Enterococci in Food Fermentations: Functional and Safety Aspects

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Summary and Conclusions

Enterococci are natural residents of the human and animal gastrointestinal tracts; many species are also found in soil, plants and food. These organisms also form an important part of the microflora of many cheeses, especially those made in Southern Europe, where they can reach levels of 107 - 108 cfu/g. There is contradictory information on their role in flavour development in cheese with some studies showing that they have a positive effect and others a negative one. *Enterococcus faecalis*, *Ec. faecium* and *Ec. durans* are the important species found in cheese, though recent results from our laboratory show that *Ec. casseliflavus* may also be important (see below). Many of these species withstand pasteurisation. Their presence in food has been questioned because they are responsible for many nosocomial infections in hospitals. They are also promiscuous and easily transfer antibiotic resistance to other organisms and acquire resistance to vancomycin themselves.

Cheddar cheese has a complex microflora and is conducive to growth of many bacteria, especially lactic acid bacteria. Enterococci are facultative anaerobes, which ferment lactose and can grow in high salt concentrations. Therefore, they should grow in cheese if they are present in the raw milk. Phenotypically they can be confused with starter lactococci. Traditionally, they are separated from lactococci by their ability to grow at 45°C and in 6.5% salt. However, these tests have serious drawbacks since some species of enterococci cannot grow at 45°C and some lactococci can grow at 45°C and in 6.5% salt. The effect of enterococci on flavour development in Cheddar cheese has not been studied to any great extent.

The overall objectives of this collaborative project were to investigate the taxonomic relationships between food, veterinary and clinical isolates of enterococci, their virulence, their ability to produce toxins, their antibiotic resistance and their technological performance in cheesemaking.

The specific objectives of the Moorepark team were to study the co-metabolism of citrate and sugar by enterococci, develop a DNA probe to distinguish between *Enterococcus* and *Lactococcus* and evaluate the contribution of enterococci to flavour development in Cheddar cheese.

Main Conclusions and Achievements

- A total of 405 strains of enterococci, collected from different sources (food, human and veterinary) and from different geographical regions of Europe, were identified by classical and molecular techniques. The majority was either Ec. faecalis (170 strains) or *Ec. faecium* (154 strains). The next most common species was *Ec. durans* (31 strains).

(A catalogue of these strains, called the FAIR-E collection, is available from BCCM™/ LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium).

- A genus specific probe was developed which discriminates between *Enterococcus* and *Lactococcus* species.
- Ec. faecalis FAIR E-236, FAIR E-279 and FAIR E-315, *Ec. faecium* FAIR E-24 and FAIR E-243 (common strain), *Ec. casseliflavus* FAIR E-230 and *Ec. durans* FAIR E-140, were used to make cheese.
- Initial levels of enterococci in the milk were ~ 105 cfu/ml and growth occurred during manufacture to 106 cfu/g of cheese at pressing. There was no significant decrease in numbers during ripening. There was no difference in pH 4.6 soluble N, which is a measure of rennet activity, in the cheesesduring ripening, but there was a significant effect on the PTA soluble N, which essentially is a measure of starter peptidase activity.
- Cheese made with Ec. faecalis FAIR E-24 scored best in each of the 2 trials while *Ec. casseliflavus* FAIR E-230 scored better than the control (without enterococci) in each of the two trials at 7 months but at 11 months the effect was much less marked.
- Cheese was also made withbacteriocin-producing *Ec. faecium* FAIR E-171 and bacteriocin production was detected in the cheese, which inhibited non-starter lactic acid bacteria (NSLAB). Bacteriocins are proteins produced by some micro-organisms which have the ability to inhibit other organisms, including pathogens.
- Small amounts of biogenic amines were produced by all strains.
- *Ec. faecalis* FAIR E-239 metabolised citrate and pyruvate but the presence of a fermentable carbohydrate at levels of 10mM prevented metabolism of citrate but not pyruvate. Glucose also prevented citrate metabolism in *Ec. faecalis* FAIR E-237, FAIR E-259 and NCDO 610 and in *Ec. faecium* FAIR E-338 and FAIR E-371.
- Formate and acetate were the major products of citrate metabolism. No acetoin was detected.
- There was a curvilinear relationship between pyruvate and glucose utilisation.
- In a mixture of citrate and pyruvate, pyruvate utilisation was slowed down until all the citrate was used.
- Addition of glucose to cells of *Ec-faecalis* FAIR E-239 growing on citrate resulted in immediate metabolism of glucose but the rate of metabolism of citrate did not slow down for ~ 45 minutes after the addition.

