



## Optimal growth of *Lactobacillus casei* in a Cheddar cheese ripening model system requires exogenous fatty acids<sup>1</sup>

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### ABSTRACT

Flavor development in ripening Cheddar cheese depends on complex microbial and biochemical processes that are difficult to study in natural cheese. Thus, our group has developed Cheddar cheese extract (CCE) as a model system to study these processes. In previous work, we found that CCE supported growth of *Lactobacillus casei*, one of the most prominent nonstarter lactic acid bacteria (NSLAB) species found in ripening Cheddar cheese, to a final cell density of  $10^8$  cfu/mL at 37°C. However, when similar growth experiments were performed at 8°C in CCE derived from 4-mo-old cheese (4mCCE), the final cell densities obtained were only about  $10^6$  cfu/mL, which is at the lower end of the range of the NSLAB population expected in ripening Cheddar cheese. Here, we report that addition of Tween 80 to CCE resulted in a significant increase in the final cell density of *L. casei* during growth at 8°C and produced concomitant changes in cytoplasmic membrane fatty acid (CMFA) composition. Although the effect was not as dramatic, addition of milk fat or a monoacylglycerol (MAG) mixture based on the MAG profile of milk fat to 4mCCE also led to an increased final cell density of *L. casei* in CCE at 8°C and changes in CMFA composition. These observations suggest that optimal growth of *L. casei* in CCE at low temperature requires supplementation with a source of fatty acids (FA). We hypothesize that *L. casei* incorporates environmental FA into its CMFA, thereby reducing its energy requirement for growth. The exogenous FA may then be modified or supplemented with FA from de novo synthesis to arrive at a CMFA composition that yields the functionality (i.e., viscosity) required for growth in specific conditions. Additional studies utilizing the CCE model to investigate microbial contribu-

tions to cheese ripening should be conducted in CCE supplemented with 1% milk fat.

**Key words:** nonstarter lactic acid bacteria, *Lactobacillus casei*, Cheddar cheese model system, cytoplasmic membrane fatty acid

### INTRODUCTION

Lactic acid bacteria (LAB) present in ripening cheese include deliberately added starter LAB (SLAB) and adjunct cultures, as well as a large adventitious microbiota referred to as nonstarter LAB (NSLAB). Whereas SLAB and adjunct cultures are intentionally added to cheese milk, adventitious NSLAB enter cheese through the cheese milk or via contamination from the manufacturing environment. The NSLAB typically dominate the cheese microbiota after the first few weeks of ripening, increasing in number from 10 to  $10^4$  cfu/g to about  $10^6$  to  $10^8$  cfu/g within 2 to 3 mo of ripening (Fox et al., 1998). Hence, unlike SLAB and adjuncts, the types and numbers of “wild” NSLAB cannot be easily controlled. Substantial heterogeneity exists within the NSLAB microbiota, at both the species and strain level, between different processing facilities and even between different manufacturing days within the same processing facility (Jordan and Cogan, 1993). Additionally, the NSLAB microbiota, through their metabolic end products and enzymes, are known to significantly contribute to Cheddar cheese flavor development, including flavor defects in Cheddar cheese. Therefore, this uncontrolled and variable microbiota can result in inconsistent cheese quality (Fox et al., 1998; Broadbent et al., 2003).

Flavor development in ripening Cheddar cheese is a complex microbial and biochemical process that is difficult to study in natural cheese. Therefore, the development of a model system that mimics the natural environment of cheese is required to analyze strain-specific contributions of SLAB and NSLAB to Cheddar cheese flavor development, as well as the effects of cheese composition and ripening regimen. In previous studies (Diaz-Muniz et al., 2006; Budinich et al., 2011), our laboratory developed a model system for cheese

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ripening based on Cheddar cheese extract (**CCE**), the aqueous fraction of cheese. Use of this model is founded on the assumption that the microorganisms that contribute to Cheddar cheese ripening utilize the water-soluble components present in the liquid phase of the cheese to meet their physiological needs. These studies evaluated growth of *Lactobacillus casei*, one of the most prominent NSLAB species (Mayra-Makinen and Bigret, 1998), in CCE at 37°C, rather than at temperatures used for cheese ripening. However, the mesophilic bacteria that dominate the Cheddar cheese microbiota during ripening must be capable of growth at the lower ripening temperatures. The objective of this study was to evaluate the utility of this model system at 8°C, a temperature typical of ripening Cheddar cheese.

## MATERIALS AND METHODS

### Bacterial Strains

The 4 *L. casei* strains used in this study were all isolated from ripening cheese. *Lactobacillus casei* ATCC 334 was obtained from the American Type Culture Collection (Rockville, MD), *L. casei* M36 (Boucher et al., 2006) and UW-4 were obtained from the Steele culture collection at the Department of Food Science of University of Wisconsin-Madison, and *L. casei* 7R1 was obtained from Department of Food Science of Royal Veterinary and Agriculture University (Frederiksberg C, Denmark). Stock cultures were maintained at -80°C in de Man, Rogosa, and Sharpe (**MRS**) broth medium (Difco Laboratories, Detroit, MI) with 25% (vol/vol) glycerol. Working cultures were prepared from frozen stocks by consecutive transfers in MRS broth supplemented with 2% (wt/vol) galactose (Sigma-Aldrich Co., St. Louis, MO) and CCE with 2% (wt/vol) galactose broth. Incubations were conducted statically at 37°C for 24 and 16 to 18 h, respectively.

### CCE and Growth Conditions

Preparation of 4-mo-old CCE (**4mCCE**) was conducted essentially as described previously (Díaz-Muñiz and Steele, 2006; Budinich et al., 2011) using grade A quality, 4-mo-old aged Cheddar cheese with low lactose, galactose, and moisture contents (Kiel Cheese LLC, St. Paul, MN). Freeze-dried 4mCCE was stored at 4°C, reconstituted in distilled H<sub>2</sub>O (95g/L) when needed, and stirred for 15 to 20 min. The salt concentration of the 4mCCE was determined to be 3.1% using chloride QuanTab test strips (Hach, Loveland, CO). The citrate concentration of 4mCCE was determined to be 0.2 mM using the Citric Acid test kit (R-Biopharm Inc., Marshall, MI). If required, the pH of the 4mCCE was

adjusted to  $5.2 \pm 0.1$  with 1 N HCl. To achieve sterility, 4mCCE was filter-sterilized by using 0.2- $\mu$ m polyether-sulfone membrane filter units (Nalgene, Rochester, NY) and stored at 4°C for not more than 24 h before use.

