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Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in raw sausage fermentations

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It is our choices that show what we truly are, far more than our abilities.

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J. K. Rowling

Abbreviations

°C	degree Celsius (centrigrade)
µ	micro (10^{-6})
ACN	acetonitrile
ADI	arginine deiminase
AF4-MALS	asymmetric Flow Field-Flow Fractionation with Multi-Angle Light Scattering
AMP	antimicrobial peptides
a_w	water activity
BA	biogenic amines
BADGE	BlAst Diagnostic Gene Finder
BLAST	basic local alignment search tool
BP	Baird Parker Medium
BTS	bacterial test standard
bp	base pairs
CE	competitive exclusion
CFU	colony forming units
CHCA	alpha-cyano-4-hydroxy cinnamic acid
Cip	cold induced proteins
C.m.	competing microbiota
CNC	coagulase negative cocci
CPC	coagulase positive cocci
CPS	cold shock proteins
CR	colonization resistance
Csp	cold-shock proteins
Da	Dalton
dH_2O	deionized water
DNA	desoxyribonucleic acid
dNTP	desoxy nucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E_h	redox potential
$E_{kin.}$	kinetic energy
EMP	Embden-Meyerhof-Parnas
ESI	electrospray ionization
FAB	fast atom bombardment
g	gram
GSH	glutathione
h	hour
HCA	hierarchical cluster analysis
H_2O_2	hydrogen peroxide
Hz	hertz
kDa	kilodalton
Kg	kilogram
kV	kilo volt
L.	Lactobacillus
l	liter
LAB	lactic acid bacteria
LC-MS/MS	liquid chromatography with tandem mass spectrometry
M	molar (mol/l)
m	milli (10^{-3}), meter
MALDI-TOF MS	matrix-assisted laser desorption-ionisation time of flight mass spectrometry
min	minute (‘)
MLST	multilocus sequence typing

mM	milimolar
mMSM	modified meat simulation medium
mMRS	modified de Man, Rogosa and Sharpe medium
MSP	mass spectrometry profile
n	nano (10^{-9})
nm	nano meter
NCBI	National Center for Biotechnology Information
NPS	nitrite curing salt
nri	not reliable identified
nt	nucleotides
OD	optical density
$OD_{max.}$	maximum optical density
OD_{590}	optical density at 590 nm wavelength
O/N	over night
ORF	open reading frame
ORP	redox potential
p	probability value
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
pH	negative decimal logarithm of hydrogen ion activity
PHAST	PHAge Search Tool
PHASTER	PHAge Search Tool Enhanced Release
PMF	peptide mass fingerprint
RAPD	randomly amplified polymorphic DNA
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
s	second (")
SE	standard error
Ssp.	species
TBE	Tris-borate-EDTA buffer
TMW	Technische Mikrobiologie Weihenstephan
TFA	triflouroacetic acid
TSA	tryptic soy agar
TS+	Tryptic-Soy Medium
TVC	total viable count
U	units
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

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1 Introduction

In this thesis, the assertiveness of *Lactobacillus* (*L.*) *sakei* and *L. curvatus* in raw sausage fermentations was investigated. This chapter provides a comprehensive overview with basic knowledge and latest scientific insights about the history of food fermentation, the ecosystem of sausage fermentations, the economic relevance of the project and factors that may influence the assertiveness and predominance of strains within the ecological niche raw fermented sausages.

1.1 Economic relevance of meat and raw fermented sausages

Global meat consumption patterns worldwide have dramatically changed over the past 50 years (Vranken, 2014). As a result of the “livestock revolution” the worldwide average of meat consumption increased from 33.7 to 41.9 kg per capita (Henchion et al., 2014). Nowadays, the meat consumption is characterized by over 300 million tons of meat supply annually (Delgado, 2003; Hansen, 2018). In Germany, the per capita consumption of meat and meat products is even higher with about 60 kg per year (BLE, 2018) corresponding to an annual revenue of about 19 billion (BVDF, 2017). Therefore, in Europe and particularly in Germany, the meat industry is of great economically importance and meat is an essential part of the daily diet.

The production of raw fermented sausages as traditional meat products has a long tradition in Europe and in Germany. By the variation of meat, calibers, degrees of chopping and production processes like ripening times and/or -conditions, fermented sausages are characterized by a large variety (Holck et al., 2017). In Germany, the tradition of sausage production is further reflected by a huge variety of different sausages types and regional specialties like Braunschweiger Mettwurst, Bregenwurst or Ahle Wurst. Still, the most popular sausage type is the German salami with a per capita consumption of 2.6 kg per year (BVDF, 2017).

One major problem with processed meat products like raw fermented sausages is the fact, that the raw meat is not sterile and harbors an autochthonous microbiota, which is introduced mostly during slaughtering and processing (Semedo-Lemsaddek et al., 2016). As the sausages only undergo a fermentation process, this natural contamination may harbor a potential risk. The prevalence of *Salmonella spp.*, *Listeria monocytogenes* and other pathogenic bacteria like *Staphylococcus aureus* with special emphasis to MRSA or *E. coli* O157:H17 were reported several times (Jackson et al., 2013; Levine et al., 2001; Martin et al., 2011; Meloni, 2015; Omer et al., 2018; van Loo et al., 2007) and thus, represent potential health risks for consumers, especially for elderly or those which undergo immunosuppression. With the implementation of the hurdle technology, the autochthonous microbiota is formed in

a desired direction, meaning the favoring of lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNC) (Leistner, 2000). Nevertheless, the occurrence of potentially pathogenic or spoilage organisms cannot be completely excluded. Therefore, it is highly important for the meat industry to identify key parameters inhibiting spoilage and pathogenic organisms to minimize the risks for consumers as well as risks for malfermentations, which may result in huge economical losses. In consequence, the need for process standardization as well as quality assurance strategies (Vignolo et al., 2010) has led to the most promising approach: the use of competitive and assertive starter cultures. Unfortunately, in the lack of time and cost-effective methods to track those starters at strain level, their assertiveness upon meat fermentation is widely unknown and remains to be elucidated.

1.2 History of food preservation and fermentation

Around 10.000 years ago, since the beginning of the Neolithic period, food storage and early preservation techniques that prolonged storage period times became a decisive prerequisite for the development of mankind and society (Bourdichon et al., 2012; Nair and Prajapati, 2003). Chemical analyses in the village in Henan province in China revealed the production of a prehistoric fermented beverage consisting of rice, honey and fruit (hawthorn fruit and/or grape) in the seventh millennium before Christ (McGovern et al., 2004).

However, the fermentation process and its mechanisms remained widely unclear until the findings of Louis Pasteur in the late 19th century, who first uncovered the process and described the fermentation as microbial life “without air” (Pasteur, 1876). Subsequently, various food fermentations were the target for intensively ongoing research and underwent continuous improvements. Scientists were able to isolate predominant bacteria from fermented beverages and food, which were well adapted to the respective environment.

Those predominant bacteria often occur in typical associations, developed under different environmental conditions thus representing the natural biodiversity of the respective product. This typical microbiota differ with the respective substrate and is nowadays commonly known as autochthonous microbiota. Nevertheless, malfermentations occurred due to unfixed fermentation and ripening conditions and events of unexpected contamination. In order to minimize the risks of malfermentations, fermented substrate was used to inoculate new fermentations (Hutkins, 2006). This principle is nowadays known as backslopping and it is still applied, e.g. for sourdough and vinegar (Hutkins, 2006; Vrancken et al., 2011). The inoculum undergoes a huge selection and is practically composed of solely dominant bacteria, driving the desired fermentation (undefined cultures) (Hutkins, 2006).

In 1873, Joseph Lister was able to isolate the first bacterial pure culture, which is nowadays classified as *Lactococcus lactis* (Narvhus and Axelsson, 2003). However, in the following further developments and research led to the direct use of previously isolated and intensively characterized bacteria, which are commonly known as starter cultures.

Today, only small manufacturers rely on the traditional method of spontaneous fermentation. In contrast, starter cultures became indispensable in today's manufacturing processes. Especially, large-scale production of fermented foods and beverages depends almost entirely on the use of such defined starter cultures (Ross et al., 2002). Due to their largely investigated and predictable performance, the application of defined and characterized starter cultures enable a consistency in product quality and safety (Laranjo et al., 2017). As the production takes place in a commercially sterile and controlled environment and fermentation as well as ripening are carried out in air-conditioned rooms and ripening chambers, following sequences of defined conditions including temperature and moisture, safety and hygiene was further improved to meet customers' demands concerning hygienic quality and safety.

1.3 Sausage fermentations

As fresh meat is a highly perishable product, great effort was made and various methods were established to increase shelf life and storage times. Traditional preservation techniques include salting, smoking, drying and fermentation (Adegocke and Olapade, 2012). The latter was practiced over hundreds of years in Asia and Europe (Selhub et al., 2014).

The history of fermented sausages already started in ancient Roman times and raw sausages were produced by Romans and Greeks (Toldrá, 2017). They form a nutrient rich but still restricted ecological niche, which is defined by comminuted meat and (pork back) fat, mixed with salt, nitrate and/or nitrite, sugar and spices like black pepper, which is stuffed into casings and subjected to a fermentation and drying process (Cocolin et al., 2006; Hugas and Monfort, 1996). Relevant biochemical changes take place by the activity of enzymes from both muscle and microbial origin enabling the enzymatic breakdown of carbohydrates, proteins and lipids (Toldrá, 2017). Free amino acids are relevant not only for the taste but for aroma as they can generate volatile compounds through Strecker degradations and Maillard reactions (Toldrá, 2017). Physical changes are mainly characterized by acid gelation of meat proteins and drying (Toldrá, 2017).

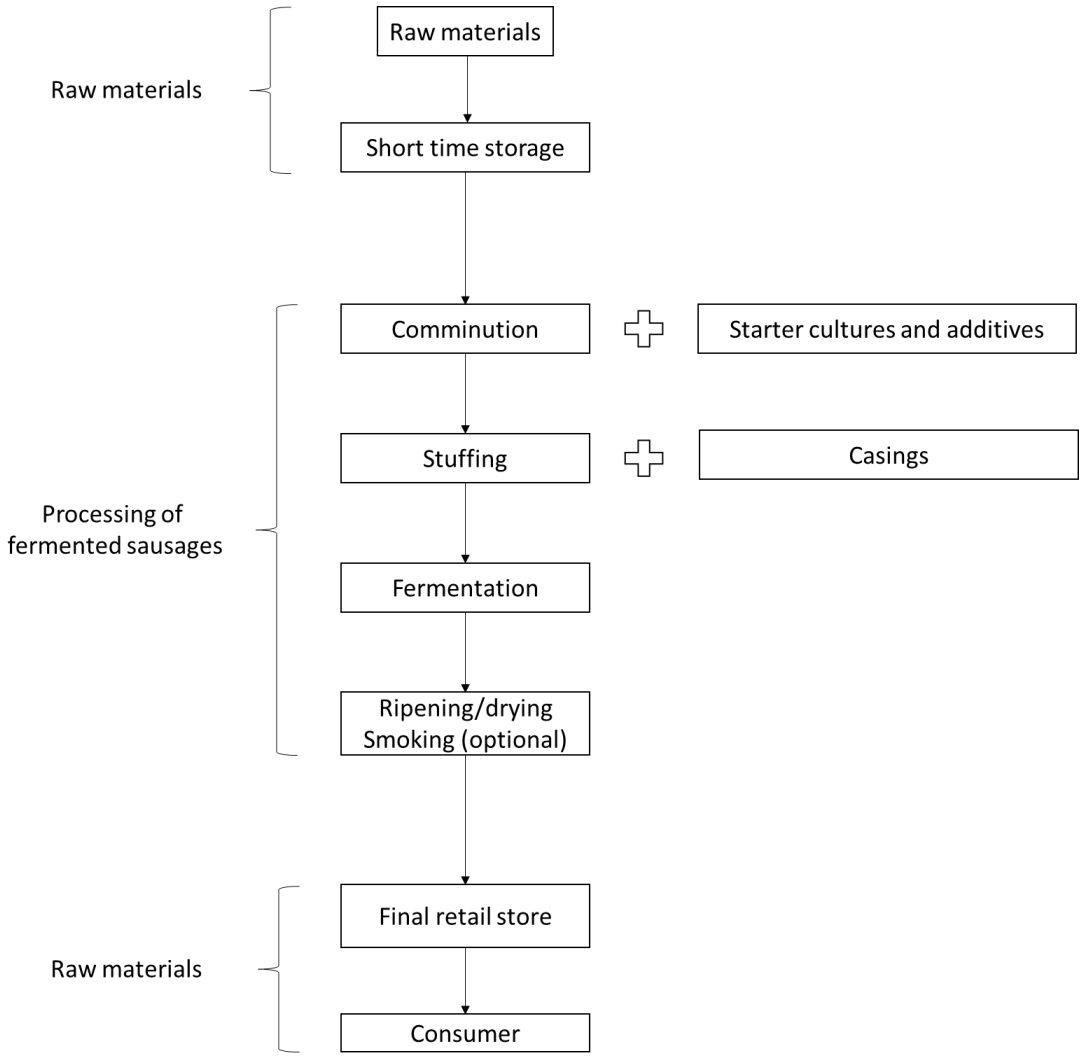

Figure 1: Process flow diagram for the processing of fermented sausages. Adapted from Toldra (Toldrá, 2017).

The extent of fermentation and drying varies depending on the climate and region. European dry-fermented sausages are consumed raw and without further heating, while in the US and China fermented sausages are normally consumed after heating (Toldrá, 2017). Nowadays, it is commonly known that the stability of a food product like raw fermented sausage is based on intrinsic as well on extrinsic factors. Leistner discovered that a sequence of hurdles (Toldrá and Reig, 2007) enables a stable fermentation and the extension of shelf-life for raw fermented sausages, e.g. salami (Leistner and Gorris, 1995). The hurdle technology is a combination of different preservation techniques to provide shelf life stability and long term storage by inhibition of potentially pathogenic and/or spoilage microorganisms (Figure 2). The hurdles include nitrite curing salt as a preservative, the reduction of redox potential (E_h), the favoring of a desirable microbiota consisting mainly of LAB, the rapid reduction of pH and the decrease of water activity (a_w).

At the beginning the addition of nitrite curing salt lowers the a_w-value in the sausage batter by the adsorption of available water and displays the first initial hurdle for spoilage and pathogenic bacteria (Verluyten et al., 2003). Furthermore, the salt functions as a vehicle for the curing agent sodium nitrite (Toldrá, 2017; Toldrá and Reig, 2007), which is mainly responsible for the red cured meat color and the inhibition of pseudomonads and other gram-negative oxidative bacteria. The addition of 0.2-0.7 % sugar, mostly glucose, enables an accelerated growth of desirable lactic acid bacteria (Lücke, 1994). By the metabolization of sugar under anaerobic conditions, lactic acid accumulates in the sausage batter leading to a distinct reduction in pH and to the development of the typical flavor (Hutkins, 2006; Toldrá, 2017). Simultaneously, the metabolization of nutrients under aerobic conditions and oxygen consumption reduces the redox potential. Reduced redox potentials favor the growth and the development of the desirable LAB. Finally, during fermentation and drying, the water activity value decreases with time and is largely responsible for long-term stability (Leistner, 2000; Toldrá, 2017).

The fermentation and drying times are usually fixed in the respective sausage formulations and vary depending on the product. In general, fermentation takes place at relatively high temperatures of 24-20°C and a relative humidity of approximately 90 % (Heinz and Hautzinger, 2007; Toldrá and Reig, 2007). The drying period is carried out at lower temperatures of 20°C or lower and at a relative humidity of around 70-85 % (Heinz and Hautzinger, 2007). Traditional manufacturing is mostly characterized by maturing times up to several months. However, industrially fermented sausages are finished within only three to four weeks to meet the customers' demands for fast and inexpensive meat supply (Heinz and Hautzinger, 2007).

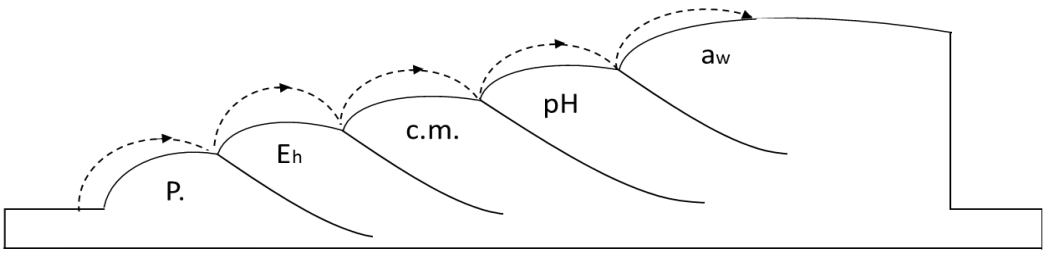

Figure 2: Hurdle technology in raw sausage fermentations, e.g. salami, adapted from Toldrá.(Toldrá, 2017) (P) Preservative like nitrite curing salt, (E_h) redox potential, (c.m.) competing microbiota,(pH) increases with time and the amount of produced lactic acid, (a_w) water activity decreases with time by drying.

1.3.1 Starter cultures for sausage fermentations

Despite the implemented hurdle-technology, the rise of unwanted, potentially pathogenic and spoilage microorganisms cannot be completely excluded. Contamination of raw ingredients used in the production of fermented sausages can take place during primary production as well as during processing and storage (De Filippis et al., 2013; Kameník, 2013; Nout, 1194; Nychas et al., 2008). Pork is known to function as a reservoir for *Yersinia enterocolitica* (Bowman et al., 2007; Terentjeva and Berzins, 2010) and *Salmonella spp.* (Berends et al., 1997; Lammerding et al., 1988; Zhao et al., 2001), whereas beef meat is often contaminated with haemorrhagic *Escherichia coli* (STEC/EHEC) (Elder et al., 2000; Rhoades et al., 2009; Vogt and Dippold, 2005) and *Listeria monocytogenes* (Fantelli and Stephan, 2001; Jay, 1996; Rhoades et al., 2009). For example, in 2002, Sweden reported a large outbreak of enterohaemorrhagic *E. coli* infection, which was traced back to the consumption of fermented sausages (Sartz et al., 2008). Moreover, the prevalence of *Clostridium perfringens* (Ladiges et al., 1974; Taormina et al., 2003), *Staphylococcus aureus* (De Boer et al., 2009; Waters et al., 2011) and *Campylobacter spp.* are widely reported (Dallal et al., 2010; Zhao et al., 2001). Further risks emerging from the spontaneous growth of autochthonous microbiota refer to their production of biogenic amines or the spread of resistances to antimicrobial substances through mobile genetic elements. Other unwanted metabolic traits of spoilage organisms may have negative impacts on the final product like discoloration, off-odor or slime production (Borch et al., 1996; Dainty et al., 1989; Nychas et al., 2008). The prevalence of such organisms do not meet the requirements to hygienic quality, which is clearly defined as absence of pathogenic and spoilage organisms. In order to reduce the prevalence of such microorganisms, starter cultures with defined properties are added additionally to the meat batter. Starter cultures, however, are preparations of living or dormant microorganisms, whose metabolic activity has desired effects in the fermenting substrate (Hammes and Knauf, 1994). The use of defined and adequately characterized starter cultures, which are well adapted to the respective substrate, not only minimize potential risks deriving from members of the autochthonous microbiota, but enable a directed and controlled fermentation process and deliver a harmonized product quality (Ammor and Mayo, 2007; Hugas and Monfort, 1996; Lücke, 2000).

Considering food fermentations, lactic acid bacteria (LAB), especially species of the genus *Lactobacillus* are primarily responsible for microbial transformations during the fermentation of the respective substrate (Ross et al., 2002). Thereof, it is unsurprising that the vast majority of starter cultures for food fermentation belong to this bacterial group. However, commercially available starter cultures also encompass species belonging to the genera *Pediococcus*, *Kocuria* and *Staphylococcus* (Hammes, 1990; Lücke, 1994). For each product, its desired taste and texture, a certain starter culture or a certain combination of starter cultures have to be

selected (Leroy, 2004). Indeed, the vast majority of sausage fermentations is driven by a combination of starter cultures, mostly lactobacilli and CNC such as *Staphylococcus carnosus* (Laranjo et al., 2017). According their metabolic characteristics Lactobacilli are used for rapid acidification while staphylococci are needed for the reduction of nitrate and nitrite leading to the desired stable red curing color (Hammes, 2012). Moreover, Staphylococci are helpful for reducing the risk of fat rancidity and contribute to the development of flavor and texture by degradation of proteins and amino acids. The introduction of competitive LAB starter strains is known as an important method suggested to retard the formation of biogenic amines (BA) by preventing the growth of amine-producing bacteria in meat products, which leads to health-related benefits (Ammor and Mayo, 2007; Sarkadi, 2016).

1.3.2 *Lactobacillus sakei* and *Lactobacillus curvatus* as starter cultures

The genus *Lactobacillus* is considered the most abundant bacterial group in fermented sausages and belongs to the order of LAB (Hutkins, 2006; Kröckel, 2013). In general, lactobacilli are characterized as rod- and cocci-shaped, gram-positive, non-sporing and facultatively anaerobic or microaerophilic bacteria with a maximal size of 2 µm (Hutkins, 2006). According to their metabolic pathway and the resulting end-products, lactobacilli are commonly classified either as homo- or heterofermentative (Marth and Steele, 2001).

According to the generally accepted definition given by Orla-Jensen (1919) and Kandler and Weiss (1986) as well as Hammes and Vogel (1995) obligate homofermentative lactobacilli ferment hexoses almost exclusively via the Embden-Meyerhof-Parnas (EMP) pathway to the only end-product lactic acid, whereas heterofermentative lactobacilli are specialized for pentose degradation via the pentose phosphate pathway, leading to the production of lactic acid, acetate or ethanol and CO_2. However, facultative heterofermentative lactobacilli are capable of using both pathways. Hexoses are degraded using the EMP-pathway, whereas pentoses are metabolized via the phosphoketolase pathway described for heterofermentative lactobacilli. However, the variety in their metabolism reflects their biodiversity. The genus *Lactobacillus* is characterized by a large degree of diversity as it encompasses more than 200) different species (Catalogue of microorganisms, DSMZ) and 17 subspecies, which partly differ widely in their genomic and metabolic properties (Goldstein et al., 2015).

Lactobacillus (*L.*) *sakei* and *L. curvatus* are facultatively heterofermentative bacteria and display the most abundant species in meat and fermented sausages (Kröckel, 2013). However, they can be isolated from a variety of habitats besides meat including sauerkraut, sourdough and fish (Arendt et al., 2007; Champomier-Verges et al., 2001; De Vuyst et al., 2014; Lhomme et al., 2014; Vogel et al., 1993).

L. sakei and *L. curvatus* are predominantly used as starter cultures and their main functions in sausage fermentation are the acidification and the inhibition of undesired autochthonous microbiota by the production of organic acids, mainly lactic acid (Ammor and Mayo, 2007; Lücke, 2000; Samelis et al., 1994). While acidifying the meat batter, LAB contribute to the coagulation of muscle proteins, resulting in increased slice stability, firmness and cohesiveness of the final product (Ammor and Mayo, 2007; Hugas and Monfort, 1997; Ordóñez et al., 1999). Additionally, they enhance the spontaneous reduction of nitrite to nitric oxides, which reacts with myoglobin to nitrosomyoglobin (Ammor and Mayo, 2007).

Both, *L. sakei* and *L. curvatus* are well adapted to the conditions in sausage fermentation or the niche meat in general (Hammes and Knauf, 1994). Especially for *L. sakei* the adaptation to meat and fermented sausage as well as the ability to use nutrients encountered in meat is well described (Zagorec and Champomier-Verges, 2017). *L. sakei* was shown to use a wide variety of amino acids and peptides from proteolysis, and ribose, which originates from abundant RNA. Furthermore, *L. sakei* can handle stresses like curing, refrigeration or oxidative stress that can occur during meat mixing or storage under oxygen enriched modified atmospheres (Leistner, 2000; Zagorec and Champomier-Verges, 2017). In 1996, *L. sakei* has been divided into two subspecies: *L. sakei* subsp. *carnosus* and *L. sakei* subsp. *sakei* (Torriani et al., 1996) and some recent studies revealed several clusters within the species (Chaillou et al., 2009; Chaillou et al., 2013) suggesting a heterogeneity and high intraspecies diversity, which may therefore lead to differences in adaptation and the ability to compete within the environment of fermented sausage. The further subdivision of *L. curvatus* in *L. curvatus* subsp. *curvatus* and *L. curvatus* subsp. *melibiosus* was shown to be obsolete (Koort et al., 2004). Nevertheless, a high intraspecies diversity is clearly indicated by a variety of RAPD-PCR studies (Aznar and Chenoll, 2006; Berthier and Ehrlich, 1999).

1.3.3 Assertiveness of starter cultures in sausage fermentation

The assertiveness of starter cultures and the ability to override competitors deriving from the natural autochthonous microbiota within the environment meat and during sausage fermentation is affected and influenced by several factors and conditions. Therefore, one major challenge for improving and controlling industrial fermentation processes is the uncovering of adaptation and assertiveness mechanisms.

Meat is a restricted niche (Labadie, 1999). In general, meat as well as raw fermented sausages are characterized by relatively small amounts of available carbohydrates like glucose but harbors high amounts of proteins and free amino acids (Hernández-Macedo et al., 2011; Labadie, 1999; Ray and Bhunia, 2013).

Furthermore it is widely reported that meat is characterized by varying glycogen levels of up to 1.8 % in resting muscle tissues (Immonen and Puolanne, 2000; Immonen et al., 2000), which increases the availability of glucose. Triacylglycerides can be cleaved by endogenous meat lipases leading to the release of free fatty acids and more glycerol (Dave and Ghaly, 2011). The availability of ribose is given by the post-mortem breakdown of nucleotides (Bendall, 1973; Eskin and Shahidi, 2012; Lee and Newbold, 1963).

Only bacteria, which have adapted their metabolism to these conditions are able to grow, compete and may dominate the meat batter in the end. Moreover, within this ecological niche, the inhabiting bacteria have to cope with challenging environmental conditions such as rapid reduction of pH, high salt contents and relatively excessive changes in temperature profiles.

As *L. sakei* and *L. curvatus* strains are known to produce lactic acid as main product of their metabolism, they are relatively resistant to low pH. Furthermore, they usually possess acid resistance mechanisms like F1F0-ATPase, glutamate decarboxylases or arginine deiminase (ADI) systems, which protect them against produced acids (Wang et al., 2018). Moreover, their resistance to high salt contents and changing temperature profiles were investigated before (Kask et al., 2003). Ammor et al. (2005) reported that *L. sakei* can grow at 4°C, in the presence of 6.5 % NaCl and at a pH of 4.2. Its psychotrophic character may be explained by the effective accumulation of osmo- and cryoprotective solutes such as betaine and carnitine (Lahtinen et al., 2011).

However, a suitable starter culture should be able to counteract various stress factors including not only salt and osmotic stress but also cold stress and oxidative stress. Stress can be defined as a change in the environment that results in a decrease in the growth rate or survival of microorganisms (Spano and Massa, 2006; Tsakalidou and Papadimitriou, 2011). In order to survive changing conditions and growth-restricting stimuli, bacteria have evolved several stress response mechanisms regulated at various levels including transcription, translation and metabolite level (van de Guchte et al., 2002) that may be key parameters responsible for the assertiveness within restricted niches.

LAB are facultatively aerobic microorganisms, which reduce pyruvate to lactate to regenerate NAD+ from NADH (Serrazanetti et al., 2013). They do not need oxygen for growth and in fact, a negative effect of oxygen on these bacteria has been observed several times. This negative effect of oxygen can be explained by the formation of reactive oxygen species (ROS), which have significant effects on growing cells. The ability to respond to ROS with the activation of specific enzymes enabling a detoxification may be of crucial importance within sausage meat batter and during fermentations as oxygen is introduced during meat mixing or storage (Zagorec and Champomier-Verges, 2017).

The cytotoxicity as well as reported DNA damage of H_2O_2 was widely reported (Imlay and Linn, 1988; Winterbourn, 1995) and reported to be based on the further partial reduction to hydroxyl radical (·OH) and hydroxid ions (HO^-)(Linley et al., 2012; Martín and Suárez, 2010). The latter reaction requires iron (Fe^{2+}) and is commonly known as Fenton reaction (Winterbourn, 1995). As raw fermented sausages provide big amounts of iron, the reduction of hydrogen peroxide to cell-damaging ROS is likely. Hydroxyl radicals are potentially oxidizing agents leading to mutations, deletions, single strand breaks and other types of DNA damage (Cadet et al., 1999; Tsunoda et al., 2010) as well to damages of bacterial proteins (Ezraty et al., 2017) and lipids (Kashmiri and Mankar, 2014; Ortiz de Orué Lucana et al., 2012).

In general, ROS molecules can be detoxified and neutralized by conversion into water by non-enzymatic antioxidants such as NADPH and NADH pools, β-carotene, ascorbic acid, α-tocopherol and glutathione (GSH) (Cabiscol Català et al., 2000). Hydrogen peroxide can be detoxified via various enzymatic pathways, e.g. NADH peroxidase or catalase activity (Mishra and Imlay, 2013). Lactobacilli are known to encode heme-dependent or manganese-dependent catalases as well as for NADH oxidases (Delwiche, 1961; Johnston and Delwiche, 1965; Whittaker, 2012), enabling the decomposition of H_2O_2 but not all strains are necessarily able to express those enzymes. For these strains the secondary defenses including DNA repair system and proteolytic and lipolytic enzymes is another possible adaptation to oxidative stress (Cabiscol Català et al., 2000). However, the adaptation to oxidative stress and development of primary as well as secondary defense mechanisms may play a crucial role for the assertiveness of strains during sausage fermentation and needs to be elucidated.

Cold-shock response of bacteria refers to an abrupt shift of an exponentially growing culture from its optimum temperature to a lower temperature and the cellular response to these changes (Phadtare and Severinov, 2010). As starter cultures are mostly used as freeze-dried powder (Carvalho et al., 2004), the cultures previously underwent a massive cold-shock before they are added to the still frozen meat batter. The described temperature downshift decreases the fluidity of membranes, affecting active transport and protein secretion (Phadtare and Severinov, 2010). In addition, the efficacy of transcription and translation is reduced due to the stabilization of secondary structures of DNA and RNA. Protein folding is inefficient as well and, ribosomes need to be adapted before they can function properly (Phadtare, 2004) leading to a transient arrest of cell growth for 3 to 6 hours (Graumann and Marahiel, 1996; Phadtare, 2004). The cold-shock lead to a transient induction of cold-induced proteins (Cips) such as cold shock proteins (Csp). After this acclimation phase, cells become adapted to low temperatures and resume growth at a lower growth rate (Phadtare, 2004). The adaptation to cold-shock and the resumption of cell growth is an essential ability in raw sausage fermentation and its respective environment and may significantly influence the assertiveness of a strain.

Besides their resistance towards oxidative stress and cold stress, other expressed traits may play a role in the assertiveness of a strain. The production of antimicrobial peptides and proteins (AMPs) of bacterial origin may lead to an intensively inhibition of competitors, enabling a selective advantage for the producer strain (Li et al., 2012), but was not documented for sausage fermentations so far. AMPs are a class of naturally occurring molecules produced as first line of defense (Zhang and Gallo, 2016). Bacteriocins are small antimicrobial peptides (AMPs), which acts mostly due to the inhibition of cell wall synthesis or the destruction of the latter by pore formation (Hassan et al., 2012). In recent times, bacteriocins have attracted interest for their use as safe food preservatives, as they are easily digested by the human gastrointestinal tract (Mills et al., 2011). The application for biopreservation demands usually includes the following approaches: inoculation of food with the bacteriocin-producing strain, addition of purified or semi-purified bacteriocins food additive and the use of a product previously fermented with an bacteriocin-producing strain as an ingredient in food processing (Chen and Hoover, 2003; Silva et al., 2018). Nisin is one of the most popular bacteriocins frequently used in food industry in cheese and pasteurized cheese. The activity spectrum include numerous gram-positive bacteria including LAB, pathogens such as *Listeria ssp*. and *Staphylococcus spp.* as well as the spore forming bacteria *Bacillus* and *Clostridium* (Chen and Hoover, 2003). Due to the inhibition of competitors, the production of bacteriocins can enable a selective advantage of the producer cells and may be a decisive reason for the dominance of a strain.

One other possible assertiveness determining property are viruses that specifically infect only bacteria. Those viruses are commonly known as bacteriophages and account together with prophage-like elements, pathogenicity islands and phage morons for most of the genetic diversity of a species. Phages are likely to be numerically the most prominent biological systems on earth, with an estimated population size of $\geq 10^{30}$ phage particles (Chibani-Chennoufi et al., 2004). The infection of bacteria with phages can display a serious problem in industry processes due to complete or partial losses of bioproducts and the spread of bacteriophages throughout the whole laboratory (Czyz et al., 2001) as susceptible bacteria are lysed.

Controversial discussions about the role of prophages within bacterial genomes and their impact on the assertiveness of a strain are still ongoing. Phages have various possible life cycles which dictate their role in bacterial biology (Clokie et al., 2011). The lytic life is characterized by a rapid infection and killing of host cells, thereby shaping bacterial population dynamics and occasionally assisting in their long term evolution via generalized transduction (Clokie et al., 2011). The lysogenic life cycle in contrast, characterized by a phage infection

without the direct killing of their host. The phage genome is integrated into the host genome, or exist as plasmids within their host cell (Clokie et al., 2011).

First of all, prophages constitute a metabolic burden for the bacteria as they need additional energy to replicate the prophage genome. Long time, it was undiscovered how prophages may compensate the additional costs for their host. Nowadays, beneficial effects of prophages on their host are described and reported. The explanation is based on the lysogenic conversion (Clokie et al., 2011). The prophage can alter the phenotype of the host bacterium by expressing genes that positively influence the fitness and competitiveness of the strain. One popular example for prophage-encoded metabolic traits is the gene associated with *Vibrio cholerae* which encodes the toxins that cause cholera symptoms (Clokie et al., 2011). Another example can be found in the temperate phage EV3 (Picozzi et al., 2015), whose dextranase may contribute actively to the fitness of its host *L. sanfranciscensis*.

So far, it is unclear if prophage influence the assertiveness of starter cultures in sausage fermentation. Nevertheless, literature reported that they may contribute to the fitness of a bacterium in its respective environment and may need confirmation with regard to possible markers for the assertiveness of strains.

The mentioned and described factors may all contribute to the fitness and assertiveness of bacteria in their respective environments. However, it was not investigated whether these factors are also important for starter cultures during sausage fermentation and if these factors are mutually responsible for the assertiveness of a strain or if only one of them is driving the assertiveness.

1.4 Matrix-Assisted-Laser-Desorption/Ionization-Time-Of-Flight Mass Spectrometry

The above discussion about starter cultures and their pivotal role in meat and sausage fermentations as well as the mentioned spoilage organisms and bacteria of potential pathogenic character, illustrate the importance of methods to track those starter cultures and autochthonous bacteria within the fermentation process to enhance food safety and quality.

Whereas it is generally sufficient to identify spoilage bacteria to the species level, the complex technological features of starter cultures are strain specific and require higher taxonomic resolution and sensitivity (Pavlovic et al., 2013). Especially, the uncovering of strategies in the assertiveness of strains demand for reliable methods to determine their competitiveness and assertiveness within the niche fermented sausage. In the following section, light is shed on

traditional identification methods in comparison to the potential of MALDI-TOF-MS (matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry).

1.4.1 Identification of bacterial isolates by conventional strain typing techniques

Conventional species differentiation methods mostly rely on biochemical criteria and require time-consuming and laborious pre-experiments (Wieser et al., 2012). Still, in the daily routine of food microbiology laboratories, traditional culture-based methods are used to identify the vast majority of food-associated bacteria (Pavlovic et al., 2013; Pavlovic et al., 2015). A discrimination on strain level is rather difficult and affords specific techniques. Concerning the value of strain typing methods the three main characteristics that need to be considered are its typeability, reproducibility and discriminatory power (Hunter, 1990). In the following, common strain typing methods are described and discussed.

One of the still most popular methods to routinely identify bacterial isolates is based on polymerase chain reaction (PCR), which enables a rapid detection, identification and differentiation (Adzitey et al., 2013; Settanni and Corsetti, 2007). PCR is still considered the gold standard with respect to the identification of isolates on species level using universal 16S rRNA primer, which is followed by sequencing of the generated PCR product (Olsen et al., 1992; Olsen and Woese, 1993). The 16S rRNA gene of bacteria contains highly conserved as well as hypervariable regions. For species identification, those highly conserved regions are used as target to produce species-specific PCR products, which can be further analyzed by sequencing and bioinformatic analysis. Besides, RAPD PCR works with arbitrary primers (typically 10-mer primer) and is used to amplify segments of target DNA under low-stringency conditions (Adzitey et al., 2012; Hadrys et al., 1992; Wassenaar and Newell, 2000). It is useful for the differentiation of different species within samples but lacks discriminatory power on strain level for the vast majority of isolates. Reliable identifications below species level using PCR demand for knowledge about strain specific genes, which are only accessible after laborious whole genome sequencing and bioinformatic analysis. Moreover, the technique can get very costly when high-throughput identifications are demanded and colony-PCR, which is easier to handle than laborious DNA extraction protocols, are not always sufficient for each species and strain. Other techniques include the pulsed field gel electrophoresis (PFGE), applicable for separating larger pieces of DNA by applying electrical current that periodically changes directions in a gel matrix (Adzitey et al., 2013; Parizad et al., 2016). PFGE is considered the gold standard typing method by many researches around the world for foodborne pathogen outbreak investigations (Adzitey et al., 2013; Alonso et al., 2005). This method is characterized by its reproducibility and its discriminatory power but it is sensitive to genetic instability, has limited availability and requires at least 3-4 days to complete a test

(Wassenaar and Newell, 2000). Multilocus sequence typing (MLST) is based on the identification of multiple housekeeping genes within a species but is not practicable for strain differentiation. One of the oldest techniques is the plasmid profile analysis, especially used of epidemiological investigations (Wachsmuth et al., 1991). Plasmid DNA is extracted and separated using simple agarose gel electrophoresis. The technique is easy in handling but suffer from poor reproducibility as plasmids are extrachromosomal elements that can spontaneously be lost or readily acquired by bacteria and thus isolates that are related can easily display differences in their plasmid profile. The most reproducible and reliable method for strain differentiation and identification is the whole genome sequencing. In the recent time, DNA sequencing is widely used for identifications of bacterial strains but the method is still time-consuming, laborious, and costly and requires deep knowledge and analysis skills, which cannot be provided in industrial environments.

In summary, the previously described methods do not meet the requirements of the food industry for a rapid, easy-to-handle and reliable high-throughput identification on strain level.

Identification of bacterial isolates by MALDI-TOF MS

Mass spectrometry is an analytical technique in which chemical compounds are ionized into charged molecules and their ratio of mass to charge (m/z) is determined (Singhal et al., 2015). Mass spectrometry has been used for decades in chemistry, but Anhalt and Fenselau (1975) were the first to propose the use of mass spectrometry with respect to the characterization and identification of bacteria. In their study, they were able to observe that extracts from bacteria of different species produced unique mass spectra. In the following, other scientists explored the use of a variety of other mass spectrometry techniques such as fast-atom bombardment (FAB) and electrospray ionization (ESI) (Ruelle et al., 2004).

Karas et al. (1987) first proposed the use of MALDI-TOF-MS, which revolutionized the identification of microbial isolates on species level respectively (Karas et al., 1987). The method is based on the analysis of sub-proteome patterns and allows a rapid and cost-effective differentiation and identification for a broad range of microorganisms (Sauer and Kliem, 2010) and can fulfill the requirements for high-throughput analysis.

The general concept seems fairly simple: the analyte (bacterial sample) is mixed with a matrix, resulting in a co-crystallization, which is loaded onto a stainless steel target plate. The whole target is introduced into the machine, where it is transported to the measuring chamber. The individual samples are exposed to short laser pulses, leading to the vaporization of analyte and matrix and in the following to the ionization of ribosomal proteins. An electromagnetic field, created by a potential of about 20 kV, accelerates the ions before they enter a vacuum flight

tube. The potential energy E_p is related to the charge of an ion (z) and the potential difference of the field (U). When the ions enter the field-free vacuum-flight tube the potential energy is converted to kinetic energy $E_{kin.}$, which is depended on the mass (m) and velocity (v) of the respective ion. The velocity however, is defined as the quotient of the way in the tube (d) and the required time (t) for its flight.

$$E_p = z \cdot U \qquad E_{kin} = \frac{1}{2} m v^2 \qquad v = d/t \;\gg z \cdot U = 1/2\, m \left(\frac{d}{t}\right)^2 \gg t = \frac{d}{\sqrt{2U}} \cdot \sqrt{\frac{m}{z}}$$

The time period the ions need to reach the end of the tube, is precisely measured by a detector. The degree of ionization as well as the mass and charge of the proteins determines their individual time-of-flight (TOF). Based on these TOF information, a characteristic spectrum is recorded and constitutes a specific peptide mass fingerprint (PMF) unique for a given species and/or strain (Singhal et al., 2015; Wieser et al., 2012). The identification of the analyte (bacterium in the case of microbiological analysis) takes place by comparison of determined PMF to a database. The identification of bacterial isolates a typical mass range m/z of 2-20 kDa is used (Wieser et al., 2012). This range is strongly dominated by highly abundant ribosomal proteins and few housekeeping genes, which represent 60-70 % of the dry weight of a microbial cell (Murray, 2012; Singhal et al., 2015).

In the past, different sample preparation methods have been evaluated for different groups of microorganisms. Some bacteria can be identified by direct cell profiling, were a single colony is directly spotted onto the target plate, whereas other bacteria need elaborate extraction methods (Singhal et al., 2015). Extraction methods were proven beneficial for identifying gram-positive species as they are characterized by a thicker cell wall (20-80 nm) than gram-negative bacteria (<10 nm) (Mai-Prochnow et al., 2016). It is commonly known, that these differences in the cell envelope confer different responses to external stress signals including heat, UV irradiation and antibiotics (Mai-Prochnow et al., 2016). A comprehensive review of the diverse sample-preparation methods used in bacterial profiling via MALDI is available (Šedo et al., 2011).

The first MALDI-TOF MS system capable of microbial identification was developed by Bruker Daltonics (Singhal et al., 2015). The “MALDI Biotyper” consisted of a basic MALDI-TOF platform, operating and analysis software, an onsite database and simple methods for extraction and preparation (Seng et al., 2009). In collaboration with bioMérieux, Shimadzu introduced another MALDI platform with the analysis software “Launchpad” and the centralized database “SARAMIS” (Clark et al., 2013). Later, Andromas were able to develop a different

kind of software and database, which was compatible with both Bruker and Shimadzu instruments (Emonet et al., 2010).

The greatest break-through was achieved with its regulatory approval for routine identification of bacteria and fungi in clinical microbiological laboratories (Singhal et al., 2015). MALDI Biotyper CA Systems from Bruker Daltonics Inc. have been approved by the US Food and Drug Administration (FDA) for the identification of cultures bacteria from humans specimens (Singhal et al., 2015). Besides, the Vitek MS system from bioMerieux Inc. was also approved by the FDA for the identification of cultures bacteria and yeasts (Patel, 2015; Singhal et al., 2015). Nowadays, both systems are approved in China as well (Luo et al., 2015; Singhal et al., 2015). With ongoing research MALDI-TOF MS, with special emphasis on the databases as key components, was continually improved. Both, the Bruker and Shimadzu systems offer a large collection of organisms, which are continually updated and expanded (Singhal et al., 2015).

Nowadays, MALDI-TOF MS is implemented as a powerful tool for bacterial identification and its success at genus and species level is widely reported and manifold (Duskova et al., 2012; Munoz et al., 2011; Nacef et al., 2017). Its success at strain level has proven more elusive (Sandrin et al., 2013) but its potential for differentiation below species level has been demonstrated (Dieckmann et al., 2008; Dieckmann and Malorny, 2011; Kern et al., 2014; Lartigue et al., 2009; Nagy et al., 2011). Arnold and Reilly reported strain level profiling with MALDI-TOF MS for E. coli by the use of a mathemathical algorithm that relied upon statistical correlation (Arnold and Reilly, 1998). Dieckmann et al. used biomarker peaks and weighted pattern matching (Dieckmann et al., 2008), whereas Teramonto et al. focused on ribosomal subunit proteins of *Pseudomonas putida* strains as biomarkers (Teramoto et al., 2007).

1.5 Motivation, aim and working hypotheses

MALDI-TOF MS is an emerging high-throughput tool, which has revolutionized the identification of bacterial isolates. Compared with conventional identification methods, this technique has proven to be time- and cost-effective as well as easy in handling. Although this technology has the potential to enable not only species but strain-level differentiation and identification, its use for this particular approach remains widely unnoticed. One major reason for this can be found in the fact that strains of the same species often display quite homogenous sub proteome mass spectra, which increases difficulties in differentiation and identification. The necessity of the establishment of an adequate database with respective database entries is indeed time-consuming and laborious. While the monitoring of diverse species within microbial populations of raw fermented sausages were investigated, the fate of the individual single starter strain of a species remains widely unclear. Studies investigating the development of single strains are scarce and mostly based on elaborate techniques, which require DNA extraction and respective knowledge. Since detailed studies investigating the fate of certain starter cultures or bacteria in general are completely missing, there exists a substantial lack of knowledge regarding strategies and key parameters enabling the observable assertiveness of a strain. In addition, the development of new starter cultures, which are of great economically importance, is a further promising approach. The unmasking of key parameters or even markers enabling the assertiveness and an efficient inhibition of autochthonous microbiota may be of great interest not only for the research field but for the industry working with sausage fermentations and/or starter cultures. By the determination of such markers newly isolated starter culture candidates could be determined and their value for the starter culture industry assessed. The implementation of MALDI-TOF MS as a reliable tool in standard analysis protocols could revolutionize safety aspects as risks deriving from the autochthonous microbiota could be strongly reduced due to a rapid in-house detection.

Against this background, the overall aim of this study was to contribute to the closing of the essential gap of knowledge regarding the assertiveness of strains in general and the assertiveness of *L. sakei* and *L. curvatus* strain in raw fermented sausages in particular. Moreover the uncovering of strategies and key parameters enabling the expression of dominance and assertiveness was of major concern to enable respective improvements in fermentation and the development of new starter cultures.

The work accomplished in this thesis can be divided into three sections: (I) implementation of MALDI-TOF MS as a reliable tool for the identification of bacterial isolates on strain-level, (II) determination of intra- and interspecies assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations and (III) the uncovering of strategies and key parameters responsible for assertiveness in sausage fermentations.

(I) MALDI-TOF MS serves as a high-throughput technology for monitoring of *L. curvatus* and *L. sakei* on strain level

The aim within this chapter was to prove MALDI-TOF MS suitability and discriminatory power for the purpose of isolate identification and differentiation on strain level. The method was subsequently used for the monitoring of population changes within the microbiota of sausage fermentation.

Working hypotheses:

- A database can be developed, which enables MALDI-TOF MS for rapid, high-throughput identification monitoring of introduced starter cultures with sufficient discriminatory power and accuracy. The main limits towards a wider use of MALDI-TOF MS for strain identification is based on database entries, not on the depth of field of the technology.
- Spectra of different strains of a given species are unique enough to enable a strain-specific differentiation and identification
- The sub proteome spectra are of dynamic character and vary with culture conditions thus highlighting the importance of standardized sampling and preparation procedures.

(II) Assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations

The results on the first chapter enabled to focus on the exploration of the assertiveness of *L. sakei* and *L. curvatus* in raw fermented sausages. Consequently the aim of this chapter was the assessment of intra- as well as interspecies assertiveness of both species and suppression of the autochthonous microbiota and unmasking of responsible strategies. For small scale fermentations in the laboratory, a sausage model system was established.

Working hypotheses:

- The established sausage model system can provide insights into the assertiveness of strains and can successfully be verified among industrial large scale fermentations.

- The assertiveness of single strains can be determined using MALDI-TOF MS
- *L. sakei* as well as *L. curvatus* show dominance over the autochthonous meat microbiota
- Strains of both species, *L. sakei* and *L. curvatus*, are not equally adapted and display intraspecies variations in their assertiveness in raw sausage fermentations.
- Raw sausage fermentations are dominated by one competitive strain through competitive exclusion.
- Assertiveness varies not only depending on the strain, but on the species used. *L. sakei* strains are generally more adapted to the niche than *L. curvatus* strains, leading to distinct differences in their assertiveness.

(III) Unmasking of strategies and key parameters responsible for the assertiveness of a strain

The aim of the last chapter of this thesis was to uncover strategies and key parameters responsible for the assertiveness of strains within the sausage environment. Those parameters could be used as markers for the development of new future starter cultures.

Working hypotheses:

- Assertiveness derives from the strains adaptation to the respective environment and can be traced back to single parameters or a combinations of parameters like general growth or stress response mechanisms. Insights into assertiveness in raw sausages may be gained by the investigation of bacterial physiology in a respective meat simulation medium.
- Antimicrobial substances may play a pivotal role in the expression of assertiveness and dominance of a strain as it enables single dominance by the inhibition and killing of competitors.
- Bacteriophages are a major driving force of bacterial evolution and diversity and contribute to the fitness of their bacterial hosts by carrying beneficial genes.

2 Materials und Methods

In this chapter, only the general methodology is described unless stated otherwise. Special experimental designs will be addressed within the corresponding result chapters.

2.1 Microorganisms, media and culture conditions

2.1.1 Microorganisms

Table 1: Bacterial strains included in this thesis.

Species	Strain TMW No.	Isolation source
L. sakei	1.2	sausage
	1.3	sausage
	1.4	sausage
	1.13	starter culture
	1.22	starter culture
	1.23	sausage
	1.30	unknown
	1.46	starter culture
	1.114	starter culture
	1.147	sausage
	1.148	unknown
	1.149	unknown
	1.150	unknown
	1.151	unknown
	1.152	unknown
	1.153	unknown
	1.154	unknown
	1.155	unknown
	1.161	starter culture
	1.163	starter culture
	1.165	unknown
	1.402	sauerkraut
	1.411	sauerkraut
	1.412	sauerkraut
	1.417	starter culture
	1.454	sausage
	1.578	starter culture
	1.579	starter culture
	1.588	starter culture
	1.589	starter culture
	1.1189	DSM 20017 T
	1.1239	sourdough
	1.1240	sourdough
	1.1290	sausage
	1.1322	meat
	1.1365	unknown
	1.1366	starter culture
	1.1383	starter culture
	1.1385	starter culture
	1.1386	starter culture

	1.1388	starter culture
	1.1392	starter culture
	1.1393	starter culture
	1.1395	starter culture
	1.1396	starter culture
	1.1397	starter culture
	1.1398	starter culture
	1.1399	starter culture
	1.1407	fermented fish
	1.1474	sauerkraut
L. curvatus	1.7	DSM 20019 T
	1.27	unknown
	1.48	starter culture
	1.49	starter culture
	1.50	starter culture
	1.51	starter culture
	1.166	unknown
	1.167	unknown
	1.401	sauerkraut
	1.407	sauerkraut
	1.408	sauerkraut
	1.421	sausage
	1.439	sausage
	1.440	salami
	1.587	starter culture
	1.591	unknown
	1.594	starter culture
	1.595	starter culture
	1.596	starter culture
	1.624	sausage
	1.1291	sausage
	1.1365	unknown
	1.1381	starter culture
	1.1382	starter culture
	1.1389	starter culture
	1.1390	starter culture
	1.1391	starter culture
	1.1408	sauerkraut

2.1.2 Media and culture conditions

All media were autoclaved for 20 min at 121°C.

2.1.2.1 Baird-Parker Agar (BP)

Baird-Parker medium was used for the cultivation of Staphylococci and for CNC and CPS in general. The basic medium contained 10 g/l peptone from casein, 5 g/l meat extract, 1 g/l yeast extract, 5 g/l lithium chloride, 12 g/l glycine and 10 g/l sodium pyruvate. Additionally, 0.24 g/l potassium tellurite and 50 ml egg yolk suspension (5 ml egg yolk from fresh eggs and 45 ml dH_2O) were included. Lithium chloride and potassium tellurite suppress the growth of accompanying flora, while pyruvate and glycine selectively facilitate the growth of staphylococci. The use of egg yolk enables the detection of lecithinase activity indicated by clear zones around visible colonies. Due to tellurite reduction staphylococci produce dark grey or black colonies. For solid media 20 g/l agar was added to the medium. The pH was adjusted to a final pH of 6.8 ± 0.2. Staphylococci were incubated aerobically at their optimal growth temperature of 37°C.

2.1.2.2 Modified De Man, Rogosa and Sharpe Medium (mMRS)

De Man, Rogosa and Sharpe developed the MRS media, which serves to cultivate lactic acid bacteria in general including the species *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* (De Man et al., 1960). The slightly modification was used to enable a better growth of the strains included in this thesis. The basic mMRS contained 10 g/l peptone from casein, 10 g/l meat extract, 5 g/l yeast extract, 2 g/l dipotassium hydrogen phosphate, 5 g/l sodium acetate, 2 g/l ammonium citrate and 1 g/l Tween 80®. For the production of one liter mMRS-media, all components were dissolved in 800 ml dH_2O and autoclaved at 121°C for 20 min. magnesium sulfate (0.2 g/l) and manganese sulfate (0.05 g/l) were prepared separately as stock cultures and added after the sterilization of the media by sterile filtration. For routine growth and due to manifold requirements of lactic acid bacteria the media was enriched with 20 g/l glucose as carbon source. The glucose was also autoclaved and added separately to avoid the formation of Maillard-products. For biodiversity experiments another modified MRS-medium was introduced and characterized by a low amount of 0.02 % glucose. For solid media 15 g/l agar was added to the medium. The pH was adjusted to 6.25. In order to guarantee an anaerobic environment liquid cultures were incubated in tightly closed bottles or plastic tubes, whereas agar plates were incubated using Anaerocult systems (Oxoid, Basingstoke, United Kingdom). For preconditioning experiments the basic mMRS recipe was modified by the addition of either 2.9 % (w/v) nitrite curing salt, 0.7 % (w/v) glucose or both.

2.1.2.3 **Modified meat simulation medium (mMSM)**

The meat simulation medium, inspired by the recipe of Verluyten (Verluyten et al., 2003) and the recipe of sausage meat batter derived from a producer in Germany, was developed to simulate growth conditions during raw sausage fermentation. The medium contained 20 g/l peptone from casein, 16 g/l Lab Lemco, 8 g/l Yeast extract, 29 g/l nitrite curing salt, 7 g/l glucose, 0.4 g sodium ascorbate and 1 g/l Tween80® as well as 0.2 g magnesium sulfate ($MgSO_4$*7 H_2O) and manganese sulfate ($MnSO_4$*H_2O). The pH of the modified meat simulation medium was adjusted using lactic acid to a final pH of 6.25, the incubation temperature applied for the cultivation of lactobacilli on meat simulation medium was set to 24°C.

2.1.2.4 **Tryptone-Solution (TS+)**

The Tryptone-Solution (TS+) contains 15 g/l tryptone from casein, 8.5 g/l NaCl and 0.1 ml Antifoam B as foaming controlling agent. The pH was adjusted to 7.0 ± 0.1. TS+ was used to prepare dilutions and inocula for this study.

2.1.2.5 **SM-Buffer**

SM-Buffer contains 5.8 g NaCl, 2 g $MgSO_4$*7 H_2O and 50 ml TRIS-HCl (pH 7.5) and was stored at room temperature.

2.1.2.6 **Culture conditions and storage**

If not stated otherwise, cultivation and incubation was carried out in mMRS with a pH of 6.25 at 30°C. Reaction tubes were filled to the maximum with the respective medium to ensure an oxygen-reduced nearly anaerobic environment. Microtiter plates were overlaid with paraffin oil and agar plates were incubated using the Anaerocult system (both Merck Millipore, Billerica, USA).

All strains were streaked out on mMRS agar plates at 30°C. Single colonies were used for the verification of the strains correct annotation. Subsequently, 15 ml of mMRS in reaction tubes were inoculated with a single colony and incubated at 30°C for 24 h. Afterwards cells were harvested by centrifugation and re-suspended in 1.8 ml fresh mMRS. Portions of 800 µl were then mixed with 800 µl glycerol for long-term storage at -80°C. From those glycerol stock cultures, strains were reconstituted for usage by streaking on mMRS agar plates.

2.1.2.7 **Preparation of pre-cultures**

Usually, mMRS liquid pre-cultures were used to obtain cell suspensions for all kind of experiments. 15 ml of mMRS with a pH of 6.25 were inoculated with a single colony and incubated for 24 h at 30°C.

2.2 Bacterial physiology and stress response

2.2.1 Growth kinetics

Growth kinetics were determined either by hand using a standard photometer or using microplate readers. Measurements in microplate readers were performed either with a spectrophotometer Novaspec II (Pharmacia Biotech, Uppsala, Sweden) or a Tecan Sunrise Plate Reader (Männedorf, Swiss).

2.2.1.1 Stress response to oxidative stress

To determine the oxidative stress tolerance, all 78 Lactobacillus strains were challenged with 8 different concentrations of H_2O_2 (0.0 mM, 1.53 mM, 3.13 mM, 6.25 mM, 12.5 mM, 25.0 mM, 50.0 mM & 100 mM) to test their ability to cope with the presence of oxidative stress. Overnight cultures of the strains were prepared to inoculate microtiter plates (to an initial OD of 0.1), which were previously filled with 200 µl of hydrogen peroxide containing mMRS-media. To prevent the plate from drying 50 µl of paraffin oil were added. Bacterial growth was measured once a day by the determination of the optical density at 590 nm with FLUOstar (BMG Labtech, Ortenberg, Germany). Collected data was analyzed for the specific time point where the maximum OD_{590nm} was reached. Hydrogen peroxide was purchased as a 30% aqueous solution from Sigma-Aldrich (Merck, Darmstadt, Germany). Raw data were analyzed and visualized using MS Excel (Microsoft, Redmond, USA). Growth parameters like lag-phase, growth rate and maximum OD were determined using the Grofit-script for R-Studio (Kahm et al., 2010).

2.2.1.2 Stress response to cold stress

For the induction of cold stress and possible stress responses O/N cultures of *L. sakei* and *L. curvatus* strains were used for inoculation of two 50 ml medium containing main cultures. One of the cultures is directly used for growth detection, the other culture was first frozen for 3 hours at -20°C and then used for growth detection via OD measurement at 590 nm. The completely frozen culture was defrosted using a 25°C tempered water bath. Samples were taken at regular time points every hour for OD and pH determination. Raw data were analyzed and visualized using MS Excel (Microsoft, Redmond, USA). Growth parameters like lag-phase, growth rate and maximum OD were determined using the Grofit-script for R-Studio (Kahm et al., 2010).

2.3 Proteomics – Matrix-assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry

The inter- and intraspecific biodiversity within the sub proteome of the species are determined using MALDI-TOF MS (matrix-assisted laser desorption ionization – time of flight – mass spectrometry). This method, based on the analysis of ribosomal proteins, allows a rapid and cost-effective differentiation and identification of microorganisms. MALDI-TOF MS is considered as a powerful instrument for bacterial identification on species level, whereas an identification on strain level is not well established so far. This could be due to the fact that protein profiles do not differ as strongly as the genome of the strains. Nevertheless, strains of a species show specific protein expression profiles, which may vary depending on the state of the culture, the media or the abiotic conditions. However, due to natural differences in the protein expression these profiles also differ when culture conditions are standardized. These differences on sub proteome level normally correlate with differences in their genetic level and are part of the biodiversity which should be depicted in this study. For isolate identification via MALDI-TOF MS two general methods were applied. On the one hand colonies can be applied directly onto the stainless steel MALDI target enabling a fast isolate identification, on the other hand colonies can be treated with an extraction method like ethanol extraction enabling a better resolution concerning the protein mass spectra.

2.3.1 Main spectrum profiles for database creation

For the establishment of a reference mass spectra database containing “Main Spectra Libraries” of all strains, lactobacilli were cultivated anaerobically on mMRS agar supplemented with 2 % glucose (v/w) at 30°C. Database entries were determined after 2 and 3 days of incubation by direct application and after 2 days of incubation using an ethanol extraction protocol.

2.3.1.1 MSP-creation using direct application

A total of 12 colonies per strain were picked with a sterile toothpick onto stainless steel MALDI target plate. Spots were covered with 1 µl of 70% formic acid (FA) and then overlaid with 1 µl of alpha-cyano-4-hydroxy- cinnamic acid (α-CHCA matrix for MALDI-TOF MS ≥ 99.9 %, Sigma Aldrich, Darmstadt, Germany), which was previously prepared with a final concentration of 10 mg/ml (50.0 % acetonitrile (ACN), 2.5 % trifluoroacetic acid (TFA) and 47.5 % dH_2O). All colonies were measured three times in a row to obtain a total of 24 protein mass spectra for each strain.

2.3.1.2 MSP creation using ethanol extraction

Due to the observed robustness of lactobacilli cell walls the traditional method of Kern (Kern et al., 2013) was modified concerning incubation times. For the cell extraction procedure cell material of the respective culture was harvested using a sterile incubation loop and resuspended in 300 µl dH_2O while vortexing. Bacteria were inactivated by the addition of 900 µl ethanol (70%) and proteins were extracted using 50 µl formic acid (70%) and 50 µl acetonitrile. Cell debris was then spun down and 1 µl of the supernatant was spotted onto a clean MALDI target plate.

2.3.2 Routine sample preparation

Isolate identification on species- and on strain-level during experiments other than for MSP creation was achieved using the direct application method. Colonies were picked with a sterile toothpick onto a stainless steel MALDI target plate. All spots were covered first with 1 µl of 70 % formic acid and then overlaid with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid, saturated solution in 2.5% TFA / 50% ACN).

2.3.3 MALDI-TOF MS parameters and data processing

Mass spectra were acquired using an Microflex LT MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm) at a laser frequency of 60 Hz operating in a linear positive ion detection mode under the FLEX CONTROL software version 3.4 (Bruker Daltonics GmbH).The mass range covers an area from 2.000 to 20.000 Da at a voltage of 20.00 kV (ion source I), 16.80 kV (ion source II), 6.00 kV (lens) and 2939 kV (linear detector). The laser power was adjusted between 38 to 40 % with an offset ranging from 48 to 50 %. Calibration of MALDI-TOF MS was performed once a week with a bacterial test standard (BTS) calibrate mixture, which is based on modified *Escherischia coli*. BTS (Bruker Daltonics, Bremen, Germany) was resuspended in 100 µl organic solvent (50.0 % ACN, 2.5 % TFA and 47.2 % dH_2O) and stored at -20°C.

Each spectrum was obtained by averaging 240 single spectra recorded by 40 laser shots steps in an automatic mode at the minimum laser power necessary for the ionization of the sample. The identification takes place by comparing collected spectra to the Bruker reference database containing a variety of especially medically relevant isolates. The obtained spectra function as a “molecular fingerprint” and are species- and probably strain- specific. Raw data of mass spectra were exported and processed with flexanalysis 3.4. All exported mass spectra were pre-processed by subtracting the baseline, smoothing and normalizing signal intensities using a self-tailored MASCAP (Mantini et al., 2010), which was implemented in octave software. Pre-

processed mass spectra were used for peak detection by picking peaks which show the highest intensity among their nearest points.

For hierarchical clustering of reference MSPs (HCA), 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.

2.4 Assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations

The assertiveness of *L. sakei* and *L. curvatus* was investigated by two different approaches. First, the assertiveness was determined using a fermented sausage model to simulate the conditions during sausage fermentation, followed by an industrial ring trial approach, where salami was produced in pilot scale fermentations.

2.4.1 Assertiveness of *L. sakei* and *L. curvatus* in a fermented sausage model system

2.4.1.1 Fermented sausage model system

The experimental setup of the competition studies included a fermented sausage model system to simulate the conditions during the first 5 days of fermentation, during which growth and primary metabolism are prominent. Frozen standardized salami meat batter with a contamination of ca. 10^3 to 10^4 CFU g^{-1} on mMRS media (enabling growth of LAB and CNS) were obtained directly from a producer in Germany and stored at -20°C until usage. For each experiment 150 g salami meat batter were transferred into sterile polypropylene cups (Kuehnle, Karlsruhe, Germany) and inoculated with a total of 10^6 cells of *L. sakei* or *L. curvatus* strains. All cultures for competition studies were maintained in glycerol stocks. Depending on their previously determined CFU ml^{-1}, a certain amount of the latter was added to TS+ Medium to obtain a total CFU/g sausage batter of 10^6 cells. A conventional mixer was used to spread the TS+ (total volume of 3 ml) within the meat. To simulate the conditions of sausage fermentation, an equal number of 10^6 cells of *Staphylococcus carnosus ssp. carnosus* TMW 2.212 were added additionally to the meat batter. This strain showed a very reproducible behavior in sausage models of a different study (data not shown) and was selected for its assertiveness.

