Influence of a Probiotic Adjunct Culture of *Enterococcus faecium* on the Quality of Cheddar Cheese

Gillian E. Gardiner,[†,§] R. Paul Ross,[†] Jean M. Wallace,[†] Francis P. Scanlan,[‡] Paul P. J. M. Jägers,[‡] Gerald F. Fitzgerald,[§] J. Kevin Collins,[§] and Catherine Stanton*[,†]

Teagasc, Dairy Products Research Center, Moorepark, Fermoy, Co., Cork, Ireland, Quest International, Naarden, The Netherlands, and Department of Microbiology, University College Cork, Ireland

Cheddar cheese has previously been shown to be an effective vehicle for delivery of viable cells of a probiotic *Enterococcus faecium* strain to the gastrointestinal tract. The particular strain, *E. faecium* PR88, has proven efficacy in the treatment of irritable bowel syndrome, and in this study it was evaluated for suitability as a starter adjunct for Cheddar cheese manufacture. When added to cheesemilk at an inoculum of 2×10^7 cfu/mL, the enterococcal adjunct maintained viability in Cheddar cheese at levels of up to 3×10^8 cfu/g during 9 months of ripening. Increased proteolysis and higher levels of some odor-active volatile compounds were observed in Cheddar cheeses containing the PR88 adjunct compared with the control throughout the ripening period. In addition, the enterococcal adjunct strain did not affect cheese composition. Although sensory evaluation showed no significant difference in flavor/aroma and body/texture scores between control and experimental cheeses, repeated comments by the commercial grader consistently described the cheeses containing PR88 as 'more advanced than the control' and as having 'better flavor'. These findings indicate that the presence of the PR88 adjunct strain in Cheddar cheese at levels of $\geq 10^8$ cfu/g may positively influence Cheddar flavor.

Keywords: *Probiotic; Enterococcus; cheese; flavor; adjunct*

INTRODUCTION

Controversy currently surrounds the use of enterococci in dairy fermentations, although undoubtedly they have played an important role in artisanal-type fermentations for millennia and have a safe history in food use. Enterococci are an indigenous part of many undefined starter cultures, and many strains are used commercially, e.g., *Enterococcus faecium* K77D has been approved for use as a starter culture in Denmark (Giraffa et al., 1997; Adams, 1997). The concept of incorporation of probiotic lactic acid bacteria into foods is gaining momentum, and the market for food products containing such microorganisms is growing rapidly (Stanton et al., in press). However, the deliberate addition of enterococci to foods has raised concerns, as a result of their relatively frequent association with human infections, particularly endocarditis, urinary tract infections, and nosocomial infections, and the increased frequency of resistance to antibiotics such as vancomycin observed among members of the genus (Aguirre and Collins, 1993; Adams and Marteau, 1995). In addition, enterococci are relatively 'promiscuous' microorganisms with many of the traits contributing to their virulence residing on conjugative plasmids, which may be readily transferred between microorganisms (Jett et al., 1994).

A long history of food use with no reported infection contracted from the consumption thereof is one significant point in favor of the use of enterococci in fermented foods (Adams, 1997). Furthermore, many food-related strains of enterococci offer a means of improving food safety, as they have been shown to produce antibacterial proteins (bacteriocins), and when added to foods, including cheese, during manufacture these strains offer potential for the inhibition of food pathogens such as *Listeria monocytogenes* (Giraffa et al., 1997). However, it was recommended at an international meeting of the Lactic Acid Bacteria Industrial Platform that enterococci should only be used in foods if there are demonstrable benefits, given their association with human infection (van der Kamp, 1996). Probiotics are defined as 'living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition' (Guarner and Schaafsma, 1998). Consumption of the particular strain, *E. faecium* PR88 (Quest International, Naarden, The Netherlands), which was used in this study had previously been demonstrated to alleviate the symptoms of irritable bowel syndrome in humans (Allen et al., 1996) and so demonstrates potential for use as a probiotic strain. The data presented in this study suggest a further beneficial role for this *Enterococcus* strain, in relation to the improvement of Cheddar cheese quality, when employed as a starter adjunct.

Cheese ripening represents a lengthy and expensive process for the manufacturer, and consequently there has long been an interest in acceleration of the process. Several approaches to achieve this end have been investigated, including enzyme addition during Cheddar cheese manufacture, modification of ripening temperature, and addition of cultures as adjuncts to the starter strain (for review, see Fox et al., 1996). Various types of cultures, including strains of lactobacilli, micrococci,

* Corresponding author (telephone, 353-25-42442; fax, 353-25-42340; e-mail, cstanton@moorepark.teagasc.ie).
[†] Teagasc.
[‡] Quest International.
[§] University College Cork.

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pediococci, and propionibacteria, have been added to Cheddar cheese as adjuncts (Bhowmik et al., 1990; Peterson and Marshall, 1990; Fernandez-Espla and Fox, 1998). The resultant effects on cheese quality have been highly variable and are dependent on the particular strain used; nevertheless, many of these starter adjuncts are commercially available (Stanton et al., 1998). In this respect, enterococci have been reported as part of the adventitious flora of many raw and pasteurized milk cheeses, and are thought to play an important role in the ripening of such products (for review, see Giraffa et al., 1997). Consequently, enterococci have previously been deliberately incorporated into many cheese varieties, either as starter or adjunct cultures. Dahlberg and Kosikowski (1948) found Cheddar cheese made with the addition of an *E. faecalis* starter adjunct ripened at an accelerated rate and had a more intense Cheddar flavor than that of a control cheese made with starter culture alone. Similarly, in a study conducted by Jensen et al. (1973), an *E. durans* adjunct strain was found to increase concentrations of free fatty acids and improve flavor in Cheddar cheese. In order to standardize Italian farmhouse cheese production, Neviani et al. (1982) employed enterococci as a starter culture and observed increased proteolysis and improved organoleptic characteristics in comparison to control cheeses. In a similar type of study, Casalta and Zennaro (1997) compared *Lactococcus lactis* subsp. *lactis* with *E. faecalis* as a starter for Venaco, a soft Corsican cheese, and found increased proteolysis in cheeses made with the enterococcal starter.

Previous results have demonstrated that the *E. faecium* PR88 strain is capable of surviving to high levels during Cheddar cheese ripening and, furthermore, that cheese is an effective vehicle for delivery of this probiotic *Enterococcus* to the gastrointestinal tract (GIT) (Gardiner et al., 1999). The aim of the present study was to investigate any influence which this enterococcal strain may have on cheese quality, when employed as a starter adjunct during Cheddar cheese manufacture. The results demonstrate that this enterococcal strain is particularly suitable for Cheddar cheese applications, remaining viable at high levels ($>10^8$ cfu/g cheese) even after 9 months of ripening and having negligible effects on cheese composition, texture, and appearance. However, cheese containing the PR88 strain contained higher levels of free amino acids (FAA) and some odor-active volatile compounds than the control. In addition, the PR88 strain may contribute in a positive way to Cheddar flavor.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *E. faecium* PR88, a well characterized probiotic strain (Allen et al., 1996), was obtained from Quest International (Naarden, The Netherlands) in the form of a spray-dried powder, containing $>2 \times 10^{10}$ cfu/g, and added in this form to experimental Cheddar cheeses as outlined below. In addition, the *Enterococcus* strain isolated from this powder, using Kanamycin Azide Aesculin Agar (KAAA; Oxoid Ltd, Basingstoke, Hampshire, U.K.), and stocked in LM17 (Terzaghi and Sandine, 1975; Difco Laboratories, Detroit, MI) containing 40% glycerol was routinely cultured in LM17 broth at 37 °C. Starters used for cheesemaking (*Lactococcus lactis ssp. cremoris* strains 227 and 223) were obtained from Chr. Hansen's Laboratories (Little Island, Cork, Ireland) in the form of freeze-dried pellets. These were grown overnight at 21 °C in heat-treated (90 °C for 30 min) 10% (w/v) reconstituted skim milk before addition to the cheese vat.

