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Development of enterococci and production of tyramine during the manufacture and ripening of Cheddar cheese

M.C. Rea[1], C.M.A.P. Franz[2], W.H. Holzapfel[2] and T.M. Cogan[1]†
[1]*Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork*
[2]*Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition (BFE), Haid-und-Neu-Str 9, D-76131 Karlsruhe, Germany*

The effect of six strains of enterococci (three strains of *Enterococcus faecalis*, and one strain each of *Ec. faecium, Ec. durans* and *Ec. casseliflavus*) on flavour development and tyramine production in Cheddar cheese during manufacture and ripening was studied in two trials. No strain produced gelatinase or haemolysin and all of them grew well during manufacture reaching 10^7 colony forming units (cfu)/g in 6 h, after which they remained more or less constant during at least 48 weeks of ripening. There was no relationship between tyramine production in a broth containing tyrosine and tyramine production in the cheese. All strains, except *Ec. casseliflavus*, produced tyramine in the cheese, with the greatest concentration (162 mg/kg) being produced by *Ec. durans* after 9 months ripening at 8 °C. There was no statistically significant difference ($P > 0.05$) between the flavour of the control cheese and any cheese containing an enterococcus. Nevertheless, cheese made with *Ec. faecium* E-24 received the best score in each trial at both time points. No off-flavours were found. Regarding proteolysis, only *Ec. faecalis* E-140 showed significant ($P < 0.05$) increases in both phosphotungstic acid and pH 4.6 soluble N. It is concluded that enterococci have little effect on the flavour of Cheddar cheese.

Keywords: Cheese manufacture; cheese ripening; enterococci; tyramine production

†Corresponding author: tcogan@moorepark.teagasc.ie

Introduction

Enterococci are normal inhabitants of the gastro-intestinal tract (GIT) of many warm-blooded animals and are also commonly found in soil and on plants. Because they are common in the GIT, it is generally assumed that the enterococci found in raw

milk originate in the cows' GIT. This has been contradicted recently by Gelsomino *et al.* (2001, 2002) who conducted an extensive study of the enterococci isolated from bovine and human faeces, raw milk and cheese in a small farmhouse cheese-making operation. Relatively few enterococci were isolated from the bovine faeces, and *Enterococcus faecium* was the dominant species, while *Ec. faecalis* and *Ec. casseliflavus* dominated the milk, cheese and human faeces. Thus, bovine faeces was not the source of the enterococci, but the same strains were found in the milk, cheese and in human faeces. Some of the strains found in the milk were also isolated from the milking equipment, implying that it was the most likely source of the enterococci; however, their ultimate source was not determined.

Enterococci are opportunistic pathogens and have been implicated in several diseases including endocarditis and also biliary tract, sinus, eye, ear and peridontal tissue infections (Franz, Holzapfel and Stiles, 1999). They are the major cause of nosocomial infections in hospitals primarily because of their increasing resistance to antibiotics, particularly vancomycin. Enterococci are also very promiscuous and can transfer antibiotic resistance phenotypes, via plasmids and transposons, to more virulent pathogens including multiple-drug resistant *Staphylococcus aureus.* Consequently, their use in food has been questioned (Franz *et al.,* 1999, 2001).

Although at least 20 species of enterococci have been described, the species found most commonly in foods continue to be *Ec. faecalis, Ec. faecium* and probably *Ec. durans.* The most common enterococcus in an Irish-farmhouse, Cheddar-type cheese was *Ec. casseliflavus* (Gelsomino *et al.,* 2001). Enterococci grow poorly in milk (Sarantinopoulos *et al.,* 2001), which precludes their use as starter cultures for cheese manufacture. Instead, they must be used as adjunct cultures.

