Lait (1997) 77, 371-381

Original article

Aromatic amino acid catabolism by lactococci*

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Summary — While catabolism of amino acids is believed to play an important role in cheese flavor development, the pathways present in cheese microflora are poorly understood. To determine the pathways of aromatic amino acid catabolism in lactococci and effects of Cheddar cheese ripening conditions on catabolic enzymes and products, eight starter lactococcal strains were screened. Cell-free extracts prepared from these strains were found to contain an α-ketoglutarate-dependent aminotransferase activity with tryptophan, tyrosine and phenylalanine. Tryptophan, tyrosine and phenylalanine aminotransferase specific activities (μmol product formed/mg protein/min) ranged from 0.30 to 2.8 10^{-3}, 0.93 to 7.3 10^{-3} and 1.5 to 7.2 10^{-3}, respectively. Metabolites produced from tryptophan by a cell-free extract of *Lactococcus lactis* S3 were indolepyruvic acid, indoleacetic acid and indole-3-aldehyde. Indoleacetic acid and indole-3-aldehyde can form spontaneously from indolepyruvic acid under the conditions employed. A defined medium was used to determine whether the aminotransferase(s) was expressed and which metabolite(s) accumulate under conditions that simulated those of ripening Cheddar cheese in terms of pH, salt, temperature and carbohydrate starvation. The results indicated that the aminotransferase(s) was expressed and stable under these conditions. The tryptophan metabolites that accumulated were determined to be strain-specific.

lactococcus / aminotransferase / aromatic amino acid / catabolism / cheese flavor

Résumé — Catabolisme des acides aminés aromatiques des lactocoques. Alors que le catabolisme des acides aminés est perçu comme jouant un rôle important dans le développement de la flaveur du fromage; les voies métaboliques liées à la microflore du fromage ne sont que très partiellement établies. Pour déterminer les voies du catabolisme des acides aminés aromatiques dans les lactocoques, et les effets des conditions d'affinage du Cheddar sur les enzymes cataboliques et sur les produits, huit souches de levains lactocoques on été analysées. Des extraits de cellules préparées à partir de ces souches possédaient une activité aminotransférase α–ketoglutarate dépendante du tryptophane, de la tyrosine et de la phénylalanine. Les activités aminotransférases spécifiques du tryptophane, de la

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* Oral communication at the IDF Symposium 'Ripening and Quality of Cheeses', Besançon, France, February 26–28, 1996

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tyrosine et de la phénylalanine variaient respectivement de 0,30 à 2,8 10^{-3} et 1,5 à 7,2 10^{-3} (µmol de produit formé/mg de protéine à la minute). Les métabolites du tryptophane produits à partir d'extraits cellulaires de *Lactococcus lactis* S3 étaient de l'acide indolepyruvique, de l'acide indoleacétique et de l'indole-3-aldéhyde. L'acide indoleacétique et l'indole-3-aldéhyde peuvent se former spontanément à partir de l'acide indolepyruvique dans les conditions employées. Un milieu défini était utilisé pour déterminer si la ou les aminotransférases étaient exprimées et quel(s) étai(en)t le (les) métabolite(s) accumulé(s) dans des conditions qui simulent celles de l'affinage du Cheddar en terme de pH, sel, température et privation en hydrate de carbone. Les résultats indiquent que la (les) aminotransférase(s) étai(en)t exprimée(s) et stable(s) dans ces conditions. Les métabolites du tryptophane qui s'accumulent ont été trouvés comme étant spécifiques des souches.

lactocoque / aminotransférase / acide aminé aromatique / catabolisme / flaveur du fromage

INTRODUCTION

The development of flavor in bacterial-ripened cheese varieties is a complex and poorly understood process. The best characterized process in the development of flavor in these products is proteolysis. The enzymes involved in the hydrolysis of milk proteins have been relatively well characterized. Additionally, our knowledge of the peptides and amino acids that accumulate in ripening cheese is rapidly expanding (Fox et al, 1993; Law and Mulholland, 1995). However, there is no definitive evidence to support the widely-held belief that amino acids and peptides are directly involved in beneficial cheese flavors. Therefore, further research on the mechanism(s) by which peptides and amino acids serve as flavor precursors in cheese is required. The catabolism of amino acids by cheese microflora is one mechanism by which products of proteolysis are thought to serve as flavor precursors in cheese. However, very little is known concerning the metabolism of amino acids from cheese-related microorganisms. The best characterized example is the production of methanethiol from methionine by a lactococcal cystathionine, β-lyase (Alting et al, 1995). Possible mechanisms of amino acid catabolism include decarboxylation, deamination, transamination, desulphuration and cleavage of the amino acid side chain. A general scheme of amino acid catabolism by cheese-related bacteria has been reviewed by Hemme et al (1982) and Law (1987).