Arginine Degradation Test. Arginine broth (Abd-el-Malek and Gibson, 1948) with 0.0016% bromocresol purple added as a pH indicator was inoculated with the *E. faecium* PR88 culture, incubated at 37 °C, and examined daily for 5 days. A positive result was indicated by development of a purple color, due to the degradation of arginine and the resultant production of ammonia, while a yellow color indicated a negative result, due to metabolism of glucose only.

Cheddar Cheese Manufacture. Pilot-scale Cheddar cheeses were manufactured in triplicate, with each vat containing 450 L of standardized (fat:protein = 1) pasteurized whole milk. A 1.5% inoculum of the commercial starter cultures, *L. lactis* spp. *cremoris* strains 227 and 223, was added to all vats. The control vats contained starter culture only, while the experimental cheeses contained an additional 0.1% (w/v) inoculum of the *E. faecium* PR88 strain added in powder form as an adjunct to the starter culture, as described in a previous study (Gardiner et al., 1999). Cheddar cheeses were manufactured according to standard procedures (Gardiner et al., 1998) with the main parameters as follows: rennet (Chr. Hansen's Laboratories) was added at a concentration of 0.07 mL/L, curds were cooked to 38 °C, drained at pH 6.1, milled at pH 5.3, and salted at the rate of 2.7% (w/w). Following pressing overnight at approximately 413 kPa, cheeses were removed from the molds, vacuum-packed, and ripened at 8 °C for 9 months.

Bacteriological Analyses. Cheddar cheeses were aseptically sampled in duplicate at intervals during the ripening period for bacteriological analyses. Samples were emulsified in sterile 2% (w/v) trisodium citrate and diluted in maximum recovery diluent (Oxoid), and appropriate dilutions were pour-plated. Enterococci (including the PR88 adjunct strain) were enumerated in the cheeses during ripening by plating on KAAA following overnight incubation at 37 °C. To show that the selective pressure of the KAAA medium did not affect enterococcal viability, cell numbers obtained using this medium were compared with those obtained using the nonselective LM17 agar. Viability of nonstarter lactic acid bacteria (NSLAB) and starter lactococci was determined on LM17 agar after 3 days of incubation at 30 °C and on LBS agar (Rogosa et al., 1951; Becton Dickinson, Cockeysville, MD) following 5 days of incubation at 30 °C under anaerobic conditions (anaerobic jars with 'Anaerocult A' gas packs; Merck, Darmstadt, Germany), respectively.

Cheese Compositional and Sensory Analyses. Grated samples of 14-day-old cheeses were analyzed in duplicate for salt (potentiometric method), fat (Gerber method), moisture (oven-drying at 102 °C), pH, as previously described (Gardiner et al., 1998), and protein as determined by the Kjeldahl method (IDF, 1964). Sensory analyses were performed after 3, 6, and 9 months of ripening by a commercial grader. Cheeses were graded blind for flavor/aroma and body/texture, receiving minimum−maximum scores of 38−45 and 31−40, respectively.

Assessment of Proteolysis in Cheddar Cheese. Proteolysis was monitored in the cheeses throughout ripening by measuring pH 4.6 SN (i.e., the proportion of total N, as measured by Kjehdahl, soluble in water at pH 4.6, as described by Kuchroo and Fox, 1982). Individual FAA in the water-soluble extracts were determined using a Beckman System 6300 High Performance Analyzer (Beckman Instruments Ltd, High Wycombe, Buckinghamshire, U.K.) equipped with a Beckman P-N 338052 Na^+ column (12 cm × 0.5 cm) as described by Gardiner et al. (1998). Amino acid concentrations were converted to microgram per gram of cheese.

Gas Chromatography-Olfactometry (GC-O) and Gas Chromatography-Mass Spectrometry (GC-MS) Analyses. Evaluation of volatiles was performed by dynamic headspace analysis. One hundred grams of cheese was cut into cubes ($\sim 2 \times 2 \times 2$ mm^3), placed in a conical flask, and mixed in order to obtain a representative and uniform sample. Fifteen grams of this sample was tempered in the headspace apparatus for 1 h at 25 °C, then flushed with ultrapure N_2 (approximately 16 mL/min) for 10 min. Volatile compounds were trapped using a glass liner (l = 9.3 cm, od = 2 mm) filled with Tenax adsorbent. The glass liner was removed from the sampling

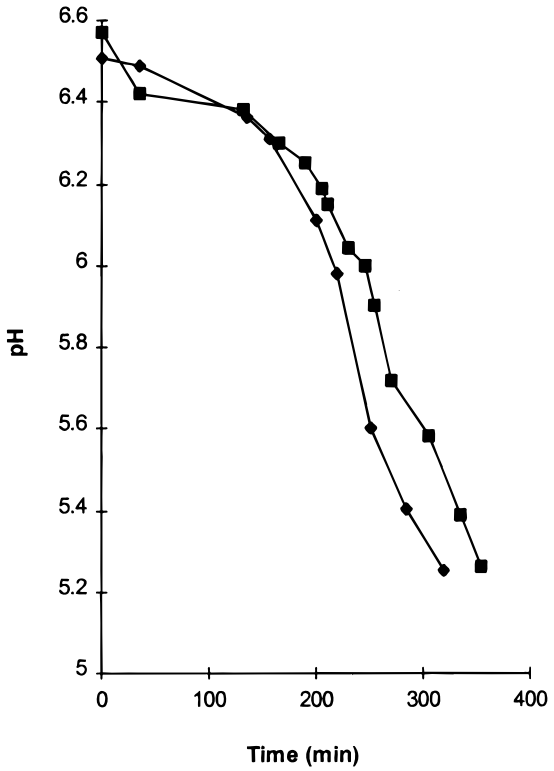

Figure 1. Acid development during pilot-scale manufacture of control cheese (■) and cheese containing *E. faecium* PR88 starter adjunct (◆).

apparatus and placed in the CIS (cooled injection system) inlet of the GC injector port (Gerstel, Mulheim, Germany), then desorbed at 275 °C for 4 min. The GC was programmed from 40 to 240 °C at a rate of 3 °C/min. Separation was performed on an apolar HP-5 capillary column (50 m × 0.32 mm, film thickness 1 μm; Hewlett-Packard, Wilmington, DE). The capillary column was connected to a two-way splitter, whereby the effluent was split between the nose and flame ionization detector (FID) in a 90:10 ratio to facilitate sniffing of the sample. The sniffing port was humidified, and the FID was held at 250 °C. Peaks were identified using retention indices (RI) and comparison with standards. Since sampling and analytical conditions were identical for all cheeses, comparisons of peak areas were used to identify differences. MS was used to identify headspace compounds using a Finnigan TSQ70 mass spectrometer (San Jose, CA). Ionization was by electron impact at 70 eV, and an in-house mass spectral library (Quest International, Naarden, The Netherlands) was used for identification of peaks.

