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„ Phage P100 in food industry: Critical view on the current state of affairs and innovative solutions based on ionic liquids “

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Content

1 General introduction

All underlying issues addressed in this thesis are directly or indirectly concerned with bio-monitoring in food safety. The tools used and the focus on methodology development were mainly based on ionic liquids.

1.1 Food safety

Food safety is globally a public health priority and the availability of safe food is a basic human right (http://whqlibdoc.who.int/publications/9241545747.pdf accessed on 2015/08/11). With progressive globalization of the food industry, food safety has become an important issue, not only in respect of medicine or economics, but it is also a highly topical issue on political agendas, in the media, in food policy, in the food industry and in research (Grunert, 2005; Verhoef et al., 2009). Food-borne disease is a major public health problem worldwide and it carries with it significant social and economic costs. In the US alone there are 76 million cases of food-borne diseases reported each year, and for the EU over 320,000 cases are reported (Newell et al., 2010, http://www.efsa.europa.eu/de/topics/topic/foodbornezoonoticdiseases 2015/12/02). Over the past 20 years there have been several efforts, partly implemented by international standards and legislation, to improve food safety. Quality management systems and food inspection systems have been developed (Röhr et al., 2005) and monitoring systems and methods for diagnosis and the detection of food-borne pathogens have been improved (Newell et al., 2010). Nevertheless, the success has been limited so far and product-associated disease outbreaks have increased in recent years (Rawsthorne et al., 2009; Newell et al., 2010). On the one hand increased testing may result in an increased number of food-borne disease outbreaks detected, but other factors additionally play a role. Increasing numbers of food-borne diseases are not caused by the fact that well-established pathogens were not kept under control, but most probably because new food-borne pathogens have emerged and food-borne infections have changed over time (Tauxe, 2002). Of particular concern is that a remarkable proportion of food-borne diseases results from unknown factors or the causative agent cannot be identified (Tauxe, 2002; Scallan et al., 2011). The increased number of food-

borne diseases has also been attributed to changes in human behaviour, such as the production and consumption of high risk foods (raw or lightly cooked food and fresh fruits or vegetables produced in countries without proper safety procedures) and an overall aging of the population associated with diminished immunities (Koopmans et al., 2002; Newell et al., 2010). Moreover, longer transport time, changing farming practices, intrusion on native wildlife habitats and climate change may increase the risk of food-borne diseases occurring. These factors could contribute to the introduction of novel vectors into temperate regions (Koopmans et al., 2002; Newell et al., 2010). Changing ecology and new technology can also link potential pathogens with the food chain and may be causing a shifting spectrum of pathogens.

A major role in the changing landscape of food-borne diseases is played by pandemic forms of pathogens that are globally distributed (Tauxe, 2002). This is especially true for *Salmonella* Enteritidis, *Salmonella* Typhimurium DT104 strains, *Yersinia enterocolitica* strains with the serotypes O3 and O9 and *Listeria monocytogenes*. Additional problems relate to food-borne bacteria that become more and more resistant to antimicrobials (see also 1.1.1) (Sabour and Griffiths, 2010). These bacteria often carry AMR (anti-microbial resistance) genes coupled with virulence genes that lead to increased bacterial pathogenicity (Martínez and Baquero, 2002).

Until now, more than 200 microbial, chemical or physical agents are known to cause food-borne disease (Acheson, 1999). Microbiological hazards include not only pathogenic microorganisms, but also parasites (most common: *Giardia lamblia*, *Toxoplasma gondii, Cryptosproidium parvum, Cyclospora cayetanensis*) (Mead et al., 1999). Further, viruses and prions can now be included as food-borne pathogens.

Chemical hazards include natural toxicants (e.g. mycotoxins) and environmental contaminants (e.g. mercury), food additives, pesticides and veterinary drug residues. In addition to physical factors, new technologies such as genetic engineering and food irradiation can be potentially hazardous.

1.1.1 Food-related microbiological hazards

1.1.1.1 Food-borne bacterial agents

One of the main concerns in food safety is the microbiological hazard produced by pathogenic microorganisms. Most food-borne illnesses comprise intestinal infectious diseases and bacterial agents are the most investigated and monitored causes in this respect.

Before 1900, *Bruchella*, *Clostridium botulinium*, *Salmonella typhi*, and toxigenic *V. cholerae* were the major bacterial food pathogens (Tauxe, 2002). Since 1990, *Campylobacter*, non-typhoid *Salmonella* and *Clostridium perfringens* have been the most common bacterial causes of food-borne disease in the US (Mead et al., 1999). Moreover, *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* play important roles. Furthermore, *Shigella, Yersinia enterocolitica, Streptococci and Listeria monocytogenes* continue to be challenges in food safety.

Campylobacter spp. cause the highest number of acute bacterial food poisoning cases in the European Union (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3129.pdf accessed on 2012/08/17) and these are found in particular in poultry meat. *Campylobacter spp*. are Gram-negative, microaerophilic, thermophilic bacteria, which include also antimicrobial (especially quinolones) resistant strains (Newell et al., 2010).

Salmonella spp. are also frequently found in food. These are Gram-negative, anaerobic bacteria that are able to infect all major livestock species such as poultry, cattle and pigs, subclinically (http://www.foodsafetywatch.org/factsheets/salmonella/ accessed on 2015/08/17). Their low infection dose for humans, their long-term survival and remarkable adaptability make *Salmonella spp.* especially difficult to control. Serotype-dependent resistance and multiple resistances, especially in *S.* Typhimurium*,* have been reported and these are most probably caused by the use of antimicrobial agents on farms and in meat production (Newell et al., 2010).

Another important food-borne bacterium is *Clostridium perfringens.* This is a spore-forming, Gram-positive bacterium found in many environments, including the intestines

of humans and animals, and certain foods such as raw meat and poultry (http://www.cdc.gov/foodsafety/clostridium-perfingens.html accessed on 2015/08/17).

E. coli is a common and usually harmless gut coloniser in many endotherm species. However there are six groups of diarrhoeagenic *E. coli* (DEC) that possess specific virulence factors. DEC *E. coli* strains can be enteropathogenic, enterotoxigenic, enteroinvasive, diffusely adherent, Vero cyctotoxin- or Shiga toxin-producing *E. coli* (Newell et al., 2010). *E. coli O157:H7,* one of the most important food-borne *E.coli*, belongs to the Vero toxin (VTEC) producers that have been found with geographical different prevalence and serotype distributions (Wick et al., 2005). Antimicrobial (multi-) resistance development in *E. coli* has become a serious public health issue. Commensal *E. coli* can be a source of resistance genes for pathogenic *E.coli.* Moreover, resistance genes can be shared or spread between animal and human *E.coli,* between bacterial clones, *E.coli* and *Salmonella* (Newell et al., 2010).

Another important food-borne bacterium that is in the focus of this thesis is *Listeria monocytogenes*, which is increasingly found in food processing plants.

1.1.1.1.1 *Listeria monocytogenes*

In the beginning of 21st century (2003-2006) *Listeria monocytogenes* was increasingly identified to be the cause of serious food-borne disease in the European Union (Allerberger and Wagner, 2010). *L. monocytogenes* is a Gram-positive, non-spore forming rod shaped bacterium with a size of approximately 0.5 – 2 µm x 0.4 – 0.5 µm. It is microaerophilic, psychotrophic and ubiquitous but especially found in soil, surface water, plants and certain foods (Newell et al., 2010). Food sources that commonly harbour *L. monocytogenes* are ready-to-eat (RTE) foods of animal origin such as milk products, meat, poultry, fish and seafood, and also fresh vegetables (Berrang et al., 1989; Farber, 1991; Furrer et al., 1991; Greenwood et al., 1991; Ojeniyi et al., 1996; Jorgensen et al., 2002; Gombas et al., 2003). In the European milk industry two thirds of all product recalls are due to the presence of *L. monocytogenes* (https://webgate.ec.europa.eu/rasff-window/portal/? event=SearchForm&cleanSearch=1 accessed on 2015/11/30). *L. monocytogenes* is particularly problematic with cheese. During the cheese ripening

process the pH rises and this facilitates the growth of *L. monocytogenes*, resulting in contamination level that can reach as high as 10^7 CFU/ g cheese (Farber and Losos, 1988). Healthy individuals who are infected with *L. monocytogenes* normally only develop a mild gastroenteritis and may not notice the association with the symptoms and having eaten contaminated food. However, immunocompromised people and pregnant women are highly susceptible to listeriosis. On the population scale listeriosis is a relatively rare disease, but the infection has a high fatality rate for individuals (20-30 %) (Newell et al., 2010). Since 2009 the number of listeriosis cases in Germany has been increasing disturbingly and in 2014 the number of listeriosis cases was found to be increased to 30 % (http://www.rki.de/DE/Content/Infekt/Jahrbuch/Jahrbuch_2014.pdf?__blob=publication File accessed on 2015/10/21). Although there are 13 different serotypes of *L. monocytogenes*, the majority of human listeriosis cases are caused by only three serotypes: 1/2a, 1/2b and 4b (McLauchlin et al., 1990).

L. monocytogenes is able to adapt to harsh environmental conditions and can grow between 1 - 45 °C, in aqueous environments comprising up to 10 % sodium chloride and is capable of growing over a pH range from 4.5 - 9 (Farber and Losos, 1988; Takhistov and George, 2004). *L. monocytogenes* is also able to form biofilms and to develop resistance to chemical disinfectants (Blackman and Frank, 1996; Folsom et al., 2006; Kostaki et al., 2012). This makes it especially difficult to control and there always remains the risk that it can persist in food production and processing plants despite cleaning measures (Lunden et al., 2003; Kim et al., 2008; Linke et al., 2014). Alternatives to commonly used antimicrobials and disinfection are described in sections 1.3 and 1.4.1.

1.1.1.2 Food-related viruses

1.1.1.2.1 Bacteriophages

Bacteriophages are the most abundant biological entities on earth and they are ubiquitous in nature (Breitbart et al., 2002). Although bacteriophages can burden food-processing plants by destroying starter or fermenter cultures, they are more and more used for monitoring and the biocontrol of pathogens. They find applications in both medicine and food production. On one hand they can be used in the laboratory for typing

bacterial strains based on varying phage susceptibility (Wilson and Atkinson, 1945). For most of the important bacterial food-borne pathogens, such as *L. monocytogenes,* typing phages are described (Loessner and Busse, 1990). On the other hand, phages can be used directly for the detection of pathogens. In the case of *L. monocytogenes* for instance, a luminescence-based detection system has been developed, where the Luciferase reporter phage A511::luxAB induces bacterial bioluminescence into infected cells (Loessner et al., 1997). Moreover, lysins of phages have been suggested as agents to control microbial pathogens in the food industry, biotechnology or medicine (Loessner, 2005). Most commonly in the food industry the phage itself is used for the biocontrol of bacteria (see 1.3.1).

1.1.1.2.2 *Listeria* phages and phage P100

The first *Listeria*-specific bacteriophage (*Listeria* phage) was described in 1945 and by 2008, 400 *Listeria* phages had been described (Kim and Kathariou, 2009). All of the known *Listeria* phages belong to the order *Caudovirales* (Hagens and Loessner, 2007a). *Caudovirales* are tailed phages with double-stranded DNA comprising between 18 – 500 kbp and an icosahedral capsid of between 45 – 170 nm. Most *Listeria* phages belong to the *Siphoviridae* family, which have long, flexible, non-contractile tails (Hagens and Loessner, 2007a). A minority of the *Listeria* phages belong to the *Myoviridae* family, which also have long, inflexible contractile tails. The majority of *Listeria* phages are temperate and extremely host-specific, for instance, phages A118 and PSA (Loessner et al., 2000; Zimmer et al., 2003).

In contrast, far fewer lytic *Listeria* phages are known. One example of a lytic *Listeria* phage of the *Myoviridae* family is phage A511, which infects the majority of *L. monocytogenes* strains (Hagens and Loessner, 2007a). Very similar to phage A511 is phage P100, which was originally isolated from the sewage of a dairy plant (Kim et al., 2008). The genome of phage P100 has a size of 131 kbp and comprises 174 open reading frames (Carlton et al., 2005). The molecular weight of the phage particle is about 1.2×10^8 Daltons and its length is approximately 300 nm.

Listeria phages are used for typing, they have been genomically characterised, their transducing potential studied and they have been tested for biotechnological applications

and the biocontrol of food pathogens (Kim and Kathariou, 2009). For the use as antimicrobial agents exclusively lytic phages can be used in order to avoid gene transfer. Phage P100 is now frequently used in the food industry to control *L. monocytogenes* (see section 1.3.1.1).

1.1.1.2.3 Food-borne viruses

Food-borne viruses are greatly troubling and are the major causes of food-borne illnesses in humans in both developed and developing countries (Koopmans et al., 2002; Guevremont et al., 2006). Viruses present in foods can remain infectious under most circumstances for several days or weeks, they are much more stable to chemical and UV disinfection and pasteurisation than bacteria and currently used food processing methods are often not able to inactive them (Grohmann and Lee, 1997; Koopmans and Duizer, 2004; Fumian et al., 2009; Scherer, 2009). Additionally, there is presently no systematic surveillance system suited to detect and monitor food-borne viruses (Newell et al., 2010). Foodstuffs are usually contaminated with these viruses via the fecal-oral route as common food-borne viruses are highly infectious and spread easily from person to person. This leads to a high rate of transmission and makes identification of the infectious source difficult (Koopmans et al., 2002).

Viruses that can be transmitted by the food-borne route can cause a wide range of diseases. Infection with most of them (for instance *Adenoviridae*, *Parvoviridae*, *Reoviridae*, *Astroviridae* and *Caliciviridae*) is associated with vomiting and diarrhoea. Some others can also produce other symptoms, such as influenza-like illnesses (*Orthomyxoviridae*, *Paramyxoviridae* and *Flaviviridae*), neurological symptoms (*Parayxoviridae*, *Flaviviridae*, *Picornaviridae*) or hepatitis (*Hepeviridae*). Skin, eye and respiratory infections, meningitis and myalgia can also result from food-borne viruses (Grohmann and Lee, 1997; Newell et al., 2010). However, due to limited detection methods (see 1.2) food-borne viral gastroenteritis is often not diagnosed. Moreover, many viral infections are asymptomatic (Grohmann and Lee, 1997). Therefore further inadvertent spread of implicated viruses is possible.

The highest number of food-related illnesses is caused by the calicivirus group, which has both human and animal reservoirs (Ando et al., 2000). Noroviruses are the most

important food-borne agents of this group (Grohmann and Lee, 1997; Tauxe, 2002; Koopmans and Duizer, 2004). Human caliciviruses are non-enveloped spherical viruses with a size of between 28 - 35 nm containing a positive sensed single-stranded RNA genome of 7.3-7.6 kb (Koopmans, 2005). They are highly diverse genetically with II.4 (GII4) being the most predominant genogroup. Human noroviruses cannot be detected in cell culture based systems, but only by PCR (Polymerase Chain Reaction). However, PCR is not able to distinguish between infective and non-infective particles. Therefore, as noroviruses are highly infective, norovirus surrogates are commonly used to develop, establish and evaluate new concentration and detection systems. The most important surrogates are *E. coli* phage MS2 and feline and murine caliciviruses (Dawson et al., 2005; Dreier et al., 2005; Cannon et al., 2006; Bae and Schwab, 2008; Rzezutka et al., 2008; Mattison et al., 2009).

Other important food-borne viruses are the hepatitis viruses. Only the two enteric hepatitis viruses, hepatitis A virus and hepatitis E virus, are causative agents for food-borne disease (Grohmann and Lee, 1997; Koopmans et al., 2002). The most common as food-borne virus is the hepatitis A virus. It is a member of the *Picornaviridae* family that also includes the Enteroviruses (Groups A-E), which are also occasionally the cause of food-borne disease (Grohmann and Lee, 1997; Koopmans and Duizer, 2004). Picornaviruses have a size of 25 - 30 nm and are non-enveloped single (positive-) stranded RNA viruses. The hepatitis A virus is very resistant to drying, heating, UV light and low levels of chlorine and ozone (Grohmann and Lee, 1997). In contrast, the hepatitis E virus is much more labile. The hepatitis E virus belongs to the *Hepeviridae* family and is similar to the caliciviruses. In older classification systems, the hepatitis E virus was even integrated into the calicivirus group.

Further occasionally found food-borne viruses are the Astroviruses, which are also small, single-stranded RNA viruses and the Rotaviruses that are double-stranded RNA viruses. Especially in developing countries, Rotaviruses are the most common cause of viral gastroenteritis in children and infection is associated with high morbidity and mortality rates (Grohmann and Lee, 1997).

1.2 Detection methods

Over the past several years there has been an enormous research effort to improve analytical methods for detecting food-borne pathogens. Rapid detection and subsequent identification of food-borne pathogens have become major issues in food safety in order to limit health risks and financial losses. Ideally detection methods should be quantitative and detection should be sensitive, fast and cost effective. Detection methods include three main steps: sample preparation, detection and typing (Mattison and Bidawid, 2009). While there are several (standardized) detection and quantification protocols available for food-borne microbiological agents at present, this does not yet hold true for viruses (Stevens and Jaykus, 2004; Brehm-Stecher et al., 2009; Rossmanith and Wagner, 2010).

1.2.1 Microbiological detection methods

Since the last decade of the nineteenth century, detection of microbes was mostly based on the growth of microbiological cultures. In the course of the time microbiological detection methods have significantly changed and improved. Nevertheless they still rely mostly on enrichment of the target cell, followed by subsequent isolation and identification of the pathogen. Therefore, the main drawback of traditional microbiological detection systems is the need of growing pathogen cultures. Consequently, non-cultivable microorganisms and pathogens in a non-cultivable state are neglected and remain undetectable with such systems. Microbiological methods are time consuming although detection times can be shortened, for instance by the use of chromogenic media, DNA hybridizations and immune assays or biochemical methods. However, such microbiological methods are usually not quantitative (due to the enrichment step) and expensive.

Another drawback of microbiological growth-based methods is that they cannot detect (all) viruses that play an increasing role in food safety (as described in chapter 1.1.1.2.3). Foods that are contaminated with viruses cannot be identified by smell or taste (Koopmans et al., 2002) and some enteric viruses cannot be multiplied tissue culture (Koopmans et al., 2002; Di Pasquale et al., 2010b). Neither can biochemical methods be

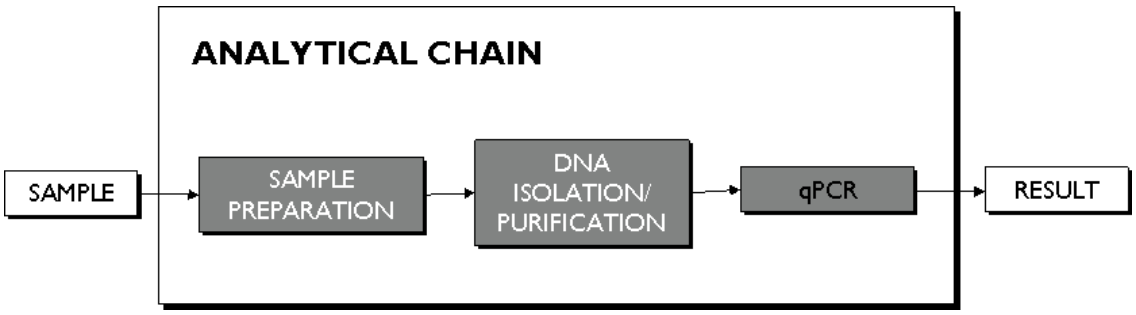
used to detect metabolism-specific products due to the inability of viruses to produce their own energy and synthesise their own proteins. Therefore, most traditional microbiological methods are not suitable for the detection of food-borne viruses and alternative molecular biological detection methods must be used.

1.2.2 Molecular biological detection methods and the analytical chain

Molecular biological and biochemical detection methods for food-borne pathogens include PCR, microarrays, chip-based PCR or parallel sequencing. PCR is one of the most important molecular biological detection method currently used in food safety and permits specific detection of pathogens without enrichment. Cultivability and proliferation of the target pathogen is not necessary. Therefore inactive or dead pathogens can also be detected as long as the nucleic acids are intact. Further development of PCR has also allowed quantitative detection of target DNA (qPCR or real-time PCR). As molecular biological methods in general and PCR in particular are often derived from basic research programmes, methods have to be extended or changed to match the requirements of biomonitoring of food-borne pathogens (Radstrom et al., 2003). Most of the molecular biological methods were developed to work under ideal laboratory conditions with high numbers of target bacteria (or viruses). In environmental and food samples however, the heterogeneous micro-environment of the samples and unknown numbers of targets have to be matched to the requirements of molecular biological methods. To achieve stable laboratory conditions and to allow quantitative detection, proper sample preparation is crucial (Stevens and Jaykus, 2004; Brehm-Stecher et al., 2009; Rossmanith and Wagner, 2010). Furthermore, highly purified nucleic acids are necessary for most molecular biological methods, especially for qPCR. Therefore, DNA isolation and purification are important steps in the detection of food-borne pathogens (Radstrom et al., 2004). Additionally, the detection assay itself has certain limits that have to be taken into account when results of the detection methods are interpreted (Rossmanith and Wagner, 2011). All of these steps in the detection chain (sample preparation, DNA isolation and purification and the core detection assay) can be termed the “analytical chain” (figure 1) (Rossmanith and Wagner, 2010).

During the first project in which this PhD thesis was included, the CD-MOFA (Christian-Doppler Laboratory for Molecularbiological Food Analysis), the analytical chain was the main focus of attention. A carefully validated analytical chain is based on analysis of all individual steps. This can be achieved with the "analytical trihedron" and includes validation of the detection method *per se*, separation of the target from the food matrix and process controls for all steps (Rossmanith and Wagner, 2010). Optimisation of all parameters should guarantee optimum performance and high confidence in the detection method.

For bacteria there are already several (standardized) detection and quantification protocols available (Stevens and Jaykus, 2004; Brehm-Stecher et al., 2009; Rossmanith and Wagner, 2010). In contrast, validated consensus protocols for virus detection in food do not exist, with the exception of shellfish (Koopmans et al., 2002; Croci et al., 2008). Attempts to develop virus detection methods suitable for food resulted in methods that are limited on particular foodstuffs or vary from virus to virus (Guevremont et al., 2006; Dubois et al., 2007; Croci et al., 2008; Mattison and Bidawid, 2009; Morton et al., 2009). Nevertheless, there is still a need for further development and standardisation of molecular biological detection methods for food-borne viruses (Grohmann and Lee, 1997; Croci et al., 2008). Moreover, research and development into new prevention, detection and identification methods relevant to both food-borne bacteria and viruses will be necessary to guarantee future food safety and to reduce the prevalence of food-borne disease (http://whqlibdoc.who.int/publications/9241545747.pdf accessed on 2015/08/11).

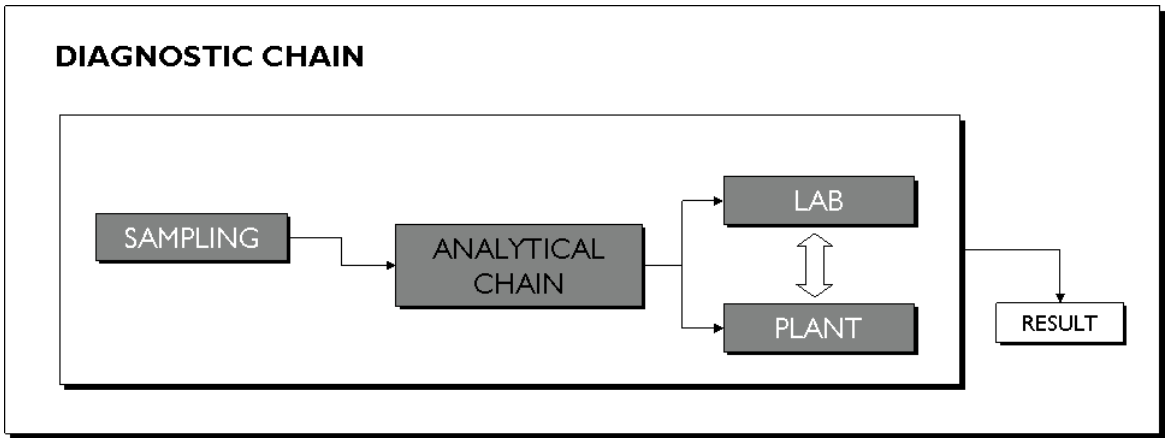


Figue 1: The analytical chain.

1.2.3 The diagnostic chain

Establishing the analytical chain has helped to understand and identify the prerequisites for reliable molecular biological pathogen detection. However, in the analytical chain only factors that influenced the methods in the laboratory were taken into consideration, while influences associated with food-production and processing, which take place before sample preparation, were neglected. Therefore, the focus of the second project, which concerned this thesis, was on the interactions of factors pertinent to the plant and the laboratory. These interactions, together with the previously described analytical chain, have been summarized as “diagnostic chain” (figure 2). Factors that influence the results of the detection of pathogens before sample preparation especially include the use of cleaning agents as well as the sampling process itself. Cleaning mainly relies on detergents, which can hinder subsequent detection by molecular methods (Schrader et al., 2012). Sampling on the other hand can influence detection results when not all targets find their way into the analytical chain. For instance, this is the case when they are not sufficiently absorbed onto the sampling material (e.g. swab), or when the pathogens adhere to the sampling material and are not accessible for the subsequent detection method.

In order to guarantee safe food a whole systems approach is necessary. This implies not only detection, but also removal or inactivation of pathogens. Cleansing and disinfection are possibility ways to remove food-borne pathogens. However, increasing resistance of bacteria to disinfectants and antimicrobial substances may call for alternative treatment strategies. Phages and vaccines are the most promising alternatives to antimicrobial agents (Boerlin et al., 2010). Other relatively new and promising approaches include ionic liquids as antimicrobial agents (Mester et al., 2015).



1.3 Biocontrol

1.3.1 Bacteriophages for biocontrol of food-borne pathogens

As stated in chapter 1.1.1.2.1., phage lysins could be possible agents to control microbial pathogens. Bacteriophage lysins degrade the polypeptidoglycan structure and lead to bacterial cell lysis (Weidenmaier and Peschel, 2008). However, most commonly, the phage itself is used for biocontrol of bacteria in the food industry. Phage use as an alternate class of antibacterial agents against food-borne pathogens is gaining popularity for several reasons, such as high host specificity and as they do not change the quality and sensory characteristics of the food (Hagens and Loessner, 2007b, 2010; Mahony et al., 2011; Ali et al., 2012). Further, increased antibiotic resistance in bacteria makes them a more and more popular alternative to physical and chemical treatments (Monk et al., 2010; Mahony et al., 2011). Currently there are now commercially available phage products against nearly all important food-borne bacteria (Hagens and Loessner, 2007b; Mahony et al., 2011; Maura and Debarbieux, 2011).

Antibacterial applications for bacteriophages include pre-harvest and post-harvest strategies (Mahony et al., 2011). An example of a pre-harvest strategy is the treatment of meat before it enters the food-production plant. Post-harvest strategies in contrast include the application of phages to meat or already processed food. Moreover, active and passive phage applications can be distinguished (Gill et al., 2006). Active treatments are based on applied low phage concentrations. Here the phage should replicate within the host cells, followed by destruction of the cells and the release of new infective phage particles. Repeated infection cycles should lead to eradication of the bacterial pathogen.

Passive treatments are based on the application of high phage concentrations. On the one hand, phage infection should lead to cessation of bacterial protein synthesis culminating in an end to host cell replication. On the other hand, a scenario described as “lysis from without (LWO)” could occur (Abedon, 2011). Hereby multiple phages infect one cell and lead to its lysis without further replication within the cell. When the passive approach is used, MOIs (multiplicity of infection; ratio of phages to bacteria) of ≥ 10 are necessary to achieve 99.999 % cell infection and a major reduction in bacterial numbers (Kasman et al., 2002).

Independently of which strategy is used, there are some facts that should be considered in advance before phages are used to control food-borne pathogens. In food protection plants and processed food it is crucial to consider that heat, cold, dryness, nutrition deficit, temperature, pH and water activity and exposure to chemical detergents or disinfectants may influence the stability of phages, the survival of bacteria and as a consequence also the host- virus interaction and therefore the success of phage treatments (Garcia et al., 2008; Jończyk et al., 2011; Arachchi et al., 2013; Denes and Wiedmann, 2014; Ly-Chatain, 2014). Further, the presence of inhibitory compounds, such as antibodies, whey proteins and bacteriocins can reduce the effectiveness of phage treatments (Maura and Debarbieux, 2011; Tessema et al., 2011; Abedon, 2012; Vongkamjan et al., 2013; Ly-Chatain, 2014). Moreover, the accessibility of the target bacteria can be limited when phages are applied in solid matrices or when the phage concentration, dose and time of application are not optimal (Ly-Chatain, 2014).

When bacterial fitness is affected by environmental conditions, transcriptional responses can lead to reduced phage susceptibility caused by changes in cell wall structures that are the docking sites for phages (Denes and Wiedmann, 2014). Moreover, phages cannot replicate in bacterial cells during their stationary growth phase, which must be taken into account if phages are used for active application strategies (Chibani-Chennoufi et al., 2004; Denes and Wiedmann, 2014). Further, bacteria possess a wide range of phage-defence systems. They include, for example, several restriction modification and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems and these prevent either entrance into the host cell or phage replication (Abedon, 2012).

Reduced success of phage treatments, their suboptimal application and repeated exposure can lead to bacterial adaptation and resistance development (Bach et al., 2003; Hagens and Loessner, 2010; Vongkamjan et al., 2013). Therefore, before phages are used for biocontrol purposes, the influence of environmental factors on phage treatments as well as establishment of pre-existing resistance should be tested. Likewise, inactivation of the phages following their use should be considered in order to avoid the development of resistance.