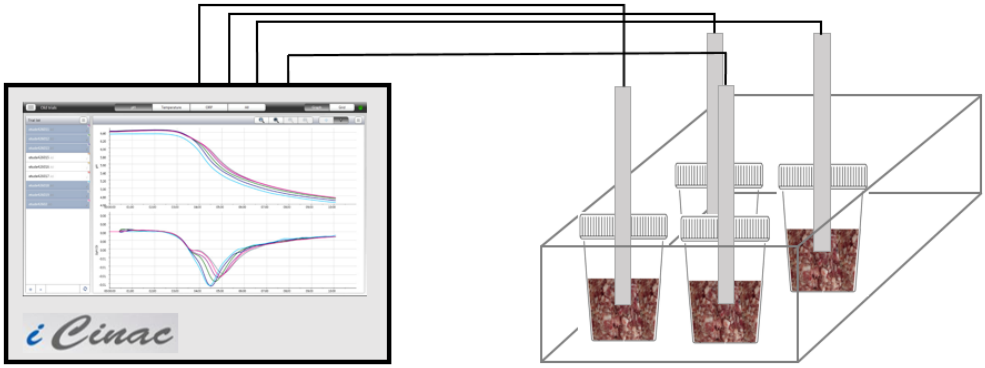

Figure 3: Fermenting sausage model system. The implemented model system consists of an iCinac analyzer and a water bath to ensure the temperatures needed for fermentation are controlled. pH and redox potential was monitored continuously over a fermentation time of 5 days.

2.4.1.2 **Combination of starter sets**

For evaluation of the competitiveness of strains in model fermentations four different sets of strains were combined (Table 2). In order to depict a representative biodiversity of the species, strains were chosen from different isolation sources such as sauerkraut, sour dough, meat or starter cultures. Traceability, i.e. differentiation, of the strains during the fermentation process was considered as key criterion for the selection and determination of starter sets for competition experiments. At the same time different proteome based MALDI-TOF MS patterns suggest different metabolic settings. The strains were therefore selected in consideration of best strain-specific traceability given by the findings of the validation of the established “in-house” database. To warrant optimal recognition specificity incubation time were 2 days. Log_{10} CFU g^{-1} for lactobacilli was determined on mMRS agar after 2 days of anaerobic incubation, whereas staphylococci were determined on Baird-Parker (BP) after 3 days of aerobic incubation.

Table 2: Combination of starter sets defined for competition studies. Selection was based on previous evaluation of strain recognition rates via MALDI-TOF MS.

Lactobacillus curvatus group		*Lactobacillus sakei* group	
set I	set II	set III	set IV
1.27	1.27	1.3	1.46
1.401	1.407	1.114	1.578
1.421	1.439	1.417	1.1189
1.624	1.595	1.1239	1.1396
1.1381	1.1390	1.1398	

2.4.1.3 **Sampling**

The pH and redox potential (ORP) were monitored continuously by using the iCinac system (AMS Systea, France). Samples were withdrawn at regular intervals on day 0, 2 and 5 and analyzed for the growth of lactobacilli by spreading appropriate dilutions onto mMRS using glass beats (diameter 2.85 – 3.45 mm, Roth, Karlsruhe, Germany). At each sampling point 10 g of the salami meat batter were homogenized with 90 ml TS+ solution for 1 minute in a BagMixer® 400 W (Interscience International, Saint Nom, France). For comparison a starter free meat batter was used and analyzed the same way. After 2 days of incubation a total of 96 colonies per plate were analyzed via MALDI-TOF-MS on species- or strain-level to determine the relative abundance of species and strains of the total microbiota as a parameter of the competitiveness. All experiments were repeated at least two times (biological replicates).

2.4.2 Assertiveness of *L. sakei* and *L. curvatus* in pilot scale fermentations

Large scale fermentations were conducted in cooperation with three different companies, which either produce starter cultures or fermented sausages.

2.4.2.1 Selection of strains and strain sets

Based on the findings made upon database evaluation, four sets of starters were defined for the determination of assertiveness during raw fermented sausage fermentation (Table 3). The strains selected had proven assertive in the competition studies using a fermented sausage model. Each set contained *S. carnosus ssp. carnosus* TMW 2.212 and either only one, two or three strains of lactobacilli. For each *Lactobacillus* species one set with a single-dominant strain and one set with a combination of co-dominating strains were employed. All starter cultures were maintained at -80°C in glycerol stocks with a previously determined CFU ml^{-1}. All participating companies received pre-mixes of respective strains or combination of strains. Bacteria were cultivated either on modified MRS (mMRS) medium (De Man et al., 1960) for lactobacilli or on BP medium (Baird-Parker, 1962) for growth determination of staphylococci. Salamis were produced with set I-IV. Each set was analyzed twice by two different companies to obtain two biological replicates. The composition of included sets is given in Table 3. Furthermore, every company was advised to produce a control batch without the addition of any starter culture.

Table 3: Strain sets included in ring-trial experiments.

Set	***L. curvatus***				***L. sakei***			***S. carnosus***
	1.624	**1.417**	**1.1390**	**1.3**	**1.417**	**1.1398**	**1.1396**	**2.212**
Set I	●	●	●	●	●	●	●	●
Set II	●	●	●	●	●	●	●	●
Set III	●	●	●	●	●	●	●	●
Set IV	●	●	●	●	●	●	●	●

2.4.2.2 **Meat**

Meat batter for salami production by all companies was obtained from one producer in Germany, which also participated in the ring trial experiments (company I). The meat batter was produced with the following ingredients: 52.4 % pork meat, 28.4 % beef meat,15.3 % bacon, 2.9 % NPS, 0.4 % dried glucose syrup, 0.3 % dextrose, 0.3 % pepper and 0.04 sodium ascorbate. Cellulose fibrous casings were used for sausage filling to a total weight of 300 g. The caliber used was 60 mm. Batch sizes varied depending on the company from 10 kg to 20 kg.

2.4.2.3 **Salami manufacturing and fermentation conditions**

Every company included in the ring trial experiments participated in the production of raw fermented sausages (salami). The contribution of experiments with the sets I-IV are listed in Table 4.

Table 4: Distribution of experiments listed for set I-IV along participating companies.

Company 1	Company 2	Company 3
Set II	Set I	Set I
Set IV	Set II	Set III
	Set III	Set IV

Every company was instructed to clean and disinfect all equipment used in the production process of raw fermented sausages before and after each ring trial experiment to avoid cross contaminations. The inoculum for the experiment was prepared by mixing the premix glycerol-culture of the strain or the strain mixture with 200 ml TS+ medium to a total CFU g^{-1} salami of 10^6 cells each for lactobacilli and staphylococci. Afterwards the inoculum was spread and chopped evenly within the minced meat. Residual amounts of inoculum were transferred using sterile Pasteur pipettes. The meat batter were subsequently filled into fibrous casings to a total weight of 300 g. Ripening was carried out in a climatic chamber. The applied fermentation and ripening conditions are listed in Table 5. Parameters like ventilation were defined individually based on the experience of the respective company.

Table 5: Fermentation scheme or ring trial experiments over a total time of 21 days.

day	temperature [°C]	humidity [%]
0-1	24	92
1-2	20	91
2-3	16	88
3-5	16	86
5-6	16	84
6-7	16	81
7-14	16	78
15-21	16	78

2.4.2.4 **Sampling and sample analysis**

Samples (two sausages for each experiment and set) were withdrawn at regular intervals on day 0 (production day), 5, 12 and 21 and shipped cooled via express delivery. About 10 g, representing the entire cross section of the individual salami, were taken using sterile scalpels. Salami samples were homogenized with 90 ml TS+ and blended for 1 minute using a BagMixer (Interscience, Saint Nom, France). Samples were then analyzed for the growth of both lactobacilli and staphylococci by spreading appropriate dilutions onto mMRS or BP. After two (lactobacilli) or three (staphylococci) days of incubation a total of 96 colonies per plate were analyzed via MALDI-TOF MS on species- or strain-level to determine the relative abundance of species and strains of the total microbiota as a parameter of assertiveness. Results were calculated as the mean of log colony-forming units (log_{10} CFU g^{-1}) and the mean of relative abundance [%]. Moreover samples were analyzed for weight, water activity using a LabMaster a_w (Novasina, Lachen, Switzerland) and for pH using a handheld stick pH-meter.

2.5 Bacteriocin production

Genbank files of the strains including whole genomes sequences were available including predicted open reading frames (ORFS) and protein translations. Genomes were used for the identification of putative bacteriocin clusters with the web server tool BAGEL3 (http://bagel.molgenrug.nl/).

Lactobacillus cultures were screened for bacteriocin production by an agar spot test slightly modified from van Reenen (van Reenen et al., 1998). All cultures as potentially bacteriocin-producing strains were grown in mMRS broth for 24 h at 30°C. After 24 h the supernatant was harvested by centrifuging the culture. A cell-free solution was obtained by filtration through a 0.2 µm-pore-size filter (Sarstedt, Nümbrecht, Germany). The supernatant was then treated

with catalase (3000 U ml^{-1}) for 30 minutes to avoid an inhibition due to possibly produced hydrogen peroxide. Aliquots (5 µl) of the sterile supernatant were placed 4-times on agar plates previously seeded with the indicator bacteria (approximately 10^6 CFU ml^{-1}). After incubation in an anaerobic Gas-Pack system (AnaeroGen compact, Oxoid, Basingstoke, UK) at 30°C for 24 h, all plates were examined for inhibition zones and existing zone diameters were measured.

The proteinaceous nature of the inhibitory compounds was confirmed by testing their sensitivity to the proteolytic enzymes Trypsin (Sigma, USA) and Proteinase K (Sigma, USA).

2.6 Bacterial prophages

2.6.1 Detection of prophages within bacterial genomes

All strains were sequenced at the Ziel institute (Freising, Germany) using a MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA). Genbank files of the strains including whole genome sequences were available, including predicted ORFS and protein translations (Eisenbach et al., 2019; Eisenbach et al., 2018). Whole prophage sequences and prophage related genes within the bacterial genomes were identified using PHAST (PHAge Search Tool) and PHASTER (PHage Search Tool Enhanced Release), a web server tool for rapid identification and annotation of prophages sequences (http://phaster.ca/). The program is able to distinguish between incomplete, questionable and intact prophage sequences and allows an annotation of genes. Predicted proteins were additionally compared with the Blastp tools of NCBI database. One strain was selected for further analysis. The presence of integrated prophages was confirmed by a strain-specific colony-PCR designed for the prophage gene XRE-regulator (Table 6).

2.6.2 Induction of prophages

The functionality of integrated prophages was verified by the induction and observed partial cell lysis using either UV-light (203 nm) or mitomycin C.

2.6.2.1 **Prophage induction with UV-light**

Prophage induction was conducted using UV-light. Growth and prophage induction were observed by the measurement of optical density at 590 nm in standardized microtiter plates. A volume of 100 µl of a previously prepared O/N culture (1%) was used to inoculate 10 ml mMRS medium. 200 µl of this respective main culture were then transferred into a microtiter plate and incubated at 30°C in a microplate reader either Novaspec II (Pharmacia Biotech, Uppsala, Sweden) or Tecan Sunrise Plate Reader (Männedorf, Swiss).

The time interval for OD-measurement was set to 5 minutes for 120 cycles. The UV-light irradiation was realized using a UV-transilluminator when ΔOD_{590nm} reached 0.2-0.3. Different

doses of UV- radiation were applied using an impervious black cardboard. As control and reference the culture was also grown without the application of UV-radiation. After prophage induction the microtiter plate was placed back into the microplate reader for further growth and lysis detection.

In order to harvest phage lysate, prophage induction was conducted using conical flasks. Again, 100 µl of a previously prepared O/N culture was used for the inoculation of 10 ml mMRS-medium. Optical density was determined every 30 minutes manually using a photometer. Again, UV-radiation was applied when ΔOD_{590nm} reached a difference of 0.2-0.3. To ensure UV-light exposure for all cells, liquid cultures were constantly shaken at 90 rpm using a standard laboratory shaker, which was placed beneath the UV-transilluminator. Afterwards, further growth and prophage induction were observed using the microplate reader by determination of optical densities at 590 nm. Incubation temperature was set to 30°C for both, microtiter late and flask in a separate incubator.

2.6.2.2 **Prophage induction using mitomycin C**

Prophage induction was determined using various concentrations of mitomycin C. Mitomycin C was added to growing cultures in exponential phase. Turbidity was measured photometrical at 590 nm to determine cell growth and a possible induction of prophages indicated by the partial lysis of the respective culture.

2.6.2.3 **Production of phage lysate**

Prophage induction was carried out according to 2.6.2.1. The culture was then transferred to a sterile 15 ml reaction tube (Sarstedt, Nümbrecht, Germany) and centrifuged for 15 minutes at 5000 rpm. Supernatant was harvested and filtrated using 0.2 µm pore size membrane filters (Merck Millipore, Burlington, MA, USA). Phage lysate was stored at -80°C for further analysis.

2.6.3 Determination of prophage proteins via LC-MS/MS

For phage protein analysis via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)10 ml of phage lysate were treated with trichloracetic acid (TCA) to a final end concentration of TCA 6.25 % (w/v) for 10 minutes at 4°C. Lysate was then centrifuged at 3500 rpm for 4 minutes at 4°C. The supernatant was discarded and the protein pellet washed twice with 5 ml acetone. After drying, pellet was frozen in liquid nitrogen and stored at -80°C until further usage.

Before separation with liquid chromatography (LC), phage lysate was tryptically digested by the addition of 50 mM DTT, constantly shaken at 400 rpm for 30 minutes at 32°C and 55 mM CAA incubated or 60 minutes at 25°C in the dark.

Protein analysis was carried out with a nanoflow LC-MS/MS (Eksigent nanoLC-Ultra 1D+ system, Eksigent Technologies, Dublin, USA). For enrichment tryptic digested prophage proteins were dissolved in formic acid buffer and applied to the trap-column. Afterwards peptides were transferred to the analytical column. Separation continued with a flow of 300 nl/min and a solvent gradient of solution B to A of 4-32% for a total separation time of 60 minutes. Measurement was conducted data dependent at BayBioMS. Results were preprocessed and summarized at BayBioMs.

2.6.4 Determination of prophage proteins using AF4-MALS

Asymmetric Flow Field-Flow Fractionation (AF4) with Multi-Angle Light Scattering (MALS) was used to determine prophage proteins in UV-treated samples of *L. sakei* TMW 1.1398. AF4 consists of a flat separation channel without any stationary phase. The channel is equipped with an impermeable upper channel plate and a bottom plate with an ultra-filtration membrane. The separation is based by differences in mobility in the flow field induced by liquid flow over the membrane and across the channel. Within the channel a parabolic flow profile is created due to the applied laminar flow of the liquid. The application of the cross-flow as perpendicular force, analytes are driven towards the boundary layer at the channel bottom (accumulation wall). Diffusion forces analytes back towards the center creating a counteracting motion. Smaller particles with higher diffusion rates reach distinctly higher positions in the channel than larger particles or proteins. The longitudinal flow at the channel center is more rapidly than the flow closer to the bottom resulting in an earlier elution of small particles or proteins.

Phages proteins were separated using asymmetric flow field-flow fractionation (AF4). The determination of corresponding molecular weights and sizes of the respective molecule (radiuses of gyration RGi) the AF4 system was combined with a multi-angle laser light scattering (MALS) and a refractive index as quantitative detector.

Phage proteins were separated on a 5 kDa regenerated cellulose membrane with 0.05 M sodium nitrate as carrier eluent. Flow conditions were as follows: injection (0.2 ml/min), elution (1.5ml/min) and cross flow (starting from 3 ml/min to 1 ml/min within 30 minutes). After measurement, the cross flow was kept at 0.1 ml/min for another 30 minutes and then reduced to 0 ml/min within 18 minutes. The flow control was maintained with Wyatt Dawn HELEOS II. The particle size of separated molecules were determined by intensity measurements of scattered light of 18 different scattering angles in the range of 10-160°. Data resulting from AF4-MALS-RI were computationally evaluated and processed using the software ASTRA V 5.3.4.19. Spacer foil: 10-500 µm.

2.6.5 Prophage Electron Microscopy

Phage lysate was produced according to 2.6.2.3 and precipitated with polyethylene glycol (PEG). For phage precipitation, 3 ml of PEG 8000 (20%) and 2.5 M NaCl were added to 12 ml of phage lysate. The solution was mixed and incubated for one hour on ice. Afterwards the precipitated phage solution was centrifuged at 13.000 x g for 10 min and the pellet was resuspended in 1.2 ml SM-Buffer.

The concentrated phage lysate was used for electron microscopy, which was conducted by Prof. Dr. A. Klingl, Chair of Plant Development, Ludwig-Maximilians University, Munich.

2.7 Molecular Biology

2.7.1 Isolation of genomic DNA

DNA was isolated using the “E.Z.N.A® Bacterial DNA Kit” (Omega Bio-Tek Inc., Norcross, USA) according to manufacturer’s instructions. Bacteria were harvested in their late log-phase. Due to centrifugation, enzymatic digestion and degradation by proteases, cells were lysed, cell walls destroyed and removed. After cell lysis, the DNA containing supernatant was applied onto a HiBind column (Omega Bio-Tek Inc., Norcross, USA) and washed to remove micronutrients like salt and protein contaminations. Finally, DNA was eluted and stored at -20°C for further analysis.

Quantification and purity control of isolated DNA was conducted using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). Prior to DNA quantification measurements, the device was initialized with 2 µl dH_2O.

2.7.2 Polymerase-Chain-Reaction

Polymerase-Chain-Reaction (PCR) was mostly conducted without prior isolation of DNA. Single colonies were directly smeared into PCR tubes.

2.7.2.1 Primer Design

Gene sequences of target genes for colony-duplex-PCR were obtained from Eisenbach et al. (Eisenbach et al., 2019; Eisenbach et al., 2018; Janßen et al., 2018). Primers with suitable characteristics like length, amplicon size, G+C content and T_M were determined using the free online tool “SDSC workbench” according to generally accepted rules for primer design.

2.7.2.2 Colony-Duplex-PCR

A strain specific colony-duplex PCR was developed for the most assertive strains in this study, and used for the validation of the accuracy of strain recognition via MALDI-TOF-MS. Strains

used in this study, were tested for strain specific genes (Eisenbach et al., 2017). In brief, pan and core genomes were compared via BlAst Diagnostic Gene findEr (BADGE) (Behr et al., 2016) with default settings, but “megablast percent identity cut” and “megablast within group qscov” were changed to 90 or 0.90 and “min DMG occurrence” was set at 0.1.

MALDI-TOF MS results were successfully validated by the combination of specific and universal primers. The latter was designed for the *dnaK* gene and allowed to test DNA accessibility and to avoid false negative results. Single colonies were picked for strain identification via MALDI-TOF-MS and used for inoculation of master-plates. These were incubated anaerobically at 30°C and used for colony-duplex PCR. Primers were obtained from Eurofins (Ebersberg, Germany) and are listed in Table 6. The amplification was carried out in strips (Braun, Wertheim, Germany) on a gradient Master Thermocycler (Eppendorff, Hamburg, Germany) in a total volume of 50 µl. The optimum annealing temperature of 58°C was determined by gradient PCR between 50 and 60°C with 1.1°C increments. Conditions for the duplex PCR assay were as follows: one single colony was picked and smeared into a 50 µl PCR tube. The mastermix consisted of 5 µl of 10 x reaction buffer ($MgCl_2$ included), 1 µl of a deoxynucleoside triphosphate mix (100 nM each), 0.25 µl of *Taq* polymerase (0.5 U; Amersham Pharmacia Biotech, Piscataway, N.J.), 0.25 µl each of the specific and universal primers (100 pmol) and deionized H_2O to a final volume of 50 µl. The PCR program was as follows: initial denaturation (95°C for 60 s) followed by 32 cycles of denaturation (95°C for 20 s), annealing (58°C for 60 s), and extension (72°C for 120 s). All other PCR reactions were conducted the same way by switching to other primer pairs.

Table 6: Overview of universal and strain specific primers applied in the PCR reactions.

oligo	sequence [5´-3´]	amplificate size	target gene
1.624F	AATGTCAGGGACAAACAGCC	429 bp	peptide ABC transporter ATP-binding protein
1.624R	CCTATGATGCCAAACCCAGT		
1.1390F	TACTATGTTGCTTGTCGGCG	213 bp	glycosyl transferase
1.1390R	TGGTCCCATACGACATGCTA		
1.3F	GAGTATGCTTCGGGTGGTGT	236 bp	hypothetical protein
1.3R	TGAATCACGTCCGGATTGTA		
1.417F	CAACGCCGCTATTAATGGTT	670 bp	sodium proton antiporter
1.417R	CACACCAACGATCAACCAAG		
1.1396F	AAGCGAGATAATCAAGGCGA	306 bp	exopolysaccharide biosynthesis protein
1.1396R	AAGCCCTTCGATATGGTGTG		
1.1398F	GCTTCTTTAACAGGAGTTGC	408 bp	XRE family transcriptional regulator
1.1398R	TGCTTCGGTGTCACCATTTA		
dnaK_F	CGTATTAGAAGGCGGACAAC	937 bp	dnaK
dnaK_R	GCCTGTCCAGTTCTTAACAG		
4300_F	AAGGAGTATTATTTCTATG	975 bp	hyp. Protein (prophage TMW 1.1398)
Int_R	TACTTATCGCGTTAGAGAC		integrase (prophage TMW 1.1398)
GnatF	CGTTTGGACATTGATGATGC	1670 bp	phage excision
LysR	CATCAACGTGCCATCAATCAA		

2.7.2.3 Analytical agarose gel electrophoresis

Nucleic acids were separated using 1.0-1.3 % agarose gels (in 0.5 x TBE-buffer). By application of an electric tension of 90-130 V in 0.5 x TBE buffer separation of nucleic acids was accomplished. Different gene rulers were used according to the expected size of fragments. Loading dye (6x) was used to achieve the necessary density of the PCR reaction and to visualize the progress of separation. A total volume of 2.5 µl of loading dye were mixed with 10 µl of the PCR reaction and 10 µl were applied to the gel. The staining was carried out using dimidium bromide. Results were documented with pictures taken with a UVT-28M transilluminator (Herolab, Wiesloch, Germany) and an AxioCam camera (Carl Zeiss, Göttingen, Germany).

2.7.2.4 Purification and sequencing of PCR products

PCR products were purified for sequencing using the E.Z.N.A® Cycle Pure Kit (Omega Bio-Tek Inc., Norcross, USA) according to manufacturer's instructions. The purified products were sequenced at GATC Biotech GmbH (Konstanz, Germany) using Sanger`s chain-termination method (Sanger et al., 1977).

3 Results

Since 18-78 strains were characterized within the respective experiments, the majority of data is summarized in tables and/or figures. The result chapters are featured by a short summary of the important findings, followed by detailed subsections and descriptions of the actual data. Moreover, experimental design will be briefly addressed. The results of this chapter partly overlap with or refer to the results of the PhD thesis of Lara Eisenbach, Technical University Munich, as a result of a joint basic study diversifying into different theses.

3.1 Strain-specific monitoring of *L. sakei* and *L. curvatus* using MALDI-TOF MS

Regarding strain-specific monitoring during sausage fermentation MALDI-TOF MS may be of great potential. Nevertheless, it has to be ensured that *L. sakei* and *L. curvatus* strains display enough differences in their sub proteome pattern to enable a reliable differentiation. For this purpose, the biodiversity of sub proteome pattern were investigated aiming to reduce the number of strains prior to database implementation and determination of its discriminatory power.

3.1.1 Sub proteome biodiversity of *L. sakei* and *L. curvatus*

MALDI-TOF-MS profiling was used to depict the biodiversity of the species *L. sakei* and *L. curvatus*. Sub proteome patterns of 49 *L. sakei* and 29 *L. curvatus* strains were acquired after 48 h to ensure proper growth and the formation of big colonies. Summarized spectra were used for cluster analysis according to Chapter 2.3.3. Upon grouping based on differences in sub proteome mass spectra, the biodiversity was depicted. Figure 4 and Figure 5 illustrate the sub proteome biodiversity of *L. curvatus* and *L. sakei*. The distance of the phylogenetic lines within the tree correspond to the similarity of sub proteome pattern. The longer and more branched the lines, the less related are the respective strains. As we just aimed to visualize the relative degree of the strain's relationship, no scale was provided. Whereas for *L. sakei* 3 main groups were found, only 2 main groups were determined for *L. curvatus*.

According to the main aim of this study, the determination of assertiveness in raw sausage fermentations using MALDI-TOF MS, a strain selection was determined according to the biodiversity in sub proteome pattern. By selecting strains of different MALDI-TOF MS groups the probability of a successful discrimination of isolates not only on species- but on strain-level was increased. Moreover, the selection was also checked for covering different isolation sources, resulting in a set with maximum diversity. Finally, 9 *L. sakei* and 9 *L. curvatus* strains were selected for further detailed characterization and determination of the strain-specific assertiveness in raw fermented sausages.

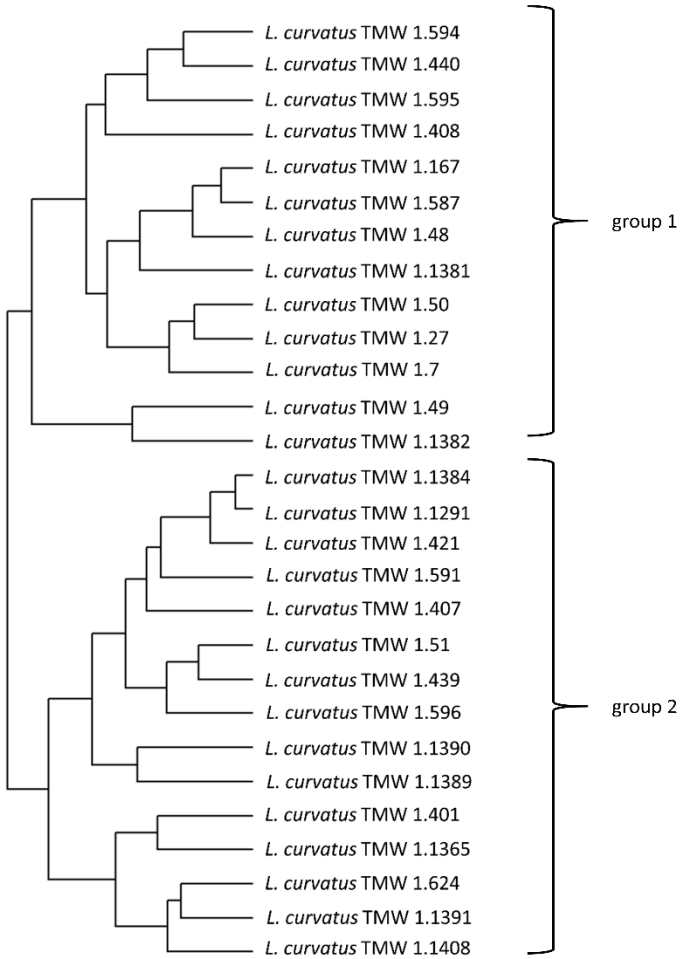

Figure 4: Sub proteome diversity of *L. curvatus*. The figure shows the MALDI-TOF MS cluster analysis of consensus spectra. All consensus spectra were clustered based on Euclidian distance. The results are summarized and shown as dendrogram. The dendrogram encompasses all 29 *L. curvatus* strains, grouped upon their similarity in sub proteome pattern obtained after 48 h incubation on standard mMRS medium.

L. curvatus strains were sorted into two main groups, displaying large diversity also within the groups. Strains were selected for further experiments upon their displayed grouping. Strains, showing similar sub proteome mass pattern should be avoided in order to increase the recognition capacity using MALDI-TOF MS. The *L. curvatus* strains were selected as follows: TMW 1.27, TMW 1.401, TMW 1.407, TMW 1.421, TMW 1.439, TMW 1.595, TMW 1.624, TMW 1.1381 and TMW 1.1390.

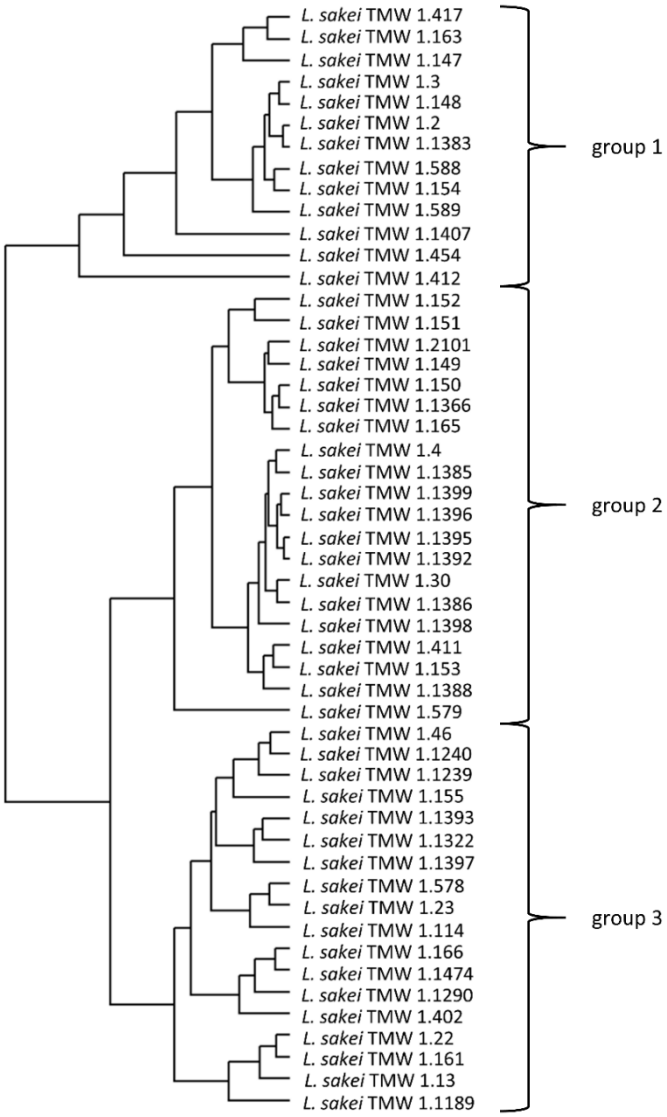

Figure 5: Sub proteome diversity of *L. sakei*. The figure shows the MALDI-TOF MS cluster analysis of consensus spectra. All consensus spectra were clustered based on Euclidian distance. The results are summarized and shown as dendrogram. The dendrogram encompasses all 78 *L. sakei* strains, grouped upon their similarity in sub proteome pattern obtained after 48 h incubation n standard mMRS medium.

Similar results were observed for *L. sakei*. The strains were sorted into three main groups, again displaying large diversity also within the groups. The *L. sakei* strains were selected as follows: TMW 1.3, TMW 1.46, TMW 1.114, TMW 1.417, TMW 1.578, TMW 1.1189, TMW 1.1239, TMW 1.1396 and TMW 1.1398.

3.1.2 Discriminatory power of MALDI-TOF MS on strain level

Discriminatory power of MALDI-TOF MS was evaluated with 9 isolates of *L. sakei* and 9 isolates of *L. curvatus*, previously selected based on biodiversity assessment (see Chapter 3.1.1). Therefore, MSP´s of all strains were acquired and implemented into an in-house database, enlarging the commercially available database of Bruker (Bruker Daltonics). Database entries were generated based on 20-24 high quality spectra. In order to cover up, small changes in sub proteome pattern, MSPs were acquired using two different methods (direct application and ethanol extraction) and incubation times, varying from 2 to 3 days. After implementation of all necessary reference spectra, the established in-house database was validated by 96 measurements per strain using direct application.

Table 7 and Table 8 show the results of similarity calculation for each strain used and the hit rate [%] of the test spectra in comparison to database entries after 2 (Table 7) and 3 (Table 8) days of anaerobic incubation. The green marked boxes are correctly identified samples on strain level and display the correct recognition rate of the strain, whereas the white ones show misidentifications with other database entries. The data confirms that strain recognition with MALDI-TOF MS and a precisely discrimination of strains within the same species is generally possible. The differentiation after 2 days of incubation between the species was achieved by almost 100 % but also the correct strain recognition rates reached values up to 100 % (e.g. for *L. curvatus* TMW 1.595) and only three strains (*L. curvatus* TMW 1.439, *L. curvatus* TMW 1.1381 and *L. sakei* TMW 1.578) showed recognition rates below 70 %. Vice versa, the rate of misidentification ranged from 0 % (e.g. for TMW 1.595) to 15.6 % (e.g. for TMW 1.439) (Table 7). However, for the majority of strains the recognition rates decreased with prolonged incubation times (Table 8). Most of the strains showed lower recognition rates after 3 instead of 2 days of incubation (Table 8), with the sole exceptions of *L. curvatus* strains TMW 1.27 and TMW 1.1390 and the *L. sakei* strains TMW 1.114. TMW 1.578 and TMW 1.1189. Simultaneously, the number of misidentifications with other TMW strains increased. For *L. curvatus* TMW 1.1381 misidentification with *L. curvatus* TMW 1.1390 increased from 4.2 % after two days of incubation to 32.3 % on day 3. In summary, prolonged incubation times led to less distinguishable sub proteome mass spectra. In order to confirm the suitability of MALDI-TOF MS for the purpose of strain identification, a strain specific colony duplex PCR was developed for *L. curvatus* strains TMW 1.624 and TMW 1.1390 and *L. sakei* strains TMW 1.3, TMW 1.417, TMW 1.1396 and TMW 1.1398 (Figure S.1). With the respective PCRs, bacterial isolates, each of MALDI-TOF MS recognized isolates were correctly identified, supporting the validity of MALDI TOF MS recognition at strain level.

Table 7: MALDI-TOF MS database accuracy and similarity calculation after 2 days of anaerobic incubation. Test spectra of the *L. curvatus* and *L. sakei* strains were compared to database entries. The resulting hit rate (%) is displayed after two days of anaerobic incubation as follows: green boxes show the exact strain match (named "recognition rate"), whereas the white boxes show misidentifications of the strain with other embedded spectra of the database. The table is compartmentalized according to the species. The term "nri" stands for not reliable identification.

		L. curvatus									*L. sakei*												
		1.27	1.401	1.407	1.421	1.439	1.595	1.624	1.1381	1.1390	1.3	1.46	1.114	1.417	1.578	1.1189	1.1239	1.1396	1.1398	*L. curvatus*	*L. sakei*	*L. graminis*	nri
L. curvatus	1.27	99.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
	1.401	0.0	88.5	0.0	1.0	3.1	1.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	2.1
	1.407	0.0	0.0	91.7	1.0	3.1	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0
	1.421	0.0	0.0	0.0	95.8	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0
	1.439	0.0	1.0	0.0	0.0	67.7	0.0	1.0	15.6	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.5	0.0	0.0	0.0
	1.595	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.624	0.0	0.0	0.0	2.1	0.0	3.1	93.8	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.1381	0.0	0.0	0.0	1.0	1.0	1.0	0.0	67.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0	0.0
	1.1390	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	96.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0
L. sakei	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	97.9	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0
	1.46	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.0	6.3	1.0	5.2	5.2	0.0	0.0	0.0	0.0	6.3	0.0	0.0
	1.114	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	91.7	0.0	6.3	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.417	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.4	70.8	3.1	10.4	0.0	0.0	0.0	0.0	6.3	0.0	0.0
	1.578	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.2	0.0	66.7	1.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0
	1.1189	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	8.3	3.1	83.3	1.0	0.0	0.0	0.0	2.1	0.0	0.0
	1.1239	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	1.0	2.1	94.8	0.0	0.0	0.0	0.0	0.0	0.0
	1.1396	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	2.1	0.0	1.0	0.0	0.0	92.7	0.0	0.0	2.1	0.0	0.0
	1.1398	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	6.3	92.7	0.0	0.0	0.0	0.0

Table 8: MALDI-TOF MS database accuracy and similarity calculation after 3 days of anaerobic incubation. Test spectra of the *L. curvatus* and *L. sakei* strains were compared to database entries. The resulting hit rate (%) is displayed after three days of anaerobic incubation as follows: green boxes show the exact strain match (named "recognition rate"), whereas the white boxes show misidentifications of the strain with other embedded spectra of the database. The table is compartmentalized according to the species. The term "nri" stands for not reliable identification.

		1.27	1.401	1.407	1.421	1.439	1.595	1.624	1.1381	1.1390	1.3	1.46	1.114	1.417	1.578	1.1189	1.1239	1.1396	1.1398	*L. curvatus*	*L. sakei*	*L. graminis*	nri
L. curvatus	1.27	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0
	1.401	1.0	87.5	0.0	0.0	2.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	6.3
	1.407	0.0	2.1	69.8	4.2	1.0	8.3	1.0	0.0	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.3	0.0	0.0	0.0
	1.421	0.0	0.0	0.0	91.7	3.1	0.0	1.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0
	1.439	1.0	3.1	0.0	0.0	57.3	0.0	0.0	21.9	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.5	0.0	1.0	1.0
	1.595	0.0	0.0	0.0	0.0	0.0	99.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
	1.624	0.0	5.2	0.0	1.0	7.3	2.1	79.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	1.0
	1.1381	0.0	0.0	0.0	2.1	0.0	0.0	0.0	54.2	32.3	2.08	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.4	0.0	0.0	0.0
	1.1390	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	99.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0.0	0	0
L. sakei	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.9	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0
	1.46	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	68.8	9.4	1.0	12.5	4.2	0.0	0.0	0.0	0.0	4.2	0.0	0.0
	1.114	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	93.8	0.0	4.2	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.417	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	8.3	68.8	1.0	2.1	2.1	0.0	1.0	0.0	13.5	0.0	0.0
	1.578	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	29.2	1.0	69.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.1189	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.0	1.0	95.8	1.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.1239	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	7.3	0.0	2.1	8.3	72.9	0.0	0.0	0.0	5.2	0.0	2.1
	1.1396	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0	88.5	6.3	0.0	1.0	0.0	1.0
	1.1398	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	1.0	6.3	90.6	0.0	0.0	0.0	0.0

3.2 Assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations

As presented in Chapter 3.1 the discriminatory power of MALDI-TOF MS enables a reliable differentiation of *L. sakei* and *L. curvatus* on strain-level. In the following, the assertiveness of the respective strains was investigated using an implemented sausage model in combination with MALDI-TOF MS as well as pilot scale fermentations. The results of this chapter have been published in Janssen et al., 2018 (1) and (2).

3.2.1 Intraspecies assertiveness in a raw fermented sausage model system

The intraspecies assertiveness of *L. sakei* and *L. curvatus* strains was assessed using a fermenting sausage model system, imitating raw sausage fermentation conditions. Strains were used in set combinations for inoculation and fermentation of salami meat batter, which was provided from an industrial partner company. The parameters pH and redox potential were monitored continuously using the iCinac system, whereas samples were taken on day 0, 2 and 5 for plating and MALDI-TOF MS analysis.

3.2.1.1 pH and redox potential

Detailed growth dynamics of fermented sausage were monitored for a total of 5 days. In all fermentations (set I-IV), pH decreased from around 6.2 to a final pH of around 4.9. The overall development of the redox potential followed the same typical profile in all fermentations. The redox potential rapidly decreased to a minimum of -40 to -50 mV with all strain sets. After 18 to 24 h of fermentation the ORP starts to increase to a final ORP value of around -3 to -5 mV. The pH and redox potential developments are provided in the supplementary figures (Figure S.2, Figure S.3, Figure S.4, Figure S.5) as they do not contain any noticeable differences.

3.2.1.2 Microbiota development

The identification of isolates based on their low molecular weight sub-proteome MSPs revealed the typical development of microbiota composition in fermentation processes.

Figure 6 shows the relative species abundance and $\log_{10}$ CFU g^{-1} of the sausage batter of samples of set I-IV. At initial and early stage, the sausage microbiota consisted mainly of the inoculated *Lactobacillus* strains. Noticeably, not all inoculated strains were necessarily detectable at day 0 and the $\log_{10}$ CFU g^{-1} determined on mMRS do not correlate with the inoculation of 10^6 cells g^{-1} sausage batter. Furthermore, staphylococci, especially the inoculated strain TMW 2.212, were also able to grow on mMRS so that a part of the $\log_{10}$ CFU g^{-1} on day 0 must be accredited to the detected *Staphylococcus carnosus* strain TMW 2.212. In all experiments (set I-IV) the $\log_{10}$ CFU g^{-1} sausage batter on day 5 reached values of 10^9

or 10^{10} cells per gram for lactobacilli, whereas numbers of staphylococci increased from initial 10^6 to final 10^8 cells per gram in all fermentations. After 5 days of fermentation the autochthonous microbiota was mainly dominated by a selection of the *L. sakei* or *L. curvatus* strains introduced as starters of the respective competitor set. The most abundant and dominating strain recognized among the staphylococci was the introduced *S. carnosus* strain TMW 2.212 by an average of 89 % (Figure S.7).

With regard to the assertiveness of lactobacilli in sausage fermentation, generally two different scenarios were observed in the experiments. Either one strain outcompeted all other members of the starter set and the autochthonous microbiota (competitive exclusion) or two or more strains cooperated to establish a stable dominance (colonization resistance).

Set I (Figure 6, A) and IV (Figure 6, D) are examples for the first situation, where a single strain is capable to dominate the meat microbiota, whereas set II and III are examples for established co-dominances within the sausage batter.

In order to get more detailed information about the strategies within the sausage environment and the dynamics of microbial consortia, experiments were repeated without the most assertive strains.

This omission then seemed to favour the growth of the previously second best strains. For set I (Figure 7, A) *L. curvatus* TMW 1.421 only reached a relative abundance of 11.8 % when tested with the dominant *L. curvatus* strain TMW 1.624. Upon omission of the latter strain, TMW 1.421 prevailed much better and reached a relative abundance of 51 % at day 5 (Figure 7, B).

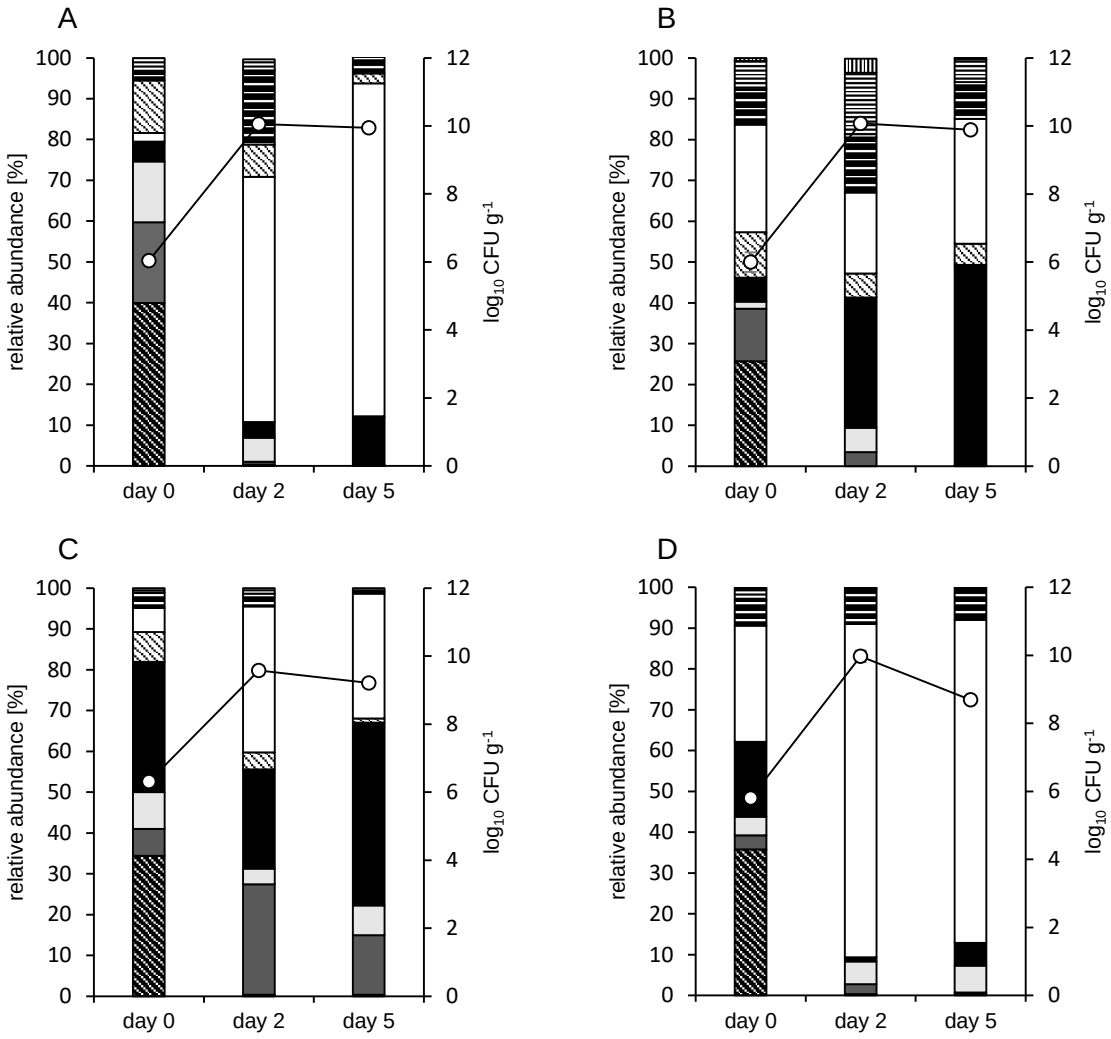

Figure 6: Microbiota dynamics in the sausage model. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 2 and 5] during fermentation. The figure shows the average of three biological replicates with standard deviation of set I (A), II (B), III (C) and IV (D). Detection limit log_{10} 1 CFU g^{-1}. The acronym nri stands for "not reliable identification". Species and strains were identified with MALDI-TOF MS as follows:

Set I (A): (■) *L. curvatus* TMW 1.27, (■) *L. curvatus* TMW 1.401, (■) *L. curvatus* TMW 1.421, (□) *L. curvatus* TMW 1.624, (▨) *L. curvatus* TMW 1.1381, (▤) *L. curvatus* other TMW (▨) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

Set II (B): (■) *L. curvatus* TMW 1.27, (■) *L. curvatus* TMW 1.407, (■) *L. curvatus* TMW 1.439, (▨) *L. curvatus* TMW 1.595, (□) *L. curvatus* TMW 1.1390, (▤) *L. curvatus* other TMW (▨) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

Set III (C): (■) *L. sakei* TMW 1.3, (■) *L. sakei* TMW 1.114, (■) *L. sakei* TMW 1.417, (▨) *L. sakei* TMW 1.1239, (□) *L. sakei* TMW 1.1398, (▤) *L. sakei* other TMW (▨) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

Set IV (D): (■) *L. sakei* TMW 1.46, (■) *L. sakei* TMW 1.578, (■) *L. sakei* TMW 1.1189, (▨) *L. sakei* TMW 1.1396, (▤) *L. sakei* other TMW (▨) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

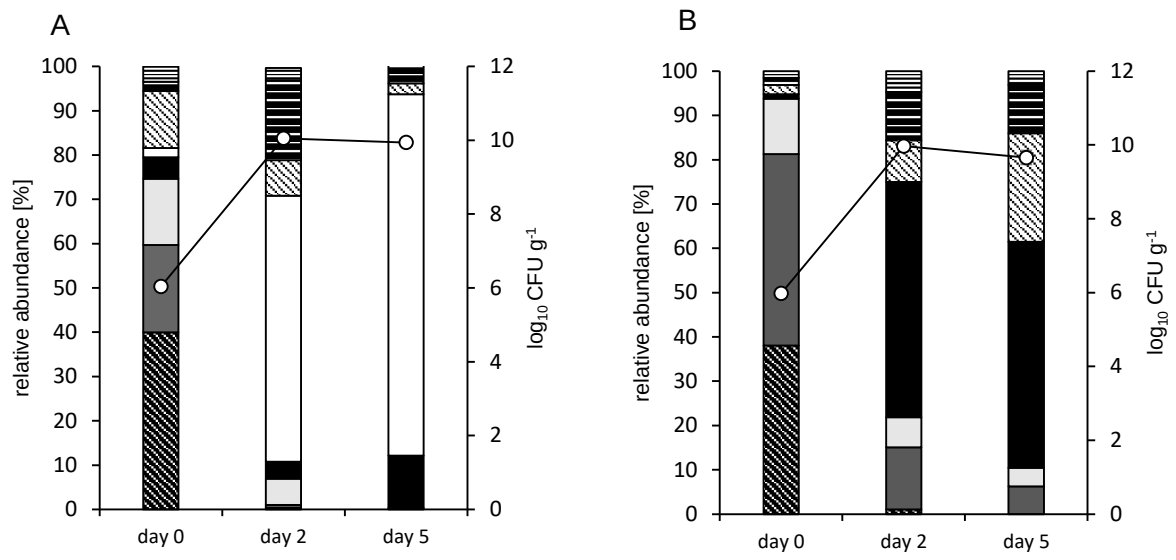

Figure 7: Microbiota dynamics in the sausage model employing starter set I. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 2 and 5] during fermentation of set I (A, average of three biological replicates) and set I without TMW 1.624 (B, average of two biological replicates). The acronym nri stands for "not reliable identification". Species and strains were identified with MALDI-TOF MS as follows: (■) *L. curvatus* TMW 1.27, (■) *L. curvatus* TMW 1.401, (■) *L. curvatus* TMW 1.421, (□) *L. curvatus* TMW 1.624, (▨) *L. curvatus* TMW 1.1381, (▤) *L. curvatus* other TMW (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

This phenomenon was also observed with *L. sakei* strains of Set IV (Figure 6, D and Figure 8, A). *L. sakei* TMW 1.1396, as the most assertive strain, dominated the microbiota with a total relative abundance of around 80 %, whereas the second strongest party TMW 1.578 was able to grow to relatively high cell numbers (67.2 %) when *L. sakei* TMW 1.1396 was omitted in the respective starter set (Figure 8, B). In all cases, members of the autochthonous microbiota were efficiently inhibited.

Differently to the winner strains, which were omitted, the dominance of the "second best strains" was not as expressed, and it was moreover possible to identify other TMW strains, which were also able to grow to relatively high cell numbers. Upon omission of *L. curvatus* TMW 1.624 in set I, it was *L. curvatus* TMW 1.1381 who was capable to grow together with *L. curvatus* TMW 1.421 (Figure 7, B) and for set IV it was *L. sakei* TMW 1.46 who grew together with the second-best strain *L. sakei* TMW 1.578 (Figure 8, B).

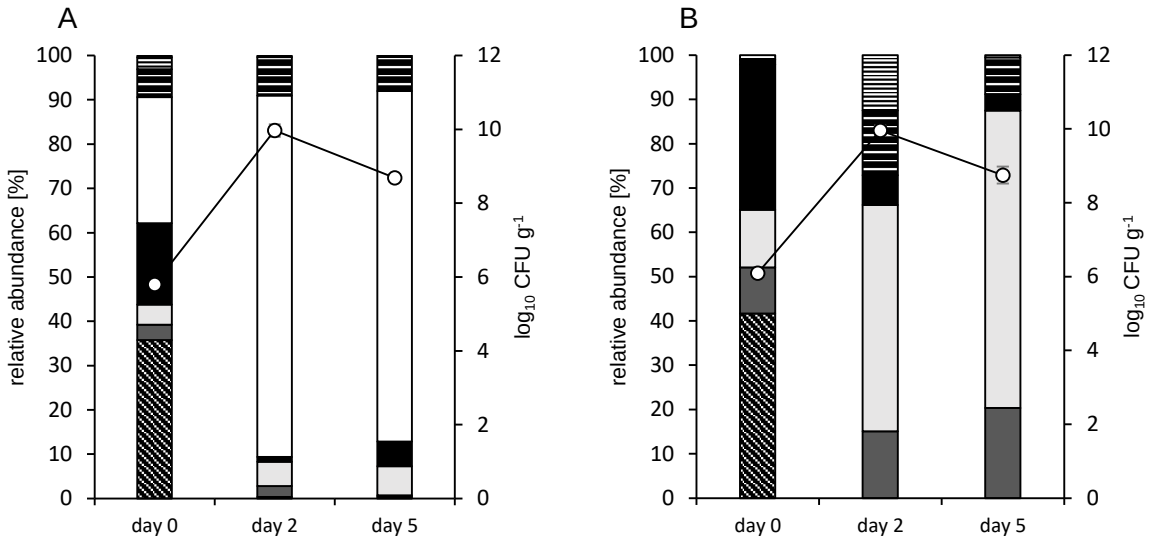

Figure 8: Microbiota dynamics in the sausage model employing starter set IV. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 2 and 5] during fermentation of set IV (A, average of three biological replicates) and set IV without TMW 1.1396 (B, average of two biological replicates). The acronym nri stands for "not reliable identification". Species and strains were identified with MALDI-TOF MS as follows: (■) *L. sakei* TMW 1.46, (□) *L. sakei* TMW 1.578, (■) *L. sakei* TMW 1.1189, (□) *L. sakei* TMW 1.1396, (▤) *L. sakei* other TMW, (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

These findings suggest a second possible strategy to overcome the autochthonous microbiota in fermented sausages, in which the strains were able to establish a co-dominance or even a cooperation. The latter could be observed within strains of both species. In set II (Figure 6, B and Figure 9, A) the *L. curvatus* strains TMW 1.439 and TMW 1.1390 were able to establish co-dominance on day 2 and 5, whereas in set III (Figure 6, C) a total of 3 strains co-dominated the microbiota.

Further experiments were carried out with set II. The omission of respectively one of the assertive strains, either TMW 1.439 or TMW 1.1390, revealed dependencies between them (Figure 9). *L. curvatus* TMW 1.439 alone was not able to dominate the meat microbiota over the whole fermentation time. The acquired dominance on day 2 was not retained and the relative abundance of the strain was reduced to only 6.8 % after 5 days of fermentation (Figure 9, B). *L. curvatus* TMW 1.1390, however, was capable to dominate without *L. curvatus* TMW 1.439 even though to a better relative abundance than originally measured (Figure 9, C).

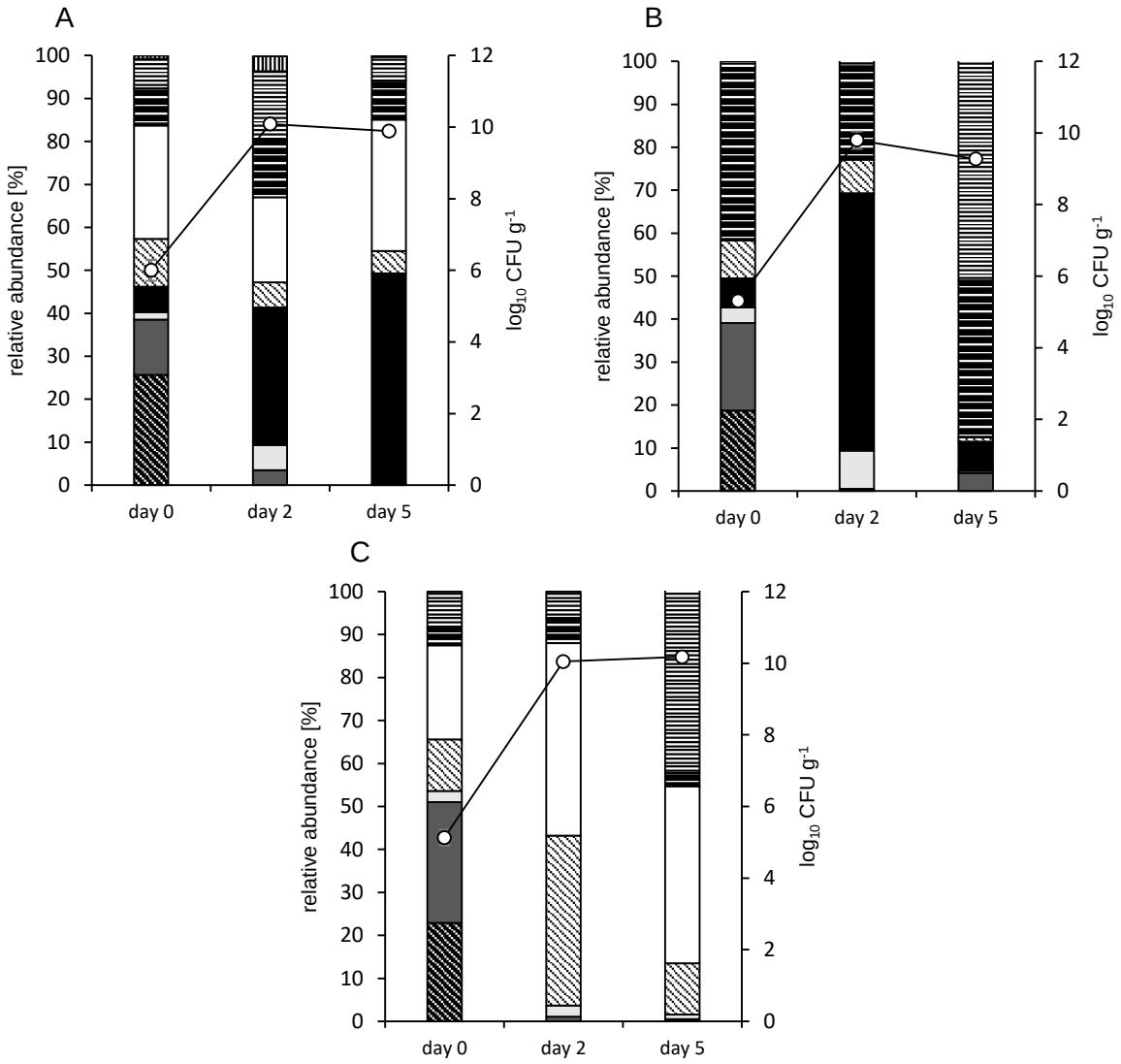

Figure 9: Microbiota dynamics in the sausage model employing starter set II. Total viable count $\log_{10}$ CFU g^{-1}] mMRS and microbiota composition as the relative abundance [%] at defined time points [days 0, 2 and 5] during fermentation of set II (A, average of three biological replicates), set II without TMW 1.1390 (B, average of two biological replicates) and set II without TMW 1.439 (C, average of two biological replicates). The acronym nri stands for "not reliable identification". Species and strains were identified with MALDI-TOF MS as follows: (■) *L. curvatus* TMW 1.27, (□) *L. curvatus* TMW 1.407, (■) *L. curvatus* TMW 1.439, (▨) *L. curvatus* TMW 1.595, (□) *L. curvatus* TMW 1.1390, (▤) *L. curvatus* other TMW (▨) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

L. curvatus TMW 1.439 appeared to be dependent on the presence of *L. curvatus* TMW 1.1390 in the sausage batter and is only assertive due to the presumed cooperation. Indeed, in both fermentation batches, various members of the autochthonous microbiota (e.g. *Lactococcus garvieae. Leuconostoc citreum. Enterococcus feacalis* and *Raoultella ornithinolytica*), named "other", were able to grow to relatively high numbers and represented a large part of the sausage microbiota at day 5. This might be a hint, that *L. curvatus* TMW 1.439 as well as *L. curvatus* TMW 1.1390 can only dominate the microbiota when they are used in combination.

The log_{10} CFU g^{-1} sausage batter of the non-inoculated control sausage batter increased from initial 1.8 10^4 to 10^9 log_{10} CFU g^{-1} on day 5. As expected, the microbiota composition of this batch was more diverse (Figure S.6) than those inoculated with starter sets. In this case, autochthonous strains of *L. sakei* and *L. curvatus* were able to grow and assert over the documented fermentation time of 5 days. *L. curvatus* reached a relative abundance of 33.8 %, whereas *L. sakei* made up the major part of the microbiota with 52.3 % relative abundance. Other parts of the autochthonous microbiota were therefore similarly inhibited as in the inoculated samples.

3.2.2 Intraspecies assertiveness in large-scale fermentations

The intraspecies assertiveness, observed in model system fermentations, were validated by the execution of ring trial experiments. The most assertive and dominant strains of the sets I-IV were used for the production of raw fermented sausages (salami) by three different German-based companies (see Chapter 2.4.2).

3.2.2.1 pH and a_w measurements

The physico-chemical analysis revealed a progressive decrease in water activity (a_w) and pH as shown in Table 9 and Figure 10. At the start of the fermentation, the a_w-value was 0.945 ± 0.005. The observed drop in water activity was progressive during maturation reaching final values of 0.88 ± 0.1. The pH showed a clearly decrease from 5.95 ±0.05 to 5.0 ± 0.1. In general, the development of water activity was quite homogenous even when inoculated and not inoculated batches were compared. In contrast, especially during the first 5 days of fermentation, differences in pH development were clearly visible. The sets of the species *L. curvatus* (set I and II) showed a more rapid decrease in pH than sets of the species *L. sakei* (set III and IV). On day 5 all sets of *L. curvatus* showed an average pH of 4.9, whereas all sets of *L. sakei* species had an average pH of 5.1. The pH of produced control batches was slightly higher reaching an overall minimum pH of 5.1. With the end of fermentation and maturation all fermentations showed a slightly increase in pH due to proteolytic activities.

Table 9: Water activity development during large-scale fermentations. Control sausages as well as sausages produced with set I-IV starters are incorporated according to the company I-III.

Sample day	control			set I		set II		set III		set IV	
	c I	c II	c III	c II	c III	c I	c III	c II	c III	c I	c II
0	0.95	0.94	0.95	0.94	0.94	0.95	0.95	0.94	0.95	0.95	0.94
5	0.94	0.94	0.94	0.93	0.94	0.95	0.94	0.94	0.93	0.94	0.93
12	0.90	0.90	0.91	0.90	0.90	0.90	0.91	0.91	0.91	0.90	0.90
21	0.88	0.88	0.89	0.87	0.89	0.88	0.89	0.88	0.89	0.88	0.88

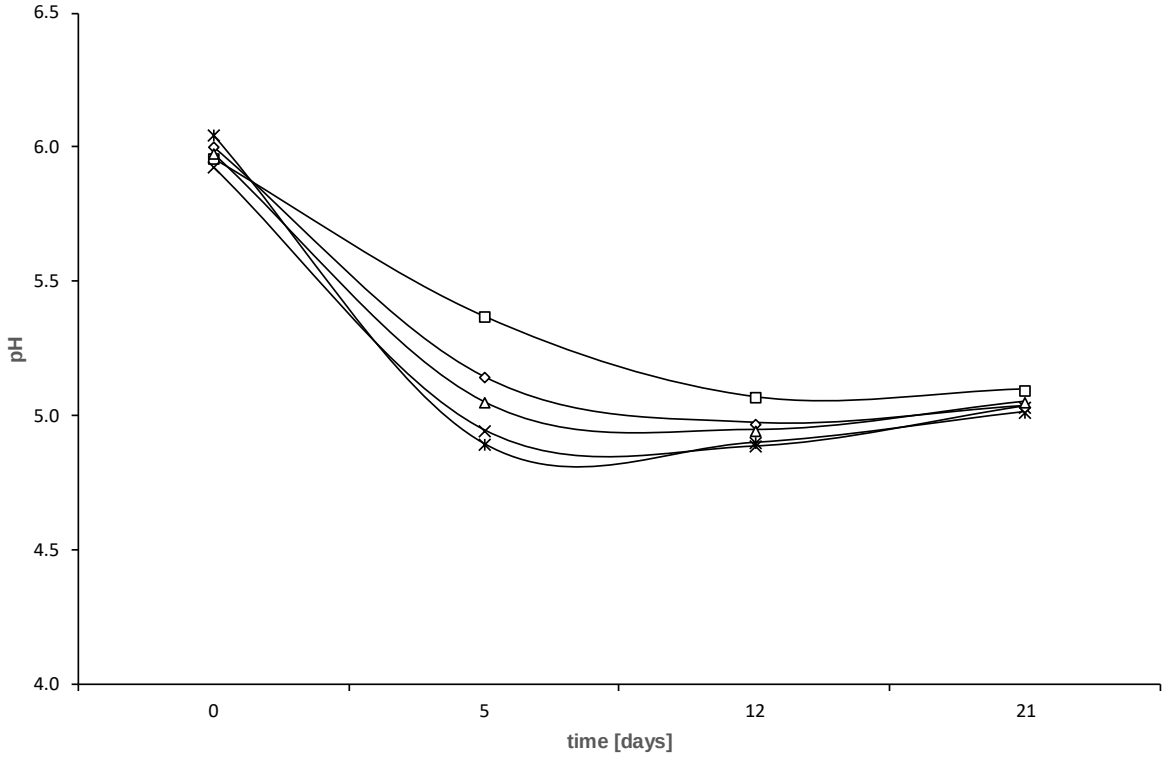

Figure 10: pH development over the total fermentation time of 21 days. The mean pH of control meat batches [□], set I [∗], set II [×], set III [◇] and set IV [△] are shown. The pH was measured only once on every sampling day.

