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Christopher S. Baird for the degree of Master of Science in Food Science & Technology presented on December 12, 2016.

Title: Contribution of Farm Level Milk Sourcing on Non-Starter Lactic Acid Bacteria (NSLAB) in Cheddar Cheese, and the Assessment of High Resolution Melt Analysis used for Species Identification.

Abstract approved:

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Non-starter lactic acid bacteria (NSLAB) are found at low levels in fresh raw milk and are important to the dairy industry because of their potential impact on the flavor and texture of yogurt, sour cream, and cheese. The purpose of this study was to evaluate three methods for the identification of NSLAB and investigate NSLAB contribution from raw milk sourced from different farms on the microbiological profile of Cheddar cheese during aging. Three methods were evaluated: P1V1/P2V1 PCR with HRM analysis, 16s rDNA sequencing using MicroSEQ® 500, and API 50 CHL. HRM analysis and API 50 CHL were evaluated using NSLAB reference strains which included *Lb. paracasei, Lb. casei, Lb. plantarum, Lb. rhamnosus,* and *L. lactis* subsp. *lactis*. In addition, 16s rDNA sequencing and HRM analysis of the P1V1/P2V1 amplicon were used for the identification of unknown isolates from raw milk and 6.5 mo aged Cheddar cheese. Five out of seven reference strains were correctly identified using API 50 CHL. HRM analysis was in agreement with 16s rDNA sequencing 75% and 66% of the time for *L. lactis* and *Lb. paracasei,*

respectively. Melt curves of isolates identified as *Lb. paracasei* and *L. lactis* subps. *lactis* by 16s rDNA sequencing showed a significant amount of overlap. Farm level contribution of NSLAB was investigated by making Cheddar cheeses using a standardized recipe with raw milk sourced from dairies on the Oregon Coast and in the Willamette Valley and aged. Isolates were selected for preliminary speciation using HRM analysis and further subtyped using a second HRM repetitive sequence-based polymerase chain reaction (rep-PCR). *Lb. paracasei/L. lactis* were identified in raw milk and cheeses sourced from the Oregon Coast and from the Willamette Valley. *Lb. curvatus* was only identified in raw milk samples. Species diversity decreased throughout aging in all cheeses with the exception of cheese made from milk sourced from the southern Oregon Coast. After 6.5 mo of aging, the predominant species across all cheeses was *Lb. paracasei/L. lactis* (70.79% of isolates). Strain diversity was highest in milk sourced from the northern Oregon Coast. 16S rDNA sequencing identified additional species not reported using HRM analysis which included *Lb. brevis, L. lactis cremoris, E. saccharolyticus, E. faecalis, S. bovis, S. chromogenes, Leuc. lactis,* and *W. paramesenteroides*. Based on comparison to 16s rDNA identification, HRM analysis of the P1V1/P2V1 amplicon is not suitable for unknown NSLAB identification. For NSLAB contribution, evidence suggests that milk sourcing at the farm level contributes to the strain diversity of NSLAB present in raw milk and Cheddar cheese. This information can help larger producers understand the contribution their farmers have on their product and steps they may need to take to mitigate it or potentially capitalize on the differences in a small batch capacity.

Contribution of Farm Level Milk Sourcing on Non-Starter Lactic Acid Bacteria
(NSLAB) in Cheddar Cheese, and the Assessment of High Resolution Melt Analysis
used for Species Identification.

by
Christopher S. Baird

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Christopher S. Baird, Author

ACKNOWLEDGEMENTS

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1. Introduction

Non-starter lactic acid bacteria (NSLAB) are a group of bacteria that include lactobacilli or lactococci which have not been intentionally added to milk and dairy products. In cheese, NSLAB have been found to be an important part of the microflora because of their impact on texture and flavor (Chou et al., 2003; Ortacki et al., 2015; O'Sullivan et al., 2016). The impact of NSLAB on cheese quality can be either positive or negative depending on the species present. Specific species of NSLAB have been found to reduce bitterness (Oberg et al., 2011), increase buttery aroma (Milesi et al., 2010), or in general improve the sensory quality of cheese (Lynch et al., 1999). Rather than acting alone, NSLAB have been found to utilize byproducts produced by other NSLAB or starter culture to impact cheese quality. An example is when *Streptococcus thermophilus* is used as part of the starter culture with *Lactobacillus curvatus* present in the milk. *S. thermophilus* uses lactose and produces galactose as a byproduct (Michel et al., 2001) which in turn can be utilized by strains of *Lb. curvatus*. Utilizing galactose, *Lb. curvatus* releases CO_2 gas which forms undesirable slits in cheese. (Porcellato et al., 2015). Situations like this caused by *Lb. curvatus* and *S. thermophilus* lead to economic loss making it important to isolate and identify NSLAB as part of quality control programs.

There are several methods for the identification of NSLAB which include both phenotypic and genotypic approaches. API 50 CHL is a phenotypic method that identifies NSLAB through the fermentation of different carbohydrates. Since API 50 CHL comes in a kit it is a convenient tool for NSLAB identification, but its use is limited by the cost per strip and low throughput. Additionally, API 50 CHL has been

found to misidentify NSLAB (Broadbent et al., 2003). Another potential tool for the

identification of NSLAB is a genotypic method that uses high resolution melt analysis

(HRM) of real-time polymerase chain reaction (PCR) products. HRM analysis is

based on monitoring the decrease in florescence emitted by dsDNA as it is denatured

into ssDNA through the application of heat. The melt curves generated through HRM

analysis are characterized by the melt curve peak which represents the temperature

(T_m) at which 50% of the dsDNA is denatured. The ability of HRM to be used to

identify species is through the differences in T_m which reflect differences in

nucleotide content of the given PCR product. Porcellato et al. (2012a) developed a

HRM method for NSLAB species identification. Recently this method has been

adapted by Iacumin et al. (2015) for further sensitivity in species identification for

Lactobacillus paracasei, Lactobacillus casei, and *Lactobacillus rhamnosus.*

DNA sequencing is another genotypic method that can also be used for the

identification of NSLAB. MicroSEQ® 500 is one DNA sequencing system that

amplifies and sequences the first 500 base pairs of the 16s rRNA gene for species

identification. This method is able to identify species by comparing the sequenced

base pairs to the MicroSEQ® 500 database or GenBank. One limitation of

MicroSEQ® 500 is the need to expand its database so that rare or newer species can

be identified with the system (Woo et al., 2003). One thing that each of these methods

relies on is the cultivation and isolation of bacteria through cultured methods.

For the cultivation of NSLAB, samples are plated on MRS agar and then

incubated anaerobically at 30°C for 48 hrs. Mesophilic lactobacilli are able to grow at

these conditions, but MRS has been found to lack selectivity allowing other genus,

such as lactococci, to grow (Couret et al., 2003). Additionally, lactobacilli are not limited to growth at 30°C. Once such species of lactobacilli is *Lb. wasatchensis* which grows slowly at an incubation of 23°C for 5 d (Ortacki et al., 2015). Without the inclusion of additional incubation temperatures diversity in NSLAB populations may be missed.

Cultured methods also rely on selecting NSLAB isolates that are present at the higher population concentrations. When plated on MRS, Cheddar cheese samples have been found to have population concentrations between 7-8 log CFU/ml (Broadbent et al., 2013). Isolates selected would only reflect NSLAB present at the previously mentioned concentration. If other NSLAB were present at lower concentrations, such as 4 log CFU/ml, the likelihood that they would be isolated from the same samples is low. Cultured methods thus present a challenge when investigating NSLAB diversity in dairy foods such as cheese.

Culture-independent methods able to overcome the challenges presented by cultured methods do exist. One immediate benefit that culture-independent methods provide is the ability to detect and identify uncultivable bacteria (Ndoye et al., 2011). Similar to real time PCR, culture-independent methods use amplified fragments of DNA to identify organisms present, but instead DNA is extracted from a sample of product, such as cheese, rather than a single isolate. Quigly et al. (2012) examined the microbial communities of several Irish cheeses by amplifying the V4 region of the 16s gene and analyzing the amplicons using high throughput sequencing. By testing both cheese and rind the authors found several genera not previously associated with cheese which included *Faecalibacterium*, *Prevotella*, and *Helcococcus*. In a more

recent study, Kable et al. (2016) investigated the microbial populations of raw milk in both tanker trucks and silos using a similar method and found that taxa detected at less than 1% abundance made up over 50% of bacteria present. If cultured methods were used alone much of the microbial diversity would have been missed when investigating cheese and especially milk. However, it is important to note that as strong of a tool as culture-independent methods are they are not without limitations. By culturing samples, isolates can be selected and kept frozen for future analysis, which cannot be done with culture-independent methods. Crow et al. (2001) were able to screen NSLAB for potential adjunct use in cheese which would not have been possible if isolates were not grown using traditional cultured methods. Additionally, Feurer et al. (2004) found that using both cultured and culture-independent methods together gave a more complete picture of the microbial diversity in cheese. Though there is benefit to employing a polyphasic approach it may not be a possibility for producers that are trying to monitor the NSLAB in their milk and subsequent products.

Sources for NSLAB found in cheese include raw milk as well as the processing equipment and the facility in which the cheese was made. NSLAB are found initially at low levels in raw milk (Fitzsimons et al., 2001; Montel et al. 2014). Pasteurized milk can also be a source, as NSLAB have been detected in milk post-pasteurization. Interestingly, some NSLAB have been found to survive high temperature short time (HTST), but not low temperature long time (LTLT) pasteurization (Ortacki et al., 2015).

NSLAB have been found at the farm, on the cows, and at the processing facilities (Somers et al., 2001; Agarwal et al. 2006; Vacheyrou et al., 2011; Verdier-Metz et al. 2012; Braem et al., 2013; McMahon et al. 2014; Bouchard et al., 2105). This suggests that NSLAB can be introduced at each stage of milk handling and processing. In Europe, an emphasis has been placed on the impact of NSLAB on cheese. Cheese flavor was found to be different based on the source of the milk (Demaringy et al., 1997; Callon et al., 2005). It was also suggested that NSLAB unique to Comté processing facilities were most likely from the raw milk (Berthier et al., 2001).

In Cheddar cheese, flavor has been found to be different between countries and within the United States different regions (Drake et al., 2005; Drake et al., 2008), but no information was gathered about NSLAB populations present in the Cheddar cheeses tested. Additionally, these Cheddar cheeses were made using different processes which can impact flavor. Similar results were found by a consumer sensory study in which participants were able to distinguish Cheddar cheeses based on the milk source (Turbes et al., 2016). Each Cheddar cheese used by Turbes et al. (2016) was made using the same process in the same facility with the milk source and heat treatment being the primary differences. Turbes et al. (2014, 2016) also cultivated NSLAB from the Cheddar cheeses used in the sensory study and identified them with API 50 CHL. NSLAB isolates from each milk source produced a distinct fermentation profile (Turbes et al., 2014). Within this current study looked to further investigate the contribution of different milk sources on NSLAB in Cheddar.

The aim of this study is to investigate the potential for P1V1/P2V1 PCR with HRM analysis as a tool for producers to monitor the NSLAB in raw milk and subsequent cheese. If applicable, HRM analysis of the P1V1/P2V1 amplicon will be a fast and inexpensive tool allowing producers to analyze NSLAB leading to more consistent, quality products. Additionally, this study aims to investigate the contribution of farm level milk sourcing on NSLAB in Cheddar cheese from farms on the Oregon Coast and in the Willamette Valley. These findings could help producers understand the contribution that different farms have on the NSLAB and lead to better quality control or potentially capitalize on the differences in a small batch capacity.

2. Assessment of high resolution melt analysis of P1V1/P2V1 PCR as a tool for differentiating and identifying non-starter lactic acid bacteria species.

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2.1 Abstract.

Non-starter lactic acid bacteria (NSLAB) are important to the dairy industry because of their ability to impact acid production and flavor in fermented products. In this study three methods for identifying NSLAB were evaluated; P1V1/P2V1 polymerase chain reaction (PCR) with high resolution melt (HRM) analysis, 16s rDNA sequencing using MicroSEQ® 500, and API 50 CHL. HRM analysis and API 50 CHL were evaluated using known reference strains; *Lactobacillus paracasei, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Lactococus lactis* subsp. *lactis*. In addition, HRM analysis using the previously mentioned reference strains were used for the identification of unknown isolates from raw milk (n=8) and 6.5 mo aged Cheddar cheese (n=19) and compared to 16s rDNA sequencing identification results. Five out of seven reference strains were correctly identified using API 50 CHL. Both reference strains that were incorrectly identified were *L. lactis* subsp. *lactis*. HRM analysis was in agreement with 16s rDNA sequencing 75% and 66% of the time for *L. lactis* and *Lb. paracasei,* respectively. Comparing the HRM melt curves of unknown isolates identified as *Lb. paracasei* and *L. lactis* subps. *lactis* by 16s rDNA sequencing showed a significant amount of overlap. This suggests that HRM analysis of the P1V1/P2V1 amplicon is not able to correctly identify isolates as *Lb. paracasei* and *L. lactis* subsp. *lactis*. Future work is still needed to develop an assay accessible for producers so they can more effectively monitor the NSLAB present in their raw milk, facility, and final products.

2.2 Introduction

Lactic acid bacteria (LAB) are important to the food and dairy industry. The addition of starter LAB (SLAB) drives much of the acid production in fermented dairy foods such as cheese and yogurt. The presence of non-starter LAB (NSLAB) are also important as contributors to flavor development in aged cheeses (Franklin and Sharpe 1963; Milesi and others 2010). Although NSLAB can have a positive effect on cheese flavor (Whetstine and others 2006) some strains have been found to negatively impact finished cheese quality (Ortakci and others 2015). With the ability for different NSLAB to impact the quality of cheese, producers are increasingly in need of tools to identify the species present and how they differ at the strain level.

Phenotypic identification through the analysis of carbohydrate fermentation of NSLAB using API 50 CHL strips has been employed in the past (Demarigny and others 1996; Williams and Banks 1997; Fitzsimmons and others 1999). Though easy to use, these strips may be cost- and time-prohibitive when identifying large numbers of NSLAB isolates. NSLAB identification using API 50 CHL has also been found to be inaccurate (Broadbent and others 2003; Boyd and others 2005; Bezeková and others 2013), and this is potentially caused by high variability in fermentation profiles within NSLAB species (Tynkkynen and others 1999) coupled with the relatively small number of isolates of each species used to create the API library database (Coeuret and others. 2003).

Genotypic identification of NLSAB using polymerase chain reaction (PCR) has increased since the late 1990s (Fitzsimons and others 1999; Berthier and others

2001). PCR has gained traction as a primary tool for identification of NSLAB because of its accuracy compared to API 50 CHL (Andrighetto and others 1998). Advances in technology led to the development of real-time PCR assays, which have been of particular interest to the food and dairy industry because of their potential for high throughput (Bhagwat 2003; Nam and others 2005). Additionally, secondary analysis of the real-time PCR products, such as high resolution melt (HRM) curve analysis, can be used for further confirmation or differentiation. Porcellato and others (2012a) developed a HRM method for NSLAB species identification. Recently this method has been adapted by Iacumin and others (2015) for further sensitivity in species identification for *Lactobacillus paracasei, Lactobacillus casei,* and *Lactobacillus rhamnosus*.

The purpose of this study was to evaluate the use of P1V1/P2V1 PCR with HRM analysis for species identification of NSLAB. Known NSLAB reference strains were tested to determine the ability for HRM analysis of the P1V1/P2V1 amplicon to differentiate species outside of those stated above. Unknown NSLAB isolated from raw milk and experimental raw Cheddar cheese alongside a control library of known cheese NSLAB were analyzed as well.

2.3 Materials and Methods

2.3.1 Culture Preparation and DNA Extraction:

Reference strains (Table 1) and cultures previously isolated from cheese and raw milk were resuscitated from freezer stocks by transferring to MRS broth (Becton, Dickinson and Company, Franklin Lakes, NJ) with incubation at 30°C for 24 hrs.

Selected reference strains were evaluated for their fermentation profile using the API

50 CHL (BioMérieux, Durham, NC). Inoculum preparation and incubation conditions

were performed according to manufacturer's instructions. A crude DNA extraction

was prepared from an aliquot of each MRS broth culture (1 ml) and initially

centrifuged at 12,000 rpm for 3 minutes. Supernatant was discarded and the pellet

was resuspended in an equal volume of 0.85% NaCl. Suspension was then

centrifuged and resulting pellet was resuspended in sterile deionized water.

Suspension was heated at 100°C for 10 min and then transferred to ice to chill for 5

min. Chilled lysate was centrifuged at 12,000 rpm for 1 min and the resulting

supernatant was reserved as DNA template. Templates were stored at -20°C for later

use.

2.3.2 P1V1/P2V1 PCR with HRM Analysis

Species identification of NSLAB was adapted from the method developed by

Iacumin and others (2015) using High Resolution Melt Analysis (HRM)-PCR. P1V1

(5′-GCGGCGTGCCTAATACATGC-3′) and P2V1 (5′-

TTCCCCACGCGTTACTCACC-3′) sequences were previously described by Klijn et

al (1991). A single PCR reaction contained 4 µl nuclease free water (EMD Chemicals

Inc., Gibbstown, NJ), 5 µl DNA template, 12.5 µl 2x MeltDoctor HRM Master Mix

(Thermo Fisher Scientific, Waltham, MA), and 0.7 µM of each primer (Integrated

DNA Technologies, Coralville, Iowa). Amplification was performed using the

Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific,

Waltham, MA) operated by the SDS software (version 1.4) with the following

conditions: 95°C for 1 min followed by 45 cycles at 95°C for 10 s, 55°C for 30 s, and

72°C for 20 s. Dissociation was performed from 60°C to 95°C increasing 4.7°C per 2

s. Resulting melt curves were analyzed using the HRM software (version 2.0).

Derivative data was exported for further analysis using Excel (Microsoft Corporation,

Bellevue, WA) and JMP Pro (version 12; SAS Institute, Cary, NC). Derivative data

was normalized between 75-85°C using the maximum florescence output per sample.

Comparison of isolates was performed using Pearson correlation with hierarchal

cluster analysis. Isolates with poor or no amplification were excluded from analysis.

2.3.3 DNA Preparation and Isolate Identification by 16S rDNA Sequencing

Select NSLAB isolates from raw milk (n = 23) and 6.5 mo aged Cheddar

cheese (n = 26) samples were grown anaerobically on MRS and incubated for 48 hrs.

Plates were sealed and shipped overnight air to the FDA Pacific Regional Laboratory.

Forty isolates were subcultured on Tryptic Soy Broth with Yeast Extract (TSB-YE)

and incubated overnight at 36°C. The DNA was extracted from 1.0 ml overnight

broth culture using PrepMan Ultra following the manufacturer's protocol (Applied

Biosystems, Inc (ABI), Foster City, CA). Isolates were identified by MicroSEQ® 500

16S rDNA Identification (ABI) using a modified version of the manufacturer's

protocol. The protocol was modified by using one third of the sample and master mix

volumes in the amplification PCR and one half the volumes in the sequencing PCR,

resulting in 10 µl reactions for both assays. The amplification PCR reactions were

cleaned up by adding 4 µl of ExoSAP-IT (Affymetrix, Santa Clara CA) to each 10 µl

reaction, following the manufacturer's protocol. Sequencing PCR reactions were

cleaned up using Performa DTR gel filtration cartridges (EdgeBio, Gaithersburg,

MD) following the manufacturer's protocol. Sequencing reactions were performed in

triplicate on the 3500 Genetic Analyzer (ABI) platform with POP-6 polymer. The resulting forward and reverse sequences for each sample were assembled using the MicroSEQ® 500 ID Microbial Identification software v3.0 and compared to the MicroSEQ® 500 ID 16S rDNA 500 Library v2013. Based on the MicroSEQ® 500 ID Microbial Identification criteria, identifications of ≥99.0% were considered to the species level, identifications of ≥97.0% were to the genus level, and an interspecies difference of >0.2% was required to differentiate closely related species from each other.

2.4 Results and Discussion:

The P1V1/P2V1 region of NSLAB reference strains used in this study were evaluated using high resolution melt analysis. Melting point temperatures for reference strains were estimated based on P1V1/P2V1 amplicon sequences obtained using NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Estimated melting peak temperatures of the P1V1/P2V1 amplicon for *Lb. rhamnosus, Lb. casei* and *Lb. paracasei, Lb. plantarum,* and *L. lactis* subsp. *lactis* were 78.00°C, 81.10°C, 81.10°C, 78.10°C, and 80.60°C, respectively (Table 2). Observed melting temperatures for references strains (Table 2) and those previously reported by Iacumin et al (2015) were above the estimated values by 0.69-1.43°F and 2.59-3.66°F, respectively. *Lb. casei, Lb. paracasei, Lb. rhamnosus, Lb. plantarum*, and *L. lactis* subsp. *lactis* had melting point temperatures averaging 81.79°C, 82.01°C, 79.43°C, 79.34°C, and 81.69°C, respectively (Table 2). Melting point temperatures for *Lb. casei, Lb. paracasei,* and *L. lactis* subsp. *lactis* were not significantly different (p-value>0.05) (Table 2). *Lb. rhamnosus* and *Lb. plantarum* were also found to be not significantly

different from each other, but were from the previously mentioned NSLAB (p-value<0.05) (Table 2). *Lb. casei, Lb. paracasei* and *Lb. rhamnosus* were lower on average by 1.5 and 2.0°C, respectively, than previously reported (Iacumin et al., 2015) (Table 2).

API 50 CHL was also evaluated, using the same reference strains, as a tool for the identification of NSLAB. Reference strains IDs were compared to API 50 results to determine its effectiveness for properly identifying NSLAB. Five out of seven reference strains were correctly identified by API 50 CHL (Table 2). *Lb. paracasei* JWC-2118, *Lb. rhamnosus* JWC-2123, and *Lb. plantarum* JWC-2142 were identified correctly with a percent correct ID greater than 99.00% (Table 2). *Lactococcus lactis* subsp. *lactis* JWC-2155 was identified correctly with a percent correct ID of 93.6%, but *L. lactis* subsp. *lactis* JWC-2108 and JWC-2156 were misidentified as *Lb. paracasei* subsp. *paracasei* with a percent correct ID of 97% or more (Table 2). Both misidentified *L. lactis* spp. *lactis* isolates were able to utilize d-melezitose (Table 3) which is found as a positive in *L. lactis* 1 and *L. lactis* 2 1.0% and 0.0% of the time, respectively, whereas in *Lb. paracasei* 1 and 2 it is found as a positive reaction over 90% of the time (Table 3).

Reference strains were grouped based on melt curves generated through HRM analysis of the P1V1/P2V1 amplicon. Melt curves for *Lb. paracasei,* and *Lb. casei* grouped together above 80.0°C with *Lb. plantarum* and *Lb. rhamnosus* grouped below 80.0°C (Figure 1). Through Pearson correlation of the HRM output the reference strains further separated into groups based on species (Figure 1).

