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PSYCHROTROPHIC LACTIC ACID BACTERIA (LAB) AS A SOURCE OF FAST SPOILAGE OCCURING ON PACKAGED AND COLD-STORED FOOD PRODUCTS

Thesis submitted in fulfillment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Thesis title in Dutch:

Psychrotrofe melkzuurbacteriën als bron van snel bederf van gekoelde, verpakte levensmiddelen.

Illustration on cover:

Genome of type strain *Leuconostoc gelidum* subsp. *gasicomitatum* LMG 18811^{T} (Johansson et al., 2011)

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FOREWORD

As my journey in the Laboratory of Food Microbiology and Food Preservation reaches its end and my doctoral study its fulfillment, I feel a sincere need to reminisce all those who contributed to my work and therefore acknowledge and appraise their help.

First and foremost, I would like to thank my promotor, Prof. Dr. ir. Frank Devlieghere. Not only did you give me the opportunity to come to Belgium and work in a laboratory of the highest standards but you also encouraged me with your trust, your support and your patience to perform this research study providing me as well with the financial means and stimulation. I am deeply grateful to you.

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My thesis jury, Prof. Dr. George-John Nychas, Prof. Dr. Danilo Ercolini, Prof. Dr. Lieven De Zutter, Prof. Dr. ir. Nico Boon, Prof. Dr. Geert Huys and Prof. Dr. ir. Frank Devlieghere for all the critical comments in the manuscript.

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This whole dissertation is a product of hard, teamwork. Thank you all for your scientific help and contribution, you are all very dear to me.

My family. My father, my mother Sophia and my siblings Kostas and Eirini for their moral support that propelled me to accomplish this. Thank you for believing in me, for your constant presence, encouragement and love, regardless of how far we may be.

Την οικογένεια μου. Τον πατέρα μου, τη μητέρα μου Σοφία, τα αδέρφια μου Κώστα και Ειρήνη για την υποστήριξη, την ενθάρρυνση και την αγάπη τους.

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ABBREVIATIONS

(m/r/t)RNA	(messanger/ribosomal/transfer) ribonucleic acid
(SPME)GC-MS	Solid Phase Microextraction Gas Chromatography Mass Spectrometry
AFLP	Amplified Fragment Length Polymorphism
a_w	Water activity
BA	Biogenic amines
BCCM	Belgian Co-Ordinated Collection of Microorganisms
BOPP	Biaxially-Oriented Polypropylene
C.	*Carnobacterium*
CFU	Colony Forming Unit
CSP(s)	Cold shock protein(s)
DNA	Deoxyribonucleic acid
E.	*Enterococcus*
EDTA	Ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
EVOH	Ethylene vinyl alcohol
GRAS	generally regarded as safe
HTS	High-throughput sequencing
ISO	International Organization for Standardization
K	Reaction rate coefficients
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
Le.	*Leuconostoc*
MA(P)	Modified atmosphere (packaging)
MLST	Multi locus sequence typing
MRS	de Man-Rogosa-Sharpe
N_{max}	maximum cell population
OTR	Oxygen transmission rate
PA	Polyamide
PCA	Plate Count agar
PE	Polyethylene
PP	Polypropylene
PPS	Peptone physiological solution

RCA	Reinforced Clostridial agar
rep-PCR	Repetitive element polymerase chain reaction
RH	Relative humidity
RTE	Ready-to-eat
SBP	Sweet bell pepper
SDS	Sodium dodecyl sulfate
SIFT-MS	Selected Ion Flow Tube Mass Spectrometry
SS	Stainless steel
SSO	Specific Spoilage Organism
TAE	Tris base, acetic acid & EDTA
TS(A/B)	Trypton Soya (agar/broth)
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
VOC(s)	Volatile organic compound(s)
W.	*Weissella*
μ_{max}	maximum specific growth rate

AIM OF THE STUDY

During the past decades, great advances in the field of food preservation have paved the way towards ensuring food safety to a greater extent. Still many obstacles need to be surpassed and improvements to be made as microbial issues related to pathogen exposure incidents and spoilage still occur. In both cases the outcome is negative leading inevitably to foodborne illness outbreaks endangering susceptible consumers and food loss, respectively.

Nowadays, the market is engaging to products, which are healthy and highly nutritious, convenient and easy to-use. At the same time, market demand is shifting towards products that contain less additives and preservatives, with functional properties and subjected to non invasive technological handlings. Balancing all these aspirations without compromising the safety aspects is challenging.

The safety of foodstuffs constitutes priority for food industry, and hence legislation is strict and entails consequences for companies that do not conform. On the other hand, assessment of properties associated with the quality of the products is more flexible and in general subject to cultural background and personal preferences. However, shelf-life is determined by organoleptic parameters, which are evaluated through sensorial analysis and correlated to microbial activity. A food product is considered spoiled when the levels of microbial contamination result in alterations of the predetermined aroma, flavor, color or texture rendering it unfit for consumption.

The quality of raw materials, cold chain maintenance, good hygiene practices and an effective hurdle technology prevent unexpected cases of spoilage within the anticipated shelf-life however, certain microbial groups still manage to proliferate and dominate in the food matrix.

Food packaging and low-temperature storage are two of the most frequently applied techniques for fresh products of meat and vegetable origin. This way respiring and cold-sensitive, spoilage-related microbes are suppressed avoiding production of offensive metabolites. Subsequently, other microbial groups that possess the ability to thrive under refrigeration temperatures and low oxygen availability grow faster. Lactic acid bacteria (LAB) represent one characteristic group of microorganisms that is selected by the implemented preservation methods.

During the last twenty years, it has become clear that psychrotrophic microbes play a decisive role in reported cases of spoilage occurring on packaged and chilled-stored food products. Seemingly unimportant taxa and hitherto unknown species have emerged as competent spoilers and were put in the spotlight. Their ability to outgrow all members of the microbial consortium and eventually dictate spoilage soon became subject of study.

Still many aspects of their physiology remain unclear. Firstly, the origin and abundance of these microbial taxa in food related habitats. The routes through which they are introduced in food processing environments and presumptive adaptation to equipment and premises. Moreover, their ability to grow very fast from low initial numbers and become dominant at the end of shelf-life. Evaluation of the currently applied microbiological analyses with respect to the assessment of these psychrotrophic LAB. Lastly, the determination of their spoilage potential and the contribution of oxygen to the spectrum of metabolic products.

All these subjects are addressed in the present study aiming to elucidate the role of these microbes in spoilage manifestations.

OVERVIEW OF THE THESIS

In **Chapter 1,** a thorough outline of the problem is attempted. All significant parameters related to the topic are explained in detail and the current knowledge concerning the concept of microbial spoilage, the relation between lactic acid bacteria and food as well as an overview of reported spoilage studies involving psychrotrophic LAB taxa are presented.

The first study conducted for the purposes of the present thesis was a screening of the Belgian market presented in **Chapter 2.** A systematic sampling of retail, packaged and cold-stored food products evaluated the occurrence of psychrotrophic LAB at the end of shelf-life. The currently implemented mesophilic enumeration methods were also assessed with respect to their ability to efficiently determine psychrotrophic populations. The dominant psychrotrophic microbes that were underestimated when mesophilic incubation was applied were isolated and constituted the first collection of presumptive spoilage, psychrotrophic LAB.

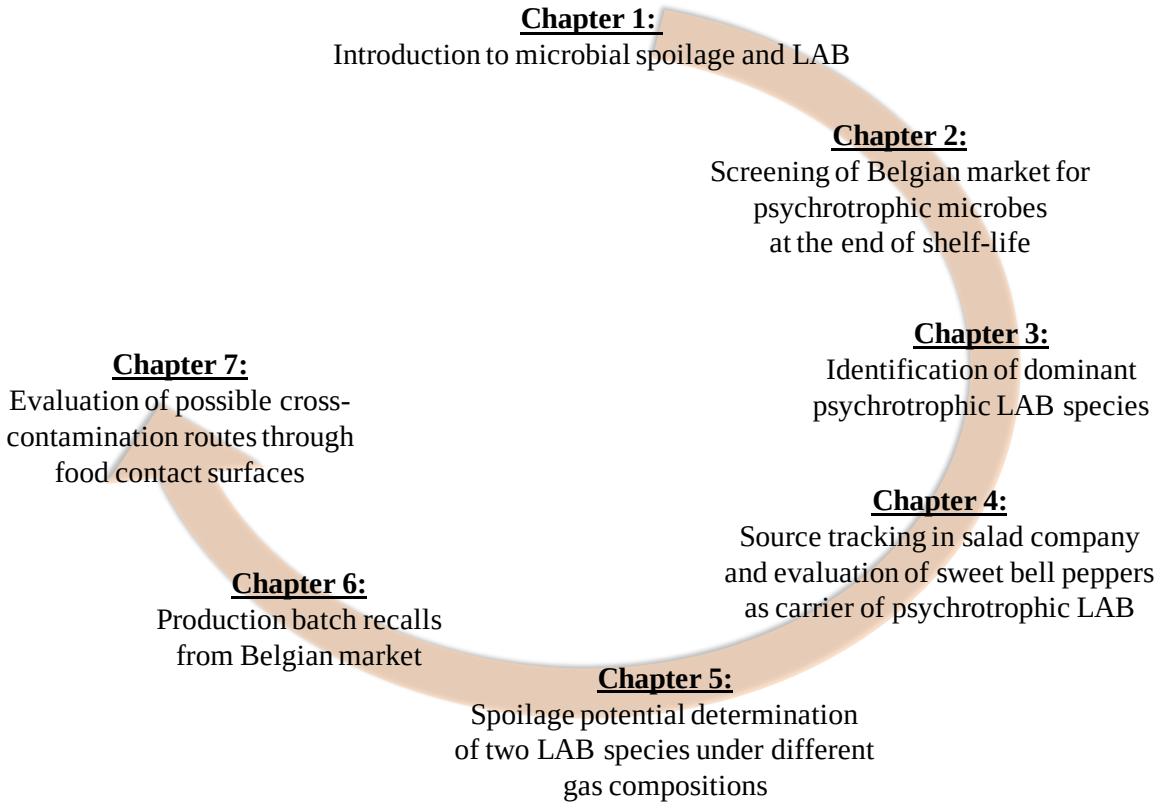
Chapter 3 corresponds to the characterization and identification of the isolated microbes. Their inability to grow at 30 ^{o}C was evaluated confirming strictly psychrotrophic character and their taxonomic position was determined by means of DNA fingerprint typing and sequencing of conserved genomic domains. The wide selection of samples analyzed in **Chapter 2** resulted in a very limited species diversity underpinning the role of few LAB genera.

After evaluating how widespread the problem is a study in a processing plant was conducted in order to investigate the ecological source of these microbes. In **Chapter 4** the results of a source tracking performed in a ready-to-eat (RTE) vegetable salad processing environment facilitated the assessment of possible cross-contamination routes and emphasized on the role of sweet bell peppers as ecological niche harboring psychrotrophic LAB species. Sampling of the entire production facility showed prevalence of different LAB genera on surfaces, air, raw material, intermediate products and water samples. Nonetheless, only genus *Leuconostoc* became dominant after storage at the end of shelf-life exhibiting the highest frequency of isolation. Noteworthy, different biotypes of *Leuconostoc* spp. were recovered from harsh environments like acid baths and disinfected surfaces.

Having correlated the presence of the most frequently isolated psychrotrophic LAB to sweet bell peppers a selection of isolates from food samples (**Chapter 3 & 6**) allocated to species *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium* were tested on sweet bell pepper simulation medium with respect to their metabolic patterns and growth dynamics under different packaging conditions, in **Chapter 5**. The two species showed different growth dynamics, interspecies diversities and adaptation to oxidative stress, however the spectrum of produced metabolites was very similar among the spoilage-related strains mainly focusing on acetic acid and ethanol while induction of buttery off-odor VOCs (like diacetyl) was observed under aeration.

During the period between 2010 and 2014 several case studies were analyzed in our laboratory shown in **Chapter 6**. These case studies were actual production batch recalls that resulted in loss of tons of products caused by unexpected, early spoilage manifestation prior to the end of shelf-life. The responsible microbiota were determined by 16S rRNA gene high-throughput sequencing (HTS) confirming all previous studies. The same LAB species recovered from the screening of the Belgian market **Chapter 2** and the source tracking **Chapter 4** that were found dominant at the end of shelf-life were implicated in actual cases of spoilage.

Lastly, in **Chapter 7** physiological traits of species *Le. gelidum* subsp. *gasicomitatum* are studied with respect to surface attachment. A selection of strains (**Chapter 4 & 6**) were tested in different substrates and type of food contact surfaces in order to evaluate the intraspecies diversity, the impact of nutrients and the substratum on the attachment of cells. Possible attachment of cells that remain embedded on surfaces could explain cross-contamination in a processing environment (**Chapter 4**). Once present in the food matrix this microbe could grow competently (**Chapter 5**) and become dominant during storage reaching high population (**Chapter 2**) resulting in unexpected spoilage defects before the end of shelf-life (**Chapter 6**).



OVERVIEW OF THE LITERATURE
Spoilage of packaged and chilled-stored food products caused by psychrotrophic LAB genera *Leuconostoc* and *Lactococcus*

1. **Food spoilage**
    - **1.1 Introduction of microbes to foodstuff**
    - **1.2 Microbial spoilage**
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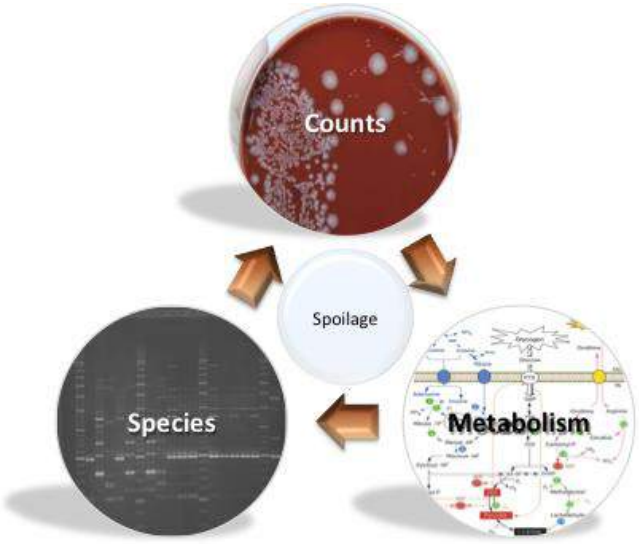

1. Food spoilage

Food is a very susceptible material, therefore bound to decompose in time. Food spoilage is a general term that corresponds to any quality deterioration of food products that leads to its rejection from the consumers, due to a wide range of reactions including some that are physical, some chemical, other enzymatic as well as microbial (FAO, 2011). Physical spoilage refers to damages of the final commercial product that constitute a deviation from its predetermined form and properties, associated with structural damages or loss of texture and flavor. Chemical spoilage is related to oxidative phenomena like rancidity and discoloration, non-enzymatic browning and nutrient degradation. On the other hand enzymatic spoilage involves reactions occurring in the food matrix facilitated by endogenous enzymes of the biological tissue comprising hydrolytic reactions catalyzed by lipases and proteases, rancidity caused by lipoxygenases and enzymatic browning (Gould, 2000). However, the most significant type of spoilage is microbial spoilage carried out by microorganisms (i.e. bacteria, yeasts and moulds) that grow by utilization of food nutrients.

1.1 Introduction of microbes to foodstuff

Our world is dominated by microbes that practically inhabit every nook and cranny of the earth. They possess a remarkable ability to thrive in almost any kind of ecological niche as they can be found in mammalian gastrointestinal tract or in deep sea and through their evolution have developed great adaptability to extreme conditions from polar aquatic environments to thermal vents.

In terms of biomass, bacteria are the most abundant form of life (Kallmeyer et al., 2012) and they are associated with all environments where foodstuffs originate. The priority of bacteria is survival and growth through energy yield, for this reason bacteria have developed a strict control of metabolic processes (Luesink, 1998; Titgemeyer & Hillen, 2002), since the expression of genes encoding unnecessary proteins would lead to an energetically and competitively unsustainable strain on the organism.

Practically, fresh produce, seeds, grains, livestock deriving products, carcass and fishery are contaminated by the microbes present in their natural ecosystems and throughout all steps of transportation, manufacturing and supply are further exposed to microorganisms.

Food is a commodity prone to microbial contamination that provides necessary nutrients for cell proliferation. However, the intrinsic parameters of the food matrix (i.e. pH, a_w, redox potential, antimicrobial constituents etc.), the production technology they are subjected, the extrinsic parameters related to their storage (i.e. O_2 availability, temperature, relative humidity etc.) and the interactions (i.e. synergism, antagonism, amensalism, Jameson effect etc.) among all the different members of the diverse microbial association - referred to as implicit factors - exert selection pressure regarding which microbial group will dominate during storage (Adams & Moss, 2008; Jameson, 1962; Jay, 2000).

Apparently, microbial growth can have either a favorable contribution to the organoleptic properties of a food product like in the case of fermented goods or can lead to detrimental alteration when causing spoilage.

1.2 Microbial spoilage

Microbial spoilage is a common and cumbersome phenomenon for food manufacturing companies, cost-demanding to control and inflicts an immense loss of goods worldwide. It comprises any bioconversion of nutrients into spoilage-related metabolites attributing an uninviting profile to the product. Numerous microbial taxa, related to food spoilage are ubiquitous in soil and vegetable tissues (Chen et al., 2005), animal skin and epithelia (Rieder et al., 2012), abiotic surfaces and food processing plants (Bokulich & Mills, 2013) thus form a heterogeneous microbial consortium introduced in food and eventually thriving in it. As previously mentioned, based on origin, nutritional content, storage conditions and technological aspect, food is a complex ecological niche given the fact that numerous extrinsic, intrinsic and implicit factors can influence microbial growth (Adams & Moss, 2008; Jay, 2000). These specific parameters in combination establish either a stressful or favorable environment for proliferation depending on the requirements of each member of the microbial association.

Microbial spoilage being the main reason for the rejection of foodstuffs concerning the organoleptic as well as nutritional properties actually determines the shelf-life (Borch et al., 1996; Huis in't Veld, 1996).

It comprises any quality deterioration associated with the growth and metabolic activity of microorganisms and is the main factor that leads to rejection of production batches because of qualitative alteration. Hence the overriding priority of most preservation techniques is notably to delay or prevent the growth of microbes (Gould, 1996). The withdrawal of products has a huge economical effect on the industries (Huis in't Veld, 1996) and it has been estimated that 25% of all foods produced globally is being discarded because of post harvest, post slaughter or post manufacture contamination (Anonymous, 1985; Rolle, 2006; Stuart, 2009). Nonetheless, nowadays the advanced expertise and technological evolution in the field of food manufacturing and food preservation can ensure the quality of products in terms of hygiene and cold-chain maintenance subsequently prolongation of the anticipated shelf-life has been achieved (Gould, 2000), yet there is much more to know concerning the coordination and physiology of the microbial communities in food.

The microbial spoilage manifestations influence the flavor, aroma, color and texture through a wide range of off-taste and off-odor compounds (Gram et al., 2002). Emission of volatile organic compounds (VOC), acidification caused by secretion of acids, slime formation due to production of exopolysaccharides, putrefaction, discoloration and gas production are the primal alterations (Nychas et al., 2008). In many cases the gas metabolites produced provoke defects to the packaging like slight blowing, bulging, swelling or even disruption.

Academic discussion concerning the concept of spoilage and the terms to describe it can be summarized in Figure 1.1. Food is considered a complex microbial commodity where different groups of microorganisms proliferate representing the consortium. From this heterogeneous microbial community only few species have the ability to dominate and dictate the spoilage manifestations that will occur constituting the Specific Spoilage Organism (SSO). All the microbial populations of the consortium grow during the storage period however the SSO is determining the end of the storage period since the spoilage-related metabolites it produces accumulate. The point where the concentration of these offensive

compounds become noticeable from the consumers is called Chemical Spoilage Index and corresponds to a specific population of SSO known as Minimal Spoilage Level, signalizing the end of the shelf-life. These thresholds depend on the type of product, microbial species and can vary greatly.

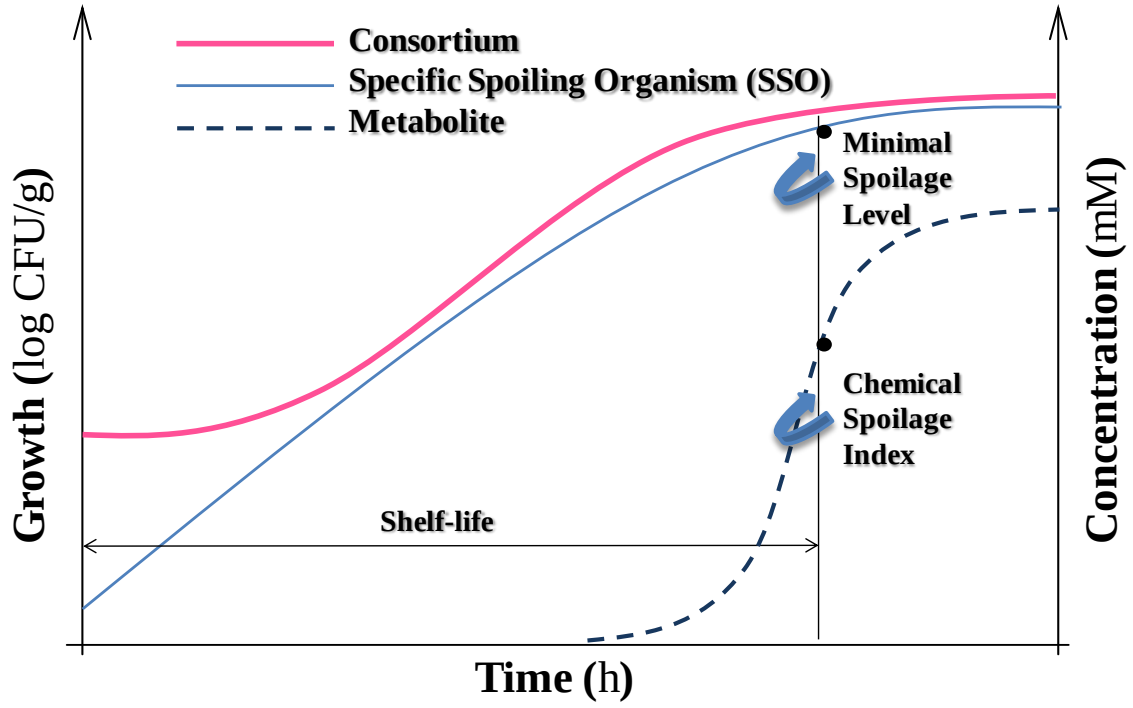

Figure 1.1: *The concept of microbial spoilage correlated to the metabolic activity of a spoilage specific microorganism (SSO) thriving in the food matrix (Huis in't Veld, 1996).*

Food manufacturing process plants have to conform to the microbiological requirements of the legislation that concern both safety and quality aspects. The protection of consumers from pathogenic microorganisms is a priority hence the microbiological standards dealing with virulent species that could jeopardize public health are very strict. There are specific threshold limits that industries have to comply with in order to avoid pathogenic outbreaks (EFSA, 2010). On the contrary defining microbiological standards associated with the quality properties is challenging. Microbial spoilage in perishable food commodities is a poly-parametric phenomenon mainly influenced by three factors: the contamination levels of the food product (microbial load/consortium), the species of the microbiota (presence of SSO) and the metabolism under the specific conditions of storage.

In industrial practice the evaluation of a product throughout its shelf-life is conducted by microbiological and sensory analysis. The contamination levels can extend between 10^6 and 10^8 CFU/g of total viable counts (AFSCA, 2012; Uyttendaele et al., 2010). However, the textural, nutritional and organoleptic (flavor, aroma, color) characteristics should be acceptable in all cases (Borch et al., 1996).

1.3 Advances in food production

In earlier times, people used to farm and cultivate crops, raise livestock and practice husbandry in order to consume what they produced. Soon, regional food commerce progressed but the means to preserve food were limited (Truninger, 2013). Mainly smoking or drying, use of salt and brine, acidification or fermentation were applied. During the 20th century food production and supply became more methodical (Oddy & Drouard, 2013) and since refrigeration was available in every household shelf-life of food extended significantly, facilitating massive production of pioneering, convenience goods, like packaged and chilled-stored foodstuffs.

Nowadays, with economical globalization food products are manufactured in processing facilities characterized by significant degree of automation and computerization (Welch & Mitchell, 2000). This systematic food production has corroborated the role of industrial food environments and technology upon the consortium of the end-product. A reciprocal relation between the raw material handled in the plant and the actual production environment has been developed. Unprocessed food materials containing an indigenous microflora are being introduced in the production facilities and the house microbiota of the plant is contaminating the intermediate or final foodstuffs, respectively. It has been proven both in the case of fermented food deriving from spontaneous fermentations or back-slopping (Abriouel et al., 2008; Bokulich & Mills, 2013; Gori et al., 2012; Santos et al., 1998; Scheirlinck et al., 2007, 2008) and documented spoilage reports (De Filippis et al., 2013; Rahkila et al., 2011; Vihavainen et al., 2007) that the raw material and the microbes adapted to the processing environment play a decisive role in microbial succession and eventually dominance.

1.4 Packaged and chilled-stored food products

Packaging (i.e. covering in an impermeable plastic/polymer wrapping) is a frequently applied hurdle in order to protect food products from the environment. Of course, apart from the fact that the package is acting as a barrier a selected gas composition can be used depending on the nature of the product and the purpose. A combination of three gases is used: N_2, which is a filer gas/inert, O_2 and CO_2 (Cutter, 2002).

Today, packaging has minimum processing effort, consumes little amounts of energy and ensures nutritional value and flavor. It can enhance product quality and freshness, while extending the shelf-life of the product providing convenience (Singh et al., 2011).

Especially modified atmosphere packaging (MAP) has become a widely implemented food preservation technique with minimal effect on fresh product characteristics, and therefore fits well with the recent consumer's preference for additive-free foods (Gould, 2000). In the case of fresh-cut produce low O_2 and moderately high CO_2 levels reduce deteriorations by suppressing the respiration rate of the living tissues, ripening and microbial growth of aerobic microbes (Conesa et al., 2007; Jacxsens et al., 2001). The choice of packaging material with appropriate permeability is also emphasized in order to keep an adequate gas composition in the package. Meat requires high O_2 levels in order to protect the color by keeping the heme

pigment in the oxymyoglobin form and avoid brown discolorations that render the products uninviting for consumption (Lorenzo & Gómez, 2012; Mcmillin, 2008).
Packaging is very often coupled with low-temperature storage. Products are usually kept at temperatures between 2 and 7 ^{o}C and this way the growth of many microbes (e.g. yeasts, moulds, cold-sensitive mesophiles, thermophiles) is retarded (Jay, 2000). Also the production process is performed under cold chain. All steps from packaging to retail are also significant as it is important for all operations to be done under chilling temperatures (Kreyenschmidt et al., 2010; Welch & Mitchell, 2000). The inhibition of quality deterioration is more effective at low temperature as well, since CO_2 solubility to the aqueous phase of the product increases (Lopez-Hernandez et al., 1996).
However, these preservation techniques exert a selection pressure towards psychrotrophic and strictly or facultative anaerobic microbes, like lactic acid bacteria (LAB) and *Brochothrix thermosphacta* (Cayré et al., 2005; Kotzekidou & Bloukas, 1996; Labadie, 1999) since most respiring microbes are inhibited (i.e. *Pseudomonas, Moraxella, Acinetobacter, Aeronomas, Photobacterium, Enterobacteriaceae,* yeasts and moulds).

1.5 Psychrotrophic microbes

Most food related microbes are mesophiles (bacteria, yeasts and moulds) and have an optimal-growth temperature range extending between 30 and 37 ^{o}C. Still, the majority (*Proteobacteria*, LAB, *Bacilli* and some pathogens) are cold-acclimatized and in the case of refrigerated products, they play a decisive role (Adams & Moss, 2007). Psychrophiles can grow from subzero temperatures till 20 ^{o}C (Feller & Gerday, 1994) while psychrotrophs grow competently from below 5 ^{o}C, till 25-30 ^{o}C (Jay, 2000). In Figure 1.2 the temperature ranges of these microbial groups are shown.

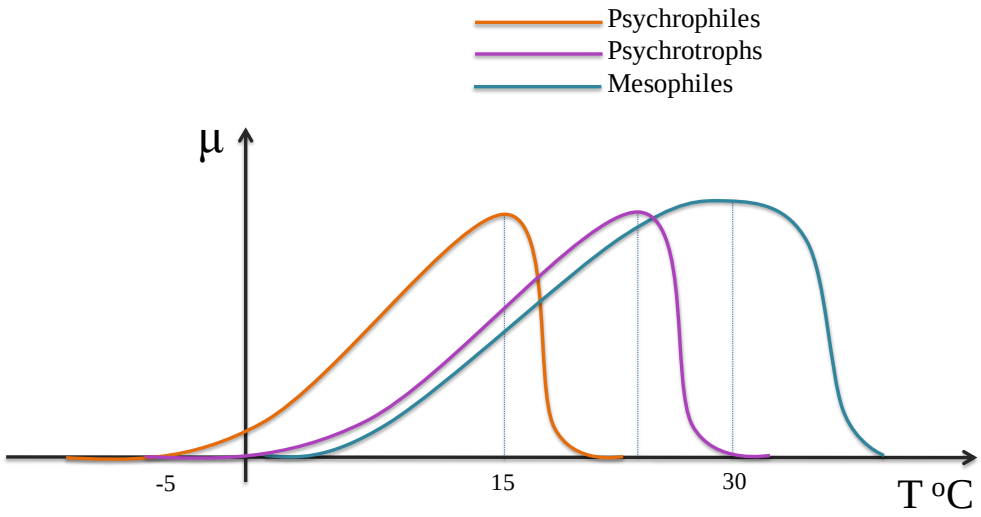

Figure 1.2: *Representation of growth rate in function of temperature (Adams & Moss, 2007; Matamoros, 2008).*

2. Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) emerged around 3 billion years ago, probably before the apparition of photosynthetic cyanobacteria. Together with related taxa LAB evolved as individual lines of descent about 1.5-2 billion years ago when the earth passed from an anaerobic to an aerobic environment (Stackebrandt & Teuber, 1988). They began to expand along with the early stages of presence of milk producing mammals on the planet, over 65 million years ago (Champomier-Vergès et al., 2002). Lactic acid bacteria (LAB) constitute a diverse group of microorganisms in the *Clostridium* phylogenetic subdivision of prokaryotes or else the low-G+C subdivision. According to the current taxonomic classification they belong to the phylum *Furmicutes*, class *Bacilli* and order *Lactobacillales*. The different families include *Aerococcaceae* (genus: *Aerococcus*), *Carnobacteriaceae* (genus: *Carnobacterium*), *Enterococcaceae* (genera: *Enterococcus, Tetragenococcus, Vagococcus*), *Lactobacillaceae* (genera: *Lactobacillus, Pediococcus*), *Leuconostocaceae* (genera: *Leuconostoc, Oenococcus, Weissella, Fructobacillus*) and *Streptococcaceae* (genera: *Lactococcus, Streptococcus*). These genera are related to food commodities but overall, approximately 40 genera have been described. They are *Bacilli*-like microorganisms originating from soil that adapted to nutritionally rich ecosystems (Makarova et al., 2006).

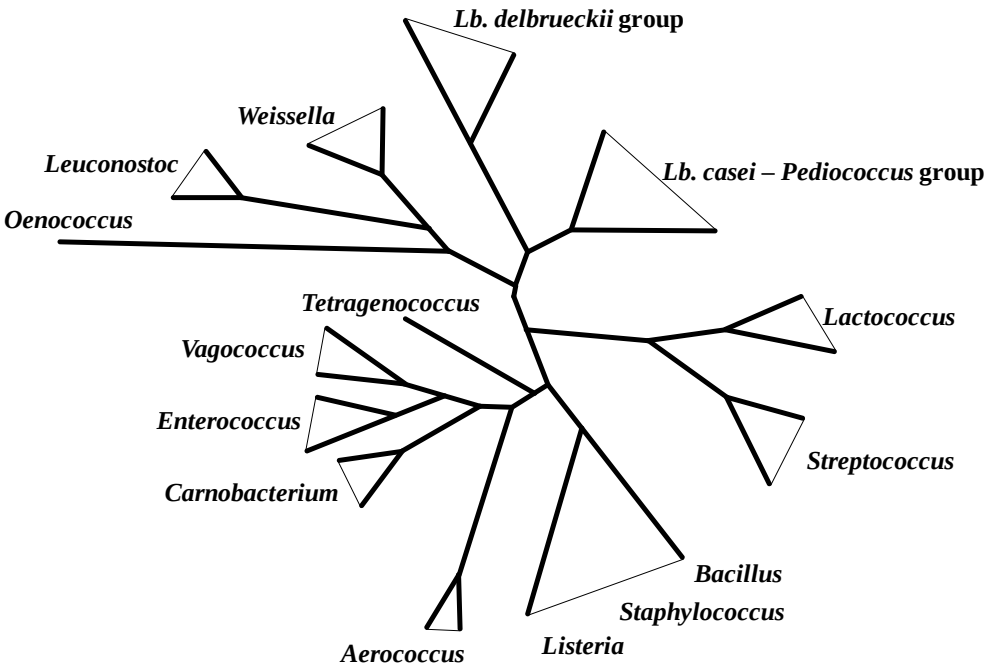

Figure 1.3: *Phylogenetic tree of lactic acid bacteria (Axelsson, 2004).* ***NOTE****: Evolutionary distances are approximate.*

LAB comprise Gram positive, oxidase and catalase negative cocci or rods, non-motile, non-sporulating, non-respiring, facultative anaerobic bacteria that mainly produce lactic acid as end-product during the fermentation of carbohydrates (Axelsson, 2004). They reside in

diverse natural habitats and are involved in the production process of various foods attributing beneficial properties to them.
They are isolated from soil, plants, the oral cavity or intestinal tract of animal species and are not pathogenic except for some species in genera *Streptococcus, Lactococcus, Enterococcus* and *Carnobacterium* that have been investigated to inflict pathogenic conditions to humans or animals (Cunningham, 2000; Morrison et al., 1997; Toranzo et al., 1993; Vendrell et al., 2006).
The LAB have a small genome of 1.8-3.3 Mbp (Kleerebezem et al., 2003) and a rather simplistic metabolism reflecting both auxotrophic and prototrophic properties. Comparative phylogenetic analyses of the genomic content across the group and reconstruction of ancestral gene sets revealed a combination of gene loss and gene gain during the co-evolution of LAB with animals and the foods they consumed. They have a wide range of biosynthetic capabilities and are indigenous to food habitats including milk, vegetable, wine, silage, cocoa, sourdough (Makarova et al., 2006).
Apparently, lactic acid bacteria evolved from soil to food microbiota by means of gene losses attributing metabolic activities. The loss of this genetic material has been compensated by evolution of efficient fermentation systems, organic acid production, transport mechanisms and acidic stress tolerance.

2.1 Role of LAB in human societies

Prokaryotes and ancestors of bacteria were among the first life forms to appear on earth around 2-3 billion years ago (Doolittle, 2000). According to the Darwinian theory of evolution, human ancestors - the first primates and hominids - evolved in the world hundreds of thousands of years ago when bacteria were abundant in water and soil, in numbers reaching millions of cells per mL or gram (Rook, 2010). During the neolithic era (10,000–3,000 BC), humans were consuming high amounts of microorganisms through their food, thus a strong support upon the hypothesis that lactobacilli and plant-based LAB were highly present in the so called paleo-diet (de Vos, 2011), could possibly explain the close interactions between mammalian cells and certain LAB taxa that developed an interesting symbiosis (Saxelin et al., 2005). This way they are associated with health-promoting nutritional aspects acting as probiotics apart from their relation to food and feed fermentations already from the antiquity. They are historically connected to food preservation, beverage and milk fermentation as well as numerous other culinary traditions (Stiles & Holzapfel, 1997).
Currently, LAB have a huge technological and economical importance in the domain of food industry (Bron & Kleerebezem, 2011; Papagianni, 2012). However, they have also emerged as spoilage bacteria for various industrially manufactured products.

2.2 Psychrotrophic LAB species involved in cases of spoilage

Especially, in the case of packaged and refrigerated food products the partial or complete exclusion of O_2 from the headspace allows the growth (Labadie, 1999) of cold-acclimatized

LAB. This way psychrotrophic LAB species become dominant and depict the spoilage manifestations at the end of the shelf-life as previously reported in many studies on various types of food products carried out during the past two decades (Table 1.1).

Table 1.1: *Studies with the most frequently implemented enumeration techniques for the assessment of microbial consortia in spoiled food products performed during the past 2 decades.*
*(**BL:** Glucose-Blood-Liver agar; **IA:** Iron agar; **LH:** Long and Hammer agar with 1% NaCl, **MRS:** de Man-Rogosa-Sharpe agar; **MRS-S:** MRS agar with 0.1% sorbic acid; **MRST:** MRS agar with 0.1% thallous acetate; **suppl. MRS:** MRS agar supplemented with 0.1% cysteine hydrochloride and 0.02% potassium sorbate; **NA:** Nutrient Agar; **PCA:** Plate Count Agar; **SNA:** Standard one Nutrient Agar; **TJA**: Tomato Juice Agar; **TSA:** Tryptic Soy Agar.)*

Food product	**Microbiological analysis for LAB**	**Enumeration method**		Highlighted species	**Reference**
		Psychrotrophic	**Mesophilic**		
Fresh raw meat					
beef	**MRS (7°C, 14d) BL (7°C, 14d) TSA (7°C, 7d)**	7°C		*Lb. algidus*	(Kato et al., 2000)
tomato marinated broiler meat	**MRS (25°C, 5d)**	25°C		*Le. gelidum* subsp. *gasicomitatum*	(Björkroth et al., 2000)
beef	**MRS (7°C, 14d) TSA (7°C, 7d)**	7°C		*Lb. fuchuensis* *Le. gelidum* subsp. *gelidum* *Lc. piscium*	(Sakala et al., 2002a,b)
marinated broiler meat strips	**PCA (25°C, 3d) MRS (25°C, 5-6d) TJA (25°C, 5-6d)**	25°C		*Le. gelidum* subsp. *gasicomitatum*	(Susiluoto, et al., 2003)
marinated broiler legs	**PCA (30°C, 3d) MRS (25°C, 5-6d)**	In parallel		*E. faecalis* *C. maltaromaticum* *C. divergens*	(Björkroth et al., 2005)
pork	**PCA (25°C, 3d) MRS (25°C, 3d)**	25°C		ND	(Liu et al., 2006)
beef	**PCA (5°C, 10d & 30°C, 3d) MRS (30°C, 2d)**	In parallel		*C. divergens* *Lb. sakei*	(Ercolini et al., 2006)
beef steaks	**MRS (25°C, 5d)**	25°C		*Le. gelidum* subsp. *gasicomitatum & gelidum*	(Vihavainen & Björkroth, 2007)
marinated pork products	**PCA (30°C, 2d) BL (15 & 30°C, 7 & 2d) MRS (30°C, 2d)**	In parallel		*Lb. algidus* *Lb. sakei* *Lb. curvatus*	(Schirmer et al., 2009)
pork	**PCA (30°C, 2d) MRS (30°C, 2d)**		30°C	*Lb. sakei* *C. divergens* *Lc. piscium*	(Jiang et al., 2010)
minced beef	**MRS (30°C, 3d)**		30°C	*Leuconostoc* spp. *Lb. sakei*	(Doulgeraki et al., 2010)
minced meat (pork, beef)	**MRS (25°C, 5d)**	25°C		*Le. gelidum* subsp. *gasicomitatum & gelidum* *Carnobacterium* spp.	(Nieminen et al., 2011)
beef	**MRS (30°C, 2d)**		30°C	*C. divergens* *Leuconostoc* spp.	(Pennacchia et al., 2011)
marinated/ unmarinated broiler meat	**MRS (25°C, 5d)**	25°C		*Le. gelidum* subsp. *gasicomitatum & gelidum* *Lactobacillus* spp.	(Nieminen et al., 2012)
broiler chicken meat	**PCA (37°C, 2d) MRS (30°C, 2d)**		30 & 37°C	*Carnobacterium* spp. *Weissella* spp.	(Zhang et al., 2012)
Cooked meat					
Vienna sausage	**SNA (25°C, 3d) suppl. MRS (25°C, 3d)**	25°C		*Lactobacillus* spp. *Leuconostoc* spp.	(Dykes et al., 1991; von Holy et al., 1991)

delicatessen meat products	**PCA (35°C, 2d) MRST (25°C, 3d)**	In parallel		ND	(Holley & McKellar, 1996)
sliced cooked meat products	**MRS-S (30°C)**		30°C	*Lb. sakei*	(Björkroth & Korkeala, 1996)
sliced cooked whole-meat products	**MRS (25°C, 5d)**	25°C		*Le. carnosum*	(Björkroth & Korkeala, 1997; Björkroth et al, 1998)
salami, cooked ham	**PCA (30°C, 3d) MRS (30°C, 3d)**		30°C	*Lb. sakei* *Le. mesenteroides* *C. divergens*	(Samelis et al., 1998a,b)
cured, cooked turkey breasts prepared	**PCA (30°C, 3d) MRS (30°C, 3d)**		30°C	*Lb. sakei* subsp. *carnosus* *Le. mesenteroides*	(Samelis et al., 2000)
"morcilla de Burgos" blood sausage	**MRS (30°C, 2-3d)**		30°C	*W. viridescens* *Leuconostoc* spp. *W. confusa*	(Santos et al., 2005)
cooked sausage	**MRS (30°C, 3d)**		30°C	ND	(Cayré et al., 2005)
cooked cured meat products	**PCA (30°C, 3d) MRS (30°C, 3d)**		30°C	ND	(Mataragas et al., 2006)
cooked ham	**PCA (30°C, 3d) MRS (30°C, 3d)**		30°C	*Le. carnosum* *E. faecalis* *Carnobacterium* spp.	(Vasilopoulos et al., 2008, 2010)
sliced cooked meat products	**PCA (20, 28 & 37°C) MRS (20, 28 & 37°C)**	In parallel		*Lb. sakei* *Lb. fuchuensis* *Le. carnosum*	(Audenaert et al., 2010)
Fresh vegetables					
RTE vegetable salad	**PCA (7°C, 10d) MRS (30°C, 2d)**	In parallel		*Lb. brevis* *Le. mesenteroides* *Lc. paracasei*	(García-Gimeno & Zurera-Cosano, 1997)
fresh-cut produce	**PCA (22°C, 5d) MRS (30°C, 3d)**	In parallel		ND	(Jacxsens et al., 2001)
RTE vegetable salad	**NA (15°C, 7d) MRS (15°C, 7d)**	15°C		*Le. gelidum* subsp. *gelidum* *Le. citreum* *Lc. piscium*	(Rudi et al., 2002)
minimally-processed fresh produce	**PCA (30°C, 3d) MRS (30°C, 3d)**	In parallel		ND	(Abadias et al., 2008)
fresh-cut salad	**PCA (21°C, 2-5d) MRS (32°C, 1-2d)**	In parallel		ND	(Randazzo et al., 2009)
Others					
"gravad" rainbow trout slices	**IA (21°C,3d) TSA (10°C, 5d) MRS (20°C, 5-7d)**	10 & 20°C		ND	(Lyhs et al., 2001)
acetic-acid herring preserve	**MRS (25°C, 5d)**	25°C		*Le. gelidum* subsp. *gasicomitatum & gelidum*	(Lyhs et al., 2004)
salmon fillets	**MRS (15°C, 7d)**	15°C		*C. maltaromaticum* *C. divergens*	(Rudi et al., 2004)
maatjes herring	**IA (21°C, 3d) MRS (21°C, 5-7d)**	21°C		*Lb. sakei* *Lb. curvatus* *Lb. fuchuensis*	(Lyhs & Björkroth, 2008; Lyhs et al. 2007)
raw salmon steaks	**LH (15°C, 7d) Elliker (20°C, 3d)**	15 & 20°C		*C. maltaromaticum* *C. divergens* *Lc. piscium*	(Macé et al., 2012)
RTE vegetable salad, raw & cooked meat, composite food	**PCA (22°C, 5d) MRS (22°C, 5d) RCA (22°C, 5d)**	22°C		*Le. gelidum* subsp. *gasicomitatum & gelidum* *Lc. piscium*	(**Chapter 3**)

Generally, LAB are very strong competitors when present in heterogeneous microbial consortia in this type of foods (Gram et al., 2002). Their ability to produce and tolerate great concentrations of lactic acid, the encoding of short antimicrobial peptides called bacteriocins or compounds with antimicrobial effect (e.g. H_2O_2, diacetyl) and the antagonistic growth rates explain their prevalence (Alakomi et al., 2000; Cayré et al., 2005; Yang et al., 2012).
It is clear that for quality aspects the assessment of the microbiota and especially LAB is imposed for packaged and chilled-stored food products (AFSCA, 2012; Uyttendaele et al., 2010). Several ways to evaluate the contamination levels and the diversity have been applied. These methods are implementing: traditional plating on (selective) media (ISO 1998, 2003), culture-dependent molecular techniques and culture-independent techniques based on polymerase chain reaction (PCR) assays (Ercolini, 2004; Jiang et al., 2010; Samelis et al., 2006), while more recently high-throughput sequencing methods and metagenomics could be applied (Ercolini, 2013; Nieminen et al., 2012).
Based on Table 1.1, LAB genera *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Carnobacterium* and *Weissella* have the greatest prevalence in cases of spoilage reported for packaged and chilled-stored food products.

2.3 LAB populations related to spoilage

Indications that counts cannot adequately describe or delineate spoilage occurrence were previously substantiated in the case of meat (Borch et al., 1996). In general, refrigerated meat and cooked meat products were considered to reach maximum contamination levels of 10^7-10^8 and 10^8-10^9 CFU/g, respectively. However, the maximum threshold that most frequently was associated with LAB populations was 10^7 CFU/g, at cold storage temperatures (Ercolini et al., 2006). Several attempts to model the growth of LAB in cooked meat matrices and correlate their levels to sensory profile scores in order to predict the end of the shelf-life have been performed. Based on quality parameters, like organoleptic properties and pH value, the LAB rejection limit was set to a value of 10^6 (Stekelenburg & Kant-Muermans, 2001; Stolzenbach et al., 2009) or 10^7 CFU/g (Kreyenschmidt et al., 2010; Slongo et al., 2009).
Sliced, cooked, whole-meat products packaged under vacuum, deriving from a meat processing plant, contaminated mainly with *Le. carnosum* to a level of 10^7 CFU/g were spoiled and thus rejected based on their appearance (Björkroth et al., 1998; Björkroth & Korkeala, 1997). Traditional Spanish blood sausages *"morcilla de Burgos"* at LAB population reaching 10^7 CFU/g are considered unsuitable for consumption as at that point typical signs of spoilage may start appearing (Diez et al., 2009a). When inoculated with *Le. mesenteroides* and *Weissella viridescens* they showed the first signs of spoilage when exceeding 10^7 and 10^8 CFU/g , after 8 and 13 days of storage, respectively (Diez et al., 2009b). Additionally, when high hydrostatic pressure was applied as preservation method spoilage occurred at LAB levels of 10^8 CFU/g (Diez et al., 2008).
Le. gelidum subsp. *gasicomitatum*, *Le. gelidum* subsp. *gelidum*, *Lb. algidus*, *Lb. sakei* and *C. divergens* were found to predominate in retail beef steaks and the contamination levels were greater than 10^8 CFU/g, when premature spoilage had occurred (Vihavainen & Björkroth, 2007).

C. maltaromaticum was inoculated in cooked and peeled tropical shrimps and based on sensorial evaluation spoilage was confirmed on Day 16 of the shelf-life experiment at contamination levels higher than 10^9 CFU/g. Other bacterial species (i.e. *Vagococcus penaei*, *C. divergens* and *C. alterfunditum*-like) tested were mainly classified as non-spoiling and weakly or moderately spoiling since no spoilage manifestations occurred until the end of the storage period although they had achieved high populations around 10^9 CFU/g (Jaffrès et al., 2011).

Tropical cooked peeled shrimp were also inoculated with *Lactococcus piscium,* which did not spoil the product, except on Day 38 when very slight "floor cloth" odor was detected. *Lc. piscium* had achieved a microbial density ranging between 10^8-10^9 CFU/g already from Day 4. This observation corroborated the bioprotective potential of the species (Fall et al., 2012; Fall et al., 2010). Two *Lc. piscium* strains were inoculated on pork meat stripes in order to evaluate their spoilage potential. They achieved contamination levels exceeding 10^7-10^8 CFU/g on Day 14 or 16 when the first significant sensory defects started appearing. *Brochothrix thermosphacta* was used as positive control and caused spoilage already from Day 10. The *Lc. piscium* strains that were inoculated in the pork samples coexisted with the initial contaminants of the background flora belonging to *Leuconostoc* spp. and albeit the initial 2-3 logs of difference in counts, the leuconostocs were able to reach the level of lactococci in a relatively short time because they did not exhibit a lag phase during the beginning of the shelf-life experiments and they also had higher growth rate (Rahkila et al., 2012). *Lc. piscium* showed again a moderate spoilage character that cannot be compared to other notorious LAB species thriving at the same commodities.

MA packaged, marinated broiler chicken legs stored at 6 ^{o}C were analyzed 5 days past the end of shelf-life in which *C. maltaromaticum*, *C. divergens* and some lactobacilli in levels of approximately 10^9 CFU/g were the SSO having outcompeted enterococci that were more abundant in the beginning of the storage experiment. Evidently spoilage had occurred and all analyzed samples were considered unfit for consumption (Björkroth et al., 2005). Severely spoiled, MA packaged, raw, tomato-marinated broiler meat strips associated with gas formation and vast LAB counts up to 10^{10} CFU/g were the first isolation commodity of formerly unknown *Le. gelidum* subsp. *gasicomitatum* (Björkroth et al., 2000). Despite the susceptibility of marinated products to spoilage, in the case of retail, marinated broiler meat strips where *Le. gelidum* subsp. *gasicomitatum* was found as predominant species with contamination levels at the end of the storage period around 3 x 10^8 CFU/g recovered on tomato juice agar medium (TJA) and 2 x 10^8 CFU/g on MRS no significant changes such as unpleasant odor, slime formation and gas production were found (Björkroth, 2005; Susiluoto et al., 2003).

All these studies corroborate that a universal threshold of contamination based on which products could be rejected does not exist. LAB species present on different substrates that provide other nutrients have dissimilar dynamics and different metabolic patterns that may or may not result in spoilage (Nychas et al., 2008; **Chapter 3**). Thus quality aspects in practice are not solely judged upon microbiological standards but on overall sensorial scores.

3. Spoilage-related LAB genera: *Leuconostoc* and *Lactococcus*

Clearly, *Leuconostoc* spp. are the most widespread psychrotrophic LAB involved in spoilage of all sorts of food products and therefore the most interesting genus for extensive study. *Lactococcus* on the other hand, is more related to food for the functional contribution of *Lactococcus* spp. in dairy industry application, biopreservation and less for spoilage. For this reason the genus is considered special as only one species (i.e. *Lactococcus piscium*) has a documented spoilage potential.