3.2.2.2 **Microbiota development of LAB**

In Chapter 3.2.1, we successfully established a system to document the microbiota development on strain level using MALDI-TOF MS. The results suggested two different strategies in competition and assertiveness. The first strategy includes an outcompeting of all other members of the meat microbiota and can be considered as a form of contest competition, where only one strain prevails and inhibit the autochthonous microbiota by the establishment of a single dominance (competitive exclusion). Conversely, the second strategy is characterized by the establishment of a co-dominance or even a cooperation, where more than one starter strain prevails and enables a colonization resistance (CR).

For the validation of these findings the predominating “winner” strain or strains were used in an upscaled ring trial experiment to produce raw fermented sausages under real conditions. For both species we included one strain, which was shown to be assertive by outcompeting all other members of the meat microbiota and one strain set, consisting of either two or three strain, where an establishment of co-dominance was observed.

Overall, the characterization of ring-trial produced sausages revealed that the microbiota of all inoculated batches was dominated by the used starter culture, either *L. curvatus* or *L. sakei*. The predictions deriving from the established model system were shown to be reliable and trustworthy.

The first set used for the production of raw fermented sausages included the *L. curvatus* strain TMW 1.624 and the *S. carnosus* strain TMW 2.212. The development of the microbiota over time followed our prediction based on the findings of the first competitions experiments using the implemented model system (Figure 11). The added *L. curvatus* starter TMW 1.624 was able to establish a stable dominance and became the predominant strain within the sausage environment. The autochthonous microbiota, called either “*L. curvatus*”, “*L. sakei*” or “other”, was efficiently inhibited by the dominance of the strain, which varied depending on the company from 93.3 % (company II, Figure 11, A) and 96.6 % (company III, Figure 11, B) Colonies of the added *S. carnosus* starter TMW 2.212 were recognized on production day partly to high relative abundance of 82.3 % (company II, Figure 11, A). Moreover, in samples of company II low amounts of *L. sakei* have been detected. Colony forming units reached values starting from $\log_{10}$ 5.25 ± 0.05 CFU g^{-1} on day 0 to $\log_{10}$ 9.6 ± 0.1 CFU g^{-1} on day 21. Like predicted before, the *L. curvatus* strain TMW 1.624 was successful in establishing a stable dominance even in the longer fermentation time of 21 days.

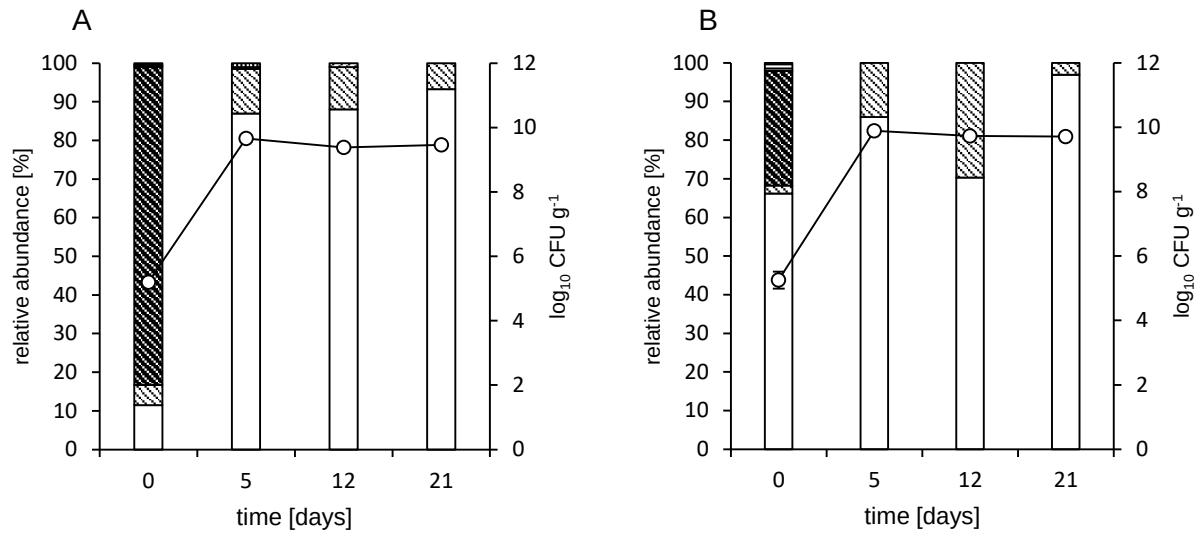

Figure 11: Microbiota dynamics and development of set I over the total fermentation time of 21 days determined on mMRS. The experiment was conducted by company II (A) and company III (B). Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species and strains were identified with MALDI-TOF MS as follows: (□) *L. curvatus* TMW 1.624, (▧) *L. curvatus*, (▨) *L. sakei*, (▩) *S. carnosus* TMW 2.212, (▤) other, (▥) nri. The acronym nri stands for not reliable identification.

Set II included a *L. curvatus* strain set of TMW 1.439 and TMW 1.1390, which were able to establish a co-dominance in the model system experiments. Both starter cultures, which were added prior to fermentation as co-dominant starter cultures, were able to grow to high cell counts representing the main microbiota within the sausage during the whole fermentation time of 21 days (Figure 12). Their dominance reached values up to 98.3 % (company III, Figure 12, B). whereby only 11.5 % can be credited to *L. curvatus* TMW 1.1390. Similar was observed with company I (Figure 12, A). The total proportion of both strains reached 89.3 % whereby only 4.8 % can be credited to TMW 1.1390. The cell counts started at log_{10} 5.9 ± 0.1 on day 0 and ended at a determined log_{10} CFU g^{-1} of 9.25 ± 0.25 on day 21 at the end of the ripening process. For both companies a slight reduction in CFU g^{-1} was visible after day 5. Again, like described before, on production day (day 0) the previously added starter *S. carnosus ssp. carnosus* TMW 2.212 was detected.

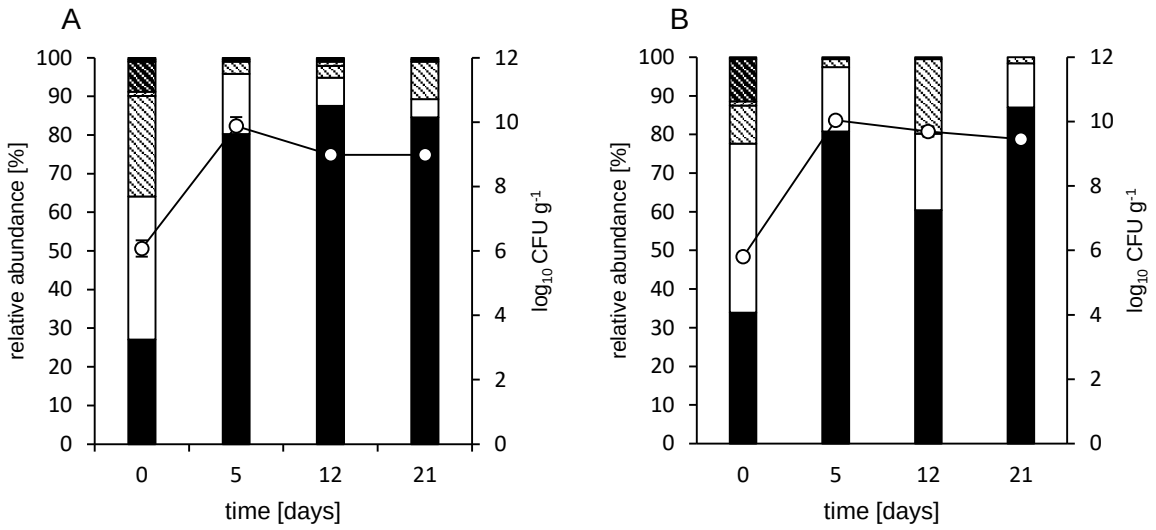

Figure 12: Microbiota dynamics and development of set II over the total fermentation time of 21 days determined on mMRS. The experiment was conducted by company I (A) and company III (B). Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species and strains were identified as follows: (■) *L. curvatus* TMW 1.439, (□) *L. curvatus* TMW 1.1390, (▧) *L. curvatus*, (▨) *L. sakei*, (▩) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

The strain collection, consisting of *L. sakei* TMW 1.3. TMW 1.417 and TMW 1.1398 was shown to be co-dominating the sausage microbiota in our model system. This expression of co-dominance was successfully validated by the ring trial experiments of this study (Figure 13). All of the added *L. sakei* starter cultures have been detected over the whole fermentation time of 21 days. Their total predominance reached values up to 93.8 % (company II, Figure 13, A) and 97.7 % (company III, Figure 13, B). In both batches, the strain TMW 1.1398 displayed the major part of the total microbiota with a range from 62.5 % (company III) to 77.1 % (company II), followed by TMW 1.417 ranging from 14.6 % (company II) to 33.3 % (company III) and TMW 1.3, which only reached a relative abundance of 2.1 % at the end of ripening. Cell counts reached log CFU g^{-1} of 9.55 ± 0.35 at day 21, starting from approximately log_{10} 6.15 ± 0.45 CFU g^{-1}. In both batches a reduction of cell counts is visible either after 5 days (company II) or 12 days (company III).

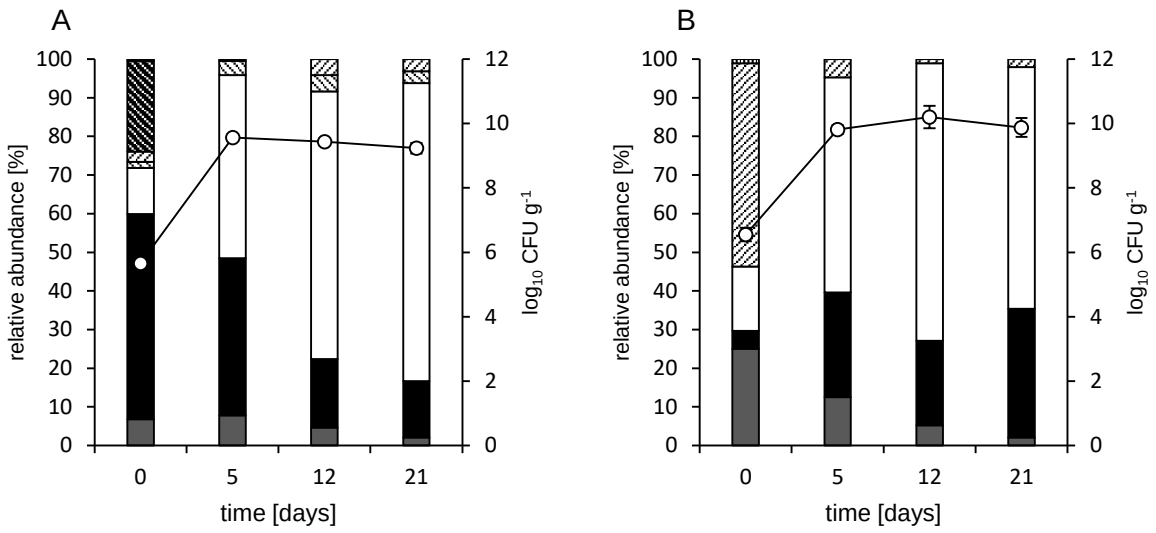

Figure 13: Microbiota dynamics and development of set III over the total fermentation time of 21 days determined on mMRS. The experiment was conducted by company II (A) and company III (B). Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species and strains were identified as follows: (■) *L. sakei* TMW 1.3. (■) *L. sakei* TMW 1.417, (□) *L. sakei* TMW 1.1398, (▧) *L. curvatus*, (▨) *L. sakei*, (▩) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

The starter used in the fourth ring trial experiment established a single dominance within the model system. The *L. sakei* TMW 1.1396 was assertive and able to inhibit the autochthonous microbiota alone. The performance of the strain TMW 1.1396 in the ring trial experiments was proven to be similar to the model system (Figure 14). Already after 5 days of fermentation the relative abundance of the strain reached values of 92.7 % (company I, Figure 14, A) and 95.8 % (company II, Figure 14, B). With the end of ripening on day 21 the total dominance determined was and 97.9 % (company I) 98.4 % (company II). Simultaneously, the colony-forming unit reached a log_{10} CFU g^{-1} of 9.1 ±0.2. For the batch of company I a distinct decrease in log_{10} CFU g^{-1} was recorded on day 5. The observed reduction could be partly explained by the relatively high standard deviation.

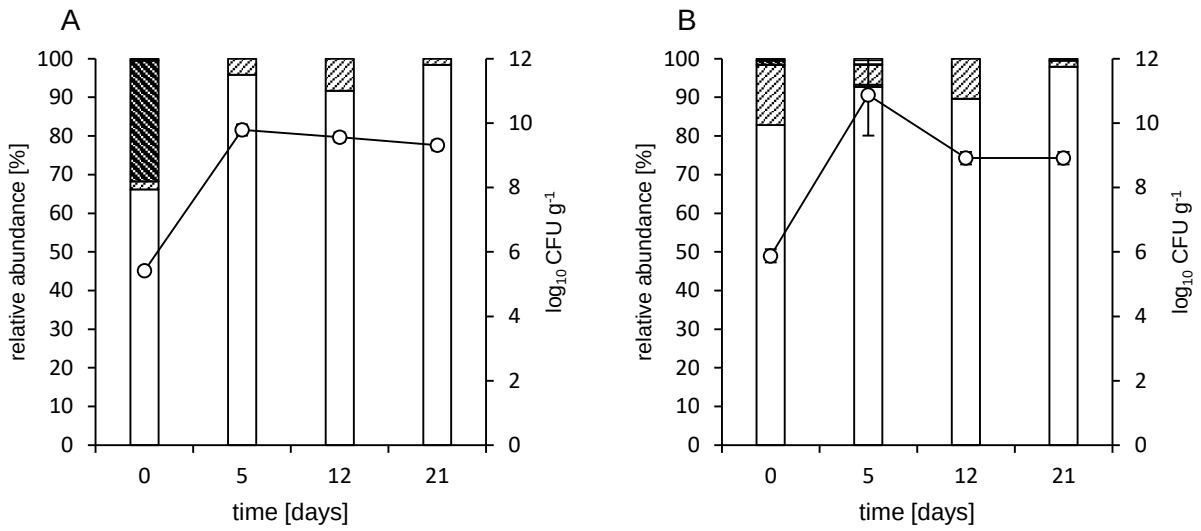

Figure 14: Microbiota dynamics and development of set IV over the total fermentation time of 21 days determined on mMRS. The experiment was conducted by company I (A) and company II (B). Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species and strains were identified with MALDI-TOF MS as follows: (□) *L. sakei* TMW 1.1396, (▨) *L. sakei*, (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for "not reliable identification".

Besides the experiments with starter cultures, all companies were advised to produce control sausages without the addition of any starter culture. Figure 15 shows the microbiota development of all control batches on mMRS. The microbiota of the sausage without starter cultures was shown to be way more diverse on production day than the microbiota of the inoculated sausages (Table 10). Nevertheless. *L. sakei* could take over the dominance within the meat and became the predominant species. This microbiota development has been observed for all control sausages of all participating companies. The colony forming units determined on production day started at log_{10} CFU g^{-1} of 4.3 ± 0.3 and ended up at log_{10} CFU g^{-1} of 9.1 ± 0.4. The observed reduction in colony forming units after fermentation day 5 varied depending on the respective company.

Table 10: Species presence in control batches and inoculated batches on mMRS over the whole fermentation and ripening time. Control batches tend to show a greater diversity of species than inoculated batches. The green circle (●) represents a positive result, the red circle (●) represents a negative result, meaning the respective species were not found.

	Ø control batches	Ø inoculated batches
L. sakei	●	●
L. curvatus	●	●
L. garvieae	●	●
L. plantarum	●	●
L. lactis	●	●
L. citreum	●	●
L. fructivorians	●	●
P. acidilacticii	●	●
P. pentosaceus	●	●
S. carnosus sp. carnosus	●	●
S. carnosus ssp. utilis	●	●
S. aureus	●	●
S. saprophyticus	●	●
S. saprophyticus ssp. bovis	●	●
S. pasteuri	●	●
E. feacalis	●	●
E. devriesei	●	●
C. maltaromaticum	●	●
C. freundii	●	●
C. divergens	●	●
M. caseolyticus	●	●
B. thermosphacta	●	●
K. varians	●	●
K. kristinea	●	●
Y. lipolytica	●	●
L. mesenteroides	●	●

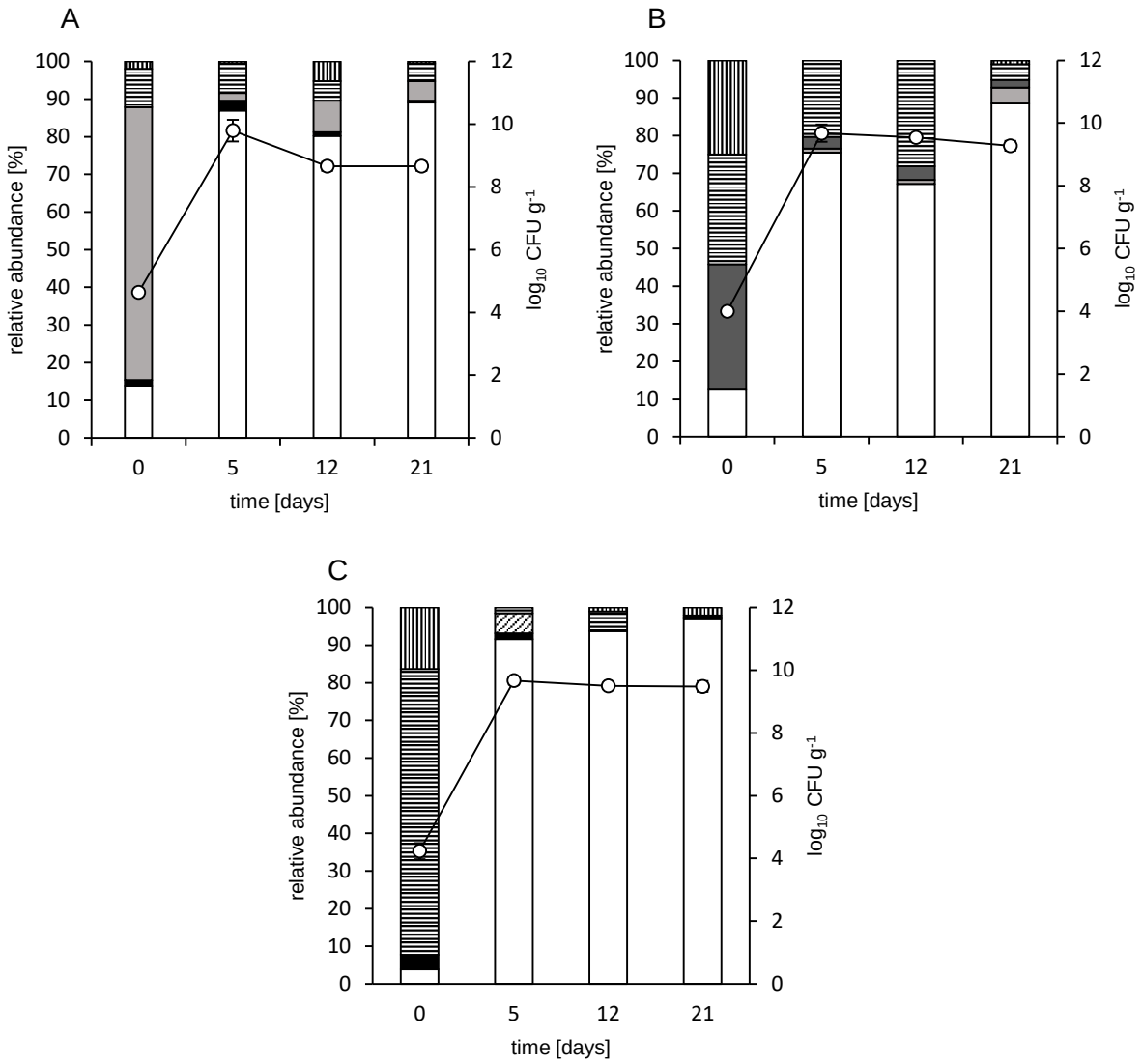

Figure 15: Microbiota development of spontaneous fermented control sausages determined on mMRS. Control sausages were produced by company I (A), company II (B) and company III (C). Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *L.sakei*, (■) *L. curvatus*, (▩) *L. plantarum*, (▨) *L. garviae*, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

3.2.2.3 **Microbiota development of CNS/CPS**

Besides microbiota monitoring of lactic acid bacteria, coagulase negative and coagulase positive staphylococci were monitored during fermentation. Like described before, the autochthonous microbiota of uninoculated batches showed a slightly greater diversity than those batches inoculated with starter cultures (Table 11). Furthermore, the microbiota of the spontaneous fermented sausages showed great variation between the participating companies and the control batches themselves (Figure 16). The control batch of company III showed high relative abundances of *S. aureus* with percentages of 45.8 % (6.8 x 10^9 CFU g^{-1}) on day 21 (Figure 16, C), whereas the control batch of company II revealed high abundances of *S. saprophyticus* with values up to 79.2 % (4.3 x 10^6 CFU g^{-1}) on day 21 at the end of fermentation (Figure 16, B). The species *S. carnosus* was highly underrepresented. Only the development of the control batch of company I showed a desirable microbiota consisting mainly of *S. carnosus ssp. carnosus* (Figure 16, A). The relative abundance of the latter species reached 86.5 % (5.76 x 10^6 cells g^{-1}) of the total sausage microbiota. *S. aureus* as a pathogenic and enterotoxigenic bacterium was identified on day 12 and day 21 and reached a relative abundance of maximal 7.3 %, corresponding to a CFU g^{-1} of 3.4 x 10^5, which is still considered very high. High relative abundances of 51.0 % of *S. saprophyticus* (7.7 x 10^6 CFU g^{-1}) were detectable on day 5, but were then rapidly reduced to 3.13 % on day 12.

Compared to these findings, the microbiota of inoculated meat batches, determined on selective BP-plates, showed the predicted and desired development. The *S. carnosus ssp. carnosus* strain TMW 2.212 was able to grow and displayed the majority of the total microbiota (Figure 17 - Figure 20). *S. saprophyticus* could have been detected, whereas *S. aureus* was nearly completely extinguished. The relative abundances of *S. carnosus ssp. carnosus* TMW 2.212 were as follows: 65.65 ± 3.15 % (set I, Figure 17, A and B). 79.2 ± 7.3 % (set II, Figure 18, A and B). 55.2 ± 9.4 % (set III, Figure 19, A and B) and 68.2 ± 15.1 % (set IV, Figure 20, A and B).

The dominance of the introduced starter strain TMW 2.212 was not always expressed in the same way and to the same relative abundance. Especially set IV is characterized by a great variance within both companies performing the experiment. Furthermore company-specific differences in their microbiota profiles have been visualized. The samples of company I are characterized by the development of relatively high abundances of *S. saprophyticus* on day 5 together with a rapidly increase and decrease in $\log_{10}$ CFU g^{-1} (Figure 18, A and Figure 20; A), whereas company II showed a steadily increase of *S. saprophyticus* over time (Figure 17, A; Figure 19, A and Figure 20, B). Microbiota results of company III always show a significant increase in $\log_{10}$ CFU g^{-1} between 12 and 21 days (Figure 17, B; Figure 18, B and Figure 19,

B). However, the autochthonous microbiota was efficiently inhibited and the microbiota mainly consisted of *S. carnosus*.

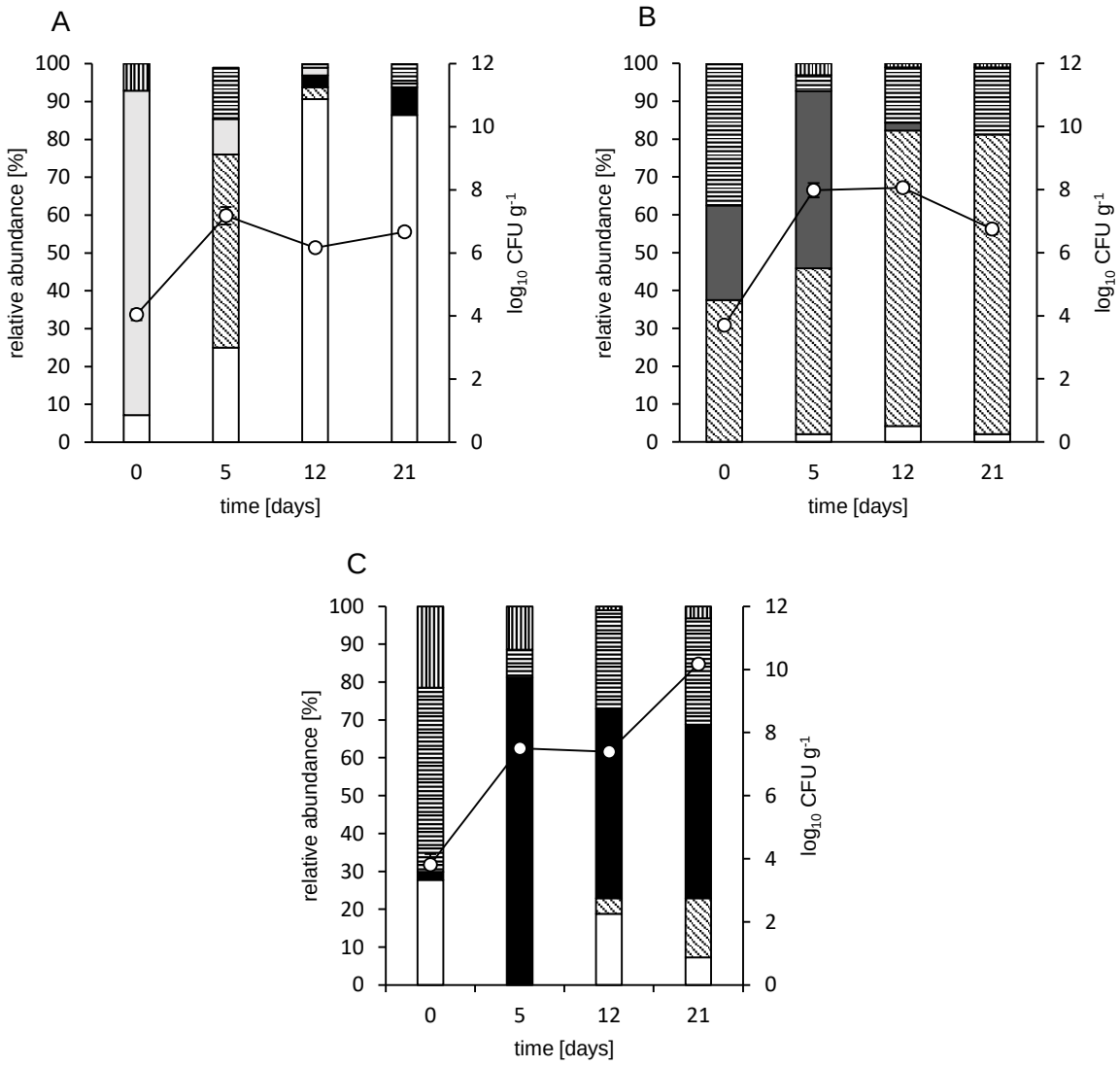

Figure 16: Microbiota development of spontaneous fermented control sausages determined on BP. Control sausages were produced by company I (A), company II (B) and company II (C). Total viable count [$\log_{10}$ CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit $\log_{10}$ 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus*, (■) *S. aureus*, (▨) *S. saprophyticus*, (▢) *S. xylosus*, (▩) *E. feacalis*, (▤) other and (▥) nri. The acronym nri stand for “not reliable identification”.

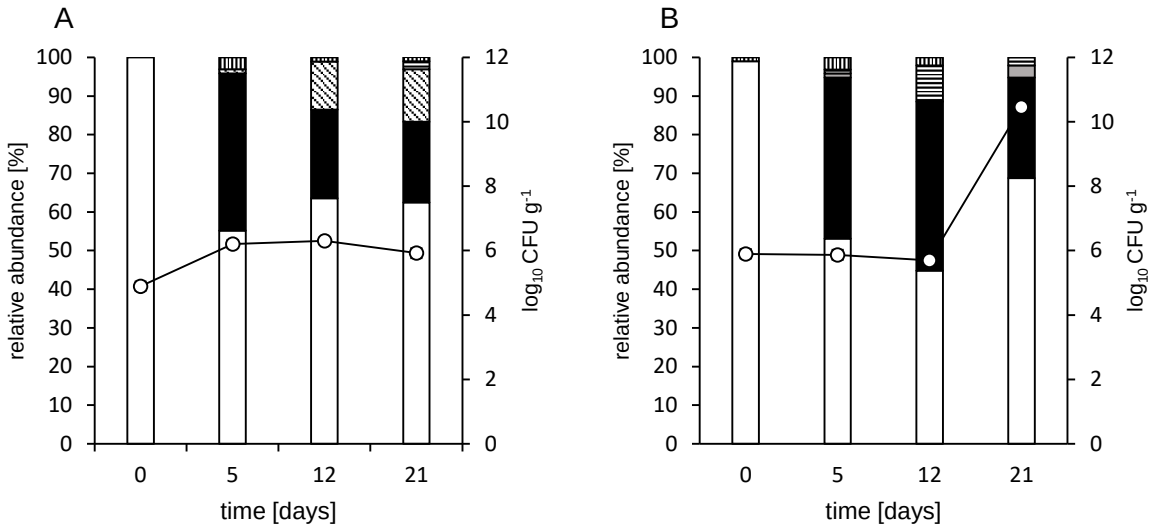

Figure 17: Microbiota development of set I over the total fermentation time of 21 days determined on BP. The experiments for set I were conducted by company II (A), company III (B). Total viable count [$\log_{10}$ CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit $\log_{10}$ 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus* TMW 2.212, (■) *S. carnosus*, (▨) *S. saprophyticus*, (▫) *S. carnosus ssp. utilis*, (▪) *E. feacalis*, (▤) other and (▥) nri. The acronym nri stand for “not reliable identification”.

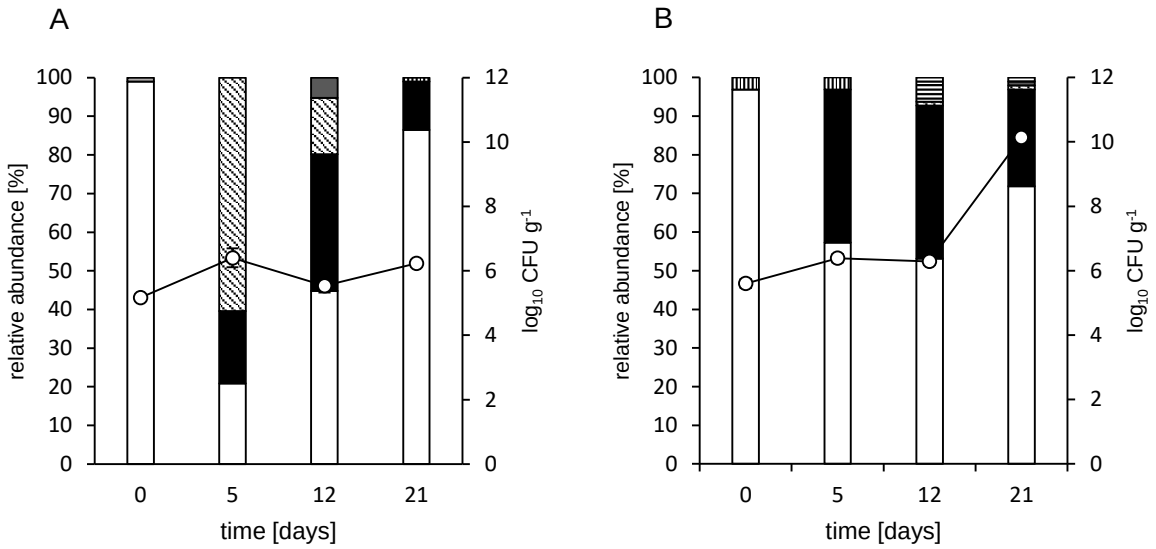

Figure 18: Microbiota development of set II over the total fermentation time of 21 days determined on BP. The experiments for set II were conducted by company I (A), company III (B). Total viable count [$\log_{10}$ CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit $\log_{10}$ 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus* TMW 2.212, (■) *S. carnosus*, (▨) *S. saprophyticus*, (▫) S. carnosus ssp. utilis, (▪) *E. feacalis*, (▤) other and (▥) nri. The acronym nri stand for “not reliable identification”.

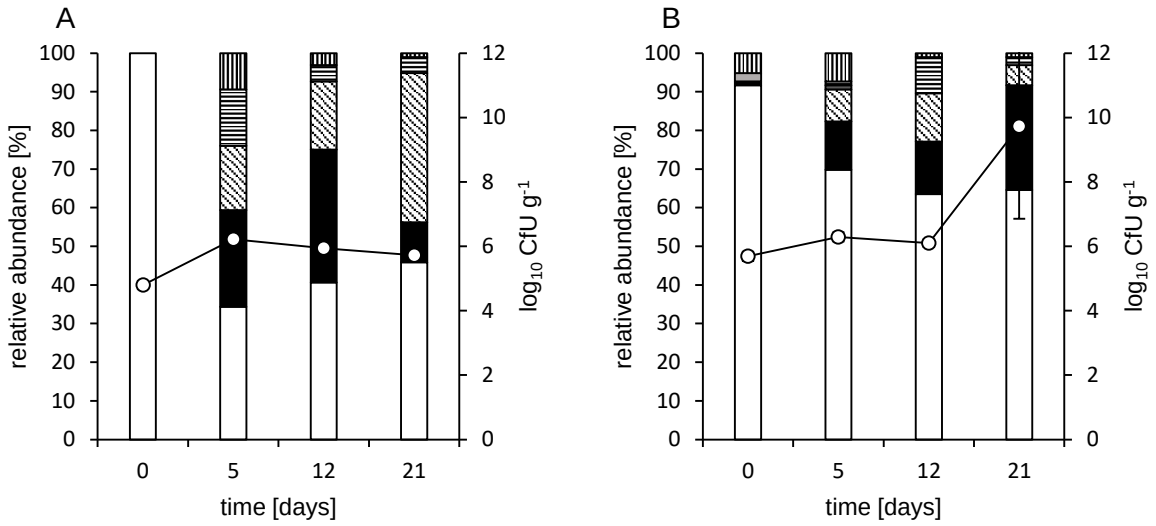

Figure 19: Microbiota development of set III over the total fermentation time of 21 days determined on BP. The experiments for set III were conducted by company II (A), company III (B). Total viable count [$\log_{10}$ CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit $\log_{10}$ 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus* TMW 2.212, (■) *S. carnosus*, (▨) *S. saprophyticus*, (▫) *S. carnosus ssp. utilis*, (▩) *E. feacalis*, (▤) other and (▥) nri. The acronym nri stand for “not reliable identification”.

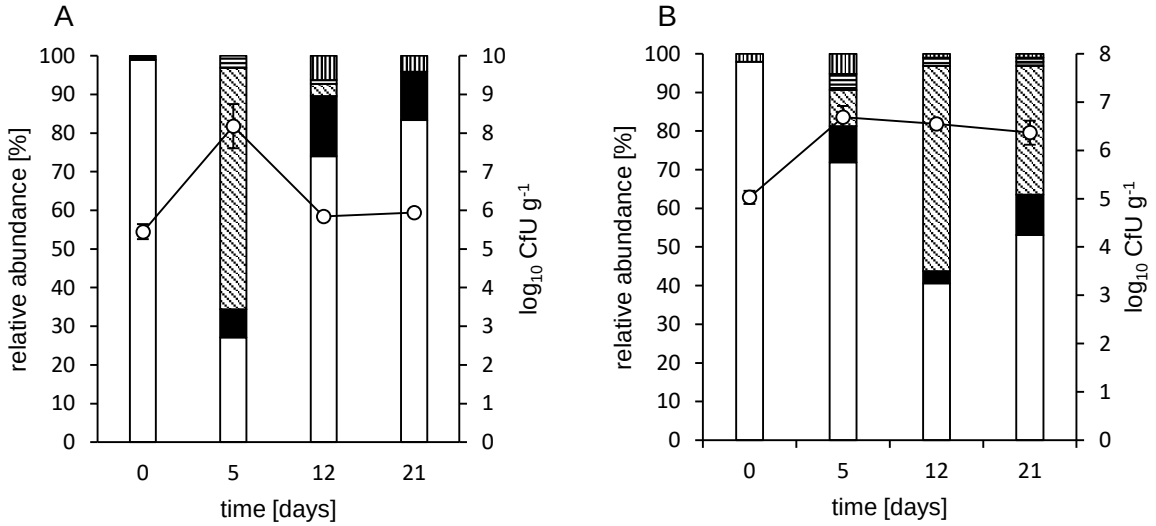

Figure 20: Microbiota development of set IV over the total fermentation time of 21 days determined on BP. The experiments for set IV were conducted by company I (A), company II (B). Total viable count [$\log_{10}$ CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of two biological replicates for each company with standard deviation. Detection limit $\log_{10}$ 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus* TMW 2.212, (■) *S. carnosus*, (▨) *S. saprophyticus*, (▫) *S. carnosus ssp. utilis*, (▩) *E. feacalis*, (▤) other and (▥) nri. The acronym nri stand for “not reliable identification”.

Table 11: Species presence in control batches and inoculated batches on BP over the whole fermentation and ripening time. Control batches tend to show a greater diversity of species than inoculated batches. The green circle (●) represents a positive result, the red circle (●) represents a negative result, meaning the respective species were not found.

species	Ø control batches	Ø inoculated batches
S. carnosus sp. carnosus	●	●
S. xylosus	●	●
S. aureus	●	●
S. saprophyticus	●	●
S. saprophyticus ssp. bovis	●	●
S. pasteuri	●	●
S. warneri	●	●
S. epidermidis	●	●
S. equorum	●	●
S. sciuri	●	●
E. feacalis	●	●
M. caseolyticus	●	●
M. luteus	●	●
C. parapsilosis	●	●
C. variabile	●	●
C. testudinoris	●	●
A. johnsonii	●	●
B. thermosphacta	●	●
P. fluorescens	●	●
P. acidilactici	●	●
T. japonicum	●	●
Y. lipolytica	●	●

3.2.3 Interspecies assertiveness in a raw fermented sausage model system

Interspecies assertiveness in the implemented sausage model was assessed by competition studies of *L. curvatus* set II against the *L. sakei* sets III and IV. For these experiments, only the previously dominant and relevant strains were included. The competition experiments including also the first *L. curvatus* set I are discussed later in the following Chapter 3.4.

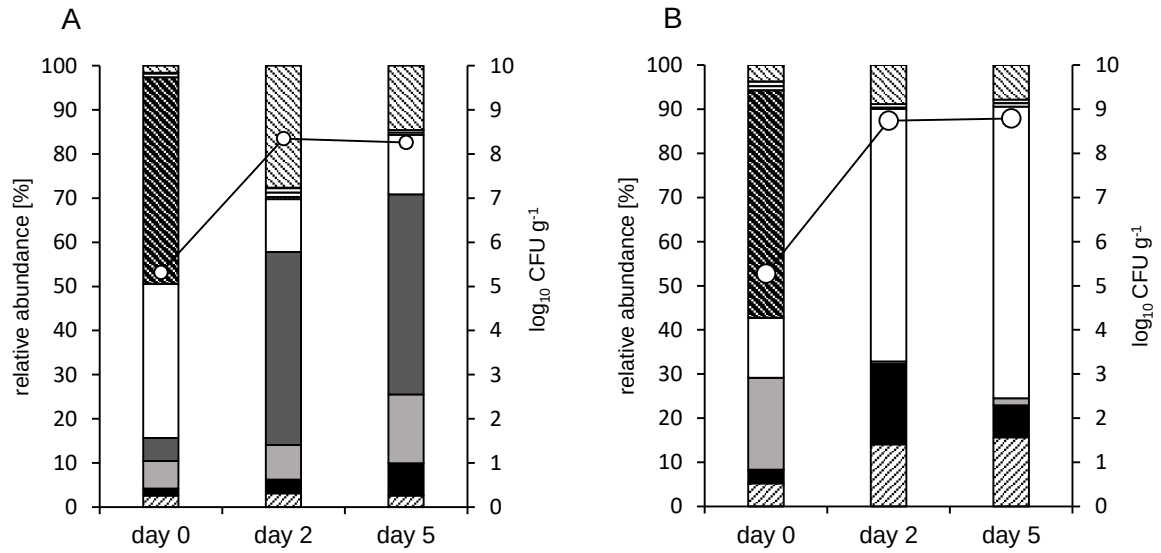

Figure 21: Microbiota development of set II versus set III (A), set II versus set IV (B) over the total fermentation time of 5 days determined on MRS. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points. The figure shows the average of two biological replicates for each set tested with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows:

(A) - (■) *L. curvatus* TMW 1.439, (▨) *L. curvatus* TMW 1.1390, (■) *L. sakei* TMW 1.3 (□) *L. sakei* TMW 1.417, (■) *L. sakei* TMW 1.1398, (▧) *L. sakei* other; (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

(B) - (■) *L. curvatus* TMW 1.439, (▨) *L. curvatus* TMW 1.1390, (■) *L. sakei* TMW 1.1189 (□) *L. sakei* TMW 1.1396, (▧) *L. sakei* other; (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

The results clearly indicate a higher assertiveness of *L. sakei* strains compared to tested *L. curvatus* strains, which were shown to be dominant and assertive within their intraspecies sets but not within interspecies defined sets.

The results of the conducted interspecies competition experiments are depicted in Figure 21. Like observed in the intraspecies competition experiment with set III, the combination of *L. sakei* TMW 1.3, TMW 1.417 and 1.1398 was able to develop a stable co-dominance within the sausage batter. The previously dominant *L. curvatus* strains in set II, namely TMW 1.439 and

TMW 1.1390 were shown to be overgrown by the predominance of the introduced *L. sakei* strains. In fact, on day 5 with the end of fermentation, the two *L. curvatus* strains had only a relative abundance of 9.9 % (7.3 % *L. curvatus* TMW 1.439 and 2.6 % *L. curvatus* TMW 1.1390), whereas the dominant *L. sakei* combination were able to establish a relative abundance of 74.4 % in total (13.5 % *L. sakei* TMW 1.417, 15.6 % *L. sakei* TMW 1.1398 and 45.3 % TMW 1.3).

Similar results were gained in competition studies conducted with set II and set IV. Again, the dominant *L. curvatus* strains TMW 1.439 (7.3 %) and *L. curvatus* TMW 1.1390 (15.6 %) were overgrown by the respective dominant starter strain of set IV, *L. sakei* TMW 1.1396 (66.1 %).

3.3 Determination of possible physiological markers for the assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations

A selection of 18 *Lactobacillus* strains were characterized in detail regarding their growth kinetics in general, their tolerance to oxidative and cold stress, their potential to produce bacteriocins and the occurrence of potentially inducible prophages within their genomes.

3.3.1 Growth kinetics of *L. sakei* and *L. curvatus*

Growth kinetics were conducted in standard mMRS medium as well as in mMSM, to simulate the conditions during sausage fermentation. For all strains, growth curves were recorded and the growth parameters lag-phase, growth rate (μ) and the maximum OD ($OD_{max.}$) were determined. Furthermore, using R-Studio and Anova, it was investigated whether significant differences were found.

Figure 22 shows the determined boxplots for lag-phases, maximum OD and growth rate for all of the strains in mMRS (Figure 22, A-C) and mMSM (Figure 22, D-F). On the first glance, the results show more noticeably differences in the strains behaviour in the more restricted medium mMSM, simulating the conditions during raw sausage fermentation.

As the performance of the strains in mMRS seems to be more similar, without clear indicatable differences or hints for correlations to the assertiveness, the results are presented only shortly. Considering mMRS, shortest lag-phases were recorded for the strains *L. curvatus* TMW 1.401 (3) und *L. sakei* TMW 1.46 (12), TMW 1.114 (13), TMW 1.578 (15) und TMW 1.1239 (16). With an average lag-phase of 5.9 h *L. sakei* TMW 1.3 (11) was shown to have the significant longest lag-phase of all strains.

TMW 1.27 seem to be of slightly outstanding character with regard to the parameters growth rates as it displayed the highest µ, followed by TMW 1.1390 and TMW 1.3, TMW 1.114 and TMW 1.578. The lowest growth rate was recorded for TMW 1.421. Besides, also the parameter $OD_{max.}$ was found to be very unpromising concerning the detection of markers. The strains *L. curvatus* TMW 1.27 (1), TMW 1.401 (3) und TMW 1.1381 (9) as well as *L. sakei* TMW 1.3 (11), TMW 1.114 (13), TMW 1.578 (15), TMW 1.1239 (16) and TMW 1.1398 (18) showed a significant higher final $OD_{max.}$ than the strains of the species *L. curvatus* TMW 1.439 (6), TMW 1.595 (7), TMW 1.624 (8) und TMW 1.1390 (10) as well as *L. sakei* TMW 1.46 (12), TMW 1.1396 (17) and TMW 1.1189 (19). The *L. curvatus* strain 1.421 (5) showed the lowest $OD_{max.,}$ followed by TMW 1.407.

However, with regard to mMSM it is possible to recognize a slightly correlation of the parameter growth rate µ and the determined $OD_{max.}$ and the observed assertiveness of the respective strains. The assertive strains *L. curvatus* TMW 1.1390, *L. sakei* TMW 1.3, TMW 1.417, TMW 1.1396 and TMW 1.1398 were shown to be characterized by high growth rates and relatively high final $OD_{max.}$, only the *L. curvatus* strains TMW 1.439 and TMW 1.624 showed relatively low growth rates and OD. In fact, TMW 1.1396 and TMW 1.1398 showed the significant highest growth rates for all strains investigated in this study. Nevertheless, due to the fact, that not all competitive strains, e.g. *L. curvatus* TMW 1.439 and TMW 1.624, were characterized by high growth rates, the growth in mMSM as well as the determination of the key parameter µ are only indicative and cannot be used as sole markers for the assertiveness.

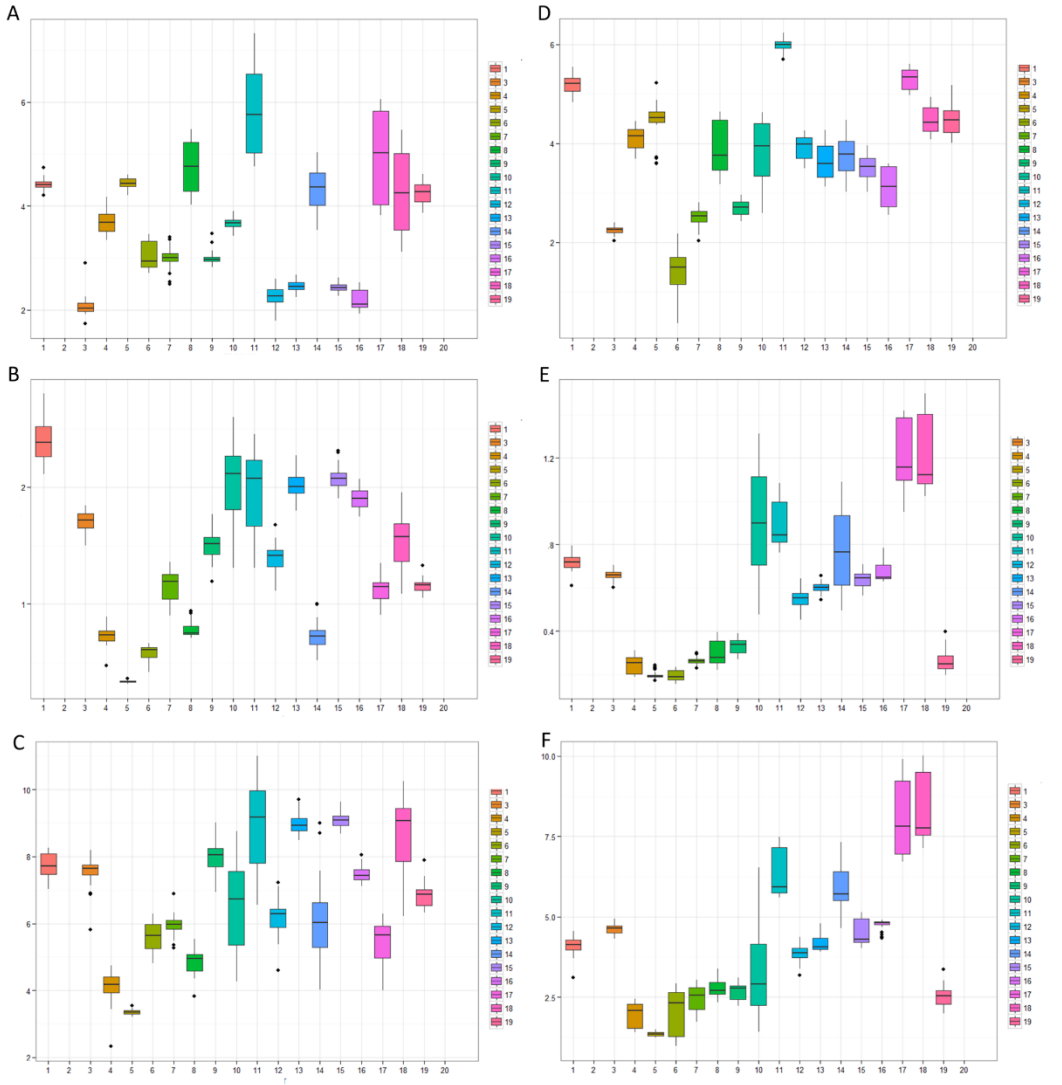

Figure 22: Boxplots of the growth parameters lag-phase, growth rate (μ) and OD_{max}. of all strains [1-19] in mMRS (A-C) and mMSM (D-F). To enhance the clarity of the figure, series numbers were used instead of TMW-numbers. (A) boxplot for lag-phase in mMRS (B) growth rate in mMRS (C) maximum OD in mMRS. (D) boxplot for lag-phase in mMSM, (E) growth rate in mMSM and (F) maximum OD in mMSM. The strains are displayed as follows: *L. curvatus* strains (1) TMW 1.27, (3) TMW 1.401, (4) TMW 1.407, (5) TMW 1.421, (6) TMW 1.439, (7) TMW 1.595, (8) TMW 1.624, (9) TMW 1.1381 and (10) TMW 1.1390, *L. sakei* strains (11) TMW 1.3, (12) TMW 1.46, (13) TMW 1. 114, (14) TMW 1.417, (15) TMW 1.578, (16) TMW 1.1239, (17) TMW 1.1396, (18) TMW 1.1398 and (19) TMW 1.1189.

3.3.2 Stress tolerance

The whole strain selection, consisting of 9 *L. curvatus* and 9 *L. sakei* strains were tested for their growth behaviour under two different stress conditions, which may occur during raw sausage fermentation.

3.3.2.1 Oxidative stress tolerance

All 9 *L. curvatus* strains and all 9 *L. sakei* strains were tested for growth in the presence of different concentrations of hydrogen peroxide, starting from 0.0 mM to 100 mM. The data (Figure 23 and Figure 24) indicates a high biodiversity in stress management for both species. However, the ability to grow in the presence of hydrogen peroxide differed not only between the species, but also within the strains.

In general, the addition of hydrogen peroxide mostly led to an extension of lag times and to a concurrent delay of logarithmic growth phases, whereas parameters like growth rates (μ) and OD ($OD_{max.}$) were only partially reduced. A hydrogen peroxide concentration of 1.56 mM was generally tolerated by both species. Most of the strains started to grow immediately after inoculation or needed one day of lag-phase to enter the logarithmic growth phase. A sorting of *L. sakei* and *L. curvatus* strains into different groups based on their stress response should enlighten the tolerance of the strains.

Group I contains the strains *L. curvatus* TMW 1.401 and TMW 1.1390 as well as the *L. sakei* strains TMW 1.114, TMW 1.578, TMW 1.1189, TMW 1.1396 and TMW 1.1398, whose growth remains widely unaffected by the addition of 1.56 mM hydrogen peroxide. Group II, however, encompasses *L. curvatus* TMW 1.27 and TMW 1.624 as well as *L. sakei* TMW 1.3 and is characterized by clearly reduced growth rates and lower maximum OD-values. Group III contains strains, which showed an extension of lag-times of 24 hours but with no significant effects on growth rate and OD, and encompasses *L. curvatus* TMW 1.407, TMW 1.421, TMW 1.439, TMW 1.595, TMW1.1381 and the *L. sakei* strain TMW 1.417. Group IV shows an extension of lag-times as well as reduced growth rates and OD and compromises *L. sakei* TMW 1.1239 as sole representative, whereas group V with *L. sakei* TMW 1.46 shows an extended lag-phase and a higher growth rate and $OD_{max.}$. In summary, the vast majority of *L. sakei* strains showed an overall unaffected growth, whereas *L. curvatus* was characterized by an extension of lag-times up to 24 hours. Therefore, the results clearly indicate a higher vulnerability of *L. curvatus* strains to hydrogen peroxide compared to investigated *L. sakei* strains.

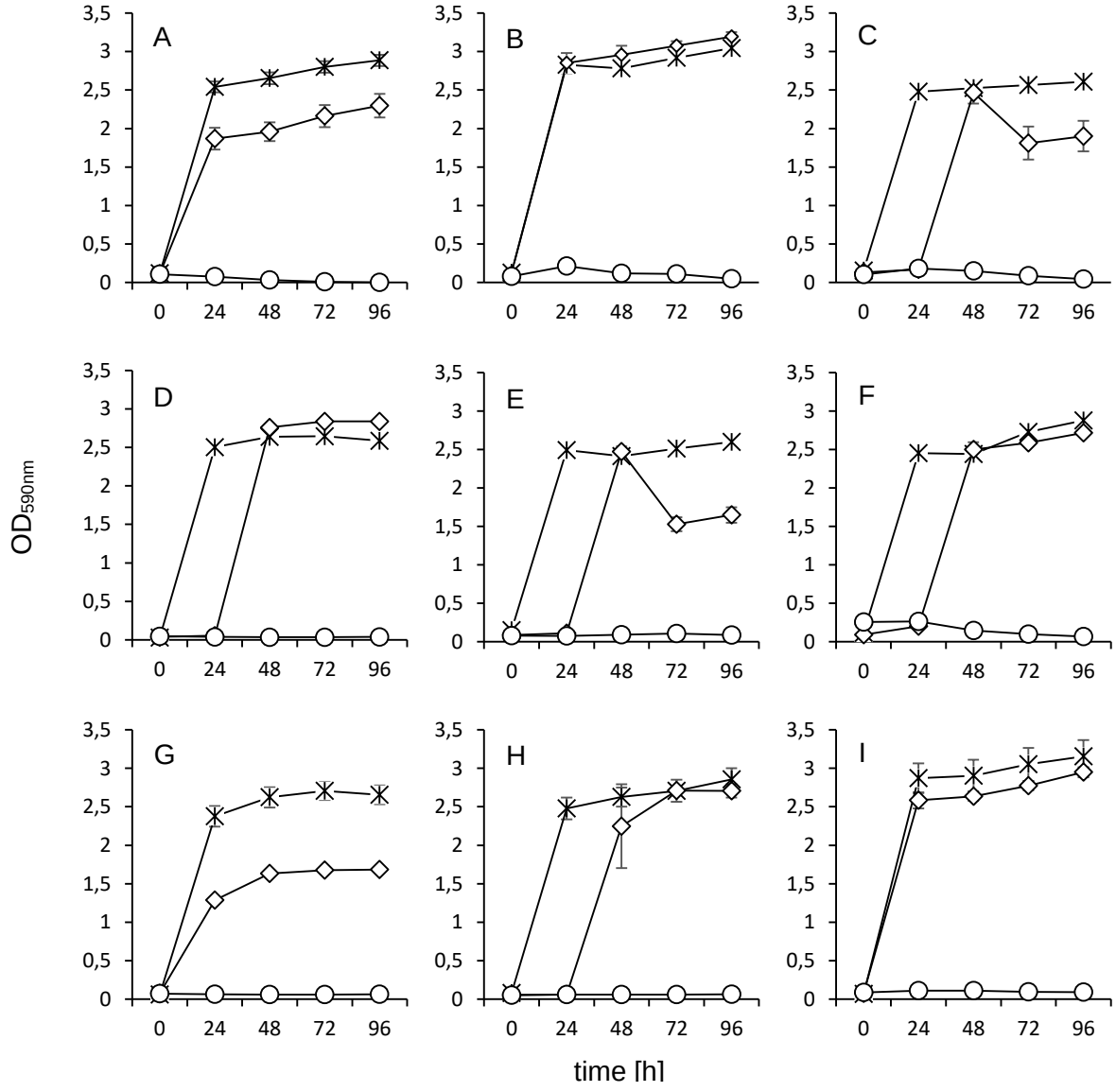

Figure 23: Oxidative stress response of *L. curvatus* strains. The figures show the growth upon three different concentrations of hydrogen peroxide. The hydrogen peroxide concentration are displayed as follows: (x) 0 mM, (◇) 1.56 mM and (○) 3.13 mM for the strains (A) TMW 1.27, (B) TMW 1.401, (C) TMW 1.407, (D) TMW 1.421, (E) TMW 1.439, (F) TMW 1.595, (G) TMW 1.624, (H) TMW 1.1381 and (I) TMW 1.1390.

These findings are strengthened looking at the higher hydrogen peroxide concentrations used in this study. None of the tested Lactobacillus strains were able to grow at hydrogen peroxide concentrations above 3.13 mM. Indeed, for the vast majority of *Lactobacillus* strains, a concentration of 3.13 mM was shown to be already lethal. Only two *L. sakei* strains, namely TMW 1.1189 and 1.1398, were capable to cope with this hydrogen peroxide concentration. However, both strains showed a clear extension of lag-times like described before.

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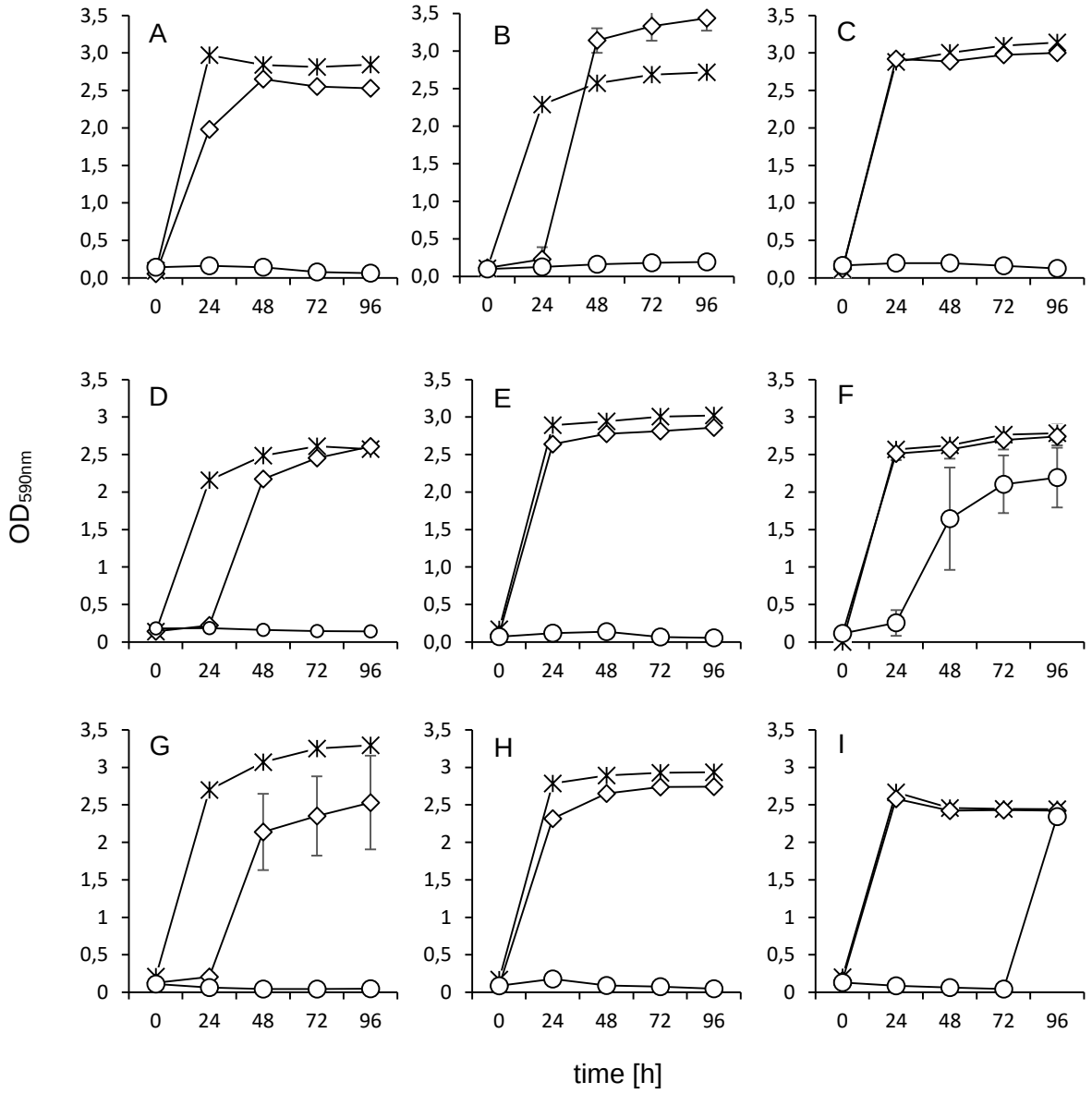

Figure 24: Oxidative stress response of *L. sakei* strains. The figures show the growth upon three different concentrations of hydrogen peroxide. The hydrogen peroxide concentration are displayed as follows: (x) 0 mM, (◇) 1.56 mM and (○) 3.13 mM for the strains (A) TMW 1.3, (B) TMW 1.46, (C) TMW 1.114, (D) TMW 1.417, (E) TMW 1.578, (F) TMW 1.1189, (G) TMW 1.1239, (H) TMW 1.1396 and (I) TMW 1.1398.

In summary, also the tolerance to oxidative stress cannot accomplish to serve as a marker for the assertiveness of *L. curvatus* and *L. sakei* in sausage fermentations. Again, like described for the stress tolerance to oxidative stress, no clear correlations can be found linking the response to cold stress to the expression of assertiveness in raw sausage fermentations.

3.3.2.2 **Cold shock tolerance**

The effect of cold stress on growing cells and the strains growth behaviour in mMRS with 0.2 % glucose was investigated. Growth behaviour in general and growth behaviour with the prior induction of cold shock was compared to gain insights into the diversity of cold shock stress response.

Growth was mainly characterized by the parameters lag-phase, growth rate [µ] and maximum OD ($OD_{max.}$). Table 12 lists all strains and the development of growth parameters with and without the induction of cold shock.

Recorded growth curves with and without cold shock of *L. curvatus* (Figure 25) and *L. sakei* (Figure 26) revealed a high variability within the species contributing to their predicted diversity. Moreover, the results demonstrate that stress response and adaptation is strongly strain specific.

