Tryptophan Catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* Cheese Flavor Adjuncts[1]

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ABSTRACT

Microbial degradation of Trp is thought to promote the formation of aromatic compounds that impart putrid, fecal, or unclean flavors in cheese, but pathways for their production have not been established. This study investigated Trp catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts under carbohydrate starvation (pH 6.5, 30 or 37°C, no sugar) and near cheese-ripening (pH 5.2, 4% NaCl, 15°C, no sugar) conditions. Enzyme assays of cell-free extracts indicated that both species of *Lactobacillus* catabolized Trp to indole-3-lactic acid, and micellar electrokinetic capillary chromatography of culture supernatants showed this reaction occurred via successive transamination and dehydrogenation reactions. Tryptophan decarboxylase activity was also detected in all *Lactobacillus* cell-free extracts, but tryptamine was not detected in culture supernatants. Micellar electrokinetic capillary chromatography showed that Trp metabolism in *Lactobacillus casei* LC301 and LC202 was similar under both incubation conditions and that those catabolic reactions were reversible (i.e., conversion of indole-3-lactic acid to Trp). In contrast, Trp catabolism by *Lactobacillus helveticus* LH212 was only detected under near cheese ripening conditions. Cells of *Lactobacillus helveticus* CNRZ32 did not catabolize Trp in either condition but did convert indole-3-pyruvic acid to Trp in carbohydrate starvation medium and to Trp and indole-3-lactic acid under near cheese ripening conditions.

(**Key words:** *Lactobacillus*, amino acid catabolism, tryptophan, cheese flavor)

Abbreviation key: A = absorptance (used with number indicating wavelength), **AAA** = aromatic AA, **ATase** = aminotransferase, **CDM** = chemically defined AA medium, **CFE** = cell-free extract, **CS** = carbohydrate starvation conditions, **DCOOHase** = decarboxylase, **IAA** = indole-3-acetic acid, **ILA** = indole-3-lactic acid, **ILDHase** = indole-3-lactic acid dehydrogenase, **IPyA** = indole-3-pyruvic acid, **MECC** = micellar electrokinetic capillary chromatography, **NADH** = reduced NAD, **NCR** = near cheese-ripening conditions.

INTRODUCTION

During Cheddar cheese maturation, the amino acids produced from casein degradation may be catabolized by the microorganisms in cheese into compounds that have a strong effect on cheese flavor (7, 8, 13, 17, 26). Even though many of these reactions make positive contributions to cheese flavor (1, 28), the catabolism of aromatic amino acids (**AAA**) is thought to promote off-flavor development (5, 10, 12). Tryptophan catabolism, for example, may lead to the formation of indole and skatole (3-methylindole), which impart putrid, fecal, and unclean flavors in cheese (12, 24). Pathways for the production of these compounds from Trp have been described for some bacteria (21), but mechanisms for their production in cheese have not been established.

Microbial pathways for Trp degradation may involve several different enzymes including Trp aminotransferase (**ATase**) (EC 2.6.1.27) (9, 23), Trp decarboxylase (**DCOOHase**) (EC 4.1.1.28) (22), Trp 2-monooxygenase (EC 1.13.12.3) (3, 16), indole-3-lactic acid dehydrogenase (**ILDHase**) (EC 1.1.1.110) (14), and tryptophanase (EC 4.1.99.1) (4). Gao and associates (10) showed that *Lactococcus lactis*, the starter culture used in Cheddar cheese, catabolized Trp by ATase to indole-3-pyruvic acid (**IPyA**) under conditions found in ripening cheese (pH 5.2, 4% NaCl, 13°C). They (10) also showed that nonviable starters in the cheese matrix may contribute to amino acid catabolism and noted that metabolic interconversion of Trp metabolites by starter bacteria and certain lactobacilli could lead to the production of skatole.

The contribution of lactobacilli to off-flavor development warrants further investigation because these bacteria dominate the population of nonstarter or adventitious bacteria in Cheddar cheese, and because species such as *Lactobacillus casei* and *Lactobacillus helveticus* are commonly used as adjunct cultures to

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intensify flavor development in reduced-fat varieties (6, 25). Although *Lactobacillus* spp. flavor adjuncts are generally reported to enhance cheese flavor intensity (6, 19, 20, 25), these bacteria have also been linked to the development of unclean flavors (12, 18, 24). Research by Guthrie (12), for example, showed that cheese with unclean flavor manufactured with *Lb. casei* adjuncts contained indole and other AAA metabolites. Indole has also been detected in the aroma concentrate of *Lb. helveticus* cultures and cheeses (18), and some ruminal *Lactobacillus* spp. can produce skatole from the Trp catabolite indole-3-acetic acid (**IAA**) (29). As a whole, these data suggest that the catabolism of Trp or Trp metabolites by lactobacilli may be an important source of off-flavor compounds in cheese. To further clarify the role of *Lactobacillus* spp. in cheese off-flavor development, we investigated Trp catabolism by *Lb. casei* and *Lb. helveticus* cheese flavor adjuncts under carbohydrate starvation and near cheese-ripening conditions.

