Origin and safety aspects of enterococci in Baylough, an Irish Cheddar-like cheese

Roberto Gelsomino

Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Sciences
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Roberto Gelsomino

Promoter: Prof. Dr. ir Jean Swings
Co. Promoter: Prof. Dr. Tim Cogan
Floris Claesz van DIJCK
(*1575, Haarlem; †1651, Haarlem)
Still-life
1613
Oil on wood
Frans Halsmuseum, Haarlem, The Netherlands

PhD thesis, Faculty of Sciences, Ghent University, Ghent, Belgium
Defence in Ghent, May 18\textsuperscript{th} 2004
Examination Commission

Prof. Dr. Jos Van Beeumen (acting chairman)
Faculty of Sciences, University of Ghent

Prof. Dr. ir. Jean Swings (promoter)
Faculty of Sciences, University of Ghent

Prof. Dr. Timothy M. Cogan (co-promoter)
Visiting Professor in Microbiology, UCC and Senior Principal Research Officer, Dairy Products Research Centre, Teagasc, Fermoy, Ireland

Dr. Luc A. Devriese
Faculty of Veterinary Medicine, University of Ghent, Merelbeke

Dr. Marc Heyndrickx
Centre for Agricultural Research, Ghent

Dr. Bruno Pot
Bacteriology of Ecosystems, Institut Pasteur de Lille

Dr. Marc Vancanneyt
Faculty of Sciences, University of Ghent

Prof. Dr. Peter Vandamme
Faculty of Sciences, University of Ghent

Prof. Dr. Mario Vaneechoutte
Department of Clinical Chemistry, Microbiology & Immunology, University Hospital Ghent
**DIALOGUE**

*between Madame J. L. & her children*

**Children**

Pray dearest mother if you please  
Cut up your double-curded cheese,  
The oldest of the brotherhood.  
It’s ripe, no doubt and nicely good!  
Your reputation will rise treble  
As we the luscious morsel nibble.  
Praise will flow from each partaker  
Both on the morsel and the maker!

**Madame**

Your suit is vain, -upon my word,  
You taste not yet my double-curd;  
I know the hour, -the very minute  
In which I’ll plunge my cutteau in it;  
Am I to learn of witless bairns  
How I must manage my concerns?  
As yet the fervid dog-star reigns  
And gloomy Virgo holds the reigns.  
Be quiet chicks, sedate and sober  
And house your stomachs till October;  
Then for a feast! Upon my word,  
I’ll really cut my double curd.

**Major Henry Livingstone Jr.**

(1748-1828)
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List of abbreviations

ARE Antibiotic resistant enterococci
BCCM Belgian Co-ordinated Collections of Microorganism
cfu Colony forming units
DSS Defined-strain starter
FAA Free amino acids
FAO Food and agriculture organization of the United Nations
FDM Fat in dry matter
FFA Free fatty acids
FHL Facultative heterofermentative lactobacilli
GIT Gastrointestinal tract
GRAS Generally recognized as safe
HAI Hospital acquired infections
HLR High level resistance
LAB Lactic acid bacteria
*Lb.* *Lactobacillus*
*Lc.* *Lactococcus*
LLR Low level resistance
LMG Laboratory of Microbiology Ghent
MSS Mixed-strain starter
MNFS Moisture-in-the-non-fat solids
NSLAB Non-starter lactic acid bacteria
PAB Propionic acid bacteria
PCR Polymerase chain reaction
PFGE Pulsed-field gel electrophoresis
SDS-PAGE Sodium dodecyl sulphate poly acrylamide gel electrophoresis
S/M Salt in moisture
sp./spp. species (sing./pl.)
ssp. subspecies
*Sta.* *Staphylococcus*
*Str.* *Streptococcus*
VRE Vancomycin resistant enterococci
Part I

Literature review
CHAPTER 1.

GENERAL INTRODUCTION ABOUT CHEESE

The production of cheese from milk is an ancient process, as is shown by prehistoric findings. The oldest of these findings about cheesemaking is the so-called “dairy frieze” from the third millennium BC (Fig. 1). This frieze shows Sumeric priests (the ancient cheese experts) in the various stages of cheese production (http://www.formaggio.it/storia1.htm). Also in the Bible in the book of Samuel (which is believed to have been written around 1100 BC) we read under Samuel 17,29: “And honey, and butter, and sheep, and cheese of kine, for David, and for the people that [were] with him, to eat”. Throughout centuries testimonies over cheesemaking can be found from the Romans to the Middle Ages. Also Homer (~800 BC), in book 9 of his “Ulysses”, describes Polyphem, a one-eyed giant who makes cheese from the milk he gets from his sheep and goats: “…while he into a cave > Of huge receipt his high-fed cattle drave, >All that he milk'd; ...> ...could not stir it there. > Thus making sure, he kneel'd and milk'd his ewes, > And braying goats, with all a milker's dues; > Then let in all their young. Then quick did dress > His half milk up for cheese, and in a press > Of wicker press'd it…” This is probably the first written recipe of cheesemaking (http://www.bartleby.com/111/chapman22.html, http://www.formaggio.it/storia1.htm;).

It is generally believed that cheese must have resulted from the simple observation that milk left in a ruminant’s stomach ends up by coagulating. This might be considered the first technological cheesemaking discovery (Johnson, 1989; http://www.formaggio.it/storia1.htm). Cheesemaking then developed from the need to preserve the constituents of milk.
Figure 1. One of the oldest findings about cheesemaking is the Sumeric frieze (above a detail), which has been dated to the 3rd millennium BC. Below the one-eyed giant polyphem threatening Ulysses and his friends (Annibale Carraci, 1595-1605, Palazzo Farnese, Roma)

1.1. Cheesemaking

There is no such thing as a standard cheesemaking recipe as every cheese varies in one or other manufacturing step. The only common step in all cheesemaking processes is the transformation of a liquid into a solid, or, defined differently, “a dehydration process in which the fat and casein in milk are
concentrated between 6- and 12-fold, depending on the variety” (Fox, 1993). There are, nonetheless, basic steps common to a lot of cheese varieties:

**Acidification.** Until the end of the 19th century the indigenous microflora of milk was responsible for acidification. Due to unpredictable acid production rates starter cultures were then introduced. Basically this was milk, which had been incubated until it coagulated. It was then used as starter and if it produced good quality cheese, it was sub-cultured. These starters were originally available as liquid cultures but they were subsequently made available as vacuum-dried cultures, then freeze-dried and more recently as either frozen or freeze-dried, concentrated cultures, which were suitable for direct inoculation of milk in the cheese vat. It is now the practice to add a starter culture of selected lactic acid-producing bacteria to achieve a uniform acid production. These are called “starter cultures” and will be discussed in detail later (section 2.1) (Cogan, 1996; Stanley, 2003).

**Pasteurisation:** The milk is generally pasteurised at 72°C for 15 sec for cheese making, but about 10% of all the cheese made in Europe is made from raw milk. Pasteurisation inactivates all pathogens, which might be present in the raw milk (*Mycobacterium tuberculosis, Brucella abortus, Staphylococcus aureus, Listeria monocytogenes*) and significantly reduces other adventitious bacteria present. To-day pasteurised milk is virtually sterile, and counts of $<5\times10^3$ cfu/ml are not uncommon (Manners and Craven, 2003a; Manners and Craven, 2003b)

**Coagulation.** This is the essential characteristic step in the manufacture of all cheese varieties and is achieved by

(i) addition of rennin (chymosin) or a mixture of pepsin and chymosin proteinases. Chymosin is an extract from the bovine abomasum, and is added to the milk, which is normally pre-warmed to ~30°C, after the starter culture. The curd which is formed is then cut with knives into small particles, the size of which depends on the cheese being made.

(ii) by starter acidification to pH ~4.6 and

(iii) a combination of 1 and 2 and heat (Fox, 1993; Hill, 2000).
Dehydration. Once the rennet gel is cut or broken, syneresis, i.e. contraction of the curd and expulsion of the whey, occurs rapidly. Acidification by starter cultures through lactose fermentation results in a decrease in pH, which helps to expel whey from the curd. This, in turn, reduces the moisture level and aids in preservation of the milk as cheese (Cogan, 1996). Cooking, also referred to as “scalding” (although boiling temperatures are never reached), sometimes occurs and further helps expulsion of the whey. The cooking temperature varies between 35°C in the case of soft cheeses to 55°C in the case of some hard Italian cheeses. The rate and extent of syneresis are influenced by pH of the whey, cooking temperature and rate and time of stirring. The whey is then drained, i.e., the whey and curd are separated from each other, and the curd is salted. This type of heating must be distinguished from that given to the so called “pasta filata” (or plastic curd) cheeses like Mozzarella, where the curd is heated with hot water (up to 67°C) to plasticize it and produce a more fibrous cheese (http://www.caseificioocrovino.com/schedamoz.htm). Most cheeses are then brined in 20-23% (saturated) NaCl once they are removed from the moulds. Cheddar cheese is an exception. Its curd is cut into small particles before it is moulded and it is then dry salted, moulded and pressed overnight. Brining can last for a few hours (small cheeses) to several days (large cheeses). Salt plays an important role in cheesemaking. It slows down acidification by inhibiting the starter and decreases enzymatic activity. If the curd is brined it is usually placed in moulds first and pressed; however, dry salting can occur before moulding (Cheddar) or after moulding (Gruyère) (Cogan, 1996; Banks and Horne, 2003).

Ripening. High moisture cheeses are ripened at relatively high temperatures of ~15°C for short periods, perhaps 4-8 weeks, while low moisture cheeses like Cheddar are ripened at 7-9°C for 6-12 months. During this time extremely complex biochemical changes occur through the action of (i) coagulant, (ii) indigenous milk enzymes, (iii) starter bacteria and their enzymes and (iv) the secondary microflora and their enzymes. It is due to the action of these four factors that every cheese develops its unique characteristics (Hill, 2000; Farkye, 2003). The major biochemical changes during cheese ripening are discussed in the following section.

Most if not all cheesemaking steps change according to the type of cheese made. Other factors influence the final product including the use of raw or
pasteurised milk, the use of different starter cultures and the adjustment of milk components such as milk fat or calcium content. In addition, the duration and temperature of cooking, the amount of whey drained, the duration of moulding, the amount of salt added and the duration and temperature of ripening are some of the factors that change the water activity, pH and salt content, which, in turn, influence the moisture content, acidity, firmness and texture of the cheese. The effect of these factors may not be great individually but the interaction of all of them leads to the diversity of cheese, which now exists in hundreds of different varieties (Johnson, 1998; Fox, 1993; Olson, 2003).

1.2. Biochemical pathways during cheese ripening

The extremely complex set of biochemical changes which occur during ripening include lipolysis, proteolysis, glycolysis, deamination, decarboxylation, desulphurisation of amino acids, β-oxidation of fatty acids, esterification etc. It is not my purpose to review this part of cheese development. Instead, a short overview of the principal ripening reactions is presented although much of the biochemical changes are due to microbial activity and are important for the formation of flavour compounds.

1.2.1. Lipolysis

Satisfactory flavour development is one of the biggest problems encountered in the manufacture of low-fat products implying that lipolysis is essential for flavour development. Milk fat contains short- and intermediate-chain fatty acids (butanoic, hexanoic, octanoic and decanoic acid), which contribute directly to cheese flavour (Ha and Lindsay, 1993). This is achieved by enzymatic hydrolysis of triglycerides to fatty acids and glycerol, mono- or diglycerides. Lipolysis is very high in hard Italian cheeses and blue mould cheeses. High lipolysis is undesirable in cheeses like Edam, Mozzarella, Camembert or Cheddar, where free fatty acids (FFA) may range
between 300 and 1500 mg/kg but it is highly desirable in cheeses like Parmesan or Roquefort, where FFA reach ~30000 mg/kg. Lipases responsible for lipolysis originate from different sources like milk, rennet, starter etc. (McSweeney and Sousa, 2000). These lipases can lead to off flavours such as goaty, muttony and sweatlike which derive from the volatile fatty acids 4-ethyloctanoic acid, 4-methyloctanoic acid and 3-methylbutanoic acid, respectively (Ha and Lindsay, 1993; Farkye, 2003).

1.2.2. Proteolysis

Proteolysis is probably the most important biochemical event, taking place during cheese manufacture. Proteolysis begins during cooking, and hence contributes to textural changes. But it is also important in contributing to flavour and off-flavour development, liberating free amino acids (FAA), which contribute to the background flavour of most cheese varieties (e.g., alanine, serine, and threonine are responsible for “sweetness”, valine, isoleucine and tyrosine for “bitter”). The FAA are further catabolized through enzymatic reactions involving deamination, decarboxylation, desulphurization etc. to products which are chemically transformed into the compounds responsible for flavour development (Farkye, 2003).

Initial hydrolysis of casein is caused by coagulation of the milk. The two major enzymes involved are chymosin and pepsin, which hydrolyse casein to large peptides. Most of the coagulant is lost in the whey. The non-starter microflora may play a significant role in proteolysis as they rapidly reach high numbers, especially in cheeses made from raw milk while starter bacteria are only weakly proteolytic. In many cases a secondary starter is added to the cheese in order to accelerate ripening. Such secondary starters comprise Penicillium roqueforti (for blue cheese), Penicillium camemberti (Camembert and Brie), Propionibacterium freudenreichii (Emmentaler) or Brevibacterium linens (smear- or surface-ripened cheeses). The final products of proteolysis are FAA and medium and small peptides. Further catabolism of FAA results in ammonia, amines, phenols, methanthiol and alcohols, all of which contribute to flavour (McSweeney and Sousa, 2000; Farkye, 2003).
1.2.3. Citrate metabolism

Most of the citrate contained in milk is lost in the whey during cheesemaking. Nevertheless, the low concentration in the cheese mass is of considerable importance since it is metabolised to flavour compounds by mesophilic, citrate utilizing (Cit+) starter bacteria. These bacteria (i.e. the flavour producers, usually *Leuconostoc lactis*, *Leuconostoc mesenteroides*, and Cit+ *Lactococcus lactis* ssp. *lactis*) co-metabolize citrate and lactose rather than using citrate as an energy source. The major flavour compounds in citrate metabolism are acetate and diacetyl. Citrate metabolism is especially important in Dutch cheeses, where the CO₂ formation is responsible for eye formation (Hugenholtz, 1993).

1.2.4. Glycolysis

Lactose is metabolised to L- or D- lactate or both isomers by the starter and non-starter lactic acid bacteria (NSLAB). The NSLAB of some cheeses (Cheddar and some Dutch varieties) isomerize L-lactate to D-lactate. Ca-D-lactate is less soluble than Ca-L-lactate and results in crystallization and the formation of white visible specks (e.g. in Parmigiano Reggiano). Some of these specks are also composed of calcium phosphate or tyrosine (Bottazzi, 2003). In Emmental L-lactate is metabolised to propionic acid, acetate and CO₂ by the propionic acid bacteria (Steffen et al., 1993). Acetate is an important flavour component and can be formed also from citrate or from amino acids. (Farkye, 2003). Late gas and off-flavour (mainly due to the production of butyric acid) production may result from metabolism of lactate by *Clostridium* spp. (Thomas and Crow, 1983).
1.3. Classification of cheese

As seen in section 1.1 changes in the manufacturing steps result in different products and it is the fact that cheese exists in so many varieties that makes it so difficult to classify. One of the most common methods is to classify on the basis of moisture content into hard (<40% moisture), semi-hard (40-45%) and soft cheeses (>45%). Several other classification schemes exist but most of them have deficiencies. A classification in fat content results in some cheeses being assigned to two or even three categories, for example Brie as “Cheese, soft > 60% fat”, “Cheese, soft 46 - 60% fat” and “Cheese, soft 30 - 45% fat” (for further details see also http://www.ianunwin.demon.co.uk/eurocode/dnotes/n006.htm#CheeseType). Attempts have been made to classify cheeses according to criteria which include method of production, level of fat, fat in dry matter, moisture in fat-free substance, texture, method of coagulation of proteins, the microorganisms used during manufacture and their influence during maturation and preservation techniques. In Table 1 the classification is modified from Scott (Fox, 1993), who classified cheeses primarily on the basis of moisture content and sub-divided these groups on the basis of cooking temperature and/or secondary microflora.
Table 1: Classification of cheese according to moisture content, cooking temperature and microflora. Modified from Scott (Fox, 1993) *“Scalding” is the heating of the curd to usually 35°C

### Hard cheese (moisture content 20-42%)

<table>
<thead>
<tr>
<th>Low scald*</th>
<th>Medium scald*</th>
<th>High scald*</th>
<th>Plastic curds (pasta filata)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edam</td>
<td>Cheddar</td>
<td>Parmiggiano</td>
<td>Scamorza</td>
</tr>
<tr>
<td>Fontina</td>
<td>Derby</td>
<td>Emmental</td>
<td>Provolone</td>
</tr>
<tr>
<td>Cheshire</td>
<td>Svecia</td>
<td>Beaufort</td>
<td>Mozzarella</td>
</tr>
</tbody>
</table>

### Semi-hard cheeses (moisture content 44-55%) low scald

<table>
<thead>
<tr>
<th>Normal lactic starter as only microflora</th>
<th>Smear coat</th>
<th>Blue veined internal mould</th>
</tr>
</thead>
<tbody>
<tr>
<td>St. Paulin</td>
<td>Herve</td>
<td>Roquefort</td>
</tr>
<tr>
<td>Lancashire</td>
<td>Limburger</td>
<td>Gorgonzola</td>
</tr>
<tr>
<td>Trappiste</td>
<td>Tilsit</td>
<td>Stilton</td>
</tr>
</tbody>
</table>

### Soft cheeses (moisture content >55%) very low or no scald

<table>
<thead>
<tr>
<th>Smear coat or Surface mould</th>
<th>Surface mould</th>
<th>Normal lactic starter as only microflora</th>
<th>Acid coagulated, Normally unripened, fresh cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brie</td>
<td>Camembert</td>
<td>Colwich</td>
<td>Ricotta</td>
</tr>
<tr>
<td>Bel Paese</td>
<td>Neufchatel</td>
<td>Lactic</td>
<td>Cottage</td>
</tr>
<tr>
<td>Maroilles</td>
<td>Carre d'est</td>
<td>Bondon</td>
<td>Quarg</td>
</tr>
</tbody>
</table>
1.4. The geography of cheese production

Cheese has long been recognised as one of the international dairy industry’s long-term growth sectors. The market continues to exhibit growth, spurred in particular by new product development, segmentation, and increased efforts to brand what has usually been a commodity sector. In 2000, cheese production in the European Union rose to over 6.5 million tonnes. This represented an increase of 5.4% compared with 1997 and 8.2% compared with 1995. With the exception of the Netherlands, all the major producer states recorded increases of the order of 6%. Italy, Portugal and Greece saw the sharpest increases (ranging from 12% to 23%) (http://www.eu-datashop.de/download/EN/sta_kurz/thema5/nn.02_17.pdf). A glance at the production data within the EU reveals that France, Germany, Italy and The Netherlands are the greatest cheese producers. Together they produced over 5 000 000 tonnes in the year 2001, compared to the ~7 000 000 tonnes of the EU (FAOSTAT, 2001).

But this has not always been so. In his history of Scotland, Smith (1995) narrates that cheese production declined from the early 30’s to the late 40’s decreasing from 9 800 tonnes to 3 200 tonnes. It is interesting to note that farmhouse cheese, which in 1937 had been some 4 400 tonnes, reached an absolute minimum in 1947 of 600 tonnes. But things were going better from then on for the creamery-produced cheeses thanks to mechanisation: In the 50’s and 60’s cheese production in the UK doubled. Cheese production slowed down but increased steadily afterwards: from 1970 to 1998 the average annual growth rate of cheese consumption in the USA was 4.5% (World Dairy, 1999; Smith, 1995). Farmhouse cheese-production on the other hand, still decreased (Smith, 1995). In the sixties and seventies, with the introduction of supermarkets, small businesses were displaced, especially independently owned small businesses that contributed significantly to local civic life (Shils, 1997). It is only in the last number of years that farmhouse cheese-production is rising (Lassaut, 2001; O’Neill, 2000; Bord Bia, 2003).

In the USA, Canada, New Zealand, South Africa and Australia the favourite cheeses are Cheddar, Mozzarella and processed cheeses. Together they dominate the market in the countries mentioned. Specialty cheeses like blue cheeses, Gouda, Edam, Camembert follow the hit list. (US Dairy Export Council, 2003) The varieties
produced in these countries are of European origin. Countries in Asia, Africa and Latin America produce little cheese compared with European or North American standards. However, even if on a very small scale, cheese is produced in these regions, frequently by nomads or shepherds. Very little scientific information is available on most of them. Some are produced by rennet or acid coagulation, but in many cases defined starter cultures are not used so that acid production is very variable (Phelan and Renaud, 1993). In some countries quite interesting techniques for different steps of cheese manufacturing are being used: in India concentration by boiling, sometimes with the addition of sugar, is widespread; in Indonesia coagulation happens by means of bromelain obtained from pineapples, while in Benin they add crushed stems of the plant *Bryophyllum* sp. In Cyprus, Halloumi is made by heating the whey to 80-90°C to further precipitate and remove the proteins in it and by submerging pieces of curd into the hot whey; Tulum Peyneri in Turkey is ripened for 3 to 4 months in a goat skin or, in some cases, in the rumen of a sheep or goat. In Tanzania some families filter the whey from soured milk in a piece of cloth, which is then hung over the fire place in the kitchen for about one week before being consumed.

For further information on non-European cheeses see Phelan and Renaud (1993) or the booklet issued by the FAO (1990). In addition, Table 2 gives an overview of the quantities of the most important cheese varieties produced around the world in 2001. The table was created by merging information from FAO (1990), the statistical database of the FAO (FAOSTAT, 2001), Phelan and Renaud (1993) and various information pages from the internet (US Dairy Export Council, 2003).
Table 2. Cheese production (x1000 tonnes) in the world and some representative cheese varieties (US Dairy Export Council; FAO, 1990; FAOSTAT, 2001; Phelan and Renaud, 1993).

<table>
<thead>
<tr>
<th>Country</th>
<th>Quantity</th>
<th>Cheese Varieties (e.g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Africa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egypt</td>
<td>480</td>
<td>Daani, Ras, Karish</td>
</tr>
<tr>
<td>Sudan</td>
<td>152</td>
<td>Bibbna, Mudafera,</td>
</tr>
<tr>
<td>South Africa</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Niger</td>
<td>15</td>
<td>Tchoukou</td>
</tr>
<tr>
<td>Tunisia</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Morocco</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Nigeria</td>
<td>8</td>
<td>Wara, Chukumara</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>6</td>
<td>Ayib</td>
</tr>
<tr>
<td>Botswana</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Algeria</td>
<td>2</td>
<td>Aoules, Takamart</td>
</tr>
<tr>
<td>Angola</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Mauritania</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tanzania</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Benin</td>
<td>(a)</td>
<td>Woagachi, Wahashi</td>
</tr>
<tr>
<td>Chad</td>
<td>(a)</td>
<td>Pont Belile</td>
</tr>
<tr>
<td>Kenya</td>
<td>(a)</td>
<td>Mtoreki Ya Iria</td>
</tr>
<tr>
<td>Madagascar</td>
<td>(a)</td>
<td>Fromage, Fromage Blanc</td>
</tr>
<tr>
<td>Mali</td>
<td>(a)</td>
<td>Wagashi</td>
</tr>
<tr>
<td>Zaïre</td>
<td>(a)</td>
<td>Mashanza</td>
</tr>
<tr>
<td><strong>North and Central America</strong></td>
<td>4594</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>4024</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>154</td>
<td>Coltija, Oaxaca, Chihuaua</td>
</tr>
<tr>
<td>Nicaragua</td>
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<td>Graddost, Herrgardsost, Prastost</td>
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<td>Hungary</td>
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<td>Balaton, Lajta, Liptauer</td>
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<td>Juustoleipa, Lappi, Turunmaa</td>
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<td>Gammelost, Geltost, Gjetost</td>
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<td>Evora de l'Alentejo, Rabacal</td>
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<td>Herve, Passendale, Oud Brugge</td>
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<td>Bryndza, Oschtipka</td>
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<td>Macedonia</td>
<td>1</td>
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</tr>
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</table>

(a) Although FAOSTAT does not list any cheese production in this country, Phelan and Renaud (1993) and FAO (1990) mention some cheese varieties typical for this country.
2.1. The starter culture

One of the most important processes in cheese manufacture is the acidification of the milk to the desired pH and this is the primary function of the starter bacteria. Starter cultures can be divided into three types, mesophilic, thermophilic and artisanal starters.

Mesophilic starters always contain acid producing and flavour producing strains. The acid producing strains are unable to metabolise citrate (Cit') and are \textit{Lactococcus lactis} ssp. \textit{cremoris} (mainly) and \textit{Lactococcus lactis} ssp. \textit{lactis}). These are the dominant organisms in mixed cultures and comprise 90-99\% of the microflora. The flavour producing strains in a mesophilic starter are citrate utilizing (Cit') strains of \textit{Leuconostoc lactis}, \textit{Leuconostoc mesenteroides}, and/or \textit{Lactococcus lactis} ssp. \textit{lactis}. The terms citrate utilizing and flavour producing are used interchangeably since citrate metabolism results in the production of compounds like acetate and diacetyl (see section 1.2.3) which impart flavour to the unripe or green curd (McSweeney and Sousa, 2000; Stanley, 2003). Depending on the presence or absence of flavour producer, mesophilic cultures are divided into (i) D-types containing Cit' \textit{Lactococcus lactis} ssp. \textit{lactis}, (ii) L-types containing \textit{Leuconostoc} spp., (iii) DL-types containing both species and (iv) O-types containing no flavour producer (Cogan, 1996). Table 3 gives an overview of the most important cheese starters and their desired functions.

Thermophilic starters contain both coccal and rod-shaped bacteria. The coccus is invariably \textit{Streptococcus thermophilus} whereas the rod is \textit{Lactobacillus helveticus}, \textit{Lactobacillus delbrueckii} ssp. \textit{lactis} or \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus}. \textit{Str. thermophilus}, \textit{Lactobacillus delbrueckii} ssp. \textit{lactis} and \textit{Lactobacillus
*Lactobacillus delbrueckii ssp. bulgaricus* only ferment the glucose portion of lactose. *Lactobacillus helveticus* can use galactose as energy source and is therefore used with *Str. thermophilus* because they have the ability to metabolize galactose. A symbiotic relationship exists between *Str. thermophilus* and the lactobacilli with the latter organisms producing amino acids, especially glycine, valine and histidine, as a result of proteolytic activity on the casein, which stimulates the growth of *Str. thermophilus*. The latter organism, in turn, produces formic acid and CO₂ from lactose, which stimulate the lactobacilli (Stanley, 2003). Some strains of *Lactobacillus delbrueckii ssp. lactis* are able to use galactose and these are now more commonly used than *Lb. helveticus* in thermophilic starters for cheese manufacture. Thermophilic cultures have an optimum growth temperature of 42-45°C, compared to ~27°C for mesophilic cultures (Limsowtin et al., 1996).

