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Overexpression of *Lactobacillus casei* D-Hydroxyisocaproic Acid Dehydrogenase in Cheddar Cheese†

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Metabolism of aromatic amino acids by lactic acid bacteria is an important source of off-flavor compounds in Cheddar cheese. Previous work has shown that α-keto acids produced from Trp, Tyr, and Phe by aminotransferase enzymes are chemically labile and may degrade spontaneously into a variety of off-flavor compounds. However, dairy lactobacilli can convert unstable α-keto acids to more-stable α-hydroxy acids via the action of α-keto acid dehydrogenases such as D-hydroxyisocaproic acid dehydrogenase. To further characterize the role of this enzyme in cheese flavor, the *Lactobacillus casei* D-hydroxyisocaproic acid dehydrogenase gene was cloned into the high-copy-number vector pTRKH2 and transformed into *L. casei* ATCC 334. Enzyme assays confirmed that α-keto acid dehydrogenase activity was significantly higher in pTRKH2:*dhic* transformants than in wild-type cells. Reduced-fat Cheddar cheeses were made with *Lactococcus lactis* starter only, starter plus *L. casei* ATCC 334, and starter plus *L. casei* ATCC 334 transformed with pTRKH2:*dhic*. After 3 months of aging, the cheese chemistry and flavor attributes were evaluated instrumentally by gas chromatography-mass spectrometry and by descriptive sensory analysis. The culture system used significantly affected the concentrations of various ketones, aldehydes, alcohols, and esters and one sulfur compound in cheese. Results further indicated that enhanced expression of D-hydroxyisocaproic acid dehydrogenase suppressed spontaneous degradation of α-keto acids, but sensory work indicated that this effect retarded cheese flavor development.

Microbial catabolism of amino acids generated from the degradation of milk proteins during cheese maturation is an essential and rate-limiting step in the development of cheese flavor and aroma properties (34, 38). Many of these reactions impact cheese flavor in beneficial ways. For example, the conversion of Met to methional, dimethyl sulfide, methanethiol, and other sulfur-containing compounds is thought to be essential for aroma development in many cheese varieties (35). On the other hand, compounds derived from the catabolism of aromatic amino acids (AAAs) have been implicated in the development of cheese off-flavors. Specifically, the Phe catabolites phenylacetaldehyde and 2-phenethyl alcohol have been shown to impart floral, rose-like off-flavors, and the Tyr catabolite *p*-cresol imparts barny, medicinal, or utensil-like off-flavors (15, 22, 32). Mechanisms for the production of these compounds in cheese have not been conclusively established, but AAA catabolism by lactococci and lactobacilli under simulated cheese-ripening conditions is initiated by aminotransferase (ATase) enzymes that convert AAAs into corresponding α-keto acids (17, 20, 21, 39). Moreover, the aromatic α-keto acids produced by these reactions can be nonenzymatically converted to benzaldehyde, phenylacetaldehyde, 2-phenethyl alcohol, and other aroma compounds (17, 19, 20, 21, 38). However, many lactic acid bacteria possess hydroxy acid dehydrogenases (HADH) such as D-hydroxyisocaproic acid dehydrogenase (D-Hic) that convert α-keto acids to α-hydroxy acids (7, 23), and these enzymes are active in cells incubated under cheese-ripening conditions (20, 21). Since α-hydroxy acids do not make a significant contribution to flavor development (38), this class of enzymes could be useful in controlling off-flavor development via their ability to divert the spontaneous degradation of AAA-derived α-keto acids to more-stable enzymatic products.

The D-Hic enzyme participates in stereospecific reduction of straight- and branched-chain aliphatic and aromatic α-keto acids. The substrate pool for this NAD^+-dependent enzyme includes α-ketoisocaproic acid and α-keto acids derived from other branched-chain amino acids, as well as α-keto acids derived from AAAs and Met (23). Because α-keto acids formed from Met and branched-chain amino acids are thought to make important contributions to cheese aroma (38), a potential limitation of D-Hic overexpression in cheese may be suppressed development of both undesirable and desirable cheese aromas.

To investigate the role of D-Hic in cheese flavor, we examined the effect of D-Hic overexpression in a *Lactobacillus casei* adjunct on the chemical and sensory properties of reduced-fat Cheddar cheese. Since addition of α-ketoglutarate (KG) has been shown to stimulate amino acid catabolism in cheese (6, 37), 2% KG (wt/wt) was also blended with the salt used for one-half of the curd from each vat.

