

Enterococci in Milk Products

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**Mirtha Lorena Giménez-Pereira
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Abstract

This review examined the benefits and risks of enterococci in dairy products. Enterococci are ubiquitous bacteria present in the environment and in the gastrointestinal tract of healthy animals and humans. In milk products, they are used as probiotics resulting in positive effects on human digestibility. As adjunct starter cultures, enterococci release natural antimicrobial substances inhibiting adulteration due to food-borne pathogens. Thanks to the efficient utilisation of organic acids, enterococci contribute to the development of unique sensory characteristics in fermented dairy products. In contrast to these positive roles, some enterococcal strains were suspected to have pathogenic properties for humans, mainly based on specific virulence factors found in some strains of *Enterococcus faecalis* and to a lesser extent in strains of *Enterococcus faecium*. In addition, they were regarded as being resistant to several antibiotics. Since virulence factors and antibiotic resistance were found to be genetically encoded and transmissible, they may be transmitted to other enterococcal strains and even to other bacteria species. So far however, no genetic similarities and clear strain specificities have been observed among traits isolated from clinical or food sources. Thus, a pathogenic potential could only be associated with clinical strains, not food strains. Moreover, there is currently no evidence for pathogenic effects on humans. However, evidence for pathogenicity exists from three experimental models in animals. Due to the efficient removal of enterococci during processing, enterococci may be regarded as ‘contaminants’ if found in processed dairy foods.

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Table of Contents

Abstract	i
Acknowledgements	iii
Table of Contents	v
List of Figures	vii
List of Tables	ix
List of Abbreviations	xi
Chapter 1: Introduction	1
1.1 General perception of enterococci - benefits and risks of their presence in food, especially in milk and dairy products	1
1.2 International food standards with regards to enterococci	3
1.3 Importance of a risk analysis with regards to enterococcal presence in milk and milk products	3
1.4 Aims of this study	4
Chapter 2: Taxonomy of Enterococci	7
2.1 Introduction	7
2.2 Historical taxonomy	8
2.3 Current taxonomy	12
2.4 Conclusions	14
Chapter 3: Properties of Enterococci	15
3.1 General properties	15
3.1.1 Heat resistance	17
3.1.2 pH resistance	18
3.2 Biochemical properties of technological interest	19
3.2.1 Acid production	19
3.2.2 Proteolytic and peptidolytic activities	21
3.2.3 Lipolytic and esterase activities	21
3.2.4 Citrate and pyruvate metabolism	22
3.2.5 Production of volatile compounds	27
3.2.6 Bacteriocin production	28
3.3 Conclusions	30
Chapter 4: Laboratory Identification	33
4.1 General considerations	33
4.2 Routine methods of isolation, identification and confirmation of enterococci	34
4.2.1 Isolation and identification	34
4.2.2 Confirmation	35
4.3 Other methods: genotypic-based techniques	36
4.4 Conclusions	36
Chapter 5: Sources and Reservoirs	37
5.1 Primary sources	37
5.1.1 Animals	37
5.1.2 Humans	39
5.1.3 Environment	39
5.2 Secondary sources: enterococci in foods	43
5.3 Conclusions	45

Chapter 6: Enterococci as Pathogens?	47
6.1 Evidence for clinical disease in humans due to enterococci	47
6.1.1 Clinical infections	47
6.1.2 Risk factors of host and environment that may contribute to pathogenesis... 48	
6.1.3 Pathogenesis.....	49
6.1.4 Review of Hill’s criteria for causality.....	49
6.2 Risk factors of enterococci that may contribute to pathogenesis.....	55
6.2.1 Virulence determinants	55
6.2.1.1 Identified virulence determinants.....	56
6.2.1.2 Prevalence of virulence factors among enterococci.....	58
6.2.1.3 Regulation mechanisms of the virulence expression	61
6.2.2 Biogenic amines.....	62
6.2.3 Antibiotic resistance.....	64
6.2.4 Transfer of virulence determinant and antibiotic-resistant genes	71
6.3 Conclusions.....	73
Chapter 7: Enterococci in Dairy Foods.....	75
7.1 Enterococci in milk and dairy products	75
7.1.1 Enterococci as contaminants in milk	75
7.1.2 Functions of enterococci in dairy products	76
7.1.2.1 Starter cultures	77
7.1.2.2 Adjuncts (non-starter) cultures.....	78
7.1.2.3 Probiotics	79
7.1.2.4 Protective cultures: the role of the ‘enterocins’	81
7.1.3 Sanitary regulations - raw milk for production of dairy products.....	83
7.1.4 Effects of heat treatments on survival of enterococci	84
7.1.4.1 Pasteurisation of raw milk.....	84
7.1.4.2 Manufacture of dairy products.....	88
7.1.4.2.1 Milk powder	89
7.1.4.2.2 Cheese	91
7.1.4.2.3 Casein.....	93
7.1.4.2.4 Butter.....	95
7.2 Conclusions.....	98
Executive Summary	101
Appendix A: Selective media for the isolation of enterococci in foods and dairy products	105
Appendix B: Methods for confirmation of enterococci in dairy foods.....	111
Appendix C: Enterococci in cheeses.....	113
Appendix D: Enterococci in meats	117
Appendix E: Heat treatment of raw milk	121
References.....	125

List of Figures

Figure 1: The position of <i>Enterococcus</i> between benefit and risk in medicine as well as in food and agricultural sciences.....	2
Figure 2: Sherman's classification of the <i>Streptococcus</i> genus including the sub-species that fell within the D-antigen-producing group of enterococci.....	10
Figure 3: Schleifer and Kilpper-Bälz's re-classification of the genus <i>Streptococcus</i> into three different sub-genera	10
Figure 4: Schleifer and Kilpper-Bälz's differentiation of the old genus <i>Streptococcus</i> and separation of its <i>Lactococcus</i> and <i>Enterococcus</i> sub-genera to constitute new genera	11
Figure 5: The phylogenetic position of the genus <i>Enterococcus</i> demonstrated by a 16S rRNA-dendrogram of Gram-positive genera, including <i>Streptococcus</i> and <i>Lactococcus</i>	12
Figure 6: 16S rRNA-based tree reflecting the relationship of enterococcal sub-species	13
Figure 7: pH values attained by <i>E. faecalis</i> , <i>E. faecium</i> and <i>E. durans</i> after growth in skim milk for 6 hours at 37 °C	20
Figure 8: Correlation between citrate and pyruvate utilisation after growth of enterococci in modified MRS broth for 6 hours at 37 °C	25
Figure 9: Correlation between citrate and pyruvate utilisation after growth of enterococci in modified MRS broth for 16 hours at 37 °C	26
Figure 10: Percentage of susceptible, intermediate and resistant strains to 12 commonly used antibiotics.....	66
Figure 11: The survival curve of <i>E. durans</i> at milk commercial pasteurisation temperature (72 °C/15 seconds).....	86
Figure 12: The survival curve of <i>E. faecium</i> at milk commercial pasteurisation temperature (72 °C/15 seconds).....	87
Figure 13: Typical process flow for dairy products manufacture	89
Figure 14: Typical process flow for liquid milk and WMP manufacture	90
Figure 15: Typical process flow for cheese manufacture	93
Figure 16: Process stages in acid casein manufacture	95
Figure 17: Process stages in butter manufacture.....	97

List of Tables

Table 1: Current enterococcal different ‘species groups’ and their sub-species, based on their phylogenetic relationship within the genus	8
Table 2: Characteristic physiological properties of validly described enterococcal species	16
Table 3: Concentrations of main volatile compounds produced by enterococci of food, human and animal origin.....	27
Table 4: Well-characterised bacteriocins produced by <i>E. faecium</i> and <i>E. faecalis</i> strains	30
Table 5: Occurrence of enterococci in the gastro-intestinal tract	38
Table 6: Environmental samples collected in Europe (Sweden, Denmark, Spain and the United Kingdom) and the percentage of samples containing detectable amounts of enterococci	41
Table 7: Distribution of enterococci in food.....	44
Table 8: <i>E. faecium</i> strains isolated from cheese, sheep, and hospitalised patients, with their PCR results for virulence traits genes.....	60
Table 9: Common enterococcal virulence traits and the enterococcal species where they have been found	61
Table 10: Quantified (mg/l broth) biogenic amine production by 26 enterococci isolates	63
Table 11: Antibiotic resistance behaviour of selected <i>E. faecalis</i> and <i>E. faecium</i> strains isolated from samples of sausages, ham, minced meat, and cheese	69
Table 12: Biotechnological important LAB genera.....	79
Table 13: Characteristics of the <i>E. faecium</i> SF68 strain	81
Table 14: Heat resistance of three enterococci species in whole milk and non-fat milk, at 72 °C/15 seconds.....	85
Table 15: Pre- and post-pasteurisation counts of the most common enterococci found in raw milk (HTST at 72 °C/15 seconds).....	88
Table 16: Enterococcal results of analyses performed on 84 samples from 10 New Zealand milk powder manufacturing plants.....	91
Table 17: Numbers and predominance of <i>Enterococcus</i> spp. in cheeses from Mediterranean countries.....	115

List of Abbreviations

ABAB	Azide blood agar base
Ace	Adhesin of collagen from <i>E. faecalis</i>
AD broth	Azide dextrose broth
AS	Aggregation substance
BA agar	Bile aesculin agar
Bar medium	Barnes medium
BB broth	Bromocresol purple azide broth
BEA agar	Bile aesculin azide agar
BHI broth	Brain heart infusion broth
CATC agar	Citrate azide tween carbonate agar
CFU	Colony-forming units
CHEF	Contour-clamped homogenous electric field electrophoresis
Cyl	Cytolysin
DNA	Deoxyribose nucleic acid
EfaA _{fm}	Adhesin-like <i>E. faecium</i> endocarditis antigen
EfaA _{fs}	Adhesin-like <i>E. faecalis</i> endocarditis antigen
ESD medium	<i>Enterococcus</i> selective differential medium
Esp	Enterococcal surface protein
fGTC agar	Fluorogenic gentamicin thallos carbonate agar
FSR system	<i>E. faecalis</i> regulator system
G + C	Glycine + Cytosine
Gel	Gelatinase
GeE	Gelatinase gene
GREF	Glycopeptide-resistant <i>E. faecium</i>
HTST pasteurisation	High temperature short time pasteurisation
KAA medium	Kanamycin-aesculin-azide medium
LAB	Lactic acid bacteria
LDL cholesterol	Low-density lipoprotein cholesterol
mE agar	Membrane filter <i>Enterococcus</i> agar
mmol	Millimol/es
MRS medium	Man, Rogosa and Sharpe medium
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
ppm	Parts per million
PYR test	Pyrrolidonyl- β -naphthylamide test
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SB agar	Slanetz-Bartley agar
SMP	Skim milk powder
Spr	Serine protease
SprE	Serine protease gene
SREF	Streptogramin-resistant <i>E. faecium</i>
TS broth	Trypticase soy broth
TTC	Tripheniltetrazolium chloride
UHT process	Ultra high temperature process
VRE	Vancomycin-resistant enterococci
VSE	Vancomycin-susceptible enterococci
w/v	Weight/volume
WMP	Whole milk powder

Chapter 1: Introduction

1.1 General perception of enterococci - benefits and risks of their presence in food, especially in milk and dairy products

The bacteria of the genus *Enterococcus* spp., also known as ‘enterococci’, form part of the environmental, food and clinical microbiology. They are considered, depending on the strain, as indicator, spoilage, or potential pathogenic organisms.

In the food industry, these lactic acid bacteria (LAB) are known as ‘adjunct or starter cultures’, where they play an important role thanks to their fermenting activity. This unique character makes them responsible for the development of the sensory characteristics of some cheeses and sausages (especially those originating in the Mediterranean area), resulting in products with special organoleptic attributes that not only contribute to the local cuisine and heritage of the region but are also considered as ‘delicacies’, being widely distributed and representing an appealing commodity worldwide. In the same manner, enterococci are also acknowledged as contributors to human’s digestibility and therefore are additionally known for their role as probiotics [62]. They are also associated with natural fermentations that occur in black olives [58] and may become the predominant population of in-package, heat-treated meats [59].

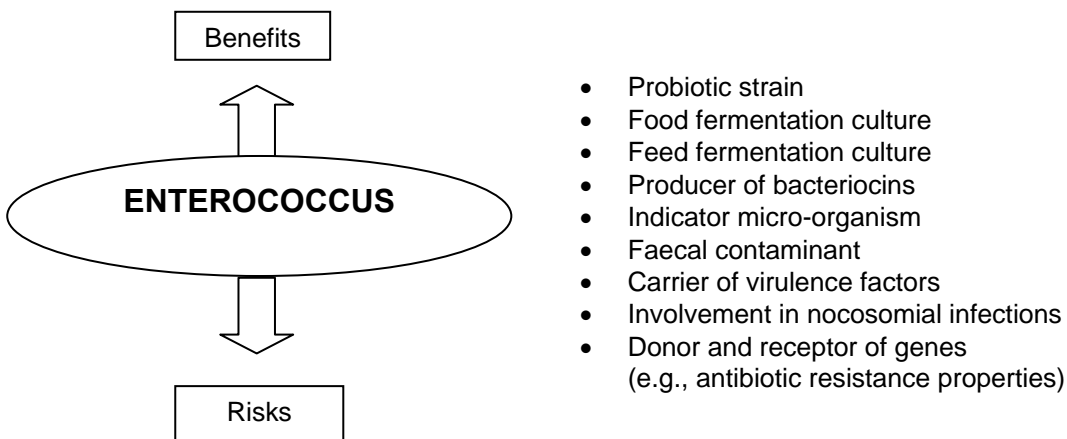
However, although they are considered to be important in foods, some strains have detrimental activities that include spoilage of foods, especially meats. For instance, *Enterococcus faecium* is markedly heat tolerant and can behave as a spoilage agent in marginally processed canned hams. In dairy products, both *Enterococcus faecalis* and *E. faecium* species are relatively heat resistant as well. Also, most of the enterococci are relatively resistant to freezing. Therefore, some investigators have associated food poisoning outbreaks with enterococcal bacteria, but definitive experiments with unequivocal positive results lack.

What is also important is that some other strains of enterococci may have an adverse role in animals and humans, behaving as typical opportunistic pathogens. This was suggested for some of the enterococcal clinical strains, especially those that have become resistant to chemotherapeutic agents, which is especially important in immunocompromised patients [62]. In the food sense, it is feared that some of these clinical

strains have already entered into the food chain and genetically contaminated the culture strains, thus becoming a further source for infection and behaving as agents of food-borne illnesses [62]. However, these hypotheses are yet to be confirmed.

As a result, enterococci may have a ‘dualistic effect’. On one hand, they play a dominant role in various fermented products but on the other, some are considered as indicators of undesirable contamination or even as micro-organisms carrying some pathogenic potential. Since the question about this apparent ‘dualistic effect’ has been raised, enterococci (and their metabolic products) have become a central issue within different research activities with regards to food safety aspects and their risk or beneficial potential as probiotics or cultures in the food industry (Figure 1). Apparently there is a general risk associated with their use as starters or probiotics, but it is necessary to know how to evaluate these risks and consequently to determine if only some enterococcal species or strains are harmful [62]. Currently, there is an approach that highlights the need to study every enterococcal food strain individually; this will allow a more accurate selection of the most suitable bacteria for starter or adjunct culturing purposes [7]. Thus, in practical terms, to comply with the food safety regulations, the food producer using an *Enterococcus* strain is responsible for evaluating the presence of all known virulence factors that the selected bacterium could harbour. Ideally the strain intended to be used as a probiotic or starter culture should have no virulence determinants and be sensitive to relevant clinical antibiotics [46, 62, 78].

Figure 1: The position of *Enterococcus* between benefit and risk in medicine as well as in food and agricultural sciences [46].



1.2 International food standards with regards to enterococci

Traditionally, the source of enterococci in foods is thought to be derived from faecal contamination. However, the ability of enterococci to grow in food processing plants, and possibly other environments, long after their introduction, as well as the observation that enterococci can establish extra-intestinal epiphytic relationships, put in doubt the reliability of enterococcal counts as a reflection of *faecal contamination* and highlight that enterococcal findings in foods are no longer exactly equivalent to 'faecal' presence [78].

On the other hand, many foods naturally contain from small to large numbers of enterococci, especially *E. faecalis* and *E. faecium* species. Relatively low levels, 10^1 to 10^3 enterococci/g, are common in a wide variety of foods, and certain varieties of cheese and fermented sausages occasionally may contain more than 10^6 enterococci/g [78].

Hence, even though they generally serve as a good index of sanitation and proper holding conditions, *no acceptable levels of enterococci can be stated for foods* because their counts vary with product, handling, time of storage, and other factors. Even though by controlling the initial numbers of enterococci the shelf life of the product could be predicted, the entire history of each product must be studied, and the culture medium and conditions must be standardised, before setting any specific criteria [78].

1.3 Importance of a risk analysis with regards to enterococcal presence in milk and milk products

Risks from microbiological hazards are of immediate and serious concern to human health. Microbiological hazards are those micro-organisms and/or their toxins capable of causing adverse health effects and which may be present in a particular food or group of foods [1].

A microbiological risk analysis is a systematic review of the hazard, exposure and consequences associated with the micro-organisms of interest, which will produce a rationale that could be of significance for governments (public health entities), organisations and companies (food industries), and other interested parties [1]. It is a key element in assuring that sound science is used to establish standards, guidelines and other recommendations for food safety to enhance consumer protection and facilitate international trade, with the overall purpose being assurance of public health protection

[1]. Although it is accepted that the formalised use of risk analysis in food microbiology is in its infancy, it is very likely that in the near future microbiological risk assessment will have a greater importance in the determination of the level of consumer protection that a government considers necessary and achievable [57].

The main goals of a food microbiological risk analysis are to provide an estimate of the risk levels of illness from a pathogen in a given population, and to understand the factors that influence it [57]. For these purposes, the analysis should explicitly consider the dynamics of bacterial growth, survival (and death) in foods and the complexity of the interaction (including consequences) between human and agent through the food chain, from primary production up to, and including consumption, as well as the potential for further spread [1]. It is also important that, when new relevant information and data become available, the programme be reassessed, rerun and updated, as part of an ongoing process [1].

With regards to enterococci, considering their ‘dualistic’ effect that has emerged in the last decades, it is important for food microbiologists to evaluate the significance of these bacteria in foods by running a risk analysis, but in an individual strain and role specific manner.

1.4 Aims of this study

Enterococci have always been important as culture bacteria. For decades, thanks to their fermenting functions, many of them have had a significant role in the food industry. But since some strains have been related to human disease lately, discussion with regards to the safety of their use as cultures for human food abounds at present.

Enterococci have traditionally served as indicators of faecal contamination as well, but this long-established role is currently running out of date, because recently the increasing ubiquitous character of the bacteria in the environment has been proven. Their role as food spoilers is well-known, as it is their role as bacteriocin producers with remarkable antibacterial action.

Enterococci seem to pose advantages and disadvantages. However, as there is a high diversity of enterococcal strains, and not all of them show the same functional characteristics, it is important to establish a clear differentiation between the beneficial

and disadvantageous strains before launching any specific and definitive damning conclusion.

This review, that includes a risk analysis approach, aimed to emphasise the most relevant characteristics, roles and applications of *Enterococcus* spp. in the dairy industry, and the importance that a good heating procedure within the process possessed, to ensure food safety regulations compliance at national and international markets. It also aimed to show that quality enterococcal cultured products for the human consumption are not to be feared. An evaluation of the suggested pathogenic effects of enterococci (based on evidence of clinical disease in humans) was also included.

Chapter 2: Taxonomy of Enterococci

2.1 Introduction

Correct bacterial identification is of considerable importance to both, medical and food microbiologists. For instance, in the clinical field, correct identification of genus, species groups and sub-species may be important for the appropriate choice of antibiotic therapy. In epidemiology, accurate identification may be useful for epidemiologic surveillance in hospitals. In food microbiology, precise identification may be important when selecting a new starter strain, when labelling of the product to which the starter is added, as well as during the testing of food for the presence of undesirable organisms, e.g. spoilers and pathogens. With the development of more sophisticated starter culture systems and the rapid changes in the taxonomy of LAB, it is of utmost importance for food microbiologists to be aware of current nomenclature [59].

In the case of the enterococci, the classical taxonomy still remains rather vague because there are no particular phenotypic criteria, which are typical of all enterococci and that as yet unequivocally distinguish this genus from other Gram-positive, catalase-negative, coccus-shaped bacterial genera [59]. This means that presumptive identification at the genus level necessarily must be followed by species identification, *viz.* when a strain shows the characteristics of an enterococcal species, only then can be presumed that it belongs to the genus *Enterococcus* [46].

Currently, there are 8 distinct *species groups* for the genus *Enterococcus*, based on ribonucleic acid (RNA) analysis (Table 1) [62]. Each of these groups, in turn, contains several phylogenetically-related *sub-species* (also commonly and simply known as *species*). Because within the same group, the sub-species can still differ from one another and because each sub-species in turn clusters several enterococci as well, the term *strain* is frequently employed for a further individualisation and differentiation of bacteria within and among sub-species of a species group. Thus, enterococcal strains are regularly named with the sub-species name plus letters and numbers, e.g. *E. faecium* SF68 (where '*E. faecium*' indicates the sub-species of the species group, and 'SF68' indicates the strain of the sub-species).

Table 1: Current enterococcal different ‘species groups’ and their sub-species, based on their phylogenetic relationship within the genus [62].

Group	Species
1. <i>E. faecium</i>	' <i>E. azikeevi</i> ' (possible new sub-species), <i>E. durans</i> , <i>E. faecium</i> , <i>E. hiraе</i> , <i>E. mundtii</i> , <i>E. porcinus</i> , <i>E. villorum</i>
2. <i>E. avium</i>	<i>E. avium</i> , <i>E. malodoratus</i> , <i>E. pseudoavium</i> , <i>E. raffinosus</i>
3. <i>E. gallinarum</i>	<i>E. casseliflavus</i> , <i>E. flavescens</i> , <i>E. gallinarum</i>
4. <i>E. dispar</i>	<i>E. asini</i> , <i>E. dispar</i>
5. <i>E. saccharolyticus</i>	<i>E. saccharolyticus</i> , <i>E. sulfureus</i>
6. <i>E. cecorum</i>	<i>E. cecorum</i> , <i>E. columbae</i>
7. <i>E. faecalis</i>	<i>E. faecalis</i> , <i>E. haemoperoxidus</i> , <i>E. moraviensis</i> , ' <i>E. rottae</i> ' (possible new sub-species)
8. <i>Tetragenococcus</i>	<i>E. solitarius</i> , <i>Tetragenococcus halophilus</i> , <i>Tetragenococcus muriaticus</i>

Nevertheless, identification of groups, sub-species or strains is still problematic. Due to the high heterogeneity in phenotypic features that enterococci possess (regardless of the origin of the isolate), the phylogenetically distinct sub-species or species groups of enterococci can differ to some extent from one another in their cell wall chemistry, physiology, growth and biochemical activity. Hence, it is difficult to unequivocally categorise isolates into one of the *Enterococcus* sub-species, based only on physiological tests [44]. This is why numerous enterococcal isolates, especially from environmental sources, often remain unidentified when recognition is based on phenotypic traits alone [75].

The phenotypic characteristics of the different sub-species have been comprehensively reviewed by Devriese *et al.* [44, 45], and their work still remains practical and valuable. It is likely that the phylogenetic system for identifying the genus *Enterococcus* and its groups and sub-species is not yet complete. More recently, new sub-species have been proposed (as discussed below) and further re-classification may be expected in the near future [46].

2.2 Historical taxonomy

The history of the taxonomy of enterococci (described according to [46, 59, 62, 75]) started in 1899, when Thiercelin first used the term ‘entérocoque’ to refer to a Gram-positive diplococcus of intestinal origin. Subsequently, the genus *Enterococcus* was proposed by Thiercelin and Jouhaud in 1903.

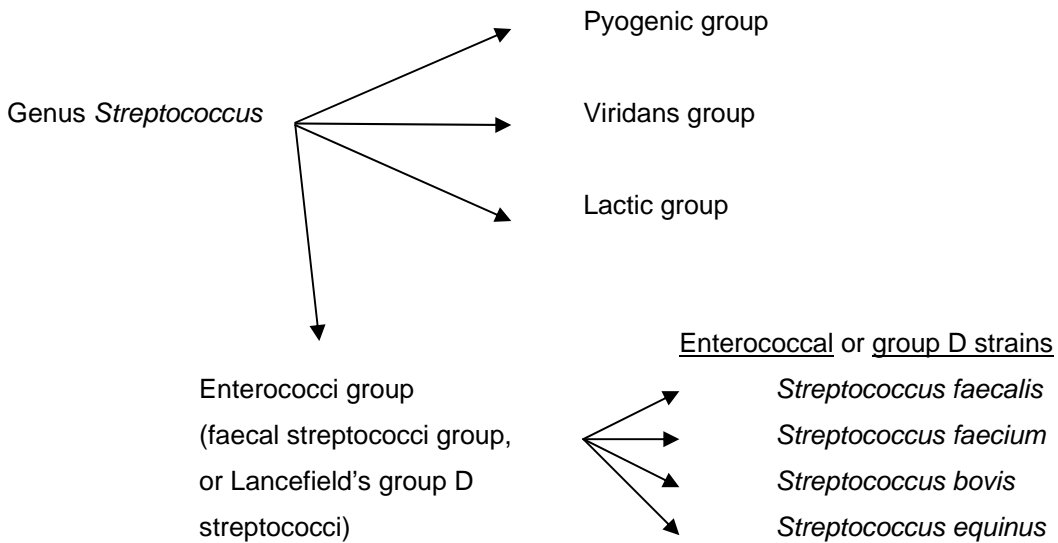
In 1906, after identifying a potentially pathogenic bacterium from a patient with endocarditis, Andrewes and Horder renamed the Thiercelin ‘entérocoque’ as

Streptococcus faecalis. The epithet 'faecalis' was suggested because of the close resemblance the organism had with strains isolated from the human intestine, leading to the assumption that the bacterium had a gastrointestinal origin.

In 1933, when applying a serological typing system, Lancefield discovered that enterococci of faecal origin were the ones possessing the 'D antigen' which reacted with group D antisera (*Enterococcus* spp. in fact can possess either group A, B, C, D, F, or G antigens, but the D antigen is the one present in those of faecal origin). This gave rise to the now well-known 'Lancefield's group D streptococci' or 'faecal streptococci' classification.

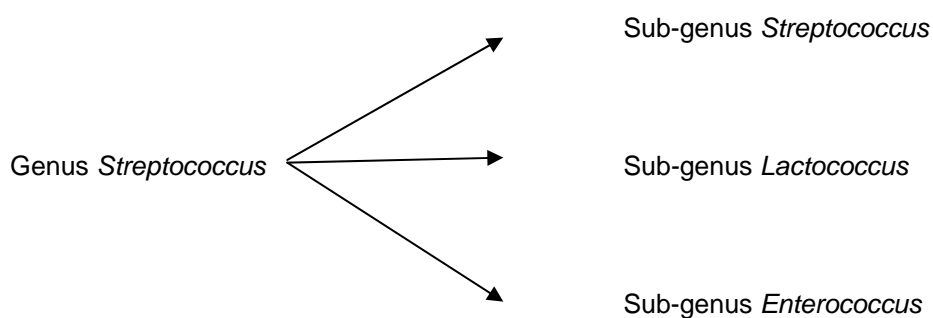
Lancefield's observation was in agreement with the classification suggested by Sherman, who in 1937 proposed a new taxonomic scheme for the genus *Streptococcus*, separating it into four divisions designated as: pyogenic, viridans, lactic, and enterococci. The enterococci group included: *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis* and *Streptococcus equinus*, and were named 'enterococcal' or 'group D' strains, as only these streptococci were believed to be of faecal origin and produced the D antigen (Figure 2). Since then, the terms 'faecal streptococci', 'enterococci' and '(Lancefield's) group D streptococci' have often been used synonymously.

Figure 2: Sherman's classification of the *Streptococcus* genus including the sub-species that fell within the D-antigen-producing group of enterococci.



Subsequent to Sherman's publications, based on modern classification techniques and serological studies, in 1984 Schleifer and Kilpper-Bälz divided the former *Streptococcus* genus into three different sub-genera: *Streptococcus*, *Lactococcus* and *Enterococcus* (Figure 3), a slow move towards establishing the enterococcal autonomy as a separate genus.

Figure 3: Schleifer and Kilpper-Bälz's re-classification of the genus *Streptococcus* into three different sub-genera.

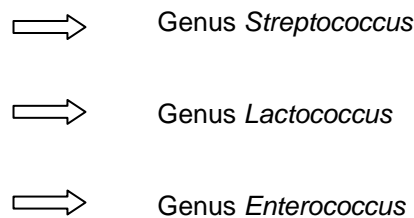


However, it was not until 1987, when applying further classification and serological techniques, that Schleifer and Kilpper-Bälz finally demonstrated that enterococci should constitute a new independent genus - *Enterococcus*. They performed molecular biology studies (including oligonucleotide cataloguing of 16S rRNA and DNA-DNA and DNA-

rRNA hybridisation) combined with physiological studies, which resulted in a more elaborate classification wherein the new genus *Enterococcus* was established.

From then on, the members of the genus *Streptococcus* that were formerly grouped as ‘enterococci’ (‘faecal streptococci’ or ‘Lancefield’s group D streptococci’) (Figure 2), or those that fell into one of the other sub-genera (Figure 3) were placed into three separate, independent and different genera: *Streptococcus*, *Lactococcus* and *Enterococcus*. The ‘new’ genus *Streptococcus* then only included the typical pathogenic sub-species (with the exception of the non-pathogenic *Streptococcus thermophilus*); the ‘new’ genus *Lactococcus* included a group of non-pathogenic and technically important sub-species, while the ‘new’ genus *Enterococcus* – still known as ‘faecal streptococci’ or ‘Lancefield’s group D streptococci’ - included those sub-species associated with, but not restricted to, the gastrointestinal tract of humans and animals, some fermented foods and a range of other habitats [62] (Figure 4).

Figure 4: Schleifer and Kilpper-Bälz’s differentiation of the old genus *Streptococcus* and separation of its *Lactococcus* and *Enterococcus* sub-genera to constitute new genera.

