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Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening*

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Summary — The influence of NaCl and reduced pH was determined for aminopeptidase, lipase/esterase and methanethiol-producing capability in selected lactic acid bacteria and brevibacteria in simulated cheese-like conditions. The observations on simulated cheese-like conditions were confirmed in 60% reduced-fat Cheddar cheese. The activity of each enzyme decreased with NaCl addition and when the pH was reduced to approximate Cheddar cheese conditions (5% NaCl, pH 5.2). Residual intracellular aminopeptidase activity was dominated by general aminopeptidase activity (aminopeptidase N and/or aminopeptidase C) in laboratory, simulated cheese-like conditions, and 60% reduced-fat Cheddar cheese curd. During cheese aging, total lipase/esterase activity peaked at 120 d then decreased, even though starter culture populations remained high. Methanethiol-producing capability occurred under cheese-like conditions in whole cells, but not in cell-free extracts. Met and Met-containing peptides induced methanethiol-producing capability for 2–3 generations and could be re-induced later in the growth cycle of *Brevibacterium linens* BL2. Aminopeptidase and lipase/esterase activity in reduced fat cheese were not correlated to an increase in Cheddar-type flavor, but a culture's methanethiol-producing capability was associated with higher cheese consumer preference scores. Results suggest that use of cheese-like conditions may aid in selecting cultures to

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increase desirable flavors for low-fat cheese manufacture. Additionally, data suggest that whole cells are important for proper flavor development in 60% reduced-fat Cheddar cheese.

cheddar cheese / ripening / flavor / lactococcus / lactobacillus / brevibacterium / methanethiol production

Résumé — Influence du NaCl et du pH sur les enzymes intracellulaires qui affectent l'affinage du cheddar. L'influence du NaCl et d'une réduction du pH a été déterminée sur les aminopeptidases, les lipases/esterases, ainsi que l'aptitude à produire du méthanéthiol de bactéries lactiques et de brevibactéries sélectionnées dans des conditions de fabrications fromagères simulées. Les observations, dans ces conditions, étaient confirmées, dans le cas du cheddar à teneur en matières grasses réduites de 60 %. Les activités de chaque enzyme diminuaient avec l'addition de NaCl et lorsque le pH était abaissé à des niveaux proches des conditions de fabrication du cheddar (NaCl 5 % et pH 5,2). L'activité aminopeptidasique intracellulaire résiduelle était dominée par l'activité aminopeptidasique générale (aminopeptidase N et/ou aminopeptidase C) au laboratoire, en conditions fromagères simulées, et dans un caillé de cheddar à teneur en MG réduite de 60 %. Pendant le vieillissement des fromages, l'activité totale lipase/esterase atteignait un pic à 120 jours puis diminuait, même si les populations de levains demeuraient élevées. L'aptitude à produire du méthanéthiol se manifestait en conditions fromagères simulées dans les cellules entières, mais non dans les extraits dépourvus de cellules. Les peptides Met et Met-contenant induisaient une aptitude à produire du méthanéthiol sur deux ou trois générations, et pouvaient être réinduits ultérieurement dans le cycle de croissance de *Brevibactérium linens* BL2. L'activité aminopeptidase et lipase/estérase dans le fromage à teneur réduite en matière grasse n'était pas corrélée avec une augmentation de la saveur de type cheddar, mais l'aptitude d'une culture à produire du méthanéthiol était associée à des notes de préférence plus élevées chez les consommateurs de fromages. Les résultats suggèrent que l'utilisation de conditions fromagères simulées peut aider à la sélection de cultures, pour augmenter les flaveurs souhaitées en fabrication de fromage à faible teneur en matière grasse. De plus, les données suggèrent que les cellules entières sont importantes dans le développement d'une flaveur correcte dans le cheddar à teneur en matière grasse réduite de 60 %.

fromage cheddar / affinage / arôme / lactocoque / lactobacille / brevibactérie / production de méthanéthiol

INTRODUCTION

Many consumers are concerned about the nutritional value of the food they consume. Demand for dairy products containing lower fat, cholesterol and saturated fatty acids is steadily increasing (Hise, 1991). However, it is difficult to manufacture lower-fat Cheddar cheese with good flavor. Defects in cheese in which fat was reduced by more than 50% include lack of flavor, off-flavors such as meaty, brothy, unclean and bitterness, as well as improper body and texture (Sherwood, 1939; Banks et al, 1989; Jameson, 1990; Hise, 1991; Johnson, 1991; Lindsay, 1991).

Cultures suitable for full-fat Cheddar cheese often do not produce acceptable low-fat Cheddar cheese (Johnson, 1991). Fat removal changes the basic biochemistry of ripening. The primary biochemical reactions in cheese ripening, which lead to cheese flavor and texture development, include glycolysis, proteolysis and lipolysis (Fox et al, 1993). Additionally, undefined secondary reactions, such as protein dephosphorylation and amino acid (AA) metabolism may be important as well (Schormüller, 1968; Reiter and Sharpe, 1971; Fox et al, 1993). Microbial enzymes strongly influence proteolysis during aging (Adda et al, 1982; Law, 1987; Aston and Creamer, 1986; Ardo et al, 1989; Olson, 1990; Seitz, 1990; El Soda and Pandian, 1991; Fox et al, 1993; McGarry et al, 1994). As a result, considerable interest has focused on the enzymol-

ogy and molecular biology of the lactococcal proteinase system and the impact that these enzymes have on desirable flavors and bitterness (Thomas and Mills, 1981; Kok, 1990; Olson, 1990). Free amino acids are thought to be precursors for background cheese flavor compounds, but the mechanism is largely undefined (McGugan, 1975; Adda et al, 1982; Law, 1987; Olson, 1990).

Adjunct bacteria have been used to boost typical Cheddar cheese flavor (Peterson and Marshall, 1990), initially focusing on non-starter lactic acid bacteria (NSLAB), since they grow to high numbers (10^7 CFU/g of cheese during storage at 8 °C) during cheese ripening, and presumably contribute to cheese flavor (Chapman and Sharpe, 1981; Broome and Hickey, 1991). Non-starter lactic acid bacteria (NSLAB) consist predominantly of *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus brevis.* In addition to lactobacilli, to a lesser extent micrococci, brevibacteria and pediococci have also been used (Chang, 1985; Peterson and Marshall, 1990). Addition of adjunct bacteria has shown some promise in manufacturing low-fat cheese with reduced defects and improved flavor (Jameson, 1990; Khalid and Marth 1990; Olson, 1990).

