5-1998

Tryptophan Catabolism by Lactobacillus spp. : Biochemistry and Implications on Flavor Development in Reduced-Fat Cheddar Cheese

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TRYPTOPHAN CATABOLISM BY *LACTOBACILLUS SPP.* : BIOCHEMISTRY AND IMPLICATIONS ON FLAVOR DEVELOPMENT IN REDUCED-FAT CHEDDAR CHEESE

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1998

ABSTRACT

Tryptophan Catabolism by *Lactobacillus spp.* : Biochemistry and Implications on Flavor Development in Reduced-Fat Cheddar Cheese

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Utah State University, 1998

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Amino acids derived from the degradation of casein in cheese serve as precursors for the generation of key flavor compounds. Microbial degradation of tryptophan (Trp) is thought to promote 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 tryptophan catabolism by *Lactobacillus casei* LC301 and LC202 and *Lactobacillus helveticus* CNRZ32 and LH212 cheese flavor adjuncts in carbohydrate starvation (pH 6.5, 30 or 37°C, no sugar) and cheese-like conditions (pH 5.2, 4% NaCl, 15°C, no sugar). Enzyme assays of cell-free extracts revealed both species of *Lactobacillus* catabolized tryptophan to indole lactic acid via indole pyruvic acid through transamination followed by dehydrogenation. Micellar electrokinetic capillary chromatography of culture supernatants showed these enzymes also catalyzed the reverse reactions, i.e., conversion of indole lactic acid to tryptophan. Tryptophan decarboxylase activity was detected in *Lactobacillus* cell-free extracts, but tryptamine was not detected in culture supernatants. Analysis of culture supernatants showed that tryptophan metabolism in *Lactobacillus casei* did not differ between the two conditions of incubation as it did in *Lactobacillus helveticus* LH212 and CNRZ32. *Lactobacillus helveticus* LH212, for example, did not catabolize Trp

in carbohydrate starvation but did in cheese-like conditions. While cells of *L. helveticus* CNRZ32 did not catabolize Trp in either condition, they catabolized indole pyruvic acid to only Trp in carbohydrate starvation and to both Trp and indole lactic acid in cheese-like conditions. Micellar electrokinetic capillary chromatography of culture supernatants incubated under either starvation or cheese-like conditions showed *Lactobacillus casei* strains produced more indole lactic acid, and *Lactobacillus helveticus* strains favored tryptophan anabolic reactions. Based on the results obtained in this study, a putative pathway for the catabolism of tryptophan by lactobacilli in cheese is proposed.

(62 pages)

To Amma and Daddy

ACKNOWLEDGMENTS

I would like to thank Dr. Jeffery R. Broadbent for providing me with such a valuable opportunity and experience. He has been a constant source of encouragement, motivation, and solution to my innumerable research problems. I would also like to acknowledge the friendship and good discussion that I received from my laboratory partners, Hua Wang, Chan Lin, Shelby Caldwell, Ling Wei, Yan Hong Kong, Farideh Chitzah, Deborah Low, and Yan Hong Zhao.

I am grateful to my graduate committee members, Drs. Bart C. Weimer and Gregory J. Pogordski, for their valuable advice and participation. I would also like to acknowledge the support and useful discussion I received from my other colleagues in the department, Mick Blake, Rathna Koka, and Ben Dias. I am thankful for the technical assistance provided by Dr. Marie S. Strickland.

I am especially thankful to Soni Ummadi, my best friend, for her friendship and unwavering encouragement. Finally, I would like to thank my parents; my sister and brother-in-law; my brother; Sinu mama and Ragini atta; my wife; and last but not least my friends for their love and support.

The research presented in this thesis was funded by Dairy Management, Inc. (through the Western Dairy Center) and the Utah Agricultural Experiment Station.

Sanjay Gummalla

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INTRODUCTION

Demand for Cheddar cheese continues to increase and has grown to include lower-fat varieties. However, reduced-fat cheeses suffer from poor Cheddar flavor intensity and greater propensity for off-flavors and bitterness (32). Off-flavors in cheese such as utensil, fecal, rosy or floral, and unclean flavors are commonly associated with characteristic compounds that may be derived from the degradation of aromatic amino acids (**AAA**) (12). Tryptophan (**Trp**) catabolism, for example, may lead to the formation of indole and skatole, which produce putrid fecal and unclean flavors in cheese (25). Pathways for the production of these compounds have been described in other bacteria (for example, many members of the enterobacter family, *Bacillus alvei*, and even some ruminal species of lactobacilli) (10, 57, 66, 67), but mechanisms for their production in cheese are not fully understood. Gao et al. showed Trp catabolism by the starter culture used in Cheddar cheese, *Lactococcus lactis*, was initiated with aminotransferasé activity under optimal or cheese-like incubation (22).

Organisms of the genus *Lactobacillus* comprise the dominating non-starter population in cheese, and *Lactobacillus casei* and *Lactobacillus helveticus* are commonly used as adjunct cultures to intensify flavor development in Cheddar cheese. Although addition of lactobacilli has been shown to enhance cheese flavor intensity (15, 55), these bacteria may also promote the development of unclean flavors (25, 38). This study investigated pathways for catabolism of tryptophan in *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts to gain an improved understanding of the biochemistry of tryptophan metabolism in lactobacilli and its implications on production of aromatic off-flavors in low-fat Cheddar cheese. Enzyme assays of cell-free extracts revealed both species of lactobacilli catabolized tryptophan to indole lactic acid via indole pyruvic acid through transamination and dehydrogenation reactions. Micellar electrokinetic capillary chromatography of culture supernatants showed these enzymes also catalyzed the reverse

reactions, i.e., conversion of indole lactic acid to tryptophan. Tryptophan decarboxylase activity was also detected in *Lactobacillus* cell-free extracts, but tryptamine was not detected in culture supernatants.

LITERATURE REVIEW

Lactic acid bacteria (**LAB**) are a group of Gram-positive, non-sporing, catalase-negative, microaerophillic, and nutritionally fastidious microorganisms that produce lactic acid as a primary product of glucose fermentation. They inhabit a variety of environments including raw agricultural commodities, fermented foods, the oral cavity, and the intestinal and reproductive tracts of humans and animals (59). This group of microorganisms has been an indispensable part of food fermentations and has exhibited potential for human health and nutritional benefits (44). Today, various cheeses, yogurt, sour cream, butter, and fermented milks are manufactured with LAB starter cultures.

Cheddar cheese, for example, is a hard, ripened cheese that is highly prized in North America, Europe, and Australia. In the United States, Cheddar and its related varieties account for 40% of total cheese consumed (23). Cheddar cheese is typically manufactured using the lactic acid bacterium *Lactococcus lactis* as the starter culture. During cheese ripening, the starter bacteria slowly die off and adventitious non-starter lactic acid bacteria (**NSLAB**) grow to levels of 10^6 to 10^7 per gram. In most Cheddar cheese, the NSLAB population is dominated by lactobacilli. The absence of Cheddar flavor development in aseptic, directly acidified cheese has established that starter, NSLAB, and their enzymes are required for proper cheese flavor development (2).

Flavor Development in Cheddar Cheese

Typical Cheddar flavor is characterized by a pleasant, sweet, and aromatic walnut sensation devoid of any single dominant note (2, 3, 40). Some studies, however, attribute Cheddar flavor to a strong sulfur note that is associated with the production of methional or methanethiol (2, 3, 40). In aged cheese, an acrid quality renders cheese sharp (36). Early theories suggested that characteristic Cheddar flavor was the result of a single compound or class of compounds, but these molecules have not been conclusively identified. At present,

the most widely accepted hypothesis for Cheddar flavor development is the Component Balance Theory (47). It states that Cheddar flavor requires numerous compounds, in the right proportions, that include fatty acids, carbonyls, amines, peptides, amino acids, and sulfur derivatives to produce Cheddar flavor characteristics. These compounds are produced from the primary breakdown of milk constituents: carbohydrates, lipids, and proteins and from secondary reactions between corresponding end-products. The concomitant development of flavor that occurs as a consequence of these reactions is called cheese ripening or maturation.

In the early stages of ripening, starter metabolism of cheese peptides, carbohydrates, and fat initiates many of the reactions required for flavor development (8). Although glycolysis and lipolysis will influence Cheddar cheese flavor, proteolysis and its secondary reactions are generally believed to play a more significant role in flavor development (18, 19). Initial caseinolysis (degradation of the major milk protein, casein) is the result of added chymosin, and to a lesser extent, native plasmin. Conversion of the large and medium-sized peptides formed by those enzymes to small peptides, however, is largely the result of microbial proteinases and peptidases (20). With the exception of bitter peptides (14, 62), low molecular mass oligopeptides have no direct flavor contribution, but further hydrolysis of these molecules by starter peptidases produces free amino acids that serve as precursors for the generation of important flavor compounds (1). A list of flavor related compounds derived from amino acids is provided in the Appendix (Table 12).

Flavor Aspects of Reduced-Fat Cheddar Cheese

In recent years, demand for Cheddar cheese has grown to include lower-fat varieties. Unfortunately, reduced-fat cheeses suffer from poor Cheddar flavor intensity and a greater propensity for off-flavors and bitterness (32). Ohren and Tuckey (51), for example, found cheese made from skim or low-fat milk did not develop Cheddar flavor. Decreased

amounts of specific fatty acids and lipolytic products like methyl ketones were noted in cheese with reduced flavor intensity (2, 4, 40, 56). Cheddar aroma was obtained in cheese where milk fat had been replaced with vegetable or mineral oil, however, which suggested that fat may influence flavor by harboring hydrophobic compounds and thereby altering the flavor perception or availability of fat-soluble flavor compounds (17). This possibility is supported by the fact that flavor thresholds of many compounds are usually lower in water than in fat.