Identification of NSLAB using P1V1/P2V1 PCR with HRM analysis was tested using reference strains and unknown isolates from raw milk and raw Cheddar cheese aged for 6.5 mo. Additionally, identification of unknown isolates using HRM analysis was compared to 16s rDNA sequencing using MicroSEQ® 500. Species identification of unknown isolates using MicroSEQ® 500 and HRM analysis of P1V1/P2V1 amplicon were in agreement 75%, 66%, and 0% of the time for *L. lactis, Lb. paracasei,* and *Lb. plantarum,* respectively (Table 4). Ranges for melting point temperatures of the P1V1/P2V1 amplicon for isolates identified as *L. lactis, Lb. paracasei, Lb. plantarum, and Lb. rhamnosus* were 79.4-83.6°C, 80.3-82.8°C, 79.2-82.1°C, and 78.5-79.0°C, respectively (Table 4). *Lb. rhamnosus* was closest to the expected melting temperature of 78.0°C (Table 3). Unknown isolates from raw milk identified as *L. lactis* subsp. *lactis* and *Lb. paracasei* using MicroSEQ® 500 were found to have T_m ranges for the P1V1/P2V1 amplicon of 79.4-83.6°C and 79.5-82.8°C, respectively (Table 4). Isolates from 6.5 mo old Cheddar cheese identified using MicroSEQ® 500 as *L. lactis* subsp. *lactis* and *Lb. paracasei* had T_m ranges for the P1V1/P2V1 amplicon of 81.4-81.9°C and 81.4-82.5°C, respectively (Table 4). Overlap of T_m of the P1V1/P2V1 amplicon of *L. lactis* subsp. *lactis* and *Lb. paracasei* are seen in Figure 1.

MicroSEQ® 500 identifications were compared to the P1V1/P2V1 amplicon melt curves for the same isolates. Melt curves for *Lb. parcasei* and *L. lactis* overlapped as was seen noted above (Figure 1 and Figure 2). Additionally, melt peaks for *Lb. plantarum* range from 79-82°C (Figure 2).

Identification of NSLAB through the use of API 50 CHL relies on the fermentation of 49 different carbohydrates. Phenotypic identification using API 50 relies on the ability for a given NSLAB species to consistently ferment all 49 carbohydrates. API 50 CHL was able to identify one out of three *L. lactis* correctly. JWC-2156, which was incorrectly identified, should have had a matching fermentation profile to JWC-2155 since they were isolated from the same single strain starter. The difference was in the fermentation of one carbohydrate, d-melezitose, which is found to be fermented by *L. lactis* 1.0% of the time or less according to the API 50 CHL kit. Tynkkynen and others (1999) encountered a similar issue with *Lb. casei* ATC 393 being identified as *Lb. rhamnosus* at a good level by fermenting a single carbohydrate: rhamnose. This discrepancy may be partly caused by the fact that the database that API relies on for identification is not regularly updated (Coeuret and others 2003) with some species missing altogether. This is also problematic since one NSLAB species has only recently been identified (Ortakci and others 2015), with the potential for more to come as isolation and identification methods are refined. Broadbent and others (2003) compared API 50 CHL and 16s rDNA sequencing and found that found NSLABs were only in agreement between both methods 31% of the time with most of the discrepancies coming from isolates that had fermentation characteristics like *Lb. curvatus* but generically closer to *Lb. casei*. Similar work by Bezková and others (2013) and Moraes and others (2013), found that identification of isolates was in agreement 83.3% and 89.7% between API 50 CHL and species specific PCR. With the inconsistencies in species identification and lack of agreement with molecular methods, API 50 CHL appears to be a poor tool for the speciation of NSLAB.

Results for P1V1/P2V1 PCR with HRM analysis for *Lb. casei, Lb. paracasei,* and *Lb. rhamnosus* were similar to results from Iacumin and others (2015). All three species were found to separate based on their melt curve. Though ability for the three species to make distinct groups were similar to Iacumin's findings, the T_m for each was markedly lower than previously reported. The most likely reason for this difference from Iacumin's findings is the use of different equipment leading to a modification of the method. Complications could arise if *Lb. plantarum* is also present as an unknown isolate since it shares similar T_m with *Lb. rhamnosus*. Pearson correlation with cluster analysis appeared to help separate out species with similar T_m, as was the issue with *Lb. rhamnosus* and *Lb. plantarum*. This method may not be appropriate for identifying unknown isolates from cheese or other dairy products since there was no significant difference in melting point temperatures between several NSLAB. As noted previously, Pearson correlation with cluster analysis helped separate out species with similar melt temperatures, but this may not be an option in day to day dairy production operations. Additionally, with the differences observed in this study and studies by Iacumin and others (2015) there is no consensus in T_m for identification of NSLAB. HRM analysis of the P1V1/P2V1 amplicon was effective at separating *Lb. casei*, *Lb. paracasei,* and *Lb. rhamnosus,* but not effective for the identification of NSLAB outside of that group. Furthermore, the melt temperature ranges of the P1V1/P2V1 amplicon for isolates identified as *L. lactis* and *Lb. parcasei* using MicroSEQ® 500 overlap. This suggests that in the samples where *L. lactis* could be present HRM analysis of the P1V1/P2V1 amplicon could not effectively or reliably identify *Lb. paracasei.* This makes HRM

analysis of the P1V1/P2V1 amplicon not a viable option for NSLAB identification in dairy products that use *L. lactis* as a starter culture.

Interestingly, HRM analysis of *Lb. plantarum* reference strains had little variation. When compared to the melting temperatures of the P1V1/P2V1 amplicon of the reference strains, unknown isolates identified as *Lb. plantarum* by MicroSEQ® 500 were over 3°C higher. This suggest that MicroSEQ® 500 may be incorrectly identifying isolates as *Lb. plantarum.* In contrast, isolates identified as *Lb. rhamnosus* by HRM analysis of the P1V1/P2V1 amplicon and using MicroSEQ® 500 were in 100% agreement.

2.5 Conclusions:

The cost and low throughput of API 50 makes it an unattractive option for NSLAB identification. In addition, API 50 was found to incorrectly identify lactococci and NSLAB. HRM analysis of Real Time PCR products is an attractive alternative because of its high throughput and also because many producers already have the necessary equipment being used for food safety applications. Though HRM analysis of P1V1/P2V1 amplicons were effective for discriminating *Lb. casei, Lb. paracasei,* and *Lb. rhamnosus* it is not discriminative enough with additional species. Future work is needed to develop an assay accessible for producers so they can more effectively monitor the NSLAB present in their raw milk, facility, and final products.

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2.7 Author Contributions:

Chris Baird designed the study, analyzed data, and drafted the manuscript. Julia Wilson assisted with the microbiological analysis of reference strains and unknown isolates using HRM analysis and API 50 CHL. Michele Moore performed sequencing on all unknown isolates included in this study. Joy Waite-Cusic and Lisbeth Goddik provided guidance on experimental design, execution, data interpretation, and writing

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Table 1: Lactic acid bacteria strains used for API 50 CHL and P1V1/P2V1

polymerase chain reaction (PCR) with high resolution melt (HRM) analysis.

Species	JWC Lab ID	Strain ID	Original Isolation Source
Lactobacillus casei	2112	AEY-A34	Swiss cheese
	2141	ATCC 334	Emmental cheese
	2117	NRRL B-1255	
Lactobacillus paracasei	2118	NRRL B-4560 (ATCC 25598)	Milking machine isolate
	2119	NRRL B-4564 (ATCC 27216)	Human saliva
Lactobacillus plantarum	2142	ATCC 10241	Sauerkraut
	2143	ATCC 8014	Unknown
	2144	MAD-337	
	2145	MAD-32	
	2146	MAD-11	
	2147	MAD-12	
	2148	AEY-140	
	2149	LB75	
	2150	LA10-11	
Lactobacillus rhamnosus	2123	NRRL B-1937 (ATCC 11982)	Unknown
	2122	NRRL B-1445 (ATCC 7469)	
	2114	AEY-H63	Swiss cheese
Lactococcus lactis subsp. *lactis*	2108	ATCC 13675	Dairy product
	2155	MD1	Commercial cheese starter culture
	2156	MD9	Commercial cheese starter culture

Table 2: Melting point temperature of the P1V1/P2V1 amplicon for lactic acid reference strains with species identification using API

50 CHL

Species	Replicates (n)	Melting Temperature by Strain (°C)[a]	Melting Temperature by Species (°C)[b,c]	Expected Melting Temperature (°C)[d]	Predicted Melting Temperature by Species (°C)[e]	API 50 CHL Identification
Lactobacillus casei JWC-2112	9	81.78 ± 0.32	81.79±0.20[A]	83.69 ± 0.03	81.10	*Lb. paracasei* subsp. *paracasei* (97%)
Lactobacillus casei JWC-2141	1	81.60				
Lactobacillus casei JWC-2117	1	82.00				
Lactobacillus paracasei JWC-2118	8	82.05 ± 0.28	82.01±0.03[A]	84.16 ± 0.04	81.10	*Lb. paracasei* subsp. *paracasei* (99.7%)
Lactobacillus paracasei JWC-2118	6	82.00 ± 0.20				
Lactobacillus paracasei JWC-2119	7	81.99±0.18				
Lactobacillus rhamnosus JWC-2123	8	78.96 ± 0.30	79.43±0.31[B]	81.66 ± 0.06	78.00	*Lb. rhamnosus* (99.8%)
Lactobacillus rhamnosus JWC-2120	7	79.30 ± 0.28				
Lactobacillus rhamnosus JWC-2122	6	79.57 ± 0.15				
Lactobacillus rhamnosus JWC-2123	7	79.67 ± 0.32				
Lactobacillus rhamnosus JWC-2114	7	79.67 ± 0.35				
Lactobacillus plantarum JWC-2142	10	79.11 ± 0.21	79.34±0.13[B]	N/A[f]	78.10	*Lb. plantarum* (99.9%)
Lactobacillus plantarum JWC-2143	1	79.20				
Lactobacillus plantarum JWC-2144	1	79.30				
Lactobacillus plantarum JWC-2145	1	79.40				
Lactobacillus plantarum JWC-2146	1	79.50				

Lactobacillus plantarum JWC-2148	1	79.40				
Lactobacillus plantarum JWC-2149	1	79.40				
Lactobacillus plantarum JWC-2150	1	79.40				
Lactococcus lactis subsp. *lactis* JWC-2108	2	81.55 ± 0.21	81.69±0.12[A]	N/A[f]	80.60	*Lb. paracasei* subsp. *paracasei* (98.9%)
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* JWC-2155	7	81.77 ± 0.21				*Lactococcus lactis* subsp. *lactis* (93.6%)
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* JWC-2166	7	81.76 ± 0.28				*Lb. paracasei* subsp. *paracasei* 3 (99.2%)

[a]Mean melting point temperature ± standard deviation.

[b]Mean melting point temperature ± standard deviation for each species. For strains with replicated melting temperatures, the mean

value was used to represent that strain.

[c]Temperatures with same superscript are not significantly different; (P-Value> 0.05)

[d]Melting temperature reported by Iacumin et al. (2015). PCR and HRM analysis was performed using the Qiagen Rotor-Gene Q

platform. Results were the average of 2 strains per species with 5 replicate assays per strain.

[e]Predicted melting point temperature of P1V1/P2V1 for NSLAB species obtained from www.ncbi.org with T_m estimates generated via

www.biophp.org set to basic T_m

[f]T_m for *Lb. plantarum* and *L. lactis* not reported by Iacumin et al. (2015).

Table 3: API 50 carbohydrate utilization reference per species with reference strain results for *Lb. casei, Lb paracasei, Lb. rhamnosus, Lb. plantarum*, and *L. lactis ssp. lactis.*

Carbohydrates[a]	GLY	LARA	SBE	RHA	MAN	SOR	MDM	MDG	AMY	MAL	MEL	SAC	INU	MLZ	RAF	GEN	TUR	TAG	GNT	API 50 Results
API Reference																				
Lb. paracasei 1	**20**	0	53	1	100	86	0	46	98	99	0	93	26	93	0	80	80	100	93	
Lb. paracasei 2	16	0	50	1	100	100	0	83	75	99	0	99	66	99	0	66	100	100	83	
Lb. paracasei 3	0	0	20	1	80	20	0	0	99	80	0	60	20	20	0	100	20	60	20	
Lb. rhamnosus	42	8	92	100	100	100	7	85	99	99	9	71	0	99	7	85	92	99	85	
Lb. plantarum 1	1	74	2	33	99	78	55	33	94	100	94	88	0	92	74	98	62	7	62	
Lb. plantarum 2	0	1	1	1	80	17	1	1	83	100	1	17	0	1	1	83	1	1	1	
L. lactis ssp. lactis 1	1	30	0	0	50	5	1	22	75	95	13	50	1	0	9	81	0	9	30	
L. lactis ssp. lactis 2	1	4	0	1	20	0	0	20	30	91	4	20	0	0	4	91	0	4	1	
Reference Strain																				
Lb. rhamnosus JWC-2123	-	-	+	+	+	+	-	+	+	?	-	-	-	+	-	+	+	+	?	*Lb. rhamnosus* (99.8%)
Lb. plantarum JWC-2142	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	*Lb. plantarum* 1 (99.9%)
L. lactis ssp. lactis JWC 2155	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	?	-	-	-	*L. lactis ssp. lactis* (93.6%)
L. lacris ssp. lactis JWC 2156	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	?	-	-	-	*Lb. paracasei ssp. paracasei* 3 (99.2%)
L. lactis ssp. lactis JWC 2108	+	-	-	-	+	-	-	-	+	+	-	+	-	+	-	+	-	+	+	*Lb. paracasei spp paracasei* (98.9%)

[a]Carbohydrates shown for reactions between reference strains that were different

Table 4: Identification of raw milk and Cheddar cheese isolates using MicroSEQ®
500 compared to HRM analysis of P1V1/P2V1 amplicon

Isolate	Source	MicroSEQ® 500 ID	% Match	Melting Temperature (°C)	HRM ID
NCC-B5	Raw Milk[a]	*Lactococcus lactis lactis*	100.00	82.0	*Unknown*[g]
NCC-B2		*Lactococcus lactis lactis*	100.00	81.1	*Unknown*[g]
NCC-B3		*Lactococcus lactis lactis*	100.00	83.6	*Unknown*[g]
NCC-A3		*Lactococcus lactis lactis*[c]	99.58	79.4	*Lb. rhamnosus*
NCA1-B1		*Lactobacillus paracasei paracasei*[d]	99.95	82.8	*Lb. paracasei*
NCC-B1		*Lactobacillus paracasei tolerans*[e]	100.0	80.3	*Lb. rhamnosus*
NCB-B1		*Lactobacillus plantarum*[f]	99.89	82.1	*Lb. paracasei*
NCB-A5		*Lactobacillus plantarum*[f]	99.9	80.5	*Lb. paracasei*
NCC-A3	Aged Cheddar[b]	*Lactococcus lactis lactis*	100.00	81.7	*L. lactis*
NCC-A1		*Lactococcus lactis lactis*	100.00	81.4	*L. lactis*
WA-A3		*Lactococcus lactis lactis*	100.00	81.4	*L. lactis*
WA-C5		*Lactococcus lactis lactis*	100.00	82.0	*Lb. paracasei*
WB-A2		*Lactococcus lactis lactis*	100.00	81.4	*L. lactis*
NCB-A5		*Lactococcus lactis lactis*	100.00	81.9	*L. lactis*
NCB-C5		*Lactococcus lactis lactis*	100.00	82.0	*Lb. paracasei*
NCB-A4		*Lactococcus lactis lactis*	100.00	81.8	*L. lactis*
NCC-C1		*Lactobacillus paracasei paracasei*[d]	99.95	81.6	*Lb. paracasei*
NCA1-A3		*Lactobacillus paracasei tolerans*[e]	100.00	82.0	*Lb. paracasei*
NCA1-A5		*Lactobacillus paracasei paracasei*[d]	99.94	82.4	*Lb. paracasei*
NCA1-B3		*Lactobacillus paracasei tolerans*[e]	99.98	82.5	*Lb. paracasei*
NCA1-A1		*Lactobacillus paracasei tolerans*[e]	100.0	81.4	*L. lactis*
NCA2-B4		*Lactobacillus paracasei tolerans*[e]	99.95	82.5	*L. lactis*
WA-A4		*Lactobacillus paracasei paracasei*[d]	99.93	81.5	*Unknown*[g]
WB-A4		*Lactobacillus paracasei tolerans*[e]	99.95	81.7	*L. lactis*
SC-A3		*Lactobacillus rhamnosus*	99.94	78.5	*Lb. rhamnosus*
SC-B1		*Lactobacillus rhamnosus*	99.98	79.0	*Lb. rhamnosus*
SC-A1		*Lactobacillus plantarum*[f]	99.88	79.2	*Unknown*[g]

[a]Raw milk sourced from individual farms on the Oregon Coast (NCA1, NCB, NCC)

[b] Isolates extracted from Cheddar cheese aged a minimum of 6.5 mo made with milk
source from the Oregon Coast (NCA1, NCA2, NCB, NCC, SC), and the Willamette
Valley (WA, WB)

[c]MicroSEQ® 500 secondary ID - *L. lactis hordniae*

[d]MicroSEQ® 500 secondary ID - *Lb. paracasei tolerans*

[e]MicroSEQ® 500 secondary ID - *Lb. paracasei*

[f] MicroSEQ® 500 secondary ID - *Lb. pentosus*

[g]Species identification not obtained through HRM analysis
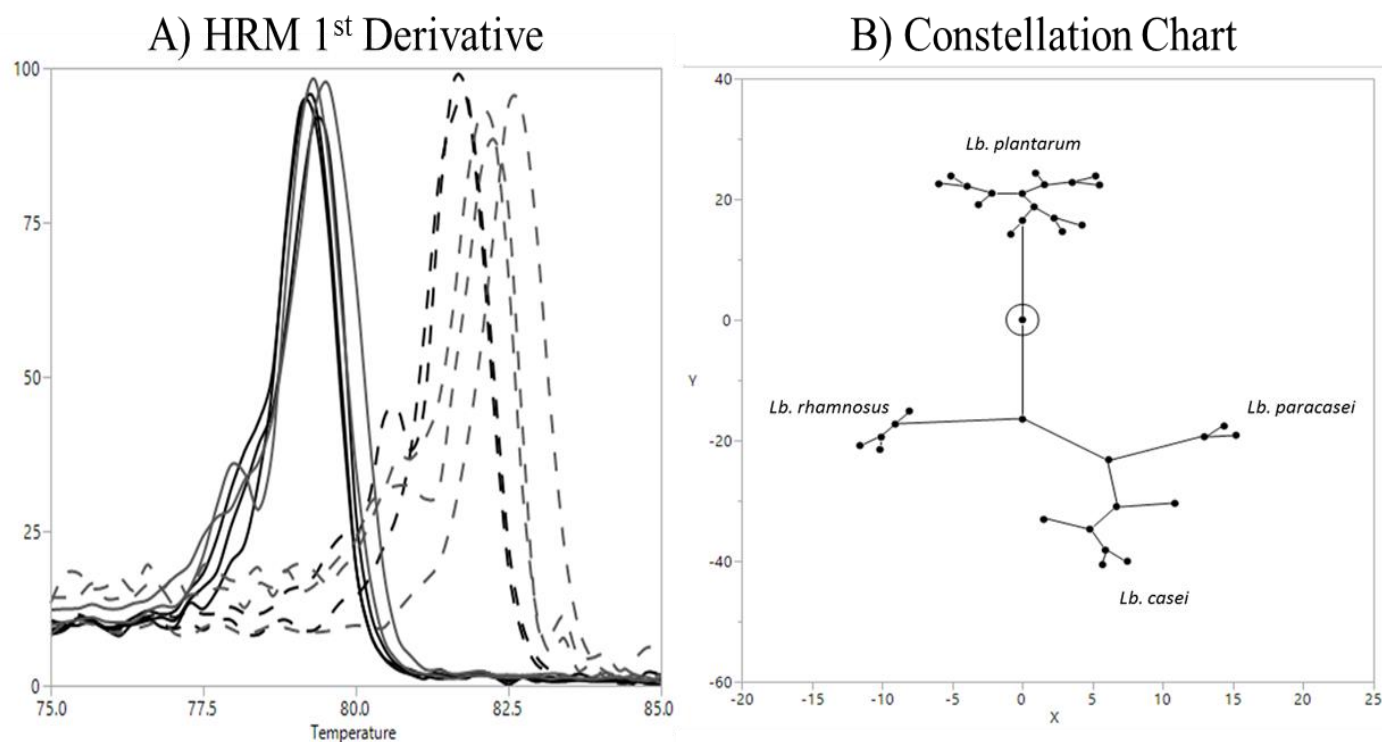
Figure 1: A) Melting point temperature curves of the P1V1/P2V1 amplicon for reference strains of *Lb. casei* (— —), *Lb. paracasei* (▬ ▬), and *Lb. rhamnosus* (▬), *Lb. plantarum* (▬). B) Relative relation of reference strains through pearson correlations of melting point temperatures of the P1V1/P2V1 amplicon.
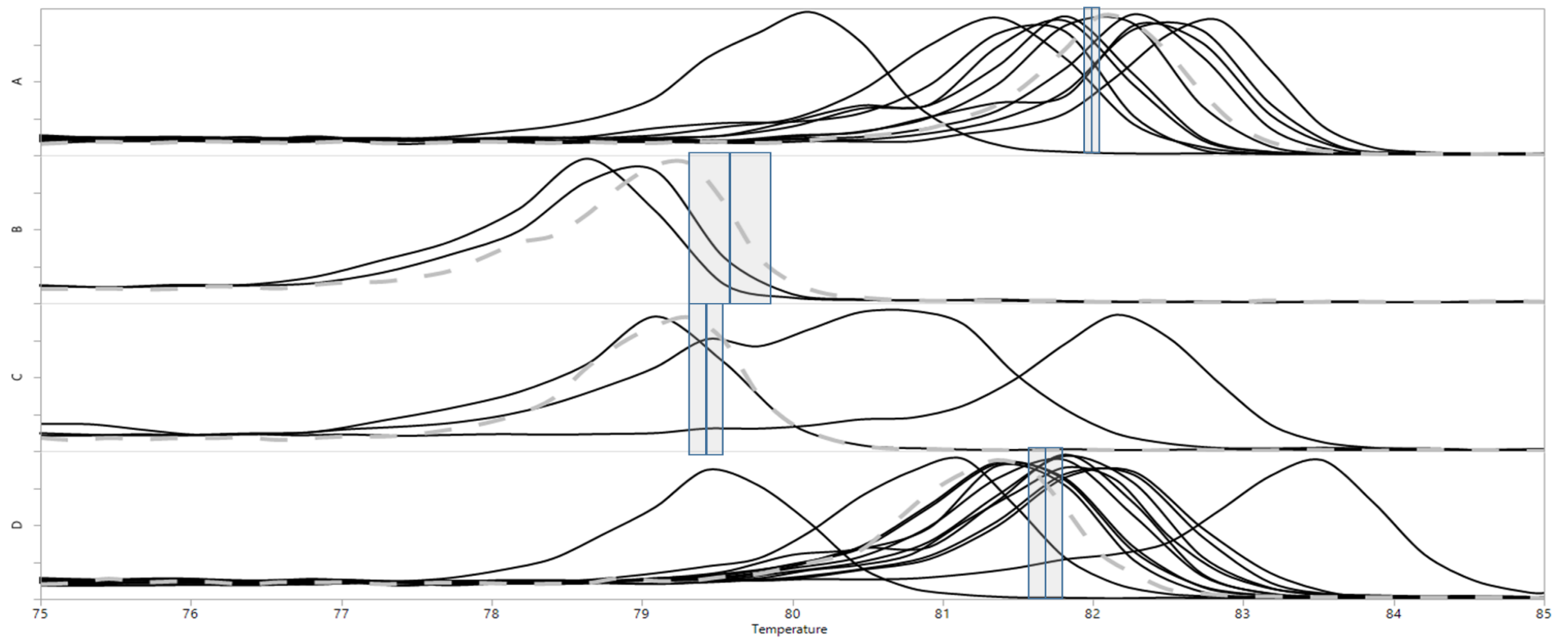
Figure 2: High resolution melt curves P1V1/P2V1 amplicon for isolates identified as A) *Lb. paracasei,* B) *Lb. rhamnosus,* C) *Lb. plantarum*, D) *L. lactis* subsp. *lactis* using 16s rDNA sequencing completed by MicroSEQ® 500 with reference strains (— —)

3. Contribution of farm level milk sourcing on non-starter lactic acid bacteria in raw milk and Cheddar cheese

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3.1 Abstract.