RESULTS AND DISCUSSION

Incorporation of *Enterococcus* Adjunct into Cheddar Cheese. The probiotic *E. faecium* strain PR88 was added to Cheddar cheese at an inoculum level of 2.3×10^7 cfu/mL of cheese milk (0.1% w/v) as an adjunct to a commercial lactococcal starter, as in a previous study (Gardiner et al., 1999). This adjunct strain survived and contributed to acid development during Cheddar cheese manufacture, as evidenced by a decrease in the time required to reach milling pH (pH 5.3) (Figure 1). In this respect, the vat containing the PR88 adjunct strain reached milling pH ~35 min faster than the control vat. This decrease in manufacturing time is a desirable trait for cheese manufacturers operating to strict production schedules. On Day 1 of ripening, the enterococcal adjunct strain was detected in the cheese at levels of 1.6×10^8 cfu/g and thereafter sustained a high level, ranging from 1.5×10^8 to 2.8×10^8 cfu/g during 9 months (252 days) of ripening (Figure 2a). Subsequent trials showed that when added at inocula of 1.4×10^6 and 1.5×10^5 cfu/mL of cheesemilk (i.e., 10 and 100 times less), concomitant levels of 3.4×10^7 and 3.2×10^6 cfu/g of PR88 were achieved in the cheeses, respectively, after 9 months (252 days) of ripening. Although enterococci were present in the control cheeses at 10^4-10^5 cfu/g throughout ripening (Figure 2a), these levels are similar to those reported for adventitious enterococci in Cheddar cheese (Clark and Reinbold, 1966). A minimum of 10^7 viable microorganisms per gram or milliter should be present in a food product in order to meet the requirements of a 'probiotic' food, as described by the Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association (Ishibashi and Shimamura, 1993). Therefore, the Cheddar cheeses manufactured in this study, using PR88 as a starter adjunct, may be described as probiotic or 'functional' foods using these criteria.

NSLAB *Lactobacillus* levels increased throughout ripening, reaching maximum numbers of 2×10^7 cfu/g after 6 months (168 days) (Figure 2b), in agreement with previous findings for mature Cheddar cheese (Peterson and Marshall, 1990; Gardiner et al., 1998). However, differences in levels of NSLAB between control and experimental cheeses were evident after only 14 days of ripening, and throughout the first 6 months (168 days) of ripening, NSLAB populations remained 10 times lower in cheeses containing PR88 than in the control cheeses, which harbored enterococci at a level of 10^4-10^5 cfu/g (Figure 2b). This finding indicates that the probiotic adjunct strain, when present at high levels ($\geq 10^8$ cfu/g) in Cheddar cheese, either competes with or inhibits the growth of NSLAB. The latter is unlikely, since deferred antagonism tests on solid media failed to show any inhibitory effect of PR88 against some representative NSLAB (data not shown). Thus, the addition of this strain to Cheddar cheese and its survival at high levels may be advantageous in reducing the undesirable properties associated with spoilage NSLAB.

Others have also demonstrated that enterococci are suited to survival in Cheddar cheese, and it has been postulated that this is due to their acid and salt tolerance and their general robust nature (Kosikowsi and Dahlberg, 1948; Clark and Reinbold, 1966). For example, *E. durans* and *E. faecalis* strains reached levels of up to $\sim 5 \times 10^8$ cfu/g in cheese after 6 months of ripening (Jensen et al., 1973). However, these previous studies involving the incorporation of enterococci into Cheddar cheese were based on the suitability of the strain for cheese manufacturing applications, whereas the *Enterococcus* strain used in this study also possesses probiotic characteristics. Although other probiotic microorganisms such as lactobacilli and bifidobacteria have previously been incorporated into Cheddar cheese (for review, see Stanton et al., 1998), this *E. faecium* strain is the first example of the use of a probiotic *Enterococcus* strain for cheese applications (Gardiner et al., 1999). Furthermore, previous in vitro and in vivo studies have shown Cheddar cheese to be an effective means of delivery of high levels of viable cells of this probiotic *E. faecium* strain to the mammalian GIT (Gardiner et al., 1999).

Cheese Compositional Analysis. The composition of control and experimental cheeses was generally found to be within the range typical for Cheddar (Table 1).

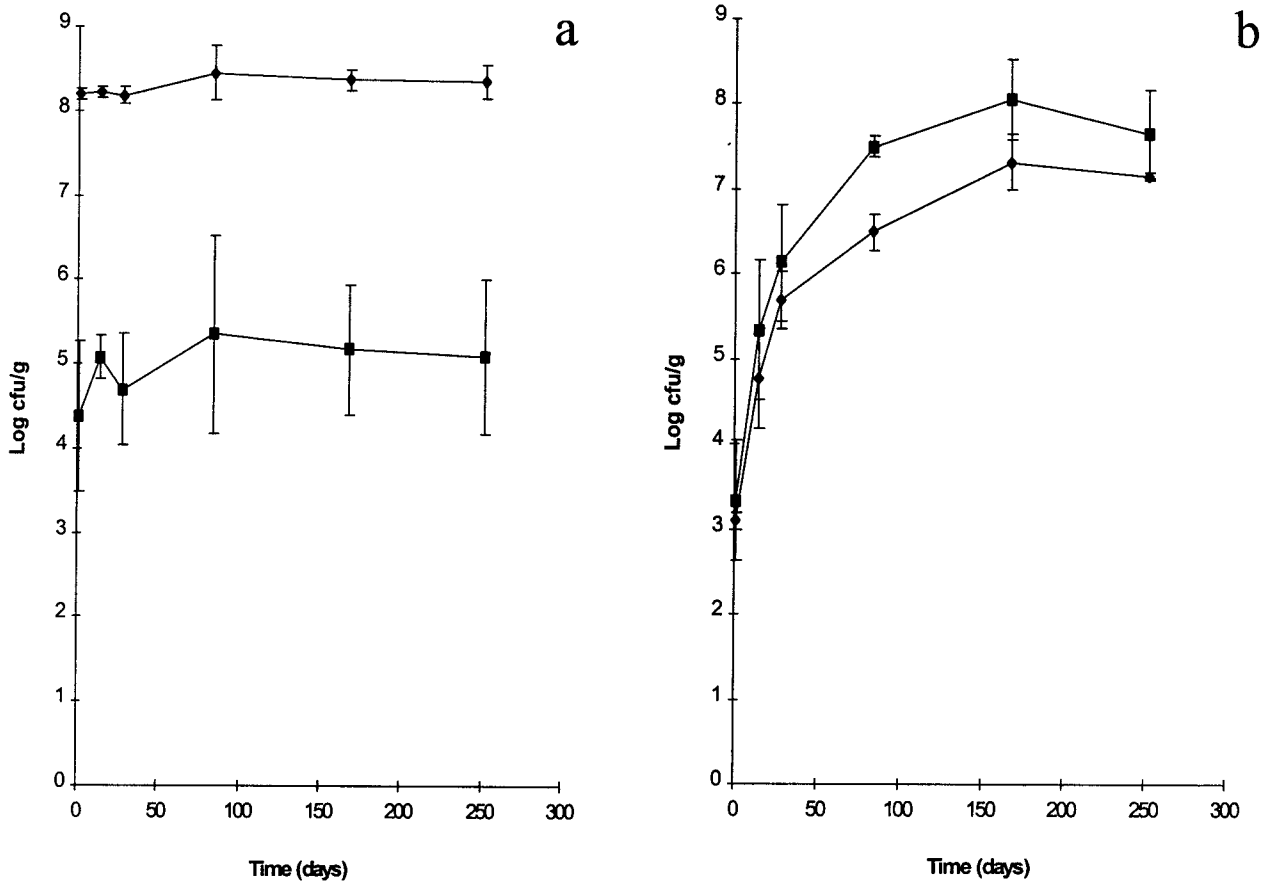

Figure 2. Survival of (a) enterococci and (b) NSLAB during ripening in pilot-scale control cheese (■) and cheese containing *E. faecium* PR88 as a starter adjunct (◆). Values are means for three cheeses, with standard deviations indicated by vertical bars.

Table 1. Composition[a] of Control and *E. faecium* PR88-Containing Cheddar Cheeses at 14 Days

cheese	pH	moisture (%)	salt (%)	S/M[b] (%)	fat (%)	protein (%)
control	5.31 ± 0.1	37.67 ± 1	1.84 ± 0.2	4.88 ± 0.6	32.5 ± 0.1	26.63 ± 0.6
+PR88	5.31 ± 0	37.46 ± 0.5	1.67 ± 0.1	4.47 ± 0.3	32.5 ± 0.8	27.19 ± 0.1

[a] Values are means ± SD of duplicate analyses of three cheeses. [b] Salt-in-moisture.