Research and Results

Genotypic Separation of Lactococcus and Enterococcus

Two sets of genus specific primers were developed: one set, the L series, was specific for *Lactococcus* species and the other, the E series, for *Enterococcus* species. On PCR the lactococcal primers gave a 570 base pair fragment with the type strains of all lactococci and no PCR products with the type strains of the 18 enterococcal species tested or with other LAB including *Streptococcus thermophilus*, *Str. salivarius*, *Lactobacillus casei*, *Lb. helveticus*, *Pediococcus acidilactici*, *Ped. pentosaceus* or *Oenococcus oenos*. The enterococcal primers gave a 733 base pair fragment with the 18 type strains of enterococci tested and did not react with the type strains of lactococci or the other species named above.

Cheddar Cheese Manufacture

The following strains of enterococci were used individually as adjunct cultures, *Ec. durans* FAIR E-240, Ec. faecalis FAIR E-236, FAIR E-279 and FAIR E-315, *Ec. faecium* FAIR E-24 and FAIR E-243 and *Ec. casseliflavus* FAIR E-230. They were grown overnight in MRS at 37°C and sufficient culture added to the milk to give initial levels of ~ 105 cfu/ml of milk. The starters used were *Lc. lactis* 223 and 227 (Chr. Hansen Laboratory). The cooking temperature was 39°C, the whey was drained at pH 6.1 and the cheese was pressed overnight at 400 kPa, when the pH decreased to 5.35. Two independent trials were undertaken.

The development of enterococci and NSLAB were determined on KAA and LBS agars after incubation at 37°C for 24 h and 30°C for 5 d, respectively. pH 4.6 sol N and PTA sol N were measured by the procedures of Reiter et al (1969) and Kuchroo and Fox (1982) respectively. The flavour was measured independently by 6 experienced graders after 7 and 11 months of ripening. Each grader was asked to rank the cheeses independently.

Acid production in some of the cheeses, particularly those containing *Ec. faecalis* FAIR E-236 and FAIR E-315, was slower than in the control. This is likely to be due to bacteriocin production by the enterococci, which inhibited the starters. The increase in time added ~ 1 h to the production of the cheese.

In Trial 1, no enterococci were found in the milk or cheese of the control while in Trial 2 low numbers (< 102 cfu/g) were found. The initial levels of enterococci added to the milk were ~ 105 cfu/ml in the experimental vats. All strains grew during manufacture to between 107 and 108 cfu/g in the curd at milling (6 h) (Fig. 1). In interpreting this data one should remember that there is a 10-fold concentration factor between milk and finished cheese.

Enterococci did not grow during ripening and the numbers remained more or less constant throughout the 11 months of ripening, indicating that little lysis was occurring (Fig. 1). As expected, the numbers of NSLAB increased from ~ 102 cfu/g at the beginning of ripening to ~ 108 cfu/g within 8 - 10 weeks. The rate of growth of NSLAB was greater in Trial 2 than in Trial 1. In Trial 2, there was a low level of enterococci (< 100 cfu/g) in the control cheese throughout ripening, whereas in Trial 1 no enterococci were found in the control cheese at any time during ripening.

