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Tyrosine and Phenylalanine Catabolism by *Lactobacillus* Cheese Flavor Adjuncts[1]

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ABSTRACT

Bacterial metabolism of Tyr and Phe has been associated with the formation of aromatic compounds that impart barny-utensil and floral off-flavors in cheese. In an effort to identify possible mechanisms for the origin of these compounds in Cheddar cheese, we investigated Tyr and Phe catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts under simulated Cheddar cheese-ripening (pH 5.2, 4% NaCl, 15°C, no sugar) conditions. Enzyme assays of cell-free extracts indicated that *L. casei* strains catabolize Tyr and Phe by successive, constitutively expressed transamination and dehydrogenation reactions. Similar results were obtained with *L. helveticus* strains, except that the dehydrogenase enzymes were induced during incubation under cheese-ripening conditions. Micellar electrokinetic capillary chromatography of supernatants from *L. casei* and *L. helveticus* strains incubated under simulated cheese-ripening conditions confirmed that Tyr and Phe transamination and dehydrogenation pathways were active in both species and also showed these reactions were reversible. Major products of Tyr catabolism were *p*-hydroxy phenyl lactic acid and *p*-hydroxy phenyl acetic acid, while Phe degradation gave rise to phenyl lactic acid, phenyl acetic acid, and benzoic acid. However, some of these products were likely formed by nonenzymatic processes, since spontaneous chemical degradation of the Tyr intermediate *p*-hydroxy phenyl pyruvic acid produced *p*-hydroxy phenyl acetic acid, *p*-hydroxy phenyl propionic acid, and *p*-hydroxy benzaldehyde, while chemical degradation of the Phe intermediate phenyl pyruvic acid gave rise to phenyl acetic acid, benzoic acid, phenethanol, phenyl propionic acid, and benzaldehyde. (**Key words:** *Lactobacillus*, amino acid catabolism, tyrosine, phenylalanine)

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Abbreviation key: A = absorptance (used with number indicating wavelength), **AAA** = aromatic AA, **ATase** = aminotransferase, **BA** = benzoic acid, **CDM** = chemically defined AA medium, **CFE** = cell-free extract, **DCOOHase** = decarboxylase, **HPAA** = *p*-hydroxy phenyl acetic acid, **HPLA** = *p*-hydroxy phenyl lactic acid, **HPLDHase** = *p*-hydroxy phenyl lactic acid dehydrogenase, **HPPA** = *p*-hydroxy phenyl pyruvic acid, **MECC** = micellar electrokinetic capillary chromatography, **NADH** = reduced NAD^+, **NCR** = near cheese ripening conditions, **PAA** = phenyl acetic acid, **PLA** = phenyl lactic acid, **PLDHase** = phenyl lactic acid dehydrogenase, **PPA** = phenyl pyruvic acid.

INTRODUCTION

During Cheddar cheese maturation, the amino acids produced from casein degradation are catabolized by the microorganisms in cheese into compounds that can have a strong effect on cheese flavor (Aston and Dulley, 1982; Keeney and Day, 1957; Urbach, 1995). While many of these reactions make positive contributions to cheese flavor, compounds that are thought to originate from the catabolism of aromatic amino acids (**AAA**) have been shown to impart pungent off-flavors (Dunn and Lindsay, 1985; Guthrie, 1993). Specifically, Dunn and Lindsay (1985) showed that the Phe catabolites phenyl acetaldehyde and phenethanol caused floral, rose-like off-flavors, and that the Tyr catabolite ρ-cresol imparted barny or utensil-like type off-flavors. Guthrie (1993) confirmed the roles of phenylacetic acid and phenethanol in off-flavor development in Cheddar cheese, and showed that another Phe metabolite, phenyl propionic acid, also contributed to this defect. Although pathways for the production of each of these molecules from Phe or Tyr have been described in some bacteria (Macfarlane and Macfarlane, 1995), mechanisms for their production in cheese have not yet been established.

Microbial degradation of Tyr and Phe may involve several different enzymes including aminotransferases (**ATase**) (EC 2.6.1.5 and EC 2.6.1.58), decarboxylases (**DCOOHase**) (EC 4.1.1.25 and EC 4.1.1.53), and aromatic hydroxy acid dehydrogenases (EC 1.1.1.222)

(Hemme et al., 1982; Hummel et al., 1986; Macfarlane and Macfarlane, 1995; Schormüller, 1968). Gao and associates (1997) showed the starter cultures used in Cheddar cheese, *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, both catabolized AAA by ATase under conditions found in ripening cheese (pH 5.2, 4% NaCl, 13°C). That study (Gao et al., 1997) also revealed that nonculturable starter bacteria may contribute to amino acid catabolism in the cheese matrix, and the authors agreed with a previous suggestion that metabolic interconversion of AAA metabolites by starter bacteria and certain lactobacilli could lead to the production of off-flavor compounds (Guthrie, 1993). Since catabolism of AAA and AAA metabolites by starter, adjunct, and nonstarter bacteria appears to be an important source of cheese flavors and off-flavors, then knowledge of AAA catabolic pathways in these organisms should reveal useful strategies to control production of the AAA-derived compounds that impart off-flavors to cheese (aromatic off-flavors). Evidence to support the latter hypothesis was recently provided by Rijnen et al. (1999), who reported that starter bacteria that had been genetically engineered to lack the lactococcal aromatic aminotransferase produced St. Paulin-type cheese that had less intense floral notes than cheese made with the wild-type parent strain. While this is an encouraging step toward the control of cheese off-flavor development, the works of Guthrie (1993) and Gao et al. (1997) suggest that full realization of this goal will require a more comprehensive understanding of AAA catabolism by some of the other bacteria that occur in ripened cheese.