High numbers of enterococci are found in many cheeses, particularly those made in Southern Europe, but there is contradictory evidence of their effect on flavour development. Some studies (Trovatelli and Schiesser, 1987; Coppola *et al.,* 1988; Tzanetakis and Litopoulou-Tzanetaki, 1992; Macedo, Malcata and Hogg, 1995; Centeno *et al.,* 1999; Suzzi *et al.,* 2000; Sarantinopoulos, Kalantzapoulos and Tsakalidou, 2002) have shown a positive effect and others (Thompson and Marth, 1986; Lopez Diaz *et al.,* 1995) a negative one. There is limited information on the effect of enterococci on the flavour of Cheddar cheese. Jensen *et al.* (1973) studied the effect of two strains each of *Ec. faecalis* and *Ec. durans* on cheese flavour. The former had no effect but the latter had a positive effect. The enterococci were enumerated on Eugonagar at 45 °C and the starter cultures on the same medium at 30 °C. Eugonagar is a non-selective medium and it is likely that non-starter lactic acid bacteria (NSLAB), which are also present in cheese at high numbers, would also grow on the medium and perhaps confound the results. More recently, circumstantial evidence for a positive effect of a probiotic enterococcus, *Ec. faecalis* PR88, on the flavour and aroma of Cheddar cheese was shown (Gardiner *et al.,* 1999).

Enterococci have also been implicated in production of biogenic amines (BA) in cheese, particularly tyramine, through amino acid (tyrosine) decarboxylase activity (Leuschner, Kurihara and Hammes, 1999). Ingestion of food containing high concentrations of biogenic amines has been implicated in various health problems (Joosten, 1988). Tyramine can cause an increase in blood pressure and cardiac output and dilation of the eyes, lacrimation and salivation (Grind *et al.,* 1986). Biogenic amines in cheese have been reviewed by Stratton, Hutkins and Taylor (1991) and Novella-

Rodriguez *et al.* (2003).

The objective of this study was to determine the effect of several strains of enterococci, isolated from different cheeses, on flavour and BA production in Cheddar cheese.

Methods and Materials

Strains

The strains of enterococci used and their sources are shown in Table 1, and were from the FAIR-E collection which is held in the BCCM/LMG bacteria collection at the University of Gent, Belgium (http://www.belspo.be/bccm) and contains 367 strains from food, veterinary and human sources.

Virulence factors

The putative virulence factors, gelatinase production, haemolysis, aggregation substance and the enterococcal surface protein were determined as outlined by Franz *et al.* (2001).

Cheese manufacture

Cheddar cheese was made in 500-L vats from pasteurised milk using *Lactococcus lactis* 223 and 227 (Chr. Hansen Laboratory, Cork), as starter cultures, which were grown overnight in 10% (w/v) heat-treated (90 °C for 30 min) reconstituted skim milk at 21 °C. After pasteurisation, the milk was cooled to 32 °C and rennet (7.7 µl/L) and starter culture (0.75%, v/v of each strain) were then added to both experimental and control vats. Enterococcal cultures were grown overnight in MRS broth (Oxoid, UK) at 37 °C and sufficient volume added (0.2%, v/v) to the milk to give initial numbers of ~10^5 colony forming units (cfu)/ml. Control cheese was made in the same way except that the enterococcus starter strain was not added. After cutting (~ 40 min), the curd was cooked to 39 °C and was pitched when the pH reached 6.1. The curd was then piled and cheddared. Once the pH reached 5.3, the curd was milled, salted at a rate of 27 g/kg of curd, packed in moulds (~18 kg) and pressed at 400 kPa overnight. In the morning, the blocks of cheese were vacuum-packed and ripened at 8 °C for 12 months. Two trials were undertaken with each strain of enterococcus and a control in each Trial.

Sampling

Cheeses were sampled, using a trier, at 0, 2 and 6 h during manufacture, after 2 days, and at weeks 1, 2, 4, 12, 24, 36 and 48 during ripening. For chemical analyses, samples were grated and frozen at –20 °C. For microbiological analyses, duplicate samples were diluted 1:10 in sterile 2% (w/w) tri-sodium citrate, emulsified in a Stomacher (Seward, Bedford, UK) for 4 min, diluted in 0.01% peptone and plated.

