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J. Dairy Sci. 86:2773–2782
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Influence of Adjunct Use and Cheese Microenvironment on Nonstarter Bacteria in Reduced-Fat Cheddar-Type Cheese[1]

J. R. Broadbent,* K. Houck,† M. E. Johnson,† and C. J. Oberg‡
*Western Dairy Center and Department of Nutrition and Food Sciences,
Utah State University, Logan, 84322-8700
†Wisconsin Center for Dairy Research,
University of Wisconsin, Madison, 53706
‡Department of Microbiology,
Weber State University, Ogden, UT 84408-2506

ABSTRACT

This study investigated population dynamics of starter, adjunct, and nonstarter lactic acid bacteria (NSLAB) in reduced-fat Cheddar and Colby cheese made with or without a *Lactobacillus casei* adjunct. Duplicate vats of cheese were manufactured and ripened at 7°C. Bacterial populations were monitored periodically by plate counts and by DNA fingerprinting of cheese isolates with the random amplified polymorphic DNA technique. Isolates that displayed a unique DNA fingerprint were identified to the species level by partial nucleotide sequence analysis of the 16S rRNA gene. Nonstarter biota in both cheese types changed over time, but populations in the Colby cheese showed a greater degree of species heterogeneity. The addition of the *L. casei* adjunct to cheese milk at 10^4 cfu/ml did not completely suppress "wild" NSLAB populations, but it did appear to reduce nonstarter species and strain diversity in Colby and young Cheddar cheese. Nonetheless, nonstarter populations in all 6-mo-old cheeses were dominated by wild *L. casei*. Interestingly, the dominant strains of *L. casei* in each 6-mo-old cheese appeared to be affected more by adjunct treatment and not cheese variety.
(**Key words:** *Lactobacillus*, nonstarter lactic acid bacteria, cheese flavor)

Abbreviation key: NSLAB = nonstarter lactic acid bacteria, **RAPD** = randomly amplified polymorphic DNA.

INTRODUCTION

Flavor development in Cheddar and other bacterial-ripened cheeses is a dynamic and complex biochemical process that requires lactic acid bacteria and enzymes (Reiter et al., 1967; Fox et al., 1993). The lactic acid bacteria that contribute to this process include deliberately added starter and adjunct cultures, as well as nonstarter lactic acid bacteria (**NSLAB**) that enter cheese through milk or via contamination of the dairy plant environment (Peterson and Marshall, 1990; Beresford et al., 2001; Somers et al., 2001). In Cheddar cheese, initial numbers of *Lactococcus lactis* starter bacteria frequently exceed 10^9 cfu/g, but the harsh cheese ripening environment (no residual lactose, pH 5.0 to 5.3, 4 to 6% salt in moisture, 5 to 13°C) causes starter viability to decline as maturation proceeds. A fraction of the dying starter cells undergo autolysis, which releases intracellular enzymes and cellular components (e.g., sugars and nucleic acids) into the cheese matrix (Fryer, 1969). At the same time, NSLAB populations (whose initial numbers are typically below 10^3 cfu/g) begin to grow and eventually plateau at cell densities of 10^7 to 10^9 cfu/g after 3 to 9 mo of aging (Peterson and Marshall, 1990). The NSLAB population in Cheddar-type cheese is typically dominated by mesophilic, facultatively heterofermentative lactobacilli (Sherwood, 1939; Fryer, 1969; Peterson and Marshall, 1990; Beresford et al., 2001). Use of *Lactobacillus* spp. NSLAB isolates as adjunct cultures for Cheddar cheese manufacture has indicated these bacteria may influence flavor in at least three ways: they may intensify (i.e., accelerate) typical flavor development; impart atypical (but desirable) flavor notes; or promote off-flavor development (Sherwood, 1939; Fryer, 1969; Broome et al., 1990b; McSweeney et al., 1994; Lynch et al., 1999; Crow et al., 2001; Swearingen et al., 2001). In addition, NSLAB can produce cheese quality defects such as open body (via gas production) and calcium lactate crystals (Fryer, 1969; Johnson et al., 1990). Because starter and NSLAB can each influence overall cheese quality, industry efforts to produce more uniform, flavorful products will require technologies to control both populations of bacteria.

While the types and numbers of starter (or adjunct) bacteria in cheese can be readily controlled, the com-

Received October 18, 2002.
Accepted February 26, 2003.
Corresponding author: J. R. Broadbent; e-mail: broadbnt@cc.usu.edu.
[1]This research was supported by the Utah Agricultural Experiment Station, Utah State University, Logan, 84322-4810. Approved as Journal Paper Number 7489.

plexity of NSLAB populations is still largely a matter of chance. The NSLAB biota of cheese is influenced by several factors including process and equipment sanitation in the manufacturing plant and milk heat treatment (Fox et al., 1998; Berthier et al., 2001; Somers et al., 2001). Several studies have demonstrated that substantial heterogeneity exists in the ratio of species and strains that dominate in a given cheese, even between products made in the same processing facility (Sherwood, 1939; Naylor and Sharpe, 1958; Jordan and Cogan, 1993; Williams and Banks, 1997; Fitzsimons et al., 1999 and 2001; Crow et al., 2001; Swearingen et al., 2001). Moreover, a succession of different *Lactobacillus* spp. and strains may dominate the NSLAB biota of Cheddar cheese at various stages of ripening (Crow et al., 2001; Fitzsimons et al., 2001).

