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MASTERARBEIT

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„Ionic Liquids- A benign alternative to commercially used quaternary ammonium compound disinfectants?“

verfasst von

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1. Introduction

1.1 Food safety

1.1.1 Food safety in general

Food can be contaminated by many agents like bacteria, viruses, parasites, and chemicals, whereas transmission of foodborne diseases can take place through contact to animals or transmission via the environment [1]. Food safety describes conditions and practices in order to prevent contaminations and foodborne illnesses [2,3,4]. Since foodborne diseases are an increasing public health problem, causing significant number of deaths, food safety is getting more important as public health issue [5,6]. In 2002, the European Union defined directives to regulate food safety. By these laws control of every single step in food production (creation, transport, feeding, and processing) is mandatory. In Europe, the EFSA (European Food Safety Authority) monitors and evaluates the risks associated with food, in order to advise politicians scientifically [7].

Concerns regarding food safety can be classified as follows [8]:

1. Microbiological hazards by pathogenic microorganisms and parasites
2. Chemical hazards induced by natural toxicants, environmental contaminants, and pesticides
3. Hazards by new technologies like genetic engineering and irradiation of food
4. Physical hazards

The present thesis will mainly focus on microbiological hazards caused by foodborne pathogens.

1.1.2 Foodborne pathogens and their prevalence

Zoonoses are naturally transmissible infections and diseases. Transmission occurs either from contaminated foodstuff, or from contact between animals and humans. Disease severity in humans is variable, from subclinical infections to life threatening [9]. Foodborne pathogens comprise bacteria, viruses, fungi, but also a number of parasites causing infections in humans via contaminated food or water. Especially foodborne bacteria such as *Escherichia coli* O157:H7, *Salmonella enterica, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Bacillus cereus, shiga toxin producing E.coli, and Vibrio spp.* are responsible for the majority of foodborne diseases [6]. As revealed by the WHO, every year 1.9 million children globally die of diarrhoeal disease [8]. Estimations suggest that in the United States foodborne diseases lead to 76 million illnesses each year, resulting in 325,000 hospitalizations and 5,000 deaths [10].

1.2 Important foodborne pathogens in this work

1.2.1 *Salmonella spp.*

The genus of *Salmonella* is composed of two species: *Salmonella enterica* and *Salmonella bongori. S. enterica* is divided into six subspecies. Out of this, the subspecies *S. enterica* is the most essential one, regarding the transmission from animal to human. The subspecies itself is further divided into serovars, which are often named according to the place of isolation [9]. A wide range of hosts and major livestock species (poultry, cattle, and horses) are colonised by *Salmonella spp.* [11]. There the pathogen can be found in the intestinal tract of these species, influencing foodstuff of animal and plant origin. Transmission often takes place when *Salmonella* is introduced into food preparation areas. There *Salmonella* proliferates in the food due to inadequate storage temperatures, cooking and inadequate handling in general. This is especially important for ready-to-eat (RTE) foods. The pathogen can also be transmitted through direct contact with infected animals or humans as well as faecal contaminated environments. Moreover infected personal handling food may act as source of contamination for foodstuff [9]. Frequently contaminated foodstuffs include for example table eggs, pig meat, broiler, and turkey meat [12]. In the European Union infections with *S.* Enteritidis and *S.* Typhimurium are those serovars mostly associated with illness in humans. Whereas *S.* Enteritidis infections are associated with consumption of contaminated eggs and poultry meat, *S.* Typhimurium infections are associated with consumption of contaminated pig and bovine meat.

Infection in animals is typically subclinical. Within a herd or a flock the pathogen can easily spread without being detected and animals can turn out as persistent carriers. Sheep, cattle, and horses may suffer from diarrhoea and abortion. In pigs, goats, and poultry the clinical signs are less apparent and no obvious signs of infection with a zoonotic serovar can be confirmed. Salmonellosis in humans is characterized by acute onset of fever, abdominal pain, nausea, and sometimes vomiting after incubation of 12-36 hours. Mostly, symptoms are mild and only last a few days. However, some patients suffer from severe disease with life threating dehydration. Mortality is usually low and less than 1% of reported *Salmonella* cases are fatal. 92,916 cases of salmonellosis in total were reported in the EU 2012. Fatality rate was 0.41% with 61 deaths, caused by *S.* Typhimurium and *S.* Enteritidis as most frequently isolated serovars [9].

1.2.2 *Escherichia coli*

Escherichia coli is a widespread commensal bacterium that colonises the gut. Six different diarrheagenic *E. coli* strains are known, each having specific phenotypic and genetic traits. These include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and Vero cytotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). Shiga toxin-producing *E. coli* are usually associated with haemolytic uremic syndrome and neurologic complications [11,13]. Food- related outbreaks are mostly associated with VTEC, to a lesser extent EPEC, ETEC, and EAggEC strains. In 1982, the first outbreaks of severe bloody diarrhea in connection with *E. coli* O157 were recorded. In the US, it has been estimated that the pathogen is responsible for 73,000 illnesses and 250 deaths every year [10]. In Europe, the first community outbreak of *E. coli* O157:H7 was in the United Kingdom in 1985 [14]. Sources of VTEC were often found in contaminated beef meat or minced meat, which is probably due to the fact that the pathogen is widespread in the guts of asymptomatic cattle. Furthermore, anything potentially in contact with ruminant faeces such as vegetables, sprouts, fruits, juices, milk (pasteurized and unpasteurized) as well as faecal contaminated drinking, recreation, and bathing water can be potential sources of VTEC [11]. In 2011 9,485 cases of VTEC were reported in the EU. A large number of these cases were contributed to the STEC/VTEC O104:H4 outbreak in Germany. The most common group was VTEC O157, followed by O104. Case fatality rate of VTEC infections was 0.75% with 56 deaths reported, of which 89% occurred in Germany [15].

1.3 *Listeria monocytogenes*

1.3.1 *L. monocytogenes* in general

Listeria monocytogenes is a gram-positive, ubiquitous pathogen occurring in various habitats such as man, sewage, soil, water, vegetation, silage, animals (wild and domesticated), and food processing plants [16,17]. *L. monocytogenes* is able to grow in a wide range of environmental conditions including refrigeration temperatures (2-4°C), low pH values, and high salt concentrations [18]. Main route of transmission is through consumption of contaminated food. Transmission from infected animals to human is possible but rare, as well as it is between humans [19]. Cutaneous listeriosis, an infection of the skin, is an example for transmission from animal to human. This disease has been especially diagnosed in veterinarians and farmers having contact with stillbirths or abortions in cattle [17].

1.3.2 Listeriosis

Due to the long incubation time (seven days to two month [20]) of listeriosis it is difficult to trace the source of contamination in terms of the specific food. The pathogen enters into the mammalian cell via phagocytosis, is then released from the membrane bound vacuole and starts to multiply. In terms of intracellular movement and cell-to-cell spread the actin polymerization helps to infect an immense range of host tissue, with the liver as main site of infection [18]. In 2012, in 18 EU member states 1,642 cases of listeriosis were reported with a fatality rate of 17.8%, resulting in 198 deaths [9]. There are two forms of listeriosis: non-invasive gastrointestinal and invasive listeriosis. Non-invasive listeriosis develops in immunocompetent individuals as a febrile gastroenteritis. In case of immunocompromised patients, as for example elderly people receiving immunosuppressive agents, listeriosis can manifest as septicaemia or meningoencephalitis. Transmission from the infected mother, via the placenta to the fetus, is also possible [21]. This can lead to abortion, stillbirth or a generalized infection (granulomatosis infantiseptica) of the infant. Therefore, during pregnancy, listeriosis is a serious threat to the unborn child. One third of the maternal-fetal infections result in abortion or stillbirth. Nevertheless, even if severely ill, the prognosis for live-born babies is good [22].

1.3.3 *L. monocytogenes*: a foodborne pathogen

Following several food-related outbreaks in the 1980s, interest in *L. monocytogenes* as foodborne pathogen grew strongly among food manufactures and government bodies [23]. In 2009, a listeriosis outbreak took place in Austria, Germany, and the Czech Republic. The outbreak was caused by consumption of the Austrian acid curd cheese "Quargel" [24]. Two *Listeria* strains, both from serotype 1/2a, were responsible for the outbreak, resulting in a total of eight fatalities [25]. *L. monocytogenes* enters food production areas from natural and urban environments, which is indicated by several listeriosis-associated findings in these respective locations [20]. The pathogen can be found in a vast variety of raw and processed foods. Products such as milk, dairy products, meat and meat products (beef, pork, fermented sausages), radish, cabbage, seafood, and fish product have been associated with *Listeria* contamination [18]. Therefore, one way to introduce *Listeria* into food production is via raw products such as meat, milk, or vegetables. Fertilization with either sewage or manure transfers *L. monocytogenes* to vegetables or crops. Furthermore, *Listeria* can infect domestic animals eating silage or grazing. The pathogen enters the food production chain via infected animals, which are transported to slaughterhouses or contamination happens via milking process [17]. Besides *Listeria* can also be introduced into the production facilities via soil on workers shoes or transport equipment [26]. In addition there is a high risk of recontamination after heating. Whereas heating itself only bears a low risk of *L. monocytogenes* surviving this treatment [27]. Furthermore cross-contamination in retailing facilities can occur, where slicing has been revealed as main factor of contamination [28].

Table 1: *Common sites of L. monocytogenes contamination in food processing plants* [27].

TABLE 1. Common sites of *L. monocytogenes* contamination
Filling or packaging equipment
Conveyors
Solutions used in chilling food
Slicers, dicers, shredders, blenders, etc. used after heating or decontaminating and before packaging
Collators used for assembling/arranging product for packaging
Racks for transporting finished product
Hand tools, gloves, aprons, etc. that contact exposed finished product
Spiral freezers/blast freezers
Containers such as bins, tubs, or baskets used for holding food while it is waiting to be further processed or packaged

As Table 1 shows, several sites of possible contamination can be found within a food processing plant. When *L. monocytogenes* has been introduced into the food processing plants, there are even more sites, where survival of this pathogen is possible [27] (see Table 2 2).

Table 2: *Reservoirs for L. monocytogenes growth within a food processing plant* [27].

TABLE 2. Examples of *L. monocytogenes* reservoirs in the plant
Equipment framework and other equipment in the area
Floors
Drains
Walls, especially if there are cracks that retain moisture
Ceilings, overhead structures, catwalks
Condensate
Insulation in walls or around pipes and cooling units that has become wet
Trolleys, forklifts, walk-alongs
Cleaning tools such as sponges, brushes, floor scrubbers
Maintenance tools

1.3.4 Resistance mechanisms relevant for persistence of *L. monocytogenes* in the food processing environment

L. monocytogenes can be found in different types of food processing plants. The pathogen has been repeatedly isolated from dairy production environments [29], meat and poultry-plants [30], RTE- food processing facilities [31], but also fish processing plants [32]. In food processing environments harsh conditions are common in order to prevent bacterial colonization. These environments are characterized by low temperatures, heat, acidic pH, high salt concentrations, drying, preservatives as well as extensive cleaning and disinfection [33]. However, *L. monocytogenes* is able to survive in food processing environments, because of its special capabilities [20]. *L. monocytogenes* withstands high levels of osmotic stress and stress caused by disinfectants. Moreover, *L. monocytogenes* can grow over a wide range of salt concentrations, pH values, and temperatures (2-45 °C).

Temperature changes lead to alterations in the lipid membrane composition in order to maintain membrane fluidity, which is required for enzyme activity and transport of solutes out of the cell. The cell membrane of *L. monocytogenes* is characterized by a high proportion of iso, anteiso, and branched-chain fatty acids [34]. At low temperatures, a main change is the increase in proportion of $C_{15:0}$ at the expense of $C_{17:0}$. In addition, the degree of unsaturated fatty acids, which helps to enhance fluidity of the membrane, facilitates growth. Shortening of the fatty acid chain length decreases carbon-carbon interaction between neighbouring chains in the cell membrane resulting in an optimum membrane fluidity at low temperatures [35].

L. monocytogenes comes into contact with low pH environment in acidic foods, during gastric passage, and in the phagosome of the macrophage [36]. Therefore using a number of stress adaption mechanisms *Listeria* can survive in low pH environments [37]. One example for a stress adaption mechanism is the production of the protein GroEL. GroEL is a chaperonin, which is induced at low pH levels. This chaperonin facilitates tasks like protein folding, renaturation, and evacuation of damaged proteins under stress conditions. Resulting in the above-mentioned tolerance of *Listeria* to low pH values. Furthermore it was demonstrated that acid-adapted *L. monocytogenes* (pH 5) show increased resistance to heat shock (52°C), alcohol stress and osmotic shock for example induced by salt (25-30% NaCl) [38].

Nevertheless, *L. monocytogenes* can generally withstand high concentrations of salt [39]. Using salt to lower water activity is a common food preservation method. Bacterial

response to osmotic stress involves physiological changes and also variations in gene expression. This process is called osmoadaption [40]. Specific salt shock proteins (Ssps) and stress acclimation proteins (Saps) were found in *Listeria*. Two stress proteins called DnaK and Ctc were identified. DnaK plays a role as heat shock protein, stabilizing cellular proteins [41] whereas Ctc is involved in the resistance of *L. monocytogenes* to high osmolarity when osmoprotectants are absent [39].

1.4 Disinfection and disinfectants

Disinfection means a 99.99 % reduction of the initial viable inoculum of bacterial cells. Therefore if an inoculum of 10^6 CFU/ml is applied, 10^2 CFU/ml remain after the disinfection process. A sterilization process on the other hand would result in only 1 CFU/ml, meaning a reduction of 10^{-6} CFU/ml. Disinfection does not necessarily kill all forms of living microorganisms, since bacterial endospores can survive disinfection. Therefore, disinfection cannot provide the same margin of safety as a sterilization process. Disinfectants in food industry are antimicrobial agents for destroying pathogenic microorganisms on non-living (floors, walls, sinks, and working surfaces) objects. They are used to sanitize equipment, working benches, floors, and other utensils [42]. The difference between the various disinfectants is the mode of action and the probability that bacteria develop resistances [43]. Table 3 summarizes the most commonly applied disinfectants in food industry.

The ideal disinfectant should fulfil certain requirements such as [44]:

- Wide antimicrobial spectrum
- Rapid killing
- Harmless to human
- Soluble in water
- No harm to the environment
- Effective in presence of organic matter and compatible with soaps, detergents, and other chemicals
- Not corrode metallic surfaces and instruments
- Leave an antimicrobial film

Disinfectants are targeting several structures in the cell, which makes these compounds highly active against microorganisms, however also potentially harmful to humans [45].

Table 3: *Various types of disinfectants including their advantages and disadvantages* [46].

Disinfectant type	Advantages	Disadvantages
Alcohols	Effective against vegetative cells, non-toxic, easy-to-use, colourless, harmless on skin, soluble in water, volatile	Microbistatic, ineffective against spores
Peracetic acid	Effective in low concentration, broad microbial spectrum, kills spores, penetrates biofilms, non-toxic ($\rightarrow$ acetic acid and water)	Corrosive, unstable
Hydrogen peroxide	Decomposes to water and oxygen, relatively non-toxic, easy to use; weakens biofilms and supports detachment	High concentrations needed, corrosive
Chlorine	Effective in low concentration, broad microbial spectrum, easy to use, supports microbial detachment, cheap	Toxic by-products, resistance development, residues, corrosive, reacts with EPS, discolouration, explosive gas
Hypochlorite	Cheap, effective in a broad microbial spectrum, easy to use, supports detachment	Unstable, toxic, oxidative, corrosive, rapid regrowth, no prevention of adhesion, discolouration of products
Chlorine dioxide	Effective in low concentration, can be produced on-site, low dependency in pH	Toxic by-products, explosive gas
Quaternary ammonium agents	Effective, non-toxic, prevents regrowth, supports microbial detachment, non-irritating, non-corrosive, odourless, flavourless	Inactivated in low pH and by salts (Ca^{2+} and Mg^{2+}), resistance development, ineffective against Gram-negative bacteria
Iodophor	Non-corrosive, easy to use, non-irritating, broad activity spectrum	Expensive, flavour, odour, forms purple compounds with starch
Ozone	Similar effect as chlorine, decomposes to oxygen, no residues, decomposes biofilm	Corrosive, inactivated easily, reacts with organics ($\rightarrow$ epoxides)
Glutaraldehyde	Effective in low concentrations, cheap, non-corrosive	Low penetration in biofilms, degrades to formic acid, increased DOC

1.4.1 Disinfection in food processing plants

Disinfection in food processing plants is necessary, especially when wet surfaces favour the growth of microbes. The most crucial aim of microbial control in food processing plants, is the prevention of product spoilage. This can only be done by following various points [46]:

1. Minimization of microbial load from sources outside
2. Control of microbial growth on sensitive sites
3. Efficient cleaning and disinfection of process lines

A microbial clean surface is a surface free of microbes or pathogens [46]. Biofilms are a fundamental problem in food processing since they adhere to working surfaces. Thus if disinfection is insufficient, the product gets contaminated [47]. Prior to the selection of an appropriate detergent or disinfectant, different criteria have to be considered such as efficacy, safety and rinseability of agents but also the effect on sensory values of the product [48].

1.4.2 Quaternary ammonium compounds as widely used disinfectants in food processing plant

Quaternary ammonium compounds (QACs) are cationic biocides that are widely and extensively used as disinfectants. These biocides are not only used in food processing environments but also in domestic, industrial, and medical applications [49,50,51]. QACs generally act on the membrane permeability. Through perturbation and disorganization of the membrane, QACs cause a cytolytic leakage of the cytoplasmic material and enzyme inhibition [45,52,53,54]. High concentrations of QACs aim for carboxylic groups causing coagulation in the bacterial cytoplasm. The toxicity mode seems to be mainly based on the long lipophilic alkyl groups that are assumed to penetrate the cytoplasmic membrane [55,43]. Besides this, the cationic nitrogen disturbs the charge of the bacterial cell membrane, resulting in destabilization [43]. QACs are primarily targeting gram-positive bacteria. In addition, gram-negative bacteria, some viruses, fungi, and protozoa can be affected [56].

1.4.3 Resistance modes of *L. monocytogenes* against QACs

QACs are often applied in food industry and are known for being effective against *L. monocytogenes* [57,58,59]. Effectiveness of a disinfectant for example can be reduced through presence of organic material [60] or biofilm growth [57,61]. Insufficient application of disinfectants can result in resistance to disinfectants due to selection or adaption through

regular exposure to sublethal concentrations [62]. Common resistance mechanisms against disinfectants are an impaired uptake or active transporters for disinfectants out of the cell [63]. In gram-positive bacteria, ethidium bromide and QACs can be substrate for the same efflux pump [64,65,66]. These efflux systems are termed multidrug efflux pumps, since they provide resistance to a range of various antibiotics, dyes, and surfactants [67]. Resistance to disinfectants has also been linked to resistance to antibiotics. Genes that encode for multidrug efflux pumps were found on plasmids containing genes for resistance to penicillin, gentamicin, trimethoprim and kanamycin [68]. *L. monocytogenes* can be completely inactivated with recommended concentrations of disinfectants like benzalkonium chloride (BC). Nevertheless, factors like food debris, biofilm formation, inadequate cleaning and disinfection, or dosage failures may essentially reduce efficiency of the respective disinfectant [60,69,70]. Between 10% - 46% of *L. monocytogenes* strains isolated from food and food processing plants can be designated BC tolerant [71,72,73]. Müller et al. (2012) identified a novel transposon, termed Tn*6188*, responsible for tolerance against BC. Tn*6188* was found in ten *L. monocytogenes* strains, in the course of screening 91 food or food-related *L. monocytogenes* strains. The MIC of BC in *L. monocytogenes* strains with Tn*6188* was significantly higher than in strains without Tn*6188*. Tn*6188* is related to Tn*554* of *Staphylococcus aureus*, as well as Tn554 like transposons such as Tn*558*, Tn*559* and Tn*5406*, which can be found in *Firmicutes*. Tn*6188* consists of 5117 base pairs and is integrated chromosomally into the *radC* gene. It consists out of three transposase genes (*tnp*A, B and C), encoding for a transcriptional regulator and QacH, a small multidrug resistance protein family transporter associated with the export of BC. There is a high correspondence in terms of the amino acid sequence of QacH to Smr/QacC from *S. aureus* and to EmrE from *E. coli* [74].

1.5 Ionic Liquids

1.5.1 Introduction

Ionic liquids (ILs) are molten salts, entirely composed of cationic and anionic species with a melting point below 100 °C [75]. Some ILs are liquid at room temperature and consequently are named room temperature ionic liquids (RTILs) [76]. The combination and choice of cations and anions has a large influence on the properties of the respective ILs. In general, ILs are built out of bulky asymmetric organic cations such as imidazolium or pyridinium with low symmetry, low charge densities, and weak intramolecular interactions [77]. As a result, these cations hinder a regular packing into the crystal lattice and the solid crystalline

state becomes less favourable in terms of energetics, leading to low melting point [78]. The implementation of anions with delocalized charge enhances this effect [79]. Modifications can be made by changing the cation, the anion, or by changing substitutes of the cation or the anion. This results in an almost unlimited number of different ILs. Moreover, their significant properties distinguish them from conventional volatile organic solvents (VOCs) [80]. These VOCs are commonly used in numerous industrial applications. However, disadvantages exist regarding their application in chemical processing industry. Their toxicity not only affects process operators, but also the environment. Besides VOCs have volatile and flammable properties, which make them a potential explosion hazard [81]. Due to these facts ILs, as benign alternatives, have gathered increasing attention in the field of "green engineering" [82,83]. ILs have unique and highly solvating properties and therefore can be applied in various types of chemical processes. Besides this, their physical properties can be designed through variations in length and branching of the alkyl side chain and the anionic precursor [84]. ILs can be built task specific to certain applications. There are limitless possibilities in structural design of ILs making them to so called "designer solvents" [85,86].
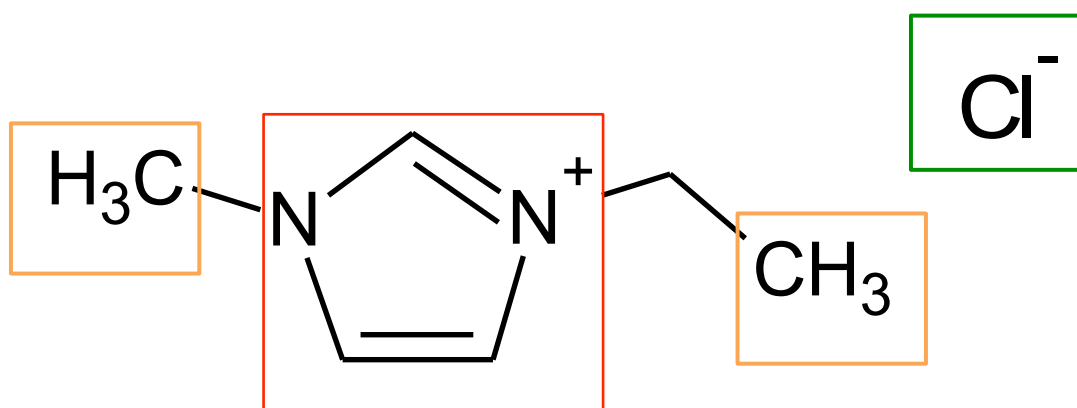
Figure 1: *Structure of an IL (1-Ethyl-3-methylimidazolium chloride). Red bordered area: cationic core, orange bordered area: variable side chains, green bordered area: variable anion.*

1.5.2 History of ILs

The first IL $[C_2H_5NH_3]^+$ $[NO_3]^-$ was described in 1914, as a salt being liquid at room temperature with a melting point of 12°C [87]. The *first generation* of ILs was developed in the 1970s and 1980s. These ILs were based on alkyl substituted imidazolium and pyridinium cations mixed with halide or tetrahalogenoaluminate anions. The first generation of ILs was primarily used as electrolytes for batteries, reaction media, and as

catalyzer for organic synthesis [88,89,90]. A major disadvantage of this first generation ILs was the moisture sensitivity and acidity/basicity. This led to the development of the *second generation* of ILs. Wilkes and Zaworotko designed ILs with 1-ethyl-3-methylimidazolium cations and neutral weakly coordinating anions like hexafluorophosphate (PF_6^-) and tetrafluoroborate (BF_4^-), enabling to extent the field of applications [88]. Nowadays, PF_6^- and BF_4^- are no longer used because of their toxicity. Therefore, less toxic anions are used like $[(CF_3SO_2)_2N]^-$ and anions with non-halogenated compounds [91]. Besides, this newly synthesized ILs possess a better electric conductivity and electrochemical stability towards oxidation and reduction [92].

