Effect of micro biocides on *Listeria monocytogenes*

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ABBREVIATIONS

ABC transporter	ATP-binding cassette transporter
AOAC	Association of Analytical Chemists
APHA	American Public Health Association
ATP	Adenosine triphosphate
BAC	Benzalkonium chloride
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pairs
BSI	British Standards Institute
CAC	Codex Alimentarius Commission
CEN	European Committee for Standardization
CFU	Colony-forming units
CKC	Cetalkonium chloride
C_T	Threshold cycle
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotides triphosphate
e.g.	for example
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMCCD	Electron-multiplying charge-coupled camera
EU	European Union

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	US Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
GABA	γ-aminobutyrate
GAD	Glutamate decarboxylase
GMP	Good manufacture practice
HACCP	Hazard analysis and critical control points
i.e.	That is
IDF	International Dairy Federation
LB	Luria and Bertani broth
LOD	Limit of detection
LOQ	Limit of quantification
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
NNS	Nisin non-susceptible
NS	Nisin susceptible
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
QAC	Quaternary ammonium compounds
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SC	Sour curd
SCC	Sour curd cheese

SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SNBI	Standard nutrient broth I
TAE	Tris-acetate-EDTA
TCS	Two-component signal transduction systems
TRIS	Tris(hydroxymethyl)-aminomethan
TSB	Tryptic soy broth
USA	United States of America
WGS	Whole genome sequencing
WHO	World Health Organisation
x g	Times gravitational force
η	Correlation ratio coefficient
τ_b	Kendall Rank Correlation Coefficient

SUMMARY

Listeria monocytogenes is a major foodborne pathogenic bacterium causing listerioses, which leads to human health problems and economic losses globally. Transmission of the disease to humans is usually due to consumption of *L. monocytogenes* contaminated food, e.g. dairy produce. Food safety management systems provide guidance to control the foodborne pathogens by a strict cleaning and sanitizing regime. Hence, micro biocides are applied in dairy production plants to prevent outgrowth of *L. monocytogenes*.

The goal of the first study of this thesis was to evaluate the efficacy of micro biocides and to determine the tolerance of micro biocides to *L. monocytogenes* field isolates. In the second study, it tackles about the response of *L. monocytogenes* field isolates of the antimicrobial preservative free nisin since it is frequently added to soft cheese to effectively inhibit *L. monocytogenes*. These data provided the prerequisites of the last study for employing nisin formulations *in vitro* and in sour curd cheese (SCC).

Two comprehensive panels with 251 and 282 *L. monocytogenes* field isolates from German raw food products, ready-to-eat foods, patient samples and food-processing environments as well as *Listeria* spp. reference strains were analyzed in the first study. For the first and last study, broth microdilution was applied as assay for efficacy and susceptibility testing. Hence, four disinfectant compounds (H_2O_2, NaOCl, benzalkonium chloride and cetalkonium chloride), two long-established antimicrobial preservatives (free nisin and $NaNO_2$) as well as one flavoring substance (citral), a potential new antimicrobial preservative, were tested against *L. monocytogenes* field isolates. An experimental design was established enabling a high comparability between all micro biocides, which were dissolved in culture broth mimicking organic debris. The efficacy of micro biocides was exceptionally unaltered in the presence of

organic compounds except for disinfection compound NaOCl. Moreover, high minimal inhibitory concentrations (MICs) of NaOCl and citral were correlated to MICs of two important therapeutic antibiotics while no correlation was found for free nisin. The majority of nisin non-susceptible *L. monocytogenes* field isolates (NNS) were serotype IIa and were found in dairy produce. This observation raised the question why NNS field isolates of serotype IIa were frequent in this environment whereas occurrence in other origin of isolation was lower.

To address this question in the second study, four nisin susceptible (NS) and two NNS *L. monocytogenes* field isolates of serotype IIa were subjected to whole genome sequencing. Subsequent analysis of genes putatively associated with nisin tolerance and its regulation resulted to DNA sequence variants (DSVs) in the *gadD2* gene encoding for the glutamate decarboxylase that differed NNS from NS field isolates. The same specific DSVs in *gadD2* were found in seven more NNS field isolates. Likewise, NNS field isolates had a substantial shorter lag phase compared to NS in presence of free nisin at pH 7.0. The GadD2 model showed that due to an amino acid substitution at position 453, aspartic acid to asparagine, the active site was not blocked at pH 7.0. Presumably, this resulted to a less pH-depended enzyme activity.

The SCC matrix was identified as an important factor reducing the antimicrobial activity of free nisin. Hence, a new nisin formulation called Neusilin UFL2-N (UFL2-N) was developed within the last study of this thesis tailoring the release of nisin from Neusilin UFL2 under a sour curd likely environment. In BHI broth, UFL2-N was competitive to free nisin over a wide pH range with similar MICs. When both nisin formulation were applied on contaminated SCC surface, UFL2-N and free nisin showed antilisterial activity and kept *L. monocytogenes* below quantification limit of qPCR at the highest applied concentration.

Collectively, the data indicated that the efficacy of micro biocides against *L. monocytogenes* was affected by organic debris while MICs of nisin were not increased. Results confirmed the ongoing discussion that DSVs in *gadD2* supports NNS state. Lastly, UFL2-N enabled a slow release and antilisterial activity *in vitro* as well as on SCC surface.

ZUSAMMENFASSUNG

Listeria monocytogenes ist ein bedeutendes, durch Lebensmittel übertragenes pathogenes Bakterium, welches Listeriosen verursachen kann und daher weltweit zu gesundheitlichen Problemen und wirtschaftlichen Verlusten führt. Der Verzehr von Lebensmitteln, die kontaminiert mit *L. monocytogenes* sind, führt zur Übertragung der Krankheit auf den Menschen, zum Beispiel durch Milchprodukte. Lebensmittelsicherheitsmanagementsysteme geben Leitlinien zur Kontrolle von Krankheitserregern, die durch Lebensmittel übertragenen werden, vor. Dies kann durch ein strenges Reinigungs- und Desinfektionsregiment erreicht werden. Mikrobiozide werden daher in Molkereiproduktionsanlagen eingesetzt, um das Wachstum von *L. monocytogenes* zu inhibieren.

Das Ziel der ersten Studie war es, die Wirksamkeit von Mikrobioziden zu bewerten und die Empfindlichkeit von *L. monocytogenes* Feldisolate gegenüber Mikrobioziden zu bestimmen. In der zweiten Studie wurde ebenfalls die Zellantwort der *L. monocytogenes* Feldisolate auf das antimikrobiell wirkende Konservierungsmittel Nisin untersucht. Häufig wird Weichkäse mit freiem Nisin versetzt, um *L. monocytogenes* wirksam zu hemmen. Diese erhobenen Daten lieferten die Grundlage für die letzte Studie, um die Nisinformulierungen *in vitro* und in Sauermilchkäse (SMK) zu erproben. Zwei Panel mit jeweils 251 und 282 *L. monocytogenes* Feldisolaten aus deutschen Rohprodukten, verzehrfertigen Lebensmitteln, Patientenproben und aus lebensmittelverarbeitenden Umgebungen sowie *Listeria* spp. Referenzstämme wurden in der ersten Studie analysiert.

In der ersten und letzten Studie wurde die Mikrodilution in Bouillon als Test für die Wirksamkeits- und Toleranzstests verwendet. Daher wurden vier Desinfektionsmittel (H_2O_2, NaOCl, Benzalkoniumchlorid und Cetalkoniumchlorid), zwei seit langem

verwendete antimikrobielle Konservierungsmittel (freies Nisin und $NaNO_2$) und ein Aromastoff (Citral), welcher ein potentiell neues antimikrobielles Konservierungsmittel darstellt, gegen *L. monocytogenes* Feldisolate getestet. Vorab wurde ein experimentelles Design entwickelt, welches eine hohe Vergleichbarkeit in Bouillon, die organische Rückstände simulierte, zwischen allen Mikrobioziden ermöglichte. Die Wirksamkeit der Mikrobioziden war in Gegenwart von organischen Verbindungen außer beim Desinfektionsmittel NaOCl unverändert. Darüber hinaus korrelierten hohe minimale Hemmkonzentrationen (MHKs) von NaOCl und Citral mit MHKs von zwei therapeutisch relevanten Antibiotika, während für freies Nisin keine Korrelation gefunden wurde. Die Mehrheit der nisintoleranten *L. monocytogenes* Feldisolate (NNS) war vom Serotyp IIa und wurde in Milchprodukten gefunden. Diese Beobachtung führte zu der Frage, warum NNS-Feldisolate des Serotyps IIa in dieser Umgebung häufig vertreten waren, während ihr Vorkommen in anderen Lebensmittelumgebungen geringer war.

Um diese Frage in der zweiten Studie zu beantworten, wurden vier Nisin empfindliche (NS) und zwei NNS *L. monocytogenes* Feldisolate des Serotyps IIa einer vollständigen Genomsequenzierung unterzogen. Die nachfolgende Analyse von Genen, die mutmaßlich mit Nisintoleranz und deren Regulation assoziiert sind, fand DNA-Sequenzvarianten (DSVs) im *gadD2*-Gen, welches für die Glutamatdecarboxylase kodiert. Dort unterschieden sich die NNS von NS Feldisolate von einander. Die gleichen spezifischen DSVs in *gadD2* wurden ebenfalls in sieben weiteren NNS Feldisolaten gefunden. In Gegenwart von freiem Nisin und bei einem pH-Wert von 7,0 hatten NNS Feldisolate im Vergleich zu NS Feldisolate eine wesentlich kürzere Verzögerungsphase in der Wachstumskurve. Modellierung des GadD2 zeigte, dass durch ein Aminosäureaustausch an Position 453, Asparaginsäure zu Asparagin, das

aktive Zentrum bei pH 7,0 nicht blockiert wurde. Dies führte vermutlich zu einer weniger pH-abhängigen enzymatischen Aktivität.

Der SMK wurde als wichtige Matrix identifiziert, die die antimikrobielle Aktivität von freiem Nisin reduzierte. Daher wurde im Rahmen der letzten Studie eine neue Nisinformulierung, Neusilin UFL2-N (UFL2-N), entwickelt. Diese ermöglichte die Freisetzung von adsorbierten Nisin aus Neusilin UFL2 in einer Umgebung, die dem pH des Sauerquarks ähnelte. In BHI-Bouillon und über einen weiten pH-Bereich war UFL2-N in der Lage, mit freiem Nisin zu konkurrieren. Die resultierenden MICs beider Nisinformulierungen waren vergleichbar. Wenn die Nisinformulierungen auf kontaminierten SMK Oberflächen aufgetragen wurden, zeigten bei der höchsten getesteten Konzentration sowohl UFL2-N als auch freies Nisin antilisteriale Aktivität und *L. monocytogenes* blieb unterhalb der Quantifizierungsgrenze der qPCR.

Insgesamt zeigten die Ergebnisse, dass die Wirksamkeit der Mikrobiozide gegen *L. monocytogenes* durch organische Rückstände beeinträchtigt wurde, während die MHKs von Nisin nicht erhöht waren. Weiterhin bestätigten die Resultate die laufende Diskussion, ob DSVs in *gadD2* die Nisintoleranz unterstützen. UFL2-N ermöglichte eine langsame Freisetzung und antilisteriale Aktivität *in vitro* sowie auf der SMK Oberfläche.

1. INTRODUCTION

1.1. *Listeria monocytogenes*

1.1.1. Phylogenetic characteristics

To date, the genus *Listeria* includes seventeen species namely *Listeria aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis*, *L. grandensis*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. newyorkensis*, *L. riparia*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis*, and *L. welshimeri* (Orsi and Wiedmann 2016). Beside *L. monocytogenes*, only *L. ivanovii* and a few *L. innocua* field isolates are considered as pathogenic mainly in animals (Johnson et al. 2004). The other five species in the group *Listeria* sensu strictu, *L. innocua, L. welshimeri, L. seeligeri*, *L. grayi* and *L. marthii*, are non-pathogenic (Orsi and Wiedmann 2016). Eleven species (*Listeria* sensu lato) have been newly identified (Orsi and Wiedmann 2016). None of *Listeria* senso lato species showed pathogenicity (Bertsch et al. 2013; den Bakker et al. 2013; den Bakker et al. 2014; Lang Halter et al. 2013; Leclercq et al. 2010; Weller et al. 2015). To differentiate and identify *L. monocytogenes* from most *Listeria* species, in the first instance its hemolytic capabilities are utilized in hemolytic tests and phosphoinositide phospholipase C activity tests. These principles are utilized in chromogenic media for detection of the pathogenic species during cultivation. The hemolytic test is based on the production of listeriolysin O leading to erythrolysis and degradation of hemoglobin (Ryser and Donnelly 2013). Phosphatidylinositol-specific phospholipase C catalyzes the hydrolysis of phosphatidylinositol and glycosylphosphatidylinositol. These lipids are anchored on the external surface of eukaryotic cells by a glycosylphosphatidylinositol moiety (Ferguson 1988; Mengaud et al. 1991). Thus, this enzyme might play a role during pathogenesis.

Listeria monocytogenes are further differentiated by serotypes based on somatic (O) and flagellar (H) antigens (Seeliger and Jones 1986). Serotyping provides a helpful description for epidemiological studies and investigations. Currently there are thirteen known serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (Allerberger 2003). These serotypes can also be grouped into molecular serotypes IIa (1/2a and 3a), IIb (1/2b, 3b and 7), IIc (1/2c and 3c), IVa (4a, 4ab and 4c), and IVb (4b, 4d, 4e) (Doumith et al. 2004). Thereof, serotypes IIa, IIb, IIc, and IVb are most likely found in contaminated food and IIa as well as IVb are commonly associated with listeriosis (Cartwright et al. 2013; Norton et al. 2001; Vallim et al. 2015). However, it is of more interest and relevance in dairy industry to trace back the origin of outbreaks. This can be achieved by genotyping also often referred to as molecular epidemiology. Discriminated *L. monocytogenes* field isolates by genetic techniques are then linked with clinical infections.

1.1.2. Listeriosis

Listeria monocytogenes can cause listeriosis, a severe illness that is globally distributed (de Noordhout et al. 2014). The aftermath of listeriosis outbreaks have a considerable impact on society and the food industry (Ivanek et al. 2004). People from risk groups such as elderly, immunocompromised people, pregnant women, and neonates suffer from symptoms like fever, diarrhea, and nausea. On the contrary, people not belonging to risk groups barely notice the infection of *L. monocytogenes* due to less severe symptoms. Once *L. monocytogenes* contaminated food is consumed, the pathogen can invade epithelial cells and surpass blood-brain barrier (Disson and Lecuit 2012). Once in blood, it causes encephalitis or meningitis with high fatality rate (Farber and Peterkin 1991). However, the documented fatal cases are low ranging from 15–30 deaths/100 cases in USA and France outbreaks (Crim et al. 2015;

Goulet et al. 2006). The bacterium can invade intracellularly its host cells and *L. monocytogenes* can evade and remain undetected by the host's immune system (Pizarro-Cerdá et al. 2012).

1.1.3. Prevalence and prevention of proliferation in food production plants

The wide diversity of risk food products are raw food (e.g. raw milk, vegetables, and meat) and ready-to-eat foods (e.g. soft cheese from pasteurized milk, sausages, smoked salmon, and salad) (Müller and Weber 1996). Ready-to-eat foods are products that are consumed raw or are minimal processed before eating, and which could allow pathogens to proliferate. However, the Gram-positive, non-sporeforming and motile bacterium can be also found in animals, plants, soil, water as well as in human and animal feces (Ryser and Donnelly 2013). Presence of *L. monocytogenes* field isolates in dairy produce like in raw milk and non-thermal treated soft cheeses continue to occur and is of paramount interest as consumption led to outbreaks (Cartwright et al. 2013; EFSA 2015).

One reason for *L. monocytogenes* to succeed in food production plants is its ability to withstand a broad temperature, salt and pH range. Moreover, the shared psychrotrophic characteristics of *Listeria* sensu strictu (Orsi and Wiedmann 2016) makes it a good competitor against other microorganisms. *Listeria monocytogenes* can survive from -0.4 °C to +50.0 °C (Farber and Peterkin 1991; Gill and Reichel 1989; Hwang et al. 2009). Although a minimum of 72.0 °C for fifteen seconds is considered effective in reducing pathogens during pasteurization (IDF 1994), some field isolates have been reported to survive this pasteurization step (Farber and Peterkin 1991).

Listeria monocytogenes also resists high salt concentration of 10.0-20.0 % (FAO/WHO 2004), low pH (O'Driscoll et al. 1996) and nitrosative stress (Hwang et al. 2009; McClure et al. 1991). Those harsh conditions limiting bacterial growth are commonly

found as intrinsic food properties. Nevertheless, the structure within the food matrix can influence local conditions, which enable proliferation of *L. monocytogenes*. Hence, foodborne contamination with pathogens enable transmission to human and upon exposure *L. monocytogenes* is virtually always responsible for reported listeriosis (Scallan et al. 2011).

Primary contaminations of unprocessed foods or ingredients e.g. starter cultures and potable water were described as recurring contamination source (Fretz et al. 2010a; Lundén et al. 2003; McIntyre et al. 2015). Thus, Lundén et al. (2003) was able to track the bacterium to many stages within the facility. Secondary contaminations were described by processing of food products (McIntyre et al. 2015; Pak et al. 2002), by employees (Montville et al. 2001; Salvat et al. 1995; Scott and Bloomfield 1990), by environment (Parisi et al. 2013; Silva et al. 2003; Spanu et al. 2015), and by surfaces (Parisi et al. 2013). In dairy production plants common recontamination occurs on objects or at sites such as cleaning tools, containers, conveyor belts, coolers, drains, floors, insulation, pumps, racks, slicers, standing water, and walls. The level of contamination on these reservoirs, which can lead to proliferation of *L. monocytogenes*, can be controlled by pre-treatment of raw milk with different technologies like pasteurization and bactofugation combined with a strict cleaning and sanitizing regime. The counteractive measures should be applied in regular intervals with disinfectants (**Table 1**).

Table 1: Areas that should be cleaned with disinfectants and common reservoirs of *L. monocytogenes*. Adopted from Tompkin et al. (1999).

Area	Frequency	Site for contamination
Drains	Daily	e.g. cracked hose
Floors	Daily	e.g. standing water, cleaning tools
Waste containers and storage	Daily	e.g. equipment, collators, racks, containers
Walls	Weekly/monthly	e.g. cracks, overhead structures, catwalks, insulation
Condensate drip pans	Weekly/monthly	Yes
Coolers	Weekly/monthly	Yes
Spiral freezers	Semi-annually	e.g. wall, crevices

Although the European Union (EU) has established microbiological limits for the prevalence of *L. monocytogenes* in different food categories (EU 2007a), food products that are recognized as contaminated with *L. monocytogenes* are withdrawn from the market. These are communicated through the Rapid Alert System for Food and Feed (RASFF) portal (EU 2011b) or are directly withdrawn from the market by the dairy production companies without public awareness. In contrast to the EU policy of determining acceptability levels of *L. monocytogenes* in risk foods, the United States follows a rigid zero tolerance policy in ready-to-eat foods.

In conclusion, presence of ubiquitous occurring *L. monocytogenes* cannot be totally avoided in dairy production plants but growth of *L. monocytogenes* can be effectively controlled by strict sanitizing regime and trained personnel.

1.2. Micro biocides and their application in dairy production plants

Meeting the mandatory microbiological regulation by governmental bodies, dairy production plants have to design and apply food safety management systems. One management tool is Hazard Analysis and Critical Control Points (HACCP) and Good Manufacture Practice (GMP). The HACCP concept was developed by a collaboration

of the Pillsbury Company and NASA in the 1960s to ensure that meals for astronauts during space flights are safe to consume (APHA 1971). The US Food and Drugs Administration (FDA) was first to include HACCP into the food production by regulating low-acid canned foods (FDA 1973). Since then it was implemented in dairy food production (CAC 2003). Based on process management principles, HACCP nowadays follows the four pillars of the Deming cycle (Plan-Do-Check-Act) (ISO 22000:2018). After hazard analysis, the critical control points (CCP) are identified (Plan). At these points, control is critical to assure food safety, e.g. pasteurization to control foodborne pathogens. The next steps are implementing critical limits of the HACCP plan, establishing a system to monitor control of CCPs, evaluating the performance (Do and Check), followed by formulating the corrective actions for improvement of a CCP if it is not under control (Act). All actions in establishing a HACCP plan are obligatory for proper verification and documentation. The ISO 22000:2018 provides internationally harmonized requirements for food safety to all types of organizations in food production including producers of cleaning agents, which can be audited (ISO 22000:2018). Moreover, the EU (2004) provides a general guidance for food business operators to implement the HACCP concept, which takes sanitizing, handling of food products, personnel training, plant design and waste management into consideration.

Secondary contaminations after pasteurization are generally controlled by a strict cleaning and disinfection regime, which can be briefly summarized in the following steps: (I) dry clean, (II) pre-rinse, (III) foam and scrub, and (IV) rinse. The cleaning and disinfection regime typically involves usage of micro biocides to prevent fungal (fungicides) or microbial (bactericides) growth. Micro biocides are defined as all chemical or natural created compounds or mixtures thereof, which destroy, inhibit or reduce growth of any harmful organism (EU 2012b). Generally, disinfectants and biocidal products are grouped into different product types (PT) by the Biocidal Products

Regulation EU 528/2012 (EU 2012b). After 2007, the European Commission identified about 350 micro biocides as available on the market (EU 2007b). A few natural substances are included in their list. However, the presumably most important biocidal active substances in terms of number of substances are within PT2 (**Table 2**).

Table 2: Overview of disinfectants and general biocidal products in main group 1 according to their product type (PT). Adopted from the Biocidal Products Regulation (EU) 528/2012 and EU 2009.

PT	No. of substances	Purpose
1	50- 99	Human hygiene biocidal products
2	150-199	Private area and public health area biocidal products
3	100-149	Veterinary and hygiene biocidal products
4	100-149	Food and feed area disinfectants
5	50- 99	Drinking water disinfectants

Across the dairy industry general disinfectant compounds such as hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl) and quaternary ammonium compounds (QACs) are applied (van Houdt and Michiels 2010). The total annual production volume of these three disinfectant compounds was about 400,000 tons within the EU (EU 2009). Commercial disinfectants for intended use in food production are often a mixture of different biocidal compounds. Prominent compounds beside QACs in commercial disinfectants include acetic acid, glutaraldehyde, peracetic acid, sodium hydroxide, and sodium peroxide (Conficoni et al. 2016; Møretrø et al. 2017). Common areas of application include food processing, distribution and retailing of dairy produce (**Figure 1**).

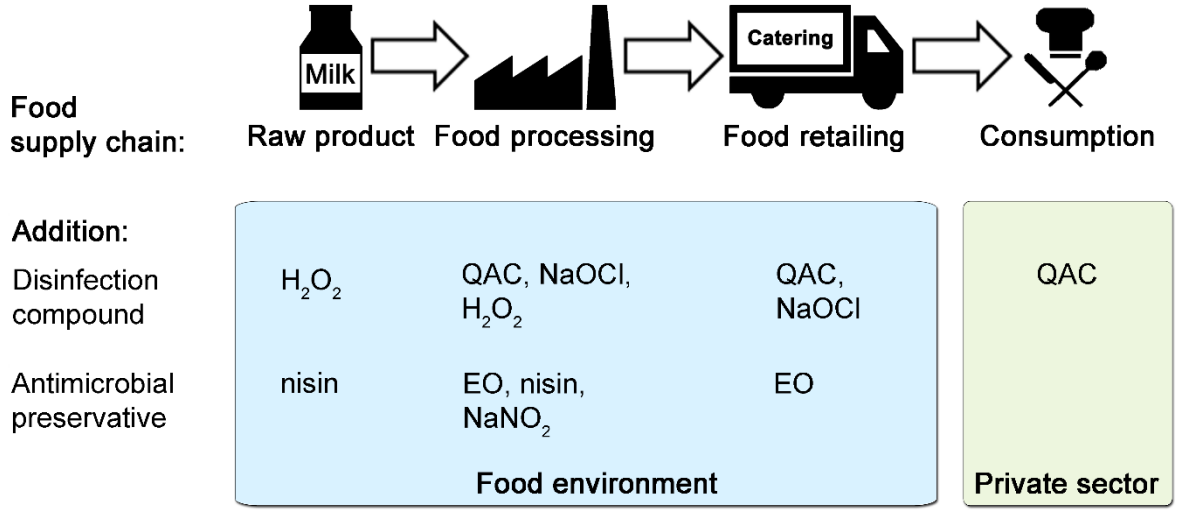

Figure 1: The farm to fork process in dairy industry. Micro biocides such as disinfection compounds and antimicrobial preservatives are applied at specific points along the food supply chain, e.g. raw milk, mixing, cutting, decontaminating, storage, logistic or personal hygiene. Usage and frequency depends on plant- or retail-specific design and food products. Abbreviation: H_2O_2 hydrogen peroxide; QAC quaternary ammonium compound; NaOCl sodium hypochlorite; EO essential oil; $NaNO_2$ sodium nitrite.

However, the choice and use of micro biocides in dairy production plant should undergo risk assessments in prerequisite programs to be primarily in compliance with the environmental conditions, e.g. non-corrosive, stability over broad ranges of pH and temperature. The mode of application such as accessibility to certain areas in a dairy production plant or machine parts, exposure time to microorganisms and concentration as well as the sensitivity of individual *L. monocytogenes* field isolates affected the bioavailability and efficacy of micro biocides (Conficoni et al. 2016). Moreover, the cleanliness of the objects and surfaces in terms of organic debris reduced the efficacy of micro biocides as shown previously (Dominguez et al. 1987; El-Kest and Marth 1988). The prerequisite programs are also advisable for the implementation chosen micro biocides as well as work instructions for the personnel in the daily routine, since

ISO 22000:2018 and EU (2004) are non-specific on appropriate usage of micro biocides. Nevertheless, findings from these programs should be defined independently from HACCP and must be documented and regularly reviewed (Sperber et al. 1998).

Organic and inorganic compounds added directly to food products as food additives or flavoring substances are also termed as micro biocides in this thesis. Food additives are any substances that are not integral part or characteristic ingredient of food products or are added for a technological purpose in the manufacture process (EU 2008a). A flavoring substance is a defined chemical substance with flavoring properties to impart or modify odor and/or taste (EU 2008b).

Antimicrobial preservatives extend the shelf life of dairy food products and on the other hand as per definition, do not interfere with the sensory food quality like off flavor or texture. Sometimes antimicrobial preservatives aid to retain a certain food characteristic. Sodium nitrite ($NaNO_2$), for example, is primary used to obtain the typical red color of meat and is added before fermentation (Cammack et al. 1999). Moreover, antimicrobial preservatives like nisin and $NaNO_2$ are beneficial in preventing food spoilage (Delves-Broughton et al. 1996; Pierson et al. 1983) and are applied to milk or during dairy processing after heating and before packaging (**Figure 1**). Unlike disinfectants, which eliminate as many organisms as possible, antimicrobial preservatives act more specific against spoilage organisms. For example, the food additive nisin interacts electrostatically with the bacterial cell membrane of Gram-positive bacteria (Bonev et al. 2004; Kordel et al. 2001). Both antimicrobial preservatives, $NaNO_2$ and nisin, are still used for preserving food products since early 20th century (Binkerd and Kolari 1975; Delves-Broughton et al. 1996; Doran 1917) and are permitted as food additives (EU 2008a). Recently, the consumer's social acceptance for long-established chemical treated food is changing and their demand

for untreated or treated with natural and sustainable compounds food products is increasing (Burt 2004). Thus, food additives that can be labeled as “natural” are frequently applied to enhance food safety (EU 2011a; JECFA 2010). Also, highly specialized starter cultures are used to meet customer demands (Benech et al. 2002a; Benech et al. 2002b; Carminati et al. 1999; Maisnier-Patin et al. 1992; Ye et al. 2018). Moreover, essential oils (EOs) and EO components have raised attention as an alternative in inhibiting foodborne pathogens in food products to avoid spoilage. EOs are volatile and fragrant compounds that are biosynthesized by plants. They are typically complex mixtures of EO components with an oily consistency. The EU has approved a variety of EO components as flavoring substances and the FDA classified the monoterpenoid citral as Generally Recognized As Safe (GRAS) (EU 2012a; FDA 2016). EOs can also be added to cheese by food retailers and before consumption in German traditional recipes such as “Handkäse mit Musik”.

1.3. Disinfectant compounds

1.3.1. Hydrogen peroxide

Hydrogen peroxide is a relatively cheap disinfectant compound and is produced in large volumes. It can be used in aseptic packaging and for surface disinfection in food production plants (PT4) (EU 2012b). The disinfectant compound H_2O_2 is also found in commercial disinfectant formulations and is effective against *L. monocytogenes* field isolates (Aarnisalo et al. 2000).

Interestingly, the FAO/WHO reviewed the application of H_2O_2 and recommended its suitability in situations, in which cooling facilities are scarce due to technical, economical or practical reasons, to counterbalance the basic food quality (WHO 2006). The addition of the substrate H_2O_2 in form of sodium percarbonate to raw milk acts as a source of H_2O_2, which can activate the lactoperoxidase system (Haddadin et al.

1996). The lactoperoxidase system is a naturally occurring antimicrobial mechanism in secretions of mammalian species like bovine raw milk. The oxidized hypothiocyanate ($OSCN^-$) has antimicrobial effects on both Gram-positive and Gram-negative bacteria (Kamau et al. 1990; Reiter et al. 1976; Wolfson and Sumner 1994). Furthermore, H_2O_2 is permitted as direct food substance in other food products (FDA 2017a). However, major use of H_2O_2 remains as a disinfectant compound in dairy production plant. For a daily routine application, Robbins et al. (2005) recommend a 3.0 % H_2O_2 solution (30.0 mg ml^{-1}) with an appropriate contact time of 10 minutes against *L. monocytogenes* Scott A to accomplish a complete elimination.

1.3.2. Sodium hypochlorite

Chlorine-based disinfectant compound NaOCl is still the most frequently used disinfectant in food industry. The advantages of NaOCl over other disinfectant compounds is that it is cheap and deactivates microorganisms without interfering with the food quality (Luo et al. 2012). Among four commercial disinfectants, NaOCl resulted in the greatest log-reduction of *L. monocytogenes* field isolates (Carruthers et al. 2012). It is very effective against this foodborne pathogen. The antimicrobial effect of NaOCl is attributed to the free chlorine content. Free chlorine refers to the chlorine, which will deactivate the microorganisms and the manufacturer usually recommends 0.2 to 0.8 mg ml^{-1} NaOCl for at least two minutes for disinfection (Mustapha and Liewen 1989). The free chlorine content is consumed by proteinaceous debris from organic materials, which leads to inconsistent efficacy (Jo et al. 2018).

1.3.3. Quaternary ammonium compounds

Historically, QACs were introduced into the market in the mid-1930s. A QAC is a cationic, surface active compound with hydrophobic and hydrophilic regions in its

molecular structure (McDonnell and Russell 1999). They are most widely used in food industry as disinfectant compounds (Hegstad et al. 2010) and they are the preferred choice because QACs do not lose efficacy in the presence of organic debris (Svoboda et al. 2016). Their antimicrobial activity depends on the amount of carbon atoms in the alkyl chain. The maximum antimicrobial activity was found with fourteen carbon atoms (Paulus 2005). The prominent and best studied representative is benzalkonium chloride (BAC), which is a mixture of benzyldimethylalkylammonium chlorides with eight to eighteen alkyl chains, as this QAC was found with notable antimicrobial activity (D'Arcy and Taylor 1962). Applied concentrations of commercial disinfectants in terms of BAC as active compound are nontoxic, non-tainting, odor-free and typically ranges from 0.5 to 1.0 mg ml^{-1} (Hegstad et al. 2010). Another example with longer alkyl chain and higher hydrophobicity is cetalkonium chloride (CKC), which has the chemical formula $C_{25}H_{46}ClN$. The positive charge and the hydrophobic region of QACs suggests interaction with the cell surface (Gilbert and Moore 2005). The antimicrobial action is thought to occur by disruption of the bacterial cell membranes and cell walls (To et al. 2002). Tested Gram-positive *L. monocytogenes* field isolate was found to be the most sensitive to QAC compared to tested Gram-negative bacteria (Fazlara and Ekhtelat 2012). In Gram-negative bacteria, modifications in the fatty acid composition of the cell wall was shown to decrease interaction with QACs (Guerin-Mechin et al. 2000).

1.4. Antimicrobial preservatives

1.4.1. Nisin

In 1928, *Lactococcus lactis* (formerly known as *Streptococcus lactis*) were described to inhibit growth of other lactic acid bacteria (Rogers 1928). A few years later novel antimicrobial preservatives from a group of *Lactococcus* spp. were found (Mattick and Hirsch 1944) and nisin was isolated for the first time.

The 34 amino acid long-chained peptide with a molecular weight of 3,500 Da is produced by several subspecies of *Lactococcus lactis* (Delves-Broughton et al. 1996; de Vos et al. 1993). Nisin has many variants based on amino acid sequence as well as on post-translational modifications (Cheigh and Pyun 2005; Field et al. 2015; Siegers et al. 1996). Thirteen amino acids are subjected to post-translational modifications (Sahl et al. 1995; Siegers et al. 1996). The unusual lanthionines in nisin determine the overall chemical structure of the peptide and form five thioether bridges (**Figure 2**). The two natural occurring forms are nisin A and Z (Gross and Morell 1971; Mulders et al. 2005), which only differ by one amino acid. In nisin Z, histidine is substituted at the 27th position by asparagine (**Figure 2**). Nisin Z is preferred in food products because the polar asparagine side chain provides better diffusion characteristics in agar (de Vos et al. 1993).