Our group is interested in the contribution of lactobacilli to AAA catabolism and cheese off-flavor development because species such as *Lactobacillus helveticus* and *Lactobacillus casei* are widely used as starters or flavor adjuncts, and members of this genus also dominate populations of nonstarter (adventitious) bacteria in virtually all ripened cheese varieties (El soda, 1993; Fox et al., 1993; Laleye et al., 1990; Lee et al., 1990). There is little question that lactobacilli can have very positive effects on cheese flavor intensity (El soda, 1993; Laleye et al., 1990; Lee et al., 1990), but Guthrie (1993) also found that certain lactobacilli, and particularly some strains of *L. casei*, were associated with unclean flavor development in Cheddar cheese. Moreover, AAA metabolites that are known to produce strong off-flavor notes (e.g., ρ-cresol, indole, and skatole) have been recovered from the aroma fractions of *L. helveticus* cultures and from unclean-flavored cheese made with *Lactobacillus* adjuncts (Guthrie, 1993; Kowaleska et al., 1985). As a whole, these data indicate that knowledge of AAA catabolic pathways in adjunct or nonstarter lactobacilli may be vital to industry efforts to control production of aromatic off-flavor compounds in Cheddar and other semihard aged cheeses.

Current understanding of the molecular genetics of AAA degradation in dairy lactobacilli is not as advanced as it is for *Lactococcus lactis*, but biochemical data indicate that AAA catabolism in both genera is almost always initiated by an aminotransferase (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999). For example, Groot et al. (1998) showed that *L. plantarum*, a species that is commonly found among cheese nonstarter bacteria (Peterson and Marshall, 1990), converts Phe to phenyl pyruvic acid (**PPA**) by transamination, and that the PPA produced in this reaction may degrade spontaneously to benzaldehyde. Our laboratory previously examined Trp catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts under simulated cheese-ripening conditions (pH 5.2, 4% NaCl, 15°C, no sugar), and found that strains which are able to catabolize Trp did so via successive transamination and dehydrogenation reactions to produce indole-3-lactic acid (Gummalla and Broadbent, 1999). Like PPA, however, the α-keto acid intermediate formed in this pathway by Trp transamination (indole pyruvic acid) was chemically labile and could degrade spontaneously into other aromatic compounds (Gao et al., 1997; Gummalla and Broadbent, 1999). In this report, we show that *L. casei* and *L. helveticus* cheese flavor adjuncts use a similar pathway for Tyr and Phe catabolism under cheese-ripening conditions.

MATERIALS AND METHODS

Bacterial Strains

Lactobacillus helveticus LH212 and *L. casei* LC301 and LC202 were obtained from Rhodia, Inc. (Madison, WI). *L. helveticus* CNRZ32 was provided by J. L. Steele at the University of Wisconsin-Madison. The cultures were propagated in APT broth (Difco, Detroit, MI) at 30°C (*L. casei*) or 37°C (*L. helveticus*), stored at 4°C, and maintained by biweekly transfer.

Preparation of Cell-Free Extracts

Individual lactobacilli were initially screened for enzymes involved in Tyr and Phe catabolism by assays of cell-free extracts (**CFE**) prepared from 10 ml of an overnight APT culture. The bacteria were harvested by centrifugation for 15 min at 4500 × *g* (4°C), washed twice with 50 m*M* potassium phosphate buffer (pH 6.5), and suspended in 1 ml of phosphate buffer. The CFE were prepared by sonic disintegration of the individual cell suspensions with a Branson cell disrupter 200 (Danbury, CT) in pulsed mode at 20 kHz for 5 min in an ice bath. Intact bacteria and cell debris were removed by centrifugation at 4500 × *g* (4°C), and the supernatant was used

as the CFE. Total protein was determined using the Pierce BCA protein assay kit (Rockford, IL) with bovine serum albumin as the protein standard.

To collect information on Tyr and Phe catabolism as it might be likely to occur in cheese, CFE were also prepared from cells that had been incubated in an environment designed to simulate some of the conditions found in ripening Cheddar cheese (Gao et al., 1997; Gummalla and Broadbent, 1999). Bacteria for these studies were harvested by centrifugation from an overnight APT culture, washed twice in a carbohydrate-free chemically defined amino acid medium (**CDM**) (Gummalla and Broadbent, 1999) that lacked L-Tyr or Phe, and suspended in 0.1 volume of the CDM. One milliliter of the cell suspension was then transferred into test tubes that contained 9 ml of CDM that lacked carbohydrate, contained 4% (wt/vol) NaCl, had been adjusted to pH 5.2 with lactic acid, and either contained or lacked 5 m*M* L-Tyr or L-Phe. Bacteria suspended in the latter medium (henceforth described as near cheese ripening conditions; **NCR**) were incubated at 15°C, then CFE were prepared by sonic disintegration as described above at time 0 (inoculation) and after 5, 10, 15, and 20 d of incubation.

Identification of Enzymes Involved in Tyr and Phe Catabolism

Tyrosine and Phe Atase activities were measured spectrophotometrically as described previously for Trp ATase activity (Gummalla and Broadbent, 1999). The reaction mixture contained 5 m*M* L-Tyr or L-Phe, 5 m*M* α-ketoglutarate, 50 μM pyridoxal phosphate, 5 m*M* sodium arsenate, and 5 m*M* EDTA in 50 m*M* sodium tetraborate (pH 8.5) buffer. The reaction was initiated by the addition of 250 μl of CFE to obtain a total volume of 1 ml, and the mixture was incubated at 30°C for 30 min. The production of *p*-hydroxy phenyl pyruvic acid (**HPPA**) from Tyr or of PPA from Phe was measured by the increase in solution absorptance at 305 nm ($\mathbf{A}_{305}$) or 300 nm (A_{300}), respectively. Specific activities for Tyr and Phe ATase were expressed as μmoles HPPA or PPA produced per milligram of protein per minute. Control reactions without substrate, without CFE, and without substrate and CFE were included in this and all other enzyme assays.

