Characterization of aromatic properties of old-style cheese starters

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ABSTRACT

Old-style cheese starters were evaluated to determine their ability to produce cheese aroma compounds. Detailed analyses of the aroma-producing potential of 13 old-style starter cultures were undertaken. The proteolytic profile of the starters was established by an accelerated ripening study using a model cheese slurry and compared with those of a commercial aromatic starter and commercial Cheddar cheeses. To evaluate the aromatic potential of the starter cultures, quantification of free amino acids liberated and volatile compounds after 15 d of ripening at 30°C as well as sensory analysis were carried out. Results showed that proteolysis patterns of all 13 starter cultures in the curd model were comparable to those of commercial Cheddar cheeses. All tested cultures demonstrated the ability to produce high amounts of amino acids recognized as precursors of aroma compounds. Several differences were observed between the starters and commercial Cheddar cheeses regarding some amino acids such as glutamate, leucine, phenylalanine, proline, and ornithine, reflecting the various enzymatic systems present in the starters. Starters Bt (control) and ULAAC-E exhibited various significant differences regarding their free amino acid profiles, as confirmed by sensory analysis. In addition, identification of volatile compounds confirmed the presence of several key molecules related to aroma, such as 3-methylbutanal and diacetyl. Besides the aroma-producing aspect, 2 starters (ULAAC-A and ULAAC-H) seem to possess an important ability to generate large amounts of γ -aminobutyric acid, which contributed up to 15% of the total amino acids present in the model curd after 15 d ripening. γ -Aminobutyric acid is an amine well-known for its antihypertensive and calming effects.

Key words: aromatic starter strain, cheese model

INTRODUCTION

The biochemical changes occurring during cheese ripening may be grouped into primary events including glycolysis, lipolysis, and proteolysis. These changes together with numerous concomitant secondary transformations taking place during ripening determine the finer aspects of the flavor and texture of cheese. Major products of carbohydrate catabolism by lactic acid bacteria (**LAB**) include lactic, acetic, pyruvic, and formic acids, but free fatty acids such as butyric acid may appear in cheese as a result of hydrolysis of milk fat during lipolysis. However, proteolysis is probably the most complex and important reaction involved in the ripening of most cheese varieties and has a primordial effect on the development of flavor, by providing free amino acids (**FAA**) that are substrates for a series of catabolic reactions that generate flavor compounds (McSweeney, 2004). Hence, the further breakdown of AA by decarboxylation, deamination, desulfurylation, and demethiolation is considered an integral part of the overall process of flavor development in cheeses (Gripson et al., 1991; Yvon and Rijnen, 2001). For example, aroma compounds derived from leucine and isoleucine such as 3-methylbutanal and 2-methylbutanal (malty and chocolate aroma) as well as 3-methylbutanoic acid and 2-methylbutanoic acid (sweaty, cheesy) are known to have significant aroma effects in cheese (Urbach, 1993).

The starter bacteria used in Cheddar cheesemaking are usually strains of *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*. The dairy industry has mainly selected starter cultures based on their performance during milk fermentation, primarily acidification rate and phage resistance (Marshall, 1991). As a result, diversity among strains used in industrial dairy fermentations is low and there is a great interest in the cheese producing industry for starters having various aromatic properties resulting in new cheese flavors and properties. This can be achieved either by genetic modification of existing strains or by isolation of new strains from natural ecological niches. For example, old-style cheese starters transferred from one day to the next in the cheese factory without modern special protection,

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in much the same way as was done in the previous century, could be considered as natural ecological niches. These starters may show unique properties that would distinguish them from current commercial starters. First, the starters might contain some form of partial resistance to bacteriophage and second, they might contain strains providing unique flavor production. Starter manufacturers and cheesemakers are constantly looking for new cultures and there is still a need to enrich the microbiota of dairy-based strains.

The aim of the present work was to explore the potential of old-style cheese starters to carry out proteolysis during ripening, producing FAA and aroma compounds. We report on the potential of chemical and sensory analyses of cheese slurries to distinguish mixed starters with respect to their ability to generate different flavor profiles.

MATERIALS AND METHODS

Starter Cultures

Thirteen old-style cheese starters collected in 1968 from various Canadian cheese factories were obtained from Agriculture and Agri-Food Canada (Ottawa, Canada) and identified as follows: ULAAC-A, ULAAC-B, ULAAC-C, ULAAC-D, ULAAC-E, ULAAC-F, ULAAC-G, ULAAC-H, ULAAC-I, ULAAC-J, ULAAC-K, ULAAC-L, and ULAAC-M. Each starter contained about 20 to 30 individual strains (isolates) of lactic acid bacteria, belonging to the genus *Lactococcus* and the species *lactis* (subspecies *lactis*, *cremoris*, and some biovar *diacetylactis*). A commercial starter with aromatic properties (starter Bt) was obtained from Rhodia Food (Sassenage, France) and was used as a commercial control. Each starter was maintained separately in sterile 11% (wt/vol) reconstituted skim milk with glycerol (20%) and kept frozen at -80°C until required.

Preparation of Starters

Each starter was inoculated into 5 mL of sterile GM17 broth (M17 broth + 0.5% wt/vol glucose) and incubated overnight at 30°C . Each culture was then subcultured twice (2% vol/vol) into 5 mL of fresh GM17 broth and incubated overnight at 30°C . Cells were harvested by centrifugation ($5,000 \times g$ for 20 min), washed with sterile 0.1 M sodium phosphate buffer (pH 6.5), and finally resuspended in 1 mL of the same buffer to obtain a total viable cell concentration of approximately 4×10^9 cfu/mL. Cell concentration was verified by serial dilutions in 0.5% (wt/vol) peptone water, and appropriate dilutions were plated onto GM17 agar plates and incubated for 48 h at 30°C .