Since reduced-fat cheeses typically have some fat replaced with water, accumulation of fat-soluble flavor compounds in the water phase may contribute to off-flavor defects such as meaty brothy, bitter, or unclean flavors which are commonly encountered in lower-fat Cheddar cheese (32). Dunn and Lindsay (12) showed that some microbially derived Strecker-type aroma compounds (compounds derived from the degradation of an amino acid to an aldehyde of one less carbon than the amino acid; for example, leucine to 3-methylbutanal and methionine to methional) were associated with unclean flavor sensations in Cheddar. Phenyl acetaldehyde and phenethanol, for example, were attributed with unclean-rosy flavors, and *p*-cresol was responsible for unclean-barny and utensil-type flavors. Guthrie (25) showed aromatic amino acid metabolites like *p*-cresol and indole imparted utensil and putrid, fecal flavors to Cheddar cheese (see also Table 12 of the Appendix) and suggests these compounds are produced by lactobacilli. This hypothesis is supported by other studies which suggest microbial degradation of Trp, tyrosine (**Tyr**), and phenylalanine (**Phe**) may lead to the formation of persisting unclean flavors in cheese (13, 42, 53, 60, 64). Preliminary experiments in our laboratory have also indicated that some NSLAB are able to produce indole (7). As a group, these studies imply that catabolism of tryptophan by *Lactobacillus* may be an important source of off-flavors in Cheddar cheese.

Metabolism of Tryptophan in Microorganisms

Tryptophan metabolism in bacteria may involve catabolic reactions that result in the formation of a variety of unique compounds (Figure 1) (65). The indole pathway (Figure 1, pathway E), for example, features the single step catalysis of Trp to indole by tryptophanase (**TNase**) (10, 27). This is a common Trp catabolic route in many enterobacteriaceae, and most TNase-producing bacteria exist in the intestinal tract of humans and animals (10). TNase has not been reported in LAB, but some species of NSLAB lactobacilli (e.g., *L. casei*) also occur in the intestine (28, 33). This suggests some lactobacilli may contain TNase and that this enzyme could contribute to indole production in cheese. Indole can also be formed from indole pyruvate, possibly via a carbon-carbon bond cleavage reaction, suggesting other indirect pathways may also contribute to indole production (61).

The aromatic pathway (Figure 1, pathway A) is found in *Bacillus megaterium* and the fungus *Neurospora crassa* (6, 46). The end-product of this pathway is usually anthranillic acid, which is also an intermediate in tryptophan biosynthesis.

The side chain pathway (Figure 1, pathway D), the indole 3-acetamide pathway (**IAM**) (Figure 1, pathway B), and the indole 3-pyruvic acid (**IPyA**) pathway (Figure 1, pathway C) all result in the formation of indole 3-acetic acid (**IAA**) (37, 50, 58). In many anaerobic bacteria, IAA is further broken down to skatole (28, 66). Aromatic aminotransferase (EC 2.6.1.27) (**ATase**) catalyzes conversion of tryptophan to indole pyruvic acid and this enzyme is found in several bacteria including the starter bacterium used in Cheddar cheese, *Lactococcus lactis* (22). More significantly, Gao and associates showed that Trp transaminase activity in *Lactococcus lactis* (22) was retained and sometimes even induced under cheese-like conditions. After transamination, IPyA may be reduced to indole lactic acid (**ILA**) by an NADH-dependent indole-lactate dehydrogenase

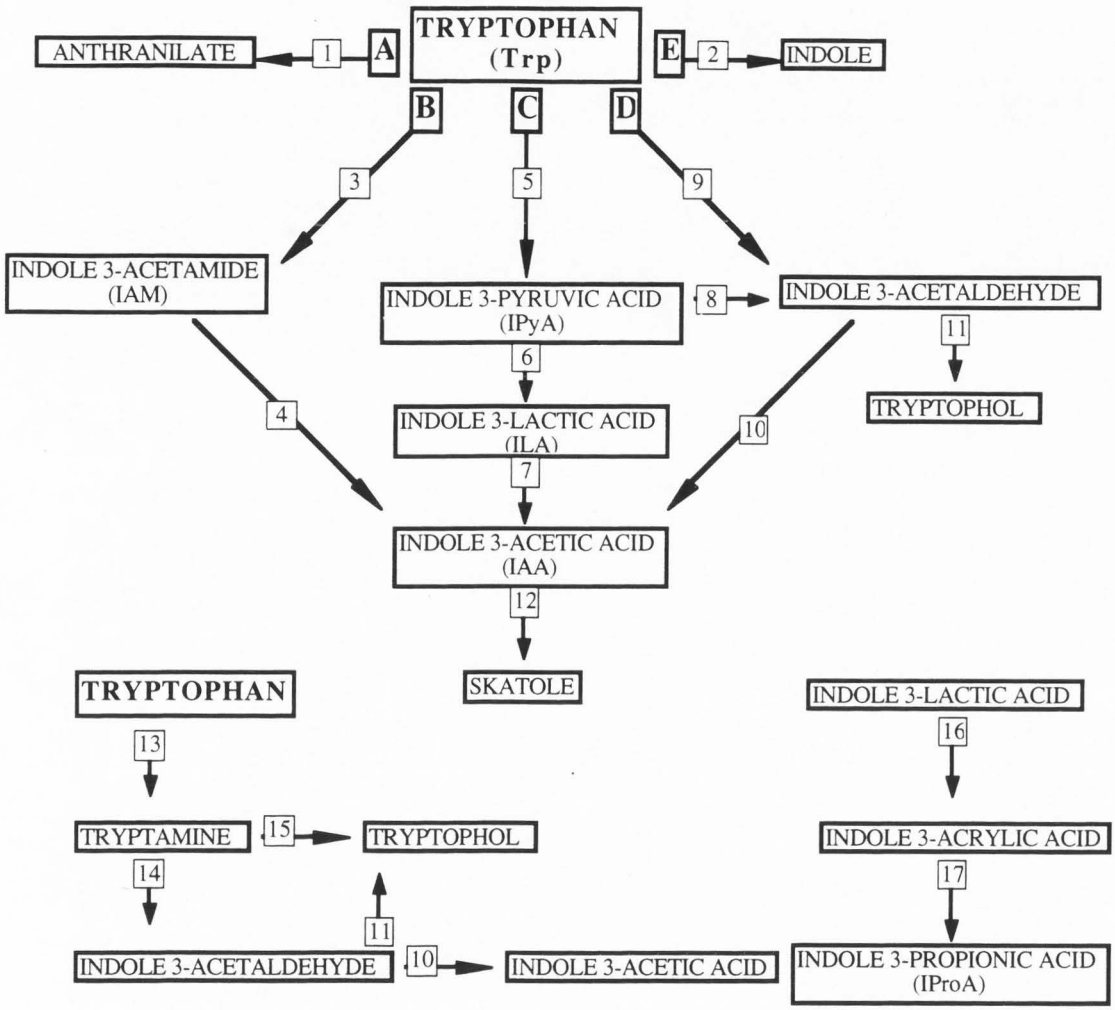

Figure 1. Tryptophan catabolism in microorganisms. Reactions and enzymes involved in these pathways include: 1, tryptophan oxygenase, kynureninase, formamidase (6); 2, tryptophanase (10, 27); 3, tryptophan monooxygenase (31); 4, indole acetamide hydrolase (37); 5, tryptophan aminotransferase (58); 6, indole lactate dehydrogenase (29,30); 7, reference 43; 8, indole pyruvic acid decarboxylase; 9, tryptophan side chain oxidase (49); 10, indole acetaldehyde oxidase (50); 11, indole ethanol dehydrogenase (54); 12, indole acetic acid decarboxylase (67); 13, tryptophan decarboxylase (48); 14, tryptaminase (5); 15, reference 5; 16, reference 52; 17, reference 16.

(EC1.1.1.110) (**ILDHase**) (29, 30). Although this reaction was not reported in *L. lactis*, IPyA in these organisms was shown to be either enzymatically or spontaneously degraded to indole 3- aldehyde and indole acetic acid (22).

In many microorganisms, an aromatic amino acid decarboxylase (EC4.1.1.28) (**DCOOHase**) catalyses the conversion of tryptophan to tryptamine (48), and tryptamine has been detected in several types of cheeses (45). In some *Arthobacter* spp., tryptophan decarboxylation represents the branch point for two catabolic routes: one path results in the formation of tryptophol, while the other produces indole 3-acetaldehyde (**I3Ac**) (5, 54). Finally, indole 3-propionic acid (**IProA**) can be formed by deamination of Trp or it may be produced from indole 3-lactic acid (**ILA**) via indole acrylic acid (16).

Role of *Lactobacillus* Adjuncts in AAA Catabolism

Although Trp catabolic pathways have been described in many microorganisms, mechanisms for the generation of aromatic off-flavor compounds in cheese or by cheese-related bacteria remain unclear. Evidence that lactobacilli have a role in these reactions includes mass spectrometry studies which detected indole in the aroma concentrate of *Lactobacillus helveticus* cultures and cheeses (38) and the fact that ruminant strains of *Lactobacillus* spp. can produce skatole or ρ-cresol, which may impart putrid or utensil flavors, respectively, from Trp or Tyr intermediates. Guthrie (25) also concluded *Lactobacillus* cultures produce aromatic off-flavor compounds in cheese, but did not elucidate the pathways responsible for their formation.

The objective of this study was to investigate Trp catabolism by two important species of dairy *Lactobacillus*, *L. helveticus* and *L. casei*, under simulated cheese conditions. It is our hypothesis that an improved understanding of Trp catabolism by lactobacilli in cheese will identify new strategies to control the production of aromatic off-flavors in low-fat Cheddar cheese.

TRYPTOPHAN CATABOLISM IN *LACTOBACILLUS CASEI* AND *LACTOBACILLUS HELVETICUS* CHEESE FLAVOR ADJUNCTS

Proteolysis in Cheddar cheese is thought to be one of the most important biochemical events during maturation (19), but peptides (except for those which are bitter) and free amino acids probably have little direct influence in cheese flavor. The amino acids that are produced through casein degradation, however, may be catabolized by microorganisms in cheese into compounds that have a strong effect on cheese flavor (20, 26, 35, 60). While many of these reactions undoubtedly make positive contributions to cheese flavor (2), the catabolism of aromatic amino acids (**AAA**) is thought to promote off-flavor development (12, 22, 25). Tryptophan (**Trp**) catabolism, for example, may lead to the formation of indole and skatole which impart putrid fecal and unclean flavors in cheese (25, 42). Pathways for the production of these compounds have been described in other bacteria (43), but mechanisms for their production in cheese are not fully understood.