Table 2. Sensory Evaluation[a] of Control and *E. faecium* PR88-Containing Cheddar Cheeses during Ripening

cheese	age (mo)	flavor/aroma score[b]	body/texture score[c]	comments
control	3	38 ± 0	33 ± 0	very mild
+ PR88	3	38.5 ± 0.5	33 ± 0	better flavor; more advanced than control
control	6	37.8 ± 1	33.5 ± 0.7	mild flavor; bitter
+ PR88	6	39 ± 1	33 ± 0	better flavor and more advanced than control; slightly bitter
control	9	39 ± 0.7	33 ± 0	not as bitter as experimental cheese
+ PR88	9	39 ± 0	32 ± 0	bitter; body cracking; over-ripe

[a] Values are means ± SD for three cheeses. [b] Maximum score = 45; minimum commercial score = 38. [c] Maximum score = 40; minimum commercial score = 31.

Although the presence of the enterococcal adjunct strain at an inoculum level of 2.3×10^7 cfu/mL of milk increased acid production during cheese manufacture (Figure la), the final pH value for this cheese was comparable with that of the control (Table 1). Overall, the comparable values observed for control and experimental cheeses indicate that incorporation of the probiotic enterococcal strain, PR88, into Cheddar cheese as a starter adjunct and its subsequent survival to high levels ($>10^8$ cfu/g) during ripening had no direct effect on cheese composition.

Sensory Evaluation. Using a commercial grader, all cheeses were described as commercial grade with respect to flavor/aroma and body/texture, achieving scores of ≥38 and 31, respectively (Table 2). Although sensory evaluation showed no significant differences in flavor/aroma and body/texture scores between control and experimental cheeses (e.g., 38 versus 38.5 ± 0.5 and 37.8 ± 1 versus 39 ± 1, respectively), repeated comments by the commercial grader consistently described the cheeses containing PR88 as 'more advanced than the control' and as having 'better flavor' (Table 2). These findings indicate that the presence of the probiotic adjunct strain in Cheddar cheese at levels of $\geq 10^8$ cfu/g may positively influence cheese flavor. However, at 9 months, these PR88-containing cheeses were described by the grader as comparable to or less desirable than the control cheeses and over-ripened (Table 2). The fact that the

flavor of the adjunct-containing cheeses at 6 months compared with that of the control at 9 months suggests a role for the probiotic PR88 strain in the acceleration of cheese ripening. Enterococci play an important role in ripening and flavor development of traditional cheeses (Trovatelli and Schiesser, 1987) and when deliberately added to cheese, they had a positive effect on sensory attributes (Dahlberg and Kosikowski, 1948; Neviani et al., 1982). However, an *E. faecalis* strain was found to cause flavor defects when added as an adjunct to Cheddar cheese, although an *E. durans* strain resulted in improved flavor (Jensen et al., 1975c), indicating the importance of strain selection.

Proteolysis in the Cheeses. An increase in pH 4.6 SN was observed in all cheeses as a result of ripening (data not shown). However, no differences were found between the control and experimental cheeses, indicating that the presence of high numbers of the PR88 strain in Cheddar cheese did not contribute to primary proteolysis. Proteolysis at this level is usually due to the action of chymosin and plasmin, and it has previously been shown not to be influenced by addition of starter adjunct (McSweeney et al., 1994; Gardiner et al., 1998; Fernandez-Espla and Fox, 1998). Total concentrations of FAA increased in all cheeses throughout ripening as expected and were substantially higher (~50%) at all time points in the cheeses containing final PR88 levels of 2.3×10^8 cfu/g compared with the corresponding controls (data not shown). Indeed, higher levels of many individual FAA were detected in the experimental cheeses compared with the controls throughout ripening (Figure 3). Results of the present study suggest that the PR88 strain is capable of releasing FAA from peptides in the cheese, thus increasing secondary proteolysis during Cheddar cheese ripening.

Although analyses showed an absence of free arginine in some of the control cheeses, due to possible degradation by NSLAB flora, this amino acid was not detected in any of the adjunct-containing cheeses at any stage during ripening (Figure 3). These data indicate the presence of the enzyme arginase, which degrades arginine to ornithine and ammonia (Broome et al., 1991) in the *Enterococcus* adjunct strain used in this study. This particular strain was tested in vitro and found to degrade arginine, in agreement with previous findings for other *E. faecium* strains (Mundt, 1989). The presence of high concentrations of arginine in Cheddar cheese has been associated with an unpleasant bittersweet taste (Puchades et al., 1989), so strains capable of degrading free arginine, including PR88, may play a positive role in terms of flavor enhancement in the cheese. Tyrosine was found at a slightly lower concentration in the cheese containing final levels of 2.3×10^8 cfu/g of PR88 than in the control cheese after 9 months of ripening (Figure 3c). This degradation of tyrosine during ripening suggests that the enterococcal adjunct strain may harbor the enzyme tyrosine decarboxylase, which would result in the formation of the potentially toxic amine tyramine (Joosten and Stadhouders, 1987). However, because degradation of this amino acid was only slight and because the resultant amine may be further degraded into harmless substances, resulting levels of tyramine in the cheese may be irrelevant in amine intoxications.

Previous additions of adjunct cultures such as lactobacilli (McSweeney et al., 1994), propionibacteria (Fernandez-Espla and Fox, 1998), and micrococci (Bhowmik et al., 1990) to Cheddar cheese have influenced proteolysis at the level of FAA, with associated improvements in Cheddar flavor. However, others have found no correlation between proteolysis and flavor development (Dacre, 1953; Law and Sharpe, 1977), and in some cases, increased proteolysis has led to development of off-flavors (Jensen et al., 1975a, 1975c; Puchades et al., 1989). The precise role of FAA in Cheddar cheese flavor is not completely understood, but it seems likely that the products of amino acid catabolism have a greater contribution to flavor than the amino acids per se (Fox and Wallace, 1997). Enterococci have previously been shown to increase proteolysis in Italian and Cheddar cheeses (Neviani et al., 1982; Jensen et al., 1975a). In this study, incorporation of the probiotic *E. faecium* PR88 as a starter adjunct resulted in increased secondary proteolysis in Cheddar cheese during ripening, with possible improvements in cheese flavor up to 6 months, based on grader's comments (Table 2). However, with further increases in concentrations of total FAA and changes in the ratio of some individual FAA, relative to the control, the flavor of the experimental cheese deteriorated, based on grader's comments, and was described at 9 months as 'over-ripe' (Table 2). Cheddar flavor is thought not to be determined by individual components of the cheese but instead by a balance of many factors, a theory referred to as the component balance theory (Mulder, 1952). Therefore, the change in FAA concentrations between 6 and 9 months may have disrupted this delicate balance, resulting in deterioration in the overall flavor quality of the cheese.

