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Combination of bacteriophage lysins and high hydrostatic pressure for the inactivation of *Listeria monocytogenes* and *Staphylococcus aureus*

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ABBREVIATIONS*

AF-4	Asymmetric flow field-flow fractionation
AI	Aggregation index
ATCC	American Type Culture Collection
B.	*Bacillus*
C.	*Clostridium*
CBD	Cell wall-binding domain
CFA	Cyclopropane-fatty-acyl
cfu	Colony forming units
Da	Dalton
E.	*Escherichia*
EAD	Enzymatically active domain
g_0	Standard acceleration of gravity (9.81 m/s^2)
GlcNAc	N-acetylglucosamine
h	Hour
HHP	High hydrostatic pressure
IPB	Imidazole phosphate buffer
L.	*Listeria*
Lac.	*Lactobacillus*
LPM	Lithium chloride phenylethanol moxalactam
M	Molar (mol/L)
min	Minute
M_r	Molecular mass
MurNAc	N-acetylmuramic acid
N	Total cell count (cells/mL)
N_0	Inoculum (cells/mL)
OD	Optical density
P.	*Pseudomonas*
PAGE	Polyacrylamide gel electrophoresis
PI	Propidium iodide

RPM	Rounds per minute
RT	Room temperature
RTE	Ready-to-eat
S.	*Staphylococcus*
Sal.	*Salmonella*
SDS	Sodium dodecyl sulfate
sec	Second
SM	Sodium magnesium
TMW	Technische Mikrobiologie Weihenstephan
TSAYE	Tryptic soy agar yeast extract
TSBYE	Tryptic soy broth yeast extract
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WSLC	Weihenstephan Strain Listeria Collection

*standard SE symbols, prefixes, and abbreviations are not provided

CONTENTS

INTRODUCTION

In this work, both bacteriophages and their lysins (i.e. endolysins) were investigated in combination with high hydrostatic pressure (HHP) for the inactivation of *Listeria (L.) monocytogenes* and *Staphylococcus (S.) aureus* in buffer and foods. This chapter provides a comprehensive overview with basic knowledge, widely accepted principles, and latest scientific insights about these foodborne pathogens, HHP inactivation of bacteria, and bacteriophages plus their lysins.

1.1 The foodborne pathogen *Listeria monocytogenes*

1.1.1 Organism

The Gram-positive, non-spore forming, facultatively anaerobic, rod shaped bacterium *L. monocytogenes* is able to survive and proliferate in a wide range of environmental conditions. The bacterium can grow in a pH range of 5-9, NaCl concentrations of up to 10%, and at temperatures ranging from <0 to 45 °C (McLauchlin, 2006). Listeria are ubiquitous present in the environment and have been isolated from many different sources, including soil, decaying vegetation, and a large variety of foods (Weis and Seeliger, 1975).

The genus *Listeria* is subdivided into six species of which *L. monocytogenes* is almost exclusively responsible for human infections. The species *L. monocytogenes* consists of 13 serotypes where numerical aspects (i.e. 1/2, 3, 4, etc.) refer to somatic antigens and the letters a, b, c, and d to flagellar antigens (Farber and Peterkin, 1991). Serotypes 1/2a, 1/2b, and 4b have been found to be responsible for most human infections (McLauchlin, 2006, Pagotto et al., 2006, EFSA, 2015). The underlying reason for this serotype specific virulence is however unclear.

1.1.2 Virulence and pathogenesis

The disease caused by *Listeria spp.* is called listeriosis and is almost exclusively the result of the consumption of contaminated food. Listeria primarily infect individuals with suppressed cellular immunity, but healthy individuals can also suffer from listerial gastroenteritis after ingestion of a high doses of cells (Paoli et al., 2005). In people with immunocompromised conditions, the most common manifestations of listeriosis include septicaemia and meningitis. Clinical presentation for pregnant women may be fever and other non-specific symptoms such as fatigue and headache. Pregnant women are also at risk of spontaneous abortion or stillbirth, whereas bacteraemia and meningitis might be present in new-borns of mothers with listeriosis. Healthy individuals may experience acute febrile gastroenteritis or the disease might pass without manifestation of clinical symptoms (Meštrović, 2015). The minimum infectious dose for listeriosis is not known, but levels of only 10 cells per gram food have already been associated with the diseases in susceptible individuals.

The primary infection site for invasive listeriosis is the gastrointestinal tract. Ingested listeria cells therefore first need to survive the harsh conditions of the stomach and upper gastrointestinal tract. In the small intestine, invasion takes place and the cells can translocate via two pathways: they either actively adhere to and invade the intestinal epithelium after which phagocytic cells engulf and transport the bacteria to the blood, or, they colonize Peyer's patches where macrophages facilitate their entry through the lymphatic tissues (Paoli et al., 2005, Pagotto et al., 2006). From here, *L. monocytogenes* can enter the lymph nodes and disseminate via the blood stream to other organs, where the infection can further spread from cell-to-cell and even pass through the blood-brain or placental barrier causing neurological or pregnancy-related complications. Although incidence of this

disease is relatively low, it is among the leading causes of death from foodborne illness (Kirk et al., 2014, EFSA, 2015) and has the highest cost-of-illness per infected case of all food pathogens (Mangen et al., 2015).

1.1.3 Epidemiology and occurrence in foods

L. monocytogenes is capable of growth over a wide pH and salt range and even survives and grows at refrigerator temperatures. This makes it of special concern for raw or minimally processed ready-to-eat (RTE) foods which are not heated prior to consumption. The European Union microbiological limit for *L. monocytogenes* in RTE foods able to support growth is set at ≤100 colony forming units (cfu) per gram food product during the product's shelf life, or, its absence in 25 g before leaving the producing food business operator (OJ, 2005). A limit of 100 cfu/g is set for RTE foods which do not support growth of *L. monocytogenes* (though absent in those foods intended for infants and special medical purposes). The legal criteria in the USA and Canada are even stricter. These countries they have adopted a zero tolerance policy for *L. monocytogenes* in all ready-to-eat foods, meaning that the complete absence of this bacterium in 25 g of sample is required (USDA FSIS, 2014). Although listeriosis is still relatively rare, it had the highest reported number of deaths of all zoonoses in the European Union in 2013; a total of 13 outbreaks were reported with 1763 confirmed cases, of which, 191 persons died (Table 1).

Table 1: Reported cases and deaths due to zoönoses in the EU, 2013. Adapted from EFSA, 2015.

Disease	Number of confirmed human cases	Hospitalization rate (%)	Reported deaths	Case-fatality rate (%)
Campylobacteriosis	214779	43.6	56	0.05
Salmonellosis	82694	36.0	59	0.14
Yersiniosis	6471	48.4	2	0.05
VTEC infections	6043	37.1	13	0.36
Listeriosis	1763	99.1	191	15.6
Echinococcosis	794	70.6	2	0.88
Q fever	648	NA	2	0.61
Brucellosis	357	70.6	1	0.99
Tularaemia	279	52.0	0	0.00
West Nile fever(a)	250	91.7	16	3.4
Trichinellosis	217	65.4	1	0.56
Rabies	1	100	1	100

The most common food categories related to *L. monocytogenes* outbreaks include raw and processed meat, dairy products, vegetables and fruits, and seafood products. A list with exemplary listeriosis outbreaks from 1981 to 2002 is provided in Table 2. Food products related to more recent major outbreaks of listeriosis include caramel apple (2014, 35 infected, 7 death) and cantaloupe (2011, 147 infected, 33 deaths; HHS CDC, 2016) in the USA, processed meats such as salami and hot dogs in Denmark (2014, 38 infected, 15 deaths; News Desk, 2014), and cold meat cuts in Canada (2008, 56 infected, 20 deaths; PHAC, 2008).

Contamination with listeria in final products can result from either incomplete elimination during processing or from contamination sites within the food production environment. In the production of smoked salmon, the slicing machines and working tables, rather than the raw material, were determined to have the highest *L. monocytogenes* contamination levels (Tocmo et al., 2014). This is especially problematic since it has been shown that, once introduced into a food processing facility, listeria can colonize floor drains, standing water, form biofilms on processing surfaces, and survive for

many years (Paoli et al., 2005). It is therefore not surprising that the food processing environment has been identified as of prime importance in prevention of listeriosis outbreaks (McLauchlin, 2006).

Table 2: Listeriosis outbreaks. Epidemiological information for a selection of published foodborne listeriosis outbreaks from 1981-2002. Adapted from Paoli et al., 2005.

Location	Year(s)	Cases	Deaths	Serotype	Food
Canada	1981	41	18	4b	Coleslaw
USA	1983	49	14	4b	Milk
Switzerland	1983-87	122	34	4b	Cheese
USA	1985	142	48	4b	Cheese
United Kingdom	1989-90	300	0	4b	Pâté
France	1992	279	88	4b	Pork tongue
France	1993	39	8	4b	Pork pâté
France	1995	36	8	4b	Soft cheese
USA	1998-99	40	4	4b	Dele meats
Finland	1988-99	25	6	3a	Butter
France	1999	29	7	n.r.*	Pork tongue
USA	2000	29	4	4b	Turkey deli meats
USA	2000-01	12	0	4b	Cheese
USA	2002	46	7	n.r	Chicken and turkey
Canada	2002	17	0	n.r	Cheese

*n.r. serotype not reported

1.2 The foodborne pathogen *Staphylococcus aureus*

1.2.1 Organism

Staphylococci are ubiquitous, commonly found in the environment (soil, water and air), and also present in the nose and on the skin of over 50% of healthy human individuals and many different animals. This mesophilic bacterium can grow in a temperature range of 7 to 48 °C (optimally 37 °C) and a pH range of 4-10 (optimally 6-7). The optimal water activity is 0.98, but growth also occurs at water activities as low as 0.83, making it one of the most halotolerant food pathogens known (Stewart et al., 2002, Stewart, 2005, FDA CFSAN, 2012).

Staphylococcal species are Gram-positive, non-motile, catalase-positive, spherical cocci, which tend to remain associated after division to form grape-like clusters. *Staphylococcus* spp. is subdivided into 32 species and subspecies. Only four species, including *S. aureus*, can produce coagulase. *S. aureus* can be further distinguished by its capability to produce thermonuclease, ferment mannitol both aerobically and anaerobically, and produce protein A and acetoin. Several staphylococci can produce enterotoxins and, although *S. intermedius* has been involved in some food outbreaks, *S. aureus* is predominantly associated with staphylococcal food poisoning (Stewart, 2005).

1.2.2 Virulence and pathogenesis

Staphylococcal food poisoning is an intoxication, not infection, caused by the ingestion of food containing enterotoxins (Stewart, 2005). Staphylococcal enterotoxins are proteins with a molecular weight of approximately 26 to 29 kDa which are produced and excreted during growth of the organism. Though, the temperature, pH, and water activity range in which enterotoxins are produced is somewhat smaller than for growth of the organism (FDA CFSAN, 2012). Staphylococcal enterotoxins are resistant towards most proteolytic enzymes and highly temperature stable; pasteurization hardly affects the enterotoxin and even boiling for 30 minutes does not inactive the toxin (Jordan et al., 1931). Most enterotoxins contain a highly flexible disulphide loop which aids in their stability and is also thought to be involved in their ability to induce vomiting (Stewart, 2005).

Onset of staphylococcal food poisoning appears around 1 to 7 hours after ingestion of contaminated food and the most common symptoms include nausea, abdominal cramping, vomiting and diarrhoea. In addition, enterotoxins also induce an inflammatory response believed to be the result of a direct binding to MHCII molecules (instead of the regular route where bacterial peptides are presented by antigen presenting cells). This direct binding activates a much higher percentage of T cells than a conventional inflammatory response induced by antigen presenting cells, which results in the production of a large amount of pro-inflammatory cytokines causing clinical symptoms such as fever and shock. The intoxication dose of staphylococcal enterotoxin is said to be less than 1 µg (FDA CFSAN, 2012), is however dependent on the individual, type of enterotoxin, and other environmental conditions such as temperature and type of ingested food.

1.2.3 Epidemiology and occurrence in foods

Staphylococcal food poisoning is considered to be one of the most common foodborne diseases worldwide (Hennekinne et al., 2012; a list of typical outbreaks from 1968 to 2009 is provided in Table 3). In 2013, a total of 393 foodborne outbreaks caused by staphylococcal enterotoxins were reported within the EU member states (2952 cases, 2 deaths; EFSA, 2015). Staphylococcal food poisoning is a short-term disease and usually results in full recovery without any medication within 1-2 days. The actual number of outbreak is, as a result of this quick recovery time, thought to be much higher than reported (Hennekinne et al., 2012).

The foods most at risk of *S. aureus* contamination are those which require considerable handling and the people which handle the food are the usual source of contamination. This makes *S. aureus* very different from most other food pathogens, including *L. monocytogenes*, where the animal, product itself, or production sites are the usual source of contamination. Improper storage of foods contaminated with staphylococci for only several hours can results in growth of the microorganism and production of enterotoxins. Although the bacterial cells can be killed by subsequent heating, the heat-stable enterotoxins will remain active. This also explains why foods involved in outbreaks might not have culturable organism when served.

The most common food categories implicated in staphylococcal food poisoning include meat and meat products such as ham or fermented sausages, poultry and egg products, salads such as tuna or chicken, bakery products such as cream-filled pastries or cream pies, and milk and dairy products. Although it is unusual for commercially processed foods to be implicated in staphylococcal food poisoning, a number of cases are present (Bergdoll and Lee Wong, 2006). These were, however, not due to failures of the preservation technique, but mistakes in the production line (e.g. not completely closed cans or elevated temperatures during storage of raw milk before pasteurization).

Table 3: Staphylococcal food poisoning outbreaks. Epidemiological information for a selection of published Staphylococcal foodborne outbreaks from 1968-2009. Adapted with permission from **Hennekinne et al., 2012**. Copyright © 2012, Oxford University Press

Location	Year	Cases	Food
Canada	1980	62	Cheese curd
Scotland	1984	27	Sheep's milk cheese
USA	1985	>1000	Chocolate milk
Thailand	1990	485	Eclairs
Brazil	1998	4000	Chicken, roast beef, rice, and beans
Japan	2000	13420	Low-fat milk
France	2006	17	Coco nut pearls
Belgium	2007	15	Hamburger
France	2008	100	Pasta Salad
Japan	2009	75	Crepes

1.3 High hydrostatic pressure processing

1.3.1 Technology description

The principle goal of any food preservation technique is to eradicate pathogenic and reduce spoilage microorganisms to enhance the product's safety and improve the shelf-life, respectively. Probably the most common food preservation method, both historically and at present, is thermal treatment. Although heating effectively reduces microorganism in foods, this process can also affect the product negatively by loss of natural taste and flavour, destruction of vitamins, and changes in colour and texture.

The higher demand for natural and fresh foods has often been described as a recent trend and reason for the increased attention for new and emerging preservation technologies. The negative aspects of thermal treatments on food quality and need for mild preservation techniques have however been recognized much longer. In 1899, Hite already described the negative impact of thermal treatment on milk's organoleptic properties and was the first to show that pressure can be used as an alternative preservation technique. Although the potential of high pressure processing as preservation technique was thus already recognized in the late nineteenth century, it was not until the early nineties from the twentiest century before the first commercial high pressure processed foods (i.e. fruit jams in plastic cups) were available on the market (Tonello, 2011). Since then, the commercial preservation of foods by high pressure technology steadily increased. Different high-pressure treated food products (e.g. fruit juices, avocado, or different RTE meat products such as tapas and dry hams) are now commercially available all over the world.

HHP is a non-thermal food sterilization and preservation technology. Industrial HHP processing is either a batch (solid and liquid products) or semi-continuous system (only liquid products). In a typical HHP batch treatment, foods are sealed, placed into a vessel filled with pressure-transmitting liquid, after which the pressure is either increased by pumps or by a reduction in the volume of the pressure chamber (e.g. due to piston movement). For semi-continuous systems, the product is pumped in and out of the processing vessel and (aseptically) filled after pressure treatment. For batch systems, the product can be treated in its final package, which eliminates the risk of recontamination by handling of the product after processing. The packaging material should however be able to withstand volume changes since the food decreases in volume as a function of the pressure applied (e.g. the volume of water is reduced by ca. 15% at 600 MPa; Heremans, 2006).

The most common pressure-transmitting fluid used is water, but fluids containing silicone oil, ethanol, or glycol are also used. The preferred pressure-transmitting fluid depends on multiple factors, including the required temperature range and viscosity of the fluid under pressure. The type of pressure-transmitting fluid can also directly influence the efficacy of microbial inactivation, for example due to differences in adiabatic heating (Balasubramanian and Balasubramaniam, 2003, Georget et al., 2015). Adiabatic heating is not only dependent on the pressure-transmitting fluid, but also on the composition of the sample under pressure. The temperature of foods containing a higher amount of fat is known to rise more compared to their low-fat equivalents (Otero et al., 2000, de Heij et al., 2003, Hogan et al., 2005, Balasubramaniam et al., 2015). Although the pressure-transmitting fluid plays a role in microbial inactivation by HHP, the main determinants are the pressure level, pressure holding time, compression and decompression rates, and treatment temperature.

Two basic theoretical principles apply to HHP processing (Hogan et al., 2005). The first principle, the Isostatic Rule, states that pressure is instantaneously and uniformly transmitted throughout a sample. Therefore, in contrast to thermal treatment, HHP acts instantaneously and uniformly throughout a mass of food independent of size, shape, and food composition. The fact that HHP works uniformly over the whole product is beneficial over other novel non-thermal food preservation methods, such as Pulsed Electric Field, which only act on the surface. The second theoretical principle applies to microbial inactivation; le Chatelier's principle states that when pressure increases, reactions with a given equilibrium will shift to the side that occupies the smallest volume. High pressure thus stimulates phenomena (e.g. phase transitions, chemical reactions, and changes in protein folding) that are accompanied by a decrease in volume. This relation between pressure and volume changes is argued to be responsible for most of the effects of pressure on microorganisms (Follonier et al., 2012).

Covalent bonds are pressure-insensitive up to values of 1000-1500 MPa (Mozhaev et al., 1996). The structure of small molecules (e.g. peptides, lipids, saccharides) and the primary structure of macromolecules (e.g. proteins, nucleic acids, polysaccharides) is therefore not expected to change in the pressure range relevant for food preservation (100-800 MPa; Follonier et al., 2012). The effect of pressure on weaker bonds depends on the type of molecular interaction and the associated volume change. For example, hydrogen bonds are known to be stabilized by pressure whereas hydrophobic interactions are weakened (Meersman and Heremans, 2008). These pressure-induced changes in weak bonds can modify the conformation of macromolecules and the interaction between them.

1.3.2 High pressure inactivation of bacteria

1.3.2.1 Cellular components

Among microorganisms, the inactivation of vegetative bacteria by pressure is most widely studied and believed to be the result of multi-factorial processes. Although pressure stress affects all levels of cellular physiology, disturbance of the cellular membrane (i.e. disturbance of the hydrophobic interactions between the phospholipids) has often been identified as one of the primary sites for pressure-induced inactivation of bacteria (McClements et al., 2001, Winter and Jeworrek, 2009,Kato et al., 2008). As a result of increasing pressure, the fluidity of phospholipid bilayers is reduced and a shift from the liquid crystalline to the gel state can be observed (Hazel and Williams, 1990, Meersman and Heremans, 2008). The pressure-induced shift towards gel state is thought to affect the functionality of the membrane at many different levels: it can weaken protein-lipid interactions required for optimal function of critical cellular processes (Winter and Jeworrek, 2009), results in the

loss of transmembrane gradients (Wouters et al., 1998), and causes physical damage as shown by leakage of ATP and uptake of membrane impermeable fluorescent dyes (Smelt et al., 1994). It is therefore generally recognized that the membrane must maintain the fluid state to sustain its critical functions and properties (Meersman et al., 2006, Rendueles et al., 2011, Mota et al., 2013).

Membranes of barophiles, barotolerants, and microorganisms better adapted to pressure stress (e.g. due to growth under specific conditions) were found to have a greater degree of unsaturated fatty acids in their membrane (Yano et al., 1998, Valentine and Valentine, 2004, Rendueles et al., 2011). Incorporation of unsaturated fatty acids can increase the fluidity of the bacterial membrane. Hence, the presence of unsaturated fatty acids in the membrane is thought to play an important role in the maintenance of fluidity under pressure and thereby pressure resistance. Casadei et al., 2002 showed that an increase in unsaturated fatty acids (induced by growth of cells at low temperature) was directly related to an increased pressure-resistance of exponential-phase *Escherichia* (*E.*) *coli* cells. Surprisingly, exactly the opposite was true for stationary-phase cells; an increase in unsaturated fatty acids correlated with a lower pressure-resistance. Hence, the authors speculated that other changes in the cellular membrane might also affect the pressure resistance of stationary-phase cells (see section 1.3.2.2.3 for further discussion). This is further strengthened by the work of Pagán and Mackey, 2000, which found a similar discrepancy between exponential- and stationary-phase *E. coli* cells for the pressure-induced loss of membrane integrity. In brief, the authors found that pressure-induced membrane damage plays a direct role in the inactivation of exponential-phase *E. coli* cells (as shown by PI staining). For stationary-phase cells, although loss of membrane integrity during pressure treatment was already observed at 100 MPa, no inactivation took place up to pressures levels of 500 MPa. Moreover, in contrast to exponential-phase cells, the membranes of stationary-phase cells became leaky during pressure treatment but could 'reseal' after decompression. This indicates a fundamental difference in the role of the membrane in the pressure inactivation of exponential- and stationary-phase *E. coli* cells. In summary, whereas the loss of membrane integrity seems to be detrimental for exponential-phase *E. coli* cells, the studies discussed above showed that it is not necessarily directly related to pressure-inactivation of stationary-phase cells and others factors might be involved.

Proteins play an essential role throughout the whole cell and pressure-induced changes in their folding and functionality affect many cellular processes, including cellular architecture and cell division, flagellar motility, transcription and translation, and energy metabolism (Mota et al., 2013). Although pressure-induced protein unfolding or denaturation is not completely understood, it is generally argued to be the result of electrostriction (higher compression of water as result of the interaction between formed ions and the water dipoles) and elimination of internal cavities present in the quaternary, and to a lesser extent tertiary, structure of the protein (Follonier et al., 2012), which is schematically depicted in Figure 1. The quaternary structure is mainly maintained by pressure-sensitive hydrophobic interactions. As such, monomeric enzymes are usually more resistant to pressure than multimeric enzymes for which the proper complex formation is mostly dependent on hydrophobic interactions (Hendrickx and Knorr, 2006). For example, the tetrameric *E. coli* phosphofructokinase dissociates reversibly under a pressure of 80 MPa (Wouters et al., 1998), whereas pressures of >500 MPa are required to inactivate the monomeric *Bacillus subtilis* α-amylase (Simpson and Gilmour, 1997).

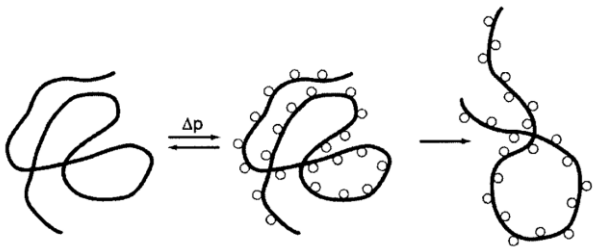


Figure 1: Elimination of protein internal cavities by HHP. Schematic representation of pressure-induced denaturation of a protein with water molecules depicted as circles. Adapted with permission from **Meersman and Heremans, 2008**.

Transcription and translation processes are also affected by pressure, especially those in which multimeric complexes that are held together by pressure sensitive interactions play a crucial role. The dissociation of ribosome subunits by pressure has, for example, been directly linked to loss of cell viability (Niven et al., 1999). DNA is relatively pressure resistant, though, replication processes were argued to be inhibited under high pressure since DNA becomes more densely packed (Mota et al., 2013). Although many more cellular changes occur upon high pressure treatment of vegetative bacterial cells (e.g. an increase in oxidative stress or reduced uptake of amino acids), the processes described above are currently recognized in scientific literature as the main mechanisms involved in the pressure-induced inactivation of these microorganisms.

1.3.2.2 High pressure inactivation and resistance

1.3.2.2.1 HHP parameters

The main parameters which determine bacterial inactivation by HHP are the pressure level, pressure holding time, compression and decompression rates, and treatment temperature. In summary, it can be stated that the inactivation of bacteria increases with higher pressure levels, longer holding times, shorter compression and decompression times, and an increase in temperature (Hogan et al., 2005, Syed et al., 2015, Smelt, 1998, Patterson and Linton, 2008). Though, several exceptions exist; it has for example been shown that higher temperatures not always lead to higher bacterial inactivation. Ritz et al., 2008 demonstrated that the inactivation of *L. monocytogenes* in smoked salmon was more effective at subzero temperatures than at 4 °C and also López-Caballero et al., 2002 found that inactivation of *P. fluorescens* was higher at a pressure treatment temperature of 5 °C than at 20 or 35 °C. These studies stress the complexity but also opportunities found in food preservation. Instead of higher temperatures (which could have a negative impact on the product), subzero treatment temperatures might be used in combination with HHP to kill microorganisms more effectively (Ponce et al., 1998; i.e. when freezing itself is not detrimental to the structure of the food product).

1.3.2.2.2 Species and strain variation

Bacterial inactivation is, in addition to the chosen HHP parameters, in large part dependent on the type of microorganism. There is a large variation between the pressure-resistance of different bacterial species and also between strains of the same species (Simpson and Gilmour, 1997, Tay et al., 2003, Patterson et al., 2011). In a study towards the inactivation of different *L. monocytogenes* and *S. aureus* strains, large differences of up to 7 log cycles by the same pressure treatment were found between strains within one species (Table 4; Alpas et al., 1999).

Table 4: Pressure inactivation of *L. monocytogenes* and *S. aureus* strains. Viability loss of multiple *L. monocytogenes* and *S. aureus* strains following pressurization at 345 MPa for 5 min at 25 °C. Adapted with permission from **Alpas et al., 1999**.

L. monocytogenes (strain)	Viability loss ($log_{10}(N_0/N)$)	*S. aureus* (strain)	Viability loss ($log_{10}(N_0/N)$)
CA	0.92	778	0.70
ScottA	0.96	485	0.70
Camp+ Beta+	1.74	743	0.86
V7	1.93	315	0.90
35091	2.10	565	1.12
117	2.48	765	1.51
103	2.49	582	7.80
$Ohio_2$	2.80		

Generally, Gram-positive bacteria are considered to be more pressure-resistance than Gram-negative bacteria as a result of the chemical composition and structural properties of their cell wall: a thick protective/stabilizing peptidoglycan layer (Hogan et al., 2005). Though, cases of highly pressure resistant Gram-negative and more susceptible Gram-positive bacteria are also known. For example, one study showed that a pressure level of 375 MPa (15 min, 20 °C) could reduce the Gram-positive bacterium *L. monocytogenes* by 10^5, whereas 700 MPa was needed to achieve a similar inactivation of the Gram-negative bacterium *E. coli* (Patterson et al., 1995).

1.3.2.2.3 Growth phase and conditions

Cells in the stationary phase have often been shown to be more pressure resistant than their exponential phase counterparts (McClements et al., 2001, Patterson, 2005, Pagán and Mackey, 2000, Hayman et al., 2007). Though, studies where exponential-phase cells were found to be more pressure resistant are also present (Juck et al., 2012). Changes in the cell envelope are thought to play a central role in the variability of pressure resistance between growth phases. In this light, it has been proposed that pressure-induced death of exponential-phase *E. coli* cells is the result of irreparable physical membrane damage, whereas death of stationary-phase cells would be due to a more subtle loss of the membrane properties (e.g. energy conservation or ion flux; Pagán and Mackey, 2000). Others found the membrane phospholipid composition to play a crucial role in the different pressure resistance of *E. coli* cells from different growth phases (Casadei et al., 2002, Charoenwong et al., 2011).

Another component argued to be involved in the increased pressure resistance of stationary-phase *E. coli* cells was found to be the RpoS-regulated cyclopropane fatty acyl phospholipid synthase (Casadei et al., 2002). The synthesis of cyclopropane fatty acids (CFA) is a modification of the membrane phospholipids that occurs in early stationary-phase and encompasses the transfer of a methylene group from S-adenosyl-methionine to the cis double bonds of unsaturated fatty acids in membrane phospholipids. The degree of CFA in the membrane of *E. coli* grown at different temperatures was shown to correlate with pressure-resistance of stationary-phase cells, whereas the CFA content did not vary much for exponential-phase cells grown at different temperatures (despite large difference in its pressure resistance; Casadei et al., 2002). By generation of *cfa*-deficient mutant strains, it was shown that the absence of CFA in the cytoplasmic membrane (and the accumulation of the unsaturated fatty acid substrate) made stationary-phase *E. coli* cells more sensitive to several environmental stressors, including HHP (Chen and Gänzle, 2016). In the same study, CFAs were predicted to enhance membrane fluidity and at the same time induce a greater degree of order than unsaturated fatty acids. This allows for both the stabilization of membranes against adverse conditions while simultaneously promoting their fluidity; two events which could be mutually responsible for the

increased pressure-resistance of stationary-phase *E. coli* cells (despite having a lower level of unsaturated fatty acids).

As already mentioned above, growth temperature largely influences pressure resistance. Juck et al., 2012 showed that *L. monocytogenes*, *E. coli*, or *Salmonella (Sal.) enterica* cells from different growth phases were generally more barotolerant when grown at 40 °C compared to cells grown at 15-35 °C. Other studies have shown a more complex influence of growth temperature on pressure inactivation. Both McClements et al., 2001 (*L. monocytogenes* and *P. fluorescence*) and Casadei et al., 2002 (*E. coli*) found that stationary-phase cells grown at higher temperatures where indeed more resistant than those grown at lower temperatures, but that exactly the opposite was true for exponential-phase cells. These differences can most likely be attributed to differences in membrane fluidity and fatty acid content as a result of the different growth temperatures.

1.3.2.2.4 Pressure resistance

Bacteria have an intrinsic pressure-tolerance but it is also a trait that can be selectively acquired. A single HHP shock could already select for a small fraction of spontaneous *L. monocytogenes* mutants (Karatzas and Bennik, 2002). This subpopulation did not only have an increased pressure tolerance with retained for at least 40 generations, but was also able to cope better with a great variety of other stresses (e.g. heat, H_2O_2, or acid). For *E. coli,* an extremely HHP resistant mutant (surviving pressure of up to 2000 MPa compared to only 600 MPa for the wildtype) was isolated by a selective enrichment approach based on consecutive cycles of increasingly severe HHP shocks (Hauben et al., 1997, Vanlint et al., 2011). Interestingly, both the *L. monocytogenes* and *E. coli* isolates with increased pressure-resistance displayed derepression of one or more heat shock genes (Karatzas et al., 2003, Aertsen et al., 2004). In this light, prior heat shock has indeed been shown to protect both *E. coli* and *L. monocytogenes* against inactivation by HHP (Pagán and Mackey, 2000, Hayman et al., 2008a), which indicates that heat shock proteins might play an important role in pressure resistance, either by preventing cellular damage and/or aid in cell recovery (Welch et al., 1993).

Interestingly, the ability of bacteria to develop high pressure resistance differ between species and even strains of the same species. In a direct comparison of multiple *E. coli* strains, it was shown that only some of the strains were capable of developing extreme HHP resistance (Vanlint et al., 2012), which was postulated to be the result of a particular set of unknown genes.

The most pressure resistant bacteria known are bacterial endospores. Within this group, the bacterial spore former *Clostridium (C.) botulinum* is among the most pressure-resistant microorganism known and combinations of heat with HHP are usually required for its inactivation (Rendueles et al., 2011). Alternatively, bacterial spores can also be stimulated to germinate at mild pressure levels (below 300 MPa), after which their vegetative counterparts can be inactivated at milder heat-pressure combinations (Lenz and Vogel, 2015).

1.3.2.3 Substrate composition

The relative resistance of microorganisms is in part determined by intrinsic parameters of the substrate such as pH, water activity, or nutrient content. The effects of these parameters on the pressure inactivation of vegetative bacteria are discussed below.

1.3.2.3.1 pH

It is well established that a reduction in the pH of the treatment medium can enhance the efficacy of high pressure inactivation (Mackey et al., 1995, Garcia-Graells et al., 1998, Alpas et al., 2000, Ritz et

al., 2000, Koseki and Yamamoto, 2006a, Somolinos et al., 2008, Ogihara et al., 2009). For example, in a study where the pH of orange juice was systematically lowered, a pressure treatment of 550 MPa (5 min, 20 °C) was sufficient to reduce an *E. coli* cell population by 6 log cycles at pH <4.5 (Linton et al., 1999). At pH >4.5, HHP needed to be combined with other processing methods such as mild heat to achieve a similar inactivation. A low pH does not only enhance high pressure inactivation, but also inhibits growth of cells sublethally damaged by the pressure treatment (Pagán et al., 2001). The prevention of growth of sublethally damaged cells at low pH thereby further enhances the efficacy of bacterial high pressure inactivation. In contrast, bacterial growth at a low pH can increase its pressure-resistance. Wouters et al., 1998 for example demonstrated that cells grown at pH 5 were more barotolerant than cells grown at pH 7. The differences in resistance was explained by the better adaptation of cells grown at pH 5 to low pH environments by higher F(0)F(1) ATPase activity, better ability to maintain a pH gradient, or a higher acid efflux.

The regulation of intracellular pH is a fundamental requirement for the survival and viability of microorganisms. HHP can affect the intracellular pH of microorganisms at two levels (Molina-Gutierrez et al., 2002b): (i) as described in section 1.3.2.1, by increasing the permeability of the cytoplasmic membrane and inactivation of the enzymes required for pH homeostasis, and, (ii) by enhancing the dissociation of weak organic acids which results in a pH decrease in most biological systems. Not only the intracellular pH, but also the pH of the treatment medium is influenced by high pressure (Quinlan and Reinhart, 2005). The dissociation constants of water and weak acids or weak bases depend on the absolute value of pressure and can result in either an increase (e.g. tris buffer) or decrease (e.g. phosphate buffer) in pH.

1.3.2.3.2 Water activity

Whereas the effect of pH on high pressure inactivation is relatively clear (a lower pH usually results in higher pressure-inactivation), the effect of the water activity is less unambiguous. On the one hand, bacteria have a higher pressure resistance at a lower water activity (Oxen and Knorr, 1993, Molina-Gutierrez et al., 2002a, Van Opstal et al., 2003, Molina-Höppner et al., 2004, Hayman et al., 2008b). On the other hand, as with a low pH, a lower water activity can also inhibit the recovery of sublethal damaged cells (Pascual et al., 2001). These opposing effects therefore make it difficult to predict the net result of a lowered water activity.

The mechanism by which a lower water activity confers baroprotection is dependent on the compound used to adjust the water activity. Smiddy et al., 2004 showed that *L. monocytogenes* cells mutated to be incapable of compatible solute uptake did not develop higher barotolerance upon NaCl stress (i.e. lower water activity), whereas wildtypes did. This suggests that baroprotection at elevated osmolarity is not a direct result of a lower water activity, but rather of compatible solute uptake as response to osmotic stress. Molina-Höppner et al., 2004 also found that ionic solutes, such as NaCl, provide asymmetric protection by inducing the intracellular accumulation of compatible solutes as a response to the osmotic stress. The mechanism by which sugars and other compatible solutes protect the cell from pressure-induced inactivation is not exactly known, but different possibilities have been suggested. The role of membrane fluidity in pressure resistance is widely recognized (1.3.2.1) and it was shown that higher pressure levels were required to induce a shift from the liquid crystalline to gel state when NaCl and sucrose were present in the treatment medium, which indicates a role for these compounds in membrane fluidity (Molina-Höppner et al., 2004). The inhibition of protein unfolding by substitution of the hydration shell through compatible solutes has also been put forward as possible

baroprotective mechanism (Smiddy et al., 2004). In summary, whereas the protective effect against HHP by ionic solutes (e.g. NaCl) probably relies on the intracellular accumulation of compatible solutes as a response to the osmotic stress, sugars can (additionally) confer a protective effect by stabilization of vital cellular components.

1.3.2.3.3 Nutrients

The presence of different nutrients is known to influence the microbial susceptibility towards high pressure. Generally, as has been shown by many groups, bacterial inactivation is less effective in foods compared to buffer systems (Patterson et al., 1995, Simpson and Gilmour, 1997, Smiddy et al., 2005). A lower inactivation by pressure in milk compared to buffer systems has been shown by multiple groups for a range of organisms, including *L. monocytogenes* (Styles et al., 1991, Dogan and Erkmen, 2004, Solomon and Hoover, 2004). Narisawa et al., 2008 investigated the protective effect of skimmed milk and its protein fractions (i.e. casein, whey, globulin, and albumin) on the inactivation of *E. coli* by HHP and postulated that the remaining solid fractions of the skimmed milk powder, rather than individual proteins tested, were responsible for this protective effect.

Black et al., 2007 could also not detect a baroprotective effect for *L. innocua* by the milk protein casein. Interestingly, micellar casein did provide a protective effect and the authors showed that this was the result of the minerals associated with the micelles (i.e. calcium, phosphate, citrate and magnesium), which were argued to stabilize the cytoplasmic membrane during pressure treatment. Another mechanism by which the micellar minerals were argued to influence high pressure inactivation of *L. innocua* is through an increase in the buffering capacity of the treatment medium, which would prevent a drop in pH during pressure treatment. The presence of Ca^{2+}, together with other divalent cations, has also been shown to reduce pressure inactivation of *E. coli* (Hauben et al., 1998). These authors suggested that Ca^{2+} could stabilize cellular targets which are normally inactivated under high pressure. Whereas the divalent cations discussed above seem to have a protective effect in vegetative bacteria, the role of Ca^{2+} in HHP resistance of spores is more ambivalent, with some studies reporting an increase in pressure resistance by removal of this mineral from mature spores (*B. subtilis*; Igura et al., 2003) and others upon their presence during sporulation (*C. botulinum*; Lenz and Vogel, 2014).

1.3.2.4 Pressure inactivation kinetics: tailing and sub-lethal damage

1.3.2.4.1 Tailing

Although there are some reports where pressure inactivation curves were described by first order kinetics (Mussa et al., 1998, Tassou et al., 2007), both vegetative bacteria and endospores usually follow non-linear inactivation kinetics (Cerf, 1977, Simpson and Gilmour, 1997, Tay et al., 2003, Sherry et al., 2004, Hayman et al., 2007). A typical pressure inactivation curve initially demonstrates a rapid decrease in total cell count followed by tailing, where there is little further inactivation with longer treatment time (Patterson and Linton, 2008, Rendueles et al., 2011). Non-linear microbial inactivation kinetics are one of the major challenges in the preservation of food products by HHP because it can result in the survival of a small subpopulation and higher pressure levels do not necessarily eliminate the tailing phenomenon (Tay et al., 2003, Jofré et al., 2010).

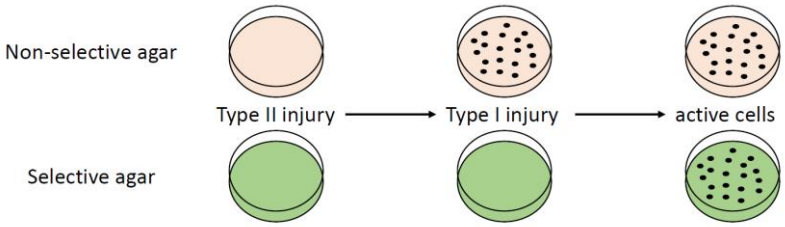
The tailing of survivor curves with increasing pressure holding times has been described already in the seventies of the last century. Nonetheless, mechanisms behind this phenomenon are still not well understood. Metrick et al., 1989 argued that tailing is the result of inherent phenotypic variation in a microbial population rather than the persistent presence of resistant cells. However, Noma et al., 2006

studied tailing in *E. coli* and found that cells isolated from the tail exhibited higher barotolerance compared to those of the original culture, indicating the existence of a pressure-resistant subpopulation mediated through genetic differences. Van Boeijen et al., 2008 also found that a small fraction of *L. monocytogenes* cells with higher pressure resistance was responsible for tailing. Genetic analyses subsequently revealed that two-thirds of the piezotolerant *L. monocytogenes* strain ScottA isolates had mutations in the c*tsR* gene, a repressor of class III heat shock genes. The role of this gene in adaptation to pressure seems to be in accordance with previous studies, which also showed that mutations in the *ctsR* gene were responsible for the increased pressure resistance of *L. monocytogenes* (Karatzas et al., 2003), and strengthens the role of heat shock proteins in the adaptation to pressure (1.3.2.2.4). However, although the *ctsR* gene might play a role in pressure resistance of the tail fraction, it cannot be solely responsible for their increased pressure since Van Boeijen et al., 2008 also isolated piezotolerant Scott A and other *L. monocytogenes* strains from the tail fraction which did not have mutations in this gene.

1.3.2.4.2 Sublethal damage

High pressure treatment is known to cause sublethal injury of cells in both buffer systems and foods and more than 99.99% of the cell population can be sublethally injured by pressure treatment (Ulmer et al., 2000, Gänzle and Vogel, 2001, Solomon and Hoover, 2004, De Lamo-Castellví et al., 2005, Koseki and Yamamoto, 2006b, Somolinos et al., 2008, Tomasula et al., 2014). The occurrence of sublethal damage of cells after pressure treatment is usually studied by selective medium plating technique. Selective media contains agents which lower the recovery rate of the bacteria, which inhibits the repair of sublethally injured cells and thereby allows for differentiation between viable and damaged cells.

Bozoglu et al., 2004 proposed the existence of two types of injury based on the pressure inactivation and recovery during storage of two Gram-positive (*L. monocytogenes* and *S. aureus*) and two Gram-negative bacteria *(E. coli* and *Sal. enteritidis*) in milk. By their definition, Type I sublethal injury is characterized by the formation of visible colonies on non-selective agar but not on selective agar. Type II injured cells are initially not able to form colonies on either non-selective or selective agar, but can recover to form colonies on non-selective agar first and subsequently also on selective agar (Figure 2). The existence of such a two-stage repair process was also hypothesized by Bull et al., 2005. However, the underlying cellular mechanism hypothesized to be involved in type I and II damage and the process of recovery opposed that of Bozoglu et al., 2004. Whereas Bozoglu and colleagues hypothesized that type I injury is the result of structural damage to the cell wall/membrane and that disturbed metabolic processes are involved in type II injury, Bull argued exactly the other way around and proposed that type I injury involves physiological damage and type II disturbance of the cytoplasmic membrane.



	Type II → Type I	Type I → active cells
Bozoglu et al., 2004	Repair of metabolic injury	Repair of structural damage to cell membrane/wall
Bull et al., 2005	Repair of the cytoplasmic membrane	Physiological repair

Figure 2: HHP-induced sublethal damage. Types of pressure-induced injuries and their ability to form colonies on non-selective and selective agar plus the underlying cellular mechanism as proposed by **Bozoglu et al., 2004** and **Bull et al., 2005**.

The degree of injured cells can differ depending on the pressure parameters and composition of the treatment medium. The effect of these factors on cellular inactivation and sublethal injury additionally depends on the species examined. For example, Kalchayanand et al., 1998b found that the degree of damaged cells increased with higher pressure level and temperature during pressure treatment for all 4 species investigated (i.e. *S. aureus*, *E. coli*, *L. monocytogenes*, and *Sal. typhimurium*), whereas variations in pressure holding time only affected the degree of injury in *S. aureus*. Somolinos et al., 2008 showed that the degree of sublethal injury was greater at pH 4.0 than at pH 7.0 (in both *E. coli* and *L. monocytogenes*). The authors additionally showed that the percentage of sublethal damaged cells was also dependent on the type of buffer used (at the same pH). The role of the treatment medium in sublethal damage and recovery of cells is also emphasized by a study towards the pressure inactivation of *S. aureus* in a ham model system (Tassou et al., 2007). Here, the authors surprisingly found a higher number of survivors on selective agar than on non-selective agar. The higher number of survival on selective plates was later argued by Rendueles et al., 2011 to be the result of the presence of substances in the food model system, such as vitamins and amino acids, which would allow for a better recovery of pressure-damaged cells. An additional factor for the fate of pressure-injured cells is the temperature of storage after pressure treatment. Both *L. monocytogenes* (Bull et al., 2005) and *E. coli* (Koseki and Yamamoto, 2006b) HHP treated cells were shown to have a higher recovery rate when they were stored at a moderate temperature (i.e. 15 or 25 °C, respectively) compared to a low (4 °C) or high (i.e. 30 or 37 °C) temperature.

With respect to the above discussion, it is important to recognize that a viable cell count of zero, when assessed directly after pressure treatment, does not mean that all cells are inactivated. Multiple groups have shown that although *L. monocytogenes* cells could not be detected directly after pressure treatment (which can also be due to the detection limit inextricably linked to the plate viability assay), sublethally damaged cells were able to recover and grow under favourable conditions (Ritz et al., 2006, Somolinos et al., 2008, Jofré et al., 2010, Tomasula et al., 2014, Stratakos et al., 2015b). For example, Jofré et al., 2010 showed that a pressure level of 400 MPa (10 min, 15 °C) was sufficient to reduce 10^9 *L. monocytogenes* cells/mL to below the detection limit (1 log cfu/mL) directly after treatment. In the same study, they also showed that these cells could recover over time and that even pressure levels

of 900 MPa (5 min, 15 °C) were not sufficient to eradicate *L. monocytogenes* completely. Hence, studies which assess viable cell counts directly after HHP treatment usually overestimate the actual number of killed cells and storage tests under appropriate conditions are required to assess the true microbiological safety of the product.

