Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media

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ABSTRACT

Various selective media for enumerating probiotic and cheese cultures were screened, with 6 media then used to study survival of probiotic bacteria in full-fat and low-fat Cheddar cheese. Commercial strains of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei*, or *Bifidobacterium lactis* were added as probiotic adjuncts. The selective media, designed to promote growth of certain lactic acid bacteria (LAB) over others or to differentiate between LAB, were used to detect individual LAB types during cheese storage. Commercial strains of *Lactococcus*, *Lactobacillus*, and *Bifidobacterium* spp. were initially screened on the 6 selective media along with nonstarter LAB (NSLAB) isolates. The microbial flora of the cheeses was analyzed during 9 mo of storage at 6°C. Many NSLAB were able to grow on media presumed selective for *Lactococcus*, *Bifidobacterium* spp., or *Lb. acidophilus*, which became apparent after 90 d of cheese storage. Between 90 and 120 d of storage, bacterial counts changed on media selective for *Bifidobacterium* spp., suggesting growth of NSLAB. Appearance of NSLAB on *Lb. casei* selective media [de man, Rogosa, and Sharpe (MRS) + vancomycin] occurred sooner (30 d) in low-fat cheese than in full-fat control cheeses. Differentiation between NSLAB and *Lactococcus* was achieved by counting after 18 to 24 h when the NSLAB colonies were only pinpoint in size. Growth of NSLAB on the various selective media during aging means that probiotic adjunct cultures added during cheesemaking can only be enumerated with confidence on selective media for up to 3 or 4 mo. After this time, growth of NSLAB obfuscates enumeration of probiotic adjuncts. When adjunct *Lb. casei* or *Lb. paracasei* cultures are added during cheesemaking, they appear to remain at high numbers for a long time (9 mo) when counted on MRS + vancomycin medium, but a reasonable probability exists that they have been overtaken by NSLAB, which also grow readily on this

medium. Enumeration using multiple selective media can provide insight into whether it is the actual adjunct culture or a NSLAB strain that is being enumerated.

Key words: probiotic, enumeration, cheese, selective media

INTRODUCTION

Dairy-based systems currently used to deliver various probiotic lactic acid bacteria (LAB) include yogurt, milk, and recently, cheese. Cheese may have benefits over other dairy delivery systems, including greater in vivo survival, longer shelf life, and being a more common component in the average diet (Vinderola et al., 2000; Ross et al., 2002; Hayes et al., 2006). One challenge to determining the efficacy of hard cheeses as a vehicle for probiotic delivery lies in determining the survival of probiotic adjunct cultures among a mixed population of existing cheese microflora. This determination is made more difficult by the growth of advantageous nonstarter LAB (NSLAB) during the cheese-ripening period particularly because many of these NSLAB are closely related to probiotic adjunct cultures (Steele et al., 2006).

Determining the survival of bacteria, whether added or advantageous, in fermented dairy products initially led to development of media containing complex substrates added to promote or maximize bacterial counts of target organisms. Differentiation between individual LAB genera and species or exclusion of specific LAB usually requires the addition of inhibitory compounds (e.g., antibiotics or heavy metals), or variations in media chemistry (e.g., pH) or incubation conditions (i.e., temperature and oxygen levels). Because LAB generally have rather fastidious growth requirements, all methods use a complex medium that is not chemically defined.

Various media for enumeration of specific bacterial cultures associated with dairy fermentations that utilize probiotic adjunct bacteria have been formulated and tested (Lankaputhra et al., 1996; Tharmaraj and Shah, 2003; Darukaradhya et al., 2006; Tabasco et al., 2007). Dave and Shah (1996) evaluated 15 media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus*

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acidophilus, and *Bifidobacterium* spp. By lowering the pH of de Man, Rogosa, and Sharpe (MRS) agar to 5.2 or that of reinforced clostridial agar (RCA) to 5.3, and incubating at 45°C for more than 72 h, these media were selective for *Lb. delbrueckii* ssp. *bulgaricus*. Moreover, MRS with sorbitol as the only carbohydrate (MRS-S) was suggested as being selective for *Lb. acidophilus*. Using MRS with a combination of inhibitory compounds added (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate; NNLP) was effective for enumerating bifidobacteria in mixed LAB cultures. Because some attenuation of target bacteria still occurs on these severely inhibitory selective media, bacterial counts are generally lower on the selective media developed for each microorganism than on media designed to optimize bacterial counts (e.g., MRS or MRS-maltose).

Evaluation of LAB enumeration media has continued in an effort to improve their ability to exclude potential competing bacteria. Tharmaraj and Shah (2003) evaluated 19 media to selectively enumerate LAB and bifidobacteria and chose MRS with vancomycin (MRS-V) for *Lactobacillus casei*, MRS-NNLP for *Bifidobacterium* spp., and MRS-S incubated at 43°C for *Lb. acidophilus* enumeration, respectively. Van de Castele et al. (2006) evaluated 11 media and also selected MRS-NNLP for *Bifidobacterium* spp. They chose MRS with clindamycin for enumerating *Lb. acidophilus* provided that *Lactobacillus rhamnosus* was not also present in the microflora (because it can also grow in the presence of clindamycin). Instead of MRS, Darukaradhya et al. (2006) modified RCA with various additives to make it more selective for specific LAB. They selected RCA with clindamycin and bromocresol green to enumerate *Lb. acidophilus*, RCA with dicloxacillin and aniline blue to select for *Bifidobacterium* spp., and RCA with vancomycin and bromocresol green (RCA-BV) to evaluate NSLAB populations (assuming these are *Lb. casei* or similar organisms). These modified RCA media performed well when enumerating pure cultures, with some discrepancies being noted when the microflora of Cheddar cheese was examined.