The *third generation* of ILs is characterized by the expression "designer solvents". They can be classified by the selection of substitutes of respective organic compounds and their counter ion. This enables customized design of ILs towards conditions like melting point, viscosity, solvation and hydrophobicity/ hydrophilicity [93].

1.5.3 Application of ILs

Due to their unique physicochemical properties, ILs can be used in a variety of applications. Especially room temperature ILs (RTILs) have been used and investigated in several fields such as catalytic synthesis [94], coordination chemistry [95], analytical chemistry [96], nanotechnology [97], lithium batteries [98], polymer materials [99], or fluorine chemistry [79]. ILs have also been successfully used as solvents for biopolymers. Cellulose for example can be dissolved up to high concentrations (~ 25% w/v) and recovered again, which is not feasible using conventional organic solvents [100]. The first successful large-scale industrial application utilizing ILs was the so-called BASIL[TM] (Biphasic Acid Scavenging utilizing Ionic Liquids) process, which was introduced by BASF AG in 2002. This process is used to produce alkoxyphenylphosphines. The original process utilized triethylamine to scavenge the acid produced in the process. The waste product of this reaction triethylammonium chloride has a high viscosity and made handling of the reaction mixture difficult. To avoid this problem, today 1-methylimidazole is used as scavenger substance resulting in the formation of 1-methylimidazolium chloride, which is separated from the reaction mixture as a discrete phase [77].

1.5.4 Characteristics of ILs

ILs are salts composed of discrete cations and anions. Basically ILs can be classified into two classes: room temperature ILs and those which are liquid at temperatures below 100°C [101,102,103]. ILs have a lot of favourable properties in comparison to conventional organic solvents [102]:

- Extremely low vapour pressure
- Wide liquid range
- Low flammability
- High ionic conductivity
- High thermal conductivity
- Good dissolution power towards several substrates
- High thermal and chemical stability
- Wide electrochemical window
- Melting point
- Polarity
- Viscosity
- Toxicity and biodegradability

The *melting point* of ILs is below 100°C. Hereby the chemical structure of ILs has a significant impact on the physical properties. Both, anions and cations, influence the melting point. Large bulky asymmetric organic cations with weakly-coordinating anions disrupt efficient packing of ions into the crystal lattice and therefore lower the melting point [104,105,106]. Therefore the liquid state of matter is thermodynamically preferred, since large size and conformational flexibility of the ions lead to small lattice enthalpies and large entropy changes, which favour the liquid state [78].

Viscosity on the other side is important concerning stirring, mixing, and pumping operations of ILs. Viscosity also influences transport properties like diffusion [85]. Although viscosity is influenced by both, cations and anions [107], especially the alkyl chain length of the cation is essential. Longer alkyl chain lengths increase the viscosity due to an increase in van der Waals forces between the cations, which increases the energy needed for molecular motion [108].

Since ILs are only composed of ions, they exert no measureable *vapour pressure*. To a large extent the Coulomb forces determine volatility. A strong cation-anion interaction results in extremely low vapour pressure and high enthalpies for vaporization. According to

this fact, air pollution is really low and furthermore other volatile components can be isolated from ILs by distillation [109].

Conductivity of a solution depends on the number of charge carriers and their mobility. Large ions in ILs reduce the mobility and therefore also reduce the conductivity [110]. Although the conductivity is barely higher in conventional non-aqueous solvents, ILs have the advantage that this property is intrinsic to pure ILs and addition of separate salt is not necessary [111].

1.5.5 Toxicity of ILs

1.5.5.1 Toxicological effects and biodegradability of ILs

ILs are often seen in a ‚green' perspective, due to the negligible vapour pressure. However, it should be considered that this characteristic alone does not make an IL ‚green'. Ideal green solvents must be intoxic and must not persist in the environment. Although ILs can minimize the risk of air pollution, some of them do have a significant solubility in water [112,113]. Therefore water is the most likely media in which ILs are released into the environment. Investigation of aqueous toxicology is crucial concerning environmental safety [114]. Additionally, some IL-properties, such as a high thermal stability and chemical stability can also cause problems due to a very limited degradation of the ILs leading to persistence in the environment [115]. Biological properties of ILs, particularly their toxicity, have been among the least investigated fields in IL-research [116,117]. The most commonly used 1-alkyl-3-methylimidazolium anions were studied in variety of systems assessing their potential environmental impact [118]. Toxicological effects of these ILs have been investigated using organisms like aquatic bacterium *Vibrio fischeri* [119], *Daphnia magna* (crustacean) [120], *Physa acuta* (freshwater snail) [121], *Caenorhabditis elegans* (nematode) [118], *Danio rerio* (zebra fish) [122], and the freshwater green algae *Pseudokirchneriella subcapitata* [123]. The so-called side chain effect was found in all used test systems from the molecular up to the organism level. Such a consistent response of the various test systems could not be confirmed for the tested anion moieties. In the majority of the investigated test systems the effect of the different anions was not as distinct as the demonstrated side chain length effect of the imidazolium cation [124]. The hazard potential of ILs can be tested in form of the flexible (eco)toxicological test battery. This system takes not only aquatic but also terrestrial compartments as well as different trophic levels into consideration [124]. Toxicity itself could be utilized in various beneficial applications, since it is a tuneable property leading to design of new and improved antiseptics, disinfectants, and antifouling reagents.

22
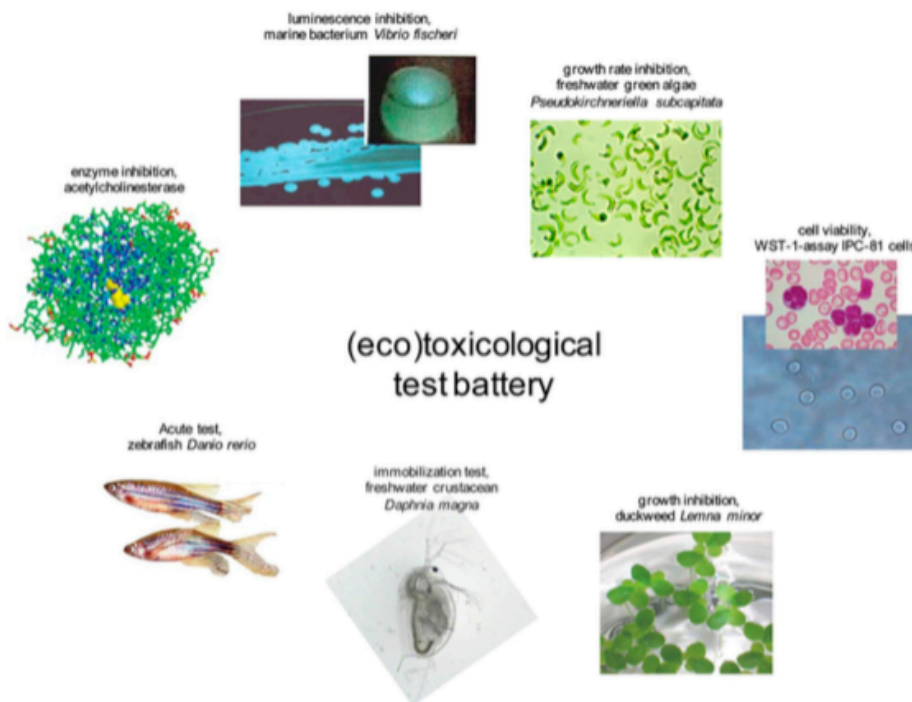
Figure 2: *Ecotoxicological test battery according to Pham et al. 2010* [124] *(adapted from Matzke et al. 2007, by permission of Royal Society of Chemistry).*

In the course of this thesis the main focus was put on the toxicity of ILs towards bacterial pathogens. The following chapter will give an overview in antibacterial effects of ILs, which have already been tested in previous studies.

1.5.5.2 Antimicrobial effects of ILs

Docherty et al. (2005) performed toxicity measurements of imidazolium and pyridinium ILs with varying alkyl chain length using *Vibrio fischeri*. It was found that an increase in alkyl group chain length as well as an increase in alkyl groups substituted on the cation ring lead to an increase in toxicity. Furthermore they examined the antimicrobial activity of butyl-, hexyl, -octyl imidazolium and pyridinium bromide on *Escherichia coli*, *Staphyloocus aureus*, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*. In this studies butyl -imidazolium and pyridinium were the least toxic ILs, whereas the ILs with longer alkyl chains (hexyl and octyl) exerted a higher toxicity towards the tested microorganisms [119]. Another study investigated 40 different ILs for their interaction between lipophilicity and (eco)toxicity. Toxicity of ILs with functionalized side chains was clearly lower compared to non-polar alkyl side chains. The influence of hydrophobic IL cations and the influence on two different lipid biological bilayers was also examined. The

study indicated that the membrane is most likely the primary target of toxicity [125]. The toxic effect of longer alkyl side chains could be explained by the lipophilicity. ILs having longer alkyl side chains are more lipophilic than ones with shorter alkyl chains. Therefore there is a higher affinity to incorporate into phospholipid bilayers, resulting in an increased toxicity due to increased membrane permeability [106,126].

Mester et al. (2015) studied the influence of different anions in imidazolium and pyrrolidinium based ILs with varying side chains, regarding their chaotropicity. They could demonstrate that in addition to the common "side-chain effect", the chaotropicity of the anion essentially influences the antimicrobial activity. Therefore ILs with an identical cationic core have an increased toxicity with regard to their strong chaotropic anions. Mester et al. concluded that the anion chaotropicity has two different toxicity mechanisms. On one hand chaotropic anions enhance the surfactant like properties of the cation, disturbing cell membranes more efficiently and on the other hand the chaotropicity represents a cation-independent antimicrobial mechanism [127].

The previously described antibacterial effects, including the side chain effect and the chaotropicity of the anion are so far the only investigated toxicity mechanisms concerning ILs. Since these are the only described and investigated toxicity mechanisms known for ILs further research in this field necessary.

Aim of this study

Since foodborne pathogens like *L. monocytogenes* are known for having defence mechanisms against QACs and since sublethal exposure to various antimicrobial substances also promotes a higher tolerance of *L. monocytogenes* cells against QACs, there is a strong desire for novel disinfectants circumventing this problem.

As presented above, ILs are a highly variable class of chemicals, which can be customized to their application. Considering the fact that certain cations of ILs do have the specific structural elements of QACs, makes them a possible alternative to this class of disinfectants.

The central question, which was addressed in the course of this study is: Can ILs with QAC- like cations be a beneficial alternative to commonly used QACs?

Therefore a set of different food associated *L. monocytogenes* strains were tested. These *L. monocytogenes* strains have been shown before to contain a specific efflux pump against QACs, which is encoded on transposon Tn*6188*. The aim was also to test these strains with respect to adaption, by exposion to sub lethal concentrations of several disinfectants.

In order to answer the central question, certain aspects were considered:

- Synthesis of new ILs incorporating quaternary ammonium structures
- Investigation of molecularly mediated tolerance of *L. monocytogenes* cells against ILs with QAC like structure
- Adaption of *L. monocytogenes* cells to ILs after sublethal exposure
- Investigation of ILs that can probably circumvent the mechanisms of the efflux pump
- Investigation of the cell membrane damage induced by the presence of ILs

Abbreviation list

L. monocytogenes	*Listeria monocytogenes*
E. coli	*Escherichia coli*
S. Typhimurium	*Salmonella enterica subsp. Enterica typhimurium serovar Typhimurium*
g	Gramm
ml	Millilitre
μl	Microlitre
CFU	Colony Forming Units
min	Minutes
sec	Seconds
rpm	Rounds per minute
v/v	Volume/Volume
w/v	Weight/ Volume
OD	Optical Density
pH	Negative log of the activity of the hydrogen ion
ddH$_2$O	Double distilled water, Milli-q water
PBS	Phosphate Puffered Saline
SDS	Sodium Dodecyl Sulfate
qPCR	Quantitative Real Time PCR
PCR	Polymerase Chain Reaction
U	Units, enzyme activity
TSA+ Y	Tryptone Soy Agar+ Yeast extract
TSB+ Y	Tryptone Soy Bouillon+ Yeast extract
BC	Benzalkonium chloride
DB	Domiphen bromide
DTAB	Dodecyltrimethylammonium bromide
prfA	Expression regulator gene of *L. monocytogenes*
DMSO	Dimethyl Sulfoxide
QACs	Quaternary Ammonium Compounds
ILs	Ionic liquids

2. Materials and Methods

2.1 Materials

The following table (Table 4) summarizes all materials and laboratory equipment used for this study.

Table 4: *Materials and laboratory equipment used in this work, listed up by category and associated manufacturer.*

Material/ Laboratory Equipment	Manufacturer
Incubator	
G24 Environmental Incubator Shaker	New Brunswick Scientific (Edison, N.J, USA)
Type BK 4266 L132	Ehret (Emmendingen, D)
Centrifuges	
Centrifuge 5424	Eppendorf (Hamburg, D)
Centrifuge 5810R	Eppendorf (Hamburg, D)
Photometer	
8452A Diode Array Spectrophotometer	Hewlett Packard (Palo Alto, CA, USA)
Fluorometer	
Qubit	Life Technologies (Waltham, MA, USA)
Thermomixer	
Thermomixer compact	Eppendorf (Hamburg, D)
Vortexer	
Vortex 3 Genius	IKA (Staufen, D)
Reax Top	Heidolph (Schwabach, D)
Microplate reader	
Infinite F2000	Tecan (Männedorf, CH)
PCR cycler	
Mx3000P	Stratagene (Santa Clara, CA, USA)
Scales	
Talent TE 214S	Sartorius (Madrid, ES)
Microscope	
DMIRB	Leica (Vienna, AUT)
Leitz Laborlux 9	Leica (Vienna, AUT)
Consumables	
Petri dishes	Greiner Bio- One (Frickenhausen, D)
50 ml Falcon tubes	Greiner Bio- One (Frickenhausen, D)
Pipette tips	Biozyme (Oldendorf, D)
Eppendorf tubes	Eppendorf (Hamburg, D)
Corning 96 well microtiter plates	Sigma (München, D)
Vacuum concentrator	
SpeedVac	Thermoscientific (Waltham, MA, USA)
Conductometer	
Mettler Toledo™ S230 SevenCompact™ Conductivity Meter	Fisher Scientific (Vienna, AUT)

2.1.1 Chemicals

Following chemicals, solvents and antibiotics were applied in the experiments:

Chemical	**Manufacturer**
QACs	
Benzalkonium chloride	Sigma (München, D)
Benzethonium chloride	Sigma (München, D)
Domiphen bromide	Sigma (München, D)
Dodecyltrimethylammonium bromide (DTAB)	Sigma (München, D)
Solvents	
Ethanol	Sigma (München, D)
Methanol	Roth (Karlsruhe, D)
DMSO	Sigma (München, D)
Antibiotics	
Ampicillin	Sigma (München, D)
Gentamicin	Sigma (München, D)
Ciprofloxacin	Sigma (München, D)
Naldixic acid	Sigma (München, D)

2.1.2 ILs used in this study

In the course of this study a broad set of ILs was used. Some of them were purchased, others were synthesized during the study.

Following nomenclature was applied for the ILs:

mim= 3- methylimidazolium

$$[\text{C}_n\text{mim}]\text{X}^-$$

n= number of carbon atoms in the alkyl side chain

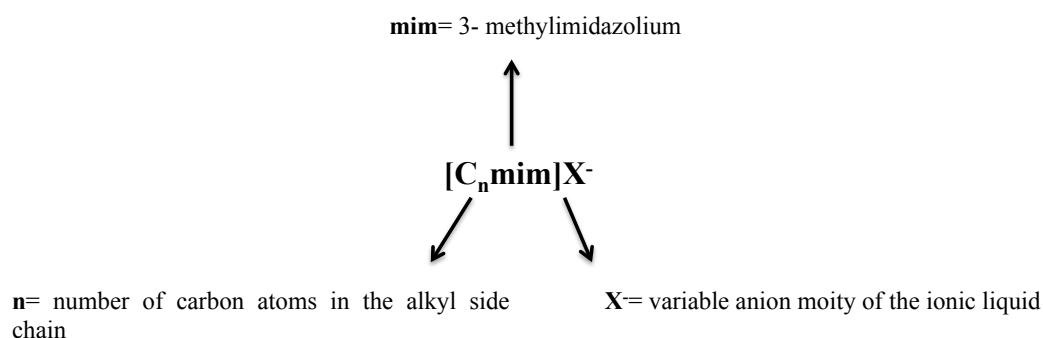**X**= variable anion moity of the ionic liquid

Figure 3: *Nomenclature of an imidazolium IL.*

Trimethylalkylammonium based ILs → **[TMC$_n$A]X⁻**, e.g: [TMC$_4$A]Cl

Triocytlmethylammonium based ILs → **[TOMA]X⁻**, e.g: [TOMA]FeCl$_4$

Commercially available ILs:

Imidazolium based ILs	Manufacturer
1-Ethyl-3-methylimidazolium chloride **[C$_2$mim]Cl**	Merck (Darmstadt, D)
1-Ethyl-3-methylimidazolium thiocyanate **[C$_2$mim]SCN**	Merck (Darmstadt, D)
1-Butyl-3-methylimidazolium chloride **[C$_4$mim]C**l	Merck (Darmstadt, D)
1-Butyl-3-methylimidazolium thiocyanate **[C$_4$mim]SCN**	Merck (Darmstadt, D)
1-Butyl-3-methylimidazolium TCA **[C$_4$mim]TCA**	Merck (Darmstadt, D)
1-Butyl-3-methylimidazolium methyl sulfate **[C$_4$mim]MeSO$_4$**	Merck (Darmstadt, D)
1-Hexyl-3-methylimidazolium chloride **[C$_6$mim]Cl**	Merck (Darmstadt, D)
1-Hexyl-3-methylimidazolium thiocyanate **[C$_6$mim]SCN**	Merck (Darmstadt, D)
1-Decyl-3-methylimidazolium chloride **[C$_{10}$mim]Cl**	Proionic (Grambach, AUT)
1-Octyl-3-methylimidazolium chloride **[C$_8$mim]Cl**	Proionic (Grambach, AUT)

Ammonium based ILs	Manufacturer
Trimethyl- butylammonium chloride **[TMC$_4$A]Cl**	Proionic (Grambach, AUT)
Trimethyl- octylammonium maleate **[TMC$_8$A]maleate**	Proionic (Grambach, AUT)
Triocytl- methylammonium ferric chloride **[TOMA]FeCl$_4$**	Proionic (Grambach, AUT)
Triocytl- methylammonium glycine **[TOMA]glycine**	Proionic (Grambach, AUT)
Triocytl- methylammonium methylcarbonate **[TOMA]MC**	Proionic (Grambach, AUT)
Triocytl- methylammonium adenosintriphosphate **[TOMA]ATP**	Proionic (Grambach, AUT)
Triocytl- methylammonium tartaric acid **[TOMA]tartaric acid**	Proionic(Grambach, AUT)
N,N- dimethyl-2 hydroxyethyl- ammonium acetate **[N,N- dimethyl-2 hydroxyethyl- ammonium]acetate**	Merck (Darmstadt, D)
N,N- dimethyl-2 hydroxyethyl- ammonium]propionate **[N,N- dimethyl-2 hydroxyethyl- ammonium]propionate**	Merck (Darmstadt, D)
[N,N- dimethyl-2 hydroxyethyl- ammonium]octanoate **[N,N- dimethyl-2 hydroxyethyl- ammonium]octanoate**	Merck (Darmstadt, D)

Synthesized ILs:

The following ILs were synthesized at our laboratory in corporation with Proionic (Grambach, Austria) applying the CBILS route with cationic precursors supplied from Proionic. The synthesis path is explained in chapter 2.2.3.1.

Trimethyl- butylammonium maleate
[TMC$_4$A]maleate
Trimethyl- octylammonium chloride
[TMC$_8$A]Cl
Trimethyl- dodecylammonium chloride
[TMC$_{12}$A]Cl
Trimethyl- dodecylammonium periodate
[TMC$_{12}$A]periodate
Trimethyl- dodecylammonium maleate
[TMC$_{12}$A]maleate
Trimethyl- hexadecylammonium chloride
[TMC$_{16}$A]Cl
Trimethyl- hexadecylammonium periodate
[TMC$_{16}$A]periodate
Trimethyl- hexadecylammonium maleate
[TMC$_{16}$A]maleate
Triocytl- methylammonium chloride
[TOMA]Cl
Triocytl- methylammonium lactate
[TOMA]lactate
Triocytl- methylammonium benzoate
[TOMA]benzoate
Triocytl- methylammonium phthalate
[TOMA]phthalate
Triocytl- methylammonium palmitate
[TOMA]palmitate
Triocytl- methylammonium stearate
[TOMA]stearate
Triocytl- methylammonium propyl
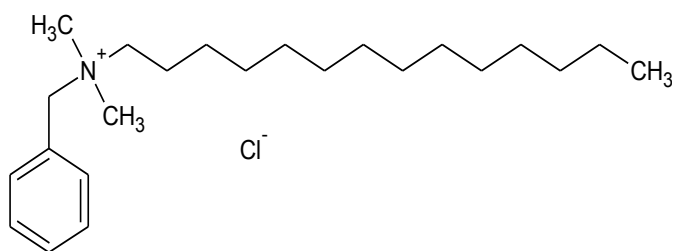**[TOMA]propyl**
Triocytl- methylammonium oxalic acid
[TOMA]oxalic acid
Triocytl- methylammonium periodic acid
[TOMA]periodate

2.1.3 Chemical structure of antimicrobial substances used in this study

Following chemical structures are exemplary for the antimicrobials used in this study. R stands for variable side chain, X^- for variable anion.

Example of QAC respesentative:
Benzalkonium chloride
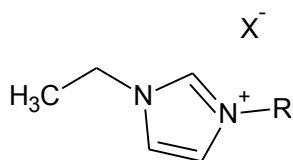
Basic structure of imidazolium based ILs: $\mathbf{[C_R mim]X^-}$

Basic structure of trimethylalkyl based ILs: $\mathbf{[TMC_R A]X^-}$

Basic structure of TOMA based ILs: $\mathbf{[TOMA]X^-}$

2.1.4 Media

The following unselective media were used during the entire study (Table 5, Table 6).
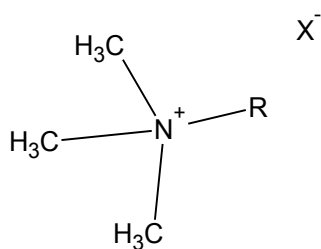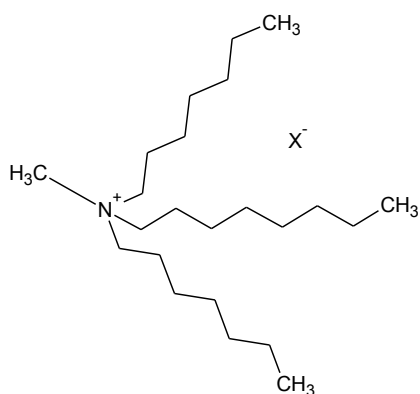
Table 5: *Composition of TSA+Y agar plates.*

Tryptone Soy Agar+ Yeast (TSA+Y, Oxoid)	
Casein peptone (pancreatic)	15.00 g
Soy peptone (pancreatic)	5.00 g
Sodium chloride	5.00 g
Yeast extract	6.00 g
Agar	15.00 g

Table 6: *Composition of TSB+Y liquid media.*

Tryptone Soy Bouillon+ Yeast (TSB+Y, Oxoid)	
Casein peptone (pancreatic)	17.00 g
Soy peptone (papain digest)	3.00 g
Sodium chloride	5.00 g
Dipotassium hydrogen phosphate	2.50 g
Glucose	2.50 g
Yeast extract	6.00 g

The components were dissolved in 1 l of ddH$_2$O and the pH was adjusted to pH 7. Afterwards the media were sterilized at 121 °C for 15 min. The TSA+Y media was filled into petri dishes to 13.5 ml volume each and the TSB+Y media was filled into 9 ml tubes.

