



**MOLECULAR MONITORING OF MEAT SPOILING *PSEUDOMONAS* SPECIES  
AND  
ANALYSIS OF STAPHYLOCOCCAL ENTEROTOXIN EXPRESSION AND FORMATION**

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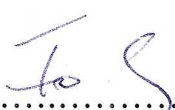
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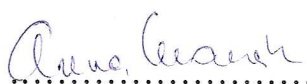
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*“I am among those who think that science has great beauty.  
A scientist in his laboratory is not only a technician: he is also  
a child placed before natural phenomena which  
impress him like a fairy tale.”*

Marie Curie  
(1867-1934)

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## LIST OF ABBREVIATIONS

Agr	accessory gene regulator
AIP	autoinducing peptide
ALCV	ammonium lactate, cristal violet
AOAC	Asociacion of Official Analytical Chemists
ARDRA	amplified rDNA restriction analysis
$a_w$	water activity
BHI	brain heart infusion
bp	basepair
BLAST	Basic Local Alignment Search Tool
<i>carA</i>	carbamoyl phosphate synthetase small subunit
CDC	Centres of Disease Controls
cDNA	complemetary DNA
CETCH	cetrimide, 2-hydroxy-2'4'4'- trichloro-o-diphenil oxide
CF	cystic fibrosis
CFC	cetrimide-fucidine-cephaloridine
CFU	colony forming unit
CN	cetrimide and naladixic acid
CPS	coagulase positive staphylococci
$C_T$ , $C_p$	cycle threshold or crossing point
ddH <sub>2</sub> O	double distilled water
DEPC	diethyl pyrocarbonate
DFD	dark, firm, pale
dNTP	Deoxyribonucleotide triphosphate
ds DNA	double stranded deoxyribonucleic acid
E	efficiency
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EFSA	European Food Safety Authority
<i>egc</i>	enterotoxin gene cluster
ELFA	enzyme linked fluorescent assay
ELISA	enzyme-linked immunosorbent assay
FRET	Fluorescence resonance energy transfer
GALT	gut associated lymphoid tissue
GH	Grant and Holt medium
GI	gastro intestinal
GSP	sodium glutamate starch, phenol red media
<i>gyrB</i>	gene of the $\beta$ subunit of DNA gyrase
HP	Herella <i>Pseudomonas</i> agar
HUS	haemolytic uraemic syndrome
ICMSF	International Commission on Microbiological Specifications for Foods
IGS	intergenic spacer region
LAB	lactic acid bacteria
MA	modified atmosphere
MGSP	modified sodium glutamate starch, phenol red media
MGV	Masourovsky medium
MLSA	multi-locus sequencing analysis

mRNA	messenger ribonucleic acid
MRS	de Man-Rogosa-Sharpe agar
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
NCBI	National Center for Biotechnology Information
OD	optical density
PAT-80	Tween <sup>TM</sup> 80 hydrolysis medium
PBP2a	penicillin binding protein
PCA	plate count agar
PCATB	tributylin agar
PCR	polymerase chain reaction
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
PVL	Panton-Valentine leukocidin
PSE	pale, soft, extrudative
Rep-PCR	repetitive extragenic palindromic PCR
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAPD	Random Amplification of Polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RDP	Ribosomal Database Project
RE	relative expression
Rot	repressor of toxin
RPLA	reverse latex agglutination
<i>rpoB</i>	gene of the $\beta$ -subunit of RNA polymerase
<i>rpoD</i>	gene of sigma subunit of RNA polymerase
SAGs	superantigens
SAPIs	<i>Staphylococcus aureus</i> pathogenicity islands
Sar	staphylococcal accessory gene regulator
<i>sea</i>	staphylococcal enterotoxin A gene
SEA	staphylococcal enterotoxin A
<i>sed</i>	staphylococcal enterotoxin D gene
SED	staphylococcal enterotoxin D
SEs	staphylococcal enterotoxins
SEI	staphylococcal enterotoxin-like
SFP	staphylococcal food poisoning
SFPOs	staphylococcal food poisoning outbreaks
SM	skim milk agar
SMC	standard methods caseinate agar
STEC	shiga toxin producing <i>Escherichia coli</i>
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-Boric acid EDTA buffer
TE	Tris-EDTA buffer
TES	Tris-EDTA-sodium chloride buffer
TSST-1	toxic shock syndrome toxin 1
<i>Tth</i>	<i>Thermus thermophilus</i>



## 1. INTRODUCTION

The consumption of red meat composes the part of human diet since the days of hunting and gathering thanks for its texture and flavour characteristics. The domestication of animals (sheep) which started at the dawn of the Neolithic revolution around 8500 BC also contributed to this habit (Adams and Moss, 2008). The red meat contains different proteins, essential amino acids and trace elements that are necessary for the human health and could not be substituted from other sources (Csapó, 2008; Kauffman, 2001).

These characteristics and the high water activity of meat also ensure a perfect medium for microbial growth. The meat is originally sterile and is contaminated after slaughtering with different psychrotroph, psychrophil and mesophil microbes from the environment (Corry, 2007). On the surface of chilled, aerobically stored meat *Pseudomonas* species play an important role and become dominant during storage. Their metabolic activity (especially proteolysis and lipolysis) contribute to the spoilage as well as off-odour and slime formation (Greaser, 2001). The characterization of the genus *Pseudomonas* faces difficulties based on their genetic heterogeneity. Reclassification of species that originally belonged to the genus of *Pseudomonas* is continuous based on the 16S rRNA studies. However, it is important to know the molecular diversity and characterization of *Pseudomonas* species to better understand the role of this type of bacteria in spoilage. In this thesis, different molecular methods were used to study the biodiversity of *Pseudomonas* species derived from pork meat which was stored at 4 and 8°C under aerobe conditions. These methods include RAPD, ARDRA, *rpoB*-RFLP, sequencing of 16S rDNA and *rpoB* genes and application of species-specific PCR. A genus-specific primer pair was also tested in parallel with the applicability of different media developed for detection of *Pseudomonas* species from various sources. To characterize the proteolytic and lipolytic activities of the isolates at different temperatures a semi-quantitative method was used.

Flavobacteria also play a role in food spoilage by composing the initial part of microflora. The characterization of these microbes derived from meat is rare because the emending of the family *Flavobacteriaceae* started in the middle of '90s when the genus *Chryseobacterium* was also established and became the member of this family (Vandamme et al., 1994; Jooste and Hugo, 1999). There is only limited information regarding *Chryseobacterium* isolates derived from spoilage. To characterize the growth of a *Chryseobacterium* isolate in parallel with a type strain, cultivations in liquid cultures were performed at different temperatures during this work. Competition behaviour was also tested with one *P. fragi* isolate combined in different ratios of inoculated cells.

Food safety is an essentially emerging problem for food industry. Consumers demand fresh food all year around containing less and less preservatives. The global distribution and consumption of these foods emphasize the importance of food safety. Contamination of food with a pathogen presents a greater economic and social problem than in the early years (Bhunia, 2008). The picture of foodborne infections changes continuously. While new pathogens appear, already existed pathogens may acquire new characteristics and occur in unexpected food matrices. The last outbreak that called the consumers' attention to the importance of food safety and prevention was detected in 2011 May in Germany and then at the end of June in France belonged to Shiga toxin 2a-producing *E. coli* serotype O104:H4. The source of infection of *E. coli* O104:H4 in both countries derived from the consumption of sprouts. The STEC outbreak reported from Germany was the second largest STEC outbreak reported in history worldwide (ECDC, 2011a; ECDC, 2011b; ECDC, 2011c).

*Staphylococcus aureus* is one of the most frequent pathogen that causes food-borne outbreaks. It is responsible for staphylococcal food poisoning (SFP) by producing heat-stable toxin. SFP was the fourth most common causative agent in foodborne illness within the EU in 2008 (EFSA, 2010). *Staphylococcus aureus* cells could be eliminated from the food but the toxin remains stable under rigorous heating. Contamination is mainly associated with improper and extensive manual handling of cooked or post-processed protein rich food, combined with inadequate heating and/or improper storage temperature of the food (Le Loir et al., 2003; Smyth et al., 2004). At present, 22 staphylococcal enterotoxin or enterotoxin-like protein were found. The two most often reported enterotoxins are however, the SEA and SED (K  rouanton et al., 2007; Wieneke et al., 1993) which are encoded on genetically different genomic constituents. SEA (staphylococcal enterotoxin A) is encoded by the *sea* gene the expression of which is linked to the life cycle of SEA-encoded prophage at a determined level (Sumby and Waldor, 2003). SED (staphylococcal enterotoxin D) is a plasmid-encoded protein that is connected to *sed* determinant and up-regulated by the accessory gene regulator (Agr) system via RNA III-mediated reduction of Rot (repressor of toxin) activity. The behaviour of pure bacteria and the gene expression of their toxins in liquid cultures may be very different from those in food matrices where interaction of other bacteria through molecular signalling is current. To better understand the effects of these circumstances on SEA and SED formation, gene expression studies of *sea* and *sed* were performed in the thesis on four processed pork products which possessed different intrinsic factors. As a control, the pure culture of *Staphylococcus aureus* SA45 was cultivated under optimal growth conditions in pH controlled batch cultures. The relative expression of *sea* and *sed* as well as the extracellular SEA and SED production were detected by the application of quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA).

## 2. LITERATURE REVIEW

### 2.1. Characterization of red meat

The category of “red meat” often involves meat derived from cattle sheep, pigs, goats, deer and horses which could be completed with meat from several other mammals (Corry, 2007). On average, most muscles should contain about 1% ash (primarily represented by the elements potassium, phosphorus, sodium, chloride, magnesium, calcium, iron and zinc), 1% carbohydrate (primarily glycogen in ante mortem), 5% lipid, 21% nitrogenous compounds (predominantly proteins), and the rest (72%) as moisture (Kauffman, 2001).

Proteins from meat origin are composed of the 22 amino acids from which 10 essential and 12 non-essential (Kauffman, 2001). The essential amino acids must be supplied from dietary intake to meet the body's needs because the human body cannot synthesize them (Kim, 2001).

Meat is a source of iron and zinc but also supports the absorption of minerals from other foods. Zinc is essential for the metabolism of nucleic acids, as well as the correct development and function of brain and immune system and responsible for membrane functions. Heme iron is mostly found in hemoglobin and myoglobin. It is present only in animal organs but not in any plant tissue (Monsen, 1988). Meat is a valuable dietary source of all the vitamin B, including thiamine, riboflavin, niacin, vitamin B6, pantothenic acid, and vitamin B12 (Kim, 2001).

After slaughtering different biochemical and physical changes start in the muscle of the animal which converts the muscle into meat. The metabolically active system turns to inextensible and moderate. The time interval and rate of post-mortem metabolism have an important effect on the properties of the muscle and its subsequent use for food (Greaser, 2001). In living muscle the pH is normally around 7.4 but after slaughtering it declines when the respiration turns to anaerobic processes (Young and West, 2001). The decline in pH is usually stopped and becomes stable at pH 5.5-5.8 before the total glucose in the muscle has been utilized. If the animal was exposed to excessive stress or exercise before slaughtering the level of muscle glycogen (glucose concentration) is very low or exhausted. The limited supply of glycogen in the muscle causes the synthesis of lower amount of lactic acid. This acidification will continue only until the glycogens run out. Red meat appears darker than normal red meat which is usually known as dark, firm, dry (DFD) meat with the final pH as 6 or above (Young and West, 2001). This is the consequence of the higher respiration rate that reduces the oxygen penetration and in parallel reduces the level of oxygenated myoglobin or oxymyoglobin (Gresaer, 1986). This gives the red colour to the meat that the consumers usually associate with freshness. The spoilage presents at lower bacterial cell densities ( $10^6$  CFU/cm<sup>2</sup>) than on normal meat especially in a modified atmosphere or in vacuum

pack (Newton and Gill, 1978). These conditions appear most often on beef but also occur on pigs or other meat animal's meat.

In contrast, pork meat may achieve a usually low pH called pale, soft, extrudative (PSE) because of releasing more moisture and possessing paler colour, softer texture than the normal meat. It is usually the result of accelerated post-mortem glycolysis while the muscle temperature is still high. The PSE meat is directly related to porcine stress syndrome in which the animals may die as a result of mild stress (Gill, 1982; Greaser, 1986; Corry, 2007).

### **2.1.1. Microbiological aspects of food spoilage especially on pork**

Several intrinsic and extrinsic factors could influence the composition of the spoilage-causing microbiota on food. Focusing on the meat, these intrinsic characteristics belong to the meat itself such as the origin of the meat (e.g. red meat, poultry meat, fish or processed meat), the structure of the meat (e.g. DFD or PSE) different  $a_w$ , pH, fat content. The extrinsic factors contain the gaseous composition of storage environment (e.g. aerobe, vacuum or modified atmosphere), type of packing and other storage conditions such as chilling at different temperatures or freezing.

Food spoilage microorganisms are those which upon growth in a food, produce undesirable flavour (odour), texture and appearance, and make the food unsuitable for human consumption (Jackson et al., 1997). The tissue of a healthy animal is protected against infection by the combination of physical barriers and the immune system. Internal organs and muscles from a freshly slaughtered carcass are relatively sterile. However, during slaughter and dressing, microorganisms contaminate the surface of carcasses and meat cuts providing perfect environment for microbial growth (Bell, 2001; Marshall and Bal'a, 2001). The most heavily colonized areas of the animal that may contaminate the fresh meat are the skin and gastrointestinal tract. Contamination from other sources also occurs such as the hide and hair of the animal, the abattoir environment (e.g. the water, air, soil) or the equipment, processing hand tools, knives as well as worker's hands during the processing (Corry, 2007). These sources contain a mixed microbial population of mesophilic, psychrophilic or psychrotrophic organisms. The temperature range for growth of these is demonstrated in Table 1. These microbes are Gram negative and Gram positive bacteria which involve mainly spoilage-causing bacteria as well as food-borne pathogens such as *Micrococcus*, *Pseudomonas*, *Moraxella*/ *Acinetobacter*, *Lactobacillus*, *Flavobacterium*, coryneforms, *Enterobacteriaceae*, *Staphylococcus*, *Streptococcus* *Bacillus* and *Brochothrix thermosphacta* (Dainty et al., 1983). Yeasts and mold could also be found in parallel (Corry, 2007). The dominance of them depends on the storage conditions after slaughtering. After dressing carcasses are washed to remove visible contamination and then it is cooled to chill temperatures. The cold shock may cause some reduction in numbers of mesophiles and psychrotrops.

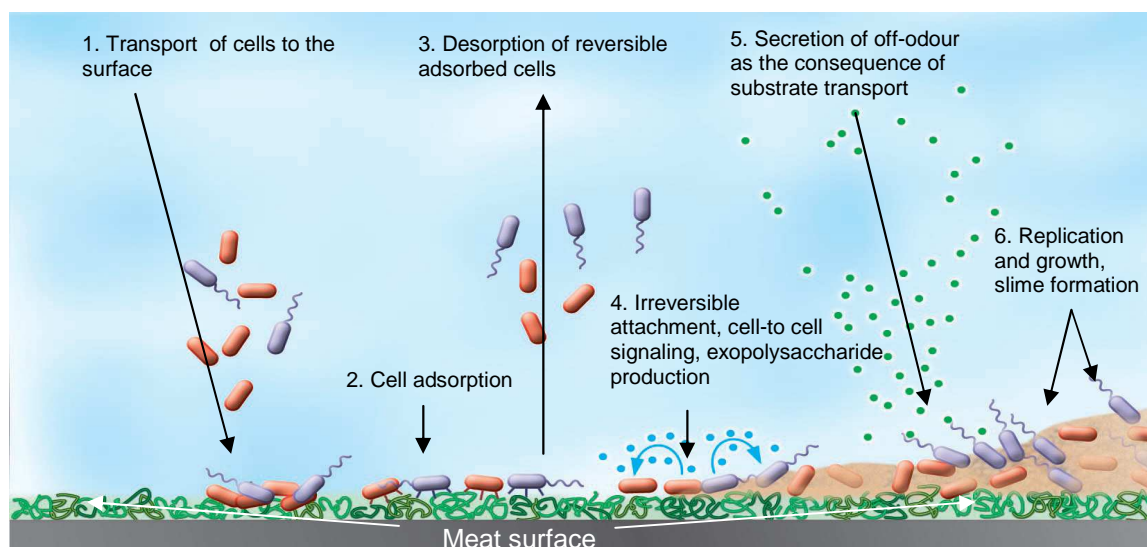
**Table 1.** Groups of microbes according to their temperature ranges they grow

Group	Temperature (°C)		
	Minimum	Optimum	Maximum
Psychrophiles (obligate psychrophiles)	-5 to +5	12-15	15-20
Psychrotrophs (facultative psychrophiles)	-5 to +5	25-30	30-35
Mesophiles	5-15	30-40	40-47

Adapted from ICMSF 1980

The initial cell count on red meat varies between  $10^2$ - $10^3$  CFU/cm<sup>2</sup> or gram (Borch et al., 1996) especially on pork is less than  $10^4$  CFU/cm<sup>2</sup> (Mackey and Roberts, 1993) and going to increase depending on the different factors that affect the microbiota.

Colonization of microbes on the muscle surface is the initial step of microbial spoilage which involves two stages (Firstenberg-Eden, 1981). In the first stage bacteria are in a tight interaction with each other thanks to Van der Waals forces or other psychochemical factors (Benedict, 1988; Dickson and Anderson, 1992; Firstenberg-Eden, 1981). In addition to the cell density, factors such as type, surface, growth phase, temperature and motility may also influence bacterial attachment to muscle surface (Chung et al., 1989; Farber and Idziak, 1984). The second stage contains an irreversible attachment to the meat surface. Attachment is facilitated by the microbial excretion of an extracellular polysaccharide layer as glycocalix (Costerson and Irvin, 1981). Bacteria already present on surfaces may influence the ability of other bacteria attachment (McEldowney and Fletcher, 1987). When the count reaches  $10^8$  CFU/cm<sup>2</sup> the off odour is followed by tackiness (Molin, 2000) that indicates the formation of bacterial slime. This slime layer is a biofilm that develops on fresh meat (Jay et al., 2005). The mechanism of attachment and slime formation is shown on Fig. 1.

**Figure 1.** Process of attachment of bacteria and slime formation on meat surface.

(Source: Breyers and Ratner, 2004, modified drawing)

To prolong the shelf life of meat and meat products three different packaging types are in use: air, vacuum and modified atmospheres (MA). Pork is generally stored aerobically or in MA while beef in a vacuum or MA due to the need for tenderization during an extended storage. Transitions between different packaging-types may be performed for retail cuts (Borch et al., 1996).

A large portion of marketed perishable meat and poultry products are stored at refrigeration temperatures. Mesophiles are generally a major component of the initial microbiota while psychrotrophic organisms form only a small percentage of that. Refrigeration restricts the growth of mesophiles and allows psychrotrophic microorganisms to grow and eventually dominate the microflora. As microbial growth occurs during storage the composition of microbiota is altered so that it is dominated by a few or often a single microbial species (Adams and Moss, 2008). The relatively rapid growth rate of *Pseudomonas* species allows successful competition with other mesophiles and psychrotrophs. Other microbes occurring in a lower number include *Acinetobacter*, *Psychrobacter*, *Flavobacterium*, *Moraxella*, *Enterobacteriaceae* and sometimes *Brochothrix thermosphacta* and belong to the meat contaminating microbiota. Psychrotrophic species belonging to the *Enterobacteriaceae* are *Serratia liquefaciens* and *Hafnia alvei* (Dainty and Mackey, 1992; Borch et al., 1996). Certain species of the genera *Flavobacterium* and *Chryseobacterium* also contributes to the initial bacterial population in a considerable ratio (Jooste and Hugo, 1999; Jackson et al., 1997) but their growth properties and role in spoilage is less examined. The genus *Chryseobacterium* is one of the genera that built on the ruins of the genus *Flavobacterium* (Bernardet et al., 1996) after the genus *Flavobacterium* set right in the middle of '90s following extensive phylogenetic investigations. Based on the 16S rRNA studies several former *Flavobacterium* species were transferred to the novel genus *Chryseobacterium* (Vandamme et al., 1994) and novel species have been described, including *Chryseobacterium joostei* (Hugo et al., 2003) from milk, *Chryseobacterium vrystaatense* sp. nov. from raw chicken (de Beer et al., 2005).

Vacuum-packaging is frequently used for cooked meat products. Generally the shelf-life for vacuum packaged meat is about 15 weeks. Initially, the oxygen permeable packaging allows the growth of *Pseudomonas* spp., but as the oxygen is consumed the restriction of growth of pseudomonads happen. When the accumulation of CO<sub>2</sub> occur inside and the package lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, and *Carnobacterium*) take over (Borch et al., 1996). If it is combined with curing salt and nitrite the growth of psychrotrophic lactic acid bacteria is also favoured (von Holy et al., 1991). *Brochothrix thermosphacta* may also be a dominant part of the bacterial flora (Nielsen, 1983). The microorganisms reach their maximum population of around 10<sup>7</sup> CFU/cm<sup>2</sup> after about a week's storage but the sour acid odour develops only slowly thereafter. The extension of shelf life produced by vacuum packing is not seen with high pH (>6.0) meat because *Shewanella putrefaciens* and psychrotrophic *Enterobacteriaceae* can grow. Storage of vacuum

packed meat at temperatures above the chill range is likely to select a microflora containing mesophilic *Clostridium* species (*Clostridium botulinum*, *C. perfringenes*) and *Enterobacteriaceae* (Ingram and Dainty, 1971) which would be highly hazardous (Corry, 2007).

Storage under modified atmospheres is also used for cooked, cured meat products. In modified atmospheres the growth of pseudomonads is inhibited by the CO<sub>2</sub> while the high levels of O<sub>2</sub> maintain the bright red colour of oxygenated myoglobin in the meat. Here the microflora depends on the type of meat, its storage temperature and whether it was vacuum packed or aerobically stored previously. The microflora and spoilage tend to follow a similar pattern to that of vacuum packed meat. As a result, the group of microbes that becomes predominant is the lactic acid bacteria with a small proportion of *Pseudomonas* spp., *Enterobacteriaceae* and sometimes *Brochotrix thermosphacta*. The product deteriorates slowly due to multiplication of *Lactobacillus* including *Carnobacterium* spp. which produce sour type odour (Gill and Newton, 1979; Adams and Moss, 2008).

## **2.2. Food spoilage-causing *Pseudomonas* species and their role on aerobically stored meat**

Storage at low temperature (0-15°C) allows the growth of psychrophils and psychrotrophs to overgrow and dominate on the spoilage microbiota (Jackson et al. 1997).

The final bacteria population during aerobically stored chilled red meat is dominated by Gram negatives, especially the members of the genus *Pseudomonas* (Dainty and Mackey, 1992; Olofsson et al., 2007). The reason why the pseudomonads become dominant is that they are able to utilize low molecular weight nitrogen compounds as energy source under highly oxidative circumstances. This is a clear competitive advantage for pseudomonads, since meat contain relatively low levels of simple sugars and more complex energy sources such as protein and fat that are not serving so significant substrates for growth until later in spoilage when high bacterial populations are attained. The first indication of spoilage in fresh meat is the production of off-odours which become apparent when microbial counts reach around 10<sup>7</sup> CFU/cm<sup>2</sup> (Olofsson et al., 2007). At this point it is believed that the microorganisms switch from the diminishing levels of glucose in the meat and amino acids as a substrate to grow. In meat with lower levels of residual glucose this stage is reached earlier 10<sup>6</sup> CFU/cm<sup>2</sup> and it accounts for the earlier onset of spoilage in high pH meat. The pseudomonads are able to grow within the usual pH range of muscle foods (pH 5.5 and 7.0) while many bacteria e.g. *Moraxella* and *Acinetobacter* species are less capable of competing under refrigeration temperatures at the lower pH in this range (Jay et al., 2005).

### 2.2.1. The genus *Pseudomonas*

The genus *Pseudomonas* belongs to the family of *Pseudomonadaceae* that composes the subclass of Gammaproteobacteria which is the most diverse class of Proteobacteria (Deák, 2010). The proposal of the new genus *Pseudomonas* belongs to Migula at the very end of the nineteenth century who described the first member of the genus (Palleroni, 2010). This is one of the most diverse genera the taxonomy of which has undergone many changes since earlier descriptions (Palleroni, 1984). The genus was redefined and its phylogenetic relationships with related groups or species that were determined previously as *Pseudomonas* have been reclassified based on 16S rDNA sequences (Kerstens et al., 1996). The rRNA sequence similarities between *Pseudomonas* species were determined initially by hybridization of DNA to ribosomal rRNA. According to the results the genus *Pseudomonas* was subdivided into five distinct “rRNA homology” groups as demonstrated in Table 2 which was based on rRNA similarities and estimations of phylogenetic connection (Palleroni et al., 1973; Palleroni, 1984). The subgroups constitute different classes of Proteobacteria (Deák, 2010).

**Table 2.** RNA “homology group” based on DNA-rRNA hybridization

RNA group	Subclasses of Proteobacteria	Results of reclassification into new genus
I	$\gamma$	<i>Pseudomonas</i>
II	$\beta$	<i>Burkholderia</i> , <i>Ralstonia</i>
III	$\beta$	<i>Comamonas</i> , <i>Acidovorax</i> , <i>Hydrogenophaga</i>
IV	$\alpha$	<i>Brevundimonas</i>
V	$\gamma$	<i>Xanthomonas</i> , <i>Stenotrophomonas</i>

The rRNA group I represents the “true” member of genus *Pseudomonas* which usually characterizes as *Pseudomonas* sensu stricto containing *P. aeruginosa*, *P. fluorescens*, *P. putida* and related species. At the time of this writing (September, 2011), 212 species and subspecies belong to this genus

([http://www.dsmz.de/microorganisms/bacterial\\_nomenclature\\_info.php?genus=PSEUDOMONAS](http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=PSEUDOMONAS)).

The genus *Pseudomonas* is a collection of species that is widely dispersed in the natural environment and living under diverse conditions. Pseudomonads are the inhabitants of soil and water ecosystems. They also compose the collection of important pathogens for plants, fungi, animals and even human (Palleroni, 1992; Lyczac et al., 2000; Locatelli et al., 2002). In public health the opportunistic pathogen *P. aeruginosa* has gained interest by affecting primarily immuno-deficient people as well as possessing high resistance against antibiotics (Locatelli et al., 2002). It also represents the type strain of the genus. Biofilms are important in *P. aeruginosa* pathogenesis, since they form on the surfaces of medical devices such as catheters, endotracheal tubes, and are



thought to contribute to chronic infection in the airways of cystic fibrosis (CF) patients (Parsek and Singh, 2003).

The universal distribution of this genus resulted that it is capable of utilizing a broad range of organic and inorganic compounds (e.g. halogenated derivatives, xenobiotic compounds). *Pseudomonas* species are well known for biofilm formation in a small or extensive form in which quorum-sensing appears to play important roles (Whiteley et al., 2001). This ability helps them surviving in various natural ecosystems (Tolker-Nielsen and Molin, 2004).

Some species are responsible for spoilage of milk (Eneroth et al., 2000) and dairy products as well as meat deterioration like red meat (Ercolini et al., 2009), poultry and fish.

The member of *Pseudomonas* genus are non-spore forming, aerobic, Gram negative, oxidase and catalase positive bacteria which are motile with few exceptions by one or several flagella. The bacteria are straight or slightly curved rods (0.5-1.0 x 1.5-5.0 µm). All species respire aerobically, using oxygen as the terminal electron acceptor for oxidative phosphorylation but some of the species can also use nitrate and able to do denitrification (dissimilatory reduction of nitrate to N<sub>2</sub>O and N<sub>2</sub>). *Pseudomonas* species dissimilate sugars. Many aerobic pseudomonads are resistant to a number of antibacterial agents such as the majority of related beta-lactam antibiotics (Garrity et al., 2005). The cells of some strains can be extremely short, while in others (*P. putida* and *P. syringae*) they may be unusually long. With few exceptions, flagellar motility is a common characteristic of *Pseudomonas* species. The pigments productions (e. g. pyoverdine, pyocyanin) by *Pseudomonas* species are also demonstrated and have an essential taxonomic marker (Meyer and Hornsperger, 1998).

#### **2.2.1.2 Impact of proteolytic and lipolytic activities of *Pseudomonas* species during spoilage**

Enzymatic activities of spoilage-causing psychrotrophic bacteria, especially *Pseudomonas* species are responsible for the deterioration of refrigerated meat and dairy products and cause defect in sensory values (Dogan and Boor, 2003). In processed fluid milk stored at 4°C mainly *P. fluorescens* becomes predominant (Eneroth et al., 2000) and its secreted enzymes are liable for proteolysis and lipolysis (Sørhaug and Stepaniak, 1997; Matselis and Roussis, 1998). The off-odour appears when the population of *Pseudomonas* spp. reaches  $2.2 \times 10^6$  to  $3.6 \times 10^7$  CFU/ml (Champagne et al., 1994) in milk. Several strains belonging to the genus *Pseudomonas* produce heat stable extracellular lipases and proteases that remain active (Garcia et al., 1989; Sørhaug and Stepaniak, 1997) even after the thermal processing step such as pasteurisation of milk. Proteases digest the casein which leads to the bitter flavour and clotting of milk while lipases degrade tributyrin and milk fat resulting free fatty acids that cause rancid, bitter taste. These hydrolysed

lipids and proteins as by-products not just reduce the organoleptic properties and the shelf-life of fluid milk products, but are responsible for the off-odour production with their volatile compounds (Dogan and Boor 2003; Ercolini et al., 2010; Rajmohan et al., 2002). These activities combined with spoilage generate significant economical losses for food industry.

On meat the proteolysis occurs only when spoilage is well advanced and when the number of bacteria at the surface reaches  $10^7$  CFU/cm<sup>2</sup> and off-odour appear (Molin, 2000). The maximum production of proteinase by psychrotrophs normally culminates in the late exponential or stationary phase of growth (Griffiths, 1989; Kohlmann et al., 1991). The majority of *Pseudomonas* species produce only one type of proteinase, typically a neutral zinc metallo-proteinase based on their functional group. The optimum pH for digestion is between 6.5-8.0 (Chen et al., 2003).

Most spoilage bacteria as well as *Pseudomonas* species utilize glucose as a carbon source. Since spoilage characteristics do not become evident until amino acids are degraded, the concentration of glucose present in the meat tissue has an essential role in the start of aerobic spoilage. The diffusion of glucose molecules starts from the deeper tissues when the glucose is consumed at the surface of meat (Gill, 1982). This procedure occurs until the glucose has been exhausted and another nutrient source becomes available for utilization such as lactate and amino acids. Degradation of amino acids such as cysteine and methionine degraded by the spoilage microflora (especially *Pseudomonas* species) results in the production of volatile sulphur compounds such as methane thiol, dimethyl sulphide and dimethyl disulphide, hydrogen sulphide, indole and other compounds. These contain the part of off-odours, flavours and colours (Edwards and Dainty, 1987; van Laak, 1994). Amino acid decarboxylation of lysine yields putrescine. Increasing putrescine levels correlate with increasing pseudomonad counts in meats (Marshall and Bal'a, 2001). In the later stages of spoilage an increase in the meat pH is seen as ammonia and a number of amines are elaborated.

Lipolytic enzymes can be defined as carboxylesterases that hydrolyse acylglycerols (Jaeger et al., 1994; Jaeger et al., 1999; Beisson et al., 2000). Those that hydrolyse acylglycerols of <10 carbon-chain fatty acids are the esterases, or carboxylases (EC 3.1.1.1); while those that hydrolyse acylglycerols of more than nine carbon-chain fatty acids are the lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3). Most bacterial lipases are extracellular and produced during the late log and early stationary phases of growth (Fox and Stepaniak, 1983; Sidhu et al., 1998). The production of lipases will be limited or inhibited by the presence of carbohydrates, lipids and proteins in the medium. Those conditions such as temperature, pH, nitrogen and lipid sources, concentration of inorganic salts and availability of oxygen all also has effect on the levels of lipase produced (Chen et al., 2003). The meat develops a sweet or fruity odour at the sensory detected stage when pseudomonads begin to increase (Adams and Moss, 2008) due to ester production. It is particularly associated with *P. fragi* which can produce ethyl esters of acetic, butyric and

hexanoic acids from glucose. This demonstrates that the off-odour is a complex mixture of volatile esters, alcohols, ketones and sulphur-containing compounds (Ercolini et al., 2009; Ercolini et al., 2010).

Many spoilage microorganisms are able to produce more than one type of extracellular lipases which catalyse the hydrolysis of triacylglycerol which may, along with their oxidation products, contribute to rancidity (Macrae, 1983). Oxidative rancidity of fat is also a part of spoilage. It occurs when unsaturated fatty acids react with oxygen from storage environment. Stable components such as aldehydes, ketones and short-chain fatty acids are produced, resulting in the eventual development of rancid flavours and odours. The procedure of autooxidation is independent from microbial activity the rate of which is determined by the proportion of unsaturated fatty acids in the fat (Formanek et al., 2002).

### **2.2.2. Detection of *Pseudomonas* species**

Traditional microbiological techniques for bacterial identification, such as observation of growth patterns on selective and differentiative media, biochemical reactions and microscopy, is very useful for pre-selection and compose the principles for isolation of different species. They are simple and relatively inexpensive, but they are time consuming. In case of *Pseudomonas* spp. the main problem with conventional phenotypic methods are that they have limited applicability in differentiating the genetically diverse genus. However, information is important for detailed analysis of representative genetic types to discover their spoilage potential (Aslam and Service, 2008). Genotypic methods such as ribotyping could help with its enhanced discriminatory power (van der Vossen and Hofstra, 1996). Ribotyping has been demonstrated to rapidly and reproducibly type bacterial isolates at genus, species and strain levels (Bruce, 1996) as well as could serve information whether all or only a few selected genetic types of *Pseudomonas* species are responsible for e.g. off-odour production or which strains become dominant (Dogan and Boor, 2003).

#### **2.2.2.1. Traditional culture-based methods**

Several selective media is available for the detection of *Pseudomonas* species however most of them are designed for the detection of the opportunistic pathogen *P. aeruginosa* and *Burkholderia cepacia*. The collection of media used for selective detection of *Pseudomonas* spp. is demonstrated in Table 3. Those media that were developed for clinically important pseudomonads are tested for the detection of food spoilage causing pseudomonads but they had poor specificity because other non- *Pseudomonas* strains considerably interfere or accompany the detection (Solberg et al., 1972;

Jeppesen, 1995; Flint and Hartley, 1996). *Pseudomonas* spp. grow well on non-selective media (e.g., plate count agar and blood agar, BHI agar) as well as on selective and differential media (e.g., MacConkey agar and eosine methylene blue agar) when incubation temperature is adequate for their growth (Jeppesen, 1995). The selectivity of these media is usually enhanced by different antibiotics which uses the resistance of the *Pseudomonas* species against these agents. For the detection of *Pseudomonas* species in fresh meat (CETCH agar) was developed by Solberg et al. (1972) which was an effective selective inhibitor of meat spoilage microorganisms and allowed the detection of *Pseudomonas* colonies after incubation at 23°C 48 h. However, *Alcaligenes*, *Citrobacter* and *Enterobacter* species were not inhibited on this medium.

Mead and Adams (1977) investigated the selectivity and productivity of four existing media for isolation of *Pseudomonas* spp. (ALCV, MGV, CETCH and Difco heart infusion agar with diamide) from poultry. These media effectively suppressed the growth of Gram positive organisms, but there were marked differences in their ability to suppress unwanted Gram negative microorganisms. ALCV medium permitted growth of most strains of Gram negative bacteria, whereas *Serratia liquefaciens* could grow on MGV and *Shewanella putrefaciens*, *Aeromonas* spp. and *S. liquefaciens* grew on CETCH. The majority of *Pseudomonas* spp. produced only small colonies on MGV and was difficult to count. The Difco medium (Verbovsky and Collins, 1973) was the least successful, inhibiting most of the non-pigmented *Pseudomonas* spp. whilst supporting the growth of certain yeasts. Driessen and Stadhouders (1972) examined these media as well as Herella *Pseudomonas* medium for detection of *Pseudomonas* spp. derived from milk. These media were found insufficiently selective. Mead and Adams (1977) developed a new selective medium called CFC. The medium successfully rolled back Gram-positive bacteria and facilitated the growth of *Pseudomonas* spp. (including *Pseudomonas aeruginosa*). It was able to inhibit other Gram negative bacteria, although *S. putrefaciens* was partially suppressed. Kristiansen (1983) made a comparison between species *P. aerogenes*, *P. fluorescens*, *Burkholderia cepacia* and *P. putida* on two selective media [cetrimide/naladixic (CN) and cetrimide/fucidin/cephaloridin (CFC)]. Only *P. aeruginosa* showed sufficient growth on these media in this trial. Tryfinopoulou et al. (2001) applied CFC medium for counting *Pseudomonas* populations on fish but the enumeration of pseudomonad populations directly on CFC medium was not adequate with fish samples. Both *Enterobacteriaceae* and *Shewanella putrefaciens* were able to grow on the CFC medium. The members of *Enterobacteriaceae* could also grow on CFC in studies relative to meat, poultry and milk (Stanbridge and Board, 1994; Flint and Hartley, 1996; Salvat et al., 1997).

**Table 3.** Collection of media developed for detection *Pseudomonas* spp.

Name of the media	Selective agents	Action of the selective agents	Application	Isolation from	References
<b>King A</b> ( <i>Pseudomonas</i> Agar P)	Potassium sulphate, magnesium chloride	stimulate the formation of pyocyanin and pyorubin	confirmation of <i>P.</i> <i>aeruginosa</i> by pyocyanin formation	no special source determined	King et al. (1954)
<b>King B</b> ( <i>Pseudomonas</i> Agar F)	magnesium sulphate	stimulates the production of fluorescein, inhibits the pyocyanin formation	Detection fluorescing bacteria, especially <i>P.</i> <i>fluorescens</i>	water, drinking water	King et al. (1954)
<b><i>Pseudomonas</i> selective agar</b> (Cetrimide agar)	cetrimide	inhibits the growth of accompanying microbial flora	isolation and differentiation of <i>P.</i> <i>aeruginosa</i>	various materials	Brown and Lowbury, (1965)
<b>ALCV</b>	ammonium lactate, crystal violet	inhibits gram positive bacteria	enumeration of <i>Pseudomonas</i> spp.	milk	Gyllenberg et al. (1963)
<b>MGV</b> (Masourovsky medium)	erythromycin, chloramphenicol	antimicrobial agents	enumeration of <i>Pseudomonas</i> spp.	food	Masourovsky et al. (1963)
<b>GH medium</b>	nitrofurantoin, nalidixic acid	primary selective agents	selective isolation of <i>Pseudomonas</i> spp.	natural habitat	Grant and Holt, (1977)
<b>CN</b>	cetrimide	selective for <i>P. aeruginosa</i>	selective media for <i>Pseudomonas</i> <i>aeruginosa</i> , used in clinical microbiology	pathological sources and (burns)	Goto and Enomoto, (1970); Lilly and Lowbury, (1972)
	naladixic acid	suppress the growth of other Gram negative bacteria			
<b>HP (Herella <i>Pseudomonas</i> agar)</b>	chloramphenicol	antimicrobial agents	enumeration of pseudomonads	milk	Driessen and Stadhouders, (1972)
<b>CETCH</b>	cetrimide	selective agent against yeasts	isolation and enumeration of	food product especially	Solberg et al. (1972)
	2-hydroxy-2',4',4'- trichloro-o-diphenyl oxide	inhibitory to a broad spectrum of microorganisms	<i>Pseudomonas</i> spp.	meat	
<b>Heart infusion with diamide</b>	diamide	antimicrobial agents	<i>P. fluorescens</i>	milk	Verbovsky and Collins, (1973)
<b>CFC</b>	cetrimide	inhibits contaminating yeasts	isolation and enumeration of <i>Pseudomonas</i> spp.	meat and meat based products	Mead and Adams, (1977); Mead (1985)
	fucidine	inhibits <i>Acinetobacter/Moraxella</i>			
	cephaloridine	inhibits enterobacteria, staphylococci and streptococci			
<b><i>Pseudomonas</i> selective isolation agar</b>	nitrofuratoin	inhibits most other Gram negative bacteria	isolation for <i>Pseudomonas</i> spp.	water and deionized water	Krueger and Sheikh, (1987)
	crystal violet	inhibits Gram positive bacteria			
<b>GSP</b>	sodium glutamate, starch, phenol red	accompanying microorganisms not metabolize them	detection <i>Pseudomonas</i> and <i>Aeromonas</i>	foodstuffs, waste water; equipment of the food industry	Kielwein, (1969; 1971)
	penicillin	selective inhibitor			
	pimaricin	antimycotic agent			
<b>MGSP</b>	Irgasan DP 300	inhibits the growth of another Gram negative bacteria	detection of <i>Pseudomonas</i> spp.	Spoilage of dairy products	Flint and Hartley, (1996)

A medium usually used for detection of fluorescent *Pseudomonas* spp. is King's medium B under UV-light. King's A medium are elective media for isolation and detection pyocyanin formation of *Pseudomonas aeruginosa*. Other media that is used for selection *Pseudomonas aeruginosa* is Cetrimide agar (Brown and Lowbury, 1965) which is specific for the detection of *P. aeruginosa* from water and used in detection of this species from clinical sources. The use of cetrimide was recommended (Lowbury, 1951) for inhibiting the growth of accompanying microbial flora and minimizing interference with the growth of *P. aeruginosa*.

Glutamate Starch Phenol-red (GSP) agar was proposed by Kielwein (1971) for the detection of *Pseudomonas* and *Aeromonas* in food sources as well as in waste water and on food handling equipment. However it was not suitable for the detection of *Pseudomonas* from milk (Driessen and Stadhouders, 1972) and a selective agent called Irgasan DP 300 was chosen to modify GSP medium (MGSP). *Pseudomonas* species grew well while other species, except *Serratia*, were inhibited (Flint and Hartley, 1996).

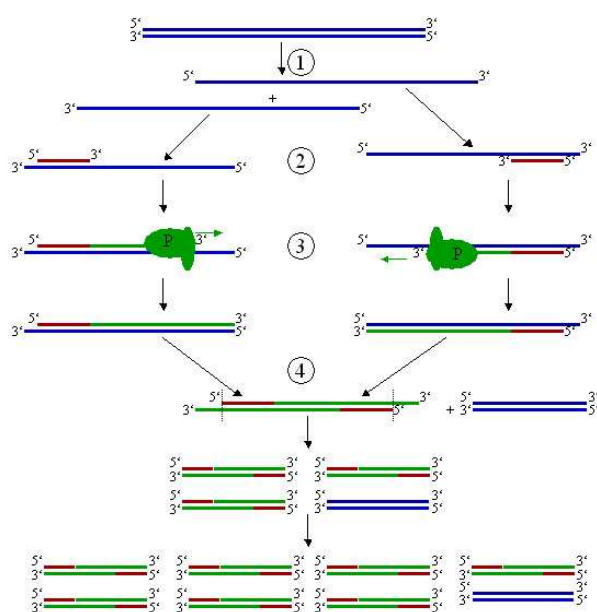
#### **2.2.2.2. Molecular methods for identification and typing of food-borne bacteria**

Molecular methods play an important role in identification and characterization of food spoilage causing bacteria. For example DNA-based typing methods are based on the polymorphism of the DNA that help to differentiate bacteria at species and strain level and make better understand the way of spoilage. The innovation of PCR (polymerase chain reaction) in 1983 by Kary Mullis revolutionised several area of molecular biology and in particular molecular diagnostics. Mullis won the Nobel Prize in Chemistry in 1993 for the invention of PCR.