The products of aromatic amino acid catabolism are believed to be involved in the development of off-flavors in cheese. Specifically, indole, skatole, *p*-cresol, and *p*-hydroxylphenylacetic acid have been reported to contribute to putrid, fecal or unclean aroma flavors in cheese (Schormüller, 1968; Dumont et al, 1974; Guthrie, 1993). The metabolic pathways involved in the formation of these compounds have been described in a number of microorganisms; however, these pathways have not been described in lactic acid bacteria. Additionally, chemical reactions may also be involved in the formation of these compounds in cheese.

It is important to examine the interactions between the starter cultures, non-starter lactic acid bacteria and adjunct cultures in order to understand how aromatic amino acid catabolites are formed in cheese. To initiate studies in this area, we have chosen to focus on characterization of the aromatic amino acid catabolism by lactococci, which are used as starter cultures in many ripened cheese varieties. The objectives of this research were to screen lactococci for enzymes capable of initiating the catabolism of aromatic amino acids and to identify the

metabolites produced by these organisms under conditions that simulate those of ripening Cheddar cheese.

MATERIALS AND METHODS

Bacterial strains and media

Lactococcus lactis spp *cremoris* HP, SK11, C2, KH, *L lactis* spp *lactis* 11454 and *L lactis* spp *lactis* biovar *diacetylactis* 11007 were obtained from LL McKay (University of Minnesota, St Paul, MN, USA). *L lactis* spp *cremoris* S1 and *L lactis* spp *lactis* S3 are industrial isolates. The strains were propagated in M17 broth containing 0.5% lactose (M17-L; Terzaghi and Sandine, 1975) for 11 to 13 h at 30 °C.

Preparation of cell-free extracts

Cells grown in M17-L broth (100 mL) were harvested by centrifugation (12 000 *g*, 10 min, 4 °C) and washed sequentially with 30 mL 0.85% NaCl and 25 mL 50 mmol/L phosphate buffer (pH 7.0) or 50 mmol/L Bis–Tris buffer (pH 6.8). All solutions used in the washes were maintained at 4 °C. Washed cells were suspended in 10 mL 50 mmol/L phosphate buffer (pH 7.0) or Bis–Tris buffer (pH 6.8) and disrupted by shaking with glass beads (150–212 µm; Sigma Chemical Co, St Louis, MO, USA) for 10 min on a paint-can shaker (Model 5410 paint mixer, Red Devil Equipment Co, Minneapolis, MN, USA). Cell debris and glass beads were removed by centrifugation (20 400 *g*, 30 min, 4 °C) and the supernatant was used as the cell-free extract (CFE).

Determination of protein concentration

Protein concentrations were determined by the method described by Bradford (1976) using bovine serum albumin (BSA) as standard.

Enzyme assays

Tryptophan aminotransferase (EC 2.6.1.27, Trp ATase) activity was assayed by measuring the formation of indolepyruvic acid (IPA) using a modification of the method described by Frankenberger and Poth (1988). The reaction mixture consisted of 5 mmol/L L-Trp, 5 mmol/L α-ketoglutarate (α-KA), 50 µmol/L pyridoxal phosphate (PLP), 0.5 mmol/L sodium arsenate, 0.5 mmol/L EDTA in 50 mmol/L sodium tetraborate (pH 8.5). The enzyme reaction was initiated by the addition of CFE and incubated for 30 min at 30 °C. The reaction was stopped by the addition of 10% trichloroacetic acid with agitation. Precipitated proteins were removed by centrifugation. Absorbance at 327 nm was determined and the concentration of IPA calculated using a standard IPA curve (Sigma) in the assay buffer. Preliminary analysis indicated that activity represents initial velocity for the reaction measured. Specific activity was expressed in µmol IPA/min/mg protein at 30 °C.