A third type of starter culture is the artisanal starter culture. This is derived from the practice of using part of a previous batch of a fermented product to inoculate a new or fresh batch. As they are produced with every cheese batch and rely on selective pressure (depending on the cheese preparation), their composition is variable and poorly defined. They can contain both mesophilic and thermophilic strains (Frank and Hassan, 1998). Starter cultures are usually deliberately added to the milk for cheese making but in some artisanal cheeses, particularly raw milk cheeses produced in Spain, no starter cultures are added. Instead the cheesemaker relies on adventitious, contaminant lactic acid bacteria present in the milk to produce the necessary acid in the cheese (Nuñez and Medina, 1979; Del Pozo et al., 1988).
Table 3: Cheese starters and their main functions (modified from Cogan, 1996).

<table>
<thead>
<tr>
<th>Starter Micro-organism</th>
<th>Lactic acid</th>
<th>Diacetyl</th>
<th>Lipolysis</th>
<th>Proteolysis</th>
<th>CO₂</th>
<th>Acetoin</th>
<th>Product</th>
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<tbody>
<tr>
<td><em>Str. thermophilus</em></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Hard cheese; pasta filata; e.g., Swiss type cheeses (with <em>Propionibacterium</em> spp.)</td>
</tr>
<tr>
<td><em>Lb. helveticus</em></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Hard cheese, high scald, e.g., Parmigiano Regiano</td>
</tr>
<tr>
<td><em>Lb. delbrueckii</em> ssp. <em>lactis</em></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Hard cheese, low scald, e.g., Cheddar</td>
</tr>
<tr>
<td><em>Lb. casei</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Cit</em> <em>Lactococcus lactis</em> ssp. <em>lactis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>Soft cheese ripened and unripened; semihard cheese; mould-ripened cheese; hard cheeses, low and medium scald, e.g., Cheddar, Gouda, Camembert</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> ssp. <em>cremoris</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Cit</em> <em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Ripened soft cheese; semihard cheese; hard cheeses, low and medium scald, e.g., Gouda, Camembert, Brie</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> ssp. <em>cremoris</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Ripened and unripened soft cheeses, e.g., Gouda, Edam</td>
</tr>
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</table>
Thermophilic and mesophilic cultures are commercially available in two types. The first are mixed-strain starters (MSS). These starter cultures derive from artisanal cultures and have been propagated under controlled conditions for several years. They are thought to contain several strains but experimental proof of this is lacking (Cogan, personal communication). However, continuous sub-culturing can lead to variability in strain and species composition. In contrast to MSS, the exact strain and species composition of defined-strain starters (DSS) is known. DSS are usually blends of two or more strains (Cogan, 1996; Limsowtin et al., 1996).

During cheese manufacture, the starter cells in the curd increase from an initial number of $10^6$ cfu/ml of milk to $10^9$ cfu/g of cheese and, in the process produce lactic acid from lactose, which causes the pH of the cheese curd to decrease to 4.6-5.3 depending on the cheese. Non-viral agents may inhibit the growth of the starter (e.g. residual antibiotics from treatment of cows for mastitis) but bacteriophages (or phage) are still the most prevalent cause of decreased starter activity in cheese manufacture. The decreased starter activity results in fermentations in which acid production is noticeably reduced or, in extreme cases, fails completely. The phage tail is initially attached to the cell wall of the host cell and then injects its DNA into the bacterial cell. The phage DNA takes over the biosynthetic machinery of the host cell to produce more phage DNA and, as a result, more phage particles are produced in a short time, perhaps 100 particles in 60 min and the cell lyses. The new phages in turn attack new cells. The net result is that very quickly the entire bacterial culture is lysed. Phage have a very narrow host range and one of the advantages of MSS is that the phage only attack closely related strains and the other strains are able to continue to grow and produce acid (Sandine, 1996; Frank and Hassan, 1998).

At the beginning of ripening, starter organisms dominate the microflora of cheese ($\sim 10^9$ cfu/g) and during ripening they loose viability, lyse and release their intracellular enzymes in a process referred to as autolysis. Interest in autolytic strains is receiving considerable attention since it was demonstrated that cheese manufactured with autolytic strains was less likely to suffer from bitter flavour defects (Beresford and Williams, 2003). Starters vary in their ability to lyse and faster lysing organisms are also thought to produce a better flavour in the cheese (Fox et al., 2000).
2.2. Non-starter bacteria

During ripening, non-starter lactic acid bacteria (NSLAB) grow from relatively small numbers of $10^2$-$10^3$ cfu/g at the beginning to $>10^7$ cfu/g after several weeks. The majority of the NSLAB are lactobacilli, but, in some cheese, pediococci and enterococci are also found. Other bacteria, which are deliberately added to some cheeses, e.g., propionibacteria to Swiss-type cheese, and coryneforms to red smear- or surface-ripened cheeses also grow (Beresford and Williams, 2003).

**Non-starter lactobacilli**: This group constitutes the majority of the non-starter bacteria in probably all cheese varieties during ripening. The dominant species found in cheese are facultatively heterofermentative lactobacilli (FHL) particularly *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*. The source of NSLAB is the raw milk or factory environment. Many of them survive pasteurisation and cooking temperatures. In contrast to the starter cells, the non-starter lactobacilli grow relatively quickly from initial levels of $10^2$ to $10^3$ cfu/ml to $10^8$ cfu/ml and die off very slowly. Despite extensive studies, the role of non-starter lactobacilli in the development of cheese flavour is still not clear (Fox et al., 2000). Some strains of *Lactobacillus plantarum* and *Lactobacillus brevis* are known to produce various off flavours, e.g., gassy, putrid, fruity (van den Berg, 2003).

**Enterococci.** Enterococci are an essential part of the natural microflora in many dairy products of the Mediterranean countries and they dominate over lactobacilli and lactococci in some cheeses, especially artisanal cheeses. The enterococcal population ranges from $10^5$–$10^7$ cfu g$^{-1}$ in cheeses from Italy, France, Spain and Greece. The species most commonly isolated in cheese are *Enterococcus faecium* and *Enterococcus faecalis* (Beresford and Williams, 2003; Delgado and Bayo, 2004).

**Pediococci.** *Pediococci* are found only occasionally as a minor part of the non-starter bacteria in some hard cheeses. Their influence on the production of cheese flavour is not clear as most pediococci do not ferment lactose and therefore do not grow very well in cheese (Litopoulou-Tzanetaki et al., 1989; Fox et al., 2000).
**Leuconostocs.** Although some *Leuconostoc* species are considered as spoilage bacteria, certain strains produce acetate and diacetyl from citrate (see above) and they are widely used as starter in L and DL cultures. However, their occurrence is not restricted to cheeses with *Leuconostoc*-containing starters. *Leuconostoc* spp. have been isolated from artisanal cheeses produced from raw milk in Greece, Italy and France (Frank and Hassan, 1998; Beresford and Williams, 2003)

**Micrococci.** *Micrococcus* are obligate aerobes. They have been isolated in low numbers from a variety of cheeses including Cheddar and Iberian cheeses. The lack of oxygen inside most cheeses inhibits their growth and this explains their low numbers. *Micrococcus* spp. are believed to contribute positively to the maturation of different cheeses (Bhowmilk and Marth, 1990; Beresford and Williams, 2003).

**Staphylococci.** *Staphylococcus* are salt-tolerant, facultative anaerobes which also grow quite well in cheese. The pathogenic *Staphylococcus aureus* is found quite often at the initial stages of ripening but dies off relatively quickly. *Staphylococci* also occur on the surface of smear-ripened cheeses. The species most commonly found are *Staphylococcus xylosus* and *Staphylococcus epidermidis* (Beresford and Williams, 2003).

**Coryneforms.** They are very salt tolerant, generally withstanding 10% NaCl, and grow on the surface of surface ripened cheeses such as Livarot, Tilsiter or Gubbeen. Yeasts on the surface of the cheese, originating from raw milk and air, metabolize the lactate causing a change in pH of the cheese surface towards neutrality. This favours the growth of coryneforms. The term ‘coryneform’ has no taxonomic status but generally includes *Arthrobacter*, *Brevibacterium*, *Brachybacterium*, *Corynebacterium* and *Microbacterium* spp., all of which have been isolated from surface ripened cheeses. The only species, which is deliberately inoculated onto the cheese surface is *Brevibacterium linens*. During ripening the microflora of the surface changes into a not yet fully characterised one where *Arthrobacter* spp., *Corynebacterium* spp., *Rhodococcus* spp., other coryneform bacteria and yeasts like *Debaryomyces hansenii* and *Geotrichum candidum* grow (Brennan et al., 2002; Beresford and Williams, 2003).

**Propionic acid bacteria.** Propionic acid bacteria (PAB) metabolise lactate to propionate, acetate, CO\(_2\) and H\(_2\)O and are found in Swiss-type cheeses. The CO\(_2\) is responsible for the eye formation while acetic acid and propionate contribute to
flavour development. They survive the high cooking temperature required for these cheeses (50°-52°C) and their growth is stimulated by increasing the ripening temperature from 12 to 22°C. PAB are found at levels of $10^8-10^9\, \text{cfu g}^{-1}$ after a few weeks of ripening. Then the temperature is decreased to limit further growth (Cogan, 1996; Frank and Hassan; 1998).

**Coliforms.** Coliforms are mostly present in raw milk cheeses. According to Bramley and McKinnon (1990), the presence of coliforms in raw milk is not evidence of direct faecal contamination as they can build up in milk residues and milking equipment or be the cause of mastitis. Coliforms are present in low levels in cheese and usually disappear or decrease after the first few weeks of ripening (Litopoulou-Tzanetaki and Tzanetakis, 1992; Macedo et al., 1995). The presence of coliforms can be the cause of blowing defect in white-brined cheeses (Abd El-Salam, 2003).

### 2.3. Source of non-starter bacteria

In the EU, over 120 million tons of milk were produced in the year 2000 (http://europa.eu.int/comm/agriculture/publi/agrep2000/2000_it.pdf). Different housing conditions, different milking equipment and different storage conditions are found on farms. Once collected, the milk is cooled and the number and types of microorganisms present depend on the temperature and duration of storage (Bramley and McKinnon, 1990; Slaghuis et al., 1991). Milk is a highly nutritious food containing valuable sources of sugar (lactose), proteins (casein and whey proteins), vitamins and minerals. It is clear that milk can be contaminated in different ways and at different times before being cooled and processed. If it is not cooled promptly the bacteria will grow and cause it to spoil (Manners and Craven, 2003a).

Unless cows suffer from mastitis, milk is sterile just before exiting the cows’ teats. The bacteria most commonly implicated in mastitis are *Streptococcus agalactiae* and *Staphylococcus aureus*, while *Streptococcus uberis* and *Escherichia coli* play a minor role. If contamination does not start from within the teats, milk becomes contaminated with microorganisms from the exterior of the udder. This
happens when cows’ teats become soiled with dung, mud, straw, sand etc. If not removed, this dirt is washed into the milk together with bacteria associated with it. These are mainly micrococci, staphylococci, streptococci, enterococci and bacilli (Bramley and McKinnon, 1990; Slaghuis et al., 1991).

Air is not an important source of microorganisms in milk (bacterial loads of air seldom exceed 200 cfu/l) although small numbers may fall into the milk. Mainly micrococci but also coryneforms, *Bacillus* spores, streptococci and Gram-negative rods are responsible for contamination from the air (Bramley and McKinnon, 1990).

Many water supplies from farms come from wells, lakes, springs. Some of them may be contaminated, e.g. with coliforms, streptococci and clostridia, if they are not properly protected from rodents, birds, insects and dust. For this reason hypochlorite is frequently added to the water if this water is used to wash and rinse equipment which may come in contact with milk, e.g. milking machines and pasteurizers. Improperly cleaned milking machines and milk storage tanks are the major sources of microorganisms in raw milk (Garg and Mital, 1991). Because of the complexity of the milking machines and their components, milk residues and microorganisms can remain trapped in crevices, joints, dead ends, ridges. A study by Mettler and Carpentier (1997) showed how easily gaskets of pasteurizers can wear and become contaminated with *Bacillus*, *Microbacterium*, *Micrococcus*, and *Lactococcus* spp., which predominate on milking equipment surfaces.

Milkers and farmers are important not only for the dirt or dust they can dislodge but also for the organisms they carry on their clothes and hands. Waes (1973) showed that bacteria on hands are not very numerous but from the ones found almost 90% belong to *Enterococcus faecalis*. Enterococci may gain entrance to the milk primarily via contaminated water supplies, dung, equipment, insects and, as mentioned, handlers. They are generally found in raw milk in the range of $10^3$ to $10^5$ cfu/ml raw milk. The most commonly found *Enterococcus* species are *E. faecalis* and *E. faecium*, which can also be the source of environmental mastitis (Jones, 1998).
2.4. Hygiene of milk and dairy products

With a product like cheese, subject to risk of microbial contamination, awareness of health hazards arising from the consumption of contaminated food has grown in the last decades. Milk is a source of nutrition and must therefore be protected from contamination or deterioration as it can lead to infections, economic loss and maybe death (http://nano.med.umich.edu/food_bacteria_background.htm). A wide variety of factors can influence the microbial safety of cheese. *Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes* are of concern in cheese. In the European council directive 92/46/EEC (Anonymous, 1992) *Listeria monocytogenes* and *Salmonella* spp. are categorized as pathogenic microorganisms, *Staphylococcus* spp. and *Escherichia coli* are categorized as “organisms indicating poor hygiene” while coliforms and the total count are guideline organisms which “should help the producers ensuring proper operation of their establishments and in implementing the system and the procedure for carrying out their own checks on their production” (Anonymous, 1992). A statistically based sampling plan and the decision criteria to be applied to a particular batch, based on examination of a prescribed number of sample units by definite methods has been defined by the EU (Table 4). The widely accepted sampling plan is the three-class plan as defined by the International Commission on Microbiological Specifications for Foods (Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health, 1999). In Table 4 the 92/46/EEC directives are described for raw milk and cheese made from raw and thermized (not pasteurised) milk. Every country belonging to the European union has to follow the above-mentioned directive (Anonymous, 1992).
Table 4. Microbiological criteria for milk and cheese made from raw and thermized milk in cfu g⁻¹ or ml⁻¹ (92/46/EEC directive, Anonymous, 1992)

<table>
<thead>
<tr>
<th>Pathogenic Bacteria</th>
<th>Raw cows’ milk (1)</th>
<th>Cheese made from raw milk and from thermized milk (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Listeria monocytogenes (3)</strong></td>
<td>n 1</td>
<td>5</td>
</tr>
<tr>
<td><strong>Salmonella (4)</strong></td>
<td>n 5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>m 500</td>
<td>1000</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>M 2000</td>
<td>1 X 10⁴</td>
</tr>
<tr>
<td><strong>Total counts</strong></td>
<td>M 1 X 10⁵</td>
<td></td>
</tr>
</tbody>
</table>

n number of sample units examined from a lot
m the threshold value for the number of bacteria (the result is considered satisfactory if the number of bacteria in all sample units does not exceed “m”)
M maximally allowed value for the number of bacteria; the result is considered unsatisfactory if the number of bacteria in one or more sample units is “M” or more
c number of sample units where the bacteria count may be between “m” and “M” the sampling being considered acceptable if the bacteria count of the other sample units is “m” or less.

(1) Raw cows’ milk intended for direct human consumption.
(2) Thermization is the heating of raw milk for at least 15 seconds at a temperature between 57°C and 68°C (Anonymous, 1992; 92/46/EEC, Chapter 1, Article 2, 6).
(3) Absent in 1g (n = 1, c = 0)
(4) Absent in 25g (n = 5, c = 0). The 25g sample has to consist of 5 specimens of 5g taken from different parts of the same food.
(5) There are no directives for total coliforms for raw cows’ milk and cheese made from raw milk and from thermized milk.
CHAPTER 3.

BAYLOUGH, AN IRISH CHEDDAR-LIKE CHEESE

3.1. Cheddar cheese

As the cheese in this study is a Cheddar-like cheese, I will discuss the major characteristics of Cheddar cheese.

Cheddar is one of the most widely produced and eaten cheeses in the world. Cheddar cheeses were originally made in England; today it is manufactured in many countries, particularly English speaking ones, all over the world. It is only made from raw or pasteurised cow’s milk. The milk is inoculated with starter strains (*Lactococcus lactis* ssp. *lactis* or *Lactococcus lactis* ssp. *cremoris*). After acidification to pH ~6.4 at 32°C, rennet is added to form a coagulum. The curd is then cut and cooked at 38°C. After the whey is drained the curd is cheddared. This means piling and re-piling blocks of warm curd on top of each other in the cheese vat for a period of time. These are worked and turned to further expel whey and also produce the characteristic fibrous texture of Cheddar cheese. During the cheddaring process, the curd also becomes further acidified (to pH 5.2-5.5) by the continuing action of the starter culture on residual lactose in the curd. After cheddaring the curd is milled and dry-salted. As salting slows down acidification, the stage at which it occurs is important in determining the fate of lactose. Cheddar cheese is dry-salted and this means that lactose is subsequently metabolised much slower than in brine-salted cheeses. This results in the presence of ~10 g lactose/kg cheese after moulding and overnight pressing. The lactose is metabolised completely within the first two weeks of ripening while, simultaneously, non-starter lactic acid bacteria, particularly facultatively heterofermentative lactobacilli (FHL), grow.
Fully cured Cheddar (curing occurs at 7-9°C for 2 month to 2 years) is a semi-hard or hard cheese shaped like a drum. Normally, the colour of Cheddar ranges from white to pale yellow. Some Cheddars, however, have a colour added, e.g. annatto, which is extracted from the Brazilian plant, *Bixa orellana*, giving the cheese a yellow-orange colour (Cogan, 1996; www.foodscience.cornell.edu/fs406/FS406-L07.ppt).

At the end of ripening, a typical Cheddar should have a pH of ~5.3. After salting the potential for further decrease in pH depends on the residual lactose level however, generally the high buffering capacity of the cheese curd prevents any further decrease in pH. The salt in moisture (S:M) ratio determines the actual pH by controlling the activity of the starter after salting. In Cheddar the S:M should lie between 4.0 and 6.0%. Other two parameters usually found to describe cheese are the fat in dry matter (FDM) and the moisture-in-the-non-fat solids (MNFS). As many cheeses now are found as “half fat”, “reduced fat” and “full fat”, the FDM value can change between ~28 and ~52 while the MNFS in Cheddar is usually ~54 +/-0.5 (Fenlon et al., 2000)

3.2. Farmhouse cheeses and Baylough

In the 60’s and 70’s supermarket shopping became a trend. The pressure from large chain stores to supply cheaper, mass-produced cheese to meet the trend towards this supermarket shopping meant that farmhouse cheesemakers went out of business. Shelf life, ease of packaging and quantity came before taste and texture (Shils, 1997). However, in the last years, farmhouse cheese-production is rising (Lassaut, 2001; O’Neill, 2000; Bord Bia, 2003).

“Farmhouse cheese” is defined as cheese made from the milk of a single farm (Pujol, 1997) and is usually made with milk from the cheesemaker’s own herd. The self-sufficient cheesemaker, assured of the high quality of their own raw materials, is given a head start over larger creameries, which blend the milk of several different herds and then pasteurise it to a bland uniformity. Regarding the issue of premises located within farmyards, the issue is the proximity of the food premises, be it meat
or cheese, livestock, manure storage or animal feed storage. “In fact, a good example is the farmhouse cheese sector where a production facility is located within the farmyard but appropriate separation ensures that there is no risk to the finished product” (O’Mahony, 2002).

Attributes such as “taste”, “traditional and artisanal quality” and “healthiness and safety of the product” have become primary factors motivating consumers to buy farmhouse products (Revell and François, 1997). In Ireland these features helped the consumption of farmhouse cheese rise from 500 tonnes in 1996 to 1000 tonnes in 2000 (O’Neill, 2000).

One of these Irish farmhouse cheeses is the Cheddar-like Baylough cheese. Although the chemical composition of Baylough fits quite well with the Cheddar characteristics, the cheese does not get a lot of cheddaring and ripening, which is why it is referred to as Cheddar-like. The farmhouse is located near Clogheen, Co. Tipperary. On the internet we read “A mixed herd of Fresians and distinctive, Red and White Dutch cows provides the full-cream milk for Baylough, a hard-pressed, waxed cheese which can mature for many months. There are several varieties of this cheese, including Oak-smoked Garlic and Herbs Fresh Garlic”) and they are sold locally in different stores around Ireland (http://www.cheese.com/Description.asp?Name=Baylough, http://www.cookingisfun.ie/letters/2002/Culinaryed18mar02.htm).

Baylough is a raw-milk Cheddar-like cheese, made at farmhouse level, from the milk of a herd of 27 cows, by the herdowner’s wife. The milking parlour is located about 1 mile from the cheese making plant to which the milk is transported in a stainless steel tank before cheesemaking. The cheese is manufactured using a proprietary method (the manufacture steps are summarized in Figure 2) and a DL mixed-strain starter culture (Flora Danica, Chr. Hansen’s Laboratory), which contains 62% *Lactococcus lactis* ssp. *cremoris*, 19% *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, 2% *Lactococcus lactis* ssp. *lactis*, 16% *Leuconostoc cremoris* and 1% unknown species (Lodics and Steenson, 1990) and was ripened for several months. Each cheese lot undergoes a microbiological control in a local laboratory. As with all raw milk cheeses, it was suspected to contain strains of the genus *Enterococcus* as part of the non-starter bacterial flora.
Figure 2. Production of the Cheddar-like cheese Baylough.

Raw Milk heated to 31°C
addition of a DL mixed strain starter culture*

acidified milk (~pH 6.4, after ~30 min.)
addition of rennet
curd formation (after ~45 min.)
cutting of the curd
cooking at 38°C (~30 min.)
draining of the whey
curd after drainage
cheddaring, salting and milling
curd in moulds
waxing step
ripening at 12°C for ~8 weeks
Baylough cheese

*the starter culture contains 62% Lc. lactis ssp. cremoris, 19% Lc. lactis ssp. lactis biovar. diacetylactis, 2% Lc. lactis ssp. lactis, 16% Leuconostoc cremoris and 1% unknown species (Lodics and Steenson, 1990).
CHAPTER 4.

ENTEROCOCCI

4.1. Description of the genus *Enterococcus*

4.1.1. Introduction

Enterococci occur widely in the environment but are mainly associated with the gastrointestinal tract and, because of this, their presence in food is seen as an indicator of poor hygiene. They are an essential part of the natural microflora in many dairy products especially in Southern European countries. The source of enterococci in cheese is not clear. However, their presence in cheese is a matter of debate since they have recently emerged as nosocomial pathogens. On the other hand, although no consensus has been reached on whether enterococci should be considered as GRAS (generally recognized as safe), enterococci from dairy products show higher sensitivity to antibiotics and they have got a long history of safe use (Giraffa et al., 1997; Franz et al., 1999; Hardie and Whiley, 1997; Beresford and Williams, 2003).

4.1.2. Taxonomy of the genus *Enterococcus*

The genus *Enterococcus* consists of Gram-positive, oxidase negative, catalase negative, non-spore forming, ovoid cells arranged in single cells, pairs or chains (Fig. 3). Enterococci are facultative anaerobes with an optimum growth temperature of 35°C. In 1937 they were described by Sherman as organisms that grow from 10 to 45°C, in 6.5% NaCl and at a pH of 9.6, survive heating at 60°C for 30 min., and react...
with Lancefield group D antisera (Stiles and Holzapfel, 1997). Since then things have changed and not all species meet all of these criteria. Exceptions include Enterococcus dispar (Collins et al., 1991), Enterococcus sulfureus (Martinez-Murcia and Collins, 1991) and Enterococcus malodoratus (Collins et al., 1984), which do not grow at 45°C and Enterococcus cecorum and Enterococcus columbae, which do not grow at 10°C (Devriese et al., 1993). Most enterococcal species are capable of growing in 6.5% NaCl. However, according to Devriese et al. (1993), E. cecorum, E. columbae and Enterococcus avium grow poorly and slowly or not at all in 6.5% salt. E. sulfureus, Enterococcus casseliflavus and Enterococcus mundtii are yellow-pigmented (Martinez-Murcia and Collins, 1991). The motility seems to create some discordance; e.g. according to Devriese et al. (1993) only Enterococcus gallinarum is motile while Schleifer and Kilpper-Bälz (1987) ascribe motility to E. gallinarum and E. casseliflavus and Morrison et al. (1997) to E. casseliflavus and Enterococcus flavescens. However, Collins et al. (1984) describe E. casseliflavus and E. gallinarum as non-motile. Haemolytic reactions may vary in accordance with the blood used (e.g. horse, sheep, calf) (Facklam and Teixeira, 1998).

Figure 3. Electronic microscope image of enterococci.

Enterococci belong to the loosely defined group of lactic acid bacteria (LAB). One of the earliest definitions of LAB was given in 1919 by Orla-Jensen as “Gram-
positive, non-motile, non-spore forming, rod- and coccus-shaped organisms that ferment carbohydrates and higher alcohols to form chiefly lactic acid”. Current genera include *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Tetragenococcus* among others (Stiles and Holzapfel, 1997).

The name “enterocoque” was first used in a paper in 1899. The name was proposed to emphasize the intestinal origin of this strain. Several enterococcal species, like *E. faecalis* and *E. faecium*, originally belonged to the genus *Streptococcus*, a term coined in 1906 (Murray, 1990; Devriese et al., 1993; Deasy, 2000). It was only in 1984 when Schleifer and Kilpper-Bälz showed by DNA-DNA and DNA-rRNA hybridisation that some species were so distantly related to streptococci that they should be transferred to another genus (Schleifer and Kilpper-Bälz, 1984). At present, 27 different *Enterococcus* species are recorded on the list of valid names. Recently newly described strains comprise *Enterococcus canis* (De Graef et al. 2003), *Enterococcus gilvus*, *Enterococcus pallens* (Tyrrell et al. 2002), *Enterococcus haemoperoxidus*, *Enterococcus moraviensis* (Svec et al. 2001), *Enterococcus phoeniculicola* (Law-Brown and Meyers 2003), *Enterococcus ratti* (Teixeira et al. 2001), and *Enterococcus villorum* (Vancanneyt et al. 2001). Further reclassification may occur in the future. *E. casseliflavus* and *E. flavescens* for instance, can barely be distinguished (Descheemaeker et al. 1997) and *Enterococcus solitarius* is more closely related to *Tetragenococcus* than to *Enterococcus* (Williams et al., 1991).