1.3.1.1 Biocontrol of *Listeria* using phages

The first reported use of phages for control of *L. monocytogenes* in food was in 2003 (Leverentz et al., 2003). Leverentz and colleagues were able to apply phages LM-103 and LMP-102 to significantly reduce *L. monocytogenes* numbers on fruits. Later experiments with phage A511 and cheese were published (Guenther and Loessner, 2011).

There are now several commercially available phage products to control food-borne pathogens. Two products have been produced to eradicate *L. monocytogenes*. LMP-102^{TM} (Listshield^{TM}), a phage cocktail containing four non-specified bacteriophages together with phages LM-103 and LMP-102, is one of which. It was developed for the treatment of ready-to-eat-foods before packaging (Ly-Chatain, 2014).

Another product is Listex^{TM} P100. It contains phage P100 as active component and received GRAS (generally recognized as safe) status by the US FDA and USDA in 2007.

Listex^{TM} P100 or phage P100 has been tested for its ability to reduce *L. monocytogenes* numbers on poultry products, fish, biofilms or on cheese and the success of these treatments was found to be dependent particularly upon the host-virus ratio (Carlton et al., 2005; Guenther et al., 2009; Soni and Nannapaneni, 2010b, a; Soni et al., 2010; Bigot et al., 2011; Guenther and Loessner, 2011; Silva et al., 2014). Although the number of bacteria could be significantly reduced most of the time, high numbers or concentrations of *Listeria* were not completely eradicated.

Even although phage P100 is already used frequently in industry for the control of *L. monocytogenes* in food, several questions remain open. These especially apply to the existence and possible development of resistance, and the impact of environmental

conditions on the susceptibility of *L. monocytogenes* is still limited (Efsa, 2009; Kim and Kathariou, 2009).

1.4 Method development for biomonitoring

1.4.1 Ionic liquids

In recent years, a new class of solvents attracted the attention of scientists and engineers: Ionic liquids (ILs). Ionic liquids are defined as organic salts with melting points below 100 °C. Ionic liquids with melting points at room temperature can be further classified as "room temperature ionic liquids" (RTILs). Ionic liquids consist entirely of ions, usually an organic cation with a charged head group and an anion (see figure 3). At least one of the ions is large and conformationally flexible, the Gibbs free energy is negative and therefore the liquid state is thermodynamically favoured (Krossing et al., 2006). It is possible to combine a high number of different cations and anions theoretically resulting in 10^{18} different ionic liquids (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Aldrich/Brochure/al_chemfile_v5_n6.pdf accessed on 2015/08/18), with unique physical and chemical characteristics. Nevertheless, there are some properties that are applicable to all ionic liquids. Due to the strong ionic interactions between the ions, all ionic liquids have a negligible vapour pressure, they are non-flammable substances and generally they are thermally (generally > 400 °C), mechanically and electrochemically stable (Chiappe and Pieraccini, 2005; Freemantle, 2010).

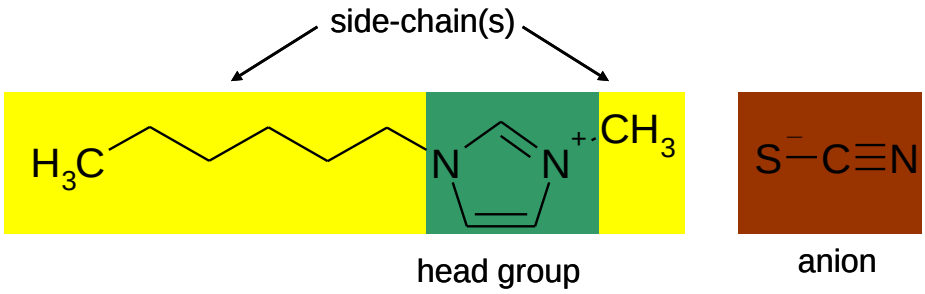


Figure 3: Typical structure of an ionic liquid (Jastorff et al., 2007)

By combining different cations and anions, ionic liquids with a wide range of water-solubilities, conductivities, densities or viscosities, toxicities and biodegradabilities can be synthesized (Olivier-Bourbigou et al., 2010). Therefore, they are sometimes described as "task-specific ionic liquids" or "designer solvents" (Freemantle, 2010; Olivier-Bourbigou et

al., 2010). Depending on their intended applications, ions with specific properties can be combined. This high degree of tunability is one of the reasons why ionic liquids are increasingly being used for numerous applications. Initially ionic liquids were particularly interesting for academic research, but now they are also implemented in a wide range of industrial processes. The most important applications of ionic liquids are summarised in figure 4 and these include use as energy sources, in biotechnology, chemistry, chemical engineering and in coatings (Thuy Pham et al., 2010). The application of ionic liquids for biological and biotechnological purposes is the sector with the highest potential of development (Quijano et al., 2010). They are already used in enzymatic assays, for the stabilization of enzymes and proteins, purification, refolding or crystallization of proteins or biomolecules and many other uses are emerging (Lozano et al., 2001; Du et al., 2007; Pei et al., 2009; Ventura et al., 2011; Vicente et al., 2014). However, applications in molecular biology or molecular diagnostics remain very limited.

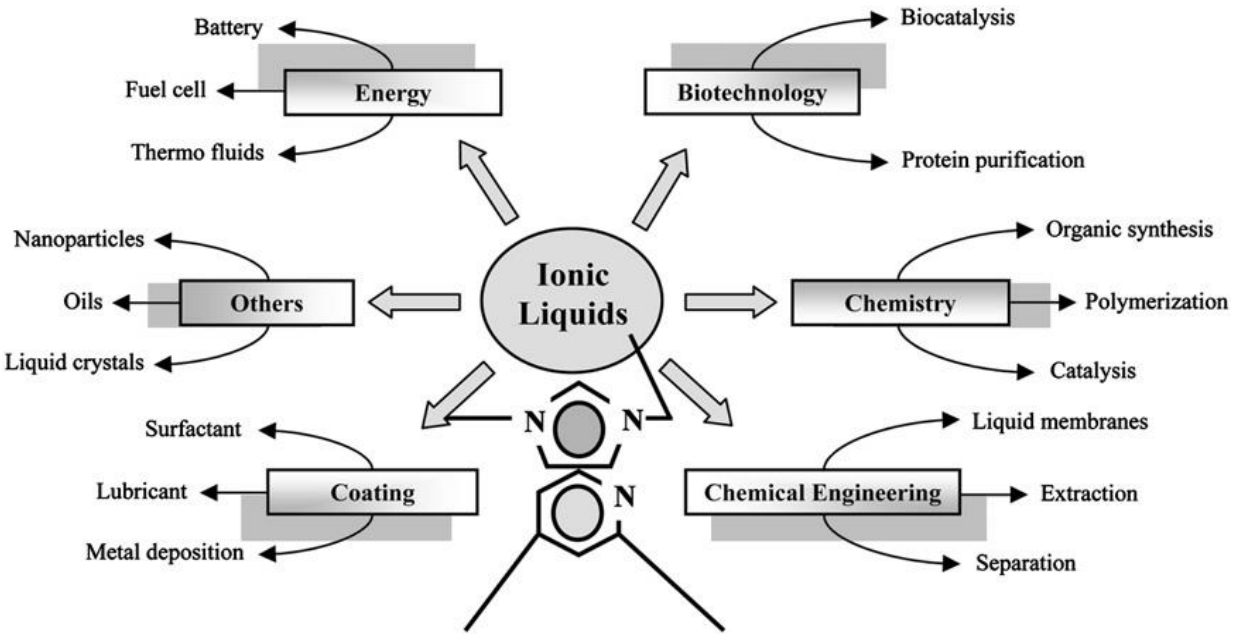


Figure 4: Ionic liquid applications (Thuy Pham et al., 2010).

The chemical and physical characteristics of ionic liquids have been well investigated. However, their biological properties and environmental effects have hardly been explored (Freemantle, 2010). Due to their negligible vapour pressures, ionic liquids were initially described as “green solvents”. Nonetheless, ionic liquids have high chemical and thermal

stabilities and many are also soluble in water. This is prompting investigators to investigate their (eco)toxicity, biodegradability and bioaccumulation (Thuy Pham et al., 2010). General statements about the toxicity of ionic liquids are not yet possible, but the structure-activity relationships (SARs) of ionic liquids tested on several biological systems (enzyme inhibition assay - to vertebrate *in vitro* tests) indicated that the toxicity of at least imidazolium based- ionic liquids increases with increasing length of the cationic alkyl-side chain (Stolte et al., 2007; Ventura et al., 2012; Mester et al., 2015). Toxic effects of ionic liquids can be due not only to the cation, but also due the anion, especially when the cationic side chain is short. For bacteria, the chaotropicity of the anion has been reported to have a major influence on ionic liquid toxicity (Mester et al., 2012, 2015).

The toxicity of ionic liquids enables their application as antimicrobial substances. Several publications have shown bactericidal effects of ionic liquids on different bacteria and biofilms (Carson et al., 2009; Busetti et al., 2010; Choi et al., 2011; Cornellas et al., 2011).

Although the effect of ionic liquids on several biological systems has been tested, viruses were not included. Until today, only one study investigating the stabilizing effect of two ionic liquids on tobacco mosaic viruses has been published (Byrne et al., 2012). Structure-activity relationship studies with ionic liquids on viruses have not yet been performed.

1.5 Summary of the state of the art

Food-borne disease is a major public health problem and results in social and economic costs worldwide. In order to guarantee food safety, two strategies have been exercised. One has attempted to avoid production of contaminated food or to prevent the contamination of already processed food. The second has attempted to prevent contaminated food being placed on the market. This can be achieved by detection and monitoring methods for food-borne pathogens.

Nevertheless, for both strategies - removal/inactivation and detection of pathogens - there are still unsolved problems and deficiencies. There are still no standardized detection systems for viruses and global food control criteria rely only on bacterial counts. On the other hand, cleansing and disinfection are becoming more and more complicated because several antibiotic and disinfection insensitive bacteria are emerging. Although the use of phages as alternative treatments is associated with advantages, there

are still several questions to be addressed, especially concerning the presence and development of bacteriophage-resistant bacteria. Further, considerations must be made in respect of the efficacy of phage treatment and long-term phage survival in the food production environment. A new strategy to remove bacteria and to inactivate viruses could involve the use of ionic liquids. However, to-date the efficacy of ionic liquids to inactivate viruses has not yet been investigated.

2 Thesis aim

The central research question of this thesis (summarized in figure 5) concerns viruses in food safety. Results of the thesis are anticipated to lead to further improvements in the diagnostic chain, which is outlined in more detail in the introduction section.

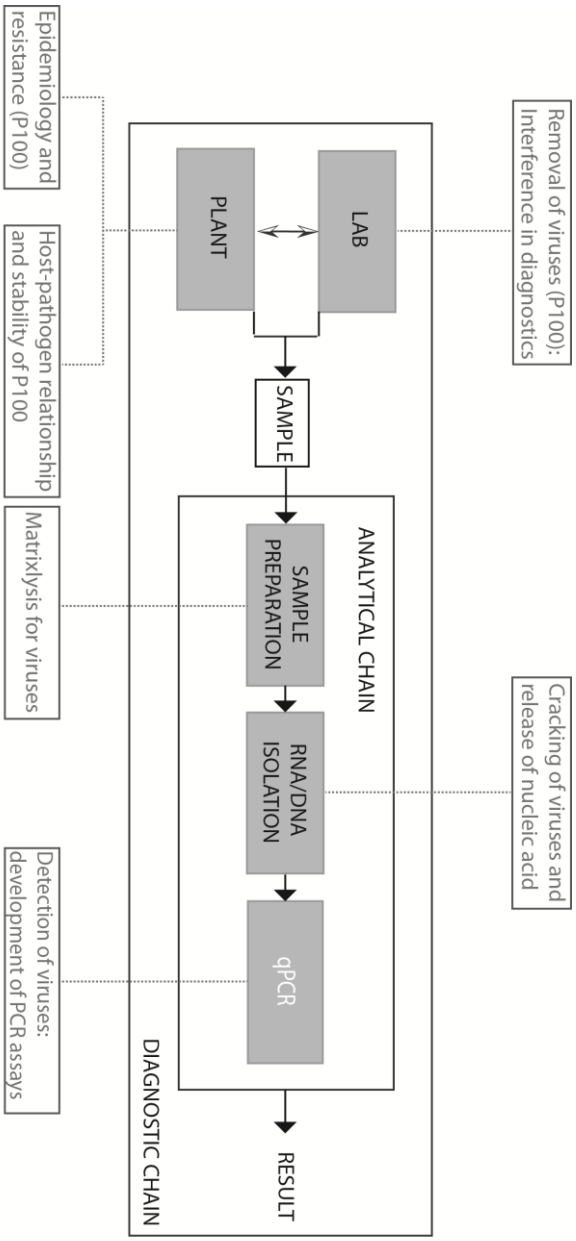


Figure 5: Overview of the central research questions of the thesis.

The essential organism of interest for this thesis is bacteriophage P100, followed by its host *L. monocytogenes*. Bacteriophage MS2 and the feline calicivirus are other viruses important to this work.

As previously stated, the scientific aims of this thesis are oriented to the structure of the diagnostic chain. The following topics are addressed:

- The applicability of P100 to the biocontrol of *L. monocytogenes*
    - Retrospective investigation of resistance formation in plant environments
    - Examination of resistant *L. monocytogenes*
- Persistence and treatment efficacy of P100 under environmental conditions
- Inactivation of bacteriophage P100 using ionic liquids
- Detection of bacteriophage P100: PCR assay development
- Development of a nucleic acid isolation method for viruses using ionic liquids
- Sample preparation for viruses out of complex food matrices

3 Scientific publications and manuscripts

Altogether four publications summarise results obtained in this thesis (see section 3). The majority of data not included in these publications were presented as posters that are attached in the appendix.

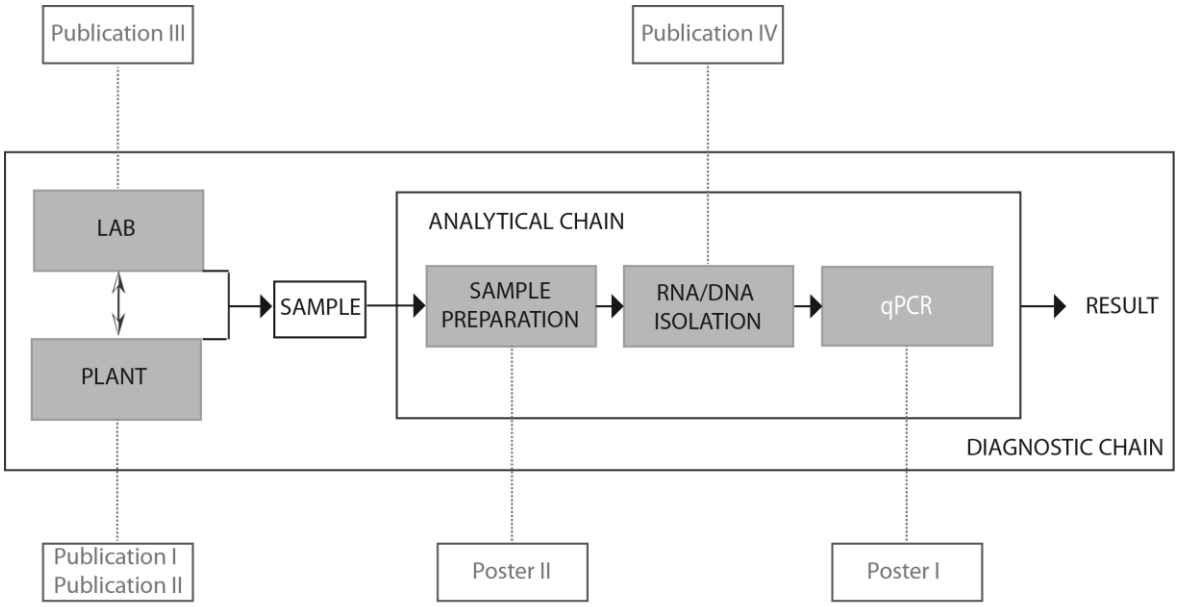


Figure 6: Overview and thematic classification of the publications and posters

3.1 Publication 1: Screening and characterisation of bacteriophage P100 insensitive *Listeria monocytogenes* isolates in Austrian dairy plants

Susanne Fister, Sabine Fuchs, Beatrix Stessl, Dagmar Schoder, Martin Wagner, Peter Rossmanith

Published: Food Control 59 (2016) 108-117

Contribution to the publication:

- Experimental design
- Performance of the majority of the experiments
- Analysis of the majority of the experiments (except PFGE)
- Drafting and revision of the manuscript

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Screening and characterisation of bacteriophage P100 insensitive *Listeria monocytogenes* isolates in Austrian dairy plants

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ABSTRACT

Listeria monocytogenes is a major health threat in food production premises. The use of bacteriophages, such as listeriaphage P100, to minimize food pathogens is a promising alternative to eliminate bacteria, as is application of Listex™ P100. However, the use of phages may result in resistant *L. monocytogenes* strains. In this study 486 *L. monocytogenes* isolates obtained from 59 dairies over 15 years were screened for the presence of P100 insensitive *L. monocytogenes*. The overall number of P100 insensitive *L. monocytogenes* isolates was 13 (2.7%) for all years and all investigated plants. Insensitivities were detected in five of 59 dairies. Detected insensitive isolates did not appear randomly, but were in connection with phage treatments.

The influence of dilution on the efficacy of P100 was tested by different host pathogen ratios. Additionally, artificially induced insensitive isolates were selected. Adsorption tests suggested that the detected insensitive *L. monocytogenes* isolates had receptor modifications.

1. Introduction

Listeria monocytogenes is a widely distributed Gram-positive bacterium that belongs to the most important food-borne pathogens. Although infections are usually harmless, listeriosis is a serious problem in people with weakened immune systems and is the third leading cause of death from food poisoning (http://www.cdc.gov/vitalsigns/listeria/accessed on: 10.12.2014). Foodstuffs are usually contaminated in the raw state or through cross contamination during fermentation, processing, packaging or storage (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005; Vazquez-Boland et al., 2001). In particular, ready to eat (RTE) foods such as cheeses are frequently implicated. *L. monocytogenes* can be found in 1–22% of cheeses and especially in those manufactured from raw milk (Guenther, Huwyler, Richard, & Loessner, 2009; Guenther & Loessner, 2011).

In 2009 a listeriosis outbreak occurred in Austria and Germany caused by contaminated acid curd cheese (Fretz et al., 2010; Schoder, Stessl, Szakmary-Brandle, Rossmanith, & Wagner, 2014). Since that time awareness of the danger in dairies has risen and greater importance has been attached to the prevention of cheese and production plant contamination through controls and monitoring, including cleansing and disinfection steps. Although there are different ways to decontaminate, physical and chemical treatments often negatively affect or change the sensory qualities of the foods they are meant to protect (Gao, Ju, & Jiang, 2006). This is particularly relevant for raw products (EFSA, 2009). Alternatives to physical and chemical treatments include the use of bacteriophages and phage products, techniques that are becoming more and more popular (Mahony, McAuliffe, Ross, & van, 2011; Monk, Rees, Barrow, Hagens, & Harper, 2010). In general, bacteriophages can be used for the bio-control of pathogens and for detection of the bacteria. Related phages are now known for all important food-borne bacteria (Hagens & Loessner, 2007; Mahony et al., 2011; Maura & Debarbieux, 2011).

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In respect of the elimination of *L. monocytogenes* from foodstuffs, different phages have been studied. The first study was performed in 2003 by Leverentz using the LMP-102 and LM-103 phages (Leverentz et al., 2003). Later phage A511 was tested for activity against *L. monocytogenes* in RTE foods and cheese (Guenther & Loessner, 2011).

Another frequently used virus to combat *Listeria* is the phage P100, which is similar to A511 and commercially available as Listex™ P100 preparation. Listex™ P100 is increasingly used in (Austrian) dairy processing facilities. Nevertheless, the use of phages for food safety purposes raises some concerns. The most important issue that needs to be resolved is whether the bacteria host strains can develop resistance to specific phages. Several antiphage systems in bacteria are known, including mechanisms that prevent either entrance to the host cell or replication of the phages (Abedon, 2012). Although several restriction modification- and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems have been reported for several bacteria (Abedon, 2012), little information is available, especially concerning any resistance of *L. monocytogenes* to P100. This includes the occurrence of resistant strains in production environments, possible mechanisms of resistance and the kinetics of the host pathogen relationship via concentration depended effects.

Consequently this work involved a thorough study of the above parameters in respect of putative resistance of *L. monocytogenes* to the bacteriophage P100.

Four hundred eighty six *L. monocytogenes* isolates, obtained from 59 Austrian dairies between 1987 and 2012, were screened for P100 resistance. The frequency of naturally occurring resistances and resistances before and after Listex™ P100 approval and introduction were examined. Subtyping of actual *L. monocytogenes* strains was performed using pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). The possibility of occurrence of artificially induced insensitive isolates was confirmed using field isolates and a recombinant strain of *L. monocytogenes* (Δ*prfA*).

The kinetics of the host pathogen relationship was investigated using different phage-to-bacteria ratios as well as the influence of dilution on the efficacy of P100. Additionally, adsorption tests were performed to provide any initial suggestion as to the type of resistance found in insensitive *L. monocytogenes* isolates.

2. Materials and methods

Fig. 1 summarises the aim of the study and study design and gives an overview of the experiments.

2.1. L. monocytogenes test strains, culture conditions and phage preparation

The 486 *L. monocytogenes* test strains included in the presented study were cryo-conserved (−80 °C, Microbank Pro-Lab Diagnostics, Round Rock, TX, USA), and available from the *Listeria* collection of the Institute of Milk Hygiene, Milk Technology and Food Science (Vetmeduni Vienna, Austria). The strain set represented sporadic or recurrent *L. monocytogenes* isolated from 59 Austrian dairy processing facilities between 1987 and 2012.

L. monocytogenes reference strains ATCC BAA-679 (EGD-e) and Δ-*prfA*, +*IAC L. monocytogenes* EGDe (DMSZ) (Fruhwirth, Fuchs, Mester, Wagner, & Rossmanith, 2012) were used in this study as phage susceptible controls.

All bacteria strains were grown overnight in tryptone soya broth (TSB) with 0.6% (w/v) yeast extract (Oxoid Ldt., Hampshire) at 37 °C. Overnight cultures were diluted ten-fold in fresh medium and incubated at 37 °C for 3–4 h to obtain a maximum number of viable cells in the logarithmic growth phase (log phase).

Phage P100 was used as the Listex™ P100 commercial preparation (EBI Food Safety Wageningen, Netherlands). PFU (plaque forming units) determination resulted in a phage titer of approx. 6×10^{10} PFU/ml.

2.2. Screening of phage insensitive plant isolates and selection of artificially induced insensitive isolates

Screening of the plant isolates for Listex™ P100 insensitive *L. monocytogenes* isolates was performed using cross streak tests (Miller, 1998). For this purpose 50 μl Listex™ P100 preparation was delivered onto tryptone soya agar plates supplemented with 0.6% (w/v) yeast extract (TSA; Oxoid Ldt., Hampshire) and allowed to run from the top to the bottom of the Petri dish. After drying the streak was crossed with log phase cultures of the *Listeria* isolates using 10 μl inoculation loops. Plates were incubated overnight at 37 °C. Insensitive isolates (which did show growth in the bacteria-phage cross of the plate) were used for a second cross streak test on PALCAM plates incubated at room temperature.

In food processing plants Listex™ P100 is often applied diluted. The supplier recommends a dilution of 0.05% in smear (1×10^8 PFU/ml; www.listex.eu/cheese-and-Listeria, accessed 2015-03-07). Therefore cross streak tests using a ten-fold dilution series of Listex™ P100 (in SM buffer: 5.8 g NaCl, 2.4 g Tris HCl, 1.0 g gelatin add. 1000 ml, pH 7.0) were carried out in order to test the effect of diluted Listex™ P100. The laboratory strain Δ-*prfA IAC*+ *L. monocytogenes* EGDe (described in 2.1), *L. monocytogenes* EGDe and two plant isolates (plant isolate 145 and 142) (all sensitive to P100) were used for these tests. Two colonies growing in the phage zone of the cross streak test (one derived from the plant isolates 145 and one from the laboratory strain Δ-*prfA*, *IAC*+ *L. monocytogenes* EGDe) were picked, named R145 and RΔ*prfA*, and used for all phage susceptibility tests.

Both, insensitive plant isolates and artificially induced insensitive isolates, were passaged for seven passages in order to test stability of insensitivity. Susceptibility of all isolates did not change significantly during this test, indicating that the insensitivity is stable.

2.3. Infection experiments using different bacterial concentrations — CFU determinations

Five hundred microliter or 1 ml aliquots of insensitive log phase *Listeria* isolates (5×10^8, 5×10^7, 5×10^3, 5×10^1 CFU/ml) were incubated with the same volume of Listex™ P100 (6×10^{10} PFU/ml; resulting in MOIs of $1.2 \times 10^{2,}$ 1.2×10^3, 1.2×10^7 and 1.2×10^9) for 30 min at 20 °C. Subsequently, the numbers of surviving bacteria were determined and compared to a non-infected control of the same isolate (treated with SM buffer instead of Listex™ P100). All plating was performed in duplicate on TSA and the experiment was repeated on two separate days. Incubation was performed overnight at 37 °C. Additionally, each isolate was plated at least once onto ALOA or PALCAM media. The phage susceptible control strain *L. monocytogenes* EGDe was integrated into the panel.

2.4. Infection experiments using different bacteriophage concentrations — OD_{600} measurements

Growth of Listex™ P100 infected plant isolates was monitored by measuring the optical density at 600 nm (OD_{600}) using a HP 8452 spectrophotometer (Hewlett Packard, Paolo Alto, CA, USA). Log phase growth of bacteria of all insensitive isolates and *L. monocytogenes* EGDe was adjusted to an OD_{600} of 0.6. One ml of

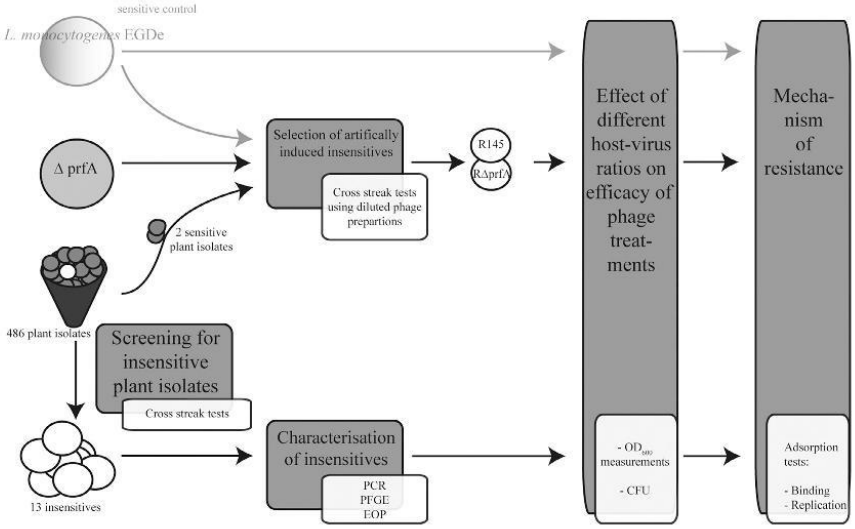

Fig. 1. Overview of the study.

the *L. monocytogenes* culture (approx. 5×10^8 CFU) was infected with MOIs of 12, 1.2 and 0.12. After 30 min incubation at 20 °C the bacteria/phage preparation was ten-fold diluted in TSB and incubated at 37 °C at 150 rpm. OD_{600} was measured after 2, 3, 4, 5, 6, 24 and 48 h. Growth curves of the infected isolates were compared with growth curves of non-infected isolates (using 1 ml SM buffer instead of Listex™ P100 preparation). All measurements were performed at least twice and repeated on two separate days. Exceptions are the experiments using *L. monocytogenes* EGDe which were repeated up to 19 times. Since measurements of optical densities are restricted to a high number of cells ($\geq 10^7$ cells/ml), EGDe infected with MOIs of 1.2 and 0.12 and the non-infected *L. monocytogenes* EGDe (for comparison) were plated onto TSA at 0.5, 1, 2, 3, 4, 5 and 6 h after infection and CFU/ml were determined.

2.5. Plaque assays and adsorption tests

PFU determinations were made using the Double Agar Overlay Plaque Assay (Kropinski et al. 2009). Five hundred microliter aliquots of the phage preparation (ten-fold dilutions in SM buffer) were mixed with 0.5 ml of log phase *L. monocytogenes* EGDe. Subsequently, 200 µl aliquots of the preparation were mixed with 3 ml of soft agar overlay (TSB supplemented with 0.4% agar and 10 mM $CaCl_2$) and immediately poured onto TSA plates. After 30 min at 20 °C the plates were incubated at 37 °C overnight. The plaques were counted and PFU/ml calculated. All PFU determinations were performed at least in duplicate with at least two different dilutions.

Numbers of plaques of *L. monocytogenes* EGDe were compared with numbers of plaques of insensitive plant isolates and efficiency of plaquing (EOP) determined as described by Kim and Kathariou (2009).

Adsorption tests were performed using log phase bacteria where the OD_{600} was adjusted to 0.6. Plant isolates and *L. monocytogenes* EGDe were infected with a MOI of 0.12. To test the binding-ability of P100 to insensitive isolates the infection was stopped after 0, 5, 10, 20 and 60 min as described by (Wendlinger, Loessner, & Scherer, 1996). Non-attached phages in the supernatant were further diluted in SM buffer and assayed using the Double Agar Overlay Plaque Assay. To monitor the ability of P100 to replicate in insensitive isolates, the infection was stopped and the number of PFUs per ml determined after 0, 1.5, 3, 4.5 and 6 h.