Table 1. Strains of enterococci used, their sources and virulence status

Strain	Source	Presence/absence of virulence factor[1]
Ec. faecalis FAIR E-236	Smear-ripened cheese, Ireland	Gel^-; Hly^-; AS^-; Esp^-
Ec. faecalis FAIR E-279	Monte Veronese cheese, Italy	Gel^-; Hly^-; AS^+; Esp^+
Ec. faecalis FAIR E-315	Natural whey culture, Italy	Gel^-; Hly^-; AS^-; Esp^-
Ec. faecium FAIR E-24	Pharmaceutical product	Gel^-; Hly^-; AS^-; Esp^-
Ec. durans FAIR E-140	Cheddar cheese, Belgium	Gel^-; Hly^-; AS^-
Ec. casseliflavus FAIR-E 230	Cheddar-type cheese, Ireland	Gel^-; Hly^-; AS^-

[1]Gel: gelatinase; Hly: hemolysin tested on blood agar; AS: aggregation substance tested in the "clumping assay"; Esp: enterococcal surface protein; +present; –absent.

Microbiological analyses

Enterococci were enumerated on Kanamycin Aesculin Azide agar (KAA, Merck), incubated at 37 °C for 24 h; NSLAB on LBS (lactobacillus selective) agar (BBL Microbiology, USA) after incubation at 30 °C for 5 days; coliforms on Violet Red Bile Agar (Merck) after 18 h incubation at 30 °C. *Ec. durans* FAIR-E-140 grew poorly on KAA agar and therefore KF agar (Merck), followed by incubation at 37 °C for 2 days, was used to enumerate this strain.

Tyramine production in broth

The amine-forming capacity of each culture was determined by qualitative (plate screening) and quantitative (HPLC) analysis. For qualitative analysis, the strains were cultured twice in MRS-decarboxylase broth containing 1.0 g/L of the precursor amino acids tyrosine (sodium salt), histidine hydrochloride, lysine hydrochloride and ornithine hydrochloride and 0.001 g/L pyridoxal-5-phosphate. The strains were then spotted (10 µl) onto Joosten and Northolt screening medium which contained (g/L) tryptone, 5.0; yeast extract, 5.0; meat extract, 5.0; NaCl, 2.5; glucose, 0.5; Tween 80, 1.0; K_2HPO_4, 2.0; ammonium citrate, 2.0; $CaCO_3$, 0.1; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; $FeSO_4.7H_2O$, 0.04; thiamine, 0.01; pyridoxal-5-phosphate, 0.05; bromcresol purple, 0.05; agar, 22.0 and one of the amino acids mentioned above, 5.0. The pH was adjusted to 5.3 for the medium containing tyrosine, 5.0 for the medium containing histidine, 5.15 for the medium containing lysine or 5.0 for the medium containing ornithine. Medium without amino acid was used as a negative control. Decarboxylase activity was indicated by a deep blue/purple discoloration of the medium after a maximum of 4 days incubation. For quantitative measurement, cultures were grown in tyrosine-decarboxylase broth. After incubation at 37 °C for 4 days, 2 ml of culture were centrifuged (12,000 rpm/5 min), and 1 ml of supernatant was mixed with 1 ml of 0.1-molar HCl and centrifuged again (12,000 rpm/5 min). This supernatant was filtered through a 0.45 µm filter (Millipore Corp., Bedford, MA, USA) and stored at –20 ºC until analysed using the high performance liquid chromatography (HPLC) method described by Hernández-Jover *et al.* (1996).

Tyramine in cheese

Cheese samples, stored at –80 °C, were thawed at room temperature for ~2 h before analysis. Each sample was cut into five portions and 10 to 15 g (omitting the edges) were taken from the inner portions, cut into small cubes of 3 to 5 mm and mixed. From this mixture, duplicate 2 g samples were taken for analysis. Each sample was homogenised in 10 ml of demineralised water by ultrasonic treatment for 10 min at 70 °C. After cooling to room temperature, acetone (12 ml) was added and the mixture was left undisturbed for 10 min. Proteins were separated by centrifugation (3000 g for 5 min). The clear supernatant was used directly for derivatisation of the biogenic amines with 9-fluorenyloxycarbonylchlorid (FMOC) (GROM, Herrenberg, Germany) and the fluorescence of the derivatised compounds detected after separation by HPLC-gradient chromatography (Scheuer, unpublished). An aliquot (20 µl) of the supernatant and 60 µl borate buffer (GROM, Herrenberg, Germany) were transferred to a glass vial and mixed by vortexing. Then 80 µl of FMOC-solution (3.87 g/L dissolved in acetone) were added and mixed by vortexing. After 45 s, 100 µl of a solution containing 3 mg/glycine per ml of acetone/borate buffer (prepared by adding 5 ml of borate buffer to 5 ml acetone) were added to trap any free FMOC. The contents of the tube

