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Virulent bacteriophages for control of *Listeria monocytogenes* and *Salmonella* Typhimurium in foods

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Abbreviations

Abbreviations of used buffers, media and solutions are listed in appendix A to C.

ATCC	American Type Culture Collection
ActA	Actin ACTivating factor
bp	Base Pairs
BSE	Bovine Spongiform Encephalopathy
CBD	Cell wall Binding Domain
CDC	Centers for Disease Control and Prevention
cfu	Colony Forming Units
CGSC	Coli Genetic Stock Center
cm	ChloraMphenicol
cm^R	Resistance to ChloraMphenicol
CNL	Centre National de Référence des Listeria
DNA	DeoxyriboNucleic Acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EAD	Enzymatically active domain
EC	European Commission
EDI	Eidgenössisches Departement des Innern
EHEC	EnteroHemorrhagic *E. Coli*
EN	European Norm
EOP	Efficiency Of Plating
GRAS	Generally Recognized As Aafe
HACCP	Hazard Analysis Critical Control Point
HIV	Human Immunodeficiency Virus
HPL	His-tagged Phage endoLysin
IDF	International Dairy Federation
IL8	Interleukin 8
ISO	International Standardization Organisation
InlA	INternaLin A
InlB	INternaLin B
LLO	ListerioLysin O
LPS	LipoPolySaccherides
Ni-NTA	Nickel-NiTrilotriacetic Acid
nt	NucleoTides
OD	Optical Density
OFR	Surface ripening culture
PCR	Polymerase Chain Reaction
PEEC	Pathogen-Elicited Epithelial Chemoattractant
PEG	PolyEhyleneGlycol
PES	PolyEther Sulfone
pfu	Plaque Forming Units
Plc	PhosphoLipase C
PLY	Phage LYsin

PrfA	Positive Regulatory Factor A
RNA	RiboNucleic Acid
rpm	Rounds Per Minute
RTE	Ready-To-Eat
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis
SLCC	Special *Listeria* Culture Collection
SPI	*Salmonella* Pathogenity Island
stm	STreptoMycin
stm^R	Resistance to STreptoMycin
SV	SeroVar
TTSS	Type III Secretion System
WSLC	Weihenstephan *Listeria* Collection
WHO	World Health Organization

Summary

The use of bacteriophages represents an innovative and promising approach for control of spoilage bacteria and pathogens in foods. Bacteriophages can be considered as the natural enemies of bacteria. Phages are of natural origin, self-perpetuating, and can be highly host specific. Some of the critical issues of biopreservation with bacteriophages are a limited host range and the emergence of phage resistant mutants. In this study, the application of high specific and broad host range phages for control of *Listeria monocytogenes* and *Salmonella* Typhimurium was evaluated during storage and ripening of different foods.

In the case of *L. monocytogenes*, 14 different foods including meat, fish, and dairy products as well as vegetables were tested during storage. Three strains of *L. monocytogenes* were used, belonging to serovars 1/2a, 1/2b and 4b. Most experiments were done with *Listeria* phage A511, but two other phages (P100, P35) were also included in some trials. The initial contamination level of *L. monocytogenes* was usually about 1×10^3 cfu/g and the application level of the *Listeria* phage about 3×10^8 pfu/g. Various parameters such as storage temperature and time, initial bacterial cell and phage concentrations, and different phage application protocols were studied.

Compared to controls, a significant reduction of *L. monocytogenes* cell counts of 0.4 to 6.0 log units was observed in phage treated foods after storage at 6°C for six days. The effect of phage A511 was comparable for the different *Listeria* strains on most foods, except for raw salmon, minced meat and mozzarella cheese brine. When foods were contaminated with a lower concentration of *L. monocytogenes* (1×10^2 cfu/g), growth inhibition was similar or higher compared to the higher contamination level (1×10^3 cfu/g). The long-term effect of A511 was shown for all *Listeria* strains in three different foods during 13 days. However, in some of the experiments phage resistant bacteria were isolated. In these cases, control effect of phage A511 was lower. After increase of the storage temperature to 20°C, reduction levels were similar or higher than at 6°C. However, at 20°C, *Listeria* cells were able to resume growth after the first days, in contrast to 6°C. A higher initial phage concentration (3×10^8 pfu/g) yielded a better control effect of *L. monocytogenes* than lower ones (3×10^7 pfu/g or 3×10^6 pfu/g). The control effect of phage A511 was improved on smoked salmon by increasing the initial phage concentration to 2×10^9 pfu/g. Efficacy of phage P100 was comparable to that of phage A511, but efficacy of phage P35 seemed to be lower. Phage concentrations were stable on foods of animal origin during 6 or 13 days. On vegetables, counts of A511 decreased by 0.6 to 1.1 log units at 6°C, and 2.0 log units at 20°C. Concentration

of P100 decreased by 0.6 log on cabbage and that of P35 by 0.8 log units on iceberg lettuce. Application of phage A511 was further tested during the ripening of white mold soft cheese and red smear soft cheese. After 3 weeks, *Listeria* cell counts were significantly decreased by 2.5 log units on white mold cheese, using a single application of A511 with 3×10^8 pfu/cm^2. Repeated application of phage A511 could not improve growth suppression, but a higher single application of 1×10^9 pfu/cm^2 was slightly more effective. On red smear soft cheese, cell numbers were significantly reduced by 3.1 to 3.6 log units after 22 days. Repeated application of phage A511 could only delay re-growth of *L. monocytogenes*, but could not further improve the control effect after 22 days. Phage concentrations decreased by 1.5 log units on white mold cheese, whereas on red smear cheese phage A511 was stable.

For experiments with *S.* Typhimurium, two antibiotic-resistant strains were either naturally selected or genetically engineered, in order to enable the determination of *Salmonella* counts in foods. *Salmonella* phage FO1-E2 was tested for growth control on six different foods including meat, fish, and dairy products as well as vegetables, at two storage temperatures (8°C and 15°C). Contamination levels of *Salmonella* and concentrations of *Salmonella* phage FO1-E2 were similar to the experiments with *Listeria.* At 8°C, no *Salmonella* could be detected after the first day by direct plating, but with selective enrichment. At 15°C, bacterial cell numbers were significantly reduced by 3.0 to 5.3 log units compared to untreated control samples after 6 days. On mung bean sprouts, no significant reduction could be observed during the whole experiment. In egg yolk, growth inhibition was significant only at day 2. Phage FO1-E2 was stable on foods of animal origin, but decreased by 0.6 log units on vegetables during the 6 day period.

The work presented here clearly demonstrates that application of bacteriophages can contribute to the control of pathogens in foods. The effect of phages was mainly influenced by the type of food, the amount of phages applied, the stability of phages on the food, and the used phage. To a minor degree, growth control was dependent on the bacterial strain, the initial contamination level, the storage temperature and time. However, emergence of phage insensitivity may represent a potential problem. Therefore, mixtures of different virulent phage cultures should be used, and application protocols must be adjusted accordingly.

Zusammenfassung

Der Einsatz von Bakteriophagen stellt einen neuartigen und viel versprechenden Ansatz zur Kontrolle von bakteriellen Verderbs- und Krankheitserregern in Lebensmitteln dar. Phagen können als die natürlichen Feinde von Bakterien angesehen werden. Sie sind natürlichen Ursprungs, selbst-replizierend und können sehr wirtsspezifisch sein. Kritische Aspekte beim Einsatz von Bakteriophagen als biologische Konservierungsmittel sind deren oftmals eingeschränkte Wirtsbereiche und die mögliche Entstehung von gegen Phagen resistenten Bakterien. In dieser Arbeit wurde der Einsatz von hoch spezifischen Phagen mit einem weitem Wirtsspektrum zur Kontrolle von *Listeria monocytogenes* und *Salmonella* Typhimurium während der Lagerung und Reifung verschiedener Lebensmittel evaluiert.

Zur Kontrolle von *L. monocytogenes* wurden 14 verschiedene Lebensmittel getestet (Fleisch-, Fisch-, Milchprodukte und Gemüse). Drei Stämme von *L. monocytogenes* der Serovare 1/2a, 1/2b und 4b wurden eingesetzt. Neben dem *Listeria* Phagen A511 wurden ebenfalls zwei weitere Phagen (P100 und P35) mit in die Experimente einbezogen. Die Anfangskontamination von *L. monocytogenes* lag bei 1×10^3 cfu/g und die Applikation der Phagen bei 3×10^8 pfu/g. Verschiedene Parameter wie die Lagerungstemperatur und -zeit, die Anfangskonzentration von Bakterien und Phagen sowie unterschiedliche Anwendungsverfahren der Phagen wurden untersucht.

Nach der Zugabe von Phage A511 zu verschiedenen Lebensmitteln wurde im Vergleich zu unbehandelten Kontrollproben eine signifikante Reduktion von 0.4 bis 6.0 Zehnerpotenzen von *L. monocytogenes* nach einer Lagerung von 6 Tagen bei 6°C beobachtet. Der Effekt von Phage A511 war für die unterschiedlichen Listerienstämme in den meisten Lebensmitteln vergleichbar, mit Ausnahme von rohem Lachs, Hackfleisch und Mozzarellalake. Wurden die Lebensmittel mit einer geringeren Listerienzellzahl kontaminiert (1×10^2 cfu/g), ergab sich im Vergleich zum höheren Kontaminationsgrad (1×10^3 cfu/g) eine ähnliche oder sogar stärkere Wachstumshemmung. Der Langzeiteffekt von A511 konnte für alle Listerienstämme in drei verschiedenen Lebensmitteln über einen Zeitraum von 13 Tagen gezeigt werden. Allerdings wurden in einigen dieser Experimente gegen Phagen resistente Bakterien entdeckt. In diesen Fällen war der Kontrolleffekt von A511 geringer. Eine Erhöhung der Lagerungstemperatur auf 20°C führte zu ähnlichen oder höheren Keimzahlreduktionen im Vergleich zur Lagerung bei 6°C. Jedoch konnten sich die Listerien bei 20°C im Gegensatz zu 6°C bereits nach den ersten Tagen erneut vermehren. Eine höhere Anfangskonzentration an Phagen (3×10^8 pfu/g) führte zur besseren Kontrolle von *L. monocytogenes* als die beiden

niedrigeren Konzentrationen (3×10^7 pfu/g or 3×10^6 pfu/g). Auf geräuchertem Lachs konnte der Effekt von A511 weiter verbessert werden, indem die Phagenkonzentration bis auf 2×10^9 pfu/g erhöht wurde. Die Effektivität von Phage P100 war vergleichbar mit der von A511, aber die Wirksamkeit von P35 schien geringer zu sein. Die Konzentration der Bakteriophagen war stabil auf allen tierischen Lebensmitteln während 6 oder 13 Tagen. Auf Eisbergsalat und Weisskohl wurde die Menge von A511 bei 6°C um 0.6 bis 1.1 Zehnerpotenzen verringert, und bei 20°C um 2 Zehnerpotenzen. Die Konzentration von P100 nahm um 0.6 Zehnerpotenzen auf Weisskohl ab und die von P35 um 0.8 auf Eisbergsalat. Die Anwendung von A511 wurde weiterhin während der Reifung von Weissschimmelkäse und Rotschmierekäse getestet. Die einmalige Applikation von 3×10^8 pfu/g auf Weissschimmelkäsen führte zu einer signifikanten Reduktion der Listerienkeimzahlen um 2.5 Zehnerpotenzen nach 3 Wochen. Eine mehrmalige Applikation von 3×10^8 pfu/g führte zu keiner signifikanten Verbesserung. Ebenfalls verbesserte eine einmalige Anwendung von 1×10^9 pfu/g die Wachstumshemmung nur geringfügig. Auf den Rotschmierekäsen wurden die Keimzahlen signifikant um 3.1 bis 3.6 Zehnerpotenzen nach 22 Tagen reduziert. Mehrmalige Anwendungen des Phagen A511 konnten das Listerienwachstum länger unterdrücken, aber keine Verbesserung der Kontrollwirkung nach 22 Tagen bewirken. Die Phagenkonzentrationen nahmen auf den Weissschimmelkäses um 1.5 Zehnerpotenzen ab, waren dagegen auf den Rotschmierekäsen weitgehend stabil.

Für die Experimente mit *S.* Typhimurium wurden zwei Antibiotika resistente Stämme entweder natürlich selektiert oder genetisch modifiziert, um die Bestimmung der Keimzahl von *Salmonella* im Lebensmittel zu ermöglichen. Der Einsatz des Salmonellenphagen FO1-E2 wurde auf 6 verschiedenen Lebensmitteln (Fleisch-, Fisch-, Milchprodukte und Gemüse) bei 2 Lagerungstemperaturen (8 und 15°C) getestet. Die Kontaminationsrate von *Salmonella* und die Phagenkonzentration waren ähnlich zu den Experimenten mit *Listeria.* Bei 8°C konnten bereits nach dem ersten Tag keine Salmonellen mehr durch direktes Ausplattieren nachgewiesen werden, aber durch selektive Anreicherung. Bei 15°C wurden die Salmonellenkeimzahlen nach 6 Tagen signifikant um 3.0 bis 5.3 Zehnerpotenzen verringert im Vergleich zu unbehandelten Kontrollproben. Auf den Mungbohnensprossen konnte während des gesamten Experiments keine signifikante Keimzahlreduktion beobachtete werden. In Eigelb war der Kontrolleffekt nur nach 2 Tagen signifikant. Phage FO1-E2 war stabil in tierischen Lebensmitteln, nahm jedoch während 6 Tagen um 0.6 Zehnerpotenzen auf den Mungbohnensprossen ab.

Die hier vorgestellte Forschungsarbeit zeigt deutlich, dass die Anwendung von Bakeriophagen zur Kontrolle von bakteriellen Krankheitserregern in Lebensmitteln beitragen kann. Die Wirkung der Phagen war im Wesentlichen beeinflusst von der Art des

Lebensmittels, der eingesetzten Phagenmenge, der Phagenstabilität im Lebensmittel und dem verwendeten Phagentyp. Zu einem geringeren Ausmass war der Kontrolleffekt abhängig vom bakteriellen Stamm, der Höhe der Anfangskontamination sowie der Lagerungstemperatur und -zeit. Allerdings stellt die Entstehung von gegen Phagen resistenten Bakterien ein mögliches Problem dar. Deshalb sollten Mischungen verschiedener Phagen eingesetzt werden und die Anwendungsverfahren entsprechend angepasst werden.

1 Introduction

1.1 The genus *Listeria*

1.1.1 Characteristics, ecology and taxonomy of *Listeria* spp.

In 1926 Murray at al. [169] isolated a bacterium from infected guinea pigs and rabbits suffering from a characteristic monocytosis. He called it *Bacterium monocytogenes.* Pirie renamed it *Listerella hepatolytica* in 1927 and, finally, *Listeria monocytogenes* in 1940 [86, 187]. First isolates from humans were obtained in 1929 [86]. Until the 1980s only sporadic cases were reported. Shortly after, however, several food-borne outbreaks attracted the attention of the food industry, of governments, and of researchers [66].

Listeria is a short, Gram-positive rod, 0.5 - 2 μm in length and 0.4 - 0.5 μm in diameter (Figure 1.1). It appears singly, in short chains, in V or Y form, or in palisades. It forms neither capsules nor spores. Peritrichous flagella enable tumbling motility between 20 and 25°C. However, *Listeria monocytogenes* is not motile at 37°C [66, 119]. Colonies of *Listeria* appear in a characteristic blue/green color, when illuminated with obliquely transmitted white light (Henry's lamp technique) [101]. *Listeria* is well adapted to environmental conditions. It is able to multiply between 1 - 45°C, with optimal proliferation occurring between 30 - 37°C. According to Seeliger and Jones [207], growth occurs in a pH range between pH 5.6 - 9.6. Optimum pH lies at neutral or slightly alkaline values [153]. However, growth was also observed at values as low as pH 4.3 at 30°C in laboratory medium [66]. The minimum pH permitting growth has, therefore, to be considered below pH 5, depending on temperature, availability of nutrients and time [66, 153]. *Listeria* tolerates high salt concentrations of more than 10% [153]. Therefore, it is able to multiply at a_w-values as low as 0.90 and survives even at lower values like 0.80. However, optimal growth occurs at a_w 0.97 [153].

Listeria is a bacterium of low G+C content of 36 - 42 mol % and is closely related to the genera *Brochothrix*, *Bacillus*, *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Clostridium* [194, 229]. The genus *Listeria* is subdivided into six species: *Listeria monocytogenes L. ivanovii L. innocua, L. seeligeri, L. welshimeri*, and *L. grayi* [194, 204]. The species *L. monocytogenes* consists of 13 serovars: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 [66, 193].

Listeria is widely distributed in the environment. The natural habitat is considered to be soil, water, and plant material undergoing decay [68]. *Listeria* has been further isolated from mud, sewage and feces of animals and humans [68, 207]. This ubiquitous occurrence together with its resistance to adverse environmental conditions and

its capability of surviving food-processing techniques makes *Listeria monocytogenes* a particular risk to human health and a big concern for the food industry.

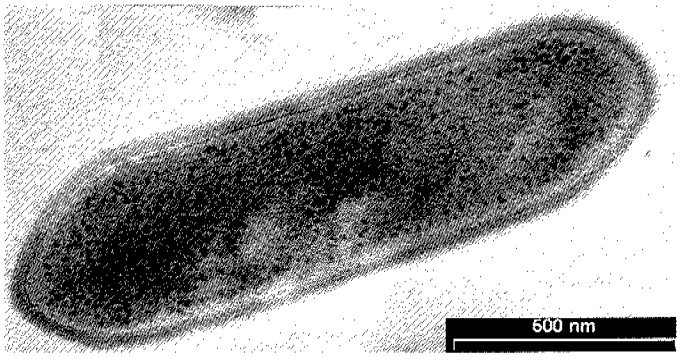


Figure 1.1: Transmission electron micrograph of *Listeria monocytogenes* (kindly provided by Simone dell'Era).

1.1.2 Virulence and pathogenesis of *Listeria monocytogenes*

L. monocytogenes and *L. ivanovii* are the only two potentially pathogenic species, whereas the most important human pathogen is *L. monocytogenes*. *L. ivanovii* is considered as animal pathogen and human cases are rather rare. *L. seeligeri* is regarded as non-pathogenic, although there is one report of human infection [229].

The disease caused by *L. monocytogenes* is called listeriosis and is transmitted almost exclusively via contaminated food. Two types of listeriosis are known in non-pregnant adults: the non-invasive gastroenteritis and the invasive infection. The non-invasive (febrile) gastroenteritis appears mostly in healthy immunocompetent persons. It is associated with diarrhea, vomiting and fever, and is characterized by very short incubation periods (20h) and high contamination levels of the food [24, 48, 75, 229]. Concentrations between $>10^6$ and 10^9 colony forming units per gram food (cfu/g) have been reported [24, 48].

The invasive infection mainly occurs in immunocompromised and weak individuals including very young and elderly persons. It is associated with a longer incubation time of up to 30 days, and lower contamination levels of the foods compared to the non-invasive gastroenteritis [229]. Usually, invasive infection leads to menigitis or menigoencephalitis (55 - 70% of cases) and generalized bacteremia or septicimea (15 - 50% of cases). Less frequent forms (5 - 10% of cases) are endocarditis, myocarditis, pneumonia, hepatitis and others [204, 229]. Invasive listeriosis is a very severe disease with a mortality rate between 15 - 40% or even higher (Table 1.1) [208, 229]. A special case is listeriosis in pregnant women. Usually, the infection is asymptomatic or present as an influenza-like illness. However, when untreated, the fetus can be infected via the pla-

centa. Consequences are abortion especially from the 5th months onwards, premature birth or early onset infection of the neonate. In the latter case the clinical presentation is sepsis or *granulomatosis infantisepticum* characterized by a high mortality (15-50%). Late neonatal listeriosis is less frequent and manifests itself several days to weeks after birth. Infection probably mainly occurs during passage through the birth channel, although also hospital acquired cases have been reported [229]. Most likely, meningitis will appear, but mortality rate is between 10 and 20% and thus lower compared to the early onset [66, 204, 229].

There are various therapeutic options for listeriosis. In general, a combination of ampicillin or amoxicillin with an aminoglycoside is recommended to achieve synergistic killing effects. Furthermore, clinical treatments have shown that the combination of amoxicillin and gentamicin are most effective [107, 108]. Treatment of uncomplicated sepsis and meningitis usually lasts for two weeks, and that of endocarditis or nonmeningitic diseases for 4 to 6 weeks [204].

The minimum infectious dose for listeriosis is not known. Estimations range between 10^2 to 10^4 cfu/g food [66, 229]. The minimal dose mainly depends on the individual immunological status of the host and on the virulence of the strain [66, 229]. Recent risk assessments suggest that low contamination levels of *L. monocytogenes* represent a low risk for healthy people [41, 206]. Some countries such as Canada and for a short time the European Union including Switzerland have risk management strategies that imply a regulatory limit for *L. monocytogenes* of up to 10^2 cfu/g in such ready-to-eat (RTE) foods that do not support growth of these bacteria [15, 16, 41, 206]. For other RTE foods absence of *L. monocytogenes* is required in $\geq 25\ g$ (zero tolerance). In the United States a petition for a similar regulatory policy has been submitted to the Food and Drug Administration (FDA) [15, 16, 41, 206, 225].

Foods are the major source for *Listeria* infections. The primary infection site is located in the gastrointestinal tract. Before reaching the intestine, bacterial cells have to pass the adverse conditions present in the stomach and the upper gastrointestinal tract. A significant number of bacteria may be destroyed here, but gastric acid tolerance factors support survival of *L. monocytogenes* [75, 229]. Surviving bacteria reach the intestine, attach to and invade the epithelial cells. Additionally, *L. monocytogenes* is able to colonize the Peyer's patches where bacteria might enter the body by penetrating M-cells in the epithelium. Furthermore, bacteria can be phagocytosed by macrophages [96, 229]. After passage through the intestine, *Listeria* cells are carried by the lymph or blood to mesenteric lymph nodes, spleen and liver. Here, bacteria mainly proliferate in the hepatocytes. If *Listeria* infection can not be controlled by an adequate immune response, multiplication causes release of bacteria into the circulation. Bacterial cells are disseminated to other organs, especially the placenta or the brain.

Major complications are - as mentioned before - menigitis or menigoencephalitis, sepsis and infection or abortion of the fetus [229].

The basic infection cycle is illustrated in Figure 1.2. Invasion of human cells is mediated by the two important virulence factors, internalin A and B (InlA, InlB). Receptors for both internalins are very specific: InlA recognizes E-cadherin (cell adhesion molecule), InlB binds to gc1q-R (complement factor) and Met (hepatocyte growth factor). Both internalins enable adhesion to the host cell surface and subsequent phagocytosis. Protein p60 is not involved in epithelial cell invasion, but might enhance phagocytosis by macrophages or fibroblasts [96, 229]. Inside the eucaryotic cell, bacteria are enclosed within a phagocytic vacuole. *L. monocytogenes* has to escape, before the vacuole fuses with a lysosome. Otherwise, bacterial cells would be killed. Disruption of the phagosome membrane is mediated mainly by the hemolysin listeriolysin O (LLO) and phospholipases C (PlcA, PlcB). Listeriolysin O is essential for lysis of the phagosome and the most important virulence factor. It is a pore-forming, cholesterol-dependent cytolysin, and active only at acidic pH (pH 5.5). Therefore it is predominating in the phagosome, but not in the cytosol. LLO has a short half-life and is degraded in the host cell cytosol rapidly after the disruption of the phagosome. This enables opening of the phagosome, but prevents disruption of the whole host cell [96, 229].

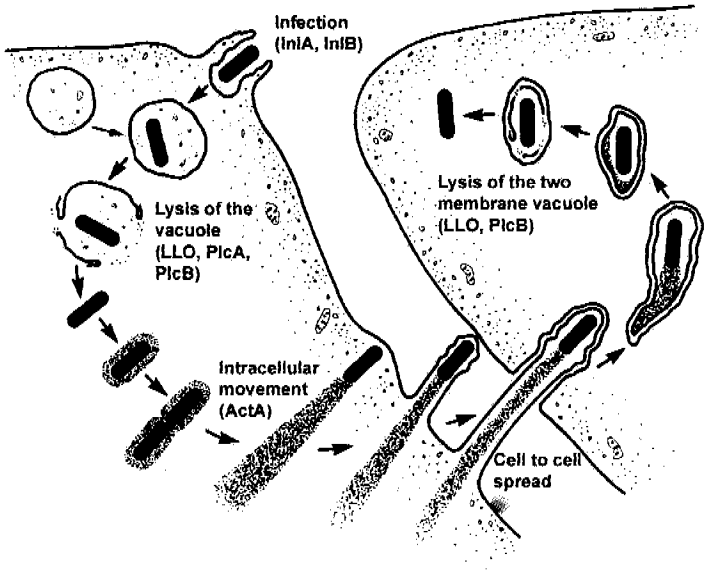


Figure 1.2: Infection cycle of *Listeria monocytogenes*. Successive steps and bacterial virulence factors are indicated. Adapted from [224].

