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TAXONOMY AND DIVERSITY OF COCCAL LACTIC ACID BACTERIA ASSOCIATED WITH MEAT AND THE MEAT PROCESSING ENVIRONMENT

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ACADEMIC DISSERTATION

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ABSTRACT

Spoilage of modified atmosphere (MAP) or vacuum-packaged meat is often caused by psychrotrophic lactic acid bacteria (LAB). LAB contamination occurs during the slaughter or processing of meat. During storage LAB become the dominant microbiota due to their ability to grow at refrigeration temperatures and to resist the microbial inhibitory effect of CO_2. Spoilage is a complex phenomenon caused by the metabolic activities and interactions of the microbes growing in late shelf-life meat which has still not been fully explained. In this thesis, the taxonomic status of unknown bacterial groups isolated from late shelf-life meat and meat processing environment was resolved by the polyphasic approach. Five isolates from a broiler processing plant represented a novel *Enterococcus* species which phylogenetic analyses showed to be located within the *Enterococcus avium* group. The name *Enterococcus viikkiensis* was proposed for this species. In addition to enterococcal studies, the taxonomy of the *Leuconostoc gelidum* group was revised. Twenty isolates from packaged meat were shown to represent a novel subspecies within *L. gelidum*, for which the name *Leuconostoc gelidum* subsp. *aenigmaticum* was proposed. The novel subspecies was closely related to both *L. gelidum* and *Leuconostoc gasicomitatum*. Phylogenetic analyses and DNA-DNA reassociation studies led to the reclassification of *Leuconostoc gelidum* and *Leuconostoc gasicomitatum* as *Leuconostoc gelidum* subsp. *gelidum* and *Leuconostoc gelidum* subsp. *gasicomitatum*. In the third part of the thesis, *Lactococcus piscium* was shown to form a significant part of the LAB population in a variety of MAP meat in late shelf-life. This formerly neglected species in meat spoilage studies grew together with leuconostocs and contributed to spoilage when inoculated into pork. Numerical analysis of ribopatterns, and/or multilocus sequence typing of several housekeeping genes were shown to differentiate species/subspecies of enterococci and lactococci well. Finally, a novel MLST scheme was developed and the population structure within 252 strains of the spoilage bacterium *Leuconostoc gelidum* subsp. *gasicomitatum* from meat and vegetable sources was investigated. Indication of niche specificity was observed, as well as a very low level of genetic material exchange within the three subpopulations.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Rahkila, R., Johansson, P., Säde, E., and Björkroth, J. (2011). Identification of enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov. Applied and Environmental Microbiology 77(4): 1196-203.

II Rahkila, R., Nieminen, T., Johansson, P., Säde, E., and Björkroth, J. (2012). Characterization and evaluation of the spoilage potential of *Lactococcus piscium* isolates from modified atmosphere packaged meat. International Journal of Food Microbiology 156(1): 50-9.

III Rahkila, R., De Bruyne, K., Johansson, P., Vandamme, P., and Björkroth, J. (2014). Reclassification of *Leuconostoc gasicomitatum* as *Leuconostoc gelidum* subsp. *gasicomitatum* comb. nov., description of *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov., designation of *Leuconostoc gelidum* subsp. *gelidum* subsp. nov., and emended description of *Leuconostoc gelidum.* International Journal of Systematic and Evolutionary Microbiology 64(Pt 4): 1290-5.

IV Rahkila, R., Johansson, P., Säde, E., Paulin, L., Auvinen, P., and Björkroth, J. (2015). Multilocus sequence typing of *Leuconostoc gelidum* subsp*. gasicomitatum,* a psychrotrophic lactic acid bacterium causing spoilage of packaged perishable foods. Applied and Environmental Microbiology 81(7): 2474-80.

ABBREVIATIONS

DNA	Deoxyribonucleic acid
HTS	High-throughput sequencing
LAB	Lactic acid bacteria
MAP	Modified atmosphere packaged
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
MRS	de Man Rogosa Sharpe
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
T-RFLP	Terminal restriction fragment length polymorphism
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WGS	Whole genome sequencing

1 INTRODUCTION

Meat is perishable, contains a lot of nutrients and is thus an excellent growth medium for bacteria. Bacterial growth results in spoilage due to the accumulation of metabolites causing off-odours, off-flavours and undesirable appearance. The economic impact of meat spoilage is enormous, and thus prevention of microbial growth is of major interest to the meat industry. Good hygienic practices during slaughter and processing, and sanitation procedures at the plants are applied to reduce the level of initial bacterial contamination. Techniques such as salting, smoking and drying have been used for centuries for meat preservation. Cold storage and modified atmosphere or vacuum packaging are modern approaches that meet the demands of today's consumers for fresh meat, but also the requirements of the industry for the extended shelf-life for meat.

The microbial ecology of meat spoilage bacteria is complex and many species or strains can contribute to spoilage. Bacterial contamination occurs during slaughter, cutting and processing at a meat plant. During cold storage, however, only a minor part of the initial microbiota is able to survive and grow and eventually cause spoilage. Interactions between different organisms can also affect the growth and spoilage activities of the whole bacterial community. Thus, the first step in understanding spoilage is to characterise the microbiota associated with meat and the meat processing environment. Taxonomy is a discipline associated with the nomenclature and classification of novel organisms. After species level identification of the organisms and naming the novel species, the relevance of each bacterial group in spoilage can be evaluated. Inoculation studies and measurements of metabolic compounds associated with spoilage are useful in evaluating the spoilage potential of strains isolated from late shelf-life meat. Reliable and reproducible culture-based and culture-independent methods are needed in detecting, identifying and characterising isolates as well as whole microbial populations. Investigation of the population structure of the major spoilage organisms can shed light on the evolution of these organisms and the possible existence of genotypes with high spoilage potential in certain food matrixes or high competitiveness in the production environment.

Refrigeration temperatures and packaging under a low-oxygen or high carbon dioxide atmosphere favours the growth of psychrotrophic lactic acid bacteria (LAB) (Nychas *et al.,* 2008). LAB are sometimes considered beneficial in foods and can be used as starters producing desirable flavour and texture, or protective cultures preventing the growth of pathogenic or fast-growing spoilage bacteria (Fadda *et al*., 2010). However, many LAB have been recognized as major spoilage organisms of packaged meat and meat products.

In previous studies by our group, we have shown that ribotyping is a valuable tool in species-level identification within many genera of LAB (Koort *et al.*, 2006, Lyhs *et al.*, 2004, Björkroth *et al.*, 1996a). A novel *Leuconostoc* species, *L. gasicomitatum* was described and shown to cause spoilage of a variety of MAP meat products (Vihavainen and Björkroth, 2007, Björkroth *et al*., 2000). A total of 384 *L. gasicomitatum* isolates from meat and vegetable sources were characterised by pulsed field electrophoresis (PFGE) typing and major meat- and vegetable-associated genotypes were identified (Vihavainen and Björkroth, 2009). During investigations of LAB in meat and at meat processing plants, several groups of bacteria were isolated that possessed similar ribopatterns, but remained unidentified in the numerical analysis of ribopatterns in comparison with LAB type and reference strains.

The purpose of the thesis was to resolve the taxonomic status of the unknown bacterial isolates and to produce novel data on the LAB associated with the manufacture of meat products. The aim was also to evaluate the usefulness of numerical analysis of ribopatterns, and/or multilocus sequence analysis of several housekeeping genes in the species/subspecies level identification of enterococci and lactococci. The fully sequenced genome of the type strain *L. gasicomitatum* LMG 18811 was utilised to establish a multilocus sequence typing (MLST) scheme for the species and the MLST data was used to evaluate the population structure of *L. gasicomitatum*.

2 REVIEW OF THE LITERATURE

2.1 MICROBIAL TAXONOMY AND PROKARYOTIC SPECIES CONCEPT

Taxonomy is a discipline that encompasses the description, identification, nomenclature and classification of organisms. Taxonomy provides a framework for the scientific community and society to understand and share knowledge about living organisms. The history of microbial taxonomy began in the late 18th century, when microscopy and the ability to cultivate micro-organisms enabled classification based first on cell morphology and later on physiological characteristics (Rossello-Mora and Amann 2001). Since then, the field has continued to develop concurrently with technological and biological innovations. The discovery of DNA in the mid-20th century finally led to the idea that microbes could be classified based on their genomic contents (Rossello-Mora and Amann 2001). The overall genomic base composition (G+C %) and DNA-DNA hybridisation became the golden standard in microbial taxonomy already in the 1970s, followed by rRNA sequence analysis (Brenner *et al*., 1969, Fox *et al*., 1977). The development of next generation sequencing technologies in the 21st century has provided scientists with the possibility to sequence the whole genome of a microbe at lower costs and in less time.

The classification system, as well as the binomial nomenclature founded by Linnaeus, was adapted to the prokaryotic taxonomy from the eukaryotic world. In microbiology, however, the concept of a species is still not clear. A common definition describes bacterial species as “a group of strains that show a high degree of overall similarity and differ considerably from related strain groups with respect to many independent characteristics” (Colwell *et al*., 1995). Horizontal gene transfers pose a major challenge for prokaryotic taxonomy and have led some scientists to doubt whether such a thing as bacterial species actually exists (Doolittle and Papke 2006). The current recommendation for bacterial species circumscription by *ad hoc* committee for the re-evaluation of the species definition in bacteriology applies a polyphasic approach and defines a species as a group of strains with more than 97% rRNA sequence similarity (nowadays 98.7% similarity; Stackebrandt and Ebers 2006) and approximately 70% or greater DNA-DNA relatedness and/or 5°C or less ΔTm, and can be differentiated from the closest phylogenetic relatives by one or more phenotypic characteristic (Wayne *et al*., 1987). This pragmatic definition is universally applicable and widely accepted by microbiologists as the basis for classification in spite of the commonly acknowledged pitfalls of the methods (Rossello-Mora, 2012). In recent years, the quest for methods that could substitute the outdated DNA-DNA hybridisation has been successful. Multilocus sequence analysis

(MLSA), which uses several housekeeping genes as molecular markers, provides substantially higher resolution than 16S rRNA gene sequence analysis and is easily applicable (Martens *et al*., 2008). The average nucleotide identity (ANI) of the shared genes between two strains is the parameter that will most probably replace DNA-DNA hybridisation in the near future and hopefully advance the current species definition for prokaryotes (Rossello-Mora, 2012, Konstantinidis and Tiedje, 2004).

2.2 TAXONOMY AND HABITATS OF COCCAL LAB FROM GENERA *ENTEROCOCCUS*, *LACTOCOCCUS* AND *LEUCONOSTOC*

Enterococci, lactococci and leuconostocs are all Gram-positive, catalase-negative, facultatively anaerobic, coccal LAB. Phylogenetically LAB belong to class *Bacilli* and order *Lactobacillales* of phylum *Firmicutes.* Fig. 1 shows the phylogenetic position of the genera *Enterococcus*, *Lactococcus* and *Leuconostoc* within LAB. All LAB exhibit DNA G+C content of less than 50 mol% and produce lactate as the main product of carbohydrate metabolism. In addition to the genera *Enterococcus*, *Lactococcus* and *Leuconostoc,* the LAB of importance in foods belong to the genera *Carnobacterium, Lactobacillus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Doyle *et al.,* 2013).

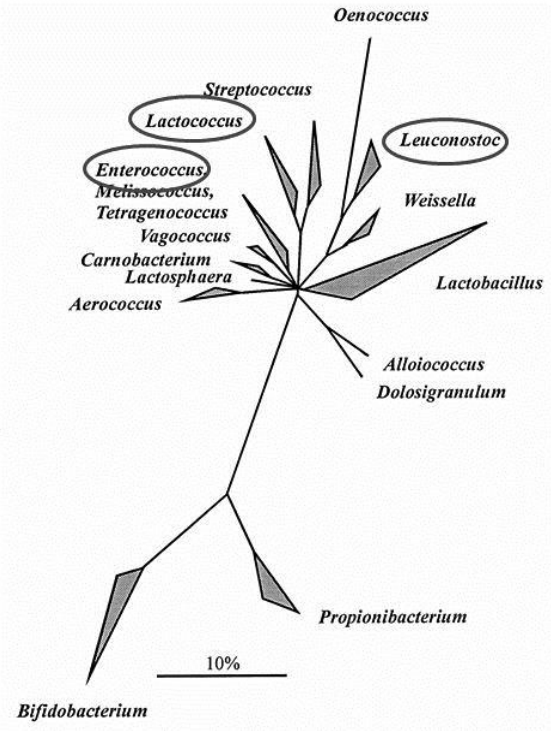

Fig. 1. The position of genera *Enterococcus*, *Lactococcus* and *Leuconostoc* in the phylogenetic tree of lactic acid bacteria based on 16S rRNA gene sequences. (adapted from Holzapfel et al., 2001).