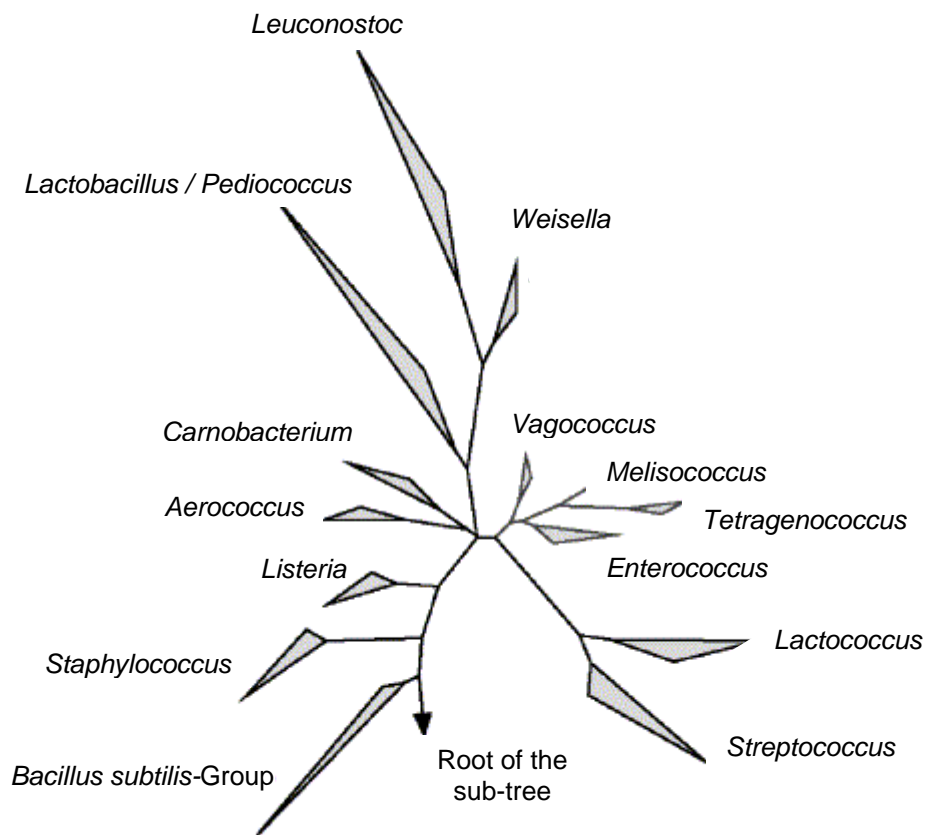


All enterococcal bacteria were described as usually growing at 45 °C, in 6.5% sodium chloride (NaCl), and at pH of 9.6, with most growing at 10 °C, susceptible to vancomycin, and very few producing gas from glycerol. *S. bovis* and *S. equinus*, which are negative for two or more of these properties, were assigned to a miscellaneous group of ‘Other Streptococci’[78].

2.3 Current taxonomy

At present, new methods of bacterial differentiation allow to show the actual phylogenetic position of the genus *Enterococcus*, which can best be demonstrated by 16S rRNA sequence comparisons and construction of a 16S rRNA-dendrogram, in which *Streptococcus* and *Lactococcus* also appear (Figure 5) [102].

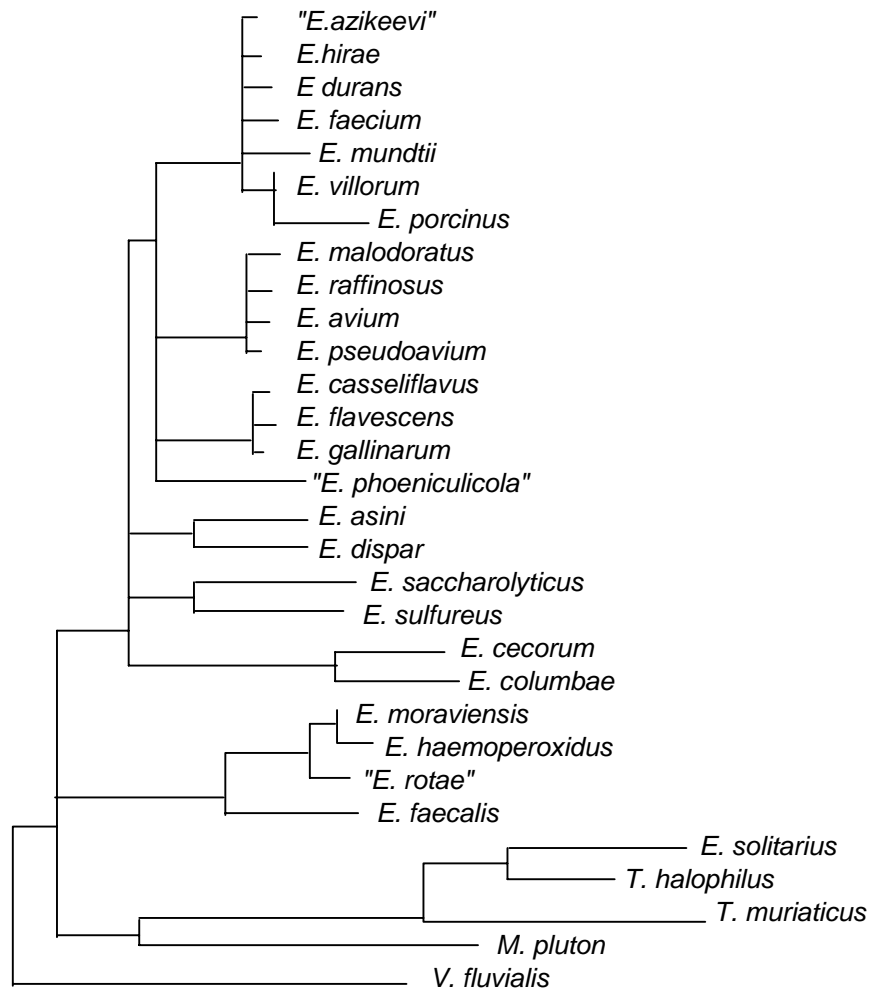
Figure 5: The phylogenetic position of the genus *Enterococcus* demonstrated by a 16S rRNA-dendrogram of Gram-positive genera, including *Streptococcus* and *Lactococcus*. The length of the branches indicates 10% estimated sequence divergence [102].



10% Sequence divergence: _____

Since 1987, chemotaxonomic and phylogenetic studies have also resulted in the assignment of 25 sub-species, grouped within 8 species groups, to the genus *Enterococcus* [44], as explained and shown in Table 1. The phylogenetic relationship of the different sub-species within the genus *Enterococcus* has been determined by comparative sequence analysis of their RNA genes. A 16S rRNA-based phylogenetic tree of enterococcal sub-species is depicted in Figure 6 [62].

Figure 6: 16S rRNA-based tree reflecting the relationship of enterococcal sub-species. The length bar indicates 5% estimated sequence divergence [62].



5% Sequence divergence: _____

Recently, three possible new sub-species, *E. azikeevi*, *E. phoeniculicola*, and *E. rottae*, have been submitted to GenBank for inclusion within the genus [62], but *E. phoeniculicola* has not been placed into any of these groups yet and apparently it will be placed into a new and different group. *E. solitarius* is validly published and based on molecular data it appears to belong to the genus *Tetragenococcus* [47].

Although 25 *Enterococcus* sub-species are now recognised, *E. faecium* and *E. faecalis* are still the two most prominent, playing the important roles in fermented foods and in probiotics, and debatably associated with human diseases, as will become apparent later in this document [62].

2.4 Conclusions

Classification of the enterococci is in a state of flux. Based on recent RNA analyses, the current taxonomy denotes *Enterococcus* spp. as a separate genus and recognises 25 sub-species, which are included within 8 species groups, according to their phylogenetic relationships. Other species are also being proposed and studied for addition. Of all the sub-species, *E. faecium* and *E. faecalis* stand out for their roles in clinical and food microbiology.

Chapter 3: Properties of Enterococci

3.1 General properties

According to RNA sequence analysis, the genus *Enterococcus* belongs to the Gram-positive bacteria with low (≤ 50 mol %) glycine and cytosine (G + C) content in the DNA, like clostridia and bacilli. Members of the genus *Enterococcus* are catalase-negative, facultatively anaerobic cocci, which can appear arranged in pairs or short chains. They are chemo-organotrophic and can ferment sugars to produce mainly lactic acid [62].

A remarkable aspect of enterococci is that they can grow in a wide range of temperatures and in restrictive environments such as high salt content and low pH. Consequently, enterococci can be easily distinguished from other Gram-positive, catalase-negative, homofermentative cocci (e.g. streptococci and lactococci) by their ability to grow between 10 and 45 °C, between 5 and 10% NaCl, in the presence of 40% bile and sodium azide, and at a pH between 4 and 9.6 [62]. Table 2 gives an overview of the typical physiological properties of valid enterococcal species, according to Domig *et al.* [47].

On the other hand, many of the more recently described enterococcal species vary in their physiological properties from those of typical enterococci. In this regard, there are contradictory reports on the detectable group D antigen in different species (Table 2), some authors argue that there are some strains that may not react with group D antiserum (therefore assuming that are not capable of producing group D antigen) [62]; other authors state that all enterococci effectively produce the group D antigen but the problem actually lies in the laboratory techniques employed which fail to demonstrate its presence in some isolates [78]. Moreover, the current *Streptococcus* spp. (*S. bovis*, *S. suis* and *S. alactolyticus*), as well as pediococci and certain *Leuconostoc* strains, also react with Lancefield's group D antiserum, bringing more confusion to enterococcal identification schemes. In the same variability context, some strains of enterococci in fact cannot grow in the presence of 6.5% NaCl (Table 2) while some strains of lactococci, pediococci, aerococci and leuconostocs do grow in its presence. Finally, growth at 45 °C is not limited to enterococcal species, pediococci and some lactococci also grow at that temperature, at which even some enterococci do not (Table 2); growth

at 10 °C is also not typical of enterococcal species only, most lactococci, leuconostocs and some streptococci also grow at that temperature, and some enterococci do not (table 2) [59].

Table 2: Characteristic physiological properties of validly described enterococcal species [47].

Species	Growth at		Growth in the presence of				Aesculin hydrolysis	Group D antigen
	10 °C	45 °C	pH 9.6	6.5% NaCl	40% bile	0.04% sodium azide		
<i>E. asini</i>	(+)	(+)	n.d.	-	+	n.d.	+	+
<i>E. avium</i>	V	+	+	V	V/+	n.d.	+	+
<i>E. casseliflavus</i>	+	+	+	V/+	+	+	+	+
<i>E. cecorum</i>	-	+	(+)	-	(+)	-	+	-
<i>E. columbae</i>	-	n.d.	n.d.	-	(+)	-	+	-
<i>E. dispar</i>	+	-	n.d.	+/-	+	-	+	-
<i>E. durans</i>	+	+	+	+	+	+	+	(+)
<i>E. faecalis</i>	+	+	+	+	+	+	+	+
<i>E. faecium</i>	+	+	+	+	+	+	+	V
<i>E. flavescens</i>	V/-	V/+	n.d.	+	+	+	+	+
<i>E. gallinarum</i>	+	+	+	+	+	+	+	+
<i>E. haemoperoxidus</i>	+	-	n.d.	+	+	+	+	+
<i>E. hirae</i>	+	+	+	+	+	+	+	V
<i>E. malodoratus</i>	+	-	+	+	+	n.d.	+	+
<i>E. moraviensis</i>	+	-	n.d.	+	+	+	+	+
<i>E. mundtii</i>	+	+	+	+	+	+	+	+
<i>E. porcinus</i>	+	+	n.d.	+	n.d.	n.d.	+	+
<i>E. pseudoavium</i>	+	+	+	+/-	V/+	n.d.	+	-
<i>E. raffinosus</i>	(+)	+	+	+	V/+	n.d.	+	n.d.
<i>E. ratti</i>	+	+	n.d.	+	n.d.	n.d.	+	(+)
<i>E. saccharolyticus</i>	+	+	n.d.	(+)	+	n.d.	+	-
<i>E. solitarius</i>	+	+	n.d.	+	+	n.d.	+	+
<i>E. sulfureus</i>	+	-	n.d.	+	+	n.d.	+	-
<i>E. villorum</i>	n.d.	n.d.	n.d.	+	+	+	+	n.d.

n.d.: not determined; (+): weak positive; V: variable; +/-: differing reports in literature

Since the most common species found in animal derived food products are *E. faecalis* and *E. faecium*, and less often *E. durans*, such inconsistencies are less important for the purpose of this review and, after having a closer look at Table 2, we can conclude that these three species strains grow between 10 and 45 °C, at a pH of between 4.0 and 9.6, and in the presence of 6.5% NaCl, 40% bile, and 0.04% sodium azide. Detection of the group D antigen however is variable, possibly as a result of strains variations in reaction to the group D antiserum – only *E. faecalis* strains reacts positively, while *E. faecium* strains can give variable results and *E. durans* strains are normally weak positives. Temperature and pH resistances are of particular interest in this review, given that

variations of these parameters directly affect enterococcal survival and growth abilities during dairy food production.

3.1.1 Heat resistance

Enterococci can grow in a wide range of temperatures. It has been reported that enterococci could grow at temperatures of between 0 to 50 °C [69], however they grow best at temperatures of between 10 and 45 °C [62, 75, 150].

Because they can withstand high heating-temperatures, they are recognised as the most thermo-resistant among the non-sporulated bacteria [150]. In this respect, it has been suggested that enterococci can survive 60 °C for 30 minutes, in a neutral medium [69]. However, a variety of factors such as time-temperature combination, number of enterococci, the age of the strains, and pH, nutrient composition and protective effect of the suspending media, influence their thermal resistance [69].

In fact, most enterococci grow at 10 °C [78]. The highest temperature at which enterococci can grow well (maximum heat resistance) is up to 45 °C [75], and at a pH range of between 6.0 and 8.0 [171]. If this temperature (45 °C) is maintained for 15 minutes, enterococci already start to develop an increased sensitivity to salt, leakage of their cell membranes, and an increased growth lag [69]. Heating at 55 °C already damages cell membranes severely, as evidenced by further loss of enterococcal membrane compounds [69]. At 60 °C/4 minutes, enterococci develop further increased growth lag, with a remarkable sensitivity to potassium (and potassium chloride), magnesium chloride, and tellurite; if this temperature is sustained for 15-30 minutes, enterococci become more sensitive to salt, pH, temperature, and have a further increasing growth lag. At 76 °C, enterococci become increasingly sensitive to sodium azide [69].

On the other hand, although it has been stated that enterococci can still grow at 0 °C [69], the growth rate and activity of enterococci between 5-8 °C, and below that temperature, starts to be severely limited [150].

Normally, the heat resistance of enterococci is measured in D-values.¹ Several studies [34, 171, 193, 194] have been done in different media that attempted to describe the heating survivability of enterococci, but only few [13, 14, 69, 150, 171] have reported enterococcal heat-resistance behaviour in milk media [14]. In 1982, Pérez *et al.* [150] carried out a study that showed that at 64 °C, 90% of *E. durans*, *E. faecium* and *E. faecalis* were inactivated at 13.4, 6.3, and 4.5 minutes, respectively in whole milk at a pH of 6.6. If the temperature was raised to 72 °C, 90% of these species were inactivated at 9.7, 2.4, and 0.88 seconds, respectively. Further aspects with regards to enterococcal heat-resistance in milk (with a risk analysis approach) are discussed on Chapter 7.

3.1.2 pH resistance

The pH of the medium where enterococci are sustained, considerably influences their survivability [194]. Enterococci can normally withstand pH ranges of between 4.0 and 9.6 [75], depending on the species. In 1996, Franz *et al.* [58] studied the growth and bacteriocin² activity of a *E. faecium* strain in Man, Rogosa and Sharpe (MRS) medium and noticed that the strain had the maximum growth (8.81-9.26 CFU/ml⁻¹) and bacteriocin production activity in the neutral or slightly alkaline range: pH 6.0-9.0. At a pH of 10.0 the strain was still able to grow at high levels (9.28 CFU/ml⁻¹), but its bacteriocin production was reduced by 50%. At a pH of 4.0 and 5.0, the strain decreased in growth (6.91-8.14 CFU/ml⁻¹) but had 0% bacteriocin production activity, whereas at a pH of 3.0 the strain was not capable of growth (0 CFU/ml⁻¹) nor have bacteriocin production activity [58].

In 1962, White [194] has carried out a study on three strains of *E. faecalis* exposed to heat (60 °C) in phosphate and citrate-phosphate buffer solutions, at various pH levels. The enterococcal resistance was calculated using D-values. The results concluded that *E. faecalis* had a maximum survivability usually at a pH of 6.8 (close to neutrality). On both sides of 6.8, its sensitivity was sharply increased. At other temperatures (50, 55, and 65 °C), the results were similar [194].

¹The D-value can be defined as the time of heat treatment required at a certain temperature to destroy 90% of the bacterial cells [171]. It is also known as ‘decimal reduction time’, and is obtained from the relationship between the logarithm of the number of bacterial survivors and time [194].

² Bacteriocins are small, ribosomally synthesised, extracellularly released, antibacterial peptides or proteins that display a limited inhibitory spectrum towards other Gram-positive bacteria [112] and that can be used as natural food preservatives to enhance the shelf life and safety of food products [25]. The bacteriocins secreted by enterococci are known as ‘enterocins’.

In milk, the Pérez *et al.* study published that *E. durans* and *E. faecium* had their maximum resistance at a pH of 6.0, while *E. faecalis* maximum survival was at a pH of 6.6, when different heating temperatures were applied [150].

3.2 Biochemical properties of technological interest

Biochemical properties of technological interest refer to the enterococcal acidification ability, proteolytic and lipolytic activity, carbohydrates metabolism, as well as their production of volatile compounds and bacteriocins. The evaluation of these biochemical properties, in respect of enterococcal origin and species, allows an initial selection of enterococcal strains to be used as cultures in food fermentations [161].

The principal inherent biochemical properties of the three most common enterococcal species – *E. faecalis*, *E. faecium*, and *E. durans* - are described below, along with their functions in the dairy industry.

3.2.1 Acid production

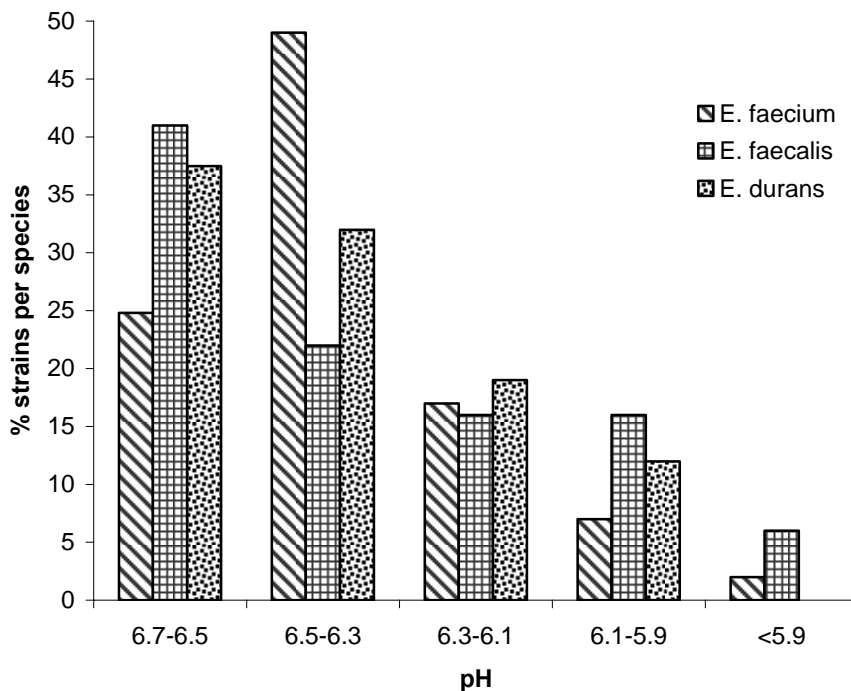
Acid production is an appreciable characteristic that results in the development of appealing sensory attributes in certain types of cheese. Milk normally has an initial pH of ~6.6, and for cheese manufacture this pH has to be reduced at the end of ripening to <6.0, depending on the type of cheese [161]. A rapid decrease in pH during the initial steps of cheese preparation is crucial in cheese manufacture since it is essential for coagulation and the prevention or reduction of the growth of adventitious microflora [161]. These beneficial roles have suggested the inclusion of some enterococcal strains as certain starter cultures.³

In general enterococci exhibit low acidifying ability, at least in the milk studies done so far [75, 161]. In 1999, Morea *et al.* [134] showed that the pH of milk, 24 hours after inoculation with enterococcal strains isolated from Mozzarella cheese, did not drop below 5.5. The poor acidifying activity of *Enterococcus* spp. species was also confirmed by Villani and Coppola [188], Andrighetto *et al.* [5], and Durlu-Ozkaya *et al.* [49].

³ A *starter culture* is the one that is added at the beginning of the manufacture of dairy products (cheeses, especially) exclusively for the purpose of acid production. An *adjunct culture*, on the other hand, is added in subsequent stages of dairy manufacture, for the purpose of amelioration or acceleration of the ripening stage, to enhance the product's sensory properties, or to act as living micro-organisms (probiotics) destined to benefit the consumer's health [75].

Also, in 2001, Sarantinopoulos *et al.* [161] (who ran the most complete experiment with regards to enterococcal biochemical properties) ran a study⁴ where they have demonstrated that, after 16 hours, at an incubation temperature of 37 °C, only 21 isolates, out of 129 studied, decreased the milk's pH below 5.0. The lowest pH values were reached mainly by *E. faecalis* strains of food origin, compared to *E. faecium* and *E. durans* strains. *E. faecalis* isolates were therefore the most rapid acidifiers [161]. Figure 7 shows this study's attained pH values in skim milk, after the first 6 hours of addition of the three enterococcal species [161]. In 2003, Giraffa *et al.* [75], also reported the high acidifying potential of *E. faecalis* compared to other enterococcal species, when in skim milk a pH lowering to about 4.5 was gained after 24 hours fermentation, using *E. faecalis* strains isolated from an artisan Italian cheese [75]. The higher acidifying potential of *E. faecalis* has also been confirmed in previous findings, by Villani and Coppola [188] and Suzzi *et al.* [175].

Figure 7: pH values attained by *E. faecalis*, *E. faecium* and *E. durans* after growth in skim milk for 6 hours at 37 °C [161].



⁴ In this study, 129 isolates were selected (57 were *E. faecium*, 56 were *E. faecalis*, and 16 were *E. durans* isolates), of which 106 were from food sources (99 from Greek, Italian and Irish dairy products), 12 from veterinary sources, and 11 from human isolates [161].

3.2.2 Proteolytic and peptidolytic activities

Casein degradation in relation to proteolytic and peptidolytic activities of microorganisms is important in cheese ripening, contributing to its texture [75]. Some peptides also contribute to the formation of flavour, whereas others (undesirable bitter-tasting peptides) can lead to off-flavour formation [161].

The majority of enterococcal strains, however, exhibit low extracellular proteolytic activity [75], in accordance with several studies done by Arizcun *et al.* [7], Tsakalidou *et al.* [182], Andrighetto *et al.* [5] and Sarantinopoulos *et al.* [161].

There is conflicting literature with regards to what species are more proteolytic, and it also appears that there is a marked strain-to-strain variation of this phenotypic trait [75]. What all the authors are in agreement with is that the most active strains are, either of food origin, or are *E. faecalis*. In this regard, there are reports of a relevant proteolytic activity within *E. faecium*, *E. faecalis*, and *E. durans* strains isolated from various cheeses [31, 175, 192], but the most active strains (> 2 mmol) usually belong to the species *E. faecalis*, especially of food origin [161]. Lesser degrees of activity are detected in *E. durans* strains, and the lowest proteolytic activity are for *E. faecium* strains [75, 161]. Yet, the extensive studies on proteinase and the fewer studies on peptidase activities in *Enterococcus* spp. suggest that proteolytic activities are generally low [75] or even nil [161].

According to the observations of Durlu-Ozkaya *et al.* [49] and Suzzi *et al.* [175], and the conclusions of Giraffa [75], there may be a correlation between enterococcal proteolytic and acidification activities, i.e., the more of an acidifier a strain is, the more proteolytic it would also be. No clear relationship has been established so far and more studies need to be done to confirm this hypothesis.

3.2.3 Lipolytic and esterase activities

According to Giraffa [75], *esterases* are arbitrarily classified as enzymes hydrolysing substrates in solution whereas *lipases* hydrolyse substrates in emulsion. The possible contribution of lipases and esterases to the cheese ripening process is not well defined yet, but the esterases are linked to cheese flavour development and texture through lipolysis of milk fat and further conversion of produced free fatty acids to methylketones and thioesters [75]. Even though lipolysis is not directly involved in

cheese rheology, partial glycerides are tensio-active compounds that influence molecular organisation and hence indirectly contribute to cheese texture as well [75].

It is generally accepted that LAB, and thus enterococci, are only weakly lipolytic, though limited and often contradictory information exists with regards to this [161]. Apparently, a number of factors (origin and/or species) can have an influence on *Enterococcus* lipolytic activity; in this regard, enterococci of food origin are considered the most lipolytic, especially *E. faecalis* species, followed by either *E. durans* or *E. faecium* [161]. It can also depend on the substrate where enterococci are present [161] – in 1994, Villani and Coppola [188] observed that enterococcal lipolytic activity was very low when grown in whole milk, suggesting a substrate specificity.

Furthermore, it is not totally clear either if lipolytic activity is strain specific or not [75, 161], i.e. whether some *E. faecalis*, *E. durans* or *E. faecium* strains could have more activity than others. On the whole, the lipolytic activity of generally all enterococci appears to be very low, although a greater enterococcal activity than lactococci, when compared to other LAB bacteria, has been reported [161].

The esterolytic activity of enterococci is also rather complex, but it is more efficient than the lipolytic activity [75]. In Sarantinopoulos *et al.* study [161] all *E. faecalis*, *E. faecium* and *E. durans* isolates were active, independent of the origin, with *E. faecium* being the most esterolytic species, and with the broader substrate specificity, of all enterococci. Esterolytic activity of enterococci is also considered higher than most other LAB (e.g. lactococci), according to a study done by Tsakalidou *et al.* in 1994 [183].

3.2.4 Citrate and pyruvate metabolism

Enterococcus main characteristic is the ability to produce L-lactic acid (lactate) from hexoses by means of homofermentative lactic acid fermentation [154]. This is the reason why they are acknowledged as ‘homofermentative LAB’.

Although the main product of enterococci is lactate, they can also produce significant amounts of acetate, formate and ethanol, depending on the growth conditions [154]. Like lactate, acetate and the others are recognised as ‘flavour compounds’ since they are important in determining the taste of many fermented dairy products [75].

The intermediate precursor of acetate, formate and ethanol is the pyruvate. The ability of enterococci to metabolise pyruvate has been extensively described. However, little is known about the ability of enterococci to metabolise citrate, in which pyruvate is also an intermediate [154]. Citrate and pyruvate metabolisms are important technological steps since many LAB species metabolise citrate and pyruvate into the above mentioned flavour compounds [161].

Until some time ago, all that was known was the ability of enterococci to metabolise citrate (as the sole carbon source) to acetate and formate [154]. Now, it is known that when enterococci are studied in the presence of other substrates, such as glucose or lactose, the situation may differ, and this could also depend on the growth media where the strain is contained [75]. In this regard, recent in-depth studies of enterococcal citrate metabolism done by Sarantinopoulos *et al.* in 2001 [162], revealed that the strain *E. faecalis* FAIR-E 229 could co-metabolise lactose and citrate in milk containing yeast extract, but could not however co-metabolise glucose (or lactose) and citrate in a more complex medium such as MRS broth, even though growth was stimulated. Like the other authors, Sarantinopoulos *et al.*, in another study [161], could obtain the metabolism into acetate and formate when citrate was present as the sole carbon source. In agreement with Sarantinopoulos *et al.* findings, a newer contribution to the studies of enterococcal citrate metabolism was done by Rea and Cogan in 2003 [154], who revealed that glucose actually prevents citrate metabolism by several strains of *E. faecalis* and *E. faecium*, suggesting some form of repression.

Finally, among the enterococcal species, *E. faecalis*, *E. faecium* and *E. durans* isolated from foods or other sources are variably capable to utilise citrate or pyruvate as the sole carbon sources, with strain-to-strain variations. Sarantinopoulos *et al.* [161] found in their study that generally *E. faecalis* isolated from foods were always faster than the others in the organic acid utilisation, which confirmed a previous study result in Picante cheese reported in 1999 by Freitas *et al.* [63].

Figures 8 and 9 show the correlation between citrate and pyruvate metabolisms by these different species in the study done by Sarantinopoulos *et al.* [161]. In this study, almost all isolates of *E. faecalis* utilised >84% of the pyruvate and citrate after 6 hours, and after 16 hours utilisation was complete (Figures 8a and 9a). *E. faecium* isolates showed a variable utilisation of citrate and pyruvate after 6 hours; no correlation was observed

between the ability to metabolise both substrates after 16 hours of incubation (Figures 8b and 9b). For *E. durans* isolates, there was no relationship either between the ability of the strains to metabolise citrate and pyruvate after 6 or 16 hours (Figures 8c and 9c) [161].

Figure 8: Correlation between citrate and pyruvate utilisation after growth of enterococci in modified MRS broth (containing 30 mmol/L⁻¹ of citrate or pyruvate) for 6 hours at 37 °C. (a) *E. faecalis*, (b) *E. faecium* and (c) *E. durans* strains [161].