Methanethiol is a degradation product of the sulfur-containing amino acid, methionine, and has been associated with good Cheddar cheese flavor (Aston and Dulley, 1982; Hemme et al, 1982). Methanethiol is the only compound that correlates to highly flavorful Cheddar cheese, but alone or in excess it produces atypical flavors (Aston and Dulley, 1982). Other compounds associated with good cheese flavor have not been conclusively elucidated. Based on this observation, Law (1987) accelerated Cheddar cheese flavor development by adding cell-free extracts of brevibacteria, which produce large amounts of methanethiol. Flavors typical of 6-month-old Cheddar cheese were observed in about 2 months. However, the cheese rapidly over-ripened and had flavor and texture defects. Thus, addition of methanethiol-producing bacteria into lower-fat cheese may increase the amount of positive, typical Cheddar-type flavors, but strains must be carefully selected to avoid defects.

The mechanism for the production of methanethiol by bacteria is not fully understood, but is linked to at least two enzymes – methionine γ-lyase (Collin and Law, 1989) and cystathionine β-lyase (Alting et al 1995). These degradation steps begin with deaminases, decarboxylases, transaminase, aminotransferases, and by the conversion of amino acid side chains in which methionine γ-lyase and cystathionine β-lyase are part of a larger metabolic pathway. Little is known of the enzymes, pathways and mechanisms involved in the formation of methanethiol during cheese ripening. This study investigated the influence of ripening conditions on aminopeptidase, lipase/esterase and methanethiol production during aging.

MATERIALS AND METHODS

Bacterial strains

Lactococcus lactis ssp *cremoris* S1 (Marschall Products, Madison, WI) and SK11 (Steele laboratory collection), and *L lactis* ssp *lactis* S3 (Marschall Products) were grown in Elliker's broth (Difco, Detroit, MI) at 30 °C for 16 to 18 h before use. *Brevibacterium linens* BL1 and BL2 (Weimer laboratory collection) were grown in trypticase soy broth (TSB; Becton Dickinson, Cockeysville, MD) at 25 °C with aeration at 250 rpm for 24 to 36 h before use. *Lactobacillus casei* LC301 (Marschall Products) and JC202 (Marschall Products) were grown in MRS broth (Difco) at 30 °C for 16 to 18 h before use. *Lactobacillus helveticus* LH212 (Marschall Products) and CNRZ 32 (Steele laboratory collection) were grown in MRS broth (Difco) at 37 °C for 16 to 18 h before use. Lactococci and lactobacilli were frozen in 10% non-fat dry milk (NFDM) containing 30% glycerol while brevibacteria were frozen in TSB containing 30% glycerol. All strains were stored at –70 °C until further use. Before each use, frozen stock cul-

tures were thawed and grown in their respective media at their respective temperatures for two transfers prior to inoculation into 100 mL growth medium for further testing.

Preparation of cell-free extracts

Cultures were incubated to mid-logarithmic phase and harvested from 100 mL media by centrifugation at 7000 *g* for 15 min at 4 °C. The pellet was collected and washed twice with 0.05 mol/L potassium phosphate buffer (pH 7.2) before resuspension in 5 mL of 0.01 mol/L potassium phosphate buffer (pH 7.2). Cell-free extract (CFE) was prepared by vortexing at high speed with glass beads (Sigma Chemicals, St Louis, MO) for 2 min at 25 °C. The CFE was collected by centrifuging at 5000 *g* for 30 min and the volume was made up to 15 mL with sterile water and added directly to either 0.05 mol/L potassium citrate (pH 5.2) or 0.05 mol/L potassium phosphate (pH 6.8).

Protein assay

The protein content in each CFE was determined using the bicinchoninic acid assay (Pierce Chemical Co, Rockford, IL). Bovine serum albumin (BSA) was used to obtain a standard curve.

Proteinase characterization

The proteinase activity of each strain was determined using BODIPY FL-labelled casein (Molecular Probes, Eugene, OR) according to the Enzchek kit instructions. Cells in mid-logarithmic phase were added (50 µL) to the substrate and incubated for 24 h at the culture's respective growth temperature in the dark. The replicate fluorescence tests measured the relative fluorescence units (RFU), which were adjusted for the plate count before and after incubation.

Aminopeptidase and lipase/esterase assays

Aminopeptidase (AP) activity was measured using automated reflectance colorimetry as described by Dias and Weimer (1995) with 10 mmol/L stock solutions of Arg-, Leu-, Lys-, Ala-, Met-, Asp-, Val-, Gly-, Pro- and γ-glutamyl-*p*-nitroanilide (*p*-NA) L-amino acid derivatives (Sigma Chemicals) made up in sterile water at 30 °C. *p*-Nitroanilide L-amino acid derivatives (Sigma Chemicals) of Tyr-, Phe- and Trp- were dissolved in 0.5 mL *N, N*-dimethyl formamide before addition to sterile water to a final volume of 10 mL.

Lipase/esterase (LE) activity was determined at 420 nm in a model DU-65 spectrophotometer (Beckman Instruments, Fullerton, CA) with *p*-nitrophenyl derivatives (Sigma Chemicals) of butyrate and caprylate, prepared as described by Blake et al (1996). The method was modified for cheese-like conditions to include a step to quench the reaction with SDS at a final concentration of 3.7%, followed by addition of 1 mol/L sodium phosphate (pH 6.8) to increase the pH. This adjustment allowed *p*-nitroanilide to be used as the indicator even though it is colorless at pH 5.2.

For salt/pH studies, either 0.05 mol/L potassium citrate (pH 5.2) or 0.05 mol/L potassium phosphate (pH 6.8) was used for the reaction buffer. The enzyme assay mixture contained 100 µL of 1 mmol/L chromogenic substrate in the assay buffer and 100 µL CFE. Assays were conducted at 30 °C for lactococci, *L casei* and brevibacteria, and at 37 °C for *L helveticus* in 96-well microtiter plates (Baxter Diagnostic Inc, Deerfield, IL) with sterile tape coverings. Substrates were pre-incubated at 30 °C for 15 min in microtiter plates before addition of CFE. Hydrolysis of the chromogenic substrates was measured with reflectance colorimetry by monitoring an increase in yellow color (b*) using an Omnispec® 4000 bioactivity monitor (Wescor Inc, Logan, UT). Readings were taken every 5 min and assays were carried out in duplicate. Wells containing 100 µL of 1 mmol/L of each substrate in the assay buffer and 100 µL of each buffer were used as controls. Assays were adjusted for the amount of protein added to the assay mixture in the CFE. The AP activity was defined as rate of color change (Δb*/mg protein/h) in the intracellular fraction. LE activity was defined as A_{420}/mg protein/h.