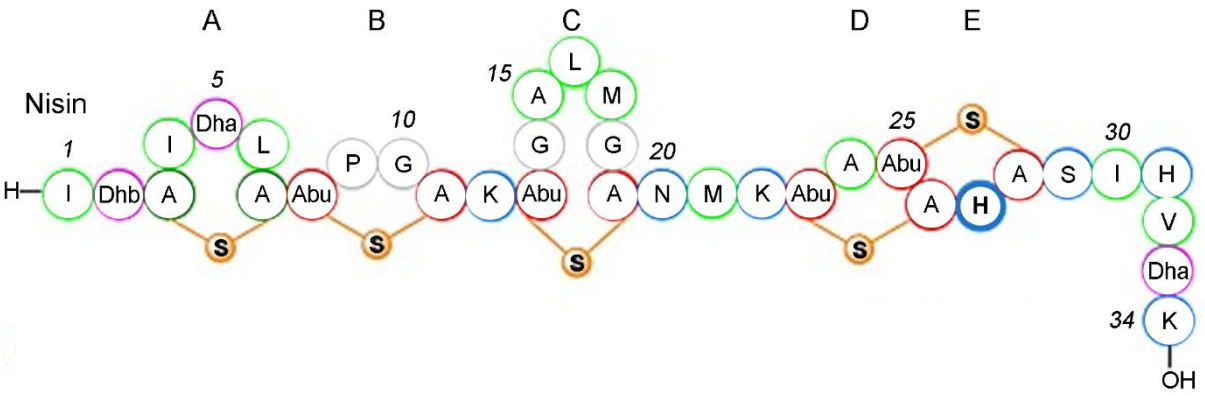

Figure 2: Schematic chemical structure of nisin A adopted from Slootweg et al. (2013). The amino residue at position 27 is asparagine in nisin Z (bold outline). Numbers above amino acids are counting the peptide length. Abbreviation: A alanine; Abu aminobutyric acid; Dha dehydroalanine; Dhb dehydrobutyrine; G glycine; H histidine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; P proline; S serine; V valine. Capitalized letters (A to E) indicate thioether bridges (A-S-A lanthionine; Abu-S-A β-methyllanthionine).

In addition, the chemical properties regarding dissociation in solvent and antimicrobial activity of nisin are unusual as the stability and antimicrobial activity of nisin is increasing with decreasing pH. Furthermore, nisin is stable when heated to 70.0 °C at pH 2.0 (Delves-Broughton et al. 1996; Liu and Hansen 1990).

1.4.1.1. Application of nisin to food products

Nisin is used for a long time by the food industries and it is one of the few natural peptides with GRAS status (FDA 1998). It is classified as nonhazardous by animal consumption (Frazer et al. 1962). Currently, application of nisin is allowed in 50 countries including the US (Delves-Broughton et al. 1996). However, the maximum nisin concentration in the final product is individually regulated in each country for different food products. In ripened or processed cheese, the commercial usage of nisin in the EU is allowed up to a limit of 12.5 mg kg^{-1} (EU 2011a).

Commercial nisin formulations contain 2.5 % nisin, which are stabilized in a mixture of non-fat milk solids and NaCl with a minimum content of 50.0 % (EC 2012). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) advised that in practical food applications, nisin should not be used in pure form because of its high activity against all Gram-positive bacteria including lactic acid bacteria (JECFA 1969). The JECFA panel defined that 0.025 µg of nisin is equal to one International Unit (IU) (JECFA 2013). Thus, 1.0 µg of nisin correspond to 40.0 IU. The activity of commercial nisin formulation was standardized by the EU and should contain not less than 900.0 IU mg^{-1} (EC 2012). Moreover, the production of nisin in *Lactococcus lactis* subsp*. lactis* is considered as a natural process. This is important for food production operators as nisin can be labeled as a natural antimicrobial preservative.

The antilisterial properties of nisin has been shown in milk, camembert and cheddar cheese (da Silva Malheiros et al. 2010; Benech et al. 2002a; Benech et al. 2002b; Jung

et al. 1992; Sulzer and Busse 1991). Due to minor importance, the application of nisin in surface-ripened cheese has been studied to a lesser extent.

Nevertheless, the practical application of nisin in cheese is limited by uncontrolled interactions with fat content, lipids, proteins or other organic components (Aasen et al. 2003; Bhatti et al. 2004; Chollet et al. 2008; Jung et al. 1992), thermostability (Liu and Hansen 1990) and proteolytic degradation processes (Sun et al. 2009).

1.4.1.2. Immobilization strategy of nisin

Since centuries, humanity use antimicrobial substances, drugs or physical preservation techniques to protect food from spoilage. To avoid chemical and microbial degradation of nisin (Schneider et al. 2011; Sun et al. 2009) and to maintain nisin activity (Gruskiene et al. 2017), novel technologies are continuously developed to immobilize nisin for a targeted, prolonged and cost-effective nisin release from carrier materials to food matrices.

There are several techniques for this purpose. For instance, the encapsulation in porous materials with a prolonged release or the smart technology of Layer-by-Layer (LbL) encapsulation (Donath et al. 1998; Peyratout and Dähne 2004; Pinheiro et al. 2015). The LbL technology exploits the alternating deposition of oppositely charged polymers, resulting in nanometer thin films of defined multi-functionality (Decher 1997). Capsules based on LbL-films can be finely tuned in respect of permeability enabling a controlled or triggered release of the encapsulated molecules with molecular weight above 1.0 kD (Peyratout and Dähne 2004). Other approaches include emulsions (Bae et al. 2005), nanoparticles (Almeida and Souto 2007; Chan et al. 2011; Mu and Feng 2003; Prombutara et al. 2012), biodegradable or non-biodegradable hydrogels (Bhattarai et al. 2010; Córdoba et al. 2013; Hoare and Kohane 2008) and nanofibers (Cui et al. 2006; Kim et al. 2007). Studies on active antimicrobial packaging employ

polymers, for instance starch and poly (vinyl alcohol) (PVA) (Meira et al. 2017; Wang et al. 2015).

The drawback of those renewable and mostly biodegradable polymers is the poor mechanical or thermal stability, color, transparency, and porous and hydrophilic properties leading to rapid release of encapsulated molecules (Chan et al. 2011; Córdoba et al. 2013; Martin et al. 2013).

Alternatively, the hydrophobic peptide nisin can adsorb to surfaces (Bower et al. 1995; Daeschel et al. 1992; Joosten and Nuñez 1995; Lante et al. 1994), particles (Dawson et al. 2005; Janes et al. 1998) or clays (Ibarguren et al. 2014; Meira et al. 2015) providing a simple and cost-effective delivery system. For some proposed materials the practical food application is not conforming to current regulation. To date, the EU allows only limited materials to be applied to food products to enhance food safety.

Neusilines are highly porous materials, which are developed as excipients in pharmaceutical formulations. They are based on magnesium aluminometasilicate. Neusilin particles were successfully used as excipient in pharmaceutical formulations (Mallappa et al. 2015). Neusilin particles like Neusilin UFL2 (UFL2) hold already an approved drug master file by the FDA for excipients and display potential characteristics for food intended usage. Neusilin particles provide several advantages such as adjustable size, large surface area and negative zeta potential for a pH-dependent release in SCC. More importantly, the production of UFL2 is standardized under strict GMP conditions. Once nisin is electrostatically loaded to UFL2, the carrier material allows protection against proteolytic degradation because enzymes can hardly enter small pores of diameter below 10.0 nm (Orosco et al. 2009). Moreover, interaction of food components with immobilized nisin in UFL2 can be reduced.

In order to achieve an effective loading and sufficient release of nisin in porous particles, charge interactions were used (**Figure 3**). For the loading process, the

porous particles should exhibit a high negatively charged surface for ionic interactions with positively charged nisin, but for the release, the surface charge should become zero or positive. Above the isoelectric point of nisin at pH 8.5 (zeta potential equals 0.0 mV) nisin will become negatively charged (Bactibase, Hammami et al. 2010). No denaturation of nisin at high pH was observed and its activity was fully restored at low pH (Liu and Hansen 1990). These chemical properties of nisin allows a wide pH range for electrostatic interaction. UFL2 had a negative zeta potential between pH 5.0-8.0, which resulted into attraction of nisin (**Figure 3**; green). If the pH decreases below the isoelectric point of UFL2 (around pH 5.3), it becomes positively charged leading to repulsion of nisin (**Figure 3**; blue). Thus, nisin will be released into the environment.

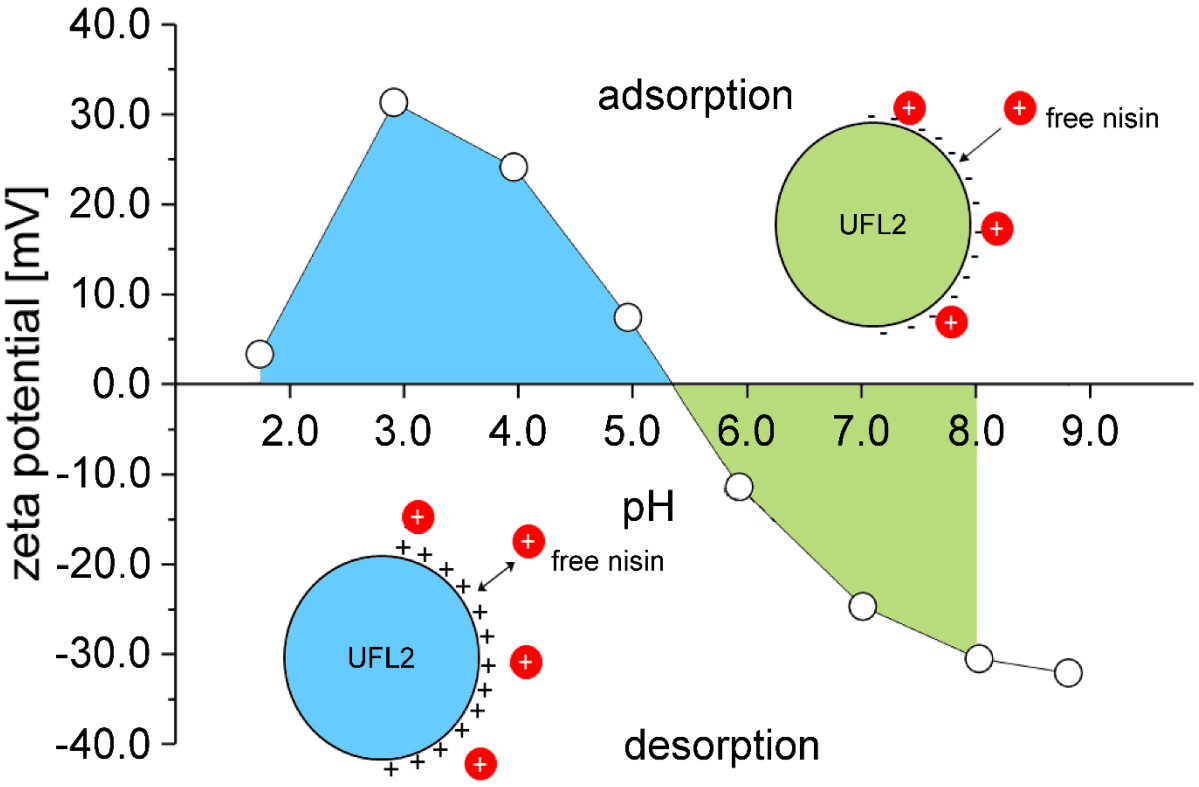

Figure 3: Schematic overview of adsorption (green) and desorption (blue) of nisin (red) as a function of pH. Loading of positively charged nisin to negatively charged Neusilin UFL2 (UFL2) during pH range of >5.3 to 8.0 (see text for explanation) and release of positively charged nisin from positively charged UFL2 at pH <5.3 (see text for explanation). The raw data of UFL2's zeta potential was provided by our collaboration partner Dr. Lars Dähne (Surflay Nanotec GmbH, Germany).

1.4.1.3. Antimicrobial activity of nisin

The peptide nisin inhibits primarily Gram-positive bacteria and spores of those. Other organisms such as Gram-negative bacteria, yeasts or molds are generally not affected by nisin (Bauer and Dicks 2005; Rayman et al. 1981; Stevens et al. 1991). Susceptibility of *L. monocytogenes* field isolates to nisin has been reported frequently (Benkerroum and Sandine 1988; Delves-Broughton et al. 1996; Ferreira and Lund 1996; Rasch and Knøchel 1998). The susceptibility was evaluated by the growth of *L.*

monocytogenes in liquid and solid media and depending on the method, bacterial growth was inhibited between $4.9x10^{-5}$ and 0.026 mg ml^{-1} nisin (Benkerroum and Sandine 1988; Rasch and Knøchel 1998; Ukuku and Shelef 1997). At the same time, reduced susceptibility of individual *L. monocytogenes* field isolates has been reported (Benkerroum and Sandine 1988; Ferreira and Lund 1996; Iancu et al. 2012; Katla et al. 2003; Mota-Meira et al. 2000; Rasch and Knøchel 1998; Ukuku and Shelef 1997).

1.4.1.4. Mode of action

The positively charged nisin is affecting the negatively charged phospholipid groups on the bacterial cell membrane of Gram-positive bacteria (Bonev et al. 2004; Kordel et al. 2001). The didehydroalanine residue in position five was attributed to the inhibition of spore outgrowth (Bauer and Dicks 2005; Pol et al. 2001).

To the current knowledge, nisin will complex with Lipid II, which is a precursor molecule involved in the biosynthesis of the cell wall. Subsequently, the peptidoglycan biosynthesis is inhibited (Kramer et al. 2006). Several nisin-Lipid II-complexes will incorporate itself into the cytoplasmic membrane resulting in pore formation (**Figure 4A**). The negatively charged cell membrane will cause a conformational change in nisin. The positively charged side chains of the amino acids will then interact with the membrane to form pores (**Figure 4A**). As a result, low molecular weight substances like amino acids, ATP and protons will leak from the cell (Breukink et al. 1999; Brötz et al. 2002; Hsu et al. 2004; Wiedemann et al. 2001). Consequently, the proton motive force will deplete.

A model illustrating the interaction between Neusilin UFL2 loaded with nisin (UFL2-N) and the cell wall is presented in **Figure 4B**. Around pH 5.3, the positively charged UFL2-N will attract negatively charged bacteria. This will allow electrostatic binding to the cell wall. Additionally, nisin will be slowly released and those peptides, which do

reach the cytoplasmic membrane, will bind to Lipid II (**Figure 4**). Free nisin, however, may be hindered by phenotypic alterations to interact with the cytoplasmic membrane. Divalent cations may decrease the overall net negative charge of the cell wall and/or the cytoplasmic membrane and may as well prevent free nisin from binding to its target site (**Figure 4B**). Due to UFL2-N and its continuous release of nisin in close proximity to the cytoplasmic membrane, the bioavailability of the antimicrobial peptide will be greatly increased.

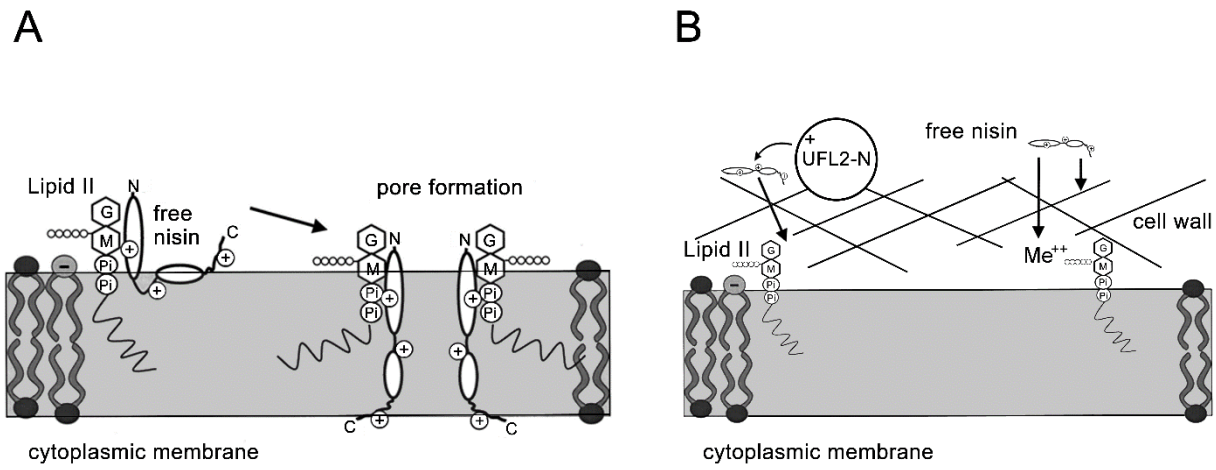

Figure 4: Models illustrating free nisin's and UFL2-N's (nisin electrostatically loaded to Neusilin UFL2) interaction with the cell wall and cytoplasmic membrane. **A** Lipid II-mediated nisin pore formation at the cytoplasmic membrane. Model was adapted from Wiedemann et al. (2001). **B** Conceptual framework for the interaction of free nisin and UFL2-N with the cell wall. Abbreviation: G *N*-Acetylglucosamine; M *N*-Acetylmuramic acid; Me^{++} divalent metal cation; P_i phosphate.

1.4.1.5. Phenotypic and genotypic adaption of *L. monocytogenes* to nisin

Nisin susceptibility is characterized by a complex phenotype. In laboratory experiments, the nisin non-susceptible (NNS) state was acquired spontaneously,

which occurred at a frequency of 10^{-6}-10^{-8} (Harris et al. 1991; Ming and Daeschel 1993). The NNS state was shown to be inducible under chilling temperature and salt stress (Bergholz et al. 2013; De Martinis et al. 1997; Mazzotta and Montville 1997). Moreover, the increased susceptibility was stable in NNS variants (De Martinis et al. 1997).

In spontaneous NNS variants of *L. monocytogenes* field isolates, alterations in phospholipid composition and membrane fatty acid composition have been observed (Crandall and Montville 1998; Davies et al. 1996; Verheul et al. 1997). This led evidentially to a difference in the fluidity of the cell membrane and resulted into decreased net negative charge of NNS field isolates (Mazzotta and Montville 1997; Verheul et al. 1997). This more positive net charge of the cell membrane could hinder the binding of nisin.

While phenotypic alterations in defense strategies are well described, the molecular mechanisms and their regulations are poorly understood. Some loci and genes have been so far associated with inherent nisin tolerance mechanisms. Regarding cell wall biogenesis and membrane composition, the *dltABCD* operon, involved in the D-alanylation of lipoteichoic acid, and *mprF*, catalyzing lysine esterification of phosphatidylglycerol, were found to lead to NNS state in *L. monocytogenes* mutants (Abi Khattar et al. 2009; Kovacs et al. 2006; Peschel et al. 1999; Thedieck et al. 2006). Homologs to a putative penicillin binding protein have been associated to nisin tolerance (Gravesen et al. 2001). To date, several two-component signal transduction systems (TCS), similar to the BceRS-BceAB TCS that can be found in *Bacillus subtilis* (Dintner et al. 2011), have raised attention in *L. monocytogenes*. These TCS were recently attributed to nisin tolerance. Generally, TCS contribute greatly to the ability of *L. monocytogenes* field isolates to sense and respond to its environment (Gottschalk et al. 2008; Mandin et al. 2005). These systems consist of a membrane-bound histidine

kinase component and a cytoplasmic response regulator, which sense the external environment conditions and affect the appropriate response required to improve survival (Gao and Stock 2009). For example, genes coding for TCS and TCS regulators are *liaRS*, *lisRK* and *virRS*. These three TCS and TCS regulators have been associated to nisin tolerance of *L. monocytogenes* field isolaste (Collins et al. 2012; Cotter et al. 2002; Fritsch et al. 2011; Mandin et al. 2005). LisRK is described to be involved into responses to environmental stresses (Cotter et al. 1999; Sleator and Hill 2005), *liaRS* codes for proteins acting as transcriptional regulatory proteins (Fritsch et al. 2011) and *virRS* encodes for regulatory proteins for tolerance to cationic peptides (Mandin et al. 2005). In addition, multidrug resistance transporter such as AnrB was found as contributor to reduce nisin susceptibility (Collins et al. 2010). Other proteins involved in nisin tolerance are alternative sigma factors (Begley et al. 2006; Palmer et al. 2009; Zhou et al. 2012) and glutamate decarboxylase (Begley et al. 2010).

The glutamate decarboxylase (GAD) system contributes significantly to the survival of *L. monocytogenes* field isolates in acidic conditions (Cotter et al. 2001). When the pathogen is exposed to low pH conditions, the GAD system imports a molecule of extracellular glutamate and converts it to γ-aminobutyrate (GABA) before exporting the GABA in exchange for another glutamate. This reaction cycle consumes an intracellular proton and thereby counteracts the acidification of the cytoplasm. GABA is alkaline and raises subsequently the external pH when released into the environment (Small and Waterman 1998). The GAD system is comprised of three decarboxylases (encoded by *gadD1*, *gadD2*, and *gadD3*) and two antiporters (encoded by *gadT1* and *gadT2*). The *gadD1* in strain *L. monocytogenes* LO28 was reported to be associated with enhanced nisin tolerance (Begley et al. 2010; Collins et al. 2011). In mild pH conditions, *gadD1* is required for growth whereas *gadD2* becomes important under severe pH conditions (Cotter et al. 2001; Cotter et al. 2005b).

1.4.2. Citral

The use of natural products such as EO or EO components to control foodborne bacteria has been vastly investigated and constitutes a promising approach. Most of the EO or EO components have been classified with GRAS status and are approved to use as flavoring substances (EU 2012a; FDA 2016). Extraction from plants is typically performed by distillation, solvent extraction or other physical techniques (Bassolé and Juliani 2012). Both in direct oil and vapor form, EO or EO components inhibit a range of Gram-positive and Gram-negative bacteria (Apolónio et al. 2014; Fisher and Phillips 2006; Friedman et al. 2002; Hyldgaard et al. 2012; Silva-Angulo et al. 2015). The EO components with antimicrobial activity are mainly found in terpenes and terpenoids as well as in phenolic compounds (Burt 2004). The basis of terpenes are isoprene units (2-Methylbuta-1,3-diene), while the class of terpenoids are derived from terpenes with oxygenated functional groups. Friedman et al. (2002) ranked EO components in descending order according to their antilisterial activity. Those were cinnamaldehyde, eugenol, thymol, carvacrol, citral, geraniol, perillaldehyde, carvone S, estragole, and salicylaldehyde. Cinnamaldehyde and its derivatives were incorporated in ready-to-eat frankfurters to inhibit growth of *L. monocytogenes* field isolates (Upadhyay et al. 2013) or eugenol was used similarly on ready-to-eat meat products (Hao et al. 1998a, 1998b). Carvacrol also had antilisterial properties on frankfurters (Upadhyay et al. 2013) and on catfish filets (Desai et al. 2012).

The monoterpenoid citral (3,7-dimethyl-2,6-octadienal) showed antimicrobial activity to *L. monocytogenes* field isolates (Apolónio et al. 2014; Kim et al. 1995; Onawunmi 1989). The EO component can be extracted from leaves and fruits of citrus plants like myrtle trees, basil, lemon, lime, lemongrass, orange and bergamot (Fisher and Phillips 2006; Hyldgaard et al. 2012). In general, it is a mixture of the stereoisomers geranial (trans-citral) and neral (cis-citral; Benvenuti et al. 2001). Potential application in dairy

industry was tested by release of citral from cellulose acetate films on coalho cheese (Oliveira et al. 2017). Important application of EO or EO components was also shown for ice cream (Ramadan et al. 2013).

At the same time, usage of these flavoring substances in dairy produce would answer the consumer's demand for fresh, organic and sustainable produce. The concerns about regulatory approval for intended food application (e.g. citral) are withdrawn for food business operators by their GRAS status (EU 2012a; FDA 2016).

1.4.3. Sodium nitrite

Since 1917, $NaNO_2$ is used for curing and preserving of meat and fish products (Binkerd and Kolari 1975; Doran 1917). It can be added to some types of cheese like Feta cheese, Pasta Filata cheese and semi-hard cheeses (FAO FAO/TCP/KEN/6611 Project; Korenekova et al. 2000). Today, $NaNO_2$ is considered as an indirect antimicrobial preservative. It means that in food industry its major function in meat products is for flavoring purposes and not to inhibit microbial growth (Nair et al. 2016). Historically, $NaNO_2$ was used to inhibit *Clostridium botulinum* (Nair et al. 2016). Besides possessing antimicrobial activity against *C. botulinum*, $NaNO_2$ was found inhibitory against *L. monocytogenes* field isolates (Duffy et al. 1994; Müller-Herbst et al. 2016). Moreover, under acidic conditions $NaNO_2$ leads to reactive species like nitric oxide, which show more bacterial inhibition than the undissociated form (Cammack et al. 1999).

1.5. *Listeria monocytogenes* and its tolerance to micro biocides

A common *in vitro* method to measure the susceptibility of *L. monocytogenes* to micro biocides is testing the minimal inhibitory concentration (MIC). The MIC is the lowest concentration of a micro biocide required to inhibit growth of a bacterium. Other wide

spread methods include disk diffusion, colony-forming units (CFU) or broth microdilution. Standardization in testing procedures for disinfectant compounds analogous to The European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2017) was formulated by national institutes, some of them are the Association of Analytical Chemists (AOAC 2002), the British Standards Institute (BSI 1991), the European Committee for Standardization (EN 1275, CEN 2002) and the Deutsche Gesellschaft für Hygiene und Mikrobiologie (Gebel et al. 2001). Unfortunately, clinical breakpoints, which are established for antibiotics or epidemiological cut-off values, are literally non-existent for disinfectant compounds or antimicrobial preservatives. Clinical breakpoints set cut-off values to categorize a bacterium as susceptible, intermediate or resistant. There are several definitions of antimicrobial resistance. All definitions have in common that resistance is the ability of microorganisms to withstand treatments with a micro biocide (Scientific Committee on Emerging and Newly Identified Health Risks 2009). For example, some researcher defined resistant *L. monocytogenes* field isolates to BAC if the MIC was greater than or equal to 0.004 mg ml^{-1} (Romanova et al. 2006; To et al. 2002), 0.008 mg ml^{-1} (Mereghetti et al. 2000), 0.016 mg ml^{-1} (Xu et al. 2014) or the double concentration of MIC_{50} (Meier et al. 2017). These tremendously different cut-off values do not allow the foundation of a harmonized system. A more appropriate method to measure the resistance to a micro biocide is the determination of the minimum bactericidal concentrations (MBCs). The MBC is the lowest concentration of a micro biocide needed to kill a bacterium. It allows the comparisons between the deactivated or are inhibited cells and the resistant cells.

Cerf et al. (2010) proposed to refer to “resistance” when the effect of micro biocides is killing and to “tolerance” when it is adaption to MICs characterized by a raised value. Throughout this thesis, the term “tolerance” will be used when referring to susceptibility

of *L. monocytogenes* field isolates to micro biocides. For clarity, the term “resistance” will be still used in context of the susceptibility of *L. monocytogenes* field isolates to antibiotics based on the used terminology in peer-reviewed literature.

The reasons that have led to tolerance in complex environments like food or dairy production plants remains often unknown. However, the cell response usual turns out to change gene expression providing tolerance against the individual stressor like a micro biocide. *Listeria monocytogenes* has intrinsic survival strategies conferred by the bacterial genome. The cell envelope is considered as an intrinsic trait. The individual sensitivity and tolerance of *L. monocytogenes* field isolates depend on the composition of the cell envelope. Alternatively, tolerance to disinfectant compounds or antimicrobial preservatives has been acquired. This can be caused by the selection pressure on the entire bacterial population. *Listeria monocytogenes* have a considerable advantage over those who have not undergone mutation or harbor plasmids with genetic elements, which enhance the tolerance to micro biocides. The four generic mechanisms corresponding to intrinsic tolerance of bacterial cells to micro biocides are: (I) formation of biofilms as a phenotypic defense strategy to reduce diffusion for certain micro biocides (Gandhi and Chikindas 2007; Schulte et al. 2005), (II) alterations in the cell envelope to decrease membrane permeability (Dubois-Brissonnet et al. 2011; To et al. 2002; Verheul et al. 1997), (III) expression of efflux pumps to affect the intracellular concentration (Bae et al. 2014; Godreuil et al. 2003; Komora et al. 2017; Romanova et al. 2006), and lastly (IV) modification or overproduction of the target molecules as well as production of enzymes that are capable to reduce effectiveness of the micro biocide (Kapoor et al. 2017).

In dairy production plants, acquired tolerance is of major importance. Especially in the production plants where the rotation or concentration of micro biocides is limited due to the plant design. Hence, tolerant *L. monocytogenes* field isolates may be selected

by decreased bioavailability of the disinfectant compounds in these plants. The reasons for the selection of tolerant field isolates could be sub-inhibitory concentrations.

To date, the frequently used BAC is the best studied example among the disinfectant compounds regarding the description of MIC and tolerance mechanism to *L. monocytogenes*. The pathogen was not found to become highly tolerant to BAC in food production plants and the majority of tested field isolates were susceptible to low concentrations (Aase et al. 2000; Mereghetti et al. 2000). This is in contradiction to laboratory conditions under which *L. monocytogenes* field isolates quickly adapted to BAC (Romanova et al. 2006; To et al. 2002). Other frequently used disinfectant compounds like NaOCl did not show increased MICs (Bloomfield and Miller 1989; Mustapha and Liewen 1989). Similarly, the antimicrobial preservatives nisin and nitrites used for a long period of time in food industry did not cause high tolerances in *L. monocytogenes* field isolates (Ghabraie et al. 2016; Müller-Herbst et al. 2016).

1.5.1. Correlation of micro biocides to respective antibiotic resistance of L. monocytogenes field isolates

Once the gene expression as part of the cell response is altered, tolerance of *L. monocytogenes* against a single micro biocide can, in return, provide survival methods, which allows tolerance to different micro biocides with similar or unrelated modes of action (Bergholz et al. 2013; Cebrián et al. 2010; Lou and Yousef 1997; McMahon et al. 2007a). A co-selection process is especially concerning when micro biocides tolerance select for antibiotic resistances. This could involve efflux pumps as mechanism of target cell tolerance or resistance, respectively. Often efflux pumps are not molecule specific but rather generic to different unrelated molecules or heavy metals (Mullapudi et al. 2008; Rakic-Martinez et al. 2011; Xu et al. 2014). These

unrelated molecules can include antibiotics as well. Therefore, high importance is attributed to the presence of efflux pumps in *L. monocytogenes*. On the other hand, decreased uptake due to alteration in peptidoglycan biosynthesis could contribute to a micro biocide tolerance or antibiotic resistance. Once *L. monocytogenes* has altered its cell wall to micro biocides, conceptually cells should be less susceptible to antibiotic classes targeting a specific pathway of bacterial cell wall biosynthesis like β-lactams and glycopeptides (Kapoor et al. 2017). The likelihood of this situation to occur should be low under complex environmental conditions. However, co-selection cannot be excluded and several studies could show opposite results under laboratory conditions (Christensen et al. 2011; Hammer et al. 2012; McMahon et al. 2007b; McMahon et al. 2008; Nielsen et al. 2013). Data from food industry examining possible tolerance concerns associated with antimicrobial preservatives is scarce even if they have been used over years (Davidson and Harrison 2002). The usage of nisin has led so far in few individual *L. monocytogenes* field isolates to nisin tolerance (Cotter et al. 2005a; Delves-Broughton et al. 1996). A few sporadic studies have reported that nisin tolerant *L. monocytogenes* field isolates were less susceptible to different β-lactams and cephalosporins (Cotter et al. 2002; Gravesen et al. 2001). The European Food Safety Authority (EFSA) commissioned a survey of nisin-induced co-selection to therapeutic antibiotics. The panel concluded that this association is not of concern when applying nisin in food products (Davidson and Harrison 2002). Their consensus was that the co-selection to antibiotics did not occur because of the differences in the mode of action between nisin and antibiotics. While nisin mostly results into pore formation and require Lipid II as a docking molecule, antibiotics require a specific target in either the cell membrane or inside the cell (EFSA 2006).

1.6. Cheese products

Due to many listeriosis outbreaks with several deaths and diseases, food safety of different types of soft cheese is of paramount interest (Bille et al. 2006; Büla et al. 1995; Carrique-Mas et al. 2003; Goulet et al. 1995; Linnan et al. 1988; MacDonald et al. 2005; Makino et al. 2005; McIntyre et al. 2015). Compared to other food products, listeriosis outbreaks from dairy produce were reported to show no decrease in frequency in the US (Cartwright et al. 2013). In the EU, consumption of a *L. monocytogenes* contaminated sour curd type of cheese led in Austria and Germany to an outbreak and the cheese was voluntarily withdrawn from the market one year later (Fretz et al. 2010b). Therefore, raw and non-thermal treated food products require particular attention in risk assessment of food safety.