Microbial pathways for Trp degradation may involve several different enzymes including tryptophan aminotransferase (**ATase**) (EC 2.6.1.27) (21, 52), Trp decarboxylase (**DCOOHase**) (EC 4.1.1.28) (48), Trp 2-monooxygenase (EC 1.13.12.3) (9), indole lactate dehydrogenase (**ILDHase**) (EC 1.1.1.110) (30), and tryptophanase (**TNase**) (EC 4.1.99.1) (10) (Figure 1). Gao and associates showed that Trp catabolism by the starter culture used in Cheddar cheese, *Lactococcus lactis*, was initiated by ATase under optimal and cheese-like conditions and produced indole pyruvic acid (**IPyA**) (22). Knowledge of Trp catabolism in other important dairy lactic acid bacteria such as *Lactobacillus* spp., however, is still lacking. This situation is unfortunate because lactobacilli may have an important role in Trp catabolism in cheese. For that reason, this study has investigated Trp degradation by two important species of dairy *Lactobacillus*,

L. casei and *L. helveticus*, in conditions that simulated the environment in Cheddar cheese. Lactobacilli dominate the non-starter or adventitious population in Cheddar cheese and species such as *Lactobacillus casei* and *Lactobacillus helveticus* are commonly used as adjunct cultures to intensify flavor development in reduced-fat varieties. Although *Lactobacillus* flavor adjuncts usually enhance cheese flavor intensity (15, 55), these bacteria may sometimes promote the development of unclean flavors (38, 39, 41, 64). Research by Guthrie (25), for example, showed cheese manufactured with *Lactobacillus* adjuncts including *Lactobacillus casei* contained AAA metabolites like indole and *p*-cresol, which are associated with unclean and medicinal flavors, respectively. In addition, mass spectroscopy studies have detected indole in the aroma concentrate of *L. helveticus* cultures and cheeses (38) and some ruminal species of a *Lactobacillus* spp. can produce skatole, a compound that imparts putrid flavors and *p*-cresol (67). Because Trp catabolism by lactobacilli may be an important source of off-flavor compounds in cheese, a detailed understanding of Trp catabolism in these bacteria should provide new strategies to control off-flavor production in ripened cheese.

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 Dr. J. L. Steele at the University of Wisconsin-Madison. The cultures were propagated in Difco APT broth (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

Lactobacillus enzymes involved in Trp catabolism were identified using assays with 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 a carbohydrate deficient chemically defined amino acid medium (**CDM**) (Table 1) (34) that lacked L-Trp, then suspended in 25 ml of the same medium. For studies of Trp catabolism under carbohydrate starvation conditions, 1 ml of the cell suspension was transferred into each of 12 test tubes that contained 9 ml of CDM either with (4 test tubes) or without (4 test tubes) 5 m*M* L-Trp. The tubes were incubated at 30° (*L. casei*) or 37°C (*L. helveticus*) and samples were collected for the preparation of CFE at time 0, and 7, 14 and 21 d. To determine whether lactobacilli produced Trp degradative enzymes in the harsh environment that typifies ripening cheese, CFE were also prepared from cells incubated at 15°C in 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 (cheese-like conditions).

Cells incubated under starvation or cheese-like conditions were harvested by centrifugation at 4°C, washed twice with 50 m*M* phosphate buffer (pH 6.5), and suspended in 1 ml of the same buffer. Extracts were prepared by sonic disintegration of the cell suspension with a Branson cell disruptor 200 (Danbury, CT) at 20 kHz in pulsed

mode 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 (11).

Identification of Enzymes Involved in Tryptophan Catabolism

Tryptophan ATase was measured spectrophotometrically as described by Frankenberger and Poth (21). The reaction mixture contained 5 m*M* L-Trp, 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 CFE to obtain a total reaction volume of 1 ml, and the reaction mixture was incubated at 30°C for 30 min. The formation of indole pyruvic acid (**IPyA**) was measured as the increase in absorbance at 327 nm (A_{327}) at 30°C, and Trp ATase specific activity was expressed as µmoles IPyA/mg protein per minute. Control reactions without substrate, without enzyme, and without substrate and enzyme were also included.

After transamination, IPyA may be reduced to indole lactic acid (**ILA**) by ILDHase (29). Cell free extracts were assayed for ILDhase by the spectrophotometric method of Hummel and coworkers, which measures the rate of decrease in NADH at 340 nm(A_{340}) (30). The reaction mixtures contained 50 m*M* sodium phosphate (pH 6.5), 0.2 m*M* indole pyruvic acid as the substrate and 0.3 m*M* NADH in a total reaction volume of 1 ml. Specific activity was reported as µmoles NADH consumed/mg protein per minute. Changes in A_{340} were also measured in control reactions that lacked substrate, CFE, or substrate and CFE.

Tryptophan decarboxylase catalyzes the decarboxylation of tryptophan to tryptamine. Activity was measured by the spectrophotometric assay of Nakazawa and associates (48),

TABLE 1. Chemically defined medium used in this study.

Constituent	mg/L	Constituent	g or ml/L
L-Alanine	100	Sodium acetate	5
L-Arginine	50	Sodium citrate	2
L-Asparagine	100	Potassium phosphate (Monobasic)	1
L-Cysteine	20	Potassium phosphate (Dibasic)	1
Glycine	100	Sodium chloride	0. 2
L-Glutamine	100	Calcium chloride	0.2
L-Histidine	50	Magnesium sulfate	0.2
L-Isoleucine	20	Manganese sulfate	0.05
L-Leucine	20	Pyridoxal	0.005
L-Lysine	100	Pyridoxamine	0.005
DL-Methionine	20	Adenine[b]	0.025
L-Proline	200	Guanine[b]	0.025
DL-Serine	100	Uracil[b]	0.025
DL-Threonine	100	Xanthine[b]	0.025
DL-Valine	100	Tween 80	1
L-Asparatic acid[a]	100	Tween 20	1
L-Glutamic acid[a]	400	Glycerol	1
L-Tryptophan[a]	5 m*M*	10% Mevalonic acid lactone	0.1
L-Phenylalanine[b]	20	RPMI vitamin stock[c]	20
L-Tyrosine[b]	25	Trace element stock[d]	2.5

[a]Dissolved in 5 ml 1 N HCl prior to addition into medium.
[b]Dissolved in 1 ml 1 N NaOH prior to addition into medium.
[c]Sigma B-7256 vitamin mixture.
[d]Trace element Stock: 2.85 g H_3BO_3, 1.8 g $MnCl_2.4H_2O$, 1.36 g $FeSO_4.7H_2O$, 1.77 g Sodium tartarate, 26.9 mg $CuCl_2.2H_2O$, 20.8 mg $ZnCl_2$, 40.4 mg $CoCl_2.6H_2O$, 25.2 mg $Na_2MoO_4.2H_20$ dissolved in 1000 ml distilled water and pH adjusted to 4.0 with H_2SO_4.

which detects the production of tryptamine from L-Trp in NH_4OH-NH_4Cl (pH 9.0) at 580 nm (A_{580}). A reaction volume of 1 ml contained 20 m*M* L-Trp, 1 m*M* pyridoxal phosphate, and 250 m*M* NH_4OH-NH_4Cl. After the addition of 250 µl CFE, the mixture was incubated at 30°C for 30 min. Then, 3 ml of a freshly prepared solution of 2% *p*-dimethyl aminobenzaldehyde in HCl-ethanol (25:75) was added to 0.5 ml of the enzyme reaction mixture and heated at 50°C for 40 min in a water bath after which the absorbance was measured. Specific activity was expressed as µmoles tryptamine formed/mg protein per minute.

Lactobacillus CFE were also assayed for Trp 2-monooxygenase and TNase activities. The CFE were screened for Trp 2-monooxygenase using the spectrophotometric assay of Hutcheson and Kosuge (31), and TNase assays were performed using a spectrophotometric procedure provided by Sigma Chemical Co. (St. Louis, MO).

All values for enzyme specific activities expressed in this study represent the mean from duplicate experiments replicated on two separate days. The effect of time (day 0 and day 21) and incubation condition (starvation and cheese-like on days 0 and 21) on enzyme specific activities were evaluated by statistical t-test comparisions between respective means at $\alpha = 0.05$ using Microsoft excel software (Redmond, WA).

Identification of Tryptophan 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 an overnight APT culture. 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 tryptophan catabolites: indole pyruvic acid (IPyA), indole lactic acid (ILA), indole acetic acid (IAA), indole acetamide (IAM), or indole propionic acid (IProA). The cells were incubated at

30°C (*L. casei*) or 37°C (*L. helveticus*), and 3 ml of the suspension, collected at time 0 (inoculation) and again 1-6 wk thereafter, was used for MECC analysis. Viable cell counts were obtained at each sampling time by plating on APT agar with anaerobic incubation for 48 h. The pH of the supernatant was then recorded using Baxter SP pH indicator strips (pH range 4.5 to 10.0) (McGaw Park, IL), and the samples were centrifuged to pellet cells, passed through a Corning 0.20 µm cellulose acetate syringe mounted filter (Palo Alto, CA), diafiltered through a Filtron 1 K cut-off Microsep (Northborough, MA) concentrator, and then diluted 1:5 in a 50 m*M* sodium tetraborate buffer immediately prior to injection. The MECC was performed using 60 m*M* SDS - 100 m*M* sodium tetra borate as described by Strickland and associates (63) with a Beckman Instruments P/ACE 2000 (Fullerton, CA) automated capillary electrophoresis system. This method detects and efficiently separates L-Trp and 11 other Trp metabolites (Figure 2). The presence of Trp catabolites in culture supernatant was initially investigated by comparisions between electropherograms obtained from cells incubated with and without 5 m*M* L-Trp or a catabolite. Compounds present in these peaks were then identified by coinjection with pure standards and a strong correlation ($r > 0.9$) between the absorbance spectrum of the unknown peaks to pure standard compounds. Cell-free control tubes that contained CDM with 5m*M* of L- Trp or individual Trp catabolites were also included in the experiment under the same incubation conditions to detect spontaneous chemical reactions.