Manufacture of Starter-Free Cheese Curd

A starter-free cheese curd was produced as described by Farkye et al. (1995) with modifications. About 375 L of fresh whole milk was used for manufacture of the cheese. Milk fat was previously collected by centrifugation and pasteurized for 18 s at 85°C . The remaining skim milk was microfiltered at 50°C on a 1.4- μm pore size membrane (Bactocatch process, Bactocatch, Alfa Laval Dairy Systems, Lund, Sweden). Pasteurized milk fat was then recombined with skim milk to obtain a final value of 3.65% (wt/wt) milk fat. This standardized milk was further pasteurized for 16 s at 72°C before being poured into a cheese vat and brought to 30°C . A 33% (wt/vol) calcium chloride solution was added to the milk (20 mL/100 L of milk). A solution consisting of 1.27 kg of glucono- δ -lactone (Aldrich, St-Louis, MO) and 75 mL of single-strength calf rennet (Ferme S.M.A., Beauport, Canada) was prepared in 5 L of water. Milk was agitated for 1 min after renneting and left to coagulate for 30 min. Cooking of the coagulum began immediately after cutting, and consisted of increasing the temperature under gentle agitation from 30 to 38°C over a period of 30 min. Once the final temperature was reached, it was held for 30 min and the whey was then drained (pH around 5.70). The unsalted curd obtained was placed in square molds covered with cheesecloths and pressed at 300 kPa for 2 h at room temperature. After milling, the curd was frozen at -32°C before lyophilization. The lyophilized starter-free cheese was then reduced in a powder with a disc grinder adjusted to the finest grind (GlenMills Inc., Clifton, NJ). The cheese powder (300-g portions) was packed in sterile plastics bags and kept frozen (-32°C) until its use.

Preparation of Cheese Slurries

Cheese slurries simulating Cheddar cheese composition were prepared as described by Farkye et al. (1995) with some modifications. Thirty-two grams of unsalted cheese powder and 46 mL of sterile autoclaved NaCl solution 20% (wt/vol) were blended together in a sterile plastic bag to obtain a cheese paste (80 g). Starter suspensions in the phosphate buffer (2×1 mL containing approximately 4×10^9 cfu/mL in total) were then added to the cheese paste and homogenized for 3 min at 25°C (Stomacher, Seward, UK). The inoculated cheese paste was transferred aseptically into 100-mL amber bottles fitted with a funnel. Bottles were capped with sterile butyl rubber caps, sealed hermetically with an aluminum seal cap (Wheaton, Millville, NJ), and incubated at 30°C for 15 d. Cheese slurries containing the Bt starter were prepared using the same procedure. Two control slurries were also prepared: an uninoculated unripened

slurry (control) and an uninoculated slurry containing 0.2% (wt/wt) sodium azide ripened 15 d at 30°C (control + azide). The latter control allowed evaluation of the contribution of residual chymosin or plasmin to proteolysis. Unless otherwise stated, “control” will refer to the slurries containing azide and chymosin. Six cheese slurries were prepared per starter.

Composition of the Slurries

Cheese slurries were analyzed for nitrogen content (FP-528, Leco, Mississauga, Canada) with a 9.57% nitrogen solid EDTA standard. Fat content was determined by the Babcock method (Marth, 1978) and moisture by microwave drying (Smart System 5, CEM Corporation, Matthews, NC). The pH of a mixture of 10 g of cheese slurry in 10 mL of deionized water (1:1) was measured with a PHM 84 Research pH meter (Radiometer, Copenhagen, Denmark). All determinations were made in triplicate.

Prior to sensory analysis, the slurries were analyzed for total aerobic count, coliforms, and yeast and mold populations. They were obtained by plating on plate count agar (Difco, Detroit MI), violet red bile agar (Difco), and acidified potato dextrose agar (Difco), respectively.

Proteolysis Assessment

Primary proteolysis was assessed by extracting and measuring the water-soluble nitrogen (**WSN**) of the cheese slurries by the method of Kuchroo and Fox (1982). Further fractionation of the nitrogenous extract was made using 12% TCA (**TCA-SN**) to evaluate secondary proteolysis (Gripon et al., 1975), and with 5% phosphotungstic acid (**PTA-SN**) for peptidolysis (Jarett et al., 1982). Determination of the nitrogen content (FP-528, Leco) of the WSN, TCA-SN, and PTA-SN was made with a 0.45% nitrogen liquid EDTA standard. Nitrogen content of the extracts was expressed as percentage of total nitrogen (**TN**). Three commercial Cheddar cheeses made from pasteurized milk (mild, old, and extra-old “Sélection Mérite” brand, Metro Inc., Montréal, QC, Canada) were purchased from a local supermarket and analyzed for comparison purposes.

Free AA Quantification

Portions of 20 g of cheese slurries were mixed with 40 mL of deionized water and WSN was extracted (Kuchroo and Fox, 1982). After centrifugation (5,000 × *g* for 15 min, 4°C), the supernatants were pipetted into cryovials and kept frozen until further analysis.

Derivatization of these samples was made with the EZ-Faast kit (Phenomenex, Torrance, CA) following the manufacturer’s recommendations. Derivatized samples were then analyzed by gas chromatography (GC 5890A gas chromatograph, Hewlett Packard, Avondale, PA) with flame-ionization detection port. Two microliters of the each sample was injected in the Zebron ZB-AAA GC column (Zebron, Phenomenex) with a split ratio of 1:15 and helium as carrier gas (1.5 mL/min). The temperature oven program started at 110°C and increased to 320°C at a rate of 32°C/min. Calibration curves were made with standard AA (5 nmol/100 µL to 40 nmol/100 µL) and norvaline (20 nmol/100 µL) as internal standard. Because the amount of FAA found in commercial Cheddar cheeses (old and extra-old) was about 4 to 5 times higher than that found in the slurries, FAA were expressed as percentage of total FAA. This conversion allowed the comparison between cheeses of different ripening stages.