3.1 Phylogeny of genus *Leuconostoc*

The three genera: *Leuconostoc*, *Oenococcus* and *Weissella* belonging to the lactic acid bacteria (LAB) are very closely related (Björkroth & Holzapfel, 2006). Previously genus *Leuconostoc* [< Gr. neut. n. λευκός (=white) + N. L. neut. n. nostoc (=algal)] was defined as a total of heterofermentative, coccoid LAB producing only D(-)-lactate when depleting glucose and incapable of forming ammonia from arginine. The characteristic trait of this genus was the heterofermentative metabolism and some morphological properties that distinguished them from other cocci and from the heterofermentative lactobacilli. Nonetheless, some leuconostocs (*Le. paramesenteroides*) shared many common aspects with some "coccoid rods" belonging to the *Lactobacillus* genus at that time (*Lb. viridescens, Lb. halotolerans, Lb. chandler, Lb. minor* and *Lb. confusus*). Phylogenetic studies that followed had proved that those bacteria formed the core of a new LAB genus hence the name *Weissella* was suggested for reclassifying these "leuconostoc-like" species that originated from meat (Axelsson, 2004; Collins et al., 1993).

Table 1.2: *Validly published names of all* Leuconostoc *spp.*

Species designation	Isolation origin of type strain	Reference
Le. carnosum	Chilled-stored meats	(Shaw & Harding, 1989)
Le. citreum	Honey dew of rye ear	(Farrow et al., 1989)
Le. fallax	Sauerkraut	(Martinez-Murcia & Collins, 1991)
*Le. garlicum**	Garlic*	Unpublished name from 2003*
Le. gelidum	Chilled-stored meats	(Shaw & Harding, 1989)
Le. holzapfelii	Coffee fermentation	(De Bruyne et al., 2007)
Le. inhae	Kimchi	(Kim, 2003)
Le. kimchii	Kimchi	(Kim et al., 2000)
Le. lactis	Milk	(Garvie, 1960)
Le. mesenteroides	Sugar beet solution	(Tsenkovskii 1878; Wilkinson & Garvie, 1983)
Le. miyukkimchii	Brown algae kimchi	(Lee et al., 2012)
Le. palmae	Palm wine	(Ehrmann et al., 2009)
Le. pseudomesenteroides	Cane juice	(Farrow et al., 1989)

On the other hand, wine bacteria belonging to the genus *Oenococcus* (Dicks et al., 1995) had been previously classified as *Le. gracile, Le. citrovorum* and *Le. oenos* (Garvie, 1967; Kunkee, 1968; Pilone et al., 1966) but studies based on the phylogenetic characteristics of these strains revealed that *Le. oenos* represented a distinct subline.
For this reason the *Leuconostoc* genus consists of three distinct evolutionary lines as it was shown by 16S and 23S rRNA analysis (Martinez-Murcia & Collins, 1990, 1991; Martinez-Murcia et al., 1993): *Leuconostoc sensu stricto*, *Le. paramesenteroides* and *Le. oenos* lines. Presently, 12 species of the genus *Leuconostoc* are recognized constituting the *Leuconostoc sensu stricto* group. The former species *Le. cremoris* and *Le. dextranicum* are currently attributed as two subspecies of *Le. mesenteroides* (Garvie, 1979) together with subspecies *mesenteroides* and subspecies *suionicum* (Gu et al., 2012).
Recently, the close relatedness among the species *Le. gelidum, Le. gasicomitatum* (Björkroth et al., 2000) was further investigated and resulted in the proposal to create three new subspecies in the species *Le. gelidum,* i.e. *Le. gelidum* subsp. *gelidum, Le. gelidum* subsp. *gasicomitatum* and *Le. gelidum* subsp. *aenigmaticum* (Rahkila et al., 2014). Lastly, the species *Le. amelibiosum* and *Le. argentinum* proved to be synonymous with *Le. citreum* (Takahashi et al., 1992) and *Le. lactis* (Vancanneyt et al., 2006) respectively. Likewise, the hitherto *Le. fructosum* subcluster comprising *Le. durionis*, *Le. ficulneum*, *Le. fructosum* and *Le. pseudoficulneum*, was reassigned to *Fructobacillus* gen. nov. (Endo & Okada, 2008). *Leuconostoc* spp. play an important role as food fermenting cultures. They perform many biochemical conversions of nutrients to flavor or odor-related compounds enhancing greatly the organoleptic profile of fermented vegetables or dairy products (Abriouel et al., 2008; Gori et al., 2012; Wouters et al., 2013).

3.2 *Leuconostoc* spp. as human pathogens

Similarly to most LAB, *Leuconostoc* spp. are considered non-pathogenic and therefore are generally regarded as safe (GRAS) microorganisms. Still human infections have been reported (Handwerger et al., 1990), nevertheless these cases are rare and leuconostocs always behave as opportunistic nosocomial pathogens (Bou et al., 2008; Zinner, 1999).
These infections develop upon critically ill patients the majority of whom present several common aspects that constitute predisposing factors such as severe underlying diseases, previous long-term vancomycin treatment, malignancy, indwelling intravascular catheter, prolonged parenteral feeding, prolonged hospitalization or a general immunocompromised status (Huber et al., 2007; Ishiyama et al., 2011; Shin et al., 2011; Svec et al., 2007; Yamazaki et al., 2009). Leuconostocs have attenuated virulence but can inflict bacteremia to patients with a certain immunodeficiency.
Le. lactis (Deng et al., 2012; Lee et al., 2011), *Le. mesenteroides, Le. garlicum* (Kumar et al., 2012) have been isolated from blood samples as well as *Leuconostoc* spp. (Florescu et al., 2008) that have often been misidentified as streptococci during clinical tests (Dhodapkar & Henry, 1996; Janow et al., 2009).
The occurrence of amino acid-decarboxylase activity in *Le. mesenteroides* subsp. *cremoris* and the former *Le. paramesenteroides* in a tyrosine supplemented milk substrate corroborates

a presumptive harmful profile based on production of biogenic amines (BA) such as tyramine (González de Llano et al., 1998). Additionally, *Le. mesenteroides* was investigated as putrescine former in sherry-type wine (Moreno-Arribas & Polo, 2008) to concentration levels that could cause a potential health risk for consumers. On the other hand numerous studies have highlighted the inability of leuconostocs to produce biogenic amines (Bover-Cid & Holzapfel, 1999; Straub et al., 1995) making it unclear whether this genus can actually contribute to the accumulation of tyramine or histamine like other potent BA formers: *Lactobacillus curvatus, Lb. brevis, Lb. buchneri, Pediococcus palvurus* (Landete et al., 2005) carnobacteria and streptococci.

3.3 *Leuconostoc gelidum* subsp. *gasicomitatum*

Leuconostoc gelidum subsp. *gasicomitatum* [ga.si.co.mi.tà.tum< N. L. neut. n. gasium (=gas) + N. L. adj. comitatum (=accompanied), implying that its growth co-occurs with gas formation] is one of the most recently identified though very notorious spoilage LAB.
The complete genome of type strain *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T has already been sequenced elucidating properties related to the expression of its spoiling potential (Johansson et al., 2011). The genome contains approximately 2 Mbp with an average GC content of 37% and has no plasmids. The cells are spherical or oval shaped varying between 0.5-1 μm in diameter and form small grayish white colonies (Björkroth et al., 2000).
Le. gelidum subsp. *gasicomitatum* is a psychrotrophic bacterium able to grow really fast at temperatures below the optimal growth temperature of the mesophilic LAB (4, 15 and 25 ^oC) and shows no growth at 30 ^oC and 37 ^oC. This could be the reason why it is not frequently isolated from spoiled food products when the mesophilic enumeration method is implemented as a shelf-life parameter assessing the contamination levels (**Chapter 3**). It is related to cases of spoilage in raw meat, cooked meat, marinated fishery products, ready-to-eat (RTE) minimally processed vegetable salads, vegetable based products as well as RTE-meals (Lyhs et al., 2004; Vihavainen & Björkroth, 2009). *Le. gelidum* subsp. *gasicomitatum* has been involved in few studies but all of them showed it dominates the food matrices it thrives in. The common characteristic of all these susceptible products is the low-temperature storage and MA packaging. Apparently, it possesses the ability to grow efficiently at 4 ^oC reaching exceptionally high populations outcompeting all the other LAB species present determining the spoilage manifestation that occurs. The low concentration of oxygen or its complete exclusion as well as high O_2 concentrations seem to favor the proliferation of the species (Jääskeläinen et al., 2013). Slime formation, unpleasant buttery odor, green discoloration, extreme gas production and bulging of the packages tend to be the most specific alterations related to the metabolic activities of *Le. gelidum* subsp. *gasicomitatum* in packaged, chilled-stored raw beef steaks and vegetable sausages (Säde, 2011).
Many studies emphasize on the fact that despite the very high populations it reaches by the end of the shelf-life of a chill-stored product (10^7-10^{10} CFU/g), the initial contamination levels are very low (Björkroth, 2005) and sometimes below the detection threshold of the microbiological analysis performed (<10 CFU/g). Attempts to trace the contamination source in food processing plants have brought poor results corroborating that a very low number of

cells can proliferate greatly and eventually dominate the microbial consortium providing that the storage and packaging technology of the products favour its growth.

Table 1.3: *Conditions promoting the dominance of* Leuconostoc *spp. among the members of a microbial association in food.*

Conditions inducing dominance of *Leuconostoc* spp. in a microbial consortium	**Specific promoting parameters or constituents**	**Products involved**	**References**
Vacuum or MA packaging & Low-temperature storage	high concentration of CO_2 & temperature 4-7^oC	Meat, vegetable sausages/salads	(Korkeala & Björkroth, 1997)
Marinade	glucose, NaCl, lactic acid, acetic acid, tomato juice, spices	Raw broiler meat strips	(Susiluoto et al., 2003; Vihavainen et al., 2007)
Brine	NaCl, citrate	Herring preserve, Boiled eggs	(Lyhs et al., 2004; **Chapter 6**)
Sweet bell peppers	sucrose, glucose	RTE minimally processed vegetable salads	(**Chapter 4 & 5**)
High oxygen packaging	Heme, O_2	Meat, sweet bell peppers	(Jääskeläinen et al., 2012; **Chapter 5**)

3.4 Genus *Lactococcus*

Lactococcus is a very significant genus of LAB playing role in many biotechnological and industrial applications. The currently known species comprise: *Lc. lactis* (subsp. *cremoris*, subsp. *hordniae*, subsp. *lactis*, subsp. *tructae*,), *Lc. garvieae, Lc. raffinolactis, Lc. plantarum, Lc. piscium, Lc. formosensis, Lc. taiwanensis, Lc. fujiensis* and *Lc. chungangensis* (Chen et al., 2013, 2014; Cho et al., 2008; Jin et al., 2011; Teuber & Geis, 2006). The most common habitats of lactococci are found in the various niches in dairy processing environments or plant material but they were also recovered from different species of domesticated animals, mainly in tonsils of ruminants, canine and feline (Pot et al., 1996).

They are considered homofermentative and microaerophilic concerning their O_2 demands although complete genome sequence obtained from *Lc. lactis* subsp. *lactis* IL1403 (Bolotin et al., 2001) showed presence of a complete cytochrome d oxidase resulting in respiration when heme is externally supplemented (Lan et al., 2006).

Species *Lc. lactic* subsp. *cremoris,* subsp. *lactis* and subsp. *lactis* biovar. *diacetylactis* have been used extensively as starter cultures for production of cheese and other dairy products. They have great biochemical and technological importance as they ferment lactose producing lactate, form diacetyl from citrate uptake and contribute to ripening through proteolysis and formation of aroma compounds (Jokovic et al., 2008; Teuber & Geis, 2006). *Lc. garvieae* is an animal and fish pathogen causing bovine mastitis and fish lactococcosis (Morita et al., 2011; Vendrell et al., 2006). *Lc. piscium* was also firstly isolated from diseased salmonoid

fish (Williams et al., 1990) and has been used as bioprotective culture for fishery products (Fall et al., 2010; Fall et al., 2012). However, *Lc. piscium* has been mainly the only *Lactococcus* sp. associated with food spoilage (Table 1.1).

3.5 Genera *Lactobacillus* and *Carnobacterium*

Lactobacillus is the LAB genus that encompasses the greatest number of species. It is significantly heterogeneous, as the different species possess a large variety of phenotypic, biochemical, and physiological attributes. Lactobacilli are widespread in nature, and many strains are economically important and greatly related to food industries dealing with dairy products, fermented sausages, pickled vegetables, table olives, sourdough or probiotic culture supplements (Stiles & Holzapfel, 1997). They are generally among the most acid-tolerant LAB and thus, terminate many spontaneous lactic fermentations such as silage and vegetable fermentations (Wouters et al., 2013). Lactobacilli are frequently isolated from the oral cavity, gastrointestinal tract, and vagina of humans and animals (Axelsson, 2004).
They are divided in three groups according to their metabolic properties: homofermentative, heterofermentative and facultative heterofermentative lactobacilli. Overall they can utilize sugars, amino acids, glycerol and give rise to several flavor compounds that can be associated to spoilage as well. The species more frequently reported in product defects are: *Lb. sakei* (Audenaert et al., 2010; Lyhs & Björkroth, 2008), *Lb. curvatus* (Gori et al., 2012; Santos et al., 1998), *Lb. algidus* (Kato et al., 2000), *Lb fuchuensis* (Sakala et al., 2002) and *Lb. alimentarius* (Lyhs et al., 2001).
Previously, carnobacteria were assigned to genus *Lactobacillus* (Collins et al., 1987). Food-related carnobacteria are associated with livestock, fishery and products of animal or fish origin (Afzal et al., 2010). There are also certain species present in polar aquatic environments as they can sustain low temperatures and serial freeze-thaw cycles (Leisner et al., 2007). Generally, carnobacteria grow at relatively high pH and are acetate-sensitive (Axelsson, 2004). Species *C. maltaromaticum* and *C. divergens* are related to spoilage of meat and meat products, cheese and milk, shell-fish and fishery (Table 1.1; Afzal et al., 2010; Grothlaursen, 2005; Noseda et al., 2012).

4. Metabolism of LAB

It was mentioned previously that apart from the cell densities and the microbial taxa present in a food matrix, metabolism plays a role in the development of sensorial defects. LAB present in food commodities are using the available nutrients based on their gene repertoires in order to grow outcompeting other microbes while their byproducts accumulate attributing uninviting properties to food (Huis in't Veld, 1996). Nutritional requirements vary among different LAB species, as well as comparing different strains within the same species (Axelsson, 2004). In general though, LAB are fastidious (i.e. nutritionally highly demanding) microorganisms. Their primary source of energy and carbon sources are sugars. They ferment hexoses (i.e. glucose) via the glycolysis pathway producing lactate and the pentose

phosphoketolase pathway producing equimolar amounts of lactate, ethanol/acetate and CO_2 (Liu, 2003).

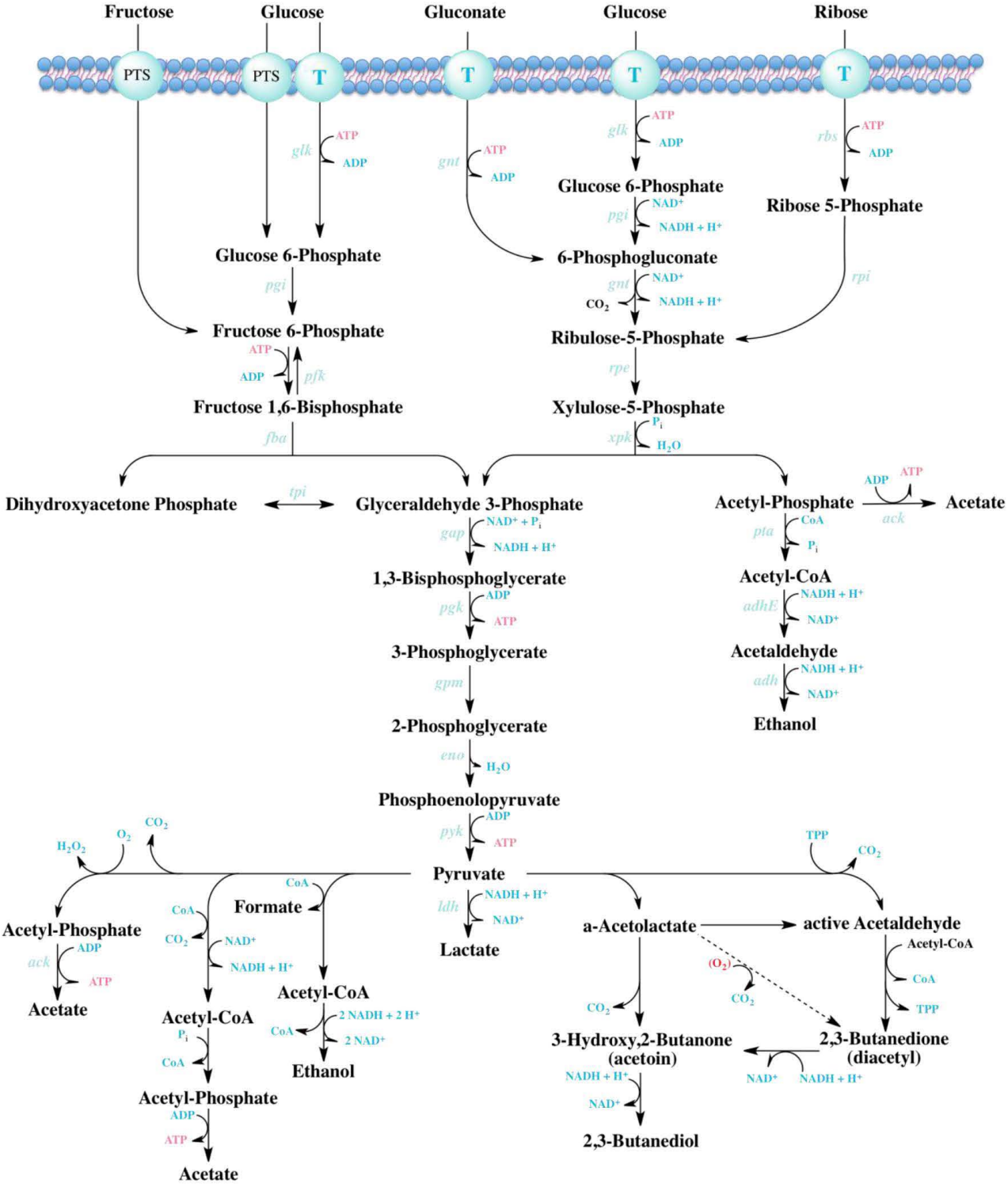

Figure 1.4: *Carbohydrate metabolism in LAB (Axelsson, 2004). Main genes encoding for enzymes,* ***glk****: glucokinase,* ***pgi****: phosphoglucoisomerase,* ***pfk****: phosphofructokinase,* ***fba****: fructose 1,6-bisphosphate aldolase,* ***tpi****: triose phosphate isomerase,* ***gap****: glyceraldehyde 3-phosphate,* ***pgk****: phosphoglycerate kinase,* ***gmp****: phosphoglycerate mutase,* ***eno****: enolase,* ***pyk****: pyruvate kinase,* ***lgh****: lactate dehydrogenase,* ***gnt****: gluconokinase,* ***gnt****: 6-phosphogluconate dehydrogenase,* ***rpe****: ribulose-phosphate 3-epimerase,* ***xpk****: xylulose 5-phosphate*

*phosphoketolase, **pta**: phosphate acetyltransferase, **ack**: acetate kinase, **adhE**: aldehyde dehydrogenase, **adh**: alcohol dehydrogenase, **rbs**: ribose pyranase, **rpi**: ribose 5-phosphate isomerase. Transport systems, **T**: transport protein, **PTS**: phosphotransferase system.*

Sugar catabolism results in energy generation through substrate oxidation steps that have glucose as starting molecule and lead to pyruvate production. Hexose fermentation for LAB can be divided into the aforementioned metabolic patterns. First of which, homofermentation (homolactic fermentation or Embden-Meyerhof-Parnas pathway) used by the majority of LAB (except leuconostocs, oenococci, weissellas and the heterofermentative lactobacilli), under normal conditions in a fermentation environment where excess of sugar is available and limited access to oxygen, involves the oxidation of glucose to fructose 1,6-bisphosphate and finally to pyruvate, which is subsequently reduced to lactic acid by a NAD^+-dependent lactate dehydrogenase (nLDH), reoxidizing the NADH generated during the earlier glycolytic steps.
A redox balance is thus obtained, lactic acid is virtually the only end-product, and the metabolism is referred to as homolactic fermentation. Theoretically, glucose homofermentation generates 2 mols of lactic acid and a net gain of 2 ATP per mol of depleted glucose.
On the other hand, heterofermentation (heterolactic fermentation or pentose phosphate pathway or 6-phosphogluconate/phosphoketolase 6-PG/PK) used by the strictly heterofermentative LAB (i.e. leuconostocs, oenococci, weissellas and heterofermentative lactobacilli) characterized by the formation of 6-phosphogluconate that is subsequently decarboxylated to pentose 6-phosphate resulting in the accumulation of lactic acid, acetic acid, CO_2 and ethanol (Axelsson, 2004; de Vos, 2011).
The key feature of bacterial fermentations include various oxidation steps based on bioconversion of a substrate that leads to energy-rich compounds. Subsequently, these intermediates can be used for ATP production through substrate-level phosphorylation (Liu, 2003). As a result NADH is formed from NAD^+, which needs to be regenerated in order to propagate the fermentation. Pyruvate serves as electron acceptor for this regeneration step in the case of both fermentation pathways used by LAB thus formation of lactate is frequent.
Under certain circumstances, LAB use alternative ways of utilizing pyruvate than the reduction to lactic acid. By over expressing NADH oxidases, O_2 could efficiently act as an electron acceptor and the surplus pyruvate could subsequently be diverted to other compounds by pathways mainly known as the alternative fates of pyruvate. This way many different organic compounds can be produced, namely acetoin, diacetyl, 2,3-butanediol and formate. (Teusink et al., 2011). Overall, factors limiting catabolism lead to fermentation patterns with multiple end-products and non-limiting catabolism with high glycolytic flux favor homofermentative metabolism (Zaunmüller et al., 2006).
However, apart from sugars other substrates can be utilized for energy yield and generation of other organic compounds. Amino acids (e.g. methionine, cysteine, arginine, leucine, isoleucine, valine, histidine, tyrosine etc.), citrate, nucleosides and nucleobases (Rimaux et al.,

Table 1.4: *Involvement of spoilage-related LAB species in documented cases of sensorial defects or studies evaluating the spoilage potential of certain LAB strains. The chemical analysis performed, the organoleptic alterations and the responsible compounds to which spoilage was attributed are presented.*

Food product	LAB species	Method of chemical analysis	Spoilage related compounds	Sensorial defect	Reference
Raw meat					
marinated broiler meat food	*Le. gelidum* subsp. *gasicomitatum*		CO_2, acetic acid	gas production, pungent off-odor	(Björkroth et al., 2000)
marinated beef	*Le. gelidum* subsp. *gasicomitatum*		H_2O_2, diacetyl	green discoloration, buttery off-odor	(Vihavainen & Björkroth, 2007)
broiler unprocessed beef, pork	*Le. gelidum* subsp. *gasicomitatum*		diacetyl	buttery off-odor	(Vihavainen & Björkroth, 2009)
beef	*C. divergens*	SPME-GC-MS ^{1}H NMR	butanoic acid, acetoin	rancid, cheese off-odor	(Ercolini et al., 2011)
beef	*C. maltaromaticum*	GC-MS	2-ethyl-1-hexanol, 2-buten-1-ol, 2-hexyl- 1-octanol, aldehydes, lactones, sulfur compounds	off-odor	(Ercolini et al., 2009)
pork	*Le. gelidum* subsp. *gasicomitatum*	SPME-GC-MS	acetic acid,ethanol diacetyl, acetoin	sour, byttery off-odor	(Jääskeläinen et al., 2013)
Cooked meat products					
heat-treated pork product	*Lb. sakei* *Le. carnosum*	HPLC	ethanol, acetic acid	sour off-odor	(Laursen et al., 2009)
artisan-type, cooked ham	*Carnobacterium* spp. *Leuconostoc* spp.	HS & SPME-GC-MS	dimethyl disulfide ethanol, acetate, acetoin	rotting off-odor	(Leroy et al., 2009)
cooked, minced sausage	*C. maltaromaticum*		a-ketoisocaproic acid, 3-methylbutanoic	fatty off-odor	(Larrouture-Thiveyrat et al., 2003)
morcilla de Burgos blood sausages	*W. viridescens* *Le. mesenteroides*	electronic nose SPME-GC-MS	ethanol, diacetyl, acetoin, hexanal, acetic acid	sour off-odor	(Diez et al., 2009)
saveloy sausages	*C. maltaromaticum*	GC-MS	2- and 3-methylbutanol, 2- and 3-methylbutanal, diacetyl, acetoin	sour, sweet off-odor	(Holm et al., 2013)
artisan-type, cooked ham	*C. divergens*	SH-GC-MS	lactic acid ethanol 3-methyl-1-butanol		(Vasilopoulos et al., 2010)
Fishery and fish products					
peeled tropical shrimps	*C. maltaromaticum*	SPME-GC-MS-FID	3- methyl-1-butanal, 2-methyl-1-butanol, 2-methyl-1-propanal, diacetyl, ethyl acetate, acetaldehyde	cheese/feet, sour/fermented and buttery off-odor	(Jaffrès et al., 2011)
acetic-acid herring	*Le. gelidum* subsp. *gasicomitatum*		dextran, CO_2	slime formation, gas production	(Lyhs et al., 2007)
raw salmon	*C. maltaromaticum*	SPME-GC-MS-FID		buttery, feet/cheese off-odor	(Macé et al., 2013)
cold-smoked salmon	*C. maltaromaticum*	GC-MS	diacetyl 2,3-pentanedione	buttery, caramel, sweet, fruity off-odor	(Joffraud et al., 2001)
shirmp	*C. divergens* *C. maltaromaticum*		ornithine, ammonia acetic acid, alcohols, aldehydes	malty, nutty, sour off-odor	(Mejlholm et al., 2005)
shrimp	*C. maltaromaticum*		ornithine, ammonia	grass/hay, weak chlorine off-odor	(Laursen et al., 2006)
Vegetable and fruit products					
vegetable sausages	*Le. gelidum* subsp. *gasicomitatum*		dextran, CO_2 lactic acid, acetic acid	slime formation, gas production, off-odor	(Vihavainen at al., 2008)
lettuce juice agar	*Le. gelidum* subsp. *gasicomitatum* *Lb. brevis*	HPLC	lactic acid, acetic acid, ethanol	sour off-odor	(Jacxsens, et al., 2003)
honeydew melon agar	*Le. mesenteroides* *Le. gelidum* subsp. *gelidum*	SIFT-MS	acetic acid, ethanol, acetoin, diacetyl	sour, buttery off-odor	(Zhang et al., 2013)

2011b) as well as glycerol can be uptaken by LAB genera contributing to a great range of metabolites (e.g. sulfur compounds, ornithine and citrulline, 2-methylbutanal, indole, histamine, tyramine etc.) that contribute to enhanced organoleptic properties in the case of fermentations or to manifestation of alterations in cases of spoilage (Ardö, 2006; Gram et al., 2002; Nychas et al., 2008; Rimaux et al., 2011a).

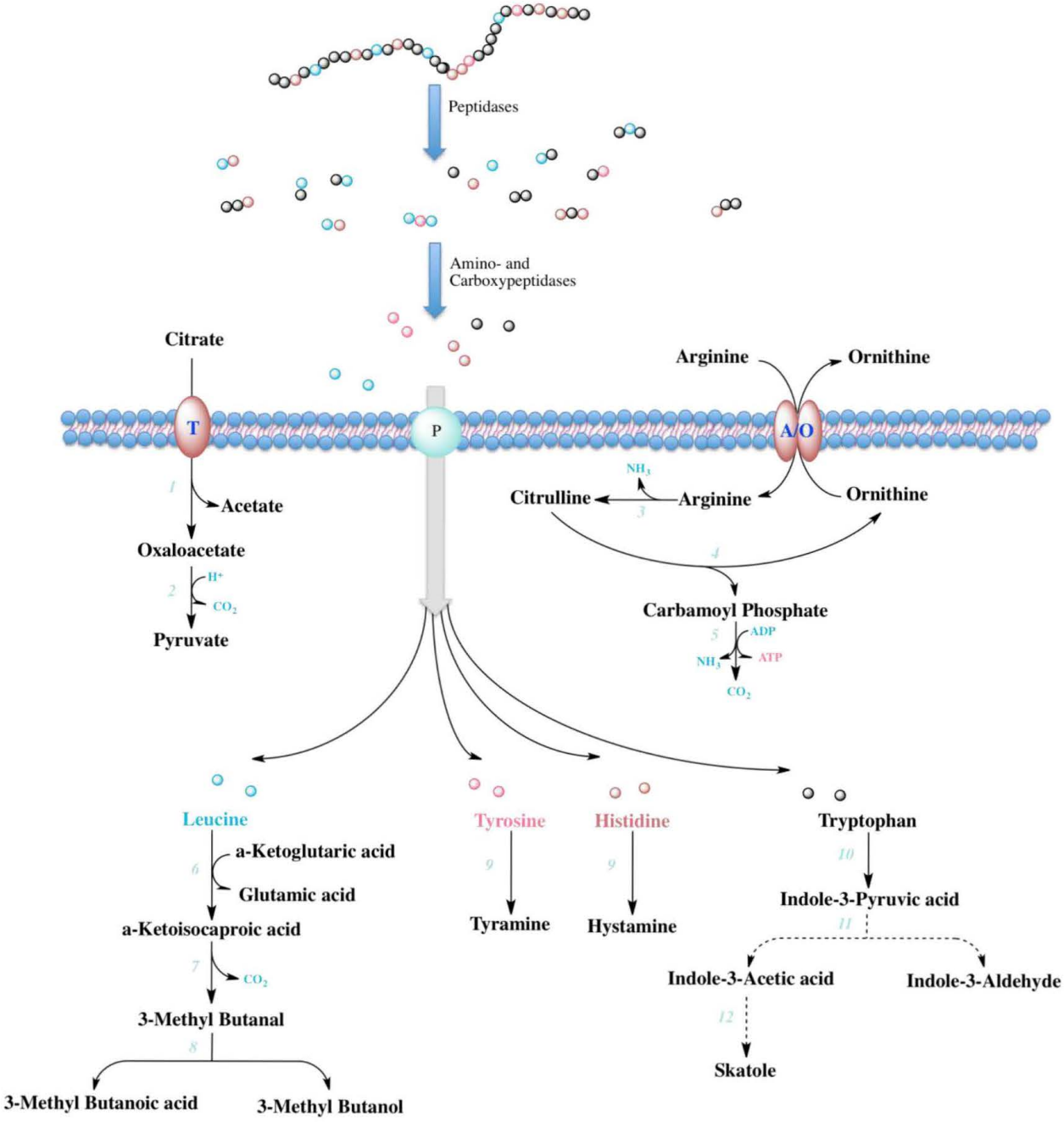

Figure 1.5: *Citrate and amino acid uptake and catabolism by LAB . Keys enzymes,* ***1****: citrate lyase,* ***2****: oxaloacetate decarboxylase,* ***3****: arginine deimenase,* ***4****: ornithine transcarbamoylase,* ***5****: carbamate kinase,* ***6****: transaminase,* ***7****: decarboxylase,* ***9****: decarboxylase,* ***10****: aminotransferase. Transport systems,* ***T****: transport protein,* ***A/O****: argine/ornithine antiporter,* ***P****: transport protein.*

The spoilage manifestations of psychrotrophic bacteria are characterized by production of organic acids, emission of off-odors, discoloration, slime formation that render a product unacceptable for consumption (Françoise, 2010). Understanding the process of microbial spoilage is vital in designing effective strategies to prevent food losses by eliminating spoilage microbes or inhibiting their growth in the case of unstable food products like packaged and chilled-stored products (Vasilopoulos et al., 2010).

5. Cold and oxidative stress for genera *Leuconostoc* and *Lactococcus*

Apart from the required adaptations to the specifications of the food matrix, the competitive interactions with the other members of the microbial consortium in the case of packaged and chilled-stored food products psychrotrophic LAB species belonging to genera *Leuconostoc* and *Lactococcus* have to adjust to low-temperature and specific gas compositions (Garnier et al., 2010; Jääskeläinen et al., 2013).

Table 1.5: *Cold stress responses of all available* Leuconostoc *and* Lactococcus *strains with entirely sequenced genomes .*

LAB strain	Origin	No. of plasmids	Genome size (Mbp)	Cold stress		Reference
				No. of CSP *genes*	CSP protein (Locus tag)	
Leuconostoc carnosum **JB16**	kimchi	4	1.773468	1	CSPA (C270_01000)	(Jung et al., 2012a)
Leuconostoc citreum **KM20**	kimchi	4	1.896614	2	CSPD (LCK_01134); CSP (LCK_01552)	(Kim et al., 2008)
Leuconostoc gelidum subsp. *gasicomitatum* **LMG 18811**	marinated broiler meat	-	1.954080	2	CSPA (LEGAS_0763); CSPC (LEGAS_1684)	(Johansson et al., 2011)
Leuconostoc gelidum subsp. *gelidum* **JB7**	kimchi	-	1.893499	1	CSP (C269_08055)	(Jung et al., 2012b)
Leuconostoc kimchii **IMSNU 11154**	kimchi	5	2.101787	2	CSPA (LKI_06770); CSPD (LKI_08955)	(Oh et al., 2010)
Leuconostoc mesenteroides subsp. *mesenteroides* **ATCC 8293**	fermented olives	1	2.075763	2	CSPA (LEUM_1878); CSPG(LEUM_0703)	www.patricbrc.org 15/2/2014
Leuconostoc mesenteroides subsp. *mesenteroides* **J18**	kimchi	5	1.896561	2	CSPA (MI1_08135); CSPG(MI1_03205)	(Jung et al., 2012c)
Leuconostoc sp. **C2**	kimchi	-	1.877273	2	(LGMK_05375); (LGMK_03160)	www.patricbrc.org 15/2/2014

Lactococcus garvieae **ATCC 49156**	yellowtail fish	-	1.950135	3	(LCGT_0544); (LCGT_1421); (LCGT_1424)	(Morita et al., 2011)
Lactococcus garvieae **Lg2**	yellowtail fish	-	1.963964	3	(LCGL_0563); (LCGL_1442); (LCGL_1445)	(Miyauchi et al., 2012)
Lactococcus lactis subsp. *cremoris* **A76**	cheese	4	2.452618	3	CSPC (LCGL_0563); CSPA (LCGL_1442); CSPB (LCGL_1445)	(Bolotin et al., 2012)
Lactococcus lactis subsp. *cremoris* **MG1363**	-	-	2.529478	6	CSPC (IImg_0180); CSPA (IImg_1847); CSPB (IImg_1238), (IImg_1256), (IImg_1846); CSPF (IImg_1255)	(Wegmann et al., 2007)
Lactococcus lactis subsp. *cremoris* **NZ9000**	-	-	2.530294	6	CSPC (LLNZ_00940); CSPA (LLNZ_09505); CSPB (LLNZ_06375), (LLNZ_06470), (LLNZ_09500); CSPF (LLNZ_06465)	(Linares et al., 2010)
Lactococcus lactis subsp. *cremoris* **SK11**	cheese	5	2.598348	5	CSPC (LACR_0174); CSPA (LACR_0755); CSPB (LACR_C47), (LACR_0756); CSPF (LACR_C48)	www.patricbrc.org 15/2/2014
Lactococcus lactis subsp. *cremoris* **UC509.9**	dairy starter	8	2.250427	2	CSPC (uc509_0161); CSPA (LACR_0718)	(Ainsworth et al., 2013)
Lactococcus lactis subsp. *lactis* **CV56**	healthy woman's urinogenital system	5	2.518737	6	CSPC (CVCAS_0146); CSPA (CVCAS_0606), (CVCAS_1647); CSPB (CVCAS_2308); CSPF (CVCAS_0605), (CVCAS_2309)	(Gao et al., 2011)
Lactococcus lactis subsp. *lactis* **Il1403**	cheese starter	-	2.365589	2	CSPC (L172505); CSPA (L117090)	www.patricbrc.org 15/2/2014
Lactococcus lactis subsp. *lactis* **KF147**	mung bean sprouts	1	2.635654	6	CSPC (LLKF_0161); CSPA (LLKF_0275), (LLKF_2285); CSPB (LLKF_0276), (LLKF_1198); CSPF (LLKF_2286)	(Siezen et al., 2010)

Both factors influence many physiological processes in LAB. However their inherent adaptability capacities trigger appropriate protective physiological processes aiming at maintaining their homeostasis (Wouters et al., 2000). Under low temperature bacterial cells are mainly confronted with problems associated with basic cellular operations. Firstly, mRNA translation, transcription and DNA replication must normally proceed uninhibitedly despite the stabilization of RNA structure and DNA supercoiling.

Secondly, bacteria need to maintain the fluidity of the cell membrane, which tends to decrease, in order to continue the physiological membrane-mediated functions (active transport, protein secretion, cell signaling). Additionally, a decrease in the efficiency of protein folding is observed and consequently a retardation of enzymatic reactions (Feller & Gerday, 1997). Hence upon temperature downshifts, cold-shock proteins (CSPs) encoded from evolutionarily, highly conserved domains play the role of chaperons while

simultaneously an increased synthesis of unsaturated fatty acids, which are incorporated into the membrane phospholipids, is observed (Yamanaka et al., 1998).
This way the structural integrity of macromolecules and macromolecule assemblies is maintained. *Escherichia coli, Bacillus subtilis* and *Lactococcus lactis* among LAB are the model organisms most extensively studied for their cold adaptation proving that Gram negative bacteria share a high degree of similarity with Gram positive concerning genetic loci encoding for cold-shock proteins (Feller & Gerday, 1997; Jones et al., 1987; Sanders et al., 1999). Additionally, CSPs contain regions with high homology to the cold shock domain of functional eukaryotic proteins that regulate transcription (Wouters et al., 2000). On the other hand, high availability of O_2 is responsible for oxidative stress in MAP packaging implementing high concentrations. As previously mentioned LAB are considered facultative anaerobic, however the most appropriate term would be aerotolerant (Axelsson, 2004). O_2 as such is generally harmless to LAB however along with all cellular processes, O_2 is reduced to water forming reactive intermediate compounds causing oxidative stress that generally affect many physiological procedures and functional macromolecules. Oxidative stress can have either a bacteriostatic or bacteriocidal effect on anaerobic or facultative anaerobic microorganisms by causing disruptions of metabolic pathways and spontaneous genetic mutations (Berlett & Stadtman, 1997; Fridovich, 1998). The highly reactive O_2 species consist of superoxide anion radical (O_2^-), hydroxyl radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2). Oxidative stress has not been studied thoroughly except for *Lc. lactis* and the metabolic patterns it adopts under aeration because of the technological importance it holds in dairy fermentations (Bolotin et al., 2001). More extensive discussion on the response mechanisms against reactive oxygen species is presented in **Chapter 5**.

6. LAB and biofouling

LAB have also been associated with biofilm formation on biotic and abiotic surfaces like industrial processing plants. In Table 1.6 all documented studies are presented.

Table 1.6: *Cases of LAB biofilm formation in processing plants and related food products.*

Food product/Plant	Surface	Species	Reference
Spanish-type Gordal green olive	fruit skin glass	*Lactobacillus pentosus* yeasts	(Domínguez-Manzano et al., 2012)
Fresh cut onion, frozen onion,	glass cover slips	*Lactobacillus plantarum* *Lactobacillus brevis* *Lactobacillus fructivorans*	(Kubota et al., 2008)
Yogurt, rice wine	glass slides	*Lactobacillus casei* subsp. *rhamnosus* *Saccharomyces cerevisiae*	(Kawarai et al., 2007)
Ice cream plant	stainless steel	*Enterobacteriaceae* *Pseudomonadaceae* *Leuconostoc* spp.	(Gunduz and Tuncel, 2006)
Poultry plant	floor drains	*Lactococcus lactis* *Streptococcus durans*	(Zhao et al., 2006)
Reduced-fat Cheddar-type cheese	ripening vats	*Lactobacillus* spp.	(Broadbent et al., 2003)
Cheddar cheese plant	equipment surfaces	*Lactobacillus curvatus* *Pediococcus acidilactici*	(Agarwal et al., 2006)

Total mesophilic counts underestimate in many cases the contamination levels of psychrotrophic lactic acid bacteria (LAB) in chilled-stored food products at the end of their shelf-life

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SUMMARY

The major objective of this study was to determine the role of psychrotrophic lactic acid bacteria (LAB) in spoilage-associated phenomena at the end of the shelf-life of 86 various packaged (air, vacuum, modified-atmosphere) chilled-stored retail food products. The current microbiological standards, which are largely based on the total viable mesophilic counts lack discriminatory capacity to detect strictly psychrotrophic LAB.

A comparison between the total viable counts on plates incubated at 30 ^{o}C (representing the mesophiles) and at 22 ^{o}C (indicating the psychrotrophs) for 86 food samples covering a wide range - ready-to-eat (RTE) vegetable salads, fresh raw meat, cooked meat products and composite food - showed that a consistent underestimation of the microbial load occurs when the total aerobic mesophilic counts are used as a shelf-life parameter. In 38 % of the samples, the psychrotrophic counts had significantly higher values (+ 0.5-3.2 log CFU/g) than the corresponding total aerobic mesophilic counts. A total of 154 lactic acid bacteria, which were unable to proliferate at 30 ^{o}C were isolated. In addition, a further 43 strains with a poor recovery at this temperature were also isolated.

This study highlights the potential fallacy of the total aerobic mesophilic count as a reference shelf-life parameter for chilled food products, as it can often underestimate the contamination levels at the end of the shelf-life.

2.1 INTRODUCTION

Food industries worldwide have to conform to microbial standards associated with the safety and the quality of their products. Safety aspects are of major importance thus are determined clearly and unambiguously. Usually strict limits up to no tolerance are implemented for pathogenic microorganisms that could cause severe health problems to consumers (E.F.S.A., 2010). Hygiene surveillance plans are dependent on norms and guidelines, which are continuously updated and reformed (Uyttendaele et al., 2010) in order to prevent foodborne illness outbreaks.

On the other hand quality, which is depicted by the sensorial, textural and nutritional properties that make a food product inviting to consumers cannot be precisely related to the counts of spoilage organisms hence cases of spoilage within the shelf-life are inevitable.

Storage and distribution at low temperature as well as packaging are two of the most regularly encountered methods of preservation for microbiologically unstable products. Low refrigeration temperatures restrain the growth of several spoilage microorganisms (Watada and Qi, 1999), while the partial or complete exclusion of oxygen inhibits the proliferation of Gram negative bacteria that are frequently isolated from spoiled products (Borch et al., 1996; Nychas et al., 2008; Willocx et al., 1993), whilst favouring the growth of Gram positive such as lactic acid bacteria (Abadias et al., 2008, Björkroth et al., 2005; Blickstad and Molin, 1984; Buta et al., 1999) and *Brochothrix thermosphacta* (Blickstad and Molin, 1984; Kotzekidou and Bloukas 1996; Mejlholm et al., 2005; Pin et al., 2002).

Quality aspects of foodstuffs are determined through microbiological analysis and sensorial evaluation. Microbiological quality standards have been established empirically by manufacturing companies complying with the microbial loads of the crude material, the production and packaging technology used, as well as the organoleptic properties demanded from the end-products. However, those standards are not applicable to all industrial processing plants and they fluctuate for numerous products of the same type. Nowadays, the competitive market of chilled-stored and packaged food products has raised the need for universal microbial criteria that would adequately indicate the acceptable (initial and) end of shelf-life contamination levels that would ensure the maintenance of quality throughout the shelf-life.

The most widely applied parameter used to determine the microbial quality of a packaged product which is stored under chilling temperature is the ISO 4833:2003 concerning the total viable mesophilic count for which incubation of the plates is performed at 30 ^{o}C. The counts of the mesophiles should not exceed a threshold that has been set by the manufacturing company. During the shelf-life of a product both its sensorial aspects and its microbial content should be acceptable (Borch et al., 1996). However, cases of sporadic spoilage occurring in a batch of packaged food products (Vasilopoulos et al., 2008) or spoilage manifestations in samples with acceptable contamination levels (Björkroth and Korkeala, 1996; Nerbrink and Borch, 1993) confirm the inefficiency of the existing microbiological standards and their interpretation.

Spoilage is indeed an immensely multifactorial phenomenon associated both with the type of microorganisms that thrive in the food matrix and the metabolic pathways used to deplete the nutrients in the packaging micro-conditions and not so much related to the total viable counts

(Ercolini et al., 2006; Gram et al., 2002; Liu et al., 2006; Pennacchia et al., 2011). This could jeopardize production batches that can potentially manifest spoilage before the end of the shelf-life, obliging companies to withdraw them under great financial losses (Huis in't Veld, 1996), in order to protect susceptible consumers.

During the last two decades many research groups dealing with spoilage-associated phenomena at low temperature storage have been implementing mesophilic incubation for the microbiological analysis they perform (Doulgeraki et al., 2010; Jiang et al., 2010; Metaxopoulos et al., 2002; Vasilopoulos et al., 2008; Vasilopoulos et al., 2010) while few have applied psychrotrophic incubation at different temperatures (Björkroth et al., 2005; Lyhs et al., 2001; Lyhs et al. 2007; Susiluoto et al., 2003; Vihavainen and Björkroth, 2009) as shown in Table 1.1 of **Chapter 1**. The objective of this comparative study was to establish a microbiological standard based on the enumeration of the psychrotrophic load. The standard would be evaluated for its efficacy and sensitivity compared to that of the total mesophilic count which is currently applied. The microbial flora of 86 packaged retail food products stored at refrigeration temperature was determined following plating out and incubation of the serial dilutions at 22 ^{o}C (favouring the growth of psychrotrophs) and at 30 ^{o}C which is the optimal growth temperature for mesophiles. It is common knowledge that the optimal temperature range for obligate and facultative psychrophiles extends between 15 and 25 ^{o}C (Adams and Moss, 2008; Jay et al., 2005). Already incubation at 22 ^{o}C is implemented by ISO 6222:1999 evaluating a wide spectrum of psychrotrophs in water and has been previously suggested to have a better recovery for lactic acid bacteria compared to other temperature values (Holley et al., 1996).

2.2 MATERIALS AND METHODS

2.2.1 Samples

A total of 86 packaged, retail food products obtained from various local companies in Belgium were purchased and the levels of microbial contamination were evaluated at the end of shelf-life (Table 2.1). The samples were packaged in air, under vacuum or modified-atmospheres (MA) and stored at temperatures from 2 ^{o}C to 7 ^{o}C. All analyzed products were kept in their original packages until the end of their self-life when they were opened for analysis. In none of the samples the packaging had been damaged in any way. For the purposes of this study the products were classified into four distinct groups: “ready-to-eat (RTE) vegetable salads”, “fresh raw meat”, “cooked meat products” and “composite foods”. They were kept at the storage temperature specified by the manufacturers until they reached the end of shelf-life after which microbial analysis and measurement of pH were performed. The initial counts of the analyzed products were not determined as the aim of the study was to evaluate the final microbial load of the products in the end of shelf-life after the packaging technology and low temperature storage would have facilitated the dominance of psychrotrophic and CO_2-tolerant microorganisms. Lastly, no sensory panel was involved in the study however, the quality deteriorations investigated were based on independent reports

given by the two scientists that performed the analysis of each product, judging on the general appearance, odour, texture and pH.

Table 2.1: *The sampled food products clustered in groups and more in detail those that proved to have greater counts at 22 °C. In* ***[bold]*** *the food products that had a quality deterioration at the end of their shelf-life.*

Samples analyzed in total	Samples with greater counts at 22 °C (% on overall samples)	Nº	Food product description	Packaging	Storage temperature
Ready-to-eat (RTE) vegetable salad					
21	**14 (67 %)**	**[1]**	**bell pepper, sweet corn, lettuce, carrot**	air	4 °C
		[2]	**bell pepper**	air	7 °C
		[3]	**lettuce, bell pepper, carrot**	air	4 °C
		[4]	**garden parsley, celery**	air	7 °C
		[5]	**bell pepper, tomato, onion, potato**	air	7 °C
		[6]	**lettuce, bell pepper, tomato, carrot, radish**	air	4 °C
		7	carrot, zucchini, radish	air	7 °C
		8	cucumber, carrot, celery, zucchini, radish	air	7 °C
		9	carrot sticks	air	4 °C
		10	lettuce	air	4 °C
		[11]	**rocket, corn salad**	air	7 °C
		12	zucchini, carrot, soybean, garden parsley	air	4 °C
		[13]	**bell pepper**	air	4 °C
		14	lettuce, corn salad	air	7 °C
Fresh raw meat					
6	**5 (87 %)**	**[15]**	**beef**	vacuum	4 °C
		16	beef	MA	4 °C
		[17]	**beef**	vacuum	4 °C
		18	beef	vacuum	4 °C
		[19]	**pork**	vacuum	4 °C
Cooked meat products					
29	**6 (21 %)**	20	blood sausages	MA	4 °C
		[21]	**ham slices**	vacuum	4 °C
		[22]	**spiced ham**	vacuum	4 °C
		[23]	**chipolata-type sausages**	MA	4 °C
		24	ham	vacuum	4 °C
		25	white sausages	MA	4 °C
Composite food					
30	**8 (27 %)**	26	spirelli-type pasta and fresh vegetable salad with dressing	air	7 °C
		[27]	**potato and fresh vegetable salad with mayonnaise sauce**	air	4 °C
		28	farfalle-type pasta with chicken and fresh vegetable salad	air	4 °C
		[29]	**tuna salad and fresh vegetable**	air	4 °C
		[30]	**niçoise-type salad**	air	4 °C
		[31]	**fusilli-type pasta and chicken with curry sauce**	air	7 °C
		32	shrimp in garlic sauce	vacuum	4 °C
		33	paella	MA	4 °C
Total					
86	**33 (38 %)**				

2.2.2 Colony counts

For the enumeration of the microbial contamination levels of the selected food products a representative sample of 15-25 g (depending on the product) was aseptically transferred into a stomacher bag and the primary decimal dilution was prepared by adding an appropriate volume of peptone physiological solution (PPS: 0.85 % w/v NaCl and 0.1 % w/v peptone in distilled water). Samples were homogenized for 60 s at 22 ^{o}C with a Colworth Stomacher 400 (Steward Laboratory, London, UK). Subsequently, decimal dilution series were prepared from the primary dilution using PPS and they were pour plated in Plate Count Agar (PCA, Biorad, Hercules, CA, USA), de Man-Rogosa-Sharpe Agar (MRS, Biorad, Hercules, CA, USA) adjusted to pH 5.9 (by addition of an appropriate volume of 12 N HCl solution) and Reinforced Clostridial Agar (RCA, Oxoid, Hampshire, UK) in order to determine the total aerobic, lactic acid bacteria and total anaerobic counts, respectively. An overlayer was added on the MRS and RCA plates to achieve micro-aerophilic conditions and the RCA plates were additionally put in anaerobic jars together with an Anaerogen sachet (Anaerogen, Oxoid, Hampshire, UK) for a completely anaerobic incubation. All plating was performed in triplicate with incubation at 22 ^{o}C for 5 days and at 30 ^{o}C for 3 days (ISO 4833:2003; ISO 15214:1998). The enumeration results of the two different incubation temperatures were compared statistically (SPSS version 21,IBM, SPSS, Chicago, IL, USA) by paired t-test in order to assess whether the two methods are generating significantly diffenrent counts.