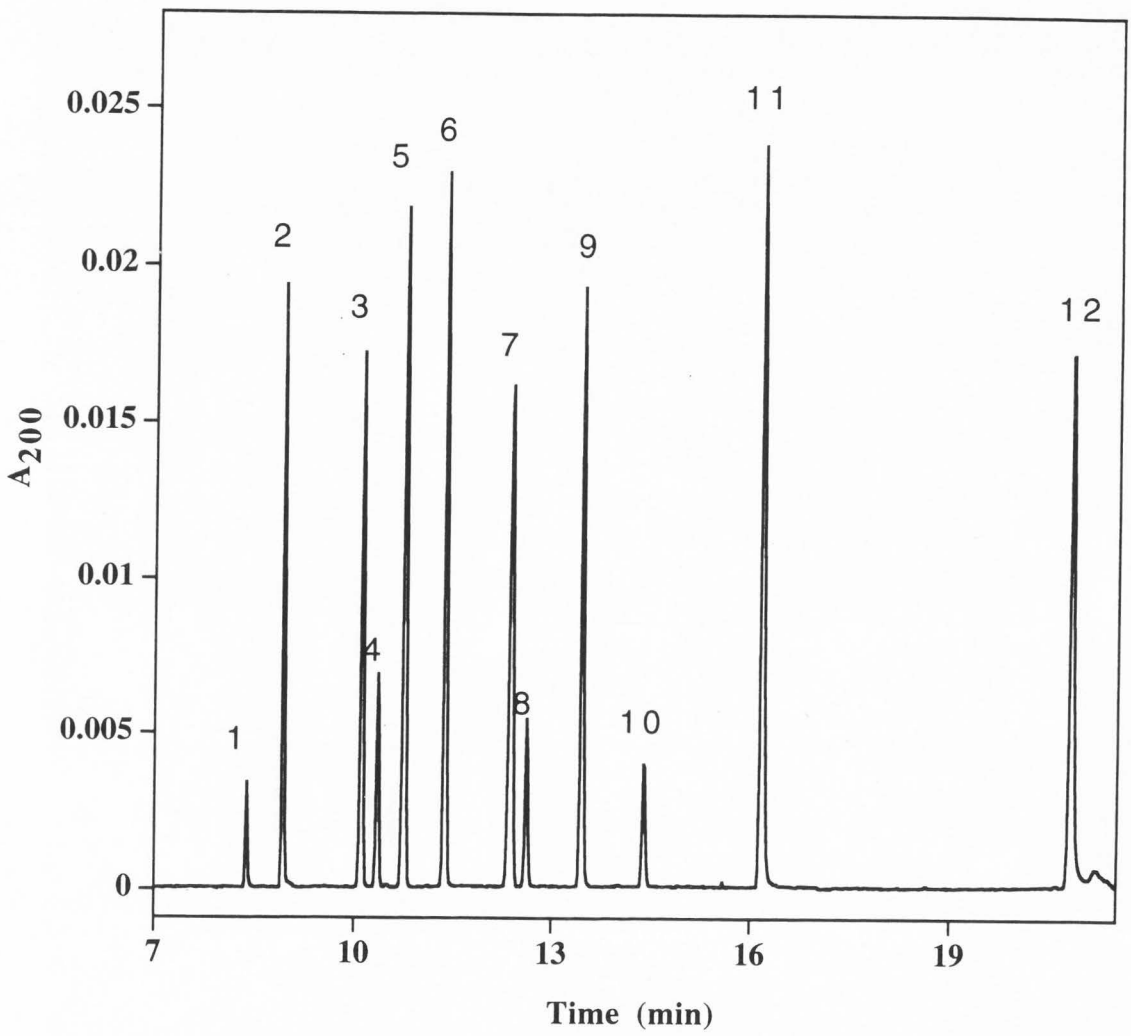

Figure 2. Micellar electrokinetic capillary chromatography f L-tryptophan and tryptophan metabolites in 60 m*M* sodium dodecyl sulfate-100 m*M* sodium tetraborate run buffer. Compounds included in the electropherogram were 1, kynurenine; 2, tryptophan; 3, indole lactic acid; 4, indole propionic acid; 5, indole acetic acid; 6, indole 3-acetamide; 7, anthranilic acid; 8, indole; 9, tryptophol; 10, indole aldehyde; 11, skatole; and 12, tryptamine.

RESULTS

Enzymes of Tryptophan Degradation in Lactobacilli

Tryptophan ATase, ILDHase, and Trp DCOOHase activities were detected in CFE from *Lactobacillus casei* LC301, *L. casei* LC202, *L. helveticus* CNRZ32, and *L. helveticus* LH212 that had been incubated under starvation or cheese-like conditions. Tryptophanase and tryptophan monooxygenase were not detected in CFE from bacteria incubated in either condition.

Specific activity of L-Trp ATase in *L. casei* LC301 and LC202 incubated in CDM with L-Trp ranged from 0.1 to 4.0×10^{-4} μmoles/mg protein per minute under starvation conditions (Table 2). A similar range of values was noted in cells incubated under cheese-like conditions (Table 3). It was observed that activities in both conditions were lowest on day 21 in all strains (less than 1.0×10^{-4} μmoles/mg protein per minute). Similarily, CFE from *L. helveticus* CNRZ32 and LH212 incubated in CDM with L-Trp under starvation and cheese-like conditions exhibited ATase specific activities that ranged between 0.3 and 3.5×10^{-4} μmoles/mg protein per minute (Tables 2 and 3) and also dropped to less than 1.0×10^{-4} μmoles/mg protein per minute by day 21.

Tryptophan ATase specific activities decreased significantly ($P < 0.05$) by d 21 in the case of *L. casei* LC202 and *L. helveticus* CNRZ32 incubated under both starvation and cheese-like conditions and in *L. casei* LC301 only when incubated under cheese-like conditions (Appendix, Tables 19 and 20). No significant differences in specific activities were observed over time in *L. casei* LC301 incubated under starvation conditions and *L. helveticus* LH212 incubated under starvation or cheese-like conditions ($P > 0.05$). Incubation condition also had no significant effect on Trp ATase specific activities at time 0 and d 21 in any of the strains except on d 21 in *L. helveticus* CNRZ32. Tryptophan ATase were similar in bacteria incubated in CDM lacking L-Trp (Appendix, Tables 13 and 14).

TABLE 2. Tryptophan aminotransferase in *Lactobacillus* spp. cells incubated in chemically defined medium with 5 m*M* L-tryptophan under starvation conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.24 ± 0.05	0.35 ± 0.01	0.16 ± 0.04	0.18 ± 0.02
7	0.19 ± 0.02	0.15 ± 0.10	0.33 ± 0.20	0.35 ± 0.13
14	0.27 ± 0.15	0.40 ±0.09	0.09 ± 0.0	0.06 ± 0.01
21	0.05 ± 0.0	0.01 ± 0.0	0.05 ± 0.0	0.03 ± 0.0

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 3. Tryptophan aminotransferase in *Lactobacillus* spp. cells incubated in chemically defined medium with 5 m*M* L-tryptophan under and cheese-like conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.30 ± 0.01	0.40 ± 0.07	0.17 ± 0.04	0.16 ± 0.01
7	0.14 ± 0.0	0.37 ± 0.10	0.22 ± 0.0	0.18 ± 0.03
14	0.19 ± 0.06	0.29 ± 0.14	0.08 ± 0.0	0.18 ± 0.06
21	0.04 ± 0.02	0.02 ± 0.0	0.04 ± 0.01	0.07 ± 0.0

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

Indole lactate dehydrogenase specific activities obtained from CFE of *L. casei* strains incubated in CDM with L-Trp under starvation and cheese-like conditions ranged from 16.3 to 65.1 $\times 10^{-3}$ µmoles/mg protein per minute, and from 6.3 to 55.2 $\times 10^{-3}$ µmoles/mg protein per minute in strains of *L. helveticus* (Tables 4 and 5). Incubation condition and time did not have a significant effect on ILDHase activities in all four strians ($P > 0.05$) (Appendix, Tables 21 and 22). Similar ILDHase activities were observed in bacteria incubated in CDM lacking L-Trp (Appendix, Tables 15 and 16).

Tryptophan decarboxylase activities in *L. casei* strains incubated in CDM with L-Trp under starvation and cheese-like conditions ranged from 0.27 to 1.48 $\times 10^{-3}$ µmoles/mg protein per minute (Tables 6 and 7) and 1.59 to 5.1 $\times 10^{-3}$ µmoles/mg protein per minute for *L. helveticus* strains (Tables 6 and 7). Activity of Trp decarboxylase did not vary significantly ($P > 0.05$) in *L. casei* LC301 and *L. helveticus* LH212 and CNRZ32 with time and incubation condition. Specific activities for *L. casei* LC202 decreased significantly ($P < 0.05$) with time under cheese-like conditions (Appendix, Tables 23 and 24). When incubated under starvation conditions, however, there was no significant ($P > 0.05$) time effect. Tryptophan decarboxylase was also active in these bacteria when incubated in CDM lacking L-Trp (Appendix, Tables 17 and 18).

MECC Analysis

No change in media pH was observed under either the cheese-like or starvation conditions during the course of this study, but cell viability was drastically reduced. Under cheese-like conditions, plate counts dropped from 10^8 to less than 10^2 cells per ml by the end of 6 wk regardless of which Trp catabolite was added to CDM (Figure 3). Under starvation conditions, however, cell numbers for all lactobacilli remained above 10^2 even after 6 wk in CDM that contained ILA (Figure 4).