1.3.2.5 High pressure in combination with other antimicrobials

There is a great number of reports where the inactivation of bacteria by HHP has been examined in combination with antimicrobials such as nisin (Hauben et al., 1996, Kalchayanand et al., 1998a, García-Graells et al., 1999, Ter Steeg et al., 1999, Masschalck et al., 2000, Chung et al., 2005, Jofré et al., 2008, Lee and Kaletunç, 2010, Hereu et al., 2012) and other bacteriocins or bacteriocin producing strains (Morgan et al., 2000, Garriga et al., 2002, Arqués et al., 2005, Rodriguez et al., 2005, Jofré et al., 2009, Hereu et al., 2012, Montiel et al., 2015), lactic and ascorbic acid (Jofré et al., 2008, Pal et al., 2008, Patterson et al., 2011, Masana et al., 2015, Mukhopadhyay et al., 2016, Lerasle et al., 2014) lactoferrin (Masschalck et al., 2001a), the lactoperoxidase system (Montiel et al., 2012), different essential oils (Karatzas et al., 2001, Stratakos et al., 2015b), other pressure tolerant bacteria with antimicrobial activity (Patterson et al., 2011) and many more food additives (Ogihara et al., 2009). Also lysozymes from different origins have been studied in combination with HHP and shown to work synergistically against both Gram-positive and Gram-negative bacteria (Hauben et al., 1996, García-Graells et al., 1999 Masschalck et al., 2000, Masschalck et al., 2002, Nakimbugwe et al., 2006a, Nakimbugwe et al., 2006b). Gram-negative bacteria are normally insensitive to lysozyme, because lysozyme's cellular target (the peptidoglycan layer) is shielded by their outer membrane. The synergy between lysozyme and HHP against Gram-negative bacteria is therefore argued to be the result of pressure-induced permeabilization of the outer membrane, which allows the peptidoglycan hydrolases to reach its cellular target and lyse the cell (Hauben et al., 1996): a mechanism also referred to as pressure-promoted uptake (Masschalck et al., 2000). Interestingly, even lysozyme-derived peptides were shown to work synergistically with HHP for the inactivation of both Gram-negative and Gram-positive bacteria (Masschalck et al., 2001b). Here, a different bactericidal mechanism must be involved because these peptides were completely devoid of enzymatic activity. The existence of such a non-enzymatic mechanism is further strengthened by work of the same group. Masschalck et al., 2002 showed that a lysozyme resistant *S. aureus* strain became sensitive to the enzyme under HHP, but without any sign of peptidoglycan hydrolysis. It was argued that either perturbation of the cytoplasmic membrane or induction of autolysis could explain this non-enzymatic inactivation mechanism. Further discussion on the non-enzymatic bactericidal activity of lysozyme and other peptidoglycan hydrolases is provided in section 1.4.2.2.3.

1.3.3 The application of HHP in foods

1.3.3.1 Consumer acceptance

The perception of a preservation technique is an integral part of consumer's acceptance towards a new product. In a study toward the consumer's attitude about novel, nonthermal processing techniques, it was found that the concern toward HHP was rated almost equal to thermal processing, whereas concerns about radio frequency sterilization or irradiation were much higher (Table 5). Consumers were found to generally have a more negative attitude towards foods processed by novel food processing techniques (i.e. without description of the technology). Interestingly, additional information about HHP technology and its benefits could significantly increase consumer acceptance.

As such, it was argued that food industry would not encounter much resistance of the general public in the marketing of HHP-treated food (Cardello, 2003 from Wright et al., 2007).

Table 5: Consumer concern about food processing techniques. Percentage of respondent that were "very" or "extremely" concerned with foods processed by novel techniques. Adapted Adapted with permission from **Wright et al., 2007**.

Food processing method	% very of extremely concerned
Genetically modified	54
Irradiation	49
Radio frequency sterilization	40
High pressure treatment	20
Microwave processing	18
Thermal processing	18
Heat pasteurization	13

1.3.3.2 Effects of HHP processing on foods

One of the most quoted benefits of HHP processing as preservation techniques is that it allows for the inactivation of pathogenic/spoilage microorganisms while leaving food quality attributes mostly intact. Although there is a great deal of reports where no negative effect of HHP on the quality attributes of foods could be detected (Vercammen et al., 2011, del Olmo et al., 2014, Stratakos et al., 2015a), this statement is only in part true since important food quality factors such as colour, flavour, texture, and nutritional value can be negatively influenced by HHP treatment (Bermúdez-Aguirre and Barbosa-Cánovas, 2011, Buckow et al., 2013, Lingham et al., 2016). Generally, the greater the pressure level and time of application, the greater the potential for changes in the appearance of selected foods. A short summary of different pressure effects on food quality attributes is provided below.

Colour is an important determinant of consumer acceptance and the colour of different food products have been reported to be affected negatively under pressure. For example, HHP processing of raw meat causes discolouration, which gives it a cooked appearance. This change in colour is described to be the result of two processes (Ludikhuyze and Hendrickx, 2006); (i) whitening in the pressure range of 200 to 350 MPa, argued to be the result of globin denaturation or haem displacement/release. (ii) A loss of red colour caused by oxidation of ferrous myoglobin into ferric myoglobin ($Fe^{2+} \rightarrow Fe^{3+}$) at or above 400 MPa. HHP processing of raw turkey breast resulted in a significant colour change at pressures just below 300 MPa (1 min, 20 °C; Figure 3a; Tintchev et al., 2010). A pressure treatment of smoked salmon also results in a cooked appearance, though critical colour changes were not observed until a pressure level of 500 MPa (1 min, 20 °C; Figure 3b). The different colour stability of meat and smoked salmon under pressure are most likely the result of differences in the pigment substance and its stability in salmon (astaxanthin) and meat (myoglobin). In contrast to smoked salmon, 150 MPa (30 min, 1-5 °C) was determined as the maximum pressure parameter which did not negatively change the colour of fresh salmon (Amanatidou et al., 2000). This indicates that prior product processing can change the stability of food quality attributes under high pressure: a phenomenon also described for raw and cooked meat products (Pandrangi and Balasubramaniam, 2005).

Figure 3: HHP-induced colour change of foods. Colour change of turkey breast (a) and smoked salmon (b) by HHP processing (0–600 MPa, 1 min, 10 °C (turkey breast) or 20 °C (smoked salmon)). Adapted with permission from **Tintchev et al., 2010**. Copyright © 2010, John Wiley and Sons.

Many colour changes and other food quality attributes are related to food enzymes. HHP processing can also positively influence food quality by the inactivation of enzymes that have a negative impact on the food product. Pressure can affect these enzymes in several ways, including (Ludikhuyze et al., 2006) (i) reversible or irreversible, partial or complete inactivation resulting from conformational changes, (ii) the inhibition or enhancement of the enzymatic activity as a result of pressure-induced volume changes, (iii) a change in substrate susceptibility, (iv) or facilitate the enzyme-substrate interaction by pressure-induced permeabilization of cellular membranes. For example, polyphenoloxidase, the enzyme responsible for enzymatic browning of damaged or sliced fruits and vegetables, can be (partially) inactivated by HHP processing (Ludikhuyze et al., 2006). The commercial production and preservation of sensory attributes of avocado's or guacamole by HHP processing is, to a certain extent, based on the inactivation of this enzyme. Enzymes not only affect food colour but also texture and flavour. The enzyme pectinmethylesterase is involved in the loss of texture in fruit juices and vegetable sauces, and lipoxygenase causes a whole range of negative changes by oxygenation of polyunsaturared fatty acids. High pressure can be used for the inactivation of these and other enzymes related to food quality, but their inactivation differs greatly depending on the enzyme and both intrinsic (e.g. the presence of salts, sugars, additives, pH, etc.) and extrinsic factors (e.g. pressure temperature, time, etc.; Pandrangi and Balasubramaniam, 2005, Ludikhuyze and Hendrickx, 2006).

HHP treatments also can change the rheological or textural properties of foods. It was, for example, demonstrated that HHP processing (500 MPa, 5-15 min, 65 °C) of cooked sausages resulted in a less firm product when compared to their heat pasteurized counterparts (Mor-Mur and Yuste, 2003). In contrast, del Olmo et al., 2014 did not find any marked effect of HHP (600 MPa, 5 min, 4 °C) on the sensory characteristics of sliced "lacón" (cured–cooked pork meat product). Pressure treatment of smoked salmon at subzero temperatures (200 MPa, -18 °C) resulted again in a product with higher hardness, gumminess, and chewiness (Lakshmanan et al., 2005). In tomatoes, texture loss and cell rupture was observed at pressures between 200 and 400 MPa (Tangwongchai et al., 2000), whereas the texture of green peas was mostly unaffected at pressure up to 900 MPa (5-10 min, 20 °C; Quaglia et al., 1996). These examples merely illustrates the high diversity of product stability under high pressure and the necessity to examine food quality attributes before commercialization of HHP-treated food products.

1.4 Bacteriophages and their lysins

Along with non-thermal food preservation methods such as HHP, the use of natural antimicrobials to extend the shelf-life of foods with minimal impact on their nutritional and organoleptic properties also received particular attention. Bacteriophages and their lysins can specifically inactivate bacteria without changing the properties of the food. In this chapter, bacteriophages, their lysins, and the application of these antimicrobials in foods are discussed.

1.4.1 Bacteriophages

1.4.1.1 Definition, morphology, and taxonomy

Bacteriophages, or simply phages, are viruses that infect Eubacteria and Archaea. The phage carries its genome from one susceptible bacterial cell to the other where it directs the production and release of more phages by the host. Most bacteriophages consist of nucleic acid surrounded by a protein shell. The vast majority contains dsDNA, but phages with ssDNA, ssRNA, or dsRNA have also been found (Ackermann, 2005). Each phage has a specific group of cells as target host. This group can be a subset of one species, but also closely related species can sometimes be infected by the same phage. Phages are classified into 13 families based on their shape, size, type of nucleic acid, the presence/absence of an envelope and whether the envelope structure contains lipids. Most of the known phages belong to *Caudovirales* order and have an icosahedral head, a tail, and double-stranded DNA (Figure 4). This order of tailed phages contains three families, subdivided according to the morphological features of their tail: *Myoviridae* with contractile tails, *Siphoviridae* with a long non-contractile tail, and *Podoviridae* with a short non-contractile tail. Whereas the head (capsid) of these phages have a cubic symmetry, their tails are true helices or stacked disks (sheaths) and possess a base plate with attached spikes or fibres for host recognition. Tailless phages are polyhedral, filamentous, or pleomorphic (lipid-containing envelop) and constitute the remaining 10 families (Ackermann, 2007).

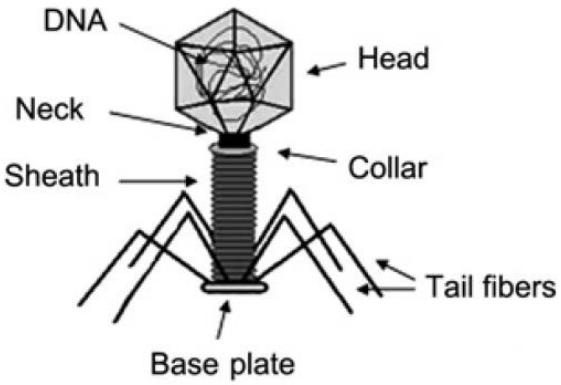


Figure 4: Structure of a typical tailed bacteriophage. Adapted with permission from **Garcia et al., 2010**. Copyright © 2010, Elsevier.

The phage propagation life cycle can be either lytic or lysogenic (Figure 5). Virulent phages can only enter the lytic phase where they multiply rapidly and lyse their host to release progeny phages. The life cycle of lytic tailed phages starts with recognition of the target bacteria by adsorption of phage fibres or spikes to specific surface molecules of the host. Whereas in Gram-negative bacteria this can be virtually any of the outer cellular components, many Gram-positive phages require clusters of one specific kind of molecule in the murein layer in order to properly position the phage tail for surface penetration (Guttman et al., 2005). After irreversible binding to the host, the phage genome is inserted

through its tail into the cell. This requires the passage of several cellular layers and membranes, generally facilitated by enzymes at the phages tail tip. Other strategies involve the use of pre-existing pores within the cellular envelope or the phage mediated release of channel forming proteins (Evans, 2009). The first step after insertion of the phage genome is its recognition by the host's RNA polymerase. This leads to the immediate transcription of genes, which reprogram the bacterial host towards the needs of the phage. In the next phase, gene transcription is responsible for the synthesis of new phage DNA and phage particles. The subsequent assembly of progeny phages is a multistep process, which involves a complex packaging process of the phage DNA into preassembled protein shells (procapsids) and further maturation of the phage head. The phage head also contains the binding site for the phage tail, which is assembled separately and attached at a later stage. The subsequent release of progeny phages is a carefully programmed and timed event. For large DNA phages, this involves two main proteins; (i) holin assembles pores in the periplasmic membrane, which allows the (ii) endolysin to reach and cleave its cellular target (i.e. the peptidoglycan). Small RNA and DNA phages have evolved a different release strategy: the production of phage-encoded proteins which interfere with bacterial host enzymes responsible for peptidoglycan biosynthesis (Fischetti, 2010).

Temperate phages are, in contrast to lytic phages, able to establish a condition called lysogeny. In this life cycle, after phage DNA is introduced into the host, it remains in a latent state and only reproduces when their bacterial host reproduces. The phage DNA either exists as a plasmid or is integrated into the host's genome. In this 'prophage' state, the phage genes are replicated and transmitted to the daughter bacterial cells together with the bacterial genes. The lysogenic or dormant stage is ended (i.e. the phage enters the lytic cycle) either spontaneously or triggered by environmental factors (e.g. stress factors or the entrance of another phage).

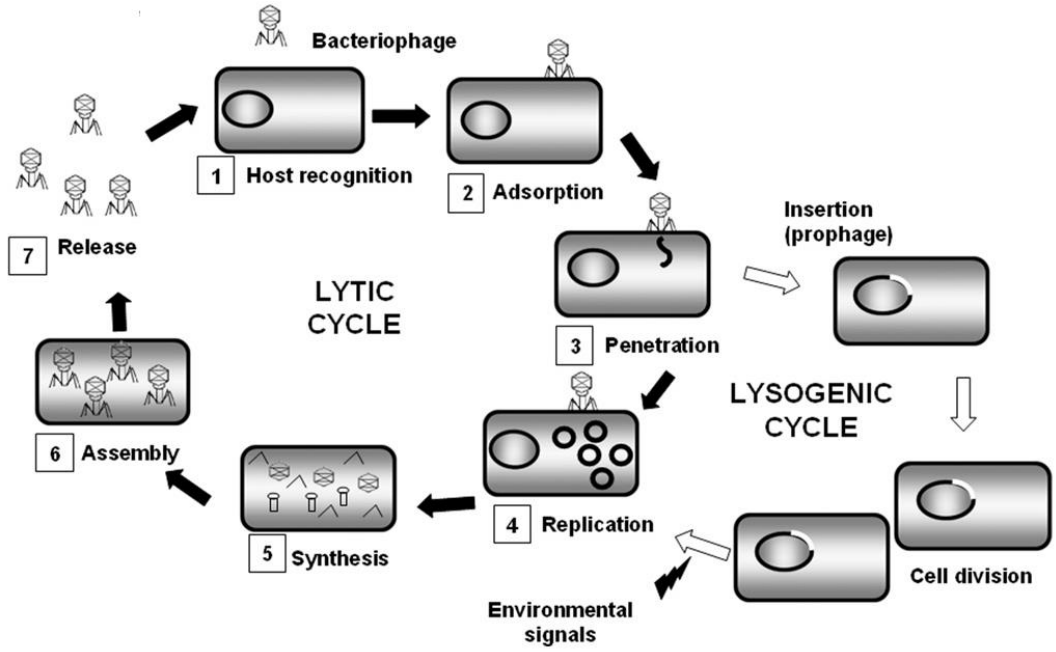


Figure 5: Steps during the bacteriophage lytic and lysogenic life cycle. Adapted with permission from **Garcia et al., 2010**. Copyright © 2010, Elsevier.

1.4.1.2 Applications

Bacteriophages find applications in different fields. After their first discovery by Twort, 1915 and d'Herelle, 1917, bacteriophages were initially only used as therapy against bacterial infections. With the discovery of antibiotics, however, phage therapy became neglected in most of the Western world. Nevertheless, in countries from the former Soviet Union and Eastern Europe, phage therapy was continued and is still an on-going practice. Nowadays, phage therapy is regaining interest in Western countries due to increasing bacterial antibiotic resistance (Brüssow, 2005, Sulakvelidze and Kutter, 2005).

The concept of bacteriophages as antimicrobial agent to control pathogens in the food industry is more recent but already finds applications in multiple fields, including water and food safety, and agriculture and animal health. In 2006, the first two bacteriophage preparations (ListShield™ (formerly LPM-102) and Listex™ P100) have been approved as a food processing aid for the control of *L. monocytogenes* in a selection of foods and received the status of Generally Recognised As Safe (U.S. Food and Drug Administration, 2006a, 2006b). In 2016, a number of bacteriophage based preparations have been approved for direct application to food in the United States, Europe, Canada, Australia, and New Zealand (Chibeu et al., 2013, Perera et al., 2015).

In this work, the phage P100 (commercially available under the name Listex™ P100, MICREOS Food Safety B.V., Wageningen, the Netherlands) was used. This phage belongs to the *Myoviridae* family, is strictly lytic, and features a very wide host range within the *Listeria* genus (Carlton et al., 2005). The efficacy of bacteriophage P100 has been investigated in multiple foods with varying results. Whereas in a single study, a complete eradication of *L. monocytogenes* on soft cheese could be achieved by the application of phage P100 (Carlton et al., 2005), most studies only found an initial reduction directly after its application and growth during storage (Guenther et al., 2009, Soni and Nannapaneni, 2010, Soni et al., 2010, Rossi et al., 2011, Soni et al., 2012, Chibeu et al., 2013, Oliveira et al., 2014, Perera et al., 2015). This is probably the result of both differences in experimental set-up (single *Listeria* strains versus cocktails, inoculation level, phage P100 dose applied, storage temperature, etc.) as well as the food examined (chemical composition of the food, specific food matrix, etc.). A similar diversity between studies and different foods has also been observed for other *Listeria* specific phages (phage A511 and P35; Guenther, 2007, Guenther and Loessner, 2011) and phage cocktails (ListShield™; Perera et al., 2015). Although an initial reduction could usually be observed, complete eradication of *L. monocytogenes* could not be achieved (i.e. listerial growth during storage).

Interestingly, after an initial reduction in cell count by the application of phage P100 in cooked turkey or roast beef, *L. monocytogenes* growth was observed despite the presence of a high concentration of infective phage particles (Chibeu et al., 2013). It was therefore argued that the inactivation of *L. monocytogenes* was only the result of the initial infecting phage population and that progeny phages do not play a major role in the efficacy of phage treatment. Other studies demonstrated that this is only inherent to phage treatment of solid but not liquid foods (Guenther et al., 2009, Oliveira et al., 2014). As such, the spatial distance between the phage and *L. monocytogenes* on solid foods limits phage infection and propagation, which allows for growth despite the presence of large numbers of phages. The combined application of phage P100 with other antimicrobials (i.e. potassium lactate, sodium diacetate, or lauric arginate) did not aid in the elimination of *L. monocytogenes* itself but could prevent growth after an initial reduction in cell count (Soni et al., 2012, Chibeu et al., 2013).

1.4.1.3 Bacterial resistance

Soon after the discovery of bacteriophages, the existence of phage-resistant bacterial mutants was observed and directly recognized as potential problem for its application as antimicrobial (described by Carlson, 2005). Since then, different resistance mechanisms through which bacteria can protect themselves against bacteriophage attack have been described and are present at each step of the phage's life cycle (Labrie et al., 2010). One such mechanism, by which bacteria acquire resistance towards phages, is the alteration of their surface structures so that phage tail fibres cannot recognize and attach to the bacterial membrane. Another described resistance mechanism is the limitation of phage spread via 'altruistic cell suicide' (i.e. an abortive infection mechanism; Dy et al., 2014), but this results in death of the bacterial cell and is therefore not relevant for possible spread of resistance through bacterial populations. The most recently discovered mechanism by which bacteria can acquire phage resistance is through the CRISPR/Cas 9 system, which allows for the recognition and cleavage of the injected phage genome (Barrangou et al., 2007). The frequency of spontaneous mutations that may confer phage-resistance has not been studied in depth, but was described to be comparable to those seen for antibiotics (Carlson, 2005). Hagens and Loessner, 2010 argued that phage resistance by random mutations does not play a role in overall effectiveness when it is applied as antimicrobial on foods because all phages and bacteria (both phage infected cells as well as resistant cells) are removed from the contamination source. The treatment of products with phages immediately prior to packing, which prevents the re-entry and establishment of a phage-resistant flora in a production environment, has also been recommended by Guenther et al., 2009 as a viable method to prevent the development of phage resistance. The authors did however recognize that phage-resistant bacterial mutants might pose a risk to the effectiveness of phage-based intervention in environments where phage selective pressure is high (e.g. when phages are used as environmental cleaning agent or to eradicate bacteria among farm animals).

The commercially available phage P100 is frequently used in food production to combat *L. monocytogenes*. Despite above argumentations of low chances on resistance development, 13 from 501 *L. monocytogenes* isolates from Austrian dairy plants, which (experimentally) used P100 were found to be insensitive to this phage (Fister et al., 2015). This is most likely caused by selective phage pressure since no P100 insensitive isolates could be recovered before industrial P100 usage. The development of phage P100 resistant isolates is thus driven by their actual application, most likely due to lack of rotation of different phages or use of phage cocktails to avoid selection (though specific information on the application of phage P100 and possibly other phages in the dairy plants was not provided). The development of P100 resistance isolates is in strong contrast with the successful application of phage therapy in the former Soviet Union and Eastern Europe for almost a century. This can however be easily explained by the use of a single phage P100 versus the use of regularly rotated and complex phage cocktails containing multiple lytic phages against the same bacterial strain. As such, it is argued that long-term resistance need not be a concern as long as adequate strategies are used and a diverse set of phages capable of infecting newly resistant strains will most likely be available from different geographical locations and evolutionary histories (Meaden and Koskella, 2015).

1.4.2 Endolysins

Endolysins are bacteriophage-encoded lytic enzymes that break down the peptidoglycan of the bacterial cell wall at the end of the lytic cycle and thereby facilitate the escape of progeny phages from the infected cell (Figure 5, step 7). However, most endolysins do not contain signal peptides to direct

them towards their substrate. For large dsDNA phages, this process in instead facilitated by holins: small hydrophobic membrane-spanning proteins that are also encoded by the phage. They assemble into oligomers in the membrane to form lesions or holes, which gives the accumulated endolysin in the cytoplasm access to the murein layer. This layer is required for the bacterium to withstand the internal cytoplasmic turgor pressure and its degradation will cause cell lysis and release of the bacteriophage progeny into the surrounding environment. Even though endolysins naturally operate from the inside of infected cells, they can also induce the lysis of bacterial cells when exogenously applied as purified recombinant proteins.

1.4.2.1 Peptidoglycan

The bacterial peptidoglycan is a protective barrier as well as a structural component of the bacterial cell wall responsible for its shape (Nelson et al., 2012). It is composed of a universal carbohydrate backbone consisting of alternating β-1,4-linked residues of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). These glycan polymers are in turn covalently linked by short peptide sequences. Whereas the glycan moiety is conserved in all bacteria, the interlinking peptide is more diverse in terms of length and composition. The typical structure of *S. aureus* and *L. monocytogenes* bacterial peptidoglycan is depicted in Figure 6. The glycan backbone of both species is linked to the stem peptide through an amide bond between MurNAc and L-alanine. The remainder of the interlinking peptide however differs greatly between these species. The third residue of the stem peptide of *S. aureus* is L-lysine. This residue is cross-linked to an opposing stem peptide on a separate glycan polymer through a glycine peptide bridge. In listeria, a *meso*-diaminopimelic acid residue is present at position number three of the peptide stem and this residue is directly linked to the opposing peptide stem (i.e. without interlinking bridge).

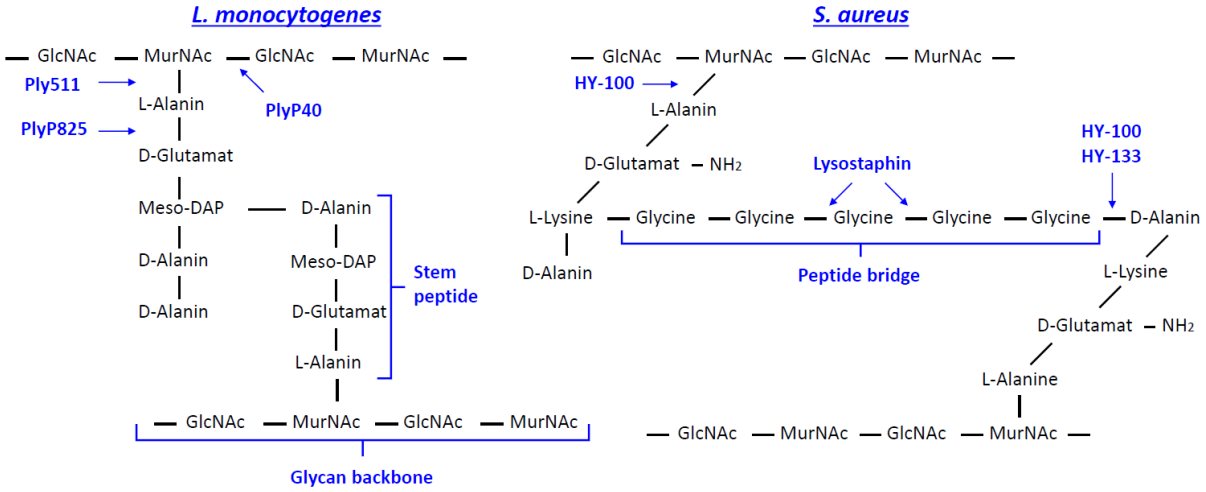


Figure 6: Peptidoglycan structure of *L. monocytogenes* and *S. aureus*. The (putative) cleavage sites of the endolysins used in this work are illustrated.

1.4.2.2 Structure and activity

Endolysins from a Gram-positive background typically have a modular structure with one or more cell wall-binding domains (CBDs) for substrate recognition at the C-terminal side and one or more enzymatically active domains (EADs) for cleavage of the peptidoglycan at the N-terminal side (Loessner, 2005). Modular domains of Gram-positive endolysins are typically connected by a flexible linker sequence (Fischetti, 2008). This linker is often described to create a relative independence of the EAD and CBD functional properties, which allows for domain swapping to create endolysins with

optimized characteristics. For example, Becker et al., 2009a fused the endopeptidase domain of the *Streptococcus agalactiae* specific endolysin λSA2 (for which the peptidoglycan cleavage site is conserved in both streptococci and staphylococci) to staphylococci specific CBDs (from the S*taphylococcus* phage lysin LysK and lysostaphin). This not only made the enzyme highly active against *S. aureus*, but it also maintained considerable streptolytic activity. Using a similar strategy, Osipovitch et al., 2015 could significantly enhance the bacteriolytic activity of *S. aureus* autolysins by replacing their CBD with that of lysostaphin. Domain swapping can also be used to optimize the isolation and purification of endolysins. Manoharadas et al., 2009 created a chimeric construct (P16-17) with which solubility problems of the wild-type endolysin could be overcome. The increased solubility of the fusion construct allowed for its purification and subsequently showed strong antimicrobial activity against *S. aureus*. Alternatively, domain swapping can also broaden or change endolysin species or strain specificity. The *Listeria* phage endolysin PlyPSA and Ply188 feature CBDs which specifically target cell walls of *L. monocytogenes* strains belonging to serovars 4, 5, and 6 (Korndörfer et al., 2006) or serovars 1/2, 3, and 7 (Loessner et al., 2002), respectively. Switching the CBDs of these two endolysins resulted in a swap of their binding and lysis specificity (Schmelcher et al., 2011).

Modular endolysins from phages that infect Gram-negative bacteria are rather exceptional. Most of the endolysins with a Gram-negative background are single (enzymatic) domain globular proteins (Nelson et al., 2012). Though, Gram-negative endolysins with a modular structure also exist, as was shown by Briers et al., 2007b for the *Pseudomonas* phage endolysins KZ144 and EL188. These endolysins however had an inverted molecular structure compared to the modular endolysins from a Gram-positive background (i.e. the EAD at the C-terminal and the CBD at the N-terminal side).

The presence of CBDs in Gram-positive phage endolysins (and their absence in most Gram-negative endolysins) is argued to have an evolutionary background (Fischetti, 2008). The peptidoglycan in Gram-positive bacteria is not protected by an outer membrane but exposed to the outer environment. Bacteriophage mediated lysis of a cell and release of produced endolysins could therefore result in the lysis of surrounding cells. This would restrict the adsorption of released phages to neighbouring host cells and the start of a new infective cycle. To circumvent killing of neighbouring cells, it is believed that Gram-positive endolysins have evolved to bind irreversible to their substrate. This would limit their activity to single-use only in the infected cell and thereby prevent lysis of surrounding cells. This hypothesis is supported by work of Loessner et al., 2002, which demonstrated that the binding affinity of listeria specific endolysin CBDs is in the nanomolar range (approaching that of an IgG molecule for its substrate). Protection to external lysins is not required for Gram-negative bacteria because they have a protective outer membrane. Hence, no evolutionary pressure was present to acquire CBDs, which probably explains the globular structure and absence of a CBD in most Gram-negative phage endolysins.

1.4.2.2.1 Enzymatically active domain

Endolysins can be divided into different classes based on their catalytic activity and the bonds they hydrolyse: glycosidases, a specific amidohydrolase, endopeptidases, and lytic transglycosylase (Nelson et al., 2012). (i) Peptidoglycan glycosidases hydrolyse the glycan component either on the reducing side of GlcNAc (i.e. N-acetylglucosaminidases) or MurNAc (i.e. N-acetylmuramidases). Most enzymes within this class, including the well-known lysozyme, are N-acetylmuramidase. These types of lysins are predicted to have a wider species spectrum than hydrolases which cleave the interlinking peptide because the glycan backbone is more widely conserved among both Gram-negative and Gram-positive

bacteria. (ii) The N-acetylmuramoyl-L-alanine amidase separates the glycan strand from the stem peptide by cleavage of the amide bond between these moieties. Amidases are therefore predicted to cause the strongest destabilization of the peptidoglycan. (iii) Endopeptidases cleave bonds between two amino acids of the interlinking peptide, either in the stem peptide or in the interpeptide bridge. (iv) Although lytic transglycosylases cleave the same bond as N-acetylmuramidases, they belong to a different mechanistic class because they do not require water and are therefore no hydrolases in a strict sense. The (putative) bonds cleaved by the endolysins used in this work are depicted in Figure 6.

The modular architecture of endolysins is not limited to a single catalytic domain. A multitude of endolysins against different species have been described to contain two or even three EADs (Nelson et al., 2012). The presence of multiple catalytic domains does however not necessarily mean that all are enzymatically active. Inactive lytic domains have been observed in both streptococcal and staphylococcal lytic endolysins (Nelson et al., 2012). For example, deletion and site-directed mutational analysis of endolysin B30 showed that a single domain of this endolysin is almost exclusively responsible for cell lysis, whereas the other domain is practically devoid of activity (Donovan et al., 2006, Cheng and Fischetti, 2007). These results are surprisingly in contrast with cut site analysis of cells treated with the same endolysin, where results indicated both catalytic domains to be active (Pritchard et al., 2004). This discrepancy between methods has been observed more often for different endolysins and is most likely the result of differences between the use of whole cells versus isolated cell wall or peptidoglycan (Nelson et al., 2012). The reasons for the presence of presumably inactive but highly conserved catalytic domains is not known, but is thought to lie in the difference between lysis from without as examined in the experiments described above (recombinant produced endolysins added to the cells) versus lysis from within (the “real-world” setting, where endolysins are expressed by the phage in the cell; Donovan and Foster-Frey, 2008). Endolysins with a dual EAD might have multiple advantages over single domain endolysins. Such advantages include a possible synergistic effect between enzymatic domains, where one EAD increases the accessibility to the cut site for the second EAD (Schmelcher et al., 2015). Dual EAD endolysins are also predicted to be more refractory to resistance development (Nelson et al., 2012), which would confer an evolutionary advantage of a phage and explain their high conservation. A similar evolutionary advantage, though much rarer, was found for endolysin PlyGRCS, for which both amidase and endopeptidase activities were detected despite having only a single EAD (Linden et al., 2014).

1.4.2.2.2 Cell wall binding domain

The CBD is responsible for targeting the protein to the bacterial cell wall and thereby directly influences lytic activity (Loessner, 2005). Multiple studies have however shown that the presence of a CBD is not an absolute requirement for catalytic activity. The role of the CBD in the lytic activity of endolysin is nicely illustrated in a study by Korndörfer et al., 2006, where it was shown that the *Listeria* phage endolysin PlyPSA is significantly hampered but not devoid of its enzymatic activity when the CBD moiety is removed. The same observation was done for the S*taphylococcus* phage endolysins PlyGRCS and PlyTW (Linden et al., 2014, Becker et al., 2015). The EADs devote of CBD displayed activity by itself, though reduced compared to the full-length protein. These studies indicate that the presence of the CBD is not essential for lytic activity, but definitely required for the endolysin to reach its maximum lytic activity.

Schmelcher et al., 2011 found that the *Listeria* phage endolysin Ply118 could efficiently lyse *L. monocytogenes* cells from serovar 4b, although the isolated CBD118 does not recognize and bind to

these cells (Loessner et al., 2002). In the same study, the authors also showed that a higher affinity of the CBD does not necessarily increase enzymatic activity. On the contrary, a duplication of the CBD in the *Listeria* phage endolysin Ply500 increased the binding affinity of the enzyme approximately 50-fold but reduced its lytic activity (Schmelcher et al., 2011). It was argued that the enzyme should retain a certain degree of surface mobility for optimal lytic activity and that steric hindrance of the EAD by the duplicated CBD might have also played a role. Interestingly, in the absence of a CBD, a positive net charge of the EAD was shown to be required for the endolysin to remain its lytic activity (Low et al., 2011). As such, it seems that the positive charge can take over the role of the CBD and direct the EAD to the negatively charged cell wall. In summary, a CBD might maximize lysis by elevating the local enzyme concentration and bringing the enzyme closer to its ligand, but is not required per se and might even hinder lytic activity.

1.4.2.2.3 Non-enzymatic activity

The antibacterial activity of peptidoglycan hydrolases is commonly ascribed to their enzymatic function. Killing of bacteria by enzyme-independent mechanisms has however also been extensively reported, especially for lysozyme (an extensive review is provided by Masschalck and Michiels, 2003). The existence of a non-enzymatic bactericidal mechanism of lysozyme has been shown by a variety of approaches, including the use of variants mutated to be catalytically inactive, heat or chemically denatured lysozyme, enzymatically digested lysozyme, and even short synthetic lysozyme-derived peptide sequences were shown to have a bactericidal effect (Ibrahim et al., 2001b Laible and Germaine, 1985, Pellegrini et al., 1992, Ibrahim et al., 1996a, Ibrahim et al., 1996b, Düring et al., 1999, Ibrahim et al., 2001a, Nakimbugwe et al., 2006a). Based on these and more studies, Masschalck and Michiels, 2003 concluded that three mechanisms exist through which lysozyme can kill Gram-positive bacteria; (i) the first mechanism of bacterial inactivation is the lytic enzymatic mode of action where lysozyme hydrolyses the peptidoglycan layer resulting in cell lysis. (ii) The second mechanism is independent from lysozyme's enzymatic activity. Here, it is proposed that autolysin activity is stimulated by the interaction of lysozyme with wall- or lipoteichoic acids or by displacement of divalent cations, which also results in cell lysis. (iii) The third and last mechanism described is non-lytic. Here, cell death is caused by direct interaction and perturbation of the cell membrane.

Although the killing of bacteria by endolysin might follow the same mechanisms as described for lysozyme, a possible non-enzymatic bactericidal activity of these enzymes has not been investigated in depth. Until now, only a single study indicated a non-enzymatic antibacterial activity of endolysin. In this study, synthesized peptides and catalytically inactive form of the *Bacillus* phage endolysin lys1521 were shown to permeabilize the outer membrane of *P. aeruginosa*, indicating some extent of non-enzymatic antibacterial activity (Orito et al., 2004).

1.4.2.3 Applications

Endolysins are recognized as highly specific antimicrobial compounds and their application has therefore been examined in different fields, including as therapeutic agent or "enzybiotic" (Loeffler et al., 2001, Nelson et al., 2001, Schuch et al., 2002, Cheng et al., 2005), in food processing environments against bacterial biofilms (Sass and Bierbaum, 2007, Son et al., 2010), or as environmental disinfectant (Hoopes et al., 2009). The specificity of CBDs from endolysins have also been exploited for rapid and highly specific detection of foodborne pathogens such as *L. monocytogenes* and *B. anthracis* (Sainathrao et al., 2009, Schmelcher et al., 2010). In this section, the use of endolysin as antimicrobial for the elimination of pathogens in food is in focus.

Most work on the inactivation of pathogens by endolysin has been done *in vitro* and only little information exist for actual application in food products. Although *in vitro* studies might provide important insights, the activity and efficacy of the endolysins in foods is not the same and cannot be extrapolated from buffer systems (Oliveira et al., 2012). For example, Mayer et al., 2010 found that the *C. tyrobutyricum* specific endolysin Ctp1l was far less effective in milk than in buffer. On the contrary, Obeso et al., 2008 demonstrated that endolysin LysH5 was highly effective against *S. aureus* growing in pasteurized milk. García et al., 2010b also examined the inactivation of *S. aureus* in milk by endolysin LysH5, though at a much lower concentration and in combination with the antimicrobial nisin. These authors concluded that LysH5 needs to be combined with nisin to achieve complete eradication of *S. aureus*. Similarly, Zhang et al., 2012 showed that the *Listeria* phage endolysin LysZ5 effectively reduced the number of different *Listeria spp.* in soya milk.

Although the studies discussed above show promising results, none of them showed the microbiological stability of endolysin treated food products. Until now, only a single publication examined the antimicrobial efficacy of endolysin by challenge storage tests. In the doctoral thesis by Guenther, 2007, different endolysins were tested for control of *L. monocytogenes* in iceberg lettuce. Although an initial reduction in cell counts could be achieved, none of the examined endolysins or combinations of different endolysin could prevent growth of listeria during storage.

1.4.2.4 Bacterial resistance

Several groups have attempted to recover bacterial strains resistant towards endolysin by repeated exposure (up to 40 cycles) to low concentrations of the enzyme in agar or liquid cultures, but none of them were successful (Loeffler et al., 2001, Schuch et al., 2002, Rodríguez-Rubio et al., 2013). This is in sharp contrast with parallel experiments, where mutant strains with antibiotic resistance could be readily identified. The development of resistance towards endolysins is believed to be rare (especially when compared to antibiotics) because of their target cell specificity, highly conserved targets in the peptidoglycan, and their application 'from without' (Fischetti, 2008, Fischetti, 2010, Oliveira et al., 2012, Schmelcher et al., 2015).

Interestingly, in a direct comparison between three (fusion) endolysins and the peptidoglycan hydrolases lysostaphin, no spontaneous resistant mutant could be recovered for the endolysins whereas a ca. 100-fold rise in resistance against lysostaphin was detected (Rodríguez-Rubio et al., 2013). The development of resistance towards lysostaphin has indeed been reported in a great number of studies (Sugai et al., 1997, Climo et al., 1998, Climo et al., 2001, Kusuma et al., 2007). Lysostaphin targets the weakyl conserved pentaglycine cross-brige (see Figure 6). This bridge is more susceptible to modifications which confer resistance (e.g. by the incoorporation of serine or truncations to tri- or monoglycine; DeHart et al., 1995, Gründling et al., 2006). A number of endolysins have been described to contain a catalytic domain that targets the same pentaglycine cross-bridge as lysostaphin, but are not prone to resistance development (Rodríguez-Rubio et al., 2013). The antimicrobial activity of these endolysins is probably ensured by the presence of an additional catalyic domain which targets a different peptidoglycan bond: a strategy which greatly reduces the risk of resistance development throughout a bacterial population.

Lysozyme is another well known peptidoglycan hydrolases for which resistance development is often reported, though the mechanistic background of resistance is different from that of lysostaphin (Bera et al., 2005, Davis and Weiser, 2011). Bacterial resistance towards lysozyme involves the modication of the glycan strand (i.e. N-deacetylation, N-glycolylation, and O-acetylation; Vollmer, 2008). Sterical

hinderance by (positively charged) wall teichoic acids and the degree of peptidoglycan cross-linking are also known to influence lysozyme susceptiblity (Bera et al., 2007, Herbert et al., 2007).

As Callewaert et al., 2011 already argued, despite endolysin resistance being very rare, the mechanism of lysozyme and lysostaphin resistance described above illustrate that bacteria can change the properties of their cell wall and there seems to be no fundamental reason why resistance towards phage lysins would not be possible. Indeed, in a recent publication, bacterial resistance against endolysin was shown for the very first time. In brief, Schmelcher et al., 2015 found a *S. aureus lyrA* mutant strain to be less susceptible to all nine peptidoglycan hydrolases examined (eight endolysins and one lysostaphin). The alteration that this mutation induces on the cell wall is not yet characterized and the mechanistic background of resistance therefore remains unclear. However, modifications of secondary cell wall structures which induce steric hindrance and prevent the access to binding or cut sites was put forward as a plausible explanation, especially because a reduced activity against the mutant strain was observed for all enzymes tested (despite having different cut sites). This hypothesis is further strengthened by the work of Gründling et al., 2006, which showed that isolated cell wall material of a different *S. aureus lyrA* mutant strain was resistant towards lysostaphin, whereas purification of the peptidoglycan (stripped of its carbohydrate, teichoic acid, and protein constituents) could restore its full susceptibility to lysostaphin.

1.5 Objectives

It was the aim of this thesis to investigate the use of endolysins or phages to enhance the pressure inactivation of Gram-positive foodborne pathogens. For this purpose, the inactivation of *L. monocytogenes* and *S. aureus* by individually HHP or endolysin/phage treatment plus their combined application should be examined. Moreover, the mechanistic background behind possible synergies of endolysin in combination with HHP should be unravelled. Finally, the combination of endolysin and HHP should be exemplary used in different *Listeria*-critical foods to examine its applicability and concomitantly establish process parameters. Beyond the evaluation of endolysins/bacteriophages in combination with HHP for the control of Gram-positive bacteria in foods, this work should improve the current understanding of the effect of these antimicrobials on the bacterial cell and the role of the cell wall in high pressure tolerance of Gram-positive bacteria.

2 MATERIAL AND METHODS

Standard microbiological compounds, chemicals, and supplies were acquired from different suppliers and certified for its intended use. If not specifically stated otherwise, deionized water was used for the preparation of media and buffers. All solutions required sterile were autoclaved at 121 °C for 15 min.

2.1 Microorganisms and solutions

2.1.1 Bacteria

All strains used were taken from the internal culture collection of the Technische Mikrobiologie Weihenstephan (TMW). Available characteristics such as serotype and source of isolation are provided in Table 6.

Table 6: *L. monocytogenes* and *S. aureus* strains.

TMW	Strain	Origin	Source	Serotype
2.597	*L. monocytogenes*	WSLC 11043	Soft cheese	4b
2.599	*L. monocytogenes*	WSLC 11048	Sausage	Unknown
2.1512	*L. monocytogenes*	ATCC 15313	Rabbit	1/2a
2.594	*L. monocytogenes*	WSLC 11017	Cheese	1/2b
2.595	*L. monocytogenes*	WSLC 11021	Faeces	1/2a
2.601	*L. monocytogenes*	WSLC 1361	Turkey	1/2c
2.49	*S. aureus*	Sabine Faulhammer	Unknown	-
2.422	*S. aureus*	ATCC 12600	Pleural fluid	-
2.424	*S. aureus*	ATCC 14458	Faeces	-

2.1.2 Buffers

Table 7: A selection of buffers used in this work. The pH of buffers was preferably set by mixing mono- and dibasic solutions and otherwise by the use of high molar NaOH and HCL solutions (2-6 M).

Buffer	Compounds	Concentration (mM)
IPB	NaCl	50.0
	NaH_2PO_4 / Na_2HPO_4	25.0
	Imidazole	25.0
IPB^{PlyP40}	NaCl	37.5
	NaH_2PO_4 / Na_2HPO_4	31.3
	Imidazole	31.3
IPB^{Ply511}	NaCl	16.7
	NaH_2PO_4 / Na_2HPO_4	41.7
	Imidazole	41.7
$IPB^{PlyP825}$	NaCl	30.0
	NaH_2PO_4 / Na_2HPO_4	35.0
	Imidazole	35.0
IPB^{HY-100}	NaCl	53.4
	NaH_2PO_4 / Na_2HPO_4	27.1
	Imidazole	27.1
IPB^{HY-133}	NaCl	25.0
	NaH_2PO_4 / Na_2HPO_4	31.3
	Imidazole	31.3
$IPB^{Lysostaphin}$	NaCl	15.0
	NaH_2PO_4 / Na_2HPO_4	37.5
	Imidazole	37.5

2.1.3 Growth media and agar

Table 8: Media and agar solutions.

