.033 OD/h and growth slowing down after 24 h but continuing through

Figure 7. Growth of *Paucilactobacillus wasatchensis* at 25°C in carbohydrate-restricted de Man, Rogosa, and Sharpe (MRS) broth (pH 5.2, 4.0% salt) containing 1.5% glucose, fructose, or *N*-acetylglucosamine (NAG) in 48-well plates with optical density measured at 600 nm (OD_{600}), error bars = SE, $n = 3$.

46 h (Figure 7). No growth of *Plb. wasatchensis* was observed when NAM was the only sugar.

In a cheese environment, lysis of starter culture cells after salting of the curd (and during pressing and storage of the cheese) would provide *Plb. wasatchensis* with at least 2 energy sources: ribose and NAG. It may be that as with other bacteria that have evolved to grow in nutrient fluctuating environments such as agricultural silage or even the human oral cavity (Moye et al., 2014), *Plb. wasatchensis* uses amino-sugars such as NAG for its own growth and it may also act to repress its own catabolic processes (Dobrogosz, 1968). Further studies are needed to determine if NAM could be used to support growth when ribose is also present to supply energy needs and then NAM and NAG could be used to support cell wall growth and cell division in the same way that galactose appears to do when ribose is also available (Ortakci et al., 2015a).

During Cheddar cheese manufacture, the starter culture lactococci reaches up to 10^8 to 10^{10} cells/g in the cheese before salting (Visser, 1977; Jordan and Cogan, 1993; Fenelon et al., 2000; McMahon et al., 2014). After salting, the number of starter culture cells in the cheese can decrease by a log within a few days. Based on the bacterial cells weighing about 6.4×10^{-13} g (Milo and Phillips, 2019) and consisting of 80% moisture, and RNA being 20% of the dry mass of bacterial cells, the amount of RNA released into the cheese serum if there were initially 10^{10} cells/g would be about 0.23 mg/g of cheese. In a cheese with 37% moisture this would provide a ribose concentration to support NSLAB growth of about 0.25 mg/mL (0.025%). Although this is much lower than the levels in MRS-R broth, given the longer time involved (60 d compared with 48 h) it would still support growth of *Plb. wasatchensis* and other NSLAB.

Cheese has a relative abundance of amino acids from the hydrolysis of the casein proteins by residual coagulant along with the starter culture and NSLAB proteases and peptidases. In addition to ribose, NAG and

other cell components from starter culture lysis would also be available. The number of starter culture cells present in the cheese curd before salting can then contribute to NSLAB growth. In previous studies (McMahon et al., 2014), we have typically observed lactococcal starter culture numbers before salting of 10^9 cfu/g with a drop to 10^8 cfu/g after pressing. For such cheese, the ribose level upon autolysis of starter cultures would only be 0.0025% and less growth of NSLAB would be supported. Adding an adjunct starter culture such as *Lactobacillus helveticus* that also undergoes lysis during storage would also add additional cellular components to support growth of NSLAB.

Ribose, along with other scavenged biocellular nutrients such as NAG as well as any residual galactose and lactose, could then support slow but ongoing growth of *Plb. wasatchensis* in the cheese. When the number of *Plb. wasatchensis* cells reaches a high level and 6-carbon sugars are used for energy, enough carbon dioxide can be produced to cause slits and cracks in the cheese.

Organic Acids

The effect of adding organic acids to MRS-R medium on growth of *Plb. wasatchensis* varied depending on the type and amount of acid added (Table 4). The level of acid added had been selected based upon our observation of the ranges at which they naturally occur in cheese (McMahon et al., 2014). In general, more inhibition of growth was observed when the medium was initially adjusted to pH 5.1. This can be considered as a reduction of growth of *Plb. wasatchensis* as pH falls below pH 5 and was most evident with organic acids added at higher molarity levels. In this part of our study, when *Plb. wasatchensis* WDC04 was grown in 48-well plates using MRS-R broth at pH 7.0 or 5.1, the OD increased about 0.6 units over 40 h (data not shown). The largest effect was when lactic acid with growth dropping by about 70% at pH 7 and virtually

Table 4. Extent of suppression of growth of *Paucilactobacillus wasatchensis* at 25°C in de Man, Rogosa, and Sharpe (MRS) broth containing 1.5% ribose in presence of organic acids at the low, middle, and high levels observed in cheese (see Table 2)

Acid (%)	Inhibition					
	pH 7.0			pH 5.1		
	Low	Mid	High	Low	Mid	High
Lactic	70	74	66	93	91	99
Formic	10	68	86	34	75	91
Propionic	14	–31	–17	–11	8	76
Citric	60	69	21	90	88	69
Acetic	–12	–22	–1	–7	–2	26

no growth at pH 5.1 medium when 560 mM lactic acid was added. Although these are the levels of lactic acid in cheese, the pH is buffered by the proteins and phosphate and the cheese remains above pH 5.1 (Ortakci et al., 2015b).

Adding 26 mM formic acid slightly inhibited *Plb. wasatchensis* growth (10 and 34% at pH 7 and 5.1, respectively) and increased as formic acid addition increased to 100 mM reached about 86% suppression at pH 7 and 91% at pH 5.1. Adding propionic acid appears to be stimulatory to growth of *Plb. wasatchensis* and showed negative inhibition at pH 7 with this effect being more apparent when 38 and 70 mM propionic acid was added. At pH 5.1, adding propionic acid at 70 mM apparently caused sufficient drop in pH to result in 76% inhibition of *Plb. wasatchensis* growth. In contrast, 12 to 15 mM addition of citric acid caused inhibition at both pH 7 and pH 5.1, suggesting its calcium chelating properties influenced growth. This is probably not an issue in cheese as there is an abundance of free calcium ions even when this amount of citrate is present. Adding acetic acid had a similar effect to adding propionic acid and either had a stimulating effect on growth at pH 7. A slight inhibition occurred with the highest acetic acid addition (17 mM) into the pH 5.1 medium.

CONCLUSIONS

When milk was processed through a HTST pasteurizer, no survival of *Paucilactobacillus wasatchensis* WDC04 was detected with an 8-log reduction in cfu/mL occurring using pasteurization (72°C for 15 s) or sub-pasteurization heating at (69°C for 15 s). Lowering pH to 5.1 and increasing salt comparable to 6% S/M slows but does not stop growth of *Plb. wasatchensis* WDC04, so, if present as part of the NSLAB population in cheese, it can still grow. In addition to ribose and galactose, *Plb. wasatchensis* WDC04 can also utilize glucose, fructose, lactose, and NAG to support growth. Growth of *Plb. wasatchensis* WDC04 was fastest with ribose, whereas growth rates with glucose, fructose, lactose, and NAG were similar to that with galactose. Both residual galactose and lactose in cheese can help support growth and could lead to unwanted gas production. Unwanted gas production in cheese is likely to occur when *Plb. wasatchensis* reaches high numbers and there is still residual galactose in the cheese. This and previous studies indicate that the risk factors for having slits and cracks in Cheddar cheese are (1) high initial NSLAB numbers that include *Plb. wasatchensis* or similar OHF lactobacilli, (2) high starter culture numbers at the end of cheesemaking followed by lysis of cells, which makes ribose and NAG available to sup-

port growth of *Plb. wasatchensis*, (3) low salt levels in the cheese, (4) high cheese pH, (5) residual galactose in the cheese, such as from use of *Strep. thermophilus* as part of the starter culture, and (6) elevated storage temperatures to accelerate cheese aging.

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