2.2.3 Isolation

Colonies were picked up from the plates incubated at 22 ^{o}C when these plates were found to have higher numbers of colonies in comparison to their equivalent incubated at 30 ^{o}C (> 0.5 log CFU/g). The number and type of single colonies selected from each plate were approximately representative of the overall distribution of colonies present on the plate. In all cases the isolates originated from the plates of the highest dilution representing the dominant microbes thriving at these food commodities selected by the intrinsic food parameters and the extrinsic factor of storage. Subsequently, the isolates from PCA, MRS and RCA were transferred aseptically to tryptone soya broth (TSB, Oxoid, Hampshire, UK), de Man-Rogosa-Sharpe broth (MRS broth, Oxoid, Hampshire, UK) and TSB (incubated under anaerobic conditions), respectively and incubated at 22 ^{o}C for 5 days. If growth was visually observed, the purity of the isolate was checked by streaking out on the same medium. All isolates underwent preliminary identification tests (i.e. Gram staining, oxidase and catalase reactions, colony morphology and microscopic observation of cell morphology).

2.2.4 Selection of psychrotrophs

The recovered microbes were subjected to growth monitoring tests in order to evaluate their ability to grow at a temperature range extending within the spectrum of all enumeration techniques currently applied for packaged and chilled-stored foodstuffs (psychrotrophic and mesophilic). Thus, all isolates - representing the dominant microbial communities growing in the food matrices of the sampled products - recovered from PCA, MRS and RCA plates

incubated at 22 ^{o}C were further inoculated in TSB, MRS broth and TSB under anaerobic conditions, respectively. The inoculated broths were then incubated at 4 ^{o}C for 10 days in order to assess their ability to grow at refrigeration temperature (4 ^{o}C was considered representative for low-temperature storage), while incubation was also done at 22 and 30 ^{o}C for 5 days to evaluate their ability to grow at these reference temperatures. The growth of the isolates at 4, 22 and 30 ^{o}C was monitored by observing the turbidity. All isolates that could grow at 4 ^{o}C were further stored at -75 ^{o}C.

2.3 RESULTS

2.3.1 Effect of incubation temperature on microbial counts

In general, the colony counts on all the food products that were analyzed indicated greater microbial counts on plates that were incubated at 22 ^{o}C, albeit the differences were not always > 0.5 log CFU/g. Colony counts were found to be significantly higher on plates incubated at 22 ^{o}C than those at 30 ^{o}C in 38 % (33 of 86) of the samples evaluated. The mesophilic counts did not exceed those of the psychrotrophs in any of the cases. The samples where a difference greater than 0.5 log CFU/g was found are shown in Table 2.2.

The RTE vegetable salads (14 in total) were in most cases mixtures of fresh-cut and minimally processed vegetables packaged in air. The psychrotrophic lactic acid bacteria counts at the end of the shelf-life of the 14 products were often very high (see Table 2.2), exceeding in 64 % (9 of 14) of the samples the shelf-life threshold of 10^{7} CFU/g. In only 8 of 14 samples (indicated in bold in Table 2.2) was a considerable degree of visible deterioration observed, whilst the majority maintained a good general appearance (Abadias et al., 2008; García-Gimeno and Zurera-Cosano, 1997). The most commonly encountered alteration was a brown discoloration. For the minimally processed salads that contained sweet bell peppers a sour odor was detected upon opening of the packages, which corresponded to the acidification observed. The initial pH value of freshly packaged sweet bell peppers was 5.5±0.2 and a drop between 0.2-0.9 units was measured. Sweet bell peppers (*Capsicum annuum*), which are botanically taxonomized as fruits were in general the most prone to spoilage, salad constituent and have been previously reported to have higher sugar concentration than other vegetables, which may explain their great susceptibility (Jacxsens et al., 2003). As can be seen in Table 2.2 the greatest difference between the counts of psychrotrophs and mesophiles was always found on MRS plates.

Although only a few fresh raw meat products (6 in total) were purchased, the results indicate that for 87 % of them (5 of 6), a considerable underestimation of the contamination level occurs when the mesophilic enumeration method is used. The psychrotrophic lactic acid bacteria present at the end of the shelf-life on fresh raw meat products were at least 10^{8} CFU/g without exception. This resulted in a sour odor and slimy texture (indicated in bold in Table 2.2). Nonetheless, no blown packages, discoloration, putrid and/or buttery off-odors were observed or detected. Differences between the recovery of psychrotrophs over the mesophiles were in most cases greater on PCA and RCA than they were on MRS.

Many cooked meat products (pasteurized, fermented, smoked, sliced or formed into whole ham logs) deriving from various companies and different batches were also analyzed. In these products the psychrotrophic LAB counts always exceeded the spoilage-associated threshold of 10^7 CFU/g and occasionally were equal to the total psychrotrophic counts. The products at the end of shelf-life were generally acceptable, except for slight slime formation and sour odor on some of the products (mentioned in the caption of Table 2.2).
The heterogeneous group of composite food comprised RTE, sauces and ready-to-heat meals. A 27 % of the examined products showed greater counts on plates incubated at 22 ^{o}C in comparison to those incubated at 30 ^{o}C.

Table 2.2: *Microbial counts of PCA, RCA and MRS (average of three independent repetitions ± standard deviation) at two temperatures, 22 ^{o}C and 30 ^{o}C. In* ***[bold]*** *the food products that had a quality deterioration at the end of shelf-life.*

[a] *The highest difference of counts investigated for each sample comparing the two temperatures. The differences always favoured the viable counts at 22 ^{o}C and the recovery of psychrotrophs.*

[b] *The medium on which the greatest difference of counts was observed.*

[c] *Average of two independent pH measurements ± standard deviation of the food product at the end of shelf-life.*

N°	log CFU/g ± SD						$\Delta\log_{max}$ ± SD_{tot} [a]	Medium [b]	pH ± SD [c]
	PCA		RCA		MRS				
	22 °C	30 °C	22 °C	30 °C	22 °C	30 °C			
Ready-to-eat (RTE) vegetable salad									
[1]	**10.03 ± 0.05**	**10.00 ± 0.04**	**9.15 ± 0.07**	**9.15 ± 0.02**	**9.09 ± 0.03**	**6.03 ± 0.23**	**3.06 ± 0.23**	**MRS**	**5.27 ± 0.12**
[2]	**9.78 ± 0.00**	**8.75 ± 0.02**	**9.81 ± 0.01**	**8.54 ± 0.03**	**9.93 ± 0.03**	**7.93 ± 0.02**	**2.01 ± 0.04**	**MRS**	**4.93 ± 0.21**
[3]	**9.55 ± 0.04**	**9.27 ± 0.03**	**8.59 ± 0.05**	**8.57 ± 0.04**	**7.98 ± 0.02**	**6.40 ± 0.17**	**1.58 ± 0.18**	**MRS**	**4.73 ± 0.19**
[4]	**9.90 ± 0.02**	**9.62 ± 0.02**	**9.87 ± 0.07**	**9.3 ± 0.14**	**9.86 ± 0.04**	**8.47 ± 0.08**	**1.39 ± 0.09**	**MRS**	**5.19 ± 0.07**
[5]	**9.37 ± 0.02**	**9.21 ± 0.02**	**8.66 ± 0.02**	**8.34 ± 0.08**	**8.45 ± 0.09**	**7.36 ± 0.11**	**1.09 ± 0.14**	**MRS**	**5.03 ± 0.12**
[6]	**9.54 ± 0.06**	**9.49 ± 0.05**	**9.23 ± 0.11**	**8.82 ± 0.04**	**8.77 ± 0.07**	**7.38 ± 0.00**	**1.39 ± 0.07**	**MRS**	**4.92 ± 0.18**
7	10.06 ± 0.08	10.06 ± 0.07	9.64 ± 0.06	9.50 ± 0.02	9.50 ± 0.08	8.84 ± 0.01	0.66 ± 0.08	MRS	5.87 ± 0.17
8	10.04 ± 0.00	9.99 ± 0.04	9.60 ± 0.02	9.45 ± 0.03	9.51 ± 0.06	8.90 ± 0.03	0.61 ± 0.06	MRS	5.52 ± 0.09
9	8.62 ± 0.05	8.61 ± 0.02	7.64 ± 0.03	7.56 ± 0.03	6.39 ± 0.13	5.77 ± 0.21	0.62 ± 0.24	MRS	5.82 ± 0.06
10	7.58 ± 0.08	7.52 ± 0.11	6.47 ± 0.02	6.38 ± 0.14	5.85 ± 0.05	4.80 ± 0.18	1.05 ± 0.19	MRS	5.91 ± 0.12
[11]	**8.74 ± 0.04**	**8.71 ± 0.09**	**7.59 ± 0.08**	**7.41 ± 0.09**	**6.16 ± 0.12**	**5.06 ± 0.20**	**1.10 ± 0.23**	**MRS**	**5.62 ± 0.08**
12	9.66 ± 0.06	9.65 ± 0.02	9.26 ± 0.07	9.18 ± 0.06	8.82 ± 0.05	8.07 ± 0.05	0.75 ± 0.07	MRS	5.73 ± 0.13
[13]	**8.09 ± 0.03**	**8.00 ± 0.07**	**7.31 ± 0.04**	**6.49 ± 0.03**	**6.87 ± 0.09**	**5.36 ± 0.10**	**1.51 ± 0.13**	**MRS**	**4.62 ± 0.21**
14	8.68 ± 0.01	8.67 ± 0.03	7.34 ± 0.07	7.34 ± 0.03	6.73 ± 0.15	5.65 ± 0.16	1.08 ± 0.22	MRS	5.53 ± 0.10
Fresh raw meat									
[15]	**9.05 ± 0.09**	**7.66 ± 0.06**	**9.08 ± 0.14**	**7.63 ± 0.05**	**8.72 ± 0.06**	**7.95 ± 0.05**	**1.44 ± 0.15**	**RCA**	**6.12 ± 0.04**
16	9.04 ± 0.01	8.23 ± 0.03	8.81 ± 0.09	8.17 ± 0.03	8.84 ± 0.05	8.18 ± 0.03	0.82 ± 0.03	PCA	6.17 ± 0.17
[17]	**9.30 ± 0.05**	**8.69 ± 0.03**	**8.95 ± 0.14**	**8.72 ± 0.03**	**8.49 ± 0.01**	**8.24 ± 0.02**	**0.61 ± 0.06**	**PCA**	**5.94 ± 0.14**
18	8.59 ± 0.03	8.26 ± 0.03	8.43 ± 0.08	7.82 ± 0.03	8.71 ± 0.06	8.37 ± 0.03	0.62 ± 0.08	RCA	6.17 ± 0.00
[19]	**7.25 ± 0.05**	**6.47 ± 0.02**	**7.22 ± 0.09**	**6.45 ± 0.05**	**8.63 ± 0.07**	**6.46 ± 0.03**	**2.16 ± 0.10**	**MRS**	**5.91 ± 0.08**
Cooked meat products									
20	8.31 ± 0.14	8.14 ± 0.09	8.12 ± 0.08	8.07 ± 0.04	7.49 ± 0.08	6.81 ± 0.03	0.68 ± 0.09	MRS	6.21 ± 0.12
[21]	**7.81 ± 0.06**	**6.14 ± 0.05**	**7.84 ± 0.12**	**7.83 ± 0.03**	**7.78 ± 0.01**	**5.36 ± 0.03**	**2.42 ± 0.13**	**MRS**	**6.01 ± 0.23**
[22]	**8.10 ± 0.06**	**5.15 ± 0.05**	**8.04 ± 0.05**	**8.04 ± 0.06**	**8.66 ± 0.04**	**8.56 ± 0.04**	**2.94 ± 0.07**	**PCA**	**6.20 ± 0.20**
[23]	**8.67 ± 0.03**	**8.50 ± 0.06**	**8.72 ± 0.09**	**8.24 ± 0.12**	**8.25 ± 0.09**	**7.06 ± 0.11**	**1.19 ± 0.15**	**MRS**	**6.03 ± 0.12**
24	7.53 ± 0.09	6.93 ± 0.05	7.39 ± 0.04	7.37 ± 0.05	7.36 ± 0.15	6.96 ± 0.02	0.59 ± 0.10	PCA	6.03 ± 0.02
25	7.70 ± 0.10	6.89 ± 0.06	7.56 ± 0.01	6.61 ± 0.10	7.75 ± 0.05	6.70 ± 0.07	1.05 ± 0.10	MRS	6.07 ± 0.06
Composite food									
26	9.48 ± 0.04	8.63 ± 0.03	9.32 ± 0.04	8.45 ± 0.09	9.49 ± 0.05	8.38 ± 0.09	1.11 ± 0.10	MRS	5.76 ± 0.07
[27]	**10.43 ± 0.05**	**10.42 ± 0.07**	**9.57 ± 0.08**	**9.53 ± 0.05**	**7.88 ± 0.09**	**6.68 ± 0.14**	**1.20 ± 0.09**	**MRS**	**4.79 ± 0.09**
28	8.99 ± 0.03	9.06 ± 0.07	9.11 ± 0.09	8.70 ± 0.04	8.83 ± 0.09	8.25 ± 0.07	0.58 ± 0.10	MRS	5.10 ± 0.13
[29]	**8.39 ± 0.08**	**8.35 ± 0.02**	**8.35 ± 0.07**	**8.05 ± 0.10**	**8.16 ± 0.05**	**7.44 ± 0.12**	**0.72 ± 0.12**	**MRS**	**4.97 ± 0.18**
[30]	**8.64 ± 0.04**	**8.14 ± 0.04**	**8.67 ± 0.02**	**7.86 ± 0.00**	**8.59 ± 0.06**	**7.84 ± 0.05**	**0.82 ± 0.05**	**RCA**	**5.15 ± 0.17**
[31]	**9.83 ± 0.04**	**9.20 ± 0.05**	**9.77 ± 0.02**	**8.96 ± 0.08**	**9.81 ± 0.04**	**8.90 ± 0.09**	**0.90 ± 0.08**	**MRS**	**5.22 ± 0.00**
32	5.64 ± 0.05	5.37 ± 0.21	5.57 ± 0.06	4.39 ± 0.36	5.99 ± 0.13	5.38 ± 0.07	1.18 ± 0.22	RCA	5.61 ± 0.08
33	9.14 ± 0.06	9.11 ± 0.01	8.13 ± 0.06	8.03 ± 0.04	8.35 ± 0.08	7.76 ± 0.03	0.59 ± 0.07	MRS	6.11 ± 0.12

The psychrotrophic LAB counts were in all samples between 10^7-10^9 CFU/g at the end of shelf-life. The most frequently observed quality deterioration was an unpleasant odor and high acidity (indicated in bold in Table 2.2). In the high majority of samples the greatest difference in counts between the plates incubated at 22 ^oC and those at 30 ^oC was observed on MRS agar with the differences in counts ranging between 0.5-3.2 log CFU/g (see Table 2.2). The results were analyzed by performance of paired t-test with a confidence level of 95%. It was shown that counts after incubation at 30 ^oC were significantly different from counts at 22 ^oC on all three media ($P \leq 0.05$). For 19 of the 33 samples that had greater counts on plates incubated at 22 ^oC, the underestimation was greater than 1 log CFU/g, while the average value of underestimation was 1.2 log CFU/g. Moreover, for 15 samples the psychrotrophic lactic acid bacteria even equaled the total psychrotrophic count suggesting that they constituted the predominant, presumptive spoilage organisms.

2.3.2 Isolates

A total of 222 isolates were recovered from the plates incubated at 22 ^oC when the counts were higher (> 0.5 log CFU/g) than those incubated at 30 ^oC. The colonies were picked up from the plates of the highest dilution. The plates with the equivalent dilutions that were incubated at 30 ^oC showed no growth in most cases, suggesting that those psychrotrophic bacteria were the actual dominant biota and they had either a poor or no ability to grow when the mesophilic enumeration method was implemented.
All isolates underwent preliminary tests (Gram staining, oxidase and catalase reaction, microscopic observation of cell morphology) and were assessed for their growth at 4, 22 and 30 ^oC in order to evaluate their psychrotrophic potential and the efficacy of the mesophilic enumeration method, respectively. Ten of the initial 222 isolates were yeasts so they were excluded from the following growth monitoring tests. The remaining 212 isolates were all Gram positive, oxidase and catalase negative. 21 were rod shaped and 191 were cocci/ovoid shaped. All 212 remaining isolates could grow at 22 ^oC in 5 days and were observed to produce the most turbid growth compared to the other temperatures. They could all grow well at 4 ^oC from the first days of their 10-day incubation. For 180 isolates the final growth at 4 and 22 ^oC was visually comparable based on the turbidity and the pellet size. On the contrary at 30 ^oC, 154 isolates showed no growth, 15 had the same turbidity as at 22 ^oC and the remaining 43 had a considerably lower growth compared to 22 ^oC. A difference between the total mesophilic and psychrotrophic counts was reported previously (Ercolini et al., 2009) and it can be attributed to psychrotrophic microbiota with poor recovery when the mesophilic enumeration technique is applied.

2.4 DISCUSSION

Many industrially manufactured, packaged and chilled-stored food products are widely consumed nowadays (Björkroth et al., 2000; Jacxsens et al., 2003). RTE meals, minimally processed vegetable salads, fresh meat portions, pasteurized or fermented meat products are becoming more popular and desirable to consumers because they perceive them as convenient

and in some cases healthy (i.e. in the case of minimally processed and fresh-cut vegetable) (Abadias et al., 2008; Garrett et al., 2003). The industry has experienced solid growth (Buta et al., 1999) and manufacturers in order to gain a competitive advantage in the market are focusing on developing and implementing microbiological standards that would eliminate cases of spoilage within the shelf-life or even prolong the storage period. However, the most widely applied microbiological enumeration procedure for routine analysis in the industries worldwide is the mesophilic enumeration technique (ISO 4833:2003 and ISO 15214:1998). By incubating at 30 ^{o}C though it is clear that psychrotolerant and cold-acclimatized mesophiles are being selected leaving strictly psychrotrophic microorganisms undetected. Thus an objective and reliable method to predict the shelf-life related to the contamination levels should be defined. Therefore it is demanded that the enumeration method which is used determines the counts of microorganisms that are able to grow in the food matrix at the storage temperature (2-7 ^{o}C). The intention of this study was to implement a general enumeration technique that would efficiently evaluate all groups of the psychrotrophic consortium (obligate and facultative psychrophiles) by means of a standard procedure that requires one incubation temperature, a relatively short incubation period and the use of the same culture media for all types of products that would cover all microbial groups thriving in food products of meat and vegetable origin.

The analyzed samples gave an insight into the inadequacies of the procedure evaluating the contamination levels, which is currently used by most food processing companies and research groups. Since the analyzed food products originated from various process plants, different production batches, diverse raw materials and different production processes the deduced outcome can be considered of general significance.

It has been shown that the total mesophilic counts cannot always give a realistic estimation of the microbial contamination levels in food. Low temperatures and packaging technology along with the intrinsic parameters of the food product select the biota that will become dominant (Labadie, 1999). Apparently, numerous psychrotrophic LAB that are ubiquitous in manufacturing process plants (Audenaert et al., 2010) or constitute autochthonous members of the indigenous microflora of raw materials (Santos et al., 2005) can proliferate greatly under such conditions and their populations could potentially lead to spoilage before these goods reach their shelf-life expectancy. Although the microbial niches from which the microorganisms derived were heterogeneous, the conclusions drawn concerning the psychrotrophic microbes agreed that LAB often unable to proliferate at 30 ^{o}C are the predominant bacterial strains in packaged food products stored at chilling temperature. Many references in the literature stress the importance of psychrotrophic LAB in cases of spoilage since when their population exceeds 10^{7} CFU/g they produce off-flavor and off-odor compounds leading to the rejection of the product (García-Gimeno and Zurera-Cosano, 1997; Jacxsens et al., 2003; Mataragas et al., 2006; Samelis et al., 1998a,b, 2000).

In most cases the high counts of psychrotrophs were not in accordance with the merely acceptable physicochemical state of the products (Vihavainen and Björkroth, 2009). Still a reassessment of the current microbial specifications and spoilage-associated strategies should be considered as a priority (García-Gimeno and Zurera-Cosano, 1997; Mataragas et al., 2006). The contamination levels, the dominant bacterial species related to spoilage and their metabolism under the specific conditions of packaging constitute the three crucial parameters

of spoilage manifestation in the food matrix. Spoilage being a complex biochemical and physicochemical procedure requires culture-dependent and culture-independent techniques in order to be adequately interpreted or predicted (Ercolini et al., 2006; Vasilopoulos et al., 2008). Nonetheless, a configuration of the microbiological criteria towards the psychrotrophs could be constructive in the evaluation of the microbial diversity of highly perishable food products.

Most of the research groups dealing with packaged food products stored at chilling temperatures and psychrotrophic spoiling floras use the mesophilic enumeration technique in order to estimate the microbial contamination level (Cayré et al., 2005; Doulgeraki et al., 2010; Metaxopoulos et al., 2002; Vasilopoulos et al., 2008; Vasilopoulos et al., 2010). From our results incubation at 30 ^{o}C for culture-dependent techniques that evaluate the microbial ecology of a packaged food product stored at chilling temperature is not sufficient. On the other hand various different approaches for the enumeration of the psychrotrophs have been suggested: incubation at 7 ^{o}C for 10 days as a complementary parameter to the mesophilic enumeration process (Ercolini et al., 2006; Ercolini et al., 2009; Sakala et al., 2002a,b), incubation at 25 ^{o}C for 5 days in the frame of a different microbiological analysis for each case study and incubation at 17 ^{o}C for 16 h and at 7 ^{o}C for 3 days. Incubation at 22 ^{o}C has been directly compared to the mesophilic enumeration technique and has proved greater reliability and accuracy. It is needless to point out that the recovered isolates of the present study unable to grow at 30 ^{o}C have a more competent growth at 4 ^{o}C and at 22 ^{o}C covering in range the lower incubation temperatures of the above mentioned psychrotrophic techniques. This fact combined with the considerable high counts determined by incubating at 22 ^{o}C leave a very weak probability of underestimation.

Food manufacturing practices demand a relatively short incubation period, achieving reliable assessment of the contamination levels, one incubation temperature and a standard methodology that are interpreted as high cost effectiveness. The assumption that a temperature value below 30 ^{o}C that lies within the growth temperature range of the mesophiles, while at the same time corresponds to the temperature requirements of the psychrotrophs is undisputed (Björkroth et al., 2005; Lyhs et al., 2001; Lyhs et al. 2007; Mejlholm et al., 2005; Susiluoto et al., 2003; Vihavainen and Björkroth, 2007; 2009). A shift in approach from exclusively mesophilic enumeration to another focusing on the psychrotrophs would not only improve the microbial analysis routine but could potentially contribute to the identification steps that follow, in order to define the species diversity with greater consistency and coherency when culture-dependent techniques are combined with culture-independent.

Psychrotrophic members of *Leuconostoc gelidum* subsp. *gasicomitatum, Leuconostoc gelidum* subsp. *gelidum* and *Lactococcus piscium* dominate at the end of shelf-life in packaged and chilled-stored food products in Belgium

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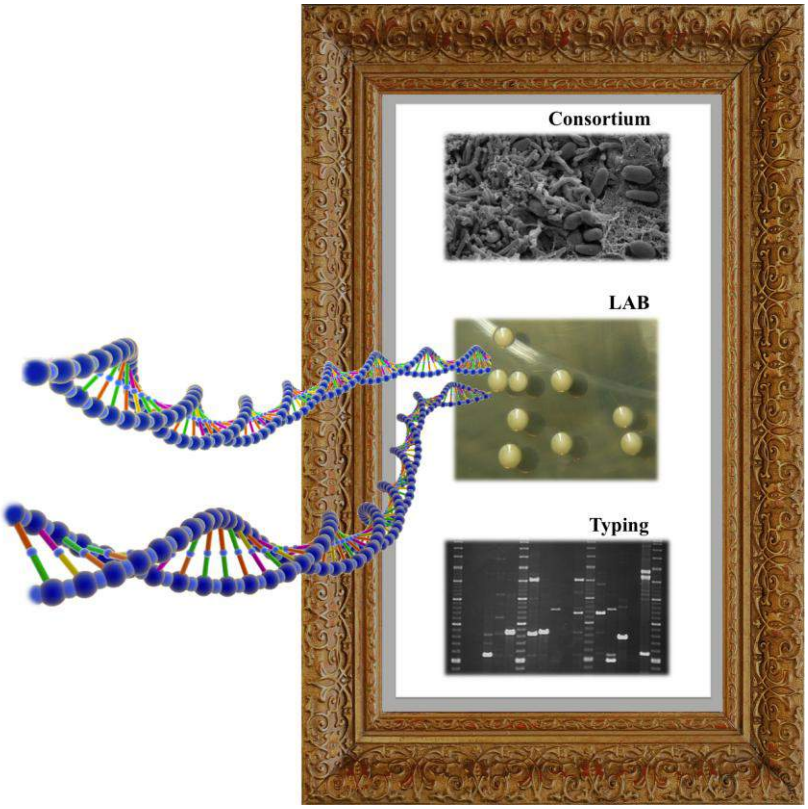


SUMMARY

Previously, a considerable underestimation (+ 0.5-3.2 log CFU/g) on the contamination levels of psychrotrophic lactic acid bacteria (LAB) was observed for 33 retail, packaged food products stored at chilling temperature when the mesophilic enumeration technique was implemented as reference shelf-life parameter (**Chapter 1**). In the present study, the microbial diversity of the dominant psychrotrophic LAB recovered after incubation of plates at 22 ^{o}C for 5 days was determined using a polyphasic taxonomic approach. A total of 212 LAB isolates were identified using a combination of rep-PCR fingerprinting, amplified fragment length polymorphism (AFLP) analysis and *pheS* gene sequencing. *Leuconostoc gelidum* subsp. *gasicomitatum*, *Leuconostoc gelidum* subsp. *gelidum*, *Leuconostoc* spp., *Lactobacillus algidus* and *Lactococcus piscium* proved to be the most competent and predominant species that may go undetected by the widely applied mesophilic enumeration protocols (ISO 4833:2003 and ISO 15214:1998).

This study has assessed the interspecific variation among potential spoilage LAB, and highlights the significance of implementing a reference shelf-life parameter based on the enumeration of the psychrotrophic load for industrial microbiological routine analyses.

Taxonomic note: recently, a taxonomic rearrangement was proposed in the genus *Leuconostoc* resulting in the proposal of three subspecies in the species *Le. gelidum*, i.e. subsp. *gelidum* (formerly *Le. gelidum*), subsp. *gasicomitatum* (formerly *Le. gasicomitatum*) and subsp. *aenigmaticum* (a newly described taxon) (Rahkila et al., 2014). The paper in this chapter still reports the former species names as its publication preceded the taxonomic proposal of Rahkila and co-workers.

3.1 INTRODUCTION

Already in **Chapter 1,** it was emphasized that food products packaged under modified atmosphere (MA) or vacuum that are stored at refrigeration temperature achieve a prolonged shelf-life and better organoleptic characteristics due to hurdle technologies that inhibit the growth and offensive metabolism of spoilage-related microbes. On the one hand, the partial or complete exclusion of oxygen from the micro-environment of the package has an inhibitory effect on respiratory Gram negative spoilage bacteria (Dainty & Mackey, 1992; Labadie, 1999; Willocx et al., 1993). In addition, low-temperature storage constitutes a hurdle which selects for psychrotrophic microbial groups (Sakala et al., 2002).
However, cases of unexpected spoilage within the storage period of packaged foods do occur (see **Chapter 6**) and usually the high contamination levels cannot be adequately evaluated as the widely applied mesophilic enumeration methods fail to differentiate strict psychrotrophs from psychrotolerant and cold-acclimatized mesophilic microorganisms (Holley & McKellar, 1996; Schirmer et al., 2009). Consequently, the microbial routine analyses performed in industrial processing plants implementing the mesophilic enumeration techniques (ISO 4833:2003 and ISO 15214:1998) largely underestimate contamination levels of psychrotrophic lactic acid bacteria (LAB). This holds major risks for the quality of packaged and chilled-stored food products (**Chapter 1**) that are mostly dominated by LAB at the end of their shelf-life (Borch et al., 1996; Cayré et al., 2005). Although the total viable mesophilic count is still used as a reference shelf-life parameter, its efficacy to depict the microbial state of a food product can often be doubtful. Until recently, research conducted on spoilage-related LAB mainly focused on mesophilic species such as *Lactobacillus sakei*, *Lactobacillus curvatus* and *Leuconostoc mesenteroides* which can successfully proliferate under refrigeration temperatures (Björkroth & Korkeala, 1996; Borch et al., 1996; Labadie, 1999; Mataragas et al., 2006; Samelis et al., 1998; Samelis et al., 2000; Vermeiren et al., 2005), and to which many cases of quality deterioration have been attributed. Considerable levels of acidification, emission of volatile organic compounds, slime formation, production of off-flavor components and other alterations of the organoleptic properties of packaged food products have been associated with their metabolic activity (Björkroth et al., 1996; Dainty & Mackey, 1992; Gram et al., 2002; Samelis et al., 2000). In addition to these species, a considerable number of hitherto unknown psychrotrophic LAB groups that grow at a lower temperature range compared to the mesophilic LAB have been reported (Björkroth et al., 2000; Kato et al., 2000; Sakala et al., 2002a; Sakala et al., 2002b). Psychrotrophic *Leuconostoc* spp. are increasingly isolated from food products deemed unfit for consumption and in which at concentrations > 10^7 CFU/g they produce unpleasant odors, form slime and cause discoloration (Björkroth et al., 2000; Doulgeraki et al., 2010; Lyhs et al., 2004; Vihavainen & Björkroth, 2007). Especially their psychrotrophic potential and their competence to proliferate at high concentrations of CO_2 has prioritized them in comparison to other LAB genera. A previous culture-dependent survey of 86 chilled-stored and packaged, retail food products in Belgium described in **Chapter 2** evaluated their microbial contamination levels through a psychrotrophic enumeration method (i.e. incubation of plates performed at 22 ^oC). Direct comparison with the mesophilic technique (incubation at 30 ^oC) revealed an underestimation (+ 0.5-3.2 log CFU/g) of potentially psychrotrophic communities

in 38 % of the analyzed products when the mesophilic enumeration was applied. In the present study, the recovered psychrotrophic isolates that could not be detected by mesophilic incubation were identified through a polyphasic taxonomic analysis attempting to elucidate the species diversity of the dominant psychrotrophic bacteria at the end of the shelf-life for food products of vegetable and meat origin.

3.2 MATERIALS AND METHODS

3.2.1 Samples, isolation procedure and growth tests

As previously described in Chapter 2, 86 various packaged (air, vacuum or modified-atmosphere) and chilled-stored (4 or 7 ^{o}C) food products were analyzed (Table 2.1 in **Chapter 2**) in the frame of a general screening of the Belgian market. For each sample a comparative enumeration was comparing the recovery of psychrotrophic microbes implementing incubation of plates at 22 ^{o}C (5 days) and 30 ^{o}C (3 days). An average underestimation of 1.2 log CFU/g was observed for 33 samples when implementing the mesophilic incubation method. For these 33 samples that showed higher counts on the plates incubated at 22 ^{o}C, single colonies were picked from plates of the highest dilutions as higher numbers of colonies in comparison to their equivalent incubated at 30 ^{o}C (> 0.5 log CFU/g) were investigated. This resulted in a collection of 212 isolates which constitute the predominant psychrotrophic population present. All isolates exhibited a typical LAB profile, i.e. Gram-positive, oxidase and catalase-negative. Growth characteristics of all isolates were visually determined at 4, 22 and 30 ^{o}C by scoring turbidity and pellet size in culture broth, and their grow characteristics at 30 ^{o}C were assessed. Isolates were stored at -75 ^{o}C on porous glass beads with glycerol as cryoprotectant. For resuscitation, a glass bead was transferred aseptically in MRS broth or tryptone soya broth (TSB, Oxoid, Hampshire, UK) tube and incubated at 22 ^{o}C for 5 days. Upon growth, purity was checked on MRS agar or tryptone soya agar (TSA, Oxoid, Hampshire, UK), by incubation at 22 ^{o}C for 5 days. TSA was used in cases where growth failed on MRS.

3.2.2 DNA extraction

The DNA extraction was performed following two different methodologies. Firstly, for the purpose of genotypic dereplication, all isolates underwent rapid alkaline lysis for use in rep-PCR fingerprinting (Niemann et al, 1997). A few pure colonies were transferred to 20 µL of lysis buffer (5 % w/v NaOH and 0.25 % w/v SDS). The cell suspension was heated at 95 ^{o}C for 15 minutes and shortly centrifuged at 13000 rpm. Lastly, 180 µL of sterile MQ water was added, the extract was centrifuged at 13000 rpm for 5 minutes and stored at -20 ^{o}C. In a second round of DNA preparation, selected isolates from the rep-PCR clusters were subjected to the phenol/chloroform extraction method (Gevers et al., 2001). The obtained extracts were used for AFLP analysis, which requires DNA of higher quality and purity.

3.2.3 rep-PCR fingerprinting

Repetitive element polymerase chain reaction (rep-PCR) was performed using the $(GTG)_5$-primer. $(GTG)_5$-PCR amplifications were conducted as previously described (Versalovic et al., 1994; Gevers et al., 2001) in a Perkin Elmer 9600 thermal cycler (Massachusetts, USA) using Goldstar DNA polymerase (Eurogentec, Liège, Belgium). The resulting PCR amplicons were separated for 16 h on a 1.5 % w/v agarose gel (20 cm x 15 cm) at 4 ^{o}C in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 55 V. After staining with ethidium bromide, $(GTG)_5$-PCR profiles were visualized under UV followed by digital image capturing using a CCD camera. Resulting fingerprints were grouped by clustering analysis in BioNumerics 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium) using the Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA). Reproducibility of PCR and electrophoresis runs were routinely assessed by inclusion of *Lactobacillus plantarum* LMG 6709^{T} as positive control and comparison of the molecular markers.

3.2.4 Amplified Fragment Length Polymorphism (AFLP) analysis

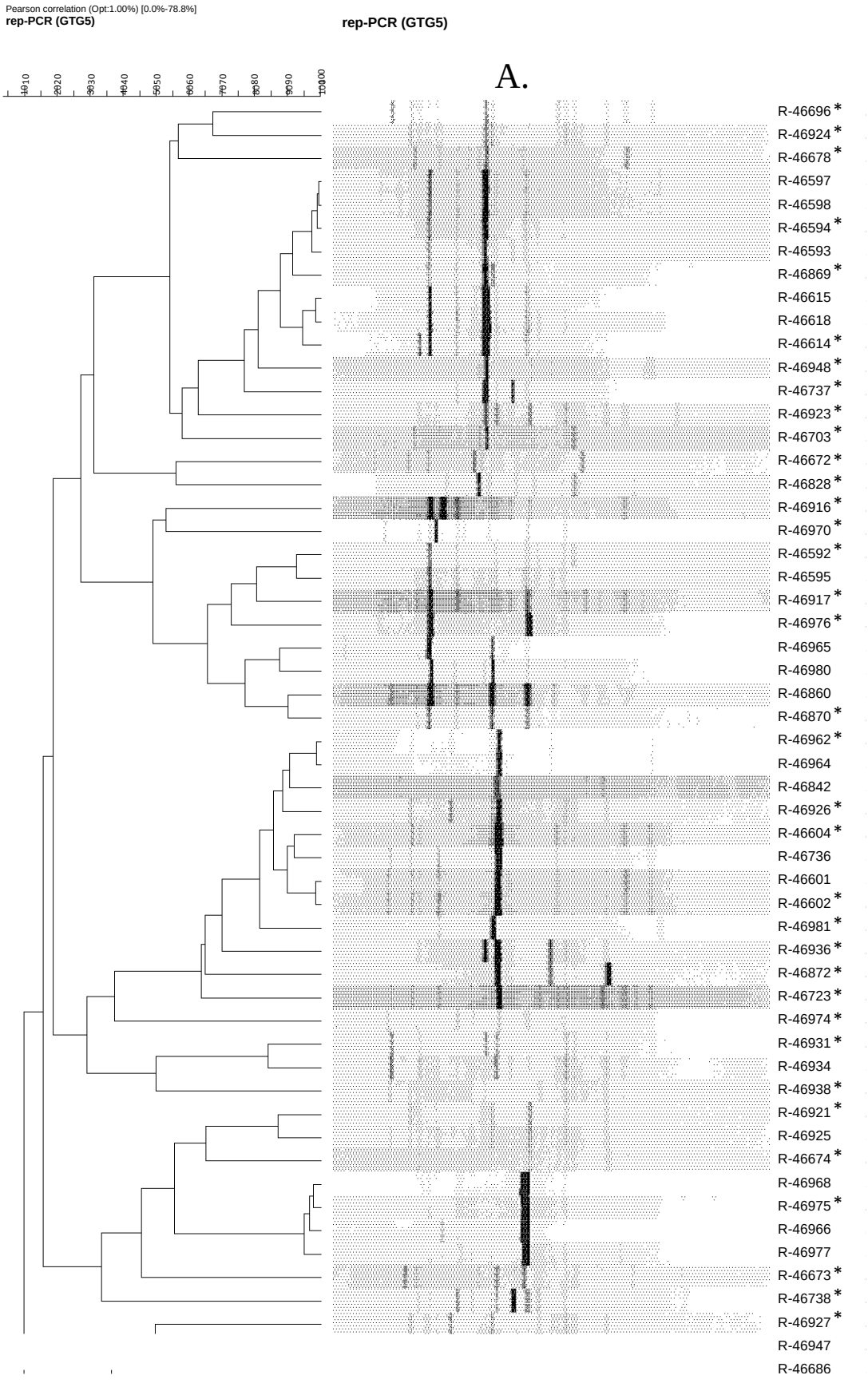
Amplified fragment length polymorphism (AFLP) was performed according to a modified version of the protocol of Thompson and co-workers (2001). DNA obtained with the phenol/chloroform extraction method (Gevers et al., 2001) was digested with the restriction enzymes *TaqI* and *EcoRI*. Fragments were amplified using the primers E01 (5'-GACTGCGTACCAATTCA-3') and T01 (5'-CGATGAGTCCTGACCGAA-3'). The selective PCR products were separated with an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, USA). The obtained electropherograms were normalized using the GeneScan 3.1 software (Applera, Norwalk, CT, USA), and peak fingerprints were processed using BioNumerics 5.10 software. The Dice coefficient and the UPGMA linkage method were used in order to compare AFLP profiles of the isolates with an in-house database containing profiles of LAB type and reference strains (BCCM/Bacteria Collection).

3.2.5 *pheS* gene sequence analysis

In cases where AFLP analysis did not result in an unambiguous species identification result, partial sequencing of *pheS* which encodes for the α-subunit of the bacterial phenylalanyl-tRNA synthase was performed. The primer combination used for the amplification and sequencing of the *pheS* gene (pheS-21-F and pheS-22/23-R), amplification conditions and details on sequencing have been described previously (Naser et al., 2005).
The annealing temperature was changed to 55 ^{o}C when aspecific *pheS* amplicons were obtained. Sequencing reactions were purified with the BigDye XTerminatorTM Purification Kit following the protocol of the supplier (Applied Biosystems, CA, USA).

3.3 RESULTS

The digitized $(GTG)_5$-PCR fingerprints of the 212 isolates were grouped in 6 clusters using


Pearson correlation (Opt:1.00%) [0.0%-78.8%]
rep-PCR (GTG5)
rep-PCR (GTG5)
A.
R-46696 *
R-46924 *
R-46678 *
R-46597
R-46598
R-46594 *
R-46593
R-46869 *
R-46615
R-46618
R-46614 *
R-46948 *
R-46737 *
R-46923 *
R-46703 *
R-46672 *
R-46828 *
R-46916 *
R-46970 *
R-46592 *
R-46595
R-46917 *
R-46976 *
R-46965
R-46980
R-46860
R-46870 *
R-46962 *
R-46964
R-46842
R-46926 *
R-46604 *
R-46736
R-46601
R-46602 *
R-46981 *
R-46936 *
R-46872 *
R-46723 *
R-46974 *
R-46931 *
R-46934
R-46938 *
R-46921 *
R-46925
R-46674 *
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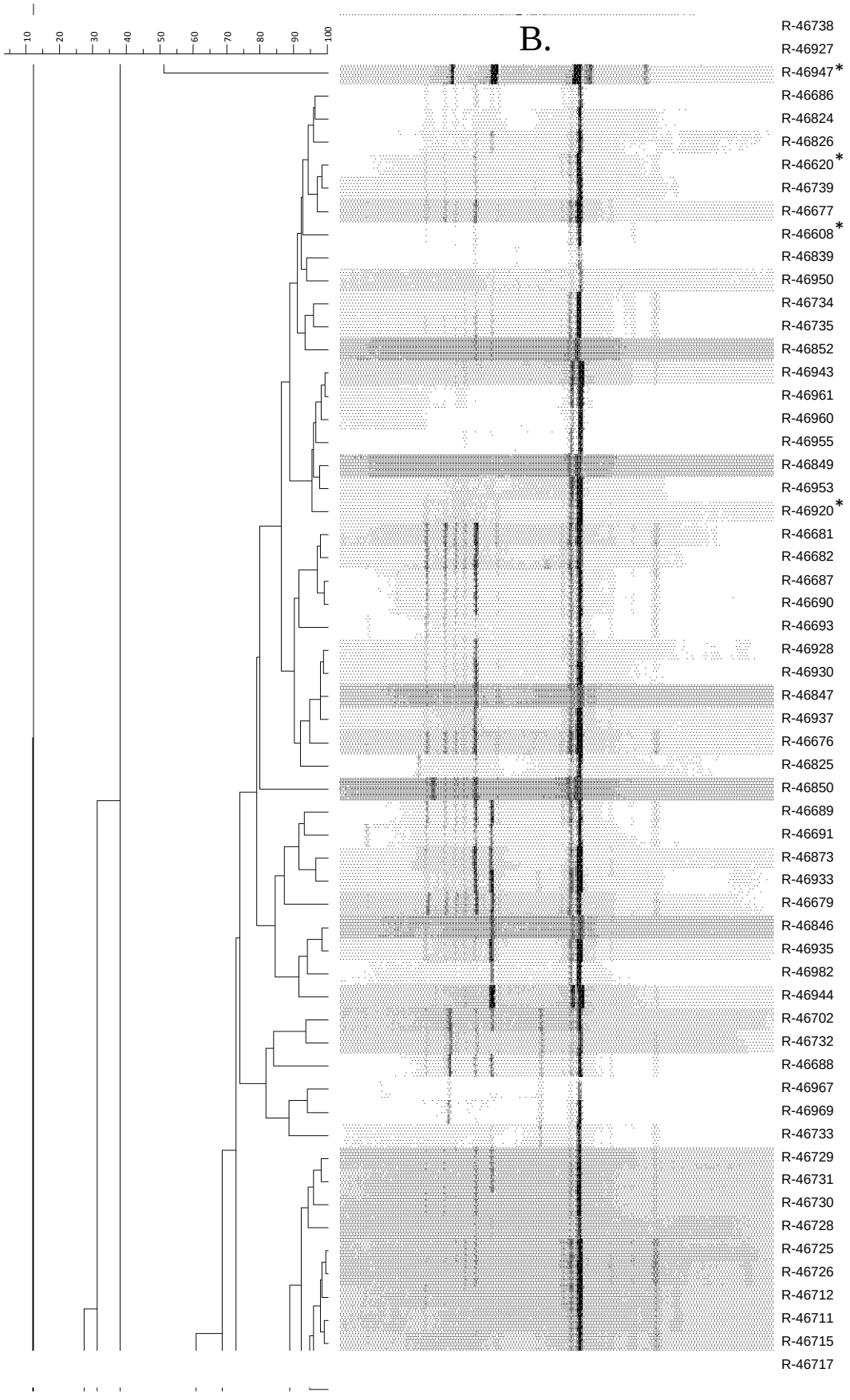
R-46673 *
R-46738 *
R-46927 *
R-46947
R-46686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R-46738
R-46927
R-46947*
R-46686
R-46824
R-46826
R-46620*
R-46739
R-46677
R-46608*
R-46839
R-46950
R-46734
R-46735
R-46852
R-46943
R-46961
R-46960
R-46955
R-46849
R-46953
R-46920*
R-46681
R-46682
R-46687
R-46690
R-46693
R-46928
R-46930
R-46847
R-46937
R-46676
R-46825
R-46850
R-46689
R-46691
R-46873
R-46933
R-46679
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R-46935
R-46982
R-46944
R-46702
R-46732
R-46688
R-46967
R-46969
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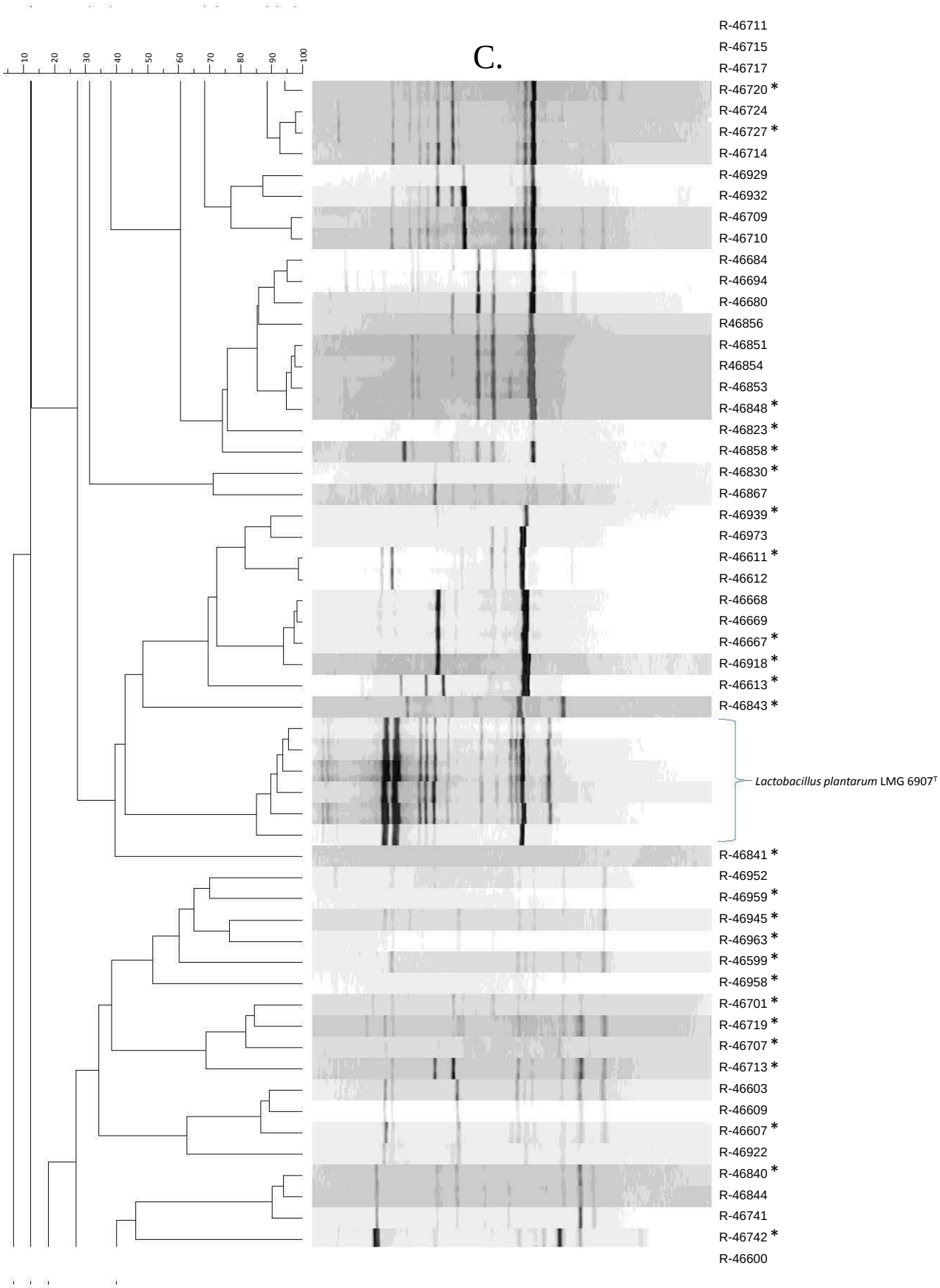
R-46717


C.
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R-46727 *
R-46714
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R-46932
R-46709
R-46710
R-46684
R-46694
R-46680
R46856
R-46851
R46854
R-46853
R-46848 *
R-46823 *
R-46858 *
R-46830 *
R-46867
R-46939 *
R-46973
R-46611 *
R-46612
R-46668
R-46669
R-46667 *
R-46918 *
R-46613 *
R-46843 *
Lactobacillus plantarum LMG 6907T
R-46841 *
R-46952
R-46959 *
R-46945 *
R-46963 *
R-46599 *
R-46958 *
R-46701 *
R-46719 *
R-46707 *
R-46713 *
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R-46607 *
R-46922
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R-46741
R-46742 *
R-46600

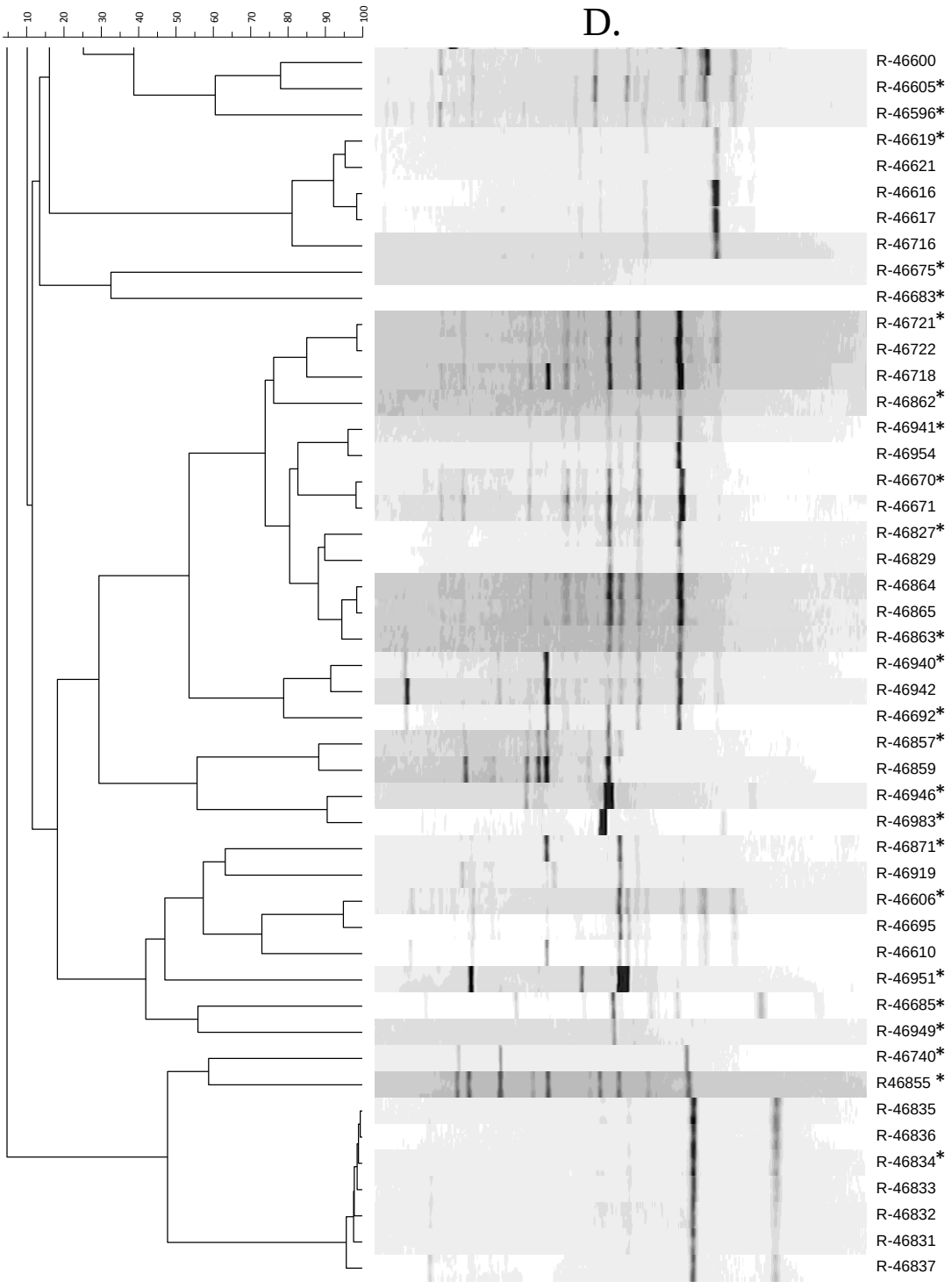


Figure 3.1 A, B, C & D: *Dendrogram generated with the digitized $(GTG)_5$-PCR fingerprints of all 212 isolates. Marked fingerprints with * were selected for AFLP analysis.*

Pearson correlation grouping and visual inspection (Figure 3.1 A, B, C & D). The largest cluster contained 64 isolates and was delineated at a Pearson correlation of 71 %. The other clusters assembled 9-20 isolates each, which were grouped with correlation values varying

between 55 and 82 %. In addition, numerous isolates remained ungrouped. Subsequently, one or more isolates from each $(GTG)_5$-PCR cluster and ungrouped isolates were selected for AFLP analysis. Following genotypic dereplication which also took into account the origins of isolation, a total of 88 isolates were subjected to AFLP analysis.