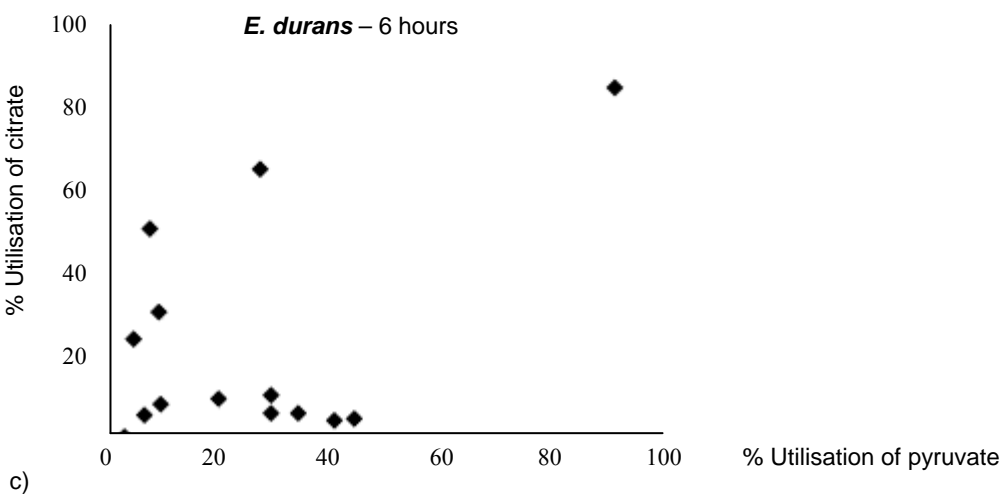
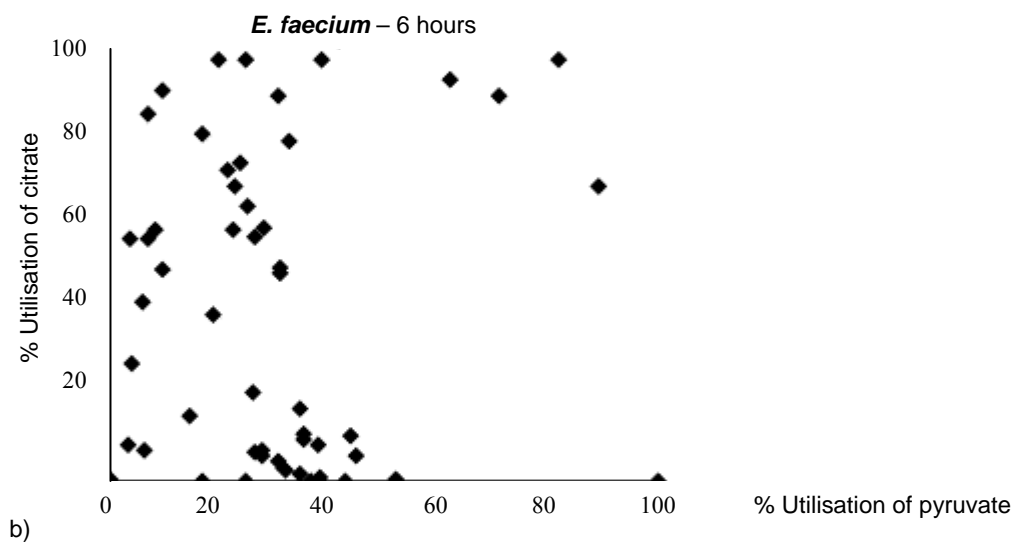
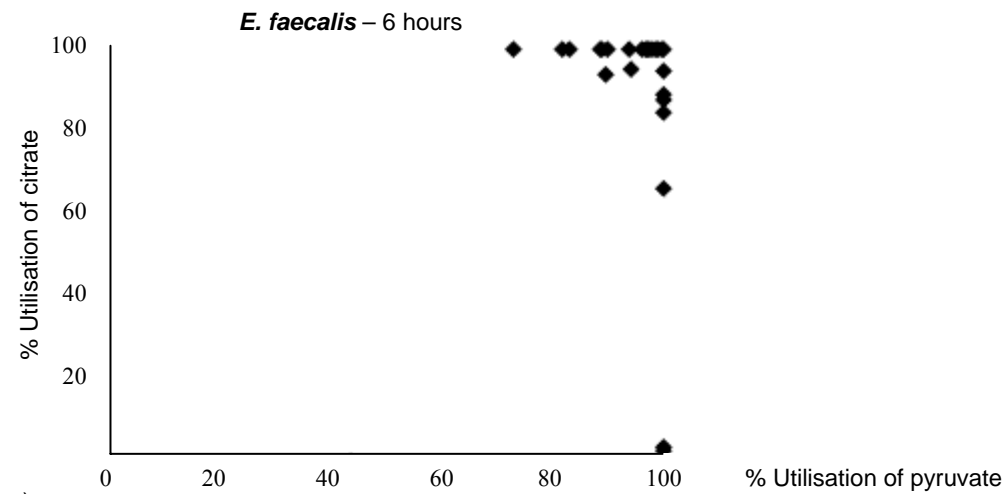
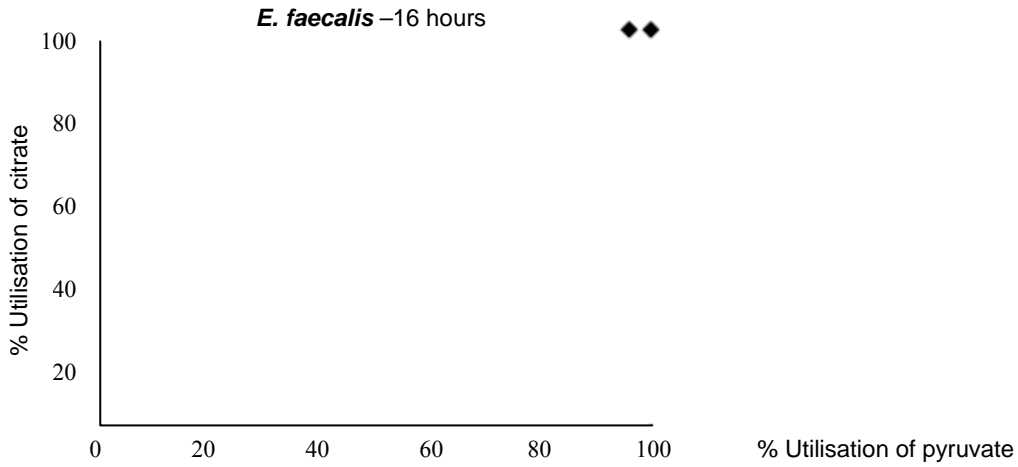
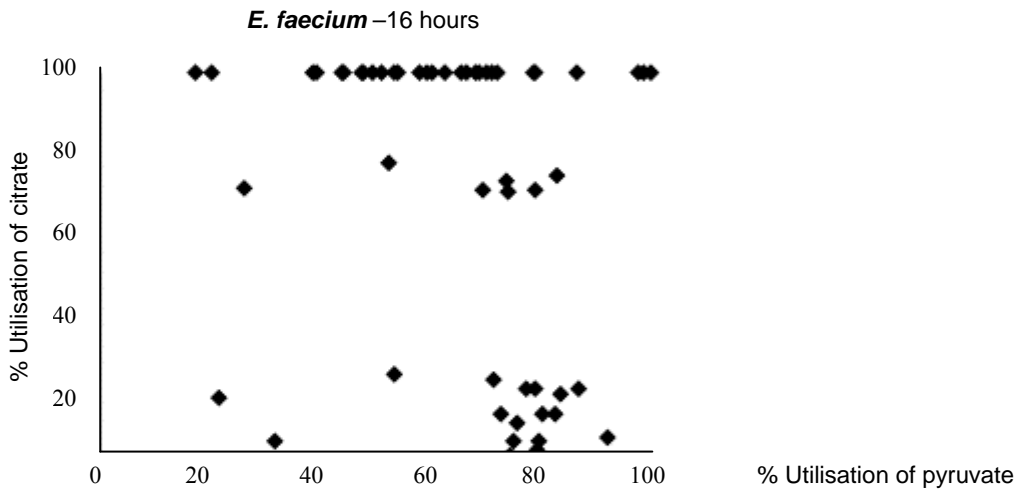


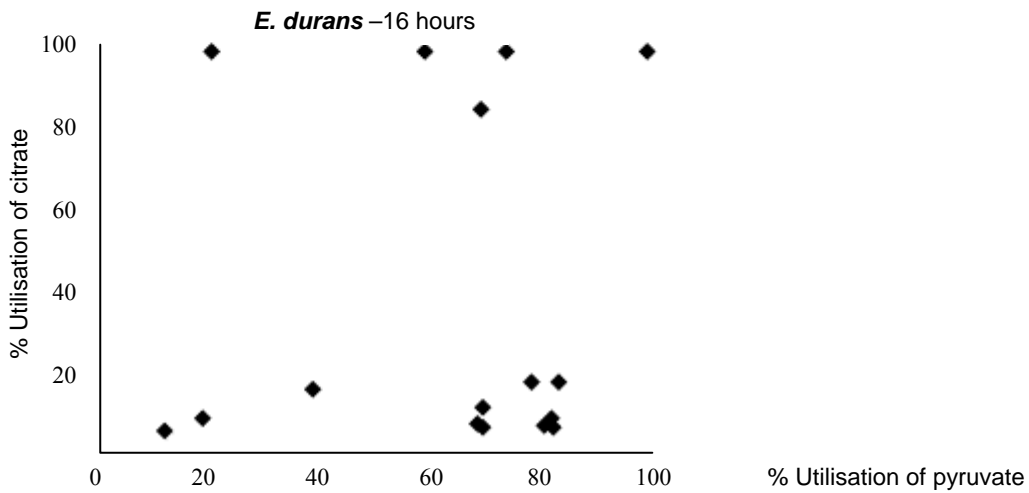
Figure 9: Correlation between citrate and pyruvate utilisation after growth of enterococci in modified MRS broth (containing 30 mmol/L⁻¹ of citrate or pyruvate) for 16 hours at 37 °C. (a) *E. faecalis*, (b) *E. faecium* and (c) *E. durans* strains [161].



a)



b)



c)

3.2.5 Production of volatile compounds

The breakdown of lactose and citrate during cheese ripening gives rise to a series of volatile compounds – acetaldehyde, ethanol, diacetyl, acetone, and acetoin, which may further contribute to flavour development of fermented dairy products. In this aspect, many *E. faecalis* and *E. faecium* strains isolated from dairy foods are shown to be good producers of mainly acetaldehyde, ethanol and acetoin (Table 3) [5, 161]. Therefore, this illustrates the importance of some enterococci as active contributors to sensory characteristics of fermented dairy products [75].

Table 3 shows the concentrations of the principal volatile compounds produced by enterococci according to the study done by Sarantinopoulos *et al.* [161]. It shows that, in general, *E. faecalis* isolates produced acetaldehyde and ethanol in the highest concentrations, while acetoin highest concentrations were produced by *E. faecium* isolates.

Table 3: Concentrations of main volatile compounds produced by enterococci of food, human and animal origin (summarised according to Sarantinopoulos *et al.* [161]).

Acetaldehyde						
Concentration range	<i>E. faecalis</i>		<i>E. faecium</i>		<i>E. durans</i>	
< 5 ppm	31	(55.4%)	57	(100%)	16	(100%)
> 5 ppm	25	(44.6%)	0		0	
Total of strains tested	56		57		16	
Ethanol						
Concentration range	<i>E. faecalis</i>		<i>E. faecium</i>		<i>E. durans</i>	
0 ppm	0		10	(17.5%)	1	(6.3%)
< 40 ppm	12	(21.4%)	45	(79.0%)	14	(87.4%)
40 - 80 ppm	33	(59.0%)	2	(3.5%)	1	(6.3%)
> 80 ppm	11	(19.6%)	0		0	
Total of strains tested	56		57		16	
Acetoin						
Concentration range	<i>E. faecalis</i>		<i>E. faecium</i>		<i>E. durans</i>	
0 ppm	2	(3.6%)	13	(22.9%)	4	(25%)
< 30 ppm	29	(51.8%)	24	(42.1%)	9	(56.2%)
30 - 60 ppm	21	(37.5%)	10	(17.5%)	3	(18.8%)
> 60 ppm	4	(7.1%)	10	(17.5%)	0	
Total of strains tested	56		57		16	

The study also reported that, regarding the origin of the isolates, *E. faecalis* isolates of food origin were the main acetaldehyde producers. Ethanol concentrations were also highest among *E. faecalis* isolates of food origin, although *E. faecium* isolates showed

more frequent production of this gas. Acetoin concentrations were found in the highest concentrations and more frequently among *E. faecium* strains of food origin. Generally, of all the three species, *E. faecalis*, and to a lesser degree *E. faecium*, produced the highest concentrations of these compounds and most of them were of food origin [161].

Finally, the production of diacetyl by *E. faecalis* cannot be ignored. In fact, it has been suggested that presence of strains of this species in Cebreiro cheese produced more diacetyl and acetoin than lactococci, *Leuconostocs* or lactobacilli [29].

3.2.6 Bacteriocin production

Enterococci's contribution to food is not limited to final taste development through their primary and secondary metabolisms, they also produce several enzymes that interact with food components and promote other important biochemical transformations linked to food bio-preservation [75]. In this regard, the enterococcal 'bacteriocins' (also called 'enterocins') are enzymes produced by numerous enterococcal strains, mostly belonging to the *E. faecalis* and *E. faecium* species associated with food systems: dairy products [55, 122, 144, 145, 146, 176, 189], sausages [10, 11, 12, 28, 32, 33, 79, 121], fish, and vegetables [17, 56, 58, 129, 187]. Especially important are the numerous bacteriocin-producing enterococci reported primarily among strains of *E. faecium* in the last 15 years [10, 28, 32, 33, 54, 55, 56, 58, 60, 71, 79, 121, 129, 137, 146, 189].

Enterocins are small, ribosomally synthesised, extracellularly released, antibacterial peptides or proteins that display a limited inhibitory spectrum towards other Gram-positive bacteria (in particular closely related strains), food-borne pathogens, and spoilage bacteria [112]. Enterocins usually belong to class II bacteriocins, i.e. they are small and heat-stable non-lantibiotics, being stable in milk and able to be produced in the temperature range of 30-37 °C. They are insensible to rennet, have a stability over a wide range of pH values, and a general compatibility with other starter LAB species [75]. Generally, the enterococcal strongest inhibitors of food-borne pathogens (especially against *Listeria monocytogenes*) belong to this class II of LAB bacteriocins [54] and are typically characterised at the biochemical level as enterocins A, B, I, and P [10, 28, 32, 56].

As suggested, since their inhibitory activity often encompasses food spoilers and food-borne pathogens, they are interesting additives for foods in the frame of natural food

preservation (bio-preservation role). Alternatively, they are also attractive to be used as co-cultures in food fermentation where they could contribute to the competitiveness of the producer strains and to the prevention of food spoilage and contamination (protective role) [112].

In the preservation context, bacteriocins produced by enterococci proved to have an interesting technological potential because almost all of them are strongly active against the food spoilers and food-borne pathogens such as *L. monocytogenes*, *Clostridium* spp. (including *C. botulinum* and *C. perfringens*), *Staphylococcus aureus*, *Bacillus* spp., *Brochothrix* spp., *Vibrio cholerae*, and spoilage LAB [25, 54, 59, 66, 75, 111, 112, 159, 160].

However, enterocins are especially active against *Listeria* and *Clostridium* [112]. For instance, inhibition of *L. monocytogenes* can be attained by the enterocin EJ97 produced by the strain *E. faecalis* EJ97 [66], and also by the enterocin 416K1 produced by the strain *E. casseliflavus* IM 416K1 [159]. Also, the strains *E. faecium* CCM 4231 and *E. faecium* RZS C13, used as starter cultures, produce bacteriocins that are strongly active against *Listeria* spp. and inactive against other LAB [25]. The strain *E. faecium* RZS C5, a natural cheese isolate, is also an interesting bacteriocin producer that has strong activity against *L. monocytogenes* [112].

Little is known about the kinetics of bacteriocin production in food ecosystems, but apparently bacteriocin production by LAB is a growth-associated process which ceases when cell growth starts to level off. For instance, it is known that enterocin production by the strain *E. faecium* RZS C5 seems to be limited to the very early growth phase [111]. Sarantinopoulos *et al.* [164] also found that *E. faecium* strain FAIR-E 198 produced enterocin throughout its growth phase, showing primary metabolite kinetics with a peak activity during the mid-exponential phase.

Taking all the advantages into account, enterocin-producers, or enterocins themselves, show a potential as bio-preservatives or protective cultures for meat and dairy foods. Further aspects of the importance of bacteriocins, specifically concerning dairy production, are discussed in Chapter 7. The bacteriocins related to meat products are discussed in Appendix D.

Table 4 shows a summary of the well-known bacteriocins produced by *E. faecalis* and *E. faecium* strains (summarised according to [59]).

Table 4: Well-characterised bacteriocins produced by *E. faecium* and *E. faecalis* strains (summarised according to [59]).

Bacteriocin	Producer organism	Reference
Enterocin A	<i>E. faecium</i>	Aymerich <i>et al.</i> [10]
Enterocin B	<i>E. faecium</i>	Casaus <i>et al.</i> [28]
Enterocin P	<i>E. faecium</i>	Cintas <i>et al.</i> [32]
Enterocin L50A	<i>E. faecium</i>	Cintas <i>et al.</i> [33]
Enterocin L50B	<i>E. faecium</i>	Cintas <i>et al.</i> [33]
Bacteriocin 31	<i>E. faecalis</i>	Tomita <i>et al.</i> [180]
AS-48	<i>E. faecalis</i>	Martínez-Bueno <i>et al.</i> [128]

3.3 Conclusions

In general, enterococci can better withstand pH levels approaching neutrality, and can better tolerate a 6.5% salt content. Their growing temperatures range between 10 and 45 °C, but most grow at 10 °C. Below and above these pH and temperature levels, enterococci experience an increased growth lag and lower activity. The pH and nutrient composition of the suspending medium, the number and age of the cells, the combination of the time-temperature of the applied heat treatment, and other factors, have considerable influence on the pH and thermal resistance of enterococci.

Among the enterococcal biochemical properties that may be of interest in the processing of fermented food products, acid production, proteolytic, lipolytic and esterase activities, citrate and pyruvate metabolisms, and production of volatile compounds and bacteriocins, have been studied in different enterococcal strains.

In the cheese industry, a rapid acid production during the initial steps of cheese preparation is a characteristic that results in the development of appealing sensory attributes and prevents the growth of adventitious microflora. For these purposes, due to the acidification abilities that some enterococcal species can have, the use of some enterococcal strains as ‘starter’ cultures has been studied. However, in spite of the acidifying potential that *E. faecalis* can offer, enterococcal strains in general are considered as poor acidifiers. Hence, their importance in the dairy industry as starter culture organisms is minimal. They may be more useful as ‘adjunct’ cultures instead.

Casein degradation in relation to proteolytic and peptidolytic activities of micro-organisms play an important role in the development of texture in cheese, which also suggested that the use of enterococci could have a beneficial effect. However, many reports on enterococcal proteolytic activities confirmed that the majority of the enterococcal species and their strains exhibit low and variable proteolytic performance. Strains of *E. faecalis* of food origin seem to be the most active. Only few reports exist on the peptidase activities of enterococci, and all of them confirm low performance as well.

Although the esterolytic and lipolytic activities of enterococci has not been well described yet, their possible contribution points out to a better flavour and texture development during cheese ripening. However, the lipolytic activity of enterococci is generally low, with *E. faecalis* strains of food origin being the most efficient. The esterolytic activity, on the other hand, seems to be more effective, especially for *E. faecium* strains of food origin.

Numerous volatile components such as lactate, acetate, formate and ethanol produced by LAB are important in cheese production because they influence on the flavour of the cheese. Thanks to the metabolism of hexoses, enterococci produce mainly lactate, but they also seem to possess the metabolic potential to produce significant amounts of acetaldehyde, ethanol, and acetoin, when grown in milk. Citrate and pyruvate metabolisms are initial steps for the production of acetate, formate and ethanol. The ability to metabolise citrate and pyruvate to produce these flavour compounds varies among the enterococcal species and strains, though organic acid utilisation by *E. faecalis* strains isolated from food seems to be faster and more effective. Production of volatile compounds could be remarkable for some enterococci, with the highest levels of production found in *E. faecalis* strains of food origin, while *E. faecium* strains could be the most frequent species in volatile compounds production.

Finally, the capability of some enterococcal strains to produce enzymes called ‘enterocins’, which can inhibit the growth of food pathogenic bacteria and food spoilage micro-organisms, has suggested their use as ‘protective cultures’ in cheese manufacture.

Chapter 4: Laboratory Identification

4.1 General considerations

The laboratory isolation of enterococci has been extensively reviewed by Hartman *et al.* [78] and Domig *et al.* [46, 47]. Due to their significance in food, feed, environmental, and clinical samples, enterococcal detection and enumeration have become an important issue not only in daily routine but also in current research activities. But even though several media have been advocated for the selective isolation and quantification of enterococci, and several protocols have been published for diverse purposes, there is no single method that universally meets all requirements yet, as all have one or more shortcomings [46].

The typical culture media employed for the estimation of enterococcal counts in water, food, feeds and clinical specimens such as the (Membrane filter) Slanetz-Bartley (SB) agar and the Kanamycin-aesculin-azide (KAA) medium are advantageously applied in the case of selective enumeration of enterococci as single components, i.e., if enterococci are the only microbial component in the product. However, like any other members of the LAB, enterococci are often found associated with a microflora of considerable diversity, and this is reflected in a much more complicated situation when samples containing such a mixed microflora have to be examined for enterococcal recovery [46].

Consequently, a number of selective agents, incubation conditions, and combinations and modifications thereof have to be used, taking into account various advantages but also drawbacks, for example, the lack of sufficient selectivity of most of these media necessary to clearly distinguish enterococci from the accompanying microflora. The use of media containing either selective chromogenic dyes or selectively inhibitory substances (e.g. antibiotics) may, however, enable some differential bacteriological enumeration [46].

Because of their requirements for several vitamins and amino acids, enterococci cannot be grown easily in synthetic media. Profuse and rapid growth is only achieved if rich

complex media such as Brain Heart Infusion (BHI) broth or Trypticase Soy (TS) broth are used [46].

In any case, the media and methods to be selected should have a good selectivity, differential ability, quantitative recovery, and relative ease of use [78].

Therefore, depending on the nature of the accompanying microflora and its level, quantitative and selective isolation methods or, in some cases, elective media are needed.

This chapter cites the most common routine methods employed for isolation and identification of enterococci in dairy products.

4.2 Routine methods of isolation, identification and confirmation of enterococci

When food samples are analysed, due to the variability and adaptability to different environments that enterococci have (e.g., strains going from aerobic to anaerobic conditions, and *vice versa*), a ‘general-purpose’ medium has been recommended for enterococcal recovery, and *physiological* as well as *serological* methods have conventionally been employed for their subsequent identification, enumeration, and confirmation [78].

4.2.1 Isolation and identification

Today, there are over 100 modifications of selective media for the isolation of enterococci from various specimens and due to the heterogeneity in the composition of the media it is impossible to recommend one universal medium [46]. The choice of a particular medium basically depends on whether enterococci are to be counted in total and whether the habitat is highly contaminated or not.

This also applies to milk products: several media for the isolation, enumeration and identification of enterococci have been reviewed, and it has been concluded that there are no ‘ideal’ media available, because most display drawbacks in terms of selectivity and recovery. As a result, the parallel use of two media, one highly, the other moderately selective, may be a reasonable way to obtain acceptable results from any food (including dairy) habitat.

At present, among the newly developed and commercially available media relevant for dairy products, a choice among KF streptococcal medium, Citrate azide agar, Citrate azide tween carbonate (CATC) agar, (Membrane-filter) SB agar, Bile aesculin azide (BEA) agar, KAA agar with or without supplements, or Bromocresol purple azide (BB) broth can be made (for details see Appendix A). *Enterococcus* selective differential (ESD) medium, Membrane filter *Enterococcus* (mE) agar, Azide dextrose (AD) broth, Barnes (Bar) medium modified, Azide blood agar base (ABAB), and Bile aesculin (BA) agar are also among the recommended media for enterococcal isolation and enumeration in dairy food [46]. An alternative medium can be the Fluorogenic gentamicin thallos carbonate (fGTC) agar [78].

In spite of the large variety of suggested media and methods with their modifications, the Citrate azide agar and the BEA agar, are the most recommended media for enterococcal isolation in dairy products [46]. Extensive screening experiments dealing with the examination of probiotic enterococcal strains contained in animal feeds have shown that especially the BEA medium seems to be the best suited for selective enumeration since it still demonstrates sufficient selective properties, even in combination with other LAB bacteria (lactobacilli and pediococci) and bifidobacteria [46].

It is always practical to bear in mind that although media for the examination of enterococci are usually incubated at 35-37 °C, when examining enterococci in dairy products a higher incubation temperature (45 °C) may be necessary to suppress the growth of the background microflora [46, 78].

Finally, in terms of identification, when *physiological* methods are employed, a spectrum of characteristics must be examined because no single, two, or three traits will establish a definitive identification [78]. For example, although all enterococci produce group D antigen, the presence of this antigen can be difficult to demonstrate in some isolates, opening the possibility of having false-negative group D reactions.

4.2.2 Confirmation

For confirmation, typical colonies can be selected, and either through conventional methods, through rapid or automated procedures, or through serological tests, the identity of the isolates could be verified [78]. For further details see Appendix B.

4.3 Other methods: genotypic-based techniques

Many of the recently described enterococcal species exhibit deviations from the so-called classical enterococci phenotypical properties [47]. For this purpose, further examination based on the application of genotypic methods may become necessary [46]. This will allow a more reliable and fast identification, especially from sources with a heterogenous microflora [102]. In particular, 16S and 23S rRNA targeted probes proved to be successful in identifying *Enterococcus* species [102]. Other methods for intra-species differentiation include protein fingerprinting, polymerase chain reaction (PCR)-based typing methods such as random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), contour-clamped homogenous electric field electrophoresis (CHEF), and restriction enzyme analysis [102]. However, apparently these methods primarily attempt to fill the gaps of the lack of a proper characterisation and designation of the diverse new enterococci within the classic taxonomy, and therefore their use within the food microbiology may still be infrequent.

4.4 Conclusions

Precise isolation and identification of enterococci in dairy foods is important. However, contradictory methodological recommendations can be found in the literature, and different media and methods have been proposed during the last two decades. Given that most of these methods emphasise on compositional details and on specific applications of the media intended to be used, there is no consensus on the most suitable medium. Yet, the most suggested media for the identification and enumeration of enterococci in dairy foods are mainly the Citrate azide agar and the BEA agar. The KF agar, the CATC medium, the (Membrane filter) SB agar, the KAA medium, and the BB broth, are also recommended. Genotyping techniques have also been recommended; however, they are more frequently used for taxonomical characterisation and sub-species classification.

Chapter 5: Sources and Reservoirs

Enterococci are ubiquitous in their occurrence, with their habitats ranging from the intestinal tract of man and a variety of farm animals to different forms of food and feed [102]. Several studies (like the one done by Kuhn *et al.* in 2003 [104] in a number of countries in Europe) demonstrated that enterococci were present almost everywhere in the food chain as well as in the environment.

This chapter deals with enterococcal sources and their reservoirs.

5.1 Primary sources

5.1.1 Animals

Enterococci constitute a large proportion of the autochthonous bacteria associated with the mammalian gastrointestinal tract. However, although it is generally believed that the primary habitat of enterococci is the intestinal contents of warm-blooded animals, the gastro-intestinal contents of cold-blooded animals, including insects and birds, constitute other important habitats as well [124].

With regards to enterococcal species found in animals, Klein [102] has suggested that certain of these species tend to have a predilection for particular animal species (Table 5). For example, in the human intestine both *E. faecium* and *E. faecalis* are the most prevalent species, and in production animals like poultry, cattle, and pigs *E. faecium* is a prevalent species. Other species also occur in high numbers, e.g. *E. faecalis* and *E. cecorum*, and less frequently *E. gallinarum* and *E. durans/hirae*. *E. mundtii* and *E. casseliflavus* are more typically of plant origin. This indicates a great diversity in the ecology of enterococci, but with tendencies to have affinities towards their hosts at the same time [102].

Table 5 shows a summary of the occurrence of enterococci in the gastro-intestinal tract of human, cattle, pig and fowl, according to Klein [102].

Table 5: Occurrence of enterococci in the gastro-intestinal tract [102].

Species	Human	Cattle	Pig	Fowl
<i>E. faecalis</i>	++	(+)	+	++
<i>E. faecium</i>	++	++	+	++
<i>E. durans/hirae</i>	(+)	-	(+)	(+)
<i>E. gallinarum</i>	(+)	-	-	(+)
<i>E. casseliflavus</i>	(+)	-	-	-
<i>E. cecorum/columbae</i>	-	+	+	++

++: usual; +: frequent; (+): occasional; -: not mentioned

While this table and other reports [70, 102, 108] agreed on that *E. faecium* is the most common species found in the intestinal tract of dairy cattle, other reports [43, 102] agreed on that *E. faecalis* can be found more often in animal faeces (and food from animal origin) than *E. faecium*. Also, a number of reports [59, 70, 108] suggested that *E. faecalis* is in fact the most common *Enterococcus* species in human faeces (as it will be discussed under section 5.1.2). Actually, the prevalence varies according to regions and countries [59] and contradictions may be in part due to difficulties in the isolation and enumeration of *E. faecium* using selective media [102].

While *Enterococcus* spp. are found in the intestinal tract of dairy cattle, they are less prevalent than other bacteria of intestinal origin in dairy cow's faeces. *Streptococcus bovis* is frequently isolated from the alimentary tract of adult cattle, sheep and other ruminants [77], and they largely predominate in dairy cows faeces as well [43].⁵ In this regard, there have been reports of finding *E. faecium*, *E. hirae* and *E. faecalis* by several authors [42, 106, 130], but all of them agreed in that *S. bovis* is in fact the most frequently occurring organism in cow's faeces [70].

In pigs, even though *E. faecalis*, *E. faecium*, *E. hirae* and *E. cecorum* are the enterococci most frequently isolated from their intestines, *E. faecium* predominates in the faecal samples [59]. The intestinal microflora of young poultry contains principally *E. faecalis* and *E. faecium*, but *E. cecorum* predominates in the intestine of chickens over 12 weeks old [59]. Not surprisingly, faeces collected from broiler chickens and fattening pigs at the farm level have been found to contain enterococci as well [23].

⁵ A study carried out by Devriese *et al.* [43] on faeces of 45 dairy cows located on 9 farms, revealed 12 *Enterococcus* spp. isolates and 90 streptococci isolates. All streptococci were identified as *S. bovis*. They concluded that enterococci were rare and that *S. bovis* largely dominated dairy cows' faeces.

Enterococci are consistently isolated from carcasses of beef cattle, poultry and pigs in abattoirs, *E. faecalis* and *E. faecium* being the most predominant species recovered [70]. Raw poultry (chicken and turkey cuts) and pork meat (pork cuts, minced meat and sausages) from food processing plants and retail outlets are also sources of enterococci [23]. However, *E. faecalis*, *E. faecium* and *E. durans* are much less frequently isolated in livestock such as pigs, cattle and sheep than from human faeces [59, 102, 108].

The occurrence of enterococci in milk and dairy products is dealt with more details in Chapter 7.

5.1.2 Humans

In humans, enterococci are part of the normal polymicrobial intestinal flora along with approximately 450 other aerobic and anaerobic bacterial species [98].

In most individuals, 10^5 - 10^7 CFU of enterococci are found per gram of stool. While this may seem a large number, it is only a fraction of the total bacterial flora of the stool (10^{10} - 10^{12} CFU/g, mainly composed of anaerobic Gram-negative rods) [98].