were mixed well by vortexing for 45 s and 140 µl of a dilution buffer added and mixed followed by another addition of this buffer, 5 min later. A 10 µl aliquot of this solution was analysed on a GROM-SIL Polyamine 2, 250 × 4-mm column (GROM, Herrenberg, Germany) by HPLC. This involved two Pharmacia pumps (LKB 2150), a gradient former (LKB LCC 2252), an autoinjector (SIL 9A, Shimadzu), a column-thermostat (VDS optilab) and a fluorescence-detector (RF-551, Shimadzu). Excitation was at 263 nm and detection at 310 nm. The eluents used were 100 ml acetonitrile plus 400 ml twice-distilled water plus 3 ml acetic acid (solution A) and 475 ml acetonitrile plus 25 ml twice-distilled water plus 3 ml acetic acid (solution B); the flow rate was 1.0 ml/min and the temperature of the column was 60 °C. The sodium borate buffer (boric acid, 0.5 mol/l) was adjusted to pH 9.0 with NaOH. The stock buffer for preparation of the dilution buffer contained 50 mmol/l sodium acetate buffer adjusted to pH 4.1 using glacial acetic acid. The dilution buffer contained 30 parts stock buffer plus 70 parts acetonitrile (v/v). Recovery rates of tyramine were 73 to 78% when known concentrations were added to Cheddar cheese. Chemicals for HPLC analysis were obtained from Merck, Darmstadt, Germany.

Other chemical analyses

The pH-4.6-soluble N and phosphotungstic acid (PTA-soluble) N were measured after 4, 12, 24, 36 and 48 weeks of ripening by the procedures of Stadhouders (1960) and Kuchroo and Fox (1982), respectively.

Flavour

The flavour of the cheese was assessed, after ripening for 28 and 36 weeks, by three commercial graders and by three graders who were members of the staff at the Dairy Products Research Centre. Each grader worked independently and was asked to rank the cheese, on a scale of 1 (worst) to 7 (best), for taste and aroma after 28 and 36 weeks of ripening at 8 °C.

Statistical analyses

Enterococci counts were log transformed before computing the averages and standard deviations. The slope of the lines relating the development of pH-4.6-soluble N and PTA-soluble N with time was obtained for each cheese in each experiment. These regression coefficients were analysed using PROC GLM of SAS (2000) with a model that included effects for trial and cheese. The grading results were averaged for each cheese at each evaluation time point and the mean scores analysed using PROC GLM with a model that included trial and age in addition to cheese. Dunnett's test was used to evaluate the statistical significance of the differences between each cheese containing enterococci and the control.

Results

Strain characterisation

None of the strains used produced gelatinase or haemolysin and one of them produced aggregation substance and the enterococcal surface protein (Table 1). The presence of enterococcal surface protein (Esp) was only evaluated in *Ec. faecalis* and *Ec. faecium* strains. None of them grew well in milk; the pH of sterile (121 °C for 5 min) reconstituted (10%) skim milk, incubated at 37 °C for 6 h, did not decrease below 6.1 for any strain. The strains used were representative of abilities to produce a range of concentrations of tyramine in broth containing tyrosine (Table 2).

pH profiles

In each trial, the pH profiles were essentially the same for the first 4 h after the addition of the enterococci and the starter culture to the milk (Figure 1), reaching pH 5.5 in ~5 h.

Table 2. Concentrations of tyramine produced in decarboxylation broth (mg/L) after 4 days incubation at 37 °C and in Cheddar cheese (mg/kg) after 4 and 36 weeks of ripening at 8 °C

Organism	Broth	Cheese			
		Trial 1		Trial 2	
		4 weeks	36 weeks	4 weeks	36 weeks
E. faecalis E-279	1052	47.1	130.1	41.2	121.8
E. faecalis E-315	440	47.4	180.1	44.8	122.3
E. faecalis E-238	436	39.2	156.9	46.4	115.3
E. faecium E-24	570	37.2	72.0	12.5	74.4
E. durans E-140	66	36.2	197.0	22.3	162.2
E. casseliflavus E-230	125	nd	43.4	nd	36.6
Control		nd	73.1	nd	47.3

nd = not detected.