In Germany, the “Käseverordnung” defines and regulates production and quality measures of cheese. The “Käseverordnung” extends classification of different cheese produce to types of milk, renneting coagulation of milk, taste and fat content in the dry matter. Different cheese produce based on water content by weight of the non-fatty matter are shown in **Table 3**.

Table 3: Grouping of cheese according to water content calculated by weight of the non-fatty matter. Adopted from “§ 6 Bundesgesetzblatt 2015 Käseverordnung”, Germany.

Cheese produce	Water content by weight of the non-fatty
Cream	>73.0 %
Soft	>67.0 %
Sour curd	>60.0-73.0 %
Semi-soft	>61.0-69.0 %
Semi-hard	>54.0-63.0 %
Hard	<56.0 %

Although surface-ripened cheeses like sour curd cheese (SCC) are of minor importance on cheese market, the “Quargel” outbreak displays significance to prevent future major outbreaks (Fretz et al. 2010b). Thus, this thesis selected SCC for evaluation of nisin loaded Neusilin UFL2 and free nisin against *L. monocytogenes*. To the best of author’s knowledge, the chemical and physical robust derived magnesium aluminometasilicate has not been applied in SCC yet. Neusilin particles were studied in the context of an adsorbent powder in animal feed administration (Ma et al. 2016) and for nutritional supplementation (Santaniello and Giannini 2016).

1.6.1. Sour curd cheese

SCC is produced from low-fat sour curd (SC) (“§1 Absatz 3 Nr. 2 Käseverordnung”) and lactic acid bacteria. They have a long tradition in Hessian, Germany and their simple production is followed by a short ripening period from a couple of days to weeks. The weight of loaf determines the assorted varieties like the well-known “Harzer Roller” and “Handkäse”. Furthermore, SCC is subdivided into cheeses with white molds (e.g. *Geotrichum candidum*) or with orange-red pigmented bacteria also called red smear like *Brevibacterium linens* (Bockelmann 2003). Since *B. linens* has its characteristical appearance, it is often referred to as the “typical red smear bacterium”. However, *Arthrobacter nicotianae*, coryneform and *staphylococci* bacteria are found on the cheese surfaces (Bockelmann 2003). SCCs that have been produced with red smear bacteria are called “Gelbkäse”. Spices or herbs can also be added as topping before or during ripening.

The basis of SCC is low-fat milk, which is coagulated at 40.0 °C, and thermophile starter lactic acid bacteria like *Streptococcus thermophilus*. During the fermentation of lactose, the pH decreases and the milk starts to thicken due to coagulation of casein (Tscheuschner 2004). In general, dairy production plants produce SC and the

intermediate good is transported to the cheese manufacturers, where it is processed without further heat treatments. Based on the SCC recipe of our collaboration partner, the production process is shown in **Figure 5**. Ripening salts sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) are added to SC to adjust salinity and pH. In other recipes, calcium carbonate is also added.

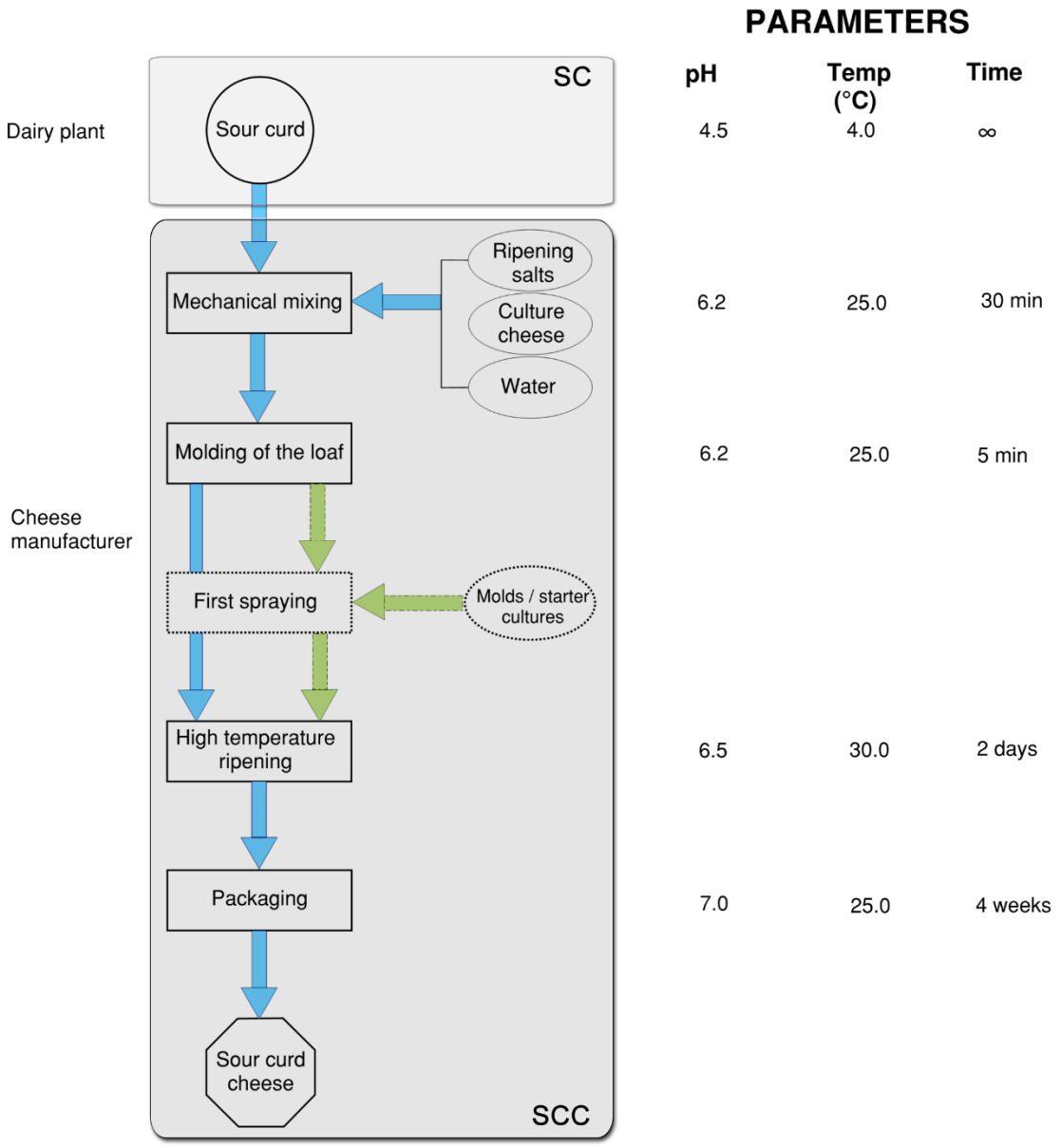

Figure 5: Sour curd cheese production process similar to the work flow of our collaboration partner. Abbreviation: SC sour curd; SCC sour curd cheese; Temp Temperature.

Apart from the indigenous microbiota from the SC and ripening salts, a special batch of two-week ripened SCC ("culture cheese") is added to initiate ripening of the cheese (**Figure 5**). This traditional method is called "old-young" smearing (adding of the "old culture cheese"). During two days of ripening, the initial white color shifts to the typical orange-yellow color of SCC on the outer surface while its white curd core remains intact. Similarly, the pH at the surface increase from approximately 4.4 to 7.2 (Belitz et al. 2001). In addition, ripening changes the attributes from a brittle, grainy texture and salty flavor to a soft, rubbery texture and intensified cheese flavor and taste. Although SCC is and will not be produced for mass market due to its strong taste, it provides interesting properties for dietary intake such as low-fat and high milk protein content (Bockelmann 2003).

1.6.2. Contamination of SCC with molds and L. monocytogenes

The traditional "old-young" smearing makes SCC prone to contamination by, for instance, molds or foodborne pathogens. High hygiene standards have to be maintained and applied to the culture cheese. The SCC surface is exposed to unsterile conditions and provides nutrients and moist content for the growth of spoilage organisms. Additionally, the sour curd and culture cheese can be seen as raw products, which do not undergo pasteurization or other mild thermal treatment. Therefore, spoilage organisms are introduced into the SCC by secondary contamination. If the balance of the red smear microbiota is disturbed, molds and bacteria were shown to immediately grow (Bockelmann 1999). Finally, the short ripening period of SCC compared to other soft cheeses is too short to compete against spoilage organisms. Carminati et al. (1999) could show that a listerial inhibition on contaminated Taleggio cheese surface was only possible at the end of ripening (15 d). In semi-soft or hard cheeses contaminated with *L. monocytogenes*, the long ripening

period led to reduction but not elimination of the pathogen at the end of ripening (Liu et al. 2007; Ramsaran et al. 1998; Sulzer and Busse 1991).

Other SCC conditions beside the intrinsic cheese properties like the temperatures in the ripening chamber, especially above average room temperature, promote growth of *L. monocytogenes*. Since SCC ripens from outside to the inside (Belitz et al. 2008), the pH of the surface will rapidly increase to slightly acidic. Therefore, the surface conditions are in favor for the growth of *L. monocytogenes*.

In general, routes of spoilage in cheese produce were described as primary contaminations or secondary contaminations. The intrinsic food conditions like low pH and low water activity, which are found in SC, do not support growth and hamper outgrowth of *L. monocytogenes* (Codex Alimentarius Commission 2009; EU 2007a). *Listeria monocytogenes* field isolates could survive for several days to months in different acidic cheese varieties (Ferreira and Lund 1996; Hystead et al. 2013; Rogga et al. 2005; Villani et al. 1996). For other ingredient such as water, primary contamination will only occur if contamination source is traced back to water supply like in one outbreak case in British Columbia, Canada (McIntyre et al. 2015). The contamination via the culture cheese is a plausible source of contamination if a batch would show prevalence of *L. monocytogenes*. Secondary contamination by surfaces and during the manufacturing process is most likely to occur. Once the surface is contaminated, *L. monocytogenes* field isolates were able to grow during ripening and under chilled conditions when packaged (Farber and Peterkin 1991; Gill and Reichel 1989; Maisnier-Patin et al. 1992).

2. MOTIVATION

The prevalence of *L. monocytogenes* field isolates in dairy production plants continues to be a health threat to the public. In fact, listeriosis outbreaks from dairy products were reported to show no decrease in frequency (Cartwright et al. 2013). Therefore, the motivation of this study was to investigate micro biocides, which act as antilisterial. Secondly, this study aimed to improve the industrial application of a selected micro biocide in an innovative way and to point out various aspects of potential tolerance developments.

While prevalence of *L. monocytogenes* cannot be eradicated during the food production process, the level of contamination can be controlled (Tompkin et al. 1999). The appropriate efficacy of micro biocides is mandatory for the control measures of *L. monocytogenes*. The susceptibility of a field isolate is often stated as MIC. MICs of disinfectant compounds and antimicrobial preservatives are not well resolved for large field isolate collections of *L. monocytogenes*, except for BAC. As a result, MICs of different studies exhibit inconsistent efficacy due to modified experimental procedures and are often not comparable. It is known that the organic debris in the environment reduce the efficacy of disinfection compounds (El-Kest and Marth 1988; Jo et al. 2018). The efficacy of the selected micro biocide nisin is, however, not reduced in broth medium. The peptide inhibits *L. monocytogenes* and is permitted in ripened or processed cheese (EU 2011a). Some cells might overcome the bacteriostatic action of nisin and change their gene expression. In return, those cells are protected against the antilisterial activity leading to reports of tolerance in few *L. monocytogenes* field isolates (Cotter et al. 2005a; Delves-Broughton et al. 1996). Some genes have been associated with nisin tolerance. For example, genes involved in the GAD system were reported to be associated with nisin tolerance (Begley et al. 2010; Collins et al. 2011).

In order to improve the industrial application of nisin and lower its limitations, the hydrophobic peptide was electrostatically adsorbed to UFL2. In this way, the carrier material allows protection against the food matrix. However, the application of nisin in SCC has been studied to a lesser extent while their surfaces provide conditions that are in favor of the growth of *L. monocytogenes*.

2.1. Hypothesis and objectives

The following hypotheses and objectives were proposed and formulated based on the observations and data mentioned above:

(I) Environmental factors such as organic compounds in culture broth (**4.1.**, see below) and pH (**4.3.**) influence efficacy of micro biocides, while certain micro biocides are correlated to specific antibiotic resistances in *L. monocytogenes* (**4.1.**).

a. Determining of MIC of *Listeria* spp. to disinfectant compounds and antimicrobial preservatives as well as antilisterial activity of Neusilin-adsorbed nisin (UFL2-N) and free nisin in dependence of factors pH and time.

b. Defining nisin susceptible (NS) and nisin non-susceptible (NNS) *L. monocytogenes* field isolates.

c. Correlating published antibiotic resistance pattern of *L. monocytogenes* field isolates to high MIC of disinfectant compounds and antimicrobial preservatives.

(II) *Listeria monocytogenes* harboring DNA-sequence variances in *gadD2* gene thrive on a genetic shift in the GadD2 protein in culture media supplemented with nisin (**4.2.**).

a. Identification of *L. monocytogenes* field isolates with DNA-sequence variances in *gadD2*.
b. Characterization of the phenotypic consequences of amino acid substitution in GadD2.

(III) The sour curd cheese (SCC) harbor stable red smear microbiota and UFL2-N as well as free nisin act antilisterial on the surface of SCC (**4.3.**).

a. Enhancing the bioavailability of nisin by a slow release.
b. Evaluation of inhibition of *L. monocytogenes* field isolates *in vitro*, in autoclaved SCC and on the surface of SCC.

At first, a screening of the efficacy and susceptibility in culture broth of different micro biocides, disinfectant compounds, antimicrobial preservatives and nisin was carried out to circumvent growth of *L. monocytogenes* field isolates (**4.1.**). Especially nisin was on focus in **4.2.** and **4.3**. Organic compounds in culture broth did not influence efficacy of UFL2-N and free nisin nor did they correlate to specific antibiotic resistances in *L. monocytogenes.* Other causes leading to nisin tolerance was studied in **4.2.**. Hence, investigating NS and NNS *L. monocytogenes* field isolates in a screening, which was based on molecular analyses, helped to identify DNA-sequence variances in *gadD2* gene. Thus, controlling the occurrence of a NS *L. monocytogenes* field isolate by enhancing the bioavailability of nisin was part of **4.3.**. The antilisterial activity of UFL2-N as well as free nisin was shown on the surface of SCC.

Parts of the results included in this doctoral thesis have been obtained at the German Federal Institute for Risk Assessment (Berlin, Germany) or are based on published data (Noll et al. 2018; Szendy et al. 2019a). Published data of the susceptibility of 282 *L. monocytogenes* field isolates to nisin (Szendy et al. 2019b) will be included into **4.1.** for reader's convenience.

3. MATERIALS AND METHODS

3.1. Efficacy and susceptibility testing of micro biocides

3.1.1. Preparation of disinfectant compounds and antimicrobial preservatives

The disinfection compounds used in this study were benzalkonium chloride (≥95.0 % BAC) (Sigma-Aldrich Chemie GmbH, Germany), cetalkonium chloride (CKC) (Sigma-Aldrich), 30.0 mg ml^{-1} hydrogen peroxide (H_2O_2) (VWR International GmbH, Germany) and 152.0 mg ml^{-1} free chlorine sodium hypochlorite solution (NaOCl) (Carl Roth Carl Roth GmbH + Co. KG, Germany). These disinfection compounds were stored at room temperature. The antimicrobial preservatives sodium nitrite (≥98.0 % $NaNO_2$) (Carl Roth) and free nisin (2.5 %) (Sigma Aldrich) were stored at room temperature as well. The EO component citral (95.0 to 98.0 %) (Carl Roth) was stored following manufacturer's instruction. Working solutions of 50.0 ml of each disinfectant compound, $NaNO_2$ and citral were prepared in BHI broth and consecutively diluted two-fold (**Table 4**). Working solutions of citral and NaOCl were prepared daily in BHI broth and citral was dispersed at room temperature to obtain a colloidal suspension. Finally, all working solutions were filter-sterilized through a 0.22 µm polyvinylidene fluoride membrane (Carl Roth). As sterile filtration lowered the antimicrobial activity of CKC, its working solution was not sterile filtrated and was routinely plated on sheep blood agar (Mast Diagnostika DM 101, Germany) instead. The agar plate was incubated for 24 h at 37.0 °C and was inspected for absent of microbial growth. If microbial growth was absent, non-filtered CKC was used. In case of citral, the colloidal suspension was rigorously dispersed before filter-sterilization and before each subsequent dilution step. In preliminary experiments, sterile filtration of citral had no effect on antimicrobial activity.

Table 4: Disinfectant compounds, antimicrobial preservatives, and their applied concentration in each well of a 96-well micro titer plate.

Biocide	Range of concentrations [mg ml^{-1}]							
Disinfectant compounds								
BAC	0.0001	0.0003	0.0005	0.001	0.002	0.004	0.008	0.016
CKC	0.0003	0.0005	0.001	0.002	0.004	0.008	0.016	
H_2O_2	0.005	0.010	0.020	0.039	0.078	0.156	0.313	0.625
NaOCl (free chlorine)	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0
Antimicrobial preservatives								
Citral	0.56	1.11	2.23	4.45	8.90	17.8	35.6	71.2
$NaNO_2$	0.063	0.125	0.25	0.5	1.0	2.0	4.0	8.0

Abbreviation: BAC benzalkonium chloride; CKC cetalkonium chloride; H_2O_2 hydrogen peroxide; NaOCl sodium hypochlorite solution; $NaNO_2$ sodium nitrite.

A stock solution of free nisin (2.5 %) (Sigma Chemical, USA) in H-medium (for fastidious microorganisms) (Merlin Diagnostika, Germany; Troxler et al., 2000) containing 3.0 mg ml^{-1} was freshly prepared before use at the German Federal Institute for Risk Assessment. Finally, free nisin was diluted to 0.0, 0.005, 0.01, 0.015, 0.02, 0.05, 0.075, 0.1, 0.15, 0.2, 0.4 and 1.5 mg ml^{-1} in H-medium, which is equivalent to 0.0, 0.0001, 0.0003, 0.0004, 0.0005, 0.001, 0.002, 0.003, 0.004, 0.005, 0.011 and 0.039 mg ml^{-1} pure nisin (NisinZ™ P 95.0 %) (Handary SA, Belgium).

3.1.2. Field isolates, reference strains and bacterial growth

251 *L. monocytogenes* field isolates have been collected from raw and pasteurized milk, meat, fish, food products, and human patients in Germany (**Table 5**). The first field isolate collection was used for efficacy testing. Molecular serotype of all field isolates were previously determined and field isolates were identified by biochemical and molecular methods (Noll et al. 2018). Similarly, 282 *L. monocytogenes* field isolates have been collected and serotyped for a second field isolate collection (**Table**

6). This second collection was used for nisin susceptibility testing. The percentage of overlapping field isolates between the collection used for nisin susceptibility testing and the collection used for micro biocide efficacy testing was 89.0 %. Additionally, 39 *Listeria* reference strains were included to the nisin susceptibility testing. *Listeria monocytogenes* field isolates were selected according to serotypes most frequently associated with human listeriosis, i.e. IIa, IIb and IVb (Allerberger 2003).

Table 5: The origin of isolation and serotypes of 251 *L. monocytogenes* field isolates and 27 *Listeria* spp. reference strains (enlisted separately in **Table 7**) for efficacy testing of disinfectant compounds and antimicrobial preservatives.

Origin of isolation	Acronym	IIa	IIb	IIc	IVa	IVb	-	Total
Crustacean and mollusc	CM	2	-	-	-	1	-	**3**
Fish and fish products	F	18	5	4	-	14	1	**42**
Human	H	6	5	3	-	8	-	**22**
Meat and meat products	M	20	14	18	2	18	-	**72**
Milk/cheese and other dairy products	MC	56	9	2	1	16	2	**86**
Other products	O	-	-	-	-	-	15	**15**
Vegetarian foods	V	2	3	1	-	4	1	**11**
Reference strains	RS	3	5	4	4	6	5	**27**
Total		**107**	**41**	**32**	**7**	**67**	**24**	**278**

Table 6: The origin of isolation and serotypes of 282 *L. monocytogenes* field isolates and 39 *Listeria* spp. reference strains (enlisted separately in **Table 7**) for susceptibility testing.

Origin of isolation	Acronym	IIa	IIb	IIc	IVa	IVb	-	Total
Environmental	E	7	2	-	1	2	-	**12**
Fish and fish products	F	12	5	3	-	14	-	**34**
Human	H	1	-	1	-	5	-	**7**
Meat and meat products	M	18	14	19	2	16	-	**69**
Milk/cheese and other dairy products	MC	99	11	2	1	21	-	**134**
Other products	O	2	1	1	-	7	5	**16**
Vegetarian foods	V	2	3	1	-	4	-	**10**
Reference strains	RS	6	5	4	5	12	7	**39**
Total		**147**	**41**	**31**	**9**	**81**	**12**	**321**

In addition, 27 reference strains from the genus *Listeria* were included for efficacy testing (**Table 7**).

Table 7: Reference strains, serotypes and sources.

Species	Molecular Serotype	Isolate collection[a]
L. innocua	-	ATCC33090[b,c]
	-	ATCC33091[b,c]; NCTC10528[c]
L. ivanovii	-	ATCC19119[b,c]
L. monocytogenes	IIa	ATCC19111[c]; ATCC19113[b,c]; CLIP74902[c]; DSM20600[Tb,c]; NCTC7973[b,c]; SLCC6190[c]
	IIb	CLIP56878[b,c]; CLIP70676[b,c]; DSM12464[b,c]; SLCC2540[b,c]; SLCC2755[b,c]
	IIc	ATCC19112[b,c]; CLIP70851[b,c]; CLIP70883[b,c]; SLCC2479[b,c]
	IVa	ATCC19114[b,c]; ATCC19116[b,c]; NCTC5214[c]; SLCC2521[b,c]; SLCC4115[b,c]
	IVb	ATCC13932[c]; ATCC19115[b,c]; ATCC19117[b,c]; ATCC19118[b,c]; ATCC13932[b]; NCTC10527[c]; SLCC3458[b,c]; SLCC3753[b,c]; SLCC6458[c]; SLCC6465[c]; SLCC9549[c]; SLCC9579[c]; SLCC9678[c]
L. seeligeri	-	ATCC35967[c]; SLCC3954[b,c]
L. welshmeri	-	ATCC35897[b,c]

[a] ATCC: American Type Culture Collection; CLIP: Listeria Collection of the Pasteur Institute; DSM: German Collection of Microorganisms and Cell Cultures; NCTC: National Collection of Type Cultures; SLCC: Special Listeria Culture Collection.
[b] Reference strains used for efficacy testing of disinfectant compounds and antimicrobial preservatives.
[c] Reference strains used for free nisin susceptibility testing.

From the *L. monocytogenes* collection (**Table 5**), eighteen *L. monocytogenes* were selected according to relevant serotypes and nisin susceptibility for further analysis (**Table 8**).

Table 8: Selected *L. monocytogenes* field isolates (n=18), isolate collection, serotypes, and MIC of free nisin in mg ml^{-1}.

BfR No.	Isolate collection[a]	Serotype	MIC [mg ml^{-1}]	Analysis[b]
L32	-	IIb	0.011	(1); (2)
L41	-	IIa	0.0001	(1); (2); (3); (4)
L192	SLCC2540	IIb	0.001	(1); (5)
L212	ATCC13932	IVb	0.039	(1)
L245	-	IIa	>0.039	(1); (2); (3); (4)
L261	-	IIa	>0.039	(1); (2); (3); (4); (5); (6)
L286	-	IVa	0.0001	(1); (7)
L308	-	IVb	0.011	(1); (2)
L330	$DSM20600^T$	IIa	0.003	(1); (2); (3); (4)
L448	-	IIa	0.003	(1); (2); (3); (4)
L451	-	IVb	0.039	(1); (2)
L493	-	IIb	0.004	(1); (2)
L548	-	IVb	0.0003	(1); (7)
L1031	-	IVb	0.0001	(1); (2); (6); (7); (8); (9); (10)
L1079	-	IIa	0.001	(1); (2); (3); (4)
L1080	-	IIa	>0.039	(1); (7)

[a] ATCC: American Type Culture Collection; DSM: German Collection of Microorganisms and Cell Cultures; SLCC: Special Listeria Culture Collection.
[b] Continuous numbering indicates analysis:
(1) Efficacy and susceptibility testing; (2) Growth curve; (3) WGS; (4) GAD assay; (5) FISH; (6) Growth curve at sub-inhibitory free nisin concentration; (7) *GadD2* sequencing; (8) Growth in autoclaved and non-autoclaved SCC; (9) QPCR; (10) Interaction with UFL2-N.

Listeria monocytogenes field isolates as well as *Listeria* spp. were maintained in brain heart infusion broth (BHI; Carl Roth). Fifteen percent glycerol (Carl Roth) was added to broth and cultures were stored at -80.0 °C until further use at University of Applied Sciences and Arts, Coburg. For subsequent cultivation in Coburg, *Listeria* spp. were routinely grown on BHI agar plate or on blood agar No. 2 (Carl Roth) supplemented with 7.0 % sheep blood (Oxoid GmbH, Germany) for 24 h at 30.0 °C. Thereafter, a single colony from an agar plate was picked and suspended in BHI broth and was incubated for 24 h at 30.0 °C under oxic conditions. The optical density (OD) at 690 nm of each bacterial overnight culture was adjusted to 0.5 McFarland standard (VWR

International) in 2.0 ml fresh broth. This working suspension was stored on ice before using them for subsequent studies.

3.1.3. In vitro efficacy and susceptibility testing of micro biocides to L. monocytogenes field isolates and Listeria spp.

At the German Federal Institute for Risk Assessment, each *Listeria* spp. (**Table 5** and **Table 7**) working suspension was diluted 1:100 in fresh BHI broth prior to efficacy testing. A total of 0.1 ml of the bacterial suspension (approximately log 5.0 CFU ml^{-1}) and 0.1 ml of BHI broth containing the respective disinfectant compound or antimicrobial preservative in double concentration (**Table 4**) were applied to each well of a sterile 96-well micro titer plate (Greiner Bio-One GmbH, Germany). Subsequently, inoculated BHI broth in each well was thoroughly mixed and the microtiter plates were incubated for 24 h at 37.0 °C. After incubation and five seconds of shaking, the OD of each well was measured at a wavelength of 595.0 nm by a microplate reader (Mithras^2 2LB 943; Berthold Technologies GmbH & Co. KG, Germany). Each bacterial suspension without any biocide was incubated as positive control, whereas the negative controls contained no bacteria. *Listeria monocytogenes* ATCC13932 was used as an external control for efficacy testing. Bacterial growth was scored positive and negative when ΔOD_{595} was >0.1 and ΔOD_{595}<0.1, respectively. When growth fell out of specification that is ΔOD_{595}<0.1 within a dilution series or showed no growth at the beginning of a dilution series but with increasing concentration, determination of MICs was repeated for this field isolate.

The influence of BHI broth on disinfectant efficacy was evaluated in preliminary experiments. Disinfectant compounds were dissolved and diluted in 50.0 mmol l^{-1} Tris(hydroxymethyl)-aminomethan buffer solution (TRIS, pH 7.2). A set of seven *L.*

monocytogenes field isolates were randomly selected (**Table 8**) and were inoculated in the same experimental set up as mentioned above.

In 2013 at the German Federal Institute for Risk Assessment, one colony of *L. monocytogenes* field isolates as well as *Listeria* spp. reference strains (**Table 6** and **Table 7**) was picked from sheep blood agar (Mast Diagnostika) and bacteria were solubilized in 0.9 % (w/v) sodium chloride to adjust OD according to McFarland standard (**3.1.2**). Thereafter, bacterial cells were diluted in 1:50 liquid H-medium. In each well of a 96-well microtiter plate, a total of 0.05 ml of the bacterial suspension and 0.05 ml of H-medium containing nisin in double concentration were shaken for 5 s and incubated for 22-24 h at 37.0 °C. Subsequently, the microtiter plates were shaked for 5 s (Dynatech MRX microplate reader; Dynatech Laboratories, USA), and the OD of each well was measured at a wavelength of 690 nm. Bacterial growth was proven by an OD_{690nm}>0.1 whereas nisin sensitive *Listeria* spp. showed an OD_{690nm}<0.1, provided that the controls including the same *Listeria* spp. without nisin and H-medium with nisin but without bacteria revealed an OD_{690nm}>0.1 and <0.1, respectively. *Lactococcus lactis* ssp*. cremoris* DSM 20069 was used as an efficacy control for the nisin susceptibility testing. NNS was defined as ≥MIC_{50} for a *L. monocytogenes* field isolate, whereas all others were classified as NS.

3.1.4. Growth curve of four NS and two NNS L. monocytogenes field isolates

From the information of nisin susceptibility of 282 *L. monocytogenes* field isolates six field isolates of serotype IIa (**Table 9**) were of interest due to their nisin susceptibility.

Table 9: Nisin susceptible (NS) and nisin non-susceptible (NNS) *L. monocytogenes* serotype IIa field isolates used for whole genome sequencing analyses.

Field isolates	MIC [mg ml^{-1}]	Classification	Source (Country), Year
BfR L41	0.004	NS	Sewage, food production environment (Germany), 1986
BfR L330	0.004		Rabbit, DSM20600 (England)
BfR L448	0.004		Smoked salmon (Germany), 2006
BfR L1079	0.004		Cheese (Germany), 2010
BfR L245	>0.039	NNS	Raw milk (Germany), 1994
BfR L261	>0.039		Raw milk (Germany), 1994

The growth of these six *L. monocytogenes* field isolates was monitored in presence of 0.011 mg ml^{-1} free nisin, which was found to be the MIC_{50} value. Each working suspension was diluted 1:100 in fresh tryptic soy broth (TSB; Carl Roth). In preliminary experiments, BHI broth was also tested. However, *L. monocytogenes* field isolates had extended lag phase in BHI broth. Thus, some field isolates with and without free nisin did not meet the criteria to reach the stationary phase within 24 h. Free nisin (NisinZ™ P 95.0 %) was prepared in a 25.0 ml stock solution of 0.132 mg ml^{-1} free nisin in TSB. Either 0.1 ml of the bacterial suspension (approximately log 5.0 CFU ml^{-1}) and 0.1 ml of TSB containing free nisin in double concentration to achieve a final concentration of 0.011 mg ml^{-1} or 0.2 ml of the bacterial suspension was applied to each well of a sterile 96-well micro titer plate (Greiner Bio-One). The microtiter plate was sealed with Breathe-Easy® membrane (Carl Roth). OD was acquired at 595.0 nm every 20 min over a period of 24 h. The FLUOstar OPTIMA microplate reader (BMG Labtech, Germany) incubated the 96-well micro titer plate at 30.0 °C. Each *L. monocytogenes* field isolate was tested with and without free nisin in five replicates of two independently overnight cultures.

3.2. Molecular analysis on nisin tolerance

3.2.1. Field isolates, reference strains and bacterial growth

Listeria monocytogenes field isolates were harvested (**3.1.2.**) and suspended in BHI broth, in Luria and Bertani broth (LB) (Carl Roth) or in TSB depending on experimental design. Incubation was carried out as described in **3.1.2.**. *Escherichia coli* K-12 ER2738 and *Lactobacillus fermentum* were maintained in LB broth or in in standard nutrient broth I (SNBI) (Carl Roth). All cultures were stored as described in **3.1.2.**. For subsequent cultivation in Coburg, *E. coli* K-12 ER2738 was grown on LB agar plates and *L. fermentum* was grown on SNBI agar plates for 24 h at 37.0 °C. Thereafter, a single colony from an agar plate was picked and suspended in respective broth, which was incubated for 24 h at optimal growth temperature of each bacterial species. The working suspensions were prepared as described in **3.1.2.**.

3.2.2. DNA preparation and whole genome sequencing

Isolation of genomic DNA (gDNA) was conducted according to manufacturer's procedures using the QIAamp DNA mini kit (Qiagen, Germany). A total of 20.0 ng of gDNA of each *L. monocytogenes* field isolate was subjected to library preparation using the Illumina Nextera® XT DNA sample preparation kit (Illumina, Germany). According to manufacturer's instructions, gDNA of each *L. monocytogenes* field isolate was tagged, pooled and paired-end sequenced. Paired-end 300.0 bp sequencing of the DNA was performed using an Illumina MiSeq (Illumina), which resulted in more than two million reads per *L. monocytogenes* field isolate. *De novo* genome assemblies of the reads were conducted using the SPAdes algorithm of the PATRIC database (www.patricbrc.org; Wattam et al., 2014) and resulted to sequence coverage of 40- to 60-fold per consensus base for each of the six *L. monocytogenes* field isolates. Initial genome annotation was performed with the automated NCBI Prokaryotic Genome

Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok; Angiuoli et al., 2008).