After transamination, HPPA or PPA may be reduced to *p*-hydroxy phenyl lactic acid (**HPLA**) or phenyl lactic acid (**PLA**), respectively, by *p*-hydroxy phenyl lactic acid dehydrogenase (**HPLDHase**) or phenyl lactic acid dehydrogenase (**PLDHase**) (Hummel et al., 1986). The CFE were assayed for each of these activities by the spectrophotometric method of Hummel and coworkers (1986), in which the decrease in reduced NAD^+ (**NADH**) is measured at A_{340}. The reaction mixture contained 250 μl CFE, 50 m*M* sodium phosphate (pH 6.5), 5.0 m*M* HPPA or PPA, and 0.3 m*M* NADH in a total volume of 1 ml. The specific activity of HPLDHase and PLDHase was reported as μmoles NADH consumed per milligram of protein per minute.

Tyrosine or Phe DCOOHases catalyze the conversion of Tyr to tyramine or Phe to phenethylamine, respectively. The presence of Tyr or Phe DCOOHase activity in CFE was investigated with a 1-ml reaction mixture that contained 5.0 m*M* L-Tyr or L-Phe, 1 m*M* pyridoxal phosphate, 250 m*M* NH_4OH-NH_4Cl (pH 9.0), and 250 μl of CFE (Nakazawa et al., 1977). The mixture was incubated at 30°C for 30 min, terminated by the addition of 0.5 ml trichloroacetic acid, then qualitatively assayed for tyramine or phenethylamine by micellar electrokinetic capillary chromatography (**MECC**) with pure standards (Strickland et al., 1996).

Finally, specific activities for Tyr ATase and HPLDHase or Phe ATase and PLDHase were also measured in CFE prepared from cells incubated under NCR for 0, 5, 10, 15, and 20 d as described above. The specific activity values presented for all enzyme assays represent the mean obtained from duplicate experiments replicated on two separate days. The effect of substrate (Tyr vs. Phe) and time (d 0 vs. 20 under NCR) on enzyme specific activities were evaluated by statistical *t*-test comparisons ($\alpha = 0.05$) between means using Microsoft Excel software (Redmond, WA).

Identification of Tyr and Phe Catabolites in Culture Supernatants

Micellar electrokinetic capillary chromatography was also used to identify Tyr and Phe catabolites in the supernatant from cells incubated up to 6 wk under NCR. Lactobacilli for these experiments were prepared from 500 ml of culture grown overnight in APT broth. The cells were harvested by centrifugation at $4500 \times g$ (4°C), washed twice in CDM that either lacked L-Tyr or L-Phe, then incubated under NCR in 50 ml of CDM that did or did not contain 5 m*M* L-Tyr or Phe or one of the following Tyr or Phe metabolites: HPPA, PPA, HPLA, PLA, *p*-hydroxy phenyl acetic acid (**HPAA**), phenyl acetic acid (**PAA**), *p*-hydroxy phenyl propionic acid, phenyl propionic acid, ρ-cresol, or phenethanol. Bacterial and control suspensions were incubated under NCR then collected for MECC at time 0 (inoculation) and at weekly intervals thereafter. Sample pH was recorded and viable cell counts determined by plating on APT agar with anaerobic incubation for 48 h. The samples were centrifuged to remove cells and the supernatants were passed through a Corning (Palo Alto, CA) 0.20-μm cellulose acetate syringe mounted filter, diafiltered through a Filtron (Northborough, MA) 1 K cut-off Microsep concentrator,

Table 1. Specific activity of Tyr and Phe aminotransferase (ATase) in cell-free extracts from lactobacilli incubated under near cheese-ripening conditions.[1]

Incubation time (d)	*Lactobacillus casei*				*Lactobacillus helveticus*			
	LC301		LC202		CNRZ32		LH212	
	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase
0	1.4 ± 0.1	4.8 ± 0.1	1.2 ± 0.0	5.7 ± 0.5	1.3 ± 0.0	5.4 ± 0.0	1.6 ± 0.1	5.0 ± 0.2
5	1.2 ± 0.1	5.2 ± 0.3	1.1 ± 0.2	5.5 ± 0.1	0.9 ± 0.1	6.9 ± 0.0	1.1 ± 0.1	5.5 ± 0.4
10	1.1 ± 0.1	5.4 ± 0.4	1.4 ± 0.1	6.3 ± 0.0	1.0 ± 0.0	6.4 ± 0.2	1.3 ± 0.1	5.8 ± 0.4
15	1.4 ± 0.0	5.6 ± 0.2	1.4 ± 0.1	5.0 ± 0.4	1.1 ± 0.0	5.9 ± 0.3	1.4 ± 0.1	5.2 ± 0.2
20	1.0 ± 0.0	4.6 ± 0.2	1.0 ± 0.1	4.0 ± 0.1	1.2 ± 0.0	4.7 ± 0.1	1.3 ± 0.1	4.9 ± 0.4

[1]Chemically defined medium with no carbohydrate, pH 5.2, 4% (wt/vol) NaCl with 5 m*M* L-Tyr or L-Phe. Cells were incubated at 15°C. Specific activity values represent μmoles *p*-hydroxy phenyl pyruvic acid or phenyl pyruvic acid produced per milligram of protein per minute $\times 10^{-3}$ (± SE). Values represent the mean from duplicate experiments replicated on 2 separate days.

then diluted 1:5 in a 50 m*M* sodium tetraborate buffer immediately before injection. The MECC was performed in 100 m*M* SDS-100 m*M* sodium tetraborate run buffer as described by Strickland et al. (1996) with a Beckman Instruments P/ACE 2000 (Fullerton, CA) automated capillary electrophoresis system with System Gold software (version 7.11). Peaks that contained Tyr or Phe catabolites were identified by comparisons between the electropherograms obtained from supernatants of cells incubated with and without 5 m*M* L-Tyr or L-Phe or a Tyr or Phe metabolite. The compound present in each of these peaks was subsequently identified by coinjection with pure standards (Sigma-Aldrich, St. Louis, MO) and by a correlation (r) greater than 0.9 between the absorption spectra from an unknown compound and a known standard. Uninoculated control tubes that contained CDM with 5 m*M* of L-Tyr or L-Phe or individual Tyr of Phe catabolites were also included in each experiment to identify any compounds that were formed by non-enzymatic degradation (Gummalla and Broadbent, 1999).