The intracellular portion of AP and LE activity was measured in the extracellular, cell wall bound, cell membrane (spheroplast) and CFE fractions. Activity on each substrate from each fraction was summed and used as the denominator to determine the percentage of total intracellular enzyme activity. The proportion due to

the intracellular activity was calculated by dividing the summed activity for the intracellular fraction by the sum of activity for each fraction multiplied by 100.

Methanethiol-producing capability

The procedure described by Ferchichi et al (1985) was used to determine the methanethiol-producing capability (MTPC). Briefly, methanethiol produced from L-methionine (substrate) reacts with 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB) to produce a yellow aryl mercaptan, which is detected spectrophotometrically at 412 nm after 1 h incubation at 25 °C for brevibacteria, 37 °C for *L helveticus,* and 30 °C for *L casei* and lactococci. Controls with only substrate or cells were used to adjust the raw data.

Induction of methanethiol-producing capability

The inducibility of MTPC was studied using methionine and methionine-containing peptides. Equimolar concentrations of Met and Met-containing peptides (2 mmol/L) were used in the growth media. The MTPC of *B linens* BL2 was monitored after incubation for 36 h at 25 °C with aeration (250 rpm).

Preparation of cheese extracts

Cheese samples (20 g) were blended in 180 mL of 0.05 mmol/L sodium phosphate buffer (pH 7.2) in a Stomacher 400 (Seward, London, UK) for 4 min on the high setting. The blended mixture was centrifuged at 10 000 *g* for 30 min (25 °C), defatted by filtering through GFA filter paper (Whatman International Ltd, Maidstone, UK), and passed through Whatman #5 filter paper before being concentrated five times by ultrafiltration (30 000 MW cut-off) with a 15 mL Centriprep® (Amicon, Inc, Beverly, MA). Erythromycin was added to a final concentration of 0.1 μg/mL to prevent additional enzyme synthesis during the assay.

Cheese making

Forty-eight vats of 60% reduced-fat Cheddar cheese were manufactured within a 2-week period at the University of Wisconsin-Madison pilot dairy processing area by a licensed Wisconsin cheese maker. Raw whole milk was skimmed to 1.3% fat and pasteurized at 73.3 °C for 16 s. Each vat of cheese was manufactured from 250 kg of milk. Two single-strain *L lactis* ssp *cremoris* S1 and SK11, and *L lactis* ssp *lactis* S3 were used to make cheese with and without flavor-adjunct bacteria. Each starter culture and flavor adjunct was grown separately in skim milk steamed for 45 min and incubated at 30 °C for 12–14 h. In preliminary trials, the rate of acid development was slightly different for each starter culture; therefore, the amount of culture used varied: 1.25% S1, 2.00% SK11 and 0.5% S3.

Six different adjunct cultures (two strains each of *L helveticus, L casei* and *B linens*) were used. Lactobacilli were grown in MRS broth (Difco Laboratories, Detroit, MI) at 37 °C for 12–14 h. Brevibacteria were propagated in medium 220 (Weimer, 1990) for 2 d at 30 °C in 500-mL flasks held in a shaking water bath (250 rpm). The adjuncts were added as follows: *L helveticus* strains 3.7×10^6 to 8.7×10^6/kg milk; *L casei* strains 2.2×10^7 to 4.0×10^7/kg milk; and *B linens* strains 6.8×10^7 to 8.4×10^7/kg milk. Starter and flavor adjunct cultures were added at the same time to the milk. After culture (15 min), calcium chloride was added (49 mL; Marschall Products). Chymax, double strength fermentation-produced chymosin (Pfizer, Milwaukee, WI), was added at a rate of 19 mL per vat.

The milk coagulum was cut with 0.95-cm knives at a milk pH of 6.5 and allowed to heal for 5 min, followed by 10 min of gentle agitation before heating. The temperature of the curd/whey slurry was raised from 32 to 37.8 °C over 25 min. After heating, a curd strainer was inserted into the vat and the whey was slowly drained. Cheese slabs were cheddared, followed by milling at pH 5.95. After milling (15 min), the curd was salted in three additions, 5 min apart, with 1.25 g flake salt/kg milk. The salted curd was packed into 9-kg Wilson-style hoops and pressed for 4 h at ambient temperature. The resulting Cheddar cheese blocks were stored at 7 °C for aging. All cheeses were made in duplicate.

Proximate analysis

Milk was analyzed for fat by the Babcock method (Richardson, 1985). Cheese was analyzed for

moisture using a moisture oven (Vanderwarn, 1989), fat was measured by the Babcock method (Richardson, 1985), pH was quantified by the quinhydrone method (Van Slyke and Price, 1979), and salt was analyzed with a sodium electrode (Johnson and Olson, 1985). Cheese was also analyzed for 12% TCA-soluble nitrogen (Adler-Nissen, 1979), 5% soluble nitrogen (Jarret et al, 1982), lactose (kit 176303; Boehringer Mannheim, Mannheim, Germany), galactose (kit 176303; Boehringer Mannheim), L(+)-lactic acid and D(–)-lactic acid (Severn et al, 1986).

Bacterial counts during cheese aging

Starter cultures and flavor adjunct bacteria were estimated after 0, 2, 4 and 6 months of aging. Brevibacteria were counted by the spread plate technique while all other bacteria were counted by the pour plate technique. Lactic acid bacteria were counted after anaerobic incubation on Elliker's agar (Difco Laboratories) at 30 °C for 2 d. Lactobacilli were enumerated after anaerobic incubation on Rogosa SL agar (Difco Laboratories, Detroit, MI) at 37 °C for 2 d. Brevibacteria were enumerated after aerobic incubation on Medium 220 agar at 30 °C for 3 d.