3.2.3. Whole genome sequence data analysis

Whole genomic DNA sequences were analyzed using the DS Gene software package (v. 2.5; Accelrys GmbH, Germany). Based on a literature search the following genes were attributed to nisin resistance and their putative regulation sequences associated with nisin resistance were identified. Genes related to cell wall modifications: *dltA, dltB, dltC* and *dltD* (D-alanyl decoration of teichoic acid) (Abi Khattar et al., 2009; Kovács et al., 2006; Peschel et al., 1999), *pbp* (lmo2229; penicillin-binding protein) (Gravesen et al., 2001), and *rmlD* (lmo1084; synthesis of dTDP-L-rhamnose) (Xuanyuan et al., 2010). Genes for cell membrane modifications: *mprF* (lmo1695; catalyzing lysine esterification of phosphatidylglycerol) (Thedieck et al., 2006). Genes coding for two-component systems (TCS) and TCS regulators: *liaSR* (lmo1021 and lmo1022; transcriptional regulatory proteins) (Collins et al., 2012; Fritsch et al., 2011), *lisRK* (involved into responses to environmental stresses) (Cotter et al., 2002), and *virRS* (lmo1741 and lmo1745; regulatory proteins for resistance to cationic peptides) (Mandin et al., 2005). Genes for ABC transporters or BceAB-like transporters: *anrB* (lmo2115; multidrug resistance transporter) (Collins et al., 2010a), lmo1746 (ABC transporter permease) and lmo1747 (ABC transporter binding protein) (Bergholz et al., 2013; Gebhard and Mascher, 2011), and *telA* (lmo1967; homologue of the tellurite resistance gene) (Bergholz et al., 2013; Collins et al., 2010b). In addition, nisin susceptibility was assigned to *gadD1, gadD2, gadD3, gadT1* and *gadT2* (lmo0447, lmo2363, lmo2434, lmo0448 and lmo2362; glutamate decarboxylase system) (Begley et al., 2010), *arcA* (lmo0043; arginine deiminase) (Kramer et al., 2006) and lmo0047 (lipoprotein) (Fritsch et al., 2011). Furthermore, the genes for alternative sigma factor *sigB* and *sigL* (general

stress response) (Begley et al., 2006; Palmer et al., 2009) were analyzed. The nucleic acid sequences of the selected genes were retrieved from whole genome sequencing (WGS) data of the six *L. monocytogenes* field isolates and were aligned by Pustel and ClustalW to elucidate DSVs. Alignments with DS Gene (Accelrys Inc.) were carried out in cases of an amino acid sequence similarity of at least 75.0 %. Bacterial promoter and transcription factor binding sites were predicted within a 300.0 bp upstream region of the target genes using BPROM (Softberry Inc., USA; Solovyev and Salamov, 2011). Putative Shine-Dalgarno (SD) sequences were identified by visual inspection of purine rich sequence stretches according to the consensus sequence provided by Shine and Dalgarno (1974).

3.2.4. Phenotypic characterization of NS and NNS L. monocytogenes field isolates via colorimetric GAD assay

Listeria monocytogenes field isolates were grown with and without 0.003 mg ml^{-1} free nisin in either BHI broth, LB broth or TSB for 24 h at 37.0 °C. Overnight cultures (1.0 ml) of *L. monocytogenes* field isolates, *E. coli* K-12 ER2738 (positive in GAD assay) and *L. fermentum* (negative in GAD assay) were centrifuged at 18,516 x g and 4.0 °C for 5 min. The supernatant was discarded and the cell pellet of *L. monocytogenes* field isolates was resuspended in 2.0 ml sterile ¼ ringer solution. Each bacterial suspension of *L. monocytogenes* field isolates was adjusted to 6.0 McFarland standard. In preliminary experiments the GAD assay failed when lower McFarland standards were used. Subsequently, the tube was centrifuged at 7,012 x g for 5 min (4.0 °C). The cell pellet of *E. coli* K-12 ER2738 and *L. fermentum* was washed with 1.0 ml ¼ ringer solution. All cell pellets were consecutively resuspended in 0.1 ml GAD reagent (1.0 g l^{-1} L-Glutamic acid [Merck]; 90.0 g sodium chloride [Carl Roth]; 3.0 ml TritonX 100 [Carl Roth]; 0.05 g bromocresol green [Carl Roth]), which was adjusted to pH 3.4 with 0.1

mol l^{-1} sodium hydroxide. Bacterial suspensions in duplicates were mixed vigorously and were incubated at 37.0 °C for up to 4 h. In intervals of 10 min, the color of the GAD assay was visually inspected. The assay was interpreted as positive if the color of GAD reagent shifts from yellow to blue indicating a pH shift from <3.8 to >5.4 (Carl Roth), respectively.

3.2.5. Sequencing of 30 NS and 30 NNS L. monocytogenes field isolates

Thirty NS and 30 NNS *L. monocytogenes* field isolates were randomly selected to broaden the DNA sequence variant (DSV) analyses of *gadD2* gene. Genomic DNA of field isolates was obtained from the German Federal Institute for Risk Assessment. Primer sequences covering the C-terminus were obtained using Primer3web (v. 4.0.0; http://primer3.ut.ee/; Koressaar and Remm 2007; Untergasser et al. 2012) and validated through BlastN. The following primers and PCR reaction conditions were used (**Table 10**).

Table 10: Sequences of *gadD2* primers and individual qPCR conditions.

Primer	Sequence [5' – 3']	qPCR set up	Time
		ID: 95.0 °C	5 min
gadD2_f	GAGCCACATCATCGGTCAATAC	D: 95.0 °C	30 s
		A: 53.1 °C	30 s
		E: 72.0 °C	1 min
gadD2_r	TAATGTGTGAAGCCGTGGACG	FE: 72.0 °C	10 min
		40.0 cycles of D/A/E	

Abbreviation: ID initial denaturation; D denaturation; A annealing; E elongation; FE final elongation.

PCR was performed in 25.0 µl reaction mixtures containing 2.0 µl of gDNA template, 300.0 nmol l^{-1} of each primer (Sigma-Aldrich), 2.5 u/µl High Fidelity PCR Enzyme Mix (Thermo Fisher Scientific GmbH, Germany), 1x High Fidelity PCR Buffer with $MgCl_2$ (Thermo Fisher Scientific), 0.2 mmol l^{-1} each dNTP Mix (Thermo Fisher Scientific), and

diethyl dicarbonate treated water (Carl Roth). Amplicons were sequenced at Seqlab GmbH (Germany). Sequences were aligned and were screened for synonymous mutations (BioNumerics v. 7.6; Applied Maths NV., Belgium). Gene sequence of *gadD2* retrieved from the 60 *L. monocytogenes* field isolates was deposited in the DDBJ/EMBL/GenBank databases under the accession numbers MF565691-MF565757.

Tree calculation was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) absolute method for DNA sequences and with Poison correction for protein sequence (DS Gene v. 2.5; Accelrys Inc., USA).

3.2.6. Growth of NS and NNS L. monocytogenes field isolates at sub-inhibitory free nisin concentration

The growth of the NS *L. monocytogenes* field isolate BfR L1031 and NNS *L. monocytogenes* field isolate BfR L261 was monitored in presence and absence of sub-inhibitory free nisin concentration (0.004 mg ml^{-1}) over time in six independent replicates. Each working suspension was prepared and added to each well of a sterile 96-well micro titer plate as described in **3.1.4.**. Free nisin (NisinZ™ P 95.0 %) was prepared in a 25.0 ml stock solution of 0.132 mg ml^{-1}. The working suspension of free nisin was prepared in pH adjusted (0.1 mol l^{-1} hydrogen chloride [HCl]) sterile BHI broth (pH 7.0 and 5.5). The microtiter plates were centrifuged at 4,000 rpm, 4 °C for 10 min. The supernatants were discarded to minimize pH changes in the wells. Cell pellets were resuspended in 0.2 ml BHI broth with 0 or 0.004 mg ml^{-1} free nisin or 0.2 ml BHI broth adjusted to pH 7.0 or 5.5. The microtiter plates were sealed with Breathe-Easy® membrane (Carl Roth) and were incubated at 37 °C. OD at 595.0 nm was measured daily for seven days using a FLUOstar OPTIMA microplate reader (BMG Labtech).

3.3. Application of electrostatic-adsorbed nisin to Neusilin particles onto surface of sour curd cheese

3.3.1. Field isolates, reference strains and bacterial growth

Listeria monocytogenes field isolates were grown as described in **3.1.2.**. *Escherichia coli* ATCC 25922, *Pseudomonas fluorescens* and *L. fermentum* were maintained in LB or SNBI. All cultures were stored like in **3.1.2.**. For subsequent cultivation in Coburg, *E. coli* ATCC 25922 and *L. fermentum* were grown as described in **3.2.1.**. *Pseudomonas fluorescens* was grown on LB agar plates for 24 h at 30.0 °C. The working suspensions were prepared as mentioned in **3.1.2.**.

3.3.2. Enumeration of L. monocytogenes and sampling procedure from non autoclaved SCC

Overnight cultures**,** working suspensions of *L. monocytogenes* or dilutions thereof were diluted ten-fold in either sterile ¼ ringer solution or sterile 1x phosphate-buffered saline (PBS). Ringer solution was used during SCC production and PBS during fluorescence *in situ* hybridization (FISH) procedure. Each 0.1 ml from the dilution series was plated in triplicate on BHI agar. The agar plates were incubated at 30.0 °C for 24 h. Subsequently, CFU ml^{-1} were enumerated to determine initial *L. monocytogenes* contamination level or to prepare *L. monocytogenes* standards for non-cultivation based methods.

From the surface of a non-autoclaved SCC loaf, thin slices were cut off with a surface area of approximately 0.45 cm^2. 0.5 g SCC was transferred to 5.0 ml ¼ ringer solution or 1x PBS. Subsequent homogenization was done using FastPrep®-24 with speed at 4.0 m s^{-1} for 30 s during each run (MP Biomedicals LLC., USA). The homogenate was ten-fold diluted in ¼ ringer solution or 1x PBS and 0.1 ml were plated on sheep blood agar (7.0 %) and on chromogenic *Listeria* agar (Oxoid) in triplicates. After incubation

at 30.0 °C for 24 h to 48 h, colonies with hemolytic activity or colonies with blue color and opaque halo were enumerated.

3.3.3. Preparation antimicrobial preservatives UFL2-N and free nisin

UFL2-N (Surflay Nanotec GmbH, Germany) and free nisin (NisinZ™ P 95.0 %) were prepared in pH adjusted (0.1 mol l^{-1} HCl) sterile BHI broth (pH 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, and 4.5) or in sterile ¼ ringer solution. 25.0 ml stock solution of 0.132 mg ml^{-1} UFL2-N or free nisin was daily prepared to omit release of nisin from UFL2 into broth or activity loss before start of the experiments. The nisin concentration in UFL2-N was calculated based on mass of nisin per mg UFL2 particle. Before susceptibility testing, UFL2-N stock solution was dispersed in ultrasonic bath (USR 30 H; Merck KGaA, Germany) at room temperature for 15 min. Routinely, 0.1 ml of the stock solution UFL2-N and free nisin was plated on BHI agar. The agar plate was incubated for 24 h at 30.0 °C and was inspected for absent of microbial growth.

3.3.4. Time- and pH-dependent in vitro nisin susceptibility testing of UFL2-N and free nisin

Inhibition of growth by UFL2-N and free nisin was tested on four NNS and two NS *L. monocytogenes* field isolates of serotypes IIa, IIb and IVb, which are associated with human listeriosis (**Table 8**). Each *L. monocytogenes* working suspension (**3.1.2.**) was diluted and added to the 96-well microtiter plate as described in **3.2.6.**. Bacterial cells were resuspended in 0.2 ml pH-adjusted BHI broth containing 0.004, 0.013, 0.026, and 0.132 mg ml^{-1} UFL2-N or free nisin. Microtiter plates were sealed with Breathe-Easy® membrane (Carl Roth) followed by incubation for seven days. At the University of Applied Sciences and Arts, Coburg, the OD of each well was measured daily at a wavelength of 595.0 nm after five seconds of shaking by FLUOstar OPTIMA microplate

reader (BMG Labtech). Controls were provided by incubating bacterial suspensions without supplementation of UFL2-N, free nisin, or UFL2. Cutoff value for bacterial growth was set to $\Delta OD_{595}>0.15$, given that the controls including the same *L. monocytogenes* field isolate also revealed $\Delta OD_{595}>0.15$. MICs were determined in six replicate measurements.

3.3.5. Growth of L. monocytogenes BfR L1031 field isolate in autoclaved SCC without time-lapsed nisin release

A commercial SCC loaf was homogenized in distilled water and was autoclaved at 121 °C for 10 min. Working suspension of *L. monocytogenes* field isolate BfR L1031 (**Table 8**) was adjusted to log 5.0 CFU ml^{-1} in sterile ¼ ringer solution and was added to 1.0 ml autoclaved SCC, which was prepared with and without 0.004 mg ml^{-1} free nisin. After one, two and three days of incubation, ten-fold dilutions of *L. monocytogenes* were enumerated on BHI agar to determine the CFU ml^{-1}.

3.3.6. Production of SCC loaf, addition of UFL2-N and free nisin, and L. monocytogenes BfR L1031 field isolate on SCC surface

Sour curd, two-week ripened SCC (“culture cheese”), sodium chloride and sodium hydrogen carbonate were manually mixed in sterile beakers at room temperature according to a traditional Hessian recipe of SCC. Loaves of SCC were formed from portions of 25.0 g by hand and were placed on racks in small boxes, which contained commercial rice. SCC loaves produced under laboratory conditions were further amended with *L. monocytogenes*. Therefore, 0.5 ml of TRIS buffer solution containing UFL2-N or free nisin were applied onto the upper surface of each loaf with 0.004, 0.013, 0.026, and 0.132 mg ml^{-1} (n=4). 0.5 ml sterile TRIS buffer solution was used as a substitute on loaves without addition of UFL2-N or free nisin (0.000 mg ml^{-1}). The

working suspension of *L. monocytogenes* BfR L1031 field isolate was diluted in ¼ ringer solution and 0.25 ml with approximately log 5.0 CFU ml^{-1} or approximately log 3.0 CFU ml^{-1} was added onto the SCC topside surface. After contamination, SCC was ripened for two days, 98.0 % relative humidity and 30.0 °C.

3.3.7. pH measurement of SCC surface

Since SCC matures from outside to inside (Belitz et al. 2008), the surface pH was measured over time. Loaves of SCC were formed and ripened as described in **3.3.6.**. After 2, 6, 20, 24, 43, 48 and 72 h of ripening the upper surface of a loaf was cut off in thin slices and 0.5 g SCC as well as 0.5 g SC (0 h) was homogenized in 10.0 ml distilled water. After continuous stirring, the pH-value was determined.

3.3.8. Non-cultivation based detection of L. monocytogenes by qPCR

3.3.8.1. Extraction of gDNA

For DNA extraction, several protocols like the polyethylene glycol (PEG) and phenol-chloroform based nucleic acid extraction as well as commercial kits like the KingFisher™ Cell and Tissue DNA Kit (Thermo Fisher Scientific), the PowerFood Microbial DNA Kit (Mo Bio Laboratories, USA) and the PowerSoil DNA Isolation Kit (Mo Bio) were tested. Finally, the KingFisher™ Cell and Tissue DNA Kit was used for preparation of the DNA standard and for DNA extraction from SCC samples (**3.3.2.**).

3.3.8.2. Quantitative PCR amplification (qPCR) of the *hlyA* gene

The *hlyA* gene, which encodes the *L. monocytogenes* specific virulence factor listeriolysin O, and the 16S rRNA gene from the gDNA was amplified in the thermal cycler CFX96 (Bio-Rad Laboratories GmbH, Germany). Published oligonucleotide primers were ordered from Sigma-Aldrich and their concentration was optimized (50.0

to 300.0 nmol l^{-1}) to achieve specific and efficient amplification (**Table 11**). Optimal conditions for target amplification was found when the single reaction had a concentration of 300.0 nmol l^{-1} of each forward and reverse primer. Theoretical average melting temperature of primers was at 60.6 °C (Bio-Rad CFX Manager v. 3.0; Bio-Rad Laboratories). However, optimal annealing temperature was found at 58.7 °C. Quantitative PCR reaction was performed in 25.0 µl reaction mixture as outlined in **Table 12**. Subsequently, the *hlyA* and 16S rRNA gene was amplified followed by a melting curve analysis. As negative controls, diethyl dicarbonate treated water (Carl Roth) was included and standard curve in duplicates was generated as previously described for every qPCR run (Nogva et al. 2000). Briefly, standard curves were generated by plotting threshold cycle values (C_T-values) of serial ten-fold dilutions of *L. monocytogenes* DNA as a function of CFU ml^{-1}. The same DNA standard was used for all qPCR runs of SCC experiments (**3.3.6.**).

Table 11: Sequences of primers and individual qPCR conditions. The *hlyA* gene amplified specifically species *L. monocytogenes*. It is present with one copy per genome (Nogva et al. 2000) while Eub341 and Eub534 target the bacterial ribosomal 16S rRNA genes.

Primer	Sequence [5' – 3']	qPCR set up	Time	Reference
LM_hlyA f	TGCAAGTCCTAAGACGCCA	ID: 95.0 °C D: 95.0 °C A: 58.7 °C E: 72.0 °C	5 min 20 s 20 s 20 s	Nogva et al. (2000)
LM_hlyA r	CACTGCATCTCCGTGGTATACTAA	40.0 cycles of D/A/E		
Eub341 f	CCTACGGGAGGCAGCAG	ID: 95.0 °C D: 95.0 °C A: 54.3 °C E: 72.0 °C	8 min 30 s 30 s 30 s	Muyzer et al. (1993)
Eub534 r	ATTACCGCGGCTGCTGG	40.0 cycles of D/A/E		

Abbreviation: ID initial denaturation; D denaturation; A annealing; E elongation.

Table 12: Components of qPCR reaction mix.

Component	Volume per reaction [µl]
2x BioRad SYBR Green Supermix[a]	12.5
5.0 µmol l^{-1} forward primer[b]	1.5
5.0 µmol l^{-1} reverse primer	1.5
Nuclease-free water	7.5
Total volume (with 2.0 µl DNA template or water)	**25.0**

[a] containing PCR buffer, $MgCl_2$ solution, SYBR green and Taq polymerase (Bio-Rad Laboratories).
[b] concentration of stock solution.

The DNA stability was evaluated after freeze and thaw cycles in four successions. The same DNA standard was used to dilute DNA ten-fold in diethyl dicarbonate treated water. The dilution series of each freeze and thaw cycle was amplified by qPCR under the same conditions and a standard curve was plotted. Based on its slope, the efficiency of qPCR was calculated. For example, a 100.0 % efficiency is obtained when the slope equals to -3.322 (Kralik and Ricchi 2017).

The sensitivity of the qPCR method that is the limit of detection (LOD) and limit of quantification (LOQ) was evaluated by measuring DNA standard curves repeatedly (n=10). The number of positive qPCR results for each dilution, e.g. 10/10, was expressed in percent e.g. 100.0 %. Hence, the LOD and LOQ were defined as the minimum number of cells that were detected in 95.0 % of the replicates (Kralik and Ricchi 2017).

The specificity of the *hlyA* primer set was tested on species within the genus *Listeria* and on unrelated bacteria. Therefore, *L. monocytogenes* field isolates (**Table 8**), strain *L. monocytogenes* SLCC2540 as well as strains *L. innocua* ATCC 33090, *Listeria ivanovii* ATCC19119, *L. seeligeri* SLCC3954, and *L. welshimeri* ATCC35897 were used. Unrelated bacteria were tested as well. Those were strain *E. coli* ATCC 25922, *L. fermentum* and *P. fluorescens*. Moreover, SC and SCC samples (**3.3.2.**) were examined for absence or presence of the target sequence. The extracted gDNA was

added to the qPCR reaction mix and the *hlyA* gene was amplified by qPCR. The *hlyA* primer set passed specificity test if only the species *L. monocytogenes* resulted in an amplification product. In summary, all *L. monocytogenes* field isolates passed specificity testing and mismatch targets were not amplified (**Table 13**).

Table 13: Specificity testing of LM_hlyA primers and their capability to amplify perfect match and mismatch targets from gDNA of phylogenetic related or unrelated bacteria.

Field isolate (n)	**Isolate collection[a]**	**DNA amplification**	**Identities [%]**	**Accession No.**
L. monocytogenes (10)				
BfR L32		+		
	SLCC2540	+	100.0	FR733645.1
BfR L261		+		
BfR L286		+		
BfR L308		+		
BfR L451		+		
BfR L493		+		
BfR L548		+		
BfR L1031		+		
BfR L1080		+		
Listeria spp. (4)				
L. innocua	ATCC 33090	-		
L. ivanovii subsp. *Ivanovii*	ATCC 19119	-	57.9	LT906478.1
L. seeligeri	SLCC 3954	-	0.0	FN557490.1
L. welshimeri	ATCC 35897	-	0.0	AM263198.1
Other microorganisms (5)				
E. coli	ATCC 25922	-	0.0	CP009072.1
L. fermentum		-		
P. fluorescens		-		
SC microbiota		-		
SCC microbiota		-		

[a] ATCC: American Type Culture Collection; SLCC: Special Listeria Culture Collection
Abbreviation: + DNA was amplified by qPCR; - DNA was not amplified by qPCR.

In preliminary experiments, the effect of organic and inorganic substances from the food matrix as well as UFL2-N on qPCR efficiency was evaluated. Therefore, extracted

gDNA of a non-autoclaved SCC sample containing 0.132 mg ml^{-1} UFL2-N and *L. monocytogenes* BfR L1031 field isolate below LOD was spiked with DNA standard (log 5.30, log 4.30 and log 3.30 CFU ml^{-1}). Different dilutions of the amended SCC sample (10:1, 1:10, 1:100 and 1:1,000) were tested. Subsequent qPCR was performed with four replicates.

3.1.1. Agarose gel electrophoresis

DNA extracts (**3.3.8.1.**) and PCR products (**3.2.5.**) were analyzed by agarose gel electrophoresis. Therefore, gel were prepared with 1.0 % agarose (Carl Roth) and 1x Tris-acetate-EDTA buffer (TAE buffer; Bio-Rad Laboratories). One µl DNA sample or 1.0 µl of PCR product was thoroughly mixed with 1.0 µl loading dye nucleic acid sample buffer (Bio-Rad Laboratories) and 4.0 µl distilled water. Afterwards, the gel was transferred into gel electrophoresis chamber (Bio-Rad Laboratories) filled with 1x TAE buffer and gel slots were loaded with sample and 2.0 µl molecular weight marker (Bio-Rad Laboratories). Finally, electrophoresis was carried out for approximately 30 min at 100.0 V. After staining with ethidium bromide and destaining with distilled water for 10 min each, gel was documented with UV light by VWR GenoPlex system (VWR).

3.1.2. Non-cultivation based detection of L. monocytogenes by fluorescence in situ hybridization (FISH)

The FISH method is based on oligonucleotide probes with tagged fluorophores to identify and locate specific bacteria in a microbiota without cultivation (Amann et al. 1990; DeLong et al. 1989). Overnight cultures were washed twice with sterile 1x PBS and were centrifuged for 5 min at 21,475 x g at room temperature. The bacterial suspension was adjusted to 0.5 McFarland in 1x PBS and was further diluted ten-fold if necessary. Samples were fixed in 0.6 ml of a mixture (1:1) of 1x PBS and 96.0 %

ethanol overnight at room temperature or stored at -20.0 °C. Afterwards, samples were washed with 1x PBS. Ten µl of fixed cells were transferred onto microscopic slides (Thermo Fisher Scientific), which were air dried. To make the cell envelope more permeable for probes, cell pellets were subjected to 10.0 µl of lysozyme (Carl Roth) for 15 min at room temperature. The reaction was stopped by rinsing the microscopic slide with sterile distilled water. After air drying, samples were dehydrated in graded ethanol series (50.0, 80.0 and 96.0 %) for 3 min each. Subsequently, samples were air dried and specific probes (ELLA Biotech, Germany) targeting *L. monocytogenes* and the majority of bacteria were used alone or in combination for hybridization (**Table 14**).

Table 14: Probes used for FISH.

Probe	Sequence [5' – 3']	Dye	Excitation/ emission [nm]	Sequence target
LIS.MONO	CGACCCTTTGTA CTATCCATTG	5´Cy5	646.0 / 664.0	*L. monocytogenes*
EUB338	GCTGCCTCCCGT AGGAGT	5´Cy3	549.0 / 562.0	Majority of bacteria (Amann et al. 1990)

The hybridization was performed by adding 10.0 µl of hybridization buffer (0.9 mol l^{-1} sodium chloride [Carl Roth]; 20.0 mmol l^{-1} TRIS-HCl buffer solution [pH 7.5; Carl Roth]; 0.01 % sodium dodecyl sulfate; and 15.0 % formamide [VWR International] for probe LIS.MONO and EUB338) to each well of microscopic slide. The hybridization buffer included the specific probes LIS.MONO and EUB338 with a final concentration of each 25.0 ng $µl^{-1}$. Incubation of microscopic slides in a 50.0 ml screw cap tube (Greiner Bio-One) was carried out in the dark at 52.0 °C for 3 h. After hybridization, microscopic slides were washed in the absence of light with 50.0 ml pre-warmed washing buffer (adjusted salt concentration of 0.3 mol l^{-1} sodium chloride to e.g. 15.0 % formamide; 20.0 mmol l^{-1} TRIS-HCl buffer solution [pH 7.5]; and 5.0 mmol l^{-1}

ethylenediaminetetraacetic acid [EDTA, pH 8.0, Carl Roth]) at 54.0 °C for 20 min. Finally, microscopic slides were removed from the washing buffer, rinsed with distilled water and were allowed to air dry. In addition, 10.0 µl of 4',6-diamidino-2-phenylindole (DAPI) (Carl Roth) was placed onto each well and incubated in the dark for 15 min. DAPI stains DNA as well as RNA and it has its excitation and emission maxima at 358.0 and 461.0 nm, respectively. After DAPI staining, the microscopic slide was washed with distilled water and was allowed to air dry. Subsequently, samples were mounted with Roti®-Mount FluorCare (Carl Roth). Strain *L. monocytogenes* SLCC2540 was used when FISH was applied to BHI broth samples. During optimization of FISH, the procedure was modified at some steps of procedure, e.g. hybridization time (1, 1.5 and 3 h) and formamide concentration (10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 %) resulting to the above stated conditions.

In preliminary experiments, the sensitivity and specificity of the probe LIS.MONO was evaluated. Working suspension of strain *L. monocytogenes* SLCC2540 was diluted ten-fold in 1x PBS (log 8.0, log 7.0, log 6.0, log 5.0, log 4.0, log 3.0, log 2.0 and log 1.0 CFU ml^{-1}) and was consecutively subjected to the FISH procedure. The specificity was tested on *L. ivanovii* ATCC19119 and *L. welshimeri* ATCC35897. For higher stringency the procedure was modified with varying formamide concentration (10.0, 15.0 and 20.0 %) in the hybridization buffer. Finally, 20.0 % formamide in the hybridization buffer was combined with either 0.3 mol l^{-1} or 0.9 mol l^{-1} sodium chloride in the washing buffer. However, these modifications could not resolve probe specificity issues and LIS.MONO hybridized to *L. ivanovii* ATCC19119 and *L. welshimeri* ATCC35897.

The FISH method was also applied to the SCC food matrix. Therefore, the SCC samples were proceeded as described in **3.3.2.**. However, one ml of homogenized SCC sample was centrifuged for 5 min at 21,475 x g at room temperature. After the

washing step and resuspending cell pellets in the 1x PBS and 96.0 % ethanol mixture (1:1), the FISH method was carried out as described above.

3.1.3. Microscopic images of FISH

Samples hybridized with probes with Cy3 and Cy5 labels (**Table 14**) were examined using the inverse fluorescence microscope AxioObserver.Z1 (Carl Zeiss AG, Germany) equipped with filter sets for Cy3, Cy5 and DAPI. Images were obtained using an electron-multiplying charge-coupled camera (EMCCD) (Carl Zeiss) and were processed using ImageJ (v. 1.50b, National Institutes of Health, USA)

3.1.4. Microscopic images of UFL2-N and L. monocytogenes BfR L1031 field isolate

Overnight culture of *L. monocytogenes* BfR L1031 field isolate was washed twice with sterile 1x PBS and centrifuged at 2,739 x g for 10 min at 4.0 °C. The supernatant was discarded and in 1x PBS bacterial suspension was adjusted to 0.5 McFarland standard. Samples were fixed in 0.6 ml of a mixture (1:1) of 1x PBS and 96.0 % ethanol overnight at room temperature. Afterwards, samples were centrifuged at 21,475 x g and 4.0 °C for 5 min. Cell pellets were resuspended in 0.6 ml 1x PBS. A UFL2-N dispersion adjusted to pH 5.0 with sodium acetate buffer solution (VWR International) was mixed with bacterial suspension to obtain a concentration of 0.005 mg ml^{-1}. 0.01 ml was spotted on a microscopic slide and was allowed to air dry. After lysing the cell envelope with lysozyme and dehydrating cells in graded ethanol series (**3.1.2.**), microscopic slide was air dried. Subsequently, *L. monocytogenes* BfR L1031 field isolate was stained by DAPI (**3.1.2.**). Images were recorded with EMCCD and were processed with Adobe Photoshop CS6.

3.2. Statistical analyses

In all cases, data sets were first evaluated for parametric or non-parametric distribution. Thereafter, adequate statistical measures like mean and standard deviation or median were applied to data. Statistical calculations were either performed in Origin 2017 (v. b9.4.1.354, Origin Lab Corporation, USA), R Studio (v. 3.3.3, R Core Team, Austria) or manually in Excel (v. 2013, Microsoft Office, USA).

3.2.1. Efficacy and susceptibility testing of micro biocides

Median values of MICs of each disinfectant compound and antimicrobial preservative were calculated for the category origin of isolation and serotype of respective *L. monocytogenes* field isolate. Categories were processed with Kruskal-Wallis test at a significance level of 95.0 %. To determine significant differences, categories were subjected to a two-tailed Wilcoxon-Mann-Whitney-Test with and without Bonferroni correction ($p<0.05$ and $p<0.002$, respectively). In addition, the non-linear correlation ratio coefficient (η; Pearson 1911) between nisin susceptibility and serotype or nisin susceptibility and origin of isolation was calculated. In contrast to correlation, η covers linear and non-linear associations and if it reaches one, there is no dispersion within the respective origin of isolation or serotype. If η becomes zero, there is no functional dependence. Two-sided Student's t tests ($\alpha=0.05$) were carried out of OD after 24, 48, 72, 96, 120, 144 and 168 h of incubation to test statistical significance of respective growth rates with and without nisin addition.

Field isolates from crustacean and mollusc, of serotype IVa (**Table 5**) and field isolates, which could not be serotyped (n=5), were omitted from statistical analysis because of low numbers. In addition, a two-tailed Wilcoxon-Mann-Whitney-Test ($p< 0.05$) was used to evaluate the presence or absence of significant differences between MIC of each disinfectant compound and antimicrobial preservative and their respective

antibiotic resistance pattern as characterized previously (Noll et al. 2018). The Kendall Rank Correlation Coefficient (τ_b) was used to estimate their relationship. However, only 236 antibiotic resistance patterns from 251 of *L. monocytogenes* field isolates were present. Susceptibility of field isolates to antibiotics were classified as susceptible (S), intermediate (I), and resistant (R) according to Noll et al. (2018).

3.2.2. Application of electrostatic-adsorbed nisin to Neusilin particles onto surface of sour curd cheese

The bacterial growth expressed in CFU ml^{-1} was log-transformed in qPCR data sets to test statistical significance by a two-tailed Wilcoxon-Mann-Whitney-Test at 95.0 % confident interval.

3.3. Contribution of other workers to this dissertation

Prof. Dr. Matthias Noll kindly provided information of antibiotic and nisin susceptibility of *L. monocytogenes* field isolates. Dr. Jens A. Hammerl analyzed the WGS data of six *L. monocytogenes* field isolates and helped with calculations constructing the phylogenetic tree based on *gadD2*. Dr. habil. Lars Dähne kindly provided UFL2-N and information about UFL2 zeta potential. Florian Westhäuser contributed in SCC experiments and within the scope of bachelor theses, Katharina Neudert and Nico Rössel were supportive during microscopy.

4. RESULTS

4.1. Study 1 – Efficacy and susceptibility testing of micro biocides

4.1.1. Disinfectant compounds

Reduced efficacy of QACs and H_2O_2 in BHI broth was not observed. Similar MICs of QACs and H_2O_2 were found in BHI broth and in 50.0 mmol l^{-1} TRIS buffer solution. The TRIS buffer solution was used in preliminary experiments (data not shown). In contrast, lower MICs of 0.5 mg ml^{-1} and consequently higher NaOCl efficacy was observed in TRIS buffer solution compared to MICs in BHI broth. The highest efficacy against *L. monocytogenes* in BHI broth was yielded by QACs (**Figure 6A** and **B**). *Listeria monocytogenes* field isolates derived from milk/cheese and other dairy products had significant lower MICs of CKC compared to those derived from fish and fish products ($p<0.002$; **Figure 6B**). Interestingly, reference strains were significantly more susceptible to H_2O_2 than *L. monocytogenes* field isolates ($p<0.05$; **Figure 6C**). MICs of NaOCl were observed for the majority of *L. monoctogenes* field isolates at 4.0 mg ml^{-1}, which were identical for almost all origins of isolation (**Figure 6D**).

