LEUCONOSTOC SPOILAGE OF REFRIGERATED, PACKAGED FOODS

Elina Säde

ACADEMIC DISSERTATION

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ABSTRACT

*Leuconostoc* spp. are lactic acid bacteria (LAB) implicated in food spoilage, especially on refrigerated, modified atmosphere packaged (MAP) meats. The overall aim of this thesis was to learn more about *Leuconostoc* spp. as food spoilage organisms with a focus on commercial products where LAB spoilage is considered a problem and the main factor limiting shelf-life. Therefore, we aimed to identify *Leuconostoc* spp. involved in food spoilage, as well as to characterise the spoilage reactions they caused and their contamination sources during poultry meat processing. In addition, we examined the distribution of strains of *Leuconostoc gasicomitatum* in different food commodities. Finally, we analysed the genome content of *L. gasicomitatum* LMG 18811T with a special focus on metabolic pathways related to food spoilage.

The findings show that *Leuconostoc gelidum* and *L. gasicomitatum* were responsible for the discoloration and off-odours developed in beef steaks. Together with *Leuconostoc mesenteroides*, these *Leuconostoc* spp., also caused spoilage of vegetable sausages. In contrast, we showed that *Leuconostoc* spp. are not important for the shelf-life or quality of non-marinated broiler products although, in marinated broiler fillet products, *Leuconostoc* spp., *gasicomitatum* in particular, are considered spoilage organisms. Furthermore, the findings of the contamination survey we carried out in a poultry processing plant indicated that spoilage *Leuconostoc* spp. are derived from the processing environment rather than from the broilers, and that air movement distributes psychrotrophic spoilage LAB, including leuconostocs, and has an important role in meat contamination during poultry processing.

Pulsed-field gel electrophoresis (PFGE) based genotyping of *L. gasicomitatum* strains demonstrated that certain genotypes are common in various meat products. In contrast, genotypes associated with meat were not recovered in vegetable-based sources. This suggests that these two food categories either become contaminated with, or favour the growth of different genotypes. Furthermore, the results indicated that the meat processing environment contributes to *L. gasicomitatum* contamination as certain genotypes were repeatedly identified from products of the same processing plant.

Finally, the sequenced and annotated genome of *L. gasicomitatum* LMG 18811T allowed us to identify the metabolic pathways and reactions resulting in food spoilage.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications


The publications are referred to in the text by their roman numerals. The published articles are reprinted with kind permission from the respective publishers.

Author’s contributions in articles included in this thesis:

I  Main responsibility for designing, experimental work, interpretation of the results and writing the paper.

II  Main responsibility for designing, experimental work, interpretation of the results and writing the paper.
| III | Participated in designing, performed sampling and part of the laboratory work. Main responsibility of interpretation of the results. The author wrote the article together with Prof. Björkroth. |
| IV | Main responsibility for designing, experimental work, interpretation of the results and writing the paper. |
| V  | Did microbiological laboratory work, and participated in interpretation of the results and writing of the manuscript |
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EPS</td>
<td>Extracellular polysaccharide</td>
</tr>
<tr>
<td>HPP</td>
<td>High pressure processing</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>MAP</td>
<td>Modified atmosphere packaged</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man Rogosa Sharpe</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group method with arithmetic averages</td>
</tr>
<tr>
<td>VP</td>
<td>Vacuum packaged</td>
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</table>
1 INTRODUCTION

Food is not generally anticipated to maintain its original and optimal quality indefinitely. During storage, some deterioration will occur that will ultimately render a product unacceptable for human consumption. The time during which food remains stable and retains its desired quality is called the shelf-life. During this time, food should remain safe, and retain its sensory, chemical, physical, and microbiological characteristics. To comply with legislative requirements (Anonymous, 2005), food operators have an obligation to determine realistic shelf-lives for their products, and label most pre-packaged food either with a “use by” or a minimum durability date.

The concepts of spoilage or “food spoilage” may entail many interpretations. In the context of this thesis, food spoilage is defined as a process leading to undesirable changes in sensory characteristics (odour, flavour, texture, appearance) and loss of acceptable quality. In addition, physical and chemical changes, such as lipid oxidation and dehydration cause losses in food production and the distribution chain. In technologically advanced countries such as Finland, where perishable foodstuffs are produced in modern facilities and distributed and sold under a strictly controlled cold chain, microbiological spoilage or “microbiological shelf-life“ have become the major factors limiting the shelf life of many foods. Understanding the process of microbial spoilage is of importance in designing effective strategies to prevent food losses. Generally, microbial growth and organoleptic spoilage result from a sequence of several events: (i) spoilage microbes need to gain access to food from one a more sources (referred to as contamination), (ii) after contamination, microbes must survive and adapt to food ecosystem, and finally (iii) the microbes need to grow to attain sufficient numbers to cause detectable changes in a particular food system. Controlling microbial spoilage may allow food operators to extend shelf-lives and the range of distribution. To reduce microbial growth, food processors apply preservation strategies including processing and packaging techniques, preservatives, and refrigeration. In addition, by paying attention to the quality of raw materials and good hygiene during food handling, microbial contamination may be minimised or prevented.

During recent decades, strict cold-chain maintenance and advances in processing hygiene and food packaging have allowed an extension of the shelf lives of many perishable food commodities, such as meat and fish. When coupled with refrigeration, packaging food under high concentrations of carbon dioxide (CO₂) suppresses food spoilage by moulds and aerobic bacteria. While the growth of aerobic microorganisms is inhibited, these storage conditions create a more or less selective environment for psychrotrophic LAB. In food, LAB ferment carbohydrates to acidic end products leading to sour odours and a drop in pH. However, these changes
are considered relatively inoffensive compared to the putrid and ammoniacal defects often attributed to aerobic spoilage bacteria. In addition, LAB spoilage often develops gradually and at later stages, compared with spoilage by aerobic microorganisms, and therefore the shelf-life of food is prolonged. However, the extended shelf-lives and distribution ranges of refrigerated and minimally processed foods, such as meat and fish, have highlighted the role of LAB in quality deterioration.

Previous studies conducted at the Department of Food Hygiene and Environmental Health at the University of Helsinki have highlighted the role of certain *Leuconostoc* spp., bacteria belonging to LAB, in the spoilage of cooked meats (Björkroth et al., 1998; Korkeala et al., 1988), marinated poultry (Björkroth et al., 2000; Susiluoto et al., 2003), and fish products (Lyhs et al., 2004). The major focus of this doctoral thesis was on elucidating the spoilage role and contamination routes of spoilage *Leuconostoc* spp. in meat and vegetable-based foods commercially produced in Finland. In addition, the need to gain genomic information on spoilage leuconostocs to link the gene content, metabolism and physiology to food spoilage process initiated the final subproject of this thesis.
2 REVIEW OF THE LITERATURE

2.1 LAB AS FOOD SPOILAGE ORGANISMS

LAB spoilage of food is favoured in MAP, chilled food ecosystems, where psychrotrophic LAB have a considerable advantage in growth rate over aerobic and facultative anaerobic, gram-negative bacteria. During the last few decades, many studies have documented or examined LAB spoilage of MAP food commodities, particularly of cooked meats. The findings of these studies, as well as an overview of our current understanding of food spoilage by LAB have been summarised in several review articles and book chapters (Björkroth, 2005; Borch et al., 1996; Dainty & Mackey, 1992; Egan, 1983; Gram, 2006; Holzapfel, 1998; Korkeala & Björkroth, 1997; Ray & Bhunia, 2008; Samelis, 2006; Schillinger et al., 2006). Academic discussion has also lead to the establishment of concepts describing the process of food spoilage (Table 1).

Table 1  Concepts related to bacterial food spoilage

<table>
<thead>
<tr>
<th>Concept</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific spoilage organism</td>
<td>Single strain or a consortium of species responsible for spoilagea</td>
</tr>
<tr>
<td>Spoilage activity</td>
<td>Ability to produce specific sensory changes in a naturally spoiling producta</td>
</tr>
<tr>
<td>Spoilage association, spoilage population</td>
<td>Bacteria dominating the microbial community at the time of sensory rejectionc</td>
</tr>
<tr>
<td>Spoilage compound, spoilage metabolite</td>
<td>Compound produced by spoilage bacteria leading to undesirable changesa,c</td>
</tr>
<tr>
<td>Spoilage domain</td>
<td>Range of conditions under within the specific spoilage organisms cause spoilagea,d</td>
</tr>
<tr>
<td>Spoilage potential</td>
<td>Ability of a pure culture to produce spoilage reaction. Can be assessed in vivo (growth medium), in vitro (sterile model food system), or in situ (&quot;natural&quot; food)a,b</td>
</tr>
<tr>
<td>Spoilage reaction</td>
<td>Conversion of substrates to spoilage compounds and subsequently to a defecta</td>
</tr>
<tr>
<td>Spoilage substrate</td>
<td>Substrate metabolised by bacteria to spoilage compounda,oe</td>
</tr>
</tbody>
</table>

Adapted from aDalgaard, 2000, bDalgaard, 1995b, cGram & Huss, 1996, dDalgaard, 1995a, and eGram et al., 2002


2.2 GENUS LEUCONOSTOC

2.2.1 PHYLOGENY AND TAXONOMY
The name *Leuconostoc* means “colourless nostoc” where “nostoc” pertains to an algal genus (Euzeby, 2009). Phylogenetically, the genus *Leuconostoc* belongs to phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*. Leuconostocs are closely related to *Fructobacillus*, *Oenococcus* and *Weissella*, and together they are commonly known as the “*Leuconostoc* group” of LAB. Originally, the LAB included in the *Leuconostoc* group were all classified as *Leuconostoc* species. In the early 1990s, findings of molecular phylogenetic analyses led to a subdivision of the group *Leuconostoc* into three distinct lineages: the genus *Leuconostoc* sensu stricto, the *Leuconostoc paramesenteroides* group, and *Leuconostoc oenos* (Martinez-Murcia & Collins, 1990; Martinez-Murcia et al., 1993). During the past 15 years, studies employing both genetic and phenotypic tests have lead to several taxonomic revisions of the group *Leuconostoc* (Collins et al., 1993; Dicks et al., 1995; Endo & Okada, 2008). The *L. paramesenteroides* group originally consisting of *L. paramesenteroides* and some atypical, heterofermentative lactobacilli, have been placed in a new genus *Weissella* (Collins et al., 1993), whereas *L. oenos* has been reclassified as *Oenococcus oeni* (Dicks et al., 1995). More recently, *Leuconostoc durionis*, *Leuconostoc ficulneum*, *Leuconostoc fructosum* and *Leuconostoc pseudoficulneum* were assigned to a new genus *Fructobacillus* (Endo & Okada, 2008). After these reclassifications, the *Leuconostoc* sensu stricto includes, at the time of writing, 11 validly published species names (Table 2) with *L. mesenteroides* being the type species (Euzeby, 2009).