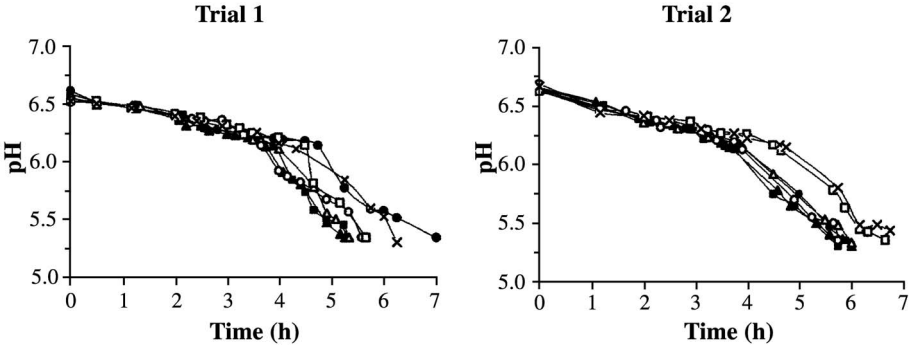

Figure 1: Development of lactic acid, measured as pH, during manufacture in each cheese in Trial 1 and Trial 2 (▲, control; ●, FAIR E-24; ■, FAIR E-140; △, FAIR E-230; □, FAIR E-236;○, FAIR E-279; ×, FAIR E-315).

Subsequently, the reduction of the pH slowed down significantly in cheese containing *Ec. faecium* FAIR-E-24 and *Ec. faecalis* FAIR-E-315 as adjuncts (Trial 1) or *Ec. faecalis* FAIR-E-236 and FAIR-E-315 (Trial 2). In these cheeses, the pH did not decrease to 5.5 for about 6 h (Figure 1).

Cheese composition

The composition of the different cheeses was determined after 2 weeks of ripening and was typical of Cheddar cheese (moisture (g/100 g) 36.5 (s.d. 1.17), 36.0 (s.d. 0.93); salt (g/100 g) 1.91 (s.d. 0.056), 2.00 (s.d. 0.64); pH, 5.34 (s.d. 0.098), 5.33 (s.d. 0.053) in Trials 1 and 2, respectively), indicating good agreement between trials.

Growth of enterococci during manufacture

Growth of the enterococci during cheese manufacture is shown in Figure 2a. The initial numbers of enterococci added to the milk ranged between 5×10^4 and 1×10^5 cfu/ml. All strains grew during manufacture reaching maximum numbers (~10^7cfu/g) 6 h after addition to the milk. There was no significant difference between Trials 1 and 2; *Ec. faecalis* FAIR-E-279 reached the highest numbers, 2.5×10^7 cfu/g, while *Ec. faecium* FAIR-E-24 reached the lowest numbers, 2.1×10^6 cfu/g. There was little or no further increase in cell numbers between 6 and 24 h

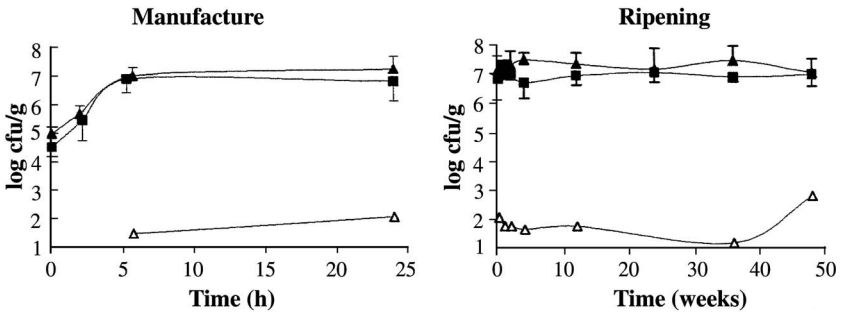

Figure 2: Growth of six enterococci during manufacture and ripening of Cheddar cheese. In each trial, the data is plotted as the average (± s.d.). No enterococci were found in the control of Trial 1 at any stage during manufacture or ripening; small numbers of enterococci were found in the control during manufacture and ripening of Trial 2 (△),(■, Trial 1; ▲, Trial

after addition of the enterococci to the milk. Consistency between strains was very good, showing less than 10% variation in growth during manufacture (data not shown). No enterococci were found at any stage during manufacture of the control cheese, to which enterococci were not added, in Trial 1, but a small number (~10^2 cfu/g) were present at 6 and 24 h in the control cheese of Trial 2.