#### **Principle of PCR**

The objective of PCR is to produce a large amount of a specific fragment of DNA from a very small amount of genomic DNA that can be used for further molecular analysis. The specificity of the method is based on an *in vitro* enzymatic reaction under controlled temperature conditions targeting and amplifying one fragment of the DNA or gene (Atlas and Bej, 1994). The PCR protocol consists of the following three steps as illustrated on Fig. 2.

The most simple and common way to detect and analyse the amplicons after PCR reaction is the electrophoresis on agarose or polyacrylamide gels (Maráz et al., 2006). Ethidium bromide, SYBR Green I could be used for staining the gels. These dyes are able to bind to the ds DNA by intercalation and can be visualized under UV light (Ausubel, 1989). SYBR Green I sufficiently sensitive to measure low concentrations (0-2 ng/ml) of DNA while ethidium bromide is only appropriate for higher concentrations (0-20 µg/ml) of DNA (Rengarajan et al., 2002).



**Figure 2.** Flow chart of polymerase chain reaction during one cycle

(Source: [http://serc.carleton.edu/microbelife/research\\_methods/genomics/pcr.html](http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html))

1. Denaturation of ds DNA at 94-95°C.
2. Annealing of oligonucleotides (primers) at 40-60°C.
3. Elongation and extension with DNA-polymerase at 72°C.
4. The double-stranded (ds) DNA completely synthesised and the first cycle is finished. The two new ds DNA molecules will be the templates in the next cycle. The number of amplified fragments of DNA is duplicated in each cycle. These three steps are repeated during 20-40 cycles.

### PCR-based techniques used for typing of bacteria: RAPD and PCR-RFLP

The **RAPD** (Random amplification of polymorphic DNA) method was developed by Williams et al. (1990) and has been initially used to detect polymorphism in genetic mapping, taxonomy and phylogenetic studies, later in genotoxicity and carcinogenesis studies (Atienzar and Jha, 2006). RAPD-PCR is the most popular typing technique applied to food ecosystems which allows simple, rapid and cost effective detection of DNA markers. The method targets the whole genome of the organism to generate randomly amplified DNA products without prior sequences information of the target DNA (Caetano-Anolles, 1997). Typically, the RAPD protocol usually works with single short (approximately 10-12 bp length), non-specific primer and applies the annealing temperature between 34-42°C. The amplified DNA is generally separated by gel electrophoresis on 1.5-2.0% agarose gels. These patterns arise from random attachment of the primer to the template DNA and several regions will be amplified if the DNA shows similarities to the primer. Polymorphism detected by these techniques can be used as taxonomic markers in population studies of a wide variety of organisms (Welsh et al., 1992). The major problem of this method is the weak reproducibility that was observed at intra- and inter-laboratory level (Maráz et al., 2006). Standardisation of the PCR conditions could be a partly solution. The reaction has to be optimized as for the quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, the brand of DNA polymerase or source as well as the brand of thermal cycler instrument (Wolff et al., 1993). RAPD technique has been successfully

applied to genetic population analysis of *Pseudomonas aeruginosa* originated from human infections (Renders et al., 1996; Campbell et al., 2000). The ecology of lactic acid bacteria (LAB) in fermented sausage was also subjected to RAPD-PCR (Urso et al., 2006). There are only few publications dealing with genetic characterization of food spoilage causing pseudomonads with RAPD analysis. *Pseudomonas* isolates are characterized by RAPD and connected to food spoilage derived from the production of pasteurised milk (Eneroth et al., 2000) or from raw and pasteurized milk where the shelf life was tested (Wiedman et al., 2000). In case of meat spoilage the RAPD method was used for genetic characterization of pseudomonads derived from retail-displayed beef (Aslam and Service, 2008) and analysing molecular diversity of pseudomonads on poultry meat during spoilage (Belák et al., 2011).

The **PCR-RFLP** (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) method is located among the first molecular techniques for fingerprinting or subtyping of organisms at molecular level. It is a reliable and relatively simple method but prior knowledge of the DNA sequence is necessary. The extracted DNA is amplified with PCR and sequence specific restriction endonucleases digest the amplicon (Maráz et al., 2006). Different fragments are developed after the enzymatic digestion number and size of which can vary among individuals, populations, and species (Semagn et al., 2006). The profiles produced are highly reproducible but the discriminatory power of the method is dependent on the selection of the restriction endonuclease (Maráz et al., 2006). After digestion the DNA appears as a mixture of linear double-stranded molecules with various lengths. Separation of the DNA fragments is performed by gel electrophoresis.

Different genes could be the target sequences for subtyping bacteria. The flagellin genes *flaA* and *flaB* which is responsible for the virulence of *Campylobacter jejuni* was successfully used in PCR-RFLP for typing (Ayling et al., 1996) while the *groEL* gene was also found to be suitable for species-specific identification of *Campylobacter* (Kärenlampi et al., 2004). The approach of PCR-RFLP was also used for identification of *Listeria monocytogenes* and *Bacillus thuringiensis* based on virulence genes (Ericsson et al., 1995; Kuo and Chak, 1996). The *recA* PCR-RFLP was useful for genotyping of *E. carotovora* (Waleron et al., 2002) that has multifunctional protein involved in homologous recombination, DNA repair and the SOS response. The heat shock protein gene *groES* was also the target of PCR-RFLP to determine *Pseudomonas spp.* from patients suffering cystic fibrosis (Clarke et al., 2003).

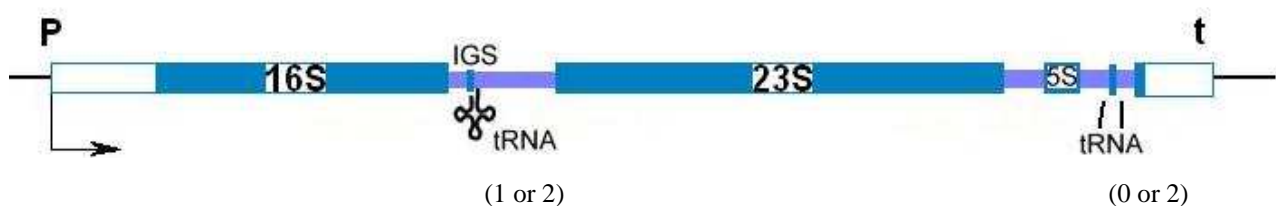
### **PCR-based techniques used for identification of bacteria**

It is important to study not just the microbial diversity, but precisely identify the key microorganisms originated from many processes and matrices in which they are present. The



development of molecular identification methods such as ARDRA, the DNA sequencing as well as species-specific primer pairs helps to assign a name to bacterial isolates. The difference between these methods is based on the level of discrimination, the time of handling and the costs. The amplified rDNA restriction analysis (ARDRA) is an easy, rapid and cost-effective method for identification purposes which possesses high reproducibility. However, it has moderate discrimination level (Giraffa and Carminati, 2008). This technique combines the PCR and RFLP methods focusing on rRNA gene.

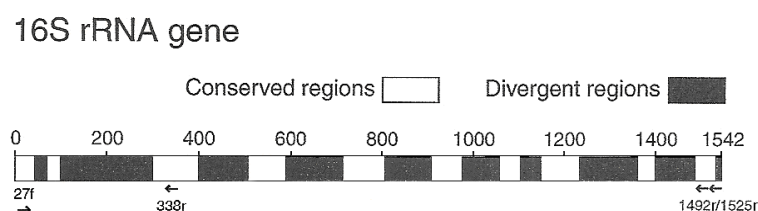
The bacterial rRNA operon (shown on Fig.3.) is most frequently used as marker in microbial ecology to characterize and determine the phylogenetic and taxonomic status and relationship of bacterial isolates. These characteristics originate from its strongly conservative and variable regions which are not affected by changes in the organism's environment. The rRNA molecules are constantly expressed molecules with restricted function which carries evolutionary importance (Uyttendaele and Debevere, 2003). The copy number of rRNA operons varies from 1 to 15 in which the rDNA genes are situated next to each other in the bacterial genome (Klappenbach et al., 2000). The bacterial ribosomal RNA (rRNA) operon itself, encompasses a 16S rRNA and 23S rRNA gene as well as an intergenic spacer (IGS) region between 16S and 23S rRNA as shown in Fig. 3.



(Source: <http://sandwalk.blogspot.com/2008/01/ribosomal-rna-genes-in-bacteria.html>; Evguenieva-Hackenberg, 2005)

**Figure 3.** Bacterial rRNA operon that starts with the promoter (P) and ending at the terminator (t) In *E. coli* rRNA operon the tRNA could vary in number and kind of tRNA sequences as it is shown in brackets (Willey et al., 2008)

Among these rRNA molecules the whole 16S rRNA or parts from its genomic coding sequences had been most often used for bacterial characterization and identification (Kolbert and Persing, 1999). It contains alternating regions of conserved and variable sequences (Woese, 1987) as shown in Fig. 4.



**Figure 4.** Conserved and variable regions of 16S rRNA gene with universal primers (27f, 338r and 1492r/1525r) attached to the conserved regions (Source: Maiwald, 2004)

The conserved regions can be used for designing universal primers for PCR reaction (Fig. 4) that anneal the same ribosomal targets from all or most bacterial species.

The ARDRA fingerprint can be used for microbial identification (Laguerre et al., 1994) or comparison of microbial communities and dynamics (Moyer et al., 1994). It was used for genotyping of bacteria community derived from reared fry of the Atlantic halibut (Jensen et al., 2002) or identification of LAB (Giraffa et al., 1998b).

The intergenic spacer region (IGS) between the small and the large ribosomal subunit also could be an ideal target for molecular identification as it is significantly variable in nucleotide sequence and length (Justé et al., 2008). A community analysis of food samples, i.e. goat milk and ensiled corn were based on IGS analysis reported by Cardinale et al. (2004). Different methods have been applied for analysis of diversity of *Pseudomonas* isolates from food origin (e.g. milk, meat, maple sap). Nevertheless, ribosomal sequences do not always show as much sequence variation as it should to make differentiation between closely related, but ecologically distinct taxa.

The increasing availability of non-ribosomal (metabolic) gene sequences has further revolutionized the PCR-based diagnostics and has facilitated the differentiation between isolates. As an example, to distinguish *Lb. delbrueckii* subsp. *lactis* from *Lb. delbrueckii* subsp. *bulgaricus* the *pepIP* and *lacZ* genes can be respectively used (Torriani et al., 1999) as well as *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* can be distinguished on the basis of primers designed on the histidine biosynthesis operon (Corroler et al., 1998).

Several authors offer to use alternative sequences of other genes such as *gyrB*, *rpoD*, *oprI* *carA* genes. These have been applied for identification and in phylogenetic studies of *Pseudomonas* spp. (Yamamoto et al., 2000, Hilario et al., 2004). Molecular phylogeny analysis based on *rpoB* sequence has been also performed on *Pseudomonas* species by Tayeb et al. (2005). It has been previously used for phylogenetic analysis for several other bacterial groups (Mollet et al., 1998; Qi et al., 2001).

DNA sequencing is considered as “the gold standard” for microbial identification (Giraffa and Carminati, 2008). DNA sequencing generally begins with PCR amplification of DNA directed at genetic regions of interest, followed by sequencing reactions. DNA sequences derived from phylogenetic studies and settled in public databases facilitate the identification of the community members originated from different ecological niche rapidly (Benson et al., 2004; Cole et al., 2005; D’Auria et al., 2006). Rapid developments in sequencing and DNA handling methods facilitate to routinely sequence partial or whole-gene amplicons as an identification technique. The 16S rDNA has extensive availability of sequences in public databases such as GenBank (Benson et al., 2004) or ‘Ribosomal Database Project’ (RDP) providing also online data analysis (Cole et al., 2005) and

permit a reliable characterisation of populations belonging to a microbial community (Chakravorty et al., 2007).

DNA sequencing method has the best accuracy and reproducibility as well as high discriminatory level between the methods used for identification. However, it is generally expensive and requires a high degree of technical competence to perform (Giraffa and Carminati, 2008).

The applicability of sequencing and the sequence alignments to the database makes it possible to design species-specific primers that are specified for the fragments e.g. the *atpD*, *recA* and *carA* gene in *Pseudomonas* species (Hilario et al., 2004). The application of *carA* gene encoding the carbamoyl phosphate synthase gave the base for further sequencing and designing of species-specific primers for detection of pseudomonads causing meat spoilage (Ercolini et al., 2007).

### **2.3. Food-borne pathogens on meat which connected to food-borne diseases**

In parallel with the food spoilage causing bacteria it is essential to take the importance of food-borne pathogens connected to meat products into account. The spread of food-borne diseases are the results of the more complex international markets, the freedom in migration as well as the different travelling possibilities (Tauxe, 2010). Food-borne diseases are caused by pathogenic microbes such as bacteria, viruses, and parasites, or their toxins present in contaminated foods that increase the universal vulnerability of human health worldwide. Bacteria that cause food-borne diseases and the most well-known biological hazards include among others *Salmonella*, *Campylobacter*, *Listeria*, pathogenic *Escherichia coli*, *Yersinia*, *Shigella*, *Enterobacter* and *Citrobacter* (EFSA, 2010, EFSA and ECDC, 2011). Many of them are commonly found in the intestines of healthy food producing animals. The risks of contamination are present from farm to fork and need to be controlled in different ways.

Approximately 30% of all newly globally emerging infections over the past 60 years include pathogens commonly transmitted through food (Jones et al., 2008). A pathogen is an organism that is able to cause cellular damage by establishing in tissue, which results in clinical signs with an outcome of either morbidity (defined by general suffering) or mortality (death). Some pathogens are designated as primary pathogens, which regularly cause disease. Others are classified as opportunistic pathogens that infect primarily immune-compromised individuals. Pathogens could be grouped as zoonotic, geonotic, or human origin based on their transmission patterns and movement among different hosts and vectors (Bhunia, 2008). There are food-borne zoonosis-diseases or infections that can be transmitted from animals to humans through food (Bhunia, 2008; EFSA and ECDC, 2011). Zoonoses also include diseases transferred to humans by other routes than food, for example by direct contact with animals. Examples of zoonotic pathogens are the pathogenic

*Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *S. serovar Enteritidis*, *Campylobacter jejuni*, *Yersinia enterocolitica*. In addition, food-borne diseases can be caused by bacterial toxins. Bacterial toxins are toxins generated by bacteria and may be highly poisonous in many cases. These include toxins from *Staphylococcus aureus*, *Clostridium botulinum* and *Bacillus cereus* (EFSA, 2010; EFSA and ECDC, 2011).

According to the report of European Food Safety Authority (EFSA, 2010), bacterial toxins were on the third place on the ranking list of pathogenicity and generated food poisoning outbreaks with 9.8% after *Salmonella spp.* (35.4%) and viruses (13.1%) in 2008. Among the bacterial toxins, staphylococcal enterotoxins (SEs) are responsible for staphylococcal food poisoning (SFP) which is the fourth most common causative agent of foodborne illness within the EU in 2008, with meat and meat products as common food vehicles (EFSA, 2010).

Based on the estimation of Centers for Disease Control (CDC) on the SEs caused food-borne diseases approximately 80 million individuals were included in the US ending with 325.000 hospitalizations and more than 5.000 deaths (Mead et al., 1999). In Korea, 30% of SFP incidents in 2001–2006 involved meat and meat products according to the Korean Food and Drug Administration and in the US, 36% of confirmed SFP outbreaks reported to CDC in 2007 involved pork meat products (CDC, 2007; Kim et al., 2009). Staphylococcal enterotoxin A (SEA) is the toxin most frequently reported to be involved in SFP (Cha et al., 2006; K  rouanton et al., 2007; Wieneke et al., 1993) worldwide but the involvement of other classical SEs has also been demonstrated. SEA is responsible for approximately 80% of the cases of food poisoning outbreaks in the USA (Atanassova et al., 2001). *Staphylococcus aureus* enterotoxin D (SED) is another of the predominant enterotoxins recovered in SFP incidents (K  rouanton et al., 2007; Wieneke et al., 1993).

### 2.3.1. The genus *Staphylococcus*

The name *Staphylococcus* derived from Alexaner Ogston who used this name first in 1882. The name is the combination of Greek nouns where 'staphyle' means a bunch of grapes while 'coccus' means a berry. Two years later a formal description of the genus *Staphylococcus* was prepared by Rosenbach. *Staphylococcus aureus* belongs to the genus *Staphylococcus* within the family *Staphylococcaceae* and the order *Bacillales*. Members of this genus form a coherent and well-defined group of related species with the exception of morphology that has similarity with the genus *Micrococcus* in few characteristics (Baird-Parker, 2000; G  tz et al., 2006). In total 70 species and several subspecies are recognized in the genus *Staphylococcus*. *Staphylococcus aureus* is currently

the	type	species	of	the	genus
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([http://www.dsmz.de/microorganisms/bacterial\\_nomenclature\\_info.php?genus=Staphylococcus&show\\_all\\_details=1](http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Staphylococcus&show_all_details=1); Baird-Parker, 2000).

*Staphylococcus* is a Gram positive, non-spore-forming, non-motile cocci (0.5–1.5 µm in diameter) that occurs in pairs, tetrads, short chains or resembling bunches of grapes. Most species are facultative anaerobes and they are positive for the catalase and negative for oxidase tests. It is multiplying very rapidly under aerobic conditions except of *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, (Götz et al., 2006). Only coagulase-positive staphylococci (CPS) strains have been obviously playing a part in food poisoning incidents. *S. aureus* subsp. *aureus* is the main causative agent described in staphylococcal food poisoning outbreaks (SFPOs). Staphylococci are living in wide spectrum of the environment such as air, dust, sewage, water, environmental surfaces, humans and animals. Their natural habitat is the nose and the skin mucous membranes of humans, other mammals, and birds (Hennekinne et al., 2010; Götz et al., 2006). A large fraction of the human population is colonized with this bacterium but *S. aureus* strains rarely cause disease. Nevertheless, *S. aureus* is one of the most frequent causes of bacterial infection in humans (Brüssow et al., 2004). It expresses a wide array of cell-associated and secreted virulence factors such as various enzymes, cytotoxins, pyrogenic exotoxins, like staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) (Novick et al., 2001) as well as exfoliative toxins.

Temperatures between 7–48°C promote the growth of *S. aureus* during processing and storage of food. The optimal temperature for growth is at 37°C. Enterotoxin production occurs in a narrower temperature range with the optimum at 35-40°C. Growth occurs optimally at pH values of 6-7 where the minimum and maximum limits are 4.0 and 9.8-10.0. The pH range of enterotoxin production is also narrower as shown in Table 4. *S. aureus* subsp. *aureus* strains have high tolerance against NaCl and reduced  $a_w$ . Growth occurs at  $a_w$  0.83 while the range for enterotoxin production has limitation at  $a_w$  0.86 (Adams and Moss, 2008).

**Table 4.** Factors for growth and enterotoxin production of *S. aureus*. (Source: Adams and Moss, 2008)

Factor	Growth		Enterotoxin production	
	Optimum	Range	Optimum	Range
Temperature (°C)	35-37	7-48	35-40	10-45
pH	6.0-7.0	4.0-9.8	SEA 5.3-6.8 others 6-7	4.8-9.0
NaCl (%)	0.5-4	0-20	0.5	0-20
$a_w$	0.98->0.99	0.83->0.99	>0.99	0.86->0.99

*S. aureus* can adapt rapidly to the selective pressure of antibiotics. Methicillin-resistant *S. aureus* (MRSA) isolates is a representing model of multi-drug resistant bacterial pathogens. Resistance to

this antibiotic is linked to the *mecA* gene that encodes a penicillin-binding protein (PBP2a), which allows the synthesis of the cell wall even at lethal concentrations of  $\beta$ -lactams (Ehlert, 1999). Most of the antibiotic-associated diarrhoea isolates of *S. aureus* are methicillin-resistant (MRSA) (Boyce and Havill, 2005) causing worldwide problem. The majority of the MRSA are toxin producing strains (TSST-1, SEA, SEB, SED) (Schmitz et al., 1997).

#### **2.3.1.1. Staphylococcal enterotoxins and their relevance in staphylococcal food poisoning**

*Staphylococcus aureus* produces a wide variety of toxins including staphylococcal enterotoxins (SEs). SEs belong to the broad family of pyrogenic toxin superantigens (SAGs), connected to significant human diseases that include food poisoning and toxic shock syndrome (Balaban and Rasooly, 2000). Superantigens stimulate large number of polyclonal T-cells resulting in release of inflammatory cytokines and symptoms of shock (Hamad et al., 1997; Orwin et al., 2002) as well as include the ability to induce emesis and gastroenteritis (Bergdoll, 1989; Su and Wong, 1995). SEs are encoded by prophages (Betley and Mekalanos, 1985), plasmids (Bayles and Iandolo, 1989) or pathogenicity islands (Yarwood et al., 2002) that allow horizontal gene transfer between strains.

Heat treatment, acidic pH can easily destroy the bacteria that produce SEs, but the protein itself is resistant to these conditions. Gastrointestinal proteases including pepsin, trypsin, rennin and papain (Le Loir et al., 2003; Bennett, 2005) are not able to degrade and inactivate SEs. Hence, these enterotoxins are able to keep their activity in the digestive tract after ingestion (Everson et al., 1988).

Staphylococcal enterotoxins have been proposed to be named by their emetic activities. Only those SAGs were defined as SEs that generate vomiting after oral administration in a primate model (Lina et al., 2004). These toxins are SEA to SEE, SEG to SEI, SER to SET). Related toxins that lack emetic activity such as (SE/L and SE/Q) or have not been tested for it like (SE/J, SE/K, SE/M to SE/P, SE/U, SE/U2 and SE/V) should be designated as staphylococcal enterotoxin-like (SE/s) superantigens (Fraser and Proft, 2008).

Until the time of writing altogether 22 staphylococcal enterotoxins (SEs) and enterotoxin-like (SE/s) types have been described as it is shown in Table 5. They are globular, single polypeptides constitute a family of structurally related exoproteins with molecular weights ranging from 22 to 29 kDa (Pinchuk et al., 2010). SEs and SE/s have been traditionally subdivided into classical enterotoxins containing SEA, SEB, SEC subdivided into three groups based on differences in minor epitopes SEC1, SEC2, SEC3; SED, SEE (Dinges et al., 2000) and new types including SEG, SEH, SEI, SE/J, SE/K, SE/L, SE/M, SE/N, SE/O, SE/P, SE/Q, SER, SES, SET, SE/U, as well as U2 and V (Thomas et al., 2006; Jarraud et al., 2001; Letertre et al., 2003; Munson et al., 1998; Omoe et al.,

2005; Omoe et al., 2003; Ono et al., 2008; Orwin et al., 2001; Orwin et al., 2003; Su and Wong, 1995; Zhang et al., 1998). The last two enterotoxin-like types are placed on an open reading frame of the enterotoxin gene cluster *egc* that encodes enterotoxin-like proteins (Thomas et al., 2006).

The various toxin serotypes (SEA though SEE, SEG, SEH) show structural similarities which are identified serologically or based on amino acid sequence differences (Thomas et al., 2007, Larkin et al., 2009). These amino acid sequence differences are also a basis of distribution of staphylococcal superantigenic toxins (Larkin et al., 2009).

**Table 5.** Characteristics of SEs and SEIs.

Toxin	Molecular Mass (kDa)	Emetic Activity	Gene	Genetic basis of SE
SEA	27.1	+	<i>sea</i>	Prophage
SEB	28.3	+	<i>seb</i>	Chromosome, plasmid, pathogenicity island
SEC	≈27.5	+	<i>sec</i>	Plasmid
SED	26.4	+	<i>sed</i>	Plasmid (pIB485)
SEE	26.4	+	<i>see</i>	Prophage
SEG	27.0	+	<i>seg</i>	<i>egc</i> , chromosome
SEH	25.2	+	<i>seh</i>	Transposon
SEI	24.9	weak	<i>sei</i>	<i>egc</i> , chromosome
SE/J	28.6	nd	<i>selj</i>	Plasmid (pIB485)
SE/K	25.5	nd	<i>selk</i>	Pathogenicity island
SE/L	24.6	– <sup>a</sup>	<i>sell</i>	Pathogenicity island
SE/M	24.8	nd	<i>selm</i>	<i>egc</i> , chromosome
SE/N	26.1	nd	<i>seln</i>	<i>egc</i> , chromosome
SE/O	26.8	nd	<i>selo</i>	<i>egc</i> , chromosome
SE/P	26.6	nd <sup>a</sup>	<i>selp</i>	Prophage (ΦSa3ms)
SE/Q	25.1	–	<i>selq</i>	Pathogenicity island
SER	27.0	+	<i>ser</i>	Plasmid (pIB485)
SES	26.2	+	<i>ses</i>	Plasmid (pIB485)
SET	22.6	weak	<i>set</i>	Plasmid (pIB485)
SE/U	27.2	nd	<i>selu</i>	<i>egc</i> , chromosome
SE/U2 (SEW)	26.7	nd	<i>selu2</i>	<i>egc</i> , chromosome
SE/V	25.0	nd	<i>selv</i>	<i>egc</i> , chromosome

nd: not determined; <sup>a</sup> Emetic activity demonstrated but not in a primate model

SFP is an intoxication caused by the consumption of food containing preformed enterotoxins that are produced by enterotoxigenic strains of coagulase positive *Staphylococcus*, mainly *Staphylococcus aureus* (Sandel and McKillip, 2004). Foods most commonly have been implicated in staphylococcal food poisoning, including meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, or sandwich fillings (Tamarapu et al., 2001; Wieneke et al., 1993). Ham, have also been implicated (Qi and Miller, 2000), according to the capacity of *S. aureus* to grow at relatively low water activity. *S. aureus* does not compete well with indigenous microbiota in raw foods. Contamination is mainly associated with improper and extensive handling of cooked or post-processed foods after heat treatment often followed by inadequate heating and/or improper storage temperature of the food (Le Loir et al., 2003; Smyth et al., 2004) which also facilitate the rapid multiplication of *S. aureus* and production of the enterotoxin(s) without those microbes that normally outcompete it (Sandel and McKillip, 2004). The main sources of contamination are humans carrying enterotoxin-producing *S. aureus* in their noses or on their hands. *S. aureus* is a common commensal of the skin and mucosal membranes of humans, where approximately 20–30% is part of the permanent and 60% is part of partial colonization (Kluytmans and Wertheim, 2005). *S. aureus* is also present in food animals, and dairy cattle, sheep and goats. Contaminations from animal origins in raw meat, sausages, raw milk, and raw milk cheese, are more frequent and due to animal infection like mastitis or animal carriage (Stewart, 2005).

Confirmation of SFP could be problematic because *S. aureus* cells could be eliminated by heat-treatment while SEs will remain in the food because of its heat-stable property (Bennett, 2005). Symptoms of SFP have a rapid onset. Initial symptoms (nausea followed by vomiting) appear within 2 h to 8 h after ingestion of contaminated food. Other commonly described symptoms include nausea, violent vomiting, abdominal cramping (Argudín et al., 2010), sometimes followed by diarrhoea after a short period of incubation and general weakness sometimes associated with a moderate fever (Le Loir et al., 2003) as a part of gastro-intestinal inflammation. The incubation period and severity of symptoms depend on the amount of enterotoxins ingested and the susceptibility of each individual. Occasionally it can be severe enough to warrant hospitalization, particularly when infants, elderly or debilitated people are concerned. Generally, symptoms are short in duration (approximately 24–48 h) (Murray, 2005). However, severe dehydration can result, necessitating intravenous fluid supplementation (Tranter, 1990).

### **2.3.1.2 Characterization of prophage-encoded *sea***

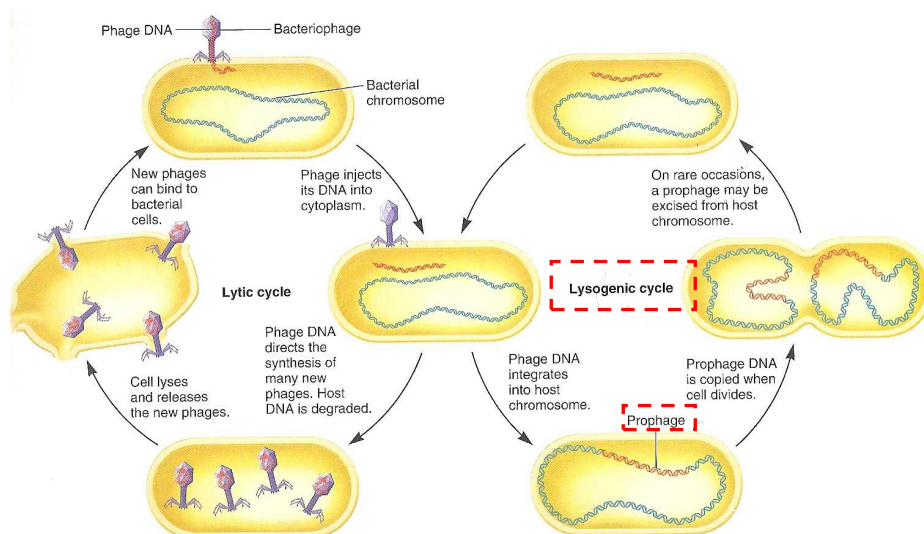
Staphylococcal superantigens as other virulence factors are encoded by mobile genetic elements including plasmids, prophages and mobile pathogenicity islands (SaPIs) which are responsible for



most of the dissimilarities between *S. aureus* strains (Baba et al., 2002; Lindsay and Holden, 2004; Betley and Mekalanos, 1985; Novick, 2003b). In particular, bacteriophages play an important role in the pathogenicity of *S. aureus* carrying accessory virulence factors such as Panton-Valentine leukocidin (PVL) (encoded by the *luk-PV* operon) (Kaneko et al., 1998; Narita et al., 2001), staphylokinase (encoded by *sak*) (Coleman et al., 1989), exfoliative toxin A (encoded by *eta*) (Yamaguchi et al., 2000) and enterotoxin A (encoded by *sea*) (Betley and Mekalanos, 1985).

Phages and their conversion is a primary vehicle in the diffusion of virulence determinants and the evolution of virulent *S. aureus* strains as well as bacterial pathogens (Brüssow et al., 2004). Spreading of virulence determinants between species enables non-pathogenic strains to become virulent and make species possible to adapt to environmental changes quickly (Burrus and Waldor, 2004; Goerke et al., 2010).

There are two life cycle of phages that they can follow as shown in Fig. 5. Virulent phages follow only a lytic cycle resulting death of host cell. In other cases, a bacteriophage infects a bacterium and follows latent state in the cell during the lysogenic cycle. Most types of phages integrate their genetic material into the chromosome of the bacterium in this cycle. This integrated phage DNA in this repressed state is called a prophage because it is not a phage but possess the potential to produce it. The prophage is replicated along with the bacterial chromosome when bacterium divides to produce two daughter cells. The extra genes from the prophage get expressed and change the properties of the bacterial cell (<http://pathmicro.med.sc.edu/mayer/phage.htm>; Brooker, 2009). At some later time, in a process called induction, a prophage may become activated to excise itself from the bacterial chromosome and enter the lytic cycle where it promotes the synthesis of new phages and finally kill the host cell. A bacteriophage that usually exists in the lysogenic life cycle is the temperate phage. Under most conditions, temperate phages do not produce new phages and will not lyse the host bacteria cell (Brooker, 2009).



**Figure 5.** Lytic and lysogenic cycle of bacteriophage (Source: Brooker, 2009)

The gene for staphylococcal enterotoxin A (*sea*) is incomparable from other staphylococcal enterotoxin coding genes such as *seb*, *sec*, and *sed* because it is carried by a polymorphic family of lysogenic or temperate phages (Betley and Mekalanos, 1985). There is still limited information about *sea* and its toxin SEA that is mainly responsible for staphylococcal food-borne intoxication (Cha et al., 2006; K  rouanton et al., 2007; Wieneke et al., 1993). The *sea* expression peaks in the late exponential growth phase (Czop and Bergdoll, 1974; Borst and Betley, 1993).

Hybridization analysis from DNA from *sea* or temperate phage PS42-D and its bacterial host suggest that this phage integrates into the bacterial chromosome by circularization and reciprocal crossover and that the gene is located near the phage attachment site (Betley and Mekalanos, 1985). Southern hybridizations revealed that the *sea* genes in staphylococcal strains were associated with a family of phages rather than with one particular phage. The potential medical importance of staphylococcal phage-encoded toxins has recently motivated a number of phage sequencing projects (Br  ssow et al., 2004).

There are examples from other bacterial species carrying phages and determine toxin production. Erythrogenic toxin of *Streptococcus pyogenes*, the structural or regulatory genes of botulinum toxins C1 and D and the shiga like toxin of *E. coli* are all encoded by temperate phage (Betley and Mekalanos, 1985). Studies with Shiga toxin-producing *E. coli* (Wagner et al., 2001) and *Streptococcus pyogenes* (Broudy et al., 2002) showed that prophage induction results an increase in production of phage-encoded virulence factors. In connection with *Staphylococcus aureus* it was established that the increased transcription and location of *sea* and *sak* upon prophage induction combined with the lysis genes are similar to the increased transcription and location of the *stxAB* genes in Shiga toxin-producing *E. coli* (Sumby and Waldor, 2003).

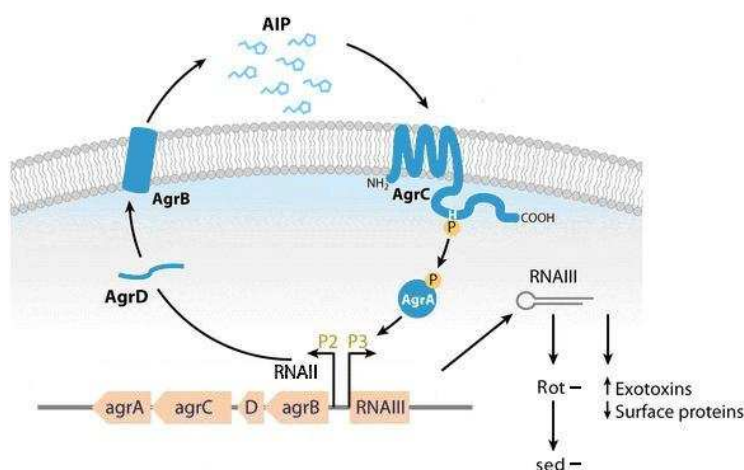
Prophage synthesis could be induced by different environmental conditions or antibiotics which induce the global bacterial response (the SOS response) to DNA damage or to the inhibition of DNA replication. These antibiotic agents could be trimethoprim: which prevents the incorporation of thymine into bacterial DNA; and ciprofloxacin: which blocks the replication fork movement by trapping DNA gyrase on DNA also induce prophages that reside on the *S. aureus* genome (Goerke et al., 2006; Kelly et al., 2009). As a result, an increasment in the number of toxin genes is produced, leading to improved toxin production (Waldor, 1998; Lindsay and Holden, 2004, Kelly et al., 2009). The same side effect was found in case of the chemotherapeutic agent mitomycin C, that induces the SOS response in lysogenic Shiga toxin-producing *E. coli* (STEC) where hemolytic uremic syndrome (HUS) appeared as side effect (Acheson and Donohue-Rolfe, 1989).

Recent studies of *S. aureus* strain MSSA476 had shown that mitomycin C induction of  $\Phi$ Sa3ms prophage resulted a transcriptional up-regulation of *sak*, *sea*, *seg2*, and *sek2* (Sumby and Waldor,

2003). Borst and Betley (1994) suggested that factors in addition to *sea* mRNA levels affected SEA production. It was determined that not just chemotherapeutic agents could induce the expression of *sea*. Identification of the effect of acetic acid as food preservative on *sea* expression and toxin production was studied at different pH by Wallin-Carlquist et al. (2010). It has shown that the acetic acid increases *sea* gene expression in *S. aureus*. At pH 6.0 and 5.5, maximal *sea* expression was observed. At pH 6.0 there was a marked shift in growth rate and phage production peaked at pH 5.5. These findings suggest prophage induction. At pH 5.0 and 4.5 the *sea* gene copy numbers increased dramatically during late stages of cultivation, but SEA levels and phage copy numbers were low indicating that protein synthesis was affected. According to hypothesis of Wallin-Carlquist et al. (2010) the acetic acid reduces the intracellular pH of *S. aureus*, inducing the temperate phage resulting upward trend in the *sea* expression. The results confirm the theory by other research groups such as Borst and Betley (1994) that prophages not only promote the spreading of virulence genes, but at the same time have contribution in the regulation of gene expressions (Wallin-Carlquist et al., 2010).

### 2.3.1.3. Regulatory system of *sed*

Several global regulators have been identified that regulate the production of virulence-associated exoproteins and cell wall components. The expression of a number of the enterotoxins including SEB, SEC, and SED are under the regulation of different regulatory systems such as the accessory gene regulator (Agr), the staphylococcal accessory regulator (Sar) and the repressor of toxin (Rot) (Bronner et al., 2004). The most characterized regulatory systems among these in *S. aureus* are the Agr system (Ji et al., 1995; Zhang et al., 2002) as shown on Fig. 6.



**Figure 6.** Agr system and its influence on *sed* expression in *Staphylococcus aureus* (Source: Novick and Geisinger, 2008 with modification)

The *agr* locus is activated when the AIP concentration reaches a threshold that is directly linked to specific cell density. This type of regulation called quorum sensing, when the bacterial population

reacts to cell density (Bronner et al., 2004; Novick and Geisinger, 2008). The *agr* operon encodes a two component signalling pathway consisting of two distinct transcripts, RNAII and RNAIII (Kornblum et al., 1990; Novick et al., 1993). RNAII encodes the structural genes of the quorum-sensing system, AgrBDCA. (Kornblum et al., 1990; Novick et al., 1993; Tseng and Stewart, 2005) while RNAIII serves as the intracellular effector of the system. AgrA is the response regulator. The production of RNAII and RNAIII is enhanced when the AgrA is activated and upregulates the expression of the P2 and P3 promoters (Benito et al., 2000). During the exponential growth phase, the AgrD encodes a propeptide, which is processed and exported as autoinducing peptide (AIP) from the cell by AgrB (Ji et al., 1995; Zhang et al., 2002; Zhang et al., 2004). The AgrC protein, that is the transmembrane receptor of the AIP, and the sensor of the system (Ji et al., 1997) detect the level of autoinducer peptides (AIP) in the environment. Accumulation of a threshold concentration of AIP in the environment leads to an activation and autophosphorylation after transferring the phosphate to AgrA (Lina et al., 1998). As a consequence, conformational changes of AgrA occur that permit to bind the agr P2 and P3 promoters. In phosphorylated form it is inducing the production of RNAII and RNA III more (Bronner et al., 2004; George and Muir, 2007). RNAIII encodes delta-hemolysin but most importantly the RNAIII itself constitute as regulatory signal of the Agr system (Yarwood and Schlievert 2003; Tseng et al., 2004). When the cell density increases in a growing culture, the intracellular level of RNAIII increases due to the activity of the Agr system. That leads to an increased transcription of many exotoxin genes and reductions of transcription of certain cell wall protein determinants.

Rot is a global transcriptional modulator of virulence genes and acts as both a negative and positive regulator of gene expression (Saïd-Salim et al., 2003). Rot is a member of the staphylococcal accessory regulator (Sar) family of transcriptional factors of *S. aureus* and is negatively regulated by the Agr system (McNamara et al., 2000). Rot is a negative regulator of *sed* expression (Tseng et al., 2004). Rot affects transcription of its target genes during the exponential phase of growth (McNamara et al., 2000). When the Agr system is induced during the post-exponential growth phase, the RNAIII regulates the synthesis of the regulator of Rot and the Rot protein is inactivated by blocking its translation. RNAIII-Rot antagonism forms and expression of *sed* happens (Novick, 2003a). This interaction controls the Agr-mediated post-exponential- phase increase in *seb* and *sed* transcription (Tseng et al., 2004; Tseng and Stewart, 2005; Geisinger et al., 2006).

It could be established that Agr regulation of the *sed* promoter is an indirect action mediated through the Agr regulation of Rot activity. Agr effect on enterotoxin D gene transcription can be explained by the control of Rot activity by the Agr system (McNamara et al., 2000).

The *sed* gene together with *sej*, *ser* are carried on the 27.6 kb penicillinase plasmid pIB485 in *S. aureus* (Tseng et al., 2004; Argudín et al., 2010). Plasmids have been long recognized as

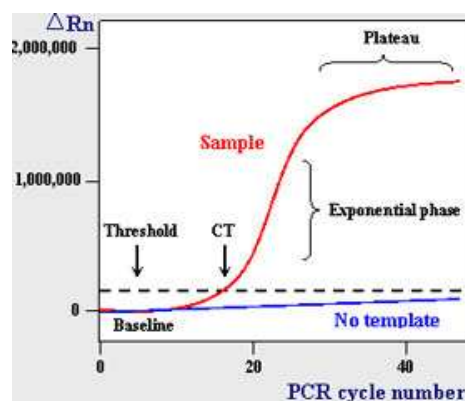
efficient vehicles for the spread of resistance and virulence determinants through horizontal gene transfer. The greatest concentration of SEB, SEC and SED is primarily produced during the transition from the exponential to the stationary phases of growth (Bergdoll, 1979; Otero et al., 1990).

### 2.3.2. Molecular methods used for studying gene expression

The combination of classical microbiology with the tools of molecular biology serves an alternative for assigning outbreaks to SEs and understand the mechanism of toxin production at molecular level. qRT-PCR-based method is suitable for monitoring the conditions when the expression of toxin gene is up- or downregulated.

#### 2.3.2.1. Real-time PCR and qRT-PCR

All real-time PCR system detect the accumulation of PCR products during amplification with fluorescent dyes and then correlate this fluorescent signal to the amount of PCR product in the reaction. Data are on line detected and can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput as well as eliminating the need for postamplification manipulation and thus reducing opportunities for contamination (Valasek and Repa, 2005; Maráz et al., 2006). All amplification composes an amplification curve which is the basis of the detection of the product in real time. Ideally the amount of product should double in each cycle of PCR, resulting in an exponential increase in fluorescence throughout the amplification process. In the first few cycles of real-time PCR, fluorescence remains at background levels and increases in fluorescence are not detectable until enough amplified product does not accumulate to yield a detectable fluorescent signal. This point is defined as the  $C_T$ , and always occurs during the exponential phase of amplification as shown on Fig. 7.



**Figure 7.** Amplification curve in real-time PCR reaction. (Source: [www.rt-pcr.com](http://www.rt-pcr.com) ); The x-axis shows the PCR cycle number while y-axis shows the detected fluorescence which is proportional to the amount of amplified product.

As the reaction is going on the amplification curve turns from the exponential phase to the plateau phase where the reaction components will be limited. Quantification is not affected in the plateau phase (Bustin, 2000). The  $C_T$  value of a reaction is determined mainly by the amount of template present at the start of the amplification reaction (Higuchi et al., 1993). Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the start of the reaction, the fewer number of amplification cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson et al., 1996). The reaction will have a high, or late  $C_T$ . This statement composes the basis for quantitative real-time PCR.