2.2 Methods

2.2.1 Microbial methods

2.2.1.1 Bacterial strains

In all conducted experiments three different bacterial species were used: *L. monocytogenes* EGD-e, *S.* Typhimurium and *E. coli*. *L. monocytogenes* EGD-e served as a representative example of gram- positive bacteria, whereas *S.* Typhimurium and *E. coli* served as gram-negative representatives. In addition, ten different *L. monocytogenes* strains were used based on a PCR targeting for the *qacH* gene conducted by Müller et al. (2012) [74]. These ten strains were divided into two groups regarding the presence of a specific transposon called Tn*6188*.

- *L. monocytogenes* strains harbouring transposon Tn*6188* (+Tn*6188*)

- *L. monocytogenes* CDL 78, serotype 3a
- *L. monocytogenes* 6179, serotype 1/2a
- *L. monocytogenes* N22-2, serotype 1/2a
- *L. monocytogenes* 4423, serotype 1/2a
- *L. monocytogenes* F17, serotype 1/2a

- *L. monocytogenes* strains without transposon Tn*6188* (-Tn*6188*)

- *L. monocytogenes* CDL 65, serotype 1/2a
- *L. monocytogenes* R479a, serotype 1/2a
- *L. monocytogenes* CDL 77, serotype 3a
- *L. monocytogenes* CDL 2, serotype 1/2a
- *L. monocytogenes* 535, serotype 4b

- *E. coli* K-12

- *Salmonella enterica subsp. Enterica typhimurium serovar* Typhimurium *(NCTC12023)*

All strains used in this work were obtained from the Institute of Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna. Bacterial strains were stored at -80°C using MicroBank^{TM} technology (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada).

2.2.1.2 Bacterial culture conditions

Bacterial cultures were established in two ways: either by taking up a colony from an agar plate or by taking up 1 ml of a well-grown overnight culture prepared in advance. Following this, the inoculum was transferred into a sterile tube containing 9 ml of TSB+Y media and agitated at 37 °C overnight. Then a fresh, sterile TSB+Y tube was inoculated with 1 ml of this overnight culture and incubated at 37 °C for approximately 3 h. Afterwards optical density of the culture was measured at 600 nm (OD_{600}) and adjusted to 0.6, depending on the subsequent experiment.

2.2.1.3 Adaption of *L. monocytogenes*

The two *L. monocytogenes* strains CDL2 (-Tn*6188*) and 6179 (+Tn6188) were adapted to sublethal concentrations of benzalkonium chloride (BC), [C$_{10}$mim]Cl, and [TMC$_{16}$A]maleate. Of the respective parental *L. monocytogenes* strains 1 ml of an overnight culture was transferred into sterile 9 ml TSB+Y tubes containing a dilution series of either BC, [C$_{10}$mim]Cl, or [TMC$_{16}$A]maleate and incubated for 24 h at 37 °C. Cultures showing growth at the highest concentration of antimicrobial substance were transferred in a 1:5 ratio to fresh TSB+Y medium including higher concentrations of antimicrobial substance to a final volume of 10 ml [128]. The adaption process was performed four consecutive times for every strain. Final adapted strains were stored at -80°C using MicroBank^{TM} technology.
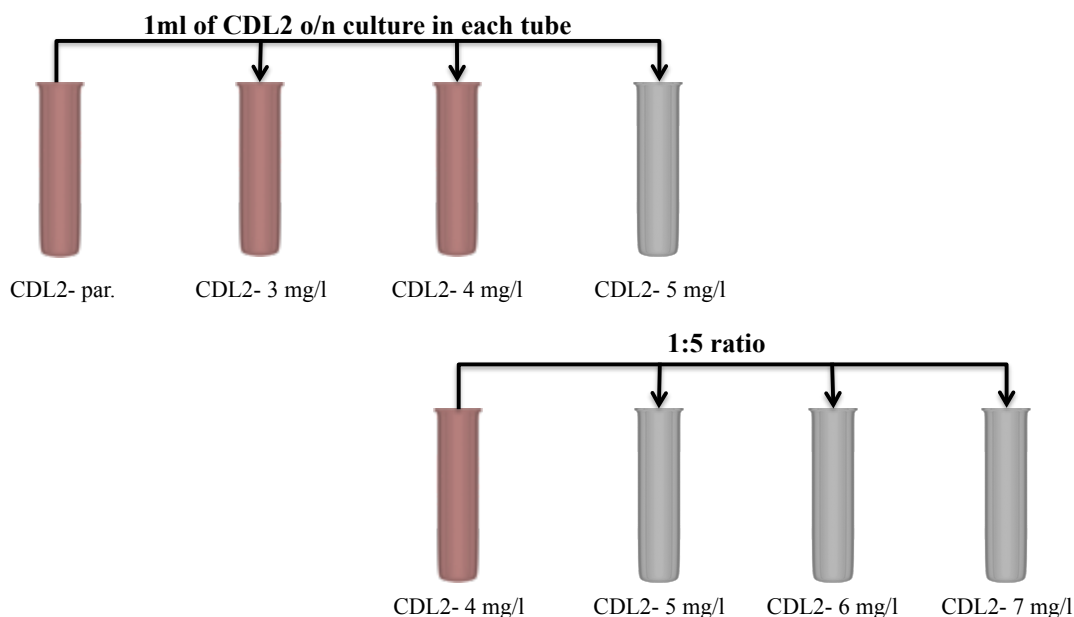
Figure 4: *Adaption process of L. monocytogenes. Parental strain of L. monocytogenes CDL 2 (CDL 2-par.) transferred to tubes with serial diluted antimicrobial substance in TSB+Y media. Red coloured tubes showing growth of bacteria, grey coloured tubes showing no growth.*

2.2.1.4 Minimal inhibitory concentration (MIC) assessment

The MIC is defined as the lowest concentration of an antimicrobial substance, which inhibits visible bacterial growth. MIC concentrations were determined by the serial dilution microtiter plate method in TSB+Y medium [129]. Starting at a definite concentration (mg/l or w/v %) of an antimicrobial substance (QACs, ILs, or antibiotics), a serial dilution of the respective substance in TSB+Y was preformed in 96 well microtiter plates. A volume of 150 µl of the respective substance plus 50 µl of bacterial inoculum was given in every single well to obtain a total volume of 200 µl. In order to ensure a constant cell status at the beginning of each experiment, 1 ml of a well-grown overnight culture was transferred into a fresh sterile 9 ml TSB+Y medium (ratio 1:10) and incubated for 3 h at 37 °C. This process provided cells in the logarithmic growth-phase. After the incubation, every well containing the serial diluted antimicrobial substance, was inoculated with $5*10^5$ of the respective bacterial strain (*L. monocytogenes*, *E. coli*, *S.* Typhimurium). Following this, the 96 well microtiter plates were measured at 610 nm wavelength in a TECAN Infinte2000 microplate reader (Tecan Austria, Groedig, Austria) to see if there is a possible interference of the given antimicrobial substance at this wavelength. After incubation of the plates for 24 h at 37 °C, microbial growth was determined by a second measurement at 610 nm. To ensure

the reproducibility and accuracy of the results, every experiment was done independently for 3 times on different days.
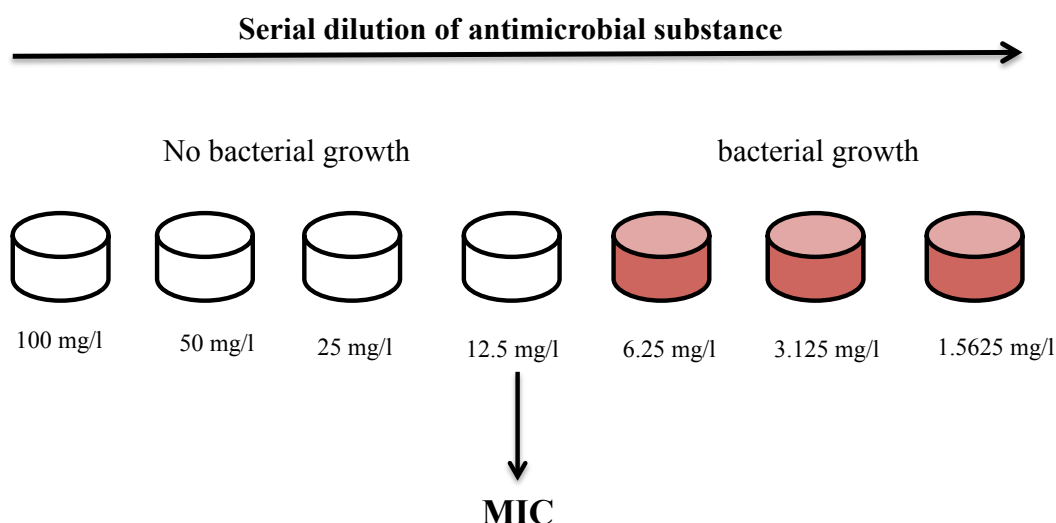
Serial dilution of antimicrobial substance

No bacterial growth   bacterial growth

| 100 mg/l | 50 mg/l | 25 mg/l | 12.5 mg/l | 6.25 mg/l | 3.125 mg/l | 1.5625 mg/l |

MIC

Figure 5: *Depiction of serial dilution microtiter plate method. White wells showing no bacterial growth, red coloured wells showing bacterial growth.*

2.2.1.5 Minimal bactericidal concentration (MBC) assessment

The MBC is defined as the lowest concentration of an antimicrobial substance that reduces the viability of the initial bacterial inoculum by 99.9%. As an antimicrobial substance can either have a bactericidal or a bacteriostatic effect, the assessment of the MIC is not able to give information about the exact state in which the bacteria are. Therefore the MBC is used to clarify this circumstance. After 24h incubation of the microtiter plates (see 2.2.1.4) and consecutive measurement the MBC to the corresponding substances was determined. For determination of the MBC four different wells of the microtiter plate were taken into consideration, the well with the MIC concentration and three wells above the MIC concentration. Out of these respective wells 50 µl were taken and mixed with 450 µl of 1x PBS. This step was performed four consecutive times in order to create a decimal serial dilution. Each of these prepared solutions was used for the plate count method later on. 50 µl of every sample were plated out on TSA+Y in duplicate (2x 50 µl). Following this the TSA+Y plates were incubated overnight at 37 °C and subsequently enumerated by counting the colonies on the plate. Since the plate out method is rather time consuming, another more resource conserving technique was applied, the so-called drop plate out method. The method has economic advantages concerning material and labour [130]. For this method no serial decimal dilution was performed, instead 5 µl of each well were directly transferred

onto a TSA+Y plate and incubated overnight at 37 °C. The method was only applied to detect if there is bacterial growth in the wells containing the MIC or not. However this is a method which has not been standardized so far, although used in laboratory for many years [131].
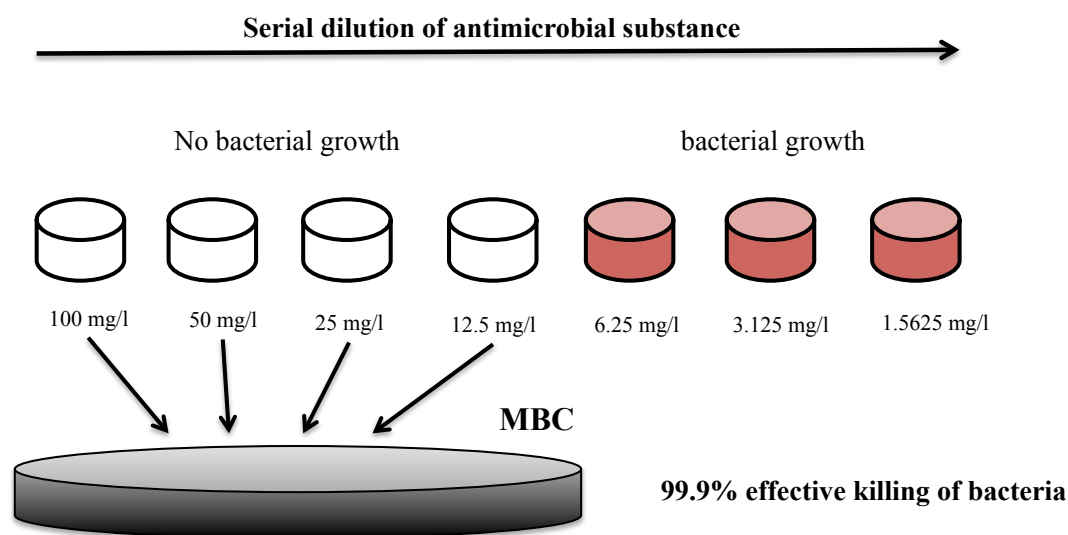
Serial dilution of antimicrobial substance

No bacterial growth   bacterial growth

100 mg/l   50 mg/l   25 mg/l   12.5 mg/l   6.25 mg/l   3.125 mg/l   1.5625 mg/l

MBC

99.9% effective killing of bacteria

Figure 6: *Depiction of MBC determination by plating out method. White wells including MIC are plated out.*

2.2.1.6 BacLight

The LIVE/DEAD© BacLight^{TM} Bacterial Viability Kit (Invitrogen, Carlsbad, USA) is a method that enables distinction between alive and dead cells under the microscope. The principle underlying the LIVE/DEAD© BacLight^{TM} Kit is, that bacteria can be stained specifically based on their membrane integrity. Two different dyes are applied, which differ with respect of their membrane permeability and fluorescence: SYTO 9 stain- a green fluorescent dye and propidium iodide- a red fluorescent dye. SYTO 9 is used for assessment of total cell counts, since it is able to enter all cells. Propidium iodide in contrast can only enter cells with damaged cytoplasmic membrane. As a result, the viable cells with an intact cell membrane are stained with SYTO9 and display green colour under the microscope. Damaged cells with a disrupted cell membrane appear red since being stained by propidium iodide. Although the kit is used by a broad scientific community, this method does not always display distinct results. It is quite probable that bacteria with a damaged cell membrane are dead. However, it does not necessarily imply that cells having their full membrane integrity are alive or can still grow [132]. For all experiments, 500 µl of the processed bacterial culture were stained with 1µl of each dye (propidium iodide and

SYTO 9). After mixing the probe by vortexing for 5-10 sec, it was incubated for 15 min in complete darkness. After incubation, the sample was filtered and the filter was placed on an object slide. Analysis of the stained cells was performed at an inverse microscope (Leitz Laborlux 9, Leica) with a 1000 times magnification. The number of bacteria per filter in 15 optical fields was counted and the bacteria/ml were calculated as follows:

Bacteria per ml= average of counted bacteria*2*5230*$(1/10^{-n})$

n= dilution factor

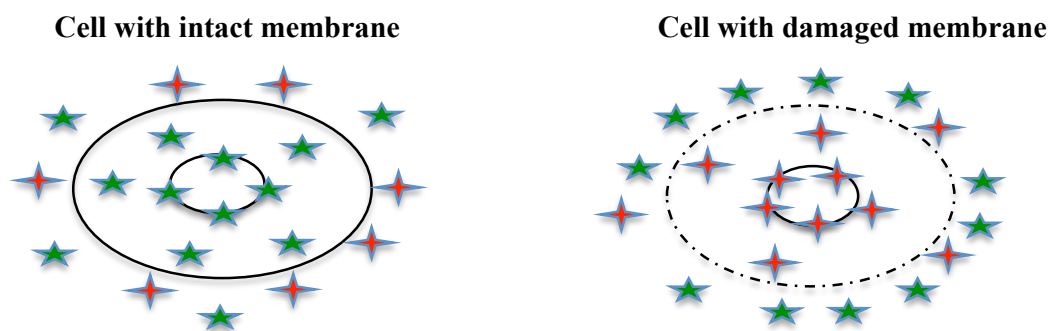**Cell with intact membrane**   **Cell with damaged membrane**

Figure 7: *Schematic depiction of BacLight stains showing alive cell (intact membrane) and dead cell (damaged membrane).*

2.2.1.7 Plate count/ viable count

The plate count method is a microbial method for estimation of cell numbers. It is based on the fact that a viable cell will form a colony on an agar plate. The number of cells is then depicted as CFU (colony forming units) per ml. Of any given sample decimal dilution series were prepared, and the respective dilutions were spread out in duplicate on TSA plates. Afterwards, the plates were incubated for 24-48 h at 37 °C. For calculation of CFU/ml the average of the counted colonies was multiplied with the appropriate dilution factors as shown in the following example.

Table 7: *Calculation of CFU/ml using weight function.*

dilution factor	CFU 1	CFU 2
10^3	400	420
10^4	40	42

Formula: **CFU/ml**= $(400+420+40+42)/2.2*50*10^3 = 4.55*10^6$

2.2.2 Molecular biological methods

2.2.2.1 Real-Time PCR (qPCR)

Real-Time PCR was first introduced by Higuchi et al., and has been extensively used since 1992 [133]. This technique allows precise quantification of specific nucleic acids in a complex mixture, even if low concentrations are present. A respective target sequence is monitored in real-time using fluorescent dyes [134]. The amount of DNA is determined during exponential amplification phase. Fluorescence changes are either measured through fluorescent labelled binding probes or a DNA binding dye. Starting from a dilution series with known target DNA concentration as standard, the amount of unknown sample can be calculated. The principle is that the fluorescent signal increases proportionally to the number of target DNA molecules. However, due to the background fluorescence the exponential phase cannot be observed before a certain number of DNA molecules is reached. The C_T-value is the cycle in which fluorescence exceeds the threshold, which is above the background fluorescence. By comparison of C_T-values from the samples to a standard of defined DNA amounts, the exact number of copies can be calculated by means of a calibration line [135].

In this study the so-called TaqMan system was applied. This system uses the DNA polymerase of *Thermus aquaticus*, which possesses 5' to 3' exonuclease activity. Part of this system is an oligonucleotide probe, complementary to the DNA sequence in between the two primers. This oligonucleotide probe is labelled with a fluorophore and a quencher, which inhibits that fluorescence when the two are in vicinity to each other. At the moment the TaqMan probe binds to a specific sequence on the DNA, the fluorophore is released from the quencher due to the 5'-3' exonuclease activity of the DNA polymerase. It is a really specific method, since the fluorescent signal is only released when the probe is bound to the DNA and degraded.

In course of this work three different qPCR assays were applied, according to the respective bacterial species (*L. monocytogenes, E. coli, S.* Typhimurium). Each mastermix was prepared as multiple of a single reactions amount and later distributed equally to PCR reaction tubes, each containing 20 µl afterwards. At the end 5 µl of respective template DNA were added. All qPCRs were performed in a Mx3000p thermocycler (Stratagene, La Jolla, CA, USA).

The following table (Table 8) summarizes all primer and probe sequences of the different qPCRs applied in the course of this work. Table 9 (see next page) should give an example of how a mastermix batch for a single reaction tube was constituted, in this case for *L. monocytogenes*.

Table 8: *Primer and probe sequences for all qPCRs applied in this study.*

Name	bp	Sequence 5'- 3'
E.coli **qPCR**		
ert2F	27	ACT GGA ATA CTT CGG ATT CAG ATA CGT
ert2R	25	ATC CCT ACA GAT TCA TTC CAC GAA A
ert2 probe	28	FAM-CAG CAG CTG GGT TGG CAT CAG TTA TTC G-BHQ1
L. monocytogenes **qPCR**		
LIP1	21	GAT ACA GAA ACA TCG GTT GGC
LIP2	25	GTG TAA TCT TGA TGC CAT CAG G
LIP probe 2	21	FAM-CAG GAT TAA AAG TTG ACC GCA-MCB
S. **Typhimurium qPCR**		
fimAF1	20	CCT TTC TCC ATC GTC CTG AA
fimAR1	17	TGG TGT TAT CTG CCT GA
fimAS1	19	FAM- TGC GAT CCG AAA GTG GCG G- BHQ1

Table 9: *Mastermix batch of a single reaction tube for L. monocytogenes qPCR.*

	Stock concentration	End concentration	µl/reaction
Aqua dest.			8.2
PCR buffer	10x	1x	2.5
MgCl$_2$	50 mM	3.5 mM	1.75
Primer rev.	5 µM	500 nM	2.5
Primer fwd.	5 µM	500 nM	2.5
Probe	5 µM	250 nM	1.25
dNTP's	20 µM	200 µM	1
Taq- pol.	5 U	1.5 U	0.3
Volume of Mastermix			20
Template DNA			5
Final volume			**25**

2.2.2.2 DNA isolation

2.2.2.2.1 Nucleospin

Genomic DNA extraction was performed using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, D). In order to collect the cells, a centrifugation step was performed at 5000 g for 5 min. After removing the supernatant, the remaining pellet was resuspended in 180 µl of pre-lysis buffer with 10 mg/ml lysozyme (Sigma, München, D) and incubated for 60 min at 37 °C. Especially for gram-positive bacteria this step, including the treatment with the lytic enzyme, is important for complete lysis of the cell. Following this incubation step, 25µl of ProteinaseK solution were added to the sample and incubated at 56 °C overnight. Afterwards, 200 µl of B3 buffer were added to the sample and incubated at 70 °C for 10 min. After centrifugation at 11000 g for 5 min the sample was mixed with 210 µl of 96 % EtOH, mixed thoroughly for 5-10 sec and completely loaded on the NuceloSpin® Tissue column. The column was then centrifuged at 11000 g for 2 min and the remaining flow through was discarded. The first washing step of the membrane was performed with 500 µl of BW solution. The second washing step was done with 650 µl of B5 buffer. Centrifugation for both washing steps was performed at 11000 g for 2 min. To ensure that the B5 buffer is fully removed from the membrane, another centrifugation step for 1 min at 11000 g was done. The column was now transferred into a new sterile 1.5 ml eppendorf tube and 50 µl of pre-warmed (70 °C) ddH$_2$O were added to the column. After an

incubation step for 1 min, another centrifugation at 11000 g was done. This procedure was repeated a second time. The eluate was either directly used or stored at -20°C for later use.

Table 10: *Composition of pre-lysis buffer (pH 8.0) for NucleoSpin® Tissue Kit.*

Tris HCl	20 mM
EDTA	2 mM
Triton X-100	1 % (v/v)
Lysozym	20 mg/ml

2.2.2.3 Determination of DNA concentration

2.2.2.3.1 Qubit Assay

The Qubit fluorometer (Life Technologies, Thermofisher Scientific, Waltham, MA, USA) can be used for quantification of DNA, RNA and protein. This method utilizes fluorescent dyes to determine the concentration of either nucleic acids or proteins. The concentration of nucleic acid/ protein is calculated by measuring the difference between bound and unbound dye and comparing it to a standard curve in order to determine the exact concentration. In order to do a measurement, firstly the assay tubes for the standard and the samples had to be prepared. The Qubit working solution was prepared by diluting the Qubit reagent 1: 200 in Qubit buffer. After that, 195 µl of the working solution were mixed with 5µl of sample. Besides 10 µl of each standard were mixed with 190 µl of the working solution to obtain a total volume of 200 µl. After thoroughly mixing the samples for 5 sec, the tubes were inserted into the Qubit 2.0 fluorometer for measurement. First, a standard curve was created with the two different standards and then the samples were measured to determine the stock concentration.

2.2.2.3.2 Determination of DNA concentration via photometer

DNA concentration of the samples was also measured with the 8452A Diode Array Spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) without using fluorescent reagents. Absorbance was measured at 260 nm and 280 nm using a half micro quartz cuvette. As blank, 500µl of sterile water were used. The samples were measured in 1:10, 1:20 and 1:50 dilutions to reach an absorbance between 0.1 and 0.9.