The objectives of our research were to determine the effectiveness of selective media for enumerating probiotic LAB such as *Lb. acidophilus*, *Bifidobacterium* spp., and *Lb. casei*, when they are used as adjunct cultures in the manufacture of cheese, and to determine their survivability in cheese during 9-mo storage of full-fat and low-fat Cheddar. Several published media were initially screened for their ability to differentiate between selected LAB genera and species, with 6 selected for cheese microflora enumeration. These media were then used to determine the survivability of probiotic adjunct cultures in Cheddar cheese.

MATERIALS AND METHODS

Bacterial Cultures and Propagation

Bacterial cultures used in this study are listed in Table 1. Starter and probiotic adjunct cultures were obtained from commercial sources, whereas NSLAB cultures that had previously been isolated from cheese came from the LAB Culture Collection at Utah State University (Logan) and as gifts from other university researchers. Lactococcal cultures were grown in M17 lactose (M17-L) broth (Difco, Becton, Dickinson and Co., Sparks, MD), bifidobacteria strains were maintained in MRS broth (Criterion, Hardy Diagnostics, Santa Maria, CA) supplemented with 0.5% cysteine (Fisher Scientific, Fair Lawn, NJ), and all other LAB cultures were propagated in MRS broth. *Lactococcus* was incubated at 30°C and the rest of the cultures were grown at 37°C.

Testing of Enumeration Media

Six media were selected for enumeration of specific bacterial types in probiotic cheese as listed in Table 2 along with their incubation parameters. Two base media, M17-L and MRS, were used with several additives including lactose (Fisher Scientific), sorbitol (Acros Organics, Morris Plains, NJ), vancomycin (Sigma-Aldrich Inc., St. Louis, MO), bromocresol green (J.T. Baker Chemical Co., Phillipsburg, NJ), cysteine (Fisher Scientific), nalidixic acid (MP Biomedicals LLC, Aurora, OH), neomycine sulfate (Sigma-Aldrich Inc.), lithium chloride (Fisher Scientific), and paromomycin sulfate (Sigma-Aldrich Inc.). The 6 media were M17-L for lactococci, MRS plus 10 g/L sorbitol (MRS+S) for total LAB, MRS-V and RCA-BV for *Lb. casei/paracasei* and other lactobacilli NSLAB, MRS-NNLP for bifidobacteria, and MRS-S (MRS made with sorbitol instead of dextrose) for *Lb. acidophilus*. Incubation parameters for each media are listed in Table 2.

Each culture was grown for 24 h in the appropriate medium at the optimum incubation temperature. Cultures were diluted using 9-mL dilution blanks containing sterile 0.1% (wt/vol) peptone phosphate buffer. Each dilution was plated in triplicate using the pour-plate method with each of the selected media. Lactococci were incubated at 30°C aerobically. Lactobacilli and bifidobacteria strains were incubated in 2.5-L anaerobic jars containing Oxoid Anerogengas generators (Oxoid Ltd., Basingstoke, UK) at 37°C. *Lactobacillus* strains were also incubated anaerobically at 45°C and 50°C to determine the effect of incubation temperature on growth. M17-Lactose plates were examined at 24 and 48 h, whereas all other media were examined at 48 and

Table 1. Bacterial cultures used in this study

Culture	Species	Function	Source ¹
DVS850	<i>Lactococcus lactis</i> ssp. <i>lactis</i>	Starter	Chr. Hansen
LH32	<i>Lactobacillus helveticus</i>	Flavor	Chr. Hansen
LA-5	<i>Lactobacillus acidophilus</i>	Probiotic	Chr. Hansen
L 10	<i>Lb. acidophilus</i>	Probiotic	DSM
Bif-6	<i>Bifidobacterium lactis</i>	Probiotic	Cargill
BB-12	<i>Bf. lactis</i>	Probiotic	Chr. Hansen
CRL 431	<i>Lactobacillus casei</i>	Probiotic	Chr. Hansen
L26	<i>Lb. casei</i>	Probiotic	DSM
F19	<i>Lb. casei/paracasei</i>	Probiotic	Medipharm
367	<i>Lactobacillus brevis</i>	NSLAB ²	USU
1364P	<i>Lb. brevis</i>	NSLAB	USU
M36	<i>Lb. casei</i>	NSLAB	USU
37	<i>Lb. casei</i>	NSLAB	USU
25598	<i>Lb. casei</i> ssp. <i>pseudopiantarum</i>	NSLAB	ATCC
LiLa	<i>Lactobacillus paracasei</i>	NSLAB	UW
14931	<i>Lactobacillus fermentum</i>	NSLAB	ATCC
14957	<i>Lactobacillus rhamnosus</i>	NSLAB	ATCC
7469	<i>Lb. rhamnosus</i>	NSLAB	ATCC

¹Chr. Hansen Inc., Milwaukee, WI; DSM = DSM Food Specialties USA Inc., Eagleville, PA; Cargill BioActives US, LLC, Waukesha, WI; Medipharm USA, Des Moines, IA; USU = Utah State University Lactic Acid Bacteria Culture Collection, courtesy of J. Broadbent, Utah State University, Logan; ATCC = American Type Culture Collection, Manassas, VA; UW = Wisconsin Center for Dairy Research Culture Collection, courtesy of J. Steele, University of Wisconsin, Madison.

²Nonstarter lactic acid bacteria.