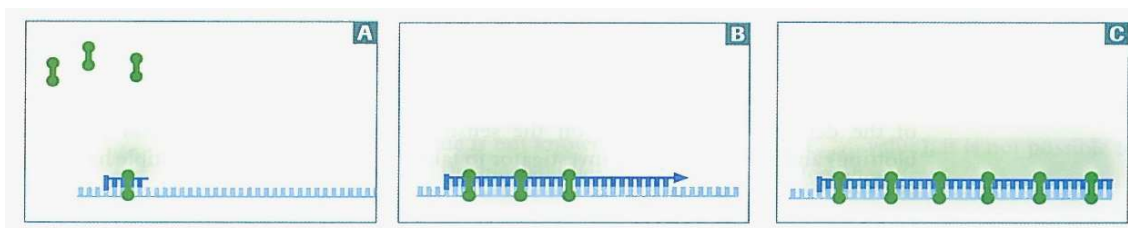
The fluorescent dye reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The dyes could be sequence independent and dependent which differ in their specificity and their unique characteristics (Valasek and Repa, 2005). **SYBR Green I** is a sequence independent intercalating dye that binds to all dsDNA molecules regardless of sequence (Fig. 8. (1)). The primary concern of that is specificity. Melting curve analysis is necessary to make discrimination between specific products, primer-dimers and other artefacts based on their specific melting temperatures.

The most commonly used sequence specific fluorophore labelled DNA oligonucleotides are hydrolysis probes (Holland et al., 1991), hybridization probes (Woo et al., 1997) and molecular beacons (Tyagi et al., 1996). The assay fluorescence increases only if the specific target is present in the reaction and primer-dimers or PCR by-products will not be detected. Most sequence specific probe formats are based on the Fluorescence Resonance Energy Transfer (FRET) that is based on the transfer of energy from one fluorescent molecule to another adjacent fluorescent molecule (Valasek and Repa, 2005).

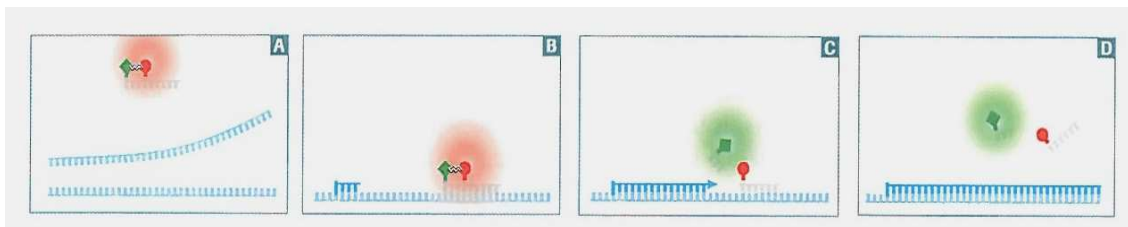
In case of **hydrolysis probes** (Fig. 8. (2)) one fluorophore is termed the quencher and the other is the reporter. When the quencher and reporter are in close proximity, they are both attached to the same short oligonucleotide and the quencher absorbs the signal from the reporter.

**Hybridization probes** are based on the use of two specially designed probes to maximise the specificity (Wittwer et al., 1997). Both probe molecules are labelled with different dyes (Fig. 8. (3)). One of the probes is labelled at the 3' end called fluorescein donor, while the acceptor is labelled at the 5' end. The energy of the donor dye from the first probe can excite the acceptor dye on the second probe which then emits fluorescent light at a different wavelength. That emission is detected by the instrument.

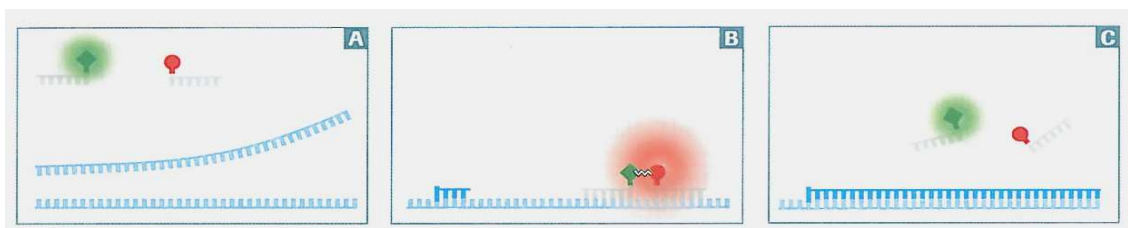
(1)



(2)



(3)



**Figure 8. Fluorescent dyes, (1) SYBR Green I, (2) hydrolysis probes (TaqMan ), (3) hybridization probes (Source: Degen et al., 2006)**

(1) When dsDNA forms (Panel A) and is synthesized (Panel B) SYBR Green I binds the dsDNA and the fluorescent signal from the bound SYBR Green I (green light) increases. At the end of elongation (Panel C) all DNA is double stranded and the maximum amount of SYBR Green I is bounded.

(2) Separation of target DNA occurs during denaturation step (Panel A); primers and probes specifically anneal to the target sequence (Panel B), the hydrolysis probe is phosphorylated at the 3' end to avoid extension of that part; as the DNA polymerase extends the primer (Panel C) the 5' endonuclease activity of the enzyme will cleave the probe allowing the reporter dye to emit green fluorescence; (Panel D) The probe fragments are then displaced from the target. The fluorescent signal of the reporter dye is detected at the end of each elongation phase.

(3) Hybridization does not occur during the denaturation step (Panel A); (Panel B) the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, the two fluorescent dyes close to each other. Fluorescein is excited by blue light which causes to emit green fluorescent light. The emitted energy excites the red acceptor dye (second probe). The red fluorescence is measured at the end of each annealing step when the fluorescence intensity is the greatest; (Panel C) elongation and displacement of the probes appear.

Real-time PCR with TaqMan probe was applied previously for detection *Staphylococcus aureus sea* and *sed* genes isolated from hospital patients (Klotz et al., 2003).

### Application of qRT-PCR for detection of gene expression

The major goal of genomics is to gain an exhaustive understanding of the structure and the function of the genomes. The area of genomics is basically divided into two fields such as structural genomics dealing with the characterisation of the physical nature of whole genomes and the functional genomics focusing on the characterisation of overall patterns of gene expression. Functional genomics works with the key molecules which give life to the cells: RNA (which



correspond to the part of DNA which is expressed and hence biologically active), proteins and also metabolites (which are both biologically active molecules within cells and tissues). Functional genomics permits the detection of genes that are up- or downregulated at any given time depending on environmental factors (Hocquette, 2005).

RNA studies hide pitfalls originating from the natural structure of RNA molecule. RNA is occurring in a single stranded form in bacterial cells and therefore it is very sensitive to degradation by RNase enzymes present in the cell or in the environment. However, it is widely used for quantification of transcription with Northern blotting and *in situ* hybridisation (Parker and Barnes, 1999), RNase protection assays (Hod, 1992; Saccomanno et al., 1992), the reverse transcription polymerase chain reaction (RT-PCR) (Weis et al., 1992) and cDNA arrays (Bucher, 1999).

qRT-PCR is the most sensitive and flexible tool from the quantification methods (Wang and Brown, 1999) to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure (Bustin, 2000). As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into complementary DNA (cDNA) using reverse transcriptase. Reverse transcriptases are enzymes used in nature by retroviruses, including human immunodeficiency virus and hepatitis C virus to generate DNA from viral RNA. There are several commonly used reverse transcriptases especially engineered enzymes that enhance polymerase activity or decrease unwanted nuclease activities (e.g. Omniscript, PowerScript, StrataScript, SuperScript II) (Valasek and Repa, 2005). The RT step can be primed using specific primers, random hexamers or oligo-dT primers. The use of mRNA-specific primers decreases background priming, whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analysed from a small sample of RNA (Zhang and Byrne, 1999; Bustin and Mueller, 2005). Reverse transcription combined with real-time PCR has become the most popular method of quantitating steady-state mRNA levels (Bustin, 2000). It is most often applied as a useful technique to determine gene expression (Orlando et al., 1998; Bustin, 2002) or to validate the results of DNA microarrays. Because of the high specificity and sensitivity of real-time RT-PCR, even slight and tender changes in gene expression can be detected (Valasek and Repa, 2005) or expression of multiple genes in various growth conditions can be monitored (Derzelle et al., 2009).

In practice qRT-PCR was developed for monitoring and determining the level of neurotoxin gene (*cntB*) in *Clostridium botulinum* (Lövenklev et al., 2004a,b) or other botulinum neurotoxin types E (Artin et al., 2007) as well as following virulence expression of *Listeria monocytogenes* in salmon at different storage temperature (Duodu et al., 2010). Bore et al. (2007) studied global gene expression in *Staphylococcus aureus* for acid-shock response where the pattern of the regulation



was confirmed by qRT-PCR. Monitoring of *sea* expression in *Staphylococcus aureus* for acetic acid at different pH adjustments was established as well by Wallin-Carlquist et al. (2010).

Generally two different methods exist for analysing data from real-time RT-PCR which are the **absolute** and **relative quantifications** (Pfaffl, 2001; Livak and Schmittgen, 2001).

To investigate the physiological changes in gene expression the relative expression ratio is adequate for the most purposes. **Relative quantification** describes the change in expression of the target gene versus a reference gene as the normalizer. To determine the relative expression of a target gene in the test and calibrator samples, the expression levels of both the target and the reference genes need to be determined using qRT-PCR.

For the calculation of relative quantification a new mathematical model were developed by Pfaffl (2001):

$$RE = \frac{(E_{target})^{\Delta C_p \text{ target (calibrator-test sample)}}}{(E_{ref})^{\Delta C_p \text{ ref (calibrator-test sample)}}}$$

where RE is a relative expression ratio between the target and the reference gene;  $E_{target}$  is the amplification efficiency of target gene transcript in the real-time PCR;  $E_{ref}$  is the amplification efficiency of a reference gene transcript in real-time PCR;  $\Delta C_p$  is a crossing point deviation of an unknown or test sample versus the calibrator or control sample.

Amplification efficiency (E) is calculated from the slope of standard curve in the log-linear range of the amplification (Klein et al., 1999):

$$E = 10^{(-1/\text{slope})}$$

The ideal reference gene used as a standard or normalizer should always be expressed at a constant level in all relevant times and conditions tested (Giulietti et al., 2001) and have to be stable against the experimental treatment and secure unregulated transcript.

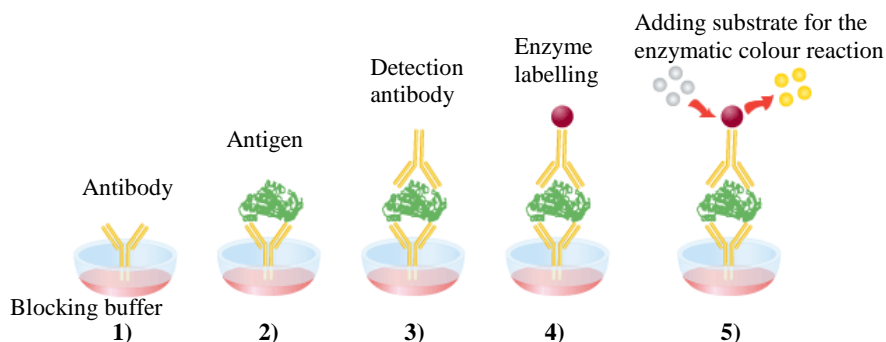
**Absolute quantification** is usually applied for viral load determination, quantification of microbial diversity in an environmental sample, and chromosome or gene copy number determination. In this assay, the concentration of the target molecule is expressed as an absolute value (e.g. copies,  $\mu\text{g}/\text{ul}$ ) This quantification determines how much of a target gene is present in a particular sample without reference to other samples (Livak and Schmittgen, 2001). The quantification is based either on an internal or an external calibration curve (Pfaffl, 2001). It involves comparing the  $C_T$  values of test samples to those of standards of known quantity plotted on a standard curve. Usually, the quantity is normalized to a unit amount of sample, such as number of cells, volume, or total amount of nucleic acid.

### 2.3.2.2. Immunological methods for investigation of staphylococcal enterotoxin production

Heat treatment commonly used in food processing can destroy viable *S. aureus* cells but not inactivate heat stable enterotoxins that can survive at high temperatures. SEA and SED the most frequently recovered from food involved in SFP (Balaban and Rasooly, 2000; Le Loir et al., 2003). It was detected that low amount of 100 to 200 ng staphylococcal enterotoxin caused symptoms of intoxication determined from chocolate milk (Evenson et al., 1988) but also there is information that as low amount as 1ng/g to more than 50 ng/g is also enough to cause symptoms (Meyrand et al., 1999). There are number of methods to detect staphylococcal enterotoxigenicity including biological and immunological assays. The traditional use of animal tests has been replaced by sensitive and convenient immunological assays. For detection of enterotoxins the AOAC (Association of Official Analytical Chemists) approved microslide test method (Horowitz, 2000). Enterotoxins could be detected directly in culture and in contaminated foods with immunological methods such as enzyme immunoassay (EIA) methods in particular enzyme-linked immunosorbent assay (ELISA) (Fey et al., 1984) enzyme-linked fluorescent assay (ELFA) and reverse passive latex agglutination (RPLA) (Rose et al., 1989).

The **ELISA** was first introduced by Saunders and Barlett in 1977 for the detection of SEA in foods (Su and Wong, 1997). In general two formats can be distinguished based on the detection principle. It could be competitive or non-competitive ELISAs such as sandwich ELISA (Kauffman, 1980; Stiffler-Rosenberg and Fey, 1978). The so-called **sandwich ELISA** involves a capture antibody specific to the protein of interest (toxin) which is immobilized on a solid phase (e.g. microtiter plate or multiple well strips). The enterotoxin in the sample is captured by the immobilized antibody and detected by an enzyme-labelled (conjugated) secondary enterotoxin-specific antibody which binds to the protein of interest by forming a “sandwich”. The enzyme acts on a suitable substrate if it is added producing a colour reaction. The intensity of the colour reaction is proportional to the amount of toxin in the sample (Schubert-Ullrich et al., 2009). Absorbance data can be generated with a single or dual wavelength microtiter plate reader. An example for sandwich ELISA is demonstrated in Fig. 9. The commercially available kits are usually sandwich ELISAs. The competitive format is applicable also to the detection of small analytes (Schubert-Ullrich et al., 2009). Polyclonal antibody technology relies on the production of a range of antibodies, either a single protein of interest or to all the proteins within the food, depending on the preparation which is used. It is able to recognize different epitopes of the antigen (toxin) in the food mixture and more tolerant to small changes in the nature of antigen. This provides a detection system which is less likely to fail completely to identify the presence of proteins of interest. The main limitations of polyclonal technology are variable affinity, limited production and a requirement for extensive

purification procedures to eliminate cross-reactivity resulting antiserum to ensure specificity (Asensio et al., 2008; Koppelman and Hefle, 2006).



**Figure 9: Detection of SE with sandwich ELISA**

(Source of the drawing: <http://blog.leinco.com/2010/sandwich-elisa-step-by-step-diagram>)

1) capture antibody is fixed on the surface of the wells; 2) sample is added and the antigen is captured by the bounded antibody creating the first complex; 3) second, detection antibody is added that is specific for the antigen; 4) enzyme is added that binds to the detection antibody creating a complex; 5) substrate is added that reacts with the enzyme producing a colour reaction; Absorbance of the colour reaction is detected on microtiter plate reader.

There are several commercially available kits based on (ELISA) methods as well as enzyme-linked fluorescent assay (ELFA) and reverse passive latex agglutination (RPLA) as shown in Table 6. The advantage of ELISA methods are that they ensure rapid detection and screening of the range of staphylococcal enterotoxins such as A, B, C1, C2, C3, D, E from the food sample at the same time. VIDAS<sup>TM</sup> SET was used for detection enterotoxin in dairy products (Meyrand et al., 1999; Vernozy-Rozand et al., 2004). In comparison with the methods it was found that detection of different purified staphylococcal enterotoxins (A, B, C2, D and E) differed. VIDAS<sup>TM</sup> SET 2 had a greater specificity (100%) and sensitivity than VIDAS<sup>TM</sup> SET and TRANSIA PLATE Staphylococcal Enterotoxins (Meyrand et al., 1999; Vernozy-Rozand et al., 2004). The drawback of these kits is that false positive results could be obtained with foods in which non-*S. aureus* grew. In that case the kit suffered from a lack of specificity as it was detected with TECRA kit by Park et al. (1992).

Several in-house ELISA exist for enterotoxin detection from food as well. Morrisette et al. (1991) used sandwich ELISA for detection of SEB from cheese. Lapeyre et al. (1988) developed indirect double sandwich ELISA for detection of SEA to SED with monoclonal antibodies in food samples. The commercial kits are focusing on the screening different enterotoxins from food at the same time. For detection gene expression of only one toxin from food a more cost effective in house ELISA methods are more preferable. For detection *sea* expression with SEA production under acetic acid stress a slightly modified ELISA method from Poli et al. (2002) was used by Wallin-Carlquist et al. (2010). That in-house method ensures a simple, viable, sensitive, accurate and cost

effective detection of enterotoxin A and B. The minimum detection level of that system for the toxins was 0.5 ng/ml and cross reactivity between serotypes was negligible.

**Table 6.** Commercially available kits for staphylococcal enterotoxin detection

Name of the kit	Type of the method/ Antibody	Detected antigens	Reaction surface	Sensitivity (to the toxin)	Specificity	Time of detection	References
Bommeli Kit	EIA / (m.)	to SEA-to SEE	plastic balls for enterotoxin capture	0.1-10 ng/ml	false + and – appear; interference with food occurs	1-2 days, preliminary food extraction is needed	Park et al.(1994); Baird-Parker, (2000)
TECRA kit (3M™ Tecra Staph Enterotoxins identification tests)	EIA or ELISA / (p.)	SEA,SEB, SEC, SED, SEE	standard microtitre plate	1 ng/ml of sample extract	false + and – appear; interference with food occurs	4 h	<a href="http://www.noackgroup.com">http://www.noackgroup.com</a> ; Park et al.(1992); Park et al.(1994)
TECRA Staphylococcal Enterotoxin Visual Immunoassay (VIA™)	EIA or ELISA / (p.)	SEA,SEB, SEC1,SEC2, SEC3, SED,SEE	standard microtitre plate	>1 ng/ml of prepared sample	>96%	4 h	<a href="http://www.noackgroup.com">http://www.noackgroup.com</a> Bennett, (2005)
TRANSIA® Tube Staphylococcal Enterotoxins	ELISA / (combination of m. and p.)	SEA,SEB, SEC1,SEC2, SEC3, SED,SEE	tubes	0.5 ng /g sample	high specificity, no false + reaction detected	90 min	<a href="http://jornades.uab.ca">http://jornades.uab.ca</a> ; Bennett, (2005)
TRANSIA® Plate Staphylococcal Enterotoxins and TRANSIA Plate Staphylococcal Enterotoxins Plus	ELISA / (combination of m. and p.)	SEA,SEB, SEC1,SEC2, SEC3, SED,SEE	standard microtitre plate	0.25 ng /g sample	false + results were detected in meats, seafood products; no false + results in liquid, canned/dehydrated food	2 h	<a href="http://www.biocontrolsys.com">http://www.biocontrolsys.com</a> ; <a href="http://jornades.uab.ca">http://jornades.uab.ca</a> ; Vernozy-Rozand et al. (2004)
VIDAS™ SET and SET2 staphylococcal enterotoxin kit	ELFA (automated) / (m.)	SEA,SEB, SEC1,SEC2, SEC3, SED,SEE	testing strip coated with antibodies	1 ng/ml and 0.5 ng/g food	false + and - are rare	80min	<a href="http://www.biomerieux-industry.com">http://www.biomerieux-industry.com</a> ; Asao et al. (2003); Vernozy-Rozand et al. (2004); Ostyn et al. (2011)
Oxoid kit (SET-RPLA kit)	reverse passive latex agglutination (RPLA) / (m.)	to SEA-to SED	latex particles (antibody is attached to its surface) on microtiter plates	semi-quantitativ; 0.5 ng/ml for test extract; for food 1 ng/g	lacking of specificity; food components interfere with the test	20-24 h preliminary sample preparation is acquired	<a href="http://www.selectscience.net">http://www.selectscience.net</a> ; <a href="http://www.oxoid.com/">http://www.oxoid.com/</a>
RISASCREEN SET A,B,C,D,E	ELISA / (m.)	SEA,SEB, SEC, SED, SEE	standard microtitre plate	0.25 ng/ml solid samples to 2 ng/ml	cross reactivity occurs (10-20%) and food samples can increase or decrease it	2 h 45min	<a href="http://seafood.ucdavis.edu">http://seafood.ucdavis.edu</a> ; <a href="http://www.r-biopharm.com">http://www.r-biopharm.com</a> ; Tasci et al. (2011); Park et al.(1994)
RISASCREEN SET Total Screen for <i>Staphylococcus</i> Enterotoxin	ELISA / (m.)	SEA,SEB, SEC, SED, SEE	standard microtitre plate	0.25 ng/ml	high specificity, no cross reactivities known	2 h 45min, preliminary sample preparation is necessary	<a href="http://www.r-biopharm.com">http://www.r-biopharm.com</a> Ostyn et al. (2011)

(m.): monoclonal antibody; (p.) polyclonal antibody.

### 3. OBJECTIVES

The overall objective of this work was to study the dominant meat-associated bacteria involved in meat spoilage (especially *Pseudomonas* species) and the important food poisoning *Staphylococcus aureus*. In the case of *Pseudomonas* species, the main objectives were (i) to work out a comprehensive methodology for molecular typing and identification and (ii) to determine and characterise the diversity of the spoilage-causing microbiota on pork meat using conventional and molecular methods. Regarding *Staphylococcus aureus*, the objectives were (i) to monitor the relative gene expression of enterotoxins, (ii) to determine the production of enterotoxins *in situ* and (iii) to compare the enterotoxin formation in food with batch cultures of pure *Staphylococcus aureus* using molecular and immunological methods.

The following steps were determined for reaching objectives:

1. Evaluation of different *Pseudomonas* selective media for detection of pseudomonads
2. Molecular identification and assessment of genetic diversity of *Pseudomonas* spp. based on different PCR-methods
  - a) evaluation of a *Pseudomonas* genus-specific primer pair published by Purohit et al. (2003)
  - b) molecular typing and comparison of the isolates with RAPD
  - c) identification of the *Pseudomonas* isolates with species-specific PCR primers
  - d) analysis of 16S *rDNA* and *rpoB* genes by using PCR-RFLP and DNA sequencing
3. Determination of proteolytic and lipolytic activities of the bacterial isolates to assess their spoiling potential
4. Study the spoiling potential and competitiveness of a *Chryseobacterium antarcticum* isolate
5. Study of the growth behaviour of *Staphylococcus aureus* SA45 strain
  - a) on different meat products
  - b) in pH controlled batch fermentations
  - c) as well as studying the expression of the enterotoxin encoding genes *sea* and *sed* in *S. aureus* by using qRT-PCR (referring to the circumstances in 5.a-b)
  - d) and determine the extracellular SEA and SED production with ELISA (referring to the circumstances in 5.a-b)

## 4. MATERIALS AND METHODS

### 4.1. Bacterial strains

*Pseudomonas* strains that were derived from the spoiling microbiota of pork chops during 6 or 8 days of chilled storage were isolated at the Department of Microbiology, Central Food Research Institute, Budapest, Hungary. Professors József Farkas and Éva Andrásy provided the availability of these isolates for my study. In Tables 7 and 8, different isolates, type strains as well as reference strains are listed.

**Table 7.** Pork chop isolates used for biochemical characterization and molecular analysis.

Day of Isolation	Code	Isolated from	Storage temperature of meat
8	F 1443/1	PCA agar	4°C
8	F 1443/2 *	PCA agar	4°C
8	F 1443/4 *	PCA agar	4°C
8	F 1443/5	PCA agar	4°C
8	F 1443/7	Cetrimide agar	4°C
8	F 1443/8	Cetrimide agar	4°C
8	F 1443/9	Cetrimide agar	4°C
8	F 1443/10 *	Cetrimide agar	4°C
8	F 1443/11	Cetrimide agar	4°C
8	F 1443/12 *	Cetrimide agar	4°C
8	F 1443/13 *	Cetrimide agar	4°C
0	F 1445/1 *	PCA agar	8°C
0	F 1445/3	PCA agar	8°C
0	F 1445/4	PCA agar	8°C
0	F 1445/5	PCA agar	8°C
0	F 1445/6	Cetrimide agar	8°C
0	F 1445/8 *	Cetrimide agar	8°C
6	F 1445/9 *	PCA agar	8°C
6	F 1445/10	PCA agar	8°C
6	F 1445/12	PCA agar	8°C
6	F 1445/14 *	Cetrimide agar	8°C
6	F 1445/15 *	Cetrimide agar	8°C

\* In case of some isolates differences were found in the morphology and these strains were named a, b, or c as subclones in further molecular identification analysis.

**Table 8.** List of type and reference strains used

Species	Strain	Origin and isolated from
<i>Pseudomonas aeruginosa</i>	ATCC 10145 <sup>T</sup>	ATCC <sup>a</sup> , origin not known
<i>Pseudomonas aeruginosa</i>	ATCC 9027	ATCC, outer ear infection
<i>Pseudomonas alcaligenes</i>	B. 02011 <sup>T</sup>	NCAIM <sup>g</sup> , swimming-pool water
<i>Pseudomonas fluorescens</i>	CCM 2115 <sup>T</sup>	CCM <sup>c</sup> , pre-filter tanks
<i>Pseudomonas fluorescens</i>	CCM 3899	CCM, spoilage-stage beef
<i>Pseudomonas fluorescens</i>	B. 01969	NCAIM, origin not known
<i>Pseudomonas fragi</i>	CCM 1974 <sup>T</sup>	CCM, origin not known
<i>Pseudomonas fragi</i>	CCM 3703	CCM, beef
<i>Pseudomonas fragi</i>	CCM 3704	CCM, pork
<i>Pseudomonas fragi</i>	CCM 3903	CCM, spoilage-stage beef
<i>Pseudomonas fragi</i>	TM-9	CUB <sup>b</sup> , chicken
<i>Pseudomonas lundensis</i>	CCM 3503 <sup>T</sup>	CCM, prepacked beef
<i>Pseudomonas lundensis</i>	CCM 3906	CCM, spoilage-stage beef
<i>Pseudomonas lundensis</i>	CCM 3907	CCM, spoilage-stage beef
<i>Pseudomonas marginalis</i>	CCM 4969 <sup>T</sup>	CCM, chicory
<i>Pseudomonas mendocina</i>	CCM 3590 <sup>T</sup>	CCM, soil enrichment with ethanol as carbon source
<i>Pseudomonas putida</i>	B. 01634 <sup>T</sup>	NCAIM, origin not known
<i>Pseudomonas putida</i>	DSM 291 <sup>T</sup>	DSM <sup>d</sup> , lactate enrichment
<i>Pseudomonas stutzeri</i>	CCM4557 <sup>T</sup>	CCM, spinal fluid
<i>Pseudomonas taetrolens</i>	CCM 1982 <sup>T</sup>	CCM, cause of mustiness in eggs
<i>Aeromonas sobria</i>	CCM 2807 <sup>T</sup>	CCM, carp ( <i>Cyprinus carpio</i> )
<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	CCM 7232 <sup>T</sup>	CCM, tin of milk with a fishy odour
<i>Bacillus subtilis</i>	MBT-B1	CUB, origin not known
<i>Brevundimonas diminuta</i> (syn.: <i>P. diminuta</i> )	B. 01118 <sup>T</sup>	NCAIM, water
<i>Burkholderia cepacia</i> (syn.: <i>P. cepacia</i> )	B. 01621	NCAIM, onion
<i>Campylobacter jejuni</i>	CCM 6214 <sup>T</sup>	CCM, origin not known
<i>Citrobacter braakii</i>	TS-8	CUB, chicken
<i>Chryseobacterium antarcticum</i>	JMC 12381 <sup>T</sup>	JMC <sup>e</sup> , soil of penguin habitats near the King Sejong Station on King George Island, Antarctica
<i>Chryseobacterium jeonii</i>	JMC 12382 <sup>T</sup>	JCM, moss of penguin habitats near the King Sejong Station on King George Island, Antarctica
<i>Escherichia coli</i>	ATCC 8739	ATCC, feces
<i>Escherichia coli</i> (O157:H7)	MBT-E1	CUB, origin not known
<i>Hafnia alvei</i>	TM-8	CUB, chicken
<i>Listeria monocytogenes</i>	CCM 4699	CCM, sheep
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	CCM 5884 <sup>T</sup>	CCM, sheep
<i>Serratia liquefaciens</i>	TM-3	CUB, chicken
<i>Stenotrophomonas maltophilia</i>	B. 01119 <sup>T</sup>	NCAIM, orophar. reg. of patient with mouth cancer
<i>Streptococcus thermophilus</i>	B. 02290	NCAIM, origin not known
<i>Yersinia enterocolitica</i>	HNCMB 98001	NCE <sup>f</sup> , human mesenteric lymph node, acute terminal ileitis
<b>Species for studying food-borne illness</b>	<b>Strain</b>	<b>Origin and isolated from</b>
<i>Staphylococcus aureus</i>	SA45	SIK <sup>h</sup> , boiled ham in a food-poisoning outbreak

<sup>a</sup>American Type Culture Collection, USA; <sup>b</sup>Corvinus University of Budapest, Department of Microbiology and Biotechnology, Budapest, Hungary; <sup>c</sup>Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic; <sup>d</sup>Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; <sup>e</sup>Microbe Division / Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan; <sup>f</sup>National Center for Epidemiology, Budapest, Hungary; <sup>g</sup>National Collection of Agricultural and Industrial Microorganisms, Corvinus University of Budapest, Budapest, Hungary; <sup>h</sup>Swedish Institute for Food and Biotechnology (SIK), Gothenburg, Sweden.

## 4.2. Media and broths

### **Brain heart broth (Merck)**

(Merck 110493; if necessary supplement with 15 g/l agar for agar plates)

### **Brain heart infusion (BHI) (BD Diagnostic)**

Bacto™ Brain Heart Infusion (Cat. No. 237500, BD Diagnostic)

### **Plate Count Agar (PCA) (Tryptone Glucose Yeast Agar)**

Glucose	1 g/l
Yeast extract	2.5 g/l
Peptone	5 g/l
Agar	15 g/l

### **Pseudomonas selective agar base, Cetrимide agar (Merck)**

(Merck 1.05284 .0500; supplemented with Glycerol, Merck 1.04094.0500)

UV lamp at 366 nm is necessary for detection of fluorescence.

### **GSP Agar (Pseudomonas, Aeromonas Selective Agar Base) (Merck)**

(Merck 1.10230.0500; supplemented with Penicillin G 100,000 IU, Merck 516104)

### **Pseudomonas Agar F, Base (Merck)**

(Merck 1.10989.0500; supplemented with Glycerol, Merck 1.04094.0500)

UV lamp at 366 nm is necessary for detection of fluorescence.

### **Pseudomonas Agar P, Base (Merck)**

(Merck 1.10988.0500; supplemented with Glycerol, Merck 1.04094.0500)

UV lamp at 366 nm is necessary for detection of fluorescence.

### **BAIRD- PARKER Agar (Merck)**

(Merck 1.05406.0500; supplemented with egg-yolk tellurite emulsion, Merck 1.03785.0001)

### **de Man-Rogosa-Sharpe (MRS) agar (Merck)**

(Merck 11.10660 .0500)



**Skim Milk Agar (SM): according to ATLAS (1995)**

Glucose	1 g/l
Yeast extract	2.5 g/l
Peptone	5 g/l
Agar	15 g/l

Skim milk powder 5 g/l dissolved in 100ml distilled water and autoclaved separately from the medium

**Standard Methods Caseinate Agar (SMC): according to ATLAS (1995)**

1.)	Peptone	5 g/l
	Yeast extract	2.5 g/l
	Glucose	1 g/l
	Agar	15 g/l
2.)	Sodium-citrate	4.41 g/l
	Sodium-caseinate	5 g/l
3.)	CaCl <sub>2</sub> *2H <sub>2</sub> O	2.94g/l

Solution 1, 2 and 3 of the media have to be autoclaved separately and then cool down to 60°C. After cooling down pour together solution 1 and 2 and finally solution 3.

**Tributyrin Agar (PCATB) for testing lipase activity: according to ATLAS (1995)**

Yeast extract	3 g/l
Peptone	5 g/l
Tributyrin (glyceryl tributyrate)	10 g/l
Agar	15 g/l

After autoclaving and cooling down the media has to be shaken quite vigorously.

**Tween<sup>TM</sup> 80 Hydrolysis Medium (PAT-80) for testing esterase activity: according to ATLAS (1995)**

Peptone	10 g/l
NaCl	5 g/l
CaCl <sub>2</sub> *2H <sub>2</sub> O	0.1 g/l
Agar	12 g/l
Tween <sup>TM</sup> 80	5 g/l

### 4.3. Solutions

#### 4.3.1. Solutions for DNA extraction

##### **Tris-EDTA (TE) buffer (pH 8)**

Tris-HCl	10 mM
Na <sub>2</sub> -EDTA	1 mM

##### **Mixture of phenol-chlorophorm-isoamyl-alcohol (PCIA) (pH 8)**

Buffered phenol: chlorophorm: isoamyl-alcohol

Ratio: v:v:v= 25:24:1

##### **Breaking buffer for cell lysis (pH 8)**

Sodium-dodecil-sulfate (SDS)	1% (w/v)
TritonX-100	2% (v/v)
NaCl	100 mM
Tris-HCl	10 mM
Na <sub>2</sub> -EDTA	1 mM

#### 4.3.2. Solutions for gel electrophoresis

##### **10x Tris- Boric acid-EDTA (TBE)**

Tris-base	108 g/l
Boric acid	54 g/l
EDTA (0,5 M, pH 8)	10 ml

##### **Loading buffer**

Sacharose	5 g
EDTA 50 mM, pH8	1 ml
Brome-phenolblue indicator	10 mg
SDS (10%)	10 µl
Distilled water	up to 10ml

##### **Agarose gel (1.5%)**

Agarose	1.8 g
TBE buffer (0,5 x)	120 ml
Ethidium-bromide (10 mg/ml)	5.5 µl

## **Molecular markers**

### **For RAPD analysis:**

-DNA Molecular Weight Marker VI. (Boehringer, Mannheim, Germany)

### **For 16S rDNA-RFLP, *rpoB*- RFLP and species-specific PCR:**

-GeneRuler™ 100 bp DNA Ladder Plus ready-to-use (Fermentas)

### **For *Pseudomonas* genus-specific PCR:**

-100 bp marker (New England BioLabs)

## **4.3.3. Solutions for RNA extraction**

### **RNase away solution (Surface decontaminants)**

Molecular BioProducts, Cat. no. 7000

### **DNA away solution (Surface decontaminants)**

Molecular BioProducts, Cat. no. 7010

### **TES buffer (pH 7.5)**

Tris	50 mM
EDTA	5 mM
NaCl	50 mM

### **Acidic phenol**

(Aquaphenol, Art. No.: AQUAPH; Saveen & Werner AB, Malmö, Sweden)

### **Chloroform**

(Sigma-Aldrich, Cat. no. 496189)

### **DEPC water**

DEPC (diethyl pyrocarbonate)	1000 µl
ddH <sub>2</sub> O	1000 ml

Incubate at 37°C for 12 hours and then autoclave

**3 M Sodium acetate (200 ml) pH 4.8 (adjusted with 3 M acetic acid)**

Sodium Acetate	81.6 g
DEPC water	200 ml

**95% Ethanol (100 ml)**

99.5% Ethanol	95.5 ml
DEPC water	4.5 ml

**70% Ethanol (100 ml)**

99.5% Ethanol	70.4 ml
DEPC water	29.6 ml

**RNA storage solution**

Applied Biosystems, Cat. no. AM7001

**4.3.4. Solutions for ELISA****PBS (100 mM) (pH 8.4)**

NaCl	80 g/l
KCl	2 g/l
Na <sub>2</sub> HPO <sub>4</sub>	14.4 g/l

**Coating buffer (pH 9.6)**

Na <sub>2</sub> CO <sub>3</sub>	10.6 g/l
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Store at +4°C for less than 1 month.

**Washing buffer**

PBS (10 mM)	1000 ml
Tween 20	500 µl

Store at +4°C for less than 1 month.

**Blocking buffer (for 100 ml)**

Washing buffer	100 ml
Skim milk powder	5 g

(Semper, Sundbyberg, Sweden)

#### **Assay buffer (for 100 ml)**

PBS 50 mM	100 ml
Bovine Serum Albumin (BSA)	10 mg
Tween 20	100 µl
Thimerosal	10 mg

Store at +4°C for less than 1 month.

#### **4.3.5. Reagents for ELISA**

##### **Antibodies**

- anti- Staphylococcal enterotoxin A IgG (Cat. no.: SLAI101 Toxin Technology, Inc.; Sarasota, FL)
- anti- Staphylococcal enterotoxin A IgG, affinity purified and conjugated to biotin (Cat. no.: SBAC101 Toxin Technology, Inc.; Sarasota, FL)
- anti- Staphylococcal enterotoxin D IgG (Cat. no.: SLDI303 Toxin Technology, Inc.; Sarasota, FL)
- anti- Staphylococcal enterotoxin D IgG, affinity purified and conjugated to biotin (Cat. no.: SBDC303 Toxin Technology, Inc.; Sarasota, FL)

##### **Antigens**

- Staphylococcal Enterotoxin A, highly purified (Cat. no. AT 101 Toxin Technology Inc.; Sarasota, FL)
- Staphylococcal Enterotoxin D, highly purified (Cat. no. DT 303 Toxin Technology Inc.; Sarasota, FL)

##### **Enzyme and substrate**

- NeutrAvidin™-linked alkaline phosphatase (ImmunoPure NeutrAvidin™, alkaline phosphatase conjugated, 0.9 mg/ml, Pierce)
- SIGMAFAST™ p-nitrophenyl phosphate (tablets Sigma-Aldrich N2770)

#### **4.3.6. Processed pork products for *Staphylococcus aureus* experiments**

For studying *sea* and *sed* gene expressions as well as enterotoxin formation of *Staphylococcus aureus* SA45 different processed pork products were tested. The intrinsic and extrinsic factors are listed in Table 9.

**Table 9.** Meat products and their properties for *Staphylococcus aureus* studies

Products	aw	Nitrite content (mg/kg)	NaCl content (%)	pH at packing	Gas composition of modified atmosphere	Thickness of the meat slices (mm)
Boiled ham	0.99	150	2.8	5.8	70% nitrogen + 30% carbon dioxide	1.2
Smoked ham	0.99	150	2.26	5.8		1.2
Serrano ham	0.94	no data	no data	no data		0.8-0.9
Black pepper salami	0.9	no data	no data	no data		0.8-0.9

#### 4.4. Methods

##### 4.4.1. Methods for the studies of *Pseudomonas* and *Chryseobacterium* species

##### 4.4.1.1. Isolation and characterization of bacterial strains from pork chops

Isolation of bacteria from pork chops (without bone and fat) was the following: Pork chops were sliced into 5 cm<sup>2</sup> pieces and stored in sterile Petri dishes at 4°C and 8°C for 6 and 8 days. Samples from Petri dishes were weighted and as much 0.9% NaCl solution was added to the samples as it was necessary to reach ten times dilution. Samples were homogenised with BagMixer (Interscience, France) and decimal dilutions were made in 0.9% NaCl solution. 100µl from the dilutions were spreaded on PCA and Cetrimide agar. After inoculation of the media the plates were incubated at 30°C for 24-48 h and CFU g<sup>-1</sup> of the original samples were determined. In case of pork chops stored at 4°C representative bacterial colonies were collected at day 8 while samples that were stored at 8°C representative colonies were gained from day 0 and 6 which derived from PCA and Cetrimide plates. Bacterial isolates were characterised by morphological examination, Gram stain, spore staining as well as some of the biochemical tests (oxidase, catalase tests, KOH probe) according to Pollack et al. (2009). Some isolates produced colonies of slightly different morphology therefore representative colonies were isolated and named as “a”, “b” or “c”.

Growth and fluorescent ability were tested on selective (Cetrimide agar, GSP agar) and elective media (*Pseudomonas* Agar P and F) at 20°C and 30°C after 72h incubation. Fluorescence was determined under UV light at 366nm. As control media PCA was used.

##### 4.4.1.2. Detection of proteolytic and lipolytic activities

For determination of protease activity derivatives of milk were used in SMC (standard methods caseinate) agar and SM (skim milk) agar according to Atlas (1995). Protease activity was determined by inoculation of the cells as macrocolonies grew onto the surface of the agar plates. Inoculation was performed with 10 µl of cell suspensions of the isolates OD of which were adjusted

to 0.5 at 600 nm. The cell suspensions were dropped to SMC and SM plates in duplicates. Plates were incubated for 7 days at 15, 20, 25 and 30°C in parallel and diameters of the clearing zones were checked after 48 h and measured after 3 and 7 days. Evaluation of the plates was done by the measurement of halos surrounding the colonies.

For detection of esterase activity Tween<sup>TM</sup> 80 Hydrolysis Medium (PAT-80) was used while for checking the lipase activity plate count agar supplemented with tributyrin (PCATB) was applied according to Atlas (1995). Inoculation of the plates was performed as in case of protease activity tests. Plates were incubated for 7 days at 15, 20, 25 and 30°C and diameters of turbid or clearing zones around the colonies on PAT-80 and PCATB plates were measured. Tests were prepared in parallel and repeated two times.

#### **4.4.1.3. Genomic DNA isolation**

DNA extraction from bacterial cells was done by the modified method of Hoffman and Winston (1987). Cells from overnight cultures were transferred into Eppendorf tubes containing 1.0 ml sterile ultrapure water and centrifuged at 14 000 rpm for 5 min. The supernatant was discarded and 200 µl breaking buffer, 0.3 g glass beads (0.425-0.6 mm, Sartorius) and 200 µl PCIA were added to the tubes. After vigorous mixing for 3 min 200 µl TE buffer was added into the samples, mixed shortly and centrifugated at 14 000 rpm for 5 min. The upper phase was transferred to a new Eppendorf tube and 800 µl of 96% ice-cold ethanol was added to the samples. The tubes were placed into a -20°C freezer for 10 min to precipitated nucleic acids. After centrifugation and discarding the supernatant 50 µl TE buffer and 0.6 mg ml<sup>-1</sup> RNase (Sigma) were added to the samples for digestion of RNA molecules at 60°C for 30 min. The enzymatic reaction was stopped by measuring 100 µl of 96% ice-cold ethanol to the tubes that were put into the freezer for 10 min. The repeated spinning and discarding were followed by drying the DNA samples in vacuum dryer (DNA mini, Heto) and 30 µl of TE buffer was added to the tubes. DNA samples were stored at -20°C until using.

#### **4.4.1.4. *Pseudomonas* genus-specific PCR assay**

Amplification of using Psf and Psr primers (Table 10) was performed by the modified protocol of Purohit et al. (2003). The reaction mixtures contained 1 x DNA polymerase buffer, 1.25 mM MgCl<sub>2</sub>, 0.1 mM dNTP, 0.25 pM of each primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzyme) and 1 µl of 100ng template DNA. The final volume of the reaction mixture was 25 µl. The PCR reactions were performed in ESCO SWIFT<sup>TM</sup> Thermal Cycler. The conditions of the PCR were the following: pre-denaturation at 95°C for 5 min, amplification (35 cycles): denaturation at 94°C for 30 s, primer annealing at 64°C for 15 s and primer extension at 72°C for 30 s; final

extension at 72°C for 4 min. The amplicons were separated by gel electrophoresis (3 µl loading buffer and 6 µl PCR product) applying 1.5% agarose gel. Gel electrophoresis was done at 120 V for 60 min in 0.5 x TBE buffer.

