



Hot topic: Geographical distribution and strain diversity of *Lactobacillus wasatchensis* isolated from cheese with unwanted gas formation

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ABSTRACT

Lactobacillus wasatchensis, an obligate heterofermentative nonstarter lactic acid bacteria (NSLAB) implicated in causing gas defects in aged cheeses, was originally isolated from an aged Cheddar produced in Logan, Utah. To determine the geographical distribution of this organism, we isolated slow-growing NSLAB from cheeses collected in different regions of the United States, Australia, New Zealand, and Ireland. Seven of the cheeses showed significant gas defects and 12 did not. Nonstarter lactic acid bacteria were isolated from these cheeses on de Man, Rogosa, and Sharpe medium supplemented with ribose, a preferred substrate for *Lb. wasatchensis*. Identification was confirmed with 16S rRNA gene sequencing and the API50CH (bioMérieux, Marcy l'Etoile, France) carbohydrate panel. Isolates were also compared with one another by using repetitive element sequence-based PCR (rep-PCR). *Lactobacillus wasatchensis* was isolated only from cheeses demonstrating late-gas development and was found in samples from 6 of the 7 cheeses. This supports laboratory evidence that this organism is a causative agent of late gas production defects. The rep-PCR analysis produced distinct genetic fingerprints for isolates from each cheese, indicating that *Lb. wasatchensis* is found in several regions across the United States and is not a local phenomenon.

Key words: gas defect, *Lactobacillus wasatchensis*, nonstarter lactic acid bacteria

Hot Topic

It was recently reported that a new species of nonstarter lactic acid bacteria (NSLAB) was isolated from cheese and implicated in unwanted gas formation during the storage of Cheddar cheese (Ortakci et al.,

2015b,c). This obligatory heterofermentative (OHF) lactobacillus was designated as *Lactobacillus wasatchensis* (Oberg et al., 2016); it co-utilizes ribose and galactose (Ortakci et al., 2015a) to support growth, and produces CO₂ when 6-carbon sugars are present and utilized for energy production. The following risk factors for unwanted gas production and formation of splits and cracks during storage of Cheddar cheese were identified:

- Presence of high levels of *Lb. wasatchensis* as part of the NSLAB population in the cheese (Ortakci et al., 2015a)
- Presence of high levels of residual 6-carbon sugars in the cheese (Ortakci et al., 2015b)
- Use of *Streptococcus thermophilus* in the starter culture used for making Cheddar cheese (Ortakci et al., 2015c)
- High levels of starter culture lysis during the early stages of cheese storage (Ortakci et al., 2015a)
- Use of elevated storage temperatures to accelerate cheese ripening (Ortakci et al., 2015b)

Lactobacillus wasatchensis uses ribose as a preferential carbohydrate and forms very small pinpoint colonies after 48 h on de Man, Rogosa, and Sharpe (MRS) agar supplemented with 1.5% ribose (MRS+R). Because enumeration and identification of NSLAB has generally been restricted to species that form easily observed colonies within 2 d at 30 or 37°C on MRS or Rogosa agar with glucose as the sole carbohydrate source (Oberg et al., 2011), *Lb. wasatchensis* and other slow-growing OHF lactobacilli have been overlooked in past attempts to identify the cause of unwanted gas problems and textural defects in aging Cheddar cheese.

Isolation and enumeration of *Lb. wasatchensis* as a NSLAB can be problematic because (1) it grows slower than facultative heterofermentative (FHF) NSLAB; (2) it requires the medium to be supplemented with a 5-carbon sugar such as ribose for growth; and (3) its preferred temperature for growth is ~23°C, with

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only marginal growth at 37°C (the typical incubation temperature used for enumerating NSLAB; Oberg et al., 2016). The current method for enumerating *Lb. wasatchensis* involves serially diluting a cheese homogenate and plating on MRS+R agar followed by anaerobic incubation at 25°C. Colonies of rapidly growing FHF NSLAB appearing after 2 d are marked on the Petri plates and then the plates are re-incubated at 25°C for another 5 d. Newly appearing colonies that match the appearance of *Lb. wasatchensis* [white, punctiform to small (0.05 to 1 mm in diameter), circular colonies with a smooth texture] can then be counted (Ortakci et al., 2015a).