Phenylalanine aminotransferase (Phe ATase) activity was determined using the same reaction mixture as described above but with L-Phe instead of L-Trp. Absorbance at 300 nm was determined and the concentration of phenylpyruvic acid (PPA) calculated using a standard PPA curve (Sigma) in the assay buffer. Specific activity was expressed in µmol PPA/min/mg protein at 30 °C.

Tyrosine aminotransferase (Tyr ATase) activity was measured in the same reaction mixture as above but with L-Tyr instead of L-Trp. Absorbance at 330 nm was determined and the concentration of 4-hydroxylphenylpyruvate (HPPA) calculated using a standard HPPA curve (Sigma) in the assay buffer. Specific activity was expressed in µmol HPPA/min/mg protein at 30 °C.

Additionally, ATase activities were determined using the same reaction mixtures described above but using 150 mmol/L Bis–Tris (pH 6.8) buffer.

Tryptophan monooxygenase activity was assayed as described by Comai and Kosuge (1980). High-performance liquid chromatography (HPLC) was used to detect the reaction product indoleacetamide as described below. Tryptophan side-chain oxidase was assayed by measuring the formation of *N*-acetyl-α,β,-didehydrotryptophanamide from *N*-acetyl-L-tryptophanamide (Sigma), as described by Narumiya et al (1979). Tryptophan decarboxylase activity was assayed as described by Nakezawa et al (1977). All specific activities reported were calculated from duplicate analysis on duplicate samples.

Isolation and determination of L-Trp metabolites

The reaction mixture (2.6 mL) employed contained 0.25 mg protein (CFE), 22.6 mmol/L L-Trp, 18.9 mmol/L α-KA, 0.1 mmol/L PLP and 150 mmol/L Bis–Tris buffer (pH 6.8). The reaction was initiated by the addition of CFE and stirred at room temperature (25 °C) for 72 h. During this time, 0.5-mL aliquots of the reaction mixture were removed, acidified to pH 3 with 2 N HCl, and extracted with 1 mL ethyl acetate. The metabolites were identified by comparison with appropriate standard compounds on thin-layer chromatography (TLC) and HPLC as described below.

Aromatic ATase zymograms

Native polyacrylamide gel electrophoresis (PAGE) was conducted with a Bio-Rad Mini-Protean unit using the method described by Davis (1964) with minor modifications. A 4% acrylamide stacking gel and 9% separating gel were employed. Samples were prepared by mixing CFE (100 μg) with sample buffer containing bromophenol blue as the tracking dye. Electrophoresis was carried out in Tris–boric acid–EDTA buffer at 120 V for 2 h at 4 °C. Gels were stained for aromatic ATase activities by a modification of the procedure described by Ryan et al (1972). The assay mixture contained 7.5 mmol/L α-KA, 10 mmol/L of the appropriate amino acid, 3 mmol/L NAD^+, 0.7 mmol/L nitroblue tetrazolium, 0.13 mmol/L phenazine methosulfate, 0.4 mmol/L PLP, 6–9 U of glutamate dehydrogenase (bovine liver), 100 mmol/L Bis–Tris (pH 6.8 for Trp and Phe, pH 7.1 for Tyr) and 1% agar. Bands of aromatic ATase activity appeared after incubation at 35 °C for 2.0 to 2.5 h.

Defined medium used to simulate conditions of Cheddar cheese ripening in terms of pH, salt, temperature and carbohydrate starvation

A defined medium for lactococci based on the medium described by Jensen and Hammer (1993) using the salt solution described by Jenness and Koops (1962) was employed. Modifications included reducing the amount of lactose to 0.2% (w/v), adding 20 mmol/L β-glycerophosphate, and minor modification of amino acid concentrations. The pH was adjusted to 6.6 with HCl and the medium was autoclaved. After cooling, the medium was supplemented with trace element solution Ho-Le (described in the ATCC catalogue, 17th edn, 1989, p 300) and vitamin mixture (R-7256, Sigma).