AFLP fingerprints of the 88 isolates were compared to an in-house database generated for the identification of LAB. Among these, 76 isolates clearly grouped in an AFLP cluster containing type and reference strains of a known LAB species and were assigned to that species. The remaining 12 isolates (6 % overall) remained unclassified as their AFLP patterns displayed low similarities with any of the reference strains of the database and their taxonomic position could not be confirmed. All these unidentified isolates were also ungrouped in the cumulative $(GTG)_5$-PCR dendrogram and did not cluster along with the rest of the retrieved dominant LAB of the food products previously sampled. The majority of isolates (136 out of 212, 64 % of all isolates) were assigned to the genus *Leuconostoc* with *Leuconostoc gelidum* subsp. *gasicomitatum* (64 isolates, 30 % of all isolates) and *Leuconostoc gelidum* subsp. *gelidum* (45 isolates, 21 %) being the most dominant followed by *Leuconostoc carnosum* (15 isolates, 7 %) and *Leuconostoc inhae*-like (10 isolates, 5 %) as shown in Table 3.1.

Table 3.1: *Taxonomic designation and growth characteristics of recovered LAB isolates at 30 °C. All isolates showed growth at 22 °C. (−**:** no growth observed ; **(+) :** slight growth ; **(++) :** turbid growth ; **(+++) :** turbid growth and formation of pellet ; **(++++) :** very turbid growth and formation of thick pellet).*

Identification			**Growth at 30 °C**				
Species	**Number of isolates (%)**		−	+	++	+++	++++
Leuconostoc	**136**	**(64 %)**					
Le. gelidum* subsp. *gasicomitatum	**64**	**(30 %)**	58	4	2		
Le. gelidum* subsp. *gelidum	**45**	**(21 %)**	40	4	1		
Le. carnosum	**15**	**(7 %)**		6	7	2	
Le. inhae	**10**	**(5 %)**	10				
Le. mesenteroides	**1**	**(0.5 %)**				1	
Le. lactis	**1**	**(0.5 %)**				1	
Lactococcus	**29**	**(14 %)**					
Lc. piscium	**29**	**(14 %)**	24	1	4		
Lactobacillus	**21**	**(10 %)**					
Lb. algidus	**10**	**(5 %)**	10				
Lb. fuchuensis	**6**	**(3 %)**	2			3	1
Lb. sakei	**3**	**(1 %)**		1	1	1	
Lb. oligofermentans	**1**	**(0.5 %)**			1		
Lb. sanfranciscensis	**1**	**(0.5 %)**	1				
Others	**16**	**(7 %)**					
Carnobacterium divergens	**7**	**(3 %)**	1	2	4		
Enterococcus raffinosus	**4**	**(2 %)**		2	2		
***Weissella* sp.**	**3**	**(1 %)**		3			
Unidentified	**12**	**(6 %)**	8	2	2		
Total	**212**		**154 (73 %)**	**25 (12 %)**	**24 (11 %)**	**8 (3.7 %)**	**1 (0.3 %)**

Other LAB species were assigned to *Lactococcus piscium*-like (13 % of all isolates), *Lactobacillus algidus* (5 %) and *Carnobacterium divergens* (4 %). The remaining LAB

species were present in less than 3 % and had an occasional isolation frequency. For the *Leuconostoc inhae*-like and the *Lactococcus piscium*-like groups, additional *pheS* gene sequencing was conducted for 3 isolates of each cluster, and this confirmed their respective taxonomic designations (De Bruyne et al., 2007).

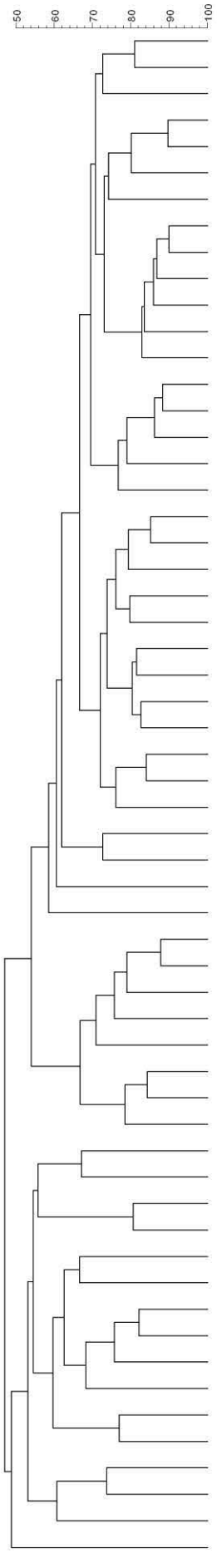
The majority of isolates (154 out of 212, 73 % of all isolates) were unable to grow at 30 ^{o}C whereas 43 isolates showed a weak growth when compared with the turbidity and pellet size obtained at 22 ^{o}C. Only 15 isolates in total, showed the same growth capacities at both temperatures. Most isolates assigned to *Le. gelidum* subsp. *gasicomitatum*, *Le. gelidum* subsp. *gelidum*, *Le. inhae*, *Lc. piscium* and *Lb. algidus* were unable to grow at 30 ^{o}C (Table 3.1), indicating that these species have a potent psychrotrophic character.

Table 3.2: *Interspecies diversity of the isolated LAB and distribution among the different groups of food products. The number of samples contaminated with each of the LAB taxa and the number of recovered isolates per food group are presented.*

Species	Number of isolates	Ready-to-eat (RTE) vegetable salads		Fresh raw meat products		Cooked meat products		Composite foods	
		Samples	Isolates	Samples	Isolates	Samples	Isolates	Samples	Isolates
Le. gelidum* subsp. *gasicomitatum	64	7	25	2	3	3	6	5	30
Le. gelidum* subsp. *gelidum	45	5	22	3	6	2	13	4	4
Le. carnosum	15					3	12	2	3
Le. inhae	10	5	5					1	5
Le. mesenteroides	1	1	1						
Le. lactis	1	1	1						
Lc. piscium	29	3	15	2	13	1	1		
Lb. algidus	10	1	2	1	7			1	1
Lb. fuchuensis	6			1	4			1	2
Lb. sakei	3			1	2	1	1		
Lb. oligofermentans	1			1	1				
Lb. sanfranciscensis	1	1	1						
C. divergens	7	1	1	1	4			1	2
E. raffinosus	4			1	4				
***Weissella* sp.**	3	1	1					1	2
Unidentified	12	1	4	1	2	2	4	2	2
Total	**212**		**78**		**46**		**37**		**51**

Le. gelidum subsp. *gasicomitatum* was found to dominate in 7 ready-to-eat (RTE) minimally processed vegetable salads and 5 composite food products. Members of this species were isolated from 17 out of the 33 samples from which psychrotrophic LAB were isolated (Table 3.2). *Le. gelidum* subsp. *gelidum* was recovered from a total of 14 samples, with highest populations found in 5 RTE salads and 2 cooked meat products.

Lc. piscium was highly present in 3 RTE salad samples and 2 raw meat products (Table 3.2). Interestingly, resuscitation of *Lc. piscium* isolates was retarded or even inhibited when inoculated in MRS broth as a lag phase of 2-3 days and a weak growth was commonly observed. In most cases, *Lc. piscium* isolates were originally recovered from food products on PCA medium.



R-46602 blood sausages
R-46604 blood sausages
R-46607 blood sausages
R-46921 salad (bell pepper)
R-46963 salad (bell pepper)
R-46924 salad (bell pepper)
R-46945 salad (zucchini, carrot, soybean, garden parsley)
R-46926 salad (bell pepper)
R-46962 salad (bell pepper)
R-46683 spirelli-type pasta and fresh vegetable salad with dressing
R-46927 salad (bell pepper)
R-46696 salad (lettuce, bell pepper, carrot)
R-46719 farfalles-type pasta with chicken and fresh vegetable salad
R-46871 salad (bell pepper)
R-46872 salad (bell pepper)
R-46938 salad (lettuce, corn salad)
R-46936 salad (lettuce, corn salad)
R-46599 beef
R-46605 blood sausages
R-46674 beef
R-46948 salad (zucchini, carrot, soybean, garden parsley)
R-46600 beef
LMG 18297^{T}
R-46958 white sausages
R-46713 potato and fresh vegetable salad with mayonnaise sauce
R-46830 pork
R-46959 white sausages
R-46923 salad (bell pepper)
R-46931 salad (bell pepper)
R-46701 salad (bell pepper)
R-46596 beef
R-46606 blood sausages
R-46603 blood sausages
R-46610 blood sausages
R-46720 farfalles-type pasta with chicken and fresh vegetable salad
R-46620 beef
R-46920 salad (bell pepper)
R-46608 blood sausages
R-46727 nicoise-type salad and tuna
R-46955 white sausages
R-46687 salad (bell pepper)
LMG 18811^{T}
R-46970 salad (bell pepper)
R-46975 salad (bell pepper)
R-46916 salad (lettuce)
R-46917 salad (rocket, corn salad)
*R-46869 salad (bell pepper)
*R-46870 salad (bell pepper)
R-46737 pork
R-46738 pork
R-46614 beef
R-46976 salad (bell pepper)
R-46592 beef
R-46594 beef
R-46974 salad (bell pepper)
*R-46981 salad (bell pepper)
R-46611 beef
LMG 23383^{T}

Figure 3.2: *AFLP dendrogram of selected isolates belonging to the three major LAB species:* Leuconostoc gelidum *subsp.* gasicomitatum, Leuconostoc gelidum *subsp.* gelidum *and* Lactococcus piscium*. The dendrogram was constructed using the Dice coefficient and UPGMA clustering method. Accession number of deposited culture and origin of isolation are stated. The type strains are also included (*Le. gelidum *subsp.* gelidum *LMG 18297^T,* Le. gelidum *subsp.* gasicomitatum *LMG 18811^T and* Lc. piscium *LMG 23383^T).*
Isolates that were further identified by means of pheS *gene sequencing are marked with an asterisk (*).*

In general, RTE vegetable salads were mostly contaminated by *Leuconostoc* spp. with *Le. gelidum* subsp. *gasicomitatum, Le. gelidum* subsp. *gelidum, Le. inhae* as well as *Lc. piscium* being the most frequently found LAB species. In fresh raw meat products, the species *Lc. piscium, Le. gelidum* subsp. *gelidum* and *Le. gelidum* subsp. *gasicomitatum* were recovered from more than one sample. In addition, lactobacilli identified as *Lb. algidus* and *Lb. fuchuensis* were dominant in single samples of this food product as was also the case for *Carnobacterium divergens* and *Enterococcus raffinosus*. In the group of the cooked meat samples *Le. carnosum, Le. gelidum* subsp. *gelidum* and *Le. gelidum* subsp. *gasicomitatum* encompassed 31 out of 33 isolates. In the composite food products, *Le. gelidum* subsp. *gasicomitatum* was the predominant species.
In Figure 3.2, all the isolates that were allocated to the three major species *Le. gelidum* subsp. *gasicomitatum, Le. gelidum* subsp. *gelidum* and *Lc. piscium* from the 88 isolates selected to undergo AFLP typing are presented in a dendrogram. The intraspecies diversity varied among the three species. Despite the fact that *Le. gelidum* subsp. *gasicomitatum* strains originated from 17 different samples and comprised 64 isolates, they formed a homogeneous $(GTG)_5$-PCR cluster (Figure 3.1 B) and required a selection of very few representatives for AFLP analysis to confirm their designation. The obtained AFLP fingerprints also showed great similarity among them and with the type strain as shown in Figure 3.2, regardless of origin. The opposite was observed for the other two taxa i.e. *Le. gelidum* subsp. *gelidum* and *Lc. piscium*. Noteworthy, not all isolates assigned to *Le. gelidum* subsp. *gelidum* and *Lc. piscium,* respectively, clustered together in $(GTG)_5$-PCR. This corroborates the greater diversity of strains presumably adapted to different ecological niches concerning *Le. gelidum* subsp. *gelidum* and *Lc. piscium*. The lowest similarity among strains and the greatest phylogenetic distance from the type strain was found for *Lc. piscium* explaining the uncertain identification with AFLP typing and the need for the sequencing of *pheS* house keeping gene (isolates marked with an asterisk in Figure 3.2) that provided an accurate identification.

3.4 DISCUSSION

This study unequivocally shows that the underestimation of psychrotrophic LAB at 30 ^oC is common in chilled-stored and packaged food products. Also, this observation necessitates the taxonomic characterization of this spoilage-related populations which would remain completely undetected during routine microbiological analyses in industry (following ISO 4833:2003 and ISO 15214:1998) as 73 % of the isolates were unable to grow at the optimal mesophilic temperature. Results of this taxonomic study indicate that the large majority of isolates belonged to psychrotrophic members of the species *Le. gelidum* and *Lc. piscium*.

From the species identified, *Le. gelidum* subsp. *gasicomitatum*, *Le. gelidum* subsp. *gelidum*, *Le. inhae*, *Lc. piscium* and *Lb. algidus* are more consistent towards an obligate psychrotrophic character, as all isolates allocated to these taxa were unable to grow at 30 ^{o}C. Nonetheless, the growth profile at different temperatures for isolates of the same species is not always identical, which suggests an intraspecific diversity in their psychrotrophic character possibly as a result of differences in adaptation to particular ecosystems (Table 3.1 and Figure 3.1). The growth characteristics of *Le. carnosum*, *C. divergens* and *Lb. fuchuensis* isolates from the present study extend between a weak growth or a slight inhibition at 30 ^{o}C compared to growth at 22 ^{o}C showing a great phenotypical variation.

Psychrotrophic LAB have proven to be the microbes that appear at the highest populations and constitute the microbial group exclusively dominating in the end of the shelf-life, thus they could presumably constitute the specific spoilage organisms (SSO) of these unstable foodstuffs. The occurrence of cold-acclimatized LAB as members of the consortium in the first place presupposes their adaptation to the environment of food manufacturing facilities. Their prevalence could be attributed to adjustment in the conditions of industrial practice (e.g. cold chain maintenance, adaptation to sanitation methods) or even to opportunistic outgrowth after introduction in the plant mediated by the raw materials and would require further investigation in order to trace down their origin.

The taxonomic study revealed a disproportionate phylogenetic distribution among the isolates compared to the wide range of analyzed food samples in **Chapter 2**. This suggests that only few bacterial species predominate at the end of the shelf-life of chilled-stored, packaged food products in Belgium. The finding demonstrates the need of an efficient assessment of psychrotrophic LAB contamination levels for routine analyses conducted in food industries. Additionally, this high heterogeneity of isolation niches and the small number of different LAB species underpins the significance of these seemingly persistent bacteria in industrial manufacturing processing plants. The retail food samples were obtained from different companies, different production batches and the end products comprised dissimilar raw materials that had been subjected to different production procedures and handlings.

Despite the variety of ecosystem characteristics, the common aspect about all samples is the low-temperature storage (4 and 7 ^{o}C) and the packaging technology (low oxygen availability), which in combination seem to facilitate the outgrowth of very specific microbial groups. Consequently, it can be concluded that the extrinsic parameters of gaseous composition and chilling temperature have a greater impact on the microbial evolution and exert a more severe selection pressure during the shelf-life compared to the intrinsic factors of the food matrices. Hence, LAB species with CO_2-tolerance and a competent psychrotrophic character were strongly favored. According to other studies, the psychrotrophic capacity along with the high competence of these predominant *Leuconostoc* spp. in proliferating at high CO_2 concentrations have assigned them among the most frequently isolated LAB (Björkroth & Holzapfel, 2006; Sakala et al., 2002a; Säde, 2011).

Our results also point to the need for a psychrotrophic reference shelf-life parameter estimating the contamination levels of this type of products in order to adequately determine the microbial load. A standard enumeration procedure that can be generally implemented for microbiological analyses and that shows selectivity and sensitivity towards obligate and facultative psychrophiles should be considered a prerequisite for an efficient culture-

dependent approach. Furthermore, our identification results suggest a crucial and dominant role of mainly *Leuconostoc* spp. in packaged food products stored at chilling temperature. Members of this genus are becoming increasingly important as cumbersome spoilage-related organisms constituting an arising problem for food companies. In previous studies, *Le. gelidum* subsp. *gasicomitatum*, *Le. gelidum* subsp. *gelidum* and *Le. carnosum* are the species of the *Leuconostoc sensu stricto* phylogenetic group that have been related to quality fluctuations in chilled-stored products nowadays (Björkroth et al., 1998; K. J. Björkroth et al., 2000; Lyhs et al., 2004; Santos et al., 2005; Susiluoto et al., 2003; Vasilopoulos et al., 2008; Vihavainen & Björkroth, 2007).

Next to leuconostocs, also members of the *Lc. piscium* were isolated in significant amounts. This organism has been thoroughly studied for its ability to outcompete other bacterial species as a protective culture (Fall et al., 2010; Matamoros et al., 2009) and to sustain low temperatures (Garnier et al., 2010). Also, it has been associated with spoilage cases in fresh beef in the past (Sakala et al., 2002a; Sakala et al., 2002b). The two *Lactobacillus* spp. that were recovered as single case contaminants, *Lb. algidus* and *Lb. fuchuensis*, have a potent psychrotrophic character and have been associated with spoilage manifestations (Belfiore et al., 2010; Lyhs & Björkroth, 2008; Nieminen et al., 2011; Schirmer et al., 2009; Vihavainen & Björkroth, 2007).

In many samples it was observed that *Leuconostoc* spp. and more specifically *Le. gelidum* co-existed with *Lc. piscium* like previously documented (Rahkila et al., 2012). It could be assumed that some sort of interaction could have developed among the two *Le. gelidum* subspecies and *Lc. piscium* that would require further research.

The implemented molecular techniques based on genomic fingerprinting proved to be very effective to determine the taxonomic position of most LAB species. Isolates of *Lc. piscium* and *Le. inhae* were initially not clearly recognized with rep-PCR and AFLP due to a low taxonomical relatedness to the reference strains of the in-house database. To confirm species identity for these isolates, *pheS* gene sequencing was used. As previously shown, this gene is a valuable phylogenetic marker for identification of various LAB (De Bruyne et al., 2007; Naser et al., 2007; Rahkila et al., 2012). Additionally, AFLP analysis faciliatates the evaluation of the entire genome providing an image of conserved domains of core genes and accessory genes reflecting adaptations to lifestyle conditions. Previous studies have proved the ability of AFLP to differentiate microbes to strain level and have highlighted its discriminatory capacity in comparison with multi locus sequence techniques (MLST) that require sequencing of numerous genes in order to provide the same resolution (Litvintseva et al., 2006; Macdonald et al., 2011; Melles et al., 2007; Mietze et al., 2011; Parisi et al., 2010).

Source tracking of psychrotrophic lactic acid bacteria (LAB) in a ready-to-eat (RTE) vegetable salad production environment

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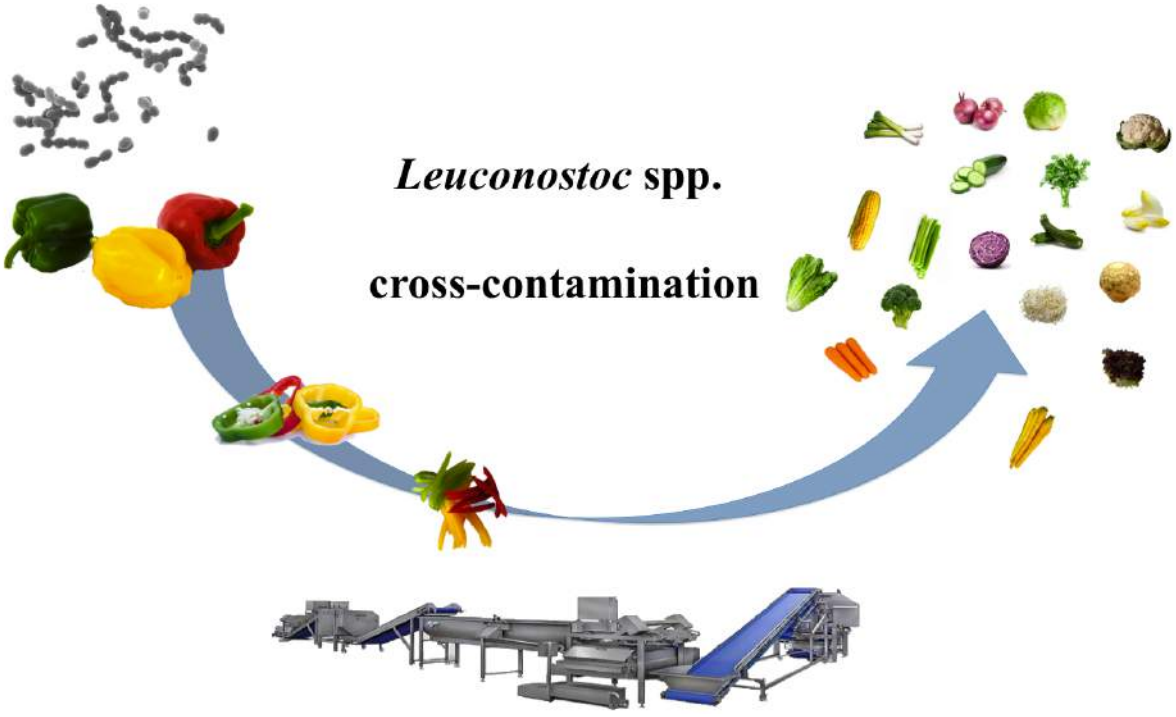


SUMMARY

After having specified the overlooked LAB taxa and characterized them in **Chapter 3** an analysis focusing on their presumptive sources in a processing environment was designed. A source tracking analysis was conducted in a company producing fresh, minimally processed, packaged and ready-to-eat (RTE) vegetable salads (stored at 4 ^{o}C) in order to investigate the origin of a widespread lactic acid bacterium (LAB) contamination. Initially, high microbial counts exceeding the established psychrotrophic thresholds set by the company ($>10^{7}$-10^{8} CFU/g) and spoilage manifestations before the end of the shelf-life (7 days) occurred in products containing an assortment of sliced and diced vegetables, but within a one year period this contamination problem became prevalent in the entire processing plant. Environmental sampling and microbiological analyses of the raw materials and final products throughout the manufacturing process accentuated the presence of high numbers of *Leuconostoc* spp. in unprocessed, fresh sweet bell peppers provided by the supplier. A combination of two DNA fingerprinting techniques facilitated the assessment of the species diversity of LAB circulating in the processing environment along with the critical point of their introduction in the production facility. Probably through air-mediation and surface adhesion, mainly members of the strictly psychrotrophic species *Leuconostoc gelidum* subsp. *gasicomitatum* and *Leuconostoc gelidum* subsp. *gelidum* were responsible for the cross-contamination of every vegetable handled within the plant.

4.1 INTRODUCTION

Technologies limiting the concentration of available oxygen (e.g. vacuum, modified atmosphere-MA) and cold storage are currently being implemented as main bacteriostatic hurdles in food packaging (Labadie, 1999; Willocx et al., 1993). This way, most notorious Gram negative, respiring, spoilage-related bacteria are inhibited and there is an increasing selection of facultative anaerobic or aerotolerant psychrotrophs (**Chapter 1**). The most competitive bacterial group growing under such preservation conditions are mainly cold-acclimatized lactic acid bacteria (Borch et al., 1996; Huis in't Veld, 1996). Although this problem concerns food products of different origin and from various production processes, it remains unclear how these LAB are introduced in production processing installations (Vihavainen et al., 2007). Possible routes of introduction include adhesion on surfaces (**Chapter 7**) and air-mediated contamination (Audenaert et al., 2010; Johansson et al., 2011).
Members of the LAB genus *Leuconostoc* have been isolated from retail products that were deemed unfit for consumption before the end of their shelf-life (Björkroth et al., 2000; Kato et al., 2000; Lyhs et al., 2004; Sakala et al., 2002a; Sakala et al., 2002b; Vihavainen & Björkroth, 2007; **Chapter 3**) and that showed severe spoilage manifestations (Lyhs et al., 2004; Susiluoto et al., 2003; Säde, 2011). Source tracking analyses attempting to elucidate the origin of *Leuconostoc* spp. along the food production line have displayed low recoveries (Vihavainen et al., 2007). This observation may suggest that contamination levels are initially low and thus cannot always be accurately and reliably determined by means of culture-dependent techniques. In addition, the implementation of an enrichment method for leuconostocs against a taxonomically heterogeneous microbial background exploiting differentiating growth traits could be compromised.
Previously, psychrotrophic LAB unable to grow at 30 ^{o}C and thus remaining undetermined during routine microbiological analysis according to the mesophilic enumeration methods (ISO 4833:2003 and ISO 15214:1998) were found to be highly prevalent at the end of shelf-life in various samples of minimally processed, ready-to-eat (RTE) vegetable salads (**Chapter 2**). *Leuconostoc* spp. were the most frequently isolated taxa exhibiting a strict psychrotrophic character and in all cases had become dominant. The present study attempted to trace the origin of the contamination in a vegetable processing plant and to identify the ecological niches in which these organisms can survive.

4.2 MATERIALS AND METHODS

4.2.1 The processing plant and its products

This source tracking analysis was carried out in a Belgian vegetable processing plant. The company is producing fresh, minimally processed, RTE vegetable salads that are being packaged in modified atmosphere (MA) or air and that are subsequently stored at refrigeration temperature (4 ^{o}C) until the end of shelf-life (7 days). The products cover a wide range of vegetables (beet, broccoli, cabbage, carrots, cauliflower, celeriac, celery, corn, cucumber, endive, leek, lettuce, onion, parsley, radish, soy germs, sweet bell peppers, zucchini etc.) and

are commercially packaged as single-vegetable or assortment salads. All end-products are sealed in biaxially-oriented polypropylene (BOPP) film bags with 35 micron thickness and an O_2 permeability of 1400 cm^3/m^2.d. The majority of salads are filled with air except for strongly respiring vegetables (i.e. celery, mixed leafy green salad, iceberg lettuce, cucumber, radish) that are packaged under MA (5-10 % O_2: 90-95 % N_2).

For the purpose of this study, the processing plant was divided in distinct domains (Figure 4.1 and Table 4.1) related to areas exclusively allocated to the handling of specific materials or corresponding to certain practices independent of the material (dicing, mixing, loading, packaging etc.). Domains 1 and 2 correspond to the cold storage rooms where all the raw materials and sometimes, intermediate products were kept until processed or assembled with other vegetables, respectively. Domain 3 was exclusively used for coarse and fine chopping as well as washing of leafy green vegetables, whereas Domain 4 was destined for chopping and washing leek. In the latter two domains, three garbage bins were always positioned in proximity for disposing of lettuce cores and leek taproots. Domain 5 was strictly limited to an automatic rotating fine chopper for parsley. Moreover, Domains 6 and 7 occupied an area where different types of vegetables were handled through a 10 mm and 6 mm dicer, respectively. Apart from the actual machines, palettes, a balance, loading baskets and hand scoops were also in use at this specific part of the plant. Domain 8 comprised three acid baths (i.e. ascorbic acid, lactic acid, acetic acid) for dipping cabbage, cauliflower, broccoli and beets and a manual centrifuge. Lastly, Domains 9, 10, 11 and 12 corresponded to the four packaging lines consisting of a mixing basket, an ascending conveyor belt and the packaging/sealing machine.

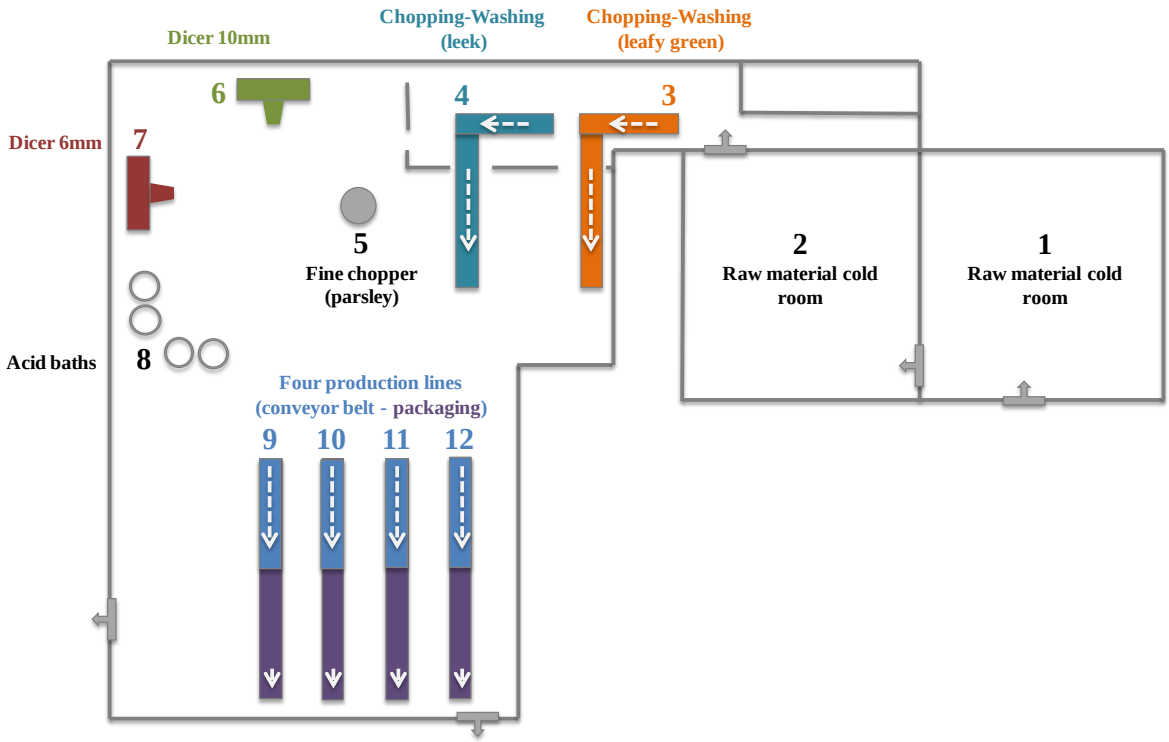


Figure 4.1: *Plan of processing area separated in twelve domains (detailed description of each domain of the plant is presented in Table 4.1).*

4.2.2 Sampling

Air samples were taken to evaluate a possible air-mediated contamination, equipment and surfaces were swabbed, water samples from washing baths and tanks, raw materials, intermediate products after each handling and packaged end-products were also taken aseptically from all production lines. Sampling started at 3:30 AM ahead of the production, right after the completion of the decontamination of the plant and ended at 14:00 PM. Surface, air, vegetable and water samples were taken from each domain of the plant (Table 4.1) repeatedly during 10 hours.

Table 4.1: *Domains of the processing plant allocated to handling exclusively one vegetable or performing a particular manufacturing practice correlated to number and type of samples taken, during the entire sampling period. In total 129 samples from air, surfaces, water, raw material and intermediate products were retrieved.*

Domain	Sampling point	Number of samples			
		Vegetable	Surface	Air	Water
1	Raw material cold room 1	2	1	2	
2	Raw material cold room 2	6	2	1	
3	Chopping board for leafy green vegetable	5	3	3	
	Washing bath for leafy green vegetable		3		2
4	Chopping board for leek	2	3	3	1
	Washing bath for leek		3	1	2
5	Fine parsley chopper	1	1		
6	Vegetable dicer (10mm)	3	4	3	
7	Vegetable dicer (6mm)	3	4	2	
8	Acid solution baths				3
9	Conveyor belt 1	2	2	3	
	Packaging 1				1
10	Conveyor belt 2	3	7	4	
	Packaging 2		2	2	1
11	Conveyor belt 3	1	5	1	
	Packaging 3		1	1	1
12	Conveyor belt 4	1	4	4	
	Packaging 4		2	2	1
Chlorination unit – Water tanks			4		5
Total		29	51	32	17

4.2.2.1 Sampling of surfaces

For the surfaces and the equipment, sterile rayon swabs with plastic shaft in individual tubes (Cultiplast, Code: 111598 rayon) were used. Peptone physiological solution (PPS: 0.85 % NaCl and 0.1 % peptone in distilled water) was added in the tube to moisten the head of the swab. Approximately 25 cm^2 were swabbed each time and the rayon head along with the plastic shaft was directly transferred aseptically in de Man-Rogosa-Sharpe broth for assessing LAB growth, supplemented with sorbic acid (MRS-S) in order to inhibit the growth of yeasts. Samples were incubated anaerobically at 4 ^oC for 20 days. The MRS-S broth was prepared from its individual components (yeast extract: 4 g/L; Lab-Lemco powder: 8 g/L; peptone: 10 g/L; sorbitan mono-oleate (Tween 80): 1 ml/L; dipotassium hydrogen phosphate: 2 g/L;

sodium acetate: 5 g/L; triammonium citrate: 2 g/L; magnesium sulphate: 0.2 g/L; manganese sulphate: 0.05 g/L) with the addition of 1 % sorbic acid, pH was adjusted to 5.7 and autoclaved separately from the glucose (20 g/L) avoiding Maillard reactions that would alter the final pH. After autoclaving, the glucose was added in the rest of MRS-S broth and distributed in sterile glass tubes.

4.2.2.2 Sampling of air

The air contamination was evaluated by using a Spin air - Air sampler (IUL instruments, Spain) set at an air flow of 100 L/min and a rotation speed of 3 rpm during 8 min resulting in the sampling of 800 L air. The 90 mm petri plates used for air analysis containing MRS-S agar. After each sampling, the rotating petri plate holder and its cover were cleaned with ethanol. All plates were incubated anaerobically, at 22 ^{o}C for 5 days.

4.2.2.3 Sampling of water

Water samples from the drilling prior to the chlorination, after the chlorination and from the different wash baths, tanks and acid solutions were taken aseptically with sterile disposable Pasteur pipettes. Samples of 1 mL were inoculated in MRS-S broth (9 mL) and incubated anaerobically at 4 ^{o}C for 20 days.

4.2.2.4 Sampling of vegetable material - Enrichment procedure for psychrotrophic LAB

Samples from the raw materials and the intermediate products obtained after each handling were collected for almost every type of vegetable salad. Additionally, final products in the original packages were kept at 4 ^{o}C until they reached the end of the shelf-life when they were eventually sampled. For the raw materials and the intermediate products, microbiological analysis was carried out immediately for psychrotrophic and mesophilic microbes by incubation under anaerobic conditions at 22 ^{o}C and 30 ^{o}C, respectively. This way, the possible presence of high psychrotrophic LAB counts would be detected if an underestimation with the mesophilic enumeration method would occur (**Chapter 2**).
On the other hand, another part of the sample was incubated anaerobically, at 4 ^{o}C for 10 days aiming at the selection of psychrotrophic LAB in case that their initial contamination levels would be very low and remain undetected through plating. In general, incubation at 4 ^{o}C under anaerobic conditions constitutes an enrichment procedure for the psychrotrophic LAB since it clearly favours the growth of cold-acclimatized and CO_2-tolerant microorganisms. All end products were analyzed at the end of their shelf-life following the indication of the manufacturers according to a mesophilic and psychrotrophic enumeration technique (incubating plates at 30 and 22 ^{o}C, respectively).
For the enumeration of the microbial contamination levels, a representative vegetable sample of 15 g was taken aseptically and the primary decimal dilution was prepared by adding 135 g of PPS. Samples were homogenized for 60 s at 22 ^{o}C with a Colworth Stomacher 400 (Steward Laboratory, London, UK). Subsequently, decimal dilution series were prepared from the primary dilution using PPS and they were pour plated in Plate Count Agar (PCA, Biorad,

Hercules, CA, USA), de Man-Rogosa-Sharpe Agar (MRS, Biorad, Hercules, CA, USA) the pH adjusted to 5.9 and Reinforced Clostridial Agar (RCA, Oxoid, Hampshire, UK) in order to determine the total aerobic, lactic acid bacteria and total anaerobic counts, respectively. An overlayer was added on the MRS and RCA plates to achieve micro-aerophilic conditions and the RCA plates were additionally put in anaerobic jars together with an Anaerogen sachet (Anaerogen, Oxoid, Hampshire, UK) for a completely anaerobic incubation. All plating was performed in triplicate with incubation at 22 ^{o}C for 5 days and at 30 ^{o}C for 3 days.

4.2.3 Isolation

For the air samples, a number of single colonies (i.e. 3-10) were taken always representatively to the overall grown colonies generated on the MRS-S plates (based on morphology). For the swabs and the water samples inoculated in tubes containing MRS-S broth, isolation of single colonies was carried out after streaking out on MRS-S agar plates at the end of the 20 days incubation period. Likewise, a representative amount of colonies to the overall grown were isolated based on morphology. For vegetable samples, colonies were picked up from the plates incubated at 22 ^{o}C when these plates were found to have higher numbers of colonies compared to their equivalent incubated at 30 ^{o}C (>0.5 log CFU/g).
The number and type of single colonies selected from each plate were approximately relative to the distribution of colonies present on the plate. In all cases, isolates originated from the plates of the highest dilution in order to select the predominant microbes. Subsequently, isolates selected from PCA, MRS and RCA were transferred aseptically to Tryptone Soya Broth (TSB, Oxoid, Hampshire, UK), de Man-Rogosa-Sharpe broth (MRS broth, Oxoid, Hampshire, UK) and TSB (incubated under anaerobic conditions), respectively and incubated at 22 ^{o}C for 5 days. If growth was observed, the purity of the isolate was checked by streaking out on the same medium. All isolates were stored in glycerol at -75 ^{o}C for further identification.

4.2.4 DNA extraction

For dereplication purposes, DNA extraction from all isolates was initially performed with alkaline lysis in order to rapidly acquire crude DNA material (Niemann et al., 1997). A few pure colonies were transferred from MRS agar or Tryptone Soya Agar medium to an eppendorf tube with the back of a sterile, plastic microbiological loop after which 20 μL of lysis buffer (5 % w/v NAOH and 0.25 % w/v SDS) was added. The cell suspension was heated at 95 ^{o}C for 15 min, and then shortly centrifuged at 13000 rpm. Finally, 180 μL of sterile MQ water were added, the extract containing the DNA was centrifuged at 13000 rpm for 5 min and then stored at -20 ^{o}C.
For the selection of 81 isolates that were subjected to amplified fragment length polymorphism (AFLP) analysis, DNA extraction was performed a second time now following a modified protocol of the phenol/chloroform/isoamyl alcohol method described by Gevers et al., (2001) in order to obtain a DNA extract of high quality and purity.

4.2.5 rep-PCR genomic fingerprinting

All isolates were subjected to repetitive element PCR using the $(GTG)_5$ primer, i.e. $(GTG)_5$-PCR. Amplifications were conducted according to Versalovic et al., (1994) in a DNA thermal cycler (Perkin Elmer 9600, Massachusetts, USA) using Goldstar DNA polymerase (Eurogentec, Liège, Belgium). The PCR amplicons were separated in 1.5 % w/v agarose gel (20 cm x 15 cm), using 1 x TAE buffer for 16 h at 55 V and at constant temperature of 4 ^{o}C. After ethidium bromide staining, $(GTG)_5$-PCR profiles were visualized under ultraviolet light followed by digital image capturing using a CCD camera. The resulting genomic fingerprints were analyzed using the BioNumerics 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium) for clustering analysis with the Unweighted Pair Group Method with Arithmetic Mean and the Pearson correlation coefficient.

4.2.6 Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP analysis of 81 isolates selected after dereplication was conducted based on a modified protocol of Thompson et al., (2001). AFLP was performed using *TaqI* and *EcoRI* as restriction enzymes and primers E01 (5'-GACTGCGTACCAATTCA-3') and T01 (5'-CGATGAGTCCTGACCGAA-3'). The selective PCR products were separated with an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, USA). The profiles obtained after electrophoresis were normalized using the GeneScan 3.1 software (Applera, Norwalk, CT, USA), and the peak fingerprints were processed using the BioNumerics 5.10 software. AFLP patterns of the isolates of the present study were compared with profiles of type and reference LAB strains of an in-house database (BCCM/Bacteria Collection) using the Dice coefficient and UPGMA clustering.

4.3 RESULTS

4.3.1 Isolation of psychrotrophic LAB

From the contamination levels detected in the air samples using MRS-S plating, circulation of LAB was observed which either belonged to the autochthonous "house microbiota" of the processing plant or spreading through air-mediation from the raw materials introduced to the production area. As shown in Table 4.2, in the beginning of the production day (5:05 AM) air contamination levels were low. The samples taken from Domains 3, 4, 11 and 12 before initiation of production showed no growth, whereas samples from Domains 1, 2, 6 and 9 indicated LAB presence varying between 11-22 CFU/800 L of filtered air. Unlike the other areas of the processing environment, Domain 10 had a very high initial contamination (>120 CFU/800 L air). As production proceeds, the circulation of LAB increased presumably due to personnel activity, raw material transportation and beginning of handlings. Especially for areas with dynamic activities (e.g. ascending conveyor belts, mixing baskets and moving palettes), counts were increasing (Domains 1, 10, 11 and 12) and random fluctuation of contamination levels was observed (40-100 CFU/800 L).

Table 4.2: *Air samples taken from the various domains of the vegetable processing plant. The contamination levels on MRS-S medium are expressed in CFU/800 L of air.*

Domain	Sample	Time	Sampling point	Contamination (CFU/800 L)
1	**1.1**	5:05	next to the palettes of lettuce	15
	1.5	8:30	next to the palettes of lettuce	103
2	**2.2**	5:15	next to the palettes of sweet bell peppers	11
3	**3.2**	4:30	next to the chopping board	0
	3.7	10:00	next to the chopping board	38
	3.12	12:00	next to the chopping board	28
4	**4.1**	4:15	next to the chopping board	0
	4.4	6:15	next to the chopping board	10
	4.8	9:30	from conveyor belt after slicing	44
	4.12	10:20	next to conveyor belt after drying	43
6	**6.1**	4:30	from the top of the dicer	22
	6.3	9:00	next to the standing palette	31
	6.9	12:10	next to the balance	25
7	**7.3**	9:30	from the top of the dicer	31
	7.5	9:30	from the top of the dicer	26
9	**9.2**	6:30	while loading the salad constituents in the basket	15
	9.6	8:00	next to the conveyor belt	36
	9.7	13:00	next to the conveyor belt	27
10	**10.3**	6:50	from the beginning of the conveyor belt	122
	10.7	7:40	next to the mixing basket	128
	10.10	8:20	next to the conveyor belt before packaging	0
	10.13	12:20	next to the conveyor belt with beetroot, carrot and cucumber	31
	10.15	13:00	at packaging	16
	10.17	13:30	at packaging	46
11	**11.1**	4:40	next to the mixing basket	0
	11.9	13:00	at packaging	47
12	**12.1**	4:50	at packaging	0
	12.3	7:00	at packaging	83
	12.6	7:30	next to the mixing basket while loading carrot sticks	25
	12.9	9:00	next to the mixing basket	43
	12.12	11:00	at packaging	53
	12.13	13:00	at packaging	19

Also for the majority of samples recovered by swabbing surfaces and equipment parts (Domains 1, 3, 4, 7, 10, 11 and 12), no growth was observed (Table 4.3) after enrichment in MRS-S broth before initiation of production. Strikingly, two samples (i.e. 5.2 and 6.2 in Table 4.3) from equipment blades at time points before the equipment was used showed growth. Because swabs obtained after sampling a surface were immediately transferred in new tubes containing MRS-S culture medium and not in their original container filled with PPS, true contamination levels were not assessed. We have chosen this procedure to anticipate previous observations (Björkroth & Korkeala, 1997) that initial LAB contamination levels can be below the detection threshold of culture-dependent techniques (<10 CFU/g).

No LAB were detected in the water samples that were taken from the two drillings, the chlorination units, the drilling pipes, the water tanks and the water bath pipes of Domains 3 and 4. The only water samples contaminated with LAB were 11.5 (red cabbage water bath), 12.7 (carrot water bath) and the actual acid solutions used in Domain 8 for avoiding browning of the vegetable tissue, i.e. lactic acid (acid bath 1) and acetic acid (acid bath 2).

Table 4.3: *Surface samples (swabs) from surfaces and equipment parts, from the various domains of the processing area. The contamination is expressed as growth/no growth observed in the tubes of MRS-S that the swabs were inoculated and incubated anaerobically for 20 days at 4 ^{o}C. (+ : growth, — : no growth).*

Domain	Sample	Time	Sampling point	Contamination
1	**1.2**	5:15	from the palettes of lettuce	—
2	**2.1**	5:10	from the palettes of sweet bell peppers	+
	2.3	5:20	from the palettes	+
3	**3.1**	4:15	from conveyor belt after chopping	—
	3.3	9:10	chopping knives	+
	3.8	10:00	from conveyor belt after slicing and before washing	+
	3.11	11:50	chopping board	+
	3.13	13:00	garbage bin 1	+
	3.14	13:00	garbage bin 2	+
4	**4.2**	4:30	from conveyor belt after chopping	—
	4.3	6:15	from conveyor belt after washing	+
	4.5	9:00	from conveyor belt after chopping	+
	4.7	9:20	from conveyor belt after slicing	+
	4.10	10:10	from conveyor belt after drying	+
	4.13	13:00	garbage bin 3	+
5	**5.2**	6:20	from chopping blade	+
6	**6.2**	4:35	from dicing blades	+
	6.4	9:00	from the standing palette	+
	6.8	12:00	hand scoop	+
	6.10	13:00	Baskets	+
7	**7.1**	4:35	from the inlet of the dicer	—
	7.2	9:00	from the outfeed of the dicer	+
	7.4	9:30	from dicing blades	+
	7.9	12:10	vegetable centrifuge	+
9	**9.1**	4:55	mixing basket	+
	9.5	7:40	from the conveyor belt before packaging	+
10	**10.1**	4:45	from the conveyor belt	—
	10.2	6:30	from mixing basket while loading soja germs	+
	10.6	7:30	baskets with sweet bell peppers, leek, soy germs and onion	+
	10.8	8:00	conveyor belt with sweet bell peppers, onion and zucchini	+
	10.9	8:15	from the conveyor belt before packaging	+
	10.12	9:00	from mixing basket while loading sliced cabbage and carrot sticks	+
	10.14	12:20	from conveyor belt with beetroot, carrot and cucumber	+
	10.16	13:00	at packaging	+
	10.18	13:30	at packaging	—
11	**11.2**	4:40	from the conveyor belt	—
	11.3	7:00	conveyor belt with sliced cabbage	+
	11.4	8:00	conveyor belt with sliced cabbage	+
	11.6	9:00	mixing basket with washed celeriac sticks	+
	11.8	9:45	from the conveyor belt with sliced leek	+
	11.10	13:00	at packaging	+
12	**12.2**	4:50	next to the mixing basket	—
	12.4	7:00	from the conveyor belt with carrot sticks	+
	12.5	7:30	from the conveyor belt with carrot sticks	+
	12.10	10:30	at packaging	+
	12.11	10:30	at packaging	+
	12.14	13:15	at packaging	—

The psychrotrophic and mesophilic counts of the raw materials and the intermediate products were assessed after each handling (Table 4.4). For 12 out of the 29 samples, contamination levels (according to the pre-enrichment analysis) determined with the two enumeration procedures were comparable. This implies the absence of strictly psychrotrophic populations in these samples. For all samples containing sweet bell peppers (13 out of 29), however, the

comparison of psychrotrophic and mesophilic counts indicated a considerable underestimation of contamination levels when incubating at 30 ^{o}C. In addition, also samples of iceberg lettuce, carrot sticks, onion cubes and celeriac (i.e. 3.9, 5.1, 11.7 and 12.8, respectively) showed a similar underestimation.