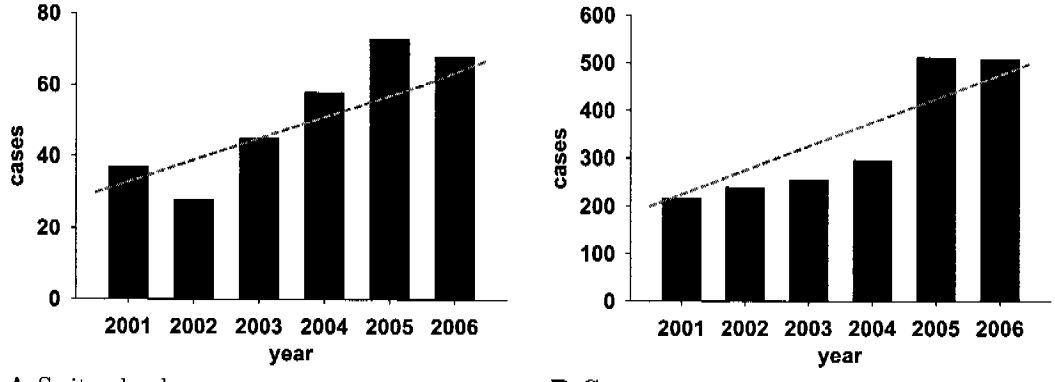
In the cytosol, bacteria multiply with a doubling time of 1 h. Movement in the cell requires actin polymerization, and several host proteins are involved. Bacteria are

surrounded by protein ActA, which is arranged to a tail of up to 40 μm in length at one of the bacterial poles. Protein ActA is anchored to the cytoplasmatic membrane via its C-terminal domain [128]. Random cell movement occurs with a mean speed of 0.3 μm/s. When reaching the cell periphery the membrane is pushed outward, pseudopods emerge and penetrate the neighboring cell. Phagocytosis takes place and secondary phagosomes with a double membrane are formed. *Listeria* escapes again with the additional help of phospholipase C (PlcB) [96, 130, 229].

Most virulence genes are grouped together in the genome in pathogenicity islands. They are all strictly regulated by PrfA (positive regulatory factor). It is a transcriptional activator that depends on growth phase of the cells and the conditions in the surrounding environment [96, 229].

1.1.3 Epidemiology and occurrence of *Listeria monocytogenes* in foods

Listeriosis is one of the most fatal bacterial infections currently known, although case incidence is rather low [229]. There are 2 - 15 cases per million inhabitants in Europe [127, 194, 229]. However, incidence increased in some countries during the last years, e.g. in Switzerland or Germany (Figure 1.3) .



A Switzerland **B** Germany

Figure 1.3: Case numbers of listeriosis in Switzerland and Germany since 2001 [18, 21].

Listeria has been isolated from a broad variety of foods: milk, cheese and other dairy products, meat and meat products, poultry and eggs, fish, fish products and seafood, vegetables, and salad [66, 196]. Of the 13 known serovars of *Listeria monocytogenes* only three (1/2a, 1/2b, 4b) are responsible for more than 90% of *Listeria* infections, although other serovars such as 1/2c are often isolated from food [66, 204]. Table 1.1 provides an overview of the most important food-related epidemiological outbreaks of *L. monocytogenes* since 1980, in which at least 10 persons were infected.

Table 1.1: Synopsis of *Listeria* outbreaks since 1980 (number of cases $\geq$ 10).

Year	Location	Number of cases (deaths)*	Mortality rate [%]*	Food source	Reference
1980	New Zealand	22 (6)	27	Raw fish	[134]
1981	England	11 (n.s.)	-	Cream	[196]
1981	Slovakia	49 (n.s.)	-	Unknown	[196]
1981	Canada	41 (17)	41	Coleslaw	[196, 203]
1983	USA	49 (14)	29	Pasteurized milk	[72]
1983-1987	Switzerland	122 (34)	28	Vacherin Mont D'Or cheese	[30]
1985	USA	142 (48)	34	Mexican-style cheese	[141]
1985-1987	Denmark	35 (n.s.)	-	Unknown	[202]
1986	Austria	20 (n.s.)	-	Raw milk, vegetables	[227]
1986-1987	USA	154 (43)	28	Hot dogs, chicken	[205]
1987	USA	11 (n.s.)	-	Butter	[196]
1987	England	23 (n.s.)	-	Unknown	[164]
1987-1989	Great Britain	350 (n.s.)	-	Pâté	[196]
1989	USA	10 (1)	10	Shrimps	[192, 196]
1989-1990	Denmark	26 (6)	23	Blue-mold or hard cheese	[196]
1990	Australia	11 (n.s.)	-	Processed meat or pâté	[196]
1992	France	279 (85)	30	Jellied pork tongue	[201]
1993	France	38 (n.s.)	-	Pork pâté "rillettes"	[84]
1993	Italy	18 (0**)	-	Rice salad	[200]
1994	USA	45 (0**)	-	Chocolate milk	[48]
1995	France	36 (11)	31	Brie de Meaux cheese	[52]
1997	France	14 (n.s.)	-	Livarot, soft cheese	[52]
1997	Italy	1566 (0**)	-	Sweet corn	[24]
1998-1999	USA	108 (18)	17	Hot dogs	[8, 85]
1998-1999	Finland	25 (6)	24	Butter	[156]
1999-2000	France	10 (1)	10	Pork pâté "rillettes"	[56]
1999-2000	France	32 (10)	31	Jellied pork tongue	[56]
1999-2000	Finland	23 (4)	17	Fish products	[99]
2000	USA	30 (7)	23	Turkey deli meat	[9, 176]
2000	USA	12 (5)	42	Mexican-style cheese	[12]
2000	Spain	15 (n.s.)	-	n.s.	[55]
2001	Japan	86 (0**)	-	Cheese	[159]
2001	USA	16 (0**)	-	Deli meat	[73]
2002	Canada	17 (n.s.)	-	Raw-milk cheese	[77]
2002	France	11 (n.s.)	-	Spreadable raw sausage	[55]
2002	USA	54 (11)	20	Deli turkey meat	[83]
2005	Switzerland	10 (5)	50	Soft cheese	[31]
2006	Italy	n.s. (n.s.)	-	Blue-veined cheese	[79]

*number of deaths and mortality rate include miscarriages and stillbirths; **predominately gastroenteritis; n.s. not specified

1.2 The genus *Salmonella*

1.2.1 Characteristics, ecology and taxonomy of *Salmonella* spp.

Salmonella was first described by K. J. Eberth in 1880 and was successfully cultured by G. Gaffky in 1884 [50]. In 1900, the term "*Salmonella*" was suggested in honor of the American veterinarian Daniel E. Salmon (1850-1918) [29, 47, 223]. *Salmonella* is a Gram-negative rod, 2.0 - 5.0 μm in length and 0.7 - 1.5 μm in diameter (Figure 1.4). It does not form spores and is usually motile by peritrichous flagella [47, 132]. *Salmonella* grows between 7 and 48°C with an optimum growth at 37°C. Proliferation below 7°C has only been observed under laboratory conditions and not on foods. However, *Salmonella* can survive well at low temperatures. *Salmonella* multiplies at pH-values between pH 4.5 and 9.5 with optimal conditions between pH 6.5 and 7.5. A few strains grow below pH 4.5. *Salmonella* is sensitive to salt concentration of 3 - 4 % and therefore grows at a_w-values >0.93 [29, 47, 132].

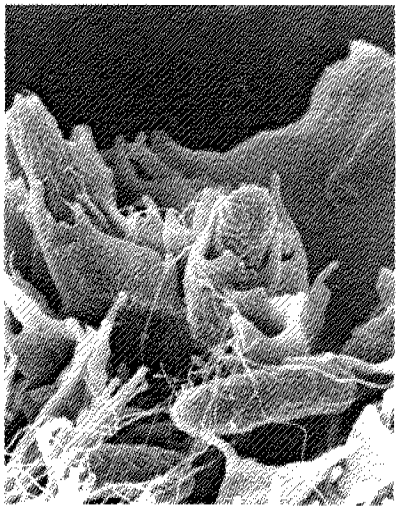


Figure 1.4: Scanning electron micrograph of *Salmonella* Typhimurium entering a Hep-2 cell [175]

Salmonella belongs to the family *Enterobacteriacae* and is closely related to *E. coli* and *Shigella* [132]. The genus *Salmonella* consists of the two species *Salmonella bongori* (V) and *Salmonella enterica*. The latter is divided into 6 subspecies (I, II, IIIa, IIIb, IV, VI) containing more than 2500 serovars [188, 231]. The majority of about 60 % belong to *S. enterica* subspecies I that is responsible for 99% of *Salmonella* infections [34, 231]. For differentiation of *Salmonella* three different types of antigens are used (Kauffmann-White scheme): *Salmonella* O antigens (somatic), H antigens phase 1 and 2 (flagellar), and Vi antigens (capsular) [34, 47, 231]. Nomenclature of *Salmonella* is

complex and different systems have been used in literature. In this work, *Salmonella enterica* subsp. *enterica* serovar Typhimurium is written as *Salmonella* Typhimurium or *S.* Typhimurium [34]. This is in agreement with the current system adopted by the Centers for Disease Control (CDC) and the WHO Collaborating Centre [34].

1.2.2 Virulence and pathogenesis of *Salmonella* spp.

Salmonella causes two distinct disease syndromes: typhoid fever and non-typhoid salmonellosis. *Salmonella* Typhi, *Salmonella* Paratyphi and *Salmonella* Sendai only infect humans and lead to enteric (typhoid) fever. This systemic illness is characterized by a low infection dose, a long incubation period (8 - 28 days), a duration of up to 30 days, and a mortality rate of 1 - 15 % [49, 175]. Symptoms are high fever, abdominal pain, watery diarrhea (or constipation) and occasionally cutaneous rose spots with underlying bacteremia [47, 49, 175]. For treatment, antibiotics are recommended. Usually, antibiotics such as chloramphenicol, ampicillin, and trimethoprim/sulfamethoxazole have been used. Due to the appearance of more and more antibiotic resistant *Salmonella* strains, research and development of alternative antibiotics has to be encouraged. At the moment fluoroquinalones are very effective [180], but resistence has already been observed [222].

Salmonellosis from non-Typhi *Salmonella* like *S.* Typhimurium or *S.* Enteritidis can occur in a wide range of animals and in humans. The infective dose is usually high (10^5 to 10^9 cfu, Chapter 1.2.3), incubation time is short (8 - 72 h) and case fatality is usually low (0.1 - 0.2%) [47]. Infection is normally restricted to the intestinal mucosa and mesenteric lymph nodes and is self-limiting within 3 - 7 days. However, excretion of *Salmonella* can last for 4 - 5 weeks or even several months [47, 49]. Symptoms are strong abdominal pains, diarrhea (watery and occasionally bloody) and nausea. Anorexia, vomiting and headache are also not uncommon and fever is possible in the first two days [47, 49, 175, 250]. Additionally, infection with non-tyhoidal *Salmonella* can lead to bacteremia and other complications, especially in children under one year of age, in elderly or immunocompromised persons such as HIV patients. For these people, mortality rate is much higher, and case antibiotic treatment is recommended. For enteritidis, an oral rehydration therapy is sufficient [47, 49].

Usually, salmonellosis is transmitted via contaminated food or water. First, bacterial cells have to combat with the adverse conditions of the low pH in the stomach. Some components of foods such as high fat or protein content protect bacteria and contribute to their survival. Additionally, *Salmonella* itself posses an adaptive acid-tolerance mechanism [76]. After stomach passage, bacteria reach the intestine and adhere to epithelial cells and M-cells via expression of different types of fimbriae or pili [50]. After adhesion, *Salmonella* cells induce bacterial-mediated endocytosis in-

cluding membrane ruffling and macropinocytosis. This step is mainly influenced by proteins of the type III secretion system 1 (TTSS-1). In general, protein secretion systems like TTSS are responsible for coordinated secretion and translocation of effector proteins from the bacterial cell into an eucaryotic host cell [236]. Structurally and functionally, they are related to flagella assembly systems containing more than 20 subunits. They are located in the inner and outer membrane, cytosol and periplasm of the bacterial cell [98]. For *Salmonella*, at least two systems are known, TTSS-1 and TTSS-2. Secreted proteins consist of two groups, the translocators and the translocated effectors. They are not considered as classical toxins, because they do not have typical receptor-binding domains [236]. Genectic information for these proteins and the TTSS-1 apparatus are grouped together within a distinct region on the chromosome - the *Salmonella* pathogenity island-1 (SPI-1) [175].

In addition to this invasion, *Salmonella* induces production of the cytokine interleukin-8 (IL8) and the pathogen-elicited epithelial chemoattractant (PEEC) by eptithelial cells [162, 250]. This leads to transmigration of neutrophils into the intestinal lumen and is associated with an increase in vascular permeability and necrosis of the ileal mucosa. Finally, inflammation causes massive fluid efflux into the lumen [175, 250]. Additionally, prostaglandins are released by neutrophil infiltration and trigger the increase of adenylate cylase activity in intestinal epithelial cells. This also leads to higher Cl^- secretion, inhibition of Na^+ absorption, and therefore fluid efflux. Besides, SPI-1 encoded effectors increase intracellular inositolphosphate level resulting also in Cl^- secretion [50]. It is controversially discussed if a cholera-like enterotoxin plays an important role in fluid accumulation in the intestine [50, 236, 250]. *Salmonella* deletion mutants for this toxin showed no difference in fluid secretion compared to wild-type strains. It rather seems that TTSS-1 is the prime virulence factor for enterocolitis [175, 236, 250].

After passing the intestinal layer, invasive *Salmonella* strains can be phagocytosed by macrophages and thereby disseminated over the whole body. Recent data suggest that *Salmonella* is transported from the gastrointestinal tract to the bloodstream by CD18-expressing phagocytes [230]. For survival in macrophages, different defense mechanisms are required to resist or evade antimicrobial effectors such as reactive oxygen or antimicrobial peptides. The two-component response regulator PhoP/PhoQ is essential for survival. The system regulates more than 40 genes involved in resistance against any adverse conditions in the macrophages. TTSS-2 encoded by SPI-2 is also necessary [98, 175].

Overall, 12 SPI have been described at present. Contributions to pathogenesis can be different, but several common motifs are shared [102]. In addition to the chromosome, virulence plasmids (45 - 95 kbp) of *Salmonella* encode further virulence fac-

tors. Gene products seem to enhance intracellular growth and systemic dissemination [43, 47]. Additional virulence factors are the lipopolysaccherides (LPS) of the outer membrane, siderophores and antibiotic resistance plasmids (R-plasmids). The ability to evade the host complement system is directly linked to the length of serotypic (O) chains of LPS [49]. Siderophores are essential for collecting the necessary inorganic iron needed for growth [49]. R-plasmids are often inter- and intra-generically transferred, leading to more and more multiply antibiotic resistant *Salmonella* strains (Chapter 1.2.3) [49].

1.2.3 Epidemiology and occurrence of *Salmonella* spp. in foods

To date, about 30,000 to 40,000 cases per year of non-typhoid salmonellosis are reported to the Centers for Disease Control and Prevention in the USA [91, 92, 109, 110, 115, 165]. However, taking into account the degree of under-reporting, approximately 1.4 million infections per year are estimated, resulting in about 400 - 600 deaths [17, 165, 233]. The total cost associated with *Salmonella* infections is estimated to be US$ 3 billion annually in the USA [17]. More than 50% of human cases in the USA are caused by the three serovars *S.* Typhimurium, *S.* Enteritidis, *S.* Newport [115]. In Germany, approximately 60,000 cases are reported annually, on average [21]. It is assumed that this figure represents only 10 - 20 % of real salmonellosis. Furthermore, 90% of foodborne infections are estimated to be caused by *Salmonella* [10]. Predominant serovars are again *S.* Enteritidis and *S.* Typhimurium [190]. In Switzerland, there are on average 2,200 cases reported annually [19]. Most important serovars are again *S.* Enteritidis (44% in 2005) and *S.* Typhimurium (21% in 2005) [170]. According to these data, non-typhoid salmonellosis is concerned to be the most common cause of foodborne death worldwide [190].

The primary natural habitat of *Salmonella* is the intestinal tract of animals (birds, reptils, insects and mammals) and humans. *Salmonella* is also widespread in nature including soil and water [29, 47]. Consequently, the main food vehicles are animal-derived products or vegetables contaminated by feces. Major food-borne outbreaks are associated with meat products (pork, beef, turkey, chicken), dairy products (milk, chocolate, ice cream, soft cheese), eggs and egg products, as well as vegetables and fruits (sprouts, honey melon) [29, 47].

The infectious dose necessary to cause salmonellosis is estimated to be 10^5 to 10^9 bacteria. However, several outbreaks show that it can be significantly lower (10 - 100 cfu). The minimum dosis depends on the protective effects of the particular food (Chapter 1.2.2), the virulence of the strain, and the physiological state of the infected person [29, 47, 50, 50, 114]. The legislation regarding the presence of *Salmonella* in

foods is very strict in most countries. In Switzerland, e.g., no *Salmonella* must be detected in 10 g or 25 g of the particular food, for example ready-to-eat foods, minced meat, raw-milk products, etc. [16].

In the last decades, the occurrence of multiply antibiotic resistant *Salmonella* strains has dramatically increased in various countries [190, 222, 231]. One example is phage type *S.* Typhimurium DT104. This strain is extremely virulent and shows resistance against various antibiotics including ampicillin, choramphenicol, streptomycin, sulphonamides, and tetracycline [190, 222, 231]. It was first detected in the United Kingdom. Then, it has emerged in several other countries such as Germany, The Netherlands, in the United Arab Emirates, and the USA. This illustrates the potential of these strains to be disseminated worldwide and to become a global threat for human health.

1.3 Bacteriophages

1.3.1 Definition, morphology and taxonomy of bacteriophages

Bacteriophages are viruses that only infect bacterial cells. They were independently discovered by Frederick Twort in 1915 and Felix d'Herelle in 1917 [37, 214]. F. d'Herelle proposed the term "bacteriophage" including the words "bacteria" and "phagein" (to eat or devour, in Greek), and systematically investigated the nature of bacteriophages. Bacteriophages are classified in one order, 13 families, and 31 genera. Phages consist mostly of nucleic acid (DNA or RNA), and a protein shell. Virions are tailed, polyhedral, filamentous, or pleomorphic [1, 2]. More than 96% of all known phages are tailed phages and belong to the order *Caudovirales*. Their heads (capsids) are mostly regular icosahedra or sometimes prolate. Capsids consists of 5 - 6 subunits (capsomers). Phage tails possess terminal adsorption structures such as base plates, spikes, or fibres [1, 2]. Tailed phages are subdivided into three families: *Myoviridae* (25%), *Siphoviridae* (61%) and *Podoviridae* (14%). *Myoviridae* have contractile tails, *Siphoviridae* have long, non-contractile tails, *Podoviridae* have short, non-contractile tails. Tailless phages are polyhedral, filamentous or pleomorphic, and include less than 4% of all known bacterial viruses and are classified into 10 families [1, 2]. Phage propagation cycles can be lytic or lysogenic (Figure 1.5). Virulent phages are only able to use the lytic life cyle in which host cells are destroyed. This life style is characterized by adsorption, infection, DNA replication, protein synthesis, particle assembly and release by host lysis. Temperate phages, however, can perform additionally the lysogenic life style. In this case, phage DNA can be introduced into the host genome after DNA was injected

into the host cell, and stay in a dormant stage. The phage genome integrated into the host genome is called prophage. The dormant stage is ended spontaneously or under the effect of inducing agents such as UV light or mitomycin C, and phages can enter the lytic life cycle [93].

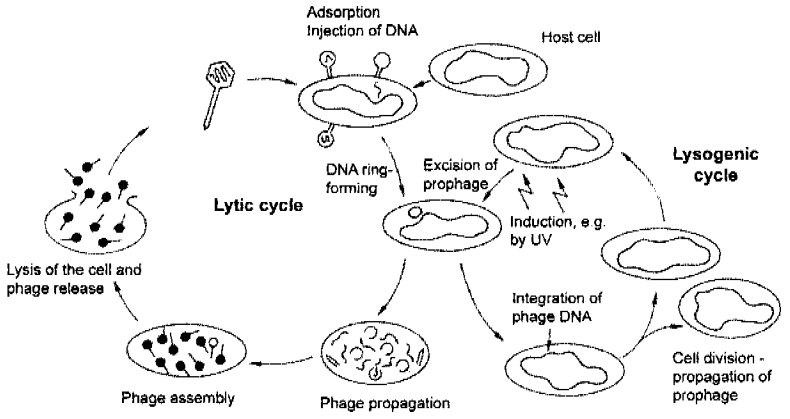


Figure 1.5: Lytic and lysogenic life cycle of bacteriophages (adapted from [125]).

1.3.2 *Listeria* bacteriophages

Bacteriophages of *Listeria* were first described in 1945 [146]. To date, more than 400 *Listeria* phages have been isolated and characterized, infecting *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*, but not *L. grayi*. Most of them belong to the *Siphoviridae* family, only few to the *Myoviridae* family. All *Listeria* phages have double-stranded DNA with genomes ranging from 36 to 135 kbp. They are well adapted to their host bacteria, and replicate over a wide temperature range (10 - 37°C). *Listeria* phages are strictly genus specific. Temperate phages infect specific serovar groups whereas virulent ones can usually infect species from all serovars [147]. In this work, the virulent *Listeria* phages A511, P100 and P35 were evaluated for control of *Listeria monocytogenes* in foods (Figure 1.6).

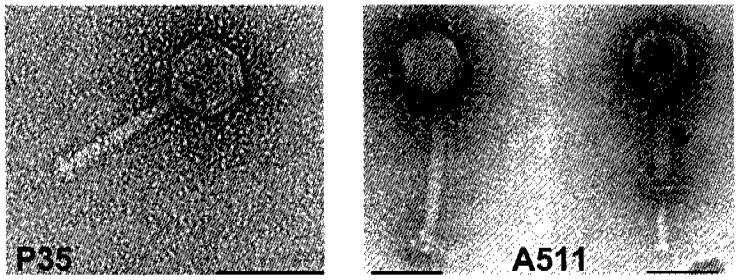


Figure 1.6: Electron micrograph of *Listeria* phages P35 [unpublished] and A511 [251] (bars=100nm).

Bacteriophage A511 was isolated in 1990 from a sample taken at a sewage purification plant [146]. A511 shows a shorter latent phase compared to temperate phages, a large burst size of 40 virions per cell and its genome is 134 kbp in size [147]. Phage A511 as well as P100 are characterized by strict specificity for the genus *Listeria* and their wide host range within the genus. Approximately 95% of *L. monocytogenes* strains belonging to serovar 1/2 and 4 are susceptible [38, 146]. Phage P35 was isolated from silage [106]. It belongs to the familiy of *Siphoviridae* and is also considered as a virlulent phage, because it's genome lacks the module of lysogeny control [58]. P35 can only infect serovar 1/2 of *L. monocytogenes* [106].

1.3.3 *Listeria* bacteriophage endolysins

Endolysins are bacteriophage-encoded enzymes produced during the late phase of gene expression in the lytic cycle of phage propagation in order to degrade the bacterial peptidoglycan and release new progeny virions. They can be divided into five classes (Figure 1.7): (i) N-acetylmuramidases (lysozymes), (ii) endo-β-N-acetylglucosaminidases, (iii) lytic transglycosylases, (iv) endopeptidases and (v) N-acetylmuramoyl-L-alanine amidases.

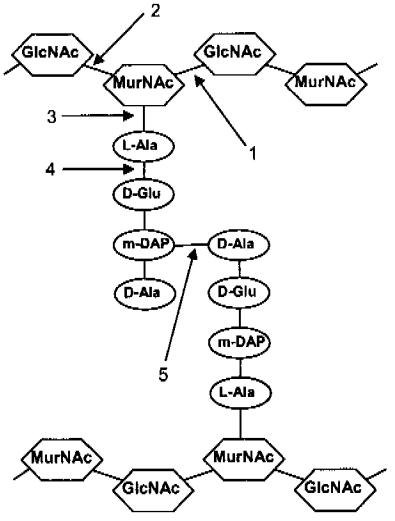


Figure 1.7: Fine structure of type A1γ-peptidoglycan. Main components are the two alternating amino sugars N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). They are cross-linked by interpetide bonds. These tetrapeptides are composed of L-alanine (L-Ala), D-glutamic acid (D-Glu), mesodiaminopimelic acid (m-DAP), and D-alanine (L-Ala) with crosslinks between m-DAP and D-Ala. The sites of cleavage by major classes of endolysins are indicated with numbers. 1, muramidases and transglycosylases; 2, glucosaminidases; 3, amidases; 4 and 5, endopetidases; adapted from [145].

All endolysins are hydrolases, except for transglycosylases. Endolysins have a modular structure with distinct functional domains. *Listeria* phage endolysins consist of two domains. The enzymatically active domain (EAD) is located at the N-terminus, and the cell-wall binding domain (CBD) is located at the C-terminus [32, 145]. Most endolysins show a high specificity of lytic activity. In the present work, endolysins of *Listeria* phages A511, A118 and A500 were used for control of *L. monocytogenes* in foods. Endolysin PLY118 is a 30.8 kDa protein, acts as a L-alanoyl-D-glutamate-peptidase and is specific for serovars 1/2, 3 and "7". The peptidase PLY500 has a molecular weight of 33.4 kDa and is specific for serovars 4, 5 and 6. In contrast to the first two enzymes, PLY511 is an N-acetylmuramoyl-L-alanine amidase of 36.5 kDa and is able to bind and to lyse almost all strains from all *Listeria* serovars [151, 242].

1.3.4 *Salmonella* bacteriophages

In this work *Salmonella* phage FO1-E2 was used for control of *S.* Typhimurium in foods. Bacteriophage FO1 belongs to the family of the *Myoviridae*, and was first referenced by Felix and Callow (Figure 1.8) [67]. It is characterized by a wide host range within the genus *Salmonella* and also by a high specificity for this genus.