Table 2  
*Current composition of the genus Leuconostoc*

<table>
<thead>
<tr>
<th>Species designation</th>
<th>Source of type strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. carnosum</em></td>
<td>Chilled meat</td>
<td>Shaw &amp; Harding, 1989</td>
</tr>
<tr>
<td><em>L. citreum</em></td>
<td>Honey-dew of rye ear</td>
<td>Farrow et al., 1989</td>
</tr>
<tr>
<td><em>L. fallax</em></td>
<td>Sauerkraut</td>
<td>Martinez-Murcia &amp; Collins, 1991</td>
</tr>
<tr>
<td><em>L. gasicomitatum</em></td>
<td>Marinated broiler</td>
<td>Björkroth et al., 2000</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>Chilled meat</td>
<td>Shaw &amp; Harding, 1989</td>
</tr>
<tr>
<td><em>L. holzapfelii</em></td>
<td>Coffee fermentation</td>
<td>De Bruyne et al., 2007</td>
</tr>
<tr>
<td><em>L. inhae</em></td>
<td>Kimchi</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td><em>L. kimchii</em></td>
<td>Kimchi</td>
<td>Kim et al., 2000</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>Milk</td>
<td>Garvie, 1986</td>
</tr>
<tr>
<td><em>L. mesenteroides</em></td>
<td>Sugar beet solution</td>
<td>Garvie, 1986</td>
</tr>
<tr>
<td><em>L. palmae</em></td>
<td>Palm wine</td>
<td>Ehrmann et al., 2009</td>
</tr>
<tr>
<td><em>L. pseudomesenteroides</em></td>
<td>Cane juice</td>
<td>Farrow et al., 1989</td>
</tr>
</tbody>
</table>

Recent phylogenetic investigations of *Leuconostoc* spp. have confirmed the close evolutionary relationship among *Leuconostoc* spp. demonstrating that *Leuconostoc* is a well-defined genus (Chelo et al., 2007; Endo & Okada,
2008). With the exception of *L. fallax*, the nucleotide sequence similarities of 16S rRNA gene among the type strains of *Leuconostoc* spp. are relatively high, ranging from 97.3% to 99.5% (Björkroth & Holzapfel, 2006). A closer look at the phylogeny of *Leuconostoc* spp., as revealed by 16S rRNA gene sequence analysis, further divides them into three evolutionary branches including *L. citreum*, *L. holzapfelli*, *L. lactis* and *L. palmae* in the first branch, *L. mesenteroides* and *L. pseudomesenteroides* in the second and *L. carnosum*, *L. gasicomitatum*, *L. gelidum*, *L. inhae* and *L. kimchii* in the third branch, whereas *L. fallax* is genetically more distinct from the other *Leuconostoc* species (Björkroth & Holzapfel, 2006).

### 2.2.2 GENERAL CHARACTERISTICS

Garvie described the characteristics of the genus *Leuconostoc* in the previous edition (9th) of Bergey’s manual of systematic bacteriology (Garvie, 1986). *Leuconostoc* cells are Gram positive, small, regular, ovoid cocci occurring in pairs or short chains. The cells are non-motile and do not form spores. *Leuconostoc* spp. are considered psychrotrophic mesophiles with optimal growth at 14-30°C. The temperature limits for growth vary among species and strains ranging from 1-10°C to 30-40°C. The ability to grow at chilled temperatures (4°C or below) is particularly characteristic for strains of *L. carnosum*, *L. gasicomitatum*, *L. gelidum*, and *L. inhae* (Holzapfel et al., 2009). Other characteristics are resistance to vancomycin and a lack of L-arginine dihydrolase and catalase activities.

### 2.2.3 PHYSIOLOGY AND ENERGY METABOLISM

Leuconostocs are referred to as nutritionally “fastidious”, revealing their limited biosynthetic capacity and their requirements for multiple growth factors, preformed amino acids, purine and pyrimidine bases and many other nutrients. Sugars are primary energy and carbon sources for *Leuconostoc* spp., and most species are able to utilise a wide variety of mono- and disaccharides (Björkroth & Holzapfel, 2006). In contrast to many other LAB, leuconostocs lack Embden-Meyerhof-Parnas pathway, the most common type of glycolysis. Instead, leuconostocs are obligate heterofermentative organisms, which ferment glucose via the phosphoketolase pathway and produce equimolar amounts of lactic acid, CO₂ and ethanol (Figure 1). Under anaerobic conditions, the reduction of acetyl-phosphate (acetyl-P) to ethanol is essential and serves to oxidise the NADH generated during the conversion of hexoses to pentoses (Cogan & Jordan, 1994). When leuconostocs grow on pentoses, the NADH is not produced and acetyl-P is directed to the acetate branch of phosphoketolase pathway yielding acetate and extra ATP. In addition, leuconostocs may utilise alternative routes for NADH reoxidation (Axelsson, 2003; Zaunmüller et al., 2006). Many *Leuconostoc* strains also possess NADH oxidases which take over NAD⁺ regeneration in the presence...
of oxygen (Lucey & Condon, 1986). Furthermore, *Leuconostoc* use pyruvate, fructose or citrate as electron acceptors and subsequently generate acetate instead of ethanol (Erten, 1998; Zaunmüller et al., 2006). This so-called co-fermentation or co-metabolism of multiple carbon sources and growth in the presence of oxygen generates more ATP from glucose and leads to an increase in growth yield and specific growth rates (Borch & Molin, 1989; Cogan & Jordan, 1994; Lucey & Condon, 1986; Zaunmüller et al., 2006).

Other characteristic metabolic features of *Leuconostoc* species include production of D(-) lactate enantiomer, fermentation of fructose to mannitol and acetate, and co-metabolism of citrate and carbohydrate under reducing conditions to diacetyl (2,3-butanedione), CO₂ and acetoin (3-hydroxy-2-butanone) (Cogan & Jordan, 1994).

![Diagram of glucose metabolism in Leuconostocs](image)

**Figure 1** The phosphoketolase or heterofermentative pathway for glucose metabolism in leuconostocs. The acetate branch in dashed box takes place only in the presence of external electron acceptors. Modified from Cogan and Jordan (1994).
2.2.4 HABITATS
Leuconostocs thrive in decaying plant material and grow well in various fermented vegetable products, such as cucumber, kimchi, cabbage and olives (Kim & Chun, 2005; Mäki, 2004). Leuconostocs are also present in low numbers in green vegetation and roots (Hemme & Foucaud-Scheunemann, 2004; Mundt et al., 1967). Consequently, due to their presence in plant material, leuconostocs are frequently encountered in foods of animal origin, including raw milk and dairy products, meat, poultry and fish (Björkroth & Holzapfel, 2006). Healthy warm-blooded animals, including humans, are rarely reported to carry *Leuconostoc* in the microbiota of their gut or mucous membranes. However, *Leuconostoc* species have been recovered from the intestines of fish (Balcázar et al., 2007) and fresh anchovy (Belfiore et al., 2010), but not as a dominant bacterial species.

2.3 LEUCONOSTOCS IN FOODS AND FOOD PRODUCTION

2.3.1 BENEFICIAL ROLES

2.3.1.1 Food fermentation
Lactic fermentation is one of the oldest forms of food preparation and preservation and LAB, including leuconostocs, have a long history of safe use in foods. The positive effects of leuconostocs on dairy products were recognised already in the early 20th century, when researchers found that leuconostocs were responsible for a buttery aroma, a desirable characteristic of many dairy products (Dessart & Steenson, 1995; Thunell, 1995). Today, leuconostocs are important in the manufacture of fermented dairy, vegetable and cereal foods, and they contribute to the taste, texture, nutritional value and safety of fermented foods (Hemme & Foucaud-Scheunemann, 2004; Vedamuthu, 1994). When used as dairy starters, leuconostocs are often combined with acid-producing *Lactococcus lactis* strains whereas *Leuconostoc* strains are used due to their role in the formation of the buttery aroma (Vedamuthu, 1994).

2.3.1.2 Food biopreservation
Biopreservation is defined as the use of antagonistic microbes and their metabolites to inhibit undesired microbes to increase safety and shelf-life (Stiles, 1996). The ability of *Leuconostoc* spp. to promote safety or quality is linked to excreted organic acids and subsequent pH reduction, and many other antimicrobial compounds such as CO2, diacetyl and hydrogen peroxide.
Review of the literature

(H$_2$O$_2$) (Björkroth & Holzapfel, 2006; Hemme & Foucaud-Scheunemann, 2004; Stiles, 1996). In addition, the ability to produce bacteriocins, e.g. peptides with inhibitory effects on other LAB and closely related Gram positive bacteria, is well-described for Leuconostocs spp. (Björkroth & Holzapfel, 2006; Hastings et al., 1994; Hemme & Foucaud-Scheunemann, 2004; Parente et al., 1996; Stiles, 1994; Stiles, 1996; Xiraphi et al., 2008). The biochemically or genetically characterised bacteriocins from Leuconostoc spp. have proven to be heat-stable, nonlanthionine-containing, unmodified peptides (class II; Cotter et al. 2005), with the majority of them being classified as pediocinlike peptides (class IIa; Cotter et al. 2005). Leuconostoc bacteriocins often inhibit Listeria monocytogenes, and subsequently, most studies have focused on their anti-listerial properties on various food including meat, fish, fruits and vegetables (Jeppesen & Huss, 1993a; Jeppesen & Huss, 1993b; Trias et al., 2008). For instance, L. carnosum 4010, a strain producing leucocin A and leucocin C, inhibits L. monocytogenes and is used as a protective culture on cold-stored vacuum-packaged (VP) cooked meat products (Budde et al., 2003; Jacobsen et al., 2003). This strain has been patented by the Danish Meat Research Institute and commercialised as a bioprotective culture for packaged meat products by Chr. Hansen. In addition, bacteriocinogenic strains of L. gelidum (leucocin A-producing) and L. mesenteroides (an unidentified bacteriocin) are reported to inhibit spoilage bacteria and extend the shelf-life of VP beef (Leisner et al., 1996), sausages (Metaxopoulos et al., 2002) and sea food (Matamoros et al., 2009).