Sensory analysis

Analyses were carried out by descriptive trained taste panels at the University of Wisconsin-Madison; panels consisted of six to ten experienced judges who evaluated the cheese for flavor and body characteristics using category scaling and giving a consensus rating in all categories except for flavor, body and texture preference. One of the assessors marked the consensus rating on a ballot sheet and then gave a copy of the marked ballot sheet to each judge. Random numbers were assigned to the cheeses, and judges tasted them on a blind basis. Cheese attributes were scored using the reference (consensus) as a guideline; the following flavor attributes were evaluated: Cheddar flavor intensity (1 = none to 7 = aged), bitter (1 = none to 7 = pronounced), off-flavor intensity (1 = none to 7 = pronounced), and overall flavor preference (1 = dislike very much to 7 = like very much). Body and texture analysis included: body (1 = very soft to 7 = very firm), body breakdown (1 = very curdy to 7 = very smooth) and overall body texture and preference (1 = dislike very much to 7 = like very much).

Analyses by consumer taste panels were carried out at Utah State University. Each consumer panel (> 100 judges) evaluated flavor, texture and overall preference using a 9-point hedonic scale. Six to eight samples (3–4 g), coded with a random number, were evaluated at room temperature under red lights in individual testing booths. Sample order was randomized between judges to avoid positional bias. Water was available for mouth-rinsing between samples.

Statistical analysis

Laboratory enzyme assays were performed and analyzed in a completely randomized design using JMP® statistical software (SAS Institute Inc, Cary, NC). Cheese manufacture, cheese enzyme assays and flavor evaluation used a completely randomized split-plot design with repeated measures (eq 1). Starter culture was the whole-plot factor and flavor adjunct was the sub-plot factor. The time during aging was the repeated measure. These analyses were carried out using Minitab (Minitab Inc, State College, PA), and canonical analysis of trained sensory scores was made with JMP statistical software (SAS Institute Inc, Cary, NC).

$$\begin{aligned} Y_{jkl} = {} & \text{Overall mean} + \text{starter culture}_j + \\ & \text{error } (a)_j + \text{flavor adjunct}_k + \\ & (\text{starter culture by flavor adjunct})_{jk} + \\ & \text{error } (b)_{jk} + \text{time}_l + \text{error } (c)_{jkl} + \\ & (\text{starter culture by time})_{jl} + \\ & (\text{flavor adjunct by time})_{kl} + \\ & (\text{starter culture by flavor adjunct} \\ & \text{by time})_{jkl} + \text{error } (d)_{jkl} \end{aligned} \quad [1]$$

RESULTS

Protease activity

Each strain varied in its proteinase, total AP and LE activity (table I). *L lactis* ssp *cremoris* S1 and *L helveticus* LH212 were more proteolytic than other strains examined (table I). *L lactis* ssp *lactis* S3 was less proteolytic on a per cell basis, but produced bitter cheese within 2 months of aging. After 6 months of aging, this cheese was elimi-

Table I. Laboratory characterization of the bacteria used in cheese making.
Caractérisation au laboratoire des bactéries utilisées en fabrication fromagère.

Strain	*Proteinase activity (RFU[a]/cell) × 10[5]*	*Intracellular AP activity (%)[b]*	*Intracellular LE activity (%)[b]*
Lactococcal starters			
L lactis subsp *cremoris* S1	17.82	84	84
L lactis subsp *cremoris* SK11	0.69	85	83
L lactis subsp *lactis* S3	0.02	89	87
Flavor adjunct bacteria			
B linens BL1	0.12[c]	86	80
B linens BL2	0.82[c]	69	91
L casei LC301	0.67	96	93
L casei LC202	0.57	89	65
L helveticus LH212	19.7	96	51
L helveticus CNRZ32	0.11	98	30

[a] Relative fluorescence units (see the description of the proteinase assay in the *Materials and methods* section); [b] % intracellular activity = total intracellular enzyme activity/Σ (total activity in the extracellular, cell wall, cell membrane and intracellular fraction); [c] proteinase activity found in culture supernatant.
[a] *Unité relative de fluorescence (voir la description du test d'activité de la protéinase dans la partie* Materials and methods; [b] *% activité intracellulaire : activité enzymatique intracellulaire totale/Σ (activité totale dans les fractions extra- et intracellulaire, la paroi et la membrance ;* [c] *activité protéinase trouvée dans le surnageant de culture.*

nated from taste panels owing to extreme bitterness. While the proteinase activity per cell was low in *L lactis* ssp *lactis* S3, the amount of proteolysis during cheese ripening was similar to other starter cultures, presumably because the cell numbers were above 10^9 CFU/g of cheese.

Aminopeptidase activity

The magnitude of total AP activity was a variable trait among the bacteria tested. *Lactobacillus helveticus* strains had an AP activity between 3.7 and 72 times higher than other bacteria tested under laboratory conditions (fig 1A) with ≥ 95% of the activity in the intracellular fraction (table I). Addition of NaCl had little influence on intracellular AP activity in all strains except *L helveticus,* in which activity decreased significantly ($P < 0.05$). Activity at pH 5.2 was substantially reduced compared to activity at pH 6.8. Total intracellular AP activity of most strains decreased further when assayed under cheese-like conditions of pH 5.2 and 5% NaCl; however, activity in *B linens* BL2 were activated under this condition compared to pH 5.2 (fig 1A).

In laboratory and simulated cheese-like conditions, substrate hydrolysis patterns were consistent with high general AP (aminopeptidase N and aminopeptidase C) activity. At pH 6.8 without salt, AP activity was similar for Arg, Leu and Lys hydrolysis; Ala and Met were hydrolyzed at half the rate, but accounted for > 98% of the total intracellular AP activity. In cheese-like con-