Table 4.4: *Microbiological analysis of the raw materials and intermediate products at 22 ^{o}C and 30 ^{o}C.*
[a] *The pre-enrichment analysis was performed immediately after recovery of the samples.*
[b] *The post-enrichment analysis was performed after an enrichment incubation of the sample for 10 days at 4 ^{o}C selecting for psychrotrophic LAB.*
[c] *The counts are expressed in log CFU/g as the mean value of a triplicate repetition. The standard deviation (S.D.) is not shown as in all cases was below ± 0.08 log CFU/g.*

Domain	Sample	Time	Sampling point	Pre-enrichment Analysis [a] Counts (log CFU/g) [c]						Post-enrichment Analysis [b]
				PCA		MRS		RCA		
				22 °C	30 °C	22 °C	30 °C	22 °C	30 °C	Underestimation at 30 °C
1	1.3	5:40	Lettuce	6.96	6.92	3.11	3.05	4.17	4.10	No	Yes
	1.4	5:40	Endive	6.35	6.31	2.60	2.63	5.04	4.97	No	No
2	2.4	5:40	onion cubes	7.37	7.29	6.52	5.79	6.92	6.87	Yes	Yes
	2.5	5:40	mixed sweet bell pepper cubes	7.52	7.39	6.95	6.53	6.87	6.65	Yes	Yes
	2.6	6:00	mixed sweet bell pepper, leek and carrot cubes	7.22	7.07	6.45	5.77	6.55	6.44	Yes	Yes
	2.7	6:00	sliced red sweet bell pepper	7.47	7.35	7.11	6.35	7.12	6.81	Yes	Yes
	2.8	9:00	yellow sweet bell pepper (cut in halves)	7.76	7.72	7.65	5.52	7.71	6.71	Yes	Yes
	2.9	9:20	mixed sweet bell peppers, leek, onion and broccoli	7.25	6.88	6.20	5.52	6.61	6.58	Yes	Yes
3	3.4	10:00	iceberg lettuce after chopping	6.98	6.90	3.10	3.10	5.92	5.86	No	No
	3.5	10:00	iceberg lettuce after slicing	6.75	6.70	4.40	4.11	5.72	5.70	No	No
	3.6	10:00	Endive	< 3	< 3	< 3	< 3	< 3	< 3	No	No
	3.9	11:00	washed iceberg lettuce	7.14	6.62	4.82	4.20	4.82	4.45	Yes	Yes
	3.10	11:00	washed iceberg lettuce before drying	5.68	5.61	3.42	3.10	3.84	3.49	No	No
4	4.9	9:10	leek after washing	5.74	5.73	3.42	3.42	5.23	5.01	No	Yes
	4.11	10:20	leek from conveyor belt after drying	4.64	4.60	3.26	3.10	3.98	3.96	No	No
5	5.1	6:00	finely chopped parsley	7.16	7.13	4.27	3.59	5.74	5.58	Yes	Yes
6	6.5	9:00	mixed sweet bell pepper, onion cubes	7.72	7.33	7.52	6.62	7.70	6.92	Yes	Yes
	6.6	10:30	yellow sweet bell pepper (cut in halves)	7.28	6.74	5.49	4.61	5.66	5.29	Yes	Yes
	6.7	10:30	red sweet bell pepper (cut in halves)	7.39	7.29	6.68	5.82	6.76	6.74	Yes	Yes
7	7.6	9:35	cucumber after drying	4.68	4.64	3.46	3.26	3.36	3.00	No	Yes
	7.7	9:40	cucumber cubes	5.62	5.44	3.46	3.26	3.84	3.52	No	Yes
	7.8	9:40	mixed sweet bell pepper cubes	6.87	6.84	6.14	5.16	6.25	5.40	Yes	Yes
9	9.3	7:00	sliced red sweet bell pepper	7.11	7.08	6.31	5.33	6.38	6.14	Yes	Yes
	9.4	7:20	sliced green sweet bell pepper	6.69	6.60	5.54	4.38	5.51	5.18	Yes	Yes
10	10.4	7:00	soy germs	7.86	7.83	5.55	5.51	6.65	6.62	No	No
	10.5	7:00	sliced onion	6.47	6.37	4.53	4.49	6.54	6.43	No	Yes
	10.11	8:20	mixed sweet bell peppers, onion and zucchini	7.46	7.33	6.55	5.78	6.74	7.06	Yes	Yes
11	11.7	9:10	washed celeriac sticks from the conveyor belt	6.14	6.00	5.20	4.70	5.52	5.28	Yes	Yes
12	12.8	8:30	carrot sticks	6.46	6.39	5.48	4.62	5.79	5.69	Yes	Yes

After a psychrotrophic enrichment incubation the contamination levels were determined again (post-enrichment analysis) to investigate if a difference between counts at 22 and 30 ^{o}C had occurred for any of the 12 samples that initially showed no indication of psychrotrophic LAB presence. For samples that showed no underestimation in both cases before and after enrichment, strictly psychrotrophic LAB were probably absent. For samples that showed lower counts when the mesophilic enumeration was implemented as a microbial count parameter in both analyses, it can be deduced that psychrotrophic LAB were already present from the beginning. Possibly, this can either be attributed to the endogenous microbiota or to

a previous contamination from the supplier of the raw material. However, the most interesting ecological evolution profile comes for the samples that showed no underestimation in the first microbiological analysis but it appeared after the enrichment incubation.
This fact suggests a very low contaminating population of LAB that was not evident in the beginning but outcompeted the other groups of the consortium during cold, anaerobic storage. This contamination could have occurred within the processing plant.
This was observed for samples 1.3, 4.9, 7.6, 7.7, 10.5 that did not contain sweet bell peppers and most of the other samples belonging to the same vegetable type did not have the same profile.
The majority of the samples (21 out of 25) from the end-products showed an underestimation of counts when the mesophilic method is used as a shelf-life parameter (Table 4.5). All types of vegetable salads were most likely subjected to cross-contamination events since the raw materials did not show evidence of detectable psychrotrophic LAB levels.

Table 4.5: *Microbiological analysis of the end-products stored at 4 ^{o}C until the end of their shelf-life (7 days) according to manufacturing specifications. All samples were analyzed in the end of their shelf-life. The average underestimation of the samples is approximately 1.42 log CFU/g.*
[a]The counts are expressed in log CFU/g as the mean value of a triplicate repetition. The standard deviation (S.D.) is not shown as in all cases was below ± 0.12 log CFU/g.

Sample	Description	Packaging	Counts (log CFU/g) [a]						Underestimation at 30 °C	$\Delta\log_{max}$	Medium
			PCA		MRS		RCA				
			22 °C	30 °C	22 °C	30 °C	22 °C	30 °C			
A.1	Finely sliced leek	air	7.16	7.11	5.70	5.67	7.10	7.09	No	-	-
A.2	Roughly chopped leek	air	8.48	7.75	8.56	7.64	7.63	7.62	Yes	0.93	MRS
A.3	Mixed leek, celery, carrot and onion in slices	air	8.63	8.05	8.60	7.15	8.49	7.67	Yes	1.45	MRS
A.4	Carrot cubes	air	7.71	7.25	7.77	6.46	7.67	6.45	Yes	1.30	MRS
A.5	Sliced celery	MAP	8.51	7.53	8.49	7.24	8.26	7.63	Yes	1.25	MRS
A.6	Cabbage cubes	air	8.00	6.76	8.09	6.33	7.91	6.71	Yes	1.76	MRS
A.7	Sliced cabbage	air	7.64	7.32	7.42	5.86	7.51	7.26	Yes	1.55	MRS
A.8	Cucumber cubes	MAP	7.87	7.09	8.01	6.59	8.31	7.03	Yes	1.42	MRS
A.9	Mixed sweet bell peppers, carrot and corn in cubes	air	9.21	8.29	9.10	7.66	8.74	7.65	Yes	1.44	MRS
A.10	Mixed sliced cabbage and carrot sticks	air	8.90	6.68	8.79	7.98	8.74	7.92	Yes	2.22	PCA
A.11	Mixed endive, radicchio and wild chicory chopped	MAP	7.60	6.87	6.59	6.11	6.71	6.36	Yes	0.73	PCA
A.12	Mixed sweet bell peppers in slices	air	8.91	8.84	8.01	6.83	8.01	7.95	Yes	1.18	MRS
A.13	Mixed leek, celery, carrot and onion in slices	air	9.01	7.71	8.19	7.61	8.18	7.43	Yes	1.30	PCA
A.14	Mixed sweet bell peppers in cubes	air	8.95	8.87	7.65	6.52	7.62	7.58	Yes	1.13	MRS
A.15	Carrot sticks	air	7.98	7.93	7.64	6.52	7.89	7.86	No	-	-
A.16	Mixed sweet bell peppers, carrot and wild chicory	air	8.73	6.90	8.76	7.53	8.71	7.72	Yes	1.83	PCA
A.17	Zucchini cubes	air	8.25	5.65	8.09	6.69	8.14	6.86	Yes	2.60	PCA
A.18	Mixed iceberg lettuce, cucumber and radish	MAP	8.57	7.24	8.53	7.33	8.47	7.69	Yes	1.34	PCA
A.19	Celeriac sticks	MAP	8.10	7.10	8.30	6.69	8.27	7.44	Yes	1.61	MRS
A.20	Washed soy germs	air	9.09	9.07	7.73	7.64	8.78	8.50	No	-	-
A.21	Iceberg lettuce chopped	MAP	8.48	7.78	8.29	7.31	8.34	7.57	Yes	0.98	MRS
A.22	Sliced cucumber	MAP	8.29	6.36	8.08	6.58	8.27	6.69	Yes	1.94	PCA
A.23	Mixed endive, radicchio and "lollo rosso" lettuce	MAP	7.23	6.63	6.25	5.23	6.12	5.59	Yes	1.02	MRS
A.24	Finely chopped parsley	air	8.04	7.98	6.58	5.72	6.29	5.90	Yes	0.86	MRS
A.25	Chopped endive	MAP	7.18	7.01	5.59	5.52	6.03	5.87	No	-	-

The results of Tables 4.4 and 4.5 were statistically analyzed by performance of paired t-test with a confidence level of 95% prooving that counts after incubation at 30 ^{o}C were significantly different from counts at 22 ^{o}C on all three media ($P \leq 0.05$).

4.3.2 Identification of isolates

In total, 442 isolates were selected from plates inoculated with the air samples, the MRS-S broth inoculated with the swabs, the water samples and the plates of the vegetable samples that were incubated at 22 ^{o}C and showed greater counts compared to their equivalent incubated at 30 ^{o}C. Initially, all recovered isolates from air, surface, water and vegetable samples were subjected to $(GTG)_5$-PCR fingerprinting as means of dereplication. Following clustering analysis, three major $(GTG)_5$-PCR groups comprising 153, 129 and 48 isolates, respectively were recognized (Figure 4.2.A-H).

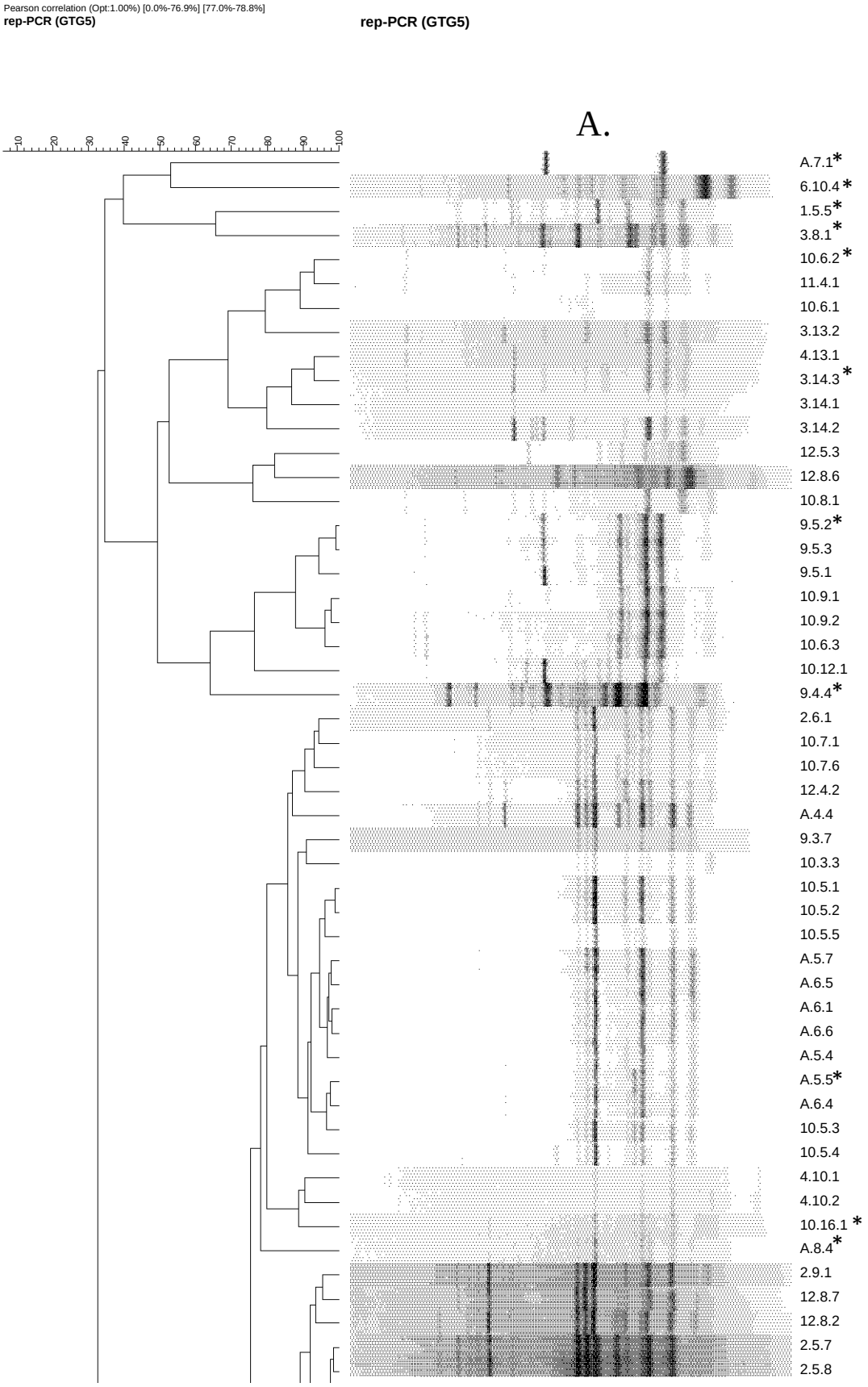
In addition, smaller groups were found containing 3-15 isolates and 42 isolates remained ungrouped. The profiles within the 3 major clusters contained isolates from all sample types. The genotypic diversity within each of these major clusters was low, and could in most cases be attributed to differences in band intensities. A certain level of heterogeneity concerning the fingerprints could be attributed to different band intensities. Nonetheless, a concrete profile of standard band positioning could be investigated by visual inspection suggesting a small species diversity.

In a second identification step, a selection of 81 isolates from all clusters and the ungrouped isolates were subjected to AFLP analysis (Table 4.6).

In this way, the three largest clusters initially delineated in $(GTG)_5$-PCR at a Pearson similarity level of 68 %, 72 % and 75 % were assigned to *Leuconostoc gelidum* subsp. *gasicomitatum* (n=153), *Leuconostoc gelidum* subsp. *gelidum* (n=129) and *Leuconostoc inhae* (n=48). Overall, *Leuconostoc* spp. were dominant among the set of isolates under study (353 out of 442 or 80 %), and only a few other genera were identified each with a low number of isolated representatives i.e. *Pediococcus, Lactococcu*s and *Lactobacillus*. A clear dominance of specific strain types throughout the handlings was not detected at AFLP level. On the contrary, and in contrast to the $(GTG)_5$-PCR fingerprints, a high intraspecific diversity was observed for the two major taxa *Le. gelidum* subsp. *gasicomitatum* and *Le. gelidum* subsp. *gelidum* suggesting the presence of a genotypically rich LAB pool in the plant environment.

Le. gelidum subsp. *gasicomitatum* was the most frequently isolated LAB species from all sample types, being recovered from 21 raw and intermediate products, 13 swabs, 11 air samples and 2 water samples (Table 4.6). Also *Le. gelidum* subsp. *gelidum* was widespread in many sample types, including 17 vegetable samples, 10 swabbed surfaces, 9 air samples and 3 water samples. The third predominant species, *Le. inhae,* was isolated from 6 vegetable and 8 air samples. Noteworthy, *Lactococcus piscium* was found in one intermediate product and the end-product but was not isolated from any other source. The remaining identified species occurred only sporadically throughout the source tracking study.

The only psychrotrophic LAB that were found at the end of the shelf-life and prevailed in all salad types belonged to the species *Le. gelidum* subsp. *gelidum, Le. gelidum* subsp. *gasicomitatum* and *Le. inhae* which dominated in 17, 14 and 9 end-products, respectively. This proves the competence of these taxa compared to other LAB species during shelf-life as cold-storage and anoxic conditions favor their growth. The population of this group of psychrotrophic LAB exceeded the threshold of 7 log CFU/g in all cases (Table 4.5), and an average underestimation of approximately 1.4 log CFU/g was occurring when applying the


Pearson correlation (Opt:1.00%) [0.0%-76.9%] [77.0%-78.8%]
rep-PCR (GTG5)
rep-PCR (GTG5)
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6.10.4*
1.5.5*
3.8.1*
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10.7.1
10.7.6
12.4.2
A.4.4
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A.6.1
A.6.6
A.5.4
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10.5.4
4.10.1
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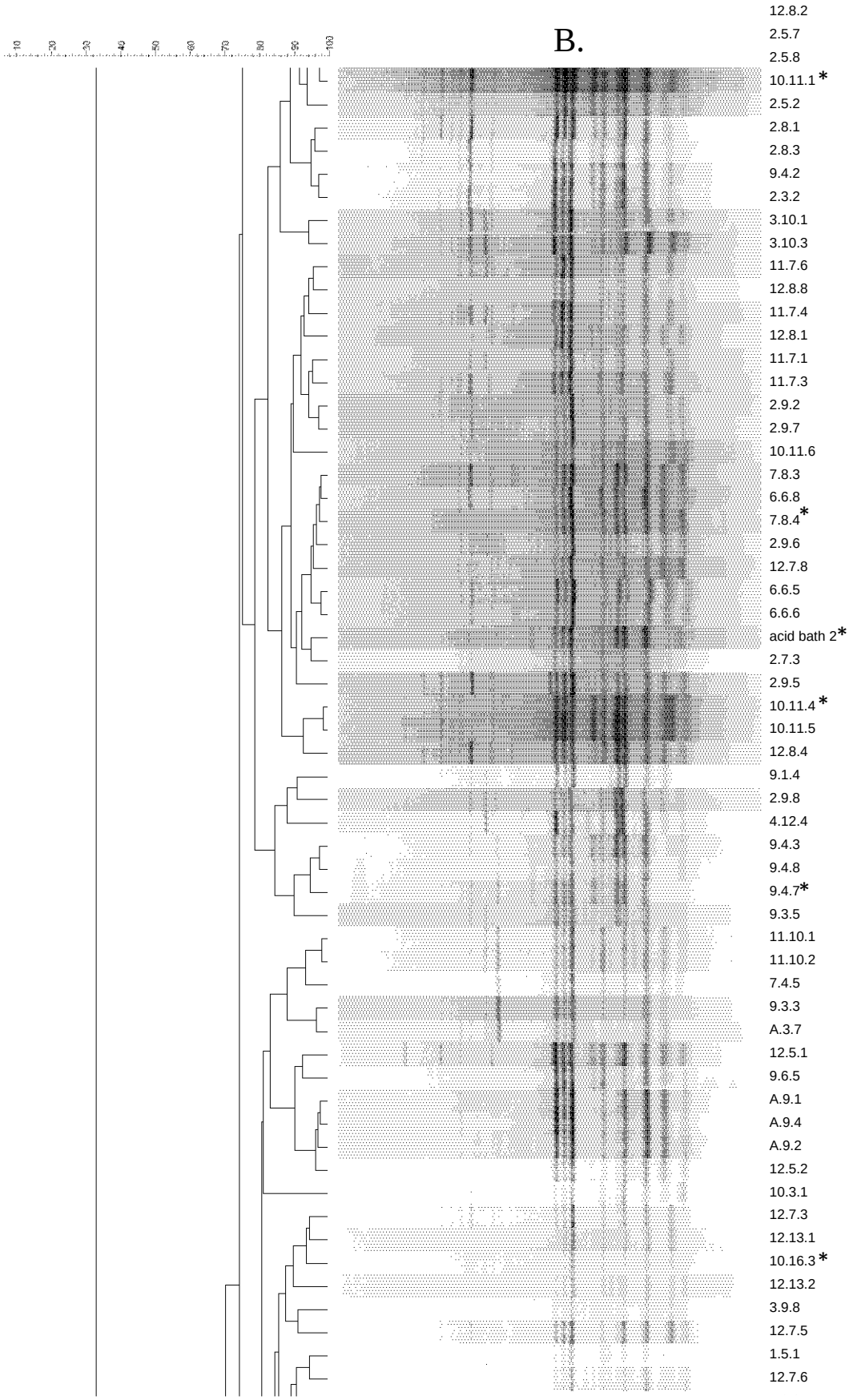
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2.8.3
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acid bath 2*
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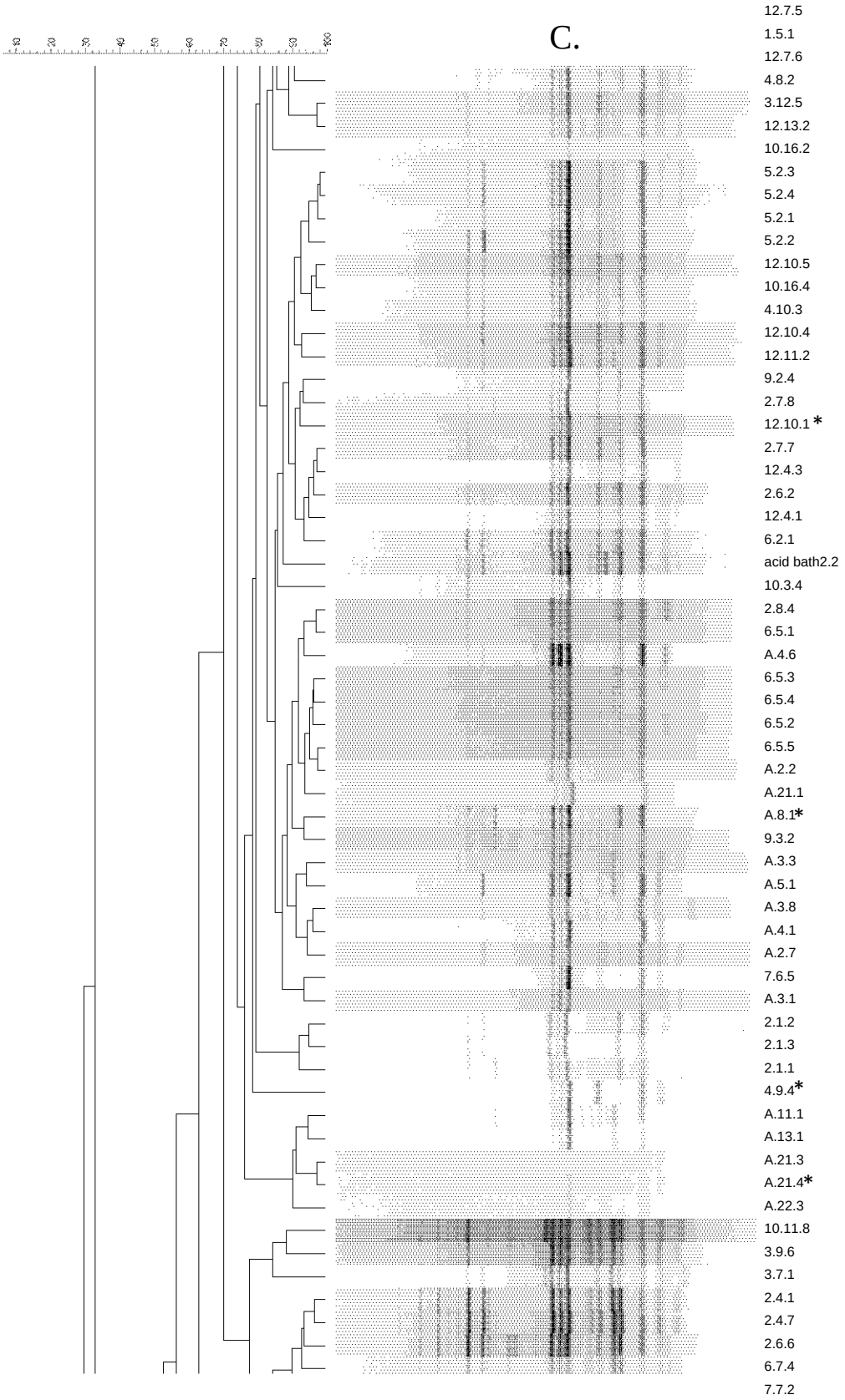
C.
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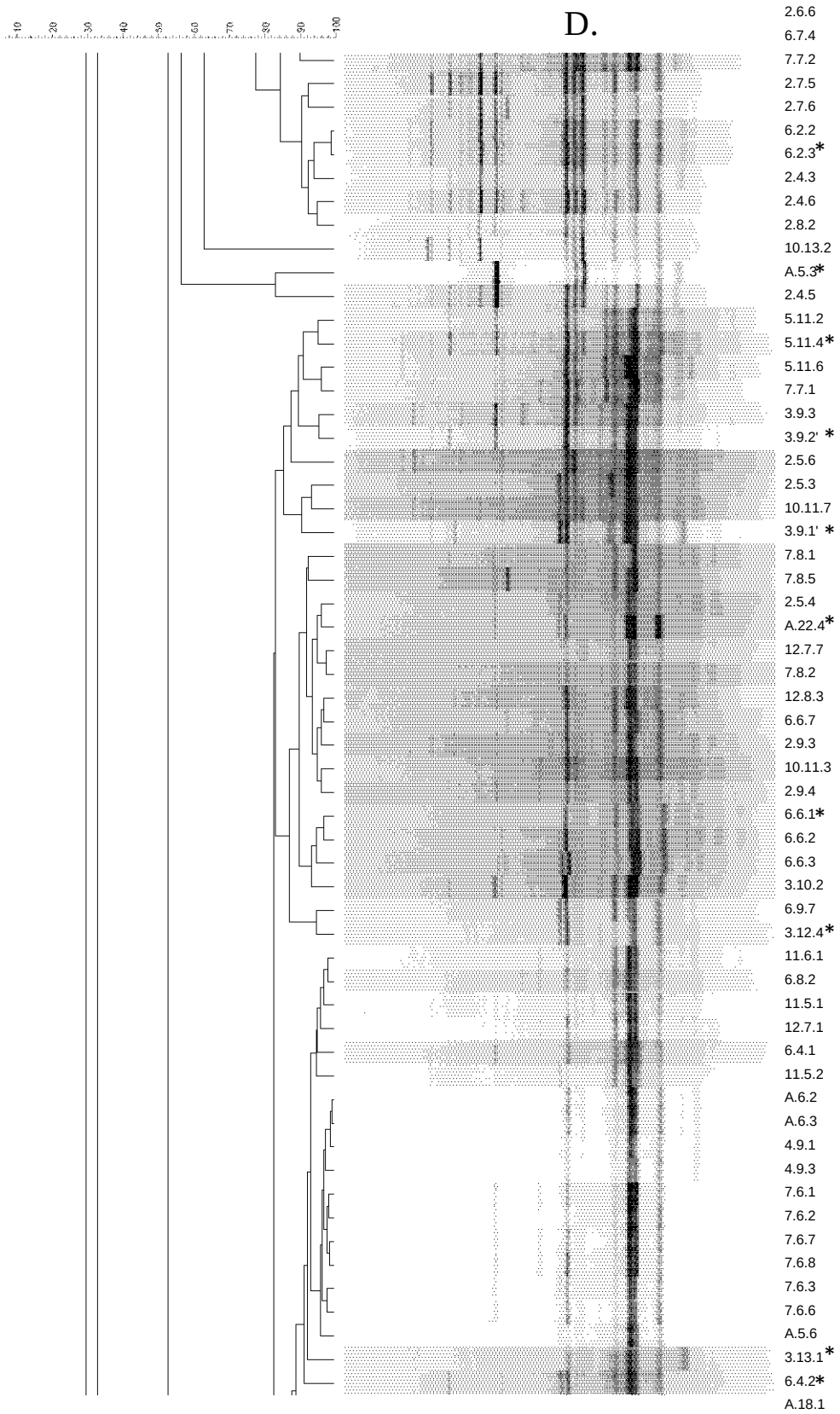
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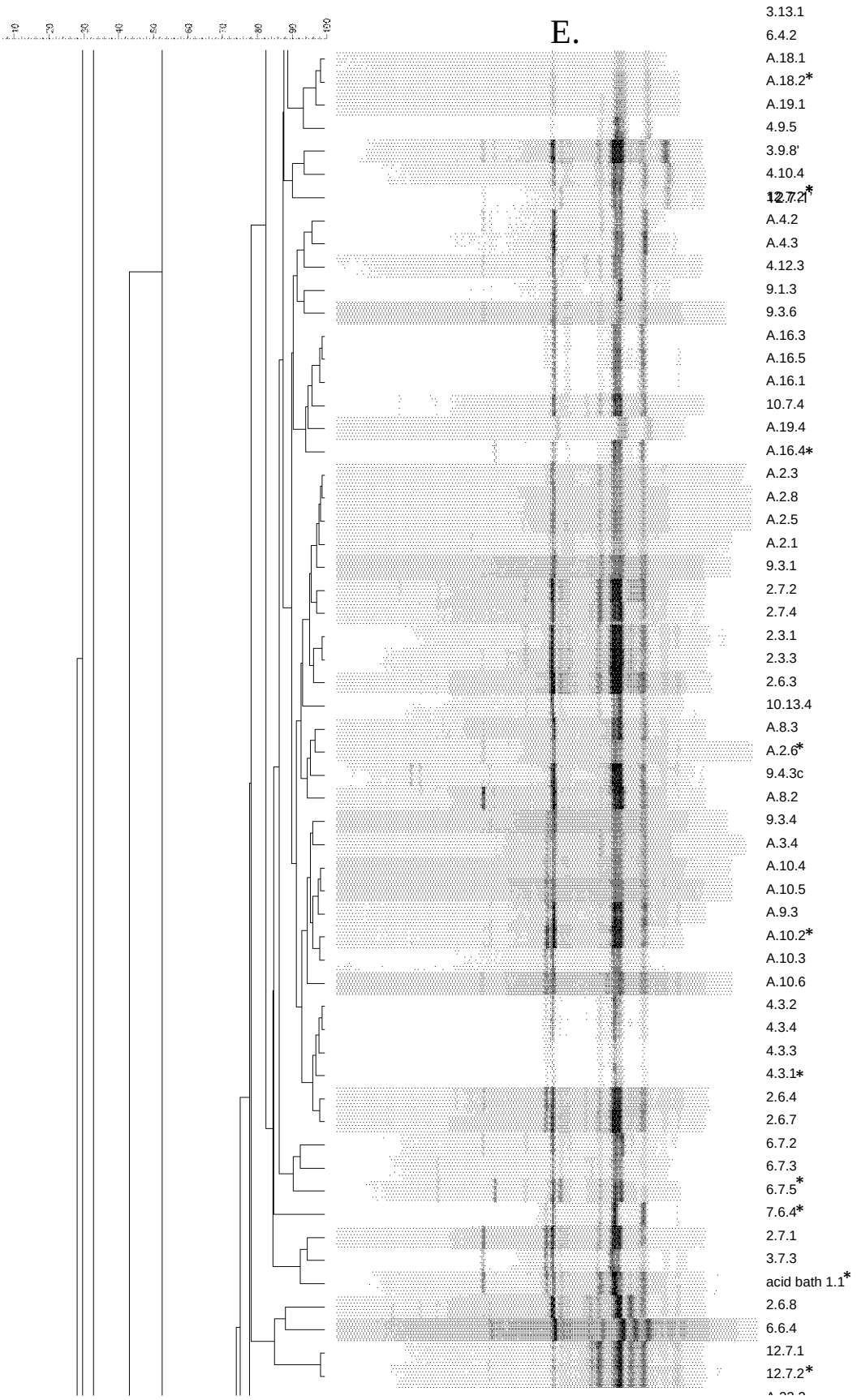
3.13.1*
6.4.2*
A.18.1


E.
3.13.1
6.4.2
A.18.1
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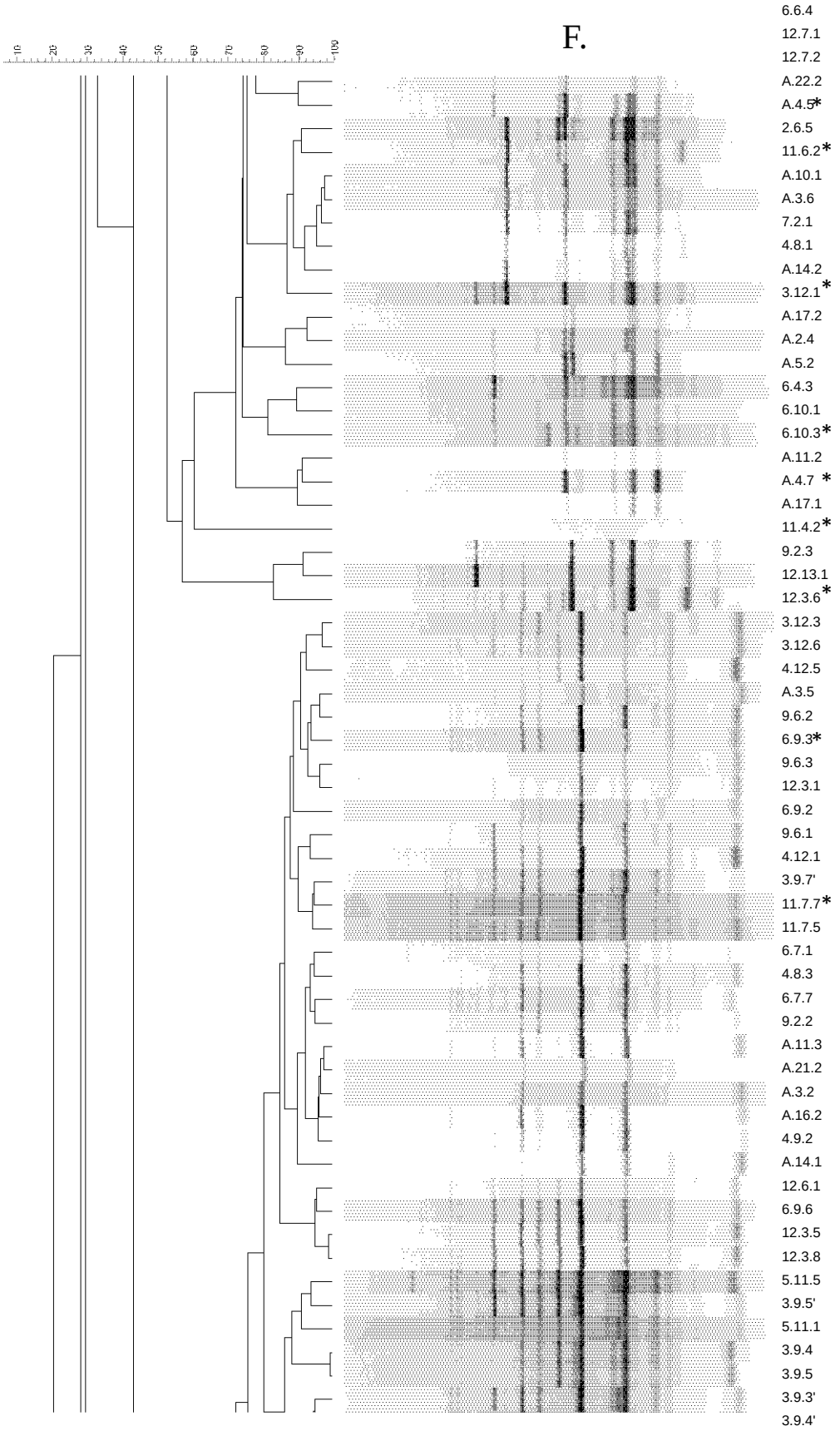
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A.3.6
7.2.1
4.8.1
A.14.2
3.12.1*
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A.2.4
A.5.2
6.4.3
6.10.1
6.10.3*
A.11.2
A.4.7 *
A.17.1
11.4.2*
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A.3.5
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6.9.3*
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9.6.1
4.12.1
3.9.7'
11.7.7*
11.7.5
6.7.1
4.8.3
6.7.7
9.2.2
A.11.3
A.21.2
A.3.2
A.16.2
4.9.2
A.14.1
12.6.1
6.9.6
12.3.5
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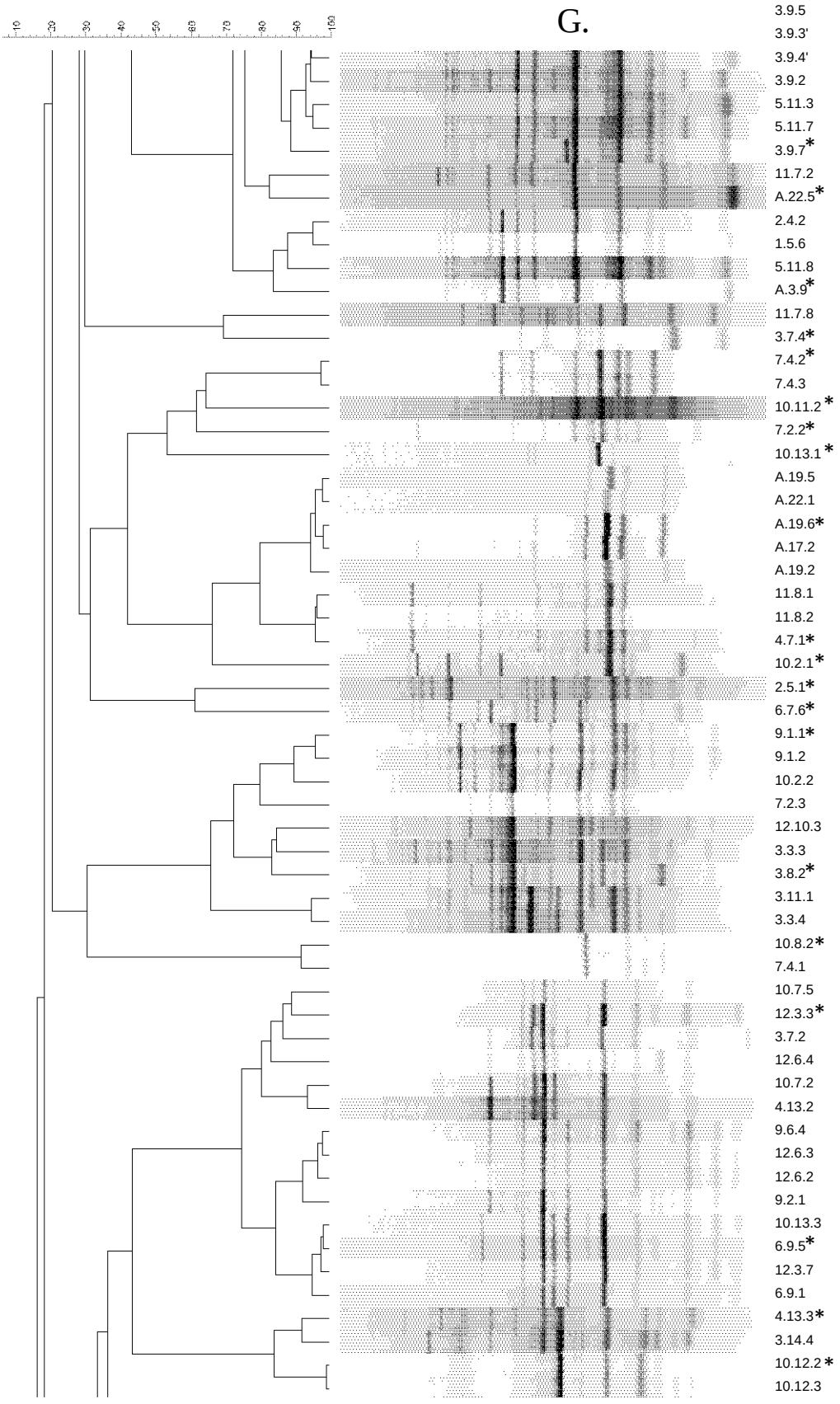
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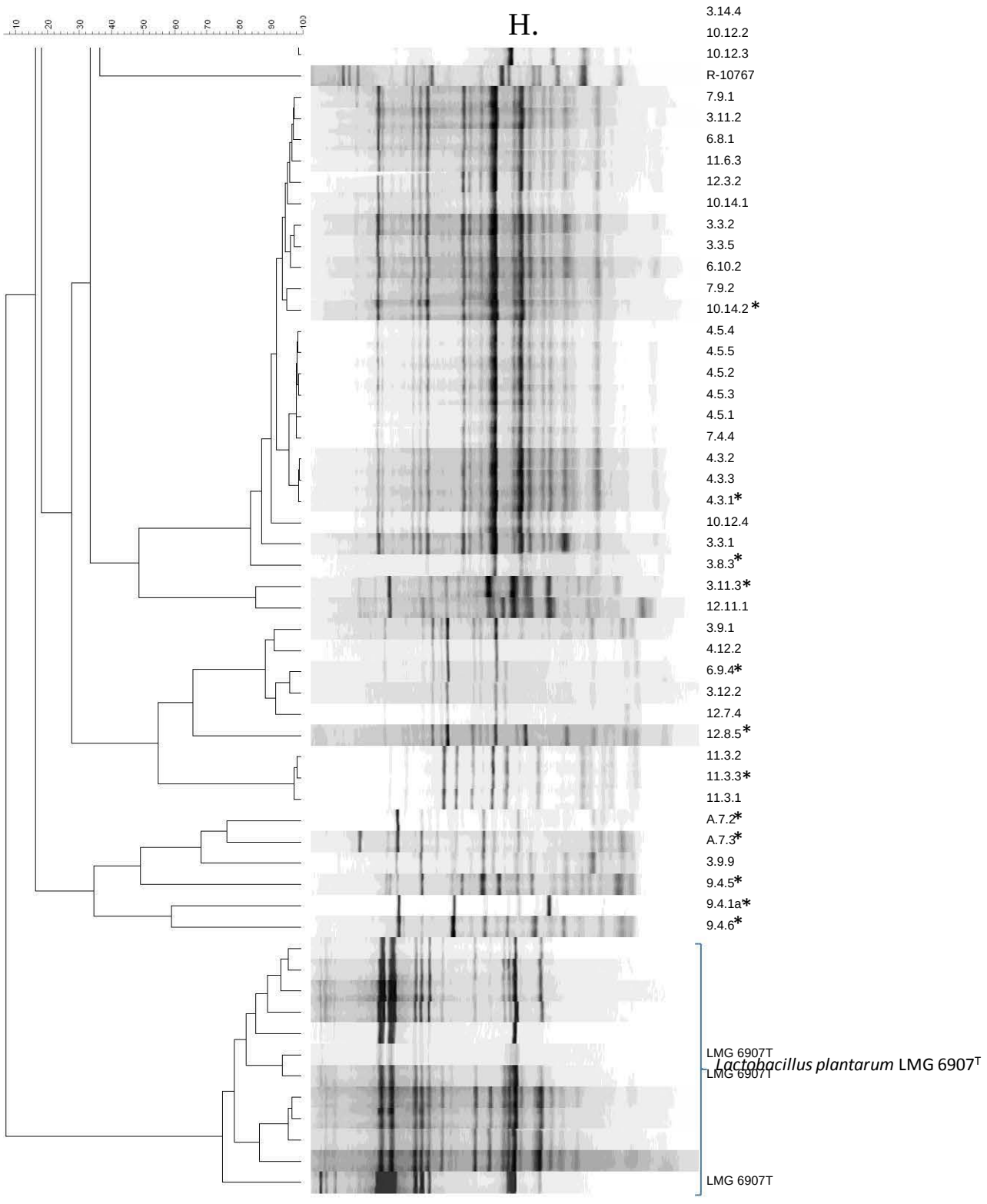
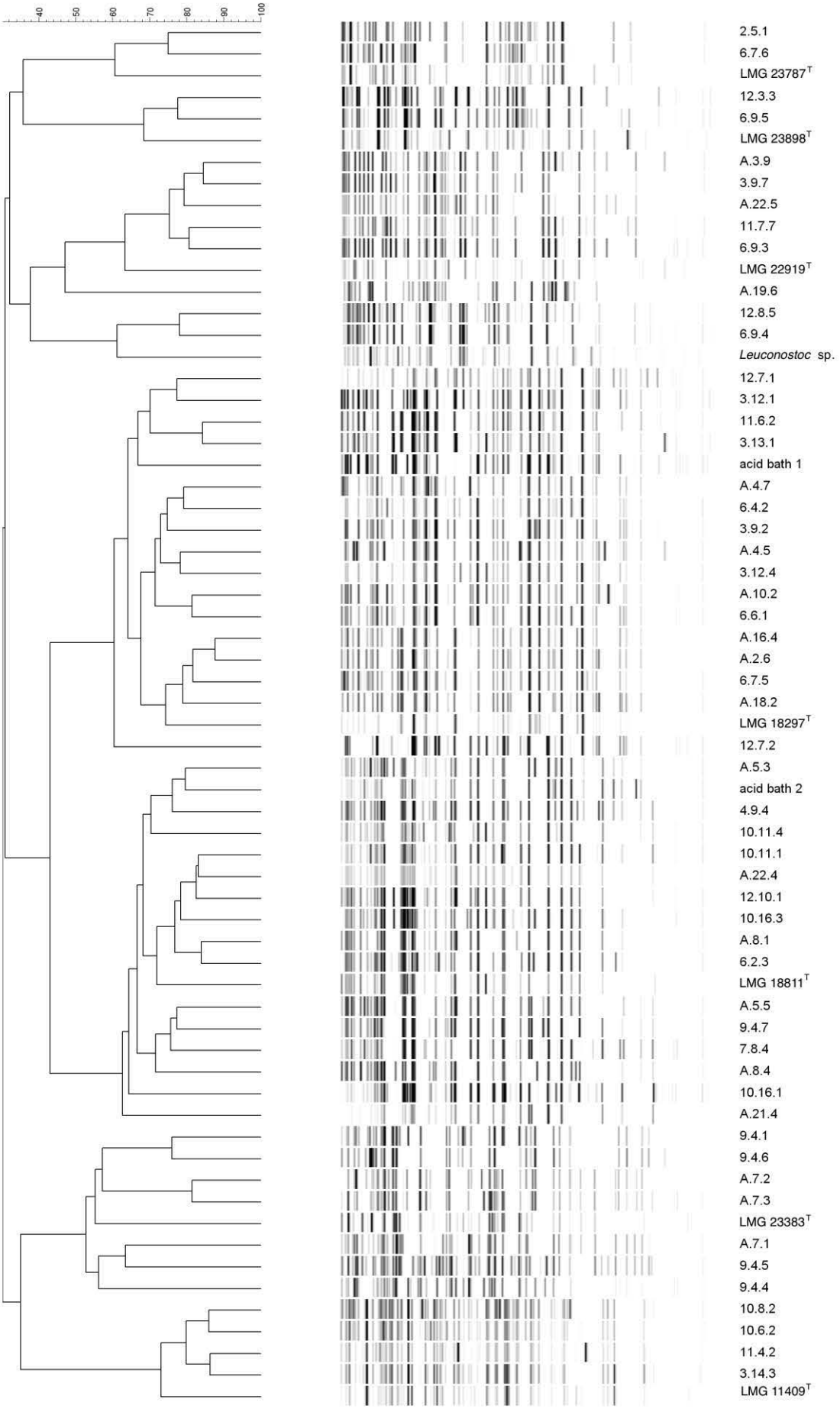


Figure 4.2 A, B, C, D, E, F, G & H: *Dendrogram generated with the digitized $(GTG)_5$-PCR fingerprints of all 442 isolates. Marked fingerprints with * were selected for AFLP analysis.*

Table 4.6: *Species designations of the recovered isolates from the processing plant, occurrence and source of isolation.*

Species	Number of isolates (% on total isolates)	Origin of isolation				
		Vegetable	Surface	Air	Water	End-products
Leuconostoc	**353 (79.9 %)**					
Le. gelidum* subsp. *gasicomitatum	153 (34.6 %)	2.4; 2.5; 2.6; 2.7; 2.8; 2.9; 3.9; 3.10; 4.9; 6.5; 6.6; 6.7; 7.6; 7.7; 7.8; 9.3; 9.4; 10.5; 10.11; 11.7; 12.8	2.1; 2.3; 4.10; 5.2; 6.2; 7.4; 9.1; 10.16; 11.10; 12.4; 12.5; 12.10; 12.11	1.5; 3.7; 3.12; 4.8; 4.12; 9.2; 9.6; 10.3; 10.7; 10.13; 12.13	acid bath 2; 12.7	A.2; A.3; A.4; A.5; A.6; A.8; A.9; A.11; A.12; A.13; A.21; A.22; A.23; A.24
Le. gelidum* subsp. *gelidum	129 (29.2 %)	2.5; 2.6; 2.7; 2.9; 3.9; 3.10; 4.9; 5.1; 6.6; 6.7; 7.6; 7.7; 7.8; 9.3; 9.4; 10.11; 12.8	2.3; 3.13; 4.3; 4.10; 6.4; 6.8; 6.10; 7.2; 9.1; 11.6	3.7; 3.12; 4.8; 4.12; 9.2; 10.7; 10.13; 12.3; 12.13	acid bath 1; 11.5; 12.7	A.2; A.3; A.4; A.5; A.6; A.8; A.9; A.10; A.11; A.12; A.14; A.16; A.17; A.18; A.19; A.22; A.23
Le. inhae	48 (10.9 %)	2.4; 3.9; 4.9; 5.1; 6.7; 11.7		1.5; 3.12; 4.8; 4.12; 9.2; 9.6; 12.3; 12.6		A.3; A.11; A.14; A.16; A.17; A.19; A.21; A.22; A.24
Le. carnosum	15 (3.4 %)		4.13	3.7; 6.9; 9.2; 9.6; 10.7; 10.13; 12.3; 12.6		
***Leuconostoc* sp.**	6 (1.4 %)	3.9; 11.4		3.12; 4.12; 6.9	12.7	
Le. kimchii	2 (0.5 %)	2.5; 6.7				
Lactobacillus	**11 (2.5 %)**					
Lb. oligofermentans	4 (0.9 %)		3.14; 4.13; 10.12			
Lb. sakei	3 (0.7 %)		4.7; 11.8			
Lb. curvatus	3 (0.7 %)		11.3			
Lb. coryniformis	1 (0.2 %)		3.8			
Other						
Pediococcus inopinatus	10 (2.2 %)		3.13; 3.14; 4.13; 7.4; 10.6; 10.8; 11.4			
Lactococcus piscium	8 (1.8 %)	9.4				A.7
Unidentified	60 (13.5 %)					
Total	**442**					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50
60
70
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2.5.1
6.7.6
LMG 23787T
12.3.3
6.9.5
LMG 23898T
A.3.9
3.9.7
A.22.5
11.7.7
6.9.3
LMG 22919T
A.19.6
12.8.5
6.9.4
Leuconostoc sp.
12.7.1
3.12.1
11.6.2
3.13.1
acid bath 1
A.4.7
6.4.2
3.9.2
A.4.5
3.12.4
A.10.2
6.6.1
A.16.4
A.2.6
6.7.5
A.18.2
LMG 18297T
12.7.2
A.5.3
acid bath 2
4.9.4
10.11.4
10.11.1
A.22.4
12.10.1
10.16.3
A.8.1
6.2.3
LMG 18811T
A.5.5
9.4.7
7.8.4
A.8.4
10.16.1
A.21.4
9.4.1
9.4.6
A.7.2
A.7.3
LMG 23383T
A.7.1
9.4.5
9.4.4
10.8.2
10.6.2
11.4.2
3.14.3
LMG 11409T

Figure 4.3: *AFLP dendrogram with the fingerprints of a selection of isolates subjected to AFLP analysis, that were allocated of the three major genera of the source tracking (*Leuconostoc, Pediococcus and Lactococcus*). The dendrogram was calculated with the Dice coefficient using UPGMA clustering method. The designation of each isolate (X.X._) corresponds to the sample from which it was recovered. The type strains (T) are also included in the dendrogram (*Le. kimchii *LMG 23787*^{T}, Le. carnosum *LMG* 239898^T, Le. inhae *LMG 22919*^{T}, Le. gelidum *subsp.* gelidum *LMG 18297*^{T}, Le. gelidum *subsp.* gasicomitatum *LMG 18811* ^{T}, Lc. piscium *LMG 23383*^{T}, Pediococcus inopinatus *LMG 11409*^{T}*).*

mesophilic ISO protocols. Only one end-product (i.e A.7) containing sliced cabbage was also dominated by *Lc. piscium*. Only members of *Le. gelidum* subsp. *gasicomitatum* were cultured from all samples surviving the stress of sanitation, namely the swabbed blades (i.e. 5.2, 6.2) and the microbial air load of Domain 10 (i.e. 10.3, 10.7) right after the decontamination and prior to production. Moreover, members of the same species were also found in the acetic acid bath (Table 4.6). Likewise, *Le. gelidum* subsp. *gelidum* was recovered from Domain 10 (i.e. 10.7) and the lactic acid bath.