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MATERIALS AND METHODS

Chemicals. Amino acids, α-keto acids, NADH, pyridoxal 5-phosphate, KG, erythromycin (ERY), lysozyme, diethyl ether, *N*-undecalactone, and tridecane were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Bacterial strains and plasmids. *Lactococcus lactis* D11 (8) and *L. casei* LC202 were obtained from Rhodia, Inc. (Madison, Wis.), *L. casei* ATCC 334 was obtained from the American Type Culture Collection (Manassas, Va.), and *Escherichia coli* SURE was obtained from Promega Corp. (Madison, Wis.). Stocks of each culture were maintained at −80°C, and working samples were prepared from frozen stocks by two transfers in appropriate broth medium. *Lactococcus lactis* D11 was propagated at 30°C in sterile reconstituted skim milk, while lactobacilli were grown at 37°C in MRS broth (Difco, Detriot, Mich.). *E. coli* was grown at 37°C in Luria-Bertani broth (30) with shaking. Plasmid pTRKH2 (29) was obtained from T. R. Klaenhammer of North Carolina State University, Raleigh.

Isogenic strain construction. The gene encoding *L. casei* LC202 D-Hic (*dhic*) was isolated by PCR and cloned into the high-copy-number vector pTRKH2. Amplification was performed with Expand High Fidelity DNA polymerase (Roche Diagnostics, Indianapolis, Ind.), using 31-mer forward (5′-AAGCACT CGAGATACCGGTGACTTACCATGG-3′) and reverse (5′-CGTTATCTGCA GATTGCCGTCTCCTTGTTCG-3′) oligonucleotide primers designed from the *L. casei dhic* sequence (24) and concatenated with XhoI and PstI linkers, respectively. Template DNA for PCR was isolated as previously described (25), and then amplification of a 1.5-kbp DNA fragment encoding *dhic* was performed in a Hybaid Thermal Reactor (National Labnet Co., Woodbridge, N.J.) programmed for 35 cycles of 92°C for 30 s, 55°C for 30 s, and 68°C for 180 s. The amplicon was cut with XhoI and PstI, ligated into XhoI and PstI double-digested pTRKH2, and then transformed into *E. coli* SURE by electroporation using standard laboratory methods (30). Transformants were selected on Luria-Bertani agar that contained 500 μg of ERY per ml, plasmid DNA was isolated from Ery^r CFU by the alkaline lysis method (30), and the presence of *dhic* insert DNA was confirmed by agarose gel electrophoresis and DNA sequence analysis. The pTRKH2:*dhic* plasmid construct from a representative clone was selected for further work and designated pHADH.

Transformation of *L. casei* ATCC 334 was performed essentially as described by Ahrne et al. (2). Briefly, stationary-phase cells were inoculated at 2% into 500 ml of MRS (Difco) broth and incubated at 37°C until the suspension reached an A_{600} of 0.8. The cells were harvested by centrifugation at 5,000 × *g*, washed twice with sterile, distilled water, and suspended in 2.5 ml of ice-cold, sterile 30% polyethylene glycol 1450 (Sigma Chemical Co.). Three microliters of pHADH or pTRKH2 was mixed with 100 μl of cell suspension in a 0.2-cm electroporation cuvette and placed on ice for 3 min. An electric pulse was delivered in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) set to the following parameters: 2.5 kV, 25 μF, and 200 Ω. After electroporation, 0.9 ml of warmed (37°C) MRS broth was added, and the cells were incubated at 37°C for 2 h. Transformants were collected on MRS agar that contained 5 μg of ERY per ml, and then cell lysates were prepared by the method of Anderson and McKay (3) and uptake of pTRKH2 or pHADH was confirmed by agarose gel electrophoresis. Representative isolates of *L. casei* ATCC 334 transformed with pTRKH2 or pHADH were selected for further work and designated *L. casei* 334e and the *L. casei* HADH strain, respectively.

D-Hic activity. The D-Hic activity in cell lysates from *L. casei* 334e and the *L. casei* HADH strain was measured spectrophotometrically as previously described (20) with phenylpyruvic acid (PPA), indole pyruvic acid, *p*-hydroxyphenylpyruvic acid, and 2-ketoisocaproate as substrates. Specific activity was expressed as micromoles of NADH consumed per milligram of protein per minute, and the values reported represent the means from duplicate experiments replicated on two separate days.

Cheese manufacture. Frozen cell preparations of *L. casei* 334e and the *L. casei* HADH strain (approximately 10^8 CFU per ml after thawing) were prepared by Rhodia, Inc., and then duplicate vats of 50% reduced-fat Cheddar cheese were manufactured on the same day and from the same milk supply at the University of Wisconsin—Madison from 250-kg lots of pasteurized milk (1.3% fat) as described previously (8). Cheese was made with three different starter culture blends: 1.5% (wt/wt) *Lactococcus lactis* D11 bulk starter grown overnight in skim milk without pH control (pH ~4.6), 1.5% D11 starter plus 25 ml of *L. casei* 334e cell preparation, and 1.5% D11 starter plus 25 ml of *L. casei* strain HADH cell preparation. After milling, one half of the curd from each vat was dry salted with 2.8% sodium chloride (wt/wt), while the other half was salted with 2.8% NaCl plus 2% (wt/wt) KG. The cheeses were hooped into 9-kg blocks, pressed overnight, and then vacuum packaged and ripened at 7°C.