MATERIALS AND METHODS

Bacterial Strains

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

Preparation of Cell-Free Extracts

Lactobacillus enzymes involved in Trp catabolism were identified from assays of cell-free extracts (**CFE**) prepared from 200 ml of an overnight APT culture. The bacteria were harvested by centrifugation for 15 min at 3500 × *g* (4°C); washed twice in carbohydrate-free, chemically defined amino acid medium (**CDM**) (10) that lacked L-Trp; and then suspended in 25 ml of the CDM. For studies of Trp catabolism under carbohydrate starvation conditions (**CS**), 1 ml of the cell suspension was transferred into each of 4 test tubes that contained 9 ml of CDM with or without 5 m*M* L-Trp (8 tubes total), then the test tubes were incubated at 30 (*Lb. casei*) or 37°C (*Lb. helveticus*). To investigate Trp catabolism in an environment that simulated some of the conditions found in ripening cheese (10), 1 ml of the cell suspension was also transferred into each of 4 test tubes that contained 9 ml of CDM that lacked sugar, contained 4% salt, had been adjusted to pH 5.2 with lactic acid, and either contained or lacked 5 m*M* L-Trp. Bacteria suspended in the latter medium (henceforth described as near cheese-ripening conditions; **NCR**) were incubated at 15°C.

Cells incubated under CS or NCR were harvested by centrifugation at time 0 (inoculation) and after 7, 14, and 21 d of incubation, then washed twice with 50 m*M* phosphate buffer (pH 6.5), and suspended in 1 ml of phosphate buffer. Cell-free extracts were prepared by sonic disintegration of the cell suspension with a Branson cell disruptor 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 3500 × *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.

Identification of Enzymes Involved in Trp Catabolism

Tryptophan ATase was measured spectrophotometrically at an absorptance of 327 nm ($\mathbf{A_{327}}$) as described by Frankenberger and Poth (9). The reaction mixture contained 5 m*M* L-Trp, 5 m*M* α-ketoglutarate, 50 μ*M* 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 CFE to obtain a total volume of 1 ml, and the reaction mixture was incubated at 30°C for 30 min. The production of IPyA was measured by the increase in A_{327}, and Trp ATase specific activity was expressed as micromoles of IPyA per milligram of protein per min. Control reactions without substrate, without CFE, and without substrate and CFE were included in this and all other enzyme experiments.

After transamination, IPyA may be reduced to indole-3-lactic acid (**ILA**) by ILDHase (15). Cell-free extracts were assayed for ILDhase by the spectrophotometric method of Hummel and coworkers (14) in which the decrease in reduced NAD (**NADH**) is measured at A_{340}. The reaction mixtures contained 250 μl CFE, 50 m*M* sodium phosphate (pH 6.5), 0.2 m*M* IPyA, and 0.3 m*M* NADH in a total volume of 1 ml. The specific activity of ILDHase was reported as micromoles of NADH consumed per milligram of protein per min.

Tryptophan decarboxylase catalyzes the conversion of Trp to tryptamine. The Trp DCOOHase activity was measured by the spectrophotometric assay of Nakazawa and associates (22) in which the production of tryptamine from L-Trp in NH_4OH-NH_4Cl (pH 9.0) is detected at A_{580}. A 1-ml reaction mixture that contained 20 m*M* L-Trp, 1 m*M* pyridoxal phosphate, 250 m*M* NH_4OH-NH_4Cl, and 250 μl CFE was prepared, and then the mixture was incubated at 30°C for 30

min. Then, 0.5 ml of the enzyme reaction mixture was added to 3 ml of a freshly prepared solution of 2% *p*-dimethyl aminobenzaldehyde in concentrated HCl-ethanol (1:3) and heated at 50°C for 40 min in a water bath. The specific activity of Trp DCOOHase was expressed as micromoles of tryptamine formed per milligram of protein per min.