2.3.2 FOOD SPOILAGE

The first observation on Leuconostoc food spoilage probably dates back to 1861, the early days of bacteriology, when Louis Pasteur discovered that gelification in cane sugar syrup was due to small, bead-like bacterial cells (Leathers, 2005). In 1878, another French natural scientist, Philippe van Tieghem, studied these slime-forming bacteria and named them “Leuconostoc mesenteroides” (Euzeby, 2009). To date, Leuconostoc spp. are implicated in the spoilage of packaged, refrigerated foods, particularly of meat and meat products (Table 3). In most of the reported cases of Leuconostoc food spoilage, LAB numbers in the spoiled food had reached levels above 10$^7$ CFU/g.
Table 3  
Reports on Leuconostoc food spoilage

<table>
<thead>
<tr>
<th>Food involved</th>
<th>Defect</th>
<th>Species involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw meat and poultry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marinated broiler fillet</td>
<td>Blowing</td>
<td>L. gasicomitatum</td>
<td>Björkroth et al., 2000</td>
</tr>
<tr>
<td>Marinated pork</td>
<td>Off-odour</td>
<td>L. mesenteroides</td>
<td>Schirmer et al., 2009</td>
</tr>
<tr>
<td>VP heat-processed meat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood sausage</td>
<td>Exudate, sour odour</td>
<td>L. mesenteroides</td>
<td>Diez et al., 2008</td>
</tr>
<tr>
<td>Cured frankfurters</td>
<td>Discolouration</td>
<td>L. mesenteroides</td>
<td>Anifantaki et al., 2002</td>
</tr>
<tr>
<td>Sausages</td>
<td>Discolouration</td>
<td>L. gelidum</td>
<td>Kröckel, 2006</td>
</tr>
<tr>
<td>Sausages</td>
<td>Slime</td>
<td>L. mesenteroides</td>
<td>Korkeala et al., 1988</td>
</tr>
<tr>
<td>Sliced, cured ham</td>
<td>Blowing, slime</td>
<td>L. mesenteroides</td>
<td>Samelis et al., 2000a</td>
</tr>
<tr>
<td>Sliced, cured ham</td>
<td>Slime, blowing</td>
<td>L. carnosum, L. mesenteroides</td>
<td>Yang &amp; Ray, 1994</td>
</tr>
<tr>
<td>Sliced ham</td>
<td>Discolouration</td>
<td>L. gelidum</td>
<td>Cai et al., 1998</td>
</tr>
<tr>
<td>Sliced ham</td>
<td>Sour odour</td>
<td>L. carnosum</td>
<td>Björkroth et al., 1998</td>
</tr>
<tr>
<td>Vienna sausages</td>
<td>Blowing, slime</td>
<td>L. gelidum, L. mesenteroides</td>
<td>Dykes et al., 1994</td>
</tr>
<tr>
<td>Whole ham</td>
<td>Blowing</td>
<td>L. carnosum</td>
<td>Samelis et al., 2006</td>
</tr>
<tr>
<td>Fruits and vegetables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrots</td>
<td>Exudation</td>
<td>L. mesenteroides</td>
<td>Torriani et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. citreum</td>
<td></td>
</tr>
<tr>
<td>Fruit juices</td>
<td>Slime, off-odour</td>
<td>Leuconostoc spp.</td>
<td>Hays, 1951</td>
</tr>
<tr>
<td>Kimchi</td>
<td>Slime</td>
<td>L. lactis</td>
<td>Kim et al., 2001</td>
</tr>
<tr>
<td>Thawed, frozen peas</td>
<td>Discolouration,</td>
<td>Leuconostoc spp.</td>
<td>Cavett et al., 1965</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandwiches</td>
<td>Blowing</td>
<td>L. mesenteroides</td>
<td>Smith et al., 1983</td>
</tr>
<tr>
<td>Marinated fish</td>
<td>Slime, off-odour</td>
<td>L. gasicomitatum, L. gelidum</td>
<td>Lyhs et al., 2004</td>
</tr>
<tr>
<td>semi-preserve</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\text{\textit{VP}}=\text{Vacuum packaged}\)

### 2.3.3 SAFETY HAZARDS

Due to their long history in food fermentation, leuconostocs are considered harmless for human consumption and have a “generally recognised as safe” status (Dessart & Steenson, 1995). Nevertheless, *L. citreum, L. lactis, L. mesenteroides* and *L. pseudomesenteroides* have caused human infections, most of them affecting neonates or health-compromised patients (Bou et al., 2008; Florescu et al., 2008; Ogier et al., 2008; Yamazaki et al., 2009). Based on these reports and reviews, the predisposing risk factors for *Leuconostoc* infections include the use of enteral or central venous catheter, enteral tube and long-term vancomycin therapy. Furthermore, contaminated infant or enteral formula have been identified as sources of *Leuconostoc* infections, whereas no human cases have been directly associated with the consumption of fermented or spoiled food.

In addition to infections, certain *Leuconostoc* spp. may form biogenic amines with vasoactive and psychoactive properties and thus represent a possible health risk for the consumer when this occurs in a food system (de Llano et al., 1998; Ouwehand & Salminen, 2003). Researchers have
examined the ability of *Leuconostoc* spp. associated with food fermentation or spoilage to produce biogenic amines (Bover-Cid & Holzapfel, 1999; de Llano et al., 1998; Moreno-Arribas et al., 2003; Murros, 2004; Nieto-Arribas et al., 2010; Pircher et al., 2007). These studies have revealed that while many strains do not produce biogenic amine, specific strains of *L. mesenteroides*, *L. lactis* and *L. carnosum* may form tyramine or histamine to amounts associated with health hazards. In addition to strain-specific amine-formation capacity, food-related factors such as the pH, NaCl concentration, availability of substrate, etc., influence the build-up of biogenic amines in a food system.

Additionally, the D (-) lactate produced by all leuconostocs during food fermentation may, in high doses, lead to metabolic acidosis, particularly in infants and patients suffering from short bowel syndrome or other absorption conditions (Ouwehand & Salminen, 2003). However, for healthy adults, normal consumption of fermented foods is not hazardous.

### 2.4 LEUCONOSTOCS IN FOOD SPOILAGE

#### 2.4.1 SPECIES INVOLVED IN SPOILAGE OF COLD-STORED FOODS

Although many *Leuconostoc* species may cause food spoilage (Table 3), leuconostocs clearly differ in their ability to survive, compete and cause spoilage reactions in chilled foods (Björkroth et al., 1998; Hamasaki et al., 2003; Samelis et al., 1998; Samelis, 2006; Samelis et al., 2000a; Samelis et al., 2000b). In general, factors such as product characteristics and processing and storage conditions are likely to play a major role in determining which species or strains become dominant or cause spoilage. In addition, species variation in raw materials and the possible differences in contamination patterns may contribute to the predominance of specific species in a food system. On the other hand, Björkroth et al. (1998) recovered several contaminating *L. carnosum* strains from a meat-processing environment, but found that only a single one prevailed in the spoiled ham produced at this plant. These authors concluded that the predominance of this particular spoilage strain was due to its physiological characteristics improving its adaptation and competence in cooked, VP ham.

In addition, microbial interaction has been suggested to play a crucial role in development of the spoilage population (Borch et al., 1996; Zhang & Holley, 1999). Indeed, a recent study showed that co-culturing *Leuconostoc* strains with other LAB affected their activity and the results from co-culture model systems differed from those expected based on pure culture studies (Diez et al., 2009a). Furthermore, researchers have also proposed that bacteriocin production is an important attribute of spoilage leuconostocs (Borch et al., 1996; Osmanagaoglu, 2003; Yang & Ray, 1994; Zhang & Holley, 1999). In contrast to these studies, Björkroth et al. (1998) reported that a
*L. carnosum* strain associated with ham spoilage did not produce bacteriocins.

### 2.4.2 FACTORS AFFECTING LEUCONOSTOC GROWTH IN FOODS

An understanding of why leuconostocs dominate in the spoilage process would allow manipulation of the bacterial dominance patterns and support of species with low spoilage potential. Therefore, researchers have aimed to describe how changes in storage conditions and product characteristics affect the growth of spoilage leuconostocs (see references cited in footnotes in Table 4). In addition, researchers have developed a kinetic model for the growth response of *L. mesenteroides* on a cooked-meat model system (García-Gimeno et al., 2005). Although the growth limiting conditions vary depending on species and strain and the food system, the findings of studies on *Leuconostoc* spoilage of heat-processed meats (summarised in Table 4) provide background for an understanding of why certain foods favour the growth of leuconostocs making them susceptible to spoilage.

#### Table 4  Important factors affecting the growth of leuconostocs in chilled meat products

<table>
<thead>
<tr>
<th>Character</th>
<th>Effect on Leuconostoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental factors</td>
<td></td>
</tr>
<tr>
<td>Gas composition</td>
<td>Elevated levels of CO(_2) are not inhibitory. O(_2) increases growth and</td>
</tr>
<tr>
<td></td>
<td>predisposes to discolouration and off-odours(^{a,b})</td>
</tr>
<tr>
<td>Temperature</td>
<td>Decreasing storage temperature decreases growth(^a). Certain strains may grow at -1.5°C(^c)</td>
</tr>
<tr>
<td>Product related factors</td>
<td></td>
</tr>
<tr>
<td>Brine</td>
<td>Increasing concentration in meat product improves growth(^d)</td>
</tr>
<tr>
<td>Glucose</td>
<td>Increasing concentration increases rate of growth and spoilage(^{a,e,f})</td>
</tr>
<tr>
<td>NaCl</td>
<td>Increasing concentration increases adaptation stage and decreases growth rate(^b)</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Effect varies among species(^{b,g})</td>
</tr>
<tr>
<td>Smoke (phenol)</td>
<td>Retards growth(^{a,c,i,h})</td>
</tr>
</tbody>
</table>

Modified from: \(^{a}\)Samelis et al., 2000a, \(^{b}\)Zurera-Cosano et al., 2006, \(^{c}\)Schillinger et al., 2006, \(^{d}\)Samelis et al., 2000b, \(^{e}\)Jacobsen et al., 2003, \(^{f}\)Samelis et al., 1998, \(^{g}\)Hamasaki et al., 2003, and \(^{h}\)Anifantaki et al., 2002

### 2.4.3 FOOD SPOILAGE REACTIONS

*Leuconostoc* food spoilage may appear in diverse forms, all being consequences of the accumulation of spoilage compounds. Thus, the spoilage potential of leuconostocs is not solely dependent on their growth, but also on their metabolic activities. The spoilage metabolites produced reflect the physiological activities of the cell and hence vary depending on the conditions of the food system, including oxygen tension, pH and carbon sources available. Furthermore, metabolic differences among species and
strains affect their spoilage potential and the spoilage reactions produced in a
given food system.