**Table 10.** List of primers used in PCR reactions

Application	Primers	Sequences (5'-3')	Reference
RAPD-PCR	OPA 4	AATCGGGCTG	Operon Technologies, Inc., USA
	OPA7	GAAACGGGTG	
	OPA13	CAGCACCCAC	
	primer272	AGCGGGCCAA	Mahenthiralingam et al., 1996
<i>Pseudomonas</i> genus- specific PCR	PsF	CTACGGGAGGCAGCAGTGG	Purohit et al., 2003
	PsR	TCGGTAACGTCAAAACAGCAAAGT	
PCR amplification of 16S rDNA for RFLP	F27	AGAGTTTGATCMTGGCTCAG	Eden et al., 1991
	R1492	TACGGYTACCTTGTTACGACTT	Edwards et al., 1989 Weisburg et al., 1991
	R1525	AAGGAGGTGWTCCARCC	Edwards et al., 1989
	R338	GCTGCCTCCCGTAGGAGT	Amann et al., 1990
<i>rpoB</i> gene PCR (RFLP)	LAPS	TGGCCGAGAACCAGTTCGCGT	Tayeb et al., 2005
	LAPS 27	CGGCTTCGTCCAGCTTGTTTCAG	
<i>P. fragi</i> specific PCR	<i>fra-F</i>	CGTCAGCACCGAAAAAGCC	Ercolini et al., 2007
<i>P. lundensis</i> specific PCR	<i>lun-F</i>	TGTGGCGATTGCAGGCATT	
<i>Pseudomonas</i> species-specific PCR	<i>carA-R</i>	TGATGRCCSAGGCAGATRCC	Hilario et al., 2004

#### 4.4.1.5. Typing of bacterial isolates by RAPD-PCR

For RAPD-PCR fingerprinting OPA 4, OPA13, OPA7 and primer 272 oligonucleotide primers were used in case of the *Pseudomonas* isolates (Table 10) The 25 µl of PCR mixture contained 1 x DNA polymerase buffer, 1.25 mM MgCl<sub>2</sub>, 0.3 mM dNTP, 0.2 µM of primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzymes) and 1 µl of the template DNA. The PCR reactions were performed in Hybaid PCR Thermal Cycler. Conditions of the PCR were the following: DNA denaturation for 5 min at 95°C; followed by amplification (35 cycles): pre-denaturation at 94°C for 45 s, annealing at 40°C for OPA 4, OPA13, OPA7 and 38°C for primer 272 for 30 s and extension at 72°C for 1 min; final extension took 4 min at 72°C. PCR reactions were carried out with Hybaid PCR Thermal Cycler. The amplicons were separated by gel electrophoresis (3 µl loading buffer and 6 µl PCR product) applying 1.5% agarose gel. Gel electrophoresis was done at 120 V for 60 min in 0.5 x TBE buffer. Gel pictures were analysed by GelCompare II (Applied Maths, Belgium) software. Cluster analysis of the pairwise values was generated using UPGMA algorithm.



#### 4.4.1.6. 16S rDNA-RFLP analysis of the *Pseudomonas* isolates

The PCR reaction for rDNA amplification was performed in 25 µl reaction volume after optimization. The reaction mixture contained 1 × DNA polymerase buffer, 1 mM of MgCl<sub>2</sub>, 0.15 mM dNTP, 2 pM of each of the primers F27 and R1492 (Table 10), 1 U *Taq* DNA polymerase (DyNAzyme, Finnzymes) and 1 µl template DNA. The PCR reactions were performed in ESCO SWIFT™ Thermal Cycler. PCR reaction was as follows: DNA pre-denaturation for 4 min at 95°C; amplification (25 cycles): 94°C for 30 s, 56°C for 30 s and 72°C for 40 s, and final extension: 72°C for 10 min. The size of PCR amplicons were approximately 1465bp. PCR products were digested with the restriction enzymes *AluI*, *HaeIII*, *RsaI* and *MspI* at 37°C for 4 h. The components of reaction mixture in 10 µl reaction volumes were the following: 2 µl PCR product, 1 µl incubation buffer (Tango buffer, Fermentas), 6.8 µl ultrapure water and 0.2 µl (10 U/µl) of the restriction enzyme *AluI* and *RsaI* (Fermentas). In case of *HaeIII* and *MspI* (Promega) the reaction mixture contained 0.1 µl BSA (10 mg/ml). Fragments were separated by electrophoresis using 1.5% agarose gels at 100 V for 2 h. The bands were visualized using ethidium-bromide staining and UV transillumination. The electrophoretic patterns were analysed by GelCompare II (AppliedMaths, Belgium) software.

#### 4.4.1.7. *rpoB*-RFLP analysis of the *Pseudomonas* isolates

Amplification of the *rpoB* gene using the LAPS and LAPS27 primers (Table 10) was done by the modified protocol of Tayeb and co-workers (2005). The reaction mixtures contained 1 × DNA polymerase buffer, 1.5 mM MgCl<sub>2</sub>, 0.1 mM dNTP, 0.2 µM of each primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzyme) and 1 µl of template DNA. The final volume of the reaction mixture was 25 µl. The PCR reactions were performed in ESCO SWIFT™ Thermal Cycler. The conditions of the PCR were the following: pre-denaturation at 94°C for 90 s, amplification (40 cycles): denaturation at 94°C for 10 s, primer annealing at 56°C for 20 s and primer extension at 72°C for 50 s; final extension at 72°C for 5 min. PCR products were digested with the restriction enzymes *AluI*, *HaeIII*, *RsaI* and *MspI* according to the same reaction conditions as in chapter 4.4.1.6.

#### 4.4.1.8. Sequencing of 16S rDNA and *rpoB* amplicons and constructing phylogenetic trees

For preparation of 16S rDNA PCR products for sequencing the same reaction conditions and reaction mixture were applied as in chapter 4.4.1.6 except for the reverse primer which was R1525. In case of amplification of *rpoB* gene for sequencing the same parameters and mixture were followed as in chapter 4.4.1.7. Both cases the amplified DNA was purified using PCR-Advanced™ PCR Clean Up System (Viogene) and the sequencing was performed by the Biological Research Centre (Szeged, Hungary) using the F27, R338 and R1525 primers and by Biomi Ltd. (Gödöllő,

Hungary) using the LAPS and LAPS27 PCR primers. Sequencing was performed by using the ABI 3100 sequencer in both cases. For evaluating the chromatograms Chromaslite 2.06 and MEGA4 software were applied. Sequences were aligned to the database of NCBI (<http://blast.ncbi.nlm.nih.gov>). One isolate was aligned to the database of EzTaxon Server 2.1. ([www.eztaxon.org](http://www.eztaxon.org)). Phylogenetic trees based on 16S rDNA and *rpoB* sequences were constructed by neighbour joining method after ClusterW analysis in MEGA4 software. Bootstraps values were calculated for 500 phylogenetic trees. Nucleotide sequences of the closest related type strains, and strains derived from the GenBank with accession numbers were the following for 16S rDNA: *P. fragi* ATCC 4973<sup>T</sup> (AF094733.1), *P. fluorescens* CCM 2115<sup>T</sup> (DQ207731.2), *P. fluorescens* strain 1582 (JN679853.1), *C. antarcticum* AT1013<sup>T</sup> (NR\_025809.1). In case of constructing phylogenetic tree from *rpoB* sequences the following additional sequences were used from the GenBank: *P. fluorescens* LMG 14674 (HE586418), *P. fluorescens* LMG 6812 (AJ748138), *P. fragi* LMG 2191T (AJ717444), *P. fluorescens* LMG 14577 (HE586423).

#### **4.4.1.9. Identification of *P. lundensis*, *P. fragi* isolates by species-specific PCR**

Distinct sequences of the carbamoyl phosphate synthase gene (*carA*) were amplified with species-specific primer sets (Table 10) for the identification of *P. lundensis* a *P. fragi* isolates as described by Ercolini and co-workers (2007) with slight modifications as follows: The reaction mixtures contained 1 × DNA polymerase buffer, 1.25 mM MgCl<sub>2</sub>, 0.1 mM dNTP, 0.2 µM of each primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzyme) and 1 µl of template DNA. The final volume of the reaction mixtures was 25 µl. The conditions of the PCR were the following: pre-denaturation at 95°C for 4 min; amplification (25 cycles): denaturation at 94°C for 30 s, primer annealing at 56°C for 20 s and primer extension at 72°C for 40 s; final extension was performed at 72°C for 3 min. The amplicons were detected by gel electrophoresis using 1.5% agarose gel.

#### **4.4.1.10. Cultivation and characterization of growth of *Chryseobacterium antarcticum* at different temperatures**

*Chryseobacterium antarcticum* type strain and one of the isolates called F1445/3 that was determined with 16S rDNA sequencing as *Chryseobacterium antarticum* were plated on brain heart infusion agar and incubated at 20°C for 5 days. 3 colonies were picked from the plates and cultivated in BHI broth for 36 h at 20°C with 180 rpm agitation. BHI broth was inoculated with a sufficient volume of cells from overnight culture to give 10<sup>6</sup> CFU/ml initial cell concentration. Cultivations were performed in paralell at 5, 10, 15, 20, 25 and 30°C for 72 h. Colony counts were determined after inoculation of BHI broth and after 24, 48, 72 h of incubation. Serial tenfold dilutions from the flasks in physiological saline solution were performed. The dilutions were plated

on BHI agar to determine the total cell count. Agar plates were incubated at 20°C for 4 and 5 days and the number of CFU was calculated.

#### 4.4.1.11. Competition analysis between *C. antarcticum* and *P. fragi* strains

*Chryseobacterium antarcticum* (F1445/3) and *P. fragi* (F1445/1) strains were plated on brain heart infusion agar and incubated at 20°C for 4 days. One colony was picked from the plates and cultivated in BHI broth for 36h at 20°C with 180 rpm agitation. BHI broth was inoculated with different cell ratio of the 2 different bacteria (experiment A, B, C) showed in Table 11. Cultivation were performed in parallell combined with control samples (experiment D, E, F, G) at 20°C for 72 h. Colony counts were determined after inoculation of BHI broth and after 24, 48, 72, 96 h of incubation. Serial tenfold dilutions from the flasks in physiological saline solution were performed. The dilutions were plated on BHI agar to determine the total cell count. Agar plates were incubated at 20°C for 4 and 5 days and the number of CFU was calculated.

**Table 11.** Cell ratio of *Chryseobacterium antarcticum* and *Pseudomonas fragi* in BHI broth

Code of experiment	<i>Chryseobacterium antarcticum</i> (F1445/3) CFU/ml	<i>Pseudomonas fragi</i> (F1445/1b) CFU/ml
A	10 <sup>6</sup>	10 <sup>6</sup>
B	10 <sup>8</sup>	10 <sup>6</sup>
C	10 <sup>8</sup>	10 <sup>7</sup>
D (control)	-	10 <sup>6</sup>
E (control)	-	10 <sup>7</sup>
F (control)	10 <sup>6</sup>	-
G (control)	10 <sup>8</sup>	-

#### 4.4.2. Methods for the studies with *Staphylococcus aureus*

##### 4.4.2.1. Cultivation of *Staphylococcus aureus* SA45

From the stock solution of *S. aureus* SA45 stored at -80°C, cells were plated on BHI agar and incubated at 37°C overnight. One colony was picked from the agar and cultivated in BHI broth overnight at 37°C, 160 rpm agitation. To study the behavior of cells between optimal conditions, 400 ml BHI broth was inoculated with an overnight culture of *S. aureus* SA45 as much volume as it was necessary to reach an optical density at 620 nm (OD<sub>620</sub>) of 0.1 for the start. The pH was adjusted to 7.0 using acetic acid (Merck). The broth was incubated at 37°C, 200 rpm, for seven days, using an in-house fermentor. The pH was maintained by automatic titration by adding sodium hydroxide (Merck). The OD<sub>620</sub> was measured using a U-1800 spectrophotometer (Hitachi High Technologies Inc., Pleasanton, CA) to follow the growth. Samples of broth were collected from the fermentor for qRT-PCR and ELISA analysis every hour up to and including hour eight, then after 12, 24, 48, 72, 120 and 168 h.

#### 4.4.2.2. Cultivation of *S. aureus* SA45 *in situ* on meat products

To study sessile cells, the same pre-cultivation steps were repeated until inoculation of the fermentor as in chapter 4.4.2.1. 250 ml Erlenmeyer flasks with 50 ml BHI broth were inoculated with the overnight culture of *S. aureus* SA45 and incubated at 37°C, 160 rpm. Cells were harvested in the exponential growth phase and washed twice with 0.85% NaCl. Washed cells were resuspended into the same volume of physiological saline as the initial volume was and 100 µl of this solution containing  $10^7$  colony-forming units (CFU) per ml was evenly distributed over the pieces of meat by pipette. For the study of smoked ham, 10 µl of the stock solution of *S. aureus* SA45 was added directly to BHI broth for overnight cultivation without the agar cultivation step. After inoculation, the meat products were incubated at room temperature (23°C) for seven days. Uninoculated meat products were used as controls. Samples were taken from each type of meat directly after inoculation and after incubation on day 1, 2, 3, 5 and day 7 of incubation for qRT-PCR and ELISA analysis, viable count determination and pH measurements. Three replicate samples were collected from each meat product at each sampling time. Each sample of meat was transferred to a BagPage® F filter bag (400 ml, 60 µm porosity, Interscience, Paris, France). Physiological saline solution was added to each sample (9:1 w/v). Cells were rinsed off in a Stomacher Lab-Blender 400 (Seward Medical Ltd. London, UK) for 1 min. The pH of the stomacher liquid from both inoculated meat and control meat samples were measured with a pH meter after stomaching (VWR symPHony Meters).

Viable counts were determined immediately after inoculation of the meat samples and after the first, second, third, fifth and seventh day of incubation. Serial tenfold dilutions of stomacher liquid in physiological saline solution were performed. The dilutions were plated on BHI agar to determine the total cell count, and on Baird Parker agar to determine the *S. aureus* cell count. Agar plates were incubated at 37°C overnight and the number of CFU was calculated. The cell count was expressed in terms of CFU/cm<sup>2</sup> since only the surface of the meat had been inoculated with bacteria. Cells were also plated on MRS agar for detecting *Lactobacillus* immediately after meat inoculation and after seven days of incubation, except for smoked ham, where plating on MRS agar was only performed after the seventh day of incubation. The MRS agar plates were incubated in an Anerocult anaerobic chamber system (Merck) at 30°C for 48 h.

#### 4.4.2.3. RNA extraction and reverse transcription

Total RNA was extracted using phenol and chloroform as described by Lövenklev et al. (2004b), except that the RNA was re-suspended in 100 µl RNA storage solution. DNA was degraded according to Lövenklev et al. (2004b) and total RNA concentrations were measured spectrophotometrically using a BioPhotometer (Eppendorf AG, Hamburg, Germany). Reverse

transcription was performed according to Artin et al. (2008). First-strand cDNA was synthesized in two separate reverse-transcription assays using reverse primers specific to SEA and SED (Table 12) and the reference gene 16S rRNA, as described previously (Lövenklev et al., 2004b) with the following reaction mixture: 0.5 µg RNA in the reference gene assay and 0.1 µg RNA in the toxin gene assay, 0.5 mM each of dATP, dTTP, dCTP and dGTP (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM primer (MWG Biotech AG, Ebersberg, Germany), 20 U RNasin<sup>®</sup> ribonuclease inhibitor (Promega GmbH, Mannheim, Germany), 10 mM DTT (Invitrogen, Carlsbad, CA), 1 × first-strand buffer (Invitrogen) and 100 U Super Script<sup>™</sup> II RNase H<sup>-</sup> reverse transcriptase (Invitrogen).

#### 4.4.2.4. Primer and probe design

The forward primer specific to *sea* was identified from the literature (Rosec and Gigaud, 2002). The reverse primer was designed in-house in Lund University using LightCycler Probe Design<sup>®</sup> software ver. 1.0 (Roche Diagnostics GmbH) (Table 12).

**Table 12.** Sequences and fluorescent dyes of primers and hybridization probes

Target	Primer/probe	Nucleotide sequence (5'→3')
<i>sea</i>	ESA-1	ACGATCAATTTTACAGC
	ToxA reverse	CCGAAGGTTCTGTAGAAGT
	ToxA-Fluo1	CCTTTGGAAACGGTTAAAACGAATAAGAAA-FL <sup>a</sup>
	ToxA-Red1	LC-R640-TGTAAGTGTTCAGGAGTTGGATCTCA-p <sup>b</sup>
<i>sed</i>	SED-1	CTAGTTTGGTAATATCTCCT
	GSEDR-2	ATTGGTATTTTTTTTCGTTC
	entD-FL	TACCCTATAAGATATAGCATTAAATTGTT-FL <sup>a</sup>
	entD-LC	LC-Red640-TGGTGGTGAAATAGATAGGACTGCTTG-p <sup>b</sup>
<i>rrn</i>	rRNA forward	TGTCGTGAGATGTTGGG
	rRNA reverse	ACTAGCGATTCCAGCTT
	Probe 1	GGACAATACAAAGGGCAGCG-FL
	Probe 2	LC-R705-ACCGCGAGGTCAAGCA-p <sup>c</sup>

<sup>a</sup> The donor probe is labeled with fluorescein (FL) at the 3' end.; <sup>b</sup> The acceptor probe is labeled with LC Red640 (LC-R640) at the 5' end and the 3' hydroxy group is phosphorylated (p); <sup>c</sup> The acceptor probe is labeled with LC Red705 (LC-R705) at the 5' end and the 3' hydroxy group is phosphorylated (p).

#### 4.4.2.5. The real-time PCR assay

PCR amplification was carried out on a LightCycler™ 2.0 instrument (Roche Diagnostics GmbH). The total volume of PCR mixture was 20 µl including 4 µl of template cDNA. The *sea* PCR mixture consisted of 1 × PCR buffer, 4.6 mM MgCl<sub>2</sub>, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.5 µM each of the forward and reverse primers, 0.05 U *Tth* DNA polymerase and 0.3 µM of each hybridization probe. The *sed* PCR mixture consisted of 1 × PCR buffer, 2.75 mM MgCl<sub>2</sub>, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.5 µM each of the forward and reverse primers, 0.05 U *Tth* DNA polymerase and 0.15 µM of each hybridization probe. The *rrn* PCR mixture was the same as the *sed* PCR mixture. All reagents except the primers and probes were obtained from Roche Diagnostics GmbH. The water used was autoclaved ultrapure water. In order to detect the amplification of possible contaminations, a negative control consisting of water instead of DNA was added to the PCR. Genomic DNA was used as a positive control. The following PCR protocol was used: initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 0 s (no hold at this temperature), primer annealing at 46°C (*sea*) or 48°C (*rrn* and *sed*) for 5 s and extension at 72°C for 25 s, with a single fluorescence measurement at the end of the extension step. The crossing point cycle for each transcript was determined using the second derivative maximum mathematical model in the LightCycler™ software (ver. 4.1) (Roche Diagnostics GmbH), and the amplification efficiency was calculated by the software.

#### 4.4.2.6. Relative quantification

The relative expression of *sea* and *sed* was calculated by relating the toxin gene expression to the constant expression of a reference gene, the 16S rRNA gene (Pfaffl, 2001). To determine the amplification efficiency and the log-linear range of amplification for each real-time PCR assay, the total RNA was serially diluted. The dilutions were reverse transcribed and amplified in the LightCycler™ instrument three times to obtain standard curves. Samples were also amplified three times. Equal amounts of total RNA from each sample were reverse transcribed to quantify the transcript levels of *sea* and *sed*.

#### 4.4.2.7. ELISA

A modified protocol was used for ELISA analysis of SEA and SED (Poli et al., 2002). The ELISA was developed for the specific monitoring of SEA and SED using affinity-purified sheep polyclonal antibodies (IgG). The enterotoxin was detected using biotinylated secondary antibodies (IgG), NeutrAvidin™-linked alkaline phosphatase and the substrate p-nitrophenyl phosphate. The colour developed was measured spectrophotometrically at 405 nm after a pre-defined incubation time.

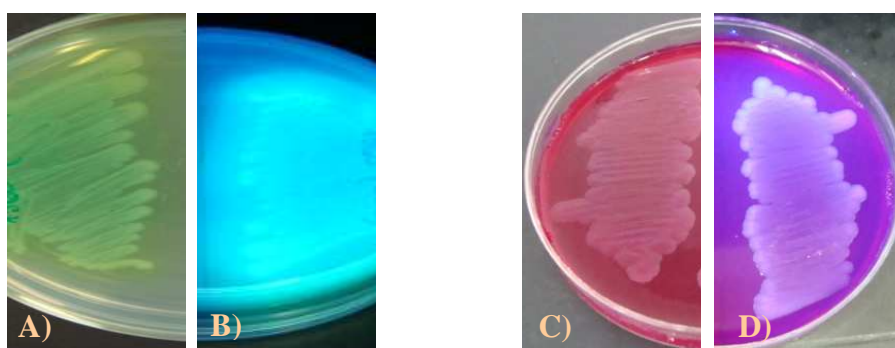
Quantification was based on a standard curve prepared using serial dilution in an appropriate matrix of highly purified SEA and SED. Absorbance values (mean of triplicate wells) were plotted against toxin concentration, and values were determined from linear regression. A microtiter plate (Immulon® 2HB polystyrene, Flat Bottom Microtiter® Plates, 96 wells solid; Thermo Electron Corporation; Waltham, MA) was coated with 100 µl/well of a solution containing 2 µg/ml anti-SEA or SED IgG antibody in coating buffer and left at 37°C overnight. All sites were then blocked with 185 µl blocking buffer for one hour at 37°C and at least one hour at 4°C. The plate was washed four times with washing buffer. Standards, stomacher liquid or culture supernatants were loaded onto the plate (100 µl/well) at appropriate dilutions and incubated for 90 min at 37°C. The plate was then washed. The biotinylated anti- SEA or SED IgG, diluted 2000 × in assay buffer was added (100 µl/well). The plate was incubated for one hour at 37°C and then washed. NeutrAvidin™-linked alkaline phosphatase (diluted 1000 × in assay buffer, no milk powder included) was added (100 µl/well), and the plate was incubated for 30 minutes at 37°C. The plate was washed and substrate (p-nitrophenyl phosphate) was added (100 µl/well). The color was then allowed to develop for 45 min in darkness. The optical density was determined using a microplate reader with a filter at 405 nm (Multiskan Ascent, Thermo Electron Corporation).

## 5. RESULTS AND DISCUSSION

### 5.1. Characterization of *Pseudomonas* isolates

The objective was to characterize the biological properties of representative bacterial strains isolated from pork meat stored at 4 and 8°C. Pure cultures of cold tolerant bacteria were spread on PCA and Cetrimide plates as listed in Table 7 in chapter 4.1. in Materials and methods. Heterogeneity of some isolates was determined after few days of storage on PCA plates. Some of the isolates were named as “a”, “b” or “c” that indicated the differences in colony morphology. Altogether 34 psychrotrophic/psychrophilic bacteria isolates were characterised according to Pollack et al. (2009) and listed in Table 13 in chapter A2. Appendix (Table). All isolates were Gram negative, catalase and oxidase positive, non-spore forming and KOH positive. One isolate namely F1445/3 had a negative oxidase test indicating inability to use oxygen for energy production.

In order to determine the selectivity of different *Pseudomonas* media, GSP, Cetrimide, *Pseudomonas* Agar P and F were tested for growth and fluorescence of the isolates at 20 and 30°C for 72 h. PCA was used as a control media. GSP agar contains glutamate and starch as a unique nutrient sources and phenol-red as indicator. *Aeromonas* species are able to degrade the starch with acid production causing color changing in phenol red turning yellow. *Pseudomonas* species do not possess this ability. Selectivity of the media for *Pseudomonas* species is improved by addition of Penicillin G. In case of Cetrimide agar the cetrimide (cetyltrimethylammonium bromide) largely inhibits the growth of the accompanying microflora according to Lowbury (1951) and Brown, Lowbury (1965). As it was expected *Pseudomonas aeruginosa* type strain produced a greenish yellow pigment (pyocyanin) on Cetrimide agar (Fig. 10 A) while on GSP media red-violet colonies were formed (Fig. 10 C). Fluorescence was detected under UV light in both cases (Figs. 10 B and 10 D).

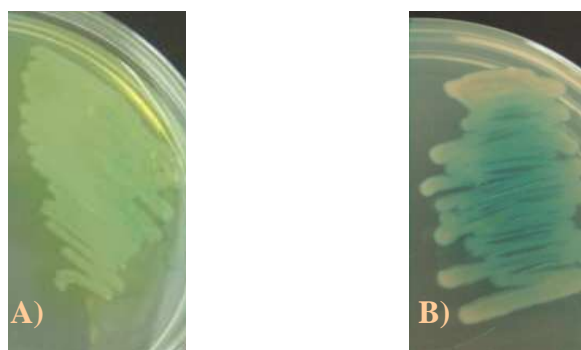


**Figure 10.** Growth of *Pseudomonas aeruginosa* ATCC 10145<sup>T</sup> on selective media **A)** growth on Cetrimide agar; **B)** Fluorescence on Cetrimide agar under UV light; **C)** Growth on GSP agar; **D)** Fluorescence on GSP agar under UV light



As an elective culture media recommended by King, Ward and Raney (1954) for the isolation and differentiation of *Pseudomonas* species, *Pseudomonas* Agar P and F were applied. *Pseudomonas* Agar P stimulates the formation of pyocyanin and/or pyorubin and rolls back of fluorescein (pyoverdine), whereas *Pseudomonas* Agar F induces the production of fluorescein (pyoverdine) and reduces the pyocyanin and/or pyorubin creation. It is important to notice that the name fluorescein extended the name of pyoverdine after the *Pseudomonas* Agar F was developed. The name of pyoverdine contains all pigments that are produced by fluorescent pseudomonads (Meyer and Abdallah, 1978). Pyoverdines are a group of structurally related siderophores produced by fluorescent *Pseudomonas* species (Visca et al., 2006) which are a powerful iron (III) scavenger and an effective iron transporter. The synthesis of pyoverdine is strongly related to iron starvation (Meyer, 2000). These molecules also serve as signalling molecules controlling gene expression inside the bacterial cells (Lamont and Martin, 2003). *P. aeruginosa* pyoverdines function as signal molecules for synthesis of virulence factors as well as crucial for biofilm development (Visca et al., 2006). These properties allowed the characterization of pyoverdine as a potent siderophore for the fluorescent pseudomonads (Meyer, 2000).

*Pseudomonas aeruginosa* type strain appears on *Pseudomonas* Agar F as colonies surrounded by greenish-yellow zone resulting from pyoverdine production. If pyocyanin is also synthesized, a bright green colour is produced (Fig. 11 A) which fluoresces under UV light. *Pseudomonas aeruginosa* can grow on *Pseudomonas* Agar P and form colonies surrounded by a blue to green zone due to pyocyanin formation (as shown in Fig. 11 B) or with a red to dark brown zone due to pyorubin synthesis.



**Figure 11.** Growth of *Pseudomonas aeruginosa* ATCC 10145<sup>T</sup> on elective media, **A)** growth on *Pseudomonas* Agar F; **B)** Growth on *Pseudomonas* Agar P

Results of the growth of isolates as well as type and non *Pseudomonas* strains on different media are shown in Table 14. All the isolates had growth on the different media at 20 and 30°C except F1445/3 that showed weak growth at 30°C (Table 14). Non *Pseudomonas* species had weak growth on all media at both temperatures. Although growth of the isolates was quite uniform there was difference in fluorescence ability and pigment production on the media at 20 and 30°C.

**Table 14.** Summary of testing growth and fluorescence ability on different media for *Pseudomonas* isolates from 4°C and 8°C and non *Pseudomonas* strains

Isolates	PCA		GSP agar		Cetrimide agar		Pseudomonas Agar P		Pseudomonas Agar F	
	growth/ UV		growth/ UV		growth/ UV		growth/ UV		growth/ UV	
	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C
F 1443/1	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1443/2a	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+
F 1443/2b	+/-	+/+	+/-	+/(+)	+/-	+/-	+/-	+/-	+/-	+/-
F1443/4a	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+
F 1443/4b	+/-	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/-	+/-
F 1443/4c	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+
F 1443/5	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+
F 1443/7	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+
F 1443/8	+/(+)	+/-	+/+	+/-	+/+	+/-	+/-	+/-	+/+	+/-
F 1443/9	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1443/10b	+/-	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-
F 1443/10c	+/-	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-
F 1443/11	+/-	+/-	+/+	+/+	+/+	+/+	+/-	+/-	+/+	+/+
F 1443/12a	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-
F 1443/12b	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-
F 1443/12c	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-	+/+	+/-
F 1443/13a	+/-	+/(+)	+/-	+/+	+/+	+/(+)	+/-	+/-	+/+	+/-
F 1443/13b	+/+	+/(+)	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/-
F 1445/1a	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/1b	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F1445/3	+/-	(+)/-	+/-	(+)/-	+/-	(+)/-	+/-	+/-	+/-	(+)/-
F 1445/4	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/5	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/6	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
F 1445/8a	+/+	+/((+))	+/+	+/-	+/+	+/+	+/-	+/-	+/+	+/+
F 1445/8b	+/+	+/((+))	+/+	+/-	+/+	+/+	+/-	+/-	+/+	+/+
F 1445/9a	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/9b	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/10	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/12	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
F 1445/14a	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/-	+/+	+/-
F 1445/14b	+/(+)	+/-	+/(+)	+/-	+/+	+/-	+/-	+/-	+/+	+/-
F 1445/15a	+/+	+/-	+/(+)	+/+	+/+	+/-	+/-	+/-	+/+	+/-
F 1445/15b	+/(+)	+/-	+/+	+/-	+/+	+/-	+/-	+/-	+/+	+/-
<i>P. aeruginosa</i> ATCC 10145 <sup>T</sup>	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
<i>P. fluorescens</i> CCM 3899	+/-	+/-	+/-	+/-	+/+	+/+	+/-	+/-	+/+	+/+
<i>P. fragi</i> CCM 1974 <sup>T</sup>	+/+	+/+	+/(+)	+/+	+/(+)	+/-	+/(+)	+/-	+/+	+/+
<i>P. lundensis</i> CCM 3503 <sup>T</sup>	+/+	+/+	+/(+)	+/+	-/(+)	-/-	+/+	+/-	+/+	+/+
<i>P. putida</i> B. 01634 <sup>T</sup>	+/-	+/-	-/-	(+)/-	-/-	+/+	+/-	(+)/-	+/-	+/-
<i>C. jeonii</i> JCM 12382 <sup>T</sup>	(+)/-	(+)/-	-/-	(+)/-	(+)/-	(+)/-	(+)/-	(+)/-	+/-	+/-
<i>C. antarcticum</i> JCM 12381 <sup>T</sup>	+/-	(+)/-	(+)/-	-/-	(+)/-	(+)/-	(+)/-	-/-	+/-	((+))/-

UV: fluorescence under UV light (366 nm)

Five isolates (F1443/4c, F1443/5, F1443/6, F1443/7, F1443/11) had greenish-yellow color on Cetrimide and *Pseudomonas* Agar F. These isolates showed fluorescence more intensively under UV light at 366 nm on these media as well as on GSP agar than other isolates (Figs.12 A, B, C).



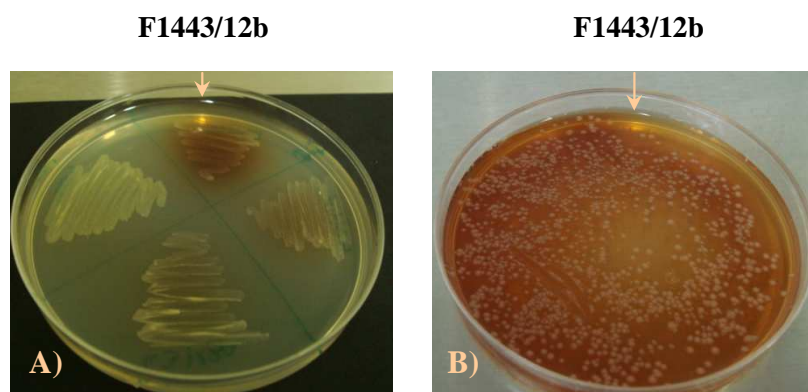
**Figure 12.** Fluoresce of different isolates under UV light on **A)** Cetrimide agar **B)** *Pseudomonas* Agar F and **C)** GSP agar

Majority of the isolates did not show fluorescence at 20 and 30°C on *Pseudomonas* Agar P and PCA and almost one third of them did not fluoresce on GSP, Cetrimide and *Pseudomonas* Agar F. In case of F1443/10b and c as well as F1443/12a, b, c fluoresced on all media at 20°C but not at 30°C except F1443/12a on GSP and F1443/10b, c on PCA. The same fluorescence pattern was detected in case of F1443/8, F1445/14a, F1445/15a and b but there was no fluorescence detected on *Pseudomonas* Agar P. Fluorescence was detected at both incubation temperatures on all media in case of F1443/4c, F1443/5, F1443/7, F1443/8, F1443/13b as well as with F445/6, F1445/8a and b except that the latter two isolates had fluoresce only at 20°C on GSP. There was no fluorescence on *Pseudomonas* Agar P or just on 20°C in case of the other isolates as demonstrated in Table 14.

It was found that pyoverdine production is connected to the growth phase of the cells. The development of the fluorescent pigment happens in the early stage of growth (Harris, 1950) and the excretion of pyoverdine stops as the culture entered the stationary phase of growth while pyocyanin is not formed until the maximum growth has occurred (Meyer and Abdallah, 1978). This suggests that the pyocyanin does not have essential role in growth but seems to accumulate and function as a secondary metabolite. During cultivation of the isolates none of them formed pyocyanin except the control strain *P. aeruginosa* ATCC 10145<sup>T</sup> (Fig. 11 B) on *Pseudomonas* Agar P.

It was found that the native pyoverdine pigment is labile in aqueous solution especially under mildly alkaline conditions (above pH 7) (Meyer and Abdallah, 1978). When the cells reach the stationary phase the cell lysis is starting which changes the pH of the environment to the alkaline stage. Although the cultivation of the *Pseudomonas* isolates was on a solid phase at 20 and 30°C in

this study the reduction and disappearance of the fluorescence ability at 30°C could be the consequence of reaching the stationary phase earlier at 30°C. The additional pyoverdine is not excreted there as well as the cell lysis has already begun to change the pH to alkaline conditions. Four isolates (F1443/4b, F1443/4a, F1443/2a and F1445/9b) formed light brown colour on *Pseudomonas* Agar P indicating pyorubin production. Four isolates (F1443/12b, F1443/13a and b, F1445/4) had dark brown pigment production on *Pseudomonas* Agar F (Fig. 13 A) while the same colour changing was observed on BHI agar after 3 days of incubation at 20 or 30°C (Fig. 13 B).



**Figure 13.** Pigment production on **A)** *Pseudomonas* Agar F; **B)** BHI agar

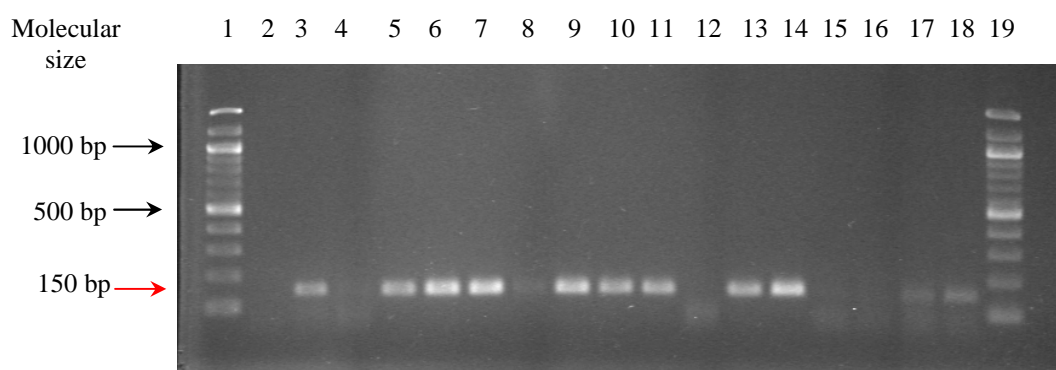
The reason of the brown colour changes after cultivation could be that some species of pseudomonads can also synthesize additional siderophores because until the beginning of this century close to 40 structurally different pyoverdines were identified (Meyer, 2000). To determine precisely this type of pigment and the pathway of its formation further characterization is essential.

These results indicated that all the media was suitable for growth of *Pseudomonas* strains and distinction from other non *Pseudomonas* species according to their pigment production and fluorescence ability. According to the data, the incubation temperatures may influence the pigment production ability. That observation could be the base of further investigations in molecular way.

## 5.2. Molecular characterization of *Pseudomonas* isolates

### 5.2.1. Testing the applicability of a *Pseudomonas* genus-specific primer pair

At the beginning of meat storage *Pseudomonas* species compose the small part of the microbiota under aerobic and refrigerated condition. However, during storage they successfully compete with other bacteria and become dominant. Finding a genus-specific primer pair could facilitate and reduce the identification and detection time of spoilage-causing *Pseudomonas* species. At the same time it could help and facilitate the typing and identification tasks. It could be also the base for developing quantitative-PCR for differentiating them in time from other spoilage causing microbes. However, designing genus-specific primers is challenging due to the heterogeneity of the genus *Pseudomonas*. For screening the food spoilage-causing *Pseudomonas* strains, a genus-specific primer pair was tested which was designed by Purohit et al. (2003). The construction of primer pair based on the 16S rDNA sequences of 50 *Pseudomonas* strains found in the rDNA database of the GenBank. The primer pair targeted a specific region of 16S rDNA which conserved among *Pseudomonas* species. As the consequence of optimization, reaction conditions of PCR were modified. Lower primer concentrations (0.25 pM) were used which altered from the originally recommended form by Purohit et al. (2003). The annealing temperature was increased from 62 to 64°C and a final extension step was added to the reaction. For testing the specificity and sensitivity of the system, 16 *Pseudomonas* reference and type strains as well as 18 non *Pseudomonas* strains were used. After gel electrophoresis the expected amplicon size, 150 bp, was detected as shown on Figure 14. Overview of PCR results is found in Table 15.



**Figure 14.** PCR products generated with *Pseudomonas* specific primers

1. 100bp Marker (BioLabs), 2. Negative control, 3. *Pseudomonas lundensis* CCM 3503<sup>T</sup>, 4. *Pseudomonas fragi* CCM 3903, 5. *Pseudomonas putida* DSM 291<sup>T</sup>, 6. *Pseudomonas taetrolens* CCM 1982<sup>T</sup>, 7. *Pseudomonas mendocina* CCM 3590<sup>T</sup>, 8. *Pseudomonas fragi* CCM 3704, 9. *Pseudomonas stutzeri* CCM 4557<sup>T</sup>, 10. *Pseudomonas lundensis* CCM 3907, 11. *Pseudomonas fluorescens* CCM 3899, 12. *Brevundimonas diminuta* B. 01118<sup>T</sup>, 13. *Pseudomonas aeruginosa* ATCC 10145<sup>T</sup>, 14. *Pseudomonas putida* B. 01634<sup>T</sup>, 15. *Burkholderia cepacia* B. 01621, 16. *Aeromonas hydrophila* subsp. *hydrophila* CCM 7232<sup>T</sup>, 17. *Aeromonas sobria* CCM 2807<sup>T</sup>, 18. *E. coli* ATCC 8739, 19. 100bp Marker (BioLabs)

**Table 15.** Summary of PCR products amplified with Psf-Psr primer pair

Amplification with Psf-Psr primer pair for <i>Pseudomonas</i> species							
Number	Species	Strain	PCR product	Number	Species	Strain	PCR product
1	<i>Pseudomonas aeruginosa</i>	ATCC10145 <sup>T</sup>	+	9	<i>Pseudomonas lundensis</i>	CCM 3503 <sup>T</sup>	+
2	<i>Pseudomonas aeruginosa</i>	ATCC 9027	+	10	<i>Pseudomonas lundensis</i>	CCM 3907	+
3	<i>Pseudomonas fluorescens</i>	CCM 2115 <sup>T</sup>	+	11	<i>Pseudomonas marginalis</i>	CCM 4969 <sup>T</sup>	+
4	<i>Pseudomonas fluorescens</i>	CCM 3899	+	12	<i>Pseudomonas mendocina</i>	CCM 3590 <sup>T</sup>	+
5	<i>Pseudomonas fragi</i>	CCM 1974 <sup>T</sup>	+	13	<i>Pseudomonas putida</i>	B. 01634 <sup>T</sup>	+
6	<i>Pseudomonas fragi</i>	CCM 3703	+	14	<i>Pseudomonas putida</i>	DSM 291 <sup>T</sup>	+
7	<i>Pseudomonas fragi</i>	CCM 3704	-	15	<i>Pseudomonas stutzeri</i>	CCM 4557 <sup>T</sup>	+
8	<i>Pseudomonas fragi</i>	CCM 3903	-	16	<i>Pseudomonas taetrolens</i>	CCM 1982 <sup>T</sup>	+
Amplification with Psf-Psr primer pair for non- <i>Pseudomonas</i> species							
Number	Species	Strain	PCR product	Number	Species	Strain	PCR product
1	<i>Stenotrophomonas maltophilia</i>	B. 01119 <sup>T</sup>	-	10	<i>Listeria monocytogenes</i>	CCM 4699	-
2	<i>Brevundimonas diminuta</i> (syn.: <i>P. diminuta</i> )	B. 01118 <sup>T</sup>	-	11	<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	CCM 5884 <sup>T</sup>	-
3	<i>Burkholderia cepacia</i> (syn.: <i>P. cepacia</i> )	B. 01621	-	12	<i>Streptococcus thermophilus</i>	B. 02290	-
4	<i>Aeromonas sobria</i>	CCM 2807 <sup>T</sup>	+	13	<i>Bacillus subtilis</i>	MBT-B1	-
5	<i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	CCM 7232 <sup>T</sup>	-	14	<i>Chryseobacterium antarcticum</i>	JMC 12381 <sup>T</sup>	+
6	<i>E. coli</i>	ATCC 8739	+	15	<i>Chryseobacterium jeonii</i>	JMC 12382 <sup>T</sup>	+
7	<i>E. coli</i> (O157:H7)	MBT-E1	-	16	<i>Hafnia alvei</i>	TM-8	+
8	<i>Campylobacter jejuni</i>	CCM 6214 <sup>T</sup>	-	17	<i>Serratia liquefaciens</i>	TM3	+
9	<i>Yersinia enterocolitica</i>	HNCMB 98001	-	18	<i>Citrobacter braakii</i>	TS-8	-

Altogether 14 *Pseudomonas* type and reference strains gave positive reactions with the Psf-Psr primer pair (Table 15). Two *Pseudomonas* reference strains were negative in the test and six non-*Pseudomonas* species were positive in the PCR reaction.