72 h. In addition to examining all culture strains used in the manufacture of probiotic-enhanced cheese, some NSLAB strains obtained from various sources (see Table 1) were enumerated to determine their ability to grow on the 6 selective media. Lactococcal media was incubated aerobically at 30°C, *Lb. acidophilus* media was incubated at 45°C in a gaspak (i.e., anaerobically), with all other media incubated at 37°C in a gaspak.

Cheese Manufacture

Fresh cow milk was obtained from the George B. Caine Dairy Research and Teaching Center (Wellsville, UT) and standardized to protein-to-fat ratios of 0.83 and 5.0 for making full-fat and low-fat cheeses, respectively, and pasteurized at 73°C for 15 s in the

Gary Haight Richardson Dairy Products Laboratory at Utah State University (Logan). Aliquots of 136 kg of milk were pumped into open stainless steel vats with the milk for making the low-fat cheese cooled to 10°C and acidified to pH 6.4 with vinegar. For the full-fat cheeses, the milk was warmed to the set temperature. Cheese manufacturing followed as outlined in Table 3.

The same starter culture (see Table 1) was used for all cheeses, and the adjunct probiotic cultures (see Table 1) were added to milk at a rate of approximately 4×10^9 cells/kg, which was calculated to yield 1×10^9 cells per 28-g serving of resultant cheese. Each probiotic culture was used 2 times for each cheese fat level during the cheese making trials.

After ripening, the cheese was renneted and the resultant curd cut with wire harps. The curd was allowed

Table 2. Agar media used to determine specific lactic acid bacteria (LAB) in cheese

Media ¹	Incubation		Presumptive LAB selection	Reference
	Temperature	Oxygen		
M17-L	30°C	Aerobic	<i>Lactococcus</i>	Terzaghi and Sandine (1975)
MRS+S	37°C	Anaerobic	Total LAB	Unpublished data
MRS-S	45°C	Anaerobic	<i>Lactobacillus acidophilus</i>	Dave and Shah (1996)
RCA-BV	37°C	Anaerobic	<i>Lactobacillus casei</i>	Darukaradhy et al. (2006)
MRS-V	37°C	Anaerobic	<i>Lb. casei</i>	Tharmaraj and Shah (2003)
MRS-NNLP	37°C	Anaerobic	<i>Bifidobacterium</i>	Laroia and Martin (1991)

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate.

Table 3. Cheese manufacturing procedures used for making full-fat and low-fat cheeses using 136 kg of standardized milk

Cheesemaking step/parameter	Full fat	Low fat
Milk pH ¹	6.65	6.40
Starter culture, g	17	26
Ripening time, min	45	45
Set temperature, °C	31	32
Calcium chloride, ² mL	18	0
Annatto, ³ mL	12	18
Rennet, ⁴ mL	12	12
Set-to-cut time, min	30	25
Curd size	Small	Large
Set-to-start of cook time, min	65	35
Cooking temperature (time), °C (min)	39 (35)	38 (15)
Set-to-drain time, ⁵ min	135	75
Drain pH	6.30	6.00
Wash water temperature, °C	NA ⁶	18
Curd wash temperature, °C	NA	27
Set-to-salt time, ⁵ min	275	190
Salting pH	5.40	5.90
Salt, g/kg of curd	27 ⁷	26
Pressing, kPa	100	55

¹Milk for making low-fat cheese was preacidified before renneting with distilled white vinegar (Heinz North America, Pittsburgh, PA).

²35% calcium chloride solution.

³Single-strength annatto solution (DSM Food Specialties USA Inc., Eagleville, PA).

⁴Double-strength chymosin (Maxiren, ~650 international milk clotting units/mL, DSM Food Specialties USA Inc., Eagleville, PA).

⁵Typical times with some variation based on rate of acid development.

⁶Not applicable.

⁷Approximate value for full-fat cheese; salt actually added at a rate of 2.95 g/kg of milk.

to heal for a few minutes and then manually stirred and heated before draining. For the full-fat cheeses, the curd was allowed to mat together and then cut into slabs, cheddared, and milled. For the low-fat cheeses, the curd was washed (using approximately 15 kg of cool water and held in the water for 10 min) and manually stirred to prevent matting. When the curd reached the desired pH, the curd was salted using 3 applications at 5-min intervals, and then filled into a cheesecloth-lined, stainless steel hoop and pressed overnight into a nominal 10-kg block. Each cheese then was cut into approximately 1-kg blocks that were vacuumed packaged and stored at 6°C.

Cheese Analysis

Proximate composition of the cheeses was determined after about 5 d as described by McMahon et al. (2009). Moisture content was by weight loss using a microwave oven, fat content by a modified Babcock method, salt by chloride measurement, and pH by glass electrode. Samples of cheese were shipped to Weber State University (Ogden, UT) for microbial analysis at 1 wk, 1, 2, 3, 4, 5, and 6 mo.

Microbial Enumeration in Cheese

Eleven grams of cheese was added to 99 mL of sterile 2% (wt/wt) sodium citrate buffer and stomached for 4 min at 260 rpm using a Stomacher 400 Circulator (Seward Laboratory Systems Inc., Bohema, NY). Further dilutions were made of the homogenized cheese sample by adding 1 mL of the previous dilution to 99 mL of sterile Butterfield phosphate buffer dilution blanks (Hardy Diagnostics). One milliliter of each dilution was placed in a sterile Petri plate and the pour-plate method was used to enumerate the microbial count. Inoculated Petri plates were incubated as described above and in Table 2 for each selective media. Each dilution was plated in duplicate and, after the specified incubation time, individual colonies were counted to calculate the total number of microorganisms for each cheese sample.