RESULTS

Enzymes Involved in Tyr and Phe Degradation in Lactobacilli

Tyrosine and Phe ATase activities were detected in CFE collected from all four *Lactobacillus* adjuncts after overnight incubation in APT broth. The specific activity of Tyr ATase was 1.0×10^{-3} μmoles/mg of protein per minute in both strains of *L. helveticus*, and ranged from 1.4 to 1.7×10^{-3} μmoles/mg of protein per minute in *L. casei* strains. The specific activity of Phe ATase was significantly higher ($P < 0.05$) than L-Tyr ATase activity in all strains, and ranged from 3.5 to 4.1×10^{-3} μmoles/mg of protein per minute in *L. casei*, and 6.0 to 8.0×10^{-3} μmoles/mg protein/min in *L. helveticus*. As shown in Table 1, Phe ATase activity was also significantly ($P < 0.05$) higher than Tyr ATase activity when cells were incubated under NCR. The effect of time on Tyr and Phe ATase enzyme activities varied among strains. Incubation for 20 d under NCR did not affect Phe ATase activity in *L. helveticus* LH212 or Tyr ATase activity in *L. casei* LC202 and *L. helveticus* CNRZ32, but the specific activities of these enzymes in the other lactobacilli showed a small but statistically significant ($P < 0.05$) decrease over that period.

Cell-free extracts of *L. casei* strains collected after overnight incubation in APT broth also exhibited HPLDHase (8.4 to 11.9×10^{-1} μmoles/mg of protein per minute) and PLDHase (6.2 to 7.0×10^{-1} μmoles/mg of protein per minute) activities, but neither activity was detected in CFE from *L. helveticus* LH212 or CNRZ32. When *L. casei* strains were incubated under NCR, HPLDHase activity ranged from 5.9 to 12.5×10^{-1} μmoles/mg of protein per minute, while PLDHase activity was 14.4 to 19.9×10^{-1} μmoles/mg of protein per minute (Table 2). Interestingly, HPLDHase activity in these cells showed a significant ($P < 0.05$) decrease by d 20, but PLDHase activity increased significantly ($P < 0.05$) over the same period.

Although no HPLDHase or PLDHase activity was detected in CFE from *L. helveticus* cells incubated for 0 or 5 d under NCR, specific activities ranging from 1.1 to 2.4×10^{-1} for HPLDHase and from 1.1 to 4.4×10^{-1} μmoles/mg of protein per minute for PLDHase were found in cells incubated for 10, 15, and 20 d (Table 2). Moreover, HPLDHase activity in *L. helveticus* LH212 and PLDHase activity in both *L. helveticus* strains increased significantly ($P < 0.05$) from d 10 to 20.

Finally, production of tyramine or phenethylamine was not detected from Tyr or Phe DCOOHase assays of CFE from any of the *Lactobacillus* spp. used in this study.

MECC Analysis

When lactobacilli were incubated under NCR, media pH remained relatively constant (pH 5.2 ± 0.5), but num-

bers of culturable cells fell from approximately 10^9 to 10^5 or fewer cfu/ml over a 6-wk period (Figures 1 and 2). As is summarized in Table 3, MECC analysis of culture supernatants showed all four lactobacilli catabolized Tyr under NCR by same pathway; Tyr was converted to HPLA and HPAA (Figure 1), and incubations in CDM with HPLA led to the production of HPAA and Tyr. As expected, supernatant from cells incubated in CDM with HPPA contained Tyr, HPLA, and HPAA. Production of *p*-cresol was not detected by any of the *Lactobacillus* strains tested, but other compounds including *p*-hydroxy phenyl propionic acid and *p*-hydroxy benzaldehyde were found in CDM with HPPA. These products were also detected in cell-free control tubes, however, which showed they were formed by nonenzymatic degradation of HPPA. Further catabolism of *p*-hydroxy phenyl acetic acid or *p*-hydroxy phenyl propionic acid by lactobacilli was not detected (Table 3).

Studies of Phe metabolism under NCR yielded similar results (Table 4). All four lactobacilli converted Phe to PLA, PAA, and benzoic acid (**BA**) (Figure 2), and incubations in CDM with PLA led to the production of PAA, BA, and Phe. Supernatant from cells incubated in CDM with PPA yielded Phe, PLA, PAA, and BA. Phenethanol, phenyl propionic acid, and benzaldehyde were also detected in CDM with PPA, but these compounds also appeared in cell-free control tubes, which confirmed they were formed by spontaneous chemical degradation of PPA (Gao et al., 1997; Groot et al., 1998; Urbach, 1995). Further degradation of phenyl acetic acid, phenethanol, or phenyl propionic acid was not detected.

Although pathways for Tyr and Phe catabolism under NCR were similar in all four lactobacilli, levels of HPLA, HPAA, PLA, and PAA that accumulated in CDM spiked with Tyr or Phe differed between *L. casei* and *L. helveticus* adjuncts, and also between strains of *L. helveticus*. In CDM spiked with Tyr, for example, HPLA was detected 1 wk earlier in supernatant from *L. casei* versus *L. helveticus* adjuncts, and the former species produced about two- to fourfold more HPLA than the latter species (Figure 1). In contrast, HPAA was detected in supernatant from *L. helveticus* strains 2 wk before it was found in supernatant from *L. casei* LC301 or LC202. However, the concentration of HPAA in culture supernatants after 6 wk of incubation under NCR was similar for all strains except *L. helveticus* CNRZ32, which produced two- to threefold more HPAA than any of the other lactobacilli. Among *L. helveticus* adjuncts, strain LH212 produced more than twice the level of HPLA detected with CNRZ32, while the opposite was true for HPAA (Figure 1C versus 1D). *L. helveticus* CNRZ32 was also the only strain that produced more HPAA than HPLA.