Without the induction of cold shock, lag phases ranged from 0.86 to 5.64 h (average lag-phase of 2.75 h) for *L. curvatus* and from 1.3 to 4.57 h (average lag-phase of 2.9 h) for *L. sakei*. The shortest lag-phase was recorded for the *L. curvatus* strain TMW 1.401 (0.86 h), the longest lag-phase for *L. curvatus* TMW 1.421 (5.64 h). Growth rates varied from 0.07 to 0.52 (average) for *L. curvatus* and from 0.05 to 0.75 for *L. sakei*. The overall maximum recorded for growth rate was 0.75 of *L. sakei* TMW 1.1398. OD values varied from 0.42 to 1.62 *for L. curvatus* and from 0.60 to 1.61 for *L. sakei*.

Interestingly, the induction of cold stress does not necessarily weaken the growth and the performance of the strain. On the contrary: some strains demonstrably showed an enhancement of growth, which is clearly indicated by the affected growth parameters lag-phase, growth rate and maximum OD. Though, a simultaneous enhancement of all three parameters is rare and was only proven for the *L. sakei* strains TMW 1.1239 and TMW 1.1396. Nevertheless, parameters were clearly affected by the induction of cold stress resulting in strain-specific stress responses.

In total, 5 of 9 *L. curvatus* (TMW .27, TMW 1.421, TMW 1.439, TMW 1.1381 and TMW 1.1390) and 5 of 9 *L. sakei (*TMW 1.46, TMW 1.114, TMW 1.1239, TMW 1.1396 and TMW 1.1398*)* strains showed a shortened lag-phase after cold stress treatment. However, the reduction of lag-phases were distinctly stronger for *L. curvatus* with an average reduction of lag-phase 1.1 h than for *L. sakei with* an average reduction of lag-phase of 0.424. Growth rates rose slightly for 3 *L. curvatus* (TMW 1.401, TMW 1.407 and TMW 1.439) and strongly for only 1 *L. curvatus* (TMW 1.624) but for 3 *L. sakei* (TMW 1.417, TMW 1.1239 and TMW 1.1396). The maximum OD was mostly negatively affected. Only 2 *L. sakei* strains showed a distinct increase in OD (TMW 1.1396 and TMW 1.1398).

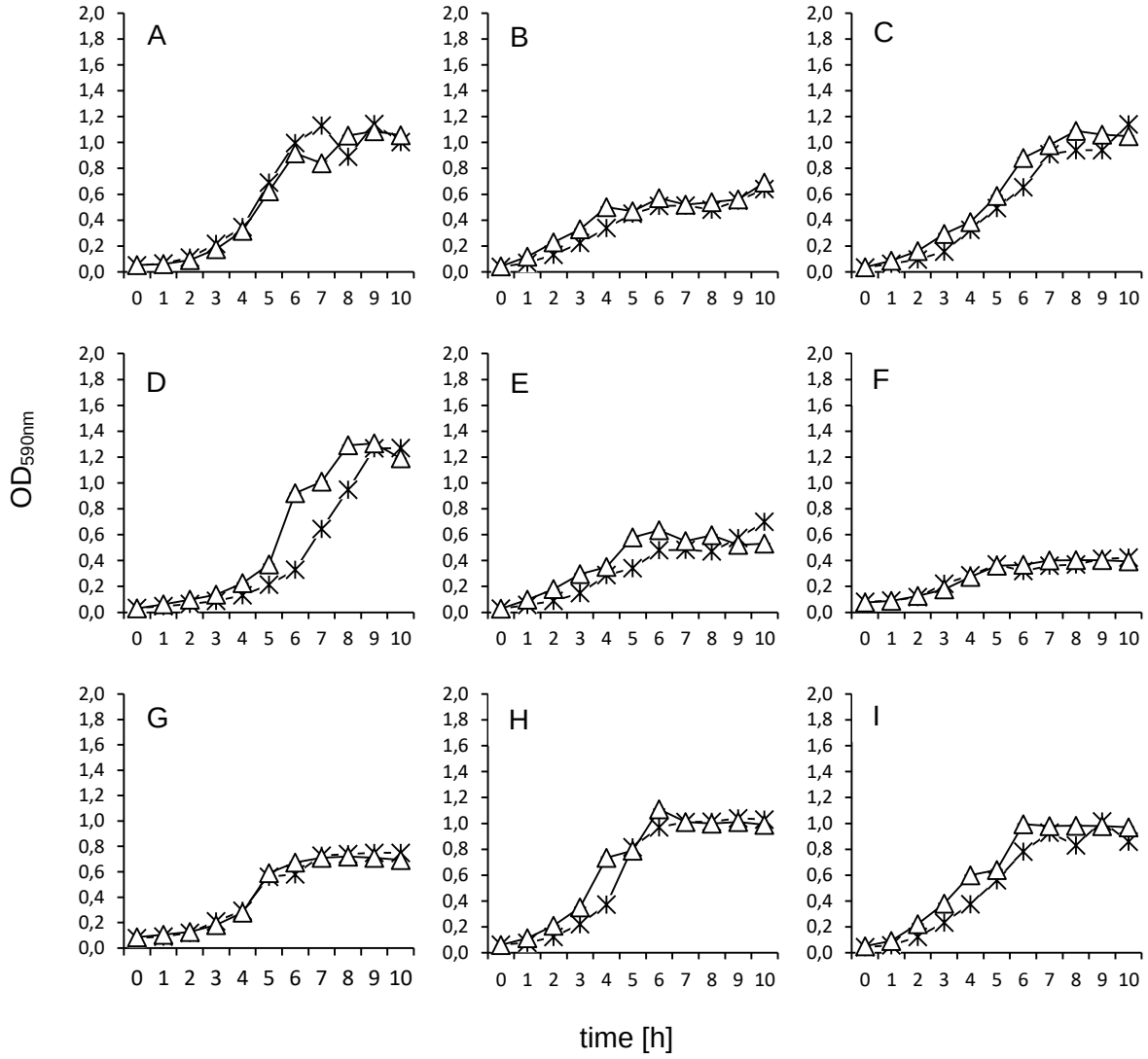

Figure 25: Cold stress response growth kinetics of *L. curvatus*. The growth curves are displayed as follows: (x) without cold shock, (△) with induced cold-shock for the strains (A) TMW 1.27, (B) TMW 1.401, (C) TMW 1.407, (D) TMW 1.421, (E) TMW 1.439, (F) TMW 1.595, (G) TMW 1.624, (H) TMW 1.1381 and (I) TMW 1.1390.

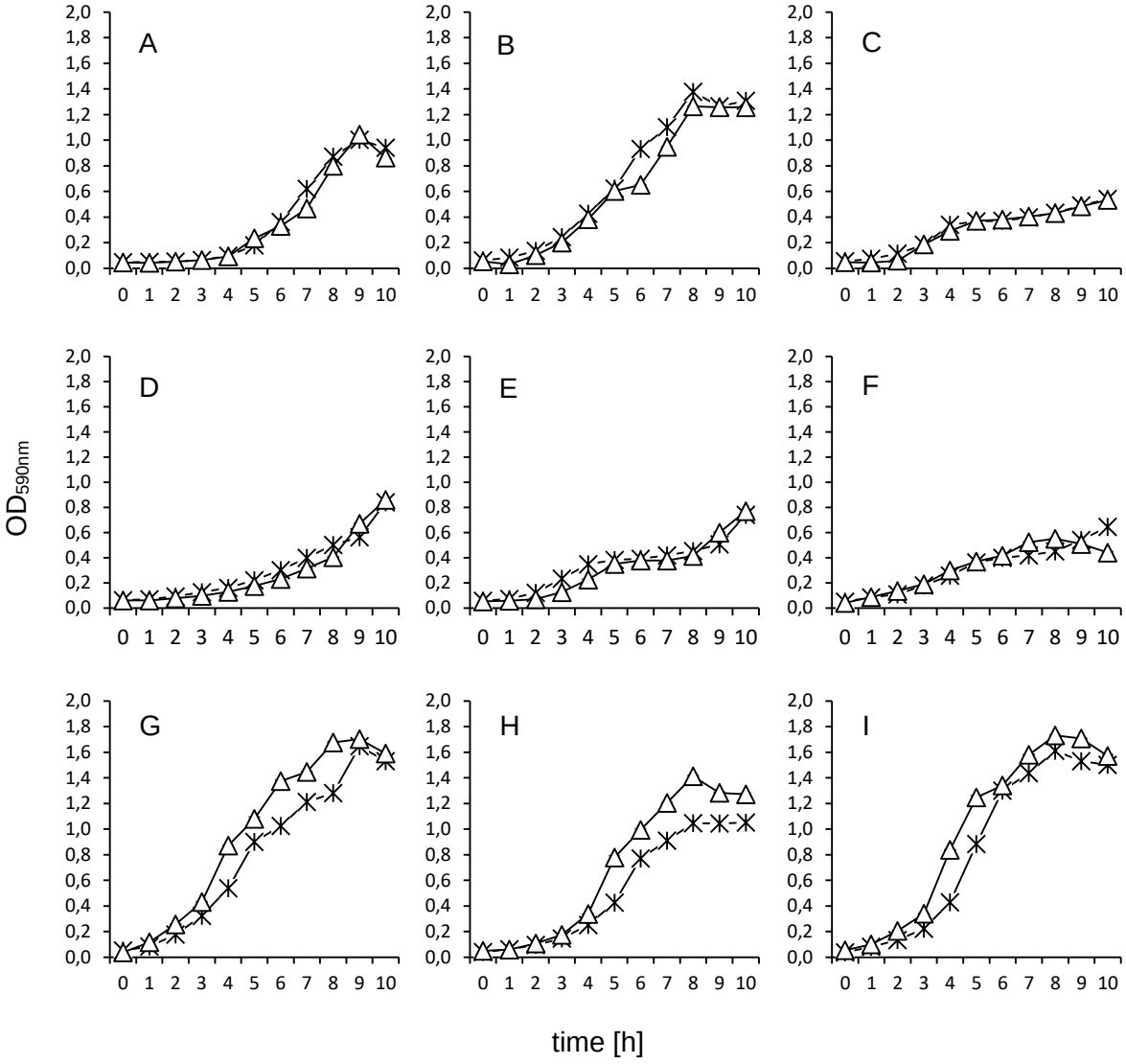

Figure 26 : Cold stress response growth kinetics of *L. sakei*. The growth curves are displayed as follows: (x) without cold shock, (△) with induced cold-shock for the strains. (A) TMW 1.3, (B) TMW 1.46, (C) TMW 1.114, (D) TMW 1.417, (E) TMW 1.578, (F) TMW 1.1189, (G) TMW 1.1239, (H) TMW 1.1396 and (I) TMW 1.1398.

Table 12: Grofit-Evaluation of *L. curvatus* and *L. sakei* with special emphasis to lag-phase, growth rate and $OD_{max.}$ The figure is compartmentalized according to the species and the respective growth parameters.

			1.27	1.401	1.407	1.421	1.439	1.595	1.624	1.1381	1.1390
L. curvatus	lag phase	wo cs	3.5	0.86	2.46	5.64	1.94	1.88	2.63	3.36	2.49
		w cs	3.09	1.06	2.73	3.9	1.26	1.93	3.14	1.88	1.31
		Δ lag	-0.42	0.2	0.27	-1.74	-0.68	0.05	0.51	-1.47	-1.19
	growth rate μ	wo cs	0.41	0.07	0.19	0.44	0.1	0.11	0.16	0.52	0.24
		w cs	0.27	0.15	0.25	0.36	0.14	0.09	0.39	0.26	0.18
		Δμ	-0.13	0.08	0.06	-0.08	0.04	-0.02	0.23	-0.26	-0.06
	$OD_{max.}$	wo CS	1.15	0.92	1.17	1.27	0.7	0.42	0.75	1.04	1.02
		w CS	1.09	0.87	1.09	1.31	0.63	0.4	0.72	1.11	0.99
		$\Delta OD_{max.}$	-0.06	-0.05	-0.08	0.04	-0.07	-0.02	-0.03	0.07	-0.02
			1.3	1.46	1.114	1.417	1.578	1.1189	1.1239	1.1396	1.1398
L. sakei	lag phase	wo cs	4.44	2.93	2.69	4.57	1.33	1.3	1.94	3.62	3.28
		w cs	4.84	2.89	2.11	6.6	2.68	1.5	1.78	3.23	2.33
		Δ lag	0.39	-0.04	-0.58	2.02	1.35	0.19	-0.16	-0.39	-0.95
	growth rate μ	wo cs	0.26	0.39	0.09	0.11	0.05	0.04	0.38	0.29	0.75
		w cs	0.23	0.29	0.07	0.22	0.06	0.05	0.57	0.48	0.7
		Δμ	-0.03	-0.11	-0.02	0.11	0.01	0	0.19	0.2	-0.05
	$OD_{max.}$	wo CS	1.35	1.38	0.60	0.95	0.79	0.65	1.65	1.05	1.61
		w CS	1.04	1.26	0.56	0.95	0.81	0.55	1.7	1.41	1.73
		$\Delta OD_{max.}$	-0.31	-0.11	-0.04	0	0.02	-0.1	0.06	0.36	0.12

3.4 Bacteriocins

The possible formation of bacteriocins was investigated to gain insights into their potential effect on the assertiveness of strains within sausage fermentations.

3.4.1 Bacteriocin cluster distribution within bacterial genomes

Bacteriocin formation was investigated by two approaches. First, strains were analysed regarding the presence of possible bacteriocin clusters within their genomes. For this purpose, the online tool BAGEL3 was used. Table S.1 displays the found gene clusters relying to bacteriocin formation for both species.

The occurrence of gene clusters associated with bacteriocin formation was found to be generally more abundant in *L. curvatus* than in *L. sakei*. Only 4 *L. sakei* strains were found to encompass one bacteriocin cluster within their genomes. Compared to these findings, only 2 *L. curvatus* strain do not possess any gene clusters associated with bacteriocin formation. *L. curvatus* strains usually own gene clusters for Sakacin Q as well as putative bacteriocin clusters. The strain *L. curvatus* TMW 1.624 seems especially noteworthy as it is associated with 5 possible bacteriocin clusters, including Enterocin NKR-5-3A and SakT α. However, the occurrence of bacteriocins within *L. sakei* strains is more diverse than in *L. curvatus*. TMW 1.46 is characterized by the presence of a carnocin gene cluster, TMW 1.114 as well as TMW 1.578 owns genes for Sakacin Q and TMW 1.3 is associated with a gene cluster for a not further described bacteriocin.

3.4.2 In vivo bacteriocin production on mMRS

To test the contribution of bacteriocin formation in vivo, culture supernatants from 9 *L. curvatus* and 9 *L. sakei* strains, derived from cultivation in standard mMRS medium, were examined for growth inhibition of other strains. Table 13 summarizes the distribution of bacteriocin production along the species *L. sakei* and *L. curvatus*. In vivo, none of the tested *L. sakei* and only one *L. curvatus* strain was tested positive for bacteriocin production. However, *L. curvatus* TMW 1.624 was able to produce one or more active bacteriocins, which inhibited all other tested 8 *L. curvatus* strains but none of the 9 *L. sakei* strains. Indeed, the activity spectrum of TMW 1.624 produced bacteriocin or bacteriocins appeared to be limited to the same species as no inhibition zones were detected for *L. sakei* indicator strains.

Table 13: In vivo expression of bacteriocin activity of *L. curvatus* and *L. sakei* strains in liquid mMRS. All indicator strains were grown on mMRS plates. (●) no bacteriocin production, (●) verifiable bacteriocin production

Species	TMW	bacteriocin production	Species	TMW	bacteriocin production
L. curvatus	1.27	●	*L. sakei*	1.3	●
	1.401	●		1.46	●
	1.407	●		1.114	●
	1.421	●		1.417	●
	1.439	●		1.578	●
	1.595	●		1.1189	●
	1.624	●		1.1239	●
	1.1381	●		1.1396	●
	1.1390	●		1.1398	●

3.4.3 Assertiveness of bacteriocin producing *L. curvatus* TMW 1.624

As described in Chapter 3.4.2 *L. curvatus* TMW 1.624 was one of the strains able to dominate the autochthonous microbiota and the meat environment without cooperating with other lactobacilli. By a not further known strategy it dominated the meat batter by competitive exclusion. Bacteriocin formation of TMW 1.624 could be demonstrated along fast agar drop tests using traditional mMRS medium (Chapter 3.4.2). Thus, the activity spectrum of the detected bacteriocin appeared limited to members of the same species.

However, the analysis of its genome with BAGEL3 revealed 5 bacteriocin clusters. So far, it is still not clear whether only one bacteriocin is produced or if the strain is also capable of producing more than one active bacteriocin(s), which could also be active against *L. sakei* strains. Those bacteriocins may only be detectable using other selective media for enrichment and expression.

As a first hint and as the bacteriocin production suggests itself for a crucial role in the assertiveness of strains, another competition study series was conducted. The *L. curvatus*

strain TMW 1.624 had to compete not only with members of the same species but with members of the species *L. sakei*. Therefore, the competition series including set I with its major dominant and potentially bacteriocin producing strain TMW 1.624 and either set II, III or IV were conducted.

The results of this competition study series are presented in Figure 27. The results clearly illustrate, that *L. curvatus* TMW 1.624 dominated all competition experiments irrespective of the type of competitors used. The previously dominant winner strains of set II (TMW 1.1390 and TMW 1.439), III (TMW 1.3, TMW 1.417 and TMW 1.1398) and IV (TMW 1.1396) were lucidly overgrown and reduced in their relative abundance due to the presence and predominance of TMW 1.624. Its dominance was remarkably reflected by its determined relative abundance at day 5 of 86.5 % (set I vs. set II), 60.4 % (set I vs. set III) and 62.5 % (set I vs. set IV). Nevertheless, the dominance of *L. curvatus* TMW 1.624 was not as expressed in combinations with *L. sakei* strains.

As TMW 1.624 was shown to possess a wide variety of bacteriocin clusters within its genome, it can be speculated that the high assertiveness is strongly based on bacteriocin formation. This, therefore suggests a strong impact of bacteriocins on the assertiveness of strains in general. In the special case of the present competition studies, it can be assumed that TMW 1.624 is able to produce antimicrobial substances, likely bacteriocins, during raw sausage fermentation, which are active and harmful against not only *L. curvatus* but *L. sakei* as well.

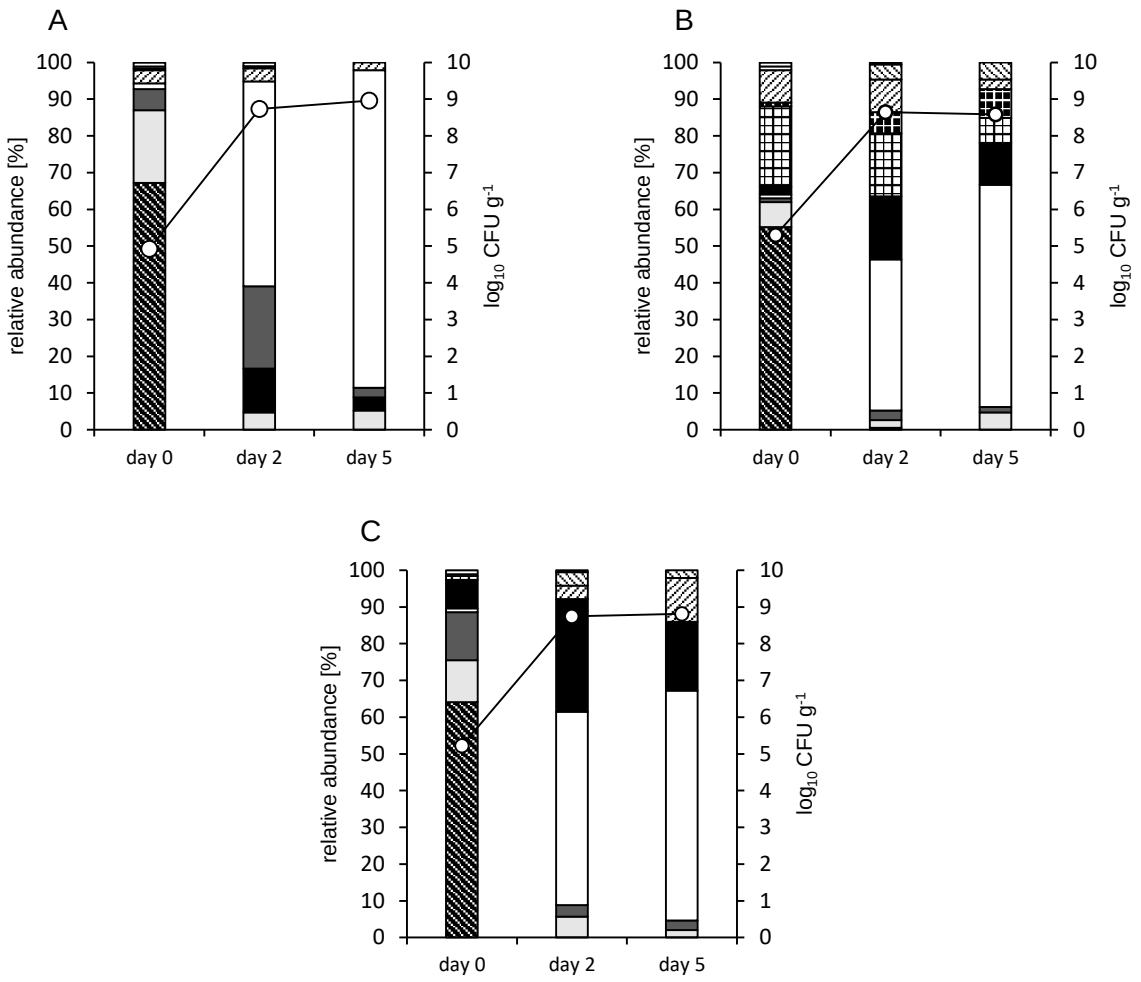

Figure 27: Microbiota development of set I versus set II (A), set I versus set III (B) and set I versus set IV (C) over the total fermentation time of 5 days determined on MRS. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points. The figure shows the average of two biological replicates for each set tested with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows:

(A) - (□) *L. curvatus* TMW 1.624, (■) *L. curvatus* TMW 1.439, (■) *L. curvatus* TMW 1.595, (▨) *L. curvatus* TMW 1.1390, (□) *L. curvatus* starter other (▧) *L. curvatus*, (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

(B) - (□) *L. curvatus* TMW 1.624, (■) *L. sakei* TMW 1.3, (▦) *L. sakei* TMW 1.417, (▦) *L. sakei* TMW 1.398, (■) *L. sakei* starter other, (□) *L. curvatus* starter other, (▨) *L. sakei*, (▧) *L. curvatus*, (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

(C) - (□) *L. curvatus* TMW 1.624, (■) *L. sakei* TMW 1.396, (■) *L. sakei* starter other, (□) *L. curvatus* starter other, (▨) *L. sakei*, (▧) *L. curvatus*, (▧) *S. carnosus* TMW 2.212, (▤) other and (▥) nri.

3.4.4 Bacteriocin production of TMW 1.624

In vivo bacteriocin production of *L. curvatus* TMW 1.624 using traditional mMRS medium for enrichment and plating was shown before (Chapter 3.4.2). However, the bacteriocins activity spectrum remains limited to *L. curvatus* strains. As the genome analysis revealed several clusters within *L. curvatus* TMW 1.624, another set of competitions studies were conducted to enlighten the assertiveness of TMW 1.624 not only in set I but in combination with set II, III and IV. The strain TMW 1.624 was always dominant, irrespective from the competitor set used. These findings elucidate the assumption that bacteriocins may be the essential factor responsible for the assertiveness of *L. curvatus* TMW 1.624. So far, we were not able to show the expression of bacteriocins inhibiting *L. sakei* strains. By the use of mMSM, the expression of bacteriocins, limiting the growth of *L. sakei* should be achieved.

For this purpose, competition studies were conducted in liquid media. This time, *L. curvatus* TMW 1.624 was grown in co-cultures with one other *L. sakei* strain. Upon plating and MALDI-TOF MS analysis, the relative abundances of the introduced strains could be depicted (Table 14). Again, the results clearly demonstrate the dominance of TMW 1.624. Only *L. sakei* TMW 1.1239 was able to compete with *L. curvatus* TMW 1.624 as their relative abundances were very similar, e.g. 52 % for *L. sakei* and 48 % for TMW 1.624. Furthermore, *L. sakei* TMW 1.1239 was also able not only to compete, but to overgrow TMW 1.624 when either catalase, proteinase K or trypsin was added to the medium, indicating hydrogen peroxide as well as an inhibiting proteinaceous agent as part of the assertiveness strategy of *L. curvatus* TMW 1.624.

Besides this exception, the dominance of *L. curvatus* TMW 1.624 was verified within the liquid medium. By the addition of catalase, the dominance of TMW 1.624 was mostly not affected, which proves that the dominance of TMW 1.624 and the concomitant inhibition of the *L. sakei* strain is not based on the formation of hydrogen peroxide. Only the *L. sakei* strain TMW 1.417 were shown to be more abundant in experiments with added catalase (57 % 1.417 and 43 % TMW 1.624). This highlights the production of hydrogen peroxide as one reason for assertiveness of TMW 1.624. However, this observation was only made for this particular strain, whereas all other *L. sakei* strains did not change in their relative abundance. Interestingly, the dominance of TMW 1.624 was not reduced upon the addition of either proteinase K or trypsin.

Table 14: Results of the co-culture competition experiments. The bacteriocin producing strain TMW 1.624 was grown in co-culture with its respective *L. sakei* partner strain given in the first column. The strain were co-cultures either in mMSM medium without any additive or in mMRS medium with either catalase, Proteinase K or Trypsin. By plating and analysis with MALDI-TOF MS their relative abundances (%) were determined. The green marked relative abundance belongs to the dominant strain. The bacteriocin producing strain TMW 1.624 is always underlined.

	Relative abundance [%] in mMSM medium with:							
	without additive		3000 U/ml catalase		1 mg/ml proteinase K		1 mg/ml trypsin	
1.417	1.624	97	1.624	43	1.624	98	1.624	100
	1.417	3	1.417	57	1.417	2	1.417	0
1.578	1.624	100	1.624	100	1.624	97	1.624	95
	1.578	0	1.578	0	1.578	2	1.578	5
1.1189	1.624	100	1.624	100	1.624	98	1.624	98
	1.1189	0	1.1189	0	1.1189	2	1.1189	2
1.1239	1.624	48	1.624	1	1.624	17	1.624	20
	1.1239	52	1.1239	99	1.1239	83	1.1239	80
1.1396	1.624	100	1.624	99	1.624	97	1.624	84
	1.1396	0	1.1396	1	1.1396	3	1.1396	16
1.1398	1.624	100	1.624	100	1.624	98	1.624	98
	1.1398	0	1.1398	0	1.1398	2	1.1398	2

Upon fast agar spot tests (see Chapter 2.5), the proteinaceous nature of the inhibiting agent, produced by TMW 1.624, and could be elucidated. According to the given hypothesis, the majority of *L. sakei* strains were inhibited by the supernatant, harvested along the co-cultivation of TMW 1.624 and the respective partner *L. sakei* strain. Table 14 summarizes the results of the fast agar spot test. In most cases, the addition of proteinase K led to a suppression of the previously observed inhibition. The treatment with trypsin was not that successful and worked only in two cases. However, differently to the gained results in liquid co-culture competition tests, the strain TMW 1.417 was not inhibited at all. In most cases, except for the treatment with catalase, the strain was completely overgrown in liquid media, but could withstand the supernatant in the fast agar spot test as no inhibition zones could be identified. *L. sakei* TMW 1.1239, which showed similar abundance like TMW 1.624 or dominance in liquid media tests, was not inhibited by the supernatant.

Table 15: Results of the fast agar spot test with supernatant of co-cultures of TMW 1.624 and its respective co-cultured strain TMW 1.417, TMW 1.578, TMW 1.1189, TMW 1.1239, TMW 1.1396 and TMW 1.1398. The supernatant was used either without any previously treatment or with catalase, proteinase K or trypsin treatment prior to the agar spot test. (●) indicates an inhibition of the respective indicator strain, whereas (●) stand for no inhibition.

	Supernatant treated with			
	No additive	3000 U/ml catalase	1 mg/ml proteinase K	1 mg/ml trypsin
TMW 1.471	●	●	●	●
TMW 1.578	●	●	●	●
TMW 1.1189	●	●	●	●
TMW 1.1239	●	●	●	●
TMW 1.1396	●	●	●	●
TMW 1.1398	●	●	●	●

In conclusion, bacteriocin formation seems to play a pivotal role in the assertiveness of *L. curvatus* TMW 1.624. Beside the exceptions of *L. sakei* TMW 1.417 and TMW 1.1239 all other tested *L. sakei* strains were inhibited in liquid culture as well as on fast agar spot test using the harvested supernatant. However, due to the observed exceptions, bacteriocin formation is not the only reason driving the assertiveness of the strain. Also the formation of H202 suggests itself for a substantial role, indicating the assertiveness of a strain cannot be traced back to single parameters.

3.5 Prophages

3.5.1 Prophage distribution along *L. sakei* and *L. curvatus* strains

The presence of prophages within bacterial genomes was checked using the web tool PHASTER. Whole genome sequences were provided by Lara Eisenbach (Eisenbach et al., 2019; Eisenbach et al., 2018). The results show, that all of the 18 *L. sakei* and *L. curvatus* strains possess prophage related genes and sequences within their genome (Figure 28).

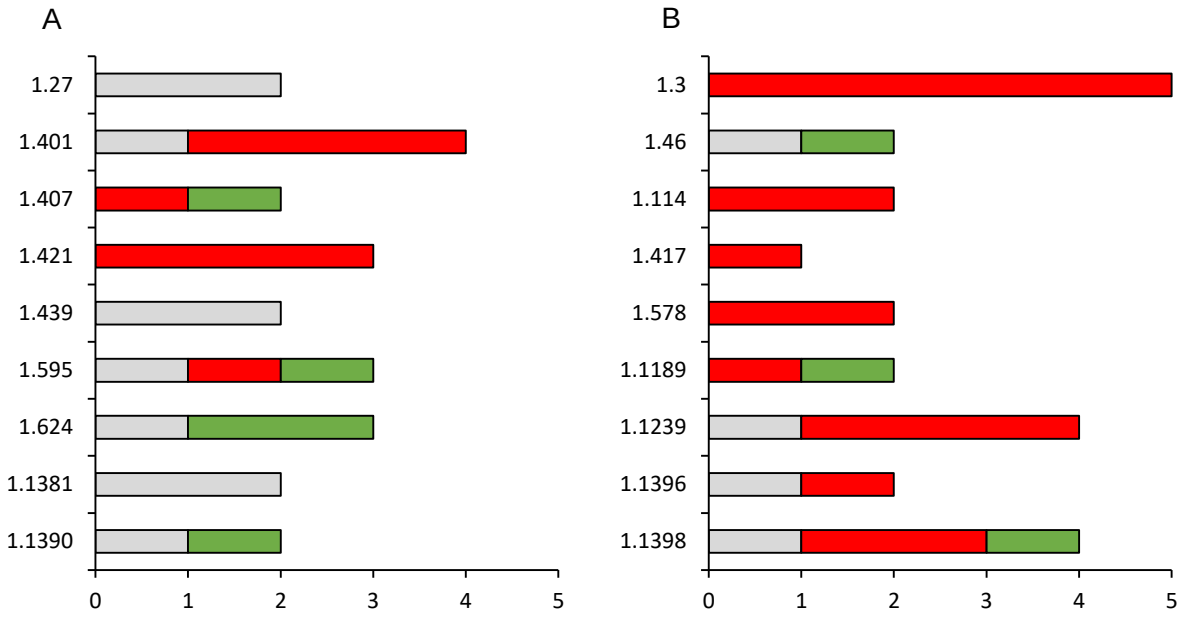

Figure 28: Presence of prophages within the genome of 9 *L. curvatus* (A) and 9 *L. sakei* strains (B). (□) questionable prophage, (■) incomplete prophage, (■) intact prophage.

Found prophages or prophage elements were annotated either as questionable, incomplete or intact based on the PHASTER completeness score (proportion of phage genes in the identified region). Intact prophages were only found in 3 *L. sakei* and 4 *L. curvatus* strains. The vast majority of strains are characterized by incomplete and/or questionable prophages. However, the presence of intact but also incomplete prophage sequences indicates that the insertion of prophages as mobile elements is very common. Due to mutation, various genome rearrangements and gradual decay, the prophage genome undergoes modifications and partly deletions leading to defective, cryptic prophages (identified as incomplete or questionable).

3.5.2 Prophage characterization of TMW 1.1398

Due to variable results in strain-specific colony-PCR indicating the spontaneous loss of the prophage, *L. sakei* TMW 1.1398, was selected for further investigations.

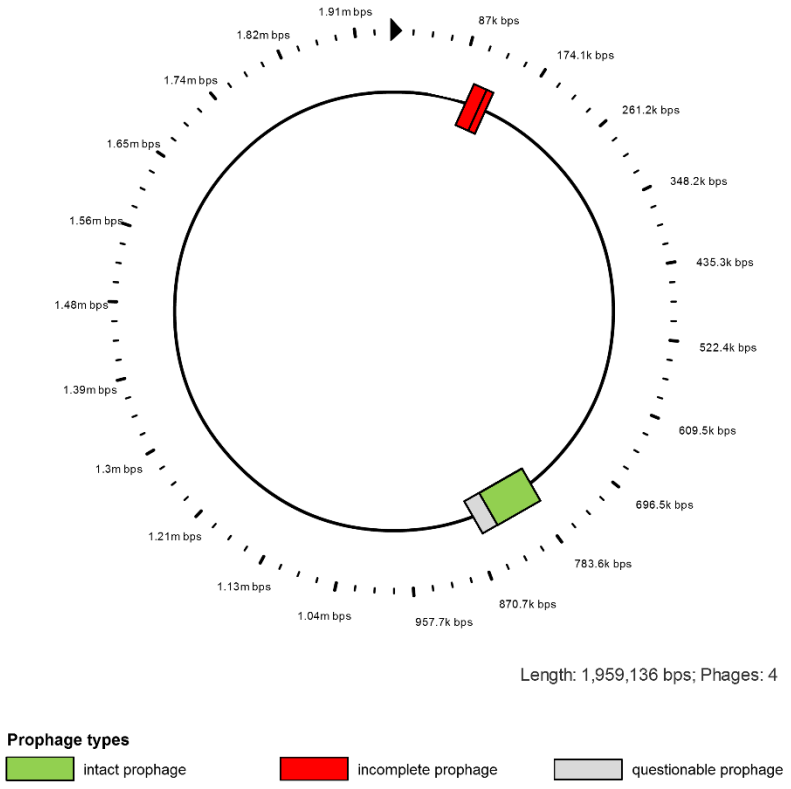

Figure 29: Localization of prophage genomes or prophage regions within the genome of *L. sakei* TMW 1.1398. The analysis with PHASTER revealed one intact (■), two incomplete (■) and one questionable (□) prophage region.

According to the annotation with PHASTER, the *L. sakei* strain TMW 1.1398 is characterized by the presence of 4 phage regions of which only one was predicted as an intact prophage. The other 3 regions were categorized as either incomplete (2) or questionable (1). The localization of the mentioned phage regions within the genome of TMW 1.1398 was determined with PHASTER and is depicted in Figure 29.

The further analysed intact prophage genome of TMW 1.1398 is 40.8 kb in length and contains 55 ORF´s (see Table 16) with an overall G+C content of 39.29 %. The prophage genome is flanked by 11 bp long attachment-sites attL and attR with the nucleotide sequence of AATTTGACCAT.

To investigate the affiliation of the prophage, the amino acid sequence of the phage integrase was compared to other database entries using blastp. The highest homology was found for

Bacillus phage phi4J1 (ALO79832.1) with 37 % identity, followed by a 32 % identity of the *L. sanfranciscensis* phage Ev3 (CUQ99446.1), of which both are described as prophages belonging to the family of *Siphoviridae* in the order of *Caudovirales* with a non-contractile tail, indicating the same affiliation for the prophage of TMW 1.1398, which was hereafter named φ-DJ1812.

By comparison of PHASTER output and the Genebank file of TMW 1.1398, it was possible to construct a full gene map of the prophage (Figure 30). Additionally, all protein amino acid sequences were used for blasting using “blastp” to confirm the annotation of the respective gene/protein.

Figure 30 displays the whole genome composition and structure of the integrated prophage φ-DJ1812 of TMW 1.1398. All predicted genes are indicated as arrows.

All necessary gene cassettes were found within the genome of φ-DJ1812 including DNA regulation module, packaging module, phage structural proteins module, host lysis protein module, lysis/lysogeny module and diverse hypothetical proteins of unknown function. Genome annotation with PHASTER revealed that 30.9 % of the open reading frames correspond to hypothetical protein with unknown identity and function.

Moreover, it was possible to detect insertion elements within the prophage genome of φ-DJ1812. One of the found transposases was integrated into one of the major tail genes, which drastically increases the probability of defects and defect cryptic phages. It can be assumed that the assembly of phages in the later stage of prophage induction, which is followed by the lysis of the cell, cannot be completed. Therefore, only inactive phages would be released with no infectious nature.

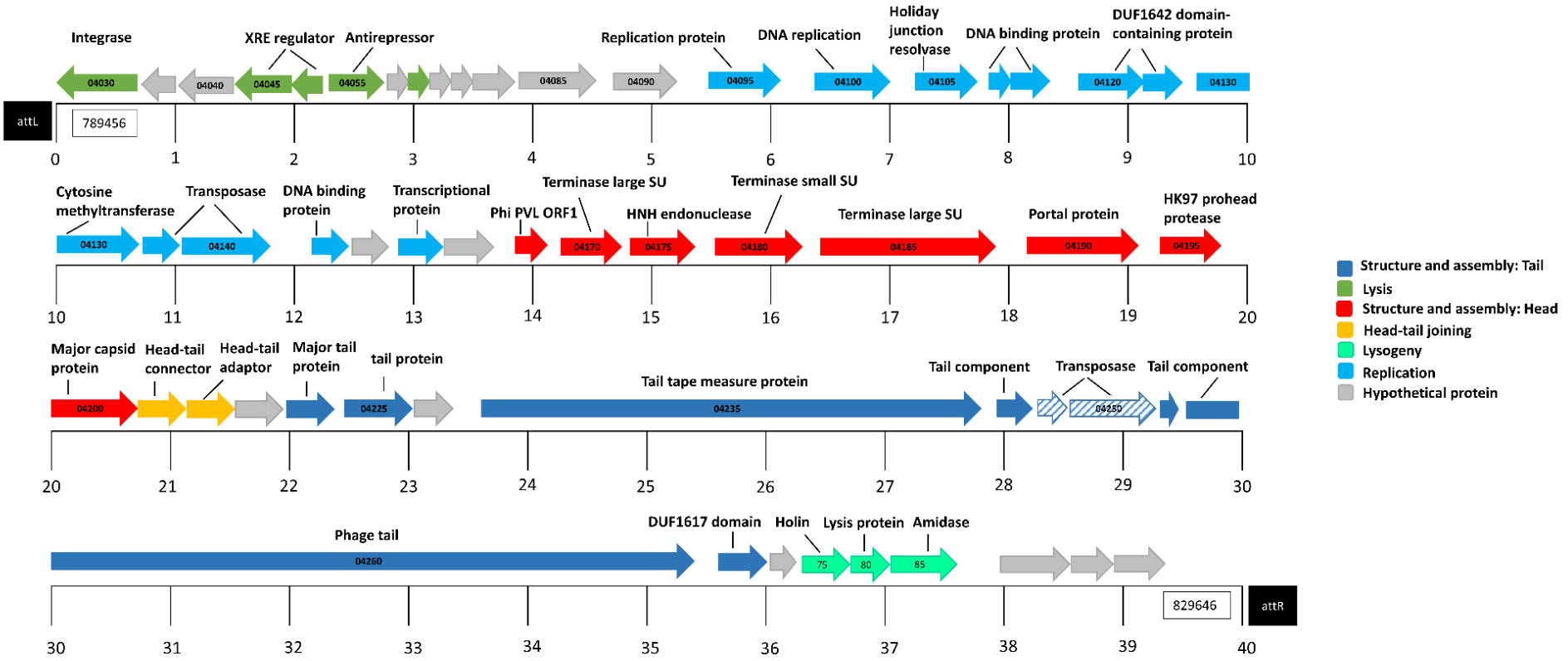

Figure 30: Genome map of φ-DJ1812 as it is integrated in the bacterial chromosome. The arrows mark the transcribed prophage genes. The length of the prophage genome is given in kb including the start and end position within the bacterial genome.

3.5.3 Induction of the temperate prophage φ-DJ1812 of *L. sakei* TMW 1.1398

Induction of the temperate prophage φ-DJ1812 was conducted using UV irradiation and mitomycin C treatment. Both treatments are discussed in detail in the respective chapter.

3.5.3.1 Prophage induction using UV irradiation

The temperate phage of TMW 1.1398 was induced using different doses of UV-irradiation. Radiation was applied at a cell turbidity of 0.3-0.4 OD_{590nm}, whereas UV doses varied from 2 to 10 min. As an internal control, the culture was also grown without prior UV treatment. Successful prophage induction was clearly indicated by a partial cell lysis of the growing culture. Growth and potential cell lysis were monitored by the measurement of cell turbidity at 590 nm every 5 minutes.

The results are summarized in Figure 31 and lucidly show the inducibility of TMW 1.1398 prophage using UV-irradiation. However, the induction strongly depends on the applied irradiation time. The greatest impact on the growing culture was achieved and observed with UV doses of 2 and 4 minutes. Partial cell lysis was always observed after 2-3 hours and was indicated by the decrease of cell turbidity at 590 nm.

The application of an UV dose of 2 min led to a distinct decrease in cell turbidity at 590 nm of 0.3. Afterwards, the culture was able to re-enter the logarithmic phase. Similar was observed for a higher UV dose of 4 minutes. However, after partial cell lysis, the culture was not able to re-enter the exponential growth phase again.

Despite the slightly observed decrease in OD, UV doses of 6 minutes or longer resulted in a more general growth inhibition without a clearly indicatable lysis. Cell density, which is proportional to the measured cell turbidity, did not decrease and remain at the same level as directly after induction.

As the most effective impact and visualization of prophage induction and cell lysis was achieved using a UV dose of 2 minutes, this UV irradiation was implemented as standard induction method for further experiments.

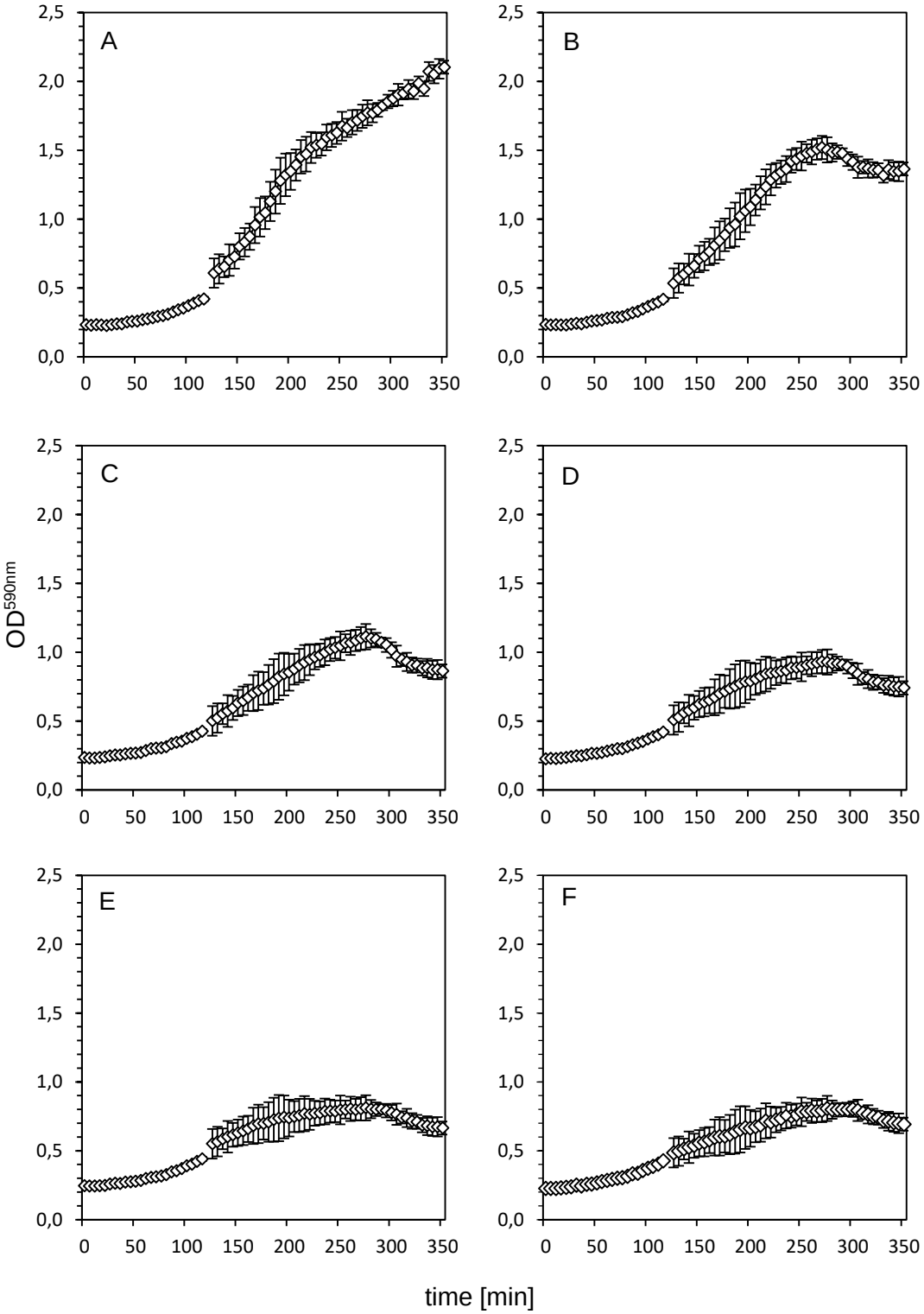

Figure 31: UV-induction of *L. sakei* TMW 1.1398 using different UV-doses of (A) 0 min, (B) 2 min, (C) 4 min, (D) 6 min, (E) 8 min and (F) 10 min. The optical density was measured in an interval of 5 minutes to depict cell-lysis and prophage induction. After 120 minutes at an OD of 0.3-0.4 the cultures were induced.

Besides the determination of the most effective UV-dose, the impact of incubation periods on induction was investigated. Therefore, *L. sakei* TMW 1.1398 was incubated for different incubation

Besides the determination of the most effective UV-dose, the impact of incubation periods on induction was investigated. Therefore, *L. sakei* TMW 1.1398 was incubated for different incubation periods starting from 0 h, meaning the use of a traditional overnight culture, to a freshly inoculated culture of 2 to 8 hours. Prophages were induced at an OD_{590nm} of 0.3 with an UV-dose of 2 minutes. Growth curves were monitored using a microplate reader over a total time frame of 10 hours.

The determined growth kinetics are summarized in Figure 32 and reveal a strong impact of incubation times on the induceability of the temperate phage φ-DJ1812.

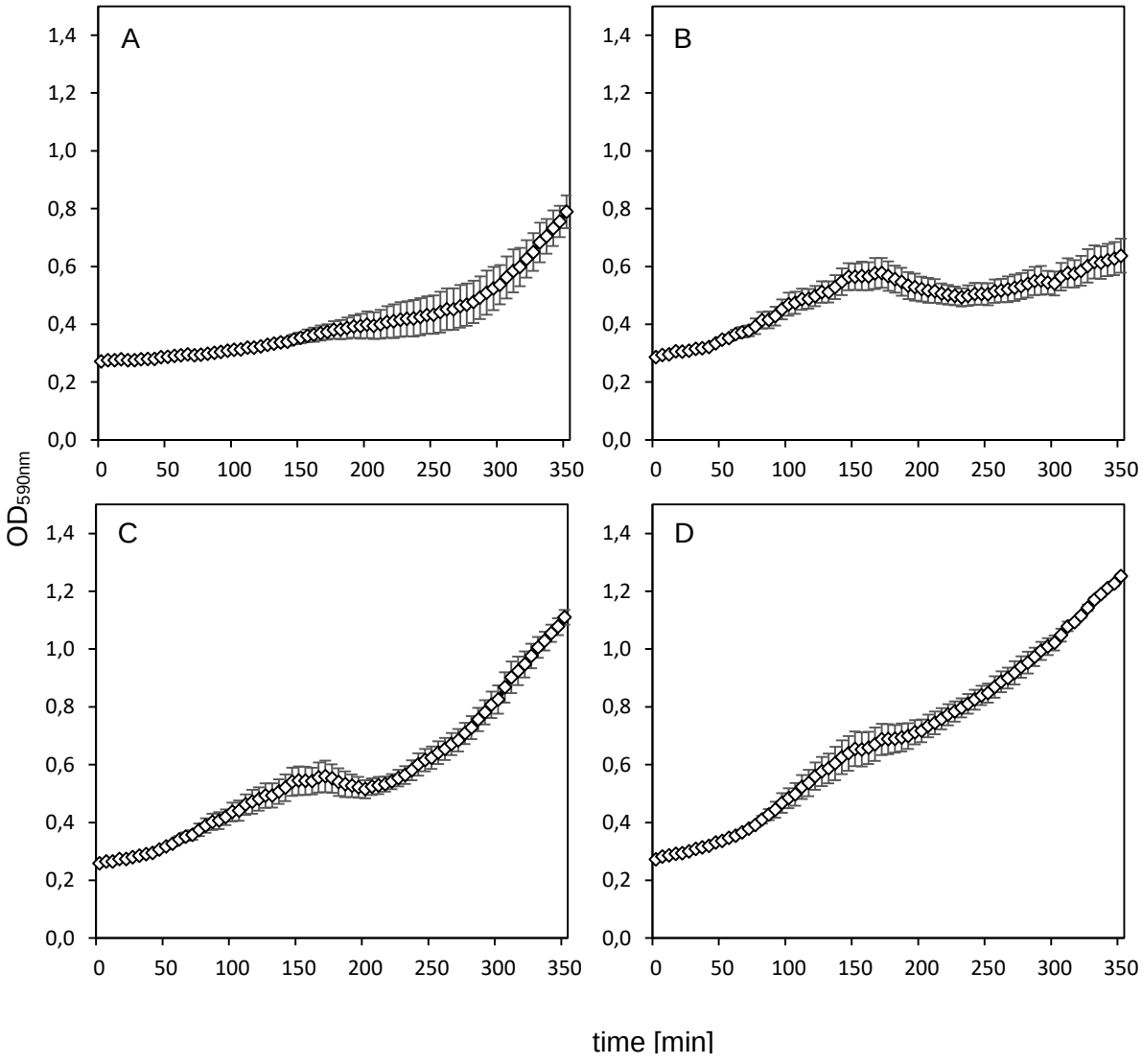

Figure 32: Effect of UV-induction on cultures with different incubation times.The figure shows growth curves after UV induction with a dose of 2 min for (A) the pre-culture, (B) 2 hours, (C) 4 hours and (C) 6 hours.

Freshly inoculated and young cultures in the exponential growth phase tended to be more vulnerable to prophage induction than older cultures and those, which already entered the stationary growth phase. This is clearly indicated by the recorded growth curves in Figure 32. Only cultures with incubation times up to 4 hours were susceptible to the applied UV induction. Cultures with incubation times longer than 4 hours were not inducible anymore. For those cultures, growth was not inhibited at all and tend to be very similar to the growth curve reported for the pre-culture, which was also not inducible and growth was not affected. Furthermore, the latter culture was characterized by a long lag-phase before. Clearly visible prophage induction resulting in partial cell lysis was only observed with cell cultures grown between 2 and 4 hours.

3.5.3.2 **Prophage induction using mitomycin C**

Besides the use of UV irradiation, mitomycin C was tested for the application of prophage induction. As the mitomycin C concentration for prophage induction seems to be species- or even strain specific (Humphrey et al., 1995; Lorenz et al., 2016; Xu et al., 2018a), different concentrations were used and evaluated.

In general, irrespective of the mitomycin C concentration used, no clear prophage induction was observed (Figure 33). The standard concentration of 0.5 µg/ml, found in most of the available literature (Aertsen et al., 2005; Chen et al., 2006; Oliveira et al., 2017), and the higher concentrations of 2.0 and 5.0 µg/ml did not lead to a successful prophage induction. Only when higher mitomycin C concentrations were applied, an effect on the growing culture could be visualized. However, even such high concentrations of mitomycin C did not induce the temperate phage of TMW 1.1398. Only a general inhibition of growth, indicated by the decrease of cell turbidity, was observed (Figure 33, D).

In summary, the results show that prophage induction using mitomycin C failed completely and cannot be included as a standard method for the purpose of prophage induction in the case of *L. sakei* TMW 1.1398.

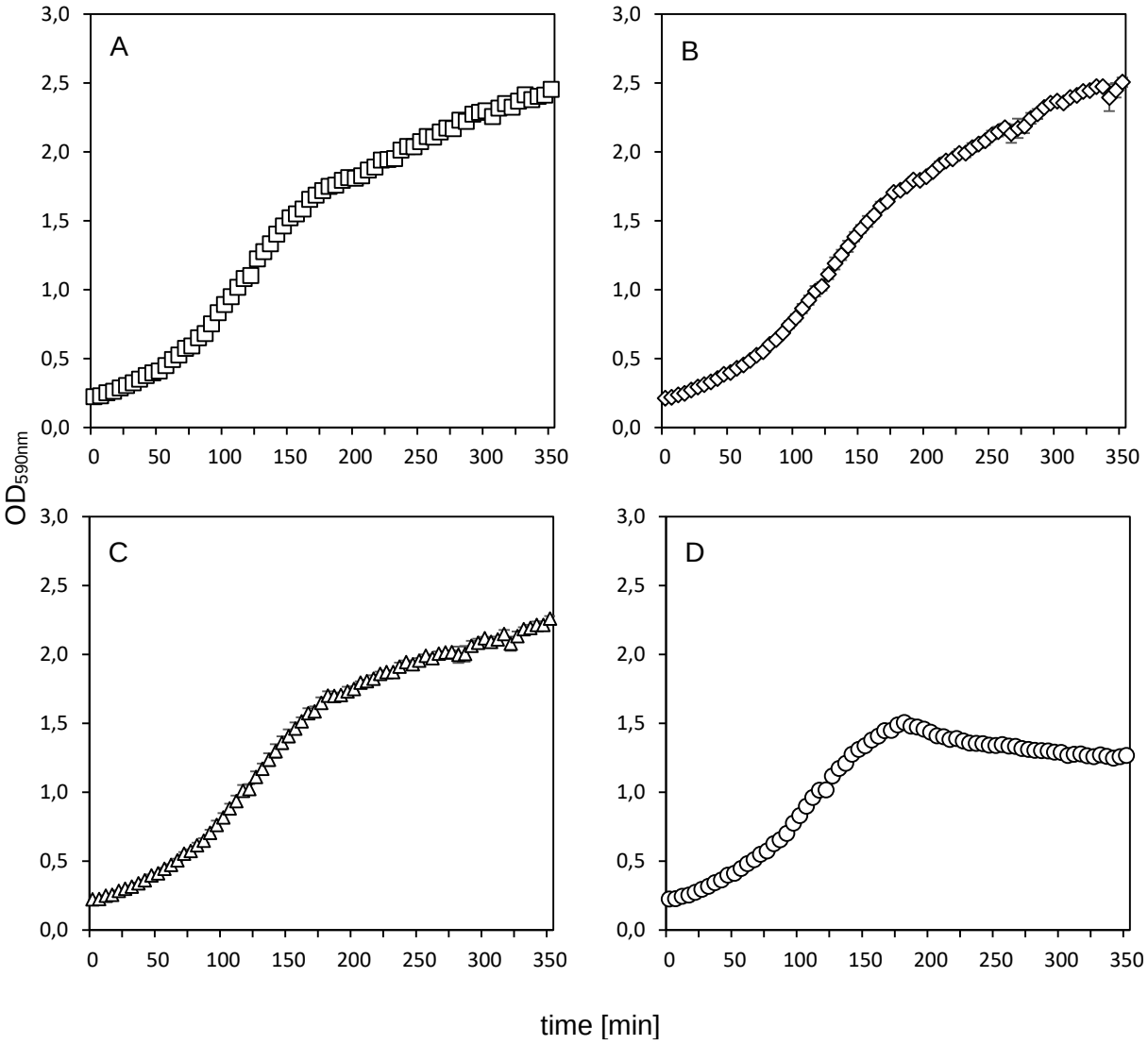

Figure 33: Mitomycin C treatment of TMW 1.1398.Mitomycin C was applied with different concentrations as follows: (□) without Mitomycin C (A), (◇) 0.5 µg/ml (B), (△) 2.0 µg/ml (C) and (○) 5.0 µg/ml (D).

3.5.3.3 **Confirmation of prophage induction**

The proteome analysis of phage lysate using LC-MS/MS is presented in Figure 34. The output of expressed proteins is given within the category “Fasta headers” and correspond to the gene locus in the available Genebank file (see Table 16). By comparison of gene locus numbers, it was possible to determine all expressed proteins in the UV-induced phage lysate of TMW 1.1398. Table 16 summarizes all phage ORFs including their start and stop position in the bacterial genome, their length [bp] as well as their predicted protein size [aa] and their status if they are expressed or not.

The proteome analysis revealed the expression of various phage structure genes, including capsid and tail proteins, proving the induction of the temperate prophage. However, not all proteins encoded by the genes were found to be expressed. An expression of the major tail protein with a predicted size of 1455 bp was not detected. Only minor tail proteins could be determined with this technique. Furthermore, the protein N-acetylmuramoyl-L-alanine amidase was the only protein of the lysis cassette, which was expressed to relatively high amounts.

iBAQ NI	Unique peptides I	Unique peptides NI	Peptides	Sequence coverage [%]	Unique sequence coverage [%]	Mol. weight [kDa]	Intensity	Protein IDs	Fasta headers
20.0654	23.75073	5	8	8	29.30	29.30	30.579	289030000	04040 hypothetical protein
0	24.73081	0	2	2	38.20	38.20	8.6858	83530000	04050 transcriptional regulator
0	22.69045	0	4	4	17.40	17.40	29.375	108300000	04055 phage antirepressor
0	22.71408	0	2	2	37.50	37.50	7.542	27522000	04065 hypothetical protein
0	25.59545	0	8	8	43.40	43.40	22.506	506990000	04080 hypothetical protein
13.90285	23.48321	1	8	8	28.60	28.60	36.267	234820000	04085 hypothetical protein
0	18.8805	0	1	1	3.10	3.10	26.567	4343500	04090 hypothetical protein
0	24.18465	0	7	7	27.50	27.50	30.399	266950000	04095 replication protein
0	20.18459	0	2	2	10.10	10.10	31.079	15492000	04100 DNA replication protein
0	23.03681	0	1	1	10.20	10.20	15.638	51632000	04105 Holliday junction resolvase
0	25.4562	0	5	5	72.90	72.90	8.271	230170000	04110 hypothetical protein
20.44213	29.06044	5	10	12	71.40	57.80	16.316	5051300000	04115 single-stranded DNA-binding protein
0	22.13411	0	1	1	13.20	13.20	12.093	18411000	04145 hypothetical protein
0	24.64378	0	3	3	25.50	25.50	16.277	157280000	04155 hypothetical protein
0	23.32567	0	3	3	41.00	41.00	7.5994	31539000	04165 hypothetical protein
0	27.306	0	4	4	56.80	56.80	9.1375	995550000	04170 hypothetical protein
0	24.69221	0	10	10	69.30	69.30	17.344	271080000	04180 hypothetical protein
0	23.25451	0	11	11	24.80	24.80	65.419	250190000	04185 terminase
15.76764	25.60266	1	17	17	56.60	56.60	44.881	1122200000	04190 phage portal protein
0	24.93369	0	8	8	61.10	61.10	20.188	416610000	04195 hypothetical protein
21.98798	30.57073	12	27	27	58.10	58.10	41.833	25583000000	04200 hypothetical protein
0	24.90545	0	6	6	78.00	78.00	12.621	219980000	04205 hypothetical protein
0	27.33892	0	6	6	61.50	61.50	12.265	848820000	04210 hypothetical protein
0	22.47266	0	2	2	14.40	14.40	13.069	40742000	04215 hypothetical protein
19.56231	28.81546	6	19	19	68.30	68.30	20.788	5678200000	04225 phage tail protein
0	26.07914	0	5	5	31.00	31.00	11.427	496250000	04230 hypothetical protein
16.7628	20.88956	1	13	13	10.30	10.30	143.25	168410000	04235 phage tail tape measure protein
0	27.14802	0	6	6	26.20	26.20	16.703	892340000	04265 hypothetical protein
0	25.93046	0	4	4	38.20	38.20	17.725	511610000	04285 hypothetical protein
0	26.81262	0	2	2	29.20	29.20	7.035	353600000	04300 hypothetical protein
1.758.189	20.7913	1	1	2	6.30	6.30	42.19	16087000	04350 beta-ketoacyl-[acyl-carrier-protein] synthase II

Figure 34. Results of the LC-MS/MS measurement of UV-induced cell lysate of TMW 1.1398

Table 16: Open reading frames deduced from the genome of *L. sakei* phage φ-DJ1812 and their predicted sizes and functions. The proteome analysis using LC-MS/MS revealed the expression of various phage genes. (●) not expressed, (●) expressed.

ORF	locus-taq	protein	start [bp]	stop [bp]}	length bp	predicted protein size [aa]	expressed
		attL					
1	4030	Integrase	789456	790550	1094	364	●
2	4035	hyp. Protein	790753	791043	290	96	●
3	4040	hyp. Protein	791058	791888	830	276	●
4	4045	XRE family transcriptor	792055	792675	620	206	●
5	4050	XRE transcriptional regulator	792834	793064	230	76	●
6	4055	Phage antirepressor	793081	793860	779	259	●
7	4060	hyp. Protein	793872	794081	209	39	●
8	4065	helix-turn-helix domain-containing protein oder XRE	794330	794524	194	64	●
9	4070	hyp. protein	794528	794755	227	75	●
10	4075	hyp. protein	794755	794934	179	59	●
11	4080	hyp. protein	795045	795641	596	198	●
12	4085	hyp. protein	795641	796609	968	322	●
13	4090	hyp. protein	796609	797286	677	226	●
14	4095	replication protein	797279	798067	788	262	●
15	4100	DNA replication protein	798048	798884	836	278	●
16	4105	holiday junction resolvase	798993	799406	413	137	●
17	4110	DNA binding protein	799396	799608	212	70	●
18	4115	single stranded DNA binding protein	799620	800063	443	147	●
19	4120	hyp. protein	800076	800783	707	235	●
20	4125	DUF1642 domain-containing protein	800803	801225	422	140	●
21	4130	DNA cytosine methyltransferase	801285	802481	1196	398	●
22	4135	transposase	802471	802722	251	83	●
23	4140	IS3-like element IS1520 family transposase	802743	803540	797	265	●
24	4145	DNA binding protein	804003	804323	320	106	●
25	4150	hyp. protein	804320	804649	329	109	●
26	4155	transcriptional protein	804794	805207	413	137	●

27	4160	hyp. protein	805573	806160	587	195	
28	4165	phi PVL ORF	806549	806734	185	61	
29	4170	terminase large subunit	806703	806948	245	81	
30	4175	HNH endonuclease	807068	807433	365	121	
31	4180	terminase small su	807558	808019	461	153	
32	4185	terminase large subunit	808012	809721	1709	569	
33	4190	phage portal protein	809902	811092	1190	396	
34	4195	HK97 family prohead protease	811073	811615	542	183	
35	4200	phage major capsid protein	811651	812778	1127	375	
36	4205	phage gp6-like head-tail connector protein	812789	813118	329	109	
37	4210	head tail adaptor protein	813096	813410	314	104	
38	4215	head tail joining protein	813407	813763	356	118	
39	4220	major tail protein	813756	814178	422	140	
40	4225	tail protein	814165	814725	302	100	
41	4230	hyp. protein	815045	815347	302	100	
42	4235	phage tail measure protein	815566	819576	4010	1336	
43	4240	phage tail protein	819569	820003	434	49	
44	4245	transposase	820054	820317	263	87	
45	4250	putative transposase	820356	821123	767	255	
46	4255	hyp. protein	821220	821468	248	144	
47	4260	tail protein	821465	825817	4352	1455	
48	4265	DUF1617 family protein	825814	826263	449	149	
49	4270	hyp. protein	826263	826490	227	75	
50	4275	holin	826530	826982	452	150	
51	4280	lysin	826979	827257	278	92	
52	4285	putative N-acetylmuramoyl-L-alanine amidase	827257	827751	494	164	
53	4290	hyp. protein	828204	828803	599	199	
54	4295	hyp. protein	828851	829207	356	118	
55	4300	hyp. protein	829449	829646	197	65	
		AttR					

3.5.4 Bacterial memory

In this study we were able to demonstrate the memory of bacterial cells after an UV-based induction of the temperate prophage φ-DJ1812. For this purpose *L. sakei* TMW 1.1398 was treated with different doses of UV-irradiation, starting from 0 to 8 minutes, aiming to induce the integrated prophage φ-DJ1812. After UV exposure, the cells were harvested and immediately frozen at -80°C to ensure temporary dormancy. Those cells were then used for growth kinetics analysis using freshly inoculated mMRS medium at the optimum temperature of 30°C. The results are summarized in Figure 35, A.