*Lactobacillus casei* growth studies were conducted in sterile 2-mL oxygen-free, crimped-top vials (National Scientific, Rockwood, TN). Bacterial cells from working cultures were washed twice using a 0.9% NaCl solution containing 0.025% of 20% Triton-X-100, a nonionic wetting agent that decreases the surface tension between cells (Mallmann and Broitman, 1956; Sigma-Aldrich Co.), at  $3,000 \times g$  for 8 min at 25°C and suspended in 1 mL of saline. Optical densities of the suspended cells were measured using SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA) and adjusted to 1.0; the cultures were subsequently diluted 100 times to achieve approximately  $10^4$  to  $10^5$  cfu/mL of washed culture. About 10% (vol/vol) of culture was inoculated into filter-sterilized 4mCCE, and then 1-mL aliquots were placed into each of the crimped-top vials. Additionally, uninoculated 4mCCE controls were prepared and confirmed to be sterile at the end of all growth experiments. A vial was removed from the 8°C water bath every week or the 37°C incubator every 3 to 6 h, diluted, and enumerated via the drop plate method on MRS agar (Mallmann and Broitman, 1956). Drop plating was conducted in triplicate using 20  $\mu$ L per drop, and plates were incubated aerobically at 37°C for 48 h before enumeration.

When needed, various supplements were added to 4mCCE. Addition of protease peptone no.3 (10 g/L; Becton Dickinson and Co., Sparks, MD), yeast extract (5 g/L; Becton Dickinson and Co.), Tween 80 (1 mL/L), D-galactose (20 g/L), glycerol (1 mL/L), sodium acetate (trihydrate; 5 g/L), or sodium citrate (dehydrate; 2 g/L; all from Sigma-Aldrich Co.) was accomplished by dissolving the supplement in distilled H<sub>2</sub>O, sterilizing at 121°C for 15 min, adding 4mCCE (95 g/L), and finally filter-sterilizing. Addition of pyridoxal HCl (5 mg/L), pyridoxamine (5 mg/L), RPMI-1640 vitamin mixture (20 mL/L), pre-sterilized stock solution of DL-mevalonic acid lactone (10  $\mu$ L/L; Sigma-Aldrich Co.), or a milk-based monoacylglycerol (**MAG**) mixture was accomplished by adding these components directly into the reconstituted 4mCCE, followed by filter-sterilization. Addition of milk fat was done directly into the vials.

### Milk Fat Preparation

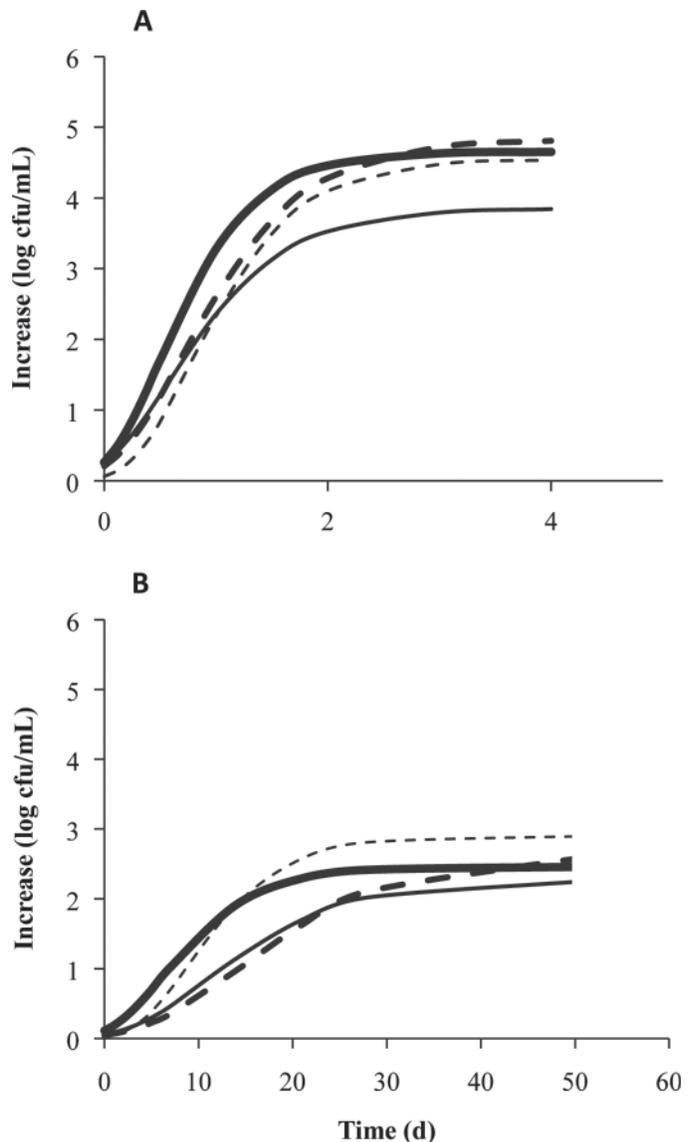
Milk fat was prepared from cream (Babcock Dairy, Department of Food Science, University of Wisconsin-Madison) using a procedure based upon Patton and Huston's (1986) method, modified by the incorpora-

tion of additional washes. Cream was washed at room temperature with sterile PBS using a cream separator to remove any remaining milk. The cream was churned and washed twice with sterile PBS at 37°C for 15 min to separate the fat. The fat layer was separated and washed twice consecutively with distilled water and PBS at 4°C. The solid milk fat was steamed twice for 20 min with a 2-h incubation at 37°C between treatments, and stored at -20°C until used.