2.2.1 GENUS *ENTEROCOCCUS*

The genus was described in 1984, when Schleifer and Kilpper-Bältz (1984) proposed that the species *Streptococcus faecalis* and *Streptococcus faecium* should be transferred to a novel genus *Enterococcus*. Enterococci are actually phylogenetically more closely related to the genera *Vagococcus*, *Carnobacterium* and *Tetragenococcus* than species presently comprising the genus *Streptococcus* (Fig. 1). During the past ten years, the genus has expanded and 54 *Enterococcus* species are currently recognised (Euzeby, 1997; latest full update 7 November 2014). Based on 16S rRNA gene sequence analysis, several phylogenetic groups have been distinguished (*Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus dispar*, *Enterococcus saccharolyticus* and *Enterococcus cecorum* species groups) (Klein, 2003, Williams *et al*., 1991).

E. faecium and *E. faecalis* are the most frequently found intestinal enterococci in humans and many animals, and these species are notorious nosocomial pathogens with both intrinsic and acquired resistance to antibiotics (Devriese *et al.,* 2006). Some species, such as *E. mundtii* and *E. casseliflavus*, are clearly plant-associated, whereas the habitat of the species in the *E. avium* group is largely unknown (Devriese *et al.,* 2006, Klein, 2003). Despite their pathogenic features, enterococci are also present in artisanally fermented foods, as well as used as probiotics (Moreno *et al.,* 2006).

2.2.2 GENUS *LACTOCOCCUS*

Schleifer *et al.,* (1985) continued revision of the taxonomy of catalase-negative, facultatively anaerobic, Gram-positive cocci by proposing that the lactic streptococci of *Lancefield* group N should be classified in a new genus, *Lactococcus*. This genus currently comprises two phylogenetic groups: species *Lactococcus lactis* (*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *tructae*), *Lactococcus taiwanensis*, *Lactococcus fujiensis*, *Lactococcus formosensis* and *Lactococcus garvieae* are clearly separated from the closely related species *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis* and *Lactococcus chungangensis* (Euzeby, 1997). Lactococci belong to the family *Streptococaceae* and are closely related to species in the genus *Streptococcus* (Fig. 1).

Species of the genus *Lactococcus* are commonly present in various fermented foods, the dairy environment and in plant and animal sources, but usually not in faecal material or soil (Teuber and Geis, 2006). Plant material is most probably the original habitat of lactococci and the adaptation from a plant to a dairy environment is a more recent event (Siezen *et al.,* 2008). *L. lactis* has been used for decades as a model organism for gram-positive bacteria and has thus been extensively studied, whereas the other species of the genus have received less attention. *L. piscium* was described by Williams

et al., (1990) more than 20 years ago, but the main habitat of the species has remained unknown.

2.2.3 GENUS *LEUCONOSTOC*

The type species of the genus, *Leuconostoc mesenteroides*, was among the first bacteria described (van Tieghem, 1878). After several taxonomic revisions (Endo & Okada 2008, Dicks *et al.,* 1995, Collins *et al.,* 1993), the genus *Leuconostoc* currently comprises 14 species (Euzeby, 1997). Based on 16S rRNA gene-based phylogeny, the species in the genus are divided into three evolutionary branches: *L. mesenteroides*, *Leuconostoc lactis* and *Leuconostoc gelidum* species groups. *Leuconostoc fallax* is phylogenetically distant from the other leuconostocs. The most closely related genera are *Fructobacillus*, *Weissella* and *Oenococcus*, which all belong to the family *Leuconostocaceae* (Fig. 1).

Leuconostocs are commonly found in decaying plant material, which is probably their natural habitat, as well as in meat, dairy foods and in various fermented foods (Björkroth and Holzapfel 2006). Except *Leuconostoc kimchii*, species in the *L. gelidum* group can grow at chilled temperatures and thus thrive in cold-stored foods and eventually cause spoilage (Björkroth and Holzapfel 2006). Leuconostocs can occasionally cause infections in immunocompromised humans (Deng *et al* 2012).

2.3 LAB IN MEAT AND THE MEAT PROCESSING ENVIRONMENT

LAB are nutritionally fastidious and require external sources of several amino acids and vitamins. Meat is rich in nutrients and water, has near-neutral pH and thus provides an excellent medium for the growth of LAB and other bacteria. Meat processing plants, however, are harsh niches, where only few bacterial species are able to survive.

2.3.1 LAB SPECIES IN MEAT AND MEAT PRODUCTS

The initial microbial contamination of meat occurs at the slaughterhouse and meat processing plant. LAB often form only a minor part of the initial microbiota of fresh meat, whereas bacteria from the genera *Acinetobacter*, *Brochothrix*, *Flavobacterium*, *Pseudomonas*, *Psychrobacter*, *Moraxella*, *Staphlycoccus*, *Micrococcus* and family *Enterobacteriaceae* usually dominate (Chaillou *et al.,* 2014, Doulgeraki *et al.,* 2012). Microbiota originating from the skin and gastro-intestinal tract of slaughter animals (species belonging to genera *Lactobacillus*, *Enterococcus*, *Clostridium*, *Corynebacterium*, *Propionibacterium*, and *Streptococcus*) were found to be less common in fresh meat than microbes originating from environmental reservoirs (species

belonging to the genera *Acinetobacter*, *Pseudomonas*, *Vagococcus*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, and *Brochothrix*) (Chaillou *et al.,* 2014). The latter group mainly consisted of psychrotrophic bacteria, whereas the bacteria originating from animals are mesophils.

During storage, the microbial community in meat undergoes a selection process and only a small fraction of the initial microbiota survives until the end of the shelf-life, even though the number of microbes rises exponentially. The bacterial richness in meat and meat products was shown to decrease circa 10-fold when fresh and spoiled samples were studied by pyrosequencing (Chaillou *et al.,* 2014). Species composition of the microbiota at the end of the shelf-life/at the time of spoilage depends on the composition of the initial contamination and the storage conditions, primarily storage temperature and the atmosphere in the package. Vacuum and modified atmosphere packaging and cold-storage favours the dominance of psychrotrophic LAB, and occasionally *Brochothrix thermospacta* and clostridia, whereas aerobic storage favours faster-growing, gram-negative organisms such as *Pseudomonas* spp. (Chaillou *et al*., 2014, Nychas and Skandamis 2005). In meat products, the shift in the microbiota from mainly Gram-negative to Gram-positive bacteria, mostly LAB, can occur after grinding and the addition of additives such as salt and nitrite (Samelis *et al*., 1998).

LAB from the genera *Carnobacterium*, *Enterococcus, Lactobacillus*, *Leuconostoc* and *Weissella* prevail in fresh meat and meat products, whereas, until recently, lactococci have only rarely been detected (Björkroth *et al.,* 2005, Champomier-Verges *et al.,* 2001). Table 1 shows the LAB species associated with packaged, late shelf-life meat. Many psychrotrophic LAB species have been overlooked in spoilage studies due to implementation of mesophilic plate counting methods or the growth medium (Pothakos *et al*., 2012, Ercolini *et al*., 2009). Recently, studies implementing novel high-throughput sequencing as well as psychrotrophic plate counting methods have shown the high prevalence of LAB species such as *Leuconostoc gelidum* and *Lactococcus piscium* in late shelf-life meat and meat products (Pothakos *et al*., 2014a, 2014b).

Within the genus *Leuconostoc*, *L. carnosum* and *L. mesenteroides*, in addition to *L. gelidum* subsp. *gasicomitatum* and *gelidum,* are common organisms in beef, pork, poultry and minced meat, as well as in processed meat products at the end of their shelf-life (Pothakos *et al.,* 2014b, Nieminen *et al*., 2011, Doulgeraki *et al.,* 2010, Schirmer *et al.,* 2009, Yang *et al*., 2009, Sakala *et al.,* 2002b, Samelis *et al.,* 2000). *L. gelidum* subsp*. gasicomitatum* was originally isolated from spoiled, marinated broiler fillet (Björkroth *et al*., 2000) and has since been detected as the dominant spoilage organism in MAP beefsteaks (Vihavainen and Björkroth, 2007), as well as in cooked meat products and several vegetable products (Pothakos *et al*., 2014a, 2014b, Vihavainen *et al.*, 2008). *L. gelidum* subsp*. gasicomitatum* is able to respire and thus improve growth and stress resistance in high-oxygen MAP

meats (Jääskeläinen *et al*., 2013, Johansson *et al*., 2011). The ability of *Leuconostoc* species in the *L. gelidum* group to grow at chilled temperatures partly explains their competitiveness in cold-stored meats (Björkroth and Holzapfel, 2006).

Lactococci, more precisely species *L. piscium* and *L. raffinolactis*, have increasingly been detected in late shelf-life meat (Xiao *et al*., 2013, Nieminen *et al*., 2012, 2011, Penacchia *et al.,* 2011, Jiang *et al.,* 2010, Sakala *et al*., 2002a, Barakat *et al*., 2000). *L. raffinolactis* and *L. piscium* formed part of the predominant microbiota in cooked, MAP poultry and vacuum packaged beef, respectively (Sakala *et al.,* 2002a, Barakat *et al.,* 2000). *L. piscium* also dominated in late shelf-life of a raw meat product in Belgium (Pothakos *et al*., 2014a). Lactococci may have earlier been overlooked in meat due to the use of mesophilic plating techniques and lack of identification methods, and the spoilage potential of these bacteria is still scarcely known.

Carnobacteria and lactobacilli, mostly the species *Carnobacterium piscicola*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens*, *Lactobacillus sakei, Lactobacillus algidus* and *Lactobacillus curvatus*, are often found within the predominant microbiota of packaged meat at the end of shelf-life (Liang *et al.,* 2012, Nieminen *et al*., 2012, Ercolini *et al*., 2011, 2009, Doulgeraki *et al*., 2010, Jiang *et al*., 2010, Schirmer *et al*., 2009, Yost and Nattress, 2002). Lactobacilli and leuconostocs are considered highly competitive in meat, whereas carnobacteria are less tolerant to low pH and can be overgrown during storage (Yang *et al*., 2009, Leisner *et al*., 2007). *C. divergens*, however, has been detected as the dominant organism in aerobically stored, vacuum-packaged, and antimicrobially packaged beef at all stages of storage (Ercolini *et al.,* 2011, Penacchia *et al*., 2011). *Weissella viridescens* is often associated with other LAB such as lactobacilli and leuconostocs when growing in late shelf-life meat (Han *et al*., 2011, Samelis *et al*., 2000).

Enterococci are commonly found in fresh meat at the beginning of storage. This may either indicate hygiene problems in meat slaughtering and processing or concern due to the antibiotic resistance of these organisms (Hammerum, 2012, Moreno *et al.*, 2006). During storage, enterococci are usually overgrown by other, more competitive bacteria and are thus not very likely to cause spoilage (Björkroth *et al.*, 2005). However, there are few reports on the association of enterococci, notably *E. faecalis* and *E. faecium*, with the spoilage of meat products (Vasilopoulos *et al*., 2008, Foulquié-Moreno *et al*., 2006).

2.3.2 LAB IN THE MEAT PROCESSING ENVIRONMENT

Since fresh meat from a healthy animal is sterile, LAB contamination of meat occurs at the slaughterhouse and the meat processing plant. It is currently unknown how LAB enter the plant: animal hides, silage, airflows and employers are suggested to be possible carriers (De Filippis *et al*., 2013,

Vihavainen *et al*., 2007, Björkroth and Korkeala 1997). Psychrotrophic spoilage LAB such as leuconostocs or *Lactococcus piscium* are not common habitants of the gastro-intestinal tract of warm-blooded animals and are thus likely to originate from environmental reservoirs. *Leuconostoc* contamination in a poultry processing plant was shown to spread via the air, whereas these spoilage bacteria were not detected in the skin or feathers of the birds entering the plant (Vihavainen *et al*., 2007). In a vegetable production environment, spoilage-causing leuconostocs were isolated from the air of the plant and few harbourage sites in the premises prior to production (Pothakos *et al*., 2014c). Contamination was estimated to mostly originate from the constant introduction of these organisms into the plant.