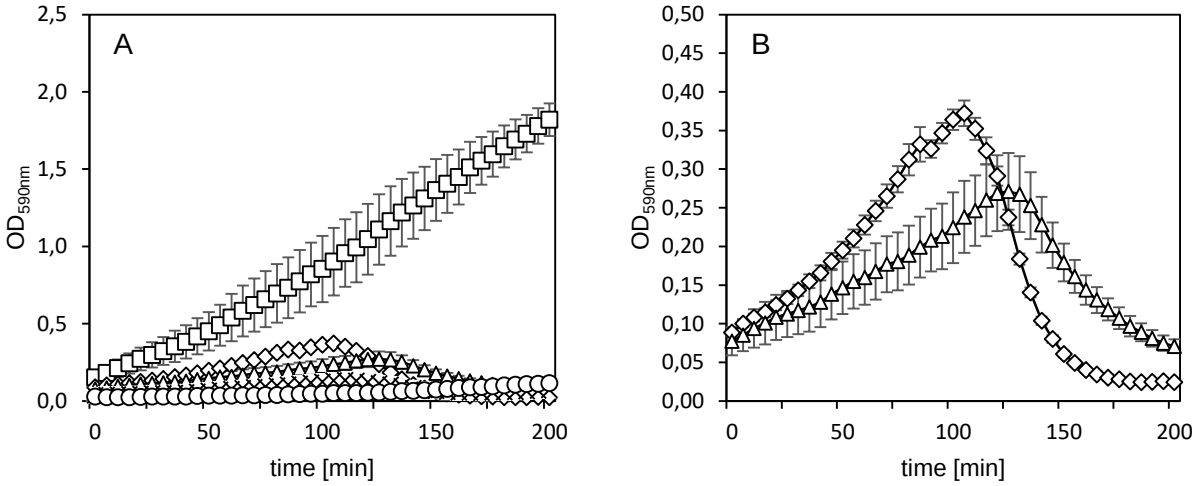

Figure 35: UV treatment of TMW 1.1398. UV irradiation was applied with an UV-doses as follows: (□) without UV irradiation, (◇) 2 min, (△) 4 min, (x) 6 min, (○) 8 min. (A) shows all of the concentrations used, whereas (B) shows only the UV-doses of 2 and 4 min leading to distinct decreases in OD590nm indicating cell lysis.

The results clearly demonstrate the memory of bacterial cells. The previously induced prophage excision led to cell lysis in the newly inoculated medium after temperate dormancy at -80°C. Like described before, UV-doses of 6 and 8 minutes generally led to an inhibition of growth rather than to an actual induction of the prophage φ-DJ1812 (Figure 35, A). However, the determined growth curves for the cultures exposed to 2 and 4 minutes showed the typical lysis after around 2 hours and is visualized in Figure 35, B.

3.5.5 Determination of phage particles using field flow fractionation (AF4-Mals)

Field Flow Fractionation was applied for the separation and the detection of phage particles of an UV-induced phage lysate of TMW 1.1398. The use of respective fitting models should enable the determination of phage capsids and tails.

The separation of macromolecules was achieved by the specific diffusion coefficient of the respective proteins over a time of 40 min with a laminar cross flow of 1 ml/min, which was continually reduced to 0.1 ml/min and a pressure gradient starting from 5.8 to 4.9 bar. First, the UV-induced supernatant of TMW 1.1398 was compared to a normal, untreated supernatant of TMW 1.1398 (Figure 36). The results of the respective field flow fractioning are depicted in Figure 36. The first peak, detected after 6 minutes, is caused by introduced air during manual switching from cleaning flow to the actual measurement. The detected peak after 16 minutes is significantly higher in the UV-treated supernatant than in the control supernatant of TMW 1.1398. It seems obvious, that the UV irradiation results in the release of high amounts of diverse macromolecules and macromolecular proteins within the strains supernatant.

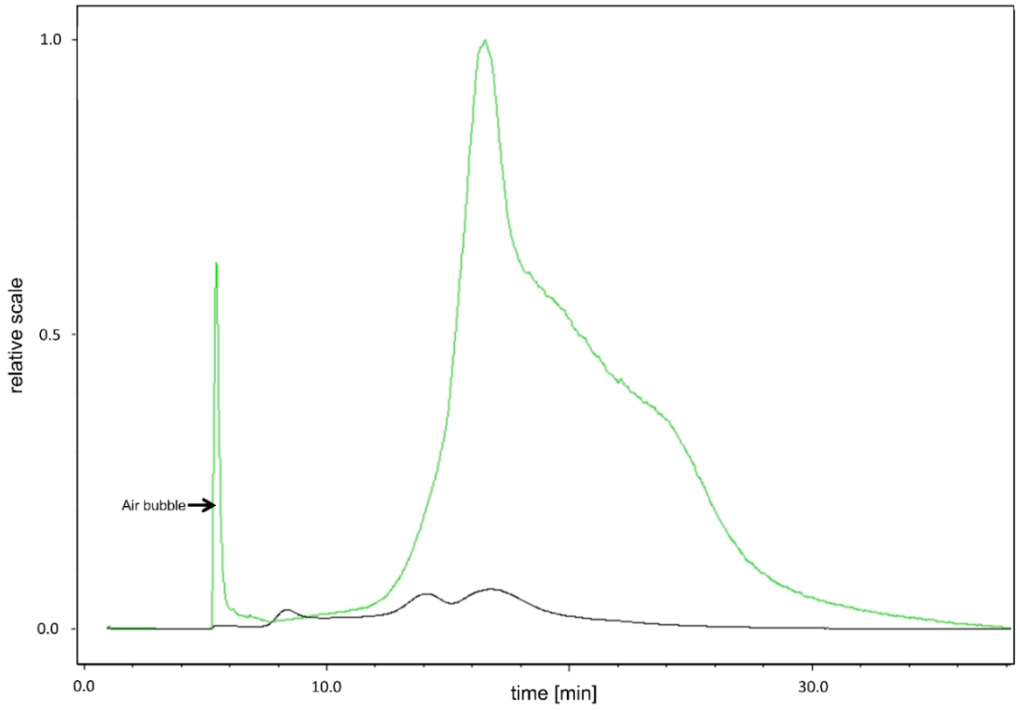

Figure 36: Field Flow Fractioning (AF4-MALS) using phage lysate of TMW 1.1398 (green) as well as unthreatened supernatant (black) of the latter strain. Proteins/macromolecules with a certain elution time is presented on a relative scale.

The comparison of phage lysate of *L. sakei* TMW 1.1398 and a purified phage lysate of *L. lactis* TMW 2.162 should clarify the peaks origin. In order to achieve higher resolutions, the injection volume was decreased to 10 µl. The results are visualized in Figure 37.

The first obvious difference between the phage lysate of TMW 1.1398 and the purified phage lysate of *L. lactis* TMW 2.162 is the massive peak after 16 minutes, which cannot be found within the purified phage lysate of TMW 1.162. Interestingly, one smaller protein peak can be visualized with the technique and the reduced injection volume. The smaller peak of phage lysate of *L. sakei* TMW 1.1398, eluting after 13 minutes, corresponds to proteins that may be dedicated to phage particles as the peak (marked in black) displayed a similar value on the relative scale as the purified phage peak of *L. lactis* TMW 2.162 (marked in green).

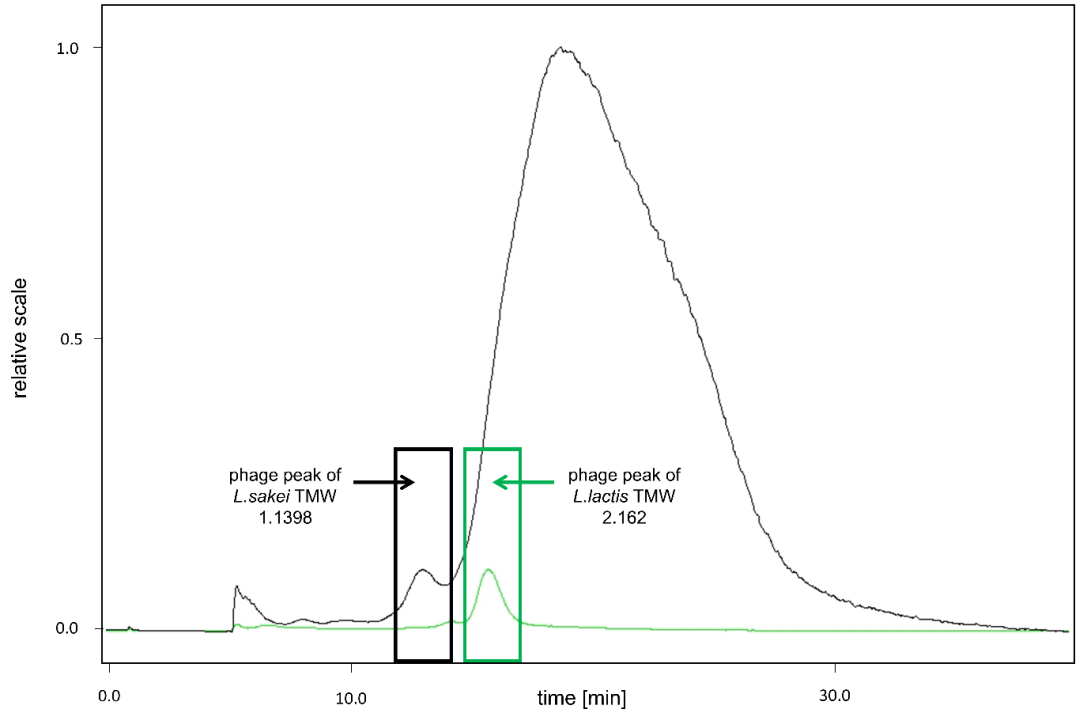

Figure 37: Field flow fractioning of a UV induced phage lysate of TMW 1.1398 and a control phage lysate originating from *L. lactis* TMW 2.162. Proteins/macromolecules with a certain elution time are presented on a relative scale.

Subsequent to these findings, Figure 38 illustrates the determination of presumed phage heads of *L. sakei* TMW 1.1398 and *L. lactis* TMW 2.162. Using a spherical fitting model it was possible to determine the radii of putative phage heads. The control phage of *L. lactis* TMW 2.162 was determined with a size of 95 nm, whereas the presumed phage head of *L. sakei* TMW 1.1398 showed a noticeably smaller phage capsid with a determined size of 70 nm. The attempt to determine the length of the phage´s tail led only for *L. lactis* phages to valid results with a length of 155 nm, which clearly stands in line with the current literature. For *L. sakei* TMW 1.1398, it was not possible to fit the used rod model, which suggested the absence of assembled phage tails, while indicating a complete failure of phage assembly.

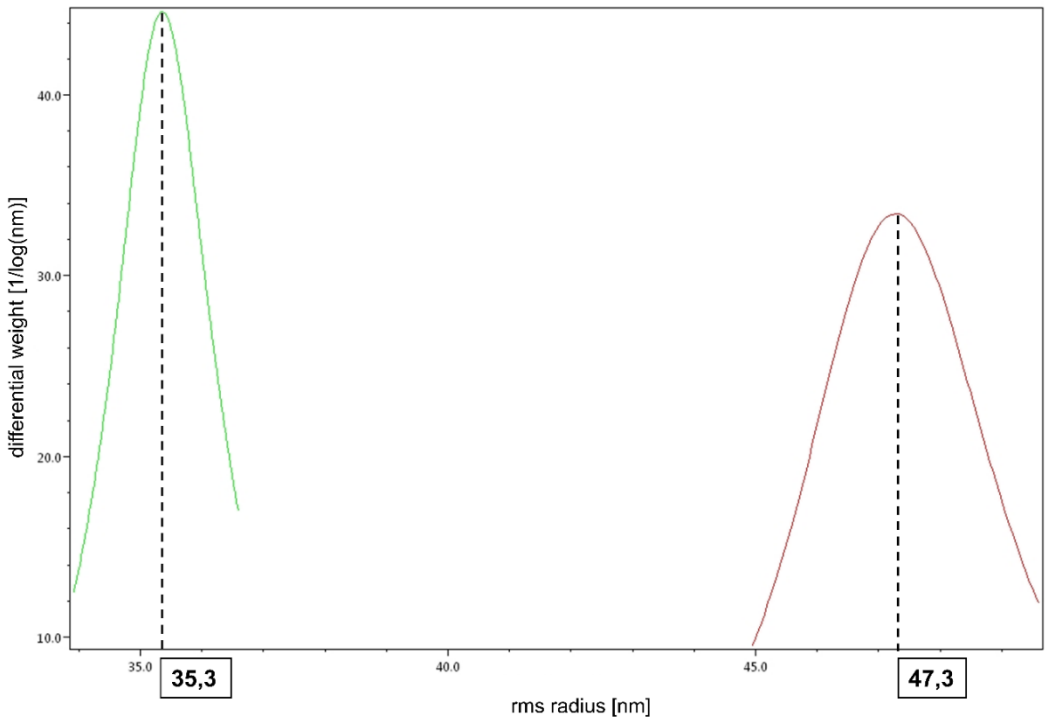

Figure 38: Field flow fractioning of a UV induced phage lysate of *L. sakei* TMW 1.1398 and a control phage lysate originating from *L. lactis* TMW 2.162.A spherical fitting model was applied to shed light on the size of phage heads. The supposed phage head of *L. sakei* TMW 1.1398 (-) shows a smaller size of 35.3 nm, whereas the phage head of *L. lactis* (-) showed a size of 47.3 nm.

3.5.6 Bacteriophage electron microscopy

Phage lysate was used for electron microscopy to validate the findings gained with AF4-Mals. The results of the microscopy are presented in Figure 39. The Figure shows different phage assembly intermediates, probably proheads. The depicted phage heads intermediates are of different sizes ranging between 100 and 200 nm. Some of the phage head intermediates looked uneven and damaged (Figure 39, C and E), while none of them showed the typical icosahedral head/capsid.

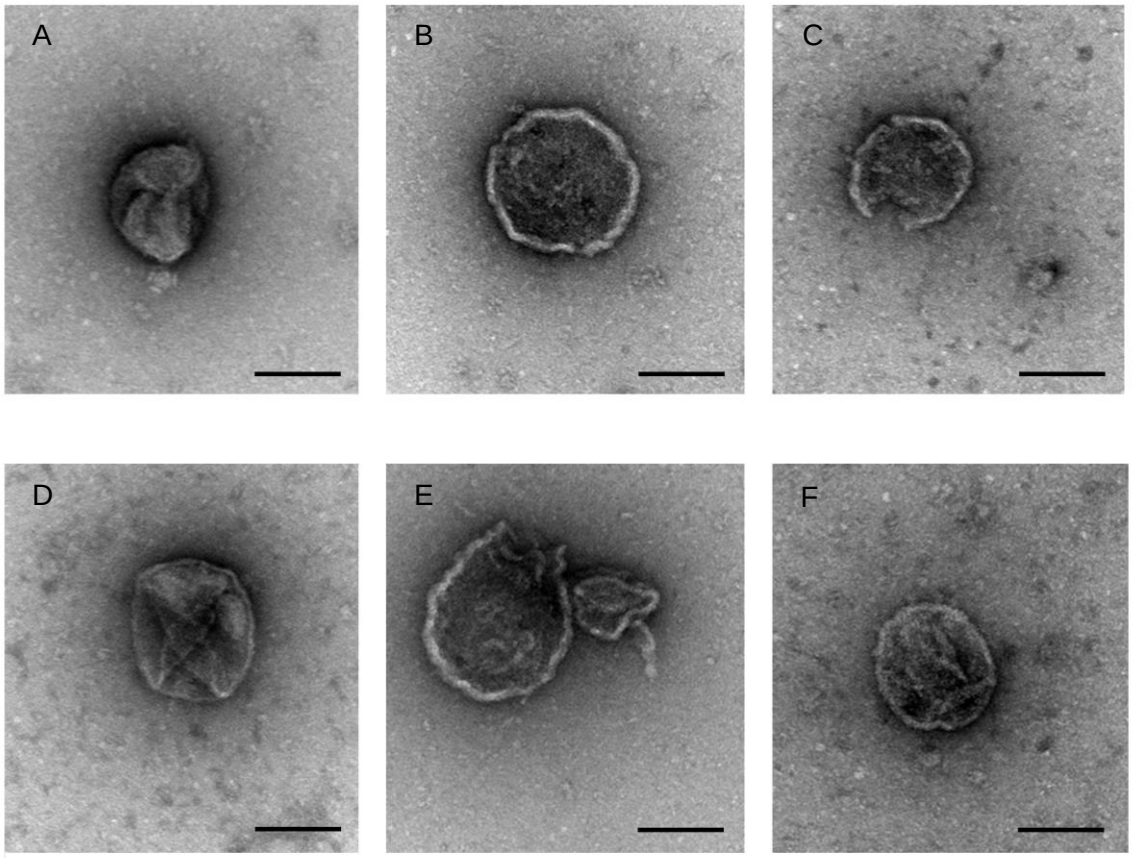

Figure 39: Electron micrograph of bacteriophage *L. sakei* φ-DJ1812. The figure shows phage heads (only assembly intermediates). Bar: 100 nm.

3.5.7 Bacterial Challenge Assay – Lysis potential of φ-DJ1812

The annotation of the phage genome of φ-DJ1812 indicated an inactive phage due to the presence of a transposase within one of the major tail proteins (Table 16). Moreover, the results gained from AF4-MALS analysis and electron microscopy confirmed the hypothesis that the phage cannot assemble to intact phages. Nevertheless, LC/LC-MS analysis reported the presence of the N-acetylmuramoyl-L-alanine amidase of the lysis cassette within the phage lysate. That enzyme is known to cleave the link between N- acetylmuramoyl residues and L-amino acid residues in cell-wall glycopeptides, which should result in cell lysis. However, it is not clear whether the phage lysate is able to provoke lysis within other *L. sakei* strains. Therefore, phage lysate was produced and harvested according to Chapter 2.6.2.3 and used for the infection of 48 other *L. sakei* strains. The latter strains were grown in microtiter plates in mMRS (200 µl) and infected after 2 h of incubation with 10 µl of phage lysate. Growth was determined with a microplate reader for a total time of 10 hours. In order to guarantee the visualization of possible cell lysis, measurements were made every 10 min. Even despite the presence of N-acetylmuramoyl-L-alanine amidase in the prophage genome, Figure 40 - Figure 43 clearly illustrate the failure of lysis induction with phage lysate of TMW 1.1398. For none of the tested cultures, decreases in cell turbidity (OD_{590nm}) were observed. The failure of lysis and suggest an inactive, cryptic and not assembled phage. The previously detected N-acetylmuramoyl-L-alanine amidase was also not able to induce cell lysis within the 48 infected *L. sakei* strains.

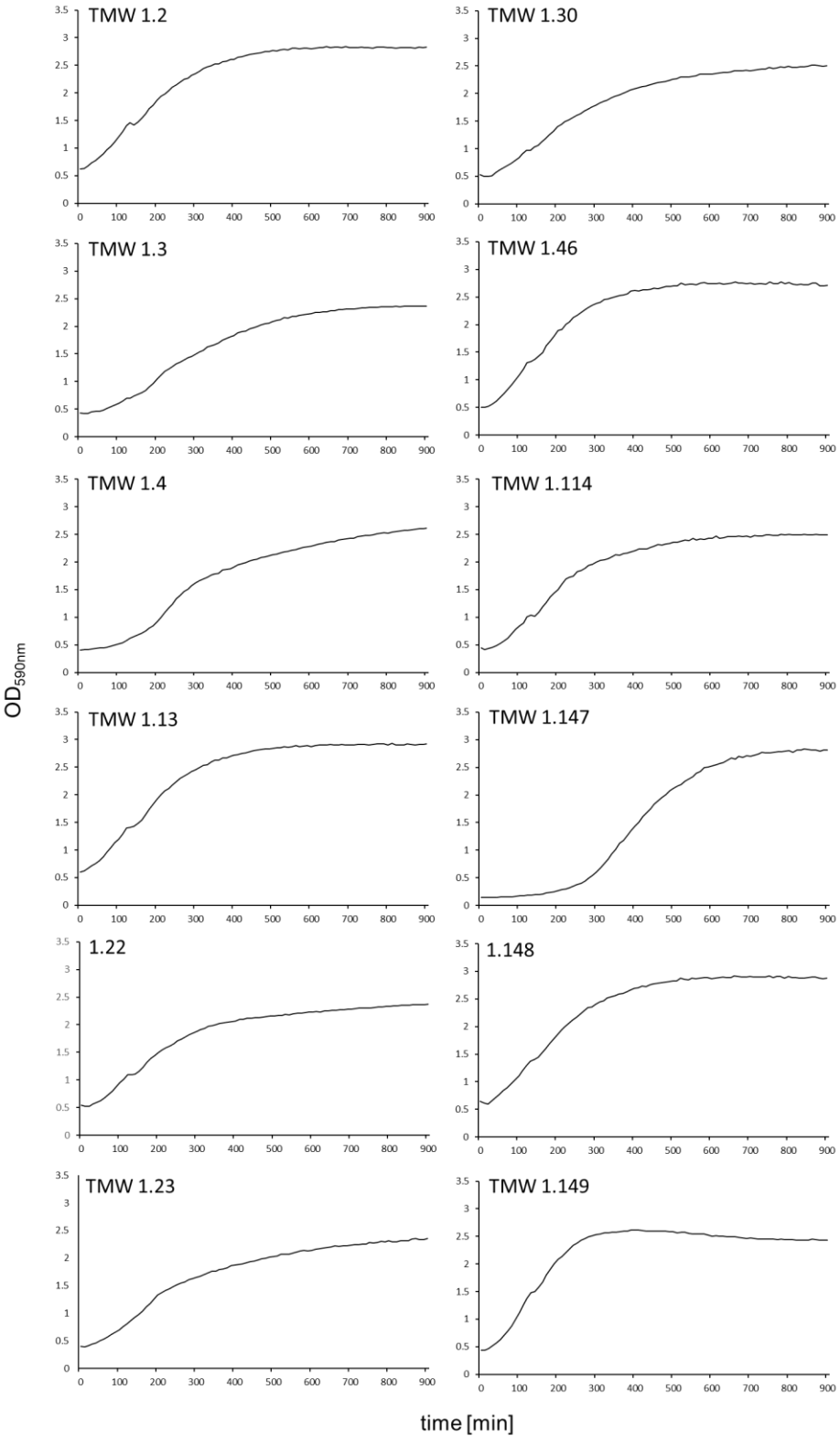

Figure 40: Lysis potential of UV-induced phage lysate of *L. sakei* TMW 1.1398 for the *L. sakei* strains TMW 1.2 – TMW 1.149.

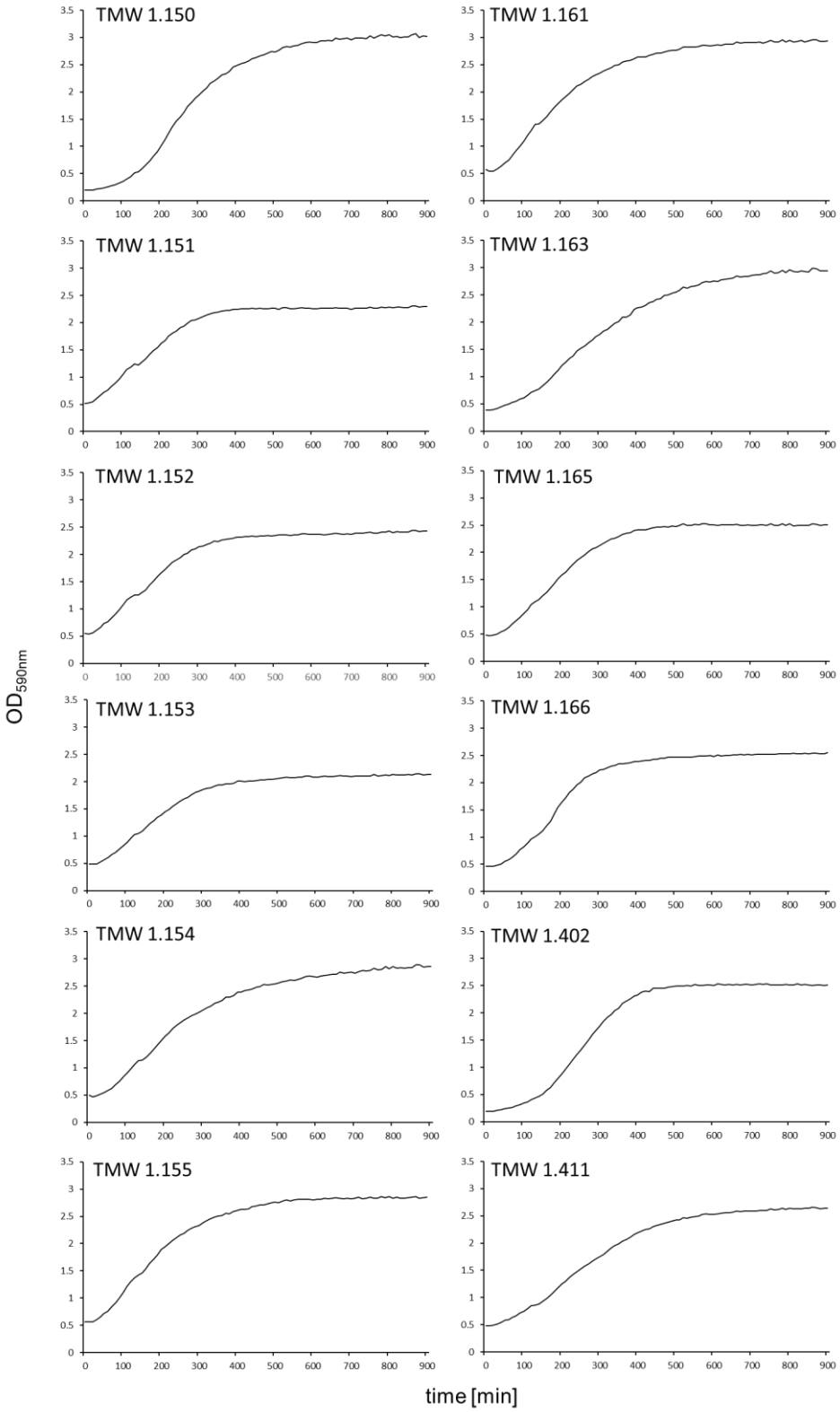

Figure 41: Lysis potential of UV-induced phage lysate of *L. sakei* TMW 1.1398 for the *L. sakei* strains TMW 1.150 – TMW 1.411.

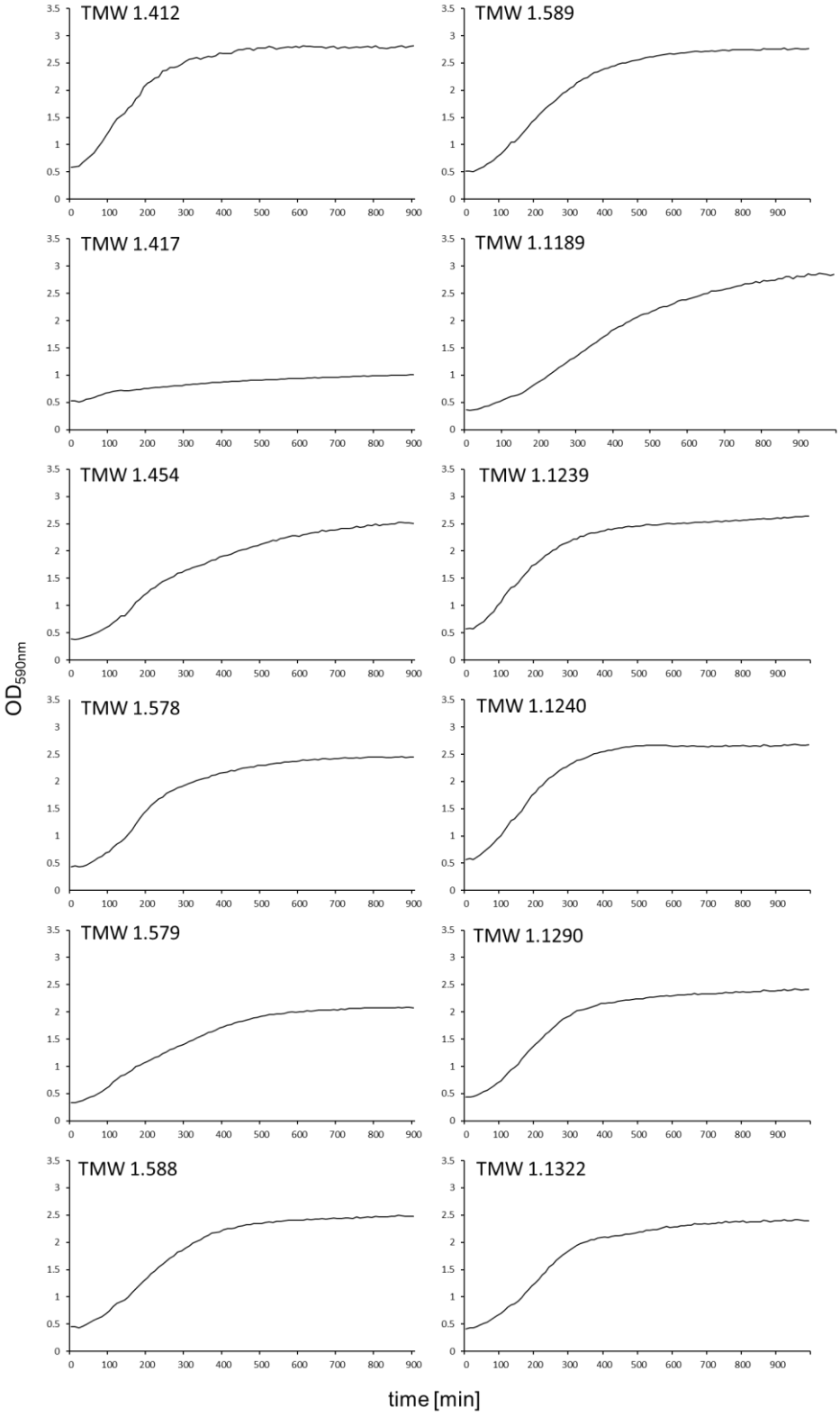

Figure 42: Lysis potential of UV-induced phage lysate of *L. sakei* TMW 1.1398 for the *L. sakei* strains TMW 1.412 – TMW 1.1322.

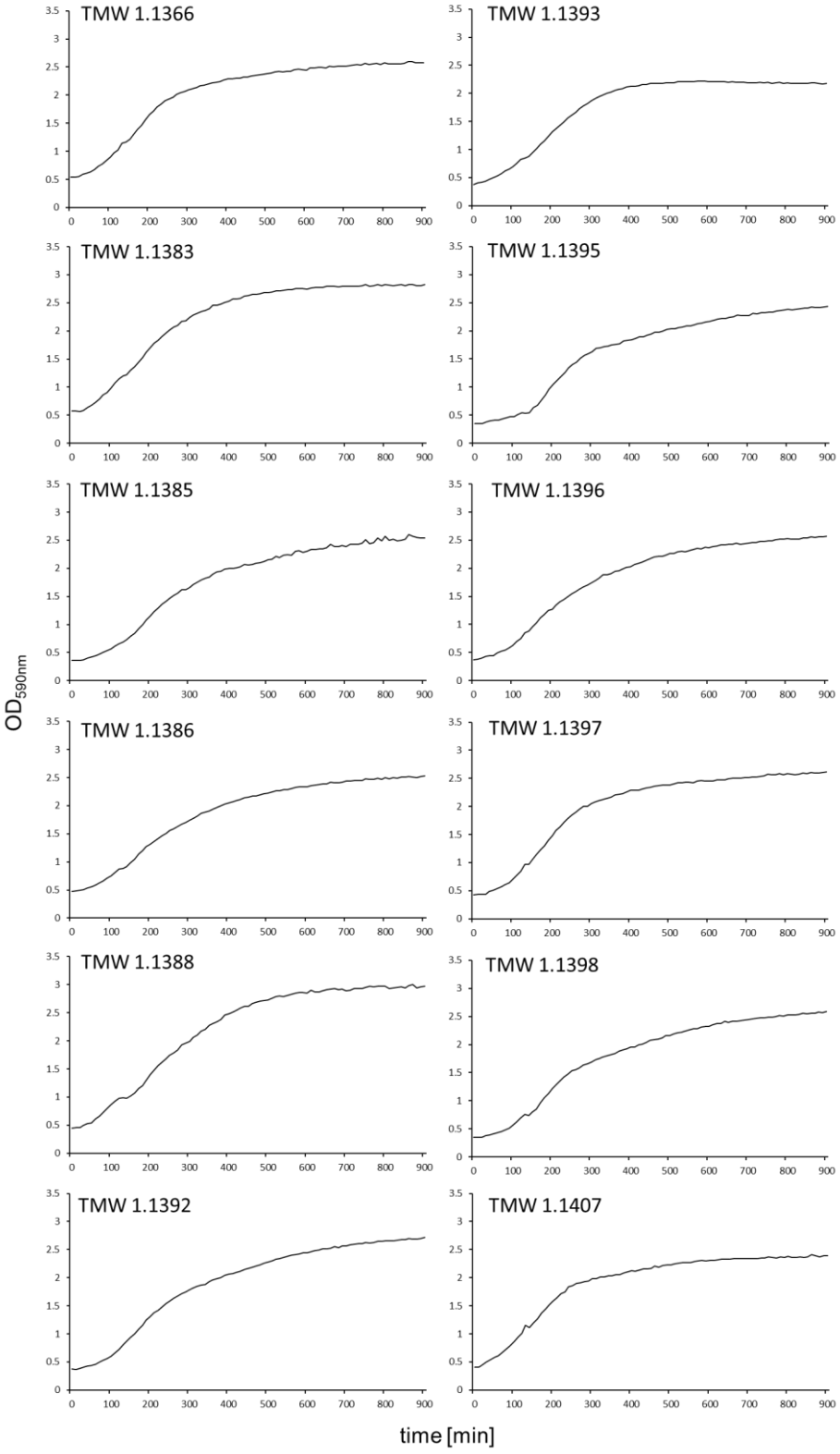

Figure 43: Lysis potential of UV-induced phage lysate of *L. sakei* TMW 1.1398 for the *L. sakei* strains TMW 1.1366 – TMW 1.1407.

3.5.8 Generation of prophage-cured strain TMW 1.2292

A mutant strain lacking the complete prophage genome of φ-DJ1812 should be generated along UV treatment. A two minutes UV treated, induced culture of TMW 1.1398 was used for dilution and plating on mMRS agar plates. Colonies were picked and sub cultured on master plates for further analysis. After two days of anaerobic incubation, cell material of each streaked colony was taken for strain-specific colony-PCR, using the strain specific primer pair 1.1398F and 1.1398R designed for the XRE family regulator in the integrated prophage genome (see Table 6).

Negative results suggested the loss of the prophage and a successful induction of the latter. Finally, it was possible to find 3 likely candidate clones, which were negative for the strain-specific PCR. In the following, all of these 3 candidates were further characterized by a second PCR with the primer pair GnatF and LysR (Table 6). Only a complete excision of the prophage genome lead to positive results with a PCR product of the size 1670 bp (Figure 44).

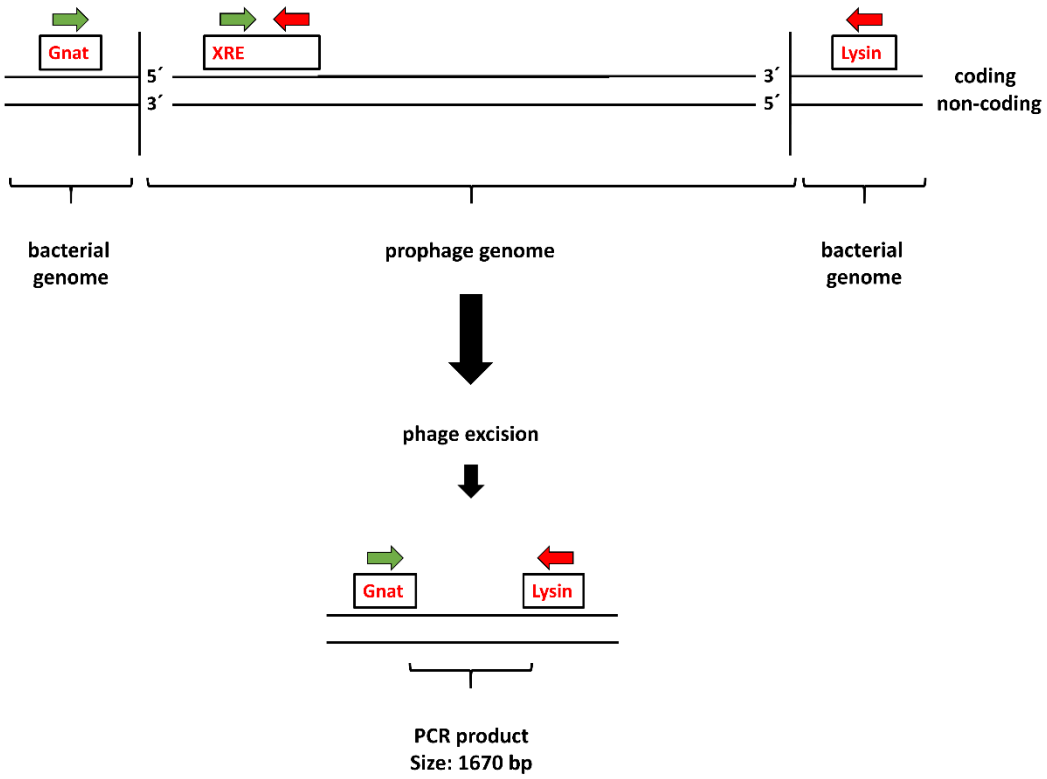

Figure 44: Primer Design for identification of possible prophage-cured clones of TMW 1.1398.

Only one of the previously selected colonies showed the desired PCR amplicon. The latter was used for sequencing and demonstration of excision. The analysis of the PCR product sequence revealed that the prophage was successfully excised and that only previously annotated and determined attachment-sites remained.

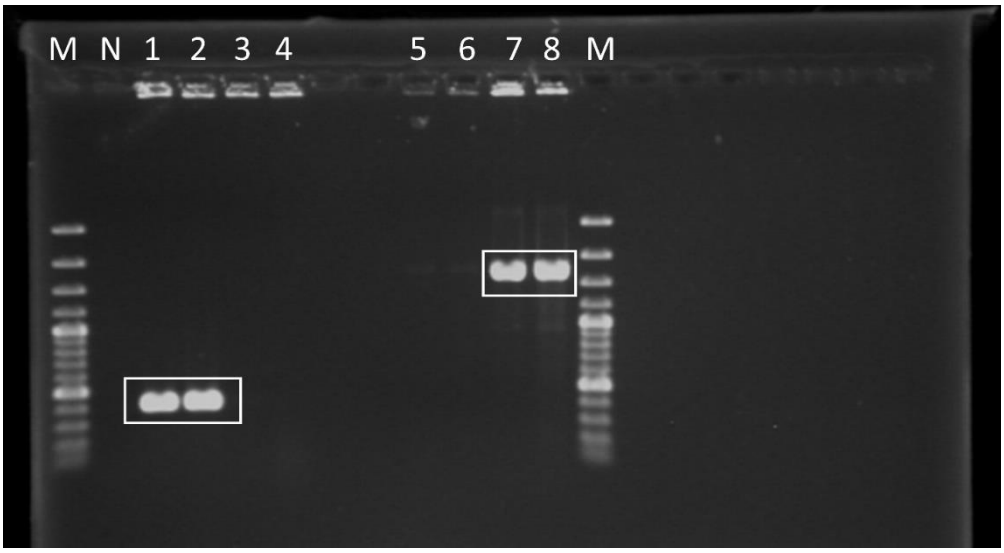

Figure 45: PCR assay for the identification of *L. sakei* TMW 1.1398 clones. Two different primer pairs were used. XRE-family regulator primers were used for the identification of possible candidates lacking the prophage φ-DJ1812. The original strain *L. sakei* TMW 1.1398 were used as well as a possible prophage-cured mutant of TMW 1.1398. Lane 1-2 contains PCR products of the original strain TMW 1.1398 using the XRE primer pair. Lane 3-4 contains PCR products of the possible revertant. Lane 5-6 again contains the original TMW 1.1398, Lane 7-8 the revertant. Lane M contains the used size marker 100 bp.

The last evidence for the successful excision of the whole prophage genome was proven by growth kinetics of the presumably prophage-cured strain. As control the wild-type strain TMW 1.1398 was used to verify the conducted experiment. Both strains were grown overnight in mMRS medium and used for the inoculation of fresh mMRS. After reaching OD values of about 0.4, both strains were exposed to UV-irradiation according Chapter 3.5.3.1. Growth was determined using a microtiter plate reader at the usual wavelength of 590 nm. The results of the growth kinetics are depicted in Figure 46 and show the failure of induction of the prophage-cured strain, which was designated TMW 1.2292. Like predicted, no induction was observed. The strain kept growing until stationary phase.

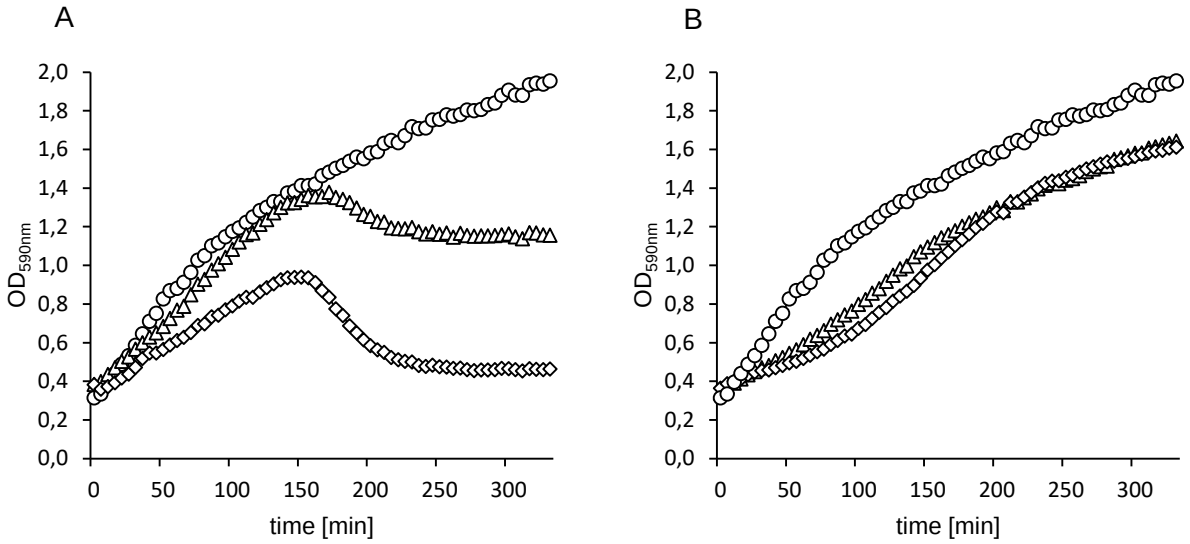

Figure 46: UV-Induction of *L. sakei* TMW 1.1398 (A) and *L. sakei* TMW 1.2292 (B). As internal control, the original wildtype-strain of TMW 1.1398 was grown without the prior UV induction (○). For induction purpose, an UV dose of 2 min (△) and 4 min (◇) was applied.

To investigate the physiological impact of the prophage loss, growth kinetics of both, TMW 1.1398 and TMW 1.2292 were determined. Both strains were grown in an overnight culture and used for inoculation of new fresh mMRS medium to an initial OD of 0.05. The results of the growth dynamics are depicted in Figure 47 and clearly demonstrate phenotypic differences. The prophage-cured strain TMW 1.2292 showed a prolonged lag-time and a slightly reduced growth rate. The maximum OD appeared to be slightly higher than for the wild-type strain.

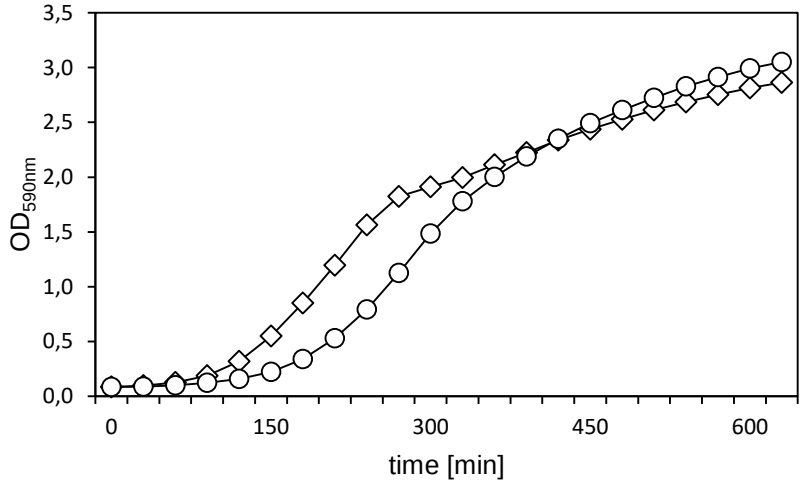

Figure 47: Growth kinetics of TMW 1.1398 (◇) and its prophage-cured clone TMW 1.2292 (○) in standard mMRS at an optimum temperature of 30°C.

3.5.9 Genetic instability of prophage φ-DJ1812

Spontaneous excision and circularization of prophage φ-DJ1812 was demonstrated by the use of a PCR approach with a specific primer pair (see Table 6). The primers were designed for the target genes integrase with the locus tag 4030 and the last hypothetical protein within the prophage genome with the locus tag 4300 (see Table 16). The PCR was conducted with isolated DNA of the original strain TMW 1.1398 and the new prophage-cured mutant strain TMW 1.2292.

The results clearly show, that parts of the population of TMW 1.1398 are characterized by a permanent excision of the prophage without prior external induction. The prophage φ-DJ1812 verifiable undergo a circularization, which was demonstrated along PCR and gel electrophoresis (Figure 48). Primers were designed specifically to obtain products of 975 bp only when the prophage genome underwent the predicted circularization. Figure 48, B displays the results of both PCR approaches visualized by agarose gel electrophoresis.

Lane 1 contains the negative control without bacterial DNA, Lane 2, 4 and 5 contain DNA of the original strain TMW 1.1398, whereas Lane 3 contains DNA of the generated mutant strain TMW 1.2292. A spontaneous excision and circularization cannot take place within the mutant strain lacking the complete prophage genome, but was successfully reported for the original strain TMW 1.1398.

After a successful excision and prior to the proven circularization, it can be assumed that the prophage genome exists in a linear intermediate form. Unfortunately, this intermediate cannot be visualized with standard PCR methods.

According to theory, the prophage is excised at the determined att-sites attL and attR, which promotes and performs the latter circularization. The sequencing of PCR products revealed the excision sites. The alignment of theoretical excision and circularization sequence and the actual sequence of the PCR product with Clustal Omega (see Table 17) confirmed the correctness of the theory. The complete mechanisms of spontaneous excision and circularization is visualized in Figure 48. Different to the presumption, only one att-site remained after excision (see Table 17, highlighted in red colour).