RESULTS

Media Evaluation

From the initial screening of selective media, it was observed that adding 10 g/L sorbitol to MRS (designated MRS+S) agar that was incubated anaerobically at 37°C for 48 h provided the highest overall bacterial counts for the starter and adjunct cultures used in this study. The MRS+S medium was subsequently used to obtain the total LAB count in the cheese samples. All of the bacterial strains grew on media developed specifically for their enumeration and fit the expected profile of growth or inhibition (Table 4) but these media were not exclusionary. For example, growth of *Lactobacillus helveticus*, which is commonly used as a flavor adjunct in Cheddar cheese to prevent bitterness, was unattenuated on medium designed for *Lb. acidophilus* in which sorbitol was the sole sugar constituent (MRS-S) and incubated at 45°C. This would be expected given the close relationship between these 2 species (Slattery et al., 2010). Interestingly, *Lb. casei* F-19 also grew on this medium at the elevated incubation temperature, whereas the other 2 *Lb. casei* adjuncts, CRL 431 and L26, did not grow. All 3 of the *Lb. casei* and *Lb. paracasei* adjunct cultures similarly grew on the MRS-NNLP medium that had been proposed as selective for bifidobacteria, although usually as very small colonies. One strain of *Lb. casei/paracasei* also grew on the MRS-S medium (Table 4).

The MRS+S medium gave higher bacterial counts in all test periods even above *Lb. casei* specific media in aged cheese, indicating it could be used to obtain the total LAB count for cheese. It is known (Cogan and Beresford, 2002) that NSLAB can grow on M17-L

Table 4. Growth of cultures added during cheesemaking on selective enumeration media

Culture ¹	Media ²					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
DVS850	2.2×10^9	1.9×10^9	$<10^4$	$<10^4$	$<10^4$	$<10^4$
LH 32	$<10^4$	5.1×10^7	$<10^4$	$<10^4$	5.1×10^7	$<10^4$
LA 5	$<10^4$	9.2×10^7	$<10^4$	$<10^4$	8.0×10^8	9.2×10^7
L 10	$<10^4$	1.8×10^9	$<10^4$	2.0×10^9	3.4×10^9	$<10^4$
L 26	$<10^4$	2.4×10^9	2.2×10^9	3.5×10^9	$<10^4$	1.9×10^9
CRL431	$<10^4$	2.2×10^9	2.2×10^9	2.6×10^9	$<10^4$	2.4×10^9
F-19	$<10^4$	6.0×10^8	1.2×10^9	2.0×10^9	5.0×10^8	8.0×10^8
Bif 6	$<10^4$	1.4×10^9	$<10^4$	8.0×10^8	$<10^4$	$<10^4$
BB 12	$<10^4$	1.8×10^9	$<10^4$	9.0×10^8	$<10^4$	$<10^4$

¹See Table 1 for culture descriptions.

²M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

medium, making it difficult to count starter culture populations during cheese storage. However, when M17-L medium was inspected for colonies at 24 h we found that it could be used for lactococcal enumeration. The *Lb. casei/paracasei* cultures also grew on the M17-L medium but much more slowly, and they could be disregarded as pinpoint colonies at 24 h. Counting the M17-L plates after 24 h rather than 48 h of aerobic incubation at 30°C thus allowed for a direct measure of starter culture lactococcal numbers. The commonly used alternative is to calculate lactococcal numbers by using a medium such as Rogosa SL to selectively grow lactobacilli and then subtracting the *Lactobacillus* count from total LAB counts (Broadbent et al., 2003).

However, with longer incubation times on M17-L, *Lb. casei/paracasei* colonies continued to grow and became indistinguishable from the lactococcal colonies after 48 to 72 h. When a representative collection of NSLAB strains (see Table 1) was grown on the selective enumeration media, the same general phenomenon was also observed, demonstrating that a variety of *Lb. casei*, *Lb. paracasei*, *Lactobacillus fermentum*, and *Lactobacillus rhamnosus* NSLAB strains are capable of growing and forming colonies when incubated on lactococcal M17-L, *Bifidobacterium* spp. MRS-NNLP, and *Lb. acidophilus* MRS-S media after 48 h of incubation (Table 5). Only the *Lactobacillus brevis* NSLAB strains did not grow on either the *Bifidobacterium* spp. or *Lb. acidophilus* media.

The only bacteria that grew on the MRS-V medium were the 3 *Lb. casei/paracasei* probiotic cultures and all the NSLAB strains (regardless of their species). The level of vancomycin used in this medium (1 mg/L) was at the minimal inhibitory concentration for bifidobacteria but 1,000 times less than that needed to inhibit *Lb. casei* (D'Aimmo et al., 2007).

Cheese Analysis

In the control full-fat Cheddar cheese (i.e., no probiotic adjunct culture added), a general trend was observed of decreasing counts on M17-L (lactococci) and increasing counts on MRS-V and RCA-BV. This increasing population over storage time was assumed to be indigenous NSLAB obtained through contact with cheesemaking equipment or the environment (no NSLAB adjunct cultures were added during cheese manufacture; Table 6). This is the generally recognized trend in cheese microflora during the initial months of Cheddar cheese storage (Peterson and Marshall, 1990; McSweeney et al., 1993). Considerable variations in this trend have been observed depending on the autolytic behavior of the lactococcal starter culture (Hannon et al., 2003), their salt-sensitivity, salt content of the cheese (Guinee and Fox, 2004), and cheese storage temperature (Cromie et al., 1987). The difficulty in differentiating between NSLAB and lactobacilli added as adjunct cultures (Boylston et al., 2004; Bergamini et al., 2005, 2006; Shakeel-ur-Rehman et al., 2008) also has caused variations in reports about cheese microflora populations.