After entering the chilled processing environment, LAB are able to survive and spread via surfaces, air or personnel (Vasilopoulos *et al*., 2010, Vihavainen *et al*., 2007, Samelis *et al*., 1998, Björkroth and Korkeala 1997). The microbiota of a meat processing environment is highly complex, with LAB representing only a minor element (De Filippis *et al*., 2013, Hultman *et al*., 2015). LAB can, however, prevail in slicing or grinding and packaging devices, and contaminate meat and meat products during processing (Vasilopoulos *et al*., 2010). LAB, with the exception of enterococci, are generally not very resistant to heat and disinfection, and survival of these microbes in a harsh processing plant environment evokes many questions. The ability of spoilage strains to adhere to surfaces and form biofilms may contribute to their survival (Giaouris *et al*., 2014, Johansson *et al*., 2011). Within *L. gelidum* subsp. *gasicomitatum*, the ability to attach to surfaces was shown to vary remarkably among the strains studied (Pothakos *et al*., 2015). Good hygiene practices are essential in meat processing plants to reduce the amounts of LAB and other spoilage organisms, and thus minimise the risk of early spoilage.

2.3.3 LAB SPOILAGE OF MEAT

Spoilage is defined as the deterioration of original nutritional value, texture, and/or flavour of food that makes it unfit for human consumption. Microbial activity, as well as autolytic enzymatic reactions and lipid oxidation, can contribute to the spoilage of food, although microbial action is considered to precede the latter. Only the microbiota that survives until the end of storage is considered as the main cause of spoilage and is called ephemeral/specific spoilage organisms (E(S)SO) (*Nychas et al*., 2008). The spoilage process, however, consists of complex interactions between bacteria, the food and the environment, and is not fully elucidated.

Spoilage potential is the quantitative ability of a micro-organism to produce metabolites that are associated with the spoilage of a particular product (Ellis and Goodacre, 2006). Spoilage potential can vary within strains representing the same species, which seems to be the case for e.g. *L. piscium* (Pothakos *et al*., 2014d). However, within species such as *L.*

gelidum and *Brochothrix thermospacta*, all strains can be considered as spoilage organisms. LAB cause food spoilage when extrinsic and/or intrinsic factors prevent the growth of fast-growing, gram-negative bacteria. In addition to vacuum and modified atmosphere packaging, low pH and low temperature, as well as the addition of sugar, salt or nitrite, are factors that the food industry uses to extend the shelf-life of food and at the same time these factors favour the growth of LAB.

The LAB species associated with spoilage hitherto belong to the genera *Carnobacterium, Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc,* and *Weissella* (Table 1.). LAB spoilage of meat and meat products is often associated with off-odours and flavours that are described as sour, acid, buttery or cheesy (Schirmer *et al.,* 2009, Diez *et al.,* 2008, Vihavainen and Björkroth, 2007, Holley *et al*., 2004, Susiluoto *et al.,* 2003, Björkroth *et al.,* 1998). These unpleasant changes are the result of the metabolism of SSO when utilising the substrates available in meat. The metabolic activities of bacteria are species or even strain specific (Ercolini *et al.,* 2011, Vihavainen and Björkroth, 2007).

LAB can utilise at least glucose, glucose-6-P, ribose, lactate, nucleosides and amino acids (Casaburi *et al*., 2015, Jääskeläinen *et al*., 2014). Some spoilage LAB, such as leuconostocs, *Weissella* spp. and *Carnobacteria* spp., are obligatory heterofermentative producing lactic acid, acetic acid, CO_2 and ethanol. Leuconostocs also co-metabolise citrate and carbohydrate to diacetyl, CO_2 and acetoin under reducing conditions. *L. gelidum* subsp. *gasicomitatum* produced significant amounts of diacetyl and acetoin when growing on citrate-including media with inosine or ribose, whereas no production of these buttery odour compounds was detected with glucose (Jääskeläinen *et al*., 2014). *L. gelidum* subsp. *gasicomitatum* is able to respire in the presence of exogenous heme and oxygen, and thus increase the growth and production of acetoin and diacetyl (Jääskeläinen *et al*., 2013). Facultatively heterofermentative LAB, such as *Lactobacillus sakei*, produce lactate from glucose, but are also able to utilise pentoses via the phosphoketolase pathway. Lactococci and enterococci are considered to mainly ferment glucose to lactic acid via the Embden-Meyerhof pathway. Most lactococci, however, possess genes for the phosphoketolase pathway in their genomes (Andrevskaya *et al.* 2015). Production of acetic acid, butanoic acid, acetoin and diacetyl are often associated with sensorial changes of meat (Casaburi *et al*., 2015, Jääskeläinen *et al*., 2013, Ercolini *et al.,* 2011, Vihavainen and Björkroth, 2007). The odour of acetoin and diacetyl is described as buttery creamy, whereas acetic acid and butanoic acid give meat an acetic aroma, respectively (Casaburi *et al*., 2015).

LAB, especially lactobacilli and leuconostocs, can also cause discoloration such as greening of meat, swelling of the package due to gas (mostly CO_2) production, or slime formation, especially in cooked meat products (Vihavainen and Björkroth, 2007, Björkroth et al., 2000, Samelis et

Table 1. LAB species associated with packaged, late shelf-life meat.

Species	Type of meat	Reference
Carnobacterium	VP/MAP beef	Ercolini *et al*. 2011
divergens/ maltaromaticum	VP beef	Penacchia *et al.* 2011
	MAP minced meat	Nieminen *et al*. 2011
	Marinated pork	Schirmer *et al.* 2009
	Cooked ham	Vasilopoulos *et al*. 2008
Enterococcus faecalis	Cooked ham	Vasilopoulos *et al.* 2008
Lactobacillus algidus	Fresh meat products	Pothakos *et al.* 2014a
	MAP minced meat	Nieminen *et al.* 2011
	Marinated pork	Schirmer *et al.* 2009
	VP beef	Kato *et al.* 2000
Lactobacillus fuchuensis	Fresh meat products	Pothakos *et al*. 2014a
	VP beef	Sakala *et al.* 2002b
Lactobacillus curvatus/*sakei*	MAP minced beef	Doulgeraki *et al.* 2010
	VP beef	Ercolini *et al.* 2011
	Marinated pork	Schirmer *et al*. 2009
Lactococcus spp.	VP beef	Ercolini *et al.* 2011
	MAP minced meat	Nieminen *et al.* 2011
Lactococcus piscium	Raw meat products	Pothakos *et al.* 2014a
	VP beef	Sakala *et al.* 2002a
Leuconostoc spp.	MAP beef	Doulgeraki *et al.* 2010
Leuconostoc carnosum	Cooked meat products	Pothakos *et al*. 2014a
	Marinated pork	Schirmer *et al*. 2009
	Cooked ham	Vasilopoulos *et al.* 2008
	Cooked ham	Samelis *et al*. 2006
	Cooked ham	Björkroth *et al.* 1998
Leuconostoc gelidum subsp.	Cooked turkey slice	Pothakos *et al*. 2014a
gasicomitatum /gelidum	Cooked meat products	Pothakos *et al*. 2014a
	MAP minced meat	Nieminen *et al*. 2011
	MAP beef	Vihavainen *et al*. 2007b
	MAP marinated broiler	Björkroth *et al.* 2000
Leuconostoc inhae	Cooked turkey slice	Pothakos *et al.* 2014a
Leuconostoc mesenteroides	VP beef	Yang et al. 2009
Weissella spp.	Cooked turkey slice	Pothakos *et al*. 2014a
	MAP minced meat	Nieminen *et al.* 2011
	MAP beef	Ercolini *et al.* 2011

al., 2000, Eagan et al., 1989). Greening is caused by hydrogen peroxide produced by certain LAB strains in the presence of oxygen reacting with myoglobin in meat, whereas slime is extracellular polysaccharide synthesised from carbohydrates present in meat (Vihavainen et al., 2008, Vihavainen and Björkroth, 2007). Accumulation of lactic acid results in a decrease in pH and decreased water-holding capacity and thus cloudy liquid in the meat package.

2.3.4 THE DUAL ROLE OF LAB IN MEAT

Spoilage caused by LAB occurs more slowly than and is not as offensive as spoilage caused by proteolytic Gram-negative bacteria. Thus, LAB can be used as protective cultures to prevent the growth of other spoilage and pathogenic bacteria in meat and meat products (Koo *et al*., 2012, Jones *et al*., 2008, Hugas *et al*., 2003). The use of LAB in bioprotection is still scarce in fresh meat due to acidification (Vasilopoulos *et al*., 2010). However, LAB are widely used as starters in meat fermentation, where acidification and change in aroma and texture in addition to bioprotection are desirable (Fadda *et al*., 2010, Leroy and Vuyst, 2005). The LAB strains used as protective cultures or in fermentation of meat should be tested for virulence traits, antibiotic resistance and spoilage potential, since these traits are clearly strain dependent (Casaburi *et al*., 2011, Doulgeraki *et al*., 2010, Vasilopoulos *et al*., 2010, Hugas *et al*., 2003). Moreover, inhibition tests should be performed in the food matrix instead of laboratory media, since bacteriocins can lose their bioactivity in meat due to adsorption to fat and protein particles (Leroy and Vuyst, 2005). Because of the strain variation in spoilage potential, a LAB species can be considered as a spoilage organism, a protective organism or an innocuous member of the microbiota of meat (Casaburi *et al*., 2011, Doulgeraki *et al*., 2010, Ercolini *et al*., 2009). *L. piscium*, for instance, is used for bioprotection in seafood, whereas when growing in meat and vegetables certain strains are considered as part of the spoilage association (Pothakos *et al*., 2014b, 2014d, Fall *et al*., 2012). Interactions of micro-organisms also affect the production of spoilage metabolites, which complicates the classification of LAB species/strains as “spoilers” or “non-spoilers” (Ercolini *et al*., 2009).

2.3.5 INTERACTIONS OF LAB DURING GROWTH IN MEAT

In addition to external conditions, interactions between bacteria have an effect on the development of the microbiota on meat during storage (Gram *et al*., 2002). At the time of spoilage, the levels of LAB in packaged meat are often 7 to 8$\log_{10}$ (c.f.u. g^{-1}). During growth, the microbes can influence each other’s growth and metabolism by antagonism, metabiosis or cell-to-cell communication (Gramm *et al*., 2002). LAB antagonise other bacteria by lowering the pH of meat by producing lactic acid and bacteriocins, and by

outcompeting on essential nutrients (Ivey et al., 2013, Qimenez and Dalgaard, 2004). Metabiosis between LAB and *Enterobacteriaceae* in meat has been detected in several studies. Some LAB are able to utilise arginine as an energy source and co-culturing these strains with putrescine-forming *Enterobacteriaceae* results in higher levels of biogenic amines than in monocultures (Borch *et al*., 1996, Dainty *et al*., 1986). Cell-to-cell communication of LAB at the transcriptome and proteome level has been studied in sourdough production and milk fermentation (Herve-Jimenez *et al*., 2009, Di Cagno *et al*., 2007). This type of bacterial interaction probably occurs during succession in meat as well. *Leuconostoc* spp. isolates from MAP-minced meat exhibited autoinducer-2-like activity indicating intra- and interspecies communication (Blana *et al.,* 2011). Modern transcriptomics and proteomics methods provide tools for studying bacterial interactions and hopefully new data on the subject will be available in the near future.

2.4 METHODS FOR IDENTIFICATION, CHARACTERISATION, AND POPULATION STUDIES OF LAB

The classification of LAB was originally based on morphology, sugar fermentation patterns, temperature range of growth and mode of glucose fermentation (Von Wright and Axelsson, 2012). These properties are still used in the differentiation and characterization of LAB, but modern genotypic and sequence-based methods are often needed for species level identification (Michel *et al*., 2007, Naser *et al*., 2005, Facklam and Elliot 1995). The development of high-throughput sequencing methods has significantly reduced the time and money required for whole genome sequencing (WGS) of bacteria and in future, WGS may be considered a routine tool in bacteria identification and characterisation (Köser *et al*., 2012).

2.4.1 PHENOTYPIC METHODS

All LAB are Gram-positive, catalase negative, facultatively anaerobic and non-sporulating (Von Wright and Axelsson, 2012). LAB can be either coccal or rod-shaped; coccal LAB can sometimes be confused with short rod-shaped bacteria such as lactobacilli (Facklam and Elliot, 1995). Enterococci, lactococci and leuconostocs divide in one plain and thus form pairs and eventually chains if the cells remain attached (Facklam and Elliot, 1995). Table 2 shows the “Classical” phenotypic characteristics for each genus of LAB.

Table 2. "Classical" phenotypic characteristics of LAB genera associated with meat. Modified from Axelsson *et al*., 2004.

