universität wien

MASTERARBEIT

Titel der Masterarbeit

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

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc.)

Wien, 2015

Studienkennzahl lt. Studienblatt:	A 066 830
Studienrichtung lt. Studienblatt:	Master Molekulare Mikrobiologie und Immunologie
Betreut von:	Prof Dr. Martin Wagner

Acknowledgement

First of all I want to thank Dr. Peter Rossmanith for the possibility to work in his lab and offering me an interesting topic for my master thesis.
I would particularly like to thank Susanne Fister for the excellent functional assistance during lab-activity, during writing the thesis and for constructive critique and interesting discussions, which helped to finish this study.
Also I want to thank Dr. Patrick Mester, Dr. Anna Jehle, Christine Leeb and Tobias Gundolf for the backup, the help with my thesis and friendly atmosphere in the lab and my parents for the financial support during the whole education period.
Last but not least I want to thank Prof. Dr. Martin Wagner for giving me the opportunity to work at the Institute for Milk Hygiene and supervising this thesis.

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List of Abbreviations

$CaCl_2$	Calcium chloride
CFU	Colony Forming Units
DNA	Desoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EOP	Effiency of plaquing
FDA	US Food and Drug Administration
g	Gram
GRAS	Generally Regarded As Safe
Kbp	Kilobase pair
L. monocytogenes	*Listeria monocytogenes*
$MgCl_2$	Magnesium chloride
µl	Microlitre
ml	Millilitre
min	Minutes
MOI	Multiplicity of infection
NaCl	Sodium chloride
Nm	Nanometre
No.	Number
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PFU	Plaque Forming Units
pH	Pondus Hydrogenii
qPCR	Quantitative Real Time PCR
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
sec	Seconds
SW	Smear water
TBE	TRIS-borate EDTA buffer
TRIS	Tris-(hydroxymethyl)aminomethane
TSA+ Y	Tryptone Soy Agar+ Yeast extract
TSB+ Y	Tryptone Soy Bouillon+ Yeast extract
UV	Ultraviolet
YOPI	Young, old, pregnant, immunocompromised

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1 Introduction

1.1 Bacteria and food safety

Bacteria are ubiquitously occurring microorganisms, which consist of one single cell. They are present everywhere in our body and in the surrounding environment. Bacteria can multiply themselve and they are not dependent on host-cell machinery like viruses. Bacterial growth and multiplication occurs by cell division and formation of autonomous cells.

Bacteria are generally important for humans, for example in the context of food safety, where bacteria can protect against growth of pathogens. These are called protective cultures and are defined as cultures consisting of live bacteria (pure cultures or culture concentrates) that are applied to foods with the intention of reducing risks by pathogens [1]. On the other hand, bacteria can become pathogens when they enter parts of the human body, where they are not intended to be. This is called zoonosis, caused by pathogens, which are transmitted from animals to humans [2]. This is especially relevant for immunocompromised patients. Zoonotic pathogens often reach the human body by contaminated food.

Food safety is a problem that concerns everyone. The more the globalization has progressed over the past decades, the more difficult it has become to retrace every step of the food products. In 2002, the European Union made control of every food-processing step mandatory. These steps are production, processing, storage, distribution, transport and animal feeding [3].

One of the main concerns in food safety is the microbiological hazard by pathogenic microorganisms such as *Brucella*, *Campylobacter*, vero-toxin producing *Escherichia coli* (VTEC), *Listeria*, *Mycobacterium bovis* and *Salmonella* (Table 1). Therefore surveillance of these bacteria in humans is obligatory.

Table 1: Reported hospitalisation and case-fatality rates due to zoonosis in confirmed human cases in the EU, 2013 (EFSA, ECDC EU summary report 2013); NA: not available

Disease	Number of confirmed human cases	Hospitalised cases	Hospitalisation rate (%)	Reported deaths	Case fatality rate (%)
Campylobacteriosis	214,779	11,922	43.6	56	0.05
Salmonellosis	82,694	7,841	36.0	59	0.14
VTEC infections	6,043	922	37.1	13	0.36
Listeriosis	1,763	735	99.1	191	15.6
Q fever	648	NA	NA	2	0.61
Brucellosis	357	139	70.6	1	0.99
Trichinellosis	217	106	65.4	1	0.56

The methods, which are currently available to control pathogenic microorganisms in food production are for instance irradiation, thermal inactivation, microwave heating, high pressure processing, pulsed-light technology, cold plasma technology or protective cultures. However, they are insufficient to achieve full eradication or have influence on ingredients of food products by changing quality or sensory properties [4]. Hence there is a need for new approaches to prevent food contamination [5].

1.2 Listeria monocytogenes

The organism, we focus on in this work, is *Listeria monocytogenes*. It is a Gram-positive bacterium and belongs to the genus *Listeria*, which belongs to the class of *Bacilli.* The class of Bacilli belong to the order of *Bacillales*, which also contains *Bacillus* and *Staphylococcus*. Several different species are included to the genus *Listeria*, but only *L. monocytogenes* is associated with human disease [6]. *L. monocytogenes* is a nonspore-forming, motile, facultative anaerobic, rod-shaped intracellular pathogen, which grows between -0.4 and 45°C. It is catalase positive and oxidase negative and expresses a β-hemolysin, which causes damage of red blood cells [7, 8].

High tolerance to extreme pH, temperature and salt conditions is a reason for a wide distribution of *L. monocytogenes* in the environment. It can occur in soil, water, effluents and food [9, 10]. A high prevalence of *L. monocytogenes* in dairy farm environments is a reason why it is mostly transmitted to humans and animals via contaminated foods [11].

There are thirteen serotypes of *L.monocytogenes*, which can cause disease. However, over 90 % of human *L. monocytogenes* isolates belong to three serotypes: 1/2a, 1/2b and 4b. Serotype 4b is responsible for 33 to 50% of human cases and for all major outbreaks in Europe and the US since 1980 [12]. Invasive infection with *L. monocytogenes*, calles listeriosis, primarily occurs in very young, old, pregnant and immunocompromised individuals. This risk group is commonly referred to as YOPIs [13, 14]. However, *L. monocytogenes* infection can also occur in healthy people without predisposing factors [15].

Listeriosis may result in meningitis, septicaemia, encephalitis or loss of the fetus during pregnancy. It is lethal in 15 to 40% of the cases [16].

As shown in Table 2, several multistate outbreaks in the US and outbreaks in Canada, Japan and Europe, caused by contaminated cheeses, made control of *L. monocytogenes* a great public health concern [17]. Therefore many countries have strict regulations for *L. monocytogenes* quantities that are allowed to appear in certain foods. Zero tolerance policies are present in the US and Oceania [13]. In the European Union there is a limit of 100 CFU/g for 25 g of ready-to-eat foods, that do not support growth of pathogens [18].

Table 2: Reported listeriosis outbreaks associated with different types of cheese [18].

Year	Location	No. of cases	No. of deaths	Suspected/implicated food	Serotype
1985	USA	142	48	Mexican-style cheese	4b
1987	Switzerland	122	34	Vacherin Mont d'Or cheese	4b
1990	Denmark	26	7	Blue mold cheese	4b
1995	France	37	11	Soft cheese	4b
1998	France	14	0	Soft cheese	4b
2000	USA	13	5	Mexican-style cheese	4b
2001	Sweden	120	NA	Soft cheese	NA
2001	Japan	19	0	Cheese	1/2b
2002	Canada	17	0	Raw milk soft cheese	NA
2003	USA	41	5	Mexican-style cheese	4b
2005	Switzerland	8	3	Tomme cheese	NA
2006	Germany	189	26	Soft cheese	4b
2007	Norway	17	3	Camembert cheese	NA
2008	Canada	92	NA	Cheese	NA
2009	Austria	34	8	Acid curd cheese	1/2a
2012	USA	22	4	Ricotta cheese	NA

NA — Not available information.

1.3 Bacteriophages

In theory a promising approach to control *L. monocytogenes* is the use of bacteriophages. Bacteriophages are viruses that infect bacteria. They are ubiquitous in nature and likely to be numerically the most abundant organism with an estimated population of 10^{30} phage particles worldwide [19].

The first step of bacteriophage infection is adsorption to the cell surface by tail fibers or spikes of the phage, by binding to specific surface molecules on the host cell. Gram-positive bacteria, like *L. monocytogenes*, offer many different binding sites due to their complex murein structure [20].

Bacteriophages can be divided into two major groups: *lytic* and *lysogenic* phages. After entering the host cell, *lytic* phages immediately use host metabolism for production of genome and structural proteins for progeny virions. These new phages are released by rupturing the cell wall causing cell death within minutes to hours after initial infection [21, 22].

In contrast, *lysogenic* phages integrate their genetic material into the genome of the bacterial host cell. Genes, which are detrimental to the bacterial cell, are not expressed in this state. The integrated phage DNA is replicated along with the host cell genome and is called a prophage. Prophages can emerge inside a new host cell. Stress can trigger a process called induction, which leads to expression of genes required to enter the *lytic* state. Once the *lytic* cycle is entered, virions are produced and released from the cell. Only *lysogenic* phages, which are able to enter the bacterial genome, can participate in horizontal gene transfer between different bacterial populations.

Genetic transfer is not wanted in antibacterial applications. Therefore *lytic* phages, which have the ability to immediately lyse bacterial cells, are used [22].

In every food processing facility, bacteria and a wide variety of phages are naturally present. Those phages can have a positive effect (killing dangerous bacteria) or a negative one (killing certain desirable bacteria) [23].

Bacteriophages generally do not cross species or genus boundaries. This makes them theoretically ideal candidates for control of food pathogens without interfering with the resident microflora or microorganisms that are required for production of fermented foods [5, 24].

1.4 *Listeria* phages

Listeria phages have been isolated from sewage plants, silage, *lysogenic* strains and food processing environments [25]. All known *Listeria* phages belong to the order Caudavirales, characterised as tailed viruses with double-stranded DNA. The families of Podoviruses, myoviruses and siphoviruses belong to this order. While podoviruses have very short tails, myoviruses have long non-flexible tails and siphoviruses have long and flexible tails. The majority of *Listeria* phages are temperate phages, being able to integrate into the host genome [13]. The less frequent *lytic* phages of *Listeria* can be divided into three groups: the large myoviruses (around 140kb) P100 and A511, which infect broad host ranges, while smaller siphoviruses P35 and P40 (40kb) and P70 (70kb) still infect most members of given serovars [5, 26]. *Podoviridae*, which infect *Listeria*, have not been found yet. Generalized transducing phages like P35 are, due to their ability to transfer its genetic material including the virulence factors, not preferable for application in food safety. [27, 28]. Therefore myoviruses are the preferred viruses for elimination of *Listeria* in food industry.

The elimination of *Listeria* by phages has to occur before the bacteria enter the human body. Once Listeriosis is acute, phage therapy is not effective, because *Listeria* multiplies as an intracellular pathogen and cannot be reached by phages. Phage therapy is currently forbidden in most countries anyway [13]. Therefore, prevention of the disease by anti-*Listeria* control in food and food processing environments is highly desirable. Bacteriophages, which are used for biocontrol purposes should be virulent, have a broad host range and infect and kill as many targets as possible. They must be unable to transduce and should be able to be propagated on a non-pathogenic production strain [13]. This eliminates the possibility of *lysogenic* conversion, which means generation of *lysogenic* hosts that would be immune to superinfection by the same virus. Thereby acquirement of new, phage encoded, properties is prevented [29].

1.5 *Listeria* phage P100

P100 is commercially available under the trade name Listex™ P100. It is a *lytic* phage for intervention of *L. monocytogenes* contaminations in food production environments and is produced by EBI Food Safety (Wageningen, Netherlands). The phage P100 was originally isolated from sewage of dairy plants [25]. It belongs to the family of *Myoviridae*, which are composed of phages that have contractile tails for injection of their genomic double-stranded DNA into the bacterial host cell. The genome of P100 (131kbp) consists of 174 open reading frames (Gene Bank: DQ004855) [5, 30]. The molecular weight of one P100 particle is about 1.2 x 10^8 Dalton and its length is approximately 300nm.

P100 infects Gram-positive *Listeria* and is strictly virulent. It is characterized by its broad spectrum towards *Listeria* strains and has been confirmed as Generally Regarded as Safe (GRAS) by the US Food and Drug Administration (FDA).

Listex™ P100 is produced in a fermentation process using non-pathogenic *Listeria innocua* for propagation. Subsequently, bacteria and cell debris are removed by multiple filtration steps. Listex™ is a water-formulated product that contains P100 phages with a concentration of 2 x 10^{11} PFU/ml. The shelf life of Listex™ P100 is indicated to be six months [23, 31].

According to the MICREOS Product Data Sheet, Listex™ P100 should have the ability to, after becoming the dominant phage in a food matrix or food processing environment, bring down the number of viable *L. monocytogenes* cells in the food processing environments [23]. According to EFSA (2012), Listex™ P100 is theoretically most effective within the limits of *L. monocytogenes* growth [31]:

- Temperature: optimum 30 °C (1°C – 35°C)
- PH: optimum 7.7 (5.5 – 9.5)
- Water activity: optimum 0.99 (minimum 0.92)
- Sodium chloride: high tolerance up to saturated solutions

1.6 Effectiveness of phage treatment

It has been demonstrated that targeted elimination of specific pathogens can be very effective under certain conditions. In general terms, the higher the ratio of phages to bacterial cells, the greater is the reduction. However, the most currently available phage based decontamination treatments can only reduce contamination levels, but not completely eliminate pathogens from foods or food processing environments [32]. Remaining bacteria may become bacteriophage insensitive mutants by losing the phage receptor, acquiring restriction modification systems that degrade viral nucleic acids or by gene mutations which affect phage replication [22]. The frequency of these mutations varies according to bacteriophages, conditions of application, target bacteria and environmental conditions [32].

The same applies to the persistence of bacteriophages in food and in food processing environments. In general, bacteriophages are able to remain in the environment for long time periods due to their lack of metabolism [32].

Nevertheless, applications in phage treatment were not always successful. The efficacy of treatment with bacteriophages against pathogenic bacteria depends on many different factors, such as the phage-bacteria ratio, physical and chemical factors (salinity, pH, detergents, temperature), phage neutralization and tolerance against phages [33]. Furthermore, the data obtained from *in vitro* experiments cannot be directly applied to *in vivo* situations, nor can data, derived from studies with one phage, be transferred to another phage. Critical parameters, which have influence on phage therapy, are adsorption rate, initial phage dose, clearance rate of phage particles from body fluids or phage-neutralizing antibodies, burst size and the latent period [34, 35]. The key factors that affect efficacy of phage treatment are shown in Figure 1.

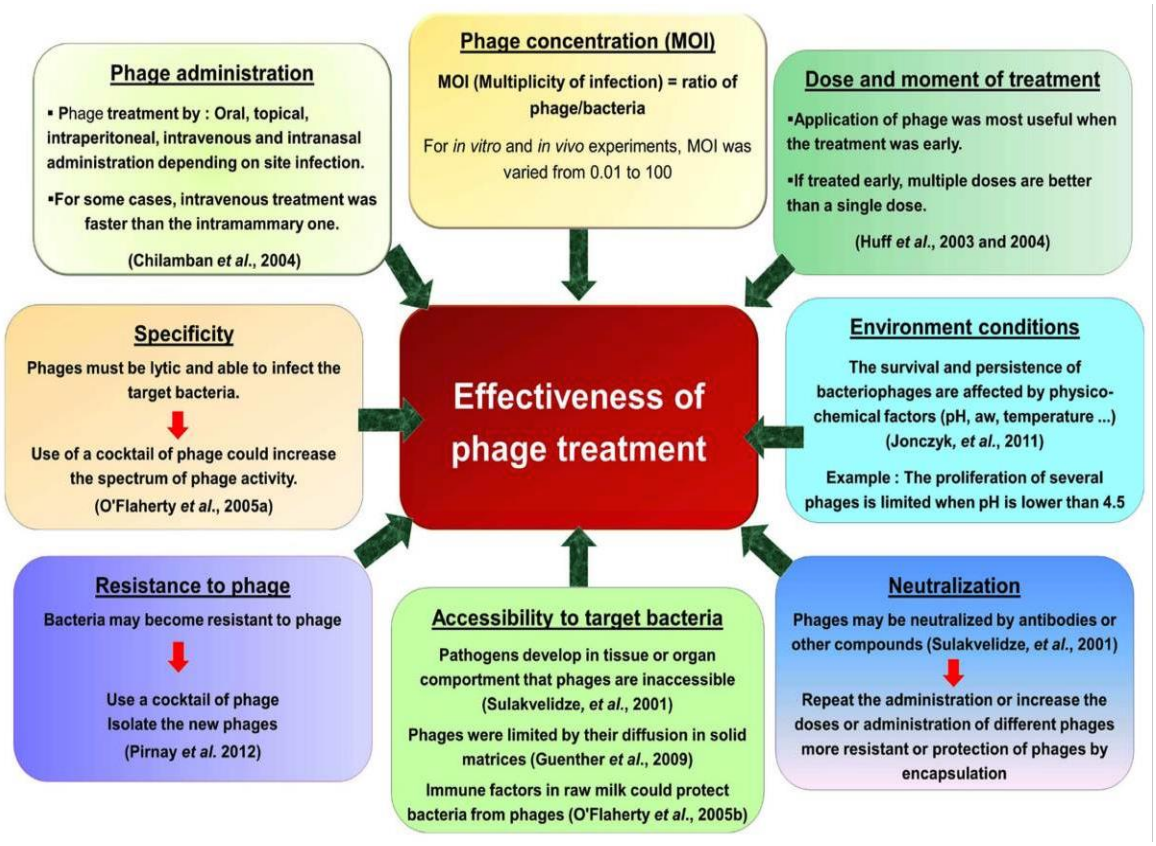


Figure 1: Factors affecting the effectiveness of phage use against pathogenic bacteria [22]

1. Phage – bacteria ratio: There are two possibilities to use bacteriophages, one active and one passive approach. The active approach is based on the addition of small amounts of phages, which therefore require phage replication. The passive approach is predicated on addition of as many bacteriophages into the system as required to ensure that all target bacteria are lysed in a short time period [36]. The passive approach is more efficient than the active approach. The phage-bacteria ratio is expressed as MOI (multiplicity of infection), which is the number of virus per cell during infection. Generally, for the passive approach a MOI of 100 is used [22].

2. Environmental conditions: Phages can be tolerant against unfavourable physico-chemical factors such as salinity, pH, temperature and ions. This is important for consistency of phage preparations but can be a problem for industry when maintenance of bacterial strains is required, regarding to starter

cultures [33]. However, low pH (pH < 5) can reduce phage titre of some phages like T4 (*Myoviridae* family) and high ionic strength can increase aggregation of phages [37]. For example exposure of T4 to pH 5.0 medium for one hour causes loss of complete activity.

3. Dose and moment of treatment: Several studies ([38, 39]) show that multiple doses are more effective than a single dose and early phage administration (up to five hours post infection) is much more efficient than late treatment [22].

4. Resistance to phage: The first step of phage infection is adsorption on bacterial surface by receptor proteins. If the bacterium loses a cell wall receptor, or if the cell wall receptor is modified, it becomes resistant to the phage. Bacteria may also obtain restriction modifications, which can inhibit specific steps of cell metabolism, resulting in inability of bacteriophages to proliferate [40, 41]. Resistance may also be caused by gene mutations, which affect pathways essential for phage replication or assembly [22].

This study focuses on the influence of environmental conditions on bacteriophage-host interactions. Different conditions referred to different food processing environments, including pH, temperature, salt concentration, detergents such as Lutensol a non-ionic detergent or SDS and smear water were used to determine influence on effectiveness of bacteriophage treatment to eliminate *L. monocytogenes*. Other factors, which are relevant in this study and possibly could influence the effectiveness of phage treatment, are phage concentration, the dose and moment of treatment and bacterial resistance to phage infections.

1.7 Aims of this study

The aim of the study was to investigate the influence of chemical and physical factors on the survival and effectiveness of the phage P100 for control of *L. monocytogenes* in dairy plants. Moreover previously detected P100 insensitive isolates were characterized and the formation and development of resistances was studied. In addition the use of the phage P100 for lysis of *L. monocytogenes* and subsequent detection of them by PCR was tested. The following aims were set:

1. Characterization of insensitive *Listeria monocytogenes* isolates obtained from dairy plants. Pulsed Field Gel Electrophoresis (PFGE), Multiplex PCR and determination of efficacy of plating (EOP [42]) were used for serotyping and characterization of the insensitivity.

2. Determination of the influence of temperature, pH, salt and detergents on phage-host interactions and efficacy of phage treatment. Adsorption tests, cell forming unit (CFU) and plaque forming unit (PFU) determinations can give an assessment over effectiveness of phage treatment.

3. Determination of the stability of P100 in different food processing environments of dairy companies was tested by PFU determinations during a time period of 117 days.