Analysis of Volatile Compounds

Volatile compounds were analyzed as described by Morales et al. (2003) with some modifications. For each ripened cheese slurry, a 3-g sample was placed into a 20-mL headspace vial (Agilent Technologies, Palo Alto, CA) and 5 g of ammonium sulfate was added. Vials were sealed with a 20-mm aluminum crimp cap with polytetrafluoroethylene/Si septum (Agilent Technologies). Separation and identification of volatiles was made using a ZB-WAX column, 100% polyethylene glycol (60 m × 0.25 mm × 0.25 µm; Zebron, Phenomenex) with a GC/MS (GC MS 5973 Network Mass Selective Detector, Agilent Technologies) connected to a headspace device (G1888 Network Headspace Sampler; Agilent Technologies). Chromatographic conditions were as follows. The injection port was maintained at 225°C. The oven temperature was held at 35°C for 1 min and then increased to 200°C at a rate of 6°C/min and held for 3 min at 200°C. The temperature was then increased to 250°C at a rate of 10°C/min and held for 4 min. Helium was used as the carrier gas at a flow rate of 2.0 mL/min and a split ratio of 1:10. The electron impact energy was set at 70 eV and the data were collected in the range of 30 to 200 atomic mass units. Components were identified by comparison of their mass spectra with those in the National Institute of Standards and Technology Mass Spectral Database (NIST, 1998). The headspace parameters were as follows: oven temperature = 95°C, loop temperature = 160°C, transfer line temperature = 170°C; vial equilibrate time = 30 min, pressurization time = 0.5 min, loop fill time = 0.25 min, loop equilibrate time = 0.05 min, injection time 1.0 min.

Figure 1. Water-soluble N (WSN; a), TCA-soluble N (TCA-SN; b), and phosphotungstic acid-soluble N (PTA-SN; c) in ripened slurries and in commercial cheeses. TN = total N. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

Table 1. pH, moisture, and free AA (FAA) in ripened slurries (15 d at 30°C) and commercial Cheddar cheeses

Starter/cheese ¹	pH	Moisture (%)	Total FAA (mmol/kg)
ULAAC-A	5.06	60.6	20.1
ULAAC-B	5.13	57.4	17.1
ULAAC-C	5.13	56.8	17
ULAAC-D	5.06	62.5	20
ULAAC-E	5.27	63.8	19.7
ULAAC-F	4.84	59.4	19.5
ULAAC-G	4.84	59.6	19.7
ULAAC-H	4.91	63.2	19.8
ULAAC-I	5.13	58.3	19.2
ULAAC-J	4.89	60.1	21.3
ULAAC-K	5.06	57.6	19.2
ULAAC-L	5.10	59.2	24.3
ULAAC-M	5.30	62.0	20.9
Bt	5.14	59.2	20.4
Control + azide	5.97	60.0	ND ²
Control	6.01	59.6	<1
Mild Cheddar	5.09	36.8	17.2
Old Cheddar	5.08	35.7	116.8
Extra-old Cheddar	5.06	32.8	84.0

¹ULAAC-A to ULAAC-M are 13 old-style cheese starters collected in 1968 from various Canadian cheese factories and obtained for this study from Agriculture and Agri-Food Canada (Ottawa, Canada). A commercial starter, starter Bt, with aromatic properties was obtained from Rhodia Food (Sassenage, France) and was used as a commercial control. Two control slurries were also prepared: an uninoculated, unripened slurry (control) and an uninoculated slurry containing 0.2% (wt/wt) sodium azide ripened 15 d at 30°C (control + azide).

²ND = not detected.

Sensory Analysis

To evaluate the potential aromatic (flavoring) properties of 13 old-style cheese starters, slurries ripened for 15 d at 30°C with old-style starters were compared with a commercial starter. Three commercial Cheddar cheeses (mild, old, extra-old) were also analyzed to estimate the real ripening stage of the slurries. Each slurry was divided into 8 portions of 10 g each and allowed to reach 20°C before the sniffing. Analyses were done in triplicate. A control made by rehydrating cheese curd powder with water was named “reference.”

The sensory panel was composed of 8 experienced tasters; they were invited to choose a maximum of 10 attributes to describe their perception of the slurries using the cheese flavor wheel (Bérodier et al., 1996). A training session involving the sniffing of 9 dairy products allowed the assessors to establish a list of 4 descriptors for each dairy product, these descriptors being used afterward for the characterization of the slurries. Aroma analysis was made using the free-choice profiling procedure, which allowed the assessors to choose their own vocabulary to describe the cheeses (Deliza et al., 2005). The assessors were required to detect differences between the samples, verbally describe the perceived attributes, and quantify them (Oreskovich et al., 1991). This procedure avoided the two major conventional flavor profiling disadvantages; that is, the need to use a common vocabulary of attributes to describe the

samples and a preestablished measure of agreement among the panelists on the interpretation and meaning of the terms they will employ. Each assessor generated between 9 and 10 attributes and many common terms were found among the group.

Statistical Analysis

For the assessment of proteolysis in cheese slurries, 3 independent repetitions were carried out for each starter. Furthermore, WSN, TCA-SN, and PTA-SN determinations were made in duplicate for each sample. The means obtained were examined by ANOVA conducted with the Tukey's test with a significance level of 5%, using the SAS/Stat 8.01 (proc GLM) package (SAS Institute Inc., Cary, NC). For quantification of FAA, 6 slurries were ripened with each starter and analyzed in duplicate. The means obtained were examined by ANOVA conducted with the Tukey's test with a significance level of 5%.

For sensory analysis, 3 independent repetitions were carried out and data obtained with the panel were analyzed using Generalized Procrustean Analysis. This analysis is based on the average scores of the 3 tests by all tasters, by taster and by product. To investigate the differences between subjects, the Procrustes ANOVA was used, determining the differences between individual and consensus configurations, as well as between samples. Results of ANOVA were expressed as a

proportion of the total amount of variance, which was previously rescaled to 100.