Because *Lb. wasatchensis* was originally isolated from aged Cheddar cheese manufactured in the creamery at Utah State University (Logan), it is not known whether its presence in cheese is widespread or whether it is a local phenomenon related to the microbiota within a single facility. It has been reported that NSLAB in cheese include mostly FHF lactobacilli such as *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*, and occasionally OHF lactobacilli, such as *Lactobacillus brevis* and *Lactobacillus fermentum* (Fox et al., 1998; Crow et al., 2001). Species and strains can vary between countries, between factories, within a factory, across vats, and even within a block of cheese during ripening (Williams and Banks, 1997; Crow et al., 2001, 2002; Fitzsimons et al., 2001).

The NSLAB are either concomitant in milk or enter the cheese during processing due to contamination from the production environment (Peterson and Marshall, 1990; McSweeney et al., 1993). Initially, NSLAB are found in low numbers but surpass the declining numbers of starter lactococci during aging of Cheddar cheese. The conditions during semi-hard cheese ripening restrict the growth of many bacteria, with a temperature between 6 and 12°C, pH between 4.8 and 5.6, and a salt-in-water concentration ranging from 3.5 to 5.5%. In addition, lactose has been consumed, which limits microbial energy source options to other molecules such as citrate, nucleic acids, proteins, and lipids that remain in the cheese (Martley and Crow, 1993; Fox et al., 1998; Williams et al., 2000). Starter cultures are less likely to thrive in these conditions (especially at the higher salt levels), whereas the more oligotrophic FHF NSLAB are better adapted to the cheese environment.

Late gas formation (“gassy defect”) in Cheddar cheese continues to be a concern to cheese manufacturers, especially during accelerated ripening. It tends to be sporadic but recurrent and it has likely been experienced in most cheese-making plants (Mullan, 2000). Slits and cracks are usually not evident until the cheese is graded and, although it may not create a specific

sensory defect, it can affect cutting and slicing of the cheese. If the defect is severe, the block can crumble upon cutting, increasing cutting losses from 10% (non-defective) up to 50% (Donnelly et al., 2014). Such cheese is downgraded and sold at a lower price.

Seven cheeses with obvious unwanted gas production (puffy bags) were obtained from the manufacturers or purchased in supermarkets, along with 12 cheeses with no apparent gassiness that had been manufactured in Australia, Ireland, New Zealand, and the United States. For each sample, an 11-g piece of cheese was placed in a bag with 99 mL of sterile 2% (wt/vol) sodium citrate and homogenized in a stomacher (Stomacher 400 Circulator, Seward Ltd., West Sussex, UK) at 260 rpm for 4 min. The homogenate was serially diluted and plated on MRS+R agar and then incubated at 25°C under anaerobic conditions using a GasPak EZ pouch system (Becton Dickinson Inc., Sparks, MD). The rapidly growing NSLAB colonies were identified as described above. After another 5 d of incubation, newly appearing colonies that matched the appearance of *Lb. wasatchensis* (Oberg et al., 2016) were selected, streaked to check for purity on MRS+R agar, and then grown in MRS+R broth. Between 5 and 15 isolates were sampled from each cheese from the 10⁻³ to 10⁻⁷ dilutions.