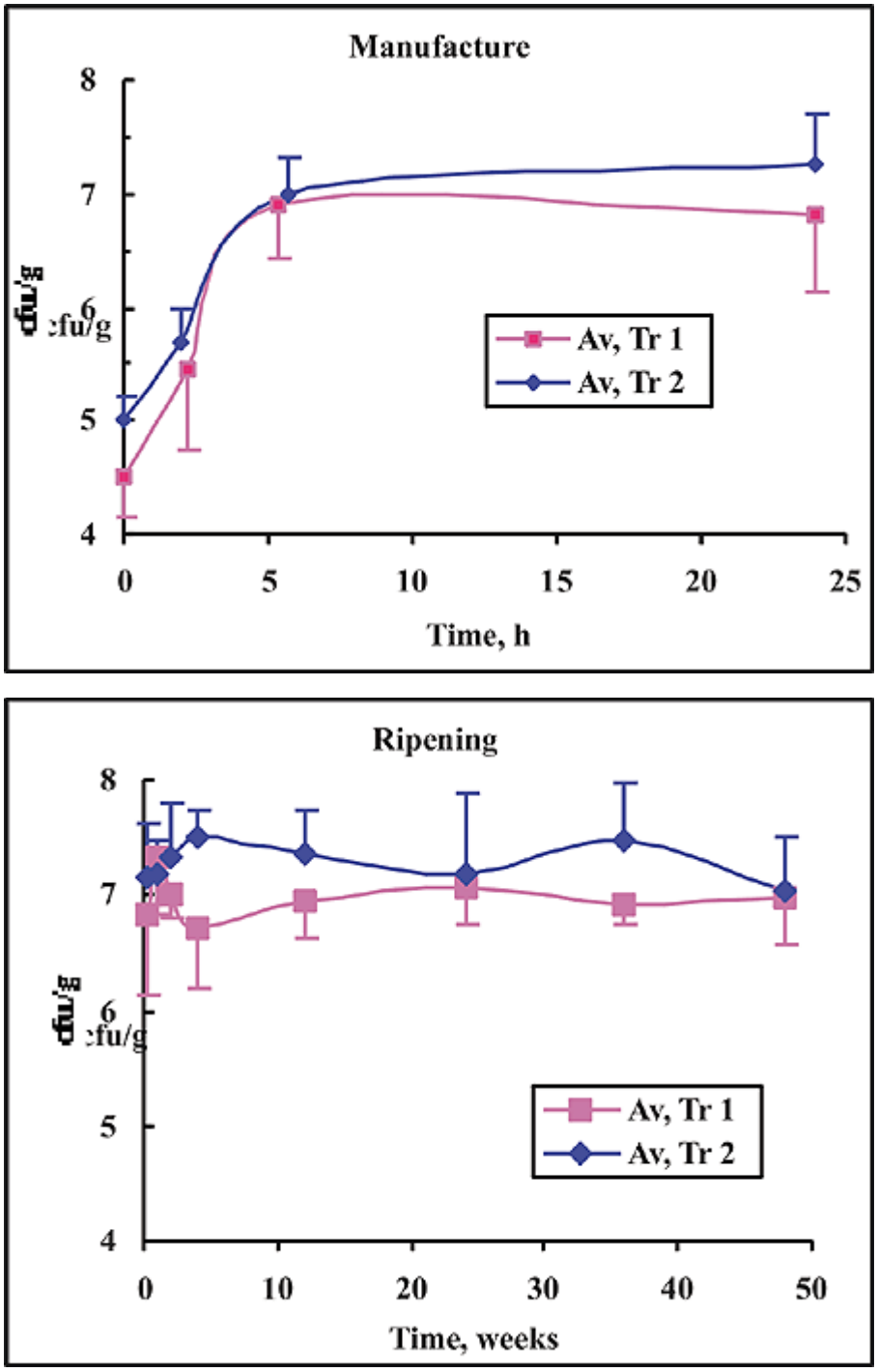

Figure 1. Growth of several enterococci in Cheddar cheese during manufacture and ripening. The data for each trial is plotted as the average ± standard deviation.