### Milk Fat Analysis

Separation of the 5 lipid classes [triacylglycerol (TAG), FFA, diacylglycerol (DAG), phospholipids (PL), and MAG] present in a milk fat isolate was conducted using a series of steps that began with lipid extraction. To start, 100 mg of milk fat was mixed with 100  $\mu$ L of surrogate standards for each lipid class. Each surrogate standard was composed of a unique FA not typically found in milk. The lipids were then extracted using the Folch method (Folch et al., 1957), and the lower lipid-containing phase was collected. Next, the lipid classes were separated by thin layer chromatography. The silica plate was placed in 100°C oven for 10 min to activate the silica. The developing solvent was an 80:20:2 solution of hexane, diethyl ether, and formic acid, respectively. The bands were visualized by exposing the plate to iodine vapor and identified according to their reported relative mobility in previous studies (Christie, 2000). Additionally, identities of bands were confirmed by the presence of the unique FA derived from the surrogate standard. Silica was scraped and the recovered individual classes of lipids from the thin-layer chromatography were analyzed as methyl esters by mixing scraped silica from TLC plate with 0.8 mL of hexane and 1.2 mL of 10% acetyl chloride in methanol. The mixture was incubated at 100°C for 40 min; then, the solution was cooled to room temperature and 2 mL of 6% sodium carbonate solution and 0.4 mL of hexane were added. Fatty acid methyl esters (FAME) were extracted with 100  $\mu$ L of hexane and transferred to a GC vial. Two GC methods were employed using a Shimadzu GC (model QP2010, Shimadzu, Columbia, MD) with flame-ionization detector: one method for the TAG lipid class and another for the other lipid classes. The TAG method used a split injection with a ratio of 1:50 with an injection temperature of 250°C. The temperature program was as follows: 35°C for 2 min, heated to 175°C at a rate of 40°C/min, holding for 4 min, heated to 250°C at a rate of 3.5°C/min, and holding for 10 min. For the other lipids classes, the injection temperature was 250°C with a split ratio of 1:10. The carrier gas was helium with a linear velocity of 56.4 cm/s. The temperature program was as follows:

50°C for 0.29 min, heated to 180°C at a rate of 82°C/min, holding for 4 min, heated to 220°C at a rate of 13.8°C/min, heated to 250°C at a rate of 35.54°C/min, and holding for 2 min. The peaks were identified using an authentic methyl FA standard (Nu-Chek Prep Inc., Elysian, MN). The amount of each FFA in each class was determined by dividing the area of each FFA by the lipid class internal standard and multiplying that by the concentration of the internal standard.



**Figure 1.** Effect of temperatures, (A) 37°C and (B) 8°C, on growth of *Lactobacillus casei* strains in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE). Initial numbers ranged from approximately  $3 \times 10^3$  to  $1 \times 10^4$  cfu/mL. Best fit lines for cell density during growth in Cheddar cheese extracts at 37 and 8°C of *L. casei* ATCC 334 (thin black line), M36 (thick black line), UW4 (short-dashed line), and 7R1 (long-dashed line) were obtained using the Gompertz growth model.

### Preparation of MAG Stock Solution

The MAG stock solution (19.2 mg/mL) was prepared by dissolving MAG salts [1-monolaurin (C<sub>12:0</sub>; 0.75 mg/mL), monomyristine (C<sub>14:0</sub>; 2.67 mg/mL),  $\alpha$ -monopalmitin (C<sub>16:0</sub>; 9.26 mg/mL), monostearin (C<sub>18:0</sub>; 3.45 mg/mL), and monoolein (C<sub>18:1n9c</sub>, where c = *cis*; 3.12 mg/mL)] in dimethylsulfoxide (Sigma-Aldrich Co.) at the same concentration (density of 0.88 g/mL) as DAG and MAG were measured in the milk fat sample (Supplemental Table S1, available online at <http://www.journalofdairyscience.org/>). Dilutions of stock solutions were prepared using dimethylsulfoxide as the diluent.

### Cytoplasmic Membrane Fatty Acid Analysis

Bacterial FA were analyzed as methyl esters according to the method of O'Fallon et al. (2007). Bacterial pellets were resuspended in 530  $\mu$ L of methanol and transferred to a 1.5-mL amber glass vial; 70  $\mu$ L of 10 N KOH was added. Samples were vortexed for 30 s and incubated in a shaking water bath at 150 rpm at 55°C for 1.5 h. After incubation, samples were cooled and 58  $\mu$ L of 24 N H<sub>2</sub>SO<sub>4</sub> was added, and the samples were incubated for another 1.5 h in a shaking water bath at 150 rpm at 55°C. The FAME were extracted with hexane (300  $\mu$ L) and transferred into a 2-mL GC vial. The FAME were analyzed by GC with a flame-ionization detector (model QP2010, Shimadzu Co.) using a standard method for FAME (O'Fallon et al., 2007). Samples containing methyl esters in hexane (1  $\mu$ L) were injected onto an HP-88 fused silica 100 m  $\times$  0.25 mm column, with 0.20- $\mu$ m film thickness (Agilent Technologies, Palo Alto, CA). The injection port was maintained at 250°C in the split mode, and the sample was split at a 10:1 ratio with a 3.0 mL/min purge flow.

Hydrogen was used as the carrier gas at a linear velocity of 41.1 cm/s. The temperature program was as follows: initial temperature 35°C and hold for 2 min, increase at 40°C/min to 175°C and hold for 4 min, increase at 3.5°C/min to 240°C and hold for 25 min. The detector was operated at 250°C and makeup gas was nitrogen 30 mL/min. Air and hydrogen flow to the detector was 450 and 40 mL/min, respectively. Total run time was 53.07 min/sample. Peaks were identified according to retention time utilizing several standard FAME mixes (Nu-Chek Prep Inc.). Raw peak areas were converted to concentrations using response factors generated with the standard FAME mixtures.

### Statistical Analysis

Growth studies were conducted in triplicate. Bacterial numbers were calculated as the mean values from triplicate samples. Growth parameters (maximum cell density, growth rate, and lag phase) and curve model fitting were obtained with Gompertz growth model (Zwietering et al., 1990) and derived using Tablecurve 2D, version 5.02 (Systat Software Inc., San Jose, CA) as described by Budinich et al. (2011). Statistical analysis of the final cell densities (cfu/mL) was done using Student's *t*-test, ANOVA, and Tukey's multiple comparison tests (Tukey, 1949). Results are reported as significantly different when the *P*-value was <0.05.