Lactobacillus CFE were also assayed for tryptophanase and Trp 2-monooxygenase using the spectrophotometric assays described by Sigma Chemical Co. (St. Louis, MO) and by Hutcheson and Kosuge (16), respectively. Enzyme-specific activities presented in this study represent the mean from duplicate experiments replicated on 2 separate d. The effect of time (d 0 versus 21) and incubation environment (CS versus NCR on d 0 and 21) on enzyme specific activities were evaluated by statistical *t* tests (α = 0.05) using Microsoft Excel software (Redmond, WA).

Identification of Trp Catabolites in Culture Supernatants

Micellar electrokinetic capillary chromatography (**MECC**) was used to detect Trp catabolites in culture supernatants. Cells for MECC studies were prepared from 200 ml of culture grown overnight in APT broth. The bacteria were harvested by centrifugation at 4500 × g (4°C), washed twice in CDM that lacked Trp, then suspended in CDM that lacked carbohydrate and either did or did not contain 5 m*M* L-Trp or one of the following Trp catabolites: IPyA, ILA, IAA, indole-3-acetamide, and indole-3-propionic acid. The cells were incubated at 30 (*Lb. casei*) or 37°C (*Lb. helveticus*), and 3 ml of the suspension was collected for MECC at time 0 (inoculation) and at six weekly intervals thereafter. Sample pH was also recorded, and viable cell counts were obtained from each sample 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 syringe mounted filter (0.20-μm, cellulose acetate; Corning, Palo Alto, CA), diafiltered through a Filtron (Northborough, MA) 1 K cut-off Microsep concentrator, and then diluted 1:5 in a 50 m*M* sodium tetraborate buffer immediately prior to injection. The MECC was performed as described by Strickland and associates (27) with an automated capillary electrophoresis system (Beckman Instruments P/ACE 2000; Fullerton, CA) with System Gold software (version 7.11). Peaks that contained Trp catabolites were identified by comparison between the electropherograms obtained from the supernatants of cells incubated with and without 5 m*M* L-Trp or a Trp catabolite. The compound present in each of those peaks was subsequently identified by coinjection with pure standards and by a correlation (r) greater than 0.9 between the absorption spectrum for an unknown compound to that of a known standard. Uninoculated tubes that contained CDM with 5 m*M* of L-Trp or individual Trp catabolites were also included in each experiment as controls to detect nonenzymatic chemical degradations.

RESULTS

Enzymes Involved with Trp Degradation in Lactobacilli

Tryptophan ATase, ILDHase, and Trp DCOOHase activities were detected in CFE from *Lb. casei* LC301, *Lb. casei* LC202, *Lb. helveticus* CNRZ32, and *L. helveticus* LH212 that had been incubated in CDM with L-Trp under CS or NCR. Tryptophanase and Trp monooxygenase were not detected in CFE from lactobacilli incubated in either condition.

TABLE 1. Specific activity of tryptophan aminotransferase[1] in cell-free extracts from lactobacilli incubated in chemically defined carbohydrate starvation[2] (CS) or near cheese-ripening[3] (NCR) medium spiked with 5 m*M* L-tryptophan.

Incubation time (d)	*Lactobacillus casei*				*Lactobacillus helveticus*			
	LC301		LC202		LH212		CNRZ32	
	CS	NCR	CS	NCR	CS	NCR	CS	NCR
0	2.4 ± 0.5	3.0 ± 0.1	3.5 ± 0.1	4.0 ± 0.7	1.6 ± 0.4	1.7 ± 0.4	1.8 ± 0.2	1.6 ± 0.1
7	1.9 ± 0.2	1.4 ± 0.0	1.5 ± 1.0	3.7 ± 1.0	3.3 ± 2.0	2.2 ± 0.0	3.5 ± 1.3	1.8 ± 0.3
14	2.7 ± 1.5	1.9 ± 0.6	4.0 ± 0.9	2.9 ± 1.4	0.9 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	1.8 ± 0.6
21	0.5 ± 0.0	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.7 ± 0.0

[1]Micromoles of indole pyruvic acid produced per milligram of protein per minute × 10^{-4} (±SD). Values represent the mean from duplicate experiments replicated on 2 separate d.

[2]Chemically defined medium with no carbohydrate, pH 6.5, no salt. Cells were incubated at 30°C (*Lb. casei*) or 37°C (*Lb. helveticus*).

[3]Chemically defined medium with no carbohydrate, pH 5.2, with 4% (wt/vol) NaCl. Cells were incubated at 15°C.