All experiments were performed at least twice in duplicate; as a control and for comparison, *L. monocytogenes* EGDe was included in the test panel.

2.6. Identification and characterization of insensitive plant isolates

Insensitive *L. monocytogenes* isolates were confirmed by three PCR assays. Two conventional PCR assays were performed as described by Border et al. (Border, Howard, Plastow, & Siggens, 1990) and Bubert et al. (Bubert et al., 1999), targeting the *iap* and *hly* locus of *Listeria*. Additionally, isolates were identified by real-time PCR, targeting the *prfA* locus of *L. monocytogenes*, according to Rossmanith et al. (Rossmanith, Krassnig, Wagner, & Hein, 2006).

L. monocytogenes serogroups were defined using a multiplex PCR targeting the specific target genes *lmo0737*, *lmo1118*, ORF2819, ORF2110 and *Listeria* spp. specific *prs* as published by Doumith et al. (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004).

DNA isolation and PFGE were performed following the current International Standard PulseNet protocol with the following modifications: applying 40 U *Asc*I and 50 U *Apa*I restriction enzymes incubated for 3 h at 37 °C and 25 °C, respectively (Pulsenet International, 2013; http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL04_ListeriaPFGEProtocol.pdf accessed on 30.09.2014). Restricted samples were used for electrophoresis on 1% SeaKem Gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C, and an inclined angle of 120° were applied for 22.5 h. The gels were stained with ethidium bromide and digitally photographed with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). The TIFF images were compared using the Fingerprinting II Cluster Analysis (Bio-Rad), and normalized using the PFGE global standard *Salmonella* ser. Braenderup H98124. Pattern clustering was performed using algorithms within Fingerprinting II Cluster Analysis (Bio-Rad). Specifically, the unweighted

pair group method, using arithmetic averages (UPGMA) and the Dice correlation coefficient with a position tolerance of 1.0%, wase applied. In order to identify identical PFGE types, a Dice coefficient similarity of 100% was used. When any differences in PFGE patterns were observable, the patterns were reported as different (Barrett, Gerner-Smidt, & Swaminathan, 2006).

2.7. Definitions

L. monocytogenes isolates that are susceptible to the phage P100 are referred to as "sensitive". *L. monocytogenes* plant isolates detected in the screening (2.2), which showed no or highly reduced susceptibility to the phage P100 in subsequent experiments (2.3 and 3.4), were referred to as "insensitive plant isolates". The degree of susceptibility (if there was one at all) was determined by plaque assays and calculation of EOPs (2.5). Results were summarized in Fig. 3. *L. monocytogenes* isolates (laboratory strains and plant strains) which were initially sensitive and were selected from single colonies in experiment 3.3 were referred to as "artificial induced insensitive isolates".

3. Results

3.1. Screening and origin of insensitive plant isolates

This study investigated *L. monocytogenes* isolates from 59 Austrian dairy processing facilities over a 15 year period (1987; 1998-1999; 2001–2012). In total, 486 isolates were processed. All data are summarised in Table S1.

In the cross streak test it could be observed that there were three different phenotypes: those insensitive to Listex™ P100, which were growing completely through the zone pretreated with P100, sensitive isolates which showed no growth in this zone (Fig. S1), and some isolates where growth was reduced and only some colonies could be seen in the phage zone (Fig. 2). Thirteen isolates that were growing completely through the phage zone were defined as insensitive and further tested.

Listex™ P100 insensitive *L. monocytogenes* isolates were found in the years 2001, 2011 and 2012. In 2001 seven out of 204 (3.4%) isolates were insensitive. Out of 158 *L. monocytogenes* isolates sampled in 2011 and 2012, six (3.8%) were insensitive to Listex™ P100. Nearly half of the investigated isolates came from one processing facility (I, 234 isolates). There were no Listex™ P100 insensitive *Listeria* in a screening of 85 isolates taken from this plant between 1987 and 2010. In contrast, six out of 152 (3.9%) plant isolates from this dairy obtained in 2011 and 2012 were insensitive.

The majority of the investigated isolates derive from cheese or cheese products. From 124 cheese isolates, two (1.6%) turned out to be insensitive. A similar number of isolates derived from samples that were summarized as 'Others', and included *L. monocytogenes* found in wash water or swabs. Here six (4.2%) out of 140 isolates showed reduced sensitivity to Listex™ P100. Of 180 isolates originating from smear water, five (2.8%) were insensitive. Additionally, there were 31 isolates from drain water and 11 isolates taken from brine. Here no Listex™ P100 insensitive isolates could be detected.

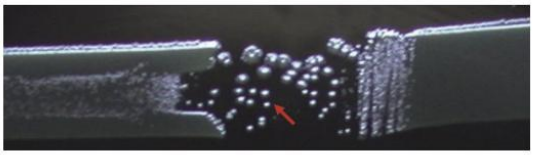

Fig. 2. *L. monocytogenes* clones growing in the phage zone of a cross-streak test.

3.2. Characterisation of insensitive isolates

EOP determinations showed that in all three years (2001, 2011 and 2012) two groups of insensitive isolates were found (Fig. 3): one group, which showed no susceptibility to the phage P100 and formed no plaques at all, and one group, which was able to produce plaques, but had a reduced susceptibility to the phage P100 compared to the sensitive control (*L. monocytogenes* EGDe).

The multiplex-PCR assay revealed that the 13 insensitive *L. monocytogenes* isolates clustered in two distinct PCR serogroups 1/2a–3a (genetic lineage II) and 1/2b–3c (genetic lineage I) (Wiedmann et al., 1997).

L. monocytogenes isolates were typeable with both macrorestriction enzymes (*Asc*I and *Apa*I). Each representative PFGE type was included in further cluster analyses. The generated *Asc*I and *Apa*I patterns divided the strains into two and three major clusters diverging at a similarity level of 50 and below 40% (Fig. 3 and Fig. 4). In detail, five *L. monocytogenes* (Wo2) isolates during 2011–2012 in cheese processing facility I shared the identical PFGE pattern I.1. Further, two insensitive *L. monocytogenes* (Wo1) isolates from cheese processing facility I shared the PFGE pattern I.2. Both PFGE patterns I.1. and I.2 showed a Dice coefficient similarity of 71%. The PFGE profile II.1 was represented in six *L. monocytogenes* isolates (Ro1) detected during 2001 in processing facilities II, III and IV. The latter hard cheese producers are from the same geographic region and very likely shared the same ripening cellar; and *L. monocytogenes* was spread between cheeses due to cross-contamination during smearing. Ro2 with the PFGE pattern II.2 was only isolated once. Moreover, the PFGE subtypes correlate with the EOP types – Ro2 and Wo2 had a highly reduced susceptibility to the phage P100 compared to the phage sensitive control *L. monocytogenes* EGDe; Ro1 and Wo1 did not produce plaques at all.

3.3. Cross streak tests with different phage concentrations and selection of artificially induced insensitive isolates

Log phase cultures of four P100 sensitive *L. monocytogenes* isolates, two of them plant isolates, were used to cross the phage infected zone with virus concentrations ranging from 6×10^{10} to 6×10^{5} PFU/ml. It could be observed that initially sensitive isolates were clearly starting to grow through the zone when the phage concentration was reduced to 6×10^{8} PFU/ml or less (Fig. 5). Two single colonies of this zone were selected. They remained insensitive even at high phage concentrations and derived from plant isolate L145, which was initially sensitive, and the cloned lab strain Δ-*prfA L. monocytogenes* EGDe, which was initially also sensitive. Thus, two insensitive strains were produced *in vitro*. All experiments regarding the effect of different host-virus ratios on efficacy of phage treatments and mechanism of resistance performed with the insensitive plant isolates were repeated with these artificially induced insensitive isolates (named R145 and R*ΔprfA*).

3.4. Effect of different host-virus ratios measured by CFU determinations

To confirm insensitivity indicated by the cross streak test and to study the effect of different host-virus ratios on phage treatments, liquid log-phase cultures of relevant *L. monocytogenes* plant as well as artificially induced insensitive isolates were infected. Results of insensitive infected *L. monocytogenes* and insensitive non-infected *L. monocytogenes* isolates did not differ significantly and were reproducible with different initial bacteria concentrations (approx. 5×10^{8} to 5×10^{1} CFU/ml infected with approx. 6×10^{10} PFU/ml), indicating that observed insensitivity is not concentration

Plant	Total all Years[a]	Isolate	Isolation Date	Serotype (PCR)	PFGE	EOP[b]
I	6 (237)[c]	L99 39.40	2011	**1/2a**, 3a	Wo1 R	no plaques
		L99 65.66	2011	**1/2a**, 3a	Wo1 R	no plaques
		L93 45.46	2011	**1/2a**, 3a	Wo2 R	8.32×10^{-4}
		L97 7.8	2011	**1/2a**, 3a	Wo2 R	2.28×10^{-4}
		L98 17.18	2011	**1/2a**, 3a	Wo2 R	6.24×10^{-4}
		L102 71.72	2012	**1/2a**, 3a	Wo2 R	3.14×10^{-4}
II	4 (6)	L49 77.78	2001	**1/2b**, 3b	Ro1 R	no plaques
		L50 29.30	2001	**1/2b**, 3b	Ro1 R	no plaques
		L50 31.32	2001	**1/2b**, 3b	Ro1 R	no plaques
		L51 39.40	2001	**1/2b**, 3b	Ro1 R	no plaques
III	1 (1)	L51 31.32	2001	**1/2b**, 3b	Ro1 R	no plaques
IV	1 (1)	L51 59.60	2001	**1/2b**, 3b	Ro1 R	no plaques
V	1 (3)	L57 53.54	2001	**1/2b**, 3b	Ro2 R	5.78×10^{-4}

[a]Only plants and years in which P100 insensitive *Listeria monocytogenes* were detected are listed
[b]Efficiency of plaquing (ratio of plaques formed on plant strain over plaques formed on sensitive *L.monocytogenes* EGDe)
[c]Number of insensitve isolates (number of total isolates)
All dark grey highlighted plants (II-V) shared one ripening cellar. Plant II - IV are located in the same geograpic region

Fig. 3. Overview of dairy, isolation year, serotype, PFGE type and EOP of insensitive *L. monocytogenes* plant isolates.

dependent (Fig. 6). In contrast, growth of *L. monocytogenes* EGDe was at least three log scales reduced and no colonies were obtained when 5×10^3 or 5×10^1 CFU/ml were infected with Listex™ P100.

3.5. Growth curves using different phage infection doses

Comparison of infected and non-infected insensitive plant isolates and *L. monocytogenes* EGDe revealed that all 13 isolates from the dairies grew similarly, irrespective of whether they were infected or not (Fig. 7A). It could be observed that growth is affected only when they are infected with high doses of Listex™ P100 (MOI of 12) (Fig. 7A). Growth curves of artificially induced insensitive isolates (Fig. 7B and D) revealed a not significant reduced growth compared with insensitive plant isolates. When growth curves of insensitive plant isolates were compared with the ones of infected phage sensitive *L. monocytogenes* EGDe, there is a significant difference (Fig. 7C): Growth of *L. monocytogenes* EGDe was clearly reduced when it was infected with a MOI of 0.12 and was not measurable ($OD_{600} \leq 0.05$) when infected with the phage P100 with MOIs of 12 or 1.2 within in the first six hours. After 24 h *L. monocytogenes* EGDe infected with a MOI of 1.2 was measurable ($OD_{600} > 0.05$). After 48 h *L. monocytogenes* EGDe infected with a MOI of 12 was measurable and growth of non-infected

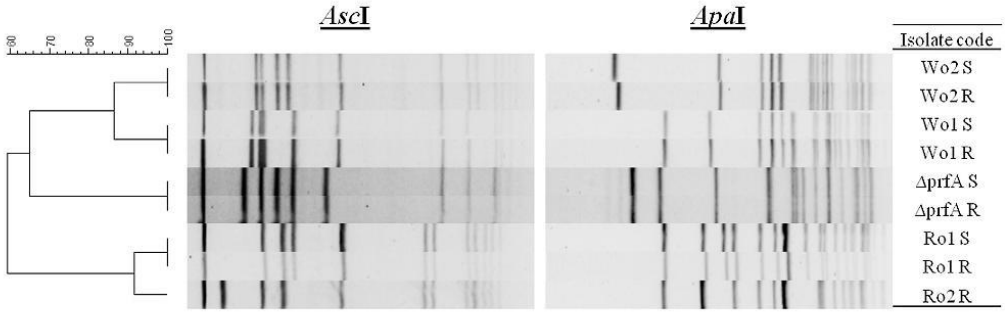

Fig. 4. Combined PFGE cluster using Asc I and Apal. Insensitive 2001 isolates are PFGE Types Ro, insensitive isolates from plant I found in 2011/12 are PFGE Types Wo. S summarizes patterns of phage sensitive isolates, R summarizes patterns of phage insensitive isolates. Δ*prfA* S is the wild type Δ*prfA L. monocytogenes* EGDe strain and Δ*prfA* R is the artificially induced insensitive counterpart isolate RΔ*prfA*. Wo2 S includes the plant isolate L145 and Wo2 R includes the artificially induced insensitive isolate R145.

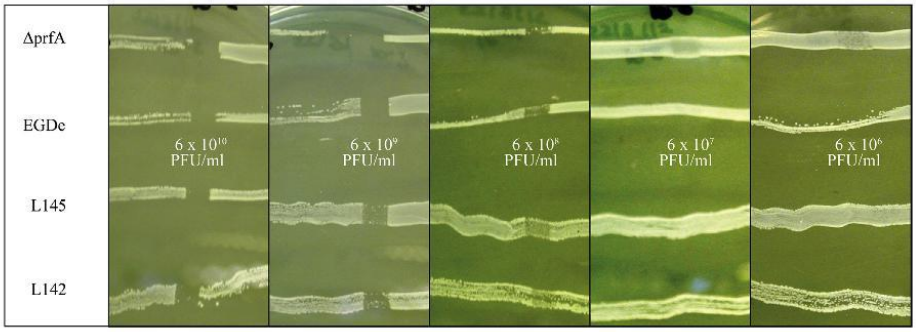

Fig. 5. Cross-streak test on TSA-Y agar demonstrating the effect of decreasing Listex™ P100 concentrations on the growth of *L. monocytogenes* EGDe, *L. monocytogenes* ΔprfA and two plant isolates, L145 and L142.

L. monocytogenes EGDe and samples infected with MOIs of 12 or 1.2 was very similar.

In a second experiment liquid log phase cultures of *L. monocytogenes* EGDe were infected with two different infection doses and CFUs were determined over six hours. The isolate infected with a MOI of 0.12 showed slightly reduced growth compared to the non-infected isolate. However, after two hours regrowth could be observed and no difference emerged (Fig. 8). When *L. monocytogenes* EGDe was infected with a MOI of 1.2 the number of CFU decreased up to five log scales within the first four hours (Fig. 8). Afterwards the bacteria cell number began to increase again and after 24 h OD_{600} was again measurable (Fig. 7C).

3.6. Phage adsorption and replication

Short time phage adsorption tests were conducted to investigate binding and attachment of the phage particles to the infected bacteria (Fig. 9A). The concentration of free phages after infection of both *L. monocytogenes* EGDe decreased within the first 20 min by about three log scales. In contrast, this could not be seen when insensitive plant isolates or artificially induced insensitive isolates were infected. The number of free viruses was stable, indicating that phages were not able to bind to these isolates.

To test phage replication, phage sensitive *L. monocytogenes* EGDe and insensitive plant isolates were investigated in long time infections (6 h) as seen in Fig. 9B. The number of phages in the supernatant increased by about three log scales over the time in sensitive *L. monocytogenes* EGDe, whereas it stayed at the same starting log scale when insensitive *L. monocytogenes* isolates and the artificially induced insensitive isolate R*ΔprfA* were infected. This shows that phages were not able to replicate in insensitive plant isolates. Infection of R145 resulted in one log-scale increase of virus concentrations during the first six hours (Fig. 9B). Nevertheless, the phage quantity was still significantly lower than when sensitive isolates or *L. monocytogenes* EGDe were infected and the quantity of phages did not further increase over time.

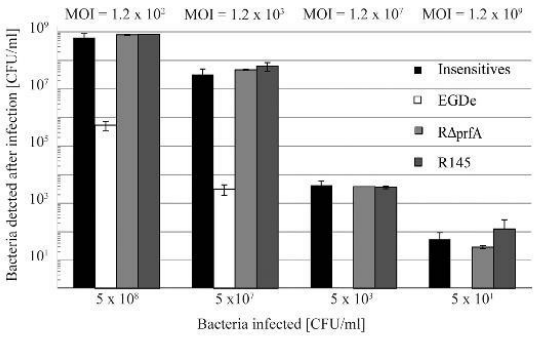

Fig. 6. Effect of Listex™ P100 using different bacteria concentrations for infection. Growth of the insensitive *L. monocytogenes* dairy isolates (average of all 13 isolates = insensitives, black), artificially induced insensitive isolates R145 and R*ΔprfA* (grey and light grey) and *L. monocytogenes* EGDe (white) were observed. *L. monocytogenes* EGDe was not detected when 5×10^3 or less CFU/ml were infected. Plating was performed 30 min after infection with P100 and CFUs were determined after 24 h of incubation. All isolates were infected twice in duplicate. Mean values and standard deviations are shown.

4. Discussion

The underlying objective for the use of bacteriophages in food production is to limit the presence of food pathogens in foodstuffs. The high specificity of phages for their hosts and the corresponding lack of other effects, whether on consumer health or on the quality characteristics of the product, make this a reasonable tool (Mahony et al., 2011; Monk et al., 2010).

Nevertheless, from an evolutionary perspective, complete elimination of a host is not desirable as this would entail simultaneous elimination of the phage. Moreover, it can be accepted that bacteria are very successful at responding to exogenous challenges, whether physical, chemical or biological. This can be achieved by modifying internal parameters to establish, if necessary, a state of resistance. Therefore it is reasonable to speculate that the use of products such as Listex™ P100 in food production could lead to resistant *L. monocytogenes* strains.

In respect of food-related *L. monocytogenes* isolates, adaptation against bacteriophages by a temperature depended resistance (Kim & Kathariou, 2009), as well as by the production of new restriction modification systems (Kim et al., 2012; Lee, Ward, Siletzky, & Kathariou, 2012) have been reported. Indeed, resistance resulting from cell wall- and receptor modifications (Nir-Paz, Eugster, Zeiman, Loessner, & Calendar, 2012) appears to be common. However, other forms of resistance can result from spontaneous mutations, which is also a specialized phage defence mechanism (O'Flynn, Ross, Fitzgerald, & Coffey, 2004). Insensitivity of *Listeria* found in turkey processing plants to *Listeria* phages isolated from these plants is very high; up to 63% (Kim, Siletzky, & Kathariou,

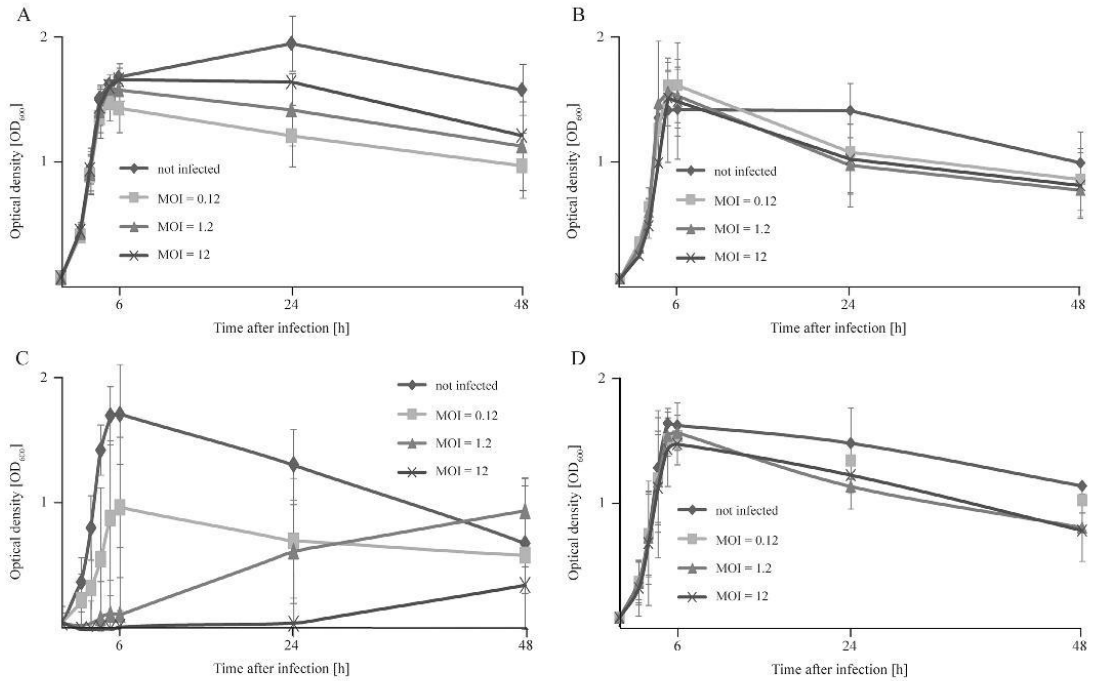

Fig. 7. Growth kinetics of *Listeria* after treatment with Listex™ P100: 0–48 h. (A) Insensitive plant isolates (average value, n = 13). (B) Artificially induced insensitive isolate R145. (C) Sensitive *L. monocytogenes* EGDe. (D) Artificially induced insensitive isolate RΔ*prfA*. All isolates were infected twice and in duplicate. Rhombi (◆) indicate controls, which display growth of non-infected *L. monocytogenes* isolates. Initial inocula of bacteria were approximately 5×10^8 CFU/ml. Concentrations of P100 used are depicted in the diagrams (■, ▲, and **x**). Bacterial growth was monitored by measuring the optical density (OD_{600}). Mean values of at least two experiments and standard deviations are shown.

2008). Unfortunately, no epidemiological data concerning P100 resistant *Listeria* in food plants is available to supplement this report.

This was the precedent for the presented study whereby 486 *L. monocytogenes* isolates obtained from 59 dairies and 15 different years were screened for the presence of P100 insensitive *L. monocytogenes* (Table S1). The central question was whether those appear randomly by natural exposure to bacteriophages or whether insensitivity is triggered specifically by exposure to Listex P100. The resulting pattern of insensitivity of *L. monocytogenes* isolates in this study showed an interesting distribution indicating a clear trend; the overall number of P100 insensitive *L. monocytogenes* isolates was thirteen (2.7%) for all years and all investigated plants, and in five of 59 dairies insensitives were detected. Assuming random events, one would then expect this to occur in thirteen different plants concerned (22%) and insensitives in thirteen out of 15 years (86%). Factually, insensitives occurred in three years (2001, 2011, 2012; 20%) and in five plants (I–V; 8.5%). These five plants could be divided into plant I, which was most representative, because nearly half of the isolates (234/486) came from that cheese processing facility, and in plants II–V, which shared one ripening cellar. If the occurrence of P100 insensitive *L. monocytogenes* isolates was based on random events, it would have been very likely to detect insensitive isolates not only in five of 59 dairies.

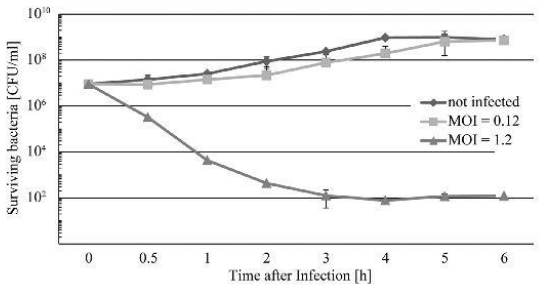

Fig. 8. Growth kinetics of sensitive *L. monocytogenes* after treatment with Listex™ P100: 0–6 h. Rhombi (◆) indicate controls, which display growth of non-infected *L. monocytogenes* EGDe isolates. Initial inocula of bacteria were 5×10^8 CFU/ml. Concentrations of P100 used are given in the diagrams (■ = 6×10^7 PFU/ml, ▲ = 6×10^8 PFU/ml). Bacterial growth was monitored by the plate count method on TSY-A agar (See also Fig. 7C). The experiment was conducted twice in duplicate and mean values and standard deviation are shown.

In plant I, in which Listex™ P100 treatments were introduced in 2010, the only P100 insensitive *L. monocytogenes* were obtained in 2011 and 2012 after introduction of Listex™ P100 treatment. 152 isolates from these years included six that were insensitive (3.9%), whereas 85 isolates obtained from 1987 to 2010 from this plant did not include any that were insensitive (0%). In plants II–V insensitive isolates were found during 2001. Likewise, this was not likely a random event. These plants shared the same ripening cellar. Conclusively, insensitive Listeria isolates were found in plants II–V only in cheese and smear water, which corresponds to the

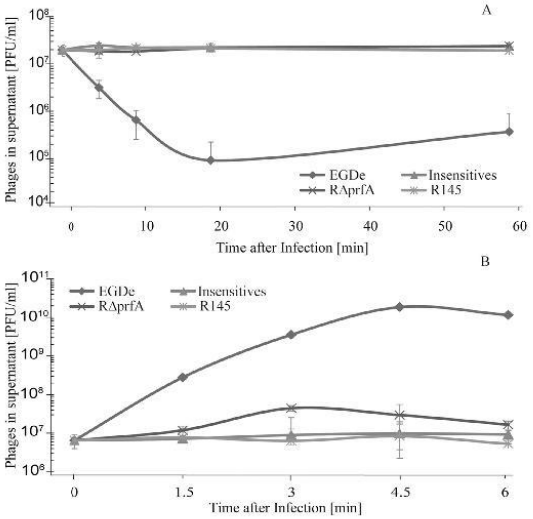

Fig. 9. Adsorption of P100 to *L. monocytogenes*. (A) Titre of phages in the supernatant indicating attachment 0–60 min after infection. (B) Titre of phages in the supernatant indicating attachment 0–6 h after infection. (◆) Sensitive *L. monocytogenes* EGDe. (x) Artificially induced insensitive isolate RΔ*prfA*. (▲) Insensitive *Listeria* plant isolates (summarised results of all isolates, n = 13). (ж) Artificially induced insensitive isolate R145. Phage numbers were determined by the plaque count method. The experiment was conducted twice in duplicate and mean values and standard deviation are shown.

experimental application of the bacteriophage P100 at that time (confirmed by personal communication).

Serotyping by PCR and PFGE resulted in the identification of two insensitive serotypes in plant I (Wo1 and Wo2) and two different serotypes in plants II–V (Ro1 and Ro2) (Figs. 3 and 4). Serotype Ro1 was related to plants II–IV, which are small facilities in the Vorarlberg federal state, and Ro2 was related to plant V, which is a small Tyrolean facility. Although resistance could be a result of selection of naturally occurring insensitive *Listeria* and reduced susceptibility to the phage P100 can be caused by bacteriocin produced by lactic acid bacteria during dairy product procession (Tessema, Moretro, Snipen, Axelsson, & Naterstad, 2011), our data indicate that the insensitive *Listeria* isolates from both, plant I and the ripening cellar, are a result of directional selection within the production sites. This hypothesis is supported by the fact that recurrently detected *L. monocytogenes* pulsotypes were among the phage P100 sensitive and insensitive isolates (Fig. 4). No large-scale genomic re-arrangements in genomic islands or mobile elements resulting in the gain or loss of restriction sites was detected when comparing sensitive and insensitive *L. monocytogenes* (Fig. 4). This also indicates an adaptation of the *L. monocytogenes* strains due to persistent contact with P100. Moreover, by testing artificially induced insensitive isolates, which is discussed in more detail in the following section, it could be shown that development of insensitive strains is induced relatively easily. The risk of resistance developing probably could be reduced by not using Listex™ P100 continuously over a long time period, but only as an emergency treatment. Some authors recommend treatments with phage cocktails or alternative use of different phages (EFSA, 2009; Garcia, Martinez, Obeso, & Rodriguez, 2008; Guenther et al., 2009; Soni & Nannapaneni, 2010b). However, according to Vongkamjan et al. (Vongkamjan, Roof, Stasiewicz, & Wiedmann, 2013), isolates that survived treatment with a phage cocktail showed decreased susceptibility to individual phages included in the phage mix.

The second part of the study examined the adaptation of *L. monocytogenes* to P100 and the effect of different phage – bacteria ratios on efficacy of phage treatments and development of resistance. Resistance development was investigated by generating artificially induced insensitive isolates. O'Flynn et al. (O'Flynn et al., 2004) found that BIMs in *Escherichia coli* O157:H7 emerged with a frequency of 10^{-4} to 10^{-6} CFU, indicating that mutations leading to phage resistance are, at least for *E. coli* O157:H7, rare. In our study cross streak tests with decreasing phage concentrations were carried out. This was performed as plants often use diluted phage preparations in practice. The resulting low phage pressure, especially on select host niches and reservoirs, could lead to resistance (Hagens & Loessner, 2010). We showed in cross streak tests that, starting with a 10^{-1} diluted Listex™ P100 preparation (6×10^9 PFU/ml), complete elimination of *L. monocytogenes* is inhibited (Fig. 5). Further experiments using two selected artificially induced insensitive isolates revealed that these isolates remained insensitive, even when higher phage concentrations were used. These data indicate that selection of insensitive *L. monocytogenes* isolates may be normally rare statistically, but easily induced if sufficient bacteria counts are involved (high concentration of bacteria and low concentration of phages).

Furthermore, we investigated the effect of different phage concentrations on the phage sensitive *L. monocytogenes* isolates. We showed that regrowth of infected sensitive *L. monocytogenes* EGDe occurred depending on the applied amount of phages (Fig. 7C) and were able to show that high concentrations of bacteria were reduced by three and five log scales, but not completely eradicated (Fig. 6).