As is shown in Figure 2, *L. casei* strains incubated under NCR in CDM spiked with Phe produced PLA more rapidly than *L. helveticus* strains, and the concentration of PLA in supernatant from the former species remained several fold higher than that detected in comparable samples from *L. helveticus* strains. Another difference between these two species was that supernatant from *L. casei* always contained more PLA than PAA, while the opposite was true of *L. helveticus*. Supernatant from *L. helveticus* adjuncts also contained more BA than was found in samples from either strain of *L. casei* (Figure 2).

Concentrations of Phe metabolites in supernatant from *L. casei* adjuncts incubated 6 wk under NCR were relatively similar (Figure 2A versus 2B), but substantive differences were noted between *L. helveticus* strains. For example, PLA production by *L. helveticus* CNRZ32 was not detected until the 3rd wk of incubation under NCR, and the concentration of this and all other Phe metabolites in supernatant from cells incubated 6 wks under NCR was less than half that found for *L. helveticus* LH212 (or for either *L. casei* adjunct) (Figure 2).

Finally, the higher Phe versus Tyr ATase activity noted in CFE from cells incubated under NCR was also reflected in the cumulative concentrations of Phe and

Table 2. Specific activity of *p*-hydroxy phenyl lactic acid dehydrogenase (HPLDHase) and phenyl lactic acid dehydrogenase (PLDHase) in cell-free extracts from lactobacilli incubated under near cheese-ripening conditions.[1]

Incubation time (d)	*Lactobacillus casei*				*Lactobacillus helveticus*			
	LC301		LC202		CNRZ32		LH212	
	HPLDHase	PLDHase	HPLDHase	PLDHase	HPLDHase	PLDHase	HPLDHase	PLDHase
0	12.5 ± 0.3	14.4 ± 1.1	11.8 ± 1.3	11.5 ± 1.3	ND[2]	ND[2]	ND[2]	ND[2]
5	8.0 ± 0.1	17.2 ± 1.0	10.8 ± 2.6	18.2 ± 0.5	ND[2]	ND[2]	ND[2]	ND[2]
10	7.4 ± 0.2	18.1 ± 0.3	8.6 ± 1.3	17.1 ± 0.4	0.7 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
15	6.2 ± 0.4	19.9 ± 1.8	7.0 ± 0.2	18.0 ± 0.6	1.5 ± 0.3	1.1 ± 0.1	1.1 ± 0.1	3.4 ± 0.2
20	6.1 ± 0.1	19.0 ± 0.9	5.9 ± 0.5	18.0 ± 0.8	2.4 ± 0.5	4.4 ± 0.2	1.9 ± 0.2	3.0 ± 0.2

[1]Chemically defined medium with no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, with 5 m*M* L-Tyr or L-Phe. Cells were incubated at 15°C. Specific activity values represent μmoles NADH consumed per milligram of protein per minute $\times 10^{-1}$ (± SE). Values represent the mean from duplicate experiments replicated on two separate days.

[2]Not detected.