RESULTS AND DISCUSSION

Slurries Composition

Protein, moisture, and fat contents of the lyophilized cheese powder were, respectively, 38.2%, 1.9%, and 50.8% (means of triplicates). Moisture of the slurries ripened for 15 d at 30°C varied slightly between 57 and 63% (Table 1). Cheddar cheeses and ripened slurries had approximately the same pH values, ranging from 4.8 to 5.1, except for the controls, which had a pH close to 6.0. The cheese curd powder used to reconstitute slurries did not contain detectable amounts of coliforms (<10 cfu/g) or yeast or molds (<10 cfu/g). Total mesophilic bacteria were about 2×10^4 cfu/g.

Proteolysis

WSN. The ratio WSN:TN is an index of the extent of proteolysis in cheeses and increases with ripening. No significant differences were found among the 13 starters or between the 13 starters and the commercial starter Bt. In the latter, WSN represented about 9% of TN (Figure 1a). This result was expected because many authors have studied the peptide profile of the WSN fraction of fresh cheeses and concluded that the high-molecular-weight peptides in this fraction are mainly produced by the residual action of chymosin, primarily on α_{S1} -casein (Gripon et al., 1975) as shown by the significantly higher WSN content (12%) in the azide control slurry. The higher proteolytic activity in the azide control is presumably linked to the pH of the products. The slurries that contained the starters reached pH levels below 5.0, which is a range where the activity of chymosin is not optimum. The further degradation of the WSN fraction in inoculated cheese slurries could also explain these differences. The higher WSN content in the azide slurry (pH 6.0) is in agreement with the study of Gripon et al. (1991), in which soluble nitrogen represented about 18% of total nitrogen and increased continuously during 48 d of ripening in a slurry.

Significant differences in WSN between the starter slurries and commercial Cheddar were observed (Figure 1a) and could be explained by the elevated ripening temperature (30°C) and moisture content of the slurries (60%), which obviously favored the proteolytic activity. By comparing WSN/TN in commercial Cheddar cheeses with slurries containing the starters (Figure 1a), it is possible to estimate that 15 d of ripening in the slurries at 30°C was slightly higher than ripening of mild, old, and extra-old commercial cheeses.

Harper and Kristoffersen (1970) have found that a 4-d ripening period in Cheddar cheese slurries was equivalent to that of a 3-mo Cheddar cheese. Many other authors have used model cheeses and all have found a correlation between flavor development in the models and commercial cheeses (Noomen, 1978; Harper et al., 1989; Thakar and Upadhyay, 1992; Muehlenkamp and Warthesen, 1999).

TCA-SN. The TCA-SN fraction represents secondary proteolysis (small peptides and FAA) and contains peptides varying in size from 2 to 22 AA residues (600 < molecular weight < 15,000 Da) but also contains FAA. The results in Figure 1b showed that extra-old Cheddar cheese tended to contain higher amounts of medium and short peptides (~55% of WSN) than most of the slurries (40–50% of WSN). The extra-old Cheddar cheeses probably contained a secondary flora with peptidolytic activity (Williams and Banks, 1997).

Unlike the WSN fraction, several significant differences in TCA-SN were found between the starters. In particular, starters ULAAC-D, ULAAC-F, ULAAC-H, and ULAAC-I seemed to contain active proteinases, as reflected by their high TCA-SN content, which was not significantly different from that of extra-old Cheddar and was higher than that of the other starters.

PTA-SN. The PTA-SN fraction reflects the further hydrolysis of medium and small peptides into dipeptides, tripeptides, and FAA. Half of the starters exhibited a PTA-SN fraction equivalent to that of the extra-old Cheddar cheese (30 to 35% of WSN), indicating a high activity of peptidases and aminopeptidases (Figure 1c). This was the case for starters Bt, ULAAC-C, ULAAC-E, ULAAC-G, ULAAC-J, and ULAAC-L, which contained low levels of TCA-SN but high levels of PTA-SN. Aston et al. (1983) observed the same correlation between TCA and PTA fractions in Cheddar cheeses. The low levels of FAA (Table 1) in controls confirm the low contribution of the coagulant to this level of proteolysis.

During this study, several significant differences were found regarding the different nitrogenous fractions analyzed. However, the interpretation of cheese ripening indices based on WSN, TCA-SN, and PTA-SN must be taken with caution, especially when correlations with flavor are sought. Aston et al. (1983) showed that TCA-SN is a good index of total flavor intensity in young cheese but correlates poorly as cheese matures. This was the case in this study because a correlation coefficient (*r*) of only 0.44 was obtained between the TCA-SN (Figure 1b) and PTA-SN (Figure 1c) data. Therefore, these nitrogen-soluble indices represent a general application in the assessment of cheese age, but organoleptic methods remain essential for assessing flavor quality. Nevertheless, these indices allowed

Figure 2. Percentage of glutamic acid in the slurries after 15 d of ripening and in commercial Cheddar cheeses. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

observation of different proteolytic patterns between the starters.

Total FAA

Free AA are mainly released by the action of microbial enzymes from starter cultures (Fox and Wallace, 1997a). In this regard, quantification of FAA is of great importance to distinguish the different enzymatic activities and flavor-forming abilities of the 13 starters. The results (Table 1) show that levels of FAA found in the slurries after 15 d of ripening at 30°C (approximately 17 to 24 mmol/kg) were similar to those found in a 3- to 4-mo mild Cheddar cheese (approximately 17 mmol/kg). Therefore, the cheese model slurry used in this study seems to be appropriate to simulate and accelerate the cheese-ripening process. Moreover, the levels of FAA in the slurries corresponded to those observed by Kieronczyk et al. (2004), who studied FAA catabolism in a cheese model ripened for 6 wk at 13°C. Moreover, it has been suggested that slurries should be used to screen organisms for their ripening potential and not to directly predict cheese ripening (Muehlenkamp and Warthesen, 1999).