4. Screening of newly formed resistances was performed by CFU determinations, followed by cross-streak tests and qPCR.

2 Materials

Phages	Source
Listex P100	MICREOS Food Safety, Netherlands
***Listeria* phage vB_LmoM_AG20**	Obtained by Prof. Griffith, University of Guelph ON, Canada
Bacterial strains	
***Listeria monocytogenes* EGDe**	ATCC BAA-679 (EGD-e)
***Listeria monocytogenes* L57 53.54**	
***Listeria monocytogenes* L93 45.46**	
***Listeria monocytogenes* L97 7.8**	Listeria collection of the Institute of Milk Hygiene
***Listeria monocytogenes* L99 39.40**	Milk Technology and Food Science
***Listeria monocytogenes* L99 65.66**	(Vetmeduni Vienna, Austria)
***Listeria monocytogenes* L102 71.72**	
***Listeria monocytogenes* R145**	
***Listeria monocytogenes* ΔprfA**	Δ-*prf*A: cloned strain, *prf*A gene was knocked out, +IAC *L. monocytogenes* EGDe (DMSZ)[43]
Kits	
Nucleospin Nucleic Acid Purification Kit	Macherey-Nagel, Germany
Pure Link viral DNA Mini Kit	Invitrogen, USA
Standards	
Gene Ruler 1kb DNA ladder	Thermo Fisher Scientific, USA
Gene Ruler 50bp DNA ladder	Thermo Fisher Scientific, USA
Enzymes	
***Apa*I**	Thermo Fisher Scientific, USA
***Asc*I**	Thermo Fisher Scientific, USA
Lysozyme	Sigma, Germany
Platinum Taq-Polymerase	Thermo Fisher Scientific, USA

Proteinase K	Thermo Fisher Scientific, USA
XbaI	Thermo Fisher Scientific, USA

Chemicals and reagents

Agar	Merck, Germany
Agarose, universal	peqGOLD, USA
Calcium chloride	Merck, Germany
Carrier RNA	Thermo Fisher Scientific, USA
Chelex	BioRad, USA
dNTP-Set (ATP, CTP, GTP, TTP)	Thermo Fisher Scientific, USA
Ethanol	Sigma, Germany
Ethidiumbromide	Thermo Fisher Scientific, USA
Ethylenediaminetetraacetic acid (EDTA)	Merck, Germany
Glycerol	Thermo Fisher Scientific, USA
Loading Dye	Thermo Fisher Scientific, USA
Lutensol AO-7	BASF, Germany
Magnesium chloride ($MgCl_2$)	Sigma, Germany
Magnesium sulfate ($MgSO_4$)	Merck, Germany
Methanol	Roth, Germany
Peptone	Merck, Germany
PCR buffer 10x	Thermo Fisher Scientific, USA
Restriction buffer 1x	Thermo Fisher Scientific, USA
Sodium chloride (NaCl)	Merck, Germany
Sea Kem Gold Agarose	VWR International GmbH, Germany
Sodium dodecylsulfate (SDS)	Thermo Fisher Scientific, USA
Soytone	Merck, Germany
SYBR Safe	Thermo Fisher Scientific, USA
Tris(hydroxymethyl)-aminomethane (TRIS)	Merck, Germany
TRIS-hydrochloride (TRIS-HCl)	Merck, Germany
Trizma base	Merck, Germany
Tryptone	Merck, Germany

Yeast Extract	Merck, Germany

Buffer, media and solutions	Composition
Agar Listeria acc. to Ottaviani & Agosti (ALOA)	Meat peptone, 18 g/l Tryptone, 6 g/l Yeast Extract, 10 g/l Sodium pyruvate, 2 g/l Glucose, 2 g/l Magnesium glycerophosphate, 1 g/l Magnesium sulphate, 0.5 g/l Sodium chloride, 5 g/l Lithium chloride, 10 g/l Disodium hydrogen phosphate anhydrous, 2.5 g/l 5-bromo-4-chloro-3-indyl-ß-D-glucopyranoside, 0.05 g/l Agar, 13.5 g/l
ALOA Selective Supplement	Nalidixic acid, 20 mg/l Ceftazidime, 20 mg/l Cycloheximide, 50 mg/l Polymyxin B, 76700 IU/l
ALOA Enrichment Supplement	L-α-fosphatidylinositol, 2 g/l
Cell lysis buffer	50mM TRIS 50mM EDTA pH 8.0
Chelex solution	0.01M TRIS-HCl 0.25g Chelex 9.5ml H_2O
Fraser broth	Meat peptone, 5 g/l Tryptone peptide digest of casein, 5 g/l Beef extract, 5 g/l

	Yeast extract, 5 g/l
	NaCl, 20 g/l
	Disodium hydrogen phosphate x H2O, 12 g/l
	Potassium dihydrogen phosphate, 1.35 g/l
	Aesculin, 1 g/l
	Lithium chloride, 3 g/l
	Sodium salt of nalidixic acid, 0.02 g/l
PALCAM basic medium	Columbia blood agar basis, 39 g/l
	Glucose, 0.5 g/l
	Mannitol, 10 g/l
	Eskulin, 1 g/l
	Ammonium iron (III)-Citrate, 0.5 g/l
	Lithium chloride, 15 g/l
	Phenol red, 0.08 g/l
	Agar, 2 g/l
PALCAM additional antimicrobial supplements	Acriflavin-HCl, 0.005 g/l
	Polymyxin-B-Sulfate, 0.01 g/l
	Ceftazidim, 0.008 g/l
SM buffer	100mM NaCl
	8mM MgSO4 x 7H2O
	50mM TRIS-HCl
TE buffer	10mM TRIS, pH 8
	1mM EDTA
TRIS-Borate-EDTA-buffer (TBE)	TRIS Base, 10.8 g/l
	Boric Acid, 5.5g/l
	EDTA-Na2, 0.7g/l
Tryptone Soy Agar (TSA)	Casein peptone 15 g/l
	Soy peptone, 5 g/l
	NaCl, 5 g/l
	Agar 15g/l
Tryptone Soy Bouillon + Yeast (TSB+Y)	Casein peptone 17g/l

	Dipotassium hydrogen phosphate, 2.5 g/l
	Glucose 2.5g/l
	Soya peptone, 3 g/l
	NaCl, 5 g/l

PCR primer	Sequence (5' - 3')
Lip1 Forward	GATACAGAAACATCGGTTGGC
Lip2 Reverse	GTGTAATCTTGATGCCATCAGG
lmo0737 Forward	AGGGCTTCAAGGACTTACCC
lmo0737 Reverse	ACGATTTCTGCTTGCCATTC
lmo1118 Forward	AGGGGTCTTAAATCCTGGAA
lmo1118 Reverse	CGGCTTGTTCGGCATACTTA
ORF2110 Forward	AGTGGACAATTGATTGGTGAA
ORF2110 Reverse	CATCCATCCCTTACTTTGGAC
ORF2819 Forward	AGCAAAATGCCAAAACTCGT
ORF2819 Reverse	CATCACTAAAGCCTCCCATTG
prs F	GCTGAAGAGATTGCGAAAGAAG
prs R	CAAAGAAACCTTGGATTTGCGG

PCR probes	
Pluc 5 probe	HEX-TTCGAAATGTCCGTTCGGTTGGC-BHQ
LIP probe 2	FAM-CAGGATTAAAAGTTGACCGCA-BHQ

3 Methods

3.1 Bacterial culture

To establish standardised conditions and bacteria in the log phase, for several experiments 9 ml of TSB+Y were inoculated with the respective *L. monocytogenes* strain or isolate and agitated over night at 37 °C. One ml of this over-night culture was added to 9 ml of fresh TSB+Y. After 3 hours, the optical density was measured at 600 nm (OD_{600}) and adjusted to 0.6.

3.2 DNA Isolation

3.2.1 Viral and bacterial DNA for qPCR standards

Purified viral and bacterial DNA was needed as a standard for qPCR. Viral DNA was isolated by using the Invitrogen pure link viral DNA Mini Kit and bacterial DNA was isolated by using the Nucleic Acid and Protein Purification NucleoSpin® Kit for bacterial DNA.
The isolation procedure was carried out according to the instruction manuals.

3.2.2 Handling of DNA for Multiplex PCR template

To obtain DNA, which can be used as template for Multiplex PCR, one bacterial colony grown on TSA was added directly to the mastermix and the initial step at 94 °C was extended to 10 min to obtain sufficient lysis of bacterial cells.

3.3 Determination of DNA concentration

To set the standard for PCR amplification, DNA concentration was determined with the Invitrogen Qubit® 2.0 Fluorometer according to the instruction manual (Quick Reference Card).

3.4 Multiplex PCR for differentiation of major *L. monocytogenes* serovars

The polymerase chain reaction (PCR) is a technique to amplify defined DNA regions using specific oligo-nucleotides (primers). The method is based on thermal cycling of repeated heating and cooling for DNA denaturation, annealing and extension and enzymatic replication of the DNA.

Multiplex polymerase chain reaction (Multiplex PCR) is a modification of the PCR in which two or more targets can be amplified in the same reaction by using more than one pair of primers. It can be used to analyse gene deletions and mutations, for quantitative analysis or reverse transcription PCR [44].

In this work a multiplex PCR assay was carried out in order to distinguish between the major *L. monocytogenes* serovars 1/2a and 3a, 1/2b and 3b, 1/2c and 4b. The marker genes for this multiplex PCR assay were *lmo0737* and *lmo1118*, identified in the sequenced *L. monocytogenes* EGDe strain, as well as ORF2819 and ORF2110, identified in a partial sequence of a the 4b strain (CLIP 80459) [7]. The *prs* gene, which is specific for all strains of the genus *Listeria*, was used as an internal amplification control.

In this assignment the multiplex PCR reaction was composed as follows:

Table 3: PCR reaction with final concentrations of all components in a single reaction tube

Serovar PCR	Final conc.	Stock conc.	1 sample [µl]
Aqua dest.			12.0
PCR buffer	1x		2.5
$MgCl_2$	2 mM	50 mM	1.0
lmo0737-F	1 µM	100 µM	0.25
lmo0737-R	1 µM	100 µM	0.25
ORF2819-F	1 µM	100 µM	0.25
ORF2819-R	1 µM	100 µM	0.25
ORF2110-F	1 µM	100 µM	0.25
ORF2110-R	1 µM	100 µM	0.25
lmo1118-F	1.5 µM	100 µM	0.375
lmo1118-R	1.5 µM	100 µM	0.375
prs-F	0.2 µM	10 µM	0.5
prs-R	0.2 µM	10 µM	0.5
dNTP	0.2 mM	5 mM	1
Taq Polymerase	2 U	5 U/µl	0.3
Total			20
Template			5
Reaction volume 1x			25

The PCR programme was the following:

94 °C 3’; 35 × (94 °C 40’’, 53°C 75’’, 72 °C 75’’); 72 °C 7’

Finally the PCR reactions were analysed with agarose gel electrophoresis.

3.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method to separate DNA and RNA fragments according to their charge. After the PCR reactions, products were analysed with agarose gel electrophoresis to determine the length of the DNA fragments. The concentration of agarose in the gel was 1.5% in 1 x TBE buffer. To melt agarose in buffer, the solution was heated in a microwave until the solution was clear.

Finally SYBR Safe, a dye, which fluoresces under UV light after incorporation into the DNA, was added to the gel solution. For 100 ml gel solution, 3.5 µl SYBR Safe were added. The PCR reaction was mixed with 6x loading dye (1 µl loading dye + 5 µl sample), loaded on the gel and the electrophoresis was carried out in 1 x TBE at 120 V. Gene Ruler 1 kb DNA ladder was used as marker. The gels were photographed with the BIORAD Gel Doc 2000 Imaging System and the software Quantity One 4.3.1.

3.6 Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis (PFGE) is an effective molecular typing method based on restriction fragment length polymorphism of bacterial DNA. Bacterial DNA is digested with restriction enzymes and resulting DNA fragments are separated by agarose gel electrophoresis [10]. In contrast to conventional agarose gel electrophoresis, PFGE allows separation of much DNA fragments. In PFGE, the direction of the current is altered at regular intervals resulting in a higher level of fragment resolution.

Pulsed field gel-electrophoresis was performed according to the most actual Centers of Disease Control and Prevention (CDC) Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes* [45]. For restriction analysis of *L. monocytogenes*, the CDC recommends to use *AscI* as the primary and *ApaI* as secondary enzyme.

The restriction enzyme master mix was prepared according to the following tables:

*Asc*I:

Reagent	µl/ Plug Slice
Aqua dest.	175.5
10x Restriction Buffer	20
BSA (10mg/ml)	2
*Asc*I (10U/µl)	2.5
Total Volume	200

*Apa*I:

Reagent	µl/ Plug Slice
Aqua dest.	177.5
10x Restriction Buffer	20
BSA (10mg/ml)	2
*Apa*I (50U/µl)	0.5
Total Volume	200

For electrophoresis the following conditions were selected on a CHEF-DR III system:

Initital switch time: 4 s

Final Switch time: 40 s

Voltage: 6 V

Included Angle: 120°

Run time: 19 hours

For analysis the BioNumerics software program (Applied Maths, Belgium) was used.

3.7 PFU Determination and efficiency of plaquing

Viral plaque assays can be used to determine the number of plaque forming units (PFU) in a virus sample, which is one measure of virus quantity.

PFU determinations were done using the Double Agar Overlay Plaque Assay [46]. The double agar method, in which the phage, susceptible cells, and agar are combined prior to pouring the overlay, enables better mixing and greater uniformity of plaques than in plating methods, which do not utilise overlays. Plaque size is also increased because of greater rates of phage diffusion in the overlay. Diffusion is increased because the overlay contains a lower concentration of agar than the basal medium [47].

In short, 100 µl of the phage solution (10-fold dilutions in SM buffer) and 200 µl of log phase *L. monocytogenes* EGDe were mixed. Then 300 µl of the solution were mixed with 3 ml soft agar overlay (TSB supplemented with 0.4 % Agar and 10 mM $CaCl_2$) and immediately poured on TSA plates. After 30 min at room temperature the plates were incubated at 37 °C over night. The plaques were counted and PFUs / ml were calculated. All PFU determinations were done in duplicate with at least two different dilutions.

The efficiency of plaquing (EOP) is defined as the plaque count obtained under a certain set of conditions, relative to the plaque count obtained under standard conditions and a standard host strain. As a control and standard host strain, the strain with the highest plaque count is used.
In this assignment the EOP was determined to analyze the susceptibility of different insensitive *L. monocytogenes* isolates to P100 at different temperatures. The procedure was already described in Kim et al (2009) [42].

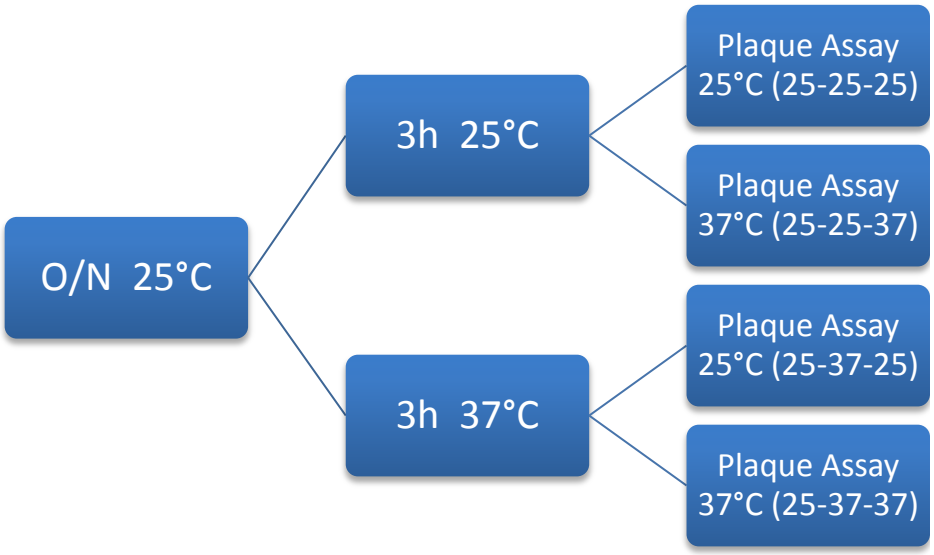


Figure 2: Procedure of EOP determination

Strains and isolates, which were analysed with this procedure, were *L. monocytogenes* EGDe as a control and the insensitive plant isolates *L. monocytogenes* L57, L93, L97, L99 39.40, L99 65.66, L102, R145 and RΔprfA. An overnight culture at 25 °C was inoculated. The day after, 1 ml of this culture was added to new TSB medium and incubated for 3 hours at 25 °C (25-25) or 37 °C (25-37). PFU determination was carried out and also plates were incubated at 25 °C or 37 °C (Figure 2). The number

of plaques was counted and the EOP was calculated. These results were used for further comparison with results obtained using 37 °C overnight culture (37-37-37).

3.8 Passaging of P100 virus particles

Passaging was done to evolve bacteriophages and to determine the influence of infection-cycles and mutations on sustainability of phages during application. Passaging involves transferring a phage to a new bacterial culture. Repeated serial passages were used to accumulate mutations.

Therefore a plaque from a former Plaque-Assay was cut out of the plate and put in an 1.5 ml Eppendorf tube. The separated plaque was covered with SM buffer and incubated for 3 hours at 37°C and 400 rpm. After shaking, this mixture was centrifuged for 5 min at 12000 rpm and filtrated with a 0.22 µm filter. The filtrate was used to infect a new *L. monocytogenes* EGDe host strain and afterwards stored at -20 °C.

3.9 Adsorption Tests

To investigate binding and replication characteristics of P100 to and in *L. monocytogenes,* adsorption tests were performed under different environmental conditions. The tested variable conditions were salt concentration, pH, concentration of detergents and temperature.

For adsorption tests, log phase bacteria (OD_{600}: 0.6) were used. 200 µl *L. monocytogenes* EGDe were infected with 200 µl of a 1000-fold dilution of Listex^{TM} P100 (approximately 6×10^7 PFU/ml in SM buffer supplemented with 10 mM $CaCl_2$). Concentration of phages was about 1 $\log_{10}$ lower (MOI = 10) than concentration of bacteria (approximately 5×10^8 CFU / ml) to make sure that there are enough bacterial cells for the phages, which can be used for attachment. To test the binding-ability of the phage P100 to EGDe, the infection was stopped by adding ice-cold TSB and centrifuging for two min at 8000 rpm after 0, 10, 20, 30, 40, 50 and 60 min as described in Wendlinger et al. (1996) [48]. To determine the replication-ability of P100 in EGDe the number of PFUs per ml supernatant was determined 0, 1, 3 and 6 hours after infection.

Phages in the supernatant, which did not attach to the bacteria, were further diluted in SM buffer and quantified using the Double Agar Overlay Plaque Assay.

For determination of adsorption characteristics at different salt concentrations, the log phase bacteria were centrifuged at 8000 × g for 5 min and then resuspended in same amount of TSB medium with a specific salt concentration (0, 0.05, 0.1, 0.5, 1 and 2 M). The dilution of Listex^{TM} P100 was also done in the same salt-containing medium. The experiment was continued as described above.

This procedure is congruent for determination of adsorption characteristics with different pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) and different detergent concentrations (Lutensol AO7 and SDS – 0, 0.1, 0.25, 0.5, 1.25, 2.5, 5%).

3.10 Influence of environmental conditions on efficacy of phage treatment

To analyse the influence of environmental and chemical conditions on efficacy of phage treatment, the *L. monocytogenes* strain EGDe was infected with Listex^{TM} P100. This test was done for different salt- (0, 0.05, 0.1, 0.5, 1 and 2 M) and detergent concentrations (0, 0.1, 0.25, 0.5, 1.25, 2.5 and 5% Lutensol AO7 / SDS) as well as at different pH values (3 - 11) in TSB medium and in fraser broth and smear water.

As control 100 µl log phase EGDe cells (OD_{600}: 0.6) were added to 1.8 ml of TSB medium containing the salt or detergent concentration that should be tested (control). A second Eppendorf tube with 1.8 ml of TSB was filled with 100 µl EGDe and 100 µl of a 1:10 dilution of P100 (diluted in SM buffer). This corresponds to 2×10^{10} PFU/ml (MOI = 10). 200 µl of this mixture were added to another 1.8 ml of TSB, making a 1:100 dilution of Listex^{TM} P100 (2×10^{9} PFU/ml; MOI = 10) and EGDe.

200 µl of these dilutions were used for a 96-well microtiter plate, shown in Table 4, and for measuring OD_{600} for 24 hours every single hour.