The DNA from these isolates was extracted using the MoBio UltraClean 129 Microbial DNA Isolation Kit (#12224, MoBio, Carlsbad, CA). The 16S rRNA gene was amplified using the bacteria-specific primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-3'). Each 50-μL reaction contained 200 nmol/L of each primer, 200 μM of the PCR nucleotide mix (Promega Corp., Madison, WI), 1 U of GoTaq Taq DNA Polymerase (Promega Corp.), 10 μL of 5× GoTaq reaction buffer (Promega Corp.), and 2 μL of DNA template. The amplification parameters were 94°C for 3 min; 25 cycles consisting of 94°C for 45 s, 57°C for 1 min, 72°C for 2 min; and a final extension step at 72°C for 7 min. The PCR products were purified and precipitated using ethanol extraction and resuspended in sterile water. Sequencing was done at the Idaho State University Molecular Research Core Facility (Pocatello). MacVector (MacVector Inc., Apex, NC) was used for sequence editing and alignment. Sequences were compared with the National Center for Biotechnology Information (NCBI) Genbank database (<https://www.ncbi.nlm.nih.gov/genbank/>) using nucleotide BLAST against the 16S ribosomal RNA database (<https://blast.ncbi.nlm.nih.gov>) and against the 16S rRNA gene of *Lb. wasatchensis* WDC04 (GenBank accession number AWTT000000000). Isolates that had 100% sequence identity with the WDC04 16S rRNA gene were designated as *Lb. wasatchensis*.

Table 1. Slow-growing species of lactic acid bacteria (LAB) isolated from gassy cheeses based on 16S rRNA gene sequencing

Cheese type	Location	<i>Lactobacillus wasatchensis</i>	Other slow-growing LAB
Flavored Cheddar	West	Yes	<i>Lactobacillus paracasei</i>
Flavored Cheddar	West	Yes	<i>Lactobacillus casei</i>
Aged Cheddar	West	Yes	<i>Lactobacillus buchneri</i>
Cheddar	West	Yes	<i>Lactobacillus rhamnosus</i>
Extra-sharp Cheddar	Midwest	No	<i>Lb. casei</i>
Dill Havarti	Midwest	Yes	<i>Lb. casei</i> <i>Lb. paracasei</i> <i>Lactobacillus curvatus</i>
Monterey Jack	Midwest	Yes	<i>Lb. casei</i> <i>Lactococcus lactis</i>

Six of the 7 gassy cheeses contained *Lb. wasatchensis* (Table 1). This included 4 cheeses manufactured in western states and 2 from the Midwest. These isolates were also screened for carbohydrate use with the API 50CH carbohydrate panel (bioMérieux Inc., Marcy l'Etoile, France), and all *Lb. wasatchensis* isolates only tested positive for utilization of ribose. In comparison, isolates that were not *Lb. wasatchensis*, including *Lb. curvatus* WSU1, which was isolated from the same cheese as WDC04, tested positive for utilization of ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdaline, cellobiose, β -gentiobiose, and D-tagatose.

None of the isolates from the non-gassy cheeses were identified as *Lb. wasatchensis*. This does not imply an absence of *Lb. wasatchensis* because the current detection method for isolating *Lb. wasatchensis* requires that it be present in the cheese at a level not less than ~ 1.5 log cfu/g of the predominant faster-growing NSLAB. And because the non-gassy cheeses examined were aged

cheeses and had fast-growing NSLAB, with levels $\geq 10^6$ cfu/g, any slow-growing NSLAB such as *Lb. wasatchensis* needed to be at levels of $\sim 10^5$ or higher for any colonies to be apparent after the second incubation. In addition to *Lb. wasatchensis*, some of the slow-growing isolates were identified as *Lb. casei*, *Lb. paracasei*, *Lb. curvatus*, *Lb. rhamnosus*, *Lactobacillus buchneri*, and *Lactococcus lactis* (Table 1).