4.4 DISCUSSION

This source tracking study clearly documents the prevalence of psychrotrophic LAB species mainly belonging to the genus *Leuconostoc* in the RTE vegetable salad manufacturing environment under study, and thus again underpins the capacity of these microbes to adapt in food production lines and premises (**Chapter 7**).

Our results also indicate that the psychrotrophic LAB contamination probably does not originate from the house-microbiota since the spoilage-associated LAB were scarcely recovered prior to production. In addition, this demonstrates that disinfection of the processing environment prior to production was conducted efficiently as part of the plant's hygienic surveillance plan. However, some harborage sites (i.e. rotating blades of dicers) were investigated where sanitation proved to be difficult to achieve. The LAB contamination problem in the company initially occurred only sporadically in salads containing sweet bell peppers or other carbohydrate-rich vegetables (**Chapter 2**), but in an one year period it developed in a systematic problem affecting every type of product.

Possibly, the psychrotrophic LAB were introduced in the production facility through the raw sweet bell peppers that were provided halved and unseeded by the supplier. The internal tissue of sweet bell peppers being spongy and permeable to water would not allow any mild decontamination handlings before entering the production lines, and the use of whole fruits would require a long procedure for removal of seeds. Quality deteriorations within the self-life were sporadic in the beginning, but became more common during the year that products were analyzed and concerned a broader range of vegetables.

Previously, in source tracking studies conducted in meat processing environments, correlations between certain taxa of the initial microbial load of unprocessed material and the dominant microbial populations of the final products at the end of shelf-life have been documented (De Filippis et al., 2013; Koo et al., 2013; Vihavainen et al., 2007). These findings emphasize on the probable cross-contamination between crude material and end-

products. Nowadays, the application of metagenomic approaches and high-throughput sequencing (HTS) techniques facilitate the in depth tracking of microbial species circulating in production environments (Ercolini, 2013), however the recovery of isolates enables the typing of the strains and possibly the further study of their psysiology (Nieminen et al., 2012).
The species *Le. gelidum* subsp. *gasicomitatum* and *Le. gelidum* subsp. *gelidum* are known spoilage organisms associated with meat processing environments (Lyhs et al., 2004; Shaw & Harding, 1989; Susiluoto et al., 2003; Vihavainen & Björkroth, 2007), but little is known about their presence as spoilers in vegetable-based products (Vihavaine et al., 2008). *Leuconostoc* spp. are mostly isolated from fermented vegetables in the case of many typical, Asian delicacies (Kim, 2003; Kim et al., 2000a; Kim et al., 2000b), where their occurrence is favorable for their contribution to organoleptic properties. In the present study, contamination with psychrotrophic *Leuconostoc* spp. concerned all salad types in a very short time period and lies at the basis of the fluctuating quality profile of the products.
The success of some *Leuconostoc* spp. as spoilers in ready-to-eat vegetable salads could be due to their broad range of ecological niches, varying between carbohydrate-rich substrates like sweet bell peppers and leafy green vegetables that only have low amounts of sugars. Moreover, strains of *Le. gelidum* subsp. *gasicomitatum* can grow equally well under anaerobic conditions and in the presence of oxygen in the case of MA packaged products (Jääskeläinen et al., 2012).
Additionally, *Le. gelidum* subsp. *gasicomitatum* and *Le. gelidum* subsp. *gelidum* grow very fast at low-temperature storage even from very low populations and cannot be cultured when mesophilic incubation is implemented hence grow at a lower temperature range unlike most leuconostocs (Björkroth & Holzapfel, 2006). As the analysis of the genome sequence of *Le. gelidum* subsp. *gasicomitatum* LMG 18811^{T} is available, it would be interesting to evaluate their adaptation in harsh conditions and possibly explain their ability to adhere on surfaces and sustain acid stress, through propagation of physiological mechanisms (Johansson et al., 2011). The ability of certain strains to adhere on surfaces will be assessed in **Chapter 7**.
Concerning the persistence of the problem, although only one sampling was performed at the processing plant, several spoilage cases before the end of the shelf-life were reported for the products as extreme acidification was usually evaluated. Significant improvement of the sensorial properties of the salads was only observed when whole sweet bell peppers were used in-house and handled manually suggesting that indeed the unseeded and halved sweet bell peppers were responsible for the high initial contaminations of spoilage-related LAB.

Spoilage potential of psychrotrophic lactic acid bacteria (LAB) species: *Le. gelidum* subsp. *gasicomitatum* and *Lactococcus piscium*, on sweet bell pepper (SBP) simulation medium under different gas compositions

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SUMMARY

Sweet bell peppers are a significant constituent of retail, chilled-stored and packaged food products like fresh salads, marinades and ready-to-eat (RTE) meals. Previously, through general screening of the Belgian market (**Chapter 2 & 3**) and by means of source tracking analysis (**Chapter 4**) in a plant manufacturing minimally processed, vegetable salads the susceptibility of fresh-cut sweet bell peppers to lactic acid bacterium (LAB) contamination was substantiated. The determination of the metabolic profiles of *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium*, two major psychrotrophic, spoilage-related LAB species, on sweet bell pepper (SBP) simulation medium under different packaging conditions – 1.) vacuum: 100% N_2, 2.) air: 21% O_2, 79% N_2, 3.) MAP_1: 30% CO_2, 70% N_2 and 4.) MAP_2: 50% O_2, 50% CO_2 – facilitated a better understanding of the spoilage potential of these microbes as well as the presumptive contribution of O_2 in the spectrum of produced volatile organic compounds (VOCs) associated with poor organoleptic properties of food products. Generally, none of the applied gas compositions inhibited the growth of the 4 *Leuconostoc gelidum* subsp. *gasicomitatum* isolates, however presence of O_2 resulted in buttery off-odors by inducing primarily accumulation of diacetyl and pungent "vinegar" smell due to acetic acid. The 3 tested isolates of *Lactococcus piscium* varied greatly among their growth dynamics and inhibition at MAP_2. They exhibited either weak spoilage profile or very offensive metabolism confirming significant intraspecies diversity.

5.1 INTRODUCTION

Species *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium* have been involved in numerous quality deteriorations associated with products of meat, fishery and to a lesser extent vegetable origin in Northern Europe (Lyhs et al., 2004; Rahkila et al., 2012; Säde, 2011; Vihavainen et al., 2008). However, in Belgium they have been recently investigated as persistent psychrotrophic microbes with high prevalence in chilled-stored foodstuffs, notably vegetables. Psychrotrophic members of these species were isolated as predominant, presumptive specific spoilage organisms (SSO) from a large selection of retail products at the end of shelf-life (**Chapter 3 & 4**). Fresh salads and ready-to-eat (RTE) meals, both containing fresh-cut sweet bell peppers, had the highest frequencies of isolation concerning these two taxa. Additionally, in the frame of a source tracking analysis in a company manufacturing fresh, RTE salads, raw sweet bell peppers provided by the supplier were the carrier of psychrotrophic leuconostocs in the plant.

Psychrotrophic LAB have been adapted to industrial environments and can easily be introduced in food products during their manufacturing process (Björkroth, 2005). Their dominance at the end of shelf-life suggests fast growth under the applied storage conditions starting even from low initial populations (Säde, 2011). This requires the evaluation of their spoilage potential as they reach high levels outcompeting the cold-adapted mesophilic microbes and constitute a threat for food quality aspects. Moreover, the interesting correlation between the occurrence of these psychrotrophic LAB species and sweet bell peppers which are widely used in fresh salads, marinades and ready-to-eat (RTE) meals demands the application of an efficient preservation method for this type of susceptible products.

Sweet bell pepper (cultivar *Capsicum annuum*) is one of the five crops with the greatest economical importance in most European and North American countries (Van Poucke et al., 2012). Novel packaging technologies for fresh produce implementing combination of super-atmospheric O_2 and high concentrations of CO_2 for modified atmosphere (MA) packaging were proven promising concerning the inhibition of Gram negative bacteria (*Pseudomonas* spp., *Enterobacteriaceae*) and yeasts, also in the case of freshly-cut peppers (Conesa et al., 2007).

The present study attempted to determine the spoilage potential of *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium* under four packaging conditions by means of single strain inoculation of a selection of isolates belonging to these two LAB species, on sweet bell pepper simulation medium. Additionally, the role of O_2 and CO_2 on growth dynamics as well as the spectrum of spoilage-related volatile organic compounds (VOCs) was evaluated along with the possibility of implementing high O_2 and CO_2 concentrations in order to eliminate these microbes.

5.2 MATERIALS AND METHODS

5.2.1 Cultivation of *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium* strains

Four selected *Le. gelidum* subsp. *gasicomitatum* and three *Lc. piscium* strains from the laboratory collection were resuscitated for the needs of the inoculation experiments (Table 5.1). All strains were previously isolated as dominant microbes from retail food products when implementing a psychrotrophic enumeration technique based on incubation of plates at 22 ^{o}C (**Chapter 2**). Identification of the strains was performed by means of amplified fragment length polymorphism (AFLP) typing and sequencing of *pheS* house-keeping gene (**Chapter 3 & 6**). Apart from the various origins of isolation, they differ in their genotypic fingerprints and possess characteristic phenotypes representing the intraspecies diversity.
For the resuscitation of the *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium* isolates one glass bead from -80 ^{o}C was transferred in de Man-Rogosa-Sharpe (MRS, Oxoid, Hampshire, UK) broth medium and Tryptone Soya Broth (TSB, Oxoid, Hampshire, UK) respectively and incubated at 22 ^{o}C overnight. After 24 hours 100 μL were transferred to a new broth tube and kept for another 24 hours at 22 ^{o}C.

Table 5.1: *Isolates belonging to species* Leuconostoc gelidum *subsp.* gasicomitatum *and* Lactococcus piscium *tested in the present study. The origin of isolation and the differentiating phenotypic characteristics of each strain are presented (−: no growth or no slime formation, +: weak growth, ++++: extreme formation of slime). Also see Table 3.1 for explanation of the phenotypic characteristics.*

Species	Isolate	Origin	Packaging	Growth at 30 °C	Slime formation
Leuconostoc gelidum subsp. *gasicomitatum*	R-46608	blood sausages	MAP	+	−
	R-46620	beef	vacuum	−	−
	R-46920	sweet bell pepper salad	air	−	−
	HS27	boiled eggs in brine	vacuum	+	++++
Lactococcus piscium	R-46592	beef	vacuum	−	−
	R-46738	pork	vacuum	−	−
	R-46976	sweet bell pepper salad	air	−	−

5.2.2 Preparation of Sweet Bell Pepper (SBP) simulation medium

Fresh sweet bell peppers (cultivar *Capsicum annuum*) were purchased from the market and stored in the laboratory at 4 ^{o}C. Equal amounts of green, red and yellow peppers were coarsely chopped under aseptic conditions and packaged in sterile bags in order to avoid further contamination. Peppers of each color were handled separately. The chopped peppers were subjected to a freezing-thawing cycle (24-48 h) to facilitate softening of the vegetable tissue and maximum juice extraction. Subsequently the peppers were juiced with the aid of a juice extractor and a cloth. Finally the extract was passed through a fine kitchen sieve to remove vegetable particles and seeds. Equal ratios of juice from the peppers of each color were homogeneously mixed. A large volume of mixed juice was prepared and frozen in order to be used for the needs of all the inoculation experiments and avoid differences in the composition of the medium due to quality fluctuations of the raw material. For each inoculation experiment approximately 3 L of SBP medium was required. Three hundred mL of the mixture were poured in sterile bottles (1 L) with the addition of 1.5 % w/v of bacteriological agar (Oxoid, Hampshire, UK). The mixture was brought to a boil over the

flame and sterilized for 2 min. Twenty g ± 0.2 were poured in petri plates and then kept at 4 ^{o}C until the moment of inoculation.

5.2.3 Inoculation of SBP simulation agar medium

The inoculum for each strain was prepared after two consecutive subcultures for 24 hours at 22 ^{o}C. The second culture was kept at 7 ^{o}C for 1 h prior to inoculation in order to stimulate the adaptation to refrigeration temperature. Subsequently, a series of decimal dilutions for the second culture was prepared in peptone physiological solution (PPS: 0.85 % w/v NaCl and 0.1 % w/v bacteriological peptone). The appropriate dilution was used for the spread inoculation of the SBP agar plates (100 μL) obtaining a level of approximately 10^3-10^4 viable cells per gram of simulation agar. The dilution series of the inoculum during every biological repetition of the experiment was plated out in order to evaluate the initial population (Day 0).

5.2.4 Packaging of SBP simulation agar plates

The inoculated SBP agar plates were packaged in plastic trays (volume = 269 ml, oxygen transmission rate (OTR) = 0.5 – 13 cm^3/m^2.d, 1 bar of O_2 at 23 ^{o}C, 0 % relative humidity (RH), PP/EVOH/PP, DecaPac NV, Herentals, Belgium) at four different conditions: 1.) vacuum: 100% N_2, 2.) air: 21% O_2, 79% N_2, 3.) MAP_1: 30% CO_2, 70% N_2 and 4.) MAP_2: 50% O_2, 50% CO_2. The trays were packaged using a Tray sealer MECA900 (DecaTechnic, Herentals, Belgium). Multilayer packaging material (PA/PE/EVOH/PE) was used as packaging top foil having low gas permeability. The barrier film had thickness of 90 micron and allowed an oxygen transmission rate of 2.0 cm^3/m^2.d, 1 bar of O_2 at 23 ^{o}C, 85 % RH. After packaging all plates were stored at 7 ^{o}C for 20 days.

5.2.5 Enumeration of inoculated bacterial strains

For each inoculation experiment the counts were determined on Days 1, 2, 4, 5, 7, 9, 13 and 20. Two SBP plates from each packaging condition were sampled by aseptically transferring 10 g of agar in a sterile stomacher bag and adding 90 g of PPS. Subsequently they were homogenized for 1 min using a Stomacher 400 Lab Blender (LED Techno, Heusden-Zolder, Belgium). Two independent decimal dilution series were prepared in PPS and aliquots were spread-plated in duplicate on MRS or Plate Count Agar (PCA, Oxoid, Hampshire, UK) in the case of *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium*, respectively. The plates were incubated at 22 ^{o}C for 5 days. Each isolate/strain represents a biological repetition of the experiment for the corresponding species. In the case of each single strain inoculation the microbiological analysis performed generated two technical replicates. Thus each time point in Figure 1 corresponds to the mean value of 8 and 6 independent measurements for *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium*, respectively.

5.2.6 Determination of growth parameters

The growth parameters of maximum specific growth rate (μ_{max}), lag phase and maximum bacterial level (N_{max}) were estimated for both *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium* by fitting the MRS and PCA colony counts respectively, in the model describing microbial growth developed by Baranyi & Roberts, (1994) using SPSS Statistics 21 software (SPSS Inc., Chicago, IL, USA).

5.2.7 Analysis of headspace gas composition and pH measurement

The analysis of the gas composition in the headspace of the packaged SBP plates concerning O_2 and CO_2 levels was performed with a Checkmate 9900 O_2/CO_2 (PBI Dansensor A/S, Ringsted, Denmark) in triplicate for each time point and each condition. The pH was measured in duplicate on a 20 g sample from 3 plates by means of a pH-electrode (InLab427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected with a pH meter (SevenEasy, metler Toledo GmbH). The initial pH of the SBP medium was 5.4 ± 0.2.

5.2.8 Qualitative determination of the headspace by means of Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) analysis

For each strain inoculation, the volatile compounds were qualitatively determined on Days 0, 2, 5, 7, 9 and 13. For each packaging condition 4 g of simulation agar were placed in a 20 mL glass vial, 3 g of NaCl were added and then mashed with a sterile glass stick. The vials were closed with a PTFE-faced silicone septum screw cap (Agilent Technologies, Diegem, Belgium) and stored at -20 ^{o}C until they were analyzed.
Chromatographic analysis was performed in an Agilent 7890A GC equipped with a CTC PAL autosampler and a 5975C Mass Spectrometer. Extraction of the analytes was carried out from the head space of the vial at 50 ^{o}C for 10 min with a carboxen-polydimethylsiloxane (CAR-PDMS) SPME fiber 75 µm, 23Ga (Supelco, Bellefonte, USA). During extraction the vial was agitated at 500 rpm. Prior to use, the fiber was conditioned at 270 ^{o}C for 1 h as recommended by the manufacturer. After each desorption, the fiber was post-conditioned for 20 min, at 250 ^{o}C to avoid carry-over. The analytes were desorbed from the fiber in a PTV inlet at 250 ^{o}C in splitless mode for 1 min and chromatographic separation was carried out in a DB 624 60 m x 0.25 mm ID, with 1.4 µm film thickness.
The temperature program was set as follows: 5 min at 45 ^{o}C, ramp 7 ^{o}C /min to 220 ^{o}C, 10 min at 220 ^{o}C and 10 min post run at 230 ^{o}C. Carrier gas was He at a flow rate of 1 mL/min. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating mode scan from m/z 36–500; scan rate 3.64 cycles/second. The data were processed by the MSD Chemstation software package (D.01.02.16, Agilent Technologies, Santa Clara, CA, USA) and identification of the observed peaks was carried out on the basis of spectra comparison with the NIST 05 Library.

5.2.9 Quantitative determination of the headspace by means of Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS) analysis

The quantification of the VOCs was performed by means of SIFT-MS analysis. Apart from the great convenience and rapid analysis, SIFT-MS facilitated the assessment of the actual headspace without opening the packaging and consequently altering the concentrations of volatiles or losing the minor metabolites that is possible during sample preparation with other methods (Holm et al., 2013). In this sense the analysis evaluated the headspace simulating the consumer's impression upon opening a packaged salad.

On Days 0, 2, 5, 7, 9, 13 and 20 three plates of SBP medium from each gas composition, sealed in the original trays were stored at -20 ^{o}C until the end of the storage period. When all samples were collected they were put at 4 ^{o}C overnight to melt prior to measuring the concentration of VOCs with the Selected Ion Flow Tube Mass Spectrometer (Voice 200, Syft Technologies). An open system was created by applying septa on the two opposite corners of the tray: one pierced by the inlet of the apparatus and the other by a syringe needle in order to avoid hypo-pressure in the tray while measuring. The headspace was sampled over a time period of 120 s at a flow rate of 77.3 Pa.L/s. Volatile organic compounds (VOCs) were introduced through the heated inlet into the flow tube, where reactions with precursor ions H_3O^+,NO^+,O_2^+ resulted in ionized masses. The produced masses were monitored by a mass spectrometer, located at the downstream end of the flow tube.

Table 5.2: *Volatile organic compounds (VOCs) measured in the headspace of the trays by means of SIFT-MS analysis.*

Volatile organic compounds (VOCs)		Precursor	*K*	Branching ration (%)	(m/z)	Characteristic product ion
Alcohols						
	methanol	H_3O^+	2,70E-09	100	33	CH_5O^+
		NO^+	1,00E-11	100	62	$NO^+.CH_3OH^+$
	ethanol	NO^+	1,20E-09	100	45	$C_2H_5O^+$
	1,3-propanediol	H_3O^+	3,50E-09	70	59	$C_3H_7O^+$
		NO^+	1,60E-09	100	75	$C_3H_7O_2^+$
	3-methyl-1-butanol	H_3O^+	2,80E-09	100	71	$C_5H_{11}^+$
	2,3-butanediol	NO^+	2,3E-9	100	89	$C_4H_9O_2^+$
Aldehydes						
	formaldehyde	H_3O^+	3,40E-09	100	31	CH_3O^+
	acetaldehyde	O_2^+	2,3E-9	55	44	$C_2H_4O^+$
Ketones						
	2,3-butanedione (diacetyl)	H_3O^+	1,70E-09	100	87	$C_4H_7O_2^+$
		NO^+	1,3E-9	65	86	$C_4H_6O_2^+$
		O_2^+	1,4E-9	20	86	$C_4H_6O_2^+$
	3-hydroxybutanone (acetoin)	NO^+	2,5E-9	100	118	$C_4H_8O_2.NO^+$
		O_2^+	2,5E-9	20	88	$C_4H_8O_2^+$
Organic acids						
	acetic acid	NO^+	9,0E -10	100	90	$NO^+.CH_3COOH$
	3-methyl-butanoic acid	H_3O^+	3,00E-09	95	103	$C_5H_{11}O_2^+$
		NO^+	2,5E-9	70	132	$C_5H_{10}O_2.NO^+$

A selection of volatile compounds were targeted through multiple ion monitoring mode (MIM), while the quantification of the VOCs was carried out using the reaction rate coefficients (*K*) and the branching ratios of the reaction between selected compounds and precursor ions that generated specific ionized masses. The compounds that were monitored were selected based on the SPME-GC-MS analysis. Analysis of the air in the room where the measurements of the sealed trays in an open system were performed, confirmed absence of the selected VOCs from the environment in all cases.

5.3 RESULTS

5.3.1 Growth characteristics of *Le. gelidum* subsp. *gasicomitatum* isolates

The four *Le. gelidum* subsp. *gasicomitatum* isolates demonstrated very similar growth dynamics with slight differences (Figure 5.1 I). The initial population with which the SBP simulation agar plates were inoculated ranged between 3.1 and 4.1 logs CFU/g. All isolates entered the exponential phase immediately and no adaptation/lag phase was observed. They reached 8 logs CFU/g already from Day 4 or 5 showing fast growth (μ_{max}=1.026-1.868 log CFU/g/day). Under vacuum, in air and MAP_1 the isolates remained in the static phase until Day 20. Only in the case of MAP_2 isolates R-46608 (blood sausages) and R-46620 (beef) after Day 13 showed a 3 logs reduction in counts. Noteworthy, none of the strains tested showed inhibition under super-atmospheric O_2 concentration.

The highest populations (N_{max}) reached for the 4 isolates ranged between 8.6 and 9.6 logs CFU/g. Isolate HS27 (boiled eggs in brine) was the strain that had the highest growth and from Day 7 sticky, ropy slime was formed on the surface of the plates. Out of all the isolates, *Le. gelidum* subsp. *gasicomitatum* HS27 was the only strain that demonstrated capacity for extreme slime formation in laboratory media (Table 5.1).

The initial pH of SBP medium was 5.4 and from Day 5 a sharp decrease was observed (Figure 5.1 I). For the plates stored under vacuum and in MAP_1 in which oxygen was absent, the acidification proceeded faster to a final value between 3.6 and 3.8 on Day 20. On the other hand, in the case of air packaging and MAP_2 the final pH was 3.7-4.4 on Day 20. Concerning the gas composition in the headspace, CO_2 and O_2 started being produced and consumed respectively, on Day 5. Under vacuum an average of 11.5 % CO_2 was measured at the end of storage. In air packaging, all O_2 was exhausted on Day 9 or 13 and a final concentration of 27.5 % CO_2 was produced. In the case of MAP_1 8.5 % CO_2 was produced and in MAP_2 a utilization of 26.3 % O_2 resulted in the formation of an additional 22.5 % CO_2, approximately. The 4 isolates had very low intraspecies diversity in their growth profiles and behaved similarly under the four packaging conditions.

Normally for ready-to-eat (RTE) salads containing peppers a shelf-life of 7-8 days is suggested by the manufacturers. In the case of the inoculation experiments performed the average contamination levels on Day 7, extended between 8.7 and 9.4 logs CFU/g, the pH was 4.3-4.6 and slight blowing of the packages was observed under all four packaging conditions.

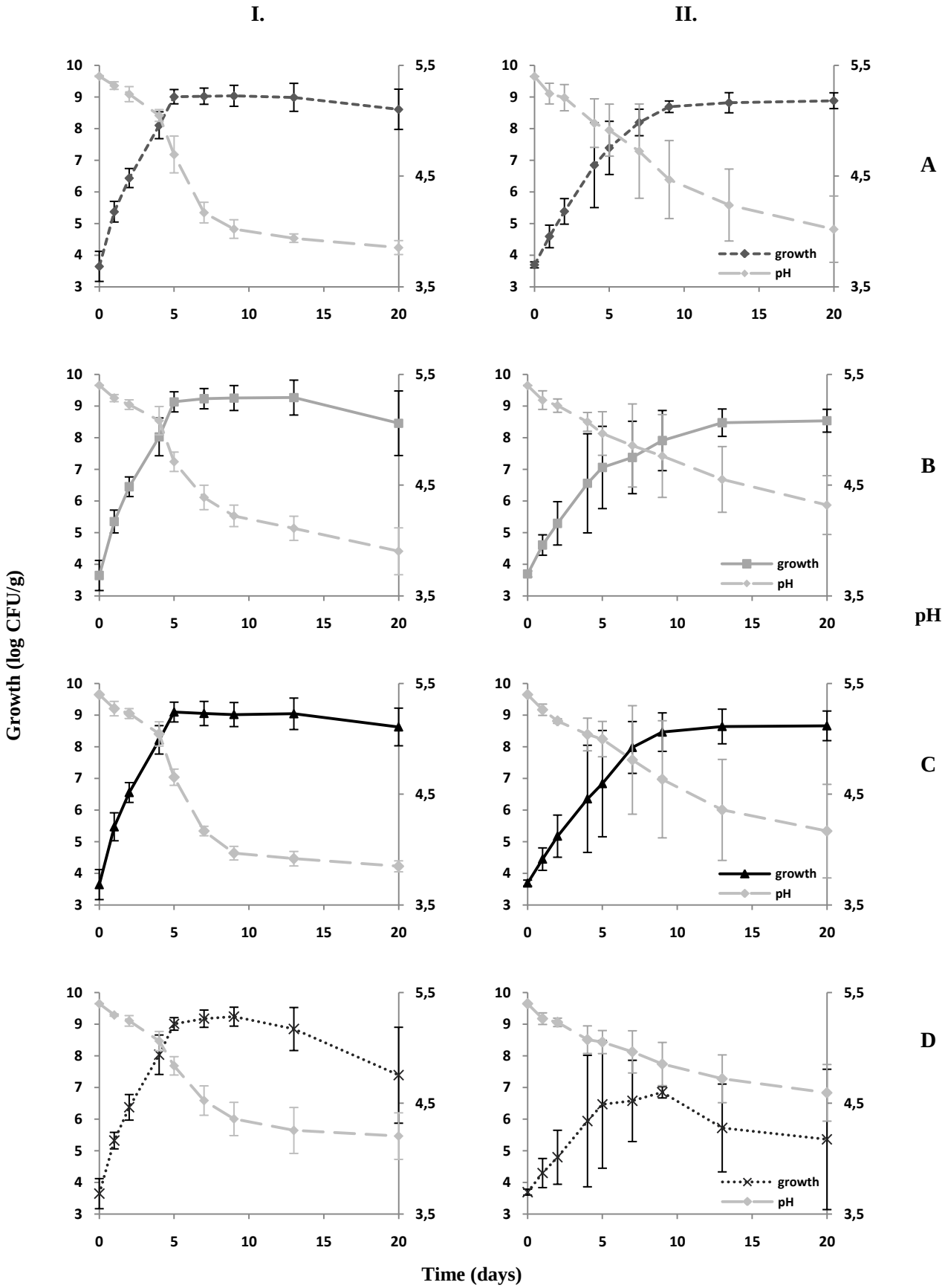
I.
II.
A
B
C
D
Growth (log CFU/g)
pH
Time (days)
growth
pH

Figure 5.1: *Growth curves and evolution of acidification for species* Leuconostoc gelidum *subsp.* gasicomitatum *(I.) and* Lactococcus piscium *(II.), under four packaging conditions: A.) vacuum: 100% N_2, B.) air: 21% O_2, 79% N_2, C.) MAP_1: 30% CO_2, 70% N_2 and D.) MAP_2: 50% O_2, 50% CO_2. Each time point corresponds to the mean of 4 (n=8) and 3 (n=6) independent biological repetitions measured in duplicate, respectively.*

5.3.2 Growth characteristics of *Lc. piscium* isolates

In the case of species *Lc. piscium* the findings were quite heterogeneous. The three tested isolates had great intraspecies diversity concerning their spoilage profiles and behaved differently (Figure 5.1 II). The initial population with which the SBP simulation agar plates were inoculated ranged from 3.6 to 3.8 logs CFU/g.

Isolate R-46592 (beef) was the strain with the most potent spoilage-related character as it reached a population density of 8.4 logs on Day 4, without having any lag phase under all packaging conditions. In the case of vacuum, air and MAP_1 the isolate remained in the static phase until the end of the storage period. On the contrary, in MAP_2 once reaching the highest population of 8.6 logs CFU/g on Day 5, it immediately entered the death phase. Under vacuum and in air a final concentration of 9 and 25 % CO_2 was produced respectively, although the O_2 in air packaging was not fully exhausted at the end of the storage. In MAP_1 and MAP_2 an additional 5 % of CO_2 was generated.

Isolate R-46738 (pork) was the slowest of the three strains and had a weak growth. It demonstrated an actual lag phase of 4.7 days in MAP_2 but not in the other packaging conditions. It also had a low growth rate (μ_{max}=0.346–0.709 log CFU/g/day) reaching 8 logs CFU/g on Day 13 under vacuum, in air and MAP_1. In MAP_2 it did not exceed this threshold during the 20 days of storage. Under vacuum and in air a final concentration of 8 and 9 % CO_2 was produced respectively, and the O_2 in air packaging was reduced by 4 %. In MAP_1 an amount of 4 % of CO_2 was additionally produced, while in MAP_2 practically no change in the headspace composition was observed.

Isolate R-46976 (sweet bell peppers) had an average growth and reached 8 logs on Day 7 or 9 under vacuum, in air and MAP_1, albeit it was indigenous to sweet bell peppers and was expected to be well adapted in this environment. In MAP_2 after 3.9 days of lag phase, it reached 6.8 logs and subsequently entered the death phase. Under vacuum and in air a final concentration of 7 and 2 % CO_2 was produced respectively and the O_2 in air packaging decreased by 4 %. In MAP_1 an amount of 2 % of CO_2 was produced on top of the existing, while in MAP_2 the gas concentrations in the headspace remained stable throughout the 20 days.

The acidification varied as well among the biological replicates but showed similar trends being more acute under anaerobic conditions (vacuum, MAP_1) reaching final pH values between 3.7 and 4.3 and milder in air and MAP_2 ranging at pH values 4.2-5 at the end of the storage (Figure 5.1 II).

The growth patterns of the 3 isolates were not comparable. However, they were all sensitive to a certain extent to MAP_2. Apparently, for *Lc. piscium* the combination of high O_2 and CO_2 concentrations had significant inhibitory effect (Figure 5.1 II.D).

5.3.3 Volatile organic compounds (VOCs)

The range of volatile organic compounds (VOCs) produced by the two species is quite limited. SBP medium has high sugar content that provides glucose, fructose and sucrose (USDA, 2012) as substrates for these fermenting microbes. Based on the results from the qualitative analysis of the VOCs assessed by means of SPME-GC-MS the peaks of acetic acid and ethanol were the most abundant, whereas peaks for 2,3-butanedione (diacetyl), 3-hydroxybutanone (acetoin), 2,3-butanediol and acetaldehyde were also present.

Monitoring VOCs by SIFT-MS without opening of the original trays resulted in a very realistic evaluation of the evolution of metabolites. For species *Le. gelidum* subsp. *gasicomitatum* there is a very clear metabolic pattern concerning the compounds produced under each of the four packaging conditions, that is confirmed by all the strains tested in the present study. The differentiating factor that modifies the catabolic trends and favors the production of certain metabolites as end-products is O_2. All isolates produced very high amounts of acetic acid (> 10000 μg/m^3 in the headspace, already from Day 7), which was the primal VOC detected and it can be concluded that when O_2 was available in the headspace the concentration was greater (Figure 5.3 A). Only isolate R-46620 (beef) could produce ethanol as well (> 25000 μg/m^3 on Day 5 as shown on Figure 5.3 E), especially under anaerobic conditions (vacuum, MAP_1) while the other *Le. gelidum* subsp. *gasicomitatum* strains did not. Clearly, diacetyl, 2,3-butanediol and acetaldehyde (Figure 5.3 B, C, D) were only or mainly produced when O_2 was present in the packaging environment (air, MAP_2). Diacetyl production in MAP_2 was twofold or greater compared to air (Figure 5.3 B). Production of diacetyl and acetoin are both related to buttery off-odor which is a distinct and desirable aroma in dairy fermentations enhanced by aeration and presence of citrate (Bassit et al., 1993; Christensen & Pederson, 1958).

For species *Lc. piscium* the profile of produced VOCs was strain-dependent. Isolate R-46592 (beef), which grew very competently in all conditions, was the strain that produced the greatest concentrations of VOCs. As shown in Figure 5.4 A and C, ethanol and acetic acid were the primal metabolites. Ethanol was generated in larger amounts under anaerobic storage (> 30000 μg/m^3 on Day 7) while acetic acid was more abundant in air packaging (> 10000 μg/m^3 on Day 7). In MAP_2 the concentrations of acetic acid and ethanol peaked on Day 5 and 7 but did not increase further as a gradual decline in counts was observed. Noteworthy, diacetyl concentration kept rising till Day 13, reaching a significant 25000 μg/m^3 approximately (Figure 5.4 B).

In Figure 5.2 the capacity of VOC production for each strain is presented in a heatmap. This plot generated in relation to the highest VOC concentrations measured with SIFT-MS, after Day 9 of storage, aims at the comparison among the spoilage potential of the different isolates.

The midpoint (threshold) values were selected for each compound after carefully comparing all data. Acetic acid was produced by all isolates that exhibited a spoilage character and 10000 μg/m^3 was considered a "baseline". For ethanol a background concentration (15000-20000 μg/m^3) was always generated in the headspace immediately after packaging most probably attributed to the natural aroma of pureed sweet bell peppers as previously documented (Wampler & Barringer, 2012). For diacetyl, 2,3-butanediol and acetaldehyde the

thresholds were set at very low values: 1500 μg/m^3, 2000 μg/m^3, 400 μg/m^3, respectively in order to point out their high affinity to aerobic storage. Lastly, for acetoin although no trend was evaluated a low value was also chosen at 4000 μg/m^3.
Isolate R-46738 (pork) did not produce any VOCs, while R-46976 (sweet bell peppers) gave rise to ethanol that had a final concentration of 25000 μg/m^3 on Day 20, in MAP$_1$ (Figure 5.2). Moreover, very low concentration of diacetyl and 2,3-butanediol were produced.

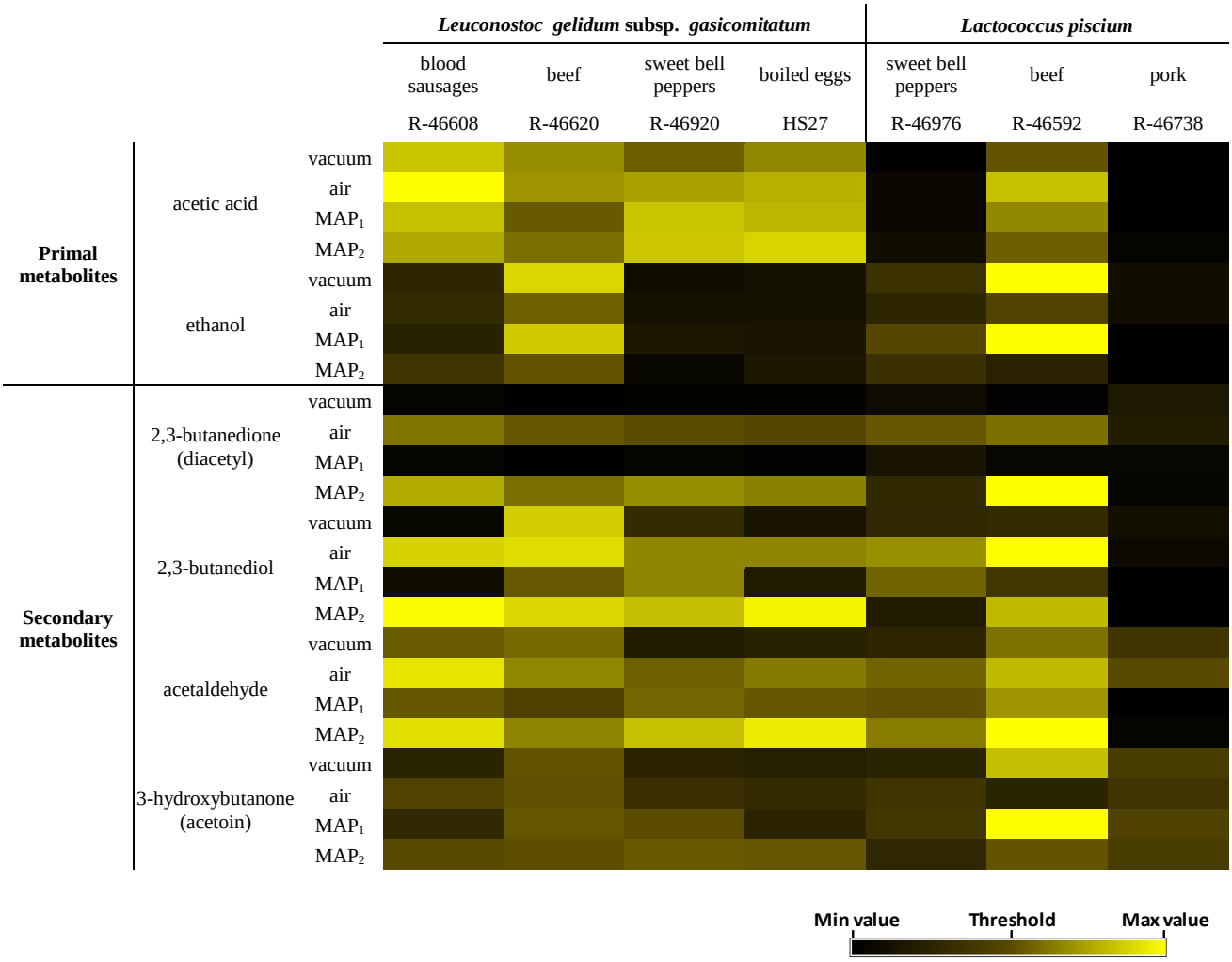

Figure 5.2: *Metabolic patterns for* Leuconostoc gelidum *subsp.* gasicomitatum *and* Lactococcus piscium *isolates under the four tested packaging conditions. The VOCs were measured with SIFT-MS and the maximum concentrations after Day 9 of storage were visualized by heatmap.*
Color change indicates the generation of larger amounts of metabolites compared to a "spoilage-baseline" in order to facilitate intra- and interspecies comparison among the 7 strains.
*The (minimum-**threshold**-maximum) μg/m^3 concentrations for each VOC are: acetic acid (362-**10000**-42088); ethanol (2797-**20000**-45716); diacetyl (50-**1500**-20792); 2,3-butanediol (213-**2000**-8505); acetaldehyde (148-**400**-969); acetoin (947-**4000**-59143).*

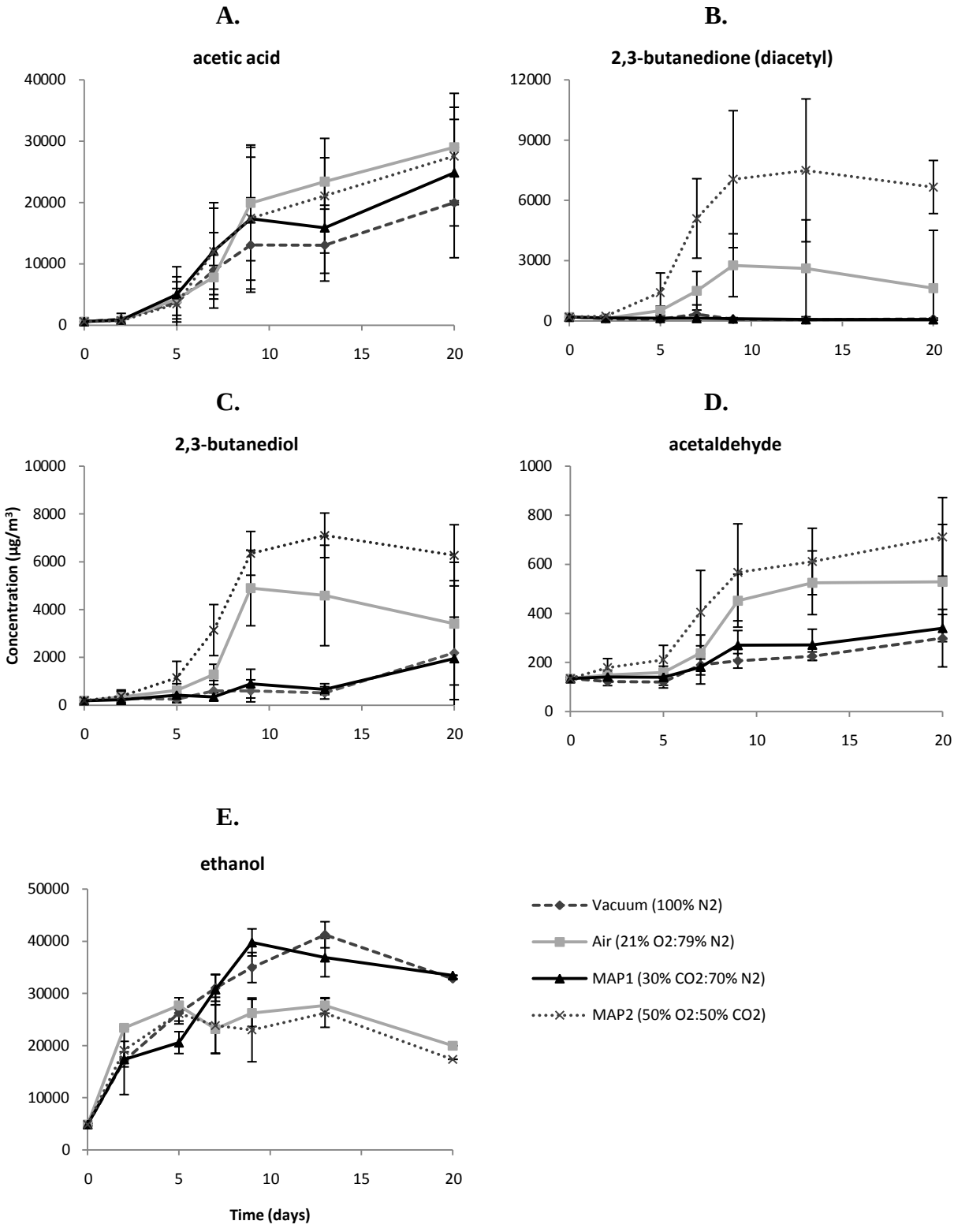

Figure 5.3: *Quantification of primal and secondary VOCs (A, B, C, D) with SIFT-MS in function with storage time for all 4* Le. gelidum *subsp.* gasicomitatum *isolates tested. (E) Ethanol production referring to isolate R-46620 (beef) that was the only* Le. gelidum *subsp.* gasicomitatum *strain that generated considerable amounts*

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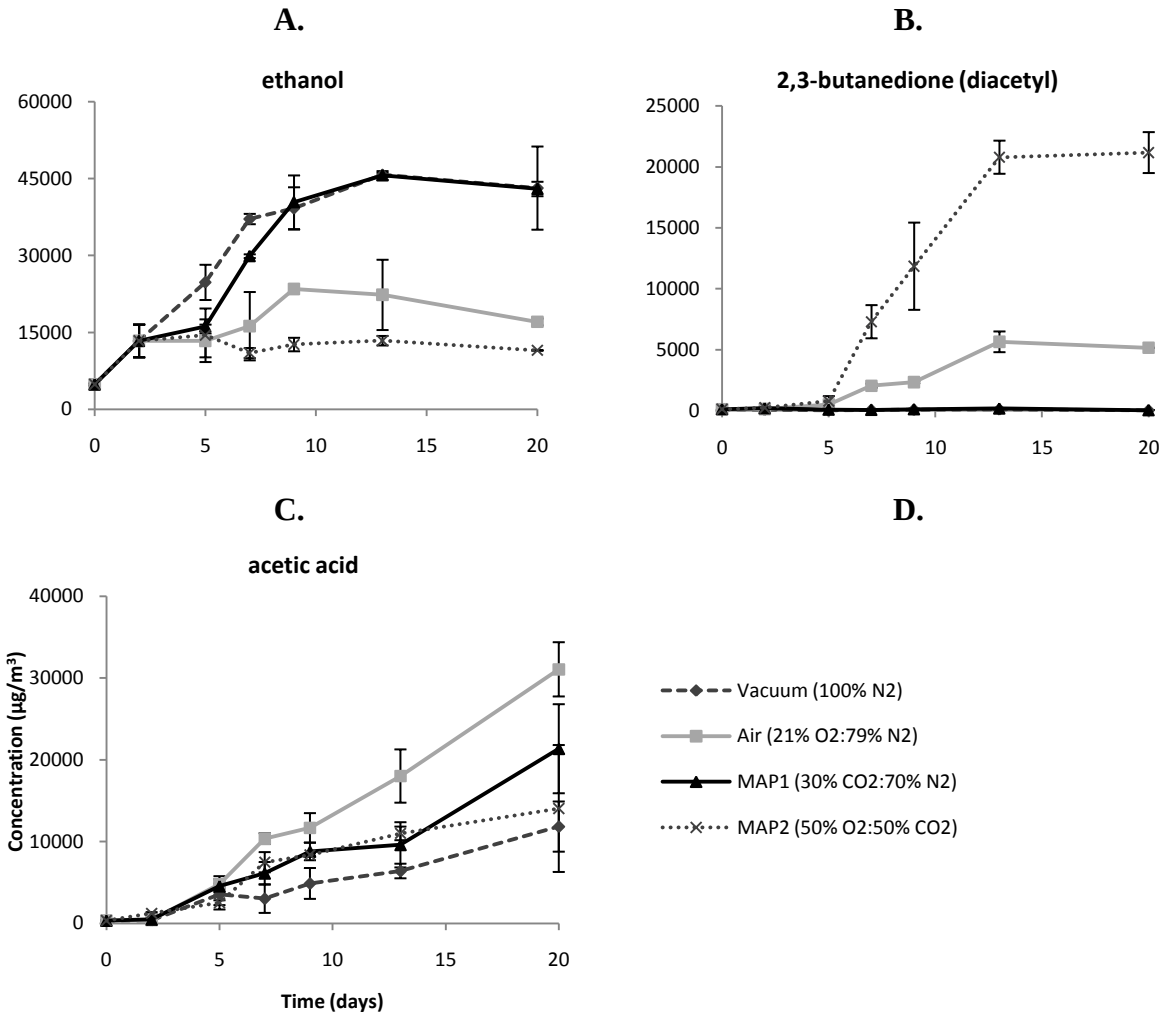


Figure 5.4: *Quantification of primal and secondary VOCs with SIFT-MS as a function of storage time for* Lc. piscium *isolate R-46592 (beef) that was the only strain demonstrating significant spoilage capacity.*

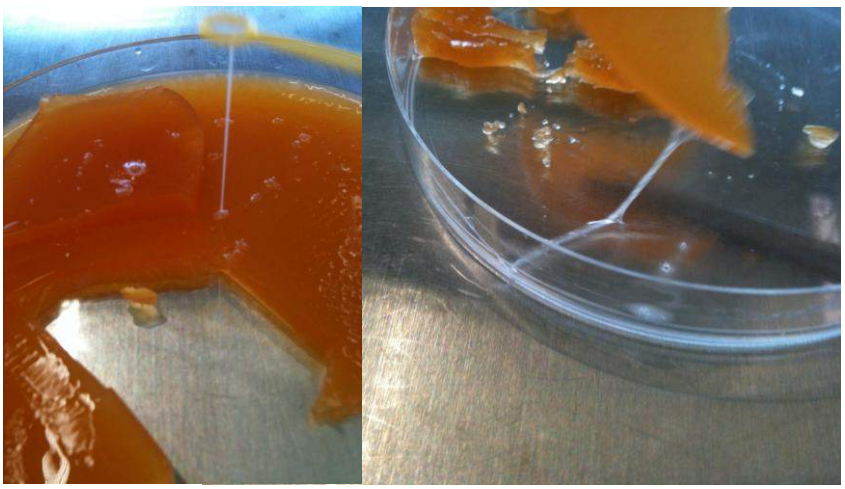


Figure 5.5: *Slime formation on the surface of the SBP agar plates due to growth of* Le. gelidum *subsp.* gasicomitatum *HS27.*

5.4 DISCUSSION

Recent advances in the application of MA packaging, have facilitated the limitation of food losses attributed to microbial growth and have achieved a prolongation of the shelf-life of fresh horticultural crops in intact and minimally processed forms (Amanatidou et al., 1999; Kader, 1993). Implementing combination of super-atmospheric O_2 levels with high concentrations of CO_2 has emerged as very promising preservation technique coupled to cold-storage (Allende et al., 2004; Amanatidou et al., 2000). The majority of Gram negative microbes as well as yeasts are efficiently inhibited (Geysen et al., 2005; Wszelaki & Mitcham, 2000; Zhang et al., 2013) hence suppliers and distributors eliminate the possibility of quality deteriorations caused by their metabolism. However, as shown in the present study *Le. gelidum* subsp. *gasicomitatum* is able to sustain the oxidative and cold stress dominating till the end of shelf-life of cold-stored and packaged products constituting a persistent problem for the industry (Susiluoto et al., 2003; Vihavainen & Björkroth, 2007).