Table 4: Pipet scheme of 96-well plate representative for different salt (0 - 2 M) PFU and *L. monocytogenes* concentrations, E: EGDe, 1:10 and 1:100 (MOI = 10): PFU / *Listeria* dilutions, b: blank, each well 200µl

	1	2	3	4	5	6	7
A	2 M E	2 M E	2 M 1:10	2 M 1:10	2 M 1:100	2 M 1:100	2 M b
B	1 M E	1 M E	1 M 1:10	1 M 1:10	1 M 1:100	1 M 1:100	1 M b
C	0.5 M E	0.5 M E	0.5 M 1:10	0.5 M 1:10	0.5 M 1:100	0.5 M 1:100	0.5 M b
D	0.1 M E	0.1 M E	0.1 M 1:10	0.1 M 1:10	0.1 M 1:100	0.1 M 1:100	0.1 M b
E	0.05 M E	0.05 M E	0.05 M 1:10	0.05 M 1:10	0.05M 1:100	0.05M 1:100	0.05 M b
F	0 M E	0 M E	0 M 1:10	0 M 1:10	0 M 1:100	0 M 1:100	0 M b

The measurement was done with a TECAN Infinite® 200 PRO microplate reader (Tecan Group, Switzerland).

3.11 Survival of P100 in different environments

To investigate the survival of P100 in the environment, 5×10^{10} PFU/ml were added to two different kinds of smear water (two different samples obtained from a dairy company) and to SM-buffer as control. Before adding phages, smear water was either autoclaved, centrifuged for 5 min at 8000 × g in order to remove cells, or enriched with *L. monocytogenes* EGDe (1 ml of 5×10^8). Additionally the untreated smear water was tested. The samples were stored at 4 °C and 10 °C. For a total period of 117 days, every 10 days, the PFU were determined.

3.12 Plate count method

Colony forming unit (CFU) is a unit to quantify microorganisms in a sample. The intention of plate counting is to determine the number of cells, on the basis of their ability to generate colonies under specific conditions of medium, temperature and time. The plate count method was carried out according to Fister et al. (2015) [49].
Decimal dilution series were prepared and spread out on TSA+Y plates with a spatula. Plates were incubated for 24 - 48 hours and CFU were determined by multiplying the counted colonies with their analogous dilution step.

3.13 Influence of temperature on long-term efficacy of phage treatment

To examine the influence of temperature on efficacy of phage treatment, 500 µl of a log phase *L. monocytogenes* EGDe culture was incubated with the same volume of Listex™ P100 (2×10^{10} and 2×10^{9} PFU/ml) for 30 min at room temperature. Then 5 ml TSB was added and stored at 4 °C, 10 °C and 20 °C. Following this, the number of bacteria was determined weekly over a time period of 17 weeks, using the plate count method and compared to non-infected control of the same isolate (treated with SM buffer instead of Listex™ P100). All plating was performed in duplicates on TSA with at least two different dilutions and incubation was over night at 37 °C.

3.14 Screening for newly formed resistances

The screening was done with regrowing culture of experiment 3.13 to determine if regrowth occurred because of newly formed resistances. It was done by cross streak tests as follows: 50 µl of undiluted Listex™ P100 (2×10^{11} PFU/ml) were added in form of a streak on a TSA+Y plate. After drying, the streak was crossed with log phase culture of the regrowing *L. monocytogenes* strains. *L. monocytogenes* EGDe was used as control strain. Plates were incubated over night at 37 °C. Resistant isolates, which could grow on areas treated with P100, were plated on ALOA and PALCAM plates and qPCR was performed to confirm the insensitive strains were *L. monocytogenes*.

3.15 Quantitative Real time PCR (qPCR)

QPCR is used to amplify and simultaneously detect and quantify a targeted DNA molecule. By using fluorescent probes, the accumulation of the target DNA is monitored and the dye fluoresces only when the double-stranded DNA, obtained from the correct sequence, accumulates [50]. The intensity of the fluorescence emitted during qPCR correlates to the amount of DNA product formed. There is no post-processing necessary because the process is monitored in real-time. This leads to a faster assay in comparison to conventional PCR and to the possibility to directly confirm successful amplification within a reaction. Therefore qPCR represents a promising tool in food pathogen detection [51].

QPCR was carried out to detect or approve *L. monocytogenes* in samples and to investigate the ability of phages to lyse *L. monocytogenes*. It was carried out according to Rossmanith et al. (2006) [52].

Table 5: PCR reaction with final concentrations of all components in a single reaction tube

qPCR prfA	Final conc.	Stock conc.	1 sample [µl]
Aqua dest.			5.95
PCR Buffer	1 x		2.5
$MgCl_2$	3.5 mM	50 mM	1.75
LIP 1	500 nM	5 µM	2.5
LIP 2	500 nM	5 µM	2.5
LIP Probe 2	250 nM	5 µM	1.25
IPC Probe	250 nM	5 µM	1.25
dNTP	200 µM	20 mM	1
Taq Polymerase	1.5 U	5 U	0.3
IPC	250 copies	250 copies/µl	1
Template			5
Total			25
Reaction volume 1x			25

The PCR programme was the following:

94 °C 2’; 45 × (94 °C 15’’, 64 °C 60’’)

3.16 Phage dissociation ability to set free bacterial DNA

In order to determine the ability of P100 phages to lyse *L. monocytogenes* and set free its DNA as an alternative DNA isolation method, qPCR was performed. Therefore *L. monocytogenes* was lysed with phages, by incubating 1 ml of culture (10^9 cells/ml), centrifugation for 5 min at 8000 × g and the pellet was resuspended in 100 µl TSB medium and 100 µl undiluted Listex^{TM} P100. After one hour of incubation at RT, 5 µl of this mixture were used for dilution series for qPCR. To evaluate dissociation efficiency, for comparison cells were also directly taken from culture and DNA was isolated with a Nucleospin kit and with Chelex DNA isolation procedure (3.2). The PCR was carried out as described in 3.15.

4 Results

4.1 Serovar and PFGE type characterization of putative insensitive *L. monocytogenes* strains

Prior to this work, Fister et al. (2015) screened P100 insensitive *L. monocytogenes* isolates in Austrian dairy plants to examine the frequency of naturally occurring resistances before and after approval of Listex^{TM} P100 [49].

To characterize these putative insensitive *L. monocytogenes* strains, Multiplex PCR and PFGE were done.

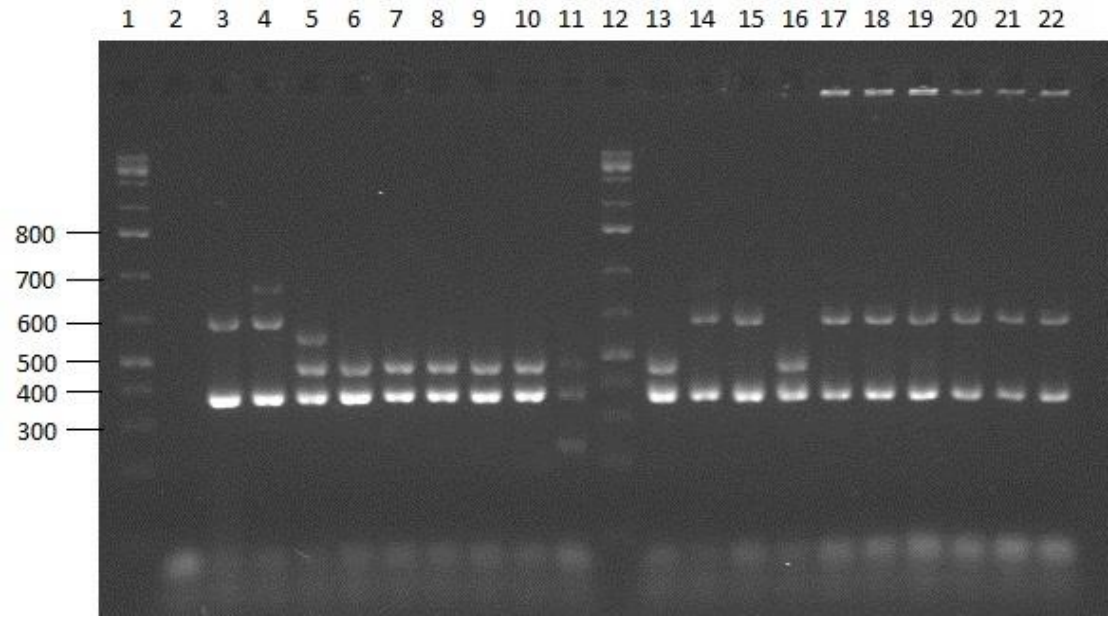


Figure 3: Agarose gel electrophoresis of DNA fragments generated by multiplex PCR;
Lane 1: 1 kb ladder; 2: negative control; 3: positive control (PC) 178; 4: PC 182; 5: PC 20; 6: PC 131; 7-11 and 13-22 *L. monocytogenes* insensitive strains (in order: L49, L50 29.30, L50 31.32, L51 39.40, L51 59.60, L57, ΔprfA, R146, L51 31.32, L93, L97, L98, L99 39.40, L99 65.66, L102); 12: 1 kb ladder

Figure 3 shows the multiplex PCR amplification with *L. monocytogenes* isolates of different serovars. Amplification of the 370 bp *prs* gene fragment, occurring with all isolates confirms that all strains belong to the genus *Listeria*. Isolates, which amplified the *lmo0737* fragment (691 bp), belong to serovars 1/2a or 3a (insensitive isolates RΔprfa, R146, L93 45.46, L97 7.8, L98 17.18, L99 39.40 and L102 71.72).

The ORF2819 fragment was amplified in isolates, which belong to serovars 1/2b or 3b. Isolates L49 77.78, L50 29.30, L50 31.32, L51 39.40, L51 59.60, L57 53.54 and L51 31.32 belong to this group.

To confirm these results and further subclassification of the different serovars, PFGE was done. PFGE has become the standard subtyping method to separate *L. monocytogenes* serovars into distinct groups (1/2a, 1/2b, 1/2c and 4b) and is useful to track the origin of specific insensitive isolates.

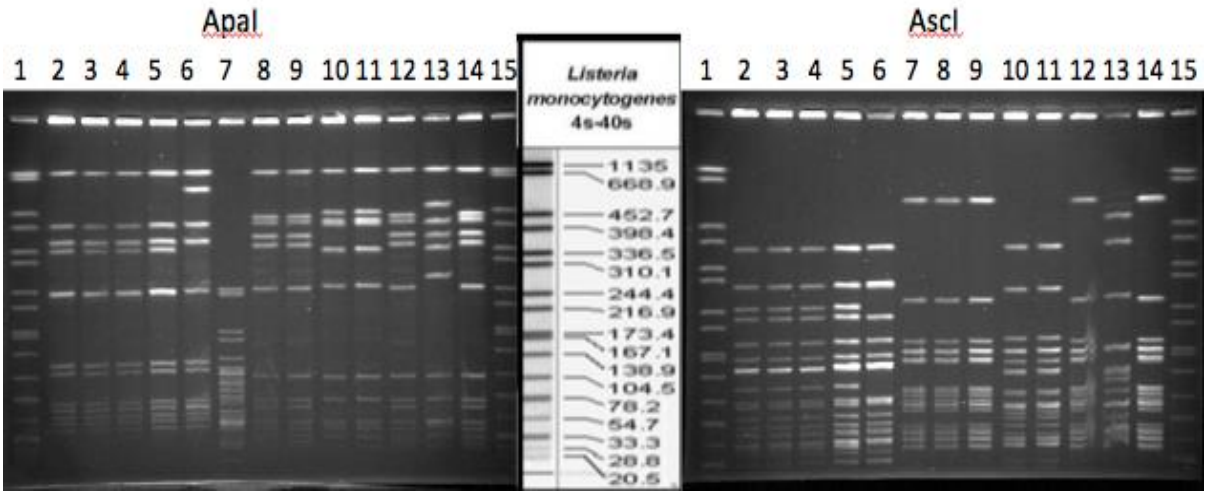
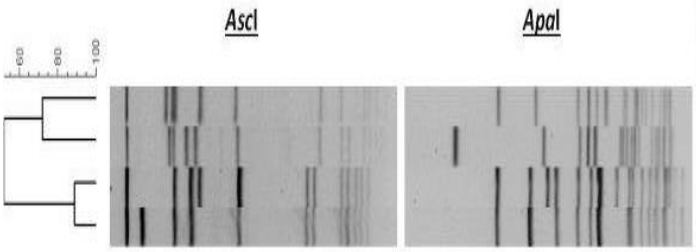


Figure 4: PFGE Cluster for *Apa*I and *Asc*I;
Lane 1,15: marker; 2: L49 77.78; 3: L51 39.40; 4: L51 31.32; 5: L51 59.60; 6: L57 53.54; 7: L93 45.46; 8: L97 7.8; 9: L98 17.18; 10: L99 39.40; 11: L99 65.66; 12: L102 71.72; 13: 145/6/111; 14: 14561/201

Figure 4 shows the distinctive banding patterns of *ApaI* and *AscI* restriction digest. Analysed by means of these banding patterns the different *L. monocytogenes* serovars could be confirmed and a PFGE type could be assigned.



Isolate code	Isolated during	PCR-Serogroup	Processing facility	PFGE profile AscI	PFGE profile ApaI
Wo1	2011	1/2a, 3a	I	I.1	I.1
Wo2	2011-2012	1/2a, 3a	I	I.2	I.2
Ro1	2001	1/2b, 3b	II, III, IV	II.1	II.1
Ro2	2001	1/2b, 3b	V	II.2	II.2

Figure 5: Combined PFGE Cluster using ASCI and ApaI

The two identified serovars 1/2a, 3a and 1/2b, 3b could be further classified into four PFGE types. PFGE types Ro1 (L49 77.78, L51 39.40, L51 31.32, L51 59.60) and Ro2 (L57 53.54) belong to serotype 1/2b, 3b and Wo1 (L99 39.40, L99 65.66) and Wo2 (L93 45.46, L97 7.8, L98 17.18, L102 71.72) belong to serotype 1/2a, 3a (Figure 5).

4.2 Phage susceptibility of insensitive isolates at different temperatures

To investigate phage susceptibility of insensitive isolates, which were subtyped in 4.1, we assessed the relative efficiency of plaquing (EOP) at different temperatures compared to the maximum phage titre observed. Table 6 shows the average EOP values for *L. monocytogenes* infected with P100 at different temperatures. The values were compared with the control strain EGDe. The EOP of the control strain was set to 1 as default value and the other values were based on that to show the extent of reduction.

Table 6: Dependence of *L. monocytogenes* susceptibility to phage infection on the growth temperature of host cell

Strain	Serotype	25-25-25	25-25-37	25-37-25	25-37-37	37-37-37
EGDe	1/2a, 3a	1	1	1	1	1
L57	1/2b, 3b	7.07^{-4}	5.20^{-4}	1.39^{-4}	2.69^{-4}	4.87^{-4}
L57.2	1/2b, 3b	8.04^{-5}	6.24^{-5}	6.26^{-5}	1.33^{-4}	1.79^{-4}
L93	1/2a, 3a	9.24^{-4}	5.88^{-5}	1.17^{-4}	1.60^{-4}	1.12^{-4}
L97	1/2a, 3a	2.96^{-5}	2.02^{-5}	1.07^{-3}	3.00^{-3}	5.60^{-4}
ΔprfA	1/2a, 3a	4.46^{-5}	2.77^{-5}	8.53^{-5}	1.26^{-4}	1.78^{-4}
R145	1/2a, 3a	3.70^{-5}	1.71^{-5}	7.73^{-6}	7.07^{-6}	1.64^{-4}
L102	1/2a, 3a	3.52^{-5}	2.84^{-5}	1.51^{-3}	3.81^{-3}	1.68^{-4}
L102.2	1/2a, 3a	9.40^{-5}	6.16^{-5}	7.77^{-5}	1.10^{-4}	4.59^{-4}
L99 39.40	1/2a, 3a	0	0	5.59^{-6}	8.89^{-6}	0
L99 65.66	1/2a, 3a	0	0	0	0	0

*EOP values represent the average of duplicate determinations from one representative experiment

Isolates L93, L97 and L102 seemed to be slightly more resistant at 25 °C. This could be observed for an even lesser extent for isolate L57. Isolate L99 39.40 grown at 25 °C was completely resistant to the phage P100, but susceptible to phages at 37 °C. This could not be proven with an over-night culture at 37 °C. Thus, temperature during infection and incubation has only little influence on phage susceptibility.

4.3 Passaging Listex™ P100

Passaging was done to propagate bacteriophages and to determine the influence of infection-cycles and mutations on sustainability of phages during application.
After passaging P100 for seven generations, the number of plaques was reduced from generation to generation until no plaques were visible anymore at eleventh generation of P100 phages.

4.4 Adsorption- and replication ability of phages

The very first step of phage infection requires the recognition and adsorption to specific surface receptors. Phage adsorption to *L. monocytogenes* under different environmental conditions was carried out to investigate binding, attachment and replication characteristics. Short time adsorption tests (0 – 60 min) were conducted to investigate binding ability of phages to bacteria by measuring the PFU/ml in supernatant every 10 min.

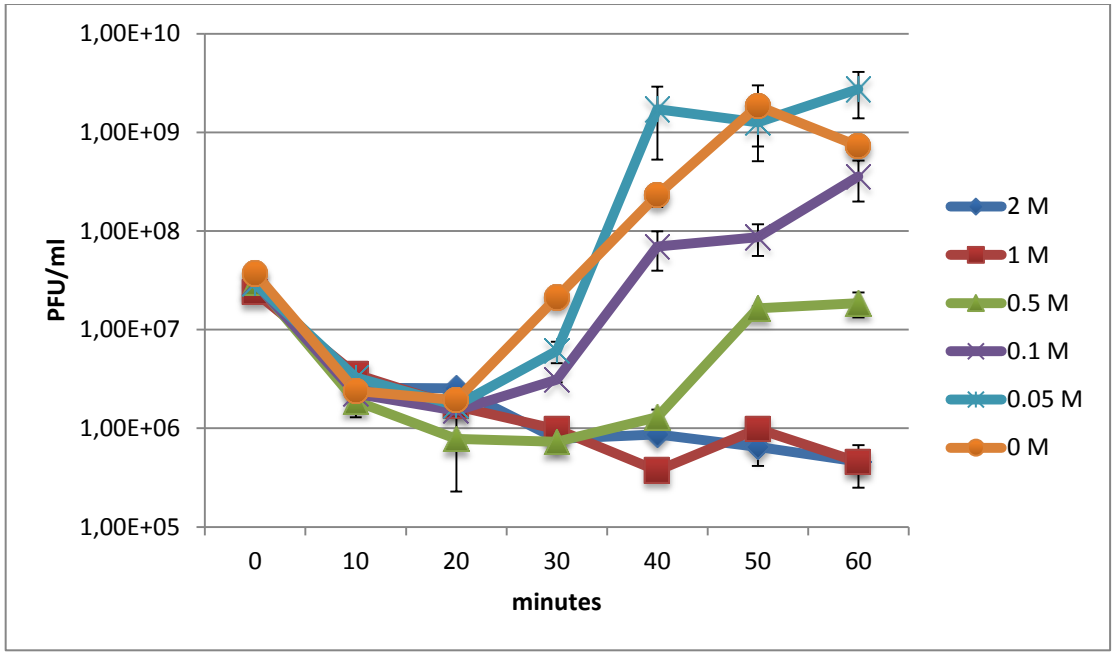


Figure 6: Influence of different NaCl concentrations on binding ability of P100 to *L. monocytogenes*

First the influence of different NaCl − concentrations on binding ability of P100 was tested (Figure 6). The concentration of free phages in supernatant was decreasing about 1.5 log scales for all concentrations within the first 20 min. After 20 min the PFU/ml increased and indicates that phages in low-salt medium (0 – 0.1 M) started to

replicate. In medium with higher salt concentrations the replication started later (0.5 M, 30 min). The higher the salt concentration in the medium was, the later the replication started. Date indicates that no replication occurred in 1 M and 2 M NaCl – medium within 60 min.

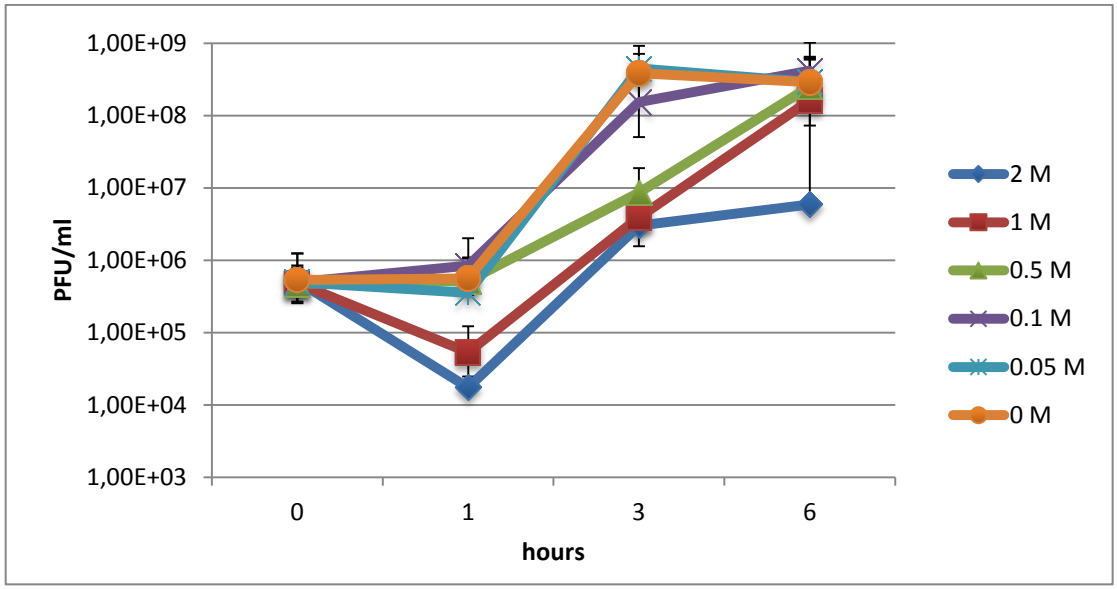


Figure 7: Influence of different NaCl concentrations on replication ability of P100

The replication ability of phages in NaCl medium was investigated by analysing the PFU/ml in supernatant over six hours (Figure 7). After an initial reduction of PFU/ml (Figure 6), the phage concentration in the supernatant was increasing up to 3 $\log_{10}$ within six hours at salt concentrations in the range of 0 M to 1 M, suggesting phage replication. The replication of P100 could also be observed at 2 M after 1 - 3 hours. The binding and attachment ability of P100 to *L. monocytogenes* was also tested for different pH and detergent concentrations and the respective results are shown in Figure 8 and 9.