In the control full-fat Cheddar cheese, growth of NSLAB after 180 d of storage was measured at 4×10^7 cfu/g (Table 6) as expected on the 2 media specifically used for enumerating *Lb. casei/paracasei* (i.e., MRS-V and RCA-BV). However, NSLAB grew to almost the same level (8×10^6) on MRS-NNLP, which was the medium proposed for selectively counting bifidobacteria, and to 4×10^7 on MRS-S incubated at 45°C and used for selectively counting *Lb. acidophilus* (Table 6). Both *Bifidobacterium* and *Lb. casei* have some intrinsic antibiotic resistance but it was expected that *Lb. casei* would not grow on MRS-NNLP. According

Table 5. Growth of nonstarter lactic acid type bacterial strains on presumptive selective media

Strain ¹	Media ²					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
367	5.5×10^8	ND ³	1.0×10^8	$<10^2$	$<10^2$	7.1×10^8
1364P	$>10^7$	2.0×10^9	1.7×10^9	$<10^2$	$<10^2$	1.5×10^9
M36	$>10^7$	2.7×10^9	3.1×10^9	3.3×10^9	2.2×10^9	3.3×10^9
M36	3.1×10^9	ND	2.4×10^9	1.9×10^9	1.0×10^7	2.4×10^9
37	2.5×10^9	ND	2.7×10^9	2.5×10^9	1.0×10^7	2.6×10^9
25598	1.1×10^9	ND	1.2×10^9	1.1×10^9	1.1×10^9	8.6×10^8
LiLa	2.9×10^9	ND	2.4×10^9	1.0×10^7	$<10^2$	2.6×10^9
14931	$<10^2$	1.5×10^9	1.6×10^9	$>1.0 \times 10^7$	1.6×10^9	1.9×10^9
14957	$>10^7$	2.0×10^9	1.9×10^9	1.9×10^9	1.9×10^9	2.0×10^9
7469	$>10^7$	6.0×10^8	6.0×10^8	$>10^7$	7.0×10^8	7.0×10^8

¹See Table 1 for culture descriptions.²M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycin sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.³Not done.

to D'Aimmo et al. (2007), *Bifidobacterium* have more resistance than *Lb. casei* to neomycin and paromomycin and similar resistance to nalidixic acid. The levels of neomycin and paromomycin in MRS-NNLP were well above the minimal inhibitory concentration of the *Lb. casei* they tested but this did not prevent growth of the NSLAB *Lb. casei* strains present in our aged cheeses.

By counting colonies on M17-L agar by 24 h, the confounding effect of the NSLAB population was removed. It was then observed that the lactococcal numbers in the control full-fat cheese (as counted on M17-L media) decreased from a level of 1×10^7 cfu/g at d 7 to 5×10^2 cfu/g after 180 d of storage even in the presence of a high NSLAB population. In contrast, the lactococci stayed at higher levels in the low-fat cheese (10^5 cfu/g) throughout 180 d of storage (Table 7). The indigenous NSLAB in the low-fat cheese also grew more rapidly and reached 10^6 within 30 d of storage and were $>10^7$ cfu/g after 90 d (Table 7). We have observed similar survival of lactococcal starter cultures in other low-fat

Cheddar cheeses (unpublished data). The reason for this difference has not been determined although it has been assumed to be a function of the lower salt-in-moisture levels present in low-fat cheese compared with full-fat cheese (Table 8).

Differences in microbial populations between replicate batches of low-fat and full-fat cheese were also observed, even though the same level of starter culture was used each time, and the cheese was made in the same set of vats and from pasteurized milk obtained from the same dairy farm. Such differences in cheese biota (especially in indigenous NSLAB populations) within an individual cheese manufacturing facility have been reported previously (Broadbent et al., 2003). These can arise from differences in cleaning and sanitation of cheese vats as well as differences in cheesemaking history of the vats (i.e., what cheeses had been made, and what cultures had previously been used in the vats).

Given the confounding nature of NSLAB growth on media supposedly selective for probiotic adjunct cul-

Table 6. Microbiological analysis of full-fat Cheddar cheese during 180 d of storage at 6°C using presumptive selective media for enumerating various lactic acid bacteria

Time (d)	Media ¹					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
7	1.0×10^7	2.1×10^7	3.1×10^3	1.7×10^2	1.3×10^4	6.0×10^3
30	1.4×10^6	9.0×10^5	2.0×10^4	$<10^2$	8.2×10^2	1.3×10^4
60	5.9×10^5	7.3×10^5	5.9×10^5	$<10^2$	6.3×10^3	6.4×10^5
90	1.4×10^4	1.7×10^6	6.0×10^6	2.6×10^5	1.7×10^6	3.0×10^6
120	1.4×10^4	1.3×10^7	1.3×10^7	4.0×10^6	4.8×10^6	1.5×10^7
180	5.0×10^2	3.9×10^7	4.2×10^7	8.0×10^6	4.5×10^7	4.1×10^7

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycin sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

Table 7. Microbiological analysis of low-fat Cheddar cheese during 180 d of storage at 6°C using presumptive selective media² for enumerating various lactic acid bacteria