Genus	Cell shape	CO_2 from glucose	Growth at 10°C	45°C	6,5% NaCl	pH 4,4	pH 9,6
Carnobacterium	rods	-	+	-	ND	ND	-
Lactobacillus	rods	D	D	D	D	D	-
Lactococcus	cocci	-	+	-	-	D	-
Leuconostoc	cocci	+	+	-	D	D	-
Enterococcus	cocci	-	+	D	+	+	+
Weissella	rods/cocci	+	+	-	D	-	-

D, strain-dependent; ND, not detected

The classical characteristics for distinguishing enterocci from other Gram-positive, catalase negative, facultatively anaerobic cocci include their ability to grow at 10 and 45°C, in 6.5% NaCl, and at pH 9.6, and the presence of Lancefield group D antigen (Devriese *et al*., 1993). However, even genus-level identification can be misleading for the recently-described species in the *E. avium* species group that do not grow at 45°C or react with Lancefield group D antisera (Koort *et al*., 2004, Svec *et al.*, 2001). In addition, species from the genera *Streptococcus, Lactococcus, Leuconostoc, Pediococcus* and *Aerococcus* may give positive results in some of the "classical tests" mentioned above (Devriese *et al*., 1993).

Even though lactococci are phylogenetically closer to streptococci than to enterococci (Fig. 1), they can be confused with enterococci if only phenotypic tests are used for identification (Facklam and Elliott, 1995). Some lactococci, such as *L. garvieae* strains, can grow at 45°C, pH 9.6 and in 6.5% NaCl, and not all strains possess the Lancefield group N antigen (Eldar *et al*., 1999, Facklam and Elliott 1995).

Members of the genus *Leuconoctoc* are resistant to vancomycin, produce gas from glucose, are unable to hydrolyze arginine and produce only D(-) isomer of lactic acid from glucose (Björkroth and Holzapfel, 2006). Distinguishing leuconostocs from weissellas can be challenging and requires several carbohydrate fermentation tests (Björkroth and Holzapfel, 2006).

Differentiation of *Enterococcus, Lactococcus* and *Leuconostoc* species based on phenotypic tests is laborious and of limited use due high strain variation (Michel *et al*., 2007, Björkroth and Holzapfel, 2006, Naser *et al*., 2005, Facklam and Elliott, 1995, Knudtson *et al*., 1992).

LAB were previously thought to lack the cytochromes of the respiratory chain, but recent studies have shown the presence of cytochrome oxidase genes in the genomes of many LAB (Brooijmans *et al*., 2009, Bolotin *et al*., 2001). Many LAB species are able to respire in the presence of heme and thus improve their growth and stress resistance (Johansson *et al*., 2011, Brooijmans *et al*., 2009).

2.4.2 GENOTYPIC METHODS

Among the traditional molecular characterisation techniques, ribotyping has been reported to be a reliable tool for species level identification of lactococci, enterococci and leuconostocs (Lang *et al*., 2001, Svec *et al*., 2001, Björkroth *et al*., 2000, Rodrigues *et al.,* 1991, Hall *et al*., 1992). However, previous studies on lactococci and enterococci have included only a limited number of strains/species and the method has not yet been used to establish species identification libraries in these genera. In ribotyping, genomic DNA is digested, the DNA fragments are separated by electrophoresis, blotted onto a membrane and finally only bands containing rDNA sequence are visualised by hybridisation to a labelled probe. Ribotyping provides high discriminatory power at the species/subspecies level, but is usually not discriminatory enough at the strain level. The discriminatory power of ribotyping can be increased by using multiple restriction enzymes and combining the data using numerical analyses.

Other DNA fingerprinting methods often applied to LAB include pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Ben Amor *et al.,* 2007). PFGE is time-consuming, but highly discriminatory, whereas RAPD is rapid, sensitive and inexpensive, but has low reproducibility. Additional limitations of these genotypic methods are their low cost/time-effectiveness and the fact that before typing the organism must be isolated. However, these methods are still often needed for strain level studies, as well as for proper species level identification for LAB with highly conserved 16S rRNA gene sequences. DNA fingerprinting methods can also be useful in in identifying large numbers of unknown LAB isolates in studies where isolates are picked for further analyses.

2.4.3 GENE-BASED APPROACHES

Contrary to genotype-based methods, gene-based approaches provide evolutionary data on the bacteria studied. Sequence analysis of single or multiple genes has been widely applied to bacterial taxonomy since the 1970s, when in his pioneer work, Carl Woese showed that 16S rRNA sequence is a useful phylogenetic marker present throughout the prokaryotic world (Woese and Fox, 1977). The 16S rRNA gene is highly conserved, but also contains variable regions with species-specific signature sequences. Public databases provide an enormous amount of 16S rRNA gene sequence data and also quality-controlled data is available in several databases (McDonald *et al*., 2012, Pruesse *et al*., 2007, DeSantis *et al*., 2006). However, the discriminatory power of 16S rRNA sequence is too low for species level identification in some bacterial groups: e.g. for species within *Enterococcus avium* and *Leuconostoc gelidum* species groups (Svec *et al*., 2005, Björkroth *et al*., 2000, Patel *et al*., 1998, Williams *et al*., 1991). For

newly described taxa, 16s rRNA sequence data is still required (Stackebrandt *et al*., 2002).

Recently, the usefulness of protein coding housekeeping genes in bacterial taxonomy and phylogeny has been recognised. To obtain informative data, the genes chosen for sequence analysis should be under stabilising selection, located at diverse chromosomal loci and widely present among taxa (Stackebrandt *et al*., 2002). In multilocus sequence analysis (MLSA), sequences of internal fragments of several (typically three to eight) housekeeping genes are concatenated and the sequence data are used to delineate microbial species or to assess the phylogenetic position of the strains studied. MLSA is suitable for studying bacterial relationships at a wide range of evolutionary distances, from intraspecies to the genus level (Gevers *et al*., 2005). The ad hoc committee for re-evaluation of the species definition regarded MLSA as a method of great promise for prokaryotic systematics (Stackebrandt *et al*., 2002).

Within LAB, MLSA has been successfully used in the species delineation of enterococci, lactobacilli and lactococci (Rademaker *et al*., 2007, Naser *et al*., 2007, 2005). Sequence analysis of DNA-directed RNA polymerase subunit A (*rpoA*) and phenylalanyl tRNA synthetase α chain (*pheS*) genes has been shown to differentiate species of enterococci and lactobacilli, but to our knowledge there are no reports on the suitability of these genes for species level identification of lactococci (Naser *et al*., 2007, 2005). Instead, Perez *et al*., (2011) showed that DNA-directed RNA polymerase subunit B (*rpoB*) and DNA recombination protein (*recA*) genes are highly useful in in identifying lactococci at the species level.

2.4.4 MLST

MLST is a typing scheme based on the DNA sequence of typically four to ten loci in a bacterial genome to identify and classify bacterial strains, and to assess population genetics and epidemiology of the species. Contrary to MLSA, most downstream analyses are based on sequence types (STs) assigned by allele numbers of the loci: each unique allele is given an arbitrary number and strains that share alleles at all loci represent the same ST (Maiden *et al*., 1998). Thus, both point mutation and recombination are considered as one genetic event. The latter mechanism often poses a problem when attempting to infer ancestral relationships of bacterial strains, since in recombination several nucleotides change at once. Recombination events are thus overweighted compared to point mutations when applying sequence-based approaches without the ability to recognise sequences gained by this mechanism.

ST designations can be used in definitions of strains or in population genetic approaches by grouping STs into groups with common ancestral origin. The relationships between STs that differ at more than three out of seven loci are likely to be unreliable (Enright and Spratt, 1999). eBurst (Feil

et al., 2004) is a commonly used algorithm which divides MLST datasets into groups of related isolates and clonal complexes (CC). eBurst relies on the model according to which a founding genotype first multiplies within the population and then gradually diversifies into single-locus variants (SLV), double-locus variants (DLV) and triple-locus variants (TLV). eBurst subdivides STs into groups, recognises the founding genotypes, assigns levels of confidence in these primary founders and displays the most parsimonious patterns of descent of STs within each clonal complex from the primary founder. eBurst only shows the relationships of strains that have diverged very recently and is mostly suited for exploratory data analysis rather than exact inference of population structure.

Bayesian models infer the population structure using sequence data instead of allele numbers. Bayesian analysis of population structure (BAPS) (Corander *et al.,* 2003) divides the population into subgroups based on sufficiently similar nucleotide frequencies and infers the level of genetic admixture between the subgroups. ClonalFrame (Didelot and Falush, 2006) is another common Bayesian-based method to assess the clonal relationships of bacteria, to estimate the frequency of recombination and mutation, and to predict the age of the common ancestor. Bayesian-based methods are able to predict whether changes in sequence result from recombination or mutation and are thus more accurate than traditional phylogenetic methods in estimating bacterial genealogies.

MLST is typically applied to typing strains within one species. Even within genera, it is often necessary to develop multiple MLST schemes since housekeeping genes vary among bacterial species/genera. However, since a small number of housekeeping genes only represent a fraction of the genome of an organism, they can only provide a limited insight into the bacterial evolution. Owing to rapidly developing next generation sequencing technology, the MLST approach can be amended by utilising the genes encoding ribosomal proteins (ribosomal MLST, rMLST) or even the whole genome sequence data (whole-genome multilocus typing, wgMLST) (Maiden *et al*., 2013). Whole genome sequence data as a basis for either allele-based or sequence-based approaches will probably replace the “traditional” MLST in the future. This, however, requires the development of model-based statistical analysis approaches such as BAPS and ClonalFrame for the analysis of these enormous datasets.

Within the genus *Leuconostoc*, MLST has previously been applied only to the species *Leuconostoc lactis* (Dan *et al*., 2013). MLST analyses revealed that the *L. lactis* population studied was highly clonal, with indication of genetic exchange only within the subpopulations. Genomes of leuconostocs are known to contain several restriction modification systems, which can limit the genetic exchange and may explain the clonal population structure (Roberts *et al*., 2013, Johansson *et al*., 2011).

2.4.5 WHOLE GENOME SEQUENCING

Genome analysis and comparison provide insights into the metabolic potential, characteristics and evolution of LAB (Pfeiler and Klaenhammer, 2007, Siezen *et al*., 2004). The falling costs and less time for whole genome sequencing (WGS) have already resulted in the application of this method in diagnostic microbiology and surveillance (Grad *et al*., 2011, Rasko *et al*., 2011). Whole genome sequences are also useful in functional genomics studies for mapping the RNA sequence reads (Sorek and Cossart, 2010). WGS can be considered as the ultimate source of information and complete, closed genome sequences as permanent, valuable scientific resources (Fraser et al., 2002). In genomic studies of spoilage bacteria, identifying metabolic pathways/genes associated with spoilage reactions is essential, as is functional analyses utilising cloning techniques, transcriptomics and metabolomics (Remenant *et al*., 2015).

Comparative genomics of fully-sequenced LAB genomes have revealed that the genomes of these organisms are relatively small, between 1.8 to 3.3 Mb, with the number of genes in the range of 1200 to 3000 (Makarova and Koonin 2007, Pfeiler and Klaenhammer 2007). Characteristic for the divergence of *Lactobacillales* from their ancestor *Bacilli* was substantial loss of genes, including genes for biosynthetic enzymes and for sporulation, due to adaptation to more nutrient-rich environments (Makarova and Koonin, 2007, Pfeiler and Klaenhammer, 2007). The majority of the genome sequences used in these comparative genomics studies represented the genus *Lactobacillus*, whereas only one *Leuconostoc* and a few *Lactococcus* genomes were included (Makarova and Koonin, 2007, Pfeiler and Klaenhammer, 2007). Within the genus *Lactococcus*, whole genome sequences are only available for strains of the species *L. lactis* and recently, *L. garvieae*, whereas the genome of *L. piscium* is still lacking (Ricci *et al*., 2013, Ainsworth *et al*., 2013, Kato *et al*., 2012, Ricci *et al*., 2012, Gao *et al*., 2011, Siezen et al., 2010, Wegmann *et al*., 2007, Bolotin *et al*., 2001). Within the genus *Leuconostoc* species relevant in meat environment, the genomes of *L. gasicomitatum* and *L. gelidum* have recently been published (Jung *et al*., 2012, Johansson *et al*., 2011). The genome of *L. gasicomitatum* possessed genes required for the utilisation of ribose, external nucleotides, nucleosides and nucleobases, which all are abundant in meat. The pathways/genes associated with buttery off-odour, greening of meat and slime formation were recognised, as well as genes associated with platelet binding and collagen adhesion (Johansson *et al*., 2011). The growing number of fully-sequenced genomes of LAB will provide a basis for more comprehensive genomic studies in the future.