Medium / agar	Ingredients	Weight (g/L)	Remarks
TSBYE	Casein peptone	17.0	Glucose was autoclaved
pH 7.3 ± 0.2*	Soy peptone	3.0	separately.
	NaCl	5.0	
	K_2HPO_4	2.5	
	Yeast extract	6.0	
	Glucose	5.0	
(TSAYE)	(Agar)	(15.0)	
CASO agar	Casein peptone	17.0	CASO broth was prepared
pH 7.3 ± 0.2	Soy peptone	3.0	according to supplier
	NaCl	5.0	instructions (Carl Roth
	K_2HPO_4	2.5	GmbH, Karlsruhe,
	Yeast extract	6.0	Germany). Ready mixture.
	Glucose	5.0	Glucose was not autoclaved
	Agar	15.0	separately.
Lithium chloride Phenylethanol	Beef extract	3.0	LPM agar and antibiotic
Moxalactam (LPM) agar	Casein peptone (pancreatic)	5.0	supplement were prepared
pH 7.3 ± 0.2	Glycine anhydride	10.0	according to supplier
	LiCl	5.0	instructions (Fluka
	Peptone (animal)	5.0	Analytical, Sigma-Aldrich
	Phenylethyl alcohol	2.5	Chemie GmbH, Buchs,
	NaCl	5.0	Switzerland).
	Agar	15.0	
	Moxalactam	0.020	
PALCAM Listeria Agar	Peptone (vegetable)	23.0	PALCAM agar was prepared
pH 7.0 ± 0.2	Starch	1.0	according to supplier
	NaCl	5.0	instructions (Carl Roth
	Mannitol	10.0	GmbH, Karlsruhe,
	Ammonium ferric citrate	0.5	Germany). Antibiotic
	Esculin	0.8	supplement was either
	Dextrose	0.5	prepared according to
	LiCl	15.0	supplier instructions or
	Phenol red	0.08	individually added (after
	Agar	13.0	dissolving in a small amount of HPLC grade
	Acriflavine Hydrochloride	0.005	water and sterile filtration).
	Ceftazidime	0.020	
	Polymyxin B sulfate.	0.010	

*the pH was adjusted using 2-6 M NaOH/HCL solutions

2.2 Microbiological methods

2.2.1 Preparation of stock cultures

Bacterial strains from the TMW internal strain collection were streak plated on tryptic soy agar supplemented with yeast extract (TSAYE). A single colony was picked, inoculated into tryptic soy broth supplemented with yeast extract (TSBYE), and grown overnight in air permeable Erlenmeyer flasks (37 °C, shaking at 100-200 rpm). On the next day, fresh TSBYE medium was inoculated with 1% (v/v) of the overnight culture and incubated for another 24 h. The cells were harvested by centrifugation (7000 × g_0, 10 min, RT), taken up in fresh TSBYE (20% of the initial culture volume), mixed 1:1 with 80% glycerol,

and stored in cryotube vails at -80 °C. Species identity was confirmed by MALDI-TOF MS analyses (Microflex LT, Bruker Daltronics, Bremen, Germany).

2.2.2 Culture conditions

TSBYE was inoculated from the stock culture and cells were allowed to grow aerobically overnight (37 °C, shaking at 100-200 rpm). On the next day, fresh TSBYE medium was inoculated with 1% (v/v) of the overnight culture and further incubated at 37 °C. The cells were harvested by centrifugation (7000 × g_0, 10 min, RT) in either the exponential (OD_{590} of 0.8 ± 0.1) or stationary growth phase (24 h).

2.2.3 Determination of viable cell count

Liquid samples were serially diluted using an isotonic tryptone solution supplemented with Antifoam B (145 mM NaCl, 14 g/L tryptone, 0.01 % (v/v) Antifoam B Emulsion; TS+). Solid (smoked salmon) or solid in liquid (mozzarella) samples were first transferred to a stomacher bag with TS+ and homogenized for 1-2 minutes. Serial dilutions were plated in duplicate and evenly spread on the agar using glass beads. Agar plates were incubated for 5-7 days at 37 °C until the colonies were counted. The viable cell count is either presented as the average of log transferred cell counts ($\log_{10}(N)$) or log reduction ($\log_{10}(N_0/N)$).

2.2.4 Microscopic examination by LIVE/DEAD staining

Samples were examined under an Axiostar plus microscope (Carl Zeiss Microscopy GmbH, Germany) equipped with phase-contrast optics and an epifluorescence unit consisting of a mercury vapour lamp as light source and two interchangeable filter sets (Table 9).

Table 9: Epifluorescent filter set specifications and LIVE/DEAD staining dyes.

Filter set #	Excitation	Beam Splitter	Emission	Dye	Excitation/emission
9	BP 450-490	FT 510	LP 515	SYTO 9	480/500
15	BP 546/12	FT 580	LP 590	PI	490/635

A mixture of the green-fluorescent nucleic acid stain SYTO 9 and the red-fluorescent nucleic acid stain propidium iodide (PI) were utilized for the LIVE/DEAD staining. SYTO 9 generally labels all bacteria with both intact and damaged membranes. PI on the other hand only penetrates bacteria with damaged membranes which causes a reduction in the SYTO 9 stain fluorescence when both dyes are present. As such, LIVE/DEAD staining of bacteria results in a fluorescent green colour for bacteria with intact cell membranes and a fluorescent red colour for bacteria with compromised membranes.

The samples were stained according to supplier instructions (Bacterial Viability Kit L7012 or L13152, Thermo Fisher Scientific Inc., Eugene, OR, USA). In short, a mixture of SYTO 9 and PI was added to the bacterial suspension (final dye concentration of 5 and 30 µM, respectively), thoroughly mixed and incubated at room temperature in the dark for 15 minutes. About 8-10 µL of the stained cell suspensions was trapped between a microscopic glass slide and square coverslip for microscopic analysis.

A 100x objective (Zeiss Aplan 100x, numerical aperture 1.25, oil, cover glass thickness ∞/0.17 mm) was used with immersion oil to give a 1000-fold magnification. Images were captured with a 1.388- by 1.038-pixel RGB camera (AxioCam ICc1) and the supporting AxioVS40 V 4.8.2.0 software (Carl Zeiss MicroImaging GmbH, Germany). Images with filter set 9 and 15 were taken of the same microscopic field (changed quickly after each other to minimize photobleaching). Optionally, a bright field microscopic image of the same microscopic field was also taken. Image processing for visual

interpretation was done as following: the image that was taken with filter set 15 was made 50% transparent and overlaid on the image taken with filter set 9.

2.2.5 Turbidity reduction assay

The turbidity reduction assay (TRA) is used to quantify the catabolic activity of peptidoglycan hydrolases. It is based on the decrease in light scattering of a suspension with whole live cells, whole non-viable cells, or a cell wall preparation. The addition of peptidoglycan hydrolases causes a decrease in optical density over time, which can be used as a measure for its enzymatic activity (ΔOD/(time × quantity of enzyme)). In this work, two different substrate preparations were used for the TRA: either isolated cell wall material or whole live cells. The experimental protocol and data analysis for the TRA with live whole cells and isolated cell wall material was slightly different and will therefore be described separately.

Live whole cells

Cells of a 500 mL exponential-phase cell culture (strain TMW 2.1512 for *L. monocytogenes* and TMW 2.422 for *S. aureus*) were harvested in aliquots of 50 mL by centrifugation (7000 × g_0, 10 min, RT) and washed once using imidazole phosphate buffer (IPB, pH 6.2; Table 7). The washed cell pellet was quickly frozen in a liquid nitrogen bath and stored at -80 °C until used.

Endolysin stock solutions were thawed and serially diluted with IPB to double the desired concentration. From each endolysin dilution, 100 µL aliquots were added to three separate wells of a flat-bottom 96-well polystyrene microtiter plate. The frozen cell pellet substrate was diluted with IPB to an initial OD_{600} of approximately 1.0 in a standard 1 cm polystyrene cuvette (taking the addition of an equal volume of endolysin into account). An aliquot of 100 µL of the cell suspension was then added to each well of the 96-well plate prefilled with endolysin solution or IPB as negative control and OD_{600} was measured over time (every 60 seconds for 1 hour minimum, 30 °C; path length correction to 1 cm; Spectrostar Nano, Peqlab Biotechnologie GmbH, Erlangen, Germany).

Two different methods were used to calculate the slope of the turbidity descent (as direct measure for the activity of the endolysin in the sample): (i) in the approach of Korndörfer et al., 2006, curves were normalized by dividing the measured OD_{600} values by the OD_{600} at t_0, corrected by the negative control values, and fitted to the following formula using Sigmaplot 12.5 (*Systat Software GmbH):*

$$f = y_0 + \frac{a}{\left(1 + e^{\frac{-(x_0 - x)}{b}}\right)^c}$$

The fitted curve and corresponding formula were used to determine the maximum slope. (ii) In the approach of Briers et al., 2007a, the region of linear descent is determined by linear regression analyses in which R^2 is maximized. An Excel spreadsheet provided by these authors was used to calculate maximized R^2 data sets and corresponding slopes.

The slopes as calculated using both approaches (in ΔOD_{600}/min) were divided by the endolysin molar concentration (M). The activities calculated at endolysin concentrations where a linear relationship with the slope of the linear descent was present were averaged to end up with the endolysin specific activity (ΔOD_{600}/(min × M)).

Isolated cell wall material

Cells of a 500 mL exponential-phase cell culture (strain TMW 2.1512 for *L. monocytogenes* and TMW 2.422 for *S. aureus*) were harvested by centrifugation (7000 × g_0, 10 min, RT), taken up in 50 mL sterile water (10% of the initial volume), inactivated in a steam bath for ca. 30 minutes, and lysed by sonication (10 min, pulse 1, 20 kHz, 90%, Sonopuls-Homogenatisor HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The lysed cell solution was again centrifuged (15,000 × g_0, 15 min, RT) and the pellet was taken up in 10 mL SM buffer (100 mM NaCl, 8 mM $MgSO_4 \bullet 7H_2O$, 50 mM Tris-Cl, pH 7.5). The sample was incubated with DNAse (0.1 mg/mL) and RNAse (0.1 mg/mL) at 37 °C for 3h. Degradation of proteins was done by incubating overnight at 53 °C with proteinase K (1 mg/mL). The enzymes were subsequently inactivated by taking up the pellet (after centrifugation at 15,000 × g_0, 30 min, RT) in a 4% sodium dodecyl sulfate (SDS) solution and incubating in the steam bath for 30 minutes. Finally, the sample was washed with IPB 3x to remove excess SDS. Aliquots of the isolated cell wall material were stored at -21 °C until used.

Endolysin stock solutions were thawed and serially diluted with IPB to 10-fold the desired concentration. The frozen cell wall suspension was diluted with IPB to the desired OD_{600}.The diluted endolysin (or IPB as control) was added 1:10 to the cell wall suspension and thoroughly mixed. Samples were subsequently divided into two aliquots: a reference sample incubated at 30 °C while the other sample was HHP treated at 300 MPa (10 min, 30 °C). After pressure treatment, 200 µL aliquots of each sample were filled in separate wells of a flat-bottom 96-well polystyrene microtiter plate. OD_{600}-measurements were started exactly 30 min after the addition of endolysin (every 60 seconds for 2 hours, 30 °C).

2.3 Protein analytical methods

2.3.1 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN® Tetra Cell Electrophoresis System (Bio-Rad Laboratories GmbH, München, Germany) according to the method of Schägger and Von Jagow, 1987 with minor modifications. The stock solutions prepared for SDS-PAGE gel electrophoresis are given in Table 10.

Table 10: Stock solutions for SDS-PAGE.

Solution	Compound(s)	Concentration	pH
Anode buffer	Tris	0.2 M	8.9
Cathode buffer	Tris	0.1 M	8.25*
	Tricine	0.1 M	
	SDS	5 g/L	
Gel buffer**	Tris	3.0 M	8.45
Sample buffer**	Tris	0.1 M	8.45
	SDS	0.1% (w/v)	
5x loading buffer**	Tris	0.25 M	8.00
	SDS	7.5% (w/v)	
	Glycerol	25% (v/v)	
	Bromophenol blue	0.25 g/L	
	2-Mercaptoethanol	12.5% (v/v)	

*no correction of pH required, **prepared with HPLC grade water

Handcast gels (8.3 x 7.3 cm, 1 mm thickness) were prepared according to the pipetting scheme in Table 11. Separating gels were prepared by mixing acrylamide:bisacrylamide solution (29:1, 30% w/v, 3.3% C; SERVA Electrophoresis GmbH, Heidelberg, Germany), gel buffer, deionized water, and 25% SDS solution (w/v). Gels were poured after the addition of fresh (<1 month) 10% ammonium persulfate (APS) solution and N,N,N',N'-tetramethylethylendiamine (TEMED), overlaid with isopropanol, and allowed to polymerize. Subsequently, the isopropanol layer was discarded and a 4% stacking gel was prepared. The stacking gel was filled onto the separating gel, a comb was inserted to form sample cavities, and the gel was allowed to polymerize. The acrylamide solution, 10% APS, and TEMED were stored at 4 °C. All other solutions were kept at RT. For electrophoresis, the electrophoresis apparatus was assembled and running reagents were poured into the tank (anode buffer) and gel chamber (cathode buffer).

Table 11: Composition of separating and stacking SDS-PAGE gels.

Solution	12 % Separating gel	4% Stacking gel
30% Acrylamide	5.30 mL	0.68 mL
Gel buffer	3.34 mL	1.29 mL
Water*	1.26 mL	3.21 mL
25% SDS	40 µL	16 µL
10% APS	50 µL	33 µL
TEMED	7 µL	7 µL

*HPLC grade water was used

Sample preparation was done as following; the endolysins where thawed on ice and diluted to the desired concentration with the sample buffer. The samples were mixed 1:5 with the 5x loading buffer and denatured at ca. 95 °C for 5-10 minutes. Afterwards, 10 µL of each sample was loaded into a separate gel well. A molecular weight marker (1 µL) was loaded simultaneously with the samples in each run (PageRuler Plus Prestained Protein Ladder, 10-250 kDa; Thermo Fisher Scientific Inc., Rockford, IL USA). Electrophoresis commenced with 5 min at 80 V followed by a constant voltage of 100 V for ca. 90 minutes. After the electrophoretic run, gels were silver stained (2.3.3).

2.3.2 Acidic native PAGE

Acidic native PAGE was performed in the same gel electrophoresis system as used for SDS-PAGE. Importantly, the polarity of the leads was reversed as the proteins will be positively charged at an acidic pH and run to the cathode (see isoelectric point in Table 16). The stock solutions prepared for acidic native PAGE gel electrophoresis are given in Table 12.

Table 12: Stock solutions for acidic native PAGE.

Solution	Compound(s)	Conc.	pH
Anode and cathode buffer	β-Alanine Acetic acid	0.35 M 0.14 M	4.3*
Separating gel buffer***	Acetic acid	1.5 M	4.3**
Stacking gel buffer***	Acetic acid	0.25 M	6.8**

*no correction of pH required, **pH adjusted with 1 M KOH, ***prepared with HPLC grade water

The separating and stacking gel were principally prepared in the same fashion as described for SDS-PAGE. The pipetting scheme of both separating and stacking gels is given in Table 13. The acrylamide solution, 10% APS, and TEMED were stored at 4 °C. All other solutions were kept at RT.

Table 13: Composition of separating and stacking native PAGE gels.

Solution	10 % Separating gel	4% Stacking gel
Separating gel buffer	3.35 mL	-
Stacking gel buffer	-	1.25 mL
Glycerol 50% (v/v)	3.00 mL	-
Water*	2.10 mL	3.10 mL
30% Acrylamide	4.40 mL	0.67 mL
10% APS	160 µL	50 µL
TEMED	20 µL	5 µL

*HPLC grade water was used

The acidic native PAGE loading buffer (5x) was prepared by mixing 1.45 mL 50% glycerol (v/v) and 0.5 mL separating gel buffer with traces of fuchsine as tracking dye. Aliquots of the loading buffer (5x) were kept at -21 °C. Sample preparation was performed on ice. Endolysins were thawed and diluted to the desired concentration with the stacking gel buffer, mixed 1:5 with the 5x loading buffer, and loaded immediately onto the gel. Electrophoresis started by 5 min at 20 A followed by a constant current of 40 A for ca. 120 minutes. After the electrophoretic run, gels were silver stained (2.3.3).

2.3.3 Silver staining

Proteins on acidic native PAGE and SDS-PAGE gels were silver stained according to the method of Blum et al., 1987 with modifications. The composition of reagents and procedure are given in Table 14. All steps were performed at RT on a platform shaker or by gentle manual shaking. Gels were scanned directly after completion of the staining procedure (Bio-5000 VIS Gel Scanner, SERVA Electrophoresis, Heidelberg, Germany).

Table 14: Silver staining protocol. Silver staining according to Blum et al., 1987 with modifications.

Step	Reagent	Duration
Fixation	40% ethanol* 10% acetic acid	overnight
Wash	30% ethanol	2 x 20 min
Wash	deionised water	1 x 20 min
Sensitization	0.2 g/L sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$)	1 min
Wash	deionised water	3 x 20 sec
Silver impregnation	2 g/L silver nitrate ($AgNO_3$)	20 min
Wash	deionised water	3 x 20 sec
Development	30 g/L sodium carbonate (Na_2CO_3) 4 mg/L sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$) 0.05% formaldehyde (H_2CO; 37%)**	3-5 min
Wash	deionised water	3 x 20 sec
Stop	5 g/L glycine	5 min
Wash	deionised water	3 x 10 min

*Ethanol used was HPLC grade. **Formaldehyde was added to the developer shortly before used.

2.3.4 UV-spectrophotometry for protein concentration and aggregation

Endolysin concentration and possible aggregation were determined by UV-spectrophotometry with a microscale spectrometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA).

2.3.4.1 Determination of endolysin concentration

The molar extinction coefficient at 280 nm (ε_{280}) of *Listeria* phage endolysins was calculated based on the amino acid sequences using the following formula (Gill and Von Hippel, 1989):

$$\varepsilon_{280} = \Sigma\text{Trp} \times 5500 + \Sigma\text{Tyr} \times 1490 + \Sigma\text{Cysteine} \times 125$$

The specific attenuation coefficient ($^{0.1\%}A_{280}$) could subsequently be calculated with the molecular mass (M_r) of the endolysins as provided by the supplier:

$$^{0.1\%}\text{A}_{280} = \frac{\varepsilon_{280}}{\text{M}_\text{r}}$$

Protein concentrations could be determined by applying the Beer-Lambert law:

$$\text{c} = \frac{\text{A}_{280}}{^{0.1\%}\text{A}_{280} \times \text{d}}$$

where *c* is the concentration (mg/mL), A_{280} the measured absorption at 280 nm, and *d* the cuvette thickness (cm). Molecular mass and molar extinction coefficient are given in Table 15.

Table 15: Molar extinction coefficient and molecular mass of *Listeria* phage endolysins.

Endolysin	Molecular extinction coefficient ε* (M^{-1} cm^{-1})	Molecular mass M_r (kDa)
PlyP40	60,195	38.2
Ply511	56,060	36.5
PlyP825	54,235	34.2

2.3.4.2 Protein aggregation

Determination of the ratio between the absorbance of a sample at 280 and 340 (i.e. absorption index) is a quick and easy method to measure the extent of protein aggregation. The peak of protein absorbance usually lies at 280 nm due to absorption of light by the aromatic side chains. Aggregated protein samples scatter light, causing the absorption measurement to display a hyperbole shape. Typical absorbance spectra for precipitated and soluble forms of a protein are shown in Figure 7.

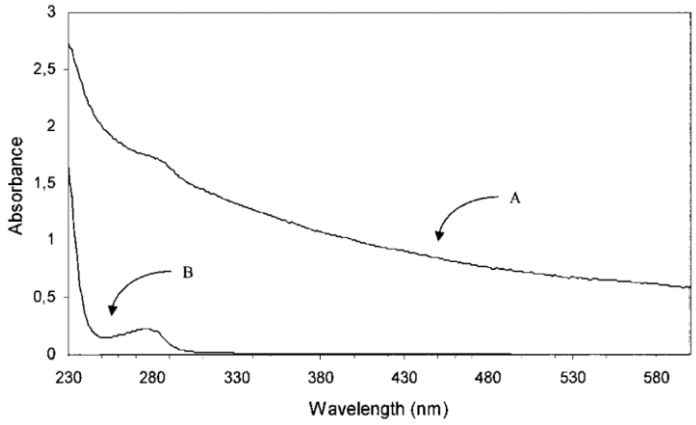


Figure 7: UV-abosrbance of protein aggregates. The UV-absorbance spectrum of the precipitated (a) and soluble (b) form of BSA. Adapted with permission from Vincentelli et al., 2004. Copyright © 2004, John Wiley and Sons.

2.3.5 Asymmetric flow field-flow fractionation

Asymmetric flow field-flow fractionation (AF-4) is a one-phase chromatography technique which allows for the separation of macromolecules based on colloidal size. Smaller particles are transported more rapidly along the channel than larger particles, which theoretically allows for the differentiation between protein monomers and aggregated protein complexes. As such, possible aggregation of endolysin PlyP825 was investigated by AF-4 (Wyatt Technology, Germany) coupled to multi-angle laser light scattering (MALS) (Dawn Heleos II, Wyatt Technology, Germany) and UV-detection (Dionex Ultimate 3000, Thermo Fisher Scientific, USA). Experimental procedure was performed under the supervision of Dr. F. Jakob (Lehrstuhl für Technische Mikrobiologie, Technische Universität München) as described in Jakob, 2014 and Ua-Arak et al., 2016 with minimal modifications. In short, endolysin PlyP825 was serially diluted with IPB and different concentrations (ranging from 5 to 200 µg/mL) were injected into the separation channel. The injection and elution flow were set to 1 and 5 mL/min, respectively. The gradient cross flow rate was kept constant at 5 mL/min. The separations were performed on 10 kDa regenerated cellulose membranes (Superon GmbH, Germany) with IPB (pH 6.2) as eluent solution. The data was collected and analysed using ASTRA 6.1 software (Wyatt Technology, Germany).

2.4 Antimicrobial agents and processing methods

2.4.1 Endolysins

All endolysins and lysostaphin were provided by Hyglos GmbH (Bernried am Starnberger See, Germany) as part of a corporation in project AZ-1051-12 funded by the Bayerische Forschungsstiftung (Munich, Germany). The endolysins were stored at -21 °C. General information on endolysin characteristics, concentration, and storage buffer is provided in Table 16.

Table 16: Endoysins used in this work. Overivew of endolysins characteristics, properties, and storage buffers as provided by the supplier.

Endolysin	Isoelectric point*	Molecular mass (kDa)	Stock concentration (mg/mL)	(µM**)	Storage buffer	Patent reference
PlyP40	9.69	38.2	1.0	26.28	40 mM Tris 100 mM NaCl pH 7.0	WO 2010/010192 (Loessner et al., 1996)
Ply511	9.77	36.5	0.46	12.60	40 mM Tris 100 mM NaCl pH 8.0	WO 1996/007756 (Loessner and Schmelcher, 2010)
PlyP825	9.80	34.2	3.5	102.3	40 mM Tris 100 mM NaCl pH 7.0	WO 2012/159774 (Grallert et al., 2012)
HY-100	9.08	55.1	12.9	234.1	25 mM Tris 10 mM CaCl2 10 mM Citrate 300 mM Arginine pH 7.5	WO 2009/150171 (Buchberger et al., 2009) (under trivial name PRF-100)
HY-133	9.45	31.1	5.0	160.8	25 mM Hepes 150 mM NaCl 300 mM Arginine 10 mM CaCl2 pH 8.0	WO 2011/076432 (Grallert and Leopoldseder, 2011; under trivial name PRF-133)
Lysostaphin	-	-	3.0	-	25 mM NaPi 120 mM NaCl pH 7.5	-

*the isoelectric point was computed based on the amino acid sequences (only available for *Listeria* phage endolysins) using an online tool from the SIB Bioinformatics Resource Portal (http://www.expasy.org/). **The molar concentration was calculated using the molecular mass and mass concentration.

The modular organization of the endolysins used in this work is schematically illustrated in Figure 8. Specific information on the (putative) cleavage and bindings sites of the different EADs and CBDs is provided in the figure caption.

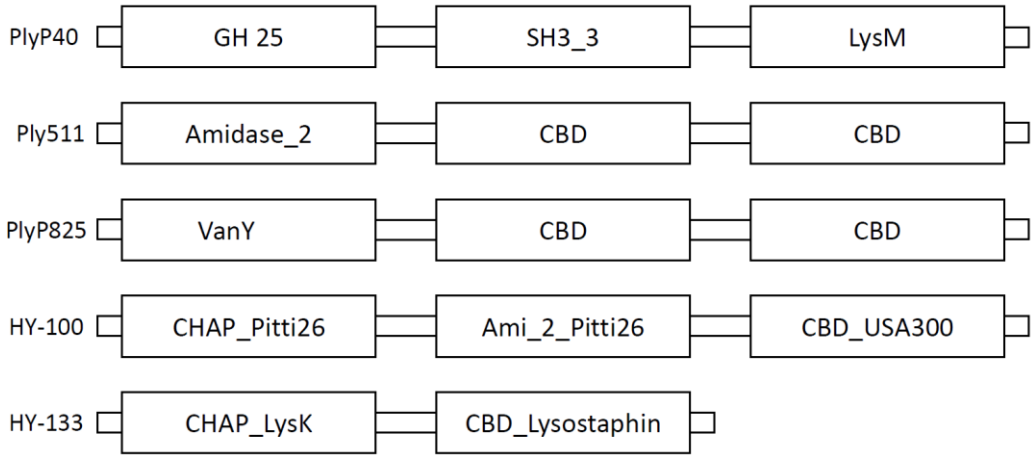


Figure 8: Schematic representation of the modular organization of endolysins used in this work. PlyP40 contains the GH_25 EAD with putative lysozyme activity (N-acetylmuramidase; Scherzinger, 2011). Ply511 was characterised as a N-acetylmuramoyl-L-alanine amidase (Loessner et al., 1995). The EAD of PlyP825 has very high sequence similarity to the VanY domain (75% sequence identity, 86% sequence homology; Scherzinger, 2011). Ply500 also contains this domain and was characterised as a L-alanoyl-D-glutamate endopeptidase (Loessner et al., 1995). Hence, PlyP825 is expected to have endopeptidase activity as well. The CBDs of endolysin PlyP40 and Ply511 were shown to specifically recognize the peptidoglycan backbone structure of *Listeria* (Eugster and Loessner, 2012). The CBD of PlyP825 has high sequence similarity with the CBD of Ply511 (61% sequence identity, 73% sequence homology; Scherzinger, 2011) and is therefore expected to bind directly to the peptidoglycan as well. HY-100 refers to the "Modul 3" chimeric constructs described by Forchheim, 2009. The CHAP_Pitti26 and Amidase_2_Pitti26 are an endopeptidase and amidase, respectively. HY-133 contains an endopeptidase CHAP_LysK EAD which cleaves between the D-alanine of the stem peptide and glycine of the cross-bridge peptide (Becker et al., 2009b). The CBD_Lysostaphin of HY-133 binds to the pentaglycine interpeptides of the peptidoglycan (Gründling and Schneewind, 2006). Specific information on the binding site of CBD_USA300 (from the prophage phi Sa2USA300) is not available. This schematic representation does not reflect the relative size of the endolysins/domains.

2.4.2 Bacteriophage

LISTEX™ P100 (viable phage count of $2x10^{11}$ pfu/mL) was kindly provided by Amrei Metz from MICREOS Food Safety B.V. (Wageningen, the Netherlands) and stored protected from light at 4 °C. See section 1.4.1.2 for further characteristics of phage P100.

2.4.3 HHP

The pressure unit TMW-RB (Knam Schneidetechnik GmbH, Langenargen, Germany) has been described before by Lenz and Vogel, 2014 and Lenz et al., 2015. Shortly, the pressure unit consists out of two parallel linked 7 mL pressure vessels equipped with thermostating jackets regulated by a recirculating thermostat (FC 600; JULABO Labortechnik GmbH, Germany). A mixture of 70% polyethylene glycol 400 (Roth, Karlsruhe, Germany) and 30% deionized water was used as pressure-transmitting fluid.

Samples for HHP processing were either filled into cryotubes with an internal thread (Thermo Fisher Scientific, Bonn, Germany) or vacuum sealed. The presence of air (bubbles) was avoided as much as possible. Samples were placed into a preheated high pressure vessel ca. 5 minutes prior to start of the pressure ramp. Reference samples without HHP treatment were simultaneously incubated at the same temperature as the pressure vessel. Compression and decompression rates were kept constant at 200 MPa/min. The pressure temperature, pressure level, and pressure holding time highly varied and are therefore described for each experiment separately in the results section.

2.5 Experimental set-up inactivation studies

A schematic representation of the experimental set-up to study the inactivation of *L. monocytogenes* and *S. aureus* by endolysin and HHP is provided in Figure 9. However, experimental procedures (e.g. preparation of the inoculum, coincubation time of cells with endolysin, time until microbiological analysis, etc.) differed greatly between experiments in buffer and food and are therefore described separately below (2.5.1 and 2.5.2, respectively) The experimental procedures to examine the inactivation of *L. monocytogenes* by phages and HHP were again different and therefore also described separately (2.5.3).

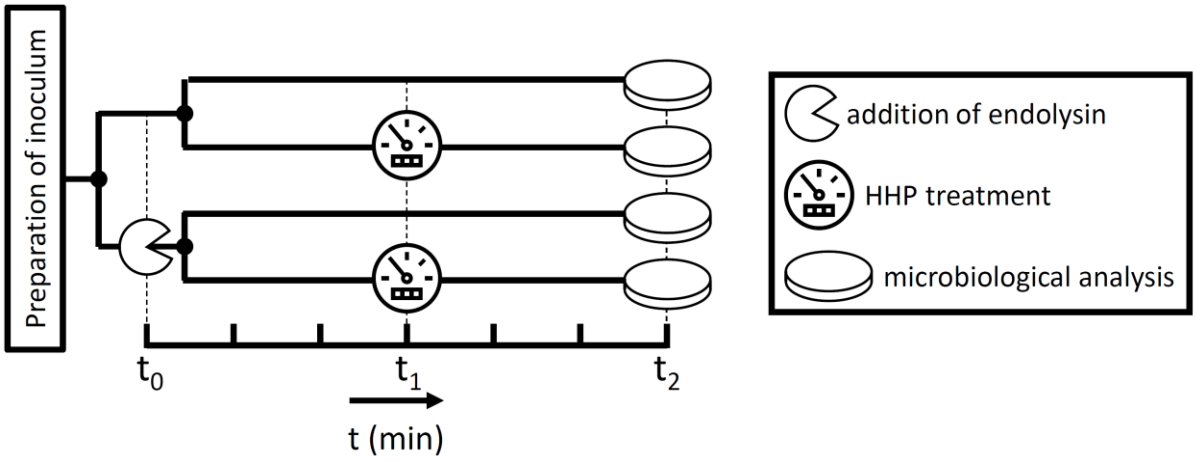


Figure 9: Experimental procedure for combined endolysin and HHP treatment. Schematic representation of the experimental set-up to study the inactivation of *L. monocytogenes* and *S. aureus* by a combination of endolysin and HHP processing.

2.5.1 Endolysin and HHP in buffer

All experiments in this section were performed in IPB[1] (pH 6.2; Table 7) with single *L. monocytogenes* and *S. aureus* strains. Individual strains were harvested in either the exponential or stationary growth phase and serially diluted with IPB to the desired inoculation level. Cells were kept at RT until further processed (within 30 – 60 min).

The endolysin stock solutions were thawed and serially diluted with IPB to 10-fold the desired concentration. Each endolysin was contained in a different storage buffer (Table 16). Hence, different buffers were used for the first dilution step of the different endolysins (IPB^{PlyP40}, IPB^{Ply511}, $IPB^{PlyP825}$, IPB^{HY-100}, IPB^{HY-133}, or $IPB^{Lysostaphin}$; Table 7). This allowed to have each endolysin in the same buffer composition (IPB, pH 6.2) after one single dilution step.

The diluted endolysin (or IPB as control) was added 1:10 to the cell suspension and thoroughly mixed. An aliquot of each samples was filled into 0.5 mL cryotubes for HHP treatment and incubated at 30 °C until further processed. All HHP treatments were performed at 30 °C, though the pressure level and holding time varied per assay and are therefore individually described for each experiment in the result section. After HHP treatment, samples were put back in the incubator at 30 °C until determination of viable cell count. The coincubation time of cells with endolysin until HHP and total coincubation time

[1]Imidazole and phosphate respectively have a positive and negative $\Delta pH/\Delta P$ (Quinlan and Reinhart, 2005). The pH of the combined buffer is therefore relatively pressure stable, minimizing possible effects of pressure-induced pH changes on bacterial inactivation.

until microbiological analyses also varied per experiment and are therefore also described for each assay individually in the result section.

2.5.2 Endolysin and HHP in foods

All experiments in this section were done using a cocktail of five separately cultured *L. monocytogenes* strains from the stationary growth phase (TMW 2.594, 2.595, 2.597, 2.601, and 2.1512). The concentration of cells in a stationary-phase cell culture was determined for each strain. This allowed for the preparation of a cocktail with equal number of cells per strain. The inoculum to weight sample ratio (only for solid foods) was approximately 1:80, thereby avoiding any significant change on product characteristics such as the water activity as much as possible.

HHP processing was performed at 25 °C for all foods examined. The pressure level, pressure holding time, and packaging method (cryotube versus vacuum packaging) varied depending on the food product used. Endolysin incubation time until HHP and total incubation time until microbiological analyses were kept constant at 3 and 4 hours, respectively. Foods used in this section were purchased in a local supermarket (Tengelmann, Freising, Germany). Specific product information as described on the food label as well as measured pH and water activities are provided in Table 17. Since the food products had different consistencies (liquid versus solid in liquid versus solid), different sample inoculation and preparation procedures were used (described below):

Table 17: Food label specification. Food products used to study the inactivation of *L. monocytogenes* by endolysin and HHP. Food specifications according to the food label including measured water activity and pH are provided.

	Bio H-Milch 3,5% (Gmundner Molkerei reg.Gen.m.b.H., Gmunden, Österreich)	Bio-Mozzarella (Die Ökobauern, Domspitzmilch eG, Regensburg, Germany)*		Schottischer Räucherlachs, (Käfer Feinkost, Gottfried Friedrichs KG, Hamburg, Germany)
Fat (per 100 gram)	3.5 g	19.0 g		12 g
-of which saturated fatty acids	2.3 g	12.0 g		2.4 g
Carbohydrates (per 100 gram)	4.8 g	1.0 g		0 g
-of which sugars	4.8 g	1.0 g		0 g
Protein (per 100 gram)	3.3 g	18 g		22 g
Salt (per 100 gram)	0.13 g	0.50 g		4.0 g
a_w	0.997	0.789 (cheese)	0.952 (liquid)	0.893
pH	6.67	6.04	6.17	6.08

*this mozzarella product was chosen because it does not contain any adiculants (e.g. citric acid).

Milk

The harvested cell pellet of each strain was taken up in milk, mixed to a cocktail, and diluted to the desired inoculation level. Endolysins were taken from the stock culture, thawed, and diluted with milk to 10-fold the desired concentration. The diluted endolysin (or milk as control) was added 1:10 to the cell suspension and rotated overhead at RT until further processed. HHP samples were transferred to 0.5 mL cryotubes shortly before HHP processing. Experimental repetitions were done with the same milk package which was kept sterile at 4 °C for maximum 2 weeks.

Mozzarella

The mozzarella brine was used to prepare the *L. monocytogenes* cocktail. Mozzarella samples were prepared beforehand by aseptically slicing the cheese into stripes (3 x 0.5 x 0.5 cm; ca. 0.7 g) which

were stored in mozzarella brine at -21 °C until used. Samples where thawed overnight at 4 °C before use. The mozzarella stripes (i.e. without brine) were inoculated with 10 µL of the *Listeria* cocktail and allowed to dry under the laminar flow for 15 min. Endolysins were thawed and diluted in mozzarella fluid to their final concentration. The mozzarella stripes were put into 1.8 mL cryotubes (Thermo Fisher Scientific, Bonn, Germany) filled with mozzarella brine (either with or without endolysin) and rotated overhead until further processed.

Smoked salmon

The *L. monocytogenes* cocktail and endolysin dilutions were prepared with a lactic acid buffer with similar characteristics as the product itself (i.e. the same salt content and buffer pH, adjusted by the food additive lactic acid; 513 mM NaCl, 10 mM lactic acid / sodium lactate, pH 6.3). Smoked salmon samples were aseptically sliced into stripes (3 x 0.5 x 0.25 cm; ca. 0.85 g) and stored at -21 °C until used. Samples where thawed overnight at 4 °C before use. Salmon stripes were inoculated with 10 µL of the *Listeria* cocktail and allowed to dry under the laminar flow for 15 minutes. The diluted endolysin (or buffer as control) was applied by spreading 50 µL to the exact same inoculation spot. Samples were vacuum packed and kept at RT until further processing.

2.5.3 Phage P100 and HHP in buffer and milk

Milk used for experiments in this section was purchased in a local supermarket (Aldi Süd, Freising, Germany). Specific product information as described on the food label is depicted in Table 18. IPB at pH 7.5 was used for assays in buffer. All experiments in this section were done with a single *L. monocytogenes* strain (TMW 2.1512) from the stationary growth phase. The bacteriophage P100 stock solution was diluted to 10-fold the desired concentration with either milk or IPB. The diluted phage (or buffer/milk as control) was added 1:10 to the cell suspension either prior to or after HHP processing (Figure 10). HHP processing was performed at 25 °C for 1 min either shortly before or 1.5 hours after the addition of phages to the cells. The pressure level varied per assay and is therefore described separately per experiment in the results section. The total coincubation time of cells with the phages (i.e. until microbiological analyses) was kept constant at 2 hours (Figure 10).

In order to examine the effect of the milk's fat-content on the inactivation of *L. monocytogenes* by bacteriophage P100, milk samples with different fat contents were created as followed: the fat-fraction was separated from the liquid milk fraction by centrifugation (7000 x g for 20 min) and collected in a separate container. This process was repeated once more and the remaining liquid milk fraction was defined as 0% fat. Milk with different fat contents (i.e. 1.5, 3.5 or 10% (v/v)) were subsequently prepared and thoroughly mixed using an Ultra-Turrax at 30,000 rpm for several seconds (D-8, MICCRA GmbH, Müllheim, Deutschland). The established milk-fat emulsions were stable at RT for at least a couple of hours. Milk samples with different fat contents were stored at -21 °C until used.

Table 18: Food label specifications. Milk product used to study the inactivation of *L. monocytogenes* by phage P100 and HHP.

	Haltbare Vollmilch 3,5 % Fett (Molkerei Gropper GmbH & Co. KG, Bissingen, Germany)
Fat (per 100 gram)	3.6 g
-of which saturated fatty acids	2.5 g
Carbohydrates (per 100 gram)	4.9 g
-of which sugars	4.9 g
Protein (per 100 gram)	3.4 g
Salt* (per 100 gram)	0.13 g

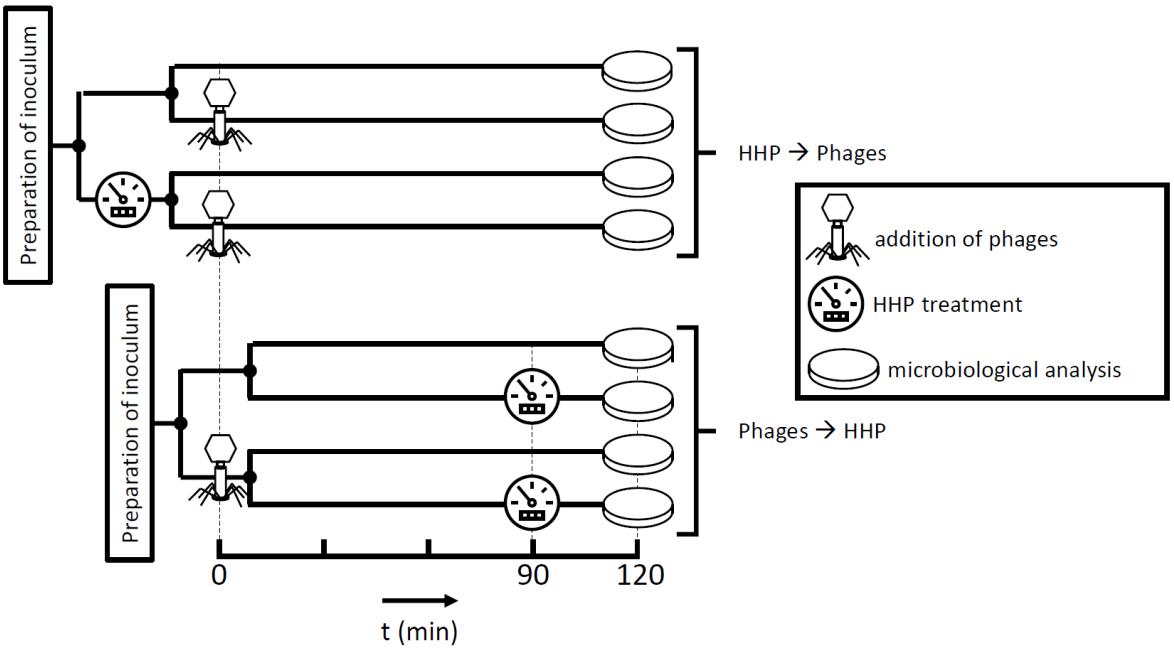


Figure 10: Experimental procedure for the combined phages and HHP treatment. Schematic representation for the inactivation of *L. monocytogenes* by phages added prior to (lower part) or after (uppert part) HHP processing.

2.6 Software and data analysis

Software

Table 19: List of software packages used in this work.

Adobe Acrobat Reader DC 2015	© Adobe Systems Incorporated
ASTRA 6.1.2.84	© Wyatt Technology Corporation
AxioVS40 V 4.8.2.0	© Carl Zeiss MicroImaging GmbH
Endnote X7.4	© Thomson Reuters
GIMP 2.8.10	© Spencer Kimball, Peter Mattis and the GIMP Development Team
Microsoft Office 2013	© Microsoft Corporation
SigmaPlot 12.5	© Systat Software

Statistics

Statistical analyses of viable cell counts were done with $\log_{10}$ transferred values (Jarvis, 2008). The statistical test used was dependent on the number of groups compared, normality of the data, and equality of variance. Shapiro-Wilk was used to test for normal distribution of the data and the Spearman rank correlation was computed to check for equal variance. For normally distributed data

with equal variance, the Student's t-test was used for the comparison of two groups. The comparison of more than two groups was done by analysis of variance (ANOVA) and the post-hoc Student-Newman-Keuls Method for paired comparison where ANOVA values were significant. In case the data was not normally distributed or did not have equal variance, the non-parametric Mann-Whitney rank sum test (two groups) or Kruskal-Wallis one way ANOVA on ranks (>two groups) was used alternatively. Groups were considered significantly different with a one-tailed p-value < 0.05. Importantly, although results might be presented as log reduction for ease of interpretation, all statistical tests were performed with the $\log_{10}$ transformation of the total cell count.

Synergy

The synergistic effect was defined as the additional log reduction provoked by a combined endolysin and high pressure treatment compared to the sum of the individual effects:

synergistic effect = $\log_{10}(N_{HHP} * N_{endolysin})/(N_0 * N_{HHP+endolysin})$.

3 RESULTS

3.1 Characterization of endolysins

The results in this chapter originate in part from a recent publication (Van Nassau et al., 2017).

3.1.1 Molecular size and protein concentration

For all endolysins used in this work, information about the stock concentrations and molecular size were provided by the supplier (see Table 16). These data were confirmed for *Listeria* phage endolysins by UV-spectroscopy at 280 nm and SDS-PAGE. The experimentally determined concentration of endolysin PlyP40, Ply511, and PlyP825 stock solutions were 1.02, 0.40, and 3.46 mg/mL, which was more or less in line with the supplier's product data sheets (except for a small deviation for endolysin Ply511). The size of proteins was roughly determined to be 44.5, 43.2, and 41.1 kDa respectively (Figure 11; calculated by plotting $\log_{10}$ of the molecular weight versus the relative migration distance). This is somewhat higher than the molecular weight as can be calculated from the amino acid sequences (Table 16), though it does reflect the size ratio between the individual endolysins, and SDS-PAGE is known to only give a rough indication of the molecular weight.

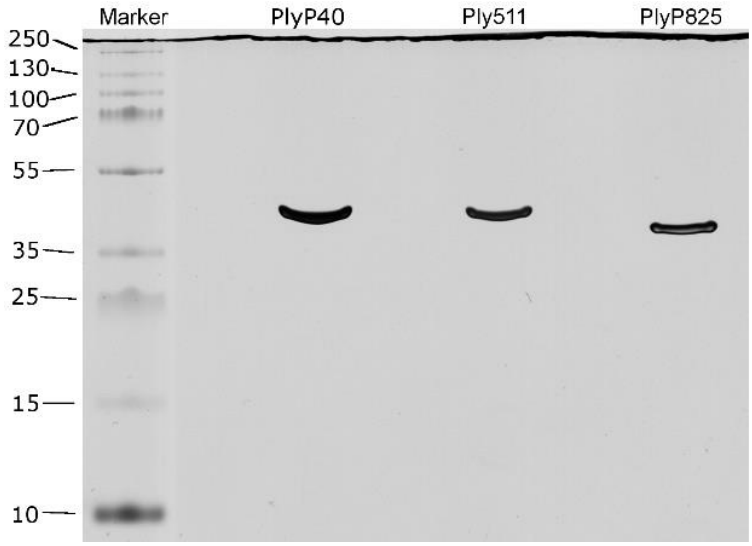


Figure 11: SDS-PAGE of endolysin PlyP40, Ply511, and PlyP825. The molecular weight of the marker proteins bands are indicated in kDa.