The physiology, biochemistry and genetics of *L. mesenteroides* and
*L. citreum* have gained attention owing to the economic importance of these
species in dairy and vegetable fermentation. In contrast, the metabolic
activities of *Leuconostoc* species associated with meat spoilage have received
less attention. However, information gained about the *Leuconostoc* strains
used for food fermentation help the researcher to understand the metabolic
background of spoilage reactions caused by *Leuconostoc* spp.

2.4.3.1 Sour off-odours and flavours

Sour odours characteristic to *Leuconostoc* spoilage arise from the
accumulation of D(-) lactic and acetic acid (Borch & Agerhem, 1992; Dainty,
1996; Drosinos & Board, 1995; Kakouri & Nychas, 1994; Laursen et al.,
2009). Acetic acid, in particular, has a sharp, vinegar-like flavour, which in
certain foods, such as meats, has an undesirable impact on the sensory
properties even at low levels (Samelis, 2006; Vermeiren et al., 2005). In
general, acetate formation is enhanced in the presence of oxygen (Nuraida et
al., 1992; Sakamoto & Komagata, 1996; Samelis et al., 2000a; Zaunmüller et
al., 2006). The amount of acetic acid formed varies depending on the species
and strain, as well as the substrates available (citrate, fructose, ribose) in the
food system (Borch & Molin, 1989; Nychas et al., 1998; Vermeiren et al.,
2005; Zaunmüller et al., 2006).

2.4.3.2 Gas formation

Gas formation and undesirable extension (blowing) of the package is often
the first indication of food spoilage by leuconostocs and other
heterofermentative LAB (Borch et al., 1996; Korkeala et al., 1990; Ray &
Bhunia, 2008; Samelis et al., 2000a; Yang & Ray, 1994). This spoilage
reaction is characteristic to VP processed meats such as ham (Table 3). The
gas is mainly CO₂ which *Leuconostoc* spp. form from various carbon sources.

2.4.3.3 Buttery off-odour

Leuconostocs produce diacetyl (2,3-butanedione), the compound mainly
responsible for the desirable buttery flavour in many dairy products.
However, in fruit juices, beer and meat and fish products, a buttery odour is
often undesirable (Borch et al., 1996; Lawlor et al., 2009; Lyhs et al., 2004).
Along with diacetyl, acetoin (3-hydroxybutanone) and 2,3-butanediol, which
are derived from diacetyl by reduction, contribute to the formation of a
buttery flavour. Studies on diacetyl and acetoin production by *Leuconostoc*
spp. have mainly included strains of dairy importance, or focused food
containing citrate (fruits, milk, vegetables) or fructose (fruits or vegetables). The diacetyl generated by *Leuconostoc* spp. originates from the chemical oxidative decarboxylation of α-acetolactate, which explains why oxygen often stimulates diacetyl formation (Axelsson, 2003).

### 2.4.3.4 Milky purge

Release of a milky or cloudy liquid, often referred to as drip or purge, is a common characteristic of LAB spoilage of cooked meats (Diez et al., 2009a). In meat, the drip loss results from a reduction in pH and decreased water holding capacity of meat proteins. Occasionally, lactic acid may change the appearance of the drip from transparent to white or grey (Hamasaki et al., 2003; Korkeala & Björkroth, 1997).

### 2.4.3.5 Slime formation

Leuconostocs producing slime have long been recognised as troublesome contaminants in sugar refineries, where slime plugging the filtration machinery disrupts the refining process. In addition, slime formation by leuconostocs is a common defect in meat and vegetable products (Table 3). In general, slime or viscosity produced by LAB is due to the secretion of long-chain, gelling extracellular polysaccharides (EPS) (Korkeala et al., 1988; Leathers, 2005). The EPS formed by leuconostocs in sugar refineries and sucrose-containing meat products is dextran, a glucose homopolysaccharide synthesised from sucrose (Leathers, 2005).

### 2.4.3.6 Meat discoloration

Colour is the major quality criterion consumers use when purchasing meat and meat products. In cured, cooked meats certain hydrogen peroxide-producing LAB, including leuconostocs, may cause greenish discolorations (Evans & Niven, 1951). This defect arises from the reaction of H₂O₂ with myoglobin (nitrosohemochrome) in cured meat and leads to the formation of greenish choleomyoglobin (Borch et al., 1996; Egan, 1983; Evans & Niven, 1951). Because leuconostocs generate H₂O₂ only in the presence of oxygen (Kandler, 1983; Lucey & Condon, 1986), discoloration typically appears when meat products are exposed to air, *e.g.* after opening of the packages (Anifantaki et al., 2002; Borch et al., 1996; Samelis et al., 1998).

In addition, *L. gelidum* has been reported to cause yellow spots on VP uncured meat products (Cai et al., 1998; Kröckel, 2006). However, the chemical identity of the yellow compound is unknown.
2.5 STRATEGIES TO CONTROL LEUCONOSTOC FOOD SPOILAGE

2.5.1 MANAGING CONTAMINATION DURING MEAT PROCESSING

It is currently unclear how spoilage leuconostocs enter the meat production chain and what the role of meat animals is in contamination of the processing area. Leuconostocs have rarely been recovered from healthy, warm-blooded animals and thus leuconostocs in meat are suggested to be environmental contaminants originating from the processing environment or animal hides (Hemme & Foucaud-Scheunemann, 2004). However, once present on meat, the contaminated meat may act as a carrier of leuconostocs throughout the processing line (Vasilopoulos et al., 2010). Although several researchers have investigated LAB contamination at different stages of meat processing, only a few surveys have reported the species recovered (Björkroth et al., 1998; Samelis et al., 2000b; Vasilopoulos et al., 2010). Collectively, these studies have demonstrated the presence of Leuconostoc spp. in raw meat and meat masses, and revealed that air-flows and cross contamination during production (curing, slicing, packing) spread leuconostocs. Production line and practices need to be organized to minimize and prevent meat contamination particularly during the handling of cooked products (Björkroth et al., 1998; Vasilopoulos et al., 2010). The results of these contamination studies have also lead the researchers to speculate that the refrigerated meat processing facilities provide conditions which allow for the survival or even thriving of psychrotrophic LAB including leuconostocs (Samelis et al., 2000b).

2.5.2 PRODUCT REFORMULATION

When applicable, manipulation of product formulation may inhibit spoilage activities of leuconostocs. For instance, as sucrose is the precursor for dextran (slime) production, the substitution of sucrose with another sweetener such as glucose, sugar alcohols or artificial sweeteners prevents dextran formation (Aymerich et al., 2002; Deibel & Niven, 1959; Magnusson & Møller, 1985).

Occasionally, minor food ingredients such as spices, herbs and sweeteners may stimulate the growth of leuconostocs. Kivanç et al. (1991) reported that cumin stimulated the growth of L. mesenteroides in a broth culture. Furthermore, tomato-based foods (i.e. juice, paste, extracts) contain D-pantothenate (also known as “tomato juice growth factor”), a substrate required by Leuconostoc spp. (Foucaud et al., 1997; Zaunmüller et al., 2006). Indeed, findings on Leuconostoc spoilage of marinated broiler fillets strips suggested that the marinade containing tomatoes and sugars stimulated the growth of L. gasicomitatum and a subsequent voluptuous in-package gas formation (Björkroth et al., 2000).
2.5.3 PROCESSING TECHNIQUES

Researchers continue to explore and examine the potential of both traditional and novel processing techniques to control spoilage leuconostocs in foods. These strategies alone may not entirely prevent *Leuconostoc* growth, but limit the rate and nature of spoilage and allow an extension of shelf-life.

2.5.3.1 Thermal processing

Heating causes irreversible damage to cell membrane, ribosomes and proteins, and thus leads to cell inactivation. Most LAB are killed at 60°C, making thermal processing, when applicable, an effective preservation method. In general, traditional cooking or smoke-cooking processes applied to meat products inactivates leuconostocs (Franz & Von Holy, 1996b; Samelis et al., 2000b). Previous findings have demonstrated that surviving (culturable) leuconostocs are not detected from cooked meats sampled immediately after heat treatment (Björkroth & Korkeala, 1997; Björkroth et al., 1998; Samelis et al., 1998; Vasilopoulos et al., 2010). However, based on the observations on the spoilage of VP cooked ham, Samelis et al. (2006) suggested that a small number of *L. carnosum* cells are likely to have survived cooking although they remain unculturable directly after heat treatment.

In addition to cooking processes, researchers have investigated the potential of in-packaging thermal pasteurisation to inactivate LAB recontamination on VP cooked meats (Diez et al., 2009b; Franz & Von Holy, 1996a; Franz & Von Holy, 1996b). Depending on the severity of the treatment, in-package pasteurisation may be effective in reducing the growth of *Leuconostoc* spp. and delaying product spoilage (Diez et al., 2009b; Franz & Von Holy, 1996a). However, as in-package pasteurisation may promote the growth of spore-forming bacteria, researchers have warned that this technique might compromise product safety (Franz & Von Holy, 1996a; Samelis et al., 1998).

2.5.3.2 Processing using high pressure

Researchers and the food industry are actively investigating the applicability of high hydrostatic pressure as a non-thermal food preservation method. High pressure processing (HPP) is currently commercially applied mainly for fruit juices and deli style meats. Similarly with thermal processing, HPP treatment damages the cell membranes as well as ribosomes and cytoplasmic proteins (Kaletunc et al., 2004). Previous studies have examined the inactivation of *Leuconostoc* spp. in HPP treated orange juice (Basak et al., 2002) and on cooked meat (Diez et al., 2008; Diez et al., 2009b; Jofré et al., 2009). In the case of VP blood sausages, HPP treatment extended the shelf-
life of the products by delaying the growth of *L. mesenteroides* and *Weissella viridescens* (Diez et al., 2008; Diez et al., 2009b). However, these authors noticed that sublethally injured *L. mesenteroides* cells were able to recover from HPP within 35 days of refrigerated storage. Similarly, *L. carnosum* recovered on chilled, VP ham treated previously with HPP (Jofré et al., 2009).