The composition of the cheeses was satisfactory, except that the pH of the control in Trial 1 was somewhat low (pH 5.14). It is unlikely that this difference would make a major difference to the results.

The production of pH 4.6 sol N, which is mainly a measure of chymosin activity, was not influenced by the enterococcus. However, the production of PTA sol N, which is a measure of peptidase activity of the starter and non-starter bacteria, increased during ripening, especially in cheese containing *Ec. faecium* FAIR E-243 and *Ec. durans* FAIR E-140 as adjuncts in both trials (Fig. 2).

Small amounts (< 200 mg/kg) of tyramine were produced in all cheeses including the control and the levels increased during ripening.

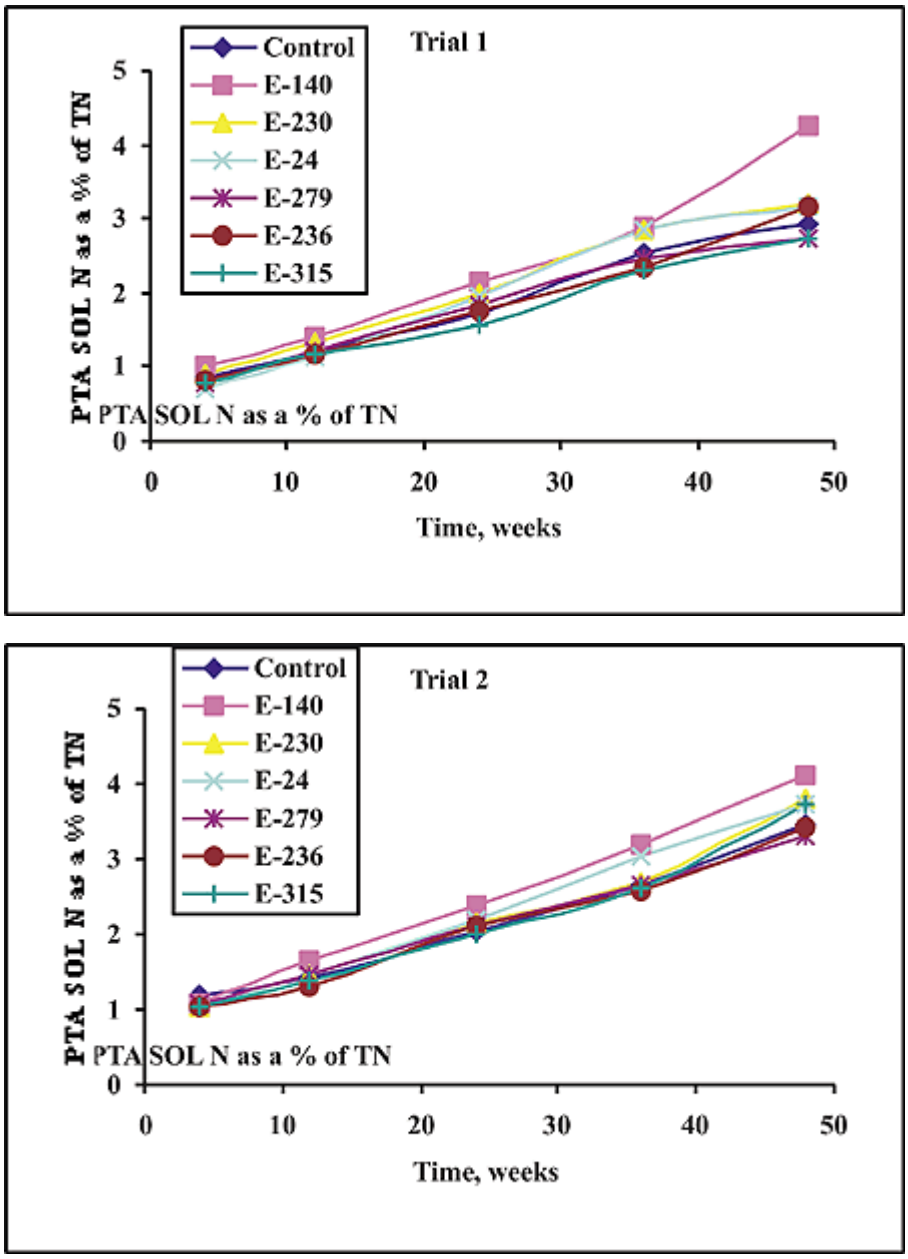

Figure 2. Development of PTA soluble Nitrogen as a percentage of Total nitrogen during ripening in each trial.