Calculation of the concentration was performed as follows:

$$OD_{260nm}= 50 \text{ µg DNA/ml solution}= 50 \text{ ng/µl solution}$$

Therefore, the actual DNA concentration can be calculated:

OD measured* dilution of the sample* 50 ng/µl= DNA concentration (ng/µl)

2.2.3 Chemical Methods

2.2.3.1 Synthesis of ILs

Synthesis of ILs was preformed according to the CBILS® (Carbonate Based Ionic Liquid Synthesis) route. Following this specific protocol, certain requirements have to be considered [136]:

- Conversion of the IL-precursors can be done with acids having pKa value < 9 or ammonium salts

- Only organic solvents or water as solvent can be used, no lye should be used

- To ensure that the reaction works out properly, the precursors and acid have to be applied in an equimolar ratio

Below, an example for IL-synthesis and the according calculation is given:

Ionic liquid precursor: $[C_2mim]MC= 1.68$ M

Stearic acid: 1 M solution (needs to be prepared in advance)

2 ml $[C_2mim]MC + 2*1.68$ ml Stearic acid

When these requirements are fulfilled, synthesis is performed as follows:

1. A defined amount of acid (pure or solved) is given into a reaction tube
2. Equimolar amount of IL-precursor is given cautiously to the acid
3. If CO_2 is released, the reaction takes place
4. In order to accelerate the reaction, a defined amount of solvents (Methanol) can be given into the reaction tube
5. Further acceleration of the process is done by heat treatment
6. The reaction is over when no further CO_2 is released and a homogenic solution is present
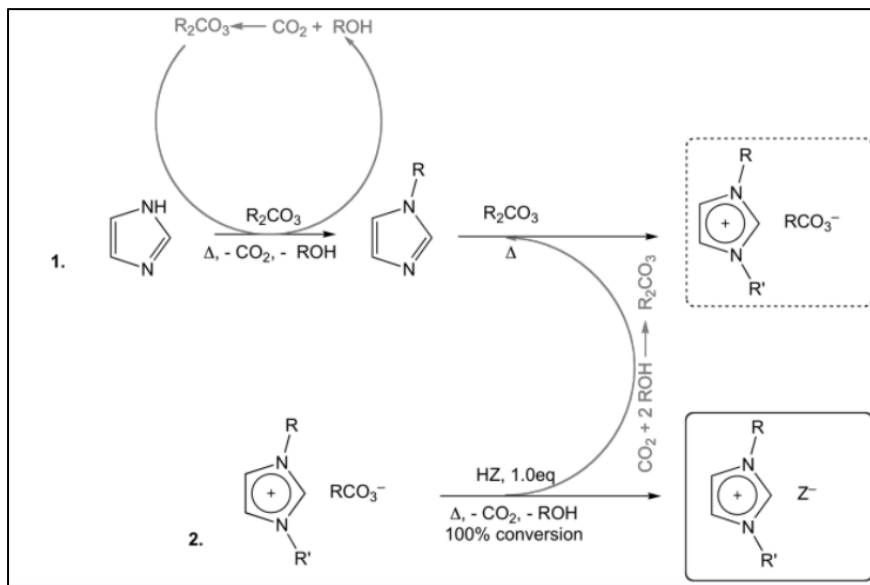7. At the end, the reaction tubes are given into the SpeedVac in order to remove remaining solvents

Figure 8: *Principle of CBILS-route* [136].

2.2.3.2 Conductivity measurement of ILs

The conductivity of ILs was measured in order to create Walden Plots. Walden plots are used to estimate the ionicity of ILs [137]. KCl is believed to be an completely dissolved salt and is therefore applied for the establishment of a calibration line to which ILs are compared in a Walden plot [138]. Therefore, it was measured in first place in order to create a calibration line. The starting concentration for KCl and every IL was 50 mM. During the measurement, Milli-Q water was used for rinsing of the electrode since normal water is composed of ions, which would influence the measurement. Certain ILs could not be solved in water, therefore the IL was primarily solved in a small amount of 99 % Methanol and filled up with water to desired volume.

Measurement was conducted in following steps:

1. 10 ml of the respective solution with a concentration of 50 mM (KCl or IL) was prepared

2. After finishing the first measurement the electrode had to be rinsed/cleaned with Milli-Q water, until the display showed 0.2 µS (this step was performed after every measurement)

3. 10 ml of Milli-Q water were added to the solution, mixed and a second measurement was performed

4. After cleaning, 20 ml of Milli-Q water were added, a third measurement was performed

5. 30 ml were discarded and again 10 ml of Milli-Q were added to the falcon tube for measuring the fourth time.

3. Results

3.1 Synthesis of ILs

In general synthesis of ILs is a complicated and time-consuming process. Therefore an easier and more timesaving method was chosen. In the course of this study a novel set of ILs was synthesized according to the CBILS (Carbonate Based Ionic Liquid Synthesis) route (see 2.2.3.1):

- [TMC$_{12}$A]Cl

- [TMC$_{12}$A]periodate

- [TMC$_{16}$A]Cl

- [TMC$_{16}$A]periodate

- [TOMA]Cl

- [TOMA]lactate

- [TOMA]benzoate

- [TOMA]phthalate

- [TOMA]stearate

- [TOMA]palmitate

Periodate

Lactate

Palmitate

[TOMA]X⁻: basic structure of [TOMA] based IL

After synthesis of the respective ILs, certain parameters (state of matter at room temperature, miscibility in water, and conductivity) were determined. These parameters were then integrated into an IL-database. Figure 9 shows the molar conductivity of KCl compared to [C$_2$mim]Cl, [TMC$_{12}$A]periodate, and [TOMA]palmitate. In conductivity measurements KCl is referred to as ideally dissolved salt, to which other substances can be compared. Meaning that the more dissolved an IL is, less ion pairs exist and the more cations and anions are free in solution. As depicted in Figure 9, [C$_2$mim]Cl has almost a

similiar conductivity as KCl, whereas [TOMA]palmitate shows a conductivity which is much lower as the conductivity of KCl or [C$_2$mim]Cl.
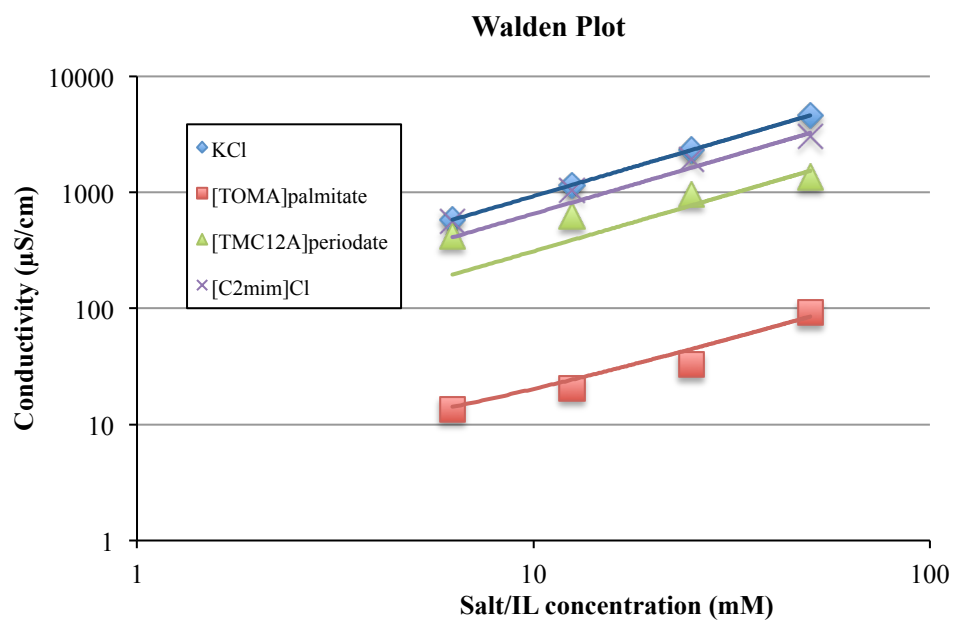
Walden Plot

Figure 9: *Conductivity of different ILs compared to KCL.*

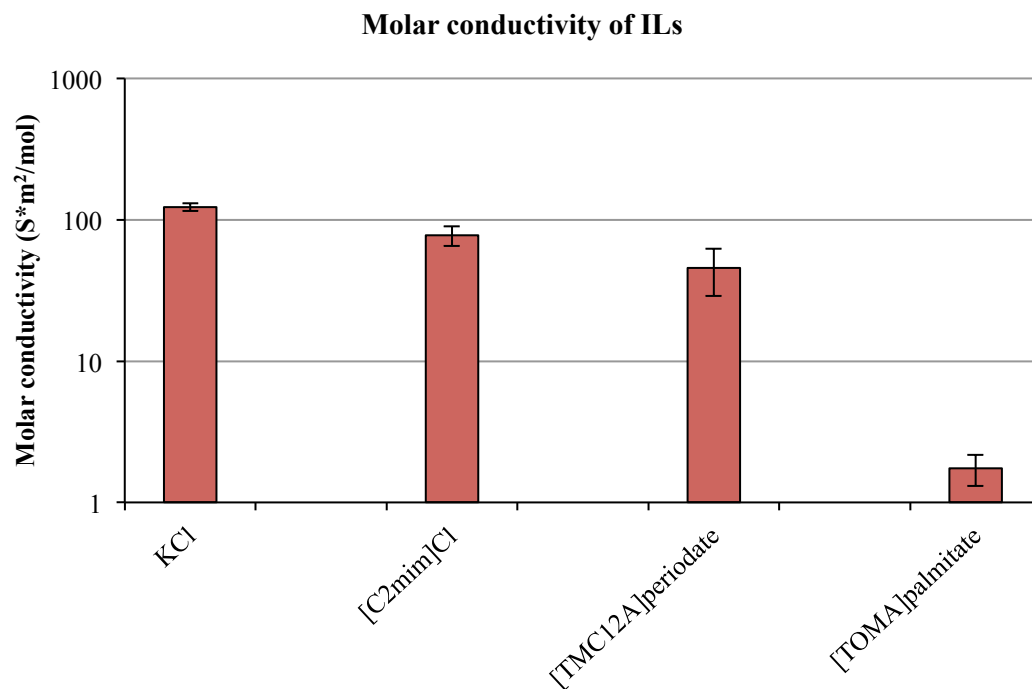**Molar conductivity of ILs**

Figure 10: Molar c*onductivity of synthesized [TOMA] based ILs*

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Figure 10 shows exemplary the conductivity of the synthesized ILs. The conductivity changes with alkyl side chain length of the anion moiety, and choice of the cationic head group. [TOMA]palmitate had the lowest conductivity of all tested [TOMA] based ILs, and the lowest conductivity of the ILs in Figure 10.

Table 11 summarizes the tested parameters (state of matter at room temperature, miscibility in water, and molar conductivity) for all synthesized ILs.

Table 11: *Synthesized ILs and their parameters.*

Synthesized IL	State of matter at room temperature	Miscibility in water	Molar conductivity ($S*m^2$/mol)
[TOMA]Cl	viscose/ jellylike	not miscible	35.18 ± 17.12
[TOMA]lactate	viscose/ jellylike	not miscible	29.526 ± 6.81
[TOMA]benzoate	viscose/ jellylike	not miscible	7.87 ± 2.08
[TOMA]phthalate	viscose/ jellylike	not miscible	6.13 ± 2.94
[TOMA]stearate	viscose/ jellylike	not miscible	2.08 ± 0.40
[TOMA]palmitate	viscose/ jellylike	not miscible	1.74 ± 0.44
$[TMC_{12}A]Cl$	viscose	miscible	50.20 ± 28.69
$[TMC_{12}A]periodate$	viscose	miscible	45.59 ± 24.04
$[TMC_{16}A]Cl$	viscose/ powdery	not miscible	37.58 ± 15.68
$[TMC_{16}A]periodate$	viscose/ powdery	not miscible	40.02 ± 18.59

3.2 Antibacterial effects of varying alkyl side chain lengths in the anion

In the course of these experiments the antibacterial effect of ILs with [C$_2$mim] and [N,N-dimethyl-2 hydroxyethyl-ammonium] as cation and alternating anions were examined. *L. monocytogenes* and *E. coli* served as model organisms, in order to have a representative pathogen of both gram-positive and gram-negative origin. In addition to the MIC, the MBC was also determined.
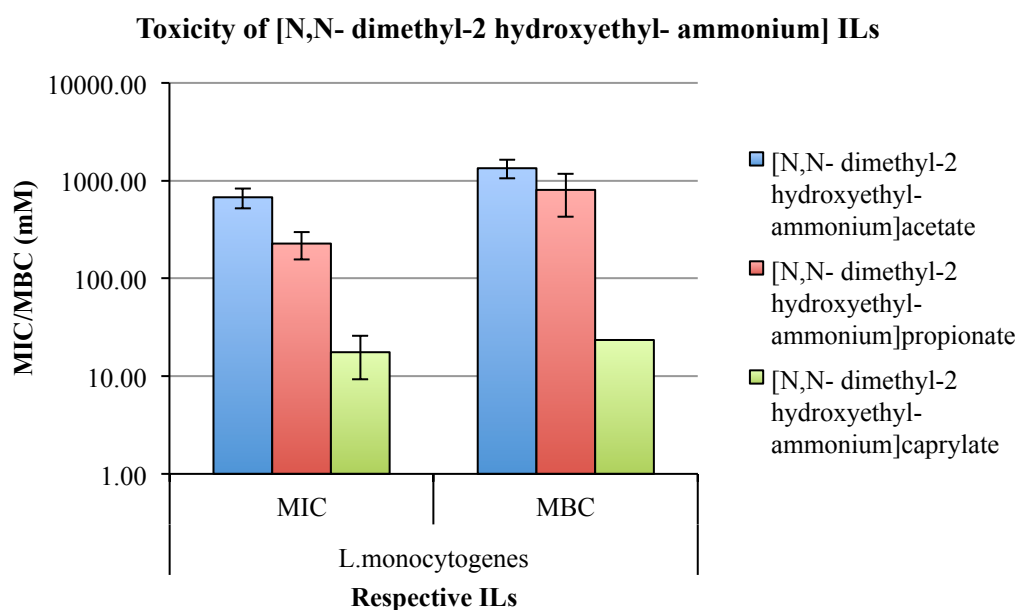

Figure 11: *MIC/MBC of [N,N-dimethyl-2 hydroxyethyl-ammonium]based ILs with altering anion moiety for L. monocytogenes EGD-e.*

Results in Figure 11 show that toxicity of [N,N-dimethyl-2 hydroxyethyl-ammonium] ILs increased from [N,N-dimethyl-2 hydroxyethyl-ammonium]acetate to [N,N-dimethyl-2 hydroxyethyl-ammonium]caprylate. The difference between these [N,N-dimethyl-2 hydroxyethyl-ammonium] ILs is the alkyl side chain length of the anion. *E. coli* showed similar results, although the MIC was lower. The exact results obtained for *L. monocytogenes* and *E. coli* are shown in the Table 12 below.

Table 12: *Mean MIC values (mM) after 24h of [N,N-dimethyl-2 hydroxyethyl-ammonium] ILs for L. monocytogenes* **EGD-e** *and E. coli* **K-12.**

	ILs	MIC (mM)	MBC (mM)
L. monocytogenes	[N,N- dimethyl-2 hydroxyethyl- ammonium]acetate	675.94 ± 154.98	1340.48 ± 290.22
	[N,N- dimethyl-2 hydroxyethyl- ammonium]propionate	227.16 ± 70.87	804.23 ± 379.12
	[N,N- dimethyl-2 hydroxyethyl- ammonium]capyrlate	17.57 ± 8.28	23.43 ± 0
E. coli	[N,N- dimethyl-2 hydroxyethyl- ammonium]acetate	310.68 ± 207.35	879.69 ± 414.69
	[N,N- dimethyl-2 hydroxyethyl- ammonium]propionate	284.03 ± 244.53	778.7 ± 508.54
	[N,N- dimethyl-2 hydroxyethyl- ammonium]caprylate	8.79 ± 4.14	17.57 ± 8.28

Imidazolium based ILs, which constitute a widely used class of ILs, were also investigated with respect to their toxicity. The used imidazolium ILs had a [C_2mim] cationic core.
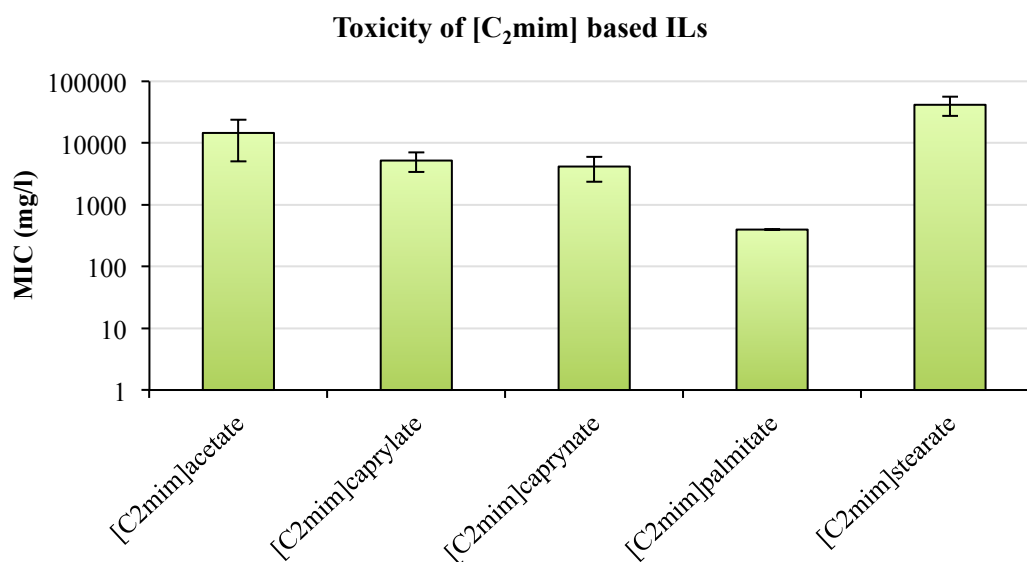
Figure 12: *MIC after 24h of imidazolium based ILs with [C_2mim] as cationic core and altering anions.*

The MIC of [C$_2$mim] imidazolium based ILs changed with anion side chain length, as it was also observed in [N,N-dimethyl-2 hydroxyethyl-ammonium] ILs (see Figure 11.). [C$_2$mim]acetate opposed the least toxic effect against *L. monocytogenes*. [C$_2$mim]palmitate on the other side was the most toxic substance. *E. coli* was also tested against those ILs and similar results were obtained. However, for most of the tested ILs the MIC was lower as the MICs for *L. monocytogenes*. Exact results for both strains are listed in the table below (Table 13).

Table 13: *Mean MIC values (mg/l) of [C$_2$mim] imidazolium based ILs for L. monocytogenes* **EGD-e** *and E. coli* **K-12.**

	ILs	MIC (mg/l)
	[C$_2$mim]acetate	14583 ± 9547
	[C$_2$mim]caprylate	5208 ± 1804
L. monocytogenes	[C$_2$mim]caprynate	3125 ± 0
	[C$_2$mim]palmitate	393 ± 5
	[C$_2$mim]stearate	41666 ± 14433
	[C$_2$mim]acetate	8333 ± 3608
	[C$_2$mim]caprylate	4166 ± 1804
E. coli	[C$_2$mim]caprynate	3645 ± 2386
	[C$_2$mim]stearate	37500 ± 21650
	[C$_2$mim]palmitate	391 ± 0

3.3 Toxicity of ILs using different *L. monocytogenes* strains with a QAC specific efflux pump

L. monocytogenes is able to withstand disinfectants like benzalkonium chloride, due to having specific efflux pumps against this class of antimicrobial substance. ILs on the otherside are also known for having antibacterial effects, however they have never been investigated with respect to specific efflux pumps and their consequences.

Therefore, the aim of the following experiments was to test, if a QAC specific efflux pump in *L. monocytogenes* strains could provide increased tolerance to ILs with quaternary ammonium structures. In order to investigate this tolerance mechanism, ten different *L.* monocytogenes strains, five harbouring a QAC specific efflux pump, encoded by the transposon Tn*6188,* and five strains without an the Tn*6188* encoded efflux pump were tested in presence of those specific ILs. Additionally the different *L. monocytogenes* strains

were tested against a different set of QACs (BC, Benzethonium chloride, Domiphen bromide and DTAB) in order to investigate the tolerance mechanism of the efflux pump and to compare the obtained results to those of ILs.

3.3.1 Determination of MIC for QACs using +Tn*6188*/-Tn*6188 L. monocytogenes* strains

As already described by Müller et al. [74] (2012) *L. monocytogenes* strains harbouring the transposon Tn*6188* show increased MIC levels against QACs compared to *L. monocytogenes* strains lacking this transposon.

Tolerance of *L. monocytogenes* strains to BC

L. monocytogenes strains
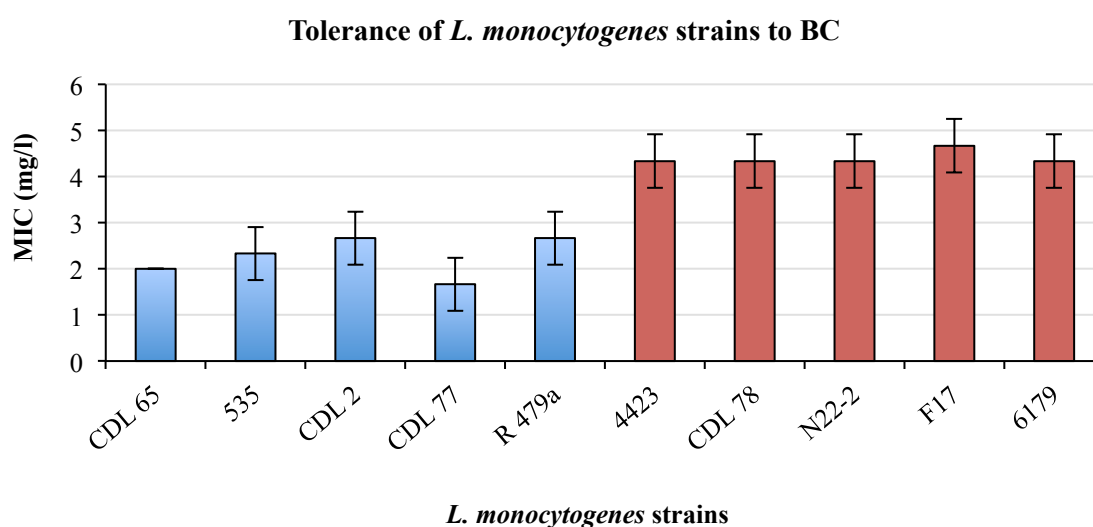
Figure 13: *Mean MIC values (mg/l) after 24h of BC for ten different L. monocytogenes strains after 24h incubation. +Tn6188 strains in red, -Tn6188 strains in blue.*

The strains were divided into two groups. One group includes five strains harbouring transposon Tn*6188* (+Tn*6188:* 4423, CDL 78, N22-2, F17, 6179) and the second group includes five strains without transposon Tn*6188* (-Tn*6188*: CDL 65, CDL 2, CDL 77, 535, R479a). Figure 13 shows the MIC values of +Tn*6188* and –Tn*6188 L. monocytogenes* strains against BC. As already described by Müller et al. (2012) all +Tn*6188 Listeria* strains demonstrated a higher tolerance to BC in comparison to the –Tn*6188* counterparts. *L. monocytogenes* strain F17 showed the highest MIC with 4.67 mg/l, whereas CDL 77 showed the lowest MIC with 1.67 mg/l.