Table 17: Alignment of theoretical phage sequence after excision with the actual sequenced phage sequence using Clustal Omega.

```
circular_theory       AAGGAGTATTATTTCTATGATATCTGGATTAGATTCAGCCCAAACGGTATTATTACCAAC    60
circularization       --------------CTATGATATCTGGATTAGATTCAGCCcAAaCGGTATTATTACCAAC    46
                                    ********************************************

circular_theory       AATCAATACTACGGATCTATTTGGAAACTAGATTTAAAAAGCTAATCTCATTTAATTGTG    120
circularization       AATCAATACTACGGATCTATTTGGAAACTAGATTTAAAAAGCTAATCTCATTTAATTGTG    106
                      ************************************************************

circular_theory       GGATTAGATTTTTTTACTCTAAAATATCGGAGTTTGAGAGCTTATACATTGAATAGCAAA    180
circularization       GGATTAGATTTTTTTACTCTAAAATATCGGAGTTTGAGAGCTTATACATTGAATAGCAAA    166
                      ************************************************************

circular_theory       TAACGTTAAGTACAGTCTTTGAGAATCGTGGTAGTTATATGAATAGGGTGGGTTTGATCA    240
circularization       TAACGTTAAGTACAGTCTTTGAGAATCGTGGTAGTTATATGAATAGGGTGGGTTTGATCA    226
                      ************************************************************

circular_theory       CCTATCCGAGTTCTACCTATATTATAAAAGCCTAACTCACTAAATTGTGGGTTAGGCTTG    300
circularization       CCTATCCGAGTTCTACCTATATTATAAAAGCCTAACTCACTAAATTGTGGGTTAGGCTTG    286
                      ************************************************************

circular_theory       TTTTTTTGCAACTCTATTCGAATCAATCTTGGGGACATTTTGGGGACATACTGGCATAAA    360
circularization       TTTTTTTGCAACTCTATTCGAATCAATCTTGGGGACATTTTGGGGACATACTGGCATAAA    346
                      ************************************************************

circular_theory       TTTATCGTTATATATCGTATTTTTAAAAATTAAAACCCCGATATAACGGGATATTAGTAG    420
circularization       TTTATCGTTATATATCGTATTTTTAAAAATTAAAACCCCGATATAACGGGATATTAGTAG    406
                      ************************************************************

circular_theory       ATGTATCTATGGCTATGCAATTTGACCATAATTTGACCATAATTAATTACCCTTTGCTAT    480
circularization       ATGTATCTATGGCTATGCAATTTGACCAT-----------AATTAATTACCCTTTGCTAT    455
                      ***************************           ********************

circular_theory       ATCAATGTTTATAGCGTTTTTGGGGACATTTGGGGACATGGACAAAACGTCCAACGCTTT    540
circularization       ATCAATGTTTATAGCGTTTTTGGGGACATTTGGGGACATGGACAAAACGTCCAACGCTTT    515
                      ************************************************************

circular_theory       ATCAGTTTCTGAAATCCGTTCTTCTTTTAGCATATGGGCGTAAACTTCTTGAGTGATTGA    600
circularization       ATCAGTTTCTGAAATCCGTTCTTCTTTTAGCATATGGGCGTAAACTTCTTGAGTGATTGA    575
                      ************************************************************

circular_theory       AGTGTTGGCATGACCTAATCTTTTTGAGATATAGTTGATTGAAACGCCTTTATATAATAA    660
circularization       AGTGTTGGCATGACCTAATCTTTTTGAGATATAGTTGATTGAAACGCCTTTATATAATAA    635
                      ************************************************************

circular_theory       ATATGATGCATGAGAATGCCTTAAACCGTGAACACTGAGTTTACTGTAAATATTAAGCGA    720
circularization       ATATGATGCATGAGAATGCCTTAAACCGTGAACACTGAGTTTACTGTAAATATTAAGCGA    695
                      ************************************************************

circular_theory       TTTAGTAACTCGCTGTATTCTTTTTAGAGCCGCTTGGTGTGTATGATCGAATATTCTTCC    780
circularization       TTTAGTAACTCGCTGTATTCTTTTTAGAGCCGCTTGGTGTGTATGATCGAATATTCTTCC    755
                      ************************************************************

circular_theory       AGAACTCCCAGAATTAAAGATAACTGCGGTTAATTCTTTAGTGATTCTAACATCGCGATA    840
circularization       AGAACTCCCAGAATTAAAGATAACTGCGGTTAATTCTTTAGTGATTCTAACATCGCGATA    815
                      ************************************************************

circular_theory       TGAGTTTTTGTTTTTTGGCGGCGTAATTTCACGACTAGCAGCTGTGAGAGATTTATCAAC    900
circularization       TGAGTTTTTGTTTTTTGGCGGCGTAATTTCACGACTAGCAGCTGTGAGAGATTTATCAAC    875
                      ************************************************************

circular_theory       ATGAATGTTATTAAACGGCGCACTAACATCCTCATAGTTTAGCGCTAAAACCTCGCCTAT    960
circularization       ATGAATGTTATTAAACGGCGCACTAACATCCTCATAGTTTAGCGCTAAAACCTCGCCTAT    934
                      ************************************************************

circular_theory       TCGCATACCAGTCTCTAACGCGATAAGTA-     989
circularization       TCGCATACCAGTCTCTAACGGCATAAGTA-     964
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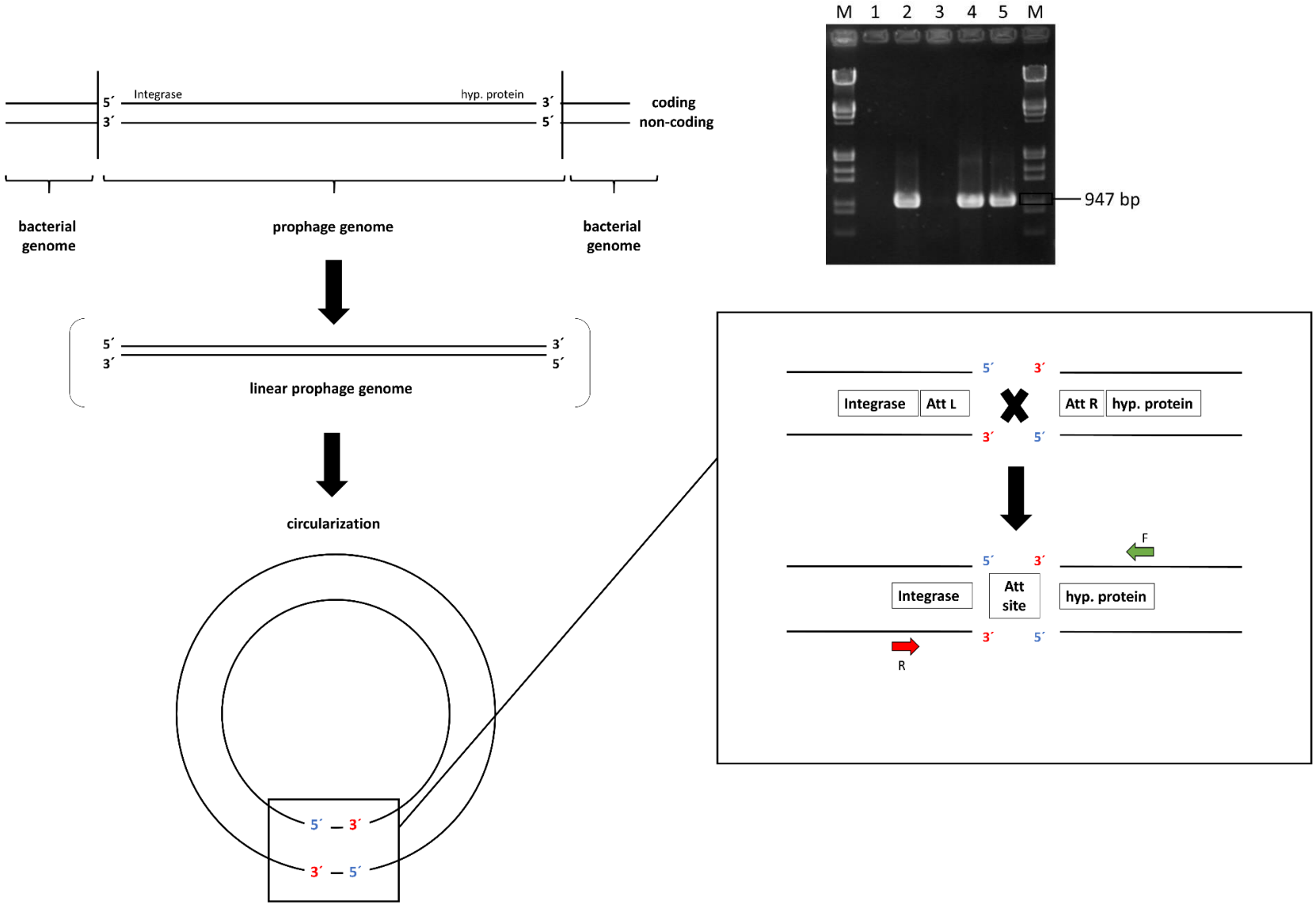

Figure 48: Mechanism of prophage excision and circularization. PCR gel electrophoresis visualize PCR products with a size of 947 bp obtained with primer pair Int_R and 4300_F.

3.5.10 Impact of φ-DJ1812 on the assertiveness of *L. sakei* TMW 1.1398 in sausage fermentations

Due to the mutant strain TMW 1.2292 lacking the prophage genome of φ-DJ1812 it was possible to conduct an experiment in order to enlighten the impact of prophages on the assertiveness of a strain. Therefore, competition experiments were carried out simultaneously either with the original strain TMW 1.1398 or with the mutant strain TMW 1.2292. Both, the original and the mutant strain of TMW 1.1398 were tested in their set arranged at the beginning of this thesis. The only difference is the presence or absence of φ-DJ1812. The performance of the strain in direct comparison can shed light on the impact of prophages within bacterial genomes and their assertiveness in raw fermented sausages.

The results of the present competition study clearly indicate an impact of the respective prophage φ-DJ1812 on the assertiveness of *L. sakei* TMW 1.1398. The excision of φ-DJ1812 led to an in generally better performance of TMW 1.2292 during the fermentation of sausage meat batter.

Figure 49 provides detailed information about the development of microbiota. As expected due to previous conducted experiments (see Chapter 3.2), the three *L. sakei* strains TMW 1.3, TMW 1.417 and TMW 1.1398 established a co-dominance within the sausage microbiota. The other introduced *L. sakei* strains, namely TMW 1.114 and TMW 1.1239 did not play a pivotal role in the fermentation. For reasons of clarity and due to low numbers of colonies of set members TMW 1.114 and TMW 1.1239 their relative abundance is summarized in "*L. sakei*".

However, despite similar $\log_{10}$ CFU g^{-1} values (starting from $\log_{10}$ 4.95 ± 0.05 to $\log_{10}$ 7.1 ± 0.03), the microbiota of the sausage batter fermented with set III and the original TMW 1.1398 was different than the microbiota developing using set III and the mutant strain of TMW 1.2292.

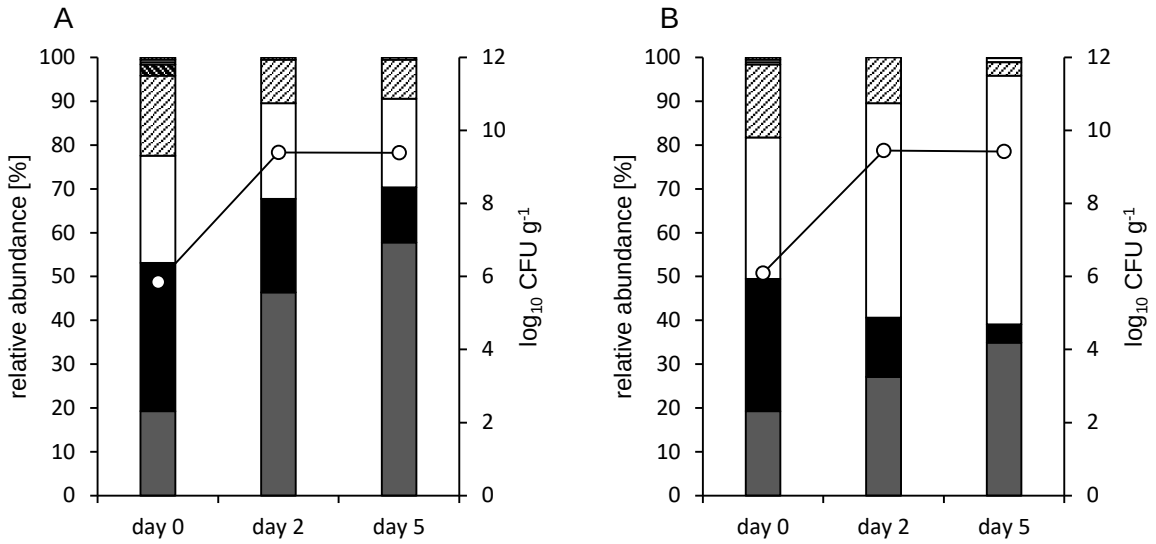

Figure 49: Microbiota dynamics and development of set I (A) and set I* including the new prophage-cured *L. sakei* TMW 1.2292 (B) over the total fermentation time of 5 days determined on mMRS. Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points. The figure shows the average of two biological replicates with standard deviation. Detection limit log_{10} 1 CFU g^{-1}. Species and strains were identified with MALDI-TOF MS as follows: (■) *L. sakei* TMW 1.3, (■) *L. sakei* TMW 1.417 (□) *L. sakei* TMW 1.1398/1.2292, (▧) *L. curvatus*, (▨) *L. sakei*, (▤) other, (▥) nri. The acronym nri stands for not reliable

The sausage batter fermented with set III and TMW 1.1398 showed a distinct domination of TMW 1.3 with a total relative abundance at day 5 of 57.8%. TMW 1.1398 grew only to a final relative abundance of 20%. The other co-dominant strain TMW 1.417 was shown to reach only 12.5 % of the total microbiota.

Whereas the microbiota of set III and the original strain was clearly dominated by the introduced starter strain TMW 1.3, the microbiota of set III and the mutant strain lacking the prophage φ-DJ1812, was dominated by the strain of interest TMW 1.2292 with a final relative abundance of 56.8 %. In comparison with the other experiment, the order of the strains switched regarding their relative abundances, discriminating TMW 1.3, which is now only the second strongest party with a relative abundance of 34.9 %. Again, like described for the first approach using the original TMW 1.1398, TMW 1.417 can only prevail during fermentation without holding larger shares (4.2 %).

In summary, the results on competition experiments with the new prophage-cured strain TMW 1.2292 suggest a clear impact of prophages on the assertiveness of strains. This time, the prophage appeared to function as a burden as the prophage-cured strain exhibits an enhanced performance.

4 Discussion

Salami is consumed raw without prior heating and harbors autochthonous microbiota. Despite the implemented hurdle technology and the widespread use of starter cultures, inhibiting the growth of undesired potentially pathogenic bacteria and spoilage organisms, the product is not necessarily free of such microorganisms. Standard microbiological analysis of producers are mostly based on the sole use of selective media and respective colony counting. Still, it remains unclear whether the introduced starter culture was able to establish a stable dominance and suppress competitors deriving from the autochthonous microbiota sufficiently, or if parts of the latter have arisen. This work demonstrates the necessity of verification of the assertiveness of a starter strain at strain level and potential of implemented MALDI-TOF MS as a new standard method enabling a “real time” monitoring of population changes. Moreover, the uncovering of assertiveness as well as the unmasking of strategies and markers enabling the observed assertiveness pave the way for an application with special emphasis to the development of future starter cultures as single strains or defined consortia.

In this work, these tasks were accomplished by the testing of the initial working hypotheses resulting in the following theses along the three sections of this study.

(I) MALDI-TOF MS serves as a high-throughput technology for monitoring of *L. curvatus* and *L. sakei* on strain level

Theses:

- The database, containing sub proteome mass spectra of *L. sakei* and *L. curvatus* strains, enables high-resolution monitoring of population changes during fermentation on strain level. Database development and the implementation of adequate database entries (MSPs) are of crucial importance towards a wider use of this technology.
- Specific strain identification and recognition with MALDI-TOF MS strongly depends on the strain of interest and the applied incubation time. Small changes in incubation and preparation routine can strongly influence the recognition rates leading to misidentifications and false negative results. In general, closely related strains increase the probability of misidentifications, false negative or false positive results. Therefore, the fruitful use of this technology is limited to monitoring of clearly distinguishable strains.

Future research will focus on the optimization of technological properties of MALDI-TOF MS enabling a sharper distinction between the peaks of bacterial sub proteome pattern, thus improving the differentiation of isolates on strain level. Moreover, other species could be tested for their differentiation using MALDI-TOF MS.

(II) Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in raw sausage fermentations

Theses**:**

- The fermented sausage model system can serve as a tool imitating real fermentation scenarios, while providing valuable information about the strains behaviour within sausage fermentations. The results derived from the model system can be successfully extrapolated to industrial ring trial experiments.
- The drying process of sausage fermentation has little or no effect on the behaviour of the development of the LAB microbiome in fermented sausages.
- Considering interspecies assertiveness, *L. sakei* strains are mostly dominant over *L. curvatus* strains, which is probably explained by the adaptation to the niche including higher tolerance to oxidative stress as well as the expression of special metabolic traits like ADI pathway. The presence of catalases or pseudo-catalases contribute and affect the resistance to oxidative stress.
- Not all strains of a species are equally adapted to the environment sausage fermentation, which is represented by a high intraspecies variation in their assertiveness.
- The fermentation is rarely dominated by a single strain. In general, two strategies are common in establishing a dominance within the meat matrix: Single-dominance, where competitors were overgrown or actively harmed, leading to competitive exclusion or co-dominance or even cooperation, leading to colonization resistance.
- A concept targeted at colonization resistance is favoured over a concept of competitive exclusion

Future research will focus on the mechanisms and strategies behind co-operational traits as well on the mechanisms enabling single-dominance. New projects may put attention on other starter cultures by the investigation of their assertiveness in sausage fermentation. Moreover, also different types of fermentations could be considered. It is conceivable that industrial partners may change their starter culture set ups upon the results gained along this type of experiments.

(III) Unmasking of strategies and key parameters responsible for the assertiveness of strains

Theses:

- Assertiveness cannot necessarily be predicted upon the presence or absence of single markers. Neither the adaptation to cold stress, oxidative stress or general growth characteristics can explain the assertiveness within raw sausage fermentations. The expression of assertiveness is rather dynamic and multifactorial. Nevertheless, specific traits can strongly influence the performance of a strain.
- The investigations of growth characteristics in mMSM allow first hints on the assertiveness of the strains in raw sausages. Strains which show high growth rates (μ) and high ODs ($OD_{max.}$) are probably more dominant within the sausage meat batter.
- Bacteriocins can play a major role in the assertiveness of a strain as they enable selective advantages by bacteriocin-mediated killing of competitors. Bacteriocin production can lead to the establishment of single dominance even though the strain has no other extraordinary metabolic or physiologic properties.
- The occurrence of prophages within bacterial genomes can play a decisive role in the assertiveness of a strain. Prophages, without genes coding for enzymes or proteins improving the fitness of a strain, can be considered a genomic and metabolic burden, which negatively influences the assertiveness of the respective strain.

A thorough understanding of the mechanisms behind the assertiveness opens possibilities for the development of new starter cultures and contribute to microbiota dynamics in a wide variety of habitats. As the assertiveness is multifactorial, a wide variety of other aspects like salt or nitrite-tolerance can be considered and investigated. The impact of prophages on the assertiveness of strains is not fully understood and investigated. Future research could close such gaps of knowledge by the investigation of other strains encompassing other prophages within their genomes. Moreover, phages could be the subject in a future research project as they seem to influence the strains in different ways. Furthermore, the bacteriocin formation, can be of great interest for the application in industry fermentations as the producer strain is not only dominant but may contribute to the inhibition of pathogenic bacteria including *Listeria spp*. as well leading to improved safety and hygienic safety aspects of the product. Taken together, the results of this study demonstrate the dynamics of sausage fermentations. A stable dominance within this niche can be accomplished by single strains or by cooperation, which might be more stable and less vulnerable than dominance of single strains. While assertiveness is multifactorial, the prediction of assertiveness cannot be made upon single markers. However, parameters like bacteriocin formation or prophages can strongly influence the assertiveness of a strain and have to be elucidated in following studies.

The presented theses are based on the results obtained in this study, which are discussed in detail in the following chapters.

4.1 MALDI-TOF MS serves as a high throughput technology for monitoring of *L. curvatus* and *L. sakei* on strain level

4.1.1 Sub proteome biodiversity of *L. sakei* and *L. curvatus*

In this study, we aimed to implement MALDI-TOF MS as a reliable tool for the identification of *L. sakei* and *L. curvatus* not only on species but on strain-level. The first step towards a respective use of this technology, was the determination of the species biodiversity.

Orla-Jensen (1919) coined the term “lactic acid bacteria” in her monograph. The criteria include cellular morphology, mode of glucose fermentation, temperature ranges and sugar utilization patterns (Orla-Jensen, 1919). The genus *Lactobacillus* was first described by Beijerinck in 1901 and belongs to the order of lactic acid bacteria (LAB) (Beijerinck, 1901). Orla-Jensen advised a subdivision into three subgenera – Thermobacterium, Streptobacterium and Betabacterium (Orla-Jensen, 1919).

Nowadays, *Lactobacillus* species are commonly classified according to their metabolic pathways and their resulting end products. This phenotype-based nomenclature enables the classification into obligately homofermentative, facultatively heterofermentative and obligately heterofermentative (Kandler and Weiss, 1986). The process of taxonomic change is continuous and ongoing (Bernardeau et al., 2006), displaying a relatively large degree of diversity (Goldstein et al., 2015). New species are continually described; other established species are intensively renamed according to new insights and findings. To date, more than 170 different species and 17 subspecies are known that may partially differ widely in their genomic and metabolic properties (Goldstein et al., 2015). The biodiversity of the genus *Lactobacillus* is also reflected by and within the species *L. curvatus* and *L. sakei*. Both can be isolated from a wide variety of habitats including meat, fish, sourdough, fermented plant material, milk, sauerkraut and silage (Berthier and Ehrlich, 1999; Michel et al., 2016; Tohno, Kobayashi, Nomura, Kitahara, et al., 2012; Tohno, Kobayashi, Nomura, Uegaki, et al., 2012) suggesting a broad metabolic potential. The phenotypic and genotypic diversities within *L. curvatus* and *L. sakei* have been revealed and reported in several studies (Albano et al., 2009; Berthier and Ehrlich, 1999; McLeod et al., 2008; Nyquist et al., 2011). The current state of taxonomy describes *L. sakei* spp. *sakei* as well as *L. sakei* spp. *carnosus* (Torriani et al., 1996) and *L. curvatus* without any further subspecies division (Koort et al., 2004; Torriani et al., 1996). So far, substantial differences of their biodiversity have not been depicted.

The analysis of sub proteome mass spectra enabled a determination of the species sub proteome biodiversity. A hierarchical clustering and sorting of recorded sub proteome mass spectra should enable the selection of differentiable strains. Therefore, in order to depict the biodiversity of the species *L. curvatus* and *L. sakei*, the sub proteome mass spectra of 49 *L. sakei* and 29 *L. curvatus* strains were acquired using MALDI-TOF MS. The analysis of sub proteome mass spectra in this study enabled a sorting into three different groups for *L. sakei* and into two different groups for *L. curvatus*. These “MALDI-types” may refer to possible subspecies and indicate a higher biodiversity than expected based on the current available literature. These findings are in line with Chaillou, who reported that the inclusion of genetic markers for *L. sakei* indicated the existence of different biotypes that are not only reflected by the two known and accepted subspecies and suggested that members of *L. sakei* derived from three ancestral lineages (Chaillou et al., 2013). Neither Chaillou’s investigations nor our studies match the findings of Torriani et al, who described only two *L. sakei* species based on diverse typing technologies (Torriani et al., 1996). Available literature related to biodiversity of *L. curvatus* is scarce, only indicative and mostly based on pattern analysis (Aznar and Chenoll, 2006). However, correlating to the indications of Chaillou, the results on sub proteome diversity suggests a wider biodiversity than currently reported. Indeed, results on sub proteome diversity of this study rather propagate a classification and division into several groups for both species. Further studies may enable statements if these determined “MALDI-types” may correlate to possible subspecies of *L. sakei* and *L. curvatus*, while it remains unclear what future genome based classification of sub-species will then be.

Still, the determination of species biodiversity on sub proteome level with MALDI-TOF MS was successful and enabled a selection of strains for further studies and the next step towards a use of MALDI for the strain-specific monitoring of *L. sakei* and *L. curvatus* during sausage fermentation.

4.1.2 Discriminatory power of MALDI-TOF MS on strain level

This study ascertains MALDI-TOF MS to be a reliable and rapid high-throughput technology for microbiota monitoring during raw sausage fermentations on strain-level. This identification on strain level requires respective reference protein mass spectra. Those reference protein mass spectra (MSPs) of all *L. sakei* and *L. curvatus* strains were acquired and implemented in an in-house database. With the establishment of the latter, it was possible to distinguish between different strains of the same species. This differentiation was possible for both species.

It was often reported, that MALDI-TOF MS mass spectra become more reliable and easier to distinguish when extraction methods are used. Although, monitoring of microbiota development during raw sausage fermentation demands a fast high-throughput identification

and cannot be realized by elaborate extraction methods. This work successfully verifies direct application approaches for secure and fast identification of isolates on strain level. It is not always necessary to rely on extraction methods in order to gain defined and distinguishable sub proteome mass spectra. In this study, direct application was used to enable a faster identification and an easy handling. The reliability of MALDI-TOF MS and the implemented in-house database was validated by the determination of recognition rates for each of the strains of interest. Strain identification and strain specific recognition rates were shown to be strongly influenced by several factors. We were able to show, that the rate of strain recognition depended on the respective strain and the time of incubation. While some strains showed recognition rates up to 100 % and appeared to be more unique, other strains were characterized by multiple misidentifications. In fact, it was possible to detect accumulations of such misidentifications for certain strains, which seem to have very similar protein mass spectra. Those similarities are responsible for difficulties in the differentiation of the strains. Therefore, for these species or strains, the possibility of correct strain differentiation cannot be generalized. Still, by composition of specific strain sets, combinations can be found, which enable tracking and assessment of assertiveness of a strain of interest compared to others.

Moreover, incubation times were shown to have a strong effect on the recognition rates. Longer incubation times led to a declining identification accuracy. These findings correlate with the findings of Wieser et al. (2012), who observed that older cultures tend to have weaker and less distinguishable peaks (Wieser et al., 2012). Generally, MALDI-TOF-MS spectra are dominated by ribosomal proteins (2-20 kDa), which ionize well, provide accurate spectra and are only minimally affected by microbial growth conditions (Wieser et al., 2012). However, upon ageing of the culture many of proteins needed for growth may be degraded, and the number of ribosomal proteins decreases. This may explain less defined spectra of older cultures and stationary phase cells reducing recognition rates at strain level. While it remains unclear, which proteins are responsible for the observed differences in strain specific protein mass spectra, we can in principle conclude that proteins suitable for strain differentiation may preferentially be found upon exponential growth when strains express strain specific traits, and can also vary with changes in growth conditions (Sauer and Kliem, 2010). Simultaneously to the observation of declining recognition rates due to the applied incubation time, we observed that unspecific identification on species level increased after three days of incubation. This could be due to the production of common stress response enzymes in a later stage of the culture. In fact, it seems that protein expression profiles react dynamically to environmental changes such as nutrient limitation, decreasing pH values or altering of the culture. Sauer et al. also stated that the same bacteria can give different mass spectra owing to the use of different culture conditions (Sauer and Kliem, 2010), which strengthens our findings and shows how crucial standardized sample preparations are for ensuring the reproducibility of this method. This

work also corroborates previous findings that reliable identifications require a database containing not only reference mass spectra of all species or strains of interest, but also mass fingerprints of multiple strains of each species (Lartigue et al., 2009). Therefore, strain specific monitoring of microbiota to date remains restricted to specific microbiota for which respective databases exist. Apart from the meat environment Kern et al. were able to demonstrate the discriminatory power of MALDI-TOF MS along beer spoilage bacteria and yeasts (Kern et al., 2013). In our study we additionally succeeded to validate the discriminatory power by the establishment and use of a colony-duplex PCR for the assertive *L. sakei* and *L. curvatus* strains used in this study. Colonies of our strains, which resulted in correct identification via MALDI-TOF-MS, were back checked on strain level via strain-specific primers in combination with universal primers for the dnaK gene to avoid false negative results. As validation was positive for all strains tested, it is likely that strain specific MALDI-TOF-MS identification is also a valid method for other strains with high recognition rates, for tracking of starter strains in complex environments.

Taken together, the results of the similarity calculation as well as the results on the validation of the discriminatory power of MALDI-TOF MS using colony-duplex PCR, successfully verified our initial hypothesis that MALDI-TOF MS can serve as a reliable, high-throughput identification tool for *L. sakei* and *L. curvatus* on strain level. The application for other species remains uncertain and has to be evaluated separately. Especially non-ubiquistic species like *L. sanfransiscensis* and *L. lindneri* may not provide enough differences in their MALDI spectra. A closer look on their accessory and core genomes may provide a prediction of the likeliness of strain specific MALDI tracking.

4.2 Assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations

Sausage fermentation is a well-known and well-studied microbial process. The basic knowledge of microbial populations developing during fermentation has been acquired in the past. Pioneering studies such as Lerche and Reuter (Lerche and Reuter, 1960; Reuter, 1972) revealed LAB and CNC as the two main bacterial groups technologically relevant in sausage fermentation. Indeed, the LAB community is often characterized by the presence of lactobacilli (Hugas et al., 1993; Leroy et al., 2006; Papamanoli et al., 2003). Especially, the species *L. sakei* and *L. curvatus* were found to predominate the LAB community in raw fermented sausages (Hammes, 1990). However, a characterization of microbiota and the determination of population changes not only on species but on strain level is an important issue and can play a pivotal role in the comprehension of microbiota dynamics. Unfortunately, the vast majority of studies described population changes in reference to microbial groups rather than for particular species or strains, which would be more useful for a better comprehension of final

microbiological properties (Giraffa, 2004). Giraffa et al. stated that suitable starter cultures must be selected at strain level since not all strains are equally well adapted to the food environment or substrate (Giraffa, 2004). In fact, different strains of a species are characterized by different genetic make-ups and therefore produce a unique set of enzymes and proteins. In conclusion, the expression of assertiveness is strain-specific as well. MALDI-TOF MS can visualize those mentioned differences in the sub proteome expression profile enabling a strain-specific tracking during fermentation and maturation. Thus, the intraspecies variety of a species and therefore the intraspecies-dependent expression of assertiveness is not-well documented so far.

In order to close the substantial gap of knowledge concerning microbiota development as well as the fate of the individual starter strain during raw sausage fermentation, competition studies were conducted. With the newly implemented model system and additional large scale fermentations including the identification not only on species- but on strain-level, general statements and insights into the dynamics of assertiveness in raw sausage fermentations were gained.

4.2.1 Intraspecies assertiveness in a fermenting sausage model system

In this thesis we determined the intraspecies assertiveness of 9 *L. sakei* and 9 *L. curvatus* strains in a fermented sausage model system using high-throughput MALDI-TOF MS validated by strain specific colony-PCR with primers derived from comparative genomics. With the enhanced MALDI-TOF MS database (Chapter 4.1), we were able to track the used starter cultures on strain level in competition studies. Starter cultures were combined in compatible sets to enable a reliable tracking of the respective starters on strain level.

Beside $\log_{10}$ CFU g^{-1}, the relative abundance of strains of the total microbiota was determined as the essential criterion for assertiveness. However, even small percentages in relative abundance may correspond to high CFU g^{-1} for the respective strain or species. Nevertheless, it was possible to reproduce the results and findings at species and strain level at this narrow range, which highlights the suitability of the established sausage model and strain tracking and identification tool MALDI-TOF-MS.

The development of $\log_{10}$ CFU g^{-1} is typical starting from approximately 10^6 cells per gram sausage batter to 10^8 or 10^9 cells. However, the determined $\log_{10}$ CFU g^{-1} sausage batter on day 0 did not always correlate with the initially inoculated cell number of 10^6 cells per gram salami meat batter. Moreover, not all of the inoculated TMW strains were found on day 0. One possible explanation for this observation could be the so called “viable but not culturable” (VBNC) state, which has been observed and described before (Colwell, 2000; Ducret et al., 2014; Fakruddin et al., 2013; Oliver, 2005, 2010). Upon use of mMRS the recovery rate could

be improved, but not increased to match inoculation levels for all strains. The observed reduction in CFU g^{-1} sausage batter on day 5 occurred in all sets and can be probably traced back to nutrient limitation and low pH values.

The results of the competition studies clearly suggest two different strategies in competition and assertiveness. The first strategy is to outcompete all other members of the meat microbiota by a specific strategy enabling single dominance. It can be speculated that this may be based on physiological properties like short lag-phase, high growth rates, production of antimicrobial substances such as bacteriocins or the release of phages, which may efficiently inhibit the growth of competitors. This strategy can be considered as a form of contest competition (Hibbing et al., 2010), in which one competitor actively harms the other. This concept is part of the competitive exclusion principle (Nurmi concept), which was first described by Nurmi in (Nurmi et al., 1992) and has been widely proposed for probiotic bacteria inhabiting the intestinal tract (Callaway et al., 2008; Fuller, 1989; Nuotio et al., 1992; Patterson and Burkholder, 2003), while it has not been proposed or demonstrated for fermenting foods before.

The competitive exclusion principle in general states, that two species or strains, which are in direct competition about the same resources, cannot stably coexist (Nurmi et al., 1992). Due to the fact that one species or strain will always show, even only slightly better performance on the respective substrate, long term competition will always lead to the extinction of the other. This phenomenon was observed for the individual starter sets of set I and IV (see Chapter 3.2.1 and 3.2.2).

Set II and III, however, do not fit in this principle. The sets were characterized by co-dominances rather than the sole dominance of a single strain. This observation may be explained by the principle of colonization resistance (CR), which was first described for complex microbial communities in the intestinal tract and can be defined as a consequence of competitive exclusion. Constant competition about the same pool of resources led to diversification and in consequence to the occupation of different niches. Colonization resistance describes the inhabitation of different niches, while preventing the occupation of the respective niche by competitors such as pathogens. The occupation of different niches, especially with regard to sausage fermentations, requires enormous variability in metabolism, which cannot necessarily be provided by one single strain. In consequence, the results on the assertiveness driven by co-dominances, shows how colonization resistance can be achieved without the production of inhibiting agents like bacteriocins. The further investigated rise and fall of *L. curvatus* TMW 1.439 of set II revealed dependencies on concomitant growth of *L. curvatus* TMW 1.1390 and could implicate co-operational traits or even mutualism as a key strategy to overcome challenges by members of the autochthonous microbiota. Fermentations,

driven by both of them, were shown to be stable as the autochthonous microbiota was efficiently inhibited. However, competition studies without the concomitant partner strain led to a completely different outcome and the complete displacement of *L. curvatus* TMW 1.439, whereas *L. curvatus* TMW 1.1390 was able to grow and prevail. Furthermore, while reaching similar dominance, the strain was looking for another "partner" strain, e.g. *L. sakei* TMW 1.595. This finding, however, indicates a co-operational trait, where especially TMW 1.439 profits from the presence and presumably metabolism of TMW 1.1390. One can speculate that the assumed cooperation between *L. curvatus* TMW 1.439 and *L. curvatus* TMW 1.1390 is a simple case of mutualism, where *L. curvatus* TMW 1.439 acts as a sponger and that *L. curvatus* TMW 1.1390 only produces metabolites as by-products (Hibbing et al., 2010). The fact that only *L. curvatus* TMW 1.439 is displaced in the absence of *L. curvatus* TMW 1.1390, whereas *L. curvatus* TMW 1.1390 is able to grow without *L. curvatus* TMW 1.439 could support the mutualism theory.

A closer look at the results of the omission experiments, however, leads to the assumption of a real cooperation of the two strains as the autochthonous microbiota is not that efficiently inhibited when TMW 1.1390 is competing without TMW 1.439 as when they are both used in combination. Even, when *L. curvatus* TMW 1.1390 was able to grow and was not displaced on day 5 of the fermentation, the autochthonous microbiota was able to emerge up to 45.8%. These findings clearly indicate that the highly stable and controlled state of assertiveness and dominance is only expressed when both strains were combined.

In summary, co-dominances were observed when single-dominant strains were absent. The strategy of co-dominance enabling colonization resistance seems to be a widespread strategy for the establishment of dominance within a habitat rather than the strategy of competitive exclusion demanding high individual fitness of the respective strain or the production of inhibiting agents like bacteriocins.

4.2.2 Intraspecies assertiveness in large scale ring trial fermentations

With the implemented fermenting sausage model system we were able to present a method for a reliable tracking of *L. sakei* and *L. curvatus* on strain level using MALDI-TOF MS allowing the depiction of population changes in fermented sausage on strain level. Moreover, we were able to gain generally insights into the mechanisms of assertiveness and the strategies enabling the establishment of dominance within sausage fermentations. Beside the opportunity to outgrow all other members of the autochthonous microbiota, our results suggested an alternative strategy for bacteria for the establishment of a stable dominance within the meat environment by co-domination or even cooperation with other strains. The intention of the current ring trial experiments was to verify those previously gained findings about the

mechanisms of assertiveness and to get generally insights about the microbiota development in raw fermented sausages using different starter organisms or sets of starters.

In order to validate these findings on strain specific assertiveness gained in model based competition studies, large scale ring trial fermentations were conducted in cooperation with industrial partners from Germany. A total of three different German-based companies that either produce starter cultures or raw fermented sausages, participated in the ring trial fermentations. All companies were advised to fill the casings to a total weight of 300 g per sausage. Unfortunately, this request was partly misunderstood. One of the three participating companies filled the casings to approximately 425 g, to achieve a final weight after drying of 300 g. However, diameter was fixed and samples of 10 g were taken over the entire cross section of the sausage so that this should not have a serious impact on the results.

The major task of the ring trial experiments was the monitoring of LAB microbiota during the whole fermentation. The LAB communities were completely dominated by the strain or the strain set introduced prior to fermentation. For each lactobacilli species one set with a single-dominant strain and one set with a combination of co-dominating strains, consisting of either two or three strains, were included. Moreover, reproducibility was guaranteed by performing two biologically and two technically replicates for each set tested.

The results of set I (Figure 11) clearly demonstrate and verify the assertiveness of the used *L. curvatus* starter strain TMW 1.624, which was already proved to express a high assertiveness within the model system. The occurrence and dominance of the strain was already established after 5 days of fermentation with a relative abundance of 86.46 ± 0.52 %, reaching final values of 95.05 ± 1.82 % at day 21. Compared to the expressed dominance in the model system, the state of assertiveness was even consolidated by an increase of 4.86 percentage points. The autochthonous microbiota, represented by “in-house” *L. curvatus* species, was efficiently inhibited to a minimum in relative abundance of 4.94 % after 21 days of maturation.

Set II, consisting of *L. curvatus* strains TMW 1.439 and TMW 1.1390, proved our findings on the co-dominance mechanisms in meat to establish a stable state of assertiveness and inhibition of the autochthonous microbiota (Figure 12). In our previous study, dependencies between the *L. curvatus* strains TMW 1.439 and TMW 1.1390 were found suggesting not only a codominance but co-operational traits. Especially the fact that TMW 1.439 was not able to prevail without the partner starter culture TMW 1.1390 seemed remarkable. Eisenbach et al. showed that both *L. curvatus* strains are characterized by a complementary genome, meaning that one strain do not possess genes the other strain have and vice versa (Eisenbach et al., 2018). Therefore, both strains seem to complement each other concerning their genetic make-up. The assumption that genes, the strain TMW 1.1390 possess, are important for the survival of TMW 1.439 seemed obvious. Moreover, it can be assumed that the strains may occupy

different niches within the sausage environment. Nevertheless, the results of the study showed that TMW 1.439 is able to establish a higher relative abundance than its co-dominating partner strain TMW 1.1390. However, the ring trial experiments, using this combination of starter cultures now for an upscaled fermentation, showed a clear indicatable shift in the determined relative abundances towards TMW 1.439, discriminating its partner TMW 1.1390.

The dominance of both co-dominating strains (company III) reached values up to 98.3 %, whereby only 11.5 % can be credited to *L. curvatus* TMW 1.1390. For the second company performing the fermentation with set II similar observations were made. The total proportion of both strains reached 89.3 %, whereby only 4.8 % can be credited to TMW 1.1390. Again, the shift in percentage distribution is clearly visible. A comparison of both studies concerning the relative abundances is only valid looking on results determined on day 5 due to the fact that our model system could only depict the first 5 days of fermentation. On average the shift, which is already present on day 5, resulted in a significant increase concerning the relative abundance of TMW 1.439 of 31.5 %, whereas TMW 1.1390 lost an average percentage of 14.5 %. This shift gets even more significant with longer fermentation times. In conclusion, the conditions of the ring trial experiments seemed to favor the microbiota development towards the previously more vulnerable strain TMW 1.439.

The *L. sakei* strain collection consisting of TMW 1.3, TMW 1.417 and TMW 1.1398 recorded a similar shift in microbiota development concerning the determined relative abundances of the used starters (Figure 13). Contrary to the observed shift in set II, the shift in relative abundances is even more distinct. Originally, in the model system, the *L. sakei* strain TMW 1.417 displayed the major part of the LAB microbiota with an average relative abundance of 44.8 %, followed by TMW 1.1398 with 30.56 % and TMW 1.3 with 14.58 %. The latter strain displayed the least competitive strain in the ring trial experiments with an average relative abundance of only 2.1 %. In addition, the order of the remaining starters, namely TMW 1.417 and TMW 1.1398, switched completely. Instead of TMW 1.417, the strain TMW 1.1398 took over the major part of the total LAB microbiota with an average relative abundance of 69.8 %. It replaced TMW 1.417, which could only grow to an average relative abundance of 24.0 %, meaning an increase of approximately 39.2 % for TMW 1.1398 and a decrease of 20.8 % for TMW 1.417. An influence of drying on the survival and the expression of strain-specific assertiveness would be reasonable.

Set IV, consisted of *L. sakei* TMW 1.1396, proved the previously determined assertiveness of the strain. Similar to results of the competition studies in the model system its dominance was already established on day 5 and prevailed to the end of the fermentation (Figure 14). The relative abundance on day 5 reached values of an average relative abundance of 98.15 %. The final determined relative abundance on day 21 with the end of the fermentation was 93 %,

whereas the autochthonous microbiota, represented by the autochthonous species *L. sakei* and species with unknown identity (“nri”), was inhibited and reduced to a relative abundance of only 1.86 % on day 21.

In conclusion, this study successfully confirmed the reliability of the established model system presented in Chapter 3.2.1. The model system can provide valuable information about the assertiveness of certain starter strains and enables the development of new possible starter organisms. Due to possible differences in large industrial scale fermentation conditions, a shift in relative species abundances may occur. However, in general, obtained statements about the assertiveness proved valid.

Moreover, this study highlights the importance of starter cultures in meat fermentations. LAB are usually present in high hygienic quality raw meat at low numbers but it was shown that they rapidly dominate the fermentation due to their adaptation to the anaerobic and restricted environment (Rantsiou and Cocolin, 2006). Especially, for *L. sakei* and *L. curvatus* their ability to grow and to dominate the microbiota in traditional meat fermentation is well documented. The results on microbiota dynamics of produced control meat batters show a desirable development of LAB community. Interestingly, despite the presence of *L. curvatus*, the LAB community on control meat batters was always dominated by the species *L. sakei*. These findings do not correlate with those gained in conducted model system experiments where control batches encompassed both lactobacilli species to relatively high abundances (see Figure S.6).

However, in contrast to these findings, the development of CNS microbiota within control meat batters is highly questionable and demonstrate the importance of the utilization of suitable starter organisms. The control batch of company III showed high relative abundances of *S. aureus* with a relative abundance of 45.8 % (6.8 x 10^9 CFU g^{-1}) on day 21, whereas the control batch of company II revealed high abundances of *S. saprophyticus* with values up to 79.2 % (4.3 x 10^6 CFU g^{-1}) on day 21 at the end of fermentation. Nonetheless, no clear sensory irregularities were noticeable. The German Society of hygiene and microbiology refers to a warning level of 2 x 10^4 cells per gram for raw fermented sausages (DGHM, status 2010, https://www.dghm-richt-warnwerte.de). In consequence, the reported levels in all of the produced spontaneous control batches of all participating companies were too high and display a possible safety risk for the consumer.

Since fermented foods are not commercially produced in an environment free of contaminating microorganisms (Giraffa, 2004) the autochthonous microbiota may include also potential spoilage and pathogenic bacteria, which have to be limited or inhibited in their growth. Besides salmonella and listeria, enterotoxin producing staphylococci surviving the dry curing process are a major concern for the safety of the final product (Messier et al., 1989). In fact,

staphylococcal food poisoning (SFP) is one of the most common food-borne diseases and results from the ingestion of enterotoxins produced by enterotoxigenic strains of coagulase-positive staphylococci such as *S. aureus* (Hennekinne et al., 2012). The presence of *S. saprophyticus* is less critical but the bacterium can be associated with urinary tract infections (Hovelius and Mardh, 1984).

Normally, in order to prevent the growth of undesired microorganisms like *S. aureus* or *S. saprophyticus*, a fast acidification and decrease in water activity is aspired. Interestingly, even within control meat batches and in accordance with available literature, water activity- and pH values followed a desirable development over the fermentation and maturation time, so that in consequence the final product can be considered as safe (Table 9). Nevertheless, *S. aureus* as well as *S. saprophyticus* grew to high cell counts, indicating a poor microbiological stability. In general, bacterial growth of *S. aureus* is reported above a_w-values of 0.86 (Qi and Miller, 2000). However, growth as well as the critical enterotoxin formation strongly depends not only on water activity but also on pH, temperature and oxygen content as it usually shows exceeding growth and therefore produces higher yields of enterotoxin in an aerobic environment rather than in an anaerobic environment (Belay and Rasooly, 2002). In the current study, water activity decreased only to values of 0.87 after 21 days of fermentation. Nevertheless, neither the development of *S. aureus* nor *S. saprophyticus* was shown for the control batch of company I with the same course concerning pH and water activity (a_w). This particular company produced and provided the minced meat for salami production for all participating companies. Contamination during transport or the following storage could be a possible explanation for the observed differences in microbiota development in control batches. A significant influence of the ripening technique, which is considered a meaningful parameter influencing the microbiota development (Rantsiou and Cocolin, 2006), can be excluded due to fixed ripening profiles in the ring trial experiments.

However, with the introduction of the *S. carnosus spp. carnosus* starter TMW 2.212 both *S. aureus* and *S. saprophyticus* could be inhibited. Nevertheless, the used *S. carnosus ssp. carnosus* starter TMW 2.212, was not able to inhibit the rise of those undesired microorganisms completely. In consequence, the CNS community of set III, performed by company II, showed a relative abundance for *S. saprophyticus* of 38.5 % at the end of the fermentation.

Beside microbiological analysis, the development of pH and drying followed a typical profile in all investigated fermentations and confirmed the stability of the ripening process. The observed drop in pH and the progressive drying resulted in a high acid (final pH lower than 5.3) and short-ripened (< 45 days) salami (Incze, 1998). In consequence and as a result of the described acidification of the meat other microbial groups than LAB and CNS never exceeded the status of background microbiota. Usually, fresh meat is characterized by an a_w-value above

0.99 (Feiner, 2006). The particularly lower initial a_w of 0.946 ± 0.0035 may be explained by the lard content in raw sausages, like described before (Aquilanti et al., 2007).

In order to allow the activity of microorganisms other than LAB and CNC, which may increase the level of volatile compounds such as yeasts, an extension of the ripening period might be beneficial (Rantsiou and Cocolin, 2006).

In summary, ring trial experiments successfully verified the suitability of the newly implemented fermenting sausage model system and the findings gained upon the use of the system. Despite observable shifts, probably exposed due to the drying, which could not be depicted upon the model system, all dominant strains proved to remain dominant in large-scale fermentations and efficiently inhibit the autochthonous microbiota. The investigation of control sausages highlighted the importance of starter cultures with special emphasis on the development of CNC and CPC as those species were significantly more abundant compared to batches inoculated with starter cultures.

4.2.3 Interspecies assertiveness in raw fermented sausages

Competitive exclusion as well as colonization resistance were shown to be common strategies in establishment of dominance and assertiveness in raw sausage fermentations. In order to gain insights not only into intra- but also into interspecies dynamics and assertiveness in raw fermented sausages, competition studies were conducted by the combination of *L. curvatus* and *L. sakei* sets.

For fermented sausages, Dossmann et al. stated that *L. sakei* is more competitive than other lactobacilli, showing shorter lag phases higher growth rates and higher cell numbers (Dossmann et al., 1996). This hypothesis could be verified with interspecies competition studies using the *L. curvatus* strain set II and the *L. sakei* strain sets II and IV (see Chapter 3.2.3). In nearly all experiments and set combinations, *L. sakei* were dominant and overgrew the introduced *L. curvatus* starters. This phenomenon may be explained by the adaptation of *L. sakei* to meat. It is frequently reported that *L. sakei* seems to be more adapted to the conditions during sausage fermentation. Indeed, we were able to show better growth performances of *L. sakei* strains compared to *L. curvatus* in a modified meat simulation medium. Moreover, the adaptation of *L. sakei* is further manifested by the conducted interspecies competition experiments, resulting in a predominance of *L. sakei* strains. The performance of the strain may be explained by different physiological properties, which are discussed in the following.

At the beginning of sausage fermentation concomitant availability of oxygen and heme can enable a respiratory metabolism of LAB (Brooijmans et al., 2009), which reduces oxidative stress and is energetically favorable (Lechardeur et al., 2011). Nevertheless, hydrogen

peroxide may accumulate due to the action of flavoprotein oxidases expressed by some LAB. Especially for *L. sakei* it is widely reported that the strains are well equipped with heme-dependent catalases as well as with pseudocatalases, which prevent oxidative stress by scavenging the reactive oxygen species (Knauf et al., 1992).

However, the oxygen is readily consumed, leading to the reduction of the E_h, while favoring the growth of the respective LAB starter cultures. During sausage fermentation the primarily preferred substrate is glucose and glycolytic intermediates with varying availability depending on residual glycogen content (Ferguson and Warner, 2008; Pösö and Puolanne, 2005). With the use of lactobacilli starter cultures these carbohydrates are metabolized homofermentatively leading to the formation of lactic acid, which lowers the pH. Finally, after carbohydrate depletion, secondary proteinaceous or triacylglyceride-derived substrates are metabolized (Gill, 1983). In fact, a recent publication addressed triacyclgylceride metabolism in meat spoilers. Höll et al. ((Höll et al., 2019)) reported that gram-positive and gram-negative meat spoilers, especially *Photobacterium* spp. are capable of using glycerol for gluconeogenesis.

Bacterial enzymes have only a minor participation in protein breakdown. The initial degradation of myosin and actin into peptides is due to the activity of cathepsin D. The later decomposition of peptides into free amino acids is then catalyzed by bacterial peptidases (Freiding et al., 2011).

However, it is reported that amino acids, play a key role in explaining the dominance, survival and growth of *L. sakei* in meat (Montanari et al., 2018). The species is auxotrophic for all amino acids except aspartic and glutamic acids, which can be obtained by the deamination as asparagine and glutamine (Chaillou et al., 2005). All other amino acids have to be up-taken as free amino acids or short peptides from the meat (Sinz and Schwab, 2012). The absence of metabolic pathways responsible for amino acid synthesis and the absence of transaminases underlines the adaptation of *L. sakei* to the environment meat (Montanari et al., 2018). Besides their role in protein synthesis, amino acids can be involved in other pathways concerning the overall cell metabolism. In detail, amino acids can contribute to energy generation, which was well described by Fernandez and Zuniga (2006) as well as to the production of aroma compounds (Smid and Kleerebezem, 2014). However, as well as *L. sakei*, *L. curvatus* can use free amino acids, which illustrate the importance of other metabolic traits enabling the dominance of *L. sakei* (Sinha, 2007).

One possible explanation for the observed dominance of *L. sakei* over *L. curvatus* may result from the purine nucleoside metabolism of *L. sakei* (McLeod et al., 2017; Rimaux, Vrancken, Vuylsteke, et al., 2011) as ribose, deriving from nucleosides, is an important carbon and energy source of meat associated bacteria (Chaillou et al., 2005; Eisenbach et al., 2018). However,

Eisenbach et al. (2018) were able to show that all of the *L. curvatus* strains indeed were able to use ribose as sole carbon source like described for *L. sakei*.

The metabolization of arginine may be another possible selective advantage of *L. sakei* over *L. curvatus*. Arginine is highly abundant in beef (Holló et al., 2001a; Schweigert and Payne, 1956) and therefore also in raw fermented sausages, whose main ingredient is fat and beef meat. The ability of *L. sakei* to utilize arginine through the arginine deaminase (ADI) pathways, resulting in additional ATP is widely described (Rimaux et al., 2012; Rimaux, Vrancken, Pothakos, et al., 2011) and represents a competitive benefit (McLeod et al., 2017). Indeed, Eisenbach et al. (2018) reported that, in contrast to *L. sakei*, the *L. curvatus* pan and core genome analysis revealed no genes involved in ADI pathway, which underlines this particular adaptation of *L. sakei* as decisive advantage. Moreover, we were able to demonstrate a tendency of growth dynamics in mMSM and the observed assertiveness determined in this study (see Chapter 3.3.1). In general, *L. sakei* strains showed an overall increased performance in comparison to the majority of *L. curvatus* strains. Especially, the dominant strains within the sets were characterized by high growth rates corresponding to higher final OD-values. So far, it is still unclear whether these increased performance can be traced back to the mentioned metabolization of arginine and needs to be investigated in further approaches.

Lastly, it has to be noticed that the results presented in this study are based on starter culture combinations with the *S. carnosus* strain TMW 2.212. Results may differ when other staphylococci, e.g. *S. xylosus* are introduced as a starter culture partner.

However, despite the predominance of *L. sakei* in the conducted interspecies competitions studies, one *L. curvatus* was able not only to survive but to dominate the whole sausage microbiota. The intraspecies experiments, which were conducted with set I and its respective dominant starter strains *L. curvatus* TMW 1.624 always ended with overgrown *L. sakei* strains and TMW 1.624 as overall winner. Therefore, a strong dominance and a high degree of assertiveness is indicated for the *L. curvatus* strain TMW 1.624 irrespective of the competitors used. The supposed reason for the observed assertiveness is discussed in detail in Chapter 4.4.

4.3 Unmasking of physiological markers enabling assertiveness in sausage fermentations

The implemented sausage model and the establishment and enlargement of the MALDI-TOF MS database enabled a reliable tracking of known starter cultures during fermentation in model systems as well as in large-scale ring trial experiments, which were conducted in cooperation with industrial partners. In this study, we succeeded in the revealing of strain specific assertiveness as well as in the determination of strategies enabling the established dominance within sausage fermentations, which were not documented so far. Neither the reasons for single dominance and assertiveness nor the background of observed co-operational traits in sausage fermentations are reported so far. Gained physiological data on growth performance partly under harsh conditions are discussed one by one in the following and as a summary at the end of this thesis with special emphasis on correlations to the assertiveness in sausage fermentations.

4.3.1 Key parameters for the assertiveness of *L. sakei* and *L. curvatus*

Possible markers, explaining the assertiveness of a strain, may be found upon adaptation to the respective environment. Microorganisms are challenged by a huge variety of fluctuating biotic and abiotic factors, which include changes in pH, inter-and intra-specific competition, nutrients and resource availability (Bleuven and Landry, 2016). Special life challenges posed by a given application, first lead to an induction of specific stress responses and further to a specific adaptation of the starter strain to the respective challenge and environment, e.g. on physiological, metabolic and genetic level (Boor, 2006; Hussain et al., 2013; Taddei et al., 1997). In general, microbial stress responses are characterized by the transient induction of general and specific proteins and physiological changes that allow the bacteria to withstand adverse environmental conditions (Hussain et al., 2013). Raw fermented sausages are considered a restricted niche (Labadie, 1999) and confer various challenges for bacteria inhabiting its respective cavities.

Physiological data for the previously determined strain selection of *L. sakei* and *L. curvatus* were collected to uncover the mechanisms of assertiveness in raw fermented sausages. The investigation of growth, partly under harsh conditions that commonly occur during fermentation processes should enable a correlation analysis and the determination of key parameters responsible for the establishment of dominance and assertiveness. Unfortunately, it was not possible to find a significant and distinct correlation between the observed assertiveness and the physiological data of the respective strains (Chapter 3.3). On the contrary, the assertiveness of a strain within the raw sausage environment appeared rather multifactorial and cannot be traced back to single phenotypical characteristics.

4.3.1.1 Growth kinetics as indicator for assertiveness

Right after mincing and stuffing, the autochthonous bacteria as well as the introduced starter cultures start competing for nutrients and place within the cavities (Katsaras and Leistner, 1991). Growth performing tests using different media including a meat simulation medium should enlighten the importance of the strains ability to use the nutrients encountered in meat for their growth and a fast occupation of available niches.

In general, the performance tests verified the wide diversity of the strains used in this study.

The analysis of growth kinetics in mMRS medium did not reveal strong markers and growth was shown to be quite similar for all of the strains used, irrespective of the species. However, growth kinetics determined in the more restricted mMSM medium did reveal slight correlations of the parameter growth rate, the determined $OD_{max.}$ and the observed assertiveness of the respective strain. In general, *L. sakei* strains were able to develop higher growth rates as well as higher final ODs than the respective strains of the species *L. curvatus*. So far it is unclear, whether these observation may be traced back to the described ability to metabolize arginine (see Chapter 4.2.3). Nevertheless, with the conduction of growth kinetics experiments, we were able to confirm the findings of Dossmann, who stated that *L. sakei* shows higher growth rates and cell numbers in sausage fermentations (Dossmann et al., 1996). This hypothesis could be verified with interspecies competition studies using the *L. curvatus* strain set II and the *L. sakei* strain sets II and IV (see Chapter 3.2.3).

However, one other important finding is that strains, which were shown to be dominant in competition experiments, were simultaneously characterized by high growth rates and high $OD_{max.}$ in mMSM. Only the dominant strains *L. curvatus* TMW 1.439 and TMW 1.624 showed low growth rates and maximum OD. However, due to the gained findings in this study, this observation is not in contradiction to the observed assertiveness in sausage fermentations, as *L. curvatus* TMW 1.439 was suggested to act as a possible sponger or social cheater (Hibbing et al., 2010) and TMW 1.624 was shown to produce antimicrobial agents, presumably bacteriocins, which strongly enhance its assertiveness in the sausage matrix (Chapter 3.4). However, it remains to be elucidated how other *L. sakei* and *L. curvatus* strains perform in this special mMSM medium. It has to be noted, that the described situation is a current snapshot and may be different when more strains are included. Nevertheless, in order to get first insights into the suitability of new strains for possible starter organisms, a pre-testing could be based on this medium and may provide valuable information indicating a strains performance during fermentation.

4.3.1.2 **Oxidative stress as indicator for assertiveness**

A suitable starter culture should be able to counteract various stresses. The development of nutrient depletion as well as acidic conditions are generally considered as a result of the bacteria's own metabolism. Other environmental stresses can occur independently from the bacteria's own activity such as oxidative stress or cold stress. Both, oxidative as well as cold stress tolerance, may play an essential role for the assertiveness of bacteria inhabiting raw meat and the cavities within raw fermented sausages.

The ability to respond to reactive oxygen species (ROS) with the activation of specific enzymes enabling a detoxification may be of crucial importance within sausage meat batter and during fermentations as oxygen is introduced during meat mixing or storage (Zagorec and Champomier-Verges, 2017). The reduction of molecular oxygen lead to the formation of superoxide, which is the precursor of most other reactive oxygen species. In the following, the dismutation of superoxide can lead to the generation of hydrogen peroxide (Culotta et al., 2006; Turrens, 2003). Moreover, hydrogen peroxide is widely produced in central carbon and energy metabolism of bacteria, including LAB, by the oxidation of pyruvate with phosphate to acetylphosphate via pyruvate oxidase (Pox), or the oxidation of L-lactate to pyruvate by lactate oxidase (Lox) and by the activity of NADH oxidases (Nox) (Condon, 1987; Hertzberger et al., 2014; Hugas and Monfort, 1996; Liu et al., 2012; Nishiyama et al., 2001).