Only few other Gram-negative bacteria like, e.g., *E. coli*, are sensitive to FO1 [105, 241]. Prerequisite for phage infection of *Salmonella* is the availability of the terminal N-acetylglucosamine side branch of the lipopolysaccharid core [112]. Therefore strain mutants which make incomplete core LPS are resistent against phage FO1 [112].

Figure 1.8: Electron micrograph of *Salmonella* phage Felix FO1 (bar=50nm) [3].

1.3.5 Application of bacteriophages

Bacteriophages have been applied for therapy of bacterial infections since they have been discovered. The initial works on phage therapy, however, delivered unreliable

and inconsistent results due to a poor understanding of bacterial pathogenesis and the nature of phage-host interactions. Furthermore, after the discovery of antibiotics, research in phage therapy has almost died out in the west, and only continued in Eastern Europe and the former Soviet Union. With the increase of multi drug resistant bacteria, however, bacteriophage based therapies of humans and animals have become of new interest in recent years. There is a considerable potential for the use of bacteriophages at least against some kinds of bacterial infections in humans and/or animals [26, 27, 37, 40, 44, 160, 210, 214, 216].

Furthermore, the potential for use of phages in research and biotechnology has been recognized in the last years. Bacteriophages have been used as bacterial typing or detection systems, as cloning vectors, and as sources of enzymes. Furthermore, the new technique of phage display provides a powerful tool for the characterization and optimization of antibodies and other biomolecules. In the environment, phages have been applied as tracers, as indicators of pollution, for monitoring and validation, and for the control of biofilms [44, 160].

The use of bacteriophages for control of foodborne pathogens represents an innovative and promising approach to the problem of microbial food contamination. Bacteriophages can be considered as the natural enemies of bacteria and offer a number of advantages: (i) they are designed to kill their host cells, (ii) they usually are highly specific and do not cross species or genus boundaries, (iii) they are self-replicating and self-limiting, and (iv) they are ubiquitous in nature and are normal commensals of humans and animals [38, 89, 160]. Accordingly, the idea of phage application against spoilage bacteria and/or pathogens in foods has also received more interest in research in the last years. The specificity of phages allows a selective and well targeted modifying of the bacterial flora in foods. Therefore, undesirable bacteria can be eliminated, but not the desirable ones. This is important for product quality, e.g., of fermented foods [87]. Bacteriophages are highly distributed in the environment and they are the most abundant self-replicating units in the world. Estimations assume 10^{30} to 10^{32} phages worldwide [35, 42]. Phages have been isolated from a wide range of different foods [121, 244, 245]. Examples are ground beef [111, 122, 244, 245], pork, chicken and other meat products [111, 122], chilled and frozen crabs [57], fermented dairy products like cheese or yoghurt [123, 213], as well as lettuce or mushrooms [103, 121]. Therefore, they are part of the natural microbiological flora of foods. These facts argue for the evaluation of the specific use of virulent bacteriophages as new biocontrol agents against pathogens in foods. Recent food studies support this approach. Phage treatment in foods was mostly successfully tested for *Campylobacter* [82, 142], *E. coli* [174], *Pseudomonas* [62, 87], *Brochothrix* [90], *Salmonella* [135, 166, 179, 243] and *L. monocytogenes* [38, 60, 136, 138].

1.4 Aim of this study

The aim of this study was a comprehensive evaluation of bacteriophages and their lytic enzymes for control of two bacterial pathogens in food. First, biocontrol of *Listeria monocytogenes* was investigated in foods. Here, the application of different *Listeria* bacteriophages (A511, P100 and P35) as well as *Listeria* phage endolysins against various strains of *L. monocytogenes* was investigated in different foods. Various parameters such as storage temperature and time, initial bacterial cell and phage concentrations, and different phage application protocols were studied.

Second, the application of phage FO1-E2 was tested to combat *S.* Typhimurium in different foods at two different storage temperatures. For this purpose, determination of *Salmonella* cell counts in foods had to be improved, by generation of antibiotic-resistant phage-indicator strains.

2 Material and Methods

2.1 Materials, microorganisms and foods

2.1.1 Composition of media, buffers and other solutions

For composition of media, buffers and other solutions and the abbreviations used in this study see appendix A to C.

2.1.2 Bacteriophages

All bacteriophages used in this study are listed in Table 2.1.

Table 2.1: Bacteriophages

Bacteriophage	Host	Virulent /Temperate	Source	Purpose
A511	*Listeria*	Virulent	[146]	Food experiments
P100	*Listeria*	Virulent	[38, 146]	Food experiments
P35	*Listeria*	Virulent	[106]	Food experiments
FO1-E2	*Salmonella*	Virulent	Lab.	Food experiments

Lab. = Laboratory stock

2.1.3 Bacterial strains

All bacterial strains used in this study are listed in Table 2.2. Strain CNL 103/2005 was isolated in 2005 from contaminated swiss soft cheese ("Tomme") that caused a minor listeriosis outbreak. More than 10 people were involved, 3 elder patients died and two miscarriages occurred. The causative strain belongs to servovar 1/2a and was subsequently included in this study.

The *Listeria* strains were maintained on BHI agar plates incubated at 30°C for 1 - 2 days and stored at 4°C for 2 - 4 weeks. For all experiments *Listeria* was grown in BHI 1/2 medium, at 30°C for approximately 15 h. Overnight cultures were subsequently diluted 1:5 in fresh medium (BHI 1/2) and incubated for another 2 - 3 h at 30°C. Strains of *Salmonella* Typhimurium and *E. coli* were maintained on LB agar plates containing the appropriate antibiotic. Liquid cultures were grown in LB at 37°C under shaking for approximately 15 h. Overnight cultures were diluted tenfold in fresh LB medium and incubated for another 1 - 3 h at 37°C. For *Staphylococcus carnosus*, B medium was used.

Table 2.2: Bacterial strains

Strain	**Source/ First description**	**Purpose**
Listeria ivanovii WSLC 3009	SLCC 4769	Propagation of phage A511
Listeria ivanovii WSLC 3009 cm^R (SV 5)	Lab.	Selective plaque assay, indicator strain
Listeria monocytogenes WSLC 1001 (SV 1/2c)	ATCC 19112	Food experiments
Listeria monocytogenes WSLC 1685 (Scott A) (SV 4b)	Clinical isolate [72]	Food experiments
Listeria monocytogenes CNL 103/2005 (SV 1/2a)	Soft cheese [31]	Food experiments
Salmonella Typhimurium DB7155	[247]	Host strain of phage FO1-E2
Salmonella Typhimurium DB7155 (pKD46)	This work	Red Recombinase system
Salmonella Typhimurium DB7155 cm^R	This work	Food experiments, chloramphenicol resistance
Salmonella Typhimurium DB7155 stm^R	This work	Food experiments, streptomycin resistance
Escherichia coli BW25113/pKD46	CGSC [51]	Plasmid pKD46, Red Recombinase system
Escherichia coli BW25141/pKD3	CGSC [51]	Plasmid pKD3, chloramphenicol resistance
Escherichia coli strains JM109	DSMZ 3129 [248]	Production of phage endolysins
Staphylococcus carnosus (pPSHG7-*ply*511)	Genmedics / This work	Production of phage endolysins
Staphylococcus carnosus TM300	Genmedics / This work	Control strain

[ATCC=American Type Culture Collection, USA], [CNL=Centre National de Référence des Listeria, CH], [DSMZ=German Collection of Microorganisms and Cell Cultures GmbH], [SLCC=Special *Listeria* Culture Collection, D], [WSLC=Weihenstephan *Listeria* Collection, D], [CGSC=Coli Genetic Stock Center, USA], Lab. = Laboratory stock

2.2 Propagation of bacteriophages

For large scale propagation, bacteriophage A511 was propagated using the liquid culture method. Here, 20 ml of an overnight culture of *Listeria ivanovii* WSLC 3009 and approximately 1×10^7 pfu of phage A511 were added to 1 l of prewarmed (30°C) BHI 1/2. The culture was incubated at 30°C for 3 - 4 h. Then, additional 2×10^9 pfu were added. After incubation at 30°C for another 3 - 4 h, the solution cleared almost completely due to apparent lysis. The liquid was centrifuged (6,000 g, 15 min, 4°C) and the supernatant sterilized by vacuum filtration using bottle top filters (0.22 μm PES membrane). For concentration, phage particles were precipitated by adding 0.5 M NaCl and 10% polyethylene-glycol (Molecular mass = 8,000) at 4°C for 15 h. After centrifugation (10,000 g, 15 min, 4°C) the pellet was redissolved in 10 ml of a 1:1 mixture of BHI 1/2 and SM buffer. The phage suspension was dialyzed (50 kDa cut-off) against two changes of 2 l buffer (1:1 mixture of SM buffer and BHI 1/2), sterilized by filtration and stored at 4°C.

Phage P35 was propagated using the soft agar double layer technique [4, 217]. In short, forty LC agar plates with semi-confluent lysis were scraped by adding 8 - 10 ml SM buffer to each plate. The eluate was kept on 4°C for 4 - 5 h to enable diffusion of phage out of the soft agar. After centrifugation (7,000 g, 20 min, 4°C) and filter sterilization, phages were precipitated, resuspended (SM buffer), dialyzed (2 l 1:1 mixture of SM buffer and LB), and stored at 4°C.

Salmonella phage Felix FO1-E2 was propagated similar to phage P35, but LB agar and LB soft agar were used. Additionally, the method was modified. The soft agar was not scraped from the plates. Instead, added SM buffer was left overnight on the plates under slightly agitation. Then, buffer was removed and sampled. Phages were concentrated and purified as described before.

2.3 Production of *Listeria* phage endolysins

2.3.1 Production of recombinant endolysins HPL118, HPL500 and HPL511 in *E. coli*

Production and one-step purification of phage endolysins PLY118, PLY500 and PLY511 have already been reported [150]. The genes *ply*118, *ply*500 and *ply*511 have been cloned downstream of a hexa-His box in expression vector pQE-30 (Qiagen) in *E. coli*.

Precultures of *E. coli* JM 109 (pHPL118), *E. coli* JM 109 (pHPL500), and *E. coli* JM 109 (pHPL511) were prepared in 5 ml LB containing 100 μg/ml ampicillin at 37°C and shaken at 180 rounds per minute (rpm). After 8 - 10 h bacteria were transferred to 100 ml LB-PE medium containing 100 μg/ml ampicillin to ensure

selection of plasmid-bearing cells. Cultures were incubated for 16 h at 37°C and 180 rpm. For overexpression 10 ml of overnight cultures were transferred to 500 ml of prewarmed LB-PE medium (30°C) containing 100 μg/ml ampicillin and incubated at 32°C or 35°C with shaking (180 rpm). After measurement of an optical density of 0.5 at 600 nm (OD_{600}), protein expression was induced by adding IPTG (1 mM). Cultures were incubated for another 4 h. Samples for a sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) were taken at 0 h, 1 h, 2 h, and 4 h post induction, and stored at -20°C. Cells were harvested after 4 h by centrifugation (7,000 g, 4°C, 15 min). Each pellet out of 500 ml expression medium was resuspended in 10 ml buffer A and stored overnight at -20°C. After thawing cells were disrupted by double passage through a French pressure cell (SLM Aminco, 20 K cell) at 100 MPa, and cell debris was removed by centrifugation (20,000 g, 40 min, 4°C).

The supernatant containing the endolysin (crude cell extract) was sterilized by filtration(0.22 μm PES membrane). It was purified by metal chelate affinity chromatography with nickel-nitrilotriacetic acid superflow resin (Ni-NTA) in a fast performance liquid chromatography system ("Äkta Purifier", Amersham Biosciences, CH). Proteins with neighboring histidine residues were bound tightly but reversibly to the Ni-NTA ligands of the column.

During the purification, further samples were taken at different steps and stored at -20°C for SDS-PAGE: the flow-through fraction containing unbound proteins, the wash fraction containing loosely bound proteins, and the eluate containing the endolysin and other tightly bound proteins. Proteins from 15 ml eluate were concentrated to a final volume of 3 - 5 ml using centrifugal concentrators (10 kDa cut-off, PES membrane) and dialyzed (10 kDa cut-off) against two changes of 2 l of dialysis buffer at 4°C for 16 h. Aliquoted proteins were stored at -20°C until use.

2.3.2 Production of endolysin PLY511 by *Staphylococcus carnosus*

Strain *Staphylococcus carnosus* (pPSHG7-*ply*511) (Tr. 81) was provided by genmedics (Germany). The strain is an auxotrophic mutant and carries the plasmid pPSHG7 with *ply*511. Overexpression of endolysin PLY511 was induced by galactose. The expression medium (1.5 % galactose) was inoculated 100fold with cells from an overnight culture in B-medium and shaken at 37°C and 180 rpm for 15 h to 17 h. After overexpression, bacterial cells were removed by centrifugation (7,000 g, 10 min, 4°C). The supernatant containing endolysin PLY511 was filter sterilized (0.22 μm, PES membrane) and kept on ice. Purification and concentration was either done by ammonium sulfate precipitation (fraction 50-100% ammonium sulfate) or ultrafiltration (10 kDa, PES membrane).

For optimization of endolysin production, different parameters were tested: (i) ad-

dition of the inducer after 0 h or 2 h, (ii) expression time for 10 to 17 h, (iii) addition of glycerol of 0 % to 1 %, and (iv) galactose concentration of 0.5 to 2 %. The lytic activities of PLY511 in the supernatants were determined after the different production protocols and compared (Chapter 2.3.3).

2.3.3 Determination of endolysin concentration and lytic activity

Protein concentration was determined at 280 nm using a microscale spectrometer ("Nanodrop ND1000"; Nanodrop Technologies, USA). Final endolysin concentrations were calculated according to their estimated purity and their molar extinction coefficients at 280 nm calculated from amino acid sequence data (software Vector NTI, Invitrogen, CH) [80]. Following formula was used:

$$A = \epsilon \cdot c \cdot d$$

where A is the absorbance, ϵ is the molar extinction coefficient [M^{-1}/cm^{-1}], c the molarity [mol/l], and d the cuvette thickness [cm]. The molarity c is the ratio of protein concentration c_p [g/l] and the molecular mass M_r [kDa]. Table 2.3 summarizes extinction coefficients and molecular mass of all used endolysins. SDS-PAGE was used to estimate the purity of endolysin preparations.

Table 2.3: Molar extinction coefficient and molecular mass of different *Listeria* phage endolysins

Endolysin	**Extinction coefficient** ϵ [M^{-1}/cm^{-1}]	**Molecular mass** M_r [kDa]
HPL118	43,360	34.819
HPL500	93,780	37.872
HPL511	53,010	32.279

To determine enzymatic activity, lysis assays were performed in a spectrophotometer (Biochrom, GB). One unit (1U) of lytic enzymatic activity was defined as the decrease of OD_{600} by 0.01/min of a substrate cell suspension in a volume of 1 ml at 30°C and pH 8.0 [149]. Screening cells of the appropriate strain (WSLC 1001 or Scott A) were mixed with PBS in a semi-micro cuvette resulting in an OD_{600} of 1.0±0.2. The endolysin sample was added, and OD_{600} was measured every 15 sec for 600 sec. Assays were repeated 1 - 3 times. Photometric curves were normalized to the starting value of OD_{600}=1.0 and corrected by the subtraction of the corresponding control values (cells in PBS). The curves were fitted using the following function

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

Software SigmaPlot 9.0 was used for curve fitting and a Visual Basic macro (Microsoft Excel) for searching the maximum slope of the function, which corresponds to the enzymatic activity according to the definition given above [129].

2.4 Food experiments with *Listeria monocytogenes*

Foods used in in this study were purchased at local groceries. All foods were first tested for *Listeria* spp. according to IDF-Standard 143A:1995 [6] or EN ISO 11290-1 [7]. Remaining samples were divided in equivalent portions and stored in sterile polypropylene bags at -80°C . Fresh foods like iceberg lettuce, cabbage or unripened cheeses were analyzed in parallel to the experiment. Briefly, samples of 25 g were homogenized with 50 ml citrate buffer in sterile polypropylene bags using a stomacher laboratory blender (Seward, UK). The suspension was mixed with TSB-ANC enrichment broth and incubated at 30°C for 48±2 h. One loop-full (approximately 10 μl) was directly streaked on Oxford agar plates and further incubated at 37°C for 48 h. Typical *Listeria* colonies formed brown-green colored colonies with a black halo. In the case of *Listeria* positive samples, the food sample was discarded and not used in the experiments.

2.4.1 Control of *Listeria monocytogenes* during the storage of foods

First, experiments were performed with different types of foods contaminated with approximately 1×10^3 cfu/g or cfu/ml of *L. monocytogenes* WSLC 1001, Scott A or CNL 103/2005, treated with approximately 3×10^8 pfu/g or pfu/ml of phage A511 and stored at 6°C for 6 days (Figure 2.1).

For this purpose, foods were thawed overnight at 4°C prior to the experiments. For inoculation of the foods an overnight culture of *L. monocytogenes* was diluted fivefold in fresh medium and incubated for 2 - 3 h at 30°C. OD_{600} was determined and the culture was subsequently diluted in PBSm buffer to the number of cells required. The total inoculum was 150 μl for 10 g of solid food or 10 μl to 50 μl for 10 ml of liquid food samples. Samples were stored at 6°C for 1 hour while *Listeria* cells adapted to the conditions in the foods. Then, phage A511 was applied in SM buffer to one sample (phage treated sample) and pure SM buffer to the control sample. The total quantity

was again 150 μl for 10 g or 10 μl to 50 μl for 10 ml food sample. Foods were stored further at 6°C.

Listeria cell concentration was determined immediately after inoculation with *L. monocytogenes*, phage concentration immediately after application, and both after 6 hours and 1, 2, 3 and 6 days after phage application. For this purpose, 10 g of solid foods were homogenized with 90 ml Citrate buffer in sterile polypropylene bags using a stomacher laboratory blender (Seward, UK). When foods were contaminated with lower levels (1×10^2 cfu/g) of *Listeria*, 45 ml were used. For determination of *Listeria* cell counts, volumes of 1 ml of the homogenate were surface plated on 145 mm Oxford agar plates, or 100 μl of decimal dilutions were plated on 94 mm plates, in duplicates. Liquid foods were directly diluted and plated. Plates were incubated for 48 h at 37°C until typical *Listeria* colonies could be enumerated, and cell concentrations were calculated.

Phage concentrations were determined using the soft agar double layer technique, in duplicates [4]. To avoid microbial contamination of the plates, the chloramphenicol-resistant host strain *Listeria ivanovii* WSLC 3009 cm^R was used as indicator strain. 100 μl of decimal dilutions of the food sample were mixed with 200 μl of log-phase host cells and 4 ml BHI soft agar containing 7.5 μl/ml chloramphenicol, in dublicates. The suspension was poured on BHI agar plates and incubated overnight at 25°C. Resulting plaques were counted, and phage concentrations were calculated.

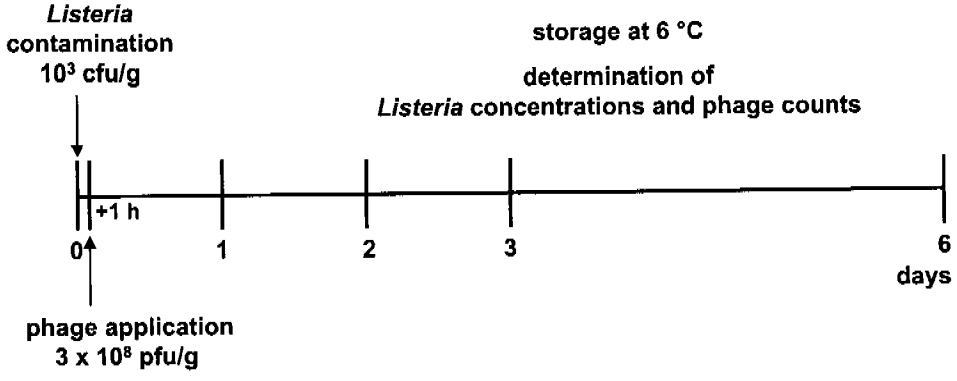


Figure 2.1: Procedure of food experiments with *L. monocytogenes* and phage A511 over 6 days at 6°C.

To evaluate the influence of parameters such as storage temperature or phage concentration, several conditions were tested: (i) the initial contamination level was reduced to 1×10^2 cfu/g or cfu/ml, (ii) storage time was extended to 13 days, (iii) storage temperature was shifted to 20°C, (iv) different phage concentration levels were used (3×10^8 pfu/g, 3×10^7 pfu/g, 3×10^6 pfu/g), (v) *Listeria* phage P35 and phage P100 were applied.

In order to optimize control effect of phage A511 on *L. monocytogenes* on smoked salmon, phage treatment was modified. First, application of phage A511 (3×10^8 pfu/g) was done (i) 1 h before *Listeria* contamination or (ii) 1, 7, 25 h after *Listeria* contamination. Furthermore, smoked salmon was dipped into the phage suspension, instead of pipetting the phage onto the surface of salmon. Additionally, smoked salmon was treated as solid blocks instead of being sliced into 10 g-pieces. Finally, a higher initial phage concentration of 2×10^9 pfu/g was used. The last two application protocols were also tested with *L. monocytogenes* Scott A.

2.4.2 Control of *Listeria monocytogenes* during the ripening of soft cheeses

Unripened soft cheeses were obtained from a swiss dairy company. Cheeses were ripened under laboratory conditions as described previously [215]. They were placed on racks of stainless steel in glass desiccators. For a relative humidity of the air of 95 %, 1 l of a 7.95 % sodium chloride solution was poured onto the bottom of the desiccators.

2.4.2.1 White mold soft cheese

White mold soft cheeses (1.7 kg, 55 % fat dry matter, 22 cm diameter) were obtained post-brining, from a swiss industrial cheese production company. They were divided into 12 equal pieces, and each piece was further cut horizontally in half. Only the upper surface (24 cm^2) was treated.

In order to achieve a concentration of about 1×10^3 cfu/cm^2, the subculture of Scott A was diluted, and a volume of 50 μl was spread onto the surface and rubbed in using sterile gloves. Desiccators were stored at 12 - 13°C for 1 h. Then, the cheeses were treated with A511 by rubbing the phage suspension (100 μl) over the cheese surface. Control cheeses were only treated with SM buffer. Cheeses were ripened for 10 days at 12 - 13°C. At day 10, they were packed in aluminum foil and stored at 6°C for further 10 days. The whole procedure is shown in Figure 2.2.

Determination of *Listeria* cell count, phage titer and pH was done immediately after treatment of the cheeses, and after 1, 3, 6, 8, 10, 13, 15, 17 and 21 days. At each measurement 3 cheeses were analysed. For this purpose, 30 g (24 cm^2) were cut from the surface and homogenized with 90 ml citrate buffer in sterile polypropylene bags using a stomacher laboratory blender (Seward, UK). Determination of cell count and phage count was done as described in Chapter 2.4.1. The pH was measured at five different places on the surface using a surface electrode (InLab426, Mettler Toledo, Germany).

Phage application was done by adding (i) 3×10^8 cfu/cm^2 1 h after contamination,

(ii) 3×10^8 cfu/cm^2 1 h and 20 h after contamination, and (iii) 1×10^9 cfu/cm^2 1 h after contamination.

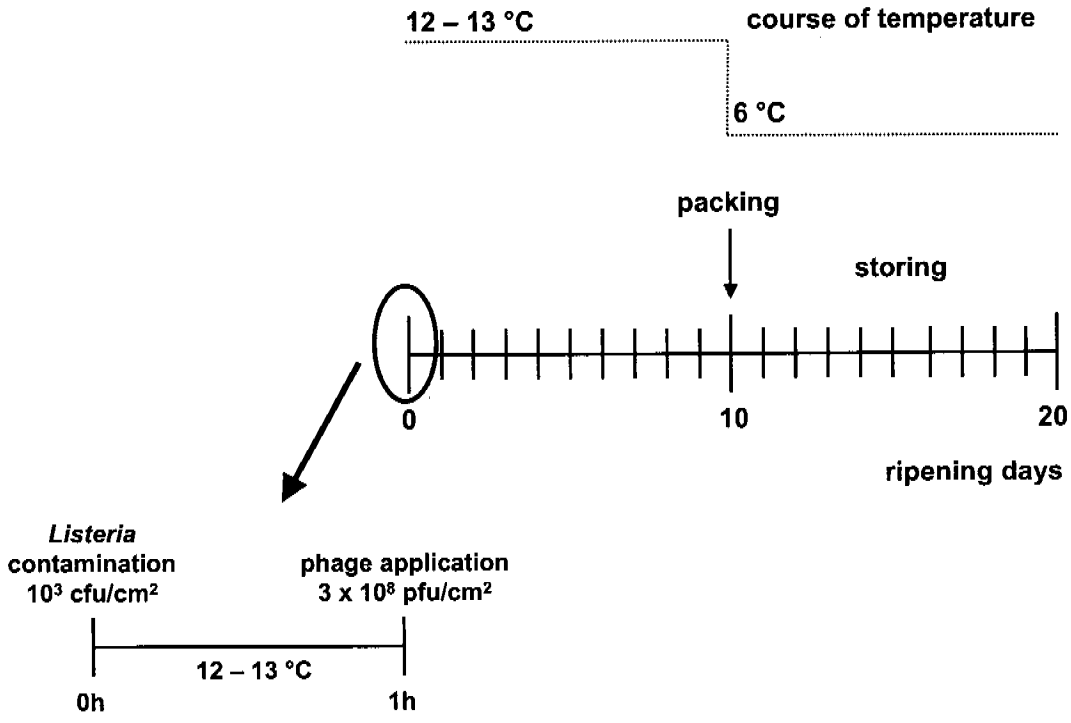


Figure 2.2: Ripening and treatment of white mold cheeses using *L. monocytogenes* and phage A511

2.4.2.2 Red smear soft cheese

Red smear soft cheeses (200 g, 55 % fat dry matter, 8.5 cm diameter) were also obtained post-brining, from a swiss industrial cheese production company. They were cut into four equal pieces.