Non-starter lactic acid bacteria (NSLAB), which include lactobacilli, are found at low levels in fresh raw milk. Lactobacilli are important to the dairy industry because of their potential impact on the flavor and texture of yogurt, sour cream, and cheese. The objective of this study was to investigate the contribution of NSLAB from raw milk sourced from different farms on the microbiological profile of Cheddar cheese during aging (0-6.5 mo). Using a standardized recipe, Cheddar cheeses were made with raw milk sourced from dairies on the Oregon Coast (n =4) and in the Willamette Valley (n = 2) and aged up to 6.5 mo at 12°C. Lactic acid bacteria counts (LAB) were determined in raw milk and cheese samples using standard serial dilution and spread plating techniques on Lactobacilli MRS Agar with anaerobic incubation at 30°C for 48 hrs. Isolates (n=5-10/sample) were selected for preliminary speciation using High Resolution Melt Analysis (HRM) PCR assay targeting the V1 region of the 16S rDNA. Strains were further subtyped using a second HRM repetitive sequence-based PCR (rep-PCR). *Lactobacillus curvatus* and *Lb. paracasei/L. lactis* were identified in raw milk sourced from the Oregon Coast. *Lb. paracasei/L. lactis* were also identified in the Willamette Valley cheeses. Species diversity decreased throughout aging in all cheeses with the exception of cheese made from milk sourced from a single dairy on the southern Oregon Coast. After 6.5 mo of aging, the predominant species across all cheeses was *Lb. paracasei/L. lactis* (70.79% of the identified isolates). Strain diversity was highest in milk sourced from the northern Oregon Coast with 18 unique strains of *Lb. paracasei/L. lactis*. Furthermore, through the use of 16s rDNA sequencing, additional NSLAB were identified in both raw milk

and 6.5 mo Cheddar cheese samples which included *Lb. brevis, L. lactis cremoris, Enterococcus saccharolyticus, Enterococcus faecalis, Staphylococcus bovis, Staphylococcus chromogenes, Leuconostoc lactis,* and *Weissella paramesenteroides.* Evidence suggests that milk sourcing at the farm level impacts the strain diversity of NSLAB present in raw milk and Cheddar cheese. Additional work using both cultured and non-cultured methods is needed to further investigate NSLAB differences between farms and days of milk collection. Producers can use these findings for steps to help control quality at the farm level or capitalize on the differences in NSLAB between farms in single farm limited small batch runs.

3.2 Introduction

Cheddar cheese is the second most consumed cheese in the United States (USDA, 2015). Although the general process for making Cheddar is the same, a trained sensory panel separated Cheddar cheeses from the United States, United Kingdom, and New Zealand into distinct groups suggesting international flavor differences (Drake et al., 2005). Furthermore, Cheddar cheese flavor in the United States was found to be distinct between the Northwest, Midwest, and Northeast regions with the observed differences found to be related to facility specific flavors (Drake et al., 2008). Eliminating the production facility as a variable, Cheddar cheese flavor was found to be distinct based on the farm the milk was sourced from (Turbes et al., 2016). A potential source for these farm to farm flavor differences could be the indigenous microflora found in raw milk (Berthier et al., 1997) which include non-starter lactic acid bacteria (NSLAB).

Potential sources for NSLAB in raw milk used for Cheddar cheese manufacturing include biofilms on production equipment (Beresford et al., 2001; Somers et al., 2001; Agarwal et al., 2006; Ortacki et al., 2015), the farm environment, teat skin (Vacheyrou et al., 2011), and teat canal (Gill et al. 2006; Bouchard et al., 2015). One potential source for NSLAB found on the teat skin is the bedding used in the barn (Joandel, E. 2007). Additionally, pasture grasses could also be a source of NSLAB on the teat skin as dairy cows graze, but for NSLAB only *Lactoccocus lactis* subsp. *lactis* have been isolated from grasses thus far (Denis and Desmasures, 2005). This could be part of why *L. lactis* subsp. *lactis* strains isolated from raw milk were found to be farm specific (Corroler et al., 1998). Though lactobacilli have not been

isolated from pasture grasses they do appear after the fermentation of the grasses during silage production (Boutan et al., 2007). This suggest that both farm facilities and pasture are a source for NSLAB diversity in raw milk.

NSLAB differences between farms have been observed by Desmasures and Guegeun (1995) which suggested that farms could potentially be identified based on the microflora present in the raw milk produced. This was observed in raw milk Camembert where strains of lactococci were found to be specific to the farm the milk was sourced from (Correler et al. 1998). General sanitation practices between farms, such as hand washing routines, have also been found to influence the NSLAB present in raw milk (Michel et al. 2001; Verdier-Metz et al., 2009).

Demarigny et al. (1996, 1997) produced Swiss-type cheese using the same microfiltered raw milk inoculated with microbial retentates sourced from different Comté producing facilities. Trained sensory panelists were able to effectively discriminate the Swiss-type cheeses into groups with the source of the microbial retentate as the defining factor (Demarigny, et al. 1997). The authors suggested that differences in microflora, mainly facultatively heterofermentive lactobacilli and propionibacteria, were a source of flavor differences in Swiss-type cheese. Callon et al. (2005) found similar results in Salers-type cheese using the method described above, but with retentates filtered from raw milk sourced directly from the farm. This suggests that cheese flavor is impacted by the microflora in raw milk from different farms or processing facilities. NSLAB impact on Cheddar cheese characteristics is well documented (Crow et al., 2001; Swearingen et al., 2001; Broadbent et al., 2003; Briggiler-Macró et al., 2007; Oberg et al. 2011), but studies investigating the farm

level contribution of NSLAB have been limited to raw milk and European cheeses (Callon et al. 2005; Franciosi et al., 2009; Vacheyrou et al., 2011)

The purpose of this study was to investigate the contribution of NSLAB in Cheddar cheese from raw milk sourced from different farms throughout Oregon's Coast and Willamette Valley. Identification and fingerprinting of NSLAB in raw milk and Cheddar cheese samples were carried out using a modified Real-Time PCR assays with HRM analysis outlined by Iacumin et al. (2015) and Porcellato et a. (2012), respectively.

3.3 Methods and Materials

3.3.1 Farm Selection

Dairy farms were selected from Oregon's Willamette Valley and the Oregon Coast regions. Preference was given to farms with herds made up primarily of Jersey cows. All herds were on pasture for a minimum of one month prior to milk collection. Three farms, no more than 16.8 miles apart, were selected from the North Oregon Coast; North Coast-A (NC-A), North Coast-B (NC-B), and North Coast-C (NC-C), two farms from the Willamette Valley, no more than 25 miles apart; Willamette Valley-A (W-A) and Willamette Valley-B (W-B), and one processing facility on the Southern Oregon Coast (SC) with a single farm supplier. Phone interviews with farms were conducted to collect details about pasture composition, feed, and silage use at the time of milk collection, which are detailed in Table 5.

3.3.2 Milk Collection

Raw milk was transferred from the bulk tank into sanitized 37.9- l stainless steel milk cans. NCA was collected twice within 3 days, and SC was collected directly out of the balance tank of the HTST pasteurizer. Approximately 100 l of raw milk was collected per milk source. Milk cans were transported on ice during transportation to the Arbuthnot Dairy Center at Oregon State University (Corvallis, OR). Cheddar cheese was produced on the day of raw milk collection and all cheeses were made within five weeks in May and June of 2015.

3.3.3 Cheese Production

A minimum of 95 kg of standardized raw milk (0.83 protein-to-fat ratio) was transferred into a 120 l steam-jacketed cheese vat. Raw milk samples (50 ml; 2 samples/milk source) were stored under refrigeration for no more than 1 day for later analysis. Milk was heated to 32.2°C, at which point starter cultures (Danisco A/S, Copenhagen, Denmark) were added. The mixture was maintained at 32.2°C for 40 min with constant agitation with a target pH between 6.50-6.55 (Table 6). pH was monitored throughout each step using an Exstik handheld pH probe (Extech Instruments, Waltham, MA). Following the fermentation step, 33% (v/v) calcium chloride (4.4 ml/100.0 kg milk; Dairy Connection, Madison WI) and single strength calf rennet (16.5 ml/100.0 kg milk; Dairy Connection, Madison, WI) diluted with 40x (v/v) distilled water were added. Milk was stirred manually for 30 seconds and left undisturbed to allow curd to set for 30 min.

Curd was cut into cubes with a target size of 6 mm^3, then allowed to rest for 5 min prior to cooking. Temperature of curd was slowly raised from 32.2°C to 39.2°C (45 min), with constant agitation. Approximately half of the whey volume was drained, discharging through a stainless steel valve. Remaining whey and curd were continuously stirred until the target pH (6.30) was reached. The remaining whey was drained and the curd was pushed to opposite sides of the vat to form two slabs, approximately 10 cm deep.

Curd mats were undisturbed for an additional 10 mins before being cut into wide loaves (10 cm wide) to maximize whey drainage. Drained curd loaves were transferred to the pre-warmed cheddaring vat, which was set to maintain a target curd mat temperature of 37.8°C. The cheddaring process was performed with flipping every 15 min until the curd mat pH reached 5.35 (average cheddaring time: 1 hr, 40 min). Cheddar loaves were milled into 12.7 mm thick curds. Milled curds were salted (2.5% w/w) and transferred into 4.5 kg cheese molds, then pressed with 8.4 kg for 1 hr. Cheddar cheese was removed from the molds, flipped, and pressed for an additional 18 hrs. Cheddar cheese was removed from the molds, with an average pH of 5.05, and cut into 101 x 152 x 25mm blocks and vacuum sealed in plastic bags (3 mm thickness) (Ultrasource LLC., Kansas City, MO). Packaged cheeses were transferred to the aging room and stacked three high. Aging occurred in two stages: i) 11.7°C for 3 mo and ii) 5.6°C for 3.5 mo. Cheese samples were collected for microbiological analysis at the following time points: 1 d (after pressing), 1 mo, 3 mo, and 6.5 mo.

3.3.4 Cheese Composition Analysis

Analysis of fat, salt, protein, and moisture was completed in 3 samples per cheese after 3 mo of aging using a NIR FoodScan™ Dairy Analyzer (ISO# 21544; FOSS, Hillerød, Denmark). ANOVA of fat, salt, protein, and moisture was conducted to determine differences between samples. Tukey multiple comparison post-hoc test was performed for fat, salt, protein, and moisture to determine which samples were most similar.

3.3.5 Microbiological Analysis - Raw Milk

Lactic acid bacteria counts were determined for raw milk samples using standard serial dilutions and spread-plating methods. All dilutions were prepared in 0.45% sodium citrate and plated on MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ). Plates were incubated anaerobically for 30°C for 48 h prior to enumeration. Following enumeration, ten colonies were selected per raw milk source for further characterization. Selection of isolates was based on capturing a range of colony color and morphology. Selected colonies were purified with subsequent isolation on MRS agar using previous incubation conditions. Isolated colonies were visually characterized by color, shape, size, and opacity. Isolates were transferred to MRS broth (Becton, Dickinson and Company) and incubated at 30°C for 24 h. Broth cultures were used to prepare stock cultures and for DNA extraction followed by PCR analysis (see below). Stock cultures were prepared by mixing broth culture with sterile 30% (v/v) glycerol to achieve a final concentration of 15% (v/v) and stored at -80°C.

3.3.6 Microbiological Analysis - Cheese

Cheese samples were analyzed for lactic acid bacteria count at the following time points: 1 d (out of the press), 1 mo, 3 mo, and 6.5 mo. For each cheese and time point, three 1 lb packages were randomly selected for aseptic extraction of plugs using stainless steel triers. From the extracted plugs, 11 g of cheese were aseptically transferred to a sterile glass jar (400 ml) and blended with 99 ml of warm 0.45% (w/v) sodium citrate. Serial dilution, spread-plating, incubation, and enumeration procedures were followed as described above. A total of 15 isolates per cheese per time point were selected, isolated, characterized, prepared for PCR (see below), and used to make culture stocks as described above.

3.3.7 Microbiological Analysis – Starter Culture

Starter cultures (1 g) used in cheese production (Table 6) were transferred to MRS broth (50 ml) and incubated at 30°C for 24 h. Broth cultures were streaked for isolation onto MRS agar and incubated anaerobically at 30°C for 48 h. Five isolates from each starter culture were selected for further characterization.

3.3.8 DNA Extraction

MRS broth cultures (1 ml) for each raw milk, cheese, and starter culture were transferred to microcentrifuge tubes and centrifuged at 12,000 rpm for 3 min to create a cell pellet. The supernatant was discarded and the pellet was resuspended in 0.85% NaCl. The suspension was centrifuged, supernatant decanted, and the pellet was resuspended in sterile distilled water. The resulting cell suspension was heated at 100°C for 10 min. The heated suspensions were transferred to an ice bath for 5 min. The chilled lysate was centrifuged at 12,000 rpm for 1 min, and the supernatant

served as the DNA template for subsequent PCR reactions. DNA extracts were stored at -20°C.

3.3.9 P1V1/P2V1 PCR with HRM for Species Identification

Species identification of lactobacilli using High Resolution Melt Analysis (HRM)-PCR was adapted from Iacumin et al (2015). Primer set used for Real Time PCR-HRM amplification were P1V1/P2V1 (Integrated DNA Technologies, Coralville, Iowa). Final mixture for amplification contained 4 µl nucleotide free water, 5 µl DNA, 12.5 µl 2x MeltDoctor HRM Master Mix (Thermo Fisher Scientific, Waltham, MA), and 0.7 µM of each primer. Amplification was performed in an Applied Biosystems 7200 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) under the following conditions: 95°C for 1 min followed by 45 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 20 s. HRM analysis was performed from 65°C to 95°C increasing 0.1°C/ 2 s.

3.3.10 Strain Level Differentiation – Rep-PCR HRM

A method for strain differentiation of NLSAB isolates using Rep-PCR High Resolution Melt (HRM) analysis was adapted from Porcellato et al. (2012). The mixture used for Rep-PCR amplification contained 2 µM GTG_5, 5 µl DNA template, 1 µl nuclease free water, and 10 µl 2x MeltDoctor HRM Master Mix (Thermo Fisher Scientific, Waltham, MA) for a final reaction volume of 20 µL. Amplification was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) with the following conditions: 95°C for 10 min, 40 cycles at 95°C for 30s, 40°C for 1 min, and 72°C for 3 min. HRM analysis was

performed from 65°C to 95°C increasing 0.01°C/s for 50 measurements for every 1°C increase.

3.3.11 Species and Strain Level Melt Curve Analysis:

Identification of unknown isolates was determined by comparison to known controls for *Lb. casei/paracasei, Lb. plantarum, Lb. rhamnosus,* and *Lb. curvatus*. Isolates were picked as additional controls from starter culture, and previous Cheddar cheese produced at Oregon State University. Species identification of unknown isolates was determined by using HRM v2.0 (Thermo Fisher Scientific, Waltham, MA). Each run consisted of isolates that were grouped based on time of sample collection. Differences in species between region and farm were visually analyzed.

Isolates were grouped by species identification. Precursory analysis of strain level melt curves was performed visually using HRM v2.0. Pearson correlation and cluster analysis was used to determine strain similarity using JMP Pro 12. Strain level melt curves were visually compared to clusters to ensure proper groupings. Analysis was performed between 70 to 90°C with HRM output of each melt curve normalized within itself.

3.3.12 DNA Preparation and Isolate Identification by 16S rDNA Sequencing:

Selection criteria for sequencing was based on collection time of isolate and melt temperature of the P1V1/P2V1 amplicon for the given isolate. Isolates were picked to cover the range of melt temperatures observed during HRM analysis of the P1V1/P2V1 amplicon to verify the species diversity seen. Select NSLAB isolates from raw milk (n=23) and 6.5 mo aged Cheddar cheese (n=26) samples were grown

anaerobically on MRS and incubated for 48 hr. Plates were sealed and shipped overnight air to the FDA Pacific Regional Laboratory. Forty isolates were subcultured on TSB-YE and incubated aerobically overnight at 36ºC. The DNA was extracted from 1.0 ml overnight broth culture using PrepMan Ultra following the manufacturer's protocol (Applied Biosytems, Inc (ABI), Foster City, CA). Isolates were identified by MicroSEQ® 500 16S rDNA Identification (ABI) using a modified version of the manufacturer's protocol. The protocol was modified by using one third of the sample and master mix volumes in the amplification PCR and one half the volumes in the sequencing PCR, resulting in 10 µl reactions for both PCR's. The amplification PCR reactions were cleaned up adding by 4 ul of ExoSAP-IT (Affymetrix, Santa Clara CA) to each 10 ul reaction, following the manufacturer's protocol. Sequencing PCR reactions were cleaned up using Performa DTR gel filtration cartridges (EdgeBio, Gaithersburg, MD), following the manufacturer's protocol. Sequencing reactions were run in a 3500 Genetic Analyzer (ABI) with POP-6 polymer, with each reaction being run in triplicate. The resulting forward and reverse sequences for each sample were assembled using the MicroSEQ® 500 ID Microbial Identification software v3.0 and compared to the MicroSEQ® 500 ID 16S rDNA 500 Library v2013. Based on the MicroSEQ® 500 ID Microbial Identification criteria, identifications of ≥99.0% were considered to the species level, identifications of ≥97.0% were to the genus level, and an interspecies difference of >0.2% was required to differentiate closely related species from each other.

3.4 Results:

The average fat, protein, moisture, and salt contents of all cheeses were 33.81%, 27.38%, 35.18%, and 1.83%, respectively (Table 7). There was no significant difference in each of the following components between all the cheeses: salt, moisture and raw milk pH. Cheddar cheese from W-A had the lowest fat and highest protein contents at 32.89% and 28.96%, respectively. Highest fat content of Cheddar cheeses was observed from NC-C samples at 34.16%, with samples from W-B having the lowest protein content at 25.43%.

NSLAB levels in raw milk samples were between 1.5-3.0 log CFU/ml (Figure 3). Cheese samples directly from freshly pressed blocks already showed higher than expected levels of NSLAB between 8-9 log CFU/ml. NSLAB levels remained high throughout aging to 6.5 mo, where levels began to taper off for NCA2 and SC with levels below 8 log CFU/ml. For both SC raw milk and freshly pressed cheese samples, NSLAB levels were higher than expected and estimates of 3 and 9 log(CFU/ml) were reported.

HRM-analysis of the P1V1/P2V1 amplicon of NSLAB isolated from raw milk and Cheddar cheese samples identified five species present: *Lactobacillus paracasei/Lactococcus lactis*, *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus curvatus,* and an unidentified NSLAB species also isolated from a prior Cheddar cheese study at Oregon State University, noted as Lab species A. *Lb. paracasei/L. lactis* made up the majority of isolates identified in raw milk. Lab species A was the dominant NSLAB accounting for 82.14% of isolates from 3 mo Cheddar cheese samples (Table 8). *Lb. paracasei/L. lactis* were once again dominant in samples from 6.5 mo aged Cheddar cheese. *Lb. curvatus*, *Lb. plantarum*, and *Lb.*

rhamnosus were not identified in all samples, with *Lb. curvatus* only found in the raw milk (Table 8). *Lb. curvatus* was only identified from raw milk sourced from the north Oregon coast while *Lb. plantarum* was only isolated from SC samples (Table 8). Unidentified isolates accounted for 47.54% of total raw milk isolates. The total of isolates that could not be identified using HRM analysis decreased to 27.19%, 15.32%, 0.00%, and 1.71% at 1 d, 1 mo, 3 mo, and 6.5 mo, respectively.

Rep-PCR of isolates identified 71 different NSLAB strains. A total of 41 and 15 different strains were identified for *Lb. paracasei/L. lactis,* and Lab Species A, respectively (Figure 4 and 5). NC and W farms had 10 and 5 unique strains of *Lb. paracasei/ L. lactis respectively* (Figure 4). Two strains of *Lb. paracasei/L. lactis* were found in Cheddar cheese samples at 1mo, 3 mo, and 6.5 mo. *Lb. paracasei/L. lactis* was also found in Cheddar cheese samples at 1 d, and then again at 6.5 mo (Figure 4). Additionally, 8 and 4 different strains of *L. paracasei/L. lactis* were identified in samples from NC and W samples, respectively (Figure 4). A total of 3, 6, and 1 different Lab Species A strains were identified in W, NC, and SC samples, respectively (Figure 5). Two strains of *Lb. curvatus* were identified from NCC samples, and 1 strain from NCA samples (Figure 6). Finally, 5 strains of *Lb. rhamnosus* were identified in NC, W, and SC samples at 2, 2, and 1 strain, respectively (Figure 6)

HRM analysis of the P1V1/P2V1 amplicon for select isolates of *Lb. paracasei/L. lactis* I, XL, and Lab Species A XV had similar Rep-PCR melt curves (Figure 4, and 5). The melt temperatures of the P1V1/P2V1 amplicon for these strains (Figure 7) had a range of over 2.0°C.

HRM analysis of the P1V1/P2V1 amplicon revealed variation in NSLAB

species in milk collected from the NC-A on two different milk collections (Figure 8).