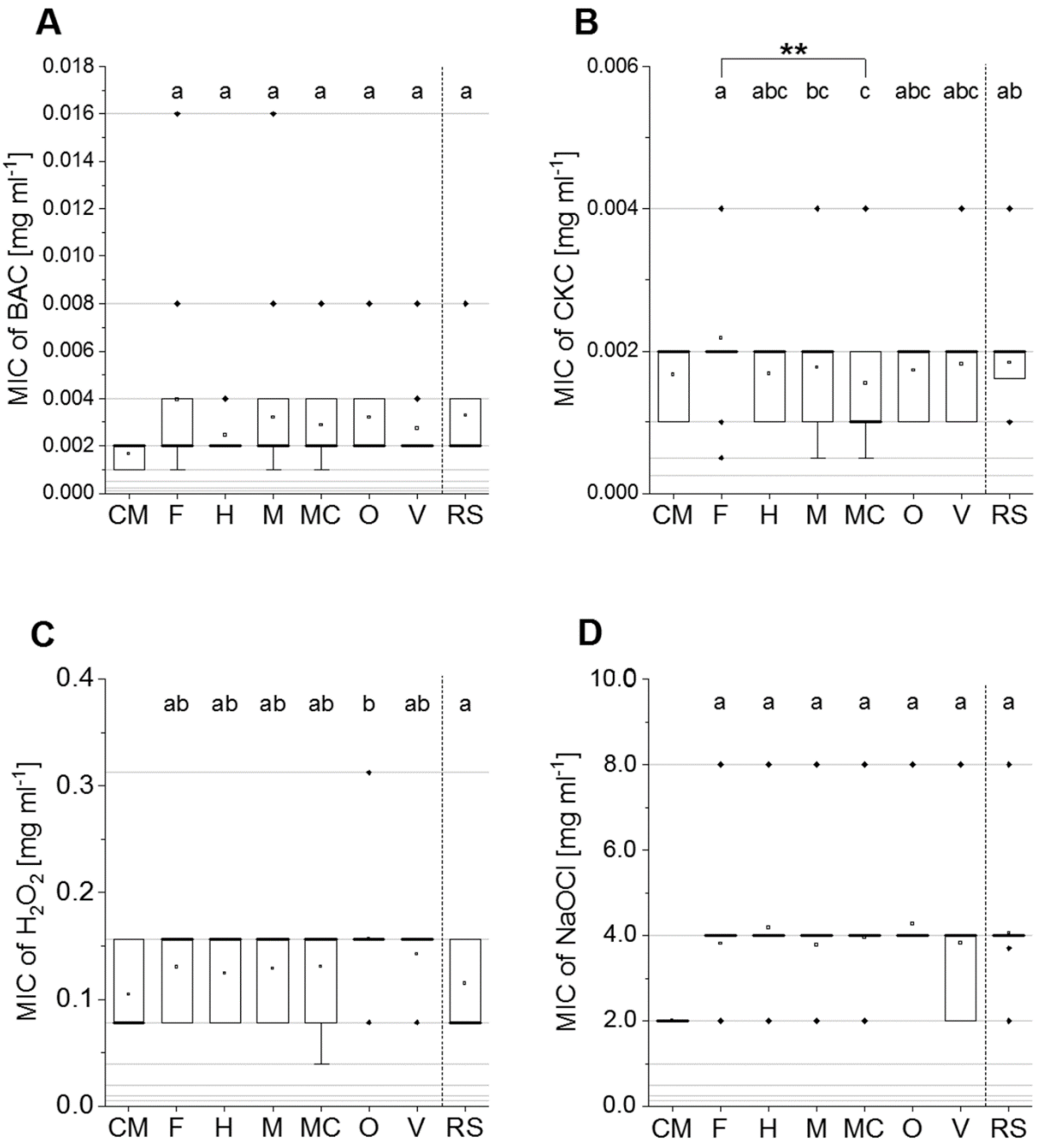

Figure 6: MICs of disinfectant compounds as a function of the origin of isolation of *L. monocytogenes* field isolates and *Listeria* spp. reference strains. **A** Benzalkonium chloride (BAC), **B** cetalkonium chloride (CKC), **C** hydrogen peroxide (H_2O_2), **D** sodium hypochlorite (NaOCl). For acronyms of CM, F, H, M, MC, O, RS, V and respective collected field isolates within each origin of isolation see **Table 5**. Gray horizontal bars denote individual dilution range of disinfectant compounds. CM was excluded from statistical analysis (n=3). Rank sums sharing the same superscript are not significantly

different from each other ($p<0.05$). ** Significance after Bonferroni correction ($p<0.002$).

4.1.2. Antimicrobial preservatives

The nisin susceptibility of 282 *L. monocytogenes* field isolates and 39 *Listeria* reference strains was characterized (**Table 6** and **Table 7**). On the other hand, MICs of free nisin were obtained from Szendy et al. (2019b). The majority of *L. monocytogenes* field isolates were already susceptible at 0.011 mg ml^{-1} (MIC_{50}). *Listeria monocytogenes* field isolates were classified to NS ($MIC<MIC_{50}$) and NNS ($MIC\geq MIC_{50}$). MIC_{90} was at 0.039 mg ml^{-1}. Nine NNS *L. monocytogenes* field isolates were able to grow on 0.039 mg ml^{-1} while seven NS *L. monocytogenes* field isolates were very sensitive towards free nisin (<0.0001 mg ml^{-1}). According to the correlation ratio coefficient, nisin susceptibility of *L. monocytogenes* field isolates was associated to their origin of isolation ($\eta=0.761$). *Listeria monocytogenes* field isolates retrieved from milk/cheese and other dairy products were inhibited at significantly higher free nisin concentrations than field isolates from other origins of isolation ($p<0.002$). The suspension containing citral turned milky after thoroughly dispersing in BHI broth. Friedman et al. (2002) described this behavior of EO compounds in culture broth previously. Nevertheless, neither the efficacy of citral nor the efficacy of $NaNO_2$ did seem to be affected by BHI broth. MICs of citral varied among the 251 *L. monocytogenes* field isolates and *Listeria* spp. reference strains. Depending on the origin of isolation, the majority of field isolates were inhibited at 8.9 to 17.8 mg ml^{-1}, respectively (**Figure 7A**). The efficacy control *L. monocytogenes* ATCC13932 was inhibited at a MIC of 8.9 mg ml^{-1}. Throughout efficacy testing, this strain was inhibited within two two-fold dilutions, 2.23 mg ml^{-1} and 17.8 mg ml^{-1} respectively.

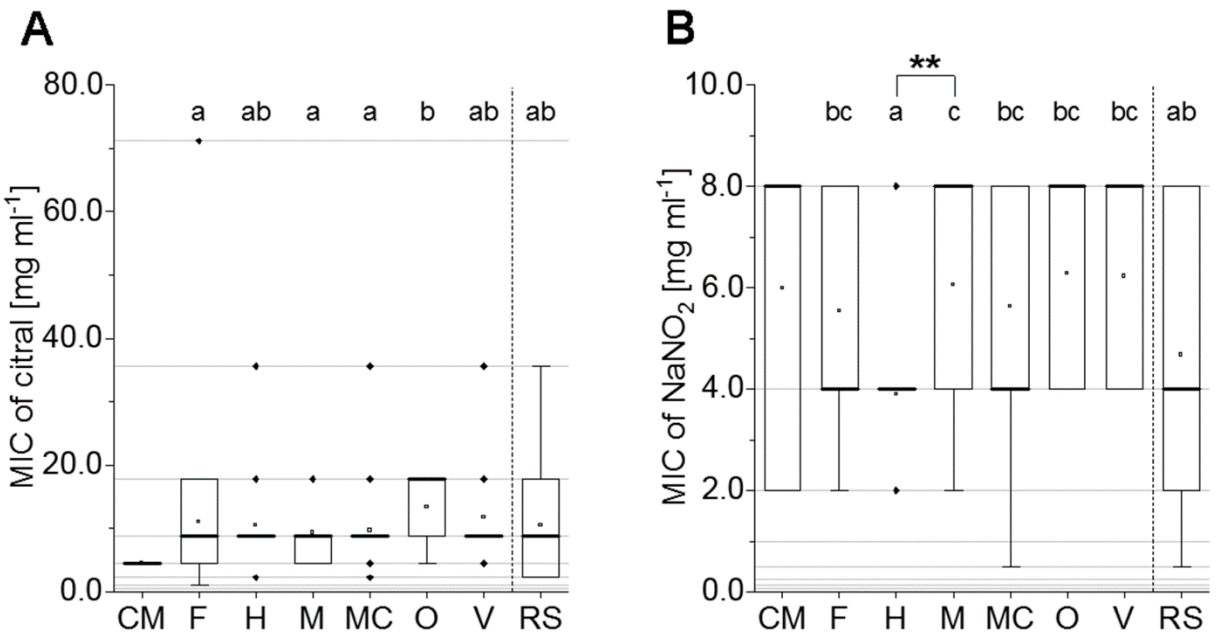

Figure 7: MICs of antimicrobial preservatives as a function of the origin of isolation of *L. monocytogenes* field isolates and *Listeria* spp. reference strains. **A** Citral and **B** sodium nitrite ($NaNO_2$). For acronyms of CM, F, H, M, MC, O, RS, V and respective collected field isolates within each origin of isolation see **Table 5**. Gray horizontal bars denote individual dilution range of antimicrobial preservatives. CM was excluded from statistical analysis (n=3). Rank sums sharing the same superscript are not significantly different from each other ($p<0.05$). ** Significance after Bonferroni correction ($p<0.002$).

While MICs of $NaNO_2$ from meat and meat products were observed at 8.0 mg ml^{-1}, MICs of *L. monocytogenes* field isolates from human were significantly lower ($p<0.002$; **Figure 7B**). In addition, *L. monocytogenes* field isolates from humans had the lowest median ($p<0.05$). Compared to citral, efficacy testing with $NaNO_2$ led to a reproducible MIC of *L. monocytogenes* ATCC13932. Moreover, reference strains were significantly more susceptible to $NaNO_2$ than the field isolates ($p<0.05$).

Overall, no association between MICs and serotype was found for disinfectant compounds, citral and $NaNO_2$. In contrast, nisin susceptibility was associated to serotype (η=0.769). MICs of serotype IIa were significantly higher compared to IIb, IIc, and IVb ($p<0.05$) and the ratio of serotypes to MIC_{50} and MIC_{90} is shown in **Figure 8**.

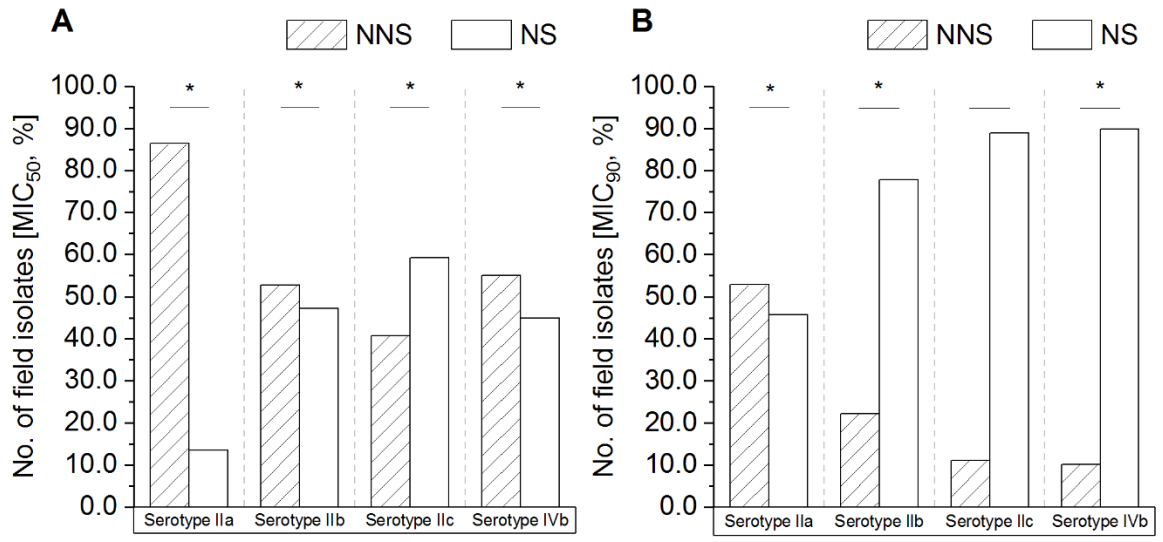

Figure 8: Distribution of 282 nisin susceptible (NS) and nisin non-susceptible (NNS) *L. monocytogenes* field isolates corresponding to their serotype and relative abundance, MIC_{50} (**A**) and MIC_{90} (**B**) respectively. Asterisk indicates significant difference between NS and NNS ($p<0.05$). Information of nisin susceptibility was provided by Prof. Dr. Matthias Noll and the German Federal Institute for Risk Assessment (Szendy et al. 2019b).

Interestingly, a high abundance of serotype IIa in milk/cheese and other dairy products was found according to the high frequency of NNS *L. monocytogenes* field isolates. Hence, six field isolates of serotype IIa were selected (**Table 8**). Four NS and two NNS *L. monocytogenes* field isolates. Both NNS *L. monocytogenes* had MICs of >0.039 mg ml^{-1} and were isolated from raw milk. To investigate the bacteriostatic nature of nisin, growth curves of field isolates were analyzed in absence and presence of free nisin

(**Figure 9**). In absence of free nisin, the lag phase of NNS *L. monocytogenes* BfR L245 field isolate was shorter than the lag phase of NNS *L. monocytogenes* BfR L261 field isolate. However, when free nisin was added, the conditions changed in favor of field isolate BfR L261 since the lag phase was shorter compared to field isolate BfR L245. With addition of free nisin, only NNS *L. monocytogenes* field isolates were able to grow into exponential phase after delayed lag phase (**Figure 9**).

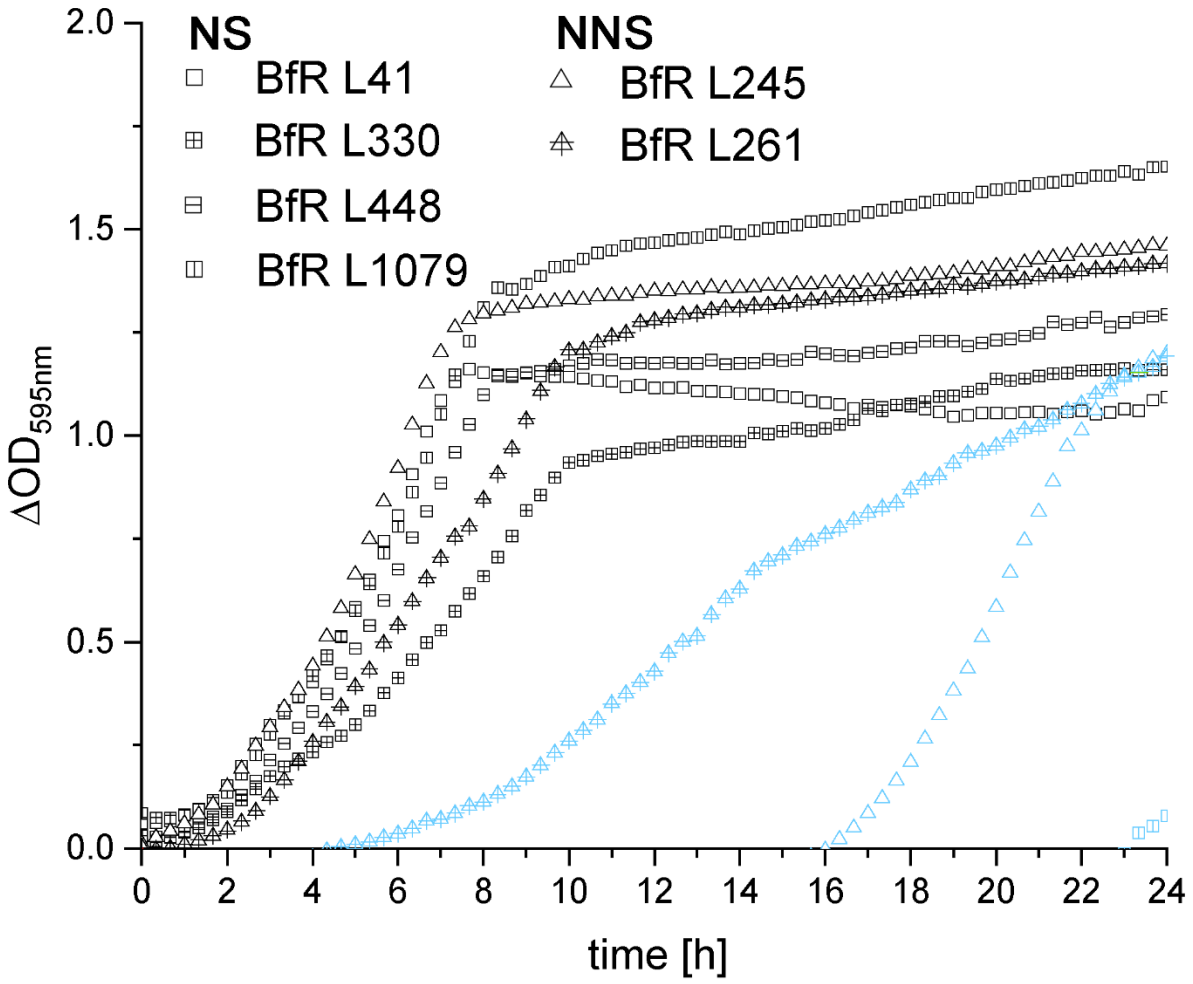

Figure 9: Growth curves of nisin susceptible (NS) (□) and nisin non-susceptible (NNS) (△) *L. monocytogenes* field isolates with (blue) and without supplementation of 0.011 mg ml^{-1} free nisin (black) in TSB (pH 7.3). Symbols are denoted in figure legend. Abbreviation: OD optical density. n=10

4.1.3. Correlation of micro biocides to respective antibiotic resistance of L. monocytogenes field isolates

The first choice to treat human listeriosis is a combination of β-lactam antibiotics and aminoglycosides (e.g. gentamicin) (Boisivon et al. 1990; Hof 2004; Temple and Nahata 2000). The MICs of 14 antibiotics from 259 *L. monocytogenes* field isolates were obtained from Noll et al. (2018). Comparison of antibiotic resistances to MICs of disinfectant compounds and antimicrobial preservatives from the field isolate collection used for micro biocide efficacy testing (**Table 5**) revealed that NaOCl was significantly associated to gentamicin, tetracycline and trimethoprim/sulfamethoxazole ($p<0.05$). Citral was significantly associated to gentamicin, meropenem, rifampicin, tetracycline and trimethoprim/sulfamethoxazole ($p<0.05$; **Table 8.2-1**, **Table 8.2-2** and **Table 8.2-3**). In **Table 15**, antibiotics and micro biocides were selected to have similar mode of action as well as bacterial resistance, which is based on modified bacterial cell components or other resistance mechanisms in these target regions (Nair et al. 2016; Walsh 2003). According to the Kendall rank correlation coefficient (τ_b), NaOCl was highly correlated to gentamicin ($\tau_b=0.92$) and trimethoprim/sulfamethoxazole ($\tau_b=0.80$), which *L. monocytogenes* field isolates were resistant to. Similarly, correlation coefficient τ_b for citral and gentamicin was 0.81. Citral and trimethoprim/sulfamethoxazole had a lower τ_b of 0.58. Moreover, increasing MICs of NaOCl and citral were skewed to *L. monocytogenes* field isolates with multi-antibiotic resistances (**Figure 10**).

Table 15: Comparison of antibiotic resistances to MICs of NaOCl and citral. Number of *L. monocytogenes* field isolates (n=236) inhibited by various antibiotics are arranged in susceptible (S), intermediate (I) or resistant (R) according to Noll et al. (2018).

Biocide	MIC [mg ml^{-1}]	ERY[a]			GEN[a]			LIZ[a]			RAM[a]			SXT[a]			TET[a]			TGC[a]		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
	8.0	16	-	1	13	-	4	17	-	-	4	9	4	13	-	4	-	10	7	-	10	7
NaOCl	4.0	168	-	1	162	-	7	169	-	-	41	126	2	160	-	9	35	93	41	1	104	64
	2.0	48	-	2	50	-	-	50	-	-	10	39	1	50	-	-	21	24	5	2	33	15
Signf.[b]			ns			*						ns			*			*				
	71.20	1	-	-	-	-	1	1	-	-	-	-	1	-	-	1	-	-	1	-	-	1
	35.60	3	-	1	2	-	2	4	-	-	-	2	2	2	-	2	-	2	2	-	2	2
	17.80	41	-	-	37	-	4	41	-	-	7	32	2	37	-	4	9	18	14	-	21	20
Citral	8.90	128	-	1	125	-	4	129	-	-	32	96	1	125	-	4	29	74	26	2	88	39
	4.45	49	-	1	50	-	-	50	-	-	12	38	-	48	-	2	10	31	9	-	31	19
	2.23	9	-	1	10	-	-	10	-	-	4	5	1	10	-	-	7	2	1	1	5	4
	1.11	1	-	-	1	-	-	1	-	-	-	1	-	1	-	-	1	-	-	-	-	1
Signf.			ns			*						*			*			*				

[a] ERY: Erythromycin; GEN: Gentamicin; LIZ: Linezolid; RAM: Rifampicin; SXT: Trimethoprim/sulfamethoxazole; TET: Tetracycline; TGC: Tigecycline.

[b] Comparison between S and R field isolates; in empty cells comparison was not possible, ns and * indicate not significant and significant at $p<0.05$, respectively.

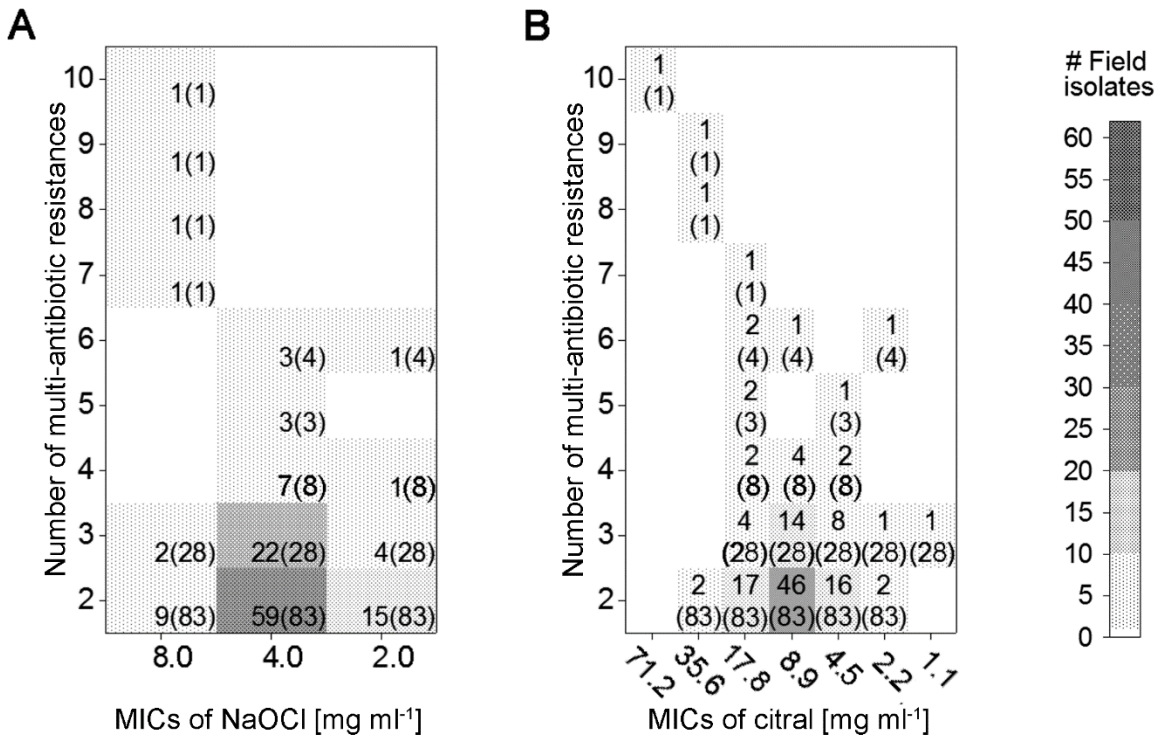

Figure 10: MICs of micro biocides were compared to 130 *L. monocytogenes* field isolates with two or more antibiotic resistances. **A** Sodium hypochlorite solution (NaOCl) and **B** citral. Numbers indicate abundance. Grayscale represents sample size of field isolates. Information of antibiotic resistance was provided by Noll et al. (2018).

Although the skewness in **Figure 10** is apparent, *L. monocytogenes* field isolates combining high MICs of micro biocides and six to ten antibiotic resistances remained sensitive to other disinfection compounds, CKC and H_2O_2 respectively (**Table 16**). On the other hand, those field isolates showed tolerance to BAC, $NaNO_2$ and free nisin.

Table 16: MICs of micro biocides of *L. monocytogenes* field isolates with multi-antibiotic resistances and resistance (R) to gentamicin (GEN) and trimethoprim/sulfamethoxazole (SXT) (Noll et al. 2018). Highlighted columns are micro biocides correlating to GEN and SXT resistance.

No. of R	BfR No.	MIC [mg ml^{-1}]						
		BAC	CKC	H_2O_2	NaOCl	Free nisin	citral	$NaNO_2$
10	L1528	0.004	0.002	0.156	8.0	0.011	71,2	>8.0
9	L1886	0.008	0.004	0.156	8.0	-	35,6	>8.0
8	L737	0.008	0.002	0.156	8.0	0.011	35,6	>8.0
7	L642	0.002	0.002	0.156	8.0	0.011	17,8	>8.0
6	LI00006	0.002	0.002	0.156	4.0	-	17,8	4.0

Abbreviation: BAC benzalkonium chloride; CKC cetalkonium chloride; H_2O_2 hydrogen peroxide; NaOCl sodium hypochlorite; $NaNO_2$ sodium nitrite.

However, free nisin susceptibility of *L. monocytogenes* field isolates neither showed skewness to multi-antibiotic resistances (**Table 8.2-4**) nor were NNS *L. monocytogenes* field isolates resistant to the antibiotic classes penicillin and carbapenem of the β-lactam antibiotics, which have their mode of action against cell wall (Kapoor et al. 2017). The information of antibiotic and nisin susceptibility of four NNS *L. monocytogenes* field isolates were available. In fact, those field isolates were sensitive to first and third generation of penicillin benzylpenicillin, ampicillin and the carbapenem meropenem, respectively. Third generation cephalosporin ceftriaxone was not very efficient in inhibiting growth of NNS *L. monocytogenes* field isolates. All NNS field isolates remained sensitive to the glycopeptide antibiotic vancomycin, which inhibits cell wall synthesis (Kapoor et al. 2017). However, NNS *L. monocytogenes* field isolates were resistant to daptomycin, a lipopeptide antibiotic targeting the cell membrane (Kapoor et al. 2017). Regarding the susceptibility to gentamicin, the NNS field isolates remained sensitive to the antibiotic, which has its mode of action as an inhibitor of protein synthesis. The antibiotic resistance of two NNS *L. monocytogenes*

field isolates to trimethoprim/sulfamethoxazole, which blocks the folic acid formation, is intriguing.

4.2. Study 2 – Molecular analyses on nisin tolerance

The whole genome of six *L. monocytogens* field isolates (**Figure 9** and **Table 9**) had been sequenced. The WGS data was analyzed for DNA sequence variants (DSVs) in genes putatively associated with nisin susceptibility and its gene regulation. The bioinformatical work was collaborated with the German Federal Institute for Risk Assessment (Szendy et al. 2019b). Genes involved in cell wall modifications, BceAB-like ABC transporter, genes encoding for TCS and TCS regulators as well as alternative sigma factors showed no association with nisin susceptibility (**Table 8.2-5** and **Table 8.2-6**). Based on DNA and its derived protein sequence, both NNS *L. monocytogenes* field isolates differed from NS field isolates in the *gadD2* gene encoding for the GAD system (**Table 17**). The DSV in *gadD2* resulted in an amino acid substitution from aspartic acid (D) to asparagine (N) at the end of the GadD2 C-terminus (protein position 453).

To screen more NNS *L. monocytogenes* field isolates for this specific finding in the *gadD2* gene, randomly selected field isolates were analyzed by sequencing of the *gadD2*. The German Federal Institute for Risk Assessment contributed to the DNA extraction of the selected field isolates, which are deposited at the institute, and helped with calculating a phylogenetic tree based on *gadD2*.

Table 17: Comparison of the gad operon in NS and NNS *L. monocytogenes* field isolates (referred to BfR L41). The amino acid (aa) substitution in GadD2 (453: D→N) is marked in bold. Data was provided by the German Federal Institute for Risk Assessment (Szendy et al. 2019b).

Protein (Size)	**Predicted function**	**NS *L. monocytogenes* field isolates**				**NNS *L. monocytogenes* field isolates**	
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261
GadD1 (463 aa)	Catalyze decarboxylation of glutamate	+	n.d.	n.d.	+	n.d.	n.d.
GadD2 (464 aa)	Catalyze decarboxylation of glutamate	-	380: N→D	380: N→D	-	-	-
		-	385: K→N	385: K→N	-	-	-
		-	-	-	-	**453: D→N**	**453: D→N**
		-	454: T→N	-	-	-	-
GadD3 (467 aa)	Catalyze decarboxylation of glutamate	-	-	-	-	-	145: E→K
		-	187: N→D	-	-	-	187: N→D
		-	207: V→I	-	-	-	207: V→I
		-	-	353: L→I	-	353: L→I	-
GadT1 (484 aa)	Antiporter in decarboxylation of glutamate	+	n.d.	n.d.	+	n.d.	n.d.
GadT2 (507 aa)	Antiporter in decarboxylation of glutamate	-	-	-	278: G→D	-	-
		-	-	409: V→I	-	409: V→I	-
		-	-	-	-	416: V→I	-
		-	-	419: V→I	419: V→I	**-**	-
		-	-	438: M→T	438: M→T	**-**	-
		-	-	441: I→M	441: I→M	**-**	-

Abbreviation: + protein present; - protein absent; D aspartic acid; E glutamic acid; G glycine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; n.d. not detected; NS nisin susceptible; NNS nisin non-susceptible; T threonine; V valine.

The C-terminal end of *gadD2* displayed minor sequence variability and clustered on DNA sequence level and protein level according to their lineage affiliation (**Figure 11**). About 12.0 % (7/60) of the screened *L. monocytogenes* field isolates showed the specific DSV in *gadD2* and were all serotype IIa from a total of 22 serotype IIa NNS field isolates (red frame, **Figure 11A**). None of the NS field isolates showed the specific DSV in *gadD2*. After translation of the C-terminus region to amino acid level, NNS *L. monocytogenes* field isolates clustered in the same group as the NNS field isolates subjected to WGS (**Figure 11B**). From this group of *L. monocytogenes* field isolates 91.0 % were isolated from milk/cheese and other dairy products.

Moreover, the identified group shared the same C-terminus protein sequence (“HNTQQ”) of GadD2 (**Figure 11B** and **Table 8.2-7**). Two more unique protein sequences were found. The protein sequence of “HDNQQ” was indicative for serotype IIb. On the other hand, the third sequence “HDTQQ” was shared mainly among serotypes IIa and serotype IVb. Thus, the C-terminus end may not be highly conserved allowing amino acid substitutions among different serotypes.

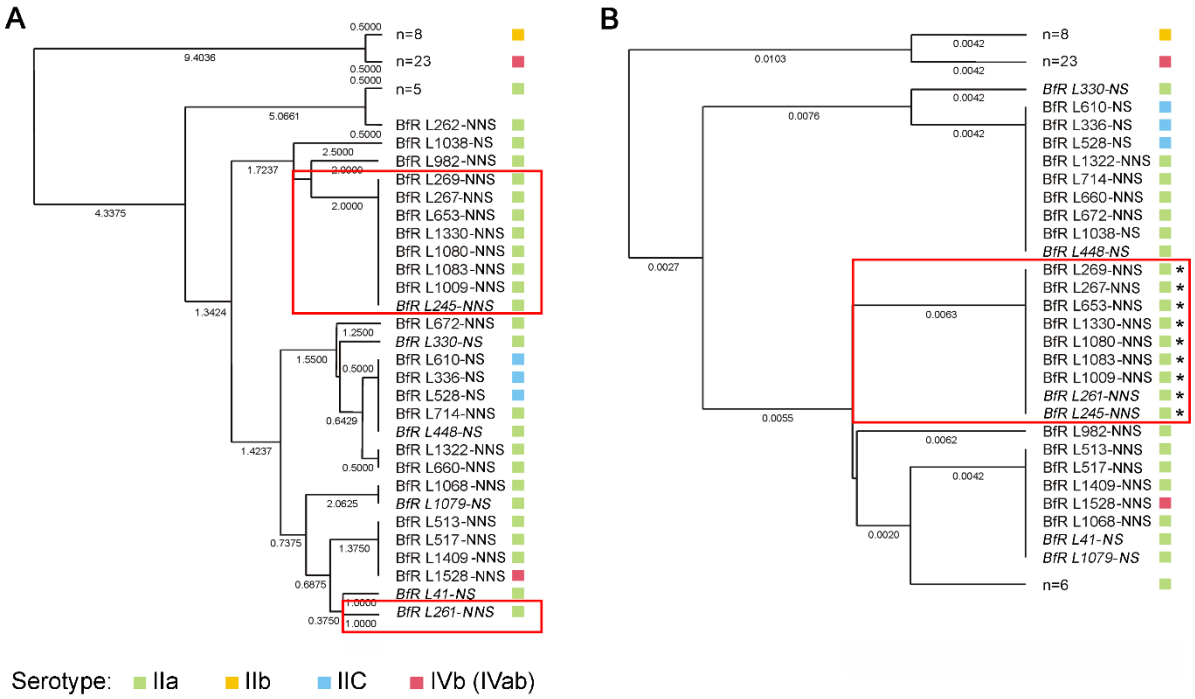

Figure 11: Clustering the *gadD2* sequences of *L. monocytogenes* field isolates based on DNA sequence level (**A**) and protein level (**B**) using UPGMA clustering method. Italicized field isolates have been subjected to whole genome sequencing (WGS) (**Table 9**). Color scheme next to field isolate name represents individual serotype (see figure legend). Red frames highlight field isolates with specific DNA sequence variant (DSV) in *gadD2* resulting in amino acid substitution $D_{453}N$. Asterisks indicate the shared protein sequence of the C-terminus ("HNTQQ") based on GadD2 of *L. monocytogenes* ATCC BAA-679 (start at 381 aa; end at 464 aa; UniProt accession number: Q9EYW9). Calculated phylogenetic tree based on *gadD2* was adopted from the German Federal Institute for Risk Assessment (Hammerl 2017; personal communication). Abbreviation: aa amino acid; D aspartic acid; N asparagine; NS nisin susceptible; NNS nisin non-susceptible.