## RESULTS AND DISCUSSION

### Effect of Temperature on Growth of *L. casei* Strains in 4mCCE

Ripening Cheddar cheese is a complex and dynamic environment, which makes it difficult to determine the strain-specific contributions of SLAB and NSLAB to

**Table 1.** Growth parameters (means and standards deviations in parentheses) of *Lactobacillus casei* strains incubated at 8 and 37°C for 49 and 4 d, respectively, in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE)

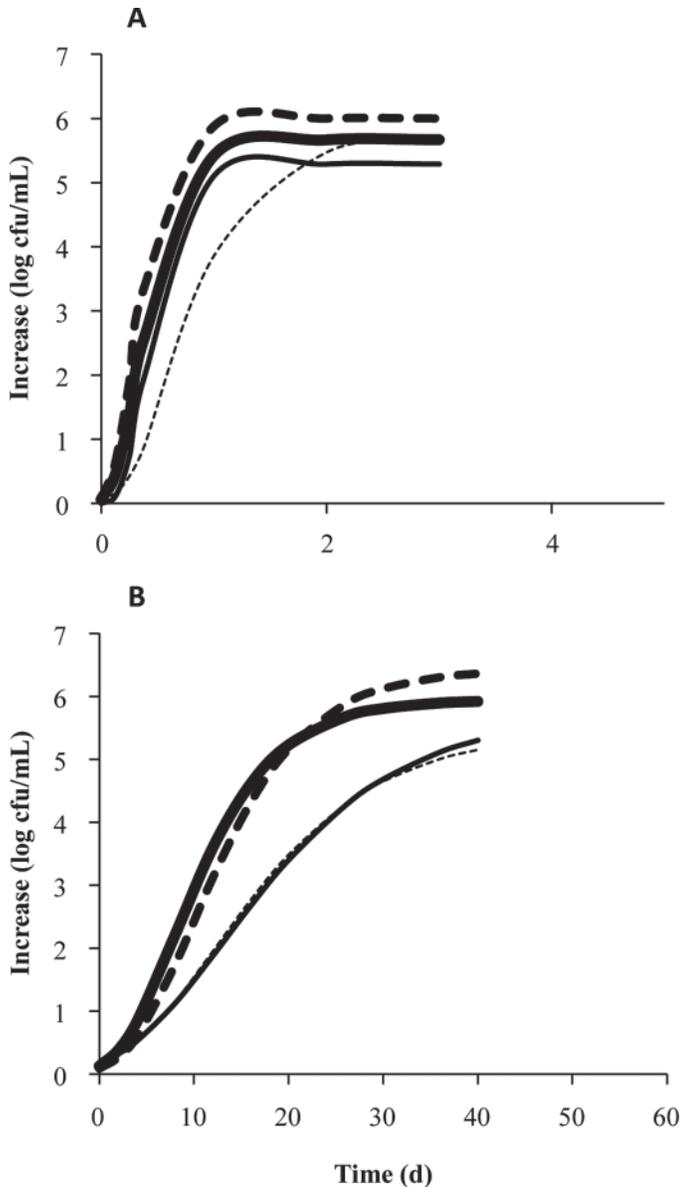
Growth parameter <sup>2</sup>	<i>Lactobacillus casei</i> strain <sup>1</sup>							
	ATCC 334		M36		UW4		7R1	
	8°C	37°C	8°C	37°C	8°C	37°C	8°C	37°C
Lag phase (d)	62.2 (26.3) <sup>a</sup>	0.0 (4.51) <sup>b</sup>	16.8 (13.3) <sup>a</sup>	0.6 (1.7) <sup>b</sup>	80.0 (27.1) <sup>a</sup>	5.9 (2.4) <sup>b</sup>	100.0 (36.2) <sup>a</sup>	2.2 (2.2) <sup>b</sup>
Growth rate <sup>3</sup> (log cfu/mL per day)	0.0 (0.0) <sup>a</sup>	0.1 (0.0) <sup>b</sup>	0.0 (0.0) <sup>a</sup>	0.2 (0.0) <sup>b</sup>	0.0 (0.0) <sup>a</sup>	0.1 (0.0) <sup>b</sup>	0.0 (0.0) <sup>a</sup>	0.1 (0.0) <sup>b</sup>
Maximum cell density increase (log cfu/mL)	2.3 (0.1) <sup>a</sup>	3.9 (0.2) <sup>b</sup>	2.5 (0.0) <sup>a</sup>	4.7 (0.1) <sup>b</sup>	2.9 (0.1) <sup>a</sup>	4.6 (0.2) <sup>b</sup>	2.7 (0.2) <sup>a</sup>	4.8 (0.2) <sup>b</sup>

<sup>a,b</sup>Means in each row within each strain with different superscripts differ (*P* < 0.05).

<sup>1</sup>Initial numbers ranged from approximately  $3 \times 10^3$  to  $1 \times 10^4$  cfu/mL.

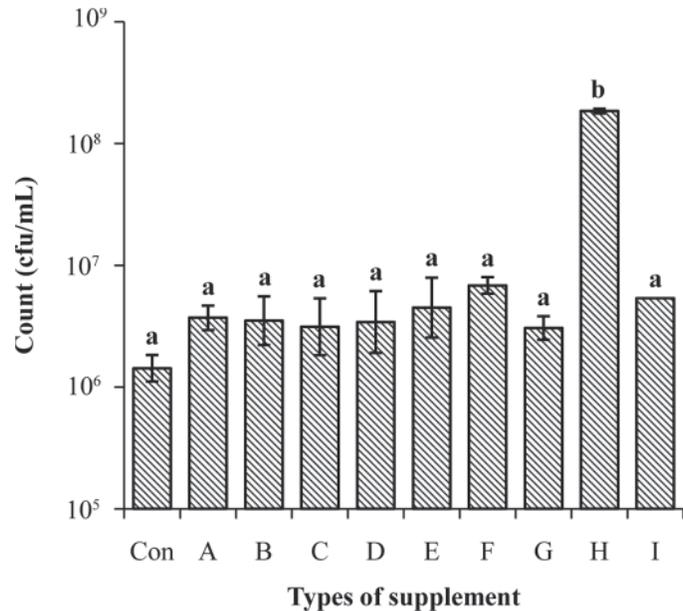
<sup>2</sup>Growth parameters were obtained using Gompertz growth model.