### 4.1.3. Habitat

The habitat of enterococci is very diverse as the nature of these bacteria allows them to survive and grow also in harsh environments. The main habitat is the gastrointestinal tract (GIT) of mammals (Franz et al., 1999). Enterococci are the predominant, Gram-positive cocci in human stools with $10^5$-$10^8$ cfu/g faeces (Jett et al., 1994; Kleessen et al., 2000). *E. faecalis* is often the dominant species found in human faeces (Murray, 1990; Facklam and Teixeira, 1998; Franz et al., 1999). *E. faecium*, *Enterococcus hirae*, *E. avium* and *Enterococcus durans* have also been found (Murray, 1990; Franz et al., 1999; Kühn et al., 2003). Some authors (Murray,
1990; Franz et al., 1999; Kühn et al., 2003) suggest that the presence of *E. faecium* and *E. faecalis* in humans is dependent on the geographical location. Some enterococci are also found in the upper GIT, the lower and upper urogenital tracts and in the oral cavity (Murray, 1990; Morrison et al., 1997).

Although enterococci are prevalent in the human intestinal tract, they are also frequently isolated from the animal intestinal tract. *E. faecium* is the most frequently occurring *Enterococcus* species in dairy cows (Latham et al., 1978; Devriese et al., 1992. Leclerc et al., 1996) but *E. faecalis*, *E. hirae* and *E. casseliflavus* are also found (Devriese et al., 1987; Devriese et al., 1992). In addition, enterococci are found in water, soil, plants, vegetables, birds and insects (Martin and Mundt, 1972; Devriese et al., 1992; Godfree et al., 1997; Facklam and Teixeira, 1998; Franz et al., 1999).

### 4.2. The occurrence of enterococci

#### 4.2.1. Presence in cheese

The two most important food products, which contain enterococci, are meat and cheese. The presence of enterococci in the GIT of animals implies a high potential of contamination of the meat at the time of slaughter. This can cause spoilage in raw meat like salami but also in cooked and processed meat, as enterococci are able to survive heat processing (Franz et al. 1999, Giraffa, 2002). In contrast, the presence of enterococci in cheese may be desirable and is, in some cases, seen as an essential part of the microflora. Enterococci are commonly found in high numbers in cheeses produced in Italy, France, Portugal, Spain and Greece from raw or pasteurized goats’, ewes’, water buffaloes’ or cows’ milk (Cogan et al., 1997) (Table 5). Different species of enterococci are found in cheese but *E. faecalis* and *E. faecium* are the species most commonly isolated (Neviani et al., 1982; Trovatelli et al., 1987; Fontecha et al., 1990; Litopoulou-Tzanetaki and Tzanetakis, 1992; Devriese et al., 1995; Arizcun et al., 1997; Cogan et al., 1997; Suzzi et al., 2000). The numbers of enterococci in cheese vary between $10^4$ and $10^8$ cfu/g cheese (Carrasco de Mendoza
et al., 1992; Arizcun et al., 1997; Franz et al., 1999). In a survey of 48 samples of fresh, soft and ripened semi-hard cheeses, enterococci were found in more than 96% of them (Giraffa et al., 1997). Some workers have reported that they cause deterioration in flavour (Lopez-Diaz et al., 1995; Thompson and Marth, 1986), while others report enterococci to play a major role in improving flavour development and cheese quality (Dahlberg and Kosikowski, 1948; Clark and Reinbold, 1966; Kurman, 1968; Neviani et al., 1982; Litopoulou-Tzanetaki et al., 1993). This is achieved by the production of flavour components such as acetaldehyde, acetoin and diacetyl (Trovatelli et al., 1987) and through proteolysis, lipolysis and citrate metabolism. These pathways were already discussed and only the contribution of enterococci will be mentioned here.

Lipolysis and proteolysis are seen as the principal reactions responsible for the flavour development in Cheddar cheese (Dovat et al., 1970; Jensen et al., 1975a). Generally enterococci show very weak proteolytic activity, *E. faecalis* being the most proteolytic species (Jensen et al., 1975a; Arizcun et al., 1997; Suzzi et al., 2000). High proteolytic activities by enterococci have been reported in such different cheeses as Arzúa (Centeno et al., 1995), Cebreiro (Centeno et al., 1999) and Feta (Sarantinopoulos et al., 2002). Fayed et al. (1989) produced a yogurt-like product with *Enterococcus* spp. and found an overall high quality of the product as well as high proteolytic values.

The lipolytic activity, i.e. the ability to hydrolyse milk triglycerides, is essential in bacteria used in cheese as it provides aroma due to liberation of volatile fatty acids. The lipolytic activity in enterococci is very variable; only *E. faecalis* showing a degree of lipolysis worthy of incorporating into the cheese (Carrasco de Mendoza et al., 1992). An increase in fatty acids has been observed in Cheddar cheese (Jensen et al., 1975b), Feta cheese (Sarantinopoulos et al., 2002), Picante cheese (Freitas et al., 1999) and Cebreiro cheese (Centeno et al., 1999) while none of the strains isolated by Suzzi et al. (2000) from Semicotto Caprino showed lipolytic activity.
Table 5. European cheeses in which enterococci have been found.

<table>
<thead>
<tr>
<th>Country</th>
<th>Cheese</th>
<th>Milk</th>
<th>Dominant Enterococcus spp.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>Comté</td>
<td>Cow</td>
<td>Enterococcus spp.</td>
<td>Bouton et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Beaufort</td>
<td>Cow</td>
<td>Enterococcus spp.</td>
<td>Cogan and Rea, 1996</td>
</tr>
<tr>
<td></td>
<td>Mont d'Or</td>
<td>Cow</td>
<td>Enterococcus spp.</td>
<td>Sozzi and Maret, 1973</td>
</tr>
<tr>
<td></td>
<td>Roquefort</td>
<td>Ewe</td>
<td>E. faecalis</td>
<td>Devoyod, 1969</td>
</tr>
<tr>
<td>Greece</td>
<td>Pichtogalo Chanion</td>
<td>Ewe/Goat</td>
<td>Enterococcus spp.</td>
<td>Papageorgiou et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Feta/Teleme</td>
<td>Ewe</td>
<td>Enterococcus spp.</td>
<td>Tzanetakis and Litopoulou-Tzanetaki, 1992</td>
</tr>
<tr>
<td></td>
<td>Orinotyri</td>
<td>Ewe</td>
<td>E. faecalis</td>
<td>Prodromou et al., 2001</td>
</tr>
<tr>
<td>Ireland</td>
<td>Cheddar-like</td>
<td>Cow</td>
<td>E. casseliiflavus/E. faecalis</td>
<td>present study</td>
</tr>
<tr>
<td>Italy</td>
<td>Fiore Sardo</td>
<td>Sheep</td>
<td>E. faecium</td>
<td>Ledda et al., 1978</td>
</tr>
<tr>
<td></td>
<td>Casu Axedu</td>
<td>Goat</td>
<td>E. faecium</td>
<td>Cogan and Rea, 1996</td>
</tr>
<tr>
<td></td>
<td>Fontina</td>
<td>Cow</td>
<td>E. faecium</td>
<td>Cogan and Rea, 1996</td>
</tr>
<tr>
<td></td>
<td>Toma</td>
<td>Cow</td>
<td>E. faecium</td>
<td>Caserio et al., 1976</td>
</tr>
<tr>
<td></td>
<td>Mozzarella</td>
<td>Cow</td>
<td>Enterococcus spp.</td>
<td>Morea et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Caciotta</td>
<td>Cow-Sheep</td>
<td>Enterococcus spp.</td>
<td>Cogan and Rea, 1996</td>
</tr>
<tr>
<td></td>
<td>Semicotto caprino</td>
<td>Goat</td>
<td>E. faecium/E. faecalis</td>
<td>Suzzi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Pecorino Sardo</td>
<td>Ewe</td>
<td>Enterococcus spp.</td>
<td>Mannu et al., 1999</td>
</tr>
<tr>
<td>Portugal</td>
<td>Picante da Beira Baixa</td>
<td>Goat+Ewe</td>
<td>E. faecium/E. faecalis</td>
<td>Freitas et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Serra da Estrela</td>
<td>Ewe</td>
<td>E. faecium</td>
<td>Macedo et al. 1995</td>
</tr>
<tr>
<td>Spain</td>
<td>Manchego</td>
<td>Cow</td>
<td>E. faecium</td>
<td>Martinez-Moreno, 1976</td>
</tr>
<tr>
<td></td>
<td>Arzúa</td>
<td>Cow</td>
<td>E. faecalis</td>
<td>Centeno et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Cabrales</td>
<td>Cow + Ewe/Goat</td>
<td>E. faecium</td>
<td>Nunez and Medina, 1979</td>
</tr>
<tr>
<td></td>
<td>La Serena</td>
<td>Ewe</td>
<td>E. faecium</td>
<td>Del Pozo et al., 1988</td>
</tr>
<tr>
<td></td>
<td>Majorero</td>
<td>Goat</td>
<td>E. faecalis</td>
<td>Fontecha et al., 1990</td>
</tr>
</tbody>
</table>
Little is known about the ability of enterococci to metabolize citrate. Early reports (Campbell and Gunsalus, 1944; Coventry, 1978) showed that enterococci metabolized citrate to acetate and formate. There appear to be no further detailed investigations of the metabolism of citrate by enterococci. Rea and Cogan (2003) found that enterococci metabolise citrate only when glucose has been exhausted.

4.2.2. Bacteriocin production

Bacteriocins, called enterocins in the case of enterococci, are, according to Klaenhammer (1993), small proteins produced by microorganisms that can have narrow inhibit or broad-host ranges (McAuliffe et al., 2001). Enterocins belong to classes I, IIa, and IIc. These are described as follows:

- Class I: small peptides (<5 kD) containing the unusual amino acids, lanthionine or/and β-methyl lanthionine (cyl LԦ and cyl L₈),
- Class IIa: heat stable, non-lanthionine containing, Listeria-active peptides (<10 kD; Enterocin A, Enterocin CRL 35, Enterocin 31, Enterocin P, Enterocin SE-K4, Mundticine, Bacteriocin RC714), and

Several authors have successfully used Enterococcus spp. (usually as co-cultures) or the isolated enterocins in food production to reduce the occurrence of Listeria monocytogenes in cheese and meat (Giraffa et al., 1995; Nuñez et al., 1997; Farias et al., 1999; Aymerich et al., 2000a; Aymerich et al., 2000b; Callewaert et al., 2000; Laukovà and Czikkovà, 2001; Rodriguez et al., 2001). Other authors have observed that enterocins lyse starter cells and free intracellular enzymes and thus accelerate the cheese ripening and increase flavour production (Garde et al., 1997; Oumer et al., 2001). Enterococci seem to produce enterocins also against Staphylococcus aureus, Clostridium spp. and Vibrio cholerae (Giraffa, 2003).
4.2.3. Further applications of enterococci

Enterococci have also been considered as indicators of faecal contamination of water and food (Kenner et al., 1961; Lopez et al., 1995, Audicana et al., 1995). Godfree et al. (1997) advise every microbiologist to decide which indicator system is more appropriate for the particular problem under consideration as several groups of microbes have been employed as indicator of faecal pollution. In the UK, enterococci are used as indicators of secondary faecal contamination in samples where *E. coli* are absent (Anonymous, 2002).

Enterococci have also been suggested as starter adjuncts for their flavour and texture improving activity and have successfully been used as part of starter cultures in a variety of cheeses (Franz et al., 1999; Sarantinopoulos et al., 2002) and a yoghurt-like product (Fayed et al., 1989). Enterococci are usually part of a starter (as so-called starter-adjuncts) as they have a low acidifying activity (Carrasco de Mendoza et al., 1992; Giraffa, 2003). Seale (1986) reported the use of enterococci as silage inoculants.

Several authors describe enterococci as organisms with properties that allow them to be used as probiotics (Fuller, 1989; Dunne et al., 2001; Franz et al. 2001). The reduction of cholesterol levels was studied in vitro by Rossi et al. (1994, 1999) with *E. faecium* CRL 183. They showed a reduction of over 50% in the level of cholesterol. Another strain, which has also been thoroughly studied, is *E. faecium* SF68, which reduces the risk of developing antibiotic-associated diarrhoea or acute diarrhoea (Högenauer et al., 1998, Vanderhoof and Young, 1998) and hepatic encephalopathy (Loguerocio et al., 1995). A study by Benyacoub et al. (2003) proves a higher immune response in dogs after they received *E. faecium* SF68. Klein et al. (1998) state that *E. faecium* has mainly been used as an animal and human probiotic and *E. faecalis* primarily as a human probiotic. However, the FAO/WHO report (2001) recommends that enterococci not be used as a probiotics for humans due to their high level resistance to vancomycin and their association with nosocomial infections.
4.3. Acquired and intrinsic antibiotic resistance of enterococci

Antimicrobial resistance can be divided into two types: acquired and intrinsic resistance. Acquired resistance results from either a mutation in the host DNA or DNA acquired from an external source. Intrinsic (or inherent) is a species-specific resistance, in which the responsible genes are believed to reside in the chromosome (Murray, 1990).

4.3.1. Acquired antibiotic resistance and vancomycin resistance mechanism

If acquired resistance does not occur by mutation, it occurs by acquisition of new DNA either by transduction (the transfer of genetic information between cells through the mediation of a virus [phage] particle), transformation (the transfer of genetic information from a donor to a recipient using naked DNA, a process not known to occur in enterococci), and conjugation (the unidirectional transfer of genetic information between cells by cell-to-cell contact; Murray, 1998). Murray (1990) quotes various papers where up to 80% of the enterococci have shown resistance to tetracycline. Several genes that encode antibiotic resistance have been found in Mycoplasma spp., Campylobacter spp., Neisseria gonorrhoeae and Clostridium spp., among others. Resistance to chloramphenicol and erythromycin and high-level resistance (HLR) to clindamycin have been reported (Morrison et al., 1997; Murray, 1998). HLR to glycopeptides such as vancomycin has been of particular concern as it confers resistance to an antibiotic, which has proven bactericidal against enterococci. An even bigger problem is represented by the threat of vancomycin resistant enterococci (VRE) which spread their resistant genes to more dangerous organisms such as methicillin-resistant Sta. aureus (MRSA) (Morrison et al., 1997). Noble et al. (1992) demonstrated that the vancomycin resistance from E. faecalis NCTC 12201 could be transferred by conjugation to Sta. aureus B111.
Five patterns of vancomycin resistance are known: VanA, VanB, VanC, VanD and VanE. VanC and VanD are intrinsic and will be discussed in the following section (4.3.2.). VanA shows inducible high level resistance to the glycopeptides vancomycin (MIC >512µg/ml) and teicoplanin (MIC >64µg/ml, both are glycopeptides) and VanB, produces moderate to high level resistance to vancomycin and low-level resistance (LLR) to teicoplanin (0.5µg/ml). VanA and VanB are most commonly found in *E. faecium* and *E. faecalis*, they are acquired and inducible and have also been found in lactococci, *Oerskonia*, arcanobacteria and *Streptococcus bovis* (Murray, 1998).

Vancomycin, a highly efficient antibiotic against the majority of Gram-positive bacteria, is a glycopeptide, which inhibits cell-wall synthesis. A peptidoglycan precursor in enterococci (as in most Gram positive bacteria) has a terminal D-alanyl-D-alanine group (D-Ala-D-Ala) to which vancomycin binds inhibiting cell-wall synthesis by interfering with the attachment of further building blocks to the cell wall (Gholizadeh and Courvalin, 2000). In vancomycin resistant enterococci *vanA*, which is a complex of genes (*vanH*, *vanR*, *vanS*, and *vanX*), produces, in the presence of vancomycin, a substrate with a terminal D-Ala-D-X, where X is usually lactate. This leads to a 1 000-fold drop in binding affinity of vancomycin to the D-Ala-D-Lac terminus than to the D-Ala-D-Ala terminus and is thus not able to block the building of the cell wall. At the molecular level, VanA and VanB resistance result from the same substitution at the termini of peptidoglycan chains involved in cell wall synthesis. They are acquired resistances and are transferable via transposons belonging to the Tn3 family (VanA) and to transposon Tn 1547 (VanB) (Gholizadeh and Courvalin, 2000). The *vanD* gene, which is related to *vanA* and *vanB*, also encodes D-Ala-D-Lac and is expressed constitutively. It is characterized by moderate levels of vancomycin and LLR to teicoplanin in *E. faecalis* and *E. faecium* (Park et al., 1997; Perichon et al., 1997).

*vanE*, which is related to *vanC*, synthesizes D-Ala-D-Ser peptidoglycan precursors (Fines et al., 1999; Gholizadeh and Courvalin, 2000; Park et al., 1997; Perichon et al., 1997). Fraimow et al. (1994) reported the findings of vancomycin dependent enterococci (VDE).
4.3.2. Intrinsic antibiotic resistance

Enterococci are naturally resistant to a series of β-lactams like penicillin, ampicillin, semisynthetic penicillins, and cephalosporins (MIC’s 2 to 32 µg/ml, 1-4 µg/ml, 8-50 µg/ml, 6.3->100 µg/ml, respectively). The resistance to clindamycin, and its related lincomycin, is another feature of enterococci with low-level resistance MICs ranging between 12.5 to 100 µg/ml. Another important group of antibiotics comprises the aminoglycosides to which *E. faecalis* has low-level resistance e.g. kanamycin, streptomycin (250 µg/ml), and gentamicin (8-64 µg/ml) (Murray, 1990).

The constitutive, non-transmissible VanC resistance is responsible for low-level resistance to vancomycin and teicoplanin. VanC occurs naturally in *E. gallinarum* (*vanC-1* gene), *E. casseliflavus* (*vanC-2* gene), and *E. flavescens* (*vanC-3* gene). VanC resistance involves a switch to a D-Ala-D-ser terminus rather than a D-Ala-D-Lac terminus (Patel, 1999).

4.4. Virulence factors

Enterococci are believed to be able to colonise the GIT as they are commensals of the human intestine. Colonization is not seen as a virulence factor but it may increase the potential pathogenicity of other strains. Eaton and Gasson (2001) and Franz et al. (2001) found more virulence traits in *E. faecalis* than in other *Enterococcus* species.

Adherence to host tissue is considered crucial for infection and a first step towards colonization. Potential recipient strains (e.g., plasmid-free strains of *E. faecalis*) secrete small peptide signal molecules known as sex pheromones. These pheromones induce, in strains carrying a particular plasmid (donor cells), genes leading to the production of aggregation substance (Agg) that facilitates formation of mating aggregates with nearby recipients. An example of this system is the highly transmissible, pheromone responsive plasmid pAD1, which also carries the cytolysin determinants (Gilmore et al., 1994). Once a copy of the plasmid has been acquired, the recipient shuts down production of that pheromone but continues to secrete
pheromones specific for other plasmids (Clewell, 1993). Without adhesion substances like Agg and surface carbohydrates, enterococci would be swept away by gastrointestinal motility. Agg may mediate adherence to urinary tract epithelial cells, resulting in urinary tract infection (UTI), and may promote adherence to endocardial tissue, resulting in endocarditis. Enterococci are believed to originate in the intestinal tract. They exit the epithelial cells spreading to distant sites (Jett et al., 1994). Agg mediates contact to other cells to facilitate plasmid transfer. It has also been shown that in some cases Agg promotes greater increases in enterococcal adherence to macrophages and phagocytosis. Once phagocytosed, enterococci promote intracellular survival due to inhibition of the oxidative burst (production by the phagocyte of large quantities of reactive oxygen species which are released into the phagocytic vesicle eliciting microbicidal results): an adhesin and invasin at the same time (Franz et al., 1999; Rakita et al., 1999; Süßmuth et al., 2000). Agg was found in *E. faecalis* strains in both food and clinical isolates (Eaton and Gasson, 2001; Franz et al., 2001; Semedo et al., 2003).

Esp and Ace are two adhesins similar to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which are protein adhesins that mediate adherence.

**Esp (enterococcal surface protein)** is a chromosomally encoded enterococcal adhesin. The incidence of Esp was shown to be higher among clinical strains (i.e. from patients with bacteremia or endocarditis) of *E. faecalis* than isolates from healthy individuals (i.e. individuals without any history of hospitalization). Esp may help enterococci to evade detection by the immune system (Shankar et al., 1999). Franz et al. (2003) suggest that Esp promotes colonization also due to its similarity with MSCRAMM.

Another putative virulent agent may be the *Enterococcus faecalis* adhesin Ace (adhesin of collagen from *E. faecalis*). Nallapareddy et al. (2000) showed that Ace was expressed by enterococci during human infections and that *E. faecalis* can adhere to different types of collagen and laminin (which are found in the extracellular matrix and used by microbes for colonization) but additional studies are needed to evaluate the contribution of Ace to the virulence of *E. faecalis*.

Very little is also known about adhesin-like antigens (*EfaA*<sub>fs</sub> and *EfaA*<sub>fm</sub> of *E. faecalis* and *E. faecium* antigen A). So far *EfaA*<sub>fs</sub> is the only one of the two
antigens showing pathogenicity in animal models and is suggested to play a role in endocarditis (Singh et al., 1998). In some cases EfaA is the only virulence determinant present in food strains (Eaton and Gasson, 2001; Mannu et al., 2003). At present, there is no evidence of whether virulence determinants other than Agg play a role in invasion of translocation of enterococci (Franz et al., 2003; Temedo et al., 2003).

**Cytolysin** (haemolysin) is a toxin, which enhances virulence and is expressed by *E. faecalis* strains associated with disease. It causes rupture of a variety of target membranes, including bacterial cells, erythrocytes, and other mammalian cells. Its activity is observed as a haemolytic zone on blood agar (Huycke et al, 1998). Its operon is transmissible by plasmids. Cytolysin is located on the highly transmissible pheromone-responsive conjugative plasmid, pAD1 (Jett et al., 1994). Cytolysin is involved in the majority of infections, it is also found in enterococci isolated from healthy humans but can also be absent from enterococci causing disease. Cytolysin is therefore not a prerequisite for virulence (Johnson, 1994).

**Gelatinase** (*GelE*) and **serine protease** (*SprE*) are two proteases, which are important in the pathogenicity in animal models. Pillai et al. (2002) studied the presence of *fsr*, the genes that regulate the expression of *gelE* and *sprE*. The *fsr* locus is present in 70% of *E. faecalis* clinical isolates and is, according to Pillai et al. (2002), an indication for a virulent sub-population within enterococci, which generally have a low pathogenic potential. No studies have yet been carried out on the association of the hyaluronidase of enterococci and infection (Jett et al., 1994; Franz et al., 1999; Mundy et al., 2000).

4.5. The spread of VRE in Europe and the USA

Over the last two decades enterococci have gained importance as pathogens for nosocomial infections (hospital acquired infections or HAI). They are the cause of a variety of infections including endocarditis and neonatal, central nervous system or respiratory tract infections (Hardie and Whiley, 1997). They may also infect the abdomen, biliary tract, burn wounds, soft tissues, paranasal sinuses, ear, eye, and
periodontal tissue (Holdeman et al., 1976). Of major concern are the sources of hospital-acquired infections. Many of these have been identified but a high percentage remains obscure in origin. Some presumably originate from the GIT. It is believed that enterococci translocate across an intact intestinal tract and spread hematogenously to distant sites (Graninger and Ragette, 1992; Jett et al., 1994; Adams, 1999). Enterococci have become the focus of attention also due to their increasing resistance to antibiotics, especially vancomycin. In Europe the first VRE were reported in 1986 and in the USA in 1988 (Morrison et al., 1997). In 1993, VRE were the third leading cause of nosocomial infections in the USA (Aguirre and Collins, 1993), second in 1995 (Morris et al, 1995), and first in 1998 (Huycke et al, 1998), accounting for ~10% of such cases (Witte, 1997; Franz et al., 1999; Kim, 1999). In Europe their incidence is much lower. In 1995 VRE accounted for <1% for the infections in Belgium and Italy, 1-5% in Germany, The Netherlands, Portugal, Spain and Switzerland, with only France showing high levels (9.1%) (Witte, 1997). A more recent study by Goossens et al. (2003) shows that the UK and Italy were the countries with the highest rate of VRE with 10.4% and 19.6%, respectively. VRE are mostly endemic and monoclonal, favouring the suggestion of a common source or cross-contamination (e.g., the use of rectal thermometers). From 1990 to 1992 a clone of VanB phenotype *E. faecium* was isolated from nine patients at three hospitals in the USA proving evidence of an inter-hospital clonal transmission (Kim, 1999). Although the degree to which VRE contribute to mortality has proven difficult to estimate because of co-morbidities often present simultaneously (Chavers et al., 2003), about 85-90% of the enterococcal infections are thought to be caused by *E. faecalis* while 5-10% are caused by *E. faecium* (Patel, 1999, Adams, 1999). According to Coque et al. (1996), in the USA VRE are characterized by nosocomial outbreaks, while in Europe, the isolation of VRE from healthy humans, animals and environmental sources is considered to indicate that these organisms are part of the normal flora and suggest that the food chain may be the origin.

Since the first outbreak of VRE the use of vancomycin has constantly risen. In 1996, 11 200 kg of vancomycin were used in the US, where it was introduced in the 1950s, while only a quarter of that amount was used in France, Germany, Italy, UK and the Netherlands combined (Kirst et al., 1998). Due to the minor use of
antibiotics in Europe the problem of VRE has not grown to the same proportions as in the US (Wegener et al., 1999).