For contamination, *Listeria monocytogenes* strains Scott A or CNL 103/2005 were used. Figure 2.3 illustrates the procedure. Using sterile gloves, cheeses were dipped into buffer (PBSm) containing *Listeria* in order to achieve a final concentration of about 1×10^3 cfu/cm^2. The liquid was manually rubbed over the whole cheese surface. Cheeses were placed back into the desiccators and stored for 1 h at 12 - 13°C. Then, phages or SM buffer were added to the smearing solution. Cheeses were dipped into the smearing solution and the liquid was manually rubbed over the whole cheese surface using sterile gloves. Smearing solution contained sodium chloride (3.0±0.3 %) and a commercial ripening culture (OFR9, Danisco, Denmark) ($1 - 4 \times 10^7$ cfu/ml). The ripening culture consisted of *Brevibacterium casei*,*Brevibacterium linens*, *Debaryomyces hansenii*, *Candida utilis*, and *Geotrichum candidum*. In general, cheeses were smeared three times (day 0, 3 and 6) and turned upside-down every 1 - 3 days. Cheeses were

ripened for 11 days at 12 - 13°C. At day 11 cheeses were packed in aluminum foil and stored at 6°C for further 11 days.

Listeria cell counts, phage counts and pH were determined immediately after treatment of the cheeses and after 1, 3, 6, 8, 12, 15, 19, and 22 days. At each measurement three cheeses were analyzed. For this purpose, 20 g (28 cm^2) were cut from the surface and homogenized with 180 ml citrate buffer in sterile polypropylene bags using a stomacher laboratory blender (Seward, UK). Determination of *Listeria* cell numbers and phage counts was done as described in Chapter 2.4.1. The pH was measured at five different places on the surface using a surface electrode (InLab426, Mettler Toledo, Germany).

Phage application was done with approximately 3×10^8 cfu/cm^2 either (i) 1 h after contamination, (ii) 1 h after contamination and on day 6, or (iii) 1 h after contamination, on day 3 and day 6.

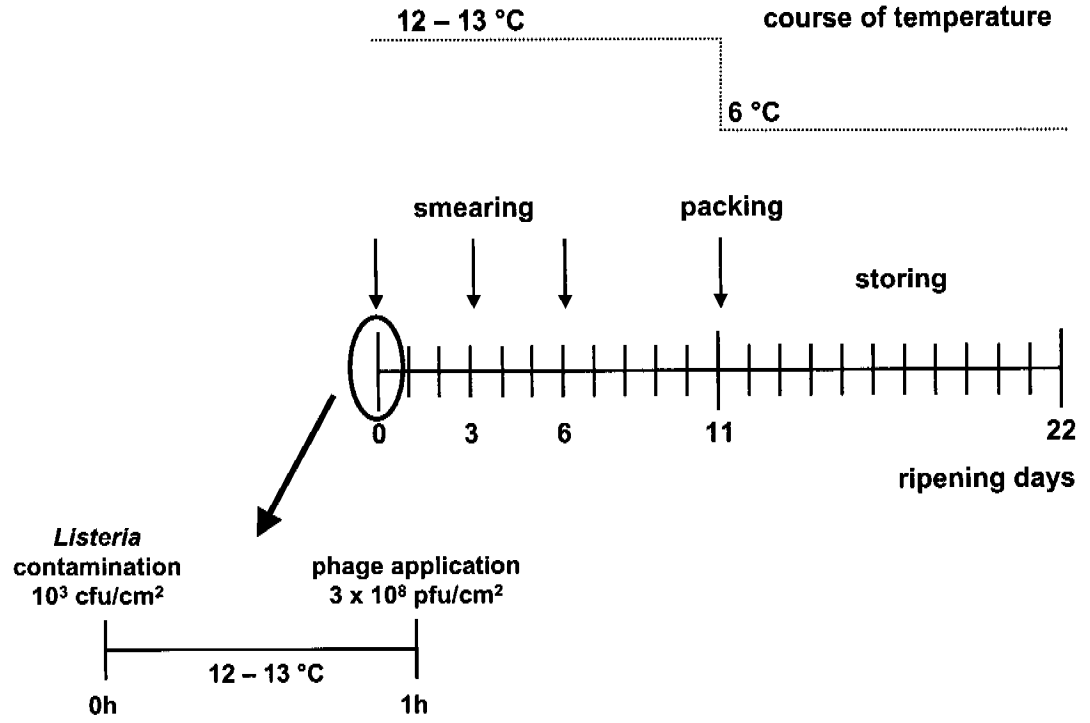


Figure 2.3: Ripening and treatment of red smear cheeses using *L. monocytogenes* and phage A511.

2.4.3 Application of *Listeria* phage endolysins for control of *L. monocytogenes*

Food experiments using phage endolysins for control of *L. monocytogenes* were performed similarly to storage experiments with *Listeria* phages. Differences are described

as follows. Endolysins were applied in PBS to the food samples 1 h after contamination. For experiments with *L. monocytogenes* WSLC 1001, endolysins HPL118, HPL511 and PLY511 were used; for experiments with *L. monocytogenes* Scott A, endolysins HPL500 and HPL511 were applied. Approximately 35 to 50 μg endolysin per g food were used corresponding to an enzymatic activity of 1,200 to 3,000 U/g. Storage temperature was 6°C and 12°C. *Listeria* cell counts were analyzed directly after contamination and after 1, 2, 5 and 6 days. For this purpose, 5 g samples of food were homogenized with 45 ml Citrate buffer, in duplicates.

2.5 Food experiments with *Salmonella* Typhimurium

2.5.1 Construction of mutants of *Salmonella* Typhimurium for quantification in foods

Until now no adequate selective media are available for quantitative detection of *Salmonella* spp. from foods by direct plating. Different selective media have been developed relying on biochemical features such as the non-fermentation of lactose and the production of hydrogen sulfite. However, the specificity of these media is rather poor. Newer media are based on the incorporation of chromogenic substrates. They show higher specificity, but also false-negative or false-positive results [157]. The most important issue with direct plating of *Salmonella* from foods is the problem of the presence of other bacteria, which are also able to grow on common media for *Salmonella* diagnostics. Though they can be distinguished, e.g., by the color from *Salmonella* colonies, they are not inhibited in growth. In the case of a high background of other bacteria, colonies of *Salmonella* could be overgrown and no longer be distinguishable or quantitatively detectable.

One possibility to deal with this problem is the pretreatment of sample foods with, e.g., heat, ethanol or irradiation, in order to reduce the bacterial contamination levels. However, conditions on such "sterile" foods would not correspond to realistic situations and influence the outcome. A more suitable approach is the use of *Salmonella* indicator strains resistant to specific antibiotics. There are several reports about the use of spontaneous mutants of *Salmonella* strains against different kinds of antibiotics for quantification in food experiments. Examples are nalidixic acid, rifampicin, streptomycin or combinations [69, 82, 135, 137, 154, 209, 226]. However, data about stability or growth characteristics of the mutants are not available.

2.5.1.1 Resistance against streptomycin

There are several possibilities for creation of antibiotic resistant bacteria. One method is transformation of plasmids carrying resistance genes. However, plasmid-stability is rather low in the absence of the selective antibiotic.

A simple and rather rapid technique is the selection of the emergence of spontaneous antibiotic resistances. This approach was used for *Salmonella* Typhimurium strain DB7155 stm^R. For this purpose, 100 μl of an overnight culture of wild type strain *S.* Typhimurium DB7155 were plated on LB agar containing different concentrations of streptomycin (10 μg/ml to 100 μg/ml) and incubated overnight at 37°C. One colony was picked from LB agar containing 100 μg/ml and incubated overnight in LB containing 100 μg/ml streptomycin at 37°C. Again, 100 μl were plated on LB agar plates containing 200 μg/ml to 500 μg/ml ($LBstm_{500}$). After overnight incubation at 37°C one colony was picked from $LBstm_{500}$ agar and streaked again on $LBstm_{500}$ agar. This was repeated twice. The resulting strain was cultivated and stored in 13%-glycerol stocks at -80°C. The problem here again is a possible instability of resistance under non-selective conditions. Furthermore, spontaneous mutations can have other side effects, e.g., on virulence or growth properties. Therefore, stability of resistance and growth properties were studied (Chapter 2.5.1.3).

2.5.1.2 Resistance against chloramphenicol

2.5.1.2.1 Concepts

Another approach is the integration of resistance genes into the bacterial chromosome by, e.g., homologous recombination. This can be achieved by recombinase mediated, targeted replacement of a specific, non-essential region on the genome of *Salmonella* with linear DNA. This method is based on the technique of one-step inactivation of chromosomal genes developed by Datsenko and Wanner [51] and is illustrated in Figure 2.4. The system relies on the red-recombinase-system of phage λ and a FLP-mediated, homologous recombination. The FLP recombinase acts on the FLP recombination target (FRT sites). In general, transformation of linear DNA is difficult, as bacteria possess intracellular exonucleases that degrade linear DNA before recombination. The red-recombinase-system consists of three genes of which one gene product (γ) inhibits the exonuclease activity of the host and prevents the degradation of the transformed DNA. The other two genes (β, *exo*) support homologous recombination [51, 168]. For this purpose, the low-copy plasmid pKD46 [51] containing the red-recombinase-system and ampicillin resistance gene was transformed into *Salmonella* Typhimurium DB 7155. Genes for the red-recombinase-system are under control of a P_{ara}BAD promotor that is inducible by L-arabinose [51, 94]. The temperature

sensitive plasmid can be eliminated at non-permissive temperature (37°C) after successful recombination. The linear DNA fragment containing antibiotic resistance was amplified by PCR. The template was pKD3 [51] containing the chloramphenicol resistence cassette. The designed primers were complementary to pKD3 (p1 and p2) and contained sequences (H1 and H2) complementary to the ends of the target gene in the bacterial chromosome.

The target genes on the chromosome were *phoN* and *stm1666*. Gene *phoN* encodes a non-specific acid phosphatase [117]. Homologous recombination with the linear DNA fragment was not successful at the *phoN* locus. Gene *stm1666* is a pseudogene and has an in frame stop located after codon 24. A promotor is located upward of the gene and 17 bp within the gene that were not affected by gene replacement. Another promotor is located in reverse direction inside the gene, but no gene is located upstream from the promotor in this direction. Downstream of *stm1666* there is a gap of 104 nucleotides before the next gene is located in reverse direction. Analyses were done using genome annotation of *S.* Typhimurium [39] and Promoter Prediction by Neural Network [20].

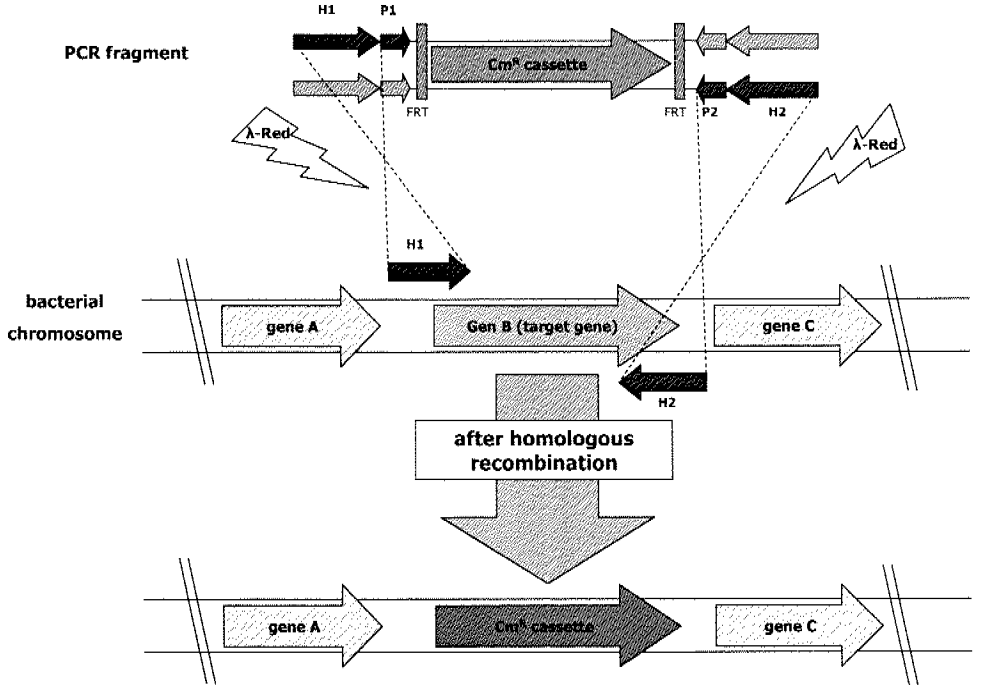


Figure 2.4: Homologous recombination of *cat* and target gene of *Salmonella* (adapted from [126]). H1 and H2 refer to the homology regions on the genome of *Salmonella*. P1 and P2 refer to priming sites complementary to pKD3.

2.5.1.2.2 Polymerase chain reaction (PCR)

For fragment multiplication polymerase chain reaction (PCR) was applied using purified plasmid pKD3 (Plasmid extraction kit, Sigma, USA) as a template (120 ng). Primers are listed in Table 2.4. Primers for fragment multiplication contained 50 nucleotides (nt) homologous to the target gene and 19nt or 20nt regions complementary to pKD3 [51]. The PCR product was digested with restriction enzyme *Dpn*I to eliminate methylated and not amplified template DNA [51]. The product was purified using phenol extraction and ethanol precipitation.

In order to confirm the insertion of the linear DNA fragment colony PCR was done using oligonucleotides binding up- and downstream of the target region: a single colony was resuspended in 100 μl deionised water (MilliQ) and 2 μl were added to the PCR reaction. Furthermore, genetic modification was verified by subsequent sequencing of the target site of one mutant. All primer sequences were chosen according to genome annotation of *S.* Typhimurium [39].

Table 2.4: Oligonucleotide primers for amplification

Primer name	Sequence 5'-3'	Target sequence	Purpose
STMforw	TTATTTTCGAAAAAAAAGAGTACGA CGAAGAGTTTTATCATGAATTGAGC GTGTAGGCTGGAGCTGCTTC	*stm1666*	Amplification of the chloramphenicol resistance cassette
STMrev	TTACGCATAAGGAACTGAAAATGAT GCCAGCGGATGGGGCAGGTTAACGT CATATGAATATCCTCCTTA	*stm1666*	Amplification of the chloramphenicol resistance cassette
Seqfwd	ATGTCGCTGTTTCTGACAAG	2nt upstream of *stm1666*	Colony PCR, sequencing
Seqrev	ATTCTCTTGTGTGCCCTCAC	22nt downstream of *stm1666*	Colony PCR, sequencing

2.5.1.2.3 Transformation

For plasmid transformation (pKD46), an overnight culture of *Salmonella* DB7155 was diluted 1:10 in 10 ml fresh LB medium and incubated again for 1-2 h at 37°C and 180 rpm. Cells were harvested by centrifugation (7,000 g, 4°C, 5 min), washed three times in 10 ml ice-cold 10%-glycerin solution and resuspended in 40 μl ice-cold 10%-glycerin. Cells were mixed with 5 μl (100 - 200 ng) of plasmid pKD46 from *E. coli* BW25113/pKD46. After electroporation (2.5kV, 200 Ohm, 25 μF), cells were regenerated in LB under gentle shaking at 30°C for 1 to 2 h, because pKD46 is sensitive to higher temperatures. Bacteria were plated on LB agar containing ampicillin (100 μg/ml) and incubated at 30°C for 1 - 2 days. Colonies were picked and analysed for the plasmid.

For transformation of the linear PCR fragment, cells were prepared differently. 100 ml LB containing ampicillin (100 μg/ml) and L(+)-arabinose (1-10 mM) were inoculated with 1:100 of an overnight culture of *S.* Typhimurium DB7155 (pKD46) and grown at 30°C and 180 rpm. At an OD_{600} of 0.5, the culture was cooled down on ice for 15 min. Cells were harvested by centrifugation (7,000 g , 4°C , 10 min) and the pellet was resuspended in 100 ml ice-cold 5%-glycerol solution. This was repeated twice, resupending the pellet in 50 ml and 4 ml 15%-glycerol solution, repectively. After the last centrifugation, cells were resuspended in 400 μl of 10%-glycerol solution. Aliquots of 40 μl were mixed with the purified PCR-fragments (1 - 2 μg) and again transformed by electroporation. Cells were regenerated at 37°C for 1 to2 h. Cells were plated on LB agar containig chloramphenicol (25 μg/ml) (LB_{cm25})and incubated at 37°C for 24 to 48 h. Colonies were picked, streaked 2 - 3 times on LB_{cm25} and verified by Colony-PCR and sequencing (Chapter 2.5.1.2.2).

2.5.1.3 Stability of antibiotic resistance, phage sensitivity and growth properties

Both *Salmonella* strains DB7155 stm^R and DB7155 cm^R were tested for stability of antibiotic resistance under non-selective conditions. An overnight culture in LB containing the antibiotic was diluted 1:3000 in fresh LB media containing no antibiotic and incubated again at 37°C and 180 rpm. After 7 h, *Salmonella* cell counts were determined on LB agar plates with and without the antibiotic. This was done 5 times. Furthermore, stability was tested on sterilized hot dogs. The sausages were heated to 121°C for 15 min. They were contaminated with 1×10^3 cfu/g of *Salmonella* and stored at 20°C. Determination of *Salmonella* cell numbers were done immediately after contamination and after 6 days on LB agar with and without the antibiotic.

The strains were also tested for phage sensitivity in comparison to the wild type. An appropriate dilution of FO1-E2 was plated on DB7155, DB7155 stm^R, and DB7155 cm^R, phage counts were calculated and compared.

Growth properties of both mutants DB7155 stm^R and DB7155 cm^R were evaluated and compared with the wild type strain. Overnight cultures were diluted 1:1000 in fresh LB media containing no antibiotic. Cultures were incubated at 37°C and 180 rpm. OD_{600} measurements were performed until the cultures reached stationary growth phase. Data refer to three independent experiments.

2.5.2 Control of *Salmonella* Typhimurium on foods

Foods tested here, were hot dogs, sliced turkey breast, minced meat, mixed seafood, mung bean sprouts, chocolate milk and egg yolk. Purchased foods were first tested for *Salmonella* spp. according to ISO 6579:2002 [13]. Briefly, samples of 25 g were

homogenized and mixed with 225 ml buffered peptone water and incubated at 37°C for 18±2 h. Then, 1 ml of the non-selective enrichment culture was transferred to 10 ml MKTTn and incubated at 37°C for 24±3 h. Another 0.1 ml were transferred to 10 ml RVS and incubated at 41.5°C for 24±3 h. One loop-full (approximately 10 μl) of each selective enrichment broth was directly streaked on XLD and BBL^{TM} agar plates and further incubated at 37°C for 24±3 h. Typical *Salmonella* colonies were black in color with a red background on XLD agar due to presence of H_2S and missing fermentation of lactose. On chromogenic BBL^{TM} agar, colonies of *Salmonella* were mauve or blue-violet colored. Further test for confirmation were done with Poly-O-Agglutination or *Salmonella* specific phage. In the case of positive *Salmonella* samples, the food sample was discarded and not used in the experiments.

For the experiments with phage FO1-E2 three samples were used: (i) the negative control, (ii) the positive control containing *S.* Typhimurium, and (iii) the phage sample containing *S.* Typhimurium and phages. *S.* Typhimurium was always grown in LB media containing the appropriate antibiotic at 37°C. Cell counts of *S.* Typhimurium were determined on LB agar containing the appropriate antibiotic. Phage titer was determined using 100 μl of the antibiotic resistant strain as host. Measurements of bacterial cell counts and phage counts were done immediately after contamination or phage application and after 1, 2, 5, and 6 days. Storage temperatures were 8°C or 15°C.

2.6 Development of insensitivity against bacteriophages in foods

In order to investigate emergence of phage resistance in foods, re-isolated *Listeria* colonies were tested for phage susceptibility. For this purpose, 10 colonies were picked from selective agar and streaked on non-selective agar. For plaque assay, 200 μl (in the case of *Listeria*) or 100 μl (in the case of *Salmonella*) of a subculture were mixed with molten soft agar and poured onto the appropriate agar plate. After solidifying and drying 10 μl of different dilutions of phage preparations were dropped on the soft agar. In general, phage dilutions contained 10^9 pfu/ml, 10^6 pfu/ml and 10^4 pfu/ml. Furthermore, *Listeria* isolates insensitive against phage A511 were tested for resistance to other *Listeria* phages according to the procedure explained before.

Three temperate phages, A118, A620 and A006, and the new isolated phages 18, 19, 20, and P40 as well as P100 and P35 were used.

2.7 Statistical analysis

Determination of bacterial cell counts and phage numbers was performed in duplicates. Each experiment was independently repeated once to five times. The mean bacterial cell counts of all experiments were calculated and plotted. The standard deviation of the mean was determined, and included as error bars. Student's t-test (unpaired, two-side, heteroscedastic) was applied to determine the significance of cell count differences between untreated control and phage treated samples. Significance was based on an α level of 5 % ($p<0.05$).

3 Results

3.1 Effect of bacteriophages on growth of *Listeria monocytogenes* in foods

Using large scale propagation, high titer stock suspensions of 3×10^{11} pfu/ml of *Listeria* phage A511 and P35 were obtained. For experiments with *Listeria* phage P100, a lysate of 2×10^{10} pfu/ml was used.

In experiments with solid foods, the detection limit by direct plating was 10 cfu/g, and with liquid foods 1 cfu/ml, concerning one single measurement. In experiments with the lower initial contamination level (1×10^2 cfu/g), detection limit for solid foods was 5 cfu/g. In experiments with white mold soft cheeses the lower detection limit was 5 cfu/cm^2, with red smear soft cheeses the limit was 7 cfu/cm^2. If no cell was detected by direct plating, the result was valuated as being negative. This was marked as n.d. (not detectable) in the Figures. Due to an averaged determination of bacterial cell counts over several independent experiments, values between n.d. and the direct detection limits such as 10 cfu/g were obtained.

Statistical analysis confirmed that most of the results were significant based on an α level of 5 % ($p<0.05$). Exceptions are indicated in the text.

3.1.1 Control of *Listeria monocytogenes* during storage of foods

The effect of *Listeria* phage A511 on the three different *Listeria monocytogenes* strains WSLC 1001 (serovar 1/2c), Scott A (serovar 4b) and CNL 103/2005 (serovar 1/2a) was studied in 14 different foods. Strain CNL 103/2005 (serovar 1/2a) was first isolated in 2005 and tested in 9 out of the 14 foods.

3.1.1.1 Meat and meat products

Four different meat products were used to evaluate the effect of phage A511 on growth of *L. monocytogenes* during storage at 6°C for six days. The results are shown in Figures 3.1 and 3.2.

3.1.1.1.1 Hot dogs and minced meat

On hot dogs (Figure 3.1, Column A), all *Listeria* strains multiplied by more than one log unit after six days at 6°C when no phages were added (control samples). On samples containing approximately 3×10^8 pfu/g of A511 contamination levels below 10 cfu/g were detected after six hours and the following day. Using strain WSLC 1001 no bacterial colony was found. This was also observed in one out of two experiments with CNL 103/2005. The reduction was therefore more than 4 log units compared to the control after 6 days. In the case of Scott A, this decrease was 2.9 log units.

On minced meat, growth of *L. monocytogenes* was similar to that on hot dogs (Figure 3.1, Column B). On phage treated samples *Listeria* cell counts decreased by more than one log unit in the beginning, followed by a minor increase. In experiments with strain WSLC 1001 and Scott A bacterial cell concentrations were approximately 2 log units lower compared to control samples after 6 days. However, using strain CNL 103/2005, bacterial cell counts were not significantly different from the control on day 6 ($p>0.05$).

