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Spoilage-associated psychrotrophic and psychrophilic microbiota on modified atmosphere packaged beef

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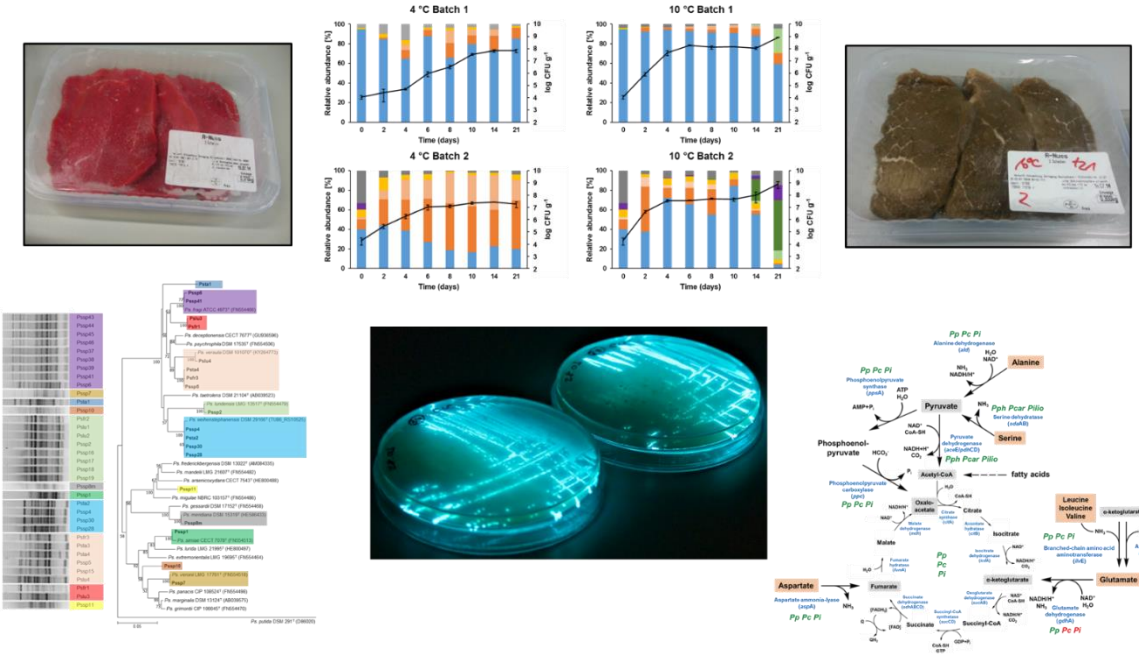

'Everything is everywhere, but, *the environment selects*'

- Lourens Gerhard Marinus Baas Becking,
inspired by Martinus Willem Beijerinck

Doctoral thesis

Freising, 2018

- This thesis is dedicated to my beloved parents -

Abbreviations

°C	degree Celsius (centrigrade)
µ	micro (10^{-6})
A	ampere
ANI	average nucleotide identity
a_w	water activity
B.	*Brochothrix*
BHI	brain-heart infusion medium
BLAST	basic local alignment search tool
bp	base pairs
C.	*Carnobacterium*
CFC	cephalothin-fucidin-cetrimide medium
CFU	colony forming units
contig	contiguous consensus DNA sequence
Da	dalton
diH_2O	deionized water
DNA	desoxyribonucleic acid
dNTP	desoxy nucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	ethylenediaminetetraacetic acid
g	gram
H.	*Hafnia*
h	hour
HCA	hierarchical cluster analysis
L	liter
LAB	lactic acid bacteria
LB	lysogenic broth
Lc.	*Lactococcus*
Le.	*Leuconostoc*
M	molar (mol/L)
m	milli (10^{-3}), meter
M	molarity
MALDI-TOF MS	matrix-assisted laser desorption-ionisation time of flight mass spectrometry
MAP	modified atmosphere packaged
Mb	mega base pairs
MB	marine broth
MEGA	Molecular Evolutionary Genetics Analysis
min	minute (')
MSL	minimum shelf life
MSM	meat simulation medium
MSP	mass spectrometry profile
n	nano (10^{-9})
NCBI	National Center for Biotechnology Information
nri	not reliable identified
nt	nucleotides
OD_{590}	optical density at 590 nm wavelength
P.	*Photobacterium*
p	probability value
PB	*Photobacterium* broth
PCR	polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
pH	negative decimal logarithm of hydrogen ion activity
ppbv	parts per billion by volume
Ps.	*Pseudomonas*

PTR-MS	proton transfer reaction mass spectrometry
RAPD	randomly amplified polymorphic DNA
RAST	Rapid Annotations using Subsystems Technology
s	second (")
S.	*Serratia*
SE	standard error
SMRT	single molecule real time
SSO	specific spoilage organism(s)
STAA	streptomycin-thallous acetate-actidione agar
Taq	*Thermus aquaticus*
TBE	Tris-HCl, boric acid, EDTA (buffer)
TCA cycle	tricarboxylic acid cycle
TCBS	thiosulfate citrate bile salts sucrose medium
TMW	Technische Mikrobiologie Weihenstephan
TSA	tryptic soy agar
TVC	total viable count
V	volt
v/v	volume per volume
VP	vacuum packaged
W	watt
w/v	weight per volume
w/w	weight per weight
WGS	whole genome shotgun

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1 Introduction

1.1 Background and economic relevance

Global meat consumption and industry has increased substantially with over 300 million tons of meat supply annually, and the per capita consumption has doubled in the last decades, especially in developing countries as part of the “livestock revolution” (Delgado 2003; Hansen 2018). The FAO has further reported that a massive amount of the produced meat (20%) is wasted globally along the production, distribution and sales (Gustavsson *et al.* 2011).

The meat industry is a highly significant and economically relevant part of the food industry especially in Germany. The annual revenue for slaughter and meat processing amounts to 36 billions euro in 2016 (BVDF 2016). The per capita consumption of meat and meat products is about 60 kg per year in Germany (BLE 2015; Fleischer-Verband 2017). The German federal ministry of Food and Agriculture has reported that a substantial amount (7.1%) of spoiled meat and meat products is discarded due to its perishability and contribute to 6.3% to all avoidable waste (Kranert *et al.* 2012; Chemnitz 2014). This means that 350.000 tons of meat end up as avoidable waste annually in Germany and this represents a commercial value of billions of euro. This also implies that the meat of millions of animals will not be consumed and that they are therefore unnecessarily slaughtered.

The shelf life of foods, including meats, is indicated by the manufacturer either by a minimum shelf life (MSL, also stated as “best before” date) or a use-by date as required by the EU parliament regulations (European Parliament 2002; 2011). Specifically, the minimum shelf life refers to the date until maintenance of specific organoleptics are guaranteed by the manufacturer, whereas the use-by date indicates the date after which the food is putatively unsafe to consum. A big issue arises from the erroneous assignment of the minimum shelf life or use-by date. If the date is assigned too long, retailers and customers will store the meat for longer time in shelves or at home, respectively, and will discard it when visibly spoiled. If the shelf life is assigned too short, retailers and customers will discard the package once the MSL or use-by date is reached while it is still edible. It is highly important for the meat industry to identify key spoilage organisms and understand their spoilage mechanisms in order to establish a rationale for determining the shelf life more precisely and for controlling of the contaminants, and thereby, minimizing the amount of avoidable waste and financial loss.

1.2 Modified atmosphere packaging

A recent ongoing and further growing trend of customers is the purchase of packaged meat in retail rather than buying unpackaged meat at the butchers. The demanded huge availability of diverse fresh meats and meat products in retail requires elaborated logistics and storage conditions since fresh meat is a highly perishable product, which has to meet high customer

demands towards hygienic safety, color, organoleptics and sensorics. In order to extend the shelf life and to limit the growth of pathogens and spoilers, modified atmosphere packaging (MAP) is widely employed by food manufacturers (Farber 1991; Church 1994; McMillin *et al.* 1999). MAP can be defined as the replacement of the atmosphere surrounding the product before sealing in vapor-barrier materials in oder to maintain a higher quality of perishable foods (Young *et al.* 1988; McMillin *et al.* 1999).

Red meats, e.g. beef and beef products are packaged under high oxygen O_2/CO_2 MAP (>70%/>20%) in order to retain oxymyoglobin levels responsible for the desirable bright red color (Young *et al.* 1988; Church and Parsons 1995; Phillips 1996; Mancini and Hunt 2005; McMillin 2008). In e.g. Norway, addition of approx. 0.5-1% carbon monoxide to the modified atmosphere is widely used by manufacturers due to its color stabilizing and antibacterial properties (Luño *et al.* 1998; Sørheim *et al.* 1999; Hunt *et al.* 2004; Djenane and Roncalés 2018). However, in the USA and EU, addition of carbon monoxide is prohibited (Cornforth 1994; Directive 1995; Van Rooyen *et al.* 2017). White meats e.g. poultry are usually packaged under N_2/CO_2 (70%/30%) modified atmospheres (Sante *et al.* 1994; Dangel 2006), although it is reported that many German producers also use high oxygen atmospheres (unpublished data IQ-Freshlabel project, (Rossaint *et al.* 2014)). Another widely used packaging method to enhance shelf life is vacuum skin packaging (VP, (Seideman and Durland 1983; Farber 1991; Borch *et al.* 1996)). However, the dark-purplish color of beef packaged under vacuum caused by deoxymyoglobin formation is not accepted by all consumers (McMillin 2008; Ščetar *et al.* 2010) and residual oxygen levels higher than 0.15% trapped within the product can even cause browning via metmyoglobin formation (Mancini and Hunt 2005). The absence of oxygen however can favor strict anaerobes (Church and Parsons 1995). Furthermore, some products e.g. minced beef cannot be packaged under vacuum due to their structural properties.

1.3 Ecosystem of cold stored MAP beef

Beef is an ecological niche (Labadie 1999) and represents a substrate-rich environment with high water activity (0.99) and a moderate pH (5.5±0.3) that supports bacterial growth leading to spoilage of the product (Gill 1983; Lambert *et al.* 1991; Lawrie and Ledward 2006; Eskin and Shahidi 2012). Meat spoilage can be defined as deterioration towards rejectable organoleptic properties e.g. discoloration, off-odor, slime and excessive exudation (Gill 1983; Borch *et al.* 1996; Gram *et al.* 2002; Mancini and Hunt 2005), and is influenced by various intrinsic and extrinsic factors (Rahman 1999). The moderate pH is caused via post-mortem glycolysis to lactate by endogenous meat enzymes, whereas reduced water holding activity results from reaching the isoelectric point of major meat proteins e.g. myosin as well as post-mortem changes of myofibrillar structures that lead to high water activity (Hamilton-Paterson and Johnson 1940; Huff-Lonergan and Sosnicki 2002; Huff-Lonergan and Lonergan 2005; Eskin and Shahidi 2012).

Beef consists of approx. 75% water, 20% protein, peptides and free amino acids as well as carbohydrates and fat with varying concentrations (Täufel *et al.* 1993; Weber 1996; Lawrie and Ledward 2006; Heinz and Hautzinger 2007; Wood *et al.* 2008; Krämer 2011). Whereas beef steaks possess high package to package fat content variation, minced beef has a defined fat content of 20% in Germany and minced beef products sold as "light" contain 10-12% fat.

Carbohydrates represent an important substrate source and comprise mostly glucose, ribose and glycolytic intermediates in concentrations of approx. 1.2% (Mottram and Nobrega 1998; Lawrie and Ledward 2006; Nychas *et al.* 2007; Koutsidis *et al.* 2008b; Eskin and Shahidi 2012). However, varying glycogen levels up to 1.8% in resting muscle tissues (Immonen and Puolanne 2000; Immonen *et al.* 2000; Ninios *et al.* 2014) depending on pre-slaughter stress and endogenous postmortem glycolysis influence the availability of glucose for spoilage microorganisms (Pösö and Puolanne 2005; Ferguson and Warner 2008). Post-mortem endogenous breakdown of nucleotides also leads to the availability of free ribose (Lee and Newbold 1963; Bendall 1973; Eskin and Shahidi 2012). Proteinaceous substances constitute another relevant substrate group. It is suggested that these substrates are metabolized after depletion of carbohydrates by spoilage organisms (Gill 1983; Nychas *et al.* 2007), because degradation of amino acids can be catabolically suppressed by sugars e.g. glucose as previously demonstrated for *Pseudomonas* and *Lactobacillus* (Marquis *et al.* 1987; Montel and Champomier 1987). However, it remains unclear whether local glucose concentration is high enough, and therefore whether repression is active in all numerically relevant meat spoilers. Free amino acids can arise from post-mortem proteolysis (autolysis) also contributing to tenderness of beef (Koohmaraie *et al.* 1991; Huff-Lonergan and Lonergan 2005; Koohmaraie and Geesink 2006; Lawrie and Ledward 2006; Dave and Ghaly 2011). Switch from sugar to amino acid metabolism frequently results in formation of off-odorous and harmful products e.g. biogenic amines, as well as nitrogen and sulfur compounds (Dainty and Mackey 1992; Stanbridge and Davies 1998; Nychas *et al.* 2008; Galgano *et al.* 2009). The third group of substrates, triacylglycerides, can also be autolytically cleaved post-mortem by endogenous meat lipases, releasing free fatty acids and glycerol (Tauro *et al.* 1986; Dave and Ghaly 2011).

Given the abundance of readily useable substrates, concomitant abundance of high levels of oxygen and carbon dioxide in high O_2 MAP sets a selective barrier for spoilage organisms. Besides retaining desirable red oxymyoglobin, oxygen also inhibits strict anaerobes, e.g. clostridia (Farber 1991; Church 1994). While high oxygen levels generally stimulates aerobes, carbon dioxide inhibits aerobic respiratory growth (Gill and Tan 1980). Mechanisms involve direct inhibition of enzymes and alteration of cell membrane function, intracellular pH and physico-chemical protein properties (Daniels *et al.* 1985; Dixon and Kell 1989; Farber 1991).

Besides the modified atmosphere, low storage temperature is another major selective hurdle determining the microbiota composition and dynamics of stored meat. To describe

different capabilities of bacteria to grow at low temperatures, the terms psychrotrophic and psychrophilic have been proposed (Morita 1975; Reichardt and Morita 1982; Jay *et al.* 2005). According to their definitions, psychrophiles have the ability to grow at subzero temperatures, reaching a growth optimum of 15 °C or below, and a maximum growth temperature of about 20 °C. Psychrotrophic bacteria have a growth optimum and maximum above 20 and 25 °C, respectively (Morita 1975; Scherer and Neuhaus 2006; Kameník 2013). Within these principal temperature ranges principally enabling growth of a strain, dynamic answers to a temperature decrease can result in “cold tolerance” or “cold adaptation”. Upon cold storage of beef products, psychrotrophic bacteria have been frequently found (Nychas *et al.* 2008; Doulgeraki *et al.* 2012; Pothakos *et al.* 2015). For meat products it has therefore been proposed to use lower incubation temperatures as compared to the ISO standard procedures for mesophilic counts (30 °C) to validly enumerate psychrotrophic spoilage-associated microbiota (Jay 2002; Ercolini *et al.* 2009; Pothakos *et al.* 2012; Pothakos *et al.* 2015). However, in such studies, it widely remains unclear, whether psychrophilic strains were among these isolates and therefore also if psychrophilic organisms contribute to meat spoilage.

1.4 Spoilage-associated microbiota on meat

Although modified atmosphere and cold storage set selective pressure on bacteria, spoilage-associated microorganisms commonly found on meat products are highly diverse and originate from various contamination sources (Gram *et al.* 2002; Jay *et al.* 2005; Nychas *et al.* 2007; Doulgeraki *et al.* 2012). Autochthonous contaminants arise during slaughter from hide and intestines, and input of allochthonous contaminants occurs from environmental niches e.g. slaughter houses, processing plants and workers during transport as well as primary and secondary processing of meat (Newton *et al.* 1978; Rahkio and Korkeala 1997; Rahman 1999; De Filippis *et al.* 2013; Kameník 2013). The fraction of organisms that becomes dominant under the selective conditions and cause meat spoilage are the so called ephemeral or specific spoilage organisms (E/SSO) (Nychas *et al.* 2008). Reports in the literature have shown a high diversity of microorganisms found on meats packaged under different atmospheres, though. The interactions of different bacteria on meat can be antagonistically with competition for nutrients or secretion of e.g. siderophores or mutualistic by the supply of nutrients for other spoilers, called metabiosis (Dainty *et al.* 1986; Jørgensen *et al.* 2000b; Gram *et al.* 2002). On aerobically stored meats, various psychrotolerant *Pseudomonas* (*Ps.*) spp. and *Entero-bacterales* have been described as the dominant spoilage organism (Nychas *et al.* 1998; Labadie 1999; Ercolini *et al.* 2007; Ercolini *et al.* 2010a; Doulgeraki *et al.* 2012). On meat packaged under modified atmosphere or vacuum, facultatively aerobic psychrotrophic LAB, especially *Lactobacillus* (*Lb.*) spp., *Carnobacterium* (*C.*) spp., *Leuconostoc* (*Le.*) spp. as well as *Serratia* (*S.*) spp. and *Brochothrix* (*B.*) *thermosphacta* have been often found (Lambert *et al.* 1991; Lambropoulou *et al.* 1996; Stanbridge and Davies 1998; Cantoni *et al.* 2000; Nychas

and Skandamis 2005; Ercolini *et al.* 2006; Doulgeraki *et al.* 2012). In vacuum packaged meats, additionally *Shewanella putrefaciens* and strict anaerobic clostridia have been reported as a cause of spoilage and blown packs, respectively (Molin and Ternström 1982; Borch *et al.* 1996; Broda *et al.* 1996; Nychas *et al.* 2008; Adam *et al.* 2010). Previous reviews by Doulgeraki *et al.* (2012) and Nychas *et al.* (2008) have reported that over 70 species and 42 genera are associated with meat spoilage, respectively, which makes it difficult to understand respective contribution of specific organisms to meat spoilage. Furthermore, the dynamics of spoilage organisms and development of microbiota composition remain widely unknown.

1.5 Identification and differentiation of spoilage associated microbiota

The culture-dependent and -independent approaches to identify spoilage-associated bacteria are as diverse as the reported species. Reports on culture-dependent methods have shown that a great variety of media and temperature is used as reviewed by Jay *et al.* (2002). The advantage of culture-dependent approaches is the cost-efficiency and actual recovery of bacteria for subsequent analysis. Within the culture-dependent approaches, there are two strategies, either to use selective or non-selective agar bases. The use of different selective media to obtain fast, selective counts for e.g. LAB, *B. thermosphacta*, *Enterobacteriaceae* or *Pseudomonas* is often employed (Samelis *et al.* 2000; Ercolini *et al.* 2006; Russo *et al.* 2006; Ercolini *et al.* 2010b), while correct selectivity or ability of all strains of the respective group to grow on the respective media is questionable. The approach with a complex media will favor cultivation of diverse microbiota, but needs subsequent time-consuming and low-throughput identification and differentiation. As a consequence, these approaches have very low-resolution regarding the development of spoilage-microbiota over time. MALDI-TOF MS constitutes a recent method for identification of microorganisms with the potential of high throughput in contrast to hitherto time-consuming and low-throughput identification by conventional methods e.g. phenotyping and gene sequencing. MALDI-TOF MS has successfully been used to identify bacteria directly from colonies on culture plates based on their low-molecular sub-proteomic fingerprint (Conway *et al.* 2001; Lay 2001; Wieser *et al.* 2012). The analysis of the mass to charge ratio is based on ionization of co-crystallized sample material by laser pulses followed by acceleration of ions and determination of their time of flight in a vacuum flight tube (Wieser *et al.* 2012). Previous studies have demonstrated application for classification of food-borne bacteria isolated from different food products (Mazzeo *et al.* 2006; Fernández-No *et al.* 2010; Angelakis *et al.* 2011; Böhme *et al.* 2011; Doan *et al.* 2012). However, the discriminatory power and accuracy of MALDI-TOF MS analysis below species level remains widely unknown and the suitability of available databases for reliable high-

throughput identification of spoilage-associated microbiota on beef has not yet been demonstrated.

On the other hand, different culture-independent approaches have been employed to characterize spoilage-associated microbiota using e.g. (T)-RFLP (Björkroth *et al.* 2005; Nieminen *et al.* 2011), PCR-DGGE (Ercolini *et al.* 2006; Audenaert *et al.* 2010) and species-specific real-time PCR (Pennacchia *et al.* 2009; Doulgeraki *et al.* 2010) as reviewed by Doulgeraki *et al.* (2012). Also, in recent studies, the use of high-throughput sequencing has been employed (Ercolini *et al.* 2011; Nieminen *et al.* 2012; De Filippis *et al.* 2013). The advantage of this culture-independent methods is the detection of potentially fastidious or unculturable bacteria that will not be recovered using conventional culture-dependent approaches. The disadvantage is the costly analysis, poor phylogenetic resolution that allows no distinct species affiliation and the predictive nature of the analysis with no isolates recovered.

However, highly interestingly, the presence of *Photobacterium* (*P.*) spp. has been recently reported in several culture-independent studies on vacuum and air stored beef (Pennacchia *et al.* 2011), MAP minced beef (Stoops *et al.* 2015) and MAP pork (Nieminen *et al.* 2016). Photobacteria are known as deep-sea bacteria and are limited to marine environments (Urbanczyk *et al.* 2011; Labella *et al.* 2017). Certain species *i.e. P. phosphoreum* and *P. iliopiscarium* have also been described as potent spoilers on MAP fish and seafood (Dalgaard *et al.* 1997; Jørgensen *et al.* 2000a; Emborg *et al.* 2002; Ast and Dunlap 2005), but it was previously unknown that they are predictively also abundant on MAP meat. However, due to the lack of a suitable detection and isolation method, the actual presence and distribution of photobacteria on meats has not yet been demonstrated.

1.6 Controversial role of LAB in meat spoilage

Psychrotrophic lactic acid bacteria have been frequently found as spoilers in MAP and VP meat. Their role or contribution to meat spoilage however is controversial as reviewed by Pothakos *et al.* (2015). *Lactobacillus sakei*, *Lb. curvatus*, *Lb. algidus* and *Leuconostoc* spp. have been reported to be associated with spoilage of VP and MAP meat (Borch and Molin 1988; Stanbridge and Davies 1998; Labadie 1999; Kato *et al.* 2000; Fontana *et al.* 2006; Chenoll *et al.* 2007; Nychas *et al.* 2008; Doulgeraki *et al.* 2010; Doulgeraki *et al.* 2012). Other important spoilage bacteria on meat are *C. divergens*, *C. maltaromaticum*, *Le. gelidum* subsp. *gelidum* & *gasicomitatum* (Björkroth *et al.* 2000; Vihavainen and Björkroth 2007b; Casaburi *et al.* 2011). Controversially, potential bioprotective behavior mainly against *L. monocytogenes* has been reported for *Le. mesenteroides*, *Le. carnosum*, *Lb. sakei*, *Le. gelidum* and *Carno-bacterium* spp. (Laursen *et al.* 2005; Jones *et al.* 2008; Trias *et al.* 2008; Vasilopoulos *et al.* 2010; Chaillou *et al.* 2014) demonstrating intra-species diversity and variability (Pothakos *et al.* 2015).

Another relevant psychrotrophic LAB is the homofermentative *Lactococcus* (*Lc.*) *piscium* (Williams *et al.* 1990a). This bacterium has been previously found on vacuum packaged beef (Sakala *et al.* 2002), broiler products (Vihavainen *et al.* 2007a) and pork (Rahkila *et al.* 2012). It has not been reported that *Lc. piscium* causes e.g. meat greening in contrast to other LAB *i.e. Le. gelidum* subsp., though. (Borch and Molin 1989; Vihavainen and Björkroth 2007b). However, the specific contribution of *Lc. piscium* strains to MAP beef spoilage still remains unclear. Recent studies have reported variable spoilage behavior or a bioprotective potential of *Lc. piscium* strains mainly for seafood products and vegetables, recently reviewed by Saraoui and colleagues (Saraoui *et al.* 2016a). On shrimp, shelf life extension and the potential to inhibit pathogenic *Listeria monocytogenes*, *Staphylococcus aureus*, as well as meat-spoiling *B. thermosphacta* and fish-spoiling *Photobacterium phosphoreum* has been reported (Matamoros *et al.* 2009a; Matamoros *et al.* 2009b; Fall *et al.* 2010a; Fall *et al.* 2010b; Fall *et al.* 2012; Leroi *et al.* 2015; Saraoui *et al.* 2017). Contrary to these findings, a shortening of the shelf life is reported on pork (Rahkila *et al.* 2012). On sweet bell pepper, *Lc. piscium* shows a strain-dependent weak or strong spoilage character (Pothakos *et al.* 2014). Also, strain-dependent spoilage behavior and inhibition of spoilage bacteria in salmon and cod juice has been recently reported (Wiernasz *et al.* 2017). However, any bioprotective potential of *Lc. piscium* on meat has not been demonstrated, yet.

2 Hypotheses

The aim of this study was to provide insight in the role of psychrotrophic, psychrophilic and previously uncultured bacteria in meat spoilage and offer approaches for their detection, and suppression or exploitation towards a retarded spoilage process. These results should initiate a process enabling reduction of the amount of wasted meat in the production and distribution chain, and at the consumer. Specifically, the following working hypotheses should be evaluated and probed along four sections of this work:

Chapter 1: Growth dynamics analysis of spoilage-associated microbiota on modified-atmosphere packaged beef steaks and minced beef

The aim within this chapter was to explore and validate the suitability and discriminatory power of MALDI-TOF MS for identification of the spoilage-associated microbiota. The method was subsequently used to enable designation of key spoilage organisms and monitoring their temporal development upon storage of MAP beef steaks and minced beef.

Working hypotheses:

- A database can be developed, which enables MALDI-TOF MS for rapid, high-throughput identification of meat spoilage bacteria with sufficient discriminatory power and accuracy.
- The onset of meat spoilage dynamics is triggered by initial contamination with key spoilers.
- Psychrotrophic and psychrophilic organisms are hitherto underestimated in regard to their role in meat spoilage.

Chapter 2: Assertiveness of *Lactococcus piscium* and evaluation of its potential to exhibit bioprotective characteristics on beef

The results of the first chapter enabled to subsequently focus on the exploration of *Lactococcus piscium* (*Lc.*), which exhibited assertiveness upon MAP beef steak storage during growth dynamics analyses and resulted in a delayed spoilage. Consequentially, the aim of this part was the assessment of intraspecies assertiveness of *Lc. piscium* strains and suppression of other prominent spoilers towards a bioprotective potential *in vitro* and evaluating the dominance of *Lc. piscium* and its exhibited volatilome *in situ*.

Working hypotheses:

- *Lc. piscium* strains show prevalence on meat over the indigenous microbiota.
- *Lc. piscium* exhibits a volatilome without rejectable sensorial impression on beef.
- Meat-borne *Lc. piscium* strains display intraspecies variations in their assertiveness and suppression of competitive meat spoilers.

Chapter 3: Photobacteria involved in meat spoilage

The aim of this part was to demonstrate the abundance of hitherto uncultured photobacteria and probe their distribution on meats employing a novel culture-dependent approach for their recovery.

Working hypotheses:

- An adapted isolation procedure can be developed, which allows isolation of *Photobacterium* spp. from food.
- *Photobacterium* spp. are common spoilers on meats and are hitherto neglected in regard to their role in meat spoilage.

Chapter 4: Lifestyle of spoilage-associated bacteria

The aim of this part was to predict the lifestyle of specific spoilage organisms (SSO) on meat and assess their respective individual spoilage potential employing a comparative genomics approach.

Working hypotheses:

- Important SSO harbor genes for metabolizing diverse substrates groups present in meat.
- Each SSO has its individual spoilage potential.

3 Material and methods

3.1 Microorganisms

Bacteria isolated from meat or obtained from strain collections were kept as cryopreserved stock cultures in the respective medium and 34% w/v glycerol at -80 °C until use. Relevant strains isolated in this study and used for subsequent analyses are shown in Table 1.

Table 1| Microorganisms used in experiments.

Species	Strain[1]	Source
Lactococcus (Lc.) piscium	TMW2.1612	MAP beef steaks
Lc. piscium	TMW2.1613	MAP beef steaks
Lc. piscium	TMW2.1614	MAP beef steaks
Lc. piscium	TMW2.1615	MAP beef steaks
Lc. piscium	TMW2.1893	MAP minced beef
Lc. piscium	TMW2.1894	MAP minced beef
Lc. piscium	TMW2.1895	MAP minced beef
Lc. piscium	TMW2.1896	MAP minced beef
Lc. piscium	TMW2.1897	MAP minced beef
Lc. piscium	TMW2.1898	MAP minced beef
Lc. piscium	TMW2.1899	MAP beef steaks
Lc. piscium	TMW2.1900	MAP beef steaks
Lc. piscium	TMW2.1901	MAP beef steaks
Lc. piscium	TMW2.1902	MAP minced beef
Lc. piscium	TMW2.1903	MAP minced beef
Hafnia (*H.*) *alvei*	TMW2.1904	MAP beef steaks
Serratia (*S.*) *liquefaciens*	TMW2.1905	MAP beef steaks
Brochothrix (*B.*) *thermosphacta*	TMW2.1906	MAP minced beef
Carnobacterium (*C.*) *divergens*	TMW2.1907	MAP beef steaks
Leuconostoc (*Le.*) *gelidum* subsp. *gelidum*	TMW2.1618	MAP beef steaks
Le. gelidum subsp. *gelidum*	TMW2.1998	MAP beef steaks
Le. gelidum subsp. *gasicomitatum*	TMW2.1619	MAP beef steaks
Pseudomonas (*Ps.*) *weihenstephanensis*	TMW2.1728	MAP beef steaks
Ps. weihenstephanensis	TMW2.2077	MAP minced beef
Ps. weihenstephanensis	TMW2.2078	MAP minced beef
Ps. weihenstephanensis	TMW2.2084	MAP minced beef
Ps. lundensis	TMW2.2076	MAP minced beef
Ps. lundensis^T	TMW2.1623	DSMZ[2], DSM 6252^T
Ps. fragi	TMW2.2079	MAP minced beef
Ps. fragi	TMW2.2080	MAP minced beef

Species	Strain[1]	Source
Ps. fragi	TMW2.2081	MAP minced beef
Ps. fragi	TMW2.2082	MAP minced beef
Ps. fragi^T	TMW2.181	DSMZ, DSM 3456^T
Ps. versuta	TMW2.2083	MAP minced beef
Pseudomonas sp. MH1	TMW2.2087	MAP minced beef
Pseudomonas sp. MH1	TMW2.2088	MAP minced beef
Pseudomonas sp. MH1	TMW2.2089	MAP minced beef
Pseudomonas sp. MH2	TMW2.2090	MAP minced beef
Pseudomonas sp. MH3	TMW2.2091	MAP minced beef
Photobacterium (*P.*) *aquimaris*^{T}	TMW2.1991	DSMZ, DSM 23343^T
P. iliopiscarium^T	TMW2.1992	DSMZ, DSM 9896^T
P. phosphoreum^T	TMW2.1993	DSMZ, DSM 15556^T
P. angustum^T	TMW2.1994	DSMZ, DSM 19184^T
P. kishitanii^T	TMW2.1995	DSMZ, DSM 19954^T
P. leiognathii^T	TMW2.1997	DSMZ, DSM 21260^T
P. piscicola^T	TMW2.2044	BCCM/LMG[3], LMG 27681^T
P. carnosum^T	TMW2.2021	MAP chicken breast
P. carnosum	TMW2.2022	MAP chicken breast
P. carnosum	TMW2.2029	MAP chicken breast
P. carnosum	TMW2.2030	MAP chicken breast

[1]TMW, Technische Mikrobiologie Weihenstephan.
[2]DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.
[3]BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms, Laboratory for Microbiology of the Faculty of Sciences of the Ghent University.

3.2 Isolation and cultivation of spoilage-associated microbiota

3.2.1 Meat samples, sampling and sensory evaluation

3.2.1.1 Meat samples

Fresh beef steak and minced beef packages were obtained directly from a producer in Germany for growth dynamics monitoring of spoilage-associated microbiota. Beef steak samples originated from the thick flank and minced beef samples contained a fat content of 10-12%. Samples were packaged under high-oxygen modified atmosphere (>70% O_2, >20% CO_2). Two different sample batches were investigated for beef steaks and minced beef, respectively and stored at proper (4 °C) and inadequate (10 °C) temperature in Lovibond thermostatic cabinets (Tintometer GmbH, Dortmund, Germany). Fresh beef steaks were also obtained from the same producer for deliberate inoculation with selected spoilage-associated strains. Additionally, random samples of beef steaks, minced beef, pork, chicken and salmon were obtained at different supermarkets in the local area of Freising, Bavaria, Germany,

respectively. All meat samples were transported to the lab in polystyrene boxes filled with ice to avoid bias by interruption of the cold chain and stored until sampling in thermostatic cabinets.

3.2.1.2 Inoculation of beef samples and repackaging

Fresh beef steaks from the thick flank packaged under high O_2 MAP were obtained directly from a producer in Germany. Packages were opened aseptically and circular pieces of beef (area of 20 cm^2) were cut out with a sterile scalpel. These circular pieces were inoculated on top and bottom with 100 µl of bacteria strains (6 log_{10} CFU) from cryopreserved stock cultures washed and resuspended in quarter strength Ringer's solution, prepared by dissolving 1 tablet (Merck, Darmstadt, Deutschland) in 500 ml deionized water (diH_2O). Stock cultures were cold-adapted at 4 °C for 4h before conservation at -80 °C. Control was treated with 100 µl quarter strength Ringer's solution only. One beef piece was placed in a polypropylene tray (205/160/40 mm; O_2-transmission rate: <234 cm^3 (STP)/m^2*d*bar)), average material thickness: 270 µm, ES-Plastic GmbH, Hutthurm, Germany), respectively and repackaged under high oxygen MAP (>70% O_2, >20% CO_2) with a PET/PA/EVOH/PP lid film (Südpack, Ochsenhausen, Germany, O_2 transmission rate: 3 cm^3 (STP)/(m^2*d*bar)) using a Multivac T250 tray sealer (Multivac, Wolfertschwenden, Germany). For volatilome analysis conducted by Corinna Franke using PTR-MS (3.4), three circular pieces each were transferred into three perfluoralkoxy (PFA) beakers (1 l, Savillex, Eden Prairie, MN, USA) and connected to the PTR-MS (hs-PTR-MS, IONICON Analytik GmbH, Innsbruck, Austria) measurement setup. Samples were subjected to microbiological analysis at the end of the measurement.

3.2.1.3 Sampling procedure

Sampling procedure of growth dynamics monitoring of spoilage-associated microbiota is visualized in Figure 1. Three packages were taken into analysis of each storage temperature for each of the sampling dates, respectively. Headspace atmosphere composition was measured with a Dansensor gas analyzer (Mocon, Neuwied, Germany) equipped with an oxygen and carbon dioxide sensor. Subsequently, each package was opened and a circular piece of beef (area of 6.6 cm^2) from each slice of a package was trepanned aseptically using a sterilized metal tube and pooled into a sterile lateral filter bag (Interscience, Saint Nom, France). Physiological Ringer's solution (100 ml) was added to the beef pieces (approx. 20 g) and samples were homogenized for 2 minutes in a stomacher 400 paddle blender (Seward, Worthing, UK). Decimal serial dilutions were carried out in quarter strength Ringer's solution followed by plating out using glass beats (diameter 2.85 – 3.45 mm; Roth, Karlsruhe, Germany). Afterwards, pH values were determined directly from each beef with a pH meter and a puncture electrode (Mettler-Toledo, Gießen, Germany).

Sampling of beef steaks deliberately inoculated with bacteria as well as random retail samples from local supermarkets was conducted analogously. For the adapted isolation

procedure of photobacteria from meat, quarter strength Ringer's solution was replaced by marine broth (3.2.5.7) for resuspension and dilution of meat samples.

After cultivation of isolated microbiota and determination of total viable count (3.2.2), microbiota was identified by MALDI-TOF MS (3.3).

3.2.1.4 Sensorial evaluation

Sensorial evaluation of samples was performed by an untrained panel in the lab. The evaluation of perceptible spoilage was focused on acceptance or rejection based on discoloration or off-odor without assigning distinct scale attributes.

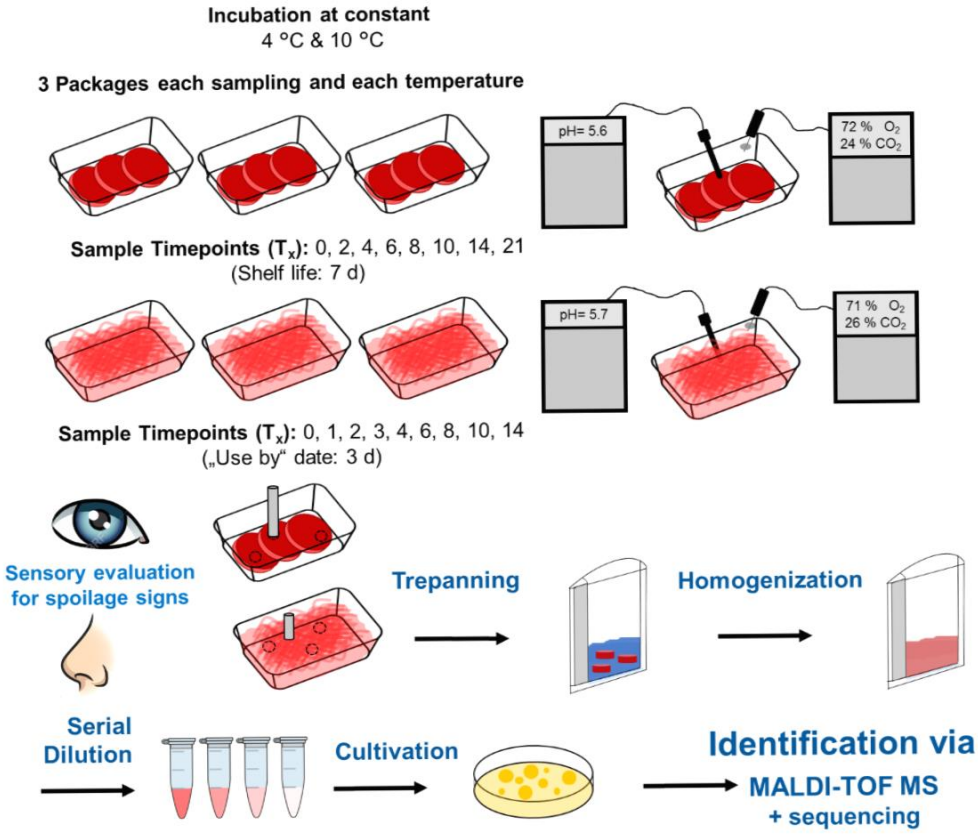

Figure 1| Sampling procedure of meat samples during growth dynamics experiments. Sampling of randomly obtained samples and samples from volatilome analysis was conducted analogously. Identification of relevant bacteria was carried out via sequencing of 16S rRNA/*rpo*D/*phe*S genes whenever the organism was absent in the MALDI-TOF database and their reference MSP was subsequently included in the database.

3.2.2 Cultivation of bacteria

3.2.2.1 Cultivation and enumeration of psychrotrophic microorganisms

Bacteria were cultivated on Brain-Heart-Infusion (BHI, 3.2.5.1) agar (1.6% w/v) and incubated for 48 h at 25 °C for Total Viable Count (TVC) as standard procedure. This temperature is dissenting from the ISO 4833-1 norm for enumeration of mesophilic bacteria to

enable isolation of psychrotrophic bacteria not able to grow at 30 °C. Plates containing 25 - 250 colonies were chosen for reliable enumeration of total viable count (Maturin and Peeler 2001; Sutton 2011).

3.2.2.2 Cultivation, enumeration and growth analysis of potentially psychrophilic microorganisms

During growth dynamics monitoring of spoilage-associated microbiota on beef steak batch samples, isolation of potentially psychrophilic bacteria not able to grow at 25 °C was additionally carried out with cultivation on BHI agar (1.6% w/v) at 4 °C for 14 days at two sample time points from beef stored at 4 °C. From these plates, 571 potentially psychrophilic isolates were transferred to new agar plates and cultivated in parallel at 4 and 25 °C, respectively. After another 14 days, plates were screened for isolates that show growth at 4 °C, but not at 25 °C. Growth potential of the isolates in a temperature range from -5 to +30 °C was conducted in liquid BHI media with 5% glycerol (w/w) to prevent freezing and incubated statically in 15 ml screw cap tubes (Sarstedt, Nümbrecht, Germany). Growth in liquid media was quantified by monitoring the optimal density at 590 nm using a Novaspec Plus spectrophotometer (GE Healthcare Europe, Freiburg, Germany).

3.2.3 Cultivation and growth evaluation of meat spoilers and photobacteria

For growth tests, relevant meat-borne isolates of *B. thermosphacta* TMW2.1906, *Ps. weihenstephanensis* TMW2.1728, *S. liquefaciens* TMW2.1905, *H. alvei* TMW2.1904, *C. divergens* TMW2.1907, *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 and *Lc. piscium* TMW2.1612, and photobacteria (*P. aquimaris*^{T}, *P. iliopiscarium*^{T}, *P. phosphoreum*^{T}, *P. angustum*^{T}, *P. kishitanii*^{T}, *P. profundum*^{T}, *P. leiognathi*^{T}) were pre-cultured for 48h in either BHI (3.2.5.1) or marine broth (3.2.5.7), respectively and then plated on different media with varying additives. All bacteria were inoculated at 25 °C with exception of *P. profundum*, which was incubated at 10 °C. Growth was evaluated after 48-72h.

Table 2| Antibiotics used in susceptibility tests.

Discs		Strips (0.016-256ug/ml)
Penicillin G (P)	10U	Tetracycline C (TC)
Ampicillin (AMP)	10 µg	Chloramphenicol (CL)
Linezolid (LZD)	30 µg	
Oxacillin (OX)	1 µg	
Erythromycin (E)	15 µg	
Vancomycin (VA)	30 µg	
Gentamicin (CN)	10 µg	
Teicoplanin (TEC)	30 µg	
Tetracycline C (TC)	30 µg	
Chloramphenicol (CL)	30 µg	

3.2.4 Antibiotic susceptibility testing

Susceptibility to different antibiotics was evaluated by the diffusion method (Bauer *et al.* 1966). Therefore, liquid overnight cultures were spread evenly on an agar plate using a cotton swab and antibiotic disks (Oxoid, Thermo Scientific, Dreieich, Germany) or strips (Etest, bioMérieux, Marcy-l'Étoile, France) containing different concentrations were placed onto the plate (Table 2). After growth of the bacteria, size of inhibitory zones were measured for discs and inhibitory concentration was determined for strips.

3.2.5 Cultivation media

All media for cultivation of bacteria were prepared with deionized water (diH_2O) and either boiled to dissolve completely and/or autoclaved (121 °C, 15-20 min, 1 bar overpressure) to ensure sterile conditions. Heat labile substances were sterile-filtered and added after cooling of the media. Solid medium was prepared from broth by addition of 1.6% (w/v) agar-agar (Roth, Karsruhe, Germany) and poured aseptically into petri dishes (7.8 cm diameter; Sarstedt, Nümbrecht, Germany) when cooled below 50 °C. For cultivation under oxic conditions using solid media, petri dishes were put into plastic bags with an opening at top to avoid drying of plates. Cultivation on solid media under anoxic conditions was performed using anaerobic gas generating bags (Oxoid AnaeroGen 2.5L, Thermo Scientific, Dreieich, Germany). Anoxic conditions were controlled with an Anaerotest strip (Merck, Darmstadt, Germany). When using liquid media, cells were incubated statically in completely filled 15 ml screw cap tubes (Sarstedt, Nümbrecht, Germany) for anoxic conditions or shaken in narrow-neck Erlenmeyer flasks for oxic conditions.

Lovibond thermostatic cabinets (Tintometer GmbH, Dortmund, Germany) were used to ensure constant incubation temperatures from 4-30 °C. Cultivation of psychrophilic strains at -5 and 0 °C was performed in a refrigerator with integrated freezer (Bomann GmbH, Kempen, Germany). Correct temperature was monitored with a Xavax thermometer (Hama GmbH, Monheim, Germany) that recorded the maximum and minimum temperature.

3.2.5.1 Brain-Heart-Infusion medium

Brain-Heart-Infusion (BHI) broth (Roth, Karlsruhe, Germany) was used for general (sub-) cultivation, preparation of cryo-preserved cultures and non-selective isolation representing a Total Viable Count (TVC). Therefore, 37.5 g of broth were dissolved in 1 Liter of diH_2O and pH was adjusted to 7.4.

BHI contact plates were used for determination of initial contamination of beef steaks. Therefore, BHI broth agar was poured into small agar plates (5.5 cm diameter; Sarstedt, Nümbrecht, Germany) in a slightly convex shape. A rubber band was stretched over the petri dish to avoid contact of agar with the lid.

Selective cultivation of *Le. gelidum* subsp. *gasicomitatum* and *C. divergens* in competition experiments with *Lc. piscium* strains was carried out on BHI agar containing vancomycin hydrochloride (15 mg/l; AppliChem GmbH, Darmstadt, Germany) and oxacillin (2 mg/l; Sigma-Aldrich, St. Louis, MO, USA), respectively that were added aseptically after autoclaving and cooling below 50 °C.

Glycerol was added (5% v/v) to BHI broth and plates for growth investigation of psychrophilic isolates to prevent freezing at (sub-)zero centigrade. Water activity of the medium was determined with a LabMaster-aw (Novasina, Lachen, Switzerland).

3.2.5.2 Commercial meat simulation medium

Meat Simulation Medium (MSM) was used for intraspecies assertiveness of *Lactococcus piscium* strains and in competition experiments. MSM consisted of 100 g meat extract (Merck, Darmstadt, Germany) that were suspended in 1000 ml diH_2O with a pH adjusted to 5.8 with concentrated lactic acid.

3.2.5.3 Self-prepared meat simulation medium

An additional meat simulation medium was prepared based on minced beef extract. Minced beef was resuspended in diH_2O, homogenized in a paddle blender and roughly filtered through coffee filters. Subsequently it was centrifuged, sterile filtered and stored at 4 °C. The medium was used to observe discoloration by meat spoilers. For *in vitro* experiments, the commercial meat simulation medium was chosen over the self-prepared medium due to the time-consuming and costly preparation as well as its limited stability.

3.2.5.4 Violet red bile glucose agar

Violet Red Bile Dextrose (glucose) Agar (VRBDA) was used for selective cultivation and enumeration of enterobacteria. 39.5 g of ready-to-use VRBDA (Merck, Darmstadt, Germany) and additional 3 g agar-agar were suspended in 1000 ml diH_2O and boiled to dissolve completely, but not autoclaved. pH was adjusted to 7.3. The agar plates were stored in the dark at 4 °C until use.

3.2.5.5 Pseudomonas selective CFC agar

Cephalothin-Fucidin-Cetrimide selective agar was used for selective cultivation and enumeration of *Pseudomonas* spp. 24.2 g of *Pseudomonas* agar base (Merck, Darmstadt, Germany) and 5 ml glycerol were suspended in 500 ml diH_2O and boiled to dissolve completely prior to autoclaving. pH was adjusted to 7.1. 2 ml of a 50% (v/v) ethanol solution was added to an ampulla of CFC supplement (Merck, Darmstadt, Germany) and aseptically added to the agar base after cooling below 50 °C. The agar plates were stored in the dark at 4 °C until use.

3.2.5.6 STAA agar

Streptomycin-Thallous acetate-Actidione (cycloheximide) Agar (STAA) was used for selective cultivation and enumeration of *Brochothrix thermosphacta*. 18.5 g of agar base (Oxoid, Wesel, Germany) was suspended in 500 ml diH_2O and boiled to dissolve completely. pH was adjusted to 7.0. Subsequently, 7.5 g of glycerol was added prior to autoclaving. After cooling below 50 °C, a vial of STAA selective supplement (Oxoid, Wesel, Germany) was added aseptically to the agar base. The agar plates were stored in the dark at 4 °C until use.

3.2.5.7 Marine Broth

Marine Broth 2216 (MB, Difco, Becton Dickinson GmbH, Franklin Lakes, NJ, USA) was used for (sub-)cultivation, enumeration and isolation of photobacteria. Therefore, 37.4 g broth were dissolved in 1 Liter of diH_2O and pH was adjusted to 7.6. Selective cultivation was carried out with an adapted medium and isolation procedure with Marine Broth supplemented with 3 g/L meat extract (Merck, Darmstadt, Germany) and vancomycin hydrochloride (7 mg/l) AppliChem GmbH, Darmstadt, Germany) that was added aseptically after cooling of the media.

3.2.5.8 *Photobacterium* Broth

Photobacterium Broth (PB, Sigma-Aldrich, St. Louis, MO, USA) was used for cultivation of photobacteria and demonstration of bioluminescence. Therefore, 65.6 g broth were dissolved in 1 Liter of diH_2O and pH was adjusted to 7.0. In a comparative isolation approach for photobacteria, 3 g/L meat extract (Merck, Darmstadt, Germany) and vancomycin hydrochloride (7 mg/l) AppliChem GmbH, Darmstadt, Germany) were additionally added aseptically after cooling of the media.

3.2.5.9 TCBS agar

Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar was used for selective isolation of *Vibrionaceae*. 88 g of ready-to-use TCBS agar (Roth, Karlsruhe, Germany) was suspended in 1000 ml diH_2O and boiled to dissolve completely, but not autoclaved. The agar plates were stored in the dark at 4 °C until use.

3.2.5.10 TSA

Tryptic Soy Agar (TSA) with casein peptone (15 g/l) and soy peptone (5 g/l) was used for phenotypic characterization of photobacteria with variable pH and sodium chloride concentrations. Motility of photobacteria was tested using the soft agar stabbing method (Adler 1966) in TSA supplemented with 2% (w/v) NaCl and 0.3% (w/v) agar.

3.2.5.11 LB

Lysogeny broth (LB) agar was used for growth tests of meat spoilers and photobacteria. 10 g of tryptone, 5 g yeast extract and varying amounts of NaCl were suspended in 1000 ml diH_2O.

3.2.5.12 Decarboxylation medium

Screening for production of biogenic amines was conducted with an optimized decarboxylation medium previously published (Bover-Cid and Holzapfel 1999). Decarboxylation potential was tested with amino acids phenylalanine, ornithine, histidine, lysine and tyrosine and indicated by color change of bromocresol purple from colorless to purple.

3.2.5.13 Plate count agar

Commercial Roti Conti-Plate contact plates (Roth, Karlsruhe, Germany) with plate count agar were used for surface sampling in addition to self-made BHI contact plates.

3.3 Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS was used for identification and proteomic comparison of spoilage-associated microbiota as well as routine verification of all used bacterial strains based on their low molecular weight subproteome fingerprint (mass range of 2 – 20 kDa).

3.3.1 Sample preparation

MALDI-TOF analysis was performed with a direct transfer method including “on target extraction” or with a liquid extraction method (Kern *et al.* 2013; Usbeck *et al.* 2013).

For the direct transfer method, colony material was smeared onto a MSP 96 polished steel target (Bruker Corporation, Billerica, MA, USA) spot using a sterile toothpick. Afterwards, each target spot was covered with 1 µl of 70% (v/v) formic acid and subsequently overlaid with 1 µl freshly-mixed matrix solution of α-4-hydroxy-cinnamic acid (HCCA, 10 mg/ml) dissolved in organic solvent consisting of acetonitrile, diH_2O and trifluoroacetic acid (ratio 50:47.5:2.5%, v/v).

For liquid extraction, 1 ml of liquid over-night culture was transferred to a 1.5 ml reaction tube (Sarstedt, Nümbrecht, Germany) and centrifuged in a tabletop mini-centrifuge MCF-1350 (LMS, Tokyo, Japan) for 2 min at 11.400 *g*. The supernatant was discarded and the pellet was resuspended in 300 µl diH_2O using a vibration shaker (Vortex Genie 2, Scientific Instruments, Bohemia, NY, USA). Subsequently, 900 µl absolute ethanol were added and mixed, followed by another centrifugation step (11.400 *g*, 2 min). The supernatant was discarded and the pellet was air-dried under a fume hood. Afterwards, 50 µl of formic acid (70% v/v) were added and mixed thoroughly. Then, 50 µl acetonitrile were added and mixed again followed by a centrifugation step (11.400 *g*, 2 min). Finally, 1 µl of the supernatant was spotted on a MSP 96 polished steel target (Bruker Corporation, Billerica, MA, USA) and overlaid with 1 µl HCCA matrix solution.

3.3.2 MALDI-TOF MS analysis and identification

Analysis was performed on a Microflex LT spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a nitrogen laser (λ= 337 nm) and operating in linear ion detection mode under Biotyper Automation Flex Control 3.4 and Real Time Classification 3.1 (Bruker Corporation, Billerica, MA, USA). Mass spectrometry profiles (MSPs) were accumulated from 240 single spectra. These sum spectra were matched with an internal Bruker database as well as a self-constructed in-house database specifically for common meat spoilage bacteria including several reference MSPs per species for reliable identification on species level and below. External calibration was performed using a Bacterial Test Standard (Bruker Corporation, Billerica, MA, USA).

3.3.3 Reference MSPs

An in-house data base containing common meat spoilers was established for reliable identification of spoilage-associated microbiota. Organisms were identified based on 16S rDNA gene or house-keeping gene sequences whenever 16S rDNA did not provide enough taxonomic discriminatory power (3.5.2). The reference MSPs were obtained using the liquid extraction method (3.3.1). The acquired MSPs (at least 24 per isolate) were analyzed in FlexAnalysis 3.4 (Bruker Corporation, Billerica, MA, USA) applying baseline subtraction and peak smoothing. The *m/z* value deviation for the eight highest peaks from 2-10 kDa were analyzed. MSPs with *m/z* deviation of 0.05% or higher at any peak were eliminated from the reference spectra. Reference MSPs comprising 20-24 sum spectra were finally included into the database.

3.3.4 Data analysis and processing

Accuracy of identification was assured by score values given by MALDI Biotyper Real Time Classification wizard 3.1 (Bruker Corporation, Billerica, MA, USA) (Clark *et al.* 2013). Score values of the measured MSP are calculated by the software based on the presence of peaks, *m*/*z* deviation as well as intensity compared to the dedicated reference MSP in the database.

In addition to identification based on MALDI Biotyper score values, MSPs were clustered in a hierarchical cluster analysis (HCA) to assure consistent species identification and allow selection of isolates subjected to subsequent molecular analysis. MSPs were exported using FlexAnalysis 3.4 (Bruker Corporation, Billerica, MA, USA). Only peaks with a width of 0.5 Da, a signal-to-noise-ratio >3, maximum variance of 600 ppm of *m/z* value (Fushiki *et al.* 2006) and a peak detection rate >0.4 were taken into analysis for each *m/z* value (Mantini *et al.* 2007; Usbeck *et al.* 2013). The data set was processed with an in-house software pipeline (Kern *et al.* 2014) based on MASCAP (Mantini *et al.* 2010) and implemented in GNU Octave (Eaton and Rawlings 2003). Mass spectrometry profiles were matched into clusters based on Euclidean distance matrices and visualized using RStudio 3.0.3 (RStudio, Boston, MA, USA).

For hierarchical clustering of reference MSPs, the spectra were exported via MALDI Biotyper Real Time Classification wizard 3.1 (Bruker Corporation, Billerica, MA, USA) and imported into Bionumerics V7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were constructed with Unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient.

3.4 Proton-transfer-reaction mass spectrometry (PTR-MS)

Volatilome analysis of beef steaks deliberately inoculated with *Lc. piscium* TMW2.1615 via online monitoring of their volatile organic compounds was carried out by Corinna Franke at the Fraunhofer IVV, Freising using PTR-MS (Franke 2018). The analytical setup of PTR-MS is explained in detail by Franke and Beauchamp (2017). The setup was placed in a compression cooled incubator (ICP 110, Memmert, Schwabach, Germany) held at 4 °C.

The analytical setup allowed the measurement of four samples simultaneously where the fourth beaker was used for determining the background signal, serving as the control. The switching from one sample to the other was reached by incorporating two-way solenoid valves that also prevented a potential cross contamination between the samples After incorporating the samples in the analytical setup, initial conditions were obtained by flushing each beaker for 1.5 min with modified atmosphere (30% CO_2, 70% O_2) (5 L/min) (Messer, Bad Soden, Germany) to fully exchange the air in the headspaces. During the measurement, samples of the headspace atmosphere were taken at a constant flow rate of 10 mL/min so the carrier gas was enriched with the VOC released meanwhile. Each sample was measured for 5 cycles using scan mode from m/z 20 to m/z 160 with a dwell time of 500 ms per m/z. This results in a measuring time of 5.9 min each sample. The samples were monitored in series every 250 cycles (4.9 h). The carrier gas containing the VOCs was then diluted by dilution air which was set to 100 ml/min before entering the PTR-MS inlet. The PTR-MS drift tube was set to 600 V, 60 °C and 2.2 mbar. All information regarding the setup originate from Corinna Franke (Franke 2018).

3.5 Molecular biological methods

3.5.1 Isolation of genomic DNA

Isolation of genomic DNA for PCR amplification and whole genome shotgun (WGS) sequencing was performed with an E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manual with minor modifications. TE-Buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8) was used for resuspending overnight cultures. The cell wall lysis step was prolonged to 2 hours.

For genome sequencing using the SMRT sequencing technology, high-molecular weight DNA was isolated with the QIAGEN Genomic-Tip 100/G kit and QIAGEN Genomic DNA buffer

set (QIAGEN, Hilden, Germany) according to the manual with minor modifications. The amounts of lysozyme and Proteinase K per sample were raised to 72 mg and 2 mg, respectively. The cell wall lysis step was prolonged to 4 hours. Subsequent denaturation and proteolysis step was also prolonged to 2 hours to obtain a clear lysate. Precipitated DNA was spooled using a glass rod and resuspended in 250 µl elution buffer. Quantitation and purity control of isolated DNA was carried out with a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). The device was initialized with 2 µl diH_2O followed by a blank measurement with the respective buffer.

3.5.2 PCR amplification

Polymerase Chain Reaction (Saiki *et al.* 1988) was performed to specifically amplify target genes (16S rRNA and housekeeping genes for multi locus sequence analysis (MLSA)) or in order to obtain Randomly Amplified Polymorphic DNA (RAPD). Primer sequences are shown in Table 3. Reaction mix and thermoprotocol of PCR approaches are shown in Table 4.

PCR reaction mix was set up on ice and performed in a PCR cycler (Eppendorf AG, Hamburg, Germany) at 50 µL scale in a 200 µL reaction tube (Sarstedt, Nümbrecht, Germany). Template was either direct or processed colony material, or isolated DNA (3.5.1).

Table 3| Primer sequences used in PCR amplifications.

Gene	Primer	Sequence	Reference
RAPD	M13V	GTT TTC CCA GTC ACG AC	(Ehrmann *et al.* 2003)
16S rRNA	27F	AGA GTT TGA TCM TGG CTC AG	(Lane 1991)
	1507R	TAC CTT GTT ACG ACT TCA CCC CAG	(Sandström *et al.* 2001)
	97K	CTG CTG CCT CCC GTA	(Stackebrandt and Charfreitag 1990)
	607V	GGG CTA CAC ACG TGC TAC	(Müller *et al.* 2000)
	609R	ACT ACY VGG GTA TCT AAK CC	
***gyr*B**	22F	GAA GTT ATC ATG ACG GTA CTT C	(Ast and Dunlap 2004)
	1240R	AGC GTA CGA ATG TGA GAA CC	
***rpo*D**	70Fs	ACG ACT GAC CCG GTA CGC ATG TA	(Yamamoto *et al.* 2000)
	70Rs	ATA GAA ATA ACC AGA CGT AAG TT	
***rec*A**	recAfor	TGG ACG AGA ATA AAC AGA AGG C	(Thompson *et al.* 2005)
	recArev	CCG TTA TAG CTG TAC CAA GCG CCC	
	recAforkish	TCT TGC CGC AGC GTT AGG CCA G	(Ast *et al.* 2007)
	recArevkish	GCT TTT TCA ACT AAT TTA TGC TTC AC	
***car*A**	carArev	TGA TGR CCS AGG CAG ATR CC	(Hilario *et al.* 2004)
	fraF	CGT CAG CAC CGA AAA AGC C	(Ercolini *et al.* 2007)
	lunF	TGT GGC GAT TGC AGG CAT T	
***phe*S**	21F	CAY CCN GCH CGY GAY ATG C	(Naser *et al.* 2005)
	23R	GGR TGR ACC ATV CCN GCH CC	

Table 4| Reaction mix and thermoprotocol of PCR amplifications.

Reagent[a]	RAPD	16S rDNA	*rpo*D	*car*A	*gyr*B	*rec*A	*phe*S
$MgCl_2$	5 mM	2.5 mM	5 mM	2.5 mM	2.5 mM	2.5 mM	5 mM
dNTPs	0.8 mM	0.4 mM	0.8 mM	0.8 mM	0.4 mM	0.4 mM	0.4 mM
Taq-Polymerase	1.5 U	1.25 U	1.25 U	1.25 U	1.25 U	1.25 U	1.25 U
Primer	1 µM	0.5 µM	0.5 µM	0.3 µM	0.5 µM	0.5 µM	1 µM
Template	20 - 100 ng						
# Step	Heat lid to 103 °C						
1 Initial denaturation	94 °C / 5'						
2 Denaturation	94 °C / 3'	-	-	-	-	-	94 °C / 1'
3 Annealing	40 °C / 5'	-	-	-	-	-	55 °C / 2'15"
4 Extension	72 °C / 5'	-	-	-	-	-	72 °C / 75'
Cycle Step 2-4	3x	-	-	-	-	-	3x
5 Denaturation	94 °C / 1'	94 °C / 45"	94 °C / 1'	94 °C / 1'	94 °C / 45"	94 °C / 60"	94 °C / 35"
6 Annealing	60 °C / 2'	52 °C / 90'	57 °C / 1'	59 °C / 1'	48 °C / 90"	45 °C / 60"	55 °C / 75"
7 Extension	72 °C / 3'	72 °C / 2'	72 °C / 90"	72 °C / 30"	72 °C / 2'	72 °C / 90'	72 °C / 75"
Cycle Step 5-7	32x	34x	35x	30x	34x	35x	30x
8 Final extension	72 °C / 5'	72 °C / 5'	72 °C / 10'	72 °C / 10'	72 °C / 7'	72 °C / 7'	72 °C / 7'

[a]*Taq* Core Kit 10 (M.P Biomedicals, Irvine, CA, USA)

3.5.3 Analytical agarose gel electrophoresis

Analysis of PCR products was performed by analytical agarose gel electrophoresis (Sambrook *et al.* 1989). PCR amplicons were mixed with loading dye (Thermo Fisher Scientific, Waltham, MA, USA) and applied on a 1% (w/w) agarose gel in 0.5x TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA). Electric current was applied with an Electrophoresis Power Supply (EPS 300, Pharmacia Biotech, Uppsala, Sweden) for electrophoretic separation of amplicons. Dimidium bromide was used for DNA staining and visualization was performed with an UVT-28M transilluminator (Herolab, Wiesloch, Germany). RAPD-PCR products and λ-DNA/*Eco*RI+*Hind*III ladder (Thermo Fisher Scientific, Waltham, MA, USA) were separated on a 1.4% (w/w) agarose gel and an electric current with 150 V, 400 mA and 11 W for 2.5 h. For all other PCR approaches, amplicons and GeneRuler 100 bp Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) were separated on a 1% (w/w) agarose gel and an electric current with 100 V and 200 mA and 11 W.

3.5.4 Purification and Sequencing

PCR products were purified using the E.Z.N.A cycle pure kit (Omega Bio-Tek, Norcross, GA, USA) according to the manual. Purified PCR products of 16S rRNA and housekeeping genes were sequenced at GATC Biotech (Konstanz, Germany) using Sanger's chain-termination method (Sanger *et al.* 1977).

3.6 Sequence and fingerprinting analysis

3.6.1 Phylogenetic sequence analysis

Sequences from PCR products were downloaded from GATC Biotech (Konstanz, Germany) and imported into MEGA v7 (Kumar *et al.* 2016). Chromatograms were checked for correct interpretation of signal peaks and reliability of sequence data. Erroneous bases were manually corrected or deleted. BLAST algorithm (https://blast.ncbi.nlm.nih.gov) (Altschul *et al.* 1990; Camacho *et al.* 2009) and the curated EZBioCloud database was used to identify closest related taxa. Sequences were homologized and aligned using ClustalW (Thompson *et al.* 2002) as implemented in MEGA v7. Dendrograms were constructed in MEGA v7 using the neighbor joining (Saitou and Nei 1987) or maximum likelihood (Felsenstein 1981) algorithm with the Jukes-Cantor model (Jukes and Cantor 1969) or maximum parsimony using subtree-pruning-regrafting algorithm (Nei and Kumar 2000) with 100-1000 bootstrap simulations (Felsenstein 1985), respectively.

3.6.2 RAPD-PCR fingerprinting

Intraspecies diversity assessment and differentiation of strains was performed with RAPD-PCR (3.5.2). Gel images were imported into Bionumerics V7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium). Bands were automatically identified, but manually curated. The DNA ladder was used to normalize sample lanes to enable a correct comparison. RAPD-PCR fingerprint pattern similarities were analyzed with UPGMA and Dice's similarity coefficient was used for hierarchical cluster analysis. RAPD-PCR fingerprinting analysis was performed at least twice per strain to assure correct unique random band patterns formed during amplification.

For reliable intraspecies discrimination of *Lc. piscium*, different methods were tested to obtain suitable templates: DNA extraction, liquid overnight cultures and colony material. Colony-based approaches involved direct transfer, FastPrep cell disruptor treatment (M.P Biomedicals, Irvine, CA, USA) or ultrasonic treatment in a Sonorex Super ultrasonic bath (Bandelin, Berlin, Germany).

3.7 Genome analysis

The genomes of two strains of *Lc. piscium* (TMW2.1612 / TMW2.1615), *Le. gelidum* subsp. *gelidum* strain TMW2.1618 and *Le. gelidum* subsp. *gasicomitatum* strain TMW2.1619 were sequenced using Single Molecule Real Time (SMRT) sequencing (3.7.1). The genomes of two strains of *P. carnosum* (TMW2.2021^{T} and TMW2.2029), two strains of *P. phosphoreum* (TMW2.2033 and TMW2.2034), and *P. iliopiscarium* TMW2.2035 were sequenced using Whole Genome Shotgun (WGS, 3.7.2).