<sup>3</sup>A value of 0.0 indicates that growth rate was <0.1 log cfu/mL per day.



**Figure 2.** Effect of temperatures, 37°C (A) and 8°C (B), on the growth of *Lactobacillus casei* strains in de Man, Rogosa, and Sharpe media. Initial numbers ranged from approximately  $3 \times 10^3$  to  $1 \times 10^4$  cfu/mL. Best fit lines for cell density during growth in Cheddar cheese extracts at 37 and 8°C of *L. casei* ATCC 334 (thin black line), M36 (thick black line), UW4 (short-dashed line), and 7R1 (long-dashed line) were obtained using the Gompertz growth model.

Cheddar cheese flavor development, as well as the effects of cheese composition and ripening regimen on Cheddar cheese microbiota and flavor development. To address these challenges, we developed the CCE model system and demonstrated that it supported growth of *L. casei* ATCC 334 to final cell densities of  $10^8$  to  $10^9$  cfu/mL at 37°C (Díaz-Muñiz et al., 2006). In this study, we compared growth of several *L. casei* strains in 4mCCE at 37°C for 4 d and 8°C for 49 d (Figure



**Figure 3.** Effect of supplementation on the final cell density attained by *Lactobacillus casei* M36 in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE) at 8°C. Initial numbers were approximately  $1 \times 10^4$  cfu/mL and incubation was conducted for 7 wk. Error bars indicate standard deviation of data obtained in triplicate. Statistical analyses between treatments were conducted with Tukey's multiple comparison test. Treatments with different letters are statistically different from each other ( $P < 0.05$ ). Control (Con) = no supplement; A = D-galactose; B = mixture of glycerol and DL-mevalonic acid lactone; C = protease peptone no. 3; D = mixture of pyridoxal HCl and pyridoxamine; E = RPMI-1640 vitamin mixture; F = mixture of sodium acetate and sodium citrate; G = yeast extract; H = Tween 80; I = 1% milk fat.

1; Table 1). The growth parameters lag phase, growth rate ( $\mu_{max}$ ), and final cell density were obtained using the Gompertz growth model (Zwietering et al., 1990). Statistical analysis of growth parameters for each strain at 8 and 37°C were conducted using paired Student *t*-test. All growth parameters of all 4 strains examined at 8 and 37°C were statistically different ( $P < 0.05$ ). These results demonstrate that temperature had a significant effect on the final cell densities attained in 4mCCE.

The final cell densities attained at 8°C (an average of  $4.3 \times 10^6$  cfu/mL) were at the lower end of the range of the NSLAB population expected in ripening Cheddar cheese, which typically reach  $10^6$  to  $10^8$  cfu/g within several weeks of ripening (Fox et al., 1998). This level is equivalent to approximately  $2 \times 10^6$  to  $2 \times 10^8$  cfu/mL in 4mCCE, accounting for the fact that Cheddar cheese contains 37% moisture. As expected, the length of the lag phase was significantly ( $P < 0.05$ ) longer and growth rates significantly ( $P < 0.05$ ) lower in 4mCCE at 8 versus 37°C (Table 1). However, when similar growth studies were conducted in MRS at 8 and 37°C, no significant difference in the final cell densities was

observed (Figure 2). These results suggest that growth of *L. casei* strains in 4mCCE at 8°C was limited by the absence of one or more essential nutrients and that this nutrient was lost during CCE preparation.

### Effect of Various Supplements on Growth of *L. casei* M36 in 4mCCE

To identify the limiting nutrient for growth of *L. casei* in 4mCCE at 8°C, growth studies were conducted at 8°C in 4mCCE containing a variety of supplements known to influence the growth of lactobacilli. *Lactobacillus casei* M36 was selected as the test organism based upon its growth characteristics in 4mCCE (Figure 1B). Supplements were selected from components of MRS (de Man et al., 1960) or the lactobacilli chemically defined media described by Christensen and Steele (2003). The supplements evaluated were protease peptone no. 3, yeast extract, D-galactose, Tween 80, glycerol, sodium acetate (trihydrate), sodium citrate, pyridoxal HCl, pyridoxamine, RPMI-1640 vitamin mixture, and DL-mevalonic acid lactone. The effect of these nutrients on growth of *L. casei* M36 at 8°C in 4mCCE is presented in Figure 3. Interestingly, addition of galactose to 4mCCE did not significantly increase the final cell density of *L. casei* M36, suggesting that energy is not limiting for growth in 4mCCE at 8°C. This result is in agreement with the observation by Budinich et al. (2011) that CCE lacking detectable levels of lactose and galactose was able to support the growth of *L. casei* ATCC 334 to final cell densities of  $>1 \times 10^8$  cfu/mL. As shown in Figure 3, the only supplement that significantly ( $P < 0.05$ ) enhanced growth of *L. casei* M36 in 4mCCE at 8°C was Tween 80. Tween 80 is a mixture of FA esters of polyoxyethelene sorbitan that contain approximately 20% FA by mass. Supplementation with Tween 80 produced a 100-fold increase final cell density compared with the control, and more than 50-fold increase in final cell density compared with the other supplements examined. The finding that Tween 80 is required for *L. casei* M36 to attain a final cell density in 4mCCE at 8°C typical of NSLAB populations in cheese suggests that exogenous FA are important for growth of *L. casei* in ripening cheese.

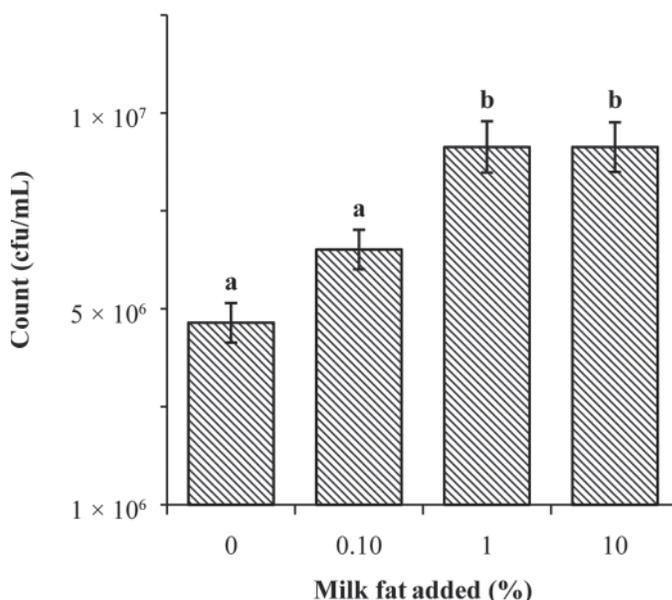
### Effect of Milk Fat and MAG Supplementation on Growth of *L. casei* Strains in 4mCCE