In Europe and Australia antibiotics such as avoparcin (avoparcin and vancomycin belong to the same class of glycopeptides) are used to a great extent as growth promoters for animals. In the USA avoparcin has not been approved for use in animals but the human use of vancomycin is far greater than in Europe (Collignon, 1999). Evidence that feeding antibiotics to chickens induces selection of antibiotic resistant enterococci (ARE), is provided by the study where the antibiotic resistance levels were high when antibiotics were used (up to 100%), while the resistance level reaches 0% when the antibiotics were not been used (Collignon, 1999; Teuber et al., 1999). Bager et al. (1997) report that there is a link between the use of avoparcin and VRE in animals. From the animals, ARE are transmitted to food as proven by numerous studies where ARE, resistant to a whole range of antibiotics (Teuber et al., 1999), are found in dairy products (Teuber, 1996 and 1999; Giraffa et al., 2000), meat products (Wegener et al., 1997; Quednau et al., 1998; Son et al., 1999) and even vegetables such as salads, tomatoes and peppers (Corpet, 1998). But ARE are not only isolated from food but also from livestock, animal carcasses, hospitalised patients and non-hospitalized patients (McDonald et al., 1997; Witte, 1997). McDonald et al. (1997) report a higher prevalence of VRE among people working on animal farms or meat processing plants than among urban residents in The Netherlands. This study, together with those of van den Braak et al. (1998) and Collignon (1999), suggests that VRE enter the human GIT by working with or ingesting contaminated food.

In Europe several antibiotics used as growth promoters have been banned: avoparcin in January 1997, ardamacin in January 1998 and bacitracin zinc, virginiamycin, tylosin phosphate and spiramycin in December 1998. After that ban a sharp drop of VRE in food has occurred in Europe (Teuber et al., 1999).

There are only four substances still authorized as growth promoting agents and none of them belong to classes used in human or veterinary medicine (EU-communication, 2001). The four substances are monensin sodium, salinomycin sodium, flavophospholipol and avilamycin (EU-Council regulation, 1998) but there are plans to ban also these four growth promoters from 1 January 2006 (EU-common position, 2003).
4.6. Enterococci in food: yes or no?

LAB are ubiquitous in fermented foods and are common components of the human GIT. For hundreds of years humans have eaten food containing LAB and this has led to the general conclusion that LAB are safe. There have been a number of reports of human infection caused by LAB. Not only enterococci, but also lactobacilli, leuconostocs, pediococci, lactococci and aerococci have been isolated from humans and animals and considered as pathogens (Rogasi et al., 1998; Scano et al., 1999; Barton et al., 2001; Ebnother et al., 2002; Gillespie et al., 2002; Cheng et al., 2003; Schmidtke and Carson, 2003). As has been observed in the previous sections, enterococci occupy a special position among LAB. Virulence factors, which seem to be strain specific (Franz et al., 2001), have often been found in enterococci isolated from different sources, also from food (Eaton and Gasson, 2001; Franz et al., 2001). Franz et al. (2003) argue that strains with multiple virulence factors pose a higher risk than strains with a single one but that clear data are lacking on this issue. They raise the question whether food strains possess an intrinsic lower pathogenic potential or not. A view also shared by Semedo et al. (2003) who argue that a more permissive attitude regarding their use in artisanal cheeses may be pursued as only a low risk is involved. As it is, knowledge on virulence factors is not satisfactory, and comparative studies between food and clinical isolates are limited. For Huycke et al. (1998) the problem lies in the limited knowledge concerning colonization. But while some time ago enterococci were put forward as co-cultures in food, now scientists are aware of the possible health hazard enterococci can pose. “Can cause human disease and might be a hazard to workers” is cited from the last directive from the European Parliament, which puts Enterococcus spp. into risk group 2 (EU-directive, 2000). Enterococci are still considered for use as probiotics or starter cultures, but with reservations: strains should carefully be evaluated for the presence of all known virulence traits, suggest Franz et al. (2003) and Giraffa (2002). Both scientists, however, are aware that enterococci may readily acquire virulence determinants due to their gene transfer mechanism, even in meat and cheese, as shown by Cocconcelli et al (2003). Teuber et al. (1996, 2003) find it irresponsible to feed ARE to people, knowing that they possess gene transfer mechanisms able to transmit resistances to
other microorganisms. They suggest that cheese should be produced only with pasteurized or microfiltered milk. The European Union looks at this problem on a higher scale creating awareness on the use and misuse of antimicrobial agents (EU-communication, 2001) as there is evidence that enterococci become vancomycin resistant. This view is shared also by other scientists (Bager et al., 1997; McDonald et al., 1997; Collignon, 1999; Teuber et al., 1999). Morbidity of enterococcal infections is described differently. According to Franz et al. (2003) and Garbutt et al. (2000) morbidity is low, although Jett et al. (1994) and Graninger and Ragette (1992) report mortality rates of 31% and 43%, respectively, to enterococcal bacteremia. Peters et al. (2003) isolated enterococci from food in Germany and found both enterococci that were sensitive to antibiotics used to treat enterococcal bacteremia and enterococci that were resistant towards tetracyclin, which the authors regard as a cause of concern.

Several aspects still remain to be elucidated: which and how virulence factors contribute to a disease and whether virulence traits-containing enterococci can be harmless. The path of transmission in the environment, the strategy of enterococci to cause diseases and whether enterococci from food possess an intrinsically lower pathogenicity are still not clear. Another important question is whether VRE acquisition increases a patient’s severity of illness or whether a patient’s severity of illness increases the likelihood of VRE acquisition (Chavers et al., 2003). Only after answering these questions, can a risk assessment be made and control measures taken (Huycke et al., 1998).

Cimons (2001) has already an apocalyptic view (“Imagine a world without fine, aged cheeses made from raw milk – no Gruyère, no Parmigiano Reggiano, no Roquefort, no Vermont Shepherd) having in mind a world, where milk unavoidably has to be pasteurized or even, more dramatically, where these cheeses have to be removed from the shops altogether.
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CHAPTER 5.

AIM OF THIS STUDY

Cheese has existed for thousands of years. Its consumption in developed nations has been steadily increasing over the past 20 years. In the US, average annual consumption per head is 12.7 kilograms a year, while in Europe it is 17 kilograms. The cheese microflora differs from cheese to cheese but can comprise starter cultures, non-starter-lactic acid bacteria, staphylococci, and coliforms. A taxon frequently found in cheese, especially in raw-milk cheeses, is *Enterococcus*. The source of enterococci in raw milk cheese, however, is not clear. Enterococci are ubiquitous and therefore found quite easily in the GIT of mammals, in the farming environment and in raw milk cheese. In recent years, attention has focused on enterococci, as they have become the cause of a variety of infections due to their increasing resistance to antibiotics. They might be able to colonize the human GIT or spread their genes to bacteria that normally reside in the GIT. The overall objective of this work was to produce knowledge on the origin and safety of enterococci found on an Irish farm and in the cheese produced on that farm.

The first step is to determine the diversity of enterococci in an Irish farmhouse raw-milk Cheddar-type cheese by identifying enterococci isolated from the raw-milk, the cheese, the cheese-making environment and the faeces of the cows and humans associated with the cheesemaking. At the same time valuable information will be obtained about the additional microflora of this particular cheese. The three trials will be undertaken in July 1998, in September 1998 and in October 1999. Enterococci will be isolated from KAA agar and identified using RAPD and SDS-PAGE.

Furthermore the origin of enterococci in this cheese will be investigated. Strains found in cheese will be typed and compared with isolates from the human
faecal samples, the milking equipment and the environment using Pulsed Field Gel Electrophoresis (PFGE). This method has shown to be highly discriminatory and is generally accepted as the method of choice for typing strains of enterococci.

There is evidence suggesting that enterococci colonize the GIT only transiently as they are unable to compete with the indigenous flora but this conclusion is not well documented. Studies exist about the passage of lactobacilli and bifidobacteria but not about enterococci. To determine whether enterococci can endure the passage through the human GIT, the effect that consumption of cheese containing enterococci has on the enterococcal flora will also be studied. The faeces of three healthy humans will be investigated before, during and after consumption of cheese. Enterococci obtained from the isolation will be characterised and compared using PFGE.

Finally the safety aspects of the representative enterococcal strains isolated from cheese will be studied. Resistances to the most common antibiotics will be tested phenotypically. The presence of resistance genes encoding resistance to tetracycline ($tet(M)$ and $tet(L)$) and/or erythromycin ($erm(B)$) will be investigated. In addition, all strains will be phenotypically and genotypically tested for the presence of (potential) virulence factors (Agg, Gel, Cyl, Esp, Ace, Efa$A_{fs}$ and Efa$A_{fm}$).

This study should allow us to get an insight into the ecology of enterococci present on this particular farm and the source of enterococci in the cheese. It is intended to learn something about enterococcal levels of the GIT of the persons living on the farm and involved with the cheesemaking, where they spread, and how safe enterococci in cheese are.
Part II

Experimental work
CHAPTER 6.

ENTEROCOCCAL DIVERSITY IN THE ENVIRONMENT OF AN IRISH CHEDDAR-TYPE CHEESEMAKING FACTORY

Gelsomino R.\textsuperscript{a, b}, M. Vancanneyt\textsuperscript{b}, S. Condon\textsuperscript{c}, J. Swings\textsuperscript{b}, and T. M. Cogan.\textsuperscript{a*}

\textsuperscript{a} Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland
\textsuperscript{b} BCCM/LMG Culture Collection, University of Gent, Ledeganckstraat 35, Belgium
\textsuperscript{c} Department of Microbiology, University College Cork, Cork, Ireland

Summary

Enterococci are natural residents of human and animal intestinal tracts and grow to high levels in a variety of artisanal cheeses. The aim of this study was to determine the diversity of enterococci in a farmhouse raw-milk cheese production unit. Putative enterococci were isolated from the faeces of all the cows and all the people associated with the cheesemaking, from the milk and cheese during manufacture and ripening and from the environment in three separate trials. Almost 1400 isolates were screened using a genus specific primer. The results indicated that all human, milk, curd and cheese isolates but only 33.7%, 6.7% and 4.4% of the bovine isolates from the 3 trials, respectively, were members of the genus Enterococcus. RAPD-PCR was used to type the enterococcal isolates. In general, only E. faecium was found in the bovine faecal samples while E. casseliflavus dominated the human faeces, milk and cheese followed by lower numbers of E. faecalis. Environmental sampling of the water in the milking parlour and rinses of the cows’ teats, the bulk-milk storage tank and the milking machine corroborated these results as E. casseliflavus and E. faecalis were the only Enterococcus species found in these samples. The putative vancomycin-resistant enterococci (VRE), isolated in Trial 1, were shown to be Pediococcus ssp. by genotypic and phenotypic analysis.
Introduction

Enterococci are ubiquitous, Gram positive, catalase-negative cocci that occur and grow in a variety of dairy and other food products. They have been used as silage inoculants (Seal, 1986), dairy starter cultures (Giraffa et al., 1997) and probiotics (Fuller, 1989) and although they do not grow well in milk (Cogan et al., 1997) they seem to play a major role in improving flavour development and quality of cheese (Dahlberg et al., 1948; Walter et al., 1956; Clark et al., 1966; Kurmann, 1968; Neviani et al., 1982a). Nevertheless, they are not considered GRAS (generally recognized as safe) organisms (Giraffa et al., 1997).

Enterococci are commonly found in high levels in a variety of cheeses produced in Italy, Portugal, Spain and Greece from raw or pasteurized goats’, ewes’, water buffaloes or cows’ milk (Cogan et al., 1997). Although they can be found on insects, in soil and on plants, their natural habitat is the intestinal tract of humans and animals (Garg et al., 1991). Previous studies have shown that Enterococcus faecalis is the most commonly found Enterococcus species in human faeces (Cooper et al., 1955; Saramago Stern et al., 1994; Leclerc et al., 1996; Facklam et al., 1998) while E. faecium is the most common in the intestinal tract of dairy cattle (Leclerc et al., 1996). Enterococci are less prevalent in dairy cows’ faeces but there have been reports of finding E. faecium, Enterococcus hirae and E. faecalis (Medrek et al., 1962; Mieth, 1962; Latham et al., 1978; Devriese et al., 1992); these workers also report that Streptococcus bovis is the most frequently occurring organism in cows’ faeces.

Enterococci are used as indicators of faecal contamination (Kenner et al., 1961; Lopez et al., 1995) and they are known to cause food spoilage, urinary tract infections, endocarditis and neonatal, central nervous system and respiratory tract infections. The ability of enterococci to exchange extrachromosomal elements which encode antibiotic resistance genes, particularly vancomycin, have been of particular concern during the last 10 years (Hardie et al., 1997). Vancomycin Resistant Enterococci (VRE) and a case of a Vancomycin Dependant Enterococcus (VDE) have been reported (Fraimow et al., 1994).

These organisms may enter the milk either directly from human or animal faeces or indirectly from contaminated water sources, exterior of the animal and/or
from the milking equipment and bulk-milk storage tank. Different species of enterococci are found in cheese but *E. faecalis* and *E. faecium* are the species most commonly isolated (Franklin et al., 1963; Fryer, 1969; Neviani et al., 1982a; Trovatelli et al., 1987; Fontecha et al., 1990; Devriese et al., 1995; Arizcun et al., 1997; Cogan et al., 1997; Suzzi et al., 2000).

The aim of this study was to determine the diversity of enterococci in an Irish raw-milk, farmhouse Cheddar-type cheese by typing enterococci isolated from the raw-milk, the cheese and the cheese-making environment and the faeces of the cows and humans associated with the cheesemaking.
Material and methods

Cheese production: Bay Lough cheese was used. This is a raw milk Cheddar-type cheese, made at farmhouse level, from the milk of a herd of 27 cows, by the herdowner’s wife. The milking parlour is located about 1 mile from the cheese making plant to which the milk was transported in a stainless steel tank before cheese-making. The cheese was manufactured using a proprietary method with a DL mixed-strain starter culture (Flora Danica, Chr. Hansen’s Laboratory) which contains 62% Lactococcus lactis ssp. cremoris, 19% Lactococcus lactis ssp. lactis biovar. diacetylactis, 2% Lactococcus lactis ssp. lactis, 16% Leuconostoc cremoris and 1% unknown species. (Lodics et al., 1990) and was ripened for several months.

Sample collection: The milk in the bulk tank, the milk in the cheese vat, the starter, the curd after cooking and at moulding and the cheese the following day were sampled. The cheese was also sampled after 1, 2, 3, 4, 6 and 8 weeks of ripening. Three trials were undertaken: the first in July 1998, the second in September 1998 and the third in October 1999. Bovine faecal samples of all the 27 dairy cows on the farm were obtained by manual rectal retrieval and transferred to sterile containers. The family involved in cheesemaking (2 adults and 2 teenagers) was also asked to collect their faecal samples in sterile containers as close as possible to the time the cows and milk were sampled. All samples were chilled and transported to the laboratory.

During the third trial the following environmental samples were also taken: A) Five hundred milliliters of the tap water in the milking parlour was membrane filtered. B) The milking machine was rinsed with ~50 l of water and sampled, it was then washed with water containing sodium hypochlorite (12ml sodium hypochlorite in 50 l water), sampled again, and then with 50 l of water containing 0.002% sodium thiosulfate to inactivate residual chlorine and sampled again. Five hundred milliliters of the chlorinated water were membrane filtered. C) The bulk tank was rinsed with 5 l of Maximum Recovery Diluent (MRD, Oxoid) and sampled. D) Milk samples were also taken at different stages from the bulk tank: after the first filling in the morning, before the second filling in the evening, before the third filling in the morning, after the third filling and shortly before the milk was put into the cheese vat (2 h later).
The four teats of each cow were rinsed with 80 ml MRD and all teat-rinses were mixed together. The environmental samples were diluted and plated on KF agar.

**Bacteriological analysis:** Curd and cheese were emulsified 1:10 in 2% (w/v) trisodium citrate, pH 8.75, and plated, with the milk samples, on the following media: coliforms on Violet Red Bile Agar (VRB, Merck) incubated for 18h at 30°C (IDF 73B, 1998); non starter lactic acid bacteria (NSLAB) on Lactobacillus selective agar (LBS, Becton Dickinson Microbiology Systems, USA) incubated for 5 days at 30°C; starter cells on LM17 agar (Difco) incubated for 3 days at 30°C; staphylococci on Baird Parker-Agar (Merck) incubated at 37°C for 3 days; enterococci on Kanamycin Aesculin Azide Agar (KAA) incubated at 37°C for 24h; total counts on Plate Count Agar (PCA, Merck) incubated at 30°C for 72h.

Faecal samples were emulsified 1:10 in sterile MRD and plated on KAA, KF-Streptococcus–agar (Difco) supplemented with 2,3,5-triphenyl-tetrazolium chloride (TTC, final concentration 100 µg/ml, BDH) and vancomycin (final concentration 6µg/ml, Sigma) was used to determine vancomycin resistant enterococci (VRE). VRE were determined only during Trial 1. An additional test to check for VRE consisted of adding 0.1ml of a 1:10 dilution of all samples to 5ml Buffered Peptone Water containing 6µg Vancomycin/ml. After incubation for 18h at 37°C, a loopful was streaked on KF-plates containing Vancomycin. KAA and KF plates were incubated at 37°C for one and two days respectively. KF agar was used instead of KAA to isolate VRE to avoid any interaction that might occur between the two antibiotics, vancomycin and kanamycin.

In Trial 3, KF-Streptococcus agar was used instead of KAA for the detection of enterococci in all samples and was incubated for 2 days at 37°C

**Isolation of strains:** Twenty colonies were picked from the highest dilution of each sample taken from the bulk tank, the cheese vat, the curd before moulding and from the cheese the following day and at 2, 4, 6 and 8 weeks of ripening. Ten colonies were picked from the highest dilution of each faecal sample. The colonies were purified twice on KAA and grown overnight in Basal Medium (BM) which contained 2% Tryptose (Oxoid), 0.5% NaCl (AnalaR), 0.5% Yeast extract (Merck), 0.5% Glucose (AnalaR), pH 6.85. Cultures were maintained at -80°C in a 1:2 glycerol-BM mixture. Isolates from stock were streaked on BM plates and a single colony was transferred to broth.
**Phenotypic characterisation:** All 1449 isolates were examined microscopically and checked for catalase production using 3% (v/v) H₂O₂ on single colonies grown overnight on BM agar at 37°C. Growth of catalase negative cocci was measured in BM broth containing 6.5% salt after 7 days, in 10% sterile (121°C, 5 min) reconstituted skim milk (RSM) after 6h, 16h and 7 days, in BM broth at 10°C after 2, 5 and 7 days and at 45°C after 2 days. Growth was determined by measuring the pH. A pH difference of at least 0.5 between the uninoculated control and the culture was considered to indicate growth. Columbia blood agar plates containing 0.1g/100ml aesculin and 7ml/100ml calf blood were used to record haemolytic reactions on strains identified as enterococci from the first trial.

**Compositional analyses:** Standard procedures were used to analyse the content of fat (IDF 5B, 1986), NaCl (IDF 88A, 1988), protein (IDF 20B, 1993) and moisture (IDF 4A, 1982) in the cheese after 8 weeks of ripening. The pH was measured on a cheese slurry prepared from 20g of cheese and 12g water (British Standard 770, 1976).

**PCR with genus-specific primer:** Genomic DNA from all strains was extracted from overnight cultures using the procedure described of Coakley et al. (1996). DNA amplification was performed using the procedures of Deasy et al. (2000) for enterococci and Deasy (2000) for pediococci.

**RAPD typing:** DNA was prepared using the rapid procedure described by Pitcher et al. (1989). Random primer D11344 was used to type enterococcal isolates to species level. DNA amplification was performed using the procedure described by Descheemaker et al. (1997). Numerical analysis was performed using the Pearson coefficient and the UPGMA (unweighted pair group method) with the GelCompar software, version 4.2 (Applied Maths, Kortrijk, Belgium).

**Fatty Acid Analysis:** Cells were grown twice for 24 h on Trypticase Soy agar (BBL, Becton Dickinson Microbiology Systems, USA) supplemented with 15g Bacto Agar (Difco) at 28°C. Fatty acids were extracted, separated and analyzed using the procedure described by Vancanneyt et al. (1996). Profiles were identified using the commercial TSBA 40 library (MIDI Inc. Newark, Delaware).

**Identification by SDS-PAGE:** Cells were grown for 24 hours on BM-agar at 37°C. Whole cell protein extracts were prepared with a slight modification as described by Pot et al. (1994): after the washing step the cells were sonicated for 2
minutes, with an output control of 3 and a duty cycle of 50% using a Branson Sonifier 250 (Branson Ultrasonics, Danbury, USA). Conversion, normalization and further analysis were performed using the Pearson coefficient and the UPGMA with the GelCompar software, version 4.2 (Applied Maths, Kortrijk, Belgium).
Results

Development of the flora in the cheese. The total counts and the counts of the different groups of bacteria in the raw milk in the bulk tank and in the cheese vat (Table 1) showed that the raw milk in each of the 3 trials was of satisfactory quality. There was little increase in numbers between milking and placing the milk in the cheese vat ~18h later. No enterococci were found in the starter culture. The enterococci, lactobacilli, coliforms and staphylococci all grew during manufacture (data not shown). Their behavior in ripening is shown in Fig. 1. The lactobacilli were the only group of organisms to show an increase in numbers. The enterococci remained more or less constant while the numbers of coliforms, staphylococci and starters all decreased.

Table 1. Count (cfu/ml) of different groups of organisms in the milk from the bulk tank and the cheese vat in each trial.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1 Bulk tank</th>
<th>Trial 1 Cheese vat</th>
<th>Trial 2 Bulk tank</th>
<th>Trial 2 Cheese vat</th>
<th>Trial 3 Bulk tank</th>
<th>Trial 3 Cheese vat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable count</td>
<td>18400</td>
<td>18100</td>
<td>16800</td>
<td>20100</td>
<td>3000</td>
<td>3700</td>
</tr>
<tr>
<td>Enterococci</td>
<td>500</td>
<td>500</td>
<td>130</td>
<td>160</td>
<td>230</td>
<td>260</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>100</td>
<td>300</td>
<td>60</td>
<td>40</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>40</td>
<td>50</td>
<td>20</td>
<td>10</td>
<td>160</td>
<td>230</td>
</tr>
<tr>
<td>Coliforms</td>
<td>80</td>
<td>110</td>
<td>80</td>
<td>500</td>
<td>101</td>
<td>400</td>
</tr>
</tbody>
</table>

There was considerable variation in the number of the different bacterial groups between each trial and some variation in the enterococci within a trial (Figure 1). However, the trends in each trial were similar. While enterococci were present in low numbers in the raw milk (~10^2/ml) (Table 1), their number increased during cheesemaking to final numbers of 1.4 X 10^4, 2.6 X 10^4 and 5.8 X 10^3 cfu/g in Trials 1, 2 and 3 respectively (Fig. 1)
Figure 1. Development of enterococci, Non Starter Lactic Acid Bacteria (NSLAB), coliforms, *Staphylococcus aureus* and starters in each trial during ripening.

Compositional profile of the cheese. The results of the compositional analyses are shown in Table 2 and did not show significant differences between the 3 trials. The salt in moisture (S/M), the pH and the ratio of moisture to nonfat substance (MNFS) were all within the range of a First grade Cheddar cheese while
the fat in dry matter (PDM) was within 50-52 which is considered to be the range for a Premium grade Cheddar cheese (Lawrence et al., 1984).

**Table 2.** Compositional analysis of the cheeses in the three trials at 8 weeks of ripening.

<table>
<thead>
<tr>
<th>parameters</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.84 - 4.95</td>
</tr>
<tr>
<td>MNFS(^a) (%)</td>
<td>54 – 57</td>
</tr>
<tr>
<td>FDM(^b) (%)</td>
<td>50 – 52</td>
</tr>
<tr>
<td>S/M(^c) (%)</td>
<td>3.6 - 4.1</td>
</tr>
</tbody>
</table>

\(^a\)MNFS, – ratio of moisture to nonfat substance, moisture/(100-fat) x 100.
\(^b\)FDM, – fat in dry matter, fat/(100-moisture) x 100.
\(^c\)S/M, – ratio of salt to moisture, salt/moisture x 100.

**Selective isolation of the strains.** In each trial, 160 strains were isolated from the milk, the curd and the cheese and 40 strains were isolated from the human faeces. Two hundred and seventy isolates from KAA or KF were obtained from the cows’ faeces in each of Trials 1 and 2 and 250 in Trial 3, as there were 2 fewer cows in the latter trial (Table 3). In Trial 3 ~2 X 10² cfu/ml were found in the milk sampled from the bulk tank at different fillings. Five colonies were isolated from each sampling point.

Five colonies were found, and subsequently isolated, on the KF plates from the rinse of the bulk-milk storage tank. Prior to chlorination the milking machine contained ~2.9 X 10³ enterococci per ml of rinse; no enterococci were found in the chlorinated rinse while after deactivation of the chlorine the number of enterococci found was 10 per ml. Five colonies were isolated from these samples before and after
Table 3. Total numbers of strains isolated per trial and the distribution of *Enterococcus* ssp.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td><em>E.</em></td>
<td><em>E.</em></td>
<td><em>E.</em></td>
<td><em>E.</em></td>
<td><em>E.</em></td>
</tr>
<tr>
<td></td>
<td>isolates</td>
<td><em>casseliflavus</em></td>
<td><em>faecalis</em></td>
<td><em>faecium</em></td>
<td><em>hirae</em></td>
<td>isolates</td>
</tr>
<tr>
<td>Milk from the bulk tank</td>
<td>20</td>
<td>14</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk from the cheese vat</td>
<td>20</td>
<td>17</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese before moulding</td>
<td>20</td>
<td>17</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese at day 1</td>
<td>20</td>
<td>14</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese at week 2</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese at week 4</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese at week 6</td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese at week 8</td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cows faeces¹</td>
<td>270</td>
<td>90</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces human 1 (adult)</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces human 2 (adult)</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces human 3 (teenager)</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces human 4 (teenager)²</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinsed bulk tank</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milking machine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cows' teats rinses⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. All the non-enterococci, catalase negative organisms screened with SDS-PAGE (136 strains) were *Str. bovis*.
2. One strain in Trial 3 was *S. saprophyticus*.
3. Of the 4 non-enterococcal strains 2 were *S. saprophyticus*, 1 was *B. cereus* and one could not be identified.
4. 6 were *S. saprophyticus* and 2 could not be identified.
chlorination. Twenty-six colonies from the cows’ teat rinses were found on the 2.5 ml-filter. Only 8 were cocci. Six colonies were found on the 500ml-filter from the tap water from the milking parlour. Vancomycin resistant colonies were only examined in Trial 1. None were found in any of the cows’ faeces in the 1:10 dilution and only 5, 3 and 2 colonies in the milk in the vat, the cheese the following day and the cheese ripened for 11 days, respectively.