3.7.1 Single Molecule Real Time (SMRT) sequencing

Isolated high-molecular weight DNA (3.5.1) was sent to GATC Biotech (Konstanz, Germany) for SMRT sequencing (Eid *et al.* 2009; McCarthy 2010) employing a PacBio RSII sequencer. More than 200 Mb of raw data were generated from one to two SMRT cells (1x120 min movies) employing P4-C2 chemistry and using libraries with an insert size of 8-12 kb. The obtained raw data was assembled using SMRT analysis software v2.2.0 p2 (Pacific Biosciences, Menlo Park, USA) applying Hierarchical Genome Assembly Process (HGAP2/3) protocols (Chin *et al.* 2013). Assemblies were manually curated, evaluated and processed as described in detail online by PacBio (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Finishing-Bacterial-Genomes). Polished assemblies (fasta) were obtained from HGAP3 assembly protocol and subsequently split into respective contigs by BioPerl (http://www.bioperl.com) and the Bio::SeqIO system. Redundancy of contigs was tested using BLAST algorithm (Altschul *et al.* 1990; Camacho *et al.* 2009). BridgeMapper protocol as implemented in SMRT Analysis was used to check correct genome assembly. Contigs were also checked for overlapping ends, coverage behavior and mapping quality using Gepard dotplot software (Krumsiek *et al.* 2007) and SMRT-View 2.30 (Pacific Biosciences, Menlo Park, USA). Non-sense contigs and redundant ones being completely covered by another contig, were discarded. Non-redundant contigs were retained and subsequently circularized if overlapping ends were present. Circularization was carried out by manually introducing an *in silico* break into the contig and reassembled using Minimus2 as implemented in AMOS (http://amos.sourceforge.net). Correct recircularization and integrity of the obtained contigs were evaluated using BLAST algorithm and the Gepard tool. All contigs of a respective genome were merged into one file and used as a template for resequencing using the RS-Resequencing tool as implemented in SMRT analysis. The protocol was repeated until an average reference accordance of 100% was accomplished.

3.7.2 Whole genome shotgun (WGS) sequencing

Isolated genomic DNA (3.5.1) was sent to ZIEL institute (Freising, Germany) and sequencing was carried out using a MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) with PCR-free library preparation. Preparation, processing and assembly with SPAdes V3.9.0 (Bankevich *et al.* 2012) were carried out as described by Huptas *et al.* (Huptas *et al.* 2016). The received fasta file containing all contigs was used for subsequent analysis and annotation.

3.7.3 Annotation and metabolic pathways

The resulting consensus fasta sequence of each genome was used for annotation using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Angiuoli *et al.* 2008) as well as Rapid Annotations using Subsystems Technology (RAST) (Aziz *et al.* 2008; Overbeek *et al.*

2013). Correct annotation of relevant genes was evaluated manually by using smart and protein BLAST algorithm (Altschul *et al.* 1990; Camacho *et al.* 2009). EC (enzyme commission) numbers obtained by RAST were used to feed the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway map pipeline and create metabolic pathways for an early overview.

The entire final predictive metabolic pathways were created manually based on literature without using automatic pipelines. Online databases ExPASy Bioinformatics Resource Portal (https://www.expasy.org/), UniProt (http://www.uniprot.org), BRENDA (http://www.brenda-enzymes.org) and MetaCyc (https://metacyc.org/) were used for looking up correct enzyme names, synonyms, coding gene names and enzyme reactions.

3.7.4 Average nucleotide identity

Average nucleotide identity (ANI) values were calculated with pairwise genome comparison of whole genome shotgun (WGS) sequences of close related species using the ANIb algorithm (Goris *et al.* 2007) implemented within the JspeciesWS web service (Richter *et al.* 2015). A Neighbor-Joining tree based on the resulting distance matrix was constructed in MEGA v7 (Kumar *et al.* 2016).

3.8 Intraspecies assertiveness and interaction of *Lactococcus piscium* strains with spoilers *in vitro*

Intraspecies assertiveness of *Lc. piscium* strains and their interaction with spoilers were carried out in 50 ml of meat simulation medium in a 100 ml narrow-neck Erlenmeyer flask. For inoculation, 100 µl of washed and resuspended cells from cold-adapted cryopreserved stock aliquots, kept in MSM with 34% w/v glycerol at -80 °C until use, were added to each flask. Erlenmeyer flasks were incubated at 4 °C - to simulate the storage temperature of fresh beef products - for 7 days, shaking under oxic conditions with 120 rpm. At each sample time point (day 0/2/4/7), 1 ml of media was taken aseptically from each Erlenmeyer flask for enumeration and identification of microbiota. Decimal serial dilutions were prepared in quarter strength Ringer's solution followed by plating with glass beads (diameter 2.85 - 3.45 mm, Roth, Karlsruhe, Germany) on respective media and cultivation at 25 °C for 48h. For enumeration of microorganisms, plates containing 25 - 250 colonies were taken into analysis (Maturin and Peeler 2001; Sutton 2011).

Intraspecies assertiveness of *Lc. piscium* isolates was tested with 15 different meat-borne strains. These strains were randomly divided in three groups à five strains. Subsequently, a fourth experiment containing the respective most assertive strains from the three groups was conducted. For each group, strains were incubated singly and simultaneously (strain mix) in triplicates in meat simulation medium with an initial inoculation quantity of 6 $\log_{10}$ CFU ml^{-1}, respectively. The initial relative abundance of the different strains was calculated from CFU counts from singly incubated triplicates. For the final relative abundance of each strain at day

7 within the strain mix, 120 colonies (40 per replicate) were subjected to RAPD biotyping (3.6.2).

In competition experiments of *Lc. piscium* strains and different SSO, medium was inoculated with initial quantities of 6 $\log_{10}$ CFU ml^{-1} of *Lc. piscium* and 3 $\log_{10}$ CFU ml^{-1} of spoilers to simulate an average initial contamination scenario of beef carcasses, which is equal of below that value (Lahr 1996; Sofos *et al.* 1999; Zweifel *et al.* 2014; Reid *et al.* 2017). In this set, medium was inoculated in triplicates with a single organism or co-culture of a *Lc. piscium* strain and a SSO, respectively. Enumeration was carried out on BHI and respective selective media. Selectivity was validated by identifying colonies via MALDI-TOF MS (3.3).

Statistical evaluation of growth reduction data was performed with SigmaPlot v12.5 (Systat Software GmbH, Erkrath, Germany). Data was considered normally distributed if $p>0.05$ and significantly different if $p<0.05$ using the Shapiro-Wilk normality test and the *t*-test, respectively.

3.9 Chemotaxonomic analysis of photobacteria

Analysis of cellular fatty acids and respiratory quinones was carried out for *P. carnosum* TMW2.2021^T. Cellular fatty acids were also determined for other type strains of different *Photobacterium* species. Cell material for the analyses was obtained from cells grown aerobically for 72h at 15 °C in marine broth followed by freeze-drying of biomass. Fatty acid methyl esters were extracted using a modified method of Miller (Miller 1982) and Kuykendall (Kuykendall *et al.* 1988). Separation and identification was performed on a Sherlock Microbial Identification System (MIDI, Microbial ID, Newark, DE 19711 USA). Fatty acid names and percentages were calculated by the MIS Standard Software (Microbial ID). Respiratory quinones were extracted using the two stage method described by Tindall (Tindall 1990a; 1990b) and identified on a LDC Analytical (Thermo Separation RP18) HPLC. Analyses of respiratory quinones and cellular fatty acids were carried out by the Identification Service, Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

3.10 Phenotypic characterization of photobacteria

For description of the proposed novel *Photobacterium* species, *P. carnosum*, various phenotypic traits were analyzed. The four strains of the proposed *P. carnosum* sp. nov. as well as reference strains of closest related species (*P. iliopiscarium* DSM 9896^T, *P. phosphoreum* DSM 15556^T, *P. kishitanii* DSM 19954^T, *P. piscicola* LMG 27681^T, *P. aquimaris* DSM 23343^T) based on 16S rRNA genes and MLSA of housekeeping genes (Table 3, Table 4) were included for comparative phenotypic characterization. Reference strains were obtained from German (DSMZ) and Belgian (BCCM/LMG) strain collections as indicated by strain designations, respectively.

Cell morphology was investigated with a Zeiss Axiostar Plus microscope with 100x magnification and documented with an Axio Cam ICc1 and the Axio Vision Rel. 4.8 software (all Carl Zeiss Microscopy GmbH, Jena, Germany). Motility was tested with the soft agar stabbing method in tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl (0.3% (w/v) agar) at 15 ºC for 5 days. Additonally, cells in wet mounts were investigated for motility under the microscope. Bacteria were screened for luminescence by observing colonies grown on MB agar and *Photobacterium* broth agar at 15 °C for three days in a dark chamber.

The ability to grow on different media (3.2.5) was tested on MB agar, *Photobacterium* broth agar, brain-heart infusion agar, thiosulfate citrate bile salts sucrose agar and tryptic soy agar supplemented with 2% (w/v) NaCl at 15 ºC. Growth at different temperatures (0-37 ºC) and within a pH range (5-9 at 0.5 intervals) at 15 ºC was tested in MB agar. The pH of the MB agar was maintained by addition of sodium phosphate-citrate buffer for pH 5.0, Na_2HPO_4/NaH_2PO_4 buffer for pH values from 5.5-8.0 and $Na_2CO_3/NaHCO_3$ buffer for pH values from 8.5-9.0, respectively (Gomori 1955). The pH was adjusted before and after sterilization (121 ºC, 15min), if necessary. Growth in the presence of different NaCl concentrations was tested by incubation on TSA at 15 ºC, supplemented with 0-10% (w/v, at 1% intervals, including 0.5%). Growth under anoxic conditions was tested in MB agar at 15 ºC, using anaerobic gas generating bags (Oxoid AnaeroGen 2.5L, Thermo Scientific). Growth within the different phenotypic tests was monitored for 20 days, however results did not change from day 6 on.

Utilization of various carbon sources was assessed using the API 50 CH test (bioMérieux, Marcy-l'Étoile, France), performed according to the instructions from the manufacturer, except the inoculum was prepared by suspending the cells from an overnight culture in marine broth, supplemented with 0.17g/L bromocresol purple, 1 ml/L Tween 80 and 56 mg/L manganese sulphate. Cultures were overlaid with mineral oil to obtain anoxic conditions. Enzymatic activities were investigated with the API ZYM test (bioMérieux, Marcy-l'Étoile, France), performed according to the instructions from the manufacturer, suspending the cells from an overnight culture in a 0.85% (w/v) NaCl solution, and incubating the strips for 5.5h at 15 ºC. Catalase activity was evaluated by the formation of air bubbles from colonies in contact with 3% (v/v) aqueous hydrogen peroxide solution (Smibert and Krieg 1981). Oxidase activity was tested by spreading colony material on oxidase discs, soaked in N,N-dimethyl-p-phenylenediamine oxalate and α-naphtol (Oxidase Test, Sigma-Aldrich).

4 Results

4.1 Monitoring of spoilage-associated microbiota on MAP beef

Two sample batches of MAP beef steaks were obtained directly from a producer to guarantee freshness of the samples and exclude interruption of the cooling chain. Samples of each batch were stored for 21 days at constant 4 °C and 10 °C, respectively and sampled at different time points. Headspace atmosphere composition, pH value, contamination level and composition of the spoilage-associated microbiota were determined at all time points (3.2.1), and a simple sensorial assessment was carried out.

4.1.1 Sensorial evaluation

Beef steak samples of both batches had a Minimum Shelf Life (MSL) assigned by the producer to 7 days. First signs of spoilage in terms of a beginning green discoloration and off-odor were detectable on day 10 (batch 1) and 8 (batch 2) when stored at proper temperature (4 °C) and on day 6 (batch 1) and day 4 (batch 2) when stored inadequately at 10 °C. At late stage of incubation, beef steaks displayed an evenly green discoloration and sweet-sour (4 °C) to putrid (10 °C) off-odor with the exception of one package at day 21 stored at 10 °C, which showed a dark red color and rotten smell. In some packages, slime and even visible colonies could be detected.

4.1.2 Identification of spoilage microbiota and monitoring of spoilage-associated parameters

Detailed growth dynamics of spoilage-associated microbiota on two different beef bulk samples were monitored at 4 and 10 °C for 21 days. Identification of approx. 8,000 isolates based on their low molecular weight subproteome MSPs revealed the typical spoilage-associated microbiota over time at initial, early, mid and late stage of spoilage at proper and inadequate storage temperature, respectively. In total, 32 different species from 16 different genera were identified and only 213 isolates (2.7%) remained as ‘not reliably identified’ (*nri*) based on low MALDI Biotyper score values. MALDI-TOF MS Biotyper identification accuracy of spoilage-associated microbiota was validated by comparison with a hierarchical cluster analysis (3.3.4) of their mass spectrometry profiles (Figure 2). All highly abundant species showed coherent clusters that diverge from other species thereby verifying the identification of MALDI Biotyper albeit representatives of some species were represented in multiple clusters.

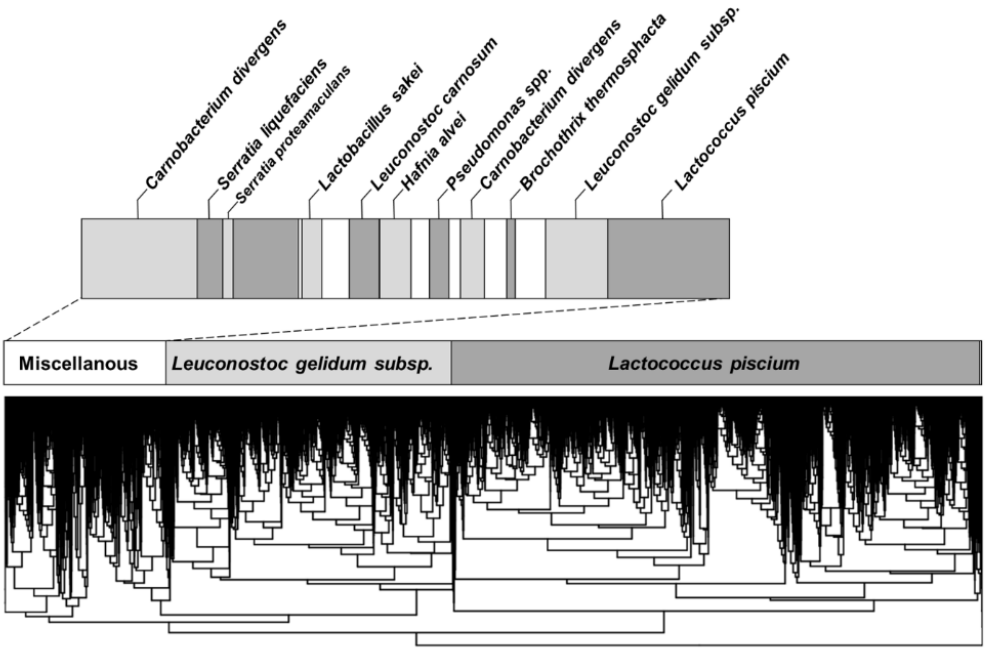

Figure 2| Hierarchical MSP cluster analysis of all isolates (n=7916) obtained via MALDI-TOF MS during growth dynamics experiments on MAP beef steaks. White areas represent mixed cluster including "not reliably identified" (nri), minor groups and singletons. MSP were matched into cluster based on different Euclidean distance matrices.

Figure 3 shows the relative species abundance and TVC of samples from both batches and incubation temperatures (batch 1: 4 °C, **A**; 10 °C, **B**; batch 2: 4 °C, **C**; 10 °C, **D**). The initial TVC was approx. 4 $\log_{10}$ CFU g^{-1} in both batches. At initial and early stage, spoilage-associated microbiota comprised of a group of organisms with minor abundance and partly not present in the database labeled as 'Others'. Organisms in this group were e.g. *Acinetobacter johnsonii* and *Stenotrophomonas* spp. This group vanished for the most part in subsequent stages of spoilage resulting in a decreased diversity. *Lactococcus* (*Lc.*) *piscium* and *Leuconostoc* (*Le.*) *gelidum* subsp. *gasicomitatum & gelidum* were identified as the most prominent spoilage-associated species in both batches during shelf life and beyond. Notably, their dominance was altering between both batches. Prevalence of *Lc. piscium* was enhanced on beef stored at 10 °C. If *Le. gelidum* subsp. were detectable as initial contaminants (batch 2), these organisms were able to dominate the spoilage-associated microbiota over three weeks of incubation at 4 °C. Also, *Carnobacterium (C.) divergens* represented a minor fraction of spoilage-associated microbiota but was present during all spoilage stages in both batches at 4 °C storage and in batch 1 at 10 °C. Starting at mid stage, a community shift was observed during late stage of spoilage on beef stored at 10 °C from LAB towards *Enterobacterales*. These bacteria were identified as *Hafnia* (*H.*) *alvei* (batch 1, Figure 2B and batch 2, Figure 2D) and *Serratia (S.) liquefaciens* (batch 2). Interestingly, *Pseudomonas* (*Ps.*) spp. were detected in one replicate in the late stage of spoilage (batch 2) concomitantly with dark red beef color and slime despite

low oxygen (~1%) and high carbon dioxide (~90%) concentrations when present as initial contaminants in the batch. The dominant biotype was identified as *Ps. weihenstephanensis* via sequencing of *rpo*D. Several *Pseudomonas* sp. isolates displayed low MALDI Biotyper score values (<2) and consistent species identification was not always possible in consecutive repeated analyses of these isolates allowing a reliable assignment only on genus level. Consequently, all isolates were defined as *Pseudomonas* spp.

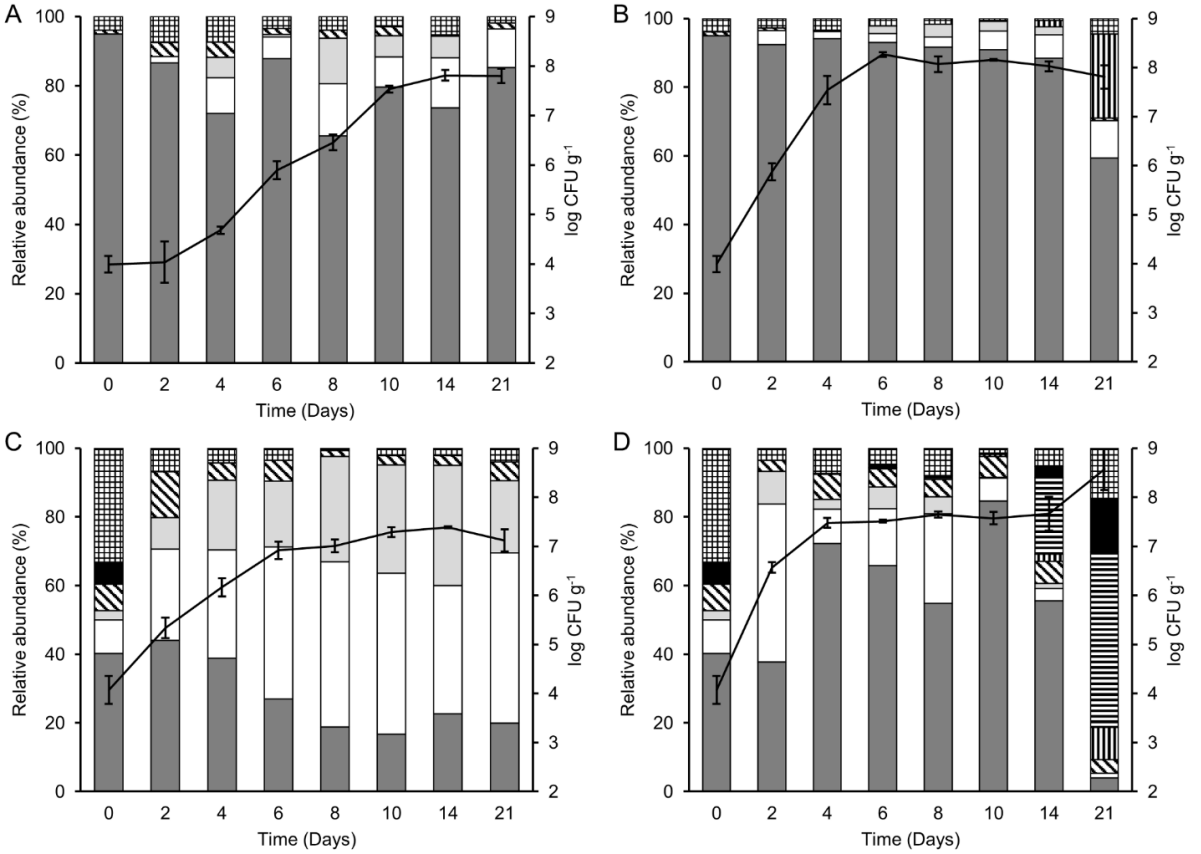

Figure 3| Relative species abundance of all isolates identified via MALDI-TOF MS during growth dynamics experiments on MAP beef steak. Two different beef batches were analyzed at 4 and 10 °C. batch 1: 4 °C, **A**; 10 °C, **B**; batch 2: 4 °C, **C**; 10 °C, **D**. CFU values are shown on secondary y-axis as mid values of triplicates with standard error. "Others" include "not reliably identified" (*nri*) and species with minor abundance. (■) *Lactococcus piscium*, (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▣) *Leuconostoc gelidum* subsp. *gelidum*, (▧) *Carnobacterium divergens*, (■) *Pseudomonas* spp., (▥) *Hafnia alvei*, (▤) *Serratia liquefaciens*, (▦) Others.

Total viable count (TVC) was carried out on BHI agar (3.2.5.1) at 25 °C. Initial contamination levels of both batches were 4 $\log_{10}$ Colony Forming Units (CFU) g^{-1}. Beef samples stored at 10 °C showed a quicker increase in CFU than samples stored at a proper temperature of 4 °C. Microbial contamination levels reached a maximum of 8 and 7 $\log_{10}$ CFU g^{-1} on day 6 to 10 following stagnation with only minor in-/decrease in CFU in batch 1 and 2, respectively. The only exception were samples of batch 2 stored at 10 °C that showed an

increase of CFU at the moment of the community shift due to strong growth of *S. liquefaciens* and *Pseudomonas* spp. during late stage spoilage.

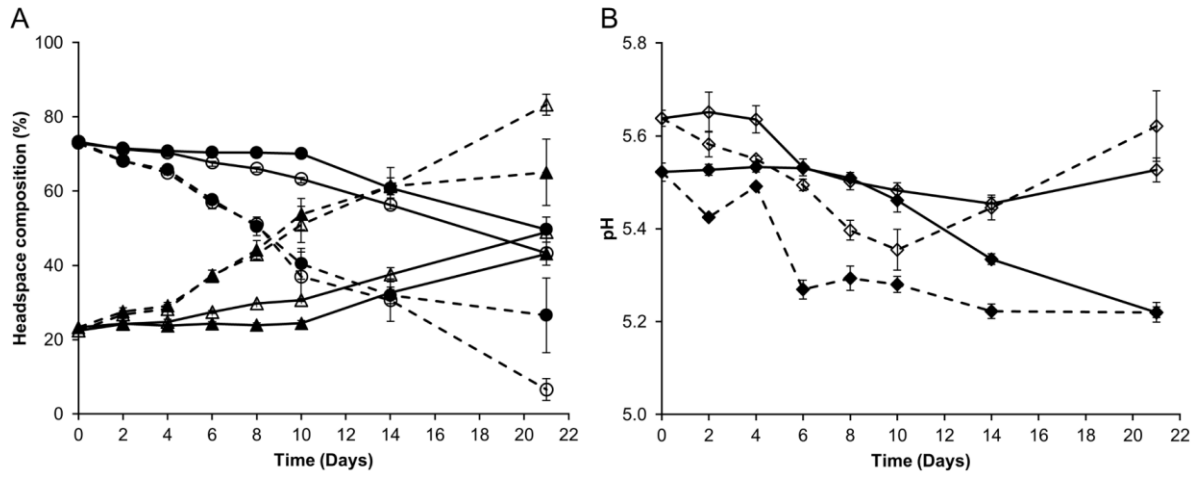

Figure 4| Development of headspace atmosphere (A) and pH (B) during growth dynamics monitoring on MAP beef steaks. Filled symbols, batch 1; empty symbols, batch 2; solid lines, 4 °C; dashed lines, 10 °C; (○) O_2; (△) CO_2; (◇) pH; values are shown as mid values of triplicates with standard error.

Development of headspace atmosphere composition was monitored during 21 days of incubation (Figure 4A). Oxygen decrement occurred with concomitant carbon dioxide evolution. Beef samples stored at 4 °C retained the initial atmospheric composition till day 6 (batch 2) and 10 (batch 1). After that, a linear decrease of oxygen and increase of carbon dioxide concentration was observed while cell counts were stagnant. Samples stored at 10 °C displayed a continuous change in headspace gas composition from the beginning of incubation while a substantial oxygen decrease occurred after day 4 in both batches. At each of these time points, the contamination level rose above 7 $\log_{10}$ CFU g^{-1}.

In addition to headspace atmosphere, pH values from beef samples were monitored during 21 days of incubation (Figure 4B). Samples from batch 1 had an initial pH value of approx. 5.5. During storage, pH values decreased at both temperatures to 5.2 at day 21. At 10 °C, decrease was steady, while samples stored at 4 °C retained the initial pH value for 8-10 days. A substantial pH value change occurred concomitantly with a change in the headspace atmosphere. Samples from batch 2 showed a different pH value development. The initial pH value of approx. 5.65 was higher compared to batch 1 and the decrease in pH was followed by a renewed increase in the late stage of storage. While the increase was only slightly in samples stored at 4 °C, a substantial rise was monitored at 10 °C incidental with the community shift from LAB to *Enterobacterales* in the late stage (Figure 3). In both batches, the change of headspace atmosphere composition and pH was concomitant with the perception of beginning onset of spoilage.

4.1.3 Cultivation of potentially psychrophilic spoilage-associated microbiota

At two sample time points (day 2 and 6) during growth dynamics analysis of batch 2, cultivation of spoilage-associated microbiota was carried out additionally at 4 °C for isolation of potentially psychrophilic bacteria present on beef samples, not being able to grow at 25 °C, and thus being neglected in routine hygiene controls. Figure 5 shows a comparison of the relative species abundance and total viable count between both cultivation temperatures. The microbiota from both cultivation temperatures consisted of the same species of *Lc. piscium*, *Le. gelidum* subsp. *gelidum & gasicomitatum* and *C. divergens*. The total cell count was slightly elevated when plates were cultivated at 4 °C. However, relative abundance of *Le. gelidum* subsp. *gelidum* was substantially increased when the lower isolation temperature was applied.

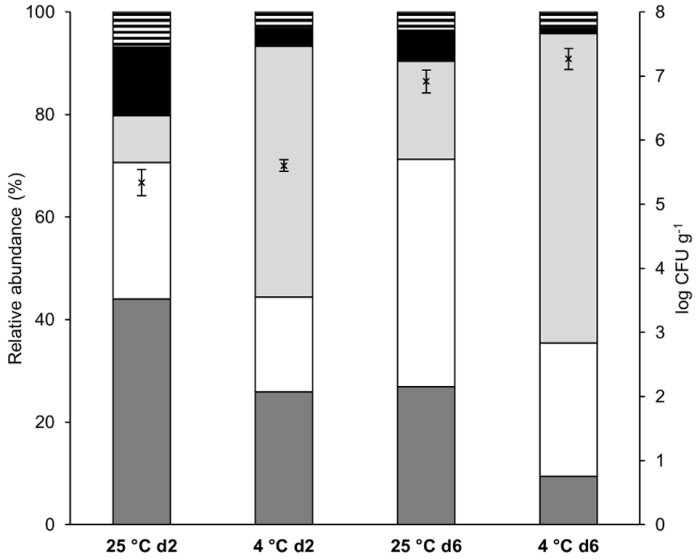

Figure 5| Composition of spoilage-associated microbiota from beef steaks isolated with different cultivation temperatures. Cross, CFU values are shown on secondary y-axis as mid values of triplicates with standard error; d, day. (■) *Lactococcus piscium,* (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) *Leuconostoc gelidum* subsp. *gelidum*, (■) *Carnobacterium divergens*, (▤) Others.

In total, 571 isolates were obtained from beef samples at 4 °C incubation. In order to test whether the differences in relative species abundance was caused by a long incubation period rather than lower temperature, all isolates were transferred to new plates and incubated in parallel again at 4 and 25 °C. Plates were screened for visible growth after 14 days of incubation. Out of 571 isolates, 39 showed growth only at 4 °C but not at 25 °C. Identification via MALDI-TOF MS revealed that all of these isolates were *Le. gelidum* subsp. *gelidum*. Subsequently, selected isolates were subjected to RAPD-PCR biotyping (3.6.2) and displayed a single band pattern divergent from the isolates cultivated at 25 °C (Figure A2). Finally, growth of the potentially psychrophilic strain (designated TMW2.1998) at different temperatures from -5 to +30 °C in 5 degree steps was quantified via monitoring of optical density OD_{590} in liquid BHI broth containing 5% glycerol to prevent freezing at (sub)zero temperatures (Figure 6).

Optimal growth temperature was 15 °C. At 4 °C, growth was delayed for four days, but reached the highest OD after 14 days of incubation. At 25 °C, growth was weaker and delayed compared to lower temperatures. No growth at 30 °C was observed. At (sub-)zero temperatures, clearly visible growth in terms of cell pellets were observed at 0 and -5 °C after three and four weeks, respectively. On plates, optimal growth was also at 15 °C, while no growth occurred at 25 °C. In contrast, other strains of *Le. gelidum* subsp. *gelidum* & *gasicomitatum* and *Lc. piscium* isolated in this study were able to grow rapidly at 30 and 25 °C in liquid medium and on plates, respectively. Water activity of plates and liquid BHI with 5% glycerol was 0.974 and 0.976.

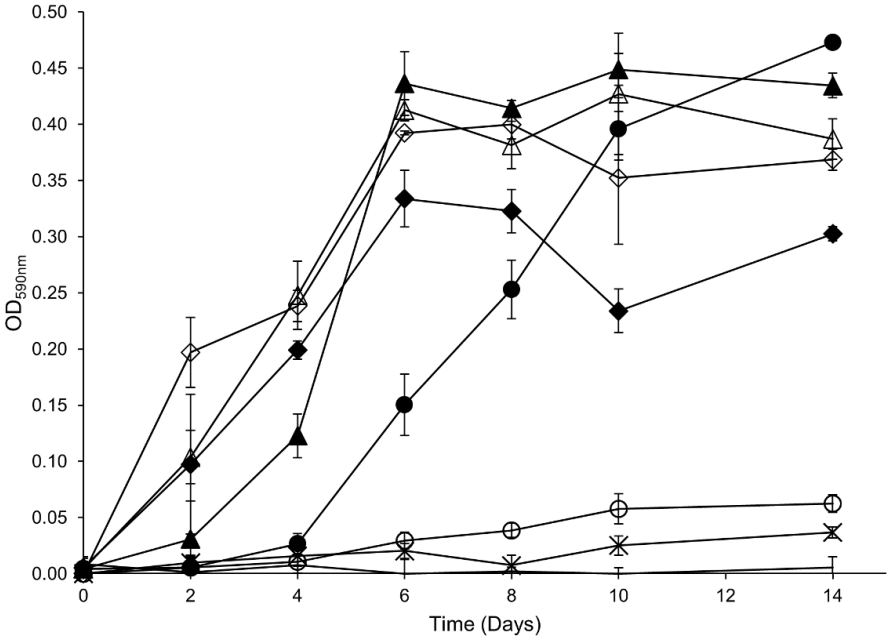

Figure 6| Growth of psychrophilic *Le. gelidum* subsp. *gelidum* isolate TMW2.1998 at different temperatures in liquid BHI broth with 5% glycerol (v/v). OD, optical density at 590 nm. Values are shown as mid values of triplicates with standard error. (**x**), -5 °C; (○) 0 °C; (●), 4 °C; (▲), 10 °C; (△), 15 °C; (◇), 20 °C; (◆), 25 °C; solid line, 30 °C.

4.1.4 Comparison of microbiota on top and bottom of beef steaks

Surface conditions for spoilage-associated microbiota may differ on top and bottom of the beef due to a suspected variant in moisture, oxygen availability and redox potential. The impact on the bacterial community was studied on four different samples during different stages of spoilage by intersecting beef steak samples from a third batch and analyzing the resulting halves separately. The relative species abundance and contamination level is shown in Figure 7. Contamination levels were slightly elevated on the upper surface of the beef in all samples analyzed. Microbial composition was highly similar on top and bottom. All species detected on top were also detected on bottom and vice versa.

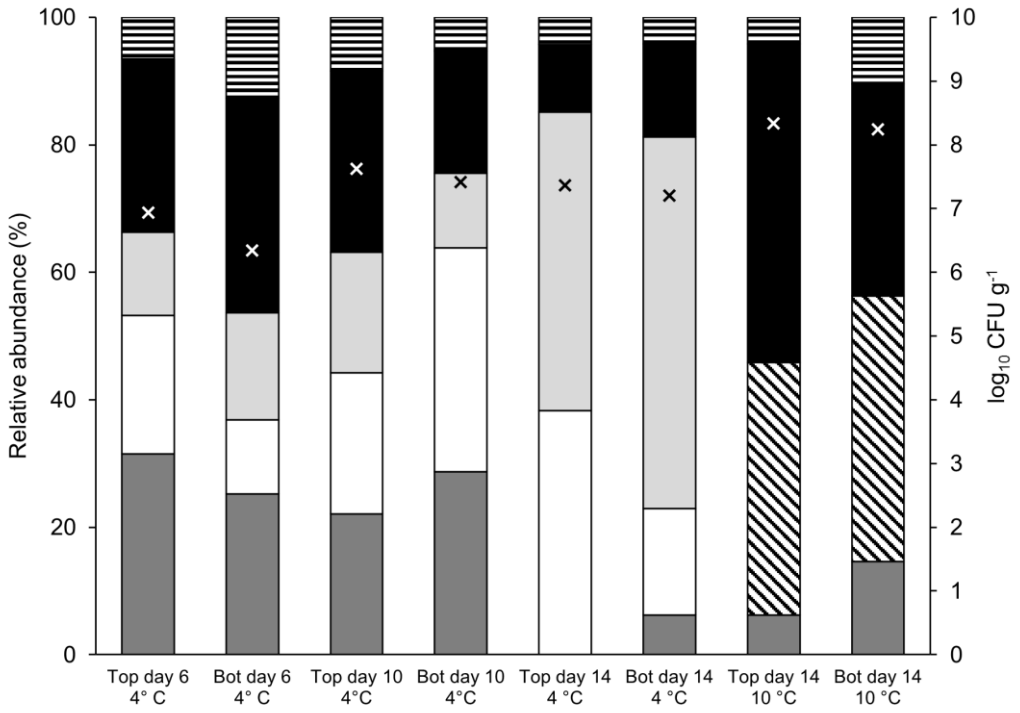

Figure 7| Comparison of spoilage-associated microbiota on top and bottom of beef steaks. Cross, CFU value (secondary y-axis); top, upper beef surface; bot, bottom side of beef surface. (■) *Lactococcus piscium,* (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▩) *Leuconostoc gelidum* subsp. *gelidum*, (■) *Carnobacterium divergens*, (▧) *Serratia liquefaciens*, (▤) Others.

4.1.5 Diversity assessment of beef steak isolates below species level

Diversity below (sub-)species level was carried out via RAPD biotyping (3.6.2). Differences in electrophoretically-separated random band patterns indicate abundance of different strains within the isolates. Isolates were chosen from sub-clusters of each species within the HCA of their low-molecular subproteome. Table 5 shows the number of unique RAPD band patterns for each species, respectively.

Lc. piscium isolates showed four different RAPD band patterns (Figure A1). All analyzed isolates from the small cluster of MALDI HCA had the same RAPD pattern, which was also formed from isolates as a sub-cluster in the big cluster. *Le. gelidum* subsp. isolates formed three unique band patterns each (Figure A2). Notably, all potentially psychrophilic isolates of *Le. gelidum* subsp. *gelidum* displayed a single pattern that was different from the patterns formed by isolates from the growth dynamics isolated at 25 °C. *Pseudomonas* spp. were isolated from one package of late spoilage stage were highly diverse (multiple species possible) and formed eight different RAPD band patterns (Figure A3). Isolates of the dominant band pattern were identified as *Ps. weihenstephanensis* via *rpo*D gene sequencing (99.6% amino acid identity). Regarding *H. alvei* and *S. liquefaciens*, only a single band pattern was detected suggesting the abundance of a single strain of the respective species (Figure A4). Isolates from both species were isolated from day 21 in batch 1 and 2, respectively.

Table 5| RAPD biotyping for diversity assessment below species level.

Species	No. of isolates analyzed	No. of unique RAPD patterns
Lactococcus piscium total	20	4
Lc. piscium SC[a]	10	1
Lc. piscium BC[b]	10	4
Leuconostoc gelidum		
subsp. *gelidum*	10	2
subsp. *gelidum* Psy[c]	11	1
subsp. *gasicomitatum*	10	3
Serratia liquefaciens	8	1
Hafnia alvei	8	1
Pseudomonas spp.	23	8

[a]small cluster of MALDI hierarchical cluster analysis
[b]big cluster of MALDI hierarchical cluster analysis
[c]psychrophilic isolates

4.1.6 Discriminatory power of MALDI-TOF MS on strain level

Discriminatory power of MALDI-TOF MS was evaluated with 14 isolates of *Lc. piscium* from the four distinct patterns obtained by RAPD-PCR biotyping (Table 5). Figure 8A shows the hierarchical cluster analysis of these 14 isolates measured as technical triplicates using liquid extraction method of overnight cultures (3.3.1). All isolates that formed a distinct RAPD-biotype also showed a cohesive clustering for each other (except for TMW2.1612, designated as isolate 7 in the analysis), but a distinct clustering from others in the hierarchical cluster analysis.

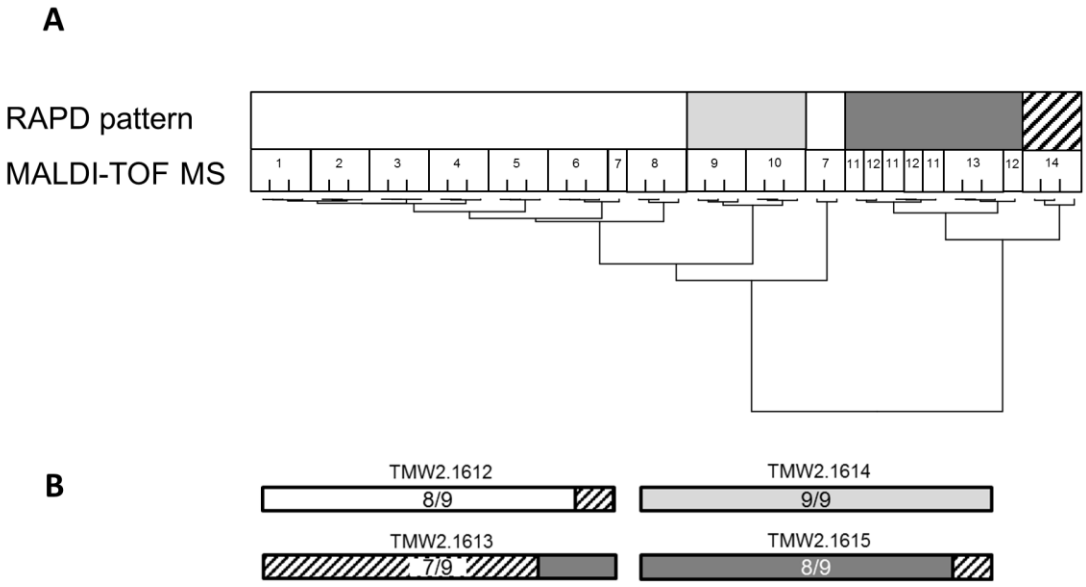

Figure 8| MSP Hierarchical cluster analysis (A) and strain identification (B) of *Lactococcus piscium* strains using a liquid extraction method for MALDI-TOF MS. All *Lc. piscium* isolates were measured in triplicates and serially numbered 1-14. RAPD patterns of isolates were compared to the MALDI-TOF MS cluster analysis (A). For strain identification (B), one representative was chosen from each RAPD biotype, respectively and measured in biological as well as technical triplicates. The number given represents the number of correct identification by MALDI-TOF biotyper. (□) TMW2.1612; (▨) TMW2.1613; (□) TMW2.1614; (■) TMW2.1615.

From each of the unique RAPD patterns, a representative isolate was chosen and measured in biological as well as technical triplicates yielding nine MSPs, respectively (Figure 8B), in order to test the correct identification rate of the MALDI Biotyper using liquid extraction. *Lc. piscium* strain TMW2.1614 was identified correctly in 9/9 instances, TMW2.1612 and TMW2.1615 in 8/9 instances and TMW2.1613 in 7/9 instances. The experiment was also conducted using the direct transfer method with on target extraction and showed that no consistent identification was possible on strain level.

4.1.7 Random retail beef samples

In addition to sample batches directly obtained from a producer, random retail beef steak samples with different use-by dates from four different supermarkets in the local area of Freising, Bavaria were obtained for comparative analysis and handled in the same manner as the batch samples (3.2.1.3).

Headspace atmosphere composition, pH, CFU and relative species abundance were determined. Samples with an expired use-by date were deliberately stored in thermostatic cabinets in the lab. An overview of the random retail samples and measured parameters is shown in Table 6.

Table 6| Overview of random retail beef steak samples.

Origin	Sample	Days to use-by date	O_2 (%)	CO_2 (%)	pH
Retailer 1	#4	5	71	18	5.55
Retailer 1	#1	2	70	25	5.51
Retailer 2	#6	6	73	22	5.57
Retailer 2	#10	5	70	18	5.72
Retailer 2	#9	3	73	18	5.5
Retailer 2	#8	1	68	18	5.51
Retailer 2	#11	-2	70	16	5.55
Retailer 2	#13	-4	61	32	5.42
Retailer 3	#7	6	70	25	5.58
Retailer 3	#5	3	69	24	5.48
Retailer 4	#2	6	70	25	5.75
Retailer 4	#12	4	69	19	5.53
Retailer 4	#3	2	70	20	5.57

The headspace atmosphere of all beef steak samples consisted of ~70% oxygen and 20-25% carbon dioxide and pH values ranged from ~5.5-5.7 except Sample #13, which displayed a reduced oxygen content (61%), elevated carbon dioxide content (32%) and a lower pH (5.42). This sample was four days over the use-by date and the only sample with perceptible spoilage signs in form of green discoloration. Contamination level and relative species abundance are shown in Figure 9 and Figure 10, respectively.

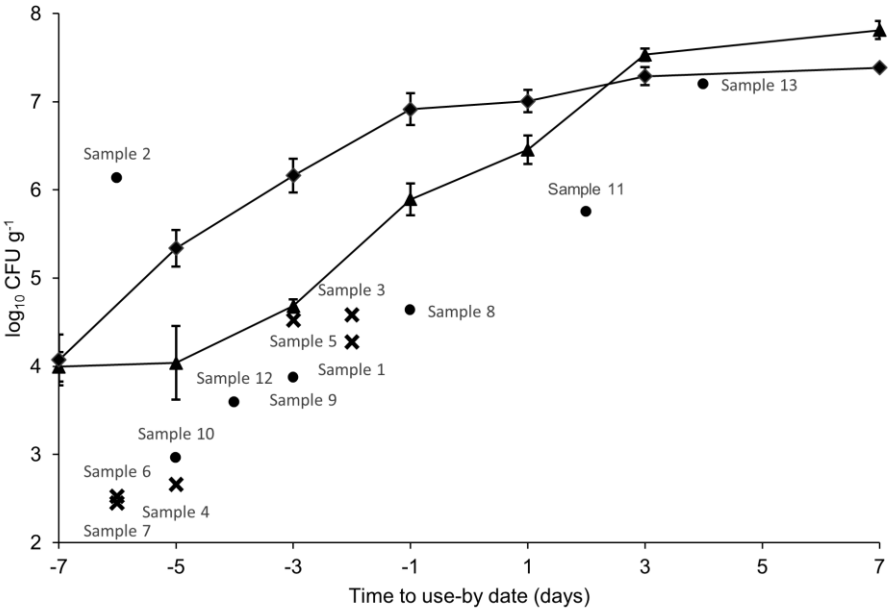

Figure 9| Contamination level of randomly obtained beef steak retail samples and batch samples from growth dynamics. (▲), batch 1; (◆), batch 2; (●) and (x), random samples; (x) shows the detection limit – actual contamination level is below that value.

Most of the randomly obtained samples showed a lower contamination level than the batch samples of the growth dynamics analysis. In about half of the random samples (6 of 13), the contamination was so low, that no bacteria were detected and only the detection limit could be assigned.

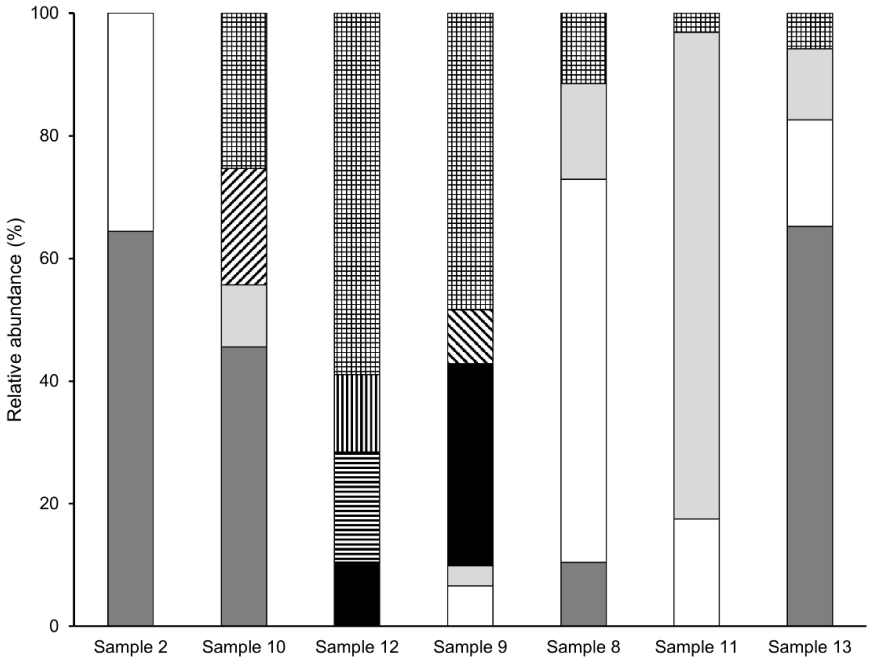

Figure 10| Relative species abundance of randomly obtained retail beef steaks. "Others" include "not reliably identified" (*nri*) and species with minor abundance. (■) *Lactococcus piscium*, (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▒) *Leuconostoc gelidum* subsp. *gelidum*, (▨) *Carnobacterium divergens*, (▧) *Chryseobacterium* spp., (■) *Arthrobacter* spp., (▥) *Rhodococcus* spp., (▤) *Pseudoclavibacter helvolus*, (▦) Others.

The majority of random beef steak samples (Sample 2, 8, 10, 11, 13) were dominated by either *Lc. piscium* or *Le. gelidum* subsp. and *C. divergens* (Sample 10). The spoilage-associated microbiota was therefore similar to the microbiota identified during detailed growth dynamics analysis of batch samples (4.1.2). Sample 9 and 12 however, showed a completely different composition with *Arthrobacter* spp., *Chryseobacterium* spp., *Rhodococcus* spp. and *Pseudoclavibacter helvolus* as spoilage-associated microbiota.

In order to locate and visualize the spacial distribution of microbiota on the beef steaks, contact plates were used for surface sampling of random as well as batch samples (Figure 11). Incubated contact plates from a random retail sample (**A-C**) showed that the bacteria are located solely at the outside of the beef steak. Spoilage e.g. green discoloration (**E**) also began to form from the edges of the beef steak. In comparison, a contact plate from a batch sample (**D**) and spoiled beef steak of the sample batch (**F**) showed an even distribution of bacteria on the surface and homogenous green discoloration.

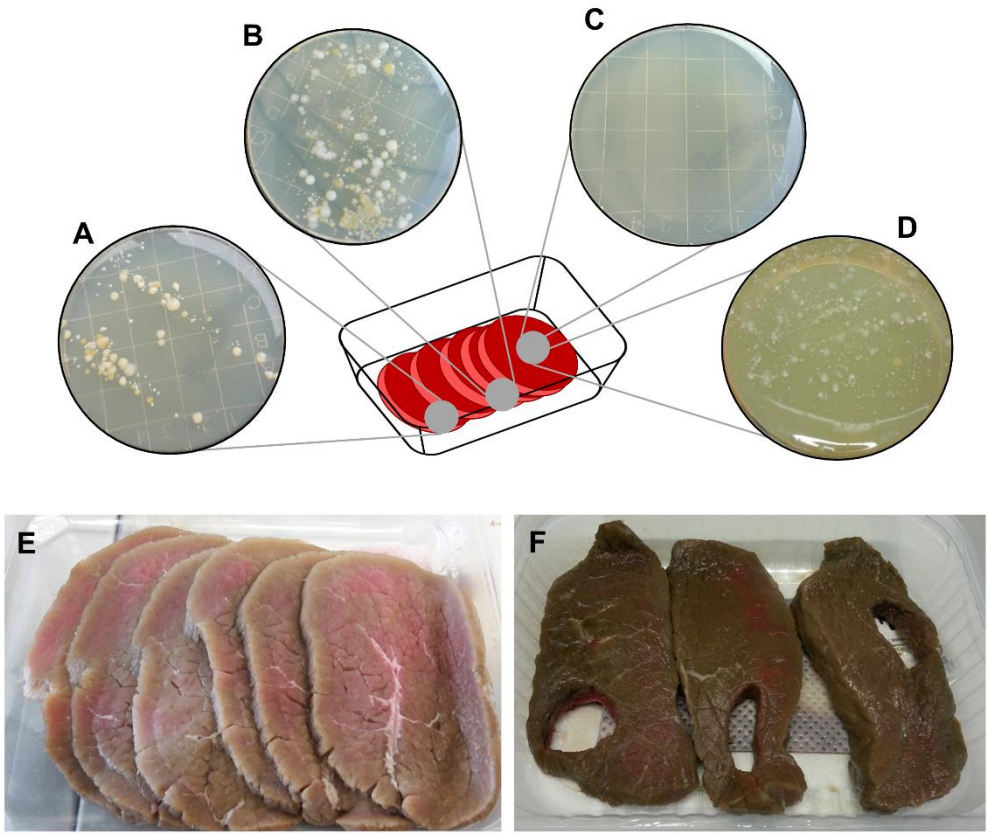

Figure 11| Localization and spacial distribution of microbiota on beef steaks. Contact samples from random retail sample (A-C) and batch sample (D); spoiled retail sample (E); spoiled batch sample (F).

4.2 Monitoring of spoilage-associated microbiota on MAP minced beef

Two sample batches of MAP minced beef with a fat content of 10-12% were analyzed in the same manner as the beef steaks (3.2). Fresh samples were obtained directly from the same producer and stored for 14 days at constant 4 °C and 10 °C, respectively and sampled at different time points. Headspace atmosphere composition, pH value, contamination level and composition of the spoilage-associated microbiota were determined at all sample time points, and a simple sensorial assessment was carried out.

4.2.1 Sensorial evaluation

Minced beef samples of both batches had a use-by date assigned by the producer to 3 days, respectively. Perceptible spoilage in terms of brownish discoloration and rancid-sweaty, sour-sweet off-odor were detected on day 8 (batch 1) and 6 (batch 2), when stored at proper temperature (4 °C) and on day 4 (batch 1) and 3 (batch 2), when stored at inadequate temperature (10 °C). At very late stage of storage, evenly brownish-grey discoloration or green discoloration of single fibers as well as putrid off-odor was perceptible.

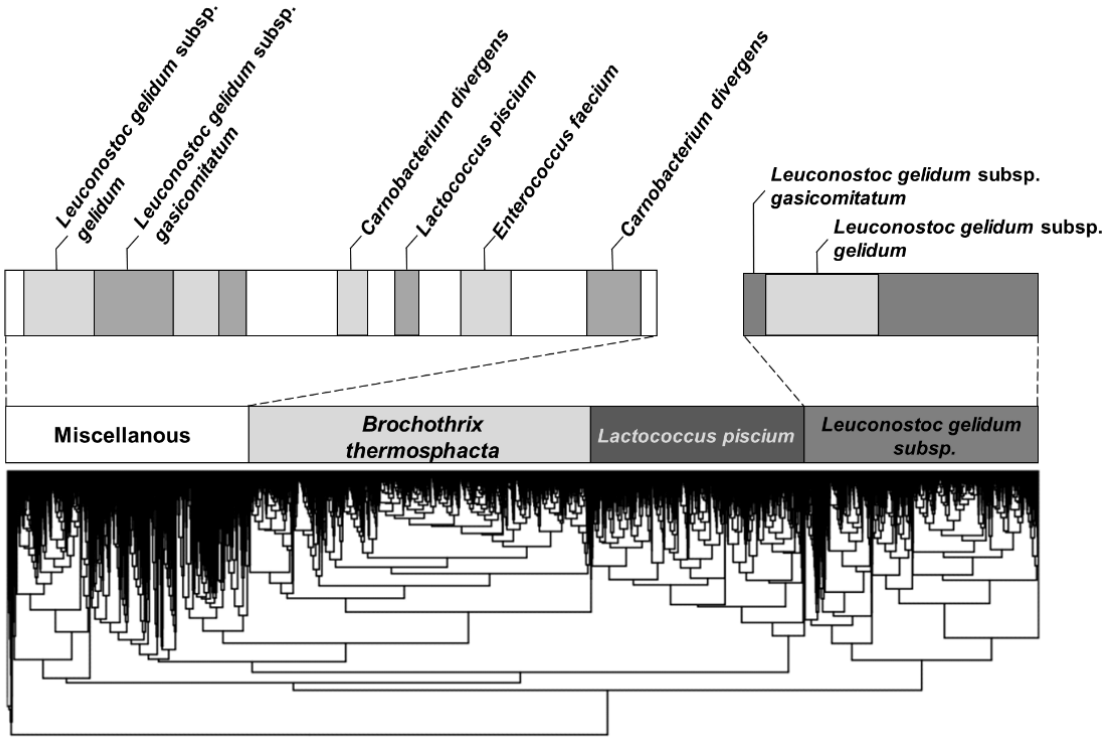

Figure 12| Hierarchical MSP cluster analysis of isolates of the dataset without *Pseudomonas* spp. (n=5801) obtained via MALDI-TOF MS during growth dynamics experiments in MAP minced beef. White areas represent mixed cluster including “not reliable identification” (nri), minor groups and singletons. MSPs were matched into cluster based on different Euclidean distance matrices.

4.2.2 Identification of spoilage-microbiota and monitoring of spoilage-associated parameters

Detailed growth dynamics of spoilage-associated microbiota on two different minced beef bulk samples were monitored at 4 and 10 °C for 14 days, respectively. Identification of approx. 9,600 isolates based on their low molecular weight subproteome MSPs revealed the typical spoilage-associated microbiota over time at initial, early, mid and late stage of spoilage at proper and inadequate storage temperature, respectively.

In total, at least 45 different species from 26 different genera were identified and 524 isolates (5.4%) remained as “not reliably identified” based on low MALDI Biotyper scores (<1.7). All MSPs were split into two different datasets due to computing power limits and subjected to a hierarchical cluster analysis (3.3.4) to validate the accuracy of the MALDI Biotyper identification. One dataset comprised spectra identified as *Pseudomonas* spp. and the other one all other species. MSPs that were not reliably identified were included in both of the data sets. All species that were highly abundant formed coherent and distinct cluster within the hierarchical cluster analysis (Figure 12), except for *Pseudomonas* spp. (Figure 13). Within this genus, MSPs were identified as 30 different species via MALDI Biotyper partially with low scores (<2) allowing no definite species identification. Most species were scattered throughout the different cluster, although certain MSPs identified as *Ps. fragi* also formed a distinct cluster. Reliable identification on species level was not possible using only MALDI-TOF MS Biotyper, although subsequent polyphasic analyses (4.2.5) allowed identification on species level. As a consequence of not reliable identification on species level by MALDI-TOF MS, isolates were combined to *Pseudomonas* spp. for assessment of relative species abundance.

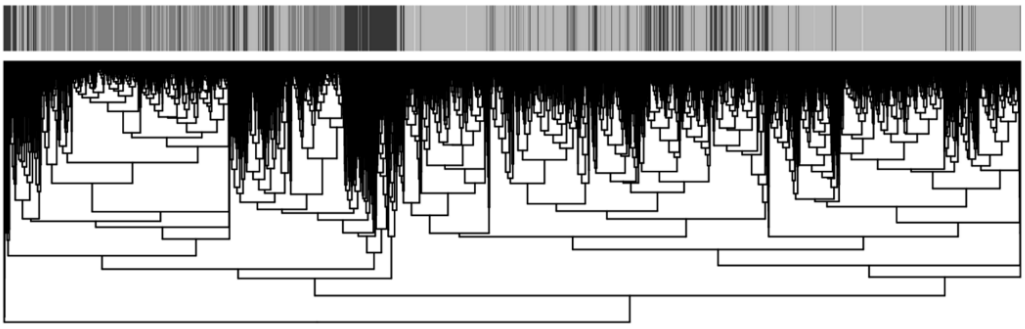

Figure 13| Hierarchical MSP cluster analysis of isolates of the dataset *Pseudomonas* spp. (n=4327) obtained via MALDI-TOF MS during growth dynamics experiments in MAP minced beef. MSP were matched into cluster based on different Euclidean distance matrices. MALDI Ident shows the identification of the Biotyper whereas each different grey scale represents different species.

Figure 14 shows the relative species abundance and TVC from both minced beef sample batches stored at different temperatures (batch 1: 4 °C, **A**; 10 °C, **B**; batch 2: 4 °C, **C**; 10 °C **D**). At the initial stage, a group of organisms labeled as “others” made up about 10% of the

microbiota. These diverse group comprises organisms not present in the database (*nri*) and minor abundant ones that vanished in later stages of spoilage e.g. *Stenotrophomonas* spp., *Janthinobacterium* spp. and *Acinetobacter* spp.

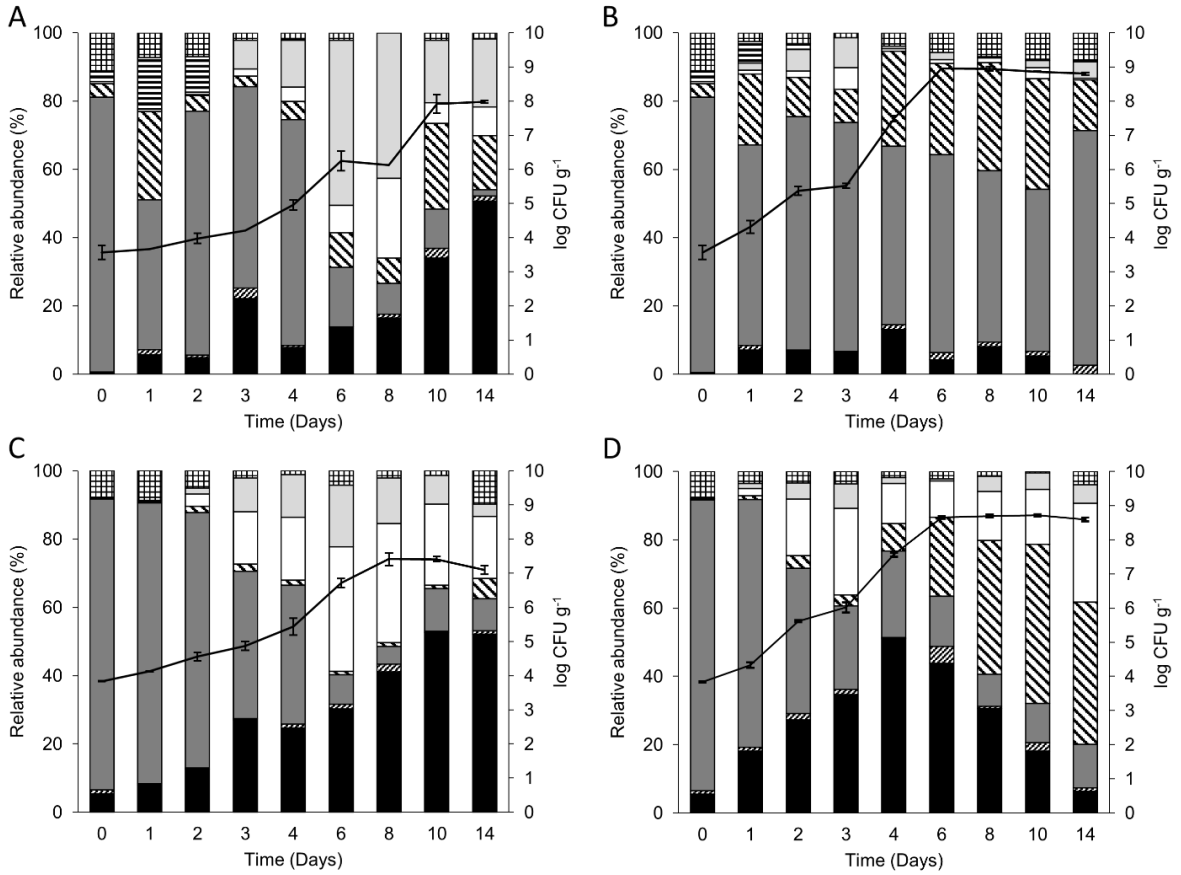

Figure 14| Relative species abundance of all isolates identified via MALDI-TOF MS during growth dynamics experiments in MAP minced beef. Two different minced beef batches were analyzed at 4 and 10 °C. Batch 1: 4 °C, **A**; 10 °C, **B**; batch 2: 4 °C, **C**; 10 °C **D**. CFU values are shown on secondary y-axis as mid values of triplicates with standard error. "Others" include "not reliable identification" (nri) and species with minor abundance. (■) *Brochothrix thermosphacta,* (■) *Pseudomonas* spp., (▧) *Lactococcus piscium*, (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) *Leuconostoc gelidum* subsp. *gelidum*, (▤) *Enterococcus faecium*, (▨) *Carnobacterium divergens*, (▦) Others.

The initial contamination microbiota was strongly dominated by *Pseudomonas* spp. in both batches. Additionally, accessory *B. thermosphacta* (batch 1) or *Lc. piscium* and *Enterococcus* (*E.*) *faecium* (batch 2) were abundant. Subsequently, abundance of *Pseudomonas* was gradually decreasing except for batch 1 at 10 °C, where *Pseudomonas* dominated the microbiota throughout 14 days of storage. Also, *E. faecium* was not detected after day 2 in batch 1 at both storage temperatures. In the mid stage of spoilage, a change in the microbiota composition to *Leuconostoc gelidum* subsp., *Lc. piscium* and *B. thermosphacta* with alternating dominance was observed. At 10 °C and if detected as initial contamination, the abundance of *Lc. piscium* was enhanced.

The abundance of *B. thermosphacta* was reciprocal to *Lc. piscium* and enhanced at later stages. *Le. gelidum* subsp. *gelidum* was dominant in the mid stage in batch 1, whereas *Le. gelidum* subsp. *gasicomitatum* was dominant in batch 2. At 10 °C, in the late stage from day 8 on, *S. liquefaciens* and *H. alvei* emerged (due to the low relative abundance grouped as "others") in both batches. *C. divergens* was detected only in minor percentages (<5%) of the spoilage-associated microbiota, however, it was present in both batches and temperatures throughout the storage period of 14 days.

Total viable count (TVC) was carried out on BHI agar at 25 °C. The initial TVCs were 3.7 (batch 1) and 3.8 $\log_{10}$ CFU (batch 2), respectively. Minced beef samples stored at 10 °C reached a maximum contamination level of approx. 8.5-9 $\log_{10}$ CFU at day 6 following stagnation. TVCs were increasing faster and were higher compared to samples stored at 4 °C, which showed a constant rise in contamination up to approx. 7 and 8 $\log_{10}$ in batch 1 and 2, respectively.

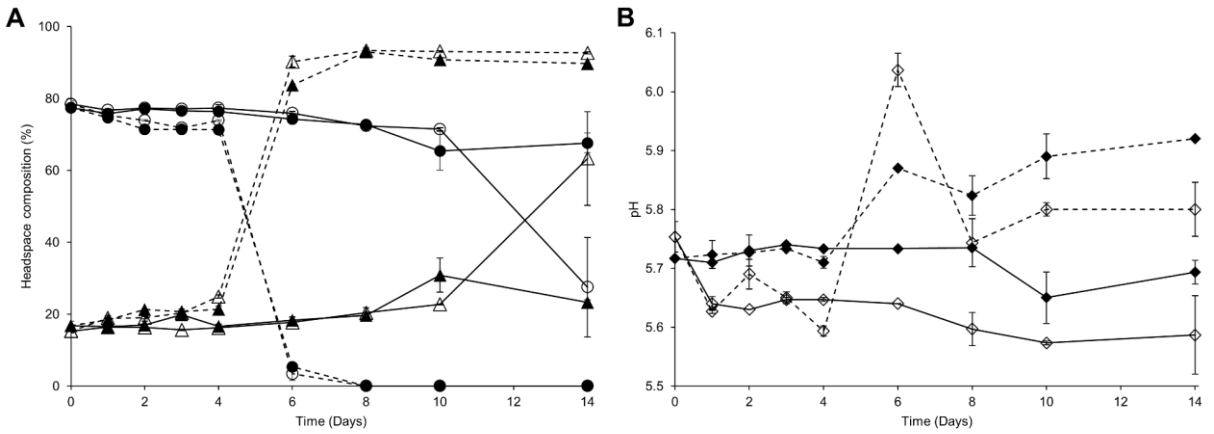

Figure 15| Development of headspace atmosphere (A) and pH (B) during growth dynamics monitoring in MAP minced beef. Filled symbols, batch 1; empty symbols, batch 2; solid lines, 4 °C; dashed lines, 10 °C; (○) O_2; (△) CO_2; (◇) pH; values are shown as mid values of triplicates with standard error.

Headspace atmosphere development was monitored during 14 days of storage time (Figure 15A). Oxygen decrease was concomitant with an increase in carbon dioxide concentration. Packages stored at 4 °C retained the atmosphere composition until day 6 where a linear decrease started to occur in both batches. Packages stored at 10 °C showed an immediate oxygen decrease and carbon dioxide increase and from day 4 to day 6, a complete alteration in the atmosphere was monitored in both batches, respectively. Furthermore, from day 8 on, the atmosphere was completely anoxic with no residual oxygen measured. In addition to headspace atmosphere composition, pH values of minced beef samples were monitored throughout the storage period (Figure 15B). The initial pH values of minced beef in both batches were approx. 5.75, respectively. In batch 1, the pH remained quite constant in

samples stored at 4 °C and gradually rose to approx. 5.9 in samples stored at 10 °C, respectively. In batch 2, samples stored at 4 °C showed a mild decrease in pH during storage to approx. 5.6. Monitored pH values of samples stored at 10 °C also decreased until day 4 followed by a rise to approx. 5.8. The peak at day 6 seems to be caused by an erroneous measurement. The anewed rise of pH in the later stage was concomitant with the complete alteration of the headspace atmosphere and the detection of *S. liquefaciens* and *H. alvei*. In botch batches, the change in headspace atmosphere composition and microbiota composition was concomitant with the perception of beginning onset of spoilage, respectively.