3.1.2 Enzymatic activity

Multiple methods have been described to study peptidoglycan hydrolases for both their enzymatic as well as antimicrobial activity (Nelson et al., 2012). In the present work, the enzymatic activity of endolysins was quantified with a standardized TRA using either purified cell wall material or whole live cells as substrate (Figure 12a). This was done in equimolar amounts in the concentration range where a linear relationship between enzyme concentration and steepest slope of the lysis curve was present (Figure 12b).

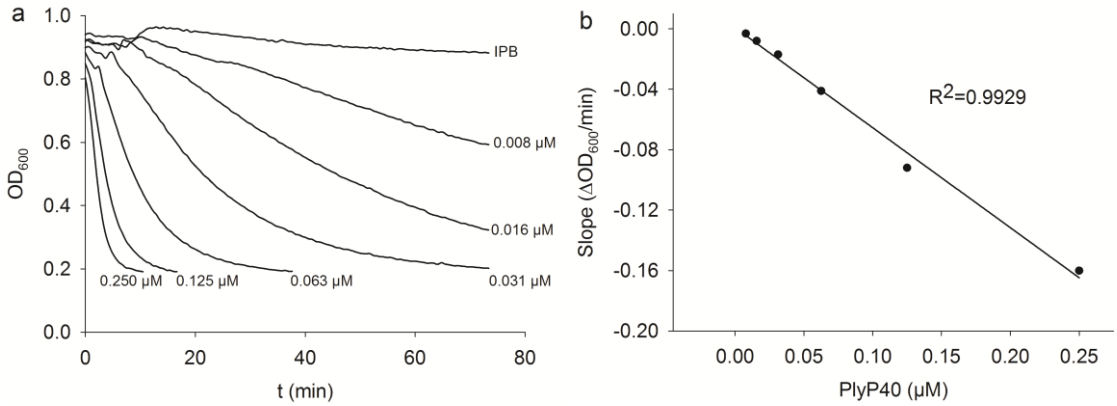


Figure 12: TRA and linear relationship of PlyP40 concentration with the slope of the linear descent. (a) Decrease in OD_{600} of a *L. monocytogenes* cell suspension (TMW 2.1512) after the addition of different PlyP40 concentrations or IPB as control (raw data). (b) The slope of the region of linear descent (of the curves in a, as determined by the method of Briers et al., 2007a) plotted against the applied PlyP40 concentration. The linear regression curve and corresponding coefficient of determination (R^2) are shown.

Two different approaches have been suggested in scientific literature to calculate the enzymatic activity from the generated OD-curves (as described in section 2.2.5). In both methods, the enzymatic activity is expressed as reduction in OD per minute per mg enzyme ($\Delta OD_{600}/(min \times mg)$). This definition of enzymatic activity only allows for an accurate comparison of enzymes with exactly the same molecular weight. Hence, endolysin's enzymatic activity is presented as $\Delta OD_{600}/(min \times \mu M)$, which reflects the enzymatic activity per mole enzyme and is therefore a more accurate description.

Although the specific enzymatic activity differed depending on the calculation method used, the ratio between the activities of the different endolysins remained more or less constant. For the *Listeria* phage endolysins examined, the activity of PlyP40 and PlyP825 was significantly higher than that of Ply511 (as calculated by the method of Korndörfer et al., 2006; Table 20). Nonetheless, enzymatic activities seemed to be in more or less the same range, especially when compared to the enzymatic activity of *S. aureus* specific peptidoglycan hydrolases, where HY-100 was found to have 3 to 5-fold higher activity than HY-133 and lysostaphin, respectively.

A direct comparison of the enzymatic activity between endolysins against different species is not relevant. The enzymatic activity is defined as the reduction in OD per time-unit and as such, the substrate and unit of measurement (i.e. absorption of light by the bacterial cell suspension) are intrinsically different between species. In addition, the enzymatic activity measured by TRA was found to be highly dependent on the substrate batch, buffer system, and strain used (data not shown), which not only limits the comparison phage endolysins in this work, but is a general limitation for the comparison of endolysins against different species and within scientific literature.

Table 20: Endolysin enzymatic activity as determined by TRA. The enzymatic activity of endolysins and lysostaphin was examined in IPB against *L. monocytogenes* strain TMW 2.1512 or *S. aureus* strain TMW 2.422 and either calculated by the method of Korndörfer et al., 2006 or Briers et al., 2007a. For *Listeria* phage endolysins, mean values ± standard deviation of three experiments are shown. Different letters within the same method denote a statistical significant difference. Values of single replicates are shown for HY-100, HY-133, and lysostaphin.

Endolysin	Specific activity (ΔOD_{600}/(min × µM))	
	Method of Korndörfer et al., 2006	Method of Briers et al., 2007
PlyP40	881.5 ± 77.9[a]	570.5 ± 30.1[a]
Ply511	532.4 ± 12.0[b]	383.2 ± 35.1[b]
PlyP825	738.7 ± 99.8[a]	492.3 ± 80.8[ab]
HY-100	-	1274,8
HY-133	-	414,9
Lysostaphin	-	238,3

3.1.3 HHP stability

Activity post HHP treatment

Endolysin PlyP40 is relative heat stable (75% residual activity after incubating for 5 minutes at 65 °C; unpublished work of Christian A. Lenz, Lehrstuhl für Technische Mikrobiologie). In this section, the pressure stability of these endolysins was examined. Endolysins were therefore diluted to a concentration of 1000 mM and pressurized at 600 MPa (10 min, 30 °C). Directly afterwards, a TRA with whole live cells as substrate was performed with the HHP processed as well as the untreated control sample. The enzymatic activities of PlyP40, Ply511, and PlyP825 control samples were determined at 435, 400, and 383 ΔOD_{600}/(min × µM), respectively. The activity of HHP-treated endolysin was equal or even somewhat higher (424, 431, and 481 ΔOD600/(min × mM), respectively), which is most likely the result of high assay and sample variability. Although this shows that a HHP treatment of 600 MPa does not permanently affect the enzymatic activity, activity might change during pressure treatment.

Activity during pressure treatment

A possible change in enzymatic activity under pressure could not be measured directly during HHP processing. Instead, the reduction in OD_{600} of a cell wall suspension was compared after samples were incubated with endolysin for 30 min and, in this time-frame, samples were either kept at atmospheric pressure or received a pressure treated at 300 MPa for 10 min (25 °C). An up- or downward shift in the TRA curves of pressure treated samples (at equal endolysin concentrations) would indicate a positive or negative effects of pressure on the enzyme's activity. However, Figure 13 shows that the pressure treatment hardly affected the reduction in OD at any of the concentration examined. A very small reduction might be initially visible, but this is also present in the control and is therefore either the effect of pressure on the cell wall suspension or caused by small sample to sample variability.

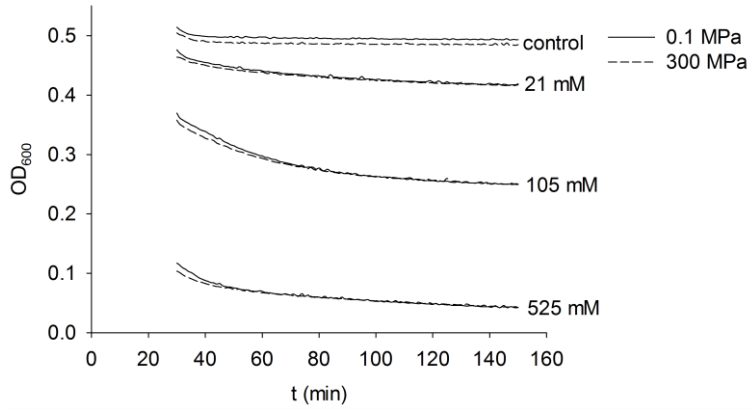


Figure 13: The effect of pressure on PlyP40's enzymatic activity as examined by TRA. The decrease in OD_{600} of a *L. monocytogenes* cell wall suspension (TMW 2.599) after the addition of different concentrations PlyP40 or IPB as control at t0. OD-measurements were started 30 min after the addition of endolysin. In this time frame, samples were either kept at atmospheric pressure (30 °C; solid line) or HHP treated (300 MPa, 10 min, 30 °C; dashed line).

3.2 The inactivation of *L. monocytogenes* and *S. aureus* by endolysin and HHP in buffer

The results in this chapter originate (in part) from a recent publication (Van Nassau et al., 2017).

3.2.1 The combined application of endolysins and HHP

The proof of principle for a synergistic effect between endolysin and HHP for the inactivation of Gram-positive bacteria was given in preliminary experiments at the chair of Technische Mikrobiologie, TU München using endolysin PlyP40 and the non-pathogenic species *L. innocua*. In this work, the combined application of these techniques against the foodborne pathogens *L. monocytogenes* and *S. aureus* was investigated in a large screening with multiple strains and different endolysins (material and methods section 2.5.1). In order to retrieve detailed insights from this screening, several preliminary experiments were performed to optimize parameters.

3.2.1.1 Preliminary experiments

3.2.1.1.1 Growth curves

A diverse set of strains used were selected from the internal strain collection based on genetic/proteomic characteristics and their diverse endolysin susceptibility (i.e. MALDI-TOF MS spectra, strain serovar and an initial screening on the effect of endolysin PlyP40 and HY-133 on lag phase, growth rate, and log reduction) to rule out any strain specific effects (preliminary data, not shown). Growth curves of selected strains were established and are provided in Figure 14. Cell cultures with an OD_{590} of 0.8 ± 0.1 were determined to be in their exponential growth phase.

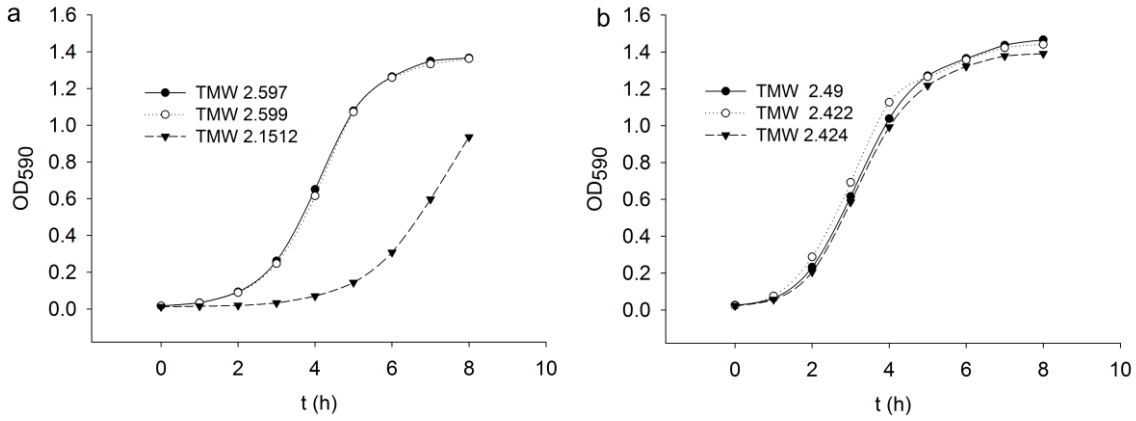


Figure 14: Growth curves of selected *L. monocytogenes* and *S. aureus* strains. The OD_{590} of *L. monocytogenes* strains TMW 2.597, 2.599, and 2.1512 (a) and *S. aureus* strains TMW 2.49, 2.422, and 2.424 (b) cell cultures as measured over time. Cells were grown in TSBYE in diagonally placed 50 mL centrifuge tubes at 37 °C while shaking at 100 rpm. OD_{590} was measured in standard 1 cm polystyrene cuvettes.

3.2.1.1.2 HHP susceptibility

To examine the inactivation of cells by a combination of endolysin and HHP processing, the maximum pressure level that did not induce a reduction of more than 1 log cfu was determined. Such treatments sensitized the cells but avoided a high level of inactivation, which would impede the detection of any additional effect when pressure and endolysin treatments were combined. The pressure resistance between strains within one species can greatly vary (1.3.2.2.2) and was therefore determined for each strain individually. The pressure holding time and initial temperature were fixed at (1 min, 30 °C) and the pressure level was incrementally increased from 200 to 500 MPa in steps of 50 MPa (Figure 15). For each strain individually, the maximum pressure levels which caused a reduction in the viable cell count of <1 log cycles was selected as parameter to investigate possible synergistic effect in combination with endolysin (summarized in Table 21).

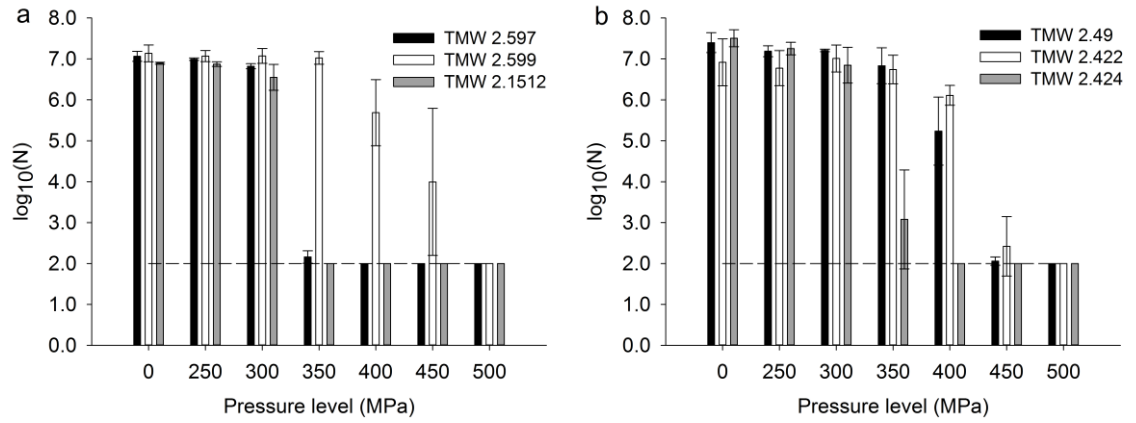


Figure 15: HHP inactivation of *L. monocytogenes* and *S. aureus* in buffer. The total cell count (log_{10}(N)) of exponential-phase *L. monocytogenes* (strain TMW 2.597, 2.599, and 2.1512) and *S. aureus* (strain TMW 2.49, 2.422, and 2.424) cells at an inoculum of ca. 10^7 cells/mL by increasing high pressure levels (200-500 MPa, increment of 50 MPa) at a fixed pressure holding time and temperature (1 min, 30 °C). Mean values ± standard deviation of three biologically independent experiments are shown (error bars). The detection limit is shown by the dashed line.

Table 21: Overview of selected pressure levels for future screening experiments. Overview of maximum pressure levels which caused a minimal reduction (<1 log cfu) in the viable cell count of different *L. monocytogenes* and *S. aureus* strains. Pressure holding and temperature were kept constant (1 min, 30 °C).

L. monocytogenes		***S. aureus***	
Strain	HHP (MPa)	Strain	HHP (MPa)
TMW 2.1512	300	TMW 2.422	350
TMW 2.597	300	TMW 2.424	250
TMW 2.599	350	TMW 2.49	300

3.2.1.1.3 Endolysin concentration-dependency

After selection of strains and HHP parameters, the process parameters for the inactivation of *L. monocytogenes* and *S. aureus* by endolysin were determined. Figure 16 shows that inactivation starts at >0.16 µg/mL for both PlyP40 and HY-133. Similar results were obtained for the other endolysins and 0.16 µg/mL was therefore chosen as lower end of the endolysin concentration range. The combination of HHP with a gradual increase in endolysin concentration (5x each step; up to either 20 µg/mL for PlyP40 and Ply511 or 100 µg/mL for PlyP825, HY-100, HY-133, and lysostaphin) was chosen to provide detailed insights into the combined application of endolysin and HHP.

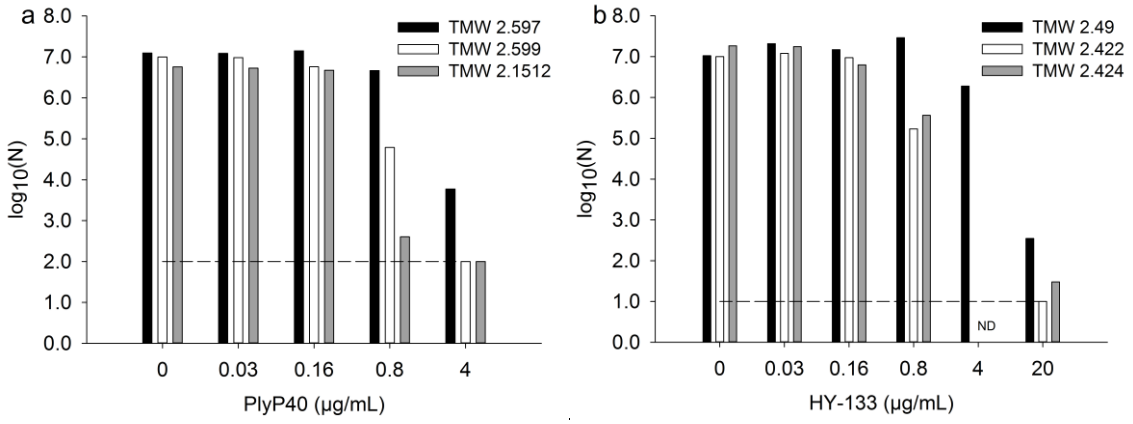


Figure 16: Inactivation of *L. monocytogenes* and *S. aureus* by PlyP40 and HY-133. The induced reduction ($log_{10}(N_0/N)$) of exponential-phase *L. monocytogenes* (TMW 2.597, 2.599, and 2.1512) and *S. aureus* strains (TMW 2.49, 2.422, and 2.424) cells at an inoculum of ca. 10^7 cells/mL coincubated with increasing endolysin PlyP40 (a) or HY-133 (b) concentrations (0.03-20 µg/mL) for 1.5h. The detection limit is shown by the dashed line. ND: not detected due to contamination.

3.2.1.1.4 Detection of sublethally damaged cells

Information on the sublethal damage of cells can provide a better understanding of the mechanism of bacterial inactivation by endolysin, HHP, or their combination. Plating on both standard TSAYE as well as TSAYE with a high NaCl concentration as selective agent allows to differentiate between viable and sublethally damaged cells (1.3.2.4.2). Hence, the maximum NaCl concentration in TSAYE which did not affect the viable cell count of a fresh exponential-phase cell culture was determined (Figure 17). Whereas a NaCl concentration above 50 g/L was sufficient to inhibit the formation of *L. monocytogenes* colonies, *S. aureus* tolerated concentrations up to 125 g/L TSAYE. As such, these concentrations were selected to examine sublethal damage.

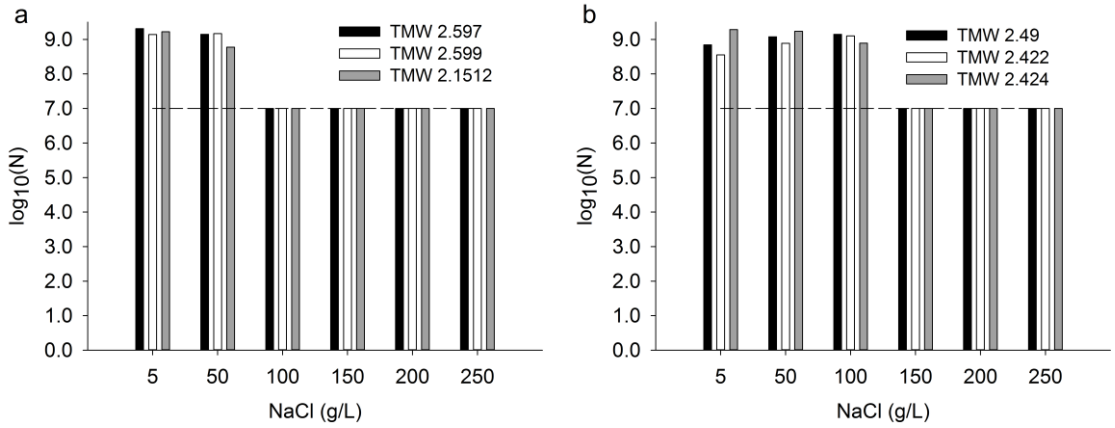


Figure 17: Determination of NaCl content for detection of sublethal damage. Growth of viable *L. monocytogenes* and *S. aureus* cells at TSAYE with icnreasing NaCl content. Exponential-phase *L. monocytogenes* (a; strains TMW 2.597, 2.599, and 2.1512) and *S. aureus* (b; strains TMW 2.49, 2.422, and 2.424) cells at an inoculum of ca. 10^9 cells/mL IPB were plated on TSAYE with increasing NaCl content. The detection limit is shown by the dashed line.

3.2.1.2 L. monocytogenes

3.2.1.2.1 Screening for synergistic effect between endolysin and HHP

Figure 18 shows the log reduction of *L. monocytogenes* strain TMW 2.1512 by HHP processing alone, endolysin alone (PlyP40, Ply511, or PlyP825), and by a combination of both treatments (plated on standard TSAYE). Since the results for the other two strains (TMW 2.597 and 2.599) were highly similar, these data are summarized in Table 22 (log reduction values are provided in Table 27 in Appendix 8.3). Results for samples plated on high salt TSAYE plates are also presented in Table 22. In Figure 18, two remarkable findings are visible; (i) the inactivation by a combined treatment is considerably higher than the sum of effects provoked by single treatments. (ii) Above a threshold concentration, even higher concentrations of endolysin PlyP40 or PlyP825 led to a lower inactivation of cells.

(i) It was found that, when *Listeria* cells were coincubated with endolysin prior to HHP treatment, a much larger inactivation could be achieved compared to the sum of effects detected after individual application of these methods (Figure 18). For example, the treatment of strain TMW 2.1512 by 0.16 µg/mL PlyP825 or HHP at 300 MPa for 1 min (30 °C) resulted in a reduction of 0.2 and 0.3 log cfu, respectively, whereas the combined application reduced the viable cell count by 5.5 log cfu (until the detection limit). The combination of techniques thus increased (i.e. synergy) the bactericidal effect by 5.0 log cfu. A synergistic effect in this order of magnitude was present for all high pressure and endolysins combinations at a concentration of 0.16 µg/mL (Table 23). At concentrations above 0.16 µg/mL endolysin, an additional high pressure treatment led, in most cases, to the inactivation of cells up to the detection limit. Since the killing by endolysin usually increased as well, the synergism by a combined treatment was obviously less pronounced. Statistical analyses of the data further strengthened the above findings (Table 22). Pressure treatments alone did not significantly change the cell count for any of the strains (with one exception, where a reduction of 0.2 log cycles was found to be significantly different from the control). However, when cells were first incubated with endolysin PlyP40, Ply511, or PlyP825 (at any concentration tested), an additional HHP treatment caused a significant reduction of the cell count as compared to its equivalent (i.e. with equal endolysin concentration) at atmospheric pressure (with only few exceptions where endolysin alone already caused an inactivation (close) to the detection limit or where data was not normally distributed with equal variance and more conservative statistical tests had to be used). This was also the case at an

endolysin concentration of 0.16 µg/mL, which by itself did also not cause a significant reduction in cell count (except for PlyP825 against strain TMW 2.597).

(ii) Considering the inactivation by only endolysin, an interesting phenomenon was observed. Figure 18a and b show that higher concentrations of endolysin PlyP40 or PlyP825 did not necessarily lead to higher inactivation. At a concentration of 100 µg/mL PlyP825, significantly less cells were killed than at a concentration of 4 or 20 µg/mL (3.8 log cycles compared to 5.1 or 4.9, respectively). Inactivation by endolysin PlyP40, but not Ply511, followed a similar trend of reduced inactivation at concentrations above 4 µg/mL and this effect was independent of the strain used (Table 22). This paradoxical phenomenon is referred to by Scherzinger, 2011 as 'Eagle' effect, named after H. Eagle, who described a similar phenomenon for the antibiotic penicillin (Eagle and Musselman, 1948). In section 3.3, the Eagle effect is examined more closely and a range of experiments were performed to test different hypotheses which could explain this phenomenon.

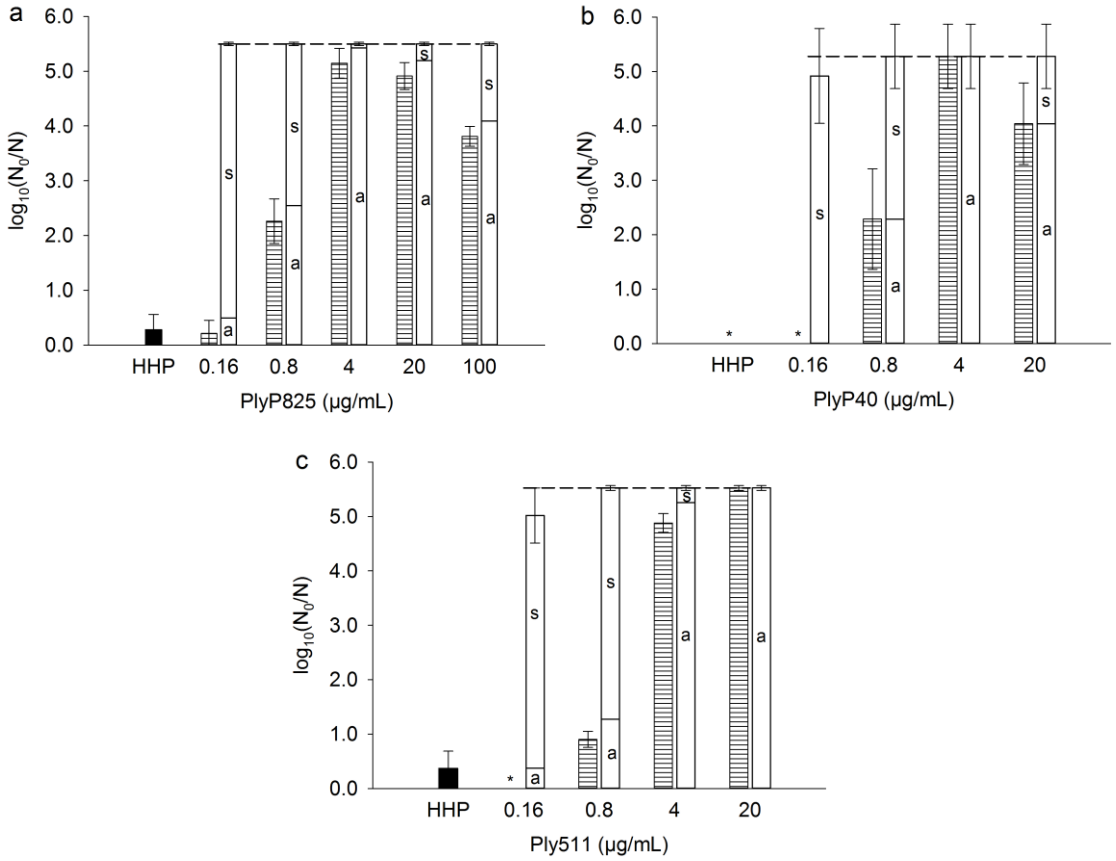


Figure 18: Inactivation of *L. monocytogenes* cells by endolysin and HHP. The induced reduction ($log_{10}(N_0/N)$) of exponential-phase cells (strain TMW 2.1512 at an inoculum of ca. 10^7 cells/mL) by high pressure alone (300 MPa, 1 min; black bars), endolysin PlyP825 (a), PlyP40 (b), or Ply511 (c) alone at different concentrations (striped bars), or a combined application (white bars). Coincubation of cells with endolysin was 0.5h until HHP treatment and 1.5h in total. The lower part of the white bars represents the calculated additive effect of individual endolysin and HHP treatments, indicated by the letter a. The upper part represents the synergistc inactivation as result of the combined treatment, indicated by the letter s. The detection limit is shown by the dashed line (1.3 log cfu). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

Table 22: Screening for a synergistic inactivation of *L. monocytogenes* by endolysin and HHP. The viable cell count (log_{10}(N)) of exponential-phase cells (TMW 2.597, 2.599, or 2.1512; inoculum of ca. 10^7 cells/mL in IPB) coincubated with different concentrations of endolysin (PlyP40, Ply511, or PlyP825), either at atmoshperic pressure (0.1 MPa) or in combination with HHP (300 or 350 MPa, 1 min, 30 °C). Coincubation of cells with endolysin was 0.5h until HHP treatment and 1.5h in total. Mean values ± standard deviation of three biologically independent experiments are shown. Cell cultures inactivated until the detection limit (1.3 log cfu) are shown in **bold**. Within each endolysin-strain combination, statistically significant differences among samples treated only with endolysin and plated on standard TSAYE are denoted by different letters (as tested by one way ANOVA or Kruskal-Wallis one way ANOVA on ranks). A grey background of HHP treated samples plated on standard TSAYE indicates a statistically significant difference with the same sample without HHP treatment (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test). A grey background of samples plated on high salt TSAYE indicates a statistically significant difference with the same sample plated on standard TSAYE (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test).

Endolysin (µg/mL)		*L. monocytogenes* strain											
		TMW 2.597				TMW 2.599				TMW 2.1512			
		Standard TSAYE		High salt TSAYE		Standard TSAYE		High salt TSAYE		Standard TSAYE		High salt TSAYE	
		0.1 MPa	300 MPa	0.1 MPa	300 MPa	0.1 MPa	350 MPa	0.1 MPa	350 MPa	0.1 MPa	300 MPa	0.1 MPa	300 MPa
PlyP40	0	7.0±0.1^{a}	6.7±0.4	6.9±0.1	5.9±0.7	7.1±0.2^{a}	6.8±0.2	6.9±0.3	6.2±0.5	6.6±0.6^{a}	6.7±0.0	6.6±0.4	4.3±0.4*
	0.16	7.0±0.2^{a}	3.1±0.5	6.9±0.1	2.4±0.7	6.8±0.2^{a}	2.8±0.5	6.9±0.3	2.1±0.8	6.8±0.0^{a}	1.7±0.3	6.7±0.0	1.4±0.2
	0.8	5.6±0.4^{ab}	**1.3±0.0**	5.5±0.2	**1.3±0.0**	5.0±0.2^{b}	1.4±0.2	5.0±0.3	**1.3±0.0**	4.3±0.4^{b}	**1.3±0.0**	4.1±0.3	**1.3±0.0**
	4	3.7±1.1^{c}	**1.3±0.0**	3.3±0.6	**1.3±0.0**	2.0±0.2^{c}	**1.3±0.0**	1.9±0.1	**1.3±0.0**	**1.3±0.0**^{c}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
	20	4.9±0.7^{bc}	**1.3±0.0**	4.8±0.7	**1.3±0.0**	3.6±1.3^{c}	**1.3±0.0**	3.5±1.2	**1.3±0.0**	2.5±0.3^{d}	**1.3±0.0**	2.5±0.3	**1.3±0.0**
Ply511	0	7.0±0.1^{a}	6.8±0.1	7.1±0.1	6.1±0.6	7.1±0.2^{a}	7.0±0.1	7.2±0.1	6.0±1.0	6.8±0.0^{a}	6.5±0.3	6.8±0.2	4.6±0.3
	0.16	6.9±0.1^{a}	2.7±0.6	6.8±0.1	2.3±0.6	7.0±0.2^{a}	3.1±2.3	7.1±0.1	2.8±2.0	6.9±0.2^{a}	1.8±0.5	6.8±0.2	**1.3±0.0**
	0.8	5.9±0.3^{b}	2.1±0.2	5.8±0.1	1.5±0.3	5.7±0.9^{a}	**1.3±0.0**	5.6±1.0	**1.3±0.0**	5.9±0.2^{b}	**1.3±0.0**	6.0±0.1	**1.3±0.0**
	4	4.0±0.1^{c}	1.8±0.3	3.8±0.2	1.4±0.1	3.2±0.9^{b}	**1.3±0.0**	2.8±1.1	**1.3±0.0**	1.9±0.2^{c}	**1.3±0.0**	1.6±0.2	**1.3±0.0**
	20	3.0±0.1^{d}	**1.3±0.0**	2.8±0.3	**1.3±0.0**	2.6±1.4^{b}	**1.3±0.0**	2.6±1.6	**1.3±0.0**	**1.3±0.0**^{d}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
PlyP825	0	7.1±0.1^{a}	6.9±0.0	7.1±0.1	6.2±0.5	7.1±0.1^{a}	6.9±0.2	7.1±0.1	5.9±1.0	6.8±0.0^{a}	6.5±0.3	6.7±0.1	4.9±0.2
	0.16	6.3±0.4^{b}	2.3±0.3	6.3±0.3	2.1±0.2	6.7±0.2^{a}	2.8±1.6	6.7±0.2	2.0±0.7	6.6±0.2^{a}	**1.3±0.0**	6.6±0.2	**1.3±0.0**
	0.8	5.4±0.1^{c}	1.8±0.5	5.4±0.1	1.7±0.2	4.7±0.1^{b}	**1.3±0.0**	4.7±0.0	**1.3±0.0**	4.5±0.4^{b}	**1.3±0.0**	4.6±0.4	**1.3±0.0**
	4	3.6±0.5^{d}	1.5±0.2	3.6±0.5	1.5±0.2	2.3±0.4^{c}	**1.3±0.0**	2.3±0.3	**1.3±0.0**	1.7±0.3^{c}	**1.3±0.0**	1.4±0.1	**1.3±0.0**
	20	3.9±0.5^{d}	1.7±0.3	3.8±0.5	1.7±0.2	2.6±0.6^{c}	**1.3±0.0**	2.6±0.5	**1.3±0.0**	1.9±0.3^{c}	**1.3±0.0**	1.6±0.3	**1.3±0.0**
	100	5.0±0.3^{c}	1.5±0.3	5.0±0.3	1.4±0.1	4.1±1.3^{b}	1.5±0.3	3.6±2.0	1.4±0.2	3.0±0.2^{d}	**1.3±0.0**	2.8±0.4	**1.3±0.0**

*only 2 replicates. $Log_{10}(N_0)$ = 6.9±0.2

Table 23: Synergy between endolysin and HHP for the inactivation of *L. monocytogenes*. The synergism between endolysin PlyP40, Ply511, or PlyP825 (at a concentration of 0.16, 0.8, 4, 20, or 100 µg/mL) and HHP (either 300 or 350 MPa for 1 min at 30 °C) for the inactivation of exponential-phase *L. monocytogenes* cells (strain TMW 2.597, 2.599, or 2.1512) expressed in log cycles cfu. Synergy was calculated for the experimental data depicted in Table 22. Negative values were set to zero.

			L. monocytogenes strain		
			TMW 2.597	TMW 2.599	TMW 2.1512
			300 MPa	350 MPa	300 MPa
Endolysin (µg/mL)	PlyP40	0.16	3.6	3.8	5.2
		0.8	4.0	3.4	3.1
		4	2.1	0.5	0.1
		20	3.3	2.0	1.3
	Ply511	0.16	4.0	3.8	4.7
		0.8	3.6	4.3	4.3
		4	2.1	1.8	0.3
		20	1.6	1.2	0.0*
	PlyP825	0.16	3.8	3.8	5.0
		0.8	3.4	3.2	3.0
		4	1.9	0.8	0.1
		20	2.0	1.1	0.3
		100	3.4	2.4	1.4

*cells were already inactivated until the detection limit by endolysin alone

3.2.1.2.2 Sublethal damage

A difference in cell count for samples plated on standard and high salt TSAYE was detected for several pressure treated samples (Table 22). The most prominent difference was observed for strain TMW 2.1512, where a HHP treatment of 300 MPa (30 °C, 1 min) caused a significant difference of >2 log cycles between counts on standard and high salt TSAYE, showing that more than 99% of the cells were sublethally damaged. About 90% of TMW 2.597 and 2.599 cells were sublethally damaged after HHP treatment, though the difference between counts on standard and high salt TSAYE were in most cases not statistically significant. Not only HHP, but also endolysin Ply511-induced sublethal damage of strain TMW 2.1512, though only at a single concentration (4 µg/mL) and with minimal difference (0.4 log cfu). This indicates that cells incubated with *Listeria* phage endolysins did not induce sublethal damage (as could be examined using NaCl as a selective agent).

3.2.1.2.3 Stationary-phase cells

In the previous section, the killing of exponential-phase cells was examined. To evaluate possible growth phase-dependent effects, the inactivation of stationary-phase TMW 2.1512 cells by endolysin PlyP40 and HHP (300 MPa, 1 min, 30 °C) was examined with the same experimental setup as used for exponential-phase cells. Although a synergistic effect between endolysin and HHP could also be detected against stationary-phase cells (Figure 19), synergism was only present at a concentration of 0.8 and 20 µg/mL (0.8 and 1.3 log cycles, respectively) and less pronounced compared to that found for exponential-phase cells (see Table 23). The Eagle effect, as discussed previously for exponential-phase cells, was also observed against stationary-phase cells. The antimicrobial activity of PlyP40 was lower at a concentration of 20 µg/mL (5.1 log cycles) than at 4 µg/mL (5.5 log cycles), demonstrating that the Eagle effect is independent on the growth phase of the cell.

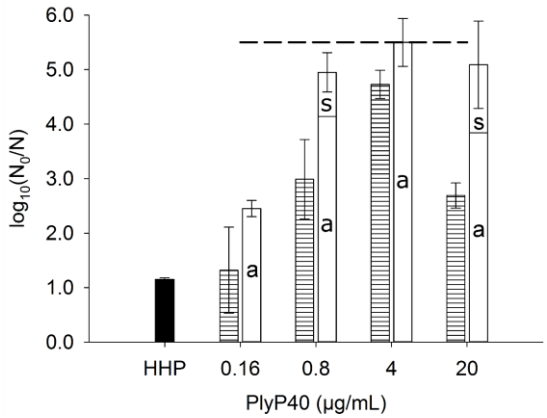


Figure 19: Inactivation of stationary-phase *L. monocytogenes* cells by endolysin PlyP40 and HHP. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase cells (strain TMW 2.1512 at an inoculum of ca. 10^7 cells/mL) by high pressure alone (300 MPa, 1 min; black bar), endolysin PlyP40 alone (striped bars), or a combined application (white bars). Coincubation of cells with PlyP40 was 0.5h until HHP treatment and 1.5h in total. The lower part of the white bars represents the calculated additive effect of individual endolysin and HHP treatments, indicated by the letter a. The upper part represents the synergistc inactivation as result of the combined treatment, indicated by the letter s. The detection limit is shown by the dashed line (1.3 log cfu). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

3.2.1.2.4 Minimal HHP parameters required for the inactivation of endolysin treated cells

A reduction of the pressure level and pressure holding time is mainly desirable for food quality, but would also make HHP economically more competitive. The minimum high pressure level and holding time required to kill PlyP40 treated *L. monocytogenes* cells were therefore examined. Figure 20 shows that a pressure level of minimum 200 MPa is required to further inactivate cells previously coincubated with endolysin; PlyP40 alone reduced the cell count by 2.3 log cfu, which was then further reduced by an additional 1.3 log cycles after a pressure treatment of 200 MPa for two minutes (▼). Longer pressure holding times at 200 MPa did not further decrease the cell count, indicating that there was only subpopulation of damaged cells which could be inactivated at this pressure level. Cells which were not treated with PlyP40 did not show any inactivation at a pressure level of 200 MPa, also not after a pressure holding time of 10 min (▽). A pressure level of 100 MPa for 10 minutes did also not influence the viable cell count, independent of the presence (●) or absence (○) of PlyP40. At 300 MPa, cells without endolysin were increasingly killed with longer pressure holding times (□), although not as readily as the cells coincubated with PlyP40 beforehand (■). Taken together, this demonstrates that prior incubation with endolysin allows for the inactivation of cells at reduced HHP parameters, but that a pressure level of 200 MPa is still minimally required.

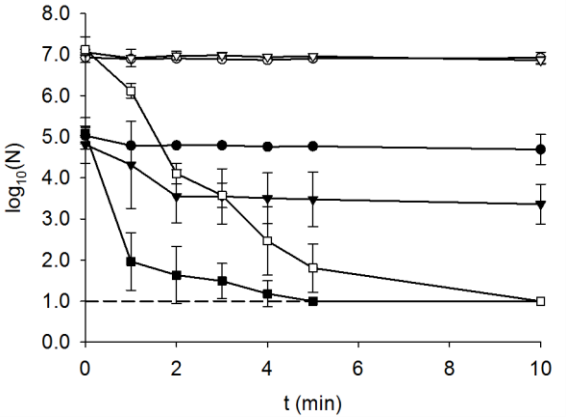


Figure 20: Critical HHP parameters for the inactivation of PlyP40-treated *L. monocytogenes* cells. Total cell count (log_{10}(N)) of exponential-phase cells (strain TMW 2.597 at an inoculum of ca. 10^7 cells/mL) in IPB (blanc symbols) or incubated with 0.16 µg/mL PlyP40 (black symbols). Coincubation of cells with PlyP40 was 1.5h until HHP treatment and 3h in total. HHP processing was done at either 100 (circles), 200 (triangles) or 300 MPa (squares) for 1, 2, 3, 4, 5 or 10 min at 30 °C. The detection limit is shown by the dashed line (1 log cfu). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

3.2.1.3 S. aureus

3.2.1.3.1 Screening for synergistic effect between endolysin and HHP

In Table 24, results for the inactivation of different *S. aureus* strains by a combination of HY-100, HY133, or lysostaphin with HHP processing are presented (log reduction values are provided in Appendix 8.3). Similar as for the *L. monocytogenes* endolysins, the combination of *Staphylococcus* endolysins or lysostaphin with HHP resulted in a higher inactivation as can be expected from the sum of individual treatments. Though, the synergism was not as strong as compared to *Listeria* phage endolysins and in some cases not present at all (Table 25). Whereas for the *Listeria* phage endolysins, a synergy in the order of 3-5 log cycles could be observed for many concentration-pressure combinations (Table 23), none of the endolysin-pressure combinations against *S. aureus* exceeded a synergy of 3 log cycles and only five combinations exceeded a synergy of 2 log cycles. Interestingly, synergy of more than 2 log cycles was only observed at concentrations where the enzyme alone already reduced the cell count substantially. This also differs from the *Listeria* phage endolysins, where a synergistic effect of 5 log cycles was also detected at an endolysin concentration that did not affect the cell count (i.e. 0.16 µg/mL). Additionally, for those samples where HHP alone did not cause a significant reduction (all except TMW 2.49 treated with HY-100 and TMW 2.422 treated with HY-133), the inactivation by a combined treatment was only significantly different from the inactivation by endolysin alone for ten samples (compared to 27 samples for the *Listeria* phage endolysins in combination with HHP). This further emphasizes the difference in efficacy of a combined endolysin and HHP treatment against *L. monocytogenes* and *S. aureus.*

Another noteworthy difference with the *Listeria* phage endolysins PlyP40 and PlyP825 is that no Eagle effect was observed for any of the *S. aureus* peptidoglycan hydrolases. Increased concentrations of HY-100, HY-133, and lysostaphin consistently led to higher inactivation until the detection limit was reached.

Table 24: Screening for a synergistic inactivation of *S. aureus* by peptidoglycan hydrolases and HHP. The viable cell count (log_{10}(N)) of exponential-phase cells (TMW 2.49, 2.422, or 2.424 at an inoculum of ca. 10^7 cells/mL in IPB) coincubated with different concentrations of peptidoglycan hydrolases (HY-100, HY-133, or lysostaphin), either at atmoshperic pressure (0.1 MPa) or in combination with HHP (250, 300, or 350 MPa, 1 min, 30 °C). Coincubation of cells with endolysin was 0.5h until HHP treatment and 1.5h in total. Mean values ± standard deviation of three or four biologically independent experiments are shown (samples within an endolysin-strain combination have the same number of replicates). Cell cultures inactivated until the detection limit (1.3 log cfu) are shown in **bold**. Within each endolysin-strain combination, statistically significant differences among samples treated only with endolysin and plated on standard TSAYE are denoted by different letters (as tested by one way ANOVA or Kruskal-Wallis one way ANOVA on ranks). A grey background of HHP treated samples plated on standard TSAYE indicates a statistically significant difference with the same sample without HHP treatment (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test). A grey background of samples plated on high salt TSAYE indicates a statistically significant difference with the same sample plated on standard TSAYE (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test).