The pattern obtained for BC could also be confirmed for other QACs like domiphen bromide. Figure 14 shows that +Tn*6188 L. monocytogenes* strains had higher MIC values compared to those strains without transposon.
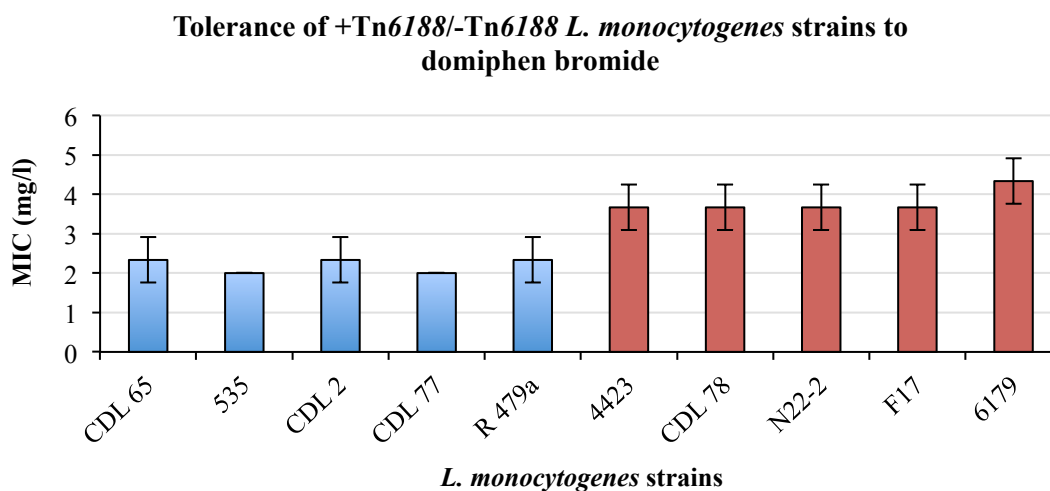

Figure 14: *Mean MIC values (mg/l) after 24h of domiphen bromide for ten different L. monocytogenes strains after 24h incubation. +Tn6188 strains in red, -Tn6188 strains in blue.*

In addition to BC and domiphen bromide other QAC species were also tested. Results are shown in Table 14. For clarity reasons the results of the individual *L. monocytogenes* strains were summarized into two groups, +Tn*6188* and −Tn*6188*. Table shows that for all tested QAC species +Tn*6188 L. monocytogenes* strains had higher MIC levels as the -Tn*6188* strains.

Table 14: *Mean MIC values (mg/l) after 24h incubation for all tested QACs. For the individual L. monocytogenes strains obtained results were summarized into two groups (+Tn6188, -Tn6188).*

	MIC [mg/l] 24h					
QACs	***L. monocytogenes* (-)Tn*6188***			***L. monocytogenes* (+)Tn*6188***		
Benzalkonium chloride	3.8	±	0.6	5.0	±	0.5
DTAB	55.0	±	14.6	95.8	±	6.7
Domiphen bromide	3.2	±	1.1	4.2	±	1.2
Benzenthonium chloride	4.2	±	0.8	5.1	±	0.6

3.3.2 Determination of MIC for different ILs with quaternary ammonium cores using +Tn*6188*/-Tn*6188 L. monocytogenes* strains

The *L. monocytogenes* strains, either harbouring the transposon or lacking the transposon were tested in presence of ILs with quaternary ammonium cores and increasing alkyl side length chains. In total, 15 ILs were tested, five of them based on a 1-alkyl- 3-methylimidazolium cation and the other ten based on a trimethylalkylammonium cation. The anions of these ILs were either chloride or maleate.

In Figure 15 the MIC values for $[C_{10}mim]Cl$ are shown as an example of imidazolium based ILs. Similar to QACs, +Tn*6188 Listeria* strains had a higher tolerance against $[C_{10}mim]Cl$ than −Tn*6188 Listeria* strains. In general the MIC was higher as the MIC values seen for QACs in 3.3.1.

Tolerance of +Tn6188/-Tn6188 *L. monocytogenes* strains to $[C_{10}mim]Cl$
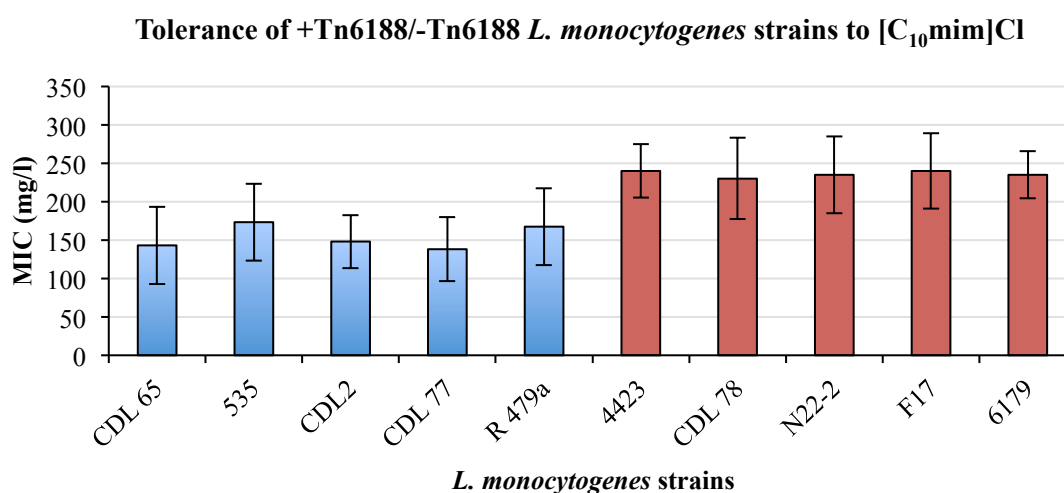
Figure 15: *Mean MIC values (mg/l) after 24h of $[C_{10}mim]Cl$ for ten different L. monocytogenes strains. +Tn6188 strains in red, -Tn6188 strains in blue.*

Other tested imidazolium based ILs with varying side chain length on the cation are presented in Table 15. $[C_{10}mim]Cl$ had the highest toxicity of all tested imidazolium based ILs. $[C_2mim]Cl$ and $[C_4mim]Cl$ on the other side had the lowest toxicity. Besides these two ILs did not show differences between the different *L. monocytogenes* strains with and without transposon Tn*6188*. Exemplary for trimethylalkylammonium ILs the results for $[TMC_{12}A]Cl$ are presented in Figure 16. As before, +Tn6188 *L. monocytogenes* strains also

showed increased tolerance to [TMC$_{12}$A]Cl. Results for all tested imidazolium and trimethylalkylammonium ILs are listed up in Table 15.
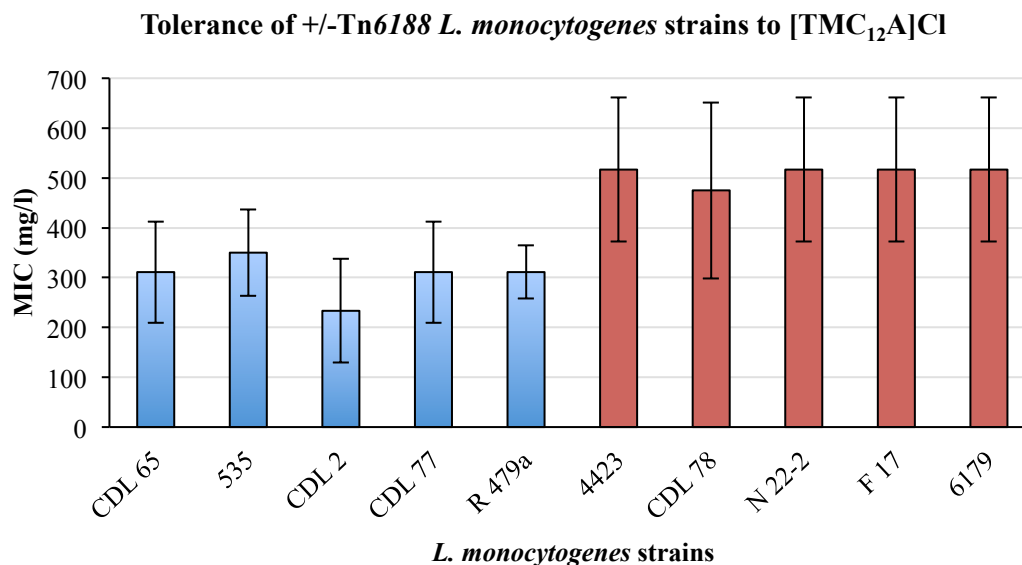

Figure 16: *Mean MIC values (mg/l) after 24h of [TMC$_{12}$A]Cl for 10 different Listeria monocytogenes strains. +Tn6188 strains in red, -Tn6188 strains in blue.*

Table 15: *Mean MIC values (mg/l) after 24h incubation for all tested imidazolium and trimethylalkylammomnium ILs.*

	MIC [mg/l] 24h			
ILs	L. monocytogenes (-)Tn6188		L. monocytogenes (+)Tn6188	
[C$_2$mim]Cl	>50000		>50000	
[C$_4$mim]Cl	>50000		>50000	
[C$_6$mim]Cl	25000	± 0	25000	± 0
[C$_8$mim]Cl	4125	± 393	4900	± 576
[C$_{10}$mim]Cl	180	± 18	235	± 13
[TMC$_4$A]Cl	25000		>25000	
[TMC$_8$A]Cl	364	± 67	523	± 85
[TMC$_{12}$A]Cl	303	± 87	511	± 124
[TMC$_{16}$A]Cl	2.9	± 1.2	5	± 0.8
[TMC$_4$A]maleate	>25000		>25000	
[TMC$_8$A]maleate	548	± 90	963	± 281
[TMC$_{12}$A]maleate	303	± 116	417	± 65
[TMC$_{16}$A]maleate	3.0	± 0.7	3.4	± 0.5
[TMC$_{12}$A]periodate	45	± 10.5	150	± 0.0
[TMC$_{16}$A]periodate	3.0	± 2.5	5.6	± 2.1

3.4 Exposure of *L. monocytogenes* to sublethal concentrations of ILs and the following consequences

The aim of the following experiments was to test, whether *L. monocytogenes* can be adapted to withstand higher concentrations of ILs, after exposure to sublethal concentrations of ILs beforehand. Because of that *L. monocytogenes* was exposed to sublethal concentrations of ILs to see if this mechanism also applies for this type of antimicrobial substances.

In the course of these experiments two different *L. monocytogenes* strains were used. *L. monocytogenes* 6179 served as representative of +Tn*6188* strains, whereas *L. monocytogenes* CDL 2 served as representative of the −Tn*6188* strains. These two strains were adapted according to the adaption protocol described in chapter 2.2.1.3. The Table 16 below summarizes all the different strains and conditions, giving explanations to the abbreviations, which were applied throughout the next chapters.

Table 16: *List of adapted L. monocytogenes strains CDL2 and 6179, adapted to different concentrations of antimicrobial substances (BC, [C$_{10}$mim]Cl, and [TMC$_{16}$A]maleate).*

L. monocytogenes strain	Abbreviation	Explanation
CDL 2	CDL 2- par.	parental *L. monocytogenes* CDL 2 strain, wild type strain
	CDL 2- ad. 4 mg/l	*L. monocytogenes* CDL 2 strain adapted to 4 mg/l of a given substance
	CDL 2 BC- ad. 4mg/l	*L. monocytogenes* CDL 2 strain adapted to 4 mg/l of BC
	CDL 2 [TMC$_{16}$A]maleate- ad. 3 mg/l	*L. monocytogenes* CDL 2 strain adapted to 3 mg/l of [TMC$_{16}$A]maleate
	CDL 2 [C$_{10}$mim]Cl- ad. 200 mg/l	*L. monocytogenes* CDL 2 strain adapted to 200 mg/l of [C$_{10}$mim]Cl
6179	CDL 6179- par.	parental *L. monocytogenes* 6179 strain, wild type strain
	CDL 6179- ad. 4 mg/l	*L. monocytogenes* 6179 strain adapted to 4 mg/l of a given substance
	CDL 6179 BC- ad. 5 mg/l	*L. monocytogenes* 6179 strain adapted to 5 mg/l of BC
	CDL 6179 [TMC$_{16}$A]maleate- ad. 3 mg/l	*L. monocytogenes* 6179 strain adapted to 3 mg/l of [TMC$_{16}$A]maleate
	CDL 6179 [C$_{10}$mim]Cl- ad. 250 mg/l	*L. monocytogenes* 6179 strain adapted to 250 mg/l of [C$_{10}$mim]Cl

3.4.1 Adaption to higher concentrations of BC

In these experiments one *L. monocytogenes* strain of each group (+Tn*6188*, -Tn*6188*) was chosen for exposure to sublethal concentrations of BC. *L. monocytogenes* strain 6179 served as representative of the +Tn*6188* group, whereas *L. monocytogenes* strain CDL 2 served as representative of the −Tn*6188* group. This was performed in order to see, if those two L. monocytogenes can be adapted to withstand higher concentration of BC and to test the efficiency of the adaption protocol. The adaption process was performed four consecutive times.

Adaption of +/-Tn6188 *L.monocytogenes* strains to BC

L. monocytogenes strains
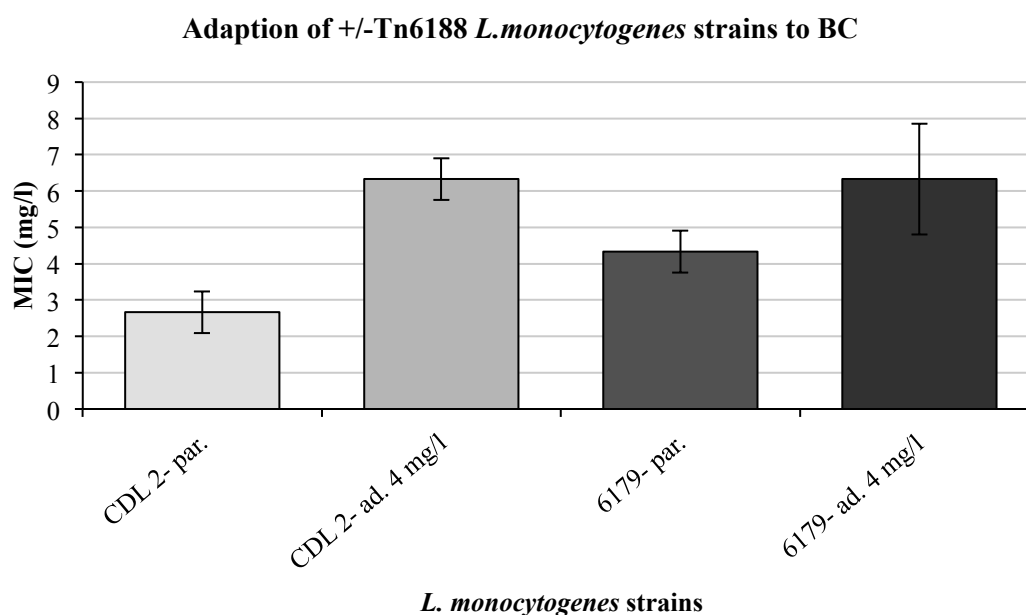
Figure 17: *Mean MIC values (mg/l) after 24h of parental (par.) and adapted (ad.) L. monocytogenes strains for benzalkonium chloride (BC).*

The data presented in Figure 17 shows that *L. monocytogenes* strains CDL 2 and 6179 could be successfully adapted to higher concentrations of benzalkonium chloride. Besides, the parental 6179 strain showed a higher MIC value than the parental CDL 2 strain. Adapted CDL 2 and 6179 strains had equal MICs.

3.4.2 Adaption to higher concentrations of ILs

The experiment explained in 3.4.1 was repeated using ILs instead of BC. The *Listeria* strains CDL2 and 6179 were exposed to sublethal concentrations of either imidazolium based or trimethylalklammonium based ILs.

As a representative of imidazolium based ILs [C_{10}mim]Cl was used for the adaption process (see Figure 18.)

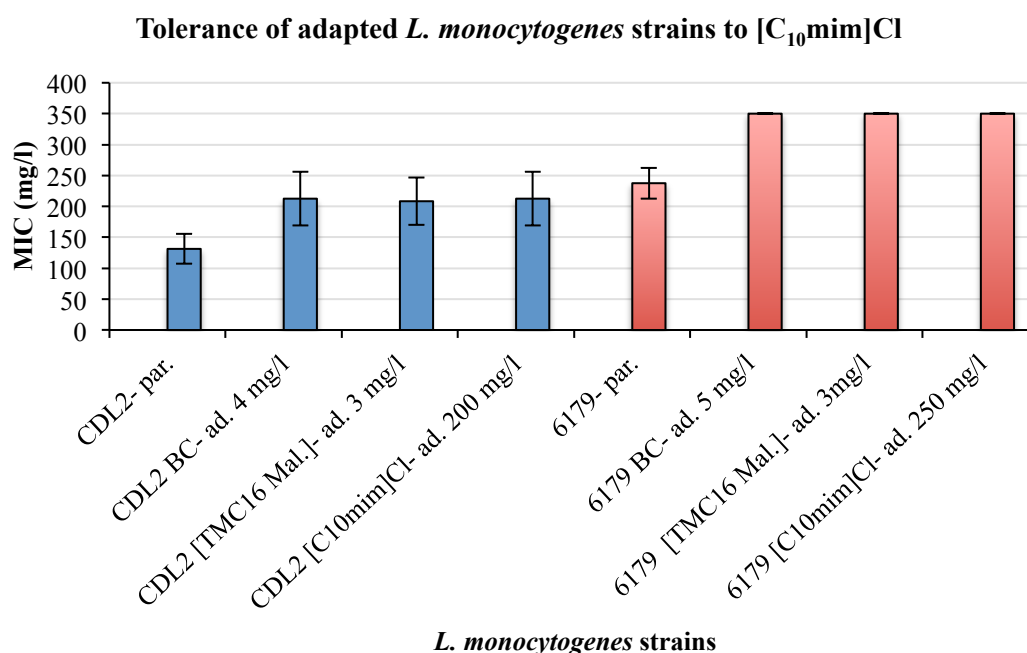**Adaption of +/- Tn*6188 L. monocytogenes* strains to [C_{10}mim]Cl**
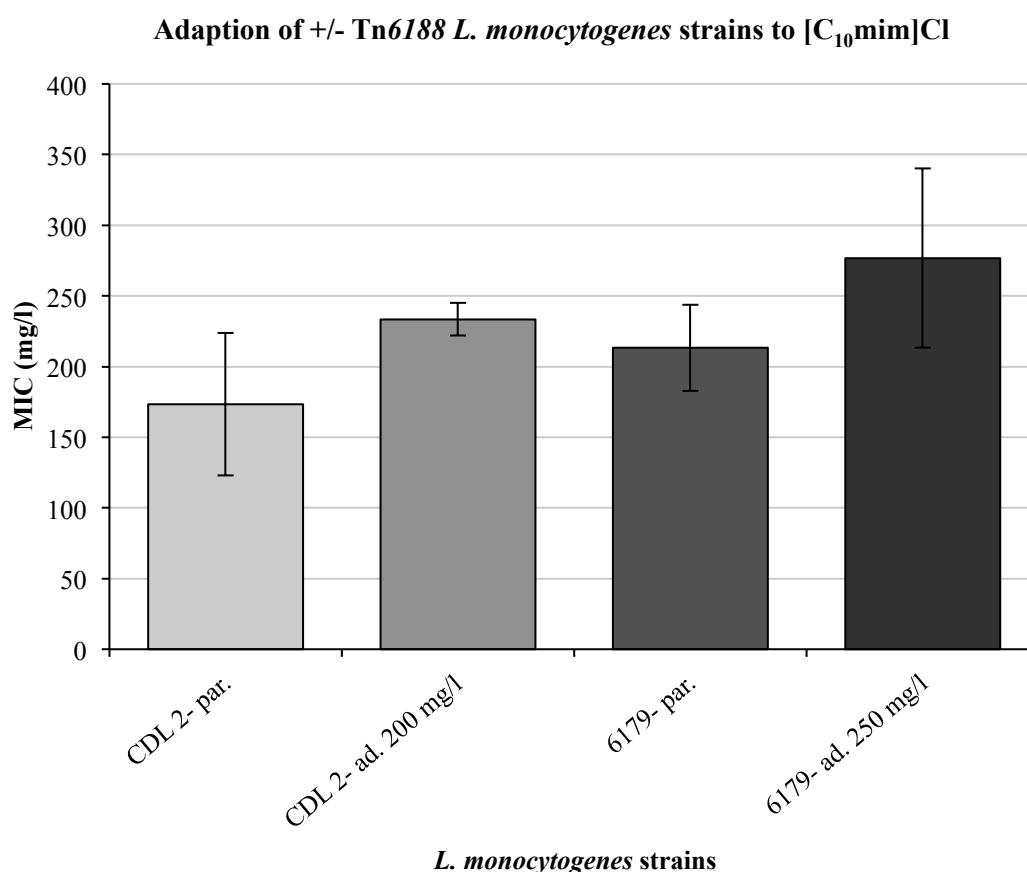
Figure 18: *Mean MIC values (mg/l) after 24h of parental and adapted L. monocytogenes strains CDL 2 and 6179 for IL [C_{10}mim]Cl.*

As already demonstrated for BC, *Listeria* strains CDL 2 and 6179 could also be successfully adapted to higher concentrations of the IL [C_{10}mim]Cl.

As a representative of trimethylalkylammonium ILs [$TMC_{16}A$]maleate was used. The adaption for [$TMC_{16}A$]maleate was also successful (see Figure 19.), showing increased MIC levels in the adapted *L. monocytogenes* strains 6179 and CDL 2.

Adaption of +/- Tn6188 *L. monocytogenes* strains to [TMC$_{16}$A]maleate
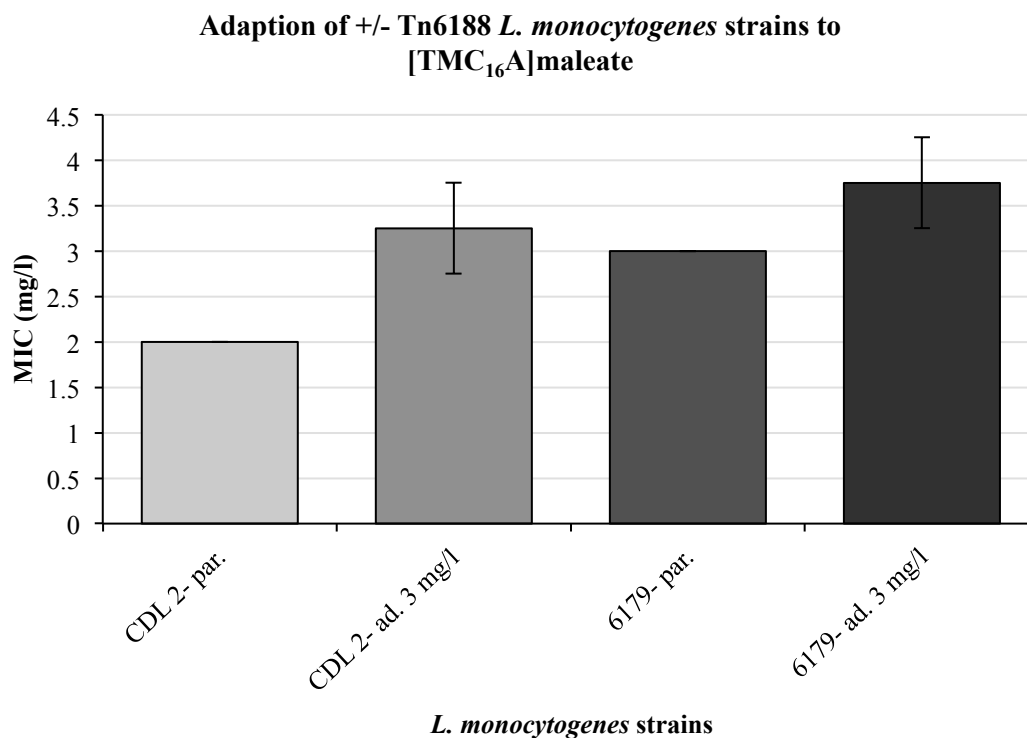
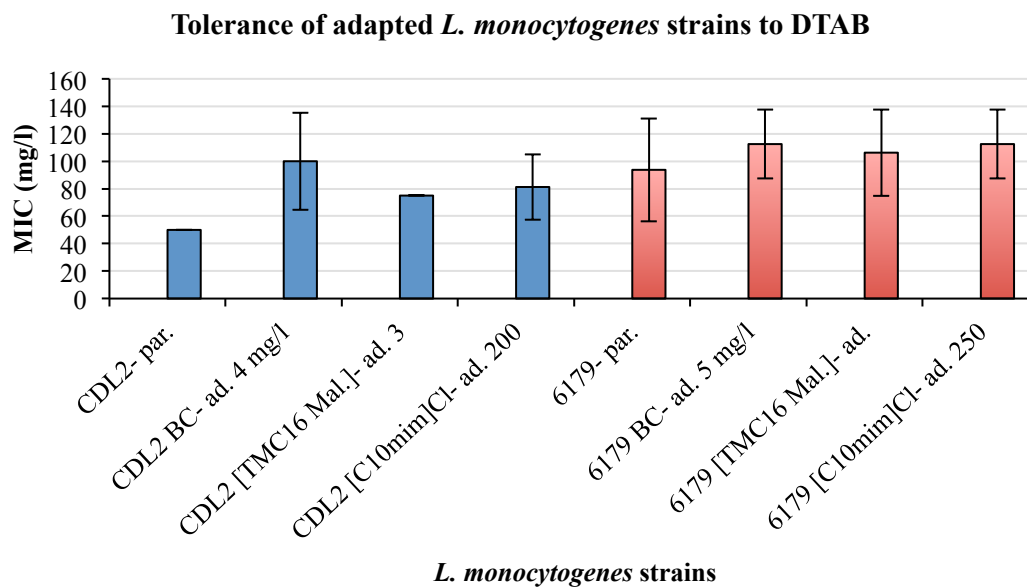**Figure 19:** *Mean MIC values (mg/l) after 24h of parental and adapted L. monocytogenes strains CDL 2 and 6179 against IL [TMC$_{16}$A]maleate.*

3.5 Tolerance of adapted *L. monocytogenes* strains to further ILs and antimicrobial substances

QAC specific efflux systems are often termed multidrug efflux pumps since they provide resistance to a range of various antibiotics, dyes and surfactants. Therefore, the previously BC and IL- adapted *L. monocytogenes* (see 3.4.1 and 3.4.2) strains CDL 2 and 6179 were tested for adaption against other ILs, QAC species, and antibiotics.