Analysis of Volatile Compounds in the Cheeses. Volatile compounds were analyzed in all cheeses throughout ripening, and those detected after 6 months are listed in Table 3. At this stage of ripening, a total of 47 volatile compounds were separated using headspace-GC analysis (Figure 4a−d) and 39 of these were identified using comparison with reference standards, calculated retention indices, and mass spectral data (Table 3). Most of these compounds have previously been found in Cheddar cheese of high quality (Urbach, 1995). One of the experimental cheeses and the corresponding control were also analyzed by GC-O (sniffing analysis) after 6 months. A total of 25 odor-active compounds were reproducibly detected among these cheeses; 12 in both the PR88 adjunct-containing and control cheeses (Table 4). Eight of these odor-active compounds were found only in the experimental cheese (Table 4) and may therefore be important for improved Cheddar flavor. In contrast, five odors were found to be specific to the control, three of which (RI = 855, 1025, 1109) are thought to be undesirable when present in high concentrations in Cheddar cheese, as they may introduce a fruity/estery note (Bills et al., 1965). Fruitiness due to esters derived from free fatty acid degradation has been recognized as a cheese flavor defect (Bills et al., 1965). Most of the odors detected in the cheeses in this study are commonly found by sniffing Cheddar extracts, but to our knowledge, odors such as anise/liquorice (control only) or naphtholic/furniture polish (experimental only) have not previously been reported in cheese aroma profiles. Although some of the odors described were not identifiable by headspace-MS, many of these descriptors have previously been used. Octen-3-ol and 1-octen-3-one have both been described as having a 'mushroom' odor (Moio et al., 1993; Milo and Reineccius, 1997; Kubickova and Grosch, 1997), while 4-methylphenol or *p*-cresol, 3-octanone or 2-methoxy-3-isopropyl

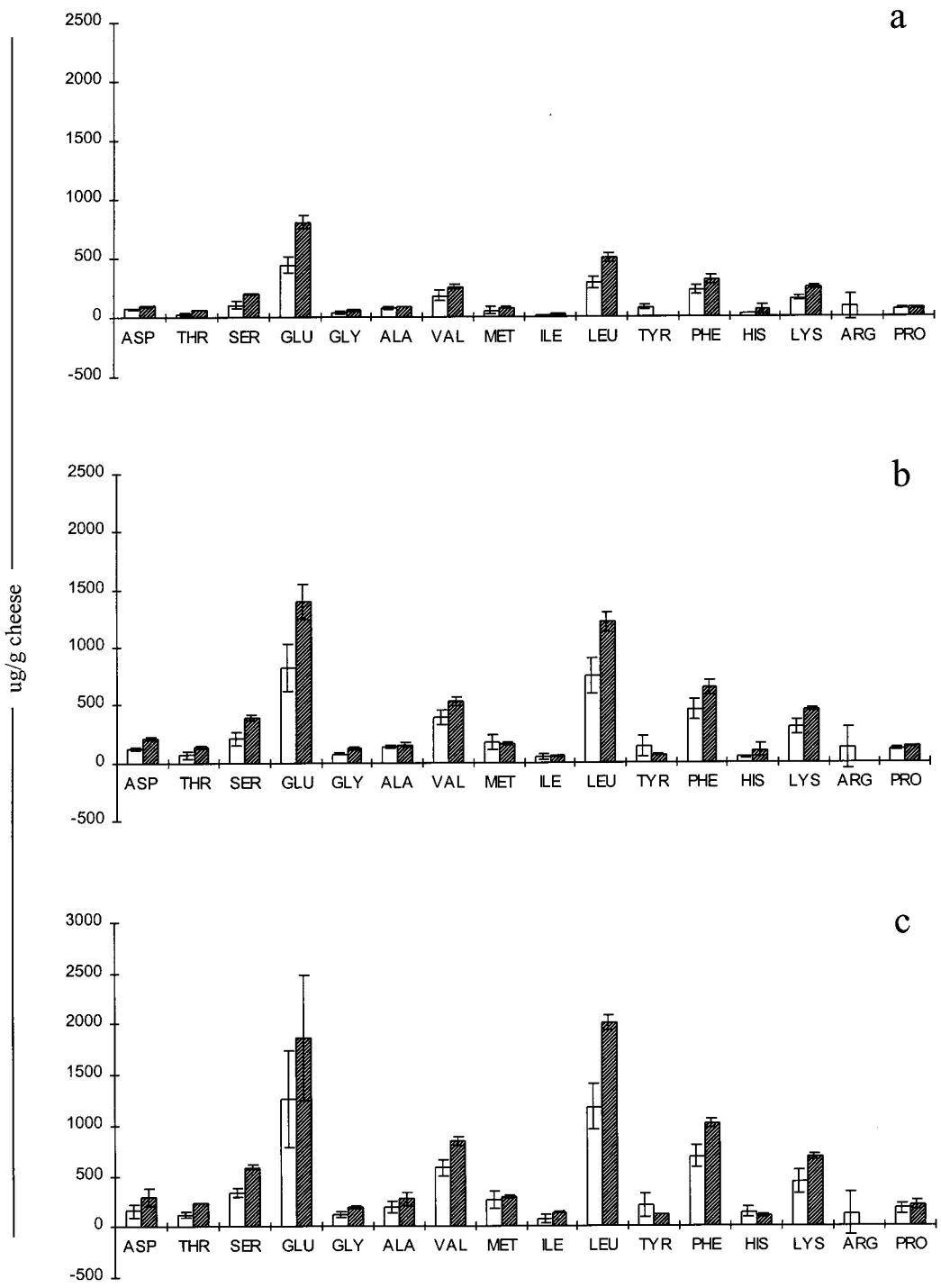

Figure 3. Concentration of individual free amino acids in water-soluble extracts of control cheese (open bars) and cheese containing *E. faecium* PR88 as a starter adjunct (hatched bars) at (a) 3, (b) 6, and (c) 9 months of ripening. Values are means for three cheeses, with standard deviations indicated by vertical bars.

pyrazine, and 3-methyl-2 butenal have been described as 'phenolic', 'earthy', and 'metallic', respectively (Moio et al., 1996; Ott et al., 1997; Kubickova and Grosch, 1997). A 'green' odor has been attributed to many different compounds, principally aldehydes and alcohols (Moio et al., 1993; Ott et al., 1997; Kubickova and Grosch, 1997), while the unidentified 'flowery' odor may be accounted for by compounds such as phenylacetaldehyde or phenylethanol (Moio et al., 1993; Kubickova and Grosch, 1997; Ott et al., 1997). Similarly, fruity odors have previously been associated with compounds such as esters, i.e., methyl-3-butylacetate, or alcohols, such as propan-2-ol (Moio et al., 1993; Kubickova and Grosch, 1997). While the GC chromatograms indicated no major qualitative differences between the cheeses throughout ripening (Figure 4a−d), there were quantitative differences between experimental and control cheeses at each ripening time investigated. Total volatile compounds were higher in the control than in the experimental cheeses following 1 day of ripening, while equal quantities were found following 3 months of ripening, and higher levels were detected in the adjunct-containing cheeses following ripening for both 6 and 9 months. This may be due to the lack of precursors for volatile flavor formation, such as FAA, in the early period of cheese ripening.

Table 3. Volatile Compounds Detected by Headspace-GC Analysis in both Control and *E. faecium* PR88-Containing Cheddar Cheeses at 6 Months

peak no.	retention time (min)	retention index	compound
1	3.67	442	acetaldehyde
2	3.73	445	unknown 1
3	3.82	450	unknown 2
4	4.21	470	ethanol
5	4.39	479	methylmercaptan
6	4.61	490	unknown 3
7	4.68	494	propional
8	4.72	496	acetone
9	4.86	503	unknown 4
10	5.37	529	dichloromethane
11	5.79	550	isobutanal
12	5.87	554	acetic acid
13	6.06	563	unknown 5
14	6.38	579	unknown 6
15	6.53	587	diacetyl
16	6.82	601	butan-2-one
17	6.88	602	butan-2-ol
18	7.28	613	ethylacetate
19	7.49	619	isobutanol
20	7.65	623	chloroform
21	8.01	635	unknown 7
22	8.51	646	3-methyl butanal
23	8.72	652	3-methyl-2-butanone
24	9.15	664	unknown 8
25	9.47	672	propionic acid
26	9.97	686	pentan-2-one
27	10.4	697	pentan-2-ol
28	10.5	700	heptane
29	10.9	708	acetoine
30	11.8	728	3-methyl butanol
31	12.1	731	2-methyl butanol
32	12.3	734	4-methyl-2-pentanone
33	13.1	750	3-methyl-2-pentanone
34	13.8	763	toluene
35	13.9	766	butyric acid
36	14.1	770	methyl 3-methyl butyrate
37	14.7	781	unknown 9
38	15.1	788	3-methyl-2-pentanol
39	15.7	800	octane/ethylbutyrate
40	19.7	868	ethyl benzene
41	20.2	875	xylene
42	21.1	891	heptan-2-one
43	21.5	898	styrene
44	21.7	901	heptanal
45	25.8	968	benzaldehyde
46	30	1037	limonene
47	33.3	1093	nonan-2-one