Growth of enterococci during ripening

The growth of enterococci during ripening is shown in Figure 2b. Some variation in cell numbers of enterococci was obtained in the first few weeks of ripening after which the numbers remained more or less constant during the remaining 48 weeks. This indicates that no cell lysis was occurring or a balance was reached between viable and dead cells. There was some variation within a trial but, overall there was little difference between Trials 1 and 2, although large variations were apparent at the 22-week sampling point in Trial 2. No enterococci were found in the control cheese in Trial 1 and a low number (<100 cfu/g) were found in the control cheese for Trial 2 (Figure 2b). This number is much too low to have had an effect on flavour formation as it is generally accepted that numbers of organisms in excess of 10^6 cfu/g are needed to cause effects on flavour and other sensory properties. No coliforms were detected in 0.1 g, the minimum weight that could be tested, of any cheese during ripening.

Growth of NSLAB during ripening

The numbers of NSLAB increased rapidly in cheeses containing enterococcal adjuncts from average initial numbers of 120 to ~10^4 cfu/g and from 300 cfu/g to 10^6 cfu/g in 4 weeks in Trials 1 and 2, respectively (Figure 3). Growth in the next 20 weeks was more gradual. After 24 weeks the numbers remained more or less constant at around ~2×10^7 cfu/g. Faster growth rates were apparent in Trial 2 cheeses than in those of Trial 1; however, final cell numbers were the same in both trials. Growth of NSLAB in the experimental cheeses in Trial 2 was similar to that in the control cheese, but the growth of NSLAB in the experimental cheese in Trial 1 was much faster than in the control cheese.

Proteolysis

The levels of pH-4.6-soluble N and PTA-soluble N increased throughout ripening but the only cheese which differed significantly ($P < 0.05$) from the control was that containing *Ec. durans* FAIR-E-140 as adjunct. The data for the PTA-soluble N in both trials is shown in Figure 4. The presence of this

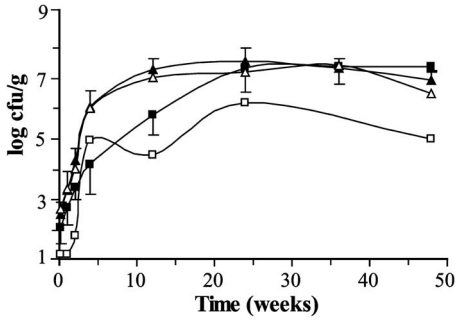

Figure 3: Development of non-starter lactic acid bacteria in Trial 1(squares) and Trial 2 (triangles) cheeses containing enterococci (closed symbols) and their controls (open symbols). (Vertical bars represent s.d.).

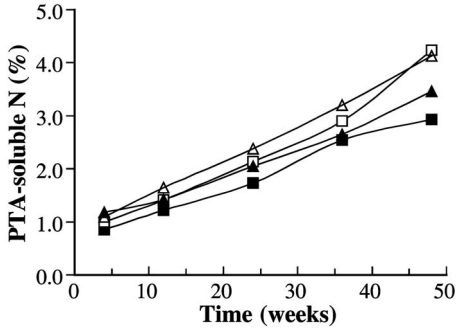

Figure 4: Development of phosphotungstic-acid (PTA)-soluble N as a percentage of total N during ripening in control cheese (closed symbols) and in cheese containing Ec. durans FAIR E-140 (open symbols) in cheeses

strain in the cheese during ripening resulted in increased proteolysis over the control and the increase was numerically greater in cheese from Trial 2 than that of Trial 1.