To determine the applicability of this primer pair specificity and sensitivity was calculated applying the following equations (De Boer and Beumer, 1999; Maráz et al., 2006):

$$\text{Sensitivity (\%)} = \frac{\text{number of true positives (p)}}{p + \text{number of false negatives}} \times 100$$

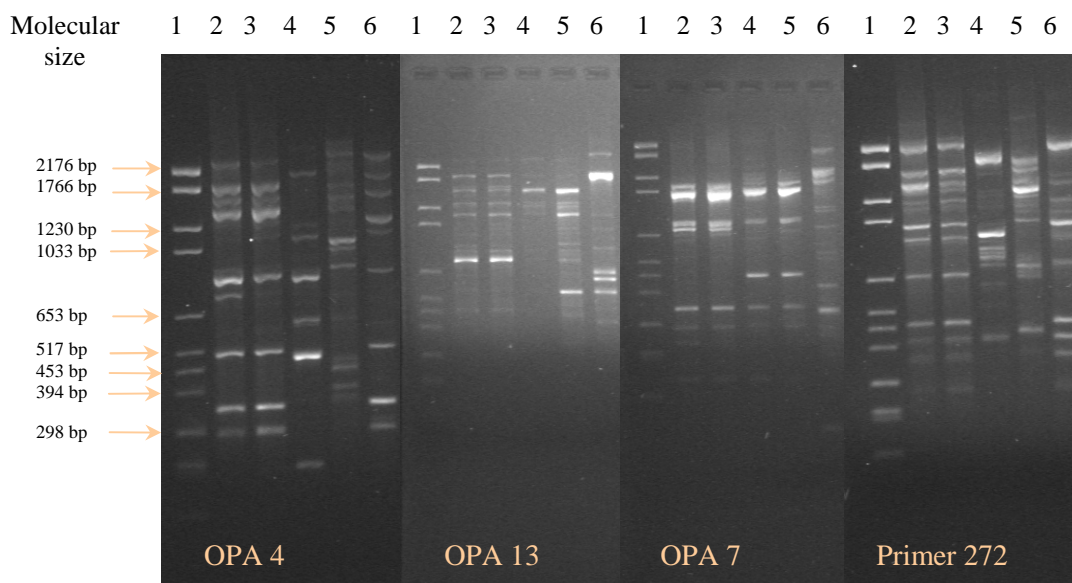
$$\text{Specificity (\%)} = \frac{\text{number of true negatives (n)}}{n + \text{number of false positives}} \times 100$$



The sensitivity was 87.5% while the specificity was only 66.6%. Sensitivity of the method was also tested with 24 *Pseudomonas* isolates represented in this thesis when the molecular identification was performed with them in chapter 5.2.4. and 5.2.5. From the 24 *Pseudomonas* species 5 isolates were negative in the PCR reaction and 19 *Pseudomonas* species had positive reaction (data not shown). The sensitivity was only 82.5% after the combination of these results with the reference strains and type strains in Table 15. According to the low specificity value the further use of this primer pair was abolished. The primers were not suitable for preliminary screening of *Pseudomonas* species due to false-positive results from other cold tolerant meat spoilage bacteria and foodborne pathogens as shown on Table 15. The primer pair was not sensitive enough for detecting all *Pseudomonas* species.

### 5.2.2. Molecular typing of *Pseudomonas* isolates by RAPD-PCR analysis

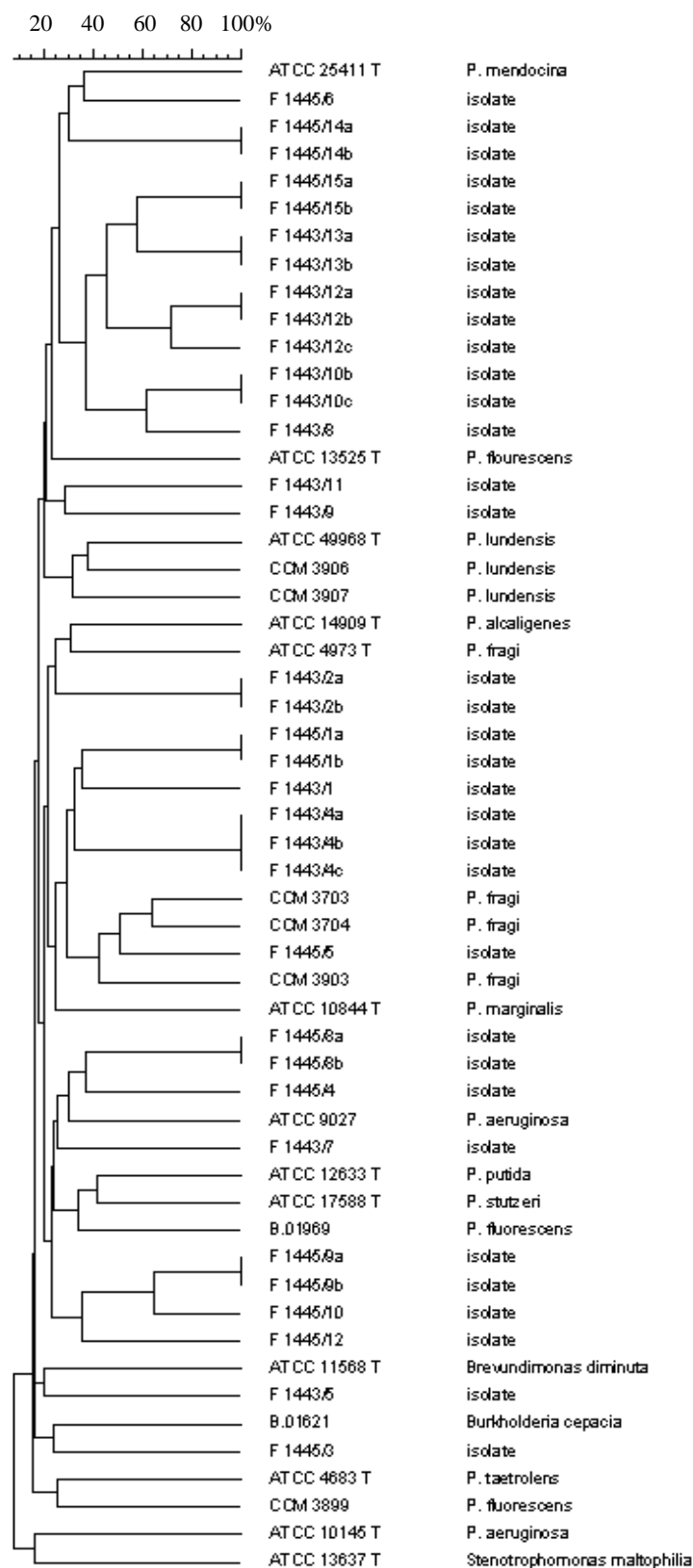
According to previous investigations RAPD was successfully used for determining the relatedness of aquatic isolates belonging to the genus *Pseudomonas* (Sazakli et al., 2005) or assessing the diversity of the rhizosphere *Pseudomonas* population (Rangarajan et al., 2002). Based on these promising results *Pseudomonas* isolates derived from meat spoilage were fingerprinted by RAPD-PCR analysis to characterize their diversity applying OPA 4, OPA13, OPA7 and primer 272 oligonucleotide primers (Table 10) as described in Materials and methods. Primers were selected previously from 8 primers according to their PCR patterns after gel electrophoresis. The patterns of randomly connecting primers chosen for typing are shown in Fig. 15.



**Figure 15.** RAPD-PCR patterns of isolates with OPA 4, OPA 13, OPA7 and Primer 272

1. Marker (MW IV.), 2. F 1445/1a, 3. F 1445/1b, 4. F 1445/3, 5. F 1445/4, 6. F1445/5

Molecular fingerprints of the isolates were evaluated with Gelcompar II software and combined dendrogram was prepared from the RAPD patterns. Similarity tree was prepared with GelCompar II software (Fig. 16).



**Figure 16.** Dendrogram of RAPD-PCR analysis of *Pseudomonas* isolates obtained with the combination of OPA 4, OPA 13, OPA7 and primer 272 patterns



The dendrogram showed very divergent RAPD patterns. There were 10 clusters that harboured strains of 100% similarity. These clusters contained always those isolates that showed different colony morphology on PCA plates and named by “a, b, c” as subclones. However, based on their RAPD analysis there was no difference in their genomic pattern that indicated their clonal identity. Therefore in the further molecular analysis only one of them was selected and studied.

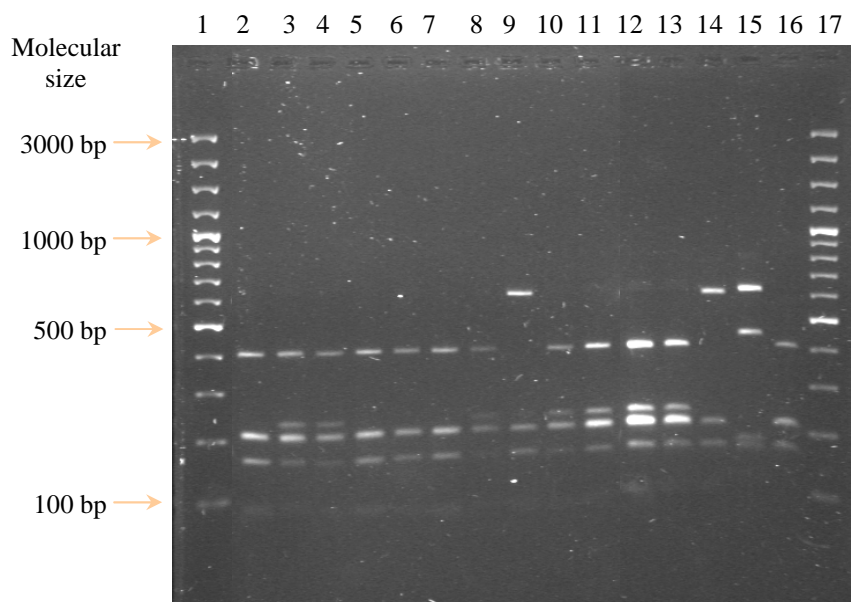
Other isolates that had no subclones had distinct position in the dendrogram and showed 60-70% similarity regardless being isolated from the same or different samples. *Pseudomonas* type and reference strains showed very low degree of similarity and they generally formed unique clusters below 50% similarity.

Based on the results it may be concluded that the strains isolated from the agar plates underwent strong population dynamic. Beside the subclones of the isolates no other high similarity clusters were formed by the isolates, therefore we could not recognize the strains that probably belonged to the same *Pseudomonas* species. As regards the discrimination power of the applied RAPD analysis it was suitable for typing *Pseudomonas* strains, similarly as in the case of *Serratia marcescens* from clinical sources (Hejazi et al., 1997) or LAB from dairy products (Coppola et al., 2006) and from traditional salami (Andrighetto et al., 2001).

### **5.2.3. Results of 16S rDNA-RFLP analysis of *Pseudomonas* isolates**

The 16S rDNA gene sequence is a widely accepted and used housekeeping genetic marker for identification of bacteria typing microorganisms (Kolbert and Persing, 1999). The 16S rDNA-RFLP (ARDRA) method has been successfully applied previously for not just the analysis of bacterial communities in environment but for food especially smoked salmon and dairy products (Giraffa et al., 1998a; Cambon-Bonavita et al., 2001). According to Lagacé et al. (2004) it has been also used for identification of the different genera and species of bacteria in maple sap in which *Pseudomonas* species could also be detected.

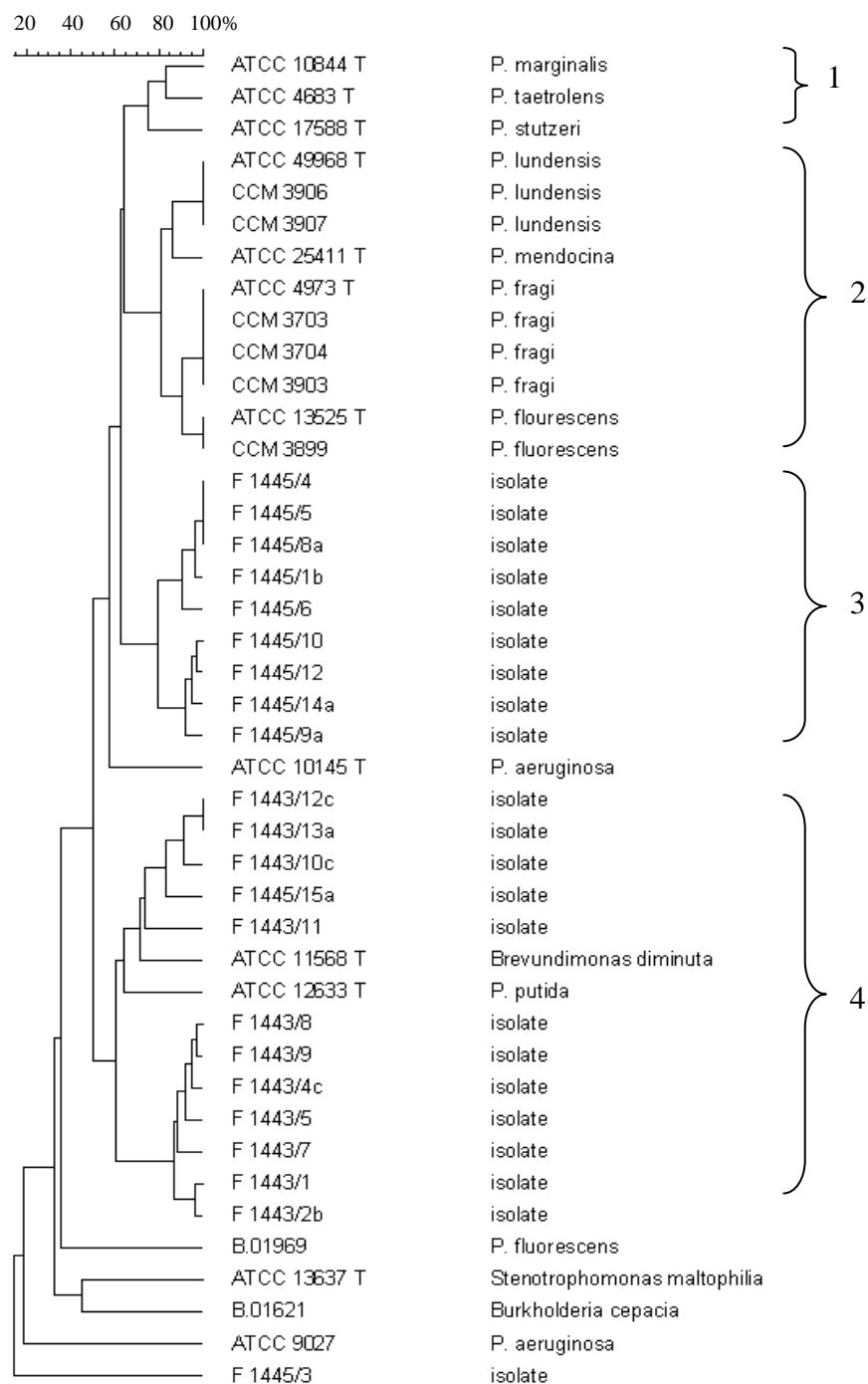
In the present study, a universal primer pair called F27- R1492 was used to amplify a large part of the 16S rDNA gene. The PCR conditions were optimized and adjusted to the used PCR instrument. All of the isolates and type strains tested in this study generated approximately a 1465 bp PCR product. In order to produce the RFLP pattern, the amplicons were digested with five different restriction enzymes (*AluI*, *HaeIII*, *MspI*, *RsaI* *ScfRI*). The enzymatic digestion with *ScfRI* yielded similar RFLP pattern and was therefore excluded in the further analysis. The most heterogenic restriction pattern was reached with *AluI* as shown in Fig. 17.



**Figure 17.** Restriction pattern of *Pseudomonas* isolates digested with *AluI* restriction enzyme  
1. GeneRuler 100bp Marker, 2. F1443/1, 3. F1443/2a, 4. F1443/2b, 5. F1443/4a, 6. F1443/4b, 7. F1443/4c, 8. F1443/5, 9. F1443/7, 10. F1443/8, 11. F1443/9, 12. F1443/10b, 13. F1443/10c, 14. F1443/11, 15. *Pseudomonas aeruginosa* ATCC 9027, 16. *Pseudomonas alcaligenes* B. 02011<sup>T</sup>, 17. GeneRuler 100bp Marker

Dendrogram was prepared by combination of the restriction patterns (Fig. 18). According to the cluster analysis four different groups could be distinguished from each other at the level of 60% similarity. All the type and reference strains that belonged to *P. fragi*, *P. lundensis* and *P. fluorescens* separated into the first two clusters. Only *P. putida* ATCC 12633<sup>T</sup> composed the part of cluster 4 but showed less than 70% similarity. Cluster 3 was composed by isolates derived from the same storage condition at 8°C. Cluster 4 contained most part of the isolates which separated into 2 main groups. Altogether 12 isolates and 2 type strains constituted cluster 4 all of which originated from 4°C except F 1445/15a.

According to these results the 16S rDNA RFLP analysis is not applicable for identification purposes in the case of *Pseudomonas* strains. The reason of it could be that the ribosomal RNA operons are present in multiple copies that harbour divergent nucleotide sequences even in a single bacterial genome (Acinas et al., 2004). For this reason further identification techniques were also tested during this work.

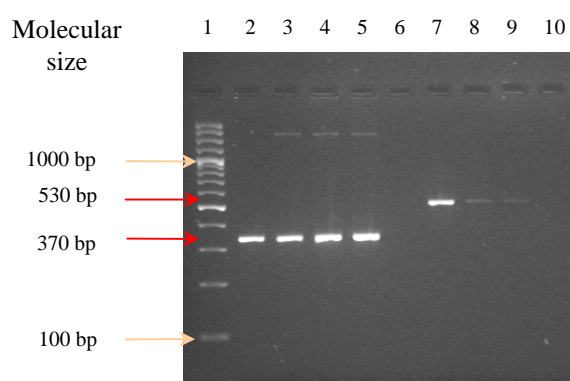


**Figure 18.** Combined 16S rDNA-RFLP dendrogram of *Pseudomonas* isolates and type and reference strains. For comparison three non-*Pseudomonas* strains were also used.

It could be established that the 16S rDNA-RFLP is not suitable for typing of spoiling *Pseudomonas* species either because majority of type and reference strains composed distinct groups within the dendrogram.

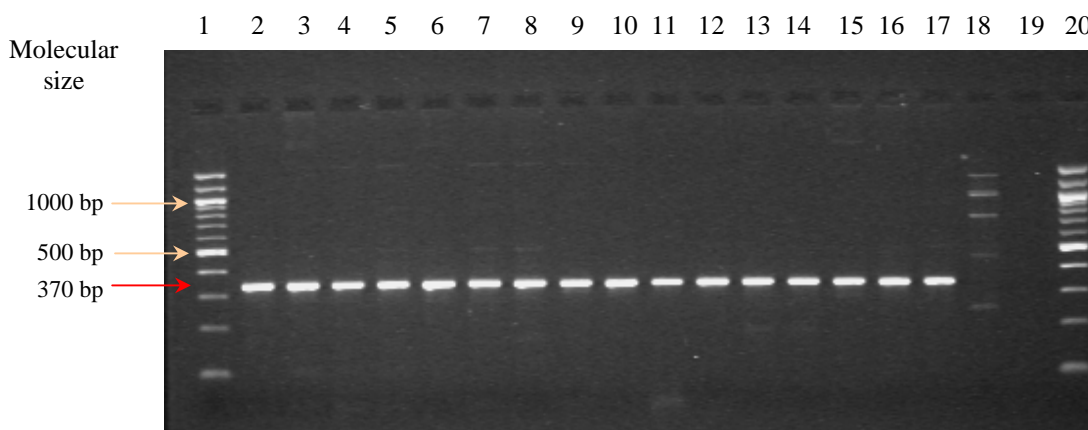
#### 5.2.4. Identification of *Pseudomonas* species using *carA* specific primers

For identification of *P. fragi* and *P. lundensis* species among the *Pseudomonas* isolates a multiplex PCR assay was used which was developed by Ercolini et al. (2007). It was specific for the *carA* gene that it was encoding for the small subunit of the carbamoyl phosphate synthase. The multiplex PCR was optimized but the species-specific primer pairs were used in separated PCR reactions. Optimization was performed with type and reference strain from CCM collection. In case of *P. fragi* the expected size of PCR product was 370 bp while for *P. lundensis* it was 530 bp, respectively shown on Figure 19. Example of positive PCR reactions in the case of type and reference *P. fragi* strains and 13 isolates from pork chop are shown on Figure 20.



**Figure 19.** PCR products with *P. fragi* and *P. lundensis* primer pairs after optimization at 56°C

1. GeneRuler 100bp Marker, 2. *Pseudomonas fragi* CCM 1974<sup>T</sup>, 3. *Pseudomonas fragi* CCM 3703, 4. *Pseudomonas fragi* CCM 3704, 5. *Pseudomonas fragi* CCM 3903, 6. Negative control, 7. *Pseudomonas lundensis* CCM 3503<sup>T</sup>, 8. *Pseudomonas lundensis* CCM 3906, 9. *Pseudomonas lundensis* CCM 3907, 10. Negative control



**Figure 20.** Positive PCR reaction in the case of *P. fragi* reference strains (lanes 15-17) and 12 *Pseudomonas* isolates (Lanes 2-14) obtained with *Pseudomonas fragi* specific primer pair

1. GeneRuler 100bp Marker, 2. F1443/1b, 3. F1443/4a, 4. F1443/4b, 5. F1443/4c, 6. F1445/1a, 7. F1445/1b, 8. F1445/5, 9. F1445/9a, 10. F1445/9b, 11. F1445/10, 12. F1445/12, 13. F1445/14a, 14. F1445/14b, 15. *Pseudomonas fragi* CCM 1974<sup>T</sup>, 16. *Pseudomonas fragi* CCM 3703, 17. *Pseudomonas fragi* CCM 3903, 18. F1443/2a, 19. Negative control, 20. GeneRuler 100bp Marker

From the 34 isolates altogether 25 strains were identified as *P. fragi*. Some of the isolates (altogether 8) generated an extra amplicon that could not eliminate with optimization. According to *rpoB* gene and 16S rDNA sequence analysis they were identified also as *P. fragi* later in chapter 5.2.5. None of the isolates generated amplicon with the *P. lundensis* specific primer pair. Summary of species-specific PCR results obtained in the case of the isolates is shown in Table 16.

**Table 16.** Summary of identification obtained with the application of *P. fragi* and *P. lundensis* specific primer pairs

Isolates from 4°C	Species-specific PCR	Isolates from 8°C	Species-specific PCR
F 1443/1	<i>P. fragi</i>	F 1445/1a	<i>P. fragi</i>
F 1443/2a	no PCR product	F 1445/1b	<i>P. fragi</i>
F 1443/2b	no PCR product	F 1445/3	no PCR product
F 1443/4 a	<i>P. fragi</i>	F 1445/4	no PCR product
F 1443/4 b	<i>P. fragi</i>	F 1445/5	<i>P. fragi</i>
F 1443/4c	<i>P. fragi</i>	F 1445/6	no PCR product
F 1443/5	<i>P. fragi</i>	F 1445/8a	no PCR product
F 1443/7	no PCR product	F 1445/8b	no PCR product
F 1443/8	<i>P. fragi</i>	F 1445/9a	<i>P. fragi</i>
F 1443/9	<i>P. fragi</i>	F 1445/9b	<i>P. fragi</i>
F 1443/10b	<i>P. fragi</i>	F 1445/10	<i>P. fragi</i>
F 1443/10c	<i>P. fragi</i>	F 1445/12	<i>P. fragi</i>
F 1443/11	no PCR product	F 1445/14a	<i>P. fragi</i>
F 1443/12a	<i>P. fragi</i>	F 1445/14b	<i>P. fragi</i>
F 1443/12b	<i>P. fragi</i>	F 1445/15a	<i>P. fragi</i>
F 1443/12c	<i>P. fragi</i>	F 1445/15b	<i>P. fragi</i>
F 1443/13a	<i>P. fragi</i>		
F 1443/13b	<i>P. fragi</i>		

### 5.2.5. Results of sequencing the 16S rDNA and *rpoB* genes

For identification of nine *Pseudomonas* isolates which were negative in the species-specific PCR reaction, direct sequencing of the *rpoB* and 16S rDNA genes were used. Eight other isolates were also analysed that originally gave positive results with *P. fragi* specific primer pair but generated extra amplicons during that reaction. After amplifying a distinct region of the 16S rDNA or *rpoB* genes in these cases the PCR amplicons have been sequenced.

The F27-R1525 primer pair was applied for amplification of 16S rDNA gene and the whole sequence was determined with the application of the F27 and R1525 primers combined with R338. Results of identification at species level were based on the sequence alignment and are shown in Table 17.

**Table 17.** Results of sequencing 16S rDNA and *rpoB* amplicons

Code	16S rDNA amplicon	Identity	<i>rpoB</i> amplicon	Identity
F 1443/4c	<i>P. fragi</i>	99%	not determined	-
F 1443/5	<i>P. fragi</i>	99%	not determined	-
F 1443/12b	<i>P. fragi</i>	100%	not determined	-
F 1445/1b	<i>P. fragi</i>	100%	not determined	-
F 1445/3	<i>Chryseobacterium antarcticum</i>	98%	not determined	-
F 1445/4	<i>P. fluorescens</i>	100%	not determined	-
F 1445/8a	<i>P. fluorescens</i>	100%	not determined	-
F 1443/2a	not determined	-	<i>P. fluorescens</i>	95%
F 1443/7	not determined	-	<i>P. fluorescens</i>	97%
F 1443/8	not determined	-	<i>P. fragi</i>	96%
F 1443/11	<i>P. fluorescens</i>	99%	<i>P. fluorescens</i>	98%
F 1443/12b	not determined	-	<i>P. fragi</i>	95%
F 1443/13b	not determined	-	<i>P. fragi</i>	96%
F 1445/6	not determined	-	<i>P. fluorescens</i>	97%
F 1445/8b	not determined	-	<i>P. fluorescens</i>	97%
F 1445/15b	not determined	-	<i>P. fragi</i>	95%

In case of 4 *P. fragi* isolates which were previously chosen because they had extra amplicons, the sequence analysis confirmed the results of species-specific PCR identification shown in Table 18. and confirmed the applicability of this primer pair for identification of the food spoilage causing *P. fragi* strains (Ercolini et al., 2007). The other 4 *P. fragi* isolates were confirmed by the *rpoB* sequencing. As the result of 16S rDNA sequencing 3 isolates were identified as *P. fluorescens*. The isolate F1445/3 was previously found different not just in phenotypic characteristics from the *Pseudomonas* isolates (e.g. production of yellow pigment, slow growth) but also altered based on the 16S rDNA sequence analysis. The sequence of the 16S rDNA amplicon from the F1445/3 isolate was aligned into the sequences deposited in the EzTaxon server 2.1 ([www.eztaxon.org](http://www.eztaxon.org)). This alignment was done in May 2008. The similarity was found 98% and 97% with *Sejongia antarctica* and *Sejongia jeonii*, respectively. Both described by Yi et al. (2005) as belonging to the newly constructed *Sejongia* genus. At that time we could not find, however, any sequences of high similarity in the GenBank of NCBI (<http://blast.ncbi.nlm.nih.gov>). Therefore the F1445/3 isolate was identified as *Sejongia antarctica*. Kämpfer et al. (2009) found that *Sejongia antarctica* and *Sejongia jeonii* are closely related to other species belonging to the genus *Chryseobacterium* and they did not find evidences for clear phenotypic differences between these organisms that justifies

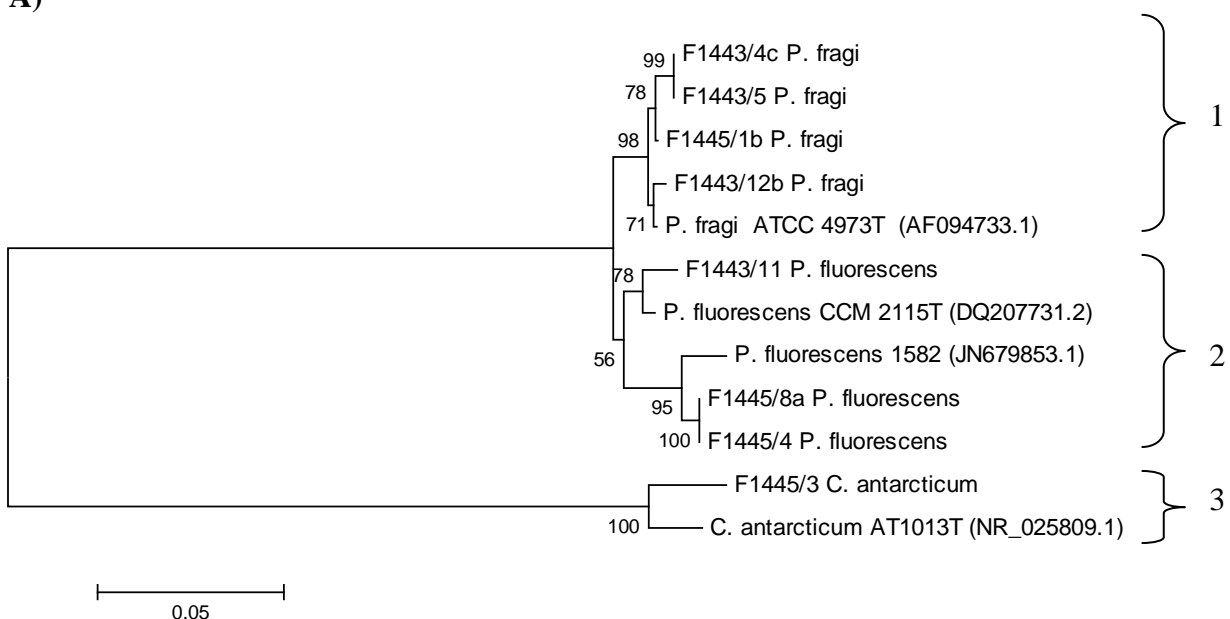
their assignment to different genera, therefore they transferred these species to the genus *Chryseobacterium* as *Chryseobacterium antarcticum* and *Chryseobacterium jeonii*. Based on this publication the F1445/3 isolate was renamed as *Chryseobacterium antarcticum* and this name was used in further investigations in my thesis.

The *rpoB* gene plays an essential role in cellular metabolism in all bacteria by encoding the  $\beta$ -subunit of RNA polymerase. It is a highly conserved housekeeping gene that evolves much faster than rDNAs. This characteristic provides higher resolution than the analysis of 16S rRNA gene because it presents in only one copy in the bacteria (Yamamoto and Harayama, 1998; Qi et al., 2001). The *rpoB* gene has been postulated to be a good candidate for phylogenetic analysis and identification of bacteria in clinical microbiology (Adékambi et al., 2009). For the analysis of *rpoB* a 1230 bp sequence was amplified with a *Pseudomonas* - *rpoB* specific primer pair designed by Tayeb et al. (2005). As the result of *rpoB* sequencing four other isolates were identified as *P. fluorescens* and four as *P. fragi*. In case of F1443/11 isolate sequencing of both genes revealed the same identification results, namely as *P. fluorescens*.

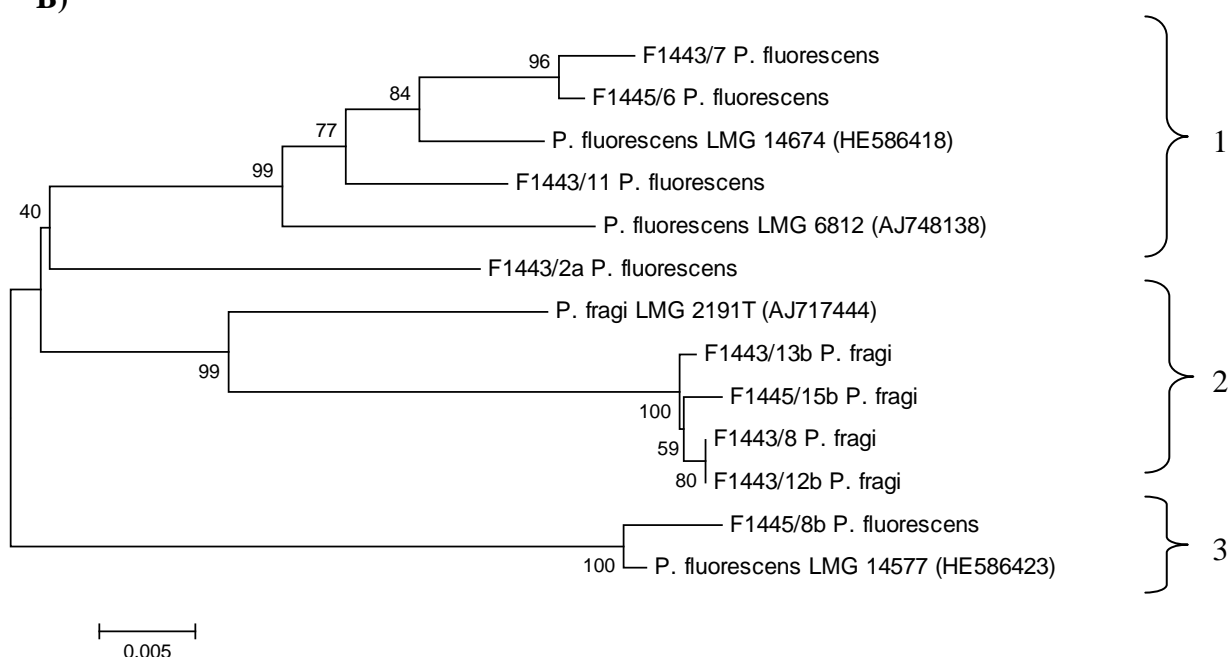
The results indicates the necessity for further development of *P. fluorescens* specific PCR. It could be more problematic according to Ercolini et al. (2007) because *P. fluorescens* strains belonging to different biotypes show very high sequence variability within the *carA* gene. Selection of another gene for identification purposes seems to be necessary.

Phylogenetic trees were also constructed based on the nucleotide sequences of 16S rDNA and *rpoB* genes of the isolates listed in Table 17. These phylogenetic trees also contain strains from the GenBank as illustrated on Figs. 21 A and B.

A)



**B)**



**Figure 21.** Phylogenetic tree based on the **A)** 16S rDNA sequences and **B)** *rpoB* sequences of isolates derived from Table 17. Closest related type strains and strains represented with accession number from GenBank are also integrated into the trees. Trees were constructed by neighbour-joining method. Bootstraps values obtained from the 500 re-samplings of data set which are given at the nodes.

The first cluster on the phylogenetic tree of 16S rDNA sequences on Fig. 21 A contained all the *P. fragi* isolates and *P. fragi* ATCC 4973<sup>T</sup>. The bootstraps value was 98 between these strains. The second major cluster collected all the *P. fluorescens* isolates as well as the *P. fluorescens* strains derived from the GenBank where the bootstraps values were 78 and 95, respectively. F1445/3 *Chryseobacterium antarcticum* was grouped separately from *Pseudomonas* strains but together with *C. antarcticum* AT1013<sup>T</sup> in cluster 3 where the bootstrap value was 100, demonstrating the highest phylogenetic relationship.

The phylogenetic tree based on the alignment of *rpoB* sequences contained also 3 major clusters as demonstrated on Fig. 21 B. The first and the third clusters contained *P. fluorescens* isolates which were combined with other *P. fluorescens* strains derived from the GenBank while the second cluster composed only *P. fragi* isolates and *P. fragi* LMG 2191<sup>T</sup>. The bootstrap value was 99 between *P. fluorescens* isolates and *P. fluorescens* LMG 6812. The same bootstrap value was obtained between *P. fragi* isolates and *P. fragi* LMG 2191<sup>T</sup>. In the third cluster the bootstrap value was 100 between the isolate and *P. fluorescens* LMG 14577.

It could be established that the trees had strong bootstrap support (>77) on main nodes and quite close phylogenetic relationship between isolates belonging to the same species were also demonstrated on both trees.

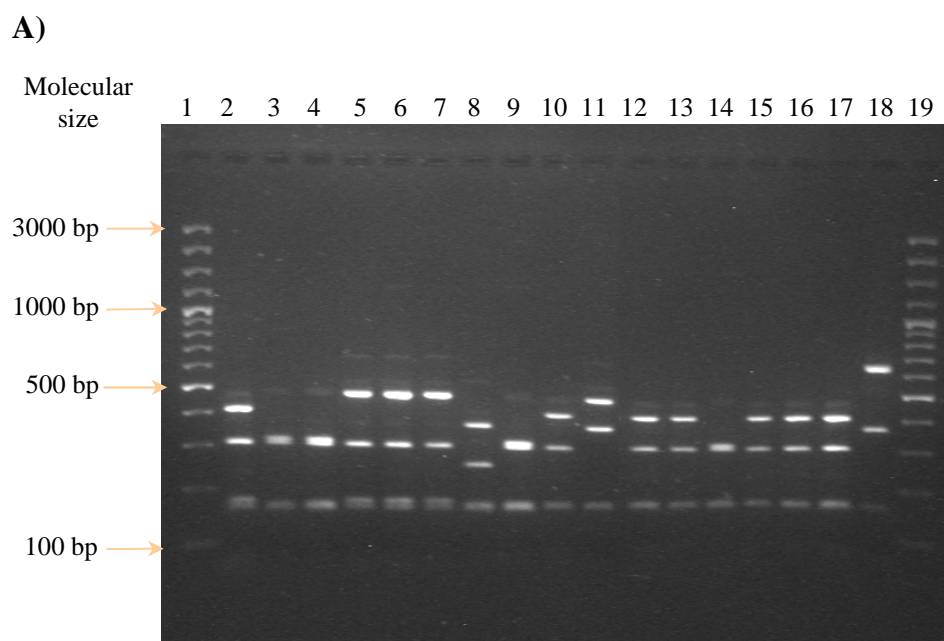


Based on the results gained with species-specific identification of the isolates the following could be established: isolates derived from the beginning of storage belonged to *Pseudomonas fluorescens* and *Pseudomonas fragi*. Moreover one *Chryseobacterium antarticum* was also isolated. Isolates derived from storage of pork meat at 4°C on day 8 also belonged to *Pseudomonas fluorescens* and *Pseudomonas fragi* while on pork meat stored at 8°C on day 6 only *P. fragi* species were isolated. These results correspond to the reports of other authors who found that *Pseudomonas fragi* is the most frequently dominating species in spoiling of red meat that is followed by *Pseudomonas fluorescens*. However, no *Pseudomonas lundensis* isolate was found which is frequently isolated with *P. fluorescens* (Dainty and Mackey, 1992; Olofsson et al., 2007).

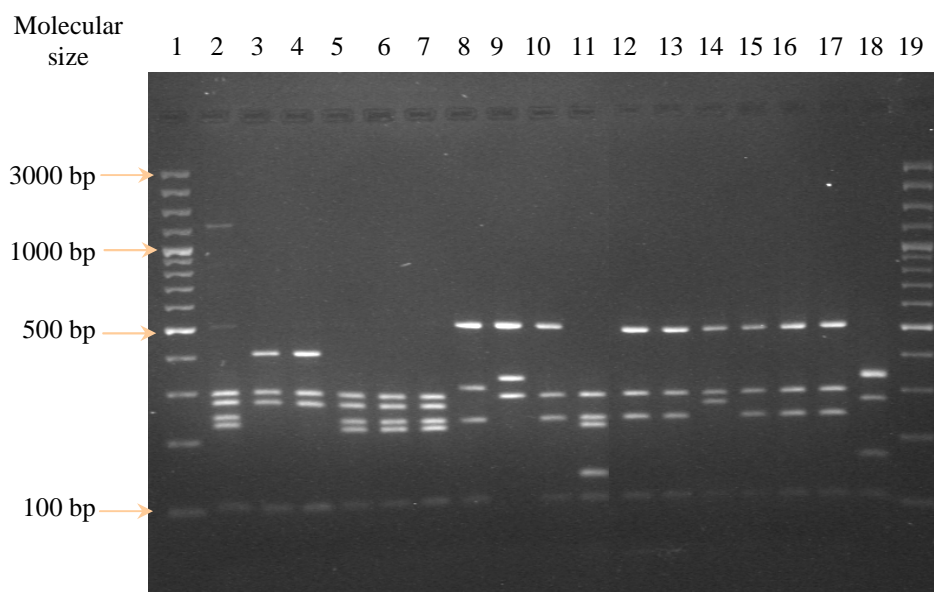
#### 5.2.6. Results of *rpoB* –RFLP

It was demonstrated that sequencing is a suitable tool for identification purposes. It provides more precise identification because it analyses the series of nucleotides in the DNA while the PCR-RFLP method focuses only few restriction sites in the DNA. Nevertheless, for establishing an in-house bacterial databank the PCR-RFLP analysis provides a more cost effective solution. The applicability of *rpoB*-RFLP for molecular identification purposes has been studied by the application of four different restriction enzymes as described in chapter 4.4.1.7. in Materials and methods.

For the analysis of *rpoB* a 1230 bp sequence was amplified with a *Pseudomonas* - *rpoB* specific primer pair as described by Tayeb et al. (2005). All the isolates and type strains were successfully amplified and resulted PCR products of the expected size. Amplicons were digested with *AluI*, *HaeIII*, *MspI* and *RsaI* restriction enzymes and separated by gel electrophoresis. Examples of RFLP patterns obtained by the *AluI* and *RsaI* are shown in Figures 22 A and B.



**B)**

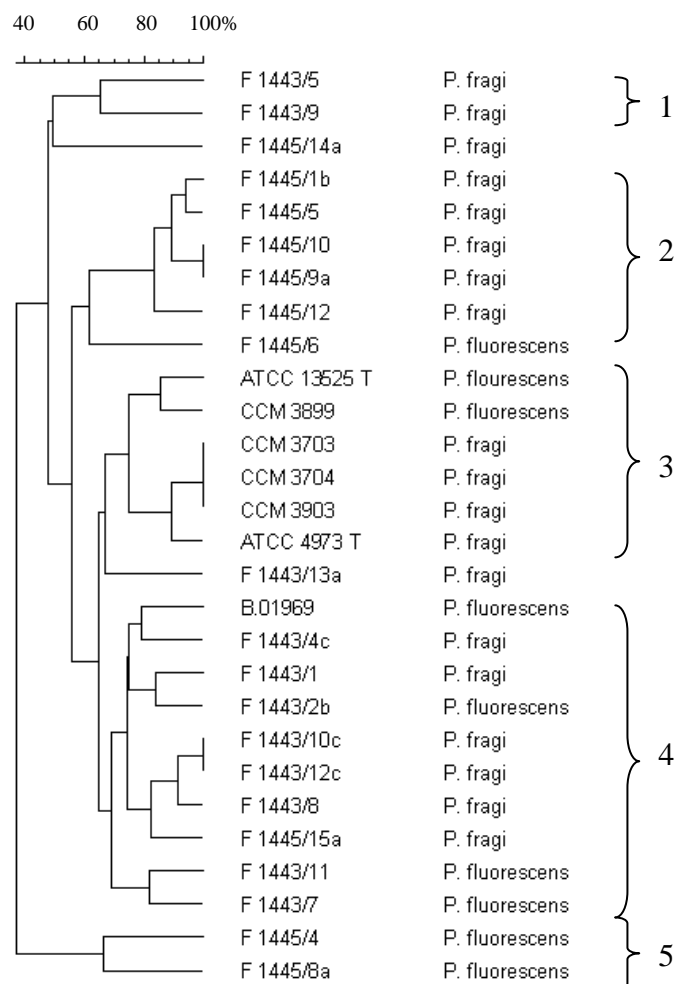


**Figure 22.** Restriction pattern of *Pseudomonas* isolates digested with A) *AluI* and B) *RsaI* restriction enzymes

1. GeneRuler 100bp Marker, 2. F1443/1, 3. F1443/2a, 4. F1443/2b, 5. F1443/4a, 6. F1443/4b, 7. F1443/4c, 8. F1443/5, 9. F1443/7, 10. F1443/8, 11. F1443/9, 12. F1443/10b, 13. F1443/10c, 14. F1443/11, 15. F1443/12a, 16. F1443/12b, 17. F1443/12c, 18. *Pseudomonas aeruginosa* ATCC 9027, 20. GeneRuler 100bp Marker

Based on the four different restriction patterns a combined dendrogram was prepared (Fig. 23). The dendrogram contains the species name of isolates which were identified in chapter 5.2.4. and 5.2.5. Altogether five clusters were formed where the similarity was higher than 60% but some solo strains have not been clustered. Cluster 1 and 2 contained *P. fragi* isolates, which derived from 4 °C and 8 °C storage temperatures, respectively. All the *P. fluorescens* and *P. fragi* type and reference strains were clustered into the same group (cluster 3) similarly as it was found previously in the 16S rDNA-RFLP analysis. Only four *P. fragi* strains (F1445/9 and F1445/10) and (F1443/10c and F1445/12c) showed 100% similarity in cluster 2 and 4 according to their restriction patterns. In clusters 4 and 5 the distinction between isolates originated from 4 and 8°C could be established except one isolate, F 1445/15a. Although the similarities between *P. fluorescens* isolates in cluster 4 (F1443/7 and F1443/11) and cluster 5 (F1445/4 and F1445/8a) were only 80% and 60% they composed distinct groups in the dendrogram.