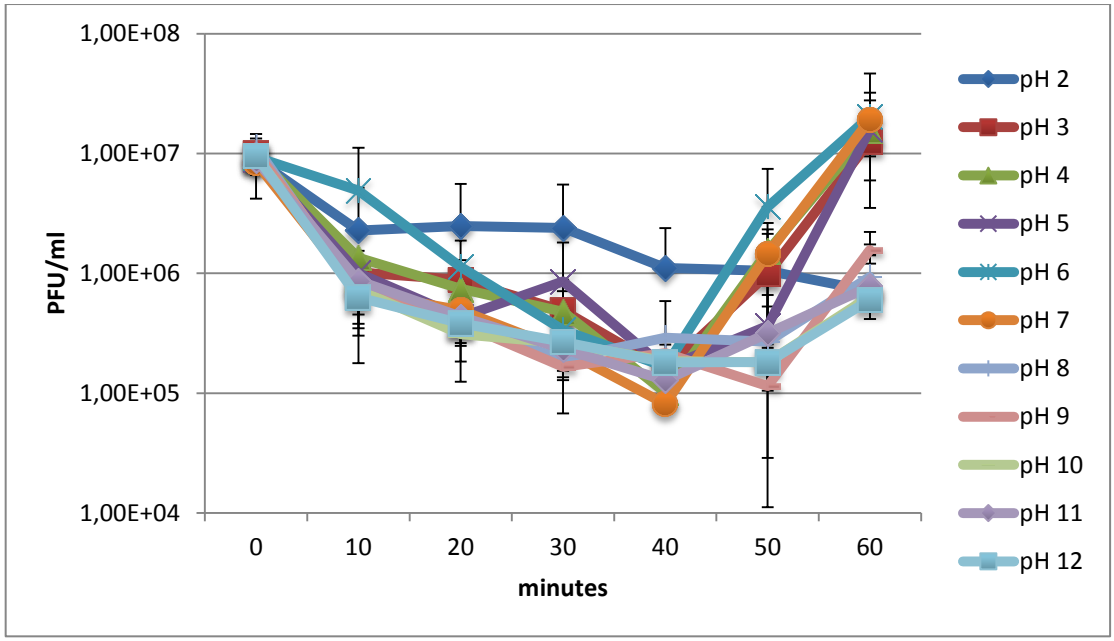


Figure 8: Influence of pH on binding ability of P100 to *L. monocytogenes*

The concentration of free phages in supernatant was decreasing at all tested pH values. An increase in phage concentration afterwards could be observed at pH 3 up to pH 11, which indicates the beginning of phage replication.

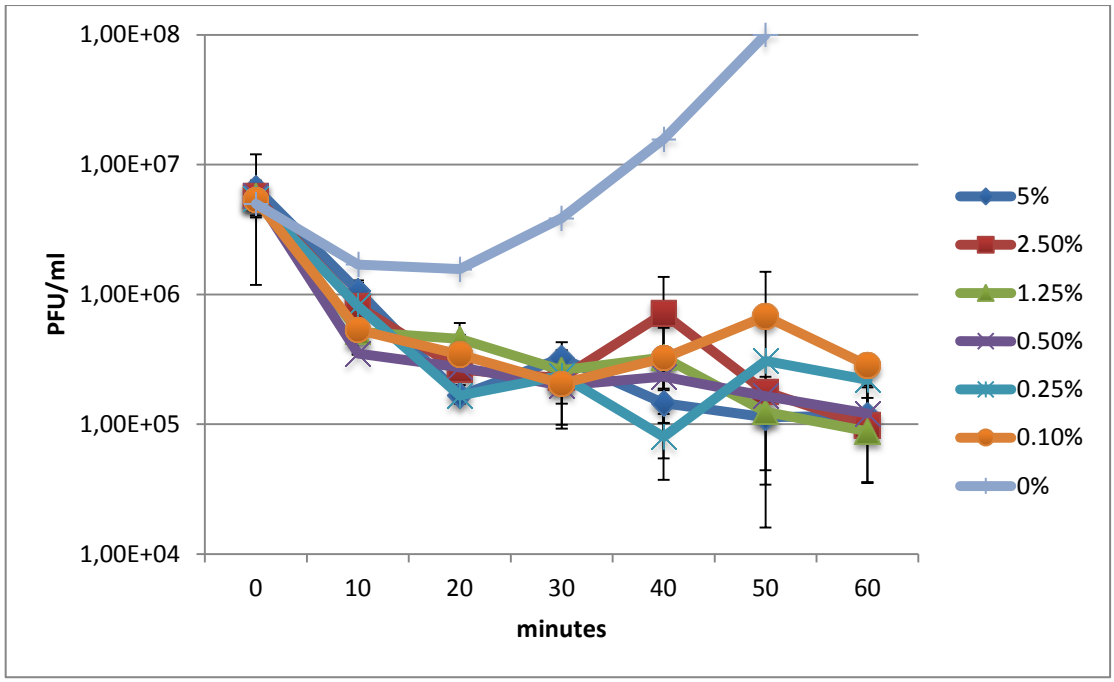


Figure 9: Influence of Lutensol AO7 on binding ability of P100 to *L. monocytogenes*

Binding ability of P100 to *L. monocytogenes* was also tested with different concentrations of the Lutensol AO7 and showed a decrease of free phages of about

1.5 $\log_{10}$ for concentrations in the range between 0.1% up to 5% AO7 within 60 min. The concentration of free phages for the control (0%) decreased only for about 0.5 $\log_{10}$ until replication started after 20 min. The replication start could not be observed for all other concentrations within one hour.

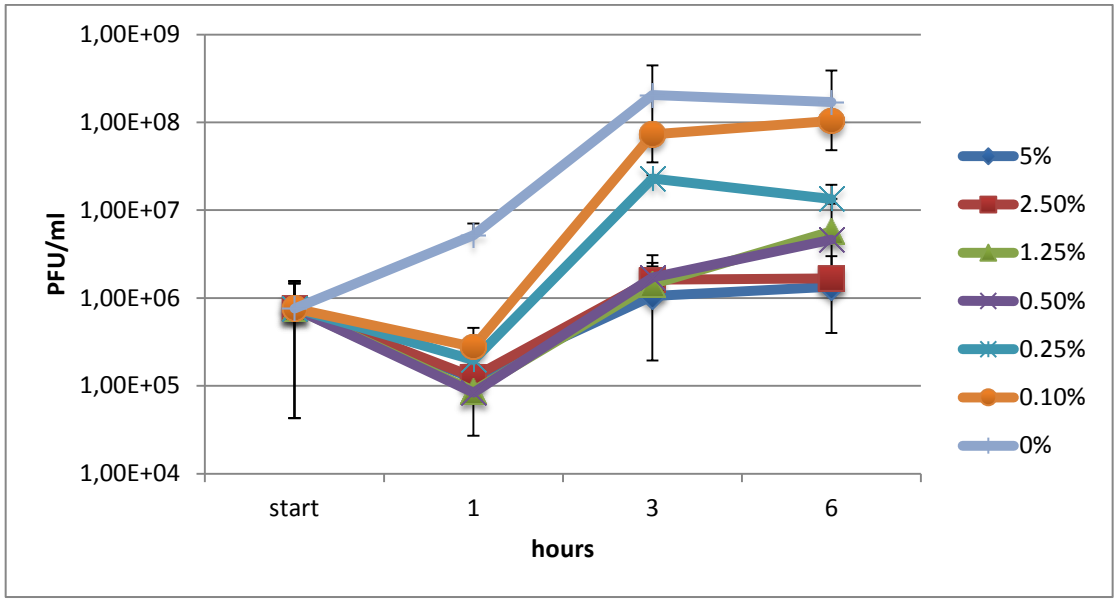


Figure 10: Influence of different Lutensol AO7 concentrations on replication ability of P100

Therefore we tested the influence of these concentrations on replication ability of P100 over six hours (Figure 10). Replication of phages could be observed with all Lutensol AO7 concentrations after 1 - 3 hours. .

Binding and replication ability was also tested for different SDS concentrations. A reduction in PFU/ml within the first 60 min indicates binding of phages. After six hours the amount of phages was below the detection limit. No replication was observed.

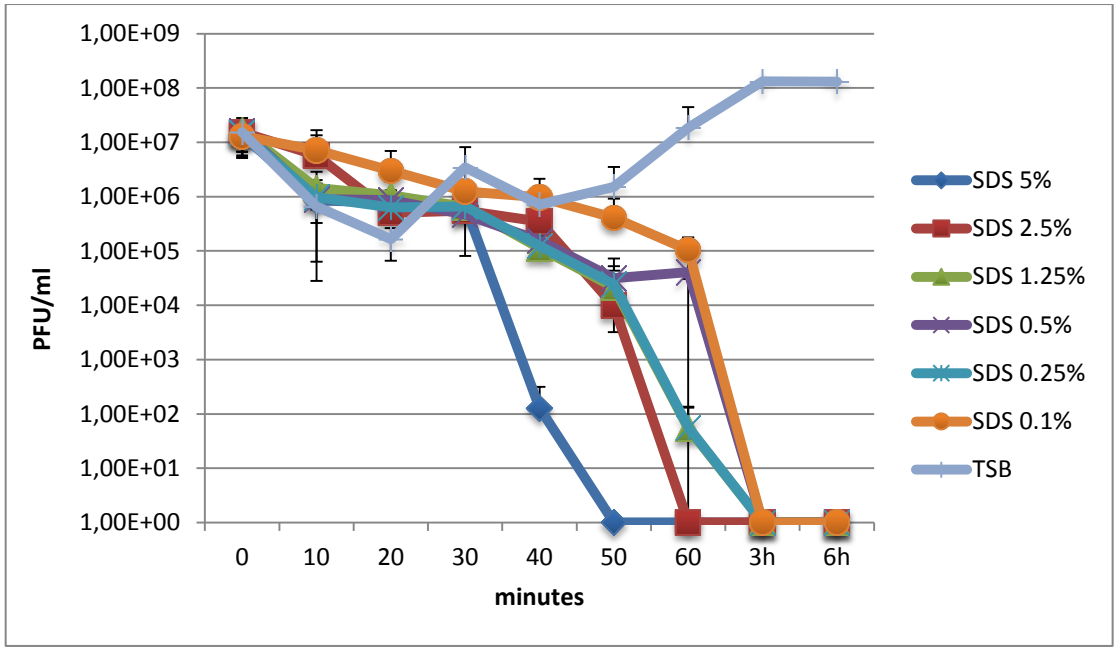


Figure 11: Influence of SDS on binding ability of P100 to *L. monocytogenes*

Phages are often used in food-processing enviroments and therefore we tested the binding ability in different kinds of smear water (SW, Figure 12).

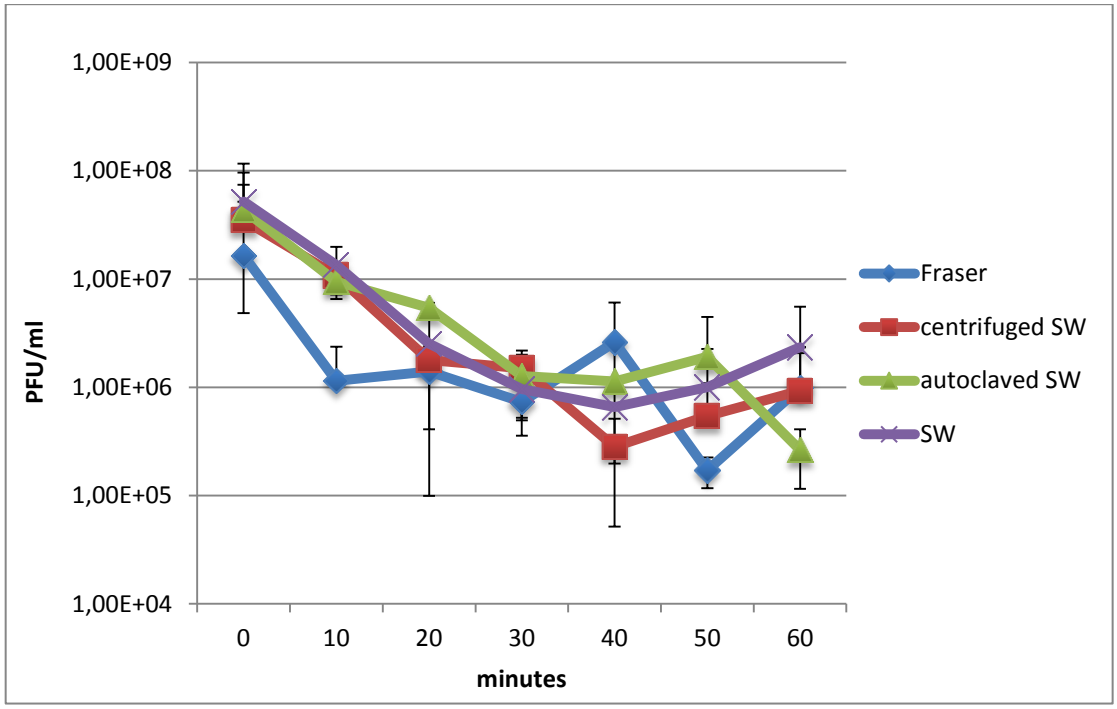


Figure 12: Influence of smear water (SW) and Fraser on binding ability of P100 to *L. monocytogenes*

Reduction in PFU/ml indicates binding to *L. monocytogenes* at autoclaved, centrifuged and untreated smear water. In summary, besides for SDS, attachment to the host cell and replication of P100 could be observed at all tested conditions.

4.5 Influence of the chemical environment on efficacy of phage treatment

In food processing environments parameters such as pH, salt or detergent concentrations are likely to change during the production process and may influence growth of *L. monocytogenes*. Therefore the influence of these environmental and chemical conditions on *L. monocytogenes* and efficacy of phage treatment was investigated by measuring OD_{600} of *L. monocytogenes* over 24 h every single hour. Bacterial growth in a specific environment, without addition of phages, was taken as control and compared with growth in presence of P100.

First the influence of NaCl on growth of *L. monocytogenes* over 24 h was tested (Figure 13).

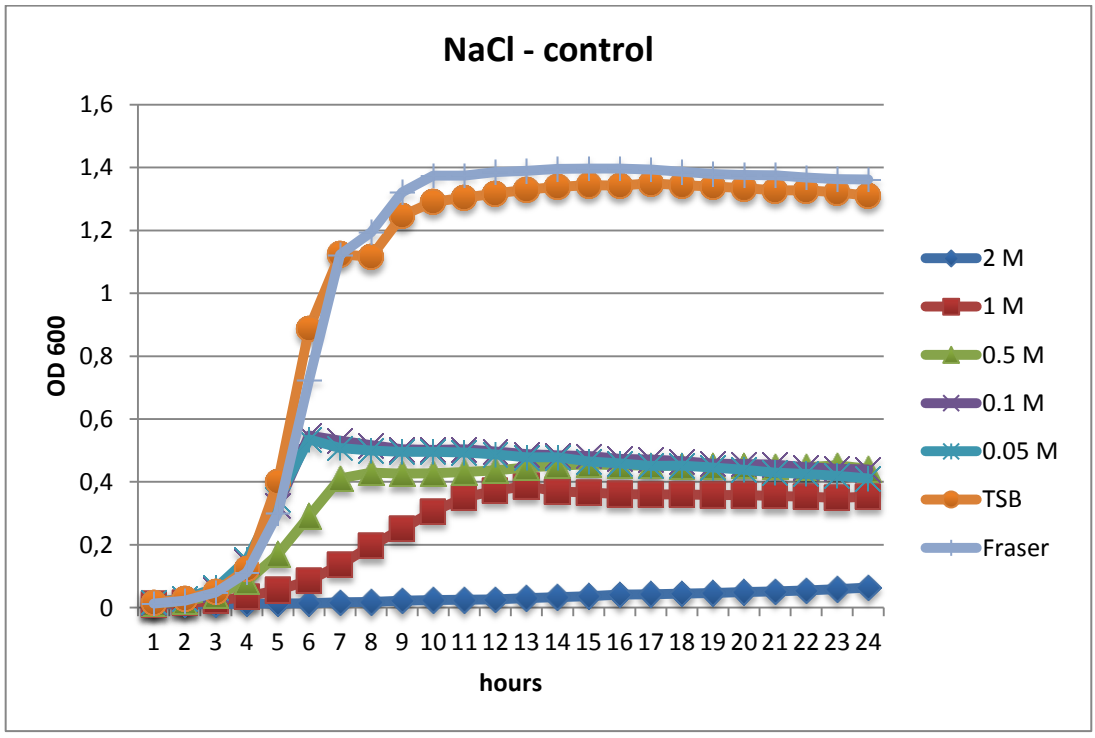


Figure 13: Influence of NaCl on growth of *L. monocytogenes* over 24 hours without addition of phages.

The following experiments were carried out two times in duplicate. All technical duplicates were very similar. Sometimes, the growth of *L. moncyotogenes* started at shifted timepoints in the second experiment. Therefore, all figures show the average

of only one experiment as representative. In the attachment of the thesis the figures of the second experiments can be found. If there were major differences in the bacterial growth within the two experiments they are described in the text.

Representative results in Figure 13 show that there is less growth of *L. monocytogenes* with 0.05 – 1 M NaCl compared with the control (TSB) and Fraser (light blue line). After addition of phages (dilution 1:10), growth of *Listeria* could not be detected for 2 M and 1 M NaCl anymore. Growth at lower NaCl concentrations (0 – 0.5 M) could be observed after 13 - 16 hours, which was 10 - 13 hours later compared with the results obtained for the control. With a dilution of 1:100, growth could be detected for the same concentrations two hours later on average (Figure not shown).

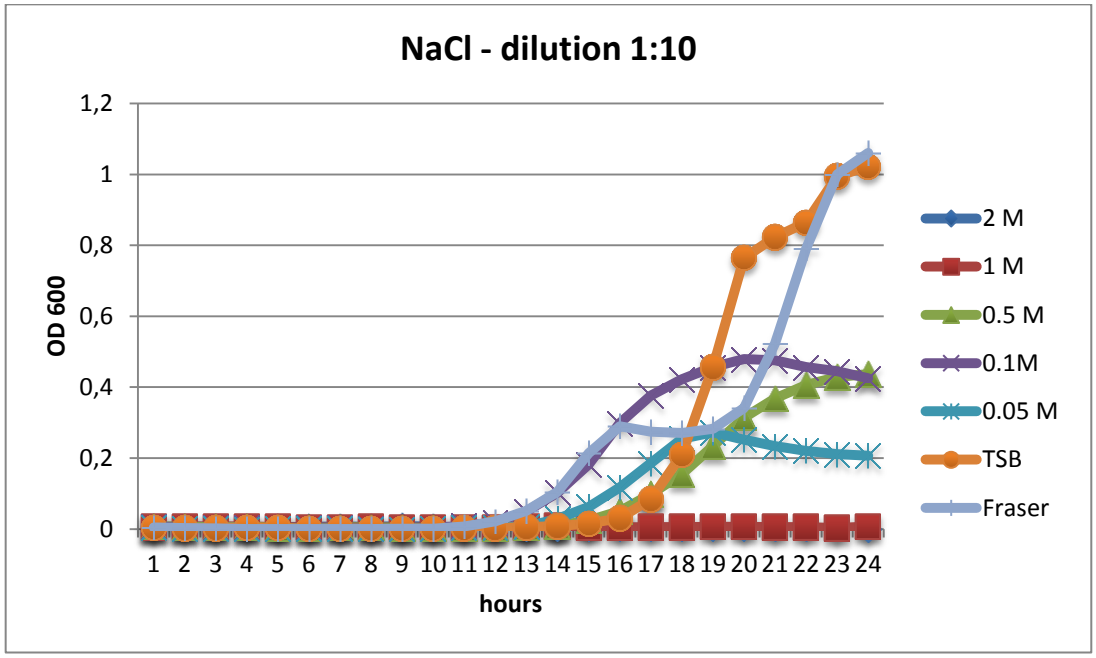


Figure 14: Influence of NaCl on efficacy of phage treatment over 24 hours, after addition of phages (2×10^{10} PFU/ml)

In a subsequent experiment the influence of pH on growth of *L. monocytogenes* was determined. The results depicted in figure 13 show that *L. monocytogenes* was not able to grow at pH 2, 3, 4, 10, 11 and 12 within 24 hours.