Of the enterococci, *E. faecalis* is often the predominant species in the human bowel, although in some individuals and in some countries, *E. faecium* outnumbers *E. faecalis* [59]. Numbers of *E. faecalis* in human faeces range from 10^5 to 10^7 CFU/g (which could be up to 100% of the enterococcal population) compared with 10^4 to 10^5 CFU/g for *E. faecium* [59]. Only *E. faecalis* has been isolated from the faeces of neonates [59].

Smaller numbers of enterococci are observed in oropharyngeal secretions, vaginal secretions, and on the skin, especially in the perineal area [98]. Thus, enterococci can be considered as normal commensals, not only of the human gastrointestinal tract, but also of the complete human organism [98].

When comparing humans to animals *E. faecalis*, *E. faecium* and *E. durans* are more frequently isolated from human faeces than from livestock such as pigs, cattle and sheep [102].

5.1.3 Environment

As well as being associated with warm-blooded animals, their faeces, animal carcasses or milk, enterococci are also able to colonise a diversity of niches, mainly because of

their exceptional aptitude and intrinsic resistance against hostile conditions. Thus, they grow and survive in extra-enteric environments under conditions that are not favourable for most bacterial species. Some enterococci, such as *E. mundtii* and *E. casseliflavus*, have even adapted to an epiphytic relationship with growing vegetation. Enterococci also abound in soil, surface waters and recipient waters, sewage water (e.g. hospital sewage), animal feed, farmland fertilised with manure, and on crops, plants and vegetables [59, 104]. A study performed in Europe by Kuhn *et al.* in 2003 [104], to compare enterococcal populations from a range of different samples of animal, human and environmental origin, showed that enterococci were present in most sample types (Table 6).

Table 6: Environmental samples collected in Europe (Sweden, Denmark, Spain and the United Kingdom) and the percentage of samples containing detectable amounts of enterococci (summarised according to [104]).

Sample type and origin	Number of samples	% of samples in which enterococci were detected
<u>Human/environmental</u>		
Urban sewage (raw)	105	100
Urban sewage (treated)	109	94
Hospital sewage	69	86
<u>Humans</u>		
Healthy humans (faecal)	63	83
Hospitalised patients (faecal)	18	100
Clinical isolates	158	99
<u>Animals in slaughterhouses</u>		
Broiler chicken (caecal)	387	83
Cattle (caecal)	328	80
Pig (caecal)	682	63
<u>Samples related to farm animals</u>		
Pig (faecal)	201	93
Pig manure	126	97
Farmland with manure	141	44
Crop from farmland with manure	31	48
Farm runoff water	15	100
Other animal farm animals	15	60
Sheep milk	65	100
<u>Mixed samples animal/human/other</u>		
Surface water	149	84
Pig feed	81	74
Farmland and crop without manure	125	30
TOTAL	2868	77

For Kuhn *et al.* study [104], 2868 samples from Sweden, Denmark, Spain and the United Kingdom, were collected during the period April 1998 to December 2000, from humans (healthy, hospitalised, and clinically ill individuals), from animals (slaughterhouse carcasses and farm animals), and from the environment (pig farms, sewage, and surface water receiving treated sewage). These samples were collected from different sites and different individuals. Later, more than 20,000 isolates were typed in total, using a rapid typing method for enterococci - the Rapid Screening PhP-Plates of the PhenePlate™ typing system⁶ – and the majority of the samples (77%)

⁶ Rapid screening plates are particular suitable for ecological studies involving large numbers of isolates, when the information of the whole population is more important than the information on each individual isolate [2].

showed the presence of presumed enterococci (Table 6 shows the number of samples collected by Kuhn *et al.* and their % of positive enterococci). According to the table, the highest percentages of enterococci, for each sample category, were found in raw urban sewage samples, in hospitalised patients and clinical isolates samples, in broiler chickens and cattle samples from slaughterhouses, in farm runoff water and sheep milk samples, and in mixed surface water samples.

The study also pointed out that, among the enterococcal isolates, the most common species found were *E. faecium* (33%), *E. faecalis* (29%), and *E. hirae* (24%), even though different enterococcal populations differed in their species distribution. In Sweden, *E. faecalis* was the most predominant enterococcal species (31%), while in Spain and in the United Kingdom *E. faecium* was the most common enterococcal species (44 and 48%, respectively). *E. hirae* was the most predominant enterococcal species found in Denmark (38%). According to the sample type and origin, *E. faecalis* was the enterococcal species mainly associated with hospitals (clinical isolates, hospital sewage and hospitalised patients). Healthy individuals and urban sewage contained less *E. faecalis*, but in healthy individuals it still was the most prevalent enterococcal species found. The enterococcal species distribution among isolates from slaughterhouses varied between animal species and also between countries [104].

However, whether this study followed a random sampling approach or not is not clearly stated, i.e. the study does not make reference of where the farm, field, animal and human samples have come from, nor how many farms and fields were sampled. Hence, it is suspected that the study was not representatively sampled and that did not follow a formal survey procedure (sampling bias).

In conclusion, the Kuhn *et al.* study [104] firstly aimed to show the abundance of enterococcal niches. Secondly, it aimed to show the high diversities and low similarities between the enterococcal populations of human versus animal origins (and even among some animal species) suggesting some form of host specificity. However, the study only accomplished the first aim (since it showed that enterococci are ubiquitous in their occurrence) but not the second (since the ‘*ad hoc*’, non-random sampling method followed made their results not repeatable and therefore no credible).

5.2 Secondary sources: enterococci in foods

In accordance with their widespread occurrence in the intestinal tract of animals, enterococci and other group D-streptococci are present in many foods, especially in those of animal origin. Therefore, the isolation of *E. faecalis* and *E. faecium* in foods has often been used to indicate a ‘primary’ contamination with faeces [75, 102].

Nevertheless, contamination of water sources, exterior of the animal and/or of milking equipment and bulk storage tanks can act as ‘secondary sources’ for food contamination.

Hence, because enterococci often have shown to be unrelated to direct faecal contamination as a result of their widespread habitat, they are now considered as *normal components of the animal derived food microflora*, and not only as indicators of poor hygiene or previous faecal contact [59, 75, 102]. Furthermore, because of their role as added cultures in meat and cheese manufactures, enterococci can be also found in important numbers in the finished products.

Table 7 gives an overview of the distribution of enterococcal species in various foods from animal origin (including raw and cultured products) [102].

Table 7: Distribution of enterococci in food [102]

Foods	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. durans/hirae</i>	<i>E. gallinarum</i>	<i>E. casseliflavus</i>	<i>E. mundtii</i>	<i>E. avium</i>	<i>E. malodoratus</i>	<i>E. pseudoavium</i>	<i>E. raffinosus</i>
Cheese	(+)	++	(+)	-	-	-	ND	ND	ND	ND
Fish/crustaceae	+	(+)	-	(+)	-	(+)	ND	ND	ND	ND
Meat	+	++	(+)	(+)	(+)	(+)	ND	ND	ND	ND
Cheese-meat combination	(+)	+	(+)	(+)	-	-	ND	ND	ND	ND
Pork carcasses	++	(+)	(+)	ND	ND	ND	ND	(+)	(+)	(+)
Fresh sausage	++	-	(+)	ND	ND	ND	ND	(+)	-	-
Expired sausage	+	-	(+)	ND	ND	ND	ND	(+)	+	(+)
Spoiled sausage	(+)	++	(+)	ND	ND	ND	ND	-	-	-
Minced beef	++	(+)	(+)	(+)	-	ND	-	ND	ND	ND
Minced pork	++	(+)	(+)	(+)	(+)	ND	(+)	ND	ND	ND

++ usual, + frequent, (+) occasional, - not mentioned, ND not investigated

5.3 Conclusions

Enterococci colonise a diverse range of niches. They primarily inhabit the gastrointestinal tract of animals, but with time they also have developed an outstanding ability to inhabit extra-intestinal environmental sources. Raw food (especially those derived from animals) also contain enterococci in low or high numbers. In a comparative study of a wide range of enterococcal populations in animals, humans, and the environment in Europe, the wide range of enterococcal sources was demonstrated.

With regards to which enterococcal species are the most common found in animals, environment, and foods, *E. faecalis* and *E. faecium* are the most prevalent. Between them, diverse opinions with regards to which is the most prevalent in cattle and humans have been reported. Although none of the enterococcal species can be considered as absolutely host specific, and that prevalence of one or other species varies according to several external factors (nutrition, region, country), there appears to be a limited exchange of enterococcal strains between human and animal species and as a result there may be some host specificity. Consequently, only certain types of enterococci may be able to establish themselves in the human gut, at least for a transient time. Unfortunately, this hypothesis remains unproven.

Chapter 6: Enterococci as Pathogens?

For many years, enterococci were considered to be harmless commensals with low pathogenic potential for humans. At present, this view is changing because of the apparent increasing role that enterococci are suggested to have in nosocomial infections, especially in patients with preceding antibiotic therapies or long and severe underlying diseases [59, 62, 74, 75, 98, 138]. The suggested pathogenicity of enterococci may be due to the presence of virulence traits in some of their strains [59, 62, 124], which may enhance the ability to evade animals and humans' immune response. Since these virulence traits can be genetically encoded, a further transmission to other strains, and possibly other bacteria, may occur [62]. It seems however, that only some strains of enterococci (mainly of clinical origin) produce these virulence traits [62]. Intrinsic and acquired antibiotic resistance to several drugs has been reported as well [18, 20, 23, 35, 39, 52, 65, 76, 95, 98, 100, 107, 110, 115, 118, 123, 124, 126, 132, 133, 138, 143, 148, 151, 156, 157, 165, 167, 168, 173, 178, 195].

Hypotheses are suggested but evidences are limited. This chapter reviews the possibility of enterococci being associated with human disease, based on available scientific evidence. It also reviews aspects of enterococcal virulence traits, biogenic amines production, and antibiotic resistance, including a possible ability for their inter-transmission.

6.1 Evidence for clinical disease in humans due to enterococci

6.1.1 Clinical infections

From a clinical perspective, enterococci have long been considered non-pathogenic bacteria, until multiple antibiotic-resistant strains were identified in the late 1970s. Since then and over the last three decades, enterococci are increasingly regarded as agents with potential pathogenicity in hospitalised patients [98, 132, 138, 165, 191], ranking fourth [191], third [95, 115, 135], or even second [132, 138] in frequency of bacteria that can be isolated from these patients in the United States of America, staphylococci and *Escherichia coli* being the most prevalent [151, 165, 191]. Apparently, enterococci are present in ~9-12% of nosocomial patients of the United

States [95, 115]. In the United Kingdom increases in enterococcal occurrence are also evident [135].

The most frequent human clinical infections that have been associated with enterococci (often as part of a polymicrobial flora) include bacteraemias (most commonly), urinary tract infections, intra-abdominal and pelvic infections, burn wound and deep tissue infections, and endocarditis [59, 98, 138]. Enterococci have rarely been associated with meningitis and respiratory tract infections [98, 132, 135, 138].

Out of the more than 20 species of the genus *Enterococcus*, only two are suggested as responsible for these infections - *E. faecalis* and *E. faecium* [95, 115, 136]. So far, *E. faecalis* is the predominant species found in human enterococcal infections, accounting for 80-90% of enterococcal isolates, while *E. faecium* accounts for the majority of the remainder [59, 62, 98, 115, 161]. However, recent data indicate an increase in the number of enterococcal infections associated with *E. faecium*, which is probably the result of their higher resistance to antimicrobials as well as the emergence of vancomycin-resistant strains [20, 52, 76, 91, 136, 157]. Other species such as *E. durans*, *E. avium*, *E. casseliflavus*, *E. gallinarum*, *E. raffinosus*, and *E. hirae* have been occasionally isolated and were therefore only rarely associated with human enterococcal infections [95, 115, 138].

6.1.2 Risk factors of host and environment that may contribute to pathogenesis

Infectious processes are always the result of interplay between determinants of pathogenicity⁷ and virulence⁸ factors of the invading organism and the host factors trying to prevent the occurrence of disease. Herein, the host immune system plays a fundamental role in avoiding aggressive factors from the intruder [98].

With respect to enterococci, extensive research concerning risk factors has been done, and more especially on those that could compromise the *host* immune system [20, 52, 65, 76, 95, 98, 115, 123, 132, 133, 138, 157, 165, 168, 191]. These studies concluded

⁷ *Pathogenicity* can be defined as the ability of an organism to cause disease, i.e. harm the host [170].

⁸ *Virulence* refers to the degree of pathology caused by the organism and may exhibit different levels [170].

that all kinds of severe immuno-suppression, like severe underlying diseases,⁹ a long hospital stay, residency in an intensive care unit, previous antimicrobial therapies,¹⁰ or the co-existence of many of these factors, could influence enterococcal colonisation in a patient.

6.1.3 Pathogenesis

Since enterococci belong to the normal gut flora, it was previously thought that enterococcal infections were ‘endogenously acquired’ from the patient’s own gastrointestinal tract. However, as noted previously, in recent years, analyses suggest that most infecting strains may be ‘exogenously acquired’ [98, 132, 135]. Hence, a suggested pathogenic process for enterococci has been proposed: in the debilitated patients, common entries for enterococci include urinary portals especially, and biliary portals [59, 98, 135, 138]; after an enterococcal strain¹¹ has entered an immuno-compromised host, the requirement for particular traits of the strain for manifest disease decreases; subsequently, the strain expands, and after tissue invasion (colonisation) in the host, disease may occur [98]. However, evidence for this hypothesis is scarce. The following section examines available studies for evidence of a role of enterococci in causing disease.

6.1.4 Review of Hill’s criteria for causality

An understanding of the causes of disease is important in health for correct diagnosis, application of correct therapies, and prevention. Hill’s list of criteria [80] is a systematic process that helps to evaluate a causal hypothesis. Several studies of enterococci isolated in the bloodstream of infected nosocomial patients [20, 52, 65, 76, 95, 107, 115,

⁹ Immuno-compromised patients with chronic ambulatory peritoneal dialysis, bowel resection, nephritic syndrome or cirrhosis, patients with underlying valvular heart disease or prosthetic valves could be at risk, as well as drug addicts and patients with prior complicated neurosurgeries or head trauma. Infected, burn or diabetic wounds, or any deep tissue infections, also could predispose the entrance for enterococci, along with other bacteria, into the blood stream. Specifically, major risk factors that could predispose infections in nosocomial patients are renal insufficiency, neutropenia, organ and bone marrow transplantation, the presence of vascular catheters [98, 107, 115, 191], and especially the preceding presence of genitourinary instrumentation or urinary tract infections [59, 95, 115, 132, 135, 138, 191].

¹⁰ An important suggested risk factor might be a preceding antibiotic therapy for other infectious diseases in nosocomial patients (such as antibiotics treatments against which enterococci possess a natural resistance or only an intermediate susceptibility) [59, 98, 115, 132, 133, 135, 143, 148, 157, 173], as well as the duration of the therapy and the use of five or more antibiotics [76, 107, 115, 133, 135, 157].

¹¹ Only limited types of these ‘exogenously acquired’ enterococci might be able to cause infections, i.e., only those enterococcal strains that are able to carry virulence traits and/or that are antibiotic resistant might be able to cause disease.

118, 123, 133, 143, 148, 157, 165, 167, 168, 173, 191] were evaluated according to Hill's criteria for causal inference.

To understand if enterococci have a causal relationship with clinical disease, firstly the location of patients infected with enterococci was evaluated. In all studies the patients spent extended periods in intensive care units of hospitals, all were terminally ill, and probably seriously immuno-suppressed. The risk of enterococcal infection increased when patients had received antibiotics to which enterococci were resistant. The hospital environment, with the burden of extensive antimicrobial use and horizontal transmission of resistant micro-organisms, increased bacterial colonisation pressure¹² of enterococci and other bacteria.

Secondly, in most patients enterococci were isolated as part of a polymicrobial flora [135, 138], which made it almost impossible to attribute a pathogenic effect to enterococci. For example, in one study of 4,367 bacteraemic nosocomial patients, enterococci were the third most common isolated bacteria with 553 (11.7%) patients carrying enterococci; however, there was not a single patient in whom enterococci were the only isolated bacterial species [95].

According to the study of Morrison *et al.* conducted in 1997 [135], up to 40% of cases enterococcal bacteraemia were accompanied by other organisms. In fact, enterococci only formed a small part (6-7%) of the polymicrobial flora isolated from the blood stream of infected nosocomial patients, *Staphylococcus aureus* and *E. coli* being the most predominant [98, 191].

Moreover, there has been too much focus on the factors that predispose the host to infection with enterococci while very little is known about the association between virulence factors of enterococci and the consequence of infection [98]. It is believed that enterococci are not particularly 'virulent' [98, 132, 135], at least in comparison with the often accompanying streptococci and staphylococci, of which many species are facultative pathogens [132]. The low pathogenicity is also evidenced in these studies by the fact that despite enterococci being isolated fairly often from sputum and other specimens from the respiratory tract of patients, these bacteria were rarely associated with respiratory tract infections [132].

¹² A high colonization pressure is said to occur when there is a high proportion of patients colonized with specific bacteria in a defined geographic area [136].

Antibiotic resistance, especially resistance of *E. faecium* strains to vancomycin, has received some attention in the literature. Enterococci are the first nosocomial pathogens to achieve pan-biotic resistance on such a widespread scale [115]. Perhaps enhanced by the imprudent use of antimicrobial agents, these organisms have acquired a remarkable ability to develop antimicrobial resistance in hospital environments. This increased enterococcal colonisation, resulting in a higher environmental load with enterococci [132]. However, antibiotic resistance alone could not explain the virulence of these bacteria [59]. For instance, vancomycin susceptibility or resistance of *E. faecium* strains have not independently increased infection risks in two studies [65, 107].

With regards to epidemiologic studies [20, 52, 65, 76, 95, 107, 115, 118, 123, 133, 143, 148, 157, 165, 167, 168, 173, 191], the reported estimates of infections and death associated with enterococcal bacteraemia vary according to study design, data analysis, patient population, case definition, control selection, and enterococcal species studied [136]. The majority of these studies were small [20, 52, 65, 76, 143, 148, 173], and only in one of the studies a control population was included, consisting of patients without bacteraemia [52]. Moreover, patients were infected with various enterococcal species [118, 143, 167, 173] for which the data have not been stratified. This may have introduced bias into these studies. More importantly, none of the studies examined blood isolates of patients for potential enterococcal virulence traits that might have contributed to disease. The reason was that in the studies blood samples always contained several different bacteria species (staphylococci, streptococci, and others), and this complicated the evaluation of the effect that enterococci and their virulence factors might have had on the severity of illness [136].

Taking all the preceding facts into account, a review of Hill's causal criteria follows.

Strength of association

Most of the studies did not quantitatively evaluate associations between isolation of enterococci and clinical disease symptoms. The studied patients were among the most severely ill in the hospitalised population, the hospitals were environmentally contaminated with enterococci and other nosocomial bacteria, and many patients had previously been treated with antimicrobials. As a result, the strength of associations inferred by some studies might have seemed moderately strong when in reality it was not. The only large study conducted in the U.S. by Weinstein *et al.* [191] examined

1,267 patients in 3 hospitals during the period 1992-1993. Eight hundred and forty-three (843) patients had critical septicaemia, of which 90.6% were unimicrobial for bacteria other than enterococci, and 9.4% were polymicrobial cases. In total, 944 different micro-organisms were isolated, and among these only 65 were enterococci. All the enterococci were isolated from the polymicrobial cases, where staphylococci and *E. coli* were predominant. Of the 65 enterococci, only 70% were judged as 'clinically significant' (with the ability of causing bacteraemia); the other 30% were contaminants or from unknown sources. The categorical decision to name an enterococcal isolate as 'clinically significant' was made after the patient's clinical history, physical findings, body temperature at the time of the blood culture, leukocyte count and differential cell counts, number of positive blood cultures out of the total number performed, results of cultures of specimens from other sites, imaging results, histopathologic findings, and clinical course and response to therapy were taken into account. If the clinical significance of the positive culture was not clear on the basis of the available information, the isolate was categorised as being of 'unknown significance'. The study reported a non-significant relative risk of death due to enterococci of 2.4. However, the reference category of patients not infected with enterococci was not clear. The latter presumably consisted of a patient group infected with other bacteria species. Since other characteristics of patients infected and non-infected with enterococci were not described, the study could not show convincing evidence for an increasing effect of enterococci on case fatalities. No post-mortem findings were reported, either. Therefore, it is not clear whether enterococci were the cause of the bacteraemia and mortality in the patients. No other study provided relevant evidence about the strength of an association.

Consistency

A large number of studies inferred from bacteraemic patients that enterococci were associated with disease. However, all studies were prone to bias due to absence of appropriate controls, unclear reference groups, and a lack of analytical methods to control various types of confounding and bias. While most conclusions were largely consistent, so were the types of bias. Consequently, none of the studies provided credible evidence for consistent literature reports about causal effects of enterococci on the type or severity of clinical disease.

Specificity

The question here is whether enterococci were associated with specific disease symptoms or syndromes, and whether these symptoms disappeared when enterococci were removed. Studies relating numerous infectious agents (including enterococci) to nosocomial infections attributed a fragile role of enterococci to disease. None of the studies performed in bacteraemic patients had enterococci as single bacteria. For instance, Jones *et al.* [95] carried out a study with a large number of patients (4,367) at 41 U.S. hospitals during the period of 1995-1996. The data provided intended to give documentation of the increasing prevalence of enterococci [95]. However, a mere increasing prevalence of enterococci in the blood stream of nosocomial patients, without associating disease symptoms specifically to enterococci while absent in patients infected by other bacteria, provides little scientific evidence for a specific pathogenic role. Moreover, in polymicrobial septicaemic patients the elimination of bacteria other than enterococci by antibiotic therapy not active against enterococci often cured the infections [98, 135, 191]. Thus, the observed cure was attributable to those other bacteria.

Temporality

Only one prospective study among humans was available to evaluate the effect of infection with enterococci on the fatality of disease [191]. The study resulted in an increased risk of death for patients infected with enterococci. Infection was diagnosed in live patients and they were followed up to release from hospital or death. However, the comparison group, although not clearly described, appeared to be patients with other infections without appropriate adjustment for confounding factors. Thus, infection with enterococci could have been caused by venal or urethral catheters that might have been absent from the reference group, facilitating iatrogenic inoculation of more severely diseased patients. In conclusion, there is little temporal evidence for a pathogenic role of enterococci in humans.

Experimental evidence

Experimentally, in 1992 and 1994, Jett *et al.* [90, 91] demonstrated that some virulence traits produced by strains of *E. faecalis* could induce retinal tissue damage in an endophthalmitis model developed in New Zealand White rabbits. Similarly, these traits also increased mortality in an endocarditis rabbit model, caused systemic toxicity, and decreased the time to death in a murine peritoneal infection model. The virulence factor

responsible for most toxigenic effects was mainly the *cytolysin (cyl)*. Singh *et al.* [170] also suggested that virulence factors produced by *E. faecalis* strains induced peritoneal infection, according to a mouse peritonitis model result [170]. These data suggested that the presence of encoded virulence traits in some strains of enterococci could have pathogenic effects in infected animals. These were the only available studies to demonstrate evidence for a pathogenic role of enterococcal strains possessing specific virulence factors in an animal model.

Dose-response relationship

In the New Zealand White rabbit's endophthalmitis model, only the cytolytic *E. faecalis* strains were able to develop retinal damage, as compared with non-cytolytic strains which produced few or no destructive changes. Transmission electron microscopy revealed tissue destruction in retinal layers as early as 6 hours post-infection with cytolytic *E. faecalis*, and light microscopy revealed near-total destruction of retinal architecture at 24 hours post-infection. *In vivo* and *in vitro* growth rates of cytolytic and non-cytolytic enterococcal strains showed similar kinetics [90, 91]. An increased mortality in rabbits with endocarditis was observed especially when the *cyl* acted in combination with another virulence factor called *aggregation substance (as)*. Hence, in the experiments, combined virulence traits enhanced invasion and facilitated adherence of enterococci to host cells, resulting in an increased infection and mortality [90, 91]. This data suggested that the carriage of more than one virulence factor by enterococcal strains, and the increased level of exposure to them, could contribute to both the course and the severity of animal experimental infections.

Analogy

Based on animal responses to virulent enterococcal strains, an analogy may be expected to occur in humans. However, the symptoms described in rabbits and mice [90, 91, 170], i.e. retinal damage, endocarditis, peritonitis, toxicity, time to death, were never reported in association with enterococci in humans. Therefore, no evidence exists at present that provides an analogy for pathogenic effects between animals and humans.

Plausibility or coherence

Based on the animal models described above [90, 91], an association between enterococci and infection in humans is biologically plausible. Based on available literature however, it is much more plausible that enterococci were isolated from

patients by chance. Most studies suggest that enterococci may be regarded as opportunistic bacteria and that the attributed pathogenicity may have been the result of a biased selection of diseased patients. Furthermore, as enterococci have relatively low virulence and enterococcal colonisation is fostered in immune-compromised, critically ill patients, their apparent clinical effect may be misinterpreted as causal [115, 135]. However, given that the virulence of enterococci is not completely known, and since there is not a comprehensive assessment about the association between virulence determinants and severity of human illness, one cannot rule out pathogenic properties, especially in immuno-compromised people.

In conclusion, a causal role of enterococci in nosocomial disease among humans remains controversial. Given that huge patient series have been studied without finding a single unibacterial infection with enterococci, and without finding patterns of states or symptoms of disease specifically associated with enterococci, the pathogenicity of these bacteria for humans appears unlikely. However, pathogenic effects seen in rabbits and mice provide an animal model in favour of a pathogenic role. Therefore, studies are required that specifically evaluate the presence of enterococci possessing specific virulence factors in humans with retinal damage, endocarditis, systemic toxicity and peritoneal infection, and other related patterns of disease symptoms.

6.2 Risk factors of enterococci that may contribute to pathogenesis

Although extensive investigations have been carried out, there is still little knowledge about the factors that contribute to the suggested virulence of enterococci. The study of virulence determinants and antibiotic resistance of enterococci has received special attention.

6.2.1 Virulence determinants

Virulence determinants (also known as virulence traits or virulence factors) are factors that are genetically encoded in some strains of some bacteria and that confer pathogenic effect on mammalian tissues and/or resistance against specific and non-specific defence mechanisms. As a result, virulence factors enable the bacteria to act as ‘opportunistic pathogens’.

Although in the past, the virulence determinants were not easy to identify because of their subtle characteristics, today considerable progress has been made in determining enterococcal virulence traits from clinical isolates. In this regard, Jett *et al.* [90, 91, 92, 93] have done extensive research on enterococci virulence factors and their effects on animals. The study of virulence factors from clinical enterococcal isolates is helping to clarify the prevalence of virulence determinants among food isolates as well, determining whether there is a difference in virulence potential between food and medical strains and evaluating the safety of the strains intended for use as probiotics or starter cultures.

So far, according to the studies conducted on animals, it is suggested that virulence traits are associated with one or more stages of infection, which can follow a common sequence of events involving invasion, adhesion and colonisation of host tissues, translocation of enterococci through cell layers, lyses of cells, and/or resistance to both specific and non-specific defence mechanisms mobilised by the host. In order to follow this process, enterococci would need to evade the natural host immune response, which might be accomplished by possession of a capsular polysaccharide that confers on them the required protection to elude immune cells; thus enterococci could resist phagocytosis and increase their intracellular survival in host's macrophages and neutrophils [85]. The presence of this capsule has been reported among clinical isolates but not among food or probiotic enterococci [62]. Another strategy used by enterococci that may help to avoid the host immune response is the production of the cellular toxin *cyl*, which could lyse cells of the immune system [131].

6.2.1.1 Identified virulence determinants

Enterococcal virulence determinants receive their names depending on the observed effects in the host, e.g. 'adhesin', 'invasin' or 'haemolysin' factor.

The known virulence factors are:

- *Aggregation substance (AS)*: this is an *adhesin* that promotes adhesion to a variety of eukaryotic cells, including macrophages and neutrophils and different intestinal cells. *AS* is capable of binding to extra-cellular matrix proteins such as fibronectin, laminin, thrombospondin, vitronectin and collagen type I, which in turn promotes bacterial translocation. Thus, thanks to *AS* properties enterococci

may be able to adhere to the intestinal or genito-urinary epithelium, encountering the basal membrane and extracellular matrix proteins, and then penetrate and enter the lymphatic and/or vascular system [62].

Especially in cases of intestinal lesions, wound infections, and bacterial endocarditis, the ability of AS to adhere to ‘exposed’ extra-cellular matrix proteins is thought to promote that bacterial translocation. Hence, where sub-endothelial extra-cellular matrix proteins are exposed, the possibilities for enterococci to penetrate the blood stream may increase [62].

- *Enterococcal surface protein (Esp)*: this is also an enterococcal *adhesin* that, like AS, was suggested to contribute to binding of enterococcal strains to the host extracellular matrix proteins. Furthermore, *Esp* also may confer colonisation and persistence properties to enterococci and could also be important in increasing cell hydrophobicity. *Esp* could even confer adherence to abiotic surfaces and biofilms, which may be of importance for patients with medical implants. However, it has not been demonstrated that *Esp* could influence histopathological changes yet, at least not in experiments done on animals [62], like in the study performed by Shankar *et al.* in 2001 [166].
- *Adhesin of collagen from E. faecalis (Ace)*: is also an *adhesin*, which binds to both collagen protein types I and IV, and to laminin [62].
- *β-haemolysin/bacteriocin or Cytolysin (Cyl)*: is also a confirmed virulence factor [62, 170]. It is a cellular toxin that mostly shows a haemolytic phenotype, and therefore is thought to be the most frequently involved virulence trait in haemolytic infectious activities [62], although not in all cases since other non-haemolytic strains of enterococci may also induce this type of infection [94]. Experimentally, it has been shown that *cyl* could induce tissue damage. In the Jett *et al.* study [90], rabbits were inoculated with cytolytic and non-cytolytic enterococcal strains; after three days, 99% loss of retinal function was detected in the rabbits that received the strain encoding *cyl*, while no or few destructive changes were detected in the rabbits that received non-cytolytic strains [90].
- *Adhesin-like E. faecalis and E. faecium endocarditis antigens (EfaA_{fs}, EfaA_{fm})*: these are considered as potential virulence factors. *EfaA_{fs}* antigen was once

suggested to have the function of an adhesin in one human endocarditis case [117]. In the experimental model performed in mice by Singh *et al.* [170], *EfaA_{fs}* antigen has been suggested to influence pathogenicity.