2.4.6 HIGH-THROUGHPUT SEQUENCING APPROACHES

The first culture-independent methods for studying microbial communities were denaturing gradient gel electrophoresis (DGGE), terminal restriction

fragment length polymorphism (T-RFLP) and DNA microarrays (Ben Amor *et al.,* 2007). The low sensitivity in detecting rare members of the community, as well as the low discriminatory power, lack of quantitative data and low sample throughput are the disadvantages of both DGGE and T-RFLP, and the methods are most useful in comparing community structural changes (Nieminen *et al.,* 2011, Ben Amor *et al*., 2007, Ercolini 2004, Temmermann *et al.,* 2004). The major limitation of DNA microarrays is that they can only detect species that are known to prevail in the community and for which the probes of the array are targeted (Roh *et al*., 2010). High-throughput sequencing (HTS), including pyrosequencing (454 Life Sciences, Inc.) provides cost-effective, rapid sequencing of high numbers of DNA from complex samples and has mostly replaced other approaches (Roh *et al*., 2010). The most important feature of HTS is the ability to discover novel gene diversity without previous knowledge of the microbial community studied (Roh *et al*., 2010). In addition, HTS analysis is considered quantitative, even though nucleic acid extraction and PCR steps can alter the proportion of the micro-organisms and thus bias the results (Ercolini *et al*., 2013).

Pyrosequencing of short hypervariable regions of SSU rRNA was first used to characterise microbial diversity in the deep sea (Sogin *et al.,* 2006). Following the advances in environmental microbiology, rRNA amplicon sequencing has been applied to study the microbial ecology of food, mostly food fermentation (Alegria *et al*., 2012, Jung *et al*., 2012, Kim *et al*., 2011, Sakamoto et al., 2011, Humblot and Guyot, 2009). In food spoilage research, Ercolini *et al*., (2011) studied the changes in the microbiota of beef during storage in different atmospheres by pyrosequencing and showed that the changes in microbiota of the meat resulted in complex shifts in the metabolites produced. De Filippis *et al*., (2013) studied the microbial diversity of beefsteaks and the sources of spoilage bacteria by examining samples from beef, carcasses and the production plant by pyrosequencing. The carcasses were shown to carry the spoilage microbes to the processing environment, where they became part of the resident microbiota (De Filippis *et al*., 2013).

In rRNA amplicon sequencing, the taxonomic resolution varies depending on the length of the amplicon (150-500 bp), as well as the level of conservation in the rRNA gene within the genus (Ercolini *et al*., 2013). Usually species-level identification, and thus long sequence reads, is required. The reliability of taxonomic assignment also depends on the quality of the reference database against which the sequences are compared and only curated databases should be used (McDonald *et al*., 2012, Pruesse *et al*., 2007, DeSantis *et al*., 2006). Sample coverage should be adjusted to the environment studied and can be determined by rarefaction analysis of sequencing data (Ercolini *et al.,* 2013).

High-throughput sequencing approaches will mostly replace traditional culture-based methods in microbial community studies. However, culture-

based methods are still needed for more detailed studies of individual isolates.

3 AIMS OF THE STUDY

The objectives of the present thesis were to study the taxonomy and diversity of psychrotrophic, coccal LAB associated with meat and meat production.

The specific aims of this thesis were as follows:

1. To resolve the taxonomic status of unknown coccal LAB from meat and the meat processing environment

2. To clarify the taxonomy of *Leuconostoc gelidum* and *Leuconostoc gasicomitatum*

3. To assess the suitability of numerical analysis of ribopatterns in species level identification of lactococci and enterococci associated with meat and meat production

4. To assess the suitability of sequence analysis of two housekeeping genes in identification of species in the genus *Lactococcus*

5. To evaluate the spoilage potential of *Lactococcus* strains isolated from MAP meat

6. To develop an MLST scheme for *Leuconostoc gelidum* subsp. *gasicomitatum* and study the genetic diversity of *L. gelidum* subsp. *gasicomitatum* strains from meat and vegetable sources

4 MATERIALS AND METHODS

4.1 BACTERIAL STRAINS AND CULTURING (I, II, III, IV)

In study I, 36 isolates that were presumptively identified as enterococci based on numerical analysis of *Hin*dIII ribopatterns were picked from previous studies for further identification (Vihavainen *et al*., 2007, Björkroth *et al*., 2005). Strains isolated from the air of a broiler processing facility originated from a study by Vihavainen *et al.,* (2007). They had been plated using Reuter centrifugal air samplers (RCS sampler; Biotest AG, Dreieich, Germany) on a strip of MRS agar (Oxoid, Basingstoke, United Kingdom). Samples from broiler carcasses had been psychrotrophically enriched by incubation in MRS broth at 6°C for 38 days. LAB from MAP broiler products (Vihavainen *et al*., 2007, Björkroth *et al*., 2005) had been isolated using MRS medium and anaerobic incubation at 25°C for 5-6 days.

In study II, 222 strains from MAP meat with similar *Hin*dIII ribopatterns were chosen for further identification (Nieminen *et al*., 2011, Vihavainen *et al*., 2007, Björkroth *et al*., 2005). In addition to the strains isolated during previous studies, further strains were isolated from porcine *Musculus masseter* and MAP turkey. The strains from *Musculus masseter* originated from MAP meat strips cut and packaged in a small-scale plant from fresh meat transported from a slaughterhouse. One-hundred to two-hundred g of pork strips were packaged under modified atmosphere containing 70% O_2 and 30% CO_2, and stored at 6°C for 13 days prior to sampling. The strains from turkey were isolated from retail MAP turkey fillet or fillet strips from one large-scale manufacturer. Packages were stored at 6°C and examined on the use-by day (12 d). Twenty-two g of pork or turkey meat were homogenised with 0.1% peptone water using a Stomacher blender. Serial 10-fold dilutions of the homogenised samples were plated and colonies were randomly selected and picked for further studies. All strains were isolated using MRS medium (Oxoid, Basingstoke, Hampshire, England) or NAP-agar [APT-agar (Merck, Darmstadt, Germany) supplied with sodium nitrite 0.06% wt/vol, actidione (cycloheximide) 0.1% wt/vol and polymyxin-B 0.03% wt/vol] and incubated under anaerobic conditions [Anaerogen (Oxoid); 9-13% CO_2 according to the manufacturer's instructions] at 25°C for 5-6 days.

In study III, 20 LAB strains were isolated from vacuum packaged pork, vacuum packaged turkey and modified atmosphere packaged (MAP) broiler obtained from a local grocery store. The strains were isolated by homogenising 22 g of meat on the sell-by day ± 1 day with 0.1% peptone water and plating 10-fold dilutions on MRS medium at anaerobic conditions at 25°C for 5 days. The strains were chosen for the study based on similar *Hin*dIII ribopatterns. In the numerical analysis of *Hin*dIII ribopatterns, these

strains showed a high level of similarity to *Leuconostoc gasicomitatum* and *Leuconostoc gelidum*, but their taxonomic status remained unclear.

In study IV, 252 strains from our culture collection identified as *L. gelidum* subsp. *gasicomitatum* were chosen based on PFGE types, ribotypes and sources, to study the population structure of the species by MLST. Isolation was performed as described by Vihavainen and Björkroth (2009). The strains were isolated from MAP poultry, pork, beef and lamb, and salad, carrots and a fish product containing vegetables. Most strains were from Finnish products, but a few strains were from products imported from Estonia, Spain or New-Zealand.

Type and reference strains used are presented in each study (I-IV). All strains were grown in MRS broth and MRS agar or M17 broth (Oxoid) with 0.5% glucose (GM17) or 0.5% lactose (M17) and GM17 or M17 agar (Oxoid) at 25°C. The plates were incubated in anaerobic jars in a CO_2-enriched atmosphere [Anaerogen (Oxoid)]. All isolates were maintained in MRS broth (Oxoid) at -70°C.

4.2 MORPHOLOGY AND PHENOTYPIC TESTS (I, II, III)

All isolates were Gram-stained and tested with 3% hydrogen peroxide for the presence of catalase.

In study I, the growth tests at different temperatures and NaCl concentrations, carbohydrate fermentation profiles, Lancefield antigen D, hemolysis, the production of ammonia from arginine and the formation of typical colonies for enterococci were performed as described by Koort *et al*., (2004). In study II, growth was tested at temperatures of 0, 4, 10, 37 and 40°C, at pH 4.5, and 6, and in NaCl concentrations of 2, 4, and 6.5% in GM17 broth (Oxoid) for 21 days. In study III, growth was tested at temperatures of 0, 5, 10, 15, 25, 30, and 37°C, at pH 2-10, and in NaCl concentrations of 2, 4, 6.5, and 8% in MRS broth (Oxoid) grown for 21 days. Carbohydrate fermentation profiles and enzyme activities were tested using API 50CH and API 20 Strep identification systems (bioMeriéux, Marcy l'Etoile, France) according to the manufacturer's instructions (II, III). The production of ammonia from arginine was tested as described by Koort *et al*., (2004). Motility was tested by stab inoculation in semisolid media. All tests were carried out at least twice and done at 25°C unless otherwise stated.

In study II and III, the growth of four representative isolates, MKFS47, LTM33-6, JL3-4, and LTM26-2, (II) or *L. gelidum* NCFB 2775^T, *L. gasicomitatum* LMG 18811^T, and strains AMKR32, POKY4-4, and POUF4h (III) in the presence of exogenous heme was tested in GM17 broth (Oxoid) (II) or MRS broth (III) supplemented with 2 µg/ml of heme (Sigma, stock solution 0.5 mg/ml in 1:1 DMSO:H_2O). An equivalent volume of 1:1 DMSO:H_2O was added to the controls growing without heme. Aerobic conditions with a 2:10 medium/volume ratio and agitation at 200 rpm was

used. OD_{600} (optical density at 600 nm) of the cultures was measured after 48 h incubation at 25°C. The growth tests were repeated four times. *Lactococcus lactis* MG1363 was used as a positive control.

4.3 ISOLATION OF DNA (I, II, III, IV)

Cells harvested from broth culture were used for DNA isolation for ribotyping, sequence analysis, determination of the G+C content and DNA-DNA reassociation. DNA was isolated as described by Björkroth and Korkeala (1996). The guanidium thiocyanate method of Pitcher *et al*., (1989) was modified by using lysozyme (25 mg/ml) and mutanolysin (200 U/ml) in the cell lysis solution.

4.4 RIBOTYPING (I, II, III)

Ribotyping was performed as described by Björkroth and Korkeala (1996). *Eco*RI and *Hin*dIII (I) or *Eco*RI, *Hin*dIII, and *Cla*I (II) restriction enzymes were used to digest 8 µg of DNA, as specified by the manufacturer (New England Biolabs, Beverly, MA, USA). DNA fragments were separated by agarose gel electrophoresis and Southern blotting was performed using a Vacugene blotting system (Pharmacia, Uppsala, Sweden). A digoxigenin-labelled probe mixture, OligoMix5, was used for detecting the fragments containing 16S or 23S rRNA gene (Regnault et al., 1997). The membranes were hybridised at 53°C, and the labelled fragments were detected by anti-digoxigenin antibody conjugated with alkaline phosphatase and NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indonyl phosphate) as recommended by the manufacturer Roche Molecular Biochemicals, Mannheim, Germany). Scanned (Scan Jet 4c/T, Hewlett Packard, Palo Alto, CA, USA) ribopatterns were analysed using Bionumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) and compared to the corresponding patterns in the previously established database of 295 LAB type and reference strains (Björkroth and Korkeala 1996). The Dice coefficient correlation and unweighted-pair group method using average linkages (UPGMA) were used for construction of the dendrograms. Band position tolerance of 1.5% and pattern optimisation of 0.6% was allowed for the bands.

4.5 SEQUENCE ANALYSIS OF 16S RRNA, ATPA, PHES, AND RPOA GENES (I, II, III)

Sequencing of the 16S rRNA gene was performed as described by Vihavainen *et al.,* (2007). The nearly complete 16S rRNA gene was amplified using a universal primer pair F8-27 and R1541-1522. The PCR product was purified (QIAquick PCR purification kit; Qiagen) and sequenced by Sanger's

dideoxynucleotide chain termination method using two long (primers F19–38 and R1541–1522) and two shorter reactions (primers F926 and R519). Samples were run in a Global IR2 sequencing device with e-Seq (version 2.0) software (LiCor, Lincoln, NE) according to the manufacturer's instructions. The consensus sequences were created with AlignIR software (LiCor).