TABLE 2. Specific activity of indole lactate dehydrogenase[1] in cell-free extracts from lactobacilli incubated in chemically defined carbohydrate starvation[2] (CS) or near cheese-ripening[3] (NCR) medium spiked with 5 m*M* L-tryptophan.

	Lactobacillus casei				*Lactobacillus helveticus*			
	LC301		LC202		LH212		CNRZ32	
Incubation time (d)	CS	NCR	CS	NCR	CS	NCR	CS	NCR
0	1.1 ± 0.6	1.9 ± 0.5	2.4 ± 1.1	2.4 ± 1.1	1.5 ± 0.2	1.8 ± 0.2	3.6 ± 1.4	4.9 ± 0.6
7	2.4 ± 0.9	2.1 ± 0.8	6.5 ± 1.2	4.1 ± 2.0	3.0 ± 1.3	2.5 ± 1.9	5.1 ± 1.6	1.9 ± 2.1
14	4.0 ± 2.7	2.8 ± 1.7	6.5 ± 2.8	4.2 ± 1.8	3.9 ± 2.3	1.0 ± 1.1	5.5 ± 2.7	3.8 ± 2.6
21	1.7 ± 0.9	1.6 ± 0.7	1.9 ± 0.4	2.2 ± 1.1	0.4 ± 0.4	0.6 ± 0.4	1.2 ± 1.0	1.6 ± 1.0

[1]Micromoles of NADH consumed per milligram of protein per minute × 10^{-2} (±SD). Values represent the mean from duplicate experiments replicated on 2 separate d.

[2]Chemically defined medium with no carbohydrate, pH 6.5, no salt. Cells were incubated at 30°C (*Lb. casei*) or 37°C (*Lb. helveticus*).

[3]Chemically defined medium with no carbohydrate, pH 5.2, with 4% (wt/vol) NaCl. Cells were incubated at 15°C.

The mean specific activity of L-Trp ATase in *Lb. casei* and *Lb. helveticus* strains incubated in CDM with L-Trp ranged from 0.1 to 4.0 × 10^{-4} μmoles/mg of protein/min under CS or NCR (Table 1). Tryptophan ATase specific activity decreased significantly ($P < 0.05$) by d 21 in *Lb. casei* LC202 and *Lb. helveticus* CNRZ32 incubated in CDM with L-Trp under both incubation conditions, but *Lb. casei* LC301 cells only showed a decrease when incubated under NCR. No significant differences ($P > 0.05$) in specific activity were observed over time in *Lb. casei* LC301 incubated under CS or in *Lb. helveticus* LH212 incubated under CS or NCR. In general, incubation conditions did not significantly affect Trp ATase specific activity on d 0 or 21, although the enzyme activity in *Lb. helveticus* CNRZ32 was significantly ($P < 0.05$) higher on d 21 under NCR versus CS.

The mean specific activity of ILDHase in cells incubated in CDM with L-Trp under CS or NCR ranged from 1.6 to 6.5 × 10^{-2} μmoles/mg of protein/min in *Lb. casei* strains and from 0.4 to 5.5 × 10^{-2} μmoles/mg of protein/min in *Lb. helveticus* LH212 and CNRZ32 (Table 2). Incubation condition or time did not have a significant ($P > 0.05$) effect on ILDHase activity in any of the *Lactobacillus* strains tested, but this observation may be limited by the relatively high variability in enzyme activity noted at some time points.

Tryptophan decarboxylase activity in cells incubated in CDM with L-Trp under CS or NCR ranged from a mean of 0.3 to 1.4 × 10^{-3} μmoles/mg of protein/min for *Lb. casei* strains and from 1.6 to 5.1 × 10^{-3} μmoles/mg protein/min for *Lb. helveticus* strains (Table 3). Tryptophan decarboxylase activity in *Lb. casei* or *Lb. helveticus* generally did not vary significantly ($P > 0.05$) with time or incubation condition, except that specific activity in *Lb. casei* LC202 decreased significantly ($P < 0.05$) with time under NCR. However, this result may again reflect the variability in enzyme

TABLE 3. Specific activity of tryptophan decarboxylase[1] in cell-free extracts from lactobacilli incubated in chemically defined carbohydrate starvation[2] (CS) or near cheese-ripening[3] (NCR) medium spiked with 5 m*M* L-tryptophan.