- *Proteases* are believed to be involved in enterococcal pathology. One protease called *Gelatinase (gel)* acts on collagenous material in tissues [62], and its production suggested an increase of pathogenicity in the Singh *et al.* mouse model [170]. Another protease called *Serine protease (spr)* also was suggested as important by Singh *et al.* [170]. Proteases are, apparently, very common virulence traits produced by some enterococci [62, 98]. However, so far, it has not been determined yet that proteases independently influence the outcome of a possible infection [62]; therefore, only a presumed association between protease production and enterococcal virulence can be suggested [98].

6.2.1.2 Prevalence of virulence factors among enterococci

Virulence factors are mainly detected among *clinical* enterococcal isolates, although studies done on the prevalence of virulence traits among enterococcal strains isolated from *food* suggest that some strains harbour virulence traits as well [62]. In this regard, in 2001 Eaton and Gasson [50] showed through PCR and gene screening tests, that enterococcal virulence factors were present in clinical, food and starter culture isolates. Though, the prevalence was higher among clinical strains, followed by food isolates; the lowest prevalence was observed for starter isolates [50].

Among enterococcal species, according to Franz *et al.* [61] and Eaton and Gasson [50], *E. faecalis* generally harbour more and multiple virulence determinants and with much higher frequencies than *E. faecium*. Eaton and Gasson found that all of the clinical, food and starter *E. faecalis* isolates they tested possessed multiple determinants (between 6 and 11), while *E. faecium* isolates were generally free of virulence determinants, with notable exceptions [50]. In turn, Franz *et al.* found that of the 47 *E. faecalis* isolates of food origin they have tested, 78.7% were positive for one or more virulence determinants, compared to 10.4% of the 48 *E. faecium* isolates of food origin tested [61]. On the other hand, in the Franz *et al.* study, the isolates exhibiting virulence traits were not necessarily positive for all traits; thus, the prevalence of virulence factors may be considered to be *strain or isolate specific* [61]. In a similar manner, the Eaton and Gasson results showed that their identified virulence determinants had not previously

been identified, and that this may have resulted from regional differences, suggesting a strain or isolate specificity as well [50].

In 2003, Mannu *et al.* [124] conducted a study where virulence traits among 94 *E. faecium* isolates were searched. For the study, 40 isolates were obtained from 3 different traditional goat's and ewe's raw milk cheeses produced in the island of Sardinia, 26 from faeces of Sarda breed sheep, and 28 from different clinical samples from patients from different wards staying at one Sardinian hospital. The results demonstrated that of all the isolates the ones obtained from cheeses harboured less virulence determinants than those obtained from patients samples [124]. It was also found that there was a difference in the type of virulence determinants present in cheese and clinical isolates (*EfaA_{fs}* was the trait isolated in cheeses while *Esp* was found in clinical samples). No virulence traits were found in sheep faeces strains. The study also revealed that, although there was a clear difference in the type of virulence factor present in isolates of different origin, each *E. faecium* isolate did not carry more than one virulence determinant, something that the investigators considered as low in prevalence [124]. The results of this study therefore suggested that *E. faecium* from traditional Sardinian raw goat's and ewe's milk cheese should not be considered as potential 'virulence carriers' for humans, since only one virulence determinant was found in each cheese positive isolate, and, overall, they were different to the type of virulence determinant isolated in patients [124].

In respect of what type of virulence traits are frequently found in *E. faecalis* or *E. faecium*, most of the findings of the Mannu *et al.* study [124] agree with the ones obtained in previous studies [38, 50, 61, 174]. For example, in the Mannu *et al.* study [124], the gene for the *gel* virulence factor was not found in *E. faecium* strains; this result that was also obtained in the study carried out by Franz *et al.* [61], while Eaton and Gasson have only found one clinical *E. faecium* isolate harbouring this virulence trait [50]. All these findings in turn agree with Coque *et al.* [38], who actually only found this gene in *E. faecalis* strains. *AS* was also not found in the Mannu *et al.* study [124], which agrees with the fact that previously *AS* has only been described in *E. faecalis* isolates as well [50, 61, 174]. With regards to *Esp*, it is not surprising that the Mannu *et al.* [124] study had found 21 clinical isolates carrying this virulence trait, since Eaton and Gasson [50] and Franz *et al.* [61] also found *Esp* in clinical *E. faecium* strains only. The *EfaA_{fs}* found in the 19 cheese isolates in the Mannu *et al.* study [124]

also agrees with those results of Eaton and Gasson [50], who found that this was the only virulence trait present in food strains of *E. faecium*.

Therefore, according to Franz *et al.* [61, 62], Eaton and Gasson [50], and Mannu *et al.* [124], among enterococcal species, *E. faecalis* of clinical origin generally harbour more and multiple virulence determinants and with much higher frequencies than *E. faecium*, which are generally free of them. In *E. faecium* isolates of food origin, only a few have been recognised as producing either *cyl* (8.3%), *Esp* (2.1%) [61], or *EfaA_{fs}* [124]. *E. faecium* appears to pose a lower risk for use in foods since their strains are generally free of virulence determinants [50, 61, 62, 124].

Table 8 shows the results of Mannu *et al.* study [124] of *E. faecium* strains. Table 9 shows the most common virulence factors found in *E. faecalis* and *E. faecium*.

Table 8: *E. faecium* strains isolated from cheese, sheep, and hospitalised patients, with their PCR results for virulence traits genes (summarised according to [124]).

Product/Origin	Number of isolates tested	Number of positive (+) isolates to virulence determinants				
		<i>Ace</i>	<i>EfaA_{fs}</i>	<i>GelE</i>	<i>AS</i>	<i>Esp</i>
<i>(a) Ewe's cheeses</i>						
Casu Axedu ^a	2	-	-	-	-	-
Fiore Sardo ^b	30	-	16	-	-	-
Pecorino Sardo ^c	8	-	3	-	-	-
Total	40	0	19	0	0	0
<i>(b) Sheep</i>						
Sheep's faeces	26	-	-	-	-	-
Total	26	0	0	0	0	0
<i>(c) Nosocomial patients</i>						
Respirator	11	-	-	-	-	10
Drain	5	-	-	-	-	1
Anal tampon	3	-	-	-	-	2
Skin tampon	1	-	-	-	-	1
Pus	1	-	-	-	-	1
Arterial catheter	1	-	-	-	-	1
Vesical catheter	2	-	-	-	-	2
Expectoration	1	-	-	-	-	-
Bronchial lavage	1	-	-	-	-	1
Urine	2	-	-	-	-	2
Total	28	0	0	0	0	21

a. Goat's milk fresh cheese

b. Hard uncooked ewe's milk cheese

c. Semi-cooked ewe's milk cheese

Table 9: Common enterococcal virulence traits and the enterococcal species where they have been found

Virulence factor	Enterococcal species	References
AS	<i>E. faecalis</i>	Jett <i>et al.</i> [91] Süßmuth <i>et al.</i> [174] Elsner <i>et al.</i> [53] Franz <i>et al.</i> [61] Eaton and Gasson [50]
	<i>E. faecium</i>	Jett <i>et al.</i> [91] Elsner <i>et al.</i> [53]
Esp	<i>E. faecium</i>	Eaton and Gasson [50] Franz <i>et al.</i> [61] Mannu <i>et al.</i> [124]
Ace	<i>E. faecalis</i>	Nallapareddy <i>et al.</i> [140, 141]
Cyl	<i>E. faecalis</i>	Jett <i>et al.</i> [91] Huycke <i>et al.</i> [88] Elsner <i>et al.</i> [53]
	<i>E. faecium</i>	Jett <i>et al.</i> [91] Franz <i>et al.</i> [61]
EfaA _{fs}	<i>E. faecalis</i> <i>E. faecium</i>	Lowe <i>et al.</i> [117] Singh <i>et al.</i> [169] Eaton and Gasson [50] Mannu <i>et al.</i> [124]
Gel	<i>E. faecalis</i>	Jett <i>et al.</i> [91] Kuhnen <i>et al.</i> [105] Coque <i>et al.</i> [38] Elsner <i>et al.</i> [53] Franz <i>et al.</i> [61]
	<i>E. faecium</i>	Eaton and Gasson ¹³ [50]

6.2.1.3 Regulation mechanisms of the virulence expression

In clinical strains of enterococci, the regulation of the virulence expression of proteases might be accomplished by genes of a system called Fsr (for *E. faecalis* regulator). This Fsr system could act by up-regulating and down-regulating the expression of the gelatinase gene (*gelE*) and the serine protease gene (*sprE*) of *E. faecalis*. So far, it is known that the Fsr system may regulate these two genes in *E. faecalis* clinical strains. It still has to be demonstrated that the Fsr system plays an important role in global

¹³ Eaton and Gasson [50] found only one medical isolate with the *gel* gene.

regulation of virulence factors of other enterococcal clinical strains, and whether there is a similar system regulating possible virulence traits of food strains [62].

6.2.2 Biogenic amines

Biogenic amines – tyramine, histamine, putrescine, and cadaverine - are organic basic compounds that occur in different kinds of food, such as fish products, cheese, wine, beer, dry sausages and other fermented foods [74]. Among the biogenic amines, *histamine* and *tyramine* production are the most important and frequently studied, since they have vasoactive and psychoactive properties that can have toxicological effects if present in high levels in the consumed food [19]. Several problems resulting from the ingestion of food containing relatively high levels of these amines have been found [19], with symptoms that include headache, vomiting, increase of blood pressure and even allergic reactions of strong intensity [74]. Biogenic amines are also a concern related to food hygiene, since the occurrence of relatively high levels of certain biogenic amines could be considered as indicators of a deterioration process and/or defective elaboration [19].

Microbial agents involved in biogenic amine production in foods may belong to either starter or contaminating microflora [74]. In dairy products, cheeses may present a good substrate for production and accumulation of biogenic amines [74], which are mainly generated by decarboxylation of the corresponding amino acids through substrate-specific enzymes of the micro-organisms present in the cheese [19, 74].

The ability to produce biogenic amines (especially tyramine) in dairy products has been reported for bacteria of the genus *Enterococcus* [68, 72, 73, 74, 179]. The production of biogenic amines is mainly dependent on the enterococcal extent of growth [68].

According to a Tham *et al.* study [179], *E. faecalis* isolated from artisanal goat cheeses did not produce histamine, whereas *E. faecium* produced only small amounts of this amine. They have concluded that enterococci in general seem to have no relevance from a histamine intoxication point of view in cheeses made of heat-treated goat milk [179]. In fact, the only relevant biogenic amine produced from enterococci isolated from dairy products is *tyramine* [19, 68, 71, 161]. In this regard, Bover-Cid and Holzapfel [19] analysed the biogenic amines formed by enterococci of food origin, in decarboxylase agar medium and in MRS agar, and have confirmed that the only biogenic amine

produced was tyramine. The quantified tyramine concentrations found in each enterococcal species at this study is depicted in Table 10 [19], where it can be seen tyramine produced concentrations of 610 mg/l broth for *E. durans*, while levels of between 601 to 4,986 and 379 to 4,339 mg/l broth were found for *E. faecalis* and *E. faecium*, respectively [19].

Table 10: Quantified (mg/l broth) biogenic amine production by 26 enterococci isolates. Determination was made after cultivation at 37 °C for 4 days in decarboxylation broth^a (summarised according to [19]).

Species	Strains tested	BA ^b producer strains	TY ^c (mg/L broth)	HI	PU	CA
<i>E. durans</i>	1	(1)	610	-	-	-
<i>E. faecalis</i>	15	(15)	601-4,986	-	-	-
<i>E. faecium</i>	10	(10)	379-4,339	-	-	-

a. Decarboxylation broth contained 0.5% tyrosine and 0.2% of histidine, ornithine and lysine, respectively.

b. Biogenic amine

c. TY, tyramine; HI, histamine; PU, putrescine; CA, cadaverine

In agreement with these findings, Sarantinopoulos *et al.* [161] found in a study that the majority (96.1%) of the 129 *E. faecium*, *E. durans* and *E. faecalis* isolates from human, food and animal sources, tested in decarboxylase agar medium, also produced tyramine only. Regrettably, Sarantinopoulos *et al.* did not make a quantitative determination of tyramine amounts produced by the isolates of their study; therefore, they could not draw any conclusions about the possible tyramine intoxication due to the presence of enterococci in cheese [161].

On the other hand, a study conducted by Gardini *et al.* [68] in skim milk, reported that, although substantial amounts of tyramine (between 0.3 and 7.93 ppm) were detected in an enterococcal tested strain (*E. faecalis* EF37), the most important biogenic amine produced by the same strain was in fact the 2-phenylethylamine (up to 14.14 ppm) [68].

Compared to other LAB, in Bover-Cid and Holzappel study [19], mainly enterococci, carnobacteria and some strains of lactobacilli were the most intensive tyramine producers, while several other strains of lactobacilli, *Leuconostoc* spp., *Weissella* spp. and pediococci did not show any potential to produce any amines. However, even though in this study all the enterococcal strains produced tyramine, they only tested very few isolates, and this opens the suggestion of that the tyramine formation could just have been strain- or source-specific.

Finally, it may be noted that already in 1987, Joosten and Northolt [96] reported that although enterococci are considered to be notorious tyramine forming bacteria, they were not found to cause this defect in cheeses if their number was not higher than 10^7 CFU/g. Further investigations carried out by Joosten in 1988 [97], led him to conclude that only if extremely high numbers of enterococci were present in cheese, increased biogenic amine formation is observed. In reality, in the traditional and artisanal cheeses produced using raw milk, enterococci hardly ever reach more than 10^7 CFU/g levels (examples can be seen in the Appendix C); therefore, enterococci in normal levels do not seem to be a threat with regards to the formation of biogenic amines in cheese products [96].

6.2.3 Antibiotic resistance

Overuse and misuse of antimicrobials in food animals represent a public health risk as they contribute to the emergence of resistant forms of disease-causing bacteria. Such resistant bacteria can be transmitted from those food animals to humans, primarily via food. Then, infections can result that are difficult to cure since the resistant bacteria do not respond to treatment with conventional antimicrobials. In this regard, nowadays in suggested enterococcal clinical infections of immune-compromised patients there are significant treatment problems, particularly because these bacteria have apparently become resistant to a great variety of antimicrobials.¹⁴

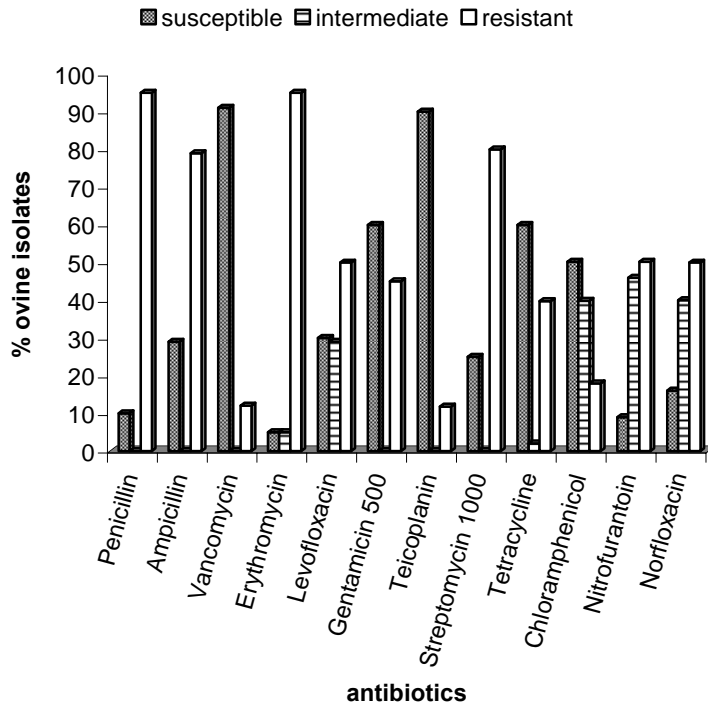
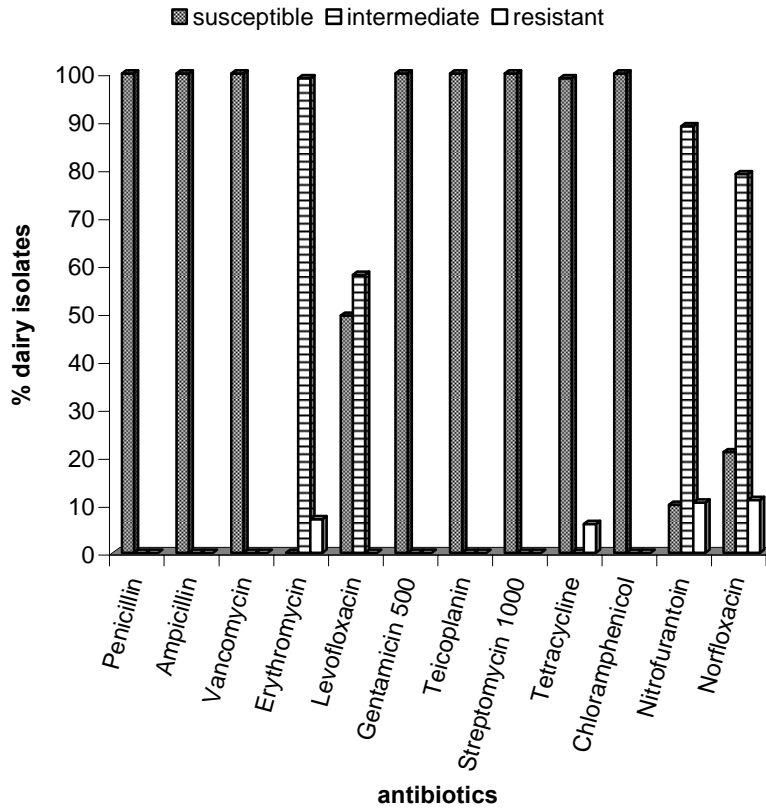
Like other Gram-positive bacteria, enterococci are ‘intrinsically resistant’ to a number of antibiotics. Similar to the virulence traits, this resistance is genetically mediated. In some strains, especially of clinical origin, there is also an ‘acquired resistance’ mediated by genes residing on plasmids or transposons [139]. Thus, enterococci possess intrinsic antibiotic resistance to cephalosporins, β -lactams, sulphonamides, and to certain levels of clindamycin and aminoglycosides, while acquired resistance exists to chloramphenicol, erythromycin, clindamycin, aminoglycosides, tetracycline, β -lactams, fluoroquinolones and glycopeptides (such as vancomycin), especially among clinical strains [109, 139]. Resistance of enterococci to vancomycin (especially among *E. faecium* strains) is of special interest as this antibiotic was the last effective resort

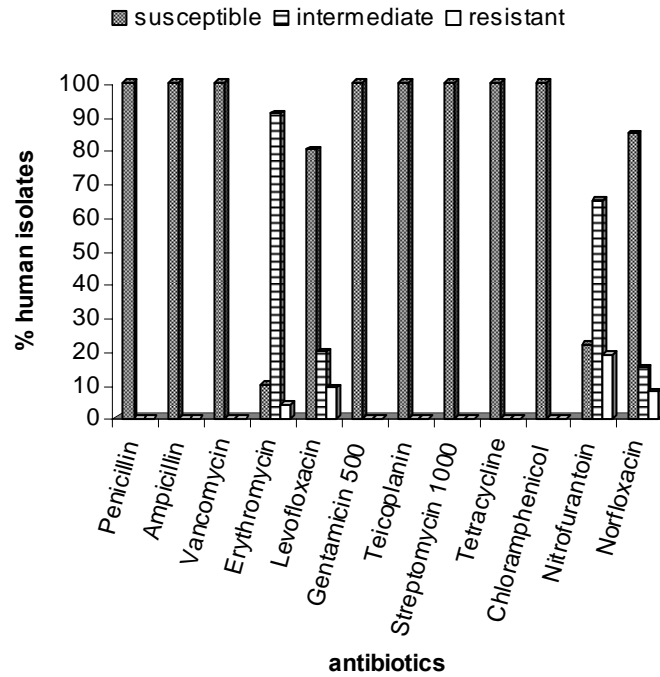
¹⁴ The epidemiological parameters that contributed to the emergence and dissemination of enterococcal antibiotic resistant species seem distinct for the United States and Europe. While in the United States injudicious use of antimicrobial agents seems to be the largest contributor to enterococcal acquired antibiotic resistance, in Europe, the use of avoparcin as a growth promoter in the form of animal feed supplement, seems to be the largest contributor [136].

available for treatment when multiple infections have already occurred, particularly in hospital patients. It has been suggested that Vancomycin-resistant enterococci (VRE) have led to infections that cannot be treated with conventional antibiotic therapies [62]. However, it is still being emphasised that almost all (99%) of enterococci are susceptible to vancomycin [78].

The increase of antibiotic-resistant enterococci among clinical isolates, especially for *E. faecium* strains [20, 52, 76, 95, 115, 136], poses the question whether enterococci that may be present in food would possess antibiotic resistance as well. However, with regards to foods derived from animals, studies on antibiotic resistance among enterococci revealed that although many of these strains showed resistance to one or more of the antibiotics, the majority of the isolates, and especially strains of *E. faecium*, are still sensitive to the clinically relevant antibiotics such as penicillin, ampicillin, streptomycin and vancomycin. In Italy, the study done by Mannu *et al.* in 2003 in Sardinian raw milk cheeses, demonstrated that these cheeses should not be considered the main source of antibiotic-resistant strains in humans (at least in the island of Sardinia, Italy) since 40 tested *E. faecium* isolates of dairy origin were susceptible to a large number of antibiotics [124]. Figure 10 shows the different susceptibility/resistance patterns to common antibiotics of 94 *E. faecium* isolates – 40 of dairy, 26 of animal, and 28 of clinical origin - found in this study [124].

Figure 10: Percentage of susceptible, intermediate and resistant strains to 12 commonly used antibiotics. (a) Strains of dairy origin; (b) strains from sheep's faeces; (c) strains of clinical origin [124].





However, antibiotic-resistant strains in food isolates have already been reported [3, 15, 61, 62, 101]. In 2000, in a study done in Spain by Robredo *et al.* [156], chicken, pork and turkey cold meat products from 18 supermarkets, and also 50 intestinal chicken samples from one slaughterhouse were examined in order to seek enterococcal resistance. The study found that ampicillin, quinupristin/dalfopristin and high level aminoglycoside resistance were frequent among the isolated enterococcal strains, and heterogeneity was observed in susceptibility patterns among VRE strains, even in those of the same species. Thus, there was a high rate of colonisation of chicken products by VRE strains (27.2%), which was also detected in 16% of intestinal chicken samples from the slaughterhouse. No VRE were found in cooked pork or turkey products however. VRE were identified as *E. durans*, *E. faecalis*, *E. faecium* and *E. hirae*. The findings therefore suggested that chicken presence in the food chain could be a source of VRE colonisation in humans [156]. Moreover, apparently, the VRE strains tend to remain in poultry carcasses for a long time (even years), especially if the birds received the glycopeptide ‘avoparcin’ as growth promoter. It is suggested that this is the result of an existing cross-resistance between vancomycin and avoparcin [18].

In agreement with the Robredo *et al.* study [156], later in 2002, in the United Kingdom, a shellfish, unchlorinated waters and chicken sampling study [195] attempted to determine the food and environmental spread of VRE. Only 1.6% and 2.7% of shellfish

were found to contain enterococci resistant to high levels of vancomycin, while 18.5% of the raw chickens contained significant VRE quantities; no VRE were found in unchlorinated water samples. All this suggested that environmental prevalence of VRE was low and that raw chickens were the ones frequently carrying VRE.

Even if in the Robredo *et al.* study [156] VRE were not found in cooked pork products, resistance to other antibiotics were confirmed in pork and their carcasses, as reported in two studies done by Martel *et al.* in 2003, in Belgium [126, 127]. In one of the studies, presence of resistance against macrolide and lincosamine were found among enterococci and streptococci [126]. The study was done with tonsillar and colon swabs from 33 pigs and 99 pork carcass swabs from animals originating from different Belgian farms. From each of the 33 pigs and in 88 of the 99 pork carcass swabs, at least one resistant strain to these antibiotics was isolated.

In 2003, Peters *et al.* [151] reported the results of a German study that attempted to determine which species of enterococci could be found in food of animal origin and their significance according to their antibiotic resistance for human beings. Between 2000 and 2002, they investigated 155 samples of food of animal origin (sausages, hams, minced meat, and cheese) bought in German retail outlets. The most frequent species isolated was *E. faecalis* (299 isolates), followed by *E. faecium* (54 isolates), *E. durans* together with *E. hirae* (24 isolates), *E. casseliflavus* (22 isolates), *E. avium* (9 isolates) and *E. gallinarum* (8 isolates). Then, they focused on the resistance patterns of 118 selected *E. faecium* and *E. faecalis* isolates to 13 antimicrobial active agents; these results can be seen in Table 11 [151].

Table 11: Antibiotic resistance behaviour of selected *E. faecalis* and *E. faecium* strains isolated from samples of sausages, ham, minced meat, and cheese [151]

Antimicrobial active agent	<i>E. faecalis</i> (n=101)			<i>E. faecium</i> (n=17)		
	Sensitive (%)	Intermediary (%)	Resistant (%)	Sensitive (%)	Intermediary (%)	Resistant (%)
Ampicillin	100	a	0	100	a	0
Amoxicillin/ clavulanic acid	100	a	0	100	a	0
Avilamycin	96	a	4	71	a	29
Chloramphenicol	57	36	7	71	29	0
Enrofloxacin	90	9	1	12	23	65
Erythromycin	36	67	7	0	82	18
Flavomycin	94	a	6	b	b	b
Gentamicin (High-level resistance)	a	a	0.8	a	a	0
Penicillin	100	a	0	94	a	6
Quinupristin/ Dalfopristin	b	b	b	59	12	29
Teicoplanin	100	0	0	100	0	0
Tetracycline	61	1	38	82	0	18
Vancomycin	100	0	0	100	0	0

a. No breakpoints defined

b. Not tested because of the intrinsic resistance of the species against this substance

In Table 11 we can see that, according to Peters *et al.* results [151], all the selected isolates were sensitive to the glycopeptide antibiotics, vancomycin and teicoplanin. Only one *E. faecalis* strain (among the 118 examined isolates) isolated from ham showed high-level resistance to gentamicin. All *E. faecalis* strains and 94% of the *E. faecium* strains were sensitive to penicillin. The study suggested that the situation of antibiotic resistance, with regards to the examined antibiotics, seemed to be favourable and that the investigated strains were sensitive to ampicillin and amoxicillin/clavulanic acid (which in combination with an aminoglycoside such as gentamicin are agents of choice for the treatment of presumptive enterococcal infections in human medicine) [151].

More recently, in 2004, a study was done by Busani *et al.* [23] with VRE. Herein, the susceptibility of vancomycin susceptible enterococci (VSE) and VRE to 10 antimicrobial agents was revised in strains that were isolated in Italy from raw meat products, farm animals, and human clinical infections in the years 1997-2000. High frequencies of resistance to tetracycline and erythromycin were observed, while chloramphenicol was the only drug that showed a relatively low rate of resistance in all the enterococcal isolates. In general, the resistance rates observed for VSE did not differ from those observed for VRE of the same species and origin. Some differences could

however be noticed among different enterococcal species - *E. faecium* strains were usually more resistant to β -lactams while *E. faecalis* strains were more resistant to gentamicin. However, the strongest differences were noticed when the strains were compared according to their source, the human isolates being usually more resistant than the isolates of animal origin. This study did not find significant differences between isolates of swine and poultry origin. Among vancomycin resistant *E. faecium*, multiple resistances to other antibiotics were much more frequent among the human strains (90%) than among poultry (48.9%) and swine (26.5%). The results of the study showed that in Italy, VRE isolates from human clinical origin are usually more resistant to antimicrobials than isolates from meat products and farm animals, and possess different antimicrobial resistance profiles [23].

Even though it is claimed that *E. faecium* strains seem to be safer than *E. faecalis* strains with regards to possession and transmission of antibiotic resistance, in some countries it has been found that *E. faecium* have developed some resistance, and therefore a prudent use of antibiotics is urgently needed in human and veterinary medicine, especially in animal husbandry. For instance, in 2003, Klare *et al.* [100] suggested that *E. faecium* strains carry important enterococcal glycopeptide resistance genes and that these strains can be found in hospitals and outside of them, namely in European commercial animal husbandries in which the glycopeptide ‘avoparcin’ was used as growth promoter, something common in the past. Since the Klare *et al.* study has found glycopeptide-resistant *E. faecium* (GREF) spread in different ecological niches (faecal samples, animal feed and waste water samples), they have suggested that these GREF could enter the human food chain through contaminated meat products. Moreover, the same study also referred that GREF strains often harbour different plasmids and carry virulence factors. Streptogramin-resistant *E. faecium* (SREF) has also emerged as a result of the use of the streptogramin ‘virginiamycin’ as a feed additive, in European commercial husbandry in the past. SREF were already isolated in Germany from waste water of sewage treatment plants, from faecal samples and meat products of animals that were fed with the additive, and even from stools of humans and clinical samples [100].

A study conducted by Temmerman *et al.* in 2003 [177] found that among 29 *E. faecium* strains isolated from probiotic products, 97% of the isolates were resistant against erythromycin, 90% against kanamycin, 41% against penicillin G, 34% against chloramphenicol, and 24% against tetracycline. No resistance was found against

vancomycin [177]. Overall, 68.4% of the isolates showed resistance against multiple antibiotics, including intrinsic resistances [177].

6.2.4 Transfer of virulence determinant and antibiotic-resistant genes

Enterococci possess different gene transfer mechanisms: pheromone-responsive plasmids, conjugative and non-conjugative plasmids and transposons [62]. Some virulence traits are encoded on pheromone-responsive plasmids, which are capable of transfer at high frequencies. This is certainly common among strains of *E. faecalis*, which explains the fact that they have a higher incidence in virulence factors. The presence of such a plasmid transfer system in *E. faecium* has been seen and described only once [62].

In vitro experiments with virulence genes that are encoded on the pheromone-responsive plasmids showed that transfer of the traits was possible to strains of *E. faecalis* used as starter cultures in food, but it was not possible into starter culture strains of *E. faecium* [50]. However, vancomycin-resistant genes could be transferred to a probiotic *E. faecium* strain in filter mating experiments [119].