Species *Le. gelidum* subsp. *gasicomitatum* was described quite recently as spoilage-related microbe (Björkroth et al., 2000) and subsequently it has often been reported as SSO in meat products. The genome of type strain *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T is fully sequenced (Johansson et al., 2011) and the metabolic capacities of the species associated with spoilage manifestations have been outlined, comprising production of acetic acid (pyruvate dehydrogenase), buttery off-odors (α-acetolactate synthetase) and slime formation (two dextransucrases) confirming the findings of the present study. Moreover, different factors favoring its growth have been suggested in order to explain its dominance in the products where it was isolated: presence of D-pantothenate in tomato based marinades (Björkroth et al., 2000), high CO_2 concentration in MA packaged products and brine, heme supplementation in meat triggering respiration (Jääskeläinen et al., 2012) could enhance its growth. Our results underpin its ability to grow fast under 7 ^oC and the resistance to oxidative stress which could be attributed to encoding of three different peroxidases (Johansson et al., 2011). It is not clear yet, how leuconostocs adapt to oxidative stress as the trigger to physiological mechanisms has not been studied thoroughly but LAB associated with plant material such as leuconostocs and pediococci, possess manganese accumulating systems that also have scavenging effect on superoxide rendering them more resistant to O_2 toxicity (Archibald & Fridovich, 1981; Axelsson, 2004; Zhang et al., 2013). Additionally, studies on *Leuconostoc* spp. indicate the putative role of *gshA* gene encoding γ-glutamylcysteine synthetase which is the key enzyme for the biosynthesis of a low-molecular-weight thiol, against oxidative stress (Kim et al., 2008). The gene is present in the genome of *Le. citreum* LBAE C10 (LEUCOC10_06675) and the genome of *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T (LEGAS_0148) as well (http://patricbrc.org/portal/portal/patric/Home). Moreover, the expression of *trxA* gene encoding a protein of 104 amino acids very similar to thioredoxin, was found to be upregulated in *Oenococcus oeni* when exposed to hydrogen peroxide and heat shock (Jobin et al., 1999). This gene is also present in the genome of *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T (*trxA1*: LEGAS_1284 and *trxA2*: LEGAS_0382). Lastly, all strains tested had high spoilage potential indicating that regardless of isolation environment and phenotypic properties they possess an inherent, metabolically offensive character. Previously, different molecular types of *Pseudomonas fragi*, a typical, spoilage-related species for milk, raw meat,

poultry and fresh fishery (De Jonghe et al., 2011; Drosinos & Board, 1995; Ercolini et al., 2009; Miller et al., 1973) were screened concerning their proteolytic activity and VOC profiles on sterile meat tissue. It was concluded that some metabolic components could be used as markers for specific biotypes, nonetheless their overall spoilage capacities were similar, without any indication of source-specific spoilage patterns (Ercolini et al., 2010).

On the other hand, *Lc. piscium* was found for the first time in diseased rainbow trout fish (Williams et al., 1990). Afterwards, it was recovered from spoiled meat (Sakala et al., 2002) and was studied for its cold adapting capacity (Garnier et al., 2010). However, studies are divided between its occurrence in cases of meat quality fluctuations as SSO (Jiang et al., 2010; Rahkila et al., 2012) and the application of specific strains in the frame of a bioprotective strategy against pathogenic and spoilage-related microbes in fishery product (Fall et al., 2010; Fall et al., 2012; Matamoros et al., 2009). This suggests that members of species *Lc. piscium* have either a strong spoiling character or not. The results from the tested strains indeed show this intraspecies heterogeneity concerning their ability to cause spoilage and cannot be correlated to origin of isolation or phenotype. It is clear that the ability to cause alterations of the organoleptic properties on SBP simulation medium is strain-dependent. Similarly to the genomic diversity of *Lc. piscium*, *Lactobacillus sakei* constitutes a paradigm species consisting of different subtypes deriving from three distinct lineages (Chaillou et al., 2009; Chaillou et al., 2013). Some cause significant alterations like formation of ropy slime, extreme acidification and putrification (Audenaert et al., 2010; Björkroth & Korkeala, 1997; Björkroth et al., 1996; Lyhs & Björkroth, 2008; Lyhs et al., 2002; Samelis et al., 2000), while a significant amount of research has focused on its bioprotective role (Chaillou et al., 2013; Vermeiren et al., 2006). Simmilarly, *Carnobacterium maltaromaticum* strains were tested with respect to their spoilage potential on sterile meat and no spoilage was evaluated highlighting their poor offensive character (Casaburi et al., 2011).

LAB have a simplistic metabolism that is based on bio-transformation of sugars into primarily organic acids, alcohols and carbonyl compounds of low molecular mass (Teusink et al., 2011). Leuconostocs are obligate heterofermentative (Björkroth & Holzapfel, 2006), while dairy lactococci are considered homofermentative (Teuber & Geis, 2006). However, in the case of certain *Lactococcus lactis* strains aeration is stimulating heterofermentative metabolism leading to production of acetoin, diacetyl and acetic acid (de Felipe et al., 1997; Lan et al., 2006). Presence of alternative electron acceptors like O_2, facilitates the formation of other metabolites from pyruvate (Liu, 2003; Zaunmüller et al., 2006). Acetic acid was not produced by *Lactococcus piscium* CNCM I-4031 in an experiment evaluating its bioprotective role (Fall et al., 2010). Nonetheless, acetoin was produced by *Lc. piscium* isolates deriving from beef (Sakala et al., 2002) and buttery off-odor was evaluated for the studied isolates which proved a rather weak spoilage character, in comparison to Gram-negative floras (Macé et al., 2013). Slow growth and weak spoilage related to “dirty socks” and “wet dog” according to the sensorial analysis was also evaluated on MA packaged meat inoculated with two *Lc. piscium* strains that had different growth dynamics each (Rahkila et al., 2012). Currently, little is known about the gene repertoirs of spoilage related lactococci corresponding to their metabolism.

In the present study monitoring the evolution of VOCs in function with time was performed directly from the headspace. Analysis based on semi-quantitative SPME-GC-MS combined

with SIFT-MS that is capable of quantifying specific VOCs in complex mixtures were implemented in a study aiming to determine distinct volatile biomarkers emitted by genotypically different strains of *Pseudomonas aeruginosa* (Shestivska et al., 2012). It has been reported previously that opening of a package as well as handlings during the sample preparation step could result in a loss of a fraction of the most volatile metabolites from the headspace. The equilibrium that will be restored eventually will correspond to the initial but an underestimation of the concentration levels could conceal the significance of certain compounds in relation to the shelf-life (Holm et al., 2013). For the qualitative analysis of an unknown sample GC-MS provides relative advantage but quantification is influenced by the affinity of the compounds to the fibre and the values of the volatiles will not represent the real concentration in the headspace (Olivares et al., 2011). SIFT-MS is a robust, convenient, easily deployable technique, which allows monitoring of the volatile compound even at real-time (Langford et al., 2014; Noseda et al., 2010, 2012; Olivares et al., 2011). A clear trend was observed for the packaging conditions that used O_2 in the headspace as a great amount of diacetyl was produced in all cases and generation of acetic acid was induced, hence increasing the spoilage potential of the tested isolates. The toxicity though from the highly reactive oxygen species responsible for oxidative stress varied. *Le. gelidum* subsp. *gasicomitatum* showed no inhibition, while *Lc. piscium* isolates were more sensitive. Apparently, not all VOCs have the same impact on the product as the aromatic profile of the foodstuff and the olfactory threshold of each compound play a decisive role. In the present study the acidification due to acetic acid gave a characteristic "vinegar" odor that was severely pungent as prooven by the high headspace concentrations. No sensory analysis was perfomed, however during sampling the fermention odor was extremely profound.

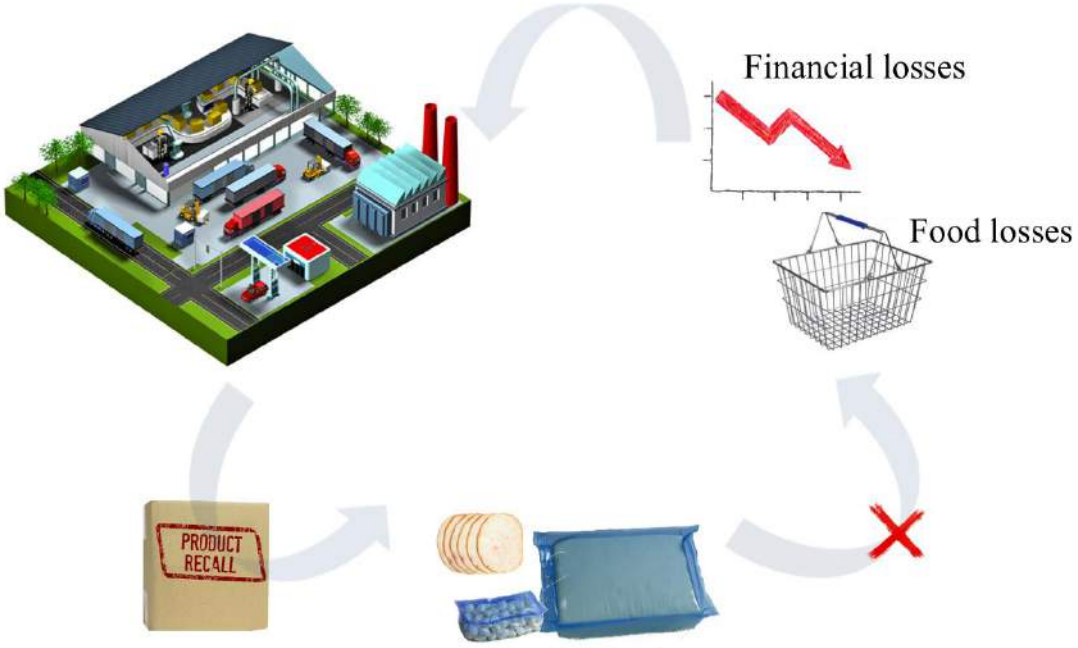
The attempt to characterize these species has elucidated their spoilage potential in carbohydrate-rich habitats. *Lc. piscium* is a heterogeneous species that encompasses members that can be protective or offensive to food. Spoilage is clearly strain-dependent and inhibition of certain strains can be achieved through a combination of elevated O_2 and CO_2 levels in the headspace. *Le. gelidum* subsp. *gasicomitatum* is a potent spoilage bacterium, since at population levels greater than 10^8 CFU/g it causes serious acidification and grows in a very wide range of gas compositions making it practically unfeasible to suppress in food matrices simply by packaging and cold-storage. The present study corroborates the concept about spoilage being complicated as well as poly-parametric. Clearly for spoilage to occur, a necessary minimum population density in the food matrix is required, differentiation of the involved microbes to nomenclatural level of subspecies and delineation of the metabolic patterns based on storage specifications and nutrient availability.

Production batch recalls and sporadic cases of product defects in Belgium, due to unexpected, early spoilage caused by psychrotrophic lactic acid bacteria (LAB) in packaged and chilled-stored foodstuffs between 2010 and 2014

This chapter is submitted as:

Pothakos V., Taminiau B., Huys G., Daube G. & Devlieghere F. (2014). *Production batch recalls and sporadic cases of product defects in Belgium, due to unexpected, early spoilage caused by psychrotrophic lactic acid bacteria (LAB) in packaged and chilled-stored foodstuffs between 2010 and 2014.*

To the International Journal of Food Microbiology



SUMMARY

During the period between 2010 and 2014 several spoilage cases occurring in retail foodstuffs prior to the end of shelf-life have been reported to our laboratory. Overall, 7 cases involved strictly psychrotrophic lactic acid bacteria (LAB) contamination in packaged and chilled-stored food products. The products derived either from recalls of entire production batches or as specimens of sporadic spoilage manifestations. Some of these samples were returned to the manufacturing companies by consumers who observed the alterations after purchasing the products. Apart from the costly, entailed replacement of products companies are confronted with subsequent brand damage and reduced trust by the market.

The products cover a wide range of foodstuffs and denote different spoilage defects. However, the microbiota determined by means of 16S rRNA gene high-throughput sequencing analysis underpin LAB genera: *Leuconostoc*, *Lactobacillus*, *Weissella* and *Lactococcus*, which are frequently encountered as specific spoilage organisms (SSO) in the screening of the Belgian market (**Chapter 3**) albeit overlooked by mesophilic enumeration methods due to their strictly psychrotrophic character (**Chapter 2**).

6.1 INTRODUCTION

An increasing part of the market is becoming engaged to packaged and chilled-stored food products (Welch & Mitchell, 2000). This industry has encountered solid growth during the last decades since this type of foods are convenient, nutritious, are considered healthy by consumers and fit the demands of modern lifestyle (Vihavainen & Björkroth, 2007). Hurdle technologies based on low-temperature storage and packaging, are less invasive but manage to inhibit the majority of highly spoiling, Gram negative, respiring microbes thus facilitate production of minimally processed foodstuffs with less additives (Gould, 1996). On the other hand, these goods are relatively unstable and greatly susceptible to microbial contamination of strictly and facultative anaerobic psychrotrophs (Audenaert et al., 2010; Borch et al., 1996; Vasilopoulos et al., 2008).

Mainly psychrotrophic members of lactic acid bacteria (LAB) dominate at the end of shelf-life while in many cases are associated with sporadic spoilage manifestations and quality fluctuations among production batches (Lyhs & Björkroth, 2008; Nieminen et al., 2011; Samelis et al., 2000; Santos et al., 2005; **Chapter 3**).

As stated in **Chapter 1**, LAB are ubiquitous in processing environments (Rahkila et al., 2011; Sidhu et al., 2001), constitute commensal microbiota of livestock (Axelsson, 2004) and are autochthonous to plant material and vegetables (Björkroth & Holzapfel, 2006). The routes of introduction to production plants are numerous and apparently certain members of LAB phylogenetic group have successfully adapted to these niches (Björkroth, 2005). Cases of spoilage attributed to LAB species are continuously reported for all sorts of products of vegetable and animal origin (Björkroth et al., 2000; Lyhs et al., 2004; Sakala et al., 2002; Vihavainen et al., 2008). They are able to grow fast and competently from low populations (Cayré et al., 2005), they thrive under different headspace compositions (Zhang et al., 2013), harsh environmental conditions trigger stress response mechanisms that render them more adaptive (Franz & Holzapfel, 2011) and finally some species have very low selectivity to substrates (**Chapter 4**), which facilitates cross-contamination.

The present study is dealing with actual cases of unexpected and fast spoilage developed on packaged and refrigerated products. The aim is to evaluate the microbial diversity of highly spoiling microbes involved in production recalls in Belgium through 16S rRNA gene high-throughput sequencing analysis and assess the ability of standard microbiological techniques based on traditional plating to determine presence of psychrotrophic spoiling microbes.

6.2 MATERIALS AND METHODS

6.2.1 Spoiled samples

A total of 7 samples were sent to our laboratory during the period 2010-2014 from different companies. All products were spoiled and were analyzed either prior to the end of shelf-life or at the end. Their packages were intact and no visible disruption was observed before evaluating the documented alterations. As presented on Table 6.1 the samples had manifested several extreme spoilage defects. In the case of the boiled eggs preserved in brine and the mozzarella cheese the

production batches before the end of shelf-life were entirely recalled from the market resulting in a loss of tons of products. Two samples of eggs and brine were analyzed from the problematic batch that had developed sour off-odor and ropy slime (Figure 6.2). The industrial mozzarella was formed in logs of 10 kg packaged in vacuum. The delivered sample had blown excessively as shown in Figure 6.1 and large hollows were formed in the entire mass of the cheese that was stored at 2 ^{o}C before reaching its shelf-life expectancy. The mayonnaise-based, fish salad and ham salad spreads were specimens of sporadic spoilage occurring in the same company at different time points. The turkey slices were returned to the company by consumers who purchased the products and observed round, convex and white colonies on the slices. Lastly, the ready-to-eat (RTE) meals (i.e. niçoise salad and salad with bacon) were greatly acidified at the end of shelf-life and very high contamination levels were assessed. This phenomenon was observed on an increasing amount of samples manufactured in the same production plant during the last year.

6.2.2 Microbiological analysis

The spoiled samples were kept at 2-7 ^{o}C after being collected by the manufacturers and sent directly to our laboratory, where they were analyzed upon arrival. Only the fish salad was delivered frozen at -18 ^{o}C. The enumeration of the samples was performed following a comparative method implementing mesophilic and psychrotrophic incubation at 30 ^{o}C and 22 ^{o}C respectively, as previously described in **Chapter 2**. The microbial counts are presented in Table 6.1.

6.2.3 Isolation and identification of dominant microbes for production recall samples

In the case of the two production recalls (i.e. boiled eggs in brine and mozzarella) 27 and 19 isolates respectively, were recovered from the plates of the highest dilutions incubated at 22 ^{o}C. All isolates were identified by means of DNA typing methods. Initially the 27 isolates originating from the boiled eggs in brine underwent a dereplication step. Repetitive element polymerase chain reaction (rep-PCR) using the $(GTG)_5$ primer was performed and the isolates were grouped in clusters. A selection of representative strains was further subjected to amplified fragment length polymorphism (AFLP) along with the 19 isolates from the mozzarella cheese. The obtained fingerprints were compared to an in-house database generated with type and reference LAB strains. The pipeline followed for the identification of the isolates was previously described in **Chapter 3**.

6.2.4 DNA extraction, Amplicon library construction, Pyrosequencing

Total DNA was isolated from each primary suspension with the DNeasy Blood & Tissue DNA extraction kit (Qiagen, Venlo, Netherlands), following the manufacturer's recommendations. The DNA was eluted into DNAse/RNAse-free water and its concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at -20 ^{o}C until used for 16S rRNA gene amplicon pyrosequencing analysis.

16S PCR libraries were generated for the samples listed in Table 6.1. The primers E9-29 and E514-430 (Brosius et al., 1981) specific for bacteria, were selected for their theoretical ability to generate the least bias of amplification capability among the various bacterial phyla (Wang et al., 2009). The oligonucleotide design included 454 Life Sciences A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5' end of each primer. The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1x enzyme reaction buffer, 200 µM dNTPs (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng of genomic DNA in final volume of 100 µl. Thermocycling conditions consisted of a denaturation step of 15 min at 94 ^{o}C followed by 25 cycles of 40 s at 94 ^{o}C, 40 s at 56 ^{o}C, 1 min at 72 ^{o}C and a final elongation step of 7 min at 72 ^{o}C. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on 1 % agarose gel electrophoresis and the DNA fragments were plugged out and purified using SV PCR purification kit (Promega Benelux, Leiden, Netherlands). The quality and quantity of the products were assessed by Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium).
All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs. All amplicons were sequenced using Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium).

6.2.5 Bioinformatics and Data analysis

The 16S rDNA sequence reads were processed with MOTHUR (Schloss et al., 2009). The quality of all sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR and filtered with the following criteria: minimum length of 425 bp, exact match to the barcode and 1 mismatch allowed to the proximal primer. The sequences were checked for presence of chimeric amplifications (Haas et al., 2011) using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS)
The obtained read sets were compared to reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse et al., 2007). The final reads were clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was attributed to each OTU by comparison to the SILVA database (80 % homogeneity cutoff).
As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA dataset 111 using BLASTN algorithm (Altschul et al., 1990). For each OTU, a consensus detailed taxonomic identification has been given based upon the identity (less than 1 % mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not).
Subsampled datasets were obtained and used to evaluate richness and microbial diversity using MOTHUR. Rarefaction curves (Colwell & Coddington, 1994), microbial biodiversity (non parametric (NP) Shannon diversity index (Chao, 2003) and richness estimation (ACE and Chao1 estimator) were calculated (Chao & Bungee, 2002) shown in Table 6.2.

Two-sided Fisher's exact test was used to highlight statistical differences in the bacterial population between each pair of samples (Rivals et al., 2007) and confidence intervals were calculated with the Newcombe-Wilson method (Newcombe, 1998) using the STAMP software.

6.3 RESULTS

6.3.1 Microbiological analysis - Identification of isolates

The microbiological analysis of all samples showed that the psychrotrophic count was significantly high as microbial populations exceeded threshold levels of 10^7-10^8 CFU/g (AFSCA, 2012; Uyttendaele et al., 2010). Noteworthy, in all cases underestimation ranging between 0.5 and 3.9 logs CFU/g was investigated when implementing mesophilic incubation at 30 ^oC similarly to all the samples presented in **Chapter 1**.

Table 6.1: *Detailed description of spoiled samples concerning the applied packaging technology and the evaluated spoilage manifestation. The contamination levels presented correspond to the psychrotrophic, microbial counts assessed by incubation of plates at 22 ^oC. The underestimation of the microbial loads at 30 ^oC is also shown.*
** Enumeration of the product was performed by implementing only psychrotrophic shelf-life parameter based on incubation of plate at 22 ^oC.*

Type of product	Description	Packaging	log CFU/g ± S.D. (Underestimation at 30 °C)			Spoilage manifestation
			PCA	MRS	RCA	
Cooked meat	turkey slices	MA (30% CO_2: 70% N_2)	8.37 ± 0.02 (1.95)	8.33 ± 0.02 (0.89)	8.20 ± 0.01 (0.60)	Colonies, Buttery off-odor
Dairy	mozzarella	Vacuum	7.72 ± 0.02 (0.18)	8.04 ± 0.04 (0.47)	7.76 ± 0.07 (0.49)	Extreme CO_2 formation, Blown package
Ready-to-eat (RTE) meal	niçoise salad	Plastic container (Air)	9.42 ± 0.09 (0.31)	8.58 ± 0.05 (0.50)	8.99 ± 0.04 (0.62)	Acidification, Sour off-odor
	salad with bacon	MA (10% O_2: 90% N_2)	9.48 ± 0.03 (1.31)	9.49 ± 0.10 (1.02)	9.47 ± 0.04 (1.98)	Acidification, Sour off-odor
Composite food (mayonnaise-based spread)	fish salad	Plastic container (Air)	5.69 ± 0.03 *	5.65 ± 0.07 *	5.12 ± 0.05 *	Putrid off-odor
	ham salad	Plastic container (Air)	7.73 ± 0.02 (3.10)	8.45 ± 0.09 (3.41)	7.77 ± 0.05 (2.19)	Acidification, Sour off-odor
Other	boiled eggs	Vacuum	7.32 ± 0.03 (0.52)	7.90 ± 0.05 (0.81)	7.86 ± 0.01 (0.66)	Acidification, Slime formation, Sour off-odor
	brine (boiled eggs)	Vacuum	8.69 ± 0.02 (3.88)	8.89 ± 0.09 (0.69)	8.71 ± 0.05 (0.55)	Extreme slime formation

LAB populations equaled total count emphasizing on the dominance of this microbial group at the moment of sampling, except for the niçoise salad for which counts on PCA were 1 log greater compared to MRS. However, the only sample that had lower counts (10^5-10^6 CFU/g) was

the fish salad spread, which was delivered frozen unlike all other products. It could be assumed that during freezing and due to subsequent stress of thawing some microbes might have lost viability.

Confirming high microbial populations, the analyzed samples had developed early spoilage that resulted in poor organoleptic properties. The mozzarella and the eggs in brine denoted extreme alterations (Figure 6.1 & 6.2) before reaching end of shelf-life, therefore characterization and identification of dominant species was performed.

The 27 colonies recovered from the highest dilution plates incubated at 22 ^{o}C during the analysis of eggs and brine were initially subjected to $(GTG)_5$ rep-PCR fingerprinting and grouped in one cluster delineated at Pearson similarity level of 91 %. A selection of 3 representative strains was further identified by means of AFLP analysis along with 19 dominant microbes isolated from the industrial mozzarella sample. As shown on the AFLP dendrogram (Figure 6.3) both spoilage cases were attributed to one bacterial species. In the case of mozzarella, *Weissella viridescens* and for the eggs in brine, *Leuconostoc gelidum* subsp. *gasicomitatum*, were identified by comparing the obtained genomic fingerprints to the type strains. *Leuconostoc gelidum* subsp. *gasicomitatum* isolates were also tested with regard to slime forming capacity. All isolates proved to be potent slime formers when growing in different laboratory media without necessarily containing sucrose.

Figure 6.1: *The alterations evaluated during sampling of the 10-kg mozzarella cheese. Blown packaging, surface irregularities and large hollows in the entire mass of the cheese give a spongy appearance to the product.*

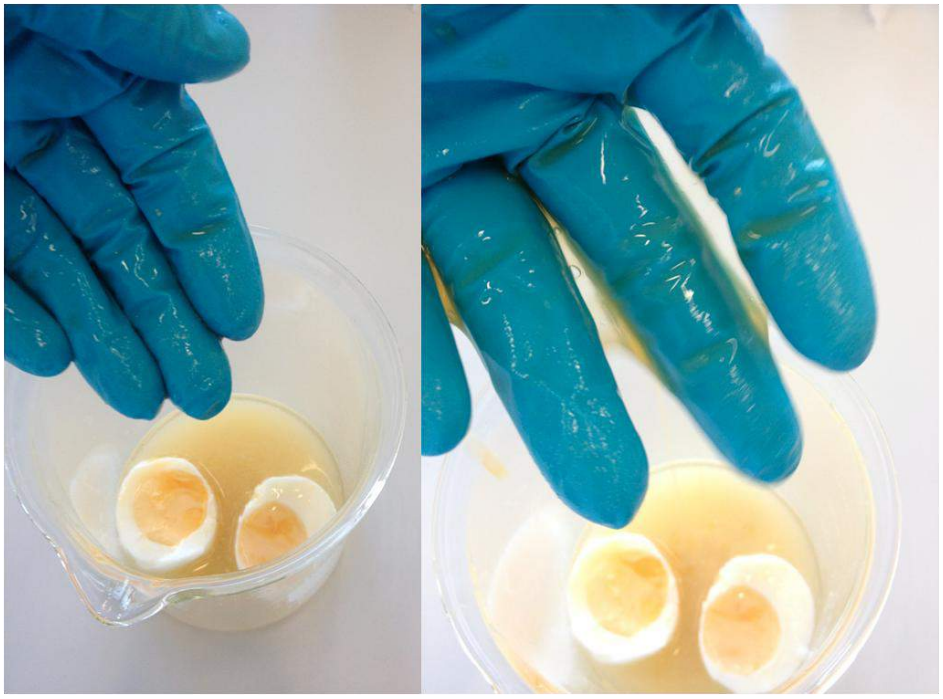


Figure 6.2: *Evaluation of the spoilage attributes of the boiled eggs preserved in brine. Apart from a pungent, sour off-odor the brine of the eggs had developed thick, viscous texture due to extreme formation of ropy slime.*

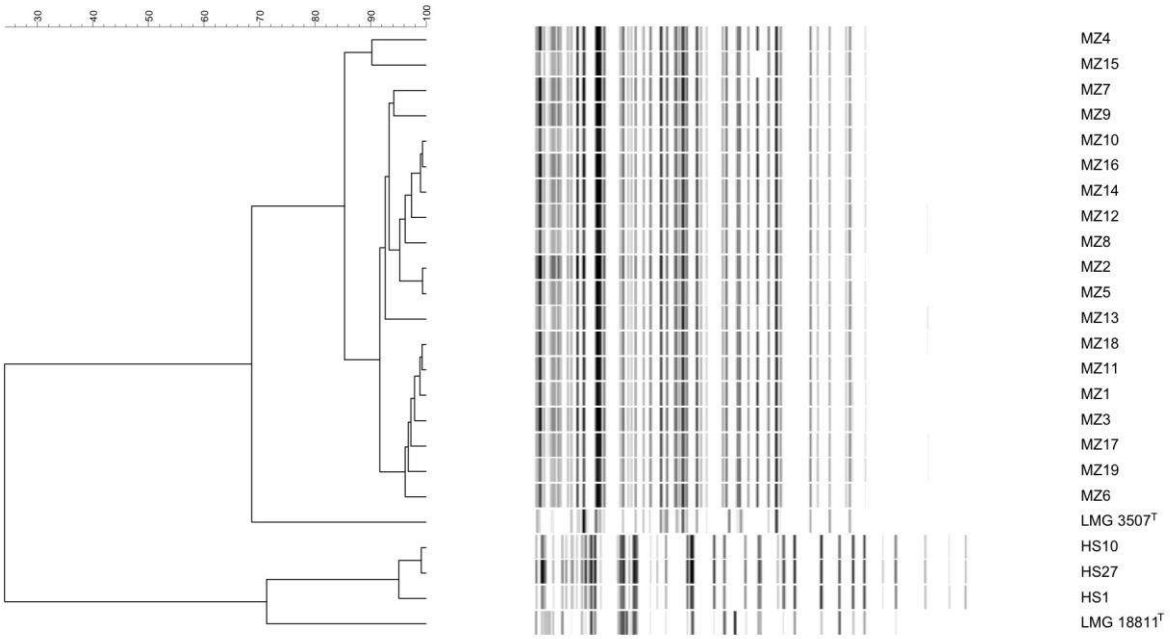


Figure 6.3: *AFLP dendrogram of strains isolated from the mozzarella cheese (MZ_) and the boiled eggs in brine (HS_). The dendrogram was constructed using the Dice coefficient and UPGMA clustering method. The type strains (*Weissella viridescens *LMG 3507^T and* Leuconostoc gelidum *subsp.* gasicomitatum *LMG 18811^T) are also included.*

6.3.2 Sequencing and Data analysis

The high-throughput sequencing results (Table 6.2 and Figure 6.4) present the low phylogenetic diversity of the communities involved in spoilage. A total of 10,419 obtained raw reads were used to recompose the microbial consortia of the case studies. The rarefaction analysis and the estimated sample coverage (ESC), which in all samples exceeded 98 % indicated satisfactory coverage with limited amount of reads/sample, which in the cases of niçoise salad, salad with bacon and fish salad spread were lower than 1000 reads/sample.

The boiled eggs were mainly dominated by *Le. gelidum* although several other bacterial genera and species were observed in low abundances. In the analyzed brine sample, *Le. gelidum* was 99 % abundant among 3 otus in total making it safe to deduce that it constituted the only specific spoilage organism (SSO). For mozzarella cheese more that 96 % of the reads corresponded to the starter culture *Streptococcus thermophilus*. However, spoilage manifestation is clearly attributed to *W. viridescens* presence of which was confirmed by typing of the dominant isolates. Turkey slices and niçoise salad were dominated by *Le. gelidum*, *Le. inhae*, *Weissella* sp., *Le. carnosum* and *Le. carnosum*, *Lactobacillus sakei* group, respectively. Both samples showed high prevalence of psychrotrophic *Leuconostoc* spp. hence explaining the microbial underestimation when implementing mesophilic incubation of the plates. Likewise, ham salad spread and salad with bacon were overall contaminated by psychrotrophic *Lactobacillus* spp. that were severely overlooked by mesophilic enumeration protocols. *Lb. sanfranciscensis* and *Lb. sakei* group, *Lb. algidus*, *Lb. sanfranciscensis*, respectively were the most predominant LAB species. In the ham salad spread *Xanthomonas hortorum* was also in high numbers although it is considered plant pathogen (i.e. causing leaf blight of carrot) and not food related microbe (Temple et al., 2013). Possibly some raw vegetable materials used as constituents of the salad might have been contaminated.Lastly, the fish salad spread had higher abundance of non-LAB taxa like *Pseudomonas fragi* (56 %) and *Brochothrix thermosphacta* (16 %), while *Le. gelidum*, *Lactococcus piscium* and *Weissella* sp. combined represented 21 % of the identified sequences.

Table 6.2: *Number of analyzed sequences, operational taxonomic units, species richness, biodiversity index and estimated sample coverage of the 16S rRNA gene amplicons were calculated at the 3 % distance level.*

Samples	Reads	OTUs	Chao (Species Richness)	Shannon (Biodiversity Index)	ESC (%)
Turkey slices	1161	31	48,5	1,10	98.71
Mozzarella	1753	7	7,3	0,19	99.89
Niçoise salad	972	7	8,5	0,29	99.69
Salad with bacon	936	12	17	1,12	99.47
Fish salad	775	31	44	1,62	98.19
Ham salad	1259	37	49	0,99	98.73
Boiled eggs	1874	53	55	3,06	99.79
Brine (boiled eggs)	1689	3	3	0,02	99.94

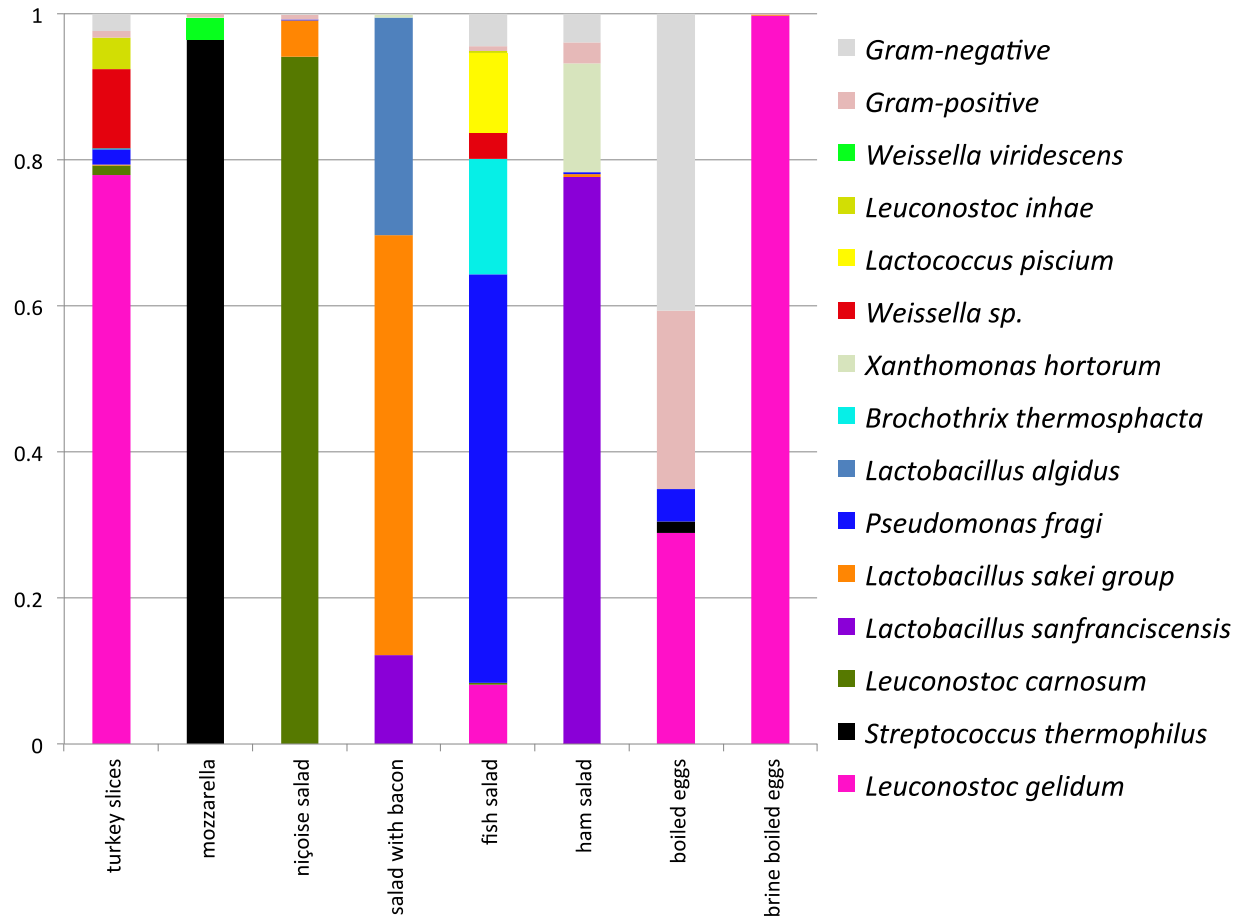


Figure 6.4: *Abundance of bacterial species in the analyzed spoiled samples. Color key legend shows bacterial taxa with abundance >2%. The group of Gram-negative and Gram-positive correspond to taxa of* Proteobacteria *and* Actinobacteria & Firmicutes *respectively, with abundances <2%.*

6.4 DISCUSSION

Good hygiene practices, cold chain maintenance and advanced in preservation methods applied in unstable products have achieved extension of the anticipated shelf-life and have managed to limit incidents of unexpected spoilage (Gould, 1996, 2000). This is mainly attributed to performance of effective surveillance plans (Säde, 2011) that eliminate hygienic indicators, implementation of innovative packaging technologies, which suppress offensive, respiring, Gram negative microbes (Allende et al., 2002; Amanatidou et al., 2000; Geysen et al., 2005) and mild treatments on minimally processed food material (Gómez-López et al., 2007; Li et al., 2001). Apart from the non-invasive techniques, heating treatments or addition of preservatives are applied in combination to the aforementioned methods in the frame of a hurdle technology. However, in the past few years it has become evident that these approaches, despite inhibiting notorious spoilage-related microbes have provided the ground for certain bacterial groups to dominate (Vasilopoulos et al., 2010). While packaging and low-temperature storage protect susceptible foodstuffs from spoiling, at the same time they exert selection pressure towards psychrotrophic, facultative anaerobic species to proliferate (Dainty & Mackey, 1992). The

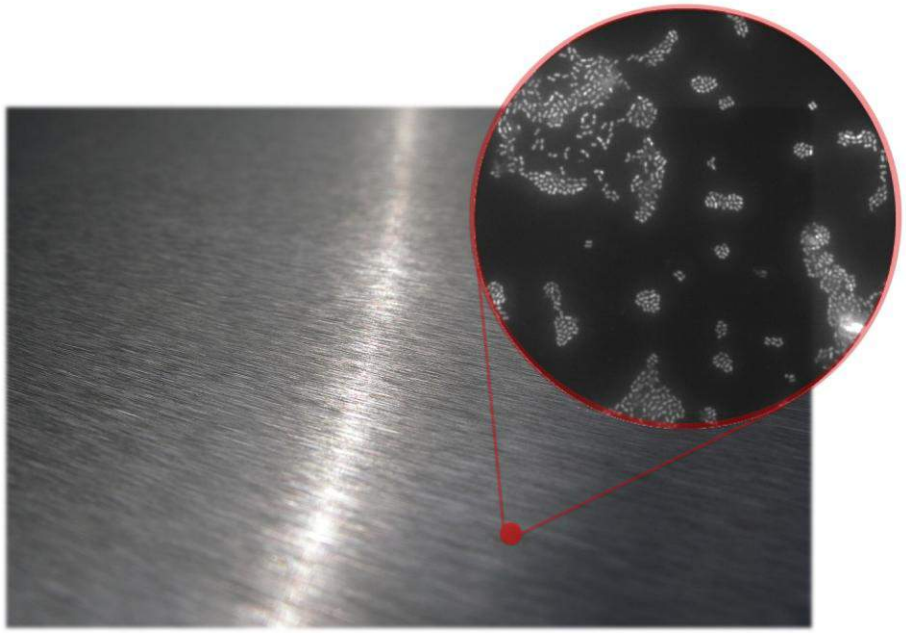
present study encompasses limited number of samples, which nonetheless constitute actual spoilage cases from retail products that had significant impact on the manufacturing companies both from financial and reliability aspects. The companies involved are dissimilar in terms of production process, manufactured end-products, topography and technology, although the confronted problems underpin the presence of psychrotrophic LAB.

Culture-dependent, culture-independent, high-throughput sequencing (HTS) and metagenomic approaches are available in order to assess the contamination levels and microbial diversity of complex matrices (Di Bella et al., 2013). In terms of convenience, taxonomic accuracy and discriminatory capacity techniques based on crude DNA extraction from the food facilitate construction of a detailed image of all members of the microbial community. However, these techniques require high level of expertise, specialized personnel and expensive equipment, therefore not generally applied (Ercolini, 2013). Plate counting methods following ISO enumeration protocols are universally used as shelf-life parameters in practice. Routine microbiological analyses conducted in production plants and accredited laboratories assess the microbial state of packaged and chilled-stored products by determining the populations of mesophilic microbes. The implemented mesophilic enumeration techniques have a limited selectivity towards certain psychrotrophic groups that are eventually underestimated (Ercolini et al., 2009; Holley & McKellar, 1996; Schirmer et al., 2009; **Chapter 3**). Thus combination of culture-dependent and culture-independent techniques is considered necessary in order to effectively evaluate the microbial taxa involved in cases of spoilage.

The present study underpins the significance of psychrotrophic genera *Leuconostoc, Lactobacillus, Weissella* and *Lactococcus* that are frequently responsible for quality fluctuations of this type of products, as previously documented in **Chapter 3**. Psychrotrophic incubation performed at 22 ^{o}C implemented as shelf-life parameter allows efficient evaluation of the microbial population drawing attention towards presumptive, spoilage LAB species (**Chapter 2**). From the data set presented here, it can also be concluded that large genotypic diversity among strains belonging to the same species inflict different spoilage alterations based on phenotype or possibly in correlation to packaging, nutrient availability and interactions among the members of the microbial association. In **Chapter 5** the spoilage potential of strain *Le. gelidum* subsp. *gasicomitatum* HS27 originating from the eggs in brine showed the spoilage potential of this microbe in comparison to other biotypes of the same subspecies, confirming as well its ability to form slime unlike the other isolates tested.

Evaluation of possible cross-contamination routes of psychrotrophic *Leuconostoc gelidum* subsp. *gasicomitatum* through adhesion to food contact surfaces

This chapter will be submitted as:
Pothakos V., Ayu Aulia Y., Van der Linde I., Uyttendaele M. & Devlieghere F. (2014). *Evaluation of possible cross-contamination routes of psychrotrophic* Leuconostoc gelidum *subsp.* gasicomitatum *through adhesion to food contact surfaces.*



SUMMARY

In **Chapters 1, 3, 4 & 5** psychrotrophic LAB species *Leuconostoc gelidum* subsp. *gasicomitatum* has been reported as specific spoilage organism (SSO) in the case of various-packaged, cold-stored food products. Raw, fresh or marinated meat, vegetable sausages, cooked meat products, vegetable salads and ready-to-eat (RTE) meals are often commodities of isolation. The competitiveness of this microbe in food matrices is substantiated and high specific growth rates at refrigeration temperature have been documented. Source tracking studies performed in food processing environments (**Chapter 4**) underpin the adaptation of *Le. gelidum* subsp. *gasicomitatum* to production lines and premises, however it is still unclear how cross-contamination occurs. Therefore, a selection of strains (**Chapter 4 & 6**) was tested on their ability to adhere on stainless steel (SS) and glass surfaces. One cocktail of three slime-formers isolated from spoiled eggs in brine and an environmental isolate recovered from disinfected blades showed very weak attachment. A strain isolated from food contact surface from a highly contaminated area of a salad production facility exhibited average attachement (5.15-6.25 CFU/cm^2). On the other hand, a strain that was surviving in an acetic acid bath in the same ready-to-eat (RTE) salad manufacturing company reached populations of attached cells exceeding 10^7 CFU/cm^2 confirming great intraspecies diversity. For the first time the ability of *Leuconostoc gelidum* subsp. *gasicomitatum* to attach on surfaces is documented.

7.1 INTRODUCTION

Psychrotrophic *Leuconostoc* spp. have been associated with several spoilage reports in raw, fresh or marinated meat, vegetable sausages, cooked meat products, vegetable salads and ready-to-eat (RTE) meals (Lyhs et al., 2004; Vihavainen & Björkroth, 2009; Vihavainen et al., 2008; **Chapter 3, 4 & 6**). A theory of adaptation to food manufacturing plants based on source tracking studies performed in processing environments, where psychrotrophic LAB contaminations were substantiated as the main problem related to quality defects, underpin the ability of leuconostocs to dominate in cold chain production lines (Björkroth, 2005; Björkroth & Korkeala, 1997). The genotypic diversity within species *Leuconostoc gelidum* that encompasses three subspecies (Rahkila et al., 2014) proves the impact of lifestyle on the gene repertoire and subsequently phenotype.

Growth dynamics in various products as well as spoilage potential have been studied intending to explain the outgrowth of these microbes once present in the food matrix (Jääskeläinen et al., 2013; **Chapter 5**). However, it remains unclear how contamination occurs in the first place. Cross-contamination through air mediation and surface adhesion from raw materials to end-products have been suggested as presumptive routes (Björkroth et al., 1998; Björkroth & Korkeala, 1997).

The occurrence of slime as metabolic product of dominant *Le. gelidum* subsp. *gasicomitatum* populations has been investigated in cases of spoilage (Johansson et al., 2011; Säde, 2011; **Chapter 6**). Exopolysaccharides (EPS) from LAB contribute to the organoleptic properties of fermented dairy products enhancing the texture and the mouthfeel or result in spoilage for different type of foodstuffs. Noteworthy, they are partially involved in biofouling processes as they accumulate on biotic and abiotic surfaces facilitating the embedment of cells (De Vuyst & Degeest, 1999).

In the current study we attempt the elucidation of potential routes facilitating the introduction of *Le. gelidum* subsp. *gasicomitatum* to food material during handlings in production environment. Through screening of selected strains allocated to this subspecies the ability to attach on food contact surfaces was evaluated.

7.2 MATERIALS AND METHODS

7.2.1 *Leuconostoc gelidum* subsp. *gasicomitatum* strains and inoculum preparation

For the purposes of the study, 6 strains of *Leuconostoc gelidum* subsp. *gasicomitatum* (i.e. HS1, HS10, HS27, 6.2.3, 10.16.3, ab2) were tested. Strains HS1, HS10 and HS27 (HS cocktail) represent food isolates with unique phenotype producing extreme slime. They were found dominating in vacuum packaged boiled eggs preserved in brine (**Chapter 6**) that exhibited spoilage defects prior to the end of shelf-life. Isolates 6.2.3, 10.16.3, ab2 were recovered from environmental sampling in a vegetable processing environment during a source tracking study (**Chapter 4**). Strain 6.2.3 was found as survivor on the blades of a vegetable dicer right after disinfection of the plant and prior to production. Isolate 10.16.3 was

swabbed from a surface of the packaging section at the end of a production line. Lastly, isolate ab2 was found growing in an acetic acid bath.

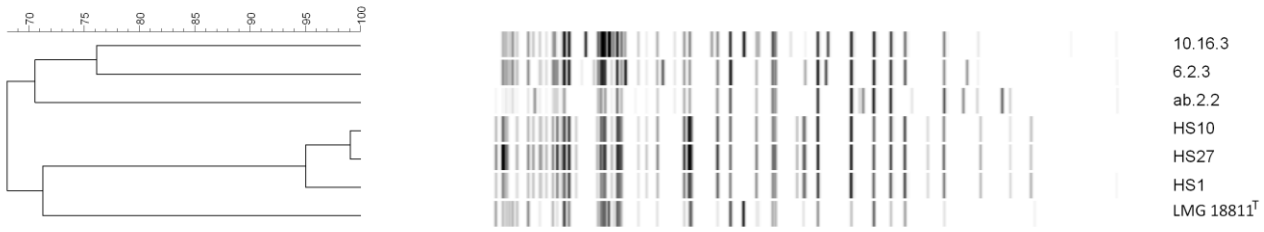


Figure 7.1: *AFLP dendrogram with the fingerprints of the* Leuconostoc gelidum *subsp.* gasicomitatum *isolates used in the present study. The dendrogram was constructed using the Dice coefficient and UPGMA clustering method. (T) The type strain* Le. gelidum *subsp.* gasicomitatum *LMG 18811^T is also included.*

The isolates were resuscitated by transferring one glass bead from the culture stored at -80 ^oC in de Man-Rogosa-Sharpe broth (MRS, Oxoid, Hampshire, UK) and incubated at 22 ^oC overnight. After 24 hours 100µL were transferred to a new broth tube and kept for another 24 hours at 22 ^oC. In the case of the HS cocktail 33 µL of each strain were transferred to the same broth tube.

7.2.2 Preparation of stainless steel (SS) and glass surfaces

Stainless steel (SS) coupons (2 cm x 5 cm, 1 mm thickness; type AISI-304; Baudoin, Mol, Belgium) were used for the attachment experiments, as stainless steel is the most frequently used material in blades, benches and for the construction of food-processing equipment. The coupons were initially soaked in acetone (overnight) to remove any debris and grease from the manufacturing process. Coupons were then washed in commercial detergent solution, rinsed thoroughly with distilled water and subsequently with ethanol, air dried, and finally sterilized by autoclaving at 121 °C for 15 min. Likewise, microscope glass slides were wiped clean with a dry piece of cloth and autoclaved at 121 °C for 15 min.

7.2.3 Preparation of Sweet bell pepper (SBP) juice and boiled eggs in brine

The sweet bell pepper juice was prepared following the protocol previously described (in **Chapter 5,** paragraph 5.2.2). After sterilizing the juice by boiling, 50 mL were aseptically distributed in sterile containers. In order to prepare boiled eggs in brine simulating the spoiled retail product, fresh eggs were covered in aluminum foil, placed in a glass jar and autoclaved at 121 °C for 15 min. Once reached room temperature, they were peeled aseptically in a biosafety cabinet and cut in halves using sterile forceps and blades. The halved boiled eggs were transferred in sterile containers (one half/container) and 50 mL of brine were added. The brine consisted of: 10 g citric acid; 20 g trisodium citrate and 17 g of NaCl suspended in 1.5 L

of distilled water. The final pH of the brine was 4.5 ± 0.1 and reached 6.0 when added to the egg.

7.2.4 Immersion of surfaces, inoculation and attachment conditions

SS coupons were immersed in SBP juice and eggs in brine, positioned in the containers accordingly so as to be totally covered by the medium. The glass slides were positioned vertically and one edge remained in contact with the headspace. The inoculum for each strain and the cocktail was prepared after two consecutive subcultures in MRS for 24 hours at 22 ^{o}C. The second culture was kept at 7 ^{o}C for 1 h prior to inoculation in order to stimulate cold-adaptation. Subsequently, a series of decimal dilutions for the second culture was prepared in peptone physiological solution (PPS: 0.85 % w/v NaCl and 0.1 % w/v bacteriological peptone). The appropriate dilution was used for the inoculation of each container (100 μL) obtaining a level of approximately 10^3-10^4 viable cells per mililiter of medium. The dilution series of the inoculum of each strain and the cocktail was plated out on MRS in order to evaluate the initial population (Day 0). The inoculated media with the surfaces were kept for 9 days at 7 ^{o}C facilitating the attachment of the cells.

7.2.5 Detachment of the attached cells from SS coupons and enumeration of suspended and attached populations

On Days 1, 2, 5, 6, 7 and 8 the population of the suspended cells was determined for all inocula on both media. Aliquots of 1 mL were extracted from the containers, series of dilutions were prepared in PPS and spread plated on MRS in duplicate. For assessing the attached populations, the vortexing method was performed as previously described (Giaouris, Chorianopoulos, & Nychas, 2005; Poimenidou et al., 2009) on Days 5, 6, 7 and 8. The SS coupons were removed from the containers and thoroughly washed with 25 mL of PPS in order to remove all loosely adhering cells as well as residual food particles, left in the biosafety cabinet until all liquid had dripped off, subsequently placed in 50 mL-centrifuge tubes filled with 10 mL PPS containing 24 glass beads (12 diameter, 3 mm and 12 diameter, 5 mm) and vortexed for 2 min at maximum intensity in order to detach the cells from the coupon.
Quantification of attached populations was performed by pour plating 1 mL aliquots of the serial dilutions of the vortexed suspension on MRS in duplicate. An overlayer of MRS was applied in order to achieve microaerophilic conditions. The formed colonies were counted after incubation at 22 °C for 5 days.

7.2.6 Microscopic observation of attached cells on glass slides

On Days 5, 6, 7, 8 and 9 the glass slides were aseptically removed from the containers, washed thoroughly with 25 mL of PPS and transferred in empty 50 mL-centrifuge tubes to dry. The BacLight dye (Molecular Probes, Eugene, OR, USA) was used for checking viability of the bacteria attached on the slides staining the live cells with Syto 9 (green) and the dead with propidium iodide (red) by means of fluorescence microscopy (Mattila, 2002).

Microscopic observation of the attached cells on the glass slides was performed through a Axio Imager A1 (Carl Zeiss NV-SA, Zaventem, Belgium) microscope and image capturing by means of an Axiocam camera.