			S. aureus strain											
			TMW 2.49				TMW 2.422				TMW 2.424			
			Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE	
			0.1 MPa	300 MPa	0.1 MPa	300 MPa	0.1 MPa	350 MPa	0.1 MPa	350 MPa	0.1 MPa	250 MPa	0.1 MPa	250 MPa
Peptidoglycan hydrolases (µg/mL)	HY-100	0	6.9±0.2^a	6.6±0.2	7.0±0.3	7.0±0.1	6.4±0.7^a	5.9±0.8	7.3±0.3	6.1±0.7	7.4±0.2^a	7.2±0.2	7.3±0.1	7.3±0.2
		0.16	7.1±0.2^a	6.8±0.3	7.2±0.3	7.0±0.2	6.5±0.8^a	6.0±0.2	7.1±0.5	6.4±0.6	7.2±0.2^a	7.2±0.1	7.2±0.2	7.1±0.2
		0.8	7.1±0.2^a	6.6±0.2	7.1±0.1	6.7±0.2	5.7±1.1^a	4.6±1.6	6.4±0.7	4.7±1.8	6.8±0.4^a	6.6±0.3	6.8±0.3	6.6±0.4
		4	6.1±0.2^b	3.6±1.6	6.1±0.2	4.5±0.7	4.8±0.9^a	1.4±0.3	5.4±0.4	1.6±0.6	5.2±0.5^b	2.9±0.6	5.0±0.6	2.8±0.8
		20	4.7±0.4^c	1.5±0.2	4.4±0.5	1.6±0.3	2.2±1.0^b	**1.3±0.0**	2.5±1.1	**1.3±0.0**	2.4±0.8^c	**1.3±0.0**	2.2±0.7	**1.3±0.0**
		100	2.0±0.8^d	**1.3±0.0**	1.7±0.5	**1.3±0.0**	1.4±0.2^b	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	1.5±0.3^d	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
	HY-133	0	6.9±0.4^a	6.6±0.4	6.7±0.1	6.3±0.6	7.1±0.3^a	6.2±0.2	7.3±0.1	6.6±0.1	6.9±0.1^a	6.5±0.4	6.8±0.0	6.4±0.2
		0.16	7.0±0.3^a	6.6±0.4	6.9±0.2	6.5±0.7	7.1±0.3^a	6.0±0.5	7.1±0.2	5.6±0.4	6.7±0.2^a	6.1±0.5	5.8±0.3	5.4±0.7
		0.8	7.1±0.2^a	6.6±0.4	6.8±0.3	5.7±1.8	6.9±0.1^a	4.8±1.2	6.6±0.5	3.5±1.0	5.8±0.4^b	5.7±0.8	4.9±0.6	4.7±0.1
		4	6.0±0.3^b	3.8±2.2	5.6±0.3	3.4±1.8	3.1±1.1^{ab}	1.5±0.3	**1.3±0.0**	**1.3±0.0**	3.8±0.6^c	3.7±1.0	1.4±0.1	1.6±0.4
		20	3.8±0.5^c	1.7±0.8	**1.3±0.0**	**1.3±0.0**	2.3±0.9^{ab}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	2.1±0.8^d	1.9±0.3	**1.3±0.0**	**1.3±0.0**
		100	**1.3±0.0**^{d}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**^{b}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	1.6±0.4^d	1.5±0.3	**1.3±0.0**	**1.3±0.0**
	Lysostaphin	0	7.0±0.3^a	6.7±0.3	7.0±0.3	7.0±0.1	6.6±0.9^a	6.0±0.9	7.2±0.3	6.1±0.7	7.4±0.2^a	7.3±0.2	7.3±0.1	7.3±0.2
		0.16	7.1±0.3^a	6.7±0.3	7.3±0.4	6.9±0.2	6.2±1.3^a	5.1±1.3	6.9±0.6	5.1±1.5	7.0±0.6^a	7.3±0.2	6.5±1.2	7.1±0.3
		0.8	6.8±0.4^a	5.3±1.0	6.7±0.4	5.9±0.5	4.3±1.4^a	**1.3±0.0**	4.6±0.7	**1.3±0.0**	5.7±0.5^b	4.5±0.4	3.8±1.8	4.2±0.8
		4	3.7±0.7^b	1.6±0.6	2.1±1.5	**1.3±0.0**	**1.3±0.0**^{a}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	2.3±0.3^c	**1.3±0.0**	1.4±0.2	1.4±0.2
		20	**1.3±0.0**^{c}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**^{a}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**^{d}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
		100	**1.3±0.0**^{c}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**^{a}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**^{d}	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**

Table 25: Synergy between peptidoglycan hydrolases and HHP for the inactivation of *S. aureus.* The synergism between HY-100, HY-133, or lysostaphin (at a concentration of 0.16, 0.8, 4, 20, or 100 µg/mL) and HHP (either 250, 300, or 350 MPa for 1 min at 30 °C) for the inactivation of exponential-phase *S. aureus* cells (TMW 2.49, 2.422, or 2.424) expressed in log cycles cfu. Synergy was calculated for the experimental data depicted in Table 24. Negative values were set to zero.

Peptidoglycan hydrolases (µg/mL)			*S. aureus* strain		
			TMW 2.49 300 MPa	TMW 2.422 350 MPa	TMW 2.424 250 MPa
HY-100		0.16	0.0	0.0	0.1
		0.8	0.2	0.6	0.2
		4	2.1	2.9	2.0
		20	2.9	0.4	1.1
		100	0.4	0.0	0.2
HY-133		0.16	0.1	0.2	0.2
		0.8	0.1	1.2	0.0
		4	1.9	0.6	0.0
		20	1.8	0.1	0.0
		100	0.0*	0.0*	0.0
Lysostaphin		0.16	0.2	0.5	0.0
		0.8	1.3	2.4	1.0
		4	1.8	0.0*	0.8
		20	0.0*	0.0*	0.0*
		100	0.0*	0.0*	0.0*

*cells were already inactivated until the detection by endolysin alone

3.2.1.3.2 Sublethal damage

In contrast to *L. monocytogenes*, treatment of the different *S. aureus* strains with HHP did not cause a statistical significant difference between samples plated on standard and high salt TSAYE (except for a single combination where the cell count was 0.5 log cycles lower on high salt TSAYE; Table 24). As such, the chosen HHP parameters did either not cause sublethal damage or the high salt agar plates (125 g/L NaCl) were not selective for this type of damage.

On the contrary, whereas the *Listeria* phage endolysins hardly caused sublethal damage of cells (only Ply511 at a single concentration), treatment of *S. aureus* with peptidoglycan hydrolases did result in sublethal damage, though highly dependent on the type of enzyme and concentration applied. HY-133 caused sublethal damage to all *S. aureus* strains with significant differences of more than 2 log cycles between the cell count on standard and high salt TSAYE. Sublethal damage in the same order was also detected after incubation with 0.8 µg/mL lysostaphin, though only for strain TMW 2.424. Endolysin HY-100 did not cause sublethally damage for any of the strains or concentrations examined. Interestingly, lysostaphin and HY-133 only have a single EAD which cleaves the glycine peptide bridge of the *S. aureus* peptidoglycan (Figure 6 and 8). HY-100 contains two EADs, of which one also cleaves the glycine peptide bridge (CHAP_Pitti26), while the second catalytic domain (Amidase_2_Pitti26) has amidase activity (just like the *Listeria* phage endolysin Ply511). It is therefore tempting to speculate that the endolysin cleavage site might be responsible for the observed sublethal damage induced by HY-133 and lysostaphin and would explain the absence of such damage in HY-100 or *Listeria* phage endolysins. Interestingly, although HY-100 did not induce sublethal damage, the synergistic effect in combination with HHP was more pronounced than HY-133 or lysostaphin (Table 25), which might indicate a more general role for the type of bond cleaved and the subsequent physiological state and pressure susceptibility of the cell.

3.2.2 Microscopic investigations

In this section, the morphology and membrane integrity of cells treated with endolysin and/or HHP were microscopically investigated to get a better understanding about the effect of endolysin on the physiological state and pressure stability of the cell.

3.2.2.1 LIVE/DEAD staining of screening experiments

Figure 21 shows a LIVE/DEAD staining of exponential-phase *L. monocytogenes* and *S. aureus* cells incubated with an increasing concentration of endolysin or lysostaphin. The experimental set-up was the same as used for the screening experiments in section 3.2.1.2.1 and 3.2.1.3.1. Since the results were similar for the different *S. aureus* and *L. monocytogenes* strains, only one single strain per species is shown. In Figure 21, it is clearly visible that the cell membrane of *S. aureus* was still intact after incubating with 0.03 µg/mL endolysin or lysostaphin (as indicated by green fluorescence). With higher concentrations, there is an increased loss of membrane integrity (as indicated by red fluorescence) which nicely corresponds to the inactivation data in Table 24.

A somewhat different trend was visible for *L. monocytogenes* cells incubated with endolysin. First, at a concentration of 0.16-0.8 µg/mL PlyP40 or PlyP825, the typical rod shaped cell morphology was lost and circular shaped cell bodies, postulated to be protoplasts, were formed. Interestingly, some of these circular cells showed no red fluorescence after LIVE/DEAD staining, indicating the formation of protoplasts with an intact cell membrane. Secondly, although the structure of every single cell was lost and showed red fluorescence after incubation with 4 µg/mL PlyP40 or PlyP825, circular shaped cells with no sign of a damaged membrane were again detected at even higher concentrations. This indicates that endolysin PlyP40/PlyP825 have a similar effect on *Listeria* cells, whether at a concentration of 0.8 or above 4 µg/mL, a phenomenon also observed in the inactivation data of the screening experiment (Table 22) and referred to as Eagle effect. For endolysin Ply511, a concentration of 0.8 and 4 µg/mL also led to the formation of circular shaped cells of which some also showed green fluorescence. Though, in contrast to endolysin PlyP40 and PlyP825, all cells had a compromised cell membrane at an even higher PlyP511 concentration (which is also in accordance with the increased inactivation at higher Ply511 concentrations as observed in the screening experiment).

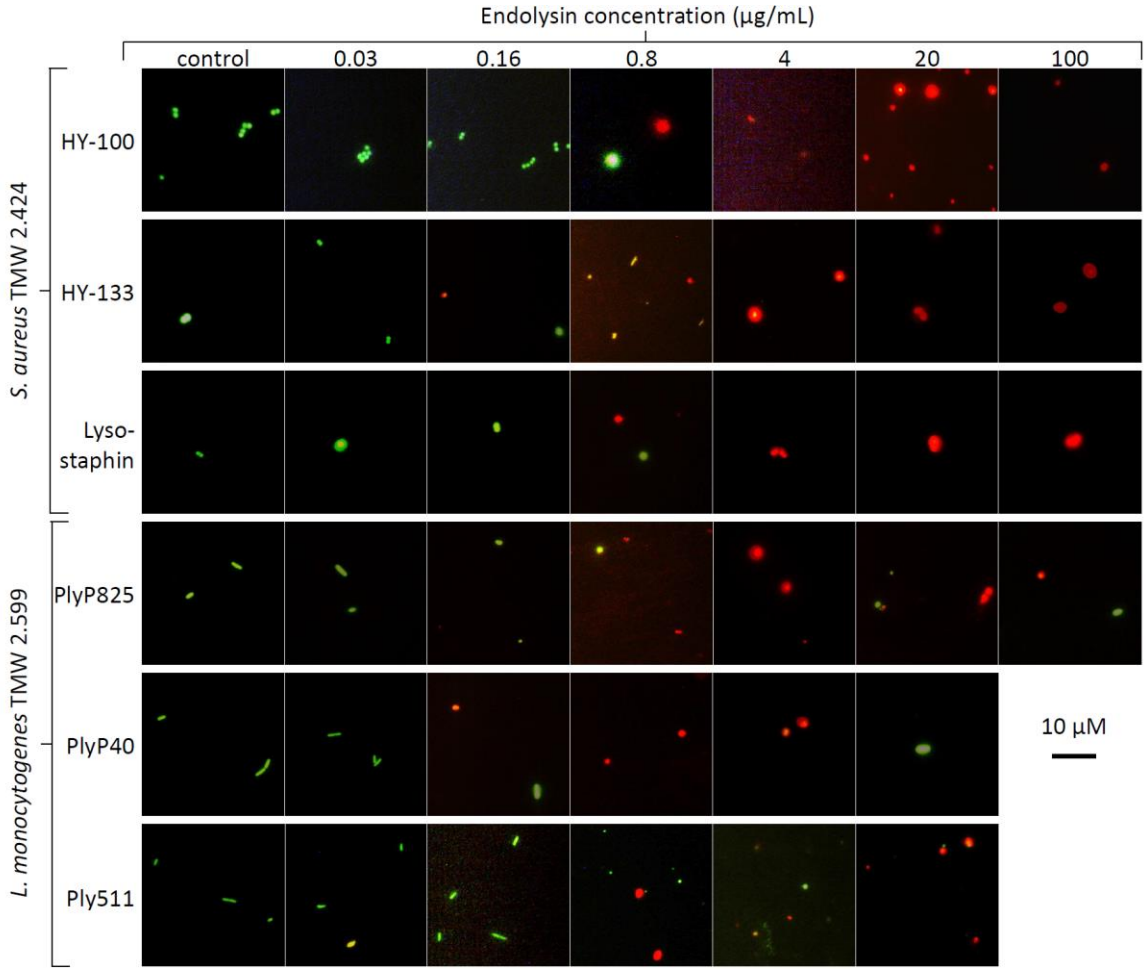


Figure 21: LIVE/DEAD staining of *L. monocytogenes* and *S. aureus* cells incubated with endolysin. Exponential-phase *L. monocytogenes* (TMW 2.599) or *S. aureus* (TMW 2.424) cells at an inoculum of ca. 10^7 cells/mL were incubated with IPB (control) or different concentrations of endolysin (0.3, 0.16, 0.8, 4, 20, or 100 µg/mL). After ca. 75 min of coincubation with endolysin, cells were stained with PI (red fluorescence) and SYTO 9 (green fluorescence), captured under a microscopic slide, and analysed with epifluorescent microscopy. Images of the same microscopic field were merged and the contrast and brightness were adjusted for optimal representation. A representative part of the images was cut out and enlarged for better visiblity. Scale bar = 10 µm.

3.2.2.2 Endolysin-induced formation of L. monocytogenes protoplasts

To examine the loss of cellular shape more closely (as seen for *Listeria* in the previous section), cells were microscopically observed while endolysin PlyP40 was added directly from the stock solution to the microscopic slide. As becomes clear from Figure 22, increasingly more cells lost their rod shape and formed circular shaped cell bodies over time. After 60 min of coincubation with a high concentration of PlyP40, most *Listeria* cells had lost their rod shape. After even longer incubations time, only circular shaped cell bodies were left.

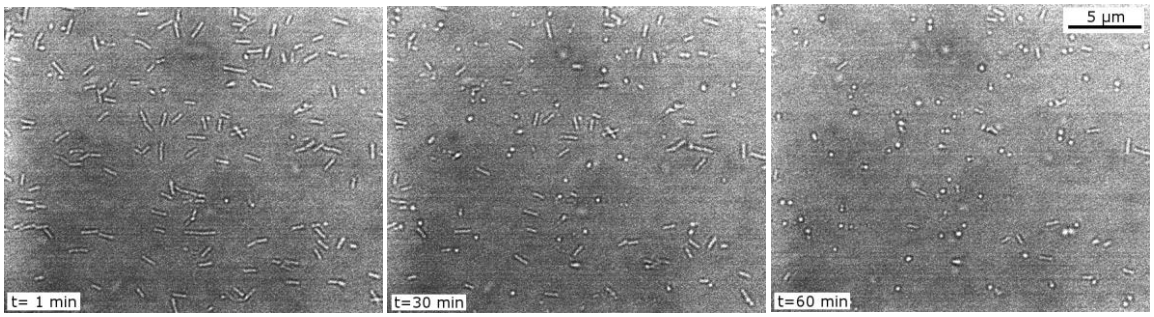


Figure 22: Time-lapse phase contrast microscopic images of *L. monocytogenes* cells incubated with PlyP40. Exponential-phase *L. monocytogenes* cells (strain TMW 2.599 at an inoculum of ca. 10^9 cells/mL IPB) were captured under a microscopic slide and PlyP40 was added directly from the stock (1 mg/mL). Microscopic images of the same microscopic field were taken every minute (only t1, t30, t60 min are provided for illustrative purposes).
Scale bar = 5 µm

In order to get more insights on the physiological state of the circular shaped cell bodies or protoplasts, their membrane integrity was examined by LIVE/DEAD staining. Cells were therefore mixed with the nucleic acid stains PI and SYTO 9 before being captured between the microscopic slide and the addition of endolysin. Figure 23 shows that coincubation of *L. monocytogenes* with endolysin Ply511 or PlyP825 for 60 minutes led to the formation of protoplasts of which most had a compromised cell membrane (as indicated by red fluorescence). Though, for some protoplasts, there was no indication of a damaged membrane (pointed out by the white arrows). These results thereby indicate that although both Ply511 and Ply825 cause *Listeria* to lose their rod-shape as a result of peptidoglycan breakdown, their action can result in circular shaped cells with an intact membrane (as could be examined by LIVE/DEAD staining).

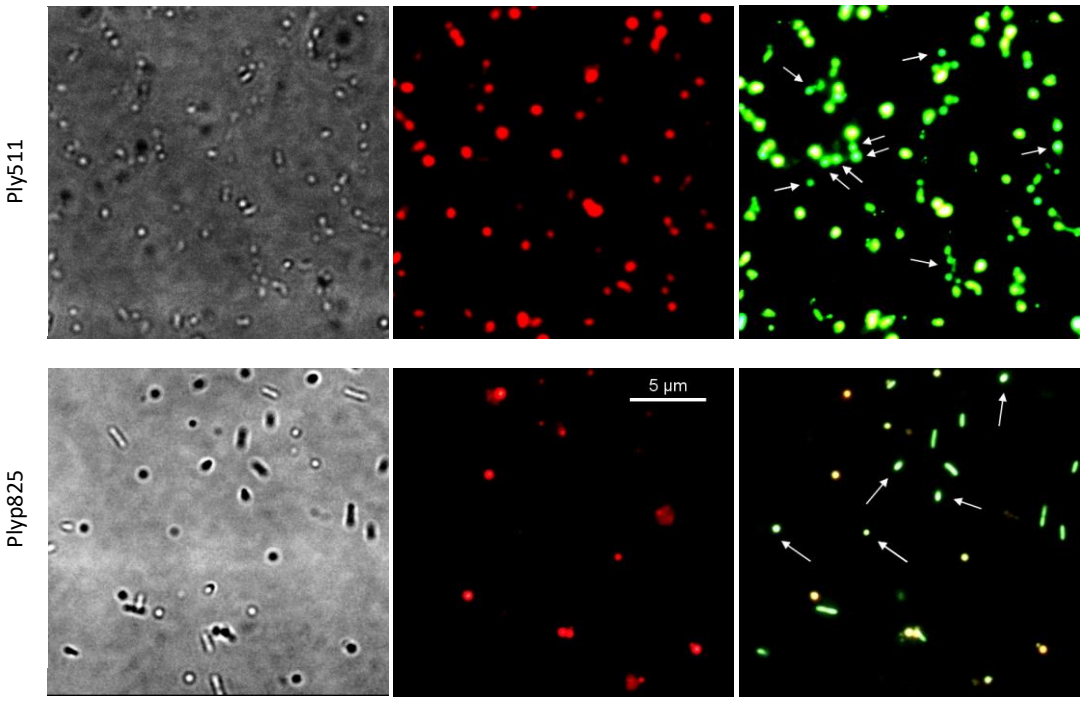


Figure 23: Membrane integrity of *L. monocytogenes* protoplast after incubation with Ply511 or PlyP825. Exponential-phase *L. monocytogenes* cells (strain TMW 2.599 at an inoculum of ca. 10^9 cells/mL IPB) were stained with PI (red fluorescence) and SYTO 9 (green fluorescence), captured under a microscopic slide, and either Ply511 (top images) or PlyP825 (bottom images) was added directly from the stock. Phase contrast and epifluorescent images of the same microscopic field were taken after 60 min of coincubation with endolysin. The contrast and brightness of epifluorescent microscopic images was adjusted for optimal representation. White arrows indicate circulate shaped cells for which no PI fluorescence was visible. Scale bar = 5 µm.

3.2.2.3 Pressure-stability of L. monocytogenes protoplasts

The effect of HHP on the membrane integrity of fresh *L. monocytogenes* cells or those incubated with a very high concentration of endolysin PlyP825 (500 µg/mL) was examined by LIVE/DEAD staining. The cells were stained after HHP treatment and only a more permanent loss of membrane integrity was therefore assessed (in contrast to the transient permeabilization of the membrane during pressure treatment). Figure 24 shows that the membrane of *L. monocytogenes* cells was stable up to a pressure level of 300 MPa (30 °C, 1 min). At higher pressure levels, the number of cells with an impaired membrane steadily increased. This is perfectly in line with the preliminary experiments described in section 3.2.1.1, where 300 MPa was found to be the maximum pressure level which did not cause major inactivation. When cells were first incubated with 500 µg/mL PlyP825 for 2 hours, mostly circular shaped cells were visible of which about half did not show red fluorescent staining (indicating an intact membrane). A pressure level of 100 MPa (30 °C, 1 min) did not change this observation. At pressure levels of 200 MPa or higher, no more circular shaped cells with an intact cellular membrane could be detected. This indicates that the membrane of these cells is impaired and more sensitive to HHP than cells without endolysin treatment.

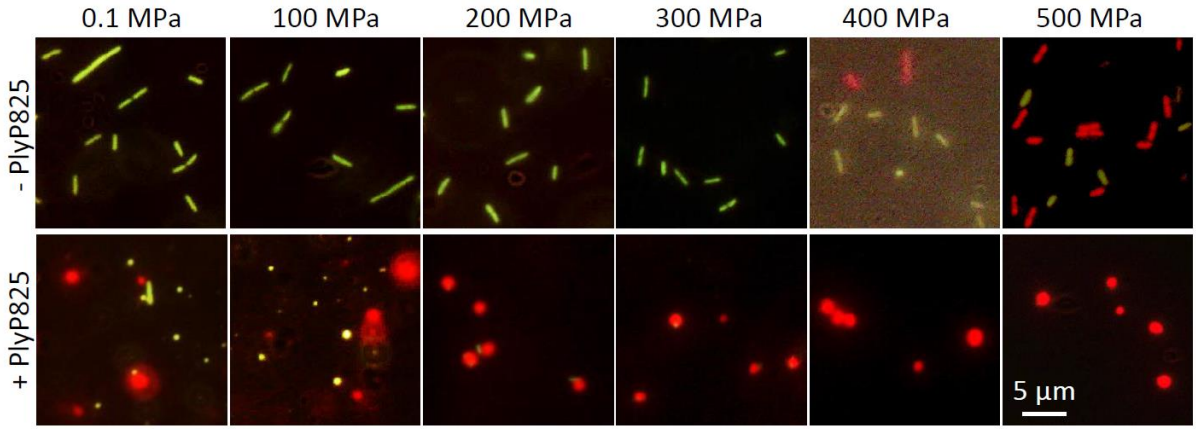


Figure 24: Membrane integrity of *L. monocytogenes* cells after HHP treatment; without or with prior PlyP825 coincubation. Exponential-phase *L. monocytogenes* cells (strain TMW 2.597 at an inoculum of ca. 10^9 cells/mL IPB) were treated with increasing HHP levels (100-500 MPa, 30 °C, 1 min) without or with previous coincubation with 500 µg/mL PlyP825 for 2h (3 mL cell suspension + 0.5 mL PlyP825 stock solution). Cells were stained with PI (red fluorescence) and SYTO 9 (green fluorescence) directly after pressure treatment, captured under a microscopic slide, and analyed by epifluorescent microscopy. Images of the same microscopic field were merged and the contrast and brightness was adjusted for optimal representation. Scale bar = 5 µm

3.3 The Eagle effect

In section 3.2.1.2, the lower inactivation of *L. monocytogenes* cells at higher PlyP40 or PlyP825 concentrations is described as the Eagle effect. Although the present work does not describe this effect for the first time (Scherzinger, 2011), it is hardly recognized in scientific literature (also not for other peptidoglycan hydrolases) and reasons for it to occur have not been clarified. In this section, two hypotheses for the Eagle effect were examined more closely: endolysin aggregation and the formation of viable protoplasts.

3.3.1 Investigations into the possible aggregation of endolysins

The Eagle effect of PlyP40 was present against different strains and both exponential- as well as stationary-phase cells, which indicates that the explanation for this phenomenon might not lie within a cellular event, but that intrinsic properties of the endolysins might be responsible. Hence, it was

hypothesized that endolysin aggregation at higher concentrations was responsible for the reduced activity. In this section, a range of experiments is described where the possible aggregation of endolysin was investigated.

3.3.1.1 Turbidity reduction assay

It was argued that if endolysin aggregation at higher concentrations is responsible for the lower inactivation of cells, this might also be reflected in its enzymatic activity. A TRA was described in this work as appropriate method to determine the enzymatic activity of endolysin. In previous experiments, the activity of endolysins was however only analysed concentrations between 0.008 and 0.25 µM and no Eagle effect was observed within this range (3.1.2). Endolysin's enzymatic activitiy was therefore determined again with concentrations up to 5 µM to examine possible concentration-dependent effects on the enzymatic activity.

The slope of the lysis curve (as direct measure for the enzymatic activity) at different concentrations PlyP40, PlyP825, or Ply511 is represented by a biphasic curve (Figure 25). For PlyP40 and PlyP825, the slope increased up to a concentration of 0,625 µM (~23 µg/mL), after which a decline was observed at higher concentrations. Interestingly, a similar trend was also visible for Ply511, with a maximal slope of 0.081 ΔOD_{600}/min at 0,3 nM. As such, whereas an Eagle effect was only visible for PlyP40 and PlyP825 in the viability plating assays, analysis of the enzymatic activity by TRA also showed a reduced activity for Ply511 at concentrations above a certain threshold. Interestingly, Hampe et al., 1982 found a similar effect of concentration on the enzymatic activity of hen egg-white lysozyme and demonstrated that this was caused by self-association of the enzyme. Similar, the concentration dependent inhibitory effect observed for endolysin PlyP40, Ply511, and PlyP825 might also be the result of self-association (i.e. reversible aggregation).

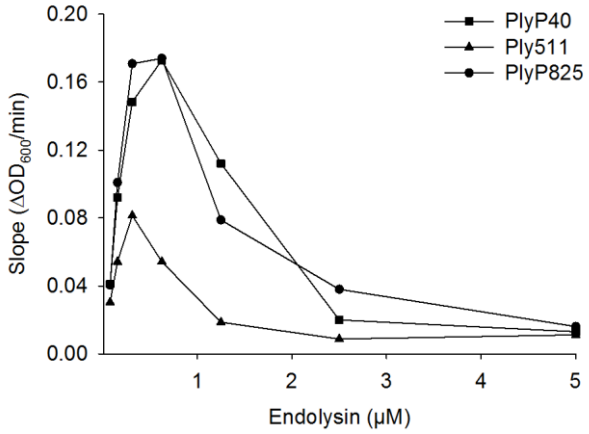


Figure 25: Effect of endolysin concentration on endolysin activity. The slope of the region of linear descent (ΔOD_{600}/min; determined by the method of Briers et al., 2007a) of PlyP40, Ply511, or PlyP825 at different concentrations measured against whole exponential-phase *L. monocytogenes* cells (TMW 2.1512) in IPB.

3.3.1.2 Acidic native page

In contrast to SDS-PAGE, native page is performed under non-denaturing conditions and therefore allows for the analyses of proteins and protein-protein complexes in their native state. The *Listeria* phage endolysins are basic proteins with an isoelectric point between 9 and 10. Their native form is thus positively charged at neutral pH and will migrate from the anode to the cathode during electrophoresis. Hence, the polarity of the leads was placed in reverse order as compared to SDS-PAGE. An acidic gel was used to further enhance the polarity and thereby migration of the proteins.

Figure 26 shows an acidic native page gel of endolysin PlyP40, Ply511, and PlyP825 at a concentration of 4000 or 2000 nM. For endolysin PlyP825, two protein bands could be distinguished and three bands were visible for Ply511, suggesting self-association into dimer and trimer protein complexes, respectively. Interestingly, no Eagle effect was observed for endolysin Ply511 in the viability plating assays and therefore no aggregation was expected. Nonetheless, Ply511 seemed to self-associate into multimeric complexes. Similar to the observation made in the previous section, the discrepancy between these assays might results from differences in the Ply511 concentration applied, which was four times higher in the acidic gel compared to the highest concentration tested in the inactivation studies. Unfortunately, acidic native page was not sensitive enough to examine the self-association of Ply511 at lower concentrations (no visible bands present). For endolysin PlyP40, only a single band was visible (at both concentrations examined), indicating that only monomeric PlyP40 was present in the acidic native gel. The absence of multiple protein bands does however not exclude self-association of PlyP40 in the viability plating assays per se; protein aggregation is highly dependent on the environmental conditions and these are highly different between the acidic native gel and buffer used in the inactivation studies.

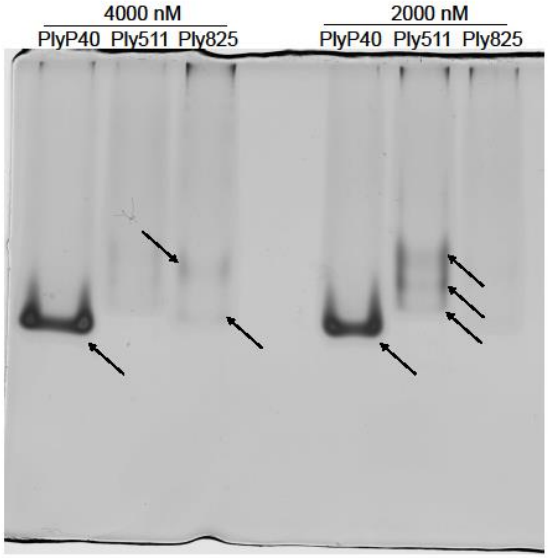


Figure 26: Acidic native page of endolysin PlyP40, Ply511, and PlyP825. Acidic native page was performed with either a 4000 or 2000 nM endolysin solution. Arrows indicate protein bands.

3.3.1.3 UV-spectroscopy

Figure 27 shows the UV-absorbance spectra of the PlyP825 stock solution and PlyP825 diluted with IPB to a concentration of 1 mg/mL. At both concentrations, PlyP825 had an absorption band in the 230 and 280 nm range, which is characteristic for the absorption by peptide bonds and aromatic residues, respectively. Absorbance above 310 nm was close to zero which indicates that the protein is completely in solution and not aggregated. The same sample was left on RT up to 24h to see if the absorption spectrum (and thereby possible aggregation) would change over time, but this was not the case (data not shown). Although no indication for aggregation could be detected by this method, it might be that the method is not sensitive enough to detect self-association into dimer or trimer complexes, as was indicated by acidic native page.

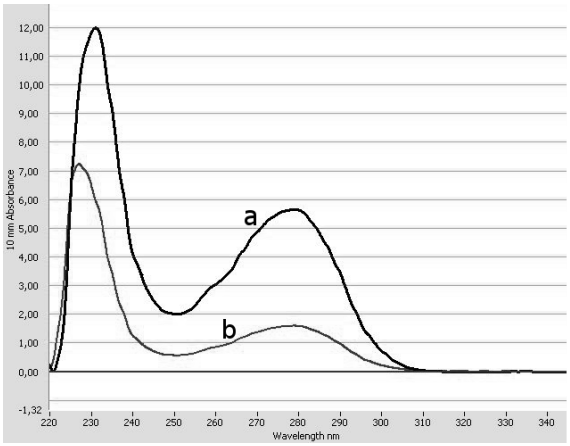


Figure 27: UV-absorbance spectra of endolysin PlyP825. The absorbance of the PlyP825 stock solution (a) and at a concentration of 1 mg/mL in IPB (b) were recorded from 220 to 340 nm. The bottom line is the IPB baseline control.

3.3.1.4 Asymmetric flow field-flow fractionation

AF-4 allows for the separation of macromolecules based on size and thereby also for the differentiation between protein monomers and multimeric complexes. The aggregation of endolysin PlyP825 was therefore additionally investigated by AF-4. Figure 28 shows both the detected voltage of the UV-channel as well as Rayleigh ratio (a measure for the amount of light scattering induced by a particle) for the AF-4 analysis of different amounts of PlyP825. In both spectra, one major peak was observed after ca. 9.3 minutes. In the UV-channel, a small increase in the signal was also observed at t≈7.5. Initially, it was hypothesized that the peak at t≈7.5 represent monomeric PlyP825 and the peak at t≈9.3 associated di- or trimers. However, this small increase at t≈7.5 was also present in the control and therefore most likely an artefact of the IPB buffer used.

To explore whether the peak at t≈9.3 represented monomeric or aggregated PlyP825, the molecular mass of the detected particle was determined. Therefore, both the PlyP825 specific UV extinction coefficient at 280 nm as well as the refractive index increment (dn/dc value) were required. The UV extinction coefficient was calculated by dividing the molar extinction coefficient through the proteins molecular weight (1.5586 mL/(mg × cm)). Determination of the dn/dc value is known to vary only minimally between proteins (personal communication with Wyatt Technology, Dernbach, Germany). The dn/dc of PlyP825 was therefore estimated at 0.180mL/g based on the known dn/dc value of bovine serum albumin in a different buffer and control AF-4 measurements of bovine serum albumin in IPB. The molecular weight of the molecule detected at t≈9.3 was subsequently determined at approximately 37 kDa using the Zimm model algorithm (suitable for small macromolecules with a radius of gyration <100nm; ASTRA, Wyatt Technology). This is therefore a strong indication that monomeric PlyP825 (and not multimeric complexes) was responsible for the observed peak in the AF-4 spectra. Since no other peaks were observed at later time-points, AF-4 analysis indicated that PlyP825 does not form multimeric protein complexes.

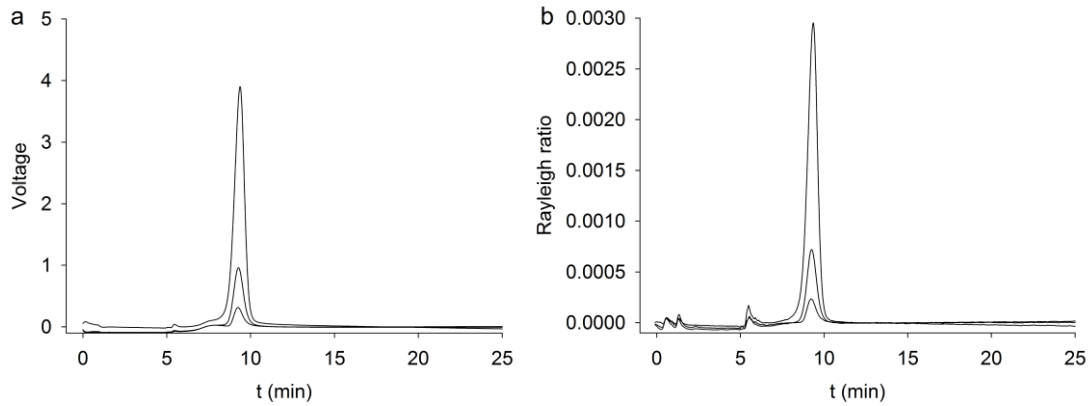


Figure 28: AF-4 analysis of PlyP825. The retention time of different amounts of endolysin PlyP825 in IPB (total amount of 200, 50, or 20 µg) against the detected voltage of the UV-channel (a) or Rayleigh ratio (b).

3.3.2 Investigations into the possible formation of protoplasts

Phase-contrast microscopic time-lapse showed the transformation of typical rod-shaped *L. monocytogenes* cells into spheres upon the addition of endolysin (Figure 22). Hence, it was speculated that the Eagle effect results from the formation of stable protoplasts as a result of the faster breakdown of the peptidoglycan layer at higher endolysin concentrations. The experiments described in this section were performed to examine this hypothesis more closely.

3.3.2.1 Inoculum-dependent inactivation of L. monocytogenes by endolysin

The formation of *Lactobacillus* (*Lac.*) *johnsonii* protoplasts incubated with lysozyme in a hypotonic phosphate buffer has been observed before by Masschalck et al., 2002 (referred to as spheroplasts by the authors). It was hypothesized that the Eagle effect could be explained by the formation of stable *L. monocytogenes* protoplasts due to an almost instantaneous breakdown of the peptidoglycan layer at higher endolysin concentrations (in contrast to pore formation and cell lysis at lower concentrations). In order to test this hypothesis, the endolysin concentration was fixed while the concentration of cells was systematically lowered. As such, more endolysin becomes available per cell and should, according to the hypothesis, result in the formation of stable protoplasts. However, instead of more survival, Figure 29 shows that the same endolysin concentration led to higher inactivation (i.e. log cycles reduction) when applied to less cells. For example, whereas 0.8 µg/mL PlyP40 caused a reduction of only 0.1 log cycles at an inoculum of 10^9 cells/mL, a reduction of 2.5 and 4.9 log cycles was achieved with the same endolysin concentration at an inoculum of 10^8 and 10^7 cells/mL, respectively. A similar trend was also observed for the other endolysin concentrations; lowering the inoculum at a fixed endolysin concentration increased the percentage of cells killed. These results thereby reject the formulated hypothesis that protoplasts formation due to a fast breakdown of the peptidoglycan layer is responsible for the Eagle effect.

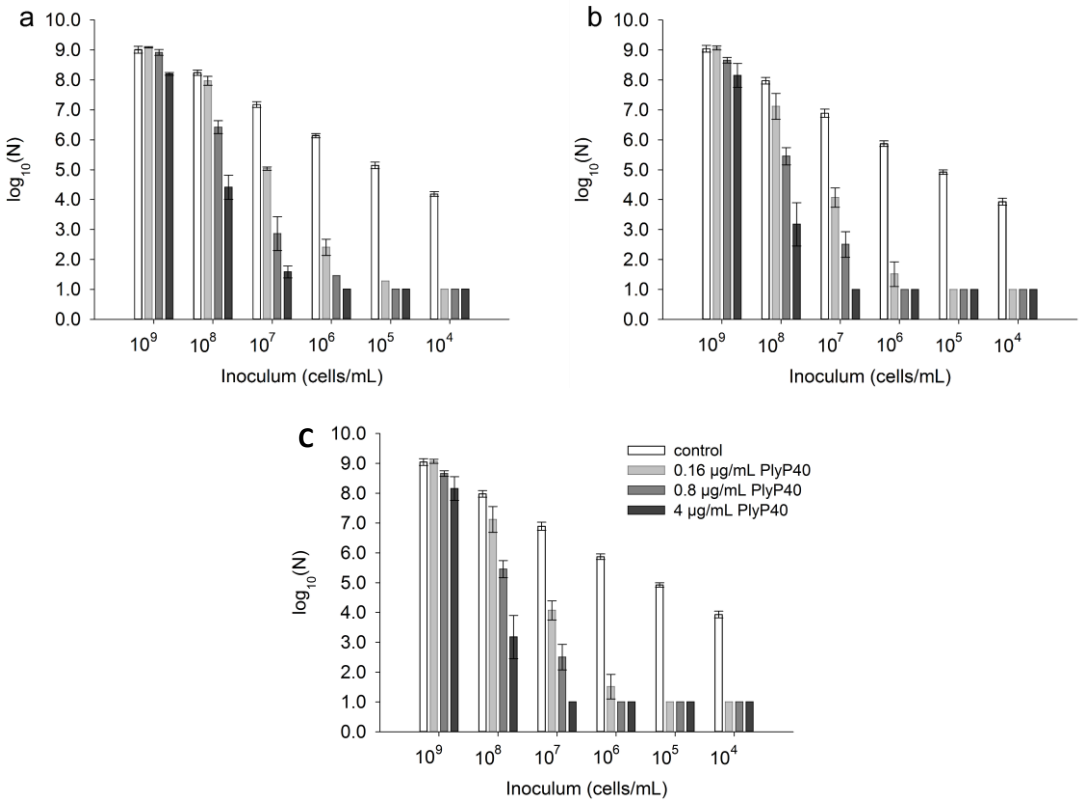


Figure 29: PlyP40-induced inactivation of *L. monocytogenes* cells at different inoculation levels. Total cell count (log_{10}(N)) of exponential-phase *L. monocytogenes* cells (TMW 2.1512) at different inocula (10^4, 10^5, 10^6, 10^7, 10^8, or 10^9 cells/mL) incubated with either IPB (control) or different concentrations of endolysin PlyP40 (0.16, 0.8, or 4 µg/mL). Coincubation of cells with endolysin was either 1 (a), 3 (b), or 24 hours (c) until microbiological analyses. The detection limit is shown by the dashed line (1.0 log cfu). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

3.3.2.2 Time-dependent inactivation by endolysin

The Eagle effect in section 3.2.1.2.1 was observed after 1.5 hours of coincubation with endolysin. The inactivation kinetics of *L. monocytogenes* by endolysin PlyP40 were studied more closely to provide more insights into the presence of the Eagle effect with varying endolysin coincubation times. As can be seen in Figure 30, the inactivation of *L. monocytogenes* by endolysin PlyP40 was not instantaneous as argued by Loeffler et al., 2001, but a timely process where cells were increasingly killed with longer endolysin coincubation times. Two more interesting observations were made:

(i) the Eagle effect diminished with longer incubation times because of the continued inactivation of cells at a concentration of 20 µg/mL. Following our initial hypotheses, this could mean two things; either protoplasts are formed after the addition of 20 µg/mL PlyP40 and slowly die because they are not stable in the buffers, or, there is a slow inactivation of cells caused by the release of monomeric endolysin from self-associated complexes.

(ii) at a concentration of 0.16 and 0.8 µg/mL PlyP40, maximum inactivation was reached after 0.5-1 hour of coincubation with endolysin, after which the cell count seemed to increase again. After 24 hours, the sample incubated with 0.16 µg/mL even had a cell count almost similar to the control. This might indicate the presence of type II sublethal damage shortly after the addition of endolysin (as described in section 1.3.2.4.2), from which cells can recover after longer incubation times.

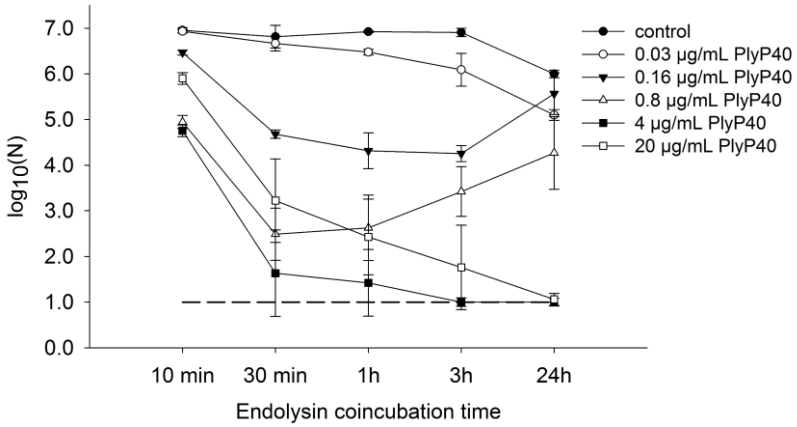


Figure 30: Time-dependent inactivation of *L. monocytogenes* inactivation by PlyP40. Total cell count (log_{10}(N)) of exponential-phase *L. monocytogenes* cells (TMW 2.1512 at an inoculum of ca. 10^7 cells/mL) plated at different time-points (10 min, 30 min, 1h, 3h, or 24h) after the addition of either IPB (control) or different concentrations of endolysin PlyP40 (0.03, 0.16, 0.8, 4, or 20 µg/mL). The detection limit is shown by the dashed line (1.0 log cfu). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

3.4 The inactivation of *L. monocytogenes* by endolysin and HHP in food systems

The results in this chapter originate (in part) from a recent publication (Misiou et al., 2018). In section 3.2, the combined application of endolysin and HHP for the inactivation of Gram-positive foodborne pathogens was investigated in buffer. Here, the combination of these techniques for the inactivation of a *L. monocytogenes* cocktail was examined in foods by both challenge lethality as well as storage tests at abuse temperatures (10 °C).

3.4.1 Preliminary experiments

3.4.1.1 Endolysin concentration dependency

The inactivation of *L. monocytogenes* by different concentrations of endolysin Ply511 or PlyP825 was examined in milk. No inactivation by either Ply511 or PlyP825 was observed until a concentration of ca. 0.35 µg/mL (Figure 31). Only at the highest endolysin concentration tested (3.5 µg/mL) cells were inactivated 0.5 and 1.5 log cycles, respectively. PlyP825 thus seemed to display a somewhat higher activity and was preferably used for future experiments. Although a direct comparison between the efficacy of endolysin in milk and IPB was not made, these data indicated that the tested endolysins are much less effective in milk (0.8 µg/mL Ply511 or PlyP825 already reduced the cell count by 1-2 log cycles in IPB; Table 22).

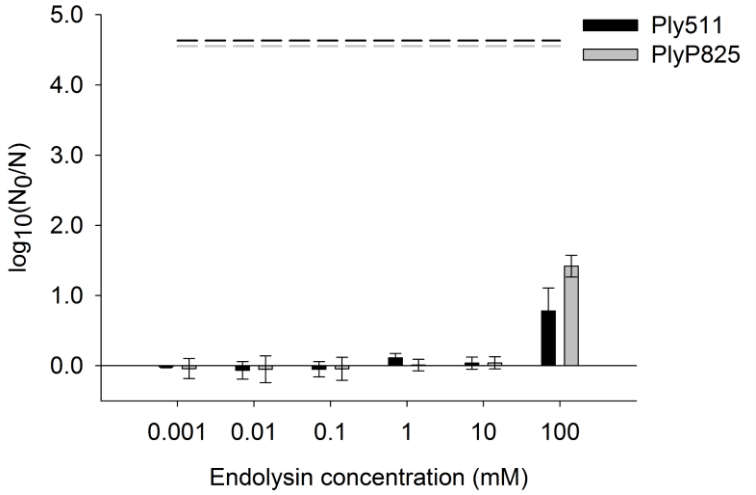


Figure 31: The inactivation of *L. monocytogenes* by Ply511 and PlyP825 in milk. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 4×10^5 cells/mL) by endolysin Ply511 (black bars) or PlyP825 (gray bars) at different concentrations (0.001-100mM; 100 mM Ply511 and PlyP825 equal 3.4 and 3.6 µg/mL, respectively). The detection limits (1 log cfu) are shown by the dashed lines. Mean values ± standard deviation of two biologically independent experiments are shown (error bars).