	Lactobacillus casei				*Lactobacillus helveticus*			
	LC301		LC202		LH212		CNRZ32	
Incubation time (d)	CS	NCR	CS	NCR	CS	NCR	CS	NCR
0	0.7 ± 0.4	1.1 ± 0.4	1.0 ± 0.2	1.0 ± 0.0	2.0 ± 0.5	1.9 ± 0.7	2.6 ± 0.9	2.2 ± 0.2
7	0.3 ± 0.2	1.2 ± 0.1	0.4 ± 0.1	1.2 ± 0.6	3.5 ± 1.0	2.4 ± 1.2	1.6 ± 0.0	1.6 ± 0.5
14	1.4 ± 0.3	0.6 ± 0.0	1.2 ± 0.0	0.5 ± 0.2	2.3 ± 1.4	2.0 ± 1.5	3.3 ± 0.7	5.1 ± 0.1
21	1.0 ± 0.4	1.5 ± 0.9	1.0 ± 0.4	0.3 ± 0.1	2.8 ± 0.8	2.8 ± 1.4	5.0 ± 2.9	4.9 ± 1.2

[1]Micromoles of tryptamine formed per milligram of protein per minute × 10^{-3} (±SD). Values represent the mean from duplicate experiments replicated on 2 separate d.

[2]Chemically defined medium with no carbohydrate, pH 6.5, no salt. Cells were incubated at 30°C (*Lb. casei*) or 37°C (*Lb. helveticus*).

[3]Chemically defined medium with no carbohydrate, pH 5.2, with 4% (wt/vol) NaCl. Cells were incubated at 15°C.

activity that was observed at some of the sampling times. The specific activities of Trp ATase, ILDHase, and Trp DCOOHase were also measured in CFE from cells incubated in CDM without L-Trp, and mean values were found to display similar trends (data not shown).

MECC Analysis

Media pH remained constant for cells incubated under CS (pH 6.5) and NCR (pH 5.2) conditions during the course of the study, but cell viability decreased substantially. Under NCR, *Lactobacillus* spp. counts dropped from 10^8 to less than 10^1 cfu/ml during the 6-wk incubation period (Figure 1, upper). A similar trend was noted in the CS medium, except that in CDM with ILA, populations of all four lactobacilli remained greater than 10^2 cfu/ml after 6 wk (Figure 1, lower).

As shown in Figure 2, MECC in 60 m*M* SDS-100 m*M* sodium tetraborate running buffer can be used to separate L-Trp and 11 other Trp metabolites. As is summarized in Table 4, MECC of culture supernatants showed that Trp metabolism by *Lb. casei* LC301 and LC202 was similar under CS and NCR. Tryptophan was converted to ILA (Figure 3, upper), and incubations in CDM with ILA led to the production of Trp (Figure 3, middle). Supernatants from cells incubated in CDM with IPyA contained Trp and ILA (Figure 3, lower). Indole-3-aldehyde and IAA were also detected in CDM with IPyA, but these compounds also appeared in cell-free control tubes, which confirmed that they were formed by spontaneous chemical degradation of IPyA (10).

Similar analyses with *Lb. helveticus* LH212 and CNRZ32 indicated that even though these bacteria did not catabolize L-Trp under CS, Trp did accumulate in the supernatant of cells incubated in CDM with ILA. Incubation of these bacteria in CDM with IPyA also resulted in the production of Trp, but ILA was not detected. A slightly different mode of Trp catabolism was noted when *Lb. helveticus* strains were incubated in the NCR medium. The *Lb. helveticus* LH212 converted Trp to ILA in CDM with L-Trp, and both *Lb. helveticus* strains produced Trp and ILA in CDM spiked with IPyA. Features of Trp catabolism by *Lb. helveticus* strains that were similar in cells incubated under NCR or CS included the absence of any detectable catabolic activity in CNRZ32 cells incubated in CDM with L-Trp and the production of Trp by LH212 and CNRZ32 in CDM with ILA.

Finally, MECC of culture supernatant from *Lb. casei* or *Lb. helveticus* strains incubated in CDM that contained IAA, indole-3-acetamide, or indole-3-propionic acid indicated that these compounds were not metabolized by *Lb. casei* or *Lb. helveticus* under CS or NCR.