Because the impact of NSLAB on cheese flavor is ultimately determined by the metabolic activities of bacteria that predominate during ripening, individual strains may, in theory, have a profound effect on cheese flavor and body characteristics (Williams et al., 2000). The unpredictable and dynamic nature of NSLAB communities is, therefore, believed to be an important source of cheese flavor defects and inconsistencies (Sherwood, 1939; Crow et al., 2001). If this hypothesis is correct, stringent control of NSLAB populations during ripening should facilitate industry efforts to produce more uniform, high-quality cheese.

One strategy to accomplish this goal is the use of *Lactobacillus* adjunct cultures that grow in ripening cheese (but do not affect the manufacturing process), possess desirable flavor-producing attributes (or at least have no negative effect on flavor), and suppress the emergence of "wild" NSLAB (Broome et al., 1990a; Crow et al., 2001). Although efforts to enhance flavor development with NSLAB adjuncts have produced mixed results (Peterson and Marshall, 1990), the overall efficacy of this approach is supported by academic studies that showed some adjuncts dominated wild NSLAB counterparts during ripening of Cheddar cheese (Broome et al., 1990b; Crow et al., 2001; Ryan et al., 2001; Swearingen et al., 2001). Furthermore, adjunct addition may help to control culture-related cheese quality defects (Layele et al., 1990; Frohlich-Wyder et al., 2002). Although adjuncts offer good promise as a tool to control cheese NSLAB and improve cheese quality, the dynamic environment of ripening cheese and the variability imparted by differences in cheese composition, added cultures or enzymes, and manufacturing or ripening regimens, suggests NSLAB control may be a difficult goal. Thus, industry efforts to utilize adjunct technology for NSLAB control would profit from a more fundamental understanding of the relationship between cheese environment, adjunct use, and NSLAB population dynamics.

Among the specific protocols used to make cheeses, curd washing or whey dilution steps offer the potential to produce dramatic differences between cheese varieties. Colby cheese is a washed, stirred-curd variety of Cheddar, and both cheeses are commonly manufactured with identical strains of *Lc. lactis* starter. In Colby manufacture, cold water is added to the curd after most of the whey has been drained. The wash treatment serves two purposes; it removes lactose and lactic acid from the curd, and cools the curd. Removal of lactic acid and lactose gives the finished cheese a lower acid content and a slightly higher pH. Cooling produces a slight increase in cheese moisture content and an initially firmer curd, which in turn produces a more open texture (Johnson, 2001). Whereas Cheddar can be aged for years, Colby is typically consumed within 3 mo since more aged Colby develops a pasty body and is often accompanied by undesirable flavor development.

Although differences in the flavor or flavor intensity of washed versus nonwashed (but otherwise identically produced) cheeses are commonly noted, the basis for this observation is unknown. One widely accepted theory is that the environment of washed curd cheese is not conducive to the development of particular flavor compounds, but many cheese technologists also believe that the changes in acid content and pH somehow alter cheese microbiology. In this study, we investigated NSLAB population dynamics in reduced-fat Cheand Colby cheese, and examined the effect of a *Lb. casei* adjunct on NSLAB populations.

MATERIALS AND METHODS

Bacterial Strains

Lactococcus lactis ssp. *cremoris* SCO213 was acquired from Chr. Hansen, Inc. (Milwaukee, WI) and propagated at 30°C in M17 broth (Difco, Beckton Dickinson, Sparks, MD). The adjunct, *L. casei* LILA, was obtained from the Wisconsin Center for Dairy Research culture collection and grown at 30°C in MRS broth (Difco). Stocks of each culture were maintained at –80°C, and working samples were prepared from frozen stocks by two transfers in broth medium.

Cheese Manufacture

Duplicate vats of 50% reduced-fat Cheddar and Colby cheese were manufactured at the University of Wisconsin-Madison from 250-kg lots of pasteurized milk (1.3% fat). The milk was inoculated with 1% *Lc. lactis* ssp. *cremoris* SCO213 starter grown at 30°C for 12 to 14 h

in steamed (88°C for 45 min) skim milk. An additional set of duplicate vats for each cheese type was inoculated with starter plus 4 ml of *Lb. casei* LILA (10^8 cfu/ml).

Fifteen minutes after starter addition, 49 ml of calcium chloride (Rhodia, Inc., Madison, WI) and 19 ml of double-strength fermentation-produced chymosin (Chr. Hansen, Inc., Milwaukee, WI) were added. The coagulum was cut at a milk pH of 6.5 with 0.95-cm knives and allowed to heal for 5 min. After 10 min of gentle agitation, the temperature of the curd and whey slurry was raised from 32°C to 37.8°C over 25 min, then the whey was slowly drained. Curd for the manufacture of Cheddar was formed into slabs, cheddared, then milled when the curd reached pH 5.95. Fifteen minutes after milling, curd for Cheddar cheese manufacture was salted in three additions, 5 min apart, with 0.275% (wt/wt) flake salt (calculated from the original milk weight). For the manufacture of Colby, the curd was washed with 32°C water for 15 min before salt addition (pH 5.86). After salt addition, the curd was packed into 9-kg rectangular stainless steel hoops, pressed for 4 h at ambient temperature, vacuum-packaged, and stored at 7°C for ripening. Cheese pH, fat, moisture, salt, and lactic acid contents were determined as described previously (Weimer et al., 1997).