Cells propagated in M17-L for 11–13 h at 30 °C were harvested by centrifugation, washed once with 0.85% NaCl, once with defined medium, and resuspended in fresh defined medium. These cells were used to inoculate (0.5%) the defined medium and the culture was incubated at 30 °C for 13 h. During the 13-h incubation period the lactose was depleted, pH was reduced to approximately 5.1, and the culture reached a stationary phase. Sterile NaCl solution was added to a final concentration of 4% NaCl. The cultures were incubated at 13 °C for 3 weeks with samples taken periodically. Samples were analyzed for number of viable cells by plating on M17-L; ATase activity was determined at pH 6.8 using CFEs prepared from harvested cells as described previously, and metabolites present in the culture supernatant were identified as described below. All analyses were made in duplicate on duplicate samples.

Extraction and quantification of metabolites

Culture supernatant (96 mL) was acidified to pH 3.0 with 2 N HCl and extracted twice with 50 mL ethyl acetate. The ethyl acetate extract was taken to near dryness using a vacuum rotary evaporator in a 37 °C water bath. The residue was quantitatively transferred to a capped vial and reconstituted to a volume of 1 mL with 95% ethanol. This reconstituted culture extract was used directly for analysis by HPLC (20 μL injection volume). Standard curves were prepared by spiking known concentrations of commercial indoleacetic acid (IAA), indole-3-aldehyde (IAld) and 4-hydroxylbenzaldehyde (HBAld) into the defined medium and using the extraction procedure described above.

High-pressure liquid chromatography (HPLC)

The HPLC equipment consisted of an Hitachi L-6200A pump and L-4500A diode array detec-

tor (Hitachi Ltd, Tokyo, Japan), and a Gilson model 231 automated sample injector (Gilson Inc, Madison, WI, USA). Data acquisition and processing was performed using the Hitachi D-6500 Chromatography Data Station Software and DAD System Manager (Hitachi Ltd). Sample separation was accomplished using a Versapak C18 analytical column (300 4.1 mm, 10 µm; Alltech Associates Inc, IL, USA) with a NewGuard C18 pre-column (15 3.2 mm, 5 µm; Applied Biosystems Inc, CA, USA). The mobile phase comprised 0.05% trifluoroacetic acid (Solvent A) and acetonitrile (Solvent B) with a flow rate of 1.0 mL min^{-1}. Elution of samples was performed using a gradient of 10 to 15% Solvent B from 0 to 25 min, followed by 15 to 30% Solvent B from 25 to 40 min and 30 to 90% Solvent B from 40 to 45 min. A 5-min treatment of 90% Solvent B was performed to elute remaining sample components following each sample injection before equilibration at initial conditions. Data were analyzed for compounds of interest detected at 278 (IAA), 290 (HBAld), and 300 nm (IAld).

Thin-layer chromatography (TLC)

Ethyl acetate extracts of reaction solution or culture supernatant were spotted onto precoated silica gel 60 plates (Merck). Standards (IAA, IAld, HBAld, IPA) were spotted onto the same plates. The plates were developed using either chloroform/ methanol/hexane/acetic acid (50:10:40:0.5) or ethyl acetate/hexane/isopropanol/acetic acid (45:45:10:0.5). Detection of compounds was accomplished via the anisaldehyde reagent (Sigma). Compounds were identified by comparing their R_f values and color to that of the standards.

RESULTS

Screening of lactococcal strains for enzymes able to catabolize tryptophan

L lactis HP, SK11, C2, KH, 11454, 11007, S1 and S3 were screened for enzymes able to catabolize L-Trp. No tryptophanase, tryptophan decarboxylase, tryptophan-2-monooxygenase or tryptophan side-chain oxidase activities were observed in any of the strains examined. Trp ATase activity was detected in all eight strains. The Trp ATase specific activities at pH 8.5 ranged from 0.30 to 2.8 10^{-3} µmol/mg protein/min (table I). With the exception of 11007, no significant differences were observed between strains. When Trp ATase specific activities were determined at pH 6.8, a 3- to 5-fold higher level of activity was observed with all strains except 11007.