The aim was to test, if the adapted CDL 2 and 6719 *Listeria* strains show higher tolerance to other ILs, QAC species, and antibiotics.

3.5.1 Tolerance of adapted *L. monocytogenes* to other ILs and QAC species

Following the prior successful adaption of *L. monocytogenes* strains 6179 and CDL 2 to BC, [C_{10}mim]Cl and [TMC_{16}A]maleate those adapted strains were tested against other ILs and QAC species.

In Figure 20 the MIC levels of adapted *L. monocytogenes* strains CDL 2 and 6179 to [C_{10}mim]Cl are depicted. The CDL 2 strains adapted to BC (CDL 2 BC-ad. 4 mg/l), [C_{10}mim]Cl (CDL 2 [C_{10}mim]Cl- ad. 200 mg/l) and [TMC_{16}A]maleate (CDL 2-[TMC_{16}A]maleate- ad. 3 mg/l) showed increased MIC levels compared to the parental CDL 2 strain Also the 6179 strains adapted to BC (6179 BC- ad. 5 mg/l), [C_{10}mim]Cl (6179 [C_{10}mim]Cl- ad. 250 mg/l) and [TMC_{16}A]maleate (6179- [TMC_{16}A]maleate ad. 3 mg/l) had increased MIC levels compared to their parental 6179 counterpart.

Figure 20: *Mean MIC values (mg/l) after 24h of parental and adapted L. monocytogenes strains CDL 2 and 6179 for IL [C_{10}mim]Cl.*

The following Figure 21 shows exemplary the MIC values of the adapted *L. monocytogenes* strains for the QAC species DTAB. As demonstrated for [C_{10}mim]Cl, all adapted CDL 2 and 6179 strains had increased MIC levels compared to the parental strains.

Figure 21: *Mean MIC values (mg/l) after 24h of parental and adapted Listeria monocytogenes strains CDL 2 and 6179 against DTAB.*

The results for all other tested ILs and QAC species are listed up below in Table 17. It illustrates that all adapted CDL 2 and 6179 strains adapted to both, ILs and QACs.

Table 17: *Mean MIC values after 24h incubation for adapted L monocytogenes strains CDL 2 and 6179 against QACs and ionic liquids.*

	MIC of L.monocytogenes strains (mg/l)				
	BC	DTAB	[C_{10}mim]Cl	[$TMC_{12}A$]maleate	[$TMC_{16}A$]maleate
CDL 2- par.	3 ± 0.82	50 ± 0	131.25 ± 23.94	275 ± 50	2 ± 0
CDL 2 BC- ad. 4mg/l	5 ± 1.00	100 ± 35.36	212.5 ± 43.30	387 ± 25	3.25 ± 0.5
CDL 2 [C_{10}mim]Cl- ad. 200 m|	4 ± 0	75 ± 0	208.3 ± 38.19	362 ± 25	3.25 ± 0.5
CDL 2 [$TMC_{16}A$] Mal- ad. 3 m|	4 ± 0	81.25 ± 23.94	212.5 ± 43.30	400 ± 81.65	3 ± 0
6179- par.	4.25 ± 0.50	93.75 ± 37.5	237 ± 25	437 ± 75	3 ± 0
6179 BC- ad. 5 mg/l	6.25 ± 1.71	112.5 ± 25	350 ± 0	550 ± 57.74	3.25 ± 0.5
6179 [C_{10}mim]Cl- ad. 250 mg/l	5 ± 0.82	106.25 ± 31.46	350 ± 0	525 ± 50	3.25 ± 0.5
6179 [$TMC_{16}A$] Mal- ad. 3 mg|	5.5 ± 1	122.5 ± 25	350 ± 0	550 ± 7.4	3.25 ± 0.5

3.5.2 Tolerance of adapted *L. monocytogenes* strains to antibiotics

Efflux pumps can cause both, resistance to disinfectants and resistance to antibiotics. Therefore the tolerance of adapted *L. monocytogenes* strains to different types of antibiotics was tested.

In Figure 22 MIC values for ampicillin of adapted and parental *L. monocytogenes* strains 6179 and CDL 2 are depicted. Adapted *L. monocytogenes* strains showed higher tolerance to ampicillin compared to the parental strains. However, the standard deviation for 6179 *L. monocytogenes* strains was quite high. Therefore, further investigations need to be conducted.
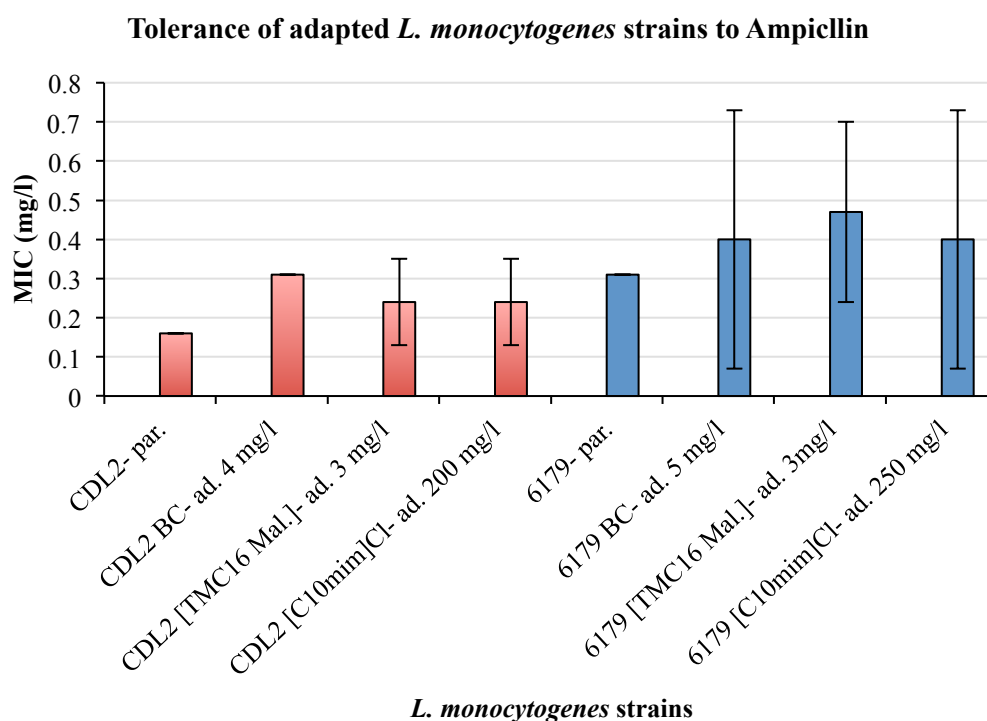
Figure 22: *Mean MIC values (mg/l) after 24h of ampicillin for adapted (ad.) and parental (par.) L. monocytogenes. +Tn6188 strains coloured in red, -Tn6188 strains coloured in blue.*

In addition to ampicillin, the antibiotics gentamicin, ciprofloxacin and nalidixic acid were investigated. Results for these antibiotics are shown in the Table below (Table 18).

Table 18: *Mean MIC values (mg/l) after 24h incubation for parental and adapted L. monocytogenes strains CDL 2 and 6179 against different antibiotics.*

	MIC of *L.monocytogenes* strains (mg/l)			
	Ampicillin	Gentamicin	Ciprofloxacin	Nalidixic acid
CDL 2- par.	0.16 ± 0	23.3 ± 23.1	10 ± 7.07	400 ± 115.5
CDL 2 BC- ad. 4mg/l	0.31 ± 0	16.7 ± 11.5	18.8 ± 14.93	400 ± 115.5
CDL 2 [C$_{10}$mim]Cl- ad. 200 mg/l	0.24 ± 0.11	20 ± 14.1	17 ± 20.42	425 ± 95.7
CDL 2 [TMC$_{16}$A] Mal- ad. 3 mg/l	0.24 ± 0.11	18.3 ± 10.4	16.3 ± 16.01	450 ± 57.7
6179- par.	0.31 ± 0	15 ± 8.7	11.7 ± 5.77	425 ± 95.7
6179 BC- ad. 5 mg/l	0.40 ± 0.33	13.3 ± 5.8	13.3 ± 7.64	425 ± 95.7
6179 [C$_{10}$mim]Cl- ad. 250 mg/l	0.47 ± 0.23	15 ± 8.7	20 ± 18.03	450 ± 57.7
6179 [TMC$_{16}$A] Mal- ad. 3 mg/l	0.40 ± 0.33	16.7 ± 11.5	15 ± 10.00	425 ± 95.7

3.6 TOMA based ILs and their behaviour in presence of an efflux pump in *L. monocytogenes*

In the course of screening various types of ILs towards their toxicity an interesting and promising class of ILs, namely [TOMA] based ILs were found. This class of ammonium based ILs is characterized by the presence of a positively charged ammonium substituted with three octyl and one methyl group in the cationic core. Since this IL-class showed high toxicity in the screenings and because of its structure it was used for further experiments. The now following results were obtained at the end of this masterwork and work with these ILs is still ongoing.

3.6.1 TOMA based ILs tested with +/- Tn*6188 L. monocytogenes* strains

Various [TOMA] based ILs were tested with focus on their toxicity and on the one hand and their influence on the Tn*6188* coded efflux pump on the other hand.

As an example for [TOMA] based ILs the MIC of [TOMA]FeCl$_4$ is illustrated in Figure 23. The data shows that both bacterial strains, *L. monocytogenes* strains harbouring Tn*6188* and *L. monocytogenes* strains without Tn*6188* had the same MIC values for [TOMA]FeCl$_4$. Furthermore, the obtained MIC values, which were between 1 and 2 mg/l, suggest that these [TOMA] based ILs are highly active antimicrobial compounds. In Figure 24 the respective MIC values of all other tested [TOMA] based ILs are depicted, which also did not show any difference between +/- Tn*6188 L. monocytogenes* strains
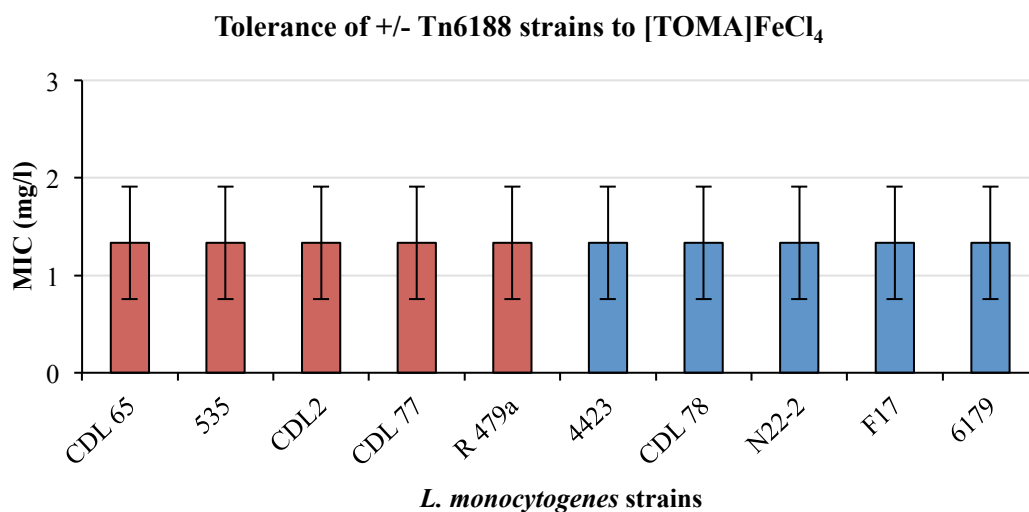
Tolerance of +/- Tn6188 strains to [TOMA]FeCl$_4$

Figure 23: *Mean MIC values (mg/l) after 24h of [TOMA]FeCl$_4$ tested against 10 different L. monocytogenes strains.*

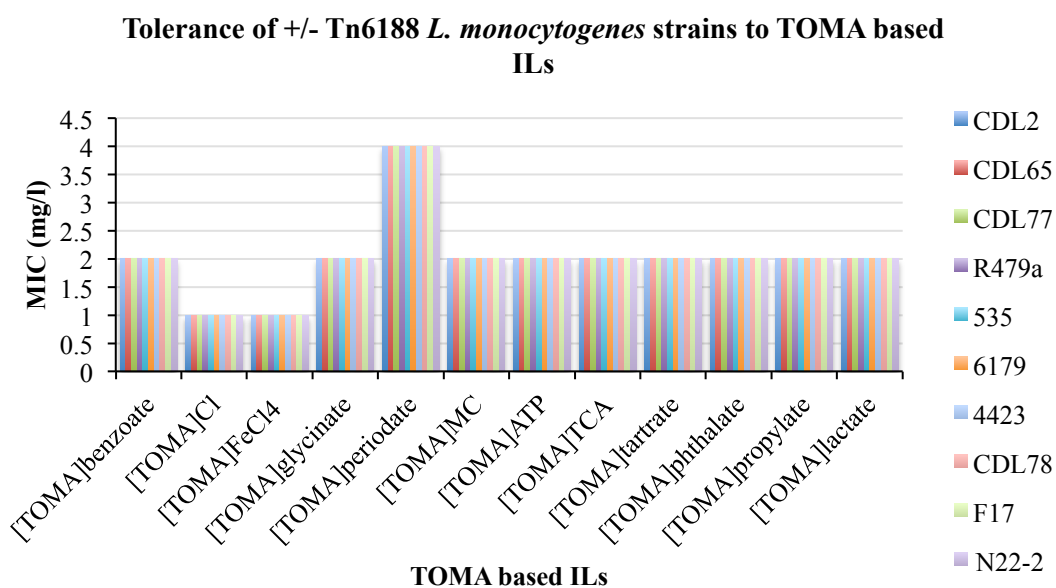**Tolerance of +/- Tn6188 *L. monocytogenes* strains to TOMA based ILs**

Figure 24: *MIC (mg/l) after 24h of various [TOMA] based ILs for ten0 different L. monocytogenes strains (+Tn6188, -Tn6188 strains).*

3.7 Molecular investigation of IL-toxicity mechanism

ILs, especially those with long alkyl side chains, are assumed to interact via incorporation into the cell membrane leading to an increased permeability. The aim of the following experiments was to show a possible destruction of the cell membrane induced by ILs using molecular biologic methods. If the cell membrane is disrupted by the presence of ILs, cellular content should be released into the supernatant. Hence the remaining pellet includes less intact cells and therefore less intact DNA, which can be detected using qPCR.

Therefore bacterial cells were incubated with ILs for a certain amount of time (1h at 30°C). After incubation, the cells had to be separated from the ILs. Therefore the treated cells were centrifuged and washed, in order to get a clear cell pellet. Following this, the cell pellet could then be used for DNA extraction using Nucleospin and the extracted DNA was used for qPCR.

QPCR results of *L. monocytogenes* treated with different antimicrobials (SDS, BC, and ILs) are presented in Figure 25. All substances were applied in a concentration of 5 % w/v. The DNA recovery rate is given in %. 1x PBS was set as reference point (100%), due to the fact that it can be assumed that treatment with 1x PBS does not affect the cell integrity.

qPCR of *L. monocytogenes* treated with different antimicrobials
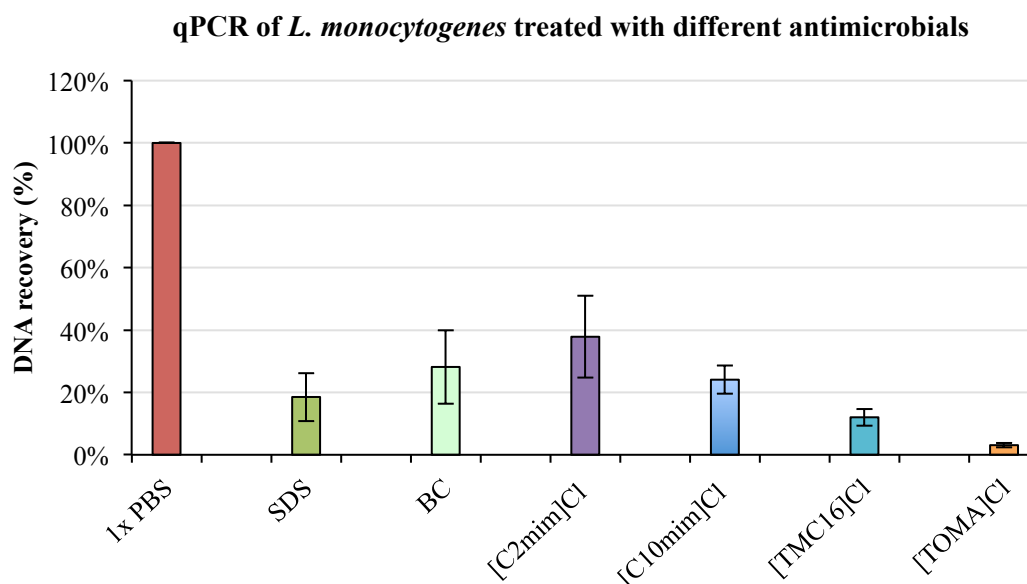
Figure 25: *QPCR results of L. monocytogenes after treatment with 5 % w/v of SDS, BC, and ILs.*

Results in Figure 25 show that the incubation with each antimicrobial substance influenced *L. monocytogenes* cell integrity and led to a loss of DNA content. The data indicate the following: SDS is known for being a strong surfactant and therefore resulted in a clear loss of DNA (about 80% of DNA). Also BC had a significant reduction in DNA concentration, since more than half of the DNA was lost after treatment. Concerning the ILs $[C_2mim]Cl$ had the highest amount of DNA content and therefore not such an effect on the cell membrane of *L. monocytogenes* compared to the other ILs. The DNA loss of $[C_{10}mim]Cl$ treated cells was increased compared to $[C_2mim]Cl$ treated cells, indicating that $[C_{10}mim]Cl$ had a more toxic effect towards the cell membrane. The highest amount DNA loss was in samples exposed to $[TMC_{16}A]Cl$ and $[TOMA]Cl$. $[TOMA]Cl$ for example, resulted in DNA loss of 97%.

qPCR of *S*. Typhimurium treated with different antimicrobials
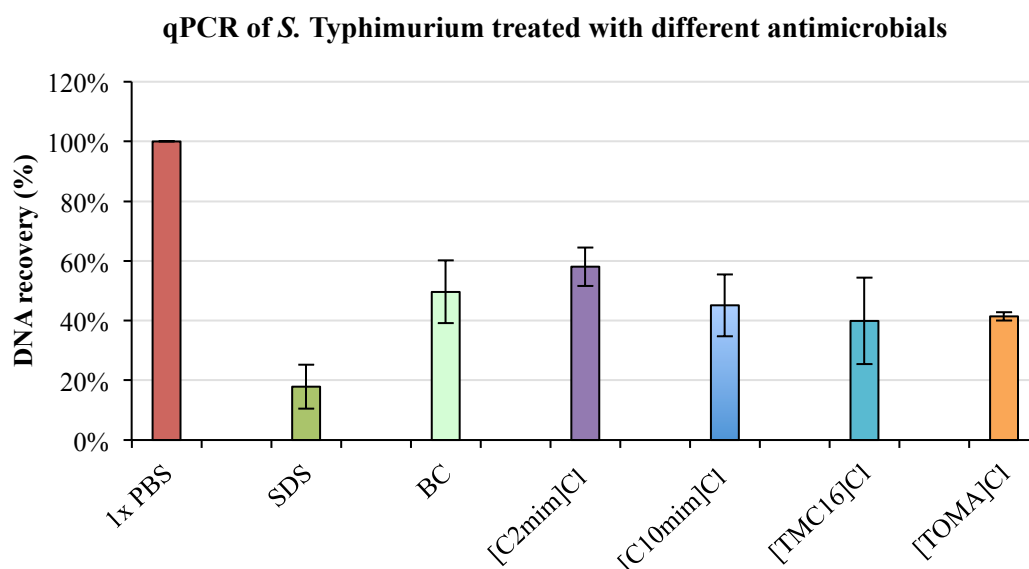
Figure 26: *QPCR results of S.* **Typhimurium** *after treatment with 5 % w/v of SDS, BC and ILs.*

S. Typhimurium showed similar results as *L. monocytogenes* (see Figure 26). All tested antimicrobial substances (SDS, BC, and ILs) had an influence on the cell integrity of *S.* Typhimurium, resulting in a reduction of *S*. Typhimurium DNA content. Incubation with $[C_2mim]Cl$ had the lowest DNA loss, whereas $[TMC_{16}A]Cl$ and $[TOMA]Cl$ showed the highest loss of DNA. Overall for all tested antimicrobial substances except SDS the DNA loss for *S*. Typhimurium was lower compared to *L.monocytogenes*.