Samples of each cheese (approximately 20 g) were collected at monthly intervals for enumeration of starter and nonstarter bacteria as described previously (8). Enumeration of the *L. casei* 334e and HADH strains in cheese was performed by incubation at 37°C on MRS agar that contained 5 μg of ERY per ml.

Cheese volatile analysis. Investigation of cheese volatile compounds was performed using gas chromatography and mass spectrometry (GC-MS) by the method of Colchin et al. (10). Approximately 100 g of each sample was collected after 3 months of ripening and stored in glass jars at −80°C until needed. Samples for GC-MS were prepared from 10 g of shredded cheese mixed with 40 ml of distilled water. *N*-Undecalactone and tridecane were added at 1 μg per g of cheese as internal standards, and cheese extracts were purged with nitrogen gas at a rate of 800 ml per min for 40 min in a circulating water bath (35 ± 1°C). Adsorbent traps (ORBO-100; Supelco, Bellefonte, Pa.) used during the sample purge were subsequently eluted with distilled diethyl ether. The first 2 ml of solvent eluate was collected and concentrated under nitrogen to approximately 100 μl for sample injection. Separation of volatile compounds collected from cheese samples was achieved using a Hewlett-Packard (Avondale, Pa.) model 6890 gas chromatograph equipped with a 60-m by 0.25-mm (inside diameter) capillary StabilWax DA column (Restek, Bellefonte, Pa.) with a 0.5-μm film thickness. The chromatography parameters included an initial temperature of 40°C for the first 4 min, which was increased at a rate of 7°C per min to a final temperature of 220°C. A column flow rate of 1.5 ml per min was maintained following injection of a 2-μl sample.

MS (Hewlett-Packard 5973 series) of cheese volatiles was performed in electron impact ionization mode with an ion source temperature of 230°C, ionization voltage of 70 eV, and mass-scan range between 29 and 400 *m/z*. Identities and quantities of volatile compounds were determined by internal-standard corrected integration responses of known standards and by comparison of mass spectra against those of a standard database (database mass spectral library, version 1.6d; National Institute of Standards and Technology, Gaithersburg, Md).

Sensory evaluation. Sensory attributes of 3-month-old cheeses were evaluated in duplicate (2 samples from each treatment × 2 evaluations per sample) by judges with more than 150 h of individual training in descriptive sensory analysis of cheese. Cheeses were evaluated for 16 flavor attributes defined by a descriptive sensory language for Cheddar cheese flavor (13, 14).

Statistics. The effects of culture treatment or addition of KG on cheese volatiles and sensory character were evaluated by statistical analysis of variance (ANOVA) with SAS software (SAS Institute, Inc., Cary, N.C.) using standardized peak areas from GC-MS data. When treatment effects were significant, least-significant-difference pairwise comparison tests were performed to identify the treatment that produced the effect. Some data were subject to nonlinear log transformations to normalize data and meet assumptions of homogenous variance.

TABLE 1. HADH activity in cell extracts of *L. casei* 334e and the HADH strain

Substrate	Activity in[a]:	
	L. casei 334e	*L. casei* HADH strain
2-Ketoisocaproic acid	1.2 ± 0.2	6.0 ± 0.2
Indolepyruvic acid	0.3 ± 0.1	1.8 ± 0.2
PPA	23.9 ± 1.1	122.6 ± 6.1
ρ-Hydroxyphenylpyruvic acid	35.1 ± 1.3	113.8 ± 3.2

[a] Micromoles of NADH consumed per milligram of protein per minute × 10^{-1} (± standard error).

RESULTS

Enzyme activity in cell lysates of *L. casei* isogens. The HADH activity in the *L. casei* HADH strain was significantly higher ($P < 0.05$) than that in *L. casei* 334e for all substrates tested (Table 1). As shown in Table 1, the difference in HADH activity between these strains ranged from three- to sixfold, depending upon the substrate.

Cheese composition. The pHs of experimental cheeses after pressing ranged from 4.7 to 5.3 and were significantly lower ($P < 0.05$) in cheeses with added KG. By day 3, the range of cheese pHs had narrowed (pH 4.8 to 5.0), but pHs continued to be significantly lower ($P < 0.05$) in cheeses with added KG.