**Phenotypic characterisation.** In total, 1449 strains were isolated i.e. 470, 470 and 450 strains from the cheese, the humans’ and cows’ faeces from Trial 1, 2 and 3 respectively, 6 from the water in the milking parlour, 10 each from the milking machine rinses before and after chlorination, 8 from the cows teats, 25 from the bulk-milk storage tank at different stages during milking and 10 from KF plates containing vancomycin.

All strains were catalase-negative cocci, except 53 bovine isolates and one human faeces isolate from Trial 3, which were catalase positive. This may have been due to the use of KF rather than KAA in this trial. The catalase negative cocci from the milk, the curd, the cheese samples and the human faeces grew at 10°C and 45°C, in broth containing 6.5% salt and in RSM after 7 days. Some of the isolates from the cows’ faeces were also able to grow at these conditions. However, numerous isolates from the latter did not grow at 10°C or in 6.5% salt. None of the enterococci showed apparent hemolytic activity. The vancomycin resistant strains were able to grow at 45°C and in broth containing 6.5% salt but not at 10°C.

Of the 8 organisms found on the cows’ teats, 2 were catalase negative and 6 were catalase positive cocci while of the 6 cocci found in the water 3 were catalase positive and 3 catalase negative. Two of the latter strains grew at 10°C and 45°C and in 6.5% salt. The third one and the 2 catalase negative strains found in the teat rinses grew in broth containing 6.5% salt but not at 10°C or 45°C.

**Genotypic analysis.** All catalase negative cocci were analyzed with the genus specific primer developed by Deasy et al. (2000). Only isolates that grew at 10°C and 45°C and in 6.5% salt reacted positively with the genus specific primer for enterococci (Figure 2) while all the strains that did not grow at 10°C and/or in 6.5% salt failed to give a band on the agarose gel. This analysis confirmed that all the isolates from the milk, the cheese and the human isolates were enterococci but
Figure 2. Agarose gel showing PCR products generated with the specific enterococcal primer developed by Deasy (2000). Mol. wt. ladders (lanes 1, 12, 23), 3 human isolates, Trial 1 (lanes 2, 3, 4), milk from the bulk tank, Trial 1 (lane 5), curd before moulding, Trial 1 (lane 6), cheese ripened for 2 weeks, Trial 1 (lane 7), human isolates, Trial 2 (lanes 8, 9, 10), milk from the bulk tank, Trial 2 (lane 11), curd before moulding, Trial 2 (lane 13), cheese ripened for 2 and 4 weeks, Trial 2 (lane 14, 15), human isolates, Trial 3 (lanes 16, 17, 18), milk from the bulk tank, Trial 3 (lane 19), curd before moulding, Trial 3 (lane 20), cheese ripened for 4 and 6 weeks, Trial 1 (lane 21, 22).

only 91, 18 and 11 out of 270, 270 and 250 isolates respectively from the animal faeces were enterococci.

The vancomycin resistant isolates from Trial 1 reacted positively with the pediococcal primer and not with the enterococcal primer.

RAPD based typing. RAPD analysis was used to type the species of enterococci in all samples. The milk and cheese samples from the different stages of cheese ripening and the human faeces were dominated by *E. casseliflavus*, followed by lower numbers of *E. faecalis*. In Trial 2 only *E. faecalis* was isolated from three of the human samples and *E. durans* was isolated from two samples in Trial 3. Two catalase negative isolates from the water sample were identified by RAPD as *E. faecalis*. The cows’ faeces were dominated by *E. faecium* in all trials (Table 3) but, in Trial 2, 10 of the 18 isolates were *E. faecalis*. One isolate of *E. hirae* was found. Figure 3 shows a selection of RAPD band
patterns of the most common Enterococcus species from different sources in different trials.

Figure 3. RAPD band patterns and corresponding dendrogram of some of the E. faecium, E. faecalis and E. casseliflavus strains found during the 3 trials. Most of the strains belong to one of the band patterns shown below. Numerical analysis was performed using the Pearson coefficient and the UPGMA (unweighted pair group method) using the GelCompar software, version 4.2 (Applied Maths, Kortrijk, Belgium).

Almost all band patterns of E. faecium, E. casseliflavus and E. faecalis found belong to one of the band patterns shown in the figure. All three clusters divide into two subclusters but this can be seen visually only for E. casseliflavus.

SDS-PAGE and Fatty Acid Analysis. Fatty Acid Profiling and SDS-PAGE were used to identify the non-enterococci. The vancomycin resistant, catalase negative
strains found to be pediococci by the genus specific primer were confirmed to be *Pediococcus pentosaceus* by SDS-PAGE.

Six colonies were isolated from the filtered tap water in the milking parlour. Three were catalase-negative and three catalase-positive. Two of the three catalase-positive isolates were shown to be *Sta. saprophyticus* and the third *Bacillus cereus* by fatty acid analysis.

Two of the catalase-negative strains were *E. faecalis* as shown by phenotypic analysis and RAPD typing while one strain, which grew in broth containing 6.5% salt but not at 10°C could not be identified.

Six of the eight colonies isolated from the cows’ teat rinses were catalase-positive. Fatty acid analysis showed that they all were *Sta. saprophyticus*. The two catalase-negative strains grew in 6.5% salt but not at 10°C and did not react with the enterococcus probe; they could not be identified.

Fifty-three catalase-positive strains were found in some of the cows’ faeces, in one human faeces and in some cheese samples. Fatty acid analysis showed them to be *Sta. saprophyticus*.

From all isolates found in the cows’ faeces that did not grow under the conditions typical for enterococci, i.e. growth at 10°C and 45°C and in broth containing 6.5% salt, 136 isolates were chosen (2 from each cow). These were all found to be *Str. bovis* after examination of whole cell proteins by SDS-PAGE. One catalase negative coccus found in the water and 2 catalase negative cocci found in the teat rinses could not be identified.
Discussion

In this study, the milk and cheese, samples from the environment and the faeces of every cow and human associated with a small artisanal cheesemaking operation were sampled for enterococci in three independent trials within a period of 15 months. Both phenotypic and genotypic analyses were used for identification of the isolates.

*E. casseliflavus* and *E. faecalis* were the most frequently isolated species in both the milk, the curd and the cheese samples and in the human faeces. This result was surprising but was confirmed in all trials. The results for the humans largely disagree with the findings of Saramago-Stern et al. (1994), Facklam and Texeira. (1989) and Leclerc et al. (1996) who stated that *E. faecalis* was the dominant enterococcal species in humans. In our study the *E. casseliflavus* population dominated not only in the humans’ intestine but also in the milk, the curd and the cheese, which also disagrees with the earlier reports of Neviani et al., 1982a; Trovatelli and Schiesser, 1987; Fontecha et al., 1990; Devriese et al., 1995; Arizcun et al., 1997; Cogan et al., 1997; Suzzi et al., 2000 for cheese. We do not have an adequate explanation for the analogous flora in both niches. The cheese used in the present study was a raw milk cheese and it is possible that *E. casseliflavus* does not withstand pasteurisation of milk and hence would not be found in pasteurised milk cheese. Human contamination of the milk may have occurred. However, the cheese is consumed by the cheesemaking family and this may have been the source of enterococci in the humans. A feeding trial would have to be undertaken to resolve this issue.

Very few enterococci were found in the cows’ faeces; they were isolated from 10, 3 and 2 cows of the 27, 27 and 25 cows in Trials 1, 2 and 3, respectively. The recovery of enterococci from the bovine faecal samples ranged from 1 to 10 isolates in those samples who contained them. In each of the 3 trials enterococci were isolated from different cows except for one cow from which 10 and 2 *E. faecium* strains were isolated in Trials 1 and 2, respectively. This poor recovery confirms the results of Devriese et al. (1992). Almost all enterococci found were *E. faecium*, which is one of the most commonly found species in the intestine of cows (Medrek and Barnes, 1962; Mieth, 1962; Latham and Jayne-Williams, 1978; Devriese et al., 1992).

The non-enterococcal catalase-negative strains found in the cows’ faeces were also typed phenotypically. They did not give the typical response for *Enterococcus* ssp.
but grew at 45°C. Two isolates from each cows’ faeces were shown to be *Str. bovis* by SDS-PAGE corroborating the findings of Medrek and Barnes (1962), Mieth (1962), Latham and Jayne-Williams (1978) and Devriese et al. (1992) that *Str. bovis* is the most common organism in the cows’ intestine.

In the three trials, the dominant species of enterococci found in the cows’ faeces, was *E. faecium*, whereas *E. faecal* and *E. casseliflavus* dominated the milk and cheese. The fact that very few enterococci were isolated from the cows’ faeces is a complicating factor in the experiments, as it is possible that *E. casseliflavus* and *E. faecalis* may have been present in low numbers in the cows’ faeces but were missed because the cows’ faeces were dominated by *Str. bovis*. No *Str. bovis* was found in the milk, the curd or the cheese although it was the dominant organism occurring in the cow’s faeces. This suggests that the cows were not the source of enterococci in the milk and cheese. A more selective medium in which enterococci can grow and *Str. bovis* cannot is currently being evaluated.

In Trial 3, KF agar was used instead of KAA agar because of the poor recovery of enterococci by KAA in the animal faeces. However, no difference was noted between both agars in the recovery of enterococci except that some catalase positive strains were isolated from the cows’ and human’s faeces from KF plates.

The vancomycin resistant organisms isolated from the milk and cheese KF agar containing vancomycin were shown to be *Pediococcus pentosaceus* by phenotypic and genotypic analyses and SDS-PAGE. This confirms the findings of Litopoulou-Tzaneki et al. (1989) and Bhowmik and Marth (1990) that *Pediococcus*, which is intrinsically resistant to vancomycin (Mackey et al., 1993), is an organism commonly found in cheese.

The milk and the cheese were of satisfactory initial microbiological quality (Table 1 and Fig. 1). Numbers of enterococci as well as staphylococci, coliforms and lactobacilli increased during manufacture. The difference in total viable counts was probably due to psychrotrophic organisms. Only lactobacilli grew subsequently during ripening. The behavior of the bacteria present in the cheese compares well with previous results. Mesophilic lactobacilli increase in numbers in all cheeses that have been examined while starter bacteria dominate the microflora of cheese at the beginning of the ripening period but their number decrease during ripening (Fox et al., 2000). Both staphylococci and coliforms decrease during ripening as reported by Fontecha et al.
(1990) and Macedo et al. (1995). Enterococci were present in small but stable numbers throughout the ripening period. Although previous studies report a decrease during curing (Clark and Reinbold, 1966; Fontecha et al., 1990) or a die-out after 30 days (Neviani et al., 1982b) the present results support those of Jensen et al. (1973) and Neviani et al. (1982a) who showed that enterococci were able to grow and survive during the ripening process.

In this study the faeces of every animal and human in contact with the cheesemaking was sampled in a small artisanal cheese factory. We are not aware of any other such studies in the literature with which to compare our results. Whether these findings can be extrapolated to other artisanal cheese producers is not clear but should be investigated. The relationship between the isolates of *E. casseliflavus* and *E. faecalis* in the human faeces and milk and cheese are not clear as RAPD typing could not give sufficient information about the relationship at the strain level. As can be seen in Fig. 3, the same band pattern occurs in almost all the strains of the same species not only from different sources but also in the 3 different trials. A study of the clonal relationships between the isolates needs to be undertaken and this is currently being examined.
Acknowledgments

This work was funded by a Teagasc Walsh Fellowship. The authors wish to thank Dick and Anne Keating for facilitating the study of their cheese and Tom Condon for his help in sampling the cows faeces. Marc Vancanneyt, Jean Swings and Tim Cogan acknowledge the European Community’s project 'Enterococci in food fermentations: Functional and safety aspects' (FAIR program FAIR-CT97-3078).
References


CHAPTER 7.

THE SOURCE OF ENTEROCOCCI IN A FARMHOUSE RAW-MILK CHEESE

Gelsomino R.\textsuperscript{a,b}, M. Vancanneyt\textsuperscript{b}, T. M. Cogan\textsuperscript{a}, S. Condon\textsuperscript{c}, and J. Swings\textsuperscript{b}

\textsuperscript{a} National Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland
\textsuperscript{b} BCCM/LMG Bacteria Culture Collection, University of Gent, Ledeganckstraat 35, Gent, Belgium
\textsuperscript{c} Department of Microbiology, University College Cork, Cork, Ireland

Summary

Enterococci are widely distributed in raw milk cheeses and are generally thought to positively affect flavour development. Their natural habitats are the human and animal intestinal tracts but they are also found in soil, on plants, in the intestine of insects and birds. The source of enterococci in raw milk cheese is unknown. In the present study, an epidemiological approach, using Pulsed-Field Gel Electrophoresis (PFGE), was used to type 646 *Enterococcus* strains which were isolated from a Cheddar-type cheese, the milk it was made from, the faeces of cows and humans, associated with the cheesemaking unit and the environment, including the milking equipment, the water used on the farm and the cows’ teats. Nine different PFGE patterns, 3 of *E. casseliflavus*, 5 of *E. faecalis* and 1 of *E. durans* were found. The same 3 clones, 1 of *E. faecalis* and 2 of *E. casseliflavus*, dominated almost all milk, cheese and human faecal samples. The two *E. casseliflavus* clones were also found in the bulk tank and the milking machine even after chlorination suggesting that a niche where enterococci could grow was present, and that contamination with enterococci begins with the milking equipment. It is likely but unproven that the enterococci present in the human faeces are due to consumption of the cheese. Cows’ faeces were not considered to be the source of enterococci in the cheese as *E. faecium* and *Str. bovis*, which largely dominated the cows’ intestinal tract, were not found in either the milk or the cheese.
Introduction

Enterococci are found in a variety of artisanal cheeses made from raw or pasteurized goats’, sheep, water-buffaloes’ or cows’ milk (Cogan et al, 1997; Franz et al., 1999). Their role in flavour development in cheese is not clear; some workers have reported that they cause deterioration in the flavour (Thompson and Marth, 1986; Lopez et al., 1995;), while others believe they play a major role in improving flavour development and cheese quality (Dahlberg and Kosikowsky, 1948; Walter et al., 1956; Clark et al., 1966, Kurman, 1968; Neviani et al., 1982). The most common enterococci in cheese are Enterococcus faecalis and Enterococcus faecium (Dave et al., 1980; Centeno et al., 1995; Arizcun et al., 1997; Cogan et al., 1997, Stiles and Holzapfel, 1997; Suzzi et al., 2000).

Enterococci have become important over the past decade as they are the most frequently encountered nosocomial pathogens and appear to have increasing antimicrobial resistance (Noskin, 1997). An even greater threat is the transfer of vancomycin resistance from vancomycin resistant enterococci (VRE) to methicillin-resistant Staphylococcus aureus (Michel and Gutman, 1997). Enterococci in food usually indicate poor bacteriological quality and poor hygiene during manufacture (Lopez et al., 1995). Therefore, there is a need to determine their source in food.

The natural habitat of enterococci is the mammalian intestinal tract (Franz et al, 1999). E. faecalis is often the dominant species found in human faeces (Murray, 1990; Facklam and Teixeira, 1998; Franz et al, 1999); however, E. faecium, Enterococcus hirae, Enterococcus avium and Enterococcus durans have also been found (Murray, 1990; Godfree et al., 1997; Franz et al, 1999). Some enterococci are also found in the lower and upper urogenital tracts and in the oral cavity (Murray, 1990; Morrison et al., 1997). Although enterococci are prevalent in the human intestinal tract, they are less frequently isolated from the animal intestinal tract. E. faecium is the most frequently occurring species in dairy cows (Medrek and Barnes, 1962; Mieth, 1962; Martin et al., 1972; Latham et al., 1978; Devriese et al., 1992) but E. faecalis, E. hirae and Enterococcus casseliflavus are also found (Devriese et al., 1987; Devriese et al., 1992). In addition, enterococci are found in water, soil,
plants, vegetables, birds and insects (Martin et al., 1972; Godfree et al., 1997; Facklam and Teixeira, 1998; Franz et al., 1999).

In a previous study (Gelsomino et al., 2001), enterococci were isolated from a Cheddar type cheese during manufacture and ripening, from the milk it was made from, from the faeces of the personnel involved in cheesemaking and from the faeces of the dairy cows present on the farm. In addition, strains were isolated from the environment and the milking equipment, the tap water, the milking machine, and the cows’ teats. All these isolates were identified to species level by RAPD (random amplified polymorphic DNA). The dominant species in milk, cheese and human faeces was *E. casseliflavus* followed by lower numbers of *E. faecalis*. The cows’ faeces contained mainly *E. faecium*. The enterococci isolated from the environmental sources were mainly *E. casseliflavus* and *E. faecalis*. As the RAPD technique was not sensitive enough to distinguish between strains, no conclusion could be drawn about the clonal relationships of the enterococci in the cheese and their putative source.

Pulsed Field Gel Electrophoresis (PFGE) has proven to be highly discriminatory and is generally accepted as the method of choice for typing strains of enterococci (Struelens et al., 1992; Gordillo et al., 1993; Maslow et al., 1993; Van Belkum et al., 1998). The aim of this study was to determine the sources of enterococci in the Cheddar cheese by comparing the strains isolated from the raw-milk and the cheese samples with the isolates from the human faecal samples and the isolates from the milking equipment and the environment using PFGE. As a result of this study valuable information has been obtained about the biodiversity of enterococci in a ripening cheese and the personnel associated with one cheese over a period of 15 months.
Materials and Methods

Isolation of strains. Six-hundred and forty-six enterococcal strains, isolated from the 3 trials described by Gelsomino et al. (Gelsomino et al., 2001) were used. These included 120 *E. casseliflavus* and 20 *E. faecalis* isolated from the milk, 304 *E. casseliflavus* and 56 *E. faecalis* isolated from the curd and the cheese, 61 *E. casseliflavus*, 49 *E. faecalis* and 9 *E. durans* isolated from the human faecal samples, 10 *E. faecalis* isolated from cows’ faeces, and 12 *E. casseliflavus* and 5 *E. faecalis* isolated from the milking equipment and the environment.

KAA containing 6.5% salt (KAAS [wt/vol]) was tested as a selective medium for enterococci. Growth of type strains of enterococci (*E. avium*, *Enterococcus cecorum*, *E. casseliflavus*, *Enterococcus columbae*, *Enterococcus dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *Enterococcus flavescens*, *Enterococcus gallinarum*, *E. hirae*, *Enterococcus malodoratus*, *Enterococcus mundtii*, *Enterococcus pseudoavium*, *Enterococcus saccharolyticus*, *Enterococcus solitarius*, *Enterococcus sulfureus*) (BCCM/LMG) and *Streptococcus bovis* (R-16101 and R-16102, Research Collection of the Laboratory of Microbiology, University of Gent, Belgium) were tested on both KAA and KAAS.

Two additional experiments were undertaken during the present study. The first consisted in mixing the first few strands of milk from each cows’ teat, which are normally disposed of, together, and plating them on KAA and KAAS.

The second additional experiment consisted of collecting faecal samples by manual rectal retrieval and plating them on KAAS as well as on KAA. Where possible 10 putative enterococci were isolated per cow.

In both of these experiments, when all 27 cows in the herd were milked, samples were taken from the bulk milk and the cheese at day 1 after manufacture. Samples were emulsified and plated and individual colonies isolated as described previously (Gelsomino et al., 2001). In this way, 131 isolates were obtained from the milk (n=30), the cheese (n=30) and the cows’ faeces, (n=71) from both KAA and KAAS plates.
RAPD typing. DNA was prepared using the rapid procedure described by Pitcher et al. (Pitcher et al., 1989). Random primer D11344 was used to identify the new isolates from the milk, the cheese, the cows’ faeces and the strands of milk to species level in the 2 additional experiments as described previously (Gelsomino et al, 2001). DNA amplification was performed using the procedure described by Descheemaeker et al. (1997).

Pulsed Field Gel Electrophoresis (PFGE). The strains were grown overnight at 37°C on BM (2% Tryptose [Oxoid, Basingstoke, England], 0.5% NaCl [Merck, Darmstadt, Germany], 0.5% Yeast Extract [Merck, Darmstadt, Germany], 0.5% Glucose [Merck, Darmstadt, Germany], pH 6.85) agar. All reagents were obtained from Sigma (St. Louis, USA) unless otherwise stated. One loopful of cells from an overnight culture was washed 3 times in 1 ml EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl [pH 8.0]). After centrifugation the pellet was resuspended in EC buffer (6 mM Tris-HCl [pH 7.6], 1 M NaCl (Merck, Darmstadt, Germany), 100 mM EDTA [pH 8.0], 0.5% Polyoxyethylene 20 cetyl ether [Brij 58], 0.2% deoxycholate, 0.5% N-laurylsarcosyl) and mixed with an equal volume of 1.6% (wt/vol) low melting-point agarose (Bio-Rad, Richmond, USA) in EC buffer and pipetted into plug molds. The solidified plugs were incubated overnight at 37°C in 1 ml EC buffer-lysozyme solution (2.88 mg lysozyme per ml of EC buffer). The lysis buffer was replaced with 1 ml of protein digestion solution (3.3 mg pronase E in 1 ml EET buffer containing 1.6% [wt/vol] SDS) and the plugs were incubated again overnight at 37°C. The agarose plugs were washed 3 times for 1 h in EET buffer, twice for 1 h in Milli-Q water and once for 1 h in the appropriate restriction buffer (Buffer Y+/Tango, MBI Fermentas, St.Leon-Rot, Germany) at room temperature. The restriction was carried out overnight at 27.5°C in 300µl restriction buffer containing 30 units Sma I (MBI Fermentas). The digestion was stopped by adding 0.5 ml of 0.5 M EDTA (pH 8.0) and the plugs were stored at 4°C. The restriction fragments were separated by PFGE in a contour clamped homogeneous electric field MAPPER system (Bio-Rad) by loading pieces of the plugs in 1% (wt/vol) Pulsed-Field Certified Agarose (Bio-Rad) gel prepared with a 0.5X TBE buffer (45 mM
Tris-HCl, 45 mM boric acid, 1 mM EDTA). Electrophoresis of *E. casseliflavus* was performed in 2 l 0.5X TBE at 14°C for 22 h at 6 V/cm, an angle of 120° with pulse times ramping linearly from 0.41 s to 15.11 s. For all the other *Enterococcus* strains pulse times ramping linearly from 5 s to 30 s were chosen.

A *Staphylococcus aureus* strain (R-6314, Department for Medical Microbiology, University of Antwerp) was used as a molecular weight marker. The genome was prepared as described above with the exception that 500 U of mutanolysin were added to the lysozyme solution. The gels were stained with ethidium bromide. Conversion, normalisation and analysis of the band patterns were performed using GelCompar software (Applied Maths, Belgium). Correlation coefficients and levels of similarity were calculated using the Dice coefficient and cluster analysis using the unweighted pair group method (UPGMA).

**Growth of *Str. bovis** in milk.** The two strains of *Str. bovis* were grown overnight in BM broth and in BM broth containing lactose instead of glucose. One ml of each culture was centrifuged and resuspended in 1 ml of Buffered Peptone Water (Oxoid). After washing, the cells were diluted in order to obtain an initial number of cells between $10^3$ and $10^6$ per ml of milk. Fifty ml of fresh raw milk and 50 ml of heat-treated milk (20 min at 95°C) were inoculated and incubated on a stirring plate at 4°C, 15°C and 37°C. Samples taken periodically during incubation for 3 days were plated on KAA and incubated for 24 h at 37°C. Control samples were also plated on Plate Count Agar (PCA, Oxoid), which was incubated at 30°C for 3 days.
Results

Selectivity of the isolation medium. The selectivity of KAA for the isolation of enterococci from cows’ faeces is very low as the vast majority of isolates from the medium were found to be Str. bovis (Gelsomino et al, 2001). The ability of 2 Str. bovis and 17 BCCM/LMG Enterococcus type strains to grow on KAA and KAAS was determined. E. avium, E. cecorum, E. columbae, E. pseudoavium, E. malodoratus did not grow or grew very poorly on KAAS (data not shown). All the other type strains grew as well on KAAS as on KAA while the 2 Str. bovis strains did not grow at all on KAAS.

Seventy-one enterococci were isolated from the faeces of 12 of the 27 cows present on the farm on KAAS at dilutions 10^{-1} to 10^{-3}. No enterococci were found in the faeces of the other 15 cows. In contrast, all samples plated on KAA showed growth up to dilution 10^{-4}, indicating the presence of Str. bovis.

No enterococci were found in the first strands of milk from all the teats of the cows either on KAA or KAAS. This corroborates the results of the previous study (Gelsomino et al, 2001).

An average of 7.3 X 10^{3} enterococci/ml was found in the bulk milk ~3 h after the cows' teats and faeces were sampled on both the KAA and KAAS while an average of 2 X 10^{5} enterococci/ml was found in the cheese on both KAA and KAAS.

Species identification. In the 2 additional experiments, the isolates on both KAA and KAAS from the milk (n=30) and the cheese (n=30), which was made shortly after the cows were milked, were shown to be E. casseliflavus (50%) and E. faecalis (50%) by RAPD. All isolates from the cows’ faeces (n=71) were shown to be E. faecium by RAPD and were not categorised any further.

Strain typing. PFGE was performed on the 646 isolates of E. casseliflavus, E. faecalis and E. durans previously isolated (Gelsomino et al, 2001). All 646 PFGE-band patterns were clustered and compared visually and with the GelCompar software. Only 9 different clones were found in the 646 isolates tested. An example of each of these is shown on Figure 1. In this figure E. casseliflavus patterns are marked with “Cs”, E. faecalis patterns with an “Fs” and E. durans patterns with a
“Ds”. The patterns marked “Fs1a”/”Fs1b”/”Fs1c” and “Cs1a”/”Cs1b” differed by only one band and were, in accordance with the definition of Tenover et al. (Tenover et al., 1995), considered to represent the same clone.

Table 1 shows the distribution of all the clones found in the milk, cheese, faeces and the environment and Fig. 2 shows the percentage of the most common clones in each of the 3 trials. Three of the 9 clones, Cs1, Cs2 and Fs1, largely dominate the milk, the cheese and the human faeces. These 3 clones, 2 E. casseliflavus and 1 E. faecalis, were found throughout the 3 trials, and 2 of them, E. casseliflavus Cs1 and Cs2, were also present in the environmental samples, i.e. the bulk tank and the milking machine rinses. E. casseliflavus Cs3 appeared less abundantly in the cheeses and in adult 1 but was not evenly distributed over the three trials.