Microbiological Sampling

In an effort to identify possible sources of cheese NSLAB, microbiological surface samples were collected as described by Somers et al. (2001) immediately before cheese manufacture from inside and outside of cheese vats, floor drains between vats, cheese mill, lab benches, milk lines, and from the stirrer motor. Samples were also collected from pasteurized (uninoculated) cheese milk, then appropriate dilutions were plated on Elliker's and Rogosa SL agars (Difco) and incubated for 2 d at 30°C.

Microbiological sampling of experimental cheese was performed at d 1, 2 wk, and once per month thereafter. Cheese samples (approx. 20 g) were collected, homogenized in sterile 2% sodium citrate (45°C) using a Seward (London, UK) model 400 Stomacher, then total bacterial counts were enumerated by the pour plate method with Elliker agar. Nonstarter and adjunct lactobacilli numbers were determined using Rogosa SL agar. Plates were incubated anaerobically for 2 d at 32°C (Elliker agar) or 37°C (Rogosa agar), and numbers of starter cfu were determined by subtracting the *Lactobacillus* ssp. count from the total bacterial count.

DNA Fingerprinting of Bacteria

Twenty isolates were selected at random from Rogosa and Elliker plates (10 per plate) collected from each cheese for microbiological counts at d 1, and at 2, 4, and 6 mo of ripening, and propagated overnight in their respective broth media. Frozen stock samples were prepared in quadruplicate by addition of 0.2-ml overnight culture to 1.8 ml of 9% reconstituted skim milk with 17% glycerol, then stored at –80°C in 2-ml cryotubes. Template DNA for PCR was isolated from 250 μl of the overnight culture as described previously (Broadbent et al., 1998) and stored at –80°C until needed.

Randomly amplified polymorphic DNA (**RAPD**) fingerprinting (Bassam et al., 1992) was used to investigate NSLAB population dynamics during ripening. The RAPD was performed in 25-μl reaction volumes that contained 2.5 m*M* Mg^{2+}, 0.4 m*M* dNTP blend, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 4 μM oligonucleotide primer (5′-TCAGCAGCCGCGG TAATTC-3′), and 4 μl of template DNA. Negative controls were prepared with sterile water as a substitute for template DNA. Samples were overlaid with 30 μl of PCR-grade sterile mineral oil (Perkin Elmer, Foster City, CA), then 40 PCR cycles were run in a Perkin-Elmer DNA Thermal Cycler model 480 set to the following parameters: denaturation at 94°C for 1 min, annealing for 1 min at 38°C, and extension at 72°C for 2 min. When the cycles were complete, the reaction was finished by incubation at 72°C for 5 min then stored overnight at 4°C. Individual band patterns were compared by visual examination after electrophoresis in 1.5% agarose gels, and representative strains (from each cheese at each sample time) that showed unique RAPD fingerprints were selected for further characterization. When necessary, additional RAPD experiments were performed to determine the similarity between isolates collected at various sample times or from different cheeses.

Identification of Bacteria

Cheese or environmental isolates that displayed a unique RAPD fingerprint were identified to the nearest known phylogenetic relative by partial nucleotide sequence analysis of the 16S rRNA gene (Baruzzi et al., 2000). One set of universal primers, UF_1 (5′-AGAGTTT-GATCCTGGCTCAG-3′) and UR_1 (5′-GCTGGCACG TAGTTAGCC-3′), were used to amplify 520 bp from the 5′ end of the 16S rRNA gene by PCR. The reactions were performed in 50-μl volumes that contained 1.5 m*M* Mg^{2+}, 0.4 m*M* dNTP blend, 1.75 U of Expand high fidelity DNA polymerase (Roche Diagnostics, Indianapolis, IN), 1 μM of each oligonucleotide primer, and 5 μl of template DNA. Negative controls were prepared with sterile water as a substitute for template DNA. Samples were overlaid with 70 μl of PCR-grade sterile mineral oil, then 40 PCR cycles were performed with the following parameters: 2-min soak at 94°C, then 30

cycles of 15 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C. The reaction was finished by incubation at 72°C for 7 min then stored overnight at 4°C. The presence of amplicon was confirmed by electrophoresis in 1.5% agarose gels, then purified with a Bio-Rad Prep-a-Gene kit (Hercules, CA) and bidirectionally sequenced by fluorescent dideoxy chain termination on a Perkin Elmer Applied Biosystems automated DNA sequencer (model 373A). Nucleotide sequence similarity searches were performed using BLAST tools available through the National Institutes of Health Center for Biotechnology Information (www.ncbi.nlm.nih.gov) to determine the species from which each amplicon was most likely derived. In some cases, nucleotide sequence analysis of the 5′ 16S rRNA gene fragment proved inconclusive, so a second set of universal primers (UF_2 5′-GCACAAGCGGTGGAG-3′; and UR_2 5′-TTGTCACCGGCAGTCT-3′) were used to amplify a 235-bp distal region of the16S rRNA gene. The PCR, nucleotide sequence determination, and homology searches were performed as described above.