3.4.1.2 The combined application of multiple endolysins

It was hypothesized that endolysins with different peptidoglycan cleavage sites might act synergistically when applied together. The inactivation of *L. monocytogenes* caused by a single endolysin was therefore compared with the inactivation caused by a cocktail of two or three different endolysins. This was done at equimolar concentrations; the inactivation by 1000 mM PlyP40 was compared to the inactivation by a mixture of 500 mM PlyP40 and 500 mM Ply511). It was found that the inactivation by a combination of different endolysins did not substantially differentiate from the inactivation induced by a single endolysin (Figure 32). Among the three endolysin examined, PlyP40 caused a higher inactivation than Ply511 and PlyP825, which is also reflected by the inactivation of PlyP40 combined with the other endolysins. Although PlyP40 in combination with PlyP825 seemed to achieve a somewhat higher inactivation than the single endolysins or other combination of endolysins, differences were only very minimal and the use of endolysin cocktails was therefore not further pursued.

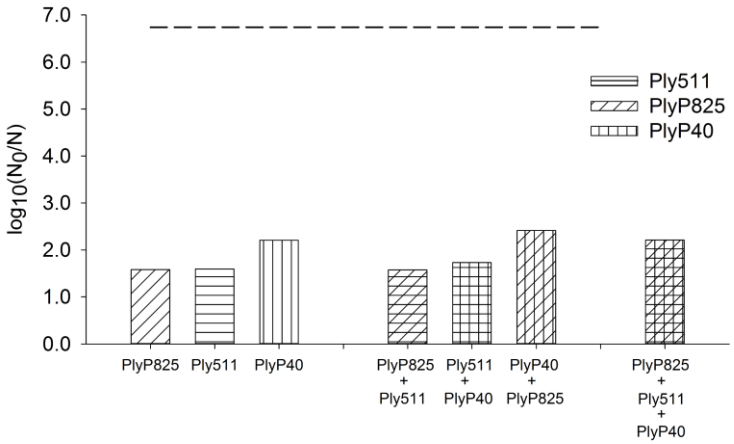


Figure 32: The combined application of multiple endolysin for the inactivation of *L. monocytogenes*. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 6×10^7 cells/mL) by endolysin PlyP825 (diagonal lines), Ply511 (horizontal lines), PlyP40 (vertical lines) applied either individually or cimbined. The total endolysin concentration was kept constant at 1000 mM with equal amounts of endolysin (either 1000, 500 or 333 mM per endolysin). The detection limit (1 log cfu) is shown by the dashed line.

3.4.1.3 HHP susceptibility in different foods

The inactivation of *L. monocytogenes* by HHP processing was directly compared in milk, mozzarella, and smoked salmon. All samples were plated on *Listeria* selective agar to allow for a direct comparison between the foods (milk samples were plated on TSAYE in other assays). No inactivation was visible in any of the foods up to a pressure level of 300 MPa (10 min, 25 °C). Whereas at 400 MPa the cell count was reduced substantially in milk and mozzarella (3-4 log cycles), a minimum pressure level of 500 MPa was required to inactivate *Listeria* cells in smoked salmon and this inactivation was still rather limited (1.5 log cycles). This demonstrates that the inactivation of *L. monocytogenes* by HHP differs greatly depending on the food vehicle used.

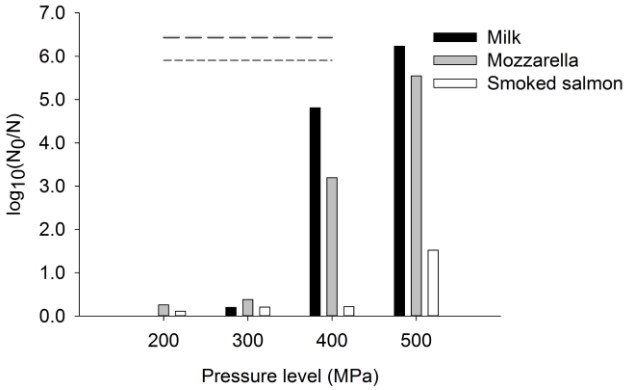


Figure 33: HHP susceptiblity of *L. monocytogenes* in milk, mozzarella, and smoked salmon. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains) in milk (black bars), mozzarella (gray bars), or smoked salmon (white bars) by HHP (10 min, 25 °C) with increasing pressure levels (200-500 MPa, increments of 100 MPa). The detection limits (1 log cfu) are shown by the long (milk or mozzarella) and short (smoked salmon) dashed lines.

3.4.2 Milk

3.4.2.1 Challenge lethality test

Figure 34 shows the combined application of multiple PlyP825 concentrations and pressure levels against a *L. monocytogenes* cocktail in milk. A synergistic effect at a pressure level of 400 MPa (10 min, 25 °C) was visible at all the PlyP825 concentrations examined. A HHP treatment of 400 MPa alone caused a reduction in cell count of 2.2 log cycles. Prior incubation with 0.34 or 3.4 µg/mL PlyP825, which applied individually did not affect the cell count, allowed for an additional reduction of 1.2 and 1.7 log cycles, respectively. Also after incubation with 34 µg/mL PlyP825, which already individually reduced the cell count by 1.5 log cycles, a synergistic effect of >1 log cycles was observed.

HHP processing at 200 MPa (25 °C, 10 min) only caused a minimal reduction of 0.3 log cycles (Figure 34). However, when cells were first incubated with 3.4 or 34 µg/mL PlyP825, a pressure treatment of 200 MPa further reduced the cell count by 2.0 or 2.1 log cycles (i.e. a synergistic reduction of 1.7 and 1.8 log cycles). Interestingly, although both 3.4 µg/mL PlyP825 and HHP processing at 200 MPa (10 min, 25 °C) individually hardly affect the cell count, their combined application is almost equally effective as a single pressure treatment of 400 MPa (10 min, 25 °C). It was already shown in buffer that the addition of endolysin allows for the inactivation of cells at a lower pressure level (3.2.1.2.4). In this section, it is demonstrated that this also applies to the inactivation of *L. monocytogenes* in actual foods.

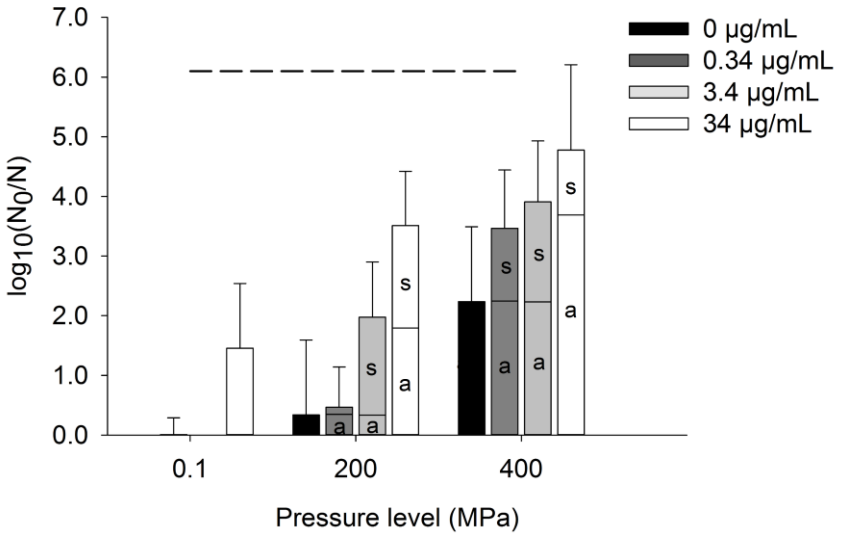


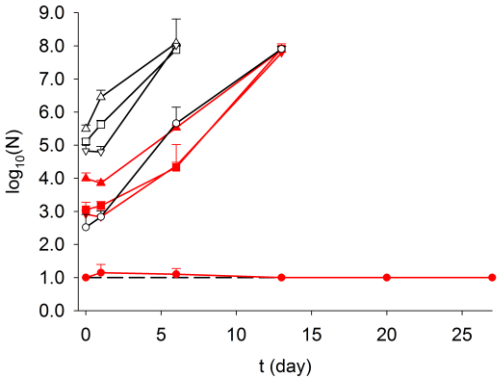
Figure 34: Challenge lethality test in milk. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 10^7 cells/mL) without endolysin (black bars), 0.34 (dark grey bars), 3.4 (light grey bars), or 34 µg/mL PlyP825 (blanc bars) at atmospheric pressure (0.1 MPa) or with an additional HHP treatment at 200 or 400 MPa (10 min, 25 °C). The lower part of the bars represents the calculated additive effect of individual endolysin and HHP treatments, indicated by the letter a. The upper part represents the synergistc inactivation as result of the combined treatment, indicated by the letter s. The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of three biologically independent experiments are shown (error bars).

3.4.2.2 Challenge storage test

Two milk storage tests were performed. In the first storage test, the efficacy of the combined PlyP825 and HHP treatment was screened at multiple pressure levels and holding times with a somewhat higher inoculation level (3×10^5 cells/mL). In the second storage test, the efficacy of the combined application was examined at a fixed pressure level and lower inoculation level (7×10^3 cells/mL).

3.4.2.2.1 High inoculum

The efficacy of a combined endolysin-HHP treatment in milk was screened with a fixed PlyP825 concentration (3.4 µg/mL), at three pressure levels (200, 300, and 400 MPa), and two pressure holding times (1 and 10 min). In Figure 35, results for a pressure holding time of 10 min are presented. A pressure holding time of 1 min was not sufficient to eliminate *Listeria* from any of the samples examined and these results are therefore presented in Appendix 8.3 (Figure 48). Treatment with only 3.4 µg/mL PlyP825 reduced the cell count by 1.5 log cycles and growth was inhibited for 1 day, after which cells steadily grew to the upper detection limit of 10^8 cells/mL at day 13 (▲). The cell count was reduced by an additional 0.9 log cycles when 3.4 µg/mLPlyP825 was combined with HHP at 200 (■) or 300 (▼) MPa, but also here rapid growth was visible after day 1. Interestingly, similar to the results in the challenge lethality test, the combination of 3.4 µg/mL PlyP825 with a pressure treatment of 200 MPa (■) was about equally effective as a pressure treatment of 400 MPa alone (○). Incubation with 3.4 µg/mL PlyP825 and subsequent pressure treatment of 400 MPa reduced the cell count until the detection limit and only 2 out of 18 samples became positive for *Listeria* during storage up to 27 days (one sample at t=1 and one at t=6; ●).



of *Listeria*-positive samples

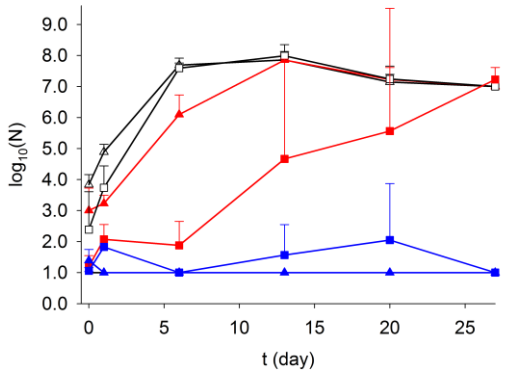
PlyP825 (µg/mL)	HHP (MPa)	Symbol	t (day) 0	1	6	13	20	27	Total
0	0.1	△	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	□	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	300	▽	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	○	3/3	3/3	3/3	3/3	3/3	3/3	18/18
3.4	0.1	▲	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	■	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	300	▼	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	●	0/3	1/3	1/3	0/3	0/3	0/3	2/18

Figure 35: Challenge storage test in milk: high inoculum. The growth (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 3×10^5 cells/mL) without endolysin (blanc symbols) or 3.4 µg/mL PlyP825 (filled red symbols) at atmospheric pressure (upward triangles) or in combination with a HHP treatment (25 °C, 10 min) of 200 (squares), 300 (downward triangles), or 400 MPa (circles). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of three biologically independent experiments are shown (error bars). The number of *Listeria*-positive samples per time-point and in total during storage is shown in the table next to the graph.

3.4.2.2.2 Low inoculum

In section 3.3.2.1, it was shown that endolysin can cause a much larger log reduction when the same concentration is applied to a lower number of cells. In the previous section, it was shown that an additional HHP treatment of 200 MPa could further reduce listerial cell count, but was not sufficient to kill all cells. Since the inoculation level in this storage test was higher than typically used to ascertain the microbiological stability of food products (i.e. $>10^5$ instead of a recommended 10^2-10^3 cells/g; FDA, 2001), the storage test was repeated with a pressure level of 200 MPa (10 min, 25 °C) and lowered inoculation level (7×10^3 cells/mL; Figure 36).

Although the combined application of 3.4 µg/mL PlyP825 and a HHP treatment of 200 MPa reduced the cell count until the detection limit in two out of three replicates directly after pressure treatment, only four out of the remaining 15 samples remained negative for *Listeria* during storage (i.e. 12/18 *Listeria*-positive samples; ■). A PlyP825 concentration of 34 µg/mL PlyP825 substantially reduced the initial cell count (until the detection limit in a single sample) and no *Listeria*-positive samples were detected at later time-points (i.e. only 2/18 *Listeria*-positive samples; ▲). Surprisingly, more samples (4/18) became positive for *Listeria* when 34 µg/mL PlyP825 was combined with 200 MPa (■). A possible explanation for the higher number of positive samples after a combined treatment versus endolysin applied individually might be that PlyP825 was (partly) inactivated by the pressure treatment. This is however rather unlikely since previous experiments showed no negative effect on the enzymatic activity of PlyP825 by a pressure treatment of 600 MPa (3.1.2). Since the number of *Listeria*-positive samples after a combined treatment is only minimal higher and still relatively low (4/18), the small number of replicates and sample variability might be a more likely explanation.



of *Listeria*-positive samples

PlyP825 (µg/mL)	HHP (MPa)	Symbol	t (day) 0	1	6	13	20	27	Total
0	0.1	△	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	□	3/3	3/3	3/3	3/3	3/3	3/3	18/18
3.4	0.1	▲	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	■	1/3	2/3	2/3	2/3	2/3	3/3	12/18
34	0.1	▲	2/3	0/3	0/3	0/3	0/3	0/3	2/18
	200	■	1/3	1/3	0/3	1/3	1/3	0/3	4/18

Figure 36: Challenge storage test in milk: low inoculum. The growth (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. $7×10^3$ cells/mL) without endolysin (blanc symbols), with 3.4 (filled red symbols), or 34 µg/mL PlyP825 (filled blue symbols) at atmospheric pressure (triangles) or in combination with a HHP treatment of 200 MPa (25 °C, 10 min; squares). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of three biologically independent experiments are shown (error bars). The number of *Listeria*-positive samples per time-point and in total during storage is shown in the table next to the graph.

3.4.3 Mozzarella

3.4.3.1 Challenge lethality test

Figure 37 shows the combined application of 34 µg/mL PlyP825 with HHP against *L. monocytogenes* in mozzarella. The combination of 34 µg/mL PlyP825 and 400 MPa (10 min, 25 °C) caused a reduction in cell count of 4.1 log cycles, which was 0.7 log cycles larger than the sum of individual effects. Whereas both 34 µg/mL PlyP825 and HHP processing at 300 MPa (10 min, 25 °C) reduced the cell count only minimally (0.2 and 0.4 log cycles, respectively), their combination caused a reduction of 1.8 log cycles (i.e. a synergism of 1.3 log cycles). This demonstrates that, similar to what was found for buffer and milk, the inactivation of *Listeria* in mozzarella can also be achieved at lower pressure levels when combined with endolysin.

At 200 MPa, the reduction in cell count by a combined treatment was only minimal (<1 log cycle). In milk, the same parameters caused a reduction of 3.5 log cycles of which 1.7 synergistically (3.4.2.1). A synergistic inactivation at 200 MPa was in milk however only observed in combination with 3.4 and 34, but not with 0.34 µg/mL PlyP825. Considering the above and that PlyP825 might generally have a lower efficacy in mozzarella, sensitization of *Listeria* in mozzarella to a pressure treatment of 200 MPa might be achieved by further increasing the PlyP825 concentration (especially because a minimal synergistic effect is already visible).

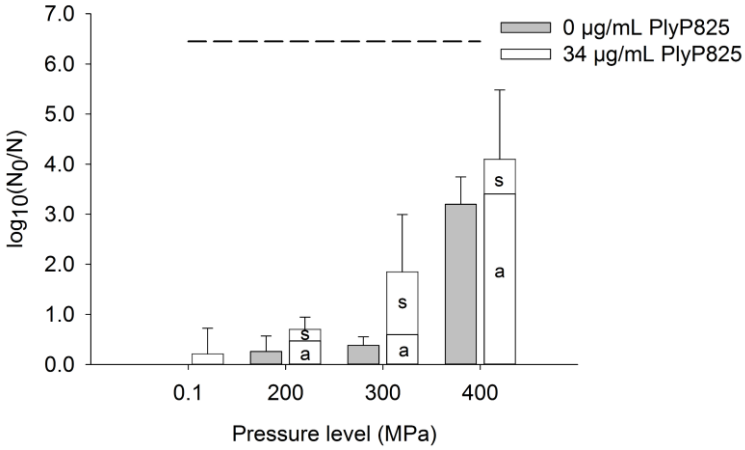
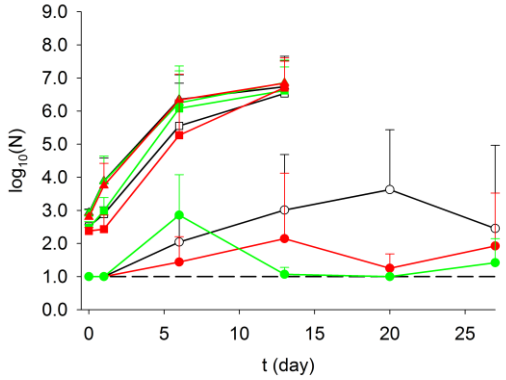


Figure 37: Challenge lethality test in mozzarella. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 10^7 cells/mL) without endolysin (gray bars) or 34 µg/mL PlyP825 (white bars) at atmospheric pressure (0.1 MPa) or in combination with a HHP treatment of 200, 300, or 400 MPa (10 min, 25 °C). The lower part of the white bars represents the calculated additive effect of individual endolysin and HHP treatments, indicated by the letter a. The upper part represents the synergistc inactivation as result of the combined treatment, indicated by the letter s. The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of three biologically independent experiments are shown (error bars).

3.4.3.2 Challenge storage test

The growth of *L. monocytogenes* in mozzarella samples after treatment with PlyP825 (0.34 or 3.4 µg/mL), HHP (200 or 400 MPa, 10 min, 25 °C), or a combination of both was observed up to 27 days (Figure 38). The application of either 0.34 (▲) or 3.4 (▲) µg/mL PlyP825 did not influence the cell count or growth kinetics. The treatment of mozzarella samples with 200 MPa alone (□) or in combination with 0.34 (■) or 3.4 (■) µg/mL PlyP825 reduced the cell count only minimally and also growth over time was hardly affected. A pressure treatment of 400 MPa (both without and with prior PlyP825 coincubation) reduced the cell count to below the detection limit and no cells were detected after 1 day of storage. At later time-points, 10 from the remaining 12 samples treated with only 400 MPa became positive for *Listeria* (○; total of 10/18 positive samples during storage). Incubation with 0.34 (●) or 3.4 (●) µg/mL PlyP825 prior to a pressure treatment of 400 MPa further reduced the total number of positive samples to 5/18 and 4/18, respectively. In summary, although the chosen parameters were not sufficient to completely eliminate *L. monocytogenes*, the challenge lethality and storage test together demonstrate that the combined application of PlyP825 and HHP enhances the efficacy of pressure treatment against *Listeria* in mozzarella cheese.



of *Listeria*-positive samples

PlyP825 (µg/mL)	HHP (MPa)	Symbol	t (day) 0	1	6	13	20	27	Total
0	0.1	△	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	□	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	○	0/3	0/3	3/3	3/3	3/3	1/3	10/18
0.34	0.1	▲	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	■	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	●	0/3	0/3	1/3	2/3	0/3	2/3	5/18
3.4	0.1	▲	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	■	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	●	0/3	0/3	1/3	1/3	1/3	1/3	4/18

Figure 38: Challenge storage test in mozzarella. The growth (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 10^3 cells/mL) without endolysin (blanc symbols), 0.34 (filled green symbols), or 3.4 µg/mL PlyP825 (filled red symbols) at atmospheric pressure (triangles) or in combination with a HHP treatment (25 °C, 10 min) of 200 (squares) or 400 MPa (circles). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of three biologically independent experiments are shown (error bars). The number of *Listeria*-positive samples per time-point and in total during storage is shown in the table next to the graph.

3.4.4 Smoked salmon

3.4.4.1 Challenge lethality test

In the preliminary experiments, it was already shown that HHP is far less effective against *L. monocytogenes* in smoked salmon compared to milk or mozzarella (3.4.1.3). Here, it was again demonstrated that a minimal pressure level of 500 MPa (10 min, 25 °C) is required for the inactivation of *L. monocytogenes* cells in vacuum packed smoked salmon stripes (Figure 39). Prior incubation with 34 µg/mL PlyP825 did not enhance pressure inactivation at any of the pressure levels examined. Moreover, even a combination of the most stringent conditions (34 µg/mL PlyP825 and 500 MPa) reduced the cell count only 1.6 log cycles (compared to a reduction of 4-5 log cycles when 34 µg/mL PlyP825 was combined with only 400 MPa in milk or mozzarella).

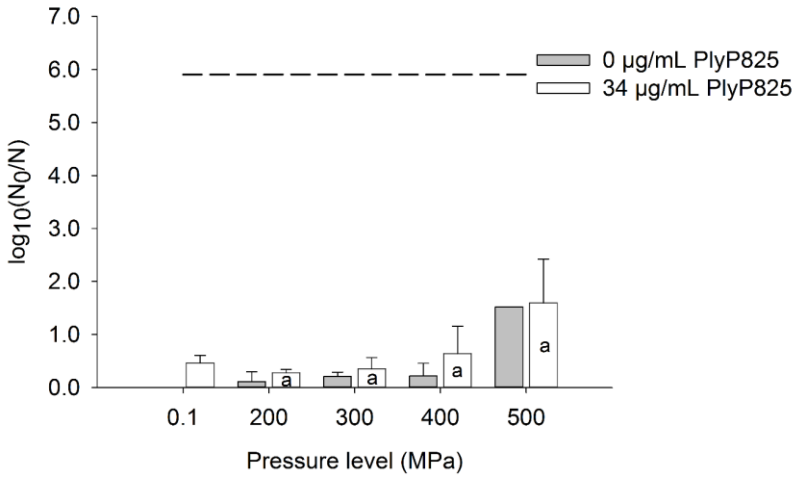
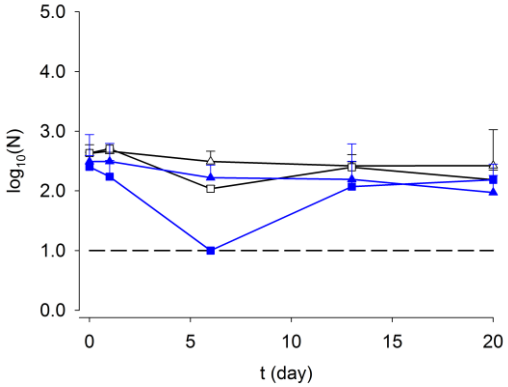


Figure 39: Challenge lethality test in smoked salmon. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 10^7 cells/g) without endolysin (gray bars) or 34 µg/mL PlyP825 (white bars) at atmospheric pressure (0.1 MPa) or in combination with a HHP treatment of 200, 300, 400, or 500 MPa (25 °C, 10 min). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of two biologically independent experiments are shown (error bars).

3.4.4.2 Challenge storage test

Figure 40 shows that, despite the low inoculation level, neither 34 µg/mL PlyP825 (▲), nor 400 MPa (□), nor their combined application (■) could eliminate *Listeria*. On the contrary, except for two

Listeria negative replicates at day 6 after a combined treatment, no reduction in cell count was observed after either individual or a combined treatment. Interestingly, whereas in milk and mozzarella substantial growth in the control was observed over time, the cell count on smoked salmon stayed constant during storage at abuse temperature in all samples. In summary, with the current experimental set-ups, no synergistic effect between PlyP825 and HHP for the inactivation of *L. monocytogenes* in smoked salmon could be detected by either challenge lethality or storage test.



of *Listeria*-positive samples

PlyP825 (µg/mL)	HHP (MPa)	Symbol	t (day) 0	1	6	13	20	Total
0	0.1	△	2/2	2/2	2/2	2/2	2/2	10/10
	400	□	2/2	2/2	2/2	2/2	2/2	10/10
34	0.1	▲	2/2	2/2	2/2	2/2	2/2	10/10
	400	■	2/2	2/2	0/2	2/2	2/2	8/10

Figure 40: Challenge storage test in smoked salmon. The growth (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 5×10^2 cells/mL) without endolysin (triangles) or 34 µg/mL PlyP825 (squares) at atmospheric pressure (blanc symbols) or in combination with a HHP treatment of 400 MPa (10 min, 25°C; blue filled symbols). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values + standard deviation of two biologically independent experiments are shown (error bars). The number of *Listeria*-positive samples per time-point and in total during storage is shown in the table next to the graph.

3.5 The inactivation of *L. monocytogenes* by phage P100 and HHP in buffer and milk

The results in this chapter originate (in part) from the work of Sabrina Bahe (8.1.2). In the previous results sections, the combination of HHP with phage endolysins for the inactivation of *L. monocytogenes* was examined. Here, a possible synergism between HHP and phage P100 was investigated in IPB (pH 7.5) and milk.

3.5.1 Preliminary experiments

3.5.1.1 Phage-concentration and inoculum-dependency

The phage-concentration and inoculum-dependent inactivation of *L. monocytogenes* strain TMW 2.1512 was examined in both IPB and milk. Figure 41 shows that a much higher phage concentration was required in milk to achieve the same activation as in IPB. For example, whereas a phage concentration of 10^8 pfu/mL was enough to inactivate 10^4 cells/mL until the detection limit in IPB, more than 10 times as much phages were required to achieve the same inactivation in milk (Figure 41a). Comparing Figure 41a and b, it can be seen that the inoculation level also influenced phage induced inactivation and was generally about 1 log cycle lower when the inoculum was increased from 10^4 to 10^7 cells/mL.

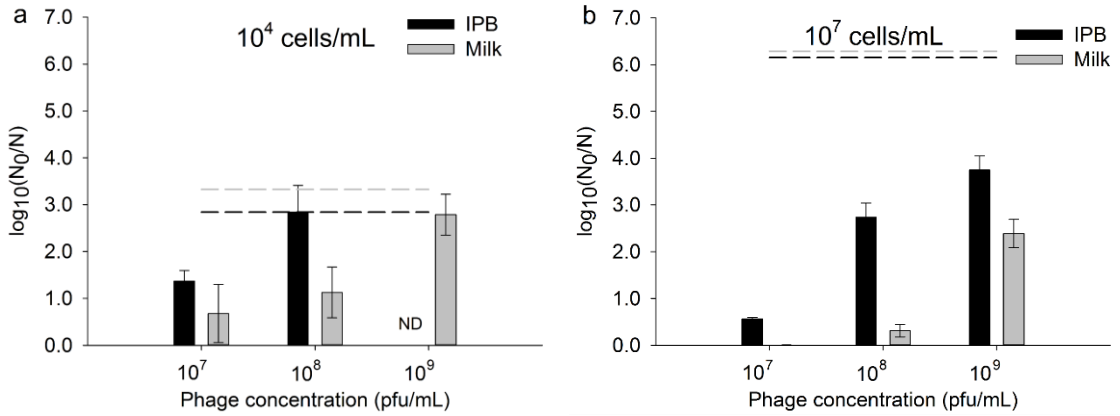


Figure 41: Phage-concentration and inoculum-dependent inactivation of *L. monocytogenes*. The induced reduction ($\log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (strain TMW 2.1512) at an inoculum of either ca. 10^4 (a) or 10^7 (b) cells/mL in IPB (black bars) or milk (gray bars) after incubation with different phage P100 concentrations (10^7, 10^8, or 10^9 pfu/mL). The detection limits (1.0 log cfu) are shown by the dashed lines. Mean values ± standard deviation of three biologically independent experiments are shown (error bars). ND not determined.

3.5.1.2 HHP susceptibility

The pressure resistance of *L. monocytogenes* TMW 2.1512 was examined in order to select parameters for a combined phage-HHP treatment. The pressure holding time and initial temperature were fixed (1 min, 25 °C) and the pressure level was incrementally increased from 250 to 600 MPa (Figure 42). Although differences between the inactivation in IPB and milk were only minimal, some inactivation was already detected for cells in IPB at 350 MPa, whereas a minimal pressure level of 400 MPa (1 min, 25 °C) was required to inactivate cells in milk. As such, in order to study the combined application of phages and HHP, both 350 MPa and 400 MPa were selected as pressure parameter for future experiments.

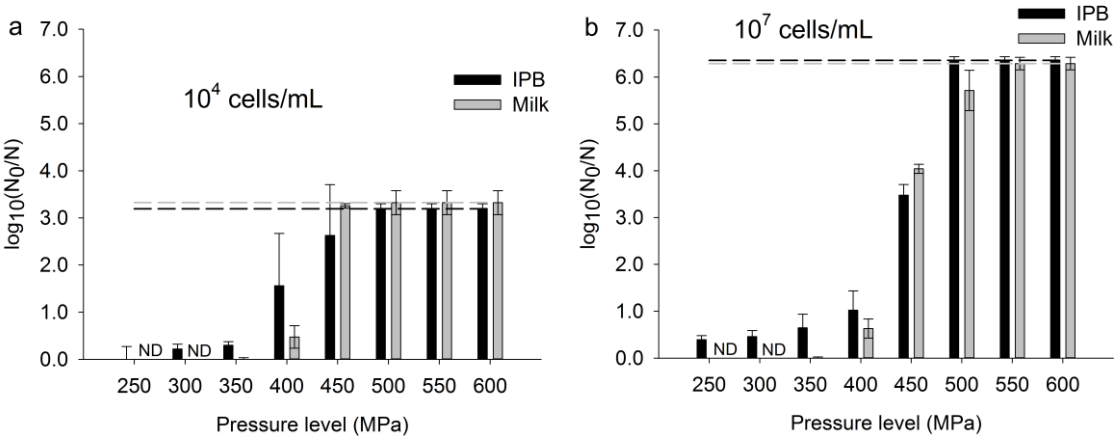


Figure 42: HHP susceptiblity of *L. monocytogenes* in IPB and milk. The induced reduction ($\log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (strain TMW 2.1512) at an inoculum of either ca. 10^4 (a) or 10^7 (b) cells/mL in IPB (black bars) or milk (gray bars) by increasing high pressure levels (250-600 MPa, increments of 50 MPa; 1 min, 25 °C). The detection limits (1.0 log cfu) are shown by the dashed lines. Mean values ± standard deviation of three biologically independent experiments are shown (error bars). ND not determined.

3.5.2 The combined application of phage P100 and HHP

The inactivation of *L. monocytogenes* by phage P100 (10^7 or 10^8 pfu/mL), HHP (350 or 400 MPa; 25 °C, 1 min), or their combined application against either 10^4 or 10^7 cells/mL in IPB or milk is summarized in Table 26. In IPB, whereas the treatment of 10^4 cells/mL by either HHP (350 or 400 MPa at 25 °C for 1 min) or phages (10^7 pfu/mL) reduced the cell count by only 1 log cycle, their combined application

inactivated cells by 3 log cycles (i.e. a synergy of ca. 1 log cycle). At the same parameters, hardly any synergism was present against an inoculum of 10^7 cells/mL. However, when the phage concentration was raised by a factor 10 (from 10^7 to 10^8 pfu/mL), the combined phage-HHP application resulted in a synergy of 1.9 (350 MPa) or 2.8 (400 MPa) log cycles. This shows that the number of applied phages in relation to the inoculum is a crucial determining factor for synergy in combination with HHP.

In the preliminary experiments (3.5.1), the reduced antimicrobial activity in milk compared to buffer was already demonstrated for individual phage or HHP treatment. The results inTable 26 show that a combined phage-HHP treatment is also less efficient in milk compared to buffer. For example, whereas the combined application of 10^8 pfu/mL phages and 400 MPa (25 °C, 1 min) reduced the cell count by 5.5 log cycles in IPB (at an inoculum of 10^7 cells/mL), the same treatment resulted in a reduction of only 1.4 log cycles in milk. Although small synergistic effects in the range of 0.1 to 0.4 log cycles were present for every phage-HHP combination in milk, the inactivation was mostly additive and much less pronounced compared to buffer. As such, the discrepancy between the synergism found for buffer and milk might be attributed to the reduced efficacy of the phage in the latter (by a factor ~10; 3.5.1.1). Considering the above and that the synergistic effect also substantially increased in buffer upon an increase in the phage concentration from 10^7 to 10^8 pfu/mL (Table 26), a stronger synergistic effect might also be present in milk at a phage concentration 10^9 pfu/mL.

In IPB, at an inoculum of 10^7 cells/mL and a phage concentration of 10^7 pfu/mL, the synergistic effect was minimal, however, substantially increased when the phage concentration was increased to 10^8 pfu/mL. Considering the factor 10 lower efficacy of P100 in milk (3.5.1.1), a stronger synergistic effects might also be present in milk at a phage concentration of 10^9 pfu/mL.

Table 26: The inactivation of *L. monocytogenes* by phage P100 and HHP. The total cell count (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (strain TMW 2.1512) at an inoculum of either ca. 10^4 or 10^7 cells/mL in IPB or milk treated with different phage concentrations (10^7 or 10^8 pfu/mL), high pressure levels (350 or 400 MPa; 1 min, 25 °C), or a combination of both treatments. The calculated synergistic inactivation is provided in brackets (syn.). The detection limit was 1.0 log cfu. Mean values ± standard deviation of three biologically independent experiments are shown. *ND not determined.

		IPB			Milk				
		0.1 MPa	350 MPa (syn.)	400 MPa (syn.)	0.1 MPa	350 MPa (syn.)	400 MPa (syn.)		
Phage concentration (pfu/mL)	0	4.2±0.2	3.5±0.2	3.1±0.1	4.5±0.1	4.5±0.2	3.7±0.0	Inoculum (cells/mL)	
	10^7	2.8±0.1	1.2±0.3 (1.0)	1.1±0.1 (0.7)	4.2±0.1	4.0±0.1 (0.2)	3.2±0.2 (0.2)	10^4	
	10^8	ND*	ND	ND	3.4±0.2	3.2±0.1 (0.2)	2.2±0.0 (0.4)		
	0	7.5±0.1	6.7±0.1	6.2±0.3	7.5±0.1	7.6±0.1	6.7±0.0		
	10^7	7.0±0.1	6.1±0.2 (0.1)	5.2±0.5 (0.5)	7.5±0.1	7.5±0.0 (0.1)	6.4±0.1 (0.3)	10^7	
	10^8	6.0±0.2	3.4±0.6 (1.9)	1.9±0.3 (2.8)	7.3±0.1	7.1±0.1 (0.3)	6.1±0.2 (0.4)		

3.5.3 Investigation into the synergy between phage P100 and HHP

It was hypothesized that the synergistic inactivation by a combination of phages and HHP was the result of phage-induced damage to the cell membrane, after which the cell could be further destabilized by high pressure. To provide more insights into this hypothesis, the order of the phage and HHP treatment was reversed (phages were added after HHP processing). Interestingly, a similar synergistic inactivation was achieved compared to cells treated with phages prior to HHP. In IPB, a

synergistic effect of 2.7 log cycles was observed when phages were added after HHP processing (Figure 43), which is highly similar to the synergism of 2.8 log cycles found at the same parameters but reversed order of treatment (Table 26). These results demonstrated that the synergy of a combined phage and pressure treatment is independent on the order in which they are applied and indicates that other or additional mechanisms of synergistic inactivation as originally hypothesized are at play.

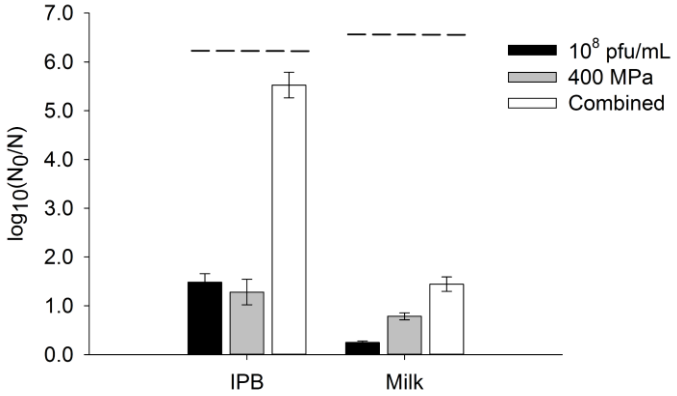


Figure 43: Inactivation by HHP and phages added after pressure treatment. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (strain TMW 2.1512 at an inoculum of ca. 10^7 cells/mL) in IPB or milk by phage P100 (10^8 pfu/mL), HHP (400 MPa, 1 min, 25 °C), or a combined application. Phages were added after HHP processing and coincubated with cells for 2 h. The detection limits (1.0 log cfu) for the experiments in IPB and milk are shown by the dashed lines. Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

3.5.4 The role of milk fat content

The role of milk fat on the inactivation of *L. monocytogenes* by HHP (400 MPa, 25 °C, 1 min), phage P100 (10^8 pfu/mL), or their combined application was examined by systemically increasing the fat content from 0 up to 10%. Whereas the inactivation by 10^8 pfu/mL phages, 400 MPa (1 min, 25 °C), or a combined treatment was much larger in IPB compared to milk, varying the fat content of milk did not significantly change the inactivation by either individual or combined treatments (Figure 44).

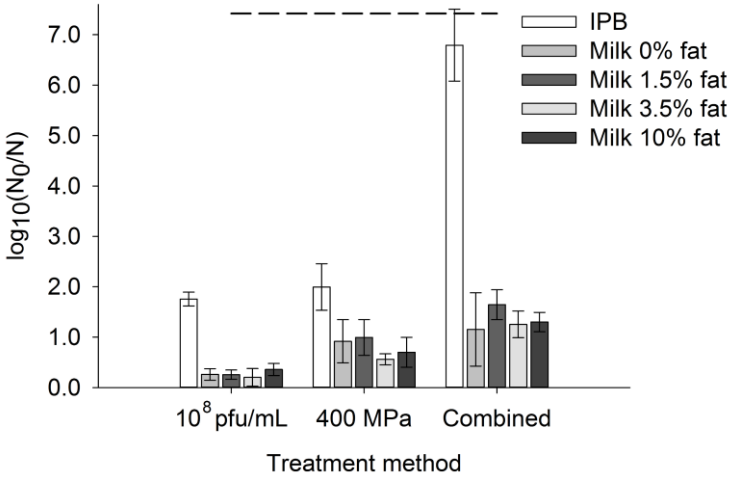


Figure 44: Role of the milk fat content on the inactivation by phages and HHP. The induced reduction ($log_{10}(N_0/N)$) of stationary-phase *L. monocytogenes* cells (strain TMW 2.1512 at an inoculum of ca. 10^7 cells/mL) in IPB (blanco bar) or milk adjusted to different fat contents (different gray tones) by phage P100 (10^8 pfu/mL), HHP (400 MPa, 1 min, 25 °C), or a combined treatment. The detection limit (1.0 log cfu) for samples in IPB is shown by the dashed line (similar for the milk samples). Mean values ± standard deviation of three biologically independent experiments are shown (error bars).

4 DISCUSSION

The present work highlights the potential of a combined endolysin and HHP treatment to inactivate Gram-positive bacteria in foods and provides new insights into the mechanistic background of synergistic inactivation by the combination of these techniques, with synergy defined as the additional inactivation provoked by a combined endolysin-HHP treatment compared to the sum of the individual effects. Moreover, this thesis deepens the understanding of endolysin's antimicrobial activity and the role of the peptidoglycan layer in bacterial high pressure resistance.

A possible synergistic inactivation of *L. monocytogenes* and *S. aureus* by the combined application of endolysin with HHP processing was initially investigated in buffer with three endolysins per species at different endolysin concentrations. It was demonstrated that the combination of these techniques can result in a substantially higher inactivation than can be expected with regard to the additive effect of individual treatments. Although strong synergistic effects were already present against *S. aureus* (up to 3 log cycles; 3.2.1.3.1), the synergistic inactivation of *L. monocytogenes* was even more pronounced (3-5 log cycles for most endolysin-HHP combinations; 3.2.1.2.1). The synergistic inactivation of *L. monocytogenes* by a combination of endolysin and HHP treatment could also be exploited in foods, where, despite major differences between different food products, the combined application increased the efficacy of these techniques in both challenge lethality and storage tests (3.4). This work thereby demonstrates that endolysins are useful to enhance the pressure inactivation of Gram-positive foodborne pathogens, both in buffer systems as well as in foods.

The extent of synergy was not only dependent on the species and medium examined, but also on the endolysin concentration applied, the bacterial growth phase, and the pressure level and holding time (3.2.1.2). Importantly, it was shown that prior incubation of *L. monocytogenes* with endolysin did not only increased the efficacy of subsequent HHP treatment, but also allowed for the inactivation of cells at a lower pressure levels (3.2.1.2.4). Different experiments throughout this work indicate that the synergistic inactivation and increased pressure susceptible of endolysin treated cells is the result of a (partial) breakdown of the peptidoglycan layer. This is suspected to cause an initial stress to the cytoplasmic membrane, which can subsequently be further destabilized by a (mild) HHP treatment and results in cell death. This work thereby not only demonstrates the enhanced inactivation of Gram-positive bacteria by endolysin and HHP, but also provides a deeper understanding of the mechanistic background behind this synergism.

The inactivation of *L. monocytogenes* in buffer by endolysin PlyP40 or PlyP825 displayed an unexpected concentration-dependent inactivation pattern; an increasing number of cells was killed until a threshold endolysin concentration, after which, less cells were killed at even higher endolysin concentrations (3.2.1.2.1). Different hypotheses were investigated and this work demonstrates that the self-association of endolysin PlyP40 and PlyP825 at higher concentrations, which reduces their efficacy, is most likely to cause this effect (3.3)

Finally, in this work, it was demonstrated that phage P100 can also enhance the pressure inactivation of *L. monocytogenes*. Interestingly, results indicate a more permanent effect of pressure on the cell envelope, which allowed for a synergistic inactivation of cells by the addition of phages both prior to and after HHP processing (3.5).

The following sections discuss these findings and other aspects in more detail and aim to enhance the current knowledge about the combined application of endolysins/phages with HHP for the inactivation of Gram-positive food pathogens.

4.1 Endolysin: bacterial inactivation mechanism and kinetics

4.1.1 Role of the binding domain

Probably one of the main factors that distinguishes the inactivation by modular endolysins from classical enzyme reactions is their binding domain, which restricts their activity to 'one-time use'. Instead of an ongoing enzymatic reaction where peptidoglycan is continuously cleaved, the endolysin binds with very high affinity to its target (in the range close to that of antibodies) and is limited to cleavage of 'nearby' bonds (Loessner et al., 2002). This one-time use of endolysin is argued to be rooted in evolution to prevent the lysis of nearby host cells after release of progeny bacteriophages (Fischetti, 2008). This limitation has two important implications for endolysin's activity as antimicrobial:

(i) longer incubation times do not necessarily lead to higher inactivation. Once bound, endolysin will stick to its target and cannot lyse other cells. This is nicely illustrated by the inactivation kinetics of viable cells upon the addition of PlyP40, where maximum inactivation by 0.16 or 0.8 µg/mL PlyP40 is achieved after ca. 30 minutes and longer incubation times did not lead to higher inactivation (Figure 30).

(ii) a fixed amount of endolysin can be expected to kill about the same number of cells (i.e. absolute number, not percentage of cells / log reduction), independent on the inoculum used. This is different from food preservation methods such as thermal or HHP processing, where a similar log reduction can generally be expected, independent on the inoculum used. This is a crucial insight for studying the antibacterial effect of endolysin, because it implies that the endolysin to cell ratio is a crucial factor for its efficacy as antimicrobial. Figure 29a and b nicely demonstrate this effect; whereas 0.8 µg/mL PlyP40 was sufficient to inactivate 10^4, 10^5, or 10^6 cells/mL until the detection limit, the same concentration affected the cell count only minimally when an inoculum of 10^9 cells/mL was used. As such, although the same absolute number of cells might have been inactivated at a low and high inoculum, the induced inactivation appears to be much more pronounced at a low inoculum due to the nature of the data processing (traditional microbiological log transformation of the cell count).

4.1.2 Inactivation kinetics and enzymatic activity unit definition

The elimination of bacteria from suspensions was previously described to happen within seconds after the addition of endolysin (Loeffler et al., 2001). In the present work, it was found that the inactivation of cells by endolysin is not an instantaneous process, but can take up to 30 minutes or even longer to reach maximal inactivation (3.3.2.2). As such, the susceptibility of the cell population to HHP after the addition of endolysin might also change over time. Though, this was not investigated in the current work and further systematic investigations into the optimal endolysin coincubation times prior to HHP would be required to ensure maximum lytic activity from their combined application.