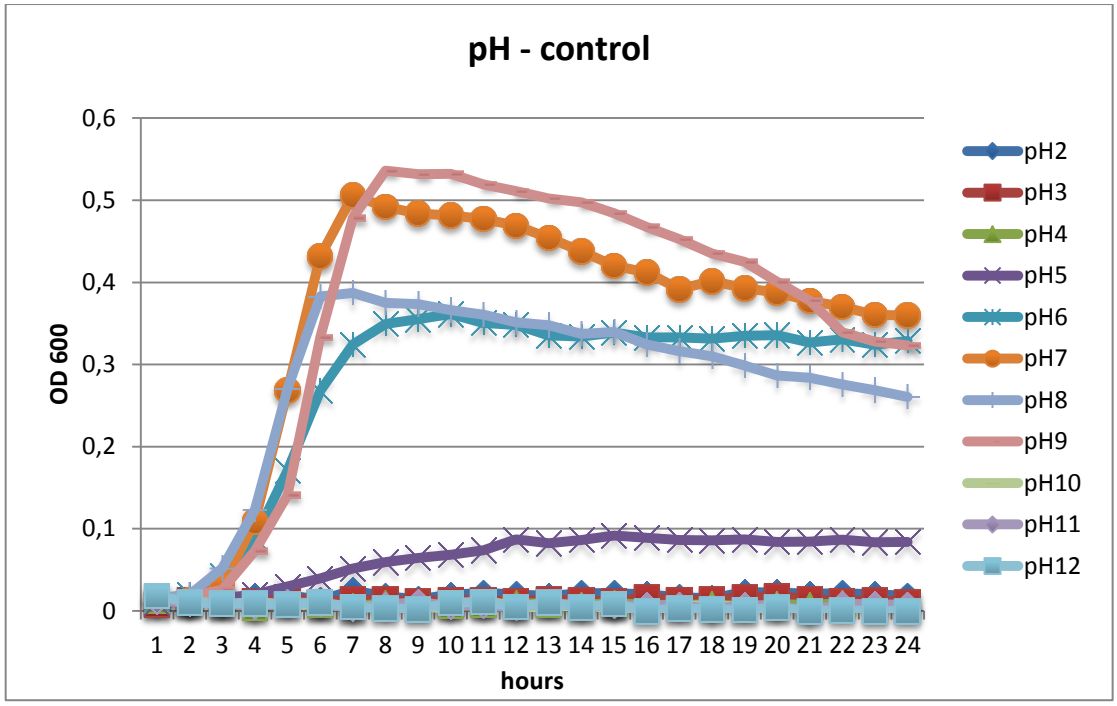


Figure 15: Influence of pH on growth of *L. monocytogenes* over 24 hours without addition of phages.

According to the results obtained in the previous experiment, determining the influence of NaCl on efficacy of phage treatment (Figure 13), growth could not be detected for samples, which were also negative in the control. Figure 16 shows that bacteriophages applied in a 1:10 dilution, lead to complete prevention of bacterial growth at all pH tested, except for pH 7, 8 and 9.

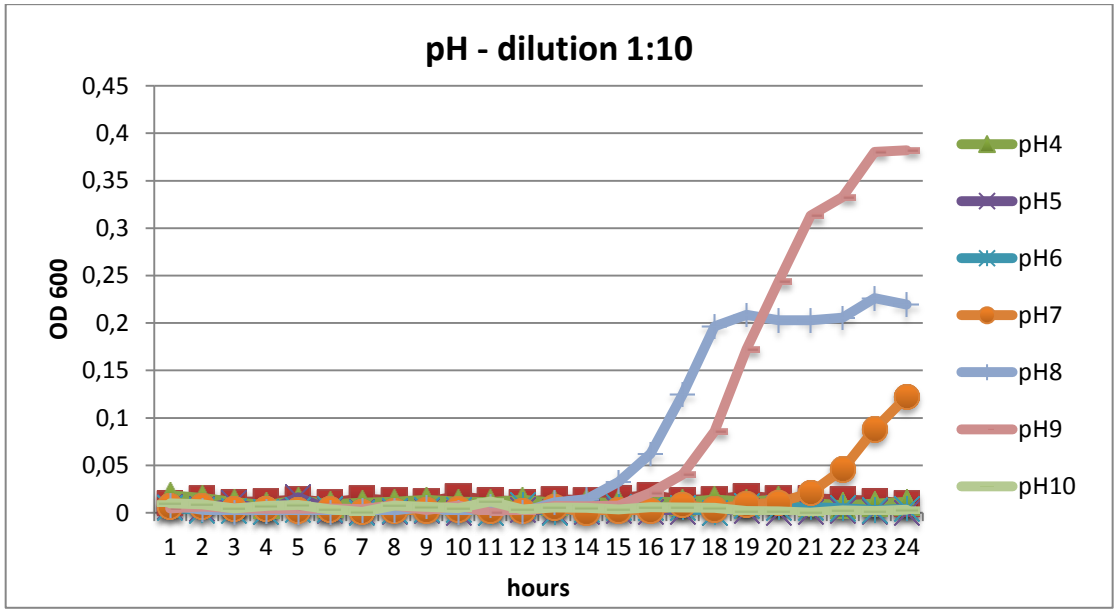


Figure 16: Influence of pH on efficacy of phage treatment over 24 hours after addition of phages (2×10^{10} PFU/ml)

Bacteria were also able to grow at pH 6 at a dilution of 1:100. This could be detected after 19 hours, which means that growth was detected six hours later than growth at pH 8 and 4 hours later than growth at pH 9 (Figure 17). Growth at pH 7 could already be detected after six hours.

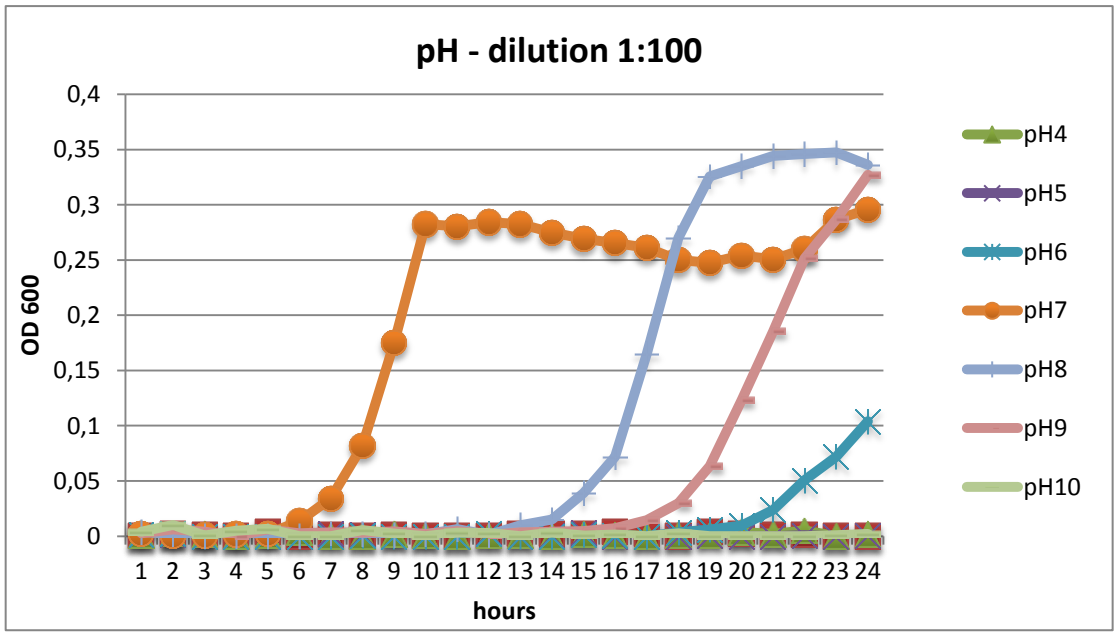


Figure 17: Influence of pH on efficacy of phage treatment over 24 hours after addition of phages (2×10^9 PFU/ml)

In a subsequent experiment the influence of the Lutensol AO7 was tested. Comparison of the growth of strains in uninfected medium showed, that Lutensol AO7 had more influence on growth of *L. monocytogenes* than NaCl and pH had. Even the lowest tested AO7 concentration (0.1 %) limited the growth to OD_{600} of 0.3 at its peak (Figure 18). Bacteria could grow in little extent at a Lutensol concentration of 5 % up to OD_{600} of 0.1 after 24 hours.

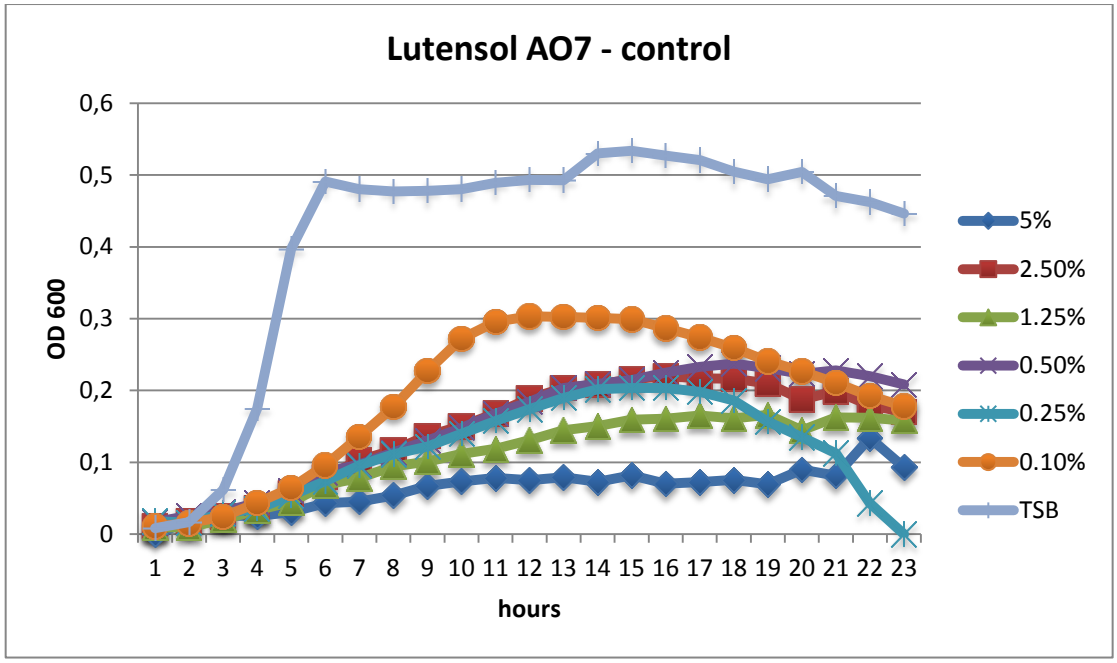


Figure 18: Influence of Lutensol concentration on growth of *L. monocytogenes* over 24 hours without addition of phages.

Phages could inhibit bacterial growth at all AO7 concentrations at a dilution of 1:10. Reducing the phage concentration (dilution 1:100) lead to growth of *L. monocytogenes* in 0.1 % Lutensol AO7 medium after 14 hours of incubation at 37°C (Figure 19). No growth was detectable for higher concentrations.

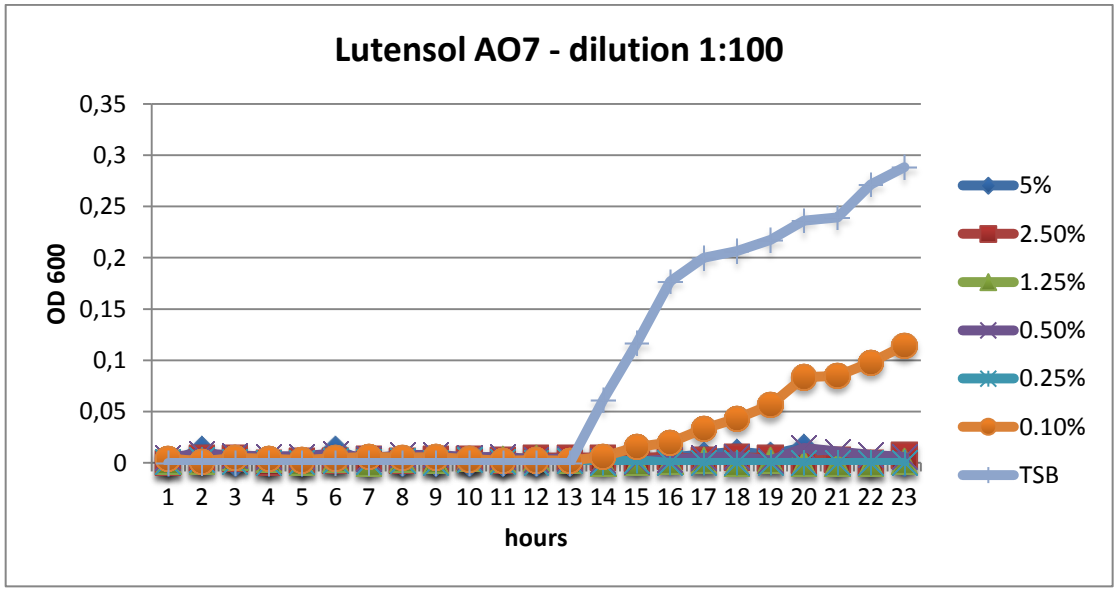


Figure 19: Influence of Lutensol on efficacy of phage treatment (1:100 = 2×10^9 PFU/ml) over 24 hours.

SDS is the second detergent that was tested and it had more influence on growth of *L. monocytogenes* than Lutensol AO7 had. It inhibited growth of *Listeria* without addition of phages even at the lowest concentration of 0.1 % (Figure 20).

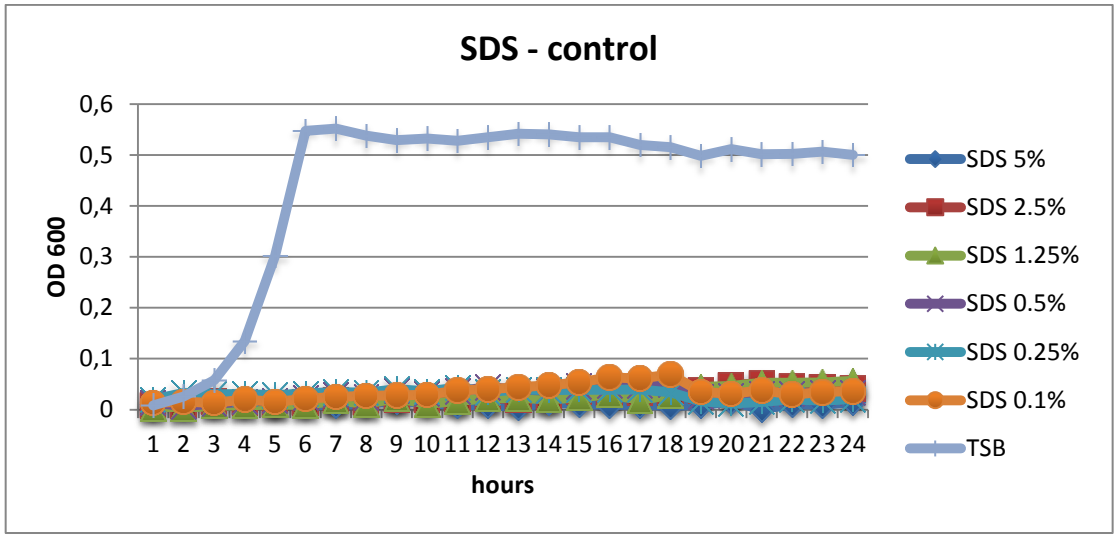


Figure 20: Influence of SDS concentration on growth of *L. monocytogenes* over 24 hours without addition of phages

In consequence, after addition of phages there was no growth of *L. monocytogenes* detectable anymore at any SDS concentration (data not shown). Altogether, results showed, that a higher phage concentration lead to a higher retention in growth of *L. monocytogenes*. This applied for all tested conditions. To determine which conditions negatively influence the activity and persistence of P100 different high-end and low-end conditions were tested just by addition of phages into the specific medium, without presence of any bacteria (Figure 21).

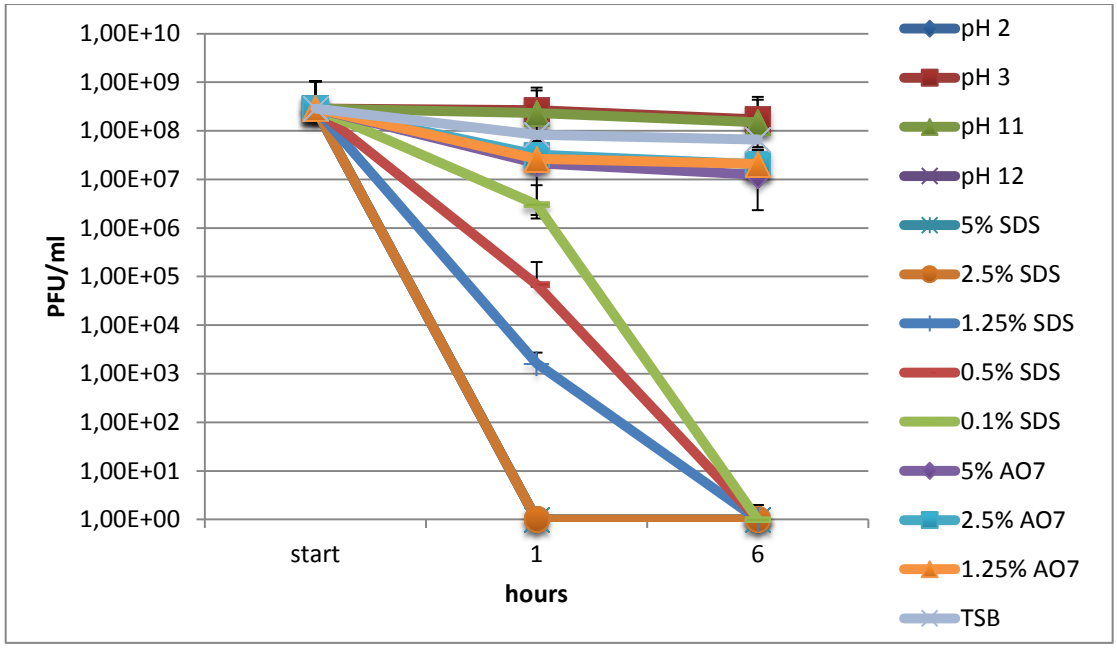


Figure 21: Persistence of P100 phages in different environments, after addition of 4×10^8 PFU/ml

Phages persist at all Lutensol AO7 and NaCl concentrations but lost infectivity at pH 2 and 12 as well as 2.5% and 5% SDS within one hour. Infectivity was lost within 6 hours for 0.1 – 1.25 % SDS.

4.6 Influence of temperature on efficacy of phage treatment (long – term experiment)

To investigate the influence of temperature and host-virus ratio on efficacy of phage treatment, *L. monocytogenes* was infected with Listex^{TM} P100 with two different concentrations (MOI 10 and 100) at 4 °C, 10 °C and 20 °C and was compared with an uninfected control over a time period of 17 weeks.