Time (d)	Media ¹					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
7	4.4×10^6	4.1×10^6	2.3×10^3	5.0×10^2	$<10^2$	1.0×10^2
30	5.7×10^5	2.7×10^6	2.4×10^6	7.0×10^5	1.0×10^6	2.4×10^6
60	6.5×10^4	1.1×10^7	4.0×10^6	5.6×10^5	4.0×10^6	5.0×10^6
90	2.7×10^5	7.1×10^7	7.4×10^7	1.2×10^6	2.5×10^3	6.7×10^7
120	1.9×10^5	5.7×10^7	5.4×10^7	4.9×10^5	2.1×10^7	5.6×10^7
180	2.9×10^5	1.0×10^8	8.5×10^7	4.5×10^7	1.2×10^8	1.1×10^8

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

tures, it is advantageous to compare bacterial counts between media selective for *Lb. casei* that are used to enumerate NSLAB (MRS-V and RCA-BV), and media used to enumerate bifidobacteria (MRS-NNLP) or *Lb. acidophilus* (MRS-S). The time point at which no clear differentiation could be made between bacterial counts on the media was termed the crossover point. Illustrative of this concept is a comparison of bacterial counts on selective enumeration media for full-fat cheese with a bifidobacteria adjunct culture (Table 9). Up to 120 d, the bacterial counts on the MRS-NNLP media were at least 1 log higher than counts on MRS-V (or RCA-BV) medium, but at 180 d the counts were almost the same for both media. In low-fat cheese supplemented with the same *Bifidobacteria* adjunct culture, the time that MRS-NNLP counts became obscured by NSLAB occurred earlier, at 90 d (Table 10).

The same phenomenon occurred in full-fat cheese supplemented with *Lb. acidophilus* adjunct culture, with MRS-S counts being much higher than counts on MRS-V or RCA-BV media until d 180. A composite table of crossover times for the probiotic culture adjuncts by cheese type shows a general trend that as cheese was made with less fat, the ability to confirm the presence of the adjunct culture in the cheese using selective media decreased with time (Table 11).

In cheeses made with *Lb. casei/paracasei* adjunct cultures, the bacterial counts on MRS-V and RCA-BV media remained much higher than on any other selective media (Table 12). As shown in Table 4, the

particular adjunct culture also grew unattenuated on both MRS-NNLP and MRS-S media. Initial total LAB counts of this cheese were almost 10^8 cfu/g on MRS-V and RCA-BV media, with perhaps less than a half log decrease on the MRS-NNLP and MRS-S media. Thus, the adjunct *Lb. casei/paracasei* culture was the initial dominant LAB species in the cheese (lactococcal counts on the M17-L media were only 10^6 cfu/g). During storage of the cheese, total LAB and MRS-V and RCA-BV counts remained constant at 10^8 cfu/g, implying that *Lb. casei/paracasei* were still present at high numbers. However, counts on MRS-NNLP medium decreased consistently to 10^3 cfu/g over 180 d, whereas counts on MRS-S medium decreased to low numbers over 60 d and then increased to 10^7 cfu/g by 180 d. This suggests that the *Lb. casei/paracasei* adjunct culture had probably died off during storage and was replaced by a growing NSLAB population that did not have the ability to grow on MRS-NNLP but could grow on MRS-S.

DISCUSSION

The obfuscation of adjunct probiotic cultures as NSLAB populations grew to high numbers during cheese storage makes it difficult to confirm the survival of probiotic bacteria in cheese during the long aging process (6 mo to 2 yr) used for Cheddar cheese, or even during the 6-mo retail shelf life commonly placed on cheese after cutting and packaging. Cheese has been reported to be a good vehicle for probiotic bacterial de-

Table 8. Average cheese composition (range in parentheses)

Cheese	Moisture (%)	Fat (%)	pH	Salt (%)	S/W ¹ (%)
Full fat	38.8 (36 to 41)	31.5 (31 to 33)	4.99 (4.8 to 5.3)	1.22 (1.0 to 1.5)	3.17 (2.3 to 4.0)
Low fat	50.5 (49 to 52)	7.5 (7 to 8)	5.35 (5.1 to 5.5)	2.04 (1.7 to 2.3)	4.03 (3.4 to 4.6)

¹Salt divided by moisture content expressed as a percentage.

Table 9. Microbiological analysis of full-fat Cheddar cheese made with added *Bifidobacteria* adjunct culture Bif-6 (see Table 1) during 180 d of storage at 6°C using presumptive selective media for enumerating various lactic acid bacteria

Time (d)	Media ¹					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
7	6.7×10^7	4.8×10^8	4.4×10^5	3.3×10^8	3.7×10^5	5.7×10^5
30	4.2×10^6	3.1×10^8	9.6×10^5	1.9×10^8	2.9×10^5	8.6×10^5
60	2.8×10^6	3.1×10^8	3.2×10^6	1.6×10^8	5.5×10^3	3.7×10^6
90	3.2×10^5	5.2×10^8	1.2×10^7	7.6×10^7	1.7×10^7	1.2×10^7
120	5.0×10^5	4.1×10^8	2.2×10^7	1.9×10^8	1.2×10^7	2.0×10^7
180	3.8×10^4	1.5×10^8	1.5×10^8	6.8×10^8	2.0×10^8	1.3×10^8

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

livery (Ross et al., 2002; Hayes et al., 2006; Ong et al., 2006). *Bifidobacterium bifidum* were reported to persist in Cheddar cheese until 24 wk at levels of 10^6 to 10^7 cfu/g (Dinakar and Mistry, 1994). Gomes and Malcata (1998) reported *Bifidobacterium lactis* at 10^7 cfu/g and *Lb. acidophilus* at 10^6 cfu/g in goat cheese after 70 d of storage. They also noted that probiotic culture growth in the cheese was dependent on the physiochemical properties of the cheese.