At two sample time points, packages originating from batch 1 stored at 4 °C were detected that showed a defective modified atmosphere with lower oxygen and carbon dioxide levels (Figure 16). The divergent atmosphere in these packages resulted in a higher TVC and higher relative abundance of *Pseudomonas* spp., and lower abundance of *Le. gelidum* subsp. *gelidum* and *gasicomitatum*, respectively.

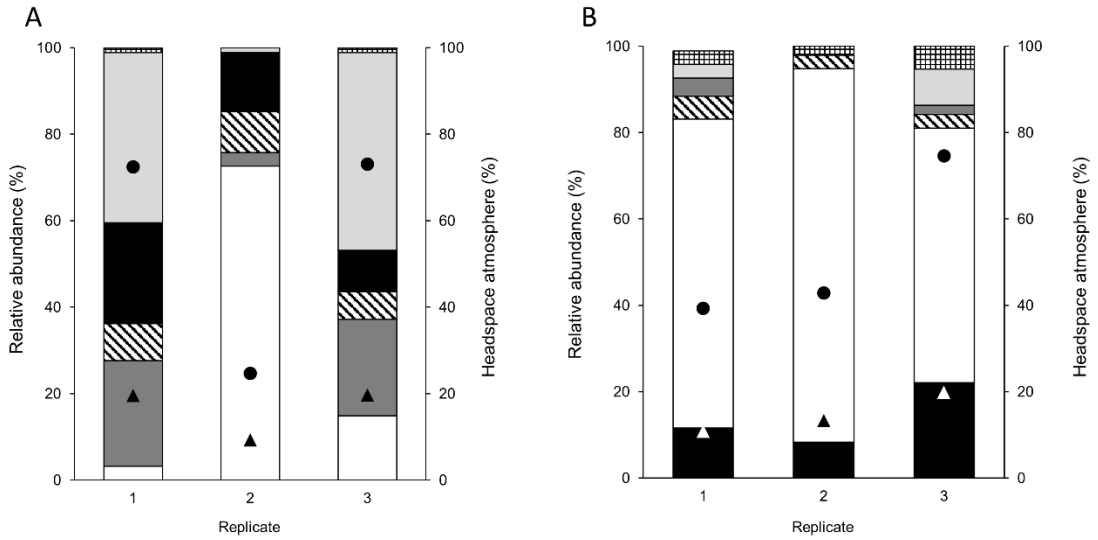

Figure 16| Relative species abundance of MAP minced beef packages with defective headspace atmosphere. A, day 3, **B**, day 8. (■) *Brochothrix thermosphacta* (□) *Pseudomonas* spp., (▧) *Lactococcus piscium*, (▩) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) *Leuconostoc gelidum* subsp. *gelidum*, (▦) Others. (○) O_2; (△) CO_2.

4.2.3 Comparison of microbiota located on the surface and within the matrix

Conditions for spoilage-associated microbiota may differ on the surface of the minced beef and within the meat matrix. In order to investigate the potential effect on the spoilage-associated microbiota, the drill cores from four minced beef samples with different use-by dates obtained by trepanning with a sterile metal rod were separated into top, mid and bottom and analyzed differentially. Relative species abundance and TVC is shown in Figure 17. All four

samples showed highly similar TVC and relative species abundance on the surfaces and within the matrix, respectively.

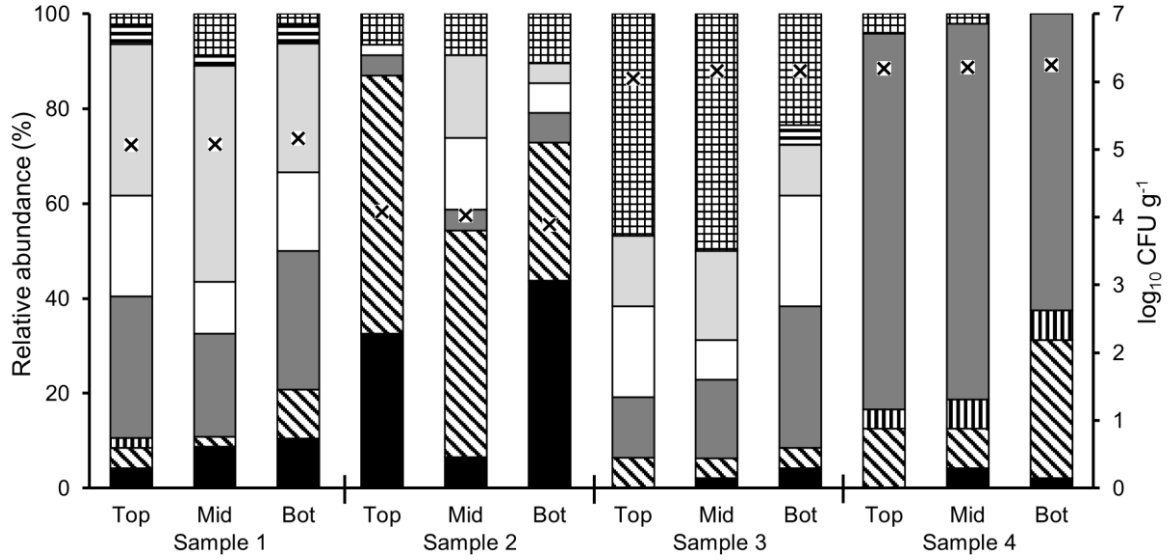

Figure 17| Comparison of spoilage microbiota on top, mid and bottom of MAP minced beef. Cross, CFU value (secondary y-axis); top, upper minced beef surface; mid, center of minced beef; bot, bottomside of minced beef surface. (■) *Brochothrix thermosphacta*, (▧) *Carnobacterium divergens*; (▩) *Lactococcus piscium*, (▥) *Carnobacterium maltaromaticum*, (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▢) *Leuconostoc gelidum* subsp. *gelidum*, (▤) *Pseudomonas* spp., (▦) Others.

4.2.4 Diversity assessment of minced beef isolates below species level

Diversity of relevant spoilage-associated microbiota below (sub-)species level was carried out via RAPD biotyping (3.6.2) as conducted for beef steak isolates (4.1.5). Differences in electrophoretically-separated random band patterns indicate abundance of different strains within the isolates. Isolates were chosen from different sub-clusters of each species within the HCA of their low-molecular subproteome (Figure 12, Figure 13). An overview of the number of unique RAPD band patterns of respective species is given in Table 7. *Lc. piscium* isolates from different cluster showed five different unique RAPD patterns, with three dominant biotype groups with isolates originating from different storage temperature and throughout the incubation period (Figure A5). *Le. gelidum* subsp. *gelidum* & *gasicomitatum* showed three and four unique RAPD patterns originating from different MALDI cluster, respectively (Figure A6). Isolates of *B. thermosphacta* showed a high diversity with at least nine unique RAPD band patterns with one dominant group of similar biotypes originating from different storage temperatures, batches and throughout the storage period (Figure A7).

Pseudomonas spp. (multiple species possible) showed the highest diversity with 17 unique biotypes from 36 selected isolates (Figure A8). In total, five different groups were formed containing isolates with identical or highly similar RAPD biotype (Group 1-5, Figure 19B).

Additionally, a lot of singletons with divergent unique RAPD patterns were detected mostly at the beginning of the storage period. Specific *Pseudomonas* isolates, subsequently identified as *Ps. fragi*, isolated from day 14 at 10 °C showed an identical RAPD biotype (Group 1, “late cluster”) and formed one consistent MALDI cluster.

Table 7| RAPD biotyping for diversity assessment below species level.

Species	No. of isolates analyzed	No. of unique RAPD patterns
Lactococcus piscium	27	5
Leuconostoc gelidum		
subsp. *gelidum*	12	3
subsp. *gasicomitatum*	12	4
Brochothrix thermosphacta	26	9
Pseudomonas spp. *total*	36	17
Pseudomonas sp. *“late cluster”*	8	1

4.2.5 Characterization of *Pseudomonas* spp. using a polyphasic approach

MALDI-TOF MS Biotyper identification of *Pseudomonas* spp. was not consistent with the hierarchical cluster analysis of the MSPs (Figure 13), but these organisms represented a major part of the spoilage-associated microbiota (Figure 14). Therefore, a polyphasic approach was conducted to enable species identification and differentiation including the hierarchical cluster analysis, RAPD biotyping, sequencing of 16S rDNA as well as *rpo*D genes, and a *car*A multiplex PCR as previously established (Ercolini *et al.* 2007).

Pseudomonas isolates from different MALDI sub-cluster were subjected for RAPD biotyping. Isolates from the resulting different RAPD biotypes were subsequently selected for sequencing of 16S rRNA, *rpo*D genes and a *car*A multiplex PCR (3.5.2). Figure 18 shows the phylogenetic neighbor-joining tree of partial 16S rRNA genes of isolates and close related *Pseudomonas* type strains identified by BLAST algorithm.

All isolates displayed sequence similarity to type strains >99%. However, type strain sequences were also highly similar to each other demonstrating poor phylogenetic discriminatory power of 16S rRNA genes within the genus *Pseudomonas*.

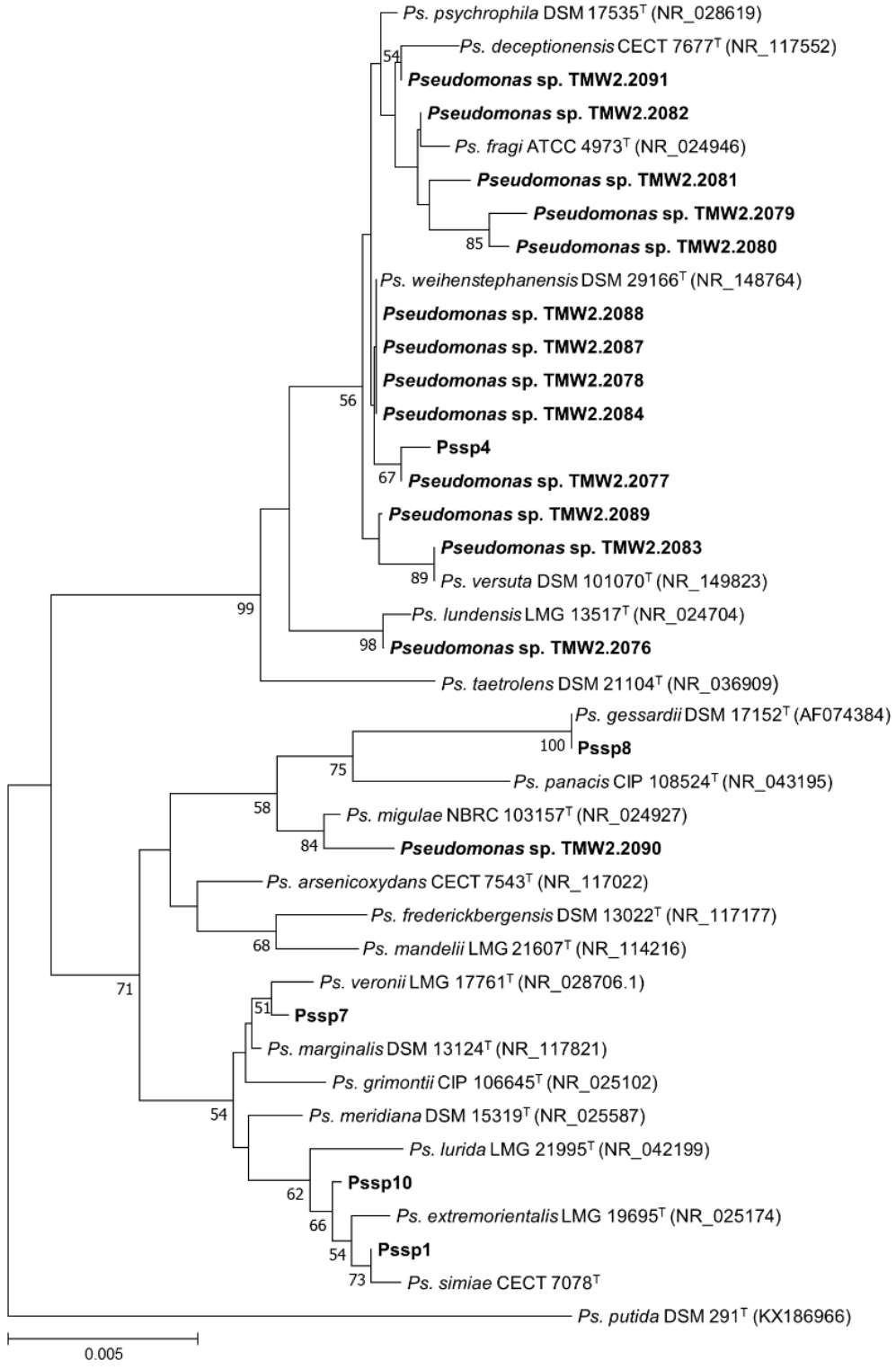

Figure 18| Phylogenetic neighbor-joining tree based on partial 16S rRNA (=1342-1346 nt) gene sequences of *Pseudomonas* spp. with 1000 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes. Scale bar indicates nucleotide substitutions per site. Isolates from these study are shown in bold. Relevant isolates that could be kept viable were included into the TMW strain collection.

In order to further resolve the phylogenetic relationship between the isolates and known type strains, *rpo*D gene sequences were also taken into analysis. The resulting phylogenetic

neighbor joining tree is shown in (Figure 19A). Analysis of *rpo*D gene sequences allowed differentiation of the isolates and affiliation to known species.

Isolates from minced beef were highly diverse and could be affiliated to *Ps. fragi*, *Ps. lundensis*, *Ps.* weihenstephanensis, *Ps. versuta*, *Ps. meridiana*, *Ps. simiae* and *Ps. veronii*. Additionally, several Pseudomonas sp. isolates (TMW2.2087, TMW2.2088, TMW2.2089, TMW2.2090 and TMW2.2091) showed high *rpo*D sequence dissimilarity to *Pseudomonas* type strains and could therefore not be assigned to known species.

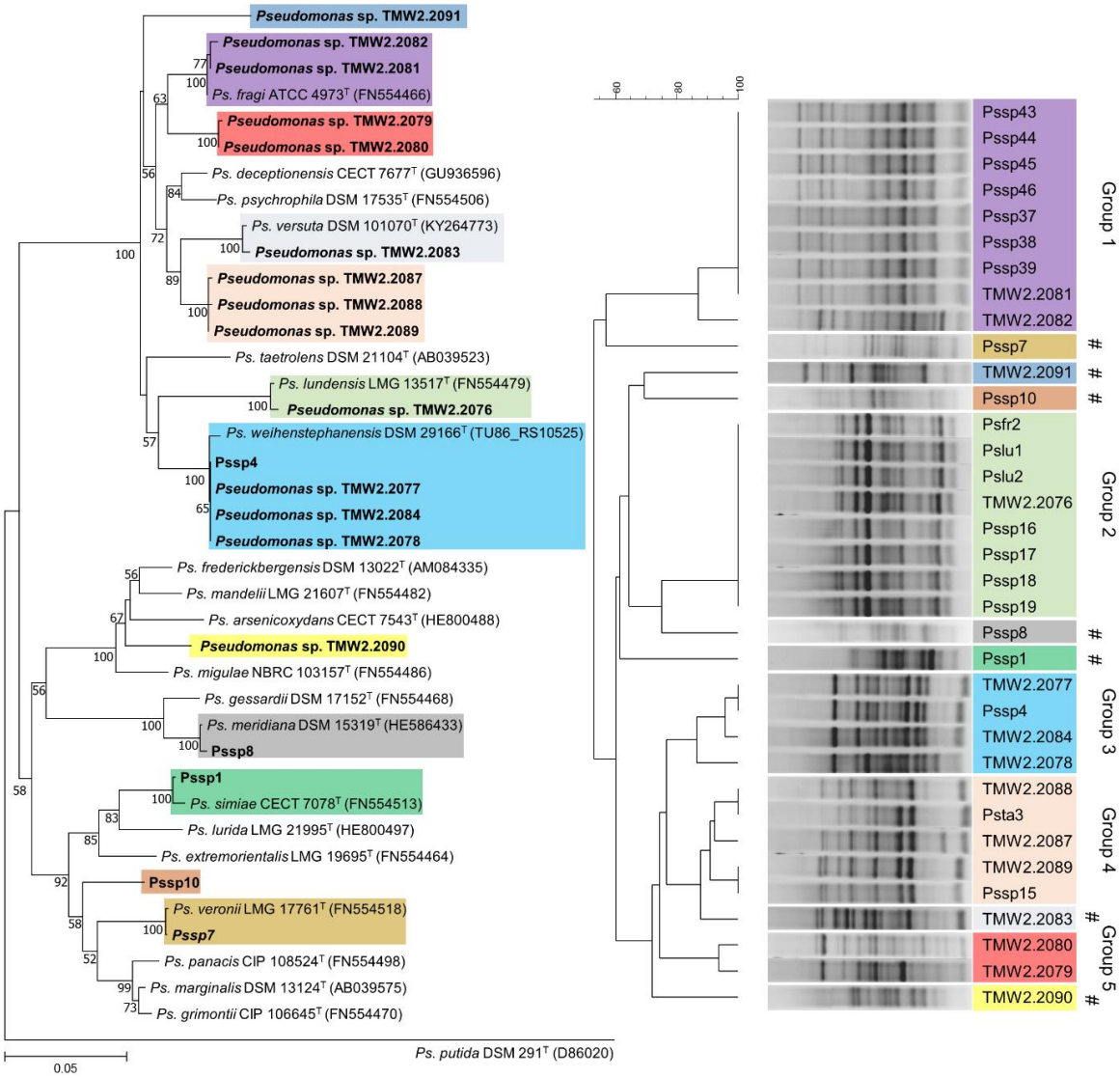

Figure 19| Phylogenetic neighbor-joining tree based on partial *rpo*D (=675-681 nt) gene sequences of *Pseudomonas* spp. with 1000 bootstraps (A) and cluster analysis of RAPD biotypes (B). **A**, gene accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes. Scale bar indicates nucleotide substitutions per site. Isolates from these study are shown in bold. **B**, Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Relevant isolates that could be kept viable were included into the TMW strain collection.

The phylogenetic relationship of the isolates based on *rpo*D gene sequence was compared to a cluster analysis of their RAPD biotypes (Figure 19B). Isolates affiliated to a respective species also displayed similar RAPD band patterns that resulted in a distinct cluster apart from other isolates and therefore supported the affiliation.

Additionally, a multiplex PCR previously developed for simultaneous detection of multiple *Pseudomonas* species via differential amplicon size (Ercolini *et al.* 2007) was performed in order to differentiate the isolates and evaluate the method. A cluster analysis based of the *car*A multiplex PCR amplicon pattern is shown in Figure 20.

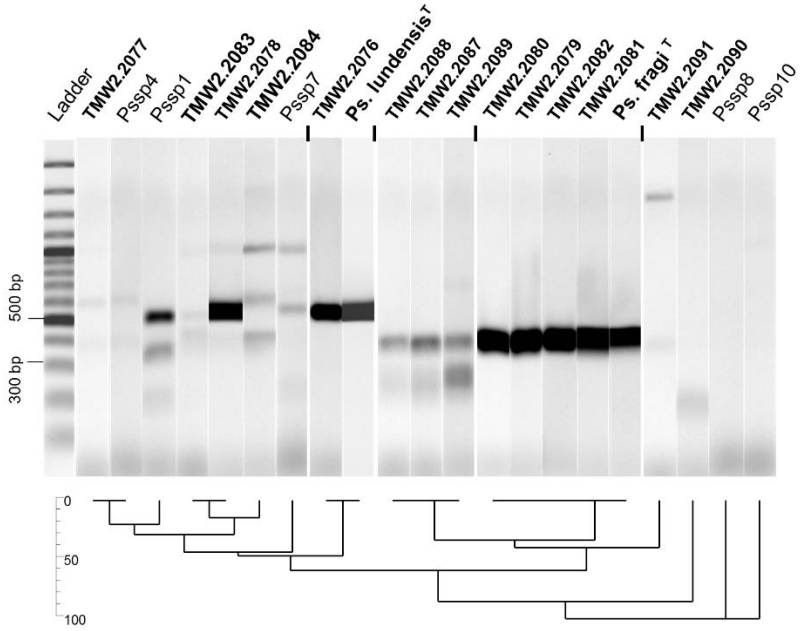

Figure 20| Cluster analysis of *car*A multiplex PCR band patterns from *Pseudomonas* spp. isolates. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice's similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Relevant isolates that could be kept viable were included into the TMW strain collection.

Type strains of *Ps. fragi* and *Ps. lundensis* were included as control and showed the desired amplicon size of 370 and 530 bp, respectively. *Ps. putida*^{T} was not available to be included as a control, however no isolates showed an amplicon of desired 230 bp as described by Ercolini *et al.* (2007). PCR amplification of isolated *Pseudomonas* sp. TMW2.2079, TMW2.2080, TMW2.2081, and TMW2.2082, resulted in a single band at 370 bp. Therefore, these isolates could be identified as *Ps. fragi*. Isolate TMW2.2076 showed only a single band at 530 bp and could be assigned to *Ps. lundensis*. All other isolates showed either no bands or a single and multiple bands of undesired sizes thus belonging to other species according to the multiplex PCR analysis.

Table 8| Concatenated polyphasic identification approach of *Pseudomonas* spp.

MALDI Cluster	RAPD	Strain[1]	MALDI Ident	Score	*car*A PCR	Sequence analysis
Cluster 1	Group 2	**2.2076**	*Ps. lundensis*	green	*Ps. lundensis*	*Ps. lundensis*
Cluster 3a	Group 3	**2.2078**	*Ps. fragi*	yellow	#	*Ps. weihenstephanensis*
		2.2084	*Ps. fragi*	yellow	#	*Ps. weihenstephanensis*
Cluster 3b		Pssp4	*Ps. taetrolens*	yellow	#	*Ps. weihenstephanensis*
		2.2077	*Ps. taetrolens*	yellow	#	*Ps. weihenstephanensis*
Cluster 4	Group 5	**2.2080**	*Ps. fragi*	green	*Ps. fragi*	*Ps. fragi*
		2.2079	*Ps. lundensis*	yellow	*Ps. fragi*	*Ps. fragi*
Cluster 5	Group 4	**2.2089**	*Ps. fragi*	yellow	#	*Pseudomonas* sp. MH1*
		2.2087	*Ps. taetrolens*	yellow	#	*Pseudomonas* sp. MH1
		2.2088	*Ps. fragi*	green	#	*Pseudomonas* sp. MH1
Cluster 6	Group 1	**2.2081**	*Ps. fragi*	yellow	*Ps. fragi*	*Ps. fragi*
		2.2082	*Ps. fragi*	yellow	*Ps. fragi*	*Ps. fragi*
Cluster M	#	Pssp1	*Ps. fluorescens*	green	#	*Ps. simiae*
Cluster M	#	Pssp10	*Ps. veronii*	yellow	neg	*Pseudomonas* sp.
Cluster M	#	**2.2090**	*Ps. frederiksbergensis*	yellow	#	*Pseudomonas* sp. MH2*
Cluster M	#	Pssp7	*Ps. veronii*	green	#	*Ps. veronii*
Cluster M	#	Pssp8	*Ps. gessardii*	green	neg	*Ps. meridiana*
Cluster M	#	**2.2091**	*Ps. taetrolens*	yellow	#	*Pseudomonas* sp. MH3*
Cluster M	#	**2.2083**	*Ps. lundensis*	yellow	#	*Ps. versuta*

[1]Relevant isolates that could be kept viable were included into the strain collection of Technische Mikrobiologie Weihenstephan (TMW).
[2]M, miscellaneous
***potential novel species; MH, Maik Hilgarth (preliminary, internal designation)**

The different identification methods for *Pseudomonas* spp. were concatenated and are shown in Table 8. The polyphasic approach revealed a high species diversity of *Pseudomonas* spp. on minced beef with multiple potential novel species designated *Pseudomonas* MH1-3 (TMW2.2087-TMW2.2091). Isolates originating from one respective MALDI cluster also grouped together in the RAPD cluster analysis. The original MALDI Biotyper identification was reliable in some cases if the score was above >2.0 (indicated as green). However, there were isolates erroneous identified as a certain species that showed divergent affiliation in the polyphasic approach. Multiple isolates were only identified on genus level (MALDI Biotyper score <2.0, indicated as yellow) and showed novelty based on sequence analysis of *rpo*D genes. The *car*A multiplex PCR was in accordance to sequence analysis. Isolates with only a single band of desired length for *Ps. fragi* or *Ps. lundensis* were identified based on their *rpo*D sequence. Other known and potential novel species showed either no, multiple and/or bands of undesired length.

Employing the polyphasic approach for identification of *Pseudomonas* spp., most of the different cluster of the MALDI hierarchical cluster analysis could be resolved and affiliated to species (Figure 21), which was not possible with the Biotyper identification alone. *Ps. weihenstephanensis*, *Ps. fragi*, *Ps. lundensis* were identified as the dominant *Pseudomonas* species.

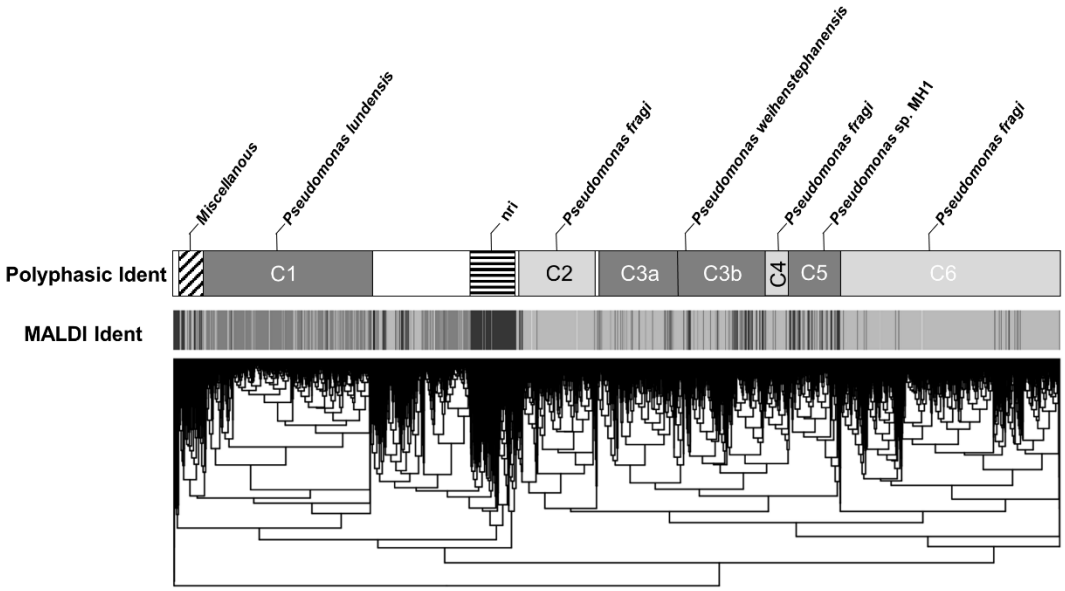

Figure 21| Affiliation of *Pseudomonas* spp. to hierarchical MSP cluster analysis using a polyphasic identification approach. MSP were matched into cluster based on different Euclidean distance matrices. Polyphasic ident shows the identification based on MALDI Biotyper, RAPD, *car*A multiplex PCR & *rpo*D gene sequences. C1-6, Cluster; White areas of polyphasic identification represent areas with nri and spectra that could not be resolved further. Cluster C2 was identified using only the *car*A multiplex PCR. Miscellaneous represents a highly diverse cluster with isolates close related to various *Pseudomonas* sp.

Pseudomonas spp. isolates of these dominant species (TMW2.20-76/78/81/82/84) isolated from the late stage of storage of minced beef, when residual oxygen was completely depleted (4.2.2), were incubated at 25 °C under anoxic conditions on BHI agar in order to test anaerobic growth. All isolates showed clear visible growth in a variable degree on agar plates after 1-2 days and therefore actual anaerobic growth of these strains was demonstrated.

4.2.6 Random retail minced beef/meat samples

In addition to minced beef sample batches directly obtained from one producer, random retail minced beef and minced meat samples with different use-by dates from different supermarkets in the local area of Freising, Bavaria were obtained for comparative analysis and handled in the same manner as the batch samples (3.2.1).

Headspace atmosphere composition, pH, CFU and relative species abundance were determined. An overview of the random retail samples and measured parameters is shown in Table 9. In addition to minced beef, mixed minced meat from beef and pork or vegetable protein, and minced meat with a reduced fat content or labeled as organic were taken into analysis.

Table 9| Overview of random retail minced meat samples.

Retail	Sample	Specification	Days to use-by date	O_2 (%)	CO_2 (%)	pH
Retailer 1	#1	minced beef (organic)	5	n.d.	n.d.	5.71
Retailer 1	#13	minced beef+vegetable protein	3	59.4	34.3	5.98
Retailer 1	#14	minced beef (organic)	3	79.9	14.2	5.79
Retailer 1	#9	minced beef+pork (organic)	2	57.5	34.8	5.99
Retailer 1	#12	minced beef (organic)	2	57.5	29.2	5.81
Retailer 2	#3	minced beef	4	n.d.	n.d.	5.79
Retailer 2	#8	minced beef (reduced fat content)	4	70.1	23.9	6.01
Retailer 2	#10	minced beef	4	55.4	18.3	5.94
Retailer 2	#4	minced beef	2	n.d.	n.d.	6.01
Retailer 2	#5	minced beef (reduced fat content)	1	64	28	5.96
Retailer 2	#6	minced beef	1	42.4	27.2	6.01
Retailer 5	#2	minced beef	5	n.d.	n.d.	5.93
Retailer 5	#7	minced beef	3	80.3	10.4	6.00
Retailer 5	#11	minced beef	1	65.9	24.6	5.68

n.d., not determined

The headspace atmosphere and pH values were varying and independent of the time until the use-by date assigned by the respective producer. A few packages showed a defective modified atmosphere – either low CO_2 concentration or a divergent composition (O_2+ CO_2 < ~90%) - e.g. Sample 10, 6 and 7. pH values ranged from 5.7 – 6.0.

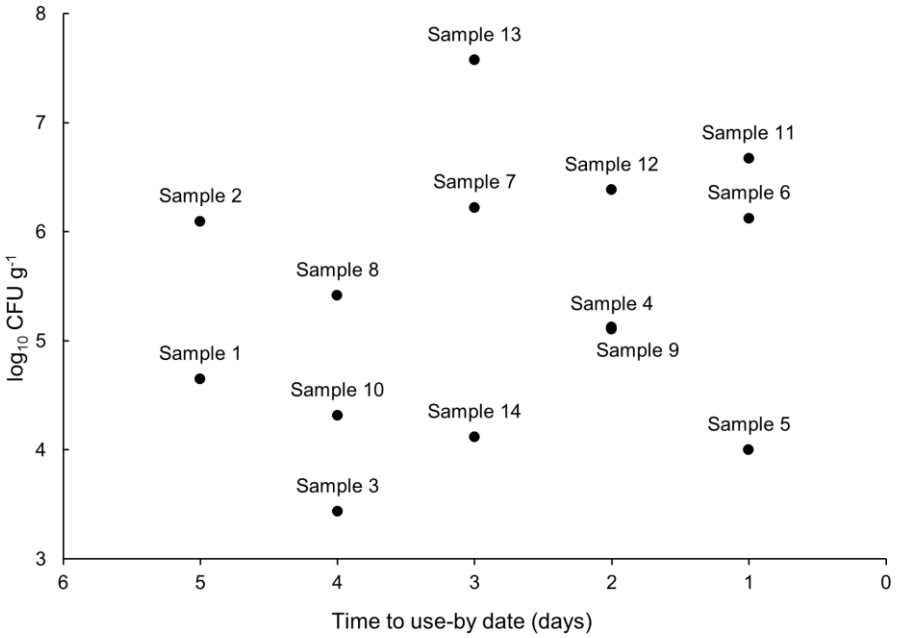

Figure 22| Contamination level in dependence of use-by date of randomly obtained MAP minced meat retail samples. Dots for Sample 4 and 9 are overlapping due to highly similar counts.

TVC of spoilage-associated microbiota of different random samples opened at one respective specific time point (no growth dynamics analysis) during shelf life are shown in Figure 22. A comparison of random samples and batch samples based on days to use-by date was not possible since the retail minced meats had the use-by date assigned to 5 days and the batch samples only to 3 days. Contamination levels were scattered and fluctuating within the use-by dates assigned by the different producers e.g. a fresh sample (Sample 2) had a high contamination (>6 $\log_{10}$ CFU g^{-1}) and a sample with only 1 day to use-by date (Sample 5) had very low levels (<4 $\log_{10}$ CFU g^{-1}).

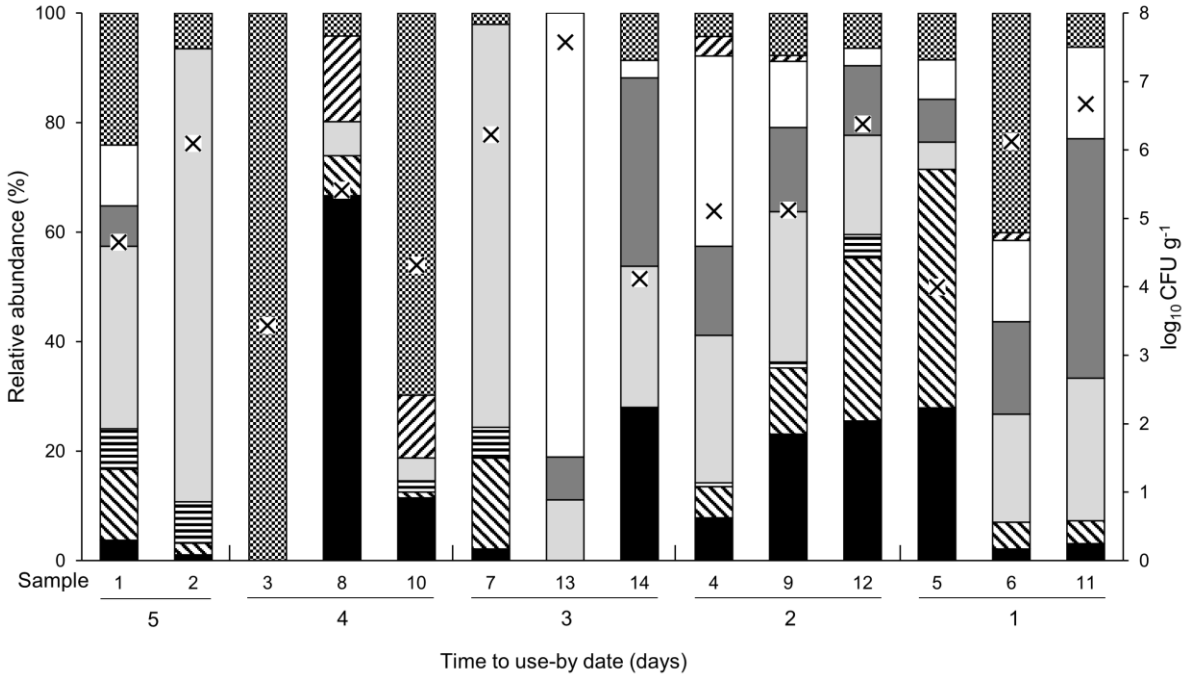

Figure 23| Relative species abundance of randomly obtained retail minced meat samples. "Others" include "not reliable identification" (*nri*) and species with minor abundance. (■) *Lactococcus piscium*, (□) *Leuconostoc gelidum* subsp. *gelidum*, (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) *Carnobacterium divergens*, (▨) *Pseudomonas* spp., (■) *Brochothrix thermosphacta*, (▤) *Carnobacterium maltaromaticum*, (▨), Others. CFU values are shown on secondary y-axis as crosses.

The relative species abundance of respective random samples are shown in Figure 23. Most random samples were dominated either by *Lc. piscium*, *Le. gelidum* subsp. *gelidum* & *gasicomitatum*, *C. divergens*, *B. thermosphacta* and/or a mix of them. Spoilage-associated microbiota composition was therefore similar to the microbiota detected in growth dynamics experiments (4.2.2). However, *Pseudomonas* spp. were either not detected or represented only a minor constituent of the microbiota in the random samples, but dominated the microbiota in the batch samples (Figure 14).

Two samples (#3 and #10) showed a divergent microbiota composition (Figure 24) with atypical organisms not detected in the other random samples e.g. *Le. citreum*, *Lc. lactis* & *garvieae* and *Macrococcus caseolyticus*.

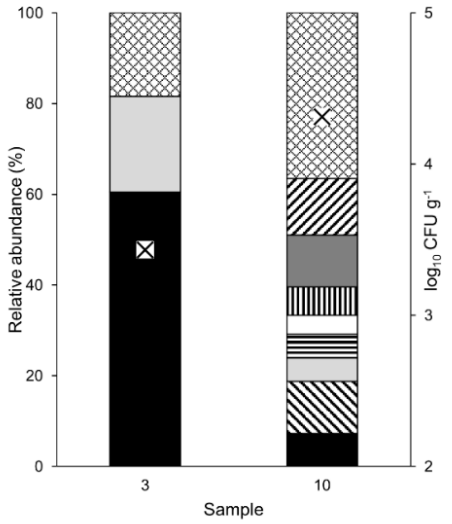

Figure 24| Relative species abundance of two random minced beef samples with atypical microbiota. "Others" include species with minor abundance. (■) *Pseudomonas* spp., (□) *Lactococcus piscium*, (▩) *Lactococcus lactis*, (▧) *Brochothrix thermosphacta*, (▨) *Acinetobacter johnsonii*, (■) *Lactococcus garvieae*, (▤) *Leuconostoc citreum*, (▥) *Macrococcus caseolyticus*, (▩) not reliably identified. CFU values are shown on secondary y-axis as crosses.

4.3 Assertiveness of *Lactococcus piscium*

4.3.1 RAPD biotyping using a colony based PCR approach

A colony-based RAPD-PCR approach was developed without necessity of DNA extraction in order to enable high-throughput identification while retaining the ability to discriminate the different strains. Ultrasonic treatment of colony material was the best method for obtaining template that yielded reproducible band patterns with minimum work amount. Therefore, colony material was directly picked from agar plates with a sterile toothpick, resuspended in 10 µl deionized water in a 1.5 ml reaction vessel and exposed to sonication for 5 minutes in an ultrasonic bath. RAPD biotyping was performed thrice per strain (3 different biological replicates in three different PCR approaches). Figure 25 shows the hierarchical cluster analysis of RAPD band patterns. Each strain resulted in a unique RAPD biotype. Respective triplicates of each strain showed a coherent cluster that was diverging from other strains thereby verifying the ability of colony-based RAPD biotyping to discriminate all *Lc. piscium* isolates on strain level.

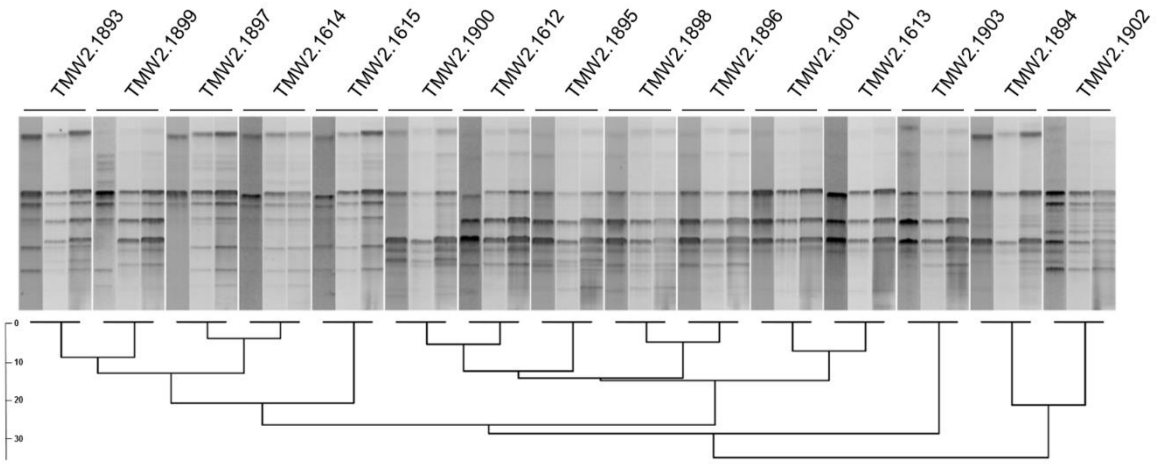

Figure 25| Hierarchical cluster analysis of RAPD band patterns of 15 different strains of *Lactococcus piscium* isolated from meat products. RAPD-PCR was performed thrice. UPGMA was used as cluster method with Dice's similarity coefficient and 3% tolerance. Bar refers to the Pearson correlation coefficient; TMW, Technische Mikrobiologie Weihenstephan.

4.3.2 Intraspecies assertiveness of *Lc. piscium*

A total of 15 meat-borne *Lc. piscium* strains with a distinctive RAPD biotype were isolated from different meat products (Table 1) and subjected to assessment of their intraspecies assertiveness. These strains were randomly divided into three groups à five strains and incubated singly and simultaneously in MSM, respectively (3.8). Figure 26 shows the initial and final relative abundance of respective strains within each group. *Lc. piscium* isolates TMW2.1897 & TMW2.1898 (Group A), TMW2.1893 (Group B) and TMW2.1614 & TMW2.1901

(Group C) were the most abundant strains at the end of the 7 day incubation period, respectively. These strains were chosen for subsequent simultaneous incubation (Group D). In this final group, *Lc. piscium* TMW2.1614 was the most assertive strain and showed the highest relative abundance of 72.5% after seven days.

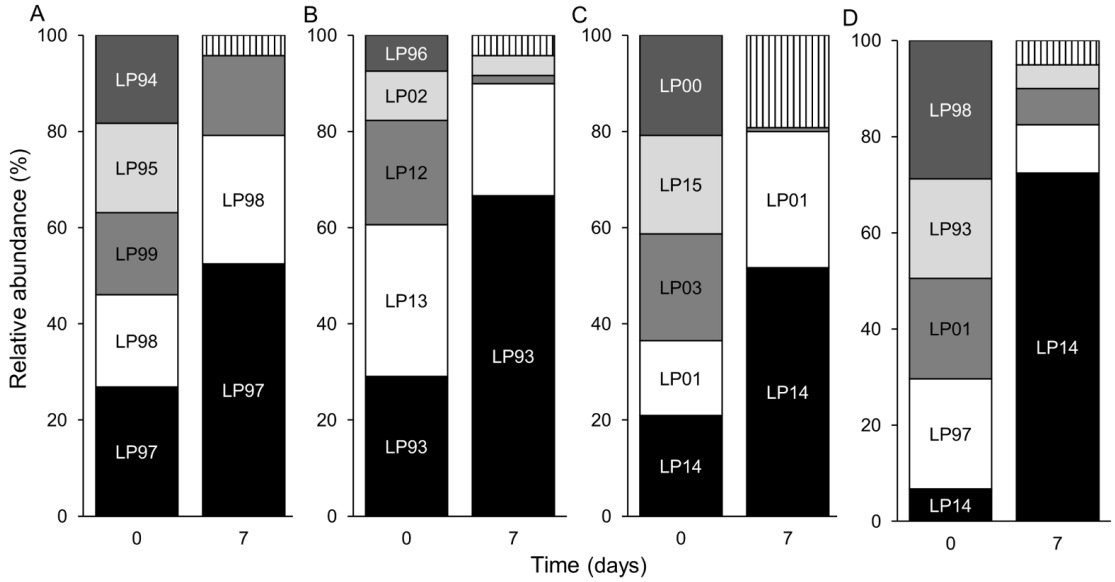

Figure 26| Relative initial and final abundance of *Lactococcus piscium* strains simultaneously incubated in groups in MSM. The 15 *Lc. piscium* (LP) strains were divided randomly in three groups (**A-C**) à five strains. A fourth group was established containing the respective most assertive strain(s) (**D**). Abundance at day 0 was calculated from CFU of singly incubated *Lc. piscium* strains. Abundance at day 7 was determined by subjecting 120 colonies to RAPD biotyping. ▥, not reliably identified (nri) RAPD pattern that could not be distinctly assigned to one specific strain. Strain designations are shortened to the last two digits.

CFU counts of growth dynamics from singly inoculated strains and altogether as a mix are shown in (Figure 27). All strains displayed similar growth behavior with different final cell levels, whereas the CFU count of singly inoculated strains did not represent their relative abundance in the mix in all cases. In group C, *Lc. piscium* TMW2.1900 and TMW2.1615 had the highest CFU count when singly inoculated, but were not detected at the end of incubation time in the simultaneous group inoculation. In group D, *Lc. piscium* TMW2.1893 had the highest CFU count at day 7 when inoculated singly, but represented only a minor fraction within the mix whereas *Lc. piscium* TMW2.1614 had the lowest CFU count when singly inoculated, but represented the most assertive strain within the mix.

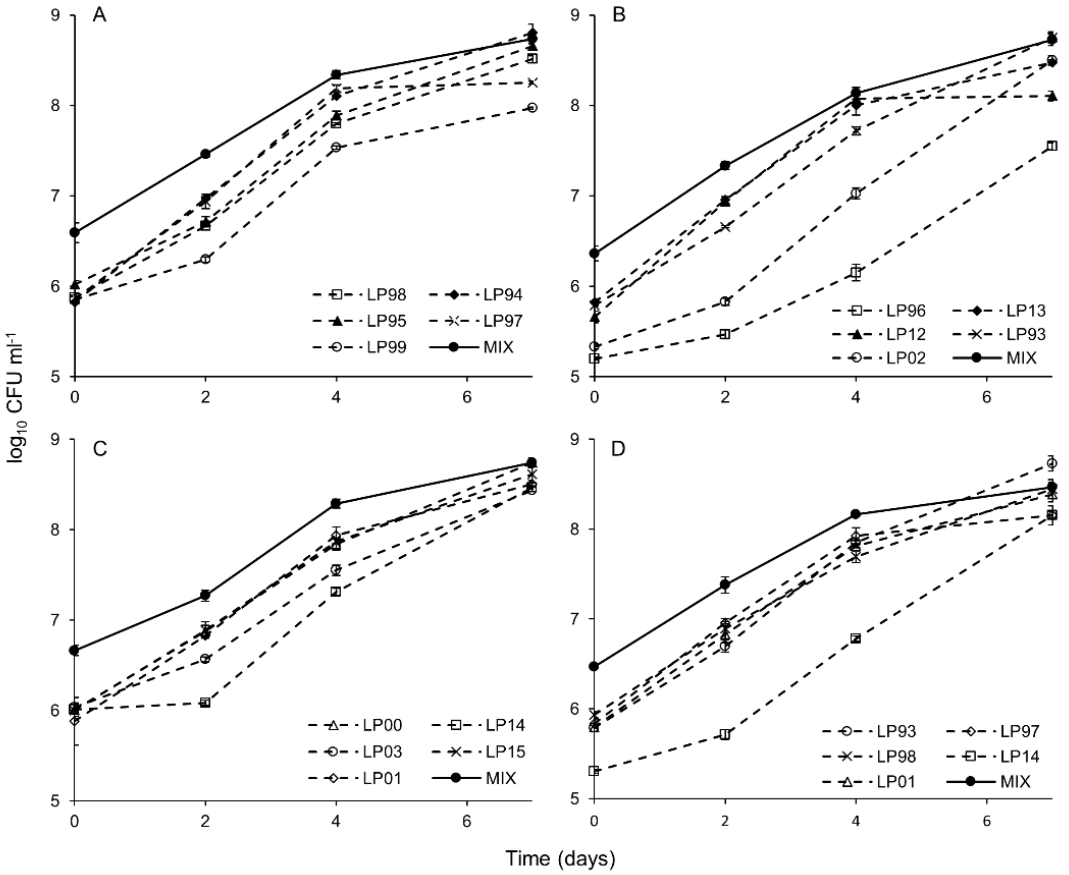

Figure 27| Growth of *Lactococcus piscium* strains singly inoculated and in different group combinations in MSM. The 15 *Lc. piscium* (LP) strains were divided randomly in three groups (**A-C**) à five strains. A fourth group was established containing the respective most assertive strain(s) (**D**). Values are displayed as logarithmic mid values of the triplicates with standard error. Strain designations are shortened to the last two digits.

4.3.3 Competitiveness of *Lc. piscium* against SSO

In addition to the most assertive strain *Lc. piscium* TMW2.1614, strains TMW2.1612 and TMW2.1615 were chosen for competition experiments due to their high abundance in growth dynamics analyses and promising results against spoilers obtained in pre-tests. Spoilage-associated species were selected based on their dominance in preceding growth dynamics monitoring experiments (4.1). For specific strain selection, isolates from the most abundant RAPD biotype was chosen (4.1.5). Competition experiments were conducted with initial quantity of 6 $\log_{10}$ CFU ml^{-1} of *Lc. piscium* strains and 3 log10 CFU ml^{-1} of the respective spoiler. Their respective growth reduction at the end of the respective experiment is shown in Figure 28.

All spoilers displayed a significantly reduced growth when co-inoculated with each *Lc. piscium* strain with the exception of *Ps. weihenstephanensis* TMW2.1728 co-inoculated with *Lc. piscium* TMW2.1615 (Table A1).

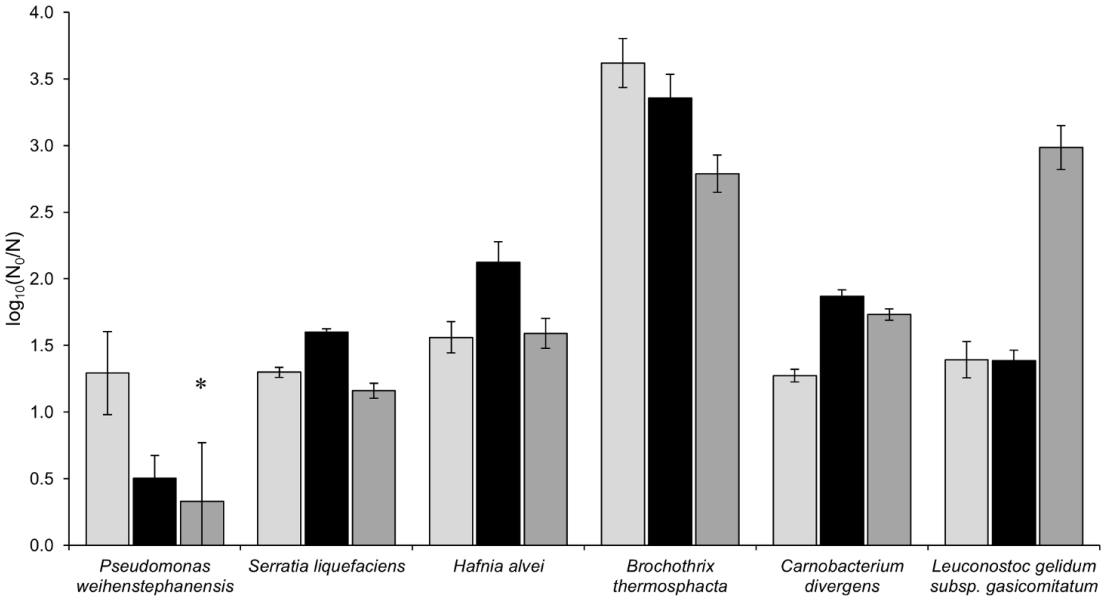

Figure 28| Growth reduction of spoilers within co-inoculation with *Lactococcus piscium* strains at the end of the incubation period. Values are displayed as quotients of mid values of single (N_0) and simultaneous inoculation (N) with propagated errors; *, no significant difference. Co-inoculation with *Lc. piscium* strain TMW2.1612 (□); TMW2.1614 (■); TMW2.1615 (▩).

The growth reduction increased with progressive incubation time and was strain- as well as spoiler-dependent. The strongest effect of the three selected *Lc. piscium* strains on spoiler growth was not exhibited by the same *Lc. piscium* strain but varied between the respective spoilers, though. *Lc. piscium* strain TMW2.1612 caused the highest growth reduction of *Ps. weihenstephanensis* TMW2.1728 and *B. thermosphacta* TMW2.1906 with a $\log_{10}$ reduction of approx. 1.3 and 3.6 magnitudes, respectively. *Lc. piscium* strain TMW2.1614 caused the highest growth reduction of *S. liquefaciens* TMW2.1905, *H. alvei* TMW2.1904 and *C. divergens* TMW2.1907 with a $\log_{10}$ reduction of approx. 1.6, 2.1 and 1.9 magnitudes, respectively. *Lc. piscium* strain TMW2.1615 caused the highest growth reduction of *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 with a $\log_{10}$ reduction of approx. 3 magnitudes.

The growth dynamics of spoilers inoculated in pure and co-culture is shown in (Figure 29). Different spoilers exhibited different spoilage inhibition patterns. Growth curves *Ps. weihenstephanensis* TMW2.1728 were parallel when inoculated singly or in co-culture, but reduced. *S. liquefaciens* TMW2.1905 showed no or only minor increase in cell counts throughout the incubation period. *H. alvei* TMW2.1904 even showed a decrease in cell counts when in co-culture from day 0 to day 2. For *B. thermosphacta* TMW2.1906, *C. divergens* TMW2.1907 and *Le. gelidum* subsp. *gasicomitatum* TMW2.1619, co-inoculation with *Lc. piscium* strains resulted only in minor suppression up to day 2 or 4, but subsequently the spoilers showed significantly reduced cell numbers compared to single culture incubation or even a drop in cell counts at the end of the incubation period.

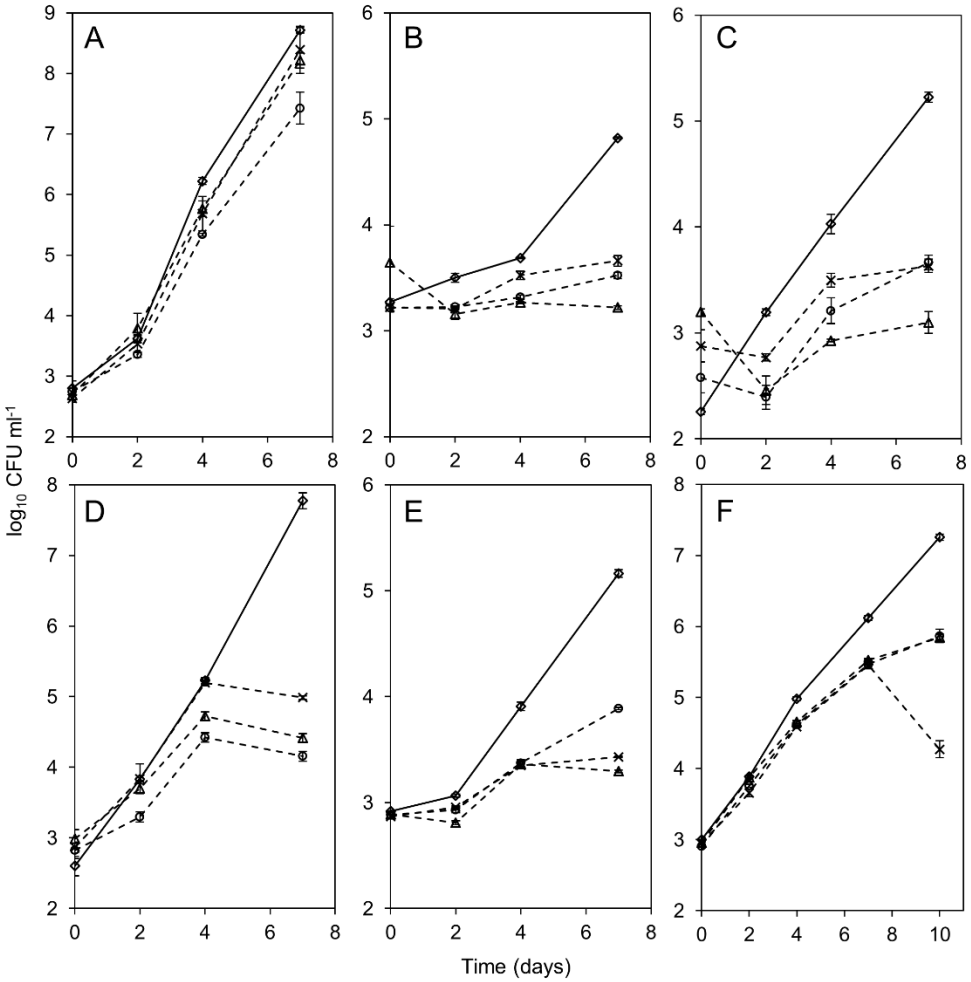

Figure 29| Single and simultaneous growth behavior of meat-spoiling bacteria co-inoculated with *Lc. piscium* strains in MSM. *Ps. weihenstephanensis* (**A**); *S. liquefaciens* (**B**); *H. alvei* (**C**); *B. thermosphacta* (**D**); *C. divergens* (**E**); *Le. gelidum* subsp. *gasicomitatum* (**F**). (◇) spoiler only; dashed lines, co-inoculation with *Lc. piscium* strains TMW2.1612 (○), TMW2.1614 (**x**) or TMW2.1615 (△). Values are displayed as mid values of triplicates with standard error.

In most experiments, growth of *Lc. piscium* was not significantly affected by co-inoculation with the respective spoiler and strains reached approx. 8.5 $\log_{10}$ CFU ml^{-1} after 7 days. Only *Lc. piscium* strain TMW2.1614 displayed a slight increase (0.45 $\log_{10}$ magnitudes) and decrease (0.2 $\log_{10}$ magnitudes) of CFU when co-inoculated with *Ps. weihenstephanensis* TMW2.1728 and *Le. gelidum* subsp. *gasicomitatum* TMW2.1619, respectively (data not shown). The development of pH values during the competition experiments is shown in (Table A2). *Lc. piscium* strains caused a slight drop of pH values in the meat simulation medium of approx. 0.3-0.4 magnitudes after one week of incubation.

In order to evaluate if nutrient availability is a limitation, meat simulation medium was also incubated with spoilers with an initial load of 6 $\log_{10}$ CFU ml^{-1}. After 7 days, harmful spoilers *Ps. weihenstephanensis* TMW2.1728 reached 10.8 $\log_{10}$ CFU ml^{-1}, *S. liquefaciens* TMW2.1905 reached 7.5 $\log_{10}$ CFU/ml and *H. alvei* TMW2.1904 7.4 $\log_{10}$ CFU ml^{-1}. Within the unwanted spoiler group, *B. thermosphacta* TMW2.1906 reached 9.3 $\log_{10}$ CFU ml^{-1}, *C. divergens*

TMW2.1907 7.83 log_{10} CFU ml^{-1} and *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 8.3 log_{10} CFU ml^{-1}, respectively.

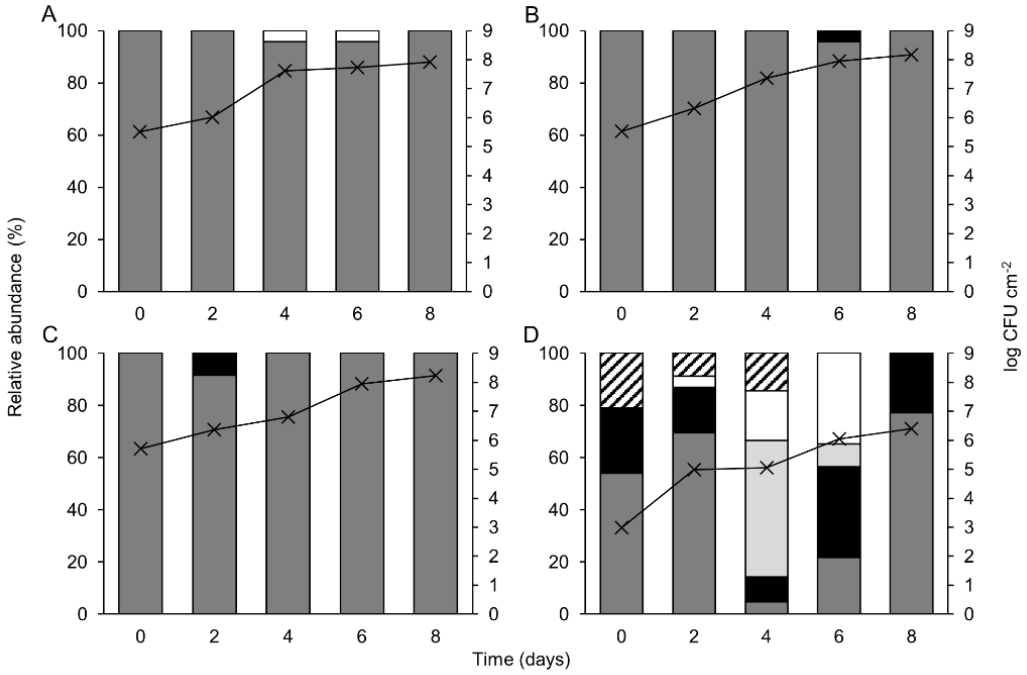

Figure 30| Relative species abundance and TVC on beef deliberately inoculated with *Lactococcus piscium.* *Lactococcus piscium* strain TMW2.1612 (**A**), TMW2.1614 (**B**), TMW2.1615 (**C**) and uninoculated control (**D**). Species were identified via MALDI-TOF MS. TVC is displayed on secondary y-axis. (■) *Lactococcus piscium*, (■) *Carnobacterium divergens*, (□) *Leuconostoc gelidum* subsp. *gelidum,* (□) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) Others (including isolates not reliably identified and minor abundant species).

4.3.4 Inoculation of beef steaks with *Lc. piscium*

Fresh beef steaks were inoculated with *Lc. piscium* strains and repackaged under high O_2 modified atmosphere (3.2.1.2) in order to investigate their growth and impact on beef. TVC and relative species abundance were monitored for an incubation period of 8 days (Figure 30).

Lc. piscium strains prevailed over the indigenous microbiota and reached a final TVC of approx. 8 log_{10} CFU cm^{-2}, respectively. Beef pH values were only slightly affected by *Lc. piscium* strains (Figure 31). Inoculation resulted in a mild acidification (TMW2.1612/ 2.1614/2.1615) followed by an anewed rise to the initial value (TMW2.1614). Inoculated beef displayed neither green discoloration nor off-odorous characteristics despite high cell counts. The internal sensorial evaluation perceived an acceptable ripened overall impression rather than rejectable organoleptics.

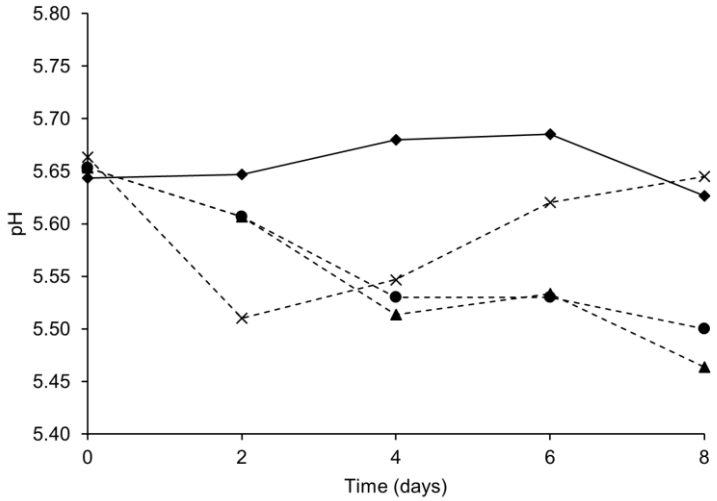

Figure 31| pH values of beef deliberately inoculated with *Lactococcus piscium* strains. (●) TMW2.1612; (**x**) TMW2.1614; (▲) TMW2.1615; (◆) un-inoculated control.

Additionally, *Lc. piscium* strains and *Le. gelidum* subsp. were inoculated in a self-prepared medium derived from extracted minced beef (3.2.5.3). After 8 days of incubation at 4 °C, *Lc. piscium* inoculated flasks showed no discoloration despite high cell counts of 8 $\log_{10}$ CFU ml^{-1}, whereas the medium with *Le. gelidum* subsp. turned green.

4.3.5 Volatilome analysis of *Lc. piscium* TMW2.1615 using PTR-MS

Volatilome analysis of *Lc. piscium* TMW2.1615 at 4 °C under high-oxygen MAP was carried out by PTR-MS (3.4). PTR analysis and calculation of volatile metabolites was performed by C. Franke at the Fraunhofer IVV within a project collaboration (Franke 2018). Fresh beef steak samples were deliberately incubated with 6 $\log_{10}$ CFU cm^{-2} cells *Lc. piscium* strain TMW2.1615 as described in 3.2.1.2. Development of microbiota was monitored for 7 days in re-packaged samples under high-oxygen MAP and also from PTR containers at the end of the incubation time. Relative species abundance and TVC are shown in Figure 32.

Lc. piscium TMW2.1615 reached a final TVC of approx. 8 $\log_{10}$ CFU cm^{-2}, equal to the result in the previous experiment (4.3.4). All colonies analyzed from inoculated beef were identified as *Lc. piscium*. TVC of the control were approx. 4 $\log_{10}$ magnitudes lower ensuring that volatile metabolites measured were produced by *Lc. piscium* TMW2.1615 and not from other contaminants. Temporal development of volatile metabolites detected from beef inoculated with *Lc. piscium* TMW2.1615 in triplicates and in the uninoculated control are shown in Figure A9. Table 10 shows an overview of detected volatiles (m/z) and the contribution of *Lc. piscium* TMW2.1615 compared to the control.

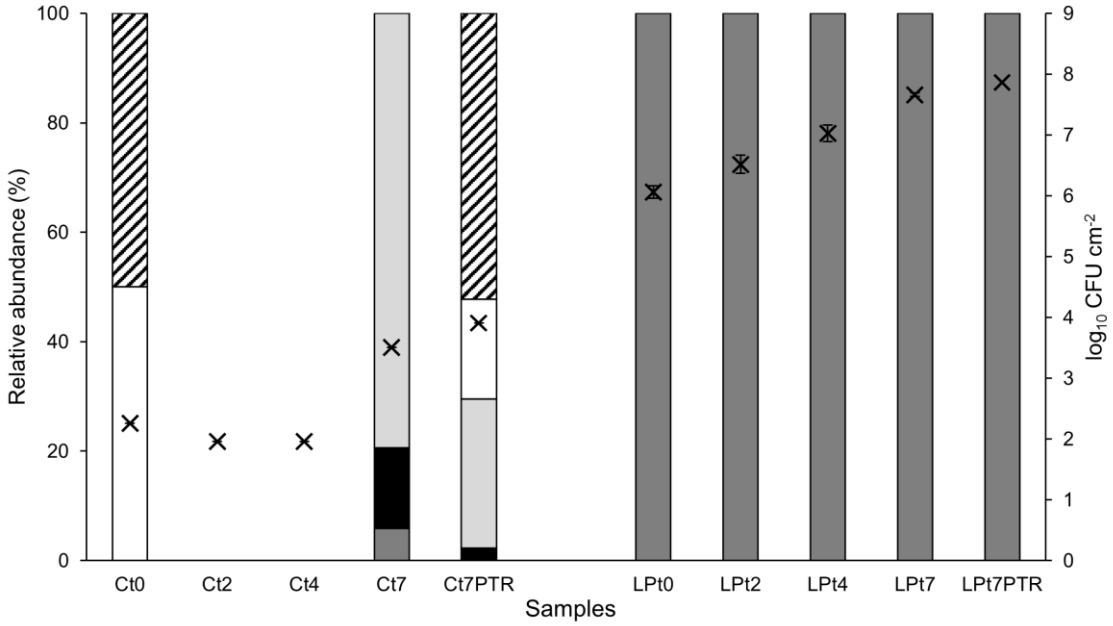

Figure 32| Relative species abundance and TVC of beef steaks deliberately inoculated with *Lactococcus piscium* TMW2.1615 within the volatilome analysis. C, un-inoculated control; Lp, inoculated with *Lc. piscium*; PTR, sample originating from the PTR container; t, time (days). Microbiota was identified via MALDI-TOF MS. (■) *Lactococcus piscium*, (□) *Staphylococcus equorum,* (▣) *Leuconostoc gelidum* subsp. *gelidum,* (■) *Leuconostoc gelidum* subsp. *gasicomitatum*, (▨) Others (including isolates not reliably identified and minor abundant species). No bacteria were detected at day 2 and 4 in the control, displayed is the detection limit.