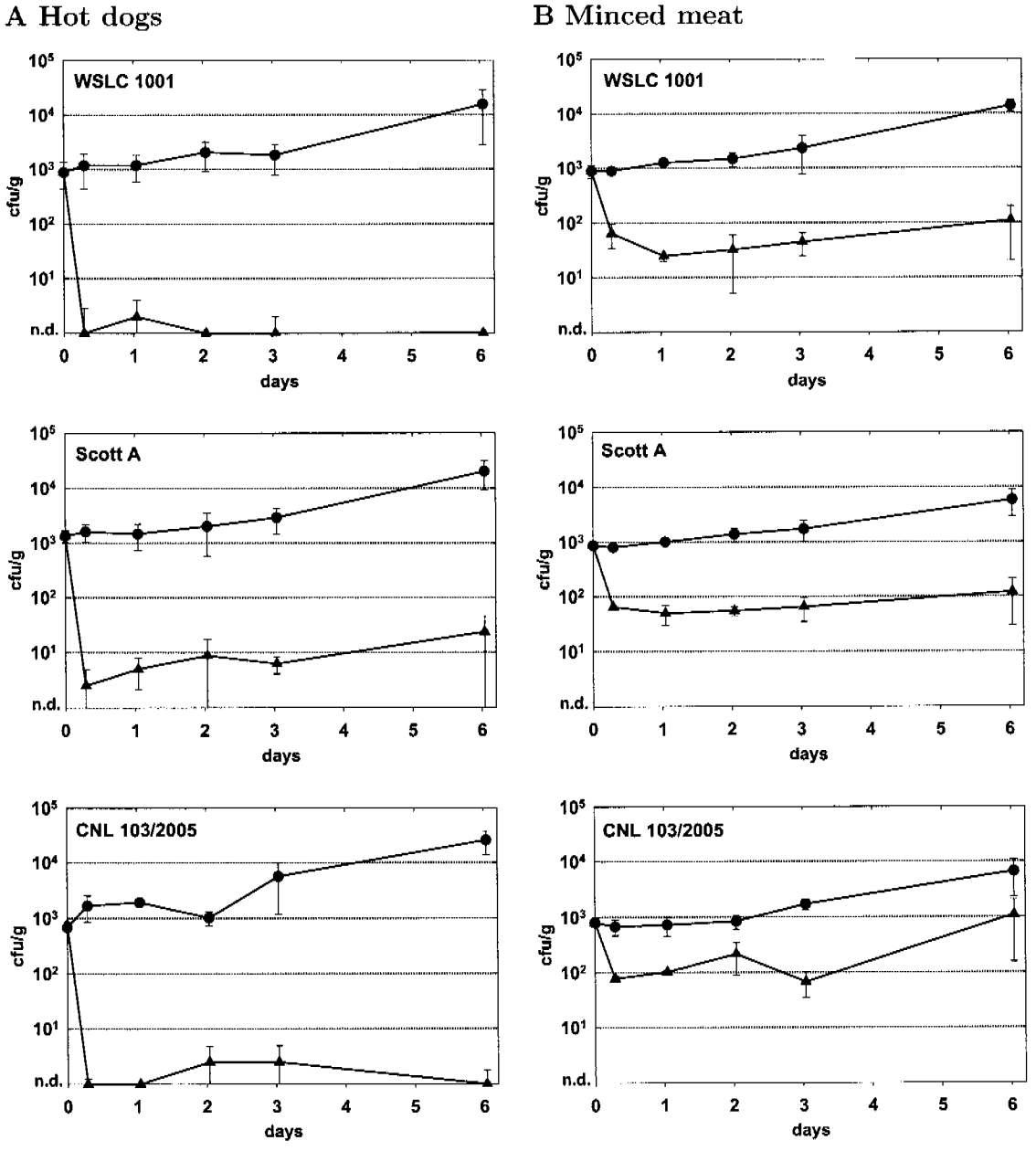


Figure 3.1: Effect of phage A511 on growth of different strains of *L. monocytogenes* on hot dogs (Column A) and minced meat (Column B) at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 1.4×10^8 pfu/g and 3.0×10^8 pfu/g after 1 hour.

3.1.1.1.2 Ham and sliced cooked turkey meat

Figure 3.2 shows the results on cooked ham (Column A) and sliced cooked turkey breast (Column B). On ham, cell counts of *L. monocytogenes* increased between 2 and 3 log units. When phage A511 was applied to the samples, bacterial numbers dropped by approximately 1 log unit within the first hours. Afterward, re-growth was observed. After 6 days the killing effect was 1.5 to 2.0 log units compared to the control samples.

On sliced cooked turkey breast, growth of the different *Listeria* strains was comparable to growth on ham. Bacterial cell numbers were reduced by about 1.5 log units after 6 days.

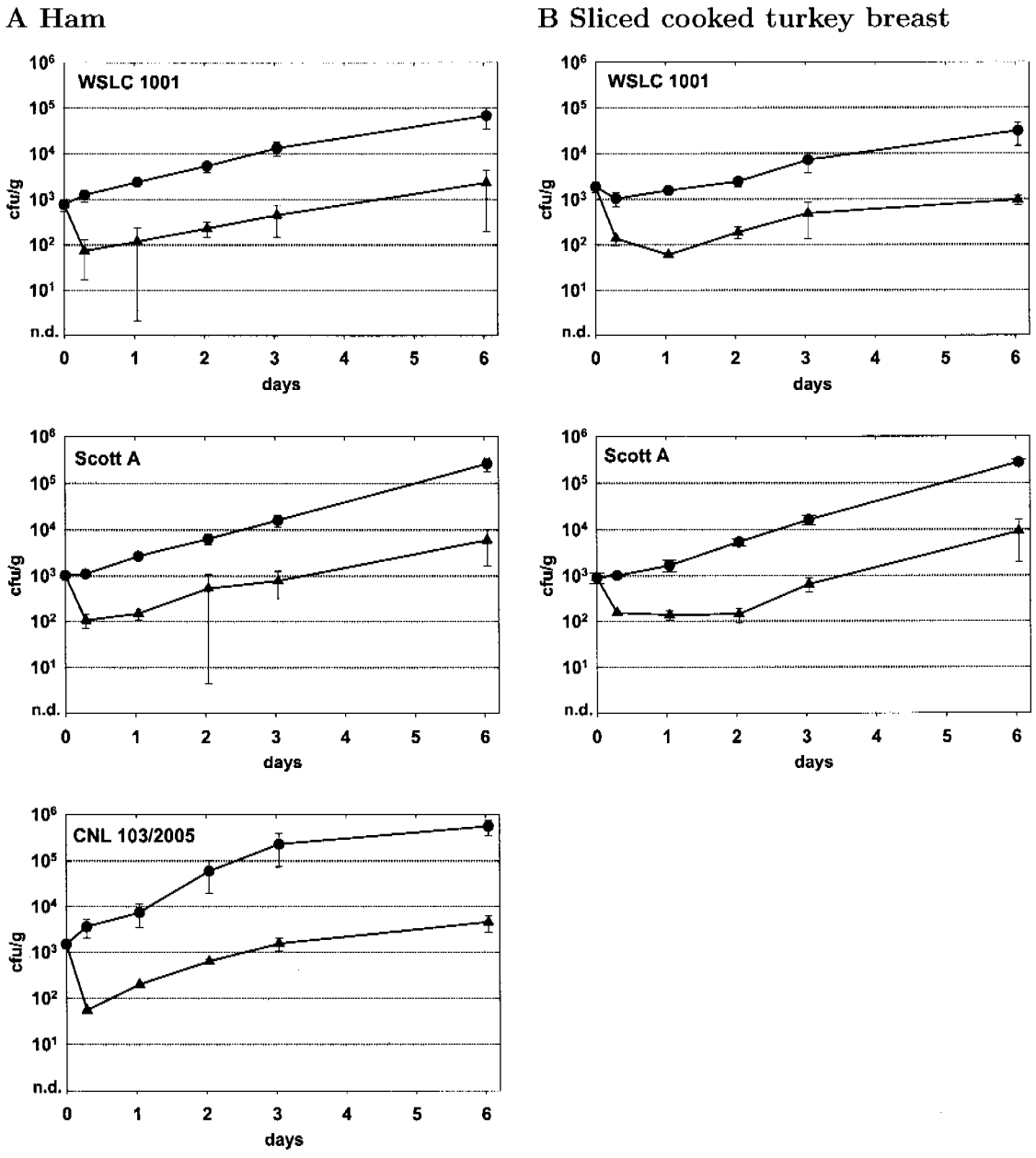


Figure 3.2: Effect of phage A511 on growth of different strains of *L. monocytogenes* on ham (Column A) and sliced cooked turkey breast (Column B) at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 2.1×10^8 pfu/g and 3.2×10^8 pfu/g after 1 hour.

3.1.1.2 Fish and seafood

Effect of phage A511 on growth of *L. monocytogenes* was investigated on six different fish products during storage at 6°C for six days (Figures 3.3 and 3.4). Strain CNL 103/2005 was tested on raw salmon and shrimps.

3.1.1.2.1 Raw and smoked fish

On smoked salmon, *Listeria* cell numbers increased by 0.9 log units on the control samples over 6 days (Figure 3.3, Column A). Suppression of growth on phage treated salmon was below 1 log unit and not significant after 6 days ($p>0.05$).

On raw salmon, *L. monocytogenes* multiplied by 1.6 to 1.9 log units (Figure 3.3, Column B). After adding phage A511, development of bacterial growth was different for each of the 3 *Listeria* strains. In the case of WSLC 1001, neither increase nor decrease of the initial contamination level was observed over the whole experiment. The difference to control samples was 2.0 log units at day 6. When Scott A was used, bacterial cell numbers dropped within the first 6 hours and again after 6 days. The killing effect was higher (3.8 log units) compared to WSLC 1001. In the case of CNL 103/2005, first after 3 days growth suppression was significant. It was 1.3 log units at day 6 and thus lower than for the remaining *Listeria* strains.

Growth of *L. monocytogenes* on non-treated smoked trout was similar to that on raw salmon (Figure 3.3, Column C). Using *Listeria* strain WSLC 1001, reduction on phage treated samples, however, was lower than 1 log unit for all data points. It was still significantly different from the control at most time points ($p< 0.05$) except on day six ($p>0.05$). Using strain Scott A, bacterial cell numbers were always significantly lower compared to the control. After 6 days, growth suppression was 1.0 log unit and slightly higher than for WSLC 1001 (0.8 log units).

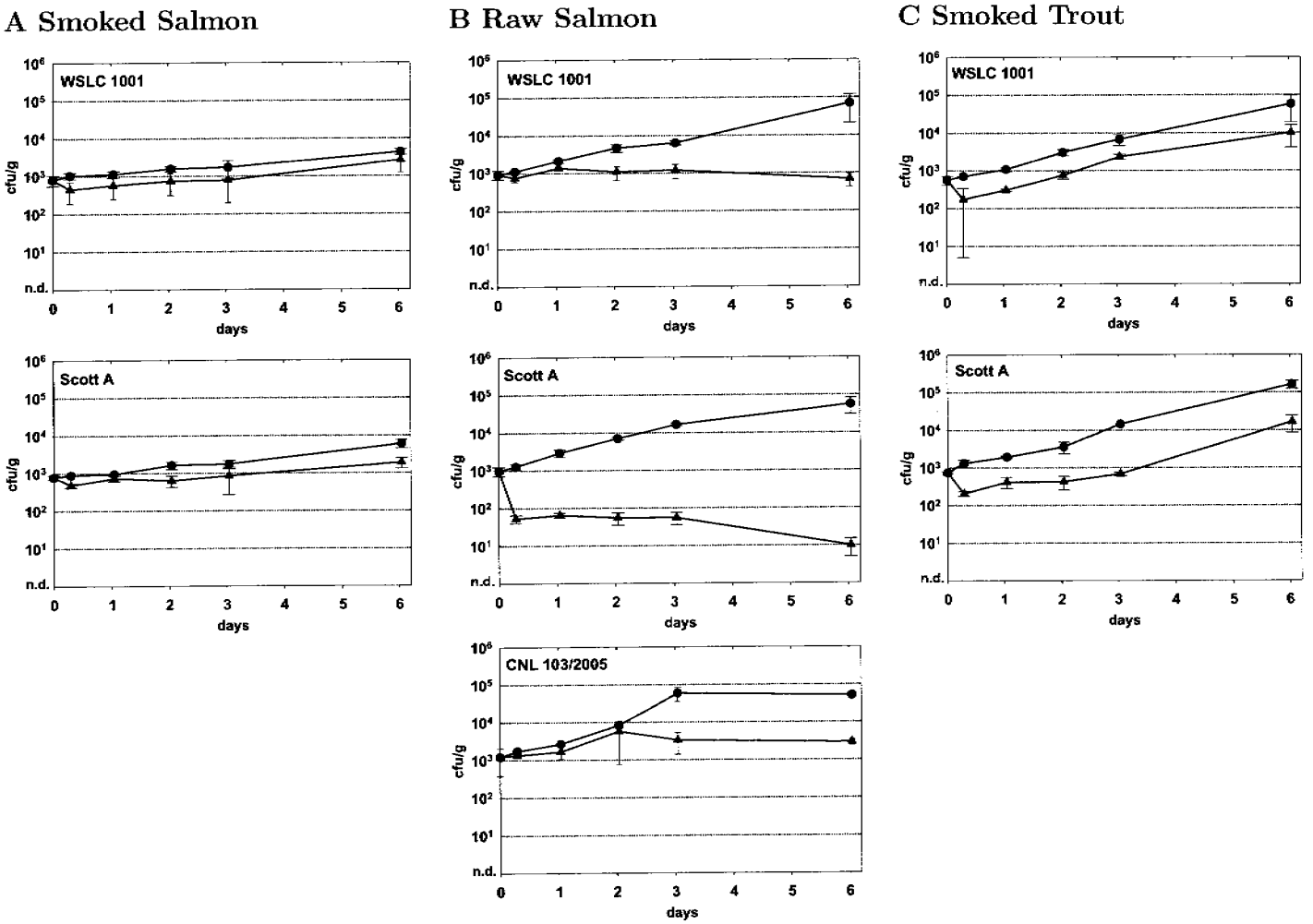


Figure 3.3: Effect of phage A511 on growth of different strains of *L. monocytogenes* on smoked salmon (Column A), raw salmon (Column B) and smoked trout (Column C) at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 1.6×10^8 pfu/g and 3.0×10^8 pfu/g after 1 hour.

3.1.1.2.2 Seafoods

On control samples of calamari, shrimps and mixed seafood, bacterial cells multiplied by 1.5 and 3.4 log units (Figure 3.4). In general, *Listeria* strain Scott A and CNL 103/2005 grew faster than strain WSLC 1001. Effect of phage A511 on growth of the 3 *Listeria* strains was quite similar on all seafoods. After a first reduction of 1.0 to 1.5 log units, bacteria started growing again. At day 6, the differences to control samples were between 1.8 and 3.0 log units. On calamari, growth inhibition was greater for strain WSLC 1001 (2.2 log units) than for Scott A (1.8 log units) after 6 days. On shrimps and mixed seafood, however, this killing effect was higher for strain Scott A (2.3 and 2.7 log units) than for WSLC 1001 (1.8 and 2.5 log units). In experiments with strain CNL 103/2005 on shrimps, this effect was even higher with 3.0 log units after 6 days.

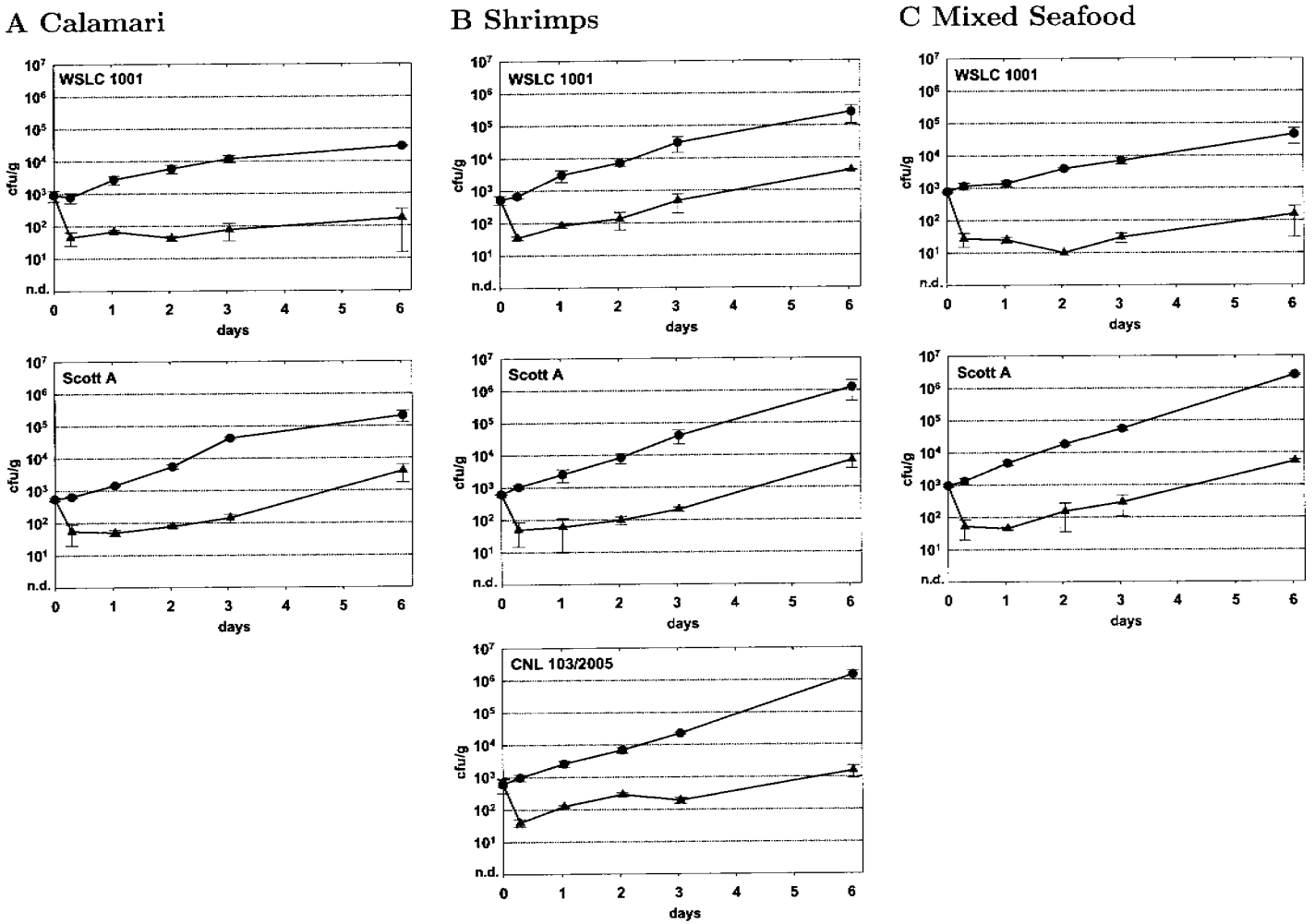


Figure 3.4: Effect of phage A511 on growth of different strains of *L. monocytogenes* on calamari (Column A), shrimps (Column B) and mixed seafood (Column C) at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 2.1×10^8 pfu/g and 3.0×10^8 pfu/g after 1 hour.

3.1.1.3 Vegetables

Figure 3.5 shows the effect of phage A511 on the three *Listeria* strains on sliced iceberg lettuce and shredded cabbage. When no phage was added, *Listeria* cell counts increased by approximately 1 log unit within six days on both vegetables. One exception was Scott A that multiplied by 2 log units on cabbage. On phage treated iceberg lettuce, bacterial cell numbers were decreased by more than 2 log units in the beginning, but then increased again (Figure 3.5, Column A). After 6 days, *Listeria* cell counts were 2.4 to 2.8 log units lower than on control samples.

Applying phage A511 to cabbage, only few (< 10 cfu/g) or even no viable cells were detected over 6 days (Figure 3.5, Column B). In comparison to the controls at the last day, suppression effects on strain WSLC 1001 and Scott A were 3.3 and 3.9 log units, respectively. With strain CNL 103/2005, no *Listeria* colonies were detected by direct plating during the whole experiment. Therefore, growth inhibition was more than 4 log units. However, the last data refer to results of only one experiment.

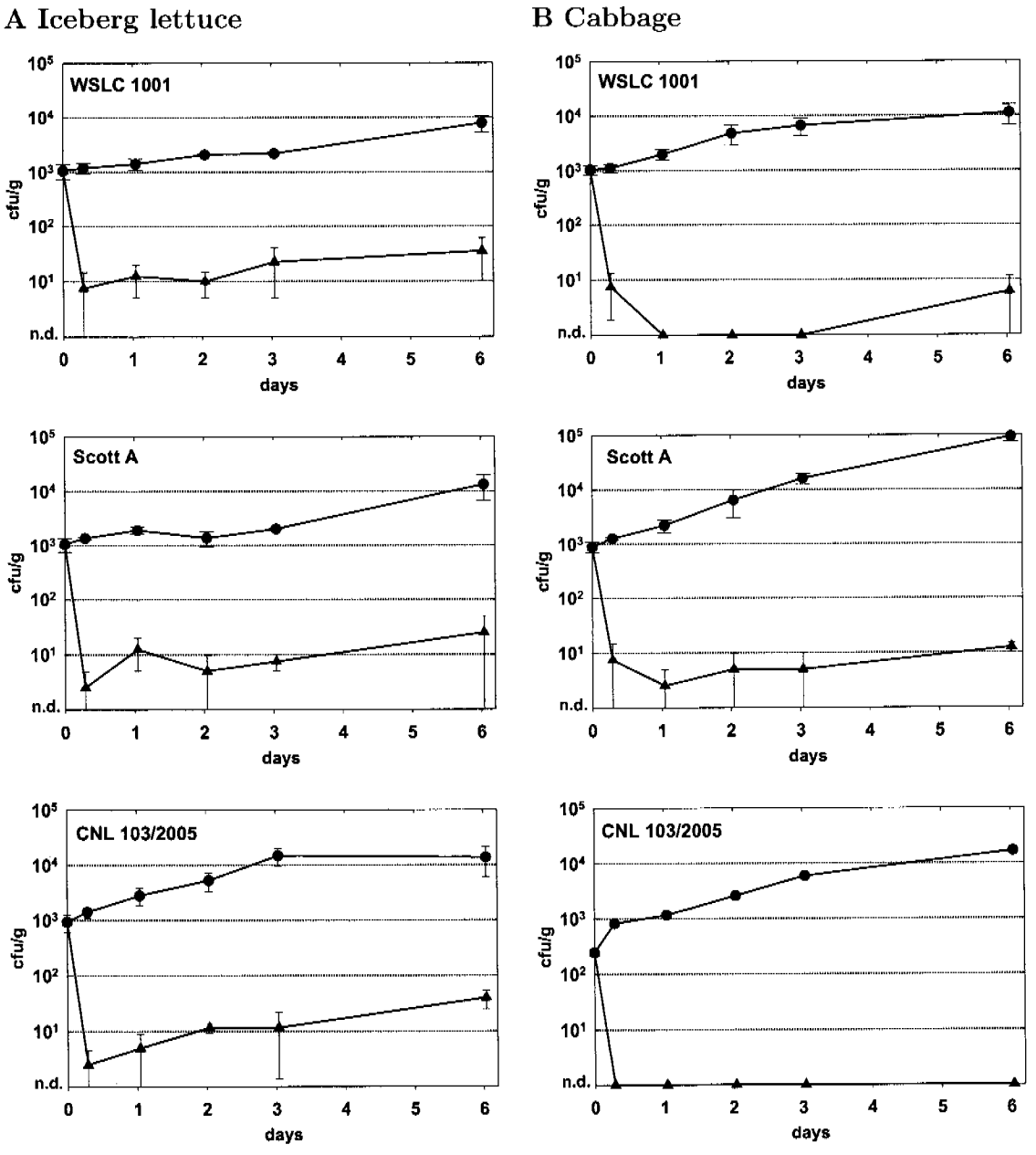


Figure 3.5: Effect of phage A511 on growth of different strains of *L. monocytogenes* on iceberg lettuce (Column A) and cabbage (Column B) at 6°C during 6 days (● control; ▲ +A511). Phage was applied in concentrations between 2.0×10^8 pfu/g and 3.6×10^8 pfu/g after 1 hour. Data for strain CNL 103/2005 on cabbage refer to only 1 experiment.

3.1.1.4 Chocolate milk and mozzarella cheese brine

In chocolate milk, cell counts of WSLC 1001 and Scott A increased by approximately 2 log units and cell numbers of CNL 103/2005 by 3 log units (Figure 3.6, Column A). After the application of phage A511, no *Listeria* cell was detected after 2 days using WSLC 1001, and after 1 day with Scott A. At day 6, growth inhibition compared to the control was 5.1 and 4.6 log units, respectively. Strain CNL 103/2005 was reduced initially by 2 log units. After the 6 day period, viable cell numbers were below 10 cfu/g and the difference to the control was 6.0 log units.

In mozzarella cheese brine, growth of *L. monocytogenes* was lower compared to chocolate milk, and bacterial cell counts increased by approximately 1 log unit over 6 days (Figure 3.6, Column B). On phage treated samples, cell numbers of WSLC 1001 were reduced below the direct detection limit after 6 days. Using Scott A, viable cells were still detectable during the whole experiment. In experiments with CNL 103/2005, a reduction of almost 2 log units was observed after the first hours. Then, bacterial cell counts were no further significantly reduced. The inhibition effect was 2.7 log units after 6 days.