TABLE 2. Numbers of viable starter, adjunct, and nonstarter lactic acid bacteria in experimental Cheddar cheeses during ripening

Cheese treatment	No. of CFU/g of cheese[a]								
	Starter			Adjunct			Nonstarter		
	3 days	1 mo	3 mo	3 days	1 mo	3 mo	3 days	1 mo	3 mo
1A	3.2×10^8	4.7×10^8	5.9×10^5	ND	ND	ND	3.7×10^1	2.0×10^4	3.2×10^7
1B	1.0×10^8	2.1×10^7	9.5×10^5	ND	ND	ND	1.6×10^1	7.8×10^4	3.1×10^7
2A	8.4×10^7	2.6×10^8	1.8×10^8	5.8×10^7	3.5×10^7	1.8×10^7	5.7×10^1	3.0×10^4	1.1×10^6
2B	1.4×10^8	1.6×10^8	1.8×10^8	4.0×10^7	2.0×10^7	6.6×10^6	2.2×10^2	3.7×10^4	2.1×10^6
3A	2.4×10^8	3.0×10^7	2.6×10^7	7.9×10^6	2.0×10^7	4.3×10^4	7.6×10^3	1.6×10^5	1.0×10^7
3B	3.0×10^8	1.9×10^7	5.0×10^6	9.7×10^6	3.2×10^7	9.0×10^3	7.7×10^3	7.6×10^4	2.4×10^7

[a] Treatments: 1, *Lactococcus lactis* D11 alone; 2, D11 plus *L. casei* 334e; 3, D11 plus *L. casei* HADH strain; A, cheese with 2% KG added; B, cheese made without KG. ND, none detected.

In contrast, there was no significant difference in the moisture, fat, and salt contents of experimental cheeses. Mean percent values (±standard error of the mean) for moisture, fat, and salt contents, respectively, were 48.5 (±1.9), 14.3 (±0.6), and 1.2 (±0.3) for D11 control cheese; 48.3 (±2.2), 14.1 (±0.6), and 1.2 (±0.2) for cheese with D11 plus *L. casei* 334e; and 47.7 (±1.9), 14.3 (±0.6), and 1.2 (±0.3) for cheese with D11 and the *L. casei* HADH strain.

As shown in Table 2, adjunct levels in reduced-fat Cheddar cheese made with *L. casei* 334e or the HADH strain approached or exceeded 10^7 CFU per g after 3 days and remained above 10^7 CFU per g at 1 month of ripening. After 3 months of ripening, however, viable counts of the *L. casei* HADH strain declined by about 3 orders of magnitude, while numbers of *L. casei* 334e showed little change. Interestingly, colonies of adjunct (ERY-resistant) lactobacilli recovered from cheeses containing KG were notably larger (>2 mm) than the pinhead-size colonies (<1 mm) obtained from cheeses without KG. Experimental cheeses also contained 10^1 to 10^3 nonstarter lactic acid bacteria per g at day 3, and the levels of these bacteria exceeded 10^6 CFU per g by 3 months in all cheeses (Table 2).

Effect of culture treatment on cheese volatile content. Cheese volatile analysis identified a variety of alcohols, ketones, aldehydes, fatty acids, and sulfur compounds in experimental cheeses (Table 3). Statistical ANOVA detected significant differences in the concentrations of several volatile compounds in cheeses made with different culture blends. Specifically, levels of 1-octen-3-ol, 1-ethylhexanol, 3-hydroxy-2-butanone (acetoin), nonanal, benzaldehyde, and acetic acid were significantly higher ($P < 0.05$) in cheese made with *Lactococcus lactis* D11 starter plus *L. casei* 334e adjunct than in cheeses made with D11 alone or D11 plus *L. casei* HADH strain adjunct (Table 3). In addition, cheeses made with *Lactococcus lactis* D11 starter plus *L. casei* 334e adjunct contained significantly ($P < 0.1$) higher levels of 2-heptanone, pentanoic (valeric) acid, and dimethyl sulfide than cheeses made with D11 plus *L. casei* HADH strain adjunct. Although the differences were not statistically significant, concentrations of most other alcohols, ketones, aldehydes, and fatty acids detected in the study were highest in cheese made with *Lactococcus lactis* D11 starter plus *L. casei* 334e adjunct (Table 3). Exceptions to this observation included 8-nonen-2-one, which was not detected in any cheese made with this culture blend, and a few compounds whose concentrations were highest in cheeses made with starter alone (3-methyl-1-butanol, 2-methyl-1-propanol, 2-heptanol, 2-phenyl alcohol, 4-methyl-3-penten-2-one, 2-ethylhexanoic acid, and 3-methyl butanoic acid) or D11 plus *L. casei* HADH strain adjunct (1-heptanol, 2-undecanone, and hexanoic acid).

Effect of added KG on cheese volatile content. Cheeses made with KG contained significantly higher ($P < 0.1$) concentrations of 1-pentanol and 3-hydroxy-2-butanone than cheeses made without KG. In addition, concentrations of many other cheese volatile compounds detected in the study were, on average, higher in cheese made with KG than in those made without added keto acid, but this effect was not as uniform as was noted for different culture treatments (Table 3).