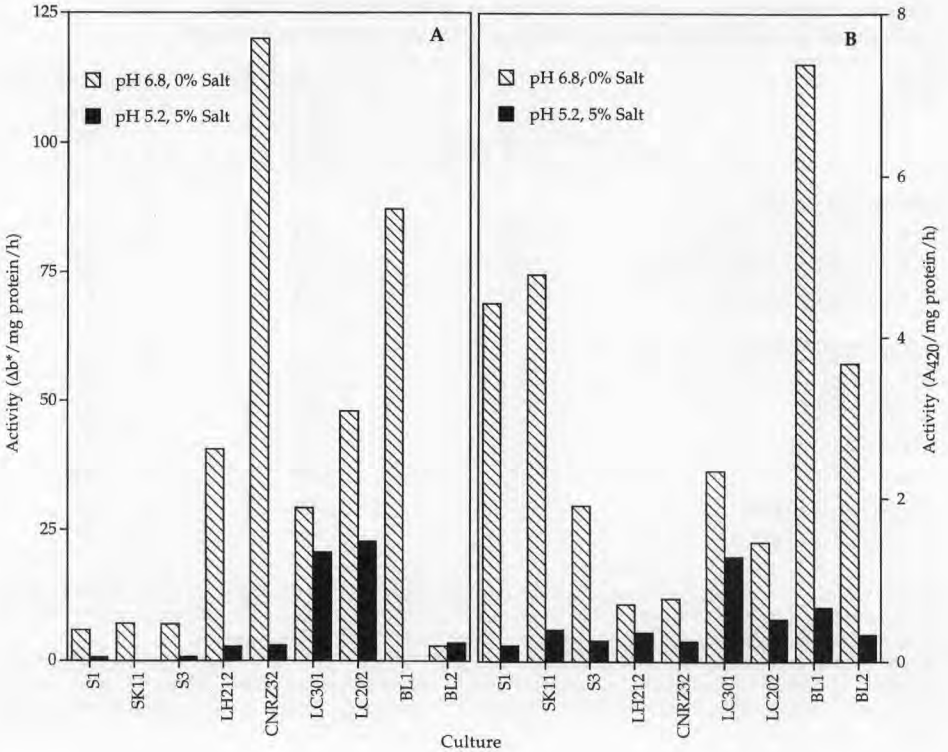

Fig 1. Influence of pH and salt on total intracellular aminopeptidase activity (panel A) and total lipase/esterase activity (panel B) of starter cultures and flavor adjunct bacteria. Bacteria tested were *L lactis* ssp *cremoris* S1 and SK11; *L lactis* ssp *lactis* S3; *L helveticus* LH212 and CNRZ32; *L casei* LC301 and LC202; and *B linens* BL1 and BL2.
Influence du pH et du sel sur l'activité totale d'une aminopeptidase intracellulaire (A) et sur l'activité totale d'une lipase/esterase (B) provenant de cultures de levains et de ferments générateurs d'arômes. Les bactéries testées sont : L lactis *ssp* cremoris *S1 et SK11 ;* L lactis *ssp* lactis *S3 ;* L helveticus *LH212 et CNRZ32 ;* L casei *LC301 et LC202 ; et* B linens *BL1 et BL2.*

ditions the individual AP substrate hydrolysis pattern changed. Hydrolysis was dominated by Arg, Leu and Ala; Lys and Met were equal, and accounted for 88 to 99% of the total AP activity. Substrate hydrolysis also shifted in cheese during ripening. Equal activity between Arg, Leu, Lys, Met and Ala was observed. In cheese, aminopeptidase N and aminopeptidase C activity accounted for approximately 56% of the total AP activity.

Lipase/esterase activity

LE activity was variable between strains at optimum assay conditions; *L helveticus* strains had the lowest total activity (fig 1B). Total intracellular LE activity was more variable between strains than AP activity (table I). The majority of LE activity was intracellular except in *L helveticus* strains. No LE activity was found in cell-free supernatants. Despite variation in activity at opti-

mum assay conditions, all cultures had low LE activity under cheese-like conditions (fig 1B).

Methanethiol production

Methanethiol-producing capability was also characterized in optimum and cheese-like conditions. Whole cells grown overnight and added to the optimum assay mixture contained various MTPC (fig 2A). The lactic acid bacteria contained varying, but lower MTPC than *B linens* BL2. Whole cells tested in cheese-like conditions contained lower MTPC than those in optimum conditions. Under cheese-like conditions *B linens* BL2 contained the highest MTPC. However, if cells from this strain were lysed and the CFE added to the assay mixture, no residual MTPC was observed at cheese-like conditions (fig 2B). Addition of Met and Met-containing peptides induced MTPC during cell growth (fig 3); however, no trend was observed owing to peptide length or composition associated with MTPC induction. Peptides formed in Cheddar cheese from the hydrolysis of β-casein by the lac-

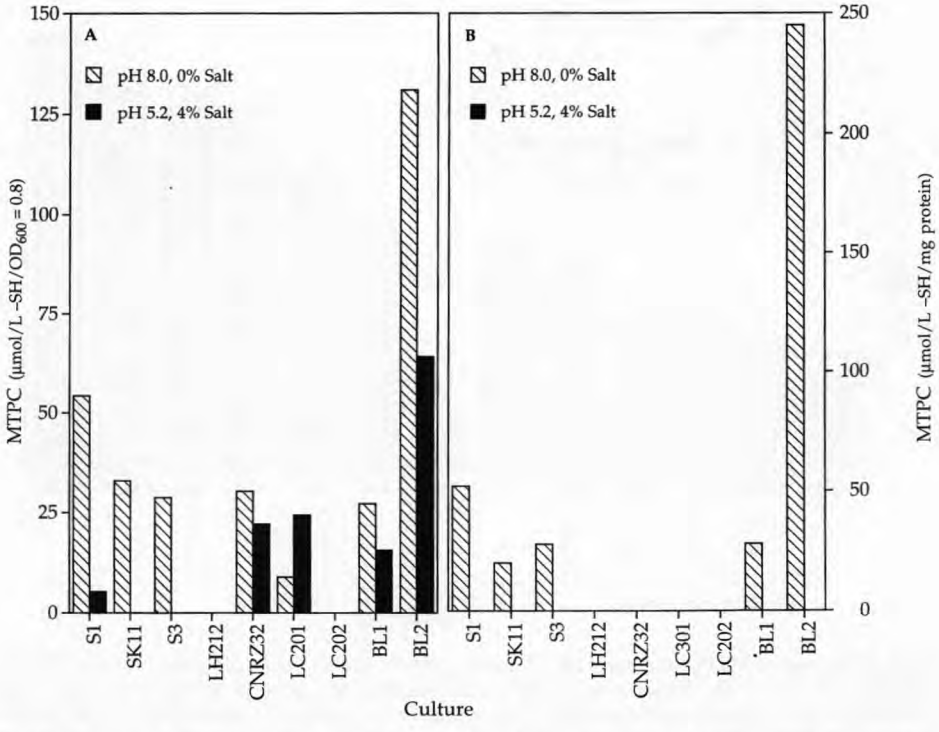

Fig 2. Influence of pH and salt on the methanethiol-producing ability of whole starter cultures and flavor adjunct bacteria (panel A) and cell-free-extracts (panel B). Bacteria tested were *L lactis* ssp *cremoris* S1 and SK11; *L lactis* ssp *lactis* S3; *L helveticus* LH212 and CNRZ32; *L casei* LC301 and LC202; and *B linens* BL1 and BL2.