Sequencing of the housekeeping genes *pheS* and *rpoA* was performed as described by Naser *et al*., (2005). Primer pairs pheS-21-F/pheS-22-R, pheS-21-F/pheS-R008, pheS-F004/pheS-R011, rpoA-21-F/rpoA-23-R and rpoA-21-F/rpoA-R009 (I, III), or rpoA-F025/rpoA-R026, pheS-F025/pheS-R025 and pheS-F026/pheS-R026 (II) were used for amplification of the genes (Table 3.). PCR was performed using PTC-200 version 3.8 (MJ Research, Massachusetts, USA). Primer pairs rpoA-21-F/rpoA-23-R and pheS-21-F/pheS-22-R (I, III) or rpoA-21F/R026, pheS-F025/R025 and pheS-F026/R026 (II) were used for sequencing. Sequencing was performed with the BigDye termination cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3700 capillary DNA sequencer (GMI, Ramsey, MN). Sequences were assembled using the Staden package (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK).

Table 3. MLSA primers used in this study.

Gene	Primer	Sequence 5´-3
16S rRNA	F8-27	AGAGTTTGATCCTGGCTGAG
	R1541-1522	AAGGAGGTGATCCAGCCGCA
	F19–38	CTGGCTCAGGAYGAACGCTG
	F926	AACTCAAAGGAATTGACGG
	R519	GTATTACCGCGGCTGCTG
*pheS**	pheS-21-F	CAYCCNGCHCGYGAYATGC
	pheS-22-R	CCWARVCCRAARGCAAARCC
	pheS-R008	CCAGCHCCHAGHACTTCAATCCA
	pheS-F004	ATGAATCTDCCWAAAGATCAYCC
	pheS-R011	TAAGAAACGTAARTCATTTTGATARAA
	pheS-F025	TATAAYTTTGARCGMATGAATCTWCC
	pheS-R025	CCTGCACCWARDAYTTCAATCCA
	pheS-F026	AAAGATCAYCCAGCKCGTGATATGCAA
	pheS-R026	GGATGGACCATWCCTGCACC
rpoA°	rpoA-21-F	ATGATYGARTTTGAAAAACC
	rpoA-23-R	ACHGTRTTRATDCCDGCRCG
	rpoA-R009	TCWARYTCTTCRATNGTCAT
	rpoA-F025	TGATTGAGTTTGAAAAACC
	rpoA-R026	TTCAAACMRTTRTAAGHACGAAC

* phenylalanyl tRNA synthetase α chain; °DNA-directed RNA polymerase subunit A

The 16S rRNA, *pheS* and *rpoA* gene sequences were subjected to the BLAST search program (Altschul *et al*., 1997) and **s**equences of representative strains from the same phylogenetic group were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The sequences were aligned using ClustalX software (Thompson *et al*., 1994). Phylogenetic trees were

constructed by using Bionumerics version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) and the neighbour-joining and maximum-parismony methods (I, II) or PALM (Chen *et al.,* 2009) by the Maximum Likelihood method and ClustalX by the neighbour-joining method (III). Bootstrap analysis was performed with 500 (I, II) or 1000 (III) replications.

4.6 DETERMINATION OF THE G+C CONTENT AND DNA-DNA REASSOCIATION (I, III)

In study I, the DNA GC content of strains IE3.2 and IE35.3 was determined as described by Xu *et al*., (2000). The melting point curves were determined in 1 x SSC with the LightCycler (Roche Diagnostics) instrument using SYBR green I dye (Roche Diagnostics). *E. devriesei* LMG 14595^T and 13603 was used as the reference organism and *E. hermanniensis* LMG 12317^T was used as the control.

DNA-DNA reassociation in studies I and III was performed by DSMZ (Braunschweig, Germany). Briefly, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al*., (1977). DNA-DNA hybridisation was carried out as described by De Ley *et al*., (1970) under consideration of the modifications described by Huss *et al*., (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

4.7 MLST (IV)

Initially, ten housekeeping genes were selected for analyses, but three of them (*atpA*, *dnaA*, and *rpoA*) were rejected because they either contributed with too little variation or were located too close to another selected gene in the chromosome. Sequencing was performed with the primers and protocol described in study IV. The genes selected for the MLST scheme were *ddl* (D-alanyl-alanine-synthetase), *dnaK* (chaperone protein DnaK), *gyrB* (DNA gyrase, subunit B), *lepA* (leader peptidase A), *pgm* (phosphoglucomutase), *pheS* (phenylalanine synthetase, alpha subunit) and *rpoC* (RNA polymerase, beta prime subunit). Multiple sequence alignment was performed using MAAFT (Katoh and Standley, 2013) and the dN/dS ratio, the pi (π), Tajima's D values and the minimum number of recombination events (Rm) were calculated using DnaSp v5.1 (Librad and Rozas 2009). goBURST (Fransisco *et al*., 2009) algorithm as implemented in PHYLOVIZ (Fransisco *et al*., 2012), and BAPS (Corander *et al*., 2003) linkage clustering and the corresponding admixture model were used for estimating the population structure of *L. gelidum* subsp. *gasicomitatum*. A phylogenetic tree of the concatenated

sequences of the 46 STs was constructed by maximum likelihood analysis by PALM (Chen *et al*., 2009). ClonalFrame (Didelot and Falush, 2006) was used to estimate the recombination ratio for the population.

4.8 INOCULATION EXPERIMENTS (II)

Two *L. piscium* strains, and for comparison, a type strain of a well-known spoilage bacterium, *Brochothrix thermospacta* CCUG 35132^T, were individually inoculated onto fresh pork at a level of 10^5 cfu/ on each side of a piece of 30 g pork fillet (*Longissimus dorsi*). The samples were packaged in high barrier film under modified atmosphere containing 71% O_2, 22% CO_2 and 7% N_2, and stored at 6°C for 22 days. Microbiological analyses were performed every other day from day 0 of storage and sensory analysis was performed every other day from day 6 of storage as described in study II. The bacterial communities of the pork samples and controls were characterised by T-RFLP after 4, 6, and 22 days of storage as described by Nieminen *et al*., (2011). After 22 days of storage, random isolates from the pork samples inoculated with the *L. piscium* strains were identified by numerical analysis of *Hin*dIII ribotypes as described above. Maximum specific growth rates (*μmax*) and maximum bacterial levels (*Nmax*) of LAB were calculated using DMfit program (Institute ofFood Research, Norwich, UK).

5 RESULTS AND DISCUSSION

5.1 IDENTIFICATION AND CHARACTERISATION OF NOVEL BACTERIAL GROUPS FROM MEAT AND THE MEAT PROCESSING ENVIRONMENT (I, III)

In studies I and III, a polyphasic approach based on phenotypic and genotypic characterisation was applied to describe unknown bacterial groups from MAP meat and the meat processing environment. Five LAB isolates from a broiler processing plant and broiler products were shown to represent a novel species *Enterococcus viikkiensis* sp. nov. within the genus *Enterococcus*. Twenty LAB originating from packaged meat were shown to represent a novel subspecies within the species *L. gelidum*, *L. gelidum* subsp. *aenigmaticum* subsp. nov. The novel subspecies was closely related to both *L. gelidum* and *L. gasicomitatum*, and the taxonomy of these species was also clarified. To understand spoilage as a phenomenon, it is important to know all the organisms present in food and the production environment. Taxonomy provides a basis for further studies on the diversity and interactions of organisms involved in spoilage or the development of the in-house microbiota of food processing plants.

Numerical analysis of *Hin*dIII and *Eco*RI ribopatterns indicated that the isolates had similar banding patterns to each other, but were clearly separated from other strains within the genuses *Enterococcus* and *Leuconostoc*. Sequence analysis of 16S rRNA gene positioned *E. viikkiensis* within the *E. avium* species group and *L. gelidum* subsp. *aenigmaticum* within the *L. gelidum* species group, but 16S rRNA gene is not discriminatory enough for species level identification within either of these species groups (Svec *et al*., 2005, Björkroth *et al*., 2000, Williams *et al*., 1991).

Sequence analysis of *pheS* and *rpoA* showed that the *E. viikkiensis* strains formed a separate cluster within the *E. avium* group, with *E. devriesei* as the closest phylogenetic neighbour (Fig. 2). Based on sequence analysis of *atpA, pheS* and *rpoA* genes, the *L. gelidum* subsp. *aenigmaticum* strains, as well as the type and reference strains of *L. gelidum* and *L. gasicomitatum*, were closely related, but formed three clearly separate subgroups (Fig. 3).

DNA-DNA hybridisation with *E. devriesei* was clearly below the threshold value of 70% for the definition of bacterial species and the name *Enterococcus viikkiensis* sp. nov. was proposed for the novel species with strain DSM 24043^T (= LMG 26075^T = IE3.2^T) as the type strain. The DNA-DNA relatedness values for type and reference strains of *L. gelidum* and *L. gasicomitatum* assigned these strains to the same species. Reclassification of *L. gasicomitatum* as *Leuconostoc gelidum* subsp. *gasicomitatum* subsp. nov. was proposed.

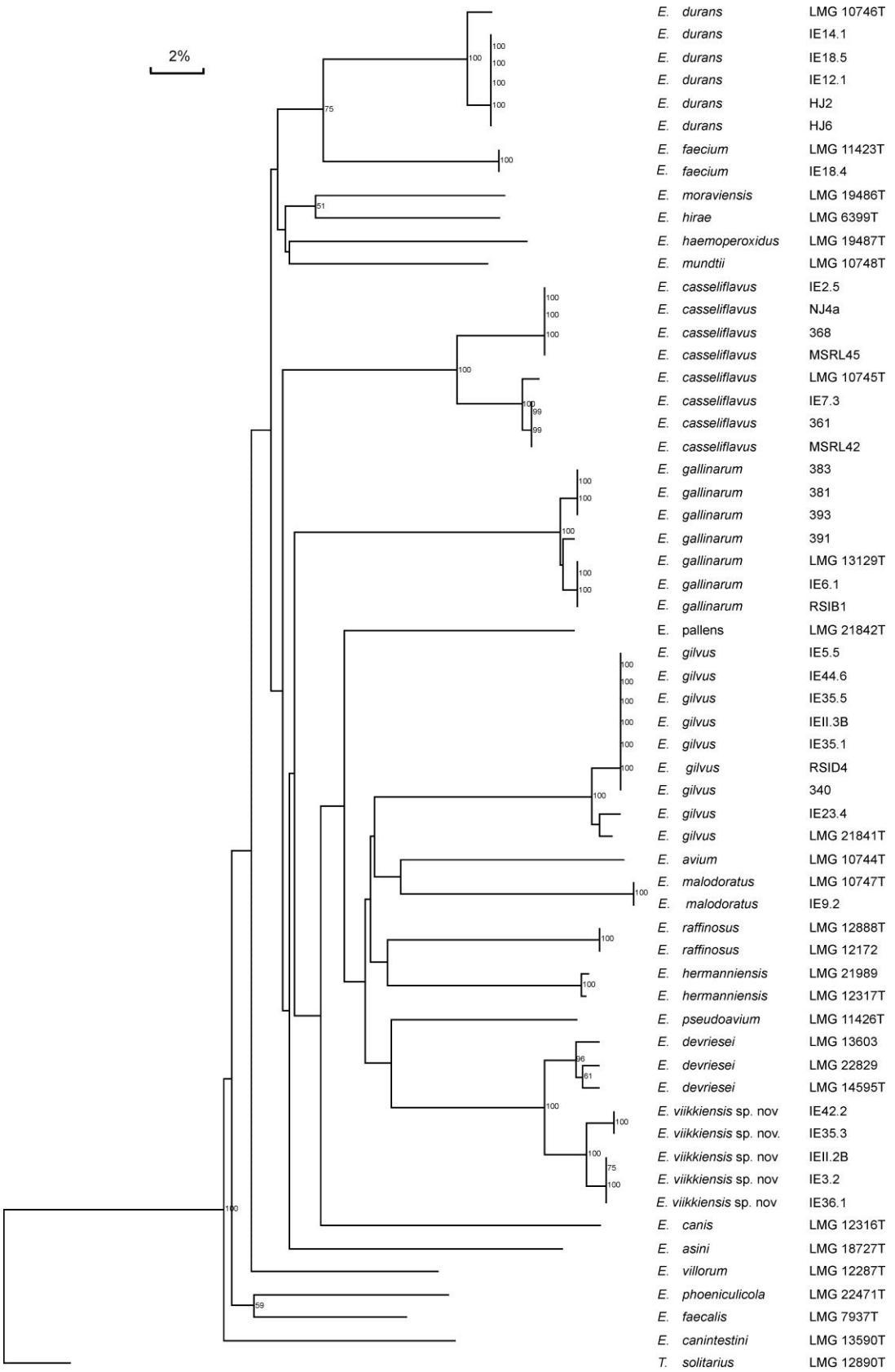

Fig. 2. Neighbour-joining dendrogram based on the *pheS* sequences of 31 *Enterococcus* type and reference strains and 33 isolates from modified atmosphere packaged (MAP) broiler products and a broiler processing plant. Bootstrap percentages (≥ 50%) after 500 replicates are shown. *Tetragenococcus solitarius* is included as an outgroup.