Two different species were repeated between both days of milk collection from NC-A

(NC-A1 and NCA-2) (Figure 8). Based on HRM analysis of the P1V1/P2V1

amplicon, differences in species were observed between NC farms with 3 and 2

different species identified in NCA and NCB samples, respectively (Figure 9).

Additionally, based on the melt curves of the P1V1/P2V1 amplicon, there were 1 and

2 different species shared between NC-A/NC-C and NC-A/NC-B, respectively

(Figure 9). Further differences in NSLAB species were found between the NC and W

regions with 10 and 5 different species, respectively (Figure 10). In addition, several

NSLAB species were common between both regions (Figure 10).

Species diversity was greatest in raw milk samples with a larger difference in

melting temperatures of the P1V1/P2V1 amplicon (Figure 11). Species diversity

decreased as Cheddar cheese samples aged, with melting temperatures more similar at

3 mo and furthermore at 6.5 mo (Figure 11). Conversely, species diversity observed

in SC samples were the opposite, with differences in melting temperatures of the

P1V1/P2V1 amplicon lowest in raw milk samples and greatest in Cheddar cheese

samples at 6.5 mo (Figure 11).

A total of 12 different species were identified using 16s rDNA sequencing in

select raw milk and aged Cheddar cheese samples (Table 9), which included *Lb.*

plantarum, Lb. paracasei, Lb. rhamnosus, Lb. brevis, L. lactis lactis, L. lactis

cremoris, Enterococcus saccharolyticus, Enterococcus faecalis, Staphylococcus

bovis, Staphylococcus chromogenes, Leuconostoc lactis, and *Weissella*

paramesenteroides. Raw milk and aged Cheddar cheese samples had 11 and 5 species identified, respectively. *Lb. paracasei* and *L. lactis lactis* made up 10.5% and 21% of sequenced raw milk isolates. Both *Lb. paracasei* and *L. lactis lactis* each accounted for 40% of the isolates identified through sequencing in aged Cheddar cheese samples.

Using 16s rDNA sequencing *W. paramesenteroides, S. bovis, L. lactis cremoris, Leuc. lactis, Lb. brevis* were only identified in North Coast samples (Table 9). Of these, only *L. lactis cremoris, Leuc. lactis, Lb. brevis,* and *S. bovis* were identified in raw milk samples. Species unique to Willamette Valley samples were *E. faecalis, E. saccharolyticus,* and *S. chromogenes*. *Lb. rhamnosus* was only identified in South Coast samples. Additionally, sample SC-A1 6.5M, which was not identified by HRM analysis, was identified as *Lb. plantarum* through 16s rDNA sequencing. Isolates identified as Lab species A by HRM analysis were also identified as a lactococci or enterococci by 16s rDNA sequencing (not shown). Isolates identified as *L. lactis lactis* and *Lb. paracasei* through 16S rDNA sequencing were in agreement with HRM analysis 66.7% and 55.6% of the time, respectively (Table 9). Comparing the melt curves of the P1V1/P2V1 amplicon of isolates identified as *L. lactis lactis* and *Lb. paracasei* illustrates that there is a large amount of overlap in melting temperatures (Appendix 6). NSLAB identified as *Lb. rhamnosus* and *Lb. plantarum* through 16s rDNA sequencing were in agreement to HRM analysis 100% and 0% of the time, respectively (Table 9).

3.5 Discussion:

de Man, Regosa, and Sharpe (MRS) media is commonly used for the cultivation of lactobacilli. Though selective for lactobacilli, it has been found that lactococci are able to grow on MRS as well (Couret et al., 2003). The author also noted that reducing agents can help make MRS more selective, but in general cultivation of lactobacilli is done with MRS. When trying to isolate and identify unknown NSLAB this becomes a challenge, as was seen in this study with the growth of *S. chromogenes,* and *Leuc. lactis* from raw milk samples. The limitations of MRS need to be a consideration when investigating NSLAB.

NSLAB levels observed in raw milk used in this study were in range of previous work by Rehman et al. (2000), Milesi et al. (2010), and Bezeková et al (2013). Observed NSLAB populations in cheese samples were in agreement to findings in previous work for both raw milk and pasteurized cheeses (De Angelis et al. 2001; Van Hoorde et al., 2008 Porcellato et al., 2015). The sharp increase in NSLAB populations observed in cheese samples taken from the press may be explained by the cultivation of starter culture on MRS, as well as enterococci and staphylococci. Additionally, lactococci levels in freshly made cheese have been found to be around 6 log CFU/ml when cultivated on M17 (Michel et al., 2001; Porcellato et al., 2013). Similar results were found by Broadbent et al. (2003), with starter populations above 9 log CFU/ml at 1 d after production in pasteurized Cheddar cheese. With lactococci identified in aged Cheddar cheese at 6.5 mo in this study, it is unlikely that the population increase noted at 1 d only was strictly an increase in NSLAB. Kable et al. (2016), using community 16s rRNA gene sequencing, found that over 50% of the taxa present in raw milk made up less than 1.0% of the total

present. Considering the microbial diversity seen in raw milk, much of the bacteria present in cheese is likely to be missed using cultured methods.

NSLAB species diversity was greatest in raw milk and declined as the Cheddar cheese aged. Crow et al. (2001) suggested that the loss of NSLAB diversity in aged cheese could be caused by non-dominant species remaining at population levels observed in raw milk, which is overshadowed by the dominant NSLAB levels at 6-9 log CFU/ml. Loss of species diversity can also be impacted by the starter/adjunct culture used by changing the available nutrients to NSLAB present throughout aging (Broadbent et al., 2003).

NSLAB found in raw milk were different between farms and between days that the milk was sourced. Desmasures and Gueguen (1997) had similar findings, suggesting that organisms useful for cheese were characteristic to the farm. Some of the differences observed in NSLAB between farms can be attributed to differences in hygienic practices during milking, as noted by Verdier-Metz et al (2009). The authors noted that lower levels of hygiene led to increased bacterial levels and species diversity. In addition to hygienic practices, refrigeration of raw milk at 4°C for 24 hr showed a decrease in bacteria present including *Lb. plantarum* (Lafarge et al., 2004). Season when raw milk was collected was also found to impact bacterial populations, with spring having the highest microbial diversity (Kable et al. 2016).

HRM analysis of the P1V1/P2V1 amplicon was found to be not sensitive enough to effectively distinguish between *Lb. paracasei* and *L. lactis lactis*. Many of the melt curves for both species were found to be very similar and potentially overlap.

This similarity in melt curves led to the misidentification of NSLAB as determined through 16s rDNA sequencing (Table 8). Porcellato et al. (2012) used a similar HRM method and noted it was necessary to have a well-developed reference library to use for each run for proper identification of NSLAB, although this is mostly useful when trying to identify target species. Without a large reference library to use per run, coupled with the wide variation observed in *Lb. paracasei* and *L. lactis lactis* melt curves (Figure 7), HRM analysis of the P1V1/P2V1 amplicon does not appear to be robust enough for NSLAB species identification.

Through additional 16s rDNA sequencing, it was found that species diversity was much greater in raw milk (Table 7) than originally determined through HRM analysis (Figure 10). The decrease in bacterial diversity was observed in both HRM analysis and 16s rDNA sequencing of 6.5-month old Cheddar samples (Table 7 and Figure 10).

3.6 Conclusion

Differences in NSLAB were observed in raw milk from each farm. Additionally, NSLAB were different in raw milk that was collected from the same farm, NCA, on two different days. Certain species of NSLAB were only identified in specific regions such as *Lb. rhamnosus* in SC samples, and *W. paramesenteroides* in NC farms suggesting a possible regional impact.

HRM analysis of the P1V1/P2V1 amplicon was determined not robust enough for the identification of unknown. Additionally, HRM analysis of the P1V1/P2V1 amplicon relies on the cultivation of isolates, which potentially skews the

results when looking at species diversity in milk or cheese. By employing a culture-independent method it is possible to identify several species below the detection limit helping to better investigate NSLAB diversity in Cheddar cheese. It is important to note that much of the diversity reported in NSLAB in aged Cheddar cheese in this study was observed at the stain level which points to a need for cultured methods still. With the considerations stated prior, future work investigating species diversity in raw milk and cheese should consider a polyphasic approach using cultured and culture-independent methods such as community 16s rDNA sequencing.

Future work is needed to investigate the contribution of day to day milking on NSLAB in raw milk and the potential for a regional contribution to NSLAB. Producers can use these findings to help control quality at the farm level or capitalize on the differences in NSLAB between farms in single farm limited small batch runs.

3.7 Acknowledgments

3.8 References.

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Table 5: Herd, feed, and pasture composition each dairy farm during time of milk collection

Farm[e]	Herd Composition per milking[b]	Feed in lbs	Time in Pasture per day	Major Pasture[c] Grass	Minor Pasture[d] Grass	Silage Type
W-A	10% Friesian/Jersey Mix 90% Jersey	4-Grain	21.6 hours	Perennial Rye Grass Orchard Grass White Clover	Plantain Herb	No Silage
W-B Grp 1[a]	32% Holsteins 68% Jersey	3.5-Alfalfa 8.5-Grain	12 hours	Orchard Grass Fescue		Corn Silage Grass Silage
Grp 2[a]	43% Holsteins 57% Jersey	7-Alfalfa 15-Grain		Rye Grass		Corn Silage
NC-A	100% Certified Jersey	5-Alfalfa 14-Grain	15 hours	Rye Grass	Clover, Native	No silage
NC-B	100% Certified Jersey	9-Alfalfa 19-Grain	14 hours	Orchard Grass	Rye Grass, Clover	Cannery
NC-C	11% Holstein 11% Aryshire 78% Jersey	20-Grain	16.5 hours	Rye Grass, Orchard Grass	Clover	Italian Rye Silage
SC	82% Holstein 6% Brown Swiss 12% Jersey	6.25-Alfalfa 12.5-Grain	19 hours	Rye Grass Fescue White Clover	Velvet Grass Canary Grass	Grass Silage

[a] Grp 1 and 2 differ based on feed given
[b] Herd composition based on survey
[c,d] Flora composition based on survey responses
[e] Farms: North Coast A (NC-A), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)

Table 6: Danisco freeze dried Choozit starter cultures RA 21 and MD 88 used in Cheddar cheese production.

Starter Blend	Species	Danisco Culture Unit (DCU)
RA Blend		3.3 DCU/100 lb
	L. lactis subsp. *lactis*	
	L. lactis subsp. *cremoris*	
	S. thermophilus	
MD		
	L. lactis subsp. lactis biovar. diacetylactis	4.5 DCU/100 lb

Table 7: Composition of Full Fat Raw Cheddar Cheese at 3.5 months of aging.

Farm[a]	Raw milk pH	Fat	Protein	Moisture	Salt
W-A	6.62	32.89[A]	28.96[A]	34.81	1.81
W-B	6.63	35.49[B]	25.43[C]	35.28	1.79
NC-A	6.59	33.45[AC]	28.40[A]	35.01	1.85
NC-A2	6.61	32.82[A]	28.72[A]	35.17	1.91
NC-B	6.59	34.11[C]	27.29[B]	35.05	1.79
NC-C	6.64	34.16[C]	27.15[B]	35.40	1.82
SC	6.61	33.73[C]	25.73[C]	35.58	1.86
Mean	6.61	33.81	27.38	35.18	1.83
Standard Deviation	0.019	0.91	1.41	0.26	0.04
Standard Error	0.007	0.345	0.532	0.098	0.017

[a] Farms: North Coast A (NC-A), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)
[b] Values within a column with different superscripts are considered significantly different; n=3 per farm (Tukey Cramer, $p < 0.05$)

Table 8: Farm level distribution of NLSAB throughout aging of Cheddar cheese through as identified by HRM analysis of the P1V1/P2V1 amplicon.

Farm	WA					WB					NCA1					NCA2					NCB					NCC					SC					Percent				
	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month	Raw	Day 1	1 Month	3 Month	6.5 Month
Lb. curvatus											1					3										4										13.11%	0.00%	0.00%	0.00%	0.00%
Lb. paracasei/L. lactis		1	4	2	10	1	3	11	1	15	6	2	3	5	13	1	2	5	2	13	3		5	3	15	2	7	8	4	10		3	2	2	8	21.31%	41.86%	30.65%	16.96%	71.79%
Lb. platarum																																	3			0.00%	0.00%	2.42%	0.00%	0.00%
Lb. rhamnosus	1																			1	2	1		1									2		6	4.92%	2.33%	1.61%	0.89%	5.98%
Lab species A*	5	9	10	13	4	3	3	2	14				10	13	4			10	13	4			10	13	4			10	13	4			10	13	4	13.11%	27.91%	50.00%	82.14%	20.51%
Unknown	4		1		1	6	4	2			2	2	4			4	2	3			4	2	1			3		2			6	2	6		1	47.54%	27.91%	15.32%	0.00%	1.71%
Total	10	10	15	15	15	10	10	15	15	15	9	4	17	18	17	8	4	18	15	18	9	3	16	17	19	9	7	20	17	14	6	5	23	15	19	100.00%	100.00%	100.00%	100.00%	100.00%

*Isolated from previous Cheddar cheese produced at Oregon State University

** Farms: North Coast A (NC-A), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)

Table 9: Identification of raw milk and Cheddar cheese isolates using MicroSEQ® 500 compared to HRM analysis of P1V1/P2V1 amplicon with associated strain ID

Sample IDª	Source	MicroSEQ® 500 ID	% Match	HRM ID w/ Strain ID	T_m (°C)
NCA1-A1	Raw	*Weissella paramesenteroides*	99.78	Unknown[b]	78.26
NCA1-B1	Raw	*Lactobacillus paracasei paracasei; Lactobacillus paracasei tolerans*	99.95; 99.88	*Lb. paracasei* XVIII	82.80
NCA2-B5	Raw	*Streptococcus bovis*	99.93	*Lb. curvatus* II	79.29
NCB-A2	Raw	*Weissella paramesenteroides*	99.76; 99.57	*Lb. rhamnosus* IV	79.03
NCB-A3	Raw	*Lactococcus lactis cremoris*	99.73; 99.74	Unknown[b]	80.29
NCB-A4	Raw	*Leuconostoc lactis*	100	Unknown[b]	82.91
NCB-A5	Raw	*Lactobacillus plantarum; Lactobacillus pentosus*	99.9; 99.82	*Lb. paracasei*	80.50
NCB-B1	Raw	*Lactobacillus plantarum; Lactobacillus pentosus*	99.89; 99.81	*Lb. paracasei*	82.10
NCB-B3	Raw	*Weissella paramesenteroides*	99.81	Unknown[b]	80.32
NCC-A1	Raw	*Lactobacillus brevis*	99.89	Unknown[b]	82.67
NCC-A3	Raw	*Lactococcus lactis lactis; Lactococcus lactis hordniae*	99.58; 99.46	*Lb. rhamnosus*	79.40
NCC-B2	Raw	*Lactococcus lactis lactis*	100.00	Unknown[b]	81.10
NCC-B3	Raw	*Lactococcus lactis lactis*	100.00	Unknown[b]	83.60
NCC-B1	Raw	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	100.0; 99.9	*Lb. rhamnosus*	80.30
NCC-B5	Raw	*Lactococcus lactis lactis*	100.00	Unknown[b]	82.00
WA-B1	Raw	*Leuconostoc lactis*	100	Lab species A	81.47
WB-A5	Raw	*Enterococcus faecalis*	99.74	Unknown[b]	81.90
WB-B1	Raw	*Staphylococcus chromogenes*	99.86	Unknown[b]	82.72
WB-B5	Raw	*Enterococcus saccharolyticus*	99.95	Lab species A XII	81.01
NCA1-A1	6.5 Month	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	100.0; 99.9	*L. lactis*	81.40
NCA1-A3	6.5 Month	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	100.00; 99.9	*Lb. paracasei* XV	82.00
NCA1-A5	6.5 Month	*Lactobacillus paracasei paracasei; Lactobacillus paracasei tolerans*	99.94; 99.89	*Lb. paracasei* XV	82.40
NCA1-B3	6.5 Month	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	99.98; 99.93	*Lb. paracasei* XIX	82.50
NCA2-B4	6.5 Month	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	99.95; 99.85	*L. lactis*	82.50
NCA2-C1	6.5 Month	*Weissella paramesenteroides*	99.7	*Lb. rhamnosus*	78.86

NCB-A4	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis* XXIX	81.80
NCB-A5	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis* XXXII	81.90
NCB-C5	6.5 Month	*Lactococcus lactis lactis*	100.00	*Lb. paracasei*	82.00
NCC-A1	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis* XXXIII	81.40
NCC-A3	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis*	81.70
NCC-C1	6.5 Month	*Lactobacillus paracasei paracasei; Lactobacillus paracasei tolerans*	99.95; 99.87	*Lb. paracasei*	81.60
SC-A1	6.5 Month	*Lactobacillus plantarum; Lactobacillus pentosus*	99.88; 99.81	Unknown[b]	79.20
SC-A3	6.5 Month	*Lactobacillus rhamnosus*	99.94	*Lb. rhamnosus*	78.50
SC-B1	6.5 Month	*Lactobacillus rhamnosus*	99.98	*Lb. rhamnosus*	79.00
WA-A3	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis*	81.40
WA-A4	6.5 Month	*Lactobacillus paracasei paracasei; Lactobacillus paracasei tolerans*	99.93; 99.92	Unknown[b]	81.50
WA-C5	6.5 Month	*Lactococcus lactis lactis*	100.00	*Lb. paracasei* S	82.00
WB-A2	6.5 Month	*Lactococcus lactis lactis*	100.00	*L. lactis*	81.40
WB-A4	6.5 Month	*Lactobacillus paracasei tolerans; Lactobacillus paracasei paracasei*	99.95; 99.85	*L. lactis* XXXII	81.70

[a] Raw milk and Cheddar cheese sample ID associated with farms: North Coast A first collection (NC-A1), North Coast A second collection (NC-A2), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)

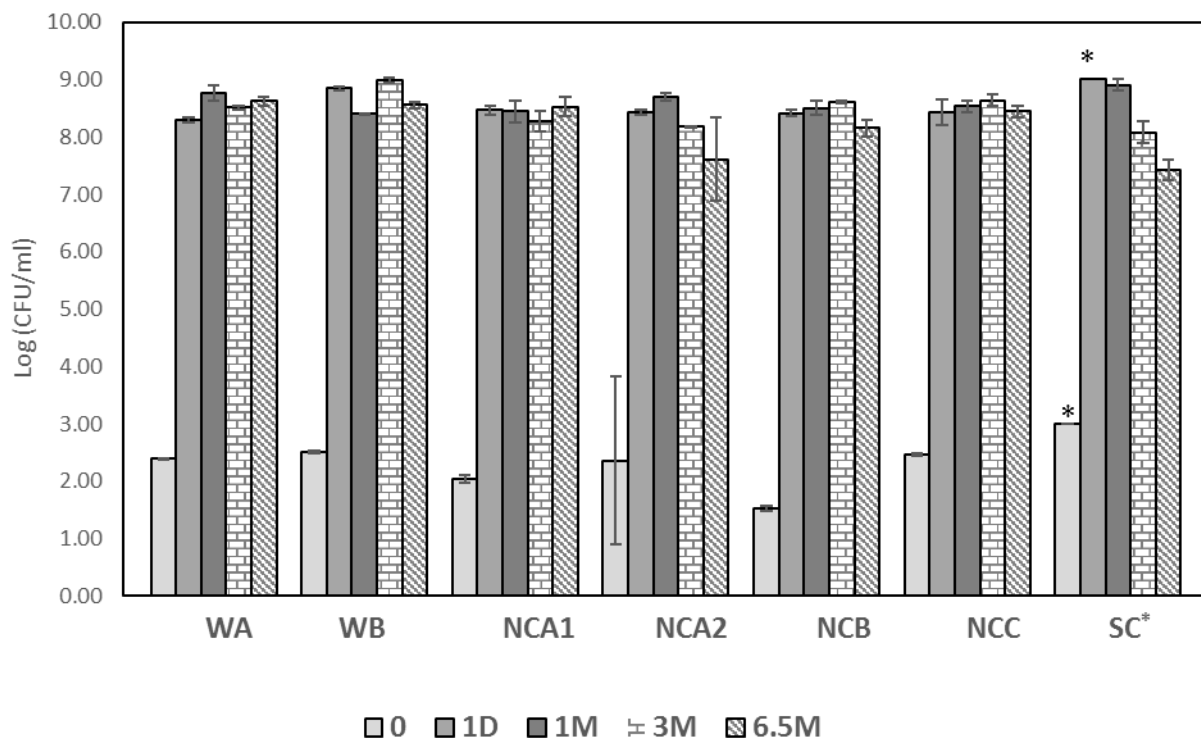[b] Isolates were not identified using HRM analysis of the P1V1/P2V1 amplicon.

Figure 3: NSLAB populations in raw milk and Cheddar cheese made with milk sourced from farms on the Oregon Coast or the Willamette Valley at different points of aging.