Treatment effects on cheese sensory properties. Reduced-fat Cheddar cheeses made in this study were evaluated by trained judges for cooked or milky, whey, milk fat, diacetyl, sulfur, nutty, fruity, brothy, barny, free fatty acid, and catty flavors and sweet, salt, bitter, and umami tastes (14). Cheeses exhibited similar intensities of cooked or milky, whey, diacetyl, and milk fat flavors and the basic tastes sweet, sour, salty, and umami (data not shown). There was not a culture-KG interaction ($P > 0.1$) for any sensory attribute except sulfur flavor, so only the effects of the main treatment (culture or KG) were investigated for other attributes. As shown in Table 4, significant differences were detected in the intensities of nutty and brothy flavors. Nutty flavor was significantly more intense ($P < 0.05$) in cheese made with *Lactococcus lactis* D11 starter alone than in cheeses made with either *L. casei* adjunct, and the intensity of brothy flavor was significantly higher ($P < 0.1$) in cheese made with D11 plus *L. casei* HADH strain adjunct than in cheeses made with D11 alone.

Incorporation of KG also had a significant effect on cheese sensory scores. Cheeses made with 2% added KG had significantly higher-intensity ($P < 0.05$) bitter taste, and significantly lower-intensity sulfur ($P < 0.05$), nutty ($P < 0.1$), brothy ($P < 0.05$), and barny ($P < 0.05$) flavors than cheeses made without added KG (Table 4).

Statistical analysis of KG-culture interactions. The ANOVA showed that the accumulation of most cheese volatile compounds was not significantly affected by the interaction between KG addition and culture system ($P > 0.1$). However, significantly higher concentrations of benzaldehyde ($P < 0.05$), phenol ($P < 0.05$), 1-pentanol ($P < 0.05$), and 1-hexanol ($P < 0.1$) were detected in cheese made with *L. casei* 334e adjunct and KG than in cheeses made with any other culture combination, with or without added KG. Similar analysis of the effects of the KG-culture system interaction on sensory scores

TABLE 3. Concentrations of volatile compounds in 3-month-old cheeses

Volatile compound	Possible source	Mean concentration in cheese (μg/g)[a]				
		Culture 1	Culture 2	Culture 3	With KG	Without KG
Alcohols						
4-Methyl-2-pentanol	Ketones	0.06 (0.07)	0.32 (0.32)	0.11 (0.05)	0.20 (0.27)	0.13 (0.16)
3-Methyl-1-butanol	Leu	3.73 (3.44)	3.01 (2.15)	3.24 (2.60)	3.30 (2.68)	3.35 (2.63)
2-Methyl-1-propanol	Val	0.32 (0.59)	0.06 (0.08)	0.04 (0.05)	0.23 (0.48)	0.06 (0.07)
1-Pentanol	Unknown	0.06 (0.08)	0.34 (0.40)	0.05 (0.05)	0.27^{A} (0.33)	0.03^{B} (0.05)
2-Heptanol	Unknown	0.05 (0.06)	0.01 (0.02)	0.02 (0.03)	0.03 (0.05)	0.02 (0.04)
1-Hexanol	Unknown	0.44 (0.21)	0.60 (0.78)	0.16 (0.09)	0.58 (0.58)	0.23 (0.28)
1-Octen-3-ol	Fatty acids	0.11^{X} (0.11)	0.81^{Y} (0.60)	0.05^{X} (0.03)	0.43 (0.51)	0.22 (0.48)
1-Heptanol	Unknown	0.01 (0.02)	0.01 (0.02)	0.02 (0.02)	0.01 (0.02)	0.02 (0.03)
2-Ethyl-1-hexanol	Fatty acids	0.36^{X} (0.47)	2.38^{Y} (1.82)	0.06^{X} (0.04)	1.27 (2.03)	0.59 (0.53)
2,3-Butanediol	Pyruvate	0.14 (0.28)	0.78 (1.55)	0.04 (0.09)	0.10 (0.23)	0.55 (1.26)
2-Phenethyl alcohol	Phe	3.77 (2.83)	3.22 (1.51)	2.10 (0.22)	3.03 (2.35)	3.03 (1.36)
Phenol	Tyr	0.94 (0.72)	1.46 (1.43)	0.27 (0.15)	0.54 (0.68)	1.24 (1.17)
Ketones						
2-Heptanone	Fatty acids	0.41^{AB} (0.31)	0.52^{A} (0.08)	0.19^{B}(0.04)	0.30 (0.13)	0.45 (0.28)
3-Hydroxy-2-butanone	Pyruvate	0.64^{X} (0.22)	1.41^{Y}(0.71)	0.61^{X} (0.29)	1.14^{A} (0.67)	0.64^{B} (0.33)
2-Nonanone	Fatty acids	0.39 (0.30)	0.82 (0.52)	0.17 (0.22)	0.37 (0.53)	0.54 (0.34)
4-Methyl-3-penten-2-one	Fatty acids	1.03 (0.83)	0.85 (0.43)	0.47 (0.05)	0.90 (0.69)	0.66 (0.37)
4-Hydroxy-4-methyl-2-pentanone	Fatty acids	0.13 (0.17)	0.50 (0.88)	0.19 (0.04)	0.45 (0.67)	0.10 (0.11)
2-Undecanone	Fatty acids	0.01 (0.01)	0.01 (0.02)	0.03 (0.03)	0.02 (0.02)	0.01 (0.03)
8-Nonen-2-one	Fatty acids	0.01 (0.02)	ND	0.01 (0.03)	ND	0.02 (0.03)
Acetophenone	Phe	0.52 (0.85)	1.36 (1.26)	0.06 (0.02)	0.60 (1.23)	0.70 (0.76)
Aldehydes						
Nonanal	Fatty acids	0.44^{X} (0.33)	1.03^{Y} (0.45)	0.23^{X} (0.13)	0.52 (0.48)	0.62 (0.49)
Decanal	Unknown	0.10 (0.15)	0.43 (0.82)	0.03 (0.05)	0.08 (0.12)	0.29 (0.67)
Hexadecanal	Unknown	0.42 (0.48)	0.55 (0.31)	0.22 (0.20)	0.27 (0.23)	0.52 (0.42)
Octadecanal	Unknown	1.45 (2.29)	1.71 (1.99)	0.06 (0.08)	1.23 (1.72)	0.91 (1.94)
Benzaldehyde	Phe	0.24^{X} (0.14)	0.94^{Y} (0.52)	0.06^{X} (0.02)	0.29 (0.21)	0.54 (0.66)
Fatty acids						
Acetic acid	Pyruvate	1.47^{X} (0.56)	3.78^{Y} (0.95)	0.38^{Z} (0.34)	2.17 (1.78)	1.59 (1.49)
Butanoic acid	Fatty acids	0.75 (0.91)	1.48 (0.70)	0.19 (0.17)	0.73 (0.92)	1.06 (1.07)
Hexanoic acid	Fatty acids	0.26 (0.33)	0.32 (0.19)	0.37 (0.07)	0.34 (0.14)	0.68 (0.95)
2-Ethylhexanoic acid	Unknown	0.20 (0.40)	0.17 (0.23)	0.11 (0.13)	0.21 (0.22)	0.11 (0.20)
Octanoic acid	Fatty acids	0.13 (0.21)	0.45 (0.33)	0.20 (0.15)	0.35 (0.22)	0.17 (0.28)
3-Methyl butanoic acid	Leu	0.49 (0.88)	0.36 (0.45)	0.11 (0.15)	0.16 (0.21)	0.48 (0.75)
Pentanoic acid	Fatty acids	0.14^{A} (0.22)	1.26^{B} (1.08)	0.05^{A} (0.04)	0.59 (0.87)	0.38 (0.82)
Sulfur						
Dimethyl sulfide	Met	0.68^{AB} (0.32)	1.36^{A} (0.68)	0.37^{B} (0.11)	0.66 (0.68)	0.94 (0.50)