These findings correlate with data of several other studies, where a *Listeria* reduction by up to five log scales and a concentration dependent effect (less phages or infection of higher bacteria densities were less effective) were described and complete eradication of the pathogen long term or eradication of high bacteria concentrations was not possible (Guenther et al., 2009; Guenther & Loessner, 2011; Silva, Figueiredo, Miranda, & de Castro Almeida, 2014; Soni & Nannapaneni, 2010a, 2010b; Soni, Nannapaneni, & Hagens, 2010). The fact that Listex™ P100 is not able to eradicate high concentrations of *L. monocytogenes* completely may enhance the risk for development of phage insensitive *Listeria* isolates.

The third part of the study dealt with the mechanism of resistance. OD_{600} measurements of infected liquid cultures of insensitive isolates over longer time periods (Fig. 7) displayed a difference between infected isolates and the control after 48 h, indicating that detected insensitive plant isolates are not completely insensitive but have highly reduced susceptibility to the phage P100. This also resulted from the EOP test which confirmed two classes of insensitive isolates: one group, which does not show any susceptibility to the phage P100 and one group of isolates which shows reduction in plaque counts of about four log scales in comparison to sensitive isolates (Fig. 3). These data refer to a cell wall modification that hinders entrance or attachment of the phage to the bacteria, but is probably not completely able to prevent infection.

Results of adsorption tests using the 13 insensitive *L. monocytogenes* isolates and the two artificially induced insensitive isolates indeed suggest a receptor modification because the phages were not able to attach to the tested plant isolates (Fig. 9). Furthermore, the number of phages remained in the same log scale up to six hours after infection, indicating that no replication of the phage took place. An exception was one artificially induced insensitive isolate, namely R145, in which the initial number of phages increased one log scale within the first three hours but did not further rise thereafter. This isolate should be investigated closer, especially since the

second artificially induced insensitive isolate did not show this phenomenon and R145 could possess a different resistance mechanism.

5. Conclusion

In summary: (i) Bacteriophage P100 insensitive *L. monocytogenes* isolates can be found in diaries. (ii) Occurrence of insensitive *L. monocytogenes* isolates is likely coupled to the application of Listex™ P100 and not the random entrance of P100 into facilities. (iii) Different serotypes of the insensitive isolates indicate adaptation of the strains to the presence of P100 in the plants.

For elimination of the bacteria the concentration of phages is crucial as well as the concentration of bacteria. Diluted phage preparations show a clearly reduced activity. Bacteria concentration has also an effect on the elimination process as higher concentrations of sensitive *Listeria* only lead to a reduction in cell counts after phage treatment up to 5 log scales; no complete elimination is observed. Finally, the underlying mechanism of the resistance is could be due to a modification of the phage receptor as P100 does not bind to the surface of insensitive *L. monocytogenes* strains.

In conclusion, the application of bacteriophage P100 could be recommended as an additional measure in an emergency, if accompanying actions are performed. These actions should prevent the bacteriophage from spreading and support the rapid elimination of P100 from the production environment. The routine application of P100 cannot be supported.

Conflict of interest statement

All authors disclose that they have no financial and personal relationships with other people or organizations that could inappropriately influence their work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2015.05.026.

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3.2 Publication 2: Influence of environmental factors on phage-bacteria interaction and on efficacy and infectivity of phage P100

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- Experimental design
- Performance of experiments (together with Christian Robben)
- Analysis of data (together with Christian Robben)
- Drafting of the manuscript

Title:

Influence of environmental factors on phage-bacteria interaction and on the efficacy and infectivity of phage P100

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3.3

22 Abstract:

23 When using bacteriophages to control food-borne bacteria in food production plants and
24 processed food, it is crucial to consider that environmental conditions influence their stability.
25 These conditions can also affect the physiological state of bacteria and consequently host-
26 virus interaction and the effectiveness of the phage ability to reduce bacteria numbers. In this
27 study we investigated the stability, binding and replication capability of phage P100 and its
28 efficacy to control *L. monocytogenes* under conditions typically encountered in dairy plants.
29 The influences of SDS, Lutensol AO 7, salt, smear water and different temperatures were
30 investigated. Results indicate that phage P100 is stable and able to bind to the host under most
31 conditions tested. Replication was dependent upon the growth of *L. monocytogenes* and
32 efficacy was higher when bacterial growth was reduced by certain environmental conditions.
33 Phages were initially able to reduce bacteria up to seven $\log_{10}$ units in long-term experiments.
34 However, thereafter re-growth and development of phage-resistant *L. monocytogenes* isolates
35 were encountered.

36

37 Keywords: Bacteriophage P100; host-phage interaction; environmental influence; resistance;
38 *L. monocytogenes;* Listex™ P100

39

40

1. Introduction

Although bacteriophages have been known for about 100 years, commercial use of lytic bacteriophages to detect and control pathogenic bacteria has increased in recent years (Mann, 2005). In particular, the use of phages as an alternate class of antibacterial agents against food-borne pathogens is of growing interest due to advantages that they offer. These include high host specificity and the fact that phages do not change the quality and sensory perceptions of food (Ali, Tuladhar, & Zevenbergen, 2012; Hagens & Loessner, 2007, 2010; Mahony, McAuliffe, Ross, & van, 2011).

Phages and phage products are now commercially available against nearly all important food-borne bacteria. A phage that is commonly used to combat *L. monocytogenes* is P100. This phage was originally isolated from the waste water of a dairy plant and is commercially available as Listex^{TM} P100. It has now been confirmed Generally Regarded As Safe (GRAS) by the US Food and Drug Administration (FDA)(Efsa, 2009).

Listeria monocytogenes is one of the most important food-borne pathogens. Due to its ability to grow or persist at low pH values, high salt concentrations, low temperatures and in environments with low water activity, it is frequently found in adverse environments, such as food production plants (Koutsoumanis & Sofos, 2005; Swaminathan & Gerner-Smidt, 2007). When phages are used to combat bacteria in food production plants it is crucial to consider that factors such as heat, cold, dryness, nutrient deficit, curing and exposure to chemical detergents or disinfectants influence phage stability, the physiological state of bacteria and consequently host-virus interactions (for instance attachment and replication of the phage) and the effectiveness of phages at reducing bacteria numbers (Arachchi, et al., 2013; Denes & Wiedmann, 2014). In food production plants external factors such as temperature, pH and water activity particularly affect the success of phage treatments (Garcia, Martinez, Obeso, & Rodriguez, 2008; Jończyk, Kłak, Międzybrodzki, & Górski, 2011; Ly-Chatain, 2014).

The first requirement for the successful application of phages for biocontrol of bacteria is phage stability. In general, bacteriophage stability is highly variable. Since bacteriophages are the most abundant biological entities on earth, their ubiquitous presence means that they play a major role on nutrition, energy and global biogeochemical cycles (Breitbart & Rohwer, 2005; Breitbart, et al., 2002; Chibani-Chennoufi, Bruttin, Dillmann, & Brüssow, 2004; Fuhrman, 1999; Mann, 2005; Wommack & Colwell, 2000). Their ubiquity and diversity means that they can be found in a range of adverse environments comprising a variety of

chemical and physical conditions (Jończyk, et al., 2011) from, for example, dry and hot environments such as the Sahara (Prigent, Leroy, Confalonieri, Dutertre, & DuBow, 2005) and hot springs (Breitbart, Wegley, Leeds, Schoenfeld, & Rohwer, 2004), to cold waters such as those in polar regions (Säwström, Lisle, Anesio, Priscu, & Laybourn-Parry, 2008), food (Lu, Breidt, Plengvidhya, & Fleming, 2003; Nel, Wingfield, Van der Meer, & Van Vuuren, 1987; Pringsulaka, Patarasinpaiboon, Suwannasai, Atthakor, & Rangsiruji, 2011) cheese factories (Bruttin, et al., 1997), to humans (Bachrach, Leizerovici-Zigmond, Zlotkin, Naor, & Steinberg, 2003), soil (Williamson, Wommack, & Radosevich, 2003) and sewage (Havelaar & Hogeboom, 1984). Nevertheless, sensitivity of individual phage classes is highly diversified (Jończyk, et al., 2011). Some authors assume a relationship between a phage's morphology and its occurrence and infectivity in adverse environments, but evidence supportive of that hypothesis and predictions that can be made is lacking (Lasobras, Muniesa, Frias, Lucena, & Jofre, 1997). However, it is known that the stability of a virus can be affected by changes of its virion or viral nucleic acid structure by various factors such as pH, ionic strength of the immediate environment, UV-light or heat (Ly-Chatain, 2014; Maura & Debarbieux, 2011). Moreover, maintenance of phage populations usually requires the presence of the bacterial host, which is also influenced by environmental factors (Maura & Debarbieux, 2011). As mentioned, bacterial fitness and physiological states can change the host-virus interaction.

The first step in host-virus interaction is attachment. On one hand attachment of the phages to bacteria and the susceptibility of the host can be reduced when bacterial fitness is compromised. A change in the physiological status of bacteria can lead to transcriptional responses that influence cell wall structures, which serve as receptors for phages (Denes & Wiedmann, 2014). Modified phage receptors could complicate or even prevent binding of the phage to the host. On the other hand, external factors, such as the presence of whey proteins, can result in non-specific binding or trapping of the phages thereby reducing host adsorption rates (J. J. Gill, Sabour, Leslie, & Griffiths, 2006). Another factor worth keeping in mind when bacteriophages are used to control food-borne bacteria, is accessibility to the target bacteria. Accessibility to the host can be limited when phages are applied in solid matrices or when the phage concentration, total numbers and time of application are not optimal (Ly-Chatain, 2014).

Following attachment of phages to the host, the phage genome is injected into the host cell and replication of the phage particle can commence. However, inadequate nutrition, poor

environments and a switch to the stationary growth phase lead to decreased productivity of phage infection and to small burst size as phage replication is dependent on host cell growth (Chibani-Chennoufi, et al., 2004; Denes & Wiedmann, 2014).

Even though attachment and replication have major influences on the effectiveness of phage treatments, external factors can directly affect the outcome of phage infections. For instance, the presence of inhibitory compounds such as antibodies, whey proteins or bacteriocins can reduce the effectiveness of phage treatments and can even lead to resistance against the phages used (S. T. Abedon, 2012; Ly-Chatain, 2014; Maura & Debarbieux, 2011; Tessema, Moretro, Snipen, Axelsson, & Naterstad, 2011; Vongkamjan, Roof, Stasiewicz, & Wiedmann, 2013). Suboptimal application of phages can also lead to adaptation of the bacteria to the phage, the development of phage resistance and consequently ineffective treatment (Hagens & Loessner, 2010).

Although it is known that several factors influence the efficacy of phage treatments against food-borne pathogens, relevant studies detailing interactions are limited and current research is still at an early stage (Arachchi, et al., 2013; Garcia, et al., 2008; Vongkamjan, et al., 2013). Until now it has not been clear as to what extent environmental factors influence bacterial susceptibility to phage infection (Denes & Wiedmann, 2014). Moreover, past failures in phage therapy were mostly caused by limited knowledge of phage biology (O'Flaherty, Ross, & Coffey, 2009). Therefore, some investigators recommend testing of phage persistence in the absence of target bacteria (Chan, Abedon, & Loc-Carrillo, 2013). Efficacy testing of phage treatments has been suggested for each type of application and under different environmental conditions on a case-by-case basis (Ganegama Arachchi, et al., 2013; Garcia, et al., 2008; Skurnik, Pajunen, & Kiljunen, 2007).

Therefore, the main focus of this study was to test the effectiveness of phage P100 against *L. monocytogenes* under conditions normally found in dairy plants. The influence of various chemical and physical factors on the infectivity and persistence of phage P100 was tested. These included the effect of different temperatures, pH values, salt and detergent concentrations, smear water and Fraser broth on the stability preinfection, the attachment on the host, and replication after injection of viral nucleic acid. Moreover, the effectiveness of phage P100 at eliminating *L. monocytogenes* under the above conditions was investigated. Long-term infection experiments were also conducted to examine the development of resistant *L. monocytogenes* isolates at different temperatures.

2. Materials and Methods

2.1 Bacterial strain, phage and growth conditions

The phage P100 susceptible *Listeri*a strain used in this study was *L. monocytogenes* reference strain ATCC BAA-679 (EGDe). To establish standardised conditions, bacteria were grown in tryptone soya broth (TSB) with 0.6 % (w/v) yeast extract (OxoidLdt., Hampshire) at 37 °C. Overnight cultures were ten-fold diluted in fresh medium and incubated at 37 °C for 3 - 4 h to obtain a maximum number of viable cells in the logarithmic growth phase (log phase).Phage P100 was supplied as the commercial preparation Listex™ P100 (EBI Food Safety Wageningen, Netherlands). PFU (plaque forming units) determination resulted in a phage titre of approximately 6 x 10^{10} PFU/ml. The Listex™ P100 preparation was diluted in SM buffer to yield phages at other concentrations.

2.2 Influence of environmental factors on the infectivity of P100

To investigate the persistence and infectivity of P100 in the dairy environment over a longer time period, phages (5 x 10^{10} PFU/ml) were added to different smear water samples stored at 4 °C and 10 °C. Two types of smear water were obtained from an Austrian dairy plant. Each smear water type was then split into four samples and each sample was pre-treated as follows: The first sample was the original untreated smear water obtained from the dairy plant. The second sample was autoclaved smear water. The third sample was smear water inoculated with *L. monocytogenes* EGDe (5 x 10^{8} CFU). The fourth sample was smear water supernatant after centrifuging for 5 min at 8,000 x g in order to remove bacterial or eukaryotic (e.g. yeast) cells. Phage infectivity was determined every ten days over a total of 117 days using the double agar overlay plaque assay. As a non-treatment control, phage infectivity following incubation in SM buffer was monitored. Each experiment with each type of smear water was performed in duplicate.

To test the short-time influence of chemicals on P100 stability in terms of infectivity, phages were incubated in TSB containing either 2 M NaCl (Fisher Scientific, Leics, UK), 5 % Lutensol AO 7 (BASF, Ludwigshafen, Germany), 5 % SDS (SIGMA-ALDRICH, Steinheim, Germany) and TSB with pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12. After 1, 6 and 24 h the number of phages was determined using the double agar overlay plaque assay (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009) as previously described (Fister, et al., 2016). For comparison and as a non-treatment control, phages were incubated in TSB. Each experiment was carried out at least in triplicate on different days.

173

174 **2.3 Adsorption tests**

175 To determine binding and replication characteristics of P100, adsorption tests were performed
176 under different environmental conditions. The variables were salt concentration, pH,
177 concentration of detergents and temperature.
178 Log - phase bacteria cultures were adjusted to an OD_{600} of 0.6. Thereafter bacteria were
179 centrifuged at 8,000 x g for 5 min. The pellet was resuspended in TSB medium representing
180 the chemical conditions described above (2.2). Phage P100 was added to a MOI (multiplicity
181 of infection, ratio of phages to bacteria) of 0.1. In order to determine adsorption of the phages
182 on the host under these conditions, the infection was stopped by the addition of ice-cold TSB
183 followed by centrifugation for two minutes at 8,000 rpm after 0, 10, 20, 30, 40, 50 and 60 min
184 as described by Wendlinger and colleagues (Wendlinger, Loessner, & Scherer, 1996).
185 Afterwards phages remaining in the supernatant were serially diluted in SM buffer and
186 quantified using the double agar overlay plaque assay. To monitor the replication ability of
187 the phage at the conditions described, the number of phages was measured after stopping the
188 infection after 0, 1, 3 and 6 hours. All experiments were carried out at least twice in duplicate.
189

190 **2.4 Chemical influence on the efficacy of phage treatment (short-term)**

191 To analyse the influence of different chemical conditions on the efficacy of phage treatments,
192 *L. monocytogenes* EGDe was infected with phage P100 and grown in Fraser Listeria Selective
193 Enrichment Broth base (Merck, Darmstadt, Germany), TSB and TSB medium with different
194 salt concentrations, detergent (Lutensol AO 7 and SDS) concentrations and in TBS adjusted
195 to different pH values (for details se 2.2). Additionally, the efficacy of phage treatment in
196 smear water, obtained from an Austrian dairy plant, was investigated. The influence of these
197 factors on the growth of uninfected *L. monocytogenes* EGDe was monitored as a control.
198 In detail, two concentrations of log-phase *L. monocytogenes* EGDe cultures (approx. 2.5×10^7
199 and 2.5×10^6 CFU/ml) were infected with P100, with a MOI of 10, and were incubated at 37
200 °C in TSB media representing the chemical conditions described above. The OD_{610} of these
201 infections was measured for 24 hours every hour in a TECAN F100 microplate reader (Tecan
202 Austria GmbH., Groeding, Austria). The experiments were performed at least twice on
203 different days and in duplicate.
204

205 **2.5 Influence of temperature on the efficacy of P100 treatments (long-term)**

206 To examine the influence of temperature on the efficacy of phage treatments, 500 µl of a log
207 phase *L. monocytogenes* EGDe culture was incubated with an equal volume of Listex^{TM} P100
208 dilutions (2 x 10^{10} and 2 x 10^{9} PFU/ml, resulting in MOIs of 10 and 100) for 30 min at room
209 temperature. Thereafter, 5 ml of TSB was added and the infected *Listeria* stored at 4 °C, 10
210 °C and 20 °C for 17 weeks. The number of surviving bacteria was determined weekly using
211 the plate count method and compared to a non-infected control of the same isolate (treated
212 with SM buffer instead of Listex^{TM} P100). All plating was performed in duplicate on TSA
213 with at least two different dilutions and plates were incubated overnight at 37 °C. The
214 experiment was performed twice.
215

216 **2.6 Screening for newly formed resistances and confirmation of insensitive isolates**

217 When regrowth of *L. monocytogenes* in the long-time temperature experiment (2.5) was
218 observed, single colonies growing on the TSA plates of the experiment were selected and
219 used for preparation of overnight cultures. These cultures were then used to screen for
220 insensitive *L. monocytogenes* isolates using cross streak tests (Miller, 1998) as follows: 50 µl
221 P100 (6 x 10^{10} PFU/ml) were allowed to run from the top to the bottom of TSA+Y plates.
222 After drying, the streak was crossed with the cultures of re-growing *L. monocytogenes*
223 isolates. *L. monocytogenes* EGDe was used as sensitive control. Plates were incubated
224 overnight at 37 °C. Suspicious isolates, which grew in the phage-zone of the plates, were
225 confirmed as *L. monocytogenes* by plating on selective ALOA and PALCAM agar.
226 Additionally, suspicious isolates were confirmed as *L. monocytogenes* by PCR as described
227 by Rossmanith *et al* (2006). Resistance and reduced susceptibility were confirmed by small
228 drop plaque assays (Mazzocco, Waddell, Lingohr, & Johnson, 2009). Stability of resistance
229 was tested by passaging the isolates for five passages.
230

231 **3. Results**

232 **3.1 Phage P100 is stable under most tested chemical conditions**

233 For the investigation of P100 stability in SM buffer and different types of smear water over a
234 period of four months, comparison revealed similar results for samples that were stored at 4
235 °C and 10 °C (Fig. 1 A and B). At both incubation temperatures the decrease in the number of
236 infective phages was lowest when P100 was stored in SM puffer, followed by phages stored
237 in autoclaved smear water. In both experimental conditions, the number of phages decreased

within the first month by about 1 - 2 $\log_{10}$ units and did not change distinctly until the end of the investigation. Phages stored in untreated smear water were reduced by about 2.5 - 3 $\log_{10}$ units. The highest phage reduction was observed in smear water containing *L. monocytogenes* EGDe. In this case P100 was reduced by 4 $\log_{10}$ units. For all experiments a successive decrease in phages was observed within the first 37 days. Afterwards the number of infective phages remained more or less stable (up to 117 days).

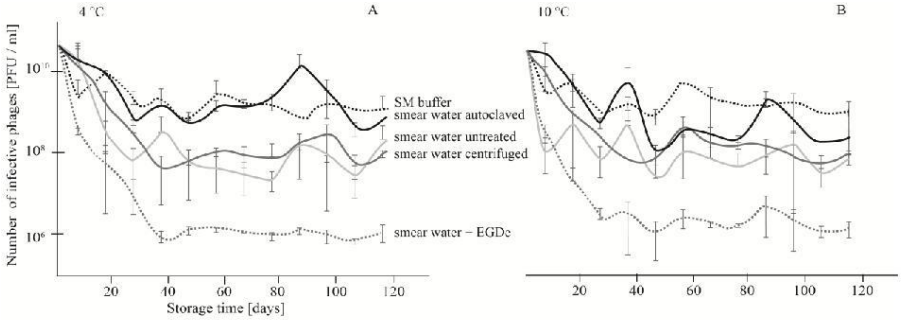

Fig. 1: Infectivity of P100 stored in smear water and SM buffer at 4 °C (A) and 10 °C (B) for 117 days. Smear water was either untreated (as obtained from the dairy plant), autoclaved or centrifuged in order to remove bacterial or eukaryotic (e.g. yeast) cells. Further, *L. monocytogenes* EGDe was added to one smear water sample.

As a next step in the short-term experiments, phage P100 was incubated in TSB containing different detergents or adjusted to different pH values. Infectivity of P100 was then determined after 1, 6 and 24 hours of incubation (Tab. 1). At pH 2, P100 numbers were rapidly reduced below the detection limit, indicating a reduction of at least 7 - 8 $\log_{10}$ units within one hour. At pH 3, P100 concentrations decreased within one hour by at least 5.4 $\log_{10}$ units and P100 numbers were reduced below the detection limit (7 - 8 $\log_{10}$) at six hours. At pH 4 – 10, P100 numbers were not distinctly reduced and even after 24 hours the decrease in phage number was $\leq$ 0.5 $\log_{10}$ units. At pH 11 phage numbers were reduced by nearly 1 $\log_{10}$ level within one hour. After 24 hours, infectivity of phages was also reduced by about 1 $\log_{10}$ units compared to the control. Five percent Lutensol AO 7 did not change the phage number within one day. The second tested detergent, SDS, reduced P100 numbers by about 0.3 $\log_{10}$

262 units, when the SDS concentration in TSB was 5 %. After six and 24 hours of incubation, the
263 phage concentration was decreased by 0.6 and 1.2 log_{10} units.

264

Table 1: Reduction of infectivity of P100 after incubation TSB adjusted to different pH values, containing NaCl and detergents

Chemical condition	Incubation time 1 hour	6 hours	24 hours
pH 2	≥ d.l.	≥ d.l.	≥ d.l.
pH 3	5.4 - d.l.	≥ d.l.	≥ d.l.
pH 4	0.1 (+/-0.1) log_{10}[a]	0.4 (+/-0.5) log_{10}	0.1 (+/-0.2) log_{10}
pH 5 - 10	≤ 0.2 log_{10}	≤ 0.3 log_{10}	≤ 0.5 log_{10}
pH 11	0.8 (+/-1.4) log_{10}	0.1 (+/-0.1) log_{10}	0.9 (+/-1.1) log_{10}
pH 12	≥ d.l.	≥ d.l.	≥ d.l.
2 M NaCl	≤ 0.5 log_{10}	≤ 0.5 log_{10}	≤ 0.5 log_{10}
5 % Lutensol AO 7	0.0 (+/- 0.1) log_{10}	-0.1 (+/- 0.8) log_{10}	-0.1 (+/- 0.2) log_{10}
5 % SDS	0.3 (+/-0.3) log_{10}	0.6 (+/-0.2) log_{10}	1.2 (+/-0.7) log_{10}

[a] log_{10} units reduction (+/- standard deviation)
≥ d.l. : Higher than the detection limit (reduction of ≥ 7 - 8 log_{10})

265

266 **3.2 Binding of phage P100 was possible at all tested chemical conditions, but replication**
267 **was not observed in the presence of detergents, at low pH values and high salt**
268 **concentrations**

269

270 In order to test the influence of different chemical conditions on the attachment of P100 to *L.*
271 *monocytogenes* EGDe, adsorption tests in different smear water samples, in Fraser medium, in
272 TSB media containing different NaCl or detergent concentrations and in TSB media with
273 different pH values were performed and the respective results are shown in Fig.2. For all
274 tested incubation conditions distinct binding of phage P100 to *L. monocytogenes*, indicated by
275 an initial drop in free phage counts in the supernatant, was found. At NaCl concentrations up

to 0.1 M an increase of free phages in the supernatant was observed after 30 minutes (Fig. 2A), indicating replication of P100. At 0.5 M NaCl the number of unattached phages increased after 40 minutes and at 1 and 2 M NaCl no increase in free phage numbers was observed within 60 minutes. In Lutensol and SDS-containing TSB medium, in all tested smear water samples and in the selective Fraser medium, there was also no detected increase in unattached phages within one hour, except in the presence of 0.1 % SDS (Fig. 2B, 2E and 2D). At all tested pH values an increase of free phages was observed 40 min after infection (Fig. 2C). The number of unattached phages 60 minutes after infection was lowest at pH 3 and between 8 - 11.

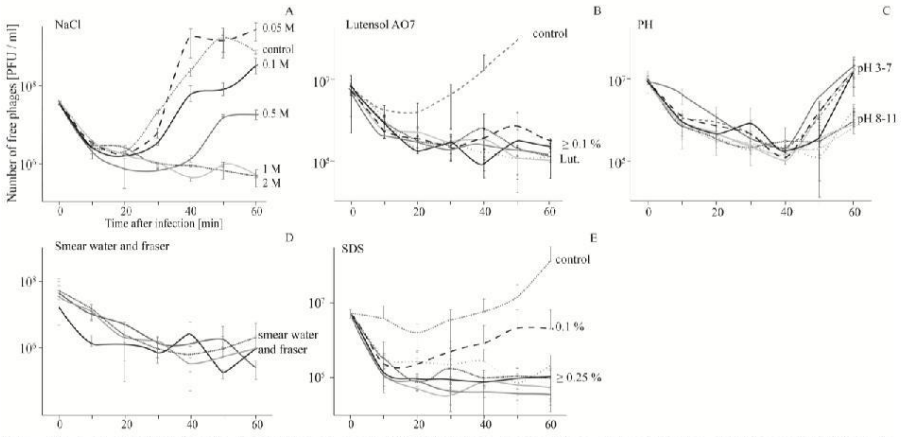

Fig. 2: Adsorption tests performed over 60 minutes indicating attachment of phage P100 to *L. monocytogenes* in TSB containing NaCl (A), Lutensol AO 7 (B), TSB adjusted to pH values 3 - 11 (C), smear water and Fraser (D) and TSB containing SDS (E).

As replication of phages was not observed at all tested conditions within one hour after infection, adsorption tests were also performed over six hours in order to obtain an indication if phage replication at different chemical conditions (described above) is possible. Results indicate that replication of phages is not possible at all tested conditions (Fig. 3). When the influence of salt was tested, the number of free phages was very similar in TSB media containing up to 0.1 M NaCl compared with the TSB control (Fig. 3A). The number of free phages in TBS containing 0.5 M or 1 M NaCl distinctly increased later as in TSB without additional NaCl. In TSB media containing 2 M NaCl an increase in free phages was observed

300 after one hour. However, after six hours phage concentration was similar to that at the
301 beginning of the experiment. These results indicate that replication of P100 is influenced by
302 increasing concentrations of NaCl. Adsorption tests in TSB media with pH values ranging
303 from 5 - 11 indicate that replication of phage P100 in *L. monocytogenes* is possible (Fig. 3B).
304 At pH value 4 the number of free phages decreased for up to three hours after infection.
305 Thereafter there was a slight increase in phages up to six hours after infection. The
306 concentration of phages measured at the beginning of the experiment was not reached
307 indicating no or only very limited replication of phage P100 at pH 4. The effect of detergents
308 on replication of phage P100 was tested by performing adsorption tests in TSB media
309 containing 0.1 - 5 % Lutensol AO 7 or SDS (Fig 3C and 3D). At all Lutensol AO 7
310 concentrations ($\leq$ 0.25 %) the number of free phages increased between one and three hours,
311 indicating that P100 replication is possible. In TSB media containing 0.5 - 5 % Lutensol AO
312 7, the concentration of free phages also increased between one and three hours, but after six
313 hours the phage concentrations had still not reached initial values. These data could either
314 indicate that phages first bind to the bacteria for about three hours and then they are set free
315 again, or that they are able to enter the cells but replication is slow. In the presence of the
316 second detergent, SDS, no increase of free phages was observed when concentrations $\geq$ 0.25
317 % are present (Fig. 3D). These data indicate that phage replication did not take place. In
318 contrast, in TSB containing 0.1 % SDS phage numbers were observed to increase after one
319 hour, indicating replication of P100.
320

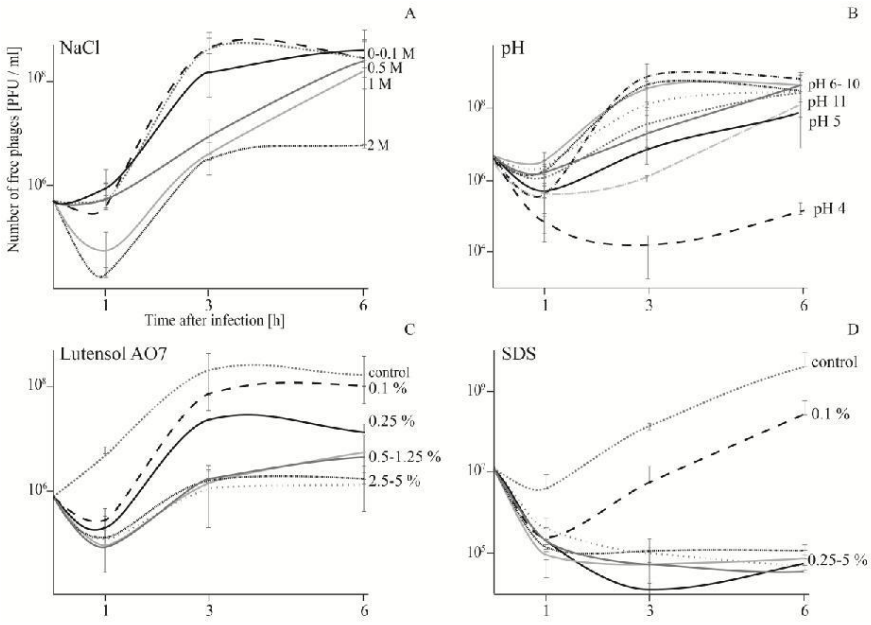

Fig. 3: Adsorption tests performed over six hours in TSB media containing NaCl (A), adjusted to different pH values (B) and containing the detergents Lutensol AO 7 (C) or SDS (D). Graphs show the number of free (unattached extracellular) phages.