*Population levels were estimated as 3 and 9 Log CFU/ml for SC at 0, and 1D since levels were TNTC at expected detection limit

** Farms: North Coast A first collection (NC-A1), North Coast A second collection (NC-A2), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)
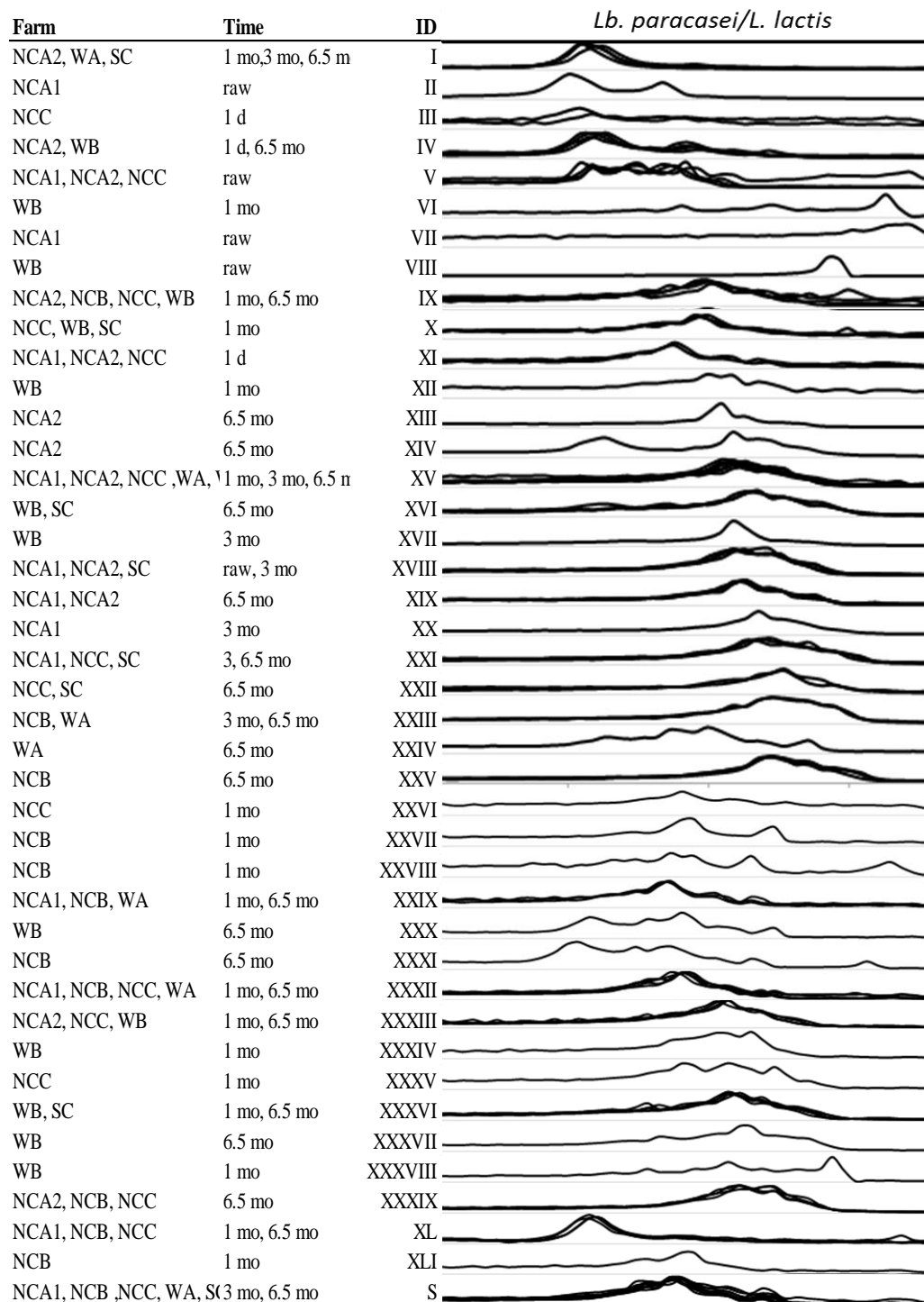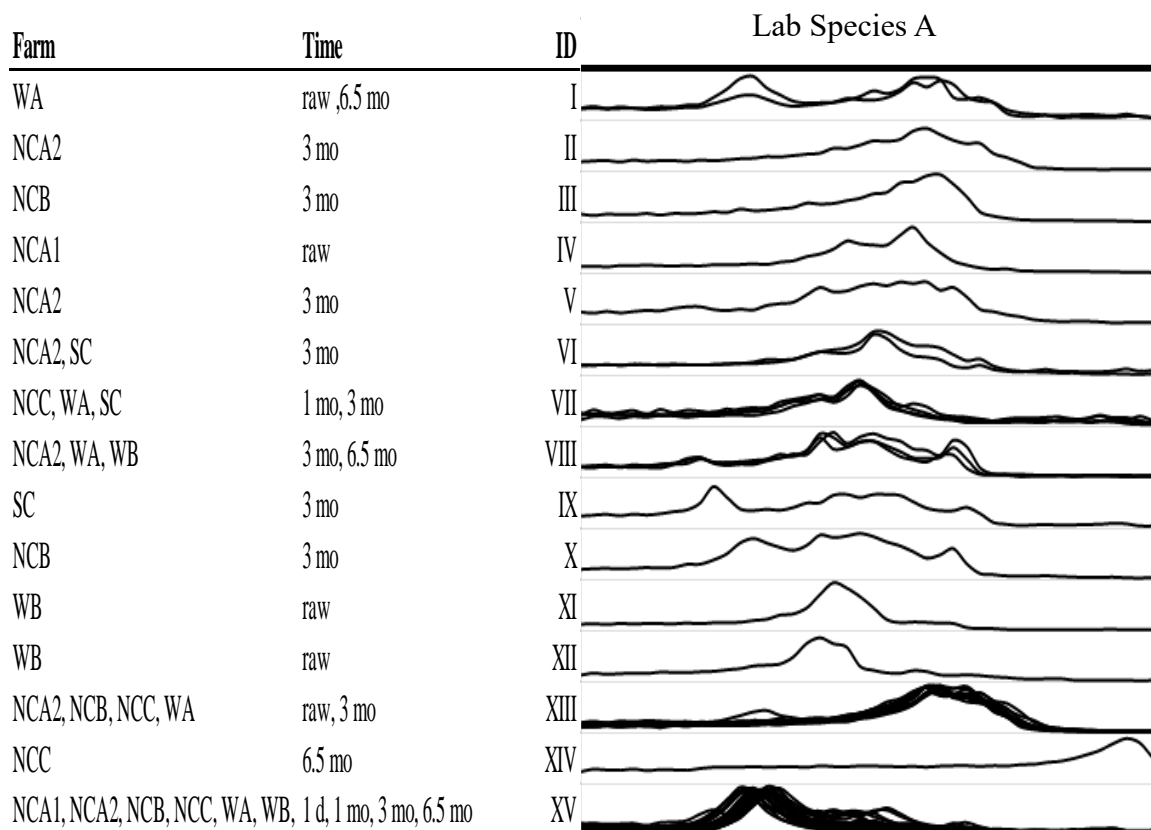
Farm	Time	ID	*Lb. paracasei/L. lactis*
NCA2, WA, SC	1 mo,3 mo, 6.5 m	I	
NCA1	raw	II	
NCC	1 d	III	
NCA2, WB	1 d, 6.5 mo	IV	
NCA1, NCA2, NCC	raw	V	
WB	1 mo	VI	
NCA1	raw	VII	
WB	raw	VIII	
NCA2, NCB, NCC, WB	1 mo, 6.5 mo	IX	
NCC, WB, SC	1 mo	X	
NCA1, NCA2, NCC	1 d	XI	
WB	1 mo	XII	
NCA2	6.5 mo	XIII	
NCA2	6.5 mo	XIV	
NCA1, NCA2, NCC ,WA, ʼ	1 mo, 3 mo, 6.5 n	XV	
WB, SC	6.5 mo	XVI	
WB	3 mo	XVII	
NCA1, NCA2, SC	raw, 3 mo	XVIII	
NCA1, NCA2	6.5 mo	XIX	
NCA1	3 mo	XX	
NCA1, NCC, SC	3, 6.5 mo	XXI	
NCC, SC	6.5 mo	XXII	
NCB, WA	3 mo, 6.5 mo	XXIII	
WA	6.5 mo	XXIV	
NCB	6.5 mo	XXV	
NCC	1 mo	XXVI	
NCB	1 mo	XXVII	
NCB	1 mo	XXVIII	
NCA1, NCB, WA	1 mo, 6.5 mo	XXIX	
WB	6.5 mo	XXX	
NCB	6.5 mo	XXXI	
NCA1, NCB, NCC, WA	1 mo, 6.5 mo	XXXII	
NCA2, NCC, WB	1 mo, 6.5 mo	XXXIII	
WB	1 mo	XXXIV	
NCC	1 mo	XXXV	
WB, SC	1 mo, 6.5 mo	XXXVI	
WB	6.5 mo	XXXVII	
WB	1 mo	XXXVIII	
NCA2, NCB, NCC	6.5 mo	XXXIX	
NCA1, NCB, NCC	1 mo, 6.5 mo	XL	
NCB	1 mo	XLI	
NCA1, NCB ,NCC, WA, S(	3 mo, 6.5 mo	S	

Figure 4: Location, time, and fingerprint Id of *Lb. paracasei/L. lactis* through REP-PCR

* Farms: North Coast A first collection (NC-A1), North Coast A second collection (NC-A2), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)
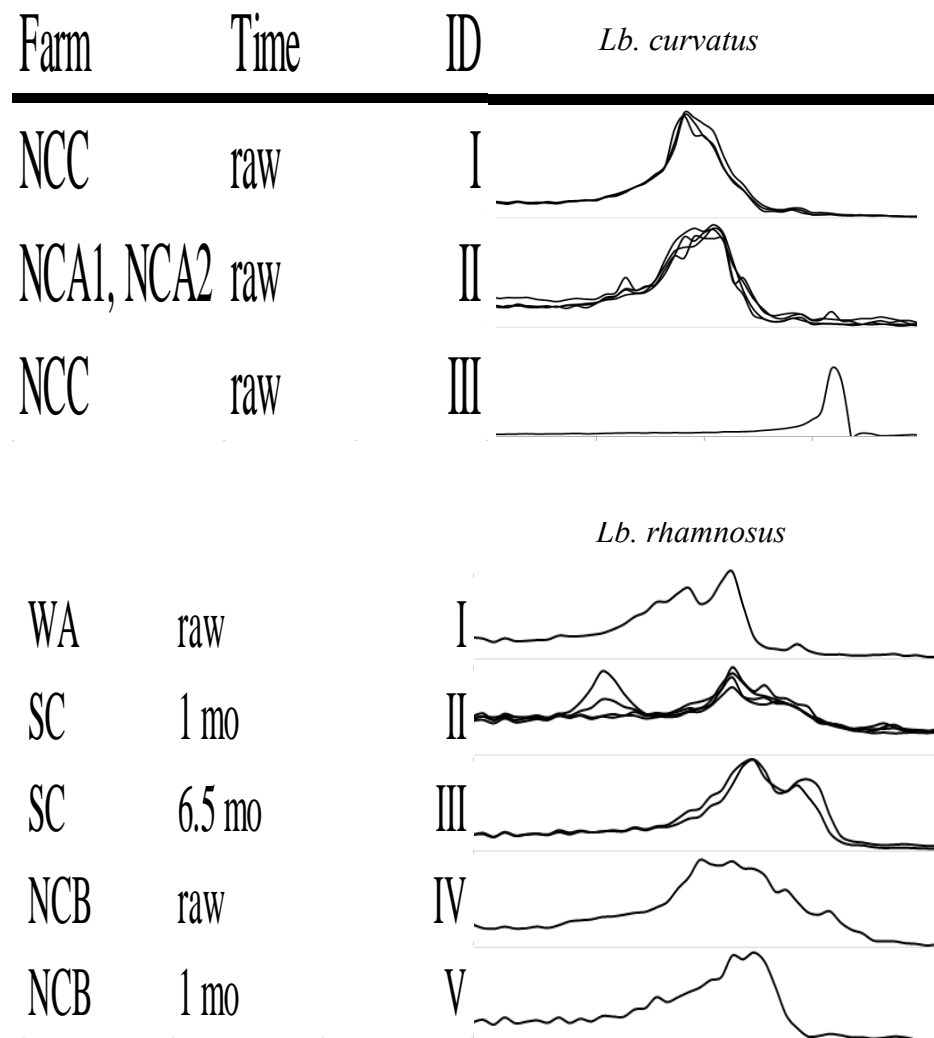
Farm	Time	ID	Lab Species A
WA	raw ,6.5 mo	I	
NCA2	3 mo	II	
NCB	3 mo	III	
NCA1	raw	IV	
NCA2	3 mo	V	
NCA2, SC	3 mo	VI	
NCC, WA, SC	1 mo, 3 mo	VII	
NCA2, WA, WB	3 mo, 6.5 mo	VIII	
SC	3 mo	IX	
NCB	3 mo	X	
WB	raw	XI	
WB	raw	XII	
NCA2, NCB, NCC, WA	raw, 3 mo	XIII	
NCC	6.5 mo	XIV	
NCA1, NCA2, NCB, NCC, WA, WB	1 d, 1 mo, 3 mo, 6.5 mo	XV	

Figure 5: Location, time, and fingerprint Id of Lab Species A isolates through REP-PCR

* Farms: North Coast A first collection (NC-A1), North Coast A second collection (NC-A2), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)

Farm	Time	ID	Lb. curvatus
NCC	raw	I	
NCA1, NCA2	raw	II	
NCC	raw	III	

Lb. rhamnosus

Farm	Time	ID	
WA	raw	I	
SC	1 mo	II	
SC	6.5 mo	III	
NCB	raw	IV	
NCB	1 mo	V	

Figure 6: Location, time, and fingerprint Id of *Lb. rhamnosus* and *Lb. curvatus* isolates through REP-PCR

* Farms: North Coast A first collection (NC-A1), North Coast A second collection (NC-A2), North Coast B (NC-B), North Coast C (NC-C), South Coast (SC) Willamette Valley A (W-A), Willamette Valley B (W-B)
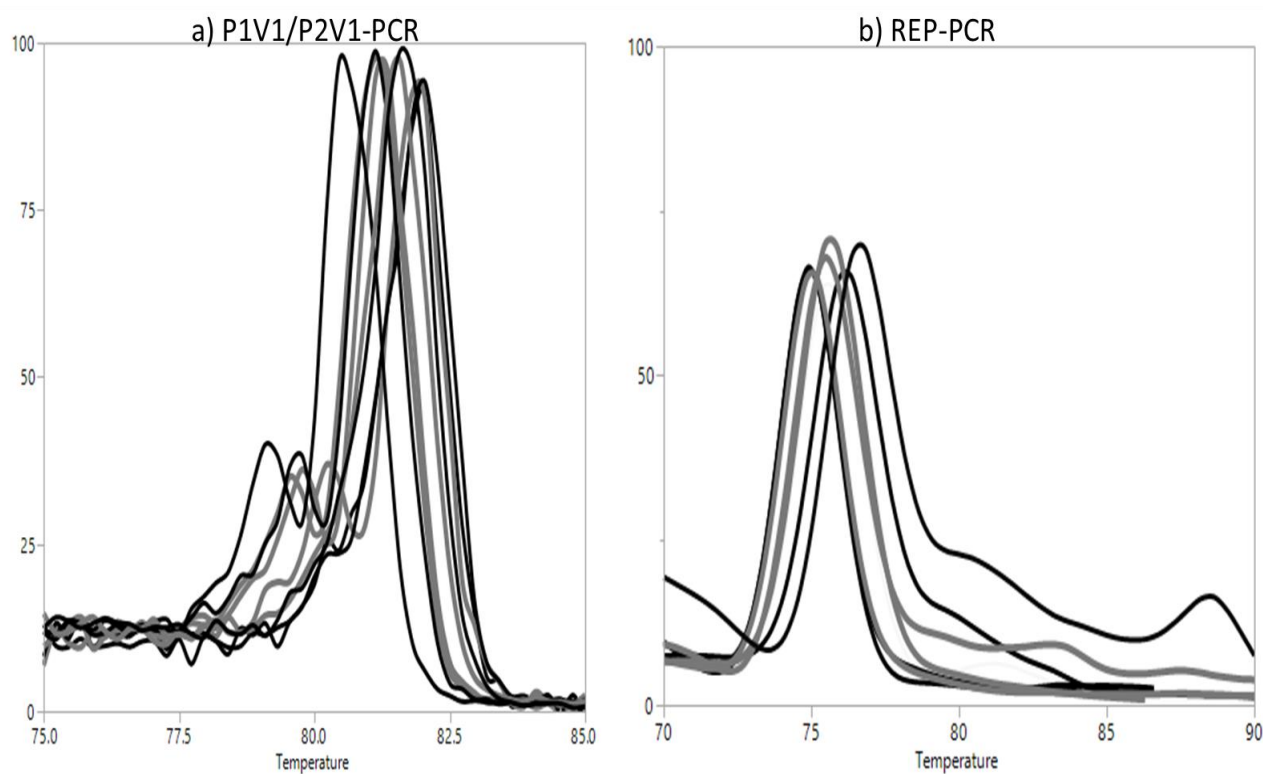
Figure 7 Comparison of HRM analysis of a) P1V1/P2V1 amplicon for *Lb. paracasei*/*L. lactis lactis* (━), and Lab Species A (━) isolates to their b) fingerprint generated through REP-PCR
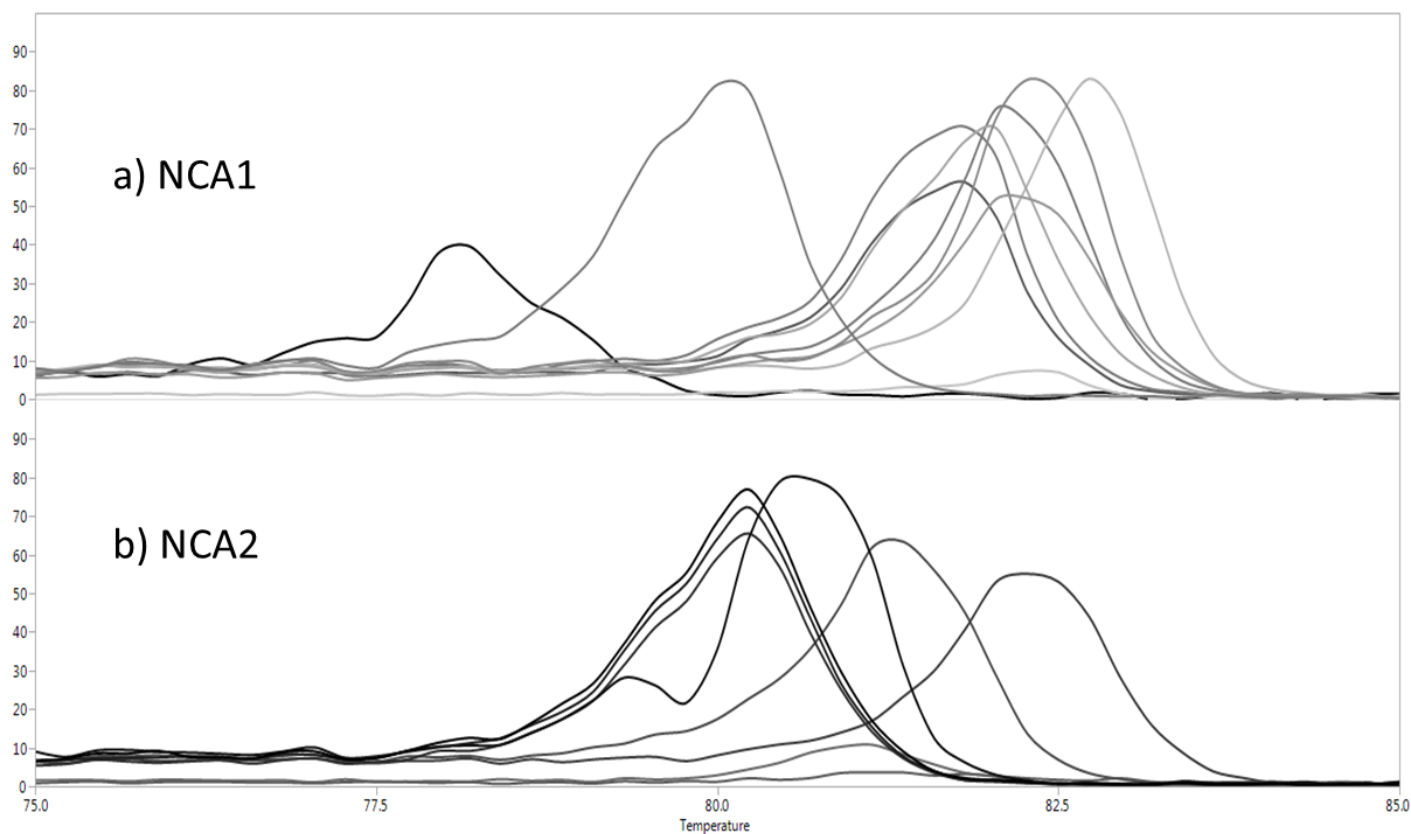
Figure 8: HRM melt curves of the P1V1/P2V1 amplicon of NSLAB present in raw milk from NCA on different days of collection. a) North Coast A first collection (NCA1); n=10 b) North Coast A second collection (NCA2); n=8.
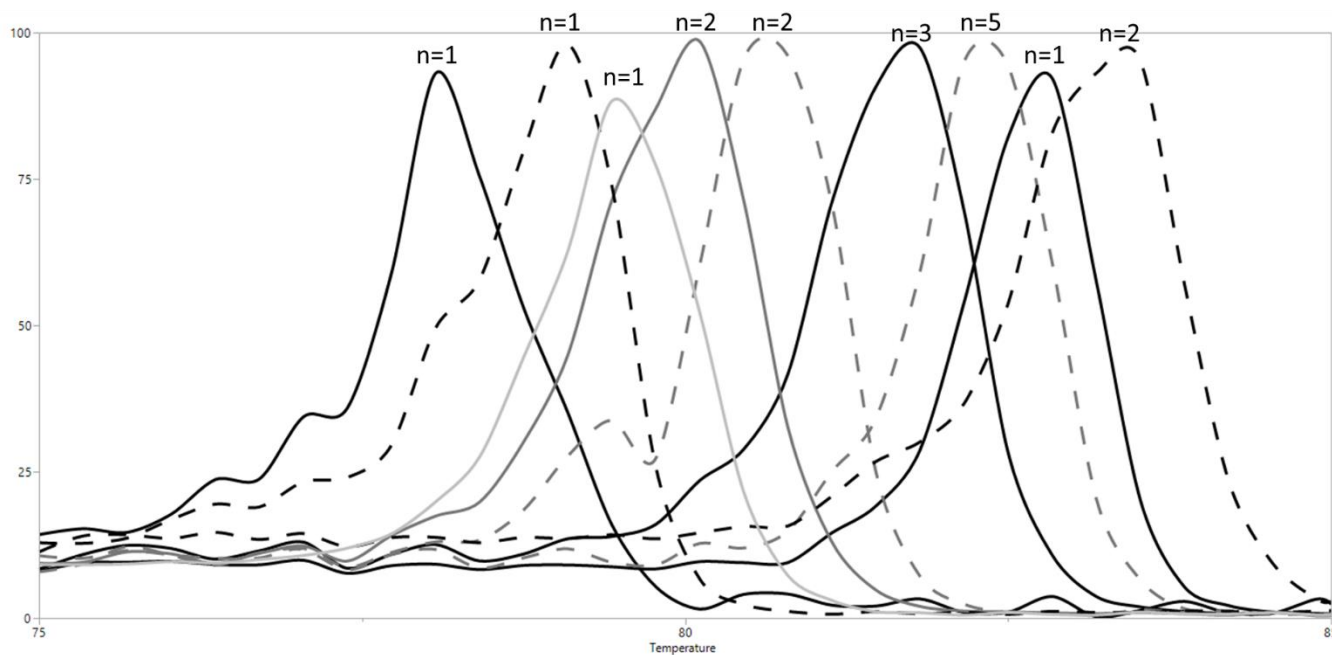
Figure 9: Melt peaks from HRM analysis of the P1V1/P2V1 amplicon of NSLAB species in raw milk from the North Oregon Coast; (●) North Coast A (NCA), (- - -) North Coast B (NCB), (●) North Coast C (NCC), (●) NCA and NCC, (- - -) NCA and NCB
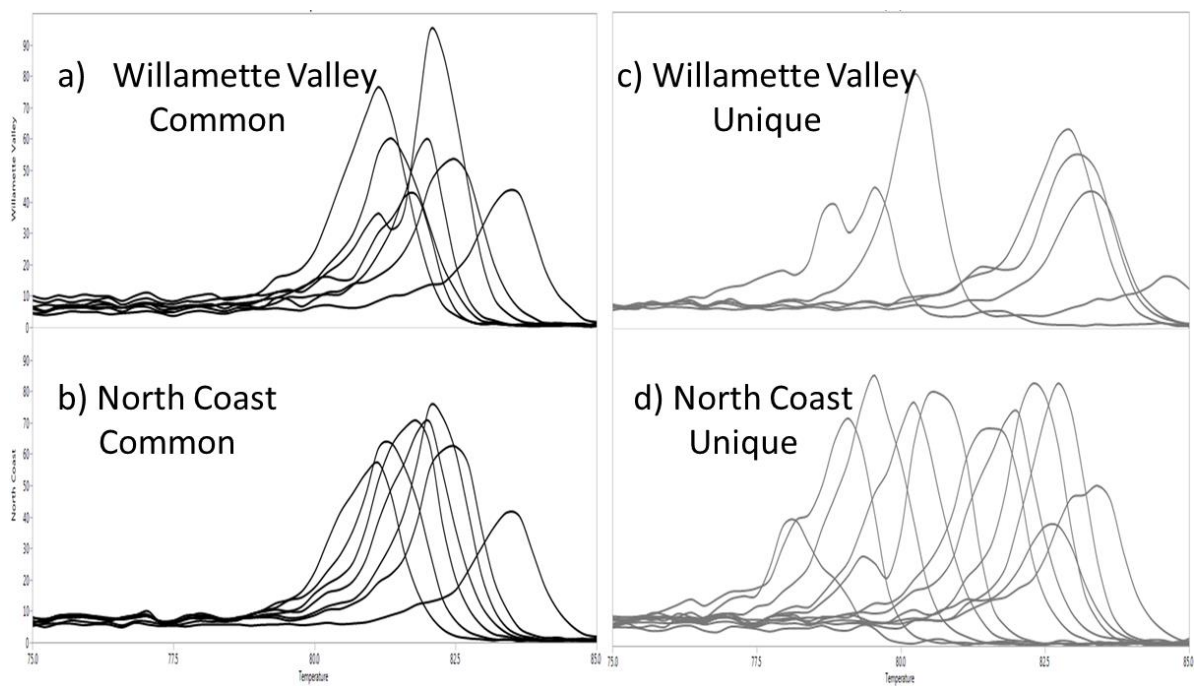
Figure 10: HRM analysis of the P1V1/P2V1 amplicon of NSLAB found in raw milk form the Willamette Valley and the North Oregon Coast for common and unique species on within this study.
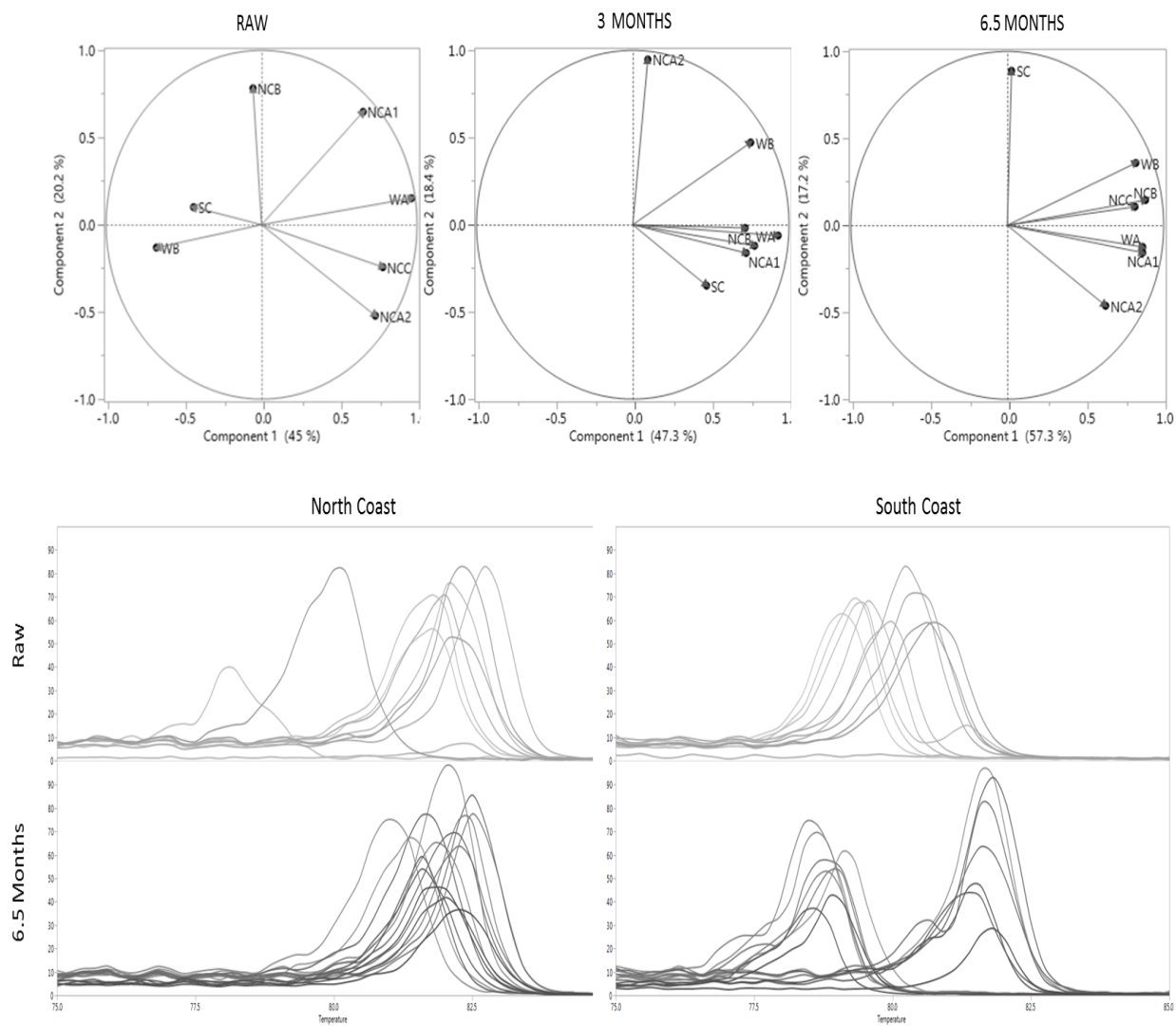
Figure 11: Farm level differences based on melting temperature of NSLAB species in raw milk and aged Cheddar cheese; raw milk n=10/farm, 3 and 6.5 months n=15/farm.