Volatile metabolites with a mass ($^{m}/_{z}$) of 87 and 89 (and 71 as a fragment of 89) matching to diacetyl or 3-methylbutanal and acetoin or methylbutanol were elevated on beef inoculated with *Lc. piscium* TMW2.1615, whereas the concentration of diacetyl 3-methyl butanal (17 ppbv) was much higher than acetoin or methylbutanol (3.3 ppbv).

Table 10| Detected volatile metabolites of *Lactococcus piscium* TMW2.16165 on beef compared to the uninoculated control using PTR-MS.

m/z obtained by PTR-MS	compound tentative identification based on GC-MS analysis results and literature reports	*Lc. piscium* TMW2.1615 compared to uninoculated control[1]
41	fragments of alcohols	▼
43	fragment of alcohols, esters and acids	►
47	ethanol	►
59	acetone	▼►
61	acetate, 1-propanol, 2-propanol	►
63	dimethyl sulfide	▼
69	1-octen-3-ol	►
71	fragment of m/z 89	▲
73	2-butanone	►
87	2,3-butanedione (diacetyl), 3-methyl butanal	▲
89	3-hydroxy-2-butanone (acetoin), 3-methylbutanol	▲

[1] **▼, lower than control; ► similar to control; ▲, higher than control**

Other detected metabolites were similar to the uninoculated control or even lower. Again, the inoculated beef showed no discoloration after 7 days and displayed a sweet, buttery, ripened impression.

4.3.6 Screening for production of biogenic amines via decarboxylation of amino acids

Production of biogenic amines by 15 *Lc. piscium* strains was tested on a decarboxylation media with amino acids phenylalanine, ornithine, histidine, lysine and tyrosine (3.2.5.12). No color change was observed for any of the tested *Lc. piscium* strains and amino acids, while *Ps. weihenstephanensis* TMW2.1728, *S. liquefaciens* TMW2.1905 and *H. alvei* TMW2.1904 caused color change of the media from yellow to dark purple.

4.4 Photobacteria as hitherto neglected spoilers on meats

4.4.1 Development of an adapted isolation procedure for *Photobacterium* spp. from food

4.4.1.1 Comparative growth screening of photobacteria and meat spoilers

In order to develop a suitable medium and isolation/cultivation procedure for photobacteria from meat, growth potentials of seven different photobacteria type strains obtained from strain collections (Table 1) and seven different species of meat-spoiling bacteria were tested (3.2.3). Photobacteria and other meat spoilers showed an optimum growth at 15-25 °C when grown in marine broth (MB, 3.2.5.7) and in brain heart infusion (BHI, 3.2.5.1) (except *P. profundum*, which grew best at 10 °C), respectively. These conditions were subsequently used for pre-culturing. At first, growth was investigated in different atmospheres under air and under a CO_2/N_2 atmosphere using anaerobic gas generating bags. Organisms showed similar growth under oxic and anoxic conditions. Therefore, plates for growth screening were subsequently incubated under aerobic conditions due to easier handling. Antibiotic susceptibility testing was conducted in order to find a suitable selective medium additive for the isolation of photobacteria from food. Growth inhibition of all tested organisms is shown in Table A3. All photobacteria were sensitive to tetracycline, chloramphenicol, linezolid and gentamycin, and resistant to oxacillin, teicoplanin and vancomycin. Susceptibility to penicillin G, ampicillin and erythromycin was variable. Vancomycin (7 mg/l) was chosen as selective agent since it inhibited the growth of gram-positive meat spoiling *B. thermosphacta*, *C. divergens* and *Lc. piscium* while it did not affect the growth of photobacteria. No antibiotic agent was found inhibiting gram-negative *Ps. weihenstephanensis*, *S. liquefaciens* and *H. alvei*, but not inhibiting photobacteria type strains at the same time.

All tested type strains of photobacteria grew well in MB and on MB agar, and an addition of 3 g l^{-1} meat extract to MB resulted in further growth enhancement of these strains (Table A4) and was therefore included for subsequent isolation approaches. Most type strains of photobacteria did not grow on agar plates of thiosulfate citrate bile salts sucrose agar (TCBS), *Photobacterium* broth (PB) with vancomycin and BHI. On MB agar containing meat extract and vancomycin, growth of *B. thermosphacta* as well as LAB isolates was completely inhibited, while growth of *S. liquefaciens* was reduced and growth of *Ps. weihenstephanensis* and *H. alvei* was unaffected.

Salt content as sodium chloride (NaCl) was also tested as a parameter for medium adaptation. Growth of photobacteria type strains and meat-spoiling isolates was tested in MB containing 3.4-10.4% NaCl (w/v) as well as lysogeny broth (LB) containing 10 and 20% NaCl, respectively (Table A4). *P. phosphoreum*^{T}, *P. iliopiscarium*^{T} and *P. profundum*^{T} showed growth up to 4.4% NaCl, *P. aquimaris*^{T} up to 5.4% and *P. angustum*^{T}, *P. kishitanii*^{T} and *P. leiognathi*^{T}

up to 6.4%, respectively. In comparison to meat spoilers tested, photobacteria did not show a higher tolerance for salt. In contrast, *B. thermosphacta* and *S. liquefaciens* showed weak growth even at 8.4% NaCl in the medium. Only *Lc. piscium* and *Le. gelidum* subsp. *gasicomitatum* displayed a higher sensitivity to NaCl on MB agar. Since an elevated NaCl content was neither favoring photobacteria nor inhibiting meat spoilers, it was not considered for a useful additive in an adapted medium for isolation of photobacteria.

Quarter strength Ringer's solution is employed for resuspending prior to homogenization and preparation of serial dilutions in standard routine for food samples as used for detailed analysis of growth dynamics (3.2.1.3). In order to test if even a short exposure time to a different and reduced salt content during decimal serial dilution results in a growth reduction, comparative CFU counts of liquid cultures of *Photobacterium* spp. type strains grown on MB with meat extract at 15 °C for 48h were performed in Ringer's solution or MB. All tested strains showed a reduced growth when diluted in Ringer's solution prior to plating with up to 2.4 $\log_{10}$ magnitudes (Figure 33). As a consequence, MB was used in the adapted final isolation procedure to resuspend and dilute food samples.

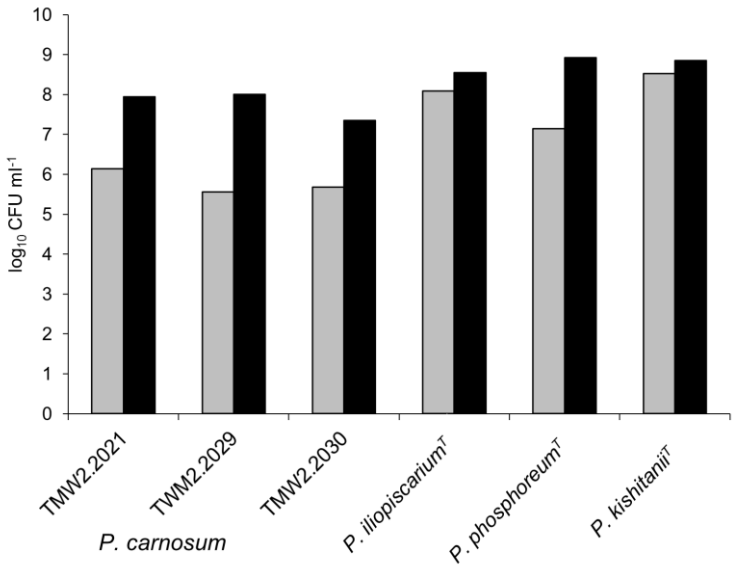

Figure 33| Comparative CFU count of photobacteria cultures using different solutions for serial dilution. (□), quarter-strength Ringer's solution; (■), marine broth.

4.4.1.2 Comparative isolation approach on different media

Different media (BHI, MB, TCBS), additives (meat extract, antibiotic agent) and temperatures (25, 15, 4 °C) were employed for a comparative isolation approach in order to demonstrate and probe the results obtained with type strains of photobacteria and meat-spoiling isolates (4.4.1.1). An overview of cultivation media and temperatures used are shown in Table 11. Meat samples were obtained from local retailers and sampled as described in 3.2.1.3. Spoilage-associated microbiota of meat samples were identified using MALDI-TOF

MS (3.3) with a database previously fed with reference MSPs of seven *Photobacterium* type strains (3.2.3). Correct identity of *Photobacterium* spp. isolates was validated by sequencing of 16S rRNA and *gyr*B genes (3.6.1). Figure 34 shows the comparative isolation approach for two poultry meat (skinless chicken breast; CB) samples (A, CB1; B, CB2) originating from different producers.

Table 11| Overview of cultivation media and temperatures used in different comparative adaptation experiments.

Media	Temperature	Time	Experiment
MB[1]	25 °C	48h	Comparative isolation approach
	15 °C	72h	
	4 °C	12 days	
BHI	25 °C	48h	
TCBS	15 °C	6 days	
PB[1]VA[2]	15 °C	72h	Evaluation of luminous count
MB[1]VA	15 °C	72h	Final adapted approach

[1]supplemented with meat extract
[2]vancomycin

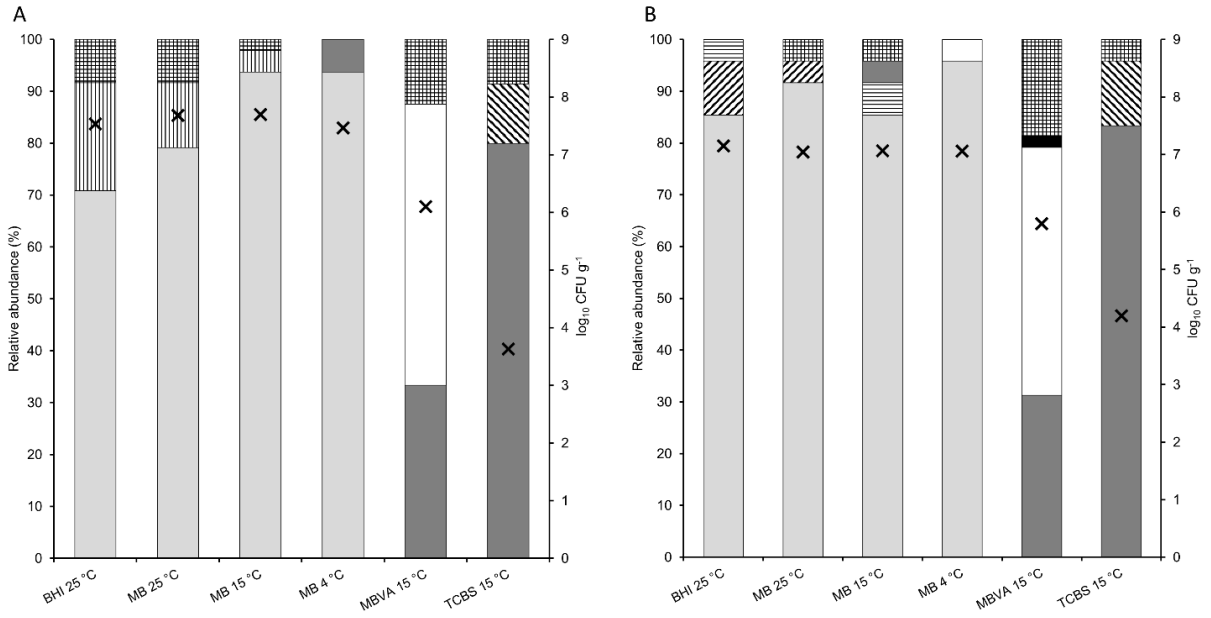

Figure 34| Comparative isolation approach of two different poultry meat samples CB1 (A) and CB2 (B). BHI, brain heart infusion agar; MB(VA), marine broth agar with meat extract (and vancomycin); TCBS, thiosulfate citrate bile salt sucrose agar; Others include organisms not reliably identified or with minor abundance; if no species name is given, identification by MALDI-TOF MS was only reliable on genus level; (■) *Photobacterium phosphoreum*, (□) *Brochothrix thermosphacta*, (■) *Pseudomonas* spp., (□) *Serratia* spp., (▨) *Lactococcus piscium*, (▧) *Yersinia* spp., (▥) *Carnobacterium divergens*, (▤) *Carnobacterium maltaromaticum*, (▦) Others.

The total viable count (TVC) was similar for BHI at 25 °C and MB at different temperatures. For these cultivation approaches, TVC of sample CB1 and CB2 was approx. 7.5 and 7 $\log_{10}$ CFU g^{-1}, respectively. On these media, the microbiota was dominated by *B. thermosphacta*

and accessory *C. divergens*, *C. maltaromaticum* and *Lc. piscium*. The relative abundance of *C. divergens* and *Lc. piscium* was reduced in MB compared to BHI. No photobacteria were recovered from these media. Vancomycin (7 mg l^{-1}) supplementation on MB cultivated at 15 °C (Figure 34) led to disappearance of Gram-positive meat-spoiling *B. thermosphacta*, *Carnobacterium* spp. and *Lc. piscium* strains, and a drop in TVC of over one log_{10} magnitude. Using MB with vancomycin and a temperature of 15 °C, microbiota of poultry sample CB1 consisted of Gram-negative *Pseudomonas* spp. and *Serratia* spp. Additionally, a *P. phosphoreum* isolate from sample CB2 was recovered on this medium. Employing TCBS media, TVC dropped to approx. 4 log_{10} CFU g^{-1} in both samples, respectively and the microbiota was dominated by *Pseudomonas* spp. and *Yersinia* spp. No photobacteria were recovered on TCBS.

4.4.1.3 Evaluation of luminous count

A comparison of total viable count (TVC) and luminous count (LC) at 15 °C was conducted between MB and PB (both with additional meat extract and vancomycin) from two chicken breast samples (CB3 and CB4) from different producers (Figure 35). *Photobacterium* spp. could be recovered from both samples and media, whereas the LC on MB agar was higher or equal compared to PB for the two poultry meat samples CB3 and CB4, respectively.

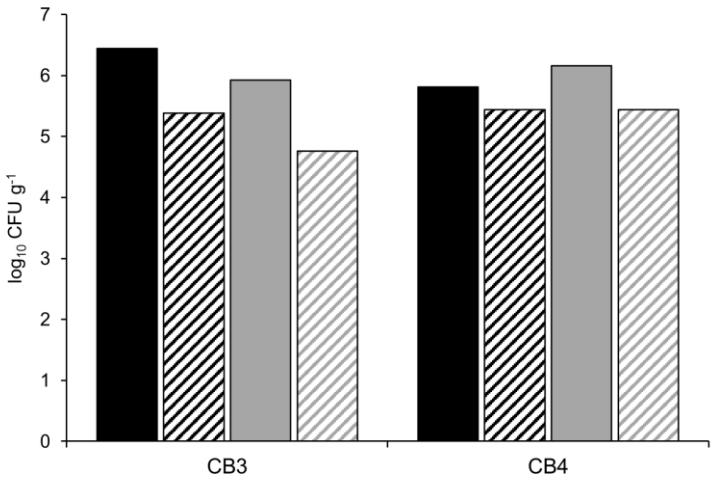

Figure 35| Total viable and luminous count on MB and PB from two different poultry meat samples. CB, chicken breast; filled bars, total viable count; diagonal upward bars, luminous count; (■) marine broth agar, (■) *Photobacterium* broth agar.

All luminous colonies on both media were identified as *P. phosphoreum* and no non-luminous isolates of *P. phosphoreum* were detected. In addition, a non-luminous *P. iliopiscarium* isolate was recovered from poultry sample CB4 on MB. Bioluminescent *P. phosphoreum* isolates from meat on plates and in liquid culture are shown in Figure 36.

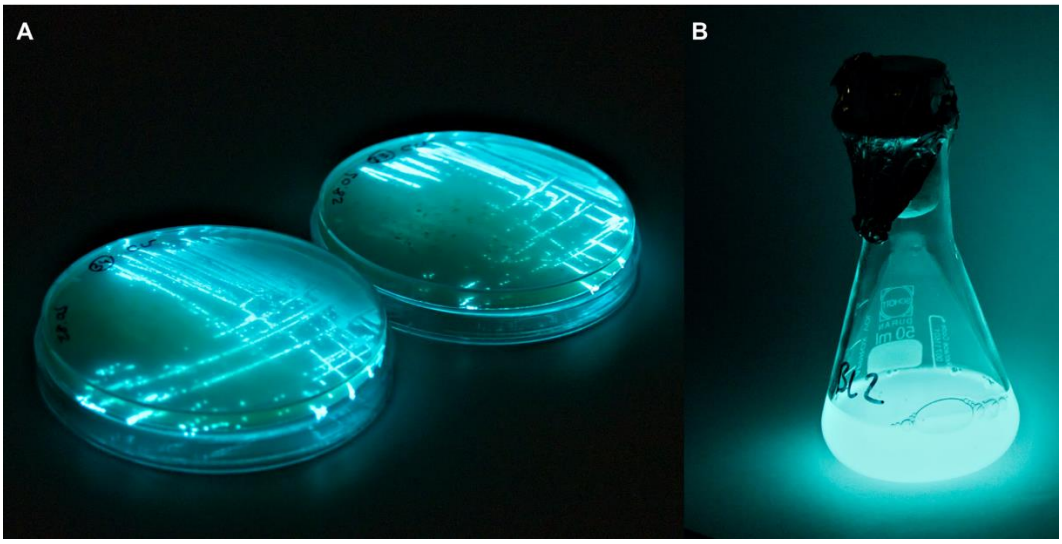

Figure 36| Bioluminescent *Photobacterium phosphoreum* isolates cultured on MB agar (A) and in liquid MB (B).

4.4.1.4 Adapted procedure for isolation of photobacteria from meat products

Growth screening of photobacteria type strains and selected meat spoilers as well as the comparative isolation approach led to development of an adapted isolation procedure for *Photobacterium* spp. from food – as demonstrated for meats: Marine broth supplemented with meat extract should be employed for resuspension and dilution of food samples followed by 72 hours aerobic incubation at 15 °C on MB agar supplemented with meat extract (3 g l^{-1}) and vancomycin (7 mg l^{-1}). Luminous as well as non-luminous isolates should be subjected to subsequent identification.

The adapted selective MB media and isolation procedure was probed for different samples and enabled highly frequent isolation of photobacteria (53% of samples; 16/30 packages) in relevant quantities up to 7 $\log_{10}$ CFU from different (un)spoiled air-stored, MAP and VP meats (beef, pork, chicken) as well as spoiled MAP salmon. *Photobacterium* spp. were found in 53% (8/15) of chicken samples, 30% (3/10) of pork samples, 100% (3/3) of beef samples and 100% (2/2) of salmon samples (Figure 37). In total, isolates from at least three different *Photobacterium* species could be recovered from meat samples, sometimes altogether on a single sample (CB5/CB6/P1/P2): *P. phosphoreum* and *P. iliopiscarium* and isolates that could not be assigned to known species. The latter isolates represented a novel species, *P. carnosum* (cf. 4.4.1). *P. carnosum* was found as predominant on MAP beef and chicken, as well as VP pork, while *P. iliopiscarium* was highly abundant on air-stored and MAP pork, and *P. phosphoreum* was dominating the microbiota on salmon. Again, luminous colonies isolated from all samples were marked and uniformly identified as *P. phosphoreum*. Furthermore, isolation of bacteria from the two poultry samples (CB5 and CB6) was conducted also under anoxic incubation conditions. The TVC was similar, but anaerobic cultivation resulted in a loss of *P. iliopiscarium* isolates.

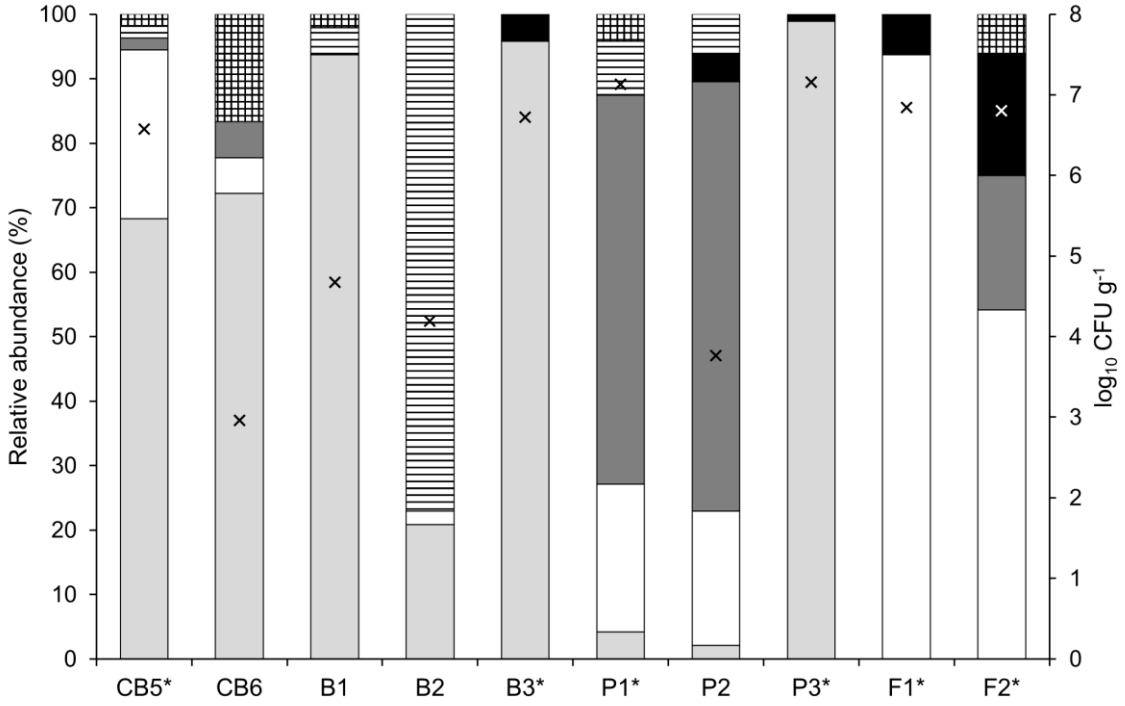

Figure 37| Recovery of photobacteria from different meats using the adapted isolation approach. Samples: skinless chicken breast (CB5/CB6), beef steaks (B1/B2/B3), pork steaks (P1/P2/P3), fish (salmon; F1/F2). Sample P1 was air-stored, samples B3 and P3 were VP, and all other products were packaged under MA. (*) indicates samples that were spoiled by off-odor and/or discoloration; others include organisms not reliably identified or with minor abundance; if no species name is given, identification by MALDI-TOF MS was only reliable on genus level. (▩) *P. carnosum*, (□) *P. phosphoreum*, (■) *P. iliopiscarium*, (■) *Photobacterium* spp., (▤) *Pseudomonas* spp., (▦) Others.

4.4.2 Characterization of *Photobacterium carnosum* sp. nov.

4.4.2.1 Isolation of strains

Bacteria isolated from retail modified-atmosphere packaged skinless poultry meat were identified via MALDI-TOF MS. Several isolates originating from different packages were not reliably identified based on the MALDI Biotyper score (<1.7), but matched best with *Photobacterium* species within the database. These isolates were further analyzed by 16S rRNA and housekeeping genes sequencing as well as RAPD biotyping (3.6). They showed high sequence and pattern similarity to each other, but significant dissimilarity to described species belonging to the genus *Photobacterium,* respectively. These analyses indicated that four different strains of the proposed *Photobacterium carnosum* sp. nov. could be isolated from spoiled (TMW2.2021^{T}, TMW2.2022, TMW2.2030) and unspoiled (TMW2.2029) poultry meat. The isolates are facultatively aerobic, Gram-staining-negative, non-luminous and non-motile. Colonies are round, convex, smooth, and white with a lightly yellow center after cultivation for 72h at 15 °C on MB agar. The cell morphology is variable with coccoidal shape as well as curved and irregular rods, single and in pairs (Figure 38).

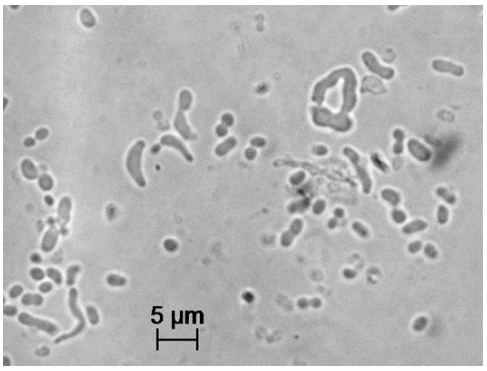

Figure 38| Cell morphology of *P. carnosum* TMW2.2021^T under the light microscope grown on marine broth agar at 15 °C for 72h.

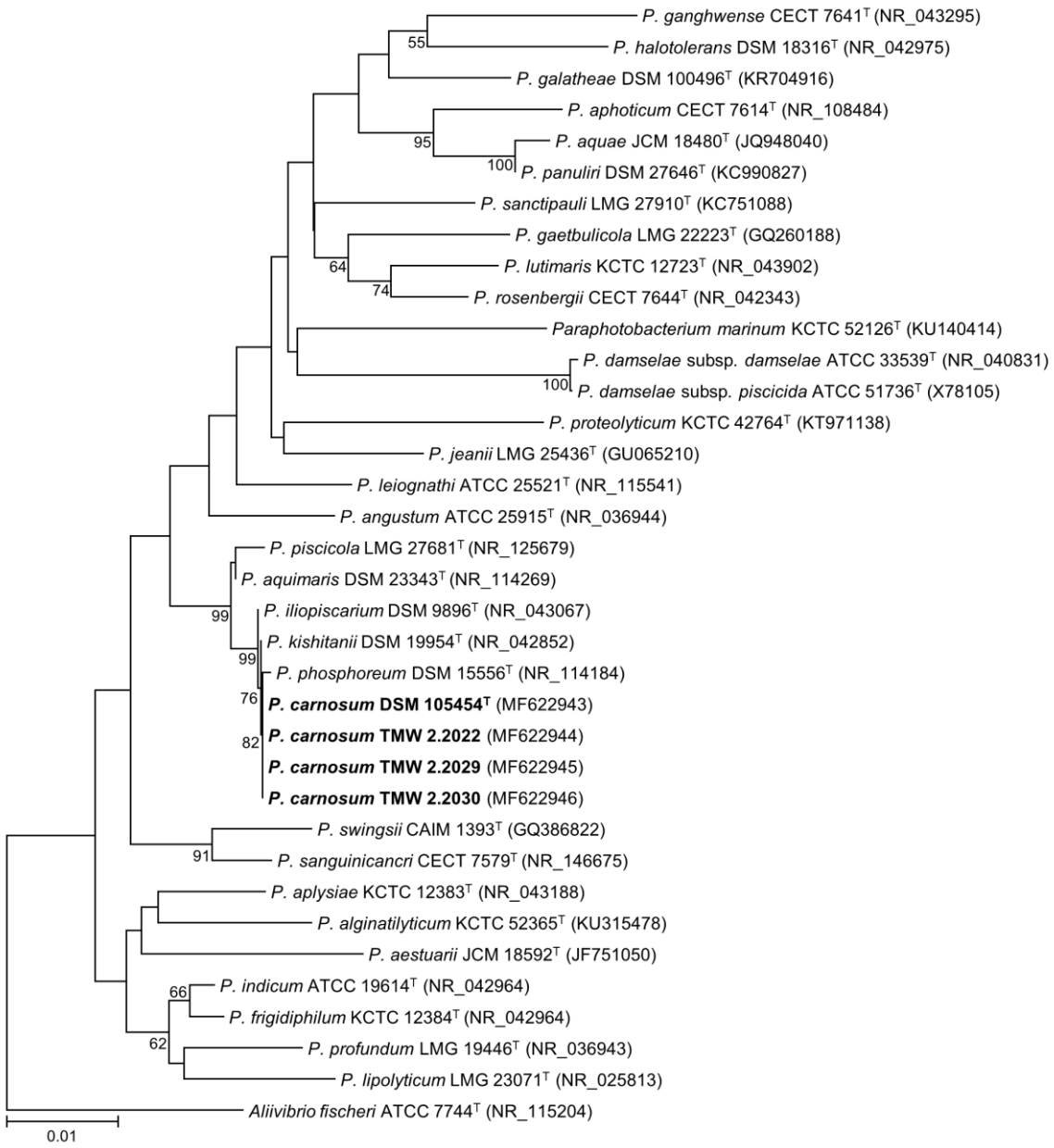

Figure 39| Phylogenetic Neighbor-joining tree of all valid *Photobacterium* species based on partial 16S rRNA gene sequences (=1341-1355 nt) with 1000 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes. Scale bar indicates nucleotide substitutions per site. *Aliivibrio fischeri* ATCC 7744^T was used as outgroup.

4.4.2.2 Phylogenetic analysis of *P. carnosum* within the genus *Photobacterium*

Analysis of partial 16S rRNA genes was used to determine the most closely related species for *P. carnosum* strains within the genus *Photobacterium*. All four strains contained identical 16S rRNA gene sequences. A phylogenetic tree based on partial 16S rRNA gene sequences (=1341-1355 nt) of all valid *Photobacterium* species calculated with neighbor joining (NJ) method (Figure 39) revealed the closest phylogenetic relationship between the strains of proposed *P. carnosum* sp. nov. and the type strains of the *P. phosphoreum* group: *P. kishitanii* DSM 19954^T (99.86% sequence similarity according to the curated EZBioCloud database), *P. phosphoreum* DSM 15556^T (99.65%), *P. iliopiscarium* DSM 9896^T (99.59%), *P. aquimaris* DSM 23343^T (99.54%) and *P. piscicola* LMG 27681^T (99.45%). These strains formed one cluster altogether supported by 99% of bootstraps. The root positioning did not change and the branching was similar when using maximum likelihood (ML) algorithm (Figure A10) and maximum parsimony (MP) algorithm (Figure A11).

To further resolve the 16S rRNA gene cluster, a MLSA (=3735-3762 nt) of selected relevant *Photobacterium* species was performed including 16S rRNA as well as housekeeping genes *gyr*B, *rec*A and *rpo*D. The phylogenetic trees using neighbor joining (Figure 40), maximum likelihood (Figure A12) and maximum parsimony (Figure A13) method display consistent root positioning and branching (with the exception of the slightly different positioning of *P. indicum*) although variations in bootstrap values are present.

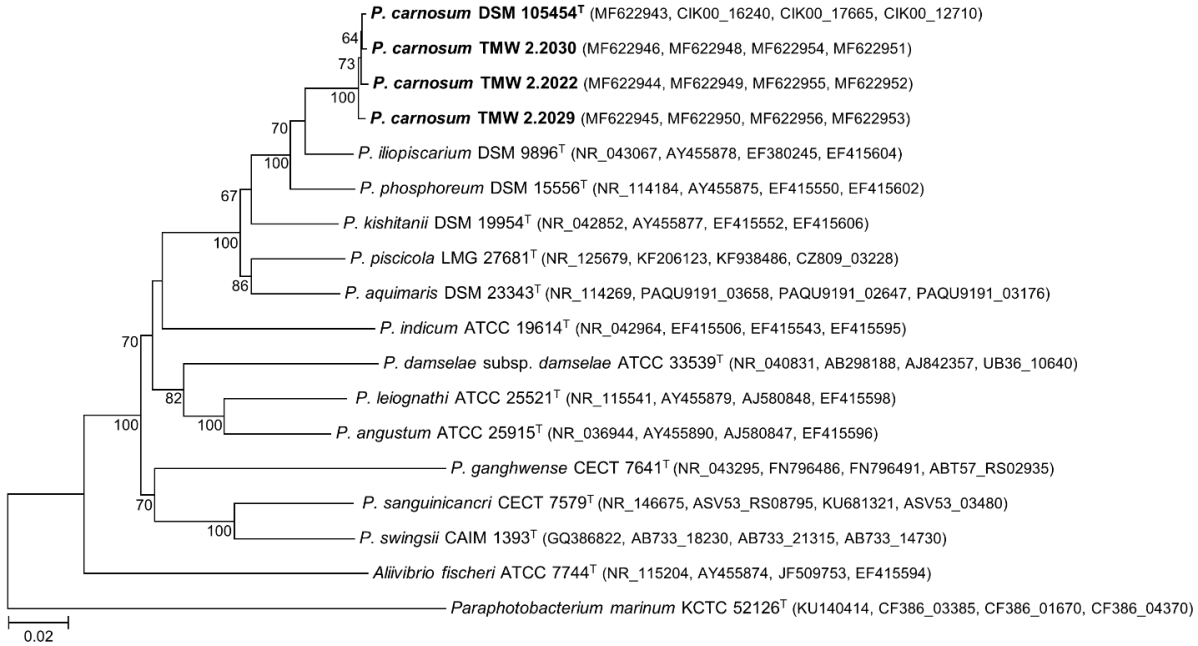

Figure 40| Phylogenetic Neighbor-joining tree of photobacteria based on concatenated partial 16S rRNA (=1341-1342) and partial housekeeping *gyr*B (=1059-1068 nt), *rec*A (=542 nt), *rpo*D (=786-810 nt) gene sequences (in this order, 3735-3762 nt total) with 1000 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes. Scale bar indicates nucleotide substitutions per site. *Paraphotobacterium marinum* KCTC 52126^T and *Aliivibrio fischeri* ATCC 7744^T were used as outgroup.

All trees based on concatenated sequence data demonstrate that the strains of proposed *P. carnosum* sp. nov. form a distinct lineage within the genus *Photobacterium* supported by a bootstrap value of 100% (NJ,ML,MP), and that these strains can be assigned to the *P. phosphoreum* (sub)clade (Gomez-Gil *et al.* 2016). Furthermore, the four strains of proposed *P. carnosum* displayed distinct genotypes in the MLSA.

The accession numbers of 16S rRNA gene sequences of *P. carnosum* strains TMW2.2021^T, TMW2.2022 TMW2.2029, TMW2.2030, are MF622943, MF622944, MF622945, MF622946, respectively. The *gyr*B sequences have the accession numbers CIK00_16240, MF622948, MF622949, MF622950, respectively. The *rpo*D sequences have the accession numbers CIK00_12710, MF622951, MF622952, MF622953, respectively. The *rec*A sequences have the accession numbers CIK00_17665, MF622954, MF622955, MF622956, respectively. The accession number of WGS assembly of strain TMW2.2021^T is NPIB01.

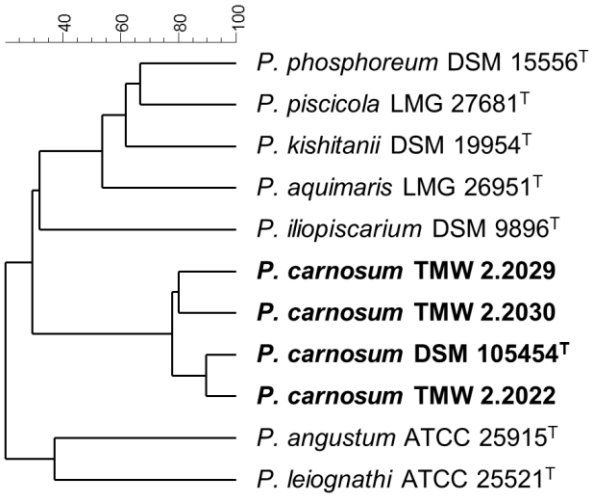

Figure 41| Dendrogram based on mass spectrometry profiles of photobacteria based on their low-molecular subproteome obtained by MALDI-TOF MS. UPGMA was used as cluster method with Dice‘s similarity coefficient. Scale bar refers to the Pearson correlation coefficient.

4.4.2.3 Fingerprinting of *P. carnosum*

Two different fingerprinting analyses were performed with strains of proposed *P. carnosum* sp. nov. and type strains of closest related species of the genus *Photobacterium* based on 16S rRNA and MLSA. Analyses were conducted based on mass spectrometry profiles of their low-molecular subproteome obtained with MALDI-TOF MS (Figure 41) as well as band pattern obtained with RAPD-PCR (Figure 42).

Both approaches demonstrated a distinct lineage of strains of *P. carnosum* resulting in a separate clustering within the hierarchical cluster analysis and support the evidence obtained

from phylogenetic analyses. Again, differences between the four strains were present allowing their differentiation.

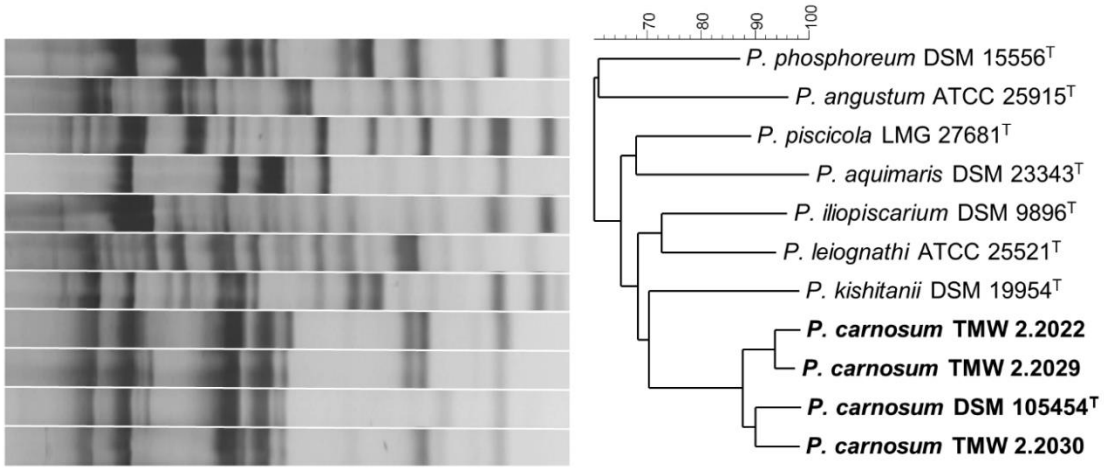

Figure 42| Dendrogram based on M13-RAPD-PCR band pattern fingerprints of photobacteria. Neighbor joining was used as cluster method with Dice's similarity coefficient. Scale bar refers to the Pearson correlation coefficient.

Table 12| Genome comparison of *Photobacterium carnosum* TMW2.2021^T and type strains of related *Photobacterium* species within the *phosphoreum* subclade based on ANIb algorithm.

Genome assembly	ANIb [%]
P. iliopiscarium DSM 9896^T (JZSQ01)	91.43
P. piscicola LMG 27681^T (FUZI01)	88.02
P. phosphoreum DSM 15556^T (JZSJ01)	86.07
P. kishitanii DSM 19954^T (JZSP01)	84.62
P. aquimaris DSM 23343^T (FYAH01)	83.88
P. angustum ATCC 25915^T (JZSO01)	75.58
P. leiognathi ATCC 25521^T (JZSK01)	75.35

Accession numbers of genome assemblies are given in parentheses.

4.4.2.4 Genome properties and ANI values

The WGS of TMW2.2021^T consists of 75 contigs, has a size of 4.56 Mb with 3998 CDS (coding) and a G+C content of 38.49 mol%. The calculated ANI values between strain TMW2.2021^T and closest related type strains of the *P. phosphoreum* (sub-)clade are shown in Table 12. The type strain of proposed *P. carnosum* and the closest related species (*P. iliopiscarium* DSM 9896^T) was 91.43% and therefore clearly below the cutoff of 95-96% for species delineation as established by Richter and Roselló-Móra (2009).

Phylogenetic relationship among *Photobacterium* type strains of the *P. phosphoreum* subclade species based on differences in ANI values is visualized in Figure 43.

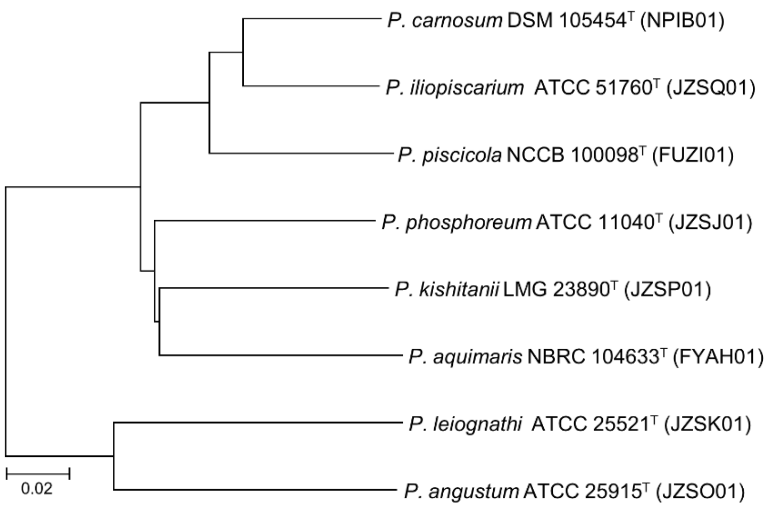

Figure 43| Phylogenetic relationship of type strains of the *Photobacterium phosphoreum* subclade based on ANI values. Neighbor-joining tree was constructed with the distance matrix obtained via ANIb algorithm implemented in JSpeciesWS. Bar indicates sequence dissimilarity. Accession numbers of WGS assemblies are given in parentheses.

Table 13| Composition of cellular fatty acids of *Photobacterium carnosum* strain TMW 2.2021^T and reference strains from closest related species of the genus *Photobacterium*.

Fatty acid[a]	1[b]	2[b]	3[b]	4[b]	5[c]	6[c]
$C_{12:0}$	3.86	3.34	4.58	4.32	4.75	5.9
$C_{12.484}$ (unknown)	0.62	0.67	0.4	0.86	0.73	<1
$C_{12:0}$ 3-OH	4.36	2.74	1.83	2.41	3.54	2.5
$C_{14:1}$ *ω5c*	tr[d]	-	-	-	<1	3.2
$C_{14:0}$	1.74	1.69	3.46	6.31	2.17	8.6
$C_{15:0}$	0.27	1.96	0.87	0.91	<1	2.5
$C_{16:0}$	**14.44**	**9.19**	**15.67**	**18.58**	**16.54**	**18.7**
$C_{17:1}$ *ω8c*	-	1.32	0.33	0.94	<1	-
$C_{17:0}$ cyclo	-	0.83	-	**15.22**	-	-
$C_{17:0}$	0.23	1.48	-	0.4	0.91	-
$C_{18:1}$ *ω9c*	0.60	0.81	-	-	<1	
$C_{18:1}$ *ω7c*	**9.88**	**21.76**	**7.88**	**7.63**	**11.20**	**2.5**
$C_{18:0}$	1.46	1.44	0.32	0.83	0.72	**10.8**
$C_{20:1}$ *ω7c*	1.39	1.62	-	-	<1	<1
$C_{20:0}$	tr[d]	-	-	-	<1	<1
Summed feature[e]						
2	**5.14**	4.39	3.54	2.11	4.73	4.9
3	**54.99**	**45.22**	**61.13**	**34.76**	**52.48**	**36.5**
5	tr[d]	-	-	-	<1	<1

[a]Specific fatty acids are shown as % of total fatty acids; major fatty acids (>5%) are shown in bold. Fatty acids abundant below 0.5% in all strains are not shown; -, not detected.
[b]Taxa: 1, strain TMW 2.2021T; 2, *P. iliopiscarium* DSM 9896^T; 3, *P. phosphoreum* DSM 15556^T; 4, *P. kishitanii* DSM 19954^T; all data from this study; cells were grown in MB for 72h at 15 °C.
[c]Taxa: 5, *P. piscicola* LMG 27681^T; 6, *P. aquimaris* DSM 23343^T; data taken from the respective species description (Yoshizawa *et al.* 2009; Figge *et al.* 2014). Values <1% were not reported in the literature.
[d]tr, trace amount that was only found in TMW2.2021^T and was below 0.5%.
[e]Summed feature contained fatty acid that could not be separated by HPLC. Summed feature 2 contains $C_{12:0}$ aldehyde and $C_{10.928}$ unkown; summed feature 3 contains $C_{16:1}$ *ω7c* and/or iso-C_{15} 3-OH; summed feature 5 contains $C_{18:2}$ *ω*6,9*c* and/or anteiso-C18:0.

4.4.2.5 Chemotaxonomic properties

Ubiquinone Q-8 was identified as the sole respiratory quinone under aerobic conditions. Analysis of cellular fatty acids of strain TMW2.2021^T revealed summed feature 3 ($C_{16:1}\omega7c$/iso-C_{15} 3-OH), $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 2 ($C_{12:0}$ aldehyde and $C_{10.928}$ unknown) as the major fatty acids.

The overall fatty acid profile of strain TMW2.2021^T was similar to reference strains of closest related species (Table 13), while the respective proportions of certain fatty acids varied between species and some compounds were found as traces only in TMW2.2021^T, but not in *P. phosphoreum* DSM 15556^T, *P. iliopiscarium* DSM 9896^T and *P. kishitanii* DSM 19954^T. Additionally, $C_{17:0}$ cyclo was not detected in strain TMW2.2021^T but represented a major compound (15.22%) in *P. kishitanii* DSM 19954^T, which is in accordance with the original data (Ast *et al.* 2007). Fatty acid $C_{18:0}$ was detected only in small amounts in strain TMW2.2021^T, while it has been reported to be a major fatty acid of *P. aquimaris* DSM 23343^T (Yoshizawa *et al.* 2009).

4.4.2.6 Phenotypic analysis

Differences in phenotypic characteristics between the four strains of proposed *P. carnosum* sp. nov. and closest related type strains of *P. iliopiscarium* DSM 9896^T, *P. phosphoreum* DSM 15556^T, *P. kishitanii* DSM 19954^T, *P. piscicola* LMG 27681^T and *P. aquimaris* DSM 23343^T are listed in Table 14. Results for carbohydrate utilization for all four strains of proposed *P. carnosum* sp. nov. were identical except for glycogen. Strain TMW2.2021^T did not produce acid from glycogen, whereas the other three strains did. All strains produced acid from glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, esculin ferric citrate, D-maltose, potassium 2-ketogluconate and starch. Enzymatic activities of the four strains were highly similar as well. All strains were tested positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. Additionally, some strains were tested positive for trypsin activity α-glucosidase (including TMW2.2021^T) and one strain weakly positive reaction for valine arylamidase. Catalase and oxidase activity was also strain dependent within the species. Strain TMW2.2021^T was tested negative for catalase and positive for oxidase.

All four strains of proposed *P. carnosum* grew at a temperature range of 0 – 20 °C with an optimum growth occurring at 10 – 15 °C. Additionally, two strains (including TMW2.2021^T) were rapidly growing at 0 °C (visible growth after three days). All strains grew within a pH range of 5.0-8.5 with an average optimum of 6 (6-8 for TMW2.2021^T). All strains required additional NaCl (0.5-1%) in the media and showed growth with up to 3-4% salt, with an optimum of 1-2%. Strain TMW2.2021^T grew in the presence of 0.5-3% NaCl, with an optimum of 1%.

Table 14| Differences in phenotypic characteristics of the four strains of *P. carnosum* and type strains of close related species within the genus *Photobacterium*.
1-4 *P. carnosum* sp. nov.; **1**, strain TMW2.2030; **2**, strain TMW2.2022; **3**, strain TMW2.2021^T; **4**, strain TMW2.2029; **5**, *P. iliopiscarium* DSM 9896^T; **6**, *P. phosphoreum* DSM 15556^T; **7**, *P. kishitanii* DSM 19954^T; **8** *P. piscicola* LMG 27681^T; **9** *P. aquimaris* DSM 23343^T.
++, complete growth/immediate, strongly positive reaction; +, growth observed/positive reaction; -, no growth/negative reaction; w, weak growth/weak or remarkably slow reaction.

Characteristics	1	2	3^T	4	5	6	7	8	9
Catalase	+	+	-	-	+	++	+	+	+
Oxidase	-	+	+	w	-	w	-	+	+
Motility	-	-	-	-	++	++	++	+	+
Growth in TCBS	-	-	-	-	+	+	+	-	-
Luminescence	-	-	-	-	-	+	++	-	+
Optimal temperature (°C)	10-15	10-15	10-15	10-15	15-25	15-25	15-25	20-25	25
Temperature									
0 °C[a]	-	w	w	-	w	-	-	-	-
25 °C	-	-	-	-	++	++	++	++	++
%NaCl									
0.5	-	-	w	w	++	w	++	++	-
4.0	w	-	-	w	++	++	++	++	++
5.0	-	-	-	-	w	+	++	+	+
pH									
5.0	w	w	w	w	w	w	++	+	+
9.0	-	-	-	-	+	w	++	-	-
API 50 CH, fermentation of									
Glycerol	w	w	w	w	+	+	w	-	-
D-cellobiose	-	-	-	-	-	-	w	-	-
D-lactose (bovine)	-	-	-	-	-	w	-	-	-
D-melibiose	-	-	-	-	-	w	-	-	-
Amidon (starch)	+	+	+	+	-	-	-	-	-
Glycogen	w	w	-	+	-	-	-	-	-
Potassium 2-ketogluconate	w	w	w	w	-	w	w	w	w
Potassium 5-ketogluconate	-	-	-	-	-	w	w	-	-
API ZYM									
α-glucosidase	-	w	+	-	-	-	-	-	-
Trypsin	+	+	-	-	w	+	+	-	-
Valine arylamidase	w	-	-	-	w	+	-	w	w
Naphtol-AS-BI-PH[b]	+	+	+	+	+	-	-	w	w
α-galactosidase	-	-	-	-	-	-	-	-	+
β-galactosidase	-	-	-	-	-	+	-	-	+
Esterase (C4)	-	-	-	-	-	-	-	w	+
Esterase Lipase (C8)	-	-	-	-	-	-	-	+	w

[a]after three days of incubation
[b]PH, phosphohydrolase

All strains grew well on MB agar (aerobically and anaerobically), *Photobacterium* broth agar and on TSA supplemented with 2% NaCl, but not on TCBS agar. The majority of strains (including TMW2.2021^T) was not able to grow on BHI agar.

4.4.2.7 Description of *Photobacterium carnosum* sp. nov.

Photobacterium carnosum (car.no`sum. L. neut. adj. *carnosum*, pertaining to flesh).

Member of this species are Gram-staining-negative, facultatively aerobic, non-motile, non-luminous and coccoid to rod shaped. All strains produced acid from starch, D-glucose and D-ribose, and the majority produced acid from glycogen. All strains showed naphtol-AS-BI-phosphohydrolase activity. Catalase, oxidase and α-glucosidase activity are strain dependent within the species and TMW 2.2021^T is catalase negative, oxidase positive, and α-glucosidase positive. The type strain grew at 0-20 °C (optimum 10-15 °C), within pH 5.0-8.5 (optimum 6-8) and in the presence of 0.5-3% (w/v) NaCl (optimum 1%). Major cellular fatty acids of TMW 2.2021^T were summed feature 3 ($C_{16:1}\omega7c$/iso-C_{15} 3-OH), $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 2 ($C_{12:0}$ aldehyde and $C_{10.928}$ unknown). Quinone Q-8 is the sole respiratory ubiquinone. The genome size of the type strain is 4.56 Mb and has a G+C content of 38.45 mol%.

The type strain is TMW 2.2021^T (=DSM 105454^T =CECT 9394^T) and was isolated from spoiled retail modified-atmosphere packaged poultry meat purchased in Freising, Germany, in 2017. Additional strains are TMW 2.2022, TMW 2.2029 and TMW 2.2030. The taxonumber of the digital protologue is TA00237. The taxonomic ID within the NCBI is 2023717.

The designation and taxonomic status of *Photobacterium carnosum* as a novel species was effectively published in the Journal Systematic and Applied microbiology (SAM) (Hilgarth *et al.* 2018) and validly published afterwards in International Journal of Systematic and Evolutionary Microbiology (IJSEM) (Oren and Garrity 2018) with *P. carnosum* TMW2.2021^T as type strain.

4.5 Whole genome sequencing analyses

Whole genome sequencing of spoilage-associated strains from relevant species and isolated from different meats in this study was carried out using either PacBio single molecule real-time sequencing (SMRT, 3.7.1) or Illumina whole genome shotgun (WGS, 3.7.2). Sequencing statistics, genome properties and accession numbers of all sequenced organisms are shown in Table 15. Chromosomal DNA of strains sequenced by PacBio SMRT could be circularized and composited to one contig. Additional contigs in case of *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 were plasmids. Genomes of strains sequenced by WGS were assembled to 47-147 contigs. Genome size varied from 1.82 – 2.16 Mb for sequenced LAB *Lc. piscium* and *Le. gelidu*m subsp., and 3.96-4.83 Mb for *Photobacterium* spp, respectively.

Table 15| Genome information, sequence statistics and accession numbers.

Strain	Accession No.[1]	Size (Mb)	Cov. (x)[2]	No. of contigs	G+C (mol %)	No. of CDSs[3]	Seq-Technique[4]
Lc. piscium TMW2.1612	CP017194	2.16	398	1	38.8	2006/ 1969	PacBio SMRT
Lc. piscium TMW2.1615	CP017195	2.27	280	1	38.2	2144/ 2108	PacBio SMRT
Le. gelidum* subsp. *gelidum TMW2.1618	CP017196	1.82	519	1	36.9	1699/ 1653	PacBio SMRT
***Le. gelidum* subsp. *gasico*[5]** TMW2.1619	CP017197-200	1.92	437	4	36.9	1860/ 1802	PacBio SMRT
P. carnosum TMW2.2021^T	NPIB01000001-75	4.56	199	75	38.5	4059/ 3998	Illumina WGS
P. carnosum TMW2.2029	NPMQ01000001-47	3.97	124	47	38.7	3483/ 3423	Illumina WGS
P. phosphoreum TMW2.2033	NQLT01000001-61	4.83	175	61	39.2	4230/ 4155	Illumina WGS
P. phosphoreum TMW2.2034	NQLU01000001-147	4.72	195	147	39.7	4179/ 4104	Illumina WGS
P. iliopiscarium TMW2.2035	NQLV01000001-77	3.96	136	77	39	3526/ 3447	Illumina WGS

[1]accession number within the NCBI database.
[2]sequencing coverage.
[3]coding sequences (total/coding)
[4]sequencing technique; SMRT, single molecule real-time; WGS, whole genome shotgun
[5]*gasicomitatum*

4.5.1 Evaluation of automatic genome annotation pipelines

Fasta files of assembled genomes were submitted to NCBI in order to obtain accession numbers as well as locus tags for genes. Genomes were sent to annotation to the Prokaryotic Genome Annotation Pipeline (PGAP), a service of National Center for Biotechnology Information (NCBI), and to the Rapid Annotations using Subsystems Technology (RAST) pipeline that additionally attributes EC numbers (enzyme commission number), respectively.

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway map pipeline was used to create pathways based on the EC numbers obtained by RAST.

Manual curation of automatic processes revealed that there are several major issues with this process. Manually re-checking via BLAST (Basic Local Alignment Search Tool) of automatic annotations obtained from pipelines revealed frequent erroneous annotation, which had to be corrected manually. If genes were not found via annotations, amino acid sequences of close related organisms were obtained from the NCBI database and checked for an alignment within the sequenced genome using protein BLAST. In order to speed up the process, respective genomes were joined into one fasta file.

Also, the KEGG database missed to find entries that e.g. have amended EC numbers in many cases. Vice versa, if an EC number could not be given specifically based on RAST annotation e.g. “EC 3.4.-“ for a peptidase, KEGG will report presence all EC entries of that class (3.4), which also leads to erroneous assignments. Furthermore, the pathways shown by KEGG are not entirely complete and do not show all enzymes/reactions involved. Manual curation of gene annotations and manual creation of metabolic pathways is therefore necessary.

4.5.2 Predictive metabolic pathways of spoilage-associated bacteria

Based on the presence or absence of genes within the genomes of the different bacteria, *in silico* predicted metabolic pathways for relevant meat-derived substrates for energy conservation were constructed. All relevant gene locus tags are listed in Table A5. In general, while strains of one respective species were highly similar regarding general metabolic routes, there were major differences between different spoilage-associated species.

4.5.2.1 Carbohydrate and nucleoside metabolism

Predictive carbohydrate metabolic routes for hexoses are shown in Figure 44. In general, all sequenced bacteria possess genes for degradation of different hexoses. *Photobacterium* spp. as well as *Lc. piscium* strains are capable of homolactic fermentation, whereas *Le. gelidum* subsp. are not due to the absence of the gene coding for 6-phospho-fructokinase (*pfk*A). Vice versa, *Le. gelidum* subsp. *gelidum* & *gasicomitatum* are capable of heterolactic fermentation, whereas the other species are not due to the absence of the gene coding for xylulose-5-phosphate phosphoketolase (*xpk*A). Strains of *Lc. piscium*, *P. carnosum* and *P. iliopiscarium* possess the gene coding for glycogen phosphorylase (*glg*P) for degradation of glycogen and additionally a gene for a glycogen debranching enzymes (*glg*X). Additionally, genome-sequenced photobacteria harbor all genes to facilitate the pentose-phosphate-pathway (Table A5). *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum* as well as *Lc. piscium* strains lack the gene coding for transaldolase (*tal*). All sequenced bacteria lack the gene for phosphogluconate dehydratase (*edd*) as part of the Entner-Doudoroff pathway.

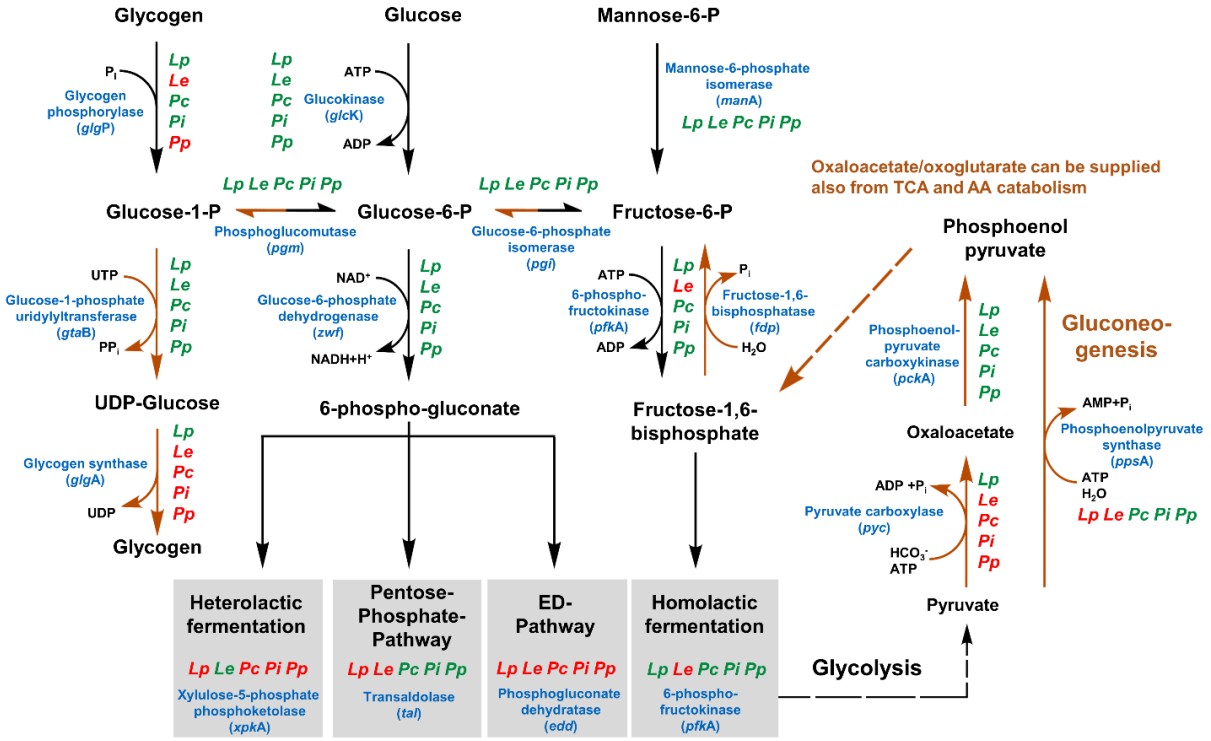

Figure 44| Predictive hexose carbohydrate metabolism of genome sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*, *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*. green, gene present in the respective genome; red, gene absent in the respective genome; black arrows, glycolytic reactions; orange, gluconeogenetic routes; enzyme names and encoding genes are shown in blue.

Genes for gluconeogenetic routes from pyruvate to phosphoenolpyruvate either directly (*Lc. piscium* strains) via phosphoenolpyruvate synthase (*pps*A) or with oxaloacetate as an intermediate (photobacteria) via pyruvate carboxylase (*pyc*) and phosphoenolpyruvate carboxykinase (*pck*A) were found in the respective genomes. These genes were absent in the genomes of both *Le. gelidum* subsp. *Lc. piscium* strains and photobacteria also possess a gene encoding a fructose-1,6-bisphosphatase (*fdp*) for the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, which can be further converted to glucose-6-phosphate and glucose-1-phosphate via glucose-6-phosphate isomerase (*pgi*) and phosphoglucomutase (*pgm*). Additionally, the gene coding for glycogen synthase (*glg*A) was found in both *Lc. piscium* genomes.

The predictive (deoxy-)nucleoside and downstream ribose metabolism of genome-sequenced organisms is shown in Figure 45. All genomes except *P. carnosum* strain TMW2.2029 possess a putative deoxyribose-/nucleoside specific ABC transporter (*nup*A/*yng*F) in the genome for uptake of these substrates. *Le. gelidum* subsp. *gasicomitatum* strain TMW2.1619 additionally possesses a gene encoding a putative ribose uptake protein (*rbs*U). All analyzed genomes contain the genes for ribonucleotide reductase (*nrd*AB) that catalyzes the conversion of nucleosides to deoxy-nucleosides.

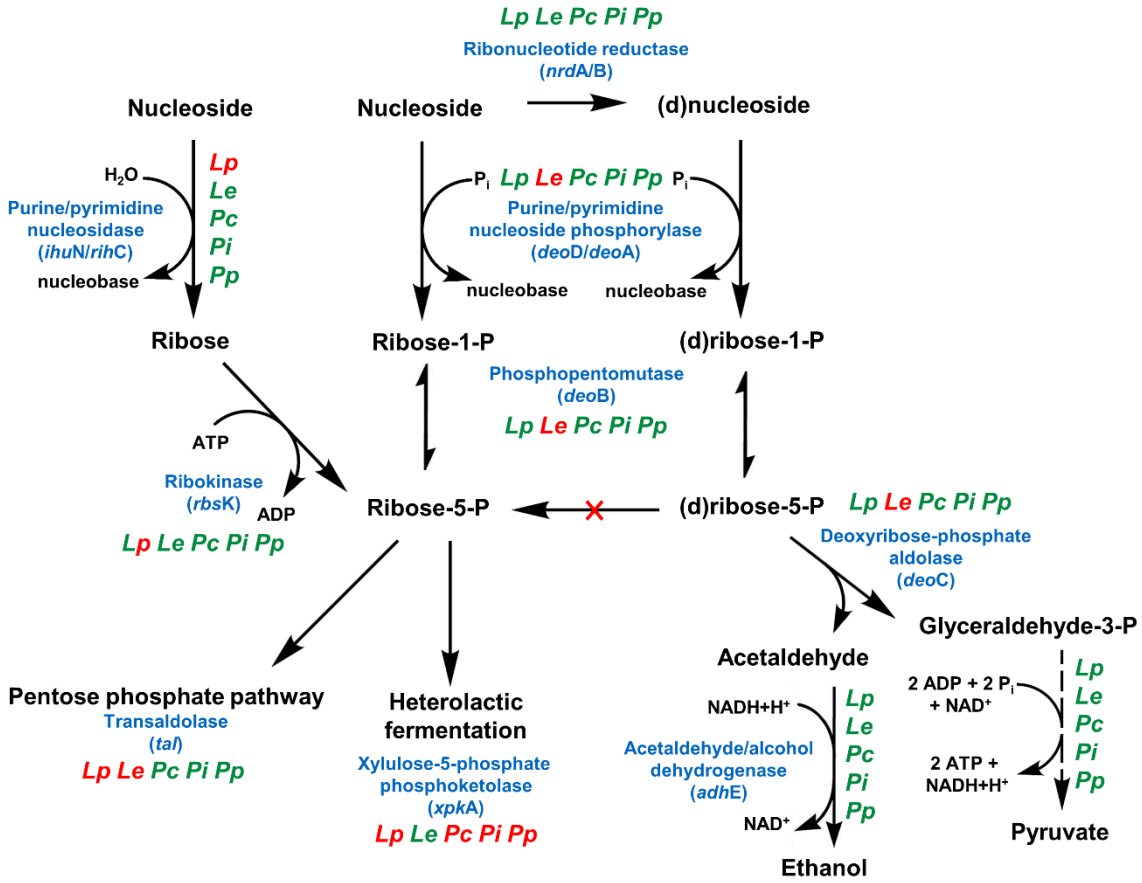

Figure 45| Predictive (deoxy-)nucleoside and ribose metabolism of genome sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*, *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*; green, gene present in the respective genome; red, gene absent in the respective genome; half green/red, gene not present in all strains; enzyme names and encoding genes are shown in blue.

Genomes of photobacteria contain genes to yield ribose-5-P from nucleosides either via purine/pyrimidine nucleosidase (*ihu*N/*rih*C) and ribokinase (*rbs*K) or purine/pyrimidine nucleoside phosphorylase (*deo*D/*deo*A) and phosphopentomutase (*deo*B). Both *Le. gelidum* subsp. possess genes for the former and *Lc. piscium* strains genes for the latter pathway. The resulting ribose-5-phosphate can be used in the pentose phosphate pathway (*Photobacterium* spp.) or in heterolactic fermentation (*Le. gelidum* subsp.). No genes were found in the genomes of *Lc. piscium* strains for using ribose-5-phosphate as a substrate. Deoxyribose-5-phosphate originating from deoxynucleosides can be potentially obtained also via *deo*D/*deo*A and further converted via deoxyribose-phosphate aldolase (*deo*C) by *Photobacterium* spp. and *Lc. piscium* strains to glycolytic glyceraldehyde-3-phosphate (G-3-P) and acetaldehyde. G-3-P can be channeled into glycolysis and acetaldehyde can be converted to ethanol via acetaldehyde/alcohol dehydrogenase (*adh*E) yielding NAD^+.