DISCUSSION

Off-flavor development is a common defect in reduced-fat Cheddar cheese, and previous work (10, 12) has indicated that microbial degradation of AAA in

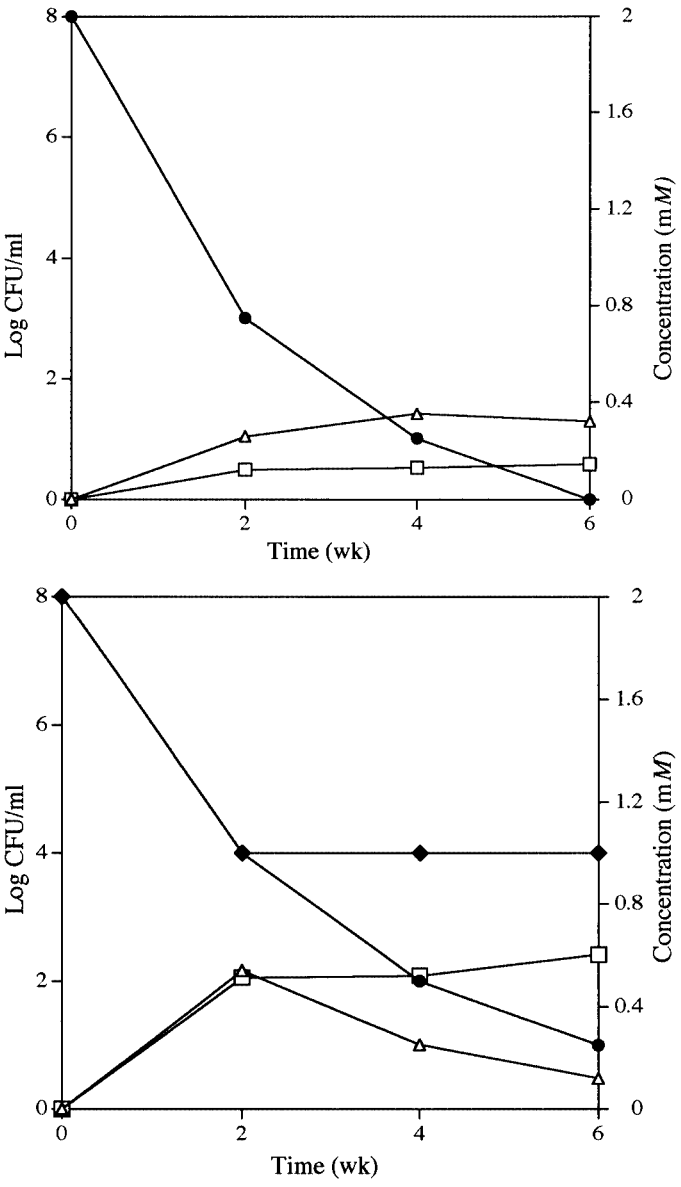

Figure 1. Metabolism of tryptophan (Trp) and indole-3-lactic acid (ILA) under near cheese-ripening (upper) and carbohydrate starvation (lower) conditions by *Lactobacillus casei* LC301. Shown are the concentration of ILA (△) produced in CDM spiked with 5 m*M* Trp and the concentration of Trp (□) produced in CDM spiked with 5 m*M* ILA. The number of colony forming units per milliliter recovered after incubation under cheese-like conditions in CDM spiked with Trp (●) or ILA (◆; lower panel) is also shown.

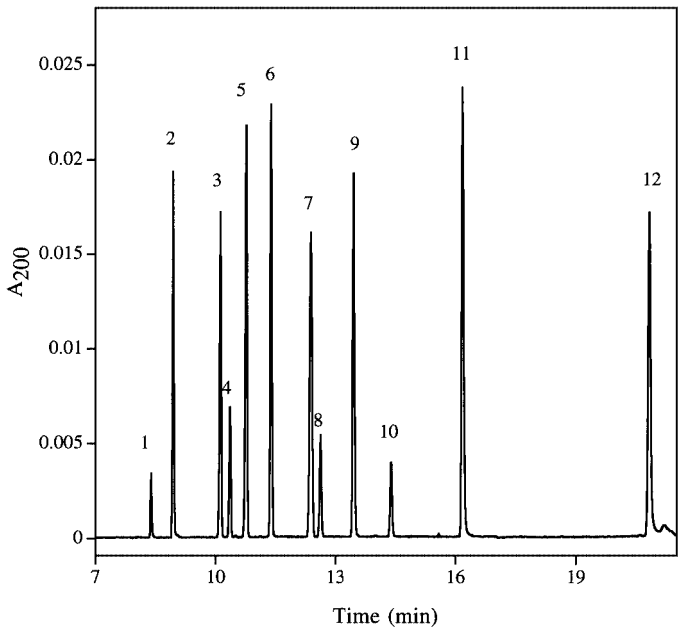

Figure 2. Micellar electrokinetic capillary chromatography of L-tryptophan and tryptophan metabolites in 60 m*M* SDS-100 m*M* sodium tetraborate running buffer. Compounds included in the electropherogram were 1, kynurenine; 2, tryptophan; 3, indole-3-lactic acid; 4, indole-3-propionic acid; 5, indole-3-acetic acid; 6, indole-3-acetamide; 7, anthranilic acid; 8, indole; 9, tryptophol; 10, indole-3-aldehyde; 11, skatole; and 12, tryptamine. A_{200} = absorptance at 200 nm.