The general AA profile obtained in the slurries and in Cheddar cheeses (results not shown) corresponded to those observed in several studies (Puchades et al., 1989; Fox and Wallace 1997b; Yvon et al., 1998). It was found that Leu, Pro, Glu, and Phe were the most abundant AA in the slurries, whereas Gly, Met, Asn,

Glu, Asp, Gln, and Lys were present in higher amounts in commercial Cheddar cheeses.

The FAA data also provide a specific picture of the ripening process and are not directly correlated with the data of the other nitrogenous fractions. Thus, r -values of 0.54 and 0.51 were obtained between the FAA data and those of TSA-SN and PTA-SN, respectively.

Proportion of FAA

Glutamate and γ -Aminobutyric Acid. Glutamate levels in cheese slurries are one of the most striking differences between the 13 starters. In Figure 2, results show that in the case of starters ULAAC-A, ULAAC-H, and ULAAC-I, glutamate levels represented only 2 to 6% of total AA compared with 10 to 15% in commercial starter Bt and other starters. Glutamate aminotransferase, glutamate dehydrogenase, and glutamate decarboxylase in lactic acid bacteria are known to be involved in glutamate catabolism (Christensen et al., 1999). The activity of the first 2 enzymes results in the formation of α -ketoglutarate, whereas glutamate decarboxylase activity produces γ -aminobutyric acid (GABA). Glutamate dehydrogenase activity is rare among *Lc. lactis* (Lapujade et al., 1998). In accordance with this observation, none of the 13 starters exhibited glutamate dehydrogenase activity with NAD as cofactor (results not shown).

The relationship observed between glutamate (Figure 2) and GABA levels (Figure 3) in cheese slurries contain-

Figure 3. Percentage of γ -aminobutyric acid in the slurries after 15 d of ripening and in commercial Cheddar cheeses. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

ing starters ULAAC-A, ULAAC-H, and ULAAC-I confirms the high glutamate decarboxylase activity present in these 3 starters. Indeed, levels of GABA produced in slurries containing starters ULAAC-A, ULAAC-H, and ULAAC-I were significantly higher than those containing other starters, and no GABA was detected in the commercial cheeses. Moreover, regression analysis showed a negative correlation ($r = -0.86$) between the

content of glutamate and that of GABA in the slurries. This is the first observation of this nature in cheese slurries involving different starters.

The physiological role of glutamate decarboxylation is likely related to the regulation of intracellular pH. When the cells are exposed to low pH, the glutamate decarboxylase system converts glutamate to GABA while consuming an intracellular proton. Consequently,

Figure 4. Percentage of methionine in the slurries after 15 d of ripening and in commercial Cheddar cheeses. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

Figure 5. Percentage of proline in the slurries after 15 d of ripening and in commercial Cheddar cheeses. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

the proton concentration within the cell is reduced, alleviating acidification of the cytoplasm (Cotter et al., 2001). Production of GABA is known to occur during the ripening of different cheeses (Nomura et al., 1998), and the concomitant production of CO_2 has been associated with the formation of eyes and cracks in Gouda cheese (Zoon and Allersma, 1996). More interestingly, GABA has been reported to lower blood pressure by intravenous administration in experimental animals (Lacerda et al., 2003). Recently, low-dose oral administration of fermented milk containing GABA has been effective to decrease blood pressure in humans (Inoue et al., 2003).

Methionine. The degradation of methionine is thought to lead to the formation of methanethiol and to other sulfur-containing compounds that have been strongly associated with desirable Cheddar cheese flavor. Figure 4 shows that the levels of methionine released by the starters were between 0.5 and 1.5% of the total FAA. These levels were significantly lower than those observed in commercial Cheddar cheeses (2 to 3% of total FAA) as reported by Christensen et al. (1995). However, starter ULAAC-E produced a significantly higher amount of methionine than 10 other starters (Figure 4).

Aspartic Acid. Decarboxylation of aspartic acid leading to the formation of alanine is known to generate metabolic energy and to regulate intracellular pH via a general mechanism common to a variety of decarboxylases (Christensen et al., 1999). Levels of

aspartic acid were similar for all 13 slurries and their relative contributions to total FAA were significantly lower than those found with commercial starter Bt and Cheddar cheeses (results not shown). Although no clear relationship was observed between aspartic acid and alanine levels, it is likely that the 13 starters possess an efficient defense system against acidic pH and used aspartic acid for pH regulation.

Proline. Except for starter ULAAC-E, the significantly higher levels of Pro found in the slurries compared with starter Bt and commercial cheeses (Figure 5), is indicative of extensive β -casein hydrolysis under the action of proline-specific peptidases (Poveda et al., 2004) and therefore indicates the presence of bacterial strains with interesting enzymatic properties among the starters. Other authors reported that the percentage of free Pro in Cheddar and Gouda cheeses was about 2 to 5% of the total FAA (Puchades et al., 1989), which corresponds to the results obtained in commercial Cheddar cheeses in the current study (2–3%). In the slurries, levels close to 8% have been observed and correspond to the levels found for long-ripened cheeses such as Grana Padano, Appenzeller, Emmentaler, and Parmigiano Reggiano (Fox and Wallace, 1997b). The effect of plasmin should also be considered in these particular cheeses because the relatively high cooking temperatures could activate plasmin activity.