3.3 Chemical conditions that reduce the growth of *L. monocytogenes* lead to higher efficacy of phage treatments

In order to test the effect of different chemical conditions on the efficacy of P100 to reduce *L. monocytogenes,* bacteria (2.5 x 10^7 and 2.5 x 10^6 CFU/ml) were infected with P100 with a MOI of 10 and the growth of bacteria in TBS media adjusted to different pH values or contained different NaCl or detergent concentrations was monitored by measurement of OD_{610} over 24 hours. All experiments were carried out twice and in duplicate. In some cases, growth of *L. monocytogenes* was observed at shifted time points or there was growth in one experiment, but not when repeated. In supplement figures S1 - 3 each single growth curve that was measured is shown. For the sake of simplicity Fig. 4 - 6 show representative growth curves for one measurement of each tested condition.

When the effects of Fraser medium and NaCl were tested on the growth of uninfected *L. monocytogenes,* a distinct reduction in growth was only observed in TSB medium containing 2 M NaCl. When 10^7 CFU/ml were infected with phage P100 (MOI = 10), growth of *L.*

monocytogenes was suppressed in TSB and TSB containing 1 and 2 M NaCl. Growth of *L. monocytogenes* in TSB containing between 0.05 M - 0.5 M NaCl and in Fraser broth was observed in all experiments. When lower bacteria concentrations (10^6 CFU/ml) were infected, in three out of four experiments growth of *L. monocytogenes* was observed in Fraser medium, in 0.05 M and 0.1 M NaCl containing TSB medium and in half of the experiments in TSB media.

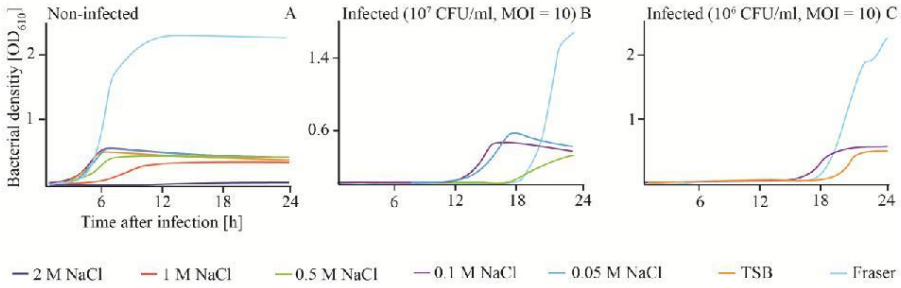

Fig. 4: Growth curves of uninfected (A) and infected (B and C; MOI = 10) *L. monocytogenes* in TSB, Fraser and NaCl containing TSB media. Bacteria concentrations at the beginning of the infections were 10^7 CFU/ml (A and B) and 10^6 CFU/ml (C).

Uninfected *L. monocytogenes* were able to grow in TSB adjusted to pH values ranging from 6 – 9 (Fig. 5A). Bacterial growth was also observed at pH 5 in three of four experiments, although lower OD values were obtained. When higher *Listeria* concentrations (10^7 CFU/ml) were infected, phage P100 was not able to suppress growth of *L. monocytogenes* at pH values 8 and 9 longer than 12 - 20 hours (Fig. 5B). In one of four repetitions at pH 7, growth of *L. monocytogenes* was observed. When lower bacteria concentrations were infected (10^6 CFU/ml), growth of *L. monocytogenes* was possible at pH 8 and 9 (Fig. 5C). At pH 7 bacteria growth was monitored in two and at pH 6 in three of four repetitions.

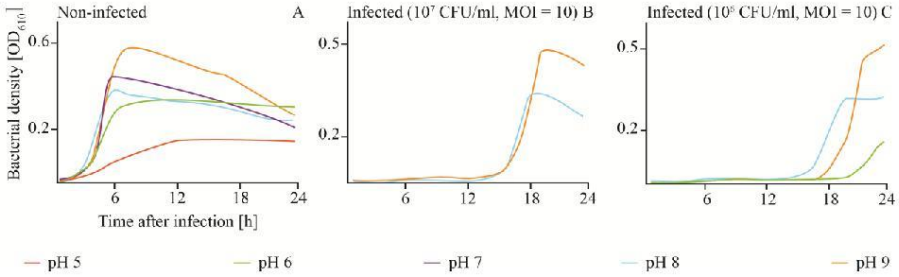

Fig. 5: Growth of uninfected (A) and infected (B and C; MOI = 10) *L. monocytogenes* in TSB adjusted to different pH values. The bacteria concentrations at the beginning of the infections were 10^7 CFU/ml (A) and (B) and 10^6 CFU/ml (C).

Detergents showed the strongest impact on the growth of both, infected and non-infected, *L. monocytogenes*.

L. monocytogenes was able to grow at all Lutensol AO 7 concentrations (0.1 - 5 %) tested. However, even at low Lutensol concentrations, growth of *L. monocytogenes* was distinctly reduced compared with growth in TSB media without Lutensol. When phages were added, growth was suppressed for at least 24 hours at all concentrations tested. The only exception was 0.1 % Lutensol in TSB. Here *L. monocytogenes* started to grow 15 hours post-infection.

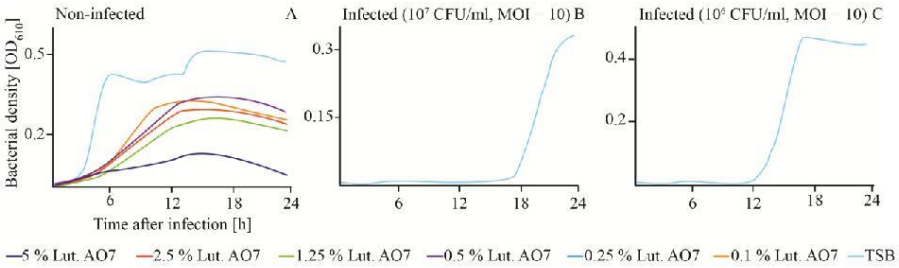

Fig. 6: Growth of uninfected (A) and infected ((B and (C); MOI = 10) *L. monocytogenes* in TSB containing different Lutensol AO 7 concentrations. Bacteria concentrations at the beginning of the infections were 10^7 CFU/ml (A) and (B) and 10^6 CFU/ml (C).

L. monocytogenes was not able to grow in TSB containing 0.1 - 5 % SDS for the first 24 hours (data not shown). Consequently, no infection experiments were performed.

As growth of *L. monocytogenes* at low temperatures is slow, the influence of temperature and host-phage ratio on the efficacy of P100 treatments was tested in longer-term experiments. *L. monocytogenes* EGDe was infected with MOIs of 10 and 100, incubated at 4 °C, 10 °C and 20 °C and the number of surviving bacteria determined weekly for 17 weeks.

At 4 °C *L. monocytogenes* was reduced by 6 and 7 $\log_{10}$ units (MOI = 10 and MOI = 100) within the first two weeks (Fig. 7A). Thereafter re-growth was observed. At 10 °C and 20 °C the reduction in *L. monocytogenes* counts was maximal at 4 - 5 $\log_{10}$ units and re-growth of *L. monocytogenes* was also observed (Fig. 7B and 7C). At all temperatures the bacteria concentration was the same irrespective of the addition of phage P100 at the end of the experiment.

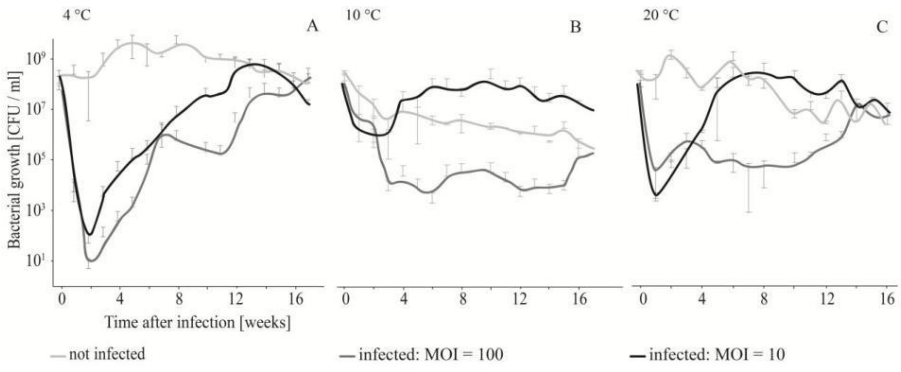

Fig. 7: Long-term infection experiment using different MOIs and temperatures for incubation. Growth of infected and non-infected *L. monocytogenes* was monitored at 4 °C (A), 10 °C (B) and 20 °C (C).

3.4 Development of insensitive *L.monocytogenes* isolates was observed at all tested temperatures

As described above, following an initial reduction of bacteria at the beginning of infection, re-growth of *L. monocytogenes* was observed at all tested temperatures and at both tested phage concentrations. In order to determine if survival and re-growing *Listeria* results from decreased efficacy of phage P100 or is caused by the resistance development, *L. monocytogenes* isolates were randomly selected at the end of the experiment. Afterwards they were tested for their susceptibility to P100. *L. monocytogenes* isolates that were obtained from

406 uninfected samples were all susceptible to phage P100. In contrast, all *L. monocytogenes*
407 isolates that were obtained from infected samples (independent of which MOI was used for
408 infection and at which temperature the infected bacteria were incubated), had reduced
409 susceptibility to P100. Moreover, susceptibility to the phage did not change over five
410 passages, indicating that insensitivity to P100 was stable.

411 **4. Discussion**

412 In food production bacteriophages have become more and more attractive as tools to eliminate
413 *L. monocytogenes* (Guenther, Huwyler, Richard, & Loessner, 2009; Guenther & Loessner,
414 2011; Oliveira, et al., 2014; Soni, Desai, Oladunjoye, Skrobot, & Nannapaneni, 2012; Soni,
415 Nannapaneni, & Hagens, 2010). In a recent publication Fister, et al. (2016) investigated the
416 basic issues regarding the occurrence of P100 resistant *Listeria* associated with the application
417 of Listex™. A connection between use of Listex™ and the presence of resistant *Listeria*
418 strains demonstrated. Additionally, the host relationship was investigated in *vitro*.
419 However, in *vivo* especially in food production plants there are harsh environmental
420 conditions. Therefore, this study took into account the influence of several of these chemical
421 and physical factors on P100 that are typically encountered in the food-production
422 environment. These conditions potentially influence the ability of P100 to reproduce by acting
423 on the phage's infection cycle. Relevant factors are: (i) the stability of the phage before
424 infection, (ii) the attachment process after contact with the host, (iii) replication after
425 attachment, injection of the phage genome, and finally (iv) the efficacy of the infection,
426 according to reduction of bacterial cell numbers. These four parameters were used to analyse
427 the efficacy of P100 against *Listeria*.
428 In following the sequence of events during the reproductive infection cycle of P100 we
429 examined the influence of pH, salt concentration and the presence of two detergents as
430 representative of chemical factors present in the production environment. The respective
431 experiments were conducted short-term (up to 24 hours). Moreover, long-term experiments
432 were conducted for 120 days. To represent naturally-occurring contaminations smear water
433 was included in the study.
434 To reflect the above mentioned succession of events, first of all the stability of the persisting
435 phage particles was investigated, as loss of phage infectivity would result in a rapid decrease
436 of overall efficacy of phage treatments (Jason J Gill, Sabour, & Griffiths, 2010).
437 We did not observed a significant reduction in phage P100 numbers in TSB media over the
438 pH range 4 - 10 within 24 hours (Tab.1). P100 numbers rapidly decreased by > 5 $\log_{10}$ units

439 within one hour, but only when the pH values were 2, 3 and 12. This data indicate that P100 is
440 stable in environments over a wide pH range. This observation is especially interesting as
441 another study showed that the phage population decreased on sliced apples due to the low pH
442 and this was why the phages were not able to prevent microbial growth (Leverentz, et al.,
443 2003). Moreover, it is known that during the cheese ripening process the pH increases and this
444 consequently supports the growth of *Listeria* (Guenther & Loessner, 2011).
445 As salt is one of the most frequent additions to food, and since it plays an important role in
446 cheese production, we tested the effect of various NaCl concentrations on phage P100. Salt is
447 known to influence osmotic pressure, leading to breaks in phage heads and tails(Jończyk, et
448 al., 2011). However, in our study no reduction in phage infectivity was observed when P100
449 was stored for up to 24 hours in TSB containing 2 M NaCl.
450 Cleaning and disinfection are also major ongoing activities in the food safety environment and
451 in food production. The most commonly used detergents used for cleaning are SDS and a
452 group of detergents summarized as *Lutensols*. Lutensols are polymeric ethoxylated aliphatic
453 alcohols that act as non-ionic tenside detergents, which means that they, like SDS, are
454 surfactants. The particular advantage of Lutensols is that they are considered to be
455 biodegradable and significantly less toxic than SDS. In our study we found no reduction in
456 phage P100 infectivity in the presence of up to 5 % Lutensol AO 7 in TSB within 24 hours,
457 whereas 5 % SDS reduced phage infectivity by about 1.2 $\log_{10}$ units within 24 hours. This
458 finding is in agreement with other studies (Howett, et al., 1999).
459 Smear water is a major application area for Listex^{TM}. Therefore we tested the influence of
460 smear water on the stability of P100 at different temperatures. As recommended by other
461 authors, phage persistence with and without target bacteria was examined (Chan, et al., 2013).
462 The infectivity of P100 was reduced faster in smear water than in buffer (Fig. 1). The
463 presence of *L. monocytogenes* increased this effect significantly, which was expressed as 2
464 log units reduction in the PFU number. This could result for different reasons. One reason
465 could be a bacteriocin, which is known to be produced by lactic acid bacteria during dairy
466 product processing (Tessema, et al., 2011). Furthermore, proteolytic activity of the smear
467 water affects the infectivity and integrity of phage particles (Guenther & Loessner, 2011).
468 Moreover, microbial load in smear water may also provide non-specific binding sides (Garcia,
469 et al., 2008). In food stuffs or raw milk, phages can be entrapped by charge or hydrophobic
470 interactions and it is known that some phages are inactivated by raw milk and bovine whey
471 proteins (Gerba, 1984; Jason J Gill, et al., 2010; J. J. Gill, et al., 2006). Non-specific binding

472 and consequently phage trapping could also occur in smear water. Additionally, bacteria have
473 several phage defence mechanisms which could reduce the P100 numbers. Interestingly,
474 temperature differences did not distinctly change phage survival, although it is known that
475 phages are generally more stable at lower temperatures (Adams, 1949; Jason J Gill, et al.,
476 2010).
477 Overall the data suggest that phage P100 is very stable under a variety of environmental
478 conditions. Therefore, P100 is most likely able to remain infective for a long period of time in
479 food production plants. Indeed, from an unpublished study we were able to detect phage P100
480 in a dairy plant three months after the cessation of Listex^{TM} P100 use.
481 Besides phage stability, attachment of the phage to its target is the next crucial step in the
482 phage infection process. Chemical and physiological factors on the other hand can influence
483 the efficacy of phage binding. It is known that bovine whey proteins, which are also likely to
484 be present in cheese and smear water, affect host adsorption of phages (Denes & Wiedmann,
485 2014). However, our data indicate that binding of P100 to *L. monocytogenes* is not affected by
486 smear water (Fig. 2). In addition, high NaCl concentrations (up to 2 M), which are commonly
487 encountered in brine baths, do not hinder P100 binding to its host. Moreover, our data indicate
488 that P100 binding is not influenced at all the pH values tested and in the presence of
489 detergents such as SDS or Lutensol AO 7.
490 Phage replication is known to depend mainly on the physiological state of the host and the
491 burst size correlates with the growth rate of the bacteria (Chibani-Chennoufi, et al., 2004;
492 Denes & Wiedmann, 2014). However, the growth rate is dependent upon stress conditions,
493 which will frequently be present in food processing environments and thus pathogens are
494 normally not found undergoing exponential growth (Denes & Wiedmann, 2014). However,
495 metabolically active bacteria are a requirement for successful use of bacteriophages for active
496 control strategies. These active attempts rely on host cell metabolism, which is necessary for
497 phage replication and consequently active disruption initiated by the bacterial cell leading to
498 release of progeny phages. Results of our study have shown that replication was highly
499 influenced by chemical parameters such as pH value, high salt concentrations and the
500 presence of surfactants (Fig. 3). Nevertheless, these parameters mainly influence the
501 metabolic condition and growth rate of host cells, therefore hindering active control strategies.
502 Although replication is necessary in the active approach for successful phage treatments,
503 passive approaches in contrast do not depend on replication (Guenther, et al., 2009; Hudson,
504 McIntyre, Billington, Sabour, & Griffiths, 2010). In this case efficacy as defined by reduced

505 bacterial cell counts is a relevant criterion for assessment and the fourth factor that was
506 investigated in this study. Passive strategies are based on high phage concentrations (MOI
507 ≥10) (Kasman, et al., 2002). On one hand phage infection should then lead to a change in host
508 synthetic machinery resulting in arrest of host replication and a bacteriostatic effect. On the
509 other hand a scenario termed "lysis from without" has been described (Stephen T. Abedon,
510 2011). This refers to infection of one bacteria cell with a high number of phages leading to its
511 passive lysis. The supplier of Listex™ P100 recommends an application of 1 x 10^8 PFU/ml
512 (www.listex.eu/cheese-and-Listeria, accessed on 2015-03-07) consistent with a passive
513 treatment strategy. Therefore, we examined the effect of chemicals on the efficacy of phage
514 treatments using infective doses of MOIs of at least 10. Results (Fig. 4 – 6, and supplementary
515 material) indicated that bacterial growth is inhibited and delayed by the environmental
516 conditions and enhanced the efficacy of phage treatment. This observation is in agreement
517 with other authors who were able to show that phage treatments are more efficacious in
518 combination with conventional disinfection and sanitation measures (Montanez-Izquierdo,
519 Salas-Vazquez, & Rodriguez-Jerez, 2012). Synergic effects were, for instance, reported in
520 combination with the bacteriocin nisin on melons and apples (Leverentz, et al., 2003).
521 However, they were not obtained on beef (Dykes & Moorhead, 2002).
522 In our study, growth was monitored over 24 hours. As proliferation of *Listeria* is generally
523 slow at 4° C, long-term experiments (120 days) were also conducted to consider the effect of
524 temperature. Results (Fig. 7) of these experiments indicate that the highest reduction in *L.*
525 *monocytogenes* counts was achieved at 4 °C. However, at all temperatures tested, re-growth
526 was observed. Thus the efficacy of phage treatment was initially increasing, followed by a
527 rapid decline after two to four weeks, depending upon the storage temperature. This is an
528 interesting observation in respect of resistance development, which was confirmed by testing
529 single colonies. Depending on the storage temperature, *L. monocytogenes* isolates were found
530 that had a reduced sensitivity to phage P100 (Fig. 7 and Table S1, supplementary data). This
531 finding is in accordance with Fister, et al. (2016) and Guenther and Loessner (2011) who
532 detected phage-insensitive clones when phage A511 was used for 22 days for the treatment of
533 red mold cheese during ripening. These findings invite us to question how this bacterial
534 adaptation is possible. Is it caused by an active reaction of the bacteria? Or does it result from
535 random errors made by the bacterial polymerase during replication, followed by natural
536 selection? It is known that a single point mutation can result in changes to the P100
537 attachment site, leading to phage resistant *L. monocytogenes* (Prof. Loessner, personal

538 communication). Assuming an error rate of the bacterial polymerase of approximately 10^{-11}
539 per base and generation (Orsi, et al., 2008), this possibility is somewhat improbable as in this
540 study a maximum of 5 x 10^8 bacteria cells were used for the respective experiments. In 10^3
541 replicates only one insensitive isolate would be expected. This supports the hypothesis that
542 development of insensitive *L. monocytogenes* is an active reaction of *Listeria* to the threat of
543 P100. This calculation, and the knowledge that alterations to the physiological status of
544 bacteria can lead to transcriptional responses that ultimately lead to cell wall structure
545 changes, indicate that the re-growing, insensitive isolates detected in the temperature
546 experiment do not result from random mutations. Together with the fact that bacteria cell
547 numbers in the environment are significantly lower than in the experiment describe above,
548 these finding indicate that formation of *L. monocytogenes* insensitive to P100 in dairy plants
549 is possible.

550 **5. Conclusion**

551 In summary, our data indicate that phage P100 is stable under most conditions typically
552 encountered in food-production environments. Results also suggest that phage attachment is
553 possible under all tested conditions. Therefore, it can be concluded that the use of phages for
554 passive approaches, which mainly rely on numerically sufficient attachment of the phages to
555 the host, are not essentially influenced by environmental conditions. In contrast, phage
556 replication, which is necessary for all active control strategies, is dependent upon host cell
557 growth, which is reduced by factors such as the presence of detergents, extreme pHs or high
558 salt concentrations. Our results demonstrate that high numbers of phages in combination with
559 environmental conditions that limit growth of bacteria are most effective. Since the permanent
560 presence of phages in the production plant is likely to support development of phage-resistant
561 bacteria, the use of phages for control of food-borne pathogens in the production plant is not
562 recommended. This conclusion is reached in agreement with other studies (Hudson, et al.,
563 2010) which found that phage use was associated with only short-term pathogen inactivation.
564 In order to avoid the development of resistance, it is suggested that phages should not be used
565 as biopreservatives.

566 **Acknowledgement**

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3.4 Publication 3: The (virucidal) effect of ionic liquids on phages P100 and MS2

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Title:

The (virucidal) effect of ionic liquids on phages P100 and MS2

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Abstract:

This study examined for the first time the effect of ionic liquids (ILs) on the infectivity of viruses. Specifically its aim was to test if structure-activity relationships of ILs, which are now known from several biological systems, are applicable to viruses. It is proposed that ILs may have future application as virucidal disinfectants. Altogether 16 different ILs were tested. The majority did not cause a reduction in virus infectivity when applied as 5% aqueous solution. Decreasing virus infectivity was observed primarily when ILs with more than one cationic alkyl side chain were applied. Tested ILs with chaotropic anions did not influence virus infectivity.

Keywords:

Ionic liquids; virus; side-chain-effect; disinfection

Introduction

Ionic liquids continue to find numerous applications, ranging from simple solvents to tools for chemical synthesis, CO_2 capture, coatings and others. With the increasing range of IL applications from laboratory to factory scale, their toxicity and environmental fate have come into focus during the last decade [8, 24]. Past research has shown that IL toxicity is not only similar to common organic solvents, but can also be attributed to the activity of highly active biocides. In almost all investigated biological test systems (from enzyme inhibition assays to vertebrate *in vitro* tests), the structure–activity relationship (SAR) outcomes of ILs have indicated that the length of the side chain is the most significant indicator of biological activity. This suggests that the anion toxicity is more relevant in short-chained, thus less toxic ILs [20, 24, 25], than in those with longer chains. With increasing numbers of publications on this subject, knowledge and predictability of IL toxicity has improved significantly over the past decade. This has led to the realization that their tuneable toxicity is of great potential and has resulted in them being research targets as new antimicrobials, antibacterial coatings and even as active pharmaceutical ingredients [2, 7, 11, 15, 17]. With such new application fields in mind, it becomes clear that one major research area has been neglected, namely the impact of ILs on viruses. So far, only one relevant study, from Byrne, Rodoni, Constable, Varghese and Davis [5] has dealt with this theme. This concerned stabilization of the tobacco mosaic virus, which is an important genetic vector in plant biotechnology, using protic ILs. They report enhanced stabilization of the virus stored in ethylammonium and diethylammonium mesylate, while storage in triethylammonium and tripropylammonium mesylate caused a change in the secondary structure of the virus particles.

What has not been studied so far is the possible application of ILs as virucides, analogous to their application as bacteriocides. While numerous disinfectants against microbes are available, reliable inactivation of viruses (especially non-enveloped viruses) is still challenging [19]. Common ethyl alcohol, phenol or quaternary ammonium compound (QAC) - based disinfectants, which are effective in killing most pathogenic bacteria and fungi, show only poor or no activity against non-enveloped viruses, such as rhinovirus, norovirus or hepatitis A. Basically the only reliable and thus widely used surface disinfectants for viruses are either chlorine-based, such as household bleach, or formaldehyde. While being effective against viruses, use of chlorine- based disinfectants is problematic in that they can burn skin and eyes, are respiratory irritants, have bleaching properties and are corrosive to metals. Use of formaldehyde-based virus disinfectants is even more problematic as it is carcinogenic and should only be applied by specialized personal.

In this study we examined potential virucidal activity of imidazolium and ammonium-based ILs against two model non-enveloped viruses: *Listeria monocytogenes* phage P100 and *Escherichia coli* phage MS2. P100 is commercially available as the active ingredient in Listex™ P100, an anti-*Listeria* intervention product designed to combat pathogen contamination in food processing plants [6, 13, 23]. P100 is a member of the *Myoviridae*, has a 131 kilo base pair genome with dsDNA, a contractile tail, a molecular weight of about 1.2×10^8 Dalton and a length of approximately 300 nm [6]. It is known to be stable at high sodium chloride concentrations (up to saturated solutions) [10]. The removal of the phage after treatment is very important in order to avoid resistances against it [12, 14, 26]. Phage MS2, a common surrogate for human noroviruses [3], has an isometric shape, +ssRNA, a diameter of 26nm [9] and is stable in monovalent salt concentrations of at least 1 M [21].

In this study, the influence of 16 ILs (table 1) on the infectivity of these two viruses was tested. Phages MS2 or P100 were mixed with each IL (final concentrations 5 - 0.1% (solid ILs w/v; liquid ILs v/v in water) and incubated for 30 min at room temperature. Thereafter, serial dilutions were prepared and virus concentration determined by the Double Agar Overlay Plaque Assay [18] using *L. monocytogenes* EGDe and *E.coli* TOP 10F' as host strains. The log10 reduction of phage concentration (plaque forming units (PFU)/ml) in comparison to the untreated control was calculated. The detection limit was about 5-6 log10 reduction (depending on the ILs and their influence on the bacteria).

Results and Discussion

The ILs tested in this study differ in several structural characteristics, such as the cation core, anion and the length of the cationic alkyl side chain (table 1). All ILs were initially tested at a concentration of 5% (w/v or v/v) in water. If a PFU reduction of >2 log units was observed, additional IL concentrations (2.5%, 1% and 0.1%) were tested. The mean results of experimental triplicates are presented in Table 1. In terms of toxicity for cellular organisms, the IL anion moiety appears to play a minor role and has also been less well investigated compared with the cation. In a previous study by our group, we could show that anion chaotropicity is one factor that influences the antimicrobial activity of ILs with cation side chain lengths of ≤6 [3]. Chaotropic anions could be promising for virus inactivation as their protective capsid consists of protein units, which can be denatured. To investigate anion chaotropicity on virucidal activity, we choose ILs with the non-virucidal cation [C_4mim] and anions representing the Hofmeister series. Results presented in Table 1 show that even strong chaotropic anions, such as trichloroacetate or thiocyanate, did not lead to significant PFU reductions, meaning that both tested phages were not inactivated. Also, the iodine containing IL showed no significant effect on virus infectivity, although iodine is known to rapidly inactivate phage MS2 [4].

The impact of an elongated alkyl side chain on IL toxicity, the so called side chain effect, has been previously described for different bacterial species and other organisms [24]. In this study this effect of elongated side chains was tested for imidazolium and ammonium based cation cores, with chloride as the common anion. Results show that the side-chain effect can also be found for inactivation of both phages, albeit to a much lesser degree than for other organisms (Table 1). In the case of imidazolium-based ILs, a PFU reduction of more than 2 log units was found for only [C_{10}mim]Cl.

The antiviral activity was concentration dependent. However, even at 5% (v/v) and an incubation time of 30 min, the requirement for use as an virucidal agent (≥99.99% PFU reduction) was not fulfilled for phage MS2 as was the case for P100 (>5 log units reduction). At lower concentrations (≤ 2.5% IL), reductions of both phages were similar. These results demonstrate the much greater stability of non-enveloped viruses in comparison to their host bacteria for the respective phages (*Listeria monocytogenes* and *Escherichia coli*), which are effectively killed (99.99% CFU reduction) by a ~500 times lower concentration of [C_{10}mim]Cl (data not shown).

Similar results were found for the ammonium based ILs concerning the side chain effect. While no PFU reduction was found for [TMC_8A]Cl, slight and concentration dependent virus inactivation was found for [$TMC_{12}A$]Cl. For this IL, phage P100 was more sensitive than MS2 (P100: 2 log reduction; MS2: 1.2 log reduction). [$TMC_{16}A$]Cl also causes higher reductions of phage P100 (3 log units at 5% IL) than of phage MS2

(1.3 log units). At lower concentrations no significant difference between the tested phages was observed. However, as for [C_{10}mim]Cl, the PFU reduction was not effective enough for the ILs to be regarded as virucidal agents. The side chain effect is normally explained due to the increase of lipophilicity and surface activity of ILs with elongated side chains, which give them a surfactant-like behaviour. For cellular organisms the increased surfactant-like behaviour leads to a non-specific disturbance of biological membranes and therefore to increased toxicity [22]. Non-enveloped viruses, such as phages P100 and MS2, however, do not possess a membrane and explains their stability in the presence of ILs with long alkyl side chains. The observed inactivation of the phages by the long chained ILs is most likely due to protein denaturation. However, it must be mentioned that from this particular experimental approach it is not clear if the whole virus particle is denatured and disintegrated or if only the specific binding sites for host recognition are compromised.