Flavour evaluation

There was no statistically significant difference between the flavour of the control cheese and any of the cheeses to which enterococci were added. Nevertheless, cheese made with *Ec. faecium* FAIR-E-24 received the best score in each trial at both time points. No off-flavours were reported in any cheese, except that two of the six graders found that cheese made with *Ec. faecalis* FAIR-E-236, E-279 and E-315 had developed slight off-flavour in Trial 1 cheese after 36 weeks of ripening.

Tyramine production

None of the strains produced the corresponding BA from histidine, lysine or ornithine in the qualitative method. The concentrations of tyramine produced in the broth after 4 days incubation and in the cheese after 4 and 36 weeks of ripening are shown in Table 2. The values are the averages of independent duplicates and the standard deviation of differences between duplicates was 7.28 mg/kg. There was no relationship between the amounts produced in broth and in the cheese especially after 1 month of ripening, e.g., the concentrations in broth ranged from 66 to 1052 mg/L while in cheese they ranged from 36 to 47 mg/g. After 36 weeks of ripening the highest concentration of tyramine in both trials was in cheese containing *Ec. durans* FAIR-E-140.

Discussion

The decrease in pH is an excellent indicator of lactic acid production by the starter during cheese manufacture. During Cheddar cheese manufacture, the pH of the curd should reach 5.5 within 5 h of addition of starter. Generally this occurred in the present study indicating that acid production by the starter was not affected by the presence of the enterococci. However, *Ec. faecalis* FAIR-E-315 retarded the growth of the starter in both trials while *Ec. faecium* FAIR-E-24 and *Ec. casseliflavus* FAIR-E-236 retarded acid production in one trial but not in the other. The source of this variation was not determined but the results suggest that some of the enterococci produced compounds, e.g., bacteriocins, which inhibited the starter culture. This aspect was not

investigated further.

The enterococci grew well during cheese manufacture from initial numbers of ~10^5 cfu/ml to 10^7 cfu/ml after 6 h. In interpreting this growth, it should be remembered that curd gradually loses moisture in the first hours of cheese making which will result in concentration of the organisms by physical entrapment (and apparent growth of the organisms). Small numbers of enterococci were found late in manufacture in the control of Trial 2. This may have been due to the use of poorly sterilised knives to cut the curd during manufacture. These low numbers persisted but did not grow during ripening and are unlikely to have affected the development of flavour or tyramine in the cheese.

Except for *Ec. durans* FAIR-E 140, the enterococci had no effect on gross proteolysis, as indicated by the increase in pH-4.6-soluble N levels, or on peptidolysis, as indicated by the increase in PTA-soluble N. This strain increased both of these parameters but did not affect flavour. It is now realised that the major flavour compounds in cheese are produced from amino acid breakdown (Yvon and Rijnen, 2001) and therefore these results imply that enterococci do not have any greater ability than starter lactococci or NSLAB to increase amino acid breakdown in cheese.

Lysis of starter cultures is considered to be very important in developing flavour production in cheese as numerous enzymes are released which help in production of the flavour compounds (Boutrou *et al.,* 1998; Lepeuple *et al.,* 1998; Meijer, Dobbelaar and Hugenholtz, 1998). Numbers of enterococci remained more or less constant during cheese ripening, implying that little lysis occurred. Despite this, *Ec. faecium* FAIR-E 24 consistently improved the flavour of the cheese in both trials at both time points, even though the effect was not statistically significant. This strain deserves to be evaluated more thoroughly.

Enterococci are found in many cheeses, particularly those made around the Mediterranean basin, and most studies suggest they are involved in flavour improvement (Trovatelli and Schiesser, 1987; Coppola *et al.,* 1988; Tzanetakis and Litopoulou-Tzanetaki, 1992; Macedo *et al.,* 1995; Centano *et al.,* 1999; Suzzi *et al.,* 2000; Sarantinopoulos *et al.,* 2002). No statistically significant effect of the presence of enterococci on flavour improvement was found in the present study. These results contrast with those of Jensen *et al.* (1973), who found that Cheddar cheese made with *Ec. faecalis* was either comparable to or less desirable than the control cheese while those made with *Ec. durans* were all more desirable. This may be due to strain differences, yet, in the present study, cheese containing *Ec. faecium* FAIR-E 24 was found to have a better flavour by most graders.