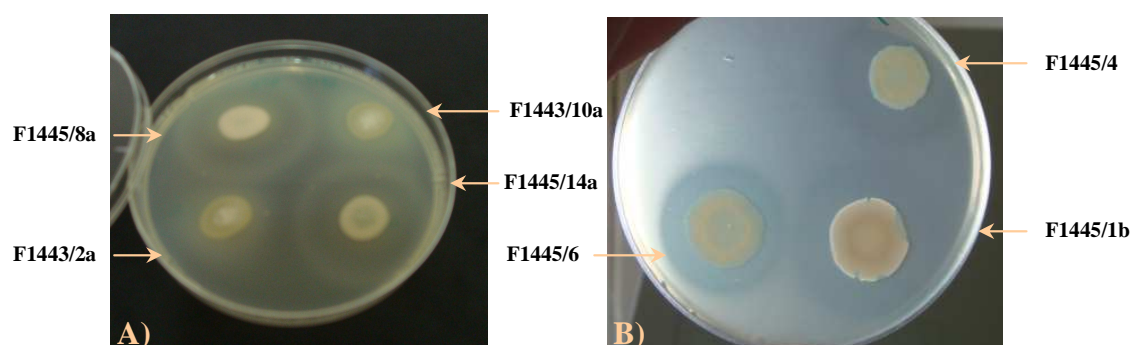
As the main conclusion it has to be established that there was no high level of similarity between the strains belonging to the same species, therefore the *rpoB*-RFLP is not suitable for identification purposes in the case of *Pseudomonas* spp.



**Figure 23.** Dendrogram of *rpoB*-RFLP with *Pseudomonas* isolates and type strains

### 5.3. Evaluation of lipolytic and proteolytic activities of the *Pseudomonas* isolates at different temperatures

Enzymatic characterization of *Pseudomonas* isolates were performed with application of a semi-quantitative method according to Atlas (1995) at the temperature of 15, 20, 25 and 30°C for 7 days. Clearing zones were checked after 24, 72 and 168 hours of incubation. Altogether 27 *Pseudomonas* isolates were tested. Protease activity was evaluated using skim milk (SM) and Standard Methods Caseinate (SMC) media as described in chapter 4.2. Clearing zones were opaque around the macrocolonies on SMC agar (Fig. 24 A) while on SM agar the zones were completely transparent and distinct (Fig. 24 B).

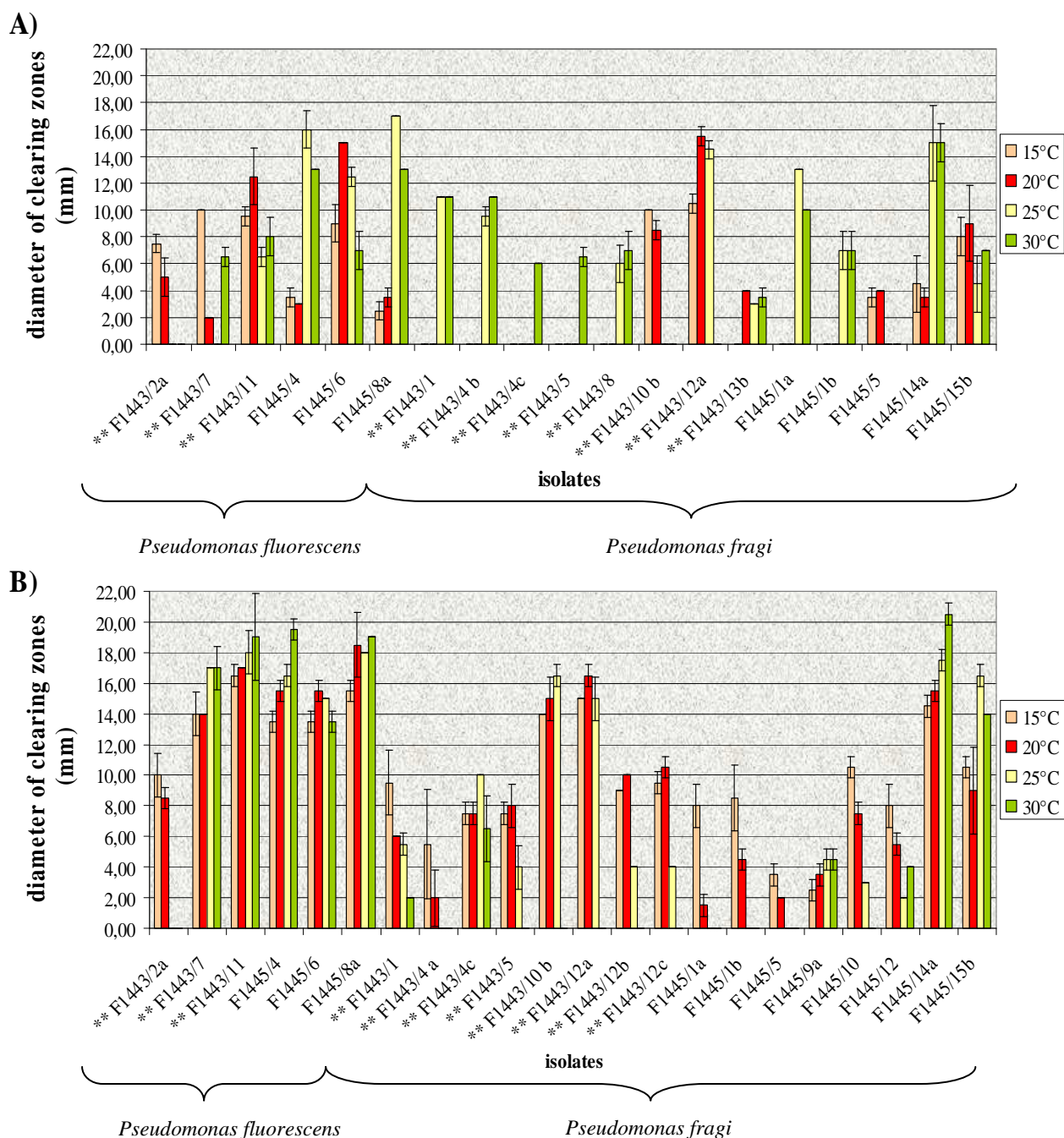


**Figure 24.** Detection of proteolytic activity **A)** Clearing zone formation on SMC and **B)** SM plates

As shown in Figures 24 A and B the proteolytic activity was different not only between isolates but at different incubation temperatures. All the isolates had growth on SMC agar but altogether 20 isolates were able to produce clearing zone on SMC agar as shown on Fig. 25 A while 7 isolates not. These isolates were F1443/4a, F1443/12b, F1443/12c F1445/9a and b, F1445/10, F1445/12 which all belonged to *P. fragi*. Six isolates had active protease synthesis in all the four different incubation temperatures. These were *P. fragi* (F1445/14a, F1445/15b) and *P. fluorescens* (F1443/11, F1445/4, F1445/6 and F1445/8a) isolates.

In case of SM plates altogether 23 isolates were able to grow and produce extracellular protease as shown on Fig. 25 B. Only 4 isolates were not able to produce proteases namely *P. fragi*, F1443/4b F1443/8, F1443/13b and F1445/9b. F1443/8 isolate had minimal protease production at 15°C. Altogether 12 isolates were able to secrete proteases at 4 different temperatures as shown on Fig. 25 B. On SM plates the proteolytic activity of *P. fluorescens* isolates namely F1443/7, F1443/11, F1445/4, F1445/6 and F1445/8a was the highest based on the diameter of clearing zones and could be detected in all incubation temperatures except in case of F1443/7.

The *Pseudomonas fluorescens* isolates show quite intense proteolytic activity on SMC plates in all incubation temperatures except F1443/2a and F1443/7 the activity of which was detected only at 15 and 20°C as shown on Fig. 25 A. The most active protease producer *P. fluorescens* strains were the same on SM plates as in case of SMC plates. Both of the media contain components from milk. SM media contains the whole part of milk while in SMC agar the only component derived from milk is Na-caseinate. It is shown on Figs. 25 A and B that the isolates hydrolysed differently these components and larger clearing zones were measured on SM plates that derives from the different origin of protein substrates in media.



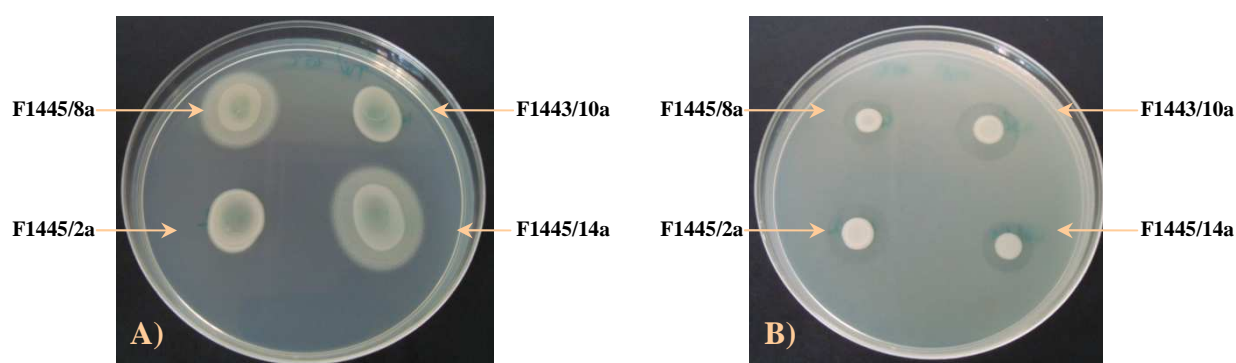
**Figure 25.** Proteolytic activities of *Pseudomonas* isolates incubated at 15, 20, 25, 30°C;

**A)** Proteolytic activity detected on SMC plates; **B)** Proteolytic activity detected on SM plates;

\*\* : isolates derived from 4°C; without sign: isolates derived from 8°C

The initial microflora of pork meat stored at 8°C originally contained different species but at the end of spoilage *P. fragi* become dominant. *P. fragi* isolates (from F1445/9a to F1445/15b) derived from the end of storage at 8°C showed higher proteolytic activities at the four different incubation temperatures than those *P. fragi* isolates that were derived the beginning of storage or the end of storage at 4°C. However almost all the *P. fragi* isolates derived from 4°C possessed relatively high proteolytic activity on SM plates (Fig. 25 B) incubated at 20, 25 and 30°C.

Lipolytic activities of *Pseudomonas* strains also have an important role in spoilage. Esterase and lipase activities of the *Pseudomonas* isolates were detected on PAT-80 and PCATB plates, respectively, as described in chapter 4.4.1.2 at 15, 20, 25 and 30°C for 7 days. Turbid or opaque halo were formed on plates surrounding the isolates as showed on Figs. 26 A and B. On PAT-80 agar the liberated fatty acids bind with the calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the inoculation site (Slifkin, 2000). On PCATB plate tributyrin was the substrate that was applied for screening purposes to detect lipolytic *Pseudomonas* species. Tributyrin is the simplest triglyceride occurring in natural fats and oils and suitable microorganisms of potential importance in foods. On the PCATB plates the enzyme activities were concluded from the diameter of zones or halos.



**Figure 26.** Detection of lipolytic activity **A)** Esterase formation on PAT-80 plates; **B)** Lipase production on PCATB plates

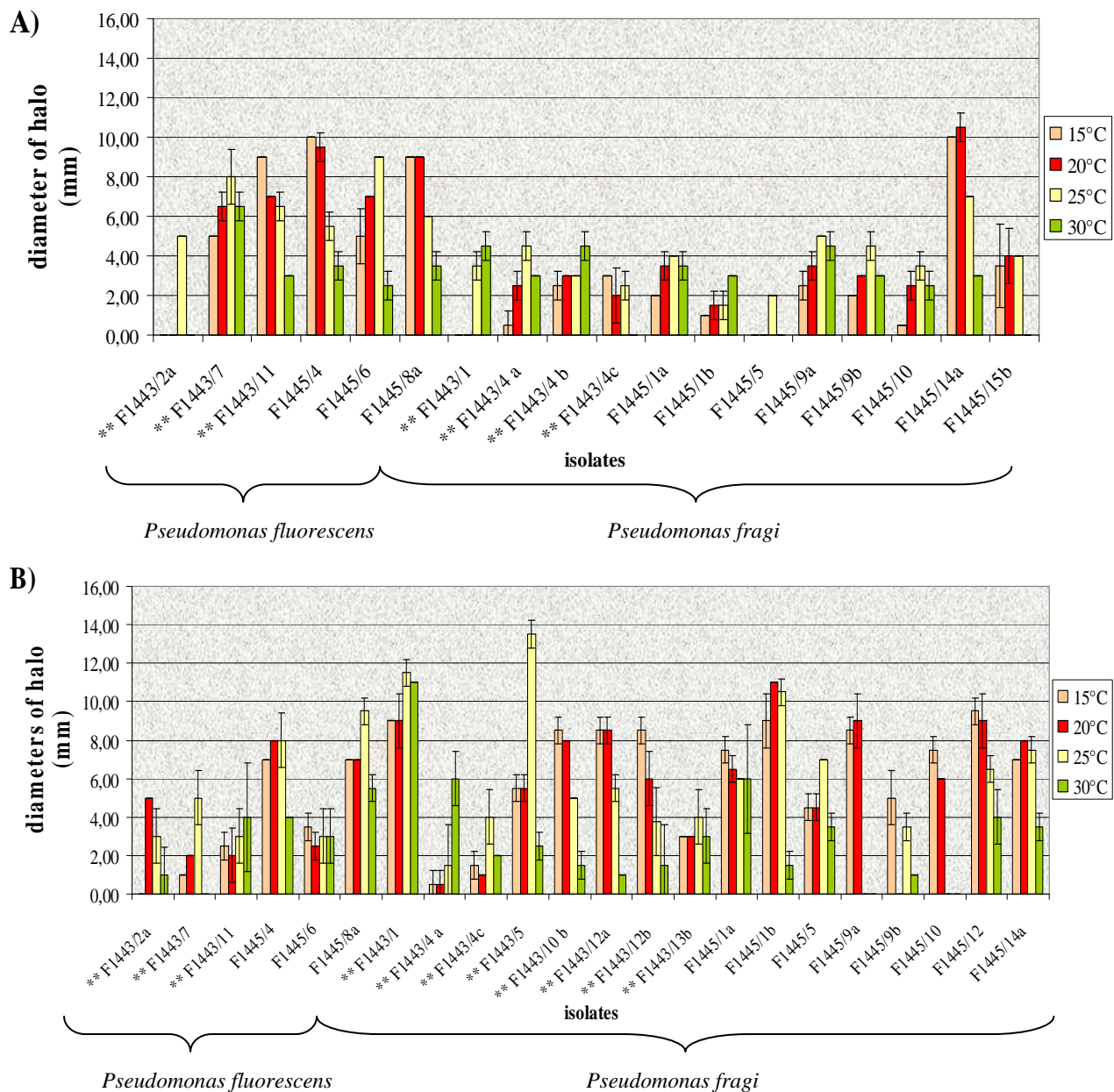
All the isolates were able to grow on PAT-80 plates and 18 isolates from 27 showed esterase activity (Fig. 27 A). Nine isolates did not produce esterase (F1443/5, F1443/8, F1443/10b, F1443/12a, b, c; F1443/13b, F1445/3 and F1445/12) which were identified as *P. fragi*. Altogether 13 isolates were able to express esterase activities at four different temperatures as it is shown on Fig. 27 A.

According to the results the esterase activity of five *P. fluorescens* isolates (F1443/7, F1443/11, F1445/4, F1445/6, F1445/8a) was also very intense at different incubation temperature as shown on Fig. 27 A. Their esterase activities were higher in all incubation temperatures than in case of *P. fragi* isolates. The exceptions were F1443/2a and F1445/14a. It was demonstrated earlier on Fig. 25 B that the proteolytic activities of these *P. fluorescens* isolates were also high. It was also found in the case of milk that *P. fluorescens* isolates contribute intensively to the spoilage by their lipolytic and proteolytic activities (Eneroth et al., 2000; Dogan and Boor 2003). The esterase activity of *P.*



*fragi* strains (from F1445/9a till F1445/15b) isolated from 8°C was also detected on the broad range of incubation temperatures. The esterase activity of *P. fragi* has an important role in spoilage. As an example, this activity usually contributes to the production of fruity and putrid odour at the end of spoilage of meat (Dainty et al., 1989; Lebert et al., 1998).

The picture was quite heterogeneous based on the lipolytic activity as it was demonstrated on Fig. 27 B.



**Figure 27.** Lipolytic activities of *Pseudomonas* isolates incubated at 15, 20, 25, 30°C;

**A)** Detection of esterase activity on PAT-80 plates; **B)** Detection of lipase activity on PCATB plates

\*\* Isolates derived from 4°C; without sign: Isolates derived from 8°C

In case of testing lipase activity 23 isolates were positive on PCATB plates (Fig. 27 B). 18 of them had activities at the four different incubation temperatures. Only four isolates (F443/4b, F1443/8, F1443/12c and F1445/15b) identified as *P. fragi* were not able to produce lipase.

The lipase activity of four *P. fluorescens* isolates (F1443/2a, F1443/7 and F1443/11 F1445/6) was lower compared to the results of *P. fragi* isolates at 15 and 20°C. Only two *P. fluorescens* isolates (F1445/4 and F1445/8a) showed as high lipase activity as the majority of *P. fragi* isolates. In case of *P. fragi* isolates there were higher and less active lipase producer isolates (Fig. 27 B). Their lipase activity was quite low when the plates were incubated at 30°C except in case of F1443/4a and c. However, it could be established that the majority of the isolates possessed lipase activity at the four different incubation temperatures.

In summary almost all of the isolates (even if it was *P. fragi* or *P. fluorescens* isolate) had protease activity as well. It was found by Belák et al. (2011) that *P. fragi* isolates from poultry meat were weak or negative protease and lipase producers. In this work the *P. fragi* isolates derived from pork meat showed an intense protease and lipase production with the application of a broader range of incubation temperatures. The contrast between these findings on poultry and pork meat could be explained by different factors of meat such as its texture, nutrition contents and freshness. As a consequence, different *Pseudomonas* species and strains could be adapted to different types of meat which have distinct characters in their enzymatic activities. The results also show and emphasize the differences in lipolytic and proteolytic activities even between the same species isolated from the same temperature which give the complexity of spoilage.

#### **5.4. *Chryseobacterium antarcticum* and investigation its spoiling potential**

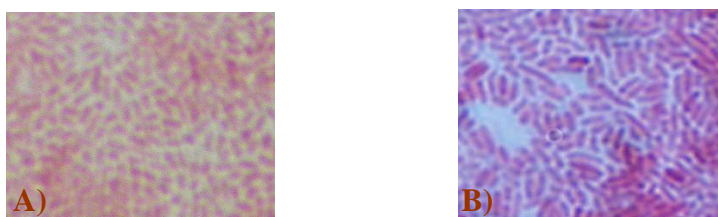
Many changes have taken place in the taxonomy of the family *Flavobacteriaceae* over the past decade and several former *Flavobacterium* species, as well as novel species, were classified in the new genus including *Chryseobacterium* which belongs to the family *Flavobacteriaceae* (Bernardet et al., 1996; Hugo et al. 2003; de Beer et al. 2005). For this reason many earlier reports need to be carefully taking into account since they deal with weakly determined “*Flavobacterium*” strains that might actually have connected to other taxa in the family *Flavobacteriaceae*. Nowadays, flavobacteria refers to the family *Flavobacteriaceae* (Jooste and Hugo, 1999). Flavobacteria are known for their psychrotrophic growth and their ability to contribute in spoilage. They have been frequently isolated from meat and poultry products as well as milk and dairy products (Jooste et al., 1985; Hugo et al., 1999). In milk product they cause off-flavors through their proteolytic and lipolytic activities. In the present study, one isolate named F1445/3 was identified as *Chryseobacterium antarcticum* according to 16S rDNA sequence analysis (see chapter 5.2.5). Because flavobacteria and especially *Chryseobacterium* species from meat sources have seldom



been precisely identified the characterization of this isolate from morphological and physiological respects was performed as well. Beside the competition of this strain and one of the *Pseudomonas fragi* strain was studied aiming to get the answer how long the *Chryseobacterium antarcticum* could persist in the presence of *Pseudomonas fragi* and how much the initial cell density can influence the population sizes of these two strains.

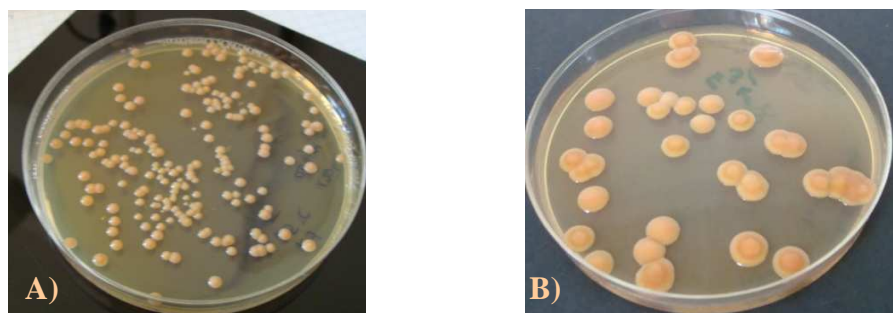
#### 5.4.1. Microscopic and colony morphology of *Chryseobacterium antarcticum* F1445/3

As a first step in characterization of *Chryseobacterium antarcticum* F1445/3 Gram stain and some biochemical tests were performed. The F1445/3 isolate was Gram negative, KOH positive, catalase positive and non-spore forming. It had a negative oxidase test indicating inability to use oxygen for energy production. To demonstrate the difference in microscopic morphology between *Chryseobacterium antarcticum* F1445/3 and *P. fragi* F1445/1b the results of Gram stains were shown on Figs. 28 A and B. In both cases the same magnification was used (100 x) for visualization the microscopic cell morphology under the oil immersion objective in bright-field microscope.



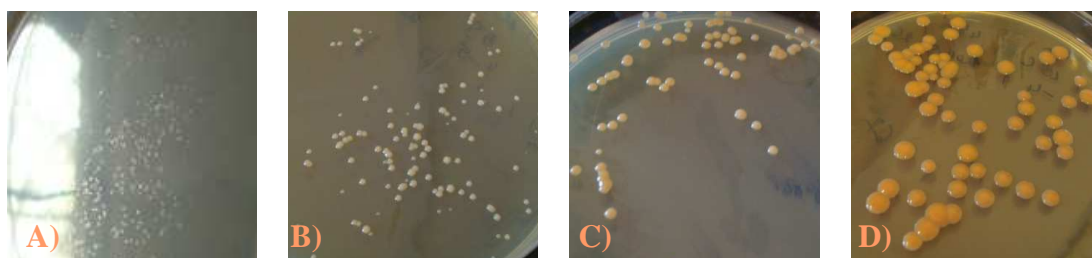
**Figure 28.** Gram stained microscopic cell morphology; **A)** *Chryseobacterium antarcticum* F1445/3; **B)** *P. fragi* F1445/1b

Growth on solid media of most members of the genus *Chryseobacterium* is typically pigmented (distinctive bright yellow to orange pigmentation with shiny appearance), but non pigmented strains also occur (Bernardet and Nakagawa, 2006; Bernardet et al., 2006). This pigment production was detected in case of F1445/3 isolate after 4 and 5 days of incubation on BHI agar, respectively. As the colonies started to age the intensity of their light orange color turned to dark orange as shown on Figs. 29 A and B.



**Figure 29.** Pigment production of *Chryseobacterium antarcticum* F1445/3 after 4 days (**A**) and after 7 days (**B**) of incubation at 20°C

In case of *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> the growth of colonies showed different phases and thus the development of pigmentation could be followed as shown in Figs. 30 A-D. The colonies of the isolate and the type strain were circular, convex to low convex, smooth, with entire edges.



**Figure 30.** Detection of growth *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> after **A)** 3 days **B)** 4 days **C)** 5 days and **D)** 7 days of incubation at 20°C

It was found by Holmes et al. (1984) as well as Hugo and Jooste (2003) that the pigment production could be influenced by the culture medium and its intensity is increasing at low temperatures, in the presence of daylight, and by such compounds as casein, milk and starch.

There was no difference in the intensity of pigment production after 7 days of incubation at 15°C and 20°C on SM plates containing skim milk as shown on Figs. 31 A and B. However, the intensity of the orange colour pigment was much lighter on SM agar (Figs. 31 A and B) than on BHI agar (Figs. 29 B and 30 D) which confirms the establishment that the pigment production could be influenced by the culture medium.



**Figure 31.** Pigment production of *Chryseobacterium antarcticum* F1445/3 and *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> on SM agar incubated at **A)** 15°C and **B)** 20°C

The presence of flexirubin type pigments in members of the genus *Chryseobacterium* is one of the key characteristics which differentiate them from members of several other genera in the family *Flavobacteriaceae*. This pigment is a non diffusible, non fluorescent bright yellow to orange pigment. The *Chryseobacterium* species produced pale cream-beige colonies (as shown on Fig. 32 A) that turned to a light brownish color when a mass of bacterial cells collected on agar and flooded with 20% KOH (Bernardet et al., 2002; Bernardet and Nakagawa, 2006; Bernardet et al., 2006). This is an easy although not absolutely specific method to demonstrate the production of flexirubin type pigments. To demonstrate the flexirubin type pigment production of the *Chryseobacterium*

*antarcticum* F445/3 and *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> the BHI plates were dropped with 20% KOH. The colour change is shown on Figs. 32 B and C.



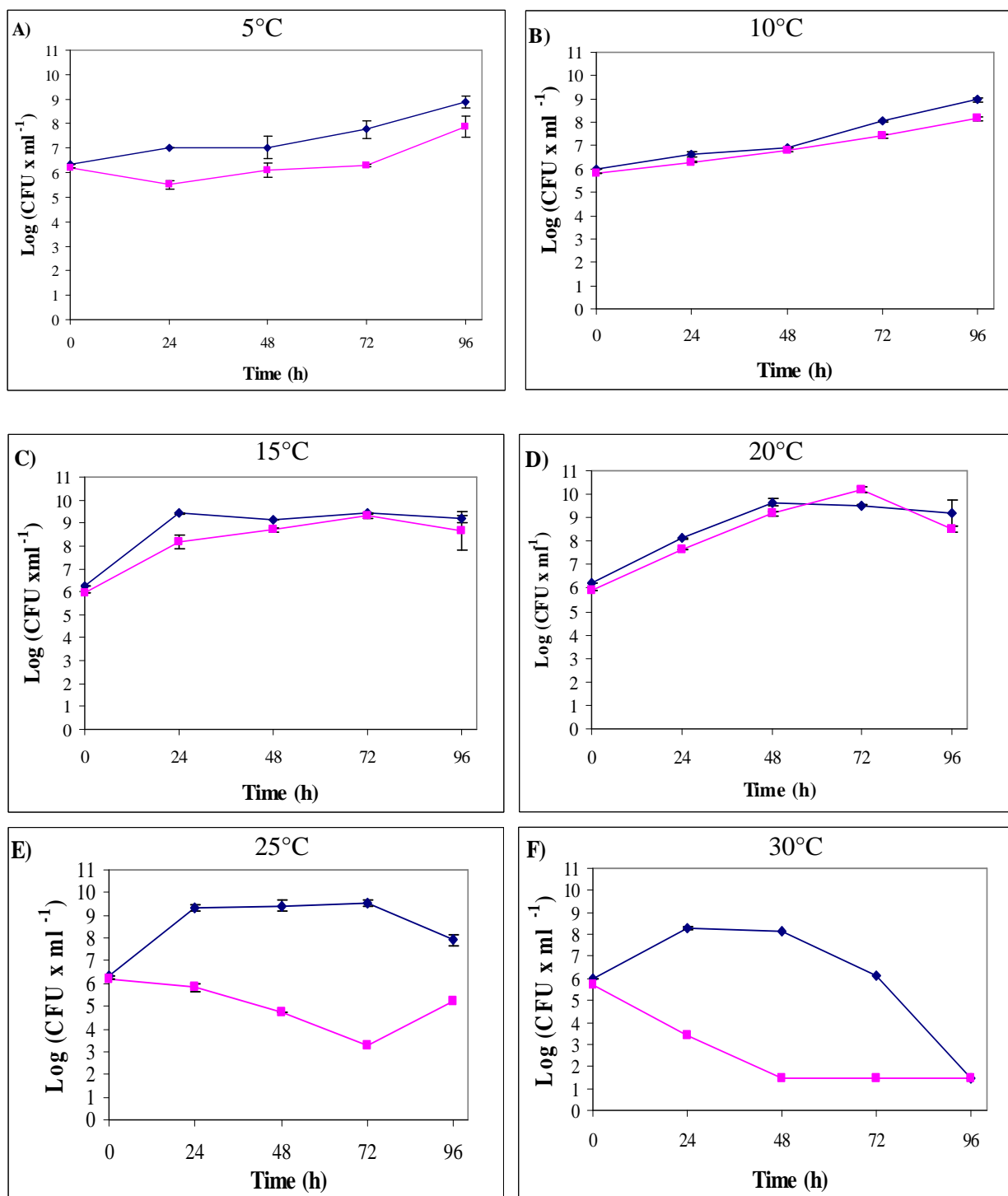
**Figure 32.** Testing flexirubin production of *Chryseobacterium antarcticum* F1445/3 and JCM 12381<sup>T</sup> strains with 20% KOH; **A)** F1445/3 before adding 20% KOH; **B)** F1445/3 after adding 20% KOH. (The lighter zones are not treated with 20%KOH); **C)** JCM 12381<sup>T</sup> with 20% KOH. The remaining area outside the circles was not flooded with 20% KOH.

It could be established that the pigment type of *Chryseobacterium antarcticum* F1445/3 isolate and the *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> belongs to the flexirubin type as consequence of the light brownish coloration observed after the addition of 20% KOH and shown on Figs. 32 B and C.

Additional conventional physiological characterization and biochemical tests were also performed on *C. jeonii* JCM 12382<sup>T</sup>, *C. antarcticum* JCM 12381<sup>T</sup> and *C. antarcticum* F1445/3 strains according to Kämpfer et al. (2011). *C. jeonii* JCM 12381<sup>T</sup> hydrolysed aesculin and had growth at 37°C as well as gave positive reaction for indole production. It was not able to utilize D-mannose, D-xylose and proprionate and grew at 5°C. Urease activity was also negative. In contrast *C. antarcticum* JCM 12381<sup>T</sup> and *C. antarcticum* F1445/3 had the same positive and negative results during the characterization in the tests. Both strains were able to produce indole from tryptophan, utilized D-mannose, D-xylose and proprionate as well as grew at 5°C while aesculin hydrolysis, urease activity and growth at 37°C were not performed. These similarities of the two strains also support the result of sequencing in chapter 5.2.5.

#### 5.4.2. Characterization of growth of *C. antarcticum* F1445/3 at different temperatures

To further characterize *C. antarcticum* F1445/3 the growth behaviour was compared with the type strain in BHI broths for 4 days at 6 different temperatures in shaking flasks (Figs. 33 A-F).



**Figure 33.** Characterization of growth of *Chryseobacterium antarcticum* F1445/3 and *Chryseobacterium antarcticum* JCM 12381<sup>T</sup>; A) Growth at 5°C, B) Growth at 10°C, C) Growth at 15°C, D) Growth at 20°C, E) Growth at 25°C, F) Growth at 30°C, (—■—) F1445/3 count in BHI broth, (—□—) JCM 12381<sup>T</sup> count in BHI broth, Values are given from 3 independent biological replicates and standard deviation of averages was equal or less than 0.5.

The initial cell concentrations of both strains were 6 log CFU/ml at 5, 10, 15, 20, 25, 30°C. At 5°C after one day of incubation the cell count of F1445/3 slightly increased while the cell count of the type strain decreased from 6 log CFU/ml to 5.5 log CFU/ml and then started to follow the same growth pattern as F1445/3 as shown in Fig. 33 A. The exponential phase of F1445/3 started from 48 h until 96 h while that phase of the type strain was shifted to 72 h. The cell count of the type strain was not as high as the isolate at the end of the experiment. The final cell count was 9.6 log CFU/ml of F1445/3 isolate and 8.8 log CFU/ml for the type strain at 96 h.

Growth curves from 10°C showed the same pattern for both strains (Fig. 33 B). The final cell count was 8.8 log CFU/ml for F1445/3 and 8.5 log CFU/ml for the type strain. The curves demonstrate a long exponential phase that started from the beginning of incubation and continuous growth was detected until 96 h.

Cultivations at 15°C and 20°C show the same growth pattern but the length of the exponential phase was different between the strains and both temperatures as shown in Fig. 33 C and D. At 15°C the F1445/3 isolate reached the end of exponential phase after 24 h and then entered to the stationary phase with 9.1 log CFU/ml. The same stage was reached by the type strain after 72 h with 9.2 log CFU/ml and then turned to the stationary phase. At 20°C both strains showed exactly the same pattern until the beginning of stationary phase. The isolate reached that phase after 48 h with the cell count of 9.2 log CFU/ml. In case of type strain the exponential phase was extended for 72 h with the cell count of 10.1 log CFU/ml.

The growth patterns were quite divergent at 25°C and 30°C for both strains. The F1445/3 isolate reached the end of exponential phase after 24 h at 25°C with 9.3 log CFU/ml and entered to the lethal phase after 72 h. The type strain showed quite long adaptation period to the environment and started to grow after 72 h of incubation. The cell count reduced until 3.3 log CFU/ml which was the initial cell count for entering the exponential phase as shown on Fig. 33 E. Cultivation at 30°C showed different patterns for both strains as represented on Fig 33 F. The isolate reached the end of exponential phase after 24 h with 8.3 log CFU/ml but decrease in cell count was started immediately. The type strain was not able to grow at 30°C and cell lysis was detected on the plates. According to the results it could be established that the optimal interval for cultivation of both strains is located between 15 and 20°C.

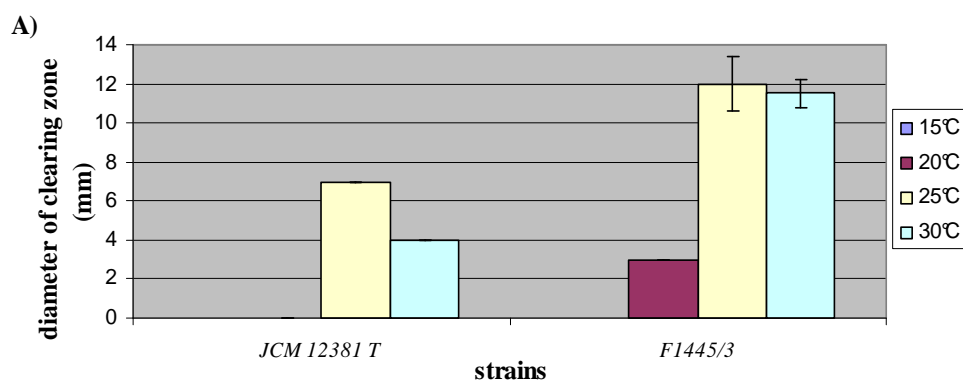
To test the growth of these strains on selective and elective media developed for *Pseudomonas* detection cultivation was performed at 15, 20, 25 and 30°C as shown in Table 14 (in chapter 5.1.) for F1445/3 and *Chryseobacterium antarcticum* JCM 12381<sup>T</sup>. Both strains were able to grow at 20°C on *Pseudomonas* Agar F and on PCA but weak growths were detected at 30°C. Although the isolate F1445/3 was able to grow on GSP, Cetrimide and *Pseudomonas* Agar P at 20°C the type strain showed quite weak growth on these media at 20°C. The growth was weak or

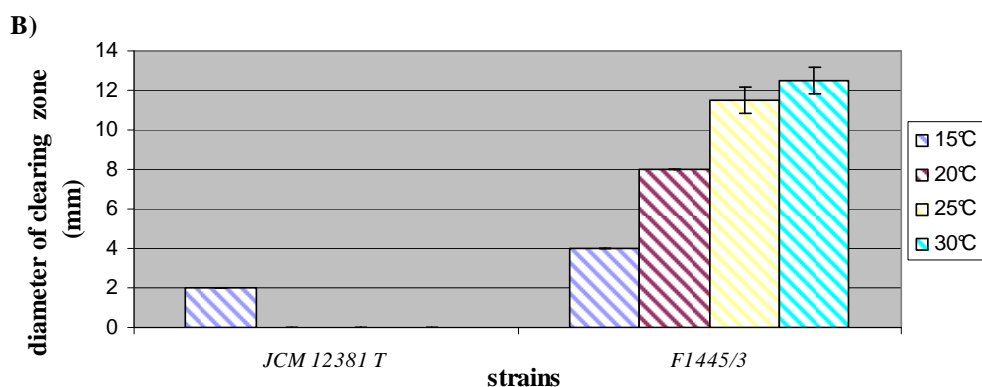
did not occur at 30°C either in both cases. From the non selective media BHI broth and agar were the most suitable for cultivation at 20°C. Further on BHI media was applied for cultivation and maintaining these strains.

According to the literature the most often used temperature range for incubation of *Chryseobacterium* strains are 20 - 25°C (Hugo et al., 2003). They are also able to grow at 4, 15, 25 and 32°C on nutrient agar. No growth occurs at 37°C or above however, some *Chryseobacterium* species are able to grow at 37°C. It could be established that growth parameters of the type strain *Sejongia antarctica* (Yi et al., 2005; Lee et al., 2007), reassigned to the genus *Chryseobacterium* (Kämpfer et al., 2009) and isolated from Antarctic soil sample of penguin habitats, did not grow above 25–31°C and had temperature optimum between 15–20°C while the F1445/3 isolate had this optimum between 15-25°C.

#### 5.4.3. Characterization of the lipolytic and proteolytic activities of *C. antarcticum* strains

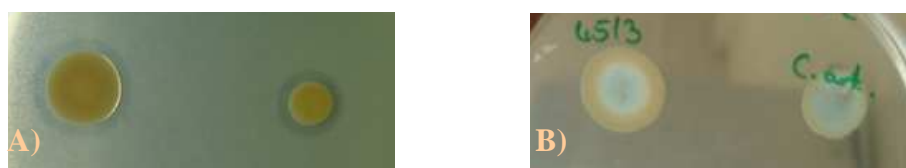
*Chryseobacterium* strains were detected in a variety of meat products, but no mention was made about their role in these products (Bernardet and Nakagawa, 2006). Proteolytic and lipolytic activities were tested for *C. antarcticum* F1445/3 and the type strain at different temperatures as shown in Fig. 34 and Fig. 36. Results were evaluated after 7 days of incubation. Experiments were repeated three times. JCM 12381<sup>T</sup> had different pattern of proteolytic activity than the F1445/3 strain. Growth and this enzymatic activity was detected at 25°C and 30°C on SMC plates while this activity was detected only at 15°C on SM plates. The type strain had less proteolytic activity than the isolate had at the same temperatures. It could be because of the different origin of the isolates. The F1445/3 strain was able to grow all temperature and produced proteases except at 15°C on SMC agar. The highest proteolytic activity of the isolate was shown at 25 and 30°C on SMC and SM agar plates.





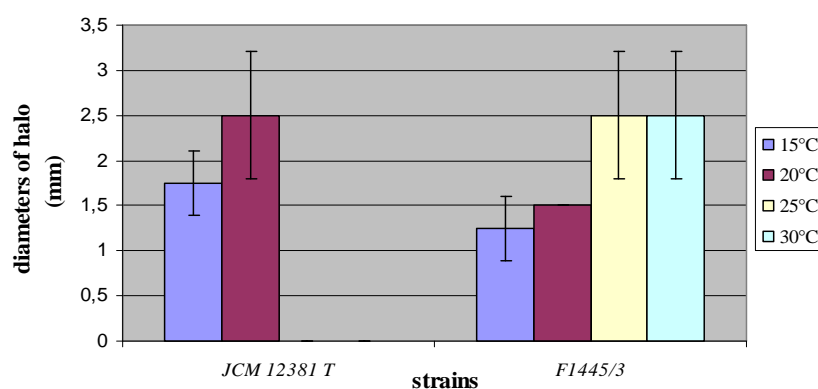
**Figure 34.** Proteolytic activities of *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> and *Chryseobacterium antarcticum* F1445/3 isolate incubated at 15, 20, 25, 30°C; **A)** Proteolytic activity detected on SMC plates; **B)** Proteolytic activity detected on SM plates.

Lipase activity on PCATB was tested as shown on Fig. 35 A while the PAT-80 agar was used for testing esterase activity of *C. antarcticum* F1445/3 isolate and the type strain as shown in Fig. 35 B. Both strains were able to grow on PAT-80 media (except at 30°C) while esterase activity was not detected (Figs. 35 A and B).



**Figure 35.** **A)** Lipase activity on PCATB and **B)** esterase activity of on PAT-80 at 20°C; *Chryseobacterium antarcticum* F1445/3 isolate on the left while *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> are on the right side of the pictures.

Both strains had lipase activity at 15 and 20°C as demonstrated on Fig. 36. The type strain showed high enzyme activity at these temperatures while growth was not detected at 20 and 30°C. *Chryseobacterium antarcticum* F1445/3 isolate was able to grow at 25 and 30°C and showed more active lipase production than at 15 and 20°C.



**Figure 36.** Lipase activity of *Chryseobacterium antarcticum* JCM 12381<sup>T</sup> and F1445/3 on PCATB plates



The analysis of lipase and protease activities of *C. antarcticum* F1445/3 showed higher intensity than the type strain. It could be because of the different source of origin which means they adapted differently to the experimental environment and conditions. Based on the results the proteolytic activity was high in all incubation temperature as it was demonstrated with *Pseudomonas* isolates in chapter 5.3. The lipase activity of the isolate couldn't be neglected either. However the lipase activity was not as high as the proteolytic activity. These characteristics ensure the ability of *C. antarcticum* to contribute actively in the procedure of spoilage of meat as well as milk, pasteurized milk and refrigerated dairy products. It was demonstrated that flavobacteria were the causing agent of surface taint and apple odour in butter (Jooste et al., 1986) off-flavours such as bitterness in milk products (Ellis and Marth, 1984; Jooste et al., 1986).