The transferability rates were also studied *in vivo* conditions, using a hamster model of enterococcal intestinal overgrowth [89]; in this study, pheromone-responsive plasmids carrying either antibiotic or *cyl* genes could be effectively transferred to other enterococcal strains in hamsters gastrointestinal tracts, even in the absence of selective pressure with antibiotics [89]. In another study using a new animal model, the streptomycin-treated mini-pigs, virulence traits genes could also be transferred in the gastrointestinal tract to other *E. faecalis* strains, once again even in the absence of selective pressure with antibiotics [114]. However, it is quite noticeable that for these gene transfer studies the pheromone-responsive plasmids were often used, which have a natural high transfer frequency and may exaggerate the transferability rates of virulence factors or antibiotic resistance genes that are located on other type of plasmids, or the transfer of non-pheromone plasmids to other enterococcal strains that generally do not harbour pheromone-responsive plasmids, such as some *E. faecium* strains [62].

Experimentally, an Italian study conducted by Cocconcelli *et al.* in 2003 [35] assessed the frequency of gene transfer of virulence determinants and antibiotic resistance factors among *E. faecalis* of clinical and food origin, during cheese and sausage fermentations.

They found that even in the absence of selective pressure with antibiotics, plasmids carrying antibiotic resistance could be transferred to food strains and that the plasmid subsequently persisted in the new receptor. Very high frequencies of transfer were observed in sausages if compared to cheese, and the highest frequencies were observed during the ripening of fermented sausages. In this study, antibiotic resistances transferred were to tetracycline and vancomycin. So, the study showed that even in the absence of selective pressure with antibiotics, mobile genetic elements carrying antibiotic resistance and virulence determinants could be transferred at high frequency to food associated enterococci, during cheese and sausage fermentation [35].

However, in ‘real life’, it appears that *clinical* strains have a higher transmission rate compared to *probiotic* strains, which rate may be, in comparison, considerably lower or even harmless. Moreover, it has been suggested that antibiotic resistance cannot be transferred from enterococcal clinical strains to enterococcal food strains. According to a report of multiple vancomycin-resistant genes found in enterococci isolated from poultry and pork in Germany by Lemcke and Bülte in 2000 [110], when comparing food isolates with human isolates by means of PFGE they did not show homologous fingerprints according to their source of origin, and therefore it is unlikely that there is a close genetic relationship between enterococcal isolates from animal foodstuff and humans. Nevertheless, enterococci in processed food still may indicate a possible route for the acquisition of antibiotic-resistant strains by vulnerable hospital patients, for example those with haematological malignancy, and precautions with them should be taken seriously [39].

Finally, with regards to possible antibiotic resistance transmission between different bacteria, enterococci from fermented food are believed to be involved in the molecular communication between Gram-positive and Gram-negative bacteria of the human and animal gastrointestinal microflora [132, 135, 136]. A study done by Teuber *et al.* in 2003 [178] showed that plasmid pRE25 of *E. faecalis* (isolated from a raw-fermented sausage) transfers resistance against several antimicrobials, and those identical resistance genes were found in other pathogens, namely *Streptococcus pyogenes*, *Streptococcus agalactiae*, *S. aureus*, *Campylobacter coli*, *Clostridium perfringens*, and *Clostridium difficile*. Given that in the gastrointestinal tract of animals and humans, a unique ecologic niche exists, where they come into close contact with other Gram-

positive or Gram-negative bacteria, it is feared that antibiotic resistance genes could be interchanged.

6.3 Conclusions

In the hospital environment, enterococci are currently suggested to be among the most common and prevalent organisms found in patients infections. The virulence attributed to enterococci is especially related to some virulence traits that they may harbour, which generally had been detected among *E. faecalis* isolates of clinical origin. However, all the animal model studies performed so far on the virulence that enterococci may harbour provide only an indirect basis for speculating whether enterococci may contribute or not to pathogenicity in human infections. A specific association between enterococci and symptoms of disease in humans has not been demonstrated yet.

On the other hand, although the virulence traits have commonly been found within clinical isolates, it is feared that some food enterococcal strains may harbour the traits as well. Nevertheless, studies reveal that so far food strains of enterococci are generally free of them.

With regards to antibiotic resistance, most resistant strains that have been found among enterococci were of clinical origin as well, especially among *E. faecium* strains. There is, however, a suspicion that food and environmental spread may be important in antibiotic resistance acquiring patterns among strains of *E. faecalis* or *E. faecium* (or even other species of enterococci) but once again, evidence to this effect is limited.

Since the suspicions are numerous but the evidences are still limited, further investigations need to be performed in order to better understand the role of the enterococcal virulence traits and if they can influence any pathological changes in humans. Also, further studies on the genetic transfer mechanisms of enterococcal virulence traits and antibiotic resistance genes would answer the question of whether these genes could be transferred to other enterococci, or to other micro-organisms in the gastrointestinal tract, where they come into close contact. Currently, in spite of the observed high strain specificity in respect of the antibiotic resistance and virulence traits that an enterococcal strain can harbour, a careful evaluation of each culture strain intended to be used in the food industry is recommended, for safety reasons.

Finally, biogenic amines produced by micro-organisms are a source of concern in the food industry, due to their toxigenic potential in humans. Enterococci in milk and cheese have proven to be tyramine formers. However, for significant concentrations to occur, enterococcal levels must reach more than 10^7 CFU per gram, which is not normally reached in cheese manufacture.

Chapter 7: Enterococci in Dairy Foods

Enterococci have different useful applications in the dairy industry. As starters or adjunct cultures, they fulfil a significant role in improving flavour development and quality of especially cheeses. As probiotics, they contribute to the improvement of microbial balance and can be used for treatment of gastroenteritis in humans and animals. Additionally, enterococci harbour other useful biotechnological traits, such as the production of bacteriocins with anti-*Listeria* activity. Nevertheless, they also have been described as spoilage micro-organisms and cross-contaminants during food processing, when their initial numbers in raw milk are high, pasteurisation is poor, or the pasteurised milk is not stored properly.¹⁵ A risk analysis approach is therefore depicted in this chapter, to show how frequently they may be present in the pasteurised milk and their sub-products when heating steps are followed during dairy products manufacturing, and if so whether they may represent a concern and a threat to human health.

7.1 Enterococci in milk and dairy products

Enterococcal presence in dairy products can have conflicting effects, of either a *risk* as a foreign or intrusive flora indicating poor hygiene during milk handling and processing (if in excessive numbers), or as a *benefit* in contributing to produce unique traditional and emerging by-products, in protecting against diverse spoilers, and as probiotics.

7.1.1 Enterococci as contaminants in milk

Enterococci are normal components of the raw milk microbiota. There are no standards set for the minimum and maximum count of enterococci because they are not normally counted in microbiological analyses. A study of the levels of enterococci in raw cow's milk from 10 New Zealand farms in 1997 [83], revealed an enterococcal minimum count of $<10^1$ CFU/ml and a maximum of 1.2×10^4 CFU/ml, though 95% of the samples of the same study had less than 1.9×10^3 CFU/ml [83]. Other sources report numbers in European raw milk varying from 10^3 cells/ml to 10^5 cells/ml, or more, without any of the species being markedly represented [150].

¹⁵ In the meat industry enterococci also perform significant roles, especially in the manufacture of some types of traditional sausages. However, like in dairy products, enterococci can also be responsible for the spoilage of some fermented meat products. (For details of the role of enterococci in meats, see Appendix D).

Enterococcal counts in goat's and ewe's milks can reach higher levels: log levels of 4.3 CFU/g⁻¹ have been reported in goat's milk in Italy [175] and 6.2 CFU/g in raw ewe's milk in Spain [41]. Higher levels of enterococci in milk are considered to be the result of contamination during the collection or processing of milk [36].

If contamination occurred, enterococci have usually been related to unhygienic conditions primarily due to contact with cow faeces. Nevertheless, this perception has now changed due to the fact that it is very common to find enterococci in other sources as well, contradicting what was formerly a sole 'faecal contamination' belief (in Chapter 5, it was seen that enterococci can enter the milk chain either primarily from human or animal faeces but also secondarily from contaminated water sources, the exterior of the animal or other contaminated milking equipment or bulk storage tanks handled in the processing plant) [75].

Therefore, it is now accepted that enterococci *naturally* occur in raw milk and whey (and consequently in their raw or semi-cooked sub-products) as part of their microbial population within normal ranges, and that high counts actually reveal poor pasteurisation or unsatisfactory hygiene practices. For details about enterococcal counts in some ripened raw and semi-cooked cheeses see Appendix C, Table 17.

7.1.2 Functions of enterococci in dairy products

On the other hand, enterococci, with other usually thermophilic LAB present in raw milk, can act as natural starter cultures, and thanks to their psychrotrophic nature and their adaptability to different substrates and growth conditions they are also able to survive during milk refrigeration¹⁶ and may survive low-heating temperatures of pasteurisation [75]. Traditionally, natural enterococcal cultures can be kept by pasteurising raw milk and incubating it at 42-44 °C for 12-15 hours, thus promoting the selection of thermophilic and heat-resistant LAB (usually *Streptococcus thermophilus* and *Enterococcus* spp.) [75].

In the same context, *Enterococcus* bacteria are often isolated as essential microflora from natural whey, which is the result of their presence in raw milk from which the whey derives, coupled with the mild acidity reached by these cultures after incubation of the whey at the end of cheese-making [75].

¹⁶ However, if the milk is maintained refrigerated at 5-8 °C, the growth rate of enterococci is markedly limited [150].

Enterococci as natural milk and whey starter cultures are appreciated in the manufacturing of a variety of cheeses, mostly artisan, produced both in Southern and Northern European countries from raw and pasteurised milk. Of several different species of enterococci found in raw milk and consequently in milk and dairy products, *E. faecalis* and *E. faecium* are the most common and with the greatest importance for the dairy industry [59, 62, 70, 75]. *E. faecium* is the most frequently and predominantly used species in dairy industry [59]. *E. durans* [161, 175] and *E. casseliflavus* may also be important [175].

Following, the different enterococcal applications within the dairy industry, as starter, adjunct, probiotic, and protective cultures, are described. Their levels and applications in the most common artisanal cheeses are summarised in Appendix C.

7.1.2.1 Starter cultures

The frequent isolation of enterococci as natural starter cultures used for the manufacture of artisan cheeses, along with the finding of strains with good acidifying and/or proteolytic properties within *E. faecium* and *E. faecalis* isolated from various cheeses (such as Cebreiro cheese by Centeno *et al.* [31] and the Italian Semicotto Caprino cheese by Suzzi *et al.* [175]) and various dairy products (raw milk, cream, butter) [192], encouraged some applications of these micro-organisms as *starter cultures* [75]. In Argentina, a recent study done with 122 strains of *E. faecium* indicated their high potential as non-traditional starter cultures in the manufacture of homemade Tafi cheese [158].

However, as it was suggested in Chapter 3, considerable variation between species and strains of the same species are continuously being recorded; this highlights the need to study individual strains when selecting the most suitable enterococcal bacteria for a starter culture [7]. Also, according to Giraffa [75], from the early works on Cheddar cheese done by Dahlberg and Kosikowski in 1948 and Thunell and Sandine in 1985, no significant research reports followed. This may be due to the fact that generally most of the enterococcal strains possess low milk acidifying and proteolytic activities (as seen in Chapter 3), which make them of minor importance as starter cultures in cheese manufacture [75, 161].

7.1.2.2 Adjuncts (non-starter) cultures

Adjunct cultures are defined as those added to cheese for purposes other than acid formation, which is exclusively devoted to the added starter. These selected ‘non-starter’ adjunct cultures can be added to accelerate ripening, to produce desirable flavour, or to act as probiotics [75].

A number of research works [27, 30, 31, 37, 142, 147, 149] have been carried out to evaluate the technological functionality of selected enterococcal species and strains in cheese production. Most of them clearly concluded that enterococci play a more fundamental role as adjunct cultures in food fermentation. For instances, *E. faecium*, *E. faecalis*, and *E. durans* strains have been proposed in combination with both mesophilic and thermophilic LAB species as part of ‘defined adjunct cultures’ for different European cheeses, e.g., Italian semi-cooked cheeses [142] and Venaco cheese [27]; for water-buffalo Mozzarella cheese, a strain of *E. faecalis* was selected with other LAB for use in an adjunct culture preparation [37, 149]; for Cebreiro cheese, enterococci with other LAB were also suggested for use in its production [30, 31], as well as for Hispanico cheese [147]. In all these studies, enterococci showed the highest performance when being added as adjuncts.

In the same manner, in 2002, Sarantinopoulos *et al.* [163] have done a study attempting to demonstrate the enhancement of microbiological, physicochemical and sensory characteristics during ripening of Greek Feta cheese when using *E. faecium* FAIR-E 198 and FAIR-E 243 strains (and their combination) as adjunct cultures. Enterococci positively affected the counts of non-starter LAB. The results demonstrated the technological significance of *E. faecium* strains and supported their use as adjunct cultures in the manufacture of Feta cheese [163]. Similarly, *E. durans* was shown to be important for aroma development in the ripening of Feta cheese when in 1993 Litopoulou-Tzanetaki *et al.* used *E. durans* strains as adjunct cultures with other LAB [116].

In all these studies, generally the presence of added enterococcal flora throughout ripening positively affected taste, aroma, colour and structure of the full-ripened cheeses, as well as the overall sensory profile. This is linked to the fact that, in cheeses made with enterococci, soluble nitrogen, total free amino acids, volatile free fatty acids,

long-chain free fatty acids, and diacetyl and acetoin contents are generally higher [59, 75].

Another aspect of enterococci as adjunct cultures is their contribution to the bacterial surface-smear ripening flora. Many European cheeses are characterised by complex bacterial surface flora generally consisting of yeasts, coryneform bacteria, and micrococci or coagulase-negative staphylococci. However, enterobacteria and enterococci are also often found as minor bacteria [75]. In a survey carried out by Carnio *et al.* in 1999 [26] on the bacterial surface ripening flora of French and German smeared cheeses, different *Enterococcus* spp. strains colonising the cheese rind were found. Half of the strains inhibited *Listeria* strains and were indicated as promising candidates for the development of a defined surface-smear ripening flora [26].

In 1996, the Advisory Committee on Novel Foods and Processes decided to approve the use of *E. faecium* strain K77D as a culture in fermented dairy products [59, 75, 161].

Clearly, the enterococci play an important role in the manufacture of cheeses typical of some regions, especially during their ripening, and their use as adjuncts has a major impact on this part of the dairy industry [59].

Table 12 shows enterococci within the different biotechnological important genera of LAB, according to Klein [102].

Table 12: Biotechnological important LAB genera [102].

Probiotics	Starter and protective cultures
<i>Lactobacillus</i>	<i>Lactobacillus</i>
<i>Enterococcus</i>	<i>Enterococcus</i>
<i>Bifidobacterium</i>	<i>Bifidobacterium</i>
	<i>Pediococcus</i>
	<i>Leuconostoc</i>
	<i>Streptococcus</i>
	<i>Carnobacterium</i>
	<i>Weisella</i>

7.1.2.3 Probiotics

Probiotics are mono- or mixed-cultures of live micro-organisms which beneficially affect the health of animals or humans health when consumed, by improving the properties of the indigenous gastrointestinal flora [186].

The positive and beneficial effects of probiotics also include inhibition of pathogenic micro-organisms, strengthening of the gut mucosal barrier, antimutagenic and anticarcinogenic activities, stimulation of the immune system and lowering of blood cholesterol levels¹⁷ [84].

Most probiotic cultures are of intestinal origin and belong to the genera *Bifidobacterium* and *Lactobacillus*, while *Enterococcus* spp. are only occasionally used as probiotics [62, 177]. However, the increasing interest for the usage of probiotics has suggested some active enterococci strains as safe candidates, along with the most common currently used strains of *Lactobacillus* and *Bifidobacterium*.

The use of cheeses as delivery systems (carriers) of safe probiotic strains has been attempted, studying the possibilities of some types of cheese for delivery of probiotic strains to the gastrointestinal tract of humans. Thus, *E. faecium* PR88 has been used as a probiotic adjunct culture in Cheddar cheese in studies carried out by Hunter *et al.* [87] and Gardiner *et al.* [67]. In these studies, *E. faecium* PR88, which possess properties required of a probiotic micro-organism including the ability to relieve irritable bowel syndrome, with intestinal origin, no pathogenicity, and tolerance to bile and acid, was used as an adjunct culture [75]. Compared with the control, increased proteolysis, and higher levels of some odour-active volatile compounds were observed in cheese containing the adjunct strain throughout the ripening period [75]. In the Gardiner *et al.* [67] study, the strain maintained viability in Cheddar cheese during 9-15 months of ripening at 8 °C. The evidence hence suggested that Cheddar cheese was a good carrier for the beneficial *E. faecium* PR88, and that the strain would not only benefit human health but would also improve intrinsic characteristics of Cheddar cheese [75].

Another strain, *E. faecium* SF68, has successfully been used in two studies [16, 113], but this time in pharmaceutical preparations, to treat certain intestinal tract disorders both in adults and children. The strain decreased the duration of diarrhoeal symptoms and the time for normalisation of patient's stools [16, 113]. The benefits were also corroborated earlier in other studies such as the ones done by Bellomo *et al.* in 1980 [16], Bruno and Frigerio in 1981 [22], and D'Apuzzo and Salzberg in 1982 [40].

¹⁷ Hypocholesterolaemic action has only been proven in the short-term with the Causido[®] culture, which consists of two strains of *S. thermophilus* and one strain of *E. faecium*. For the rest of enterococcal strains used as probiotics, including the Causido[®] culture, their clinical relevance in long-term reduction of Low-density Lipoprotein (LDL)-cholesterol levels is still uncertain [4, 120, 155].

Consequently, this strain, used in probiotic therapies in the form of pharmaceutical preparations, is considered as an alternative to antibiotic treatments and seems to be of no risk for the human health at the present time [98]. Table 13, summarised according to Kayser [98], explains why this strain is considered beneficial and harmless for the human health.

Table 13: Characteristics of the *E. faecium* SF68 strain (summarised according to [98]).

Observed characteristics	Results
Virulence factors	Examination of the strain has revealed absence of all known enterococcal virulence factors since it does not carry a pheromone responsive conjugative plasmid. Also, in agreement with this finding is the observation that the strain is unable to adhere to vascular epithelial cells and endocardial cells.
Persistence in the GI tract	Constant colonisation of the strain in the intestinal tract over a long period of time is unlikely. In animals, steady state levels in their gut could not be established. In healthy humans, the strain could be detected in the stools up to 72 hours after administration, but not after 96 hours.
Antimicrobial resistance	The strain does not yet possess acquired resistance to antimicrobials.
Human infections	No single case of human infection with this strain has ever been reported (at least not in the last 20 years).

Another probiotic *Enterococcus* is the Causido[®] culture that consists of two strains of *S. thermophilus* and one strain of *E. faecium*. This probiotic has been claimed to be hypocholesterolaemic in the short-term. Since long-term reduction of LDL-cholesterol levels was not demonstrated yet, its clinical relevance is still doubtful [62].

In spite of the well-established probiotic benefits of several enterococcal strains, and because of the controversy that they often embrace for the human health, application of enterococci as probiotics within the food industry still remains uncertain and is debatably feared [62].

7.1.2.4 Protective cultures: the role of the ‘enterocins’

Another great benefit of enterococci, especially with respect to cheeses, is that there are many strains that produce bacteriocins or *enterocins*. As discussed in Chapter 3,

enterococcal strains have long been shown to be prominent bacteriocin producers. As such, some strains may play an important role in the natural preservation of food products by controlling, competing and inhibiting the growth of undesirable bacteria, thus preventing adulteration of foods particularly caused by *Listeria monocytogenes* and other food-borne pathogens that in the past have led to severe disease outbreaks. Hence, enterococci also show a potential for dairy application as 'bio-preservatives' or 'protective cultures'.

Enterococcal strains with specific antimicrobial activity against pathogenic or spoilage bacteria belong to a particular class of adjuncts [75]. This is due to the fact that most of the enterococcal bacteriocins characterised to the biochemical level so far turned out to be either 'enterocin A' or 'enterocin B' [54]. Hence, since these A and B enterocins appear to be among the most common LAB bacteriocins, the preservative role of enterococci in terms of inhibition of spoilage and food-borne pathogens in dairy products may be widely associated with the production of these enterocins types [54]. A variety of bacteriocins produced by enterococci have already been isolated from dairy products [55, 122, 144, 145, 146, 176, 189], one of them being *bacteriocin AS-48*, a peptide produced by *E. faecalis* that is found in raw milk and dairy products and that has a broad spectrum of antimicrobial activity, including an anti-listerial action [64]. *E. faecium* WHE 81 isolated from cheese also produces these bacteriocins A and B, with the same beneficial action [54].

Enterococcal strains that are producers of bacteriocins are often used as adjunct cultures in artificial model studies attempting to inhibit target bacteria and improve the safety of cheeses. Thus, *E. faecium* and *E. faecalis* strains, effective against *L. monocytogenes* and *S. aureus*, are artificially introduced in dairy systems such as milk, soft cheeses, and soy milk with varying degrees of success [75]. In some cases [71, 144, 147], the presence of anti-*Listeria* activity of enterocins produced by protective cultures in cheeses was observed towards the end of ripening and no or minor influences were generally reported on both the commercial culture activity and the organoleptic characteristics of the products. However, in other cases, the complex curd (or cheese) environment possibly interfering with bacteriocin production levels, or the lack of growth of the enterocin-producing strain, may have affected the *in situ* bacteriocin efficiency. This was demonstrated by Sarantinopoulos *et al.* with Greek Feta cheese making: in a first trial [163], *E. faecium* FAIR-E 198 effectively contributed to sensory

characteristics of the cheese and its bacteriocin production successfully inhibited target bacteria; but when the same strain was applied as an adjunct culture in Feta cheese in a second trial [164], no enterocin activity was detected throughout ripening, even though the strain could grow well. Sarantinopoulos *et al.* hence suggested that the presence of rennet and calcium chloride, the presence of non-*Enterococcus* mixed starter cultures, and the use of skim milk as substrate, either one or all of these factors may have influenced the bacteriocin production and hence the successful inhibition of target bacteria [164]. Results obtained in this study [164] underline the frequently underestimated finding that *in vitro* production by novel bacteriocinogenic starter or co-cultures is no guarantee for *in situ* efficiency and that the complex food environment may interfere with bacteriocin production levels. The functionality of enterocin-producing strains under industrial, cheese processing conditions seems then strongly dependent on the cheese system and the technology applied [75].

In addition, even though the fact that bacteriocin production in the strains is chromosome-linked and that suggests that this antibacterial character is stable and can withstand long-term use [54], genes responsible for enterocin production may well differ between strains, and studies have yet to be carried out to identify the accessory genes required for synthesis, processing, secretion and immunity of enterocins, as well as the possible connections among them [54, 60].

7.1.3 Sanitary regulations - raw milk for production of dairy products

Raw milk destined for production and commercialisation of dairy products for human consumption should be from healthy cows, free of Brucellosis and Tuberculosis, free of any other contagious diseases for the human species and free of undesirable organoleptic characteristics of the milk. Cows used for milk production must not exhibit symptoms of disease in the genital tract (e.g. abnormal discharges from the mammary gland or from the reproductive tract), intestinal tract (diarrhoeal enteritis with fever), or udder; they also must produce more than 2 litres of milk per day, should not have been treated with substances that could be transmitted to humans, or that are, or could be, dangerous to the human health [51].

Cow's bulk raw milk assigned for thermal treatment, and therefore human consumption, must comply with the following norms:

- A bacterial count (at 30 °C) of $\leq 100,000$ cells/ml

(Observed geometric mean in a two-month period of time, with at least two samples taken per month).

- A somatic cells count of $\leq 400,000$ cells/ml

(Observed geometric mean during a three-month period of time, with at least one sample taken per month).

The regulation also points out that any milk not complying with these requirements must be separated from the milking tank and later be taken in vehicles with this legend: 'not suitable for human consumption'. This regulation is important since it gives a verifiable definition to distinguish the milk from problematic cows, allowing a better compliance of the norm [51].

The European sanitary regulations do not specifically address the presence or concentration allowed for enterococci. According to the American Public Health Association, specific acceptable levels of enterococci are not indicated for foods in the United States either, since it varies according to product, handling, time of storage, and other factors [78], as discussed in Chapter 1. In New Zealand, no standards are set for enterococci either, given that these bacteria are not routinely counted in dairy food.

7.1.4 Effects of heat treatments on survival of enterococci

7.1.4.1 Pasteurisation of raw milk

As discussed previously, the enterococcal levels in raw cow's milk are very variable. In Europe, counts may fall between 10^3 to 10^5 enterococci/ml [150]. This variability is presumably influenced by factors such as region, climate, milk production levels, breed, and handling of the milk. In a New Zealand study of raw milk from individual farms performed by Hill and Smythe in 1997 [83], counts of between $<10^1$ and 1.2×10^4 enterococci/ml were found, with 95% of the samples containing $<1.9 \times 10^3$ enterococci/ml (in reality, dilution of raw milk in tankers and factory silos is likely to lead to a much lower level in the bulk raw milk entering the pasteuriser).

To evaluate the effects of heat treatments on enterococcal survival in milk, in this review, the count of 1.9×10^3 /ml firstly was taken to represent a 'worst case' level.

Based on the D-values of the three most common enterococcal species found in raw whole milk, obtained from the study of Pérez *et al.*¹⁸ (Table 14), two survival curves were created to show the potential of the most heat-resistant enterococci (*E. durans* and *E. faecium*) to survive the heat treatment applied during commercial pasteurisation of raw cow's milk¹⁹ (Figures 11 and 12).

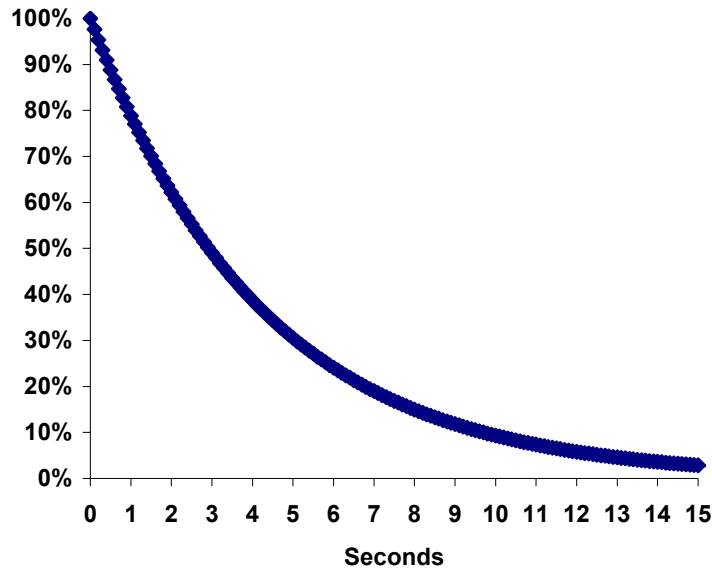
Table 14: Heat resistance of three enterococci species in whole milk and non-fat milk, at 72 °C/15 seconds (summarised according to [150]).

Species	D _{72 °C} (seconds)	
	whole milk	non-fat milk
<i>E. durans</i>	9.7	7.38
<i>E. faecium</i>	2.4	1.54
<i>E. faecalis</i>	0.88	0.57

¹⁸ In 1982, Pérez *et al.* carried out a study [150] to determine the heat resistance of enterococci in whole and skim milk. The results were given using D-values (or decimal reduction time, which is the time of heat treatment required at a certain temperature to destroy 90% of the bacterial cells [171]). In the study, it was shown that when a temperature of 72 °C is applied for 15 seconds, only 9.7 seconds were needed to destroy 90% of the bacterial cells of *E. durans*, while 2.4 and 0.88 seconds were required to destroy 90% of the *E. faecium* and *E. faecalis* bacterial cells, respectively (Table 14) [150].

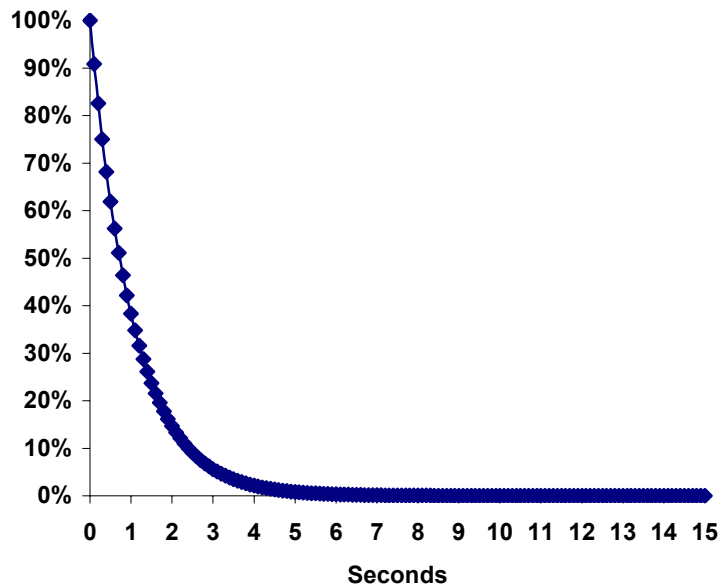
¹⁹ Commercial pasteurisation is normally carried out at 72 °C for 15 seconds, which is commonly known as 'High Temperature Short Time (HTST)' pasteurisation. This choice of temperature/time is based on the heat resistance of *Mycobacterium tuberculosis* [24, 150].

Figure 11: The survival curve of *E. durans* at milk commercial pasteurisation temperature (72 °C/15 seconds), according to Pérez *et al.* D-values information [150].²⁰



²⁰ Hypothetically, if 1,900 enterococci/ml were present in raw cow's milk and these were all *E. durans*, pasteurisation at 72 °C would destroy 90% of *E. durans* in 9.7 seconds, i.e. at this time only 190 *E. durans*/ml would survive. After 15 seconds, only about 54 *E. durans*/ml would survive (2.85%). In order to reduce *E. durans* to say < 1/ml (non-detectable levels), a 72 °C heat treatment would need to be applied for **34.8 seconds**, assuming 1,900 *E. durans*/ml were present in the initial milk. Even though *E. durans* is the most heat-resistant enterococcal species, none of its strains have exhibited any toxigenic potential yet [14].

Figure 12: The survival curve of *E. faecium* at milk commercial pasteurisation temperature (72 °C/15 seconds), according to Pérez *et al.* D-values information [150].²¹



Pérez *et al.* [150] (and other authors [14, 69, 171]) showed that *E. faecalis* is more sensitive than *E. durans* and *E. faecium* (Table 14).²² Therefore, a survival curve for this species is not presented here.

These heat kill estimates have been calculated according to the D-values obtained in *whole milk* by Pérez *et al.* [150], who showed shorter D-values when the milk was skimmed (Table 14). Hence, whole milk offers more protection against the lethal action of heat than non-fat milk [150], a fact that was corroborated in a similar study carried out by Batish *et al.* [14].