The cytotoxicity of H_2O_2 is generally reported to be based on the further partial reduction to hydroxyl radical (·OH) and hydroxid ions (HO^-) (Linley et al., 2012; Martín and Suárez, 2010). The latter reaction requires iron (Fe^{2+}) and is commonly known as Fenton reaction (Winterbourn, 1995). However, hydroxyl radicals are potentially oxidizing agents leading to mutations, deletions, single strand breaks and other types of DNA damage (Cadet et al., 1999; Tsunoda et al., 2010) as well to damages of bacterial proteins (Ezraty et al., 2017) and lipids (Kashmiri and Mankar, 2014; Ortiz de Orué Lucana et al., 2012). One of the primary effects of lipid oxidation is the reduction of membrane fluidity, which alters membrane properties and may disrupt membrane-bound proteins (Kashmiri and Mankar, 2014). In the following, polyunsaturated fatty acids are degraded to a variety of products, including aldehydes, which are reactive and can damage molecules such as proteins (Kashmiri and Mankar, 2014).

In order to protect themselves, bacteria have developed a variety of mechanisms to cope with oxidative stress and to scavenge reactive oxygen species to keep the intracellular concentration of the latter at nanomolar levels (Imlay, 2008; Mishra and Imlay, 2013; Storz and Imlayt, 1999). The neutralization of ROS can be achieved by conversion into water via non-enzymatic antioxidants like NADPH and NADH pools, glutathione (GSH), ascorbic acid, pyruvate, flavonoids, carotenoids and the activity of enzymes called peroxidases and catalases

(Cabiscol Català et al., 2000). Hydrogen peroxide can be detoxified via various enzymatic pathways, e.g. NADH peroxidase or catalase activity (Mishra and Imlay, 2013). The effectivity of detoxification is strongly influenced by the hydrogen peroxide concentration within the medium and by the expression and effectiveness of detoxifying enzymes. Increasing expression of peroxidases and catalases, which are strictly controlled by the OxyR regulon (Teramoto et al., 2013), allow faster detoxification and can be investigated by the number of genes or whole operons encoding for these catalases. The steady-state level of H_20_2 can be kept by the expression of high titers of extremely active peroxidases and catalases (Mishra and Imlay, 2013). In E. coli the expression levels are so high, that despite the rapidity of endogenous H_2O_2 formation, the level of the latter is restricted to about 20 nM (Seaver and Imlay, 2001).

The oxyR protein is a transcription factor found in many bacteria and is largely inactive until H_20_2 oxidizes a key cysteine residue, which triggers the formation of a disulfide bond that in consequence locks the protein into an active conformation (Lee et al., 2004; Zheng et al., 1998). The oxidized OxyR binds to the promoter regions of members of the regulon, including genes encoding catalases and peroxidases. In *E. coli* the regulon is oxidized by 0.1 M H_20_2 ensuring that the regulon is activated when intracellular levels rise from basal 20 nM to 400 nM (Mishra and Imlay, 2012). The importance of peroxidases and catalases is also visualized by mutants lacking those hydrogen peroxide-scavenging enzymes as they were shown to exhibit growth defects (Mishra and Imlay, 2012).

Another possible adaptation to oxidative stress is the secondary defense including DNA repair systems once the damage has taken place (Cabiscol Català et al., 2000). It is widely reported that strains without repair systems and mechanisms like recA, exonuclease II or V are more sensitive to hydrogen peroxide than the respective wild type (Imlay and Linn, 1987). As raw fermented sausages provide large amounts of iron (Holck et al., 2017), the reduction of hydrogen peroxide to cell-damaging ROS is likely and consequently the ability to detoxify those ROS or handle caused DNA-damage may play a decisive role in the survival and assertiveness of a strain.

The results deriving from growth kinetics under oxidative stress contribute to the biodiversity of *L. sakei* and *L. curvatus*. The vast majority of *L. sakei* stains showed an overall unaffected growth at a concentration of 1.51 mM, whereas *L. curvatus* was characterized by an extension of lag-times up to 24 hours. Therefore, the results indicate a higher vulnerability of *L. curvatus* strains to hydrogen peroxide, compared to investigated *L. sakei* strains. These findings were strengthened by the investigation of the next higher hydrogen peroxide level of 3.13 mM. None of the tested *L. curvatus* strains were able to cope with this concentration, whereas two *L. sakei*

strains showed a delayed growth. This observation contribute to the massive amount of publications reporting the enhanced adaptation to *L. sakei* in the ecological niche fermented sausages.

As the result of the absence of heme in the respective mMRS media, it can be assumed that no heme-dependent catalase was expressed by the lactobacilli. However, non-heme manganese catalases are widely distributed, which represent an important alternative to heme-containing catalases and may be expressed upon the presence of hydrogen peroxide. In fact, non-heme catalase was found to be widespread among strains of pediococci and Lactobacilli (Delwiche, 1961; Johnston and Delwiche, 1965; Whittaker, 2012). It can be further assumed that strains, showing no prolongation in lag-times may be equipped with a respective catalase. Strains, which were characterized by prolonged lag-times and a later resumption of growth may act on using the secondary defense mechanisms of DNA-repair. The latter, however, take some time and may explain the observed lag-times. For higher hydrogen peroxide concentrations of 3.13 mM, it seems that detoxification takes more time and resulting DNA damages may be more significant, which is reflected and likely explained by long lag-phases.

In fact, the genome analysis of the strains used in this study, revealed that all of them possess genes encoding for catalases or pseudocatalases. Unfortunately, it was not possible to distinguish between both enzymes so far (Lara Eisenbach, PhD thesis, not published yet). This, however, does not explain the differences in their resistance and behavior under oxidative stress determined in this study. In order to elucidate the exact mechanisms in oxidative stress responses, further studies must be set up.

4.3.1.3 **Cold shock**

However, for the purpose of finding markers responsible for assertiveness, the tolerance to oxidative stress is not promising and requires more detailed and intensively studies about the strains. Concerning raw sausage fermentations, knowledge of bacterial adaptation to cold stress can also be considered as a possible criterion influencing the assertiveness within this restricted niche (Labadie, 1999). Fresh meat as well as fat needed for sausage production is stored frozen until the start of manufacturing (Feiner, 2006). The frozen meat and fat are then minced and mixed with other additives like preservatives, spices and starter cultures. The latter are further mostly prepared as freeze-dried cultures, indicating a cold-shock prior to manufacturing (Feiner, 2006).

Again, also the investigation of cold-shock induced stress response did not reveal strong hints for a key parameter for dominant strains. Bacterial growth was analyzed by the determination of the growth parameters growth rate µ, lag-phase and the maximum OD. The results (Chapter

3.3.2.2) demonstrate that stress response and adaptation is strongly strain specific and diverse. Differently to the current literature available on cold-shock tolerance, *L. sakei* did not show a general better tolerance than *L. curvatus*. One main reason for the assumption was reported by Chaillou, who found more putative cold shock genes within *L. sakei* in comparison with all other lactobacilli (Chaillou et al., 2005). However, due to the results gained in this work, it seems that the sole presence of cold shock proteins can only function as indicative. Temperature was shown to have a major effect on growth rates and lag-phases and that with decreasing temperatures the specific growth rate slows down (Marceau et al., 2003). In fact, we were able to show that the growth parameters µ, lag and $OD_{max.}$ were clearly affected by the induction of cold-shock. Interestingly, the induction of cold shock does not necessarily weaken the growth and the performance of the strains. Lag-phases assertive strains *L. curvatus* TMW 1.439 and 1.1390 as well as *L. sakei* TMW 1.1396 and TMW 1.1398 were positively affected and shortened. Derzelle et al. (2003) reported similar findings, were increased cryotolerance and faster growth rates were determined after an induced cold-shock (Derzelle et al., 2003). Again, like described for oxidative stress, it was not possible to find a correlation, which could explain the assertiveness of the strains. The lag-phases of the other dominant strains TMW 1.3, 1.417 and TMW 1.624 were prolonged, not shortened. Similar observations were made for the parameter growth rate µ, were only the growth rate of TMW 1.624 and TMW 1.1396 were affected positively.

In summary, these findings elucidate the difficulty to define key parameters or physiological markers for the assertiveness of strains and highlights the theory of a multifactorial based assertiveness. No direct correlations between the expression of assertiveness and the stress resistance to oxidative stress or cold stress were found upon the experiments in this study. Due to the dynamic and manifold response of bacteria to stress factors encountered in the sausage environment, general statements are impossible. However, it may be worthy to consider an implementation of cold-shock treatments prior to freeze-drying of industrial used starter cultures to enhance their performance and to shorten lag-phases in the meat batter. This particularly is only valid for strains, which were tested for this application

Nevertheless, the performance of strains in the used mMSM medium, may be of valuable information as the parameters growth rate and $OD_{max.}$ enabled a slight correlation linking the growth kinetics with the observed assertiveness. Moreover, it may be worthy to consider an implementation of cold-shock treatments prior to freeze-drying of industrial used starter cultures to enhance their performance and to shorten lag-phases in the meat batter. This particularly is only valid for strains, which were tested for this application. Besides, we were able to determine two other parameters, which may be promising markers for the assertiveness of a strain and are characterized in detail in Chapter 4.4 and 4.5.

4.4 Bacteriocin production as key parameter affecting the assertiveness of strains

As bacteria exist in complex communities, they are constantly in keen competition for nutrients and space. The production of antimicrobial peptides, such as bacteriocins, has been shown to be a possible competitive advantage (Kommineni et al., 2015). De Vuyst et al. (2007) stated that it is believed that bacteriocins contribute to the competitiveness of producer cells. Therefore, the production of bacteriocins may be one possible criterion for and influencing the assertiveness of a strain by inhibition of other inoculated starters or members of the autochthonous microbiota and play a decisive role and key strategy to increase the fitness and competitiveness of a strain. It is estimated that 99 % of bacterial strains produce at least one bacteriocin (Cotter et al., 2005; Klaenhammer, 1993). In fact, many lactic acid bacteria, including lactobacilli, secrete anti-microbial peptides that have a bactericidal or bacteriostatic effect on other, usually closely related species (Jack et al., 1995; Mokoena, 2017; Nes et al., 1996; Vaughan et al., 2004; Venema et al., 1995). Further, with special regard to the safety of products, several studies reported the inclusion of bacteriocin producers in sausage fermentations, leading to distinct log-reductions of other bacteria, especially *L. monocytogenes* (Berry et al., 1990; de Souza Barbosa et al., 2015; Martinez et al., 2015; Pucci et al., 1988), a ubiquitous Gram-positive pathogen that has been reported to cause food related outbreaks in the last decades (de Souza Barbosa et al., 2015; Scallan et al., 2011).

Bacteriocins are ribosomally synthesized and posttranslationally modified peptides (Alvarez-Sieiro et al., 2016; Vaughan et al., 2004; Yang et al., 2012). Genes encoding bacteriocin production as well as immunity are generally organized in operon clusters (Mokoena, 2017; Zacharof and Lovitt, 2012). These clusters can be identified within bacterial genomes using special identification tools like BAGEL and BAGEL3 (de Jong et al., 2006; van Heel et al., 2013). The analysis of bacterial genomes of *L. sakei* and *L. curvatus* strains with BAGEL3 revealed higher abundances of bacteriocin clusters within the genomes of *L. curvatus*, where 7 of 9 strains possess at least one respective gene cluster. For *L. sakei* only 3 of 9 strains were found to encompass putative bacteriocin clusters within their genomes (see Chapter 3.4).

Above the mechanism of co-dominances of two or more strains in the competition studies, *L. curvatus* TMW 1.624 displayed dominance as single strain to a total of 81.6% of the microbiota. As *L. curvatus* TMW 1.624 is not characterized by an outstanding growth behavior (Chapter 3.3.1), it can be assumed that the strain must possess other strategies to establish its dominance. Using a simple agar spot test and MRS as the standard media for the enrichment of the strains, TMW 1.624 was the only strain displaying *in vitro* bacteriocin-activity. It is suggested that the production of these antimicrobial compounds is a major cause for its assertiveness. However, the activity spectrum of bacteriocin(s) produced by TMW 1.624

appears to be limited to strains of the species *L. curvatus*, as we were not able to detect inhibition zones in any of the tested closely related *L. sakei* strains. Nonetheless, the analysis of its genome revealed 5 possible bacteriocin clusters. Azevedo et al. (2015) tested 224 ruminal bacteria and found 104 of them associated with bacteriocin gene clusters. Only few of them to encompass a similar amount of bacteriocin clusters within the strains genomes. In fact, there were only three strains, characterized by 5 or 6 putative bacteriocin clusters. Most of the investigated strains possessed an average amount of 1-2 bacteriocin cluster (Azevedo et al., 2015). Such an accumulation of bacteriocin clusters seems to be scarce and with regard to the investigated *L. curvatus* strain TMW 1.624, it may be indicatable for a major role of the bacteriocin(s) on the performance of the strain.

To gain more insights into the assertiveness of the bacteriocin producing *L. curvatus* strain TMW 1.624 and in order to enlighten the impact of its bacteriocin(s) another set of competitions studies were conducted, where TMW 1.624 had to compete against the other *L. curvatus* strains of set II and the *L. sakei* strains of set III and IV.

For fermented sausages, Dossmann et al. stated that *L. sakei* is more competitive than other lactobacilli, showing shorter lag phases higher growth rates and higher cell numbers (Dossmann et al., 1996). This hypothesis could be verified with interspecies competition studies using the *L. curvatus* strain set II and the *L. sakei* strain sets II and IV (see Chapter 4.2.3). However, the inclusion of *L. curvatus* TMW 1.624 led to an entirely different outcome. In these experiments it was always *L. curvatus* TMW 1.624, not one of the introduced *L. sakei* strains, which was able to establish a stable single-dominance within the sausage environment. The previously dominant strains of set II, III and IV were all overgrown to a final total dominance of TMW 1.624 of 60-80 %. A closer look at the available physiological data on TMW 1.624 strengthens the hypothesis that bacteriocin formation is the major driving force of the assertiveness of the strain. Looking at the growth dynamics or the determined stress resistance of TMW 1.624, its performance in mMRS and mMSM can only be rated as average. Neither the growth in general nor the stress resistance concerning oxidative and cold stress seems extraordinary, especially in a direct comparison with the other tested *L. curvatus* or *L. sakei* strains. However, cold shock experiments revealed a considerable shortened lag-phase after an induced cold-shock. Though, a similar development was observed for the *L. sakei* strain TMW 1.1239. Nevertheless, the strain was shown to be miniscule during sausage fermentation and was readily overgrown and displaced.

In summary, these findings not only strengthens the suggestion of bacteriocins as a major driving force of the assertiveness of TMW 1.624 but suggests the production of bacteriocin(s), which are presumably active and harmful not only against *L. curvatus* but *L. sakei*.

The bacteriocin-mediated killing is based on membrane permeabilization of the target cell membrane, probably by forming ion-selective pores which cause dissipation of the proton motive force and depletion of intracellular ATP. The expression of bacteriocin production such may be depended on various factors and may require direct cell-to-cell contact. Some bacteriocins such as lactocin B (Barefoot and Klaenhammer, 1983), plantacin F (West and Warner, 1988), enterolysin A (Nilsen et al., 2003) and streptin (Wescombe and Tagg, 2003), are constitutively produced only on solid media suggesting that signals from cell-to-cell or cell-to-surface contact are required for the induction of bacteriocin production and Ruiz-Barba (2010) reported the bacteriocin production of *L. plantarum* NC8 gets activated by co-cultivation with specific bacteriocin production-inducing bacterial strains, e.g. *Enterococcus feacium* 6T1a-20 and *Pediococcus pentosaceaus* FBB63. Further, De Vuyst et al. reported a stimulation of bacteriocin production under unfavorable growth conditions, while Hillmann et al. (Hillman et al., 1998) as well Qi et al. (Qi et al., 2001) indicated that two bacteriocins of *S. mutans* appears to be dependent on culture conditions. Sometimes, the usage of another enrichment medium increase or enable the production of bacteriocins as shown in a study by Goh and Philip, where the activity of the bacteriocin weisellicin, produced by *Weisella confusa,* was shown to be the highest in MRS, followed by LAPTg and M17 (Goh and Philip, 2015).

Due to these findings, co-culture experiments in meat simulation medium were conducted to enable an enrichment of previously not expressed bacteriocins of TMW 1.624. Again, and as expected due to the presence of bacteriocin clusters within the strains genome, the results clearly verified the dominance of *L. curvatus* TMW 1.624. Only the *L. sakei* TMW 1.1239 was able not only to compete but to overgrow *L. curvatus* TMW 1.624 when either catalase, proteinase K or trypsin were added, indicating that also the production of hydrogen peroxide may contribute to the assertiveness of *L. curvatus* TMW 1.624. Besides this exception, the dominance of *L. curvatus* TMW 1.624 was successfully verified within liquid medium.

By the addition of catalase, the dominance was mostly not affected, which proves that the observed inhibition is not based on the formation of hydrogen peroxide. Only the strain TMW *L. sakei* 1.417 was shown to be more abundant in experiments with added catalase. Interestingly, the dominance of *L. curvatus* TMW 1.624 could not been reduced upon the addition of proteinase K and trypsin. This however, was only partly confirmed in the following fast agar spot tests. The test showed clear inhibition zones for most of the strains treated with the respective co-cultures supernatant of TMW 1.624. Again, the *L. sakei* TMW 1.1239 was not inhibited at al. Moreover, the previously inhibited TMW 1.417 did also not show any inhibition zones using the fast agar spot test. This observation may be of special interest for further investigations and has to be repeated to ensure reproducibility. However, the majority of *L. sakei* strains acted similar on the fast agar spot test in comparison to the liquid competition experiments. Upon the addition of proteinase K, the proteinaceous nature of the inhibiting

agent, produced by TMW 1.624 could be elucidated. The treatment with trypsin was not always successful and did only lead to the inactivation of inhibition for *L. sakei* TMW 1.1396 and TMW *L. sakei* 1.1398.

The failure of trypsin cleavage may be explained by the structure of bacteriocins, encoded in the genome of *L. curvatus* TMW 1.624 (

Table 18). Two of the possible clusters encode for Sakacin Tα and Lanthipeptid class II, which have only one or three enzymatic cleavage sites for trypsin. Trypsin cleaves only after arginine or lysine (Olsen et al., 2004; Simpson, 2006). This could mean that the enzyme is not appropriate for a sufficient cleavage of the respective bacteriocin. Furthermore, the mentioned cleavage sites are located at the edge of the bacteriocin sequence and if the binding site of the bacteriocins remains intact, the activity is not even influenced. Bromberg et al. (2004) reported similar for one of the bacteriocins investigated in their study. Contrary to trypsin, proteinase K degrades enzymes unspecific but were shown to have a preference for aromatic and hydrophobic amino acids in P1, like other subtilisins (Saenger, 2013). However, this could likely explain the observed inactivation of inhibition zones with proteinase K and the only partly observed inactivation with trypsin.

Table 18: bacteriocins encoded in the genome of *L. curvatus* TMW 1.624. All five putative gene clusters are depicted with their respective amino acid sequences [aa].

bacteriocin	protein sequence [aa]
enterocin NKR-5-3A	LKGSFIMRKFQKLNEQEMKRLMGG
sakacin Tα	MKNVQSLSKEELVLVVGGYTAKQCLQAIGSWGIAGTGAGAAGG PAGAFVGAHVGGIAGSAVCIGGFLGQ
type-A Lantibiotic	MIFKELSEKELQKINGGMAGNSSNFIHKIKGIFTHR
sakacin Q	MQNTKELSVVELQQILGGKRASFGKCVVGAAGLGAGVSGGLW GMAAGGIGGELAYMGANGCL
lanthipeptid class II	MNFQALSRLIVDFVQYAPLQSWPNAVSTQNIC

In summary, *L. curvatus* TMW 1.624 was shown to express bacteriocins active against other *L. curvatus* and *L. sakei* strains. Due to the findings gained in this study, the possible production of hydrogen peroxide adds another factor to the assertiveness of the strain as one of the tested *L. sakei* strains were able to grow when catalase was added to the respective

medium. However, the *L. sakei* strain TMW 1.1239 appeared to be immune to produced bacteriocins of TMW 1.1624 as no inhibition zones were detected using the fast agar spot test. Besides, also the conducted liquid competition studies showed a relative predominance of the strain in co-culture with TMW 1.624 indicating no inhibitory action of TMW 1.624 and its presumed production of bacteriocin(s).

While the molecular nature of the bacteriocins remains to be elucidated by purification and sequencing, the observed bacteriocin-activity likely explains the assertiveness of the strain TMW 1.624 observed in this study and highlights the importance of sausage environment.

Therefore, the production of inhibitory compounds adds another single strain specific strategy of assertiveness to more general strategies of the establishment of putatively multifactorial co-dominance in the occupation of this ecological niche. These findings strongly suggest the production of bacteriocins as a key parameter for the assertiveness of a strain and that bacteriocin production may also play a crucial role not only in liquid fermentations but also in solid-state fermentations like raw sausage fermentation.

As the use of bacteriocins as food additives is widely known and established (Cleveland et al., 2001) and bacteriocins are only harmful to respective susceptible bacteria (Yang et al., 2014), a deployment of *L. curvatus* TMW 1.624 as a future assertive starter organism for the meat and raw sausage industry may be considerable. The production of active bacteriocins may be of additional benefit if they were able to reduce the number of *Listeria ssp*. and other unwanted microorganisms. Bacteriocins produced from LAB are well known to exhibit activity against *L. monocytogenes* (de Souza Barbosa et al., 2015) and Vogel et al. (2010) described *Listeria* spp. as a serious problem in processed foods as the bacterium is able to cope with hurdles during manufacturing such as pH, salt and nitrite concentrations (Vogel et al., 2010). The anti-listerial activity of *L. curvatus* TMW 1.624 and its expressed bacteriocins remains to be evaluated in further studies.

4.5 The role of prophages for the assertiveness of bacteria in raw sausage fermentations

In order to elucidate the impact of prophages on the assertiveness of strains, *L. sakei* and *L. curvatus* genomes were screened for the presence of bacteriophage related proteins and integrated prophage genomes. One prophage of the *L. sakei* strain TMW 1.1398 was chosen for further studies and detailed investigation.

4.5.1 Prophage distribution

The genomes of bacteria are often littered with functional and non-functional viral chromosomes. Those viral genetic information originates from attacks of bacteriophages. (Canchaya et al., 2003; Casjens, 2003). Bacteriophages are viruses, infecting specifically only bacterial cells and can either integrate into the host genome as temperate phages or induce cell lysis and death, as well as the release of new bacteriophages into the environment (Clokie et al., 2011). Integrated and quiescent temperate phages constitute a potential risk and may cause serious problems in food fermentations (Samson and Moineau, 2013). Especially, diary fermentations constantly report partial or total losses due to growth delays or complete growth inhibition of needed LAB in milk acidifications (Garneau and Moineau, 2011; Giraffa et al., 2017; Marcó et al., 2012). So far, it is not investigated whether phage attacks or spontaneous prophage induction play also a role in solid-state fermentation like raw sausage fermentations.

Raw sausage fermentations are known and characterized as solid-state-fermentations (Gould, 2012). Due to the solid matrix, it is commonly assumed that bacteriophages do not play a pivotal role in raw fermented sausages (Champomier-Verges et al., 2001). One study dealing with sour sough as a solid-state fermentation and an *L. sanfransiscencis* phage EV3 reported that the phage is not distributed within the sour dough (Picozzi et al., 2015). However, no reports on raw sausage fermentation and phage distribution are available. Therefore, only little research and literature is available for bacteriophages in meat fermentations (Champomier-Verges et al., 2001). However, an effect of prophages cannot be completely excluded. It is important to note that members of the autochthonous microbiota as well as introduced starter cultures are immobilized in cavities of the sausage mix. As those cavities are limited in size and nutrient availability, the bacteria are in keen competition (Gould, 2012). The release of active bacteriophages by induction of a relatively small subpopulation of the respective starter strain within those cavities may lead to a lysis of other bacteria including all susceptible members of the autochthonous microbiota or other starter cultures. Furthermore, a diffusion of small virus particles cannot be excluded as well. Released prophages may diffuse and reach other bacteria in other cavities as the distance of those cavities or nests varies between only 100 and 5000 µm (Gould, 2012). This could either mean an enormous advantage for the

respective starter in competition for nutrients or a disadvantage when too much of the population becomes induced and lysed.

Recently, different publications reported effects of the presence or absence of prophages on the host physiology and its assertiveness. Several phages encompass genes, which encode enzymes or proteins advantageous for their hosts (Fortier and Sekulovic, 2013). In consequence, it can be assumed that the assertiveness of some strains is influenced on such enzymes or proteins of viral origin. However, both scenarios, where either a positive effect or a negative effect was recorded, are common.

Until now, only little is known about the occurrence and composition of prophages within the genome and prophage encoded traits in *L. curvatus* and *L. sakei* strains. In fact, prophage genomes can make up huge proportions of the total bacterial genome (Canchaya et al., 2003; Casjens, 2003; Srividhya et al., 2007). One of the most extreme cases, reported so far, is the food pathogen *E. coli* 0157:H7 strain *sakai* which encompasses 18 prophage genome elements corresponding to over 16 % of its total genome content belonging to viral DNA (Canchaya et al., 2003; Ohnishi et al., 2001). All of the investigated TMW strains in this work, were found to possess prophages and prophage related genes within their genomes. The completeness of prophage-like elements (47 in total) was estimated by PHASTER, resulting in 17 % intact, 53 % incomplete and 27 % questionable.

Nevertheless, we did not succeed in finding a direct correlation between the presence of intact prophages and the observed assertiveness of the strains described in Chapter 3.2. Indeed, all of the tested *L. sakei* and *L. curvatus* strains possess rudimental genes of bacteriophage origin and it is commonly known that bacteriophages are a major driving force of horizontal gene transfer and bacterial evolution distributing to shaping bacterial communities (Bossi et al., 2003; Fortier and Sekulovic, 2013; Koskella and Meaden, 2013; Schuster et al., 2019).

4.5.2 Prophage characterization and genome organization

Despite their biodiversity and their known role on gene transfer as mobile elements little is known about the vast majority of bacteriophages, their lifecycle and the mechanisms of integration. The low number of fully sequenced and characterized *Lactobacillus* phages can be explained by the huge variety and biodiversity of host and phages. Over 200 species are recognized within the *Lactobacillus* genus indicating a huge host heterogeneity for phages (Mahony and van Sinderen, 2014; Sun et al., 2015). Therefore, it seems unsurprising that phages infecting species of this genus are equally complex and difficult to classify (Mahony and van Sinderen, 2014). To date, phage genomes of 30 *Lactobacillus* phages have been fully sequenced and published (Mahony et al., 2017). Their genetic complexity is also reflected in their reported genome sizes ranging from approximately 31-42 kb (Mahony and van Sinderen,

2014). Despite increasing numbers of reports on dairy LAB bacteriophages, information on the meat LAB phages is only poorly documented so far (Toldrá, 2008). Very few bacteriophages using *L. sakei*, *P. pentosaceus* or L*. plantarum* as hosts were isolated from industrial meat fermentations (Toldrá, 2008).

In this thesis, a prophage of TMW 1.1398 was investigated in detail, named and its role on the assertiveness of the strain was evaluated using a mutant strain of TMW 1.1398, which lost the complete prophage sequence of φ-DJ1812. According to recent available literature 96 % of all described and known phages are tailed phages and the vast majority of *Lactobacillus* phages are classified as *Siphoviridae* (Lopes et al., 2014). The prophage φ-DJ1812 was successfully classified along its integrase as a *Siphoviridae* with a non-contractile tail. However, the annotation of the prophage genome was only partly successful. Phage genes are usually small in size (< 1kb) and only very few of them have been subjected to a detailed biochemical and functional characterization which makes the annotation of phage genomes challenging (Smith et al., 2012). Nevertheless, by the utilization of the phage detector software PHASTER and blast it was possible to get as much as possible detailed information about the genome organization of the temperate phage of TMW 1.1398. All intact prophages must encode genes involved in crucial functions like encapsulation, bacterial lysis, recombination or conjunction that are usually grouped in functional modules. For φ-DJ1812 all necessary gene cassettes were found (see Figure 30). However, the incorporation of a transposon within the gene XY encoding for the major tail protein of the phage may be noticeable as it may significantly influence the expression of the mentioned tail protein. Zou et al. (2000) showed unassembled phages of *S. aureus* PV83 due to the presence of a transposon in a head-to-tail joining gene.

4.5.3 Prophage induction

Temperate, quiescent prophages are usually controlled by a phage repressor, which binds to the phage operator sequences by repressing early promotors controlling the expression of genes involved in the lytic cycle (Fortier and Sekulovic, 2013). The induction of prophage generally requires the proteolytic cleavage of the respective repressor and its displacement (Fortier and Sekulovic, 2013). This therefore is mostly achieved upon activation of the RecA-depended SOS response following DNA damage (Fortier and Sekulovic, 2013). However, the excision and induction of the lytic cycle can be mediated by different external signals and triggers (Wang and Wood, 2016). Two of the most common signals are mitomycin C and UV irradiation (Banks et al., 2003; Barnhart et al., 1976; Berenstein, 1986; Raya and H'Bert E, 2009). Both stressors are commonly used as gold standards for prophage induction experiments (Xu et al., 2018b). Sechaud et al. showed that 23 out of 30 *L. salivarius* strains were lysogenic and produced phage particles after induction with mitomycin C (Sechaud et al., 1988). The total number of inducible prophages within bacterial genomes must be higher due

to the fact that not all signals driving the excision have been discovered yet. However, the induction of φ-DJ1812 was only successful using UV irradiation, whereas the induction with mitomycin C treatment failed. As described above, it would be reasonable to take more external signals into consideration. Also light stressors that may commonly occur during fermentation may be of possible interest. In diary industry, it is common to use several strategies to minimize the risk of a phage outbreaks and dissemination (Garneau and Moineau, 2011; Marcó et al., 2012). Culture rotation programs are applied as well as specifically characterized cultures with reduced phage susceptibly (Marco et al., 2012). Reported phage attacks in diary industry (Giraffa et al., 2017) feed the assumptions that also “light” stressors occurring during normal fermentations under more or less optimal conditions are capable of inducing quiescent phages. So far, it is not clear whether the prophage φ-DJ1812 might be also inducible under standard conditions during raw sausage fermentation. Possible stressors, which should be further investigated, could be high concentrations of nitrate, the low pH, and the limitation of sugar as carbon source, low a_w-values and more.

Successful prophage induction of φ-DJ1812 using UV irradiation was confirmed using different techniques and methods. The results of the LC-MS/MS clearly verified the induction of the prophage using UV irradiation doses of 2 min. Important major phage proteins including head and some minor tail proteins were expressed and detected along this technique. The major tail protein, however, was not detected and strengthens the suggestion of inactive and unassembled phages. The observed cell lysis in growth kinetics can be explained by the expression of the phage lysis cassette including N-acetylmuranyl-L-alanine-amidase even when not all of those lysis enzymes could be detected with LC-MS/MS. The failure of detection using LC-MS/MS may be probably explained due to the detection limit of the machine

At last, field flow fractioning was used to determine and visualize phage components like head and tail. This technique is frequently used to separate larger molecules, aggregates and particles based on their size. Due to the phage induction mediated cell lysis of the culture, it was difficult to detect definable peaks of phage origin. However, with the reduction of the injection volume, it was possible to detect a peak after approximately 13 minutes. By comparison with a purified phage sample of *L. lactis*, the peak could be classified as similar, though corresponding to a smaller size due to its earlier elution. The fitting using the spherical model enabled a prediction on phage head sizes of 70 nm for the phage φ-DJ1812 and 90 nm for *L. lactis*. According to available literature both sizes are within acceptable limits (Comeau et al., 2012). Assembled tail proteins were only found for *L. lactis* with a determined size of 155 nm. The fitting for TMW 1.1398 was not successful and phage tails were not found using the field flow fractioning. This therefore strengthens the assumption of a failure of phage assembly due to the presence of transposase within the major tail protein.

Finally, the conducted electron microscopy verified our assumption that the phage assembly of bacteriophage φ-DJ1812 failed. It was possible to detect and visualize head intermediates, (proheads) of the respective phage. Found proheads differed in size and shape and appeared to be damaged sometimes. Generally, the morphogenesis of the icosahedral head of bacteriophages like φ-DJ1812 can be divided into two main stages: the formation of the prohead and the encapsulation of phage DNA. The formation of the capsid viruses usually requires capsid proteins as well as a scaffolding protein and a portal protein/ connector. It was further reported that most capsids form aberrant structures without the respective scaffolding protein. For example, the capsid proteins T4 and lambda can assemble into long cylindrical structres. In case of DJ1812 it can be assumed that the nessessray scaffolding protein is missing, leading to different structures of the prohead as seen with electron microscopy. The relatively large size of proheads found for DJ1812 can be compared to one described bacteriophage of L. sake, whose phage PWH2 has a similar size.

4.5.4 Impact of prophage φ-DJ1812 on the assertiveness on its host TMW 1.1398

Integrated prophages undergo consistent changes and frequently suffer mutations or partial prophage deletions that destroy genes needed for lytic development (Bossi et al., 2003; Fortier and Sekulovic, 2013). If prophages were modified and defective, they are called “cryptic” (Casjens, 2003; Wang and Wood, 2016). This is also the case for prophage φ-DJ1812 where a transposon was integrated into the major tail protein leading to the assumption that no active and lytic prophages are produced. Indeed, we were able to show that the induction of φ-DJ1812 only led to non-infectious phage particles. Within a bacterial challenge assay it was not possible to induce lysis in other *L. sakei* strains using phage lysate of φ-DJ1812. However, genome annotation with PHASTER revealed that most of the open reading frames correspond to hypothetical proteins, disclosing the diversity of prophage genes and the difficulty in the annotation process. Especially those genes with unknown function and origin are of huge interest. Like described earlier, prophages may confer adaptive features to their host including genes related to virulence, antibiotic resistance cassettes or toxin genes. None of the latter genes were found in the genome of φ-DJ1812. Nevertheless, especially three of those proteins with unknown function at the end of the phage sequence could be the focus for further experiments as the last protein was found to be expressed in an induced culture of TMW 1.1398 (see Table 16). So far it is still unclear whether this is a protein influencing the host bacterium TMW 1.1398 or not. Furthermore, it is not known whether this protein has a positive or negative impact on the fitness of the strain.

In order to gain insights into the mechanisms of prophage mediated assertiveness a prophage cured mutant of TMW 1.1398 was created. We were able to find a prophage-cured mutant of

the strain TMW 1.1398, where the prophage φ-DJ1812 was induced and excised due to the exposure to UV irradiation. After prophage induction, lysis was observed after 2-3 hours. At the end of cell lysis and cell death, the culture was used for plating on mMRS agar to determine the number of survivors. Colonies were picked after two days of anaerobic incubation and were then used for specific colony-PCRs. We found one colony, which was negative for the developed strain specific PCR with the primers 1398_F and 1398_R and positive for the primer pair Gnat_F and Lys_R, which proven the excision of the prophage as the primers were designed for genes located outside from the prophage genome. With the prophage-cured strain, formerly named as TMW 1.2292 comparative competition studies were conducted to uncover the influence of the prophage φ-DJ1812 on the assertiveness of its host TMW 1.1398. Meat batter was inoculated with the respective members of set III and either the prophage containing strain TMW 1.1399 or the mutant strain TMW 1.2292 without the prophage. The results of these experiments clearly showed an enhanced performance of TMW 1.2292 without the prophage φ-DJ1812. These observations collaborate with the work of Selva et al. (2009), where phages decreased the performance of the respective strain. *Staphylococcus aureus* is displaced by its relative *Streptococcus pneumoniae* by the production of hydrogen peroxide via the Fenton reaction in the nasopharynx. The RecA-inducible prophages of *S. aureus* are induced and lead in the following to cell death and finally to the displacement of the strain (Selva et al., 2009). Interestingly, the results do not match growth experiments in mMRS of TMW 1.1398 and its prophage-cured strain TMW 1.2292, where the wildtype strain were characterized by a faster growth and respective higher growth rates. However, as described for the determination of physiological markers in Chapter 3.3, mMSM medium may provide more reliable information about the strains performance in a sausage-like environment.

However, prophages can be a selective advantage due to the transportation of specific genes, which encodes enzymes or proteins, which open up new metabolic pathways or enzymes, which enhance stress responses (Canchaya et al., 2003). Many phages associated with virulent strains encode powerful toxins, effector proteins participating in invasion or various enzymes such as superoxide dismutase, enhancing the tolerance to oxidative stress, or more general proteins increasing the resistance to superantigens, proteinases and mitogenic factors (Fortier and Sekulovic, 2013). Wang et al. (2010) reported a positive impact of prophages on the fitness of *E. coli* K-12. They deleted nine cryptic prophages, which were unable to propagate into infectious virus particles. They were able to show decreased growth rate and a greater sensitivity to antibiotics for the prophage-cured strain as well as the inability to adapt to osmotic stress and verifiable decreases in biofilm formation (Wang et al., 2010). Moreover, it was shown for *Salmonella enterica* serovar Thypimurium, which harbors four to five full-size prophages, that a spontaneous prophage induction of a small percentage of the culture can be a selective advantage when co-cultured with other strains of different origin or strains which

are prophage-cured (Nanda et al., 2015). Due to the immunity protein (repressor), the strains are not attacked by released prophages, whereas other susceptible strains, not carrying the type of prophage, are attacked and lysed. Moreover, Alexeeva et al. (2018) conducted very similar experiments to those conducted in this study with *L. lactis* strains. Competition experiments with GFP and mCherry labeled wildtype and cured strains revealed a positive impact of prophages.

In conclusion, no general statements about the effect of prophages within bacterial genomes on the fitness and assertiveness of a strain are possible. However, without the clear presence of advantageous gene clusters encoding for enzymes or proteins increasing the fitness, it can be assumed that prophages are a metabolic burden as the bacterium has to invest energy in form of ATP and nucleosides for the replication of the prophage genome. Lawrence et al. (2001) considered prophages dangerous “molecular time bombs” (Lawrence et al., 2001; Paul, 2008). The possibility of eventual induction of a relatively high percentage of the whole population overshadowed the benefits of possible selective advantages. Lawrence stated that this should favor mutations, leading to the inactivation of the induction processes or even large scale deletions, in order to reduce the metabolic burden (Lawrence et al., 2001). These mutations and partial deletions are reflected by the genomes of *L. sakei* and *L. curvatus* strains investigated in this study. Using PHASTER, we found many incomplete or questionable prophage regions within the genomes, which may be explained by these mutations. For the as intact classified prophage φ-DJ1812 of the *L. sakei* strain TMW 1.1398, no genes could be identified, which are clearly associated with enzymes or proteins enhancing the assertiveness and fitness of the strain. With regard to this observations, the results of the competition studies may be reasonable. The strain, carrying the respective prophage was not able to express the same dominance as its prophage-cured version supporting the metabolic burden theory. However, we found many genes within the annotated prophage genome of unknown function, which were not able to match current database entries. Though, this does not inevitable mean that these genes do not affect the fitness of the host.

4.5.5 Spontaneous prophage induction and circularization of φ-DJ1812

The spontaneous prophage excision (SPE) within single cells of a bacterial population without the clear presence of known external triggers is a widely unnoted phenomenon but has been described first in the early 50s of the 20th century (Alexeeva et al., 2018; Lwoff, 1953). It is commonly reported, that the spontaneous induction is linked to the activation of an SOS response system (Alexeeva et al., 2018). As well as the integration, the excision is a site-specific recombination and requires not only the integrase but an additional phage-encoded protein called excinionase (Cho et al., 2002; Liu et al., 2015). Several studies reported that the excisionase binds to the integrase thus enhancing the recognition of prophage attachment

sites. Once bound to these sites the integrase cuts the sequence and recreates the attachment sites leading to an excision of the whole prophage sequence.

In this work we were able to show that prophage excision is rather a constitute process, where a small percentage of the population constitutively undergoes an excision. Prophage excision is not necessarily dependent on strong stressors like UV irradiation or mitomycin C treatment inducing the SOS response system. The results on PCR assays using specific primer pairs (see Chapter 3.5.9) clearly show, that parts of the population of TMW 1.1398 are characterized by a permanent excision of the prophage without prior external induction. The prophage φ-DJ1812 verifiable undergoes circularization, which was demonstrated along PCR and gel electrophoresis. After a successful excision and prior to the proven circularization, it can be assumed that the prophage genome exists in a linear intermediate form. Unfortunately, this intermediate cannot be visualized with standard PCR methods. According to theory, the prophage is excised at the determined att-sites attL and attR, which promotes and performs the latter circularization. The sequencing of PCR products revealed the excision sites. The alignment of theoretical excision and circularization sequence and the actual sequence of the PCR product with Clustal Omega confirmed the correctness of the theory. In line with the theory, only one att-site remained after excision. The integrative recombination between the specific attachment site attP on the phage DNA and attB on the bacterial chromosome, generates the recombinant attR and attL sites flanking the prophage DNA. After excision, the recombinant att-sites were reversed (Cho et al., 2002). Interestingly, this spontaneous excision does not necessarily lead to an observable cell lysis of the culture. In the contrary, the prophage is excised and seemed to remain quiescent. Only few studies have so far focused on the exact mechanisms underlying this phenomenon. Some studies investigated the spontaneous prophage induction (SPI) and argued that this phenomenon could result from fluctuations in the levels of repressor proteins, where lytic genes are expressed when a certain threshold was not reached (Broussard et al., 2013). However, more signals may be of interest for further studies. The spontaneously excised prophage undergoes a circularization and remains as an extrachromosomal plasmid. Other authors reported similar observations, where prophages remain linear or as circular plasmids (Casjens et al., 2004; Heinrich et al., 1995; Ravin, 2011). With a specifically designed PCR we were able to verify these statements and observations. The regulation behind SPI phenomenon and the relevance in bacterial population remains to be elucidated.

4.6 Correlation analysis – markers for the assertiveness of *L. sakei* and *L. curvatus*

One of the main aims of this work was to identify possible markers for the assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations. Unlike the initial hypothesis, we did not succeed in finding predicted physiological markers, which connect to the observed assertiveness in this study. Neither the stress response to cold-stress or oxidative stress nor growth dynamics in standard mMRS medium revealed distinct correlations. However, the adaptation to the environment meat, imitated by the development of a modified meat simulation medium, enabled a slight correlation of the growth parameter growth rate µ.

Nearly all dominant strains were characterized by significant high growth rates, except for two *L. curvatus* strains TMW 1.439 and TMW 1.624. Both strains, are characterized as special as they use other strategies to achieve dominance and assertiveness. *L. curvatus* TMW 1.439, appeared to be dependent on the presence of its “partner-strain” TMW 1.1390 as it was displaced in fermentations were the latter was omissed. It can be speculated that TMW 1.439 acts as a sponger or social cheater. *L. curvatus* TMW 1.624 was characterized as average regarding its growth dynamics, suggesting a different strategy in assertiveness as the strain is able to outcompete all other strains investigated in this study. By detailed characterization it was possible to trace its assertiveness back to the production of potent bacteriocins, which inhibit the growth of the other competitors/starter cultures. These findings strongly suggest bacteriocin production as a major factor in competitive exclusion and influencing the assertiveness of bacteria. Moreover, we were able to show that prophages affect the assertiveness of strains within sausage fermentations. TMW 1.1398 and its prophage-cured mutant strain TMW 1.2292 were shown to be significantly different in their assertiveness during sausage fermentations. Finally, it has to be stated that assertiveness and dominance cannot necessarily be traced back to single strain characteristics. In fact, assertiveness is multifactorial and has to be investigated individually for the respective starter culture or strain of interest. Nevertheless, growth dynamics in modified meat simulation medium can be implemented as a first line basis as the vast majority of dominant strains were shown to exhibit high growth rates.

Figure 50: Word-Cloud of multifactorial-based assertiveness in sausage fermentations.

5 Summary

Raw fermented sausages, e.g. salami, are consumed raw and without prior heating. The product harbors autochthonous microbiota, which harbors potential risks for the consumer. Despite the implemented hurdle technology and the use of starter cultures in order to reduce the risks emerging from members of the autochthonous microbiota, it is unclear whether the introduced starters were able to prevail during fermentation and safely dominate the microbiota. Studies, investigating the fate of individual strain are completely missing. Since detailed studies are completely missing, there is a substantial lack of knowledge regarding the assertiveness of starter cultures. Furthermore, the mechanisms and strategies enabling assertiveness were fundamentally neglected so far.

The main aim of this study was to probe the assertiveness of single starter strains in sausage fermentation. Therefore, MALDI-TOF MS should be developed and implemented as a reliable high-throughput method to track introduced starter cultures during fermentation not only on species- but on strain level. Furthermore, the assertiveness of *L. sakei* and *L. curvatus* strains should be investigated and possible markers with special emphasis to physiological characteristics should be identified. The implementation of an in-house database containing the MSPs of all strains of interest, enabled the use of MALDI-TOF MS as an adequately suitable, high-throughput technique for the monitoring of *L. sakei* and *L. curvatus* on strain level. The recognition rates, however, depended on the strain of interest and the applied incubation time as sub proteome mass pattern tended to be less distinguishable with prolonged incubation times. The results illustrate how crucial standardized preparation procedures are in order to guarantee the reliability of the method. In order to depict the assertiveness in sausage fermentation, a fermenting sausage model system, imitating the conditions during raw sausage fermentation, was successfully implemented. The model was shown to be adequate for the first 5 days of fermentation as no drying could be provided.

In the following, the fermented sausage model in combination with MALDI-TOF MS enabled the determination of the assertiveness of the strains used in this study. Upon competition studies in defined sets, the most dominant strains were determined and selected. Interestingly, two strategies in dominance could been observed. The first strategy is based on the competitive exclusion principle, leading to the extinction of competitors while one strain is able to dominate and assert. The second strategy is the establishment of colonization resistance, which can be considered as a consequence of competitive exclusion. Both strategies were observed within both species. The conduction of competition experiments with the omission of the previously dominant strains, revealed further insights into the strategies in assertiveness.

The principle of colonization resistance by co-dominance appeared to be of remarkably importance as this phenomenon was widely observed. In fact, we were able to detect even possible co-operational if not mutualistic traits between some of the strains. These findings were successfully verified by the conduction of ring trial experiments with industrial partners from Germany. The results clearly showed that the first 5 days are sufficient with regard to the development of lactobacilli in a pilot scale sausage ripening setting including the ripening i.e. drying process.

In order to gain insights not into intraspecies but interspecies assertiveness, the strains of both species had to compete against another. These kind of experiments revealed the predominance of *L. sakei* over *L. curvatus*. It can be assumed that the assertiveness is based on the widely reported adaptation of *L. sakei* to the environment meat. In fact, we were able to demonstrate higher stress response to oxidative stress as well as the development of higher growth rates in comparison to *L. curvatus* in the implemented modified meat simulation medium. One of the most promising explanations for this observation may be manifested in the expression of ADI pathway in *L. sakei*. This, however, does not only enable a neutralization of increasing acid levels in the environment but contributes to the generation of energy. The latter can also likely explain the observed fitness expressed by differences in growth rates. However, the predominance of *L. sakei* was only valid when *L. curvatus* TMW 1.624 was excluded. By the inclusion of the latter, the outcome of competition studies changed completely. The strain was able to dominate all sets tested in this study. The strain was further investigated with special emphasis on bacteriocin formation, which is known to be one major factor driving competitive exclusion.

By the use of BAGEL3 it was possible to determine in total 5 possible putative bacteriocin clusters. This accumulation of clusters may be indicative for the role in the assertiveness of the strain. In fact, along this study we were able to demonstrate the production of potent bacteriocins, active not only against *L. curvatus* but *L. sakei*. These findings strongly suggest a decisive role for bacteriocins in the assertiveness of strains also in sausage fermentation.

The investigation of possible physiological markers, corresponding to the assertiveness was not successful. Neither stress response to oxidative stress or cold stress nor the determination of growth characteristics allowed general statements. The most promising approach was the determination of growth dynamics in the modified meat simulation medium. All dominant strains were shown to exhibit the highest growth rates in the respective medium. The only exceptions of this observation were made for the *L. curvatus* strains TMW 1.439 and TMW 1.624. Both exceptions are easily explained by the performance of other strategies. *L. curvatus*

TMW 1.624 is not of outstanding character concerning growth characteristics but harbor other potent weapons as it produces bacteriocins, enabling its assertiveness. *L. curvatus* TMW 1.439 seems to profit from the presence of *L. curvatus* TMW 1.1390 and was shown to be not able to grow in absence of its partner (sponger theory). In consequence and as a first approximation for the assertiveness in sausage fermentation, the investigation of growth dynamics in mMSM seems considerable.

Lastly, we were able to demonstrate the role of prophages on bacterial assertiveness. Besides widely described positive impacts, a prophage-cured strain of *L. sakei* TMW 1.1398 showed a significant better performance in conducted competition experiments than its respective wildtype strain. The prophage was intensively characterized and studied. We were able to report its inducibility and demonstrate the release of phage proteins using LC-MS/MS.

Taken together, the studies of the present thesis provide extensive insights into the assertiveness of *L. sakei* and *L. curvatus* in sausage fermentations. The exploration of strain-specific strategies in assertiveness represents a considerable progress of knowledge of these technologically relevant starter organisms with implications for a strategic selection of new prospective starter cultures and recommended applications for sausage production with combinations of strains rather than single starters.

6 Zusammenfassung

Rohwurst wie Salami wird ohne vorangegangenes Erhitzen verzehrt und enthält interne Kontaminanten, so genannte autochthone Mikroorganismen, die für den Konsumenten von potentieller Gefahr sind. Trotz der Implementierung der Hürdentechnologie sowie des Einsatzes von Starterkulturen, gelingt es nicht immer, die autochthone Mikrobiota vollends zu unterdrücken. Weiterhin ist es unklar, ob sich industrielle Starterkulturen nach ihrer Zugabe tatsächlich in der Rohwurst durchsetzen und diese dominieren. Studien, die sich mit dem individuellen Schicksal einer solchen Starterkultur beschäftigen sind rar und an intensiven detaillierten Beschreibungen fehlt es nahezu komplett. Darüber hinaus wurden die zugrundeliegenden Strategien der Durchsetzungsfähigkeit und Dominanz in Rohwurst weitgehend vernachlässigt. Dies ist vermutlich dem Umstand geschuldet, dass es kaum Methoden und Techniken gibt, die das Verfolgen von Starterkulturen auf Stammebene zulassen.

Das Hauptziel der Arbeit lag vor allem darin, die Durchsetzungsfähigkeit von Starterkulturen in Rohwurstfermentationen zu untersuchen. Hierfür wurde MALDI-TOF-MS als zuverlässige Hochdurchsatz-Methode für ein Verfolgen der Stämme implementiert und validiert. Weiterhin sollte die Durchsetzungsfähigkeit der *L. sakei* und *L. curvatus* Stämme mit potenziellen physiologischen Markern erklärt werden. Dank der implementierten „in-house“ Datenbank, welche die kommerziell erhältliche Datenbank von Bruker erweitert, konnten stammspezifischen Erkennungsraten der Spezies determiniert werden. Diese zeigten sich jedoch stark von dem Alter der Kulturen sowie von dem eingesetzten Stamm abhängig. Die erzielten Ergebnisse verdeutlichen, wie wichtig standardisierte Aufbereitung für die Zuverlässigkeit der Methode ist.

Mittels eines implementierten Rohwurstmodellsystems gelang es weiterhin, in Kombination mit MALDI-TOF MS, die Durchsetzungsfähigkeit der Stämme zu determinieren. Das Modell zeigte sich für die ersten 5 Tage der Fermentation verlässlich, da keine Trocknung abgebildet werden konnte. *L. sakei* und *L. curvatus* Stämme wurden in definierten Sets auf ihre Durchsetzungsfähigkeit hin überprüft. Das Modellsystem konnte durch die anschließende Durchführung von Ringversuchen in Kooperation mit Industriepartnern des Projektes verifiziert werden. Hierbei zeigte sich, dass für die Entwicklung der Laktobazillen eine Verfolgung über 5 Tage durchaus als ausreichend einzustufen ist. Auch hier zeigten sich die als dominant ausgewiesenen Stämme als durchsetzungsfähig. Insgesamt konnten zwei unterschiedliche Strategien nachgewiesen werden, die es den Stämmen erlauben eine Dominanz auszubilden. Die erste Strategie besteht darin, seine Konkurrenten zu überwachsen und diese vollständig

zu inhibieren, während die zweite Strategie in einer Ko-Dominanz begründet liegt. Hier gelingt es nicht nur einem Stamm, sondern mehreren Stämmen sich zu etablieren und so zu einer Kolonisierungsresistenz im Fleisch führen. Weitere Experimente ohne die zuvor durchsetzungsfähigen Stämme zeigten im Anschluss, dass gerade die Strategie der Ko-Dominanz vielfach verbreitet ist.

Um nicht neben der intraspezies-bedingten auch die interspezies-bedingte Durchsetzungsfähigkeit zu untersuchen, wurden die *L. curvatus* und *L. sakei* Sets miteinander kombiniert. Die Ergebnisse verwiesen hier auf eine deutliche Dominanz der *L. sakei* Stämme. Diese Dominanz lässt sich vermutlich durch die vielfach beschriebene Anpassung der Spezies *L. sakei* an die ökologische Nische Fleisch erklären. Tatsächlich konnte eine erhöhte Stressresistenz gegenüber oxidativen Stress sowie die Entwicklung von höheren Wachstumsraten in einem implementierten modifizierten Fleischsimulationsmedium feststellen. Eine der vielversprechendsten Erklärungen für diese Beobachtung könnte sich in der Expression des ADI Stoffwechselweges manifestieren, der für die *L. curvatus* Stämme nicht beschrieben und gefunden werden konnte. Der ADI Stoffwechselweg ermöglicht hierbei nicht nur die Neutralisierung im sauren Milieu, sondern trägt auch zur Energiegewinnung bei, was letztlich durch erhöhte Wachstumsraten sichtbar gemacht werden konnte. Die Dominanz der Spezies *L. sakei* konnte jedoch nur so lange beobachtet werden bis der *L. curvatus* Stamm TMW 1.624 in die Experimente inkludiert wurde. Durch die Inklusion des *L. curvatus* TMW 1.624 änderte sich das Ergebnis der Durchsetzungsstudien drastisch. Der *L. curvatus* Stamm war in der Lage, alle anderen Stämme dieser Arbeit zu überwachsen und jede Fermentation zu dominieren.

Diese Dominanz war so signifikant, dass es weiterer Untersuchungen bedurfte. Der Schwerpunkt anschließender Untersuchungen lag dabei auf der Bakteriozinbildung. Letztere wurde vielfach als eines der zentralen Elemente des „competitive exclusion“ Prinzips beschrieben. Mittels BAGEL3 konnten fünf mögliche Bacteriozin-Cluster im Genom des Stammes identifiziert werden. Diese Ansammlung von Bakteriozinen war dabei ein erster Hinweis auf die Bedeutung in Hinsicht auf die Durchsetzungsfähigkeit des Stammes. Im Folgenden gelang es, die Produktion von potenten Bakteriozinen nachzuweisen, die nicht nur gegen *L. curvatus,* sondern auch gegen die Spezies *L. sakei* wirksam sind. Die Ergebnisse legen demnach nahe, dass antimikrobiell-wirksame Bakteriozine eine zentrale und wichtige Rolle bei der Ausprägung von Dominanz und Durchsetzungsfähigkeit spielen.

Die Suche nach potentiellen Markern, welche die Durchsetzungsfähigkeit erklären und Voraussagen erlauben, blieb jedoch erfolglos. Weder Stressresistenz noch das generelle

Wachstum erlauben allgemeine Aussagen. Der vielversprechende Ansatz war die Determinierung von Wachstumsdynamiken in einem modifizierten Fleischsimulationsmedium. Nahezu alle im Rohwurstmodell dominanten Stämme zeigten hier erhöhte Wachstumsraten und damit einhergehende hohe optische Dichten. Die einzigen Ausnahmen, stellten die zuvor als dominant klassifizierten *L. curvatus* Stämme TMW 1.439 und TMW 1.624 dar. Diese Ausnahmen lassen sich jedoch relativ leicht erklären, da besagte Stämme andere Durchsetzungsstrategien verfolgen. *L. curvatus* TMW 1.439 zeigte sich in Durchsetzungsversuchen abhängig von seinem determinierten Partner-Stamm *L. curvatus* TMW 1.1390. Die Vermutung liegt nahe, dass es sich hierbei um eine tatsächliche Kooperation handelt. *L. curvatus* TMW 1.624 hingegen prägt seine Durchsetzungsfähigkeit über Bakteriozine aus, die seine Konkurrenten inhibieren und seine Defizite im Wachstum aufwiegen. Zusammenfassend lässt sich festhalten, dass für erste Näherungen die Untersuchung von Wachstumsdynamiken im Fleischsimulationsmedium zulässig ist.

Zuletzt konnten wir in dieser Arbeit den Einfluss von bakteriellen Prophagen auf die Fitness und Durchsetzungsfähigkeit eines Stammes aufzeigen. Neben dem vielfach beschriebenen positiven Einfluss, zeigte der Prophagen-kurierte *L. sakei* Stamm TMW 1.2292 ein deutlich höheres relatives Auftreten und damit eine erhöhte Durchsetzungsfähigkeit als sein Wildtyp Stamm TMW 1.1398. Der Prophage wurde intensiv charakterisiert und untersucht. Wir konnten die Induzierbarkeit des Phagen sowie die Freisetzung von Phagenproteinen mittels LC-MS/MS nachweisen.