[a] Treatments: culture 1, cheeses manufactured with *Lactococcus lactis* D11 alone; culture 2, cheeses manufactured with D11 plus *L. casei* 334e; culture 3, cheeses manufactured with D11 plus the *L. casei* HADH strain; with KG, cheeses manufactured with 2% KG added; without KG, cheeses manufactured without added KG. Values shown in the same row with different superscript letters are significantly different at P values of <0.05 (X, Y, Z) or <0.1 (A, B) from other values for the same treatment group (culture or KG). Values in parentheses denote ± standard errors ($n = 3$ for culture treatment and $n = 2$ for KG treatment). ND, not detected.

showed no effect for any flavor attribute except sulfur notes. Sulfur flavor scores for cheeses made without KG from *Lactococcus lactis* D11 starter alone or D11 plus *L. casei* 334e were significantly higher ($P < 0.05$) than those for cheeses made with any other treatment in this study. Moreover, sulfur flavor notes were significantly lower ($P < 0.05$) in cheese made with KG and D11 alone than in cheeses with KG and either *L. casei* adjunct (*L. casei* 334e or the HADH strain).

DISCUSSION

Analysis of volatile compounds in 3-month-old experimental cheeses by GC-MS detected a variety of ketones, aldehydes, alcohols, fatty acids, and esters and one sulfur compound (Table 3). Statistical analysis showed that cheese volatile composition was significantly affected ($P < 0.1$) by culture treatment, KG addition, and the culture-KG interaction. While all three factors influenced cheese volatile content, most of the statistically significant differences detected between cheese volatile profiles were associated with culture treatment (Table 3). Since prior studies have clearly shown that KG addition significantly enhanced conversion of amino acids into cheese volatile compounds (6, 37), the limited impact of this treatment on the volatile content of cheeses made in this study was somewhat unexpected. One possible explanation for this observation is that *L. casei* ATCC 334 produces glutamate dehydrogenase, which would negate the impact of exogenous KG (34). This hypothesis is supported by the discovery of a putative gene encoding glutamate dehydrogenase in the *L. casei* ATCC 334 genome sequence (accessible through www.jgi.doe.gov).