### 2.5.3.3 Processing with pulsed electric fields

Pulsed electric field processing is another non-thermal food preservation method used to inhibit microorganisms mainly in beverages, yoghurts and liquid foods. This treatment applies short, high voltage pulses to foods, producing high electric fields between two electrodes. Aronsson et al. (2001) used electron microscopy to study the effect of pulsed electric field treatment on *L. mesenteroides* and found that it damaged the cell membrane by causing it to detach from the cell wall. This treatment alone, however, was not effective against *L. mesenteroides* in orange juice concentrate, whereas pulsed electric field treatment in combination with moderate heat (60°C) reduced *L. mesenteroides* (McDonald et al., 2000).

### 2.5.4 BIOPRESERVATION AND ANTIMICROBIALS

#### 2.5.4.1 Biopreservation with bacteriocins and protective cultures

Concerning inhibition of leuconostocs using preformed bacteriocins, the interest of researchers has focused primarily on nisin. This bacteriocin, produced by *Lc. lactis* subsp. *lactis*, and is currently the only bacteriocin in widespread commercial use as a food preservative (E234). In the European Union, nisin is allowed in certain dairy products and puddings, but currently not in those food categories (such as meat, fish or vegetable foods) where *Leuconostoc* spoilage commonly occurs. However, studies have demonstrated that nisin delays or prevents *Leuconostoc* spoilage in cured or cooked meat products (Aymerich et al., 2002; Davies et al., 1999; Gill & Holley, 2000). Furthermore, Gill and Holley (2003) reported that nisin together with lysozyme effectively delayed the growth of *L. mesenteroides* on VP cooked bologna.

Another approach for biopreservation is to add antagonistic bacterial preparations as protective cultures. However, only few biopreservation studies have obtained successful results in using protective cultures towards *Leuconostoc* spp. in a model food system. For instance, inoculation of cooked ham with a specific *Lactobacillus sakei* strain inhibited the growth of *L. mesenteroides* thereby prolonging the shelf-life (Hu et al., 2008; Vermeiren et al., 2006). Additionally, *Lactobacillus plantarum* IMPC LP4
prevented the growth of spoilage-causing leuconostocs in cold-stored, shredded carrots (Torriani et al., 1999). However, to be suitable for commercial application, these technologies may face practical limitation (Rodgers, 2008). For instance, a protective culture must be commercially available, easy to apply, and give a reproducible response without negatively impacting the sensory properties (Rodgers, 2008).

### 2.5.4.2 Organic acids

Organic acids and their salts, mainly lactates and (di)acetates, are used in the food industry as preservatives. A few recent studies have evaluated the effects of lactates and acetates on meat spoilage leuconostocs (Devlieghere et al., 2009; Diez et al., 2009b; Samelis & Kakouri, 2004). According to these reports, the effects on leuconostocs depend on the product characteristics and the organic acids used. For example, mixtures of sodium acetate and sodium lactate in cured meat formulation inhibited slime-producing leuconostocs and extended the shelf-life of VP frankfurters (Samelis & Kakouri, 2004) and cooked ham (Devlieghere et al., 2009). In contrast, the inclusion of lactates in blood sausages failed to delay spoilage caused by leuconostocs (Diez et al., 2009b).

### 2.5.4.3 Plant antimicrobials

Many plant-derived compounds, such as the essential oils and phenolic compounds extracted from spices, herbs and vegetables, have antibacterial properties and have therefore been examined for their potential to preserve foods (Tassou et al., 2004). Some work has also been published on the inhibitory effects of plant-derived antimicrobials on the growth of leuconostocs (Blaszyk & Holley, 1998; Kivanç et al., 1991; Sagdic et al., 2005; Schirmer & Langsrud, 2010). For instance, eugonol, found in sage and oregano, and extract from black thyme are reported to inhibit leuconostocs in laboratory media (Blaszyk & Holley, 1998; Sagdic et al., 2005). The doses required for inhibition of leuconostocs are higher than those normally used in spiced food and may have an unacceptable impact on sensory properties (Samelis, 2006; Tassou et al., 2004). Furthermore, the levels preventing the growth on laboratory media are often insufficient to cause inhibition in food due to immobilisation of the antimicrobial by fats, proteins or carbohydrates, differences in water activity or changes in the bacterial cell (Tassou et al., 2004). Recently, Schirmer and Langsrud (2010) reported that several plant antimicrobials, such as thymol and rosemary extract, suitable for meat marinades, prevented the growth of *L. carnosum* and *L. mesenteroides* in a microplate model system, but when applied on VP pork, they neither reduced the microbial growth nor improved the shelf-life.
3 AIMS OF THE STUDY

The focus of this work was to describe the role of leuconostocs in spoilage of MAP refrigerated foods, and increase an understanding of their spoilage potential and distribution in foods. Consequently, the specific aims of this thesis were to study:

1. the role of *Leuconostoc* spp. in spoilage of MAP beef steaks (I) and in VP vegetable sausages (II)

2. whether *Leuconostoc* spp. involved in the spoilage of marinated broiler fillet products also contribute to the shelf-life quality of non-marinated broiler products (III)

3. the role of broilers and processing plant air as sources of *Leuconostoc* contamination during the processing of broiler products (III)

4. the distribution of *L. gasicomitatum* strains in different foods (IV)

5. the genome of *L. gasicomitatum* LMG 1881T with a special focus on the metabolic routes related to spoilage reactions (V)
4 MATERIALS AND METHODS

4.1 SAMPLING AND MICROBIOLOGICAL ANALYSES

4.1.1 FOOD SAMPLING AND LAB ENUMERATION (I-III)
Food products (Table 5) were received either directly from the manufacturers (I, II) or purchased from local supermarkets (III). The products were stored at 6°C and analysed at the use-by date (+/- 1 d).

A sample of either 22 g or 25 g was first homogenised in a peptone saline solution (0.9% NaCl, 0.1% peptone) and then diluted in the same solution up to 10⁻⁷. For the enumeration of LAB, 0.1 ml of dilutions were spread onto de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, UK) plates, and incubated anaerobically at 25°C for 5 days.

Table 5 Description of foods analysed during studies I-III

<table>
<thead>
<tr>
<th>Study</th>
<th>Food</th>
<th>Sensory quality</th>
<th>No. of packages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>High-oxygen MAP marinated beef steaks</td>
<td>Unacceptable</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>High-oxygen MAP moisture-enhanced beef steaks</td>
<td>Unacceptable</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>VP vegetable sausages</td>
<td>Acceptable</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>Unacceptable</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>MAP non-marinated broiler meat</td>
<td>Acceptable</td>
<td>39</td>
</tr>
</tbody>
</table>

MAP= modified atmosphere packaged; VP = Vacuum-packaged

4.1.2 CARCASS SAMPLING, AND ENRICHMENTS FOR TARGETED LAB (III)
Samples of neck skin, oropharynges and feather shafts were collected from broiler carcasses during four visits to the scalding and defeathering sections of a broiler slaughterhouse. For cold-enrichment of psychrotrophic LAB, 43 carcass samples were placed separately in 10 ml MRS broth (Difco, Detroit, USA) and incubated at 6°C. If no growth was observed after 38-days’ incubation, incubation was continued at 25°C for the recovery of LAB in general. For enrichment of leuconostocs and other vancomycin-resistant LAB, 31 samples were placed separately in tubes containing MRS broth supplemented with 10 μg/ml vancomycin (Sigma, St. Louis, USA) and incubated at 25°C for 5 days.

After the enrichment procedures described above, 10 μl from each MRS broth culture was streaked onto MRS agar plate and incubated anaerobically at 25°C for 5 days to produce individual colonies.
4.1.3 AIR SAMPLING (III)

For the recovery of airborne LAB, air samples were collected during two visits to the broiler meat processing plant of the same unit where broiler carcass sampling was conducted. Sampling sites within the processing plant included areas for air chilling, cutting, deboning and packaging. Using a Reuter centrifugal sampler (RSC sampler; Biotest Diagnostics Corp, Denville, USA), 43 air samples were collected on plastic strips filled with MRS agar. After sampling, the agar strips were incubated anaerobically at 25°C for 5 days.

4.2 LAB ISOLATES AND CULTURE CONDITIONS AND MAINTENANCE (I-III)

For each food sample, 10 to 20 colonies were randomly picked from MRS agar plates of the highest dilution. One to five colonies were picked from the MRS plates or strips cultured from the enriched carcass samples and the air samples, respectively. The colonies were purified by subculturing in MRS broth and streaking onto MRS agar. Each isolate was preserved in Cryovials (Nalgene, New York, USA) at −72°C in MRS broth. Working cultures were prepared by culturing in MRS broth and MRS agar at 25°C.

4.3 RIBOTYPING-BASED IDENTIFICATION OF LAB ISOLATES (I-III)

Ribotyping (Grimont & Grimont, 1986) refers to the analysis of restriction fragment length polymorphism patterns (ribotypes or ribopatterns) generated after hybridisation of restriction endonuclease treated DNA with probes targeting to 16S and 23S rRNA encoding regions. Identification of LAB isolates was based on comparing their ribotypes with those of LAB type strains deposited in the existing in-house ribotyping database. At the time of analysis, this database contained ribotypes of over 350 type and reference strains of relevant food-associated LAB.

4.3.1 RIBOTYPING PROCEDURE

The genomic DNA from each strain was extracted with a guanidinium isothiocyanate-based method (Pitcher et al., 1989) with modifications in the lysis protocol (Björkroth & Korkeala, 1996). DNA (8 μg) was cleaved with restriction endonuclease \textit{HindIII} (New England Biolabs, Ipswich, USA) and the restriction fragments were separated by agarose gel electrophoresis. A digoxigenin (DIG) labelled DNA Molecular Weight Marker II (Roche
Diagnostics, Penzberg, Germany) was used as a fragment size marker. The separated fragments were vacuum-transferred from the gel onto a nylon membrane and fixed to it by cross-linking with UV light.

During studies I and II, a specific set of DIG-labelled oligonucleotide probes, OligoMix5 (Regnault et al., 1997), were synthesised by Oligos Etc., Inc. (Wilsonville, Oreg.). In study III, DIG-labelled (DIG DNA labelling kit; Roche Applied Science; Mannheim, Germany) cDNA generated from 16S and 23S rRNA from *Escherichia coli* was used as probes. The membranes were hybridised and washed, after which the hybridised fragments were visualised by immunoenzymatic detection of the DIG labelled probes as instructed in the supplier's manual (DIG nucleic acid detection kit; Roche Applied Science). The ribotyping fingerprints (ribotypes) on the membranes were scanned and imported into Bionumerics (Applied Maths, Saint-Martens-Latem, Belgium) software as Tagged Image File Format files. The images of ribotypes were stored, processed and analysed using Bionumerics.