The product of the reaction using an S3 CFE with L-Trp was determined to be IPA by TLC and HPLC; this reaction mixture contained 0.5 mmol/L sodium arsenate, 0.5 mmol/L EDTA and 50 mmol/L sodium tetraborate, which have been shown previously to stabilize IPA in aqueous solutions (Frankenberger and Poth, 1988). The removal of PLP from the reaction mixture had no effect on IPA production. However,

Table I. Aromatic aminotransferase activities[a] from cell-free extracts of lactococcal strains. *Activités transférases des acides aminés aromatiques provenant d'extraits de cellules de souches de lactocoques.*

Strain	*Specific activities (µmol product formed/mg protein/min) × 10^{-3}*		
	Trp ATase	*Phe ATase*	*Tyr ATase*
HP	1.6 ± 0.4	5.1 ± 0.8	5.8 ± 0.4
SK11	2.7 ± 0.1	7.2 ± 0.6	7.3 ± 2.0
S3	1.7 ± 0.1	5.2 ± 0.7	5.7 ± 1.1
S1	1.8 ± 0.7	4.9 ± 1.1	5.2 ± 0.4
11007	0.30 ± 0.2	1.5 ± 0.4	0.93 ± 0.2
11454	2.0 ± 0.2	6.5 ± 1.3	6.5 ± 1.3
C2	2.8 ± 0.4	6.2 ± 0.6	6.7 ± 2.5
KH	2.0 ± 0.1	5.2 ± 0.8	6.1 ± 1.6

[a]Average values and ranges for duplicate cultures. All values obtained from a given sample had < 10% difference. Assays were conducted at pH 8.5 as described in *Materials and methods*.
[a]Valeurs moyennes et amplitudes pour deux cultures. Toutes les valeurs obtenues pour un échantillon donné ont une différence inférieure à 10 %. Les essais ont été réalisés avec un pH de 8,5 comme préconisé dans Materials and methods.

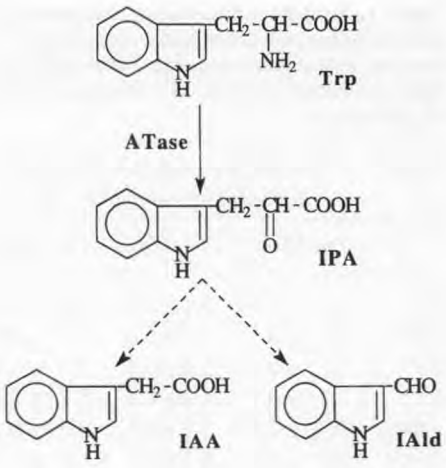

Fig 1. Scheme of tryptophan catabolic pathways in lactococci. Trp: tryptophan; ATase: aminotransferase; IPA: indole-3-pyruvic acid; IAA: indole-3-acetic acid; IAld: indole-3-aldehyde. The solid arrow represents enzymatic conversion; the dashed arrow indicates that the mechanism of conversion is unknown.
Schéma des voies cataboliques de tryptophane dans les lactocoques. Trp : tryptophane ; ATase : aminotransférase ; IPA : acide indole-3-pyruvique ; IAA : acide indole-3-acétique ; IAld : indole-3-aldéhyde. La flèche pleine représente la conversion enzymatique, la flèche en pointillés indique que le mécanisme de conversion est inconnu.

reaction mixtures lacking α-KA or using heat-inactivated CFE did not result in IPA production. When reactions were conducted using S3 CFE with L-Trp in the absence of the components known to stabilize IPA (the reaction mixture contained 22.6 mmol/L L-Trp, 18.9 mmol/L α-KA, 0.1 mmol/L PLP and 150 mmol/L Bis–Tris, pH 6.8) the products were determined by TLC and HPLC to be IPA, IAld, and IAA (fig 1). The concentration of IPA decreased as the concentrations of IAld and IAA increased. However, spontaneous decomposition of IPA to IAA and IAld was also observed under these conditions. Regardless of the reaction conditions employed, tryptophol, indoleacetaldehyde or indoleacetamide were not detected.

Screening of lactococcal strains for Tyr ATase and Phe ATase activities

The same eight lactococcal strains were also examined for Tyr ATase and Phe ATase activities. Tyr ATase and Phe ATase specific activities ranged from 0.93 to 7.3 10^{-3} and from 1.5 to 7.2 10^{-3} μmol/mg protein/min (table I), respectively. As was observed with Trp ATase activity, only 11007 differed significantly from the other strains.

The products of the reaction of S3 CFE with L-Tyr were determined by HPLC to include HPPA, 4-hydroxyl-benzaldehyde (HBAld) and 4-hydroxylphenylacetic acid (HPAA). However, spontaneous decomposition of HPPA to HBAld and HPAA was also observed under the conditions employed.