To find a relationship between the $D_{453}N$ substitution in GadD2 and nisin tolerance, the GAD system was studied in the presence of L-glutamic acid and the pH indicator bromocresol green. The choice of culture broth to promote growth of *L. monocytogenes*

during pre-cultivation influenced the time necessary to change the color of the GAD reagent (**Table 8.2-9**). Conclusively, only pre-cultivation in TSB resulted in reasonable results.

Secondly, the growth rate of *L. monocytogenes* field isolates at sub-inhibitory free nisin concentration in combination with pH 7.0 and pH 5.5 was monitored over time (**Figure 12**). In the presence of free nisin, the NNS field isolate reached significantly faster the exponential growth phase compared to the NS field isolate after 24 hours at pH 7.0 ($p<0.05$). Subsequently, growth in the stationary phase did not significantly differ between NNS and NS field isolates ($p<0.05$). At pH 5.5, however, no significant difference was observed between NNS and NS field isolates in the initial and subsequent bacterial growth phases ($p<0.05$; **Figure 12B**).

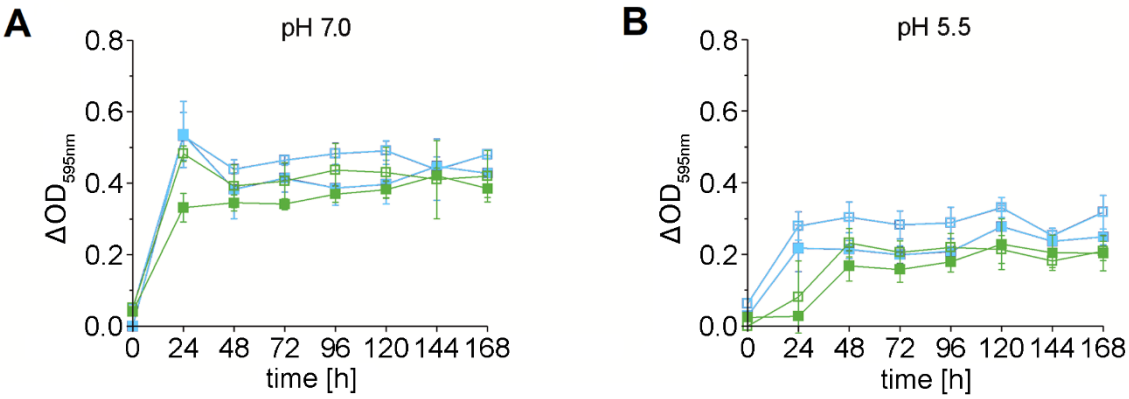

Figure 12: Optical density (ΔOD_{595nm}) of nisin susceptible (NS) *L. monocytogenes* field isolate without *gadD2* DNA sequence variant (DSV) (■) and nisin non-susceptible (NNS) with *gadD2* DSV and substitution at $D_{453}N$ (□) in BHI broth with 0 mg ml^{-1} (blue) or sub-inhibitory concentration of free nisin (0.004 mg ml^{-1}, green) at pH 7.0 (**A**) and pH 5.5 (**B**). Each curve is the mean of six replicates. Error bars represent standard error of mean. Abbreviation: D aspartic acid; N asparagine

4.3. Study 3 – Application of electrostatic-adsorbed nisin to Neusilin particles onto surface of sour curd cheese

The prerequisites for employing free nisin and UFL2-N on the surface of SCC were set in studies **4.1.** and **4.2.**. In summary, the food additive nisin was selected among all the micro biocides since its application in dairy industry is in compliance with legal requirements (EU 2011a). Proteinaceous compounds were not problematic for obtaining MICs. Furthermore, the subdivision of *L. monocytogenes* field isolates into NS and NNS laid down the criteria for the field isolate selection in study **4.3.**. The ideal *L. monocytogenes* field isolate should belong to NS. Additionally, the serotype of the field isolate should be clinically relevant. Study **4.2.** pointed out a potential tolerance development. The NS and serotype IVb field isolates did not show association of *gadD2* with nisin susceptibility based on WGS data. Consequently, a tolerance development of the selected field isolate and worst case scenario is unlikely to occur during the experiments in **4.3.**.

For the successful release of electrostatic adsorbed nisin from UFL2-N on the surface of non-autoclaved SCC, the surface pH was of paramount importance. The surface pH of SCC rapidly increased from pH 4.6 in SC to pH 6.3 after the addition of curing salts and thereafter steadily increased to pH 7.0 (**Figure 13**). The zeta potential of Neusilin UFL2 was measured by our collaboration partner. The positive zeta potential of UFL2 dropped with increasing pH until its value was equal to zero at the particle's isoelectric point, which was about pH 5.3 (**Figure 13**). Based on measurement of the surface pH, the isoelectric point of UFL2 would already be surpassed after one hour as illustrated in **Figure 13** and this would consequently reduce the nisin release.

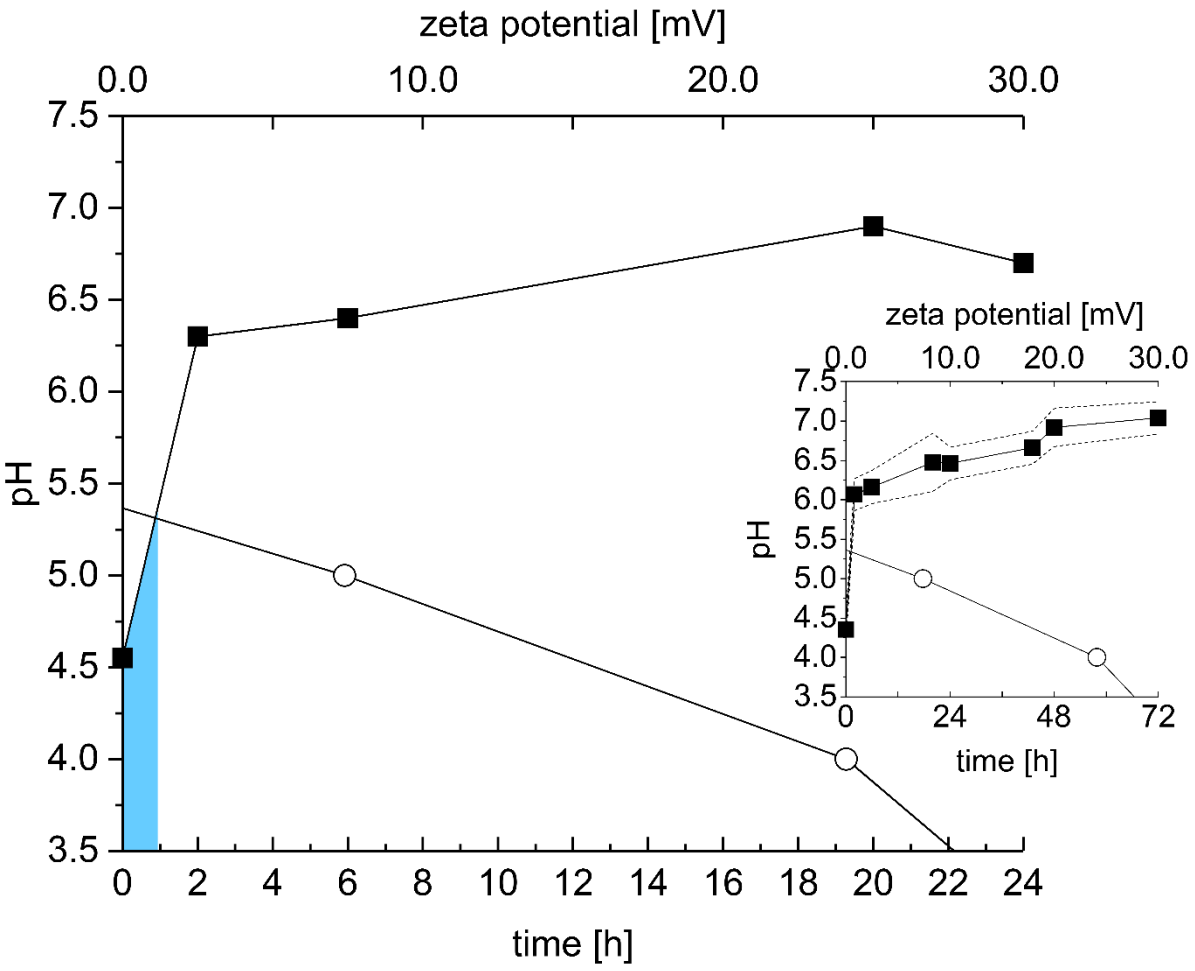

Figure 13: Experimental measurements of pH-value on SCC surface during SCC production over time (■) and zeta potential of UFL2 as a function of pH (○). Filled area under line (-■-) indicates potential timespan at which zeta potential of UFL2 would change from positive to zero during SCC production. Dashed lines in in-lay graphic refer to 95.0 % confidence interval (n=3). Zeta potential of Neusilin UFL2 was measured by our collaboration partner (Szendy et al. 2019a). n=3

4.3.1. Antilisterial activity of electrostatic adsorbed nisin and free nisin in vitro

Nisin susceptibility was tested at various nisin concentrations of UFL2-N and free nisin to six *L. monocytogenes* field isolates at a pH range from 7.5 to 4.5 over time (**Table 18**). The activity of UFL2-N and free nisin was affected by pH. Subsequently, MICs were decreased at low pH. For instance, at pH 5.0 the MICs of UFL2-N and free nisin

decreased to 0.004 mg ml^{-1} (**Table 18**). However, MICs of UFL2-N and free nisin were specific for each of the field isolates like *L. monocytogenes* BfR L1031 field isolate, which appeared to be more susceptible to both UFL2-N and free nisin compared to other field isolates. Independently from pH, antimicrobial activity of UFL2-N and free nisin decreased after seven days of incubation for some *L. monocytogenes* field isolates (**Table 18**).

Table 18: MIC of UFL2-N, free nisin or UFL2 for six *L. monocytogenes* field isolates at pH of 7.5 to 4.5 over seven days of incubation. n=6

pH-value	**BfR No.**	**MIC [mg ml^{-1}]**								
		UFL2-N			**Free nisin**			**UFL2**		
		1d	4d	7d	1d	4d	7d	1d	4d	7d
7.5	L32	0.026	0.132	0.132	0.026	0.132	0.132	+	+	+
	L261	0.026	0.132	0.132	0.013	0.132	0.132	+	+	+
	L308	0.026	0.132	0.132	0.026	0.132	0.132	+	+	+
	L451	0.026	0.132	0.132	0.026	0.026	0.026	+	+	+
	L493	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L1031	0.026	0.026	0.026	0.013	0.013	0.013	+	+	+
7.0	L32	0.026	0.132	0.132	0.026	0.026	0.026	+	+	+
	L261	0.026	0.132	0.132	0.013	0.026	0.026	+	+	+
	L308	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	L451	0.026	0.132	0.132	0.013	0.026	0.026	+	+	+
	L493	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L1031	0.026	0.026	0.026	0.013	0.013	0.013	+	+	+
6.5	L32	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	L261	0.013	0.026	0.132	0.013	0.026	0.026	+	+	+
	L308	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L451	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L493	0.013	0.026	0.026	0.013	0.026	0.026	+	+	+
	L1031	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
6.0	L32	0.026	0.026	0.026	0.026	0.026	0.026	+	+	+
	L261	0.013	0.026	0.132	0.013	0.026	0.026	+	+	+
	L308	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L451	0.026	0.026	0.026	0.013	0.026	0.026	+	+	+
	L493	0.013	0.026	0.026	0.013	0.026	0.026	+	+	+
	L1031	0.013	0.026	0.026	0.013	0.013	0.013	+	+	+

Table 18: continued.

pH-value	BfR No.	MIC [mg ml^{-1}]								
		UFL2-N			Free nisin			UFL2		
		1d	4d	7d	1d	4d	7d	1d	4d	7d
5.5	L32	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	L261	0.013	0.013	0.026	0.004	0.013	0.013	+	+	+
	L308	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	L451	0.013	0.013	0.013	0.013	0.013	0.013	+	+	+
	L493	0.013	0.013	0.013	0.004	0.013	0.013	+	+	+
	L1031	0.004	0.013	0.013	0.004	0.013	0.013	-	-	+
5.0	L32	0.004	0.004	0.004	0.000	0.013	0.013	-	-	-
	L261	0.000	0.013	0.013	0.000	0.004	0.004	-	+	+
	L308	0.000	0.013	0.013	0.004	0.004	0.004	-	-	-
	L451	0.004	0.004	0.004	0.004	0.004	0.004	-	-	-
	L493	0.004	0.013	0.013	0.004	0.013	0.013	+	+	+
	L1031	0.000	0.013	0.013	0.000	0.004	0.004	-	-	+
4.5	L32	0.000	0.004	0.004	0.000	0.004	0.004	-	-	-
	L261	0.000	0.004	0.004	0.000	0.004	0.004	-	-	+
	L308	0.000	0.004	0.004	0.000	0.004	0.004	-	+	-
	L451	0.000	0.004	0.004	0.000	0.004	0.004	-	-	-
	L493	0.000	0.004	0.004	0.000	0.000	0.000	-	-	-
	L1031	0.000	0.004	0.004	0.000	0.004	0.004	-	-	-

Abbreviation: + bacterial growth; - no bacterial growth based on Δoptical density at 595 nm.

At pH ≤ 5.5, the lag phase of *L. monocytogenes* field isolates was extended without addition of UFL2-N or free nisin (**Figure 14**). The exponential phase was especially delayed in field isolates BfR L32 and BfR L1031. An increased lag phase at low pH was also observed when only the particle UFL2 was added to BHI broth (**Table 18**).

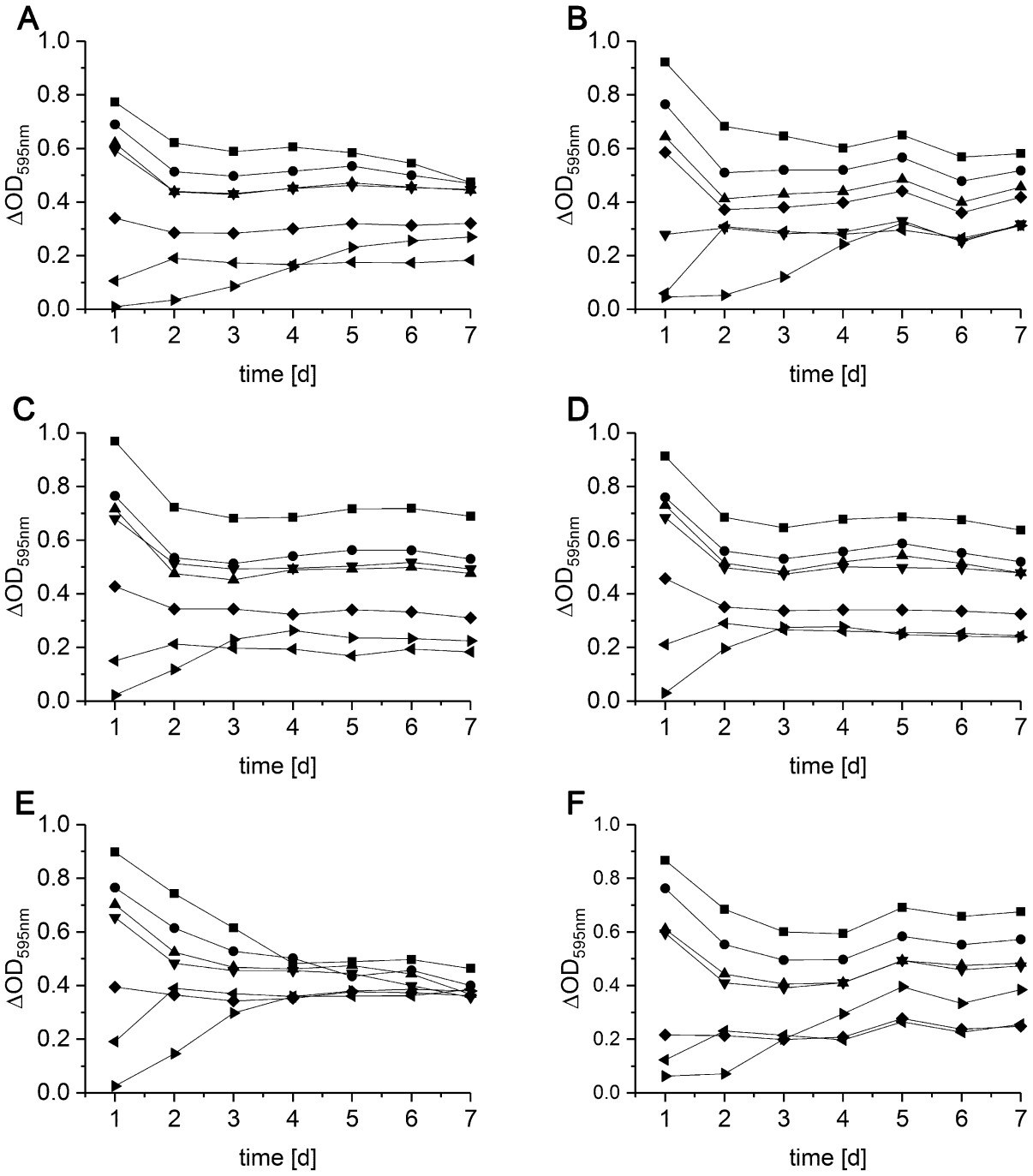

Figure 14: Growth of six *L. monocytogenes* field isolates at pH 7.5 (■), 7.0 (●), 6.5 (▲), 6.0 (▼), 5.5 (♦), 5.0 (◄), and 4.5 (►) for seven days in BHI broth at 30.0 °C. Optical density was monitored daily at 595.0 nm (ΔOD_{595nm}). **A** *L. monocytogenes* BfR L32 field isolate, **B** *L. monocytogenes* BfR L261 field isolate, **C** *L. monocytogenes* BfR L308 field isolate, **D** *L. monocytogenes* BfR L451 field isolate, **E** *L. monocytogenes* BfR L493 field isolate, and **F** *L. monocytogenes* BfR L1031 field isolate. n=4

4.3.2. Antilisterial activity of electrostatic adsorbed nisin and free nisin in autoclaved SCC

Autoclaved SCC was incubated with log 5.0 CFU ml^{-1} *L. monocytogenes* BfR L1031 field isolate for three days, which is the usual ripening time of this SCC variety. When 0.004 mg ml^{-1} of free nisin was added, the outgrowth of the field isolate was completely inhibited (**Table 8.2-10**). After the conventional ripening time, *L. monocytogenes* BfR L1031 field isolate was recovered with log 6.87 CFU ml^{-1} when free nisin was absent. Autoclaving SCC was vital for this experiment as the SCC microbiota not only readily grew on BHI agar but also grew on more selective media like sheep blood agar. Growth of the SCC microbiota resulted in large, brownish colonies on sheep blood agar. Some morphological listerial-atypical colonies formed halos. On chromogenic *Listeria* agar, growth of the SCC microbiota was not inhibited as well. Conclusively, the chromogenic *Listeria* agar failed to selectively isolate *L. monocytogenes* BfR L1031 field isolate from non-autoclaved SCC. The ISO methodology (ISO 11290:1) is suggesting the usage of Half Fraser Broth for primary enrichment. In this situation, this was not an appropriate method to enumerate field isolate BfR L1031 from artificially contaminated SCC surface. Therefore, alternative cultivation-independent methods were of interest.

4.3.3. Detection of L. monocytogenes field isolates from non-autoclaved SCC

Using the FISH method in BHI broth for detection of *L. monocytogenes*, LOD and LOQ were established at log 3.0 CFU ml^{-1} and log 5.0 CFU ml^{-1}, respectively. In non-autoclaved SCC, contamination with *L. monocytogenes* field isolates at the LOD and LOQ level could be detected when the FISH protocol was transferred to the food matrix. An example for detection of log 3.0 CFU ml^{-1} *L. monocytogenes* field isolate on the SCC surface is shown in **Figure 15**. Similarly, the LOD and LOQ of qPCR were both at log 2.64 CFU ml^{-1} and were comparable to the FISH method.

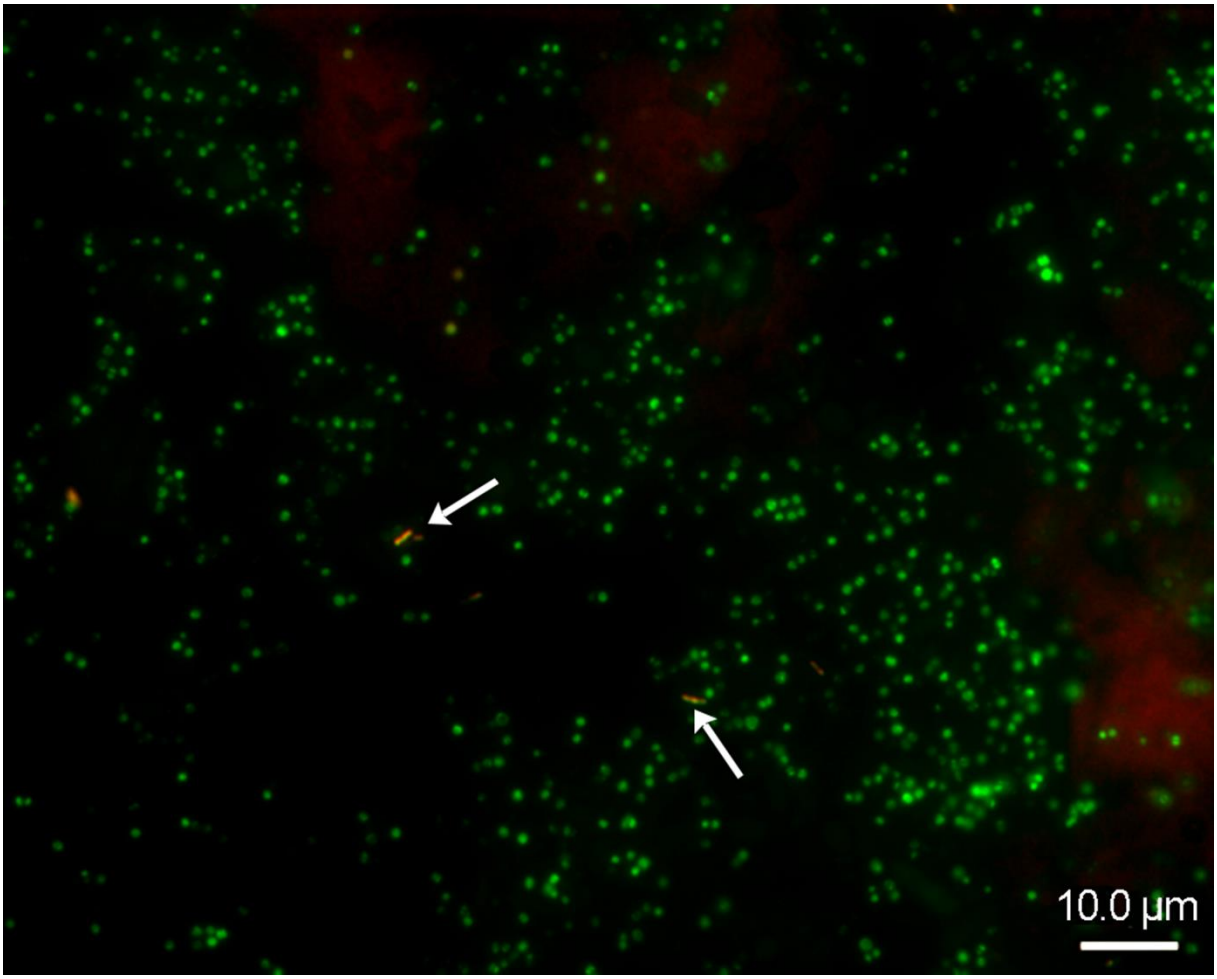

Figure 15: Probes LIS.MONO and EUB338 detected on contaminated SCC surface. Overlapping colors of LIS.MONO (red) and EUB338 (green) result into yellow color as positive for *L. monocytogenes* (arrows). Microbial microbiota in SCC is colored in green. During image processing, colors were added to the fluorescence signals. Scale bar indicates a length of 10.0 µm.

Although the LOD and LOQ levels were practical in both cultivation-independent methods, one of the drawbacks of FISH was the method's specificity. The probe LIS.MONO was not specific for *L. monocytogenes*. However, the *hlyA* target region was very specific for *L. monocytogenes* (**Table 13**). Thus, no false positive results were expected. Quantitative PCR was superior to FISH when developing a stable DNA standard for ease of use. The obtained DNA standard assured steady C_T-values and

did not fall substantial below the 95.0 % confidence interval even over multiple thaw and freeze cycles (**Figure 16**).

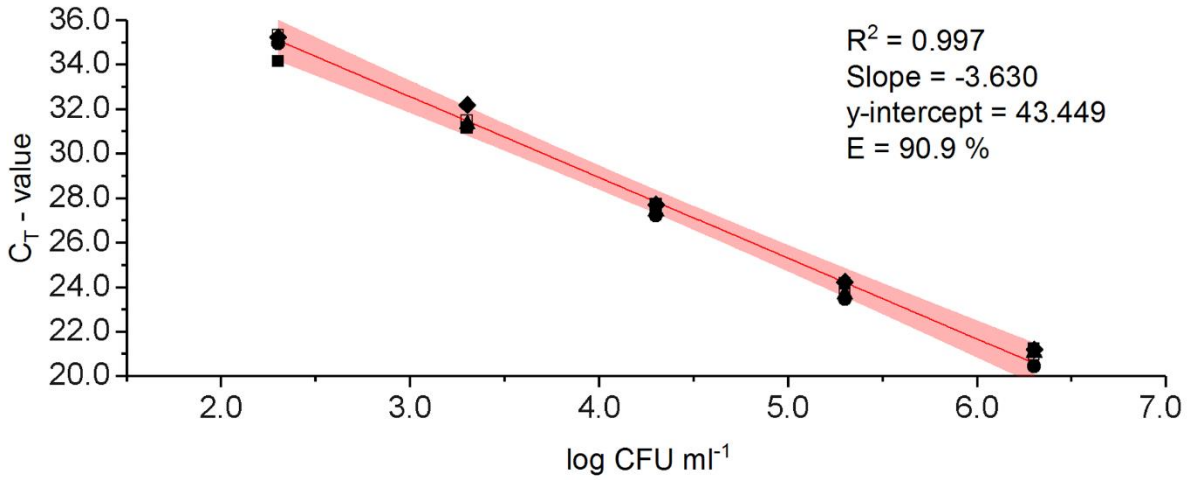

Figure 16: Logarithmic CFU ml^{-1} as a function of C_T-values of the listerial *hlyA* gene amplification by qPCR. Standard curves from same standard material over four thaw and freeze cycles of DNA (n=2). First thaw and freeze cycle (■), second thaw and freeze cycle (●), third thaw and freeze cycle (▲), fourth thaw and freeze cycle (♦), and mean of standard curves (□). Linear fit of means (red line) and 95.0 % confidence interval (red band). Abbreviation: C_T threshold cycle; R^2 coefficient of determination; E qPCR efficiency.

The qPCR method provided more advantages compared to the FISH method or the cultivation-dependent method and removed shortcomings like laborious sample preparation as well as microscopy. Therefore, qPCR was used for detection of *L. monocytogenes* BfR L1031 field isolate from non-autoclaved SCC surface.

4.3.4. Nisin formulations on non-autoclaved SCC surface contaminated with log 5.0 CFU ml^{-1} L. monocytogenes BfR L1031 field isolate

UFL2-N and free nisin at concentrations of 0.004, 0.013, 0.026 and 0.132 mg ml^{-1} were added on top of non-autoclaved SCC as those MICs were effective *in vitro* between pH 7.5 and pH 4.5 (**4.3.1.**). The addition of increasing MICs to the surface did not result in any loss of texture, structure or color of non-autoclaved SCC compared to commercial SCC. Ripening for two days resulted in a solid, rubbery, yellow-colored surface with a white core similar to the control loaf. However, the white core was smaller in some loaves compared to commercial SCC. In addition, the presence of the microflora was not substantially reduced although *L. monocytogenes* BfR L1031 field isolate and UFL2-N or free nisin were present (**Table 19**). In cases of higher MICs of UFL2-N or free nisin, log-reductions of field isolate BfR L1031 also increased and medians were significantly different ($p<0.05$; **Table 19**). By addition of 0.132 mg ml^{-1} UFL2-N or free nisin, field isolate BfR L1031 was below LOD.

Table 19: Median log-reduction of *L. monocytogenes* BfR L1031 field isolate (*hlyA* gene) and microbiota (16S rRNA gene) after addition of UFL2-N or free nisin on top of SCC and after two days of ripening. CFU ml^{-1} was derived from standard curve of the qPCR run. Logarithmic reduction is referred to samples without addition of UFL2-N or free nisin (n=4). Contamination on SCC surface was performed with log 5.12 CFU ml^{-1}.

Concentration [mg ml^{-1}]	**log-reduction in CFU ml^{-1}**			
	***hlyA* gene**		**16S rRNA gene**	
	UFL2-N	Free nisin	UFL2-N	Free nisin
0.004	0.28[a]	0.71[a]	0.04	no reduction
0.013	1.25	1.33[a]	0.08	no reduction
0.026	1.39[a]	1.07[a]	no reduction	0.001
0.132	n.d.	n.d.	0.17	0.040

[a] Significantly difference at $p<0.05$ within column.
Abbreviation: n.d. no amplification detected by qPCR or signal lay below LOD.

4.3.5. Interaction of UFL2-N with L. monocytogenes BfR L1031 field isolate

The manufacturer of UFL2-N was able to show that the density of sprayed fluorescently labeled particles was homogeneous on the SCC (Szendy et al. 2019a). Within less than one hour, released nisin diffused into particle free areas. Microscopic observations of UFL2-N with *L. monocytogenes* BfR L1031 field isolate showed that planktonic cells formed agglomerates in approximation of UFL2-N (**Figure 17**).

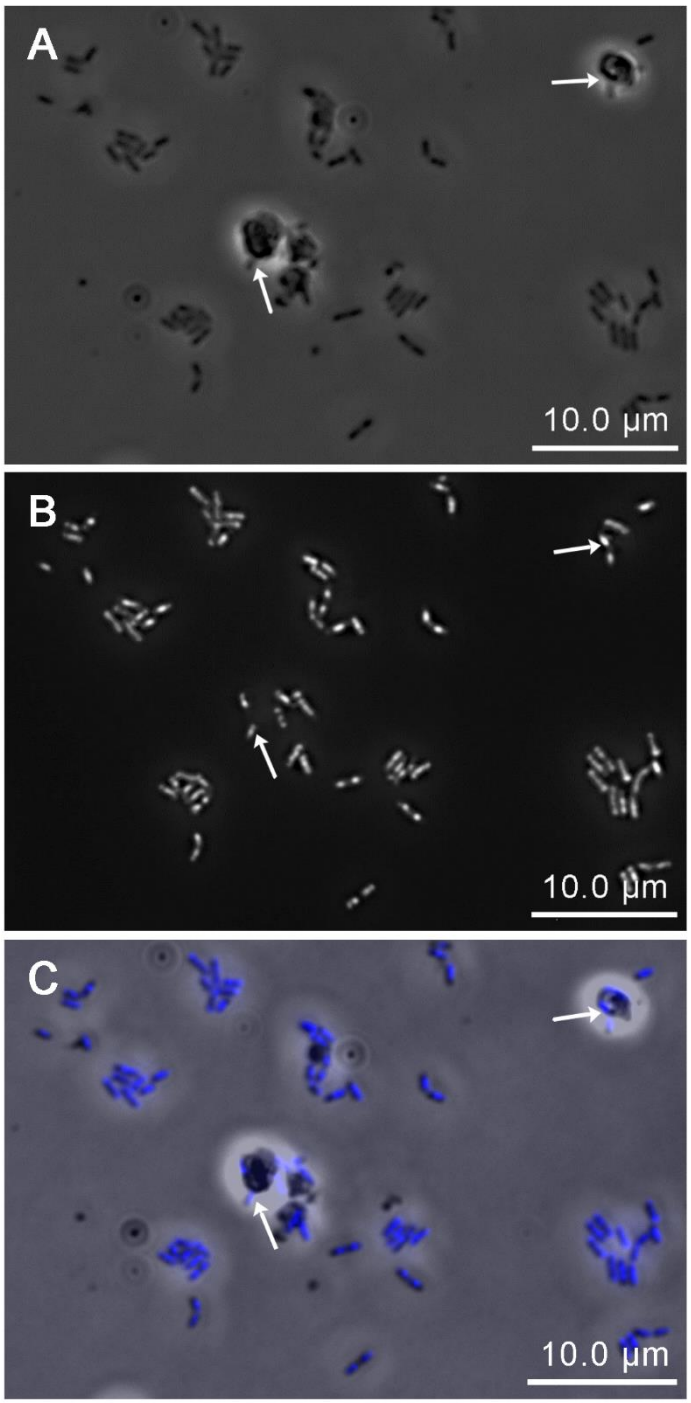

Figure 17: Microscopic image of UFL2-N and *L. monocytogenes* BfR L1031 field isolate using different excitation and emission filters. Each image was acquired separately at the same position using phase contrast (**A**) and DAPI filter (**B**). Merged digitally colored image (**C**). DAPI (blue) stained DNA of field isolate BfR L1031. Arrows indicate the Neusilin particles. Scale bar indicates a length of 10.0 µm.