After finding that *Lb. wasatchensis* was present as an unwanted gas-forming bacterium in a wide geographical region in the United States, an investigation was conducted into its strain diversity. The DNA from the *Lb. wasatchensis* isolates were subjected to repetitive element sequence (rep)-PCR analysis using the (GTG)₅ primer (which anneals to repetitive elements interspersed in the *Lactobacillus* genome) and then visualized via agarose gel electrophoresis (Figure 1). The resulting banding patterns, or fingerprints, were analyzed using BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was

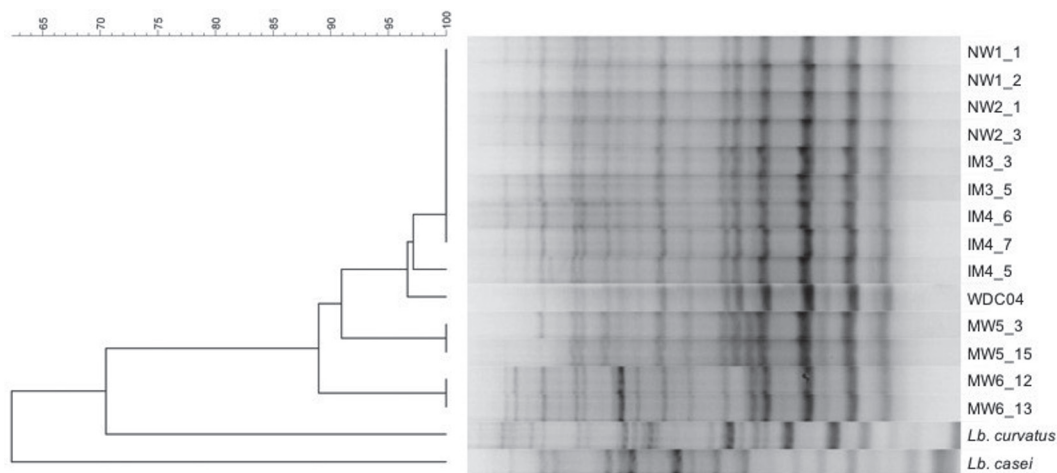


Figure 1. Cluster analysis of repetitive element sequence (rep)-PCR fingerprint patterns obtained using (GTG)₅ primers of *Lactobacillus wasatchensis* isolates from gassy cheeses manufactured in the western (IM3, IM4, NW1, NW2) and Midwest (MW5, MW6) regions of the United States compared with the type strain WDC04 as well as *Lactobacillus curvatus* and *Lactobacillus casei*.

performed using Pearson correlation, and an average linkage (UPGMA, unweighted pair group method with arithmetic mean) dendrogram was derived from the gel profiles. Distinct genetic fingerprints for the isolates were obtained, with the newly isolated strains of *Lb. wasatchensis* from cheese manufactured in the western region being identical or very similar (>95% similarity) to type strain WDC04. The exception was isolate IM4_5, which was closely related but not identical to the other isolates from the same cheese sample. *Lactobacillus wasatchensis* strains in that cheese may have some variation within the genomic sequence. Strains of *Lb. wasatchensis* isolated from gassy cheeses manufactured in the Midwest were different (~90% similarity) from WDC04 as well as from each other. Multiple isolates from an individual cheese showed only one strain present in each of the cheeses.

In conclusion, our investigation has shown that *Lb. wasatchensis* is not just a local phenomenon related to where it was first isolated but has a more general occurrence in cheese. It can be considered the presumptive bacterium responsible for unwanted gas production observed in cheese from several states across the United States. Because the detection limit in aged cheese using this double-incubation isolation method is $\sim 10^5$ cfu/g, these gassy cheeses had a high level of contamination initially or considerable growth of *Lb. wasatchensis* occurred during storage. In the creamery from which WDC04 was first isolated, the d-1 levels of total NSLAB as well as *Lb. wasatchensis* in cheese are typically $< 10^2$ cfu/g. The detection level for *Lb. wasatchensis* becomes higher as the cheese is aged and the fast-growing NSLAB increase in numbers. Hence, not being able to isolate *Lb. wasatchensis* from non-gassy cheeses does not preclude its presence at lower numbers in those cheeses. Further genomic analysis may allow us to trace the evolutionary spread of this organism between regions and manufacturing facilities. The source of *Lb. wasatchensis* contamination remains unknown, but possible sources include silage, milk, and contaminated processing equipment. This organism has the potential to have a significant commercial impact on the cheese industry, and understanding its biology and ecology will be necessary to implement effective controls.

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