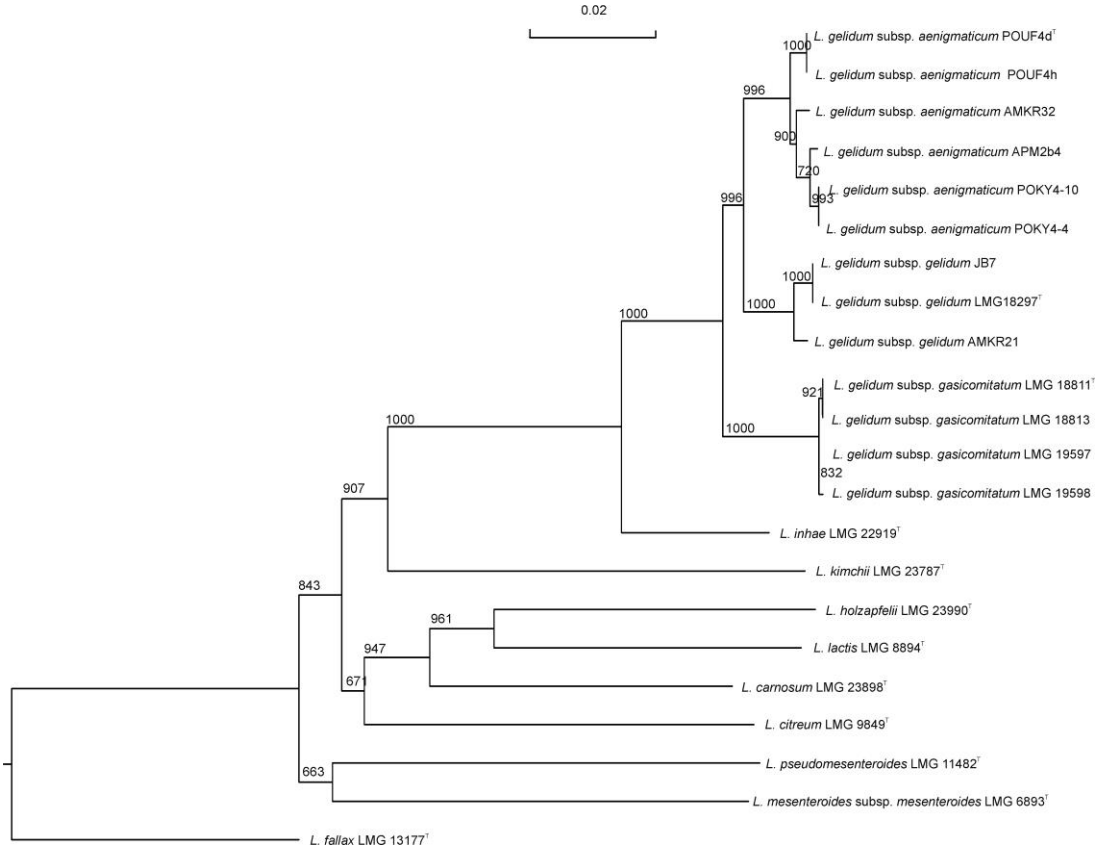

Fig. 3. Neighbour-joining tree based on concatenated *atpA*, *pheS*, and *rpoA* gene sequences of 22 *Leuconostoc* strains showing the phylogenetic position of *L. gelidum subsp. aenigmaticum* subsp. nov. within the genus. *Leuconostoc fallax* LMG 13177T is included as an outgroup and bootstrap values above 500 after 1000 resamplings are shown.

DNA-DNA reassociation value between *E. viikkiensis* and the closest phylogenetic neighbour, *E. devriesei*, was clearly below the value of 70% for species delineation, whereas the values between *L. gelidum* subsp. *aenigmaticum*, *L. gelidum* and *L. gasicomitatum* were close to the cut-off point (75-82%). The values for *L. gelidum* and *L. gasicomitatum* were higher than those reported by Björkroth *et al.,* (2000) and Kim *et al.,* (2000), and placed these strains within the same species. We thus repeated the experiment in another laboratory and confirmed the results. One of the commonly recognised pitfalls of DNA-DNA reassociation is high experimental error, which may explain the discrepancy of the reassociation results for *L. gelidum* and *L. gasicomitatum* (Rossello-Mora, 2006). In addition, *L. gasicomitatum* was further from *L. gelidum* in the phylogenetic analyses than the novel subspecies (Fig. 3). Taxonomists are constantly searching for alternative methods that could replace DNA-DNA reassociation, but the methods proposed to date have not yet achieved gold standard status (Tindall *et al*., 2010). Methods based on whole genome sequence data, such as the average nucleotide identity (ANI), will most probably substitute this outdated and labour-intensive tool in microbial taxonomy in the very near future (Richter and Rossello-Mora, 2009).

Additionally, methods recommended by *ad hoc* committee for the re-evaluation of the species definition in bacteriology, i.e. sequence analysis of housekeeping genes and ribotyping, were used to further verify the taxonomic status of the isolates studied (Stackebrandt *et al*., 2002). Several restriction enzymes for ribotyping and housekeeping genes for sequence analysis were used in both studies. Both new taxa were described based on several strains (five strains in study I and 20 strains in study II) in accordance with recommendation by the *ad hoc* committee (Stackebrandt *et al*., 2002, Christensen *et al*., 2001). At the moment, species is the lowest taxonomic unit that can be defined in phylogenetic terms (Wayne *et al*., 1987). Subspecies status can be proposed to genetically closed organisms that diverge in phenotype, but there are no clear standards for subspecies description (Wayne *et al*., 1987).

The *E. viikkiensis* strains were isolated from the air of a broiler processing plant and, as is typical for enterococci, the strains were able to grow at 37°C, but not at 4°C (Table 4.). Enterococci are common contaminants of fresh meat, but are often overgrown by other, more competitive LAB (Björkroth *et al*., 2005). Thus, *E. viikkiensis* is unlikely to cause spoilage of refrigerated, packaged meat (Table 4.). The twenty *L. gelidum* subsp. *aenigmaticum* strains described in this study were isolated from packaged meat of late shelf-life during spoilage studies within several years. The novel subspecies can thus be considered a rare contaminant of packaged meat and the spoilage potential of strains representing this subspecies is currently unknown (Table 4.).

Table 4. Characteristics of species/subspecies identified in this study.

Species/ subspecies	Source	Growth at 5°C	37°C	Spoilage potential	Restriction enzymes for ribotyping
E. viikkiensis sp. nov.	Broiler processing plant air	-	+	Unlikely to cause spoilage	*Hin*dIII, *Eco*RI
L. gelidum subsp. *aenigmaticum* subsp. nov.	Packaged meat	+	-	Unknown; rare in packaged, late shelf-life meat	*Hin*dIII, *Eco*RI
L. piscium	Packaged meat	+	D*	Strain-dependent; common in packaged, late shelf-life meat	*Eco*RI, *Cla*I

* D; strain-dependent

5.2 METHODS FOR IDENTIFICATION OF COCCAL LAB FROM MEAT (I, II)

In studies I and II, numerical analysis of ribopatterns was shown to differentiate species of enterococci and lactococci. In addition, multilocus sequence analysis of *pheS* and *rpoA* genes provided species level identification of lactococci. Despite the current development of culture-independent sequencing methods, there is still a need for robust culture-based methods for the identification of LAB isolates from food.

Identification of enterococci within the *E. avium* phylogenetic group required polyphasic taxonomic approaches due to the high level of 16S rRNA sequence similarity and variation in phenotypic characteristics. Numerical analysis of *Hin*dIII and *Eco*RI ribopatterns had a greater resolution power than the methods above in the identification of enterococci, especially species in the *E. avium* group. The species detected in MAP broiler products were considered as *Enterococcus gallinarum, E. casseliflavus* and *Enterococcus gilvus*. Strains that were isolated from the air of the broiler processing plant were assigned to the species *E. gilvus*, *E. gallinarum*, *E. casseliflavus*, *Enterococcus durans*, *Enterococcus malodoratus*, *Enterococcus hermanniensis* and *E. faecium*. *E. durans* and *E. casseliflavus* were detected in broiler carcasses (Fig. 2). Species level identification of enterococci by combined *Hin*dIII and *Eco*RI ribotyping was congruent with identification obtained by sequence analysis of *rpoA* and *pheS* genes (Naser *et al*., 2005).

Combined *Eco*RI and *Cla*I ribopattern analysis and sequence analysis of *rpoA* and *pheS* partial gene sequences clearly differentiated species of the genus *Lactococcus*. In ribotyping, the restriction enzymes *Eco*RI and *Cla*I were shown to provide enough bands for species level identification, whereas numerical analysis of *Hin*dIII ribopatterns provided only genus level identification. In the cluster analysis of both *rpoA* and *pheS* partial gene sequences, the three subspecies of *L. lactis* and *L. garvieae* formed one distinct phylogenetic group, whereas the type strains of *L. piscium*, *L. raffinolactis*, *L. plantarum*, and *L. chungangensis* clustered together corresponding with the 16S rRNA based phylogeny (Fig. 4). The 63 representative LAB isolates that were identified as *L. piscium* by ribotyping, clustered with *L. piscium* type strain. In the analyses, *pheS* provided the highest resolution power of the three genes (at least 95.3% sequence similarity within a species), whereas the discrimination with *rpoA* was substantially higher than with 16S rRNA gene (at least 98.3% sequence similarity within a species).

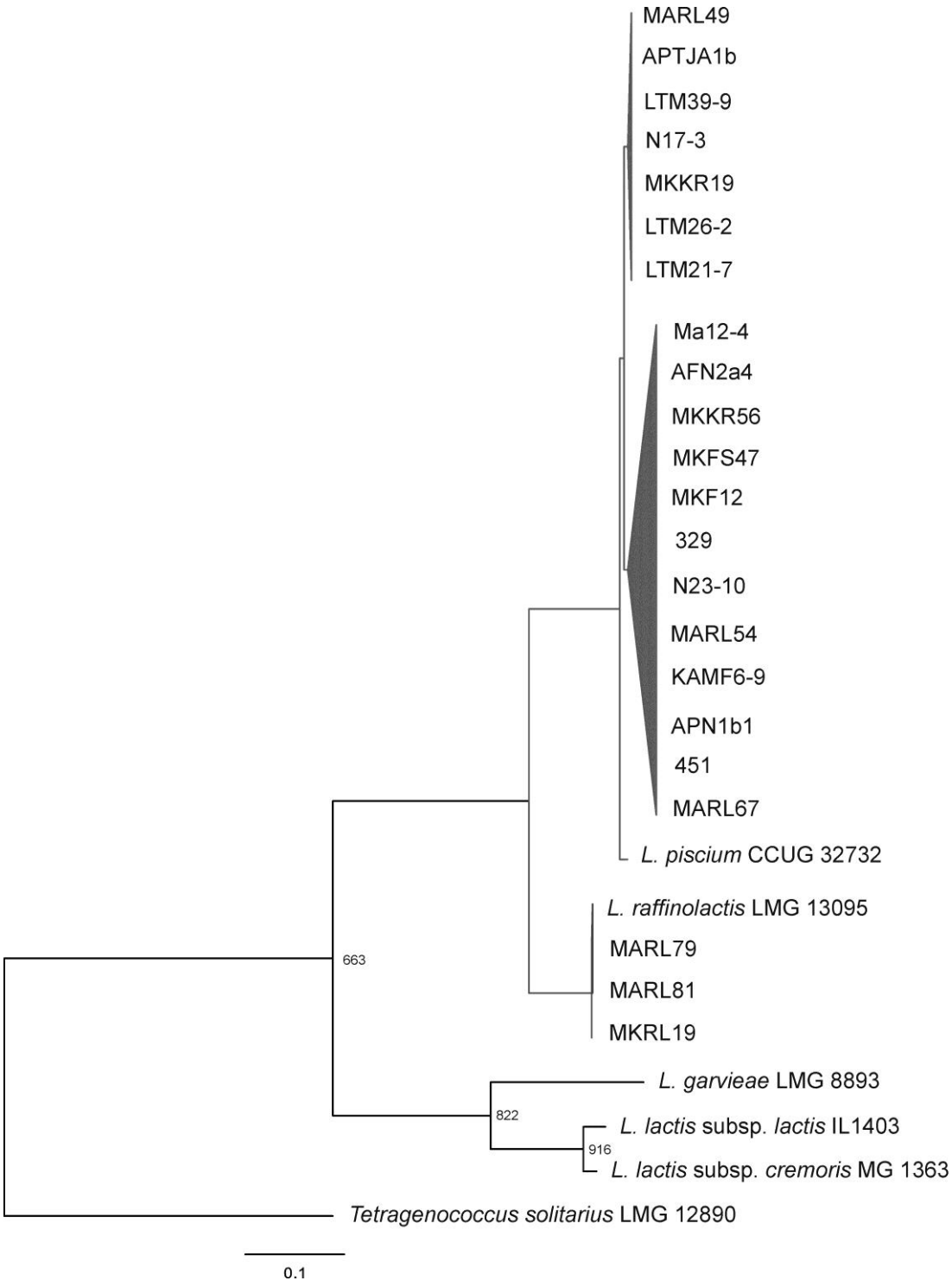

Fig. 4. Maximum-likelihood tree based on concatenated *rpoA* and *pheS* gene sequences of 22 representative *Lactococcus* isolates from MAP meat and five *Lactococcus* type strains. *Tetragenococcus solitarius* is included as an outgroup and bootstrap values above 500 after 1000 resamplings are shown.