3. General Conclusion

The ability for producers to identify the NSLAB in the raw milk and the final cheese product is an important step in controlling quality. Since not all NSLAB are affected by heat treatments the same it is important to be able to isolate and identify problem organisms. Methods utilizing real time PCR are very attractive for larger producers as many already have the equipment needed. Unfortunately, P1V1/P2V1 PCR with HRM analysis was not sensitive enough to separate out certain NSLAB, leading to misidentifications of isolates. Though attractive due to its easy use, API 50 CHL also had issues correctly identifying NSLAB and with the associated cost and time needed for the method it is a less appealing option. The best performing method for NSLAB identification was 16s rDNA sequencing, but this method out of all is least likely to be used by a producer as most would not have a sequencer on hand. To better characterize NSLAB diversity in cheese a polyphasic approach, including both culture and culture-independent methods, should be considered as many species can be found at levels below 1.0% of the total species identified in a sample.

Farm level contribution to NSLAB in both raw milk and Cheddar cheese were observed. Direct causes to NSLAB difference between farms were not identified, though possible sources are the pasture grasses and farm sanitation practices. Information was gathered on herd and pasture composition, but no direct link could be made with the differences in NSLAB between farms. In cheese, the farm level contribution was not as obvious as most of the cheeses between farms had the same NSLAB species. Though species became more homogenous between all of the

cheeses, there were still strain level differences between cheeses that could be attributed to the source of the milk. This cannot be said as a matter of fact, though, as none of the strains found in the 6.5 mo samples were observed in the raw milk. Though they were not observed in the raw milk they still could have been present, but below the level of detection.

Difficulty was also encountered when developing a research recipe for Cheddar cheese. Initial testing was only conducted using milk sourced from W-B, but when applied to other milk sources issues with acidification during production arose. It was determined that multiple milk sources should be used for testing a cheese recipe for further research.

The information reported here suggests that milk source at the farm level does contribute to the NSLAB in raw milk and cheese produced with it. More work is still needed, though, to investigate the farm level contribution to Cheddar cheese throughout the year. Additionally, further investigation is needed to determine how much fluctuation occurs in NSLAB between days of milk collection. This information can help producers understand the contribution their farmers have on their product and develop steps to mitigate it or potentially capitalize on the differences in a small batch capacity.

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Appendix:

Appendix 1: P1V1/P2V1 amplicon sequence of NCBI reference strains with estimated and experimental melting points.

NCBI Reference Strain	P1V1/P2V1 Amplicon	Est. T_m(C°)[a]	Exp. T_m(C°)[b]
Lb. casei ATCC 334	G C G G C G T G C C T A A T A C A T G C A A G T C G A A C G A G T T C T C - - G T T G A T G A T C G G T G C T T G C	81.10	81.79±0.20
Lb. paracasei N1115	G C G G C G T G C C T A A T A C A T G C A A G T C G A A C G A G T T C T C - - G T T G A A T C T C G G T G C T T G C	81.10	82.01±0.03
Lb. rhamnosus ATCC 53103	G C G G C G T G C C T A A T A C A T G C A A G T C G A A C G A G T T C T G - - A T T A T T G A A A G G T G C T T G C	78.00	79.43±0.31
Lb. plantarum WCFS1	G C G G C G T G C C T A A T A C A T G C A A G T C G A A C G A A C T C T G - - G T A - T T G A T T G G T G C T T G C	78.10	79.34±0.13
L. lactis subsp. *lactis* NBRC 100933	G C G G C G T G C C T A A T A C A T G C A A G T T G A G C G C T G A A G G - - T T G G T A C - - - - - - - - - - - -	80.60	81.69±0.12
	* * * * * * * *		
Lb. casei ATCC 334	A C C G A - - - - - - - - - - - G A T T C A A C A T G G A A C G A G T G G C G G A C G G G T G A G T A A C A C G T G G G		
Lb. paracasei N1115	A C C G A - - - - - - - - - - - G A T T C A A C A T G G A A C G A G T G G C G G A C G G G T G A G T A A C A C G T G G G		
Lb. rhamnosus NBRC 3425	A T C T T - - - - - - - - - - - G A T T T A A T T T T G A A C G A G T G G C G G A C G G G T G A G T A A C A C G T G G G		
Lb. plantarum WCFS1	A T C A T - - - - - - - - - - - G A T T T A C A T T T G A G T G A G T G G C G A A C T G G T G A G T A A C A C G T G G G		
L. lactis subsp. *lactis* 1403	- - - - - - - - - - - - - - - - T T G T A C C G A C T G G A T G A G C A G C G A A C G G G T G A G T A A C G C G T G G G		
	* * * * * * * * * * * * * * * * * * * * * * * * * *		

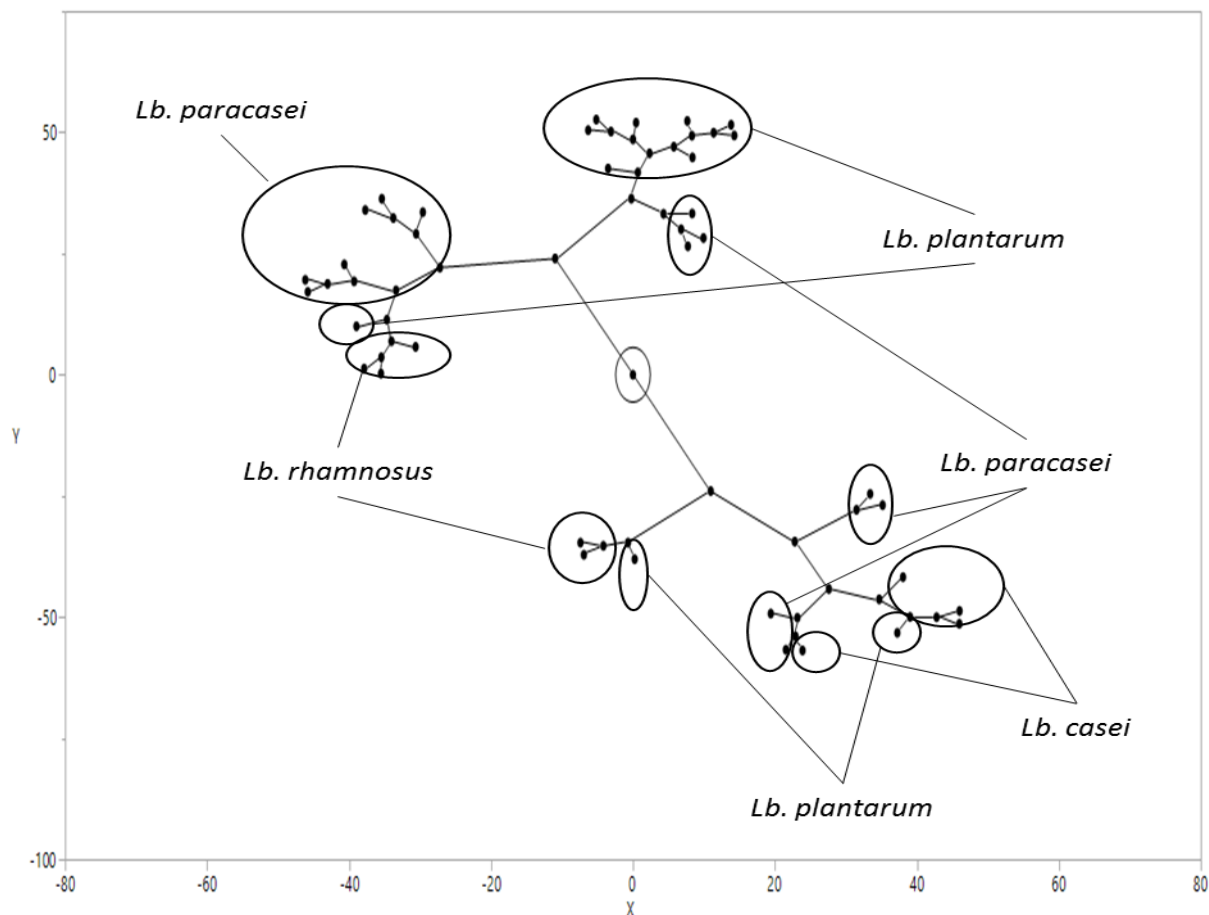[a] Estimated T_m of P1V1/P2V1 on obtained from BioPhp set for Basic T_m:
http://www.biophp.org/minitools/melting_temperature/demo.php
[b] Average T_m from Table 2
[c] Sequences acquired through NIH Blast: http://blast.ncbi.nlm.nih.gov/Blast.cgi
[e] Clustal Omega used for sequence alignment: www.ebi.ac.uk/Tools/msa/clustalo/
*Consensus of bases between all NLAB species

Appendix 2: Alignment of MicroSEQ® 500 consensus data for the P1V1/P2V1 amplicon of raw milk and Cheddar cheese isolates with estimated and experimental melting temperatures.

Species	Sample ID	P1V1/P2V1 Amplicon		Estimated T$_m$ (°C)	Experimental Tm (°C)
E. faecalis	WB-A5 raw	GCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTCCTCCCGAGTG------------	48	80.90	82.10
E.saccharolyticus	WB-B5 raw	GCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTTCCGCCGA---A-----------	46	79.80	81.30
Lb. brevis	NCC-A1 raw	GCGGCATGCCTAATACATGCAAGTCGAACGAGCTTCCRTTGAATG-ACGTGCT-------	52	78.90	82.50
Leuc. Lactis	NCB-A4 raw	GCGGCGTGCCTAATACATGCAAGTCGAACGCGCAGCGAAAGGTGCTTGCACCT-------	53	80.50	83.50
Lb. plantarum	SC-A1 6M	GCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGY-ATTGATTGGTGCT-------	52	78.10	79.20
Lb. paracasei	NCC-B1 raw	GCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTGATGATCGGTGCT-------	53	80.80	80.30
Lb.rhamnosus	SC-A3 6M	GCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTGATTATTGAARGGTGCT-------	53	78.00	78.50
W. paramesenteroides	NCA2-C1 6M	GCGGCGTGCCTAATACATGCAAGTCGAACGCTTTGTCTTTAATTGATCTGACGAGCTTGC	60	77.95	79.00
S. chromgenes	WB-B1 raw	GCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGACGAGGAGCTT-------------	46	80.90	82.9
S. bovis	NCA2-B5 raw	GCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGACTTTAGCTT-------------	46	77.30	80.3
L. lactis lactis	NCC-B3 raw	GCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGTACTT-------------	46	80.50	83.6
L. lactis cremoris	NCB-A3 raw	GCGGCGTGCCTAATACATGCAAGTTGAGCGATGAAGATTGGTGCTT-------------	46	79.10	80.4
		***** ******************** ** **			

Species	Sample ID	P1V1/P2V1 Amplicon	
E. faecalis	WB-A5 raw	---CTTGCACTCAATYGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTA	100
E.saccharolyticus	WB-B5 raw	---CTTCGGTTCATTGGAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTA	98
Lb. brevis	NCC-A1 raw	TGCACTGATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTAACACGTGGGAA	106
Leuc. Lactis	NCB-A4 raw	------------------TTCAAGCGAGTGGCGAACGGGTGAGTAACACGTGGATA	91
Lb. plantarum	SC-A1 6M	TGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAA	106
Lb. paracasei	NCC-B1 raw	TGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTA	107
Lb.rhamnosus	SC-A3 6M	TGCATCTTGATTTAATTTTGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTA	107
W. paramesenteroides	NCA2-C1 6M	TCTGATKTRATTTTATCTGACAAAGATGGCGAACGGGTGAGTAACACGTGGGTA	115
S. chromgenes	WB-B1 raw	-----------GCTCCTTTGAYGTTAGCGGCGGACGGGTGAGTAACACGTGGGTA	90
S. bovis	NCA2-B5 raw	-----------GCTAAAGTTGGAAGAGTTGCGAACGGGTGAGTAACGCGTAGGTA	90
L. lactis lactis	NCC-B3 raw	-----------GTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGA	90
L. lactis cremoris	NCB-A3 raw	-----------GCACCAATTTGAAGAGCAGCGAACGGGTGAGTAACGCGTGGGGA	90
		** *** ** *********** *** * *	

[a]Consensus data from sequenced isolates using MicroSEQ® 500

[b] Estimated T$_m$ of P1V1/P2V1 on obtained from BioPhp set for Basic T$_m$:
http://www.biophp.org/minitools/melting_temperature/demo.php

[c]Clustal Omega used for sequence alignment: www.ebi.ac.uk/Tools/msa/clustalo/

**Appendix 3**: API 50 CHL results for NSLAB reference strains in JWC culture library

	GLY 0	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG	API 50 Results
API Reference																																																		
Lb. paracasei 1	0	20	0	1	0	100	0	0	13	0	100	100	100	100	53	1	13	6	100	86	0	46	100	98	100	99	100	93	99	99	0	93	99	26	93	0	0	6	0	80	80	26	100	0	1	0	40	93	0 0	
Lb. paracasei 2	0	16	0	16	0	100	0	0	33	0	100	100	100	100	50	1	50	33	100	100	0	83	100	75	100	83	99	66	99	0	0	99	99	66	99	0	0	0	0	66	100	16	100	0	0	0	0	83	0 0	
Lb. paracasei 3	0	0	0	0	98	0	0	0	1	100	100	100	100	20	1	0	0	80	20	0	0	100	99	100	80	100	100	80	80	0	60	99	20	20	0	0	0	0	100	20	0	60	0	0	0	0	20	1	0	
Lb. rhamnosus	0	42	0	9	8	100	0	0	0	0	100	100	100	100	92	100	14	42	100	100	7	85	100	99	100	85	100	100	99	100	9	71	99	0	99	7	0	7	0	85	92	42	99	0	7	0	7	85	0 0	
Lb. plantarum 1	0	1	0	0	74	92	2	0	0	92	100	100	100	2	33	0	0	99	78	55	33	100	94	99	99	99	99	99	100	99	94	88	96	0	92	74	7	7	0	98	62	0	7	0	0	36	0	62	0 0	
Lb. plantarum 2	0	0	0	1	83	1	0	0	75	100	100	100	1	1	0	0	80	17	1	1	100	83	67	67	67	67	100	75	1	17	1	0	1	1	18	1	0	83	1	0	1	0	0	1	0	1	0	0		
L. lactis ssp. lactis 1	0	1	0	5	30	95	40	0	0	0	90	100	100	100	0	0	0	0	50	5	1	22	100	75	85	90	85	95	95	90	13	50	99	1	0	9	50	0	0	81	0	0	9	0	0	0	0	30	0 0	
L. lactis ssp. lactis 2	0	1	0	4	4	85	1	0	0	0	100	100	100	100	0	1	0	0	20	0	0	20	100	30	91	91	91	91	91	99	4	20	100	0	0	4	60	4	0	91	0	0	4	0	0	0	0	1	0 0	

Reference Strain

Strain	API 50 Results
Lb. casei JWC-2112	*Lb. paracasei ssp. paracasei* (97%)
Lb. paracasei JWC-2118	*Lb. paracasei ssp. paracasei* (99.7%)
Lb. rhamnosus JWC-2123	*Lb. rhamnosus* (99.8%)
Lb. plantarum JWC-2142	*Lb. plantarum 1* (99.9%)
L. lactis ssp. lactis JWC 2155	*Lactococcus lactis ssp. lactis* (93.6%)
L. lacris ssp. lactis JWC 2156	*Lb. paracasei ssp. paracasei 3, Lb. plantarum* (99.2%)
L. lactis ssp. lactis JWC 2108	*Lactobacillus paracasei spp paracasei* (98.9%)
Lb. curvatus JWC-2140	*Lb. curvatus* (87.1%)
T2PA1-5	*Lb. plantarum 2* (94.2%) *Lactococcus lactis 2* (3.3%)
P. pentosaceus JWC-2107	*Pediococcus pentosaceus* (99.9%)
Lb. brevis JWC-2132	*Leuc. Mesenteriodes* (94.5%) *Lb.brevis 2* (4%)
Lb. buchneri JWC-2106	*Weissella viridescens* (97.8%)
W. viridescens JWC-2105	*Lb. buchneri* (99.7%)
P. cerevisiae JWC-2154	*Pediecoccus pentosaceus* (70%) *Lactococcus lactis ssp lactis* (22.7%)
P. pentosaceus JWC-2103	*Pediococus pentosaceus* (99.6%)
P. pentosaceus JWC-2102	*Pediococus pentosaceus* (99.8%)
P. pentosaceus JWC-2104	*Pediococus pentosaceus* (99.8%)
Leuc. mesenteroides JWC-2109	*Leuconostoc mesenteroides* (97.6%)
Lb. acidophilus JWC-2133	*Lactobacillus acidophilus* (74%)

Appendix 4: Constellation clustering (right) of HRM analysis of P1V1/P2V1 PCR for *Lb. plantarum, Lb. rhamnosus, Lb. paracasei,* and *Lb. casei* of reference strains and unknown isolates identified by 16s rDNA sequencing.