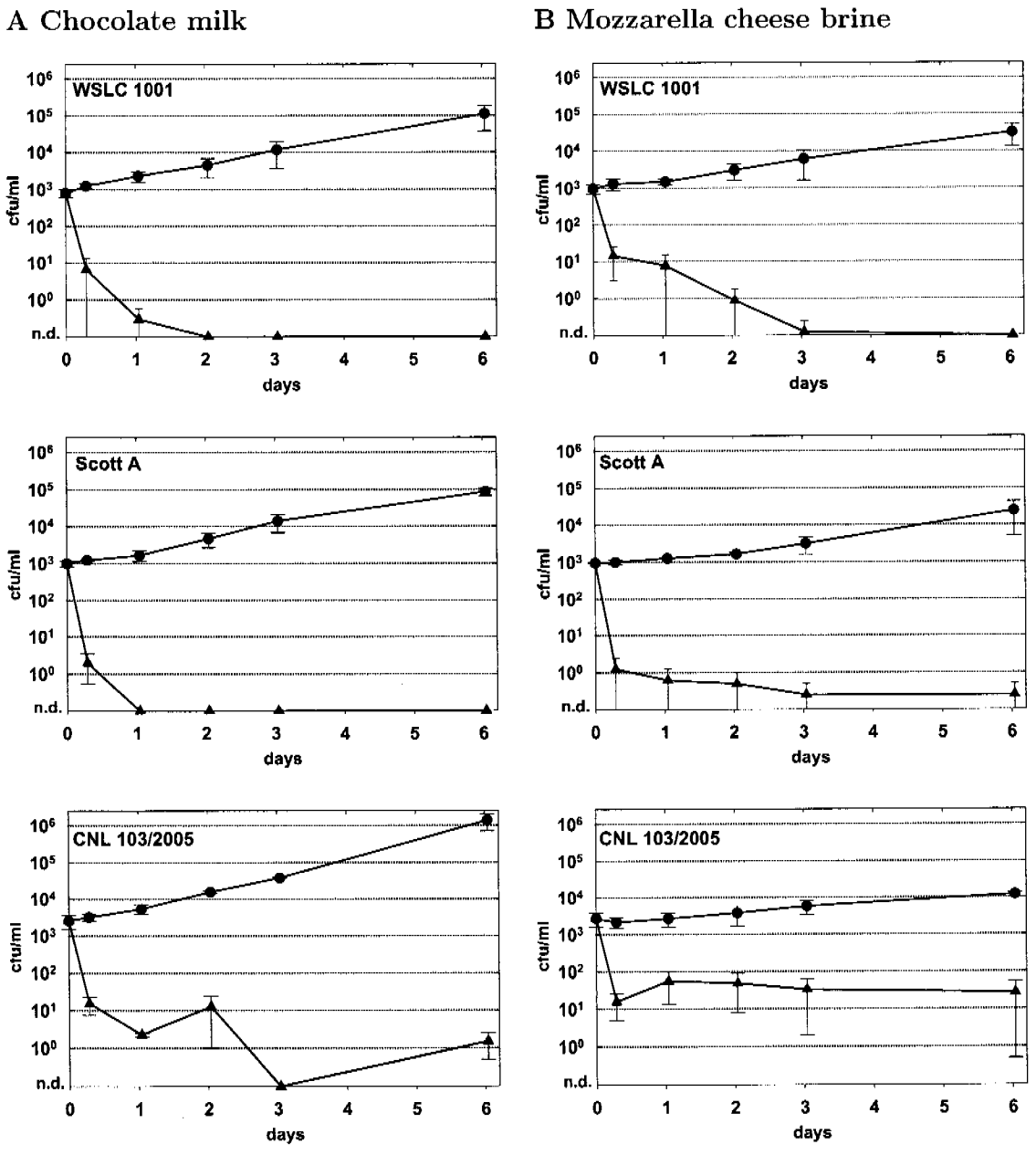


Figure 3.6: Effect of phage A511 on growth of different strains of *L. monocytogenes* in chocolate milk (Column A) and mozzarella brine cheese (Column B) at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 2.4×10^8 pfu/g and 3.4×10^8 pfu/g after 1 hour.

3.1.1.5 Contamination with low *Listeria* cell counts

The initial contamination level was reduced from 10^3 cfu/g to 10^2 cfu/g to investigate the influence of lower *Listeria* concentrations on the effect of phage application. For this purpose, four different foods and *Listeria* strain WSLC 1001 were used. Detection limit by direct plating was 5 cfu/g (Chapter 3.1).

On all food samples, *Listeria* cell counts increased by 1.2 to 1.5 log units after 6 days (Figure 3.7). When phage was added to minced meat, the difference to the control was less than 1 log unit over the whole time, but still significant. On phage treated smoked salmon, *Listeria* cell numbers decreased by 0.5 log units after the first day, but then bacteria started growing again. After 6 days, viable cell counts were 1.1 log units lower compared to the control samples. In phage samples of mozzarella brine, no viable *Listeria* were detected after 2 days, corresponding to a reduction of 3.4 log units compared to the control after 6 days. On iceberg lettuce, counts of WSLC 1001 were reduced below 10 cfu/g after the first hours. At day 6 growth inhibition was 2.5 log units compared to the control.

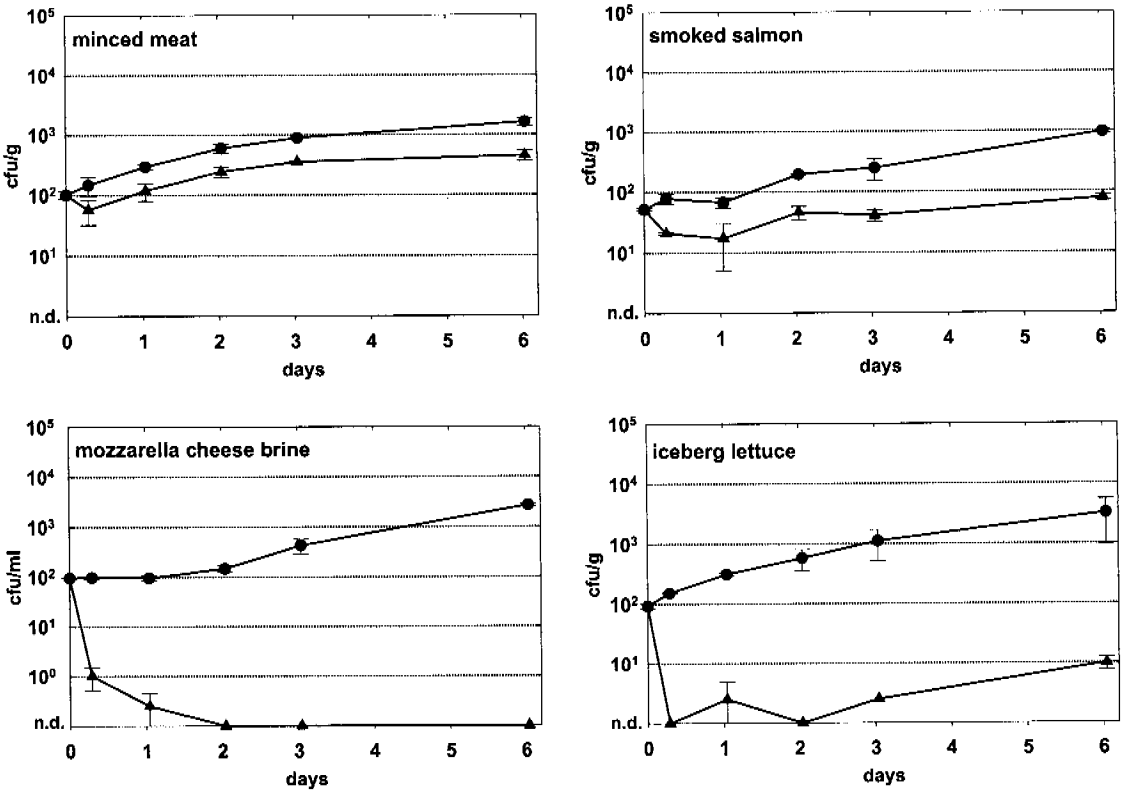


Figure 3.7: Effect of phage A511 on growth of *L. monocytogenes* WSLC 1001 after contamination of 1×10^2 cfu/g on different foods at 6°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 1.1×10^8 pfu/g and 2.7×10^8 pfu/g after 1 hour.

3.1.1.6 Increase of storage temperature

By increasing the storage temperature from 6°C to 20°C, growth of *L. monocytogenes* was enhanced on different foods (hot dogs, cabbage, chocolate milk, and mozzarella cheese brine). It was important to evaluate the influence of this temperature effect on the efficiency of phage application.

At 20°C, *L. monocytogenes* WSLC 1001 reached cell numbers of 10^6 to 10^9 cfu/g or cfu/ml on control samples (Figure 3.8). These numbers were 2 to 5 log units higher than at 6°C. On phage treated hot dogs and cabbage, WSLC 1001 was reduced below the direct detection limit in the first six hours. Then, *Listeria* started growing again almost similarly to the control samples. In chocolate milk and mozzarella cheese brine containing A511,no *Listeria* cell was detected until day 6 and day 3, respectively. Differences in *Listeria* cell counts compared to the control were similar or even higher to those at 6°C after six days (Chapter 3.1.1.1, 3.1.1.3, 3.1.1.4). However, at 20°C, cells on phage samples multiplied after the initial reduction effect, and reached higher concentrations than at 6°C.

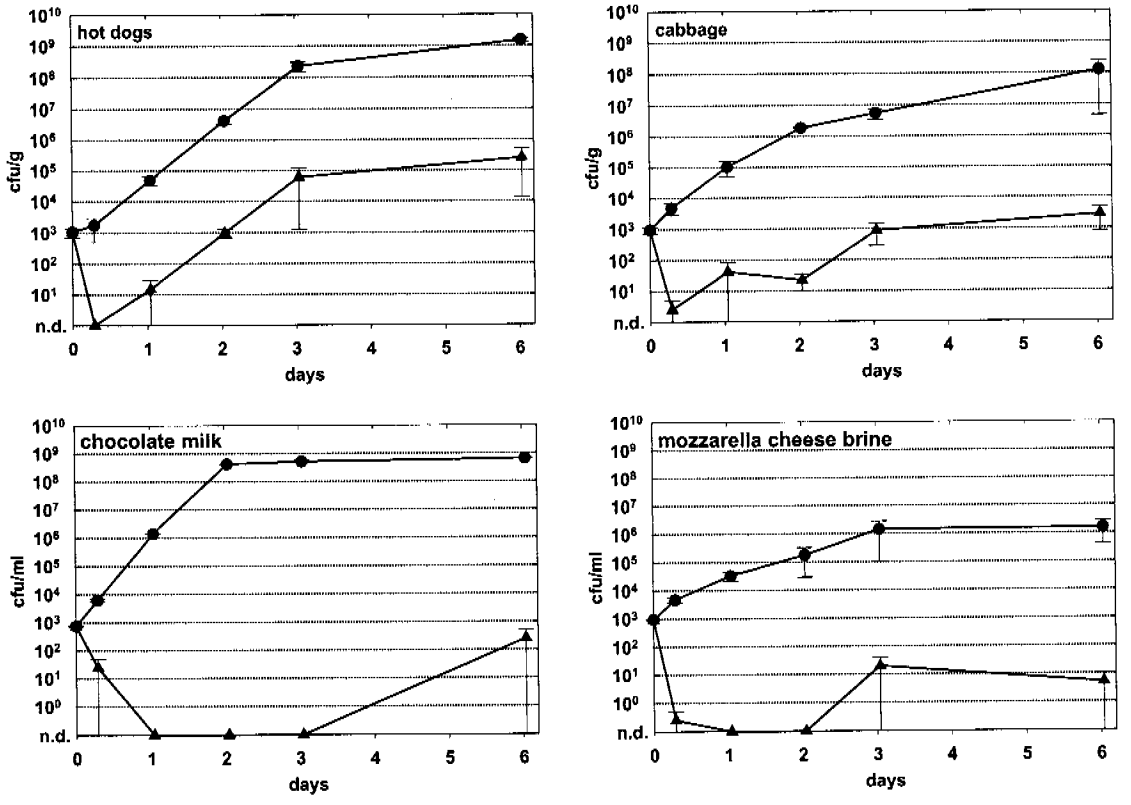


Figure 3.8: Effect of phage A511 on growth of *L. monocytogenes* WSLC 1001 on different foods at 20°C over 6 days (● control; ▲ +A511). Phage was applied in concentrations between 2.8×10^8 pfu/g and 3.8×10^8 pfu/g after 1 hour.

3.1.1.7 Extension of storage time

In order to analyze the influence of extended storage time, four foods were stored for two weeks until the end of their shelf life. Foods were contaminated with one of the three *L. monocytogenes* strains. Figures 3.9 - 3.10 show the data for all three *Listeria* strains. In the case of strain WSLC 1001, data for phage treated samples of hot dogs, chocolate milk, and mozzarella cheese brine refer to only one experiment from day 7 on. This is due to the emergence of phage resistance in a second experiment leading to completely different results. These will be presented in Chapter 3.1.3.

3.1.1.7.1 Hot dogs and minced meat

On hot dogs, bacterial cell counts reached about 10^6 cfu/g in the case of WSLC 1001, and about 10^7 cfu/g in the case of Scott A and CNL 103/2005 after 13 days (Figure 3.9, Column A). When phage A511 was applied, bacterial cells were reduced below 50 cfu/g during the whole experiment with strain WSLC 1001. This was also the case for strain Scott A, however, at day 13 a strong increase in bacterial numbers were observed in one of the experiments. Growth inhibition was 4.1 log units and lower compared to 5.7 log units for WSLC 1001. With strain CNL 103/2005, reduction of bacteria was similar to strain WSLC 1001 in the first week. After 13 days, however, CNL 103/2005 reached approximately 10^4 cfu/g corresponding to a killing effect of 4.5 log units compared to the control. In both experiments with strain CNL 103/2005 some of the re-isolated colonies were insensitive to phage A511 (Chapter 3.1.3).

On ham, a similar development of bacterial cell counts on both control and phage samples were observed for each of the three *Listeria* strains (Figure 3.9, Column B). Viable cell numbers after 13 days were similar to numbers after six days. On phage samples, also no further growth occurred after 6 days. Growth suppression was therefore similar to experiments lasting for 6 days and comparable between the three strains of *L. monocytogenes*.

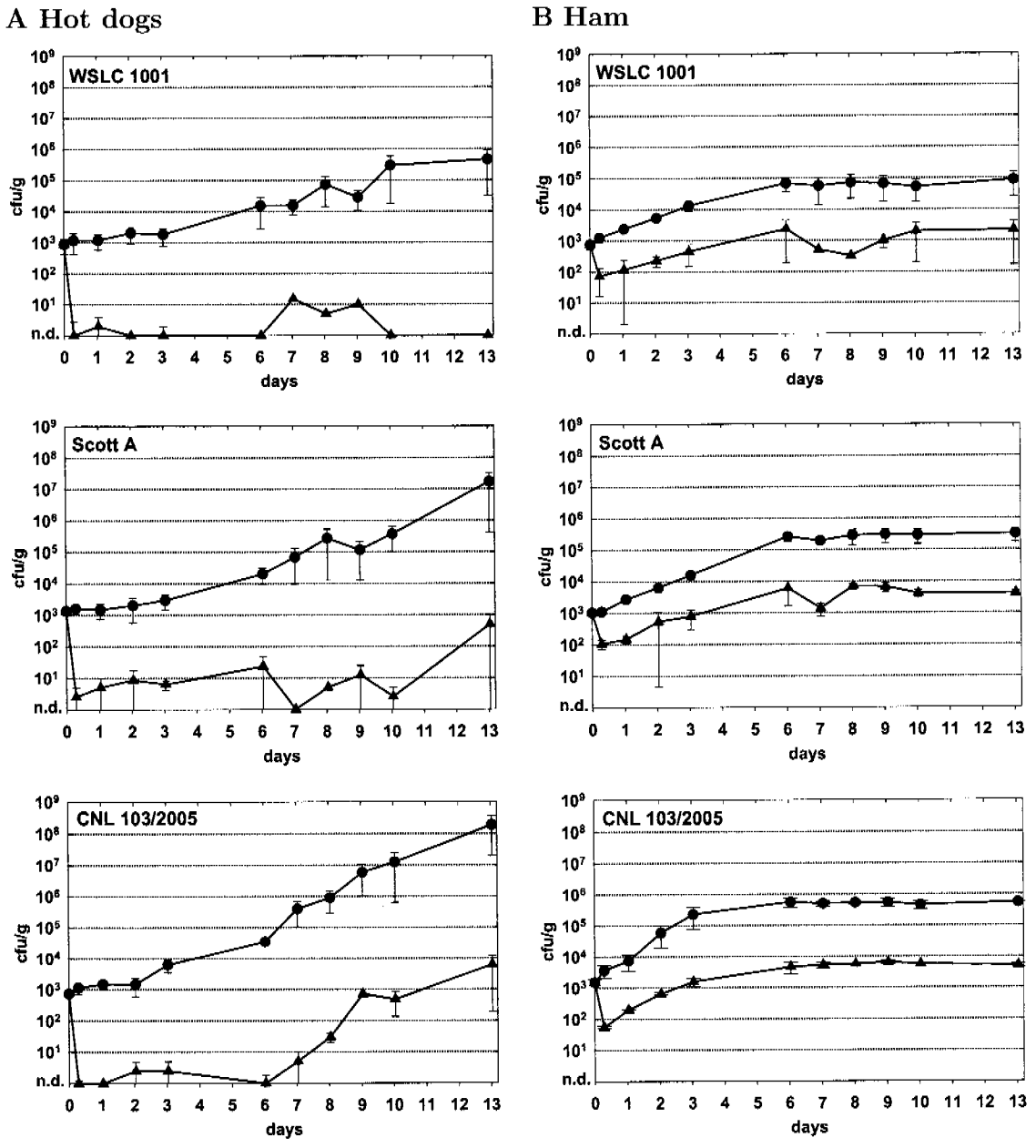


Figure 3.9: Effect of phage A511 of different strains of *L. monocytogenes* on hot dogs (Column A) and ham (Column B) at 6°C during 13 days (● control; ▲ +A511). Phage was applied in concentrations between 1.4×10^8 pfu/g and 3.0×10^8 pfu/g after 1 hour.

3.1.1.7.2 Chocolate milk and mozzarella cheese brine

In chocolate milk, strain WSLC 1001 and Scott A multiplied to more than 10^7 cfu/ml, and CNL 103/2005 to more than 10^8 cfu/ml (Figure 3.10, Column A). After application of phage A511, no viable cells were detected after 13 days in experiments with WSLC 1001 and Scott A. The killing effect was therefore more than 7 log units. Using CNL 103/2005, bacterial numbers decreased by 2 to 3 log units in the first week, but increased again in the second week to more than 1×10^2 cfu/ml. The reduction was 6.5 log units compared to the control after 13 days.

In mozzarella cheese brine (Figure 3.10, Column B), no further growth, but rather a slight reduction of strain WSLC 1001 was observed after the first week. Cell numbers of Scott A, however, further increased by approximately 1 log unit, and cell counts of CNL 103/2005 by almost 3 log units. In phage treated samples levels below 5 cfu/ml or even no viable *Listeria* cell were detected in experiments with WSLC 1001 and Scott A. The killing effect after 13 days compared to the control was 3.3 and 5.1 log units, respectively. For strain CNL 103/2005 this effect was 6.0 log units, but bacterial cell concentration were still between 10 and 100 cfu/ml over the whole experiment.

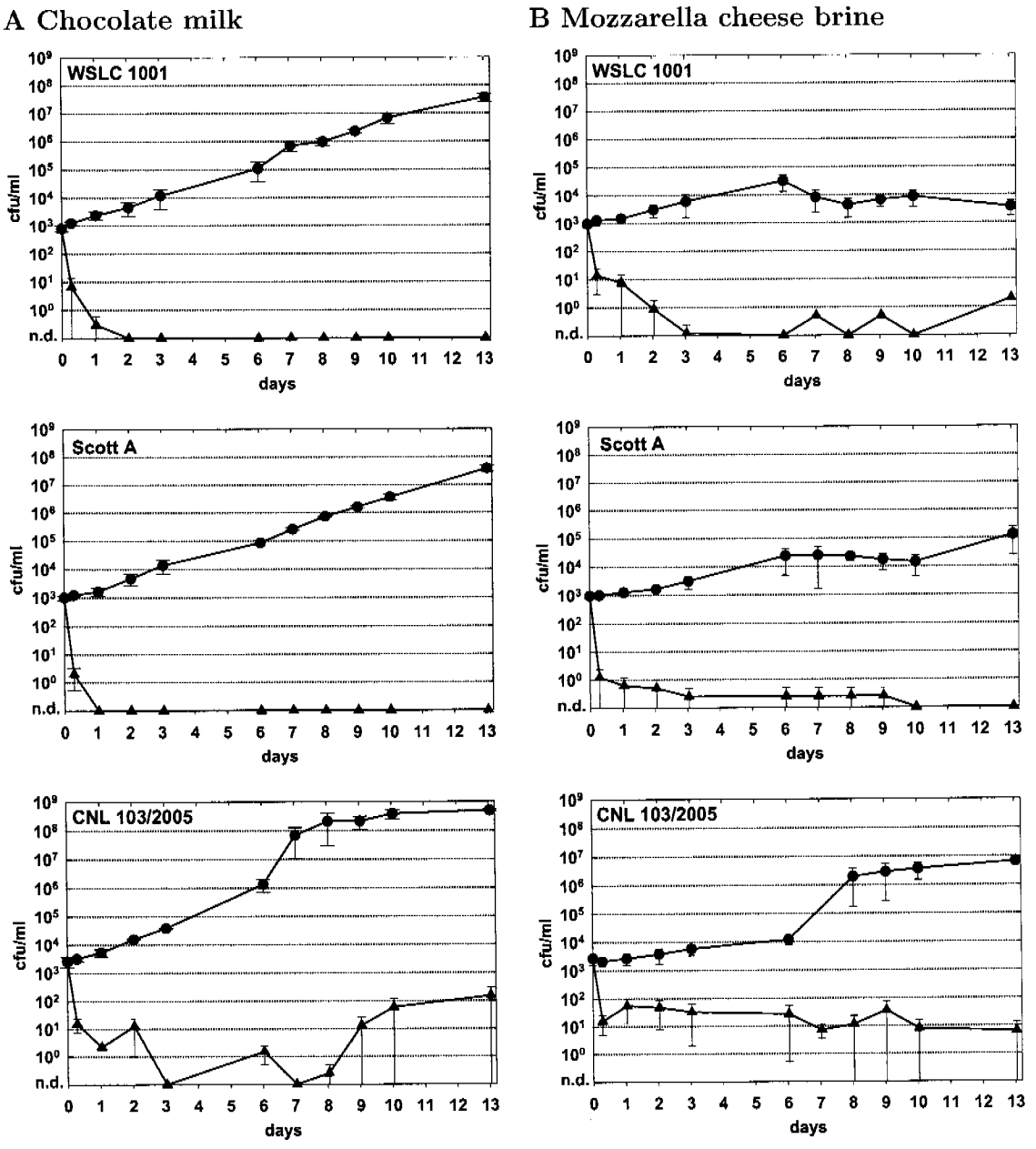


Figure 3.10: Effect of phage A511 of different strains of *L. monocytogenes* in chocolate milk (Column A) and mozzarella cheese brine (Column B) at 6°C during 13 days (● control; ▲ +A511). Phage was applied in concentrations between 2.0×10^8 pfu/g and 3.4×10^8 pfu/g after 1 hour.

3.1.1.8 Application of different phage concentrations

The effect of different phage concentrations (3×10^8 pfu/g, 3×10^7 pfu/g, 3×10^6 pfu/g) was evaluated on three foods. In general, a lower initial phage titer resulted in a lower cell count reduction (Figure 3.11).

On hot dogs, no *Listeria* cell was detected after 6 days, when 3×10^8 pfu/g were applied to the foods. With 3×10^7 pfu/g, cell counts were reduced by 2.5 log units, but then increased again by 1.1 log units until day 6. Using 3×10^6 pfu/g, a reduction of 2.0 log units until day 3 was observed, but then again an increase of 1.1 log units. The difference to the control were 4.2, 2.7 and 2.2 log units after 6 days.

In chocolate milk, no bacteria were detected after 2 days, when 3×10^8 pfu/ml were added. Applying 3×10^7 pfu/ml, a reduction of 1.4 log units was observed between day 1 and day 2. Afterward, *Listeria* cell counts decreased further to less than 10 cfu/ml. This corresponds to a difference of 4.4 log units compared to the control. Using 3×10^6 pfu/g no significant growth suppression was observed until day 3. At day 6, however, reduction was 1.0 log unit and statistically significant.

On cabbage, contamination levels were reduced below 10 cfu/g using 3×10^8 pfu/g. With 3×10^7 pfu/g viable cell numbers were decreased by 1.7 log units after one day, but then increased again to 1.5×10^2 cfu/g after 6 days. On samples containing 3×10^6 pfu/g *Listeria* cell concentration did not change. Growth inhibition compared to the control were 3.3, 1.9, and 0.9 log units at day 6.

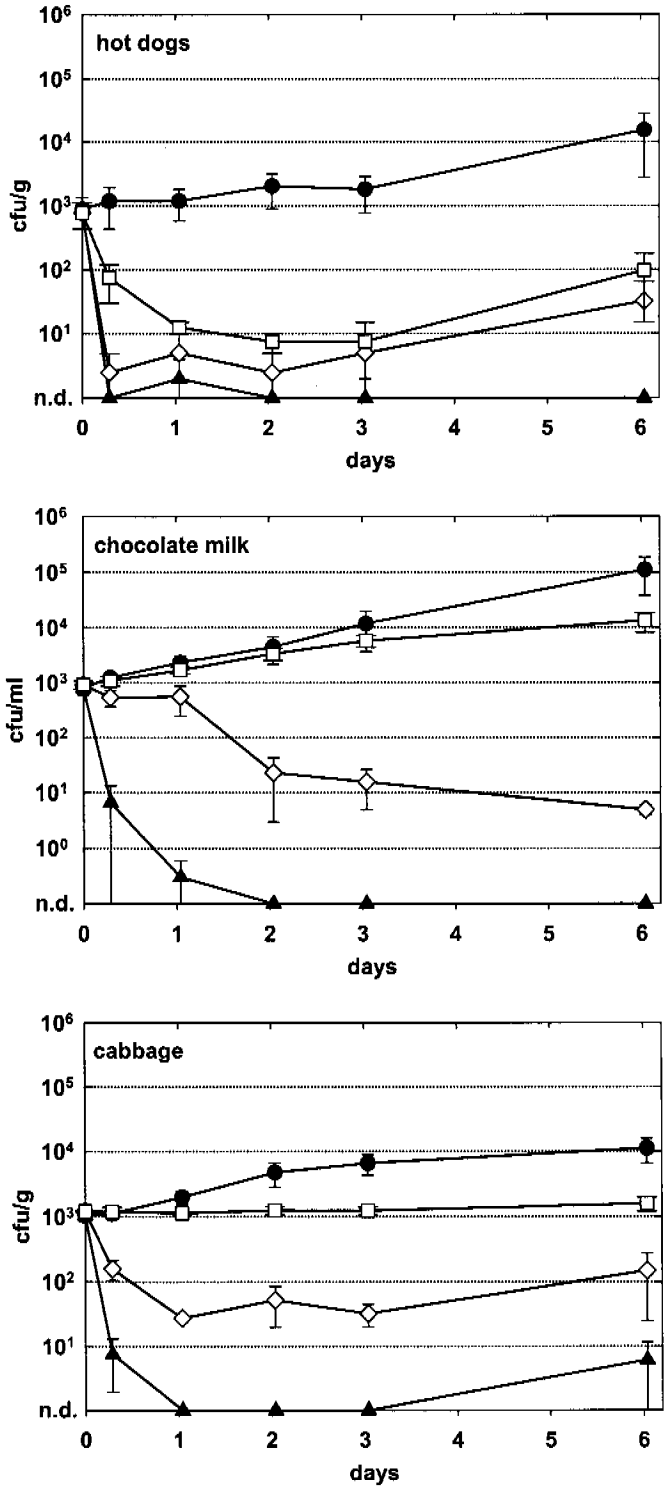


Figure 3.11: Effect of different concentrations of phage A511 on growth of *L. monocytogenes* WSLC 1001 in different foods at 6°C over 6 days (● control; ▲ $+3 \times 10^8$ pfu/g; ◇ $+3 \times 10^7$ pfu/g; □ $+3 \times 10^6$ pfu/g)).