One objective of this work was to investigate the effect of HADH overexpression on accumulation of AAA-derived volatiles in cheese. As is shown in Table 3, three of these com-

TABLE 4. Descriptive sensory analysis of 3-month-old cheeses

Flavor attribute	Mean scores for experimental cheeses[a]				
	Culture 1	Culture 2	Culture 3	With KG	Without KG
Sulfur	1.38 (0.56)	1.75 (0.76)	1.13 (0.25)	0.75^{X} (0.42)	2.08^{Y} (0.74)
Nutty	1.25^{A} (0.76)	0.25^{B} (0.50)	0.00^{B}(0.00)	0.00^{X} (0.00)	0.83^{Y} (0.68)
Brothy	1.50^{B} (0.41)	2.13^{AB} (1.03)	2.50^{A} (1.08)	1.50^{X} (0.63)	2.58^{Y} (0.86)
Barny	0.63 (0.75)	0.63 (0.95)	0.50 (0.58)	0.00^{X} (0.00)	1.17^{Y} (0.52)
Bitter	1.50^{A} (1.47)	1.50^{A} (1.08)	0.88^{B} (0.48)	2.08^{X} (0.86)	0.50^{Y} (0.32)

[a] Treatments: culture 1, cheeses manufactured with *Lactococcus lactis* D11 alone; culture 2, cheeses manufactured with D11 plus *L. casei* 334e; culture 3, cheeses manufactured with D11 plus the *L. casei* HADH strain; with KG, cheeses manufactured with 2% KG added; without KG, cheeses manufactured without added KG. Flavor and taste intensities were scored on a 10-point scale, where 0 indicates no intensity and 10 indicates extremely high intensity (26). Values shown in the same row with different superscript letters are significantly different ($P < 0.1$) from other values for the same treatment group (culture [A, B] or KG [X, Y]). There was not a culture-KG interaction ($P > 0.1$), so only effects of the main treatment are shown. Values in parentheses denote ± standard errors.

pounds, benzaldehyde, 2-phenylethanol, and phenol, were detected in cheeses made for this study. Although most differences in the concentrations of these compounds between cheeses were not large enough to be statistically significant, the mean level of each compound was lowest in cheese made with the *L. casei* HADH strain. The mechanism for phenol production by lactic acid bacteria is unknown, but benzaldehyde and 2-phenylethanol can be produced by spontaneous degradation of PPA, the α-keto acid that is derived from ATase action on Phe (19, 21). Thus, the significantly lower ($P < 0.05$) level of benzaldehyde in cheese made with the *L. casei* HADH strain versus *L. casei* 334e and the parallel trend seen with 2-phenylethanol suggest that enhanced D-Hic activity in experimental cheeses served to limit the spontaneous decomposition of AAA-derived α-keto acids.

While this observation indicates that elevated D-Hic activity in cheese could help to prevent the development of AAA-derived off-flavors, sensory data showed that cheeses manufactured with the *L. casei* HADH strain also had the lowest sensory scores for desirable nutty and sulfur flavors and the highest brothy flavor score (Table 4). In addition, cheese made with KG also had significantly reduced ($P < 0.1$) nutty, sulfur, and brothy flavor scores (Table 4). The identities of the volatile compounds responsible for brothy flavor in Cheddar cheese have not been conclusively determined (31), but this attribute has been linked to methional or furaneols (27). Nutty aroma in cheese has also been attributed to a variety of volatile compounds, including pyrazines or furans (11, 28), and Avsar et al. (4) recently showed that 2-methylpropanal and 2,3-methylbutanal produced nutty flavor in aged Cheddar cheese. Unfortunately, none of these compounds was detected in experimental cheeses prepared in this work, so the chemical basis for nutty or brothy flavors in experimental cheeses remains unclear. However, cheese flavor chemistry is very complex. Volatile-compound recovery (amount and type) varies with extraction approach and instrumentation (31). The lack of detection of nutty flavor compounds does not preclude the possibility that these specific compounds were not recovered by the methods used in the present study or the possibility that other compounds may also contribute to nutty flavor.

A correlation ($r = 0.65$) was noted between sulfur flavor scores and concentrations of dimethyl sulfide in experimental cheeses (Tables 3 and 4). Sulfur flavor is an important component of desirable aroma in aged Cheddar and many other cheese varieties, and this attribute may be produced by a number of volatile sulfur compounds (11). The only volatile sulfur compound identified in experimental cheeses from this study was dimethyl sulfide, which is reported to impart boiled cabbage or sulfurous aromas to cheese (11). Most of the known sulfur aroma compounds, including dimethyl sulfide, arise from microbial catabolism of Met during cheese ripening (12, 36). In lactic acid bacteria, Met catabolism is primarily initiated by one or more ATases to yield the α-keto acid 4-methylthio-2-oxobutyrate, which is subsequently converted into methanethiol and other volatile sulfur compounds via enzymatic and chemical processes (18, 36). However, the recent works of Gao et al. (18) and Demarigny et al. (12) indicate that dimethyl sulfide is not a product of 4-methylthio-2-oxobutyrate but instead is formed by an alternative and still unknown pathway. If dimethyl sulfide was responsible for the sulfur flavor in experimental cheeses, then production of this compound by an alternative pathway would also be consistent with the observation that KG addition (which stimulates ATase activity) led to a significant decrease ($P < 0.1$) in sulfur flavor (Table 4).