Reference strains made distinct clusters based on the melting temperature of the P1V1/P2V1 amplicon Additionally, melting point curves formed to distinct groups with *Lb. plantarum/Lb. rhamnosus* clustered around 78.0°C and *Lb. paracasei/Lb. casei* clustered around 81.0 °C. Clusters became less distinct with the addition of unknown isolates from raw milk and Cheddar cheese identified using 16s rDNA sequencing. Clusters that were originally exclusive to a single species were found to have two or more species present. With the addition of raw milk and Cheddar cheese isolates reference strains remained in their respected clusters, but included one or more additional species that were not present originally. Additionally, *Lb. paracasei* from raw milk and Cheddar cheese samples were included in all clusters. Though *Lb. plantarum* reference strains formed a tight cluster this was not reflected in the distribution of the raw milk and Cheddar cheese isolates.
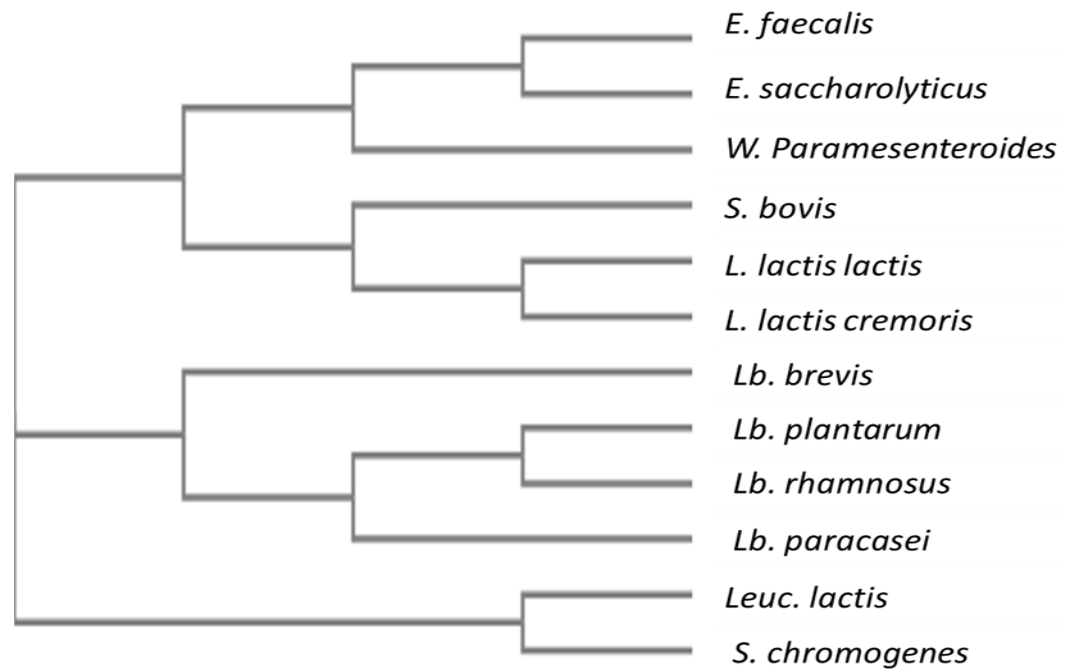
Appendix 5: Phylogenetic tree of P1V1/P2V1 amplicon of raw milk and Cheddar cheese samples as identified by MicroSEQ® 500 16s rDNA sequencing.

[a]Phylogenetic tree generated using Clustal Omega: www.ebi.ac.uk/Tools/msa/clustalo/
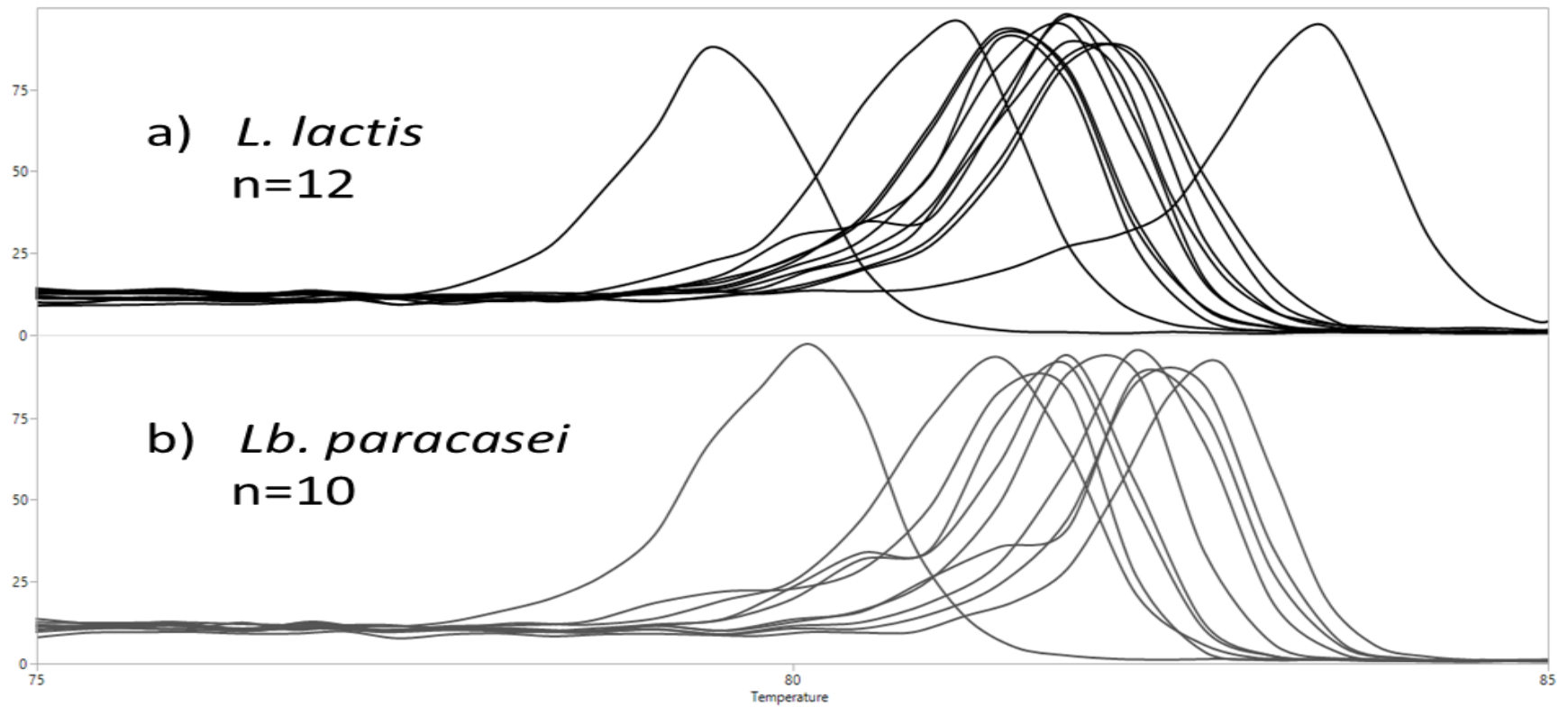
Appendix 6: HRM melt curves of the P1V1/P2V1 amplicon of isolates identified as *L. lactis* subsp. *lactis* (●) and *Lb. paracasei* (●) through 16s rDNA sequencing by MicroSEQ® 500

Appendix 7: Defense Presentation

Photo by Lynn Ketchum

NSLAB| Overview

NSLAB are a group of bacterial species that contaminate or are naturally present in milk

NSLAB can impact flavor and texture of fermented dairy products

Sources for NSLAB
- Cows
- Pasture
- Feed Lot/Farm
- Milk Truck
- Creamery/Processor

Image Source:
Jersey Cows in Field Photo by Lynn Ketchum
http://www.pakdairyinfo.com/Pic/Parallel.jpg
http://democratherald.com/news/local/students-test-market-with-osu-s-own-beaver-classic-cheese/article_6895e1cc-24a5-11e2-ae96-0019bb2963f4.html

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Evidence of *terroir* in milk sourcing and its influence on Cheddar cheese

Gregory Turbes, Tyler D. Linscott, Elizabeth Tomasino, Joy Waite-Cusic, Juyun Lim,[1] and Lisbeth Meunier-Goddik[1]
Department of Food Science and Technology, Oregon State University, Corvallis 97331

- Produced Cheddar cheese using raw milk and pasteurized milk sourced from different farms in Oregon

- Panelist grouped cheeses based on flavor
  - Groups were representative of different farms
- Flavor analysis support sensory results

- NSLAB identified using API 50 CHL
  - *Lb. paracasei* and *Lb. plantarum*

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The Hypothesis:

Regional and farm level milk sourcing contribute to the NSLAB present in raw milk and the subsequent cheese made with that raw milk.
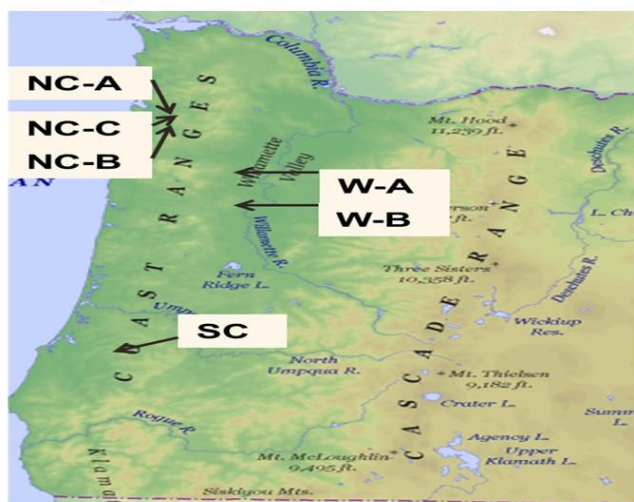
Assumptions:

1. Strains found in raw milk would be found in aged cheese

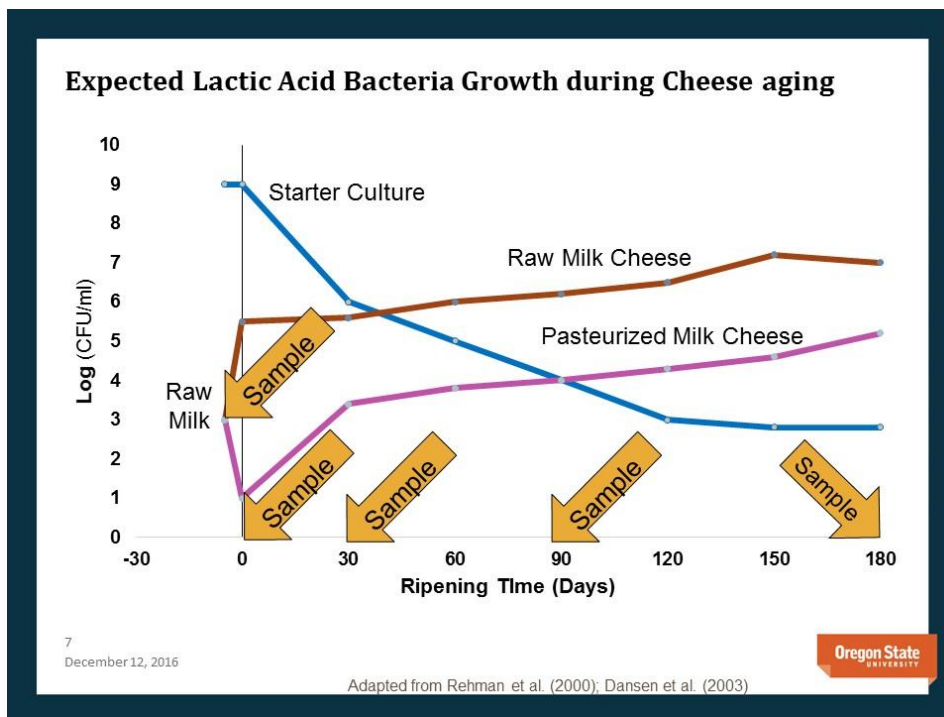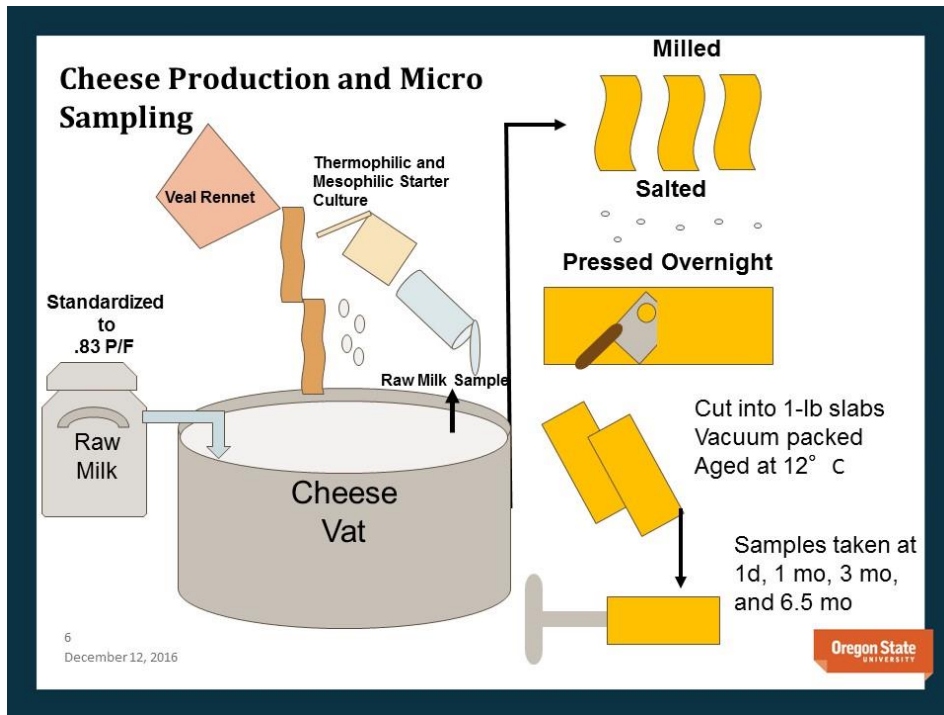*2. NSLAB would consist predominately of Lb. paracasei and Lb. plantarum*

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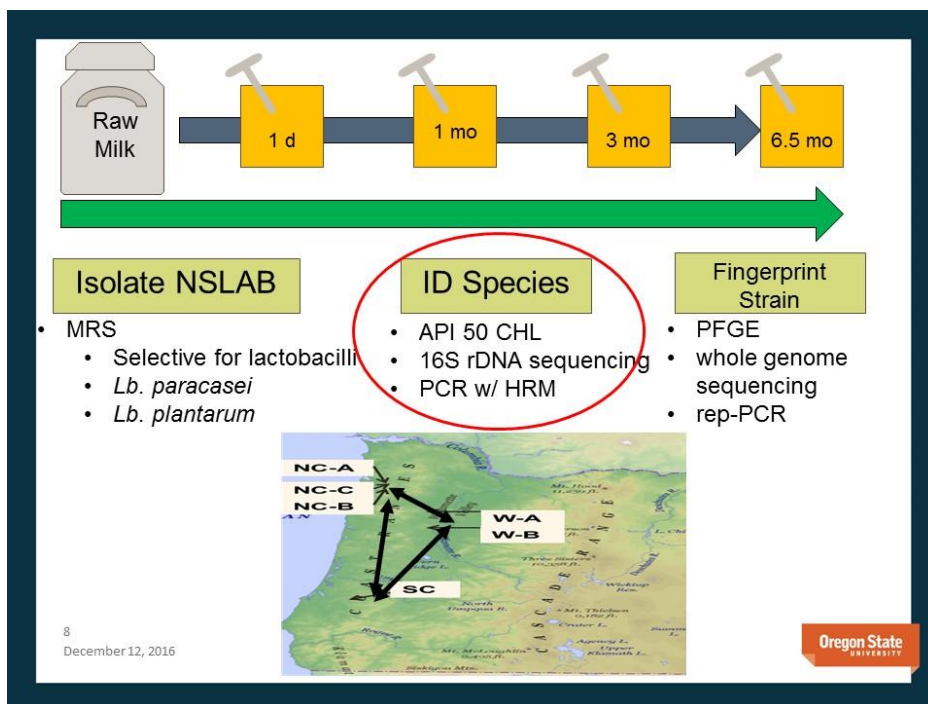**Milk Sourcing | Milk Collection and Production**

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API 50 CHL Overview

- Turbes et al. (2014)
  - *Lb. plantarum*
  - *Lb. paracasei*
    - Poor at properly identifying between *Lb. paracasei, Lb. casei,* and *Lb. rhamnosus*
- High Cost
- Reliant on API library for identification
- Low throughput
  - 10 min per strip
  - Additional 48 hr incubation after inoculation

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16S rDNA sequencing for NSLAB Identification

- 16S rDNA highly conserved in lactobacillus

- Current genomic data limited for lactobacilli

- High Cost
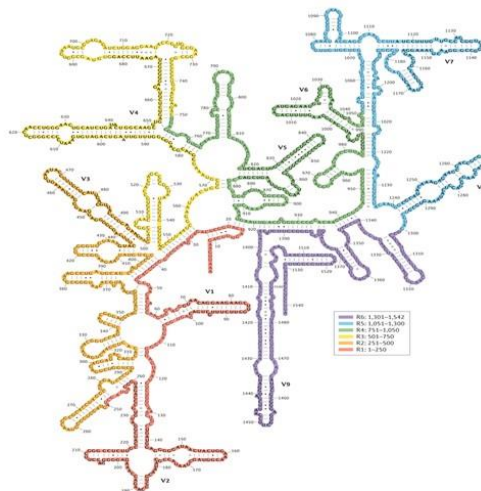
10
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Image Source: Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzéby J, Amann R, Rosselló-Móra R. 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nature Rev Microbiol, 12: 635-645

P1V1/P2V1 PCR w/ HRM Analysis

Iacumin et al (2015) found using P1V1/P2V1-PCR with HRM analysis was effective for identification of *Lb. paracasei, Lb. casei,* and *Lb. rhamnosus*

- Amplifies V1 region of 16S rDNA

- Needs library of known control organism for identification

- Identification of species through high resolution melt analysis
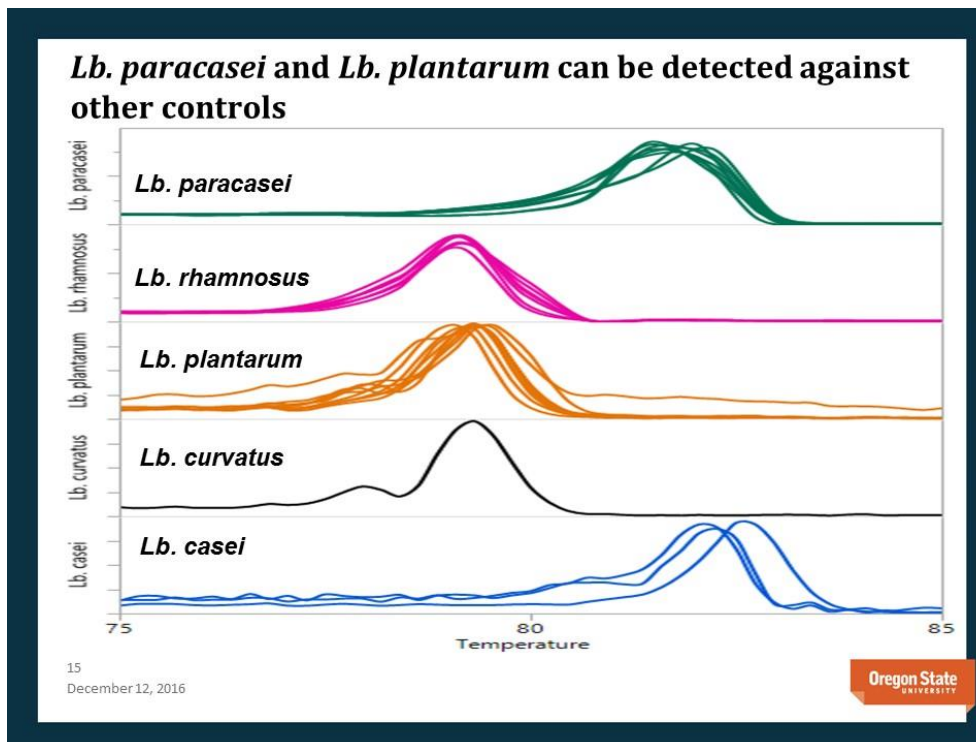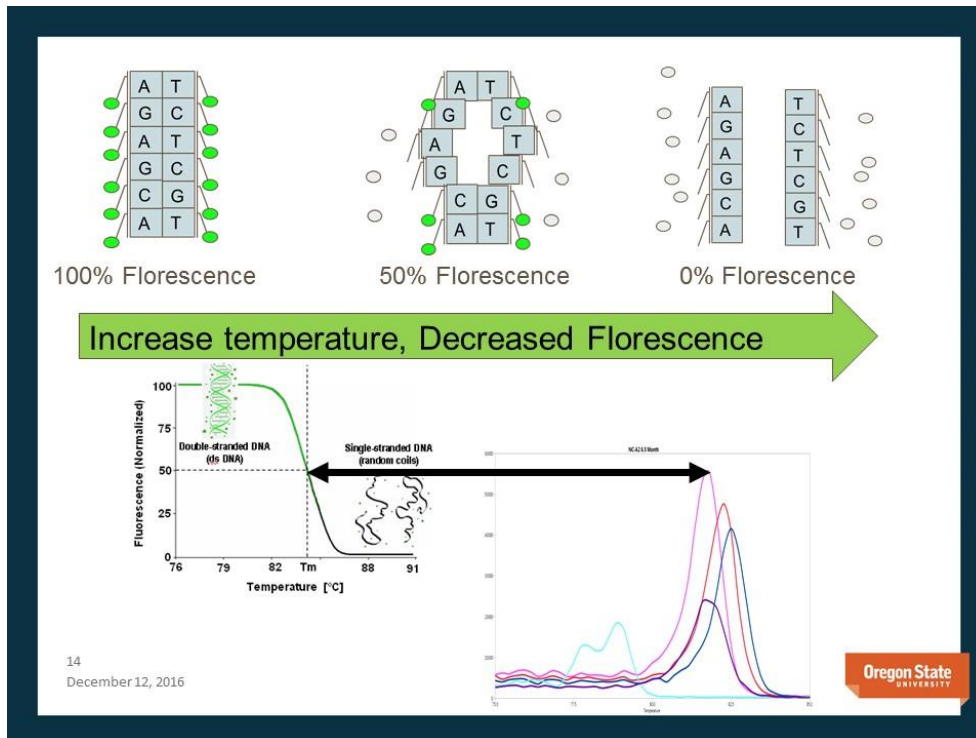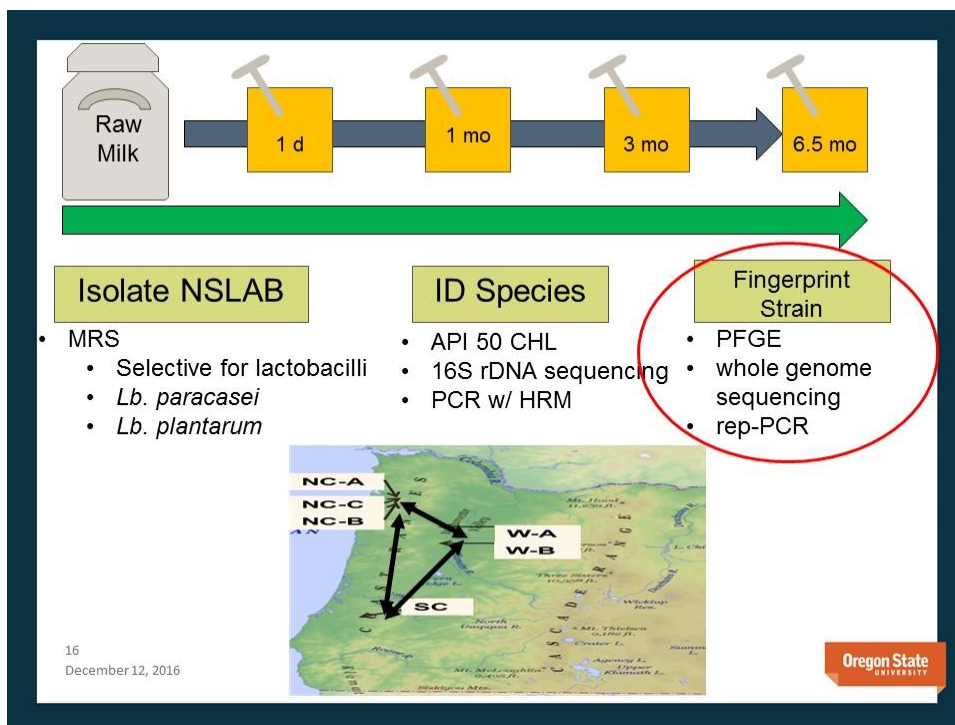

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Lactobacillus 16S rRNA
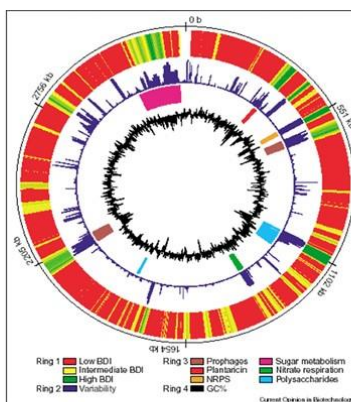Number of sequences: 608

Positions with a nucleotide in more than 95% of the sequences are shown in one of four categories:

ACGU – 98.0+% conserved
agcu – 90.0-98.0% conserved
· – 80.0-90.0% conserved
– – less than 80.0% conserved

Otherwise, the regions are represented by arcs.
Arc labels indicate the upper and lower number of nucleotides known to exist within the associated variable region.