Analysis of *L. casei* LC301 (Figure 4) and LC202 culture supernatants after incubation under starvation conditions in CDM with L-Trp over a 6-wk period showed

TABLE 4. Indole lactate dehydrogenase in *Lactobacillus* spp. cells incubated in chemically defined medium with 5 m*M* L-tryptophan under starvation conditions.[1]

Time (days)	*L.casei* Specific activity[2] LC301	*L.casei* Specific activity[2] LC202	*L.helveticus* Specific activity[2] LH212	*L.helveticus* Specific activity[2] CNRZ32
0	10.83 ± 5.96	24.35 ± 10.74	14.84 ± 2.07	36.33 ± 14.32
7	23.51 ± 9.20	64.92 ± 12.16	29.66 ± 12.86	50.66 ± 15.87
14	39.66 ± 26.57	65.17 ± 28.33	38.50 ± 22.72	55.22 ± 27.21
21	16.81 ± 8.75	18.88 ± 3.65	4.32 ± 4.07	11.47 ± 9.69

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in μmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 5. Indole lactate dehydrogenase in *Lactobacillus* spp. cells incubated in CDM with 5 m*M* L-tryptophan under cheese-like conditions.[1]

Time (days)	*L.casei* Specific activity[2] LC301	*L.casei* Specific activity[2] LC202	*L.helveticus* Specific activity[2] LH212	*L.helveticus* Specific activity[2] CNRZ32
0	18.50 ± 4.90	23.52 ± 10.58	17.59 ± 2.13	48.80 ± 6.40
7	20.83 ± 8.17	40.66 ± 20.11	24.86 ± 18.46	18.96 ± 20.85
14	27.91 ± 16.95	42.02 ± 18.30	10.00 ± 10.56	37.87 ± 25.85
21	16.37 ± 6.57	21.68 ± 10.63	6.33 ± 3.86	16.20 ± 9.70

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in μmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 6. Tryptophan (Trp) decarboxylase in *Lactobacillus* spp. cells incubated in chemically defined medium with 5 m*M* L-tryptophan under starvation conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.65 ± 0.36	1.05 ± 0.20	1.96 ± 0.53	2.56 ± 0.89
7	0.27 ± 0.16	0.41 ± 0.10	3.47 ± 1.09	1.59 ± 0.04
14	1.35 ± 0.28	1.22 ± 0.01	2.25 ± 1.42	3.31 ± 0.73
21	1.08 ± 0.42	0.99 ± 0.35	2.81 ± 0.83	5.03 ± 2.85

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 7. Tryptophan (Trp) decarboxylase in *Lactobacillus* spp. cells incubated in chemically defined medium with 5 m*M* L-tryptophan under cheese-like conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	1.11 ± 0.38	0.95 ± 0.02	1.85 ± 0.68	2.17 ± 0.19
7	1.20 ± 0.06	1.16 ± 0.59	2.44 ± 1.16	1.61 ± 0.45
14	0.62 ± 0.01	0.48 ± 0.19	2.01 ± 1.50	5.10 ± 0.07
21	1.48 ± 0.85	0.31 ± 0.05	2.83 ± 1.40	4.93 ± 1.15

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

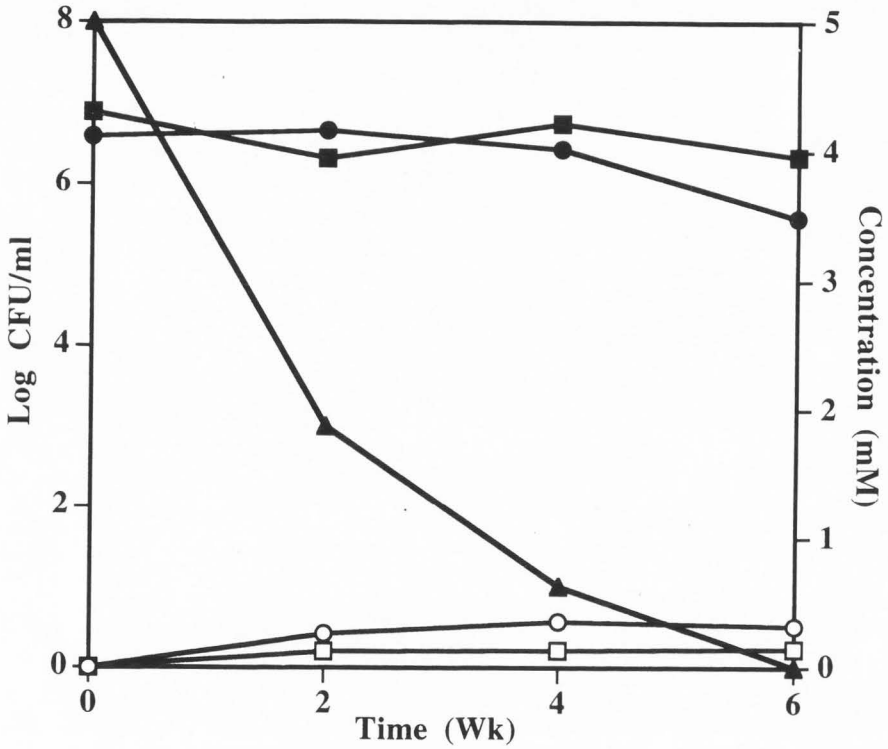

Figure 3. Metabolism of tryptophan and indole lactic acid under cheese-like conditions by *Lactobacillus casei* LC301 and cell viability in chemically defined medium. Concentration of indole lactic acid (ILA) (O) produced in CDM with Trp (■) and concentration of Trp ( ) produced in CDM with ILA (●). Reduction in cell numbers when incubated in CDM with either Trp or ILA (▲) under cheese-like conditions.

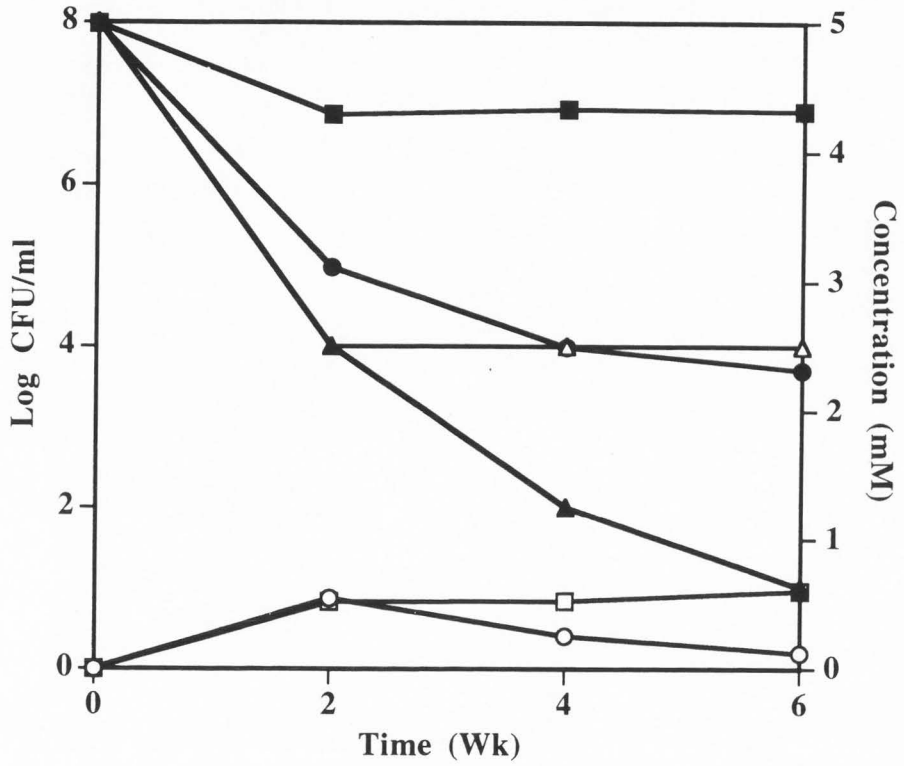

Figure 4. Metabolism of tryptophan and indole lactic acid under starvation conditions by *Lactobacillus casei* LC301 and cell viability in chemically defined medium. Concentration of indole lactic acid (ILA) (O) produced in CDM with Trp (■) and concentration of Trp ( ) produced in CDM with ILA (●). Reduction in cell numbers when incubated in CDM with either Trp (▲) or ILA (Δ) under starvation conditions.

these bacteria converted Trp to ILA (Table 8). These cells also produced Trp when cells were incubated in CDM with ILA (Table 8). *L. helveticus* LH212 and CNRZ32 did not catabolize L-Trp under starvation conditions but tryptophan did accumulate when the cells were incubated in CDM with ILA (Table 9). Supernatants from *L. casei* LC301 andLC202 incubated in CDM with IPyA contained Trp and ILA (Table 8) while *L. helveticus* LH212 and CNRZ32 supernatants contained only tryptophan (Table 9).

Tryptophan metabolism by *L. casei* LC301 (Figure 3) and LC202 incubated under cheese-like conditions was similar to that noted under carbon starvation (Table 8). Tryptophan was converted to ILA (Figure 5), and incubations in CDM with ILA led to the production of Trp (Figure 6). Supernatant from cells incubated in CDM with IPyA contained both Trp and ILA (Figure 7). In contrast, *L. helveticus* strains metabolized Trp differently under starvation versus cheese-like conditions. *L. helveticus* LH212 incubated in CDM with L-Trp under cheese-like conditions produced small amounts of indole lactic acid (Table 10) while *L. helveticus* CNRZ32 did not catabolize Trp at all (Table 11). Both *L. helveticus* strains synthesized Trp when incubated in CDM with ILA, however, and tryptophan and ILA accumulated in supernatants from both *L. helveticus* strains when these bacteria were incubated in CDM with IPyA (Tables 10 and 11).

The MECC of culture supernatant from *L. casei* and *L. helveticus* strains incubated in CDM containing other Trp catabolites like indole acetic acid, indole acetamide, and indole propionic acid showed they were not metabolized by these bacteria under starvation or cheese-like conditions.

TABLE 8. Micellar electrokinetic capillary chromatography analysis of the production of tryptophan catabolites in chemically defined media by *Lactobacillus casei* LC301 and LC202 incubated under carbohydrate starvation[1] and cheese-like[2] conditions.