The most notable quantitative difference between the experimental and control cheeses was the concentration of butyric acid, which was considerably higher in the experimental cheeses after ripening for 6 and 9 months (Figure 5). Increases in butyric acid concentrations may indicate increased lipolytic activity in cheeses with added enterococci relative to the control. Carrasco de Mendoza et al. (1992) assayed a number of *Enterococcus* strains for lipolytic activity and found most to be only weakly so. However, a number of *E. faecalis* strains had high activity against milk triglycerides. In contrast, Jensen et al. (1975b) found that cheeses manufactured with *E. faecalis* as an adjunct had a somewhat lower concentration of free fatty acids than did the control cheese but suggested that this was due to subsequent conversion of acids to neutral compounds. Therefore, the *E. faecium* PR88 strain used in this study may have contributed to lipolysis in the experimental cheeses, which may account for the increased level of butyric acid, although no evidence was obtained to indicate increases in longer chain fatty acids. The increased levels of butyric acid may not therefore be due solely to

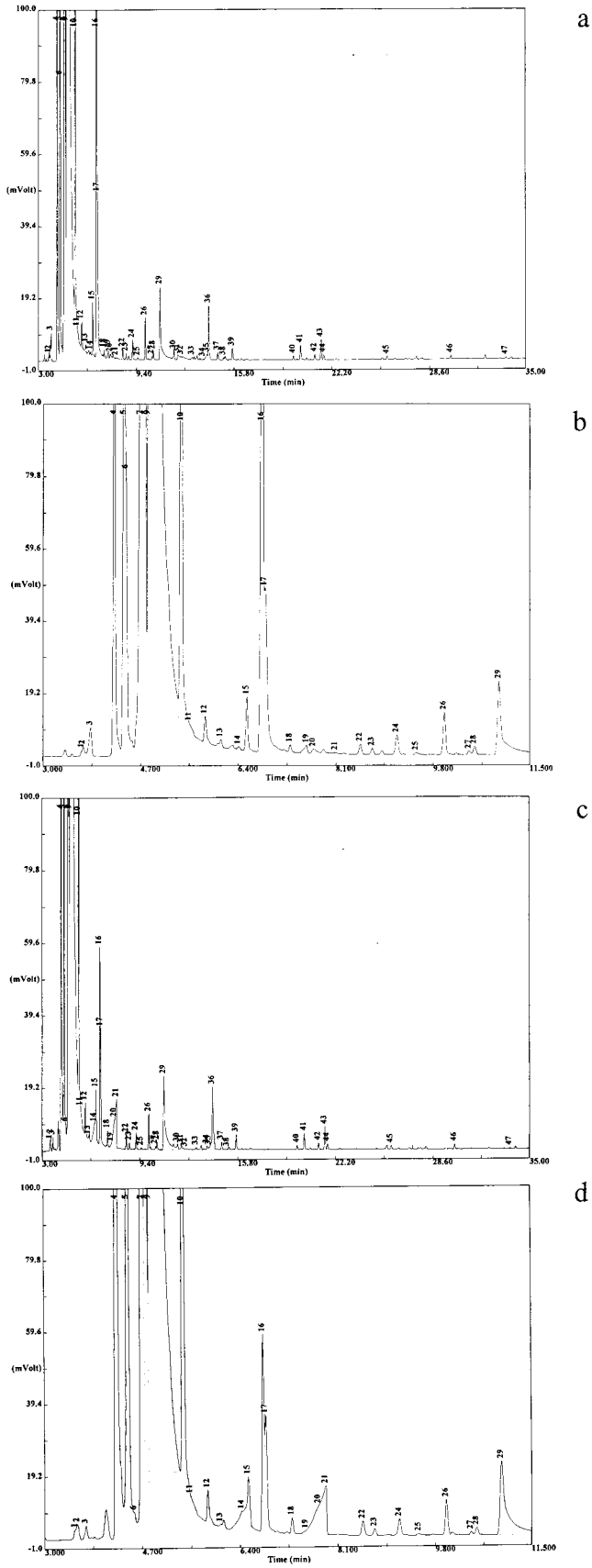

Figure 4. Chromatograms of volatile compounds detected by headspace-GC analysis in (a, b) control cheese and (c, d) cheese containing *E. faecium* PR88 adjunct after 6 months of ripening. The area of the chromatogram containing peaks 1−29 is expanded in b and d for control and PR88-containing cheeses, respectively. Peak numbers correspond to Table 3. Peaks 2, 3, 6, 9, 13, 14, 21, 24, and 37 were not identified.

Table 4. Odors Identified by GC-Olfactometry after 6 Months of Ripening in one *E. faecium* PR88-Containing and Corresponding Control Cheese

retention index	specific to control	specific to experimental	both control and experimental	headspace-MS identification
116		green, fresh		
586			buttery, diacetyl	diacetyl
681			proteinaceous, serumy	pentan-2-one
729			sweaty, fatty acid	3-methylbutanol
773			cheesy, fatty acid, buttery	butyric acid
803		green		
836			cheesy, fatty acid	3-methylbutyric acid
847			flowery, perfumy, fruity	2-methylbutyric acid
855	fruity			
946		slightly cheesy		4-methylvaleric acid
984			mushroom	2-methyl-2-hepten-6-one
989		metallic		
1010		aldehydic, fatty		octanal
1025	estery			
1057			floral, violet	isoamyl butyrate
1080		naphtholic/furniture polish		
1084			mushroom	
1102	earthy			
1106			waxy	nonanal
1109	estery/fruity			
1125		flowery, roast		
1209			earthy, aldehydic	decanal
1217			phenolic	
1220		baked, fatty, aldehydic		
1247	anise/liquorice			

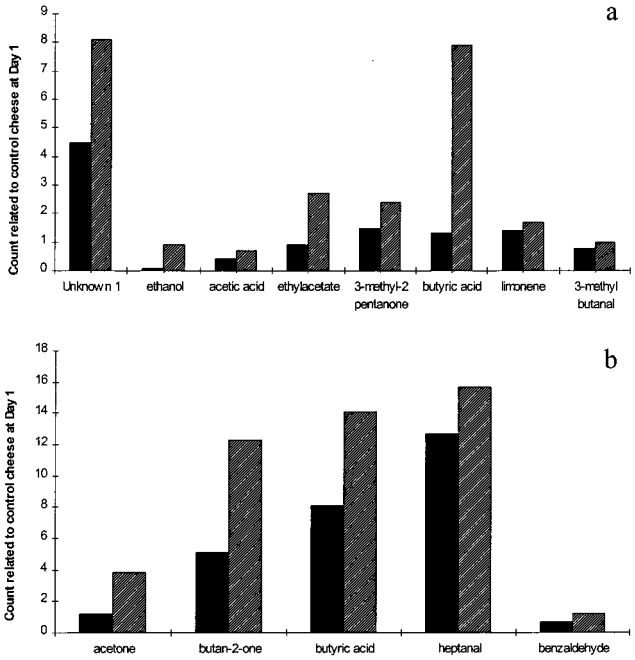

Figure 5. Individual volatile compounds detected in higher levels in cheese containing *E. faecium* PR88 adjunct (hatched bars) than in control cheese (solid bars) after (a) 6 and (b) 9 months of ripening. Volatile compounds were measured by head space-GC analysis, and levels are related to control cheese at Day 1. Unknown 7 was also detected in higher levels in experimental cheeses at 6 months, but concentrations were too high to include on this scale.