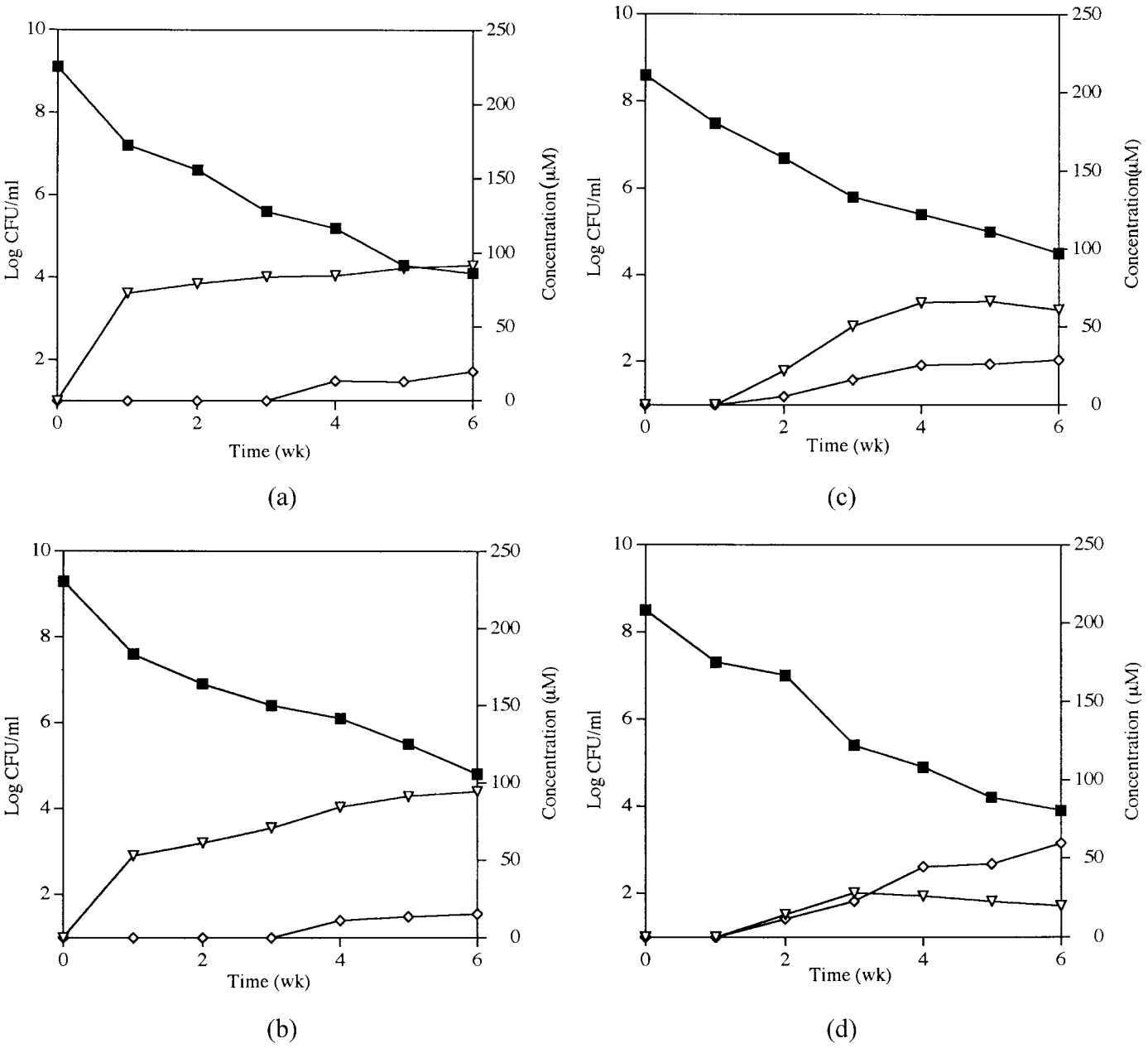

Figure 1. Tyrosine metabolism by *Lactobacillus casei* LC202 (A), *L. casei* LC301 (B), *Lactobacillus helveticus* LH212 (C), and *L. helveticus* CNRZ32 (D) incubated under near cheese-ripening conditions (NCR) in chemically defined medium spiked with 5 m*M* L-Tyr. The figures show the number of colony forming units per ml recovered (■) and the concentrations of *ρ*-hydroxy phenyl lactic acid (▽) and *ρ*-hydroxy phenyl acetic acid (✧) that accumulated in culture supernatant over a 6 wk incubation under NCR.

Tyr metabolites that accumulated in supernatant from all lactobacilli except CNRZ32. As is shown in Figures 1 and 2, levels of PLA plus PAA and BA in supernatant from LC202, LC301, and LH212 cells that had been incubated under NCR in CDM with Phe were about threefold higher than the concentration of HPLA plus HPAA in supernatant from cells incubated in CDM with Tyr.

DISCUSSION

In Cheddar cheese Phe and Tyr metabolites such as PAA, phenethanol, phenylpropionic acid, and *ρ*-cresol have been shown to impart potent floral, barny, or utensil off-flavors (Dunn and Lindsay, 1985; Guthrie, 1993). However, because pathways for the production of these compounds in cheese have not yet been established, the

individual contributions of starter, adjunct, and nonstarter bacteria to cheese off-flavor development remain unclear. To investigate the contribution of lactobacilli to off-flavor production in Cheddar cheese, our laboratory has analyzed AAA catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts (Gummalla and Broadbent, 1999). In this report, we describe pathways for Tyr and Phe catabolism by these two species in an environment that simulated some of the conditions found in ripening Cheddar cheese (e.g., no carbohydrate, suboptimal growth temperature, pH 5.2, and 4% NaCl).

Enzyme assays of CFE from *L. casei* LC202 and LC301 indicated both bacteria were likely to catabolize Tyr and Phe through successive transamination and dehydrogenation reactions. Strong evidence that this pathway is the primary route for Tyr and Phe catabolism in cheese