3.1.1.9 Using other *Listeria* phages: P35 and P100

In order to compare the efficacy of phage A511 to other virulent *Listeria* phages, the two phages P35 and P100 were also tested in different foods.

3.1.1.9.1 Application of bacteriophage P35

The effect of *Listeria* phage P35 on *L. monocytogenes* WSLC 1001 was studied on four different foods (minced meat, smoked salmon, chocolate milk and iceberg lettuce). Growth of WSLC 1001 on minced meat and smoked salmon was virtually indistinguishable between phage and control samples ($p>0.05$) (Figure 3.12). In chocolate milk and on iceberg lettuce reduction of *Listeria* cell counts was 5.3 and 2.1 log units, respectively, and thus similar to experiments with A511.

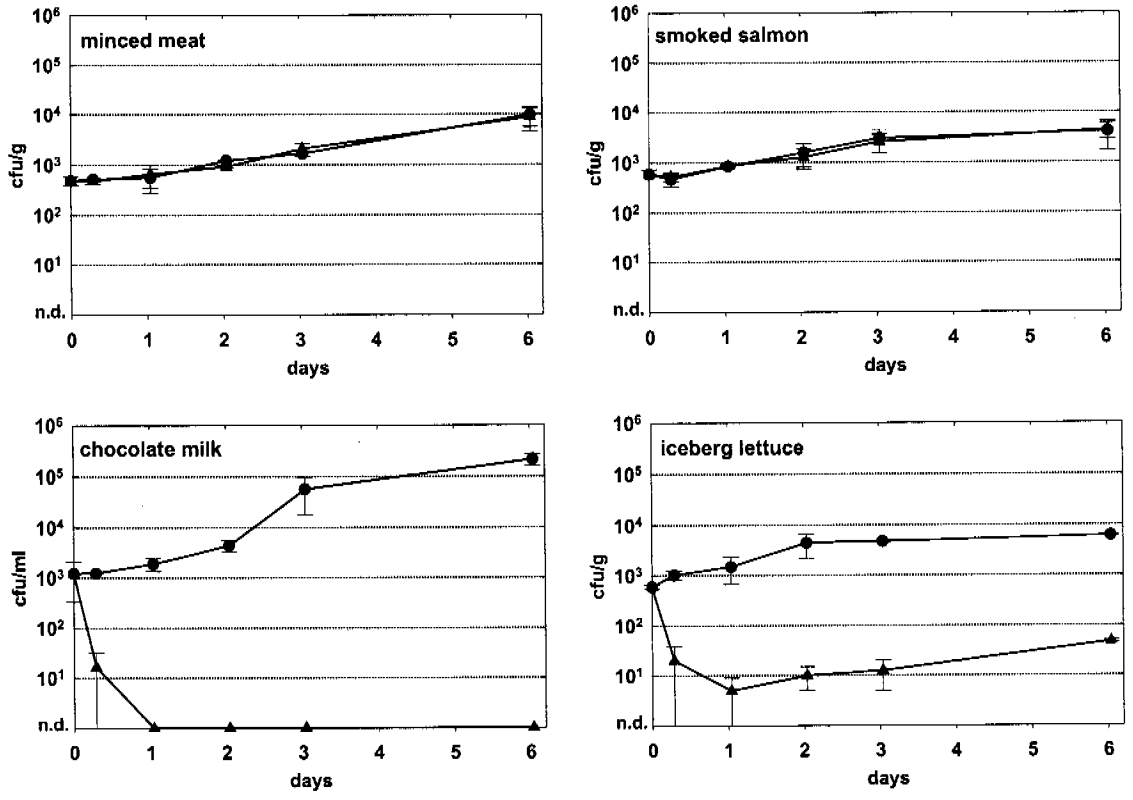


Figure 3.12: Effect of phage P35 on growth of *L. monocytogenes* WSLC 1001 on different foods at 6°C over 6 days (● control; ▲ +P35). Phage P35 was applied in concentrations between 3.2×10^8 pfu/g and 5.9×10^8 pfu/g after 1 hour.

3.1.1.9.2 Application of bacteriophage P100

Inhibiting effects of phage P1001 on WSLC 1001 were tested on five different foods: hot dogs, minced meat, smoked salmon, mixed seafood and cabbage (Figure 3.13). In general, growth suppression by phage P100 was comparable to phage A511. Minor differences were observed on hot dogs, mixed seafood and cabbage. On hot dogs, the killing effect of *L. monocytogenes* after 6 days was 0.9 log units lower for P100 compared to A511. On mixed seafood and cabbage, however, inhibiting effects were 0.4 and 0.7 log units higher using P100.

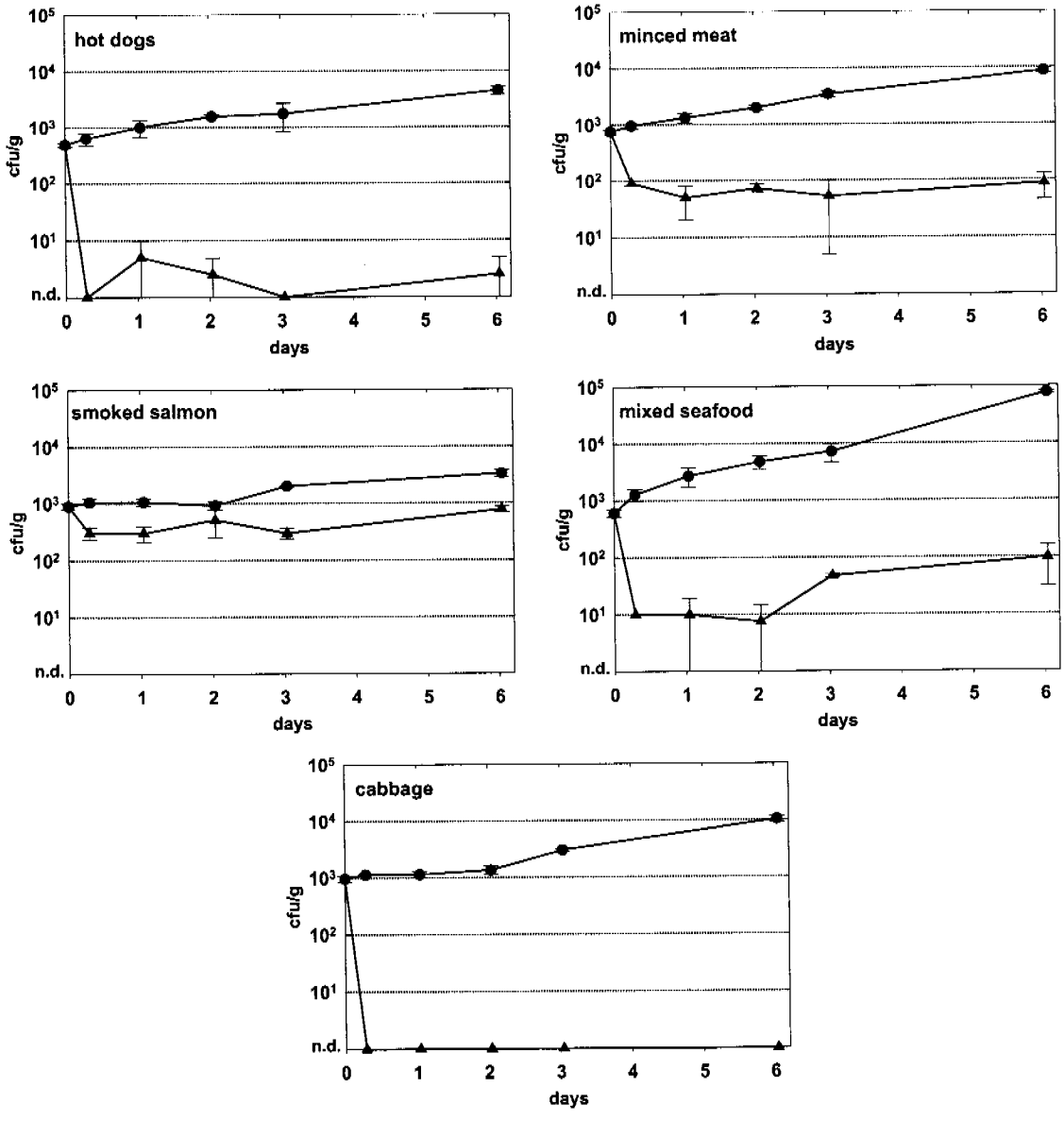
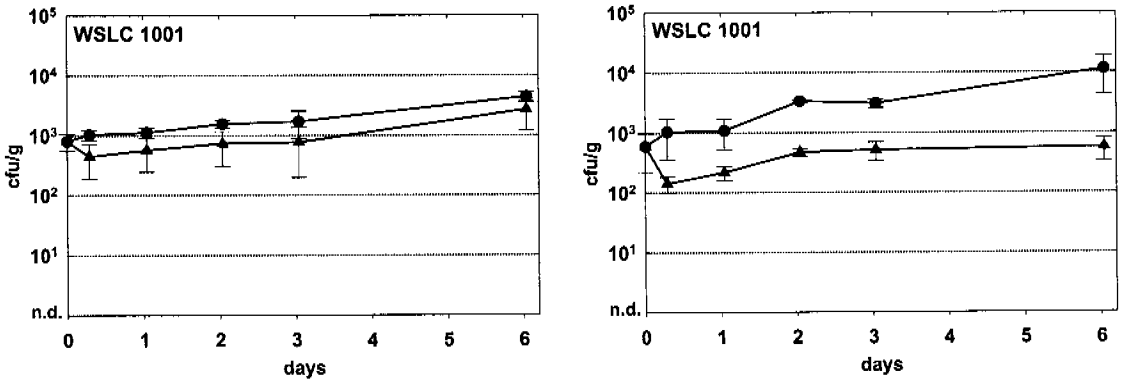


Figure 3.13: Effect of phage P100 on growth of *L. monocytogenes* WSLC 1001 in different foods at 6°C over 6 days (● control; ▲ +P100). Phage P100 was applied in concentrations between 3.8×10^8 pfu/g and 5.2×10^8 pfu/g after 1 hour.

3.1.1.10 Optimization of phage application on smoked salmon

Results of the previous chapters showed that growth inhibition of *L. monocytogenes* on smoked salmon and smoked trout was less than 1 log unit and in most cases not significant using either phage A511, P100 or P35. In order to improve the killing effect of *L. monocytogenes* on smoked salmon, different approaches were investigated using phage A511 and *Listeria* strains WSLC 1001 or Scott A (Chapter 2.4.1).

The application of phage A511 one hour before the contamination did not result in any significantly improved control effects of WSLC 1001 (data not shown). Repeated application after 1, 7 and 25 hours slightly enhanced growth suppression of *L. monocytogenes* from 0.4 to 0.9 log units after 6 days (data not shown). Dipping the sliced pieces of smoked salmon into the phage suspension improved reduction effects significantly by 1.3 log units, compared to manual spreading of A511 onto the surface of the salmon (Figure 3.14).

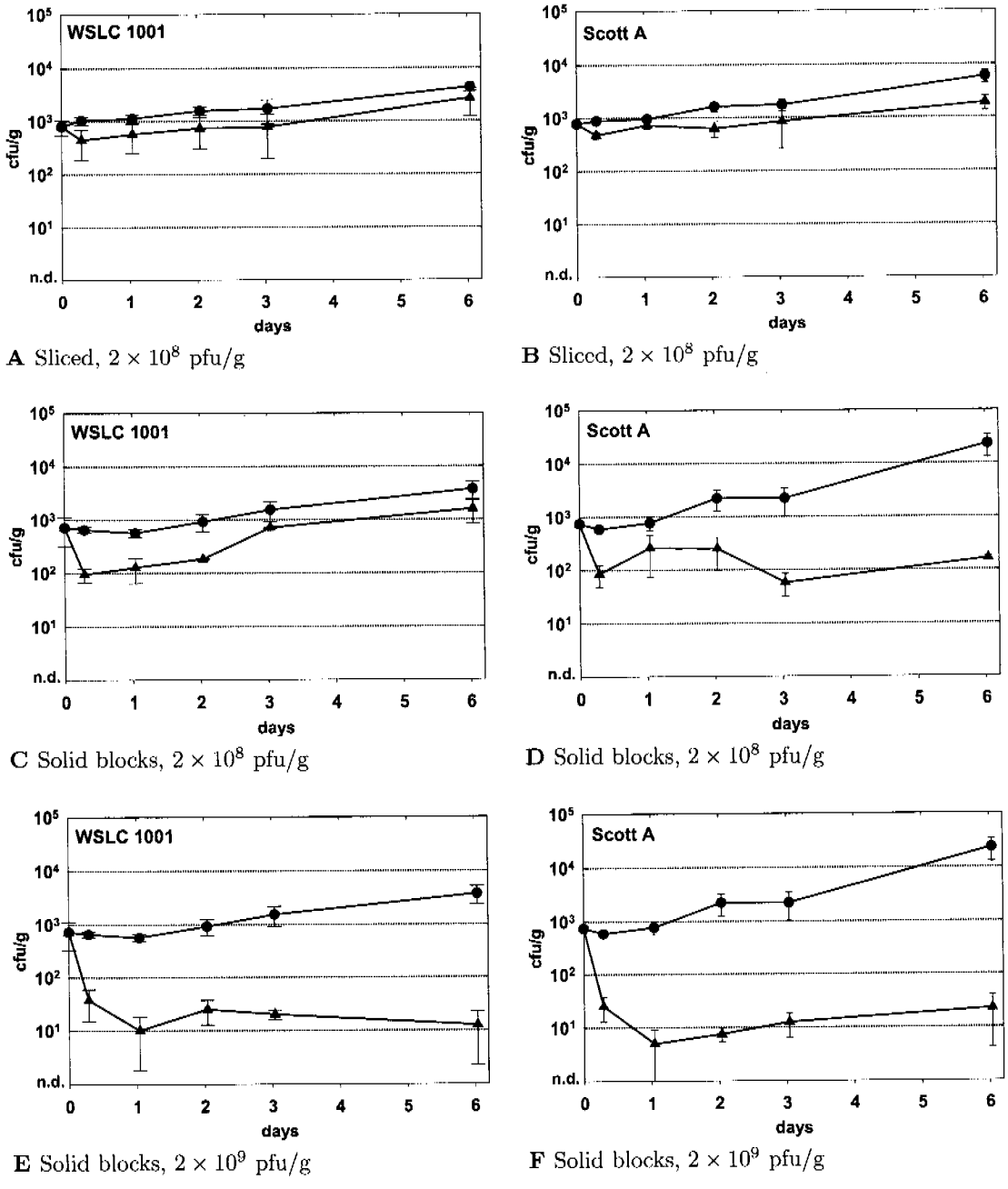


A Manual spreading **B** Dipping

Figure 3.14: Effect of different application protocols of phage A511 on growth of *L. monocytogenes* WSLC 1001 on smoked salmon at 6°C during 6 days. Phages (2×10^8 pfu/g) were manually spread onto the smoked salmon pieces (A) or salmon was dipped into the phage suspension (B) (● control; ▲ +A511).

When solid blocks of smoked salmon were used, the killing effect of WSLC 1001 was comparable to the effect on salmon sliced into 10 g pieces (Figure 3.15 C). However, using a higher initial phage concentration (2×10^9 pfu/g) on solid blocks of smoked salmon, growth suppression was improved by 1.7 log units higher after 6 days (Figure 3.15 E).

Listeria strain Scott A, was reduced by 0.5 log units on sliced smoked salmon after 6 days ($p>0.05$). However, on solid blocks of smoked salmon growth inhibition of Scott A was 2.2 log units ($p<0.05$) and thus significantly higher (Figure 3.15 D). Adding the higher initial phage concentration (2×10^9 pfu/g) on solid blocks of smoked salmon, growth inhibition was further increased to 3.0 log units after 6 days ($p<0.05$) (Figure 3.15 F). After day 1, contamination levels below 1×10^2 cfu/g were observed.



A Sliced, 2×10^8 pfu/g

B Sliced, 2×10^8 pfu/g

C Solid blocks, 2×10^8 pfu/g

D Solid blocks, 2×10^8 pfu/g

E Solid blocks, 2×10^9 pfu/g

F Solid blocks, 2×10^9 pfu/g

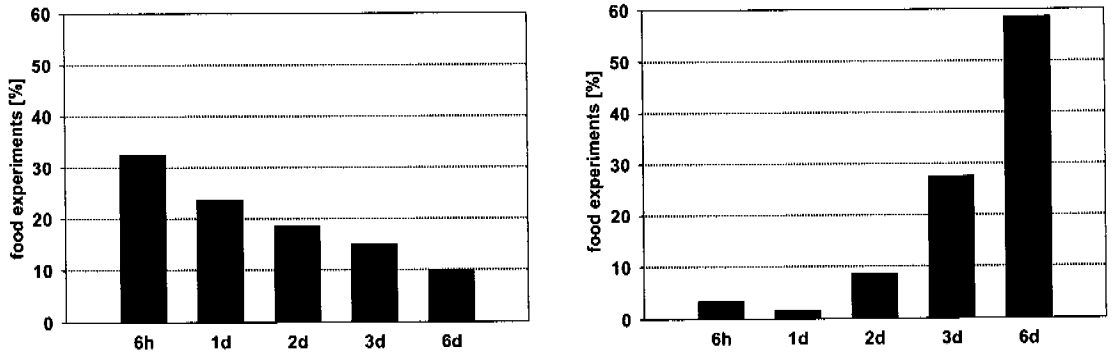
Figure 3.15: Effect of phage A511 on growth growth of *L. monocytogenes* WSLC 1001 and Scott A at 6°C during 6 days: on sliced smoked salmon (A, B) or solid blocks of smoked salmon (C-F) using 2×10^8 pfu/g (A-D) or 2×10^9 pfu/g (E,F) (● control; ▲ +A511).

3.1.1.11 Overall efficacy of *Listeria* bacteriophages in different foods

For the evaluation of the efficacy of phage on bacterial growth in foods, two criteria are important. First, the reduction of the initial contamination level, and second, the growth reduction compared to non treated foods after a certain time.

In 56% of all storage experiments with phage A511 lasting for 6 days, the highest reduction of the initial contamination level was measured after the first 6h and the first day (Figure 3.16A). There were few exceptions with highest reduction levels first after 6 days. Examples are raw salmon contaminated with strain Scott A (Figure 3.3) and mozzarella brine cheese contaminated with WSLC 1001 (Figure 3.6). However, the most striking reduction of cell number was again obeserved within the first day.

On the other hand, in 59% of all storage experiments the greatest growth inhibition in comparison to the control was observed after 6 days (Figure 3.16B). Exceptions were experiments at 20°C (Figure 3.8), where *Listeria* cells were able to propagate strongly on phage containing samples. Therefore, the difference to the control became smaller after 6 days. This was also observed in experiments with cabbage or iceberg lettuce, where phage titer decreased and re-growth occurred (Figure 3.5).



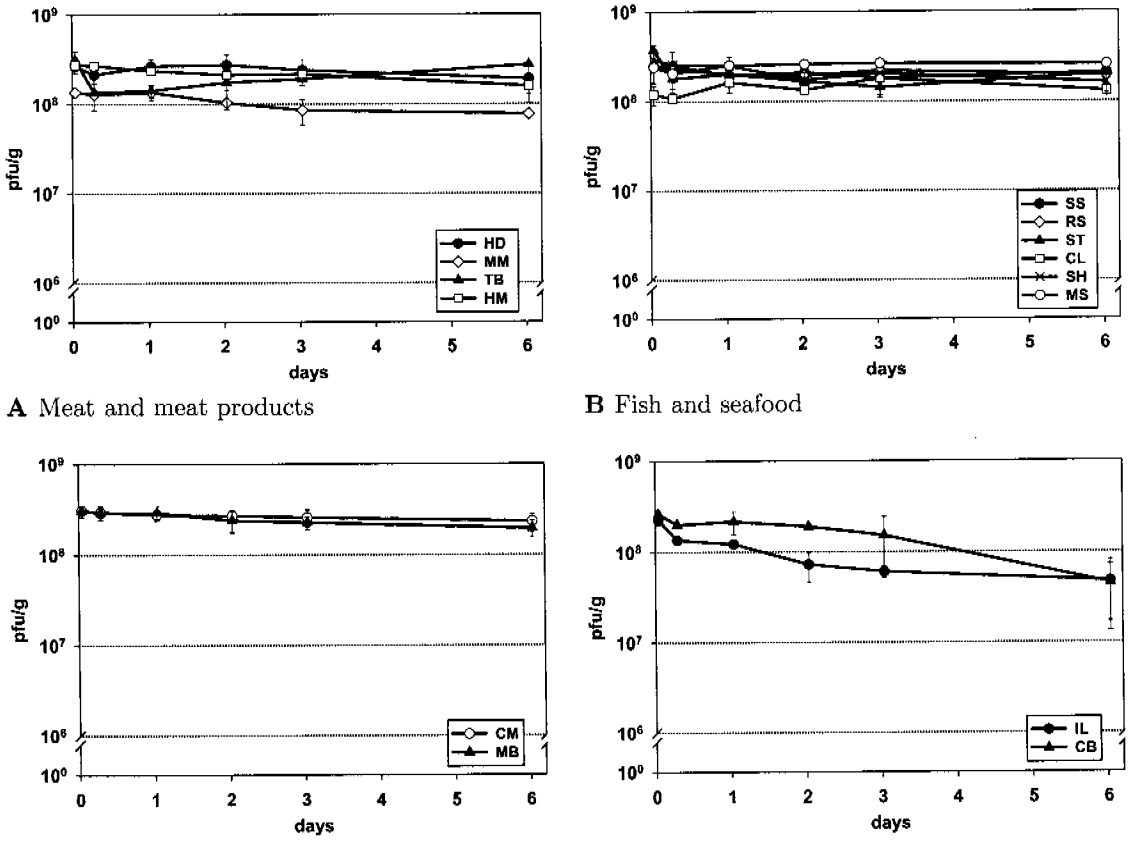
A Reduction of the initial contamination level

B Reduction compared to control samples

Figure 3.16: Number of food experiments in percent with maximal growth inhibition effects of *L. monocytogenes* after a certain time: Reduction of the initial cell contamination level of *L. monocytogenes* (A), Reduction of *Listeria* cell numbers compared to control samples (B).

3.1.1.12 Stability of *Listeria* bacteriophages in foods

In order to evaluate the stability of bacteriophages on different foods, phage counts were determined during all experiments. In general, no significant changes in concentrations of phage A511, P100 or P35 were observed in non-plant products over 6 or 13 days at different temperatures. One exception was hot dogs, where counts of phage A511 were decreased by 0.5 to 1.1 log units after 13 days. In contrast to non-plant products, a reduction of 0.6 - 1.2 log units was determined on cabbage or iceberg lettuce. This decrease was even higher on cabbage at 20°C (2.0 log units). As an example, development of concentration of phage A511 on different foods is shown in Figure 3.17. Here, foods were contaminated with *L. monocytogenes* WSLC 1001 and stored over 6 days at 6°C.



A Meat and meat products

B Fish and seafood

C Chocolate milk and mozzarella cheese brine

D Vegetables

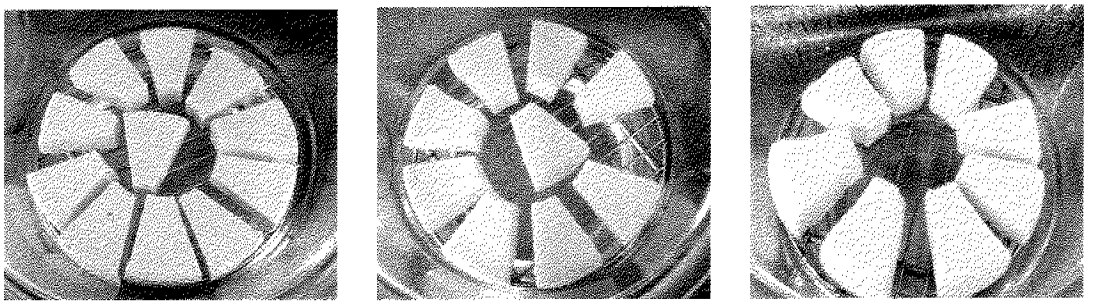
Figure 3.17: Concentration of A511 on different foods over 6 days at 6°C. HD = hot dogs, MM = minced meat, TB = sliced turkey breast, HM = ham, SS = smoked salmon, RS = raw salmon, ST = smoked trout, SH = shrimps, CL = calamari, MS = mixed seafood, CM = chocolate milk, MB = mozzarella cheese brine, IL = iceberg lettuce, CB = cabagge.