As suggested before, in reality, the risk of survival by enterococci is likely to be lower than hypothesised. Although the above modelling was based on 1,900 enterococci/ml of milk entering a pasteuriser, based on the work of Hill and Smythe [83] it is much more likely that enterococci numbers in the bulk milk will be lower, considering the comingling of lower and higher counts, and the dilution of milk from different individual

²¹ Hypothetically, if all 1,900 enterococci present/ml of raw milk were *E. faecium*, and the raw milk was pasteurised at 72 °C, only 190 *E. faecium*/ml would survive at 2.4 seconds. In order to reduce *E. faecium* to < 1/ml (non-detectable levels), a 72 °C heat treatment would need to be applied for **8.6 seconds**, assuming 1,900 *E. faecium*/ml were present in the initial milk.

²² In order to reduce *E. faecalis* to < 1/ml (non-detectable levels), a 72 °C heat treatment would need to be applied for **3.5 seconds**, assuming 1,900 *E. faecalis*/ml were present in the initial milk.

farms in bulk tankers and silos. Also, raw milk does not contain exclusively a single enterococcal species, but rather a mixture of *E. faecalis*, *E. faecium*, and *E. durans* in different proportions. It is also probable that *E. durans* will be in the minority, compared with *E. faecalis* and *E. faecium* (based on the information provided in earlier chapters and according to [59, 75, 102, 161]).

The mean value found in the Hill and Smythe study [83] was of 100 enterococci/ml in total. Consequently, if now we hypothesised that the enterococcal level in the bulk milk were of the order of 100/ml, and the population were made up of 44% *E. faecalis*, 40% *E. faecium*, and 16% *E. durans* (probable numbers according to [59, 75, 102, 161]), then after a HTST pasteurisation the enterococcal level in the pasteurised milk could be of the order of <1 enterococci/ml - not detectable - for all the species considered (see Table 15).

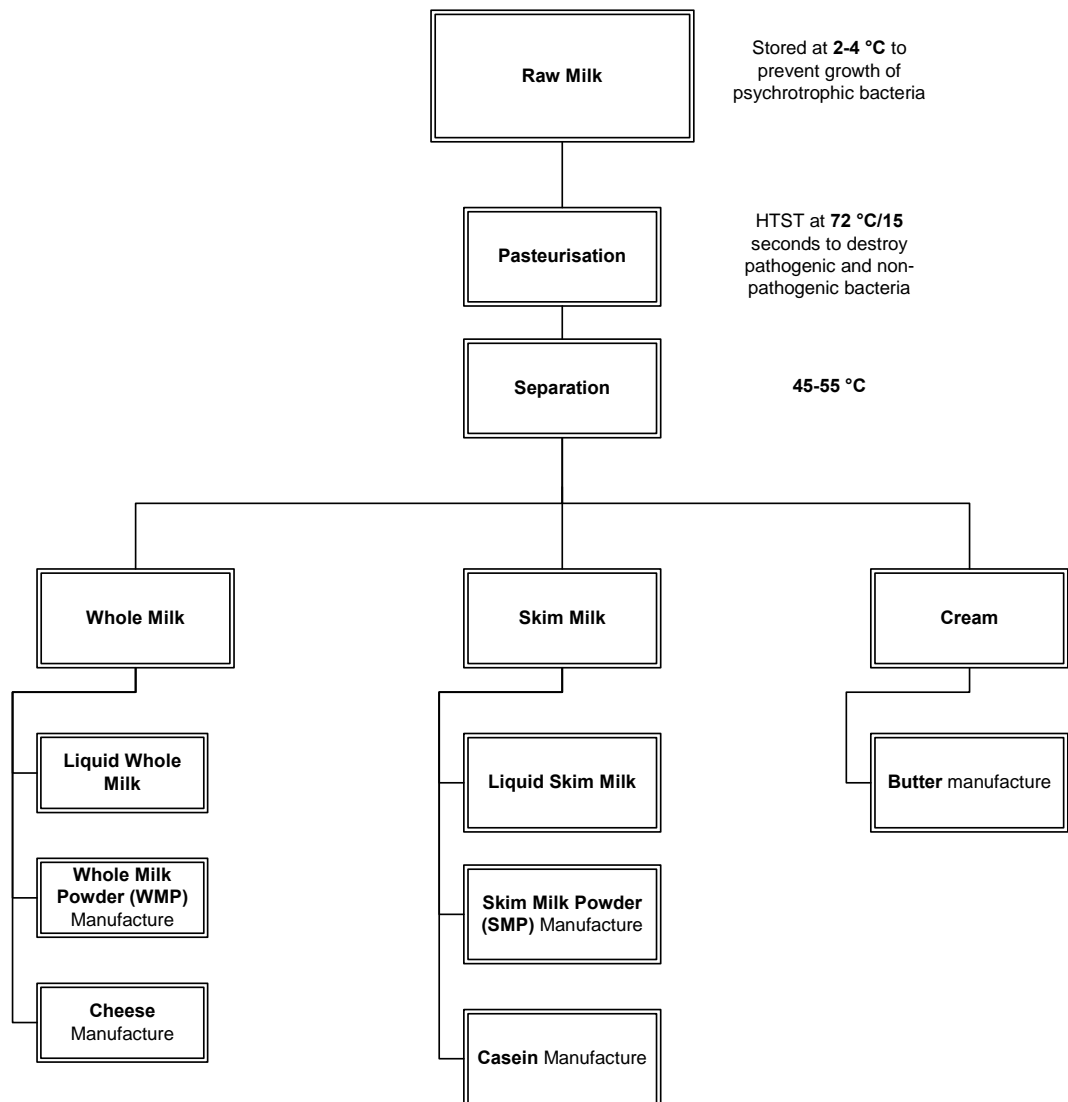
Table 15: Pre- and post-pasteurisation counts of the most common enterococci found in raw milk (HTST at 72 °C/15 seconds).

	Pre-pasteurisation counts (raw milk)	Post-pasteurisation counts and time of inactivation
<i>E. faecalis</i>	44/ml	<1/ml (1.76 seconds)
<i>E. faecium</i>	40/ml	<1/ml (4.6 seconds)
<i>E. durans</i>	16/ml	<1/ml (14.7 seconds)
Total enterococci	100/ml	<1/ml (14.7 seconds)

7.1.4.2 Manufacture of dairy products

Once the raw milk arrives in the processing plant and is stored in raw milk silos, pasteurisation takes place almost immediately (at the latest within 24 hours of milk reception) to eliminate adventitious and pathogenic flora, and to leave the milk in good condition for further processing [24]. Figure 13 shows the most common dairy products manufactured from raw cow's milk, and subsequent figures outline the steps involved in each process, where high heating temperatures also take place (For further discussion and a brief description of the importance, the process, the different types and the limitations of pasteurisation of raw milk, see Appendix E).

Figure 13: Typical process flow for dairy products manufacture.

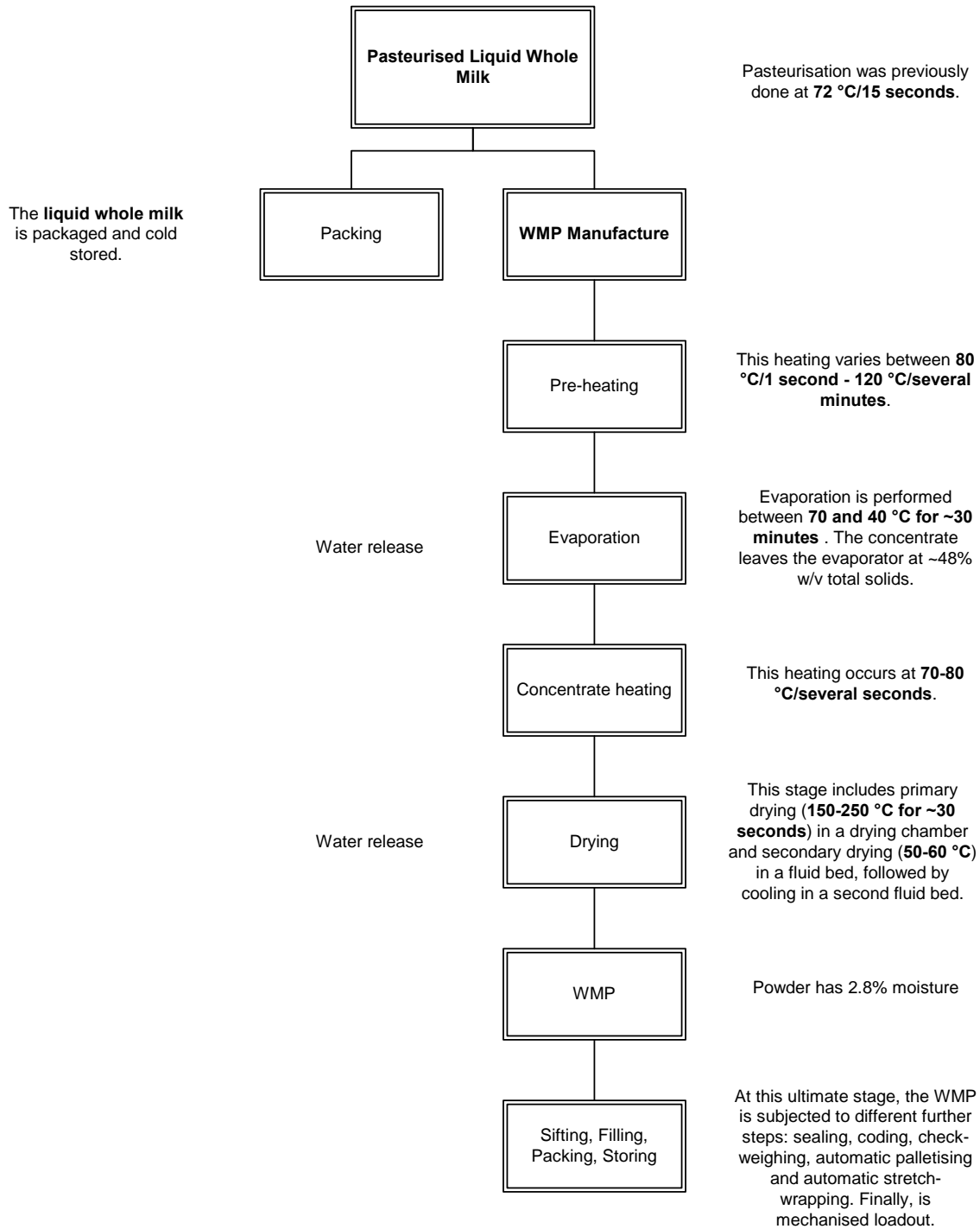


7.1.4.2.1 Milk powder

The manufacture of WMP or SMP is essentially the same, differing only in that the raw material is either whole or skim milk, respectively. It involves initial pasteurisation of the raw milk at HTST. Then, a pre-heating stage is carried out, which involves temperatures of between 80 °C/1 second – 120 °C/several minutes (depending on whether low-heat or high-heat powder is being manufactured). This is followed by the evaporation stage, when temperatures drop from 70 °C to 40 °C as the liquid passes through each evaporator effect. This stage results in a ‘concentrate’, with ~ 48% w/v of total solids. After evaporation, the concentrate is heated at 70-80 °C/several seconds, after which it is dried. The primary drying stage (in a drying chamber) provides the highest air heating temperatures, 150-250 °C, followed by secondary drying (in a fluid

bed) and then cooling (in a second fluid bed). The powder (2.8% moisture) is then sifted, filled in containers, packed, sealed, coded, weight-checked, palletised, stretch-wrapped, and stored prior to export [24]. Figure 14 shows the steps involved in the manufacture of milk powder.

Figure 14: Typical process flow for liquid milk and WMP manufacture.²³



²³ The process flow for SMP is essentially the same, but it is performed on pasteurised liquid skim milk.

From the heating steps shown in Figure 14, it can be seen that the key processing steps (pre-heating and concentrate heating) would effectively kill any remaining enterococcal bacteria that may have survived pasteurisation. Therefore, it is reasonable to assume that if enterococci are detected in milk powder, they will have been acquired as a result of contamination after the last severe heating step of the manufacturing process.

In New Zealand, a study of the microbiological composition of 84 samples from 10 milk powder manufacturing plants performed in 1993 [81] showed that most samples (79.8%) contained no detectable enterococci (<10/g) (Table 16), supporting the view that the process is severe enough to destroy any enterococci that may have been present in the raw milk. It is probable that the enterococci contained in the other 20.2% of samples (Table 16) resulted from post-heat contamination. However, the sources of contamination were not identified during the study [81]. Possible sources of enterococci contamination during milk powder manufacture include moist deposits in the fluid bed stages of powder drying, and possibly during powder transport and storage, which would only occur as a result of condensation of equipment surfaces or failure to dry the plant after cleaning.

Table 16: Enterococcal results of analyses performed on 84 samples from 10 New Zealand milk powder manufacturing plants (summarised according to [81]).

Not detected <10/g	Detected		
	10 ¹ -10 ² /g	10 ² -10 ³ /g	10 ³ -10 ⁴ /g
79.8%	14.2%	4.8%	1.2%

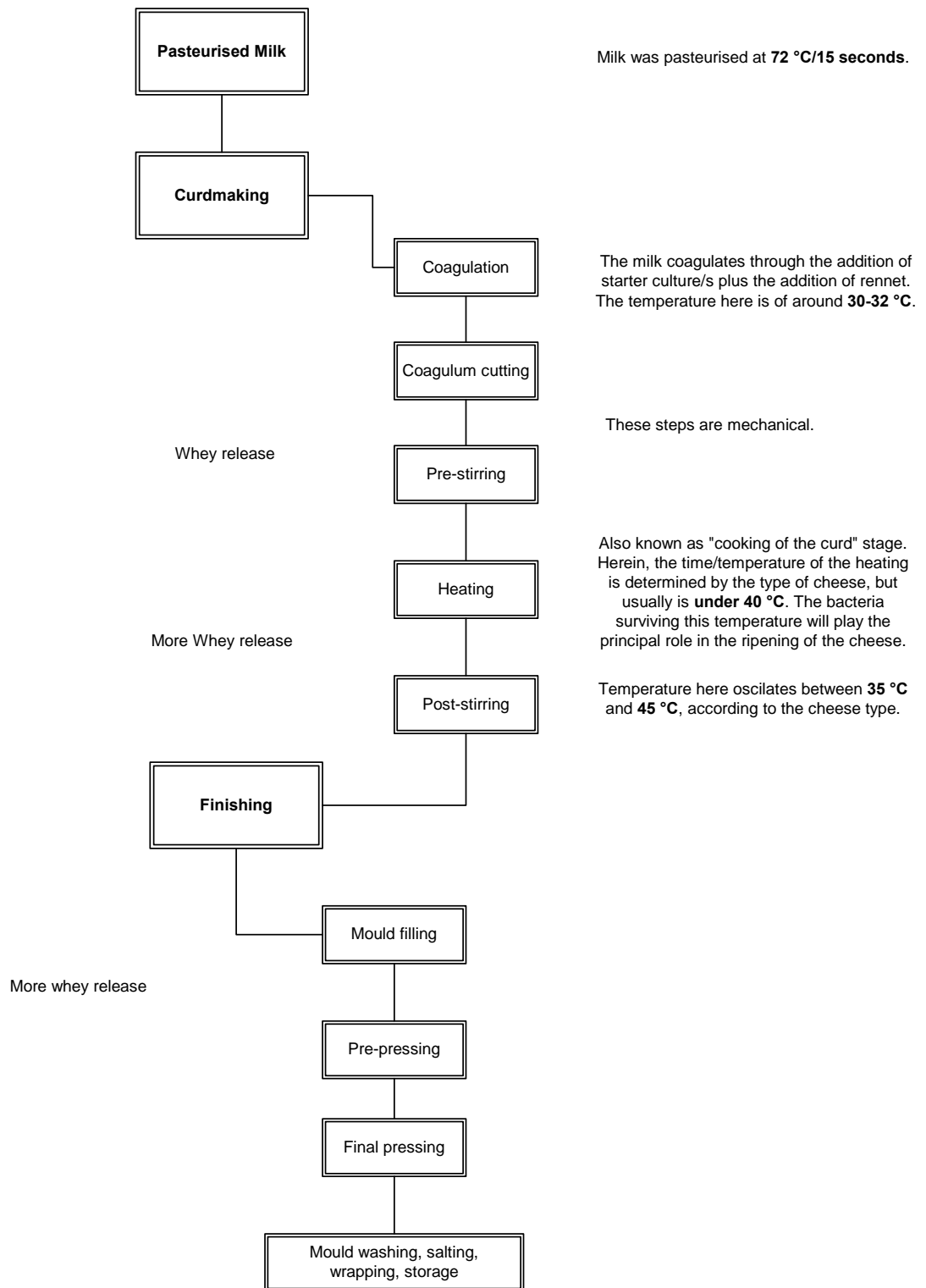
7.1.4.2.2 Cheese

For the manufacture of cheese, the pasteurised milk is subjected to coagulation through the addition of starter culture/s and rennet. After the coagulum is formed, it is cut and pre-stirred mechanically. Later, the curd is cooked, usually at <40 °C, in order to ensure starter survival as these organisms take the principal role later during cheese ripening. A post-stirring stage follows, where the temperature fluctuates between 35 and 45 °C. The final steps comprise mould filling, pre-pressing, final pressing, mould washing, salting, packaging and storage of the cheese. Figure 15 shows the typical process followed during the manufacture of cheese.

As discussed previously, pasteurising of milk ensures that the enterococci in raw milk destined for the manufacture of cheese are unlikely to survive. However, these bacteria

find the cheese environment very favourable for growth. Consequently, if re-contamination occurs after pasteurisation, enterococci are likely to reach significant numbers at the end of ripening, especially if there is a long ripening period, for example, several months [150]. Therefore, if presence of contaminant enterococci is to be avoided (and provided enterococci are not being added as cheese cultures), extreme hygiene must be maintained during the entire cheese manufacturing process.

Figure 15: Typical process flow for cheese manufacture.



7.1.4.2.3 Casein

As with the other products discussed, casein is made with pasteurised milk, but in this case it will be skimmed beforehand. Later, a mesophilic starter culture is added to the

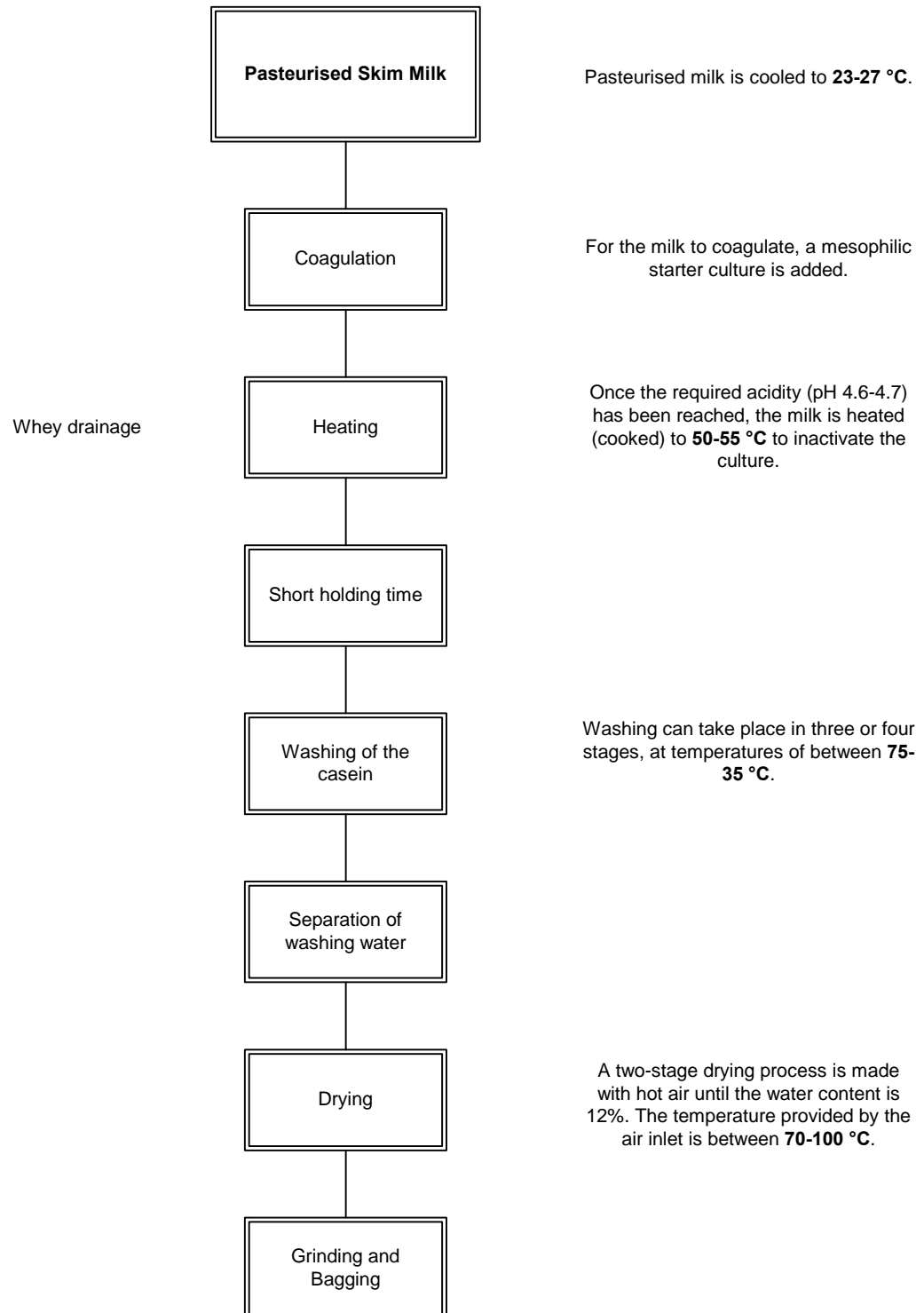
skim milk in order to coagulate it. Once the coagulum is formed, and the required acidity reached, it is cooked at 50-55 °C, to inactivate the starter. After a short holding time, the casein is washed 3 or 4 times, first at temperatures of around 75 °C and subsequently at temperatures that progressively decrease to ~35 °C. After the separation of the washing water, the washed curd is dried with hot air of ~100 °C, until the water content of the casein reaches ~12%. From the heating to the drying stages the process takes ~1 hour. Finally, the casein is ground and bagged. The complete casein process is depicted in Figure 16.

During casein manufacture, the drying stage is very severe – to the extent that any enterococci that may have survived pasteurisation will be destroyed. Therefore, it would be reasonable to expect not to find any significant numbers of enterococci in the final product, unless re-contamination or deficiencies in manufacturing techniques or storage have occurred.

A New Zealand study [82] performed on 165 caseinate samples²⁴ demonstrated that 92.7% contained <10 enterococci/g, i.e. non-detectable levels. The presence of enterococci in 7.3% of the samples was probably the result of re-contamination at the later stages of processing.

²⁴ Caseinate production is a process subsequent to casein production.

Figure 16: Process stages in acid casein manufacture.



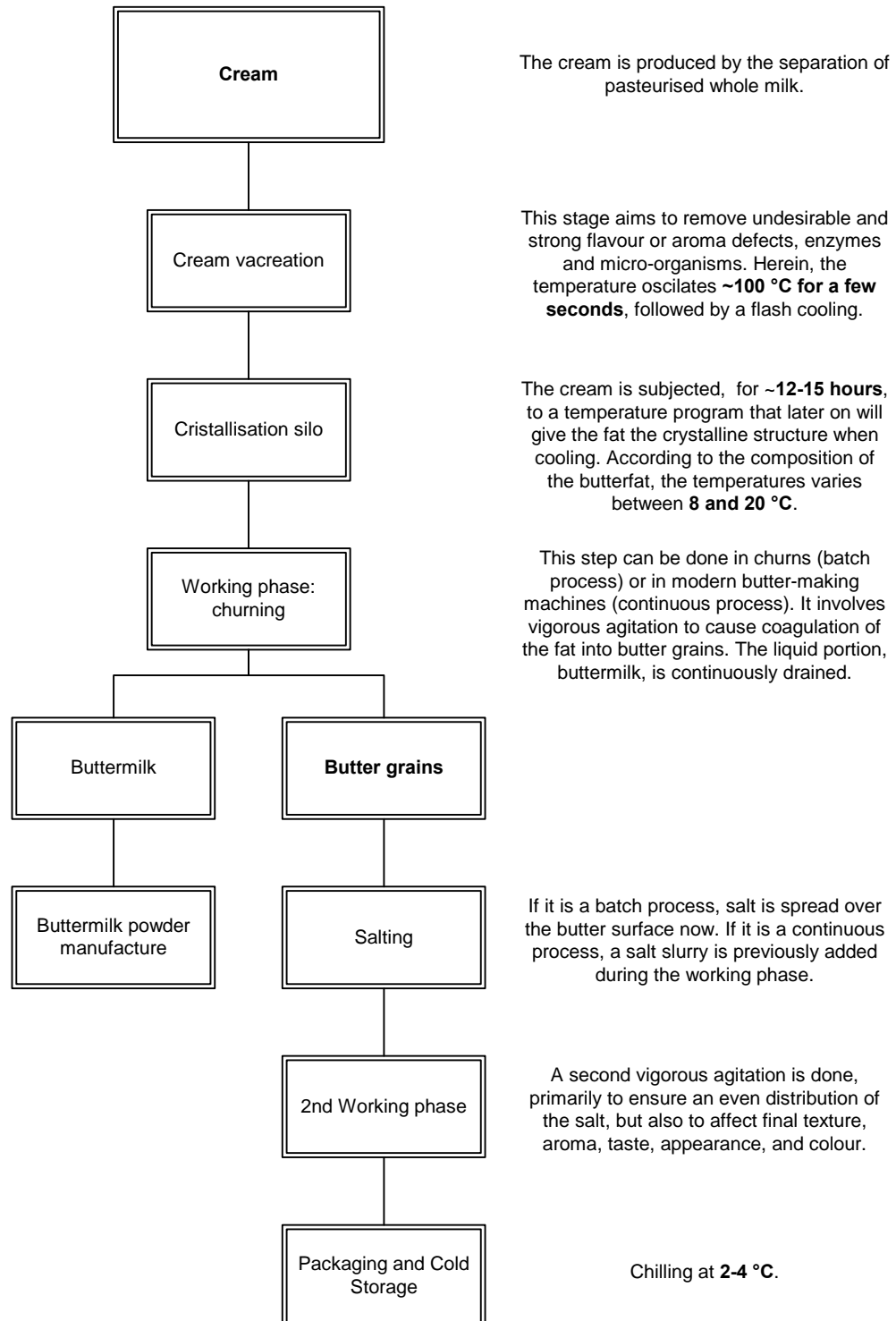
7.1.4.2.4 Butter

The manufacture of butter involves utilisation of pasteurised cream that is subsequently exposed to vacreation. The temperature at vacreation is ~100 °C, which aims to remove all sensory defects that the cream may harbour, as well as enzymes and micro-

organisms. A flash cooling follows, which aims to free any remaining entrapped gas and volatile substances. Subsequently, the cream is crystallised for ~12-15 hours at a temperature between 8 and 20 °C in a crystallisation silo. The subsequent working phase involves a vigorous agitation that seeks to coagulate the fat and transform it into butter grains, while the buttermilk is continuously drained off. After draining, the butter is salted, and again worked vigorously in order to ensure an even distribution of the salt and improve the development of its texture, aroma, taste, appearance and colour. The finished butter is discharged into the packaging unit and from there to cold storage (2-4 °C). The process is represented in Figure 17.

In the manufacture of butter, the cream vacreation emits a temperature that is high enough to kill any enterococcal bacteria. Therefore, provided good hygiene is maintained, butter should be free of enterococci. If any are present, this would indicate re-contamination during the manufacture, and after the vacreation stage.

Figure 17: Process stages in butter manufacture.



7.2 Conclusions

Enterococci naturally occur in raw milk as part of its microbial population. In cow's raw milk, ranges of $<10^1$ - 10^5 enterococci/ml are acceptable; however, levels higher than these may suggest poor pasteurisation or unsatisfactory hygiene practices. European and American sanitary regulations do not specifically address the presence or concentration allowed for enterococci, since they vary according to product, handling, time of storage, and other factors. In New Zealand, no standards are set for enterococci either, given that these bacteria are not routinely counted in dairy food.

On the other hand, enterococci are appreciated in the manufacture of a variety of cheeses as added cultures, and their role as probiotics and as protective cultures are also significant.

In this chapter, after describing the role of enterococci as dairy cultures, the focus was turned to the presence of enterococci as contaminants. Firstly, a hypothetical 'worst case' enterococcal count of 1,900 enterococci/ml in raw cow's milk was taken, based on the information provided by a study of microbiological quality of raw milk from New Zealand farms in 1997. To increase the risks, the 1,900/ml count was assigned to a single species at a time. The results suggested that, at this count, after 8.6 and 3.5 seconds of 72 °C pasteurisation no detectable *E. faecalis* or *E. faecium* levels, respectively, would be found. *E. durans* would, however, only be inactivated after applying this temperature for 34.8 seconds. Subsequently, considering the mixture of different species and the co-mingling of their counts after dilution of milk from individual farms in bulk tanks and silos, a more 'realistic' mean count of 100 enterococci/ml was chosen, based on the information provided by the same study of microbiological quality of raw milk from New Zealand farms. The results suggested that, at this count, 14.7 seconds were more than sufficient to achieve an effective *E. faecium*, *E. faecalis*, and *E. durans* inactivation. Finally, in later sections, diagrams depicting traditional steps for the manufacture of common dairy products revealed that enterococci from raw milk would not survive further post-pasteurisation heating steps either, since they are even more severe, and enough to ensure not detectable counts if they had originated from raw milk.

From the results and the hypotheses we can conclude that overall, provided quality raw milk is used, and that HTST pasteurisation (72 °C/15 seconds) is carried out, followed

by proper storing conditions that ensure no enterococcal re-growth, enterococcal presence would be extremely unlikely to occur in the pasteurised milk. In the principal dairy products – milk powder, butter, casein – their presence is not likely either, since their manufacture involves one or several heating stages that will effectively further inactivate any enterococcal bacterium. Therefore, if final products are contaminated with enterococci, these would not have originated from the raw milk, but from post-heat re-contamination and growth.

In the case of cheese manufacture, special care must be taken, given that its produce provides an appealing environment for re-growth of enterococci if re-contamination occurs.