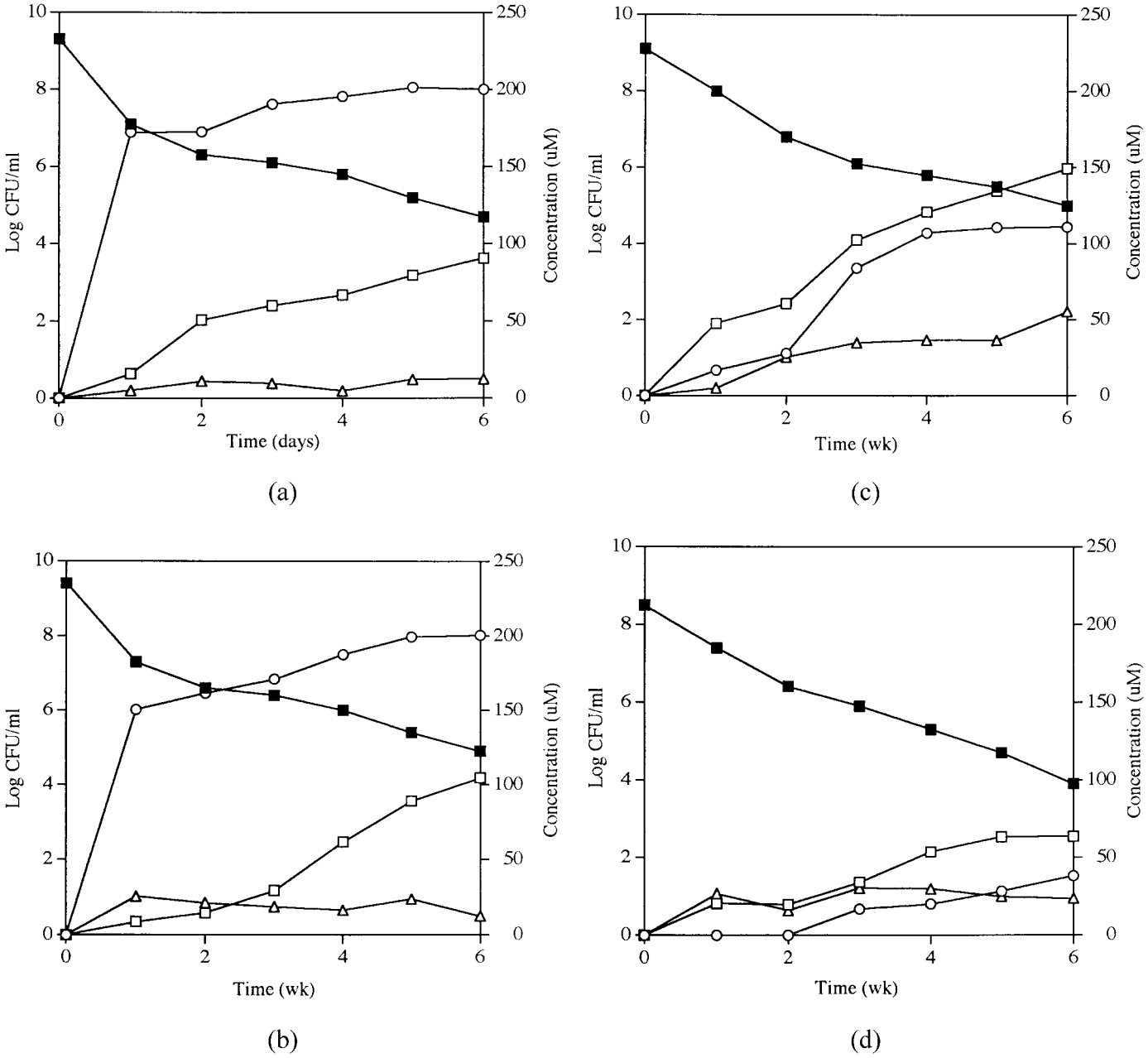

Figure 2. Phenylalanine metabolism by *Lactobacillus casei* LC202 (A), *L. casei* LC301 (B), *Lactobacillus helveticus* LH212 (C), and *L. helveticus* CNRZ32 (D) incubated under near cheese-ripening conditions (NCR) in chemically defined medium spiked with 5 m*M* L-Phe. The figures show the number of colony forming units per ml recovered (■) and the concentrations of phenyl lactic acid (○), phenyl acetic acid (□), and benzoic acid (△) that accumulated in culture supernatant over a 6 wk incubation under NCR.

Table 3. Tyrosine metabolites detected by micellar electrokinetic capillary chromatography of culture supernatant from *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts.[1]

	Metabolites detected[2]					
Incubation medium	Tyr	HPLA	HPAA	HBAld	HPProA	ρ-cresol
CDM	–	–	–	–	–	–
CDM + 5 m*M* Tyr	+	+	+	–	–	–
CDM + 5 m*M* HPPA	+	+	+[3]	+[3]	+[3]	–
CDM + 5 m*M* HPLA	+	+	+	–	–	–
CDM + 5 m*M* HPAA	–	–	+	–	–	–
CDM + 5 m*M* ρ-cresol	–	–	–	–	–	+
CDM + 5 m*M* HPProA	–	–	–	+	–	

[1]Cells were incubated in chemically defined, near cheese-ripening (no carbohydrate, pH 5.2, 4% [wt/vol] NaCl, 15°C) medium (CDM) spiked with Tyr or a Tyr metabolite.

[2]Abbreviations: HPPA, ρ-hydroxy phenyl pyruvic acid; HPLA, ρ-hydroxy phenyl lactic acid; HPAA, ρ-hydroxy phenyl acetic acid; HBAld, hydroxy benzaldehyde; HPProA, ρ-hydroxy phenyl propionic acid. Symbols identify compounds that were (+) or were not (–) detected.

[3]Cell-free controls showed this compound is produced by spontaneous chemical degradation of HPPA.

was obtained by MECC analysis of culture supernatant from cells incubated under NCR in CDM with Tyr (Table 3) or Phe (Table 4). The finding that Tyr and Phe catabolism by these strains was initiated by an ATase is consistent with our previous data for Trp catabolism by these strains and with other reports of AAA catabolism in dairy lactic acid bacteria (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999; Hemme et al., 1982). Data presented in Tables 3 and 4 also indicated that Tyr ATase, Phe ATase, HPLDHase, and PLDHase have reversible, anabolic activity because Tyr or Phe were produced from each of their respective products.

The specific activity of Phe or Tyr ATase in CFE from lactobacilli incubated under NCR for up to 20 d was relatively similar in both *Lactobacillus* species (Table 1), but HPLDHase and PLDHase expression in these bacteria differed both temporally and quantitatively. As is shown in Table 2, the latter enzyme activities were constitutively expressed in CFE from *L. casei*, but neither was initially detected in strains of *L. helveticus*. However, HPLDHase and PLDHase activities were detected in CFE from *L. helveticus* LH212 and CNRZ32 cells that had been incubated more than 5 d under NCR (Table 2). These observations indicate that *L. helveticus* HPLDHase and PLDHase are inducible under NCR, but it is not known if enzyme induction was in response to carbohydrate starvation, low temperature (15°C), salt (4% NaCl), pH (5.2), or some combination of these factors. Furthermore, enzyme specific activities from *L. helveticus* remained at least sixfold lower than those found in CFE from *L. casei* at comparable sampling times. The differences noted in HPLDHase and PLDHase activities from *L. casei* versus *L. helveticus* strains suggest that the former species likely plays a more prominent role in dehydration of aromatic α-keto acids in the cheese matrix. This distinction may be significant to cheese flavor development because: 1) the α-keto acids formed by ATase reactions are chemically labile; 2) products formed

Table 4. Phenylalanine metabolites detected by micellar electrokinetic capillary chromatography of culture supernatant from *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts.[1]

	Metabolites detected[2]						
Incubation medium	Phe	PLA	PAA	BA	Phen	BAld	PProA
CDM	–	–	–	–	–	–	–
CDM + 5 m*M* Phe	+	+	+	+	–	–	–
CDM + 5 m*M* PPA	+	+	+[3]	+[3]	+[3]	+[3]	+[3]
CDM + 5 m*M* PLA	+	+	+	+	–	–	–
CDM + 5 m*M* PAA	–	–	+	–	–	–	–
CDM + 5 m*M* Phen	–	–	–	–	+	–	–
CDM + 5 m*M* PProA	–	–	–	–	–	–	+

[1]Cells were incubated in chemically defined, near cheese-ripening (no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, 15°C) medium (CDM) spiked with Phe or a Phe metabolite.

[2]Abbreviations: PPA, phenyl pyruvic acid; PLA, phenyl lactic acid; PAA, phenyl acetic acid; BAld, benzaldehyde; BA, benzoic acid; PProA, phenyl propionic acid; Phen, phenethanol. Symbols identify compounds that were (+) or were not (–) detected.