The ability to spray fluorescently labeled UFL2 particles and UFL2-N makes the launching of UFL2-N in the dairy production feasible. The nebulization allows development and implementation of novel control strategies in semi-solid food matrices. One strategy could be the installation of nebulization devices above the conveyor belts. A nebulizer would spray UFL2-N on the surfaces of SCC. In this study, the percentage reduction achieved on the SCC surface was 99.9%. However, the dairy producer should perform risk assessments in prerequisite programs before taking action. The producer could use the data of this study with subdivided *L. monocytogenes* field isolates into NS and NNS and their frequency in food industry as well as the association of DSV in *gadD2* to NNS to score risks for the production plant. Alternatively, UFL2-N could be intruded in foils used for foil-ripening of SCC with a positive net charge, which allows diffusion of nisin to the surface. In this case, the dairy producer could omit the labeling of the food additive.

The legal requirements for free nisin are already fullfilled (EU 2011a). The UFL2-N particles have good chances to comply with legal requirements as sodium and potassium aluminium silicates are already permitted in the food industry (EU 2011a). Nevertheless, the dairy producer should implement UFL2-N at appropriate concentrations. The usage is an optional counteractive measure and should be combined with a strict cleaning and sanitizing regime.

5. DISCUSSION

5.1. Study 1 – Efficacy and susceptibility testing of micro biocides

The choice and use of micro biocides in dairy production plant are not clearly specified in food safety management systems and inadequate application can lead to reduced efficacy. Hence, disinfection compounds and antimicrobial preservatives were tested with similar media compositions and incubation conditions for all micro biocides to enable a high comparability. The culture broth was employed to mimic organic compounds, which can be found in dairy produce or debris on soiled surfaces.

The results of the efficacy testing highlight the importance of thoroughly washing and physically cleaning soiled surfaces before disinfection routine to remove organic debris. The DNA damaging NaOCl (Dennis et al. 1979) was evaluated with reduced efficacy in BHI broth or more specifically its including organic compounds by own preliminary experiments and literature. The published MICs of 0.5 to 1.0 mg ml^{-1} were reproducible in TRIS buffer solution but not in BHI broth even though NaOCl was prepared freshly before each use (Bloomfield and Miller 1989; El-Kest and Marth 1988b; Jacquet and Reynaud 1994b; Svoboda et al. 2016; Tuncan 1993). The daily preparation was important as previous results showed that protein compounds affect the availability of free chlorines (El-Kest and Marth 1988a; Jacquet and Reynaud 1994a; Jo et al. 2018). Jo et al. (2018) were able to demonstrate that beef extract and tryptone as well as peptone, which is a major component of BHI broth, depleted the free chlorine content. The formation of organohalides such as trihalomethane resulted from chemical reaction of organic compounds with chlorine (Gómez-López et al. 2017; Shen et al. 2012; Waters and Hung 2013). Thus, the chemical reaction between the free chlorine and the major components of BHI broth might have lowered the free chlorine content. Consequently, MICs of NaOCl increased to 4.0 mg ml^{-1} (**Figure 6D**).

By removing organic debris in regular intervals chlorine-based disinfectants remain active against *L. monocytogenes*. In dairy industry, the deposition of milk and proteins are usually found on soiled surfaces. In presence of milk (2.0 % fat), reduced efficacy of NaOCl has been shown (Best et al. 1990). Similarly, the inhibitory concentration of H_2O_2 had to be about three folds higher in presence of sterilized raw milk than tested in this study to inhibit growth of *L. monocytogenes* after 24 hours (Dominguez et al. 1987b).

The MICs of the other micro biocides citral, free nisin, H_2O_2, $NaNO_2$ and QAC were not affected by the organic compounds in culture broth. Although MICs of H_2O_2 were low after 24 hours and were not affected by culture broth (**Figure 6C**), the efficacy of H_2O_2 strongly depended on the exposure time as found by Ali et al. (2006). In a study by Lou and Yousef (1997), the concentration of 1.0 mg ml^{-1} was reported to be lethal after 10 hours incubation in TSB supplemented with yeast extract. For cell protection bacteria rely on its catalyzing function during H_2O_2 decomposition (Brul and Coote 1999) and all *Listeria* sensu strictu are positive for catalase (Orsi and Wiedmann 2016). Therefore, this intrinsic tolerance mechanism reduces effectiveness of H_2O_2. Consequently, the concentration or the exposure time has to increase for similar results. Beyond the capability of catalase to decompose H_2O_2, the disinfection compound will inflict DNA damage, deactivate proteins as well as lipids (Ananthaswamy and Eisenstark 1977; Crow 1992; Imlay and Linn 1988). For a daily routine application in the food industry, Robbins et al. (2005) recommend a 3.0 % H_2O_2 solution (30.0 mg ml^{-1}) with an appropriate contact time of 10 minutes to accomplish a complete elimination of *L. monocytogenes*.

Occasionally, MICs of micro biocides deviate from the MICs reported in literature. Crucial for data comparison is the experimental procedure as well as the selected *L. monocytogenes* field isolates or reference strains. For example, the experimental

procedure of Lundén et al. (2003) differed from other studies. They used the microdilution broth method instead of plating and the MICs of NaOCl were in line with the results in this thesis. Since there was no description to remove the culture broth by washing of cell pellets before NaOCl treatment (Lundén et al. 2003), the culture broth has probably reduced effectiveness of NaOCl. In other studies, the effects of emulsifiers, which were used to prepare and dilute EO compounds, on MICs have been investigated. In absence of dimethylsulfoxide, the antimicrobial activity of cinnamon increased 50-fold (Hili et al. 1997). In contrast, the presence of Tween 80 improved the antimicrobial activity of tea tree oil (Remmal et al. 1993). Due to the lack of standardization in efficacy testing of EO compounds, MICs of citral were higher compared to reported MICs, which ranged from 0.1 to 1.0 mg ml^{-1} depending on the citral suspension, field isolates and applied methods (Apolónio et al. 2014a; Kim et al. 1995; Onawunmi 1989). The mechanisms of tolerance to citral is still uncertain as the precise cellular targets of citral have not yet been identified. In *E. coli*, the cytoplasmic and the outer membrane were disrupted by citral resulting in loss of the membrane potential and ATP synthesis (Somolinos et al. 2010). So far it is known that the lipophilic character of EO compounds favors their incorporation into the cell membrane resulting to inhibition of membrane-bound enzymes (Cox et al. 2001).

However, other sensory properties like taste and after taste have to be considered. This has limited the application of antimicrobial preservatives e.g. EO and EO compounds to certain food products. In addition, information on toxicological effects have to be carefully evaluated. For example, the maximum amount of $NaNO_2$ that may be added during manufacturing in processed meat was set to 0.15 mg ml^{-1} by the EU (2011). MICs of $NaNO_2$ exceeded this maximum amount for all origins of isolation (**Figure 7B**). Although the antimicrobial activity of $NaNO_2$ was found to depend on pH (Müller-Herbst et al. 2016) as acidification leads to reactive species that have more

antimicrobial activity (Cammack et al. 1999), this parameter was not adjusted in the efficacy testing to enable high comparability between all micro biocides. Moreover, the application range could even narrow down when the antimicrobial activities decrease during physical food processing methods like pasteurization. Duffy et al. (1994) demonstrated increased and variable lag times of *L. monocytogenes* in cooked food products with added $NaNO_2$.

To prevent undesired taste and maintain sensory properties while sustain antimicrobial activity, active food contact materials are a potential field of application for EO and EO compounds. Oliveira et al. (2017) tested cellulosic films containing α, β-citral on coalho cheese to guarantee a safer food. Despite the color enhancement of cheese after citral application, the food product's texture had no changes (Oliveira et al. 2017).

MICs of QACs and free nisin were similar compared to MICs reported in literature (Benkerroum and Sandine 1988; Ferreira and Lund 1996a; Iancu et al. 2012; Katla et al. 2003; Kovacevic et al. 2013; Mereghetti et al. 2000; Møretrø et al. 2017; Mota-Meira et al. 2000; Rasch and Knøchel 1998; Romanova et al. 2002; Svoboda et al. 2016; To et al. 2002 Ukuku and Shelef 1997). The results showed that MICs of QACs remained constant through a period of 40 years. CKC with a longer alkyl chain and higher hydrophobicity had lower MICs compared to BAC. This was not unexpected as the antimicrobial activity depends on the amount of carbon atoms in the alkyl chain (Paulus 2005). Therefore, the interaction with the cell surface is enhanced as described previously (Gilbert and Moore 2005). However, the low solubility of CKC in water diminish its potential in daily routine application. In case of free nisin, the majority of the tested *L. monocytogenes* field isolates was already susceptible to 0.011 mg ml^{-1} free nisin. The abundance of NNS *L. monocytogenes* isolated from milk/cheese and other dairy products was 23.8 %. *Listeria monocytogenes* had significant higher abundance of NNS field isolates in this environment than in meat and meat products

($p<0.002$). Therefore, the persistence of NNS *L. monocytogenes* field isolates in dairy industry cannot be excluded. In previous studies, tolerance to antimicrobial peptides were induced in defined food environments leading to the selection of a sub-population of field isolates with higher nisin tolerance (Gravesen et al. 2002; Harris et al. 1991; Ming and Daeschel 1993; Wu et al. 2017). No reports have been issued up-to-now on the frequency of NNS *L. monocytogenes* field isolates in dairy produce nor on increased development of the NNS state (Davidson and Harrison 2002).

Higher nisin tolerance of *L. monocytogenes* field isolates need not to be in line with superior fitness compared to NS field isolates. Growth curve measurements in TSB indicated that NS and NNS field isolates had similar growth rates, but ΔOD indicated that NS *L. monocytogenes* field isolates could outgrow NNS field isolates if free nisin was not present (**Figure 9**). In contrast, NNS field isolates grew better within the first 48 hours compared to the NS field isolates if free nisin was present (**Figure 12**). This is in concordance with previous studies (Begley et al. 2010; Mantovani and Russell 2001). Apparently, the NNS field isolates contained some cells, which could tolerate free nisin and were able to grow in presence of the peptide. However, the growth rate might not provide a good framework for a relative index of fitness because it would not describe the complexity well enough.

Differences in the tolerance of *L. monocytogenes* field isolates to micro biocides have been suggested to influence bacterial survival strategies in respective origin of isolation, which may offer organic islands to survive. The correlation ratio η between nisin susceptibility and origins of isolation was medium to high, which indicated that the origin of isolation might have influenced the degree of nisin tolerance. Regarding citral, H_2O_2, $NaNO_2$, NaOCl and QAC, origin of isolation of *L. monocytogenes* field isolates was more important than serotype. In two studies, the BAC tolerance of *L.*

monocytogenes was encountered mainly among serotype IIa (Mereghetti et al. 2000; Mullapudi et al. 2008). Nisin susceptibility and serotype was associated by means of correlation ratio η. Previous studies have associated nisin susceptibility of *L. monocytogenes* field isolates with serotype as well (Buncic et al. 2001; Katla et al. 2003). A high frequency of NNS *L. monocytogenes* field isolates was found within serotype IIa, whose MICs were significant higher compared to other serotypes ($p<0.008$). Serotype IIa has probably undergone a phenotypic or genotypic alteration process as lineage II field isolates are generally described to be more prone to genetic recombination than lineage I field isolates (Orsi et al. 2011).

Since the dairy industry relies on usage of micro biocides for sanitation, there is concern that the common usage leads to tolerance in *L. monocytogenes*. Tolerance has been shown to develop when *L. monocytogenes* field isolates were exposed to sub-lethal concentrations (Harris et al. 1991; Ming and Daeschel 1993; Romanova et al. 2006; Rakic-Martinez et al. 2011; Kovacevic et al., 2013). Despite the fact that food business operators use micro biocides as recommended by the manufacturer, which are higher concentrations than the MIC, sub-lethal concentrations may arise for instance due to organic debris, poor accessibility or careless rinsing leaving water residues that contained micro biocides on surfaces (Møretrø et al. 2017). Tolerance of *L. monocytogenes* field isolates to a micro biocide and the possible co-selection process of field isolates to other micro biocides used in the dairy industry could lead to enhanced selection and growth advantage of those organisms. For example, a NNS *L. monocytogenes* field isolate had high MICs of BAC, citral and nisin. The three micro biocides have in common their mode of action against the cell membrane and/or cell wall (Bonev et al. 2004; Hyldgaard et al. 2012; Kordel et al. 2001; To et al. 2002). McDonnell and Russell (1999) described the co-selection process based on specific

or nonspecific cellular changes. After the alteration, the efficiency of related or unrelated micro biocides is reduced. The low MIC of NNS *L. monocytogenes* BfR L268 to citral was surprising as nisin tolerance is usually associated with an altered cell envelope (Somolinos et al. 2010). An altered cell envelope by citral was also described previously (Crandall and Montville 1998, Verheul et al. 1997). Thus, modifications in either the cell wall or the cell membrane resulted in reduced binding affinity for nisin. At the same time, this may have led to conditions in favor for the lipophilic citral, which could disrupt the cell membrane more readily. Moreover, the field isolate BfR L268 was resistant to daptomycin and meropenem, which have their mode of action against the cell membrane and the cell wall, respectively. A co-selection process to therapeutic antibiotics could result in a growth advantage for *L. monocytogenes* when specific or nonspecific cellular changes occur. Other mechanisms correspond to an intrinsic tolerance. This includes efflux pumps that affect the intracellular concentration of toxic and non-toxic compounds. The BAC tolerance was associated to efflux pump activity in several studies (Chen et al. 2010; Conficoni et al. 2016; Haubert et al. 2016; Meier et al. 2017).

In order to associate a relationship of MICs from micro biocides to certain antibiotics, the organic and inorganic compounds were divided into groups that share similar cellular targets in *L. monocytogenes* (Allen et al. 2016; Kapoor et al. 2017; Komora et al. 2017; Krawczyk-Balska and Markiewicz 2016; Nair et al. 2016; Walsh 2003). High correlation coefficients of NaOCl and citral were found with two different classes of antibiotics, gentamicin and trimethoprim/sulfamethoxazole respectively. The latter is part of a second choice antibiotic to treat human listeriosis. There are opinions that misusage of a micro biocide may select field isolates with tolerance to the respective compound and decreased susceptibility to therapeutic antibiotics. For example, BAC induced resistance in *L. monocytogenes* to both gentamicin and kanamycin when

exposed at sub-lethal concentrations (Romanova et al. 2006; Rakic-Martinez et al. 2011; Kovacevic et al. 2013). In worst case, traits remain stable as for the chlorinated aromatic compound triclosan (Christensen et al. 2011). The correlation of the micro biocides, NaOCl and citral, and antibiotics indicated presumably similar mode of interaction that is protein synthesis for gentamicin and nucleic acid synthesis for trimethoprim/sulfamethoxazole (Kapoor et al. 2017). Previously, free chlorine was shown to interact with nucleic acid due to the formation of chlorinated nucleotides (Dennis et al., 1979). Since the food industry relies on usage of NaOCl for sanitation, the correlation for NaOCl are plausible when thought of a co-selection process involving multiple mechanisms. If these correlations intensify due to overuse of NaOCl and result into higher MICs of therapeutic antibiotic, this trend is alarming for future human therapies. Allergic reactions to aminoglycosides and co-trimoxazole have been already reported with common concentrations during antibiotic treatment (Choquet-Kastylevsky et al. 2002; Sánchez-Borges et al. 2013; Spigarelli et al. 2002). Although the bacterial targets for monoterpenes were not yet further characterized, the association of MICs of citral to antibiotics may be based on the fact that citral has a multitargeted mode of action (Hyldgaard et al. 2012). However, several previous studies failed to induce tolerance to antibiotics by EO in a variety of Gram-positive and Gram-negative pathogens (Ali et al. 2005; Apolónio et al. 2014b; da Silva Luz et al. 2012; Hammer et al. 2012; Thomsen et al. 2013). In conclusion, correlations of citral to antibiotics have to be evaluated and these correlations are currently of minor importance since citral has not be approved as a food additive yet.

In general, resistances or multi resistances of *L. monocytogenes* to antibiotics did not increase over the last years according to Noll et al. (2018). However, resistances to β-lactam antibiotics were described to increase over in the past years (Morvan et al. 2010). Nisin tolerance of *L. monocytogenes* field isolates could not be associated to

antibiotics, which have their mode of action against the cell wall and/or membrane (e.g. β-lactams).

5.2. Study 2 – Molecular analyses on nisin tolerance

It is known that upon sequentially increase in free nisin concentration *L. monocytogenes* field isolates became more tolerant under laboratory conditions (Harris et al. 1991; Ming and Daeschel 1993).

In *L. monocytogenes*, the majority of coding genes and regulatory elements known to be related to nisin tolerance were not associated to the NNS state of *L. monocytogenes* field isolates except for *gadD*2 (**Table 17**, **Table 8.2-5** and **Table 8.2-6**). A C-terminal amino acid substitution of aspartic acid to asparagine at position 453 ($D_{453}N$) restricted to NNS field isolates was identified in GadD2. The relevance of the GAD system under acidic conditions and in nisin tolerance of *L. monocytogenes* has previously been described. The explanation for this association was that a link between intracellular ATP level and an intact GAD system in *L. monocytogenes* was found (Begley et al. 2010; Bonnet et al. 2006). In addition, Begley et al. (2010) proposed that NNS field isolates may benefit from additional ATP formation via the γ-aminobutyric acid shunt pathway, in which GadD2 is involved. The GAD system contributed also to the survival of the pathogen in acid foods while the glutamate enhanced the survival of the pathogen (Cotter et al. 2001). If NNS *L. monocytogenes* field isolates have an amino acid substitution, which results into higher enzyme activity of decarboxylating glutamate, and are more permeable for this substrate due to nisin, glutamate would promote survival by maintaining a constant ATP pool under acid stress. Hence, a faster neutralization of the GAD reagent by means of color change should be observed only in NNS *L. monocytogenes* field isolates. This hypothesis was tested in a GAD assay

with a standardized inoculum for all field isolates. However, the hypothesis was disproved in three different culture broths (**Table 8.2-9**).

Sequences of DSVs in the *gadD2* gene clustered according to *L. monocytogenes* lineage affiliation on DNA and protein level (**Figure 11**). Seven NNS *L. monocytogenes* field isolates showed the specific DSV in *gadD2*, were serotype IIa and had reduced nisin susceptibility. Six of them were isolated from milk/cheese and other dairy products. On protein level, the NNS field isolates shared the same C-terminus sequence (**Table 8.2-7**).

The amino acid substitution potentially results in a less pH-dependent enzyme activity. According to the modeled protein structure of GadD2, the C-terminus would not close at pH 7.0 to block the active site for NNS *L. monocytogenes* field isolates (**Figure 18**). Hence, it was proposed that by the $D_{453}N$ substitution GadD2 is permanently active. The $D_{453}N$ substitution is probably responsible for decreased nisin susceptibility by indirectly counteracting the nisin-induced pore formation of the cell membrane due to the γ-aminobutyric acid shunt pathway (Ruhr and Sahl 1985).

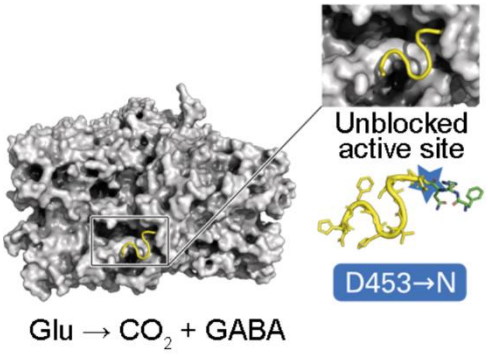

Figure 18: The modeled protein structure of GadD2 in the NNS *L. monocytogenes* field isolate with substitution of amino acid D to N at position 453 in the hinge region of the C-terminus. This substitution probably prevents the pH-dependent blockage of the active site and the enzyme activity becomes less pH-dependent. The 3D structure was provided by Prof. Dr. Dirk Labudde from the University of Applied Sciences Mittweida (Szendy et al. 2019b). Abbreviation: CO_2 carbon dioxide; D aspartic acid; GABA γ-aminobutyric acid; Glu glutamate; N asparagine.

Contrary to NNS field isolates, which contain a permanent active GadD2, the enzyme's active site in NS field isolates should be blocked at pH 7.0. Therefore, NNS would have a growth advantage over NS in culture broth. This would in part explain the phenomenon observed at pH 7.0 during the pH-dependent growth curves (**Figure 12A**). The *gadD2* is usually expressed under extreme acidity (Cotter et al. 2001). At pH 5.5, the growth of the NNS field isolate did not significantly differ from the growth of the NS field isolate ($p<0.05$) supposing that in this scenario the GAD system is active in the NNS and the NS field isolate. Moreover, NNS would also have a growth advantage in raw milk, which is usually maintained at neutral pH, and, in fact, both NNS *L. monocytogenes* field isolates were isolated from raw milk (**Table 9**). Consequently, the NNS state may be a result of co-evolution with lactic acid bacteria and their anti-Gram-positive bacteriocins such as nisin. However, real food matrices

comprise many other factors resulting in unexpected phenotypic outcome. Collins et al. (2011) compared growth in inoculated cottage cheese between a Δ*gadD1* mutant and its wild type strain. Without addition of free nisin the mutant had hardly any growth advantage over the wild type. However, survival of the Δ*gadD1* mutant was enhanced upon addition of free nisin. On the contrary, the wild type strain was recovered with higher cell numbers when monosodium glutamate was added in combination with free nisin.

5.3. Study 3 – Application of electrostatic-adsorbed nisin to Neusilin particles onto surface of sour curd cheese

The occurrence of NNS *L. monocytogenes* field isolates may be controlled by a multiple hurdle strategy. However, several restrictions have limited practical application of free nisin; these are: (I) reduced biological activity and (II) low solubility at neutral pH. Free nisin shows a low bioavailability as it interacts with food components (Aasen et al. 2003; Bhatti et al. 2004; Chollet et al. 2008). Moreover, rapid proteolytic degradation processes occur (Sun et al. 2009). Although fat content was associated with binding free nisin in cheddar cheese (Benech et al. 2002; Benech et al. 2002b; Jung et al. 1992), this is in SCC unlikely to occur as the overall fat content in SCC is as low as 0.5% according to the manufacturer.

Thus, the highly porous Neusilin was employed as the carrier material for a controlled and slow release of adsorbed free nisin (**Figure 3**) to minimize nisin's interaction with food components and exposition to degradation. The electrostatic interaction between oppositely charged nisin and UFL2 led to a nisin release behavior tailored to a SC likely environment rather than to rely on diffusion rates (Hosseini et al., 2014). Different Neusilin types were previously applied as excipient and improved solubility of less

water soluble drugs (Mallappa et al. 2015). Similarly, the poor solubility of free nisin at neutral pH was circumvented by UFL2.

In accordance with previous studies, decreasing antimicrobial activity of free nisin and UFL2-N were observed when pH was increased (Ferreira and Lund 1996; De Martinis et al. 1997). According to the manufacture, a pH ≤5.0 enabled initial high release rates of nisin from UFL2-N as pH was below the isoelectric point and the neutral surface charge of UFL2. At a pH of 5.0 and 4.5, UFL2-N showed enhanced antilisterial activity (**Table 18**). The following antilisterial action of UFL2-N in BHI broth was hypothesized. If the pH was below the isoelectric point of UFL2 (pH <5.3), the positive zeta potential would result in attraction of bacteria, which were available in high numbers with negatively charged bacterial cell walls (**Figure 17**). The electrostatic interaction between UFL2-N and the bacterial cell wall would aid the bioavailability of nisin since the diffusion path is then considerably reduced for the attached cells. In addition, the antimicrobial activity of nisin is higher at low pH as discussed earlier. As a result, the bacterial growth would be impaired. If the pH was higher than the isoelectric point, the negative zeta potential would cause electrostatic repulsion between the negatively charged cell walls and UFL2-N while at the same time the release rate of nisin from UFL2-N would be reduced. In consequence, the electrostatic repulsion prevented contact between bacterial cells and nisin as discussed previously (da Silva Malheiros et al. 2010; Were et al. 2004). On the contrary, UFL2 did not show any antilisterial activity in tested pH range where the six *L. monocytogenes* field isolates readily grew. Moreover, antilisterial activity of free nisin and UFL2-N decreased isolate specific over time indicating that either the ratio of nisin to cells changed or the bacterial cells had the capability to become NNS. Growth of *L. monocytogenes* at pH 5.0 and 4.5 was also isolate specific (**Figure 14**), which is in line with previous findings of extended lag

phase at pH 5.6 to 3.8 *in vitro* and *in vivo* (Cheroutre-Vialette et al. 1998; Rogga et al. 2005).

After evaluating the adequacy of free nisin and UFL2-N in BHI broth as well as conducting a risk assessment of genes involved in potential nisin tolerance, both nisin formulations were tested on the surface of SCC. However, isolation of *L. monocytogenes* field isolates from non-autoclaved SCC by cell counting on agar plates remained difficult without pre-enrichment. Although the ISO 11290:2017 is the gold standard for detection and/or enumeration of *L. monocytogenes*, the enrichment step was omitted to avoid re-growth of injured cells. Loessner (1991) was also challenged with growth of other microorganisms on chromogenic *Listeria* agar. He solved this issue by phage typing of *L. monocytogenes* (Loessner 1991). On sheep blood agar, SCC microbiota were hemolytic active like for instance the red smear microbiota (Boucabeille et al. 1997). Therefore, cultivation independent methods were selected for detection of *L. monocytogenes* field isolates from non-autoclaved SCC.

A popular method for detection of bacteria is FISH and allows identification on species level (Amann et al. 1990). Another advantage is that bacteria are immediately visualized *in situ*. However, the lack of specificity, the substantial amount of auto fluorescence in SCC and the laborious sample preparation made the FISH method not an ideal method to be applied to SCC.

Therefore, the less laborious and more time efficient qPCR approach was chosen. The *hlyA* gene was selected for PCR-based detection of *L. monocytogenes* BfR L1031 field isolate from non-autoclaved SCC. The *hlyA* gene is common in hemolytic *Listeria* species (Orsi and Wiedmann 2016). *Listeria monocytogenes*, *L. ivanovii* and *L. seeligeri* show hemolytic capabilities (Orsi and Wiedmann 2016). On chromogenic *Listeria* agar, *L. monocytogenes* and *L. ivanovii* are not further differentiated according

to the manufacture (Oxoid). Although these two *Listeria* species share identities by their *hlyA* gene (**Table 13**), the PCR only amplified DNA of *L. monocytogenes* in preliminary experiments. Thus, specificity of the *hlyA* primers were confirmed. The SCC matrix was not inhibitory during PCR amplification and the LOD was log 2.64 CFU ml^{-1}. This is about one log CFU ml^{-1} higher compared to earlier studies (Bassler et al. 1995; Nogva et al. 2000).

The addition of free nisin or UFL2-N up to 0.132 mg ml^{-1} to non-autoclaved SCC did not alter quality criteria when compared to commercial SCC. In addition, the presence of the microflora (e.g. starter cultures) was not significantly reduced although *L. monocytogenes* and free nisin or UFL2-N were present ($p>0.05$; **Table 19**). Results obtained from non-autoclaved SCC with log 5.0 CFU ml^{-1} showed in practice significant log-reduction ($p<0.05$; **Table 19**) to virtually complete inhibition of *L. monocytogenes* BfR L1031 field isolate at 0.132 mg ml^{-1}. More importantly, MICs did not exceed the limit of 12.5 mg kg^{-1} set by the EU (EU 2011). In a study by Benech et al. (2002), 0.008 mg ml^{-1} encapsulated nisin in liposomes inhibited outgrowth of *L. innocua* in contaminated cheddar cheese (log 5.0 to log 6.0 CFU ml^{-1}) when added before milk coagulation. The acid necessary to coagulate the milk will increase the antimicrobial activity of free nisin in cheddar cheese (Benech et al. 2002). Since SCC ripens from outside to the inside (Belitz et al. 2001), it was crucial to determine the surface pH. The data showed that the nisin release kinetics of UFL2-N were reduced after roughly one hour (**Figure 13**) and, therefore, lesser log reduction was expected. Increased salt contents did not alter nisin efficacy as shown earlier (Chollet et al. 2008; Harris et al. 1991; Pawar et al. 2000). However, De Martinis et al. (1997) and Yen et al. (1991) described a protective effect to *L. monocytogenes* after addition of similar salt concentrations, which is supported by own preliminary experiments (**Figure 8.1-1**).

The outcome of no complete inhibition of the field isolate BfR L1031 below 0.132 mg ml^{-1} might have been a combination of increased pH and slower nisin release rate from UFL2-N due to pH and salt content as well as presence of proteolytic or nisin-degrading members of the SCC microbiota (Ramsaran et al. 1998; Sulzer and Busse 1991). Moreover, the SCC surface with a pH of greater than 6.5 (**Figure 13**) and the water availability during 30°C at 98.0% humidity provided ideal conditions for the outgrowth of the *L. monocytogenes* field isolate (Liu et al. 2007; Ramsaran et al. 1998; Sulzer and Busse 1991). More importantly, UFL2-N was superior at a concentration of 0.026 mg ml^{-1} compared to free nisin. Growth of *L. monocytogenes* BfR L1031 field isolate was still present at lower UFL2-N concentrations, but at 0.132 mg ml^{-1} growth of the field isolate was inhibited (**Table 19**). In summary, an approximately three log reduction of field isolate BfR L1031 was achieved on the SCC surface.

6. CONCLUSION

Organic compounds in culture broth affected the efficacy of certain micro biocides. The reduced efficacy of NaOCl was likely caused by protein compounds. Thus, confirming the postulated Hypothesis I (**2.1.**). Further exclusive factors in the dairy supply chain that may affect the susceptibility of *L. monocytogenes* field isolates to micro biocides include environmental conditions and unique niches (**Figure 19**). Other factors and pathways not mentioned in **Figure 19** should be looked at in the future to resolve the complex interaction with each other in the dairy food chain.

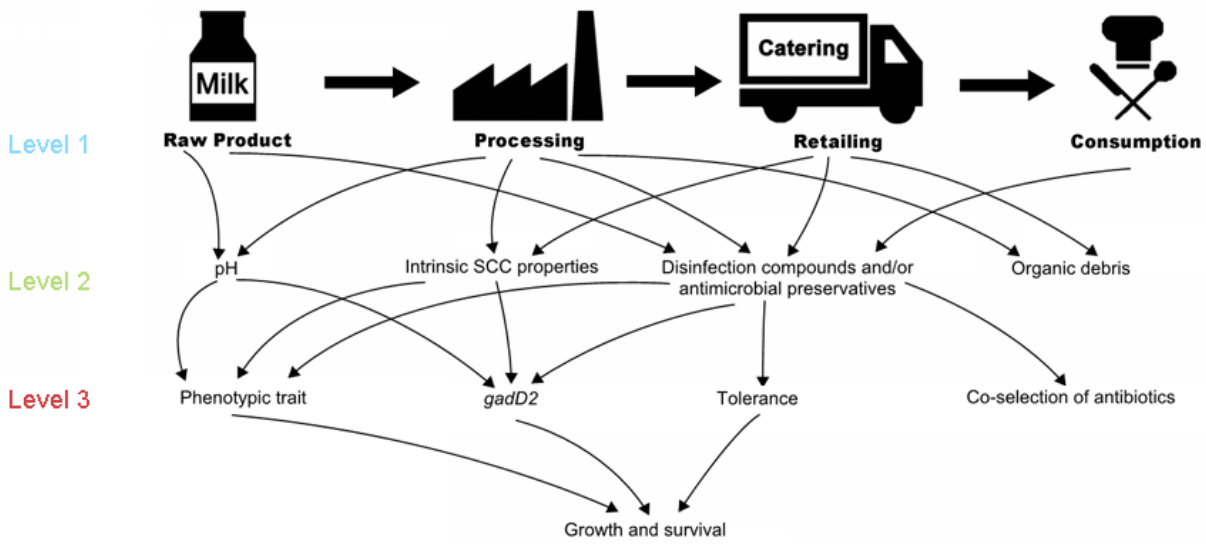

Figure 19: Summary of exclusive factors that may affect susceptibility of *L. monocytogenes* field isolates to micro biocides studied in this thesis. The dairy food chain along the farm to fork process is synopsized on level 1 (blue). This highlights how environmental conditions, factors unique in niches and/or affected micro biocides (level 2, green) may lead to growth and/or survival by specific response in *L. monocytogenes* field isolates (level 3, red). For the purpose of clarity, factors influencing each other within each level were not shown.