### 4.3.2 NUMERICAL ANALYSIS OF RIBOTYPES AND LAB IDENTIFICATION

Bionumerics software was used for processing the ribotyping data and grouping by numerical analysis the ribotypes of LAB isolates with the existing ribotype entries in the ribotyping database. To compare the ribotypes, the percentage similarity of ribotypes was calculated using the band-based Dice similarity coefficient. For visualisation of the pattern similarities, dendrograms were generated using the unweighted pair group method with arithmetic averages (UPGMA). During pattern comparison, a band position tolerance of 1.5% (III) or 1.8% (I,II) was taken into account. In the dendrograms, the ribotypes matching or clustering closely with the ribotypes of type strains were considered to represent the respective species.

### 4.4 SPOILAGE ACTIVITIES OF *LEUCONOSTOC* STRAINS (I, II)

The ability of selected strains of leuconostocs to cause spoilage reactions was tested by inoculating pure cultures onto fresh beef steaks (I) or vegetable sausages (II). The inoculated samples were packaged and stored under conditions similar to those for the commercial product. In addition, uninoculated control samples were included in the trials, to ensure that possible spoilage reactions in inoculated samples were not caused by chemical reactions or microbes developing from natural contamination. After storage, sensory panellists evaluated the sensory quality by describing the potential defects and rating the severities of spoilage reactions.
4.5 *L. GASICOMITATUM STRAINS* (II, IV, V)

The *L. gasicomitatum* strains, including *L. gasicomitatum* LMG 18811\textsuperscript{T}, were from our in-house *L. gasicomitatum* strain collection. For Study IV, the strains (*n* = 384) were chosen so as to represent foods analysed during 1997-2008 and different ribotypes obtained. Several of these strains were described earlier (Björkroth et al., 2000; Lyhs et al., 2004; Susiluoto et al., 2003, Studies I and II).

4.6 PFGE TYPING OF *L. GASICOMITATUM STRAINS* (IV)

PFGE typing was conducted as described by Björkroth et al. (1998). In short, genomic DNA was extracted from cells embedded in agarose plugs and digested overnight with 25-35 U of the restriction enzyme *SmaI* (New England Biolabs). DNA fragments were resolved by electrophoresis in 1% SeaKem Gold agarose (Lonza Rockland, Rockland, USA) using a contour-clamped homogeneous electric fields PFGE apparatus (Bio-Rad CHEF-DRIII system; Hercules, USA). Electrophoresis was carried out with the following PFGE parameters: initial switching time 0.5 s; final switching time 25 s; at 6 V/cm for 20 h. A lambda DNA ladder (New England Biolabs) was used as a molecular size marker. Tagged Image File Format images of banding patterns were analysed using Bionumerics. For construction of the dendrogram, clustering was performed using the Dice coefficient and the UPGMA method with arithmetic averages.

4.7 GENOME ANALYSIS OF *L. GASICOMITATUM* LMG 18811\textsuperscript{T} (V)

The genome of *L. gasicomitatum* LMG 18811\textsuperscript{T} was sequenced at the Institute of Biotechnology, University of Helsinki, Helsinki, Finland. From purified and sheared DNA, fosmid and plasmid libraries constructed. Sanger sequencing was carried out using Big-Dye (Applied Biosystems) terminator chemistry on ABI3700 sequencing machines. The conventional Sanger strategy was used to determine the draft sequence to ca. eight-fold coverage. Sequence reads were assembled into contigs with Phrap and finished and edited using GAP4 (Staden et al., 2000).

To identify protein coding genes, EasyGene (Nielsen & Krogh, 2005) and Glimmer (Delcher et al., 2007) gene-finding software was used, after which the predicted genes were reviewed and altered when appropriate. The initial automated assignment for gene name and function was conducted using the Manatee annotation platform (http://manatee.sourceforge.net/). Predicted protein coding genes were searches against public databases (e.g. UniProt, KEGG, Pfam, InterPro) with BLAST (Altschul et al., 1997). When
appropriate, the gene name and description of the product were manually curated based on the results of these comparisons. In addition, literature was used to identify genes and pathways involved in spoilage reactions.

The nucleotide sequence was deposited in GenBank under accession number FN822744.
Results

5 RESULTS

5.1 ROLE OF LEUCONOSTOC IN SPOILAGE (I-III)

5.1.1 LAB LEVELS AND LEUCONOSTOC DETECTED
The LAB counts in spoiled beef steaks (I) and vegetable sausages (II) were above $6 \times 10^8$ CFU/g. In Study III, LAB on non-marinated broiler products analysed at the end of shelf-life had reached counts ranging from $10^6$ to $10^8$ CFU/g. Among these predominant LAB in food products analysed during studies I-III, we identified 153 isolates either as *L. carnosum*, *L. gasicomitatum*, *L. gelidum* or *L. mesenteroides*. Figure 2 shows the dendrogram constructed from the different ribotypes obtained from the leuconostocs isolated during Studies I-III and those of the *Leuconostoc* type strains. The distribution of ribotypes into *Leuconostoc* species clusters and the sources and number of isolates representing each ribotype are shown (Figure 2).

5.1.2 ROLE IN SPOILAGE AND SHELF-LIFE QUALITY OF FOODS
Leuconostocs formed a major part of the spoilage LAB population in commercial beef steaks and vegetable sausages, but were rarely recovered among the predominating LAB in non-marinated broiler products analysed at the end of shelf-life. Table 6 lists the *Leuconostoc* species detected in different foods examined during Studies I-III, and the proportion of each species among the LAB isolates identified from the respective food group.

To determine whether the leuconostocs isolated from spoiled, commercial products were responsible for the spoilage reactions, we re-inoculated *Leuconostoc* strains onto respective fresh food samples and followed their ability to produce spoilage. The spoilage activities of the *Leuconostoc* strains tested are summarised in Table 6. Moreover, the sensory panellists scoring the intensities of the spoilage defects noticed differences in the severities of some spoilage reactions depending on the strain inoculated (see Study I, Table 2, p. 344; and Study II, Table 3, p. 2314).
Figure 2  UPGMA dendrogram showing the different *Hind*III ribotype patterns from the leuconostocs isolated during Studies I-III, and those of *Leuconostoc* type strains. The source and number of isolates representing each ribotype are listed on the right. The scale on top gives the similarity index for the ribotype patterns. Isolates clustering together with a type strains (species cluster) were considered to represent the respective *Leuconostoc* spp.

Table 6  *Leuconostocs* detected and their role in food spoilage in studies I-III

<table>
<thead>
<tr>
<th>Species detected</th>
<th>% of LAB(^a)</th>
<th>Role in spoilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marinated beef steak (I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. gascomitatum</em></td>
<td>62</td>
<td>Not determined by inoculation</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>12</td>
<td>Not determined by inoculation</td>
</tr>
<tr>
<td>Moisture-enhanced beef steak (I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. gascomitatum</em></td>
<td>64</td>
<td>Green discolouration, buttery off-odour(^b)</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>14</td>
<td>Green discolouration, buttery off-odour(^b)</td>
</tr>
<tr>
<td>Non-marinated broiler meat (III)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. carnosum</em></td>
<td>&lt;1</td>
<td>Not associated with spoilage</td>
</tr>
<tr>
<td><em>L. gascomitatum</em></td>
<td>&lt;1</td>
<td>Not associated with spoilage</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>&lt;1</td>
<td>Not associated with spoilage</td>
</tr>
<tr>
<td>Vegetable sausage (II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. gascomitatum</em></td>
<td>24</td>
<td>Gas, vinegar off-odour, slime(^b)</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>56</td>
<td>Gas, vinegar off-odour, slime(^b)</td>
</tr>
<tr>
<td><em>L. mesenteroides</em></td>
<td>6</td>
<td>Gas, vinegar off-odour, slime(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Relative proportion (%) of all lactic acid bacteria isolates.

\(^b\)Confirmed by inoculation experiments.
5.2 RECOVERY OF LEUCONOSTOCS FROM BROILER CARCASSES AND AIR (III)

None of the broiler carcass samples yielded leuconostocs. The vancomycin-resistant LAB from carcass samples were identified mainly as Weissella spp., Pediococcus spp. or Lactobacillus spp. (see Study III, Table 2, p. 1140). Furthermore, we did not observe growth for those carcass samples incubated at refrigerated conditions (6°C). After the initially cold-enriched cultures were incubated at 25°C, all showed growth yielding mainly isolates belonging to the genus Enterococcus.

From the 43 air samples taken, we picked and identified 122 LAB isolates of which 11 were identified as Leuconostoc spp. with L. citreum, L. gasicomitatum, L. lactis and L. pseudomesenteroides being the species recovered (Figure 2).

5.3 DISTRIBUTION OF L. GASICOMITATUM STRAINS IN FOODS (IV)

We applied PFGE typing to investigate the distribution of L. gasicomitatum strains from various foods mainly in relation to food group, processing plant and year of isolation. PFGE typing differentiated the 384 food-borne isolates into 68 genotypes and revealed that none of the genotypes associated with meat products was recovered from vegetable-based foods (see Study IV, Figure 1, p. 34). However, meat-derived genotypes were often linked to products of different types or processors (see Study IV, Table 1, p. 35). Furthermore, PFGE typing of several strains from the products of the same processing plant revealed that meat products analysed in different years were contaminated with indistinguishable strains (see Study IV, Table 2, p. 35).