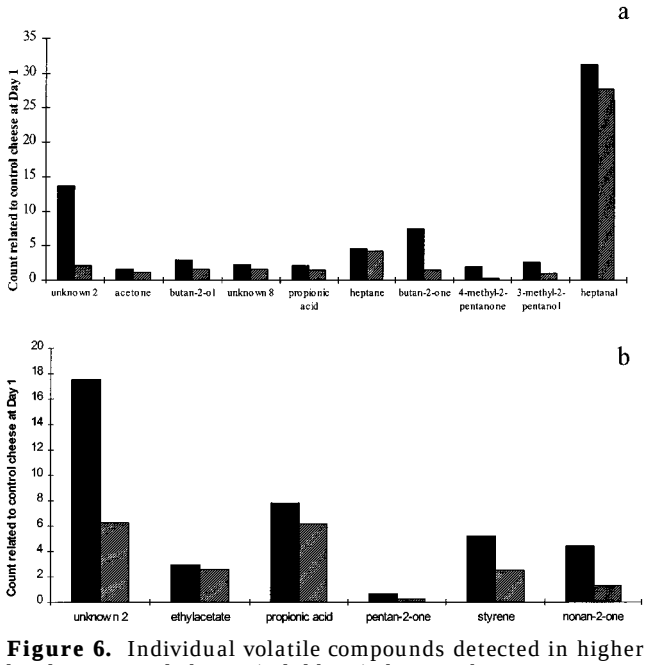

Figure 6. Individual volatile compounds detected in higher levels in control cheese (solid bars) than in cheese containing *E. faecium* PR88 adjunct (hatched bars) after (a) 6 and (b) 9 months of ripening. Volatile compounds were measured by head space-GC analysis, and levels are related to control cheese at Day 1. Unknown 7 was also detected in higher levels in the control cheese at 9 months, but concentrations were too high to include on this scale.

lipolysis but may have been formed by an alternative route. Interestingly, a novel pathway has recently been described in *E. faecalis*, which involves the catabolism of α-keto acids with concomitant formation of ATP (Ward et al., 1999). This energy-generating pathway may therefore account for the elevated levels of butyric acid found in the cheese containing the *E. faecium* PR88 adjunct strain. Butyric acid may have a strong impact on cheese flavor since it has a potent, sweaty, or cheesy odor and is commonly found in high concentrations in many cheese types, most notably Parmesan and other hard Italian cheeses (Barbeiri et al., 1994). Other related compounds such as butan-2-one, 2-methyl butyric acid, 3-methyl butanol, 3-methyl butanal, and 3-methyl butyric acid were found in both control and PR88-containing cheeses (Table 3), the latter two contributing cheesy, sweaty, and fatty acid aromas (Table 4). Some compounds were found at higher concentrations in the control compared with the experimental cheeses (Figure 6). For example, the ketone, nonan-2-one was present in higher concentrations in control compared with experimental cheeses throughout 9 months of ripening, being present at 2 and 3 times

higher concentrations following 6 and 9 months of ripening, respectively (Figure 6). Nonan-2-one has been described as having a malty or fruity flavor (Kubickova and Grosch, 1997) and thus may have had a negative impact on the flavor of the control cheese. In fact, estery/fruity notes were detected by olfactometry in 6-month-ripened control cheese (RI = 855, 1025, 1109) which were not detectable in the experimental cheeses (Table 4).

GC-FID and GC-MS analyses at 6 months showed clear differences between control and adjunct-containing cheeses. However, the differences detected by olfactometry may be most significant, since they clearly indicate different sensory characteristics in the cheeses. Since the only variation in the manufacture of the control and experimental cheeses was the incorporation of the PR88 starter adjunct, the results indicate that the differences were probably due to the metabolic activity of this strain.

CONCLUSIONS

Previous results have demonstrated that Cheddar cheese supports the viability of high numbers of the probiotic *E. faecium* strain (PR88) during ripening and, furthermore, that cheese is an effective vehicle for delivery of this probiotic *Enterococcus* to the GIT (Gardiner et al., 1999). Results of the present study demonstrate that the PR88 strain, when incorporated into Cheddar cheese during manufacture, proved particularly suitable as a starter adjunct. It resulted in a reduced manufacturing time and was capable of survival to high cell numbers in cheese during 9 months of ripening without affecting cheese composition or appearance. The adjunct strain may also have had a positive contribution to cheese flavor and a possible role to play in acceleration of the ripening process. Higher concentrations of most individual FAA were detected in experimental cheeses containing the PR88 strain, indicating an increase in secondary proteolysis by the enterococcal adjunct. In addition, higher levels of some volatile compounds, most notably butyric acid, were detected in the adjunct-containing cheeses throughout ripening. Furthermore, GC-olfactometry indicated differences in the sensory characteristics of the control and adjunct-containing cheeses. These data indicate that catabolic activity of the PR88 strain leads to increased release of FAA and volatile compounds in the experimental cheeses. Overall, when added as an adjunct to Cheddar cheese, this probiotic strain did not adversely affect product quality and indeed may positively influence certain attributes of the product, such as flavor.

ABBREVIATIONS USED

FAA, free amino acids; GIT, gastrointestinal tract; KAAA, kanamycin azide aesculin agar; NSLAB, nonstarter lactic acid bacteria; GC-O, gas chromatography-olfactometry; GC-MS, gas chromatography-mass spectrometry; FID, flame ionization detector; RI, retention index.

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LITERATURE CITED

Abd-el-Malek, Y.; Gibson, T. Studies in the bacteriology of milk. I. The streptococci of milk. *J. Dairy Res.* **1948**, *15*, 233−240.

Adams, M. R. The safety of lactic acid bacteria. *BNF Nutr. Bull.* **1997**, *22*, 91−98.

Adams, M. R.; Marteau, P. On the safety of lactic acid bacteria. *Int. J. Food Microbiol.* **1995**, *27*, 263−264.

Aguirre, M.; Collins, M. D. Lactic acid bacteria and human clinical infection. *J. Appl. Bacteriol.* **1993**, *75*, 95−107.

Allen, W. D.; Linggood, M. A.; Porter, P. European Patent 0508701B1, July 1996.

Barbeiri, G.; Bolzoni, L.; Careri, M.; Mangia, A.; Parolari, G.; Spagnoli, S.; Virgili, R. Study of the volatile fraction of Parmesan cheese. *J. Agric. Food Chem.* **1994**, *42*, 1170−1176.

Bhowmik, T.; Riesterer, R.; Van Boekel, M. A. J. S.; Marth, E. H. Characteristics of low-fat Cheddar cheese made with added *Micrococcus* or *Pediococcus* species. *Milchwissenschaft* **1990**, *45*, 230−235.

Bills, D. D.; Morgan, M. E.; Libbey, L. M.; Day, E. A. Identification of compounds responsible for fruity flavor defect of experimental Cheddar cheeses. *J. Dairy Sci.* **1965**, *48*, 1168−1173.

Broome, M. C.; Krause, D.; Hickey, M. W. The use of proteinase-negative starter and lactobacilli in Cheddar cheese. *Aust. J. Dairy Technol.* **1991**, *46*, 6−11.

Carrasco de Mendoza, M.; Scarinci, H. E.; Garat, M. H.; Simonetta, A. C. Technological properties of enterococci in lactic starters: acidifying and lipolytic activities. *Microbiol. Aliments Nutr.* **1992**, *10*, 289−293.

Casalta, E.; Zennaro, R. Effect of specific starters on microbiological, biochemical and sensory characteristics of Venaco, a Corsican soft cheese. *Sci. Aliments* **1997**, *17*, 79−94.

Clark, W. S.; Reinbold, G. W. Enterococci in young Cheddar cheese. *J. Dairy Sci.* **1966**, *49*, 1214−1218.

Dacre, J. C. Amino acids in New Zealand Cheddar cheese, their possible contribution to flavour. *J. Sci. Food Agric.* **1953**, *4*, 604−608.