In addition to ILs with a single elongated alkyl side chain, Byrne [5] tested two ammonium based ILs with either two or three octyl side-chains, triethylammonium and tripropylammonium ILs, for destabilisation of the tobacco mosaic virus. In this study, the application of [$C_8C_8C_1C_1N$][Cl] proved to be more effective against both tested phages compared to ILs with a single elongated alkyl side chain. PFU reductions of >5 log units (5% IL), 4.4 log units (2.5 and 1%) and < 1 log unit (0.1%) were determined for phage P100, while phage MS2 was less sensitive, showing a highest reduction of 3 log units after incubation with 5% IL. In good accordance with our previous results and following the trend observed by Byrne et al. (2012), [$C_8C_8C_8C_1N$][Cl] was effective against phage MS2, causing PFU reductions of about 3 log units at all tested concentrations. However, surprisingly, there was almost no effect of [$C_8C_8C_8C_1N$][Cl] on phage P100 at any of the tested concentrations. This was especially interesting as, compared with all other tested ILs, phage MS2 was more stable compared to phage P100. The reason for this could be the different structure and size of the phages. Phage P100 is much bigger and has a cubic head and a contractile tail, which consists of a sheath and a central tube. The tail could be the sensitive part of the phage as it is necessary for successful recognition and binding of the host [1]. In contrast, polyhedral phage MS2 is smaller and has no tail and this probably enhances its stability against ILs. Nevertheless, there is no evidence for the phage structure-stability hypothesis [16].

Table 1 Reduction of P100 and MS2 after 30 min incubation

Cation	Anion	Abbreviation	Tested concentration	P100 Log_{10} reduction	SD	MS2 Log_{10} reduction	SD
Effect of side chain							
Dimethylimidazolium	Chloride	[C_1mim][Cl]	5%	0.16	0.05	0.08	0.10
1-Ethyl-3-methylimidazolium	Chloride	[C_2mim][Cl]	5%	-0.09	0.06	0.13	0.08
1-Butyl-3-methylimidazolium	Chloride	[C_4mim][Cl]	5%	-0.05	0.06	0.71	0.98
1-Hexyl-3-methylimidazolium	Chloride	[C_6mim][Cl]	5%	0.03	0.20	0.14	0.15
1-Octyl-3-methylimidazolium	Chloride	[C_8mim][Cl]	5%	0.08	0.40	0.49	0.34
1-Decyl-3-methylimidazolium	Chloride	[C_{10}mim][Cl]	5%	> d.l.	-	2.48	1.17
			2.5%	2.27	0.79	1.83	0.51
			1%	1.15	1.40	1.53	1.25
			0.1%	0.23	0.51	0.85	1.60
Trimethyloctylammonium	Chloride	[TMC_8A][Cl]	5%	0.47	0.41	0.45	0.62
Trimethyldodecylammonium	Chloride	[$TMC_{12}A$][Cl]	5%	2.00	1.29	1.24	1.03
Trimethylhexadecylammonium	Chloride	[$TMC_{16}A$][Cl]	5%	3.11	1.70	1.32	0.51

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			2.5%	1.25	0.86	1.53	0.95
			1%	0.60	0.97	1.22	1.07
			0.1%	0.80	0.65	0.62	0.98
Dioctyldimethylammonium	Chloride	$[C_8C_8C_1C_1N][Cl]$	5%	4 - > d.l.	-	3.03	0.67
			2.5%	4.40	0.37	2.34	0.76
			1%	4.41	0.87	1.98	0.25
			0.1%	0.69	1.04	-0.21	0.56
Trioctylmethylammonium	Chloride	$[C_8C_8C_8C_1N][Cl]$	5%	0.79	0.21	2.83	0.62
			2.5%	-0.09	0.46	3.25	0.29
			1%	0.07	0.30	3.26	0.29
			0.1%	0.00	0.28	2.44	0.21
Effect of Hofmeister series							
1-Butyl-3-methylimidazolium	Methylsulfate	$[C_4mim][MeSO_4]$	5%	0.01	0.11	0.30	0.08
1-Butyl-3-methylimidazolium	Iodate	$[C_4mim][I]$	5%	0.38	0.32	1.04	0.17
1-Butyl-3-methylimidazolium	Dicyanamide	$[C_4mim][DCA]$	5%	0.25	0.38	0.24	0.15
1-Butyl-3-methylimidazolium	Thiocyanate	$[C_4mim][SCN]$	5%	-0.20	0.04	0.27	0.10
1-Butyl-3-methylimidazolium	Tricyanomethadine	$[C_4mim][TCM]$	5%	0.90	0.55	0.55	0.54
1-Butyl-3-methylimidazolium	Trichloracetate	$[C_4mim][TCA]$	5%	0.46	0.80	0.80	0.69

SD: standard deviation

> d.l. : reduction was higher than the detection limit (> 5-6 $\log_{10}$ reduction)

Conclusions

Results of this study demonstrate that relationships between defined IL properties and toxicity obtained from bacteria or other organisms are not readily applicable to viruses. It could be shown that: (i) the chaotropicity of the IL anion did not influence virus infectivity, and (ii) the side chain effect of imidazolium and ammonium based ILs was much less pronounced than observed in bacteria or eukaryotes. With the exception of $[C_8C_8C_8C_1N][Cl]$, the effect of ILs on phage P100 was greater compared to MS2, demonstrating the importance of including structurally different viruses for future studies. Although none of the tested ILs could be readily classified as virucide against both viruses at the tested concentrations, the results are promising. Apparently, ILs with more than one elongated side chain proved to be much more effective at inactivating viruses then those with only one elongated side chain. Besides this very interesting discovery, it is a good starting point for future studies and ILs may become interesting for disinfection and also in food industry for removal of phages.

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3.5 Publication 4: The use of ionic liquids for cracking viruses for isolation of nucleic acids

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The use of ionic liquids for cracking viruses for isolation of nucleic acids

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ABSTRACT

The most important molecules in the life sciences are nucleic acids. This is especially important in the context of virus research where nucleic acids are primarily analysed. In general, there are two methods for extracting nucleic acids: solution-based and column-based. In many cases quantitative isolation of nucleic acids is necessary. In the present paper, ionic liquids have been tested for the first time for disintegration of virus particles and separation of the nucleic acids in a liquid phase system. $[(OH)^2C_2C_1C_1NH][C_2CO_2]$ and several $[NTf_2]$-based ionic liquids were tested as well as $[C_1C_1im][C_1PO_2OH]$, $[C_8C_8C_8C_1N]$ and $[C_8C_1im]$-based ionic liquids. With $[(OH)^2C_2C_1C_1NH][NTf_2]$, $[C_6C_6C_6C_{14}P][FAP]$ and $[C_6C_1im][FAP]$ a significant higher recovery was obtained in comparison to the control. In particular, $[C_1C_1im][C_1PO_2OH]$ achieved promising results with respect to recovery rates and purity. The isolation protocol is fast, easy and column free. An additional advantage is the deactivation of nucleases such as DNase I and RNase H.

1. Introduction

In the life sciences, particularly in molecular biology, one of the most important classes of molecules are nucleic acids such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). While DNA encodes the genetic instructions used in the development and functioning of all known living organisms and some viruses, ribonucleic acid (RNA) has various biological functions such as coding, decoding, regulation, and expression of genes, and is also a DNA equivalent in some viruses. Indeed, due to enzymatic activities of RNA and the possibility of self replication, RNA is considered the likely starting molecule for evolution on earth [1]. Due to their prominent role in all known living organisms, various methods have been developed to analyse nucleic acids to address biological questions in both diagnostics and research and they are starting points for many downstream processes.

DNA was isolated from leukocytes for the first time by the Swiss physician Friedrich Miescher in 1869 [2]. Initially, extraction was complicated and time-consuming. Today there are many extraction kits available and isolation is fast and practical. In general, there are two possibilities for extracting nucleic acids: solution-based methods, that include mostly organic solvents followed by alcohol precipitation, and column-based methods [3]. Both methods require two general steps: The first step is lysis of the cell, bacterium or virus particle; the second step is separation, in which the desired nucleic acid (genomic DNA, plasmid DNA, cDNA, or genomic RNA, mRNA, mi RNA, rRNA, etc.) is extracted out of the cell debris and sample residues.

Most of the state of the art commercially available kits are column-based. After disruption of the cells or viruses, nucleic acids are bound on silica matrices, nitrocellulose or polyamide columns or membranes by hydrogen-bonding. After several washing steps the nucleic acid is eluted [3]. Column-based methods are well established and the recovered nucleic acid is usually of a high level of purity. On the other hand, these kits are not the best choice if DNA/RNA should be isolated from complex matrices, because the columns are easily clogged resulting in a biased recovery. Moreover, column-based methods provide 75–80% recovery at best and have been reported to depict a high intra-experimental deviation in actual recovery rate (User manual NucleoSpin® Extract II (Macherey–Nagel) April 2004/ Rev.01).

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For viruses, the most important conventional solution-based nucleic acid extraction method is guanidinium thiocyanate phenol chloroform extraction, which was originally developed by Ulrich et al. (1977) [3] and then further improved [4]. Liquid–liquid extraction is a simple and fast technique that is highly flexible and can achieve high yields [5], especially from complex matrices where it is the preferred method compared to column-based extraction. Moreover, the use of guanidinium thiocyanate provides some protection against nucleases, which degrade free nucleic acids and can cause to false results. This is especially true in the case of RNases, which are ubiquitously present in environmental or clinical samples and are not completely inactivated by guanidinium thiocyanate. Another disadvantage of guanidinium thiocyanate phenol chloroform based isolation methods is their toxicity and volatility, which requires special working areas with fume hoods.

In many cases, the quantitative isolation of nucleic acids is necessary. This is especially true in diagnostics where legal regulations define the number of pathogens permitted to be present in the sample. In this context, the lysis step of the cells or viruses is the most crucial one and the time that is required for this step also plays an important role.

To the best of our knowledge, ionic liquids have not previously been used for the isolation of nucleic acids from viruses. Nevertheless, their unique properties appear to make them a promising tool. Ionic liquids have been used to break up yeast cells and to extract proteins [6] as well as double stranded DNA [7]. The first successful application of ionic liquids for both lysis of cells and extraction of DNA from bacteria was presented by Fuchs et al. [8]. This group developed a lysis method for quantitative and fast DNA isolation of bacteria based on ionic liquids and high temperature. For quantitative cell lysis and DNA release, the bacteria had to be incubated at 120 °C for 1 min in $[C_4C_1pyrr][NTf_2]$. Afterwards the DNA could simply be extracted into water and the DNA-containing aqueous phase could be directly used for analysis without inhibiting any subsequent methods such as Real time PCR (qPCR).

In the paper presented here, ionic liquids have been tested for the first time for fast and quantitative disintegration of virus particles for subsequent isolation of either RNA or DNA. Based on the results of Fuchs et al. [8] the impact of different incubation temperatures was determined. Most of the ionic liquids tested in this study were water-immiscible, as from such ionic liquids the nucleic acids should be extractable into water without an additional precipitation step.

2. Material and methods

2.1. Ionic liquids

In this study, a total of 16 different ionic liquids were tested and the respective abbreviations and structures are presented in Fig. 1. Six water-immiscible ionic liquids ($[C_4C_1pyrr][NTf_2]$, $[C_6C_6C_6C_{14}P][NTf_2]$, $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$, $[(OH)^2C_2C_1C_1NH][NTf_2]$, $[C_6C_1im][FAP]$ and $[C_6C_6C_6C_{14}P][FAP]$) as well as the water-miscible ionic liquid $[(OH)^2C_2C_1C_1NH][C_2CO_2]$ were provided from Merck KGaA (Darmstadt, Germany). Water-miscible $[C_1C_1im][C_1PO_2OH]$ was provided from Solvionic (Toulouse, France). In addition, seven ionic liquids based on the $[C_8C_8C_8C_1N]^+$ cation combined with $[IO_3]^-$, $[H_2NSO_3]^-$, [orotate]$^-$, $[FeCl_4]^-$, [calconate]$^-$, $[(COOH)^4(OH)^{2,3}C_3CO_2]^-$ and $[Cl^4C_6H_6OC_1CO_2]^-$, as well as $[C_8C_1im][IO_4]$, were provided by Proionic GmbH (Grambach, Austria) with a nominal purity higher than 95%, or synthesised using the CBILS® [1] route [9]. In short, exactly one molar equivalent of the corresponding acid (iodic acid, 4-chlorophenoxyacetic acid, sulphamic acid (all Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and periodic acid, L(+)-Tartaric acid, orotic acid and calconcarboxylic acid (all Merck KGaA, Darmstadt, Germany)) was added to the $[C_8C_8C_1N][C_1CO_2]$ (provided by Proionic). Carbon dioxide was released and the ionic liquid was formed. The ionic liquids were isolated from respective solvents *in vacuo* to yield typically 98–99% of the theoretical amount using a SPC-SpeedVac® (Thermo Savant, Thermo Scientific Inc.).

2.1.1. Selection of special water-immiscible ionic liquids

In this study, our aim was to find water-immiscible ionic liquids which form two-phase systems with DNA suspended in water. For handling reasons, it is preferable to have two-phase systems in which the DNA stays in the upper phase. For this reason, several water-immiscible ionic liquids were mixed with DNA that was stained with SYBR® Safe DNA Gel Stain (Life Technologies, Carlsbad, USA). The sample was then shortly centrifuged to accelerate phase separation and photographed under UV-light. Ionic liquids which formed two-phase systems with fluorescence in the upper phase were tested for cracking of viruses and are listed in Section 2.1 and shown in Fig. 1.

2.2. Viruses

Feline calicivirus (FCV) was used as a model for RNA viruses; it being a well-established surrogate for human noroviruses [10–14], one of the most important food-borne pathogens. A virus stock and corresponding CRFK (Crandel feline kidney) cells were obtained from Dr. Reimar Johne (BfR, Berlin, Germany). The viruses were replicated in CRFK cells in DMEM (Gibco®, Life Technologies™, UK) plus 10% foetal bovine serum gold, 1% L-glutamine (both PAA Laboratories GmbH, Pasching, Austria), 2% Anti–Anti (antibiotic–antimycotic; Gibco®, Life Technologies™, Grand Island, NY, USA) and MEM Non-Essential Amino Acids (PAA Laboratories GmbH, Pasching, Austria). Harvested viruses were aliquoted and frozen at −20 °C. Working stocks were thawed and stored at 4 °C.

The phage P100 was used as a model for DNA viruses. This phage was purchased as Listex™ P100 solution (Batch 12G26, Lot: 308; Micreos, Wageningen, NL). The phage solution was used for plaque assays [15]. A single plaque was isolated and used for replication in a log-phase culture of the phage-sensitive *L. monocytogenes* EGDe (ATCC BAA-679). Thereafter the infected culture was centrifuged at 8,000 rpm for 2 min. The supernatant was treated with chloroform, aliquoted and stored at 4 °C. The phage was confirmed as phage P100 by qPCR (for details, see Section 2.5.2).

2.3. Cracking of viruses and extraction of nucleic acid isolation

2.3.1. Procedure

In a ratio of 1:10 (v/v), 5–10 µl of virus suspension (FCV: 10^4–10^5 RT qPCR units; P100: approx. 10^7 qPCR units) was added to either 45 or 90 µl of ionic liquid, mixed and incubated for either 1 min at 120 °C, 10 min at 70 °C or 10 min at room temperature. Subsequently 200 µl of ddH_2O was added, mixed by pipetting and used for precipitation of nucleic acid. If phase formation was observed (water-immiscible ionic liquids), only the upper phase was used for precipitation. All ionic liquids were first tested for cracking of DNA viruses and promising candidates also tested for lysis of RNA viruses.

All experiments included a negative control (water instead of virus suspension) and were repeated at least three times, except ionic liquids that were clearly unsuitable.

[1] CBILS® is a registered trademark of Proionic GmbH.

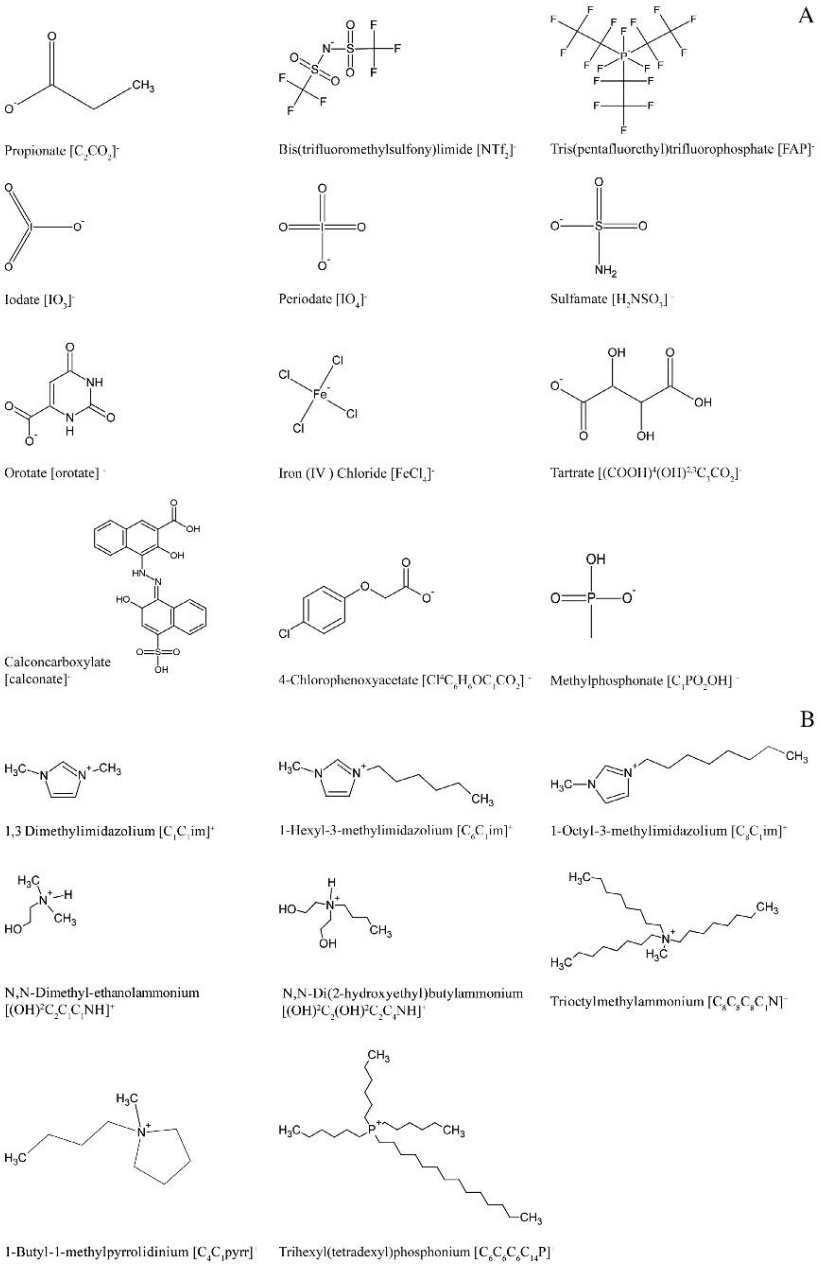

Fig. 1. Structure and abbreviations of all used anions (A) and cations (B).

2.3.2. Controls and calculation of recoveries

Due to possible different efficiency rates between individual experiments of e.g. the precipitation of nucleic acids or the reverse transcription step, recoveries of all nucleic acid isolations were compared to results obtained using the PureLink® Viral RNA/DNA Mini Kit (Invitrogen™, Life Technologies, Carlsbad, USA). In each experiment to isolate nucleic acid with ionic liquids, the same amount of virus suspension was used for the isolation of nucleic acids using the PureLink® Kit. Afterwards, nucleic acids from each attempt were precipitated as described below in Section 2.3.3 and resuspended in the same volume of water. The yield of nucleic acids was then quantified by (RT) qPCR (see Section 2.5) and the recovery was calculated using the yield from the PureLink® Kit as 100%. Each experiment was repeated at least three times enabling the calculation of the average recovery. Additionally the average ct value and standard error of all controls and for each ionic liquid were calculated. This was done in order to show intra-experimental deviations. An overview of the experimental procedure is shown in Fig. 2.

2.3.3. Nucleic acid precipitation

For purification and concentration of nucleic acids, a precipitation step was introduced because most ionic liquids inhibit subsequent enzymatic reactions such as the polymerase chain reaction (PCR). DNA was precipitated according to Sambrook et al. [16].

RNA precipitation was performed using 2-propanol and an optimised protocol: Equal volumes of 2-propanol and 0.75 µl glycogen (35 mg/µl) (peqlab, Erlangen, Germany) were added to the RNA and incubated on ice for 15 min. The sample was then centrifuged for 10 min and the volume subsequently redoubled with 100% ethanol. The sample was centrifuged again. The supernatant was removed and the pellet washed with 75% ethanol, dried and resuspended in 100 or 200 µl DEPC-treated H_2O.

2.4. Reverse transcription (RT)

C-DNA synthesis was carried out according to the protocol of the AMV Reverse Transcriptase (Invitrogen™, Life Technologies, Carlsbad, USA) using SW-FeCV-as as primer [17].

2.5. Real time PCR (qPCR)

2.5.1. FCV

Real time PCR was performed according to Johne et al. [17]. The primer and probe sequences are listed in Table 1 and can be used to amplify a 139 bp fragment of the capsid protein gene. The amplification conditions were applied as following: 0.5 µM primer, 0.25 µM probe, 3.5 mM $MgCl_2$, 0.8 mM dNTPs (all final concentrations), 0.06 U/µl Taq (final concentration), 1× PCR buffer and 5 µl cDNA/25 µl PCR reaction. The cycling conditions were 2 min 94 °C followed by 45 × (15 s 94 °C, 1 min 52 °C and 15 s 72 °C). Real time PCR was carried out using the Mx3000P™ Real time cycler (Stratagene, La Jolla, CA, USA). All qPCRs included a standard series for calculation of a standard curve and a negative amplification control. The standard curve was used for quantification of the viral nucleic acids.

2.5.2. P100 qPCR

The P100 qPCR was designed by Fister et al. (submitted) and can be used for amplification of a fragment of the *gp 104* gene. The same chemistry was used as for the mastermix of the FCV qPCR. The Mx3000P™ Real time cycler was used for amplification (Stratagene, La Jolla, CA, USA). The cycling temperatures were as follows: 2 min 94 °C, 45 × (30 s 94 °C, 30 s 59 °C, 30 s 72 °C). The average efficiency of this qPCR was 99.6% and the average Rsq was 0.998. All qPCR experiments included a standard series for calculation of a standard curve and a negative amplification control. The standard curve was used for quantification of the viral nucleic acids.

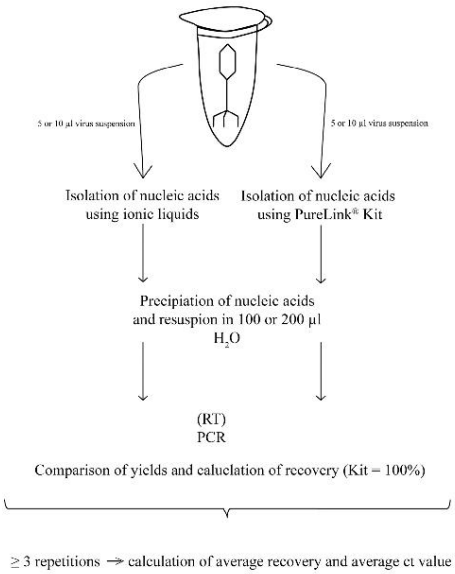

Fig. 2. Scheme of experimental procedure and calculation of recoveries.

2.6. Desoxyribonuclase I (DNase I) and Ribonuclease H (RNase H) activity in ionic liquids

Selected ionic liquids (listed below) were tested to determine whether they could provide protection against degradation of DNA or RNA by nucleases.

2.6.1. Protection against DNAse I activity

100 µl ionic liquid ($[C_1C_1im][C_1PO_2OH]$ pure and 40% (v/v), $[(OH)^2C_2C_1C_1NH]$ and $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$, $[TMC_{14}P][FAP]$, $[C_8C_8C_8C_1N]$[calconate] or water as control), 5 µl P100 DNA and 95 µl DNAse I in its buffer (components of the illustra RNAspin Mini Kit, GE Healthcare UK Limited, Buckinghamshire, UK) were mixed and incubated at 37 °C for 15 min. Thereafter, DNA was precipitated as described above and the amount of the remaining DNA quantified using qPCR. Results were compared with untreated DNA.

2.6.2. Protection against RNase H activity

40 µl ionic liquid ($[DMIM][C_1PO_2OH]$ pure and 40% (v/v), $[(OH)^2C_2C_1C_1NH][C_2CO_2]$, $[C_6C_6C_6C_{14}P][FAP]$, $[C_4C_1pyrr]$ and $[C_6C_6C_6C_{14}P][NTf_2]$ or water as control) and 40 µl FCV RNA + 0.5 µl RNase H (10 U/µl; Ambion Inc., Life Technologies, Carlsbad, CA, USA) were mixed and incubated at 37 °C for 15 min. The reaction was stopped by the addition of 10 µl EDTA (100 mM), the RNA was then precipitated as described above and the amount of the remaining RNA was quantified using RT qPCR (Sections 2.4 and 2.5.1). Results were compared with untreated RNA.

Table 1
Primer and Probes used in this study.

Name	Sequence (5′–3′)	References
SW-FeCV-s	GAA AGC CCA ACA AAT TGA ATT	Johne et al. [17]
Sw-FeCV-as	CGT GTA CCC TCT GCT GCT CAA G	
FCV Probe	6FAM-AAT GAA TCT CGA TCG CCA GGC-BHQ	
P100 Primer 1	AGCAGAGTTTGAAAAAATTGATGACTAC	Fister et al. (submitted for publication)
P100 Primer 2	TCGTCATGCGTGTTTCTATGC	
P100 Probe 1 + 2	6FAM-TGGCGGTAAAACTTATAGACTTAGTTACAAAATCACAGCA-BHQ	

3. Results

3.1. DNA viruses

All results are summarised in Table 2.

Using $[(OH)^2C_2C_1C_1NH][C_2CO_2]$, DNA recovery rates of 76% (room temperature), 21% (70 °C) and 56% (120 °C) were achieved compared to the commercially available kit. At room temperature, $[C_4C_1pyrr]$ and $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$ led to the same yield as the purchased kit and 69–80% recoveries at 70 °C and 120 °C, respectively. $[C_6C_6C_6C_{14}P][NTf_2]$ obtained comparative yields of 64–89%. $[(OH)^2C_2C_1C_1NH][NTf_2]$ returned more than 100% recovery at room temperature. However, no DNA could be extracted with this ionic liquid at 70 °C or 120 °C.

Both [FAP]-based ionic liquids that were tested achieved > 100% recovery for all temperatures, except for $[C_6C_1im][FAP]$ which obtained 75% at room temperature. With respect to most of the $[C_8C_8C_8C_1N]X$ based ionic liquids (X = $[IO_3]^-$, $[H_2NSO_3]^-$, $[orotate]^-$ and $[FeCl_4]^-$) as well as $[C_8C_1im]$ $[IO_4]$, DNA extraction did not work at all or not very well and thus no further tested was carried out (data not shown). However, two interesting ionic liquids were found: $[C_8C_8C_8C_1N][calconate]$ and $[C_8C_8C_8C_1N][Cl^4C_6H_6OC_1CO_2]$ achieved > 100% recovery at room temperature. At higher temperatures, however, recovery of DNA was lower (65–95%) than with the comparison kit.

Using $[C_1C_1im][C_1PO_2OH]$ cracking of viruses worked best (168% recovery) when pure ionic liquid was used for 1 min at 120 °C, while yields at lower temperatures or use of 40% and 80% (v/v) $[C_1C_1im][C_1PO_2OH]$ allowed recoveries < 100%.

3.2. RNA viruses

Isolation of viral RNA using ionic liquids generally resulted in poor recovery rates (Table 2).

$[(OH)^2C_2C_1C_1NH][C_2CO_2]$ resulted in approximately 40% recovery (room temperature – 120 °C) compared to the comparison kit.

At 120 °C ⩽5% RNA could be extracted using $[C_4C_1pyrr]$, $[(OH)^2C_2(OH)^2C_2C_4NH]$, $[C_6C_6C_6C_{14}P][NTf_2]$, $[C_6C_6C_6C_{14}P][FAP]$ or $[C_8C_8C_8C_1N][calconate]$. At room temperature and 70 °C yields of approximately 20% ($[C_4C_1pyrr][NTf_2]$), 60% ($[C_8C_8C_8C_1N][calconate]$) and 40% ($[C_6C_6C_6C_{14}P][FAP]$) were achieved. Recoveries of 14% and 1% ($[C_6C_6C_6C_{14}P][NTf_2]$) and 72% and 7% ($[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$) were obtained at room temperature and 70 °C, respectively.

The best results for RNA isolation were obtained when $[C_1C_1im][C_1PO_2OH]$ was used: pure ionic liquid led to recoveries of 244%, 188% and 149% at room temperature, 70 °C and 120 °C, respectively. Diluted $[C_1C_1im][C_1PO_2OH]$ (40% v/v) achieved maximum results at 70 °C (172%) and returned yields of 96% and 62% (room temperature and 120 °C, respectively).