Regarding flavour, cheese made with *Ec. faecium* FAIR E-24 and *Ec. casseliflavus* FAIR E-230 scored better than the control at 7 months in both trials, but this was not statistically significant; at 11 months the effect was much less marked. This may be due to a more dominant effect of the NSLAB late in ripening.

Cheddar cheese was also made with the bacteriocin producing strain (Bac+), *Ec. faecium* FAIR E-171. The bacteriocin producer had no effect on starter growth since the pH decreased as rapidly in the presence of the Bac+ strain as in the control. Like the Bac- strains above, the Bac+ strain also multiplied during manufacture to between 107 and 108 cfu/ml and remained at these values throughout ripening. The effect on NSLAB was inconsistent; in Trial 1, the Bac+ strain caused a significant reduction in the number of NSLAB but in Trial 2 there was no effect since NSLAB did not develop in the control cheese. This result was surprising and we have no explanation for it.

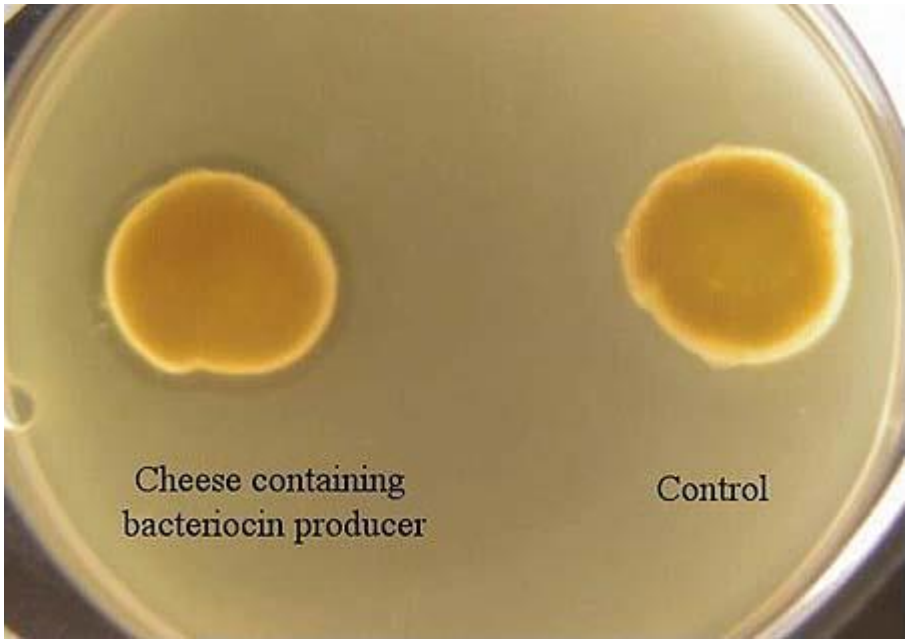

Figure 3. Photograph of a sample of cheese made with the bacteriocin producing Enterococcus as an adjunct starter showing the inhibition of Listeria innocua. The control cheese showed no evidence of inhibition (See PowerPoint file, Bac Photo)

It proved very difficult to estimate the amount of bacteriocin produced by any of the extraction techniques used (trisodium citrate, phosphate or EDTA). The only way it was detected was by placing the cheese directly on the indicator strain of Listeria innocua (Fig. 3). Under these circumstances, the control cheese showed no zone of inhibition.