Zusammengefasst bieten die Studien der vorliegenden Arbeit umfangreiche Einblicke in die Durchsetzungsfähigkeit von *L. sakei* und *L. curvatus* in Rohwurstfermentationen. Die Erforschung stamm-spezifischer Strategien der Durchsetzungsfähigkeit und Dominanz stellen einen wesentlichen Erkenntnisfortschritt dar, der sich auf die strategische Auswahl neuer potentieller Starterkulturen in der Industrie auswirkt. Weiterhin ist es möglich, dass industrielle Fermentationen, die bisher nur mittels einer Starterkultur realisiert wurden, nun auf eine dominante Kombination umstellen. Es ist denkbar, dass das etablierte Modellsystem auch für die Unternehmen der Fleischwarenindustrie oder für Starterkulturhersteller von außerordentlichen Nutzen sein kann.

7 Outlook

The studies of the present thesis provide extensive insights into the assertiveness of *L. sakei* and *L. curvatus* in sausage fermentations. The exploration of strain-specific strategies in assertiveness represents a considerable progress of knowledge of these technologically relevant starter organisms with implications for a strategic selection of new prospective starter cultures and recommended applications for sausage production with combinations of strains rather than single starters.

This knowledge opens new perspectives and forms a good base for further detailed studies with special emphasis to bacteriocin-mediated assertiveness and prophages as central elements shaping the dominance of strains.

8 References

Adegocke, G.O., Olapade, A.A., 2012. Preservation of Plant and Animal Foods: An Overview., in: R. Bhat, A.K.A.a.G.P. (Ed.), Progress in Food Preservation.

Adzitey, F., Huda, N., Ali, G.R.R., 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. 3 Biotech 3, 97-107.

Adzitey, F., Rusul, G., Huda, N., Cogan, T., Corry, J., 2012. Prevalence, antibiotic resistance and RAPD typing of Campylobacter species isolated from ducks, their rearing and processing environments in Penang, Malaysia. International journal of food microbiology 154, 197-205.

Aertsen, A., Faster, D., Michiels, C.W., 2005. Induction of Shiga toxin-converting prophage in Escherichia coli by high hydrostatic pressure. Appl Environ Microbiol 71, 1155-1162.

Albano, H., van Reenen, C.A., Todorov, S.D., Cruz, D., Fraga, L., Hogg, T., Dicks, L.M., Teixeira, P., 2009. Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from "Alheira", a traditional fermented sausage produced in Portugal. Meat Sci 82, 389-398.

Alexeeva, S., Martínez, J.A.G., Spus, M., Smid, E.J., 2018. Spontaneously induced prophages are abundant in a naturally evolved bacterial starter culture and deliver competitive advantage to the host. BMC microbiology 18, 120.

Alonso, R., Martin, A., Pelaez, T., Marin, M., Rodriguez-Creixems, M., Bouza, E., 2005. An improved protocol for pulsed-field gel electrophoresis typing of Clostridium difficile. Journal of medical microbiology 54, 155-157.

Alvarez-Sieiro, P., Montalban-Lopez, M., Mu, D., Kuipers, O.P., 2016. Bacteriocins of lactic acid bacteria: extending the family. Appl Microbiol Biotechnol 100, 2939-2951.

Ammor, M.S., Mayo, B., 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci 76, 138-146.

Ammor, S., Dufour, E., Zagorec, M., Chaillou, S., Chevallier, I., 2005. Characterization and selection of Lactobacillus sakei strains isolated from traditional dry sausage for their potential use as starter cultures. Food Microbiology 22, 529-538.

Anhalt, J.P., Fenselau, C., 1975. Identification of bacteria using mass spectrometry. Analytical Chemistry 47, 219-225.

Aquilanti, L., Santarelli, S., Silvestri, G., Osimani, A., Petruzzelli, A., Clementi, F., 2007. The microbial ecology of a typical Italian salami during its natural fermentation. Int J Food Microbiol 120, 136-145.

Arendt, E.K., Ryan, L.A., Dal Bello, F., 2007. Impact of sourdough on the texture of bread. Food Microbiol 24, 165-174.

Arnold, R.J., Reilly, J.P., 1998. Fingerprint matching of E. coli strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. Rapid Commun Mass Spectrom 12, 630-636.

Azevedo, A.C., Bento, C.B., Ruiz, J.C., Queiroz, M.V., Mantovani, H.C., 2015. Distribution and Genetic Diversity of Bacteriocin Gene Clusters in Rumen Microbial Genomes. Appl Environ Microbiol 81, 7290-7304.

Aznar, R., Chenoll, E., 2006. Intraspecific diversity of Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus sakei, and Leuconostoc mesenteroides associated with vacuum-packed meat product spoilage analyzed by randomly amplified polymorphic DNA PCR. J Food Prot 69, 2403-2410.

Baird-Parker, A.C., 1962. An improved diagnostic and selective medium for isolating coagulase positive staphylococci. Journal of Applied Microbiology 25, 12-19.

Banks, D.J., Lei, B., Musser, J.M., 2003. Prophage induction and expression of prophage-encoded virulence factors in group A Streptococcus serotype M3 strain MGAS315. Infect Immun 71, 7079-7086.

Barefoot, S.F., Klaenhammer, T.R., 1983. Detection and activity of lactacin B, a bacteriocin produced by Lactobacillus acidophilus. Applied and environmental microbiology 45, 1808-1815.

Barnhart, B., Cox, S., Jett, J., 1976. Prophage induction and inactivation by UV light. Journal of virology 18, 950-955.

Behr, J., Geissler, A.J., Schmid, J., Zehe, A., Vogel, R.F., 2016. The Identification of Novel Diagnostic Marker Genes for the Detection of Beer Spoiling Pediococcus damnosus Strains Using the BlAst Diagnostic Gene findEr. PLoS One 11, e0152747.

Beijerinck, M.W., 1901. Sur les ferments lactiques de l´industrie. Arch. Néer Sci. 6, 212-143.

Belay, N., Rasooly, A., 2002. Staphylococcus aureus growth and enterotoxin A production in an anaerobic environment. J Food Prot 65, 199-204.

Bendall, J., 1973. Postmortem changes in muscle. The structure and function of muscle 2, 243-309.

Berends, B., Van Knapen, F., Snijders, J., Mossel, D., 1997. Identification and quantification of risk factors regarding Salmonella spp. on pork carcasses. International journal of food microbiology 36, 199-206.

Berenstein, D., 1986. Prophage induction by ultraviolet light in Acinetobacter calcoaceticus. Microbiology 132, 2633-2636.

Bernardeau, M., Guguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol Rev 30, 487-513.

Berry, E.D., Liewen, M.B., Mandigo, R.W., Hutkins, R.W., 1990. Inhibition of Listeria monocytogenes by Bacteriocin-Producing Pediococcus During the Manufacture of Fermented Semidry Sausage. Journal of Food Protection 53, 194-197.

Berthier, F., Ehrlich, S.D., 1999. Genetic diversity within Lactobacillus sakei and Lactobacillus curvatus and design of PCR primers for its detection using randomly amplified polymorphic DNA. Int J Syst Bacteriol 49 Pt 3, 997-1007.

BLE, 2018. Bericht zur Markt- und Versorgungslage Fleisch 2018.

Bleuven, C., Landry, C.R., 2016. Molecular and cellular bases of adaptation to a changing environment in microorganisms. Proc Biol Sci 283.

Boor, K.J., 2006. Bacterial stress responses: what doesn't kill them can make then stronger. PLoS Biol 4, e23.

Borch, E., Kant-Muermans, M.L., Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. Int J Food Microbiol 33, 103-120.

Bossi, L., Fuentes, J.A., Mora, G., Figueroa-Bossi, N., 2003. Prophage contribution to bacterial population dynamics. Journal of bacteriology 185, 6467-6471.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., 2012. Food fermentations: microorganisms with technological beneficial use. International journal of food microbiology 154, 87-97.

Bowman, A.S., Glendening, C., Wittum, T.E., LeJeune, J.T., Stich, R.W., FUNK, U.A., 2007. Prevalence of Yersinia enterocolitica in different phases of production on swine farms. Journal of Food Protection 70, 11-16.

Bromberg, R., Moreno, I., Zaganini, C.L., Delboni, R.R., Oliveira, J.d., 2004. Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. Brazilian Journal of Microbiology 35, 137-144.

Brooijmans, R., Smit, B., Santos, F., Van Riel, J., de Vos, W.M., Hugenholtz, J., 2009. Heme and menaquinone induced electron transport in lactic acid bacteria. Microb Cell Fact 8, 28.

Broussard, G.W., Oldfield, L.M., Villanueva, V.M., Lunt, B.L., Shine, E.E., Hatfull, G.F., 2013. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Molecular cell 49, 237-248.

BVDF, 2017. Wirtschaftliche Entwicklung des deutschen Fleischsektors - Überblick, https://www.bvdf.de/presse/mgv2017_pressemeldung/.

Cabiscol Català, E., Tamarit Sumalla, J., Ros Salvador, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiology, 2000, vol. 3, núm. 1, p. 3-8.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J.-P., Ravanat, J.-L., Sauvaigo, S., 1999. Hydroxyl radicals and DNA base damage. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 424, 9-21.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W., Nisbet, D.J., 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim Health Res Rev 9, 217-225.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A., Brussow, H., 2003. Prophage genomics. Microbiol Mol Biol Rev 67, 238-276, table of contents.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P., 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. International Dairy Journal 14, 835-847.

Casjens, S., 2003. Prophages and bacterial genomics: what have we learned so far? Molecular microbiology 49, 277-300.

Casjens, S.R., Gilcrease, E.B., Huang, W.M., Bunny, K.L., Pedulla, M.L., Ford, M.E., Houtz, J.M., Hatfull, G.F., Hendrix, R.W., 2004. The pKO2 linear plasmid prophage of Klebsiella oxytoca. Journal of bacteriology 186, 1818-1832.

Chaillou, S., Champomier-Verges, M.C., Cornet, M., Crutz-Le Coq, A.M., Dudez, A.M., Martin, V., Beaufils, S., Darbon-Rongere, E., Bossy, R., Loux, V., Zagorec, M., 2005. The complete genome sequence of the meat-borne lactic acid bacterium Lactobacillus sakei 23K. Nat Biotechnol 23, 1527-1533.

Chaillou, S., Daty, M., Baraige, F., Dudez, A.M., Anglade, P., Jones, R., Alpert, C.A., Champomier-Verges, M.C., Zagorec, M., 2009. Intraspecies genomic diversity and natural population structure of the meat-borne lactic acid bacterium Lactobacillus sakei. Appl Environ Microbiol 75, 970-980.

Chaillou, S., Lucquin, I., Najjari, A., Zagorec, M., Champomier-Verges, M.C., 2013. Population genetics of Lactobacillus sakei reveals three lineages with distinct evolutionary histories. PLoS One 8, e73253.

Champomier-Verges, M.C., Chaillou, S., Cornet, M., Zagorec, M., 2001. Lactobacillus sakei: recent developments and future prospects. Res Microbiol 152, 839-848.

Chen, F., Wang, K., Stewart, J., Belas, R., 2006. Induction of multiple prophages from a marine bacterium: a genomic approach. Appl Environ Microbiol 72, 4995-5001.

Chen, H., Hoover, D., 2003. Bacteriocins and their food applications. Comprehensive reviews in food science and food safety 2, 82-100.

Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L., Brüssow, H., 2004. Phage-host interaction: an ecological perspective. Journal of bacteriology 186, 3677-3686.

Cho, E.H., Gumport, R.I., Gardner, J.F., 2002. Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. Journal of bacteriology 184, 5200-5203.

Clark, A.E., Kaleta, E.J., Arora, A., Wolk, D.M., 2013. Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clinical microbiology reviews 26, 547-603.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71, 1-20.

Clokie, M.R., Millard, A.D., Letarov, A.V., Heaphy, S., 2011. Phages in nature. Bacteriophage 1, 31-45.

Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C., Comi, G., 2006. Dynamics and characterization of yeasts during natural fermentation of Italian sausages. FEMS Yeast Res 6, 692-701.

Colwell, R.R., 2000. Viable but nonculturable bacteria: a survival strategy. J Infect Chemother 6, 121-125.

Comeau, A.M., Tremblay, D., Moineau, S., Rattei, T., Kushkina, A.I., Tovkach, F.I., Krisch, H.M., Ackermann, H.-W., 2012. Phage morphology recapitulates phylogeny: the comparative genomics of a new group of myoviruses. PLoS One 7, e40102.

Condon, S., 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiology Reviews 46, 269-280.

Cotter, P.D., Hill, C., Ross, R.P., 2005. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3, 777-788.

Culotta, V.C., Yang, M., O'Halloran, T.V., 2006. Activation of superoxide dismutases: putting the metal to the pedal. Biochim Biophys Acta 1763, 747-758.

Czyz, A., Los, M., Wrobel, B., Wegrzyn, G., 2001. Inhibition of spontaneous induction of lambdoid prophages in Escherichia coli cultures: simple procedures with possible biotechnological applications. BMC biotechnology 1, 1.

Dainty, R.H., Edwards, R.A., Hibbard, C.M., Marnewick, J.J., 1989. Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperatures. J Appl Bacteriol 66, 281-289.

Dallal, M.M.S., Doyle, M.P., Rezadehbashi, M., Dabiri, H., Sanaei, M., Modarresi, S., Bakhtiari, R., Sharifiy, K., Taremi, M., Zali, M.R., 2010. Prevalence and antimicrobial resistance profiles of Salmonella serotypes, Campylobacter and Yersinia spp. isolated from retail chicken and beef, Tehran, Iran. Food control 21, 388-392.

Dave, D., Ghaly, A.E., 2011. Meat spoilage mechanisms and preservation techniques: a critical review. American Journal of Agricultural and Biological Sciences 6, 486-510.

De Boer, E., Zwartkruis-Nahuis, J., Wit, B., Huijsdens, X., De Neeling, A., Bosch, T., Van Oosterom, R., Vila, A., Heuvelink, A., 2009. Prevalence of methicillin-resistant Staphylococcus aureus in meat. International journal of food microbiology 134, 52-56.

De Filippis, F., La Storia, A., Villani, F., Ercolini, D., 2013. Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing. PLoS One 8, e70222.

de Jong, A., van Hijum, S.A., Bijlsma, J.J., Kok, J., Kuipers, O.P., 2006. BAGEL: a web-based bacteriocin genome mining tool. Nucleic acids research 34, W273-W279.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology 23, 130-135.

de Souza Barbosa, M., Todorov, S.D., Ivanova, I., Chobert, J.M., Haertle, T., de Melo Franco, B.D.G., 2015. Improving safety of salami by application of bacteriocins produced by an autochthonous Lactobacillus curvatus isolate. Food Microbiol 46, 254-262.

De Vuyst, L., Leroy, F., 2007. Bacteriocins from lactic acid bacteria: production, purification, and food applications. J Mol Microbiol Biotechnol 13, 194-199.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H.M., Weckx, S., 2014. Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol 37, 11-29.

Delgado, C.L., 2003. Rising consumption of meat and milk in developing countries has created a new food revolution. J Nutr 133, 3907S-3910S.

Delwiche, E., 1961. Catalase of Pediococcus cerevisiae. Journal of bacteriology 81, 416.

Derzelle, S., Hallet, B., Ferain, T., Delcour, J., Hols, P., 2003. Improved adaptation to cold-shock, stationary-phase, and freezing stresses in Lactobacillus plantarum overproducing cold-shock proteins. Applied and environmental microbiology 69, 4285-4290.

Dieckmann, R., Helmuth, R., Erhard, M., Malorny, B., 2008. Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74, 7767-7778.

Dieckmann, R., Malorny, B., 2011. Rapid screening of epidemiologically important Salmonella enterica subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 77, 4136-4146.

Dossmann, M.U., Vogel, R.F., Hammes, W.P., 1996. Mathematical description of the growth of Lactobacillus sake and Lactobacillus pentosus under conditions prevailing in fermented sausages. Appl Microbiol Biotechnol 46, 334-339.

Ducret, A., Chabalier, M., Dukan, S., 2014. Characterization and resuscitation of 'non-culturable' cells of Legionella pneumophila. BMC Microbiol 14, 3.

Duskova, M., Sedo, O., Ksicova, K., Zdrahal, Z., Karpiskova, R., 2012. Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS. Int J Food Microbiol 159, 107-114.

Eisenbach, L., Geissler, A.J., Ehrmann, M.A., Vogel, R.F., 2019. Comparative genomics of Lactobacillus sakei supports the development of starter strain combinations. Microbiol Res 221, 1-9.

Eisenbach, L., Janßen, D., Ehrmann, M.A., Vogel, R.F., 2017. Comparative genomics of *Lactobacillus sakei* and *Lactobacillus curvatus* suggests the use of strain sets, in: Talon, R. (Ed.), 3rd International symposium on fermented meats Clermont-Ferrand, France, p. 16.

Eisenbach, L., Janssen, D., Ehrmann, M.A., Vogel, R.F., 2018. Comparative genomics of Lactobacillus curvatus enables prediction of traits relating to adaptation and strategies of assertiveness in sausage fermentation. Int J Food Microbiol 286, 37-47.

Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M., Laegreid, W.W., 2000. Correlation of enterohemorrhagic Escherichia coli O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proceedings of the National Academy of Sciences 97, 2999-3003.

Emonet, S., Shah, H., Cherkaoui, A., Schrenzel, J., 2010. Application and use of various mass spectrometry methods in clinical microbiology. Clinical Microbiology and Infection 16, 1604-1613.

Eskin, N.M., Shahidi, F., 2012. Biochemistry of foods. Academic Press.

Ezraty, B., Gennaris, A., Barras, F., Collet, J.-F., 2017. Oxidative stress, protein damage and repair in bacteria. Nature Reviews Microbiology 15, 385.

Fakruddin, M., Mannan, K.S., Andrews, S., 2013. Viable but nonculturable bacteria: food safety and public health perspective. ISRN Microbiol 2013, 703813.

Fantelli, K., Stephan, R., 2001. Prevalence and characteristics of shigatoxin-producing Escherichia coli and Listeria monocytogenes strains isolated from minced meat in Switzerland. International journal of food microbiology 70, 63-69.

Feiner, G., 2006. Meat products handbook: Practical science and technology. Elsevier.

Ferguson, D., Warner, R., 2008. Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? Meat Science 80, 12-19.

Fernandez, M., Zuniga, M., 2006. Amino acid catabolic pathways of lactic acid bacteria. Crit Rev Microbiol 32, 155-183.

Fortier, L.C., Sekulovic, O., 2013. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence 4, 354-365.

Freiding, S., Gutsche, K.A., Ehrmann, M.A., Vogel, R.F., 2011. Genetic screening of Lactobacillus sakei and Lactobacillus curvatus strains for their peptidolytic system and amino acid metabolism, and comparison of their volatilomes in a model system. Syst Appl Microbiol 34, 311-320.

Fuller, R., 1989. Probiotics in man and animals. J Appl Bacteriol 66, 365-378.

Garneau, J.E., Moineau, S., 2011. Bacteriophages of lactic acid bacteria and their impact on milk fermentations. Microb Cell Fact 10 Suppl 1, S20.

Gill, C., 1983. Meat spoilage and evaluation of the potential storage life of fresh meat. Journal of Food Protection 46, 444-452.

Giraffa, G., 2004. Studying the dynamics of microbial populations during food fermentation. FEMS Microbiol Rev 28, 251-260.

Giraffa, G., Zago, M., Carminati, D., 2017. Lactic acid bacteria bacteriophages in dairy products: problems and solutions, Microbiology in Dairy Processing: Challenges and Opportunities, p. 233.

Goh, H.F., Philip, K., 2015. Purification and Characterization of Bacteriocin Produced by Weissella confusa A3 of Dairy Origin. PLoS One 10, e0140434.

Goldstein, E.J., Tyrrell, K.L., Citron, D.M., 2015. Lactobacillus species: taxonomic complexity and controversial susceptibilities. Clin Infect Dis 60 Suppl 2, S98-107.

Gould, G.W., 2012. New methods of food preservation. Springer Science & Business Media.

Graumann, P., Marahiel, M.A., 1996. Some like it cold: response of microorganisms to cold shock. Archives of microbiology 166, 293-300.

Hadrys, H., Balick, M., Schierwater, B., 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molecular ecology 1, 55-63.

Hammes, W.P., 2012. Metabolism of nitrate in fermented meats: the characteristic feature of a specific group of fermented foods. Food Microbiology 29, 151-156.

Hammes, W.P., Knauf, H.J., 1994. Starters in the processing of meat products. Meat Sci 36, 155-168.

Hammes, W.P., Vogel, R.F., 1995. The genus Lactobacillus, The Genera of Lactic Acid Bacteria. Springer, Boston, MA.

Hammes, W.P.B., A.; Min, S., 1990. Lactic acid bacteria in meat fermentation. Fems Microbiology Letters 87, 165-173.

Hansen, A., 2018. Meat consumption and capitalist development: The meatification of food provision and practice in Vietnam, Geoforum, pp. 57-68.

Hassan, M., Kjos, M., Nes, I., Diep, D., Lotfipour, F., 2012. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. Journal of Applied Microbiology 113, 723-736.

Heinrich, J., Velleman, M., Schuster, H., 1995. The tripartite immunity system of phages P1 and P7. FEMS Microbiology Reviews 17, 121-126.

Heinz, G., Hautzinger, P., 2007. Meat processing technology for small to medium scale producers.

Henchion, M., McCarthy, M., Resconi, V.C., Troy, D., 2014. Meat consumption: trends and quality matters. Meat Sci 98, 561-568.

Hennekinne, J.A., De Buyser, M.L., Dragacci, S., 2012. Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol Rev 36, 815-836.

Hernández-Macedo, M.L., Barancelli, G.V., Contreras-Castillo, C.J., 2011. Microbial deterioration of vacuum-packaged chilled beef cuts and techniques for microbiota detection and characterization: a review. Brazilian Journal of Microbiology 42, 1-11.

Hertzberger, R., Arents, J., Dekker, H.L., Pridmore, R.D., Gysler, C., Kleerebezem, M., de Mattos, M.J., 2014. H(2)O(2) production in species of the Lactobacillus acidophilus group: a

central role for a novel NADH-dependent flavin reductase. Appl Environ Microbiol 80, 2229-2239.

Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 8, 15-25.

Hillman, J.D., Novak, J., Sagura, E., Gutierrez, J.A., Brooks, T.A., Crowley, P.J., Hess, M., Azizi, A., Leung, K., Cvitkovitch, D., Bleiweis, A.S., 1998. Genetic and biochemical analysis of mutacin 1140, a lantibiotic from Streptococcus mutans. Infect Immun 66, 2743-2749.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M., Heir, E., 2017. Health and Safety Considerations of Fermented Sausages. Journal of Food Quality 2017, 25.

Höll, L., Hilgarth, M., Geissler, A.J., Behr, J., Vogel, R.F., 2019. Prediction of in situ metabolism of photobacteria in modified atmosphere packaged poultry meat using metatranscriptomic data. Microbiological Research 222, 52-59.

Holló, G., Csapó, J., Szűcs, E., Tőzsér, J., Repa, I., Holló, I., 2001a. Influence of breed, slaughter weight and gender on chemical composition of beef. Part 1. Amino acid profile and biological value of proteins. Asian-Australasian Journal of Animal Sciences 14, 1555-1559.

Hovelius, B., Mardh, P.A., 1984. Staphylococcus saprophyticus as a common cause of urinary tract infections. Rev Infect Dis 6, 328-337.

Hugas, M., Garriga, M., Aymerich, T., Monfort, J.M., 1993. Biochemical characterization of lactobacilli from dry fermented sausages. Int J Food Microbiol 18, 107-113.

Hugas, M., Monfort, J.M., 1996. Bacterial starter cultures for meat fermentations. Food Chem 59, 547-554.

Hugas, M., Monfort, J.M., 1997. Bacterial starter cultures for meat fermentation. Food chemistry 59, 547-554.

Humphrey, S.B., Stanton, T.B., Jensen, N.S., 1995. Mitomycin C induction of bacteriophages from Serpulina hyodysenteriae and Serpulina innocens. Fems Microbiology Letters 134, 97-101.

Hunter, P.R., 1990. Reproducibility and indices of discriminatory power of microbial typing methods. Journal of Clinical Microbiology 28, 1903-1905.

Hussain, M.A., Hosseini Nezhad, M., Sheng, Y., Amoafo, O., 2013. Proteomics and the stressful life of lactobacilli. Fems Microbiology Letters 349, 1-8.

Hutkins, R.W., 2006. Microbiology and Technology of Fermented Foods. Wiley-Blackwell Publishing.

Imlay, J.A., 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. 77, 755-776.

Imlay, J.A., Linn, S., 1987. Mutagenesis and stress responses induced in Escherichia coli by hydrogen peroxide. Journal of bacteriology 169, 2967-2976.

Imlay, J.A., Linn, S., 1988. DNA damage and oxygen radical toxicity. Science 240, 1302-1309.

Immonen, K., Puolanne, E., 2000. Variation of residual glycogen-glucose concentration at ultimate pH values below 5.75. Meat Sci 55, 279-283.

Immonen, K., Ruusunen, M., Hissa, K., Puolanne, E., 2000. Bovine muscle glycogen concentration in relation to finishing diet, slaughter and ultimate pH. Meat Sci 55, 25-31.

Incze, K., 1998. Dry fermented sausages. Meat Science 49, S169-S177.

Jack, R.W., Tagg, J.R., Ray, B., 1995. Bacteriocins of gram-positive bacteria. Microbiol Rev 59, 171-200.

Jackson, C.R., Davis, J.A., Barrett, J.B., 2013. Prevalence and characterization of methicillin-resistant Staphylococcus aureus isolates from retail meat and humans in Georgia. J Clin Microbiol 51, 1199-1207.

Janßen, D., Eisenbach, L., Ehrmann, M.A., Vogel, R.F., 2018. Assertiveness of Lactobacillus sakei and Lactobacillus curvatus in a fermented sausage model. Int J Food Microbiol.

Jay, J.M., 1996. Prevalence of Listeria spp. in meat and poultry products. Food control 7, 209-214.

Johnston, M., Delwiche, E., 1965. Isolation and characterization of the cyanide-resistant and azide-resistant catalase of Lactobacillus plantarum. Journal of bacteriology 90, 352-356.

Kahm, M., Hasenbrink, G., Lichtenberg-Fraté, H., Ludwig, J., Kschischo, M., 2010. grofit: fitting biological growth curves with R. Journal of Statistical Software 33, 1-21.

Kameník, J., 2013. The microbiology of meat spoilage: a review. Maso International—Journal of Food Science and Technology.—2013.—P, 1-9.

Kandler, O., Weiss, N., 1986. Regular, nonsporing gram-positive rods. Bergey´s Manual of Systematic Bacteriology 2, 1208-1234.

Kandler, O.a.W., N. , 1986. Genus Lactobacillus. Bergey's manual of systematic bacteriology 2, 1209-1234.

Karas, M., Bachmann, D., Bahr, U., Hillenkamp, F., 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. International journal of mass spectrometry and ion processes 78, 53-68.

Kashmiri, Z., Mankar, S., 2014. Free radicals and oxidative stress in bacteria. Int J Curr Microbiol App Sci 3, 34-40.

Kask, S., Adamberg, K., Orłowski, A., Vogensen, F.K., Møller, P.L., Ardö, Y., Paalme, T., 2003. Physiological properties of Lactobacillus paracasei, L. danicus and *L. curvatus* strains isolated from Estonian semi-hard cheese. Food Research International 36, 1037-1046.

Katsaras, K., Leistner, L., 1991. Distribution and development of bacterial colonies in fermented sausages. Biofouling 5, 115-124.

Kern, C.C., Usbeck, J.C., Vogel, R.F., Behr, J., 2013. Optimization of Matrix-Assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry for the identification of bacterial contaminants in beverages. J Microbiol Methods 93, 185-191.

Kern, C.C., Vogel, R.F., Behr, J., 2014. Differentiation of Lactobacillus brevis strains using Matrix-Assisted-Laser-Desorption-Ionization-Time-of-Flight Mass Spectrometry with respect to their beer spoilage potential. Food Microbiol 40, 18-24.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12, 39-85.

Knauf, H.J., Vogel, R.F., Hammes, W.P., 1992. Cloning, sequence, and phenotypic expression of katA, which encodes the catalase of Lactobacillus sake LTH677. Appl Environ Microbiol 58, 832-839.

Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C.J., Salzman, N.H., 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. Nature 526, 719-722.

Koort, J., Vandamme, P., Schillinger, U., Holzapfel, W., Bjorkroth, J., 2004. Lactobacillus curvatus subsp. melibiosus is a later synonym of Lactobacillus sakei subsp. carnosus. Int J Syst Evol Microbiol 54, 1621-1626.

Koskella, B., Meaden, S., 2013. Understanding bacteriophage specificity in natural microbial communities. Viruses 5, 806-823.

Kröckel, L., 2013. The Role of Lactic Acid Bacteria in Safety and Flavour Development of Meat and Meat Products, Lactic Acid Bacteria-R & D for Food, Health and Livestock Purposes. InTech.

Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological niche. Meat Sci 52, 299-305.

Ladiges, W., Foster, J., Ganz, W., 1974. Incidence and viability of Clostridium perfringens in ground beef. Journal of Milk and Food Technology 37, 622-623.

Lahtinen, S., Ouwehand, A.C., Salminen, S., von Wright, A., 2011. Lactic acid bacteria: microbiological and functional aspects. CRC Press.

Lammerding, A., Garcia, M., Mann, E., Robinson, Y., Dorward, W., Ruscott, R., Tittiger, F., 1988. Prevalence of Salmonella and thermophilic Campylobacter in fresh pork, beef, veal and poultry in Canada. Journal of Food Protection 51, 47-52.

Laranjo, M., Elias, M., Fraqueza, 2017. The Use of Starter Cultures in Traditional Meat Products. Journal of Food Quality.

Lartigue, M.F., Hery-Arnaud, G., Haguenoer, E., Domelier, A.S., Schmit, P.O., van der Mee-Marquet, N., Lanotte, P., Mereghetti, L., Kostrzewa, M., Quentin, R., 2009. Identification of Streptococcus agalactiae isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 47, 2284-2287.

Lawrence, J.G., Hendrix, R.W., Casjens, S., 2001. Where are the pseudogenes in bacterial genomes? Trends in microbiology 9, 535-540.

Lechardeur, D., Cesselin, B., Fernandez, A., Lamberet, G., Garrigues, C., Pedersen, M., Gaudu, P., Gruss, A., 2011. Using heme as an energy boost for lactic acid bacteria. Curr Opin Biotechnol 22, 143-149.

Lee, C., Lee, S.M., Mukhopadhyay, P., Kim, S.J., Lee, S.C., Ahn, W.-S., Yu, M.-H., Storz, G., Ryu, S.E., 2004. Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. Nature Structural and Molecular Biology 11, 1179.

Lee, C., Newbold, R., 1963. The pathway of degradation of inosinic acid in bovine skeletal muscle. Biochimica et Biophysica Acta (BBA)-Specialized Section on Nucleic Acids and Related Subjects 72, 349-352.

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. Int J Food Microbiol 55, 181-186.

Leistner, L., Gorris, L.G., 1995. Food preservation by hurdle technology. Trends in Food Science & Technology 6, 41-46.

Lerche, M., Reuter, G., 1960. [A contribution to the method of isolation and differentiation of aerobic "lactobacilli" (Genus "Lactobacillus Beijerinck")]. Zentralbl Bakteriol 179, 354-370.

Leroy, F., Verluyten, J., De Vuyst, L., 2006. Functional meat starter cultures for improved sausage fermentation. Int J Food Microbiol 106, 270-285.

Levine, P., Rose, B., Green, S., Ransom, G., Hill, W., 2001. Pathogen testing of ready-to-eat meat and poultry products collected at federally inspected establishments in the United States, 1990 to 1999. J Food Prot 64, 1188-1193.

Lhomme, E., Mezaize, S., Ducasse, M.B., Chiron, H., Champomier-Verges, M.C., Chaillou, S., Zagorec, M., Dousset, X., Onno, B., 2014. A polyphasic approach to study the dynamics of

microbial population of an organic wheat sourdough during its conversion to gluten-free sourdough. Int Microbiol 17, 1-9.

Li, Y., Xiang, Q., Zhang, Q., Huang, Y., Su, Z., 2012. Overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application. Peptides 37, 207-215.

Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.-Y., 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. Journal of antimicrobial Chemotherapy 67, 1589-1596.

Liu, L., Tong, H., Dong, X., 2012. Function of the pyruvate oxidase-lactate oxidase cascade in interspecies competition between Streptococcus oligofermentans and Streptococcus mutans. Appl Environ Microbiol 78, 2120-2127.

Liu, X., Li, Y., Guo, Y., Zeng, Z., Li, B., Wood, T.K., Cai, X., Wang, X., 2015. Physiological function of rac prophage during biofilm formation and regulation of rac excision in Escherichia coli K-12. Scientific reports 5, 16074.

Lopes, A., Tavares, P., Petit, M.A., Guerois, R., Zinn-Justin, S., 2014. Automated classification of tailed bacteriophages according to their neck organization. Bmc Genomics 15, 1027.

Lorenz, N., Reiger, M., Toro-Nahuelpan, M., Brachmann, A., Poettinger, L., Plener, L., Lassak, J., Jung, K., 2016. Identification and Initial Characterization of Prophages in Vibrio campbellii. PLoS One 11, e0156010.

Lücke, F.-K., 1994. Fermented meat products. Food Research International 27, 299-307.

Lücke, F.K., 2000. Utilization of microbes to process and preserve meat. Meat Sci 56, 105-115.

Luo, Y., Siu, G.K., Yeung, A.S., Chen, J.H., Ho, P.L., Leung, K., Tsang, J.L., Cheng, V.C., Guo, L., Yang, J., 2015. Performance of the VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid bacterial identification in two diagnostic centres in China. Journal of medical microbiology 64, 18-24.

Lwoff, A., 1953. Lysogeny. Bacteriological Reviews 17, 269.

Mahony, J., Cambillau, C., Van Sinderen, D., 2017. Host recognition by lactic acid bacterial phages. FEMS Microbiology Reviews 41, S16-S26.

Mahony, J., van Sinderen, D., 2014. Current taxonomy of phages infecting lactic acid bacteria. Front Microbiol 5, 7.

Mai-Prochnow, A., Clauson, M., Hong, J., Murphy, A.B., 2016. Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. Scientific reports 6, 38610.

Mantini, D., Petrucci, F., Pieragostino, D., Del Boccio, P., Sacchetta, P., Candiano, G., Ghiggeri, G.M., Lugaresi, A., Federici, G., Di Ilio, C., Urbani, A., 2010. A computational platform for MALDI-TOF mass spectrometry data: application to serum and plasma samples. J Proteomics 73, 562-570.

Marceau, A., Zagorec, M., Champomier-Vergès, M.-C., 2003. Positive effects of growth at suboptimal temperature and high salt concentration on long-term survival of Lactobacillus sakei. Research in microbiology 154, 37-42.

Marco, M.B., Moineau, S., Quiberoni, A., 2012. Bacteriophages and dairy fermentations. Bacteriophage 2, 149-158.

Marcó, M.B., Moineau, S., Quiberoni, A., 2012. Bacteriophages and dairy fermentations. Bacteriophage 2, 149-158.

Marth, E.H., Steele, J., 2001. Applied dairy microbiology. CRC Press.

Martin, B., Garriga, M., Aymerich, T., 2011. Prevalence of Salmonella spp. and Listeria monocytogenes at small-scale spanish factories producing traditional fermented sausages. J Food Prot 74, 812-815.

Martín, R., Suárez, J.E., 2010. Biosynthesis and degradation of H2O2 by vaginal lactobacilli. Applied and environmental microbiology 76, 400-405.

Martinez, R.C., Staliano, C.D., Vieira, A.D., Villarreal, M.L., Todorov, S.D., Saad, S.M., Franco, B.D., 2015. Bacteriocin production and inhibition of Listeria monocytogenes by Lactobacillus sakei subsp. sakei 2a in a potentially synbiotic cheese spread. Food Microbiol 48, 143-152.

McGovern, P.E., Zhang, J., Tang, J., Zhang, Z., Hall, G.R., Moreau, R.A., Nunez, A., Butrym, E.D., Richards, M.P., Wang, C.S., Cheng, G., Zhao, Z., Wang, C., 2004. Fermented beverages of pre- and proto-historic China. Proc Natl Acad Sci U S A 101, 17593-17598.

McLeod, A., Mosleth, E.F., Rud, I., Branco Dos Santos, F., Snipen, L., Liland, K.H., Axelsson, L., 2017. Effects of glucose availability in Lactobacillus sakei; metabolic change and regulation of the proteome and transcriptome. PLoS One 12, e0187542.

McLeod, A., Nyquist, O.L., Snipen, L., Naterstad, K., Axelsson, L., 2008. Diversity of Lactobacillus sakei strains investigated by phenotypic and genotypic methods. Syst Appl Microbiol 31, 393-403.

Meloni, D., 2015. Presence of Listeria monocytogenes in Mediterranean-Style Dry Fermented Sausages. Foods 4, 34-50.

Messier, S., Smith, H.J., Tittiger, F., 1989. Survival of Salmonella typhimurium and Staphylococcus aureus in Genoa salami of varying salt concentration. Can J Vet Res 53, 84-86.

Michel, E., Monfort, C., Deffrasnes, M., Guezenec, S., Lhomme, E., Barret, M., Sicard, D., Dousset, X., Onno, B., 2016. Characterization of relative abundance of lactic acid bacteria species in French organic sourdough by cultural, qPCR and MiSeq high-throughput sequencing methods. Int J Food Microbiol 239, 35-43.

Mills, S., Stanton, C., Hill, C., Ross, R., 2011. New developments and applications of bacteriocins and peptides in foods. Annual review of food science and technology 2, 299-329.

Mishra, S., Imlay, J., 2012. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Archives of biochemistry and biophysics 525, 145-160.

Mishra, S., Imlay, J.A., 2013. An anaerobic bacterium, B acteroides thetaiotaomicron, uses a consortium of enzymes to scavenge hydrogen peroxide. Molecular microbiology 90, 1356-1371.

Mokoena, M.P., 2017. Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. Molecules 22.

Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., Tabanelli, G., 2018. Phenotypic Diversity of Lactobacillus sakei Strains. Front Microbiol 9, 2003.

Munoz, R., Lopez-Lopez, A., Urdiain, M., Moore, E.R., Rossello-Mora, R., 2011. Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. Syst Appl Microbiol 34, 69-75.

Murray, P.R., 2012. What is new in clinical microbiology—microbial identification by MALDI-TOF mass spectrometry: a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. The Journal of Molecular Diagnostics 14, 419-423.

Nacef, M., Chevalier, M., Chollet, S., Drider, D., Flahaut, C., 2017. MALDI-TOF mass spectrometry for the identification of lactic acid bacteria isolated from a French cheese: The Maroilles. Int J Food Microbiol 247, 2-8.

Nagy, E., Becker, S., Soki, J., Urban, E., Kostrzewa, M., 2011. Differentiation of division I (cfiA-negative) and division II (cfiA-positive) Bacteroides fragilis strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Med Microbiol 60, 1584-1590.

Nair, B.M., Prajapati, J.B., 2003. The history of fermented foods, Handbook of fermented functional foods. CRC Press, pp. 17-42.

Nanda, A.M., Thormann, K., Frunzke, J., 2015. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. Journal of bacteriology 197, 410-419.

Narvhus, J.A., Axelsson, L., 2003. Lactic Acid Bacteria, Encyclopedia of Food Sciences and Nutrition. Elsevier.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., Holo, H., 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70, 113-128.

Nilsen, T., Nes, I.F., Holo, H., 2003. Enterolysin A, a cell wall-degrading bacteriocin from Enterococcus faecalis LMG 2333. Applied and environmental microbiology 69, 2975-2984.

Nishiyama, Y., Massey, V., Takeda, K., Kawasaki, S., Sato, J., Watanabe, T., Niimura, Y., 2001. Hydrogen peroxide-forming NADH oxidase belonging to the peroxiredoxin

oxidoreductase family: existence and physiological role in bacteria. Journal of bacteriology 183, 2431-2438.

Nout, M.J.R., 1194. Fermented foods and food safety. Food Research International 27, 291-298.

Nuotio, L., Schneitz, C., Halonen, U., Nurmi, E., 1992. Use of competitive exclusion to protect newly-hatched chicks against intestinal colonisation and invasion by Salmonella enteritidis PT4. Br Poult Sci 33, 775-779.

Nurmi, E., Nuotio, L., Schneitz, C., 1992. The competitive exclusion concept: development and future. Int J Food Microbiol 15, 237-240.

Nychas, G.J., Skandamis, P.N., Tassou, C.C., Koutsoumanis, K.P., 2008. Meat spoilage during distribution. Meat Sci 78, 77-89.

Nyquist, O.L., McLeod, A., Brede, D.A., Snipen, L., Aakra, A., Nes, I.F., 2011. Comparative genomics of Lactobacillus sakei with emphasis on strains from meat. Mol Genet Genomics 285, 297-311.

Ohnishi, M., Kurokawa, K., Hayashi, T., 2001. Diversification of Escherichia coli genomes: are bacteriophages the major contributors? Trends in microbiology 9, 481-485.

Oliveira, J., Mahony, J., Hanemaaijer, L., Kouwen, T., Neve, H., MacSharry, J., van Sinderen, D., 2017. Detecting Lactococcus lactis Prophages by Mitomycin C-Mediated Induction Coupled to Flow Cytometry Analysis. Front Microbiol 8, 1343.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. J Microbiol 43 Spec No, 93-100.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34, 415-425.

Olsen, G.J., Overbeek, R., Larsen, N., Marsh, T.L., McCaughey, M.J., Maciukenas, M.A., Kuan, W.M., Macke, T.J., Xing, Y., Woese, C.R., 1992. The Ribosomal Database Project. Nucleic Acids Res 20 Suppl, 2199-2200.

Olsen, G.J., Woese, C.R., 1993. Ribosomal RNA: a key to phylogeny. The FASEB journal 7, 113-123.

Olsen, J.V., Ong, S.E., Mann, M., 2004. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics 3, 608-614.

Omer, M.K., Alvarez-Ordonez, A., Prieto, M., Skjerve, E., Asehun, T., Alvseike, O.A., 2018. A Systematic Review of Bacterial Foodborne Outbreaks Related to Red Meat and Meat Products. Foodborne Pathog Dis 15, 598-611.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M., Hoz, L.d.l., 1999. Changes in the components of dry-fermented sausages during ripening. Critical reviews in food science and nutrition 39, 329-367.

Orla-Jensen, S., 1919. The lactic acid bacteria, The lactic acid bacteria. Andr. Fred. Host & Son, Copenhagen.

Ortiz de Orué Lucana, D., Wedderhoff, I., Groves, M.R., 2012. ROS-mediated signalling in bacteria: zinc-containing Cys-XX-Cys redox centres and iron-based oxidative stress. Journal of signal transduction 2012.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E., Kotzekidou, P., 2003. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci 65, 859-867.

Parizad, E.G., Parizad, E.G., Valizadeh, A., 2016. The application of pulsed field gel electrophoresis in clinical studies. Journal of clinical and diagnostic research: JCDR 10, DE01.

Pasteur, L., 1876. Études sur la bière': ses maladies, causes qui les provoquent, procédé pour la rendre inaltérable. Gauthier-Villars.

Patel, R., 2015. MALDI-TOF MS for the diagnosis of infectious diseases. Clinical chemistry 61, 100-111.

Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poult Sci 82, 627-631.

Paul, J.H., 2008. Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas? The ISME journal 2, 579.

Pavlovic, M., Huber, I., Konrad, R., Busch, U., 2013. Application of MALDI-TOF MS for the Identification of Food Borne Bacteria. Open Microbiol J 7, 135-141.

Pavlovic, M., Wudy, C., Zeller-Peronnet, V., Maggipinto, M., Zimmermann, P., Straubinger, A., Iwobi, A., Martlbauer, E., Busch, U., Huber, I., 2015. Identification of bacteria isolated from veterinary clinical specimens using MALDI-TOF MS. Berl Munch Tierarztl Wochenschr 128, 24-30.

Phadtare, S., 2004. Recent developments in bacterial cold-shock response. Current issues in molecular biology 6, 125-136.

Phadtare, S., Severinov, K., 2010. RNA remodeling and gene regulation by cold shock proteins. RNA biology 7, 788-795.

Picozzi, C., Meissner, D., Chierici, M., Ehrmann, M.A., Vigentini, I., Foschino, R., Vogel, R.F., 2015. Phage-mediated transfer of a dextranase gene in Lactobacillus sanfranciscensis and characterization of the enzyme. Int J Food Microbiol 202, 48-53.

Pösö, A.R., Puolanne, E., 2005. Carbohydrate metabolism in meat animals. Meat Science 70, 423-434.

Pucci, M.J., Vedamuthu, E.R., Kunka, B.S., Vandenbergh, P.A., 1988. Inhibition of Listeria monocytogenes by using bacteriocin PA-1 produced by Pediococcus acidilactici PAC 1.0. Appl Environ Microbiol 54, 2349-2353.

Qi, F., Chen, P., Caufield, P.W., 2001. The group I strain of Streptococcus mutans, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol 67, 15-21.

Qi, Y., Miller, K.J., 2000. Effect of low water activity on staphylococcal enterotoxin A and B biosynthesis. J Food Prot 63, 473-478.

Rantsiou, K., Cocolin, L., 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. Int J Food Microbiol 108, 255-267.

Ravin, N.V., 2011. N15: The linear phage–plasmid. Plasmid 65, 102-109.

Ray, B., Bhunia, A., 2013. Fundamental food microbiology. CRC press.

Raya, R.R., H'Bert E, M., 2009. Isolation of Phage via Induction of Lysogens. Methods Mol Biol 501, 23-32.

Reuter, G., 1972. Experimental ripening of dry sausages using lactobacilli and micrococci starter cultures. Fleischwirtschaft 52, 465-468, 471-473.

Rhoades, J., Duffy, G., Koutsoumanis, K., 2009. Prevalence and concentration of verocytotoxigenic Escherichia coli, Salmonella enterica and Listeria monocytogenes in the beef production chain: a review. Food Microbiology 26, 357-376.

Rimaux, T., Riviere, A., Illeghems, K., Weckx, S., De Vuyst, L., Leroy, F., 2012. Expression of the arginine deiminase pathway genes in Lactobacillus sakei is strain dependent and is affected by the environmental pH. Appl Environ Microbiol 78, 4874-4883.

Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L., Leroy, F., 2011. The kinetics of the arginine deiminase pathway in the meat starter culture Lactobacillus sakei CTC 494 are pH-dependent. Food Microbiol 28, 597-604.

Rimaux, T., Vrancken, G., Vuylsteke, B., De Vuyst, L., Leroy, F., 2011. The pentose moiety of adenosine and inosine is an important energy source for the fermented-meat starter culture Lactobacillus sakei CTC 494. Appl Environ Microbiol 77, 6539-6550.

Ross, R.P., Morgan, S., Hill, C., 2002. Preservation and fermentation: past, present and future. Int J Food Microbiol 79, 3-16.

Ruelle, V., Moualij, B.E., Zorzi, W., Ledent, P., Pauw, E.D., 2004. Rapid identification of environmental bacterial strains by matrix‐assisted laser desorption/ionization time‐of‐flight mass spectrometry. Rapid Communications in Mass Spectrometry 18, 2013-2019.

Ruiz-Barba, J.L., Caballero-Guerrero, B., Maldonado-Barragan, A., Jimenez-Diaz, R., 2010. Coculture with specific bacteria enhances survival of Lactobacillus plantarum NC8, an autoinducer-regulated bacteriocin producer, in olive fermentations. Food Microbiol 27, 413-417.

Saenger, W., 2013. Proteinase k, Handbook of Proteolytic Enzymes (Third Edition). Elsevier, pp. 3240-3242.

Samelis, J., Maurogenakis, F., Metaxopoulos, J., 1994. Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. Int J Food Microbiol 23, 179-196.

Samson, J.E., Moineau, S., 2013. Bacteriophages in food fermentations: new frontiers in a continuous arms race. Annual review of food science and technology 4, 347-368.

Sandrin, T.R., Goldstein, J.E., Schumaker, S., 2013. MALDI TOF MS profiling of bacteria at the strain level: a review. Mass Spectrom Rev 32, 188-217.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences 74, 5463-5467.

Sarkadi, L.S., 2016. Biogenic Amines in Fermented Foods and Health Implications, Fermented Foods in Health and Disease Prevention. Elsevier, pp. 625-651.

Sartz, L., De Jong, B., Hjertqvist, M., Plym-Forshell, L., Alsterlund, R., Löfdahl, S., Osterman, B., Ståhl, A., Eriksson, E., Hansson, H.-B., 2008. An outbreak of Escherichia coli O157: H7 infection in southern Sweden associated with consumption of fermented sausage; aspects of sausage production that increase the risk of contamination. Epidemiology & Infection 136, 370-380.

Sauer, S., Kliem, M., 2010. Mass spectrometry tools for the classification and identification of bacteria. Nat Rev Microbiol 8, 74-82.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17, 7-15.

Schuster, J.A., Vogel, R.F., Ehrmann, M.A., 2019. Characterization and distribution of CRISPR-Cas systems in Lactobacillus sakei. Arch Microbiol.

Schweigert, B., Payne, B.J., 1956. A summary of the nutrient content of meat. American Meat Institute Foundation Bull. No. 30.

Seaver, L.C., Imlay, J.A., 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli. Journal of bacteriology 183, 7173-7181.

Sechaud, L., Cluzel, P.-J., Rousseau, M., Baumgartner, A., Accolas, J.-P., 1988. Bacteriophages of lactobacilli. Biochimie 70, 401-410.

Šedo, O., Sedláček, I., Zdráhal, Z., 2011. Sample preparation methods for MALDI‐MS profiling of bacteria. Mass spectrometry reviews 30, 417-434.

Selhub, E.M., Logan, A.C., Bested, A.C., 2014. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. Journal of physiological anthropology 33, 2.

Selva, L., Viana, D., Regev-Yochay, G., Trzcinski, K., Corpa, J.M., Novick, R.P., Penadés, J.R., 2009. Killing niche competitors by remote-control bacteriophage induction. Proceedings of the National Academy of Sciences 106, 1234-1238.

Semedo‐Lemsaddek, T., Carvalho, L., Tempera, C., Fernandes, M.H., Fernandes, M.J., Elias, M., Barreto, A.S., Fraqueza, M.J., 2016. Characterization and Technological Features of Autochthonous Coagulase‐Negative Staphylococci as Potential Starters for Portuguese Dry Fermented Sausages. Journal of food science 81, M1197-M1202.

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.E., Rolain, J.M., Raoult, D., 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49, 543-551.

Serrazanetti, D.I., Gottardi, D., Montanari, C., Gianotti, A., 2013. Dynamic stresses of lactic acid bacteria associated to fermentation processes, Lactic acid bacteria-R & D for food, health and livestock purposes. InTech.

Settanni, L., Corsetti, A., 2007. The use of multiplex PCR to detect and differentiate food-and beverage-associated microorganisms: a review. Journal of Microbiological Methods 69, 1-22.

Silva, C.C., Silva, S.P., Ribeiro, S.C., 2018. Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. Frontiers in microbiology 9, 594.

Simpson, R.J., 2006. Fragmentation of protein using trypsin. CSH Protoc 2006.

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Frontiers in microbiology 6, 791.

Sinha, N., 2007. Handbook of food products manufacturing, 2 volume set. John Wiley & Sons.

Sinz, Q., Schwab, W., 2012. Metabolism of amino acids, dipeptides and tetrapeptides by Lactobacillus sakei. Food Microbiol 29, 215-223.

Smid, E.J., Kleerebezem, M., 2014. Production of aroma compounds in lactic fermentations. Annu Rev Food Sci Technol 5, 313-326.

Smith, D.L., Rooks, D.J., Fogg, P.C., Darby, A.C., Thomson, N.R., McCarthy, A.J., Allison, H.E., 2012. Comparative genomics of Shiga toxin encoding bacteriophages. Bmc Genomics 13, 311.

Spano, G., Massa, S., 2006. Environmental stress response in wine lactic acid bacteria: beyond Bacillus subtilis. Critical reviews in microbiology 32, 77-86.

Srividhya, K., Alaguraj, V., Poornima, G., Kumar, D., Singh, G., Raghavenderan, L., Katta, A.M., Mehta, P., Krishnaswamy, S., 2007. Identification of prophages in bacterial genomes by dinucleotide relative abundance difference. PLoS One 2, e1193.

Storz, G., Imlayt, J.A., 1999. Oxidative stress. Current opinion in microbiology 2, 188-194.

Sun, Z., Harris, H.M., McCann, A., Guo, C., Argimón, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nature communications 6, 8322.

Taddei, F., Vulic, M., Radman, M., Matic, I., 1997. Genetic variability and adaptation to stress. EXS 83, 271-290.

Taormina, P.J., Bartholomew, G.W., Dorsa, W.J., 2003. Incidence of Clostridium perfringens in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. Journal of Food Protection 66, 72-81.

Teramoto, H., Inui, M., Yukawa, H., 2013. Oxy R acts as a transcriptional repressor of hydrogen peroxide ‐ inducible antioxidant genes in C orynebacterium glutamicum R. The FEBS journal 280, 3298-3312.

Teramoto, K., Sato, H., Sun, L., Torimura, M., Tao, H., Yoshikawa, H., Hotta, Y., Hosoda, A., Tamura, H., 2007. Phylogenetic classification of Pseudomonas putida strains by MALDI-MS using ribosomal subunit proteins as biomarkers. Analytical Chemistry 79, 8712-8719.

Terentjeva, M., Berzins, A., 2010. Prevalence and antimicrobial resistance of Yersinia enterocolitica and Yersinia pseudotuberculosis in slaughter pigs in Latvia. Journal of Food Protection 73, 1335-1338.

Tohno, M., Kobayashi, H., Nomura, M., Kitahara, M., Ohkuma, M., Uegaki, R., Cai, Y., 2012. Genotypic and phenotypic characterization of lactic acid bacteria isolated from Italian ryegrass silage. Anim Sci J 83, 111-120.

Tohno, M., Kobayashi, H., Nomura, M., Uegaki, R., Cai, Y., 2012. Identification and characterization of lactic acid bacteria isolated from mixed pasture of timothy and orchardgrass, and its badly preserved silage. Anim Sci J 83, 318-330.

Toldrá, F., 2008. Meat biotechnology. Springer Science & Business Media.

Toldrá, F., 2017. The Storage and Preservation of Meat: III—Meat Processing, in: Toldrá, F. (Ed.), Lawrie´s Meat Science, 8 ed. Woodhead Publishing, pp. 265-296.

Toldrá, F., Reig, M., 2007. Sausages. Handbook of food products manufacturing, 251-264.

Torriani, S., Van Reenen, G.A., Klein, G., Reuter, G., Dellaglio, F., Dicks, L.M., 1996. Lactobacillus curvatus subsp. curvatus subsp. nov. and Lactobacillus curvatus subsp. melibiosus subsp. nov. and Lactobacillus sake subsp. sake subsp. nov. and Lactobacillus sake subsp. carnosus subsp. nov., new subspecies of Lactobacillus curvatus Abo-Elnaga and Kandler 1965 and Lactobacillus sake Katagiri, Kitahara, and Fukami 1934 (Klein et al. 1996, emended descriptions), respectively. Int J Syst Bacteriol 46, 1158-1163.

Tsakalidou, E., Papadimitriou, K., 2011. Stress responses of lactic acid bacteria. Springer Science & Business Media.

Tsunoda, M., Sakaue, T., Naito, S., Sunami, T., Abe, N., Ueno, Y., Matsuda, A., Takénaka, A., 2010. Insights into the structures of DNA damaged by hydroxyl radical: crystal structures of DNA duplexes containing 5-formyluracil. Journal of nucleic acids 2010.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. J Physiol 552, 335-344.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E., 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82, 187-216.

van Heel, A.J., de Jong, A., Montalban-Lopez, M., Kok, J., Kuipers, O.P., 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-) bactericidal posttranslationally modified peptides. Nucleic acids research 41, W448-W453.

van Loo, I.H., Diederen, B.M., Savelkoul, P.H., Woudenberg, J.H., Roosendaal, R., van Belkum, A., Lemmens-den Toom, N., Verhulst, C., van Keulen, P.H., Kluytmans, J.A., 2007. Methicillin-resistant Staphylococcus aureus in meat products, the Netherlands. Emerg Infect Dis 13, 1753-1755.

van Reenen, C.A., Dicks, L.M., Chikindas, M.L., 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by Lactobacillus plantarum. J Appl Microbiol 84, 1131-1137.

Vaughan, A., J, O.M., Eijsink, V.G., M, O.C.-M., van Sinderen, D., 2004. Transcriptional analysis of bacteriocin production by malt isolate Lactobacillus sakei 5. Fems Microbiology Letters 235, 377-384.

Venema, K., Venema, G., Kok, J., 1995. Lactococcal bacteriocins: mode of action and immunity. Trends Microbiol 3, 299-304.

Verluyten, J., Messens, W., De Vuyst, L., 2003. The curing agent sodium nitrite, used in the production of fermented sausages, is less inhibiting to the bacteriocin-producing meat starter culture Lactobacillus curvatus LTH 1174 under anaerobic conditions. Appl Environ Microbiol 69, 3833-3839.

Vignolo, G., Fontana, C., Fadda, S., 2010. Semidry and dry fermented sausages. Handbook of meat processing, 379-398.

Vogel, B.F., Hansen, L.T., Mordhorst, H., Gram, L., 2010. The survival of Listeria monocytogenes during long term desiccation is facilitated by sodium chloride and organic material. Int J Food Microbiol 140, 192-200.

Vogel, R.F., Lohmann, M., Nguyen, M., Weller, A.N., Hammes, W.P., 1993. Molecular characterization of Lactobacillus curvatus and Lact. sake isolated from sauerkraut and their application in sausage fermentations. J Appl Bacteriol 74, 295-300.

Vogt, R.L., Dippold, L., 2005. Escherichia coli O157: H7 outbreak associated with consumption of ground beef, June–July 2002. Public health reports 120, 174-178.

Vrancken, G., Rimaux, T., Weckx, S., Leroy, F., De Vuyst, L., 2011. Influence of temperature and backslopping time on the microbiota of a type I propagated laboratory wheat sourdough fermentation. Appl Environ Microbiol 77, 2716-2726.

Vranken, L.A., Tessa; Petalios, Dimitrios; Erik, Mathijs, Erik, 2014. Curbing global meat concumption: Emerging evidence of a second nutrition transition 39.

Wachsmuth, I., Kiehlbauch, J., Bopp, C., Cameron, D., Strockbine, N., Wells, J., Blake, P., 1991. The use of plasmid profiles and nucleic acid probes in epidemiologic investigations of foodborne, diarrheal diseases. Int. J. Food Microbiol 12, 77-89.

Wang, W., He, J., Pan, D., Wu, Z., Guo, Y., Zeng, X., Lian, L., 2018. Metabolomics analysis of Lactobacillus plantarum ATCC 14917 adhesion activity under initial acid and alkali stress. PLoS One 13, e0196231.

Wang, X., Kim, Y., Ma, Q., Hong, S.H., Pokusaeva, K., Sturino, J.M., Wood, T.K., 2010. Cryptic prophages help bacteria cope with adverse environments. Nat Commun 1, 147.

Wang, X., Wood, T.K., 2016. Cryptic prophages as targets for drug development. Drug Resist Updat 27, 30-38.

Wassenaar, T.M., Newell, D.G., 2000. Genotyping of Campylobacterspp. Appl. Environ. Microbiol. 66, 1-9.

Waters, A.E., Contente-Cuomo, T., Buchhagen, J., Liu, C.M., Watson, L., Pearce, K., Foster, J.T., Bowers, J., Driebe, E.M., Engelthaler, D.M., 2011. Multidrug-resistant Staphylococcus aureus in US meat and poultry. Clinical Infectious Diseases 52, 1227-1230.

Wescombe, P.A., Tagg, J.R., 2003. Purification and characterization of streptin, a type A1 lantibiotic produced by Streptococcus pyogenes. Applied and environmental microbiology 69, 2737-2747.

West, C.A., Warner, P.J., 1988. Plantacin B, a bacteriocin produced by Lactobacillus plantarum NCDO 1193. Fems Microbiology Letters 49, 163-165.

Whittaker, J.W., 2012. Non-heme manganese catalase–the 'other'catalase. Archives of biochemistry and biophysics 525, 111-120.

Wieser, A., Schneider, L., Jung, J., Schubert, S., 2012. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). Appl Microbiol Biotechnol 93, 965-974.

Winterbourn, C.C., 1995. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicology letters 82, 969-974.

Xu, J., Kiesel, B., Kallies, R., Jiang, F.L., Liu, Y., Maskow, T., 2018a. A fast and reliable method for monitoring of prophage-activating chemicals. Microb Biotechnol 11, 1112-1120.

Xu, J., Kiesel, B., Kallies, R., Jiang, F.L., Liu, Y., Maskow, T., 2018b. A fast and reliable method for monitoring of prophage‐activating chemicals. Microbial biotechnology.

Yang, E., Fan, L., Jiang, Y., Doucette, C., Fillmore, S., 2012. Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. AMB Express 2, 48.

Yang, S.-C., Lin, C.-H., Sung, C.T., Fang, J.-Y., 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Frontiers in microbiology 5, 241.

Zacharof, M.P., Lovitt, R.W., 2012. Investigation of Shelf Life of Potency and Activity of the Lactobacilli Produced Bacteriocins Through Their Exposure to Various Physicochemical Stress Factors. Probiotics Antimicrob Proteins 4, 187-197.

Zagorec, M., Champomier-Verges, M.C., 2017. Lactobacillus sakei: A Starter for Sausage Fermentation, a Protective Culture for Meat Products. Microorganisms 5.

Zhang, L.-j., Gallo, R.L., 2016. Antimicrobial peptides. Current Biology 26, R14-R19.

Zhao, C., Ge, B., De Villena, J., Sudler, R., Yeh, E., Zhao, S., White, D.G., Wagner, D., Meng, J., 2001. Prevalence of Campylobacter spp., Escherichia coli, and Salmonella serovars in retail chicken, turkey, pork, and beef from the Greater Washington, DC, area. Applied and environmental microbiology 67, 5431-5436.

Zheng, M., Åslund, F., Storz, G., 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279, 1718-1722.

Zou, D., Kaneko, J., Narita, S., Kamio, Y., 2000. Prophage, φPV83-pro, carrying Panton-Valentine leukocidin genes, on the Staphylococcus aureus P83 chromosome: comparative analysis of the genome structures of φPV83-pro, φPVL, φ11, and other phages. Bioscience, biotechnology, and biochemistry 64, 2631-2643.

9 Appendix

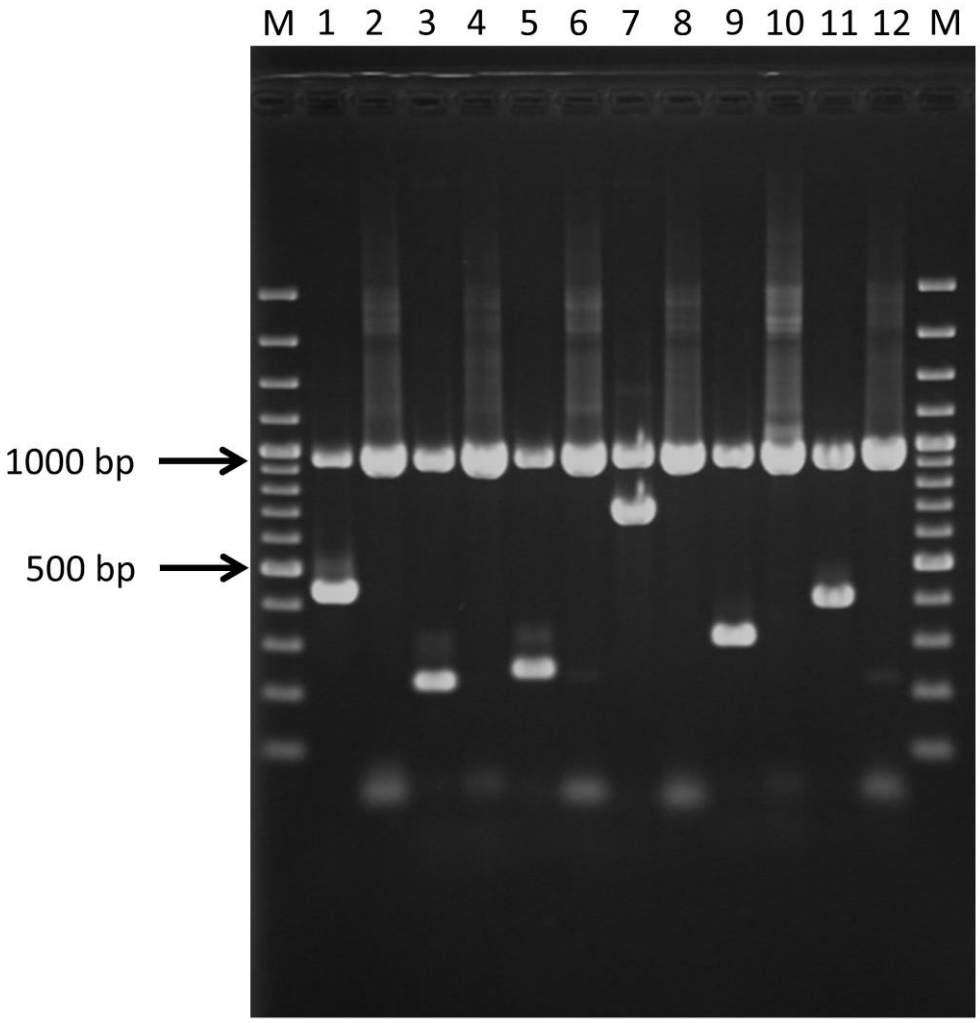

Figure S.1: Colony-duplex PCR assay for the strain-specific identification of *L. curvatus* and *L. sakei* strains. Colony-duplex PCRs were run with a specific strain and a mix of the other respective strains. Primers targeting dnaK were used as controls. *L. curvatus* TMW 1.624 (lane 1, Mix lane 2) TMW 1.139 (lane 3, Mix lane 4), *L. sakei* TMW 1.3 (lane 5, mix lane 6), TMW 1.417 (lane 7, mix lane 8), TMW 1.1396 (lane 9, mix lane 10) and TMW 1.1398 (lane 11, mix lane 12), size marker 100 bp (M).

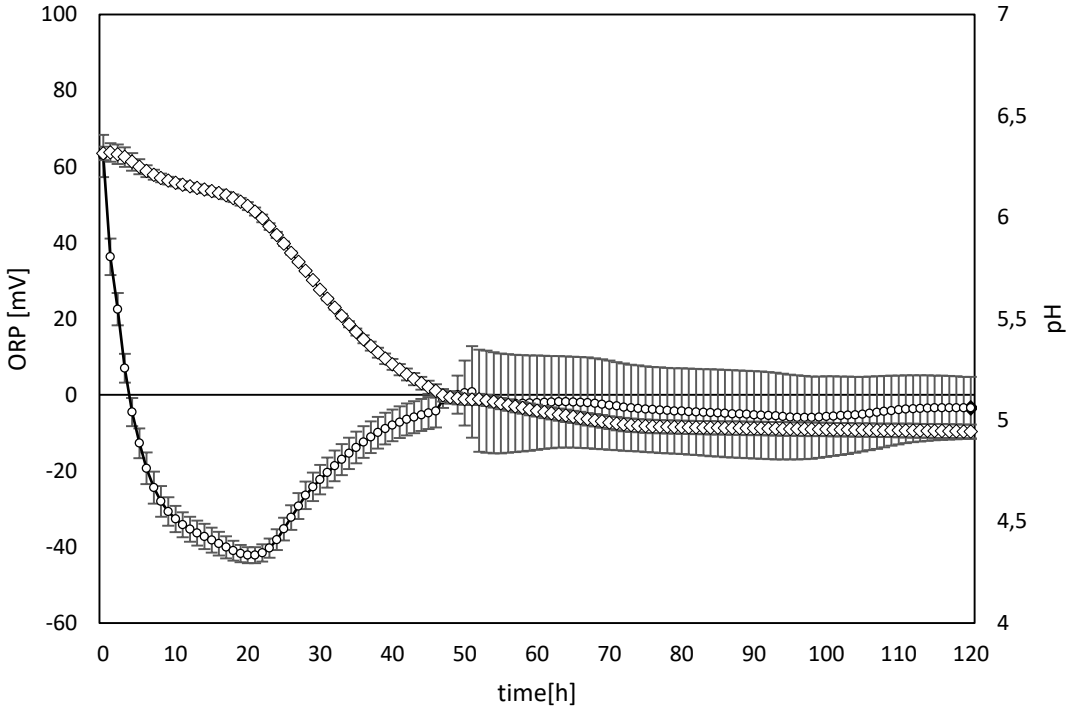

Figure S.2: Development of pH and redox potential (ORP) of set I over the whole fermentation time of 5 days. Measurements were performed hourly. The development of pH is displayed as (○) and ORP as (◇).

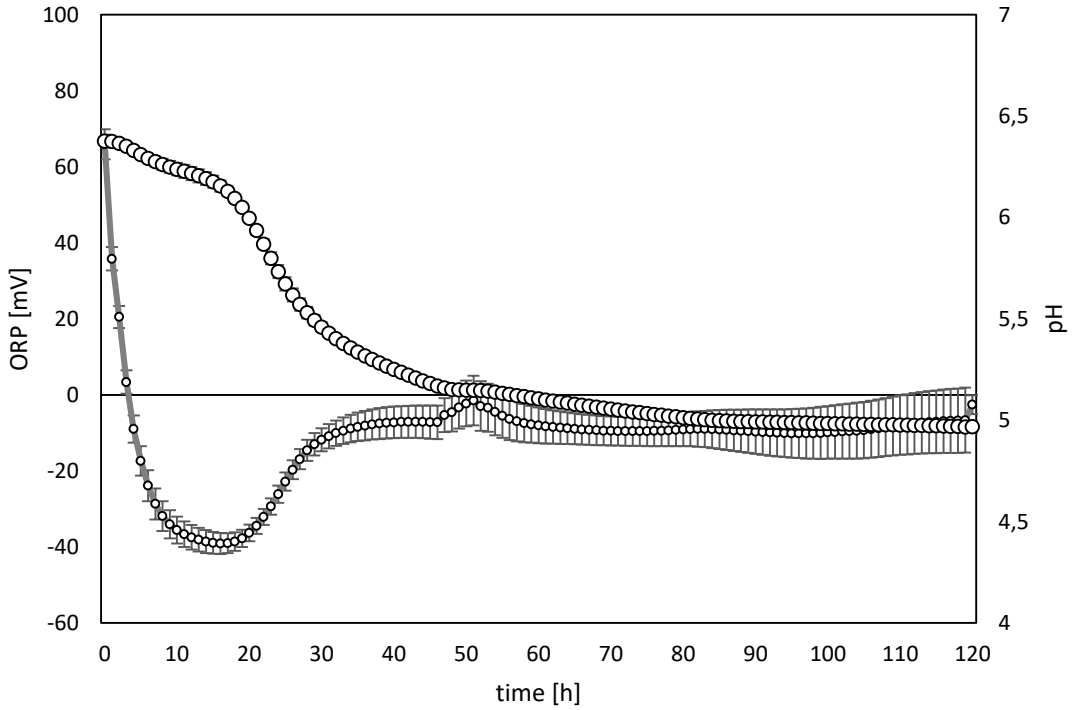

Figure S.3: Development of pH and redox potential (ORP) of set II over the whole fermentation time of 5 days. Measurements were performed hourly. The development of pH is displayed as (○) and ORP as (◇).

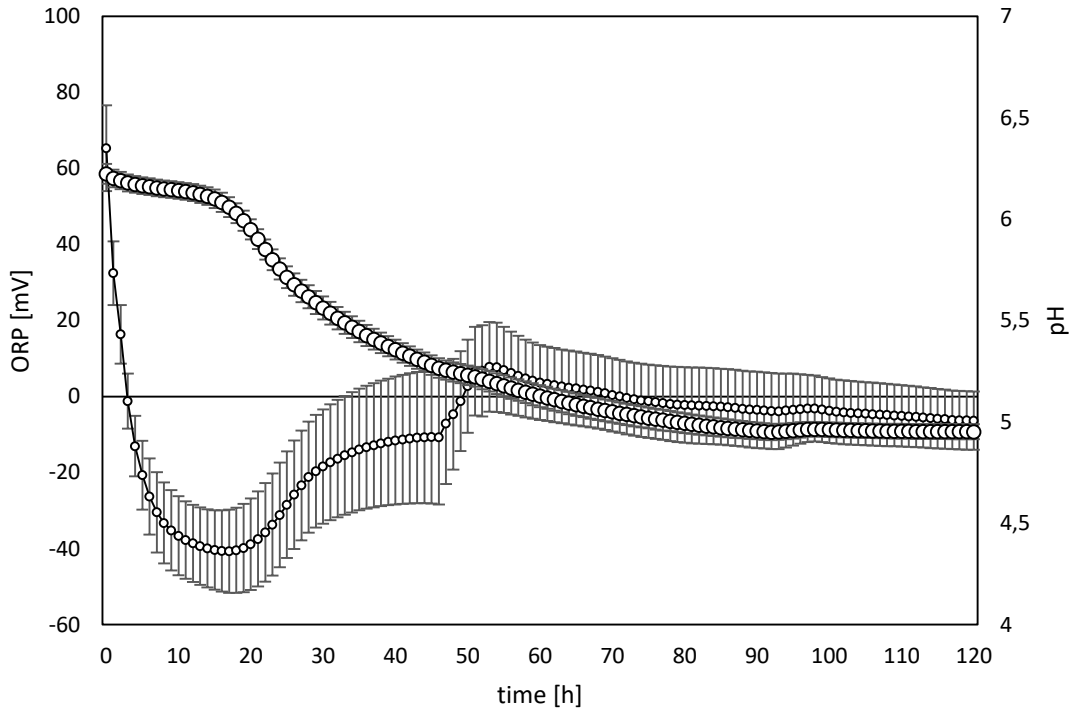

Figure S.5: Development of pH and redox potential (ORP) of set III over the whole fermentation time of 5 days. Measurements were performed hourly. The development of pH is displayed as (○) and ORP as (◇).

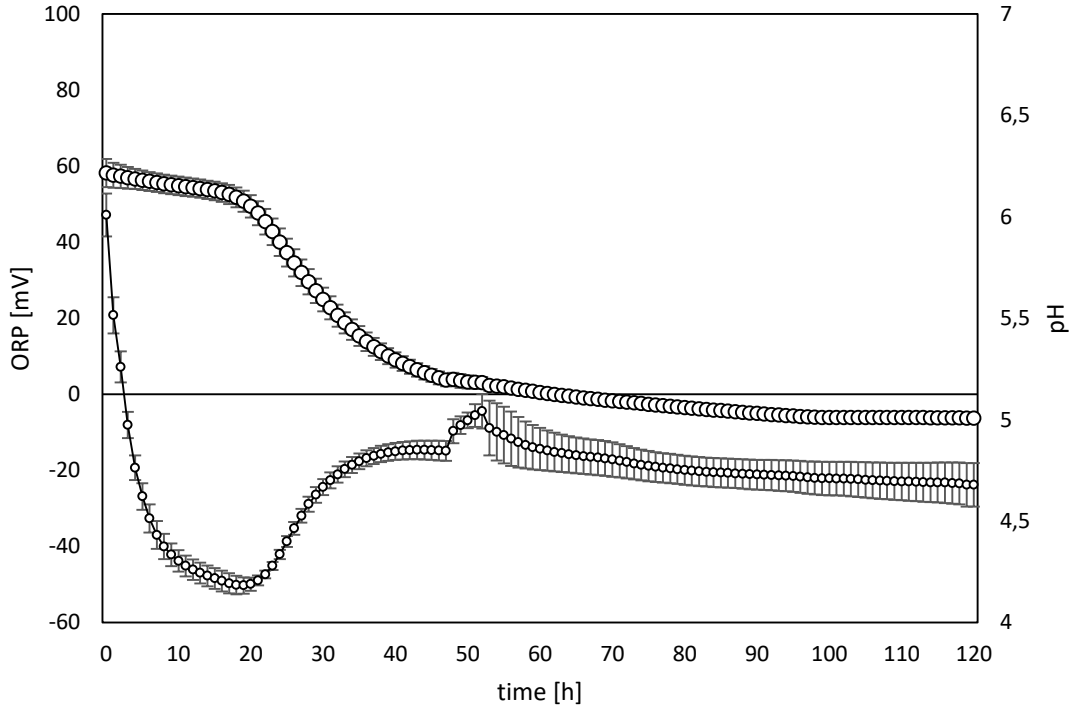

Figure S.4: Development of pH and redox potential (ORP) of set IV over the whole fermentation time of 5 days. Measurements were performed hourly. The development of pH is displayed as (○) and ORP as (◇).

Table S.1: Found gene clusters associated with the formation of bacteriocins. Genomes were analyzed using BAGEL3. (-) no bacteriocin gene clusters found within the genome (+) bacteriocin clusters found within the genome

species	TMW	Sakacin Q	Enterocin	SakT α	Carnocin	Bacteriocin	Putative bacteriocin
L. curvatus	1.27	+	-	-	-	-	+
	1.401	+	-	-	-	-	+
	1.407	-	-	-	-	-	-
	1.421	+	-	-	-	-	+
	1.439	+	-	-	-	-	+
	1.595	+	-	-	-	-	+
	1.624	+	+	+	-	+	+
	1.1381	+	-	-	-	-	+
	1.1390	-	-	-	-	-	-
L. sakei	1.3	-	-	-	-	+	-
	1.46	-	-	-	+	-	-
	1.114	+	-	-	-	-	-
	1.417	-	-	-	-	-	-
	1.578	+	-	-	-	-	-
	1.1189	-	-	-	-	-	-
	1.1239	-	-	-	-	-	-
	1.1396	-	-	-	-	-	-
	1.1398	-	-	-	-	-	-

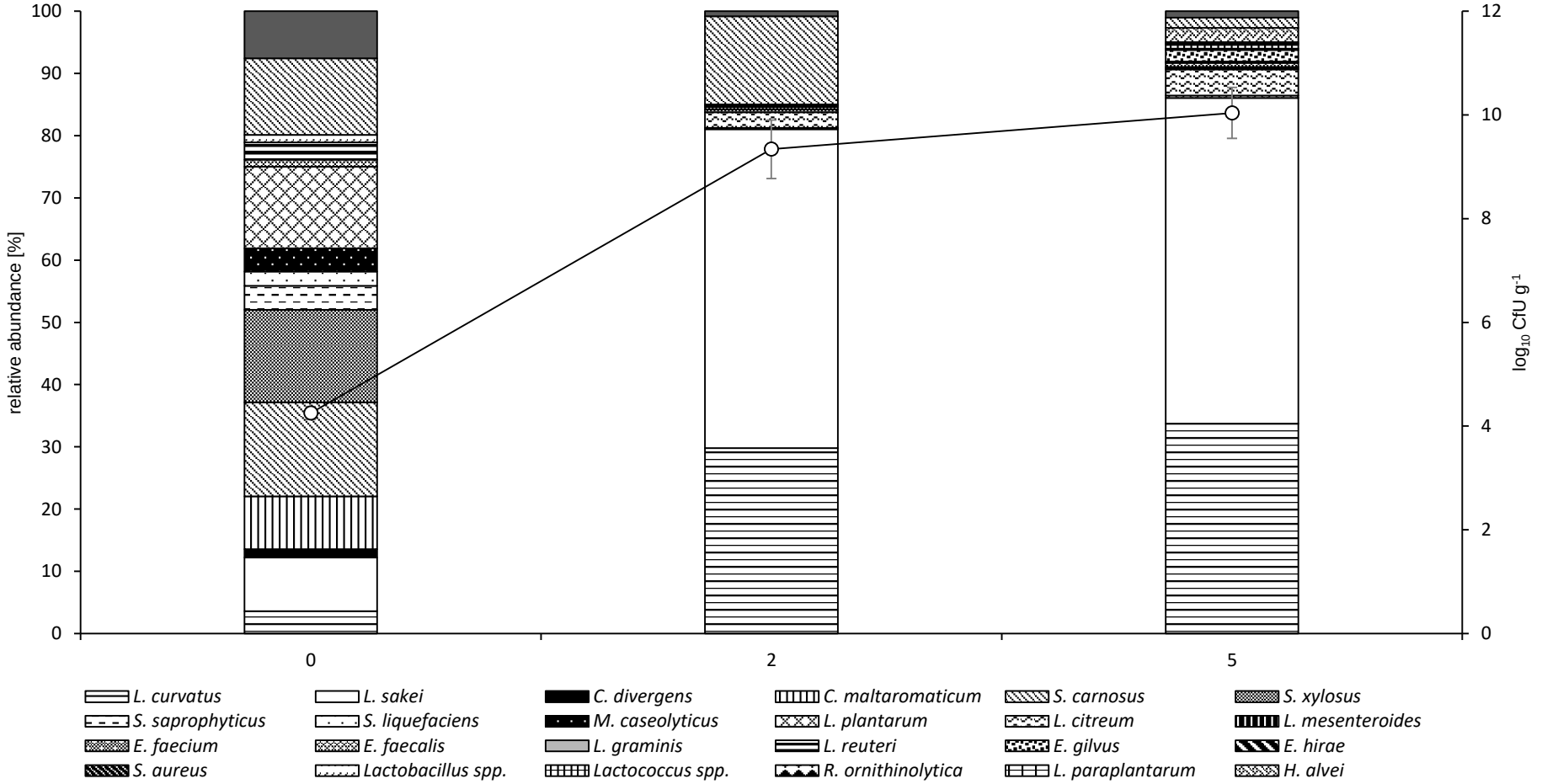

Figure S.6: Microbiota dynamics in the sausage model of control batches Total viable count [log_{10} CFU g^{-1}] on mMRS and microbiota composition as the relative abundance [%] at defined time points [day 0, 2 and 5] during fermentation (n=5).

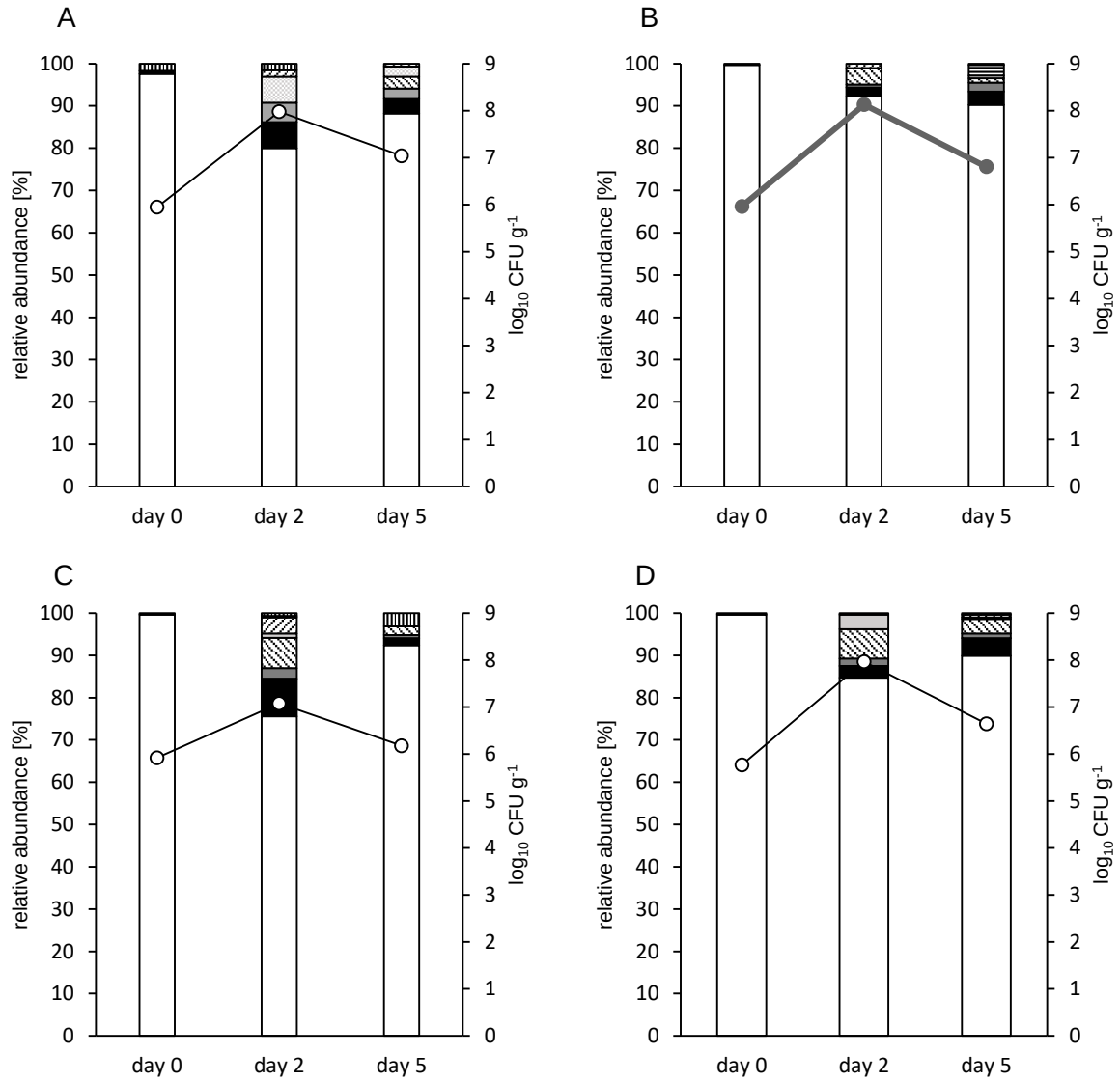

Figure S.7: Microbiota dynamics in the sausage model determined on BP. Total viable count [log_{10} CFU g^{-1}] on BP and microbiota composition as the relative abundance [%] at defined time points [day 0, 5, 12 and 21] during fermentation. The figure shows the average of three biological replicates with standard deviation of set I (A), II (B), III (C) and IV (D). Detection limit log_{10} 1 CFU g^{-1}. Species were identified with MALDI-TOF MS as follows: (□) *S. carnosus* TMW 2.212, (■) *S. carnosus*, (■) *S. xylosus*, (□) *S. aureus* (▧) *S. saprophyticus*, (▨) *M. caseolyticus*, (▤) other and (▥) nri. The acronym nri stands for “not reliable identification”.

10 List of publications

This thesis was supported by the German Federal Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn) with the project number 18552 N. The money giving party did neither influence the design of the study nor interpretation of results.

First authorship publications in peer-reviewed journals derived from this thesis:

Janßen, Dorothee, Eisenbach, Lara & Ehrmann, Matthias & Vogel, Rudi F. (2018). Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in a fermented sausage model. International Journal of Food Microbiology. 10.1016/j.ijfoodmicro.2018.04.030.

Janßen, Dorothee & Ehrmann, Matthias & Vogel, Rudi F. (2018). Monitoring of assertive *Lactobacillus sakei* and *Lactobacillus curvatus* strains using an industrial ring trial experiment. Journal of Applied Microbiology. 10.1111/jam.14144.

Further participation in peer-reviewed journals:

Eisenbach, Lara, Janßen, **Janßen, Dorothee** & Ehrmann, Matthias & F. Vogel, Rudi. (2018). Comparative genomics of *Lactobacillus curvatus* enables prediction of traits relating to adaptation and strategies of assertiveness in sausage fermentation. International Journal of Applied Food Microbiology. 10.1016/j.ijfoodmicro.2018.06.025

Prechtl, Roman M., **Janßen, Dorothee**, Behr, Jürgen, Ludwig, Christina, Küster, Bernhard, Vogel, Rudi. F., & Jakob, Frank (2018). Sucrose-induced proteomic response and carbohydrate utilization of *Lactobacillus sakei* TMW 1.411 during dextran formation. *Frontiers in Microbiology*, *9*, 2796. doi:10.3389/fmicb.2018.02796

Poster presentations at academic symposia

Janßen, D., Eisenbach L. (2016). Biodiversity of *Lactobacillus sakei* and *Lactobacillus curvatus*. 19.07.16-22.07.16, Food Micro, Dublin, P02-SB-236

Janßen, D. (2017). Determination of the assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in a fermented sausge model using MALDI-TOF MS. 27.09.17-29.09.17, Fermented Meats, Clermont-Ferrand, France, P01

Oral presentations for FEI and AiF

Janßen, Dorothee, Eisenbach, Lara (05.11.2015). Biodiversity of *L. sakei* and *L. curvatus*, Freising-Weihenstephan.

Janßen, Dorothee (28.10.16). Assertiveness of *L. sakei* and *L. curvatus* in a fermented sausage model system, Freising-Weihenstephan.

Janßen, Dorothee (15.02.18). Assertiveness of *L. sakei* and *L. curvatus* in raw fermented sausages, Freising-Weihenstephan.

Student theses and contributions to this thesis

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

Anna Widenmann – Bachelors Thesis: “Integration sites and inducibility of prophages in the genome of *L. sakei”*. 16.10.2017-15.01.2018

Lena Dworschak – Master Thesis: “Assertiveness of *L. sakei* and *L. curvatus* in raw sausage fermentations”. 11.0612018-07.12.2018

Collaborations

LC-MS/MS analysis of UV-induced samples was conducted in collaboration with BayBioMS and Jürgen Behr.

Electron Microscopy was conducted by Prof. Dr. A. Klingl at the Chair of Plant Development, Ludwig-Maximilians University, Munich.

11 Statutory declaration

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

“**Assertiveness of *Lactobacillus sakei* and *Lactobacillus curvatus* in raw sausage fermentations”**

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 “publication and contribution” section.

Freising,

Dorothee Janßen

12 Acknowledgements

At the very end I would like to take the opportunity to thank all those who have supported me in writing this thesis.

First of all, I would like to thank my doctoral supervisor Prof. Dr. Rudi F. Vogel, for all his help, the fruitful discussions and guidance along the way.

A special thanks to my mentor Prof. Dr. Matthias Ehrmann, who always supported me, especially concerning the prophage research. Thank you, for all the good suggestions and the motivation when I needed it the most.

Special thanks go to my dear colleagues Dr. Maik Hilgarth, Evelin Wigmann, Julian Schuster, Carolin Schiffer and Lena Dworschak, for the many laughs, the best office and all the good times we had at the laboratory and in our time we spent together in private life. You became friends and I will never forget what you all have done for me. It was a pleasure.

I also want to thank all the other current and former members of the department of Technical Microbiology at TUM for their great helpfulness and the great atmosphere in the lab.

I dedicate this dissertation to my mother Barbara Janßen, my father Raimund Janßen and my grand-father Heinz Janßen. Thank you for your unconditional support and your love. You always believed in me. I´ve done it also for you, grandpa, as you never had the chance.

This last word of acknowledgement I have saved for the very special person in my life, my beloved fiancé Christian Gaigl. I would have never made it without you. Thank you so much!