Executive Summary

As one of the largest exporters of dairy products in the world and member of the World Trade Agreement, New Zealand has a vital interest in the sustained quality and safety of all exported foods. Dairy products must meet food quality and safety requirements according to general rules laid out in the Sanitary and Phyto-Sanitary Standards. Although there are no specific standards for enterococci within the dairy industry, an enterococcal count was occasionally requested by food importers, since these bacteria were traditionally used as hygiene indicators. Meeting international hygiene standards would benefit local markets as well.

Therefore, this review intended to describe the most significant characteristics of enterococci in clinical and food microbiology with a special focus on their roles in dairy foods.

Enterococci are Gram-positive, facultative anaerobic bacteria. Currently, the genus *Enterococcus* comprises 25 sub-species within 8 species groups. Among all enterococci species, *E. faecalis* and *E. faecium* stand out.

There are numerous benefits from enterococci in milk and dairy products. Enterococci have always been important as food fermentation bacteria. For decades, many enterococcal strains have been used in the dairy industry to produce a variety of cheese. The benefit of enterococci for the dairy industry, especially when added as adjunct cultures, was primarily attributed to their ability to improve the sensory profile of cheese, particularly during ripening stages. In this regard, volatile components such as lactate, acetaldehyde, ethanol, diacetyl and acetoin are produced by some enterococcal species. The highest levels were found in *E. faecalis* strains, while *E. faecium* was the most frequent species in producing volatile components. The production of volatile compounds showed a marked strain specificity and appeared to be higher among strains of food origin. Other appealing attributes of fermented food products, such as acid production, proteolytic, lipolytic and esterase activities, citrate and pyruvate metabolism, were less pronounced by enterococci strains than by other LAB. Enterococci also produce a number of bacteriocins that can effectively inactivate pathogenic bacteria such as *Listeria monocytogenes*. Therefore, it was suggested that

enterococci have a role as 'protective' bacteria. Moreover, it was proposed that, as probiotics, some enterococcal strains may have beneficial effects on the health of animals and humans.

Enterococci primarily inhabit the gastrointestinal tract of mammals. However, recent ecologic studies have demonstrated that enterococci were wide spread in the environment. Considering that enterococci are predominantly ubiquitous, it may be inappropriate to regard them as indicators of faecal contamination.

Because of the ability to colonise a diverse range of niches, enterococci can be found in raw milk and their raw or semi-cooked sub-products, as part of its microbial population. Counts of 10^1 - 10^5 /ml in raw milk are regarded as normal. Higher levels of enterococci, however, were considered a result of poor handling practices or lack of a proper pasteurisation.

Isolation in dairy products can be carried out through standard laboratorial techniques. Citrate azide agar and BEA agar are the most widely used media.

In contrast to their beneficial effects on the sensory characteristics and the digestibility of dairy products, some authors suggested that enterococci may also have pathogenic properties. The detection of virulence factors and antibiotic resistance traits in enterococci isolated from severely immuno-compromised patients has led to the hypothesis that enterococci may be a cause for clinical illness. However, not a single study found patients with a unibacterial enterococcal infection. Among thousands of patients examined, those with enterococci isolated from blood also had other bacteria with known pathogenicity present. Currently, the only valid argument favouring a pathogenic role was based on three experimental studies in rabbits and mice demonstrating adverse clinical effects after artificial inoculation. Observed effects included retinal tissue damage, endocarditis, peritonitis, systemic toxicity symptoms, and reduced time to death. Therefore, the perception of enterococci as a cause for clinical disease in humans is currently not substantiated by conclusive scientific evidence.

Virulence factors and genetically encoded antibiotic resistance have been found in enterococci isolated from hospital patients with clinical disease. Because such virulence factors and antibiotic resistance traits can be transferred to other strains, it was

hypothesised that enterococcal strains in food may also be able to transmit virulence factors and antibiotic resistance to ubiquitous bacteria. However, available evidence suggested that virulence factors and antibiotic resistance traits from clinical and food strains may not be identical. Thus, these traits may not be transmitted between clinical and food strains. In addition, virulence traits were not generally found in food strains and food enterococci were relatively susceptible to antibiotics.

This review also included a risk analysis of enterococcal survival during milk processing. It was found that an initial enterococcal count of 100 CFU/ml (published mean value) subjected to pasteurisation at 72°C and a holding period of 15 seconds was sufficient to almost completely kill the most frequent enterococcal species present in raw bovine milk. With even higher temperatures and/or longer holding periods in subsequent processing steps, enterococci would be removed even more efficiently. According to model calculation based on published survival rates, processing would reduce the risk of survival of bacteria in raw milk to almost zero, even at concentrations 1,000 times higher than the above count. Hence, if adequate processing conditions were maintained, enterococci isolated from dairy products would presumably be the result of a post-heat re-contamination from the environment, for example due to a breach of hygiene standards.

Appendix A: Selective media for the isolation of enterococci in foods and dairy products

a) KF streptococcal agar

When food samples are analysed, it is widely accepted that *E. faecalis* and *E. faecium* are the most common enterococci encountered. This influences the rationale of employing a selective differential medium, the KF streptococcal agar, for the estimation of enterococci in foods. This agar employs *sodium azide* as the chief selective agent and *triphenyltetrazolium chloride* (TTC) for differential purposes. Given that *E. faecalis* and *E. faecium* species (and several others) are resistant to sodium azide, they can grow in a medium containing 0.04% of this substance [46].

Procedure: When KF streptococcal agar is used, as in other microbiological plating procedures, sample preparation is important. For example, dried foods are often reconstituted and immediately diluted and plated. Culturing by the pour plate method into duplicate Petri plates is indicated. After incubation for 48 ± 2 hours at $35 \text{ }^\circ \pm 1 \text{ }^\circ\text{C}$, a dissecting microscope with a magnification of 15 diameters or a colony counter will give us the means for counting all red and pink colonies. The colour is due to the reduction of TTC, and the intensity of this reduction varies, e.g., *E. faecalis* imparts a deep red colour to the colony whereas other group D streptococci are feebly reductive and the colonies appear light pink. The number of colonies will then be reported as the 'KF enterococcal count' [78].

Drawbacks of employing this agar: Even though many industry and regulatory agencies accept KF streptococcal agar for the quantitative estimation of enterococci in non-dairy foods, the selectivity of this agar is not absolute, the quantitative recovery is less than ideal, and preparation of the medium needs an aseptic addition of an indicator. Also, the precision parameters for this test are undefined [190].

Moreover, although when KF streptococcal agar is used most other LAB are partially or completely inhibited, some strains of *Pediococcus*, *Lactobacillus*, and *Aerococcus* may grow, producing light pink colonies, confounding with the deep red or light pink colour that enterococcal colonies form. Therefore, a 'repair-detection' procedure should be

considered when the enterococcal population of a food may contain a large proportion of injured cells [172] (for details see *Compendium of methods for the microbiological examination of foods* [48], Chapter 5 [172]) or a more selective medium or higher incubation temperature (45 °C) may be necessary in order to reduce background growth of other lactobacilli and lactic streptococci, especially for dairy products [78].

A final, major drawback in using KF streptococcal agar is that the employed *sodium azide* as the chief selective agent is not only inhibitory to other bacteria but may possibly be inhibitory to many strains of the newly named *Enterococcus* spp. [78].

b) Citrate azide agar

A variant of the KF agar is the Citrate azide agar, highly recommended for dairy products [46, 190], which employs *citrate azide* instead of *sodium azide* agar pour plates. Here, *tetrazolium blue* is added in the medium so enterococcal colonies are stained blue by it. Plates are incubated at 37 °C for 48-72 hours. Then, only blue colonies are counted [190].

c) CATC agar

CATC agar has been used for isolation of enterococci from meat and its by-products, dairy products and other foods. Pronounced growth and a brilliant formazan production can be obtained with *E. faecalis*, while colonies of *E. faecium* exhibit a weaker formazan reaction. If plates are evaluated at a defined time (after incubation for 24 hours at 37 °C) this medium is highly selective and elective for *E. faecalis*. Moreover, it is useful for culturing and detecting *E. faecium*. Enterococci colonies can be identified as appearing as bright red or pink.

Comparing the growth of pure cultures on CATC and SB agar, CATC agar has a highest specificity for enterococci after incubation for 24 hours.

Another significant advantage is that *Lactobacillus* and *Streptococcus* spp. are not able to grow when CATC agar is used [46].

d) (Membrane filter) SB agar

A complex culture medium, the (Membrane filter) SB agar, also known as M-enterococcus agar, has been widely used for the culture, isolation and enumeration of enterococci. SB agar is usually employed, in combination with membrane filtration procedures, for recovery of enterococci from dried foods, including non-fat dry milk [46, 190]. Samples can be directly plated onto the medium in order to detect and enumerate faecal streptococci.

Like the KF streptococcal agar, this medium employs TTC as the marker substance and *sodium azide* as the selective agent. Therefore, enterococcal colonies appear as pink or dark brownish-red colonies, after incubation for 48-72 hours at 37 °C.

The SB agar combined with the membrane filtration technique, has shown to possess superior performance among a multitude of media tested. The method showed a high recovery rate, precision and accuracy, as well as a good specificity [46], despite the fact that this medium may not inhibit the growth of *Lactobacillus acidophilus* and *S. bovis*, specifically [190].

e) BEA agar

An excellent medium to isolate and even confirm the presence of enterococci in foods is the BEA agar. This agar has frequently been recommended because of its ability to discriminate enterococci in a specimen containing multiple microbial components. This is due to the capability of an isolate to grow on bile (since enterococci/group D streptococci are bile-tolerant) and also to its ability to hydrolyse the aesculin fraction (since enterococcal bacteria produce an 'aesculinase' enzyme that hydrolyses aesculin and releases aesculetin, which reacts with iron in the medium to form a dark brown or black complex) [78].

On this medium, enterococci produce colonies surrounded by a black halo after 24 hours of incubation. However, even though most other bacteria either grow weakly or appear as colonies of different shape, *L. monocytogenes* may exhibit a similar colonial morphology as enterococci after 48 hours of incubation on this medium.

The sensitivity of BEA agar is comparable with that of blood or Mitis-Salivarius agar, but its selectivity seems to be superior. Also, BEA agar offers the advantage of allowing

the selection of *S. bovis* because typical results are produced earlier than on other media [46].

f) KAA medium

KAA is a commercially available medium and it is used for the isolation and enumeration of enterococci from foods, water and other specimens. It contains *sodium azide* and *kanamycin* as selective agents. The growth of the majority of other bacteria is suppressed, while targeted organisms hydrolyse aesculin, which leads to the formation of black haloes around the colonies.

The medium is usually incubated for 24 hours at 37 °C, though increased incubation temperatures (42 °C) and shorter incubation times (18 hours) may improve the selectivity. Devriese *et al.* [45] recommended using KAA incubated for 24 hours at 42 °C for the isolation of enterococcal species of animal origin.

However, unfortunately KAA also allows some partial growth of mesophilic lactobacilli, since some members are able to cleave aesculin and incubation at 42 °C does not inhibit the growth of aesculin-positive lactobacilli. Apparently, by increasing the concentration of *sodium azide* this problem can be avoided, but results in a reduced recovery rate of enterococci [46].

g) BB broth

BB broth is usually employed for the confirmation of enterococci from water, although it is also suited for the enumeration of enterococcal species in dairy products [46].

h) fGTC agar

An alternative procedure that permits the recovery of a wider variety of enterococci from foods is fGTC agar, which utilises inhibitors other than azide. However, this agar is more frequently used for isolation of enterococci from meat and fish sources [103].

Procedure: Plates with fGTC agar are prepared, cooled to 45 °C and allowed to solidify. After incubation of the plates for 18-24 hours at 35 ° ± 1 °C, observation for starch hydrolysis (a zone of clearing around a colony under visible light) and fluorescence (a zone of bright bluish fluorescence when the opened plate is held under a long-wave

ultraviolet lamp) should be made. Then, the following phenotypic groups are identifiable: (1) starch hydrolysis and fluorescence, indicative of *S. bovis*, (2) no starch hydrolysis but fluorescence, indicative of *E. faecium* and related biotypes, and (3) no starch hydrolysis or fluorescence, indicative of *E. faecalis*, *E. avium*, and other streptococci. Now, all colonies must be used to calculate the 'fGTC enterococcal count' (which can be further divided, if desired, into subgroups based on the observed starch hydrolysis and fluorescence) [78].

Drawbacks of employing this agar: Despite the fact that the incubation period for fGTC agar should only be of 18-24 hours (compared to 48 hours for KF agar), enterococcal counts from foods may be two or more orders of magnitude higher on fGTC agar than on KF agar.

In addition, the fGTC agar has not been tested for recovery of some of the newly named enterococci (nor KF agar). Therefore, the performance of both media should be re-evaluated in the light of recent advances in enterococcal classification [78].

Appendix B: Methods for confirmation of enterococci in dairy foods

a) Conventional Procedures

Five to 10 typical colonies are selected and transferred each into a separate tube of BHI broth, incubated at 35 °C for 18-24 hours, and then observed for cells showing typical enterococcal morphology. A test for catalase activity is also performed and should give a negative result.

Another good confirmatory test is to demonstrate the ability of an isolate to grow on BEA agar, as noted in Appendix A [78].

b) Rapid Methods

A 15 minute ‘aesculinase’ test [181] and a 4 hour combined NaCl tolerance-aesculin hydrolysis test [153] can be performed. Convenient tri-plates, quad-plates, and tubed media [99] for key identification tests are also available from many suppliers of prepared media and can be time-saving. Another rapid confirmatory test is the pyrrolidonyl- β -naphthylamide (PYR) test [78]. A ‘RapID STR’ kit [196] is also convenient; however, the efficacy of this kit and the data bank used with it are based on isolates from human clinical material; therefore, the efficiency may differ when isolates from other animals or food are studied [78].

c) Automated Identification

Both the Vitek AutoMicrobic Gram-positive identification system [6] and the General Diagnostics Autobac system [21] can be used for confirmation of enterococci. Also, an impedance method that detects 10 to 100 faecal streptococci in dry milk within 18 hours has been described [184].

d) Serological tests

Where serological confirmation is required, commercial grouping sera are available, as well as a variety of serological kits. However, these kits vary in efficacy, and false-negative group D reactions are common. This may be due to the difficulty in

demonstrating the presence of the group D antigen in some strain isolates [78]. This being the case, the method of group antigen testing adopted is important [152].

Appendix C: Enterococci in cheeses

If bacteria are naturally present in raw milk, then it is expected that some of them may naturally occur and grow in a variety of raw or semi-pasteurised dairy products as well. With regards to enterococci, even though high levels of contaminating enterococci usually result from poor hygienic practices during cheese manufacture, leading to deterioration of sensory properties in some cheeses (especially fresh or soft industrial ones made with pasteurised milk and selected lactic starter cultures), in other cheeses they play a major role in improving flavour development and quality. Thus, enterococci can be commonly found in high levels in a variety of artisanal cheeses produced in European countries such as Italy, Portugal, Spain and Greece (and even in other countries such as Argentina [158], Ethiopia [9] and Turkey [185]) from raw or pasteurised milk from goats, ewes, water buffaloes or cows [70]. The beneficial effect of enterococci has been attributed to the hydrolysis of milk fat by esterases and to the production of typical flavour components such as acetaldehyde, acetoin and diacetyl [161].

Numbers of enterococci in cheese curds and traditional European fully ripened cheeses range from 10^4 to 10^7 – sometimes up to 10^8 - CFU/g [59]; in ewe's milk cheeses especially, count of enterococci tends to be particularly high [7]. Levels of enterococci tend to remain stable throughout the different stages of cheese ripening [7], with some minor variations - in cheese curds, levels of enterococci may range from 10^4 to 10^6 CFU/g, whereas in the fully ripened cheeses levels can vary from 10^5 to 10^7 CFU/g [62]. Some other dairy products, like living cultured yoghurts, may yield growths from 10^5 to 10^9 CFU/g, i.e. if a culture is added [177]. Numbers of enterococci vary with their microbial load, the cheese type, the starter used, and the production season. Varying levels in different cheeses are also influenced by the survivability of the bacteria in the dairy environment (dependent on seasonal temperature) as well as the survival and growth ability under the particular conditions of cheese manufacture and ripening, i.e. the technology applied [59, 75].

Enterococci in a Mediterranean cheese may reach high levels. This may be due to the fact that cheeses, in general, have a complex microflora and are conducive to growth of many bacteria, especially LAB. Being facultative anaerobes, with the ability to ferment

lactose and grow at 45 °C and in 6.5% salt, enterococci are expected to grow in cheese if present in raw milk either as natural or added microflora (e.g. artisan cheese starter cultures) or as adventitious microflora (through environmental contamination post-pasteurisation).

Table 17 shows different counts of enterococci in Mediterranean cheeses [59]. It shows that Cebreiro, Kefalotyri, Manchego, Picante da Beira Baixa and Teleme cheeses have predominantly enterococci when are fully ripened [59]. In the other types of traditional cheeses (White-brined, La Serena ewe's milk and Serra cheeses), even though LAB such as *Lactobacillus plantarum*, *Weisella paramesenteroides*, *Leuconostoc lactis* or *Leuconostoc paracasei* predominate in the ripened product, enterococci are usually an important part of the bacterial count as well [59].

Important counts of enterococci can also be found in the Tafi [158], Roncal and Idiazábal cheeses [7, 8] as well as in the Feta [125], Turkish white cheese [185], Ayib cottage cheese [9], Armada, Majoero, Mozzarella, Monte Veronese, Fontina, Caprino, Venaco, and Comté cheeses, where they form a major part of the fresh cheese curd microflora, and in some cases predominate in the fully ripened product [75]. Other LAB found in cheeses (usually *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Weisella*) also play starter, probiotic, and/or protective roles [102, 177].

The recovery, persistence and dominance of enterococci over all the other LAB bacteria is attributable to their wide range of growth temperatures, high tolerance to heat, salt, and acid, and their production of proteolytic enzymes involved in casein degradation. Salt concentration is increased during cheese ripening, and this is an important selection factor for growth of salt-tolerant enterococci during the latter stages of ripening [59, 75].

Among enterococci, *E. faecium*, *E. faecalis*, and to a lesser extent *E. durans*, are generally the most frequent and prevalent species in cheeses [59, 75, 161], especially in those manufactured from ewe's and goat's milk, as seen in Table 17 [7].

Table 17: Numbers and predominance of *Enterococcus* spp. in cheeses from Mediterranean countries (summarised according to [59]).

Cheese	Country of origin	Milk source	Enterococci in curd (log CFU/g)	Enterococci at end of ripening (CFU/g)	Predominant bacteria in end product (% of isolates)
White-brined	Greece	Raw goat's milk or mixed goat's and ewe's milk	4.0	6.7	<i>L. plantarum</i> (47%) ^b <i>E. faecium</i> (12%) <i>L. paracasei</i> subsp. <i>paracasei</i> (10%) <i>E. faecalis</i> (9%)
Kefalotyri	Greece	Ewe's milk, cow's milk or mixed ewe's and goat's milk	4.9	5.8	<i>E. faecium</i> (35.6%) <i>L. plantarum</i> (18.4%) <i>L. casei</i> subsp. <i>casei</i> (15.8%) <i>E. durans</i> (9.2%) Pediococci (9.2%)
Teleme	Greece	Pasteurised ewe's milk	n.r. ^a	n.r.	Lactobacilli Leuconostocs Enterococci
La Serena sheep cheese	Spain	Raw ewe's milk	6.2	7.2	Lactobacilli Leuconostocs Enterococci
Manchego	Spain	Raw ewe's milk	n.r.	n.r.	Enterococci
Cebreiro	Spain	Raw cow's milk	n.r.	6.5	<i>E. faecalis</i> (30.1%) <i>E. faecalis</i> (subsp. <i>liquifaciens</i>) (11.9%) <i>Lact. lactis</i> (19.0%) <i>W. (Leuc.) paramesenteroides</i> (7.9%) <i>Leuc. mesenteroides</i> subsp. <i>mesenteroides</i> (6.3%) <i>E. faecium</i> (4.8%)
Serra	Portugal	Raw ewe's milk	n.r.	n.r.	<i>Leuc. lactis</i> , <i>Lact. lactis</i> , <i>Leuc. mesenteroides</i> subsp. <i>mesenteroides/dextranicum</i> <i>E. faecium</i>
Picante da Beira Baixa	Portugal	Mixture of raw goat's and ewe's milk	n.r.	n.r.	<i>E. faecium</i> , <i>E. faecalis</i> , <i>E. durans</i> , <i>L. plantarum</i> , <i>L. paracasei</i>

a. n.r. = not reported.

b. *L.* = *Lactobacillus*; *E.* = *Enterococcus*; *Lact.* = *Lactococcus*; *Leuc.* = *Leuconostoc*; *W.* = *Weissella*.

Appendix D: Enterococci in meats

a) Enterococci as contaminants

As discussed in Chapter 5, the presence of bacteria in the gastrointestinal tract of animals - and in the general environment - leads to a potential for contamination of meat in abattoirs at the time of slaughter. Enterococci are not the exception among these bacteria and although they are not the most predominant bacteria isolated (typical meat products isolated bacteria are *Enterobacteriaceae*, *Salmonella* spp., *S. aureus*, *Clostridium* spp., *E. coli*, *Listeria* spp., *Carnobacterium* and *Pseudomonas* spp.), *E. faecalis* and *E. faecium* can be found in raw meat products such as beef, chicken and pork cuts, and consequently in their sub-products such as pork meat sausages. The numbers of viable enterococci in contaminated poultry, pork and beef are usually in the range of 10^2 - 10^4 CFU/g⁻¹[86]. Pig carcasses from slaughtering plants can contain mean log counts of 10^4 to 10^8 enterococci per 100 cm² of carcass surface [59]. Enterococci can also derive from cross-contamination at the final stages of meat processing, such as slicing and packaging [86].

Enterococcal presence may result in spoilage of processed meats as the consequence of the relative heat resistance enterococci have (described in Chapter 3). Processed meats are typically salted or cured, and either raw or cooked. Normally, cooking of processed meats raises the core temperature of products to at least 60 °C and frequently above 70 °C. Since some enterococci can survive 30 minutes heating at 62.8 °C [75], low heating processes may result in undesirable enterococcal survival and hence may spoil cured meat products, especially if recontamination with competing bacteria is prevented beforehand, e.g. when products are heated after packaging in cans or in impermeable plastic films. It seems that the best way to prevent occasional spoilage of processed meats by enterococci, is to keep the initial contamination by these micro-organisms at a minimum, and to assign an adequate heat processing temperature based on D-values of the most heat-resistant enterococci that could be isolated from raw materials [59].

Spoilage of vacuum-packaged processed meats has to a low degree been associated with enterococci. Members of the genera *Lactobacillus* and *Leuconostoc* are usually the most frequent and predominant spoilers responsible for the deterioration of processed meats, even though varying proportions of enterococci and pediococci are often also present in

the products after heat treatment and before packaging. In order to decrease the initial bacterial load to a minimum, a secondary, in-package heat process of vacuum-packaged products is suggested, which results in a noticeable delay of spoilage. Thereafter, if spoilage eventually occurs, due to the higher tolerance pediococci possess to environmental factors, they will often be the ones predominating, with an almost zero incidence of enterococci in the products [59].

A higher count of enterococci may also occur as a result of occasionally 'reworking' already processed meat, e.g. when the packaging material is broken during heat treatment and the meat from faulty products is added to the raw materials for further processing. If the faulty product already contained heat-resistant enterococci, these may multiply during the 'reworking' and survive a second heat processing step, but this time in greater numbers [59].

b) Enterococci as fermentative cultures and enterocin producers

Enterococci fulfil a significant role in meat fermentation, although this role has not yet been extensively investigated [86].

In Mediterranean countries, many traditional and artisanal meat fermented products are manufactured without the use of starter cultures, relying on the endogenous flora to keep the traditional organoleptic qualities. As a consequence, the natural microbiota consists of a mixture of species of LAB, including enterococci and *Lactobacillus*, as well as coagulase-negative *Staphylococcus* and yeasts [86]. The dominant microbiota is constituted mainly of lactobacilli (*Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum*), and to a lesser degree by coagulase-negative staphylococci [86]. However, enterococci, and mainly *E. faecium*, represent the LAB species that can also be found in relatively high numbers during meat fermentation, contributing to it [86].

Presence of enterococci in sausages occurs in high pH environments (more than 5.0). Here, enterococci co-exist with lactobacilli as the dominant population, and the growth of the latter provides an advantageous - but also competitive - ecological niche for the former [86].

The number of enterococci during natural fermentation varies, even at the same manufacturing plant and with the same formulation and technology, especially if competitive starter cultures, like *L. curvatus*, are present in the product as well [86]. During fermentation, enterococci survive and multiply, and at the end of the ripening the concentration can reach various levels, like 10^3 to 10^5 CFU/g⁻¹ in German and Italian fermented sausages [86]. Fermented meat products like Salami and Landjäger can contain enterococci at numbers ranging from 10^2 to 2.6×10^5 CFU/g [62], while Spanish sausages can contain 1.3 to 4.48 log CFU/g⁻¹ of enterococci out of a population of total LAB ranging from 7.12 to 9.07 log CFU/g⁻¹ [86].

Despite the concern that enterococci raise for human health, *meat* enterococci, and especially strains of *E. faecium*, appear to be safer than *clinical* strains, with regards to the possible virulence potential. Moreover, meat enterococci have shown a competitive advantage over other microbiota in fermented meat, and many enterococci isolated from sausages have the beneficial ability to produce enterocins with antimicrobial activity against pathogens and spoilage micro-organisms of concern. In this sense, enterocin production by some enterococcal strains is beneficial for the preservation of fermented sausages and for sliced, vacuum-packed cooked meat products in preventing the growth of *L. monocytogenes* and slime-producing LAB [25, 75, 159]. The role of the enterococci as adjunct cultures improving food safety due to the anti-listerial activity developed by their bacteriocins has also been demonstrated in studies performed on the following meat products: cooked ham, minced pork meat, de-boned chicken breasts, paté, Spanish-style dry fermented sausages, and some other fermented foods [62].

Their inhibitory effect could further be increased when used in conjunction with particular physical and chemical processes. But these results are variable. For example, in an early study done by Aymerich *et al.* in 2000 [11], the use of bacteriocin producer strains was in doubt, as the bacteriocin production and growth of *E. faecium* was inhibited by low temperatures and the salt and pepper ingredients used in sausages recipes. But later on, other studies, including Aymerich *et al.* [12, 25], demonstrated that two bacteriocin producer strains of *E. faecium* could effectively inhibit a strain of *Listeria innocua* in a fermented sausage model, giving support to the thought of the enterococcal strain's additional biopreservative performance. However, it still seems that functionality of bacteriocin producer enterococcal strains in meat industries is

narrowly linked with the industrial processing conditions of manufacturing to which the strains are subjected.

Even though bacteriocinogenic and enterocinogenic enterococcal strains hold considerable promise as alternatives to traditional chemical preservatives, and the fact that they could be exploited for the control of serious emergent pathogens in meat products, because of all the uncertainties, current food processing regulations do not yet allow the application of these strains, nor their purified bacteriocins [86].

Appendix E: Heat treatment of raw milk

Cow's milk is the raw material for a wide range of dairy products: market milk, butter, cheese, milk powder, and others, but it is also a perfect growth medium for micro-organisms. Diseases such as tuberculosis and typhus were in the past spread by raw milk.

Regardless of what the end product will be, the milk used to make it must be treated in such a way that all pathogenic micro-organisms are killed. This is achieved by heat treatment, a process also known as 'pasteurisation'.

The primary purpose of pasteurisation is to kill all micro-organisms capable of causing diseases in humans. Pasteurised milk must be entirely free from pathogens, and to gain this a combination of temperature and holding time is very important as it determines the intensity of the heat treatment required. For instance, the same lethal effect for coliform bacteria can be obtained either at 70 °C/1 second or at 65 °C/10 seconds. Later, if infections are spread by pasteurised milk, the reason is either that heat treatment has not been properly performed or that the milk has been re-infected. It is therefore important to monitor the pasteurisation process carefully in order to make sure that all the milk is treated in the prescribed manner.

Apart from this primary reason, milk also contains other substances and non-pathogenic micro-organisms which may spoil the taste and shelf life of milk products. Hence, a secondary purpose of pasteurisation is to destroy as many as possible of these other organisms and enzymatic systems in order to safeguard product quality. This requires more intensive heat treatment than is necessary to kill the pathogenic bacteria.

The last aim of this process looks at modifying the composition of the milk to make it more suitable for subsequent manufacturing processes.

Pasteurisation of milk is required by law in most countries. In the case of some countries, selected unpasteurised milk is allowed for use in the manufacture of certain types of cheese [24].

a) Different degrees of heat treatment

Nowadays, milk is almost always pasteurised in the continuous HTST process, or sterilised in the Ultra High Temperature (UHT) process. Many dairies also apply a process called Thermisation (63-65 °C/15 seconds) when it is not possible to pasteurise and process all the milk immediately after delivery.

Normally, HTST of raw milk is used when dairy manufacturing is intended. In the HTST process, the actual time/temperature combination varies according to the quality of the raw milk, the type of product treated, and the required keeping properties. If it is milk, it usually involves heating to 72-75 °C with a 15 second holding time before it is cooled. If it is cream and cultured products, it usually involves heating to >80 °C with a holding time of about 3-5 seconds [24].

b) Limiting factors for the heat treatment

Although intensive heat treatment of milk is desirable from the microbiological point of view, such temperatures also involve a risk of adverse effects on the appearance, taste and nutritional value of the milk, and consequently its sub-products. For instance, proteins in milk are denatured at high temperatures and the cheesemaking properties of milk could therefore be drastically impaired by intensive heat treatment. Intensive heating may also result in changes in taste (cooked and burnt flavours).

The choice of time/temperature combination is therefore a matter of optimisation in which both, microbiological effects and quality aspects, must be taken into account [24].

c) The process of pasteurising raw milk

Chilled raw milk usually arrives at the plant at a temperature of 4 °C. Then, when pasteurisation takes place, it is subjected to a heating temperature of 75 °C, by means of hot water or vacuum steam, and is held at that temperature for 15 seconds (holding time). After the holding time, the temperature of the milk is checked by a sensor (set in the line), which transmits a continuous signal to a temperature control panel. The same signal is also transmitted to a recording instrument that records the entire pasteurisation process temperature. The milk is cooled to about 7 °C and then gradually further to 4 °C

by means of cold water, ice-water, or a refrigerant (glycol solution). The process must be applied as soon as the milk arrives and definitely not later than 24 hours after arrival.

After pasteurisation takes place, care must be taken to avoid any risk of re-contamination of the pasteurised product by any unpasteurised product or cooling medium [24].

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