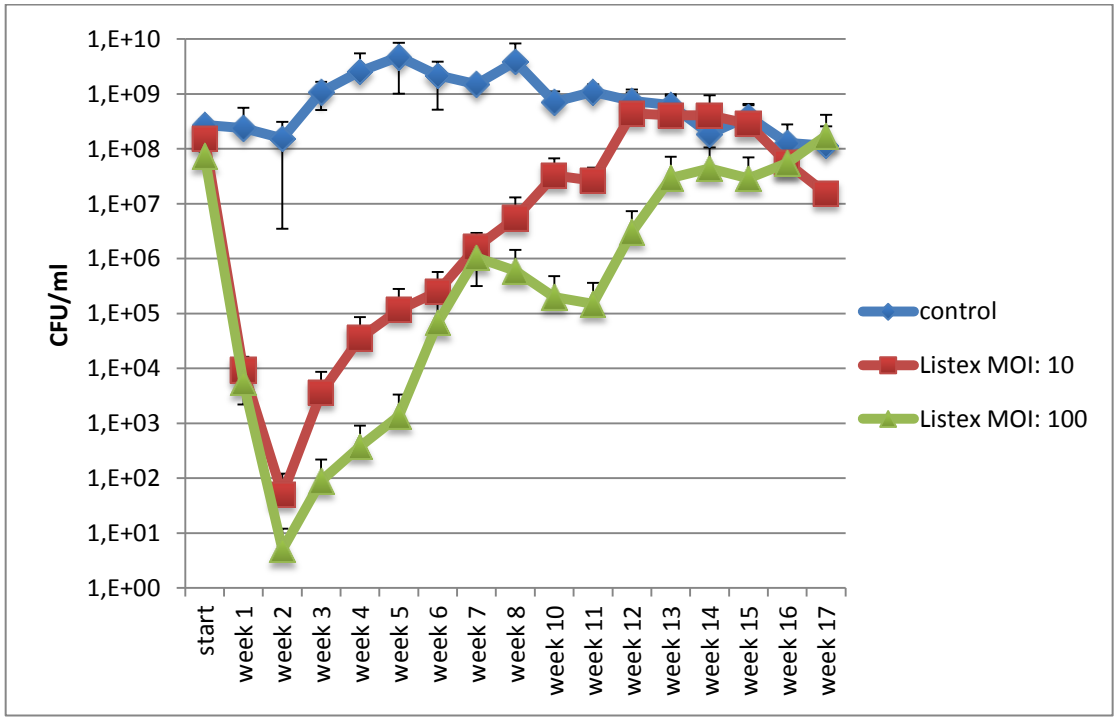


Figure 22: Influence of temperature (4 °C) on efficacy of phage treatment, control: no addition of phages

At 4 °C (Figure 22), there was an initial reduction of 6 $\log_{10}$ CFU/ml in *L. monocytogenes* cell count at a MOI of 10 and a 7 $\log_{10}$ reduction at a MOI of 100. After two weeks the amount of *L. monocytogenes* increased from 1.8 $\log_{10}$ CFU/ml or 0.8 $\log_{10}$ CFU/ml to 7.2 and 8.1 $\log_{10}$ CFU/ml during 17 week storage at 4°C. After 12 (MOI = 10) and 16 weeks (MOI = 100) the cell count of the regrowing culture obtained the same CFU / ml as the cell count of the control.

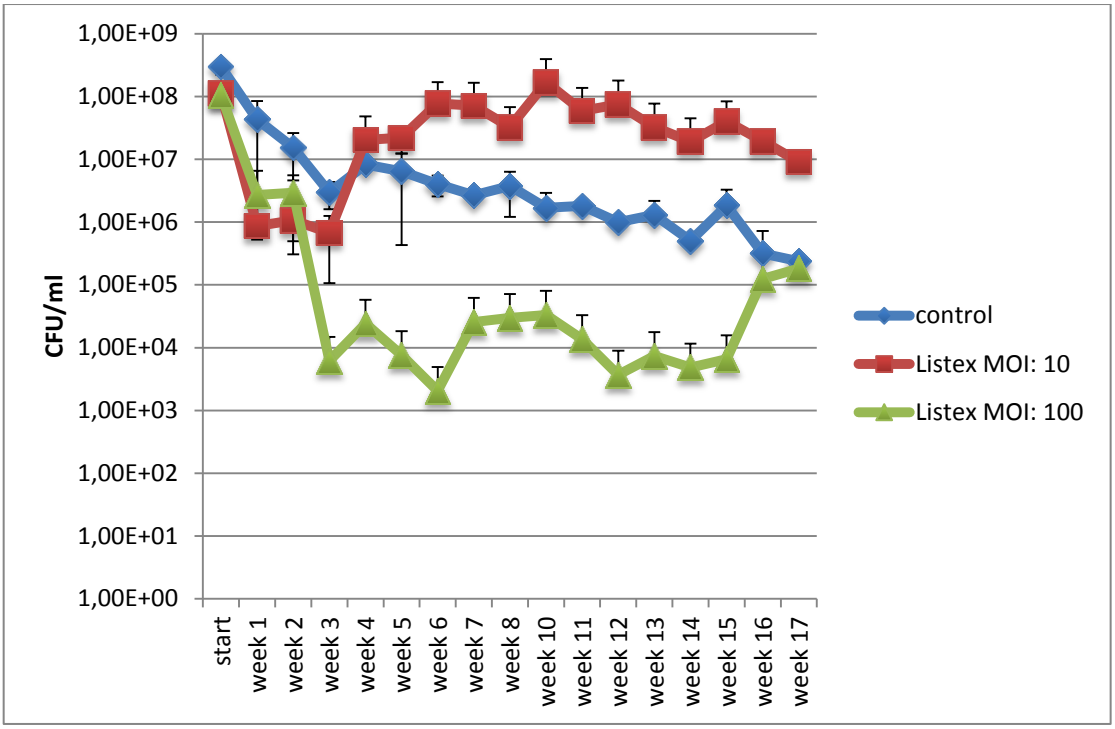


Figure 23: Influence of temperature (10 °C) on efficacy of phage treatment, control: no addition of phages

At 10 °C (Figure 23) the maximum reduction with 4.8 $\log_{10}$ CFU/ml (MOI=100) was obtained after six weeks. The regrown culture reached the cell count level of the control after 17 weeks. With a MOI of 10 the cell count decreased in about 2.0 $\log_{10}$ CFU/ml after one week. After regrowth the cell count increased to a level higher than the cell count of the control. The final cell count was 2.0 $\log_{10}$ CFU/ml higher compared with the cell count at MOI 100.

At 20 °C there was an instant reduction of *L. monocytogenes* cells of about 3.5 $\log_{10}$ CFU/ml after one week (Figure 24). Regrowth of bacteria was observed at both phage concentrations to the same amount of cells (6.8 $\log_{10}$ CFU/ml), which is 1.2 $\log_{10}$ CFU/ml less than the initial concentration. When *L. monocytogenes* were infected with a MOI of 10 at 20 °C, five weeks after infection the bacterial concentration was as high as in the uninfected control. When *L. monocytogenes* was infected with a MOI of 100, re-growth to the same bacterial concentration as in the uninfected control lasted 14 weeks.

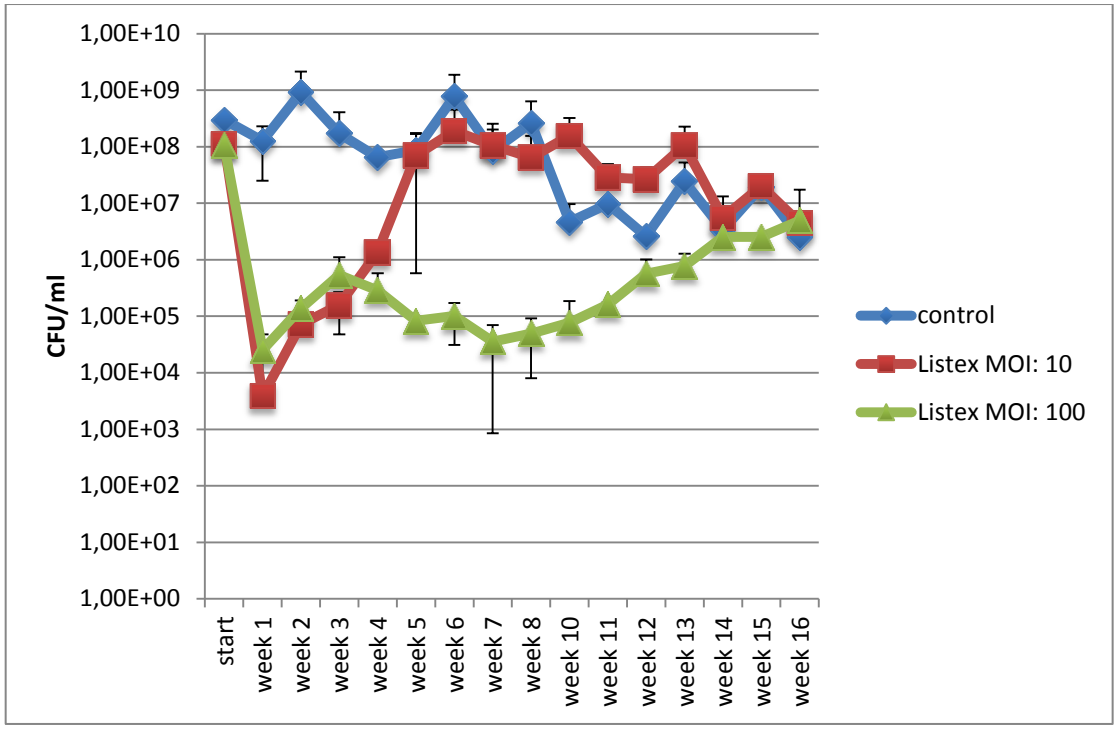


Figure 24: Influence of temperature (20°C) on efficacy of phage treatment, control: no addition of phages

The number of uninfected bacteria (control) was constant at 4 °C. However at 10 °C reduction of 3.5 log scales and two log scales at 20 °C could be observed.

In summary, an initial reduction followed by regrowth was observed in all experiments at all tested temperatures. Seventeen weeks after infection with the phage P100, the amount of *L. monocytogenes* was not reduced in comparison to the control at 4 °C, 10 °C and 20 °C.

4.7 Screening on newly formed resistances by cross streak tests

Regrown cultures, obtained from a repetition of the long-term experiment analysing the influence of temperature (4.4.), were screened for *L. monocytogenes* isolates with resistance to the phage P100.

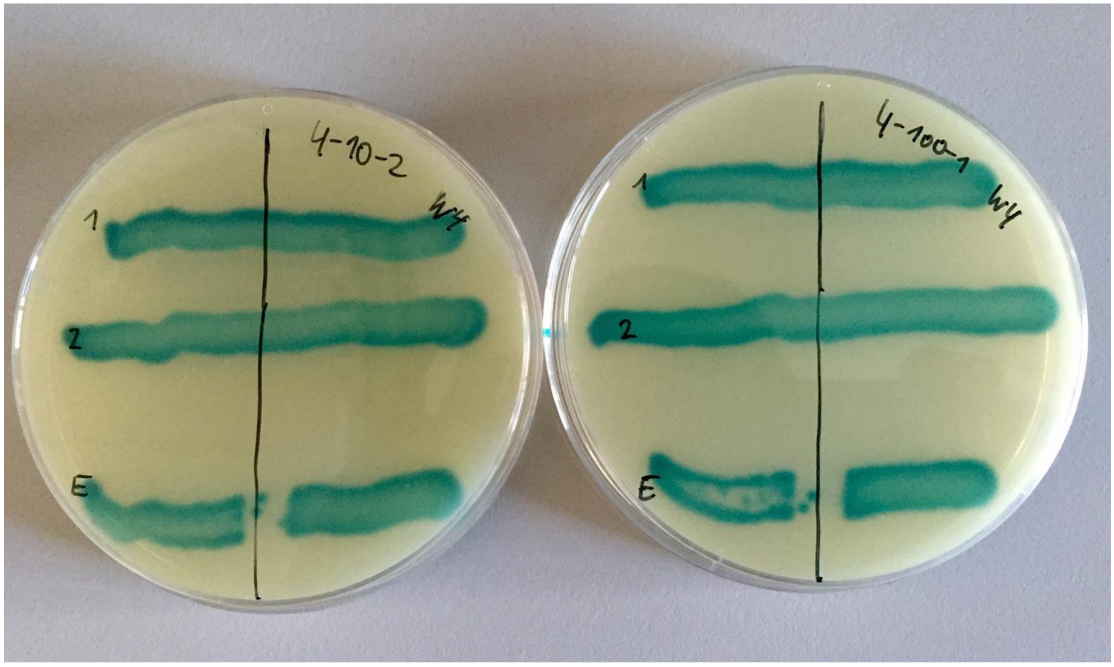


Figure 25: Cross streak test of strain growing at 4 °C on ALOA agar; Left: MOI: 100, 4 weeks; Right: MOI: 10, 4 weeks; E: *L. monocytogenes* EGDe strain (control)

Results in Figure 25 show the resistant strains obtained from the 4 °C long - term experiment (Figure 22), exemplary for all tested temperatures. Colonies from regrown cultures were growing in the phage zone whereas the control strain was lysed. The resistant isolates were confirmed as being *L. monocytogenes* by qPCR.

Plate A shows a resistant isolate obtained from the sample that was infected with a MOI of 100 and plate B shows a resistant isolate that was infected with a MOI of 10. The same test was done with regrowing culture obtained at 10 °C and 20 °C which showed similar results. In total, twelve resistant isolates were found.

4.8 Persistence of P100 phages in food-processing environments

In dairy companies P100 phages (as Listex^{TM} P100 preparation) are often added to smear water to overcome contamination with *L. monocytogenes*. Therefore, persistence of P100 in smear water that was stored at 4 °C and 10° C was investigated by PFU determinations that were carried out every 10 days. The temperatures reflect temperatures that are typical in food-processing environments. Investigated smear water was autoclaved, centrifuged, untreated or enriched with *L. monocytogenes*.

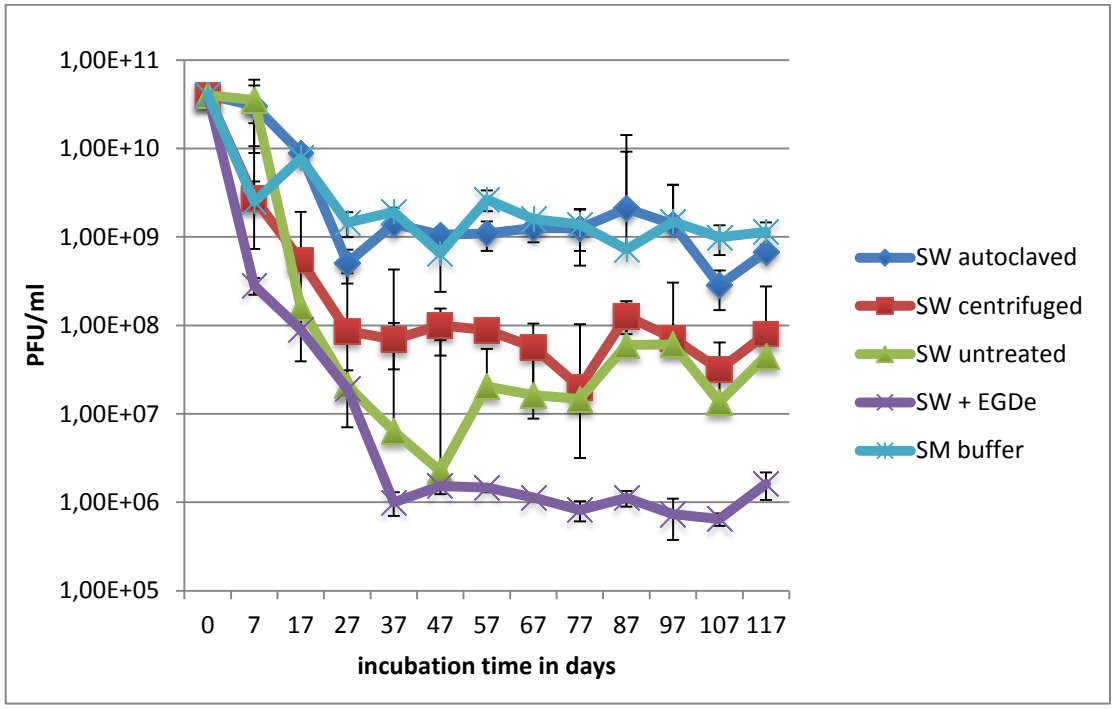


Figure 26: Persistence of P100 phages in smear water (SW) at 4 °C after addition of 5 × 10^{10} PFU/ml

When persistence of P100 was monitored at 4 °C (Figure 26), it could be observed that the number of phages in SM buffer and autoclaved smear water was reduced about 1.5 $\log_{10}$ PFU/ml. The highest reduction of phages was about 4.5 $\log_{10}$ PFU/ml that was observed in smear water with *L. monocytogenes* EGDe over 117 days. In untreated and centrifuged smear water phage titre was reduced about 3 $\log_{10}$ PFU/ml after 27 - 37 days and kept constant afterwards.

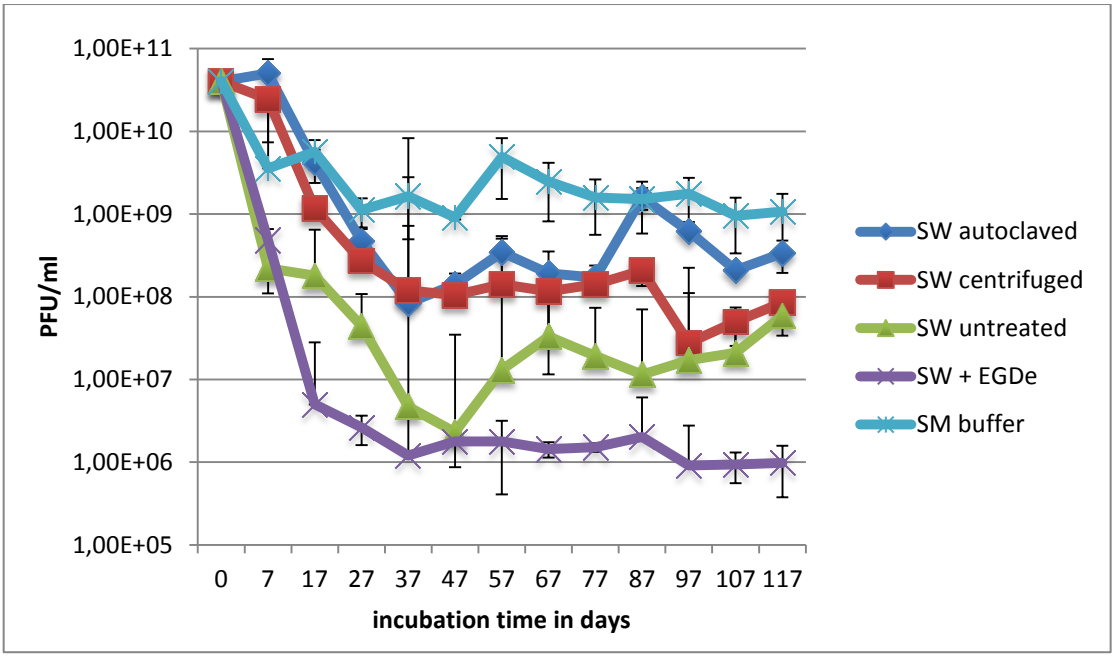


Figure 27: Survival of P100 phages in smear water at 10 °C after addition of 5 × 10^{10} PFU/ml

Figure 25 shows the respective results obtained for survival of phage P100 at 10 °C, which showed no significant difference to those obtained at 4 °C.

5 Discussion

Because of the relevance of *L. monocytogenes* due to outbreaks caused by contaminated foods, the search for alternatives to control this food pathogen became more and more important. Bacteriophages seem to be an attractive and promising approach for elimination of *L. monocytogenes* in food processing environments, because of their high tolerance to pH and NaCl [22]. Moreover, phages do not affect the flavor, taste and chemical and physical properties of the food product. Additionally, bacteriophages have a high specificity to their hosts and no effect on consumers health [53]. In this study the use of the phage P100 for elimination of *L. monocytogenes* was evaluated.

In addition phages can not only be used to control *L. monocytogenes*, but also to lyse *L. monocytogenes* and set free its DNA for subsequent molecular methods like PCR. Therefore the usability of P100 in context of isolation of *L. monocytogenes* DNA was also investigated in this study.

5.1 The occurrence of P100 insensitive *L. monocytogenes* strains is linked to the application of Listex™.

In several publications the question arose, if the repeated use of phages for biocontrol of pathogens could lead to development or increase of insensitivity of the target bacteria. Fister et al. (2015) studied the development of resistance against Listex™ P100 using 486 naturally occurring *Listeria* dairy plant isolates [49]. Overall they found 13 insensitive *L. monocytogenes* isolates.

In this study, characterization of these isolates showed that five strains belong to PFGE type Ro1 and one strain to PFGE type Ro2. All of these six strains belong to serotype 1/2b, 3b. The six other strains belong to serotype 1/2a, 3a, two of them to PFGE type Wo1 and four of them to PFGE type Wo2 (Chapter 4.1). Concluding from these data, obtained by PFGE and Multiplex PCR serotyping, it was obvious that the detected isolates did not appear randomly. The appearance of insensitive *Listeria* strains was strongly connected to the application of P100 phages. This was deduced from the fact that PFGE types Wo and Ro respectively could be related to two independent events of application of Listex™ P100. The observed adaptation of insensitive *L. monocytogenes* isolates against bacteriophages may occur by new

restriction modification systems or cell wall- and receptor modifications, caused by spontaneous mutations or it might be temperature-dependent [42].