	Product					
Medium	Tryptophan	Indole pyruvic acid	Indole lactic acid	Indole acetic acid	Indole	Skatole
CDM	-	-	-	-	-	-
CDM with 5mM Trp	-	-	++	-	-	-
CDM with 5mM IPyA	+++	-	+++	-	-	-
CDM with 5mM ILA	++	-	-	-	-	-
CDM with 5mM IAA[3]	-	-	-	-	-	-
CDM with 5mM IAM[4]	-	-	-	-	-	-
CDM with 5mM IProA[5]	-	-	-	-	-	-

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).
[2] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C
[3] Indole acetic acid
[4] Indole acetamide
[5] Indole propionic acid.

TABLE 9. Micellar electrokinetic capillary chromatography analysis of the production of tryptophan catabolites in chemically defined media by *Lactobacillus helveticus* LH212 and CNRZ32 incubated under carbohydrate starvation conditions.[1]

	Product					
Medium	Tryptophan	Indole pyruvic acid	Indole lactic acid	Indole acetic acid	Indole	Skatole
CDM	-	-	-	-	-	-
CDM with 5mM Trp	-	-	-	-	-	-
CDM with 5mM IPyA	+++	-	-	-	-	-
CDM with 5mM ILA	+	-	-	-	-	-
CDM with 5mM IAA[3]	-	-	-	-	-	-
CDM with 5mM IAM[4]	-	-	-	-	-	-
CDM with 5mM IProA[5]	-	-	-	-	-	-

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).
[2] Indole acetic acid,
[3] Indole acetamide,
[4] Indole propionic acid.

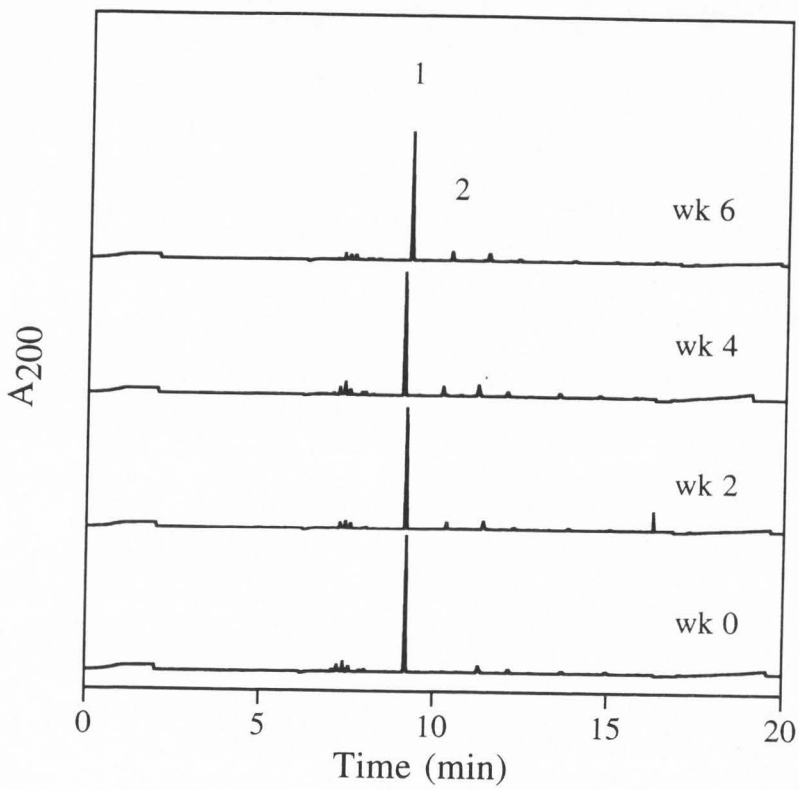

Figure 5. Micellar electrokinetic capillary chromatography analysis of *Lactobacillus casei* LC301 incubated in chemically defined medium with 5 m*M* L-tryptophan under cheese-like conditions. Peaks that were identified in the electropherogram include 1, L-tryptophan; and 2, indole lactic acid.

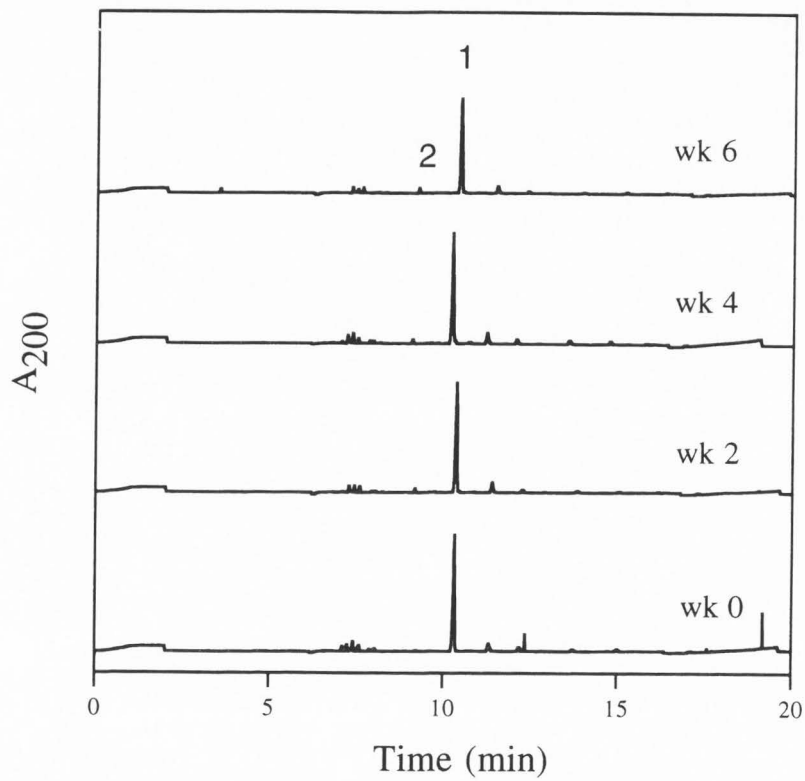

Figure 6. Micellar electrokinetic capillary chromatography analysis of *Lactobacillus casei* LC301 incubated in chemically defined medium with indole lactic acid under cheese-like conditions. Peaks that were identified in the electropherogram include 1, tryptophan; and 2, indole lactic acid.

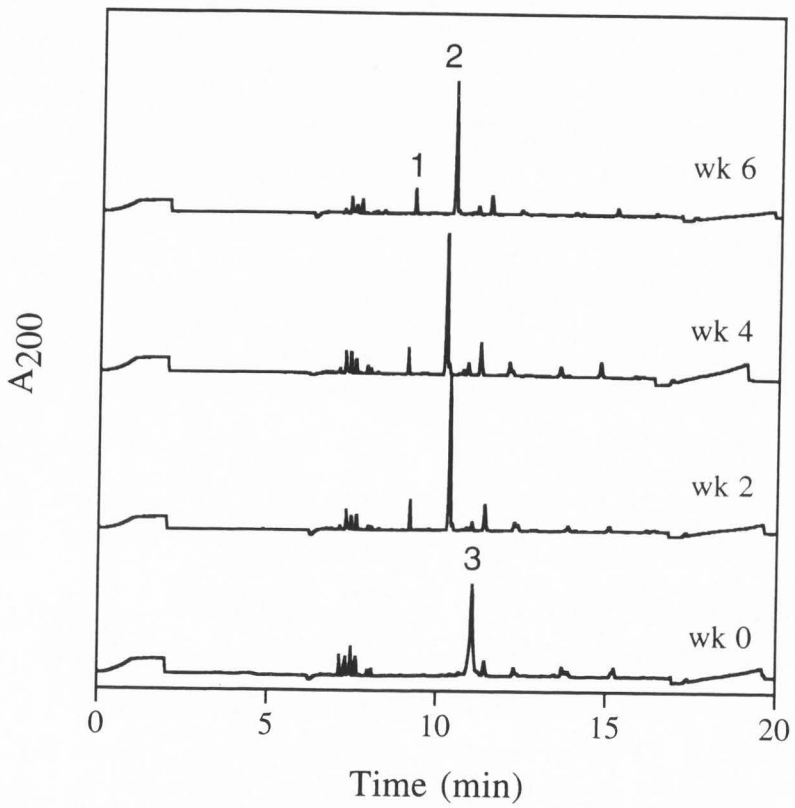

Figure 7. Micellar electrokinetic capillary chromatography analysis of *Lactobacillus casei* LC301 incubated in chemically defined medium with indole pyruvic acid under cheese-like conditions. Peaks that were identified in the electropherogram include 1, tryptophan; 2, indole lactic acid; and 3, indole pyruvic acid.

TABLE 10. Micellar electrokinetic capillary chromatography analysis of the production of tryptophan catabolites in chemically defined media by *Lactobacillus helveticus* LH212 incubated under cheese-like conditions.[1]

	Product					
Medium	Tryptophan	Indole pyruvic acid	Indole lactic acid	Indole acetic acid	Indole	Skatole
CDM	-	-	-	-	-	-
CDM with 5mM Trp	-	-	+	-	-	-
CDM with 5mM IPyA	+	-	+	-	-	-
CDM with 5mM ILA	+	-	-	-	-	-
CDM with 5mM IAA[2]	-	-	-	-	-	-
CDM with 5mM IAM[3]	-	-	-	-	-	-
CDM with 5mM IProA[4]	-	-	-	-	-	-

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C
[2] Indole acetic acid
[3] Indole acetamide
[4] Indole propionic acid

TABLE 11. Micellar electrokinetic capillary chromatography analysis of the production of tryptophan catabolites in chemically defined media by *Lactobacillus helveticus* CNRZ32 incubated under cheese-like conditions.[1]

	Product					
Medium	Tryptophan	Indole pyruvic acid	Indole lactic acid	Indole acetic acid	Indole	Skatole
CDM	-	-	-	-	-	-
CDM with 5mM Trp	-	-	-	-	-	-
CDM with 5mM IPyA	++	-	+	-	-	-
CDM with 5mM ILA	+	-	-	-	-	-
CDM with 5mM IAA[2]	-	-	-	-	-	-
CDM with 5mM IAM[3]	-	-	-	-	-	-
CDM with 5mM IProA[4]	-	-	-	-	-	-

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C
[2] Indole acetic acid
[3] indole acetamide
[4] indole propionic acid.