7.3 RESULTS

7.3.1 Suspended cells of *Le. gelidum* subsp. *gasicomitatum*

The 4 inoculated cultures of *Le. gelidum* subsp. *gasicomitatum* overall, showed the same growth dynamics suspended in both SBP juice and boiled eggs in brine without any significant statistical difference (P>0.05). As shown in Table 7.1, on Day 5 or 6 they had reached the static phase of growth (8.15-9.17 log CFU/mL) and remained at densities ranging between 8.44 and 9.37 log CFU/mL until Day 8. The only difference among the 4 tested inocula was the formation of thick and viscous, ropy slime by the HS cocktail in both matrices. Strains 6.2.3, 10.16.3 and ab2 did not form slime.

Table 7.1: *Population of suspended cells of* Le. gelidum *subsp.* gasicomitatum *strains in sweet bell pepper juice and boiled eggs in brine, where stainless steel (SS) surfaces were submerged and stored at 7 ^{o}C.*

	Suspended cells (log CFU/mL)							
	Sweet Bell Pepper juice				Boiled eggs in brine			
Time (Day)	HS cocktail	6.2.3	10.16.3	ab2	HS cocktail	6.2.3	10.16.3	ab2
0	3.60 ± 0.03	3.48 ± 0.05	3.73 ± 0.02	3.51 ± 0.04	3.60 ± 0.03	3.48 ± 0.05	3.73 ± 0.02	3.51 ± 0.04
1	4.09 ± 0.07	3.75 ± 0.27	3.85 ± 0.07	4.10 ± 0.14	3.65 ± 0.33	4.14 ± 0.09	3.82 ± 0.06	3.99 ± 0.30
2	5.35 ± 0.17	5.34 ± 0.03	4.96 ± 0.00	5.30 ± 0.03	5.24 ± 0.14	5.24 ± 0.09	5.15 ± 0.04	5.32 ± 0.09
5	9.03 ± 0.01	8.23 ± 0.26	9.17 ± 0.07	8.82 ± 0.01	8.15 ± 0.27	8.22 ± 0.06	8.82 ± 0.31	8.97 ± 0.19
6	8.94 ± 0.03	9.03 ± 0.03	8.92 ± 0.11	9.05 ± 0.07	8.72 ± 0.21	9.00 ± 0.18	9.01 ± 0.16	8.93 ± 0.03
7	9.07 ± 0.07	9.02 ± 0.03	9.20 ± 0.03	9.05 ± 0.01	8.44 ± 0.06	8.95 ± 0.08	8.87 ± 0.04	8.94 ± 0.08
8	8.96 ± 0.02	9.10 ± 0.01	9.35 ± 0.01	9.17 ± 0.01	8.95 ± 0.02	9.13 ± 0.02	9.37 ± 0.03	9.17 ± 0.01

7.3.2 Attachment on stainless steel (SS) surfaces

In terms of attachment capacities, great variance was observed among the different strains in both media (Figure 7.2). In SBP juice the HS cocktail and strain 6.2.3 showed weak attachment reaching population of 4.54 and 4.77 log CFU/cm^2, respectively on Day 8. Strain 10.16.3 reached levels of approximately 5.3 log CFU/cm^2, however strain ab2 had the highest attachment among all isolates showing hundredfold stronger adhesion of 7.0 log CFU/cm^2. The same trend in behaviors was observed in the simulation medium of the egg product. The attached cells of HS cocktail on the SS coupons remained below 4.0 log CFU/cm^2 and strain 6.2.3 did not exceed 4.5 log CFU/cm^2. On the contrary, for the environmental isolate 10.16.3 and the survivor of the acetic bath (i.e. strain ab2) a firm attachment was evaluated resulting in 6.0 and 7.4 log CFU/cm^2 of attached population, respectively.

Clearly, strain *Le. gelidum* subsp. *gasicomitatum* ab2 showed a significantly stronger attachment (P≤0.05) capacity compared to the other environmental and food isolates through statistical analysis by paired t-test, with 95% level of confidence.

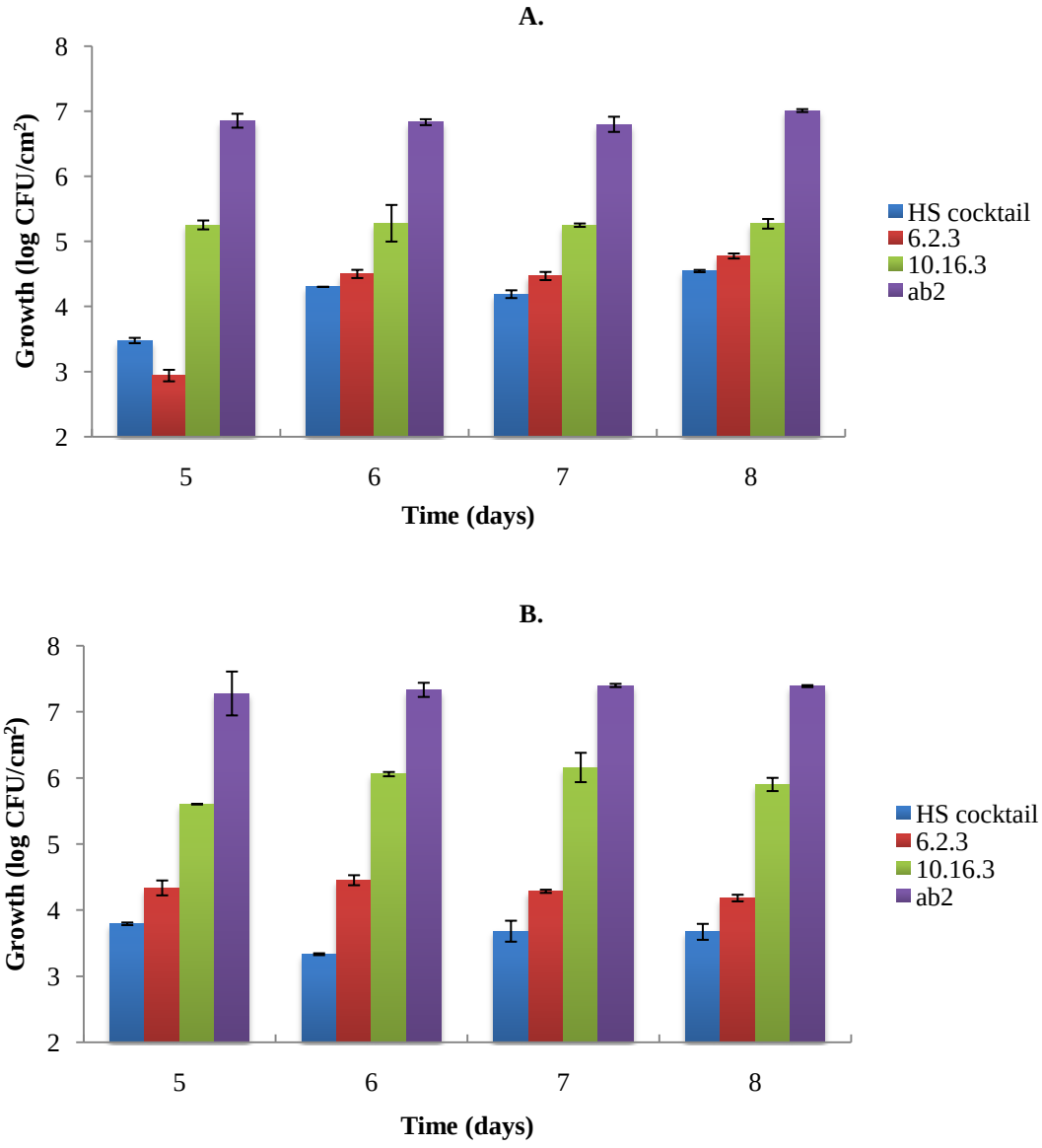


Figure 7.2: *Population of attached cells of the 4* Le. gelidum *subsp.* gasicomitatum *strains on stainless steel (SS) surfaces submerged in: A.) Sweet bell pepper (SBP) juice and B.) Boiled eggs in brine, stored at 7 °C for 8 days.*

7.3.3 Attachment on glass slide

Glass slides were selected in order to evaluate the adhesion on a different type of surface material. The slides of all isolates were stained with a live/dead dye, in order to capture the image of the viable cells. In this case the bead vortexing method for detachment of the cells was not applied but the estimation of the populations adhering was performed semi-quantitatively (Pantanella, Valenti, Natalizi, Passeri, & Berlutti, 2013). The images obtained

from the slides immersed in the suspension of HS cocktail, 6.2.3 and 10.16.3 showed a very sparse attachment of cells (not shown).

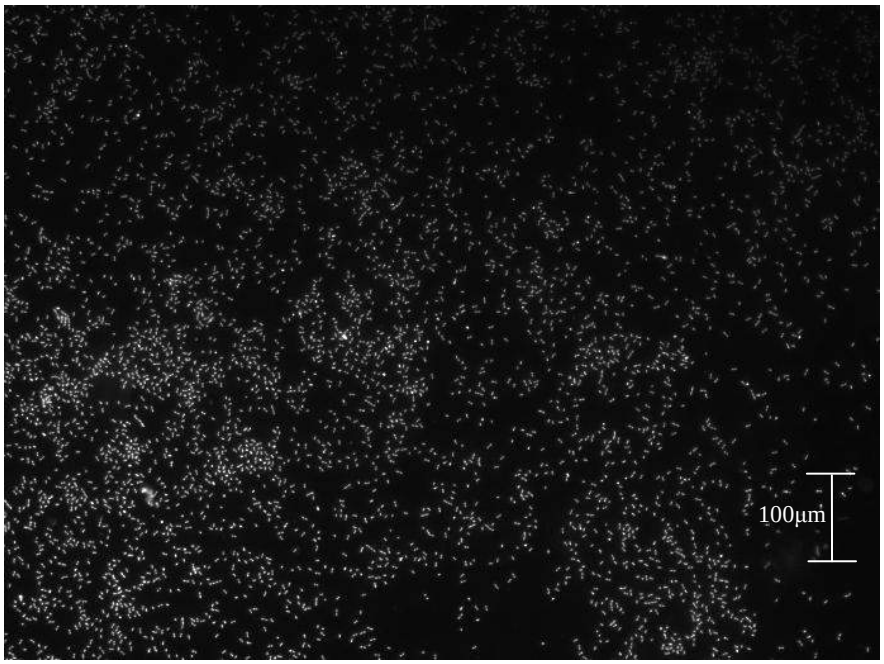


Figure 7.3: *Alive cells of strain* Le. gelidum *subsp.* gasicomitatum *ab2 attached on glass slide after 8 days of incubation at 7 ^{o}C in SBP juice. The image was captured at magnification x40.*

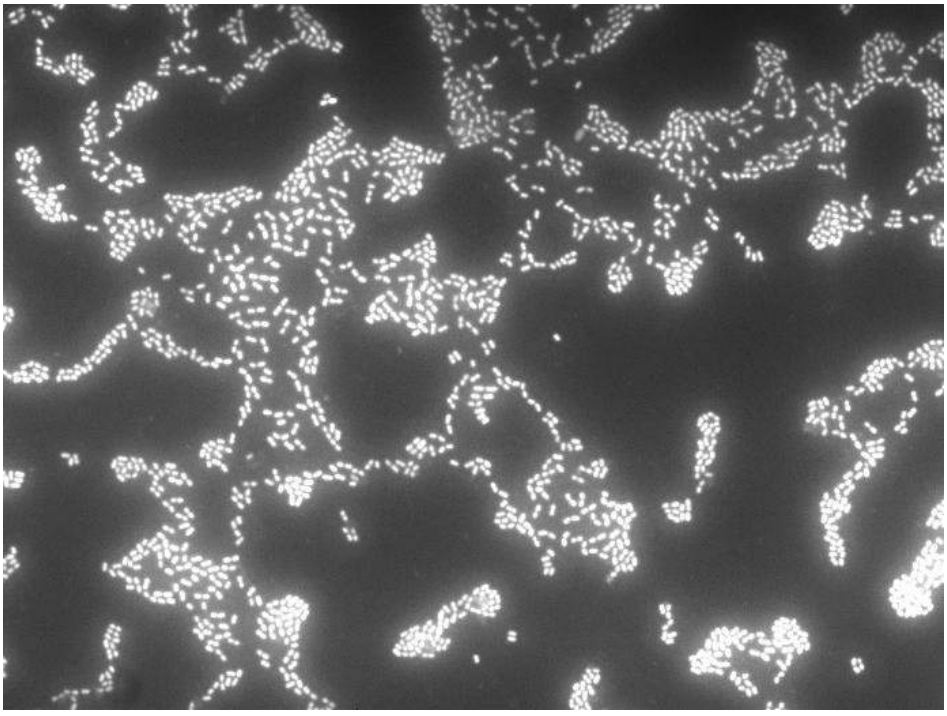


Figure 7.4: *Alive cells of strain* Le. gelidum *subsp.* gasicomitatum *ab2 attached on glass slide after 8 days of incubation at 7 ^{o}C in SBP juice. The image was captured at magnification x100.*

However, the attachment of *Le. gelidum* subsp. *gasicomitatum* ab2 was very dense as shown in Figures 7.3 & 4 in the case of SBP juice and Figure 7.5 A & B in the eggs in brine.

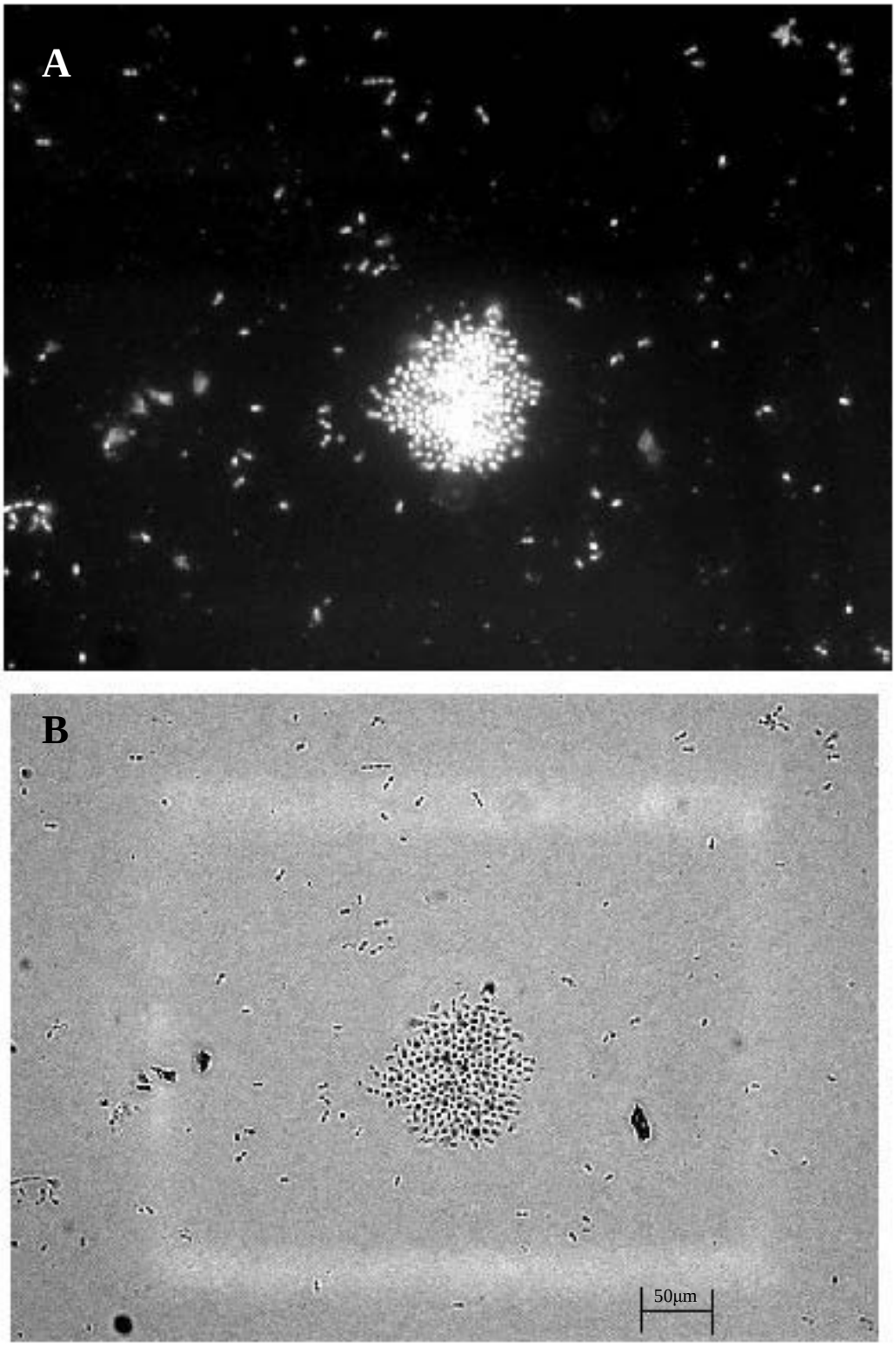


Figure 7.5: *A.) Alive cells of strain* Le. gelidum *subsp.* gasicomitatum *ab2 attached on glass slide after 9 days of incubation at 7 ^{o}C in eggs in brine. B.) Transmission image of the cells. The image was captured at magnification x100.*

7.4 DISCUSSION

Extensive research on virulent bacteria like *Listeria monocytogenes* as well as *Salmonella enterica*, *Pseudomonas aeruginosa* and other pathogenic Gram negative microbes has been conducted in order to assess their ability to attach on surfaces (Fouladkhah et al., 2013; Renier et al., 2011; Zottola & Sasahara, 1994). Embedded cells are considered more resistant to disinfection treatments and antimicrobial substances compared to planktonic cells, therefore eradication can be challenging (Habimana et al., 2010; Kreske et al., 2006; Park et al., 2012). Still the significance of eliminating their presence is crucial for the prevention of foodborne illness outbreaks among susceptible consumers (Carpentier & Cerf, 2011).

Nonetheless, microbes that adhere on surfaces whether they are virulent or spoilage-related cause economic losses to companies due to imposed thorough cleanup and equipment malfunction, such as mechanical blockage or impedance of heat transfer processes (Carpentier & Cerf, 2011). Additionally, biofilms on food contact surfaces act as source of contamination for food material due to continuous detachment of cells from the biofilm (Bai & Rai, 2011). Leuconostocs have been associated with surface biofouling in dairy companies as members of multispecies biofilms (Gunduz & Tuncel, 2006). The use of food simulating media for the evaluation of biofilm formation is a common practice assessing the putative attachment of bacterial cells on food contact surfaces where residual amounts of food material collect. Previously, dairy products, meat exudates and milk have been used ressembling dairy and meat processing environments (Fouladkhah et al., 2013; Knight et al., 2010; Poimenidou et al., 2009).

Generally, for embedded cells the secreted EPS are regarded as an essential component interms of abundance and structural significance of the matrix, although in some cases proteins and other materials predominate (Leathers & Bischoff, 2011). *Leuconostoc* strain-specificity concerning the synthesis of polymeric substances was evaluated by means of a modified randomly amplified polymorphic DNA (RAPD) protocol that incorporated specific primers designed from conserved regions of dextransucrase genes (Holt & Cote, 1998).

The findings of the present study show no correlation between slime-forming strains of *Le. gelidum* subsp. *gasicomitatum* and surface attachment. On the contrary, the strains isolated from eggs in brine - tested in cocktail culture - had the weakest attachment despite the viscous, ropy slime they produced. These results do not confirm previous studies performed. Several slime-producing *Leuconostoc* strains allocated to species *Le. mesenteroides* and *Le. citreum* have shown ability to adhere on stainless steel (SS) surfaces (Côté & Leathers, 2009; Gunduz & Tuncel, 2006). However, their biofilm-forming capacity showed high intraspecies variance (Leathers & Bischoff, 2011).

The investigated slime in the case of HS cocktail represented water-soluble polymers (Capek et al., 2011; Johansson et al., 2011) but no further analytical method was applied for determination. Similarly, *Leuconostoc gelidum* producing water-soluble polymers was proved to suppress habitation of streptococci on the dental surface by decreasing the production of insoluble glucans synthesized by *Streptococcus mutans* that generally induce the pathogenicity of oral biofilm by promoting the adhesion and accumulation of cariogenic microbiota leading to dental plaque (Kang et al., 2007).

The only case that formation of insoluble mutan-like glucan was reported for *Leuconostoc* sp. occurred by *Le. mesenteroides* NRRL B-1355 and production was enhanced when growing on surfaces (Côté & Leathers, 2009).

The genome analysis of *Le. gelidum* subsp. *gasicomitatum* LMG18811^T (Johansson et al., 2011) showed presence of two dextransucrase genes (LEGAS_0699; LEGAS_1012) responsible for slime formation. Moreover, three genes related to adhesion were found. Firstly, a gene encoding a putative mucus-binding protein, whose function remains unknown (LEGAS_0414) and which is also present in the genomes of *Le. mesenteroides* and *Le. citreum*. Secondly, a gene encoding for a serine-rich protein (LEGAS_0537) and a putative collagen-adhesion protein (LEGAS_1063) were detected. No orthologs of the latter genomic loci exist in the chromosomes or plasmids of other leuconostocs.

Serine-rich glycoproteins constitute a highly conserved family of surface-expressed proteins found in numerous Gram-positive pathogens related to formation of infectious biofilm, i.e dental caries, subacute endocarditis and otitis media (Liang et al., 2011; Sanchez et al., 2010). These surface proteins are anchored on the cell wall constituting fimbrial structures that project away from the cell surface (Garnett et al., 2012) and serve as specific docking sites to a certain substratum or as intraspecies bacterial adhesins propagating bacterial aggregation (Zhou et al, 2012; Zhu et al., 2011).

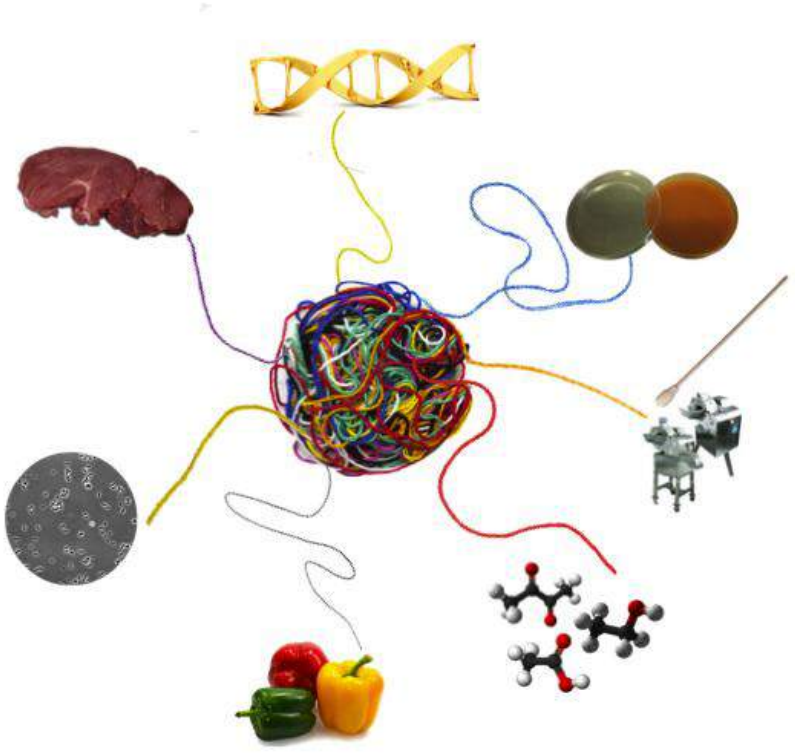
From our findings strain *Le. gelidum* subsp. *gasicomitatum* ab2 growing in both tested substrates reached very high populations (>10^7 CFU/cm^2) of attached cells unlike the rest of the strains. The history of this environmental isolate recovered from an acetic acid bath could have contributed to this phenotype. This is the first documented report in the literature involving *Le. gelidum* subsp. *gasicomitatum* in putative biofilm formation. Biofilm as a term refers to the structure but also denotes the physiological state of the cells (Donlan & Costerton, 2002), hense additional experiments are required in order to clarify the mechanism of attachment and the genes involved with respect to defining this behavior.

A microbial community can be characterized as biofilm, when the sessile cells that form it remain attached to a substratum or interface or to each other and are embedded in extracellular matrix of polymeric substances (EPS) along with proteins and DNA (Lazazzera, 2005). The attached cells do not only have a different phenotype compared to planktonic microbes but also exhibit altered growth dynamics (Poimenidou et al., 2009) and gene transcription profiles (Giaouris et al., 2013). All these parameters need to be studied in order to explain the aggregation of this subspecies on food contact surfaces and the potential effect on the spoilage character of the microbe.

GENERAL DISCUSSION & PERSPECTIVES

8. Introduction

- **8.1 Occurrence of psychrotrophic LAB**
- **8.2 Are there any patterns of microbial interactions?**
- **8.3 Micro-environment conditions in the packaging**
- **8.4 Species *Leuconostoc gelidum* and its current classification**
- **8.5 Metabolic traits of *Le. gelidum* subsp. *gasicomitatum***
- **8.6 Exploitation of LAB genome data**
- **8.7 How to study spoilage?**



8. Introduction

The prevalence of cold-acclimatized LAB as predominant microbiota in packaged meat, fishery and vegetables was encountered and described previously by many pioneering research groups (Dykes et al., 1995; Ercolini et al., 2009; Holley et al., 1996; Leisner et al., 2007; Schirmer et al., 2009; Susiluoto et al., 2003). The present work contributed to existing studies by delineating the problem in the Belgian market, disseminating the multiparametric concept of spoilage, proposing a standard psychrotrophic incubation technique as shelf-life parameter for industrial routine analyses and describing few traits of species *Le. gelidum* subsp. *gasicomitatum* and *Lc. piscium.* Our work focused on vegetables among other commodities, and eventually the relation between psychrotrophic LAB species and sweet bell peppers was documented.
Based on the findings of the present study all discussion points are summarized and future perspectives are briefly outlined.

8.1 Occurrence of psychrotrophic LAB

LAB are considered ubiquitous and are generally associated with all types of foodstuffs as mentioned in **Chapter 1.** Raw vegetables as well as carcasses have an indigenous load of microbes. Theoretically all microbial groups are present on food material and during handlings, packaging and storage certain favored taxa are being selected and thrive throughout shelf-life (Gram et al., 2002). Packaged and chilled-stored food products are frequently dominated by psychrotrophic LAB (Björkroth et al., 2000; Kato et al., 2000; Lyhs et al., 2004; Sakala et al., 2002a). They do not seem to have an affinity with one substrate since they can grow competently on a broad variety of products utilizing different nutrients as energy sources.
Already in **Chapter 2**, the problem was substantiated and the responsible microbial groups were identified. Psychrotrophic LAB unable to grow at 30 ^{o}C are the dominant, presumably spoiling microbes for refrigerated foodstuffs in Belgium. The phylogenetic diversity of psychrotrophic LAB species was assessed in **Chapter 3, 4 & 6.** Food isolates with putative spoilage role (**Chapter 3**), which outcompeted all other microbes during shelf-life, were identified as *Leuconostoc* spp., *Lactococcus* sp., *Lactobacillus* spp. and to a lesser extent *Carnobacterium* sp. and *Weissella* sp. The same species were involved in industrial recalls of spoiled production batches confirming the spoilage potential of certain LAB taxa and their strictly psychrotrophic character (**Chapter 6**). Lastly, in the case of the source tracking study performed in a salad company, presented in **Chapter 4**, mainly the genera *Leuconostoc*, *Lactobacillus* and *Lactococcus* were found circulating in the air, on the food-contact surfaces, the raw and the intermediate products of the processing plant. Members of the genus *Leuconostoc* were associated with quality defects of the retail products as it dominated all products at the end of shelf-life.
Overall, the most widespread species are: *Le. gelidum* subsp. *gasicomitatum, Le. gelidum* subsp. *gelidum, Le. inhae, Le. carnosum, Lc. piscium, Lb. algidus* and *Lb. fuchuensis.*

8.2 Are there any patterns of microbial interactions?

Our studies employed primarily traditional microbiological techniques, application of culture-dependent approaches or combination of the latter coupled with molecular techniques based on PCR assays. In **Chapter 6,** 16S rRNA gene high-throughput sequencing was implemented for a relatively small amount of samples.

Notably, the dominant LAB taxa at the end of shelf-life were assessed (**Chapters 3, 4 & 6**) and not the initial diversities. However, certain patterns of co-existing LAB species were observed. As shown for several samples in **Chapter 3**, presence of *Le. gelidum* subsp. *gasicomitatum*, *Le. gelidum* subsp. *gelidum* and *Lc. piscium* as predominant species was observed and has been previously documented (Rahkila et al., 2012). It could be hypothesized that interaction of protocooperation (i.e. beneficial interaction among species but without having the need to co-exist) is developing among these taxa that allows their co-occurring growth. If no solid and concrete evidence can support such theory then it can be postulated that food-manufacturing installations that follow similar production pipelines, use the same raw materials and have comparable hygienic plans also exhibit similarities in their microbiomes (Vihavainen et al., 2007).

Apart from antagonistic and mutualistic interactions, metabiosis (i.e. beneficial interaction among species when one species profits from this relationship) should be considered a possible scenario (Gram et al., 2002) as respiring, Gram-negative microbes widely abundant in salads and meat can use headspace oxygen and food constituents providing anaerobic environment and putative nutrients inducing the growth of psychrotrophic LAB.

High-throughput sequencing and metagenomics could facilitate the investigation of recurring patterns of microbial succession or dominance involving certain LAB taxa.

8.3 Micro-environment of conditions in the packaging

The natural diversity of LAB is considered vast among species, subspecies and strains, since habitation of specific ecosystems propagates adaptation to environmental conditions. LAB are regarded mesophilic and thrive usually under limited oxygen availability. During the years they have been referred to as anaerobic, facultative anaerobic or aerotolerant (Axelsson, 2004). Cold-acclimatized mesophiles and strictly psychrotrophic species can sustain cold stress and grow without inhibition at refrigeration temperatures. The ability of *Lc. piscum* to upregulate the expression of genes encoding for metabolic enzymes and functional proteins has been studied (Garnier et al., 2010). In **Chapter 1,** the genome domains encoding for cold shock proteins (CSPs) for leuconostocs and lactococci were presented.

In **Chapter 5,** it was shown that *Le. gelidum* subsp. *gasicomitatum* has the ability to sustain high oxygen headspace concentrations, while *Lc. piscium* had more versatile behavior. Super-atmospheric oxygen suppresses other spoilage-related microbes but has limited effect on leuconostocs (Conesa et al., 2007; Geysen et al., 2005; Zhang et al., 2013a; Zhang et al., 2013b). Oxygen also results in a broader spectrum of produced metabolites (i.e. diacetyl, 2,3-butanediol, acetaldehyde, acetoin). A study exploring the metabolic patterns in meat, where heme favors respiration for *Le. gelidum* subsp. *gasicomitatum* also stresses the production of

buttery off-odors and faster growth (Jääskeläinen et al., 2013). The growth rates of leuconostocs are high even starting from initial populations <100 CFU/g (Rahkila et al., 2012).
Elucidation of these stress response mechanisms would assist the understanding of the physiology of psychrotrophic LAB and their metabolism since the energy yielding pathways are shifting along with cold adaptation (Cocaign-Bousquet et al., 1996).

8.4 Species *Leuconostoc gelidum* and its current classification

The species *Le. gelidum* and *Le. gasicomitatum* were recently reclassified as subspecies of *Le. gelidum* along with a novel group that was allocated to a novel subspecies, namely *Le. gelidum* subsp. *aenigmaticum* (Rahkila et al., 2014). In contrast to the first two subspecies, little is known concerning the role of *Le. gelidum* subsp. *aenigmaticum* in food environments. The house-keeping genes that contain information for essential cellular functions and are subject to evolutionary constraints underpin great taxonomic relatedness, however the subspecies have adapted to various niches and apparently have adopted different lifestyles reflected by the accessory gene inventories.
Le. gelidum subsp. *gelidum* has been isolated from meat and fermented kimchi (Kim et al., 2011; Shaw & Harding, 1989). An antimicrobial, bacteriocin-like substance acting against other LAB and *Listeria monocytogenes* was produced by this subspecies in meat (Hastings & Stiles, 1991) and the resistance was plasmid mediated. Its psychrotrophic character has been substantiated through spoilage manifestations in cold-stored food products (Vihavainen & Björkroth, 2007), but was also related to high prevalence during fermentation of cabbage-based kimchi preserved throughout winter when temperatures were lower (Kim et al., 2000).
Likewise, *Le. gelidum* subsp. *gasicomitatum* is of great interest as it possesses inherent spoilage character (**Chapter 5**), it is predominant in early spoilage occurring in refrigerated foodstuffs (Säde, 2011; Susiluoto et al., 2003; **Chapter 6**). The whole-genome sequencing of the type strain LMG 18811^{T} revealed metabolic capacities and physiological attributes, which corroborate its spoilage potential, surface attachment shown in **Chapter 7** suggest biofilm formation and adaptation to food environments (Björkroth, 2005; **Chapter 4**). All these facts prioritize this subspecies among all psychrotrophic LAB taxa compelling further studies to be carried out.

8.5 Metabolic traits of *Le. gelidum* subsp. *gasicomitatum*

One case study described in **Chapter 6** resulting in production batch recall concerned boiled eggs preserved in brine and involved only *Le. gelidum* subsp. *gasicomitatum* as SSO. Extreme formation of slime was observed and very high populations exceeding 10^{8} CFU/g before the end of shelf-life suggest fast outgrowth. The retail product was reconstituted and the growth of selected *Le. gelidum* subsp. *gasicomitatum* strains was assessed in **Chapter 7**. The available nutrients in the product were limited. Sugars of the egg yolk and mainly citrate from the brine could serve as substrate for proliferation, however, the growth dynamics of the

suspended cells compared to sweet bell pepper juice that provides more nutrients (i.e. glucose, sucrose, fructose) was the same.
It would be interesting to evaluate whether sugars exert catabolic repression (Titgemeyer & Hillen, 2002) to citrate uptake when both are present in the growth medium or *Le. gelidum* subsp. *gasicomitatum* can ferment simultaneously sugars and citrate.
Dairy enterococci have been studied with respect to their metabolic patterns when citrate is provided. Some strains of *Enterococcus faecalis* and *Enterococcus faecium* use glucose and fructose until they are fully depleted and subsequently utilize citrate resulting in slower growth (Rea & Cogan, 2003b), while strain *Enterococcus faecalis* E-239 metabolized simultaneously citrate and sucrose (Rea & Cogan, 2003a). Additionally, *Enterococcus faecium* FAIR-E 198 was able to carry out co-fermentation of glucose and citrate from the early stages of growth without exhibiting catabolic repression (Sarantinopoulos et al., 2003). No suppression of citrate uptake mechanism was documented for strain *Lc. lactic* subsp. *lactis* biovar. d*iacetylactis* (Goupry et al., 2000) and *Lb. casei* or *Lb. plantarum* when co-metabolizing hexoses with citrate (Palles et al., 1998).
The investigation of these catabolic traits could possibly elucidate the high growth rates of psychrotrophic bacterium *Le. gelidum* subsp. *gasicomitatum* in tomato-based marinades (Björkroth et al., 2000), brines and vegetable material since in all matrices citric acid is present (Leyva et al., 2014).
It remains unclear how viscous slime is formed from eggs in brine in absence of sucrose, while other sugars at the same time are present in residual amounts.
The whole-genome sequencing of the type strain of *Le. gelidum* subsp. *gasicomitatum* LMG 18811^{T} showed that energy yield can occur from nucleobases and nucleosides apart from sugars and citrate (Johansson et al., 2011). Depletion of nucleosides has been studied for starter culture *Lactobacillus sakei* CTC 494 leading to accumulation of equivalent nucleobases and ribose, which is subsequently fermented through the heterolactic pathway. The ability to utilize these alternative energy sources contributes to the competitiveness of the strain in meat fermentation environment (Rimaux et al., 2011).

8.6 Exploitation of LAB genome data

Whole-genome sequencing of LAB has provided new insights in the taxonomy and evolution of these microbes that possess relatively small genomes, varying between 1.8 and 2.6 Mb, with *Lactobacillus plantarum* constituting an exception featuring a large genome of 3.3 Mb (Siezen et al., 2004). The currently available genomes show that LAB underwent loss of 600-1200 genes related to biosynthesis of nutrients, sporulation and virulence as they adapted to nutrient-rich habitats, while duplication of genes encoding metabolic enzymes and transport proteins occurred (Pfeiler & Klaenhammer, 2007). Their auxotrophic character was emphasized like other attributes associated with food-related lifestyle depicting their evolutionary adaptation (Makarova et al., 2006). Probably, smaller genome provides significant advantage in competitive niches like food.
To date great focus has been given to genomes of probiotic strains and starter cultures while more knowledge about spoilage-related species and gene repertoires they harbor is required.

8.7 How to study spoilage?

Food is a term that has biological (i.e. human nutrition) and cultural (i.e. social anthropology) substance. From the biological point, it is organic material that provides the body with essential nutrients. From the cultural side though, it is a matter of a basic cognitive process. Spoilage by definition is a recognition and interpretation of sensorial stimuli. It is a fine line that is subject to human perception. Microbial spoilage is a complicated phenomenon. In order to be described in depth understanding of its complexity is demanded, as well as combination of different disciplines and tools.

Levels of microbial populations (10^6-10^9 CFU/g) have been empirically correlated to unacceptable food alterations for each type of product, but cannot be used alone as rejection thresholds (**Chapter 2**). The dominant microbial species present in the food matrix constitute an important factor since not every microbe causes alterations at the same contamination levels. The spoilage related species have been studied systematically and the offensive SSO are known. Still, dominance of SSO is actually not a necessary and sufficient condition leading to spoilage (**Chapter 3**). The present study among numerous others showed that spoilage-related character cannot always be attributed collectively to a species, since it is strain-dependent in several cases. The metabolic patterns of a strain under the storage conditions that the substrate is kept determine the spoilage capacity. Intraspecies diversity concerning catabolic features can be significant (**Chapter 5**).

These are the three most tangible parameters of spoilage because they are determined by means of traditional microbiological plating, molecular techniques of identification and chemical/sensorial analysis, respectively.

Nevertheless, several other axes of spoilage need to be taken into account when describing or predicting spoilage.

Firstly, microbial interactions are known to be very important but it is still very difficult to explain phenomena like intra- or interspecies communication. Antagonistic or synergistic effects and quorum sensing definitely have critical impact on microbial behaviour (Bai & Rai, 2011; Gram et al., 2002; Tsigarida et al., 2003). The possible interactions that can be developed are analogous to the diversity of the food microbiota. As the number of putative interactions in the food matrix increase, the outcome of the combination of all these forces becomes more unpredictable. In the future, studies on cell-to-cell communication and mining of systematic metagenomic data of food-consortia evolution in function with storage time will allow more accurate predictions.

Secondly, it is imperative to ponder on the physiology of the cells present in the food matrix under realistic conditions. Spoilage studied *in vitro* is usually performed with optimally grown cells inoculated on food material. In reality, spoilage-related microbes are not always autochthonous to food but can contaminate it in processing environments after having endured stressful habituations that either render them less competitive or more resistant. Cells deriving from biofilms, survivors of disinfection or exposed to mild stresses are presumed to have altered phenotypes (Chorianopoulos et al., 2011; Gahan et al., 1996; Poimenidou et al., 2009; Sidhu et al., 2001). Therefore, the history of the microbes is a parameter as well.

Additionally, technological aspects of the products or inconsistencies of the synthesis can alter the available nutrients. Seasonal, geographical or climatic parameters and stage of

ripening can affect the composition and physicochemical properties of fresh produce. Lastly, microbial spoilage is recurring with chemical spoilage and many changes can be due to indigenous enzymes, respiration, maturation etc.
A more complete scheme presenting the aforementioned spoilage parameters is presented in Figure 8.1.

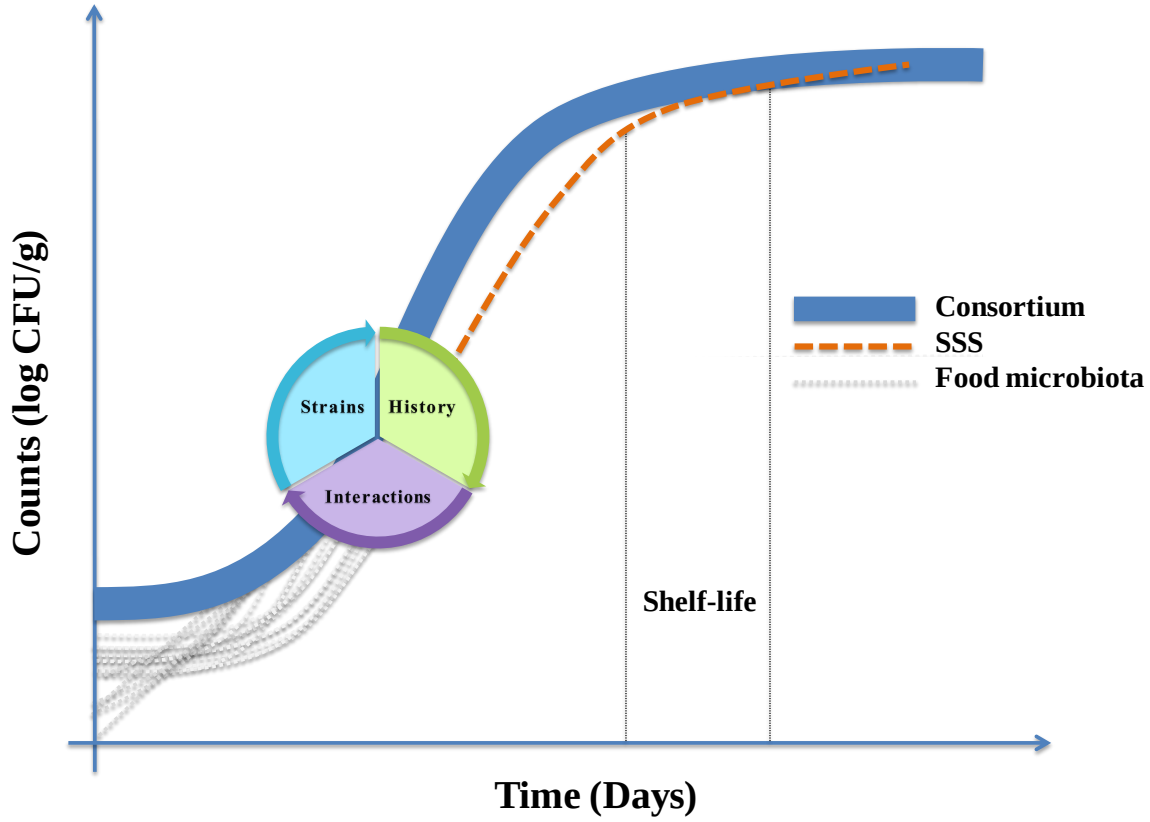


Figure 8.1: *Concept of food spoilage (modified from Huis in't Veld, 1996).*

In this scheme it is summarized that throughout the storage time different interactions will be developed among the food microbiota and based on the history and the spoilage potential of each microbe one or more specific spoilage strains (SSS) will outcompete the others and determine the spoilage pattern. The end of shelf-life is dependent on the spoilage potential of the dominant SSS(s) and can occur at an early of late stage.

CURRICULUM VITAE

Vasileios Pothakos was born on June 25, 1985 in Athens, Greece. Following an education in Sciences, he graduated High School in 2003 and admitted 1st at national level to the Department of Food Science and Technology, Agricultural University of Athens. He conducted his MSc thesis in the Research Group of Industrial Microbiology and Food Biotechnology, Vrije Universiteit Brussel in Belgium and obtained his MSc diploma in Food Science and Technology with specialization in Food Microbiology with the highest distinction in 2008. In 2010 he was granted a National Honorary Distinction from the Greek State Scholarships Foundation and started his doctoral research in the Laboratory of Food Microbiology and Food Preservation under the supervision of Prof. Dr. ir. Frank Devlieghere. Until 2014 he worked on the GOA-Project: "Fast and convenient mass spectrometry-based real-time monitoring of volatile organic compounds of biological origin", dealing with microbial spoilage caused by psychrotrophic lactic acid bacteria.

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SUMMARY

The present thesis describes the problem of fast spoilage caused by psychrotrophic lactic acid bacteria (LAB) occurring on packaged and cold-stored foodstuffs.

In **Chapter 2,** a systematic sampling of retail, packaged and cold-stored food products evaluated the occurrence of psychrotrophic LAB at the end of shelf-life. The currently implemented mesophilic enumeration methods were directly compared to a psychrotrophic shelf-life parameter. The dominant psychrotrophic microbes that were dominating in the food matrix for more than 30 % were underestimated due to their strict psychrotrophic character. These dominant and overlooked microbes were isolated and constituted the first collection of presumptive spoilage, psychrotrophic LAB.

In **Chapter 3** the characterization and identification of the isolated microbes was performed. Their inability to grow at 30 ^{o}C was evaluated confirming strictly psychrotrophic character and their taxonomic position was determined by means of DNA fingerprint typing and sequencing of conserved genomic domains. The wide selection of samples analyzed in **Chapter 2** resulted in a very limited species diversity underpinning the role of few LAB genera.

In **Chapter 4** the results of a source tracking conducted in a ready-to-eat (RTE) vegetable salad processing environment facilitated the assessment of possible cross-contamination routes and emphasized on the role of sweet bell peppers as ecological niche harboring psychrotrophic LAB species. Sampling of the entire production facility showed prevalence of different LAB genera on surfaces, air, raw material, intermediate products and water samples. Nonetheless, only genus *Leuconostoc* became dominant after storage at the end of shelf-life exhibiting the highest frequency of isolation. Noteworthy, different biotypes of *Leuconostoc* spp. were recovered from harsh environments like acid baths and disinfected surfaces.

Having correlated the presence of the most frequently isolated psychrotrophic LAB to sweet bell peppers a selection of isolates from food samples (**Chapter 3 & 6**) allocated to species *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium* were tested on sweet bell pepper simulation medium with respect to their metabolic patterns and growth dynamics under different packaging conditions, in **Chapter 5**. The two species showed different growth dynamics, interspecies diversities and adaptation to oxidative stress, however the spectrum of produced metabolites was very similar among the spoilage-related strains mainly focusing on acetic acid and ethanol while induction of buttery off-odor VOCs (like diacetyl) was observed under aeration.

During the period between 2010 and 2014 several case studies were analyzed in our laboratory shown in **Chapter 6**. These case studies were actual production batch

recalls that resulted in loss of tons of products caused by unexpected, early spoilage manifestation prior to the end of shelf-life. The responsible microbiota were determined by 16S rRNA gene high-throughput sequencing (HTS) confirming all previous studies. The same LAB species recovered from the screening of the Belgian market **Chapter 2** and the source tracking **Chapter 4** that were found dominant at the end of shelf-life were implicated in actual cases of spoilage.

Lastly, in **Chapter 7** physiological traits of species *Le. gelidum* subsp. *gasicomitatum* are studied with respect to surface attachment. A selection of strains (**Chapter 4 & 6**) were tested in different substrates and type of food contact surfaces in order to evaluate the intraspecies diversity, the impact of nutrients and the substratum on the attachment of cells. Possible attachment of cells that remain embedded on surfaces could explain cross-contamination in a processing environment (**Chapter 4**). Once present in the food matrix this microbe could grow competently (**Chapter 5**) and become dominant during storage reaching high population (**Chapter 2**) resulting in unexpected spoilage defects before the end of shelf-life (**Chapter 6**).

SAMENVATTING

De thesis beschrijft het probleem van snel bederf veroorzaakt door psychrotrofe melkzuurbacteriën (MZB) bij verpakte en koel bewaarde levensmiddelen.

In **hoofdstuk 2** werd het voorkomen van psychrotrofe MZB geëvalueerd op het einde van de houdbaarheid met behulp van een systematische monstername van retail producten. De huidig geïmplementeerde mesofiele telmethoden werden direct vergeleken met een psychrotrofe techniek. Deze dominante en vergeten bacteriën werden geïsoleerd en vormden de eerste groep die verantwoordelijk was voor presumptief bederf, namelijk psychrotrofe MZB.

In **hoofdstuk 3** werden de geïsoleerde bacteriën gekarakteriseerd en geïdentificeerd. Hun onvermogen om te groeien bij 30 °C werd geëvalueerd en bevestigde het strik psychrotroof karakter. Hun taxonomische positie werd bepaald door DNA fingerprint typing en sequencing van de geconserveerde genomische domeinen. De brede selectie van de geanalyseerde stalen in **hoofdstuk 2** resulteerde in een erg gelimiteerde diversiteit in species, welke de rol van een weinig MZB geslachten ondersteunt.

In **hoofdstuk 4** wijzen de resultaten van een bron onderzoek uitgevoerd in een salade producerende omgeving op de rol van paprika's als ecologische niche voor psychotrofe melkzuurbacteriën. Na staalname in de gehele productiefaciliteit blijkt dat verschillende genussen van melkzuurbacteriën voorkomen op oppervlakten, lucht, grondstoffen, tussenproducten en waterstalen. Niettegenstaande, enkel het genus *Leuconostoc* wordt dominant bij einde houdbaarheid, wat leidt tot de hoogste frequentie van isolatie. Opmerkelijk is dat er verschillende biotypes van *Leuconostoc* spp. werden gevonden in de fabriek.

Na correlatie van de aanwezigheid van de vaakst geisoleerde psychotrofe melkzuurbacteriën met paprika's, werden een selectie van isolaten uit voedsel stalen (**hoofdstuk 3 & 6**) toegewezen aan de species *Leuconostoc gelidum* subsp. *gasicomitatum* en *Lactococcus piscium* getest op paprika simulatie medium, rekening houdend met hun metabole patronen en groeidynamiek onder verschillende verpakkings condities, in **hoofdstuk 5**. De twee species vertonen een verschillende groeidynamiek en interspecies verschillen.

Tijdens de periode tussen 2010 en 2014 werden een aantal case studies geanalyseerd in het LFMFP, weergegeven in **hoofdstuk 6**. Deze case studies bestudeerden authentieke batches die door vroeg, onverwacht bederf voor het einde van de houdbaarheidsdatum teruggeroepen werden met het verlies van meerdere tonnen producten tot gevolg. De micro-organismen verantwoordelijk voor het bederf werden geïdentificeerd door metagenomen, wat eerdere studies bevestigt. De MZB stammen

gevonden in **hoofdstuk 2 & 4** waren bleken de voornaamste bederfveroorzakers te zijn in werkelijke situaties.

Ten slotte worden in hoofdstuk 7 de fysiologische eigenschappen van de stam *Le. gelidum* subsp. *gasicomitatum* onderzocht met betrekking op oppervlakteaanhechting. Een selectie van stammen (**hoofdstuk 4 & 6**) werd getest om de diversiteit binnen de soort en de invloed van nutriënten en het substraat op het aanhechtingsvermogen van de cellen te kunnen evalueren. De mogelijke aanhechting van cellen die achterblijven op oppervlakten kan de kruiscontaminatie in een verwerkingsomgeving verklaren (**hoofdstuk 4**). Eens terecht in een voedselmatrix kan het micro-organisme sterk uitgroeien (**hoofdstuk 5**) en dominant worden binnen de bederfflora (**hoofdstuk 2**), waardoor onverwacht bederf ontstaat voor het einde van de houdbaarheidsdatum (**hoofdstuk 6**).

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