Interestingly, culture treatment had a significant effect ($P < 0.1$) on the levels of several compounds (i.e., 1-octen-3-ol, 1-ethylhexanol, 2-heptanone, nonanal, and pentanoic acid) that are derived from fatty acids. As is shown in Table 3, concentrations of these volatiles were significantly lower ($P < 0.1$) in cheese made with the *L. casei* HADH strain than in cheese made with *L. casei* 334e. The mechanisms for production of these compounds in cheese are unclear, but 1-octen-3-ol is probably formed by oxidation of linoleic and linolenic acids, 2-heptanone is probably formed by oxidative decarboxylation of fatty acids, and straight-chain aldehydes like nonanal are probably formed via β-oxidation of unsaturated fatty acids (1, 16). The presence of ketones in Cheddar cheese can be indicative of mold contamination, but no surface mold was observed on any of the experimental cheeses over a 6-month ripening period. Since the documented action of D-Hic is limited to the reduction of straight- and branched-chain aliphatic and aromatic α-keto acids (7), the impact of D-Hic overexpression on synthesis or catabolism of these compounds was most likely indirect (e.g., a consequence of an altered redox state in adjunct lactobacilli).

A more direct role for D-Hic can be invoked as an explanation for the observation that levels of 3-hydroxy-2-butanone and acetic acid were significantly reduced in cheese made with the *L. casei* HADH strain versus *L. casei* 334e (Table 3). Each of these compounds is derived from pyruvate, which can arise

from catabolism of carbohydrate, citrate, or Ala (9). Production of 3-hydroxy-2-butanone occurs when cells experience a surplus of pyruvate relative to the need for NAD^+ regeneration, while acetate is formed via the pyruvate-formate lyase pathway as cells generate ATP from acetyl phosphate. Although D-Hic displays the greatest affinity for C3-substituted α-keto acids, Bernard et al. (7) showed that the *Lactobacillus delbrueckii* subsp. *bulgaricus* enzyme also reduced pyruvate to lactate. Thus, D-Hic overexpression by the *L. casei* HADH strain may have inhibited formation of 3-hydroxy-2-butanone and acetic acid by reducing intracellular levels of pyruvate.

The apparent inhibition of acetate production by the *L. casei* HADH strain is of particular interest, since the pyruvate-formate lyase pathway is induced by substrate limitation and an anaerobic environment (9), which are predominant conditions for ripening cheese. Acetate production through the pyruvate-formate lyase pathway could therefore be an important mechanism for energy production by *L. casei* and other bacteria that grow in ripening cheese. If this hypothesis is correct, inhibition of this pathway by D-Hic overexpression could explain the decline in viable cell counts observed for the *L. casei* HADH strain but not for *L. casei* 334e in 3-month-old cheeses (Table 2).

In summary, this study found that levels of several compounds whose synthesis may be associated with amino acid catabolism, including benzaldehyde, 3-hydroxy-2-butanone, acetic acid, and dimethyl sulfide, were significantly lower in Cheddar cheese manufactured with the *L. casei* HADH strain than in cheese made with an isogenic derivative, *L. casei* 334e. Cheddar made with the *L. casei* HADH strain also had diminished intensity of beneficial sulfur and nutty flavors, and stronger brothy flavor. Because nonstarter lactic acid bacteria populations exceeded 10^6 CFU per g by 3 months in all experimental cheeses (Table 2), some of these changes may not be due exclusively to the action of *L. casei* 334e and *L. casei* HADH strain adjunct cultures. However, statistical analysis showed that culture treatment had significant effects on the cheese volatile content and sensory properties, and the nature of these changes indicated that enhanced D-Hic activity served to redirect the chemical and biochemical conversions of α-keto acids in a manner that retarded overall flavor development. Because this effect is likely a consequence of the relatively broad substrate pool upon which D-Hic can act, it may still be possible to selectively suppress AAA-derived off-flavor production by overexpression of an alternative HADH with a more narrow specificity for AAA-derived α-keto acids. The D-(−)-mandelate dehydrogenase from *Rhodotorula graminis*, for example, displays greater selectivity for aromatic substrates such as PPA (5). Since HADH activity among lactic acid bacteria is both widespread and complex (33, 38), enzymes with similarly narrow specificities may occur in one or more of these species.

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