The product of the reaction when L-Phe was used as the substrate with an S3 CFE was determined by HPLC to be PPA. PPA was determined to be stable under the conditions employed.

Aromatic ATase zymograms

The R_f of Trp ATases from the eight strains after native PAGE were determined (fig 2). Seven strains produced a band with a similar R_f; one of these strains (KH) had an additional very faint band. No band was observed with CFE from 11007. Native PAGE followed by histochemical staining was also conducted using L-Tyr or L-Phe as substrates. In both cases, the staining patterns were very similar to that observed when L-Trp was used as the substrate (data not shown).

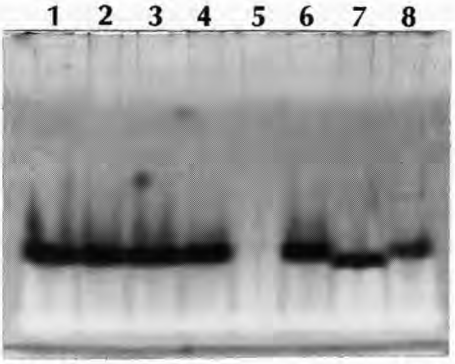

Fig 2. Identification of tryptophan aminotransferase activities in various lactococcal strains by histochemical staining of native PAGE. Lanes: 1, HP; 2, SK11; 3, S3; 4, S1; 5, 11007; 6, 11454; 7, C2; 8, KH.
Identification des activités aminotransférases du tryptophane de plusieurs souches de lactocoques par coloration histochimique des PAGE. Lignes : 1, HP ; 2, SK11 ; 3, SB ; 4, S1 ; 5, 11007 ; 6, 11454 ; 7, C2 ; 8, KH.

ATase activities and metabolites produced in defined medium

A defined medium was employed to determine whether lactococcal ATase activities were expressed under conditions that simulated those encountered by cultures in ripening Cheddar cheese in terms of pH, salt, temperature and carbohydrate starvation; and which metabolites, if any, would accumulate. The three lactococcal strains chosen for this component of the study were S3, S1 and 11007. The number of viable

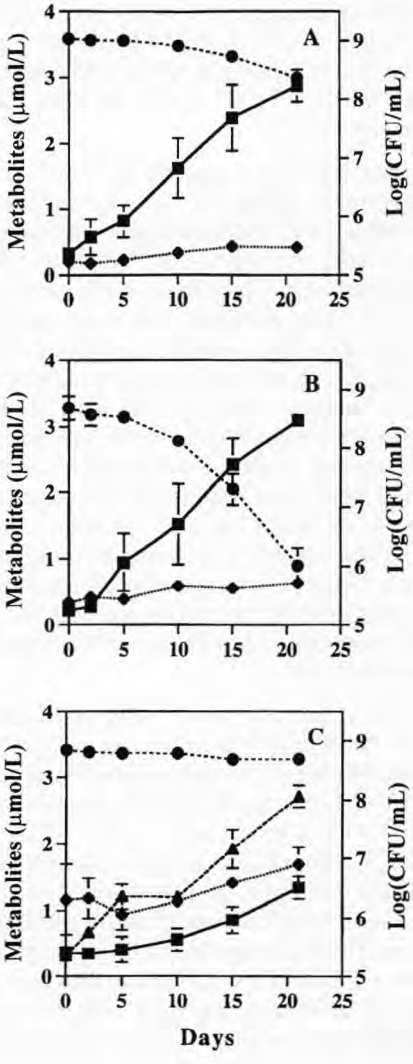

Fig 3. Accumulation of metabolites from aromatic amino acids and CFU/mL (●) during a 3-week incubation period under conditions that simulated Cheddar cheese ripening (pH 5.1, 4% NaCl, 13 °C, lactose depletion). Aromatic amino acid metabolites indole-3-aldehyde (IAld, ■), indoleacetic acid (IAA, ▲), and 4-hydroxyl-benzaldehyde (HBAld, ◆) were quantified by HPLC. The three strains of *L lactis* examined were S3 (panel A), S1 (panel B) and 11007 (panel C).
Accumulation de métabolites provenant d'acides aminés aromatiques et UFC/mL (●) après 3 semaines de maturation dans des conditions qui simulent l'affinage du Cheddar (pH 5,1 ; 4 % NaCl ; 13 °C ; déplétion du lactose). Les métabolites d'acides aminés aromatiques indole-3-aldéhyde (IAld, ■) acide indoleacétique (IAA, ▲), et hydroxyl-4-benzaldéhyde (HBAld, ◆) sont quantifiés par HPLC. Les trois souches de L lactis *examinées sont S3 (échantillon A), S1 (échantillon B) et 11007 (échantillon C).*

microorganisms, Trp ATase activities, and aromatic amino acid metabolites were determined.