5.1.1 Parameter affecting the adaptation of P100 insensitive *L. monocytogenes*

The possible *temperature-dependence* and the occurrence of *spontaneous mutations* as adaptation mechanisms were further investigated in this work. The 12 insensitive isolates obtained by Fister et al. were tested on susceptibility to phage treatment at different *temperatures*. Four out of the 12 insensitive strains showed a higher tolerance to phages grown at 25°C. At 37 °C these four strains were infected by P100 at a higher rate (Chapter 4.2). Three out of these four isolates (L93, L97, and L102) belong to PFGE type Wo2 and isolate L57 to PFGE type Ro2. These PFGE types could be traced to two events, one in 2001 and another one in 2011 including two dairy plants, which used P100 phages. Nevertheless the occurrence of those four temperature dependent insensitive *L. monocytogenes* isolates is not coupled to the location, time or PFGE type and must be assumed as a stochastic event. Therefore *spontaneous mutations*, which belong to well-known phage defence mechanisms, may result in formation of the observed resistances leading to insensitive *L. monocytogenes* strains [54]. The underlying genetic properties of these *L. monocytogenes* cells have to be further investigated.

5.2 Host – pathogen relationship of susceptible *L. monocytogenes* EGDe and P100

5.2.1 The effect of passaging on infectivity of P100 towards *L. monocytogenes* EGDe

Passaging of P100 was done to determine the influence of infection-cycles on sustainability of phages. This was done to propagate bacteriophages, possibly causing mutations, changing the infectivity of P100 towards susceptible *L. monocytogenes* EGDe.

The data obtained by these experiments showed that after eleven generations of passaging the infectivity of P100 was not given anymore. This suggests that mutations caused the loss in infectivity. Nevertheless, this is a first indication and further investigations would be necessary. Spontaneous mutations are frequently occurring

with bacteriophages and eleven cycles of passaging should significantly support mutations in the P100 genome. The underlying mechanism for spontaneous mutations is based on a given error rate of the involved DNA polymerases which leads to the observed change in phage infectivity [22].

5.2.2 The effect of physico-chemical parameters as present in the dairy environment on the host – pathogen relationship of *L. monocytogenes* EGDe and P100

Infectivity of bacteriophages could also be influenced by stress conditions of food-processing environments, not given in laboratory conditions. This was the case in the work of Fister et al. (2015) where the P100-*Listeria* host pathogen relationship was investigated only *in* vitro. Growth kinetics, MOI and adsorption of P100 was investigated both for insensitive and susceptible *L. monocytogenes* strains. In contrast it is important to determine how food production environments impact the efficacy of phage treatment.

The very first step of phage infection requires the recognition and adsorption to specific surface receptors on the bacterial cell. Different components of wall teichoic acids and acetyl groups in the peptidoglycan layer are common phage receptors for Gram-positive organisms as *L. monocytogenes.* The composition and the structure of wall teichoic acids and peptidoglycan is affected when environmental conditions change [55]. Following to adsorption, which takes places within the first 40 minutes of infection, replication occurs leading to propagation of the phages and reduction of the number of host cells.

Therefore we tested, if various conditions like pH, salt and presence of detergents have an influence on adsorption- and replication ability of phages. These environmental parameters are given in the food production environment and in the product. Two applications of Listex^{TM} are theoretically useful: Emergency treatment on the surface of the product to prevent growth of *L. monocytogenes* and application in the production environment for eradication of *L. monocytogenes.*

5.2.2.1. The effect of physical and chemical parameters on *adsorption* of P100 to *L. monocytogenes* EGDe cells

The adsorption to the host cell is the most critical step during P100 infection. Therefore a broad variety of physico-chemical parameters were tested to investigate the influence of the environment on P100 adsorption. Adsorption tests were performed in the presence of (i) the detergent Lutensol AO7 (0.1 – 5%), (ii) SDS (0.1 – 5%), (iii) NaCl (0.05 – 2 M) and (iv) at temperatures of 4 °C, 10 °C, 20 °C and (v) at pH values from 2 – 12. Moreover (vi) Fraser broth and (vii) smear water were included (Chapter 4.4).

Surprisingly P100 did bind to the *L. monocytogenes* cell surface under most tested conditions. P100 did not bind at a pH ≤ 2 and SDS concentrations of ≥ 5%, where infectivity was lost within one hour (Figure 21).

These results suggest that the affinity of the involved receptors is high and adsorption as the first step of the infection cycle of P100 is not significantly influenced by usual environmental parameters as found on food surfaces and the production facility.

5.2.2.2. The effect of physical and chemical parameters on *replication* of P100 in *L. monocytogenes* EGDe enrichments

After adsorption to the host cell there are still many mechanisms, which can prevent phage infection. Even after successful attachment, possibilities by which the cell can resist phage infection are CRISPR-Cas mediated resistance, abortive infection or restriction modification systems, as mentioned above [55].

Therefore the *replication* ability was tested. *Replication* tests were performed under the same experimental parameters as outlined in chapter 5.2.2.1. for the adsorption experiments. The data obtained by these replication experiments showed significantly different results. If Lutensol AO7 (Figure 10) is added, replication of the phages was given but with a delay up to three hours. SDS inhibited phage replication from 0.1% up to 5% (Figure 11). NaCl had no influence at concentrations from 0.05 M to 1 M (Figure 7) and 2 M NaCl phage replication is timely delayed reaching 10^7 PFU/ml after six hours in comparison to 5 x 10^8 PFU/ml at 0.05 M to 1 M NaCl. Replication of P100 was supressed at a pH value ≤ 2 (Figure 8). In presence of smear water and Fraser broth, replication was not hindered.

In summary, the environmental parameters, which hindered replication of P100 also affected the growth of the host *L. monocytogenes*. This leads to the assumption that success of phage treatment is strongly dependent on growth conditions of *L. monocytogenes*, which is also described by Guenther et al [24]. The growth of susceptible *L. monocytogenes* EGDe under the influence of P100 and environmental factors is outlined in the next chapter.

5.3 Growth of susceptible *L. monocytogenes* EGDe under the influence of P100 and environmental factors

In food processing environments parameters such as pH, salt or detergent concentrations are given by the production process and influence growth of *L. monocytogenes* [22]. In chapter 5.2.2., it was suggested that the adsorption and replication of P100 is more dependent on growth of *L. monocytogenes* than on the environmental physico-chemical parameters.

Therefore the influence of these environmental and chemical conditions on *L. monocytogenes* growth in combination with the efficacy of phage treatment was investigated by measuring OD_{600} of *L. monocytogenes* over 24 h after infection with P100.

5.3.1 Growth of *L. monocytogenes* EGDe under the effect of physico-chemical parameters in presence of P100

The influence of the environment on growth of *L. monocytogenes* was tested in the presence of (i) the detergent Lutensol AO7 (0.1 – 5%), (ii) SDS (0.1 – 5%), (iii) NaCl (0.05 – 1 M) and (v) at pH values from 2 – 12.

L. monocytogenes cells were able to grow in the presence of Lutensol AO7 of ≤ 5% (Figure 18), NaCl concentrations of ≤ 2 M (Figure 13) and at pH values between 5 and 9 (Figure 15). SDS prevented growth of *L. monocytogenes* at a concentration of ≥ 0.1% (Figure 20).

In combination with these environmental effects, the efficacy of phage treatment was then tested. In the presence of Lutensol AO7, phages could prevent growth of *L. monocytogenes* from 0.25% up to 5% (Figure 19). In combination with NaCl growth was prevented for 1 M and 2 M (Figure 14).

In general growth of *L. monocytogenes* was detected 10 – 13 hours later in comparison to the uninfected control. Delayed growth in comparison to the control was also possible at pH values of 7 – 9 (Figure 16) indicating that *L. monocytogenes* is more imperishable against phage infections in a neutral and alkaline environment. This is in accordance to the results obtained for phage replication (5.2.2.). Therefore we conclude that the efficacy of the phage treatment is dependent on the status of the *L. monocytogenes* host cells more than on environmental factors directly influencing P100.

5.3.2 Growth of *L. monocytogenes* EGDe in the presence of P100 in different concentrations

Another parameter, which is influencing the efficacy of phage treatment, is the numerical relationship between phage and host. Therefore experiments were conducted comparing the growth of *L. monocytogenes* EGDe in different concentrations in the presence of P100 in varying concentrations (4.5).

The obtained data showed that the efficacy of phage treatment was higher with a concentration of 2×10^{10} PFU/ml in comparison to 2×10^{9} PFU/ml, even if the MOI was the same. The results show that treatment with phages is more effective when conditions negatively influence bacterial growth. Denes et al showed that productivity of phage infection correlates with host growth rate [55]. Phage production is higher under conditions that promote faster growth of host cells but contrary phage treatment is more effective when growth of host cells is negatively influenced by environmental conditions. If the bacterial metabolism is completely disrupted, then replication of P100 is not possible whereas adsorption takes place. The second step after adsorption in the propagation cycle of lytic bacteriophages is the incorporation of the nucleic acid of the phage into the host cell. In a resting bacterial cell it is then more likely that the nucleic acid of the phage is degraded slowly. The resting cell would not increase metabolic activity, which is necessary for phage propagation. In this case findings make application of Listex™ not recommendable.

5.3.3 Long-term growth of *L. monocytogenes* EGDe in the presence of P100 and formation of insensitive *L. monocytogenes* strains

The influence of the MOI (10 and 100) on long-term growth of *L. monocytogenes* in presence of P100 was tested at different temperatures for 117 days.

The data obtained during these experiments show that there was no complete eradication of *L. monocytogenes* at 4 °C, 10 °C and 20 °C. At all tested temperatures, an initial reduction of bacteria was observed that was followed by regrowth of *L. monocytogenes* (Figure 22 - 24). Corresponding to the other results obtained in 5.3.2 (MOI 24h), applying a higher amount of phages leads to a higher reduction of bacteria. Regrowth occurred later when a MOI of 100 was applied in comparison to a MOI of 10, although regrowth was observed at all tested temperatures and phage concentrations. This is in agreement with data previously published for other bacteriophages [11, 22, 24]. This effect becomes even more apparent when not only the absolute number of phages is increased but also the MOI.

Assuming emergence of phage resistance, the experiment was repeated and re-growing bacteria were tested for insensitivity towards P100. As a result insensitive strains were obtained at all tested temperatures (Figure 25), indicating that the occurrence of resistant *L. monocytogenes* during long-term presence of P100 in the direct environment of the culture is a given phenomenon.

Although the exact reason for the development of phage resistance is unclear, *Listeria* is likely to become resistant by cell wall modifications or modifications of specific surface carbohydrates responsible for phage attachment [11]. Moreover O'Flynn et al. demonstrate that a phage-resistant phenotype of the bacteria is likely to revert without selective pressure which means in the absence of P100 [54].

5.4 Stability of P100 during long-term application

The fact that long-term presence of P100 in little amounts can lead to formation of resistances raises the question how long phages can persist in food processing environments[22].

Stability of phages in food processing environments is a parameter, which is important for successful phage application. As mentioned above, phages should sustain and be able to replicate for adequate dissemination and efficacy of treatment. [56]. Therefore experiments were conducted to investigate the infectivity of P100 in

in smear water as a real model for cheese making. This was performed in a long-term application including different temperatures (4 °C and 10 °C), which are common in the natural dairy environments.

The data show that infectivity of P100 is stable in autoclaved smear water but is reduced in untreated smear water (Figure 26 and 27). Lowest phage infectivity was observed after addition of *Listeria,* probably caused by mutations in the P100 genome. This is in agreement with the results obtained at passaging P100, which were not able to infect *Listeria* after eleven generations.

Tessema et al. (2011) showed that lactic acid bacteria might produce bacteriocin during dairy product processing. Exposure to bacteriocins may lead to reduced susceptibility to P100 in *L. monocytogenes* [57]. This may be the reason for reduced survival rate of P100 in non-autoclaved smear water. Another possibility is that proteolytic activity of compounds in smear water affects infectivity and integrity of phage particles [11]. Also binding to *L. monocytogenes,* which induces phage defence mechanisms, can reduce the amount of P100.

Moreover the data show that temperature did not change the survival of phage P100 significantly. Altogether, the data indicate that phages persist in food-processing environments for a very long time period, thus resistant bacteria might have an advantage over non-resistant bacteria and will probably dominate the bacterial population in phage treated environments.

6 Conclusion

The high efficacy of phage treatments in food production plants is the basis of their increased use. This thesis should assess the use of P100 phages in food production environments.

In a previous work it was shown that the occurrence of P100 insensitive *L. monocytogenes* strains is significantly linked to the application of Listex^{TM}. This was demonstrated by comparison of PFGE types of insensitive *L. monocytogenes* strains resulting in two clusters linked to two events of Listex^{TM} application.

In the next step it was demonstrated that there is no linkage between temperature and formation of P100 insensitive *L. monocytogenes* strains. Therefore spontaneous mutations within the Listeria genome as well as mediated response of the cells to P100 are likely to cause the insensitivity.

In this work the host pathogen relationship of susceptible EGDe and P100 was investigated.

Firstly it was shown that P100 is attenuated when repeatedly applied to the host. This was confirmed after eleven generations of passaging.

Secondly adsorption of the phages being the first step in infection and replication were investigated by means of the influence of physico-chemical parameters. It was demonstrated that environmental factors do not influence the adsorption significantly.

Replication on the other hand was mostly hindered at environmental parameters, which also affected growth of *L. monocytogenes* because of the lack of target cells. However, phage treatment was more effective when conditions negatively influence bacterial growth.

Furthermore it could be shown that there is dependency of the number of applied phages as well as the ratio between phages and host cells.

Experiments including long-term infection with P100 demonstrated that the formation of insensitive *L. monocytogenes* was favoured under these conditions (117 days). Development of this phage insensitivity was observed at all tested physico-chemical conditions. It was also demonstrated that P100 persists in food processing environments for a period of more than 100 days with stable infectivity.

Practically, if P100 should be used against *Listeria* contamination, an application procedure is needed which considers following factors: P100 has to be best applied in very high concentrations, should not be used for long-term treatments and should be applied in combination with other *Listeria* phages to prevent development of phage resistances. Therefore the application of P100 can be considered as emergency treatment but constant routine application of P100 in the production environment cannot be recommended. Furthermore phages can influence the in house monitoring of *L. monocytogenes*, if dissemination of phages is promoted in enrichment broth. Suppressing growth of the target bacteria is leading to false negative results.

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Zusammenfassung

Lebensmittelsicherheit ist ein Anliegen das jeden Menschen betrifft und eine große Rolle dabei spielt die Gefahr, die durch pathogene Mikroorganismen entsteht. Ein Beispiel ist *Listeria monocytogenes*, welches in der Lage ist lange in der Umgebung lebensmittelverarbeitender Industrie zu überleben. Die Methoden, die zur Zeit zur Kontrolle pathogener Mikroorganismen angewendet werden, reichen nicht um Listerien vollständig zu kontrollieren oder haben einen negativen Einfluss auf die Qualität der Lebensmittel. Eine vielversprechende neue Möglichkeit zur Kontrolle von *L. monocytogenes* sind Bakteriophagen. Die Effektivität der Phagenbehandlung wurde in einigen Publikationen beschrieben und Experimente hierzu wurden meist unter eingeschränkten Bedingungen im Labormaßstab durchgeführt. Die Virus-Wirts Interaktion, die Bindungs- und die Replikationsfähigkeit der Phagen sind jedoch abhängig von der Wachstumsrate der Bakterien und der chemisch-physikalischen Umgebung.

Ziel dieser Arbeit ist es, den Einfluss verschiedener Umweltbedingungen, wie Temperatur, Salzkonzentrationen, pH und Detergenzien auf das Überleben des P100 Phagen und auf die Virus-Wirts Interaktion zu untersuchen.

Dazu wurde mittels Keimzahlbestimmung das Wachstum von *L. monocytogenes* mit und ohne Phageninfektion untersucht. Das Überleben, die Bindungs- und die Replikationsfähigkeit der Phagen in Wirtszellen bei verschiedenen pH Werten, Salz- und Detergenzkonzentrationen wurde mit Plaque-Assays, Adsorptionstests und durch die Messung der optischen Dichte bestimmt. Zusätzlich wurde das Überleben der Phagen in Schmierwasser bei 4 °C und 10 °C untersucht.

Die Effizienz der Phagenbehandlung war abhängig von der Temperatur und des Virus-Wirts Verhältnisses. Die besten Ergebnisse wurden bei 4 °C und einer hohen Phagenkonzentration erzielt, allerdings sind die Listerien bei 4 °C als auch bei 10 °C und 20°C wieder hochgewachsen. Das Überleben der Phagen war unabhängig von der Temperatur, jedoch von der Anwesenheit von Wirts-Bakterien in Schmierwasser. Innerhalb von 37 Tagen wurde der Phagentiter um 1.5 $\log_{10}$ in SM Puffer und um 3 - 4 $\log_{10}$ in Schmierwasser reduziert. Die Infektiosität der Phagen ging bei pH 2 und pH 12, sowie bei einer SDS Konzentration ab 2,5% innerhalb einer Stunde verloren, jedoch nicht bei hohen Salz- und Lutensol-Konzentrationen. Die Phagenbindung war unter allen getesteten Bedingungen möglich, wohingegen die Replikationsfähigkeit

vom Wachstum der Listerien abhängig war. Die hohe Effektivität der Phagenbehandlung in lebensmittelproduzierenden Betrieben ist die Basis für deren Gebrauch. Diese Untersuchung zeigt, dass Umweltbedingungen einen signifikanten Einfluss auf die Effizienz der Behandlung haben. Dies muss bei der Verwendung von Bakteriophagen als Notfallbehandlung berücksichtigt werden.

Abstract

Food safety is a problem that concerns everyone. One of the main concerns of food safety is the microbiological hazard by pathogenic microorganisms such as *Listeria monocytogenes*, which is able to persist in food processing environments. The methods, which are currently available to control *L. monocytogenes* are not able to achieve full control or negatively influence the quality of food. One promising approach is the use of bacteriophages. Effectiveness of bacteriophages was described in several publications and experiments were done under restricted growth conditions on laboratory scale. However, host-virus interaction, adsorption and replication of phages are dependent on the growth of host cells and the physico-chemical environment.

The aim of this study was to investigate the influence of salt concentrations, pH, detergents and temperature on survival of the phage P100 and on host-virus interactions under these conditions.

Bacterial growth at different temperatures was determined with and without infection using plate counts. The survival of phages and their ability to attach to and to replicate on and in host cells at different pH, salt and detergent concentrations was monitored using plaque assays, adsorption tests and measurement of optical density over 24 hours. Additionally, the survival of P100 in smear water was tested at 4 °C and 10 °C.

Efficacy of the phages was dependent on temperature and host-virus ratio and best results were achieved at 4 °C with high phage concentrations. At 10 °C and 20 °C re-growth of bacteria was observed. Survival of bacteriophages was not depended on temperature but was influenced by smear water components and by presence of bacteria. Within the first 37 days the phage-titre was decreasing 1.5 $\log_{10}$ in SM buffer and 3 - 4 $\log_{10}$ in smear water. Phage infectivity was lost at pH 2 and pH 12 within one hour, but not at high salt or detergent concentrations. Attachment of the phages to the host was observed at all tested conditions, whereas replication was dependent on growth of the bacteria in these environments.

The high efficacy of phage treatments in food production plants is the basis of their use. This investigation demonstrates that environmental factors do significantly influence the performance of such treatments. This has to be considered when bacteriophages are used as emergency treatment.