5.4 GENOME ANALYSIS OF L. GASICOMITATUM LMG 18811T (V)

The genome of L. gasicomitatum LMG 18811T is plasmid-free and contains a 1 954 080-bp circular chromosome with an average guanine-cytosine content of 36.7%. The genome sequence revealed features typical for a Leuconostoc species including genes for the phosphoketolase pathway and three alternative pathways for pyruvate utilisation (Figure 3). Screening of this genome for genes encoding enzymes required for reactions and complete pathways releasing acetate, CO₂, diacetyl, and H₂O₂ revealed that these spoilage compounds are mainly generated during pyruvate and citrate metabolism (Figure 3). Furthermore, we identified genes encoding proteins involved in EPS synthesis. Table 7 lists the enzymes and the corresponding genes involved in the formation of acetate, CO₂, diacetyl, H₂O₂, or slime.
Figure 3  Predicted pathways for synthesis of spoilage metabolites via pyruvate by L. gasicomitatum LMG 18811T. Redox-neutral (normal arrows), and NADH consuming (bold arrows) and producing (white arrows) reactions are indicated. The dashed arrows illustrate reactions requiring oxygen. The spoilage metabolites are boxed, and the enzymes involved are indicated using abbreviations in circles: ACK, acetate kinase; ALD, acetolactate decarboxylase; ALS, α-acetolactate synthase; BDH, 2,3-butanediol dehydrogenase; CL, citrate lyase complex; DAR, diacetyl (acetoin) reductase; LDH, lactate dehydrogenase; OAD, oxaloacetate decarboxylase; PDH, pyruvate dehydrogenase; POX, pyruvate oxidase; PTA, phosphate acetyltransferase.

Table 7  Enzymes and coding genes in the genome of L. gasicomitatum LMG 18811T involved in the formation of spoilage compounds

<table>
<thead>
<tr>
<th>Spoilage compound and enzyme(s)</th>
<th>Gene(s)</th>
<th>Locus tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate: Acetate kinas</td>
<td>ackA1, ackA2</td>
<td>LEGAS_1085, LEGAS_1559</td>
</tr>
<tr>
<td>Citrate lyase complex</td>
<td>citCDEF</td>
<td>LEGAS_0213-0216</td>
</tr>
<tr>
<td>N-acetylglucosamine-6-phosphate Deacetylase</td>
<td>nagA</td>
<td>LEGAS_0472</td>
</tr>
<tr>
<td>CO₂: Acetolactate decarboxylase</td>
<td>alsD</td>
<td>LEGAS_1346</td>
</tr>
<tr>
<td>Oxaloacetate decarboxylase</td>
<td>citM</td>
<td>LEGAS_0212</td>
</tr>
<tr>
<td>6-phosphogluconate dehydrogenase</td>
<td>gnd1, gnd2</td>
<td>LEGAS_1343, LEGAS_0931</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase complex</td>
<td>pdhABCD</td>
<td>LEGAS_1381-1378</td>
</tr>
<tr>
<td>Pyruvate oxidase</td>
<td>poxB</td>
<td>LEGAS_1053</td>
</tr>
<tr>
<td>Diacetyl: Acetolactate synthase</td>
<td>alsS</td>
<td>LEGAS_0526</td>
</tr>
<tr>
<td>H₂O₂: Pyruvate oxidase</td>
<td>poxB</td>
<td>LEGAS_1053</td>
</tr>
<tr>
<td>Slime: Dextranucrase</td>
<td>dsrA</td>
<td>LEGAS_1012</td>
</tr>
<tr>
<td>Protein cluster related to formation of an EPS with unknown structure</td>
<td>eps cluster</td>
<td>LEGAS_0699-0710</td>
</tr>
</tbody>
</table>
6 DISCUSSION

6.1 ROLE OF LEUCONOSTOCs IN SPOILAGE OF REFRIGERATED FOODS

6.1.1 LEUCONOSTOCs INVOLVED IN SPOILAGE (I, II)
Studies I and II focused on identifying the LAB responsible for spoilage incidents encountered by two meat processors (I) and one company producing vegetable sausages (II). Common to the spoilage of these different foods was that they contained LAB population dominated by *Leuconostoc* spp. at levels above $6 \times 10^8$ CFU /g. Although the spoilage reaction developed in MAP beef steaks and VP vegetable sausages were quite different, in both cases, the prevalent species were *L. gasicomitatum* and *L. gelidum*. In addition, *L. mesenteroides* was implicated in the spoilage of vegetable sausages. These three *Leuconostoc* species often prevail in chilled-stored, packaged, nutrient-rich, foods, such as vegetables and cooked meats (Björkroth & Holzapfel, 2006, see also references in Table 3). However, our finding of *L. gasicomitatum* and *L. gelidum* from high-oxygen MAP beef is significant since previously, these species have mainly been reported in anaerobically packaged meats (Björkroth et al., 2000; Sakala et al., 2002; Shaw & Harding, 1989; Susiluoto et al., 2003).

The spoilage LAB populations in commercial products consisted of different LAB species and strains, and we therefore re-inoculated relevant strains onto fresh food samples to identify those directly responsible for the spoilage reactions. In general, all *Leuconostoc* strains tested lead to one or more spoilage reactions, although the spoilage potential varied among the strains. Similar findings have been described by others as well, (Björkroth et al., 1998; Borch & Molin, 1989; Budde et al., 2003; Hamasaki et al., 2003; Lyhs et al., 2004) demonstrating that *Leuconostoc* strains may produce different types and amounts of end products under identical growth conditions.

6.1.2 SPOILAGE OF HIGH-OXYGEN MAP BEEF STEAKS (I)
In Study I, a progressive, greenish surface discolouration on beef steaks stored under high-oxygen modified atmosphere was caused by *L. gasicomitatum* RSNS1b and *L. gelidum* RSNL1b. Such discolouration has rarely been reported in raw meat, whereas “greening” in cured meats is a well-known spoilage problem attributed to $\text{H}_2\text{O}_2$-producing LAB, including *Leuconostoc* species (Anifantaki et al., 2002; Borch et al., 1996; Samelis et al., 1998). In addition, some LAB, but not leuconostocs, may metabolise
cysteine to hydrogen sulphide to cause greening of VP beef (Egan et al., 1989; Leisner et al., 1995). Even though the biochemical background of the greenish defect on beef steaks in Study I remains to be characterised, earlier studies suggested that the oxygen containing modified atmosphere likely promoted H₂O₂ production by *Leuconostoc* spp. (Borch & Molin, 1989; Lucey & Condon, 1986).

Moreover, the spoilage of beef steaks was associated with a buttery off-odour. Buttery or “buttermilk” odours in meat have been attributed to diacetyl production by *Enterobacteriaceae* and *Brochothrix thermosphacta* (Dainty & Mackey, 1992; Dainty, 1996). In contrast, we found that in the case of moisture-enhanced beef steaks, *L. gasicomitatum* and *L. gelidum* caused the buttery off-odour. Such a defect has previously been described in association with *Leuconostoc* spoilage of fruit and vegetable-based foods (Christensen & Pederson, 1958; Lyhs et al., 2004; Sakamoto et al., 1996; Torriani et al., 1999). Diacetyl formation is an uncommon characteristic of *Leuconostoc* spoilage of meat suggesting that in a meat system, leuconostocs produce diacetyl only under certain conditions.

### 6.1.3 SPOILAGE OF VP, VEGETABLE SAUSAGES (II)

Within 12 days, the packages of vegetable sausages inoculated with *L. gasicomitatum*, *L. gelidum* or *L. mesenteroides* were loose or clearly bulging due to accumulated gas and the sausages had a strong, vinegar odour. Blowing of the packages was due to CO₂ produced during heterofermentative sugar metabolism (Björkroth & Holzapfel, 2006; Björkroth et al., 2000; Dykes et al., 1994; Yang & Ray, 1994). The vinegar-like odour was attributed to acetic acid, a metabolite formed, for instance, during fructose fermentation or glucose cofermentation with either fructose or citrate (Erten, 1998; Zaunmüller et al., 2006). The slime formed in inoculated sausages was likely to be dextran, a glucose-polysaccharide which many leuconostocs polymerise from sucrose (Björkroth & Holzapfel, 2006; Leathers, 2005).

The vegetable sausages were given heat treatment (cooking at 82°C) inactivating most vegetative cells and thus contamination with *Leuconostoc* spp. of the packaged vegetable sausages was most likely to occur after cooking. Furthermore, as carrots may contain high levels of *L. gasicomitatum*, *L. gelidum* and *L. mesenteroides* (Lyhs et al., 2004; Torriani et al., 1999), minimally processed carrots used in sausage formulation possibly exposed the processing environment and the cooked sausages to *Leuconostoc* contamination and subsequent spoilage of the finished product. Consistently, to overcome the problem, we advised the manufacturer to redesign processing practices to limit post-cooking contamination and to replace unprocessed carrots in sausage formulation.
6.2 ROLE OF LEUCONOSTOCS IN SHELF-LIFE QUALITY OF NON-MARINATED BROILER PRODUCTS (III)

*Leuconostoc* spp., *L. gasicomitatum* in particular, are the major contributors to spoilage of marinated poultry fillet products (Björkroth et al., 2000; Susiluoto et al., 2003), whereas *Carnobacterium* spp. are important in the spoilage of MAP, marinated broiler leg cuts (Björkroth et al., 2005). Similarly, in Study III, *Carnobacterium* spp. constituted the major part of the late shelf-life LAB populations (64%) in non-marinated broiler products, whereas *Leuconostoc* spp. were rarely (2.9% of LAB) recovered. These findings together indicate that *Leuconostoc* spp. mainly contribute to the shelf-life quality of marinated, skinned poultry products, but are not involved in the spoilage of other raw poultry products, including non-marinated fillets or marinated leg cuts. The reason for the predominance of *L. gasicomitatum* in marinated fillet products is not clear. However, factors explaining the growth advantage of *L. gasicomitatum* in marinated fillet products are likely to be related to the ability to utilise substrates competitively under the pH and redox conditions typical for marinated fillet products, as well as to those related to processing involved in the production of marinated, skinless fillet products.

6.3 LEUCONOSTOC CONTAMINATION RELATED TO BROILER CARCASSES AND PROCESSING PLANT AIR (III)

The psychrotrophic *Leuconostoc* spp. associated with poultry meat spoilage are not considered to be commensal organisms in the avian intestinal tract, because most strains of these species do not grow at 37°C (Björkroth & Holzapfel, 2006; Holzapfel et al., 2009). Therefore in Study III, we targeted the carcass sampling on skin and feathers, where the temperature is lower compared to the respiratory or gastrointestinal tract. Our findings indicated that broilers are not a significant source for *Leuconostoc* contamination of poultry products. On the other hand, we recovered *Leuconostoc* spp., including *L. gasicomitatum*, from air samples obtained from the processing plant. Even though earlier reports on *Leuconostoc* spp. from the poultry processing environment are lacking, our finding is consistent with those of previous surveys demonstrating that air disseminated *L. carnosum* contamination during the processing of cooked ham (Björkroth & Korkeala, 1997; Björkroth et al., 1998; Goto et al., 2004). As vegetables and plant material are considered natural habitats of leuconostocs, spices and other plant-based ingredients used in meat marinades have been suspected as a potential contamination source. However, two recent studies revealed that *Leuconostoc* associated with meat spoilage were not recovered from
marinade ingredients (Lassila, 2007; Lundström & Björkroth, 2007). Taken together, such findings, and those of Study III, indicate that spoilage leuconostocs are derived from the processing environment rather than from broiler carcasses or marinade ingredients. From manufacturing sources, *Leuconostoc* may become airborne from worker activities or water spraying during cleaning and directly contaminate the in-process poultry products, or indirectly through the processing equipment. Their routes of entry into poultry processing facilities as well as possible reservoirs in the processing environment remain to be established.