Correlation between MICs of micro biocides and therapeutic antibiotics was assessed to address concerns about therapeutic failures. Correlation coefficients revealed that high MICs of NaOCl and citral were correlated to therapeutic antibiotics, which are frequently used to treat listeriosis. This supports the postulated Hypothesis I (**2.1.**). These correlations might have been caused by the experimental design (i.e., selection of *L. monocytogenes* field isolates, origin of isolation, cultivation conditions). The impact of the correlations, however, have yet to be evaluated. The single factor pH at level 2 (**Figure 19**) was already of major importance. The pH influenced the efficacy of UFL2-N as well as free nisin and the growth of *L. monocytogenes* field isolates (**Table 18**, **Figure 14**), which is in agreement with Hypothesis I (**2.1.**). The accumulative release of nisin from UFL2-N into the environment depended on pH and was investigated in detail by Szendy et al. (2019a). Moreover, the pH controls the protein structure of GadD2, which showed association with nisin susceptibility based on WGS data of NS and NNS. However, homology modeling of GadD2 in NNS predicted a protein structure that promoted a less pH-dependent GAD activity (**Figure 18**). In culture broth supplemented with free nisin, both NNS field isolates with amino acid substitution in their GadD2 had significant faster growth rates compared to NS (**Figure 12**). This also indicated that the amino acid substitution maximize protection at neutral pH in combination with other nisin resistance mechanisms. Thus, confirming the postulated Hypothesis II (**2.1.**). Nevertheless, GAD assays based on a pH-sensitive colorimetric assay showed inconsistent results to further reinforce the Hypothesis II. Thus, one of the future tasks will be to conduct knockout mutant studies, proteome or transcriptome analyses that will hopefully help to understand nisin susceptibility in *L. monocytogenes*. Finally, the pH initiated the ripening of the SCC while the pH-shift on the SCC surface (**Figure 13**) could slow down the nisin release from UFL2-N. The total red smear microbiota was largely unaffected by the presence of UFL2-N or free nisin

(**Table 19**), which is in agreement with Hypothesis III (**2.1**). Neither the addition of UFL2-N nor the addition of free nisin to the SCC surface did alter its texture and appearance like size, color in curd core or rind when compared to commercial SCC underscoring the Hypothesis III. Antilisterial property of UFL2-N and free nisin was investigated on the SCC surface. By addition of 0.132 mg ml^{-1} UFL2-N or free nisin, *L. monocytogenes* was below limit of quantification (**Table 19**) indicating that UFL2-N enabled a slow release and antilisterial activity in SCC manufacturing. Thus, the results support Hypothesis III.

Future research should be directed towards the identification of induced co-selection processes as mentioned in **Figure 19** and the implementation of UFL2-N nebulization in dairy production.

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8. APPENDIX

8.1. Appendix Figures

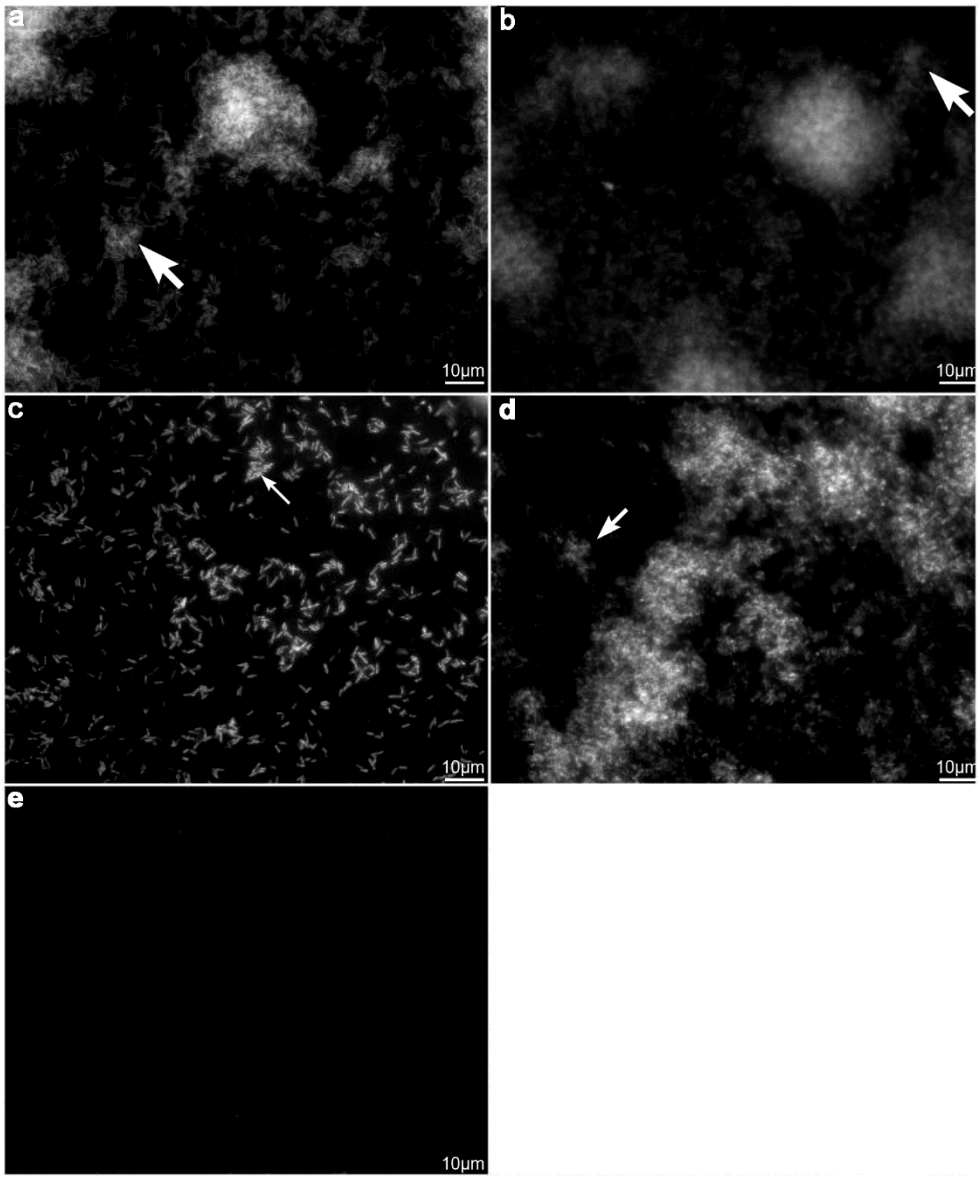

Figure 8.1-1: Fluorescence *in situ* hybridization images of *L. monocytogenes* BfR L261 field isolate (5.18 log CFU ml^{-1}) after incubation for four days in BHI broth at 30 °C. Addition of 0.74 mol l^{-1} NaCl and/or 0.004 mg ml^{-1} free nisin to BHI broth followed the traditional Hessian recipe of sour curd cheese. **A** Field isolate BfR L261. **B** Field isolate BfR L261 incubated with 0.74 mol l^{-1} NaCl, **C** with 0.004 mg ml^{-1} free nisin, and **D** with 0.74 mol l^{-1} NaCl as well as 0.004 mg ml^{-1} free nisin. **E** BHI broth without any additions.

Arrows indicate cells of field isolate while arrow widths represent density of cell conglomeration. Scale bar indicates a length of 10.0 µm.

Table 8.2-1: Comparison between antibiotic resistances to MICs of disinfectant compounds (BAC benzalkonium chloride; CKC cetalkonium chloride) and antimicrobial preservative (citral). Antibiotics and biocides were selected to have similar mode of action as well as bacterial resistance, which is based on modified cell wall and/or membrane or other resistance mechanisms in these target regions (Kapoor et al. 2017; Nair et al. 2016; Walsh 2003). Number of *L. monocytogenes* field isolates (n=238) inhibited by various antibiotics are arranged in susceptible (S), intermediate (I) or resistant (R) according to Noll et al. (2018).

Biocide	MIC [mg ml^{-1}]	AMP[a]			CRO[a]			DPT[a]			MER[a]			PEN[a]			VAN[a]		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
BAC	0.016	2	-	-	1	1	-	-	-	2	2	-	-	2	-	-	2	-	-
	0.008	22	-	-	5	14	3	-	-	22	20	-	2	21	-	1	22	-	-
	0.004	56	-	-	6	42	8	-	-	56	53	-	3	55	-	1	56	-	-
	0.002	149	-	-	21	120	8	-	-	149	146	-	3	148	-	1	149	-	-
	0.001	9	-	-	2	5	2	-	-	9	9	-	-	9	-	-	9	-	-
Signf.[b]						ns						ns							
CKC	0.004	21	-	-	4	16	1	-	-	21	20	-	1	20	-	1	21	-	-
	0.002	118	-	-	18	87	13	-	-	118	112	-	6	116	-	2	118	-	-
	0.001	95	-	-	13	77	5	-	-	95	95	-	-	95	-	-	95	-	-
	0.0005	4	-	-	-	2	2	-	-	4	3	-	1	4	-	-	4	-	-
Signf.						ns						ns							
Citral	71.20	1	-	-	-	-	1	-	-	1	-	-	1	-	-	1	1	-	-
	35.60	4	-	-	-	2	2	-	-	4	2	-	2	3	-	1	4	-	-
	17.80	42	-	-	6	31	5	-	-	42	40	-	2	41	-	1	42	-	-
	8.90	130	-	-	21	101	8	-	-	130	128	-	2	130	-	-	130	-	-
	4.45	50	-	-	6	40	4	-	-	50	49	-	1	50	-	-	50	-	-
	2.23	10	-	-	1	8	1	-	-	10	10	-	-	10	-	-	10	-	-
	1.11	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-

Table 8.2-1: Continued.

Biocide MIC [mg ml^{-1}]	**AMP[a]**			**CRO[a]**			**DPT[a]**			**MER[a]**			**PEN[a]**			**VAN[a]**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Signf.					ns						*							

[a] AMP Ampicillin; CRO Ceftriaxone; DPT Daptomycin; MER Meropenem; PEN Benzylpenicillin; VAN Vancomycin.
[b] Comparison between S and R field isolates; in empty cells comparison was not possible, ns and * indicate not significant and significant at $P < 0.05$, respectively.

Table 8.2-2: Comparison between antibiotic resistances to MICs of disinfectant compounds (BAC benzalkonium chloride; CKC cetalkonium chloride) and antimicrobial preservative (H_2O_2 hydrogen peroxide). Antibiotics and biocides were selected to have similar mode of action as well as bacterial resistance, which is based on efflux pump activity (Allen et al. 2016; Komora et al. 2017; Krawczyk-Balska and Markiewicz 2016; Nair et al. 2016; Walsh 2003). Number of *L. monocytogenes* field isolates (n=238) inhibited by various antibiotics are arranged in susceptible (S), intermediate (I) or resistant (R) according to Noll et al. (2018).

Biocide	**MIC [mg ml^{-1}]**	**AMP[a]**			**CIP[a]**			**CRO[a]**			**PEN[a]**			**TET[a]**		
		S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
	0.016	2	-	-	2	-	-	1	1	-	2	-	-	-	2	-
	0.008	22	-	-	20	-	2	5	14	3	21	-	1	7	9	6
BAC	**0.004**	56	-	-	50	-	6	6	42	8	55	-	1	14	26	16
	0.002	149	-	-	135	-	14	21	120	8	148	-	1	32	87	30
	0.001	9	-	-	8	-	1	2	5	2	9	-	-	1	6	2
Signf.[b]						ns			ns						ns	
	0.004	21	-	-	19	-	2	4	16	1	20	-	1	4	10	7
CKC	**0.002**	118	-	-	105	-	13	18	87	13	116	-	2	29	58	31
	0.001	95	-	-	87	-	8	13	77	5	95	-	-	22	58	15
	0.0005	4	-	-	4	-	-	-	2	2	4	-	-	1	2	1
Signf.						ns			ns						ns	
	0.156	158	-	-	142	-	16	22	120	16	155	-	3	32	87	39
H_2O_2	**0.078**	79	-	-	72	-	7	13	61	5	79	-	-	24	40	15
	0.039	1	-	-	1	-	-	-	1	-	1	-	-	-	1	-
Signf.						ns			ns						ns	

[a] AMP Ampicillin; CRO Ceftriaxone; CIP Ciprofloxacin; PEN Benzylpenicillin; TET Tetracycline.
[b] Comparison between S and R field isolates; in empty cells comparison was not possible, ns indicates not significant at P <

Table 8.2-3: Comparison between antibiotics resistance to MICs of disinfectant compound (H_2O_2 hydrogen peroxide) and antimicrobial preservative ($NaNO_2$ sodium nitrite). Antibiotics and biocides were selected to have similar mode of action as well as bacterial resistance, which is based on modified bacterial cell components or other resistance mechanisms in these target regions (Kapoor et al. 2017; Nair et al. 2016; Walsh 2003). Number of *L. monocytogenes* field isolates (n=238) inhibited by various antibiotics are arranged in susceptible (S), intermediate (I) or resistant (R) according to Noll et al. (2018).

Biocide	MIC [mg ml^{-1}]	ERY[a]			GEN[a]			LIZ[a]			RAM[a]			SXT[a]			TET[a]			TGC[a]		
		S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
	0.156	154	-	4	148	-	10	158	-	-	33	117	8	145	-	13	32	87	39	2	90	66
H_2O_2	**0.078**	78	-	1	78	-	1	79	-	-	22	56	1	78	-	1	24	40	15	1	56	22
	0.039	1	-	-	1	-	-	1	-	-	-	1	-	1	-	-	-	1	-	-	1	-
Signf.[b]			ns			ns						ns			ns			ns				
	>8.0	12	-	1	9	-	4	13	-	-	5	4	4	9	-	4	2	7	4	-	6	7
	8.0	102	-	1	101	-	2	103	-	-	28	73	2	99	-	4	27	62	14	2	66	35
$NaNO_2$	**4.0**	98	-	2	95	-	5	100	-	-	17	81	2	96	-	4	21	51	2	-	63	37
	2.0	18	-	-	18	-	-	18	-	-	4	14	-	17	-	1	5	7	6	1	10	7
	1.0	2	-	1	3	-	-	3	-	-	1	1	1	2	-	1	1	-	2	-	1	2
	0.5	1	-	-	1	-	-	1	-	-	-	1	-	1	-	-	-	1	-	-	1	-
Signf.			ns			ns						ns			ns			ns				

[a] ERY Erythromycin; GEN Gentamicin; LIZ Linezolid; RAM Rifampicin; SXT Trimethoprim/sulfamethoxazole; TET Tetracycline; TGC Tigecycline.
[b] Comparison between S and R field isolates; in empty cells comparison was not possible, ns indicates not significant at $P < 0.05$.

Table 8.2-4: MICs of free nisin of *L. monocytogenes* field isolates (n=117) with multi-antibiotic resistances (R) (Noll et al. 2018).

No. of R	No. of field isolates	MIC of free nisin [mg ml^{-1}]
10	1	0.011
8	1	0.011
7	1	0.011
6	2	0.032
5	2	0.032
4	6	0.011
3	26	0.011
2	78	0.011

Table 8.2-5: Comparison of nisin susceptibility associated genes in the genomes of *L. monocytogenes* field isolates (referred to BfR L41). Data was provided by the German Federal Institute for Risk Assessment (Szendy et al. 2019b).

Protein (Size)	**Predicted function**	**NS *L. monocytogenes* field isolates**				**NNS *L. monocytogenes* field isolates**	
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261
AnrB (646 aa)	ABC transporter permease	-	36: L→F	-	-	-	-
		-	-	-	-	355: E→D	-
		-	-	407: N→S	-	-	-
		-	-	439: D→N	-	-	-
ArcA (410 aa)	Deregulation of the arginine deiminase (*arc*) operon	-	144: A→D	144: A→D	144: A→D	144: A→D	144: A→D
		-	205: V→L	-	-	-	-
		-	215: I→L	215: I→L	-	215: I→L	215: I→L
DltA (510 aa)	D-Alanylation of teichoic acids	-	-	-	-	-	-
DltB (394 aa)	D-Alanylation of teichoic acids	-	-	-	80: R→Q	-	80: R→Q
		-	-	-	98: V→I	-	98: V→I
		-	-	-	109: H→N	-	109: H→N
		-	-	-	126: T→A	-	126: T→A
		-	-	-	188: F→I	-	-
DltC (79 aa)	D-Alanylation of teichoic acids	-	-	-	-	-	-
DltD (425 aa)	D-Alanylation of teichoic acids	-	398: I→M	-	-	-	-
		-	419: P→Q	-	-	-	-
LiaSR (353 aa)	Gene regulator	-	-	261: V→I	-	-	-
LisK (483 aa)	Signal transduction system	-	-	-	-	-	-
LisR (226 aa)	Signal transduction system	-	-	-	-	-	-

Table 8.2-5: Continued.

Protein (Size)	**Predicted function**	**NS *L. monocytogenes* field isolates**				**NNS *L. monocytogenes* field isolates**	
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261
lmo0047 (410 aa)		- -	144: A→D 205: V→L	144: A→D -	144: A→D -	144: A→D -	144: A→D -
lmo1746 (658 aa)	ABC transporter permease	- -	- -	571: A→G 618: V→I	- -	- -	- -
lmo1747 (255 aa)	ABC transporter binding protein	- -	- 179: A→S	- 179: A→S	134: I→L -	- 179: A→S	- -
MprF (865 aa)	Lysinylation of membrane phosphatidylglycerol (multiple peptide resistance factor)	-	664:S→P	664:S→P	664:S→P	664:S→P	664:S→P
Pbp (714 aa)	Formation of a rod-shaped peptidoglycan cell wall	-	-	-	-	-	-
RmlD (277 aa)	Precursor involved in cell wall polysaccharide backbone production	-	73: D→N	73: D→N	-	-	-
SigB (259 aa)	Regulation of stress response and virulence	-	-	-	-	-	-
SigL (447 aa)	Regulation of stress response and virulence	- -	- -	90: M→L 393: K→M	- -	- -	- -
TelA (399 aa)	Tellurite resistance	-	-	50: D→E	-	-	-
VirR (225 aa)	Gene regulator	-	-	-	-	-	-
VirS (364 aa)	Gene regulator	- -	- 261: L→S	7: L→I 261: L→S	7: L→I 261: L→S	7: L→I 261: L→S	- 261: L→S

Abbreviations: aa amino acid; A alanine; D aspartic acid; E glutamic acid; F phenylalanine; G glycine; H histidine; I isoleucine; K lysine; L leucine; M methionine; N asparagine; NS nisin susceptible; NNS nisin non-susceptible; P proline; Q glutamine; R arginine; S serine; T threonine; V valine.

Table 8.2-6: Comparison of regulatory sequences in the genomes of *L. monocytogenes* field isolates (referred to BfR L41). Data was provided by the German Federal Institute for Risk Assessment (Szendy et al. 2019b).

Target gene	Identified TF binding sites	NS *L. monocytogenes* field isolates				NNS *L. monocytogenes* field isolates		DNA sequence variance
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261	
anrB	cytR, deoR, ihf, irp (2), phoB, dnaA	-	-	-	-	-	-	-
arcA	rpoH2, lexA, argR, argR2, ihf, rpoD17	-	-	-	-	-	-	-
dltA	ihf (2), gcvA, tyrR	-	-	-	-	-	-	-
dltB	rpoD16	-	-	-	-	-	-	-
dltC	fnr, nagC, cpxR, rpoD18	-	-	-	-	-	-	-
dltD	nagC, cpxR, rpoD18, rpoD17	-	-	-	-	-	-	-
gadD2	dnaA	-	-	+	+	-	-	dnaA T(T→G)GTTATC
gadD3	Irp, argR2	-	-	-	-	-	-	-
gadT2	rpoH3, ompR crp, arcA	-	-	-	-	-	-	-
liaSR	glpR	-	-	-	-	-	-	-
lisK	hipB, rpoN, arcA, rpoD17, fnr	-	-	-	-	-	-	-
lisR	rpoD17, rpoD16	-	-	-	-	-	-	-
lmo0047	rpoH2, lexA, argR, argR2, ihf, rpoD17	-	-	-	-	-	-	-
lmo1746	rpoS17, phoB	-	-	-	-	-	-	-
lmo1747	Lrp, lexA, rpoD17, rpoD18, cpxR, cynR	-	-	-	-	-	-	-
mprF	rpoD16, fur, rpoD15	-	-	-	-	-	-	-

Table 8.2-6: Continued.

Target gene	Identified TF binding sites	NS *L. monocytogenes* field isolates				NNS *L. monocytogenes* field isolates		DNA sequence variance
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261	
pbpA	hipB, rpoN, arcA, rpoD17, fnr	-	-	-	-	-	-	-
rmlD	rpoD18, rpoN	-	-	-	-	-	-	-
sigB	argR2, crp	-	-	-	-	-	-	-
sigL	Ihf, fnr, tyrR, rpoD17	-	-	-	-	-	-	-
telA	ihf, rpoH3, rpoD18, ilvY	-	-	-	-	-	+	-10 region GCG(T→C)ACACT rpoD18
		-	-	+	-	-	-	AAAT(A→C)TAT ilvY
		-	-	+	-	-	-	AT(A→C)TATCA
virR	arcA, purR, meJ	-	-	-	-	-	-	-
virS	rpoD16 (2), fis, gcvA	-	-	-	-	-	-	-

Abbreviations: + presence of target gene, - absence of target gene; NS nisin susceptible; NNS nisin non-susceptible; TF transcription factor.

Table 8.2-7: Comparison of the three different end C-terminal protein sequences of *gadD2* in 30 nisin non-susceptible (NNS) *L. monocytogenes* field isolates ("HDTQQ", "HDNQQ" and "HNTQQ"). Shown sequences start at amino acid position 452 and end at position 456 (total of 464 amino acids; UniProt ID Q9EYW9). Protein sequences with amino acid substitution on position 453 are highlighted in bold. Additionally, the two NNS field isolates from whole genome sequencing are italicized. Data was provided by the German Federal Institute for Risk Assessment (Hammerl 2017; personal communication).

BfR No.	Protein sequence	Free nisin MIC [mg ml^{-1}]	Serptype	Source
BfR L1322	...HDTQQ...	0.011	IIa	Environmental sample
BfR L1068	...HDTQQ...	0.011	IIa	Cheese
BfR L517	...HDTQQ...	0.011	IIa	Cheese from raw milk
BfR L672	...HDTQQ...	0.011	IIa	Cheese from raw milk
BfR L271	...HDTQQ...	0.011	IIa	Raw milk
BfR L268	...HDTQQ...	>0.039	IIa	Raw milk
BfR L262	...HDTQQ...	>0.039	IIa	Raw milk
BfR L266	...HDTQQ...	0.011	IIa	Raw milk
BfR L1409	...HDTQQ...	0.011	IIa	Raw milk
BfR L233	...HDTQQ...	0.011	IIa	Raw milk
BfR L243	...HDTQQ...	>0.039	IIa	Raw milk
BfR L982	...HDTQQ...	0.011	IIa	Raw milk
BfR L714	...HDTQQ...	0.011	IIa	Ricotta salad with sheep cheese
BfR L660	...HDTQQ...	0.011	IIa	Smoked salmon
BfR L513	...HDTQQ...	0.011	IIa	Soft cheese from raw milk
BfR L55	...HDTQQ...	0.011	IVb	Cheese
BfR L380	...HDTQQ...	0.011	IVb	Raw milk
BfR L459	...HDTQQ...	0.011	IVb	Smoked salmon
BfR L1528	...HDTQQ...	0.011	IVb	Smoked salmon
BfR L554	...HDTQQ...	0.011	IVb	Tuna salad
BfR L479	...HDTQQ...	0.011	IVb	Unknown
BfR L166	...HDTQQ...	0.011	IVb	Unknown
BfR L386	...HDNQQ...	0.011	IIb	Obatzer
BfR L1009	**...HNTQQ...**	0.011	IIa	Beef
BfR L269	**...HNTQQ...**	0.011	IIa	Raw milk
BfR L267	**...HNTQQ...**	0.011	IIa	Raw milk
BfR L653	**...HNTQQ...**	0.011	IIa	Raw milk
BfR L1330	**...HNTQQ...**	0.011	IIa	Raw milk

Table 8.2-7: continued.

BfR No.	Protein sequence	Free nisin MIC [mg ml^{-1}]	Serptype	Source
BfR L1080	**...HNTQQ...**	>0.039	IIa	Soft cheese
BfR L1083	**...HNTQQ...**	0.011	IIa	Soft cheese
BfR L245	***...HNTQQ...***	*>0.039*	*IIa*	*Raw milk*
BfR L261	***...HNTQQ...***	*>0.039*	*IIa*	*Raw milk*

Abbreviation: D aspartic acid; H histidine; N asparagine; Q glutamine; T threonine.

Table 8.2-8: Comparison of the three different end C-terminal protein sequences of *gadD2* in 30 nisin susceptible (NS) *L. monocytogenes* field isolates ("HDTQQ" and "HDNQQ"). Shown sequences start at amino acid position 452 and end at position 456 (total of 464 amino acids; UniProt ID Q9EYW9). The protein sequences of field isolate BfR L600 could not be derived due to bad quality in ab1-file. Additionally, the four NS field isolates from whole genome sequencing are italicized. Data was provided by the German Federal Institute for Risk Assessment (Hammerl 2017; personal communication).

BfR No.	Protein sequence	Free nisin MIC [mg ml^{-1}]	Serptype	Source
BfR L1038	...HDTQQ...	0.004	IIa	Soft cheese
BfR L336	...HDTQQ...	0.0005	IIc	Clinic isolate
BfR L610	...HDTQQ...	0.0005	IIc	Mixed ground meat
BfR L528	...HDTQQ...	0.003	IIc	Onion Mettwurst
BfR L286	...HDTQQ...	0.000	IVab	Mettwurst
BfR L548	...HDTQQ...	0.0001	IVb	Atlantic salmon
BfR L335	...HDTQQ...	0.002	IVb	Clinic isolate
BfR L725	...HDTQQ...	0.0001	IVb	Fish
BfR L883	...HDTQQ...	0.0001	IVb	Fish
BfR L1165	...HDTQQ...	0.0005	IVb	Fleischkäse
BfR L1229	...HDTQQ...	0.0005	IVb	Meat ball
BfR L914	...HDTQQ...	0.0001	IVb	Ground beef
BfR L850	...HDTQQ...	0.003	IVb	Ground pork
BfR L934	...HDTQQ...	0.0005	IVb	Meat
BfR L292	...HDTQQ...	0.003	IVb	Onion Mettwurst
BfR L530	...HDTQQ...	0.004	IVb	Onion Mettwurst
BfR L887	...HDTQQ...	0.003	IVb	Salami
BfR L964	...HDTQQ...	0.0005	IVb	Sausauge
BfR L101	...HDTQQ...	0.0005	IVb	Spot sample
BfR L994	...HDTQQ...	0.001	IVb	Spot sample
BfR L1031	...HDTQQ...	0.000	IVb	Tilapia
BfR L1385	...HDTQQ...	0.000	IVb	Tofu
BfR L41	*...HDTQQ...*	0.0001	*IIa*	*Sewage*
BfR L448	*...HDTQQ...*	0.003	*IIa*	*Smoked salmon*
BfR L1079	*...HDTQQ...*	0.001	*IIa*	*Soft cheese*
BfR L846	...HDNQQ...	0.0005	IIb	Cured pork
BfR L1303	...HDNQQ...	0.003	IIb	Egg salad
BfR L1339	...HDNQQ...	0.003	IIb	Ham
BfR L1245	...HDNQQ...	0.0001	IIb	Raw sausage
BfR L1060	...HDNQQ...	0.003	IIb	Crumble

Table 8.2-8: continued.

BfR No.	Protein sequence	Free nisin MIC [mg ml^{-1}]	Serptype	Source
BfR L1138	...HDNQQ...	0.002	IIb	Sushi
BfR L625	...HDNQQ...	0.0005	IIb	Tuna
BfR L600	-	0.000	-	Frozen gyros
BfR L330	*...HDNQQ...*	0.003	*IIa*	*Unknown*

Abbreviation: D aspartic acid; H histidine; N asparagine; Q glutamine; T threonine.

Table 8.2-9: Comparison of how the culture broth affected the GAD system response. Nisin susceptible (NS) and nisin non-susceptible (NNS) *L. monocytogenes* field isolates were pre-cultured in three different culture broths (BHI, LB or TSB) with or without supplementation of 0.003 mg ml^{-1} free nisin, N+ and N- respectively. *Escherichia coli* K-12 ER2738 was GAD positive within 5 to 10 minutes and was used as the reference strain (+++). Field isolates were denoted as +++, if the color of the GAD reagent changed within 10 minutes plus twice its standard deviation. Alternatively, denotation followed as: ++ ≥30 min to <60 min; + ≥60 min to <90 min; - ≥90 min. n=4

	NS *L. monocytogenes* field isolates								**NNS *L. monocytogenes* field isolates**				**GAD^+**	**GAD^-**
BfR No.	**L41**		**L330**		**L448**		**L1079**		**L245**		**L261**		**EC[a]**	**LF[b]**
	N-	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-	N-
BHI	-	n.d.	++	n.d.	-	n.d.	++	n.d.	++	n.d.	+	n.d.	+++	n.d.
LB	n.d.	n.d.	+++	+++	+++	++	+++	++	+++	+++	++	+	+++	n.d.
TSB	+++	++	+++	+++	+++	++	+++	+++	+++	+++	++	++	+++	n.d.

[a] EC: *E. coli* K-12 ER2738; positive GAD reaction (GAD^+).
[b] LF: *Lactobacillus fermentum* field isolate; negative GAD reaction (GAD^-).
Abbreviation: n.d. not detected.

Table 8.2-10: Incubation of autoclaved SCC (log 5.0 CFU ml^{-1}) with *L. monocytogenes* BfR L1031 field isolate over ripening time (N-). Before incubation, autoclaved SCC was amended with 0.004 mg ml^{-1} free nisin (N+). Values in brackets represents standard deviation.

Time [d]	log CFU ml^{-1}	
	N-	N+
0	5.35 (±0.28)	5.69 (±0.25)
1	7.96 (±0.05)	5.36 (±0.19)
2	7.87 (±0.37)	5.65 (±0.23)
3	6.87 (±0.15)	5.22 (±0.26)

8.3. Publications, oral presentations and posters

Publications:

Szendy, M.; Westhäuser, W.; Baude, B.; Reims, J.; Dähne, L.; Noll, M. (2019): Time lapsed and pH-controlled release of nisin from Neusilin particles to enhance food safety of sour curd cheese. Journal of Food Science and Technology 56 (3), p. 1613–1621.

Szendy, M.; Kalkhof, S.; Bittrich, S.; Kaiser, F.; Leberecht, C.; Labudde, D.; Noll, M. (2019): Structural change in GadD2 of *Listeria monocytogenes* field isolates supports nisin resistance. International Journal of Food Microbiology 305, 10.1016/j.ijfoodmicro.2019.108240.

Szendy, M.; Rödel, A.; Horn, M.A.; Al Dahouk, S.; Dieckmann, R.; Noll, M.: Efficiency of disinfectant compounds and antimicrobial preservatives towards 253 *Listeria monocytogenes* field isolates and evaluation of antibiotic correlation; *manuscript in preparation*

Oral presentations:

17. Fachsymposium Lebensmittelmikrobiologie (2017): Intrinsische Nisintoleranz durch das Glutamat Decarboxylase System (GAD) in *Listeria monocytogenes*

16. Fachsymposium Lebensmittelmikrobiologie (2016): Wirkung von Mikrobioziden auf *Listeria monocytogenes*

Annual Conference of the Association for General and Applied Microbiology (VAAM) (2016): Effect of nisin on the survival of *Listeria monocytogenes* in sour curd cheese after artificial contamination

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10. (EIDESSTATTLICHE) VERSICHERUNG UND ERKLÄRUNGEN

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

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Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden habe.

(§ 8 Satz 2 Nr. 4 PromO Fakultät)

Hiermit erkläre ich, dass ich Hilfe von gewerblichen Promotionsberatern bzw. –vermittlern oder ähnlichen Dienstleistern weder bisher in Anspruch genommen habe noch künftig in Anspruch nehmen werde.

(§ 8 Satz 2 Nr. 7 PromO Fakultät)

Hiermit erkläre ich mein Einverständnis, dass die elektronische Fassung der Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung unterzogen werden kann.

(§ 8 Satz 2 Nr. 8 PromO Fakultät)

Hiermit erkläre ich mein Einverständnis, dass bei Verdacht wissenschaftlichen Fehlverhaltens Ermittlungen durch universitätsinterne Organe der wissenschaftlichen Selbstkontrolle stattfinden können.

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Ort, Datum, Unterschrift