Ornithine. Ornithine is not naturally present in milk. Therefore, its accumulation in cheese results from an enzymatic reaction known as the arginine deimi-

Figure 6. Percentage of ornithine in the slurries after 15 d of ripening and in commercial Cheddar cheeses. Means with the same letter are not significantly different ($\alpha = 0.05$). Bt and various ULAAC codes represent the different starters used in the preparation of the slurries; mild, old, and extra-old (X-old) represent data from commercial Cheddar cheeses.

nase pathway, which is related to an adaptive response to acidity commonly referred to as the acid tolerance response. Figure 6 shows that most of the starters were able to generate higher amounts of Orn than the commercial Cheddar cheeses, reflecting either an active arginine deiminase system naturally present in these starters or induced by the accelerated ripening conditions. Moreover, 11 starters produced higher levels of Orn as compared with those obtained with starter Bt.

Branched-Chain AA. Levels of the branched-chain AA Leu, Ile, and Val produced by the 13 starters were similar (results not shown), except in starter ULAAC-E, which released a significantly higher amount of Ile. Generally, branched-chain AA tend to accumulate in cheese as ripening progresses (Yvon et al., 1998), as observed in mild, old, and extra-old Cheddar cheeses. Levels of branched-chain AA in cheese slurries were higher than in commercial Cheddar cheeses, indicating more pronounced hydrolysis of α_{S1} -casein, which has a high content of Leu and Val (Tavaria et al., 2003; Poveda et al., 2004). Yvon et al. (1997) observed that the activity of branched-chain amino transferase was repressed by FAA. Because the ripening process in the slurries was accelerated, higher levels of branched-chain AA released could have had a repressing effect on the activity of branched-chain amino transferase enzymes, thus explaining the less important catabolism of these AA in the slurries compared with commercial Cheddar cheeses.

Volatile Compounds

A total of 38 different volatiles compounds were detected and identified in the volatile fraction of the ripened slurries and in commercial Cheddar cheeses. These compounds were classified into different chemical groups (Table 2) according to odor descriptors commonly observed in Cheddar cheeses (Gomez-Ruiz et al., 2002). It can be observed that the most abundant volatile compounds released by the starters were alcohols (9 compounds), fatty acids (8), ketones (7), aldehydes (3), esters (3), pyrazines (2), and others (7). The same trend was observed by Morales et al. (2003).

All 13 starters showed approximately the same chromatographic profile (results not shown) with ethanol, acetic acid, and the 2 isomers of 2,3-butanediol (meso and opticals) being the most abundant volatiles produced.

Because of its very low perception threshold, diacetyl is one of the major flavor compounds in fermented milk and fresh cheeses, with a typical buttery and mild Cheddar note. However, the reduction of diacetyl generates acetoin, and reduction of acetoin generates 2,3-butanediol, which can be further reduced to 2-butanone, and ultimately 2-butanol, the latter compounds being widely reported in Cheddar cheese (Urbach, 1993). The rapid appearance of 2-butanol after the synthesis of 2-butanone seems to indicate a detoxifying pathway for microorganisms (Molimard and Spinnler, 1996). Inter-

Table 2. Major volatile compounds released by the 13 starters after 15 d of ripening in the slurries¹

Compound	Odor description in cheese ²	Possible source	Target ions (by GC-MS)
Alcohols			
Ethanol	Dry, dust	Pyruvate	45, 31, 27, 15
1-Pentanol	Fruity, balsamic		70, 55, 42, 31
2,3-Butanediol (optical forms)	Fruity, onions	Pyruvate (acetoin)	90, 75, 57, 45
2,3-Butanediol (meso)		Pyruvate (acetoin)	90, 72, 57, 45
1-Propanol	Sweet (candy), alcohol		59, 42, 31
Isoamyl alcohol	Fuel oil, pomace, alcoholic, green		87, 70, 55, 42
2-Butanol	Wine	Pyruvate (diacetyl)	59, 45
1-Butanol	Medicinal, fruity		56, 41, 31
Carbitol			72, 59, 45
Aldehydes			
Acetaldehyde	Sweet, pungent, ethereal	Threonine, glucose	44, 29, 15
3-Methylbutanal	Malty, chocolate, caramel	Leucine	86, 71, 58, 53, 44, 29
Benzaldehyde	Almonds, sugar, burnt	Phenylalanine, tryptophan	105, 77, 51, 39
Ketones			
Acetone (2-propanone)	Wood pulp, hay,		58, 54, 43, 27, 15
Diacetyl (2,3-butanedione)	Butter, mild cheddar, caramel, cheesy	Pyruvate	86, 43, 42, 15
2-heptanone	Blue cheese, soapy, Gorgonzola, varnish	Lipolysis, fatty acids	114, 99, 71, 58, 43
Acetoin (2-hydroxy-3-butanone)	Sour milk	Pyruvate	88, 73, 55, 45, 27
2-nonanone	Malty, fruity, floral, peachy, hot milk	Lipolysis, fatty acids	142, 71, 58, 43
2-butanone	Butterscotch, soap, acetone	Pyruvate (diacetyl)	72, 57, 43
2-pentanone	Fruity, acetone	Lipolysis, fatty acids	43, 58, 71, 86
Esters			
Ethyl butanoate	Sweet, fruity, apple, bubble gum		116, 101, 88, 71, 60, 43
Ethyl acetate	Fruity, pineapple		88, 73, 70, 61, 45, 43
Ethyl hexanoate	Fruity, melon		99, 88, 60, 43
Carboxylic acids			
Acetic acid	Vinegar, sour, sharp, peppers, green	Pyruvate	60, 43, 29
Butyric acid	Nacho cheese, cheesy, rotten, sharp, fecal, sweaty	Lipolysis, fatty acid	88, 73, 60, 41
Isovaleric acid (3-methylbutanoic acid)	Sweet, rancid, rotten, cheesy, Swiss cheese	Leucine	87, 60
Hexanoic acid	Bad breath, popcorn, goaty	Lipolysis, fatty acids	73, 60, 41
Octanoic acid	Sweaty, rancid	Lipolysis, fatty acids	73, 60, 101
n-Decanoic acid	Stale butter, sour, goaty, aged cheese, sweet	Lipolysis, fatty acids	172, 129, 73, 60
Propanoic acid	Gas, burnt, solvent, sulfur	Pyruvate	
Isobutyric acid	Cheesy, butter rancid	Lipolysis, fatty acids	88, 73, 43,
Phenolic			
Toluene	Nutty, bitter, almonds, paint		92, 91, 65
Others			
Dimethylsulfone	Sulfurous, hot milk, burnt	Methionine	79, 81, 94
1,2-Dimethylbenzene			106, 91, 77, 65, 51,
2,3-Dimethylpyrazine	Roasted nuts, peanuts, coffee, cocoa		108, 93, 81, 67, 52, 40
Tetramethylpyrazine	Cooked aroma, raw potato, beans		136, 54, 42, 27
1,2-Dichlorobenzene			146, 148, 150, 111, 75
3-Furanmethanol	Baked		98, 81, 70, 69
Chloroform	Hay		118, 83