Blue tags indicate insertions relative to the reference sequence.
Insertions that appear are:
(1) length 1-4 in more than 10% of sequences
(2) length 5 or greater in at least one sequence
with format:
(Max. Length of Insertion:Percentage of seqs with any length insertion)

McFredericka QS, Cannoneb JJ, Gutellb RR, Kellnera K, Plowesc RM, Muellera UG. 2013. Specificity between Lactobacilli and Hymenopteran Hosts Is the Exception Rather than the Rule. Appl Environ Microbiol, 79(6): 1803-1812

100% Florescence   50% Florescence   0% Florescence

Increase temperature, Decreased Florescence

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Lb. paracasei and *Lb. plantarum* can be detected against other controls

Lb. paracasei

Lb. rhamnosus

Lb. plantarum

Lb. curvatus

Lb. casei

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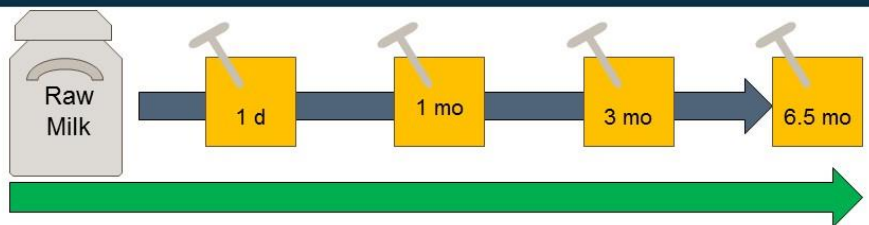
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Genome map of *Lb. plantarum*

Repetitive Extragenic Palindromic sequence (rep) PCR w/ HRM analysis

- Single primer – (GTG)5
- Produces multiple PCR products of varying length
- Melt curve with multiple melt peaks

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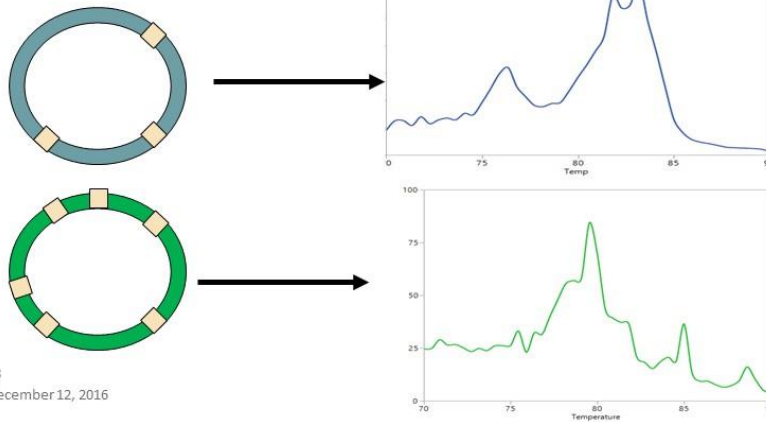
Isolate NSLAB
- MRS
  - Selective for lactobacilli
  - *Lb. paracasei*
  - *Lb. plantarum*

ID Species
- API 50 CHL
- 16S rDNA sequencing
- PCR w/ HRM

Fingerprint Strain
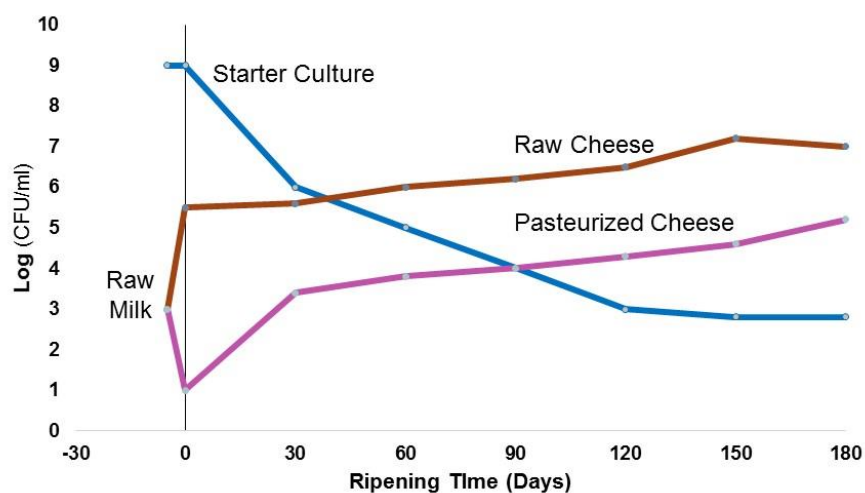- PFGE
- whole genome sequencing
- rep-PCR

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Expected Lactic Acid Bacteria Growth during Cheese aging

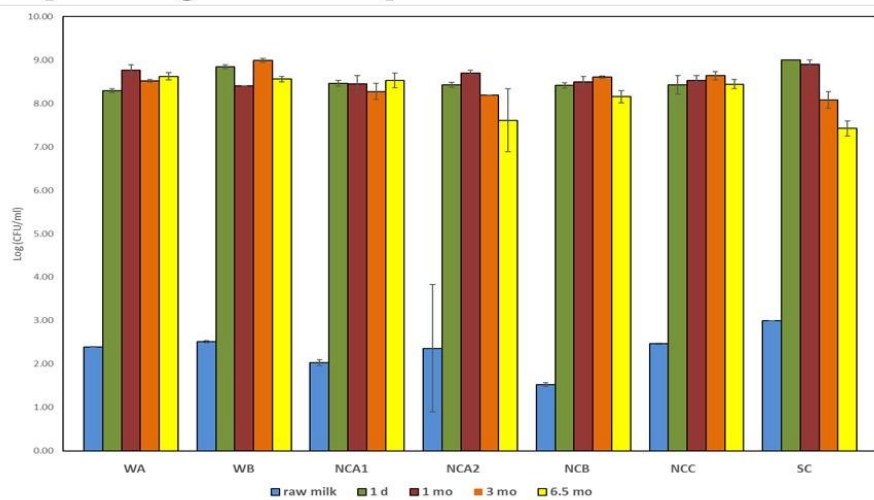NSLAB populations higher in Cheddar cheese than expected right out of the press

Large Amount of NSLAB Variability in Raw Milk

Day 2
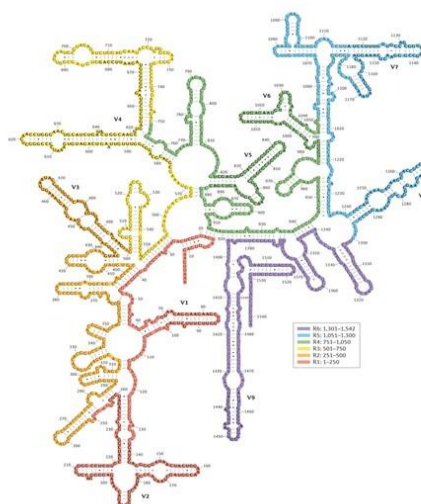
Day 1

North Coast
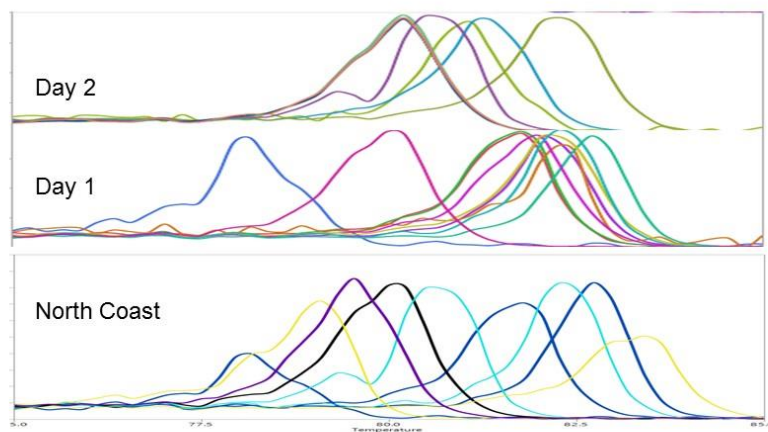
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16S rDNA Sequencing with Assistance of the FDA

Large amount of raw milk isolates unidentified using P1V1/P2V1 PCR

Partnered with FDA for 16s rDNA sequencing using the MicroSeq 500 system

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Identification of Raw Milk Isolates using 16S rDNA Sequencing

Sample ID[a]	MicroSEQ® 500 ID
NCA1-A1	Weissella paramesenteroides
NCA1-B1	Lactobacillus paracasei paracasei
NCA2-B5	Streptococcus bovis
NCB-A2	Weissella paramesenteroides
NCB-A3	Lactococcus lactis cremoris
NCB-A4	Leuconostoc lactis
NCB-A5	Lactobacillus plantarum
NCB-B1	Lactobacillus plantarum
NCB-B3	Weissella paramesenteroides
NCC-A1	Lactobacillus brevis
NCC-A3	Lactococcus lactis lactis
NCC-B2	Lactococcus lactis lactis
NCC-B3	Lactococcus lactis lactis
NCC-B1	Lactobacillus paracasei tolerans
NCC-B5	Lactococcus lactis lactis
WA-B1	Leuconostoc lactis
WB-A5	Enterococcus faecalis
WB-B1	Staphylococcus chromogenes
WB-B5	Enterococcus saccharolyticus

P1V1/P2V1 ID
Lb. paracasei

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Comparison of *L. lactis* subsp. *lactis* Melt Temperature to NSLAB Controls

Species	Melt Temperature by Species (°C)
Lactobacillus casei	81.79±0.20
Lactobacillus paracasei	82.01±0.03
Lactobacillus plantarum	79.34±0.13
Lactococcus lactis subsp. lactis	81.69±0.12

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Percent of Isolates Identified for All Samples per Time Point

Farm	Percent				
	Raw	Day 1	1 Month	3 Month	6.5 Month
Lb. curvatus					
Lb. paracasei/L. lactis					
Lb. platarum					
Lb. rhamnosus					
Lab species A*					
Unknown					
Total	100.00%	100.00%	100.00%	100.00%	100.00%

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API 50 CHL misidentification of *L. lactis*

Turbes et al. (2014)
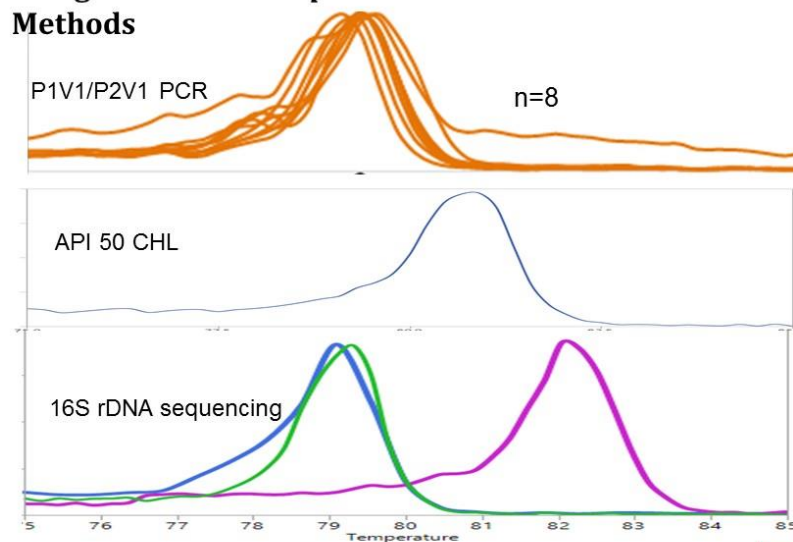- NSLAB isolates identified as *Lb. paracasei* or *Lb. plantarum* using API 50 CHL

Carbohydrates[a]	GLY	LARA	SBE	RHA	MAN	SOR	MDM	MDG	AMY	MAL	MEL	SAC	INU	MLZ	RAF	GEN	TUR	TAG	GNT	API 50 Results
Reference Strain																				
Lb. plantarum JWC-2142	-	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	+			*Lb. plantarum* 1 (99.9%)
L. lactis ssp. lactis JWC 2155	-	-	-	-	-	-	-	-	+	-	-	-	-	?	-	-	-			*L. lactis ssp. lactis* (93.6%)
L. lacris ssp. lactis JWC 2156	-	-	-	+	+	-	-	+	+	-	-	+	-	?	-	-	-			*Lb. paracasei ssp. paracasei* 3 (99.2%)
L. lactis ssp. lactis JWC 2108	+	-	-	+	-	-	-	+	+	-	+	-	+	-	+	-	+	+		*Lb. paracasei spp paracasei* (98.9%)

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No agreement on *Lb. plantarum* identification between Methods

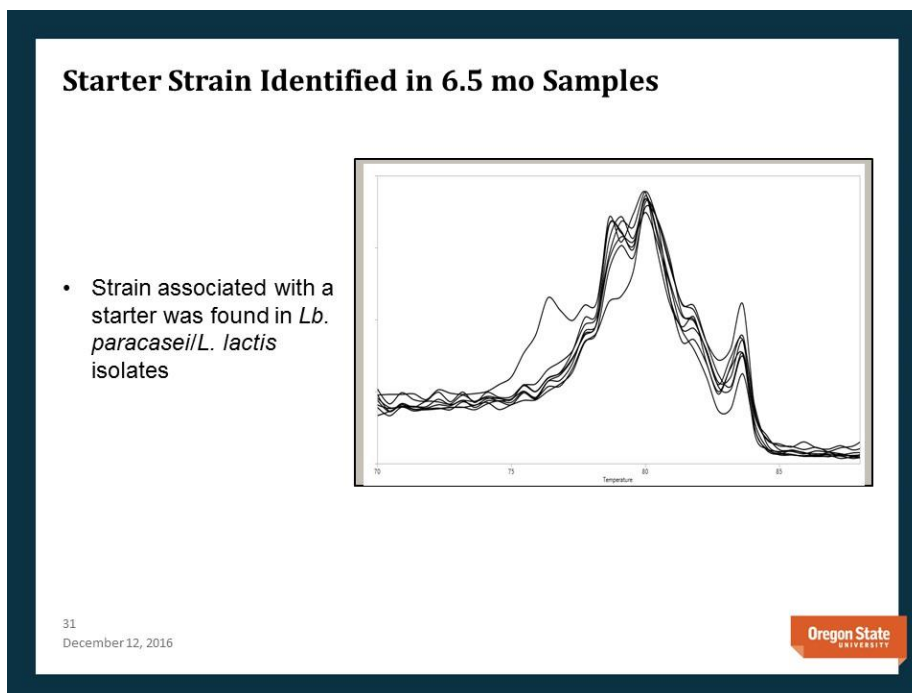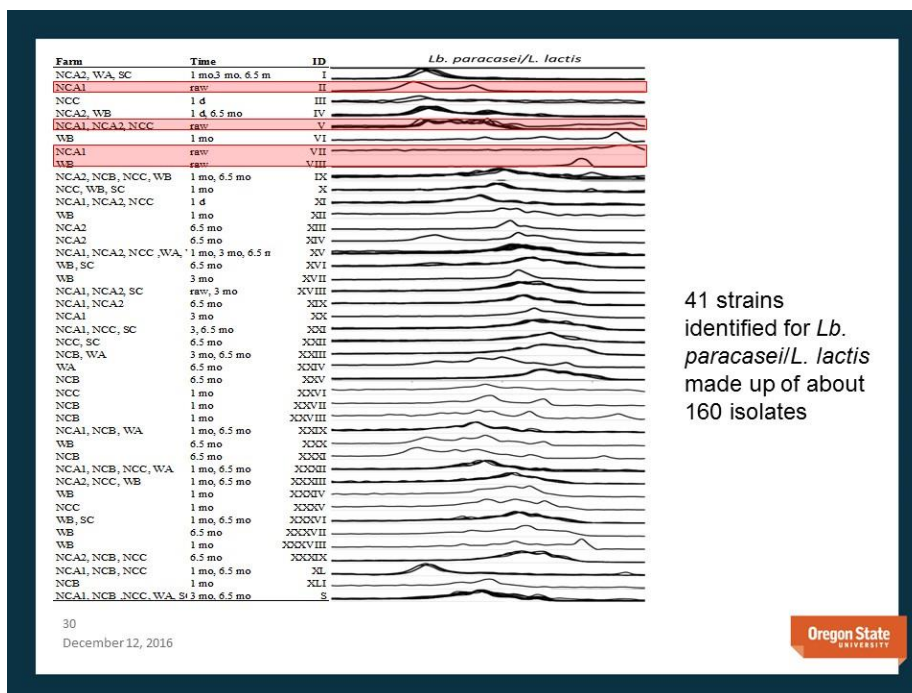P1V1/P2V1 PCR   n=8

API 50 CHL

16S rDNA sequencing

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Percent of Isolates Identified for All Samples per Time Point

Farm	Percent				
	Raw	Day 1	1 Month	3 Month	6.5 Month
Lb. curvatus	13.11%	0.00%	0.00%	0.00%	0.00%
Lb. paracasei/L. lactis	21.31%	41.86%	30.65%	16.96%	71.79%
Lb. platarum	0.00%	0.00%	2.42%	0.00%	0.00%
Lb. rhamnosus	4.92%	2.33%	1.61%	0.89%	5.98%
Lab species A*	13.11%	27.91%	50.00%	82.14%	20.51%
Unknown	47.54%	27.91%	15.32%	0.00%	1.71%
Total	100.00%	100.00%	100.00%	100.00%	100.00%

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41 strains identified for *Lb. paracasei/L. lactis* made up of about 160 isolates

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Starter Strain Identified in 6.5 mo Samples

- Strain associated with a starter was found in *Lb. paracasei/L. lactis* isolates

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The Hypothesis:

Regional and farm level milk sourcing contribute to NSLAB present in raw milk and the subsequent cheese made with that raw milk.

Assumptions:

> *1. Strains found in raw milk would be found in aged cheese*
>
> Strains identified in raw milk were not found in cheese samples
>
> *2. NSLAB would consist predominately of Lb. paracasei and Lb. plantarum*
>
> 16S rDNA sequenced isolates showed large species variability in raw milk
>
> *Lb. plantarum* was not a major NSLAB identified using either P1V1/P2V1 PCR or 16S rDNA sequencing

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Acknowledgements:
Julia Wilson, Elizabeth Tomasino, Robin Frojen, Lisbeth Goddik, Joy Waite Cusic
and my lovely wife Kimberly who has always been there for me

Photo by Lynn Ketchum

Farm	Herd Composition per milking	Feed in lbs[a]	Time in Pasture[b] per day	Major Pasture[c] Grass	Minor Pasture[d] Grass	Silage
NC-A	100% Certified Jersey	5-Alfalfa 14-Grain	15 hours	Rye Grass	Clover, Native	None
NC-B	100% Certified Jersey	9-Alfalfa 19-Grain	14 hours	Orchard Grass	Rye Grass, Clover	Cannery*
NC-C	11% Holstein 11% Aryshire 78% Jersey	20-Grain	16.5 hours	Rye Grass, Orchard Grass	Clover	Italian Rye
W-A	10% Friesian/Jersey Mix 90% Jersey	4-Grain	21.6 hours	Perennial Rye Grass, Orchard Grass	Plantain Herb, White Clover	None
W-B Grp 1	32% Holsteins 68% Jersey	3.5-Alfalfa 8.5-Grain	12 hours	Orchard Grass, Fescue, Rye Grass		Corn, Grass
Grp 2	43% Holsteins 57% Jersey	7-Alfalfa 15-Grain				Corn
SC	82% Holstein 6% Brown Swiss 12% Jersey	6.25-Alfalfa 12.5-Grain	19 hours	Rye Grass, Fescue, White Clover	Velvet Grass Canary Grass	Grass