The most likely source of FA to support growth of lactobacilli in ripening cheese is milk fat; therefore we explored whether addition of 0.1, 1, or 10% milk fat (vol/vol) to 4mCCE would enhance growth of M36 at 8°C. Results confirmed that addition of 1 or 10% milk fat produced a significant ( $P < 0.05$ ) increase in the

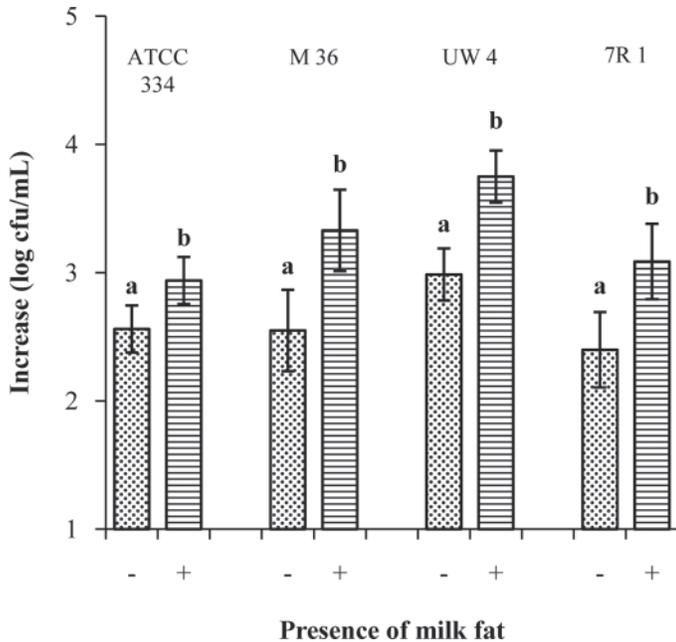
final cell densities of *L. casei* M36 in 4mCCE at 8°C, whereas no statistical change ( $P > 0.05$ ) was observed with 0.1% milk fat (Figure 4). These observations indicated that CCE supplementation with 1% milk fat allowed *L. casei* M36 to reach the final cell densities expected in ripening Cheddar cheese.

To determine if this response was strain-specific, growth studies were performed at 8°C in 4mCCE with 1% milk fat with 3 additional *L. casei* strains. As shown in Figure 5, milk fat addition significantly ( $P < 0.05$ ) enhanced the final cell density in 4mCCE of all strains tested by 5- to 10-fold over that observed in unsupplemented 4mCCE. The largest increase was observed with *L. casei* M36 (10-fold), and the smallest increase was observed with *L. casei* ATCC 334 (5-fold). These results support the hypothesis that supplementation of 4mCCE with milk fat provides a source of exogenous FA that allows *L. casei* strains to reach the final cell densities expected in ripening Cheddar cheese.

The genome sequence of *L. casei* ATCC 334 contains 4 genes encoding esterases but no gene encoding a lipase (Makarova et al., 2006; Cai et al., 2009); therefore, MAG, DAG, and FFA are the most likely components of milk fat used by *L. casei* as sources of exogenous FA. To test this hypothesis, growth studies were conducted in CCE supplemented with a MAG solution that con-



**Figure 4.** Effect of different percentage of milk fat addition on the final cell density of *Lactobacillus casei* M36 in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE) at 8°C. Initial numbers were approximately  $1 \times 10^4$  cfu/mL and incubation was conducted for 7 wk. Error bars indicate standard deviation of data obtained in triplicate. Statistical analysis between treatments was conducted with Tukey's multiple comparison test. Treatments with different letters are statistically different from each other ( $P < 0.05$ ).

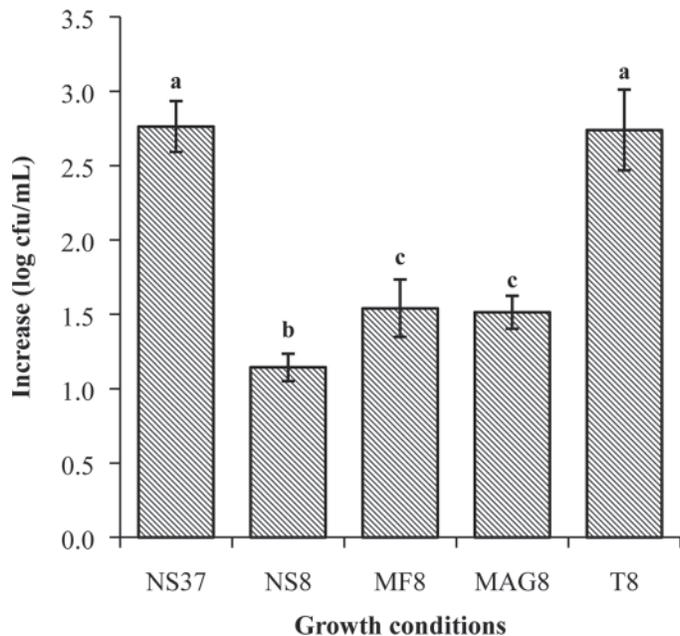


**Figure 5.** Effect of 1% milk fat addition (+) or not (-) on the final cell density of *Lactobacillus casei* strains in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE) at 8°C. Initial numbers were approximately  $5 \times 10^3$  to  $3 \times 10^4$  cfu/mL and incubation was conducted for 7 wk. Error bars indicate standard deviation of data obtained in triplicate. Statistical analysis between treatments of each strain was conducted with Student *t*-test. Treatments with different letters are statistically different from each other within each strain ( $P < 0.05$ ).