4.5.2.2 Pyruvate fates

The various possible fates of pyruvate are shown in Figure 46. Pyruvate can be converted to lactate via lactate dehydrogenase (*ldh*) yielding NAD^+, which gene is present in all analyzed genomes. The potential reverse reaction can be coupled to NADH oxidase (*nox*). A gene encoding a protein with a putative NADH oxidase like domain was found also in all genomes, but no distinct annotation for its actual function was possible in this case.

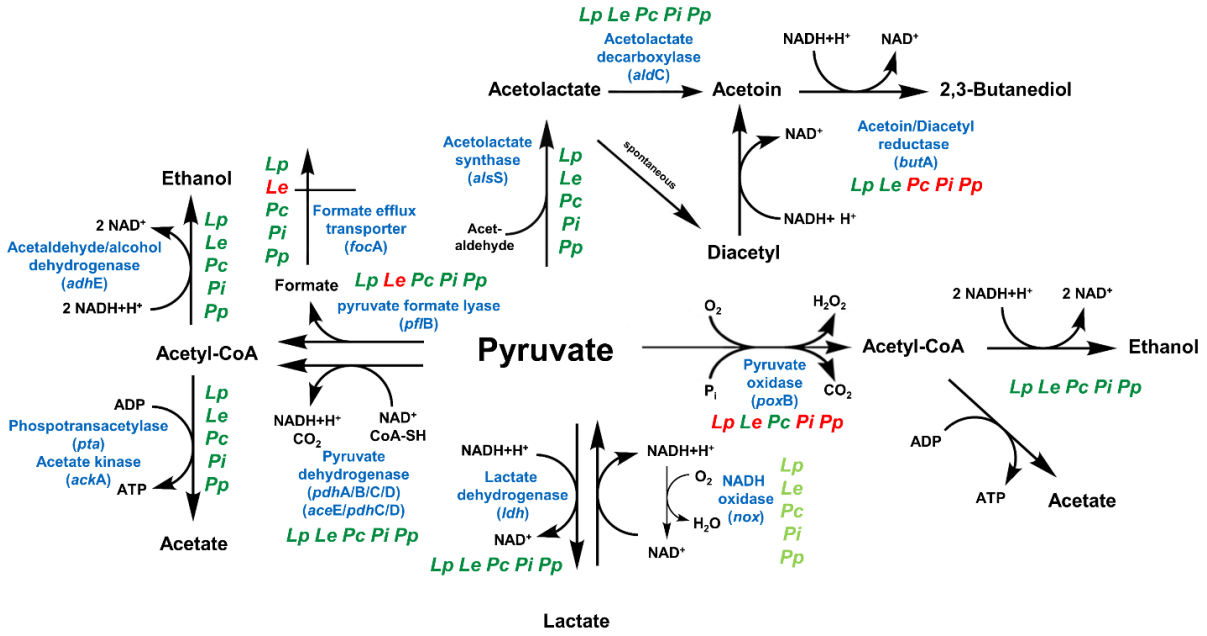

Figure 46| Predictive pyruvate fates in genome-sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*, *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*; dark green, gene present in the respective genome; light green, gene with only a putative domain present in the respective genome; red, gene absent in the respective genome; half green/red, gene not present in all strains; enzyme names and encoding genes are shown in blue.

Pyruvate can also be converted to acetyl-CoA in three different ways. The first option is via pyruvate dehydrogenase (*pdh*ABCD or *ace*E/*pdh*CD) yielding also carbon dioxide and $NADH+H^+$, which is present in all sequenced genomes. The second option is conversion via pyruvate formate lyase (*pfl*B) yielding also formate that is present in all genomes except both *Le. gelidum* subsp. An efflux transporter (*foc*A) facilitates subsequent export of formate. The third conversion option is via pyruvate oxidase (*pox*B) yielding carbon dioxide and hydrogen peroxide, which is only present in the genome of *Le. gelidum* subsp. *gasicomitatum* as well as the two strains of *P. carnosum*. The resulting acetyl-CoA can be potentially converted by all sequenced organisms to either ethanol via acetaldehyde/alcohol dehydrogenase (*adh*E) or acetate via phosphotransacetylase (*pta*) and acetate kinase (*ack*A), yielding NAD^+ and ATP, respectively.

Pyruvate can potentially also be converted to acetolactate by acetolactate synthase (*als*S) and subsequently to acetoin by acetolactate decarboxylase (*ald*C). Both genes are present in all analyzed genomes. Additionally, genomes of *Lc. piscium* strains and both *Le. gelidum* subsp. contain the gene encoding an acetoin/diacetyl reductase (*but*A), which catalyzes the reaction of diacetyl (formed spontaneous from acetolactate) to acetoin and subsequently to 2,3-butandiol with each reaction yielding NAD^+.

4.5.2.3 Triacylglyceride catabolism

Predictive triacylglyceride catabolism of genome-sequenced bacteria is shown in Figure 47. The first step of triacylglyceride breakdown is cleavage into fatty acids and glycerol, catalyzed by lipases. Genes encoding lipases were found in all photobacteria genomes, whereas the genomes of *Lc. piscium* and both *Le. gelidum* subsp. only contained a gene with a putative esterase/lipase domain, but no gene clearly identifiable as a lipase. Resulting free fatty acids can be transported into the cell by long-chain fatty acid transporter (*fad*L) and activated by long-chain fatty acid CoA ligase (*fad*D). Subsequently, fatty acids can be converted to acetyl-CoA upon aerobic β-oxidation involving acyl-CoA dehydrogenase (*fad*E), 3-hydroxyacyl-CoA dehydrogenase (*fad*B) and acetyl-CoA acyltransferase (*fad*A). Additionally, anaerobic β-oxidation of fatty acids is facilitated by long-chain fatty acid CoA ligase (*fad*K), 3-hydroxyacyl-CoA dehydrogenase (*fad*J) and acetyl-CoA acyltransferase (*fad*I). All genes encoding import proteins and enzymes for this processes were found in all genomes of sequenced photobacteria, but none in LAB. Finally, the acetyl-CoA can be channeled into the TCA cycle (4.5.2.4).

The glycerol moiety can be activated by glycerol kinase (*glp*K) to glycerol-3-phosphate, present in all genomes except both *Le. gelidum* subsp. Subsequent oxidation by either glycerol-3-phosphate dehydrogenase (*gps*A) or α-glycerophosphate oxidase (*glp*O) yields dihydroxyacetone phosphate, which can be channeled into glycolysis. The gene *gps*A was present in all analyzed genomes, whereas additional *glp*O genes were only detected in both *Lc. piscium* strains. Another possible pathway of glycerol metabolism is the gradual reduction to 3-hydroxy-propanal and 1,3-propanediol by glycerol dehydratase (*dha*B) and 1,3-propanediol dehydrogenase (*dha*T) yielding NAD^+, but genes for these reactions were not present in the analyzed genomes. Genes for glycerol uptake protein (*glp*F) were detected in all genomes (putative in photobacteria genomes).

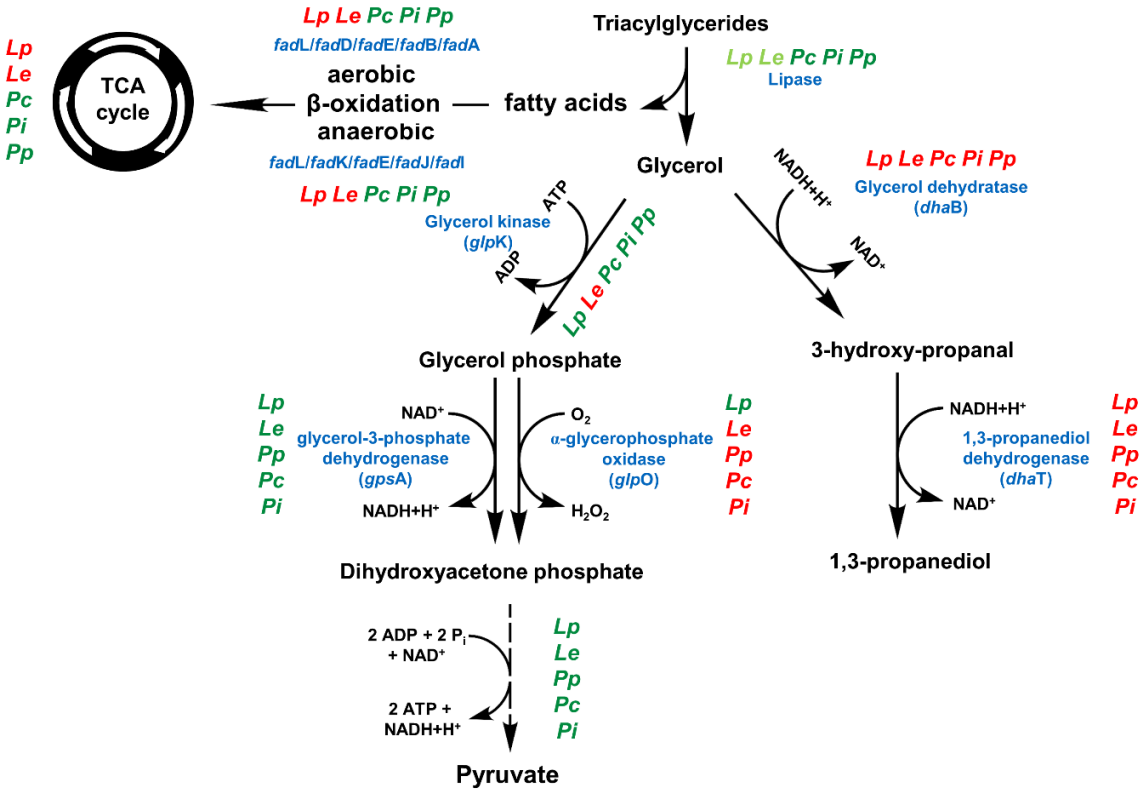

Figure 47| Predictive triacylglyceride catabolism in genome sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*, *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*; dark green, gene present in the respective genome; light green, gene encoding respective putative domain present in the respective genome; red, gene absent in the respective genome; enzyme names and encoding genes are shown in blue.

4.5.2.4 Tricarboxylic acid cycle and anaplerotic amino acid reactions

All photobacteria genomes analyzed harbor the genes necessary for the complete tricarboxylic acid (TCA, Figure 48) cycle, which are citrate synthase (*cit*A), aconitate hydratase (*cit*B), isocitrate dehydrogenase (*icd*A), oxoglutarate dehydrogenase (*suc*AB), succinyl-CoA synthetase (*suc*CD), succinate dehydrogenase (*sdh*ABCD), fumarate hydratase (*fum*A), malate dehydrogenase (*mdh*). In contrast, *Lc. piscium* strains and both *Le. gelidum* subsp. harbor only the first three out of eight genes needed for a complete TCA cycle.

Anaplerotic reactions towards acetyl-CoA are facilitated from pyruvate via pyruvate dehydrogenase complex (*ace*E/*pdh*CD) or pyruvate formate lyase (*pfl*B) present in all photobacteria genomes or via pyruvate oxidase (*pox*B), present only in the genomes of *P. carnosum* (cf. Figure 46). Also, β-oxidation of fatty acids from triacylglycerides can yield acetyl-CoA (cf. Figure 47). Anaplerotic reactions towards oxaloacetate can also be facilitated from pyruvate. While genes encoding a pyruvate carboxylase (*pyc*) are absent in all photobacteria genomes, the reaction can also be facilitated by phosphoenolpyruvate synthase (*pps*A) and

subsequent phosphoenolpyruvate carboxylase (*ppc*) reaction. These genes are present in the genomes of all photobacteria analyzed.

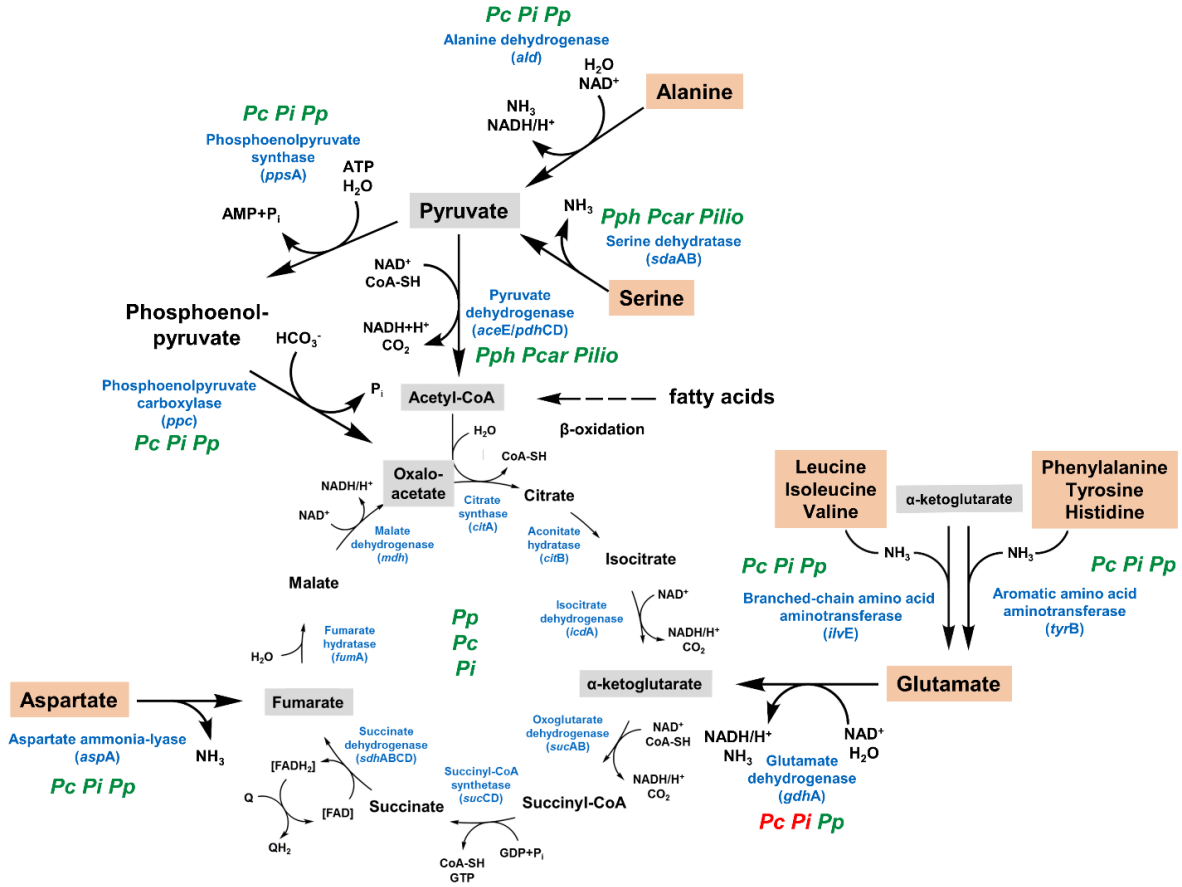

Figure 48| Predictive tricarboxylic acid cycle and anaplerotic routes of *Photobacterium* spp. *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*; green, gene present in the respective genome; red, gene absent in the respective genome; enzyme names and encoding genes are shown in blue; orange boxes, amino acid supplying the TCA cycle; grey boxes, key intermediates of the TCA cycle.

Genes for different anaplerotic routes involving amino acids are present in the genomes of *Photobacterium* spp. analyzed (Figure 48). Alanine and serine can be converted to pyruvate by alanine dehydrogenase (*ald*) and serine dehydratase (*sda*AB), respectively. Aspartate can be converted to fumarate by aspartate ammonia-lyase (*asp*A). All of these reaction release ammonia and genes needed for these reactions were found in every *Photobacterium* spp. genome. Additionally, (iso-)leucine, valine as well as phenylalanine, tyrosine, and histidine can be converted to glutamate via branched-chain (*ilv*E) and aromatic amino acid (*tyr*B) aminotransferases. Anaplerotic reaction of glutamate towards α-ketoglutarate is catalyzed by glutamate dehydrogenase (*gdh*A) and the gene is present in the genome of *P. phosphoreum*, but not in the genomes of *P. carnosum* and *P. iliopiscarium*.

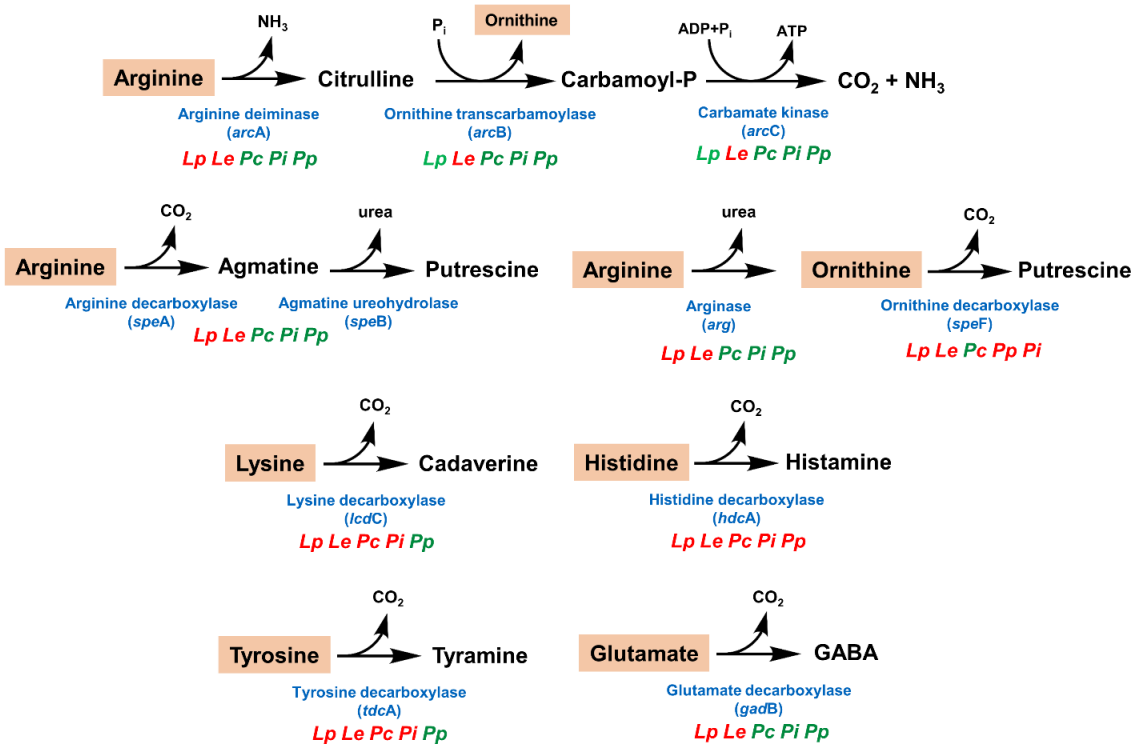

Figure 49| Predictive amino acid metabolism in genome-sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*; *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *P. phosphoreum*; green, gene present in the respective genome; red, gene absent in the respective genome; half green/red, gene not present in all strains; enzyme names and encoding genes are shown in blue.

4.5.2.5 Amino acid metabolism

Predictive amino acid metabolism of photobacteria and potential reactions producing biogenic amines are shown in Figure 49. Genes for arginine deiminase pathway were present in all *Photobacterium* spp. genomes consisting of arginine deiminase (*arc*A), ornithine transcarbmoylase (*arc*B) and carbamate kinase (*arc*C). Additionally, genes for arginase (*arg*) yielding ornithine, and for production of putrescine from arginine via arginine decarboxylase (*spe*A) and agmatine ureohydrolase (*spe*B), and for production of γ-aminobutyric acid (GABA) were present in all genomes of photobacteria. Furthermore, the genomes of both *P. phosphoreum* strains TMW2.2033/TMW2.2034 harbor genes for cadaverine production from lysine via lysine decarboxylase (*lcd*C) and tyramine production from tyrosine via tyrosine decarboxylase (*tdc*A). Production of putrescine can also originate from ornithine via ornithine decarboxylase (*spe*F). The gene *spe*F is present in the genome of *P. carnosum* TMW2.2029, but absent in the other photobacteria genomes. The gene coding for a histidine decarboxylase (*hdc*A) that catalyzes the reaction of histidine to histamine was absent in all analyzed genomes.

In the genomes of sequenced LAB *Le gelidum* subsp. and *Lc. piscium*, genes coding for enzymes of the ADI pathway were only partly present and genes coding for amino acid decarboxylases were absent (Figure 49).

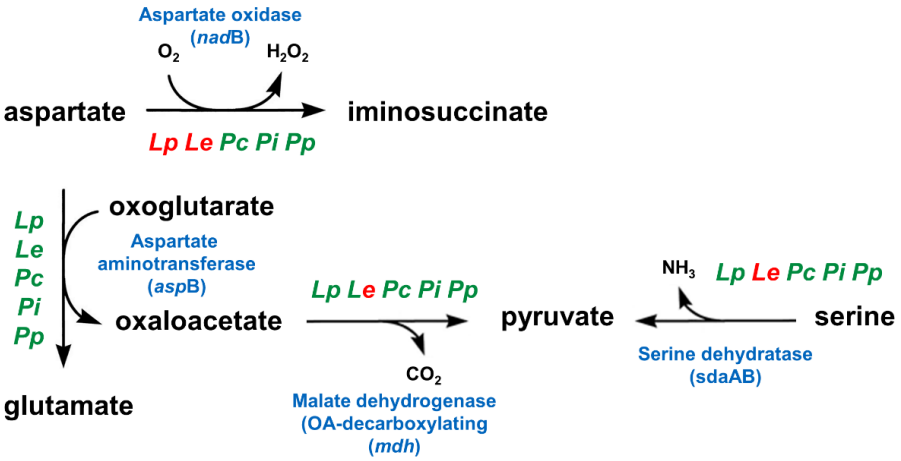

Figure 50| Predictive additional catabolic amino acid reactions in genome-sequenced spoilage-associated bacteria. *Lp*, *Lactococcus piscium*, *Le*, *Leuconostoc gelidum* subsp. *gelidum* & *gasicomitatum*; *Pc*, *Photobacterium carnosum*, *Pi*, *Photobacterium iliopiscarium*; *Pp*, *Photobacterium phosphoreum*; green, gene present in the respective genome; red, gene absent in the respective genome; half green/red, gene not present in all strains/subspecies; enzyme names and encoding genes are shown in blue.

Additional catabolic amino acid reactions in spoilage-associated bacteria are shown in Figure 50. Genome-sequenced *Lc. piscium* strains harbor the gene encoding a serine dehydratase (*sda*AB), catalyzing the reaction of serine to pyruvate. However, in one strain, the CDS contains stop codons. Furthermore, genomes of *Lc. piscium* strains as well as *Le. gelidum* subsp. *gasicomitatum* harbor genes for aspartate aminotransferase (*asp*B) and oxaloacetate-decarboxylating malate dehydrogenase (*mdh*) yielding glutamate and pyruvate from aspartate and oxoglutarate. All photobacteria genomes also contain all of these genes and additionally a gene coding for aspartate oxidase (*nad*B), converting aspartate to iminosuccinate and producing hydrogen peroxide.

4.5.2.6 Respiration

Genes were present in all analyzed photobacteria genomes to build a functional respiratory chain under oxic (Figure 51A) and anoxic conditions (Figure 51B/C).

Under oxic conditions, the first step in the respiratory chain is facilitated by NADH dehydrogenase (*ndh* or *nqr*ABCDEF) with NADH and succinate dehydrogenase (*sdh*ABCD) with fumarate as electron donor. Additionally, both *P. phosphoreum* strains harbor the gene cluster for NADH dehydrogenase (*nuo*A-N). Subsequently, ubiquinone 8 (*ubi*C/A/D/B/G/H/I/E/F) and cytochrome C reductase (*qcr*ABCD) can function as electron shuttle. Genes for all eight steps of ubiquinone biosynthesis are present in the genomes. A heme-dependent cytochrome C oxidase (*cyc*ABC) is the predictive terminal oxidase with oxygen functioning as terminal electron acceptor. An F_0F_1-ATPase complex (*atp*BFE/*atp*ADGHC) finally facilitates ATP synthesis. All 10 genes needed for the biosynthesis

of heme were also present in all sequenced photobacteria genomes (hemA/L/B/C/D/N/E/F/G/H).

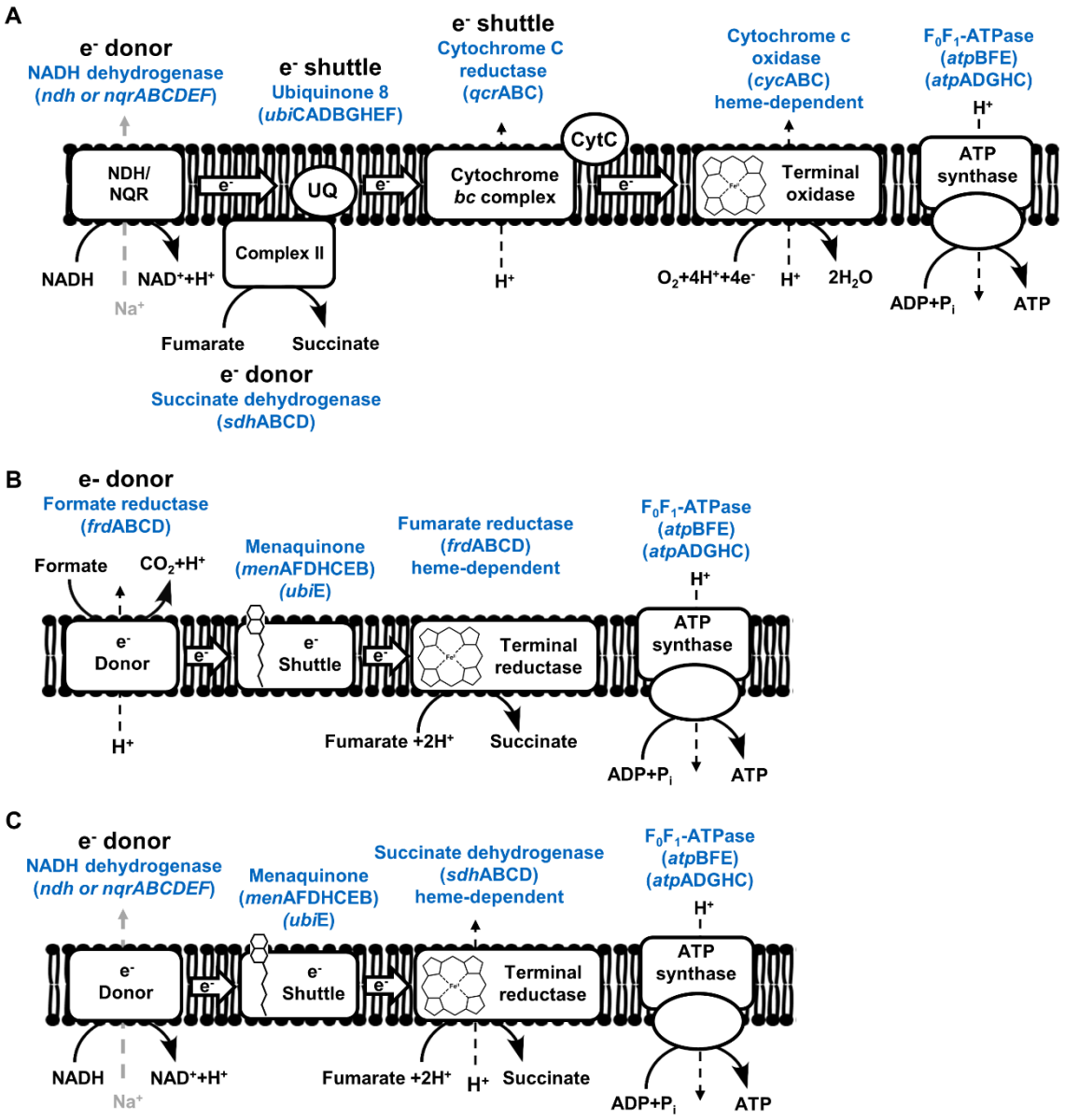

Figure 51| Predictive aerobic (A) and anaerobic (B/C) respiratory chains of *Photobacterium* spp. Enzyme names and encoding genes are shown in blue; all genes shown were present in the photobacteria genomes; e^-, electron. Strains of *P. phosphoreum* additionally harbor genes for NADH dehydrogenase *nuo*.

Under anoxic conditions, the first step in the respiratory chain is facilitated by NADH dehydrogenase (*ndh* or *nqr*ABCDEF) with NADH and formate dehydrogenase (*fdh*ABCE) with formate as electron donor. Menaquinone can function as electron shuttle and all genes needed for the biosynthesis (*men*A/F/D/H/C/E/B) are present in all photobacteria genomes. Heme-dependent fumarate reductase (*frd*ABCD) or succinate dehydrogenase (*sdh*ABCD) as terminal reductase can reduce fumarate to succinate. Additionally, genomes of all photobacteria harbor

genes encoding a hydrogenase (hydABCDE), periplasmatic nitrate reductase (napABC) and TMAO (trimethylamine N-oxide) reductase (torA). Therefore, hydrogen can potentially function as electron donor, and nitrate and trimethylamine as electron acceptors under anoxic conditions.

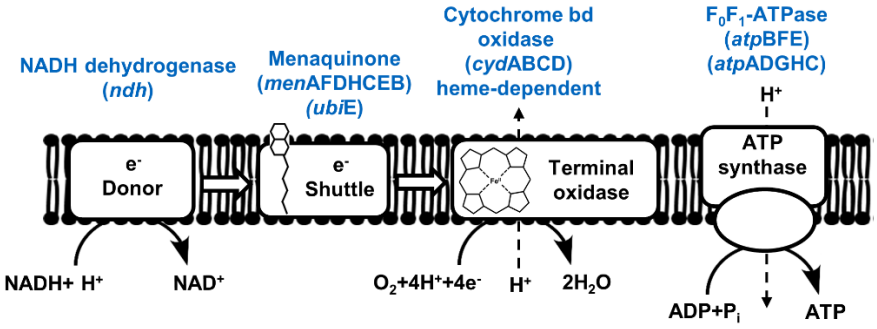

Figure 52| Predictive aerobic respiratory chains of *Le. gelidum* subsp *gelidum* & *gasicomitatum*. Enzyme names and encoding genes are shown in blue; all genes shown were present in the genomes; e^-, electron.

The genome of both *Le. gelidum* subsp. also contains genes for building a respiratory chain under oxic conditions (Figure 52). NADH can act as electron donor via NADH dehydrogenase (*ndh*). All genes are present for the biosynthesis of menaquinone, which can function as electron shuttle. The genomes of both *Le. gelidum* subsp. contain genes encoding a heme-dependent cytochrome bd terminal oxidase (*cyd*ABCD), which facilitates electron acceptor reaction of oxygen to water. Again, ATP is generated via the F_0F_1-ATPase complex (*atp*BFE/*atp*ADGHC). No genes were detected needed for the biosynthesis of heme.

In the genomes of *Lc. piscium* strains, no genes encoding a terminal oxidase or biosynthesis of heme and menaquinone were detected. Only the genes for the F_0F_1-ATPase complex (*atp*BFE/*atp*ADGHC) are present in the genome of both *Lc. piscium* strains.

4.5.2.7 Predictive lysozyme-like encoding genes of *Lc. piscium* strains

Lc. piscium strains effectively inhibited diverse gram-positive and gram-negative meat spoilers (4.3).Therefore, genomes were screened and several genes were detected in both sequenced *Lc. piscium* strains TMW2.1612 and TMW2.1615 that encode for proteins exhibiting putative antimicrobial or lysozyme activities. Namely, genes were found coding for putative lysozyme family proteins, muramidases, glycoside hydrolase family 25 proteins, LysM peptidoglycan-binding motif containing proteins and CHAP-domain containing peptidoglycan hydrolysis proteins (Table A6).

5 Discussion

The long term aim of this study is to reduce the amount of meat, which is produced and subsequently discarded, while it is still acceptable for consumption, reduce the number of slaughtered animals with respect to the amount of consumed meats and save resources of carbon dioxide, water, feedstuff and livestock on a global scale.

In this work, different strategies are highlighted, which can be followed to achieve this specified aim and control MAP meat spoilage. Indeed, some strategies are followed in subsequent approved research projects that were initiated in this work and derived from the obtained results.

These strategies follow the theses derived from this work, which emerged from the testing of the initial working hypothesis along the four sections of this study:

Chapter 1: Growth dynamics analysis of spoilage-associated microbiota on modified-atmosphere packaged beef steaks and minced beef

- The database developed in this work enables high-resolution monitoring of growth dynamics enabled by MALDI-TOF MS, which provides the designation of key spoilage bacteria to subsequently develop a rationale in order to suppress or exploit them.
- This work provides the basis for the development of ISO methods used in laboratory routine analysis towards a lowered isolation temperature, which enables recovery of underestimated psychrotrophic and psychrophilic bacteria causing hitherto unexplainable spoilage cases in the standard routine.
- It is possible to predict the onset of meat spoilage scenarios along the initial contamination by SSO that lead to different spoilage dates.
- The control of oxygen content in O_2-containing MAP can enable determination of the actual shelf lives of even individual packages to prevent avoidable waste caused by erroneously assigned shelf life or spoilage variations.

Future research will focus to develop an oxygen sensor device enabling an individualized assessment of shelf life of MAP meat packages based on the correlation of sensorially perceptible spoilage and oxygen content changes in the follow-up project: “Development of a non-invasive optical detection method for the individualized assessment of meat spoilage in modified atmosphere packaged meats” (AiF 19993N2). The project aims to develop a rationale that predicts onset of spoilage before it occurs and therefore enabling manufacturers and retailers to respond to variations in shelf life, and thereby, reducing avoidable waste and financial loss.

Chapter 2: Assertiveness of *Lactococcus piscium* and evaluation of its potential to exhibit bioprotective characteristics on beef

- *Lc. piscium* strains are assertive over other spoilers when present as initial contaminants in fresh meat.
- Different *Lc. piscium* strains exhibit variable potential towards the inhibition of different competitors.
- *Lc. piscium* TMW2.1615 expresses a sensorially acceptable volatilome on beef.
- *Lc. piscium* strains are candidates for a novel application in the control of fresh meat spoilage.

Future research will focus on the exploitation of *Lc. piscium* strains as a novel bioprotective organism and develop practical strategies for the deliberate application on meat in the follow-up project: “Exploitation of *Lactococcus piscium* for the regulation of meat spoilage and ripening in modified atmosphere and skin packages” (BLE 281A105116). The project will aim towards the development of suitable *Lc. piscium* cultures that contribute to the suppression of competitors and pathogens, and therefore enable the control of MAP spoilage, reduce avoidable waste and enhance product safety.

Chapter 3: Photobacteria involved in meat spoilage

- The newly developed isolation procedure for photobacteria demonstrates their frequent abundancy in meats and their potential contribution to meat spoilage.
- The isolation and description of *Photobacterium carnosum* sp. nov. from spoiled meat indicates occurrence of photobacteria apart from previously described marine and deep sea environments.

Future research will focus to reveal, control and minimize the entry of photobacteria in the meat production, explore their *in situ* metabolism and behavior in MA and skin packages as well as under high pressure in the follow-up project: “Control of psychrophilic photobacteria in meat spoilage” (AiF 20113N1). The project will aim to establish critical control points and preventive measures towards photobacteria that can lead to a prolonged shelf life and reduced spoilage of MAP meats.

Chapter 4: Lifestyle of spoilage-associated bacteria

- Comparative genomics analyses enabled prediction of the lifestyle of SSO along their metabolic potential to utilize diverse substrate groups present on meat.
- The genomic analyses allows a predicted individual spoilage potential of the respective SSO enabling insights in their role in spoilage, product safety and human health concerns.

A thorough understanding of the metabolic capabilities of the meat spoiling bacteria, namely including their relation to O_2 and CO_2 and their metabolic impact, opens possibilities for the development of e.g. novel preservation techniques that could be the subject in a future research project targeting the meat microbiome in a systems biology approach using (meta)omics.

Taken together, a combination of these approaches can save millions of tons of carbon dioxide, water and livestock, and lead to an enhanced food safety in regard to human health. The bases of these strategies emerging from results obtained in this study are discussed in detail in the following four chapters.

5.1 Growth dynamics monitoring of spoilage-associated microbiota on MAP beef steaks and in MAP minced beef

5.1.1 Discriminatory power of MALDI-TOF MS

This study ascertains MALDI-TOF MS biotyping as a reliable, rapid, high-throughput method for identification of spoilage-associated bacteria on beef steaks and minced beef based on mass spectrometry profiles of their low molecular weight subproteome (4.1.2/4.2.2). It has been previously shown that MALDI-TOF MS is also capable of identifying typical poultry spoilage bacteria (Höll *et al.* 2016). Accuracy based on MALDI Biotyper identification and score values was validated by a hierarchical cluster analysis (HCA). In this study, a HCA for huge data sets was employed with previously optimized peak processing of MS data (Fushiki *et al.* 2006; Mantini *et al.* 2007; Mantini *et al.* 2010; Usbeck *et al.* 2013). In general, the discriminatory power of MALDI-TOF MS allowed identification on species level. For *Le. gelidum*, differentiation was even possible for its subspecies *gelidum* and *gasicomitatum*, resulting in distinct clustering. Although the subspecies formed one big cluster when using all isolates in the HCA of beef steak isolates, they were correctly identified by MALDI Biotyper as *Le. gelidum* subsp. *gelidum* and *gasicomitatum* and clustered apart from each other when a dataset only was used containing isolates from both *Le. gelidum* subsp. for HCA. Discrimination of isolates was even possible at strain level when using the liquid extraction method (Usbeck *et al.* 2013) – not suitable for high-throughput - instead of the direct method with on target extraction as demonstrated for different *Lc. piscium* isolates (4.1.6).

On the other hand, *Pseudomonas* isolates could only be reliably identified on genus level with no distinct clustering within the HCA based on Biotyper identification and partially caused by low score values. Exopolysaccharide production of these isolates resulting in strong slime formation (Parolis *et al.* 1991) seems to interfere with the analysis to some extent.

Taken together, MALDI-TOF MS is a highly valuable tool for rapid identification of spoilage-associated microbiota, but it is crucial to rely on a database with several reference MSPs entered per species to ensure reliable identification. It should be noted that cultivation conditions of bacteria used for reference MSPs match the conditions used in subsequent experiments. Furthermore, evaluation of identification accuracy by employing a HCA proved also to be of major importance because it revealed that MALDI Biotyper identification of *Pseudomonas* spp. was only reliable on genus level. Subsequently, a polyphasic approach was employed to further characterize and identify *Pseudomonas* species from minced beef due to their high diversity and dominance in the meat environment (5.1.7).

5.1.2 The onset of meat spoilage

Growth dynamics experiments were conducted to explore in-depth development of spoilage-associated microbial composition during spoilage of beef steaks (4.1.2) and minced

beef (4.2.2) at different temperatures. In total, approx. 20,000 isolates were identified from these meats. Isolation of bacteria was carried out by cultivation at 25 °C enabling detection of psychrotrophic bacteria (Greer 1981; Jay 2002; Ercolini *et al.* 2009). In general, TVC was higher and more diverse on minced beef and samples showed earlier spoilage signs compared to beef steak samples. This might be caused by the mechanical disruption of cells resulting in a higher availability of substrates and the fact that different pieces of meat are combined during minced beef production. Observed meat spoilage of both meats occurred in different stages:

The initial contaminant composition was highly diverse on beef steaks and minced beef in both batches, respectively. This initial microbiota comprised various organisms that were not found in later stages, *i.e. Stenotrophomonas*, *Acinetobacter* and *Janthinobacterium* were only present as initial contaminants. *Acinetobacter* species have been found as common and normal inhabitants of human skin, however they are also known as nosocomial pathogens (Bergogne-Berezin and Towner 1996; Towner 2006). *Stenotrophomonas* are typical plant-associated microbes, but also responsible for nosocomial infections (Ryan *et al.* 2009). They have been occasionally found on meat as rare spoilage cases (Garcia-Lopez *et al.* 1998; Giaccone *et al.* 2008; Nychas *et al.* 2008). Additionally, *E. faecium* made up to 20% of the initial and early contamination microbiota in minced beef. Strains of these species have been found occasionally on different meats and can be multi-drug resistant (Hayes *et al.* 2003; Vignaroli *et al.* 2011). However, none of these species were assertive under high-oxygen MA in this study and are therefore most likely not relevant in regard to spoilage. Also, many of the isolates from the initial contamination were not reliably identified based on low Biotyper score value. Spectra that remained as *nri* did not form a coherent cluster, but were scattered within the HCA indicating poor spectra quality and high diversity instead of neglected but highly abundant species absent in the data base. The high diversity of microbial contamination of the carcasses detected in the initial stage can originate from diverse autochthonous and allochthonous sources during slaughtering, dissection and processing that harbor different microbiota (Rahkio and Korkeala 1997; De Filippis *et al.* 2013). The initial contamination load of beef steaks and minced was similar with approx. 4 $\log_{10}$ CFU g^{-1}. Values of similar magnitude were reported for red meat carcasses (Dainty and Mackey 1992), carcass flank (Eisel *et al.* 1997) and commercial beef cuts (Barros *et al.* 2007), respectively.

In the subsequent phase, Ephemeral/Specific Spoilage Organisms (E/SSO) (Nychas *et al.* 2008) well adapted to the ecological niche emerged and became dominant. These organisms were identified as *Lc. piscium* and *Le. gelidum* subsp. *gelidum* & *gasicomitatum* on beef steaks. Their dominance was altering between batches in dependency of their initial abundance, respectively. If detected as initial contaminants, both *Le. gelidum* subsp. successively superceded other spoilers on beef steaks stored at 4 °C throughout the storage time. If *Lc. piscium* was highly abundant in the initial stage, it could maintain its dominance throughout the

incubation period. Also, abundance of *Lc. piscium* was enhanced in both batches when beef was stored at 10 °C.

These lactic acid bacteria (LAB) were also important spoilage-associated bacteria in minced beef. Additionally, *B. thermosphacta* and especially *Pseudomonas* spp. were dominant SSO throughout the storage time. *Pseudomonas* spp. dominated the spoilage-associated microbiota at initial stage (>80%), but relative abundance was sequentially decreasing except in batch 1 at 10 °C hinting that only a subset of certain species were assertive under the MAP conditions. If detected as initial contaminants, abundance of *Lc. piscium* or *B. thermosphacta* was much higher than in the respective batch where this species was not detected at the beginning. Again, the composition at initial and early phase determined the microbial composition throughout the storage period demonstrating the importance of controlling the initial contaminants.

Lc. piscium, a homofermentative, psychrotrophic, non-motile coccus, was first isolated from trout (Williams *et al.* 1990a; Teuber and Geis 2006). This bacterium has been previously also found on vacuum packaged beef (Sakala *et al.* 2002), broiler products (Vihavainen *et al.* 2007a) and pork (Rahkila *et al.* 2012). *Le. gelidum* and *gasicomitatum* (reclassified as subsp. *gelidum* and *gasicomitatum*; (Rahkila *et al.* 2014), both heterofermentative LAB, have been first isolated from VP meat (Shaw and Harding 1989) and MAP broiler meat (Björkroth *et al.* 2000), respectively. These subspecies have been found concomitantly as part of the spoilage-associated microbiota (Vihavainen and Björkroth 2007b; Rahkila *et al.* 2014). Different *Pseudomonas* species e.g. *Ps. fragi* and *Ps. lundensis* have been frequently isolated from aerobically stored meat products and are able to dominate the spoilage-associated microbiota under oxic conditions (Church and Parsons 1995; Gram *et al.* 2002; Ercolini *et al.* 2007). *B. thermosphacta* (Sneath and Jones 1976), emended from *Microbacterium thermosphactum* (McLean and Sulzbacher 1953), has been also frequently found on meats as part of the spoilage-associated microbiota (Borch *et al.* 1996; Cantoni *et al.* 2000; Ercolini *et al.* 2006; Russo *et al.* 2006; Doulgeraki *et al.* 2012). *C. divergens* was detected only as a minor proportion of the microbial composition on beef steaks as well as in minced beef. It was however detected throughout the incubation period in all batches at 4 and 10 °C and reached a maximum cell count of approx. 6 $\log_{10}$ CFU g^{-1}. Hence *C. divergens* might have had a contribution to spoilage, though. *C. divergens* (emended from *Lactobacillus divergens*; (Collins *et al.* 1987) is a heterofermentative LAB species that has been first isolated from beef (Holzapfel and Gerber 1983). Previous studies have reported that this bacterium is associated with meat spoilage (Jones 2004; Laursen *et al.* 2005; Ercolini *et al.* 2009).

The next stage of meat spoilage is the organoleptic onset of perceptible spoilage. The MSL of beef steaks was assigned to 7 days and the use-by date of minced beef to three days by the producer. Depending on the microbiota, spoilage of beef steaks was perceptible at day 10

and 8, and spoilage of minced beef at day 6 and 8 when stored at 4 °C, respectively. This demonstrates that the producer has assigned the MSL and use-by date much too short, respectively and the erroneous assignment will likely lead to avoidable waste and economic losses.

At the point of time where spoilage was perceptible – for both, beef steaks and minced beef - a concomitant occurring change in the headspace atmosphere composition and a drop of pH values was observed hinting at a potential use of these parameters as general spoilage indicators. Additionally, a change in microbiota composition was observed in analyzed minced beef samples at this time point, from *Pseudomonas* spp. and *Lc. piscium* to *Le. gelidum* subsp. as well as *B. thermosphacta*. It is possible that the observed slight drop in pH values resulted from rising carbon dioxide levels produced by bacteria and by the beef tissue itself which were absorbed by the meat as carbonic acid and further dissociated into bicarbonate and hydrogen ions (Jakobsen and Bertelsen 2002). This effect can account for a pH drop of 0.1 units (Rousset and Renerre 1991) and is increased at lower temperatures (Gill 1988), which should not be a decisive parameter for selection of different microbiota. In addition, acidic fermentation borne metabolic end products, namely lactate and acetate, can contribute to a lowering of pH in meat (Gram *et al.* 2002) and could have caused the detected sour off-odor.

On beef steaks, spoilage occurred earlier when the microbial composition was dominated by *Le. gelidum* subsp. throughout the storage time compared to beef steaks with a microbiota dominated by *Lc. piscium*. Spoilage was perceptible in both batches of beef steaks by green discoloration and sour-sweet off-odor. Meat greening is caused by a reaction of hydrogen sulfide or hydrogen peroxide with myoglobin, altering the heme structure (Faustman and Cassens 1990; Lawrie and Ledward 2006). *Le. gelidum* subsp. and *C. divergens* are known to cause meat greening by hydrogen peroxide production (Borch and Molin 1989; Vihavainen and Björkroth 2007b). It has been reported that *Lc. piscium* strains do not produce hydrogen sulfide (Williams *et al.* 1990a), but certain strains may be able to produce hydrogen peroxide under certain conditions e.g. aerobically from glycerol (Andreevskaya *et al.* 2015). There have been no reports in the literature that *Lc. piscium* causes meat greening, though.

As observed in beef steak batches, the batch of minced beef samples showed delayed spoilage if *Lc. piscium* was a substantial part of the detected spoilage-associated microbiota. In minced beef samples, a constant change in microbiota was observed in both batches during storage time. Although *Pseudomonas* spp. were dominating the microbiota at initial stage and up to day 4 with 5 and 6 $\log_{10}$ CFU g^{-1}, minced beef was not yet spoiled. This might be due to the availability of residual carbohydrates. It has been reported that *Pseudomonas* spp. do not form off-odorous byproducts when glucose is still available (Gill 1996), but do when using amino acids presumably as a result of carbohydrate depletion (Gill 1983). Meat-spoiling *Pseudomonas* spp. get access to other substrate groups due to their proteolytic (Tarrant *et al.*

1973; Ercolini *et al.* 2009; Marchand *et al.* 2009) as well as lipolytic (Lebert *et al.* 1998; Alquati *et al.* 2002; Ercolini *et al.* 2010a) metabolic activity. At the time of spoilage onset, *Pseudomonas* spp. showed low relative abundance but still had relevant absolute numbers (>6 $\log_{10}$ CFU g^{-1}) and have likely contributed to spoilage via metabolism of these substrates. However, the community shift from *Pseudomonas* spp. and *Lc. piscium* to *Le. gelidum* subsp. as well as *B. thermosphacta* indicated that these SSO also contributed to spoilage. No meat greening was observed which was likely caused by *Le. gelidum* subsp. on beef steaks via hydrogen peroxide production, though - but rather sweaty rancidity, sour-sweet off odor and brown discoloration. The absence of green discoloration does not mean that *Le. gelidum* subsp. did not contribute to minced beef spoilage because hydrogen peroxide is able to activate metmyoglobin resulting in significant lipid oxidation and rancidity (Kanner and Harel 1985; Bhattacharya *et al.* 1988). Spoilage was likely also caused by *B. thermosphacta*, which is known to produce a mixture of iso-butyric acid, acetic acid and acetyl/acetoin, iso-valeric acids, 2-heptanone and 2-hexanone (Borch *et al.* 1996; Rattanasomboon *et al.* 1999; Mejlholm *et al.* 2005; Russo *et al.* 2006) resulting in the observed off-odor in this study.

In the very late stage of spoilage, a community change towards *Enterobacterales* & *Pseudomonas* spp. was observed on beef steak samples at improper storage temperature of 10 °C. *Enterobacterales* were also detected as part of the microbiota in minced beef in the late stage. Most of them were identified as *H. alvei* and *S. liquefaciens*. Both organisms have been described as opportunistic pathogens (Grimont and Grimont 2006; McBee and Schauer 2006) and are able to form harmful biogenic amines from amino acids on meat (Dainty *et al.* 1986; Durlu-Özkaya *et al.* 2001; Galgano *et al.* 2009). These result in putrid off-odors that were sensible when packages were opened for sampling and an elevated pH that was measured at this point of time. However, this community change or emerging abundance and a probable metabolic shift to amino acid metabolism (Gill 1983) resulting in putrid off-odor seem to have subordinate importance for spoilage indication, because the beef steaks and minced beef samples were already considered spoiled at this time point. On beef steaks, highly abundant LAB causing meat-greening and off-odor. In minced beef, the same LAB species as well as *B. thermosphacta* and *Pseudomonas* spp. caused perceptible spoilage via browning, rancidity and sour-sweet as well as sweaty odor.

5.1.3 Influence of atmosphere and substrate availability on growth of E/SSO

Although being offered a niche with easily available substrates on beef, modified-atmosphere packaging and cold storage create conditions that can be challenging for microorganisms therefore prolonging shelf life of the meat (Church and Parsons 1995). Concomitant presence of oxygen and carbon dioxide inhibits strict anaerobes and aerobes, respectively (Farber 1991). However, well-adapted psychrotrophic E/SSO – *Lc. piscium*, *Le.*

gelidum subsp., *B. thermossphacta* and *Pseudomonas* spp. - could thrive under this conditions and dominate the microbiota in this ecological niche.

A relevant substrate group in beef are carbohydrates i.e. glucose and glycolytic intermediates with varying availability depending on residual glycogen content (Pösö and Puolanne 2005; Ferguson and Warner 2008) allowing growth of spoilage bacteria up to 8 $\log_{10}$ CFU cm^{-2} (Gill 1996). These carbohydrates can be metabolized homofermentatively by highly prominent *Lc. piscium* and *B. thermosphacta* or heterofermentatively by *Le. gelidum* subsp. without or with carbon dioxide production, respectively (Pin *et al.* 2002; Sakala *et al.* 2002; Björkroth and Holzapfel 2006). On beef, packaged under high oxygen modified atmosphere, concomitant availability of oxygen and heme can enable a respiratory metabolism of LAB (Brooijmans *et al.* 2009), which is energetically favorable and reduces oxidative stress (Lechardeur *et al.* 2011). It has been previously reported that *Le. gelidum* subsp. *gasicomitatum* strains can form a functional electron transport chain (Johansson *et al.* 2011) and show enhanced growth with supplemental heme (Jääskeläinen *et al.* 2012) whereas *Le. gelidum* subsp. *gelidum* (Rahkila *et al.* 2014) and *Lc. piscium* (Rahkila *et al.* 2012) do not. Additionally, in case of *Le. gelidum* subsp., oxygen could be consumed in an alternative pyruvate shunt via pyruvate oxidase yielding ATP and CO_2, hydrogen peroxide and acetate (Sedewitz *et al.* 1984; Johansson *et al.* 2011). *B. thermosphacta* is known to be facultatively aerobic and mostly limited to carbohydrates as substrate except alanine (Dainty and Hibbard 1983), while the main fermentation product is lactic acid under anoxic conditions (Blickstad and Molin 1984; Pin *et al.* 2002), which is rather imperceptible. Under aerobic conditions, it has been reported that aerobic respiration is employed (Gil *et al.* 1992) but also more spoilage-relevant substances e.g. acetate, butyric acid, acetoin are produced (Borch *et al.* 1996; Rattanasomboon *et al.* 1999; Mejlholm *et al.* 2005; Russo *et al.* 2006). Although oxygen-consuming respiration is possible, it has been reported that *B. thermosphacta* strains do not possess a full TCA cycle (Stanborough *et al.* 2017). While *B. thermosphacta* requires high levels of oxygen, 20% carbon dioxide as in MAP seem not to block the aerobic metabolism (Blickstad and Molin 1984; Pin *et al.* 2002) and therefore it is expected that *B. thermosphacta* produces spoilage-relevant products on MAP (minced) beef.

In this study, these metabolic potentials, which lead to oxygen consumption and carbon dioxide evolution could be observed via monitoring of the headspace atmosphere indicating that respiratory activity as well as oxygen-dependent reactions were employed by the spoilage-associated microbiota. On beef steaks, in the batch dominated *Le. gelidum* subsp., a decrease of oxygen concentration and increase of carbon dioxide was observed after day 6. *Lc. piscium* on the other hand seems not to influence or interact with the headspace atmosphere. In the batch of beef steaks dominated by *Lc. piscium*, the headspace atmosphere remained constant for 10 days. A substantial change in the atmosphere occurred only when minor abundant *Le.*

gelidum subsp. also reached high cell counts, and most likely caused the observed meat greening and sour off-odor. In minced beef batches, a change of atmosphere could also be observed in both analyzed batches, when a community shift from *Lc. piscium* and *Pseudomonas* spp. towards *B. thermosphacta* and *Le. gelidum* subsp. occurred concomitantly with the perceptible onset on spoilage.

It has been reported in the literature that proteinaceous or triacylglyceride-derived substrates are metabolized after carbohydrate depletion and termed “secondary substrates” (Gill 1983; Nychas *et al.* 2007). It is also known that glucose can exert catabolic repression of e.g. arginine deiminase pathway in pseudomonades and LAB (Mercenier *et al.* 1980; Marquis *et al.* 1987; Montel and Champomier 1987). However, it remains unknown if the glucose levels present in meat are sufficient to inhibit amino acid metabolism in SSO or if both substrate groups are metabolized concomitantly and not in a sequential order. *Le. gelidum* subsp. have been reported to be not proteolytic, not hydrolyze arginine or produce biogenic amines (Johansson *et al.* 2011; Rahkila *et al.* 2014). *Lc. piscium* strains have been also reported do not produce biogenic amines and have been tested negative (Williams *et al.* 1990a; Sakala *et al.* 2002) or positive (Rahkila *et al.* 2012) for arginine hydrolysis. *B. thermosphacta* is known to be also mainly limited to carbohydrates and restricted to utilize alanine regarding amino acids (Dainty and Hibbard 1983). The fact that these LAB and *B. thermosphacta* were predominant in this work in high cell counts throughout the incubation period of two and three weeks at 4 °C on beef steaks and minced beef, respectively, the disability to metabolize arginine and a decreasing beef pH suggest that either sugar levels were sufficient for growth throughout the incubation period or other non-proteinaceous substrates were metabolized. It is also possible that other proteolytic and lipolytic microbes e.g. *C. divergens* (Leisner *et al.* 2007) or *Pseudomonas* spp. (Stead 1986; Alquati *et al.* 2002; Marchand *et al.* 2009; Stoeckel *et al.* 2016) - detected also as fraction of spoilage-associated microbiota - may have provided degradable amino acids or glycerol moieties of fats as metabiosis (Gram *et al.* 2002) for LAB via extracellular lipases and proteases. Although these reactions occur extracellular, the organisms capable of lipolysis and proteolysis are likely to have an advantage over the bacteria that are not. The higher fat content in minced beef might therefore be a reason for the detected dominance of *Pseudomonas* spp.

Pseudomonas spp. have been frequently isolated from aerobically stored meat products (Ercolini *et al.* 2007) and are able to dominate the spoilage-associated microbiota under oxic conditions supporting aerobic growth (Church and Parsons 1995; Gram *et al.* 2002). It has been reported that they do not form numerically significant proportions of the spoilage-associated microbiota in meats packaged under modified atmosphere (Stanbridge and Davies 1998). In contrast, high abundance of *Pseudomonas* spp. was demonstrated in this study especially in minced beef. The dominant *Pseudomonas* species detected in this study were

identified as *Ps. weihenstephanensis*, *Ps. fragi* and *Ps. lundensis* (cf. 5.1.7). All three species have been described as potential meat spoilers as well as raw milk spoilers (Shaw and Latty 1982; Molin and Ternström 1986; Labadie 1999; Ercolini *et al.* 2007; Von Neubeck *et al.* 2016; Lee *et al.* 2017) with reported lipolytic and proteinolytic activity (Stead 1986; Alquati *et al.* 2002; Marchand *et al.* 2009; Stoeckel *et al.* 2016).

It has also been reported that the growth of *Pseudomonas* spp. is restricted by oxygen limitation rather than substrate depletion (Gill and Newton 1978; Gill 1996) and inhibited by CO_2 (King and Nagel 1967; Enfors and Molin 1981; Fang and Lin 1994; Stanbridge and Davies 1998). Highly interesting and unexpected, *Pseudomonas* spp. were dominating the spoilage-associated microbiota in early, mid and very late spoilage stage up to day 14 in MAP minced beef stored at 10 °C, although carbon dioxide levels were high and no residual oxygen was measured after day 8. Additionally, *Pseudomonas* spp. were isolated also in late spoilage stage from MAP beef steaks containing an atmosphere with up to 90% CO_2 and low level of oxygen down to 1%. Generally, *Pseudomonas* species are recognized as obligate aerobes with solely aerobic respiratory metabolism (Palleroni 1984; Moore *et al.* 2006; Özen and Ussery 2012). However, some *Pseudomonas* sp. are capable of denitrification or dissimilatory nitrate reduction and use nitrate as the terminal electron acceptor instead of oxygen (Fewson and Nicholas 1961; Samuelsson 1985; Bedzyk *et al.* 1999; Arai 2011). A study by (Iammarino and Di Taranto 2012) has shown that the availability of nitrate in fresh beef is very low, though (mean average 13.2 mg/kg) and nitrate has been detected only in a minority of samples. Thus, it is unlikely that nitrate respiration account for detected cell counts of over 8 and 7 $\log_{10}$ CFU g^{-1} of *Pseudomonas* spp. in minced beef and beef steaks, respectively. It has also been reported that *P. aeruginosa* is capable of different kinds of anaerobic fermentation (Arai 2011). In the absence of nitrate, *P. aeruginosa* ferments arginine via arginine deiminase pathway to conserve energy (Shoesmith and Sherris 1960; Vander Wauven *et al.* 1984; Benkert *et al.* 2008). Arginine is highly abundant in beef (Schweigert and Payne 1956; Holló *et al.* 2001a) hence arginine fermentation could be an explanation for late stage *Pseudomonas* growth. In addition to nitrate respiration and arginine fermentation, *Pseudomonas aeruginosa* is capable of mixed acid pyruvate fermentation supporting long term survival under anoxic conditions (Eschbach *et al.* 2004). For *Ps. fragi*, utilization of glucose, lactate and amino acids has been described for batch cultures under oxygen limitation as well as high CO_2 concentrations (Molin 1985).

In this study, *Pseudomonas* spp. were further characterized via sequencing of 16S and *rpo*D gene sequences, and isolates from the late spoilage stage were mainly identified as *Ps. weihenstephanensis* on MAP beef steaks and additionally *Ps. fragi* and *Ps. lundensis* on MAP minced beef (for details see 5.1.7). In order to evaluate if *Pseudomonas* spp. only endure anoxic conditions or are truly able to grow anaerobically, *Ps. weihenstephanensis* TMW2.1827

from beef steaks as well as various minced beef isolates of *Ps. weihenstephanensis* (TMW2.2078/84), *Ps. fragi* (TMW 2.2081/82) and *Ps. lundensis* (TMW2.2076) from time points where residual oxygen was depleted, were incubated under anoxic conditions on BHI agar. *Ps. fragi* has been previously described as strictly aerobic (Hussong *et al.* 1937), strains of *Ps. weihenstephanensis* have been reported to be unable to grow anaerobically (Von Neubeck *et al.* 2016) and *Ps. lundensis* has been reported to be only able to facilitate respiratory but not fermentative metabolism (Molin *et al.* 1986). Highly interesting and contrary to these reports in the literature, visible anaerobic growth of tested isolates of these *Pseudomonas* species was observed on the BHI agar plates after 1-2 days of incubation, proving the ability of these strains for anaerobic growth under anoxic conditions. This observation hints that these spoilage-associated *Pseudomonas* spp. indeed possess a fermentative metabolism, which allows them to grow on beef under MAP conditions and contribute to spoilage via carbohydrate or amino acid fermentation. However, the actual fermentative mechanisms of meat-borne *Pseudomonas* spp. remain unknown for now. Future studies should be conducted to elucidate the anaerobic lifestyle of *Pseudomonas* species, which seems to be a common trait within the genus as hinted in this study. Research is hitherto mostly limited to clinically relevant strains of *Ps. aeruginosa* and therefore the anaerobic lifestyle of *Pseudomonas* is currently underexplored.

During growth dynamics experiments, observations were made that beef steaks displayed a brownish color on the bottom side or where beef slices overlapped. Conditions for spoilage-associated microbiota may differ on top and bottom surface of the beef due to a suspected variance in moisture, oxygen availability and redox potential. The observed brown discoloration can originate from oxidation of oxymyoglobin yielding metmyoglobin and occurs at low levels of oxygen (Neill and Hastings 1925; Brooks 1935; Faustman and Cassens 1990). In order to investigate the effect on the spoilage-associated microbiota, beef slices at different storage time points and temperatures were horizontally separated in half and analyzed separately (4.1.4). Additionally, minced beef samples were also split into an upper surface, mid and bottom surface of the drilling core to evaluate possible differences in microbiota distribution (4.2.3). Although the bottom side and overlapping meat pieces of beef steaks and lower surface as well as inner matrix of the minced beef could generate conditions to protect organisms from elevated levels of carbon dioxide, microbial composition and TVC on top and bottom of the beef steaks as well as top/inner matrix/ bottom of the minced beef was highly similar and comprised of the typical facultative aerobic SSO that seem well adapted to the potentially different conditions. Therefore, no direct recommended action needs to be done to e.g. enhance aeration on the bottom of the modified atmosphere package via grooving of the package base, or reducing the overlap of slices in regard to beef steaks. For sampling of beef

steaks and minced beef, a complete drill core can be obtained and needs not to be analyzed separately.

5.1.4 Intraspecies diversity assessment of spoilage-associated microbiota

Differentiation of relevant spoilage-associated organisms below species level was carried out via RAPD biotyping (Welsh and McClelland 1990; Williams *et al.* 1990b), which is a common DNA fingerprinting method that has been frequently used in LAB taxonomy (Cocconcelli *et al.* 1995; Ehrmann *et al.* 2003; Rossetti and Giraffa 2005). It has been previously employed also to characterize spoilage-associated microbiota on meat (Ercolini *et al.* 2009). Isolates of *Lc. piscium*, *Le. gelidum* subsp. and *Pseudomonas* spp. from beef steaks and minced beef, as well as *B. thermosphacta* isolates from minced beef and *S. liquefaciens* and *H. alvei* from beef steaks were selected for subjected to RAPD biotyping.

H. alvei and *S. liquefaciens* isolates from beef steaks stored at 10 °C displayed a single respective band pattern, suggesting abundance of only a single strain, respectively. These *Enterobacterales* can originate from animal gut content hence adapted to higher temperatures (Grimont and Grimont 2006; Janda and Abbott 2006). These contaminants are likely to be introduced frequently as fecal contaminations and during evisceration into processing plants. Low diversity on MAP beef suggests a strong selective pressure by cold storage towards these mesophilic organisms. In contrast to *Enterobacterales*, detected psychrotrophic bacteria (*Lc. piscium, Le. gelidum* subsp., *B. thermosphacta*) adapted to cold environments, may not be introduced as frequently into processing plants by animal sources, e.g. animal skin or intestine due to their inability to grow at higher temperatures. Specific strains of these organisms – once introduced, could be able to colonize the respective processing plant surfaces as persisting resident populations that become airborne and contaminate the beef during processing (Gustavsson and Borch 1993; Rahkio and Korkeala 1997). The detected low intraspecies diversity of psychrotrophic bacteria and isolation of different strains from random samples speculatively suggests that a persistent plant-specific on-site microbiota may be present in every processing plant.

Pseudomonas isolates subjected to RAPD-PCR from beef steaks and minced beef showed a high number of unique biotypes indicating a high diversity. The isolates from beef steaks originated from a single package at day 21 of batch 2 and the dominant biotype was identified as *Ps. weihenstephanensis*. *Pseudomonas* spp. isolates from minced beef originated from different time points and temperatures. However, some unique biotypes were found only as initial contaminants and some biotypes dominant at late storage from packages with no residual oxygen indicating the abundance of specific *Pseudomonas* spp. adapted to the MAP conditions and likely capable of anaerobic metabolism as discussed earlier. These assertive biotypes could be identified as *Ps. weihenstephanensis*, *Ps. fragi* and *Ps. lundensis* (cf. 5.1.7). *Pseudomonas* isolates displayed a higher amount of unique RAPD biotypes indicating a higher

diversity than other organisms. It should be noted that total number of biotypes originated from multiple species, though. The previously described ability of *Pseudomonas* spp. to colonize processing plant surfaces via biofilms (Chmielewski and Frank 2003) and the potential to employ an anaerobic, fermentative metabolism (Arai 2011) could enable their observed high abundance and diversity on MAP beef in this study. Furthermore, it is known that *Pseudomonas* spp. are psychrotrophic or mesophilic with a psychrotolerance enabling diverse growth at higher and lower temperatures (Molin *et al.* 1986; Moore *et al.* 2006; Ercolini *et al.* 2009; Ercolini *et al.* 2010a). The high diversity of *Pseudomonas* spp. biotypes demonstrated in this study emerging under oxygen limitation and concomitant with high levels of carbon dioxide in MAP hints that the ability to grow under these conditions seem common within the highly versatile and diverse genus of *Pseudomonas*.

Future studies should be conducted to further explore intraspecies diversity of meat-spoiling bacteria, explore their origin of contamination and identify transient or persisting niches in order to get control over the initial contaminants on meats.