cheese may contribute to this problem. This study investigated Trp catabolism by *Lb. casei* and *Lb. helveticus* cheese flavor adjuncts in an effort to improve current understanding of the contribution of dairy lactobacilli to Trp degradation and off-flavor production in Cheddar cheese. Incubations were performed under CS to collect information regarding Trp catabolism when fermentable carbohydrate was not available. The NCR was used to obtain insight into culture physiology as it is likely to occur in the complex and challenging environment of ripening cheese (e.g., absence of fermentable sugar combined with a suboptimal growth temperature, pH of 5.2, and 4% NaCl).

Enzyme assays of CFE from lactobacilli incubated under CS or NCR indicated that these bacteria could catabolize Trp to ILA through successive transamination and dehydrogenation reactions and Trp to tryptamine by decarboxylation. Analysis of culture supernatants by MECC, however, indicated decarboxylation was not an active pathway under the incubation conditions used in this study. Instead, supernatants of *Lb. casei* LC301 and LC202 cultures incubated under CS or NCR in CDM with Trp accumulated ILA, and Trp accumulated in supernatants from cells incubated in CDM with ILA. These data, combined with the finding that all four lactobacilli produced Trp and ILA in CDM spiked with IPyA, support our conclusion that the primary route for Trp catabolism by *Lb. casei* under CS or NCR involved conversion to ILA via successive transamination and dehydrogenation reactions with IPyA as the sole intermediate.

Enzyme data suggested that Trp catabolism by *Lb. casei* and *Lb. helveticus* strains would be similar, but MECC studies showed that the primary function of Trp ATase and ILDHase in *Lb. helveticus* whole cells was Trp anabolism. Nonetheless, incubation of *Lb. helveticus* strains under NCR somehow broadened the activity of these enzymes; LH212 produced ILA from Trp, and CNRZ32 converted IPyA to ILA.

Gao et al. (10) recently showed that the Cheddar cheese starter *Lc. lactis* initiated Trp catabolism via ATase under some of the conditions found in cheese but did not convert IPyA to ILA. Instead, IPyA formed by the starter underwent enzymatic or spontaneous degradation to indole-3-aldehyde and IAA (10). As

TABLE 4. Tryptophan metabolites detected by micellar electrokinetic capillary chromatography of culture supernatant from *Lactobacillus casei* LC301 or LC202.[1]

Incubation medium	Metabolites detected[2]					
	Trp	IpyA	ILA	IAA	IAld	Skatole
CDM	–	–	–	–	–	–
CDM + 5 m*M* Trp	–	–	+	–	–	–
CDM + 5 m*M* IPyA	+	–	+	+	+	–
CDM + 5 m*M* ILA	+	–	–	–	–	–
CDM + 5 m*M* IAA	–	–	–	–	–	–
CDM + 5 m*M* IAM	–	–	–	–	–	–
CDM + 5 m*M* IProA	–	–	–	–	–	–

[1]Cells were incubated in chemically defined (CDM) carbohydrate starvation medium (no carbohydrate, pH 6.5, no salt, 30°C) or near cheese-ripening medium [no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, 15°C] spiked with Trp or a Trp metabolite.

[2]IPyA = indole-3-pyruvic acid, ILA = indole-3-lactic acid, IAA = indole-3-acetic acid, IAld = indole-3-aldehyde, IAM = indole-3-acetamide, and IProA = indole-3-propionic acid. Symbols identify compounds that were (+) or were not (–) detected.