Table 1. Mean composition of Cheddar and Colby cheeses prepared in the study.[1]

Component	Cheddar	Colby
Moisture (%)	48.39 ± 0.46^a	49.35 ± 0.26^b
Salt (%)	1.50 ± 0.03^a	1.60 ± 0.05^b
Salt-in-moisture (%)	3.11 ± 0.09^a	3.23 ± 0.01^a
Fat (%)	12.33 ± 0.10^a	12.25 ± 0.16^a
Cheese pH		
Press	5.09 ± 0.04^a	5.12 ± 0.03^a
2 wk	5.07 ± 0.10^a	5.21 ± 0.02^b
1 mo	5.12 ± 0.04^a	5.27 ± 0.01^b
2 mo	5.11 ± 0.02^a	5.30 ± 0.01^b
3 mo	5.20 ± 0.03^a	5.40 ± 0.03^b
6 mo	5.25 ± 0.01^a	5.60 ± 0.05^b
Total lactate		
Day 1	1.51 ± 0.11^a	1.48 ± 0.02^a
1 mo	1.88 ± 0.02^a	1.60 ± 0.03^b
3 mo	1.97 ± 0.01^a	1.67 ± 0.02^b
4 mo	1.94 ± 0.02^a	1.66 ± 0.02^b

[1]Cheese composition was not significantly altered by addition of *Lactobacillus casei* adjunct, so values listed for each cheese type represent product made with and without the adjunct. Means with the same superscript letter in the same row were not significantly different from one another ($P > 0.05$).

Sugar Fermentation Profiles of NSLAB

The ability of selected NSLAB isolates to produce acid from different carbohydrates was determined with API 50 CHL test kits (bioMérieux, Hazelwood, Mo). The API test strips were prepared as recommended by the kit supplier and scored after incubation for 24 h at 30°C. Test results were communicated to the bioMérieux technical service department, which used the phenotypic data to predict a species identity for each isolate.

Sensory Evaluation and Statistics

Trained sensory evaluation of cheese was performed at 3 and 6 mo by six to 10 experienced judges in a randomized blind design as described previously (Weimer et al., 1997). Cheeses were judged for qualities that included Cheddar flavor intensity (1 = none, 7 = aged), lipase/rancid flavor intensity (1 = none, 7 = pronounced), off-flavor intensity (1 = none, 7 = pronounced), and overall flavor preference (1 = dislike very much, 7 = like very much). Statistical evaluation of cheese composition and sensory data was performed by ANOVA using Minitab software version 9.1 (Minitab Inc., State College, PA), and differences were declared to be significant when $P < 0.05$.

RESULTS

Cheese Composition

Mean percentages of moisture, salt, salt-in-moisture, and fat in experimental reduced-fat Cheddar and Colby cheeses are presented in Table 1. As expected, the washed Colby cheese contained significantly ($P < 0.05$) less residual lactose at press, and had a significantly higher pH and a significantly lower total lactate content (and thus a significantly lower lactic acid to moisture ratio) throughout ripening versus the nonwashed Cheddar cheese. Experimental Colby also had significantly higher average moisture and salt contents than the Cheddar cheese, but mean percentages of salt-in-moisture or fat in each cheese type were not significantly different (Table 1).

The addition of *Lb. casei* LILA did not significantly affect ($P > 0.05$) any of the compositional attributes shown in Table 1, but cheeses made with the adjunct did have a significantly ($P < 0.05$) lower D-lactate content than control cheeses at 1, 2, and 4 mo of age. The percentage of D-lactate in the total lactate content of Cheddar and Colby cheese made with *Lb. casei* LILA at d 1, and 1, 2, 3 and 4 mo of age was 2.8, 2.9, 18.6, 23.6, and 28.8%, respectively. Levels for control cheeses at the same time points were 2.7, 7.8, 30.4, 21.4, and 33.3%, respectively.

Sensory Scores

The ANOVA of sensory scores collected at 90 and 180 d by experienced judges in a randomized blind design showed neither cheese type nor adjunct addition had a significant effect ($P > 0.05$) on Cheddar flavor intensity, lipase/rancid flavor intensity, off-flavor intensity, overall flavor preference, or body/texture preference (data not shown).

Microbiological Counts

No colonies were detected on Rogosa agar from microbiological samples collected from the processing plant immediately before cheese manufacture. Plate counts on Elliker agar were also negative for most areas, but 6.8×10^2 cfu were recovered from the floor drain between cheese vats, and <10 colonies were isolated from the lab bench and stirrer motor. Similar analysis of pasteurized milk samples yielded no colonies on Rogosa agar, while counts on Elliker agar detected 2.9×10^2 cfu/ml.

Enumeration of starter and nonstarter bacteria in experimental reduced-fat cheeses showed all cheeses contained approximately 2.0×10^9 starter cfu/g at pressing, and that the number of viable starter bacteria declined about one order of magnitude during the 6 mo ripening period (Table 2). No NSLAB were detected on Rogosa agar plates from d 1 Cheddar or Colby cheeses that did not contain *Lb. casei* LILA adjunct, but numbers of adventitious lactobacilli in these cheeses was greater than 10^4 cfu/g by 4 mo (Table 2). *Lactobacillus* spp. counts in cheeses that contained *Lb. casei* LILA adjunct were greater than 10^6 cfu/g at press and remained above 10^7 cfu/g throughout the ripening period.