5.1.5 Comparison of bulk samples and random retail samples

Beef steaks and minced beef for high-resolution growth dynamics experiments were obtained directly from one producer as bulk samples. In order to compare the identified microbiota with samples from different producers and batches, approx. 30 random samples from different retailers and different time points were additionally analyzed. In most random samples, the relevant SSO were the same as identified in detailed analyses of batch samples thereby verifying the results obtained in the growth dynamics experiments. *Lc. piscium*, *Le. gelidum* subsp. and accessory *C. diverge*ns for beef steaks and additionally *B. thermosphacta* and *Pseudomonas* spp. for minced beef. Additionally, single random samples showed a completely different spoilage-associated microbiota demonstrating that the occurrence of atypical spoilage cases is possible. These bacteria were e.g. *Chryseobacterium* (de Beer *et al.* 2005; Bernardet *et al.* 2006), *Arthrobacter* spp. (Nychas *et al.* 2008), *Rhodococcus* spp. (Youssef *et al.* 2014) and *Pseudoclavibacter* (Røder *et al.* 2015), which are rarely or occasionally found on meats or the meat processing environment. Additionally, two random minced beef samples showed a high abundance of *Lc. garvieae*, which is known as a contaminant in raw milk cheese and fish pathogen causing lactococcosis (Vendrell *et al.* 2006; Fortina *et al.* 2007) indicating that it also might play a role in occasional meat spoilage cases.

Contamination levels of random minced beef samples showed a great dispersion and no correlation with days left to use-by date was observed, e.g. a “fresh” minced beef sample already had a microbiota level of 6 $\log_{10}$ CFU g^{-1} with 5 days until the use-by date and another sample with 1 days until the use-date had only a contamination level of 4 $\log_{10}$ CFU g^{-1}. A comparison of the contamination level with bulk samples was not directly possible since the producer of bulk samples assigned the use-by date to only three days, but initial/early

contamination levels were similar. Although the typical SSO organisms from bulk samples were verified by random samples, the abundance of *Pseudomonas* spp. was much lower and less frequent in the random retail samples hinting towards a contamination issue in the processing plant of the producer providing the bulk samples.

A similar MSL of bulk and random retail samples allowed comparison of beef steak samples. In general, contamination level of random retail samples were lower compared to bulk samples and in approx. 50% of random samples the contamination level was below the detection limit. In order to further investigate this observation, contact samples were taken at different spacial positions of beef steaks. Microbiota on random beef steaks was initially located mainly at the edge of the slices, whereas bacteria of bulk samples were evenly distributed on the surface. While spoilage signs e.g. greening of the former also started from the outside, the latter samples showed evenly meat greening when spoiled. During an on-site visit at the meat processing facility of the producer providing the bulk samples, it was observed that beef steak samples were cut from bigger meat pieces by hand and the microbiota presumably dispersed over the slices by the knife and hands of butchers. It could be speculated that the random samples probably originate from major food producers that use automatic cutting mechanisms instead and possibly employ more frequent disinfection of the cutting devices resulting in a lower overall contamination level.

5.1.6 Psychrotrophic and psychrophilic spoilage-associated microbiota on MAP beef steaks

Recently, cases of obviously spoiled meat within the MSL were reported from the project-affiliated meat-producing industrial partner to our department with very low numbers of spoilage microbiota suggesting that a standard analytical routine targeting only mesophilic bacteria was unable to detect the causative bacteria. The standard methods were carried out at 30 °C hence underestimating psychrotrophic and psychrophilic bacteria not able to grow at this temperature (Ercolini *et al.* 2009; Pothakos *et al.* 2012). In this study, a general cultivation temperature of 25 °C was employed instead of 30 °C to allow detection of psychrotrophic bacteria (Gill and Newton 1978; Greer 1981; Jay 2002) responsible for meat spoilage. Furthermore, isolation of psychrophilic bacteria not able to grow even at 25 °C but involved in meat spoilage was carried out at 4 °C, as used upon the storage condition of refrigerated beef. Psychrophilic bacteria are usually able to grow sub-zero and grow well at near-zero temperatures with varying growth optima below 20 °C (Morita 1975; Jay *et al.* 2005; Scherer and Neuhaus 2006). A comparison of spoilage-associated microbiota cultivated at 4 and 25 °C showed the abundance of the same psychrotrophic LAB species (4.1.3). However, abundance of *Le. gelidum* subsp. *gelidum* was enhanced. Subsequent growth screening on plates and RAPD biotyping revealed the abundance of a single psychrotrophic strain, *Le. gelidum* subsp. *gelidum* TMW2.1998, growing at 4 °C but not at 25 °C. Abundance of *Le.*

gelidum subsp. *gelidum* was even higher than the contribution of the psychrophilic strain not detected at 25 °C indicating that incubation for 14 days at 4 °C favors psychrophilic as well as slow growing *Le. gelidum* subsp. *gelidum*.

Growth was recorded as OD_{590} within a large temperature range (-5 – 30 °C) in liquid BHI broth containing glycerol (5% w/w), which was sufficient to prevent freezing at -5 °C. The growth analysis showed that *Le. gelidum* subsp. *gelidum* TMW2.1998 is a true psychrophile. Its growth behavior was clearly different from psychrotrophic strains of *Le. gelidum* subsp. *gelidum* & *gasicomitatum* and *Lc. piscium,* which were able to grow at higher temperatures. At 25 °C *Le. gelidum* subsp. *gelidum* TMW2.1998 was unable to grow on plates. In liquid media it was able to grow at 25 °C, but growth was weak as compared to growth at lower temperatures. Water activity was only slightly increased in broth compared to agar plates hinting that other factors e.g. substrate diffusion rates, oxygen availability and accumulation of by-products contributed to different growth behavior on solid and in liquid media (Cheung and Fischetti 1988). *Le. gelidum* subsp. *gelidum* TMW2.1998 showed a typical growth behavior reported for psychrophiles (Morita 1975; Jay *et al.* 2005) with a demonstrated optimum growth at 15 °C, highest optical density at 4 °C and ability to grow at sub-zero temperatures. Such psychrophilic strains are suggested as one causative for periodically occurring meat spoilage cases, which cannot be explained along the results of routine microbiological analyses, and indicates that these analyses needs to be amended towards the detection of psychrophilic organisms.

5.1.7 Characterization of *Pseudomonas* spp. from MAP minced beef using a polyphasic approach

MALDI Biotyper identification seemed not reliable for identification of *Pseudomonas* spp. below the genus level (5.1.1). Although isolates formed distinct cluster in the hierarchical cluster analysis, the species identification of the Biotyper was scattering along the clusters. In order to further characterize and identify *Pseudomonas* spp. from minced beef that were highly abundant in different stages of spoilage, a polyphasic approach was employed including MALDI Biotyper identification, a HCA of their mass spectrometry profiles (MSPs), RAPD biotyping, sequencing of 16S rDNA and *rpo*D genes as well as a *car*A multiplex PCR (4.2.5). A phylogenetic neighbor-joining tree based on the partial 16S rDNA sequences of isolates as well as type strains of close-related *Pseudomonas* spp. showed a poor phylogenetic resolution due to high sequence similarity (>99%), which allowed no assignment to a distinct species. To further resolve the phylogenetic affiliation of selected isolates, *rpo*D gene sequences were analyzed as previously recommended (Yamamoto and Harayama 1998; Yamamoto *et al.* 2000) using an optimized primer set for PCR (Mulet *et al.* 2009). The resulting phylogenetic tree of partial *rpo*D sequences of isolates and type strains of close-related *Pseudomonas* species allowed affiliation to seven different species (Table 8). Due to high sequence

dissimilarity to type strains of known species, there are supposedly also several additional novel species (designated *Pseudomonas* sp. MH1-3, TMW2.2087-2.2091). The branching of the phylogenetic tree and species affiliation of isolates correlated well with RAPD biotyping cluster analysis (Figure 19).

A *car*A multiplex PCR yielding distinct amplicon sizes to differentiate *Ps. fragi*, *Ps. lundensis* and *Ps. putida* previously established (Ercolini *et al.* 2007) was conducted to enable semi-fast identification and to probe the suitability of this method for the meat-borne isolates. All isolates identified as *Ps. fragi* and *Ps. lundensis* by sequence analysis showed the desired specific amplicon size, whereas isolates affiliated to other species showed either no bands or multiple amplicons of desired and undesired length. Therefore, the method proved suitable for differentiation of *Ps. fragi*, *Ps. lundensis* and others, and could be used to preselect isolates with medium throughput. Taken together, the polyphasic identification approach enabled resolving the HCA (Figure 21) and affiliation to species, which was not able using Biotyper identification alone.

Besides *Ps. weihenstephanensis*, *Ps. fragi*, *Ps. lundensis* that were suggested as main contributors to spoilage and highly abundant also at later spoilage stages as previously discussed in detail (5.1.2/5.1.3), isolates could be affiliated to *Ps. versuta*, *Ps. meridiana*, *Ps. simiae* and *Ps. veronii*. By now, there have been no reports of the abundance of these species on meat or contribution to spoilage. *Ps. versuta* and *Ps. meridiana* have been isolated from antarctic soil, although they are described as psychrophilic, the reported observed growth occurred at 4-30 °C (Reddy *et al.* 2004; See-Too *et al.* 2017). *Ps. veronii* has been isolated from natural mineral water (Elomari *et al.* 1996) and *Ps. simiae* from clinical samples of primates (Vela *et al.* 2006). The latter has been reported on ready-to-eat vegetables (Federico *et al.* 2015) and raw frozen seafood (Noor Uddin *et al.* 2013), however, the identification has been based on 16S rDNA sequences which was demonstrated in this study to not possess enough phylogenetic discriminatory power for *Pseudomonas* spp.

In summary, this polyphasic approach demonstrated that a highly diverse group of *Pseudomonas* species can be found on MAP minced beef and likely contribute to spoilage of the product. Recovered isolates were affiliated to species known as meat spoilers but also to species which were previously not reported to be present on meat. At the time of isolation, *Ps. weihenstephanensis* as well as *Ps. versuta* were not yet described. Still, isolates TMW2.2087-TMW2.2091 from minced beef are likely to belong to different undescribed, novel species (*Pseudomonas* sp. MH1-3, cf. Table 8) within the genus *Pseudomonas*.

5.2 *Lactococcus piscium* as a novel bioprotective organism

5.2.1 Intraspecies RAPD biotyping

The usability of colony-based RAPD biotyping for reliable, intraspecies discrimination of *Lc. piscium* strains was demonstrated enabling high-throughput tracking in mixed inoculations. While MALDI TOF-MS direct transfer method is a reliable tool for high-throughput identification on species level, the liquid extraction method able to differentiate on strain-level (4.1.6) requires pure overnight cultures and is not suitable for high throughput (Usbeck *et al.* 2013). RAPD-PCR biotyping with isolated DNA as template is also not suitable for high-throughput. The colony-based approach with ultrasonic treatment is suitable for high-throughput while retaining the ability to differentiate the different strains with reproducible band patterns (4.3.1).

5.2.2 Intraspecies assertiveness and competitiveness against spoilers

The 15 different *Lc. piscium* strains previously isolated from meat products displayed a strain-dependent assertiveness with *Lc. piscium* TMW2.1614 as the most assertive one (4.3.2). Nutrient availability seems not to be the limiting factor, since the incubation with the mixed cultures had higher cell counts than singly inoculated strains. However, the intraspecies assertiveness seems to have synergistic, antagonistic or competitive interactions, since the relative abundance in the mix did not represent the cell counts of singly inoculated strains. Hence it should be considered to establish and exploit groups of *Lc. piscium* strains acting synergistically for each other and against spoilers.

The potential of *Lc. piscium* to inhibit pathogenic or spoilage-associated bacteria has been previously reported on seafood products using an agar spot assay (Matamoros *et al.* 2009a; Matamoros *et al.* 2009b; Fall *et al.* 2010a; Fall *et al.* 2010b). This study demonstrates that co-inoculation with highly-competitive *Lc. piscium* strains TMW2.1612, TMW2.1614 and TMW2.1615 resulted in significant growth reduction of all tested relevant meat-spoiling SSO (4.3.3). Co-inoculation was conducted with a ratio of *Lc. piscium* (6 $\log_{10}$ CFU/ml) and spoilers (3 $\log_{10}$ CFU/ml) in order to simulate an average initial contamination scenario of beef carcasses, which is equal of below that value (Lahr 1996; Sofos *et al.* 1999; Zweifel *et al.* 2014; Reid *et al.* 2017). The inhibitory potential affected a broad spectrum of SSO including gram-staining negative (*Pseudomonas, Serratia, Hafnia*) as well as gram-staining positive bacteria (*Brochothrix, Carnobacterium, Leuconostoc*). Although *Lc. piscium* strains reached similar cell densities, the effectiveness regarding growth reduction of respective spoilage organisms was different. *Lc. piscium* strain TMW2.1612 was most effective against *Ps. weihenstephanensis* and *B. thermossphacta,* strain TMW2.1614 against *S. liquefaciens*, *H. alvei* and *C. divergens* and strain TMW2.1615 against *Le. gelidum* subsp. *gasicomitatum*. Strain-dependency of *Lc. piscium* regarding spoilage and inhibition potential has also been previously reported in the literature (Matamoros *et al.* 2009a; Pothakos *et al.* 2014; Leroi *et al.* 2015).

5.2.3 Putative antimicrobial mechanisms

The antimicrobial mechanisms of *Lc. piscium* strains on various pathogens and spoilers are widely unknown. A recent study reported that the inhibitory effect of a *Lc. piscium* strain on *Listeria monocytogenes* involves a cell-to-cell contact-dependent mechanism and is not due to limitation of nutrients (Saraoui *et al.* 2016b). Strain specific differences in the ability to establish respective cell-to-cell contact may explain strain dependent differences observed in this study, *i.e.* that in some cases the required cellular interaction could not take place because of difference in the cell envelopes of *Lc. piscium* or the respective spoilers. Genome analysis of sequenced *Lc. piscium* strains TMW2.1612 and TMW2.1615 indeed revealed several genes coding for putative proteins with lysozyme-like function that might cause or contribute to the observed growth inhibition of spoilers. Genes were detected coding for putative lysozyme family proteins and muramidases, potentially responsible for lysis via hydrolysis of peptidoglycan (Vollmer *et al.* 2008). Additional genes were found coding for proteins putatively belonging to glycoside hydrolase 25 family comprising CH-type lysozymes, which have been initially characterized in fungal *Chalaropsis* species (Hash and Rothlauf 1967), targeting the N-acetylglucosamine/N-acetyl muraminic acid backbone of the peptidoglycan (Vollmer *et al.* 2008). Other genes encode for proteins containing a LysM motif. This motif is known for its peptidoglycan binding activity and proteins containing this domain exhibit various cleavage specificities (Buist *et al.* 2008). Additional genes were found encoding proteins with a CHAP domain. This domain family is widely distributed in proteins also involved in peptidoglycan lysis (Bateman and Rawlings 2003). It remains to be demonstrated that these putative proteins exhibit true exoenzymatic activities and are not involved in cell-division or autolytic processes (Higgins *et al.* 1971).

In this study, singly-inoculated spoilers were able to reach higher cell counts than *Lc. piscium* strains in MSM also suggesting that nutrient availability is not the limiting factor. Another factor contributing to growth inhibition is hydrogen peroxide, which is frequently produced in food systems exhibiting antimicrobial activity (Price and Lee 1970; Gilliland 1985). Another *Lc. piscium* strain, MKFS47, is able to produce hydrogen peroxide from glycerol under aerobic conditions *in vitro* as previously reported (Andreevskaya *et al.* 2015). However, is has not been reported that *Lc. piscium* strains produce hydrogen peroxide *in situ* or that they are contributing to meat greening via hydrogen peroxide-driven choleglobin formation. That is in concordance with the finding of this study that beef steaks deliberately inoculated with *Lc. piscium* strains did not display green discoloration despite high cell counts of $\log_{10}$ 8 CFU cm^{-2} (4.3.4). It is also known that strains of *Lc. piscium* are able to form diacetyl (Sakala *et al.* 2002; Rahkila *et al.* 2012), which is a compound that exhibits antimicrobial properties (Jay 1982). Furthermore, acidification could also contribute to an inhibition of spoilers. However, only a slight drop in pH values of 0.3-0.4 units in meat simulation medium and only 0.2 units

on beef was observed that unlikely caused the observed growth reduction of spoilers. It has been previously reported that pH lowering caused by *Lc. piscium* on shrimps can contribute to growth inhibition of spoilers but does not fully explain growth inhibition (Fall *et al.* 2010a; Fall *et al.* 2010b). The observed distinct inhibition patterns of different gram-staining positive and negative SSO also hint that the mode of action may be multifactorial and that different *Lc. piscium* strains may potentially have different inhibitory mechanisms affecting SSO in a variable degree.

5.2.4 Inoculation of beef with selected *Lc. piscium* strains

Bioprotective cultures have to exhibit inhibitory behavior on spoilers while retaining acceptable organoleptics of the product. It has been previously reported that different *Lc. piscium* strains lead to shelf life extension of shrimp (Matamoros *et al.* 2009a; Matamoros *et al.* 2009b; Fall *et al.* 2010a; Fall *et al.* 2010b) and cold smoked salmon (Leroi *et al.* 2015), cause shortening of the shelf life on pork (Rahkila *et al.* 2012) and display a strain-dependent weak or strong spoilage character on sweet bell pepper (Pothakos *et al.* 2014). In this study, MAP beef steaks deliberately inoculated with *Lc. piscium* strains TMW2.1612, TMW2.1614 and TMW2.1615 displayed an acceptable ripened overall impression rather than rejectable organoleptics e.g. green discoloration or off-odor after 8 days of incubation and high cell counts of 8 $\log_{10}$ CFU cm^{-2} (4.3.4). Furthermore, all tested *Lc. piscium* strains did not produce biogenic amines on the decarboxylation media, while harmful spoilers (*Pseudomonas*, *Serratia* and *Hafnia*) did. These findings indicate the potential of *Lc. piscium* strains TMW2.1612, TMW2.1614 and TMW2.1615 to be used as bioprotective cultures on meat. Investigation of spatial distribution of microbiota on beefsteaks (cf. 4.1.7) revealed that the initial spoilage microbiota is located mainly on the edges of beef steaks suggesting a potential application of *Lc. piscium* not on steaks but on big meat pieces or the whole carcass.

5.2.5 Volatilome analysis of *Lc. piscium* on beef

Volatilome analysis of *Lc. piscium* TMW2.1615 was carried out by PTR-MS (4.3.5) and conducted by C. Franke at the Fraunhofer IVV within a project collaboration (Franke 2018). Volatile metabolites with a mass ($^{m}/_{z}$) of 87 and 89 were the only ones with higher concentration compared to the uninoculated control matching to diacetyl or 3-methyl-butanal and acetoin or methyl-butanol. Again, the beef displayed a sweet, buttery and ripened impression, therefore it is highly likely that these volatile metabolites were diacetyl and acetoin, which exhibit this aroma (Marshall 1987; Axelsson 2004) and not 3-methyl-butanal and methyl-butanol, which have been described to exhibit a malty and fermented aroma (Morgan 1976; Casaburi *et al.* 2015). No other unwanted or off-flavor generating volatile substances were detected via PTR-MS monitoring as well as in sensorial evaluation, demonstrating that *Lc. piscium* retains acceptable organoleptics of the meat without truly spoiling it despite high cell counts.

5.3 Photobacteria as hitherto neglected spoilers on MAP meats

5.3.1 Photobacteria as common spoilers on MAP meats

Member of the genus *Photobacterium* have been typically isolated from coastal, open-ocean and deep-sea environments (Urbanczyk *et al.* 2011). *Photobacteria* occur either as free-living pelagic in seawater or in association with marine organisms in pathogenic and symbiotic lifestyles (Urbanczyk *et al.* 2011; Labella *et al.* 2017). *P. phosphoreum*, a species with luminous strains, is known as an important fish spoilage organism (Dalgaard *et al.* 1997) and known to be responsible for the formation of biogenic amines (Jørgensen *et al.* 2000a; Emborg *et al.* 2002) causing e.g. histamine fish poisoning (Lehane and Olley 2000). *P. iliopiscarium* has been also found on spoiled fish (Ast and Dunlap 2005) and its ability to form histamine has been previously reported as well (Takahashi *et al.* 2015).

Recently, presence of *Photobacterium* spp. has been reported in culture-independent studies on vacuum and air stored beef (Pennacchia *et al.* 2011), modified atmosphere packaged minced beef (Stoops *et al.* 2015) and MAP pork (Nieminen *et al.* 2016). Moreover, Nieminen *et al.* were able to recover *P. phosphoreum*-like isolates from pork samples, while it is not clear if the isolates represent different strains or not. These previous studies provided evidence for the abundance of *Photobacterium* spp. on meat that seem hitherto underestimated in relevance to their role in meat spoilage. However, the previous culture independent reports on photobacteria are very rare and seem random, and only a few isolates of a single species could be isolated until now. It still remained unclear, which meats are affected, how many different species are abundant and how frequent the contamination with these organisms occur.

Therefore, this study aimed to develop a novel adapted isolation and cultivation procedure for recovery of photobacteria from foods and probe the contamination frequency of these organisms on different meats. Various media, additives and temperatures were tested beforehand with photobacteria type strains and relevant meat spoilers, and were afterwards employed for a comparative isolation approach. Additionally, reference MSPs of *Photobacterium* spp. type strains were created and included into the MALDI-TOF MS database in order to enable reliable identification. After building up a database with photobacteria reference MSPs, all not reliably identified isolates (*nri*) from the previous growth dynamics experiments of beef steaks and minced beef were re-identified to evaluate if *Photobacterium* isolates were among them and were not identified due to the absence of their reference spectra at that time. However, no match to any photobacteria was found within the *nri* spectra indicating that no *Photobacterium* spp. were isolated in the growth dynamics experiments with the isolation parameters previously used.

All tested Photobacteria did not show a higher salt tolerance compared to other meat spoilers, although halophilic species have been reported e.g. *P. ganghwense* (Park *et al.*

2006). However, *Photobacterium* type strains required a salt addition (at least 0.5%) in the medium. Furthermore, their growth was greatly reduced if quarter strength Ringer's solution was employed for preparation of serial dilutions as used in standard routine for food samples. As a consequence, Ringer's solution was substituted by marine broth used in the final adapted isolation procedure to resuspend and dilute food samples (4.4.1.1).

Marine broth 2216 (MB, Difco) was selected as one of the media to be tested. It is used as a medium for cultivation and isolation of marine heterotrophs (Atlas 2004) and also recommended as a standard medium for cultivation of various *Photobacterium* species by different culture collections (DSMZ, CECT and LMG). Furthermore, it is frequently used for routine cultivation and phenotypic analysis of *Photobacterium* spp. with varying temperatures (Seo *et al.* 2005; Ast *et al.* 2007; Yoshizawa *et al.* 2009; Liu *et al.* 2014; Lo *et al.* 2014; Gomez-Gil *et al.* 2016). MB was supplemented with meat extract (3g/L) due to its growth promoting effect. In addition to MB with and without vancomycin at different temperatures, non-selective Brain Heart Infusion agar (BHI), used for general cultivation of (non)-fastidious bacteria (Atlas 2004), and thiosulfate citrate bile salts sucrose agar (TCBS, (Kobayashi *et al.* 1963)), used for selective cultivation of *Vibrionaceae* (Donovan and Van Netten 1995) and phenotyping of photobacteria (Yoshizawa *et al.* 2009; Gomez-Gil *et al.* 2016), were included in the comparative approach (4.4.1.2).

Using BHI and MB without vancomycin, typical meat spoilers previously identified during monitoring experiments (4.1.2) were isolated, but no photobacteria. The relative abundance of *C. divergens* and *Lc. piscium* was reduced on MB agar compared to BHI, which is in accordance to observed weak growth on MB agar of pure cultures of these species. Vice versa, pure cultures of photobacteria on the other hand showed poor or no growth on BHI, which is one of the reasons that no photobacteria were detected in the previous growth dynamics monitoring experiments.

In antibiotic susceptibility pre-tests with pure cultures, vancomycin (7 g/l) was effectively inhibiting growth of common Gram-positive meat spoilers while growth of *Photobacterium* spp. was unaffected (4.4.1.1). In the comparative isolation approach, supplementation with vancomycin led to disappearance of *B. thermosphacta*, *Lc. piscium* and *Carnobacterium* spp. from the spoilage-associated microbiota accordingly and a drop in TVC of over one $\log_{10}$ magnitude compared to medium without vancomycin. Furthermore, a successful recovery of a *P. phosphoreum* isolate on marine broth agar with vancomycin was possible when employing a cultivation temperature of 15 °C. Therefore, this medium and cultivation temperature potentially favoring psychrotrophic and psychrophilic microbiota, were chosen in the final approach.

On TCBS, microbiota consisted of *Pseudomonas* spp. and *Yersinia* spp., which was unexpected because selective additives in the media (thiosulfate, bile, high pH) have been

suggested to inhibit not only gram-positive organisms but also *Pseudomonas* spp. as well as enterobacteria (Donovan and Van Netten 1995; Atlas 2004). Pure cultures of *Pseudomonas* spp., *S. liquefaciens* and *H. alvei* tested on TCBS were also growing well, whereas gram-positive spoilage-associated bacteria did not show growth at all. However, most of the tested pure cultures of *Photobacterium* reference strains also showed no or only weak growth on this media and no *Photobacterium* isolate was recovered using TCBS, suggesting a poor suitability of this medium for isolation of photobacteria from meat products or even in general.

In addition to marine broth, *Photobacterium* broth (PB) can also be used to cultivate and demonstrate luminescence of photobacteria (Doudoroff 1942; Giese 1943; Atlas 2004). In order to compare these two media (both supplemented with meat extract and vancomycin), TVC and luminous count (LC) were analyzed of two poultry samples. While photobacteria type strains showed only weak or no growth on PB agar in pre-tests, isolates of *Photobacterium* spp. could be recovered from both media in the isolation approach. However, a non-luminous *P. iliopiscarium* isolate was recovered from a poultry sample on MB whereas none was found on PB despite higher total count. MB is therefore more suitable for isolation of different *Photobacterium* spp. from meats than PB and also able to demonstrate their luminescence in the same degree. Further analysis of luminous colonies via MALDI-TOF MS and sequencing of housekeeping genes identified all as *P. phosphoreum*. Also, all genes of the *lux* cluster (*lux*CDABFEG) (Mancini *et al.* 1988) were present in both genome-sequenced *P. phosphoreum* isolates from meats (Table A5). A luminous count can therefore be used to visibly quantify *P. phosphoreum* and allows a quick pre-selection though it disregards non-luminous *P. phosphoreum* strains and dominant *P. carnosum* as well as *P. iliopiscarium* and does therefore not represent correct contamination level of meats. Consequently, luminous as well as non-luminous colonies should be subjected to subsequent identification as it has been previously recommended (Dalgaard *et al.* 1997).

In order to specifically quantify *P. phosphoreum* (including non-luminous strains) on fish and seafood, Dalgaard *et al.* (1996) have developed a specific conductance method. This method relies on a conductance change resulting from microbial reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) in a specific medium and is supposed to be sensitive and selective for *P. phosphoreum* (Dalgaard *et al.* 1996). This method has been frequently employed (Emborg *et al.* 2002; Emborg *et al.* 2005; Reynisson *et al.* 2010; Nieminen *et al.* 2016). However, comparative genome analysis in this study (4.5.2.5) revealed that also *P. carnosum* TMW2.2021^{T} and TMW2.2029 as well as *P. iliopiscarium* TMW2.2035 have a TMAO reductase encoded in their genome. Apparently, the conductance method is not specific and will give erroneous quantification. As a consequence, previous studies employing this method might have overestimated *P. phosphoreum* while neglecting other species of photobacteria.

The adapted isolation and cultivation method (4.4.1.4) developed in this study allowed frequent isolation of different *Photobacterium* species (*P. phosphoreum*, *P. iliopiscarium* and *P. carnosum*). The isolation in relevant quantities from different meats (poultry, pork, beef, salmon) as well as different packaging conditions (MAP, VP, air-stored) demonstrated that photobacteria occur not only sporadically but are a pervasive constituent of the contaminating microbiota of meats. This study provides clear evidence and novel finding that the importance of photobacteria in relevance to their role in meat spoilage is hitherto underestimated. Future research should aim to identify the source of contamination and distribution in order to develop a rationale for the control of these organisms.

Highly interesting in this regard is the fact that Martinus W. Beijerinck, who established the genus *Photobacterium* in 1889 (Beijerinck 1889), had his first encounter with this luminous bacteria back in 1888 not on fish, but when a colleague showed him a piece of salted pork that was glowing in the dark. Though he was not able to isolate the luminous bacteria from that meat, he was highly intrigued and continued with his experiments. He was finally able to isolate light-producing bacteria from fish, which he stored aerobically in his cellar using a medium based on fish and marine water (Robertson *et al.* 2010).

5.3.2 *Photobacterium carnosum* sp. nov.

Using the adapted isolation and cultivation procedure developed in this study (4.4.1.4), strains of *P. phosphoreum* and *P. iliopiscarium* were frequently isolated from meats. In addition, several bacterial strains were isolated, *inter alia*, from (un-)spoiled poultry meat that were below the cut-off recommended by the manufacturer (score value <1.7) for reliable identification via MALDI-TOF MS. However, they matched best with the photobacteria reference spectra in the database hinting that they belong to a *Photobacterium* species not present in the database. Further characterization via RAPD-PCR and MALDI-TOF MS fingerprinting revealed four different biotypes among the isolates that were divergent from fingerprints of other photobacteria species (4.4.2). Isolates of the different biotypes shared identical 16S rRNA sequences, indicating that they belong to a single species, and showed high similarity to type strains of the *P. phosphoreum* group (>99.5%). However, 16S rRNA genes alone are not a distinctive phylogenetic marker within the genus *Photobacterium* due to high sequence similarity between the species. (Ast and Dunlap 2004; 2005; Urbanczyk *et al.* 2011). To further resolve the 16S rRNA gene cluster, a multi locus sequence analysis (MLSA) of selected relevant *Photobacterium* species was performed including 16S rRNA as well as housekeeping genes *gyr*B, *rec*A and *rpo*D, which has been previously used to resolve the *P. phosphoreum* 16S group (Ast *et al.* 2007). The isolates had distinct unique genotypes and formed one cluster apart from known species of photobacteria, supporting the results of the fingerprinting analysis that four strains of a novel species were recovered from meat.

Therefore, the name *Photobacterium carnosum* (latin, *carnosum*, pertaining to flesh) was proposed.

The current gold standard for prokaryotic species delineation is the overall genome related index (OGRI) of Average Nucleotide Identity (ANI) with a cut-off of 95-96% sequence similarity (Richter and Rosselló-Móra 2009; Kim *et al.* 2014; Chun *et al.* 2018; Ciufo *et al.* 2018) replacing DNA-DNA hybridization (cut-off 70% similarity) (Wayne *et al.* 1987; Stackebrandt and Goebel 1994) and 16S rDNA cut-off of 97% sequence similarity (Hagström *et al.* 2000; Yarza *et al.* 2008). The ANIb value of *P. carnosum* TMW2.2021^T and the closest relative *P. iliopiscarium* was only 91.43% and does therefore clearly represents a new species within the genus *Photobacterium*. It could be assigned to the *P. phosphoreum* (sub-)clade (Sawabe *et al.* 2013; Gomez-Gil *et al.* 2016). *P. carnosum* was also assigned to this clade by Labella *et al.* (2018) based on a MLSA, although it is the most divergent *Photobacterium* species within this clade.

Furthermore, chemotaxonomic properties *i.e.* cellular fatty acids (Welch 1991) and respiratory quinones (Collins and Jones 1981), were determined for *P. carnosum* TMW2.2021^T and compared to type strains of other photobacteria species. Ubiquinone Q-8 was identified as the sole respiratory quinone under aerobic conditions by the DSMZ, which is consistent with previous findings of all other species within the genus *Photobacterium* (Lo *et al.* 2014). The complete gene cluster needed for biosynthesis was also detected in the genome (Table A5). The overall fatty acid profile of *P. carnosum* was very similar to other *Photobacterium* species with certain variations in the respective proportions. However, $C_{17:0}$ cyclo was not detected in *P. carnosum* TMW2.2021^T but represented a major compound (15.22%) in *P. kishitanii* DSM 19954^T, which is in accordance with the original data (Ast *et al.* 2007). Fatty acid $C_{18:0}$ was detected only in small amounts in strain TMW2.2021^T, while it has been reported to be a major fatty acid of *P. aquimaris* DSM 23343^T (Yoshizawa *et al.* 2009).

All strains of *P. carnosum* isolated in this study were non-luminous with no *lux* operon detected in the genome. The type strain of the closest related species, *P. iliopiscarium*, also possess no *lux* operon in contrast to *P. phosphoreum*^{T}, which is bioluminescent and has a *lux* operon in the genome (Urbanczyk *et al.* 2011; Machado and Gram 2017). *P. carnosum* strains were tested negative also for motility. No motility was observed from cells in wet mounts under the microscope and the soft agar stabbing test was interpreted as negative. However, type strain TMW2.2021 (in contrast to other strains) produced a minimal halo in the soft agar and a big flagellar gene cluster was detected in the genome (CIK00_02020-02295), whereas the genome of *P. carnosum* TMW2.2029^T contains none. Functionality or expression of the gene cluster remains unknown, but the negative physiological tests indicates that it is not (completely) functional or expressed under the conditions tested. Interestingly, the flagellar gene cluster of *P. carnosum* TMW2.2021^T is homologous to the one present in the genome of a shallow-water strain 3TCK of *P. profundum* for polar flagella and not to the lateral flagellar

cluster additionally present in the deep-sea piezophilic *P. profundum* strain SS9, which is expressed under high pressure conditions (Campanaro *et al.* 2005; Vezzi *et al.* 2005; Eloe *et al.* 2008). Complete loss of flagellar gene cluster as in *P. carnosum* TMW2.2029 or presence of only a cluster of a pressure-sensitive flagellum as in *P. carnosum* TMW2.2021^T hints the adaptation away from deep-sea environments.

As stated previously, member of the genus *Photobacterium* are limited to aquatic marine habitats (coastal, open-ocean and deep-sea) (Urbanczyk *et al.* 2011; Labella *et al.* 2017; Moi *et al.* 2017). For the first time, a novel species of *Photobacterium* was discovered in this study without any detected marine background. Instead, this species is associated with meat spoilage. Other members of the *Vibrionaceae* family have been isolated also from non-aquatic sources, e.g. *Vibrio casei* from cheese (Bleicher *et al.* 2010). Type strains of closest related marine photobacteria of the *P. phosphoreum* clade exhibited various phenotypic differences compared to *P. carnosum* sp. nov. Strains of this novel species appear to be potentially adapted to conditions rather found in terrestrial environments (ability to hydrolyze glycogen and starch) and show a loss of adaptations for marine environments (loss of motility, loss of luminescence, less tolerance for high salt concentration and alkaline pH) under the conditions tested. Furthermore, the growth behavior of all four isolated *P. carnosum* strains is clearly psychrophilic in contrast to all other tested related *Photobacterium* spp. with a rapid growth at zero centigrade, optimum growth at 10-15 °C and incapability to grow above 20 °C. This is further evidence for the contribution of psychrophiles to meat spoilage as demonstrated for the psychrophilic strain of *Le. gelidum* subsp. *gelidum* TMW2.1998 (5.1.6).

P. carnosum shows a clear distinct lineage from other species of the genus *Photobacterium* based on polyphasic (phylogenetic, genomic, chemotaxonomic and phenotypic) evidence. The name "*Photobacterium carnosum* sp. nov." and its taxonomic status was effectively published in the journal "Systematic and Applied Microbiology" (Hilgarth *et al.* 2018) and validly published in the Validation List 181 of the International Journal of Systematic and Evolutionary Microbiology (Oren and Garrity 2018). The name was therefore made available in the nomenclature of prokaryotes under the procedure described in the *International Code of Nomenclature of Prokaryotes* (2008 Revision) (Parker *et al.* 2015).

5.4 Predictive lifestyle of psychrotrophic and psychrophilic spoilage-associated bacteria

5.4.1 Automatic pipelines and annotations for whole genome analysis

Evaluation of automatic bioinformatic pipelines (PGAP, RAST, KEGG) used for annotation and creation of metabolic maps revealed major issues of these processes e.g. erroneous or missing annotations as well as incompleteness of automatically generated pathways. Although it is a highly time-consuming task, manual curation and re-checking of all respective gene identities as well as enzyme reactions is inevitable in order to yield correct assignments and predictive metabolic pathways. Erroneous annotation and propagated errors into secondary databases have been also previously reported in the literature (Richardson and Watson 2012).

Specific annotation and BLAST search enabled assessment of potential gene functions in relevant predictive metabolic routes on meat. However, a specific assessment of genes coding for transporters was not possible in many cases. Annotation and subsequent BLAST search often allowed only an unspecific assessment to e.g. sugar ABC transporter, amino-acid transporter, aquaporin or ABC transporter permease, which is also partly caused by the poly-specificity of these transport systems (Davidson *et al.* 2008).

5.4.2 Substrates for spoilage-associated microbiota on beef

Beef can be considered an ecological niche (Labadie 1999) and represents a substrate-rich environment with high water activity (0.99) and a moderate pH (5.5±0.3) that support diverse bacterial growth (Gill 1983; Lambert *et al.* 1991; Lawrie and Ledward 2006; Eskin and Shahidi 2012). It offers easily available diverse carbohydrates as well as protein- and triacylglyceride-derived substrates. Carbohydrate substrates comprise mostly glucose, fructose, mannose, ribose and glycolytic intermediates in concentrations up to approx. 1.2% (Mottram and Nobrega 1998; Lawrie and Ledward 2006; Nychas *et al.* 2007; Koutsidis *et al.* 2008b; Eskin and Shahidi 2012). Additionally, varying glycogen levels up to 1.8% are possible in resting muscle tissues (Pethick *et al.* 1995; Immonen and Puolanne 2000; Immonen *et al.* 2000; Ninios *et al.* 2014) and therefore, it represents an additional relevant substrate for the spoilage bacteria. Remaining levels of carbohydrates depend on pre-slaughter handling, stress and breed, and endogenous postmortem glycolysis influence the availability of glucose for spoilage microorganisms (Pösö and Puolanne 2005; Ferguson and Warner 2008; Koutsidis *et al.* 2008a). Additionally, proteins, peptides and free amino acids make up about 20-25% of meat (Weber 1996; Lawrie and Ledward 2006; Eskin and Shahidi 2012). The amino acid composition is highly diverse with relevant essential and non-essential amino acids including various aromatic and branched amino acids (Holló *et al.* 2001a). The fat content of meat various greatly between products. In this study, beef steaks and minced beef were analyzed with a respective fat content of approx. 2% and 10-12% w/w.

Given the substrates available in meats, carbohydrate, amino acid and triacylglyceride metabolism of spoilage-associated microbiota have to be considered in order to predict their lifestyle and energy conservation in this ecological niche.

In general, there are three modes of energy conserving processes to form ATP: Respiratory electron transport phosphorylation (ETP) via generation of a proton motive force (PMF) (Haddock and Jones 1977), fermentative substrate level phosphorylation (SLP) (Zehnder and Brock 1979) and flavin-based electron bifurcation (FBEB) (Buckel and Thauer 2013), while the latter represent a special mechanism found in e.g. clostridia.

5.4.3 Predictive meat-derived substrate fermentation pathways of spoilage-associated bacteria

In this study, nine strains from different species of LAB and photobacteria were selected for genome sequencing. Two strains of *Lc. piscium* (TMW2.1612, TMW2.1615) and one strain of *Le. gelidum* subsp. *gelidum* (TMW2.1618) and *gasicomitatum* (TMW2.1619) were selected, representing the major SSO identified within the growth dynamics analyses in this study. Additionally, two strains of *P. carnosum* (TMW2.2021^T / TMW2.2029), two strains of *P. phosphoreum* (TMW2.2033 / TMW2.2034) and one strain of *P. iliopiscarium* (TMW2.2035) were selected due to their high abundance on different meats as demonstrated with the adapted isolation procedure developed in this study. For an overview of sequenced strains and sequencing statistics, see Table 15. The following chapters will discuss predictive fermentative and respiratory energy conserving metabolic routes of genome-sequenced, spoilage-associated LAB as well as *Photobacterium* spp. based on the presence of genes within their genomes (4.5.2).

5.4.3.1 Fermentative pathways of LAB

General fermentation metabolisms known for LAB are the glycolytic (homolactic), phosphoketolase (heterolactic) or bifidobacterial pathways (Thompson 1987; de Vos 1996), whereas it has been reported that some LAB can switch between homo- and heterolactic fermentation based on different conditions (Thomas *et al.* 1979). The terminology is partly misleading and sometimes used differently in the literature, though. Hereinafter, homolactic fermentation (homofermentative) is used to state that the glycolytic pathway is used instead of heterolactic phosphoketolase pathway (heterofermentative) and not that lactic acid is the sole fermentation product. *Lc. piscium* has been described as a homofermentative LAB (Williams *et al.* 1990a; Teuber and Geis 2006), whereas *Le. gelidum* subsp. *gelidum* & *gasicomitatum* are known to be heterofermentative LAB (Shaw and Harding 1989; Björkroth *et al.* 2000; Rahkila *et al.* 2014). In concordance, genome analysis in this study showed that *Lc. piscium* strains possess all genes to employ homolactic fermentation, but lack a gene encoding a xylulose-5-phosphate phosphoketolase (x*pk*A) (4.5.2.1). *Lc. piscium* strains are likely to follow

the glycolytic route and produce lactic acid from pyruvate as the primary product in order to regenerate NAD^+ if no other reactions perform this step (Axelsson 2004). The net outcome is 2 mol ATP per mol glucose (Thauer *et al.* 1977; Flamholz *et al.* 2013). Vice versa, *Le. gelidum* subsp. possess all genes enabling heterolactic fermentation but lack a gene encoding a 6-phospho-fructokinase (*pfk*A). Therefore, the expected primary products of *Le. gelidum* subsp. fermentation are lactic acid, carbon dioxide and either ethanol or acetate. Conversion of the acetyl-phosphate intermediate to ethanol is needed to regenerate NAD^+ that is needed in the first steps from glucose to ribulose-5-phosphate. If other reactions cover this regeneration, acetyl-phosphate is converted to acetate resulting in ATP conservation. The net outcome of heterolactic fermentation is therefore 1-2 mol ATP per mol glucose (Axelsson and Ahrné 2000; Axelsson 2004).

Glucose, mannose, fructose and their phosphorylated forms has been described as the relevant hexose carbohydrates on meats and are present in beef (Koutsidis *et al.* 2008a; 2008b), chicken (Aliani and Farmer 2005a; 2005b), pork (Meinert *et al.* 2009a; Meinert *et al.* 2009b) and fish (Tarr 1966), contributing to the respective flavor. All of these carbohydrates can be predictively used as substrates by sequenced *Le. gelidum* subsp. as well as *Lc. piscium* strains and channeled into the respective fermentation pathway. Furthermore, both *Lc. piscium* strains possess a gene encoding a glycogen phosphorylase (*glg*P) enabling degradation of glycogen, which can be present in high concentrations in muscle meat (Trowbridge and Francis 1910; Pethick *et al.* 1995; Immonen and Puolanne 2000; Immonen *et al.* 2000; Ninios *et al.* 2014) and still be available after 21 days (Koutsidis *et al.* 2008b). The ability to use glycogen could be a selective advantage, however it is known that also endogenous meat enzymes can be active postmortem to hydrolyze glycogen (Warriss 1990; Eskin and Shahidi 2012). Other widely distributed hexose degradation pathways are the Entner-Doudoroff pathway (Entner and Doudoroff 1952; Conway 1992) or the pentose-phosphate pathway (Kandler 1983). However, both analyzed LAB species lack genes coding for phosphogluconate dehydratase (*edd*) and transaldolase (*tal*) that would be necessary to employ these pathways, respectively.

In addition to hexoses, nucleoside and derived ribose represent a relevant substrate for the spoilage-associated microbiota. Endogenous postmortem metabolism of muscle ATP leads to the formation of IMP, inosine, adenosine, hypoxanthine as well as release of free ribose (Macy *et al.* 1964a; 1964b; Macy *et al.* 1970; Lawrie and Ledward 2006; Eskin and Shahidi 2012). Additionally, nucleosides and deoxy-nucleosides could arise from endogenously degraded RNA and DNA, respectively. It is known that the pentose moiety of nucleosides represents a relevant substrate for LAB (Kilstrup *et al.* 2005; Rimaux *et al.* 2011). The predicted metabolic routes and capabilities of analyzed *Lc. piscium* and *Le. gelidum* subsp. are quite different in regard to their nucleoside metabolism. Sequenced *Le. gelidum* subsp. strains harbor genes coding for putative ribose and nucleoside transporter

(*rbs*U/*nup*A/*yng*F), purine/pyrimidine nucleosidases (*ihu*N/*rih*C), and ribokinase for import, release and activation of ribose, respectively, yielding ribose-5-phosphate. For activation, the ribose-5-phosphate needs to be in its furanose conformation, however, in aqueous solutions, ribose exists mainly in pyranose form (80%) and only to 20% in furanose form (Drew *et al.* 1998). A ribopyranase (*rbs*D), catalyzes this interconversion and the gene coding for the enzyme is present in both *Le. gelidum* subsp. genomes. The resulting ribose-5-phosphate can then be channeled into heterolactic fermentation. The advantage of ribose utilization is that no reduction equivalents has to be regenerated in contrast to glucose as a substrate and therefore the heterolactic acetate route can be used to yield more energy. Sequenced *Lc. piscium* strains on the other hand are predictively not able to ribose or ribose-5-phosphate as a substrate, because they lack genes to employ downstream pentose phosphate pathway as well as heterolactic fermentation. They can however predictively convert nucleosides to deoxy-nucleosides via ribonucleotide reductase (*nrd*A/B) and create deoxy-ribose-5-phosphate via purine/pyrimidine nucleoside phosphorylase (*deo*D/*deo*A) and phosphopentomutase (*deo*B). The deoxy-ribose-5-phosphate can further be cleaved via deoxyribose-phosphate aldolase (*deo*C) into glyceraldehyde-3-phosphate and acetaldehyde. The former can be channeled into glycolysis creating 2 ATP and the latter can be converted to ethanol to cover for the necessary regeneration of NAD^+.

Depending on the conditions, alternative fates of pyruvate (4.5.2.2) besides lactate are possible in LAB (Kandler 1983). For both analyzed LAB species, predicted alternative pyruvate fates are similar. Ethanol, acetate, carbon dioxide and diacetyl/acetoin/2,3-butanediol are the predicted products of pyruvate metabolism. Acetate and ethanol can be produced via acetyl-CoA to either conserve energy or regenerate reducing equivalents, respectively. Strains of both species can facilitate the production of acetyl-CoA via pyruvate dehydrogenase complex (*pdh*A/B/C/D). Both *Lc. piscium* strains additionally possess a gene coding for pyruvate formate lyase (*pfl*B) producing formate as a by-product, which is also known for other LAB (Mayo *et al.* 2010; Gao *et al.* 2011). The resulting formate needs to be exported due to its toxicity by a formate efflux transporter (*foc*A) (Beyer *et al.* 2013). However, pyruvate formate lyase has been reported to be highly sensitive to oxygen (Takahashi *et al.* 1987) and it is therefore unlikely to function in high-oxygen modified atmosphere. On the other hand, *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 possesses a gene (not present in *Lc. piscium* or *Le. gelidum* subsp. *gelidum* genomes) encoding an oxygen-depending pyruvate oxidase (*pox*B) that produces carbon dioxide and hydrogen peroxide as by products (Sedewitz *et al.* 1984). This reaction has the advantage that no NAD^+ must be regenerated afterwards and the acetate route can be used to conserve energy. This reaction can also explain the green discoloration of spoiled meat observed in this study (4.1.1), which also has been previously reported to be caused also by other strains of *Le. gelidum* subsp. (Vihavainen and Björkroth

2007b; Johansson *et al.* 2011). Produced hydrogen peroxide reacts with myoglobin, altering the heme structure and yielding green choleglobin (Faustman and Cassens 1990; Lawrie and Ledward 2006). Furthermore, within the analyzed genomes, a gene was found putatively coding for a NADH oxidase (*nox*). This oxygen-depending enzyme could re-oxidize lactate to pyruvate, enabling alternative fates, and is known in *Leuconostoc* sp. (Lucey and Condon 1986; Condon 1987). However, a definite annotation could not be given and therefore the actual function of this gene could not be predicted precisely. Another alternative pyruvate pathway known in LAB is the diacetyl/acetoin pathway. All genes necessary for employing this pathway (acetolactate synthase (*als*S), acetolactate decarboxylase (*ald*C), acetoin/diacetyl reductase (*but*A)) were found in the genomes of both *Lc. piscium* strains as well as *Le. gelidum* subsp. regenerating 2 NAD^+ by sequential reduction of diacetyl to acetoin and further to 2,3-butanediol (Kandler 1983; Axelsson 2004). Diacetyl has a buttery (off-)aroma and can contribute to spoilage impression if together with other compounds, while acetoin and 2,3-butandiol have been reported to have only little contribution to the aroma (Marshall 1987; Axelsson 2004). Diacetyl can also exhibit antimicrobial properties (Jay 1982). Another relevant substrate could be glycerol, which can originate from triacylglycerides on meat. Genomes of both analyzed LAB species do not possess a lipase, only a gene containing a putative esterase/lipase domain and therefore are supposedly not able to hydrolyze triacylglycerides present in meat. However, endogenous meat lipases can release the fatty acids and glycerol from fat (Hierro *et al.* 1997; Molly *et al.* 1997; Toldra 1998) or they can be made available for LAB with extracellular lipases from other organisms e.g. *Pseudomonas* (Bala *et al.* 1977; Stead 1986) or *Photobacterium* spp. The fatty acid moiety is predictively not used by both analyzed LAB species, though, because genes for β-oxidation as well as a functioning TCA cycle are absent in their genomes. The glycerol moiety can be co-fermented with other carbohydrates to 1,3-propanediol under regeneration of NAD^+ as known for lactobacilli (da Cunha and Foster 1992). However, the genes were also not present in the analyzed LAB genomes. Another possible pathway is the activation and oxidation of glycerol to dihydroxyacetone phosphate, which can subsequently be fermented facilitated by glycerol-3-phosphate dehydrogenase (*gps*A) or α-glycerophosphate oxidase (*glp*O). The *gps*A gene was present in all LAB genomes, whereas *Lc. piscium* strains also possess the *glp*O gene (5.4.6), enabling glycerol as a substrate for fermentation.

Genomes were additionally analyzed for the presence of genes for degradation of amino acids. Both species, and especially *Le. gelidum* subsp. *gelidum*, seem to have very limited ability to use amino acids of meat in contrast to photobacteria (5.4.4.2/5.4.4.4). No genes for decarboxylation of amino acids yielding biogenic amines were detected in the genomes. Arginine is highly abundant in meats (Schweigert and Payne 1956; Happich *et al.* 1975; Holló *et al.* 2001a; Lawrie and Ledward 2006) and the widely-distributed arginine deiminase pathway

(ADI) can be important also for LAB as previously reported (Cunin *et al.* 1986; Montel and Champomier 1987; Zúñiga *et al.* 2002; Rimaux *et al.* 2012). Genome analysis showed that both species lack the genes encoding an arginine deiminase (*arc*A), though. *Lc. piscium* strains possess the genes coding for ornithine transcarbamoylase (*arc*B) and carbamate kinase (*arc*C) for subsequent downstream reactions of the pathway. However, ornithine levels are very low in raw meats (Baldwin *et al.* 1976) since it is a non-proteinogenic amino acid and seems therefore not to represent a substrate with major relevance for *Lc. piscium*, but could potentially contribute to its dominance if sugar levels are very low. Two other amino acids, serine and aspartate, are also highly abundant in meat (Schweigert and Payne 1956; Holló *et al.* 2001a). Regarding aspartate metabolism, genes for conversion of aspartate and oxoglutarate to glutamate and oxaloacetate (catalyzed by aspartate aminotransferase (*asp*B)) and subsequent decarboxylation of oxaloacetate to pyruvate (catalyzed by malate dehydrogenase (*mdh*)) were detected in the genomes. Both *Lc. piscium* strains additionally possess a serine dehydratase (*sad*AB) catalyzing the conversion of serine to pyruvate. Conversion to pyruvate is performed in each case without consuming NAD^+ and therefore it can be converted to acetate yielding additional ATP.

Pyruvate production from amino acids is also highly interesting in regard to gluconeogenetic routes. Although most of the glycolytic reactions are reversible, enzymatic reaction of pyruvate kinase and phosphofructokinase have to be side-stepped in gluconeogenesis to yield phosphorylated hexoses. The analyzed strains of *Le. gelidum* subsp. are predictively not able to synthesize hexoses from pyruvate due to the absence of genes encoding the key enzymes. Pyruvate carboxylase (*pyc*) and phosphoenol-pyruvate carboxykinase (*pck*A) are needed to enable conversion of pyruvate to oxaloacetate and subsequently to phosphoenolpyruvate (Hanson and Garber 1972; Jitrapakdee and Wallace 1999). Both sequenced *Lc. piscium* strains however possess these genes in their genomes. Side-stepping of phosphofructokinase can be facilitated by the fructose-1,6-bisphosphatase (*fdp*) (Hines *et al.* 2006; Say and Fuchs 2010) and the gene is also present in both *Lc. piscium* strains. Therefore, gluconeogenesis seems possible for both *Lc. piscium* strains from pyruvate and oxaloacetate originating from free pools or the amino acids aspartate and serine.

5.4.3.2 Fermentative pathways of *Photobacterium* spp.

Genome-sequenced meat-borne photobacteria *P. carnosum* TMW2.2021 & TMW2.2029, *P. iliopiscarium* TMW2.2035 and *P. phosphoreum* TMW2.2033 & TMW2.2034 harbor genes for employing homolactic glycolysis as well as pentose-phosphate pathway as fermentation routes of relevant hexoses (glucose, mannose, fructose and their phosphorylated forms). These pathways and substrates have been discussed in depth in the previous chapter (5.4.3.1). Due to the reduced energy yield, these pathways are likely to be used only under anoxic conditions and if no alternative electron receptor is available. Analyzed photobacteria

genomes also harbor a glycogen phosphorylase gene (*glg*P) to hydrolyze glycogen (except both *P. phosphoreum* strains). Furthermore, they are predictively able to utilize nucleosides and deoxy-nucleosides and channel the resulting ribose moiety into the pentose-phosphate pathway, creating 1.67-2.67 ATP – depending on if activation of ribose is necessary or not. Alternatively, the deoxyribose-phosphate aldolase pathway can be employed yielding 2 ATP. The sequenced photobacteria lack the gene for phosphogluconate dehydratase (*edd*) and are therefore predictively not able to facilitate the Entner-Doudoroff pathway (Entner and Doudoroff 1952; Conway 1992).

Predicted alternative pyruvate fates of all five sequenced photobacteria are ethanol, acetate, formate, diacetyl and acetoin (cf. 5.4.3.1). All strains can produce acetyl-CoA from pyruvate, whereas only *P. carnosum* strains possess a pyruvate oxidase gene (*pox*B) yielding additionally hydrogen peroxide. Furthermore, all photobacteria analyzed can potentially convert the glycerol moiety of triacylglycerides to pyruvate via glycerol kinase (*glp*K) and glycerol-3-phosphate dehydrogenase (*gps*A).

In contrast to the analyzed LAB species *Lc. piscium* and *Le. gelidum* subsp., all five sequenced *Photobacterium* spp. have supposedly various capabilities to utilize amino acids. All three species of photobacteria possess the genes arginine deiminase (*arc*A), ornithine transcarbamoylase (*arc*B) and carbamate kinase (*arc*C) to enable the ADI pathway and conserve ATP (Cunin *et al.* 1986; Zúñiga *et al.* 2002). Other amino acid pathways e.g. production of biogenic amines as well as fatty acid degradation is discussed in the following chapters, since these are employed in respiratory mechanisms.

5.4.4 Aerobic and anaerobic respiratory metabolism of meat-borne *Photobacterium* spp.

Photobacterium spp. have been described as facultative aerobes that are able to ferment and respire under the respective conditions (Farmer and Hickman-Brenner 2006). In this chapter, the predictive glycolytic routes, triacylglyceride catabolism, anaplerotic routes of amino acids, production of biogenic amines as well as aerobic and anaerobic respiratory chains are discussed in detail. In general, all five sequenced strains of *P. carnosum*, *P. iliopiscarium* and *P. phosphoreum* have very similar predicted respiratory lifestyles.

5.4.4.1 Glycolysis, triacylglyceride catabolism and TCA cycle

All genomes of meat-borne photobacteria analyzed in this work harbor the genes to conduct the tricarboxylic acid cycle (TCA cycle). As a consequence, all relevant carbohydrates abundant on meat (glycogen/glucose/mannose/fructose) are suggested to be utilized in glycolysis and converted into acetyl-CoA. The resulting acetyl-CoA can be fueled into the TCA cycle and ultimately coupled to the respiratory chain under aerobic conditions or presence of

alternative electron acceptors (c.f. 5.4.4.3) in order to maximize energy yield (Thauer *et al.* 1977).

All photobacteria possess genes for lipases and can potentially hydrolyze triacylglycerides into glycerol and fatty acid moieties. For import and activation, genes for long-chain fatty acid transporter (*fad*L) and long-chain fatty acid CoA ligase (*fad*D) are needed (DiRusso and Black 1999; Black and DiRusso 2003). Subsequently, aerobic β-oxidation sequentially convert fatty acids to acetyl-CoA in each cycle involving acyl-CoA dehydrogenase (*fad*E), 3-hydroxyacyl-CoA dehydrogenase (*fad*B) and acetyl-CoA acyltransferase (*fad*A) (Kunau *et al.* 1995). Under anoxic conditions, anaerobic β-oxidation of fatty acids is also possible (Mackie *et al.* 1991; Kunau *et al.* 1995). In order to facilitate anaerobic β-oxidation, fadD, fadA and fadB have to be replaced by fadK, fadI and fadJ, respectively (Campbell *et al.* 2003). All genes for these pathways are present in all five genome-sequenced photobacteria. Their ability to degradate the fatty acids via β-oxidation represents a big competitive advantage over e.g. LAB due to the high energy yield.

Carbon fixation in photo- or chemo-lithoautotrophs is facilitated by a modified TCA cycle, called reductive or reverse TCA cycle (Evans *et al.* 1966; Buchanan and Arnon 1990; Huynen *et al.* 1999; Hügler *et al.* 2005). However, in analyzed meat-borne photobacteria, the key enzymes e.g. ATP-citrate lyase, α-ketoglutarate synthase and monomeric fumarate reductase are absent in the genomes. They possess genes for a membrane-bound fumarate reductase for fumarate respiration (Leys *et al.* 1999) though, but not for a monomeric soluble one needed for the reductive TCA cycle (Miura *et al.* 2008). Therefore, all five sequenced photobacteria are presumably not able fix carbon dioxide, which would be highly abundant in high oxygen MAP.

5.4.4.2 Anaplerotic amino acids routes and gluconeogenesis of photobacteria

The anaplerotic routes comprise reactions replenishing key intermediates of the TCA cycle e.g. oxaloactetate, α-ketoglutarate or fumarate. They can occur via carbohydrate precursors e.g. pyruvate or from amino acids via oxidative deamination (Eikmanns 2005). All three species of photobacteria analyzed in this work possess genes for aspartate ammonia-lyase (*asp*A) replenishing fumarate, and alanine dehydrogenase (*ald*) and serine dehydratase (*sda*AB) replenishing oxaloacetate via pyruvate as the precursor. The latter can also be used for fueling the TCA cycle via acetyl-CoA for energy conversion. Both *P. phosphoreum* strains additionally possess glutamate dehydrogenase (*gdh*A) to replenish α-ketoglutarate either directly from the available glutamate pool or via branched-chain / aromatic amino acid transferases (*ilv*E/*tyr*B). Aspartate, serine, alanine and glutamate are all abundant in meat (Holló *et al.* 2001a) and therefore represent relevant substrates for the spoilage bacteria. Additional amino acid metabolic reactions *i.e.* production of biogenic amines are discussed in a subsequent chapter (5.4.4.4).

The phosphoenolpyruvate-pyruvate-oxaloacetate node is known as an important switch point for carbon flux distribution in bacteria (Sauer and Eikmanns 2005). Anaplerotic reactions can be coupled to gluconeogenesis as previously discussed for LAB species (5.4.3.1). Genes coding for phosphoenol-pyruvate carboxykinase (*pck*A) and phosphoenolpyruvate (PEP) synthase (*pps*A) are present in all analyzed photobacteria genomes and enable synthesis of PEP from oxaloacetate and pyruvate, respectively. All analyzed photobacteria genomes also possess the gene coding for a fructose-1,6-bisphosphatase (*fdp*) to sidestep irreversible 6-phosphofructokinase reaction enabling gluconeogenesis of hexoses.

5.4.4.3 Aerobic and anaerobic respiratory chains

During respiration, a proton motif force is generated via translocation of protons or alkalization of the cytosol that lead to a proton gradient across the cell membrane (Haddock and Jones 1977). Subsequently, proton reflux by the F_0F_1-ATPase (*atp*BFE/*atp*ADGHC) activity conserves energy via ATP_{pmf} generation (Nakamoto *et al.* 2000). The ATP yield is drastically higher compared to fermentation. Whereas 2 mol of ATP are generated during fermentation per mol glucose, a maximum of 38 mol of ATP can be generated via respiration (Thauer *et al.* 1977).

In general, the respiratory chain consists of an electron donor, an electron shuttle or carrier and a terminal electron acceptor (Haddock and Jones 1977). The electron donor in the aerobic respiratory chain is either NADH, derived from e.g. carbohydrate catabolism, or fumarate as part of the TCA cycle. Different types of NADH:quinone oxidoreductases exist in bacteria (Steuber *et al.* 2014): (i) Proton-translocating NADH dehydrogenase (NDH) I, homologous to complex I in eukaryotes and encoded by the *nuo* operon with 14 subunits (Weidner *et al.* 1993), (ii) non-electrogenic NDH II, a single subunit enzyme encoded by the gene *ndh* (Young *et al.* 1981) and (iii) sodium translocating NADH:quinone oxidoreductases (Na^+-NQR) (Juárez and Barquera 2012). All five sequenced photobacteria genomes harbor genes for NDH II (*ndh*) and Na^+-NQR (*nqr*ABCDEF). Additionally, both *P. phosphoreum* strains possess the gene cluster (*nuo*A-N) for proton-translocating NADH dehydrogenase class I, which is absent in the genomes of *P. carnosum* strains and *P. iliopiscarium*. The sodium motive force (*smf*) created by translocation of sodium ions via Na^+-NQR is presumably not directly contributing to energy conservation since full sodium cycles mostly occur only in hyperthermophiles and alkaliphiles. However, sodium ions but can be used for energizing symport of substrates instead of protons (Mulkidjanian *et al.* 2008) and therefore contribute indirectly to energy conservation in terms of maintaining the proton gradient. However, the proton-translocating NDH (*nuo*) of *P. phosphoreum* strains could be a potential energetic advantage. All three *Photobacterium* species can potentially also use succinate as the other possible electron donor as part of the TCA cycle. Their genomes harbors all genes of the TCA cycle (5.4.4.1) including cluster *sdh*ABCD encoding the electroneutral succinate dehydrogenase (homologues to complex II in

mitochondria). The subsequent component in the respiratory chain is the electron shuttle. All five sequenced meat-borne photobacteria possess the complete gene cluster *ubi*CADBGHEF necessary for biosynthesis of ubiquinone Q8 (Meganathan 2001). HPLC analysis conducted by the DSMZ identification service also identified ubiquinone Q-8 as the sole respiratory ubiquinone of *P. carnosum* TMW2.2021^T if cultured under aerobic conditions (see species description, 4.4.1), which is in accordance with previous findings for all other *Photobacterium* species (Lo *et al.* 2014). Subsequent electron transport allowing additional proton translocation involves the cytochrome C reductase (*qcr*ABC, homologous to the mitochondrial complex III) (Berks *et al.* 1995), which encoding genes are present in all photobacteria genomes. Ultimately, the electrons are transferred to a heme-dependent terminal electron acceptor via a terminal oxidase (Haddock and Jones 1977). All five sequenced *Photobacterium* spp. possess the gene cluster *cyc*ABC coding for a cytochrome C oxidase that catalyzes the reduction of oxygen to water and generates *pmf* via consuming protons in the cytosol as well as translocating protons into the periplasm (Ostermeier *et al.* 1996). Furthermore, all genes were detected coding for proteins necessary for the biosynthesis of heme. Although it is highly available in beef (Kongkachuichai *et al.* 2002; Cross *et al.* 2012; Ramos *et al.* 2012), meat-borne photobacteria isolated in this work seem independent of an exogenous source of heme.

Under anoxic conditions, or if the aerobic growth is suppressed by high levels of CO_2, meat-borne *P. carnosum*, *P. iliopiscarium* and *P. phosphoreum* strains can predictively also build another type of respiratory chain with a different electron shuttle as well as electron donors/acceptors. Under these conditions, ubiquinone has to be replaced by menaquinone during e.g. fumarate respiration (Unden and Bongaerts 1997) because ubiquinone cannot donate the electrons to e.g. fumarate due to a more positive electron potential (Tielens and Van Hellemond 1998). The complete gene cluster *men*AFDHCEB and gene *ubi*E needed for biosynthesis of menaquinone (Bentley and Meganathan 1982) are present in the genome of all five sequenced photobacteria. Potential electron donors during anaerobic respiration are fermentation-borne NADH, formate or hydrogen. The respective gene clusters *hyd*ABCDE encoding a hydrogen uptake group 1 NiFe-hydrogenase (Vignais and Billoud 2007) and *frd*ABCD encoding a formate dehydrogenase are present in the genomes. While molecular hydrogen should not be present in a MAP and can therefore be disregarded, formate can be formed from pyruvate via PFL and transported into the periplasm via an efflux transporter (*foc*A) as discussed earlier for alternative pyruvate fates. A potential electron acceptor under anoxic conditions is fumarate. Again, all five genomes contain the gene cluster *frd*ABCD needed for respiratory fumarate reductase (Cecchini *et al.* 2002), which is multimeric in contrast to the soluble monomeric fumarate reductase used in the reductive citrate cycle (Miura *et al.* 2008). Fumarate respiration via fumarate reductase as terminal reductase is electroneutral unless coupled to hydrogen or formate oxidation. It is also possible that

succinate dehydrogenase functions as terminal reductase, facilitates the reduction of fumarate and is coupled to generation of *pmf* as previously suggested for *Bacillus subtilis* (Kröger *et al.* 2002). Furthermore, genomes of all three analyzed photobacteria species harbor genes encoding a dissimilatory periplasmatic nitrate reductase (*nap*ABC) and TMAO (trimethylamine *N*-oxide) reductase (*tor*A) that can potentially serve as additional alternative electron acceptors for anaerobic respiration as known for marine *Photobacterium* spp. and *Vibrio* spp. (Proctor and Gunsalus 2000). In contrast to energy-conserving nitrate reductase *nar*G, *nap*A is not electrogenic (Roldán *et al.* 1998; Richardson *et al.* 2001) and in order to conserve energy, nitrate reduction has to be coupled to hydrogen or formate oxidation (Jormakka *et al.* 2003). However, while nitrate is widely available for microorganisms in marine environments (Koike and Hattori 1978), it is absent or present only in very low levels on meats (Iammarino and Di Taranto 2012) and does therefore not represent an alternative electron acceptor of major importance. TMAO is used as an osmolyte by a variety of marine organisms (Yancey *et al.* 1982). Reduction of TMAO results in trimethylamine formation (Barrett and Kwan 1985), a biogenic amine with a strong fishy off-odor, which is important in regard to spoilage of seafood and fish (Gram and Dalgaard 2002). However, on meats, TMAO is not present (Cho *et al.* 2017). It remains to be demonstrated, which respiratory chain is employed by photobacteria under high oxygen MAP. It is known that other strains of *P. phosphoreum* strains are highly resistant to carbon dioxide, though and even growing in 100% carbon dioxide (Dalgaard 1995), which hints to an effective adaptation and switch to anaerobic respiratory mechanisms.