Daigle et al. (1999) added *Bifidobacterium infantis* to fermented cream to produce a Cheddar-like cheese and reported bifidobacteria at 10^6 cfu/g until 84 d of storage at 4°C. McBrearty et al. (2001) manufactured Cheddar cheese with 2 commercial strains of bifidobacteria with *Bf. lactis* Bb-12 reported at $>10^8$ cfu/g for 6 mo of storage and *Bifidobacterium longum* BB536 counts were reduced to 10^5 cfu/g. These enumerations presume that all bacteria growing on the selective media were bifidobacteria; from our study of selective media, this may not be an accurate assumption, as some NSLAB strains also grow on these media (see Table 5).

The difficulty, in cheese aging studies, of enumerating probiotic cultures from indigenous NSLAB on selective media has been suggested by other researchers.

Boylston et al. (2004) observed that the simultaneous presence of several species in a cheese matrix (starter cultures and probiotic bacteria) could make it difficult to achieve a differential or selective colony count of each individual species. When different strains of the same species are added to a cheese, traditional colony count techniques lack the resolution necessary to differentiate them. Boylston et al. (2004) suggested that when media are used to perform LAB enumeration of strains that are different from those employed to design the methodology, the results might be difficult to interpret. Recently, Vinderola et al. (2010) reviewed the challenge of enumerating probiotic bacteria and stated that “for each new combination of probiotic culture and starter LAB in a particular food matrix, an adequate screening of selective/elective/differential culture media must be carried out before determining the most appropriate one for testing viability of probiotic bacteria.”

Stanton et al. (1998) also suggested that enumeration of adjunct strains in an aged cheese requires a thorough knowledge of the residual microflora in the product. Our study confirms that probiotic enumeration in aged cheese cannot be made on an a priori basis using media developed to identify specific species or genera. In our

Table 10. Microbiological analysis of low-fat Cheddar cheese made with added *Bifidobacteria* adjunct culture Bif-6 (see Table 1) during 180 d of storage at 6°C using presumptive selective media for enumerating various lactic acid bacteria

Time (d)	Media ¹					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
7	1.4×10^8	2.5×10^4	1.3×10^7	1.6×10^4	2.4×10^4	1.4×10^8
30	3.5×10^7	1.0×10^7	2.1×10^7	6.0×10^6	7.0×10^6	3.5×10^7
60	5.3×10^7	4.1×10^7	2.0×10^7	1.1×10^5	6.2×10^7	5.3×10^7
90	1.8×10^8	1.5×10^8	8.9×10^7	1.5×10^5	1.6×10^8	1.8×10^8
120	1.3×10^8	1.5×10^8	9.0×10^4	4.7×10^7	2.0×10^8	1.3×10^8
180	2.3×10^8	1.9×10^8	8.6×10^7	6.7×10^5	2.1×10^8	2.3×10^8

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

Table 11. Crossover times in full-fat and low-fat Cheddar cheeses when the nonstarter lactic acid bacteria microbial population, enumerated on de Man, Rogosa, and Sharpe (MRS) medium with 1.0 mg/L vancomycin, has equal numbers as the population enumerated on media specific for various types of probiotic bacteria

Probiotic culture ¹	Time (mo)	
	Full-fat cheese	Low-fat cheese
L-10	3	2 to 3
LA-5	6	3
BB-12	3 to 4	3 to 4
Bif-6	4 to 6	2
F-19	6	6
CRL-431	6	6
L-26	6	6

¹See Table 1 for description of cultures.

cheeses, indigenous NSLAB were present that could grow on media presumed selective for bifidobacteria. Comparing growth on the media selected for enumerating the adjunct culture with growth on media used for enumerating *Lb. casei/paracasei* can give an indication of probiotic viability during cheese aging. If the number of NSLAB (as measured using the *Lb. casei/paracasei* MRS-V agar) is much lower than the number of probiotic adjunct cultures (e.g., as enumerated using MRS-NNLP or MRS-S agar), then the data for the probiotic culture enumeration are valid. However, as NSLAB populations increase during cheese aging, a crossover point is reached in which the NSLAB are greater in number than the probiotic adjunct culture. This crossover point (Table 11) limits the ability to determine probiotic culture viability using selective media.

When we made either full-fat or low-fat cheese containing *Lb. casei* or *Lb. paracasei* probiotic adjunct cultures, the counts of these on MRS-V (or RCA-BV) medium remained at least at the initial levels. A modest increase in cell numbers may be expected because these adjunct cultures mimic NSLAB. Other researchers have noted *Lb. casei/paracasei* viability during cheese

aging, but without sophisticated molecular techniques for strain differentiation, the adjunct culture cannot be distinguished from indigenous NSLAB.

Cheddar cheese made with *Lb. paracasei* and *Lb. casei* adjunct culture isolated from human sources was reported to retain a viability level >10⁷ cfu/g (Gardiner et al., 1998), although these researchers recognized that enumeration of *Lb. paracasei* in aged cheese is complicated by presence of NSLAB and that methods other than just a genus- or species-specific medium must be used to properly enumerate adjunct bacteria in cheese. Gardiner et al. (1998) also used the molecular identification technique of DNA fingerprinting by random amplified polymorphic DNA-PCR to confirm that the particular *Lb. paracasei* adjunct culture used during cheesemaking was the predominant bacterial species in the cheese.