Curriculum Vitae

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WORK EXPERIENCE

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- Internship at the institute for forensic medicine Bonn, 2010:

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- Project work at Saxion University Enschede, 2010:

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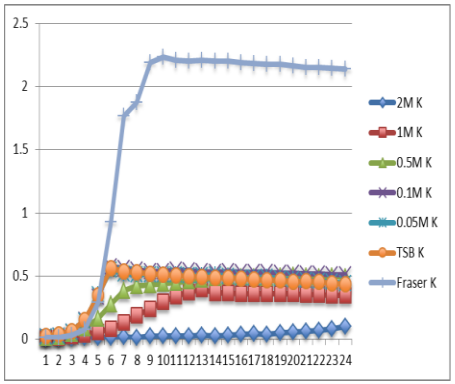
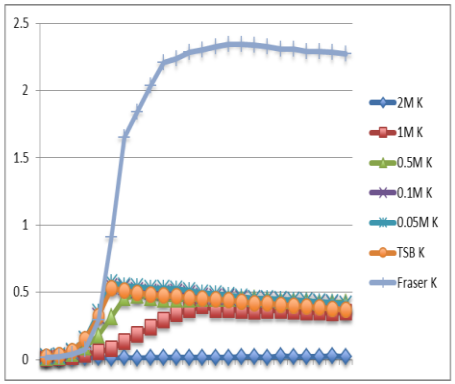
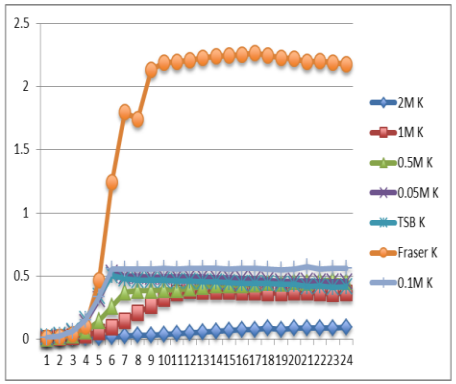
IT: Windows, Mac OS X, Word, Excel, SPSS

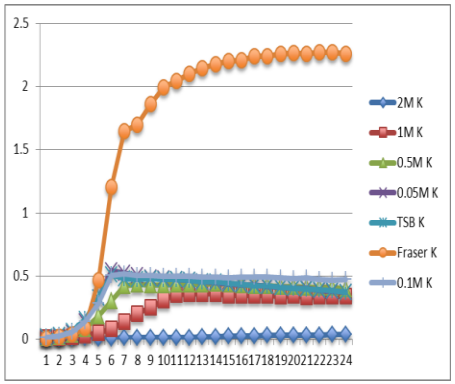
Supplements

NaCl control









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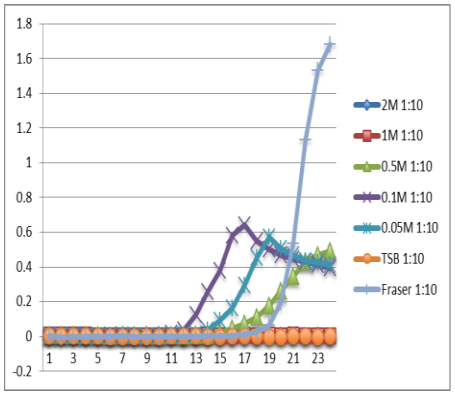

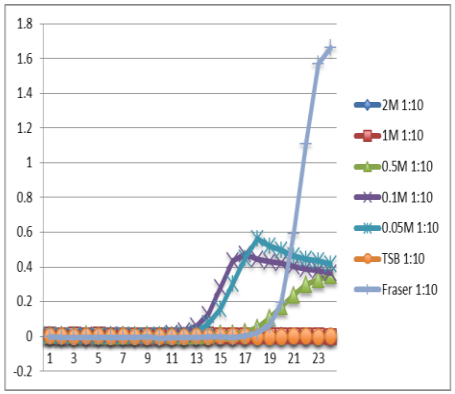

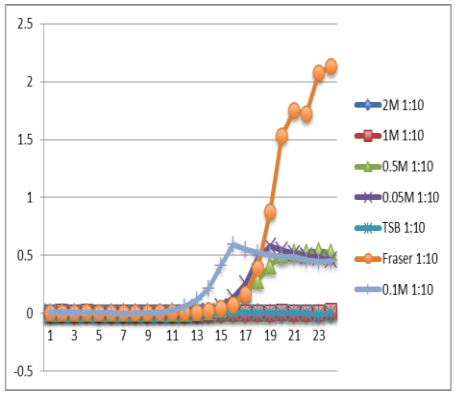

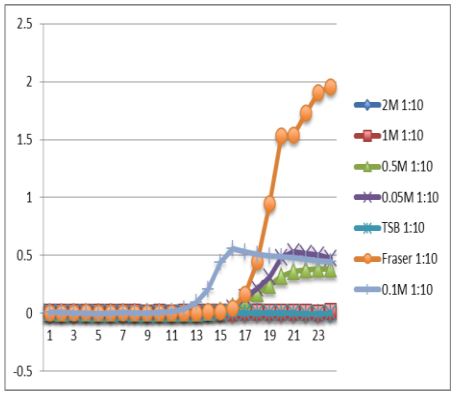

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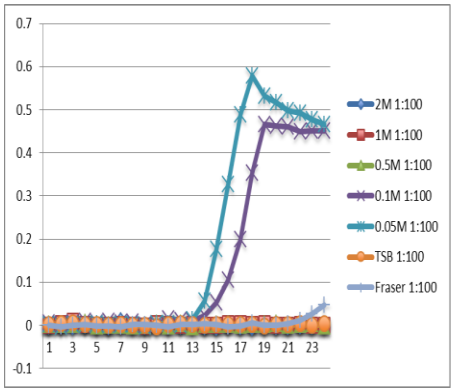

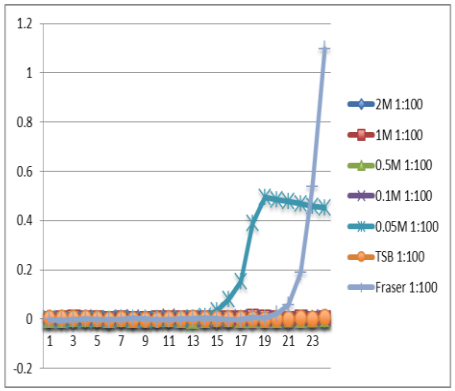

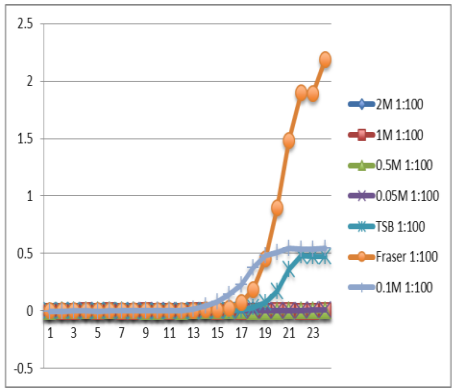

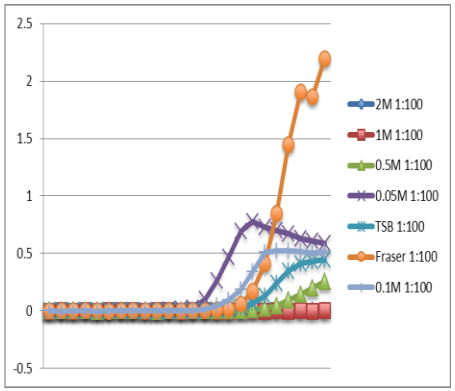

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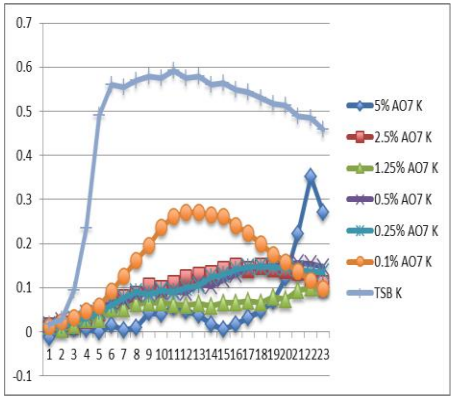

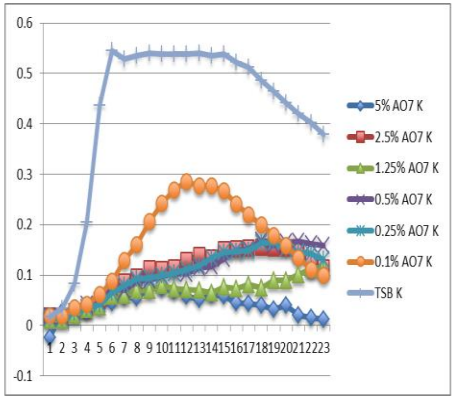

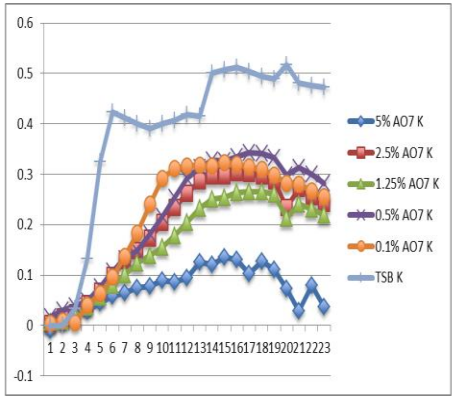

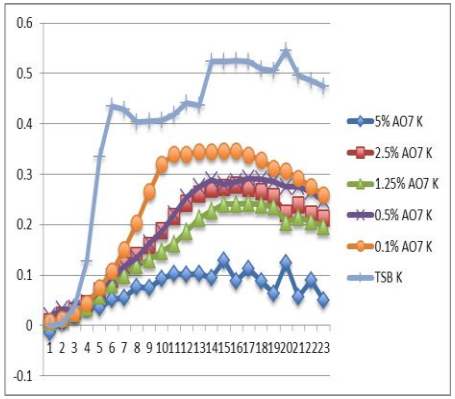

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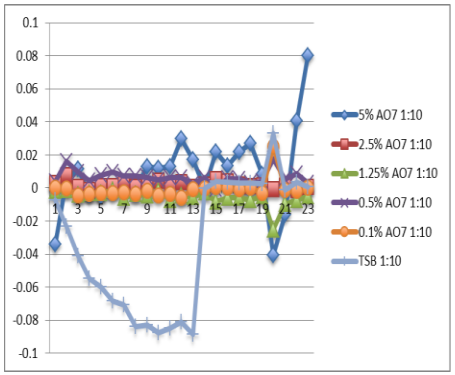

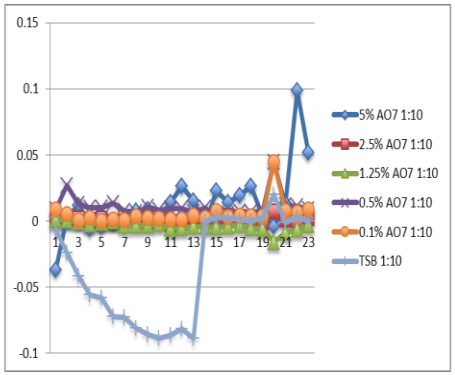

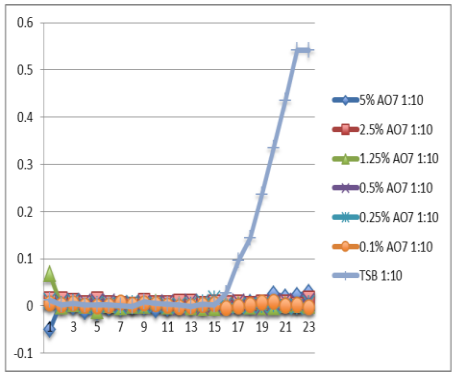

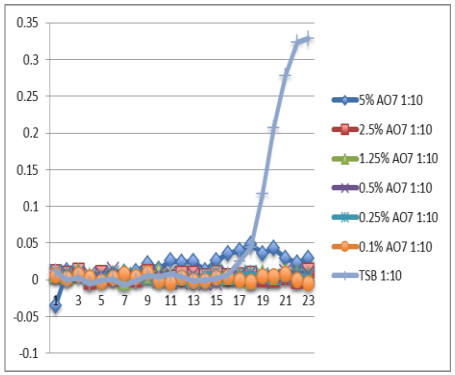

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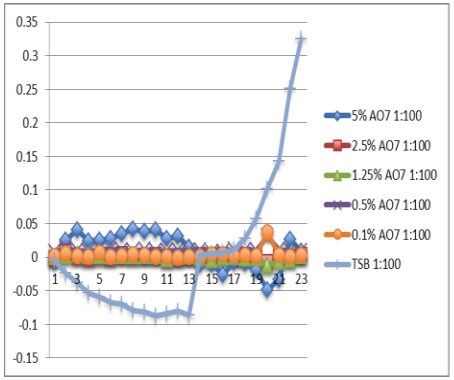
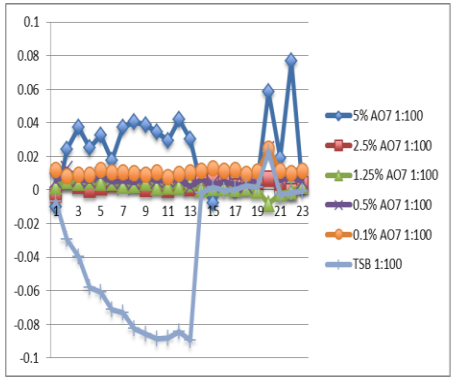
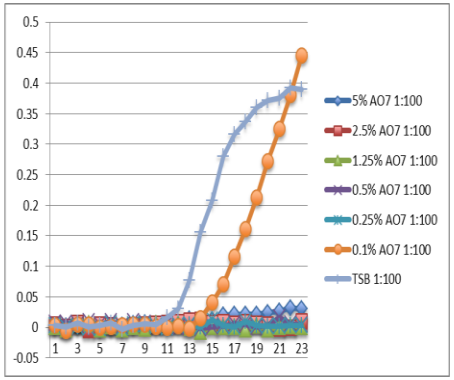
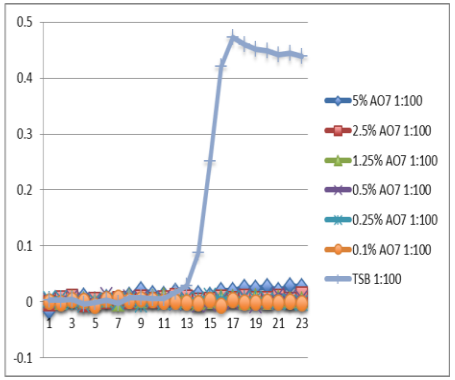
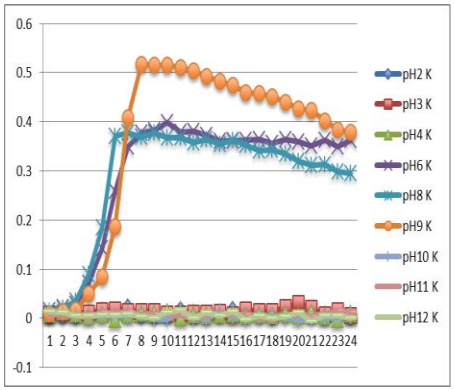


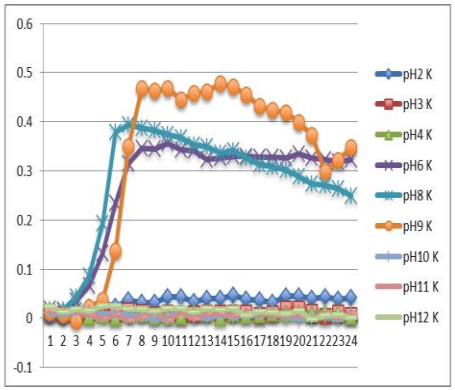


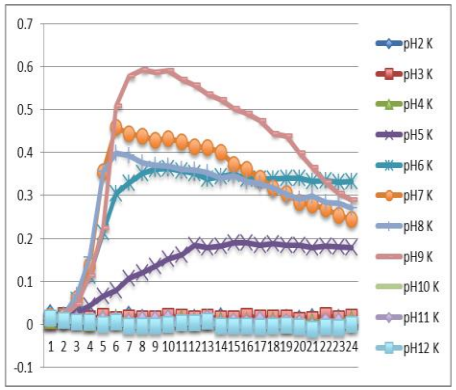


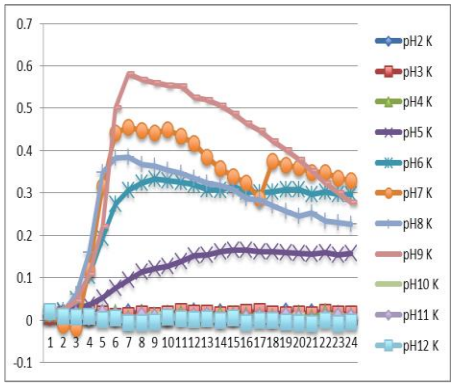


pH control

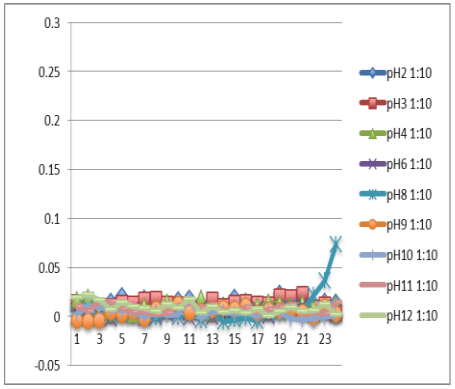


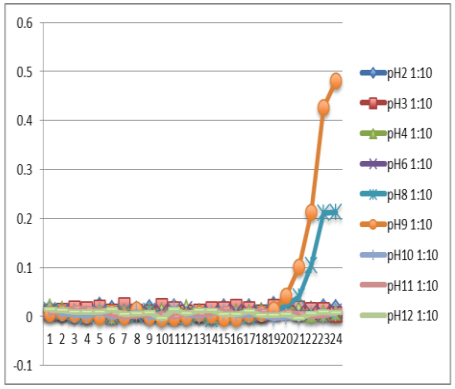


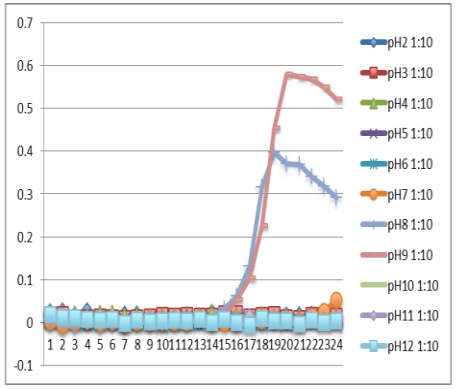


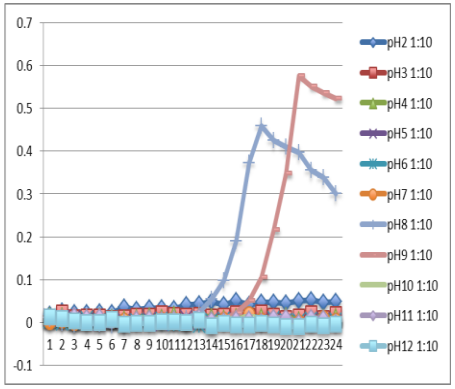


pH 1:10

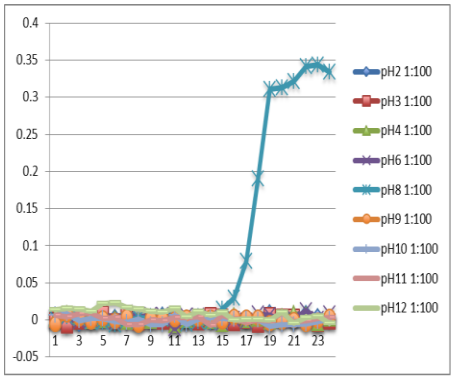








pH 1:100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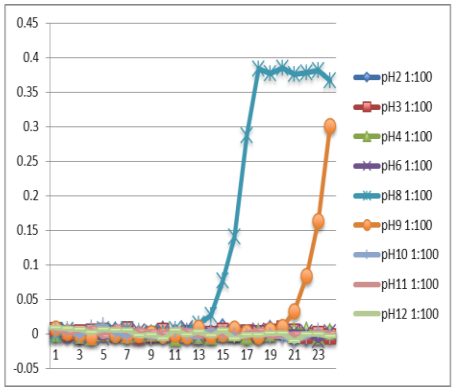
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
1 3 5 7 9 11 13 15 17 19 21 23
pH2 1:100
pH3 1:100
pH4 1:100
pH6 1:100
pH8 1:100
pH9 1:100
pH10 1:100
pH11 1:100
pH12 1:100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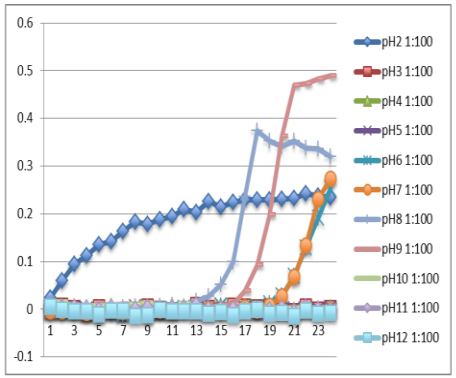
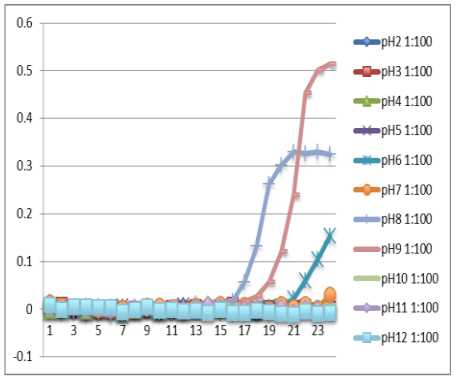
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
1 3 5 7 9 11 13 15 17 19 21 23
pH2 1:100
pH3 1:100
pH4 1:100
pH6 1:100
pH8 1:100
pH9 1:100
pH10 1:100
pH11 1:100
pH12 1:100


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
1 3 5 7 9 11 13 15 17 19 21 23
pH2 1:100
pH3 1:100
pH4 1:100
pH5 1:100
pH6 1:100
pH7 1:100
pH8 1:100
pH9 1:100
pH10 1:100
pH11 1:100
pH12 1:100


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
1 3 5 7 9 11 13 15 17 19 21 23
pH2 1:100
pH3 1:100
pH4 1:100
pH5 1:100
pH6 1:100
pH7 1:100
pH8 1:100
pH9 1:100
pH10 1:100
pH11 1:100
pH12 1:100