The number of viable microorganisms present in the defined medium during the 3-week incubation period is presented in figure 3. All three strains obtained approximately the same cell density after 13 h at 30 °C. At this point the lactose was depleted, the pH was approximately 5.1 and NaCl was added to a final concentration of 4%. Significant variation was observed in strain viability during the 3-week incubation period at 13 °C (fig 3). The percentage reduction in CFUs per mL over this period for S3, S1 and 11007 was 79, 99.8 and 29% respectively.

The Trp ATase specific activities were determined during the 3-week incubation period at 13 °C. Significant differences were observed between strains with respect to the influence of this incubation on the expression of ATase activities. An approximately 5-fold increase from 0.57 ± 0.01 to $3.0 \pm 1.2\ 10^{-3}$ (μmol/mg protein/min) in Trp ATase activity was observed with 11007. However, no notable change in Trp ATase activity was observed with either S1 or S3. Their activities remained consistent at $29 \pm 4\ 10^{-3}$ and $12 \pm 8\ 10^{-3}$ (μmol/mg protein/min), respectively. Although no significant change in Trp ATase specific activity or total activity was observed with S1, a 99.8% reduction in viability of this culture was observed.

The accumulation of L-Trp and L-Tyr derived metabolites in the culture supernatant during the 3-week incubation period at 13 °C for S3, S1 and 11007 is presented in figure 3 (A, B and C, respectively). All three strains accumulated similar quantities of HBAld during the aforementioned period at 13 °C; 11007 accumulated significantly more HBAld during the initial 30 °C incubation. Significant variation was observed in the accumulation of the L-Trp derived metabolites during the 3-week incubation. For both S3 and S1, IAld accumulation increased throughout the latter period and reached a final concentration of approximately 3 μmol/L. No accumulation of IPA or IAA was detected with either S3 or S1. In contrast, accumulation of both IAA and IAld increased throughout the 3-week incubation with 11007 and reached final concentrations of 2.8 and 1.3 μmol/L, respectively. No accumulation of IPA was detected with 11007.

DISCUSSION AND CONCLUSION

Lack of flavor development and an increase in the occurrence of off-flavors in reduced-fat Cheddar cheese has significantly reduced consumer acceptance of these products. Previous research had indicated that the intensity of off-flavors such as meaty-brothy and unclean was starter-culture specific (Johnson and Chen, 1995). This information and the knowledge that unclean flavor compounds can be produced from aromatic amino acids prompted us to investigate the catabolism of aromatic amino acids by lactococci.

Screening of eight lactococcal strains for enzymes capable of initiating catabolism of L-Trp indicated in these strains that the first step was mediated by an ATase. ATase activity was also observed with L-Tyr and L-Phe in all strains examined. Histochemical staining results also suggested that the ATase(s) detected had activity on L-Trp, L-Tyr and L-Phe. The S3 ATase activity was determined to be dependent on α-KA; however, PLP addition was not required for enzyme activity. ATases from other bacterial sources have been shown to be PLP-dependent; it is likely that the S3 ATase is also PLP-dependent but that sufficient PLP for maximum activity was present in the CFE. It is not possible from our data to determine the number of lactococcal ATases that are involved in the catabolism of aromatic amino acids. Previously, aromatic amino acid ATase activity had been demon-

strated to be widely distributed in bacteria; examples include *Brevibacterium linens* (Lee and Desmazeaud, 1985), *Rhizobium leguminosarum* (Pérez-Galdona et al, 1992), *Enterobacter cloacae* (Koga et al, 1994) and *Erwinia herbicola* (Manulis et al, 1991).