Influence du pH et du sel sur la capacité à produire du méthanéthiol de l'ensemble des levains et des ferments générateurs d'arômes (A) et des extraits de cellules libres (B). Les bactéries testées sont : L lactis *ssp* cremoris *S1 et SK11 ;* L lactis *ssp* lactis *S3 ;* L helveticus *LH212 et CNRZ32 :* L casei *LC301 et LC202 ; et* B linens *BL1 et BL2.*

tococcal proteinase system also increased MTPC. In the presence of the inducer this increase lasted two to three generations, which equals 5 to 7 h (fig 3, inset) and could be reinduced later in the growth cycle with the addition of more inducer.

Cheese making and trained sensory evaluation

To explore the relationship between AP and LE activities and flavor, each starter culture was combined with each flavor adjunct to make 60% reduced-fat Cheddar cheese. All cheeses were within expected proximate analysis parameters for Cheddar cheese, with an average moisture of 49.2 ± 0.6%, an average fat content of 13.3 ± 0.3%, an average salt-in-the-moisture of 3.4 ± 0.2%, and 0.6 ± 0.2% residual lactose at 1 d.

Enzyme activity during cheese ripening was monitored (fig 4). Total AP activity never exceeded 5 Δb*/mg protein/h in any cheese tested and usually decreased during aging. The hydrolysis patterns using chro-

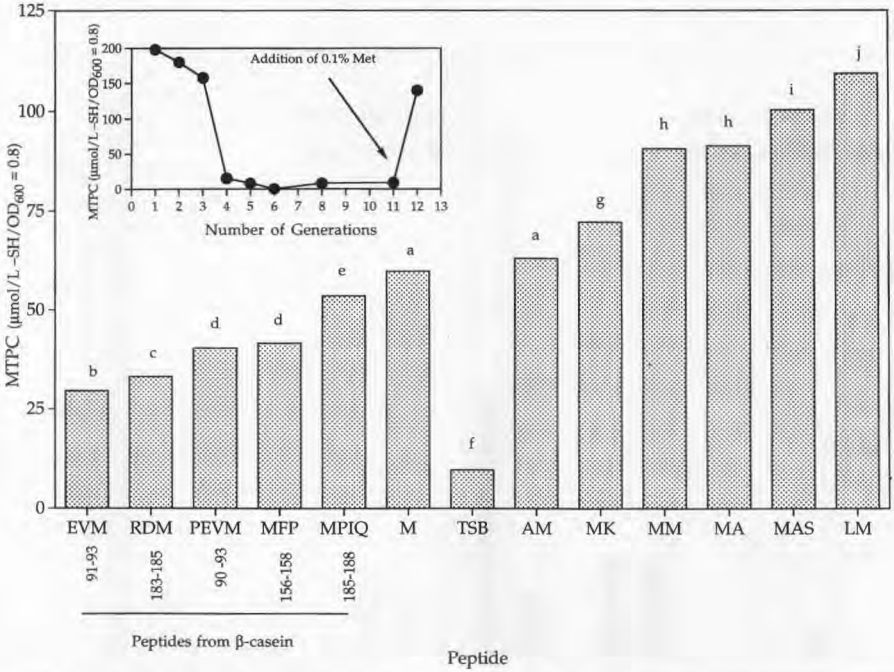

Fig 3. Induction of MTPC in *B linens* BL2 during growth. Inducer peptides were initially added to 2 mmol/L Met into TSB. The same letter above a bar indicates that no significant difference was noted ($\alpha = 0.025$) when the means were compared by a *t*-test. The peptides have been indicated with the single letter abbreviation for amino acids; A: Ala; M: Met; S: Ser; K: Lys; and L: Leu. The inset figure demonstrates the induction duration of MTPC in *B linens* BL2 during growth with a generation time of 2.5 h. *Induction de la capacité à produire du méthanéthiol (MTPC) chez* B linens *pendant sa croissance. Les peptides inducteurs étaient initialement additionnés de 2 mmol/L Met dans du TSB. La même lettre surmontée d'une barre indique qu'il n'y a pas de différence significative entre les moyennes comparées avec un test de* t *($\alpha = 0,025$). Les acides aminés des peptides sont indiqués par une seule lettre d'abréviation : A pour Ala, M pour Met, S pour Ser, K pour Lys, et L pour Leu. La figure en médaillon montre le temps d'induction de MTPC chez* B linens *BL2 au cours de sa croissance, avec un temps de génération de 2,5 heures.*