3.3. DNase and RNase activity

When activity of DNase I in ionic liquids was tested (Fig. 3A), the lowest level of DNA digestion was observed in $[C_1C_1im][C_1PO_2OH]$ (pure and 40% (v/v): 80% of the initially applied DNA was present following incubation with the enzyme. In $[(OH)^2C_2C_1C_1NH][NTf_2]$ and $[C_8C_8C_8C_1N][calconate]$ 85%, and in $[C_6C_6C_6C_{14}P][FAP]$ and $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$ less than 50% of the DNA was degraded.

The highest reduction in RNase H activity (Fig. 3B) was observed with $[C_1C_1im][C_1PO_2OH]$. Here 15–25% of the RNA was degraded (depending on the concentration of the ionic liquid used). In $[C_6C_6C_6C_{14}P][FAP]$ almost 70% and in $[C_4C_1pyrr][NTf_2]$ 60% of the initially applied RNA was present after addition of RNase H. In $[(OH)^2C_2C_1C_1NH][C_2CO_2]$ and $[C_6C_6C_6C_{14}P][NTf_2]$, as well as in the control (water), 70% of the applied RNA was digested.

4. Discussion

In this paper, we present the results of our investigation into ionic liquids tested for the first time with respect to disintegrating virus capsids and subsequent isolation of nucleic acids. Our data show that several different ionic liquids delivered promising results when compared to the performance of a commercial nucleic acid isolation kit. As there were high deviations within repetitions of the experiment (e.g. caused by the series of several experimental steps, high intra-experimental deviations of the commercial available kit that was used as control and probably also deviations in the virus titres of different virus aliquots), we decided to show the average ct values in addition to the average recoveries (Table 2).

For nucleic acid extraction from bacteriophage P100, the model virus for DNA extraction, most of the $[C_8C_8C_8C_1N]$-based ionic liquids, as well as $[C_8C_1im][IO_4]$ achieved poor yields (21–75%) and were, therefore, not tested further. Surprisingly $[(OH)^2C_2C_1C_1NH][C_2CO_2]$, which has previously been reported to effectively lyse bacterial cells for DNA extraction [8], did not achieve equivalent results compared to the commercial kit. Nevertheless, two ionic liquids produced results that were similar to those of the commercially available kit, namely $[C_6C_6C_6C_{14}P]$ with 80% recovery and $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$ with 107%. Moreover, significantly higher recoveries than those provided by the kit were obtained with two $[C_8C_8C_8C_1N]$-based ionic liquids ($[C_8C_8C_8C_1N][calconate]$ and $[Cl^4C_6H_6OC_1CO_2]$ 159% and 169%), one $[NTf_2]$-based ionic liquid ($[(OH)^2C_2C_1C_1NH][NTf_2]$ 166%), both [FAP]-based ionic liquids (143% and 152%) and $[C_1C_1im][C_1PO_2OH]$ (168%).

With respect to nucleic acid extraction from FCV, the model virus for RNA extraction, all tested ionic liquids, except $[C_1C_1im][C_1PO_2OH]$, achieved RNA recovery rates that were lower than that of the commercially available kit. Application of $[C_1C_1im]$ $[C_1PO_2OH]$ however, increased recovery of RNA around two-fold compared to the commercial RNA isolation kit. Best results were obtained when viruses were incubated at room temperature and in pure ionic liquid (244%). Decreasing recoveries at 70 °C and 120 °C were most likely caused by degradation of the extracted RNA as discussed below.

When isolating bacterial DNA, high temperature in general is known to enhance recovery rates [8,18,19]. In our experiment,

Table 2
Results of both RNA and DNA isolation experiments (Overview).

IL	RNA RT	70	120	DNA RT	70	120
Control[a]	**100%**[b] 22.32 (0.85)[c]			**100%** 13.37 (0.31)		
$[(OH)^2C_2C_1C_1NH][C_2CO_2]$	26% 24.35 (0.71)	32% 24.04 (0.54)	36%) 23.86 (1.17	13.79 (0.42) 76%	15.69 (0.49) 21%	14.25 (0.65) 56%
$[C_4C_1pyrr][NTf_2]$	25% 24.45 (0.84)	21% 24.66 (0.63)	0% 30.85 (1.57)	**102%** 13.34 (0.28)	78% 13.75 (0.78)	75% 13.8 (0.82)
$[C_6C_6C_6C_{14}P][NTf_2]$	14% 25.34 (0.99)	1% 28.84 (1.18)	0% 30.59 (1.07)	**107%** 13.26 (0.32)	80% 13.71 (0.57)	69% 13.94 (1.29)
$[(OH)^2C_2(OH)_2C_2C_4NH][NTf_2]$	72% 23.64 (0.96)	7% 26.33 (1.21)	1% 30.34 (1.10)	64% 14.04 (0.47)	89% 13.54 (0.32)	74% 13.83 (0.90)
$[(OH)^2C_2C_1C_1NH][NTf_2]$	n.t.	n.t.	n.t.	**166%** 12.61 (0.27)	0% 23.52 (0.34)	0% 23.22 (0.39)
$[C_6C_1im][FAP]$	40% 23.71 (1.10)	47% 23.46 (0.89)	5% 27 (0.40)	75% **13.8 (0.59)**	**107%** 13.27 (0.46)	**152%** 12.74 (0.39)
$[C_6C_6C_6C_{14}P][FAP]$	n.t.	n.t.	n.t.	**119%** 13.11 (0.28)	**143%** 12.84 (0.27)	**123%** 13.06 (0.12)
$[C_8C_8C_8C_1N][calconate]$	56% 23.21 (1.00)	69% 22.89 (0.72)	3% 27.82 (1.11)	**159%** 12.67 (0.11)	95% 13.44 (0.26)	0% 14.03 (0.28)
$[C_8C_8C_8C_1N][Cl^4C6H_6OC_1CO_2]$	n.t.	n.t.	n.t.	**163%** 12.64 (0.31)	79% 13.72 (0.37)	89% 13.55 (0.22)
$[C_1C_1im][C_1PO_2OH]$ pure	**244%** 20.97 (0.87)	**188%** 21.36 (0.65)	**149%** 21.72 (0.40)	85% 13.62 (0.50)	77% 13.77 (0.50)	**168%** 12.59 (0.56)
$[C_1C_1im][C_1PO_2OH]$ 40% (v/v)	n.t.	n.t.	n.t.	84% 13.64 (0.43)	51% 14.39 (0.17)	47% 14.53 (0.54)
$[C_1C_1im][C_1PO_2OH]$ 80% (v/v)	96% 22.37 (0.65)	**174%** 21.47 (0.47)	62% 23.05 (1.02)	43% 14.70 (0.63)	72% 13.87 (0.17)	75% 13.81 (0.59)

Ct (cycle threshold) is the number of cycles necessary for the signal to cross the background fluorescence. The smaller value the more target is in the sample (in perfect PCRs the target is doubled every cycle); **bold letters** mark ionic liquids that achieve higher recoveries as the tested Kit.
n.t.: not tested.
[a] PureLink® Viral RNA/DNA Mini Kit.
[b] % recovery compared to the kit.
[c] Ct values (standard error).

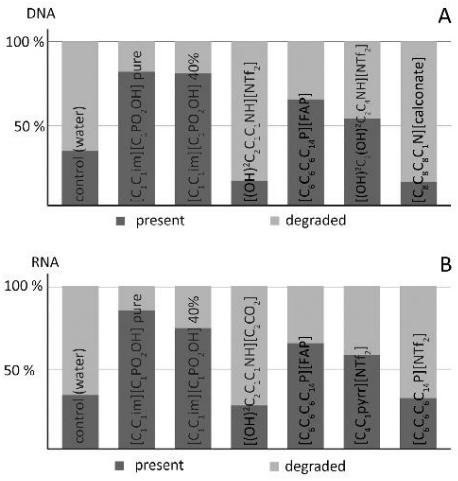

Fig. 3. Degradation of DNA by DNase I in ionic liquids (A). Degradation of RNA by RNase H in ionic liquids (B). Dark grey columns represent the amount of DNA or RNA present following incubation with DNase I or RNase H; light grey columns represent the amount of degraded DNA or RNA. Values are relative to the amount of applied DNA or RNA.

$[C_1C_1im][C_1PO_2OH]$ and $[C_6C_1im][FAP]$ performed best for DNA extraction at rising temperatures. The maximum recoveries of 168% and 152% were obtained at 120 °C. However, for most of the other ionic liquids, and in all RNA isolation experiments, the opposite effect was observed when high temperature was applied (70 °C or 120 °C), leading to lower recovery rates of nucleic acids. It is common knowledge that the stability of nucleic acids, especially RNA, decreases with temperature [3,20–22]. Therefore, it can be assumed that the decreased recovery of nucleic acid is caused by its degradation and not due to reduced disintegration of the virus particles. This hypothesis is supported by the observation in our experiments that RNA, in particular, was lost at high temperatures. Degradation of the nucleic acid was observed for the $[C_8C_8C_8C_1N]$-based ionic liquids for DNA extraction as well as for $[NTf_2]$-based ionic liquids. In RNA extraction, degradation of nucleic acid was observed in all experiments; $[C_1C_1im][C_1PO_2OH]$, which yielded the highest recovery rates, was also noted to have this effect. Furthermore, an additional degradation of nucleic acids by the ionic liquids cannot be excluded in all cases. This is supported by the data obtained with $[(OH)^2C_2C_1C_1NH][NTf_2]$. This ionic liquid itself degrades DNA completely at 70 °C and at 120 °C, a finding which was confirmed in our laboratory (data not shown). RNA is almost completely degraded even at room temperature by $[(OH)^2C_2C_1C_1NH][NTf_2]$ (4% recovery, Table 2). Nevertheless, $[(OH)^2C_2C_1C_1NH][NTf_2]$ destroys the virus capsid effectively as the recovery of DNA (166%) was determined to be significantly better than that obtained with the commercial kit, even although a certain amount of ongoing DNA degradation must be assumed at

room temperature. In conclusion, temperature in conjunction with the various ionic liquids showed several effects: (i) degradation of the yielded nucleic acid due to rising temperature, (ii) increasing yield with rising temperature due to better extraction conditions for the ionic liquids, and (iii) complete degradation of the yielded nucleic acid at rising temperatures by the ionic liquid itself.

If quantitative extraction of nucleic acids is to be achieved, inactivation of nucleases is important [3]. In this study we identified ionic liquids which reduce the activities of DNAse I and RNase H. Nevertheless, not all tested ionic liquids influenced the activity of nucleases. In $[C_1C_1im][C_1PO_2OH]$ both DNase I and RNase H activities were reduced and at least 75% of the applied nucleic acid was detected after incubation with these nucleases. These encouraging results are probably due to the fact that this ionic liquid is water-miscible. Degradation of RNA and DNA was also limited in $[C_6C_6C_6C_{14}P][FAP]$ where less than 50% of the nucleic acid was digested. This could be an advantage in comparison to other nucleic acid isolation methods, because the sample could be stored in the ionic liquid before or after nucleic acid isolation.

The benefit of the water-immiscible ionic liquids, $[C_4C_1pyrr]$ and $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$, $[C_6C_1im]$ and $[C_6C_6C_6C_{14}P][FAP]$ and $[C_8C_8C_8C_1N]$ [calconate] and $[Cl^4C_6H_6OC_1CO_2]$, could be that nucleic acids may be used directly after extraction or without precipitation, but this remains to be tested.

A specific mechanism of the method has not been identified so far. We were not able to identify chemical structures that could be responsible for the lysis of the virus particles. The disintegration of the protein capsid of the viruses may be caused by several mechanisms, as a wide range of different ionic liquids seem to be effective for nucleic acid isolation. As discussed above, a variety of effects with respect to the resulting recovery or nucleic acid degradation have been observed; especially by means of temperature and the different ionic liquids. This supports the theory of several mechanisms for virus disintegration. Moreover, for isolation of RNA and DNA different ionic liquids achieved high recovery rates and this also makes it hard to predict a particular mechanism. What has become clear is that different ionic liquids must be used for the extraction of viral nucleic acids compared to those used for the isolation of bacterial DNA.

5. Conclusion

For the first time ionic liquids have been tested for disintegration of virus particles for the purpose of the subsequent isolation of nucleic acids. Some ionic liquids, especially $[C_1C_1im][C_1PO_2OH]$, achieved promising results regarding recovery rates and purity (no inhibitory effects in qPCR). The isolation protocol detailed here is fast, easy and column-free. An additional advantage of some ionic liquids is the deactivation of nucleases such as DNase I and RNase H.

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3.6 Posters and unpublished data

3.6.1 Talks

Susanne Fister, Patrick Mester, Ilkka Kilpeläinen, Martin Wagner, Peter Rossmanith

The use of ionic liquids for cracking viruses for isolation of nucleic acids

- ILSEPT 2nd International Conference on Ionic Liquids in Separation and Purification Technology; JUN, 29-JUL 2, 2014; Toronto, CAN

Susanne Fister, Sabine Fuchs, Beatrix Stessl, Dagmar Schoder, Martin Wagner, Peter Rossmanith

Screening and Characterization of Bacteriophage P 100 Insensitive *L. monocytogenes* Isolates in Austrian Dairy Plants

- 55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der DVG Dreiländertagung; SEP 23-26, 2014; Garmisch-Partenkirchen, GER

Susanne Fister, Christian Robben, Dagmar Schoder, Beatrix Stessl, Martin Wagner, Peter Rossmanith

P100 insensitive *L. monocytogenes* plant isolates and influence of environmental factors on phage-bacteria interaction, on efficacy and survival of the phage P100

- Bacteriophages 2015 Jan 27-29, 2015, London, UK

3.6.2 Poster presentations

Susanne Fister, Sabine Fuchs, Patrick Mester, Martin Wagner, Peter Rossmanith

The Impact Of Four Buffer Systems Derived From A Matrix Solubilisation Based Sample Preparation Protocol For Bacteria On The Integrity Of Virus Particles

- IAFP´s European Symposium on Food Safety; May 18-20, 2011; Ede, NL
- 144-144.-100th Anniversary, International Association of Food Protection; Jul 30-Aug 4, 2011; Milwaukee, USA
- 1. European Congress of Applied Biotechnology (ECAB); SEPT 25-29, 2011; Berlin, GER

Peter Rossmanith, Patrick Mester, Sabine Fuchs, Susanne Fister, Martin Wagner

Matrix Lysis: A sample preparation method for recovery of bacterial targets based on solubilization of the sample matrix

- European Congress of Applied Biotechnology (ECAB); SEP 25-29, 2011; Berlin, GER

Susanne Fister, Franz Allerberger, Martin Wagner, Peter Rossmanith

The Impact Of A Hydrogen Peroxide Vapour Based Decontamination Method On the Infectivity and Detectability of Feline Caliciviruses As Surrogates For Human Noroviruses.

- 24th ECCMID; MAY 10-13, 2014; Barcelona, ESP

Susanne Fister, Christian Robben, Dagmar Schoder, Greta Göltz, Martin Wagner, Peter Rossmanith

Development, Testing and Evaluation of Conventional and Real Time PCRs for Detection of the Phage P100

- 55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft e.V.; SEP 23-26, 2014; Garmisch-Partenkirchen, GER

Susanne Fister, Christian Robben, Dagmar Schoder, Greta Göltz, Martin Wagner, Peter Rossmanith

Influence of environmental factors on phage-bacteria interaction and on efficacy and survival of the phage P100

- 55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft e.V.; SEP 23-26, 2014; Garmisch-Partenkirchen, GER

Patrick Mester, Tobias Gundolf, Susanne Fister, Martin Wagner, Peter Rossmanith

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

- ILSEPT - 2nd International Conference on Ionic Liquids in Separation and Purification Technology; JUN 29- JUL 2, 2014; Toronto, CAN

- 6th ÖGBMT Annual Meeting; SEPT 15-18, 2014; Vienna, AUT
- 55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft e.V.; SEP 23-26, 2014; Garmisch-Partenkirchen, GER

Patrick Mester, Tobias Gundolf, Susanne Fister, Martin Wagner, Peter Rossmanith

Ionic liquid induced tolerance of *Listeria monocytogenes* against disinfectants

- ILSEPT - 2nd International Conference on Ionic Liquids in Separation and Purification Technology; JUN 29- JUL 2, 2014; Toronto, CAN

Anna Kristina Jehle, Martin Bobal, Susanne Fister, Tobias Gundolf, Peter Rossmanith

CO_2 as disinfection method?

- How dead is dead IV; MAY 21-22, 2015; Dübendorf, CHE

Susanne Fister, Patrick Mester, Julia Sommer, Anna Kristina Jehle, Roland Kalb, Martin Wagner, Peter Rossmanith

The (virucidal) effect of ionic liquids on phages P100 and MS2

- 7th OEGMBT annual meeting, SEP 9-11, 2015, Salzburg, AUT

4 Summary and discussion

Microbiologically safe food for human consumption is a prerequisite for a nutritious and sustainable food supply. In order to guarantee microbiologically safe food a whole systems approach (summarised as diagnostic chain) is necessary that includes both detection and removal or inactivation of food-borne pathogens (cleansing and disinfection). Rapid detection and subsequent identification of contaminants is important to prevent contaminated food coming onto the market. Alternatively, cleansing and disinfection are important in order to avoid production of contaminated food and contamination of already processed food.

In this thesis the focus of research within the diagnostic chain was phage P100 (figure 7). Firstly, the presence and development of resistance to phage P100 was investigated. Moreover, the influence of environmental factors on the stability of the phage and on the host-virus interaction was studied. As presence of P100 also influences the analytical chain and interferes with diagnostics, ionic liquids were tested on viruses (using phage P100 as model for DNA viruses and phage MS2 as a surrogate for human noroviruses) as possible virucidal agents. Moreover, the analytical chain was further developed to cover food-borne viruses (using MS2 and FCV as models) and phage P100: For the first step of the analytical chain, the sample preparation, a method called Matrixlysis, which was developed for isolation of food-borne bacteria, was tested. For the second step, the isolation of nucleic acids, a new method for disintegration of the virus particle and subsequent RNA/DNA isolation based on ionic liquids was developed. For the third step, detection, a qPCR for detection of phage P100 was developed and established.

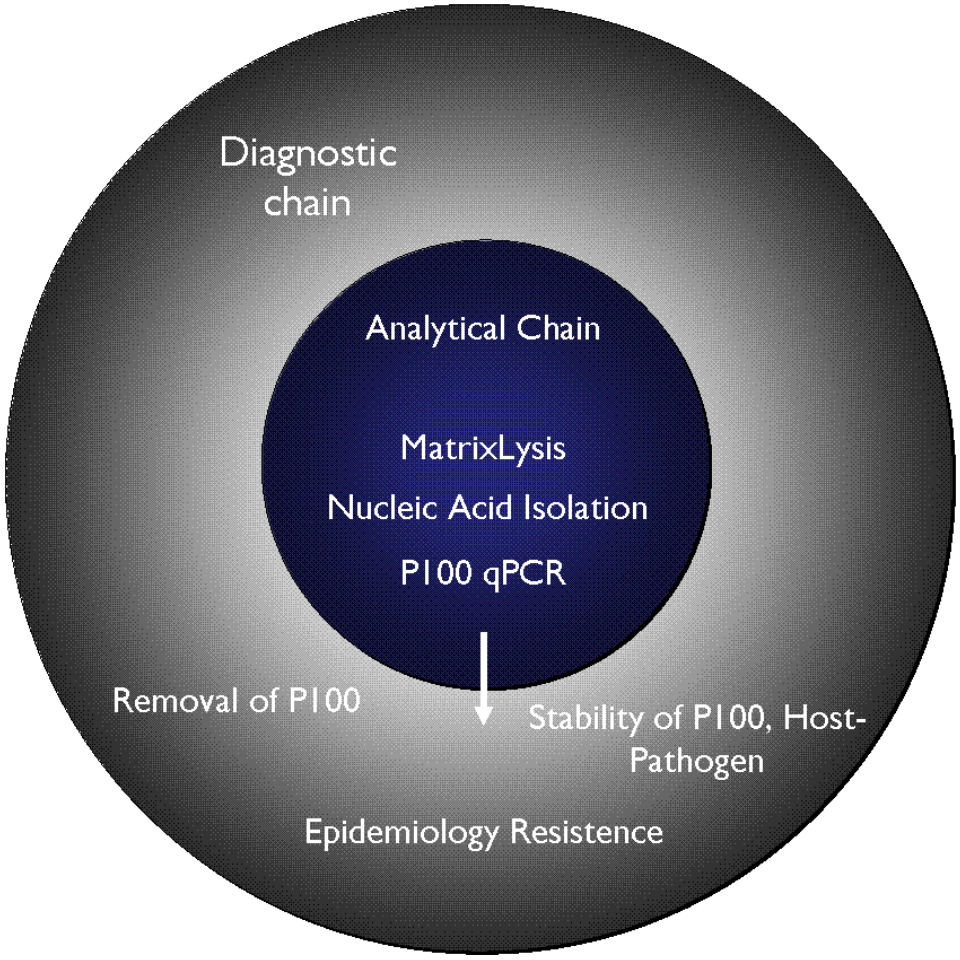

Figure 7: Overview of the thesis. Major topics were embedded in the diagnostic chain. Methodological approaches were based on the use of ionic liquids. Main organism of the thesis is phage P100.

4.1 Application of phage P100 – Resistance of *L. monocytogenes* and general usefulness (Publication I and II)

Although several phages, including phage P100, have been approved for use as anti-bacterial agents in food, there are still many unanswered questions. In particular, little is known concerning the influence of environmental factors on the efficacy of phage treatments and the presence or development of resistance to P100.

Therefore, this thesis addresses the prevalence of naturally occurring P100 resistant *L. monocytogenes* isolates in dairy plants. Screening *L. monocytogenes* plant isolates

revealed that 2.7 % of all those investigated showed no or distinctly reduced susceptibility to phage P100. Further investigation clearly indicated that all of these isolates were associated with the application of phages in the respective facilities. Characterization of phage-insensitive *L. monocytogenes* isolates revealed that they can be assigned to two different serotypes and four different PFGE types. This indicates that the detected isolates were not clones and that insensitivity to phage P100 had developed several times independently, suggesting that the development of phage resistance is not a very rare event. Moreover, although a bacteria count reduction of up to five $\log_{10}$ units could be observed when P100 was applied at high concentrations (MOI ≥ 1000), reduction of the phage to host ratio led to artificially induced insensitive bacteria that could be easily selected. Furthermore, in long term experiments (> 120 days) complete eradication of *L. monocytogenes* upon infection with P100 (MOI ≤ 100) was not observed and re-growth of the bacteria occurred. These re-growing isolates had distinctly reduced susceptibility to P100 or were completely resistant.

In respect of phage P100, limited information is available concerning the development of resistance. However, for other phages the occurrence of phage-resistant isolates after phage application has been described (Guenther and Loessner, 2011; Vongkamjan et al., 2013). Data from these publications and findings from this thesis indicate that the development of resistance or reduction of susceptibility to phage P100 is problematic for food industry.

Although mechanisms leading to resistance can be very diverse, all insensitive production plant isolates and the artificially induced insensitive mutants, which were investigated in this thesis, seem to have undergone changes to the bacteria receptor site. This is in accordance with other unpublished data, which indicate that most P100-resistant *L. monocytogenes* isolates have one point mutation that leads to modification in the receptor and therefore resistance to P100 (Prof. Martin Loessner, personal communication). Publication II illustrates that this single point mutation is likely to be actively selected by the bacteria host due to the presence of the phage.

The efficacy of phage P100 at controlling *L. monocytogenes* numbers is reduced, not only by low virus to host ratios and the presence or development of resistance, but it can also be influenced by physical and chemical parameters. Therefore, the influence of physical

and chemical parameters on the *stability*, *attachmen*t, and *replication* and *efficacy* of P100 was also studied in respect of their sequence in the reproductive cycle of P100.

Our data indicate that *stability* of phage P100 is established under most conditions that are typically found in food-production environments. Although the high stability of the phage can be an advantage and permits usage of the phage in adverse environments such as food production plants, it can also be a shortcoming. The (permanent) presence of phages in food-production plants is problematic. On one hand the development of resistance to the phage is an important topic. On the other hand the analytical chain and microbiological detection methods, for instance in *Listeria* routine testing using mini VIDAS® technology, can be affected by the presence of P100 in the sample. False negative results are then obtained as growth of the bacteria during enrichment is reduced by the phage.

When the *attachment* of phage P100 to the host cell was investigated no significant influence of the tested environmental factors was observed. This indicates that the use of phage P100 in passive approaches (see also chapter 1.3.1), which mainly rely on sufficient numerical attachment of the phages to the host, is possible under all tested conditions.

In contrast, *replication* of phage P100 was significantly influenced and could not be observed at all tested conditions. The presence of detergents (SDS and Lutensol) in particular blocked the replication of the phage. This is linked to reduced growth of the host bacteria in the presence of surfactants and indicates that use of P100 in active control approaches, which rely on the replication of the phages, is not recommended in food production environments.

When *efficacy* of phage treatment was studied, results indicate that high numbers of phages in combination with environmental conditions that limit bacterial growth caused highest reductions in *L. monocytogenes* numbers. Together with results of other publications, these findings indicate that phages could be more effective at reducing *L. monocytogenes* when used in combination with other measures, such as cleansing or disinfection. This is likely on account of synergistic effects.

In summary, the use of phage P100 for control of *L. monocytogenes* in production plants, especially when intended as an active approach, should be critically reconsidered and in future may not be promising. Moreover, additional surveillance is required if P100 is

employed. The phages easily spread in the environment and thus their detection by PCR and inactivation is subsequently necessary.

The scientific outcome of these two papers in respect of basic research is two-fold: (i) the first indication of the development of resistance of *L. monocytogenes* to P100 and its linkage to the artificial spread of phage P100 using commercial phage products is presented, and (ii) this resistance development is most probably promoted by the bacteria in response to contact with P100.

4.2 Interference of P100 in diagnostics - Inactivation of P100 using ionic liquids (Publication III)

As stated above, the presence of phages in the food production plant interferes with detection of the bacterial host by enrichment-based diagnostics. Therefore, inactivation of phages in the production plant is necessary following their application. However, legal regulations in food-production plants limit the choice of disinfectants that can be used. Further, viruses are often less sensitive to disinfection methods than bacteria and special disinfectants are needed. Thus, this thesis has investigated the application of ionic liquids as possible new virucidal agents, analogous to their application as bactericides.

Until now, the effect of ionic liquids on viruses has been poorly studied. There is only one relevant publication in which the stabilization of the tobacco mosaic virus in protic ionic liquids was reported (Byrne et al., 2012). In contrast, the general effects of ionic liquids on several other biological test systems has since been covered extensively and a structure-activity relationship ("SAR", (Jastorff et al., 2007)) has been described. Therefore, we attempted to establish structure-activity relationships for ionic liquids and viruses and particularly studied the effects of elongated cationic side chains and anionic chaotropicity on viruses.

It was shown for the first time that the so called side chain effect of imidazolium and ammonium based ionic liquids, as demonstrated for bacteria (Mester et al., 2015), is also relevant to the viruses (the phages MS2 and P100) tested. Nevertheless, this side chain effect is much less pronounced than in bacteria and the first distinct reduction of virus infectivity was observed at side chain lengths of ≥ 10. Further, bacteria were much more sensitive in respect of the concentration of the applied ionic liquids and they are

effectively killed by ~ 500 times lower concentrations than needed for the inactivation of viruses (Dr. Padde Mester, personal communication).

Ionic liquids with more than one elongated side chain had stronger effects on virus infectivity. The highest reductions obtained for both tested phages was observed with $[C_8C_8C_1C_1N][Cl]$. Interestingly, $[C_8C_8C_8C_1][Cl]$ had a reducing effect on phage MS2 but nearly no effect on phage P100, although this phage was more sensitive to all other tested ionic liquids compared with MS2. This interesting observation must be studied further, but could be due to the different sizes and structures of the phages.

The second structure-activity relationship that was studied in this publication was the effect of chaotropic anions. In contrast to the side chain effect, anion chaotropicity, which is known to enhance the antimicrobial activity of ionic liquids, did not affect virus infectivity. This was especially interesting because we expected that the protective virus capsid, which consists of protein units, could be denatured by ionic liquids with chaotropic anions. However, the results obtained indicate that even strong chaotropic anions such as trichloroacetate or thiocyanate do not reduce virus infectivity.

In summary, P100 reduction and the results of publication IV indicate that there exist ionic liquids capable of inactivating viruses. Therefore, screening for virucidal ionic liquids in future will be continued and analogue ionic liquids based on known virucidal agents will be synthesised. In order to understand the mode of action of ionic liquids on viruses, and to establish structure-activity relationships others than the side chain effect, further experiments, such as Fourier transform infrared spectroscopy (FTIR) , must be conducted. Furthermore, the combinatory effect of cations and anions has to be investigated.

4.3 Interference of P100 in diagnostics - Development of a PCR assay targeting phage P100 (Poster I)

When phages are used to control bacteria they not only prevent the growth of bacteria within the food production plant but also interfere with routine microbiological testing. Therefore detection of the phage using PCR is crucial to monitor routine diagnostics to exclude false negative results. However, no PCR suitable for phage P100 has yet been published. Consequently, we developed a qPCR to detect phage P100 (Poster I).

Altogether 12 primer pairs and four probes were designed. During testing and establishment of the PCRs, new phage sequences were published on NCBI and it transpired that some of the primer pairs were no longer specific for phage P100. Specificity testing of the remaining primer pairs was performed using *L. monocytogenes* DNA and DNA of phage vB LmoM AG20. Two primer and probe combinations remained that specifically detect phage P100.

These primer and probe combinations demonstrated good performance in PCRs and also amplified the P100 target sequences in naturally contaminated samples (swap samples and smear water samples). Moreover, the developed PCR assay provides quantitative results and has already been introduced for quantification of phage P100 DNA as in publication IV.