Identification of Environmental and Cheese Isolates

The RAPD was performed on more than 320 individual colonies collected from environmental samples and from Cheddar and Colby cheese extracts plated on Rogosa or Elliker agar after pressing (d 1), and after 2, 4, and 6 mo of ripening. At every sample point or time, unique strains present in those samples were identified by visual examination of RAPD fingerprints (Figures 1 and 2). Representative strains for each unique DNA fingerprint were then selected and identified to the nearest taxonomic relative by nucleotide sequence analysis of 16S rRNA gene fragments. As noted in the previous section, no colonies were detected on Rogosa agar from environmental samples or pasteurized cheese milk, and no facultatively heterofermentative lactobacilli were identified among the strains isolated from either source on Ellikers agar. Instead, representative strains of bacteria collected from environmental surface swabs prior to cheese making (Figure 1A) included *Ba-*

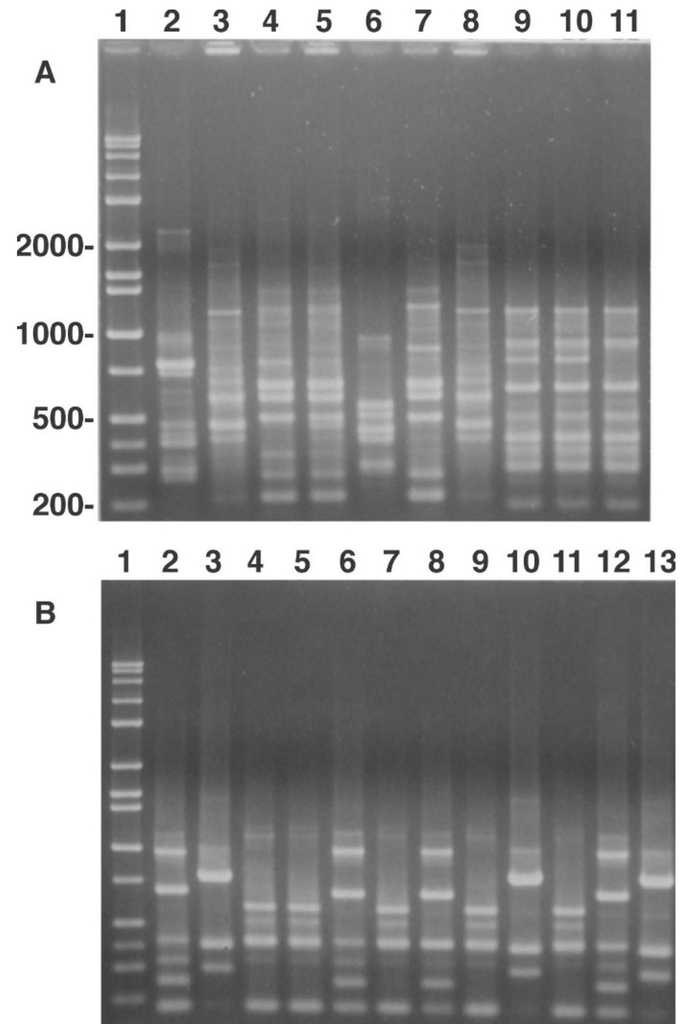

Figure 1. Agarose gel electrophoresis of randomly amplified polymorphic DNA (RAPD) from environmental bacterial isolates collected on Elliker agar. Panel A shows RAPD patterns for strains isolated from environmental surface swabs before cheese making, whereas panel B shows RAPD profiles for isolates obtained from pasteurized cheese milk prior to starter inoculation. Lane 1 in each panel contains DNA fragment size standards.

Table 2. Mean total number (cfu/g) of viable *Lactococcus lactis* starter and lactobacilli (nonstarter alone or nonstarter plus *Lactobacillus casei* LILA adjunct) in experimental cheeses during ripening.

Sample time	Cheddar		Cheddar + LILA		Colby		Colby + LILA	
	Starter	Lactobacilli	Starter	Lactobacilli	Starter	Lactobacilli	Starter	Lactobacilli
Day 1	2.0×10^9	<10	1.8×10^9	8.4×10^6	2.2×10^9	<10	2.0×10^9	4.0×10^6
2 mo	6.1×10^8	1.6×10^4	5.2×10^8	6.1×10^7	6.9×10^8	6.1×10^3	1.1×10^9	6.3×10^7
4 mo	3.5×10^8	5.8×10^4	3.7×10^8	5.6×10^7	5.6×10^8	3.5×10^5	5.4×10^8	9.2×10^7
6 mo	1.0×10^8	1.3×10^7	2.0×10^8	7.0×10^7	1.6×10^8	5.5×10^5	1.7×10^8	5.0×10^8