DISCUSSION

Persistent defects in the flavor quality of low-fat Cheddar cheese has intensified the need to identify key enzymes and chemical reactions responsible for the production of cheese flavor compounds. Previous work has indicated that microbial degradation of AAAs in cheese may promote off-flavor development, and this defect is especially common in reduced-fat varieties (64). This study investigated tryptophan catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts in an effort to gain an improved understanding of the relationship between tryptophan degradation and the production of off-flavor compounds in Cheddar cheese.

Enzyme assays of cell-free extracts from *Lactobacillus* incubated under carbohydrate starvation and cheese-like conditions (pH 5.2, 4% NaCl, no sugar, 15°C) indicated that these strains may catabolize Trp to ILA through transamination and dehydrogenation reactions or to tryptamine by decarboxylation. The MECC analysis of culture supernatants indicated decarboxylation was not an active pathway under carbohydrate starvation or cheese-like conditions but showed transamination and dehydrogenase enzymes were active under these conditions. *Lactobacillus casei* LC301 and LC202 cultures incubated under starvation or cheese-like conditions in CDM with Trp accumulated ILA in their supernatant, and cells incubated in CDM with ILA accumulated Trp. The fact that these cultures produced both Trp and ILA when incubated in CDM with IPyA supported enzyme data that indicated Trp conversion to ILA occurred via successive transamination and dehydrogenation reactions with IPyA as the intermediate. The absence of tryptamine in culture supernatant despite the presence of detectable decarboxylase activity in CFE is in line with the study of Zoon and Allersma (68), which showed that mixed cultures of *Streptococcus thermophilus* and *Lactobacillus helveticus* produced carbon dioxide and γ-amino butyric acid in cheese from decarboxylation of glutamic acid but detected no other amino acid decarboxylation products.

Enzyme data suggested Trp catabolism by *L. helveticus* strains was similar to *L. casei*, but MECC showed that in these bacteria Trp ATase and ILDHase were almost exclusively used for the Trp anabolic pathway. *L. helveticus* CNRZ32 did not catabolize Trp under either the starvation or cheese-like conditions, but *L. helveticus* LH212 was able to catabolize Trp under cheese-like conditions. From these data we have proposed a putative pathway for the catabolism of tryptophan by *Lactobacillus casei* and *Lactobacillus helveticus* under cheese-like conditions (Figure 8).

This study showed Trp catabolism by *L. casei* led to the production of indole lactate, an organic acid. If efflux of this compound occurs in a manner similar to that used for lactate, then proton symport may allow cells to create or maintain a proton motive force which, through the reverse action of the F_0 F_1 ATPase, may allow the cell to synthsize ATP (24). Since sugar-starved bacteria have a limited supply of ATP and often depend upon degradation of amino acids for the generation of energy (24), this capability may facilitate the growth of *L. casei* and other NSLAB in the harsh cheese environment. The physiological significance of these pathways, however, is still unclear. Because bacteria incubated under carbohydrate starvation have limited reducing power, an alternative possibility is that cells use the anabolic reactions to generate NADH. Thus the energy status of the cell and the NAD to NADH ratio might favor catabolic or anabolic Trp reactions.

Lactobacillus helveticus LH212 cells incubated under starvation or cheese-like conditions favored the production of Trp over ILA, but under cheese-like conditions a small amount of Trp is catabolized to ILA. Tryptophan ATase and ILDHase activity in CFE from cells incubated under cheese-like conditions and ILA production by whole cells under these conditions indicate that *L. helveticus* LH212 adjuncts may contribute to Trp

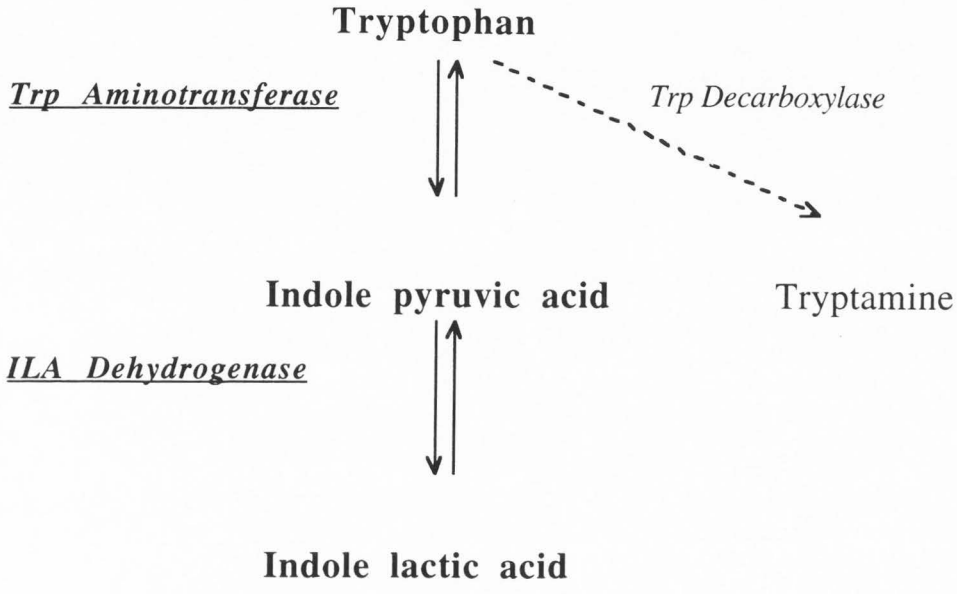

Figure 8. Proposed pathway for tryptophan catabolism by *Lactobacillus casei* and *L. helveticus*.

catabolism in ripening Cheddar cheese. Although *L. helveticus* CNRZ32 whole cells did not catabolize tryptophan, Trp DCOOHase, Trp ATase, and ILDHase were active in CFE from this bacterium. That observation suggests autolysis could contribute to Trp catabolism in the cheese matrix. However, the general preference of *L. helveticus* strains towards the anabolic pathway suggests they are more likely to convert ILA produced by other cells in the cheese environment into Trp.

Gao and associates (22) showed that the Cheddar cheese starter bacterium *Lactococcus lactis* initiated Trp catabolism via ATase under optimal and cheese-like conditions but did not convert IPyA to ILA. Our investigation showed lactobacilli

catalyzed both the Trp ATase and ILDHase reactions. The Trp ATase specific activities of lactococcal strains were about 10-fold higher than those found in this study for lactobacilli, suggesting that *Lactococcus lactis* may have a greater role in the initial conversion of Trp to IPyA in cheese, and lactobacilli may be important in ensuing reactions such as conversion of IPyA to ILA or Trp.

Indole is an important tryptophan metabolite that has been implicated with unclean flavor in dairy products. Although pathways for the production of indole have been described in some bacteria, mechanisms for its production in cheese or by cheese bacteria have not been ascertained. Indole or skatole production was not observed by MECC analysis of culture supernatant from any of the strains and in either condition used. These data and the lack of detectable tryptophanase activity in these organisms indicate *L. casei* and *L. helveticus* do not produce indole in cheese. Our laboratory has detected tryptophanase activity and indole production in some wild lactobacilli that were isolated from cheese, but this trait was unstable (unpublished data). Further studies are needed to elucidate the mechanism for indole and skatole production by lactobacilli and the origin of these compounds in cheese.

SUMMARY AND CONCLUSIONS

In the last few decades, the dairy industry has progressed rapidly; production and consumption of dairy products have reached unprecedented levels, and a wide range of speciality products has become the focus of dairy research. Manufacture of high-quality low-fat cheese for the growing number of consumers who wish to decrease their level of dietary-fat intake has become a national priority. Unfortunately, most reduced-fat Cheddar cheeses continue to suffer from poor flavor intensity, a greater propensity for off flavors, and inferior body and textural qualities. These challenges have revitalized interest in the biochemistry of cheese ripening, and a substantial amount of new information has been generated in recent years. Cheese ripening is a chemically, microbiologically, and enzymatically complex system; however, a comprehensive understanding of ripening and flavor development remains elusive.

Aromatic compounds such as indole, skatole, and *p*-cresol have been shown to contribute typical unclean sensations in cheese that are often more pronounced in reduced-fat varieties, and these compounds are believed to arise via the catabolism of aromatic amino acids by cheese bacteria. Gao and associates (22) recently showed that the starter lactic acid bacterium *Lactococcus lactis* can initiate the process by an aromatic aminotransferase, but information related to the catabolism of AAA by non-starter and adjunct lactobacilli is lacking.

This study investigated tryptophan catabolism by *Lactobacillus casei* LC301 and LC202 and *Lactobacillus helveticus* CNRZ32 and LH212 cheese flavor adjuncts under carbohydrate starvation and cheese-like conditions (pH 5.2, 4% NaCl, 15°C). The results obtained in this study can be summarized as follows:

1. Enzyme assays revealed lactobacilli catabolize tryptophan to indole lactic acid via indole pyruvic acid through transamination and dehydrogenation and to tryptamine by decarboxylation.

2. MECC confirmed the action of these enzymes and showed these organisms could also catalyze anabolic reactions, i.e., conversion of indole lactic acid to tryptophan.
3. Tryptamine was not detected by MECC, which indicated Trp decarboxylase was not active under starvation or cheese-like conditions.
4. MECC studies also showed tryptophan catabolism in *Lactobacillus casei* was similar under starvation or cheese-like conditions, but in *Lactobacillus helveticus* it varied with incubation condition.
5. *Lactobacillus* tryptophan aminotransferase activities are approximately 10-fold lower in this study than those observed for *Lactococcus lactis* (22).
6. MECC did not detect unclean compounds like indole and skatole in *Lactobacillus* supernatants, and no tryptophanase activity was detected in CFE.

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APPENDIX

Table 12. Cheddar cheese flavor compounds obtained from catabolism of amino acids (Adapted from 47, 64).