Due to the ongoing publication of new *Listeria* phage sequences and the discrepancy between bioinformatic specificity and reality, further specificity testing will be necessary. This has been problematic due to the fact that the leading scientific groups are essentially involved with the development of commercial phage products and are therefore not cooperative in sharing the necessary phage isolates.

A multiplex PCR targeting both *L. monocytogenes* and phage P100 has already been tested and could be further developed in future. As it appears to be challenging to develop a PCR specific for P100, a future consideration may be to establish a universal *Listeria* phage PCR.

4.4 Further development of the analytical chain – Nucleic acid isolation from P100 and the feline calicivirus (Publication IV)

One crucial prerequisite for reliable qPCR detection of food-related viruses and phages, such as P100, is quantitative isolation of nucleic acid of sufficient quality.

As described above, available systems for the isolation of viral nucleic acids are often not quantitative, time intensive or based on toxic chemicals. Therefore, ionic liquids were tested in respect of virus disintegration and subsequent extraction of nucleic acids.

To date the effect of ionic liquids on viruses has been poorly studied. However, results of this thesis indicate that ionic liquids can indeed be used for these purposes. Ionic liquids were identified that achieved significantly higher recoveries of both DNA and RNA

compared with that obtained by commercially available kits. Several ionic liquids were discovered suitable for viral DNA isolation. These ionic liquids include $[C_8C_8C_8C_1N]$[calconate] and $[C_{l4}C_6H_6OC_1CO_2]$ (159 % and 169 % recovery compared to the kit), $[(OH)_2C_2C_1C_1NH][NTf_2]$ (166 % recovery), two [FAP]-based ionic liquids ($[C_6C_6C_6C_{14}P]$[FAP] 143 % and $[C_6C_1mim]$[FAP] 152 % recovery) and $[C_1C_1im][C_1PO_2OH]$ (168 % recovery). In contrast, high yield viral RNA recovery could only be achieved with one ionic liquid, $[C_1C_1im]$ $[C_1PO_2OH]$ (up to 244 % recovery).

An advantage of the developed method is that it is less toxic than, for example, typically used guanidinium-phenol-chloroform extraction. Moreover, the method is a liquid phase system, meaning that there is no clogging of the columns. Additionally, some of the tested ionic liquids that performed relatively well, significantly inhibit nucleases. Thus, the use of ionic liquids to disintegrate viruses and for the subsequent isolation of nucleic acids is promising. Due to the inhibitory effect of some of the tested ionic liquids on nucleases (RNAse or DNase inhibition), ionic liquids will probably also be used in future for the storage of nucleic acids or as RNAse or DNase inhibitors.

Interestingly, DNA and RNA isolation was achieved using chemically and structurally diverse ionic liquids. Thus, so far it has not been possible to explain the mechanism underlying virus disintegration by the ionic liquids. Consequently, subsequent studies examined the general effects of ionic liquids on viruses (see. 4.2).

4.5 Further development of the analytical chain – Sample preparation targeting food borne viruses (Poster II)

A prerequisite for good quantitative PCR detection is nucleic acid isolation, which is dependent upon reliable sample preparation (Brehm-Stecher et al., 2009).

The isolation of food-borne viruses is usually achieved by elution of the virus from the food surface using different buffers or by homogenising the foodstuff mechanically. A major disadvantage of the elution approach is that they only capture viruses from the surface. Therefore, the recently developed sample preparation method called Matrixlysis system was tested as an alternative method (Appendix, Poster I).

Our results indicate that Matrixlysis buffer I (8 M Urea and 1 % SDS; Rossmanith et al., 2007) and buffer II (8 M Urea and 1 % Lutensol AO 7; Mayrl et al., 2009) decrease phage

MS2 and feline calicivirus (FCV) infectivity. Nevertheless, the detection of both viruses using PCR was possible and the detection rate ranged from 70 – 100 %. Matrixlysis buffer III (7.5 % [emim]SCN; Mester et al., 2010) and buffer IV (1 M $MgCl_2$; Mester et al., 2014) did not reduce infectivity of the viruses tested, yet detection using microbiological detection methods (plaque assays and titre determination in cell culture) and molecular biological methods (qPCR) was possible.

These results were similar to those obtained from bacteria. Matrixlysis buffers I and II caused loss of viability of the tested bacteria (*L. monocytogenes*, *S. aureus* and *B. cereus*), albeit the cells were physically intact (Rossmanith et al., 2007; Mayrl et al., 2009). In contrast, Matrixlysis buffers III and IV did not affect bacterial viability (Mester et al., 2010; Mester et al., 2014).

A disadvantage was that Matrixlysis buffers I and II can only be used to detect viruses by PCR and not with plaque assays due to reduced infectivity. Therefore, a distinction between infective and non-infective viruses is not possible. However, when a distinction between infective and non-infective viruses is necessary, Matrixlysis buffers III and IV can be used. Further, cell culture assays are still not available for several (food-borne) viruses, including the most important food-borne viruses, the human noroviruses, meaning that they can only be detected by molecular biological methods such as PCR or qPCR (Di Pasquale et al., 2010a). Consequently, infective viruses are not a requirement for sufficient detection.

The advantage of the Matrixlysis method for lysis of foodstuff and subsequent virus detection is that a broad range of foodstuff can be processed. The preparation method can also be used for other matrices, e.g. clinical samples. In addition, due to complete lysis of the foodstuff, virus detection is not limited to those confined to the food surface. Advantageously, the method is cheap and simple to perform.

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6 Abstract

Food safety is globally a public health priority and the availability of safe food is a basic human right. To guarantee microbiologically safe food a systems approach, summarised as diagnostic chain, is necessary, including detection and removal or inactivation of food-borne pathogens.

In this thesis the focus within the diagnostic chain was *Listeria* phage P100. The presence and development of resistance to P100 was investigated as well as the influence of environmental factors on the stability of the phage *per se* and on the host-phage interaction. Results indicate that phage P100 is stable and binds to the host under most conditions tested. Replication was dependent upon the growth of *L. monocytogenes* and efficacy of phage treatments was higher when bacterial growth was simultaneously reduced by appropriate environmental conditions. Experiments revealed a first evidence for resistance development to P100 in *L. monocytogenes* and its linkage to the artificial spread of commercially available phages. Furthermore, this resistance development seems to be actively promoted by the bacterial cell in response to contact with P100.

Since the presence of phages in the production plant interferes with host bacteria detection using enrichment based diagnostics, P100 inactivation by ionic liquids and their effects on viruses was examined. For the first time ionic liquids were identified inactivating phages P100 and MS2. In addition, the most commonly recognized structure-activity relationship characteristic of ionic liquids, the so called side chain effect, was also applicable to viruses. A qPCR assay for detection of P100 to monitor routine diagnostics was developed and has already been tested in naturally contaminated smear water samples. However, further specificity testing is necessary. As reliable qPCR detection requires quantitative isolation of nucleic acids, the use of ionic liquids for virus disintegration and subsequent isolation of nucleic acids was investigated. Ionic liquids transpired to be promising tools for both the isolation of viral RNA and DNA, and recovery rates higher than those achieved with commercially available kits were obtained. As some of the ionic liquids tested had inhibitory effects on nucleases, these compounds may prove useful for storage of nucleic acids or as RNAse or DNase inhibitors. Finally, adequate sample preparation is crucial to the analytical chain. Therefore, the alternative sample preparation method Matrixlysis was investigated. Depending on the buffer system used, Matrixlysis did not distinctly reduce infectivity or detectability of FCV and MS2 and may find application for subsequent virus detection with both microbiological and molecular biological detection methods.

6.1 Zusammenfassung

Weltweit ist Lebensmittelsicherheit ein wichtiges Thema und ungefährliche Nahrung ist ein grundlegendes Menschenrecht. Zur Bereitstellung mikrobiologisch unbedenklicher Lebensmittel ist ein umfassendes Konzept nötig, das sowohl die Detektion als auch die Entfernung von Lebensmittelpathogenen beinhaltet und als „diagnostische Kette“ zusammengefasst werden kann.

Innerhalb der diagnostischen Kette war der Phage P100 das zentrale Thema dieser Dissertation. Es wurde untersucht, ob Resistenzen gegen den Phagen bereits vorkommen oder sich entwickeln und wie sich Umweltfaktoren auf die Stabilität des Phagen und auf die Wirt-Phagen Interaktion auswirken. Die Ergebnisse zeigen, dass der Phage P100 unter den meisten getesteten Umweltbedingungen sehr stabil ist und an seinen Wirt binden kann. Im Gegensatz dazu war die Replikation des Phagen vom Wachstum von *L. monocytogenes* abhängig. Der Einsatz von Phagen war erfolgreicher bei Umweltbedingungen, die das Wachstum von *L. monocytogenes* einschränkten. Es wurde gezeigt, dass sich Resistenzen gegen den Phagen P100 entwickeln und dieses Phänomen mit dem Einsatz und der Verbreitung von kommerziell erhältlichen Phagenprodukten zusammenhängt. Diese Resistenzen entstehen wahrscheinlich durch eine Reaktion der Bakterien auf die Phagen.

Phagen im Betriebsumfeld oder den Proben stören die mikrobiologische Detektion des Wirtsbakteriums, da diese auf der Anreichung von Bakterien basiert. Deshalb wurde untersucht, ob sich ionische Flüssigkeiten zur Inaktivierung der Phagen eignen und wie sie auf Viren oder Phagen wirken. Erstmals wurden ionische Flüssigkeiten gefunden, die Phagen (P100 und MS2) inaktivieren. Auch der viel beschriebene Seitenketten-Effekt, die häufigste Struktur-Aktivitätsbeziehung ionischer Flüssigkeiten, konnte bei den getesteten Viren festgestellt werden. Außerdem wurde eine qPCR entwickelt, um P100 in Routineuntersuchungen zu detektieren. Diese qPCR konnte P100 in natürlich kontaminieren Schmierwasserproben detektieren. Allerdings muss die Spezifität dieser PCR noch weiter untersucht werden. Da die quantitative Isolation von Nucleinsäuren Voraussetzung für eine zuverlässige PCR ist, wurde eine Methode basierend auf ionischen Flüssigkeiten entwickelt, um Virenpartikel aufzubrechen und danach Nucleinsäuren zu isolieren. Mit verschiedenen ionischen Flüssigkeiten konnten höhere Ausbeuten an viraler RNA und viraler DNA als mit dem kommerziellen Kit erzielt werden. Weil manche dieser ionischen Flüssigkeiten Nucleasen inhibierten, könnten sie in Zukunft auch zur Lagerung von Nukleinsäuren oder als RNase oder DNase Inhibitoren verwendet werden. Innerhalb der analytischen Kette ist ebenfalls eine adäquate Probenaufarbeitungsmethode ausschlaggebend. Deshalb wurde die alternative Methode Matrixlysis ausgetestet. Die verwendeten Puffer verminderten die Infektiösität und Nachweisbarkeit der getesteten Viren (MS2 und FCV) nicht entscheidend und eignen sich deshalb für die mikrobiologische und molekularbiologische Virendetektion.

7 Curriculum Vitae

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Education

1992 – 1996 Elementary School Unterlangkampfen

1996 – 2004 Bundesrealgymnasium Kufstein

2004 – 2009 Diploma studies in biology (microbiology and genetics) at the University of Vienna

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8 Appendix

Poster I

Development and Testing of Conventional and Real Time PCRs for Detection of the Phage P100

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INTRODUCTION

The phage P100, which is commercially available as Listex™ P100, is frequently used to combat *Listeria* in food production. Routine testing for *L. monocytogenes* in food samples is mostly done by enrichment and microbiological methods therefore the use of phages can lead to false negative results.

PURPOSE

The aim of the study was to enable the detection of the phage P100 in food or environmental samples. For this reason a stable PCR was developed and established in order to realise false negative results in routine microbiological testing due to phage contamination.

MATERIAL and METHODS

Primer design

After alignment of all known listeria phages primer and probes were designed using Primer Express®. Unspecific binding of primers was checked using blast.

Specimen

The listeria phage vB LmoM AG20 was achieved by Hany Anany (University of Guelph). *L. monocytogenes* ATCC BAA-679 (EGD-e) was used for amplification of the phages and as control for specificity testing. The phage P100 was used as Listex™ P100 preparation (EBI Food Safety Wageningen, Netherlands)

DNA Extraction and DNA-Standard

The NucleoSpin© tissue kit was used for isolation of DNA according to the correlating protocol. The DNA concentration was measured and the copy number was calculated. For the DNA standard 10-fold dilution series were used.

Natural contaminated Samples

Swap samples and smear water of a dairy plant that used the phage P100 as Listex™ P100 preparation were used as natural contaminated samples.

PCR

After testing of different annealing temperatures and $MgCl_2$ concentrations PCRs were preformed as follows:
For real-time PCR 25-µl PCR reaction contained 3.5 mM $MgCl_2$, 0.5 µM of each primer, 0.25µM probe, 0.8 mM dNTPs, 1.25 U of Platinum© *Taq* DNA polymerase (Invitrogen), and 5 µl isolated DNA. Amplification following initial denaturation at 95 °C for 2 min was performed in 45 cycles, at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min.

Analysis

PCR products were analysed by gel electrophoresis and melting curve analysis. Specifictiy was tested using DNA of *L. monocytogenes* and the phages vB LmoM AG20. Other published listeria phages could not be tested yet as several authors did not respond to queries or were not prepared to send them.

RESULTS and DISCUSSION

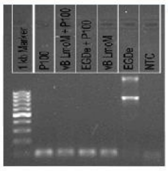
Tab. 1

Primer / Probe	Sequence	Position	Gene	Amlicon	Blast Hits (100% Query Cover and 100% Identity)															Detection in PCR
P100 Primer 1	agcagagtttgaaaaaattgatgactac	94436-94463			LP-125	vB LmoM AG20			LP-064	LP-048										
P100 Primer 2	tcgtcatgcgtgtttcttatgc	94528-94508			LP-125	vB LmoM AG20			LP-064	LP-048	LP-083-2	LP-124	LMSP-25	LMTA-94	LMTA-57					
P100 probe 1+2	tggcggtaaaacttatagcacttagttacaaaatcacagca	94465-94504	gp104	92 bp																only P100
P100 Primer 3	ggaagaaagagtattggtagactacatcag	94932-94961			LP-125				LP-064				LMSP-25	LMTA-94	LMTA-57	LMTA-34				
P100 Primer 4	gagttttctgcgtttaggttttcaa	95021-94997			LP-125				LP-064				LMSP-25	LMTA-94	LMTA-57	LMTA-34				P100 + vB LmoM AG20
P100 probe 3+4	ctagaagacgacaacggcagaaacatcatttt	94963-94994	gp105	89 bp	LP-125				LP-064				LMSP-25	LMTA-94	LMTA-57	LMTA-34				
P100 Primer 5	gcaagtaccttcatcttactaagtctaaactaa	96728-96762																		
P100 Primer 6	cgcatatataagcctttgagttgac	96866-96841			LP-125	vB LmoM AG20	AS11	List 36	LP-064	LP-048	LP-083-2	LP-124	LMSP-25	LMTA-94	LMTA-57	LMTA-34	LMTA-148			
P100 probe 5+6	ctgtaatcacctcagcatttttttcacacgg	96808-96839	unkown	138 bp																only P100
P100 Primer 7	cagaagacttagcagcacacgaatac	12709-12734	gp17 =																	
P100 Primer 8	tgtacattggagctaactcgatttcta	12849-12823	Major																	P100 + vB LmoM
P100 probe 7+8	agtctattgcaagtgaagtggctacagctacagttactg	12763-12801	capsid	140 bp	LP-125	vB LmoM AG20	AS11	List 36	LP-064	LP-048	LP-083-2	LP-124	LMSP-25	LMTA-94	LMTA-57	LMTA-34	LMTA-148	20422-1	805405-1	AG20

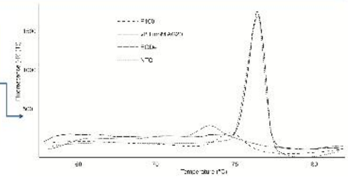
Tab. 2

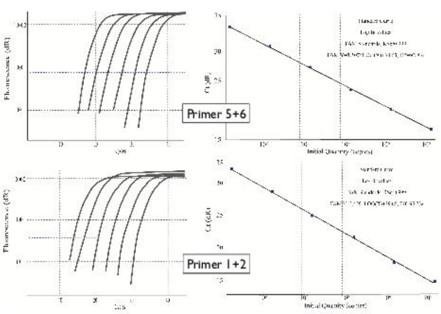
Primer	Sequence	Position	Gene	Amlicon	Blast Hits (100% Query Cover and 100% Identity)													Detection in PCR
gp3 P100 F	ggagatgccgtaaaggatga	748-767		81 bp											LMTA-34		List-36	
gp3 P100 R	atctcctgccgaaacatctg	828-809	gp3		AS11	LP-125		LMTA-94	LP-048	LP-124	LP-083-2	LP-064	LMSP-25	LMTA-57				P100 + vB LmoM AG20
gp3 P100 F1	cagatgtttcggcaggagat	809-8028		107 bp	AS11	LP-125		LMTA-94	LP-048	LP-124	LP-083-2	LP-064	LMSP-25	LMTA-57				
gp3 P100 R1	tgttcctccgctaccactatc	915-895	gp3		AS11	LP-125			LP-048	LP-124	LP-083-2	LP-064	LMSP-25	LMTA-57				P100 + vB LmoM AG20
gp4 P100 F	acatcgaccttggcaaccta	1301-1320		52 bp	AS11	LP-125	vB LmoM AG20	LMTA-94	LP-048	LP-124	LP-083-2	LP-064	LMSP-25	LMTA-57	LMTA-34	LMTA-148	Lkt36	
gp4 P100 R	agctacattcgggtgggagt	1352-1333	gp4		AS11	LP-125	vB LmoM AG20		LP-048	LP-124	LP-083-2	LP-064			LMTA-34	LMTA-148	Lkt36	P100 + vB LmoM AG20
gp4 P100 F1	tggatatgctaactccatcaaca	1198-1220		102 bp	AS11					LP-124	LP-083-2							
gp4 P100 R1	cctgcgcctttcagtagac	1299-1280	gp4		AS11					LP-124	LP-083-2							P100 + vB LmoM AG20
gp118 P100 F	cgcttatgtaccgtatgtgaga	100725-100747		57 bp					LP-048			LP-064				LMTA-148	Lkt36	
gp118 P100 R	tacccattcacctcctctgc	100781-100762	gp118						LP-048			LP-064				LMTA-148	Lkt36	P100 + vB LmoM AG20
gp118 P100 F1	aaagcacctatttcagaatgtcc	100567-100589		100 bp		LP-125		LMTA-94	LP-048	LP-124	LP-083-2	LP-064	LMSP-25	LMTA-57		LMTA-148	Lkt36	
gp118 P100 R1	cattaaatttcatccgccagt	100666-100646	gp118						LP-048			LP-064				LMTA-148	Lkt36	P100 + vB LmoM AG20
gp120 P100 F	acgtgaacggctttcctagt	101118-101137		72 bp		LP-125												
gp120 P100 R	cgctagcgaactccttgtct	101189-101170	gp120			LP-125												P100 + vB LmoM AG20
gp120 P100 F1	aattatacgtgaacggctttcc	101112-101133		80 bp		LP-125		LMTA-94				LP-064		LMTA-57				
gp120 P100 R1	tacgctagcgaactccttgtc	101191-101171	gp120			LP-125												P100 + vB LmoM AG20

At the beginning four primer pairs for real-time PCR (Tab. 1) and eight primer pairs for conventional PCR (Tab.2) were designed. After testing and establishment of the PCRs new phage sequences were published on NCBI and it turned out that some of the primer pairs were not specific for the phage P100 anymore. One primer pair (gp 3 P100 F and R) for conventional PCR and three primer pairs for real-time PCR were specific in bioinformatical analysis.



Specificity testing of the primer using the phage vB LmoM AG20 as example showed that the primer pair for conventional PCR is not able to distinguish between the phage P100 and the phage vB LmoM AG20 neither in analysis by gel electrophoresis nor in melting curve analysis.





One real-time primer pair/probe combination (P100 primer 7+8) did also detect the phage vB LmoM AG20. The two remaining combinations (P100 primer 1+2 and 5+6) gave no signal with the phage vB LmoM AG20 and were able to detect the phage P100 specifically.

Analysis of standard curves and amplification plots (Fig. on the left are examples) of real-time primer P100 primer 1+2 and 5+6 showed a good performance PCRs with a mean efficiency of 99.6 % (primer 1+2) and 92.8 % (primer 5+6) and mean RSq values of 0.998 and 0.998 (primer 1+2 and 5+6)

The developed real-time PCR was able to detect the phage P100 in natural contaminated samples even when the phage could not be detected by the plaque assay. This indicates a good sensitivity.

OUTLOOK and CONCLUSION

The design of a PCR specific for the phage P100 in order to detect false negative results in routine testing of *L. monocytogenes* is not trivial. The ongoing publication of new listeria phage sequences makes it hard to design highly specific primer and probes. Moreover there is a discrepancy between bioinformatic specificity (e.g. blast hits) and reality (detection in PCR using other listeria phages) that makes it complicate to develop a highly specific PCR. Therefore in future specificity of the remaining two primer/probe combinations has to be tested further in experiments using DNA of as many listeria phages as possible as template.

Poster II

The Impact Of Four Buffer Systems Derived From A Matrix-Solubilization Based Sample Preparation Protocol For Bacteria On The Integrity Of Virus Particles

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ABSTRACT Major causes of food-borne illnesses in humans are viruses. Accurate detection of food-borne viruses is limited on molecular diagnostics and existing methods are laborious, time consuming and not standardized. In contrast there exists a newly developed and well established protocol for molecular biological quantification for food-borne bacteria based on the solubilization of the food called MatrixLysis. The question was if this system provides the standardized environment necessary for the molecular virus detection.

MATERIAL AND METHODS

Specimen
As a surrogate for food-borne viruses MS2 bacteriophages and feline caliciviruses (FCV) were used. FCV were grown in Crandel Feline Kidney (CrFK) cells.

Buffer
Buffer I: 8M Urea and 1% SDS (Rossmanith et al., 2010); buffer II: 8M Urea and 1% Lutensol (Mayrl et al., 2009); buffer III: 7.5% Ionic Liquid: [emim]SCN) (Mester et al., 2010); buffer IV: 1M $MgCl_2$ (Mester, personal communication)

Treatment with MatrixLysis buffers
75µl phages or FCVs were incubated in 175µl MatrixLysis buffer at 37/ 45°C for 30min. Afterwards influence of the buffers was tested by titer/$TCID_{50}$ determination and RT qPCR.

RNA isolation
The PureLink Viral RNA/DNA Mini Kit (Invitrogen) was used according to the manufacturer's instructions (RNA elution was done with 20µl RNAse-free water).

cDNA synthesis (RT)
RNA was transcribed using Cloned AMV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Primers, probes and TaqMan real-time PCR (qPCR)
MS2: PCR was performed according to Dreier et al. 2005, using a FAM-labelled probe.
FCV: PCR was performed according to Johne et al. (personal comunication).

$TCID_{50}$ (Median tissue culture infective dose)
Tenfold dilution series of the (treated) viruses were incubated on CrFK cells in 96 well plates (6 wells per dilution; 3x10⁴ cells/ ml; 100µl/well) for 1h. Afterwards suspension was removed and media was added, wells displaying CPE were counted after 3 days and $TCID_{50}$ was calculated according to SPEARMAN and KÄRBER.
MEM media: 5ml 200mM (100x) L-Glutamine, 50ml Fetal Bovine Serum Gold, 5ml Antibiotic/ Antimycotic, 5ml Non Essential Amino Acids (100x), 40mM HEPES pH 6.5- 7.5 (final concentration), add. 500ml with MEM (everything PAA).

Titer determination MS2
Dilution series of the (treated) phages were made in SM-Buffer. 100µl of the phage dilution and 100µl of *E.coli* Top10F' (Invitrogen; OD_{600}=1.2) were mixed in a petri dish and incubated for 15min at room temperature. 7ml of LB-soft agar, (45°C) was added, mixed and incubated at 37°C over night. Plaque forming units/ml were calculated. SM-buffer: 5.8g NaCl, 2.4g Tris HCl, 1.0g gelatine add. 1000ml, pH7.0 (with HCl).

Concept
Influence on MS2
- four MatrixLysis buffers at 37°C and 45°C
- Titer determination
- RT qPCR
- TEM (Transmission Electron Microscopy; Figure 1)

Influence on FCV
- four MatrixLysis buffers at 37°C and 45°C
- $TCID_{50}$ determination
- RT qPCR

MS2 bacteriophage and Feline Calicivirus
The MS2 phage is an icosahedral bacteriophage of *E.coli* with a diameter of 27-34nm. It is a single stranded positive sense RNA virus of the Leviviridae family with a genome of 3,569 nucleotides.
The feline calicivirus (FCV) is also a single stranded positive sense RNA virus but of the Caliciviridae family. It has a diameter of 35-39nm and a genome of 7,700 nucleotides.
Both viruses are commonly used as surrogates of food-borne viruses especially as a model for human noroviruses which can not be grown and propagated with standard cell culture methods.

RESULTS

Titer determination of MS2 after incubation in MatrixLysis buffers
There is no or significantly decreased phage propagation in the presence of buffer I and II and phage were not able to produce plaques anymore. Compared to the control (phages without MatrixLysis buffer and temperature treatment) where 1.02E+07 PFU/ml were found incubation of phages in buffer III and IV resulted in slightly decreased infectivity with 7.85E+06 (recovery = 77%; buffer III) and 8.58E+06 PFU/ml (recovery = 84%; buffer IV; Table 1).

$TCID_{50}$ determination of FCV incubated in MatrixLysis buffers
By incubation with undiluted buffer I and buffer II CrFK cells did not survive. By using 10- or 100-fold dilutions of buffer I and II cells survived. However $TCID_{50}$ of viruses incubated in buffer I and II could not be determined because no cytopathic affect was visible at 10- or 100- fold dilution. In contrast buffer III and IV did not effect the survival of CrFK cells (when incubated for 1h with these buffers). The $TCID_{50}$ values for FCV incubated at 37°C were -5.084 (buffer III) and -5.667 (buffer IV) vs -5.332 (control: FCV not treated with heat or MatrixLysis buffer). Incubation at 45°C resulted in decreased infectivity of FCV when incubated with buffer III and buffer IV (Table 2).

Recoveries of MS2 phages after incubation in MatrixLysis buffer using RT qPCR
The best results were achieved using buffer IV and buffer I (37°C and 45°C, ≥100% recovery). Recovery rates of buffer II and III were not comparable but at 37°C ≥ 60% (Figure 2).

Recoveries of FCV after incubation in MatrixLysis buffer using RT qPCR
Although all four buffers had recovery rates of more than 60% when they were used at 37°C buffer IV was the best one (92% recovery). When viruses were incubated at 45°C recovery of buffers I and II decreased to 37% (buffer I) and 31% (buffer II). Recovery rate of buffer III was even higher at 45°C (75%) than at 37°C (63%) and buffer IV showed again the best result (86% recovery). When the viruses were only treated with temperature and water instead of MatrixLysis buffer recovery rates were 93% at 37°C and 102% at 45°C (Figure 3).

Figure 1: MS2 phages

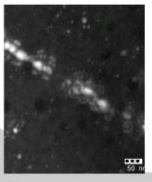


Table 1: Titer determination of MS2 phages after incubation in MatrixLysis buffers at 37°C for 30 min by plaque assay

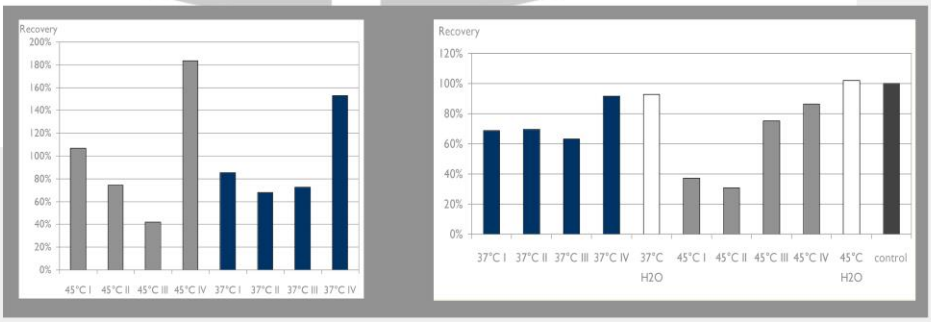
	37°C	
buffer	PFU/ml	recovery
A	0	X
B	1.00E+00	X
C	7.85E+06	77%
D	8.58E+06	84%
control	1.02E+07	100%

Table 2: Titer determination of FCV after incubation in MatrixLysis buffers at 37°C for 30 min by $TCID_{50}$

T	buffer	$TCID_{50}$
37°C	III	-5.084
37°C	IV	-5.667
RT	control	-5.332
45°C	III	<-1
45°C	IV	-3.667
RT	control	-5.165

Figure 2: RT qPCR recovery of MS2 after incubation with MatrixLysis buffers at 45°C and 37°C for 30 min.

Figure 3: RT qPCR recovery of FCV after incubation with MatrixLysis buffers at 45°C and 37°C for 30 min.



DISCUSSION AND CONCLUSION

The MatrixLysis system is a promising tool to support molecular biological detection of food-borne viruses. Buffers III and IV may be even used for conventional detection methods like titer determinations. Future work will focus on concentration of viruses subsequent to MatrixLysis and before RT qPCR detection.

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Rossmanith,P., Suss,B., Wagner,M., and Hein,I. (2007). Development of matrix lysis for concentration of gram positive bacteria from food and blood. J. Microbiol. Methods 69, 504-511.