Another approach is to produce a mutant strain of the adjunct culture that has antibiotic resistance not naturally possessed by lactobacilli or bifidobacteria. Sharp et al. (2008) followed the survival rate of a probiotic adjunct culture *Lb. casei* 334e (an erythromycin-resistant strain) during 3-mo storage of low-fat Cheddar cheese. They observed that the adjunct culture remained at 10⁷ cfu/g for 3 mo, and in a pH 2 phosphoric acid challenge study, survival rates for this *Lb. casei* culture were 4 logs higher when in low-fat cheese than when in yogurt. Although such molecular techniques have been used in research studies to evaluate LAB isolates from dairy products, most manufacturers still rely on conventional cultivation methods for enumeration. To enable cheese manufacturers to track changes in cheese microflora, any culture medium proposed for differential or selective enumeration should not be complex or time-consuming to prepare, and it should offer a good cell recovery for the microorganisms (Lim et al., 1995). From our study, it is apparent that such a goal is elusive and has the potential for producing erroneous results. Given the inherent antibiotic resistance of *Lb. casei/paracasei* and

Table 12. Microbiological analysis of low-fat Cheddar cheese made with added *Lactobacillus casei* adjunct culture F-19 (see Table 1) during 180 d of storage at 6°C using presumptive selective media for enumerating various lactic acid bacteria

Time (d)	Media ¹					
	M17-L	MRS+S	MRS-V	MRS-NNLP	MRS-S	RCA-BV
7	3.4 × 10 ⁶	9.5 × 10 ⁷	8.4 × 10 ⁷	4.5 × 10 ⁷	6.8 × 10 ⁷	9.4 × 10 ⁷
30	6.0 × 10 ⁶	1.5 × 10 ⁸	1.2 × 10 ⁸	7.1 × 10 ⁷	6.0 × 10 ⁴	1.5 × 10 ⁸
60	3.3 × 10 ⁵	1.2 × 10 ⁸	1.4 × 10 ⁸	1.1 × 10 ⁴	<10 ²	1.1 × 10 ⁸
90	3.5 × 10 ⁶	1.9 × 10 ⁸	1.5 × 10 ⁸	1.5 × 10 ³	2.4 × 10 ⁷	1.8 × 10 ⁸
120	5.0 × 10 ⁴	1.4 × 10 ⁸	1.3 × 10 ⁸	1.3 × 10 ⁴	8.0 × 10 ⁴	1.3 × 10 ⁸
180	4.6 × 10 ³	1.3 × 10 ⁸	1.5 × 10 ⁸	3.5 × 10 ³	3.7 × 10 ⁷	1.2 × 10 ⁸

¹M17-L = M17-lactose; MRS+S = de Man, Rogosa, and Sharpe (MRS) medium with 10 g/L sorbitol added; MRS-V = MRS medium with 1.0 mg/L vancomycin; MRS-NNLP = MRS medium with added cysteine and antibiotic mixture consisting of 150 mg/L nalidixic acid, 100 mg/L neomycin sulfate, 3.0 g/L lithium chloride, and 200 mg/L paromomycine sulfate; MRS-S = MRS medium with 10 g/L sorbitol substituted for dextrose; RCA-BV = reinforced clostridial agar, pH 5.5, containing 40 mg/L bromocresol green and 10 mg/L vancomycin.

the multitude of NSLAB strains that can be present in cheese, it is unlikely that a medium using antibiotics for selectivity will provide enumeration of probiotic adjunct cultures without interference from the NSLAB population that grows during cheese aging.

Several possible explanations exist for the difficulties in obtaining accurate and confirmable counts of probiotic adjunct cultures during cheese aging. The environment of cheese is much different from that of laboratory media, which can influence survival rates of starter cultures and culture adjuncts. This may result in metabolically injured cells whose resuscitation may be variable during sample preparation and enumeration. Incubation time and temperature, along with antibiotic concentrations, are important when trying to suppress various NSLAB populations in cheese. Other reasons for media failure when enumerating multiple strains of different LAB species may include the lack of recovery of one or more species, the lack of differentiation among colonies, antagonism between species (particularly the production of inhibitory compounds), and inherent problems with the pour-plate methodology in which elevated or sudden changes in temperature can kill or injure bacterial cells.

Medium containing trans-galactosylated oligosaccharides to promote the growth of bifidobacteria with propionic acid, lithium chloride, and mupirocin to inhibit growth of other LAB has been tested for enumeration of bifidobacteria in yogurt (Zitz et al., 2007). Whether this medium (Anonymous, 2010) will be sufficiently inhibitory to selectively enumerate bifidobacteria in cheese containing a high background population of NSLAB lactobacilli has yet to be determined.

CONCLUSIONS

Confounding the enumeration of starter LAB cultures or adjunct cultures is the proliferation of NSLAB during cheese storage. Although not added during the initial stages of cheese-making, these advantageous bacteria eventually become the predominant flora in aged cheese. Their growth on selective media designed to enumerative specific bacterial genera and species makes it difficult to interpret data without sophisticated and time-consuming molecular techniques that can provide the exact identity of individual bacterial isolates. We have demonstrated that the screening of starter cultures, adjunct cultures, and especially NSLAB strains on selective media before cheese-making trials is essential to avoid enumeration errors. Many lactobacilli, especially NSLAB, can grow on media that have previously been presumed to be exclusionary. Using a combination of media and comparing growth on media used for enumerating *Lb. casei* to media selective for

Bifidobacterium spp. and *Lb. acidophilus* during cheese aging can provide an indication of probiotic adjunct survival versus NSLAB growth.

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