In the previously described experiments only a specific concentration (5 % w/v) was applied. Therefore the idea was to test, if a concentration dependent effect can be observed

in these experiments. In order to test this, the antimicrobial substances (BC, and ILs) were tested in three different concentrations, which are explained below in more detail.
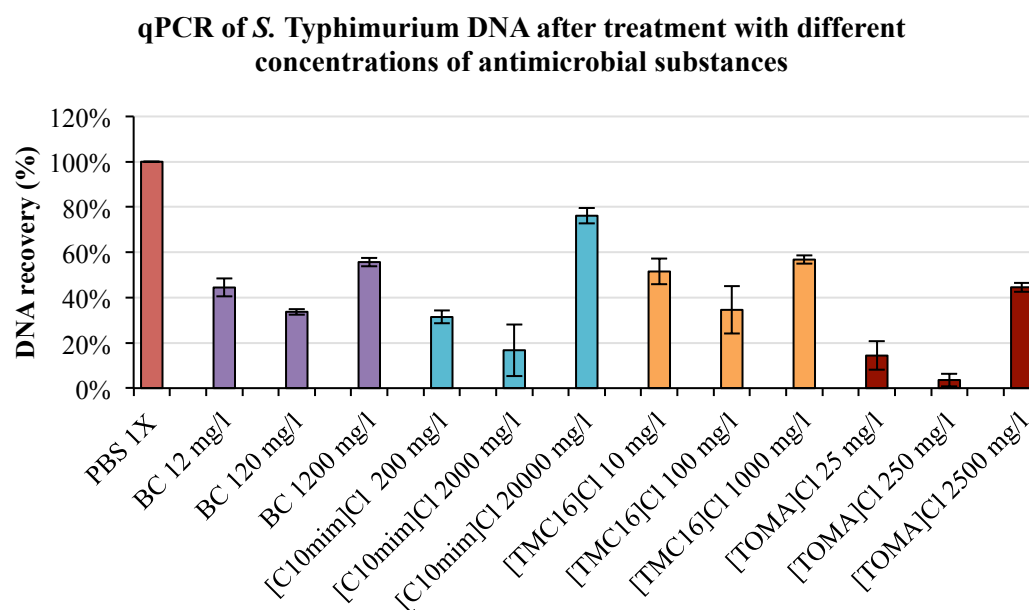
qPCR of *S.* Typhimurium DNA after treatment with different concentrations of antimicrobial substances

Figure 27: *QPCR results of S. Typhimurium after treatment of cells with different concentrations (MIC, 10x MIC, and 100x MIC) of an antimicrobial substance.*

Figure 27 shows the results for the concentration dependent experiments. In this experiment, *S.* Typhimurium was treated with three different concentrations of BC and ILs ([C_{10}mim]Cl, [TMC$_{16}$A]Cl, and [TOMA]Cl). After treatment of the *S.* Typhimurium cells with these different antimicrobials, *S.* Typhimurium DNA was extracted and used for qPCR (see 3.7.). The concentrations selected for this experiment, were the MIC, 10x MIC, and 100x MIC. The concentration of 10x MIC resulted in a higher DNA loss compared to the MIC. 100x MIC resulted in higher DNA recovery as 10x MIC and the DNA loss at 100x MIC was less than the MIC. These results were observed for all other tested antimicrobials as well.

3.8 Determination of IL-toxicity using BacLight[TM]

The principle of BacLight[TM] is the detection of live and dead cells under the microscope using two different fluorescent dyes (see chapter 2.2.1.6). The aim of this particular experiment was to give a microbiological prove of the previously obtained results from the qPCR experiments preformed in (chapter 3.7). After treating *L. monocytogenes* cells with the respective ILs, SDS and PBS it should be possible to see a pattern, which is comparable to the qPCR experiments suggesting that the cell membrane is destructed by the presence of ILs and therefore the target of the toxicity of ILs. The experiment was performed the same way as illustrated in 3.7 for the qPCR with exposing bacterial cells to antibacterial substances for 1h at 30°C. After washing and centrifugation steps the cells were directly used for BacLight[TM] staining.

BacLigth of *L. monocytogenes* after treatment with different antimicrobials
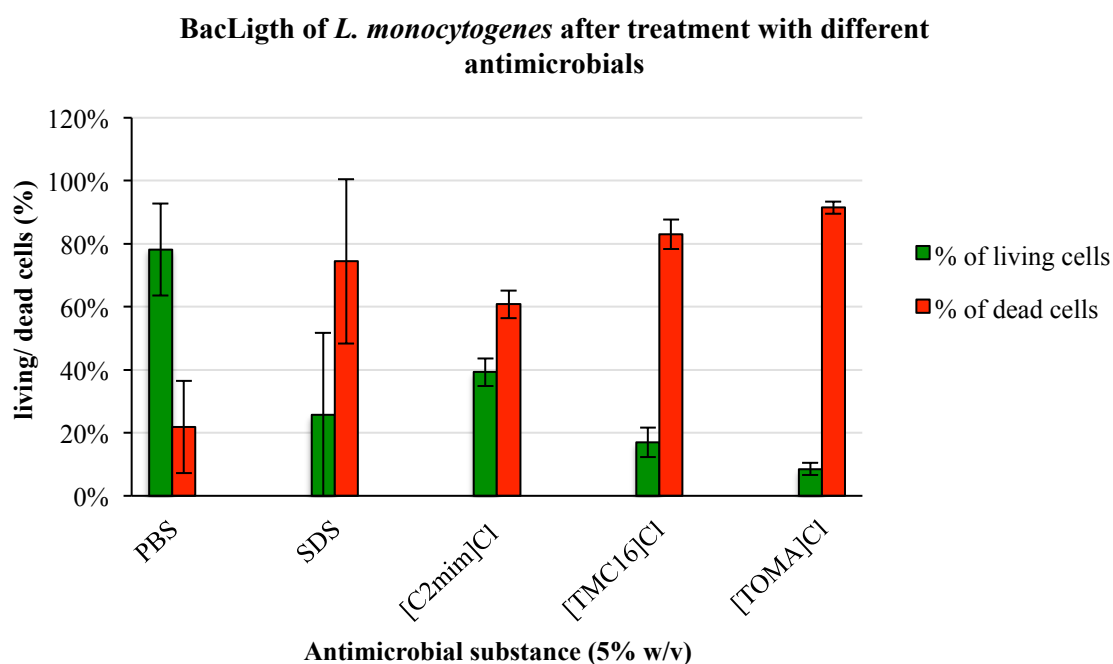
Figure 28: *Percentage of living/ dead L. monocytogenes cells after 1h exposure to antimicrobial substances (SDS, and ILs) according to BacLight[TM].*

All substances were compared to 1X PBS, which was used as reference. The substances were used in concentration of 5 % w/v. [C₂mim]Cl was the least toxic IL with almost 40% of green cells whereas [TOMA]Cl was the most toxic substance resulting in only 8.5% of green cells (Figure 28).

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4. Discussion

The central question, which was addressed in the course of this study, is: Can ILs with QAC- like cations be a beneficial alternative to commonly used QACs? Therefore following aspects were investigated:

- Synthesis of novel ILs incorporating quaternary ammonium structures
- Investigation of molecularly mediated tolerance of *L. monocytogenes* cells against ILs with QAC like structure
- Adaption of *L. monocytogenes* cells to ILs after sublethal exposure
- ILs that can probably circumvent the mechanisms of the efflux pump
- Investigation of the cell membrane damage induced by the presence of ILs

All of this task will be discussed in the following paragraphs.

4.1 Synthesis novel ILs incorporating quaternary ammonium structures

In general synthesis of ILs is a complicated process, which is normally performed by special chemical engineers. Due to the fact that our laboratory is not equipped with any specific apparatuses for IL-synthesis, there was a need to find an alternative. In corporation with the Proionic (Grambach, Austria), we managed to synthesize ILs in a laboratory scale. The Proionic invented a process that allows synthesis of novel ILs just by adding any acid with a pKa value of >9 to an IL- precursor. These two components have to be put together in a equimolar ratio to allow the reaction to work. If this is done properly, CO_2 is released. The reaction can be accelerated by adding Methanol and applying heat. At the end all remaining solvents are removed using a SpeedVac. This method is called CBILS® route. The Proionic provided us with a set of different IL-precursors in the course of this study, so we were just performing the last steps of the CBILS® route. Using this method customized ILs can easily be synthesized, if all requirements of the method are considered. Based on the novel synthesized ILs further experiments were conducted in order to create a database, which should help to evaluate the potential of ILs as possible antimicrobial agents.

In the course of this study more than 100 different ILs were synthesized using the CBILS® route. Here only a few of them are described, which were considered as promising in terms of toxicity and chemical structure. In this study ten of them are described, all incorporating

a quaternary ammonium in their cationic core. These ten ILs include [TOMA] based, [TMC$_{12}$A] based and [TMC$_{16}$A] based ILs. The synthesized [TOMA] based ILs were immiscible in water, which was probably due to the fact that the [TOMA] cation includes three octyl side chains. These structural elements contribute to a lipophilic character, and therefore prevent a complete solubility in water. For this reason, the ILs were successfully dissolved in Methanol or Ethanol, in order to create a stock solution that serves as basis for working solutions prepared with water. Also ILs with [TMC$_{16}$A] were prepared in the same way as those ILs with [TOMA] cation. Besides solubility, the most essential parameter is conductivity. Conductivity of ILs is a parameter for ion dissociation of ILs. It enables to estimate how the ions of an IL behave and allow predictions, like if the ions are acting separately in a solution or as an ion pair. Low conductivity indicates, that the ions tend to stay together as an ion pair. In the course of the screening process a correlation between conductivity and toxicity of ILs could be observed. ILs with low conductivity are mostly toxic, like for example [TOMA] based ILs. In general the conductivity of TOMA based ILs and the tested ILs with a trimethylalkylammonium cation ([TMC$_{12}$A] based and [TMC$_{16}$A]) was low, compared to KCl. As already described for [TOMA] based ILs, ILs with trimethylalkylammonium cation had also a low toxicity. The toxicological aspects of these synthesized ILs and their influence on *L. monocytogenes* is discussed later on.

4.2 Antibacterial effect of the alkyl side chain of the anion in ILs

Nowadays, especially for imidazolium based ILs, the relationship between the length of the alkyl side-chain substituted on the imidazolium ring, and the planarity of the cation have been the most studied toxicity effects of ILs against bacteria. An increase in alkyl length substituted on the imidazolium ring of the cation leads to an increase of toxicity, which is described as side-chain effect [124,119]. Mester et al. (2015) demonstrated that in imidazolium ILs [$C_n$mim] with a side chain up to n <6 (hexyl) the anion moiety, more exactly the chaotropicity of the anion, was the main factor influencing the toxicity [127]. Other studies reported that a relationship between lipophilicity of the anion or the number of fluorine atoms and their toxicity towards prokaryotic cells exist [79]. However, the majority of toxicity studies focused on the toxicity of the cationic core and length of the incorporated alkyl side chain in imidazolium ILs. Therefore, further research focussing on the anion part of ILs is necessary.

In the conducted experiments (see chapter 3.2) two different species of ILs were applied: imidazolium based ILs, where [C_2mim] constitutes the cationic core and a type of ammonium based ILs, where [N,N- dimethyl-2 hydroxyethyl- ammonium] is the cationic core. The only varying factor in these ILs was the anion. In case of the [N,N- dimethyl-2 hydroxyethyl- ammonium] ILs, three different anions where used: acetate, propionate, and caprylate. For the [C_2mim], additional anions were used: caprylate, caprynate, stearate, and palmitate. The used anions are all carboxylic acids and so far, no toxicity data of ILs incorporating carboxylic acids as anion has been described. For both IL species, the anion moiety seemed to be the essential part concerning the toxicity. The only varying structural component was alkyl side chain length of the anion moiety in these ILs. For all tested [N,N- dimethyl-2 hydroxyethyl- ammonium] and [C_2mim] ILs, toxicity increased with the length of the alkyl side chain. Therefore [N,N- dimethyl-2 hydroxyethyl- ammonium] caprylate had the highest toxicity of all tested [N,N- dimethyl-2 hydroxyethyl- ammonium] ILs. In case of the imidazolium based ILs [C_2mim]palmitate had the highest toxicity, which was suprising, since [C_2mim]palmitate does not have the longest alkyl side chain of the tested imidazolium based ILs. Results showed that the MIC of [C_2mim]stearate was significantly lower than of all other tested imidazolium based ILs, which was not expected up to this point. So far there is no explanations for these results.

Concerning the MIC the two tested bacterial species *L. monocytogenes*, and *E. coli* did not differ significantly from each other after exposure to respective ILs. These results suggest,

that the cell wall of *L. monocytogenes* does not necessarily give an increased tolerance to these substances.

Taking facts together, it was shown for the first time that the length of the alkyl side chain in anions with carboxylic acid nature, plays an essential role in the toxicity of ILs with [C$_2$mim] and [N,N- dimethyl-2 hydroxyethyl- ammonium] as cation. This is fundamental finding, because so far, no results investigating the toxicity of the anion side chain in ILs exist. Since the used anions are fatty acids and therefore biologically degradable, these ILs could be interesting for future applications. Furthermore, a toxic effect of a short chained imidazolium IL ([C$_2$mim]) with palmitate incorporated in its structure was demonstrated. The fact that [C$_2$mim]palmitate had a surprisingly high toxicity is also promising with regard to an application of this ILs as an disinfectant. Since palmitate is a naturally occurring substance and is therefore relatively easy accessible compared to synthesized substances.

4.3 Molecularly mediated tolerance of *L. monocytogenes* against ILs with QAC like structures

L. monocytogenes is known for using efflux pumps against disinfectants like BC, to persist in food processing environments. This is a major problem in food processing industry, where factors like food debris, biofilm formation, inadequate cleaning and disinfection or dosage failure promote the exposure to sublethal concentrations and therefore the development of increased tolerance to disinfectants.

Therefore based on the work from Müller et al. (2012) *L. monocytogenes* strains harbouring a QAC specific efflux pump, encoded by the transposon Tn*6188*, were used for investigation of their tolerance to ILs with QAC compound structures. In addition the consequences of exposure to sublethal concentrations of these ILs were investigated.

4.3.1 Tolerance of *L. monocytogenes with* QAC specific efflux pumps to ILs

The aim of the experiments described in chapter 3.3 was to investigate, if *L. monocytogenes* strains with a QAC specific efflux pump against BC, also show an increased tolerance to ILs.

Increased tolerance of *L. monocytogen*es strains harbouring transposon +Tn*6188* could be successfully shown for the QACs benzalkonium chloride, benzethonium chloride, domiphen bromide, and DTAB. The +Tn*6188 L. monocytogenes* strains showed elevated MIC levels compared to those *Listeria* strains lacking Tn*6188* and the encoded QAC

specific efflux pump. Imidazolium based ILs used in the study differed in the length of the alkyl side chain substituted on the cation. [C_8mim]Cl and [C_{10}mim]Cl displayed the most distinct results, showing the lowest MIC levels within the tested imidazolium ILs. *L. monocytogenes* strains harbouring the transposon +Tn*6188* showed increased MIC levels against [C_8mim]Cl and [C_{10}mim]Cl. The results indicate that the increased tolerance is caused by the encoded efflux pump. Differences concerning the MIC could be seen between these imidazolium ILs, with increasing length of the alkyl side chain. The underlying toxicity effect is the so-called side chain effect. However, for [C_2mim]Cl, [C_4mim]Cl, and [C_6mim]Cl the side chain effect could not be investigated, as their MIC levels were not within the test parameters (MIC> 50000 mg/l). Influence of Tn*6188* is therefore probably only effective for [C_8mim]Cl and [C_{10}mim]Cl. ILs with short chain lengths presumably do not interact with the membrane to such an extent and for those ILs, the influence of the anion moiety is the key toxicity mechanism [127].

Similar results were obtained when the experiment was repeated using trimethylalkylammonium ILs. Although different anion moieties were applied, the data show that altering of anion moiety had no significant influence concerning the effect of the Tn*6188* coded efflux pump. Therefore MIC levels among those trimethylalkylammonium ILs were similar. Since those trimethylalkylammonium ILs have long alkyl side chains substituted on the cation, the effect of the long alkyl side chain seems to be the main part in the toxicity of these ILs. Conductivity measurements, which were preformed with [$TMC_{12}A$]Cl, [$TMC_{12}A$]peridate, [$TMC_{16}A$]Cl, and [$TMC_{16}A$]peridate resulted in a low conductivity suggesting that the anion and cation stick together in aqueous solution, but did not interfere with the effect of the efflux pump.

In summary it could be demonstrated for the first time that bacterial defence mechanisms, like QAC specific efflux pumps, also apply for ILs with quaternary ammonium structure. This is fundamental finding for two reasons: on the one hand ILs succumb the same tolerance mechanisms as QACs, and on the other hand that the tested class of ILs (imidazolium based, and trimethylalkylammonium based) could not provide an alternative to circumvent the *L. monocytogenes* efflux pumps. Therefore further research needs to be conducted in this field, using novel ILs with different cation cores such as sulfonium or oxonium.

4.3.2 Adaption of *L. monocytogenes* strains after sublethal exposure to increased levels of ILs, QAC species, and other antimicrobial substances

L. monocytogenes strains were exposed to sublethal concentrations of ILs, with quaternary ammonium structures. The aim was to figure out, if *L. monocytogenes* strains could adapt to increased levels of ILs, QACs and other antimicrobial substances after prior exposure to sublethal concentrations of ILs.

Listeria strains CDL 2 as −Tn*6188* representative and 6179 as +Tn1688 representative strain were adapted to BC and ILs. The results indicate that the adaption protocol was successful for BC as well as for the investigated ILs. *L. monocytogenes* strains 6179 and CDL2 were successfully adapted to higher concentrations of the imidazolium based IL [C$_{10}$mim]Cl and the trimethlylalkylammonium IL [TMC$_{16}$A]maleate . The adapted *Listeria* strains were not only tested against BC, [C$_{10}$mim]Cl or [TMC$_{16}$A]maleate but also against other types of ILs, QACs and also antibiotics. The fact that the IL-adapted *Listeria* strains CDL 2 and 6179 also showed adaption against the QAC species DTAB and the IL [TMC$_{12}$A]maleate indicates, that the adaption is not only limited to same antimicrobial components. This can be explained by a mechanism better known as cross resistance. Cross resistance is typical for so called small multidrug transporter proteins like Tn6188. Therefore also increased tolerance to ILs could be observed in BC adapted *L. monocytogenes* cells.

4.3.3 The effect of TOMA based ILs on the viability of +Tn6188 *L. monocytogenes* cells

In the course of screening a diverse set of different ILs species, an interesting class of ammonium based ILs with three octyl substituted cationic side chains, namely [TOMA] based ILs, was identified. The toxicity of these ILs ranged from 1-10 mg/l. This high toxicity is likely due the presence of three octyl side chains, thus given the cation a high lipophilicity. Because of their high toxicity these [TOMA] based ILs were then tested with respect to the previously described *L. monocytogenes* strains and their QAC specific efflux pumps.

The [TOMA] based ILs showed a completely different pattern of tolerance compared to the tested imidazolium and trimethylalkylammonium ILs. For all ten tested *L. monocytogenes* strains (+/- Tn6188) the same MIC levels were found, indicating that the efflux pump did not positively influence the tolerance of the respective strains. Although so far, there is no

clear evidence for this hypothesis, it is reasonable to assume that structural differences between the ILs classes are responsible for these results. In contrast to the ILs deployed by tolerance towards +Tn6188 strains, the [TOMA] based ILs have three long alkyl (octyl) side chains. A possible explanation for their high toxicity against bacteria could be, that the [TOMA] cation is too bulky to be transported out of the cell via an efflux pump.

Nevertheless, it has to be considered that experiments with this class of ILs are still ongoing and further research is needed. However, the results are promising for two reasons: On the one hand [TOMA] based ILs exerted a high toxicity against *L. monocytogenes*. On the other hand these ILs seem to circumvent the bacterial defence, opposed by the Tn*6188* coded efflux pump of *L. monocytogenes*. Although this [TOMA] based ILs are hydrophobic, a possible application as disinfectant should not be excluded. [TOMA] based ILs can easily be dissolved in Methanol prior to the preparation of an aqueous final solution.

4.3.4 Conclusion

The results are very essential for further research. The tested quaternary ammonium containing ILs do not circumvent the defense mechanism opposed by the Tn*6188* encoded efflux pump. However, the tested [TOMA] based ILs could circumvent the efflux pump, suggesting a possible future application as disinfectant. Nevertheless, further research concerning these [TOMA] based ILs needs to conducted, including other relevant foodborne pathogens. Moreover, the (eco)toxicological effect of these ILs and the biodegradability with respect to their future application needs to be investigated.

4.4 Investigation of the cell membrane damage induced by ILs

The toxicity effect of ILs with long alkyl side chains substituted on the cationic core (like imidazolium ILs) can be explained by lipophilicity. They can be incorporated in the plasmamembrane, changing the lipid bilayer and leading to an increased permeability. This toxic effect is being described throughout the literature [124,126], however it has never been directly shown applying molecular biological methods. Therefore qPCR was applied as method to describe membrane damage by detecting the loss of DNA content out of the cell. Toxic ILs should destroy the cell membrane leading to a loss of DNA into the supernatant.

For the qPCR experiments the most toxic substances, identified in the course of the screening process, were chosen and investigated. Overall the results of qPCR were in good agreement with the MIC screenings of the respective ILs, implying that a very toxic ILs leads to high amount DNA loss and therefore significant cell membrane damage must be the case. *L. monocytogenes* cells treated with [TOMA]Cl for example, showed a loss of initial DNA amount of about 97 % in qPCR. No significant difference could be found between gram-positive and gram-negative bacteria concerning the DNA loss. The qPCR results for S. Typhimurium and *L. monocytogenes* were quite similar, suggesting that the second lipid membrane layer in gram-negative bacteria has no positive effect in terms of tolerance to antimicrobials (BC, and ILs).
Moreover, the obtained data of the qPCR are supported by the cell staining viability experiments (BacLight™ kit), which delivered a confirmation of the molecular biological data of the qPCR. This staining also demonstrates cell membrane damage and distinguishes between intact and dead cells through fluorescent dyes. In the experiments, [TOMA]Cl and [TMC$_{16}$A]Cl resulted in the highest percentage of dead cells with damaged cell membrane.

To investigate a possible concentration dependency of the membrane destabilization, the bacterial cells were exposed to different IL-concentrations: the respective MIC, 10x MIC and 100x MIC. Indeed a concentration dependent mechanism could be seen for the respective antimicrobial substances (BC and ILs). The 10x MIC of each substance compared to 1x MIC resulted in a higher DNA loss. However, surprisingly the highest MIC concentration, 100x MIC, resulted in the lowest DNA loss of all tested concentrations. This is an interesting finding, since it was supposed that the highest concentration would result in the highest loss of DNA. A possible explanation for this effect could be the formation of micelles by the ILs, due to reaching the so-called critical micelle concentration (CMC). The CMC is the concentration, above which surfactants form micelles in an aqueous solution.

This could also be the case for ILs, indicating that above a certain concentration ILs form micelles, leading to a decrease of freely available and dissolved ILs and therefore to a decrease in toxicity. Following this, the percentage of lost DNA and therefore the damage to the cell membrane is decreased. Consequently, such a biochemical effect cannot be measured by MIC, since the concentration of IL is significantly lower. So far this is only a hypothesis and therefore further experiments need to be conducted in the future.

In summary, it could be demonstrated that the results of the qPCR and BacLight™ were in good agreement with previously conducted experiments investigating the MIC. The tested ILs demonstrated that the cell membrane is affected by their presence, and is probably the target of toxicity effects of the tested ILs. Nevertheless, qPCR and BacLight™ cannot give exact information about the state of the bacteria. Therefore other studies need to be conducted in order to clarify the exact toxicity mechanism. Furthermore, the results concerning the concentration dependent toxicity of ILs are essential for future applications.

5. Conclusion

QACs (Quaternary ammonium compunds), like benzalkonium chloride are widely and extensively used throughout the food processing industry, although application of these disinfectants bears certain disadvantages. Foodborne pathogens, like *L. monocytogenes* are able to persist in food processing plants using QAC specific efflux pumps. Due to inadequate use of QACs, adaption processes occur which strongly complicate the irradication of persistent *L. monocytogenes* strains. ILs on the otherside are a class of chemicals, which are widely used in various fields of industry due to their specific and tuneable characteristics. Although known for a longer period of time, biological applications and especially studies concerning the biological activity of ILs are limited.

To overcome this problem a set ten of novel ILs was successfully synthesized and analysed.

Besides this, toxicity of the side chain of the anion moiety in ILs was investigated. It could be demonstrated that with increasing anion side length, the toxicity in [N,N- dimethyl-2 hydroxyethyl- ammonium] as well as [C_2mim] based ILs increased.

It could also be demonstrated that a QAC specific efflux pump, encoded by the transposon Tn*6188*, provided tolerance to ILs with ammonium structures. Furthermore, it could be shown that *L. monocytogenes* strains can be adapted to increased MIC levels of ILs with ammonium structures, after exposure to sublethal concentrations. The IL-adapted *L. monocytogenes* strains did also show increased tolerance to structural similar ILs, and different QAC species. IL-synthesis revealed a promising class of ILs, namely [TOMA]based ILs, which could circumvent the tolerance mechanism in *L. monocytogenes* strains mediated by the Tn*6188* encoded efflux pump.