tained the 5 FA present at the highest concentration in the PL/MAG and DAG fractions of the milk fat sample examined (Supplemental Table S1, available online at <http://www.journalofdairyscience.org/>). However, as MAG are known to be bacterial inhibitors (Wang and Johnson, 1992), preliminary growth experiments were conducted with *L. casei* M36 in CCE (due to limitations in the quantity of 4mCCE, a different batch was used in these preliminary experiments) at 37°C with a broad range of MAG concentrations (Supplemental Figure 1, available online at <http://www.journalofdairyscience.org/>). The results indicated that the MAG mixture had no effect on growth at the lowest level examined (0.19 µg/mL), stimulated growth at 1.90 µg/mL, and was inhibitory at the higher levels examined (19.0, 63.0, and 193 µg/mL). Therefore, *L. casei* M36 8°C growth experiments were conducted in 4mCCE supplemented with 1.90 µg/mL of the MAG mixture. The results of these growth experiments (Figure 6) were that the growth enhancement of M36 in 4mCCE with MAG was indistinguishable from that observed with 1% milkfat. These results strongly suggest that the *L. casei* growth-stimulating component of milk fat is MAG, DAG, or FFA and that these FA sources are important nutrients for NSLAB growth in ripening cheese.

### Membrane FA Composition of *L. casei* M36 Under Different Growth Conditions

The cytoplasmic membrane fatty acid (CMFA) composition of a bacterial cell is determined by de novo FA synthesis, incorporation of exogenous FA, and modification of existing CMFA (Zhang and Rock, 2008). To determine if *L. casei* M36 incorporated exogenous FA added as supplements to 4mCCE, we examined the FA composition of the CMFA of M36 grown with different FA containing supplements (Table 2). Oleic acid ( $C_{18:1n9c}$ ) accounted for 90% of the FA present in Tween 80, and *L. casei* is known to contain cyclopropane synthase, which catalyzes the addition of a methylene residue across the *cis* double bond of unsaturated FA such as  $C_{18:1n9c}$  to form cyclopropane fatty acids (CFA), such as dihydrosterulic acid ( $C_{19:0\Delta9c}$ ; Suutari and Laakso, 1992; Broadbent et al., 2010). Oleic and dihydrosterulic acids account for 17% of the total FA present in the CMFA of cells grown in 4mCCE at 8°C without supplementation, whereas these FA account for 62.5% of the FA present in the CMFA of cells grown



**Figure 6.** Effect of different supplementation and growth conditions on the growth of *Lactobacillus casei* M36 in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE). Initial number was approximately  $4 \times 10^4$  cfu/mL. NS37 and NS8 = no supplementation at growth temperature of 37°C and 8°C; MF8 = supplementation with 1% milk fat at growth temperature of 8°C; MAG8 = supplementation with monoglyceride solution (1.90 µg/mL) at growth temperature of 8°C; T8 = supplementation with 0.1% Tween 80 at growth temperature of 8°C. Error bars indicate standard deviation of data obtained in triplicate. Statistical analysis between treatment and control was conducted with Student *t*-test. Treatments with different letters are statistically different from each other ( $P < 0.05$ ).

in 4mCCE at 8°C supplemented with Tween 80 (Table 2). Similarly, the predominant FA present in MAG solution used in the growth experiments was palmitic acid (C<sub>16:0</sub>). This FA accounts for 29.1% of the total FA present in the CMFA of cells grown in 4mCCE at 8°C without supplementation, whereas it accounts for 60.4% of the FA present in the CMFA of cells grown in 4mCCE at 8°C supplemented with the MAG solution (Table 2). These results clearly demonstrate that *L. casei* M36 incorporates exogenous FA into its cytoplasmic membrane during growth in 4mCCE at 8°C. Because FA biosynthesis in bacteria is an energy-intensive process (Zhang and Rock, 2008), the incorporation of exogenous FA represents a significant energy savings to the cell and likely explains the growth enhancement these supplements provide at 8°C in 4mCCE.

The incorporation of exogenous FA into the cytoplasmic membrane will also have a significant effect

on membrane function. In many bacteria, the ability to grow at sub-optimal temperatures requires homoviscous adaptation of the cytoplasmic membrane, which is manifest through alterations in the CMFA profile (Farrell and Rose, 1967; Kaneda, 1991; Annou et al., 1997; Sajbidor, 1997; Chattopadhyay and Jagannadham, 2003; Hulbert, 2003; Zhang and Rock, 2008). These alterations include changes in the average FA chain length, percentage CFA, percentage branched-chain FA (BCFA), ratio of iso:anteiso (I:A), percentage PUFA, and ratio of unsaturated to saturated FA (U:S ratio). The adaptation of *L. casei* M36 to growth at 8°C, a sub-optimal temperature, is apparent by comparing the CMFA profile of this organism after growth in unsupplemented 4mCCE at 8°C to that observed after growth at 37°C. The most obvious difference was the dramatic increase in the percentage of BCFA in the cytoplasmic membrane when this organism was

**Table 2.** Cytoplasmic membrane FA profiles of *Lactobacillus casei* M36 propagated in Cheddar cheese extract prepared from cheese aged 4 mo (4mCCE) under different conditions