As was already pointed out by Nelson et al., 2012, the variation in assays between laboratories and arbitrary unit definitions often make comparisons of the lytic activity between endolysins difficult. Though, Briers et al., 2007a provided a standardized approach to quantify the activity of murein hydrolases in high-throughput assays and proposed a uniform standard definition expressed in ΔOD600/(min × µg). Although the method allows for a quick and objective determination of the

hydrolase activity, there is a fundamental issue with the proposed unit definition, since it defines the activity per mass and not mole (i.e. gram vs. number of enzyme molecules). Consequently, although two different endolysins molecules might cleave peptidoglycan bonds at the same rate, the endolysin with the lower molecular weight will appear to have a higher activity when measured per mass. The enzymatic activity expressed per mol enzyme provides a more accurate description and is therefore used in this work to determine the enzymatic activity of endolysins.

Despite the above argumentation, most viability plating assays were performed at equal mass concentrations. Since the molecular weight of the used *Listeria* phage endolysins is highly similar, their molar concentration is also more or less the same at equal mass concentration. In contrast, the molecular weight of the S*taphylococcus* phage endolysin HY-100 is almost twice that of HY-133. Since HY-100 and HY-133 were applied at equal mass concentrations in the viability plating assays, the molar concentration of HY-100 was only half that of HY-133. Nonetheless, a more or less equal antimicrobial activity was observed for HY-100 and HY-133 in the viability plating assays at equal mass concentration (3.2.1.3.1). This is most likely the result of the higher enzymatic activity of HY-100 as was determined by the turbidity reduction assay (3.1.2), which could compensate for the lower molar concentration applied. Although the above argumentation are important to take into account for the characterization and comparison of endolysins, their activity in buffer is only a first indication for the enzymes efficacy and does not guarantee antimicrobial activity in food products.

4.1.3 Non-enzymatic inactivation

Lysozyme can kill bacterial cells via a mechanism completely separate from its ability to cleave the peptidoglycan (1.4.2.2.3). Ibrahim et al., 2001b provided evidence that a lysozyme derived peptide with a helix-loop-helix structure is responsible for this non-enzymatic antimicrobial activity by the formation of channels in the cytoplasmic membrane. It was furthermore shown that the non-enzymatic bacterial inactivation by 6-15 amino acid long lysozyme derived peptide sequences is highly dependent on their positive charge and hydrophobic properties (Pellegrini et al., 1997). A disturbance of the outer membrane has also been found for catalytically inactive variants and short peptide sequences from the C-terminal domain of the *Bacillus* phage endolysin 1521 (Orito et al., 2004). Although the non-enzymatic bactericidal activity of endolysins was not examined closely in this work, online prediction tools based on the proteins amino acids sequences showed that all endolysins had a highly cationic nature (pI>9.6). Possible helix-loop-helix structures were also detected in the CBD of both PlyP40 and PlyP825 (data not shown). It is therefore interesting to speculate that the *Listeria* phage endolysins used in this work can kill cells both by enzymatic breakdown of the peptidoglycan layer as well as non-enzymatic destabilization of the cytoplasmic membrane.

Interestingly, a non-enzymatic lytic activity of endolysin might have crucial implications for studies towards the role of the CBD. In many studies, it has been shown that endolysin is almost completely devoid of lytic activity upon removal of the CBD moiety. It was logically concluded that the CBD is required to maximize lysis by elevating the local enzyme concentration and bringing the enzyme closer to its ligand. However, in the light of the above discussion, it could be speculated that a lower lytic activity upon absence of the CBD is not because the endolysin is not targeted to its substrate. Instead, the absence of antimicrobial peptide sequences conserved in the CBD might be responsible.

4.1.4 Eagle effect

An increase in the concentration of an antimicrobial substance generally results in an equal or higher inactivation of the microorganism. Although this was true for the *Staphylococcus* phage endolysins, the inactivation of *L. monocytogenes* by PlyP40 and PlyP825 only increased until a certain threshold concentration, after which higher endolysin concentrations led to a lower number of inactivated cells (3.2.1.2.1): a phenomenon referred to as Eagle effect.

In the work of Masschalck et al., 2001b, it was mentioned that "Higher concentrations of lysozyme were not tested, since these tended to cause aggregation of the bacteria, making the plate counts unreliable". Although the data referred to is not shown, this implies that these authors also observed unexpected effects when the efficacy of lysozyme was examined by plate viability assay and the aggregation of bacterial cells was put forward as explanation for this observation. Unfortunately, Masschalck and colleagues did not provide data for this argumentation and although it might be true for lysozyme, no aggregation of *L. monocytogenes* cells upon the addition of endolysin was observed in any of the microscopic investigations (3.2.2). Hence, any protective effect by the aggregation of *L. monocytogenes* cells upon higher endolysin concentrations was excluded as possible explanation for the Eagle effect observed in this work.

Two other hypotheses as possible explanation for the Eagle effect were investigated more closely (Figure 45): (i) the formation of stable protoplast due to a faster breakdown of the peptidoglycan layer at higher endolysin concentrations, and, (ii) the self-association/aggregation of endolysin at higher concentrations. Both hypothesis could explain the lower inactivation of cells at higher PlyP825/PlyP40 concentrations are discussed in more detail below.

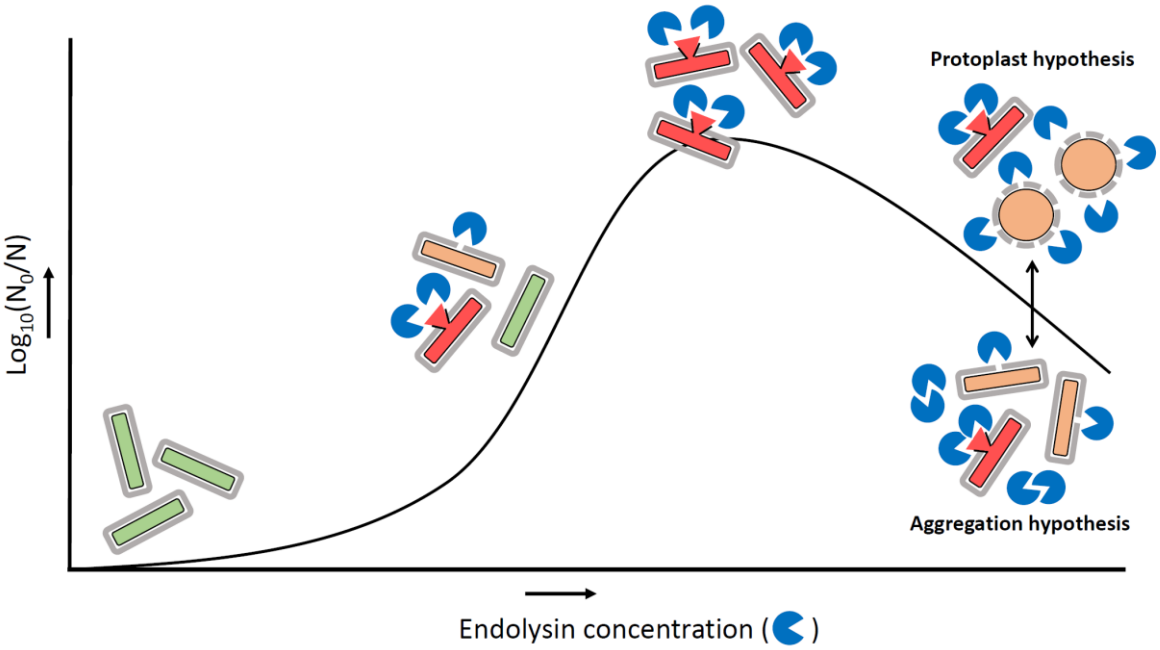


Figure 45: The Eagle effect. Schematic representation of the aggregation and protoplast hypotheses as possible explanation for the Eagle effect. Green colour indicates vital cells, orange impaired and red inactivated.

Protoplast hypothesis

A phase-contrast microscopic time-lapse showed the transformation of typical rod-shaped *L. monocytogenes* cells into spheres upon the addition of endolysin (Figure 22). The formation of spherical cells upon the addition of endolysin has been observed before for *Bacillus cereus* and *Bacillus*

anthracis cells and were described as 'ghost' cells: empty bacterial membrane vesicles without cytoplasm and DNA (Schuch et al., 2002, Fischetti, 2008). Masschalck et al., 2002 also microscopically observed the formation of protoplasts (the authors refer to it at spheroplasts; see text box "Protoplasts and spheroplasts") after incubation of *Lac. johnsonii* with lysozyme and showed by parallel viable cell counts that cells converted to protoplasts were simultaneously inactivated. This is in line with the work of Connell et al., 1988, which also found that protoplasts are generally not able to regenerate a cell wall and form colonies when plated.

Protoplasts and spheroplasts
Both spheroplasts and protoplasts refer to bacteria (or yeast) of which the shape-maintaining structure is weakened and a spherical shaped cell is formed (encyclopedia.com, 2003). Whereas protoplasts only have one membrane and refer to Gram-positive bacteria of which the murein layer is destabilized, spheroplasts refer to Gram-negative spherical bacteria with a disturbed murein layer and two membranes (inner and outer). The essential difference between protoplasts and spheroplasts is thus the presence of an additional outer membrane in the latter, which could however be removed to generate protoplasts from Gram-negative bacteria.

Nonetheless, LIVE/DEAD staining indicated that some of the *L. monocytogenes* protoplasts had an intact cell membrane (Figure 23). It was therefore speculated that the instant removal of the peptidoglycan layer by very high endolysin concentrations could result in the formation of stable protoplasts. Although this might be true, the epifluorescent microscopic observations in Figure 23 needs to be interpreted with caution. In this experiment, endolysin was added after cells were LIVE/DEAD stained and captured under a microscopic slide. In Figure 22, it was shown that *L. monocytogenes* cells are not instantaneously transformed into circular shaped cells with this approach, but that it happens over time and is thus a continuously changing system. As such, cells which lost their membrane integrity directly after the addition of endolysin will logically have bound more PI and a stronger fluorescent signal than cells which lost their membrane integrity shortly before microscopic analyses. Especially because the sample could not be mixed properly anymore and PI binding thus relied on its diffusion under the microscopic slide. The stronger PI fluorescent signal of cells which lost their membrane integrity directly after the addition of endolysin might blind the detection of those cells which were damaged/lysed in a later phase (and therefore have less PI bound and a less intense fluorescent signal). This would lead to the incorrect conclusion that circular shaped cells have an intact membrane. Unfortunately, this hypothesis could not be tested since monitoring the membrane integrity of cells in the same microscopic field for a longer time period was restricted due to photobleaching of the dye.

Despite the above argumentation, LIVE/DEAD staining indicated that the membrane of 'intact' protoplasts could be disturbed by a HHP treatment of minimal 200 MPa (compared to 400 MPa required to disturb the membrane of untreated *L. monocytogenes* cells without endolysin treatment; Figure 24), which nicely correlated with the inactivation of *L. monocytogenes* cells at 200 MPa after incubation with PlyP40 (Figure 20). It is therefore tempting to speculate that the additional inactivation achieved by a mild HHP treatment of 200 MPa after incubation with endolysin reflects the inactivation of protoplasts. Although this might be a valid statement, it is not in line with the protoplasts hypothesis as explanation for the Eagle effect; the additional inactivation by 200 MPa was observed after incubation with a relatively low PlyP40 concentration (0.16 µg/mL), whereas it was hypothesized that the formation of stable protoplasts only takes place at higher endolysin concentrations (>4 µg/mL). Moreover, protoplasts with an intact membrane were also observed at endolysin concentrations <4 µg/mL (Figure 21). Taken together, whereas the data discussed above might indicate that the synergy

between endolysin and HHP results from the inactivation of protoplasts (which is discussed in more detail in section 4.2.1), it does not support the protoplast hypothesis as explanation for the Eagle effect.

Apart from microscopic observations, viability plating assays also demonstrated that the formation of protoplasts might not be a suitable explanation for the Eagle effect. A higher concentration of endolysin was hypothesized to allow for the formation of stable protoplasts due to the instant removal of the peptidoglycan layer. In this light, lowering the number of cells at a fixed endolysin concentration, which increases the number of available endolysins per cell, should have a similar effect and also support the formation of protoplasts. Instead, less cells survived when the PlyP40 concentration was kept constant and the inoculum systematically lowered (Figure 29). Moreover, in another viability plating assay, it was observed that the Eagle effect diminishes with longer incubation times (Figure 30). After 30 minutes of coincubation with endolysin PlyP40, the inactivation by 0.8 µg/mL was higher than that by 20 µg/mL (Eagle effect). However, with longer incubation times, this effect diminished and eventually substantially more cells were inactivated by 20 µg/mL PlyP40. Although the formation of viable protoplasts after the addition of 20 µg/mL PlyP40 and their slow lysis over time might also explain these inactivation kinetics, the self-association of endolysin and slow release of monomers from these aggregates, which become available to lyse cells, might be a more suitable explanation.

Aggregation hypothesis

The presence of the Eagle effect against multiple strains and cells from different growth phases hints that the explanation for this phenomenon might not lie within a cellular event, but that intrinsic properties of the endolysins might be responsible. The decrease in lysozyme's enzymatic activity above a certain threshold concentration has been described to be the result of self-association (Figure 46; Hampe et al., 1982). In this work, a similar inhibition of the enzymatic activity at higher concentrations was observed for all endolysins (Figure 25), which was therefore speculated to be the result of self-association as well and examined using different approaches.

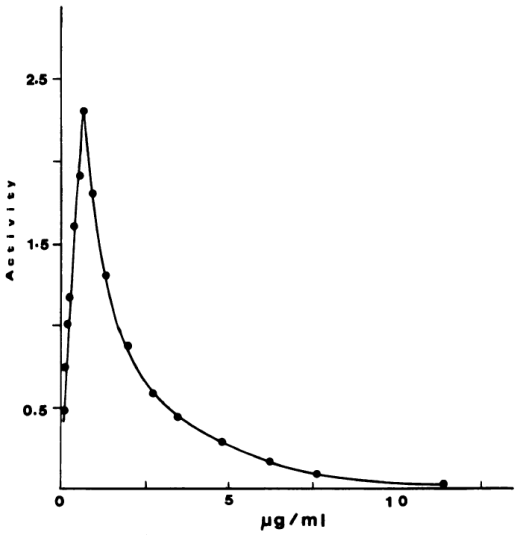


Figure 46: Effect of lysozyme concentration on lysozyme activitiy. Adapted with permission from Hampe et al., 1982.

Analysis of endolysin by acidic native page suggested self-association of endolysin PlyP825 and Ply511, but not PlyP40 (3.3.1.2). Protein aggregation is highly dependent on factors such as pH or temperature and slight changes can change this behaviour drastically. As such, whereas Ply511 and PlyP825 remained self-associated in the acidic native page, a change of the system from IPB to the acidic gel might have caused PlyP40 to dissolve into monomers. Nonetheless, the multiple bands observed in the acidic native page for Ply511 and PlyP825 demonstrate the potential of these enzymes for self-association.

In light of the aggregation hypothesis, the continued cellular inactivation kinetics by high (≥20 µg/mL) but not low (<4 µg/mL) concentrations of endolysin might be due to the release of monomeric endolysin from the self-associated complexes (Figure 30). Since endolysin is a one-time use enzyme (i.e. endolysin binds irreversible to the cell which reduces the concentration of monomeric endolysin), there might be a constant re-equilibrium from self-associated to monomeric endolysin, which results in the continuous lysis of cells over a longer time period.

No indications of PlyP825 aggregation could be detected by either UV-spectroscopy (3.3.1.3) or AF-4-MALS-UV (3.3.1.4). UV-spectroscopy is a relatively robust method and allows for the detection of larger aggregates. Since acidic native page analyses only indicated self-association of endolysin into di- or trimers, these complexes might be too small to scatter sufficient light and cause a noticeable change in the UV signal pattern. Moreover, large protein aggregates might even be macroscopically observable due to drastic turbidity increases, while the used PlyP825 protein solutions were transparent. With respect to the AF-4-MALS-UV analysis, it can be argued that the perpendicular flows caused a constant re-equilibrium, which diluted the endolysin and quickly led to the dissociation of self-associated complexes into monomers, precluding the possible detection of di- or trimers. Additionally, AF-4 has an efficient separating effect on macromolecules, which in this case might disturb and prevent the appearance of (initially) loosely associated PlyP825 oligomers in the AF-4 spectrum.

In summary, although microscopic observations indeed showed the formation of protoplasts with indications of an uncompromised cell membrane after incubation with endolysin, it remains highly questionable whether these would survive the hypotonic conditions of the buffer used (50 mM NaCl), regenerate a cell wall, and form colonies when plated. Additionally, discrepancies between endolysin PlyP40/PlyP825 and endolysin Ply511 where the Eagle effect is not visible can also not be easily explained by the protoplast hypothesis. Taken together, whereas all data discussed above could be supportive for the endolysin aggregation hypothesis and reasonable arguments can be given for those assays where no self-associated endolysin was detected, numerous data and insights from scientific literature contradict the protoplasts hypothesis.

4.2 The synergism between endolysin or bacteriophages with HHP

In the present work, a synergistic inactivation of Gram-positive bacteria was shown when HHP processing was combined with either endolysin or bacteriophages. The increased inactivation achieved by non-thermal processing techniques in combination with different antimicrobials has been defined before as synergism (Corbo et al., 2009) and used as such by multiple others (Ogihara et al., 2009, Patterson et al., 2011, Montiel et al., 2014). Although some also refer to this effect as partial enzymatic effect, sensitization, or simply additional inactivation, it was decided to clearly define the term synergy in this thesis (2.6) and refer to it as such for ease of discussion.

4.2.1 Endolysin

Masschalck et al., 2002 and Nakimbugwe et al., 2006b both showed that the peptidoglycan hydrolass lysozymes (from different origins) can make Gram-positive bacteria more susceptible to high pressure. The advantage of endolysins over lysozymes is that they are highly specific and much more unlikely to evoke resistance (1.4.2.4). A synergistic effect between HHP and endolysin has been shown before, though only once against the Gram-negative bacterium *P. aeruginosa* (Briers et al., 2008). In the paper by Van Nassau et al., 2017, which originated from this work, synergism between endolysins and HHP was demonstrated for the first time against Gram-positive bacteria.

Briers et al., 2008 found the onset of synergism between endolysin and HHP against a Gram-negative bacterium at pressure levels between 150-200 MPa. Interestingly, synergistic inactivation of PlyP40 treated *L. monocytogenes* cells started around the same pressure level (200 MPa; 3.2.1.2.4). The synergistic effect found by Briers and colleagues was in the range of 1.5–2.4 log cycles and argued to be the result of pressure-induced permeabilization of the outer membrane. This would allow the peptidoglycan hydrolases to reach its cellular target and lyse the bacterial cell. This mechanism has also been described for nisin in combination with HHP against Gram-negative bacteria and referred to as pressure-promoted uptake (Masschalck et al., 2000). Since Gram-positive bacteria do not have this natural shield (i.e. the outer membrane), the synergistic effect cannot be the result of pressure-promoted uptake and other mechanisms must be at play. In Figure 47, a variety of different mechanisms as possible explanation for the observed synergism are schematically illustrated. These different hypotheses are discussed in more depth below and can be divided as follow:

- Sensitization: incubation with endolysin makes the cells more susceptible to a subsequent HHP treatment (Figure 47a-c)
- Simultaneous: endolysin and pressure are required simultaneously for a synergistic inactivation (Figure 47d-g).

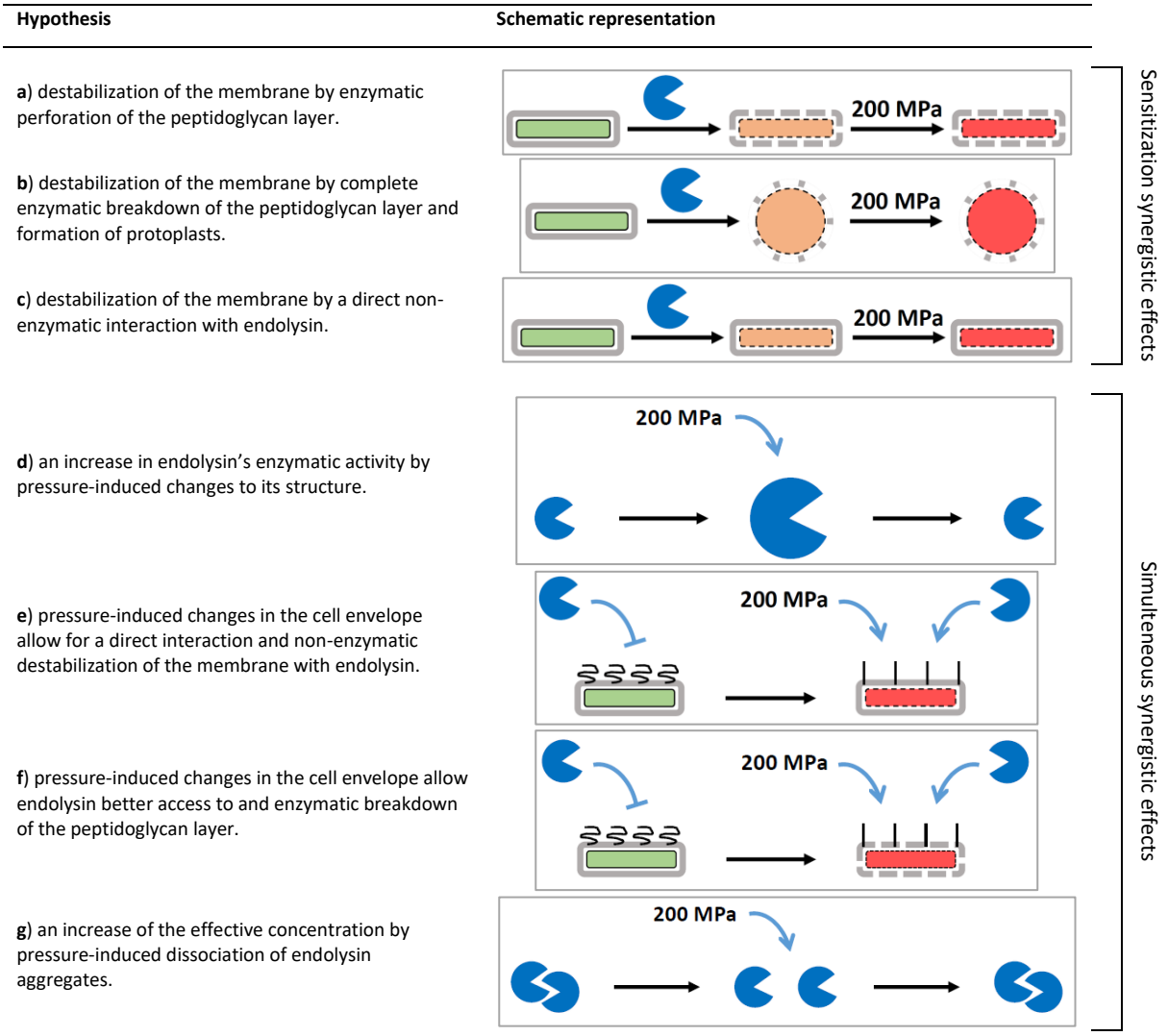


Figure 47: Hypothesis for the inactivation of Gram-positive bacteria by endolysin and HHP. Schematic representation of possible mechanism for the inactivation of *L. monocytogenes* by a combined application of endolysin and HHP.

Sensitization synergistic effects

In multiple studies, the existence of a physiologically heterogeneous population after incubation of cells with peptidoglycan hydrolases has been described (Connell et al., 1988, Mitchell et al., 2010). Generally, three physiological states are distinguished: completely vital cells, lysed cells, and compromised cells which remain intact although their cell walls are (partly) digested by the action of these enzymes. Several experiments throughout this work also indicated a physiologically heterogeneous population after incubation with endolysin. For example, it was demonstrated that there is a more pressure susceptible subpopulation after endolysin treatment, which can be readily inactivated at a lower pressure level (3.2.1.2.4). Different molecular processes might explain the increased susceptibility of this subpopulation.

A synergistic inactivation of Gram-positive bacteria by a combination of nisin and HHP has been demonstrated by multiple groups (Kalchayanand et al., 1998a, Chung et al., 2005, Jofré et al., 2008, Hereu et al., 2012). Ter Steeg et al., 1999 speculated that nisin increases the pressure susceptibility of the Gram-positive bacteria *Lac. plantarum* by binding to phospholipid head groups and local immobilization of the membrane. Interestingly, the efficacy of the *Staphylococcus* phage endolysin

LysH5 was also enhanced when it was applied in combination with nisin (García et al., 2010a), indicating that the action of endolysin caused additional membrane stress. For *E. coli*, disturbance of the cellular membrane is described as one of the main determinants for pressure inactivation of exponential- but not stationary-phase cells (1.3.2.1). Interestingly, the synergism between endolysin and HHP was found to be much stronger against exponential- than stationary-phase *L. monocytogenes* cells. These discrepancies between cells from different growth phases are another indication that membrane stress by (partial) breakdown of the peptidoglycan layer is responsible for the synergistic inactivation by HHP. In the latter scenario, synergism would be purely a physical effect: the membrane surface tension increases because it needs to compensate for the loss of stability normally provided by an intact murein layer. Further destabilization of the membrane by a pressure-induced reduction in membrane fluidity subsequently leads to cell death.

The exact state of *L. monocytogenes* cells after endolysin treatment and before their membrane is further destabilized by HHP remains unclear. The peptidoglycan layer might only be partly perforated which allows the cell to retain its typical rod-shape (Figure 47a), or, the stabilizing murein layer is completely lost which results in the formation of protoplasts (Figure 47b). The existence of protoplasts with an intact membrane and disturbance of their membrane by mild pressure was demonstrated microscopically in this work (Figure 24). However, others described that protoplasts are generally not able to regenerate a cell wall and form colonies when plated (Connell et al., 1988, Masschalck et al., 2002) and similar microscopic observations have been described before as ghost cells (Fischetti, 2008). Although *L. monocytogenes* cells could be readily inactivated after endolysin treatment, endolysin alone did not cause sublethal damage (as could be examined using high salt agar plates; Table 22), also not at endolysin concentrations where protoplasts were microscopically observed. This further strengthens the assumption that microscopically observed protoplasts after endolysin treatment were not viable. Metabolic studies might however give definite proof to this respect. Taken together, it is however rather unlikely that the pressure inactivation of protoplasts is responsible for the observed synergism.

The extent of destabilization induced by peptidoglycan hydrolases on the murein layer has been described to be dependent on the type of peptidoglycan bond cleaved (Nelson et al., 2012). Whereas HY-133 and lysostaphin caused sublethal damage, treatment by HY-100 did not (just like the listeria phage endolysins). Interestingly, whereas HY-133 and lysostaphin only cleave the interlinking glycan peptide bridge, HY-100 has an additional catalytic domain with amidase activity. This indicates that cleavage of the *L. monocytogenes* peptidoglycan generally, and specific hydrolysis of the MurNAc-L-Alanine bond in peptidoglycan of *S. aureus* (separating the glycan strand from the stem peptide), might result in a different type of stress than cleavage of the peptide bridge present in the *S. aureus* peptidoglycan. Paradoxical, the largest synergistic effects were observed for those endolysins which did not cause sublethal damage of cells (HY-100 and all *Listeria* phage endolysin). This implies that the type of peptidoglycan bond cleaved determines the type of cellular stress and thereby different pressure susceptibilities and thus synergistic effect.

Another mechanism which might be responsible for membrane destabilization is of non-enzymatic nature (Figure 47c). Several studies have shown that enzymatically inactivate lysozyme still has a bactericidal effect mediated through a direct lysozyme interaction with -and disturbance of- the cytoplasmic membrane (1.4.2.2.3). This non-enzymatic bactericidal activity of lysozyme has also been indicated to work synergistically with HHP (Masschalck et al., 2002, Nakimbugwe et al., 2006a).

Interestingly, other antimicrobials which work synergistically with HHP against Gram-positive bacteria have also been described to disturb the cytoplasmic membrane. For example, Ter Steeg et al., 1999 hypothesized that the peptide bacteriocin nisin binds to the membrane surface phospholipid head groups (as part of the pore formation process) which causes local immobilization. This was argued to decrease membrane fluidity and make the cell more susceptible to HHP. A non-enzymatic antimicrobial activity has however not been studied in depth for endolysin. Until now, it has only been shown that a catalytically inactive form of the *Bacillus* phage endolysin lys1521 can permeabilize the outer membrane of the Gram-negative bacterium *P. aeruginosa* (Orito et al., 2004). It is therefore interesting to speculate that the endolysins used in this work can interact and damage the cellular membrane independent from their enzymatic activity, but further studies will be required to investigate such effects.

Simultaneous synergistic effects

The synergistic inactivation by endolysin and HHP at lower pressure levels implicates that these cells are in a more vulnerable state (e.g. a compromised cell membrane). It is therefore rather surprising that synergism is strongest for those combinations where no sublethal damage was detected (i.e. for cells treated with *Listeria* phage endolysins and HY-100). This could either mean that NaCl as selective agent could not inhibit growth of endolysin damaged cells or that a different mechanism is at play. For example, pressure might increase the enzymatic efficacy of endolysin, either by changes in the protein structure (Figure 47d), the cell envelope (Figure 47e and f), or by dissociation of endolysin aggregates (Figure 47f). Endolysin aggregation was previously discussed as a likely explanation for the observed Eagle effect and high pressure has been described to dissociate protein aggregates (Meersman and Heremans, 2008a). The pressure-induced dissociation of endolysin aggregates might therefore be a valid explanation for the observed synergism. However, when endolysin was added to an isolated cell wall suspension and subsequently pressurized at 300 MPa (10 min, 30 °C), no differences in OD_{600} between the pressurized and the sample kept at atmospheric pressure could be observed (3.1.3: activity during pressure treatment). This indicates that neither the dissociation of aggregates under pressure nor an increase in endolysin's enzymatic activity by pressure-induced changes to its structure are responsible for the synergistic inactivation by a combined application of endolysin and HHP. However, since the activity of endolysin during pressure was only examined against isolated cell wall, it cannot be excluded that the efficacy of endolysin is increased due to structural changes in the cell wall envelope during pressure, for example due to better accessibility to the cellular membrane followed by non-enzymatic destabilization (Figure 47e) or to the peptidoglycan layer and its enzymatic breakdown (Figure 47f).

4.2.2 Bacteriophages

The application of phage P100 for the elimination of *L. monocytogenes* has been investigated by others in different foods and in combination with multiple other antimicrobials (1.4.1.2). Although the combination of phages with HHP has been investigated before (Tabla et al., 2012), this work is the first to investigate the combination of a bacteriophage preparation approved as a food processing aid in combination with HHP (3.5.2). In buffer, the cell count could be reduced 5.5 log cycles by a combined phage (10^8 pfu/mL) and HHP (400 MPa) treatment, whereas the additive effect was only 2.7 log cycles (i.e. a synergistic inactivation of 2.8 log cycles). This shows that a strong synergistic inactivation between phages and HHP is also possible, though synergy was highly dependent on the medium used and parameters applied (i.e. buffer or milk, phage concentration, inoculum, and pressure level).

The higher pressure inactivation of cells by prior incubation with phages indicates that there was a subpopulation of cells which became more susceptible to pressure, for example by phage-induced damage to the cell envelope upon infection. This could result from pores formed by the phage to inject its genome into the cell, or, by phage-induced production of cytoplasmic proteins such as holin and endolysin which normally allow for the release of progeny phages. Both mechanisms might cause destabilization of the cellular membrane, one of the main targets of bacterial pressure inactivation, and thereby increase the pressure sensitivity of cells. Alternatively, pressure might increase the efficacy of the phage lytic cycle by, for example, inducing structural changes in the cell envelope which allow for better cell recognition/adsorption or easier penetration of the phage's genome into the cell.

Surprisingly, the observed synergism between phages and HHP processing was independent from the order in which techniques were applied; i.e. a synergistic inactivation in the same order of magnitude was observed when phages were added after HHP processing (3.5.3). In line with the different hypotheses for the synergistic effect between phages and pressure discussed above, it might be that HHP processing causes a more permanent change to the cell membrane or wall, which results in cell death upon further destabilization of the membrane by phage infection or allows for better cell recognition/adsorption/penetration by the phage and thereby increases the efficacy of the lytic cycle.

Similar as was extensively discussed for endolysin (4.2.1), the synergy between bacteriophages and HHP might thus also result from a disturbance of the cytoplasmic membrane or cell wall. Though, differences in the antimicrobial mechanism of action between these two antimicrobials are also present. This is, for example, reflected by the effect of the inoculation level on the efficacy of a predetermined endolysin or phage concentration. Whereas a reduction of the inoculation level at a fixed endolysin concentration resulted in a much higher log reduction (3.3.2.1), it had a much smaller effect on the inactivation of cells by bacteriophages (3.5.1.1). This illustrates that the process by which bacteriophages disturb and inactivate Gram-positive bacteria is highly different from that of endolysin; whereas the latter causes 'instant' breakdown of the peptidoglycan layer and lysis of the cell, killing by phages precedes a complete infectious cycle. This is an important difference which needs to be considered when one tries to understand the inactivation of bacteria by the combination of these antimicrobials with HHP.

In the present work, it was shown for the first time that addition of phages after HHP treatment can also result in the synergistic inactivation of Gram-positive bacteria. Both pressure-induced membrane damage or an increased phage efficacy due to (more permanent) changes in the cell envelope might be responsible for this increase. Although this approach might provide interesting insights on the more permanent effects of HHP processing on the cell envelope, the additional use of phages after HHP processing is not relevant for its application in industry since it is a post-packaging preservation technique and the addition of phages after pressure treatment would only increase the risk of recontamination.

4.3 Application in foods

HHP processing is used as a preservation technique for a range of different food products. For sensitive foods such as fish products, the pressure levels required for the inactivation of pathogenic bacteria might frequently be too high to avoid detrimental effects on product quality. The hurdle concept of food preservation can be applied to increase the antimicrobial efficacy of HHP processing and allow for milder pressure parameters.

The efficacy of lysozyme alone or in combination with HHP has been studied in different foods. Although several endolysin have also been studied extensively, most work was done in buffer systems and only very few studies investigated the efficacy of endolysin in actual foods. Moreover, the only study which investigated the combination of endolysin with HHP against a Gram-negative bacterium was also performed in buffer. The efficacy of bacteriophage P100 has, unlike for endolysins, been investigated in many different foods, but never in combination with HHP. In the present work, the combined application of endolysin or bacteriophage P100 with HHP processing for the inactivation of *L. monocytogenes* was therefore examined in several *Listeria*-critical food products.

4.3.1 Endolysin

The application of endolysin PlyP825 alone or in combination with HHP for the inactivation of *L. monocytogenes* in milk, mozzarella, and smoked salmon was examined by both challenge lethality as well as storage tests. Although the results highly differed per food product and assay, it could be shown that the addition of PlyP825 allows for a synergistic inactivation of cells, a reduction in pressure level with equal antimicrobial efficacy, and can eliminate *L. monocytogenes* from food during storage at abuse temperatures.

Like many other antimicrobial substances and food preservation techniques, the efficacy of the combined endolysin and HHP application was substantially lower in foods compared to buffer. For example, whereas the combination of 0.16 µg/mL PlyP825 and 300 MPa at 30 °C for 1 min was sufficient to reduce the cell count of a *Listeria* suspension by ca. 5 log cycles in buffer (3.2.1.2.1), a PlyP825 concentration of 34 µg/mL in combination with a pressure treatment of 400 MPa at 25 °C for 10 min was required to achieve a similar inactivation in milk (3.4.2.1). Although this might have resulted from differences in the growth phase of cells examined or the use of a single strain versus a cocktail, the reduced efficacy of antimicrobial treatments in foods compared to buffer has been extensively documented for both for lytic enzymes as well as HHP processing and is more likely the result of differences in intrinsic properties (e.g. ionic strength, water activity, pH, etc.) or food matrix. The latter is not only generally accepted as one of the main causes for the reduced pressure susceptibility of cells in solid compared to liquid foods, but was also described as one of the main determinants for bacteriophage efficacy in foods (Guenther et al., 2009). The specific food environment, especially in liquids versus solid products, is a large determinant for the distribution of endolysin towards the cell. The spatial distance between antimicrobials and bacteria on solid foods was discussed before as one of the main reasons for the inefficacy of phage treatment in solid foods (1.4.1.2), and, is likely to be one of the main determinants for the inefficacy of endolysin in smoked salmon as well. Moreover, differences in the inoculation procedure and application of endolysin (e.g. in solution versus spot inoculation) also affect endolysin's efficacy, which further complicates and precludes a direct comparison between the different foods.

Generally, the greater the pressure level and time of application, the greater the potential for changes in the appearance of selected foods. Therefore, substances which allow for a reduction in pressure level while achieving the same antimicrobial activity are desirable, especially for pressure-sensitive foods such as smoked salmon. In buffer, it was demonstrated that prior incubation with endolysin allows for the inactivation of *L. monocytogenes* cells at lower pressure levels (3.2.1.2.4). Also in milk, prior incubation with endolysin allowed for a reduction in pressure level. For example, neither PlyP825 nor 200 MPa affected the cell count when applied individually, but were equally efficient as a HHP

treatment of 400 MPa when combined (3.4.3.1). This perfectly demonstrates the potential of applying the hurdle principle with endolysin and HHP in foods.

The efficacy of the combined endolysin-HHP method was highly dependent on the inoculation level used. In the milk storage tests, the same treatment parameters were much less efficient when a higher inoculum was applied (3.4.2.2.1 versus 3.4.2.2.2). Similarly, discrepancies between milk challenge lethality (high inoculum) and storage tests (low inoculum) at equal endolysin concentrations were observed; whereas 34 µg/mL PlyP825 was sufficient to eliminated *L. monocytogenes* in most samples during storage tests (3.4.2.2.2), the same concentration only reduced the cell count minimally in the challenge lethality test where a much higher inoculum was applied (3.4.2.1). Taken together, it was demonstrated in foods (and previously in buffer) that the efficacy of endolysin is highly dependent on the number of cells it is applied to, a finding which challenges the conventional approach for the evaluation of novel food preservation techniques. Although the standard requirements to examine novel food preservation techniques includes both challenge storage and lethality tests, it can be questioned whether the latter provides a suitable method to evaluate antimicrobials for which the ratio between the amount of substance applied and the number of bacterial cells is of crucial importance for the efficacy of the method. Although challenge lethality tests are appropriate to evaluate the extent of reduction by process lethality steps such as heat or HHP processing, it might underestimate the efficacy of antimicrobials like endolysin and lead to the incorrect conclusion that the treated formulation is microbiologically not stable. A possible solution for the evaluation of endolysin's efficacy might be to normalize the amount of endolysin per cell. This would provide an alternate form of the minimum bactericidal concentration, which relates the antimicrobial's efficacy to both dose and number of exposed microorganisms. This approach might provide a more valid measure for antimicrobials, such as endolysin, which efficacy is dependent on both dose and number of cells it is applied to.

The complete absence of *L. monocytogenes* in food is an absolute requirement from both a safety and regulatory perspective; the United States even have a "zero tolerance" policy towards *Listeria* (USDA FSIS, 2014). In light of the discussion above, the contamination level is of crucial importance to determine endolysin and HHP process parameters required to eradicate *L. monocytogenes*. Although natural contamination levels are likely to highly vary, 10^2-10^3 cells/g product is typically recommended as inoculation level to ascertain the microbiological stability of a food formulation (FDA, 2001, Beuchat et al., 2003). Although the inoculum of challenge storage tests performed in this work was usually somewhat higher, statements about product safety and process parameters can be provided within the framework of this study. The complete elimination of *L. monocytogenes* by endolysin and HHP was not achieved in any of the storage tests. However, in milk and mozzarella, the combined application of 3.4 µg/mL PlyP825 and 400 MPa was sufficient to prevent growth of *L. monocytogenes* in 89% (milk) or 78% (mozzarella) of the samples during storage (Figure 35 and Figure 38). The same pressure conditions in combination with 10-fold the PlyP825 concentration (34 µg/mL) was shown to reduce the cell count of a ~7 log culture by 4-5 log cycles in both products (Fig. 34 and 37). Considering the higher efficacy of endolysin against a lower cell numbers (see discussion above), this combination might provide process parameters which completely eradicate a lower more realistic *Listeria* contamination level from milk and mozzarella, though this will need to be confirmed in further studies.

The survival and growth of *L. monocytogenes* in foods over time was determined by plating on *Listeria* selective agar. In many samples, no viable cells could be detected. Although this might indicate that

L. monocytogenes was completely absent, especially after longer storage time at abuse temperature, the survival of *L. monocytogenes* cannot be excluded because of the detection limit of the viability plating assay. Although this assay is a valid method for the detection of listerial growth, it is also one of the main limitations of this work, since even very low levels of *L. monocytogenes* have been indicated in outbreaks and a complete elimination of this pathogen is therefore required. To confirm the complete absence of *L. monocytogenes*, one needs to culture a larger quantity of the food sample in selective enrichment broth and subsequently check for the presence of *Listeria*. Sample size in this work was however restricted by the size of the pressure vessels and did not allow for such analyses.

Other limitations of this work are also related to the experimental procedure of the assays. Although the application of endolysin and HHP was examined in real foods, most experiments were performed at RT, which does not reflect their possible application in industrial food processing. In possible follow-up studies, their application will need to be examined as an integrated part of the food production process. Moreover, the growth of cells in optimal medium and their inoculation onto food samples might not represent the state of cells during natural contamination, but this is intrinsically related to the examination of preservation methods. In addition, lower, more realistic inoculation levels should be used to get an optimal picture of the methods efficacy (10^2-10^3 cells per gram food).

In conclusion, it was demonstrated that the combined application of endolysin and HHP can provide a wider margin of safety for the control of *L. monocytogenes* in foods. In addition, with the limited set of parameters examined, it was already shown that *L. monocytogenes* could be (partly) eradicated during storage. As such, further fine-tuning of parameters might allow for its complete elimination. Moreover, the present works demonstrated the elimination of food pathogens at mild pressure levels, which promises great potential for its application in pressure-sensitive foods. The efficacy of the combined method was however highly dependent on the food vehicle examined, and its application therefore needs to be individually optimized for every single product with its particular characteristics, food processing steps, and storage conditions.

4.3.2 Bacteriophage

The inactivation of *L. monocytogenes* by phage P100 alone or in combination with HHP was examined in buffer and milk. In buffer, depending on the parameters examined, a reduction in cell count of >5 log cycles and synergistic effect of almost 3 log cycles was observed. In milk, both the reduction in cell count as well as the synergistic effect were much lower. At the most stringent conditions tested (400 MPa and 10^8 pfu/mL), the cell count was reduced only 2.3 or 1.4 log cycles, depending on the inoculum used (10^4 or 10^7 cells/mL, respectively). Although the antimicrobial efficacy of the combined method in milk was minimally additive, synergy never exceed 0.4 log cycles. The above findings are in line with other studies, which showed that HHP inactivation of *L. monocytogenes* (and other microorganism) is generally much less efficient in food products (including milk) compared to buffer systems (1.3.2.3.3).

Simpson and Gilmour, 1997 and Schnabel, 2015 showed that a higher fat content (in emulsions) can enhance the pressure resistance of *L. monocytogenes* and *C. botulinum* type E endospores, respectively. In the present work, no effect of fat content on the inactivation of *L. monocytogenes* by phages, HHP, or their combined application could be demonstrated in milk (Figure 44). The phage and HHP resistance of *L. monocytogenes* was however higher in milk compared to buffer, also in the sample with zero percent fat, which indicates that other components present in the milk's water phase (e.g. proteins, sugars, minerals, etc.) are responsible for the enhanced resistance. This could be either

mediated through a direct protective effect on the cell or by a direct interaction and inhibition of the phage's antimicrobial efficacy.

There is only a single study which examined the combination of phages with HHP against a Gram-positive pathogen (Tabla et al., 2012). In this study, the authors applied a phage cocktail (phiIPLA35 and phiIPLA88) alone or in combination with HHP against *S. aureus* cells in milk and determined the viable cell count at different time-points after treatment (up to 48 hours). Directly after treatment (similar to our experimental protocol), the application of a phage cocktail (10^8 pfu of each phage/mL), HHP (400 MPa, 10 °C, 5 min), or their combination only reduced the cell count minimally (<1 log cfu). However, when samples were analysed 48 hours after pressure treatment, the cell count was reduced until the detection limit by the combined phage-HHP treatment (compared to substantial growth in samples treated with only phages or HHP). In the present work, the cell count was only determined directly after treatment. In light of the study by Tabla et al., 2012, the efficacy of the combined phage P100 and HHP application against *L. monocytogenes* in milk might thus be severely underestimated in this work and challenge storage tests with realistic inoculation levels (i.e. 10^2 – 10^3 cells/mL) could provide further insights.

4.4 General considerations, future perspectives, and conclusion

4.4.1 Endolysins for control of foodborne pathogens

For the application of endolysin in foods, several other aspects next to its antimicrobial activity need to be considered. For example, endolysin production costs are expected to be relatively high with current technologies (Oliveira et al., 2012). Though, this is argued to be an acceptable challenge for the application of endolysin, since many different enzymes used in the food industry are produced cost-effectively on large scale. However, technological developments for more efficient endolysin production would be required to make its use as food control agent financially appealing.