Applying qPCR and BacLight^{TM} it could be shown that the bacterial cell membrane is the most probable target of ILs and their toxicity can be explained due to structural membrane damage.

Some of the results obtained in this master thesis were successfully published this year in the Journal *"Seperation and Purification Technology"*. In addition two posters were created based on this thesis, which were presented to a scientific community in Vienna (Austria) at the *"OEGMBT Annual Meeting"* (2014) and *"55. Tagung des Arbeitsgebietes Lebensmittelhygiene der DVG"* (2014) in Garmisch- Partenkirchen (Germany). Further details can be taken from the Supplemental.

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Curriculum vitae

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10/2011-now:	**University of Vienna,** Vienna/ Austria Master study in Molecular Microbiology and Immunology
10/2008- 08/2011:	**University of Innsbruck, BSc,** Innsbruck/ Austria Bachelor study in Biology
09/1999- 06/2007:	**Secondary education- humanistic high school in** Reutte/Austria A- levels in Mathematics, German, English, Physics, Biology and Latin

Work experience

08/2013- now:	**Christian Doppler Laboratory for Monitoring of Microbial Contaminants**, University for Veterinary Medicine, Vienna Univ.-Prof. Dr.med.vet. Dipl.ECVPH Martin Wagner Dr.rer.nat Peter Rossmanith
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07/2011- 08/2011:	**Internship at Tyrolean Cancer Research Intsitute (TKFI),** Innsbruck/ Austria Cell culture, Western Blot and ELISA
06/2008- 09/2010:	**Paramedic at the Red Cross,** Ehrwald/ Austria
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Personal interests

Sports: Rugby, Golf, Swimming, and Climbing

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Language skills

German: mother tongue, spoken and written

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Zusammenfassung

Erkrankungen, die durch kontaminierte Lebensmittel hervorgerufen werden, sind ein wesentliches Problem. Die Übertragung von Zoonoseerregern erfolgt einerseits über den Konsum kontaminierter Lebensmittel und anderseits über den Kontakt zu infizierten Tieren. Obwohl es bei Produktion von Lebensmitteln diverse Richtlinien gibt, kommt es immer wieder zu Krankheitsausbrüchen im Zusammenhang mit verdorbenen Lebensmitteln. Ein essentielles Lebensmittelpathogen ist *Listeria monocytogenes*. Dieses gram- positive Pathogen stellt in lebensmittelverarbeitenden Betrieben eine entscheidende Problematik dar. Trotz der Tatsache, dass lebensmittelverarbeitende Betriebe regelmäßig und extensiv gereinigt/desinfiziert werden schaffen es *Listerien* in Nischen innerhalb des Betriebes zu überleben. Mit Hilfe eines umfassenden Repertoires an Mechanismen vermag es *L. monocytogenes* in Betrieben zu persistieren und speziell den dort vorherrschenden Reinigungs- bzw. Desinfektionsdruck zu überwinden. Dabei hat sich gezeigt, dass *Listerien* welche über längere Zeit sublethalen Konzentrationen von Desinfektionsmitteln (vor allem Quaternären Ammonium Salze, QACs) ausgesetzt sind, an diese Bedingungen adaptieren und erhöhte Toleranz sowie erhöhte Virulenz entwickeln. Die wohl am häufigsten eingesetzten Desinfektionsmittel basieren auf quaternären Ammoniumsalzen, wie beispielsweise Benzalkoniumchlorid. Daher ist die Suche nach umweltfreundlichen Alternativen von entscheidender Bedeutung. Eine mögliche und durchaus vielversprechende Alternative sind ionische Flüssigkeiten.

Das Ziel der vorliegenden Arbeit war es zu untersuchen, ob jene Abwehrmechanismen die *Listerien* bei der Anwendung klassischer ammonium basierter Desinfektionsmittel (QACs) zeigen, auch im Zusammenhang mit ionischen Flüssigkeiten als alternatives Desinfektionsmittel vorherrschend sind. Außerdem ging es darum eine Methode zu finden, um die vielbeschriebene Toxizität von ionischen Flüssigkeiten zu zeigen. Im Zuge dieser Arbeit konnte dann zum ersten Mal gezeigt werden, dass Listerien mit QAC-spezifischen Effluxpumpen auch gegen ionische Flüssigkeiten eine erhöhte Toleranz zeigen sowie auch eine Adaption gegen diese Substanzklasse beobachtet wurde. Durch ein weiteres Screening von ionischen Flüssigkeiten konnte eine Klasse von ionischen Flüssigkeiten basierend auf einem [TOMA] Kation identifiziert werden, bei denen die getesteten Listerien keinen Toleranzmechanismus zeigten. Die Wirkungsweise ionischer Flüssigkeiten wurde mittels Real Time PCR untersucht. Dabei konnte die Zellmembran als mögliches Target des vorherrschenden Toxizitätsmechanismus identifiziert werden.

Abstract

Food borne disease is a problem, which is of major concern. Transmission of foodborne pathogens either occurs by consumption of contaminated food or by contact to infected animals. Although several directives regulate every single step during food production, outbreaks of food- related disease happen frequently. One of the main food borne pathogens is *Listeria monocytogenes*. This gram- positive, facultative intracellular pathogen is a major concern in food processing plants. Despite the fact that food processing plants are cleaned on a large scale and the extensive use of disinfectants, *Listeria* manages to survive in niches within the plant. Using a set of diverse defence mechanisms *L. monocytogenes* is able to persist in food processing plants and to survive the predominant disinfection stress. Especially the extensive use of disinfectants is an essential issue. One of the most applied classes of disinfectants are quaternary ammonium compounds, such as benzalkonium chloride. Following an exposure to sublethal concentrations of disinfectants over a long period of time, *L. monocytogenes* can adapt to these concentrations resulting in an increased tolerance and virulence. Therefore searching an environmentally friendly alternative with similar effects is crucial. A promising alternative to these classic disinfectants are ionic liquids (ILs). ILs constitute a relatively new class of substances and less information is currently available.

The aim of this work was to investigate, if defence mechanisms of *L*. monocytogenes against QACs also apply to ILs. Furthermore a method should be found to describe the toxicity of ILs. For the very first time it could be demonstrated that efflux pumps specific for quaternary ammonium compounds in *L. monocytogenes* show a similar tolerance pattern against ILs with quaternary ammonium structures. Besides this, an adaption process of *L. monocytogenes* to elevated concentrations of ILs could be observed. Following this, a class of ILs based on [TOMA] cations was identified, which did not show the same tolerance pattern. Using Real Time PCR the possible toxicity mechanisms of the tested ILs was determined. Results indicate that the destruction of the cellular membrane is the probable target of toxicity.

Supplemental

Molecular mechanisms mediating tolerance to ionic liquids in *Listeria monocytogenes*

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ARTICLE INFO

ABSTRACT

Increased application of ionic liquids (ILs) in various chemical and biochemical processes as well as for pharmaceuticals or antimicrobial agents has prompted researchers to assess their biological functions, especially their toxicity. However, to date the molecular mechanisms underlying tolerance of certain bacterial species to ILs has not been studied in detail. The recently found transposon Tn*6188* in *Listeria monocytogenes* has been shown to mediate tolerance to quaternary ammonium compounds (QACs) such as benzalkonium chloride, which are widely used as disinfectants in food processing plants, via QacH, a small multidrug resistance protein family (SMR) transporter. In this study the possible Tn*6188* mediated tolerance of *L. monocytogenes* to ILs was investigated. It was found that Tn*6188* confers tolerance of *L. monocytogenes* to ILs based on imidazolium and ammonium cations.

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1. Introduction

Increased applications involving ionic liquids (ILs) have led to increased knowledge about their ecotoxicity, especially their acute toxicity, biostability and biodegradability. However, the once neglected so far in both toxicological studies of ILs and possible applications as biologically active compounds, is the cellular response in terms of possible stress and defense mechanisms.

Bacteria, which are often used in these studies, are well known to possess and adapt cellular defense strategies that help them

Molecular mechanisms mediating tolerance against ionic liquids in Listeria monocytogenes

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ABSTRACT

The increasing application of ionic liquids in various chemical and biochemical applications as well as pharmaceuticals or antimicrobial agents led researchers to asses their biological functions, namely toxicity. However, so far the molecular mechanisms underlying tolerance of certain bacterial species against ILs has not been studied in detail. The recently found transposon Tn6188 in *Listeria monocytogenes* has been shown to mediate tolerance against quaternary ammonium compounds (QACs) such as benzalkonium chloride, which are widely used as disinfectants in food processing plants, via qacH a small multidrug resistance protein family (SMR) transporter. In this study the possible Tn6188 mediated tolerance of *L. monocytogenes* against ILs was investigated.

MATERIALS & METHODS

To investigate the effect of the QAC-tolerance mediating efflux pump *qacH*, located on the Tn6188 Transposon, ten different *L. monocytogenes* strains, 5 strains harboring Tn6188 (CDL78, 6179, N22-2, CDL64, F17) and 5 strains without Tn6188 (CDL65, R479a, CDL77, CDL2, 535), were used in this study. All *L. monocytogenes* strains were screened for the presence of the bcrABC resistance cassette located on Tn6188, using primers BcF5 and BcR targeting the *bcrABC* genes (Müller et al. 2013).

In vitro antibacterial activities, expressed as the minimal inhibitory concentration (MIC), of 5 imidazole based ILs ([$C_n$mim]Cl with n = 2, 4, 6, 8 and 10), 14 Trimethylalkylammonium based ILs ([TMC_nA]X with n = 4, 8, 12 and 16 and X = Cl, malate, propylbenzoate and periodat) and four QACs (benzalkonium chloride, DTAB, domiphen bromide and benzenthonium chloride) were determined in triplicate by the microbroth dilution assay.
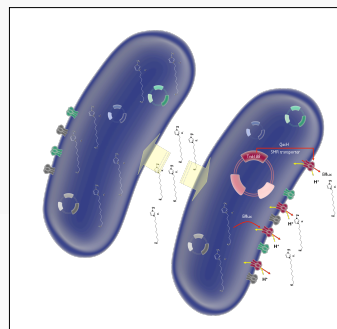

Fig. 1. Schematic representation of the role of the QAC tolerance mediating transposon Tn6188 in *Listeria monocytogenes*. This picture was created using parts of the Library of Science and Medical Illustrations by Sommersault18:14 (http://www.somersault1824.com/science-illustrations/) under the Creative Commons license CC BY-NC-SA 4.0.

RESULTS I

- *Listeria monocytogenes* strains harboring Tn6188 show higher MIC values against QACs (Fig. 2 and table 1)

- *L. monocytogenes* strains harboring Tn6188 show higher MIC values against all tested ILs with cationic side chain lengths >6 (Fig. 3 and 4 and table 1)

- The "side-chain effect" (increasing toxicity with longer cationic alkyl side chains) was found for all tested *L. monocytogenes* strains independent from Tn6188
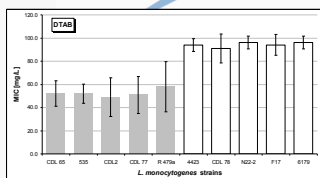

Fig. 2. Minimal inhibitory concentration [mg/L] with the respective Standard deviation of DTAB for 10 different *L. monocytogenes* strains. Strains harboring Tn6188 (+Tn6188) are shown in white and strains without Tn6188 (-Tn6188) are shown in grey.
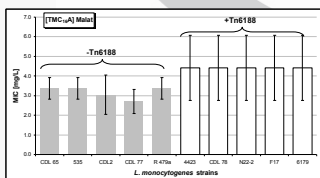
Fig. 3. Minimal inhibitory concentration [mg/L] with the respective Standard deviation of [TMC_16A]Malate for 10 different *L. monocytogenes* strains. Strains harboring Tn6188 (+Tn6188) are shown in white and strains without Tn6188 (-Tn6188) are shown in grey.

Fig. 4. Minimal inhibitory concentration [mg/L] with the respective Standard deviation of [C_10mim]Cl for 10 different *L. monocytogenes* strains. Strains harboring Tn6188 (+Tn6188) are shown in white and strains without Tn6188 (-Tn6188) are shown in grey.
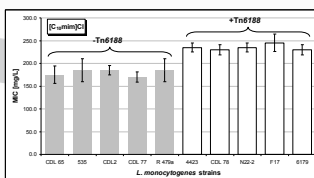
Table 1

MIC [mg/L] values after 24h of all tested ILs. The results for individual *L. monocytogenes* strains were combined into the two groups +Tn6188 or -Tn6188, dependend on the presence of Tn6188 in the respective strain.

Ionic liquid	MIC [mg/L] 24h			
	L. monocytogenes (-)Tn6188		*L. monocytogenes* (+)Tn6188	
[TMC_4A]Cl	25000.0	± 0.0	30000.0	± 11180.3
[TMC_4A]Malate	30000.0	± 10351.0	30000.0	± 10351.0
[TMC_4A]Propylbenzoate	1484.3	± 784.1	1510.3	± 751.0
[TMC_8A]Cl	345.0	± 38.7	457.5	± 23.7
[TMC_8A]Periodate	440.0	± 51.6	610.0	± 31.6
[TMC_8A]Malate	548.3	± 89.9	963.3	± 281.4
[TMC_8A]Propylbenzoate	581.7	± 52.2	883.3	± 281.4
[TMC_12A]Cl	295.0	± 95.6	475.0	± 131.8
[TMC_12A]Periodate	45.0	± 10.5	150.0	± 0.0
[TMC_12A]Malate	303.3	± 115.7	416.7	± 64.5
[TMC_12A]Propylbenzoate	433.3	± 48.8	600.0	± 207.0
[TMC_16A]Cl	2.9	± 1.2	5.0	± 0.8
[TMC_16A]Periodate	3.0	± 2.5	5.6	± 2.1
[TMC_16A]Malate	3.0	± 0.7	3.4	± 0.5
[TMC_16A]Propylbenzoate	3.2	± 0.6	4.4	± 1.4
Benzalkonium chloride	3.8	± 0.6	5.0	± 0.5
DTAB	55.0	± 14.6	95.8	± 6.7
Domiphen bromide	3.2	± 1.1	4.2	± 1.2
Benzenthonium chloride	4.2	± 0.8	5.1	± 0.6
[C_2mim]Cl	>50000		>50000	
[C_4mim]Cl	>50000		>50000	
[C_6mim]Cl	25000	± 0	25000	± 0
[C_8mim]Cl	4125.0	± 393.2	4900.0	± 575.8
[C_10mim]Cl	180.0	± 18.4	235.0	± 12.8

DISCUSSION

In good agreement with the results of Müller et al. 2013, who first identified and described Tn6188, *L. monocytogenes* strains harboring Tn6188 show a higher tolerance against "classical" QACs. The strains harboring Tn6188 achieve an higher tolerance due to the expression of the efflux pump *qacH* which belongs to the small multidrug resistance family protein (SMR) transporters. It could be shown in this study that Tn6188 also confers tolerance of *L. monocytogenes* against ionic liquids, just as it does against classical QACs. For all ILs with alkyl side chain length ≥8 the +Tn6188 strains showed a higher tolerance compared to the -Tn6188 strains. The impact was not influenced by the different anions tested in this study. The probable reason why the impact of Tn6188 can only be observed for ILs with side chain lengths ≥8 is due to the fact that the toxicity of ILs correlates with the length of the alkyl side chain, meaning that ILs with shorter side chains are simply not toxic enough to observe the influence of Tn6188.

IN CONCLUSION, the results of this study show that the efflux pump *qacH*, located on the QAC tolerance mediating transposon Tn6188, leads to a significant higher tolerance against ILs, even within the same bacterial species. It can therefore be concluded, that the same mechanisms of bacterial resistance against commonly used disinfectants also mediate tolerance against ILs, at least the ones used in this study. As it has been shown before that IL (eco)toxicity varies widely with organisms and across trophic levels, future examinations should always consider the genetic and defensive background of the test organisms.

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Ionic liquid induced tolerance of *Listeria monocytogenes* against disinfectants

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ABSTRACT

Bacterial resistance against antimicrobials is a major problem in health care and food processing environment. Exposure to sublethal concentrations of disinfectants, often based on quaternary ammonium compounds (QACs), can lead to development of tolerance not only against the same compound but also against other disinfectants as well as antibiotics (*Aase et. al 2000*). Resistance mechanisms of bacteria against antimicrobials include altering metabolic pathways, drug inactivation/modification, altering cell membrane composition and substrate specific efflux pumps. For example *Listeria monocytogenes*, a foodborne pathogen colonizing food processing environments, utilizes QAC specific efflux pumps to protect itself. Ionic liquids have been reported as effective antimicrobials comparable to commercially used QACs such as benzalkonium chloride (BC). However, so far limited information is available about the influence of e.g. efflux pumps in regard to the antimicrobial activity of ionic liquids. In this study, the influence of sublethal concentrations of different QACs and ionic liquids on the development of general tolerance against disinfectants and antibiotics was investigated. Two different strains of *L. monocytogenes*, one harboring an QAC- tolerance mediating transposon (+Tn6188) and one lacking transposon (-Tn6188) were used (*Müller et. al 2013*).

Tab. 1: List of chemical substances and antibiotics used in this study.

	Chemical Name	Abbreviation
QACs	Dodecyltrimethylammonium bromide	DTAB
	Benzalkonium chloride	BC
Ionic Liquids	1-Decyl-3-methylimidazolium chloride	DecMIM Cl
	Trimethyldodecylammonium malate	TMC$_{12}$ Malate
	Trimethylhexadecylammonium malate	TMC$_{14}$ Malate
Antibiotics	Ciprofloxacin	
	Gentamicin	
	Nalidixic acid	
	Ampicillin	

Experimental approach:
1) Does exposure to sublethal concentration of ionic liquids lead to increased tolerance against disinfectants?
2) Is there a difference in resistance between the two Listeria strains (+Tn6188, -Tn6188)?
3) Does exposure to sublethal concentration of ionic liquids show increased tolerance against antibiotics?

MATERIAL AND METHODS

Two different strains of *Listeria monocytogenes* were used: 6179 harboring the transposon (+Tn6188), coding for the QAC- tolerance mediating efflux pump *qacH*, and CDL2 (-Tn6188) lacking transposon. These two Listeria strains were adapted to sublethal concentrations of benzalkonium chloride (BC), 1-decyl-3-methylimidazolium chloride (DecMIM Cl) and trimethylhexadecylammonium malate (TMC$_{16}$ Malate). Adapted strains were tested against QACs (BC, DTAB), ionic liquids (TMC$_{12}$ Malate, DecMIM Cl) and antibiotics (Nalidixic acid, Gentamicin, Ciprofloxacin, Ampicillin). Adaption of bacteria was preformed as follows: *Listeria* were grown in TSB media containing increasing concentrations of the antimicrobial substance. Strains growing at concentrations below the MIC (Minimal Inhibitory Concentration) were inoculated into medium containing higher concentrations. For inoculation of Listeria to the next higher concentration, the ratio 1:5 (one part old medium) was applied to maintain quorum sensing signals (*Paula et al 2012*). The MIC was determined by microbroth- dilution- assay, as the lowest substrate concentration inhibiting growth (measured with TECAN microtiter plate reader at OD$_{600}$) within 24h
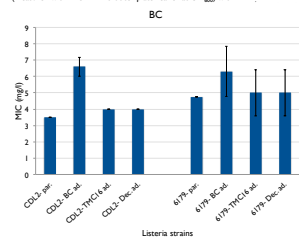

Fig. 1: Adapted and parental CDL2, 6179 strains tested against BC. BC adapted (BC- ad.), DecMIM Cl adapted (Dec. ad.), TMC$_{16}$ Malate adapted (TMC16- ad.). MIC values for BC displayed in mg/l.
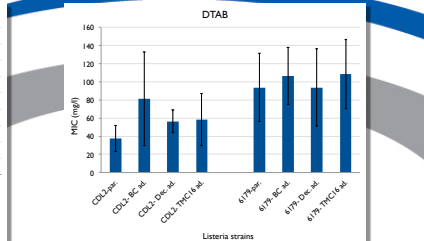

Fig. 2: Adapted and parental CDL2, 6179 strains tested against DTAB. BC adapted (BC-ad.), DecMIM Cl adapted (Dec. ad.), TMC$_{16}$ Malate adapted (TMC16- ad.). MIC values for DTAB displayed in mg/l.
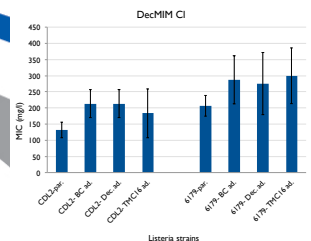

Fig. 3: Adapted and parental CDL2, 6179 strains tested against DecMIM Cl. BC adapted (BC- ad.), DecMIM Cl adapted (Dec. ad.), TMC$_{16}$ Malate adapted (TMC16- ad.). MIC values for DecMIM Cl displayed in mg/l.
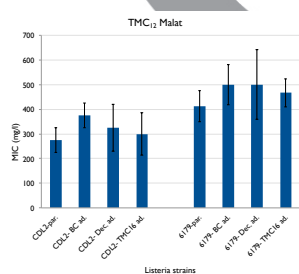

Fig. 4: Adapted and parental CDL2, 6179 strains tested against TMC$_{12}$ Malate. BC adapted (BC- ad.), DecMIM Cl adapted (Dec. ad.), TMC$_{16}$ Malate adapted (TMC16- ad.). MIC values for TMC$_{12}$ Malate displayed in mg/l.

RESULTS I

- Listeria strains adapted to sublethal concentrations of BC and ionic liquids (DecMIM Cl, TMC$_{16}$ Malate) show increased MIC values against QACs (DTAB, BC) and Ionic Liquids (DecMIM Cl, TMC$_{12}$ Malate)

- Listeria strain 6179 shows higher MIC values compared to CDL2 for all tested substances

RESULTS II

- CDL2 strains adapted against either BC or ionic liquids show higher tolerance against Ciprofloxacin

- for Gentamicin (Fig.5), Nalidixic acid and Ampicillin (data not shown) no increased tolerance could be observed for any adapted strain
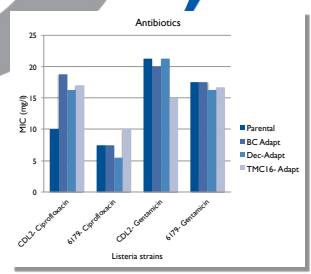

Fig. 5: Adapted and parental CDL2, 6179 strains tested against Ciprofloxacin and Gentamicin. BC adapted (BC- ad.), DecMIM Cl adapted (Dec. ad.), TMC$_{16}$ Malate adapted (TMC$_{16}$ ad.). MIC values for Ciprofloxacin and Gentamicin displayed in mg/l.

DISCUSSION

The results of this study show that *L. monocytogenes* can develop tolerance against QACs and Ionic Liquids. All adapted strains showed increased MIC values against QACs (DTAB, BC) and Ionic Liquids (DecMIM Cl, TMC$_{12}$ Malate) independent of the substance they were adapted to. An explanation for this behaviour could be the structural similarity between the tested antimicrobials. In general, Listeria strain 6179, harbouring the QAC tolerance mediating transposon Tn6188, showed higher MICs compared to CDL2 for BC and all tested ionic liquids. However, both strains showed a similar adaption against disinfectants, suggesting that more than one specific efflux pump (in this case *qacH*) plays a role in this process. Antibiotic resistance was only confirmed for the strain CDL2 in connection with Ciprofloxacin.

IN CONCLUSION, this study shows for the first time that *Listeria monocytogenes* is able to develop tolerance against different disinfectant classes if exposed to ionic liquids. Given the structural similarity between the ionic liquids applied in this study and QACs (for which such adaptations have been previously described), the results are not completely surprising and do not solve the problem of emerging resistance in pathogens. However, given the high and easy variability of ionic liquids it seems likely to identify unique structural motifs which hinder bacterial adaption processes in the future.

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