¹Identification made by comparison of mass spectra with those in the National Institute of Standards and Technology Mass Spectral Database (NIST, 1998).²Handbook of Flavor Ingredients (Burdock, 2002).

Table 3. Attributes generated by sensory assessors positively correlated with the 2 first dimensions of the Generalized Procrustean Analysis

Dimension 1	Dimension 2
Butyric acid	Roasted nuts
Propionic acid	Mild torrefaction
Ammonia	Lactic fresh
Yeast (fermentation)	Diacetyl
Yogurt	Caramel
Cowshed	
Rancid	
Lactic acid	
Sour milk	
Parmesan	
Blue cheese	

estingly, all these compounds resulting from citrate metabolism were present in the headspace of the slurries and it is assumed that the 13 starters studied possessed the enzymatic abilities to form these compounds. On the other hand, only low levels of acetoin were found in commercial Cheddar cheeses, and no traces of further degradation products such as 2-butanone, 2-butanol, and 2,3-butanediol were detected.

Free fatty acids detected in the headspace of the slurries were mainly butyric, isovaleric, hexanoic, and octanoic acids. The presence of butyric and hexanoic acids, which are known to occupy almost exclusively the *sn*-3 position of the triglyceride molecule (Parodi, 1979), may indicate the predominant activity of the α -position-specific milk lipase (Fox and Wallace, 1997a). Collins et al. (2003) established the relationship existing between the extent of starter cell autolysis and the level of lipolysis during Cheddar cheese ripening. Thus, autolysis of the starters during ripening at 30°C could occur faster, explaining the higher levels of free fatty acids and 2-ketones compared with the azide controls. However, a proportion of short-chain free fatty acids such as acetic acid can also originate from degradation of lactose and amino acids (Molimard and Spinnler, 1996).

Ketones, and particularly acetone, were found in the headspace of the slurries, commercial cheeses, and unripened fresh slurry. The presence of several methyl ketones such as 2-butanone, 2-heptanone, and 2-nonanone, although appearing at low concentration (results not shown), is important because of their low perception threshold. Methyl ketones are particularly recognized for their contribution to the flavor of mold-ripened cheeses (Sablé and Cotteceau, 1999) as well as Cheddar cheeses (Lawrence, 1963).

Several volatiles identified as having the highest odor potency in young and old Cheddar cheese were also identified in this study. These compounds are 3-methylbutanal, diacetyl, acetic acid, butyric acid, decanoic acid, and hexanoic acid, and they seemed to be more

abundant in the headspace of slurries than in commercial Cheddar cheeses. Despite the predominance of leucine in the slurries (Figure 4), all 13 starters and commercial cheeses contained low levels of 3-methylbutanal, which is a degradation product of leucine and possesses a very low perception threshold and a typical chocolate, malty, and roasted nut aroma (Curioni and Bosset, 2002; Avsar et al., 2004). Such aldehydes could also be the source of undesirable flavors.

No thiol compound, excepted dimethylsulfone, was found in the headspace of the slurries. Thiol compounds have a very low boiling point and, being volatile, they were likely lost in the early stages of the chromatographic analysis. No methanethiol was found in the slurries or in the commercial Cheddar cheeses. Dias and Weimer (1998) found that the activities of methionine-converting intracellular enzymes decreased as they were released into the cheese matrix, and that only whole cells were able to maintain their methanethiol-producing capacities under cheese-like conditions. Because of the high temperature and moisture content present during the ripening of the slurries, cell lysis presumably occurred rapidly, resulting in a decrease in catabolic activities and the production of sulfur compounds.

Sensory Analysis

Most assessors had a low residual variance (0.583), indicating homogeneity among the group. In addition,

Table 4. Multiple comparisons of the starters on the first dimension of the Generalized Procrustean Analysis consensus space described in Table 3

Starter ¹	Mean
Bt	4.504 ^a
ULAAC-J	2.002 ^b
ULAAC-C	1.399 ^{bc}
ULAAC-A	0.823 ^{bcd}
ULAAC-B	0.389 ^{cde}
ULAAC-L	-0.318 ^{def}
ULAAC-D	-0.616 ^{def}
ULAAC-H	-0.660 ^{defg}
ULAAC-G	-0.685 ^{defg}
ULAAC-M	-0.712 ^{efg}
ULAAC-I	-0.717 ^{efg}
ULAAC-K	-0.784 ^{efg}
ULAAC-E	-1.214 ^{fg}
ULAAC-F	-1.235 ^{fg}
Control	-2.178 ^g

^{a-g}Means followed by the same letter are not significantly different at the 10% significance level.

¹ULAAC-A to ULAAC-M are 13 old-style cheese starters collected in 1968 from various Canadian cheese factories and obtained for this study from Agriculture and Agri-Food Canada (Ottawa, Canada). A commercial starter, starter Bt, with aromatic properties was obtained from Rhodia Food (Sassenage, France) and was used as a commercial control.