The initial products produced by the S3 ATase(s) from L-Trp, L-Tyr and L-Phe were determined to be IPA, HPPA and PPA, respectively. IPA and HPPA are rather unstable in aqueous solution. A roughly 1:1 ratio of IAld to IAA was observed in both Trp aminotransferase reactions and reaction mixtures monitoring spontaneous degradation of IPA. Deviation from this ratio was observed when reactions were conducted under inert gas atmosphere. The spontaneous degradation of IPA has made it difficult to obtain evidence for the participation of enzymes in the conversion of IPA to either IAld or IAA (Kaper and Veldstra, 1958; Shelddrake, 1973; Koga, 1995). The stability of PPA in the reaction mixture suggests that lactococci do not express enzymes capable of the catabolism of PPA. However, it is also possible that enzymes capable of the catabolism of PPA were not expressed under the conditions in which the culture was grown, or that an enzyme capable of degrading PPA was present but not active under the conditions employed.

The observed variation among strains of lactococci in ATase activity on aromatic amino acids was insignificant, except for that observed with 11007. The lower activities with 11007 initially suggested that this strain would not produce aromatic amino acid metabolites at the levels expected from the other strains. However, 11007 produced higher levels of aromatic amino acid metabolites after a 3-week incubation period in the defined medium. These results demonstrate the potential for reaching inaccurate conclusions when results from experiments not conducted under cheese-like conditions are extrapolated to the cheese environment.

The presence of L-Trp-derived metabolites in all the supernatants from the defined medium indicates that an aromatic ATase(s) with activity on L-Trp is active under these conditions in the three strains examined. The accumulation of IAld in the absence of IAA by *L lactis* S1 and S3 in the defined medium suggests that these strains contain an enzyme capable of converting IPA to IAld or the IAA produced by spontaneous degradation of IPA is further catabolized. However, further research is required to confirm that this conversion is enzyme-mediated and not due to spontaneous degradation of IPA solely to IAld. The accumulation of both IAA and IAld by *L lactis* 11007 in the defined medium suggests that this strain produces IPA from L-Trp, which then spontaneously degrades to IAA and IAld. However, it is also possible that this culture contains both an enzyme capable of converting IPA to IAld and a pathway similar to that described in *E cloacae* for the conversion of IPA to IAA. The *E cloacae* pathway includes an indolepyruvic decarboxylase, and possibly an indole-3-acetaldehyde oxidase (Koga et al, 1992; 1994; Koga, 1995). Further research is required to elucidate the pathways involved in the catabolism of L-Trp by lactococci.

The accumulation of HBAld by *L lactis* S1, S3 and 11007 in the defined medium indicates that at least one aromatic ATase with activity on L -Tyr is active under these conditions. The mechanism for the conversion HPPA to HBAld remains unknown, and will require further investigation.

An approximately 10-fold difference was observed in the Trp ATase activities between the three strains used in the defined medium; however, only minor differences were observed in the accumulation of L-Trp-derived metabolites. These results suggest that Trp ATase activity was not the limiting factor in the production of these compounds. It seems likely that L-Trp transport or diffusion is the limiting factor, given

the fact that the carbohydrate has been depleted prior to production of the L-Trp-derived metabolites.

Comparison of results obtained with *L lactis* S1 and S3 indicate that while a significant reduction in viable S1 cells occurred, these cells remained metabolically active. *L lactis* S1 produced a quantity of IAld similar to that observed with S3. Additionally, no significant reduction occurred in S1 intracellular Trp ATase activity during the incubation period. This indicates that lysis of *L lactis* S1 did not occur in the defined medium, and suggests that non-viable lactococcal strains in the cheese matrix can influence cheese flavor development.

Investigation of possible interactions between starter cultures and non-starter lactic acid bacteria will be required to determine what, if any, influence the production of these aromatic amino acid-derived compounds have on cheese flavor. Production of IAA is of particular interest, as some strains of lactobacilli are known to convert IAA to skatole (Honeyfield and Carlson, 1990).

ACKNOWLEDGMENTS

This project was supported by the College of Agricultural and Life Sciences at the University of Wisconsin-Madison, the Wisconsin Milk Marketing Board, Utah State University Agricultural Experiment Station, and the Western Center for Dairy Protein Research and Technology through funding from the National Dairy Promotion and Research Board.

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