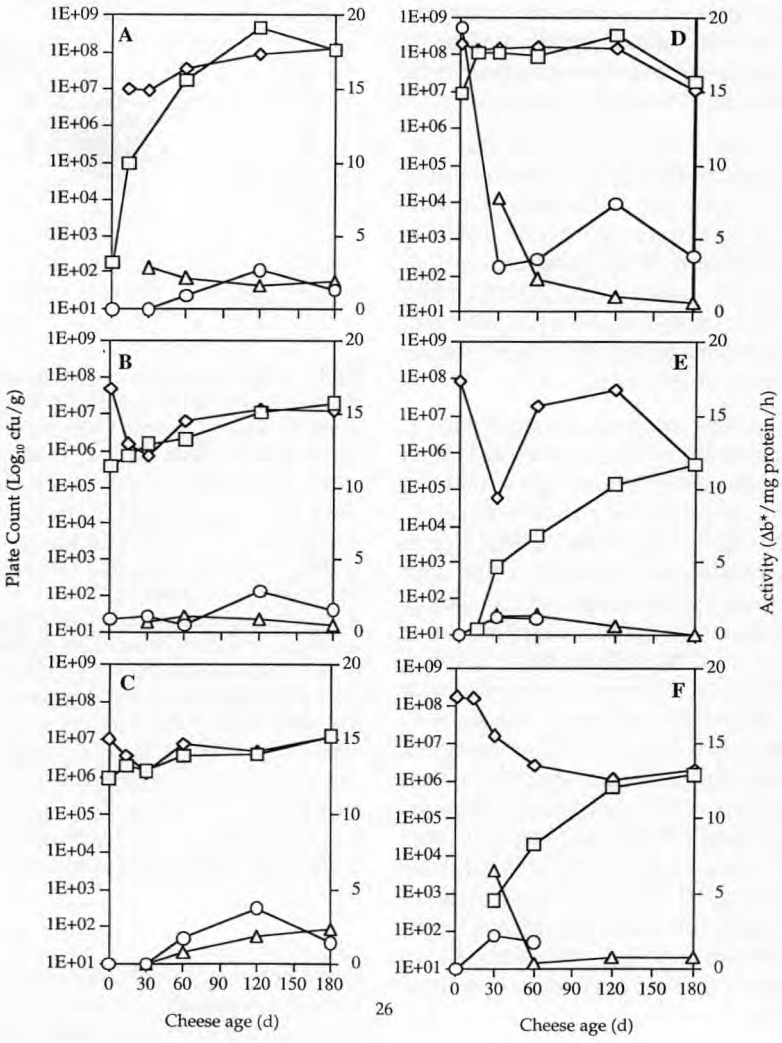

Fig 4. Starter culture count (◇), non-starter lactic acid bacteria count (□; not including brevibacteria), total aminopeptidase activity (△), and total lipase/esterase activity (○) during aging in cheeses rated with significantly better flavor by consumer sensory evaluation. Panel A represents cheese made with S1, panel B represents cheese made with S1 + *L helveticus* CNRZ32, panel C represents cheese made with S1 + *L helveticus* LH212, panel D represents cheese made with S1 + *L casei* LC301, panel E represents cheese made with S1 + *B linens* BL2, and panel F represents cheese made with SK11 + *B linens* BL2. All data are means of replicate cheese trials.

Dénombrement des levains (◇), des bactéries lactiques non levain (□, brevibactérie non incluse), activité totale aminopeptidasique (△) et activité de la lipase/estérase (○) pendant l'affinage des fromages évalués comme ayant un goût significativement meilleur par un jury de consommateurs. A représente le fromage fabriqué avec S1, B, celui fabriqué avec S1 + L helveticus *CNRZ32, C, celui fabriqué avec S1* + L helveticus *LH212, D, celui fabriqué avec S1* + L casei *LC301, E, celui fabriqué avec S1* + B linens *BL2 et F, celui fabriqué avec SK11* + B linens *BL2. Toutes les données sont des moyennes de fromages expérimentaux faits en double.*

mogenic substrates was associated with general AP activity (aminopeptidase N or C) throughout aging and were associated with an increase in TCA-soluble nitrogen.

Lipase/esterase usually rose slightly during aging and peaked at 120 d, but remained low during aging (fig 4). In cheese, the individual LE substrate hydrolysis was distributed between all the substrates tested. The hydrolytic components, in descending order, were caprylate, butyrate, laurate, myristate, palmitate, stearate and propionate, with a range of 29 to 4%.

Taste panels consiting of trained participants demonstrated that cheese made with single strain starter cultures had a mild flavor with no bitter or off-flavors after 6 months (fig 5). *L helveticus* CNRZ32 produced a mild-flavored cheese after 6 months, similar to cheese made with starter cultures alone. *B linens* BL1 produced slightly more off-flavors than other adjunct bacteria tested, while bitterness was more pronounced with *L casei* strains. Cheese made with *B linens* BL2 and *L helveticus* LH212 contained a more pronounced Cheddar-like flavor than cheese made with other flavor adjuncts. However, total AP ($R^2 = 0.16$ for consumer overall acceptance), individual AP substrates, total LE ($R^2 = 0.32$ for consumer overall acceptance), and individual LE substrates did not correlate with cheese flavor data from consumer evaluation or trained taste panel.

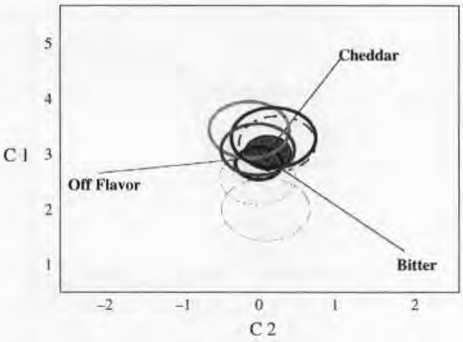

Fig 5. Canonical analysis of trained sensory evaluation after 6 months of aging. *L lactis* ssp *cremoris* S3 was not included because the samples were too bitter. Flavor profiles from *L lactis* ssp *cremoris* S1 ( ), *L lactis* ssp *cremoris* SK11 ( ), *L helveticus* CNRZ32 ( ), *L helveticus* LH212 ( ), *L casei* LC301 ( ), *L casei* LC202 ( ), *B linens* BL1 ( ) and *B linens* BL2 ( ) are shown.
Analyse canonique des résultats d'analyse sensorielle avec un jury entraîné après 6 mois d'affinage. L lactis *ssp* cremoris *S3 n'est pas incluse, car les échantillons étaient trop amers. Les profils aromatiques des bactéries* L lactis *ssp* cremoris *S1 ( ), L lactis ssp cremoris SK11 ( ), L helveticus CNRZ32 ( ), L helveticus CNRZ212 ( ), L casei LC301 ( ), L casei LC202 ( ), B linens BL1 ( ), B linens BL2 ( ) sont montrés.*

Consumer evaluation

Consumer preference for lower-fat Cheddar cheese was evaluated by untrained sensory evaluation. Consumers preferred younger more mild Cheddar cheese with significantly ($P < 0.05$) lower scores at 6 months. Starter culture ($P = 0.006$), flavor adjunct ($P = 0.003$; table II) and the interaction between starter culture and adjunct significantly ($P < 0.02$) influenced consumer flavor preference (table III). Flavor adjuncts formed two groups; *L helveticus* LH212 and *B linens* BL2 were most preferred (tables II and III). These data highlight strain variation and its importance regarding cheese flavor in starter cultures and flavor adjunct bacteria.

The interaction between the starter culture and adjunct is noticeable when comparing the relative flavor ranking of cheese made with *L helveticus* CNRZ32 and *B linens* BL2 at different ages (table III). For example, consumers significantly preferred *L helveticus* CNRZ32 and *B linens* BL2 over other adjuncts paired with *L lactis* ssp *cre-*

Table II. Consumer flavor preference for 60% reduced-fat Cheddar cheese with starter culture and adjunct flavor bacteria as independent variables.
Préférence des consommateurs en matière d'arôme pour des cheddar à teneur en matière grasse réduite de 60 % avec les levains et les bactéries génératrices d'arôme comme variables indépendantes.

Starter culture	*Flavor adjunct bacteria*
L lactis ssp *cremoris* S1[a]	*L helveticus* LH212[a]
L lactis ssp *cremoris* SK11[b]	*B linens* BL2[a]
L lactis ssp *lactis* S3[c]	*L helveticus* CNRZ32[b]
	B linens BL1[b]
	L casei LC301[b]
	L casei LC202[b]

Cultures are ranked in descending order of preference; the same letter indicates no significant difference ($\alpha = 0.01$).
Les cultures sont classées par ordre de préférence décroissante, la même lettre indiquant l'absence de différence significative ($\alpha = 0,01$).