NSLAB, which mainly comprise *Lb. casei, Lb. paracasei, Lb. plantarum* and *Lb. curvatus* in Cheddar cheese, grow from low numbers of ~10^2 cfu/g at the beginning of Cheddar cheese ripening to high numbers within a few months (Fox, McSweeney and Lynch, 1998; Fitzsimons *et al.,* 1999). The present results agree with these but there were significant differences in the rates of growth in Trials 1 and 2 where the NSLAB reached maximum numbers in 25 and 12 weeks, respectively. In addition, the growth of NSLAB in the control cheese in Trial 1 was much lower than in the experimental cheeses. The reasons for these results are not apparent. Such high numbers of NSLAB would grow on the non-selective media used in previous studies to enumerate enterococci in Cheddar cheese (Jensen *et al.,* 1973) and confound the results. NSLAB are generally considered to be adventitious contaminants although there is some evidence that they withstand pasteurisation (Jordan and Cogan, 1999).

There was no relationship between the ability of the strains to produce tyramine in broth and in cheese but the reasons are not clear. Differences were found in the tyra-

mine concentrations in each cheese (Table 2) but the concentrations produced in cheese containing the same strain were consistent. A considerable increase occurred between weeks 4 and 36. At 4 weeks, the cheeses contained low numbers of NSLAB (Figure 3) and high numbers of enterococci (Figure 2b). The major NSLAB in Cheddar cheese are homofermentative lactobacilli, *Lb. casei, Lb. paracasei, Lb. plantarum* and *Lb. curvatus* (Fox *et al.,* 1998; Fitzsimons *et al.,* 1999). There is no information about production of BAs by *Lb. casei, Lb. paracasei* or *Lb. curvatus* in cheese and one out of two strains of *Lb. plantarum* produced putrescine from arginine in a broth system (Arena and Manca de Nadra, 2001). *Lb. brevis*, an obligate heterofermenter, which is occasionally present in cheese, produced tyramine but not histamine in a broth system (Maijala, 1993). Other obligate heterofermenters, *Lb. buchneri* LBS-1 and LBS-3, were isolated from a Swiss cheese involved in an outbreak of histamine poisoning and produced large amounts of histamine (Sumner *et al.,* 1985) while histamine accumulated rapidly in a Gouda cheese made with *Lb. buchneri* St2A (Joosten and Northolt, 1989).

In the present study, no tyramine was produced in the control cheeses at 4 weeks in either trial and little at 36 weeks, implying that the NSLAB produced only small amounts of tyramine. Approximately the same amounts of tyramine were also produced by *Ec. casseliflavus* E-230, indicating that this strain also produced little tyramine in cheese. Therefore, it is concluded that the relatively high concentration in some of the experimental cheeses was due to growth of the other five strains of enterococci. Biogenic amines, particularly tyramine, have been implicated as the causes of food-induced migraines and hypertension (Beutling, 1996; Hálasz *et al.,* 1994). Higher concentrations of tyramine than histamine are almost invariably found in cheese and in Cheddar cheese the concentrations range from 90 to 270 mg/kg of cheese (Voigt and Eitenmiller, 1978). In the present study the concentrations of tyramine found ranged from 37 to 197 mg/kg after 9 months ripening and compare favourably with these concentrations. Leuschner *et al.* (1999) found 477 mg/kg of tyramine and no histamine in a Gouda-type cheese, containing two strains of *Ec. faecalis*, after 12 weeks ripening at 14 °C. There is little information on the concentrations of tyramine needed to cause a response in humans except for the study of Grind *et al.* (1986) who found that in 12 healthy people, 3 mmol (412 mg) needed to be ingested before a significant increase in systolic blood pressure was evident. Such a concentration would require ingestion of 3 to 11 kg of the cheeses in the present study, implying that the levels of tyramine determined in the cheese in this study would result in no pharmacological reactions. However, Novella-Rodriguez *et al.* (2003) state that 6 mg is the maximum tolerable dietery intake of tyramine for patients receiving non-selective monoamine oxidase inhibitor drugs to avoid headache or hypertensive crises; such a concentration would be found in a typical serving (30 g) of the cheese showing the highest concentration of tyramine in the present study.

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