5.3 THE ROLE OF *LACTOCOCCUS PISCIUM* IN MAP MEAT (II)

A majority of the 222 unknown LAB isolates (n=215) from the use-by day, MAP meat were identified as *L. piscium*, whereas seven isolates represented the species *L. raffinolactis* (Fig. 4). *L. piscium* was shown to grow in a variety of MAP meat products, including broiler, pork, turkey and minced meat made of beef and pork, where they belonged to the predominating microbiota. Previously, only few reports on the isolation of lactococci from meat have been available (Sakala *et al*., 2002a, Barakat *et al*., 2000). This study showed that *L. piscium*, in particular, is a significant member of the microbial association of late shelf-life meat and the involvement of this species in meat spoilage deserves further studies (Table 4.).

All the 21 *L. piscium* isolates tested for their phenotypic properties grew at 0.5±0.5°C and were not able to utilise ribose. Supplementation of the growth medium with heme did not increase the biomass during aerobic growth of the four *L. piscium* strains tested. This suggests that this species is not competent for heme-induced respiration (Brooijmans *et al*., 2009). Some LAB that are often detected in meat together with *L. piscium*, such as species of *Leuconostoc* and *Carnobacterium*, are potentially respiring organisms (Jääskeläinen *et al*., 2013, Lechardeur *et al*., 2011, Brooijmans *et al*., 2009). Respiration metabolism may give a competitive advantage by improved growth and survival compared to *L. piscium* when growing in high-O_2 MAP meat.

The spoilage potential and competitiveness of two *L. piscium* isolates among the initial microbial contamination of MAP pork was studied in an inoculation experiment in comparison with a well-known spoilage organism, *Brochothrix thermosphacta*. The sensory shelf-life of pork inoculated separately with *L. piscium* strains LTM33-6 and JL3-4 (14 and 16 days, respectively) was shortened compared to that of an uninoculated control (18 days), and the odour of the spoiled pork inoculated with the *L. piscium* strains was described as buttery and sour (Fig. 5). This indicates that growth of *L. piscium* contributed to spoilage. However, the spoilage potential of the *L. piscium* strains was substantially lower than that of the *B. thermosphacta* type strain used as a positive control for spoilage; the sensory shelf-life of pork inoculated with *B. thermosphacta* was only 10 days. The ability of *B. thermospacta* to cause sensory defects at a lower cell number than LAB is a well-known phenomenon (Betts *et al*., 2006).

T-RFLP and ribotyping analyses of samples showed that *L. piscium* strains were growing in pork together with *Leuconostoc gelidum* subsp. *gelidum*/*gasicomitatum* present as initial contaminants. In previous studies, these *Leuconostoc* species have been shown to flourish in MAP meat (Samelis *et al*., 2006, Björkroth *et al*., 2000). In this study, leuconostocs had a high growth rate at the beginning of storage and, unlike lactococci, they did not show a lag phase. However, lactococci were able to co-exist with

leuconostocs until the meat was condemned as spoiled. The co-existence and possible interactions of lactococci and leuconostocs in a meat environment deserves further studies.

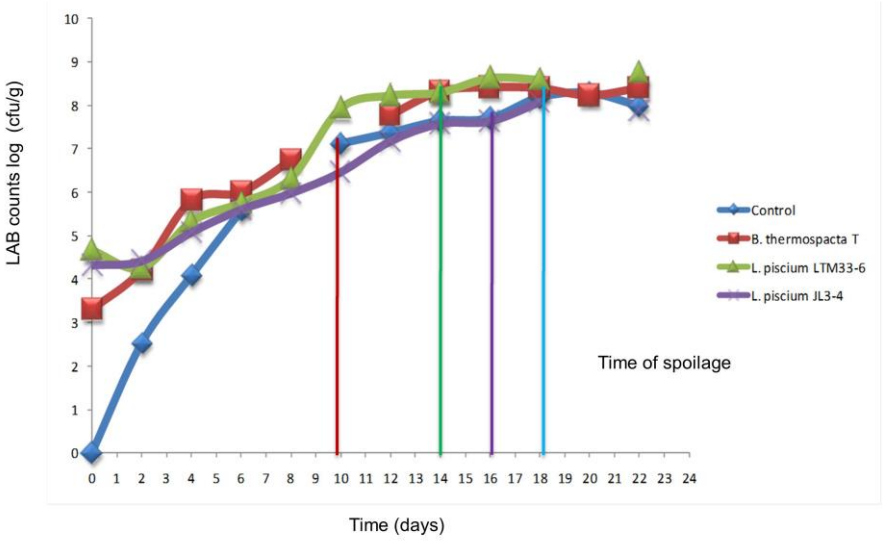

Fig. 5. LAB counts and the time of spoilage of MAP pork inoculated individually with two *L. piscium* strains (LTM33-6 and JL3-4) or *B. thermospacta* CCUG 35132T or left uninoculated (control), and stored at 6°C for 22 days. The vertical lines represent the time of spoilage.

5.4 GENETIC DIVERSITY OF *LEUCONOSTOC GELIDUM* SUBSP. *GASICOMITATUM* STRAINS FROM MEAT AND VEGETABLE SOURCES (IV)

A novel MLST scheme employing seven housekeeping genes was developed for determining the population structure within 252 *L. gelidum* subsp. *gasicomitatum* strains from meat and vegetable sources. The strains were chosen for the study based on PFGE types, ribotypes and sources, to obtain maximal genetic and ecologic diversity. Forty-six STs were recognised within the population, with a majority of the strains (> 60%) representing the three most prevalent STs, ST1 (n=86), ST2 (n=40), and ST7 (n=35). The STs were divided into three clonal complexes and 17 singletons by goeBurst at a single locus variant level, and three groups by BAPS (Fig. 6). Based on admixture analysis by BAPS, the level of recombination between the three subgroups was very low, indicating clonal expansion. *Leuconostoc lactis* population of 50 dairy isolates had a similar highly clonal population structure (Dan *et al*., 2013), whereas for *Lactobacillus sakei* recombination was shown

to be relatively common (Chaillou *et al*., 2013). Leuconostocs possess several restriction modification systems, which may limit genetic exchange and thus prevent succesful recombination and explain the tendency to clonal expansion (Roberts *et al*., 2013, Johansson *et al*., 2011).

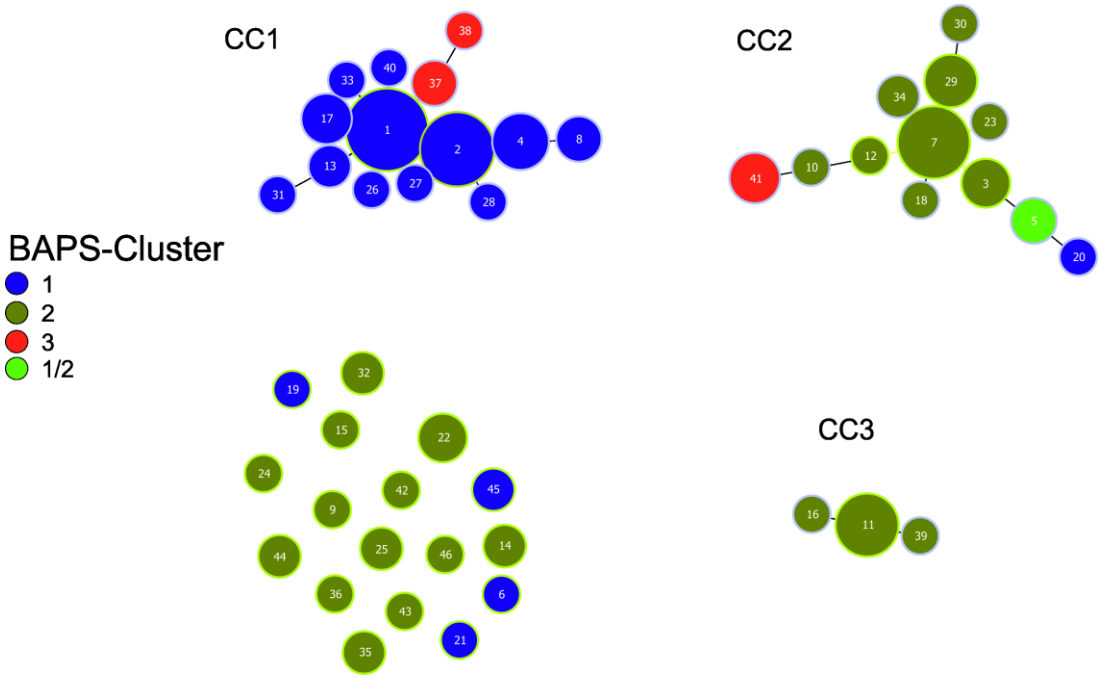

Fig. 6. Minimum spanning tree reflecting clonal relationships of 46 *Leuconostoc gelidum* subsp. *gasicomitatum* STs at SLV level constructed using goeBurst. A circle represents each ST, and the size of the circle is proportional to the number of isolates represented by that ST. The color of each circle represents the BAPS cluster of the strains belonging to that ST.

The STs in CC1 consisted mostly of strains from a variety of MAP meat sources, whereas the STs in CC2 contained strains from vegetables and MAP poultry, indicating niche specificity of the subpopulations. BAPS cluster 2 contained 97% of the vegetable strains and 45% of the MAP poultry strains, whereas most of the MAP meat strains were located in BAPS cluster 1. The results may indicate that there is a subpopulation within *L. gelidum* subsp. *gasicomitatum,* which is specialised in growing in a variety of MAP meat and/or surviving in a meat processing environment. PFGE typing of 384 *L. gelidum* subsp. *gasicomitatum* isolates from meat and vegetable sources, including the strains in this study, also supported this conclusion (Vihavainen *et al*., 2009). The few available contamination studies of leuconostocs in meat processing plants indicate that these spoilage bacteria are constantly introduced into the processing environment instead of forming an in-house microbiota (Vihavainen *et al*., 2007, Björkroth *et al*., 1996, 1997). Similar results were obtained for a vegetable processing plant (Pothakos *et al*., 2014c). The connection between vegetable and poultry strains requires further studies with more isolates from different niches. This MLST scheme

provides a tool for characterising isolates of *L. gelidum* subsp. *gasicomitatum* from spoilage studies, and the 46 STs a basis for a MLST database of this important spoilage organism.

6 CONCLUSIONS

1. The formerly unidentified LAB strains isolated from the air of a broiler processing plant represent a novel species, *Enterococcus viikkiensis* sp. nov.

2. The formerly unidentified LAB strains from vacuum/modified atmosphere packaged meat represent a novel subspecies, *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov. *Leuconostoc gasicomitatum* was reclassified as *Leuconostoc gelidum* subsp. *gasicomitatum* subsp. comb. nov. and *Leuconostoc gelidum* designated as *Leuconostoc gelidum* subsp. *gelidum* subsp. nov.

3. Numerical analysis of combined *Hin*dIII and *Eco*RI ribopatterns was of considerable assistance in species level identification of enterococci within the *E. avium* group. Numerical analyses of *Eco*RI and *Cla*I ribopatterns and phylogenetic sequence analyses of *rpoA* and *pheS* genes were reliable tools in species level identification of meat lactococci.

4. *Lactococcus piscium* formed part of the predominating microbiota in a variety of MAP meat products including broiler, turkey, pork and minced meat from beef and pork. The growth of *L. piscium* together with *Leuconostoc* spp. shortened the shelf-life of MAP meat.

5. The population of 252 *L. gelidum* subsp. *gasicomitatum* strains from meat and vegetable sources was divided into three subgroups, with evidence of niche specificity and a low level of exchange of genetic material between the subgroups.

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