Compound	Amino acid	Flavor (ppm)	Threshold
Hydrogen sulfide	Cysteine	unknown	unknown
Methional/Methanethiol	Methionine	Cheese cracker-like	unknown
Dimethylsulfide	Methionine	Cheesy	unknown
2-methyl-propanal	Valine	Malty, Grainy	unknown
3-methyl-butanal	Leucine	Malty, Solvent-like	unknown
2-methyl-butanal	Isoleucine	Malty	unknown
Ammonia	All amino acids	Bitter	unknown
Phenol	Tyrosine	Sharp, Carbolic Bitter almond	>0.1
Phenethanol	Phenyalanine	Rosy	>7.5
Tyramine	Tyrosine	unknown	unknown
Skatole	Tryptophan	Stale, Dirty	>0.3
Indole	Tryptophan	Putrid, Fecal Tarry, Repulsive	>0.1
Benzoic acid	Phenylalanine	unknown	unknown
Tryptamine	Tryptophan	unknown	unknown
p-cresol	Tyrosine	Unclean, Barny	>0.01
Acetate	Alanine, Glycine Serine	Sour Vinegar-like	unknown
Propionate	Threonine, Serine Aspartic acid	Sweet	unknown
n-Butyrate	Glutamine, Methionine, Threonine	Cheesy, Buttery	unknown

TABLE 13. Tryptophan aminotransferase in *Lactobacillus* spp. cells incubated in chemically defined medium without tryptophan under starvation conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.32 ± 0.01	0.32 ± 0.01	0.17 ± 0.04	0.17 ± 0.0
7	0.20 ± 0.04	0.21 ± 0.11	0.29 ± 0.17	0.16 ± 0.10
14	0.22 ± 0.01	0.23 ±0.09	0.04 ± 0.02	0.17 ± 0.16
21	0.01 ± 0.01	0.02 ± 0.0	0.03 ± 0.0	0.04 ± 0.0

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in μmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 14. Tryptophan aminotransferase in *Lactobacillus* spp. cells incubated in chemically defined medium without trypotphan under cheese-like conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.32 ± 0.11	0.43 ± 0.0	0.16 ± 0.03	0.17 ± 0.01
7	0.19 ± 0.0	0.38 ± 0.21	0.18 ± 0.07	0.46 ± 0.3
14	0.13 ± 0.02	0.22 ± 0.06	0.18 ± 0.11	0.19 ± 0.14
21	0.03 ± 0.0	0.03 ± 0.01	0.03 ± 0.02	0.07 ± 0.01

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in μmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 15 Indole lactate dehydrogenase in *Lactobacillus* spp. cells incubated in chemically defined medium without tryptophan under starvation conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	11.42 ± 5.35	31.21 ± 11.93	30 ± 15.22	16.39 ± 1.45
7	25.87 ± 7.43	53.13 ± 22.52	52.08 ± 19.51	36.46 ± 18.29
14	41.32 ± 23.64	71.35 ± 24.29	54.2 ± 22	48.88 ± 19.75
21	22.42 ± 7.3	32.57 ± 10.34	17 ± 8.74	10.45 ± 4.41

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 16. Indole lactate dehydrogenase in *Lactobacillus* spp. cells incubated in chemically defined medium without tryptophan under cheese-like conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	12.16 ± 0.55	32.43 ± 10.88	16.89 ± 1.91	39.91 ± 4.38
7	28.38 ± 6.86	35.77 ± 21.76	14.78 ± 16.88	29.25 ± 17.95
14	37.02 ± 15.7	23.49 ± 14.39	17.51 ± 10.69	43.13 ± 28.65
21	17.77 ± 8.07	19.52 ± 10.63	6.37 ± 3.94	7.21 ± 9.76

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 17. Tryptophandecarboxylase in *Lactobacillus* spp. cells incubated in chemically defined medium without tryptophan under starvation conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	1.1 ± 0.15	1.03 ± 0.04	1.84 ± 0.88	1.7 ± 0.48
7	0.185 ± 0.0	0.35 ± 0.09	1.27 ± 0.10	2.26 ± 1.34
14	1.03 ± 0.37	1.06 ± 0.34	1.96 ± 0.84	3.09 ± 0.68
21	0.14 ± 0.03	0.14 ± 0.0	3.05 ± 0.59	4.14 ± 1.87

[1] No carbohydrate, pH 6.5, incubated at 30°C (*L. casei*) or 37°C (*L. helveticus*).

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 18. Tryptophan decarboxylase in *Lactobacillus* spp. cells incubated in chemically defined medium without tryptophan under cheese-like conditions.[1]

	L.casei		*L.helveticus*	
Time (days)	Specific activity[2] LC301	LC202	Specific activity[2] LH212	CNRZ32
0	0.73 ± 0.39	1.09 ± 0.35	1.91 ± 0.8	2.3 ± 0.81
7	0.66 ± 0.3	0.72 ± 0.15	2.23 ± 1.44	3.77 ± 1.80
14	0.48 ± 0.15	1.54 ± 0.49	1.56 ± 0.52	4.22 ± 2.06
21	0.31 ± 0.21	0.23 ± 0.05	2.33 ± 1.05	6.8 ± 0.45

[1] No carbohydrate, pH 5.2, 4% NaCl, incubated at 15°C.

[2] Specific activity measured in µmoles/mg protein per minute × 10^{-3} (mean ± standard errror).

TABLE 19. Statistical t-test: Effect of time[1] on tryptophan aminotransferase in cells incubated under starvation and cheese-like conditons.

	With 5 m*M* L-Trp		Without Trp	
Strain	Starvation	Cheese-like	Starvation	Cheese-like
Lactobacillus casei LC301	$P = 0.12$	$P = 0.007$	$P = 0.003$	$P = 0.17$
Lactobacillus casei LC202	$P = 0.001$	$P = 0.04$	$P = 0.02$	$P = 0.001$
Lactobacillus helveticus LH212	$P = 0.16$	$P = 0.12$	$P = 0.14$	$P = 0.07$
Lactobacillus helveticus CNRZ32	$P = 0.04$	$P = 0.02$	$P = 0.12$	$P = 0.16$

[1] Researchers performed only t-test between specific activities measured on d 0 and d 21.

TABLE 20. Statistical t-test: Effect of incubation condition on tryptophan aminotransferase in cells incubated in chemically defined medium at days 0 and 21.

	With 5 m*M* L-Trp		Without Trp	
Strain	Day 0	Day 21	Day 0	Day 21
Lactobacillus casei LC301	$P = 0.37$	$P = 0.46$	$P = 0.99$	$P = 0.39$
Lactobacillus casei LC202	$P = 0.52$	$P = 0.81$	$P = 0.02$	$P = 0.57$
Lactobacillus helveticus LH212	$P = 0.95$	$P = 0.93$	$P = 0.90$	$P = 0.98$
Lactobacillus helveticus CNRZ32	$P = 0.49$	$P = 0.02$	$P = 0.95$	$P = 0.10$

TABLE 21. Statistical t-test: Effect of time[1] on indole lactate dehydrogenase in cells incubated under starvation and cheese-like conditons.

	With 5 m*M* L-Trp		Without Trp	
Strain	Starvation	Cheese-like	Starvation	Cheese-like
Lactobacillus casei LC301	$P = 0.65$	$P = 0.78$	$P = 0.02$	$P = 0.60$
Lactobacillus casei LC202	$P = 0.51$	$P = 0.67$	$P = 0.93$	$P = 0.46$
Lactobacillus helveticus LH212	$P = 0.17$	$P = 0.07$	$P = 0.10$	$P = 0.06$
Lactobacillus helveticus CNRZ32	$P = 0.12$	$P = 0.13$	$P = 0.58$	$P = 0.08$

[1] Researchers performed only t-test between specific activities measured on d 0 and d 21.

TABLE 22. Statistical t-test: Effect of incubation condition on indole lactate dehydrogenase in cells incubated in chemically defined medium at days 0 and 21.

	With 5 m*M* L-Trp		Without Trp	
Strain	Day 0	Day 21	Day 0	Day 21
Lactobacillus casei LC301	$P = 0.35$	$P = 0.97$	$P = 0.66$	$P = 0.66$
Lactobacillus casei LC202	$P = 0.91$	$P = 0.46$	$P = 0.94$	$P = 0.38$
Lactobacillus helveticus LH212	$P = 0.54$	$P = 0.71$	$P = 0.66$	$P = 0.41$
Lactobacillus helveticus CNRZ32	$P = 0.13$	$P = 0.72$	$P = 0.85$	$P = 0.26$

TABLE 23. Statistical t-test: Effect of time[1] on tryptophan decarboxylase in cells incubated under starvation and cheese-like conditons.

	With 5 m*M* L-Trp		Without Trp	
Strain	Starvation	Cheese-like	Starvation	Cheese-like
Lactobacillus casei LC301	$P = 0.40$	$P = 0.64$	$P = 0.06$	$P = 0.34$
Lactobacillus casei LC202	$P = 0.85$	$P = 0.01$	$P = 0.02$	$P = 0.17$
Lactobacillus helveticus LH212	$P = 0.36$	$P = 0.49$	$P = 0.26$	$P = 0.70$
Lactobacillus helveticus CNRZ32	$P = 0.42$	$P = 0.17$	$P = 0.30$	$P = 0.03$

[1] Researchers performed only t-test between specific activities measured on d 0 and d 21.

TABLE 24. Statistical t-test: Effect of incubation condition on tryptophan decarboxylase in cells incubated in chemically defined medium at days 0 and 21.

	With 5 m*M* L-Trp		Without Trp	
Strain	Day 0	Day 21	Day 0	Day 21
Lactobacillus casei LC301	$P = 0.35$	$P = 0.62$	$P = 0.39$	$P = 0.44$
Lactobacillus casei LC202	$P = 0.59$	$P = 0.22$	$P = 0.84$	$P = 0.25$
Lactobacillus helveticus LH212	$P = 0.87$	$P = 0.98$	$P = 0.93$	$P = 0.50$
Lactobacillus helveticus CNRZ32	$P = 0.64$	$P = 0.96$	$P = 0.48$	$P = 0.28$