Table III. Consumer flavor preference for 60% reduced-fat Cheddar cheese during aging based on the interaction between starter culture and flavor adjunct.
Préférence des consommateurs, en matière d'arôme, pour des cheddar à teneur en matière grasse réduite de 60 % pendant l'affinage, réduction fondée sur l'interaction des levains et des bactéries génératrices d'arôme.

2 Months	*4 Months*	*6 Months*
S1 + BL2[a]	S3[a]	S1 + BL2[a]
S1 + CNRZ32[a]	S1[a]	SK11 + BL2[a]
SK11 + BL2[b]	S1 + LH212[b]	S1[a]
S1 + LH212[b]	S1 + CNRZ32[b]	S1 + LC301[b]
S3[bc]	S1 + BL2[b]	S1 + LH212[b]
S1[c]	SK11 + BL2[bc]	SK11[bc]
SK11[d]	SK11[c]	S1 + CNRZ32[c]
SK11 + BL1[d]	S1 + BL1[d]	S3[c]
SK11 + LH212[d]	SK11 + LC202[d]	SK11 + LH212[c]
S1 + LC202[de]	SK11 + LH212[d]	SK11 + BL1[d]
S1 + BL1[e]	SK11 + BL1[d]	SK11 + LC202[d]
SK11 + CNRZ32[e]	S3 + LH212[e]	S1 + BL1[d]
S1 + LC301[f]	SK11 + CNRZ32[e]	SK11 + LH212[d]
S3 + LC301[f]	S1 + LC301[e]	S1 + LC202[e]
SK11 + LC202[f]	S3 + LC301[ef]	SK11 + LC301[ef]
S3 + LH212[g]	S1 + LC202[f]	S3 + LC301[f]
S3 + LC202[g]	S3 + LC202[f]	S3 + BL2[g]
SK11 + LC301[g]	S3 + BL1[g]	S3 + LH212[g]
S3 + BL1[h]	S3 + CNRZ32[g]	S3 + LC202[h]
S3 + CNRZ32[h]	S3 + BL2[g]	S3 + BL1[i]
S3 + BL2[i]	SK11 + LC301[g]	S3 + CNRZ32[j]

Cultures are ranked in descending order of preference; the same letter indicates no significant difference ($\alpha = 0.02$).
Les cultures sont classées par ordre de préférence décroissante, la même lettre indiquant l'absence de différence significative ($\alpha = 0,02$).

moris S1 at 2 months, but preferences decreased when these flavor adjuncts were paired with either of the other two starters. This type of preference switching was noticeable at all time points.

DISCUSSION AND CONCLUSION

Cheese flavor development is largely the result of microbial activity in ripening cheese (Schormüller, 1968; Reiter and Sharpe, 1971). Most investigators agree that proteinases and peptidases are important to cheese flavor development, but little is known about the relative influence of individual proteolytic enzymes (Fox et al, 1993). As a result, most cheese manufacturers rely on empirical information to select cheese making strains. We investigated the influence of AP, LE and MTPC in an attempt to link specific enzyme activities in simulated cheese-like assay conditions with desirable flavor characteristics in reduced-fat Cheddar cheese.

Intracellular AP activity decreased with increasing NaCl and decreasing pH and was not correlated with trained or consumer sensory evaluation. The residual activity was dominated by hydrolysis of substrates associated with aminopeptidases N or C, but the relative contribution of individual substrates associated with general AP activity changed depending on the condition tested. Laboratory experiments in simulated cheese-like conditions accurately reflected the total AP activity in cheese, but did not predict the contribution of specific substrates, suggesting that other parameters in addition to pH and salt-in-the-moisture influenced AP activity during aging. Cultures with higher aminopeptidases N or C-type hydrolysis patterns did not increase the flavor of low-fat cheese, which agrees with the results of McGarry et al (1994) and Christensen et al (1995).

Intracellular LE activity also decreased with the addition of NaCl and the reduction in pH to 5.2. Residual LE activity in simulated cheese-like conditions and cheese curd was dominated by shorter-chain fatty acid hydrolysis. As cheese ripened, LE activity generally decreased even though cell numbers increased, suggesting that LE activity in cheese curd was not associated with intracellular enzymes. After 120 days of ripening, LE activity was lower in all cheeses, suggesting that these enzymes have a limited life in cheese, or that inhibitors were produced during aging. Intracellular LE activity also was not correlated with increased cheese flavor during aging. The lack of AP and LE activity correlation to cheese flavor suggests that other possibly secondary metabolic reactions may play a role in converting protein and fat to desirable flavor compounds.

There was considerable variability in MTPC under cheese-like conditions; lactobacilli and brevibacteria contained significantly more MTPC than lactococci, which may explain why the addition of lactobacilli and brevibacteria improved Cheddar-type flavor as assessed by trained taste panels. Whole cells retained MTPC, while CFE contained no measurable MTPC cheese-like conditions, suggesting that the enzymes responsible for methanethiol production are not active in the cheese matrix during ripening. At least two enzymes, cystathionine β-lyase and Met γ-demethiolase, contribute to MTPC via related pathways (Ferchichi et al, 1985; Alting et al, 1995). While Alting et al (1995) claimed that they detected cystathionine β-lyase activity at pH 5.2 with 4% NaCl, we did not observe MTPC in CFE under these conditions. These results need further study to determine whether both enzymes are inhibited upon cell lysis and are released into the cheese matrix.

The ability of MTPC to be maintained and induced in whole cells suggests that methanethiol is produced during cheese

aging. Met or Met-containing peptides are required for sustained MTPC activity during cheese ripening. A possible source of Met during aging is casein-peptides derived from the action of the lactococcal cell wall proteinase (Smid et al, 1991; Exterkate et al, 1995; Reid et al, 1995) with a measured concentration of < 0.5 μmol/g to 1.6 μmol/g in the cheese matrix during aging (Christensen et al, 1995). Laboratory results for methanethiol production have confirmed the results found during aging for whole cells and CFE, suggesting that this may be an important selection criterion for use of starter cultures. In accordance with the findings of McGarry et al (1994) and Christensen et al (1995), AP activity did not appear to be a primary factor in improving cheese flavor.

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