FA (% of total)	Conditions <sup>1</sup>				
	NS 37°C	NS 8°C	1% mf, 8°C	0.01% MAG, 8°C	0.1% T80, 8°C
C <sub>14:0</sub>	6.4 (0.71)	10.1 (1.25)	10.4 (1.17)	9.6 (0.72)	10.7 (1.72)
iso-C <sub>15:0</sub>	0.1 (0.17)	2.3 (1.25)	1.6 (0.72)	0.9 (0.52)	3.1 (0.24)
anteiso-C <sub>15:0</sub>	0.3 (0.26)	2.4 (0.47)	0.9 (0.32)	0.6 (0.37)	3.6 (0.42)
C <sub>15:0</sub>	1.0 (0.48)	0.9 (0.16)	1.1 (0.23)	0.7 (0.31)	0.8 (0.08)
iso-C <sub>16:0</sub>	0.4 (0.16)	2.4 (0.39)	0.5 (0.10)	0.4 (0.19)	0.5 (0.03)
C <sub>16:0</sub> <sup>2</sup>	41.8 (2.90)	29.1 (4.25)	30.0 (1.05)	60.4 (3.08)	13.9 (1.48)
C <sub>16:1</sub>	5.3 (0.48)	BQL <sup>3</sup>	BQL	BQL	BQL
C <sub>16:1n9c</sub>	4.2 (0.66)	12.2 (1.82)	2.7 (0.04)	0.5 (0.16)	0.8 (0.11)
C <sub>17:0Δ9c</sub>	0.9 (0.21)	1.1 (0.17)	0.5 (0.03)	0.3 (0.08)	0.3 (0.05)
C <sub>18:0</sub> <sup>4</sup>	9.3 (2.00)	16.6 (4.62)	11.5 (0.25)	13.4 (0.48)	1.4 (1.07)
C <sub>18:1nt</sub>	1.9 (0.47)	0.5 (0.89)	2.3 (0.15)	0.2 (0.06)	1.6 (0.43)
C <sub>18:1n9c</sub>	3.4 (0.84)	7.7 (2.09)	28.5 (1.78)	5.4 (0.35)	41.8 (2.62)
C <sub>19:0Δ9c</sub>	1.7 (0.30)	9.3 (3.83)	1.8 (0.37)	6.9 (0.97)	20.7 (0.72)
C <sub>18:1n11c</sub>	15.3 (1.76)	2.8 (1.05)	2.8 (0.41)	0.5 (0.19)	2.0 (0.15)
C <sub>19:0Δ11c</sub>	8.8 (2.79)	3.0 (0.74)	BQL	BQL	BQL
C <sub>18:2n6</sub>	1.4 (0.62)	1.9 (0.56)	4.3 (0.34)	0.1 (0.13)	0.2 (0.04)
C <sub>18:3n3</sub>	0.1 (0.11)	0.7 (0.20)	0.9 (0.03)	0.1 (0.03)	0.1 (0.01)
% CFA <sup>5</sup>	11.4 (2.81)	13.3 (3.91)	2.3 (0.37)	7.20 (0.97)	21.0 (0.72)
% UFA <sup>6</sup>	31.5 (2.26)	25.8 (3.16)	41.6 (1.86)	6.81 (0.45)	46.5 (2.66)
% SFA <sup>7</sup>	70.6 (4.60)	77.0 (7.63)	58.4 (1.83)	93.2 (3.43)	54.9 (2.99)
% PUFA <sup>8</sup>	1.5 (0.63)	2.7 (0.60)	5.2 (0.34)	0.2 (0.13)	0.3 (0.04)
% BCFA <sup>9</sup>	0.8 (0.35)	7.1 (1.39)	3.1 (0.80)	2.0 (0.66)	7.1 (0.48)
U:S <sup>10</sup>	0.45	0.34	0.71	0.07	0.85
I:A <sup>11</sup>	1.7	2.0	2.3	2.2	1.0
Average chain length	16.6	16.5	16.8	16.3	17.0

<sup>1</sup>NS = no supplement; mf = 1% milk fat; MAG = 0.01% monoglyceride; 0.1% T80 = 0.1% Tween 80.

<sup>2</sup>Double bond position unknown.

<sup>3</sup>BQL = below quantifiable limit (0.002%).

<sup>4</sup>Mixture of isomers.

<sup>5</sup>Cyclopropane fatty acids (C<sub>17:0Δ9c</sub>, C<sub>19:0Δ9c</sub>, and C<sub>19:0Δ11c</sub>), where c = cis, in the cytoplasmic membrane.

<sup>6</sup>Unsaturated fatty acids (C<sub>16:1</sub>, C<sub>18:1</sub>, C<sub>18:2n6</sub>, and C<sub>18:3n3</sub>) in the cytoplasmic membrane.

<sup>7</sup>SFA (C<sub>14:0</sub>, iso-C<sub>15:0</sub>, anteiso-C<sub>15:0</sub>, C<sub>16:0</sub>, iso-C<sub>16:0</sub>, C<sub>17:0Δ9c</sub>, C<sub>18:0</sub>, C<sub>19:0Δ9c</sub>, and C<sub>19:0Δ11c</sub>) in the cytoplasmic membrane.

<sup>8</sup>PUFA (C<sub>18:2n6</sub> and C<sub>18:3n3</sub>) in the cytoplasmic membrane.

<sup>9</sup>Branched-chain fatty acids (iso-C<sub>15:0</sub>, anteiso-C<sub>15:0</sub>, and iso-C<sub>16:0</sub>) in the cytoplasmic membrane.

<sup>10</sup>Ratio of unsaturated to saturated FA in the cytoplasmic membrane.

<sup>11</sup>Ratio of iso- to anteiso BCFA in the cytoplasmic membrane.

grown at 8°C (7.1%) compared with 37°C (0.8%). The CMFA profiles of *L. casei* M36 grown in 4mCCE at 8°C unsupplemented or supplemented with 1% milk fat, the MAG mixture, or Tween 80 are very different. Of particular relevance are the significant alterations in percentages of CFA, BCFA, PUFA, and ratios I:A and U:S, which ranged from 2.3 to 21.0%, 2.0 to 7.1%, 0.2 to 5.2%, 1.0 to 2.3, and 0.07 to 0.85, respectively (Table 2). These results suggest that *L. casei* M36 grown in 4mCCE at 8°C uses different strategies to accomplish homoviscous adaptation depending on the FA content of the supplement.

## CONCLUSIONS

This study demonstrated that growth of *L. casei* in 4mCCE was significantly affected by temperature and that 4mCCE, without supplementation, is not suitable as a model system at temperatures representative of ripening Cheddar cheese (i.e., 8°C). The addition of Tween 80, 1% milk fat, or a MAG mixture resulted in a significant increase in the final cell density of *L. casei* in 4mCCE at 8°C, suggesting that exogenous FA are important for growth of *L. casei* under conditions present in ripening Cheddar cheese. Additionally, these results support that milk fat typically provides the required exogenous FA in ripening cheese. The exogenous FA were incorporated into the cytoplasmic membrane and hence likely stimulate growth by significantly reducing the energy needed for FA biosynthesis. The FA present in the cytoplasmic membrane, either from exogenous sources or de novo FA synthesis, are then modified to generate a CMFA profile that allowed for growth at 8°C. Subsequent studies examining the strain-specific contributions of SLAB and NSLAB to Cheddar cheese flavor development, as well as the effects of cheese composition and ripening regimen on the Cheddar cheese microbiota and flavor development, will be conducted in CCE supplemented with 1% milk fat.

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