Other clones were occasionally found. Four E. faecalis isolates of clone Fs2 were found in the milk from the bulk tank and 1, 4 and 1 isolates of this clone were found in adults 1 and 2 and teenager 2, respectively, in Trial 1. Ten isolates in the cows’ faeces during Trial 2 and one isolate from the rinse of the bulk tank were found to be clone Fs2. Only 1 isolate of clone Fs3 (E. faecalis) was found in the cheese of Trial 1, ripened for 11 days. In addition, 6 and 3 isolates of E. durans were found in the faeces of adult 2 and teenager 2, respectively, and they all show the same band pattern (Ds). The two Fs4 clones were found in the tap water while the two Fs6 clones were found in the milking machine (Figure 2 and Table 1).

A representative isolate of each clone has been deposited in the BCCM/LMG Bacteria Collection with the following numbers: LMG 19846 (Fs1), LMG 20228 (Fs2), LMG 20229 (Fs3), LMG 20230 (Fs4), LMG 20234 (Fs6), LMG 20231 (Ds), LMG 19844 (Cs1), LMG 19845 (Cs2), and LMG 20235 (Cs3).

Growth of Streptococcus bovis in milk. Large numbers of Str. bovis were found in cows’ faeces when plated on KAA but no Str. bovis isolates were found in any milk sample (Gelsomino et al, 2001). This raised the question whether Str. bovis grows in milk.
Figure 1: Agarose gel showing the PFGE-band patterns of the 9 clones found and digested with Sma I. A Staphylococcus aureus strain was used as molecular weight marker; Sc and Sf are Staph. aureus strains used as a molecular weight marker run with the PFGE parameters for *E. casseliflavus* and *E. faecalis*, respectively. Cs, Fs and Ds signify *E. casseliflavus*, *E. faecalis* and *E. durans*, respectively, while Cs1a and Cs1b and Fs1a, Fs1b and Fs1c are considered to be the same clones.
Table 1. Strain distribution of the isolates found in milk, cheese, faeces and the environment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>E. casseliflavus</th>
<th>E. faecalis</th>
<th>E. durans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cs1</td>
<td>Cs2</td>
<td>Cs3</td>
</tr>
<tr>
<td><strong>Milk</strong></td>
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<td></td>
</tr>
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<td></td>
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<tr>
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<td>19</td>
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<td></td>
</tr>
<tr>
<td>before moulding</td>
<td>29</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>at 1 day</td>
<td>16</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>at 11 days</td>
<td>35</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>at 25 days</td>
<td>19</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>at 39 days</td>
<td>35</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>at 53 days</td>
<td>22</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td><strong>Faeces</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>adult 2</td>
<td>15</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>teenager 1</td>
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<td>5</td>
<td></td>
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<td>rinse emty bulk tank</td>
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<td>rinse milking machine before chlorination</td>
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<td></td>
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<td>2</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
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</table>
Figure 2: Distribution of the clones found in each of the 3 trials (July 1998, September 1998 and October 1999). Numbers in brackets give the total number of isolates in that particular trial. Cs1, Cs2 and Cs3 are different clones of *E. casseliflavus* and Fs1 a clone of *E. faecalis*.

Both glucose-grown and lactose grown cells of the 2 strains of *Str. bovis* survived for at least 3 days at 4°C and grew at 15°C and at 37°C from 1.5 X 10^3 cfu/ml to 9.6 X 10^7 cfu/ml in 2 days in the raw and heat-treated milk indicating that the natural inhibitors present in the raw milk did not prevent the growth of *Str. bovis*. In 3 days, the total bacterial counts on Plate Count Agar increased from 4.6 X 10^4 to 7.0 X 10^6 cfu/ml in the raw milk at 4°C and from 3.2 X 10^4 to 3.5 X 10^8 cfu/ml in the raw milk at 37°C (data not shown); bacteria with which *Str. bovis* was able to compete.

No colonies were detected on KAA or PCA in the uninoculated heat-treated milk indicating that the heat treatment was sufficient to inactivate any contaminating bacteria present.
Discussion

In this study, an epidemiological approach was used to identify the sources of enterococci in cheese using PFGE. Nine different PFGE-band patterns, which were considered to be distinct clones, were found among 646 isolates from the milk, the cheese, the human faeces, the equipment and the environment (Fig. 1). Of these, 5 were produced by *E. faecalis* (Fs1-Fs4, F6), 1 by *E. durans* (Ds) and 3 by *E. casseliflavus* (Cs1-Css3).

The dominant species found in the cheese and the milk was *E. casseliflavus* (Gelsomino et al, 2001), although *E. faecalis* and *E. faecium* are reported to be the most common species of *Enterococcus* in cheese (Trovatelli and Schiesser, 1987; Devriese et al, 1995; Arizcun et al., 1997; Cogan et al., 1997; Suzzi et al., 2000) whereas *E. casseliflavus* is more often associated with plants (Devriese et al., 1992; Leclerc et al., 1996). Two *E. casseliflavus* clones, Cs1 and Cs2, accounted for 47% and 36% of the 500 isolates from the milk and cheese respectively. *E. faecalis* clone Fs1 was present in lower numbers (14%). Except for the milk and the cheese in Trial 2, where clone Fs1 was not present (Fig. 2), these 3 clones were found in all samples of the milk and the cheese throughout the 3 trials (Table 1) and constituted 97% of all isolates found in the milk and the cheese. *E. faecalis* clone Fs1 was present in lower numbers (14%). Except for the milk and the cheese in Trial 2, where clone Fs1 was not present (Fig. 2), these 3 clones were found in all samples of the milk and the cheese throughout the 3 trials (Table 1) and constituted 97% of all isolates found in the milk and the cheese. *E. casseliflavus* clone Cs3 was found quite often in the ripening cheese but not in the milk. Of the other *E. faecalis* isolates, only 4 isolates of clone Fs2 were found in the bulk tank milk while clone Fs3 was found only once, in the cheese ripened for 11 days.

The dominant species in the human faeces was *E. casseliflavus* followed by lower numbers of *E. faecalis* (Gelsomino et al, 2001). This result disagrees with previous studies which showed that *E. faecalis* is the most common species in the human intestine (Leclerc et al., 1996; Saramago et al., 1994; Facklam and Teixeira, 1998). Strain identification with PFGE showed that the same clones occurred in the milk, the cheese and the human faeces. Within the human faeces *E. faecalis* clone Fs1 was found in high numbers (36% of isolates), followed by *E. casseliflavus* clones Cs1 (28% of isolates) and Cs2 (19% of isolates). Clone Cs2 was only found in Trial 1 while clones Fs1 and Cs1 occurred in all trials (Fig. 2). *E. casseliflavus* clone Cs3 was found in adult 1 in Trial 3 while *E. faecalis* clone Fs2 was distributed over 3 of
the 4 members of the family. The 9 isolates of *E. durans* gave identical patterns and were found in adult 2 and teenager 2 in Trial 3 (Ds).

As both the milk and cheese samples and the human samples contained the same *Enterococcus* species and, more importantly, the same 3 dominant clones, human contamination could be regarded as the possible source of enterococci in cheese. Another possibility is that the enterococci in the human faeces are due to ingestion of the milk and the cheese. The latter is more likely as the family eat their own cheese.

In the previous study (Gelsomino et al, 2001) only a few enterococci were found in the cows’ faeces. All of them were *E. faecium* except 10 isolates from cow No. 27 in Trial 2. A more selective medium (KAAS) was used in the present study to isolate putative enterococci from the bovine faeces and *E. faecium* was still the only species found, and again in very low numbers; some sample of cows’ faeces showed no colonies even at dilution 10⁻¹. In contrast, growth on KAA without NaCl occurred up to dilution 10⁻⁴, indicating the presence of *Str. bovis* (Gelsomino et al, 2001).

As indicated, *E. faecalis* was isolated only from cow No. 27 in Trial 2 (This cow was present in the herd of Trials 1 and 2 but was culled in Trial 3) and all isolates belonged to clone Fs2. The same clone was found in the milk in Trial 3 and also in human faeces in Trial 1. We have no adequate explanation for this result but we believe that cows’ faeces could not have contaminated the milk, as Fs2 was already present in the human faeces in Trial 1. Enterococci were not found on the teats (Gelsomino et al, 2001) or in the udder (the present study). In addition, no clones of *E. casseliflavus* or *E. faecalis* found in the milk or the cheese, could be traced back to cows, except for the one already discussed. Furthermore, the only *Enterococcus* species found in cows’ faeces, except for the one cow mentioned above, was *E. faecium*, which was also not found in the milk or cheese. Therefore, we conclude that the cows’ faeces were not the source of enterococcal contamination of the cheese and milk. This is corroborated by the findings that *Str. bovis*, which is the most dominant species in cows’ faeces and grows quite well on KAA, can grow in milk but was not found in the milk or the cheese.

Both the milking machine and bulk tank are normally sterilized with Hydrosanᵀ before chlorination. Both were rinsed with sterile water and the rinses examined for enterococci. Ten isolates of *E. casseliflavus* clone Cs2 were found in
the rinses of the milking machine and bulk tank and 2 isolates of clone Cs1 were found in the rinse of the bulk tank. Clone Fs2 was found in the rinse of the bulk tank and in the milk of the bulk tank. \textit{E. faecalis} clones Fs4 and Fs6 were also found in the tap water and in the milking machine. The fact that the dominant clones in the milk, the cheese and the humans are found in the milking equipment strongly suggests that enterococcal contamination starts there. Although the milking machine has been rinsed and sterilized with a 0.024\% sodium hypochlorite solution (Gelsomino et al, 2001) in order to maintain good hygiene in the milking equipment, disinfection might not be fully effective, so that milk residues and bacteria are not completely eliminated from the equipment. Crevices, joints, dead ends and fittings are danger points where bacteria tend to accumulate and grow, infecting the milk directly (Bramley et al., 1990; Slaghuis et al., 1991). The possibility of a biofilm also being present (Venø, 1999) cannot be ruled out. This might explain why enterococci were found even after chlorination. It is also possible that \textit{E. casseliflavus} survives better in the milking equipment than \textit{E. faecalis}.

In conclusion, data from the present study demonstrate that enterococci survive and grow in the hidden corners of the milking machine and the bulk tank thus infecting the milk directly. From the milk the enterococci are transferred into the cheese and from there probably into the human intestinal tract by ingestion. But whether these strains were brought into the equipment via the air or poor hygienic housing conditions is not obvious. The original source of the enterococci in the milking machine is not clear.
Acknowledgments

This work was funded by a Teagasc Walsh Fellowship. The authors wish to thank Dick and Anne Keating for facilitating the study of their cheese and Tom Condon for his help in sampling the cows faeces. Marc Vancanneyt, Jean Swings and Tim Cogan acknowledge the European Community’s project 'Enterococci in food fermentations: Functional and safety aspects' (FAIR program FAIR-CT97-3078).
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CHAPTER 8.

EFFECT OF CONSUMING A RAW MILK CHEESE ON THE ENTEROCOCCAL FLORA OF HUMAN FECES

Roberto Gelsomino\textsuperscript{a}, Marc Vancanney\textsuperscript{a}, Timothy M. Cogan.\textsuperscript{b}, and Jean Swings\textsuperscript{a}

\textsuperscript{a} BCCM/LMG Bacteria Culture Collection, University of Gent, Ledeganckstraat 35, Gent, Belgium
\textsuperscript{b} National Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland

Summary

Enterococci are one of the major facultative anaerobic bacterial groups that reside in the human gastrointestinal tract (GIT). In the present study the composition of the enterococcal fecal flora in three healthy humans was analysed before, during and after the daily consumption of ~125g of a raw-milk Cheddar-type cheese containing $3.2 \times 10^4$ enterococci/g cheese. Enterococcal counts ranged between $1.4 \times 10^2$ and $2.5 \times 10^8$ cfu/g of feces and varied within subjects and from week to week. The cheese contained mainly *Enterococcus casseliflavus* and a small population of *Enterococcus faecalis*. Clonal relationships were determined by PFGE. Before and after consumption of the cheese, human samples contained mainly *Enterococcus faecium* with some of the clones being resident. During consumption of the cheese one particular transient clone of *E. faecalis*, clone Fs2, which was present in small numbers in the cheese, largely dominated the feces. Two clones of *E. casseliflavus* from the cheese were also found in the feces of one of the subjects during cheese consumption. These result suggest that a clone need not to be present in a food in high numbers to establish itself in the intestine.
Introduction

The human gastrointestinal tract (GIT) harbours a complex bacterial ecosystem. Up to $10^{14}$ bacteria may be present (Adlerberth et al., 2000; Fons et al., 2000) comprising 400-500 species although generally only a few predominate (Kleessen et al., 2000). The major bacterial groups are normally stable (Holdeman et al., 1976). Studies in which known strains were ingested showed that certain strains are detected in faeces constantly and over a long period, while others are found only occasionally (Adlerberth et al., 2000; Wallick and Stuart, 1943). Sears et al. (1949) called the persistent strains “resident” and the strains found occasionally “transient”.

The human intestinal flora is a complex network of mutual or/and antagonistic interactions. To establish in the intestine, bacteria must either adhere to the mucosa in order not to be swept away by peristalsis (Adlerberth et al., 2000) or multiply at a rate exceeding their rate of elimination (Kleessen et al., 2000); bacteria must also compete for nutrients, growth factors, binding sites and colonisation resistance towards already established bacteria (Adlerberth et al., 2000) which generate an environment that is inhibitory towards potential competitors. This can be accomplished by changes in pH and oxidation-reduction potential and by the production of $\text{H}_2\text{S}$ and volatile fatty acids (8). Recent studies have monitored the passage of lactobacilli and bifidobacteria through the GIT (Bunte et al., 2000; Kimura et al., 1997; Sørensen et al., 2001).

Enterococci are the predominant, Gram-positive cocci in human stools with $10^5$-$10^8$ cfu/g feces (Jett et al., 1994; Kleessen et al., 2000) and Enterococcus faecalis is the commonest Enterococcus species found (Facklam and Teixeira, 1998; Leclerc et al., 1966; Saramago Stern et al., 1994). Franz et al. (1999) and Murray (1990) suggest that the presence of Enterococcus faecium and E. faecalis in humans is dependent on the geographical location.

Enterococci are the cause of a variety of infections including endocarditis and neonatal, central nervous system or respiratory tract infections (Hardie and Whiley, 1997). They may also infect the abdomen biliary tract, burn wounds, soft tissues, paranasal sinuses, ear, eye and peridontal tissue (Jett et al., 1994). Despite these involvements, the study of these organisms at a strain level is limited. Of major concern are the sources of nosocomial infections. Many of these have been
identified, but a high percentage remains obscure in origin and some presumably originate from the GIT. It is believed that enterococci exit the epithelial cells or migrate in phagocytes and spread hematogenously to distant sites (Jett et al., 1994). Enterococci have become the focus of attention also due to their increasing resistance to antibiotics. Not only are they resistant to vancomycin, they are also resistant to teicoplanin, penicillins and aminoglycosides. In addition, vancomycin dependent enterococci have also been reported (Fraimow et al., 1994). The presence of vancomycin resistant enterococci (VRE) in hospitals is met with considerable apprehension (Franz et al., 1999; Gardiner et al., 2000; Morris et al., 1995; Morrison et al., 1997). A major issue of concern is the transfer of antibiotic resistance from enterococci to more virulent pathogens such as multiple-drug resistant staphylococci (Morrison et al., 1997). VRE have also been isolated from food (Franz et al., 1999; Giraffa et al., 1997; Gardiner et al., 2000; Perreten et al., 1997). From this point of view the statement by Garg and Mital (1991), “a food should be free not only from disease-producing organisms, but also from those that have the potential of causing disease” seems legitimate.

Nevertheless enterococci are used as silage inoculants (Seale, 1986), starter cultures (Giraffa et al., 1997) and probiotics (Franz et al., 1999; Fuller, 1989), possess antilisterial activities (Elotmani et al., 2002; Nunez et al., 1997) produce various metabolic compounds that can interfere with the growth of undesirable bacteria (Fonds et al., 2000) and have a beneficial role in ripening and flavor development of cheese (Clark and Reinbold, 1966; Kurmann, 1986; Neviani, et al., 1982). Finally, enterococci from dairy products show higher sensitivity to antibiotics and they had a long history of safe use (Giraffa et al., 1997).

In a previous study (Gelsomino et al., 2002) the enterococcal flora of a raw-milk, farmhouse cheese was compared with the microflora of human and bovine feces. The cheese and the human feces contained two dominant strains of Enterococcus casseliflavus and one of E. faecalis followed by lower numbers of other clones from the two species. The cows were not the source of enterococci in the cheese but the presence of both E. casseliflavus clones in the milking equipment suggested that contamination of the milk starts there. The presence of identical clones of enterococci in human feces and cheese was explained by consumption of milk and cheese by the humans, but this was not proven.
In the present paper we describe the results of a study in which 3 healthy human subjects consumed cheese containing enterococci (Gelsomino et al., 2001; Gelsomino et al., 2002). The purpose of our investigation was to determine the impact that consumption of cheese containing enterococci had on the enterococcal flora of the feces. Valuable information was also obtained about the regular enterococcal flora.
Materials and methods

Collection of samples: Four healthy Belgian subjects (2 males and 2 females) who were between 26 and 52 years old participated in this study. Fecal samples were collected weekly, in sterile containers, by the volunteers, over a 10-12 week period, except in week 4. The first 3 weeks was the control period after which three of the four volunteers began to consume between 100 and 150 g of an Irish farmhouse raw milk Cheddar-type cheese (Gelsomino et al., 2001) daily, giving a daily dose of $3.2 \times 10^6$ – $4.8 \times 10^6$ enterococci for 4 weeks. The feces were not sampled during the first week of cheese consumption. Weekly fecal samples were also collected for 3 weeks after cheese consumption had stopped. The 4th volunteer was used as a control. During this period the volunteers maintained their lifestyles and usual diet, including, in some cases, other cheeses. None of the subjects was administered antibiotics during the study period. If, for any reason, a weekly fecal sample was not obtained, the experiment was prolonged by one week.

Isolation of strains: The cheese was emulsified 1:10 in a 2% (wt/vol) trisodium citrate solution, pH 8.75, homogenized with the aid of a Stomacher and plated on Kanamycin Esculin Azide (KAA, Merck) agar.

Fecal samples (3-6g) were immediately diluted 1:10 in sterile peptone saline solution (PSS, [0.1% Peptone, Oxoid; 8.5% NaCl, Merck]), emulsified in a Stomacher, diluted in PSS and plated on KAA agar.

All plates were incubated overnight at 37°C under microaerophilic conditions (5% O$_2$, 10% CO$_2$, and 85% N$_2$). Four hundred and eighty colonies were picked from plates of the cheese showing over 100 colonies. Ten colonies were randomly picked from the highest dilution of each fecal sample. All colonies were purified twice on KAA and once on BM agar (2% Tryptose [Oxoid], 0.5% NaCl [Merck], 0.5% Yeast extract [Merck], 0.5% Glucose [Merck], pH 6.85). Cultures were maintained at -20°C in a 1:2 glycerol-BM broth mixture. Isolates from stock were streaked on BM plates.

Strain typing by Pulsed Field Gel Electrophoresis (PFGE). As the running conditions for PFGE are different for the yellow-pigmented *E. casseliflavus* than for other enterococci, all isolates were first distinguished by colony-color when grown on BM agar. The yellow colonies were assumed to be *E. casseliflavus* as shown in previous studies (Gelsomino et al., 2001; Gelsomino et al., 2002) while the white
colonies represented *E. faecalis*, *E. faecium*, *Enterococcus hirae* and other enterococci, commonly present in feces.

The strains were grown overnight at 37ºC on BM (2% Tryptose [Oxoid, Basingstoke, England], 0.5% NaCl [Merck, Darmstadt, Germany], 0.5% Yeast Extract [Merck, Darmstadt, Germany], 0.5% Glucose [Merck, Darmstadt, Germany], pH 6.85) agar. All reagents were obtained from Sigma (St. Louis, USA) unless otherwise stated. One loopful of cells from an overnight culture was washed 3 times in 1 ml EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl [pH 8.0]).

After centrifugation the pellet was resuspended in EC buffer (6 mM Tris-HCl [pH 7.6], 1 M NaCl (Merck, Darmstadt, Germany), 100 mM EDTA [pH 8.0], 0.5% Polyoxyethylene 20 cetyl ether [Brij 58], 0.2% deoxycholate, 0.5% N-laurylsarcosyl) and mixed with an equal volume of 1.6% (wt/vol) low melting-point agarose (Bio-Rad, Richmond, USA) in EC buffer and pipetted into plug molds. The solidified plugs were incubated overnight at 37ºC in 1 ml EC buffer-lysozyme solution (2.88 mg lysozyme per ml of EC buffer). The lysis buffer was replaced with 1 ml of protein digestion solution (3.3 mg pronase E in 1 ml EET buffer containing 1.6% [wt/vol] SDS) and the plugs were incubated again overnight at 37ºC. The agarose plugs were washed 3 times for 1 h in EET buffer, twice for 1 h in Milli-Q water and once for 1 h in the appropriate restriction buffer (Buffer Y+/Tango, MBI Fermentas, St.Leon-Rot, Germany) at room temperature. The restriction was carried out overnight at 27.5ºC in 300µl restriction buffer containing 30 units Sma I (MBI Fermentas). The digestion was stopped by adding 0.5 ml of 0.5 M EDTA (pH 8.0) and the plugs were stored at 4ºC. The restriction fragments were separated by PFGE in a contour clamped homogeneous electric field MAPPER system (Bio-Rad) by loading pieces of the plugs in 1% (wt/vol) Pulsed-Field Certified Agarose (Bio-Rad) gel prepared with a 0.5X TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA). Electrophoresis of *E. casseliflavus* was performed in 2 l 0.5X TBE at 14ºC for 22 h at 6 V/cm, an angle of 120º with pulse times ramping linearly from 0.41 s to 15.11 s. For all the other *Enterococcus* strains pulse times ramping linearly from 5 s to 30 s were chosen.

A *Staphylococcus aureus* strain (R-6314, Department for Medical Microbiology, University of Antwerp) was used as a molecular weight marker. The genome was prepared as described above with the exception that 500 U of
mutanolysin were added to the lysozyme solution. The gels were stained with ethidium bromide.

**Species identification by SDS-PAGE.** Cells were grown for 24 hours on BM-agar at 37°C under microaerophilic conditions. Whole cell protein extracts and protein separation was carried out as described by Gelsomino et al. (Gelsomino et al., 2002) on 1 to 3 isolates from each clone clustered by PFGE.
Results

**Total counts of enterococci in cheese and human feces.** The cheese used contained an average of $3.2 \times 10^4$ enterococci/g (sd = $5.6 \times 10^3$; data not shown). Figure 1 shows the number of enterococci in the fecal samples as well as the clonal diversity at each sampling point. A black bar on the x-axis illustrates the period of cheese consumption. The subjects started eating the cheese in week 4 during which no samples were taken.

**Figure 1.** Diagrams showing the numbers of enterococci and the species and strains recovered from the feces of single subjects before, during and after cheese consumption. (Fs = *E. faecalis*, Fm = *E. faecium*, Cs = *E. casseliflavus*, Ds = *E. durans*, He = *E. hirae*). Numbers refer to different clones. The black bars show the period of cheese consumption. Column-colors in the legend refer to the strains that were recovered from both humans and cheese.
Fig. 1. continued

Subject B

Subject C

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Recovery of enterococci in the feces varied from subject to subject and from week to week. In subject A, the level of enterococci remained stable during the first 6 weeks at $1.9 \times 10^3$ cfu/g after which it increased to $1.6 \times 10^6$ cfu/g. Subject B had stable numbers of enterococci in the first 3 weeks ($8.7 \times 10^4$ cfu/g) and in the last 3 weeks ($1.8 \times 10^5$ cfu/g) but the level peaked at $5.7 \times 10^7$ cfu/g during cheese consumption. Subject C had numbers of $\sim 1 \times 10^8$ cfu/g during the first five weeks and a lower number during the last 3 weeks ($9.1 \times 10^5$ cfu/g). The number of enterococci in the control subject increased during the first 3 weeks from $4.1 \times 10^5$ cfu/g to $3.4 \times 10^7$ cfu/g, remained stable around $7.3 \times 10^5$ cfu/g between weeks 6-8 and decreased from $5.8 \times 10^6$ cfu/g to $1.3 \times 10^4$ cfu/g in the last three weeks (Figure 2).

**Species identification by SDS-PAGE.** Four hundred and eighty colonies were isolated from plates of the cheese showing $>10^2$ colonies. These were divided into yellow-pigmented, sticky colonies ($n = 443$) and white, thick-creamy colonies ($n = 37$) and counted. Forty randomly selected yellow-pigmented colonies were identified as *E. casseliflavus* and the 37 white colonies as *E. faecalis* by SDS-PAGE.
After PFGE had been performed on the fecal isolates and the band patterns had been clustered, 1 to 3 isolates from each clone were also identified by SDS-PAGE as *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. durans* or *E. hirae*.

During the pre-consumption period (weeks 1-3) subjects A, B and C showed the presence of high numbers of *E. faecium* in their feces (73%, 70% and 100% respectively). Other species were detected in lower numbers: *E. faecalis* (16.5%), *Enterococcus durans* (6.5%) and *E. casseliflavus* (3.5%) in subject A, and *E. faecalis* (26.5%) and *E. casseliflavus* (3.5%) in subject B. In the control subject the distribution of enterococcal species remained fairly stable during the entire experiment. *E. faecium* (84.5%) was the most frequently encountered species with much lower numbers of *E. faecalis* (11.0%) and *E. hirae* (4.5%).

During cheese consumption the detection frequency of *E. faecalis* was much higher in subjects A, B and C. Subject A showed high numbers of *E. faecalis* (76.5% of isolates) followed by lower numbers of *E. casseliflavus* (23.5% of isolates) while, in subject B, *E. faecalis* was the only species detected. In subject C, 33% of the

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**Figure 2.** Total counts of enterococci found in subjects A, B, C and the control person.
isolates were still *E. faecium* and they were all found in week 6. *E. faecalis* was found in weeks 5 and 7 (67% of isolates).

In the post-consumption period *E. faecium* appeared again in subjects A, B and C (56.5%, 46.5% and 56.5% of isolates, respectively) while *E. faecalis* was present at 40%, 53.5% and 40% of isolates, respectively. *E. hirae* was present in small numbers in subjects A (3.5%) and C (3.5%).