### 6.4 DISTRIBUTION OF L. GASICOMITATUM GENOTYPES IN FOODS (IV)

Study IV included 384 *L. gasicomitatum* isolates from various meat and vegetable-based foods and employed *Sma*I PFGE typing to explore the distribution of *L. gasicomitatum* strains in foods of different origins. Based on our results, none of the *L. gasicomitatum* genotypes identified from meat were recovered from vegetable sources. The lack of common genotypes between meat and vegetable-based foods suggests that either the vegetable-derived strains were not transmitted to the meat production chain or that they are unable to grow predominantly in MAP meats. However, further studies are necessary to reveal whether *L. gasicomitatum* strains in vegetables and meat are generally niche specific, or if vegetable and meat products support the growth of different *L. gasicomitatum* strains.

Comparison of the genotypes associated with beef, broiler, pork and turkey products showed that the same *L. gasicomitatum* strains may prevail in products of different meat animal species. This also indicates that these strains are likely to follow similar contamination patterns in beef, pork, and poultry production. Furthermore, raw poultry products from one poultry processing plant repeatedly yielded isolates with indistinguishable genotypes. Similarly, a specific strain of *L. mesenteroides* was repeatedly isolated from the products of a ham processing plant (Samelis et al., 2000a). These findings raise the question of whether the continual product contamination was due to *Leuconostoc* strains established in the meat processing environment. There are currently no reports of *Leuconostoc* spp. being able to survive in food processing facilities for extended periods. In the absence of further information, it remains unclear whether specific strains may persist in the processing facilities or if they become continuously introduced to the processing chain from a currently unidentified source.
6.5 GENOME OF *L. GASICOMITATUM* LMG 18811\(^T\) (V)

6.5.1 METABOLIC FEATURES RELATED TO GROWTH IN MEAT

Information derived from the genome of *L. gasicomitatum* LMG 18811\(^T\) provides insights into the nutritional requirements and metabolism of LAB adapted to refrigerated, MAP meat systems. The three other publicly available *Leuconostoc* genomes, *L. citreum* KM20 (Kim et al., 2008), *L. kimchii* IMSNU1154 (GenBank accession numbers CP001753 to CP001758) and *L. mesenteroides* ATCC 8293\(^T\) (Makarova et al., 2006) represent strains used for vegetable fermentation. In general, with respect to the encoded metabolic pathways, the genome of *L. gasicomitatum* reveals only minor differences to *L. citreum*, *L. kimchii* or *L. mesenteroides*. Similarly to the other *Leuconostoc* spp., *L. gasicomitatum* appears to be adapted to grow at the expense of carbohydrates on a nutrient-rich environment. These features are reflected in the genome of *L. gasicomitatum*, which contains a wide repertoire of genes involved in uptake of sugars, citrate and amino acids, but lacks many genes required for the biosynthesis of amino acids, vitamins and co-factors.

As regards growth on meat, the results of the Study V suggest that being unable to obtain energy from proteinaceous substrates, lactate, or fatty acids *L. gasicomitatum* primarily utilises glucose and ribose. Furthermore, *L. gasicomitatum* harbours genes required for the energetic catabolism of nucleosides and grows well on adenosine and inosine (see Study V, supplementary table 1). Nucleosides, particularly inosine, are abundant in meat, providing a potential energy source for *L. gasicomitatum* in a meat system where glucose is exhausted. In comparison, *L. sakei* 23K, a meat-borne LAB, may release amino acids from meat proteins and utilise arginine as an energy source (Chaillou et al., 2005; Champomier Verges et al., 1999). In a meat system, these traits present a clear growth advantage to *L. sakei* (Chaillou et al., 2005; Champomier Verges et al., 1999). Nevertheless, the genes encoding these functions are absent in *L. gasicomitatum*.

Furthermore, from the food safety perspective, the genome analysis confirms that *L. gasicomitatum* LMG 18811 is unable to decarboxylate aminoacids to form biogenic amines (Murros, 2004), whereas such enzymatic activities have been reported in few other strains of *Leuconostoc* (de Llano et al., 1998; Pircher et al., 2007).

Interestingly, the genome of *L. gasicomitatum* encodes the components required for a functional respiratory chain similar to the one present in *Lc. lactis* (Gaudu et al. 2002). This suggests that in a heme containing media, such as meat, *L. gasicomitatum* may undergo respiration. For *Lc. lactis*, respiration increased both growth and survival during glucose depletion (Duwat et al., 2001; Gaudu et al., 2002). Consistently, Study V showed that addition of heme to aerated MRS medium enhanced the biomass formation of *L. gasicomitatum*. These findings suggest that respiration may promote
the growth of *L. gasicomitatum* in aerobically stored meat, although it is unclear if respiration increases the production of spoilage compounds and hence, the spoilage potential of *L. gasicomitatum*.

### 6.5.2 Metabolic Features Related to Specific Spoilage Reactions

In addition to acetate produced during metabolism of pentoses or citrate, *L. gasicomitatum* LMG 18811 harbours the central genes involved in the pyruvate-dissipating routes leading to formation of acetate and diacetyl (Figure 3). As described for other leuconostocs, *L. gasicomitatum* is likely to metabolise pyruvate to acetate or diacetyl under conditions when intracellular pyruvate exceeds the rate at which pyruvate is reduced to lactate, such as when oxygen, citrate or fructose are available (Axelsson, 2003; Zaunmüller et al., 2006). Even though regulation of pyruvate metabolism in *Leuconostoc* is poorly characterised, factors such as glucose concentration, oxygen, redox state of the cell and external pH influence the activities of the key enzymes dictating the fate of pyruvate (Axelsson, 2003; Garcia-Quintans et al., 2008). Notably, some *Lactobacillus* and *Lactococcus* strains can form diacetyl via catabolism of aspartate (Kieronczyk et al., 2004; Le Bars & Yvon, 2007), an amino acid present in meat. Genome analysis showed that also *L. gasicomitatum* possesses the genes required for aspartate catabolism, but lacks the gene for glutamate dehydrogenase considered important for the formation of α-ketoglutarate, the amino group acceptor essential for the pathway (Tanous et al., 2005). Instead, *L. gasicomitatum* LMG 18811 encodes a transporter for α-ketoglutarate suggesting that it may obtain exogenous α-ketoglutarate. Further studies are needed to assess whether *L. gasicomitatum* produces diacetyl via aspartate catabolism and, if this is found to be possible, to determine whether this occurs in meat.

We proposed in Study I that H$_2$O$_2$ production by *L. gasicomitatum* strains caused a green discolouration on beef steak and suggested that this defect was due to the activity of a H$_2$O$_2$-producing NADH oxidase. Analysis of the gene arsenal of *L. gasicomitatum* LMG 18811 revealed that it harbours an NADH oxidase reducing oxygen directly to water, and that pyruvate oxidase is the only enzyme indentified which is known to generate H$_2$O$_2$. Because *L. gasicomitatum* LMG 18811 was not included in Study I and has not been tested for this particular spoilage activity, the exact metabolic process that resulted in meat greening remains to be characterised.

Slime formation on vegetable sausages (Study II) and in a herring product (Lyhs et al., 2004) was proposed to be due to dextran production from sucrose. Consistently, *L. gasicomitatum* encodes a dextranucrase, a cell wall-associated glycosyltransferases catalysing the formation of dextran from sucrose. We also found a gene cluster homologous to the heteropolysaccharide EPS gene cluster present in *Streptococcus*.
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*thermophilus* (Minic et al., 2007). Even though information on heteropolysaccharide formation in *Leuconostoc* is lacking, in other LAB, the biosynthesis of heteropolysaccharides has been reported to be more complex compared to the formation homopolysaccharides, such as dextran (De Vuyst & Degeest, 1999; Minic et al., 2007). Hence, in the absence of experimental data, it is difficult to predict the structure or physical properties of the polysaccharide produced by this EPS gene cluster, or its possible role in food spoilage.
7 CONCLUSIONS

1. Confirmatory results from pure culture studies revealed that *L. gelidum*, *L. gasicomitatum* and *L. mesenteroides* were responsible for the unpleasant spoilage reactions developed in MAP beef steaks (I) and VP vegetable sausages (II). As regards the highly undesirable green discoloration associated with beef spoilage, our findings highlight the role of *Leuconostoc* spp. as specific spoilage organisms in meat packaged under a high-oxygen modified atmosphere, a common gas atmosphere applied for the retail packaging of red meats.

2. Only a few isolates of *Leuconostoc* were detected among the late shelf-life LAB populations in nonmarinated broiler products. Our findings reveal that *Leuconostoc* species contaminate and are able to grow in these products. However, in terms of number, leuconostocs do not contribute to shelf life quality of this product category.

3. The findings of Study III showed that the role of broilers as sources of *Leuconostoc* spp. involved in poultry spoilage is minimal indicating that contamination of poultry meat occurs during processing. This study demonstrated that air movement is an important vector of transmission and therefore, that air quality, especially in the final processing and packaging areas, may be critical for the quality of broiler products.

4. In general, strains of *L. gasicomitatum* are able to grow and predominate in various types of meat products and are likely to follow similar contamination patterns in beef, pork and poultry production. Furthermore, certain strains were repeatedly recovered from products of the same processing plant, suggesting that the processing environment may have an impact on *L. gasicomitatum* contamination of meat products. The lack of common *L. gasicomitatum* strains between meat and vegetable-based products indicate that either the vegetable-derived strains were not transmitted to meat production chain or that they are unable to grow to predominate in MAP meats.

5. Uncovering the genome content of *L. gasicomitatum* LMG 18811T allowed us to identify the metabolic pathways and reactions related to the development of specific spoilage reactions. The annotated genome of *L. gasicomitatum* will provide us with new opportunities to study the metabolic background of spoilage reactions, and the responses of this organism to processing or product conditions.
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