Table 5. Multiple comparisons of the starters on the second dimension of the Generalized Procrustean Analysis consensus space described in Table 3

Starter ¹	Mean
ULAAC-I	1.166 ^a
ULAAC-H	0.944 ^a
ULAAC-M	0.935 ^a
ULAAC-E	0.829 ^{ab}
ULAAC-G	0.750 ^{ab}
ULAAC-K	0.553 ^{abc}
ULAAC-L	0.463 ^{abc}
ULAAC-C	0.284 ^{abc}
ULAAC-D	0.180 ^{abc}
ULAAC-J	0.014 ^{abc}
ULAAC-A	-0.147 ^{abc}
ULAAC-F	-0.306 ^{abc}
ULAAC-B	-0.750 ^{bc}
Bt	-1.012 ^c
Control	-3.904 ^d

^{a-d}Means followed by the same letter are not significantly different at the 10% significance level.

¹ULAAC-A to ULAAC-M are 13 old-style cheese starters collected in 1968 from various Canadian cheese factories and obtained for this study from Agriculture and Agri-Food Canada (Ottawa, Canada). A commercial starter, starter Bt, with aromatic properties was obtained from Rhodia Food (Sassenage, France) and was used as a commercial control.

a low residual variance (0.310) over the slurries was observed, indicating that there were no products for which the judges were in discordance. Residual variances over the assessors and products provide a measurement of the consensus space for each assessor (Gonzalez Vinas et al., 2001). Individual principal component analyses and ANOVA show that the judges did not perceive much difference between the products.

From the consensus matrix obtained after Generalized Procrustean Analysis of the data, the total amount of variance explained by the first 2 dimensions accounted for 50.1 and 29.1%, respectively, and described adequately most of the variation in the data (total of 79.2%). Further dimensions explained only a small proportion of variance and were more difficult to interpret. Interpretation of the dimensions of the average space is provided in Table 3. Sensory descriptors from assessors showing the highest correlation with the 2 dimensions are given in this table.

Tables 4 and 5 show the results of the ANOVA carried on the 2 main consensus axes. The most important differences among the starters are shown in the first dimension followed by the second dimension. Dimension 1 essentially compares starter Bt against the other starters, mainly ULAAC-E and ULAAC-F. Characteristic attributes of starter Bt are those of a ripened cheese. Starter ULAAC-E was considered by the panel to be different from the others by its Cheddar and mild sulfurous attributes, which may be explained by the significantly higher amounts of methionine found in its

slurry (Figure 4). Starter ULAAC-E also showed lower levels of GABA and Pro as well as higher levels of Met, Asp, and Orn. It is noteworthy that the 2 cultures that showed the greatest differences in AA profile (Bt and ULAAC-E) were also detected as being different in the sensory analyses.

The second dimension (Table 5) categorizes starters Bt and ULAAC-B (characterized by a lactic fresh and diacetyl aroma) against ULAAC-I, ULAAC-H, and ULAAC-M. This is in line with the AA profiles of the slurries. Indeed, when comparing AA values (Figures 2 to 6) to the average levels of the other starters, starter Bt tended to have lower levels of GABA and Pro as well as higher levels of Glu, Met, and Asp. On the second dimension (Table 5), the fresh slurry (control) was also well discriminated.

The presence of several free fatty acids was well correlated with the butyric, lactic acid, sour milk, and parmesan attributes detected by the panel. Butyric and octanoic acids have very strong odors (Table 2) and probably had an important impact on the overall aroma of the slurries. Furthermore, low pH prevailing in the slurries probably favored the nondissociated form of these fatty acids, which is also the aromatic form (Molimard and Spinnler, 1996).

Although significant differences were observed between the free AA profiles, depending on the starters, the impact on the sensory attributes remains difficult to understand. It should be mentioned that FAA are not the first-limiting factor for aroma production in cheese. The bioconversions of these AA and the occurrence of α -ketoglutarate, the final acceptor for the transamination reactions leading to the aroma compounds, would be far more significant in this regard. The presence of glutamate dehydrogenase activity is required for the conversion of glutamate to α -ketoglutarate in several LAB strains commonly used in cheese manufacture and for the production of aroma compounds (Tanous et al., 2002). Whatever the starter used and the ripening time, Yvon et al. (1998) observed very low amino acid degradation without α -ketoacid acceptors (α -ketoglutarate) for AA transamination. The same observation was made by Banks et al. (2001), who found that the addition of α -ketoglutarate to cheese increased volatile components originating from the catabolism of branched-chain and aromatic AA.

CONCLUSIONS

The detailed analysis of 13 old-style cheese starter cultures revealed many differences regarding the free AA content formed during accelerated ripening in cheese slurries. These differences reflected the existence of different enzymatic systems in these starters.

For example, starters ULAAC-A and ULAAC-H were characterized by their glutamate decarboxylase activity, leading to the transformation of glutamate into GABA, an amine well known for its antihypertensive properties. On the other hand, limited correlation was observed between the TCA and PTA fractions of the WSN, as well as the FAA profiles, which points to wide variations in proteinase and peptidase activities or autolytic properties of the starters. This study has shown that links between sensory properties and chemical attributes of slurries can be made. All 13 starters were characterized by a lactic-fresh aroma with roasted nuts and chocolate attributes. Moreover, identification of volatile compounds confirmed the presence of several key molecules in relation to aroma, such as 3-methylbutanal and diacetyl. Two starters presented sensory patterns different from the others, and the only chemical analyses that also set them apart from the others were FAA patterns. Because each starter was composed of about 20 to 30 individual lactic strains, more extensive work is needed to characterize these individual strains and distinguish those having technological and organoleptic characteristics desirable for cheesemaking. Moreover, the GABA-producing capacity reported in some starters is particularly interesting and is currently under investigation with the objective to study the potential antihypertensive effect of cheeses containing GABA-producing strains. This study points to chemical analyses that would enable the discovery of cultures carrying novel sensory and health-promoting attributes.

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