**Strain typing by PFGE.** A total of 437 isolates were typed by PFGE (360 human isolates, i.e. 10 isolates picked from 4 human fecal samples over 9 sampling points and 77 isolates from the cheese, i.e. 40 yellow and 37 white colonies). In the present study we labelled the *E. faecalis* clones Fs1-Fs32, the *E. faecium* clones Fm1-Fm69, the *E. casseliflavus* clones Cs1-Cs5, the *E. durans* clone Ds1 and the *E. hirae* clones He1-He5.

Thirty-nine *E. casseliflavus* isolates were identical to clone Cs2 and 1 to clone Cs1 of *E. casseliflavus* from the previous study (Gelsomino et al., 2002), while 33 *E. faecalis* isolates were identical to clone Fs1 and 4 to clone Fs2. It was presumed that the other 403 yellow isolates from the cheese were either clone Cs1 or Cs2. All these clones, except Fs2, were also found in the cheese in the previous study proving that the flora of this particular cheese remains constant over a period of at least 3 years. Clone Fs2 was found in the milk and the human feces in the previous study. Taking into consideration that the average enterococcal content in the cheese was $3.2 \times 10^4$ cfu/g cheese, we conclude that each gram of cheese contained at least $2.7 \times 10^2$ cfu of clone Fs2, $7.4 \times 10^2$ cfu/g cheese of clone Cs1, $2.2 \times 10^3$ cfu of clone Fs1 and ~2.9 $\times 10^4$ cfu of clone Cs2.

None of the clones detected in the cheese were found in the human feces in the 3 week pre-consumption period. The clones found in this period, especially in the control and in subjects A and B, were very diverse. Subject C showed different dominant clones in each week, Fm37 in week 1, Fm38 in week 2 and Fm39 in week 3 (Fig. 1).

During consumption of the cheese, *E. faecalis* clone Fs2, which was a minor component of the cheese, dominated the feces of all cheese-consuming subjects, especially subjects A (73.5% of isolates) and B (96.5% of isolates). In addition, the feces of subject A contained *E. casseliflavus* clones Cs1 (16.5%) and Cs2 (6.5%), both of which were also found in the cheese. The latter was the dominant clone in the
cheese. Subject C’s feces contained clone Fs2 but only in weeks 5 and 7; in week 6, *E. faecium* clone Fm38, which had also been found in the pre-consumption period, was the only clone detected (Fig. 1). During the first week of cheese consumption, a number of other *E. faecalis* clones were isolated one of which (Fs20) was isolated from the feces of two different subjects (B and C).

In the post-consumption period, *E. faecalis* clone Fs2 disappeared from the feces of all subjects, except subject B, where it was detectable for one more week. New clones of *E. faecalis* appeared in all subjects and *E. faecium* appeared again in subjects A, B and C. However, all these clones were different from the ones recovered in the cheese. As in the pre-consumption period, the feces of subjects A, B and C showed dominant clones, e.g. Fm44 and Fs25 in weeks 10 and 11 in subject C. All the clones found in the feces of subject C are shown in Fig. 3. Clone Fm38 was recovered in the pre- and post-consumption periods as well as during the cheese consumption period.

Comparison of the PFGE band patterns of all fecal isolates was made with GelCompar and visually. All clones, except clone Fs20, present in the feces of one subject were unique to that subject and were not present in another subject (data not shown).
Figure 3. PFGE agarose gel showing the strains recovered from subject C. A *Sta. aureus* strain was used as molecular weight marker (SA). Clone Fm38 was found before, during and after the cheese consumption period.
Discussion

The aim of this study was to determine the common clones of enterococci in human feces and the effect that daily consumption of a raw milk cheese, containing known clones of enterococci, has on the fecal enterococcal microflora. We assumed, like Tannock et al. (Tannock et al., 2000), that the bacteria in feces reflect the bacteria in the distal large bowel although a study by Savage (1997) claims that fecal samples alone cannot reveal the composition and localization of bacterial communities in the colonic lumen. However, it is the common method used to study the flora of the GIT (Bunte et al., 2000; Kimura et al., 1997; Sears et al., 1949; Sørensen et al., 2001).

The common enterococcal flora in humans was studied during three weeks (pre-consumption period) in subjects A to C and during 12 weeks in the control subject. Only the clones isolated from the feces during the first three weeks were considered to reflect the regular flora as the effect of cheese consumption could last through the post-consumption period. Total enterococcal counts varied in each of the subjects from week to week. In the pre-consumption period, *E. faecium* was the dominant organism in all subjects (70-100%) followed by *E. faecalis*. These findings confirm the statements of Devriese et al. (1995) and Murray (1990) that *E. faecalis* and *E. faecium* are the dominant species in the human intestine. Generally, each sample of feces contained 2-7 different clones. Very often the subjects showed dominant clones or clones that are recovered over several weeks like Fm38 in subject C or Fm45 in the control (Figs. 1 and 3). According to the definition of Sears et al. (1949) these clones are resident, whereas the clones that appear only once are transient. In the only cheese-consuming subject where a resident clone could be detected (Fm 38 in subject C), it is precisely this clone that comes back first after the cheese consumption. No resident clone was found in subjects A and B. This may be due to the low numbers of isolates taken per sample. *E. hirae* clone He4 in the control subject seems to be resident too, although it was found only twice. According to Sears et al. (1949) though, resident strains eventually disappear to be replaced by other resident strains.

During cheese consumption, the subjects showed a drastic change in their fecal flora. The cheese contained an average enterococcal count of 3.2 X 10^5 cfu/g,
which is within the range \((10^3 - 10^7 \text{ cfu/g cheese})\) of enterococci found in cheese by Fryer (1969). Four clones were detected in the cheese (Fs1, Fs2, Cs1 and Cs2). Three of them (Fs1, Cs1, Cs2) were the same as those recovered from the same cheese in the previous study (Gelsomino et al., 2002). The incidence of these clones differs when compared to the previous trials.

Also during cheese consumption no correlation seemed to exist between the amount of enterococci/g ingested and the number of enterococci/g in the feces as decreasing and increasing of numbers of enterococci occurred all the time. The majority of the strains isolated from the feces during the consumption period belonged to the same clones as those detected in the cheese. Although the cheese contained primarily *E. casseliflavus* clone Cs2, almost all clones detected in the feces of subjects A, B and C belonged to *E. faecalis* clone Fs2 (Fig. 1). Why the finding of clone Fs2 was interrupted by clone Fm38 in subject C is not clear but the reasons might be found in dietary habits or in the low number of isolates taken. Clone Fs2, although a minor component of the enterococcal clones in the cheese, apparently thrived best in the human intestine as soon as cheese consumption began, and disappeared as soon as the cheese consumption came to an end. This clone most likely found optimal conditions in the bowel and proliferated as long as there was a continuous supplementation of that particular clone. It is possible that other enterococcal strains were brought into the human GIT by consuming the cheese but were missed because they were in low numbers. An example of this happening is that *E. faecalis* clone Fs20 was found twice in subject C and once in subject B. Sørensen et al. (38) proved, in a similar experiment, that enterococci in a suspension, pass the GIT, but in contrast, in this study we could prove the effect of the food, in this case cheese, as a carrier. Similar studies with other organisms (*Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*) confirm the present finding (Bunte et al., 2000; Matsumoto et al., 2000; 38).

A similar result was found in a previous study (Gelsomino et al., 2002) where isolates from the raw-milk, the cheese and the milking equipment were compared with isolates from the four cheesemakers’ fecal samples. In that study three clones, 2 of *E. casseliflavus* (Cs1 and Cs2) and 1 of *E. faecalis* (Fs1), largely dominated the milk, cheese and human fecal samples. Clone Fs2 was never isolated from the cheese in that study; however, it was found in the raw milk and in the human feces. In the
present study, this clone was present in very low numbers in the cheese but was the dominant clone isolated from the feces during the cheese consumption period. Why different clones dominate the feces of the Irish cheesemakers and the Belgian consumers is not known. This is due to the two groups living in different geographical areas, as stated by Franz et al. and Murray (Franz et al., 1999; Murray, 1990) or, that the dominant clones of the cheese also dominate the GIT of the cheesemaking family due to consumption over several years. These findings support the idea that the fecal flora of the cheesemaking family reflects consumption of the cheese rather than human fecal contamination of the milk (Gelsomino et al., 2002).

According to Franz et al. (1999), enterococci are able to colonize the GI tract as they are part of the normal intestinal flora. The allochtonous clone Fs2 (found in a place other than where it originated from) is, however, not able to colonize the intestine as it disappears quickly after cheese consumption ends. In subjects A and C, clone Fs2 disappears when cheese consumption stops while, in subject B, it remains for another week (Fig. 1). This may be subject dependent. Tannock et al. (2000) obtained similar results with a strain of *Lactobacillus rhamnosus*. The clones probably undergo a reduction in numbers due to the variable conditions in the stomach, the changing nature of the intestinal contents, the rate of movement, the competition for nutrients or binding cites etc. There may also be an antagonistic effect between the enterococci or other bacteria in the GIT and the new enterococci from the cheese such as clone Fs2 which can only be detected in the feces when relatively large amounts of it are consumed.

One of the first reports of a similar experiment was that of Sears et al. (1949). *E. coli* strains were swallowed deliberately in large numbers but they were recovered for limited periods only and were not established as residents. The present findings corroborate these results. Enterococci do not seem to adhere to the intestinal mucosa. Although adhesion is regarded by numerous authors as an essential feature for probiotics (Dunne et al., 2001; Morelli, 2001; Tannock et al., 2000) there is concern over the use of probiotic bacteria that contain antibiotic resistance genes as these could be transmissible to other bacteria especially if adhering to the mucosa. Another recommendation regards the level of viable cells. According to Ziemer and Gibson (1998) probiotics should establish and flourish in the intestine while others (Garg and Mital, 1991; Reid, 2001) recommend a daily intake of at least $10^7$ cfu/g or ml. *E.*
faecalis clone Fs1 and *E. casseliflavus* clone Cs2 were present in the cheese in relatively high numbers, 2.2 \( \times 10^3 \) and 3.0 \( \times 10^4 \) cfu/g, respectively. But it is *E. faecalis* clone Fs2 which is present in the cheese at only 267 cfu/g (giving a daily consumption of 2.6 \( \times 10^4 \) to 4.0 \( \times 10^4 \) cfu) which proliferates and colonizes the intestine during cheese consumption. These results prove that a clone present in very low numbers in cheese can establish itself in the intestine without having to be present in the food in high numbers.
Acknowledgments

This work was funded by a Walsh Fellowship from Teagasc, Dublin. The authors wish to thank Dick and Anne Keating for supplying the cheese and the 4 volunteers who eat the cheese.
References


CHAPTER 9.

ANTIBIOTIC RESISTANCE AND VIRULENCE TRAITS OF ENTEROCOCCI ISOLATED FROM AN IRISH ARTISANAL CHEESE

Roberto Gelsomino¹, Geert Huys¹, Klaas D'Haene¹, Marc Vancanneyt², Timothy M. Cogan³, Charles M. A. P. Franz⁴, and Jean Swings¹,²

¹ Laboratory of Microbiology, ² BCCM/LMG Bacteria Culture Collection, Ghent University, K. L. Ledeganckstraat 35, Gent, Belgium
³ National Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland
⁴ Federal Research Centre for Nutrition, Institute of Biotechnology and Molecular Biology, Haid-und-Neu-Strasse 9, D-76131 Karlsruhe, Germany

Submitted to the Journal of Food Protection
Abstract

A representative selection of 8 *Enterococcus* strains from a collection of over 600, previously isolated from an Irish artisanal cheese, was subjected to phenotypic and genotypic analysis of their antibiotic resistance and virulence properties. Resistance genes encoding resistance to tetracycline (*tet*(M) and *tet*(L)) and/or erythromycin (*erm*(B)) were detected in five strains. In addition, all strains were found to contain two or more virulence genes of those tested (*agg*, *gel*, *cyl*, *esp*, *ace*, *efaAfs* and *efaAm*). Further investigation into the transferability and environmental dissemination of these resistance and virulence traits will allow risk assessment and safety evaluation of the consumption of artisanal cheeses.
**Introduction**

Enterococci can be found in a variety of cheeses made from raw or pasteurised milk (Cogan et al., 1997). Some authors have reported beneficial roles for them in developing the sensory properties of cheese while others have attributed probiotic characteristics to them (Franz et al., 1999). Their application in food preservation results from their antilisterial activity based on bacteriocin production (Franz et al., 1999, Giraffa et al., 1995; Nuñez et al., 1997).

Enterococci are frequently encountered nosocomial pathogens (Noskin, 1997) and cause infections like bacteraemia, endocarditis, neonatal central nervous system and urinary tract infections (Hardie and Whiley, 1997). In addition to their intrinsic antibiotic resistance to cephalosporins and low level resistance to clindamycin and aminoglycosides, an increasing number of food enterococci have developed atypical resistances against various therapeutic antibacterial agents including vancomycin (Giraffa et al., 2002; Robredo et al., 2000; Wegener et al., 1997), tetracyclines (Teuber et al., 1999; Huys et al. 2004), gentamicin (Donabedian et al., 2003) and streptogramin (Simjee et al., 2002). The increase of vancomycin-resistant enterococci is of particular importance, as in many cases vancomycin is considered the last resort for treatment of multiple-resistant *Enterococcus* infection (Murray, 1990).

A number of virulence factors have been linked to the pathogenicity of *Enterococcus faecalis* including cytolysin (CylA), enterococcal surface protein (Esp), aggregation substance (Agg), and gelatinase (GelE). The cytolysin CylA causes rupture of a variety of membranes, including those of bacterial cells, erythrocytes, and other mammalian cells (Huycke et al, 1998). The Esp determinant can cause disease in mouse and may assist enterococci to evade detection by the immune system (Shankar et al., 1999). The aggregation substance is believed to facilitate formation of mating aggregates with nearby recipients as well as facilitate binding to eucaryotic cells, which may be crucial steps in colonization and infection (Franz et al., 2003). Other enterococcal adhesins include the adhesin of collagen in *E. faecalis* (Ace) (Nallapareddy et al., 2000) and the adhesin-like *E. faecalis* and *E. faecium* antigen A (EfaA_{fs} and EfaA_{fm}, respectively) (Franz et al., 2003). Gelatinase is a protease that hydrolyses bioactive peptides. Gelatinase producing *E. faecalis*...
were commonly found in intensive care units in Germany and in patients with endocarditis and other nosocomial infections (Kühnen et al., 1988; Coque et al., 1995). Franz et al. (2001) reported that 10.4% and 78.7% of *E. faecium* and *E. faecalis* strains, respectively, isolated from cheese, were positive for one or more of these virulence factors.

Current evidence suggests that virulence and antibiotic resistance properties in enterococci are often strain-specific (Huys et al., 2004; Eaton and Gasson, 2001; Franz et al., 2001). This finding may have important implications for the safety evaluation of industrial *Enterococcus* strains used in food manufacturing and of food products that contain enterococci as natural constituents. The objective of the present study was to determine the presence of antibiotic resistance and virulence traits among a set of eight enterococcal strains. These strains were shown to be the representative strains of the dominant enterococcal flora in the microbial ecosystem of Baylough, an Irish artisanal cheese.
Material and Methods

Bacterial strains. The strains used in this study were isolated from Baylough, an Irish artisanal cheese, and were identified and typed in the course of previous studies (Gelsomino et al., 2001; 2002). The 8 strains in question represent unique strain types observed among over 600 isolates obtained from the cheese mentioned above. Three of them, LMG 19844, LMG 19845 and LMG 20235 (original isolate nr. Cs1, Cs2 and Cs3 respectively) belong to the species *E. casseliflavus*, and 5 strains, LMG 19846, LMG 20228, LMG 20229, LMG 20230 and LMG 20234 (Fs1, Fs2, Fs3, Fs4 and Fs6, respectively) belong to *E. faecalis* (Table 1). All strains have been deposited in the BCCM™/LMG Bacteria Collection, Gent University, Gent, Belgium (http://www.belspo.be/bccm/lgm.htm#main).

Phenotypic and genotypic assessment of antibiotic resistance traits and detection of int genes. Strains were screened for phenotypic resistance to 12 antibiotics using the disc diffusion method and Mueller-Hinton agar (Oxoid). The following antibiotic discs (Oxoid) were used: tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), ampicillin (10 µg), streptomycin (10 µg), kanamycin (30 µg), rifampicin (30 µg), vancomycin (30 µg), erythromycin (15 µg), gentamicin (10 µg), trimethoprim (1.25 µg), and chloramphenicol (30 µg). When available, inhibition zones were interpreted following the guideline tables of the National Committee for Clinical Laboratory Standards (1999). All isolates were subjected to MIC testing in the range of 4-512 µg/ml tetracycline based on the micro-broth dilution method using cation-adjusted Mueller-Hinton II broth (Beckton-Dickinson 212322, Maryland) as recommended by NCCLS (1999). Following a 24 h incubation, the MIC value was visually read from a microtiter plate. In cases of doubt, the MIC was read as the concentration at which ≥80% inhibition of growth occurred. Each batch of MIC determinations included the control strain *E. faecalis* LMG 8222 (= ATCC 29212).

Total genomic DNA was prepared using a protocol based on the method of Pitcher et al. (1989). For all detection assays, a common PCR core mix (total volume, 50 µl) was used consisting of 1×PCR buffer (Applied Biosystems, Warrington, United Kingdom), deoxynucleoside triphosphates (Applied Biosystems) at a concentration of 200 µM each, 1 U of AmpliTaq DNA polymerase (Applied
Biosystems), and 20 pmol of each primer (Sigma-Genosys Ltd., Cambridgeshire, UK). A 50 ng portion of intact total DNA was used as PCR template. The presence of efflux genes \textit{tet}(K) and \textit{tet}(L) was determined as previously described (Gevers et al., 2003). The \textit{tet} genes encoding ribosomal protection (RP) proteins were detected using the RP-group specific primer pairs DI and DII (Gevers et al., 2003) and Ribo2-FW and Ribo2-RV (Aminov et al., 2001). Isolates harbouring an RP-type \textit{tet} gene were further subjected to PCR detection of \textit{tet}(M), \textit{tet}(O), and \textit{tet}(S) as previously described (Gevers et al., 2003). In addition to \textit{tet} gene detection, the presence of erythromycin resistance gene \textit{erm}(B) (Gevers et al., 2003) and of the vancomycin resistance genes \textit{van}A, \textit{van}B, \textit{van}C1 and \textit{van}C2 (Dutka-Malen et al., 1995) was investigated using previously described PCR primers. The occurrence of conjugative transposons of the Tn916-Tn1545 family was determined using the primers Int-FW and Int-RV targeting the transposon integrase (\textit{int}) gene (Doherty et al., 2000; Gevers et al., 2003). All PCR reactions were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) using previously described amplicon conditions for each primer set (Gevers et al., 2003). PCR amplicons were subjected to electrophoresis on 1% agarose gels and visualised using ethidium bromide fluorescence.

**Phenotypic and genotypic assessment of virulence traits.** Genomic DNA was prepared as described previously (Lewington et al., 1987). PCR assays were performed using pairs of previously reported primers for the detection of the following genes (positive control strains are indicated between brackets): \textit{agg} (\textit{E. faecalis} LMG 20947 [FAIR-E 404] and plasmid pAD1), \textit{gel} (\textit{E. faecalis} LMG 20869 [FAIR-E 313] and \textit{E. faecalis} LMG 20823 [FAIR-E 265]) \textit{efa}A\textsubscript{fs}, \textit{efa}A\textsubscript{fm} (\textit{E. faecalis} MMH594 and \textit{E. faecium} BFE 2262, respectively) (Eaton and Gasson, 2001), \textit{esp} (\textit{E. faecalis} MMH594) (Shankar et al., 1999), \textit{ace} (\textit{E. faecalis} V583) and \textit{cyl}A (\textit{E. faecalis} LMG 20681 [FAIR-E 77]) (Rich et al., 1999). Production of gelatinase on gelatin agar was performed as previously described (Franz et al., 2001). For the detection of haemolysin/cytolysin the strains were streaked on commercially obtained horse blood agar plates (Columbia Agar with 5% horse blood, Becton Dickinson, Heidelberg, Germany), incubated at 37°C for 3 days and examined daily for production of clear zones of beta haemolysis. All virulence traits studied have previously been found in human isolates.
Results and discussion

During previous studies (Gelsomino et al., 2001; 2002) over 600 isolates were obtained from Baylough, an Irish artisanal cheese, over a period of 15 months. All isolates were typed with Pulsed-field gel electrophoresis from which only 8 unique strain types were found. One representative strain of each of these 8 types was included in this study and considered to represent the total enterococcal flora of the Baylough cheese over a period of 15 months. Results from disc diffusion testing indicated that all strains, regardless of their species designation, were susceptible to ampicillin, vancomycin, gentamicin, rifampicin, and trimethoprim (the last two are based on inhibition zones of >21 and >23 mm, respectively, as no NCCLS guidelines are available) and showed uniform resistance to streptomycin when using the 10 µg disc. However, it should be highlighted that a disc with a higher content of streptomycin (300µg) is recommended to record high-level resistance in enterococci. The vancomycin-susceptible phenotype was confirmed by the finding that none of the strains contained the \textit{vanA}, \textit{vanB}, and \textit{vanC1} resistance genes (data not shown). The three \textit{E. casseliflavus} strains contained the \textit{vanC2} gene, which is in agreement with the results of Dutka-Malen et al. (1995) showing that this species is intrinsically resistant to low levels of vancomycin. The fact that these three strains did not show a phenotypic vancomycin resistance could be explained by the inability of the disc diffusion method to recognize low-level resistances to vancomycin. Strain-specific resistance traits were observed for the tetracyclines (5 of 8 strains), kanamycin (4 of 8 strains), chloramphenicol (3 of 8 strains), and erythromycin (1 of 8 strains). Except for \textit{E. casseliflavus} strain LMG 19845, which displayed tetracycline resistance (Tc$^R$), all observed resistances were restricted to \textit{E. faecalis} strains (Table 1). \textit{E. faecalis} strain LMG 20230 displayed multiple resistance to all four antibiotics. Teuber et al. (1999) have reported that multiple resistance to tetracycline, erythromycin, chloramphenicol, and gentamicin can occur among \textit{Enterococcus} isolates from various types of cheese.

Resistance to tetracyclines, erythromycin, and chloramphenicol are considered atypical and thus possibly acquired in enterococci (Teuber et al., 1999). In the present study, we further investigated the molecular basis of the first two mentioned resistance phenotypes. As shown in Table 1, resistance to tetracycline in four \textit{E.}}
faecalis isolates and in E. casseliflavus LMG 19845 was linked to the presence of the ribosomal protection (RP) gene tet(M) sometimes in combination with the efflux gene tet(L) (Table 1). To our knowledge, this is the first report of the tet(M) gene occurring in members of the species E. casseliflavus. The single strain displaying erythromycin resistance, E. faecalis LMG 20230, contained the macrolide resistance erm(B) gene which is widespread among food enterococci (Teuber et al., 1999). The observation that all TcR isolates carried a representative of the RP tet gene group complies with the minocycline resistance phenotype found in disc diffusion testing, which is mediated only by RP mechanisms in enterococci (Chopra and Roberts, 2001). The two E. faecalis strains that showed the highest MIC value for tetracycline (256 µg/ml) harboured both tet(M) and tet(L), whereas the remaining strains which displayed lower MIC values in the range of 32-128 µg/ml, contained only tet(M) (Table 1). This reinforcement effect has recently also been reported by Huys et al. (2004) for food enterococci and seems to indicate that strains containing both an active efflux and a RP mechanism can achieve a higher level of Tc resistance. As expected, no tet genes were detected in the strains showing the lowest MIC (E. casseliflavus LMG 19844 and LMG 20235 and E. faecalis LMG 20234).

In the majority of enterococci and streptococci originating from clinical or food sources, Tc resistance genes are more frequently located on conjugative transposons than on plasmids (Teuber et al., 1999). From the five tet(M)-carrying strains included in this study, four contained the integrase element int, indicating that they harbour a member of the broad host range Tn916-Tn1545 conjugative transposon family (Table 1). Members of this family carry the tet(M) gene, which is sometimes joined by genetic elements encoding resistance to additional antibiotics such as erythromycin and kanamycin in the case of Tn1545 (Clewell et al., 1995). Conjugal transfer of Tn916-Tn1545-associated tet(M) and of tet(L) genes by food enterococci has previously been reported for isolates from cheese and meat products (Teuber et al., 1999; Huys et al., 2004). In order to assess the potential risk involved with the presence of resistance gene-carrying enterococci in the cheese investigated in this study, detailed analyses of the potential mobility of the detected tet or erm genes are required.
Table 1. Phenotypic and genotypic virulence traits and antibiotic resistance properties of Enterococcus isolates from Baylough cheese.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LMG number</th>
<th>code</th>
<th>Gel&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Haem</th>
<th>cyl</th>
<th>agg</th>
<th>ace</th>
<th>efAfm</th>
<th>efAfs</th>
<th>Van genes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VanC2 gene</th>
<th>esp</th>
<th>Phenotypic resistance&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MIC (Tc) (mg/ml)</th>
<th>tet genes</th>
<th>Tn916</th>
<th>erm(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. casseliflavus</td>
<td>LMG 19844</td>
<td>Cs1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>St</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>LMG 19845</td>
<td>Cs2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>St, Tc</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>tet(M)</td>
<td>-</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>LMG 20235</td>
<td>Cs3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>St</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>LMG 19846</td>
<td>Fs1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>St, Tc</td>
<td>32</td>
<td>tet(M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>LMG 20228</td>
<td>Fs2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>St, Tc, Ka, Cm</td>
<td>256</td>
<td>tet(M), (L)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>LMG 20229</td>
<td>Fs3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>St, Tc, Ka, Cm</td>
<td>256</td>
<td>tet(M), (L)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>LMG 20230</td>
<td>Fs4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>St, Tc, Ka, Cm, Er</td>
<td>128</td>
<td>tet(M)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>LMG 20234</td>
<td>Fs6</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>St, Ka</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phenotypic assessment

<sup>b</sup> vanA, vanB, and vanC1

<sup>c</sup>Phenotypic resistances determined from disc diffusion susceptibility testing. Cm, chloramphenicol; Er, erythromycin; Ka, kanamycin; St, streptomycin; Tc, tetracycline.
All strains included in this study contained one or more virulence genes that have previously been found in human isolates. Except for *E. faecalis* strain LMG 20229 all strains carried the *gelE* gene, although phenotypic gelatinase activity could only be detected in two *E. faecalis* strains (LMG 19846 and LMG 20230). Similar results were obtained for haemolysis testing. All three *E. casseliflavus* strains and *E. faecalis* strain LMG 20230 showed haemolytic activity and carried the *cylA* gene, whereas two other *E. faecalis* strains (LMG 20228 and LMG 20234) displayed no haemolytic activity although they both carried the *cylA* gene. Collectively, these results seem to indicate that several of the investigated strains contained silent *gelE* and *cylA* genes. According to Eaton and Gasson (2001), there are several factors such as environmental or temporal parameters that may account for the apparent lack of phenotypic expression of enterococcal virulence genes. The same authors also reported up to 50% difference between gelatinase and haemolytic activity and the presence of GelE and CylA determinants in enterococci isolated from food. In a previous study of Franz et al. (2001), 48.9% of the investigated *E. faecalis* strains isolated from different cheeses displayed gelatinase activity. Other authors (Kühnen et al., 1988; Coque et al., 1995) have reported that 54-64% of enterococci isolated from nosocomial infections had a gelatinase positive phenotype. Haemolysin plays an important role in enterococcal virulence, and although this virulence property is believed to increase the severity of infection (Franz et al., 2001), it has also been found in faecal isolates from healthy individuals (Franz et al., 2003). Similar to the case of gelatinase, the presence of the *cylA* gene is not always linked to the phenotypic expression of haemolysin activity. It has been speculated that this phenomenon may be due to low-level gene expression or to the presence of an inactive gene product (Eaton and Gasson, 2001).