5.4.4.4 Decarboxylation of amino acids

Decarboxylation of amino acids leads to alkalization of the cytosol and therefore contributes to generation of proton motive force (Romano *et al.* 2012). It is known that photobacteria are able to produce biogenic amines on fish products (Jørgensen *et al.* 2000a; Emborg *et al.* 2002). Biogenic amines are highly off-odorous and have high significance to food safety and human health due to their toxicity and mutagenic precursor function. Food poisoning can occur by histamine poisoning and tyramine toxicity, and cadaverine as well as putrescine can cause lockjaw and enhance the toxicity of histamine (Santos 1996; Shalaby 1996).

Genome-sequenced photobacteria of all three species *P. carnosum*, *P. iliopiscarium* and *P. phosphoreum* harbor genes for production of multiple biogenic amines from amino acids. All analyzed photobacteria possess genes for production of putrescine from arginine with agmatine as intermediate via arginine decarboxylase (*spe*A) and agmatine ureohydrolase (*spe*B). *P. carnosum* TMW2.2029 is supposedly also capable to form putrescine from ornithine via ornithine decarboxylase (*spe*F). Ornithine is present in only small quantities in meat (Baldwin *et al.* 1976; Koutsidis *et al.* 2008a), but it can also originate as a by-product from the ADI pathway or via arginase (*arg*) reaction. Additionally, all analyzed photobacteria harbor the

genes for production γ-aminobutyric acid via glutamate decarboxylase (*gad*B). Both *P. phosphoreum* strains also possess the genes for tyrosine and lysine decarboxylase (*tdc*A/*lcd*C) yielding cadaverine and tyramine, respectively. Histamine decarboxylase (*hdc*A) was not found in any photobacteria genome analyzed, although it is known that other strains of *P. phosphoreum* and *P. iliopiscarium* strains can produce histamine and can cause histamine poisoning (Lehane and Olley 2000; Kanki *et al.* 2004; Kanki *et al.* 2007; Takahashi *et al.* 2015). The abundance of multiple amino acid decarboxylases in the genomes of photobacteria predicts the production of unpleasant and harmful to toxic spoilage substances from amino acids present in meat (Holló *et al.* 2001a) and indicates their role as potent meat spoilers.

5.4.5 Role of oxygen for LAB

While LAB are widely known to drive (anaerobic) food fermentations, several LAB are capable of building a minimum respiratory machinery (Lechardeur *et al.* 2011) and show a heme- and menaquinone-stimulated growth (Lan *et al.* 2006; Brooijmans *et al.* 2009). Exogenous heme is required as a cofactor of terminal cytochrome *bd* oxidase, since LAB lack enzymes for heme biosynthesis (Pedersen *et al.* 2012). In accordance, no genes coding for enzymes necessary in heme biosynthesis (*hem*A/L/B/C/D/E/N/F/G/H, (Layer *et al.* 2010)) were present in the analyzed genomes of *Lc. piscium* strains as well as both *Le. gelidum* subsp. However, exogenous heme is highly available in - especially red - meats originating from myoglobin (Kongkachuichai *et al.* 2002; Cross *et al.* 2012; Ramos *et al.* 2012).

Lc. piscium strains TMW2.1612 and TMW2.1615 seem not to be able to form a functional respiratory chain since they do not possess a cytochrome *bd* oxidase and are not capable of menaquinone biosynthesis due to absence of these genes. Also, for other strains of *Lc. piscium* (n=4), it has been reported that exogenous heme does not result in a growth enhancement (Rahkila *et al.* 2012). *Le. gelidum* subsp. *gelidum* TMW2.1618 and *gasicomitatum* TMW2.1.619 on the other hand possess cytochrome *bd* oxidase genes and the full gene cluster necessary for menaquinone biosynthesis and are therefore both potentially capable to build a functioning electron transport chain in the presence of exogenous heme. It has been reported in the literature that other *Le. gelidum* subsp. *gasicomitatum* isolates (n=4) show heme-stimulated aerobic growth, whereas *Le. gelidum* subsp. *gelidum* isolates (n=30) do not (Rahkila *et al.* 2014). Either *Le. gelidum* subsp. *gelidum* TMW2.1618 does not express the genes for menaquinone and cytochrome *bd* oxidase or represents a strain of *Le. gelidum* subsp. *gelidum* that is actually capable of aerobic respiration. For *Le. gelidum* subsp. *gasicomitatum*, it has been further reported that the growth stimulation with exogenous heme is not reduced by the addition of 20% CO_2 in the atmosphere (Johansson *et al.* 2011), as employed in high oxygen MAP, and will therefore likely take place *in situ*. Although a respiratory chain can be established, analyzed genomes of *Le. gelidum* subsp. lack genes for a functional

complete tricarboxylic acid cycle and therefore aerobic respiration will likely not be as efficient. Nevertheless, it allows effective coupling of fermentation with respiration through interconversion of NADH/NAD^+, *i.e.* fermentation-borne NADH can be respiratively converted back to NAD^+ and *vice versa*. An additional oxygen-dependent reaction predicted to be used by *Le. gelidum* subsp. *gasicomitatum* TMW2.1619 is the previously discussed pyruvate oxidase reaction, converting pyruvate to acetyl-CoA yielding hydrogen peroxide. Since no NAD^+ is oxidized in this process, it can enable the acetate shunt and therefore conserve ATP giving a competitive edge to this strain. Furthermore, all analyzed LAB genomes possess a gene putatively encoding a NADH oxidase (*nox*), which could facilitate oxygen-dependent re-oxidation of NAD^+. It remains unclear if this process is possible though, because the gene function could not be clearly assigned.

5.4.6 Predicted spoilage potential of individual SSO

Genome analysis of selected spoilage-associated microbiota enabled deep insights in the predictive lifestyle on meat, allowed assessment of the individual spoilage potential and explained their dominance in this habitat as observed within other experiments in this study.

5.4.6.1 *Le. gelidum* subsp. *gelidum* and *gasicomitatum*

Le. gelidum subsp. were identified as SSO in the growth dynamics experiments that could dominate the microbiota on MAP beef and partly on minced beef if dominant as initial contamination. These organisms were highly abundant when the meat showed clear spoilage signs, *i.e.* green discoloration, sour off-odor and lowered pH. They also caused green discoloration in a self-prepared medium based on minced beef extract. The organisms are known from previously studies for their spoilage potential on MAP beef, poultry, fish and sausages causing greening and sour off-odor (Lyhs *et al.* 2004; Vihavainen and Björkroth 2007b; Vihavainen *et al.* 2008; Vihavainen and Björkroth 2009; Jääskeläinen *et al.* 2012; Rahkila *et al.* 2014). Genomic analysis of *Le. gelidum* subsp. *gelidum* TMW2.1618 and *gasicomitatum* TMW2.1619 allowed to predict the lifestyle on meat and the spoilage-associated reactions. They can predictively feed on all free carbohydrates and free glycerol present in meats, but have very limited ability to utilize proteinaceous substrates and fat. Genome analyses predict that end products of heterolactic fermentation are lactate, carbon dioxide, ethanol, diacetyl/acetoin. *Le. gelidum* subsp. *gasicomitatum* can produce hydrogen peroxide in the oxygen-dependent pyruvate oxidase reaction that also enable the heterolactic acetate shunt. This reaction explains production of the spoilage-relevant compounds acetate and hydrogen peroxide that lead to observed meat greening via choleglobin formation, pH drop and sour off-odor. The establishment of a minimal respiratory machinery is also possible for both subspecies and allows regeneration of fermentation-borne NADH, so that energy-conserving acetate formation can be employed.

5.4.6.2 *P. carnosum, P. iliopiscarium* and *P. phosphoreum*

It is known from the literature that photobacteria are the causative of spoilage on fish products (Dalgaard *et al.* 1997; Ast and Dunlap 2005) but are hitherto highly underestimated in regard to their role in meat spoilage. This study provides evidence that diverse photobacteria are common meat spoilers and present on different types of meats and atmospheres employing a culture-dependent approach. Additional genome analysis revealed their predicted versatile fermentative as well as respiratory metabolic abilities for various carbohydrate, proteinaceous and triacylglyceride-derived substrates abundant on meat, which gives them a competitive advantage in this ecological niche. The genome analysis of different photobacteria showed similar predicted fermentative products including spoilage-relevant acetate. Additionally, hydrogen peroxide production from pyruvate (*P. carnosum*) and aspartate (all species) is predicted. Beside these unpleasant but harmless spoilage substances, all five sequenced photobacteria - including the novel species *P. carnosum* - are predictively able to produce several harmful to toxic biogenic amines from amino acids on different meats as previously only reported for fish products (Lehane and Olley 2000; Kanki *et al.* 2004; Kanki *et al.* 2007; Takahashi *et al.* 2015). Therefore, the genome analysis indicates the potential of photobacteria as potent spoilers in the meat environment.

5.4.6.3 Lactococcus piscium

Growth dynamics experiments as well as competition experiments *in situ* and *in vitro* were conducted in this study and showed that *Lc. piscium* strains are highly competitive on meat, result in a delayed spoilage and are able to substantially inhibit meat spoilers. Genomic analysis predicts that *Lc. piscium* strains employ homolactic fermentation and are mostly limited to utilize glycogen, free carbohydrates and free glycerol present on meat. Predicted alternative pyruvate fates are similar to those of *Le. gelidum* subsp. including acetate. However, no sour off-odor was perceived when *Lc. piscium* was inoculated on beef and pH values were not significantly lowered. In commercial meat simulation medium, the pH was also only slightly lower than the control in contrast to *Le. gelidum* subsp. which caused acidification. This indicates that *Lc. piscium* is likely to produce more pH neutral end products e.g. ethanol, lactate and diacetyl/acetoin. Genome analysis also showed the abundance of a *glp*O gene potentially yielding hydrogen peroxide as a byproduct. However, no meat greening was observed in this study when *Lc. piscium* strains were deliberately inoculated on beef and in a self-prepared simulation medium from minced beef extract. There have been also no reports in the literature that *Lc. piscium* contributes or causes meat greening, although other *Lc. piscium* strains are able produce hydrogen peroxide from glycerol - but not from glucose (Andreevskaya *et al.* 2015). It is therefore hypothesized that *Lc. piscium* strains TMW2.1612 and TMW2.1615 uses preferably the glycerol-3-phosphate dehydrogenase instead of the glycerophosphate oxidase to avoid the oxidative stress to itself caused by hydrogen peroxide

or that the gene is either not expressed or functional. Another possible explanation is that available glycerol levels are low due to the inability of these strains to hydrolyze triacylglycerides. Within the volatilome analysis of TMW2.1615 in this study, only diacetyl/acetoin were enhanced compared to the control, contributing to a sensorial impression of ripeness rather than spoilage. Also, no genes were found in the genomes for decarboxylation of amino acids resulting in formation of biogenic amines and a physiological test on a decarboxylation medium was negative for all amino acids tested. Furthermore, the predicted inability to use ribose as a substrate could maintain ribose levels in meat and therefore even the flavor assumed that it is not taken up for anabolism or utilized by other spoilers. Furthermore, the glutamate produced from aspartate could also contribute to flavor. Taken together, the predicted as well as perceived spoilage potential of *Lc. piscium* strains TMW2.1612 and TMW2.1615 is very limited and these strains could even lead to a ripened and enhanced sensory impression of beef while repressing other spoilers.

6 Summary

The overall aim of this study was to develop different novel strategies to reduce the amount of discarded meat, thus saving resources of carbon dioxide, water, feedstuff and livestock. This study explored the causative psychrotrophic and psychrophilic bacteria for the onset of spoilage on modified atmosphere packaged (MAP) beef steaks and in MAP minced beef. High-resolution monitoring of spoilage-associated microbiota at initial, early, mid and late stage at different storage temperatures enabled improved insights in the spoilage process. MALDI-TOF MS was employed for high-throughput identification of approx. 20,000 isolates and exhibited high discriminatory power and reliability even below species level. While initial microbiota was highly diverse, only a subset, called specific spoilage organisms (SSO), was assertive under the selective conditions on MAP beef. The SSO were identified as the psychrotrophic lactic acid bacteria *Lc. piscium*, *Le. gelidum* subsp. *gelidum* & *gasicomitatum* and *C. divergens* on beef steaks and additionally *B. thermosphacta* and *Pseudomonas* spp. on minced beef. Their prevalence throughout the storage time was greatly influenced by the respective relative abundance at initial stage demonstrating the importance of control of the initial contaminants. At the beginning of perceptible onset of spoilage, the headspace atmospheric composition of the packages and the pH of the meat changed, revealing the potential of these parameters as general spoilage indicators.

Cultivation of organisms was generally carried out at 25 °C using a complex media to enable detection of diverse psychrotrophic organism that are not able to grow at higher temperatures. Additionally, screening for psychrophilic organisms at 4 °C revealed the abundance of a true psychrophilic strain of *Le. gelidum* subsp. *gelidum* TMW2.1998. The demonstrated abundance of psychrotrophs and psychrophiles is the likely causative for hitherto unexplained spoilage cases along the results of routine microbiological analysis, which clearly need to be amended towards the detection of psychrophiles. Another spoilage group, psychrotolerant *Pseudomonas* spp., was highly diverse and assertive in MAP minced beef during all stages of spoilage. They even grew in the absence of residual oxygen in the atmosphere, although these organisms have been described as strict aerobes and sensitive to carbon dioxide. Subsequent experiments showed that *Pseudomonas* spp. isolates are clearly able to grow under anoxic conditions. It can be assumed that an anaerobic lifestyle is a common trait within the genus *Pseudomonas*, which is currently highly underexplored.

Lc. piscium showed to be highly competitive in the meat environment and batches of (minced) beef showed a delayed spoilage if microbiota were dominated by *Lc. piscium*. This study explored intraspecies assertiveness of 15 meat-borne *Lc. piscium* strains and investigated the effect of selected strains on relevant meat spoilers *in vitro*. Intraspecies assertiveness assessment revealed *Lc. piscium* TMW2.1614 as most assertive strain. Co-inoculation of three selected *Lc. piscium* strains (TMW2.1612/14/15) caused substantial

growth reduction of diverse meat spoilers while the extent was strain- and spoiler dependent. Furthermore, the growth behavior selected *Lc. piscium* strains deliberately inoculated on beef revealed prevalence over the endogenous microbiota *in situ*. Volatilome analysis of *Lc. piscium* TMW2.1615 revealed diacetyl/acetoin as sole relevant volatile metabolites and sensorial evaluation demonstrated maintenance of acceptable organoleptics with a ripened sensory impression without undesired alterations. Taken together, selected *Lc. piscium* strains exhibit high potential to be used as bioprotective cultures on beef for competitive exclusion of spoilers in order to extend minimum shelf life and enhance product safety.

Photobacteria have been described as marine deep-sea bacteria, however, some recent culture-independent studies reported presence of uncultured photobacteria on meat. Motivated by these findings, an adapted selective isolation procedure for recovery of photobacteria from food samples was developed in this study in order to probe the role of photobacteria potentially contributing to meat spoilage. The adapted isolation procedure led to discovery of a novel psychrophilic *Photobacterium*, designated *P. carnosum*, which represents the first species of *Photobacterium* without any detected marine background. Furthermore, this study demonstrated highly frequent isolation of multiple photobacteria (psychrophilic *P. carnosum*, and psychrotrophic *P. phosphoreum*, and *P. iliopiscarium*) from different meats (beef, pork, poultry, salmon) as well as different packaging conditions (MAP, VP, air-stored) that were hitherto uncultured. This provides clear evidence that photobacteria are pervasive constituents of the spoilage microbiota of meats and demonstrated that these bacteria are hitherto neglected and underestimated in relevance to their role in meat spoilage.

Genome analysis of selected SSO enabled detailed insights in their predictive lifestyles on meat and demonstrated strain- and species-dependent ability to utilize diverse substrates available on meat. *Le. gelidum* subsp. seem limited to fermentation of free carbohydrates, nucleosides and glycerol and also able to build a minimum respiratory chain. *Lc. piscium* strains seem also mainly limited to glycogen, carbohydrates, deoxy-nucleosides and glycerol fermentation, but can use certain free amino acids to increase the pyruvate pool for higher energy conservation or gluconeogenesis. Photobacteria are predictively able to additionally feed on amino acids and fatty acids and to employ diverse respiratory and fermentative mechanisms. Together with the other experiments, genome analysis allowed an assessment of an individual spoilage potential of SSO. *Le. gelidum* subsp. will predictively spoil meat via harmless but unpleasant hydrogen peroxide and acetate causing greening and sour off-odor. Photobacteria are predicted to be highly potent spoilers and to produce harmful to toxic biogenic amines causing danger to human health and putrid off odor on meats. The presence of two independent mechanisms for respiration may explain their high competitiveness in the presence of CO_2. *Lc. piscium* has very low predicted spoilage potential besides

diacetyl/acetoin production and is suggested to contribute to a ripened sensorial impression rather than being a true spoiler.

Taken together, application of the approaches developed in this study can save millions of tons of carbon dioxide, water, feedstuff and livestock on a global scale, and can lead to an enhanced food safety in regard to human health.

7 Zusammenfassung

Das übergreifende Ziel dieser Arbeit war die Entwicklung von Strategien, die zu einer Verringerung des vermeidbaren Fleischabfalls führen und somit Ressourcen wie Kohlenstoffdioxid, Wasser, Futtermittel und Viehbestand eingespart werden können. In dieser Arbeit wurden die psychrotrophen und psychrophilen Bakterien untersucht, die den Verderb von schutzgasverpackten Rindersteaks und Rinderhackfleisch verursachen. Eine hochauflösende Analyse der Entwicklung der Verderbsmikrobiota in der initialen, frühen, mittleren und späten Phase bei verschiedenen Lagertemperaturen hat einen tiefen Einblick in, sowie verbesserte Erkenntnisse über die Verderbsprozesse ermöglicht. MALDI-TOF MS wurde als Hochdurchsatzmethode für eine schnelle, verlässliche und genaue Identifizierung von ca. 20.000 Isolaten bis unterhalb der Speziesebene verwendet. Während die initiale Kontamination eine hohe Diversität aufwies, konnte sich nur ein Teil der Mikrobiota, sogenannte Spezifische Verderbsorganismen (SSO), erfolgreich unter den selektiven Bedingungen auf schutzgasverpacktem Fleisch durchsetzen. Die dominanten SSO wurden als psychrotrophe Milchsäurebakterien der Spezies *Lc. piscium*, *Le. gelidum* subsp. *gelidum* & *gasicomitatum* und *C. divergens* auf Rindersteaks und zusätzlich *B. thermosphacta* und *Pseudomonas* spp. auf Rinderhackfleisch identifiziert. Die Prävalenz über den gesamten Lagerungszeitraum hinweg war von der jeweiligen relativen Abundanz in der initialen Phase abhängig. Dies zeigt die Wichtigkeit der Kontrolle initialer Kontaminanten auf, um den Verlauf des Fleischverderbs zu bestimmen. Zum Zeitpunkt des Beginns des sinnfälligen Verderbs wurde eine gleichzeitige Veränderung der Atmosphärenzusammensetzung in den Packungen sowie des pH Wertes im Fleisch detektiert, was das Potenzial dieser Parameter als generelle Verderbsindikatoren aufzeigt.

Die Kultivierung von Organismen wurde generell bei 25 °C auf einem Komplexmedium durchgeführt, um die Detektion unterschiedlicher psychrotropher Bakterien zu ermöglichen, welche nicht bei höheren Temperaturen wachsen können. Ein zusätzliches Screening auf psychrophile Organismen bei 4 °C führte zur Entdeckung eines psychrophilen Stamms von *Le. gelidum* subsp. *gelidum* TMW2.1998. Die in dieser Arbeit gezeigte hohe Abundanz psychrotropher und psychrophiler Bakterien ist die vermutliche Ursache von Fällen des Fleischverderbs, die bisher mit mikrobiologischen Routineanalysen nicht erklärt werden konnten. Dies zeigt deutlich, dass diese Richtlinien gegenüber der Detektion von psychrophilen Bakterien überarbeitet und verbessert werden müssen. Eine andere Gruppe von Verderbsorganismen sind psychrotolerante Pseudomonaden, welche hoch divers und durchsetzungsfähig in schutzgasverpacktem Rinderhackfleisch während des gesamten Verderbsverlaufs waren. *Pseudomonas* spp. wurden selbst dann isoliert, als kein Restsauerstoff mehr in der Atmosphäre verfügbar war, obwohl diese Organismen als strikt aerob beschrieben wurden und sensitiv auf Kohlenstoffdioxid reagieren. Weitere Experimente

konnten zeigen, dass verschiedene *Pseudomonas* spp. eindeutig dazu fähig sind, unter anoxischen Verhältnissen zu wachsen. Damit kann davon ausgegangen werden, dass diese Fähigkeit zur anaeroben Lebensweise eine weitverbreitete Eigenschaft im Genus *Pseudomonas* ist, welche bisher noch kaum erforscht ist.

Lc. piscium Stämme erwiesen sich als stark konkurrenzfähig im Fleischsystem. Außerdem zeigten Fleischchargen einen verzögerten Verderb wenn *Lc. piscium* die Mikrobiota dominierte. In dieser Arbeit wurde die Durchsetzungsfähigkeit innerhalb der Spezies von 15 *Lc. piscium* Stämmen *in vitro* untersucht und die Wechselwirkung von ausgesuchten Stämmen auf andere relevante Fleischverderbsorganismen. Die Analysen zeigten, dass der *Lc. piscium* Stamm TMW2.1614 die höchste Durchsetzungsfähigkeit besaß. Co-Inokulation mit drei ausgewählten *Lc. piscium* Stämmen (TMW2.1612/14/15) verursachte eine erhebliche Verringerung des Wachstums unterschiedlicher fleischverderbender Bakterien. Dabei war die Stärke der Inhibition abhängig von dem jeweiligen Verderber und *Lc. piscium* Stamm. Außerdem wurde das Wachstumsverhalten von *Lc. piscium* Stämmen untersucht, indem Rindfleisch bewusst mit Einzelstämmen beimpft wurde. Diese Analyse zeigte, dass *Lc. piscium* Stämme auch *in situ* eine Prävalenz gegenüber der endogenen Verderbs-Mikrobiota besitzen. Eine zusätzliche Analyse des Volatiloms von *Lc. piscium* TMW2.1615 zeigte, dass Diacetyl und Acetoin die einzigen verderbsrelevanten volatilen Metaboliten sind. Eine gleichzeitige sensorische Beurteilung von angeimpften Fleischstücken bestätigte die Aufrechterhaltung von akzeptablen organoleptischen Eigenschaften und einen gereiften sensorischen Eindruck. Aus den Ergebnissen lässt sich schlussfolgern, dass ausgewählte *Lc. piscium* Stämme ein hohes Potential besitzen, um im Konkurrenz-Ausschluss Prinzip als Schutzkultur auf Fleisch gegen verderbsverursachende Bakterien eingesetzt zu werden. Dadurch kann das Haltbarkeits- bzw. Verzehrdatum verlängert und eine erhöhte Produktsicherheit gewährleistet werden.

Obwohl Photobakterien als Tiefseebakterien beschrieben wurden, haben jüngste kultivierungsunabhängige Studien vom Vorkommen nicht-kultivierter Photobakterien auf Fleisch berichtet. Motiviert durch diese Erkenntnis, wurde in dieser Arbeit ein adaptiertes, selektives Isolationsverfahren entwickelt, um Photobakterien von Fleisch zu isolieren und den potentiellen Beitrag dieser Bakterien zum Fleischverderb zu erforschen. Das adaptierte Isolationsverfahren führte zur Entdeckung einer neuen psychrophilen *Photobacterium* Art, welche *P. carnosum* benannt wurde, und die erste Photobakterien Spezies ohne detektierbare Verbindung zu Meereshabitaten repräsentiert. Darüber hinaus konnten in dieser Arbeit mehrere Photobakterien Spezies (psychrophile *P. carnosum* sowie psychrotrophe *P. phosphoreum* und *P. iliopiscarium*) von unterschiedlichsten Fleischsorten (Rindfleisch, Schweinefleisch, Hühnerfleisch und Lachs) und unterschiedlichen Verpackungstypen (schutzgasverpackt, vakuumverpackt, unverpackt) isoliert werden, die bisher nicht kultiviert wurden. Diese Arbeit beweist, dass Photobakterien pervasive Bestandsteile der

Verderbsmikrobiota auf Fleisch repräsentieren. Diese Organismengruppe wurde bisher vernachlässigt und unterschätzt, was deren Beitrag am Verderb von Fleisch betrifft.

Eine Genomanalyse von ausgewählten SSO ermöglichte darüber hinaus ein detailliertes Verständnis über die prädiktive Lebensweise auf schutzgasverpacktem Fleisch and zeigte eine spezies- und stammabhängige Fähigkeit zur Benutzung der vielfältigen Substrate, welche im Fleisch verfügbar sind. Subspezies von *Le. gelidum* scheinen auf die Fermentation von frei verfügbaren Kohlenhydraten, Nukleosiden und Glyzerin beschränkt zu sein und zusätzlich fähig, eine minimale Atmungskette ausbilden. Stämme von *Lc. piscium* scheinen ebenfalls hauptsächlich auf die Fermentation von Glykogen, frei zugänglichen Kohlenhydraten, Deoxy-Nukleosiden und Glyzerin beschränkt zu sein. Allerdings können diese Stämme voraussichtlich auch bestimmte freie Aminosäuren nutzen, um damit den Pyruvatpool zu füllen und diesen für eine höhere Energiegewinnung oder die Glukoneogenese verwenden. Photobakterien sind voraussichtlich zusätzlich dazu fähig, sich von unterschiedlichen Aminosäuren und Fettsäuren zu ernähren und unterschiedliche respiratorische sowie fermentative Mechanismen zu verwenden.

Verknüpft mit den anderen Experimenten erlaubte die Genomanalyse außerdem eine Einschätzung des individuellen Verderbpotenzials der jeweiligen SSO. Subspezies von *Le. gelidum* werden voraussichtlich den Fleischverderb durch harmlose aber unangenehme Substanzen wie Wasserstoffperoxid und Acetat verursachen, welche eine grünliche Verfärbung und sauren Fehlgeruch auslösen. Photobakterien sind voraussichtlich äußerst potente Verderber, die zusätzlich schädliche bis giftige biogene Amine produzieren, die Vergiftungen beim Menschen und Verwesungsgeruch auf Fleisch auslösen können. Das Vorhandensein von zwei voneinander unabhängigen respiratorischen Mechanismen könnte die hohe Wettbewerbsfähigkeit in der Anwesenheit von Kohlenstoffdioxid erklären. *Lc. piscium* besitzt ein sehr niedriges Verderbspotenzial abgesehen von der Produktion von Diacetyl und Actoin und trägt damit eher zu einer gereiften sensorischen Impression bei, als ein echter Fleischverderber zu sein.

Zusammengenommen könnte die Anwendung der in dieser Arbeit entwickelten Konzepte Millionen Tonnen Kohlenstoffdioxid, Wasser, Futtermittel und Viehbestand global einsparen und die Lebensmittelsicherheit für die Verbraucher erhöhen.

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9 Appendix

Table A1| Statistical evaluation of growth reduction of spoiler in co-inoculation with *Lc. piscium* strains. Displayed are CFU values of the replicates.

***Ps. weihenstephanensis* TMW2.1728**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
8.63	7.62	8.21	8.54
8.79	6.78	8.38	8.57
8.72	7.51	7.95	7.38
***t*-TEST**	0.00629	0.0161	**0.234**
Shapiro-Wilk-TEST	0.506	0.968	0.223
***S. liquefaciens* TMW2.1905**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
4.83	3.53	3.21	3.70
4.81	3.56	3.19	3.56
4.82	3.46	3.25	3.71
***t*-TEST**	0.00000182	0.00	0.00
Shapiro-Wilk-TEST	0.714	0.981	0.397
***H. alvei* TMW2.1906**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
5.13	3.64	3.28	3.75
5.29	3.54	2.96	3.59
5.24	3.78	2.97	3.54
***t* -TEST**	0.00005	0.0000473	0.0000369
Shapiro-Wilk-TEST	0.803	0.214	0.54
***B. thermosphacta* TMW2.1906**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
7.78	4.04	4.34	4.96
7.53	4.27	4.35	5.03
7.92	4.11	4.53	4.95
***t* -TEST**	0.0000113	0.0000134	0.0000193
Shapiro-Wilk-TEST	0.929	0.889	0.71
***Le. gelidum* subsp. *gasicomitatum* TMW2.1619**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
7.34	5.93	5.88	4.38
7.19	5.66	5.81	4.34
7.23	5.94	5.93	4.00
***t* -TEST**	0.00	0.00	1.98E-05
Shapiro-Wilk-TEST	0.187	0.473	0.391
***C. divergens* TMW2.1907**			
	Co-inoculation with *Lc. piscium*		
Single	**TMW2.1612**	**TMW2.1614**	**TMW2.1615**
5.21	3.89	3.31	3.43
5.09	3.87	3.27	3.42
5.18	3.90	3.29	3.44
***t* -TEST**	0.0000051	0.0000011	0.0000014
Shapiro-Wilk-TEST	0.68	0.684	0.601

Table A2| Development of pH values during competition experiments of *Lc. piscium* strains and respective SSO.

Strain[a]	Day 0		Day 7		Difference	
	pH	SE	pH	SE	ΔpH	ΔSE
LP (TMW2.1614)	5.77	0.003	5.38	0.000	0.39	0.003
LP + BT	5.73	0.007	5.33	0.003	0.40	0.010
LP + PS	5.71	0.003	5.58	0.000	0.13	0.003
LP (TMW2.1614)	5.80	0.003	5.46	0.003	0.35	0.007
LP + SL	5.78	0.000	5.42	0.012	0.36	0.012
LP + HA	5.78	0.006	5.39	0.006	0.39	0.012
LP (TMW2.1614)	5.72	0.000	5.37	0.000	0.35	0.000
LP + CA	5.72	0.000	5.36	0.003	0.36	0.003
LP (TMW2.1614)	5.73	0.003	5.35	0.006	0.38	0.009
LP + LGA	5.74	0.000	5.33	0.003	0.41	0.003
LP (TMW2.1612)	5.76	0.003	5.37	0.003	0.39	0.007
LP + BT	5.71	0.000	5.40	0.003	0.31	0.003
LP + PS	5.69	0.003	5.45	0.003	0.25	0.007
LP (TMW2.1612)	5.69	0.003	5.35	0.006	0.34	0.009
LP + SL	5.70	0.006	5.35	0.003	0.35	0.009
LP + HA	5.70	0.003	5.27	0.006	0.43	0.009
LP (TMW2.1612)	5.72	0.003	5.31	0.006	0.41	0.009
LP + CA	5.73	0.000	5.34	0.009	0.39	0.009
LP (TMW2.1612)	5.74	0.000	5.41	0.003	0.33	0.003
LP + LGA	5.74	0.003	5.34	0.003	0.39	0.007
LP (TMW2.1615)	5.76	0.003	5.34	0.003	0.42	0.007
LP + BT	5.72	0.006	5.33	0.003	0.39	0.009
LP + PS	5.70	0.006	5.39	0.052	0.31	0.058
LP (TMW2.1615)	5.80	0.003	5.31	0.003	0.49	0.007
LP + SL	5.78	0.003	5.29	0.006	0.49	0.009
LP + HA	5.80	0.000	5.29	0.006	0.51	0.006
LP (TMW2.1615)	5.72	0.000	5.29	0.003	0.43	0.003
LP + CA	5.72	0.003	5.29	0.006	0.43	0.009
LP (TMW2.1615)	5.73	0.000	5.31	0.006	0.42	0.006
LP + LGA	5.73	0.000	5.27	0.006	0.46	0.006

[a]LP, *Lc. piscium*; HA, *H. alvei*; PS, *Ps. weihenstephanensis*; SL, *S. liquefaciens*; BR, *B. thermosphacta*; LE, *Le. gelidum* subsp. *gasicomitatum*; CA, *C. divergens*.

[b]SE, standard error

Table A3| Antibiotic susceptibility of photobacteria and other meat spoiling organisms.

Strain	Strips[1]		Discs[2]									
	TC	CL	TC 30	CL 30	PG-10	AMP 10	LZD 30	OX 1	E 15	VA 30	CN 10	TEC 30
P. aquimaris TMW2.1991	1.5	0.125	n.d.	n.d.	0.75	0.7	2	-	0.65	-	0.75	-
P. iliopiscarium TMW2.1992	3	0.38	n.d.	n.d.	0.7	n.d.	1.8	-	0.6	-	1	-
P. phosphoreum TMW2.1993	6	0.5	n.d.	n.d.	0.9	n.d.	1.7	-	0.7	-	1	-
P. angustum TMW2.1994	24	0.5	n.d.	n.d.	-	-	1.55	-	0.85	-	0.5	-
P. kishitanii TMW2.1995	16	0.38	n.d.	n.d.	-	-	1.7	-	0.75	-	1.15	-
P. profundum TMW2.1996	12	0.75	n.d.	n.d.	0.9	n.d.	1.1	-	-	-	0.7	-
P. leiognathi TMW2.1997	3	0.38	n.d.	n.d.	-	-	1.4	-	0.6	-	1	-
B. thermosphacta TMW2.1906	n.d.	n.d.	1.2	1	1.2	0.7	n.d.	-	0.9	1	1	n.d.
Ps. weihenst. TMW2.1728	n.d.	n.d.	0.7	0.5	-	-	n.d.	-	0.5	-	1	-
S. liquefaciens TMW2.1905	n.d.	n.d.	0.4	0.5	-	-	n.d.	-	0.1	-	0.7	-
H. alvei TMW2.1904	n.d.	n.d.	0.5	0.4	-	-	n.d.	-	-	-	0.6	-
C. divergens TMW2.1907	n.d.	n.d.	-	1	-	n.d.	n.d.	-	1.3	1	0.5	n.d.
Le. gel gasico TMW2.1619	n.d.	n.d.	0.9	1.2	1.5	n.d.	n.d.	-	1.6	-	1.4	n.d.
Lc. piscium TMW2.1615	n.d.	n.d.	1.9	1.1	1.4	n.d.	n.d.	1	0.8	1.3	1	n.d.

TC, Tetracycline C; CL, Chloramphenicol; PG, Penicillin G; AMP, Ampicillin; LZD, Linezolid; OX, oxacillin;
E, Erythromycin; VA, Vancomycin; CN, Gentamicin; TEC, Teicoplanin
[1](0.016-256 µg/ml)
[2]µg

Table A4| Growth of photobacteria type strains and meat spoiling bacteria on different media.

	Strain	Medium															
		MB	ME	BHI	MEVA	TCBS	PBVA	MB + NaCl (%)								LB + NaCl	
								3.4	4.4	5.4	6.4	8.4	10	10.4	20	10	20
Photobacteria	*P. aquimaris* TMW2.1991	+	++	-	++	-	-	++	++	+	-	-	-	-	-	-	-
	P. iliopiscarium TMW2.1992	+	++	-	++	-	w	+	+	-	-	-	-	-	-	-	-
	P. phosphoreum TMW2.1993	+	++	-	++	-	w	++	+	-	-	-	-	-	-	-	-
	P. angustum TMW2.1994	++	++	+	++	w	-	++	++	+	+	-	-	-	-	-	-
	P. kishitanii TMW2.1995	++	++	++	++	w	-	++	++	+	+	-	-	-	-	-	-
	P. profundum TMW2.1996	+	++	-	++	w	-	++	+	-	-	-	-	-	-	-	-
	P. leiognathi TMW2.1997	++	++	++	++	-	-	++	+	+	+	-	-	-	-	-	-
Meat spoilers	*B. thermosphacta* 2.1906	+	++	++	-	-	-	++	++	++	+	w	-	-	-	-	-
	Ps. weihenst. TMW2.1728	+	++	++	++	w	w	+	+	+	+	-	-	-	-	-	-
	S. liquefaciens TMW2.1905	+	++	++	+	+	++	++	++	+	+	w	-	-	-	-	-
	H. alvei TMW2.1904	+	++	++	++	+	++	++	+	+	+	-	-	-	-	-	-
	C. divergens TMW2.1907	+	+	++	-	-	-	+	+	+	+	-	-	-	-	-	-
	Le. gel gasico TMW2.1619	-	+	++	-	-	-	w	-	-	-	-	-	-	-	-	-
	Lc. piscium TMW2.1615	-	+	++	-	-	-	w	w	-	-	-	-	-	-	-	-

Table A5| Locus tags of genes used for creation of predictive metabolic pathways.

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
Nucleoside and ribose metabolism									
ribopyranase (*rbs*D)	-	-	08770	p_9470	13550	06860	06460	00905	16990
ribose Transporter (*rbs*U)	-	-	-	p_9465	-	-	-	-	-
putative ribose/nucleoside transporter (*nup*A/*yng*F)	07670	08485	00405	00375	04630	-	08995	07645	05535
putative ribose/nucleoside transporter (*nup*A/*yng*F)	07675	08490	00410	00380	04635		09000	07650	05540
ribokinase (*rbs*K)	04630	-	03430	03380	13555	06865	06455	00890	16975
			08750	09055	13570	06880	06440		
				p_9475					
inosine-uridine nucleoside ribohydrolase (*iun*H)	-	-	01730	01715	-	-	-	-	-
			06670	06725					
			08790	09075					
ribonucleoside nucleosidase (unspecific, *rih*C)			08785	09070	04035	15920	04725	20525	00210
purine (deoxy)nucleoside phosphorylase (*deo*D)	04020	04240	-	-	07190	10275	13425	11940	07245
pyrimidine (deoxy)nucleoside phosphorylase (*deo*A)	03655	03915	-	-	07200	10265	13415	11930	07255
ribose 1,5-phosphopentomutase (*deo*B)	04015	04235	-	-	07195	10270	13420	11935	07250
ribonucleotide reductase alpha/assembly/beta (*nrd*A/*nrd*I/*nrd*B)	04935	05275	01180	01160	01015	02865	01395	04095	05760
ribonucleotide reductase alpha/assembly/beta (*nrd*A/*nrd*I/*nrd*B)			01185	01165					
deoxyribose-phosphate aldolase (*deo*C)	04930	05270	11890	01170	01020	02870	01400	04100	05760
	03660	03920	-	-	07205	10260	13410	11925	07260
Pentose phosphate Pathway	-	-	-	-	+	+	+	+	+
6-phospho-gluconolactonase (*dev*B)	05280	05570	04955	05015	16850	15305	07790	06045	17470
phosphogluconate dehydrogenase (*gnt*Z)	01460	09475	04425	04495	16855	15300	07785	06050	17475
ribulose-5-phosphate 3-epimerase (*rpe)*	07625	08435	02900	02840	06320	11125	10730	12855	03395
ribose 5-phosphate isomerase (*rpi*A)	01260	09300	03165	03110	17885	14275	18915	18660	09685
	01790	09620	07630	07915					
			08745	09050					
				p_9480					
transketolase (*tkt*A)	4700/5	06445	03740	03705	16330	06105	03420	01985	13115
	06035						21305	20330	
transaldolase (*tal*)	-	-	-	-	16325	06110	03415	01990	13110
Glycolysis									
glucokinase (*glc*K)	04055	04265	05260	05335	04150	08040	04610	18835	00325
phosphoglucomutase (*pgm*)	07655	08465	05705	05690	01655	03515	02050	04765	08105

Strain	***Lc. piscium* TMW2.1612**	***Lc. piscium* TMW2.1615**	***Le. gelidum* subsp. *gelidum* TMW2.1618**	***Le. gelidum* subsp. *gasicomitatum* TMW2.1619**	***P. carnosum* TMW2.2021**	***P. carnosum* TMW2.2029**	***P. phosphoreum* TMW2.2033**	***P. phosphoreum* TMW2.2034**	***P. iliopiscarium* TMW2.2035**
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
					05715				
glucose-6-phosphate isomerase (*pgi*)	**02710**	**02855**	**01560**	**01545**	**18350**	**16090**	**17300**	**19890**	**13935**
mannose-6-P isomerase (*man*A)	**07725**	**08525**	**06925**	**07165**	**13205**	**04110**	**13915**	**16435**	**11630**
Homolactic fermentation	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**+**	**+**
6-Phosphofructokinase (*pfk*A)	**04780**	**05055**	**-**	**-**	**06500**	**10945**	**10925**	**12660**	**03205**
fructose-1,6-bisphosphate aldolase (*fba*A)	**02475**	**02630**	**07660**	**07945**	**17905**	**14295**	**18895**	**18680**	**09705**
Heterolactic fermentation	**-**	**-**	**+**	**+**	**-**	**-**	**-**	**-**	**-**
phosphoketolase (*xpk*A)	**-**	**-**	**07495**	**07745**	**-**	**-**	**-**	**-**	**-**
glucose-6-phosphate dehydrogenase (*zwf*)	**07565**	**08380**	**08135**	**08375**	**16845**	**15310**	**07795**	**06040**	**17465**
KDPG weg	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
phosphogluconate dehydratase (*edd*)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
KDPG aldolase (*eda*)	**07230**	**-**	**-**	**-**	**13585**	**06895**	**06390**	**00840**	**16955**
Gluconeogenese									
pyruvate carboxylase (*pyc*)	**02470**	**02625**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
phosphoenolpyruvate carboxykinase (*pck*A)	**02470**	**02625**	**06470**	**06530**	**14600**	**13705**	**17545**	**15745**	**08850**
	04635	**04935**			**12515**	**13055**		**14630**	**16455**
phosphoenolpyruvate synthase (*pps*A)									
fructose-1,6-bisphosphatase (*fdp*)	**04635**	**04935**	**-**	**-**	**06480**	**10965**	**10895**	**12690**	**03230**
PEP synthase (*pps*A)	**-**	**-**	**-**	**-**	**00060**	**01905**	**00060**	**13875**	**06700**
PEPCase (*ppc*)	**-**	**-**	**06470**	**06530**	**06410**	**11035**	**10820**	**12765**	**03305**
L-Lactate dehydrogenase (*ldh*)	**04265**	**04515**			**10805**	**06150**	**03405**	**02000**	**13075**
D-Lactate dehydrogenase (*ldh*)			**00365**	**00325**					
			02215	**02180**					
			08445	**08765**					
Pyruvate dehydrogenase complex									
pyruvate dehydrogenase alpha E1 *pdh*A / *ace*E (homodimeric)	**06875**	**07450**	**02405**	**02370**	**08990**	**11795**	**15060**	**17070**	**04100**
pyruvate dehydrogenase beta E1 (*pdh*B)	**06870**	**07445**	**02410**	**02375**					
dihydrolipoamide acetyltransferase E2 (*pdh*C)	**06865**	**07440**	**02415**	**02380**	**08995**	**11800**	**15055**	**17065**	**04105**
dihydrolipoyl-dehydrogenase E3 (*pdh*D)	**06860**	**07435**	**02420**	**02385**	**09000**	**11805**	**15050**	**17060**	**04110**
Acetate									
phosphotransacetylase (*pta*)	**06120**	**06490**	**03060**	**03005**	**13060**	**03970**	**13760**	**16585**	**11790**
pyruvate oxidase (*pox*B)	**-**	**-**	**-**	**04365**	**07965**	**00970**	**-**	**-**	**-**
acetatekinase (*ack*A)	**02100**	**02245**	**01605**	**03850**	**13065**	**03975**	**13765**	**16580**	**11785**

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
			03890	**01590**					
Ethanol									
acetaldehyde dehydrogenase / Alcohol dehydrogenase (*adh*E)	**00605**	**10275**	**07945**	**08200**	**01380**	**03240**	**01770**	**04485**	**08380**
Ethanol/Acetate									
Pyruvate formate lyase (p*fl*B)	**02445**	**02600**	**-**	**-**	**13125**	**04035**	**13835**	**16515**	**11710**
formate efflux transporter / formate-nitrite transporter (*foc*A)	**01265**	**09615**	**-**	**-**	**13130**	**04040**	**13840**	**16510**	**11705**
Butanoate metabolism									
Acetolactate									
acetolactate synthase (*alsS*)	**05375**	**05625**	**06300**	**06360**	**10875**	**17260**	**07690**	**06140**	**13490**
	9260/65	**1305/10**							
Acetoin									
acetolactate decarboxylase (*ald*C)	**05310**	**05600**	**02580**	**02545**	**10870**	**17255**	**07695**	**06135**	**13495**
diacetyl reductase (Acetoin reductase)	**+**	**+**	**+**	**+**	**-**	**-**	**-**	**-**	**-**
*bud*C / *but*A / *bdh*A									
Butane-2,3-diol									
acetoin reductase (Butandiol dehydrogenase) *bud*C / *but*A / *bdh*A	**09150**	**01425**	**07955**	**08255**	**-**	**-**	**-**	**-**	**-**
Glycerol metabolism									
lipase	**-**	**-**	**-**	**-**	**08405**	**00495**	**07010**	**11165**	**04665**
putative esterase/lipase domain protein	**06985**	**07550**	**04435**	**04505**					
	07760	**08560**	**08280**	**08525**					
glycerol transporter	**-**	**-**	**03105**	**00195**	**-**	**-**	**-**	**-**	**-**
				03050					
glycerol uptake facilitator protein (*glp*F)	**02055**	**02200**	**-**	**-**	**-**	-	-	-	-
	05255	**05545**	**-**	**-**	**-**	-	-	-	-
putative glycerol uptake protein	**-**	**-**	**-**	**-**	**06470**	**10975**	**10885**	**12700**	**03240**
glycerol kinase (*glp*K)	**05265**	**05555**	**-**	**-**	**16590**	**05840**	**03700**	**01730**	**14520**
alpha-glycerophosphate oxidase (*glp*O)	**05260**	**05550**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
glycerol-3-phosphate dehydrogenase (*gps*A / *glp*D)	**04760**	**05035**	**05735**	**05720**	**06850**	**10595**	**11280**	**12305**	**02855**
Glykogen									
glycogen phosphorylase (*glg*P)	**07685**	**08495**	**-**	**-**	**18180**	**09125**	**-**	**-**	**05160**
glycogen debranching enzyme (*glg*X)	**-**	**-**	**-**	**-**	**18200**	**09145**	**-**	**-**	**05180**
UTP-glucose-1-phosphate uridylyltransferase (*gta*B)	**04755**	**05030**	**05730**	**05715**	**13355**	**04290**	**15530**	**09515**	**11130**
					08525	**11330**	**14130**	**18320**	**03600**

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
glycogen synthase (*glg*A)	**07690**	**08500**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Glycerol/Lactate Co-Fermentation									
glycerol dehydratase (*dha*B)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
1,3propanediol dehydrogenase (*dha*T)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Reoxidizing NADH (O_2)									
putative NADH oxidase (*nox*)	**02795**	**02960**	**04450**	**04520**	**17990**	**08950**	**11575**	**07070**	**04990**
	03945				**00715**	**02565**	**01105**	**03800**	**06065**
Respiration									
cytochrome bd oxidase	**-**	**-**	**+**	**+**	**-**	**-**	**-**	**-**	**-**
subunit I *cyd*A	**-**	**-**	**02640**	**02605**	**01320**	**03180**	**02455**	**05125**	**08445**
subunit II *cyd*B	**-**	**-**	**02645**	**02610**	**01315**	**03175**	**02460**	**05130**	**08450**
subunit IV *cyd*D			**02650**	**02615**					
subunit III *cyd*C			**02655**	**02620**					
NADH-quinone reductase (*nqr*ABCDEF)	**-**	**-**	**-**	**-**	**2500-20**	**12440-60**	**18705-30**	**8740-60**	**15100-25**
NADH:ubiquinone oxidoreductase (*ndh*)	**03945**	**04145**	**01115**	**01095**	**10855**	**17240**	**07710**	**06120**	**13510**
					00715	**02565**	**01105**	**03800**	**06065**
NADH-ubiquinone oxidoreductase (*nuo*ABCDEFGHIJKLMN)	**-**	**-**	**-**	**-**	**-**	**-**	**05140-05080**	**15315-15255**	**-**
menaquinone biosynthesis	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
1,4-dihydroxy-2-naphthoate prenyltransferase (*men*A)	**01825**	**09265**	**06815**	**07110**	**06460**	**10985**	**10875**	**12710**	**03255**
Isochorismate synthase (*men*F)	**-**	**-**	**04520**	**04590**	**09290**	**04465**	**20235**	**09715**	**10960**
2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase (*men*D)	**-**	**-**	**04515**	**04585**	**09295**	**04470**	**20240**	**09720**	**10955**
2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase (*men*H)	**-**	**-**	**04510**	**04580**	**09300**	**04475**	**20245**	**09725**	**10950**
o-succinylbenzoate synthase (*men*C)	**-**	**-**	**04505**	**04575**	**09305**	**04480**	**20250**	**09730**	**10945**
2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (*ubi*E)	**-**	**-**	**00270**	**00215**	**14350**	**13960**	**17795**	**15995**	**08600**
O-succinylbenzoate-CoA ligase (*men*E)	**-**	**-**	**00265**	**00210**	**09310**	**04485**	**20255**	**09735**	**10940**
1,4-dihydroxy-2-naphthoyl-CoA synthase (*men*B)	**-**	**-**	**00095**	**00095**	**10080**	**01500**	**14650**	**14230**	**01280**
cytochrome c oxidase (*cyc*ABC)									
subunit I	**-**	**-**	**-**	**-**	**06815**	**10630**	**11245**	**12340**	**02890**
subunit II	**-**	**-**	**-**	**-**	**06820**	**10625**	**11250**	**12335**	**02885**
subunit III	**-**	**-**	**-**	**-**	**06805**	**10640**	**11235**	**12350**	**02900**
Succinate dehydrogenase (*sdh*ABCD)	**-**	**-**	**-**	**-**	**1615-630**	**3475-90**	**2005-20**	**4720-35**	**8145-30**

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
cytochrome C	-	-	-	-	02390	12330	18840	08630	16815
cytochrome bc complex (*qcr*ABC)	-	-	-	-	8795-805	11600-11610	15260-50	17265-55	3875-85
fumarate reductase (*frd*ABCD)	-	-	-	-	17205-190	15755-40	17115-100	17755-70	15900-15
F_0F_1-ATP synthase (*atp*ADGHC/*atp*BFE)	6640-75	7235-70	690-725	670-705	13470-505	6780-815	4335-70	17470-505	6600-35
Quinone Q-8 biosynthesis									
Chorismate pyruvate lyase (*ubi*C)	-	-	-	-	06840	10605	11270	12315	02865
4-hydroxybenzoate octaprenyltransferase (*ubi*A)	-	-	-	-	06835	10610	11265	12320	02870
3-octaprenyl-4-hydroxybenzoate carboxy-lyase (*ubi*D)	-	-	-	-	19170	17160	20465	20155	14670
2-octaprenylphenol 6-hydroxylase (*ubi*D)	-	-	-	-	14360	13950	17785	15985	08610
3-demethylubiquinol 3-O-methyltransferase (*ubi*G)	-	-	-	-	01010	02860	01390	04090	05765
2-octaprenyl-6-methoxyphenyl hydroxylase (*ubi*H)	-	-	-	-	17860	14250	18940	18635	09660
2-methoxy-6-octaprenyl-1,4-benzoquinol methylase (*ubi*E)	-	-	-	-	14350	13960	17795	15995	08600
2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase (*ubi*F)	-	-	-	-	14525	13785	17795	15995	08600
Heme biosynthesis	-	-	-	-	+	+	+	+	+
glutamyl-tRNA reductase (*hem*A)	-	-	-	-	20405	03765	13575	16785	11960
glutamate-1-semialdehyde-2,1-aminomutase (*hem*L)	-	-	-	-	07640	09825	12950	11465	14370
aminolevulinic acid dehydratase (*hem*B)	-	-	-	-	05635	07615	17760	15575	02485
					14385	13925		15960	08635
hydroxymethylbilane synthase (*hem*C)	-	-	-	-	14420	13890	17725	15925	08670
uroporphyrinogen-III synthase (*hem*D)	-	-	-	-	14415	13895	17730	15930	08665
uroporphyrinogen decarboxylase (*hem*E)	-	-	-	-	17010	15565	16910	17960	16685
coproporphyrinogen III oxidase (oxygen independent) (*hem*N)	-	-	-	-	14540	13770	17605	15805	08790
coproporphyrinogen III oxidase (oxygen dependent) (*hem*F)	-	-	-	-	15950	14880	20910	17325	11260
Protoporphyrinogen IX dehydrogenase (protoporphyrin oxidase)	-	-	-	-	16310	15240	20090	17685	11620
Ferrochelatase (*hem*H)	-	-	-	-	01720	03580	02125	04840	15555
Citrate Cycle	-	-	-	-	+	+	+	+	+
Citrate Synthase (*cit*A)	04425	04750	-	-	01635	03495	02030	04745	08125
Aconitate hydratase (*cit*B)	04430	04755	-	-	09010	11815	15045	17055	04120

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
Isocitrate Dehydrogenase (*icd*A)	**04435**	**04760**	**-**	**-**	**09680**	**04850**	**16715**	**10175**	**12490**
Oxoglutarate dehydrogenase (*suc*AB)	**-**	**-**	**-**	**-**	**1605/10**	**3465/70**	**1995/2000**	**4710/15**	**8150/55**
Succinyl-CoA-Synthetase (*suc*CD)	**-**	**-**	**-**	**-**	**1595/1600**	**3455/60**	**1985/90**	**4700/05**	**8165/70**
Succinate dehydrogenase (complex II) (*sdh*ABCD)	**-**	**-**	**-**	**-**	**1615-30**	**3475-90**	**2005-20**	**4720-35**	**8145-30**
Fumarate hydratase (Fumarase) (*fum*A)	**-**	**-**	**-**	**-**	**00770**	**02620**	**01145**	**03840**	**06010**
Malate dehydrogenase (*mdh*)	**-**	**-**	**-**	**-**	**12535**	**13035**	**15655**	**14650**	**16435**
phosphoenolpyruvate carboxylase (*ppc*)	**-**	**-**	**06470**	**06530**	**06410**	**11035**	**10820**	**12765**	**03305**
Alternative electron donors									
NiFe hydrogenase (*hyd*ABCDE)					**11405-25**	**0405-25**	**7105-25**	**11240-260**	**4740-60**
formate dehydrogenase (*fdh*ABCE)	**-**	**-**	**-**	**-**	**19980-95**	**17400-15**	**18165-80**	**9215-30**	**10545-60**
Alternative electron receptors									
fumarate reductase (*frd*ABCD)	**-**	**-**	**-**	**-**	**17190-205**	**15740-55**	**17100-15**	**17770-85**	**15900-15**
TMAO reductase (*tor*A)	**-**	**-**	**-**	**-**	**00580**	**02430**	**00965**	**03665**	**16120**
nitrate reductase (*nap*ABCD)	**-**	**-**	**-**	**-**	**2395-410**	**12335-50**	**18835-20**	**8635-50**	**16820-35**
Aminoacid metabolism									
arginine deminiase (*arc*A)	**-**	**-**	**-**	**-**	**12880**	**12690**	**16005**	**15000**	**10150**
ornithine transcarbmoylase (*arc*B)	**02870**	**03040**	**05380**	**05445**	**12870**	**12700**	**15995**	**14990**	**10140**
carbamate kinase (*arc*C)	**00865**	**10000**	**-**	**-**	**12875**	**12695**	**16000**	**14995**	**10145**
arginine decarboxylase (*spe*A)	**-**	**-**	**-**	**-**	**01030**	**02880**	**01410**	**04110**	**05745**
agmatinase (*spe*B)	**-**	**-**	**-**	**-**	**16470**	**05965**	**03655**	**01775**	**14455**
arginase (*arg*)	**-**	**-**	**-**	**-**	**13605**	**06915**	**06360**	**00810**	**16935**
agmatine ureohydrolase (*spe*B)	**-**	**-**	**-**	**-**	**16470**	**05965**	**03655**	**01775**	**14455**
histidine decarboxyalse (*hdc*A)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
lysine decarboxylase (*lcd*C)	**-**	**-**	**-**	**-**	**-**	**-**	**16170**	**15100**	**-**
ornithine decarboxylase (*spe*F)	**-**	**-**	**-**	**-**	**-**	**17635**	**-**	**-**	**-**
tyrosine decarboxylase (*tdc*A)	**-**	**-**	**-**	**-**	**-**	**-**	**04810**	**19730**	**-**
glutamate decarboxylase (*gad*B)	**-**	**-**	**-**	**-**	**15595**	**14510**	**20690**	**16415**	**10595**
aspartate Aminofransferase (*asp*B)	**05970**	**06325**	**04740**	**03445**	**06300**	**11145**	**10710**	**12875**	**03415**
			05030	**00105**					
malate dehydrogenase (*mdh)*	**05690**	**05975**	**-**	**08240**	**13545**	**06855**	**04425**	**00910**	**16995**
glutamate dehydrogenase (*gdh*A)	**03530**	**03720**	**-**	**-**	**-**	**-**	**18470**	**10360**	**-**
serine dehydratase (*sda*AB)	**6630/35**	**7230/25**	**-**	**-**	**00790**	**02640**	**01165**	**03860**	**05990**
aromatic amino acid aminotransferase (Tyr,Phe,His) (Glu) (*tyr*B)	**10205**	**00215**	**01330**	**01315**	**04000**	**02360**	**00870**	**03570**	**00175**

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615	*Le. gelidum* subsp. *gelidum* TMW2.1618	*Le. gelidum* subsp. *gasicomitatum* TMW2.1619	*P. carnosum* TMW2.2021	*P. carnosum* TMW2.2029	*P. phosphoreum* TMW2.2033	*P. phosphoreum* TMW2.2034	*P. iliopiscarium* TMW2.2035
Biosample	**BHS00_**	**BHS01_**	**BHS02_**	**BHS03_**	**CIK00_**	**CIT27_**	**CJF25_**	**CJF26_**	**CJF27_**
					00510	**15900**		**20575**	**16195**
branched-chain aa aminotransferase (Leu,Ile,Val) (*ilv*E)	**05075**	**05365**	**04830**	**04900**	**16060**	**14990**	**21020**	**17435**	**11370**
alanine dehydrogenase (*ald*)	-	-	-	-	**11110**	**00095**	**02275**	**05030**	**13260**
aspartate ammonia-lyase (*asp*A)	**01340**	**09545**	**07635**	**07920**	**17140**	**15690**	**17055**	**17815**	**15960**
aspartate oxidase (*nad*B)	-	-	-	-	**17805**	**14195**	**19000**	**18575**	**09605**
Lux operon	-	-	-	-	-	-	+	+	-
fatty acid reductase (*lux*C)	-	-	-	-	-	-	**03295**	**02075**	-
acyl-transferase (*lux*D)	-	-	-	-	-	-	**03300**	**02080**	-
luciferase alpha subunit (*lux*A)	-	-	-	-	-	-	**03305**	**02085**	-
luciferase beta subunit (*lux*B)	-	-	-	-	-	-	**03310**	**02090**	-
nonfluorescent flavoprotein (*lux*F)	-	-	-	-	-	-	**03315**	**02095**	-
acyl-protein synthetase (*lux*E)	-	-	-	-	-	-	**03320**	**02100**	-
flavin reductase (*lux*G)	-	-	-	-	-	-	**03325**	**02105**	-
Fatty acid β-oxidation									
aerobic	-	-	-	-	+	+	+	+	+
long-chain fatty acid transporter (*fad*L)	-	-	-	-	**08400**	**00500**	**07005**	**11160**	**04660**
long-chain fatty acid CoA ligase (*fad*D)	-	-	-	-	**01510**	**03370**	**01900**	**04615**	**08250**
acyl-CoA dehydrogenase (*fad*E)	-	-	-	-	**00840**	**02690**	**01215**	**03915**	**05935**
					18565	**16275**	**19790**	**19500**	**13735**
3-hydroxyacyl-CoA dehydrogenase (*fad*B)	-	-	-	-	**16295**	**15225**	**20075**	**17670**	**11605**
acetyl-CoA acyltransferase (*fad*A)	-	-	-	-	**16290**	**15220**	**20070**	**17665**	**11600**
anaerobic	-	-	-	-	+	+	+	+	+
long-chain fatty acid CoA ligase (put. *fad*K)	-	-	-	-	**12680**	**12890**	**15805**	**14800**	**09950**
3-hydroxyacyl-CoA dehydrogenase (*fad*J)	-	-	-	-	**01935**	**12090**	**02500**	**05170**	**15335**
acetyl-CoA acyltransferase (*fad*I)	-	-	-	-	**01940**	**12095**	**02505**	**05175**	**15330**

Table A6| Locus tags of genes from sequenced *Lc. piscium* genomes encoding putative proteins with antimicrobial activity.

Strain	*Lc. piscium* TMW2.1612	*Lc. piscium* TMW2.1615
Biosample accession number	**BHS00_**	**BHS01_**
lysozyme family protein	**07150**	**07710**
muramidase	**00715**	**10150**
glycoside hydrolase family 25 protein	**04390**	**04685**
LysM peptidoglycan binding protein	**10040**	**00440**
CHAP domain containing peptidoglykan hydrolysis protein	**00756**	**07780**
	01740	**10830**

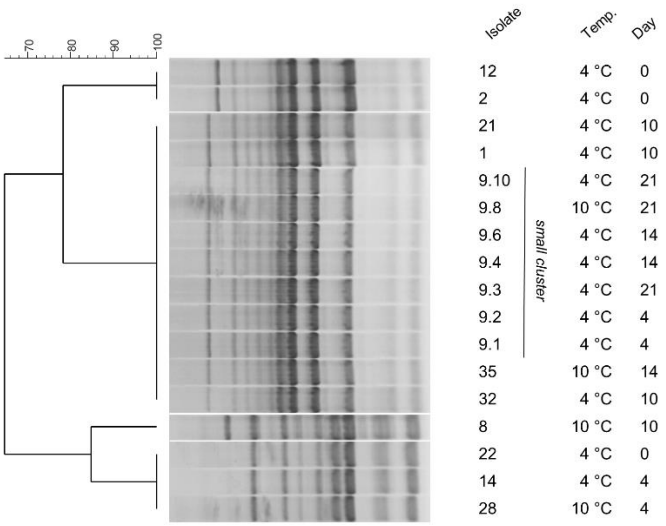


Figure A1| RAPD cluster analysis of selected *Lc. piscium* isolates from beef steaks. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.

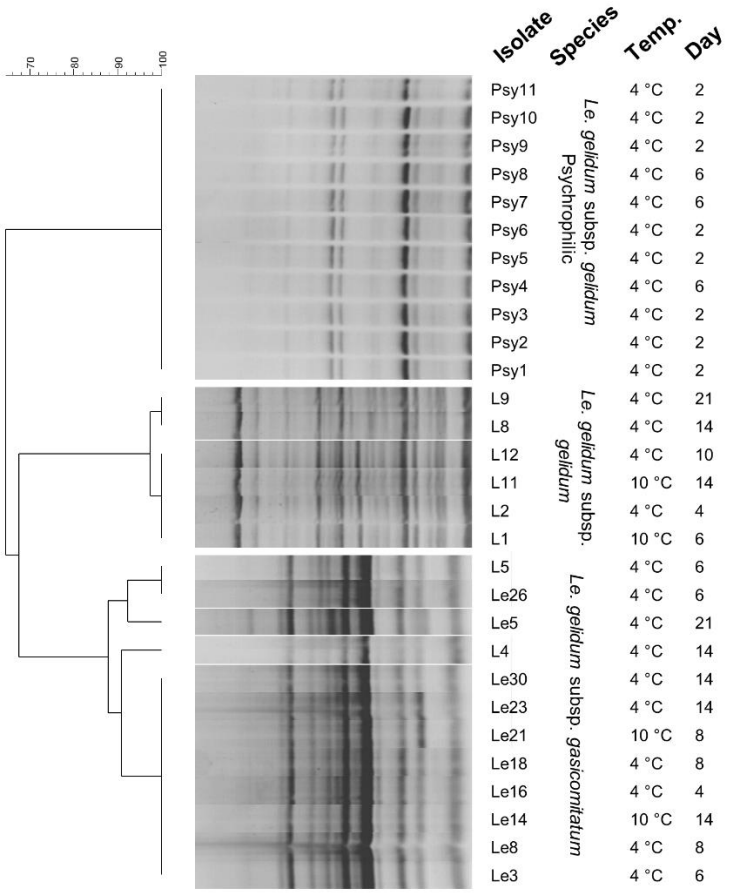


Figure A2| RAPD cluster analysis of selected *Le. gelidum* subsp. isolates from beef steaks. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.

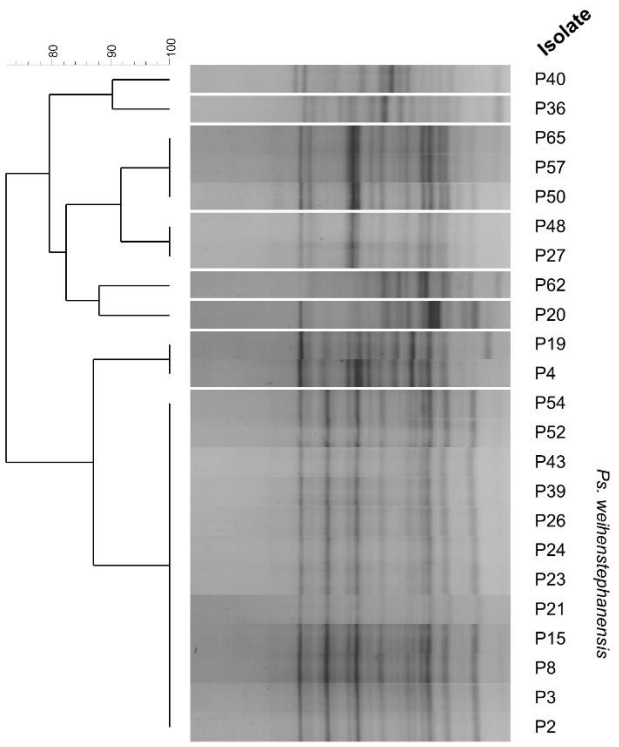


Figure A3| RAPD cluster analysis of selected *Pseudomonas* spp. isolates from beef steaks. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 3% tolerance. Scale bar refers to the Pearson correlation coefficient. Isolate names refers to internal short designations.