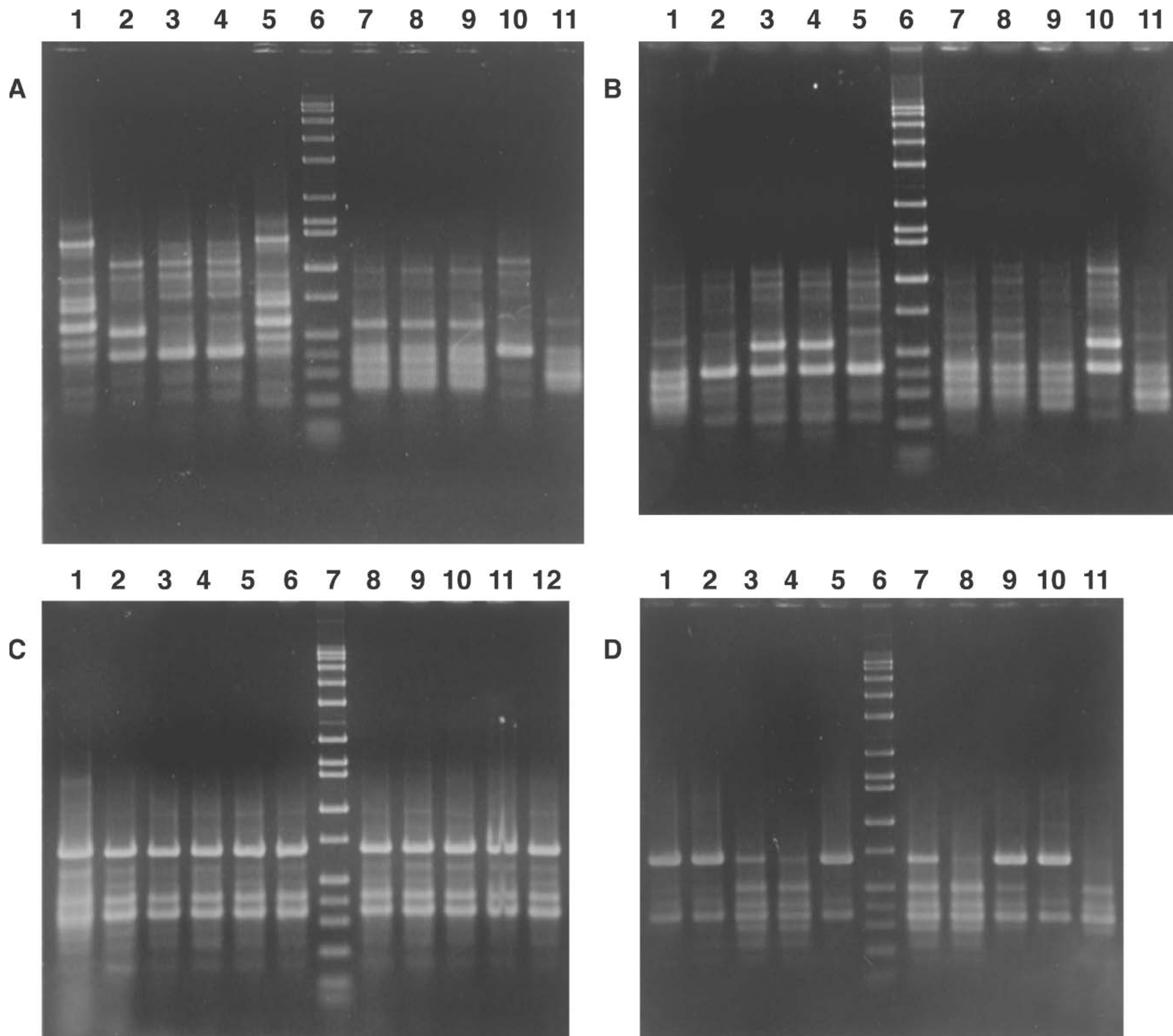

Figure 2. Agarose gel electrophoresis of randomly amplified polymorphic DNA (RAPD) from nonstarter lactic acid bacteria (NSLAB) isolated on Rogosa agar from extracts of ripened reduced-fat Cheddar or Colby cheese. Panels A and B show RAPD patterns for NSLAB isolated from 2-mo-old Cheddar and Colby cheese, respectively. Panel C shows RAPD profiles for NSLAB collected from 2-mo-old Cheddar made with *Lactobacillus casei* LILA adjunct, with the adjunct RAPD profile shown in lane 1. Panel D shows RAPD patterns for NSLAB collected from 4-mo-old Colby cheese made with *L. casei* LILA. Lane 6 in panels A, B, and D contain DNA fragment size standards, which are also present in lane 7 of panel C.

cillus licheniformis, *Enterococcus durans*, *Escherichia coli*, and *Klebsiella* sp. Isolates obtained from pasteurized cheese milk before starter inoculation (Figure 1B) included one strain of *Lc. lactis* spp. *lactis* and several strains of *Streptococcus* spp. phylogenetically related to either *S. salivarius* or *S. bovis*.

Characterization of NSLAB isolates from each cheese by RAPD and16S rDNA sequencing showed NSLAB populations in both types of control cheeses were dominated by *Lb. curvatus* and *Lb. casei* (Tables 3 and 4). By 4 mo, NSLAB isolates from control reduced-fat Cheddar cheese were all identified as *Lb. casei*, and this species was also the only one found at 6 mo (Table 3). In contrast, 4-mo-old control reduced-fat Colby contained a variety of NSLAB species including *Lb. curvatus*, *Lb. casei*, and *Enterococcus* spp., and species heterogeneity was also noted in 6-mo-old cheese (Table 4). Addition of *Lb. casei* LILA appeared to reduce NSLAB species and strain heterogeneity in ripening Colby and young Cheddar. As shown in Figure 2, the RAPD profile of the

Table 3. Population dynamics of *Lactococcus lactis* ssp. *cremoris* (*Lc. cremoris*), *Lactococcus lactis* spp. *lactis* (*Lc. lactis*), and *Lactobacillus* (*Lb.*) spp. starter and nonstarter bacteria in reduced-fat Cheddar cheese made with or without *Lb. casei* LILA adjunct.[1]

Sample time	Isolation medium	Cheddar		Cheddar + LILA	
		%[2]	Species and strains (strain ratio)[3]	%[2]	Species and strains (strain ratio)[3]
day 1	Elliker	100	*Lc. cremoris* SCO213	84	*Lc. cremoris* SCO213
				16	*Lc. cremoris* 1.5.10
	Rogosa		ND[4]	100	*Lb. casei* LILA
2 mo	Elliker	100	*Lc. cremoris* SCO213	50	*Lc. cremoris* SCO213
				50	*Lb. casei* LILA
	Rogosa	40	*Lb. casei* 2.1.5	100	*Lb. casei* LILA
		40	*Lb. curvatus* 2.1.2, 2.2.4 (3:1)		
		20	*Lb. pentosus* 2.2.5		
4 mo	Elliker	100	*Lc. cremoris* SCO213	100	*Lc. cremoris* SCO213, 4.6.6 (9:1)
	Rogosa	100	*Lb. casei* 4.2.3, 2.1.5, 4.2.1, 4.2.5 (4:3:2:1)	100	*Lb. casei* 4.5.3, 4.5.1, 4.5.2, 4.5.5 (2:1:1:1)
6 mo	Elliker	100	*Lc. cremoris* SCO213, 6.2.8 (9:1)	100	*Lc. cremoris* SCO213
	Rogosa	100	*Lc. casei* 2.1.5, 6.2.4 (1:1)	100	*Lb. casei* 6.5.2, 6.6.4, 6.5.1, 6.5.4 (6:2:1:1)