All strains contained the *agg* gene responsible for the clumping factor aggregation substance, which is often linked to haemolysin production encoded by the pheromone-responsive plasmids (Franz et al., 2001). The Agg factor has also previously been found in food and clinical isolates of *E. faecalis* (Eaton and Gasson, 2001; Franz et al., 2001). Süßmuth et al. (2002) have shown that the Agg factor promotes a greater increase in enterococcal adherence to macrophages and phagocytosis. Once phagocyted, enterococci promote intracellular survival probably due to inhibition of the respiratory burst (Süßmuth et al., 2000). In addition
to AS, some of the strains in this study also possessed the *ace* gene (4 of 8 strains) or the adhesin-like antibodies EfaA\(_{fm}\) (2 of 8 strains) and EfaA\(_{fs}\) (7 of 8 strains) (Table 1). It has been suggested that the EfaA\(_{fm}\) plays a role in endocarditis (Lowe et al., 1995).

Four strains (*E. faecalis* strains LMG 19846, LMG 20229, and LMG 20230 and *E. casseliflavus* strain LMG 19845) were found to contain the *esp* gene. The incidence of Esp+ enterococci was shown to be higher among clinical strains of *E. faecalis* than isolates from healthy individuals (Shankar et al., 1999). This was confirmed by Eaton and Gasson (2001) where the *esp* gene was detected in more than half of the clinical isolates but only rarely occurred in food enterococci.

Collectively, the current study has demonstrated that the enterococcal sub-population present in artisanal cheese types can harbour various antibiotic resistance and virulence traits. All virulence traits found in our strains have also been found in human clinical isolates. However, the current data set does not allow speculation on the safety evaluation of the cheese itself. Risk assessment requires research on the potential source and the *in-vitro* and *in-situ* transferability of these resistance and virulence properties.
Acknowledgments

This study was made possible thanks to a Walsh Fellowship from Teagasc, Dublin, Ireland. Part of this work was supported by the Fund for Scientific Research - Flanders (Belgium) (F.W.O.-Vlaanderen) (contract G.0309.01). G. Huys is a postdoctoral fellow of the Fund for Scientific Research - Flanders (Belgium) (F.W.O.-Vlaanderen).


Part III
Conclusions
10.1 Conclusions

Enterococci are present in the GIT of both humans and animals and in the farm environment. In addition, the nature of these bacteria, which allows them to survive and grow in harsh environments, makes them ideal to study the source of enterococci in raw milk cheeses.

The diversity of enterococci in an Irish farmhouse cheese and the farm’s environment were studied in order to characterize the enterococcal microflora present in the human and animal intestinal tract, the milk, the cheese and the manufacturing equipment. A further aim consisted of tracing and locating the enterococci back to their source. The following conclusions were drawn:

- The source of contamination were neither the cows nor the humans but the milking equipment. This was not expected but was not surprising either, as it is known that enterococci can accumulate in joints and fittings of milking machines infecting the milk directly (Slaghuis et al., 1991). In addition, *E. faecium* is the most frequently occurring *Enterococcus* species in dairy cows (Devriese et al., 1992) but none were found in the cheese. This was also corroborated by the abundant findings of *Streptococcus bovis* in the cows’ faeces while none were found in the cheese. The same few enterococcal clones repeatedly survive chlorine sterilization and infect the milk each time anew. We are not aware of any other study where such an epidemiological approach was used.

- The enterococcal population of the milk and cheese was constant over time as shown by the results of the three trials. The same strains present in milk and cheese dominate the enterococcal flora in the human GIT of the cheese-
making family. Three strains, two *E. casseliflavus* and one *E. faecalis*, were the most abundant. From the literature we know that *E. faecalis* is the dominant species in the human intestine and that *E. faecalis* and *E. faecium* are very commonly isolated from cheese (Murray, 1990; Cogan et al., 1997; Franz et al., 1999). A surprising finding was that *E. casseliflavus*, although never found in such large numbers, was present in cheese and in human faeces. This finding was confirmed in all trials. It was assumed that an analogous flora in the farmhouse family was due to cheese consumption.

We performed a cheese consumption study to determine the common enterococcal clones in human faeces and cheese, in which three persons consumed Baylough cheese on a daily basis for three weeks. This led to the following conclusions:

- Enterococci ingested with food survive trans-intestinal passage. Our result confirms a study by Sørensen et al. (2001) although they did not use food as a carrier, which might influence the passage through the GIT. Similar studies, but with other organisms, were performed by Bunte et al. (2000) and Matsumoto et al. (2000) with the same outcome.

- Enterococci present in very small numbers in cheese ($2.7 \times 10^2$ cfu/g) can survive and proliferate in the human GIT. It was surprising that this strain could establish itself so well and grow to such large numbers in the GIT. The presence of this strain, however, stopped when cheese consumption came to an end. Adherence and persistence in the GIT causes some dispute. Some authors regard it as an essential feature (Ziemer and Gibson, 1998; Dunne et al., 2001) while others are concerned over the use of probiotic bacteria that contain antibiotic resistance genes and recommend that strains, which do not establish in the GIT be used (Garg and Mital, 1991; Reid, 2001).

In the last phase of our study we determined antibiotic resistance (genes) and potential virulence (genes) of the enterococci from Baylough cheese and we concluded that:

- In several strains we detected resistance genes to tetracycline and erythromycin. All strains contained two or more virulence genes. Various
studies report the finding of enterococci harbouring antibiotic resistances and potential virulence traits from food in general, among which also cheese (Eaton and Gasson, 2001; Franz et al., 2001). Knowledge on the spread of antibiotic resistance and virulence genes is still very limited. As suggested by Perreten et al. (1997) epidemiological studies are necessary to clarify the contribution of antibiotic resistant microorganisms in food to the general phenomenon of the antibiotic resistance, which, in clinically important and pathogenic species, has become an almost worldwide occurrence.

To our knowledge this is the first study concerning the source of enterococci in a raw milk cheese combined with the determination of their antibiotic resistance and virulence factors. This study shows that potentially pathogenic enterococci may be found in food. We realise that the extent of risk has still to be established. Of course it has to be considered that this is a case study with one type of cheese (Baylough) from one particular farm in Ireland, and it is premature to draw conclusions about cheeses made in other farms.

10.2 Perspectives

Before resistance genes and virulence traits were discovered in them, enterococci were considered relatively innocuous lactic acid bacteria and regarded as GRAS (generally recognized as safe). The emergence of VRE has begun to worry the disease community for mainly two reasons. First of all, VRE have shown in vitro to be able to transfer their vancomycin resistance gene to Staphylococcus aureus. Secondly, enterococci resistant to vancomycin leave few other possibilities as therapeutic alternative. The spread of VRE was different in USA and Europe. While in the USA a careless and imprudent use of antibiotics in medical surroundings seemed to be the key factor (McDonald et al., 1997), in Europe the use of avoparcin as growth promoter seems to be the largest contributor to vancomycin resistance (Collignon, 1999; Teuber et al., 1999). Next to the antibiotic resistance there are
virulence traits such as cytolytic toxin, gelatinase and aggregation substance, which are believed to enhance the ability to cause disease. Several questions remain unanswered. The knowledge on the consequences of the known virulence factors is not satisfactory. It is also not yet clear whether enterococci, which possess several virulence traits, pose a higher risk than enterococci with only one virulence trait.

Surveillance, prevention, control and research are becoming increasingly important in food safety issues.

Surveillance can be done in hospitals and health care settings when specimens taken from diseased patients are routinely analysed for antibiotic resistance and virulence genes. It is important to monitor antibiotic use in human and veterinary medicine and in agriculture and link it to clinical information.

Prevention and control, in the day-to-day life, is also important in health care settings. Concern and worry was created by the study of Badri et al. (1998) who showed not only contaminated hands of gloved and ungloved health care workers but also the presence of VRE on their hands after glove removal. As shown by the Centers for Disease Control and Prevention, improved hand hygiene is able to terminate outbreaks in health care facilities, to reduce transmission of antimicrobial resistant organisms and reduce overall infection rates (http://www.cdc.gov/handhygiene/; Weinstein, 2001). Promotion of an appropriate use of antibiotics can help us to decrease the incidence of antibiotic resistant strains. In Europe this is especially important in agriculture. Several antibiotics used as growth promoters were banned between 1997 and 1998, including avoparcin, ardacin, bacitracin zinc, virginiamycin, tylosin phosphate and spiramycin. After that ban a sharp drop of VRE has occurred in Europe (Teuber et al., 1999). Some substances are still authorized as growth promoting agents but there are plans to ban also these four growth promotors (monensin sodium, salinomycin sodium, flavophospholipol and avilamycin) from 1 January 2006 (EU-common position, 2003). Another form of prevention is pasteurisation, which can reduce the number of enterococci in cheese although this will not please lovers of raw milk products. Also ultrafiltration (UF) is increasingly used in the industry. It is mainly used for milk standardisation but Teuber et al. (1996) recommend it to eliminate dangerous
bacteria, among them enterococci. One of the major problems with UF is fouling of the filtration membrane and this still requires further research (Razavi et al. 2004).

Detecting and researching enterococci has become important more recently since the discovery of virulence traits and antibiotic resistance genes. But enterococci have always been extensively used for their functional and safety properties. Research should provide genome sequences and other powerful tools to identify target genes such as antibiotic resistance and virulence trait genes. A comparison of genome sequences will help us to better understand the technological and health benefits of enterococci such as lactic acid production, proteolysis and lipolysis, production of antimicrobials and, from a medical point of view, such undesired genes as potential virulence genes, antibiotic resistance genes and biogenic amines (Franz et al., 1999; Sarantinopoulos et al., 2001). Another useful tool is DNA-arrays, which, in combination with whole genome sequences, allow sequence comparisons of a large number of strains and provide evidence of unwanted genes. The next aim for research should be to evaluate rapid, affordable and accurate diagnostic methods for human and veterinary uses, which can easily be implemented in routine settings. This way new strategies can be developed which prevent colonization and infection with enterococci containing undesirable genes. A topic, which deserves some attention, is how the source of virulence factors and antibiotic resistances in enterococci is linked to the whole bacterial population from the milk, the cheesemakers, the herd and the milking equipment and whether the virulence factors have their origin in organisms other than enterococci. This could not only reveal possible solutions in breaking the transfer chain but also draw our attention to genera that in our eyes are still innocuous, like enterococci were 20 years ago.
References


CHAPTER 11.

SUMMARY

Enterococci are natural residents of human and animal intestinal tracts and grow to high levels in a variety of artisanal cheeses. In the first part of this study we analysed the typical flora of Baylough cheese and determined the diversity of enterococci on a farm. For this purpose putative enterococci were isolated from the faeces of all the cows and all the people associated with the cheesemaking, from the milk and cheese during manufacture and ripening, and from the environment in three separate trials. In a first step almost 1800 putative enterococci were screened using a genus specific primer. The results indicated that all human, milk, curd and cheese isolates were members of the genus *Enterococcus*. RAPD-PCR was used to identify the enterococcal isolates. While the bovine faecal samples were dominated by *E. faecium*, the non-enterococcal strains of the cows were *Streptococcus bovis*. In the human faeces, milk and cheese samples *E. casseliflavus* were dominant, followed by lower numbers of *E. faecalis*. The same two species could be found in the environmental samples, the water in the milking parlour, rinses of the cows’ teats, the milking machine and the bulk-milk storage tank.

In the following part of this study PFGE (restriction enzyme *Sma*I) was used to type the enterococcal isolates to a clonal level. All strains that were isolated in the first part could be represented by nine strains: three *E. casseliflavus* (LMG 19844, LMG 19845, LMG 20235), five *E. faecalis* (LMG 19846, LMG 20228, LMG 20229, LMG 20230, LMG 20234) and one *E. durans* (LMG 20231). The same 3 clones, 1 of *E. faecalis* (LMG 19846) and 2 of *E. casseliflavus* (LMG 19844 and LMG 1945), dominated almost all milk, cheese and human faecal samples. The two *E. casseliflavus* clones were also found in the bulk tank and the milking machine even after chlorination. This suggests the presence of a niche where enterococci could grow and contaminate the milk.
The question at this point arose whether the enterococci in humans and faeces were the same due to consumption of the cheese and whether enterococci would survive the passage through the GIT. In the study that followed the composition of the enterococcal faecal flora in three healthy humans was analysed before, during and after the daily consumption of cheese made in the same plant under the same conditions. PFGE was used to determine the clonal relationship. Before and after consumption of the cheese, human samples contained mainly \textit{E. faecium}. During consumption of the cheese one particular transient clone of \textit{E. faecalis}, clone LMG 20228, which was present in small numbers in the cheese \((2.7 \times 10^2 \text{ cfu/g})\), largely dominated the faeces. \textit{E. casseliflavus} LMG 18944 and LMG 18945, dominant in the cheese \((7.4 \times 10^2 \text{ en } 2.9 \times 10^4 \text{ cfu/g cheese})\) were also found in the faeces of one of the subjects during cheese consumption.

The same nine clones recovered from cheese and the environment were subjected to phenotypic and genotypic analysis of their antibiotic resistances and virulence properties. All strains showed phenotypic resistance to streptomycin. In those five strains which showed phenotypic resistance to tetracycline, genes encoding resistance to tetracycline \((\text{tet}(M) \text{ and } \text{tet}(L))\) were detected. In the only strain which showed an erythromycin resistant phenotype, the \text{erm}(B)\) gene were detected. Additionally, all strains were found to contain two or more virulence genes of those tested \((\text{agg, gel, cyl, esp, ace, efaA_{fs} and efaA_{fm}})\) resulting in some reservations about the safety of these strains.

An important conclusion in the present study is that cows’ faeces were not considered to be the source of enterococci in the cheese. \textit{E. faecium} and \textit{Str. bovis} largely dominated the cows’ intestinal tract but were not found in either the milk or the cheese. Furthermore we deduce that the homologies in the enterococcal population between cheese and farmers are found in the cheese consumption and that the cheese was contaminated by enterococci residing in the milking machine. A significant conclusion is also that a clone needs not to be present in a food in high numbers to establish itself in the intestine. Additionally, we conclude that cheese can contain enterococci with virulence traits and resistances to clinically important antibiotics.
It is and remains a case study, so conclusions for other farms should not be drawn. Additional studies about the putative source and safety aspects of enterococci are advised. DGGE would be very helpful in establishing the similarity between samples, which could have been resulted in less sampling. Nevertheless, the methods chosen for this study served its purpose very well.

In een verdere studie werd PFGE (SmaI restrictie enzyme) gebruikt om de enterokokken isolaten tot op klonaal niveau te typeren. Alle verzamelde isolaten behoorden tot slechts 9 stammen, drie E. casseliflavus stammen (LMG 19844, LMG 19845, LMG 20235), vijf E. faecalis stammen (LMG 19846, LMG 20228, LMG 20229, LMG 20230, LMG 20234) en één E. durans stam (LMG 20231). Drie van deze stammen, E. faecalis LMG 19846 en E. casseliflavus LMG 19844 en LMG
De twee *E. casseliflavus* stammen werden echter ook na reiniging en chlorinatie in de tank van kaasbereiding en in de melkmachine gevonden. Dit suggereert de aanwezigheid van een niche waar enterokokken blijvend aanwezig zijn en telkens opnieuw de melk besmetten.

In een daaropvolgende studie waren twee belangrijke vragen aan de orde: (i) Is een analoge enterokokkenpopulatie in het darmkanaal van de kaasproducenten en in de kaas toe te schrijven aan de consumptie van de kaas en (ii) kunnen enterokokken de passage door het gastrointestinaal kanaal overleven? Hiertoe werd de enterokokken flora in drie gezonde mensen voor, tijdens en na een dagelijkse consumptie van Baylough kaas geanalyseerd. Isolaten uit fecaal materiaal werden geïdentificeerd en getypeerd. PFGE werd gebruikt om de clonale verwantschap te bepalen. Voor en na de consumptie van kaas was in elk van de proefpersonen *E. faecium* dominant. Tijdens de consumptie van kaas overheerste hoofdzakelijk de van de kaas afkomstige *E. faecalis* stam LMG 20228. Deze kloon was slechts in kleine hoeveelheden in de kaas te vinden (2.7 x 10^2 cfu/g) en verdween uit het darmkanaal na consumptie. In een van de proefpersonen werden tijdens de kaasconsumptie ook de stammen *E. casseliflavus* LMG 19844 en LMG 19845, dominant aanwezig in de kaas (7.4 x 10^2 en 2.9 x 10^4 cfu/g kaas), gevonden.

Vervolgens werden de veiligheidsaspecten van de negen stammen uit de kaas en de omgevingsstalen bepaald. Met een fenotypische en genotypische benadering werd de aanwezigheid van antibiotica resistenties en virulentiefactoren nagegaan. Alle stammen waren resistent tegen streptomycine. In vijf stammen, die resistent waren tegen tetracycline, werd het *tet*(M) en *tet*(L) gen gedetecteerd. De stam waar het *erm*(B) gen aanwezig was toonde ook een fenotypische resistentie tegen erythromycine. In alle stammen werden bovendien twee of meer virulentiegenen gedetecteerd (agg, gel, cyl, esp, ace, efaAfs en efaAfms) en heeft tot besluit dat de veiligheid van deze stammen in vraag kan gesteld worden.

De belangrijkste besluiten van dit doctoraat zijn dat koeien niet de primaire bron van enterokokken besmetting in Baylough kaas zijn vermits *E. faecium* en *Str. bovis*, die met grote hoeveelheden in het darmkanaal van koeien aanwezig zijn, noch in de melk noch in de kaas gevonden werden. De onderzochte kaas werd telkens
opnieuw door dezelfde enterokokken gecontamineerd, aanwezig in de melkmachine en melktank, waar ze de reiniging overleven. Verder besluiten we dat een zelfde enterokokken populatie in de kaas en in het gastrointestinaal kanaal van de mensen toe te schrijven is aan de consumptie van kaas. Onze resultaten tonen aan, dat een *Enterococcus* stam in lage hoeveelheden aanwezig in voeding zich in het darmkanaal dominant kan manifesteren en groeien. Onze studies tonen aan dat de enterokokken uit Baylough kaas virulentiefactoren en resistenties tegen klinisch belangrijke antibiotica bevatten, wat hun veiligheid in vraag stelt.

We moeten ervan uit gaan dat conclusies niet veralgemeend kunnen worden naar alle kaasboerderijen. Bijkomende analoge studies omtrent de mogelijke bron en veiligheidsaspecten van enterokokken in andere ambachtelijke kazen zijn aangewezen. Om de werklast te verminderen en een snellere screening naar variaties in enterokokken populaties toe te laten zou DGGE een waardig alternatief kunnen vormen voor de uitzuivering van honderden isolaten. Niettemin hebben de gekozen methoden in deze studie de vooropgestelde vragen beantwoord.
**APPENDIX 1**

List of strains isolated in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>original number</th>
<th>LMG number</th>
<th>origin</th>
<th>year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. casseliflavus</em></td>
<td>Cs1</td>
<td>LMG 19844</td>
<td>milk bulk tank</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. casseliflavus</em></td>
<td>Cs2</td>
<td>LMG 19845</td>
<td>milk bulk tank</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. casseliflavus</em></td>
<td>Cs3</td>
<td>LMG 20235</td>
<td>cheese</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>D</td>
<td>LMG 20231</td>
<td>human faeces</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Fs1</td>
<td>LMG 19846</td>
<td>milk bulk tank</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Fs2</td>
<td>LMG 20228</td>
<td>milk bulk tank</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Fs3</td>
<td>LMG 20229</td>
<td>cheese</td>
<td>1998</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Fs4</td>
<td>LMG 20230</td>
<td>water</td>
<td>1999</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Fs6</td>
<td>LMG 20234</td>
<td>milking machine</td>
<td>1999</td>
</tr>
</tbody>
</table>
Appendix 2
Curriculum Vitae

Name: Gelsomino, Roberto
Date of Birth: 6th March 1972
Place of birth: Merano (Italy)

Education
1986-1991: secondary school “A. Einstein” with scientific background in Merano (Italy)
1995-1998: thesis at the Institut für alpenländische Milchwirtschaft in Rotholz (Austria), Magister der Naturwissenschaft (MSc degree in Microbiology; Hygiene and Medical Microbiology as option). Graduated in April 1998.
1998-1999: Beginning of the PhD at Teagasc, Moorepark, Dairy Products Research Centre, Fermoy, Ireland
since September 1999 PhD at the University Gent-Laboratorium voor Mikrobiologie
since September 2001: EU-project: “Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese”

Activities
-1996/1997: Chemistry teacher at the Technical School “G. Matteotti” in Merano (Italy)
-Since October 2002: some one-hour lectures and assistance in the practical courses of microbiology
-Supervision of a Master student (Hinde Benbrahim): “Karakterisering van de microflora van roodsmeerkazen”
Research papers:


Oral presentations:


Other:

ACKNOWLEDGMENTS
RINGRAZIAMENTI

When I decided to begin my PhD I knew I would have to live most of the time in Fermoy, Co. Cork and a few months in Gent. I had never heard of either of them but my adventurous soul made me accept. For personal reasons it turned out to be exactly the opposite but I have learned to appreciate the bad and good sides of both. The person who made this possible was the man who I felt looked at me not only as a PhD student but also as a person: Prof. Dr. Tim Cogan. Tim, it is not only your expertise but also your understanding, patience, kindness, helpfulness and big humanity that added considerably to my graduate experience. I doubt that I will ever be able to convey my appreciation fully, but I owe you my eternal gratitude.

I would like to acknowledge Teagasc for Walsh Fellowship funding and the opportunity to carry out this research.

I would like to express my gratitude to Prof. Dr. ir. Jean Swings for inviting me in the Laboratorium voor Microbiologie in Gent. Thanks to you I was able to continue my studies and this means a lot to me. Furthermore you gave me the possibility to learn more about academic life, thank you very much!

I appreciate also the help of the Keiting family. Ann and Dick, thank you for all your help, assistance, and support. This study was only possible thanks to you.

A very big thanks goes to Dr. Marc Vancanneyt, to his patience, his explanations, his big help. Marc, this work would not have been possible without you.

Another big thanks goes to the people in the lab in Ireland, Mary and Finbarr, thanks for helping me with all the plates in “triplicates”, and we are talking triple digits here! To the good friends I have made there Brid D., Brid L., Noelle, Nora – thanks for
the laughs. In Belgium thanks to Cindy and Karen, you were a precious help in the beginning and throughout the PhD, thanks also for my first words in Dutch ever (“een bruin broodje met kaas en hesp”). Thanks to everybody who helped me in learning everything that I know now, and for the laughs we had in the lab, Dirk D., Margo, Maggy, Liesbeth, Ben, Geert, Moudjahid, Leen, Ann, Katleen, Nele, Katrien, Bart, and Renata.

Paul, thanks a lot to you too. You were the gateway to cultural Vlaanderen in particular and Belgium in general, you taught me my first steps in appreciating all the old stuff I am stacking at home. It’s catching dust, but it is oh so beautiful!

Mamma, papà, e Omi, è difficile rinziarvi doverosamente in poche righe. Voglio solo dirvi grazie per avere sempre mostrato, per quello che faccio, una fiducia cieca e priva di incertezze, spronandomi sempre ad andare avanti per la mia strada. Grazie per il vostro appoggio, che mi avete dato, soprattutto per telefono, perché vi ho privato troppo spesso della mia presenza a casa.

Amici, italiani, conosciuti in Belgio: Savina, Cristina, Francesco e Alberto. Un grazie affettuoso anche a voi, per avere condiviso momenti di ansia ma anche tante situazioni divertenti e gratificanti.

Un grazie di cuore anche mia sorella Antonella. Non solo per l’aiuto e l’appoggio che mi hai dato ma anche per l’allegria che sprijungi e le risate che ci facciamo insieme.

Thank you too, Pery, I should have taught you how to cook Italian, but the “arroz a la Cubana” was just too yummy.

Thanks also to Silvie, who helped me in keeping weight. Thanks for cooking French for me and for not bypassing the chocolate isle at the supermarket.

A very big hug goes to Wolfgang, Andreas, Petra, Jochen, Martin and Heidi (Waikiki-Koko). With you I shared very important and intensive moments during my PhD and you were always there; in hard times, when I was really down and you didn’t
fail to support me, and in happy and funny moments, were we shared so many laughs.
Thank you so much!!

Het laatste dankwoordje aan mijn vrienden in Belgie: Greta, Wilfried en Geert voor de lachertjes, de vakanties (met de slang), de etentjes. Jurgen, Gregory en Peter voor de kookinspiraties, Daniel en Bart voor de etentjes, de babbeltjes en de competitieve squashavonden (“stond ik in de weg?”). Een grote dank ook aan Marino voor de steun, het gezelschap en al de hulp die je me gaf. En zeker voor de wetenschappelijke informatie in Melle: ik hoop de camionbestuurder, die jouw autodeur weggezogen heeft, het weet te waarderen. Ik doe dat in iedere geval.