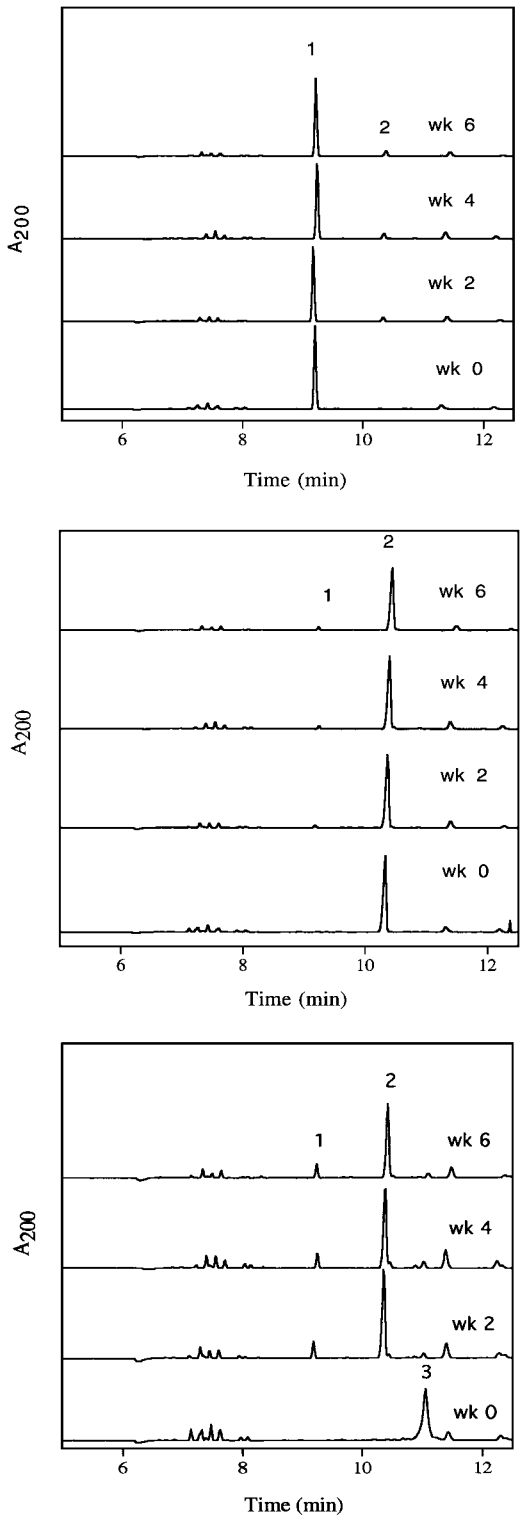

Figure 3. Micellar electrokinetic capillary chromatography of supernatant from *Lactobacillus casei* LC301 incubated under near cheese-ripening conditions in chemically defined medium spiked with 5 m*M* L-tryptophan (upper), indole-3-lactic acid (middle), or indole-3-pyruvic acid (lower). Peaks that were identified in the electropherogram include 1, tryptophan; 2, indole-3-lactic acid; and 3, indole-3-pyruvic acid. A_{200} = absorptance at 200 nm.

noted by those authors (10), these secondary reactions may be important because some lactobacilli can convert IAA into skatole, a compound that imparts very unclean flavors to cheese (12, 29). Our investigation showed that *Lb. casei* and *Lb. helveticus* catabolized Trp via ATase and ILDHase and that Trp catabolism was active in cells incubated in an environment that simulated many of the conditions found in ripening cheese. The specific activity of Trp ATase in *Lc. lactis* (10) is about 10-fold higher than was found in this study for lactobacilli. Because *Lc. lactis* is present at very high cell numbers in cheese during the early stages of ripening, and nonviable cells may contribute to amino acid catabolism (10), starter bacteria likely have a greater role in the initial conversion of Trp to IPyA in the cheese matrix. However, our results suggest that nonstarter and adjunct lactobacilli may have an important role in secondary reactions involving IPyA and other starter-derived aromatic metabolites. For example, the conversion of IPyA to ILA or Trp by *Lb. casei* or *Lb. helveticus* may restrict the production of IAA, which is a known precursor of the very unclean flavor compound skatole (29).

Finally, *Lb. casei* and other nonstarter lactobacilli grow to high numbers in the harsh cheese environment (25), but the metabolic pathways by which this is accomplished remain unclear. Although cell viability decreased substantially during the incubations used in the study, the culturability of *Lb. casei* and *Lb. helveticus* during CS (but not NCR) was clearly improved in CDM with ILA (Figure 2). The conversion of ILA to Trp was detected in supernatant from these cells and from bacteria incubated in CDM with IPyA, but improved survival was only noted in CDM with ILA. Because conversion of ILA to IPyA results in the production of NADH, our data indicate that the generation of reducing power (NADH) may be important to *Lactobacillus* spp. viability when fermentable carbohydrate is not available. Alternative energy sources in the CDM that might be used by carbon-starved lactobacilli include arginine and citrate (2, 11), but neither of these reactions would require NADH production. Some bacteria are able to generate ATP from NADH via proton translocation through the cytochromes (11), but this reaction is also unlikely because lactobacilli lack functional cytochromes (2). Thus, the basis for enhanced cell viability in CDM with ILA during CS is unknown.

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