[1]As determined by randomly amplified polymorphic DNA profile, partial nucleotide sequence analysis of the 16S rRNA gene, and API 50 CHL carbohydrate fermentation patterns.

[2]Of 10 individual isolates collected at random from each growth medium at each sampling time.

[3]Representative strain designation (relative ratio of individual strains isolated within a particular species). Strains recovered at more than one ripening time are identified by repeated use of the first designation assigned to the strain.

[4]Not determined (no colonies detected on agar plates).

adjunct was the only pattern observed among NSLAB isolates from d 1 and 2-mo-old cheeses (Figure 2C). Although the adjunct was not detected in 4- or 6-mo-old reduced-fat Cheddar and Colby cheeses, both cheese types yielded fewer strains of wild *Lb. casei* than did their respective control cheeses (Tables 3 and 4).

Interestingly, RAPD patterns and sugar fermentation profiles indicated that the dominant strains of *Lb. casei* in each cheese appeared to be most affected by adjunct treatment and not cheese variety. In control cheese, for example, *Lb. casei* isolate 2.1.5 was recovered from 2-, 4-, and 6-mo-old Cheddar and from 4- and 6-mo-old Colby cheese, but it was not detected in cheese made with *Lb. casei* LILA adjunct (Tables 3 and 4). Conversely, *Lb. casei* isolates 6.5.2 and 6.5.4 were recovered from 6-mo-old Cheddar and Colby cheeses made

Table 4. Population dynamics of *Lactococcus lactis* spp. *cremoris* (*Lc. cremoris*), *Lactococcus lactis* spp. *lactis* (Lc. lactis), and *Lactobacillus* (*Lb.*) spp. starter and nonstarter bacteria in reduced-fat Colby cheese made with or without *Lb. casei* LILA adjunct.[1]

Sample time	Isolation medium	Colby		Colby + LILA	
		%[2]	Species and strains (strain ratio)[3]	%[2]	Species and strains (strain ratio)[3]
day 1	Elliker	100	*Lc. cremoris* SCO213	79	*Lc. cremoris* SCO213
				21	*Lb. casei* LILA
	Rogosa		ND[4]	100	*Lb. casei* LILA
2 mo	Elliker	100	*Lc. cremoris* SCO213	100	*Lc. cremoris* SCO213
	Rogosa	50	*Lb. casei* 2.3.1	100	*Lb. casei* LILA
		50	*Lb. curvatus* 2.3.3, 2.3.5 (3:2)		
4 mo	Elliker	100	*Lc. cremoris* SCO213	100	*Lc. cremoris* SCO213
	Rogosa	70	*Lb. casei* 2.1.5, 4.3.3, 4.4.1 (3:2:2)	100	*Lb. casei* 4.8.5, 4.7.4, 4.7.1 (5:4:1)
		10	*Lb. curvatus* 4.3.2		
		20	*Enterococcus* sp. 4.3.1		
6 mo	Elliker	100	*Lc. cremoris* SCO213	100	*Lc. cremoris* SCO213
	Rogosa	70	*Lb. casei* 2.1.5, 6.3.2, 6.3.5, 6.4.1, 6.4.4 (3:1:1:1:1)	100	*Lb. casei* 6.7.1, 6.8.2, 6.5.2, 6.5.4 (6:1:1:1)
		30	*Lb. curvatus* 6.4.2 (2:1)		

[1]As determined by randomly amplified polymorphic DNA profile, partial nucleotide sequence analysis of the 16S rRNA gene, and API 50 CHL carbohydrate fermentation patterns.

[2]Of 10 isolates collected from each growth medium at each sampling time.

[3]Representative strain designation (relative ratio of individual strains isolated within a particular species). Strains recovered at more than one ripening time are identified by repeated use of the first designation assigned to the strain.

[4]Not determined (no colonies detected on plate counts).

with *Lb. casei* LILA, but were not found in control cheeses.

Carbohydrate Utilization by NSLAB

Analysis of carbohydrate fermentation patterns by 39 dominant NSLAB isolates collected from 2- (8 strains), 4- (16 strains), or 6- (15 strains) mo-old, reduced-fat Cheddar or Colby cheese showed all isolates fermented galactose, glucose, fructose, mannose, and *N*-acetyl glucosamine. Lactose, esculine, and tagatose were also utilized by at least 75% of the NSLAB isolates collected for characterization from 2-, 4-, or 6-mo-old cheeses, while ribose was fermented by 63 to 75% of these strains, depending on the sampling time. Finally, the ability to ferment mannitol, turanose, cellobiose, maltose, trehalose, and melezitose was slightly more prevalent in dominant strains from 4- and 6-mo-old cheese (50 to 75% positive, depending on the sampling time) than 2-mo samples (38 to 50% positive).