Another aspect which needs to be considered is consumer safety and acceptance. Although endolysins have already been successfully applied in several animal infection models of human disease (as reviewed by Nelson et al., 2012), more comprehensive and systematic investigations into the safety of these enzymes for human consumption will be required for its approval. To this respect, legislative approval for the use of endolysin on foods might pose one of the biggest challenges. Whereas phages are considered a natural product and starting to find acceptance either as additive (USA) or food processing aid (EU; Perera et al., 2015, Whitworth, 2016), endolysins are purified from genetically modified organisms, which increases the hurdles in their approval process. Despite considerable progress in recent years, the use of genetically modified organism for the production of enzymes that are added to food either as food additive or processing aid, are only slowly finding legislative approval (EFSA, 2014). Similarly, the classification of endolysin as either food processing aid or additive could greatly influence consumer acceptance. Since the production of microbiologically safe and clean label food products is one of the main advantages of HHP processing, classification of endolysin as food additive would also limit interest for their combined application. Although the concern about genetically modified food is slowly declining (Deloitte, 2010), only about one decade ago consumers were still most concerned about genetic modification among several other food processing techniques (Wright et al., 2007).

A final but very important consideration for the use of phages or their lysins as food antimicrobials is resistance development. Fister et al., 2015 isolated multiple phage P100 resistance strains from an Austrian dairy plant where the phage was experimentally used. This illustrates the importance for the intelligent use of phages as biocontrol agents of food pathogens, for example by the use of phage cocktails and rotation schemes of cocktails with different phage compositions. For endolysins, the development of bacterial resistance is, compared to antibiotics or phages, much rarer. Despite extensive efforts of several groups, a less susceptible mutant strain has only been isolated once until now (1.4.2.4). Nonetheless, to ensure long-term efficacy beyond what can be tested on a laboratory scale, similar strategies as mentioned above for the application of phages will be required. The combination of multiple endolysins with different cleavage specificities is usually put forward as strategy to prevent resistance development. Although this might reduce the risk on resistance development, the only endolysin resistant mutant strain ever isolated directly demonstrated an increased resistance towards a whole range of peptidoglycan hydrolases with different enzymatic targets (Schmelcher et al., 2015), which indicates that the use of endolysin cocktail does not eliminate the risk of resistance development. Nonetheless, the use of a combination of different endolysins is recommended because it might provide additional other benefits as well. For example, several groups have shown a synergistic inactivation of target bacteria by the combination of different peptidoglycan hydrolases (Loeffler and Fischetti, 2003, Becker et al., 2008). In the current work, the combination of different *Listeria* phage endolysin merely resulted in an additive but not synergistic effect (3.4.1.2). Although PlyP40, Ply511, and PlyP825 cleave different bonds, their CBD all specifically recognize the peptidoglycan backbone structure of *Listeria*, which might have restricted any synergistic effects. Although the combination of endolysins with different binding targets might thus be desired to achieve maximum lytic activity, highly variable structures such as the peptidoglycan's interlinking peptide sequence should be excluded as possible binding target because of the increased risk on resistance development, which limits the binding targets of interest to the highly conserved polysaccharide backbone and N-acetylmuramoyl-L-alanine amide bond. In conclusion, to reduce the risk on resistance development, cocktails containing endolysins with both different enzymatic as well as conserved binding targets are recommended.

4.4.2 Endolysin as biological tool to study the role of the cell wall

The present work investigated the direct application of endolysin to food products. The antimicrobial properties of endolysin have also been investigated as enzybiotic or in food processing environments against biofilms, while the high affinity CBD is being exploited for the rapid detection of pathogens. In several experiments throughout this work, the combined application of endolysin and HHP caused a major reduction in cell count, whereas individual treatments hardly had any effect. This synergism was discussed before to be the result of a partial breakdown of the peptidoglycan layer, which destabilizes the cytoplasmic membrane and makes the cell more susceptible to pressure (4.2.1.). This work thereby indicates that the peptidoglycan layer plays a major role in pressure resistance. Although the mechanistic background of bacterial high pressure inactivation has been extensively studied in the last century, the role of this layer in pressure resistance has been mostly neglected, which is somewhat surprising while the peptidoglycan layer is commonly put forward as the main cellular component responsible for the difference in pressure resistance between Gram-positive and -negative bacteria (Hogan et al., 2005). Considering the above, endolysin might find another interesting application as biotechnological tool to study the mostly unexplored role of the peptidoglycan layer in bacterial

pressure resistance. Modular endolysins, with their highly diverse cleavage target sites, binding properties, and the ability to swap domains creating enzymes with desired characteristics, would provide a perfect toolbox to examine the role of the murein layer in bacterial pressure resistance.

5 SUMMARY

There is an increasing consumer demand for safe and high-quality food products, which are minimally processed and additive-free. Despite industry efforts (for example by the implementation of quality control and hygiene models), contamination of food by bacterial pathogens remains an ongoing challenge. This calls for novel and innovative preservation methods which do not adversely impact product quality and nutritional attributes, but allow for the control of pathogenic microorganisms in foods.

HHP processing provides a promising alternative to conventional preservation techniques to eliminate microorganism with minimal impact on the product. However, pressure treatments alone, i.e. without other hurdles such as low pH or mild heat, are usually not sufficient to control pathogens without a negative effect of on the food product, especially in more pressure-sensitive foods. Hence, the inactivation of food pathogens by HHP has been extensively studied in combination with various antimicrobials. Although the combination of endolysin and HHP might provide a promising approach for the control of pathogens in food, this combination has never been investigated for the inactivation of Gram-positive bacteria. Similar, although the application of phages as biocontrol agent in food has been extensively studied and found acceptance by the food industry in the last decade, only a single study investigated their combination with HHP. The present work therefore highlights the combination of endolysin or bacteriophages with HHP processing for the inactivation of Gram-positive bacteria.

Inactivation of the Gram-positive bacteria *L. monocytogenes* and *S. aureus* by endolysin, HHP, or their combination was screened in buffer to investigate possible synergisms. By systematic variation of different endolysins (PlyP40, Ply511, and PlyP825 against *L. monocytogenes*; HY-100, HY-133, and lysostaphin against *S. aureus*), the endolysin concentration, and multiple strains per species, it could be demonstrated that a substantial synergistic inactivation can be achieved when endolysin is added prior to HHP processing. Although this strong synergistic effect could be demonstrated against both *S. aureus* and *L. monocytogenes*, it was most pronounced for the latter. A treatment with only 0,16 µg/mL endolysin or only 300 MPa did not affect *L. monocytogenes* cell count, but their combination reduced the number of cells by up to 5 log cycles. This strong synergistic effect could be demonstrated for all *Listeria* phage endolysins tested against different *L. monocytogenes* strains. Moreover, the addition of endolysin did not only enhance pressure inactivation, but also allowed for the inactivation of *Listeria* at reduced pressure levels (i.e. 200 MPa), which is especially promising for pressure-sensitive foods where higher pressure levels deteriorate food quality.

Since the mechanistic background of the synergistic inactivation by endolysin and HHP was unknown, several hypotheses were put forward and investigated. It is generally known that pressure can both enhance or negatively impact the activity of enzymes. Therefore, possible effects of HHP processing on the enzymatic activity of endolysin were examined with turbidity reduction assays. However, no changes in the activity of endolysin could be detected, neither during HHP processing nor a more permanent effect after pressure treatment. Instead, the synergy between these two methods is more likely the result of endolysin-induced damage to the bacterial cell. Prior incubation of cells with PlyP40 allowed for the inactivation of *L. monocytogenes* at reduced pressure levels, which indicates that endolysin sensitized the cells to pressure stress. The existence of such a damaged more vulnerable state is most strongly reflected in experiments where bacterial inactivation by endolysin was followed over time. At defined concentrations, endolysin initially caused a reduction in the viable cell count, but

with longer incubation times, cells could recover to a level almost equal to the control. This indicates that cells were in a damage state shortly after the addition of endolysin, but could recover to form colonies over time. In this damaged state, cells are likely to be more vulnerable towards other stressors, including pressure. Although other hypotheses for the damaged state and increased pressure susceptibility have been discussed, it most likely results from a perforation of the peptidoglycan layer, which destabilizes the membrane, after which cells can be readily inactivated at lower pressure levels.

To validate the applicability of endolysin and HHP as food preservation technique, their combination was examined in several *Listeria*-critical foods (i.e. milk, mozzarella cheese, and smoked salmon) by both challenge lethality and storage tests. The antimicrobial activity of the combined method was generally lower in foods compared to buffer. Nonetheless, incubation with endolysin prior to HHP also allowed for a synergistic inactivation of *Listeria* in foods and a reduction in pressure parameters with equal antimicrobial effect. Although no complete eradication of *L. monocytogenes* was achieved in any of the foods at the parameters examined, the combination of 3.4 µg/mL PlyP825 and 400 MPa reduced the number of *Listeria*-positive milk samples during storage to only 2 out of 18, whereas all samples were positive after individual endolysin or pressure treatment. Also at a pressure level of only 200 MPa, the number of *Listeria*-positive milk samples could be substantially reduced by prior incubation with PlyP825. Like milk, prior incubation of mozzarella samples with PlyP825 enhanced pressure inactivation and further reduced the number of *Listeria*-positive samples, though not to the same extent. Moreover, whereas 200 MPa was sufficient to further reduce the number of *Listeria*-positive milk samples previously treated with PlyP825, a minimal pressure level of 400 MPa was required in mozzarella samples (a difference which might be solely attributed to the lower activity of PlyP825 in mozzarella). Unfortunately, no synergism between endolysin and HHP processing could be detected for the inactivation of *L. monocytogenes* in smoked salmon, which could have resulted from differences in both the specific food matrix as well as the experimental protocol between foods. Nonetheless, the work in milk and mozzarella demonstrated that the elimination of Gram-positive food pathogens by HHP is possible at reduced pressure levels when combined with endolysin, which promises great potential for its application in pressure-sensitive foods.

Surprisingly, the antimicrobial activity of endolysin PlyP40 and PlyP825 in buffer declined above a certain threshold concentration, a phenomenon referred to as Eagle effect. The formation of protoplast and endolysin aggregation were examined in this work as possible explanations for this phenomenon. Viability plating assays indicated that the instant removal of the peptidoglycan layer by high endolysin concentrations and formation of viable protoplasts is unlikely to be responsible for the decrease in endolysin's lytic activity. On the other hand, a combination of protein analytical methods and viability plating assays indicated an equilibrium between monomeric and self-associated multimeric endolysin, a state which initially reduces the antimicrobial activity of endolysin causing the observation of an Eagle effect, but where monomeric variants are slowly released and continue to lyse cells. As such, the results obtained in this work are supportive for the aggregation hypothesis as explanation for the reduced activity of endolysin PlyP40 and PlyP825 at higher concentrations.

Not only endolysin, but also bacteriophage P100 was investigated in combination with HHP processing for the inactivation of *L. monocytogenes* in buffer and milk. Like endolysin, synergistic effects were detected but highly dependent on the inoculation level, concentration of the antimicrobial, and medium used. Whereas a substantial synergistic effect between P100 and HHP was present in buffer

(up to 3 log cycles), only additive or minimal synergistic effects were found in milk at the parameters examined. This difference can most likely be attributed to the lower activity of phage P100 in milk compared to buffer and stronger synergistic effects are expected in milk upon a further increase in the phage concentration. Moreover, other studies indicated that the antimicrobial efficacy of a combined phage and HHP treatment drastically increases with incubations times longer than was examined in this work, which indicates that the effectivity of a combined phage-HHP application is highly underestimated in the present work. The synergy between phage P100 and HHP was initially speculated result from phage-induced damage to the cell envelope, but was equally strong when the order of application was reversed (i.e. the addition phages after pressure treatment), indicating that other mechanisms such as more permanent pressure-induced changes to the cell wall or membrane must be involved.

In conclusion, the results presented in this work demonstrate for the first time that endolysin can enhance the inactivation of Gram-positive bacteria by HHP processing and allows for the elimination of food pathogens at milder pressure parameters. The combination of these two hurdles thereby provide a wider margin of safety for the control of *L. monocytogenes* in foods during storage with subordinate effect on food quality attributes. Moreover, general knowledge on the inactivation of Gram-positive bacteria by endolysin and bacteriophages, the synergistic effect of these antimicrobials in combination with HHP, and the role of the cell wall in pressure resistance was considerably enhanced.

6 ZUSAMMENFASSUNG

Die Nachfrage nach sicheren und qualitativ hochwertigen Lebensmitteln, welche zugleich nur minimal verarbeitet und frei von Zusatzstoffen sind, steigt stetig. Trotz großer Bemühungen der Industrie, sichere Lebensmittel zu produzieren (z. B. durch das Implementieren von Qualitätskontrollen und die Erhöhung von Hygienestandards), ist das Risiko einer Lebensmittelkontamination durch pathogene Bakterien allgegenwärtig. Die Entwicklung innovativer Konservierungsmethoden, welche die mikrobielle Stabilität von Lebensmittel garantieren, die Produktqualität und den Nährwert des Lebensmittels jedoch nicht negativ beeinflussen, wird dabei angestrebt.

Das hydrostatische Hochdruckverfahren (HHD) ermöglicht es, Mikroorganismen mit nur minimalen Auswirkungen auf die Produktqualität effektiv abzutöten und stellt somit eine vielversprechende Alternative zu konventionellen Konservierungsmethoden dar. Das HHD reicht jedoch alleine, d. h. ohne andere Hürden wie einem niedrigen pH-Wert oder einer Hitzebehandlung, meistens nicht aus, um die mikrobiologische Stabilität eines Produktes zu gewährleisten. Die Kombination aus HHD und anderen antimikrobiell wirksamen Methoden und Stoffen wurde deshalb bereits ausführlich in der Literatur beschrieben. Obwohl der kombinierte Einsatz von Endolysinen und HHD einen vielversprechenden Ansatz zur Kontrolle von Lebensmittelpathogenen darstellt, wurde diese Methode zur Inaktivierung von grampositiven Bakterien bisher nicht untersucht. Des Weiteren sind Bakteriophagen zwar als natürliches Konservierungsmittel ausführlich untersucht und werden bereits in der Lebensmittelindustrie eingesetzt, ihr kombinierter Einsatz mit dem HHD wurde jedoch bislang nur in einer Studie erforscht. Die vorliegende Arbeit beleuchtet daher den Einfluss von Endolysinen und Phagen in Kombination mit dem HHD auf die Inaktivierung von grampositiven Pathogenen in Lebensmitteln.

Die Inaktivierung der grampositiven Bakterienspezies *L. monocytogenes* und *S. aureus* durch Endolysine, HHD oder einer Kombination aus beidem wurde im Hinblick auf die Erforschung von synergistischen Effekten im Puffermedium untersucht. Durch die Verwendung verschiedener Endolysine (PlyP40, Ply511 und PlyP825 für *L. monocytogenes*; HY-100, HY-133 und Lysostaphin für *S. aureus*), unterschiedlicher Endolysin-Konzentrationen und mehrerer Stämme pro Spezies konnte gezeigt werden, dass es zu einer synergistischen Inaktivierung kommt, wenn die untersuchten Bakterien vor der Hochdruckbehandlung mit Endolysin inkubiert wurden. Obwohl der synergistische Effekt bei beiden Bakterienspezies gezeigt werden konnte, war dieser bei *L. monocytogenes* am stärksten. Während die Behandlung von *L. monocytogenes* alleine mit 0,16 µg/mL Endolysin bzw. alleine mit 300 MPa hydrostatischem Hochdruck zu keiner Inaktivierung führte, reduzierte die Kombination beider Methoden die Zellzahl um bis zu 5 log-Stufen. Darüber hinaus konnte gezeigt werden, dass der Einsatz von Endolysin nicht nur den antibakteriellen Effekt des Hochdruckverfahrens erhöht, sondern auch die Inaktivierung von Listerien bei niedrigeren Druckverhältnissen (z. B. 200 MPa) ermöglicht. Die kombinierte Anwendung dieser Methoden erweist sich durch diese Erkenntnisse als besonders vielversprechendes Verfahren für die Konservierung druckempfindlicher Lebensmittel.

Aufgrund des fehlenden Wissens über die zugrundeliegenden Wirkmechanismen der synergistischen Inaktivierung durch den kombinierten Einsatz von Endolysinen und HHD wurden verschiedene Hypothesen untersucht. Es ist hinreichend belegt, dass Hochdruck sowohl negative als auch positive Effekte auf die Aktivität von Enzymen haben kann. Die möglichen Auswirkungen von HHD auf die enzymatische Aktivität von Endolysinen wurden daher mit Hilfe eines photometrischen Flüssiglysetests

untersucht. Eine Änderung der Endolysinaktivität konnte aber weder während der Hochdruckbehandlung noch danach festgestellt werden. Der synergistische Effekt des kombinierten Einsatzes dieser Methoden scheint vielmehr das Ergebnis einer Endolysin-induzierten Schädigung der Bakterienzellwand zu sein. Die Tatsache, dass *L. monocytogenes* mit einem geringeren Druck abgetötet werden konnte, wenn sie zuvor mit PlyP40 inkubiert wurden, impliziert, dass die Bakterien durch den Zusatz von Endolysinen druckempfindlicher werden. Dieser instabile Zustand der Zelle zeigt sich am deutlichsten in Versuchen, in welchen der Einfluss von Endolysinen auf die Inaktivierung von *L. monocytogenes* im zeitlichen Verlauf untersucht wurde. Während sich die Zellzahl bei einigen Endolysin-Konzentrationen initial reduzierte, stieg sie bei längerer Inkubationszeit wieder beinahe auf das Level der Kontrolle an. Diese Ergebnisse deuten darauf hin, dass sich die Zellen kurz nach dem Zusatz der Endolysine in einem geschädigten Zustand befanden, sich aber über die Zeit regenerieren und wieder Kolonien formen konnten. In diesem geschädigten Zustand scheinen die Zellen empfindlicher gegenüber anderen Stressoren wie beispielsweise Hochdruck zu sein. Verschiedene Ursachen für die Schädigung bzw. Sensibilisierung der Zellen für Hochdruck wurden in dieser Arbeit diskutiert. Die Perforierung des Peptidoglykans und eine daraus resultierende Destabilisierung der Zellmembran scheint die plausibelste Antwort auf die Frage zu liefern, weshalb die untersuchten Zellen bei niedrigem Druck inaktiviert werden konnten.

Um den kombinierten Einsatz von Endolysinen und HHD als Konservierungsmethode zu validieren, wurde deren synergistischer Effekt mittels Letalitäts- und Lagertest in verschiedenen Lebensmitteln (Milch, Mozzarella und Räucherlachs) untersucht. Die Effektivität der Methode war durchgehend niedriger in Lebensmitteln als im Puffermedium. Dennoch führte die Kombination der Methoden zu einer synergistischen Inaktivierung der Listerien in Lebensmitteln und ermöglichte gleichzeitig eine Reduktion der Druckintensität bei gleichbleibendem antimikrobiellem Effekt. Obwohl es mit den untersuchten Methoden in keinem der Lebensmittel zu einer vollständigen Abtötung der Listerien kam, reduzierte der kombinierte Einsatz von 3,4 µg/mL PlyP825 und 400 MPa hydrostatischem Hochdruck die Zahl *Listeria*-positiver Milchproben von 18 auf 2. Im Gegensatz dazu wurden alle Proben, welche nur mit 3,4 µg/mL PlyP825 oder nur 400 MPa behandelt wurden, positiv auf Listerien getestet. Die Anzahl *Listeria*-positiver Milchproben konnte durch die Kombination mit PlyP825 bereits bei einem Drucklevel von nur 200 MPa weiter reduziert werden. Auch bei Mozzarella erhöhte der Einsatz von Endolysinen den antibakteriellen Effekt des Hochdruckverfahrens und reduzierte die Anzahl *Listeria*-positiver Probe, hier jedoch mit geringerer Ausprägung im Vergleich zur Milch. Während bei der Milch nach Inkubation mit PlyP825 nur noch 200 MPa nötig waren, um die Anzahl *Listeria*-positiver Proben zu reduzieren, musste der Druck bei den Mozzarellaproben auf mindestens 400 MPa erhöht werden. Dies könnte durch die vergleichsweise geringere Endolysinaktivität im Mozzarella bedingt sein. In Räucherlachs konnte dagegen keine synergistische Inaktivierung von *L. monocytogenes* gezeigt werden. Die Unterschiede in der Lebensmittelmatrix sowie der Versuchsaufbau scheinen damit entscheidend für den Erfolg der Pathogeninaktivierung zu sein. Die Experimente mit Milch und Mozzarella zeigen, dass grampositive Pathogene in Lebensmitteln durch den Einsatz von Endolysinen bei niedrigem Druck inaktiviert werden können. Daraus schlussfolgernd erscheint der Einsatz der beschriebenen Methode in (drucksensiblen) Lebensmitteln besonders vielversprechend.

Bemerkenswerterweise nahm die antimikrobielle Aktivität der Endolysine PlyP40 und PlyP825 bis zu einer Konzentration von 4 µg/mL zu, sank jedoch nach Überschreiten dieses Wertes wieder ab. In der

vorliegenden Arbeit wird auf diesen sogenannten Eagle-Effekt eingegangen und die Bildung von Protoplasten und die Aggregation der Endolysine wurden als mögliche Erklärungen hierfür untersucht. Keimzahlbestimmungen verschiedener Tests deuten darauf hin, dass die Bildung von Protoplasten durch den unmittelbaren Abbau der Peptidoglykanschicht bei hohen Endolysinkonzentrationen wohl nicht ursächlich für den Eagle-Effekt ist. Andererseits deuten die Ergebnisse anderer Versuche, darunter Keimzahlbestimmungen und proteinanalytische Methoden, daraufhin, dass es, besonders bei höheren Endolysinkonzentrationen, ein Gleichgewicht zwischen monomeren und zusammengelagerten polymeren Endolysinen gibt. In diesem Zustand ist die antimikrobielle Aktivität der Endolysine höchstwahrscheinlich reduziert. Die in dieser Arbeit erhobenen Daten unterstützen demnach die Aggregationshypothese als Erklärung für die reduzierte Aktivität der Endolysine PlyP40 und PlyP825 bei höheren Konzentrationen.

Neben Endolysinen wurde auch der Bakteriophage P100 in Kombination mit HHD für die Inaktivierung von *L. monocytogenes* in Puffer und Milch untersucht. Ähnlich wie bei den Endolysinen war die synergetische Inaktivierung stark vom Inokulum, der Konzentration des antimikrobiellen Wirkstoffes sowie vom Medium abhängig. Während der kombinierte Einsatz des Bakteriophagen P100 und HHD im Puffermedium zu einer substantiellen synergistischen Inaktivierung von bis zu 3 log-Stufen führte, konnten bei den gleichen Parametern in Milch nur additive oder minimal synergistische Effekte gezeigt werden. Die Abwesenheit eines starken synergistischen Effektes in Milch scheint auf die niedrigere Aktivität des Bakteriophagen in diesem Lebensmittel zurückzuführen sein. Stärkere synergistische Effekte sind daher bei einer Erhöhung der Phagenkonzentration zu erwarten. Darüber hinaus konnte in anderen Studien gezeigt werden, dass sich die antimikrobielle Wirkung einer kombinierten Anwendung von Phagen und HHD bei längeren Inkubationszeiten substanziell erhöht. Dies deutet darauf hin, dass die Effektivität der Phagen-HHD-Kombination in der vorliegenden Arbeit stark unterschätzt wird. Die Phagen-induzierte Schädigung der Zellwand bildete ursprünglich den Ausgangspunkt der Hypothese zum synergistischen Effekt zwischen Phage P100 und HHD. Bemerkenswerterweise war der Synergismus jedoch gleich stark, wenn die beschriebenen Methoden in umgekehrter Reihenfolge angewandt wurden (d. h. wenn die Phagen erst nach der Hochdruckbehandlung hinzugefügt wurden). Deshalb scheinen andere Mechanismen, wie zum Beispiel eine Druck-induzierte Änderungen der Zellwand oder der Zellmembrane, ebenfalls involviert zu sein.

Zusammenfassend wurden in der vorliegenden Arbeit grundlegende Erkenntnisse über die Inaktivierung grampositiver Bakterien durch Endolysine und Bakteriophagen, den synergistischen Effekt dieser antimikrobiellen Wirkstoffe in Kombination mit hydrostatischem Hochdruck und die Rolle der Zellwand bei Hochdruckresistenz gewonnen. Zum ersten Mal konnte gezeigt werden, dass der Einsatz von Endolysinen nicht nur den antibakteriellen Effekt des hydrostatischen Hochdruckverfahrens gegen grampositive Bakterien erhöht, sondern es darüber hinaus ermöglicht, sie bei reduzierter Intensität der Hochdruckbehandlung abzutöten. Die Kombination der dargestellten Methodik erweist sich damit als geeignetes Konservierungsverfahren für die Inaktivierung von *L. monocytogenes* in druckempfindlichen Lebensmitteln.

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8 APPENDIX

8.1 Publications and supervised student theses

8.1.1 List of publications derived from this work

Peer-reviewed journals

Van Nassau TJ, Lenz CA, Scherzinger A, Vogel RF (2017). Combination of endolysins and high pressure to inactivate Listeria monocytogenes. *Food Microbiology* **68**: 81-88.

Misiou O, Van Nassau TJ, Lenz CA, Scherzinger A, Vogel RF (2018). The preservation of Listeria-critical foods by a combination of endolysin and high hydrostatic pressure. *International Journal of Food Microbiology* – **266**: 355-362.

Oral presentations (speaker is underlined)

Van Nassau, Tomas J., Lenz, Christian A., Scherzinger, Anna S., and Vogel, Rudi F. (2015), "Synergy between bacteriophage-encoded peptidoglycan hydrolases and high pressure on the inactivation of *Listeria monocytogenes* and *Staphylococcus aureus*.", Bacteriophages 2015, Lecture, (London, UK).

Van Nassau, Tomas J., Lenz, Christian A., Scherzinger, Anna S., and Vogel, Rudi F. (2015) "Synergy between endolysin and high pressure on the inactivation of *Listeria monocytogenes*", 15. Fachsymposium Lebensmittelmikrobiologie, Fachgruppe Lebensmittelmikrobiologie der VAAM, Fachgruppe Lebensmittelmikrobiologie der DGHM, Lecture, (Freising, Germany).

Van Nassau, Tomas J., Lenz, Christian A., Scherzinger, Anna S., and Vogel, Rudi F. (2015). "Synergism of high pressure and bacteriophage endolysins for the preservation of sensitive foods", 9. International Conference „Forum Life Science", Lecture, (Garching, Germany).

Van Nassau, Tomas J., Lenz, Christian A., Scherzinger, Anna S., and Vogel, Rudi F. (2016), "The role of the cell wall in HHP", 9. International Conference on High Pressure Bioscience and Biotechnology (HPBB), Lecture, (Toronto, Canada).

Poster

Van Nassau, Tomas J., Lenz, Christian A., Scherzinger, Anna S., and Vogel, Rudi F. (2014), "Synergy between endolysins and high pressure on the inactivation of *Listeria monocytogenes* and *Staphylococcus aureus*", 8. International Conference on High Pressure Bioscience and Biotechnology (HPBB). 07/2014, Poster presentation, (Nantes, France).

8.1.2 Supervised student theses

Zehe, Anja (2015), „Bacteriophage Technology in Food Preservation“, Seminararbeit des Universitätsstudiengangs „Technologie und Biotechnologie der Lebensmittel“.

Bahe, Sabrina (2015), „Kombinierte Anwendung von hydrostatischem Hochdruck und Bakteriophagen zur Inaktivierung von *Listeria monocytogenes*“, Bachelorarbeit im Universitätsstudiengang „Technologie und Biotechnologie der Lebensmittel“.

Misiou, Ourania (2016), „The inactivation of *Listeria monocytogenes* by a combination of endolysin and high hydrostatic pressure in foods “, Internship report.

8.2 Endolysin amino acid sequences

● PlyP40 (retrieved from WO patent 2012/159773, King et al., 2011)

MVLVLDISKWQPTVNYSGLKEDVGFVVIRSSNGTQKYDERLEQHAKGLDKVGMPFGLYHYALFEGGQDTINEAN
MLVSAYKKCRQLGAEPTFLFLDYEEVKLKSGNVVNECQRFIDHVKGQTGVKVGLYAGDSFWKTHDLDKVKHDLRW
VARYGVDNGKPSTKPSIPYDLWQYTSKGRIKAIASPVDMNTCSSDILNKLKGSKAPVKPAPKPTPSKPAPAKPAPKTT
TKYVNTAHLNIREKASADSKVLGVLDLNDSVQVISESGGWSKLKSGNKQVYVSSKYLSKSKTTPKAKPSSKQYYTIKS
GDNLSYIAKKYKTTVKQIQNWNGIKDANKIYAGQKIRVK

● Ply511 (retrieved from WO patent 2012/159773, King et al., 2011)

MVKYTVENKIIAGLPKGKLKGANFVIAHETANSKSTIDNEVSYMTRNWKNAFVTHFVGGGGRVVQVANVNYVSW
GAGQYANSYSYAQVELCRTSNATTFKKDYEVYCQLLVDLAKKAGIPITLDSGSKTSDKGIKSHKWVADKLGGTTHQD
PYAYLSSWGISKAQFASDLAKVSGGGNTGTAPAKPSTPAPKPSTPSTNLDKLGLVDYMNAKKMDSSYSNRDKLAK
QYGIANYSGTASQNTTLLSKIKGGAPKPSTPAPKPSTSTAKKIYFPPNKGNWSVYPTNKAPVKANAIGAINPTKFGGL
TYTIQKDRGNGVYEIQTDQFGRVQVYGAPSTGAVIKK

● PlyP825 (retrieved from WO patent 2012/159773, King et al., 2011)

MALTEAWLLEKANRRLNEKGMLKEVSDKTRAVIKEMAKQCIYINVAQGFRSIAEQNELYAQGRTKPGNVVTNAKG
GQSNHNYGVAVDLCQYTQDGKDVIWAVDAKFKKIVAAMKKQGFKWGGDWKSFKDNPHFELYDWVGGERPNS
STPAKPSKPSTPAKPSGELGLVDYMNSKKMDSSFANRKVLAGKYGIKNYTGTTSQNTQLLAKIKAGAPKHATPKPPA
KPATSGMYVYFPAGKGTWSVYPLNKAPVKANAIGAINPSKFGGLTYKVEKNYGDNVLGIKTGSFGHVKVYCHPSTG
VKISNNGAGNFPNVQN

● HY-100 (retrieved from WO patent 2009/150171,Buchberger et al., 2009)

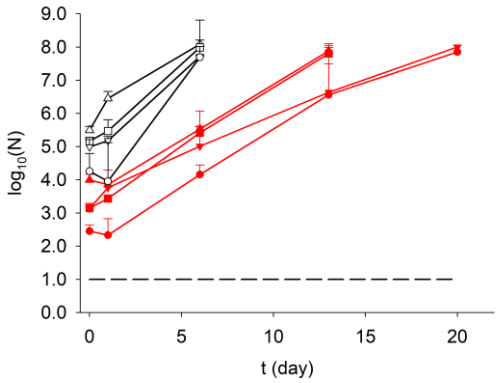
MASIIMEVATMQAKLTKKEFIEWLKTSEGKQFNVDLWYGFQCFDYANAGWKVLFGHTLKGLGAKDIPFANNFDG
LATVYQNTPDFLAQPGDMVVFGSNYGAGYGHVAWVIEATLDYIIVYEQNWLGGGWTDRIEQPGWGWEKVTRR
QHAYDFPMWFIRPNFKSATAPASIQSPTQASKKETAKPQPKAVELKIIKDVVKGHDLPKRGGNPKGIVIHNDAGSK
GATAEAYRNGLVNAPSSRLEAGIAHSYVSGNTVWQALDESQVGWHTANQLGNKYYYGIEVCQSMGADNATFLK
NEQATFQECARLLKKWGLPANRNTIRLHNEFTSTSCPHRSSVLHTGFDPVTRGLLPEDKRLQLKDYFIKQIRAYMDG
KIPVATVSNESSASSNTVKPVAELMPPVPAGYTLDKNNVPYKKEQGNYTVANVKGNNVRDGYSTNSRITGVLPNNT
TITYDGAYCINGYRWITYIANSGQRRYIATGEVDIAGNRISSFGKFSAV

● HY-133 (retrieved from WO patent 2011/076432, Grallert and Leopoldseder, 2011)

MAKTQAEINKRLDAYAKGTVDSPYRVKKATSYDPSFGVMEAGAIDADGYYHAQCQDLITDYVLWLTDNKVRTWG
NAKDQIKQSYGTGFKIHENKPSTVPKKGWIAVFTSGSYEQWGHIGIVYDGGNTSTFTILEQNWNGYANKKPTKRVD
NYYGLTHFIEIPVGGSKPGGTKPGGSKPGSTVTPTPNTGWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGV
LKAGQTIHYDEVMKQDGHVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIK

8.3 Additional results

Endolysin challenge storage test in milk: 1 min pressure holding time (3.4.2.2.1)



of *Listeria*-positive samples

PlyP825 (µg/mL)	HHP (MPa)	Symbol	t (day) 0	1	6	13	20	27	Total
0	0.1	△	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	□	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	300	▽	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	○	3/3	3/3	3/3	3/3	3/3	3/3	18/18
3.4	0.1	▲	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	200	■	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	300	▼	3/3	3/3	3/3	3/3	3/3	3/3	18/18
	400	●	3/3	3/3	3/3	3/3	3/3	3/3	18/18

Figure 48: Challenge storage test in milk: high inoculum. The growth (log_{10}(N)) of stationary-phase *L. monocytogenes* cells (cocktail with five strains at an inoculum of ca. 3×10^5 cells/mL) without endolysin (blanc symbols) or 3.4 µg/mL PlyP825 (filled red symbols) at atmospheric pressure (upward triangles) or in combination with a HHP treatment (25 °C, 1 min) of 200 (squares), 300 (downward triangles), or 400 MPa (circles). The detection limit (1.0 log cfu) is shown by the dashed line. Mean values ± standard deviation of three biologically independent experiments are shown (error bars). The number of *Listeria*-positive samples per time-point and in total is shown in the table next to the graph.

Screening for synergistic effect between endolysin and HHP: log reduction (3.2.1)

Table 27: Data of Table 22 (screening for a synergistic ianctivation of *L. monocytogenes* by endolysin and HHP) presented as log reduction. The induced reduction ($log_{10}(N_0/N)$) of exponential-phase cells (TMW 2.597, 2.599, or 2.1512; inoculum of ca. 10^7 cells/mL in IPB) coincubated with different concentrations of endolysin (PlyP40, Ply511, or PlyP825), either at atmoshperic pressure (0.1 MPa) or in combination with HHP (300 or 350 MPa, 1 min, 30 °C). Coincubation of cells with endolysin was 0.5h until HHP treatment and 1.5h in total. Mean values ± standard deviation of three biologically independent experiments are shown. Cell cultures inactivated until the detection limit (1.3 log cfu) are shown in bold. Within each endolysin-strain combination, statistically significant differences among samples treated only with endolysin and plated on standard TSAYE are denoted by different letters (as tested by one way ANOVA or Kruskal-Wallis one way ANOVA on ranks). A grey background of HHP treated samples plated on standard TSAYE indicates a statistically significant difference with the same sample without HHP treatment (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test). A grey background of samples plated on high salt TSAYE indicates a statistically significant difference with the same sample plated on standard TSAYE (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test).

			L. monocytogenes strain											
			TMW 2.597				TMW 2.599				TMW 2.1512			
			Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE	
			0.1 MPa	300 MPa	0.1 MPa	300 MPa	0.1 MPa	350 MPa	0.1 MPa	350 MPa	0.1 MPa	300 MPa	0.1 MPa	300 MPa
Endolysin (µg/mL)	PlyP40	0	7.0±0.1[a]	0.3±0.4	0.1±0.2	1.1±0.6	7.1±0.2[a]	0.2±0.0	0.1±0.1	0.9±0.4	6.6±0.6[a]	-0.1±0.6	0.0±0.2	2.6±0.2
		0.16	0.0±0.1[a]	3.9±0.4	0.1±0.1	4.6±0.7	0.2±0.0[a]	4.3±0.4	0.2±0.1	5.0±0.8	-0.2±0.6[a]	4.9±0.9	-0.1±0.6	5.1±0.8
		0.8	1.4±0.3[ab]	**5.7±0.1**	1.5±0.1	**5.7±0.1**	2.0±0.2[b]	5.6±0.2	2.0±0.1	**5.8±0.2**	2.3±0.9[b]	**5.3±0.6**	2.5±0.9	**5.3±0.6**
		4	3.3±1.1[c]	**5.7±0.1**	3.7±0.5	**5.7±0.1**	5.1±0.2[c]	**5.8±0.2**	5.1±0.3	**5.8±0.2**	**5.3±0.6[c]**	**5.3±0.6**	**5.3±0.6**	**5.3±0.6**
		20	2.1±0.7[bc]	**5.7±0.1**	2.2±0.6	**5.7±0.1**	3.5±1.4[c]	**5.8±0.2**	3.5±1.3	**5.8±0.2**	4.0±0.7[d]	**5.3±0.6**	4.1±0.8	**5.3±0.6**
	Ply511	0	7.0±0.1[a]	0.2±0.0	-0.1±0.0	0.9±0.6	7.1±0.2[a]	0.1±0.1	-0.1±0.2	1.1±1.0	6.8±0.0[a]	0.4±0.3	0.1±0.2	2.2±0.3
		0.16	0.1±0.2[a]	4.3±0.7	0.2±0.2	4.7±0.7	0.1±0.2[a]	4.0±2.5	0.0±0.2	4.3±2.1	0.0±0.2[a]	5.0±0.5	0.0±0.2	**5.5±0.0**
		0.8	1.1±0.2[b]	4.9±0.2	1.2±0.2	5.5±0.3	1.4±0.8[a]	**5.8±0.2**	1.5±0.9	**5.8±0.2**	0.9±0.1[b]	**5.5±0.0**	0.9±0.1	**5.5±0.0**
		4	2.9±0.1[c]	5.2±0.2	3.2±0.2	5.6±0.2	3.9±1.0[b]	**5.8±0.2**	4.3±1.2	**5.8±0.2**	4.9±0.2[c]	**5.5±0.0**	5.3±0.2	**5.5±0.0**
		20	3.9±0.1[d]	**5.7±0.1**	4.2±0.2	**5.7±0.1**	4.5±1.6[b]	**5.8±0.2**	4.5±1.7	**5.8±0.2**	**5.5±0.0[d]**	**5.5±0.0**	**5.5±0.0**	**5.5±0.0**
	PlyP825	0	7.1±0.1[a]	0.2±0.1	-0.1±0.1	0.9±0.6	7.1±0.1[a]	0.2±0.1	0.0±0.0	1.2±1.0	6.8±0.0[a]	0.3±0.3	0.1±0.1	1.9±0.3
		0.16	0.7±0.3[b]	4.8±0.3	0.8±0.2	5.0±0.2	0.4±0.3[a]	4.3±1.7	0.4±0.3	5.1±0.8	0.2±0.2[a]	**5.5±0.0**	0.2±0.2	**5.5±0.0**
		0.8	1.6±0.2[c]	5.2±0.5	1.7±0.2	5.3±0.2	2.4±0.1[b]	**5.8±0.1**	2.4±0.1	**5.8±0.1**	2.3±0.4[b]	**5.5±0.0**	2.2±0.4	**5.5±0.0**
		4	3.5±0.6[d]	5.6±0.2	3.4±0.5	5.6±0.3	4.8±0.3[c]	**5.8±0.1**	4.8±0.2	**5.8±0.1**	5.1±0.3[c]	**5.5±0.0**	5.4±0.1	**5.5±0.0**
		20	3.2±0.5[d]	5.4±0.3	3.3±0.6	5.3±0.3	4.5±0.5[c]	**5.8±0.1**	4.5±0.4	**5.8±0.1**	4.9±0.2[c]	**5.5±0.0**	5.2±0.3	**5.5±0.0**
		100	2.0±0.3[c]	5.6±0.3	2.0±0.3	5.7±0.1	3.0±1.3[b]	5.7±0.2	3.6±2.0	5.7±0.2	3.8±0.2[d]	**5.5±0.0**	4.0±0.4	**5.5±0.0**

*only 2 replicates. $Log_{10}(N_0)$ = 6.9±0.2

Table 28: Data of Table 24: Screening **for a synergistic inactivation of *S. aureus* by peptidoglycan hydrolases and HHP. The viable cell count (**log10(N)) of exponential-phase cells (TMW 2.49, 2.422, or 2.424 at an inoculum of ca. 107 cells/mL in IPB) coincubated with different concentrations of peptidoglycan hydrolases (HY-100, HY-133, or lysostaphin), either at atmoshperic pressure (0.1 MPa) or in combination with HHP (250, 300, or 350 MPa, 1 min, 30 °C). Coincubation of cells with endolysin was 0.5h until HHP treatment and 1.5h in total. Mean values ± standard deviation of three or four biologically independent experiments are shown (samples within an endolysin-strain combination have the same number of replicates). Cell cultures inactivated until the detection limit (1.3 log cfu) are shown in bold. Within each endolysin-strain combination, statistically significant differences among samples treated only with endolysin and plated on standard TSAYE are denoted by different letters (as tested by one way ANOVA or Kruskal-Wallis one way ANOVA on ranks). A grey background of HHP treated samples plated on standard TSAYE indicates a statistically significant difference with the same sample without HHP treatment (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test). A grey background of samples plated on high salt TSAYE indicates a statistically significant difference with the same sample plated on standard TSAYE (as tested by one-tailed Student's t-test or Mann-Whitney rank sum test).

			S. aureus strain											
			TMW 2.49				TMW 2.422				TMW 2.424			
			Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE		Standard TSAYE agar		High salt TSAYE	
			0.1 MPa	300 MPa	0.1 MPa	300 MPa	0.1 MPa	350 MPa	0.1 MPa	350 MPa	0.1 MPa	250 MPa	0.1 MPa	250 MPa
Peptidoglycan hydrolases (µg/mL)	HY-100	0	6.9±0.2a	6.6±0.2	7.0±0.3	7.0±0.1	6.4±0.7a	5.9±0.8	7.3±0.3	6.1±0.7	7.4±0.2a	7.2±0.2	7.3±0.1	7.3±0.2
		0.16	7.1±0.2a	6.8±0.3	7.2±0.3	7.0±0.2	6.5±0.8a	6.0±0.2	7.1±0.5	6.4±0.6	7.2±0.2a	7.2±0.1	7.2±0.2	7.1±0.2
		0.8	7.1±0.2a	6.6±0.2	7.1±0.1	6.7±0.2	5.7±1.1a	4.6±1.6	6.4±0.7	4.7±1.8	6.8±0.4a	6.6±0.3	6.8±0.3	6.6±0.4
		4	6.1±0.2b	3.6±1.6	6.1±0.2	4.5±0.7	4.8±0.9a	1.4±0.3	5.4±0.4	1.6±0.6	5.2±0.5b	2.9±0.6	5.0±0.6	2.8±0.8
		20	4.7±0.4c	1.5±0.2	4.4±0.5	1.6±0.3	2.2±1.0b	**1.3±0.0**	2.5±1.1	**1.3±0.0**	2.4±0.8c	**1.3±0.0**	2.2±0.7	**1.3±0.0**
		100	2.0±0.8d	**1.3±0.0**	1.7±0.5	**1.3±0.0**	1.4±0.2b	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	1.5±0.3d	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
	HY-133	0	6.9±0.4a	6.6±0.4	6.7±0.1	6.3±0.6	7.1±0.3a	6.2±0.2	7.3±0.1	6.6±0.1	6.9±0.1a	6.5±0.4	6.8±0.0	6.4±0.2
		0.16	7.0±0.3a	6.6±0.4	6.9±0.2	6.5±0.7	7.1±0.3a	6.0±0.5	7.1±0.2	5.6±0.4	6.7±0.2a	6.1±0.5	5.8±0.3	5.4±0.7
		0.8	7.1±0.2a	6.6±0.4	6.8±0.3	5.7±1.8	6.9±0.1a	4.8±1.2	6.6±0.5	3.5±1.0	5.8±0.4b	5.7±0.8	4.9±0.6	4.7±0.1
		4	6.0±0.3b	3.8±2.2	5.6±0.3	3.4±1.8	3.1±1.1ab	1.5±0.3	**1.3±0.0**	**1.3±0.0**	3.8±0.6c	3.7±1.0	**1.4±0.1**	1.6±0.4
		20	3.8±0.5c	1.7±0.8	**1.3±0.0**	**1.3±0.0**	2.3±0.9ab	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	2.1±0.8d	1.9±0.3	**1.3±0.0**	**1.3±0.0**
		100	**1.3±0.0d**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0b**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	1.6±0.4d	1.5±0.3	**1.3±0.0**	**1.3±0.0**
	Lysostaphin	0	7.0±0.3a	6.7±0.3	7.0±0.3	7.0±0.1	6.6±0.9a	6.0±0.9	7.2±0.3	6.1±0.7	7.4±0.2a	7.3±0.2	7.3±0.1	7.3±0.2
		0.16	7.1±0.3a	6.7±0.3	7.3±0.4	6.9±0.2	6.2±1.3a	5.1±1.3	6.9±0.6	5.1±1.5	7.0±0.6a	7.3±0.2	6.5±1.2	7.1±0.3
		0.8	6.8±0.4a	5.3±1.0	6.7±0.4	5.9±0.5	4.3±1.4a	**1.3±0.0**	4.6±0.7	**1.3±0.0**	5.7±0.5b	4.5±0.4	3.8±1.8	4.2±0.8
		4	3.7±0.7b	1.6±0.6	2.1±1.5	**1.3±0.0**	**1.3±0.0a**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	2.3±0.3c	**1.3±0.0**	1.4±0.2	1.4±0.2
		20	**1.3±0.0c**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0a**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0d**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**
		100	**1.3±0.0c**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0a**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0d**	**1.3±0.0**	**1.3±0.0**	**1.3±0.0**