Citrate and Pyruvate Metabolism by . faecalis FAIR E-239

MRS from which acetate, Tween 80, citrate and glucose were omitted, was used as the Basal Medium (BM). Separately sterilised solutions of glucose and citrate were added to BM as necessary. Spent medium (SM) was prepared by growing Ec. faecilis FAIR E-239 in BM, centrifuging it and filter-sterilising the supernatant.

Ec. faecalis FAIR E-239 grew in BM without citrate or glucose indicating that the medium contained fermentable carbohydrate, which was mainly associated with the yeast-extract component of the medium. The strain also metabolised citrate added to the BM but citrate metabolism was inhibited in a mixture of glucose and citrate until all the glucose was metabolised. As low as 10mM glucose was sufficient to prevent utilisation of citrate. Other metabolisable sugars e.g. fructose also prevented citrate utilisation and non-metabolisable ones e.g. lactose did not. Sucrose was metabolised slowly and resulted in slow utilisation of citrate. Glucose also prevented citrate metabolism in Ec. faecalis FAIR E-235, E-259 and NCDO 610 and in *Ec. faecium* FAIR E-338 and E-371. Ec. faecalis FAIR E-239 does not ferment lactose while Ec. faecalis NCDO 610 does; lactose also prevented citrate utilisation by Ec. faecalis NCDO 610.

Glucose did not prevent Ec. faecalis FAIR-239 from metabolising pyruvate; both substrates were co-metabolised but the relationship between them was curvilinear with greater amounts of pyruvate used as the glucose concentration decreased.

When glucose was added to cells growing activity on glucose, it was immediately catabolised. Citrate catabolism continued at the same rate for about 90 min after addition of glucose before it slowed down significantly.

When Ec. faecilis FAIR E-239 was grown on a mixture of pyruvate and citrate the rate of pyruvate utilisation slowed down until all the citrate was used after which it increased.

The major products of pyruvate and citrate metabolism were acetate and formate, indicating that the enzyme involved was pyruvate-formate lyase. These products were also produced in small amounts from glucose but the dominant product was lactate. No acetoin was detected from any substrate indicating that diacetyl was also not produced.

These results show that citrate utilisation is quite different in enterococci than in lactobacilli, lactococci or leuconostocs. In enterococci metabolisable sugars exert some type of catabolite repression of citrate but not pyruvate whereas the other organisms co-metabolise sugars and citrate.

Source of Enterococci in Cheese

The source of enterococci in milk and cheese was also studied. Putative enterococci were isolated from the faeces of all the cows and personnel associated with manufacturing of a farmhouse cheese in Ireland, from the milk and cheese and from the environment. Three trials were undertaken and almost 1400 strains were isolated.

All the isolates from the human faeces milk and cheese and only 33.7%, 6.7% and 4.4% of the bovine isolates from the three trials were enterococci when tested by the *Enterococcus* primer in a RAPD-PCR reaction. The others were mainly Streptococcus bovis.

In general, only *Ec. faecium* was found in bovine faeces, while *Ec. casseliflavus* dominated the human faeces, milk and cheese, followed by large numbers of Ec. faecalis. These results were corroborated by the environmental samples, which showed that *Ec. casseliflavus* and Ec. faecalis were present in the milking equipment, indicating that it was the likely source of the enterococci.

Pulsed Field Gel Electrophoresis (PFGE) showed that three clones, two of *Ec. casseliflavus* and one of Ec. faecalis dominated almost all the milk, cheese and human faeces samples.

A feeding trial involving 3 people demonstrated that one clone of Ec. faecalis, which was present in relatively low numbers in the cheese, was found in the faeces of the three subjects during the period in which cheese was consumed. In addition, 2 clones of *Ec. casseliflavus* from the cheese were found in the faeces of one of the subjects. These clones were not present in any subject before feeding or after cheese consumption had ceased, implying that the *Ec. casseliflavus* and the Ec. faecalis strains found in the human faeces were the result of eating the cheese.

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