As an alternative method the miniaturized biochemical tests (e.g. API 20NE) could also be applied for the identification of an isolate. In case of *Chryseobacterium* species the application of this test has several problems. These systems have been primarily developed for the identification of human pathogens, and the extremely slow growth of *Chryseobacterium antarcticum* would be problematic in the interpretation of the results.

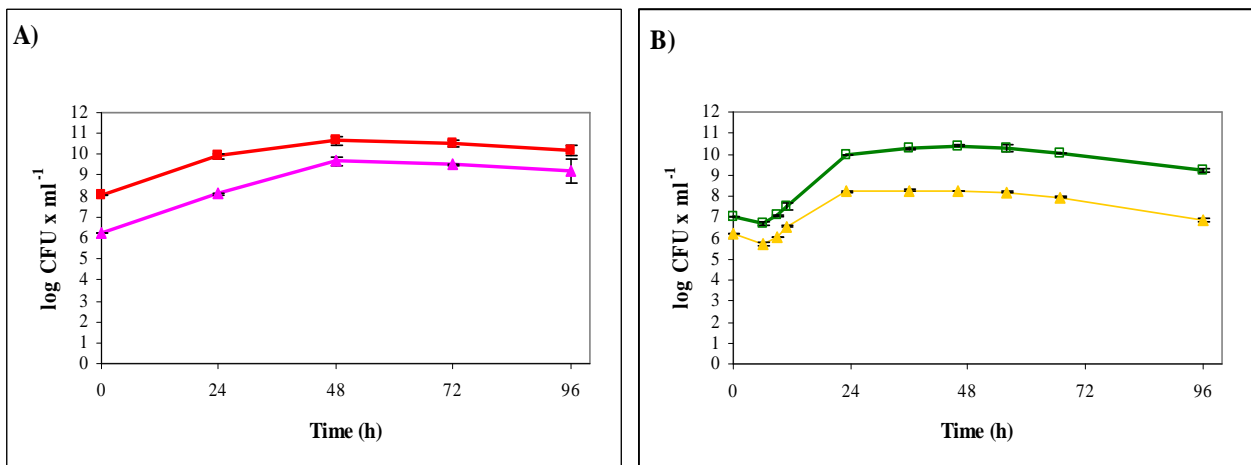
In case of API 20NE test the identification of *Chryseobacterium* strains is usually not valid before 48 h of incubation at 30°C (Bernardet and Nakagawa, 2006) that influence the final result. Because of the database mainly built on pathogen organisms and reorganization of the family of *Flavobacteriaceae* was started less than 20 years ago the database is lack of information from *Chryseobacterium* species derived from food samples and increase the uncertainty of the identification.

#### **5.4.4. Detection of competition between *C. antarcticum* and *P. fragi* strains**

It has been published that in chilled meat and poultry flavobacteria are practically compose the permanent part of the initial spoilage causing biota (McMeekin, 1982). To determine how the *Chryseobacterium antarcticum* could compete with *Pseudomonas fragi* during spoilage different cultivation set ups were performed in BHI broth. Control cultivations were performed with *Chryseobacterium antarcticum* F1445/3 and *Pseudomonas fragi* F1445/1b strains. The initial cell concentrations of F1445/3 were  $10^6$  and  $10^8$  CFU/ml in BHI broth while for *P. fragi* these concentrations were  $10^6$  and  $10^7$  CFU/ml in BHI broth at 20°C. Growth curves derived from these setting are shown in Figs. 37 A and B. Cultivation of the two different strains was performed separately in shaking flasks. In case of F1445/3 both growth curve showed the same pattern as illustrated in Fig. 37 A. The end of exponential phase was reached after 48 h incubation when the cell count was 10.6 log CFU/ml and 9.6 log CFU/ml, respectively. The growth of *P. fragi* was

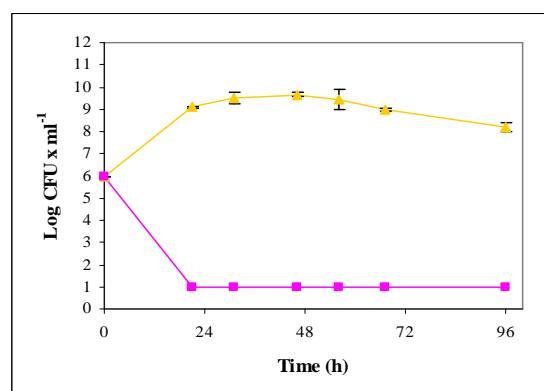


faster in case of both cultivation settings as shown in Fig. 37 B. After a short adaptation period that took approximately 6 hours cells entered to the exponential phase and reached 8.2 and 9.9 log CFU/ml cell concentration after 23 h. After 56 h incubation the cell concentration in both culture started to decline and cell disruption was detected.



**Figure 37.** **A)** Growth curves of *Chryseobacterium antarcticum* F1445/3; (—▲—) initial cell concentration is  $10^6$  CFU/ml, (—■—) initial cell concentration is  $10^8$  CFU/ml; **B)** Growth curves of *P. fragi* F1445/1b; (—▲—) initial cell concentration is  $10^6$  CFU/ml, (—□—) initial cell concentration is  $10^7$  CFU/ml. Incubations were performed at 20°C in BHI broth. Values are given from 3 independent biological replicates and standard deviation of the averages was less than 0.5.

Competition between these isolates were tested with different cell ratios as well. When the cell ratio was equal ( $10^6:10^6$  CFU/ml) at the beginning of the cultivation the following growth was detected as shown in Fig. 38. *P. fragi* isolate reached the stationary phase with 9.5 log CFU/ml after 31 h and declining in cell count was detected after 56 h as during control cultivation in Fig. 37 B.



**Figure 38.** Cultivation of *C. antarcticum* F1445/3 and *P. fragi* F1445/1b in equal ratio; (—▲—) Colony count of *P. fragi* F1445/1b; (—■—) colony count of *C. antarcticum* F1445/3; Values are given from 3 independent biological replicates and standard deviation of the averages was less than 0.5.

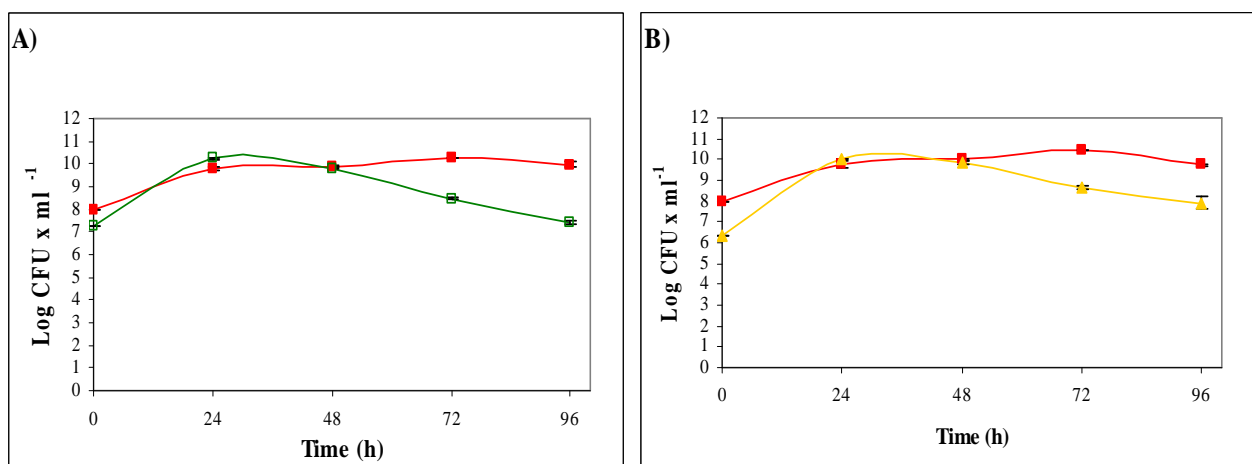
*C. antarcticum* F1445/3 was not able to compete with *P. fragi* (F1445/1b) isolate because of the shorter log phase of *P. fragi* as show in Fig. 37 B. It overgrew *C. antarcticum* F1445/3 that could not be detected later on the cultivation.

To detect the behavior of *P. fragi* F1445/1b if is cultivated in less concentration than *C. antarcticum* (F1445/3)  $10^8$ :  $10^7$  CFU/ml and  $10^8$ :  $10^6$  CFU/ml cell ratios were set at the beginning of cultivation. Competition between these strains is demonstrated in Fig. 39. while growth curves are shown on Figs. 40 A and B



**Figure 39.** Competition between *C. antarcticum* F1445/3 and *P. fragi* F1445/1b at 20°C after 6 days of incubation.

The same growth pattern could be seen in both experimental setup (Figs. 40 A and B) even if these were independent experiments. *C. antarcticum* isolate reached the stationary phase after 48 h incubation with 9.9 and 10 log CFU/ml cell count while *P. fragi* isolate had the maximum cell count at 24 h in both cases with 10.2 and 10 log CFU/ml. Further on slowly decrease started in the cell count in both cases (Figs. 40 A and B). After 48h *P. fragi* isolate started to to be lysed but *Chryseobacterium* cell concentration remained constant. *P. fragi* was not able to overgrow *C. antarcticum* F1445/3 and it is highly probable that components of cell lysates from *P. fragi* F1445/1b could be the base of further growth as nutrient source for *C. antarcticum* F445/3.



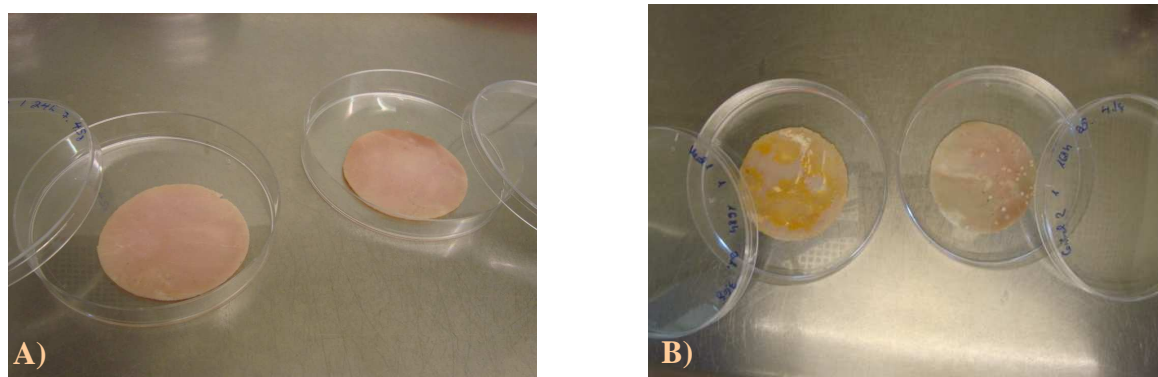
**Figure 40.** Growth curves of *C. antarcticum* F1445/3 and *P. fragi* F1445/1b isolates at 20°C **A)** cell ratio  $10^8$ :  $10^7$  CFU/ml; **B)** cell ratio  $10^8$ :  $10^6$  CFU/ml; (—■—) colony count of *C. antarcticum* F1445/3; (—□—) and (—▲—) Colony count of *P. fragi* F1445/1b; Values are given from 3 independent biological replicates and standard deviation of the averages was less than 0.5.

It was proved by the data that although the members of genus *Chryseobacterium* could compose the initial microbiota of meat and contribute to the spoilage but are unable to compete, in terms of growth with pseudomonads (McMeekin, 1982). The cell ratio of flavobacteria is usually less than the pseudomonads count on meat. Under environmental selection the number of these flavobacteria reduces and competition with pseudomonads could realize only the beginning of meat spoilage. However, high initial numbers of a slowly growing species may compete successfully with lower numbers of a species with a faster growth rate. If the number of spoilage microorganism in the initial population is high, a slower growth rate may not be an important factor, since less growth may be necessary before spoilage occurs (Jackson et al., 1997).

## 5.5. Expression and production of enterotoxin A and D of *Staphylococcus aureus* SA45

### 5.5.1. Growth and enterotoxin A and D expression in meat products

To monitor *in situ* the *sea* and *sed* expression of *Staphylococcus aureus* SA45, four different pork meat products (boiled ham, smoked ham, Serrano ham and black pepper salami) were tested. Cultivation was performed on the surface of these products at room temperatures for 7 days as shown in Figs. 41 A and B. *S. aureus* was not detected on the control meat samples of the four pork products.



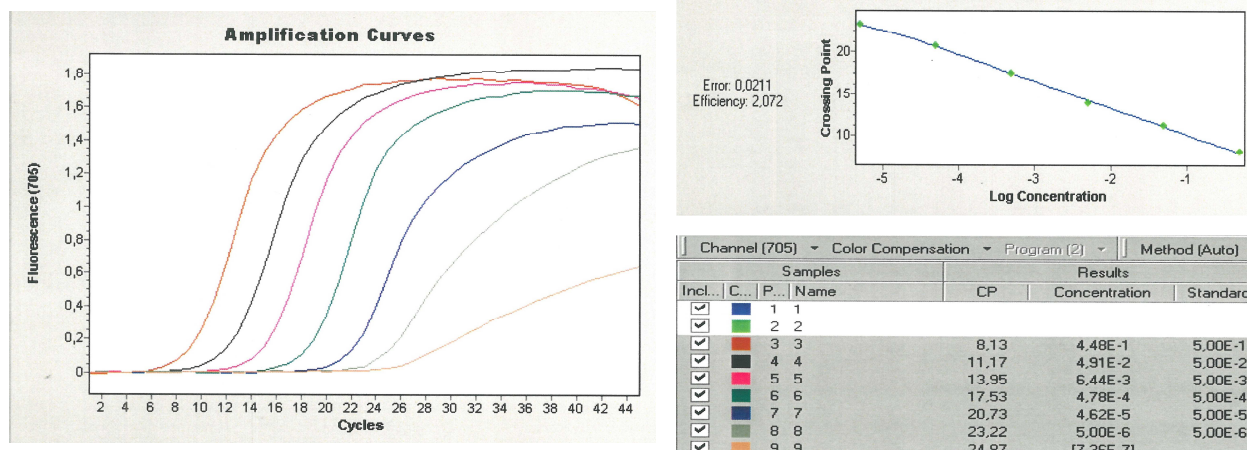
**Figure 41.** Cooked ham samples at the beginning of storage (A) and after 168h (B). Inoculated samples are on the left while control samples are on the right side of the pictures.

Relative expression of *sea* and *sed* was calculated according to Pfaffl (2001) using qRT-PCR. As reference, the expression level of 16S rRNA was chosen because of its rather constant expression. The specific amount of 16S ribosomal mRNA was found to be stable in the present experiments. The amplification efficiency (E) and the log-linear range of amplification were calculated for 16S rRNA (*rrn*) and *sea* as well as *sed*. Examples of determined standard curves for *rrn* and *sea* are shown in Figs. 42 A and B.

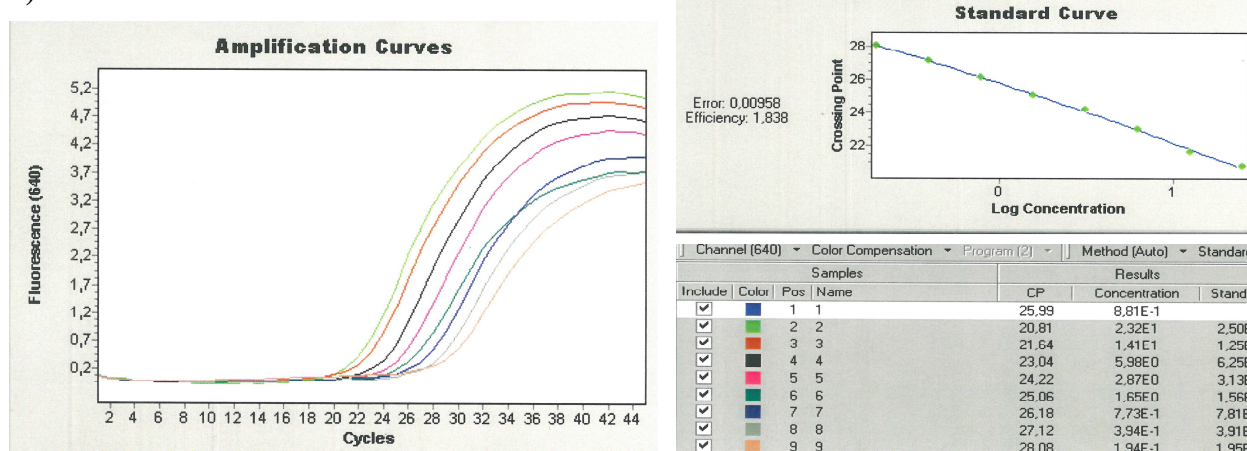
The relative expression (RE) was calculated from the amplification efficiencies for each PCR assay and the crossing point deviation ( $\Delta C_p$ ) of an unknown sample compared with the calibration sample according to the following equations.

$$RE = \frac{(1+E_{sea})^{\Delta C_{psea} \text{ (calibrator-unknown sample)}}}{(1+E_{rm})^{\Delta C_{prm} \text{ (calibrator-unknown sample)}}} \quad \text{or} \quad RE = \frac{(1+E_{sed})^{\Delta C_{psed} \text{ (calibrator-unknown sample)}}}{(1+E_{rm})^{\Delta C_{prm} \text{ (calibrator-unknown sample)}}}$$

A)



B)



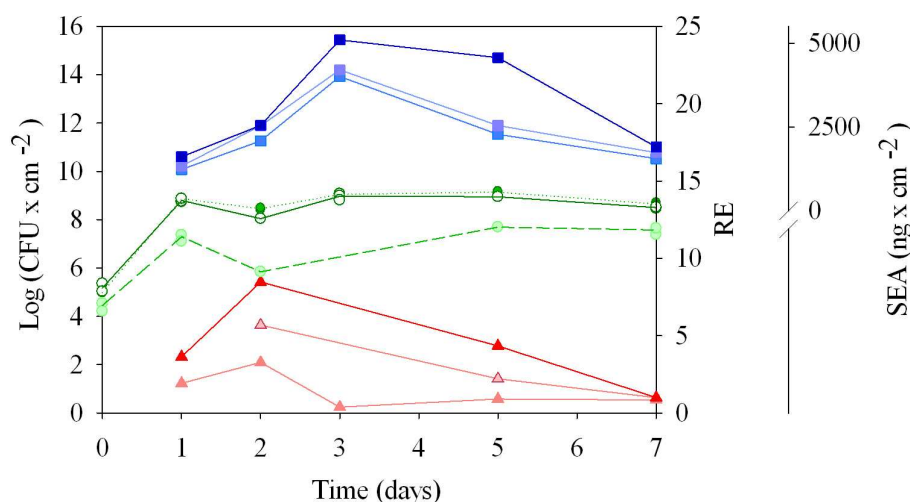
**Figure 42.** Cp values, standard curves and amplification efficiencies of **A)** 16S cDNA and **B)** *sea* cDNA.

The relative expression of enterotoxin A and D in all four pork products as well as the bacterial growth and specific amounts of SEA and SED produced are shown in Figs. 43, 44, 45 and 46. All the analysis were performed three times at each concentration. For the meat products the mean of crossing point values of biological replicates from day seven was used as calibrator in the RE calculations. The amount of synthesized SEA and SED were measured by sandwich ELISA. The independent experiments are indicated as individual curves in the figures rather than means with standard deviation due to slightly different inoculum sizes.

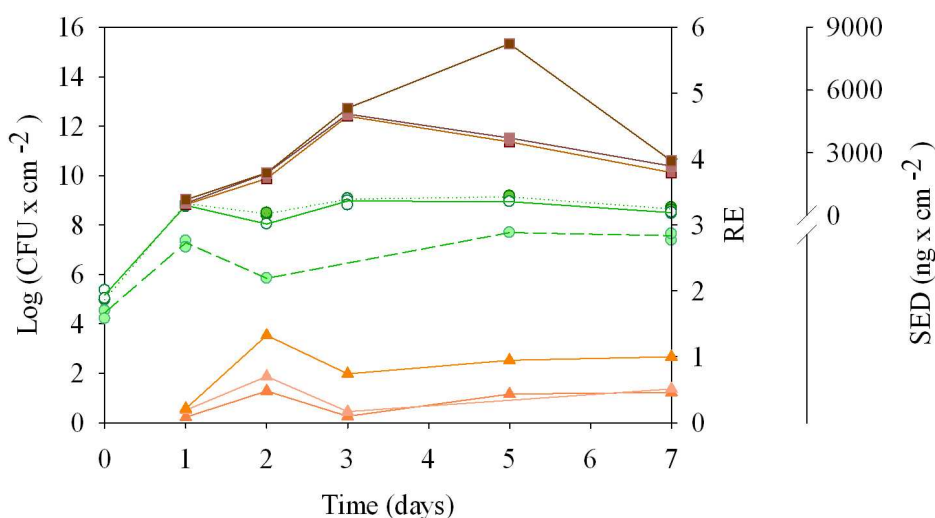
### 5.5.1.1. Boiled ham

The initial viable count of *S. aureus* on boiled ham was  $9.6 \times 10^4 \pm 2.8 \times 10^3$  CFU/cm<sup>2</sup> as shown in Figs. 43 A and B. After one day of incubation, the cell count reached  $7.4 \times 10^8$  CFU/cm<sup>2</sup>  $\pm 4.5 \times 10^7$ . The final count was  $4.5 \times 10^8 \pm 6 \times 10^7$  CFU/cm<sup>2</sup> after seven days. The total viable cell count on the inoculated ham was the same as the *S. aureus* count. The total bacterial count on the uninoculated ham was at least one log unit lower than that on the inoculated ham. *Lactobacillus* sp. was detected on inoculated ham at the beginning and end of the experiment (5 and 8 log CFU per cm<sup>2</sup>, respectively).

A)



B)



**Figure 43.** A) Growth, relative expression (RE) of *sea* and SEA production and B) growth, relative expression of *sed* and SED production of *S. aureus* SA45 on boiled ham at room temperature.

(—○—) Total cell count of inoculated meat, (.....●.....) *S. aureus* count on inoculated meat, (---●---) total cell count on uninoculated control meat, (—▲—) RE of *sea*, (—■—) extracellular SEA concentration, (—▲—) RE of *sed*, (—■—) extracellular SED concentration. Values are given from 3 independent biological replicates with different tones of red, blue, orange and brown colors. The y- axis for SEA and SED concentration curves has been shifted for better separation from the growth and RE curves.

The pH of the inoculated boiled ham increased slowly over time from the initial pH of 6.3 to pH 7.3 after seven days of incubation (Table 18).

**Table 18.** pH changes in the meat products. Data are the mean pH values of three samples combined with standard deviation.

Meat products		pH changes with standard deviations					
		Incubation time (days)					
		0	1	2	3	5	7
Boiled ham	Inoculated meat	6.3±0.040	6.4±0.010	6.8±0.060	7.3±0.12	7.3±0.13	7.3±0.038
	Control meat	6.3±0.015	6.1±0.026	6.2±0.036	6.3±0.055	6.1±0.11	6.2±0.29
Smoked ham	Inoculated meat	6.3±0.020	6.5±0.015	7.0±0.45	7.0±0.15	7.5±0.090	7.4±0.15
	Control meat	6.3±0.032	6.4±0.060	6.4±0.0058	6.0±0.075	6.0±0.17	6.3±0.26
Serrano ham	Inoculated meat	5.8±0.081	5.8±0.072	5.9±0.012	5.9±0.0	5.8±0.053	6.8±0.12
	Control meat	5.8±0.083	5.9±0.084	5.9±0.012	5.9±0.015	5.7±0.030	6.3±0.24
Black pepper salami	Inoculated meat	4.4±0.12	4.5±0.010	4.2±0.015	4.7±0.010	4.8±0.053	4.7±0.010
	Control meat	4.4±0.085	4.5±0.026	4.4±0.030	4.7±0.047	4.8±0.012	4.7±0.035

The pH of the uninoculated boiled ham remained above pH 6. It could be established that *sea* mRNA was detected on boiled ham during the entire incubation period. The relative *sea* mRNA levels increased the first two days and thereafter decreased. The concentration of extracellular SEA, as determined by the ELISA, after three days of incubation reached a maximum level of 4400 ng/cm<sup>2</sup> (n = 3) and then started to decline. In the case of *sed* expression, the relative *sed* mRNA level peaked after two days of incubation and a second increase in *sed* expression was observed after five days of incubation (Fig. 43 B). That was in contrast with the decrease of SED observed after three days of incubation.

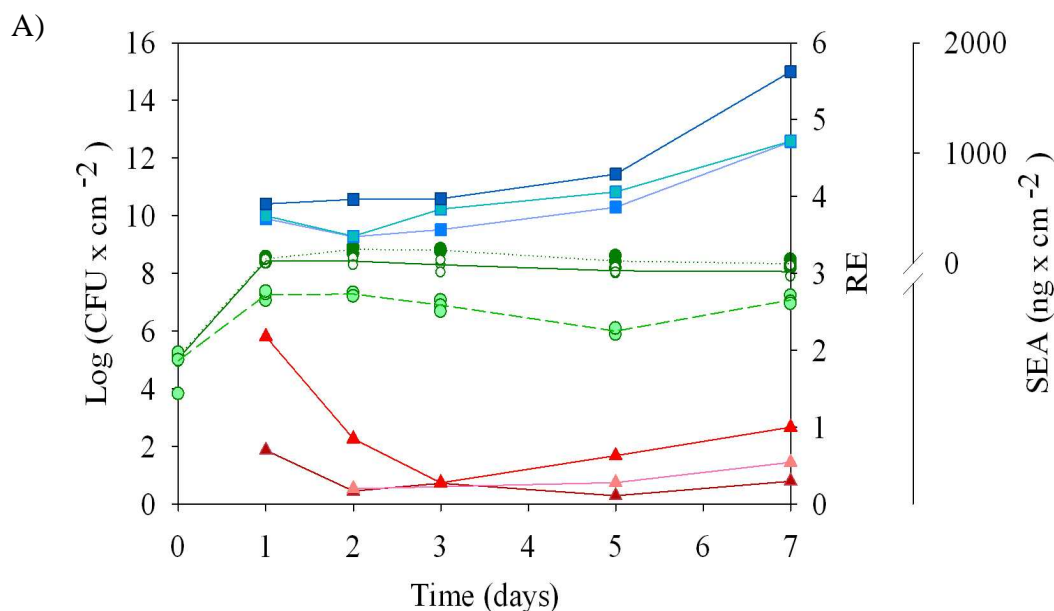
Accumulation of SEA and SED was expected on boiled ham because enterotoxins are known to be very stable. They are heat stable and resistant to the degradation by several proteases, dehydration, and stable between high pH range such as pH > 2 and pH < 12 (Denny et al., 1966; Genigeorgis, 1989; Le Loir et al., 2003). The decrease in SEA amount could be the result of lack of serological recognition, meaning that the proteins were not detectable even if the toxin were still present in the meat. Loss of biological activity is usually associated with loss of immunological activity, but exceptions have also been existed (Bennett and Berry, 1987). However, the ELISA method applied in the experiments was based on polyclonal antibodies. The probability of none of the antibodies recognizing the toxin is low. Certain bacteria, mostly lactic acid bacteria, grown in the presence of SEA combined with or without *S. aureus*, may cause disappearance of enterotoxins (Chordash and Potter, 1976; Daoud and Debevere, 1985; Donnelly et al., 1968). According to Chordash and Potter

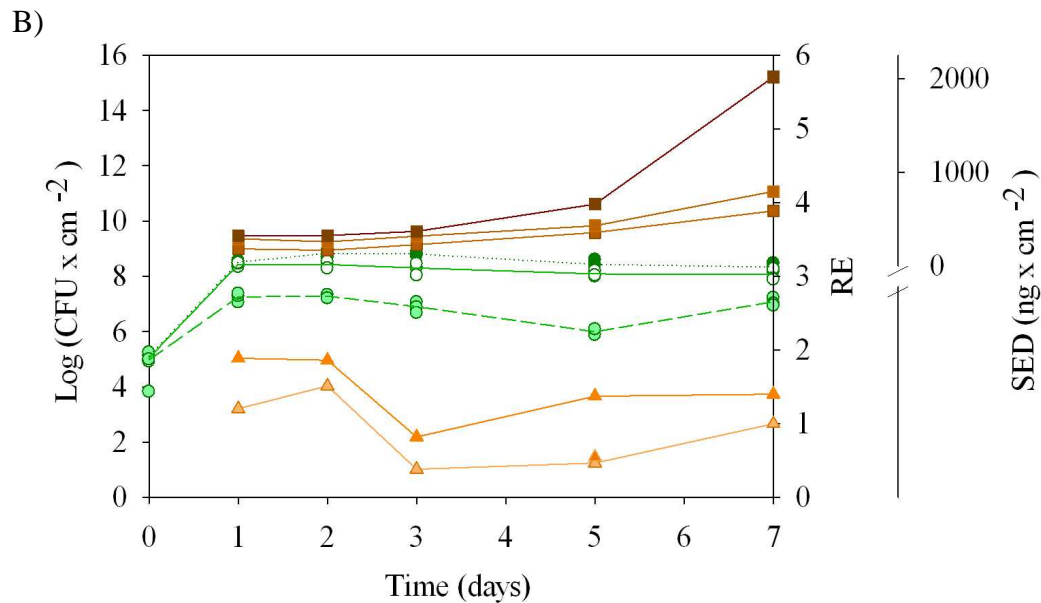


(1976) suggestion the metabolites produced by the lactic acid bacteria degraded the staphylococcal enterotoxins that did not belong to the acid production. These metabolites may be proteases. In this study, on all types of meat products including both inoculated and uninoculated ones *Lactobacillus* sp., i.e. lactic acid bacteria were detected. Another possible explanation for the declining in SEA and SED amounts could be that the enterotoxins became cell-associated with *S. aureus* (Hallis et al., 1991). It was found by Hallis et al. (1991) that the addition of glucose to de-repressed steady-state cultures of *S. aureus* using continuous culture techniques could be the cause of the cell-associated SEA.

### 5.5.1.2. Smoked ham

The initial *S. aureus* count was  $1.3 \times 10^5 \pm 3.5 \times 10^3$  CFU/cm<sup>2</sup> on smoked ham as shown in Figs. 44 A and B. After one day of incubation, the *S. aureus* count reached  $3.3 \times 10^8 \pm 7.2 \times 10^7$  CFU/cm<sup>2</sup>. The final count after seven days was  $2.2 \times 10^8 \pm 7.7 \times 10^7$  CFU/cm<sup>2</sup>. Initially, the total count on the inoculated ham was the same as the *S. aureus* count, but after two days it was slightly lower than the *S. aureus* count. The total count on the uninoculated ham was at least one log unit lower than that of the inoculated ham. *Lactobacillus* sp. was detected on inoculated ham at the end of the experiment as well as on cooked ham which was 6 log CFU/cm<sup>2</sup>.





**Figure 44. A)** Growth, relative expression (RE) of *sea* and SEA production and **B)** growth, relative expression of *sed* and SED production of *S. aureus* SA45 on smoked ham at room temperature.

(—○—) Total cell count of inoculated meat, (.....●.....) *S. aureus* count on inoculated meat, (---○---) total cell count on uninoculated control meat, (—▲—) RE of *sea*, (—■—) extracellular SEA concentration, (—▲—) RE of *sed*, (—■—) extracellular SED concentration. Values are given from 3 independent biological replicates with different tones of red, blue, orange and brown colours. The y- axis for SEA and SED concentration curves has been shifted for better separation from the growth and RE curves

The pH of the inoculated smoked ham increased with time compared to the uninoculated products (Table 18). Enterotoxin mRNA was detected on smoked ham during the entire incubation period (Figs. 44 A and B). The relative *sea* mRNA levels decreased in the middle of the incubation period but increased again towards the end. The SEA concentration increased with time. The level of SEA increased twice between day five and seven, reaching 1300 ng/cm<sup>2</sup> at the end of the experiment (Fig. 44 A). The *sed* expression profile was following the same trend as *sea*. The level of SED started to increase from 482.7 ng/cm<sup>2</sup> at day five reaching 1134 ng/cm<sup>2</sup> (n=3) at the end of the experiment. It could be established that less SED was produced than SEA.

Based on the results, *S. aureus* produced considerably lower SEA and SED amounts in smoked ham than in the boiled ham. The SEA concentration per CFU of *S. aureus* was six times less than the SEA concentration per CFU of *S. aureus* in the boiled ham after three days of incubation. In this case, a repression of *sea* expression and SEA formation was observed in smoked ham compared to boiled ham. The reverse picture was observed for the botulinum toxin of *Clostridium botulinum* where increased toxin production per cell has been found (Artin et al., 2008; Lövenklev et al., 2004a, b). Increased expression and production of type B and type E botulinum

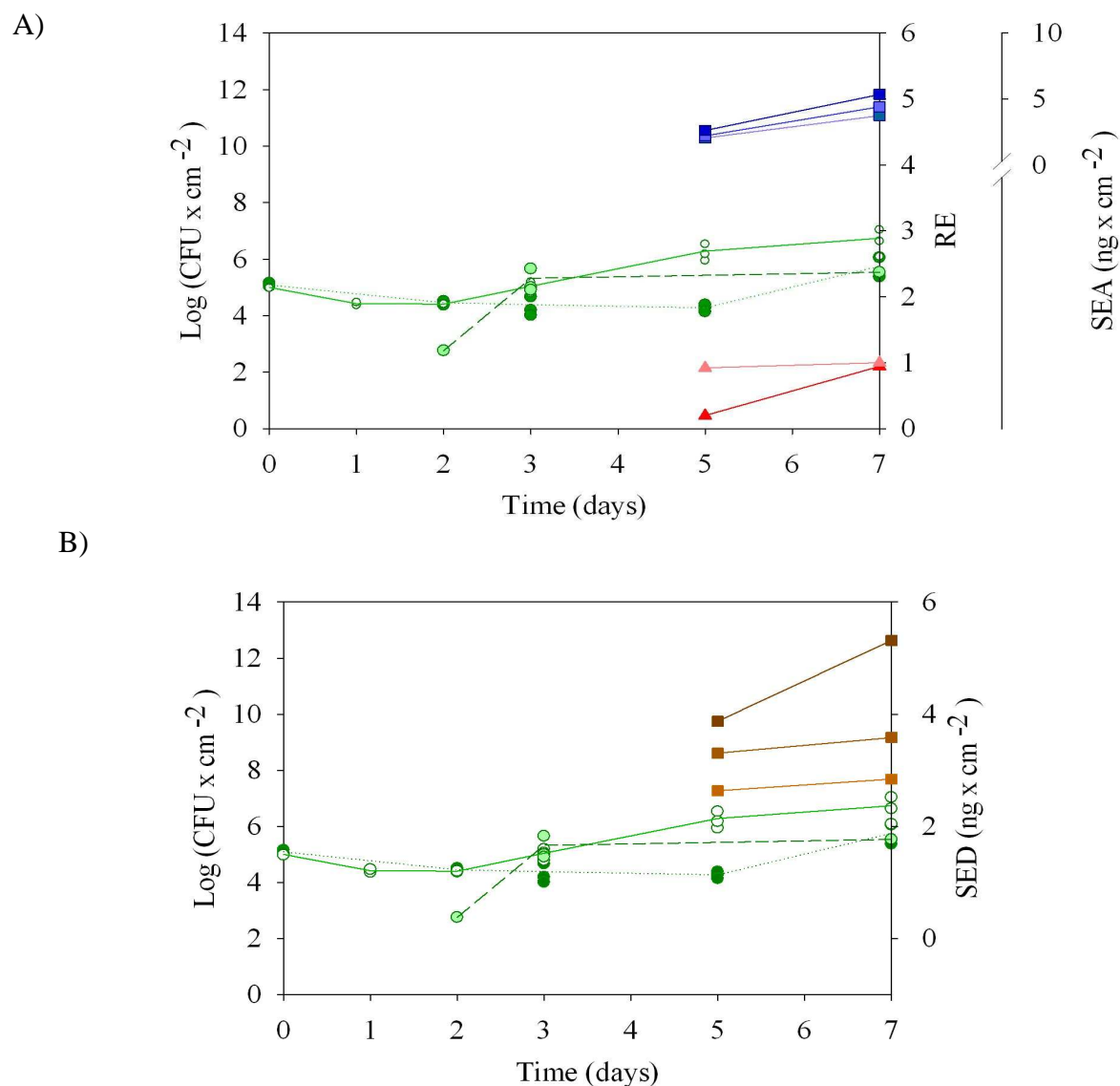


neurotoxin were observed in *C. botulinum* when high levels of carbon dioxide was applied, while growth was reduced (Artin et al., 2008; Lövenklev et al., 2004a). The *C. botulinum* viable counts were reduced by 83% when exposed to air, however the expression of type B botulinum neurotoxin was not influenced (Lövenklev et al., 2004b). For both smoked and boiled ham, *sed* expression peaked after two days of incubation and a second increase in *sed* expression was observed after five days of incubation. It could be established that active *sed* and *sea* expression occurred throughout the incubation period on the ham products. Furthermore, a similar prolonged *sed* expression has also been observed in cheese production with milk inoculated with  $10^3$  CFU/ ml of *S. aureus* (Duquenne et al., 2010). The effect of growing *S. aureus* on meat in the presence of an undefined microbiota at a lower than optimal temperature, with zones being exposed to low pH, low nutrient availability and lack of oxygen, may explain the prolonged *sea* expression observed in the boiled and smoked hams. Even et al. (2009) demonstrated continuous *sea* expression in *S. aureus* during 25 h of cultivation together with *Lactococcus lactis* using a chemically defined medium held at a constant pH and Duquenne et al. (2010) showed active *sea* expression over a 72 h experiment when cheese was inoculated with *S. aureus*.

The SED concentration per CFU of *S. aureus* was nine times less in smoked ham than in boiled ham after three days of incubation. Taormina and Bartholomew (2005) found that enterotoxin production was inhibited in smoked, cured and ground pork bellies, although *S. aureus* growth was detected, but not in un-smoked pork bellies.

#### **5.5.1.3. Serrano ham**

The initial *S. aureus* count was  $1.2 \times 10^5 \pm 1.7 \times 10^4$  CFU/cm<sup>2</sup>, and declined slowly during the first five days of incubation. At day seven, an increase in CFU was observed, giving a final count of  $5.7 \times 10^5 \pm 5.0 \times 10^5$  CFU/cm<sup>2</sup> as shown in Figs. 45 A and B. The total count during the first two days of incubation was the same or moderately less than the *S. aureus* count. The total count on the control meat increased steadily with time, and reached the same level as *S. aureus*. *Lactobacillus* sp. was detected on inoculated Serrano ham at the beginning and at the end of the experiment (1 and 4 log CFU/cm<sup>2</sup>, respectively). Mold was detected on both inoculated and uninoculated ham after seven days of incubation.



**Figure 45. A)** Growth, relative expression (RE) of *sea* and SEA production and **B)** growth and SED production of *S. aureus* SA45 on Serrano ham at room temperature.

(—○—) Total cell count of inoculated meat, (.....●.....) *S. aureus* count on inoculated meat, (---○---) total cell count on uninoculated control meat, (—▲—) RE of *sea*, (—■—) extracellular SEA concentration, (—■—) extracellular SED concentration. Values are given from 3 independent biological replicates with different tones of red, blue and brown colors. The y- axis for SEA and SED concentration curves has been shifted for better separation from the growth and RE curves

The pH of the inoculated and uninoculated Serrano ham was stable the first five days (Table 18). After seven days of incubation, the pH of both the control and inoculated product increased from 5.7 and 5.9 to 6.3 and 6.8, respectively. Detectable level of *sea* mRNA in the Serrano ham was observed after five and seven days of incubation (Fig. 45 A). The level was slightly greater on day seven. A similar increase was noticed for SEA produced from approximately 2.3 to 4.5 ng SEA per cm<sup>2</sup> (n=3). The amount of *sed* mRNA was too low to be detected by the developed *sed*-specific

qRT-PCR methodology. However, extracellular SED was slightly increased from 3.8 to 5.3 ng SED per cm<sup>2</sup> (n=3) in the end of the experiments (Fig. 45 B).

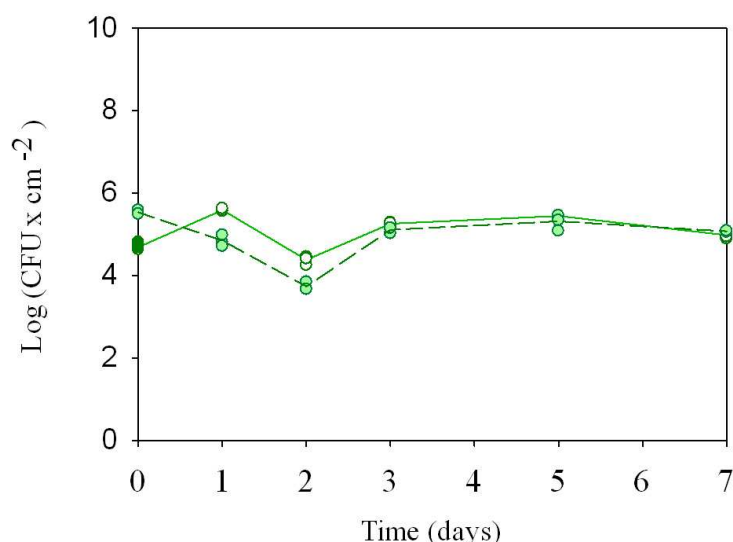
Serrano ham ensured challenges with its difficult environment for *S. aureus* growth, with high salt and fat content, low water activity, and other microorganisms. *S. aureus* growth was poor and no increase in the *S. aureus* count was observed until after seven days of incubation. Untermann and Müller (1992) observed a similar growth pattern of *S. aureus* when grown at 20°C for one week on minced dry-cured ham ready for sale. Also, decreasing water activity have been found to have an adverse effect on SED production and curing salts, even at relatively low concentrations, were found to inhibit SEB production (Ewald and Notermans, 1988; McLean et al., 1968).

The pH in the inoculated ham increased with one-unit between days five and seven. It could be the consequence of protein degradation of the meat by the *Staphylococcus* or by consumption and degradation of lactic acid by moulds (Willey et al., 2008). This changing in pH could be the reason for the observed growth. The toxin expression and the SEA levels increased between days five and seven. After five days of incubation no increase in cell numbers had yet occurred, although *sea* expression was detected. This indicates that the number of *sea* transcripts per cell had increased between days three and five, resulting in *sea* mRNA levels above the detection limit of the applied assay.

SFP can be caused by as little as 20-100 ng of enterotoxin (Asao et al., 2003). Much higher amounts were detected in smoked and boiled ham already after one day of incubation. After five days of incubation, the Serrano ham contained SED levels enough to cause SFP in sensitive individuals.

#### **5.5.1.4. Black pepper salami**

At the beginning of the experiment after inoculation of the salami the total viable count and the *S. aureus* count were equal which was  $5.4 \times 10^4$  CFU/cm<sup>2</sup>. Later on no viable *S. aureus* cells were found as shown in Fig. 46. The total viable count then increased after one day of incubation, and reached the final count as  $9.7 \times 10^4$  CFU/cm<sup>2</sup> after seven days of incubation. The total count on the control samples decreased from the start of the experiment until day three. The CFU count then increased and was the same as for the total count on inoculated meat. *Lactobacillus* sp. was detected on inoculated salami at the beginning and end of the experiment (4 and 5 log CFU/cm<sup>2</sup>, respectively).