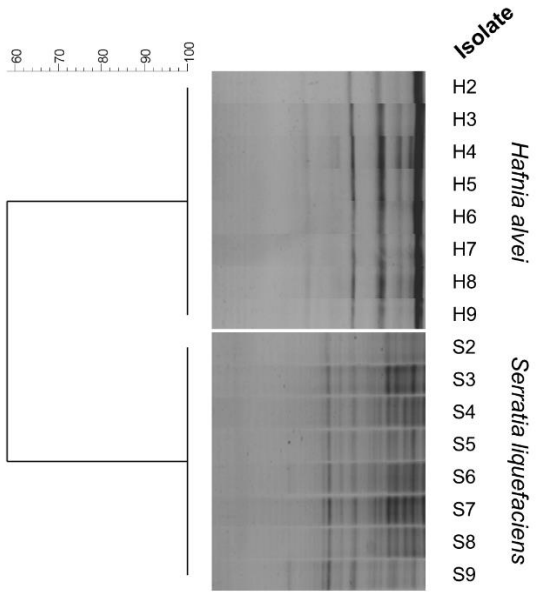
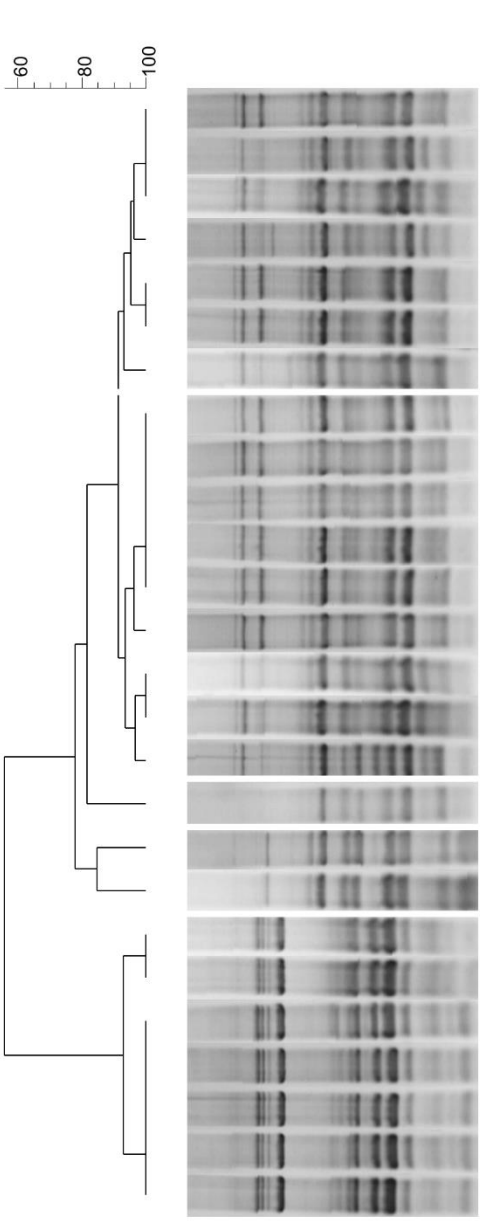


Figure A4| RAPD cluster analysis of selected *Enterobacterales* isolates from beef steaks. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Isolate names refers to internal short designations.



Isolate	Temp.	Day	Batch
Lapi10	10°C	d10	2
Lapi29	10°C	d8	1
Lapi38	4°C	d14	1
Lapi15	10°C	d4	1
Lapi33	10°C	d4	2
Lapi35	10°C	d10	2
Lapi24	10°C	d21	2
Lapi1	10°C	d10	2
Lapi3	10°C	d8	2
Lapi4	4°C	d1	1
Lapi42	10°C	d6	2
Lapi43	10°C	d14	2
Lapi28	10°C	d6	1
Lapi21	4°C	d14	1
Lapi40	4°C	d10	1
Lapi19	4°C	d14	1
Lapi26		d0	1
Lapi8	4°C	d1	1
Lapi25	10°C	d6	1
Lapi23	10°C	d2	2
Lapi45	10°C	d3	1
Lapi2	10°C	d6	2
Lapi14	10°C	d4	1
Lapi30	10°C	d21	2
Lapi32	10°C	d10	1
Lapi34	10°C	d8	2

Figure A5| RAPD cluster analysis of selected *Lc. piscium* isolates from minced beef. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.

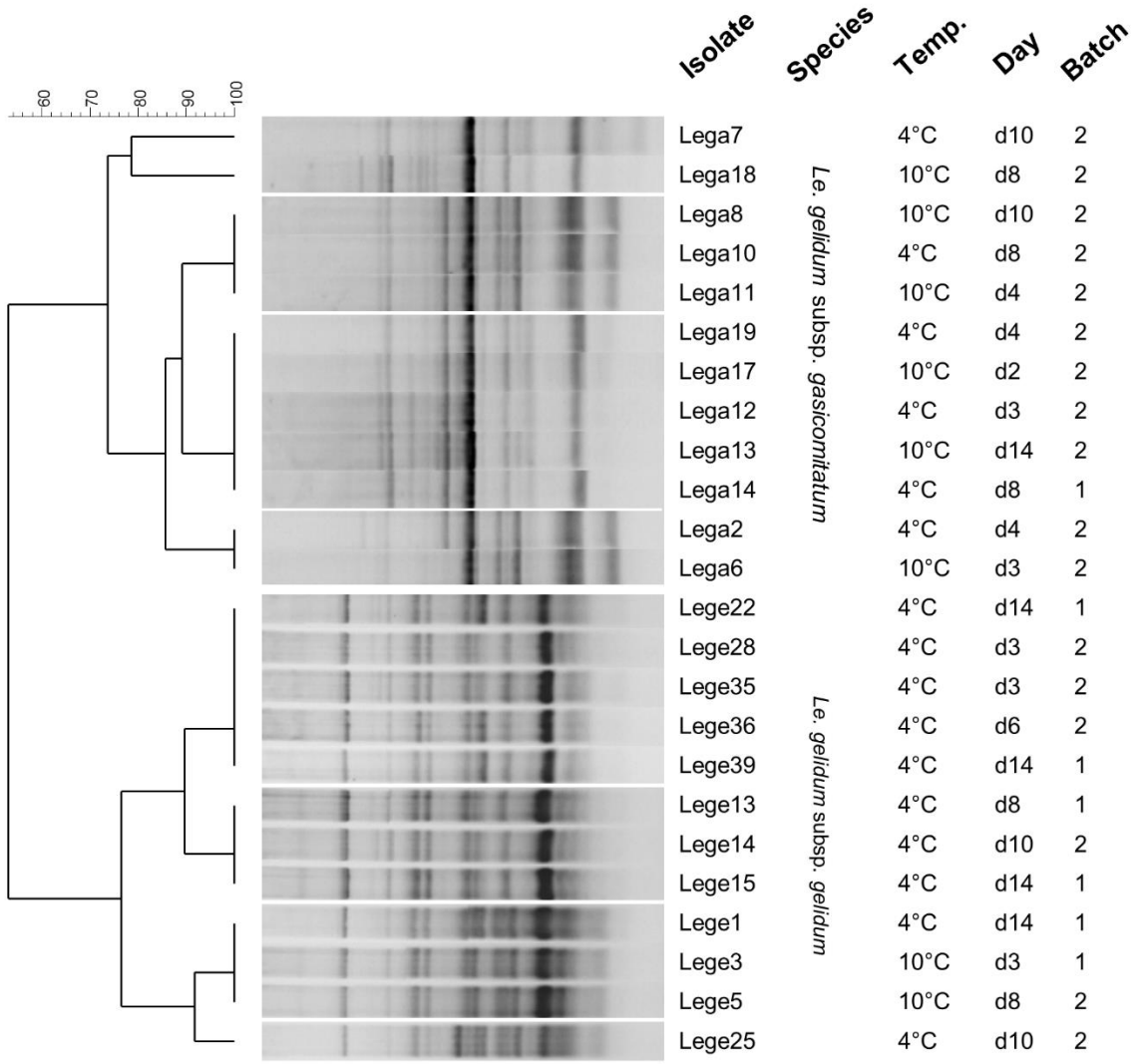


Figure A6| RAPD cluster analysis of selected *Le. gelidum* subsp. isolates from minced beef. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.

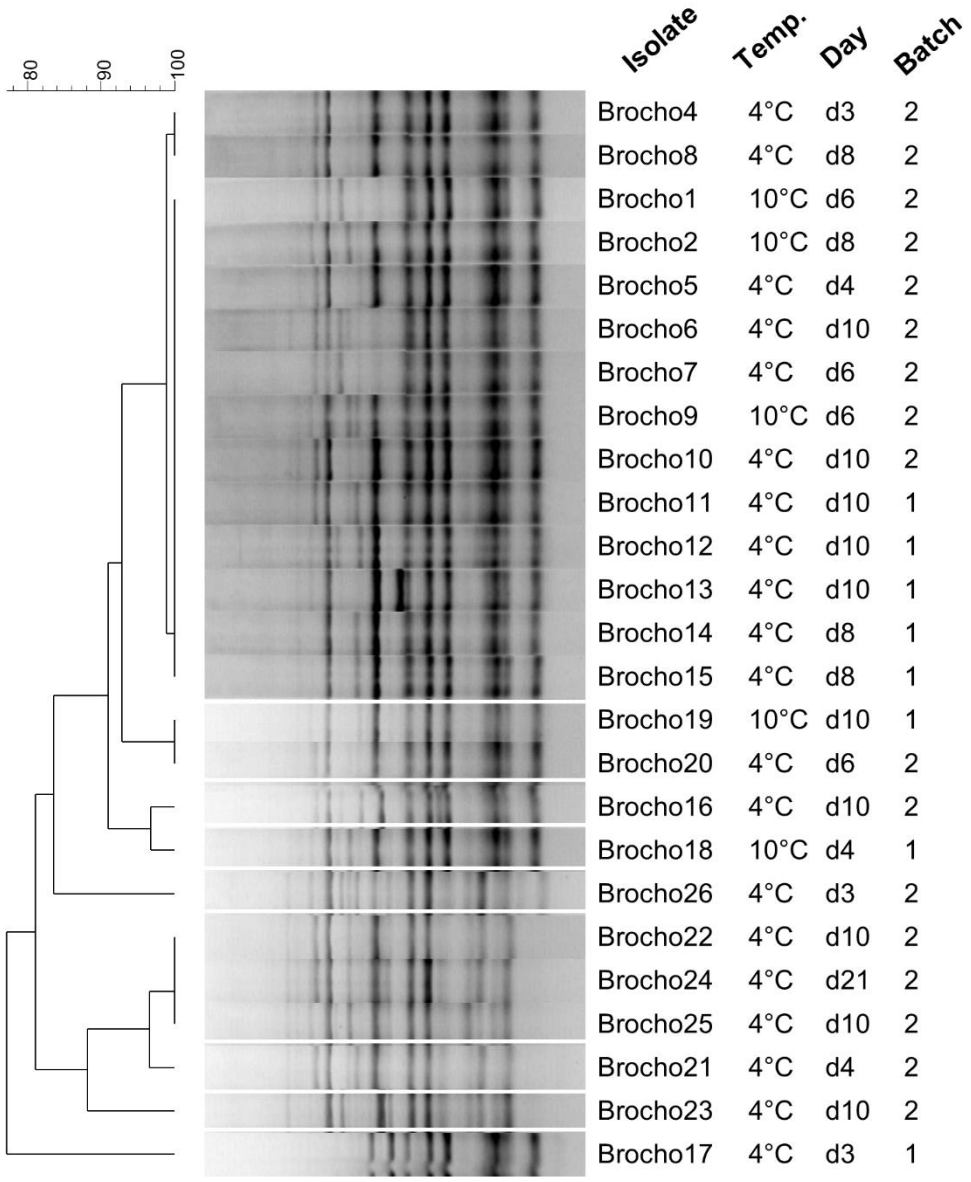


Figure A7| RAPD cluster analysis of selected *B. thermosphacta* isolates from minced beef. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.

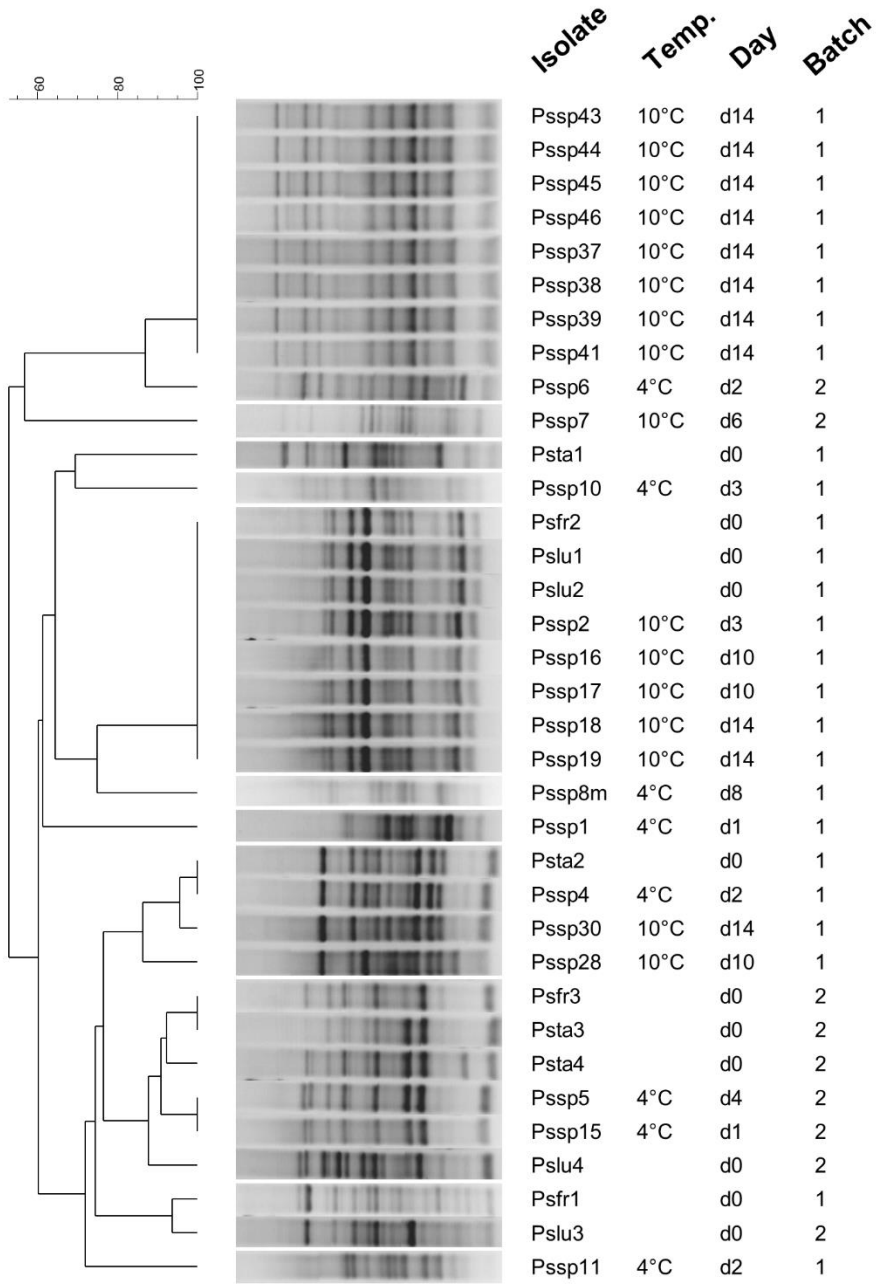
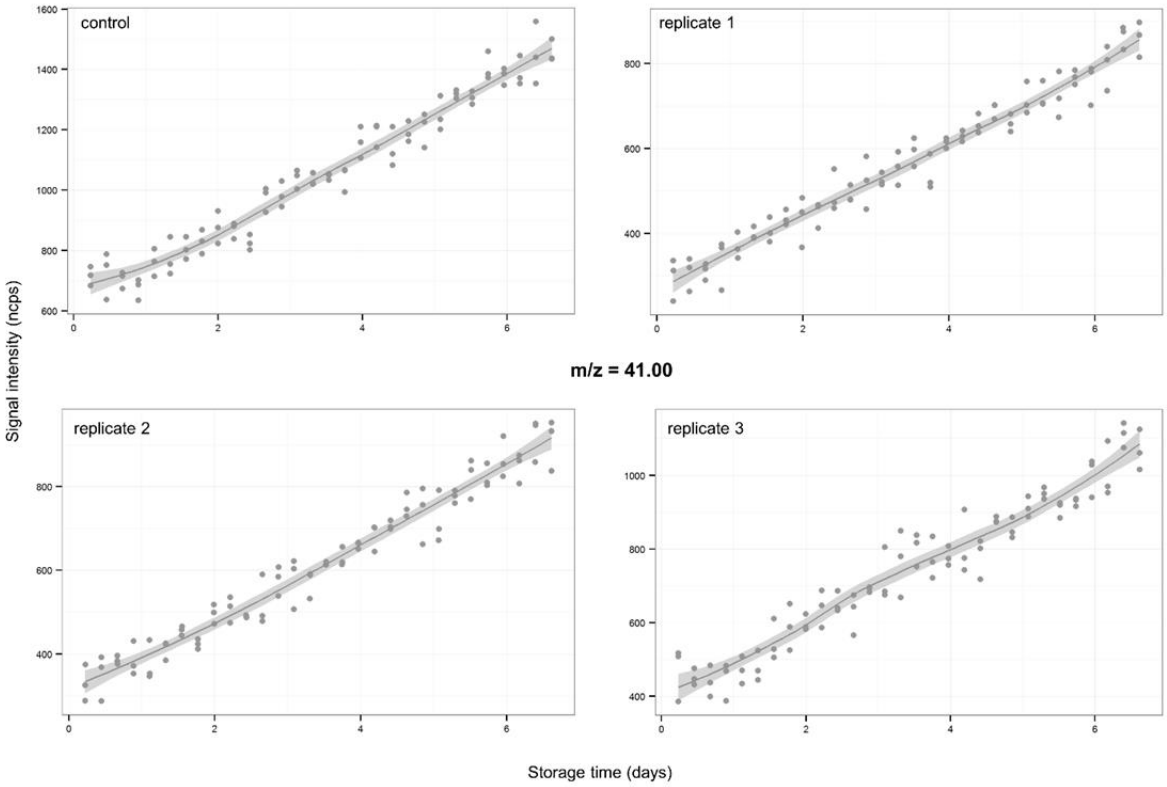


Figure A8| RAPD cluster analysis of selected *Pseudomonas* spp. isolates from minced beef. Dendrogram was calculated based on unweighted pair group method with arithmetic mean (UPGMA) as cluster method with Dice‘s similarity coefficient and 2% tolerance. Scale bar refers to the Pearson correlation coefficient. Temp., storage temperature. Isolate names refers to internal short designations.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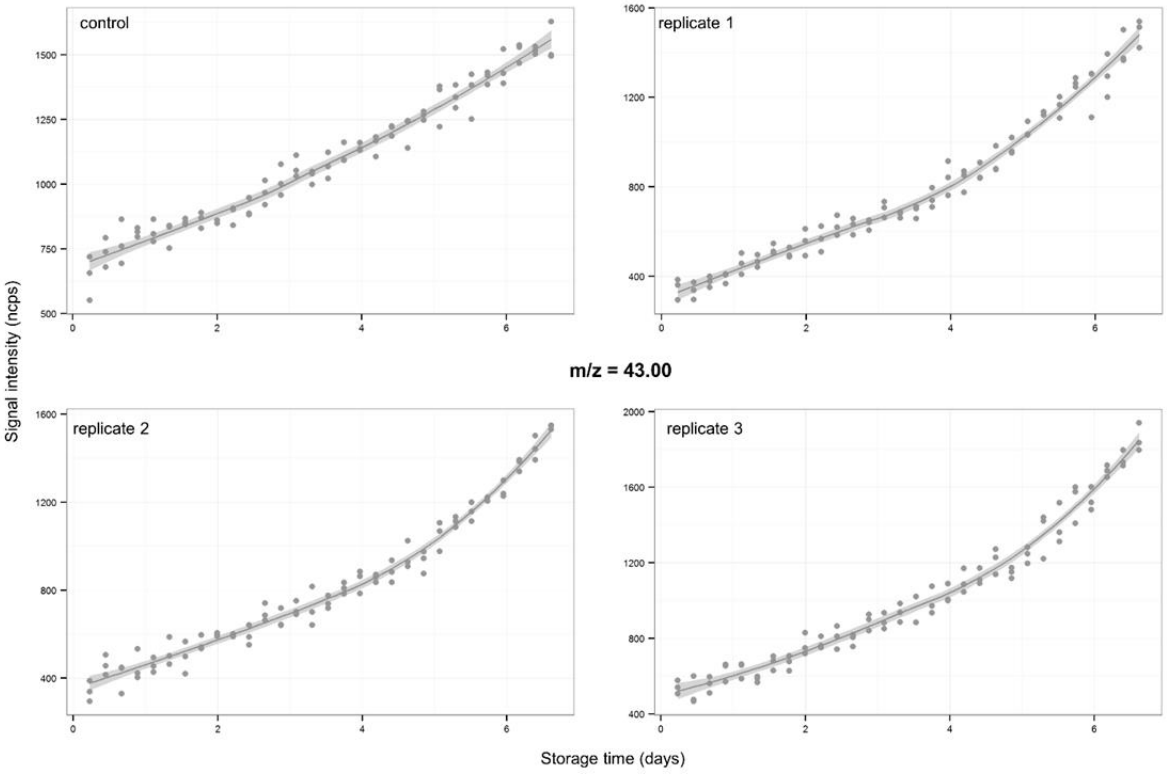
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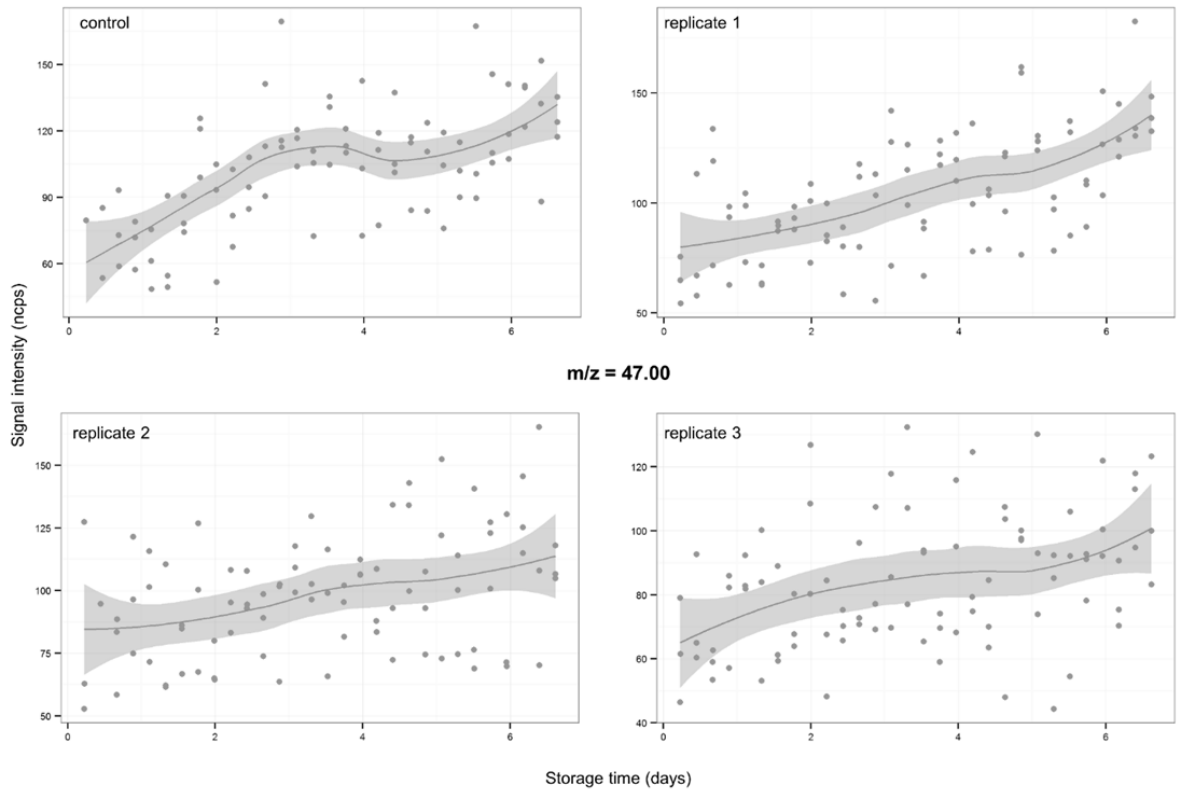
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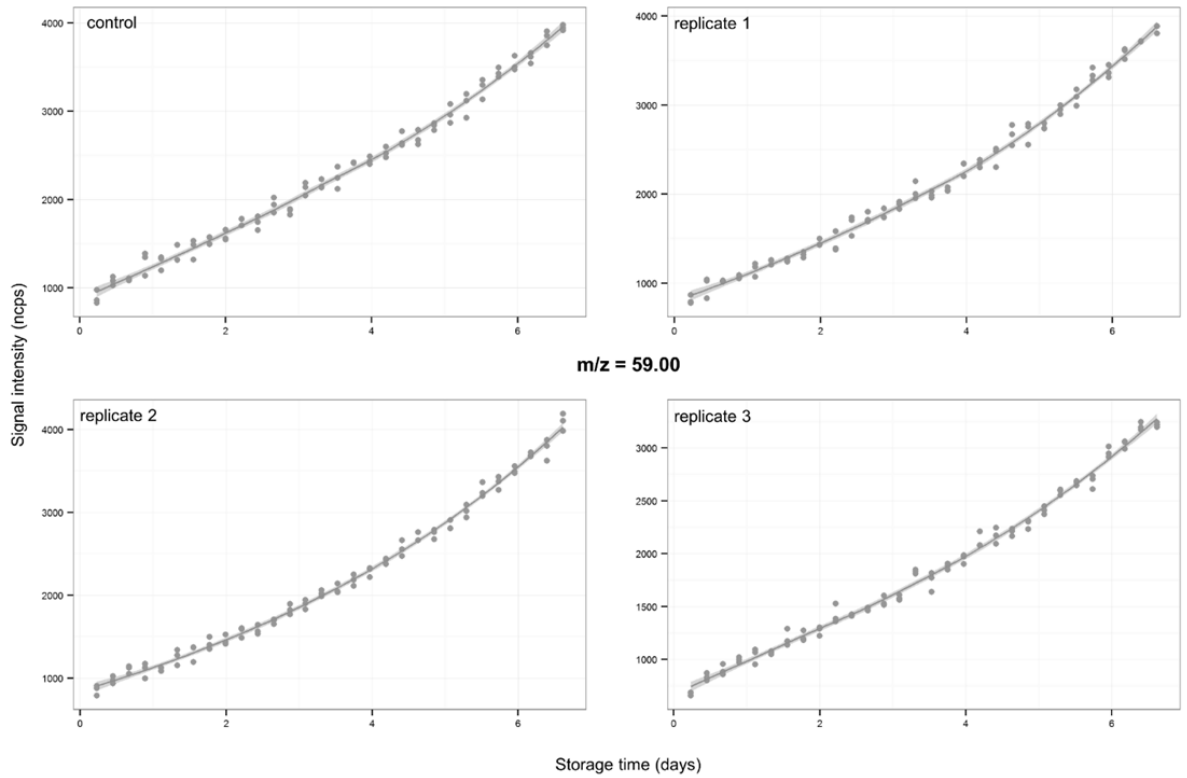
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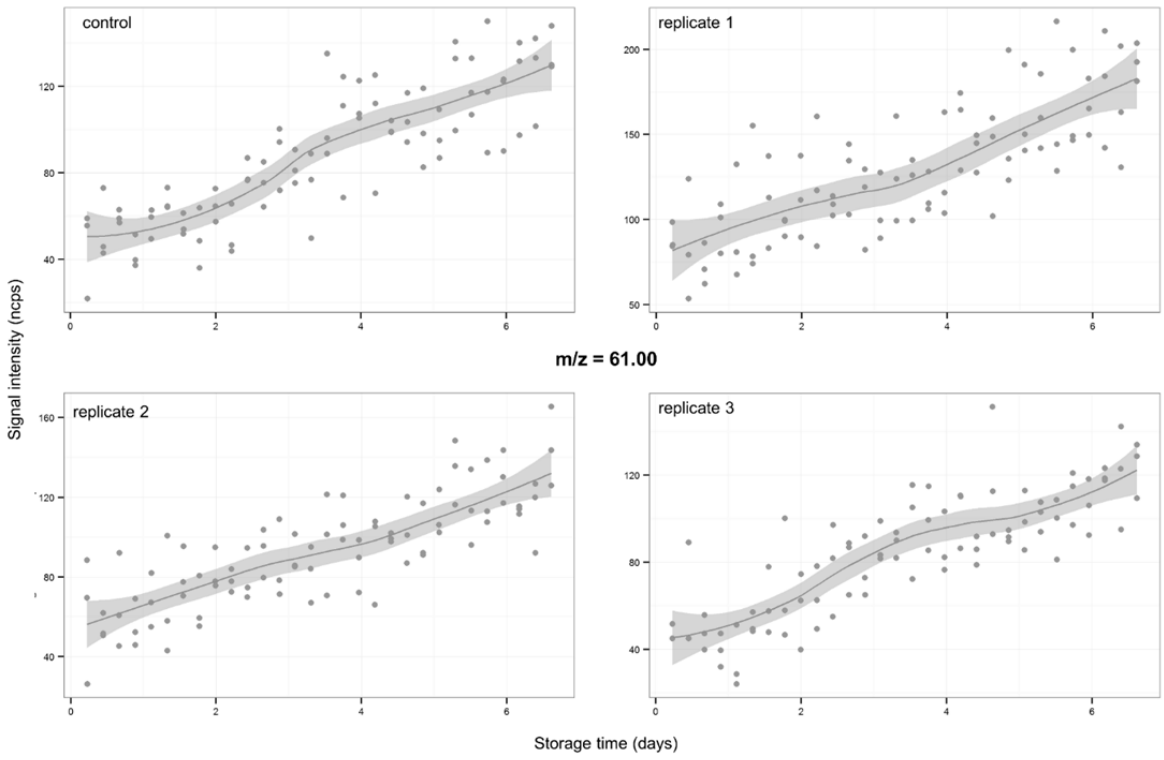
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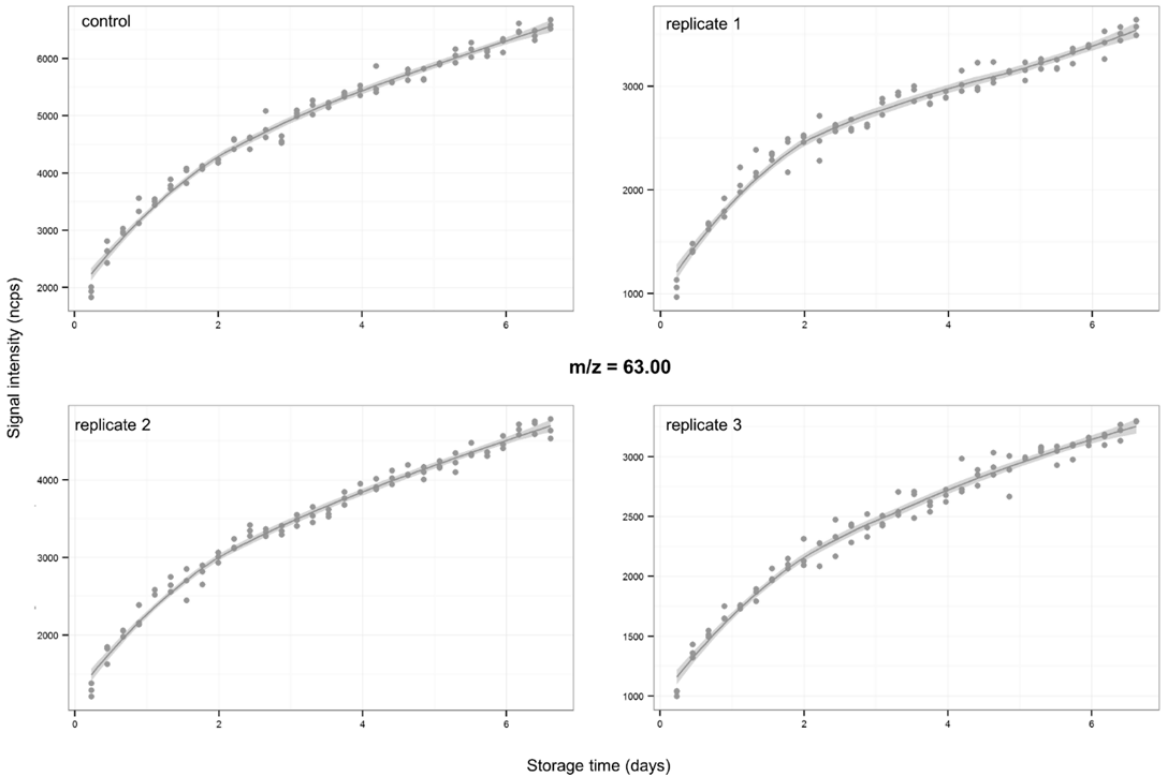
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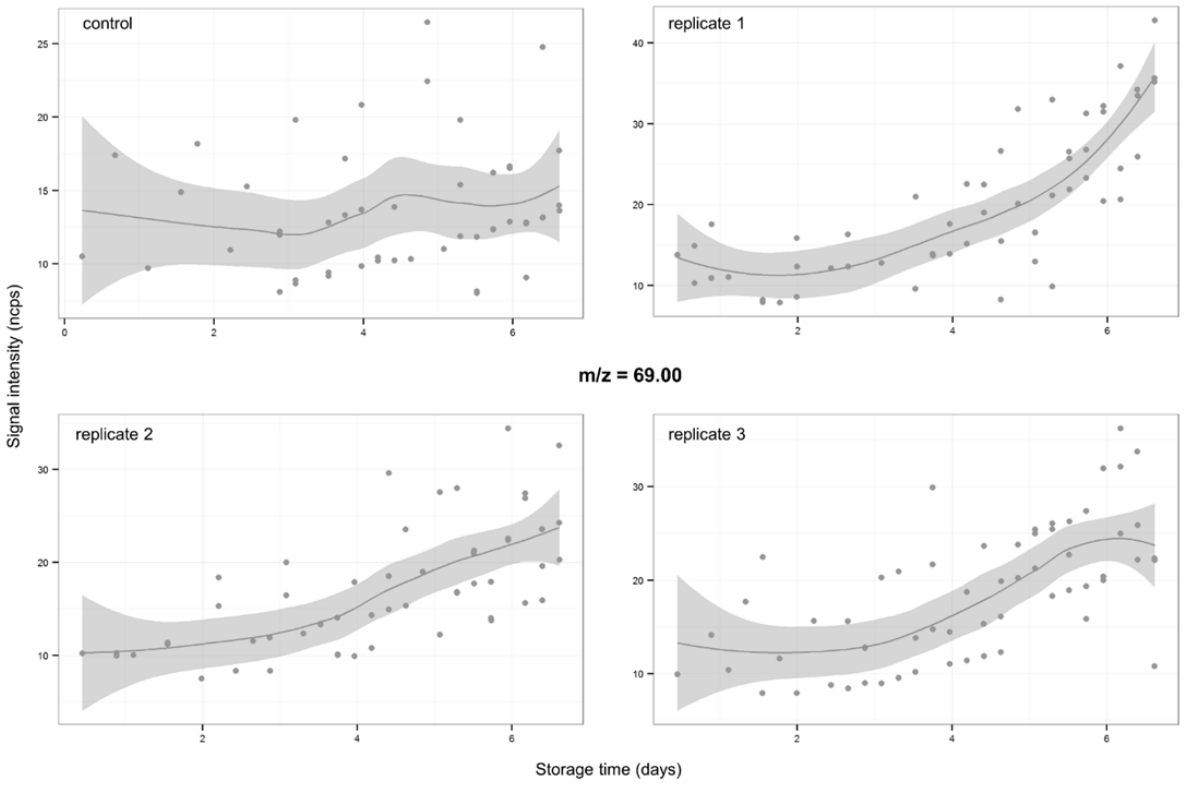
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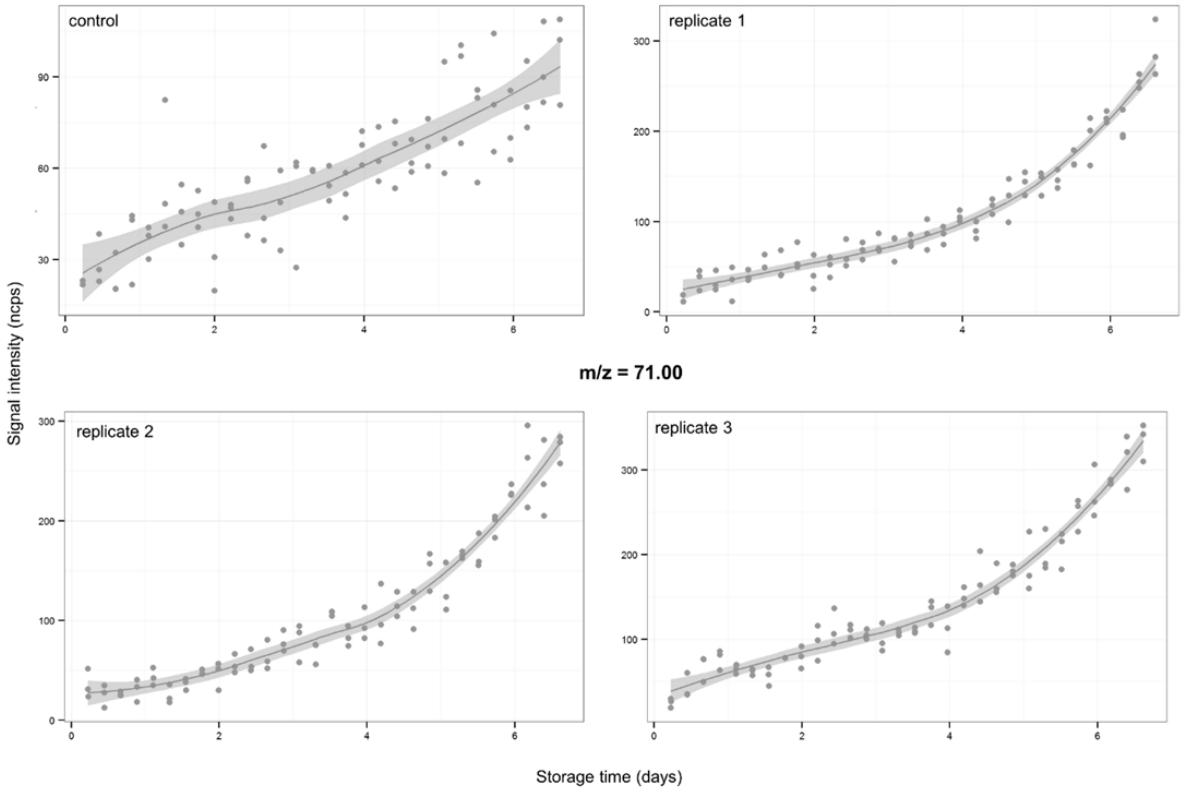
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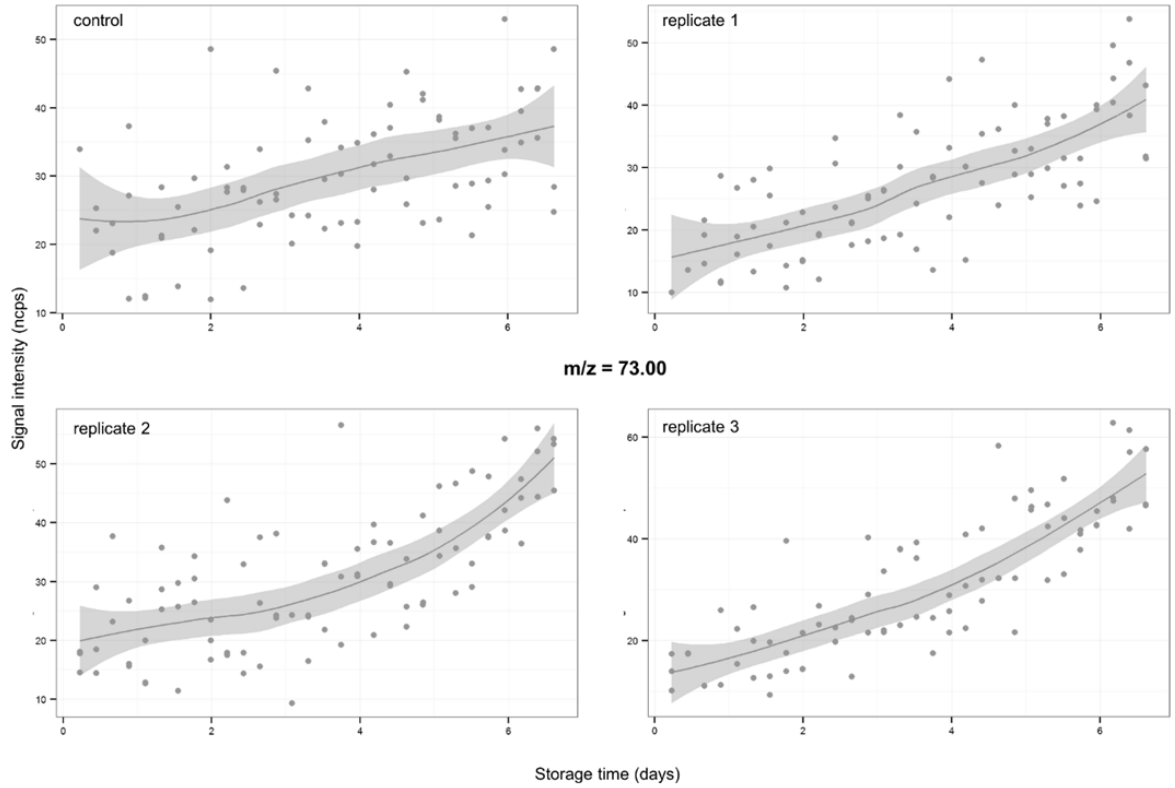
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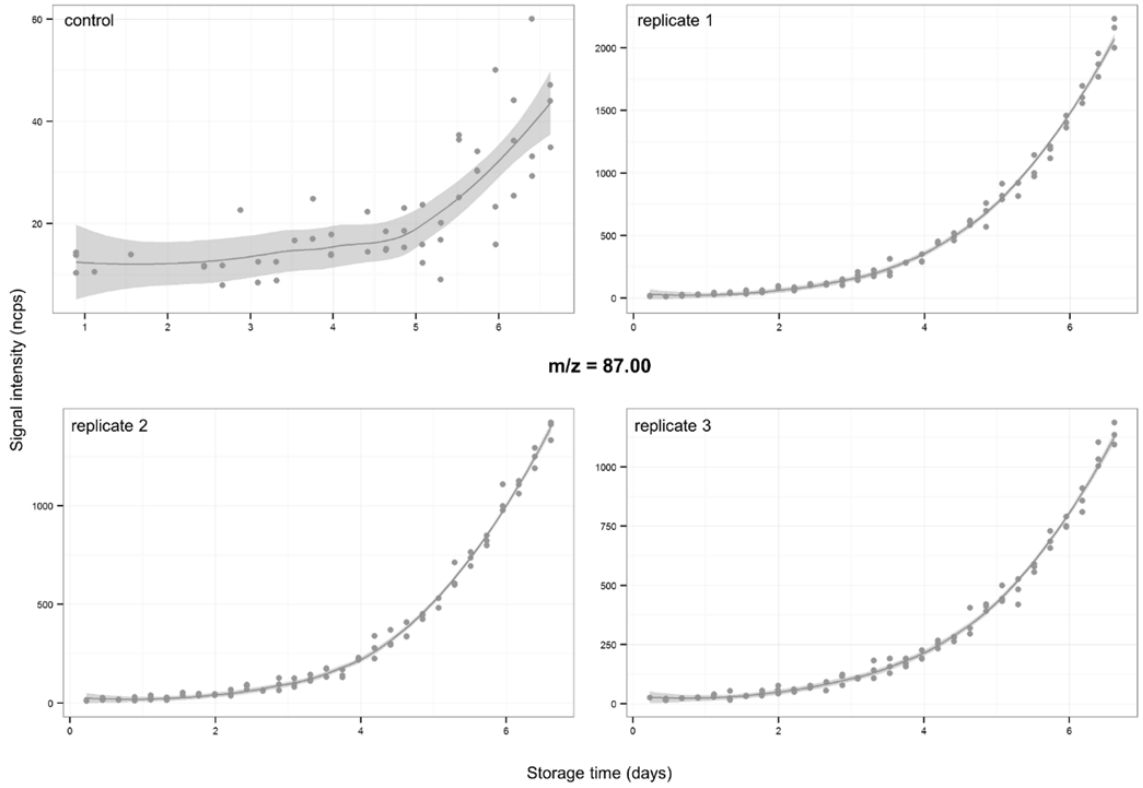
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Signal intensity (ncps)
Storage time (days)

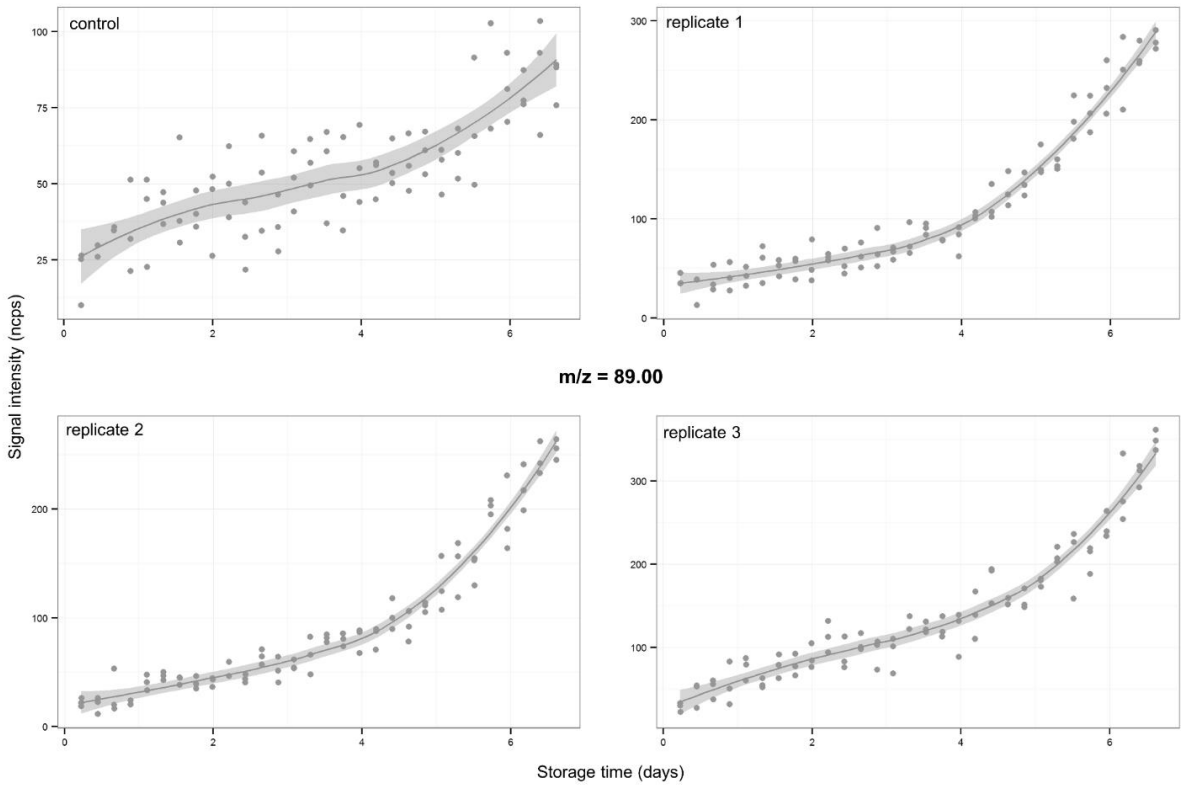

Figure A9| Development of volatile metabolites at 4 °C of beef inoculated with *Lc. piscium* TMW2.1615 monitored by PTR-MS. ncps, number of counts per second; dots represent single measurement replicates; smoothed curve is based on all single measurements.

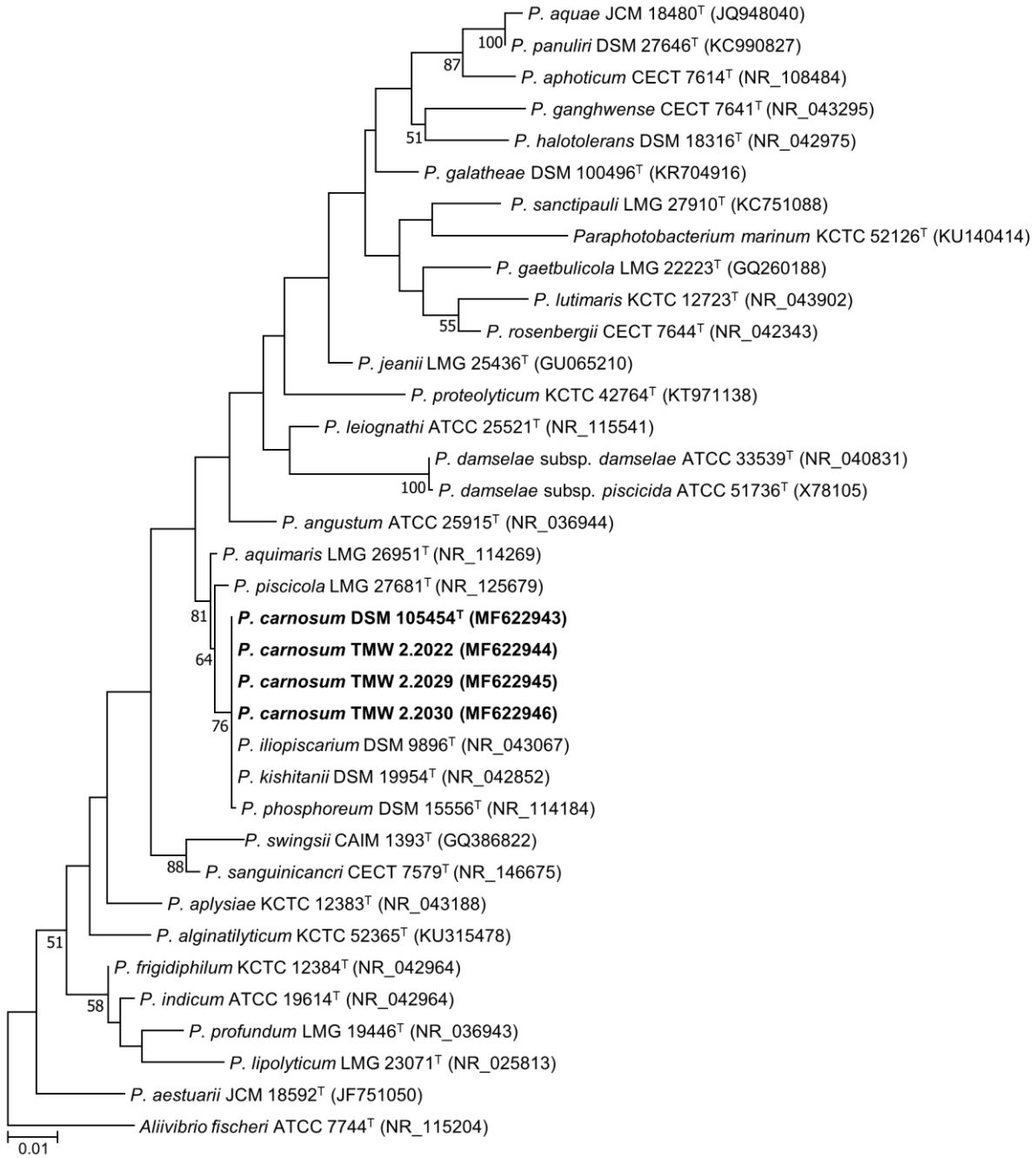

Figure A10| Phylogenetic maximum likelihood tree of *Photobacterium* spp. based on partial 16S rRNA gene sequences (=1341-1355 nt) with 100 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes. Scale bar indicates nucleotide substitutions per site.

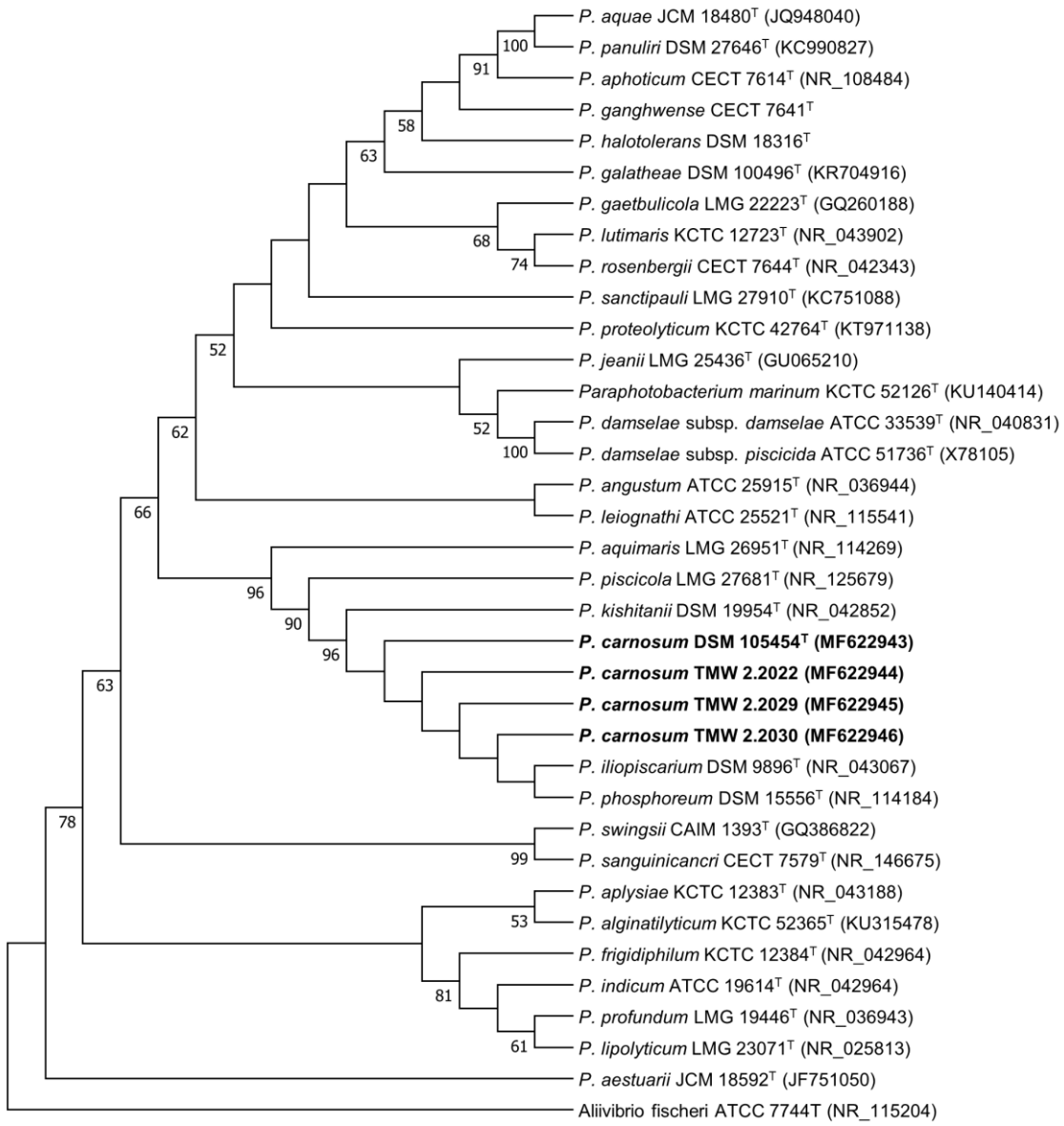

Figure A11: Phylogenetic maximum parsimony tree of *Photobacterium* spp. based on partial 16S rRNA gene sequences (=1341-1355 nt) with 100 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes.

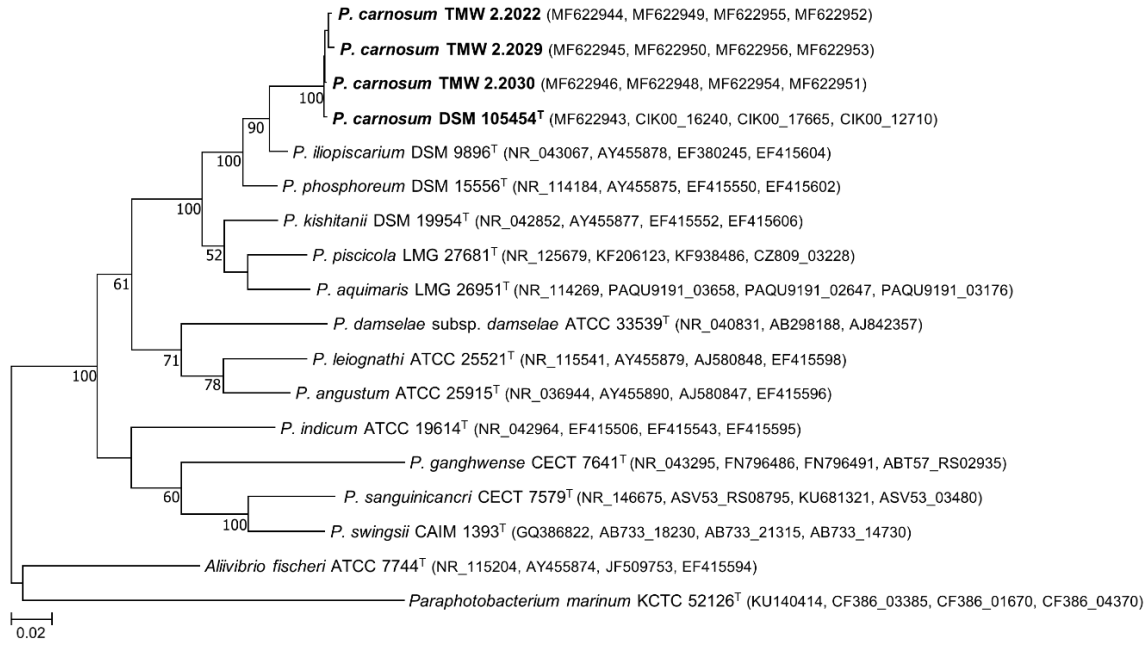

Figure A12|: Phylogenetic maximum parsimony tree of *Photobacterium* spp. based on concatenated partial 16S rRNA (=1341-1342) and partial housekeeping *gyr*B (=1059-1068 nt), *rec*A (=542 nt), *rpo*D (=786-810 nt) gene sequences (in this order, 3735-3762 nt total) with 100 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes.

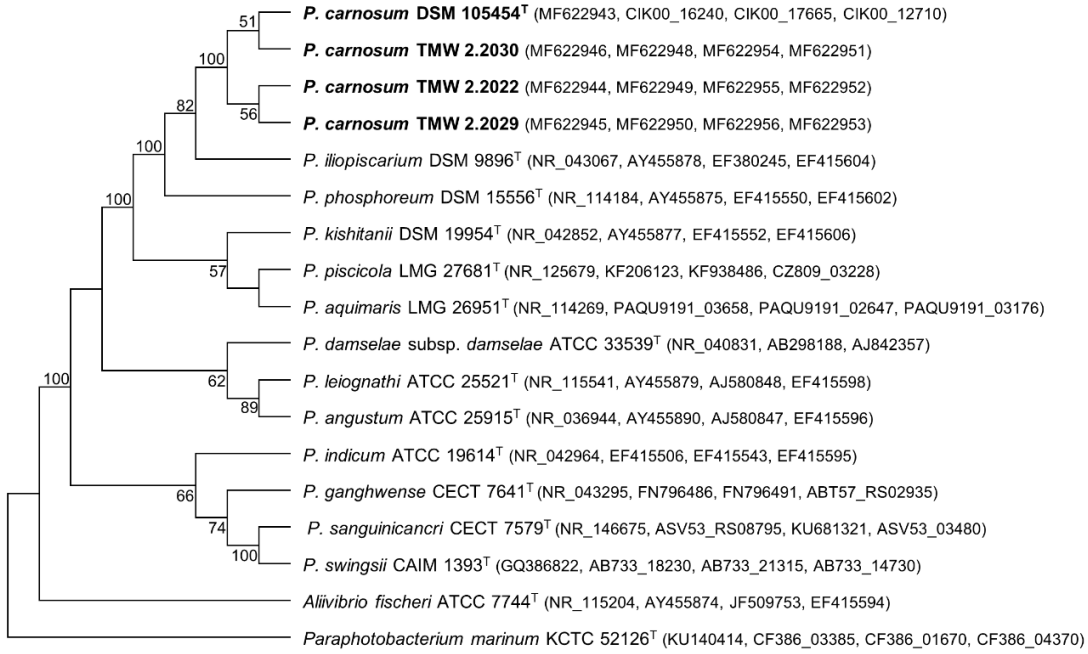

Figure A13|: Phylogenetic maximum parsimony tree of *Photobacterium* spp. based on concatenated partial 16S rRNA (=1341-1342) and partial housekeeping *gyr*B (=1059-1068 nt), *rec*A (=542 nt), *rpo*D (=786-810 nt) gene sequences (in this order, 3735-3762 nt total) with 100 bootstraps. Accession numbers are given in parentheses. Bootstrap values greater 50% are shown at nodes.

10 List of Publications, presentations, collaborations and students theses

Publications in peer-reviewed journals:

Hilgarth M, Behr J, Vogel RF. 2018. Monitoring of spoilage-associated microbiota on modified atmosphere packaged beef and differentiation of psychrophilic and psychrotrophic strains. *J Appl Microbiol* **124**:740-753.

Hilgarth M, Nani M, Vogel RF. 2018. Assertiveness of meat-borne *Lactococcus piscium* strains and their potential for competitive exclusion of spoilage bacteria in situ and in vitro. *J Appl Microbiol* **124:**1243-1253.

Hilgarth M, Fuertes-Pèrez S, Ehrmann MA, Vogel RF. 2018. *Photobacterium carnosum* sp. nov., isolated from spoiled modified atmosphere packaged poultry meat. *Syst Appl Microbiol* **41:**44-50.

Hilgarth M, Fuertes-Pèrez S, Ehrmann MA, Vogel RF. 2018. An adapted isolation procedure reveals *Photobacterium* spp. as common spoilers on modified atmosphere packaged meats. *Lett Appl Microbiol* **66:**262-267.

Höll L., **Hilgarth M**, Geissler AJ, Behr J, Vogel RF. 2018. „Metatranscriptomic analysis of modified atmosphere packaged poultry meat reveals photobacteria as spoilers" *submitted, under revision.*

Franke C, **Hilgarth M**, Vogel RV, Petermeier H, Langowski H-C. 2018. Characterization of the dynamics of volatile organic compounds from modified atmosphere packed beef by PTR-MS and non-linear regression. *in prep.*

Hilgarth M, Lehner EM, Behr J, Vogel RF. 2018. Characterization and anaerobic growth of *Pseudomonas* spp. isolated from modified atmosphere packaged minced beef. *in prep*.

Hilgarth M, Höll L, Vogel RF. 2018. Predicted lifestyle of specific spoilage organisms on modified atmosphere packaged meat using comparative genomics. *in prep*.

Oral presentations at academic symposia

Hilgarth M, Nani M, Franke C, Vogel RF. 2017. "Assertiveness of *Lactococcus (Lc.) piscium,* a novel bioprotective organism for competitive exclusion on meat" Oral presentation at "2nd Innovations in Food Packaging, Shelf Life and Food Safety" conference held on 04-06.09.2017 in Erding, Germany.

Poster presentations at academic symposia

Hilgarth M, Behr J, Vogel RF. 2015. “Dynamics of spoilage microbiota in high-oxygen modified-atmosphere packaged beef using MALDI-TOF MS.” Poster presentation at VAAM/DGHM/ZIEL “15. Fachsymposium Lebensmittelmikrobiologie” held on 15-17.04.2015 in Freising, Germany.

Hilgarth M, Behr J, Vogel RF. 2015. “*Lactococcus piscium* - A potential protective culture for MAP meat?” Poster presentation at “1st Innovations in Food Packaging, Shelf Life and Food Safety” conference held on 15-17.09.2015 in Erding, Germany.

Hilgarth M, Nani M, Vogel RF. 2016. “*Lactococcus piscium* – A potential application as a competitive excluder in MAP meat” Poster presentation at “30th EFFoST Conference - Targeted Technologies for Sustainable Food Systems” conference held on 28-30.09.2016 in Vienna, Austria.

Hilgarth M, Nani M, Vogel RF. 2017. “Assertiveness of *Lactococcus (Lc.) piscium,* a novel bioprotective organism for competitive exclusion on meat” Poster and oral presentation at “Microbial spoilers in food 2017” conference held on 28-30.06.2017 in Quimper, France.

Oral presentations at meetings of the steering committee (AiF 17803 N)

Hilgarth M, Behr J, Vogel RF. 2014. “Identification and growth dynamics of spoilage-associated microbiota on modified atmosphere packaged beef steaks using MALDI-TOF MS.” Oral presentation at the annual AiF project meeting of the steering committee (17803 N) held on 09.10.2014 in Freising, Germany.

Hilgarth M, Behr J, Vogel RF. 2015. “Development and diversity assessment of spoilage-associated microbiota on different modified atmosphere packaged beef products.” Oral presentation at the annual AiF project meeting of the steering committee (17803 N) held on 13.05.2015 in Freising, Germany.

Hilgarth M, Franke C, Vogel RF. 2016. “Detection of volatile metabolites of spoilage-associated microbiota and growth of selected spoilers *in situ* and *in vitro*.” Oral presentation at the annual AiF project meeting of the steering committee (17803 N) held on 13.16.2016 in Freising, Germany.

Student theses and contributions to this thesis

The listed student theses were supervised and respective experiments were designed. The resulting raw data obtained in the experiments were partially incorporated into this thesis.

Elisabeth Maria Lehner - Master Thesis: “Growth dynamics and characterization of spoilage-associated microbiota on MAP minced beef.” 2015.

Marco Nani - Master Thesis: “Characterization of different strains of *Lactococcus piscium*: Prevalence and interaction with other competing spoilage bacteria.” 2016.

Sandra Fuertes-Perez – Research Internship: “Characterization of *Photobacterium* ssp. involved in meat spoilage.” 2017

Collaborations

Volatilome analysis of inoculated beef was conducted together with Corinna Franke (Franke 2018) as part of a project collaboration. Repackaging of samples, PTR-MS analysis and calculation of volatile compounds was performed by Corinna Franke at the Fraunhofer Institute IVV. Sample preparation, inoculation, and microbiological analysis and evaluation was conducted by me.

11 Statutory declaration

I hereby declare that I wrote the present dissertation with the topic

“**Spoilage-associated psychrotrophic and psychrophilic microbiota on modified atmosphere packaged beef”**

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. Other contributions to this work in terms of collaboration and supervised student theses are clearly indicated and acknowledged in the “collaboration and contribution” section.

Freising,

Maik Hilgarth

12 Acknowledgements

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