[3]Cell-free controls showed this compound is produced by spontaneous chemical degradation of PPA.

by spontaneous degradation of these acids (e.g., PAA, phenethanol, and phenylpropionic acid) have been shown to impart off-flavors to Cheddar cheese; and 3) once formed, these compounds do not appear to undergo further catabolism by *L. casei* or *L. helveticus* (Tables 3 and 4). As a result, cheese made with *L. helveticus* or other bacteria with low-level HPLDHase and PLDHase activities is likely to contain higher concentrations of aromatic compounds formed spontaneously from the breakdown of HPPA and PPA than cheese made with microorganisms that more actively convert aromatic α-keto acids into aromatic α-hydroxy acids. This hypothesis is supported by data in Figures 1 and 2, which show levels of HPAA, PAA, and BA were almost always higher in supernatant from *L. helveticus* versus *L. casei* adjuncts incubated under NCR, while the opposite was true for HPLA and PLA.

Finally, some cheese-related bacteria are reported to possess AAA DCOOHase activity, and aromatic amines have been isolated from ripening cheese (Hemme et al., 1982; Moreno-Aribas, 2000; Schormüller, 1968). Data collected from cells incubated under NCR in our laboratory, however, indicate that the *L. casei* LC202 and LC301 and *L. helveticus* LH212 and CNRZ32 do not contribute to the production of tyramine, phenethylamine, or tryptamine in Cheddar cheese (Gummalla and Broadbent, 1999).

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REFERENCES

Aston, J. W., and J. R. Dulley. 1982. Cheddar cheese flavor. Aust. J. Dairy Technol. 37:59–64.

Dunn, H. C., and R. C. Lindsay. 1985. Evaluation of the role of microbial Strecker-derived aroma compounds in unclean-type flavors of Cheddar cheese. J. Dairy Sci. 68:2859–2874.

El Soda, M. A. 1993. The role of lactic acid bacteria in accelerated cheese ripening. FEMS Microbiol. Rev. 12:239–252.

Fox, P. F., J. Law, P.L.H. McSweeney, and J. Wallace. 1993. Biochemistry of Cheese ripening. Pages 389–483 *in* Cheese: Chemistry, Physics, and Microbiology. Vol. 1, 2nd ed. P. F. Fox, ed. Chapman and Hall, London, United Kingdom.

Gao, S., D. H. Oh, J. R. Broadbent, M. E. Johnson, B. C. Weimer, and J. L. Steele. 1997. Aromatic amino acid catabolism by lactococci. Lait. 77:371–381.

Groot, Masja. N. Nierop, and J.A.M. de Bont. 1998. Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64:3009–3013.

Gummalla, S., and J. R. Broadbent. 1999. Tryptophan catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts. J. Dairy Sci. 82:2070–2077.

Guthrie, B. D. 1993. Influence of cheese-related microflora on the production of unclean-flavored aromatic amino acid metabolites in Cheddar cheese. Ph.D. Diss., Univ., Wisconsin, Madison.

Hemme, D., C. Bouillanne, F. Metro, and M. J. Desmazeaud. 1982. Microbial catabolism of amino acids during cheese ripening. Sci. Des Aliments 2:113–123.

Hummel, W., N. Weiss, and M. R. Kula. 1986. Isolation and characterization of a bacterium possessing L-phenylalanine and dehydrogenase activity. Arch. Microbiol. 137:47–52.

Keeney, M., and E. A. Day. 1957. Probable role of the Strecker degradation of amino acids in development of cheese flavor. J. Dairy Sci. 40:874–875.

Kowaleska, J., H. Zelaowska, A. Babuchowski, E. G. Hammond, B. A. Glatz, and E. Ross. 1985. Isolation of aroma-bearing material from *Lactobacillus helveticus* cultures and cheese. J. Dairy Sci. 68:2165–2170.

Laleye, L. C., R. E. Simard, B. H. Lee, and R. A. Holley. 1990. Quality attributes of Cheddar cheese containing added lactobacilli. J. Food Sci. 55:114–120.

Lee, B. H., L. C. Laleye, R. E. Simard, R. A. Holley, D. B. Emmons, and R. N. Giroux. 1990. Influence of homofermentative lactobacilli on physiochemical and sensory properties of Cheddar cheese. J. Food Sci. 55:386–392.

Macfarlane, S., and G. T. Macfarlane. 1995. Proteolysis and amino acid fermentation. Pages 75–100 *in* Human colonic bacteria: role in nutrition, physiology, and pathology. G. R. Gibson and G. T. Macfarlane, ed. CRC Press, Inc., Boca Raton, FL.

Moreno-Arribas, V., S. Torlois, A. Joyeux, A. Bertrand, and A. Lonvaud-Funel. 2000. Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol. 88:584–593.

Nakazawa, H., K. Sano, H. Kumagai, and H. Yamada. 1977. Distribution and formation of aromatic L-amino acid decarboxylase in bacteria. Agric. Biol. Chem. 41:2241–2247.

Peterson, S. D., and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: a review. J. Dairy Sci. 73:1395–1410.

Rijnen, L., A. Delacroix-Buchet, J. L. Le Quere, J. C. Gripon, and M. Yvon. 1999. Inactivation of lactococcal aromatic aminotransferase prevents the formation of floral aroma compounds from aromatic amino acids in semi-hard cheese. Int. Dairy J. 9:877–885.

Schormüller, J. 1968. The chemistry and biochemistry of cheese ripening. Adv. Food Res. 16:231–242.

Strickland, M., B. C. Weimer, and J. R. Broadbent. 1996. Capillary electrophoresis of Cheddar cheese. J. Chromatogr. A. 731:305–313.

Urbach, G. 1995. Contribution of lactic acid bacteria to flavor compound formation in dairy products. Int. Dairy J. 5:877–903.