Phenotypic identification of dominant NSLAB isolates through API 50 CHL carbohydrate fermentation profiles showed little consensus with 16S rDNA sequence data, with taxonomic agreement only noted for 12 of 39 strains (31%) analyzed by both methods. Most of the discrepancy between these methods was due to 19 isolates (49% of total) that had metabolic traits most akin to *Lb. curvatus*, but whose closest phylogenetic relative by 16S rDNA sequence analysis was *Lb. casei*.

DISCUSSION

All bacterial-ripened cheeses contain NSLAB that enter cheese through processing equipment or milk and grow to high numbers during ripening (Beresford et al., 2001). Although NSLAB can have a significant effect on flavor development, little is known about the factors that influence the growth and composition of their populations. As a result, the types and numbers of NSLAB present in cheese remain largely a matter of chance. Nonetheless, the knowledge that NSLAB influence cheese quality indicates technologies to control NSLAB populations during ripening would impart greater uniformity to the overall quality of Cheddar and other bacterial-ripened cheeses.

One of the most promising strategies to control NSLAB populations is to employ well-characterized *Lactobacillus* spp. adjunct cultures that suppress the emergence of wild NSLAB (Broome et al., 1990a; Crow et al., 2001). Cheese is a dynamic environment, however, and the effectiveness of adjunct technology may be limited by differences in cheese composition or manufacturing and ripening regimens. Colby and Cheddar cheeses are commonly manufactured with identical strains of *Lc. lactis* starter, but Colby cheese is a washed, stirred-curd variety of Cheddar, and this treatment produces significant differences in the cheese microenvironment (Table 1). Because the intrinsic properties of Colby are less restrictive to microbial growth than those of Cheddar, it was our hypothesis that the environment in the former cheese would accommodate a greater diversity of NSLAB species and strains than the latter. To investigate this hypothesis, we examined NSLAB population dynamics in reduced-fat Cheddar and Colby cheese, and studied the effect of *Lb. casei* LILA on NSLAB population dynamics in each cheese.

Results showed the NSLAB biota in both cheeses changed over time, but NSLAB populations in Colby cheese were more heterogeneous for a longer period of ripening than those of Cheddar made in the same processing facility (Tables 3 and 4). Addition of the *Lb. casei* adjunct to Cheddar or Colby cheese did not influence sensory qualities or important compositional attributes, but it did appear to limit the NSLAB species and strain heterogeneity in both cheeses. In addition, even though the adjunct was not detected in 4- or 6 mo-old reduced-fat Cheddar and Colby cheeses, the dominant strains of *Lb. casei* in each cheese appeared to be most affected by adjunct treatment and not cheese variety. The reasons for these observations are unknown, but they do support the hypothesis that adjunct cultures can help to control cheese NSLAB composition during ripening.

Data from this study also indicated that phenotypic identification of cheese NSLAB may be problematic, and similar observations have been noted by other researchers (Liu et al., 1988; Nigatu, 2000). The most common discrepancy between phenotypic and 16S rDNA sequence data in this study was due to isolates that had sugar fermentation profiles most like *Lb. curvatus*, but whose 16S rDNA sequence was most closely related to *Lb. casei*. Because *Lb. curvatus* and *Lb. casei* are mesophilic, facultatively heterofermentative species with relatively similar sugar fermentation profiles (Kandler and Weiss, 1986), it is not surprising that efforts to identify these species through phenotypic data may lead to confusion.

The substrates used by NSLAB for growth in cheese have been the subject of considerable research interest and conjecture (Fryer, 1969). Previous workers have shown NSLAB can derive energy from compounds such as lactic acid, citric acid, carbohydrate moieties from glycoproteins, fatty acids, glycerol, and amino acids (Fryer, 1969; Thomas, 1986; Williams et al., 2000). Evidence also suggests that starter autolysis may be an important source of ribose and other nutrients for NSLAB growth (Thomas, 1987; Rapposch et al., 1999). Nonetheless, the substrates required by NSLAB to at-

tain final populations levels of 10^7 to 10^9 cfu/g in ripening cheese have not been conclusively identified. Examination of carbohydrate fermentation patterns for NSLAB strains isolated in this study also did not reveal any obvious differences in the sugar utilization profiles for strains isolated at different ripening periods. Although the ability to ferment some sugars was more prevalent in dominant strains from 4- and 6-mo-old cheese than 2-mo samples, the differences were not striking (50 to 75% vs. 38 to 50% positive) and could have simply been due to variation in the sample size.

CONCLUSIONS

The dynamic and complex nature of NSLAB populations in Cheddar cheese is a likely source of quality defects and inconsistencies, so technologies to control NSLAB populations during ripening should provide greater uniformity in overall cheese quality. *Lactobacillus* spp. adjuncts are a promising tool for this purpose, but their efficacy is challenged by the ecological diversity that results from differences in cheese manufacturing and ripening protocols, and in overall cheese composition. To overcome these challenges, additional research is needed to understand the impact of the cheese microenvironment on growth and dominance of NSLAB species and strains.

ACKNOWLEDGMENTS

The authors thank Rebekah Allen, Kristen Anderson, Brie-Ann McKernan, Melinda Moss, Beatriz Rodriguez, and Ann Stuhlman for technical assistance, and Charlotte Brennand for help with statistics. This work was supported by Dairy Management, Inc., through the Western Dairy Center and the Wisconsin Center for Dairy Research, and by the Utah Agricultural Experiment Station.

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