Dahlberg, A. C.; Kosikowski, F. W. The development of flavour in American Cheddar cheese made from pasteurised milk with *Streptococcus faecalis* starter. *J. Dairy Sci.* **1948**, *31*, 275−284.

Fernandez-Espla, M. D.; Fox, P. F. Effect of adding *Propionibacterium shermanii* NCDO 853 or *Lactobacillus casei* ssp. *casei* IFPL 731 on proteolysis and flavour development of Cheddar cheese. *J. Agric. Food Chem.* **1998**, *46*, 1228−1234.

Fox, P. F.; Wallace, J. M. Formation of flavour compounds in cheese. *Adv. Appl. Microbiol.* **1997**, *45*, 17−85.

Fox, P. F.; Wallace, J. M.; Morgan, S.; Lynch, C. M.; Niland, E. J.; Tobin, J. Acceleration of cheese ripening. *Antonie van Leeuwenhoek* **1996**, *70*, 271−297.

Gardiner, G.; Ross, R. P.; Collins, J. K.; Fitzgerald, G.; Stanton, C. Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. *Appl. Environ. Microbiol.* **1998**, *64*, 2192−2199.

Gardiner, G.; Stanton, C.; Lynch, P. B.; Collins, J. K.; Fitzgerald, G.; Ross, R. P. Evaluation of Cheddar cheese as a food carrier for delivery of a probiotic strain to the gastrointestinal tract. *J. Dairy Sci.* **1999**, *82*, 1379−1387.

Giraffa, G.; Carminati, D.; Neviani, E. Enterococci isolated from dairy products: a review of risks and potential technological use. *J. Food Prot.* **1997**, *6*, 732−738.

Guarner, F.; Schaafsma, G. J. Probiotics. *Int. J. Food Microbiol.* **1998**, *39*, 237−238.

IDF. Determination of the protein content of process cheese products. Standard 25, International Dairy Federation, Brussels, Belgium, 1964.

Ishibashi, N.; Shimamura, S. Bifidobacteria: research and development in Japan. *Food Technol.* **1993**, *47*, 126−135.

Jensen, J. P.; Reinbold, G. W.; Washam, C. J.; Vedamuthu, E. R. Role of enterococci in Cheddar cheese: growth of enterococci during manufacture and curing. *J. Milk Food Technol.* **1973**, *36*, 613−618.

Jensen, J. P.; Reinbold, G. W.; Washam, C. J.; Vedamuthu, E. R. Role of enterococci in Cheddar cheese: proteolytic activity and lactic acid development. *J. Milk Food Technol.* **1975a**, *38*, 3−7.

Jensen, J. P.; Reinbold, G. W.; Washam, C. J.; Vedamuthu, E. R. Role of enterococci in Cheddar cheese: free fatty acid appearance and citric acid utilisation. *J. Milk Food Technol.* **1975b**, *38*, 78−83.

Jensen, J. P.; Reinbold, G. W.; Washam, C. J.; Vedamuthu, E. R. Role of enterococci in Cheddar cheese: organoleptic considerations. *J. Milk Food Technol.* **1975c**, *38*, 142−145.

Jett, B. D.; Huycke, M. M.; Gilmore, M. S. Virulence of enterococci. *Clin. Microbiol. Rev.* **1994**, *7*, 462−478.

Joosten, H. M. L. J.; Stadhouders, J. Conditions allowing the formation of biogenic amines in cheese, 1: Decarboxylase properties of starter bacteria. *Neth. Milk Dairy J.* **1987**, *41*, 247−258.

Kosikowski, F. W.; Dahlberg, A. C. The growth and survival of *Streptococcus faecalis* in pasteurised milk American Cheddar cheese. *J. Dairy Sci.* **1948**, *31*, 285−292.

Kubickova, J.; Grosch, W. Evaluation of potent odorants of Camembert cheese by dilution and concentration techniques. *Int. Dairy J.* **1997**, *7*, 65−70.

Kuchroo, C. N.; Fox, P. F. Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft* **1982**, *37*, 331−335.

Law, B. A.; Sharpe, M. E. The influence of the microflora of Cheddar cheese on flavour development. *Dairy Ind. Int.* **1977**, *42*, 10−14.

McSweeney, P. L. H.; Walsh, E. M.; Fox, P. F.; Cogan, T. M.; Drinan, F. D.; Castelo-Gonzalez, M. A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish J. Agric. Food Res.* **1994**, *33*, 183−192.

Milo, C.; Reineccius, G. A. Identification and quantification of potent odorants in regular-fat and low-fat mild Cheddar cheese. *J. Agric. Food Chem.* **1997**, *45*, 3590−3594.

Moio, L.; Dekimpe, J.; Etievant, P. X.; Addeo, F. Comparison of the neutral volatile compounds in Mozzarella cheese made from bovine and water buffalo milk. *Ital. J. Food Sci.* **1993**, *3*, 215−225.

Moio, L.; Rillo, L.; Ledda, A.; Addeo, F. Odorous constituents of ovine milk in relationship to diet. *J. Dairy Sci.* **1996**, *79*, 1322−1331.

Mulder, H. Taste and flavour forming substances in cheese. *Neth. Milk Dairy J.* **1952**, *6*, 157−167.

Mundt, O. Enterococci. In *Bergey's Manual of Determinative Bacteriology*; Sneath, P. H. A., Ed.; The Williams & Wilkins Co: Baltimore, MD, 1989; Vol. 2.

Neviani, E.; Muchetti, G.; Contarini, G.; Carini, S. Ruolo delle enterococcaceae nei formaggi italiani. I. Loro presenza in formaggi di monte ed impiego in un innesto selezionato. *II Latte* **1982**, *7*, 722−728.

Ott, A.; Fay, L. B.; Chaintreau, A. Determination and origin of the aroma impact compounds of yogurt flavor. *J. Agric. Food Chem.* **1997**, *45*, 850−858.

Peterson, S. D.; Marshall, R. T. Non-starter lactobacilli in Cheddar cheese: a review. *J. Dairy Sci.* **1990**, *73*, 1395−1410.

Puchades, R.; Lemieux, L.; Simard, R. D. Evolution of free amino acids during the ripening of Cheddar cheese containing added lactobacilli strains. *J. Food Sci.* **1989**, *54*, 885−888.

Rogosa, M.; Mitchell, J. A.; Wiseman, R. T. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bacteriol.* **1951**, *62*, 132−133.

Stanton, C.; Gardiner, G.; Lynch, P. B.; Collins, J. K.; Fitzgerald, G.; Ross R. P. Probiotic Cheese. *Int. Dairy J.* **1998**, *8*, 491−496.

Stanton, C.; Gardiner, G.; Meehan, H.; Collins, J. K.; Fitzgerald, G.; Lynch, P. B.; Ross, R. P. Market potential for probiotics. *Am. J. Clin. Nutr.*, in press.

Terzaghi, B. E.; Sandine, W. E. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **1975**, *29*, 807−813.

Trovatelli, L. D.; Schiesser, A. Identification and significance of enterococci in hard cheese made from raw cow and sheep milk. *Milchwissenschaft* **1987**, *42*, 717−718.

Urbach, G. Contribution of lactic acid bacteria to flavour compound formation in dairy products. *Int. Dairy J.* **1995**, *5*, 877−903.

van der Kamp, J. W. Safety considerations regarding probiotic strains. Conclusions of two specialist workshops. *IDF Nutr. Newsletter* **1996**, *5*, 27−28.

Ward, D. E.; Ross, R. P.; van der Weijden, C. C.; Snoep, J. L.; Claiborne, A. Catabolism of branched-chain α-keto acids in *Enterococcus faecalis: bkd* gene cluster, enzymes and metabolic route. *J. Bacteriol.* **1999**, *181*, 5433−5442.

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