**Figure 46.** Growth of *S. aureus* SA45 on black pepper salami at room temperature.

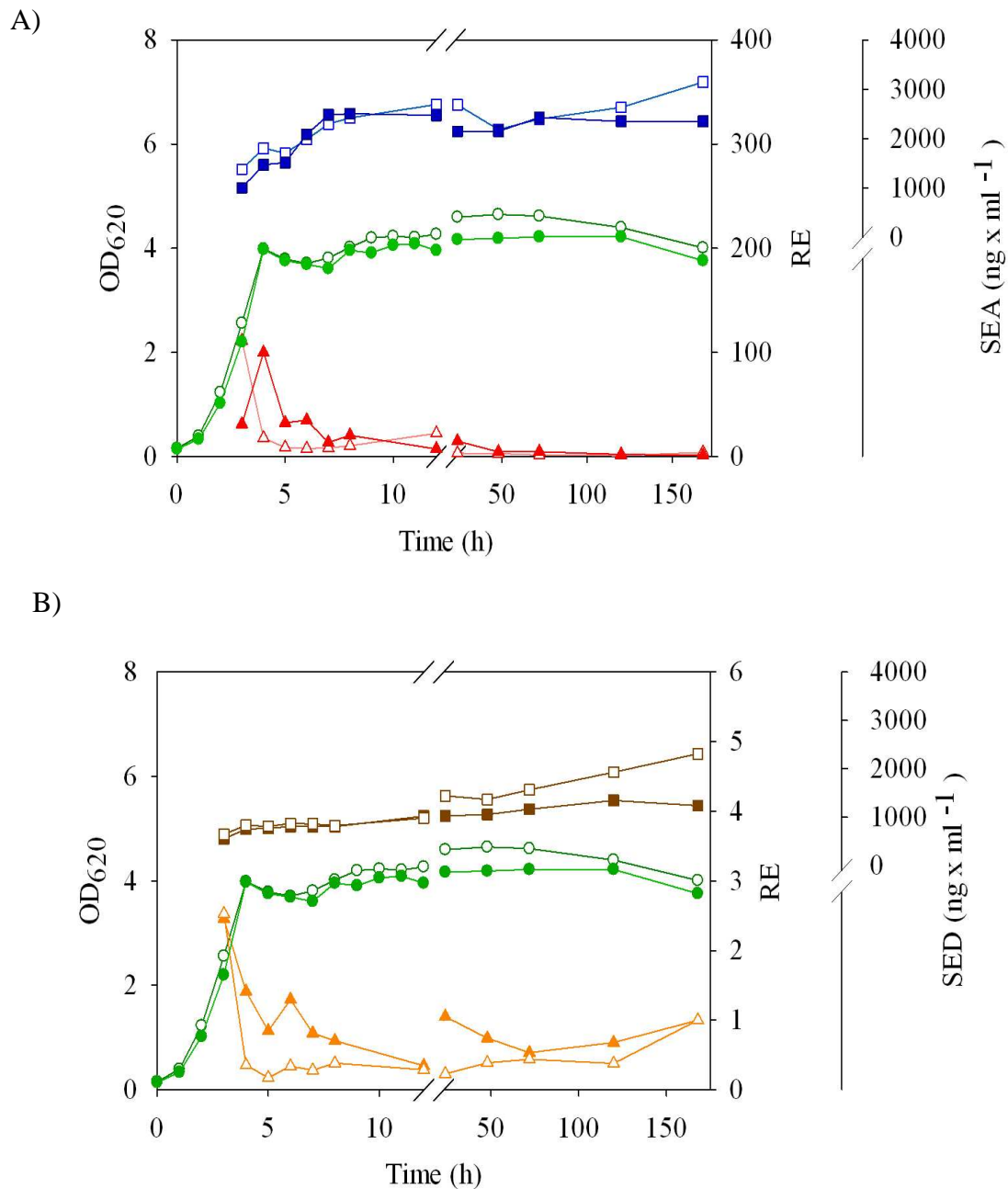
(—○—) Total cell count of inoculated meat, (●) *S. aureus* count on inoculated meat, (---○---) total cell count on uninoculated control meat

The pH of inoculated and uninoculated salami was the same, and varied between pH 4.2 and 4.8 during the incubation period as shown in Table 18. No *sea* and *sed* expression or SEA and SED could be detected. *S. aureus* was not able to survive on black pepper salami.

It could be because of the low pH, high fat content, low water activity, and the presence of competing microbiota of the product. Furthermore, black pepper has been noted to have antimicrobial properties (Dorman and Deans, 2000).

### 5.5.2. Cultivation and enterotoxin A and D formation during fermentation

For characterization of toxin production of *Staphylococcus aureus* SA45 toxin encoded by *sea* and *sed* genes fermentations were performed under favorable growth conditions. *S. aureus* 45 was cultivated in pH controlled batch cultures for 7 days at pH 7 and 37°C using BHI broth in parallel. *sea* and *sed* expression and formation were monitored using qRT-PCR and sandwich ELISA. The same RE calculation was applied as in meat experiments in chapter 5.5.1. The demonstration of the results in graphs was similar such as in case of graphs from the meat experiment. The independent experiments are represented as individual curves in Figs. 47 A and B instead of illustrate as a mean with standard deviation.



**Figure 47. A)** Growth, relative expression (RE) of *sea* and SEA production and **B)** growth, relative expression of *sed* and SED production of *S. aureus* SA45 cultivated at pH 7, 37°C.

(—●—) Growth determined by OD measurement at 620nm, (—▲—) RE of *sea*, (—■—) extracellular SEA concentration, (—▲—) RE of *sed*, (—■—) extracellular SED concentration. Values are from 2 independent biological replicates with full and empty symbols. The y- axis for SED concentration curves has been shifted for better separation from the growth and RE curves.

*S. aureus* SA45 reached the end of exponential growth phase after approximately four hours of growth (Figs. 47 A and B). Simultaneously the relative level of *sea* and *sed* mRNA decreased after the exponential growth phase and the *sea* mRNA level remained at low level as presented in Fig. 47 A. In case of *sed* mRNA increased expression was observed towards the end of incubation time

(Fig. 47 B). The second increase in *sed* expression was verified by increased SED concentration until the end of the experiment. The amount of extracellular SEA accumulated over time, with the greatest increase occurring in the exponential and early stationary growth phases shown in Fig. 47 A. The *sea* expression peaked in the transition phase from the exponential to stationary growth phase in broth cultures after 3–4 h of cultivations, linking expression to bacterial growth as well as it was shown in meat products. However, the time scale differed significantly between the two matrices. Two *sea* expression patterns were observed. In broth, the *sea* expression peaked in the late exponential phase, as established previously (Borst and Betley, 1993; Czop and Bergdoll, 1974) and then rapidly declined. The extracellular toxin amounts increased during early cultivation and equalized in the stationary growth phase which confirmed the *sea* expression observed. On boiled and smoked ham where rapid growths were observed, prolonged *sea* expression and SEA formation occurred throughout the incubation period. In boiled ham, the SEA levels increased during the first three days of incubation then suddenly decreased. In smoked ham, however, the extracellular SEA levels increased markedly towards the end of the incubation period where increased *sea* expression was also noted. The meat differs in many ways from broth, e.g. microbial content, nutrient availability, pH, salt content, and water activity that compose a more complex matrix than the broth (Valero et al., 2009). Additionally the bacteria are immobilized on meat in multicellular communities such as biofilm growth. Inside a biofilm anoxic or acidic zones could be created by the development of gradients in the inner of biofilm cell clusters (Beenken et al., 2004; Hall-Stoodley and Stoodley, 2009; Weinrick et al., 2004). These zones compose special environment which may cause cell stress. In *S. aureus* the acidic environment has been linked to oxidative stress (Clements et al., 1999) that could activate the SOS response system in staphylococcal cells. As a consequence, it would lead to a number of responses including prophage induction (Selva et al., 2009). The enterotoxin A gene is carried by prophage in the bacterial chromosome (Borst and Betley, 1994). Sumbly and Waldor (2003) have demonstrated that there is a linkage between prophage induction and increased transcription of *sea*. Increased expression was observed in case of *sed* near the end of the incubation. This second increase in expression in batch cultures, confirmed by increased SED concentrations, was not as pronounced as in the boiled and smoked hams. Boles and Horswill (2008) found that the Agr system is re-activated upon glucose depletion. This might partly explain the second increase in *sed* expression. Wright et al. (2005) also noticed a second increase in Agr activity in a mouse abscess model for *S. aureus* infection. The SED concentration increased over time throughout the experiments in ham and broth, except for in the boiled ham. This is in contrast with previously published results demonstrating maximal SED production during the post-exponential growth phase (Bronner et al., 2004; Derzelle et al., 2009).

## 6. NEW SCIENTIFIC RESULTS

### 1. Evaluation of selective and elective media for *Pseudomonas* isolates:

It has been proved that the combinations of *Pseudomonas* Agar F, *Pseudomonas* Agar P, GSP agar and Cetrimide agar were applicable for the detection and characterization of *Pseudomonas* species derived from meat spoilage when the incubation temperature was 30°C. *Chryseobacterium antarcticum*, however, could also grow on these media at 20°C. Fluorescence of the isolates at 30°C was less intensive than at 20°C that could be connected to the faster growth at 30°C and instability of pyoverdine in the stationary growth phase.

### 2. Molecular identification and typing *Pseudomonas* isolates:

The *Pseudomonas* genus-specific primer pair published by Purohit et al. (2003) had low specificity (66.6% ) and sensitivity (82.5%) as calculated by the application of *Pseudomonas* isolates, type and reference strains, therefore it could be concluded that these primers are not suitable for preliminary screening of *Pseudomonas* species belonging to this genus.

It has been concluded that the RAPD analysis was not applicable for the determination of high similarity clusters of *Pseudomonas* isolates as the consequence of high level of their molecular diversity. However it could be applied for typing of *Pseudomonas* isolates. The 16S rDNA-RFLP method was not suitable either for identification or typing of *Pseudomonas* species. The *rpoB*-RFLP was not useful for identification purposes either.

From the end of storage of pork meat at 4°C *P. fluorescens* and *P. fragi* species were identified by sequencing of 16S rDNA and *rpoB* gene and using species-specific primer pair while the initial microbiota of pork meat stored at 8°C contained not only these species but the *Chryseobacterium antarcticum* (F1445/3) as well. At the end of storage at 8°C only *P. fragi* became dominant.

### 3. Proteolytic and lipolytic enzymatic activities of *Pseudomonas* isolates:

The *Pseudomonas fluorescens* isolates showed quite intense proteolytic activity on SMC plates in 15, 20, 25 and 30°C except F1443/2a and F1443/7 which showed activity only at 15 and 20°C. However the proteolytic activity of all *P. fluorescens* isolates on SM plates was higher than on SMC agar. The esterase activity of these *P. fluorescens* isolates was also intense and higher than that of the *P. fragi* isolates in all incubation temperatures. However, the lipase activity of *P. fluorescens* isolates was lower compared to *P. fragi* isolates at 15 and 20°C. The majority of *P. fragi* isolates had both proteolytic and lipolytic activities.

#### **4. Spoilage potential of *Chryseobacterium antarcticum*:**

It was the first case that *Chryseobacterium antarcticum* was isolated from surface of chilled pork meat. The optimal growth range of *Chryseobacterium antarcticum* F1445/3 was between 15 and 25°C. This isolate had proteolytic activity at 15, 20, 25 and even at 30°C. It also produced lipase but esterase production was not detected. Measurement of the proteolytic and lipolytic activities of this strain showed that this bacterium could also contribute to the initial spoilage process.

The competition between *Chryseobacterium antarcticum* F1445/3 and *P. fragi* F1445/1b strains showed that when *Chryseobacterium antarcticum* was in higher cell ratio than the *P. fragi* F1445/1b it was able to grow and compete even after the *P. fragi* reached its maximal growth rate and started to decline. When *Chryseobacterium antarcticum* F1445/3 was in equal cell ratio with the *P. fragi* F1445/1b or *P. fragi* F1445/1b had higher cell ratio this competition between the two strains could not be demonstrated.

#### **5. Growth of *Staphylococcus aureus* combined with enterotoxin expression and formation:**

It was the first time that the *sea* and *sed* enterotoxin gene expression of *S. aureus* was detected in combination with enterotoxin production in food, especially on cooked ham, smoked ham, Serrano ham and black pepper salami. Extended *sea* and *sed* expression were observed in ham products as well as during fermentations, the pattern of these gene expressions and enterotoxin formations, however, were different. The *sea* expression was continuous under pH controlled circumstances, while the *sed* expression had second peak during the expression after 4 days. The amount of SEA toxin was higher than the SED during fermentation and both of them proved to be stable in the liquid culture medium. The ratio and the amount of SEA and SED also altered in different ham products but could be enough to potentially cause SFP. In smoked ham the amount of toxins remained stable while in cooked ham it decreased after 5 days. In black pepper salami there was no growth and enterotoxin production at all.



## 7. CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the cultivation results on selective and elective media (Cetrimide, GSP, *Pseudomonas* agar F and P) all of them were suitable for selection and pre-characterization of the *Pseudomonas* isolates from meat but cultivation at 30°C is more advisable than at 20°C. To receive broader view from pigment production of the isolates derived from meat *Pseudomonas* Agar F and P is advised. Further studies on pigment production and analysis of its function in the life cycle of the spoilage causing pseudomonads could also contribute to the deeper characterization of the isolates. The results of investigations at the beginning of this century already indicated that (Meyer, 2000). The fluorescent pigments pyoverdines are might be considered as potent taxonomic markers for the fluorescent species of the genus *Pseudomonas* (Meyer, 2000). It was also found in another investigation that different pigments have a function as signalling molecules controlling gene expression inside the bacterial cells (Lamont and Martin, 2003) as well as that in case of *P. aeruginosa* it was proved that the pyocyanin production has potential virulence function (Liu and Nizet, 2009).

It was found that the RAPD analysis could be applied for pre-selection purposes of the isolates from large number of candidates. It was demonstrated that sequencing is a suitable tool for identification purposes. It provides more precise identification because it analyses the series of nucleotides in the DNA while the PCR-RFLP method focuses only few restriction sites in the DNA. Nevertheless, for establishing an in-house bacterial databank the PCR-RFLP analysis provided a more cost effective solution until now. As a promising method the MLSA (multi-locus sequencing analysis) technique seems more reliable for detection of *Pseudomonas* species in the future based on the combination and application of *rpoB*, *gyrB* or *rpoD* genes at the same time (Mulet et al., 2010). The application of Rep-PCR (repetitive extragenic palindromic-PCR) is also an alternative method that focuses on repetitive chromosomal elements, which are randomly distributed in bacterial genomes and are the target of the PCR amplification. Based on the results gained with *Pseudomonas* specific primer pairs by Ercolini et al. (2007) the majority of the isolates mainly dominated by *Pseudomonas fragi* and sequencing of few isolates identified as *P. fragi* also supported the applicability of the species-specific primer. Designing further species-specific primer pairs e.g for *P. fluorescens* species could be a future perspective based on genes that belongs to the metabolic pathway e.g. enzyme production or pigment production. However this perspective brings more difficulties in case of *P. fluorescens* because it has 5 biovars as it was found in variation of ribotyping patterns in *P. fluorescens* (Palleroni et al., 1972).

According to the data regarding the lipolytic and proteolytic activities and physiological properties of *Chryseobacterium antarcticum* this bacterium could be the member of the spoilage causing biota

though it has only role at the beginning. Molecular characterization of species belonging to the young genus of *Chryseobacterium* could be a novel field in the identification and characterization of them. Some information is available in a molecular database from *gyrB* gene that encodes the subunit B protein of DNA gyrase but only for a few *Chryseobacterium* species.

The extended *sea* and *sed* expression observed in ham products has provided new information about SEA and SED production in food products where *S. aureus* is immobilized in multi-cellular communities. Furthermore, the *sed* gene expression pattern in ham products indicates a complex behaviour of the regulatory network controlling *sed* expression. These results may be used to enhance the assessment of SFP (staphylococcal food poisoning). However, more data is required on staphylococcal enterotoxin regulation and signal transduction to ensure the production of microbiologically safe food with high quality. Further investigations should therefore be carried out to study the effects of other intrinsic and extrinsic factors that influence the enterotoxin expression and then toxin production in *S. aureus* and growth in various food matrices. Microarray studies could be a help to understand the mechanism of the global gene regulation between these toxins and how the quorum sensing influences the toxin production on these product when other microbial community also contribute to the deterioration of the food.

## 8. SUMMARY

Meat consumption is one of the most ancient habit in human life. The structure of meat is a complex collection of proteins, essential amino acids, trace elements and vitamins that are important for human health. These excellent circumstances provide perfect media for the bacterial growth. Depending on the intrinsic and extrinsic factors belonging to the meat different microbes can adapt from the environment source of which could be the skin, hide, gastrointestinal tracts of the animal. The other source of contamination can also be the equipment used during slaughtering and the food handlers themselves. These microbes can grow on the meat but usually only one genus becomes dominant on the originally sterile meat. Under aerobic conditions and refrigerated circumstances *Pseudomonas* spp. are the most important spoilage organisms. At the beginning of storage between these conditions they present the minority of the total microbiota but the high water activity, neutral pH, suitable temperature and the good supply of oxygen favour their rapid growth and final dominance. The population of *Pseudomonas* species derived from 4°C at the end of storage and from 8°C from the beginning and at the end of aerobic storage of pork meat was identified with molecular methods during my work. Previously the applicability of different *Pseudomonas* specific elective, selective and differential media (*Pseudomonas* Agar F, *Pseudomonas* Agar P, GSP agar and Cetrimide agar) were tested at 20 and 30°C to characterize *Pseudomonas* species which were derived from meat spoilage. All the isolates were able to grow on these media but differences in fluorescence ability and pigment production were altered at the different incubation temperatures. On BHI agar and *Pseudomonas* Agar F a dark brown pigment formation was observed on both incubation temperatures that diffused into the medium. This pigment production was not induced by any selective agent derived from the media in case of *Pseudomonas* Agar F. This ability belonged to three *P. fragi* (F1443/12b, F1443/13a and b) and one *P. fluorescens* (F1445/4) species while other *P. fragi* isolates did not possess this ability. That also showed the heterogeneity of the spoilage causing population. Based on the results the pigment production and fluorescence ability is influenced by the temperature. The combination of these media was suitable for detection and characterization of the *Pseudomonas* isolates at 30°C. At 20°C the *Chryseobacterium antarcticum* isolate (F1445/3) was also able to grow. This microbe could be the part of spoilage at the beginning of storage as well.

Altogether 27 isolates were tested according to their lipolytic and proteolytic activities on SM, SMC, PCATB and PAT-80 agar plates at 15, 20, 25 and 30°C. The *Pseudomonas fluorescens* isolates show quite intense proteolytic activity on SMC plates in all incubation temperatures except F1443/2a and F1443/7 isolates the activity of which was detected only at 15 and 20°C. On SM plates the proteolytic activity of *P. fluorescens* isolates namely F1443/7, F1443/11, F1445/4,

F1445/6 and F1445/8a was higher based on the diameter of clearing zones and could be detected in all incubation temperature. The same results could be also established in case of esterase activity with the same isolates. Lipase activity of these isolates which were incubated at 15 and 20°C was lower compared to the results of *P. fragi* isolates. The majority of the *P. fragi* isolates were able to produce esterase and lipase as well as protease at 15, 20, 25 and 30°C. Most of the cases *Pseudomonas fluorescens* isolates had higher proteolytic and lipolytic activities than *P. fragi* isolates. Further analysis on the enzyme production could be advisable on molecular way by designing proteolytic and lipolytic enzyme specific primers.

Molecular methods represent a powerful tool for identifying and typing microbes. Testing the applicability of a genus-specific primer pair for meat spoilage causing pseudomonads was also performed. The sensitivity of the method was 82.5% but the selectivity was only 66.6%. According to the results this primer pair was not suitable for selection of the member of the genus *Pseudomonas* from other spoilage causing microbes.

For molecular typing of *Pseudomonas* isolates the RAPD analysis was used which was suitable for this purpose. For molecular identification the analysis with 16S rDNA-RFLP and *rpoB*-RFLP were applied. The ARDRA method was not applicable either for identification or typing of *Pseudomonas* species. The *rpoB*-RFLP was not applicable for identification at species level. Molecular identification with *P. fragi* and *P. lundensis* specific primer pairs were performed as well. Altogether 14 *P. fragi* was identified from 4°C and 11 *P. fragi* from 8°C. None of the isolates was *P. lundensis*. Further identification was performed by sequencing 16S rDNA and *rpoB* genes. Based on the results 4-4 *P. fluorescens* isolates were identified which derived from 4 and 8°C and only one isolate was identified as *Chryseobacterium antarcticum* F1445/3 according to the 16S rDNA sequence analysis. That was the first time this species was found on pork meat. This isolate composed the initial microflora of meat at 8°C and was pre-selected together with *P. fluorescens* species during the storage. At the end of storage at 8°C only *P. fragi* species became dominant. At 4°C mainly *P. fragi* species dominated but *P. fluorescens* also contributed to the microflora at the end of spoilage.

To characterize the temperature ranges of growth of *Chryseobacterium antarcticum* (F1445/3) liquid cultivations were performed parallel with the type strain. The optimal growth range was between 15 and 25°C for the isolate. Lipolytic and proteolytic activities were also tested by the semi-quantitative methods at 15, 20, 25 and 30°C. Protease and lipase activities were determined but esterase activity was not found. The development of pigment production was also detected. As the colonies became older the intensity of their colour changed from light to dark orange. Competition studies between a *P. fragi* F1445/1b and *Chryseobacterium antarcticum* F1445/3 were also performed in liquid culture with different cell ratios. When they had equal cell ratio or *P. fragi*

F1445/1b possessed higher cell number the *Chryseobacterium antarcticum* F1445/3 was not able to compete. However, if higher cell ratio of *Chryseobacterium antarcticum* F1445/3 was tested than *P. fragi* F1445/1b it was able to grow and compete with *P. fragi* F1445/1b. Further studies on *Chryseobacterium* species is advised especially on molecular way because its genetic map is lack of information. The reason is the genus *Chryseobacterium* was built on the ruins of the genus *Flavobacterium* in the middle of '90s and information of their spoilage causing ability was mainly derived from weakly identified *Flavobacterium* species before the middle of '90s.

The foodborne pathogens cause serious problems not just for the food industry but also represent a risk factor for human health which emphasises the importance of food safety. The landscape of foodborne pathogens is changing continuously. New pathogens appear while already existing ones possess new characteristics. To prevent foodborne outbreaks it is essential to know the factors that induce their ability to adapt much more efficiently to the environment. *Staphylococcus aureus* is one of these foodborne pathogens that get more attention nowadays. It is responsible for causing SFP by its heat-stable enterotoxins which behave also as superantigens. Until now 22 staphylococcal enterotoxin or enterotoxin like proteins are known but SEA and SED are the enterotoxins that are most frequently reported from the outbreaks. SEA (staphylococcal enterotoxin A) is encoded by the *sea* gene the expression of which is linked to the life cycle of SEA-encoded prophage. SED is a plasmid-encoded protein that is connected to *sed* determinant and up-regulated by the accessory gene regulator (Agr) system. During my work the gene expression of *sea* and *sed* and enterotoxin formation of SEA and SED were determined on cooked ham, smoked ham, Serrano ham and black pepper salami as well as in liquid culture by qRT-PCR and ELISA. That was the first time that these characteristics were studied parallel with food matrices, especially on processed meat products. The *sea* and *sed* expression and toxin production were altered in these food sources and in liquid cultures. On smoked and boiled ham active *sea* and *sed* expression occurred throughout the incubation time of seven days. Lower level of *sea* mRNA and SEA were found in smoked ham compared to boiled ham though the viable counts of *S. aureus* on these products were similar. In case of *sed* on these products continuous *sed* expression was observed throughout the incubation period with second increase in *sed* expression found after five days of incubation. The ratio and the amount of SEA and SED production were also different on these products but could be enough to cause SFP. In smoked ham the amount of toxins remained stable while on boiled ham SED decreased after 3 days of incubation and SEA decreased after 5 days. On Serrano ham no increase in cell number was observed until the end of the experiment and *sea* expression and SEA, SED could only be detected on day five and seven. The *sed* expression was too low to determine. However, the amount of toxins could also be enough for SFP. The *sed* expression pattern which

was observed in ham products indicates a complex behaviour of the regulatory network controlling *sed* expression.

On black pepper salami there was neither growth of *S. aureus* nor enterotoxin production due to the lactic acid bacteria content and low pH as well as antimicrobial effect of black pepper. Fermentations under pH controlled circumstances also showed differences in *sea* and *sed* expression. The *sed* expression had second peak in expression after 4 days while *sea* expression was continuous. The toxin amount of SEA was higher than SED during fermentation but both of them remained stable in the liquid culture. Based on these results it is important to know in the future how the microbiological community influences the gene expression and toxin production and how the food composition, packaging gas, preservatives, temperature effect the up- and down regulation of enterotoxin expression in *S. aureus* growing in varying food matrices. Application of microarray technique could help in understanding the global regulation and expression of toxins.

## 9. ÖSSZEFOGLALÁS

A húsfogyasztás az egyik legősibb szokás az emberiség életében. A hús, szerkezetéből eredően a fehérjék, esszenciális aminosavak, nyomelemek, illetve vitaminok komplex gyűjteményét alkotja, amelyek fontos szerepet töltenek be egészségünk megőrzése szempontjából. Ezek a kiváló körülmények tökéletes tápközeget biztosítanak a baktériumok növekedésének is. A húson különböző, a környezetből származó mikrobák képesek megtelepedni, attól függően, hogy a húshoz kötődően milyen külső és belső tényezők befolyásolják azt. Ezek a baktériumok az állati bőrről, irhából, és a béltraktusból származhatnak. További szennyezési forrásként pedig azok az eszközök is szerepelhetnek, amelyeket az állat levágásánál használnak, illetve az élelmiszer feldolgozásában részt vevő személyek is a kontaminációs lehetőségek közt vannak. Ezen forrásokból származó mikrobák bár képesek szaporodni a húson, azonban általában csupán egyetlen egy nemzetség válik dominánssá közülük az eredetileg steril hús felületén. Aerob módon és hűtött körülmények között a *Pseudomonas* fajok számítnak a legjelentősebb romlást okozó élőlényeknek. A tárolás elején az említett körülmények között a teljes mikroflórának csak kis hányadát alkotják, azonban a magas vízaktivitás, a semleges pH, a megfelelő hőmérséklet és a jó oxigén ellátás kedvezően hatnak a gyors növekedésükre és végső dominanciájukra. Munkám során a sertéshús aerob tárolásának végéről 4°C-ról, illetve a 8°C-os tárolás elejéről és végéről származó *Pseudomonas* fajok kerültek azonosításra molekuláris módszerek segítségével. Ezt megelőzően különböző *Pseudomonas* specifikus elektív, szelektív és differenciáló táptalajok (név szerint *Pseudomonas* Agar F, *Pseudomonas* Agar P, GSP agar és Cetrimid agar) alkalmazhatóságát vizsgáltam 20 és 30°C-on. Mindegyik izolátum képes volt növekedni ezeken a táptalajokon, azonban a különböző inkubációs hőmérsékleteken a fluoreszkáló képesség, illetve a pigment termelés tekintetében eltérések voltak felfedezhetők. BHI agar és *Pseudomonas* Agar F esetében sötétbarna pigmentképződés volt megfigyelhető, amely bediffundált a táptalajba az említett mindkét inkubációs hőmérsékleten. Ez a színeképzés semmilyen szelektív hatóanyaghoz köthető indukciónak nem volt tulajdonítható a *Pseudomonas* Agar F tápközegen. Ez a színeképző képesség három *P. fragi* (F1443/12b, F1443/13a and b) és egy *P. fluorescens* (F1445/4) izolátum esetében volt megfigyelhető, míg a többi *P. fragi* izolátum nem rendelkezett ezzel a tulajdonsággal, amely a romlást okozó populáció heterogenitását mutatja. Ezen tápközégek kombinációja 30°C-on alkalmasnak bizonyult a *Pseudomonas* izolátumok detektálására és jellemzésére. A 20°C-os inkubációs hőmérsékleten a *Chryseobacterium antarcticum* (F1445/3) izolátum, szintén képes volt növekedni, amely szintén közrejátszik a romlásban a tárolás elején.

Az izolátumok lipolitikus és proteolitikus aktivitásának meghatározás során összesen 27 izolátumot vizsgáltam SM, SMC, PCATB és PAT-80 agar lemezekon, amelyeket 15, 20, 25 és 30°C-on

inkubáltam. A *P. fluorescens* izolátumok meglehetősen intenzív proteolitikus aktivitást mutattak az SMC lemezekben az összes inkubációs hőmérsékletet beleértve, kivéve az F1443/2a és az F1443/7 izolátumokat, amelyek aktivitása csupán 15 és 20°C-on volt mérhető. Az SM lemezekben a *P. fluorescens* izolátumok proteolitikus aktivitása, név szerint az F1443/7, F1443/11, F1445/4, F1445/6 és F1445/8a magasabb volt a feltisztulási zóna átmérőjét tekintve, illetve ez az aktivitás mindegyik hőmérsékleten észlelhető volt. Ugyanez az eredmény állapítható meg az észteráz aktivitás esetében is szintén ezekre az izolátumokra vonatkozóan. Ezeknek az izolátumoknak a lipáz aktivitása 15 és 20°C-on alacsonyabb volt, mint a *P. fragi* izolátumoknak. A *P. fragi* izolátumok többsége termelt észteráz, lipáz, illetve proteáz enzimet 15, 20, 25 és 30°C-on is. A legtöbb esetben a *P. fluorescens* izolátumok nagyobb proteolitikus és lipolitikus aktivitással rendelkeztek, mint a *P. fragi* izolátumok. További vizsgálatként a *Pseudomonas* izolátumok enzimtermelésének molekuláris vizsgálatát javasolnám, proteolitikus és lipolitikus enzimekre specifikus primerek segítségével.

A molekuláris módszerek óriási jelentőséggel bírnak a mikrobák meghatározásával és tipizálásával kapcsolatban. Munkám során egy *Pseudomonas* nemzetség-specifikus primer pár alkalmazhatóságának tesztelését végeztem el a romlást okozó *Pseudomonas* izolátumokra. A módszer érzékenysége tekintve 82,5% volt, míg a szelektivitása csupán 66,6% -nak bizonyult. Az eredmények alapján elmondható, hogy ez a primer pár nem alkalmazható a *Pseudomonas* nemzetség tagjainak szelekciójára az egyéb romlást okozó baktériumok közül.

A *Pseudomonas* izolátumok molekuláris tipizálásához a RAPD módszert alkalmaztam, amely tipizálási célra megfelelőnek bizonyult. Molekuláris identifikálás során a 16S rDNS-RFLP és *rpoB*-RFLP analízis alkalmaztam. Az ARDRA módszer sem identifikálás sem pedig tipizálás céljából nem volt megfelelő a *Pseudomonas* fajokra nézve. Az *rpoB*-RFLP módszer sem bizonyult alkalmasnak az izolátumok faji szintű meghatározásra. A további molekuláris meghatározást faj specifikus primer párok segítségével végeztem, amelyek a *P. fragi* és *P. lundensis* fajokra voltak specifikusak. Ezen primer párok segítségével összesen 14 *P. fragi* izolátumot sikerült meghatározni a 4°C-ról származó izolátumok közül, míg a 8°C-ról származó izolátumok esetében összesen 11 *P. fragi* izolátumot identifikáltam. Az izolátum közül egyik sem volt *P. lundensis*. A további identifikálás a 16S rDNS és az *rpoB* gén szekvenálásával történt. Az eredmények alapján 4-4 *P. fluorescens* izolátumot sikerült meghatároznom, amely 4 és 8°C-ról származott. Csupán egy izolátumot, név szerint F1445/3-ot identifikáltam *Chryseobacterium antarcticum*-ként a 16S rDNS szekvencia analízis alapján. Első alkalommal identifikáltam ezt a baktériumot a disznóhús felületéről, amely a 8°C-on tárolt hús kezdeti mikroflóráját alkotta. A tárolás során viszont a *P. fluorescens* fajokkal együtt a szelekció révén a továbbiakban nem volt felfedezhető a tárolás későbbi szakaszában. A 8°C-os tárolás végén a *P. fragi* törzsek váltak dominánssá, míg 4°C-on



főképp a *P. fragi* fajok érvényesültek, azonban a *P. fluorescens* fajok a romlás végén 4°C-on szintén a mikrobióta részét képezték. A *Chryseobacterium antarcticum* (F1445/3) növekedéséhez szükséges hőmérsékleti tartomány meghatározásához folyadék tenyészeteket alkalmaztam a típus törzzsel párhuzamosan. Az optimális növekedési tartományt 15 és 25°C között állapítottam meg. A *Chryseobacterium antarcticum* F1445/3 és típustörzs lipolitikus és proteolitikus aktivitását szintén vizsgáltam fél-kvantitatív módszerek segítségével 15, 20, 25 és 30°C-on, melynek eredményeként proteáz és lipáz aktivitás kimutatható volt, míg ezzel szemben az észteráz aktivitás nem. A pigment képződés folyamatát is megfigyeltem. A telepek szín intenzitása világos narancssárga színből sötét narancssárga színűvé változott, amint a telepek öregebbé váltak. A *P. fragi* F1445/1b és *Chryseobacterium antarcticum* F1445/3 közötti kompetíció vizsgálata folyadék tenyészetekben szintén kutatásom tárgyát képezte. A kompetíció vizsgálata során különböző sejt arányokat alkalmaztam a két izolátum között. Amikor egyenlő arányban, vagy pedig a *P. fragi* F1445/1b izolátum nagyobb sejtszámban volt jelen a folyadék tenyészetben a *Chryseobacterium antarcticum* F1445/3 izolátum nem tudott versenyezni a *P. fragi* F1445/1b izolátummal. Ezzel szemben, amikor a *Chryseobacterium antarcticum* volt nagyobb sejt arányban jelen, mint a *P. fragi*, akkor képes volt a növekedésre, illetve a *P. fragi*-val történő kompetícióra. További kutatási lehetőségként *Chryseobacterium* fajok további jellemzését javasolnám molekuláris módszerek segítségével, mivel ezen fajok genetikai térképe hiányos. Ennek oka, hogy a *Chryseobacterium* nemzetség a *Flavobacterium* nemzetség romjaira épült a '90-es évek közepén és a romlást okozó képességeikről szóló információk főképp azon *Flavobacterium* fajokról származnak, amelyeket még a '90-es évek közepét megelőzően gyengén vagy alig identifikáltak.

Az ételmérgezést okozó patogének súlyos problémákat okoznak nemcsak az élelmiszeripar számára, hanem kockázati tényezőt jelentenek az egészség megőrzése szempontjából is, amely az élelmiszerbiztonság jelentőségét hangsúlyozza. Az ételmérgezést okozó patogének palettája folyamatosan változik. Új patogének bukkannak elő, míg a már meglévők új tulajdonságokkal vértetik fel magukat. Az ételmérgezések megelőzése érdekében alapvető azon tényezők ismerete, amelyek előidézik a patogének környezethez való egyre hatékonyabb adaptációját. A *Staphylococcus aureus* egyike ezen ételmérgezést okozó patogéneknek, amely egyre több figyelmet kap napjainkban. Eddig 22 sztafilokokkusz enterotoxint (SE), vagy ahhoz hasonló fehérjét ismerünk, amelyek közül a SEA és a SED enterotoxinok azok, amelyekről leggyakrabban beszámolnak az ételmérgezések során. A SEA (sztafilokokkusz enterotoxin A) fehérjét a *sea* gén kódolja, amely egy profágon található, a gén kifejeződése pedig a profág életciklusához kötött a baktériumon belül. A SED (sztafilokokkusz enterotoxin D) a plazmidon hordozott *sed* gén által kódolt fehérje, és e gén szabályozását az úgynevezett "kiegészítő szabályozó rendszer" (Agr rendszer) végzi. Munkám során a *sea* és *sed* gének kifejeződésének és a SEA, illetve SED

enterotoxinok képzésének meghatározását végeztem el főtt és füstölt sonkán, Serrano sonkán és fekete borsos szalámin, illetve folyadék tenyészetben kvantitatív reverz- transzkripció polimeráz láncreakció (qRT-PCR) és szilárd fázisú, enzimmel jelzett immunológiai módszer (ELISA) segítségével. Ez volt az első alkalom, hogy ezen tulajdonságok egymással párhuzamban képezték vizsgálat tárgyát élelmiszer mátrixban, különösképp feldolgozott hústermékekben. A *sea* és *sed* kifejeződése és SEA és SED toxinok termelése eltérést mutatott ezekben az élelmiszerekben, illetve a folyadék tenyészetekben is. A füstölt és főtt sonka esetében aktív *sea* és *sed* expresszió volt megfigyelhető a hét napos inkubációs idő alatt. Alacsonyabb szintű *sea* mRNS szintet és SEA mennyiséget detektáltam a füstölt sonka esetében, ha az eredményeket összevettem a főtt sonkánál tapasztaltakkal, annak ellenére, hogy a *S. aureus* élő sejtszáma a két terméken megegyezett. Ezen termékek esetében az inkubációs idő alatt folyamatos *sed* kifejeződés volt megfigyelhető, illetve az inkubációs idő ötödik napját követően egy másodszori növekedés volt tapasztalható a *sed* expressziójában. Bár szintén voltak eltérések a termelt SEA és SED mennyisége és aránya között ezeken a termékeken, mégis elegendő mennyiségűnek bizonyultak ahhoz, hogy esetlegesen a sztafilokokkusz által okozott ételmérgezést idézzenek elő. A füstölt sonkában jelen lévő toxin mennyisége állandónak bizonyult, míg a főtt sonkán a SED mennyisége 3 napos inkubálás után, a SEA mennyisége pedig 5 nap után kezdett el csökkenni. A Serrano sonkán nem volt tapasztalható sejtszámban való növekedés egészen a kísérlet végéig, míg a *sea* expressziója, illetve a termelt SEA és SED csupán az ötödik, illetve a hetedik napon volt detektálható. A *sed* gén kifejeződése túl alacsonynak bizonyult annak meghatározáshoz. Azonban a Serrano sonkán termelt toxin mennyisége szintén elegendő lehetne egy esetleges sztafilokokkusz által okozott ételmérgezés kiváltásához. A sonka termékeken megfigyelt *sed* gén expressziós mintázata, a *sed* expresszióját kontrolláló szabályozó hálózat összetett szerepét és viselkedését tükrözi.

A fekete borsos szalámin sem *S. aureus* növekedést, sem pedig enterotoxin termelést nem tapasztaltam a tejsavbaktériumok jelenléte, az alacsony pH, illetve a fekete bors antimikrobás hatása révén. A pH szabályzott körülmények között végzett fermentációk során szintén eltérések voltak a *sea*, illetve a *sed* gének kifejeződésében. A *sed* expressziója során a 4. napot követően egy második expressziós csúcs volt tapasztalható, míg a *sea* expressziója folyamatosnak bizonyult. A fermentációk során a termelt SEA toxin mennyisége magasabb volt, mint a SED, azonban mindkettő stabil maradt a folyadék tenyészetben. Az eredmények alapján fontos lenne tudni a jövőben, hogy a mikrobiológiai közösség miként befolyásolja a génexpressziót és a toxintermelést, illetve milyen hatása van az élelmiszer összetételének, a csomagolás gázösszetételének, a tartósítószernek, a hőmérsékletnek a *S. aureus* növekedésnél az enterotoxin expresszió alul és felülszabályozására az eltérő élelmiszer mátrixokban. Erre vonatkozóan az úgynevezett 'microarray' technika alkalmazása segíthet a toxinok globális szabályozásának és expressziójának megértésében.

## 10. APPENDIX

### A1. References

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## A2. Appendix (Table)

**Table 13.** Characterization of isolates morphologically and results of biochemical tests

Original code	Code after segregation	Colonial morphology	Microscopical morphology	KOH test	Catalase test	Oxidase test	Gram stain	Spore staining
F 1443/1	F 1443/1	ochre yellow plane	middle rods	+	+	+	Gram -	-
F 1443/2*	F 1443/2a	middle ochre yellow plane	longish rods	+	+	+	Gram -	-
	F 1443/2b	ochre yellow plane	long rods	+	+	+	Gram -	-
F 1443/4*	F 1443/4 a	big ochre yellow plane	rods	+	+	+	Gram -	-
	F 1443/4 b	big ochre yellow plane	very small rods	+	+	+	Gram -	-
	F 1443/4c	big ochre yellow plane	very small rods	+	+	+	Gram -	-
F 1443/5	F 1443/5	middle ochre yellow	middle rods	+	+	+	Gram -	-
F 1443/7	F 1443/7	ochre yellow plane	small consistent rods	+	+	+	Gram -	-
F 1443/8	F 1443/8	middle ochre yellow	small coccoid rods	+	+	+	Gram -	-
F 1443/9	F 1443/9	ochre yellow plane	small rods	+	+	+	Gram -	-
F 1443/10*	F 1443/10b	shiny ochre yellow	very small rods	+	+	+	Gram -	-
	F 1443/10c	shiny ochre yellow	middle-size rods	+	+	+	Gram -	-
F 1443/11	F 1443/11	ochre yellow	very small rods	+	+	+	Gram -	-
F 1443/12*	F 1443/12a	middle ochre yellow	small long rods	+	+	+	Gram -	-
	F 1443/12b	ochre yellow	very small rods	+	+	+	Gram -	-
	F 1443/12c	ochre yellow	small rods	+	+	+	Gram -	-
F 1443/13*	F 1443/13a	small ochre yellow	very small rods	+	+	+	Gram -	-
	F 1443/13b	ochre yellow	small rods	+	+	+	Gram -	-
F 1445/1 *	F 1445/1a	ochre yellow plane	longish rods	+	+	+	Gram -	-
	F 1445/1b	ochre yellow plane	small rods	+	+	+	Gram -	-
F 1445/3	F 1445/3	orange creamy consistence	very small rods	+	+	-	Gram -	-
F 1445/4	F 1445/4	small white	middle longish rods	+	+	+	Gram -	-
F 1445/5	F 1445/5	ochre yellow plane	very small rods	+	+	+	Gram -	-
F 1445/6	F 1445/6	ochre yellow plane	longish rods	+	+	+	Gram -	-
F 1445/8*	F 1445/8a	ochre yellow plane	small rods	+	+	+	Gram -	-
	F 1445/8b	ochre yellow plane	small rods	+	+	+	Gram -	-
F 1445/9*	F 1445/9a	ochre yellow plane	small long rods	+	+	+	Gram -	-
	F 1445/9b	ochre yellow plane	small rods	+	+	-	Gram -	-
F 1445/10	F 1445/10	ochre yellow plane	very-very long rods	+	+	+	Gram -	-
F 1445/12	F 1445/12	small creamy colour	small sphere rods	+	+	+	Gram -	-
F 1445/14*	F 1445/14a	creamy colour	very small rods	+	+	+	Gram -	-
	F 1445/14b	big ochre yellow plane	very small rods	+	+	+	Gram -	-
F 1445/15*	F 1445/15a	big ochre yellow plane	small longish rods	+	+	+	Gram -	-
	F 1445/15b	big ochre yellow plane	small short rods	+	+	+	Gram -	-

\* Heterogeneity was determined in the morphology

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*“ Alattad a föld, fölötted az ég, benned a létra.”*

Weöres Sándor  
(1913-1989)

*“ The ground is under you while the sky is above but the  
ladder is within you.”*

Sándor Weöres  
(1913-1989)