3.1.2 Control of *Listeria monocytogenes* during the ripening of soft cheeses

In order to evaluate the effect of phage A511 on growth of *L. monocytogenes* during ripening of cheeses, phages were applied to the surface of artificially contaminated soft cheeses. In preliminary experiments, growth behavior of *Listeria* strains WSLC 1001, Scott A and CNL 103/2005 was determined on the surface of soft cheeses during ripening and storage. Cell numbers of WSLC 1001 on white mold soft cheese were 1 to 3 log units lower than that of Scott A during 21 days. Therefore, WSLC 1001 was not used here.

3.1.2.1 White mold soft cheese

White mold soft cheese was chosen for ripening experiments. During ripening, the pH and the appearance of the cheeses was monitored. From the 3rd to 6th day on characteristic growth of mold appeared on the surface of the cheeses (Figure 3.18). After one week cheeses became softer inside, and the "typical" odor was developed.

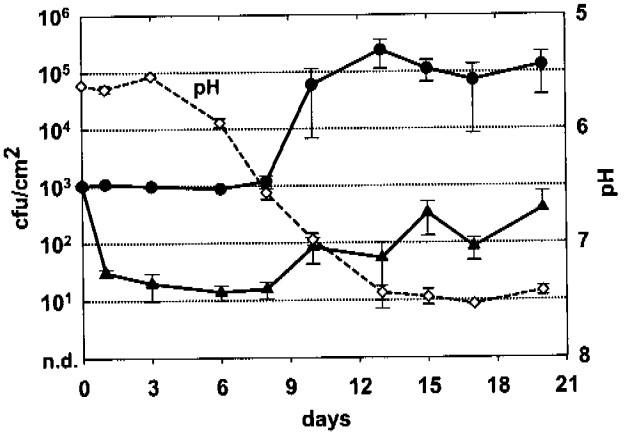


A day 1 **B** day 6 **C** day 10

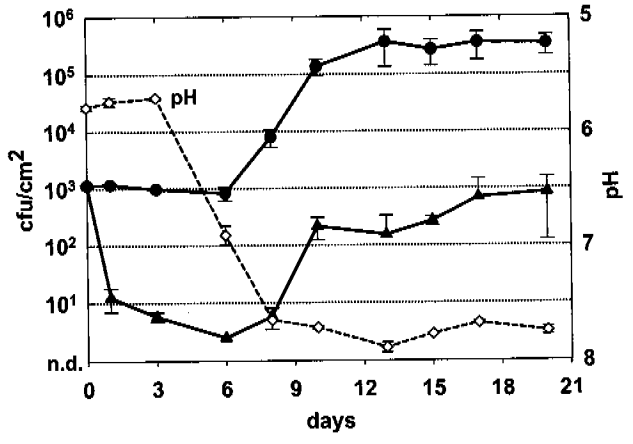
Figure 3.18: Ripening stages of white mold soft cheese at different days.

The pH on the cheese surface was pH 5.6±0.2 in the beginning, started increasing after 3 days and reached pH 7.6±0.2 after 20 days. Growth of *L. monocytogenes* Scott A started, when the pH on the cheese surface was above pH 6. Within days 6 and 13, *Listeria* cell counts rose by 2.5 log units and reached 3.1×10^5 cfu/cm^2 after 20 days (Figure 3.19). On cheeses containing 3×10^8 pfu/cm^2 of A511, *Listeria* cell counts were reduced below 1×10^2 cfu/cm^2 in the first 8 days (Figure 3.19 A). However, after 13 days contamination levels reached 4.0×10^2 cfu/cm^2. Growth inhibition of Scott A was 2.5 log units after 20 days compared to the control. When phage A511 was added twice to the cheese surface (3×10^8 pfu/cm^2 after 1 h and after 20 h), *Listeria* cell counts were decreased below 10 cfu/cm^2 after the first 3 to 8 days (Figure 3.19 B). Then, an increase of cell concentrations was observed again. At day 20 the difference to the control was 2.6 log units. Application of higher phage concentration after 1 h

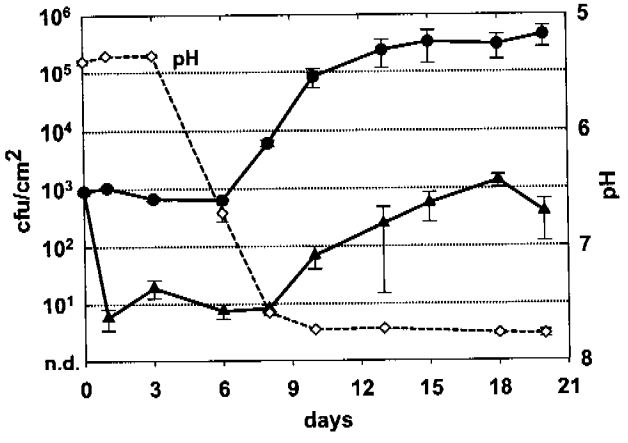
(1×10^9 pfu/cm^2) caused a similar *Listeria* cell count reduction below 10 cfu/cm^2 after the first 6 to 8 days and a similar increase afterward (Figure 3.19 C). Growth suppression was 3.1 log units after 20 days.



A Single application of 3×10^8 pfu/cm^2



B Double application of 3×10^8 pfu/cm^2



C Single application of 1×10^9 pfu/cm^2

Figure 3.19: Effect of phage A511 on growth of *L. monocytogenes* Scott A during ripening of white mold soft cheeses (● control; ▲ +A511; ◇ pH (right axis, reverse scale)).

Concentration of phage A511 decreased significantly by 1.5 log units on the surface of white mold soft cheeses. This was also the case when phages were applied twice (Figure 3.20).

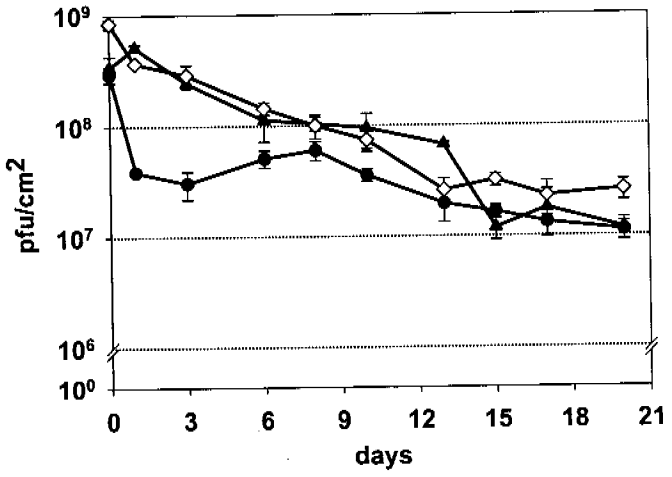


Figure 3.20: Concentration of phage A511 on the surface of white mold soft cheeses over 20 days (● 1 x (3×10^8 pfu/cm^2); ▲ 2 x (3×10^8 pfu/cm^2); ◇ 1 x (1×10^9 pfu/cm^2)).

3.1.2.2 Red smear soft cheese

The effect of phage A511 on growth of *Listeria monocytogenes* CNL 103/2005 and Scott A was also tested on red smear soft cheeses. Cheeses were smeared three times at day 0, 3 and 6 using a commercial ripening culture (OFR9). The surface developed a yellow-orange like color, partly covered with mold (Figure 3.23). Cheeses became softer inside after one week, and the "typical" odor was developed.

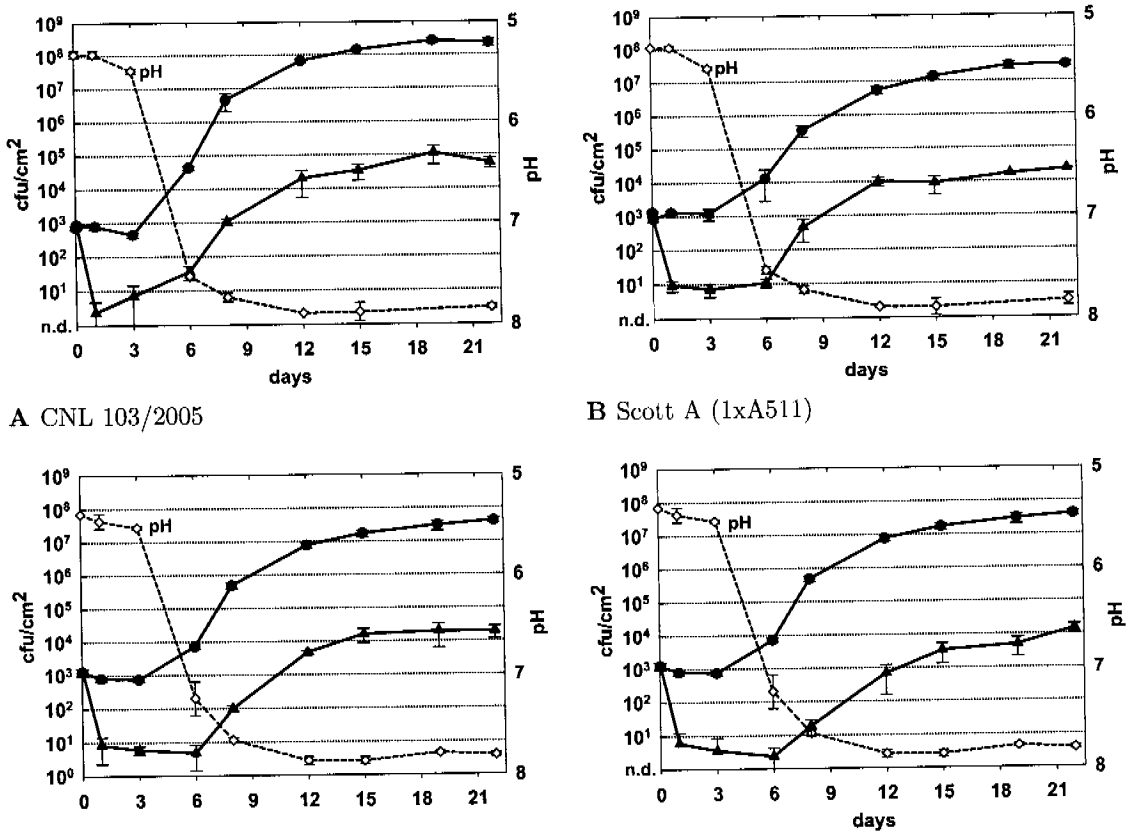
The pH on the cheese surface was pH 5.4±0.1 in the beginning, started increasing after 3 days and reached pH 7.8±0.1 after 22 days. Additionally total aerobic plate counts were determined in one of the experiments on red smear cheeses to confirm that application of phage A511 has no influence on the ripening flora. There were no significant differences in total aerobic cell numbers between control cheeses or phage treated cheeses (data not shown).

When the pH was above pH 6, *L. monocytogenes* started growing on the cheese surface (Figure 3.21). Within day 3 and 12 *Listeria* cell numbers strongly increased by 5.2 log units in the case of strain CNL 103/2005 and by 4.2 log units with Scott A. After 22 days, contamination levels were 2.3×10^8 cfu/cm^2 and 3.5×10^7 cfu/cm^2, respectively.

When phage A511 was applied once to the cheeses, cell counts were reduced below 10 cfu/cm^2 in the first 3 to 6 days (Figure 3.21 A and B). Then, re-growth occurred and bacterial numbers above 1×10^4 cfu/cm^2 were observed at day 22. Growth inhibition

was then 3.6 for strain CNL 103/2005 and 3.1 log units for Scott A.

In order to improve the killing effect of A511 on soft cheese, two additional phage application protocols were evaluated. First, phage A511 (3×10^8 pfu/cm^2) was applied twice with the smearing solution (day 0 and 6). Second, it was added three times (day 0, 3, 6). Adding A511 twice to the cheese surface, a small, but significant difference compared to single application could be detected between day 6 and day 12 (Figure 3.21 C). The triplicate application led to significant lower *Listeria* cell counts between day 8 and 19 (Figure 3.21 D). However, after 22 days no significant difference between the three application protocols was determined.



A CNL 103/2005

B Scott A (1xA511)

C Scott A (2xA511)

D Scott A (3xA511)

Figure 3.21: Effect of phage A511 on growth of *L. monocytogenes* CNL 103/2005 (A) and Scott A (B, C, D) during ripening of red smear soft cheese. Phage application was performed once (A, B), twice (C) or three times (D)(● control; ▲ +A511; ◇ pH (right axis, reverse scale)).

During the total duration of the experiments, phage concentrations on the surface of red smear soft cheeses were reduced by 0.5 log units. This reduction was lower after 22 days, when phage was added twice (0.3 log units) or three times (0.1 log units) to the cheeses (Figure 3.22).

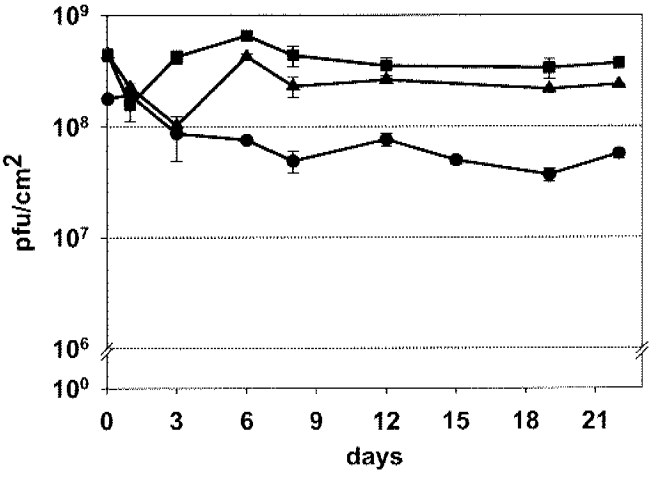
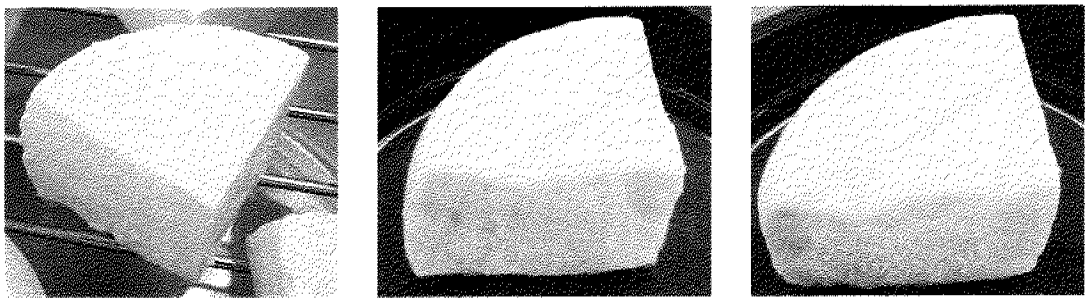


Figure 3.22: Concentration of phage A511 on the surface of red smear soft cheeses over 20 days (● 1 x (3×10^8 pfu/cm^2); ▲ 2 x (3×10^8 pfu/cm^2); ■ 3 x (3×10^8 pfu/cm^2)).



A day 1 **B** day 6 **C** day 11

Figure 3.23: Ripening stages of red smear soft cheese at different days.

3.1.3 Insensitivity against phage A511

In order to investigate emergence of phage resistance in foods, *Listeria* colonies re-isolated from phage treated food samples were tested for phage sensitivity. In experiments with *Listeria* strain Scott A, no phage resistant bacterial cells were isolated from phage treated foods. However, using strain WSLC 1001 and CNL 103/2005, isolates less sensitive or even resistant to phage A511 were found.

During longterm experiments with WSLC 1001, phage resistant isolates were detected in one out of two experiments with hot dogs, mozzarella cheese brine and chocolate milk. In these cases, growth of WSLC 1001 was not inhibited over the whole time. After an initial reduction of *Listeria* cell numbers, the bacteria multiplied similarly to bacteria on control samples (Figure 3.24). After 13 days growth suppression was 2.2 log units on hot dogs and in mozzarella cheese brine, and 2.5 log units in chocolate milk. Compared to experiments, where no phage resistance emerged, growth inhibition was 1.1, 3.5 and 5.1 log units lower, respectively (Chapter 3.1.1.7). Re-isolated *Liste-*

ria cells from hot dogs at day 13 were tested for phage resistance. Five isolates were as sensitive as the wild type strain; for three isolates efficiency of plating (EOP) was 10^{-1} - 10^{-3}. The EOP was determined by devision of the phage titer obtained using the isolate with the titer obtained using the sensitive host strain (Chapter 2.6). Two further isolates could not be infected by A511 (EOP < 10^{-7}). In the case of mozzarella cheese brine, EOP was 10^{-3} with one clone, the remaining nine isolates were not infected by A511. With chocolate milk, EOP was 10^{-3} for 4 isolates and the remaining 6 clones were insensitive to A511.

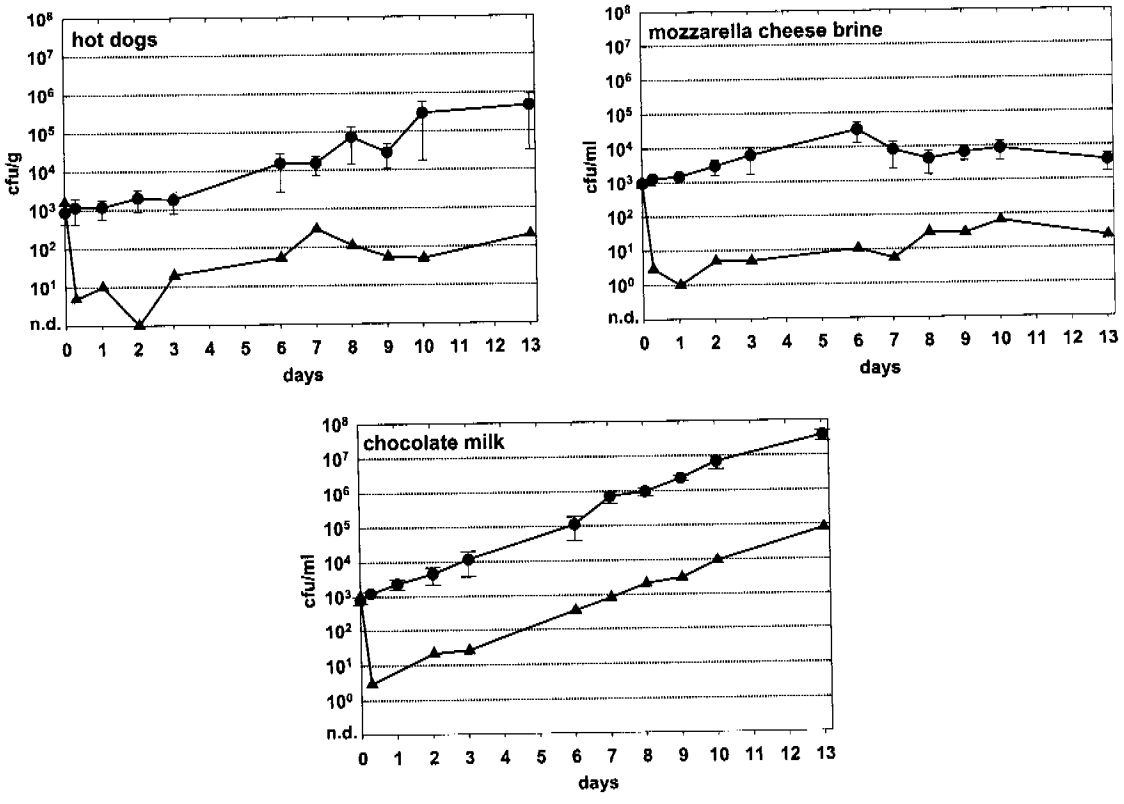
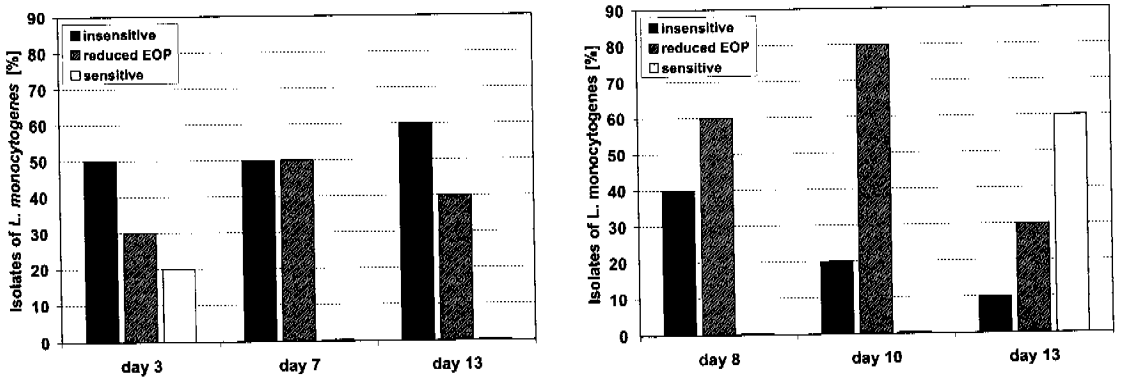


Figure 3.24: Growth of *L. monocytogenes* WSLC 1001 on hot dogs, in mozzarella cheese brine, and in chocolate milk over 13 days. Re-isolated *Listeria* cells at day 13 showed resistance against phage A511 in all cases.

In two out of five experiments in chocolate milk with CNL 103/2005 at 6 °C, higher *Listeria* cell counts were detected after 6 days. Ten re-isolated clones were tested for phage sensitivity and all showed insensitivity (EOP¡10^{-7}). During both longterm experiments on hot dogs, *Listeria* colonies less sensitive to phage A511 were detected after 13 days. In the first experiment, six isolated *Listeria* colonies were as sensitive as the wild type strain CNL 103/2005, for three clones EOP was below 10^{-6} and 1 isolate was insensitive to phage A511. In the second experiment, nine of 10 isolates were insensitive against phage A511. Furthermore, in experiments with red smear cheese, resistant *Listeria* cells were also isolated after 22 days. Three out of 10 isolates showed

insensitivity to phage A511 in this case.

Development of insensitivity was analyzed during the longtime experiments in chocolate milk with WSLC 1001 and on hot dogs with CNL 103/2005 stored at 6°C (Figure 3.25). This development was completely different for both cases. In chocolate milk the proportion of insensitive cells of WSLC 1001 increased during time while on hot dogs insensitive isolates of CNL 103/2005 seemed to disappear again.



A WSLC 1001

B CNL 103/2005

Figure 3.25: Percentage of *Listeria* cells in chocolate milk (WSLC 1001) (A) and on hot dogs (CNL 103/2005) (B) showing insensitivity to phage A511 after the indicated time. EOP=Efficiency of plating.

Listeria colonies re-isolated from foods and resistant to phage A511 were also tested for sensitivity against other *Listeria* phages. Isolates were still sensitive to P100 or P35, but EOP was reduced to 10^{-1} and 10^{-2}, respectively.

3.1.4 Use of *Listeria* phage endolysins for control of *Listeria monocytogenes* in foods

3.1.4.1 Production of *Listeria* phage endolysins HPL118, HPL511, HPL500 in *E. coli*

Recombinant *Listeria* phage endolysins were produced using *E. coli* JM 109 (pHPL118), *E. coli* JM 109 (pHPL500), and *E. coli* JM 109 (pHPL511). Purity of the purified proteins was between 75% and 85% (Table 3.1).

Table 3.1: Concentration and enzyme activity of different *Listeria* phage endolysins

Endolysin	Concentration [mg/ml]	Enzymatic activity [U/mg]
HPL118	1.8	23,000
HPL500	3.3	73,000
HPL511	4.8	48,000

3.1.4.2 Production of *Listeria* phage endolysin PLY511 by *S. carnosus* (pPSHG7-*ply*511)

Best conditions for production of PLY511 by *S. carnosus* (pPSHG7-*ply*511) were cultivation of the bacteria for 15 - 17 h at 150 rpm and 37°C in yeast extract medium containing 1% glycerol and 1% - 1.5% galactose. Enzymatic activity in the supernatant was between 1,500 and 2,500 U/ml. After ammonium sulfate precipitation, activity was about 70,000 U/ml. Compared to the concentration of HPL511 (230,000 U/ml) it was 3.3 times lower.

3.1.4.3 Application of *Listeria* phage endolysins to food

In food experiments, 35 to 50 μg endolysin per g food were used corresponding to an enzymatic activity of 1,200 to 3,000 U/g. The effect of endolysin application on growth of *Listeria* WSLC 1001 was examined on iceberg lettuce, at different temperatures, and over 6 days. At 6°C (Figure 3.26A) cell counts of WSLC 1001 slightly increased by 0.3 log units. On iceberg lettuce treated with HPL118, HPL511, or both enzymes, contamination levels were significantly reduced by 0.6 to 0.7 log units compared to the control after 6 days. There was no significant difference in growth inhibition between the three applications (HPL118, HPL511 or both endolysins).

At 12°C (Figure 3.26B), WSLC 1001 multiplied by 0.5 log units. Using HPL118 caused no significant growth inhibition ($p>0.05$). With HPL511, however, growth suppression was 1.0 log unit after 6 days ($p<0.05$), and thus significantly different from the

application of HPL118. When both endolysins were added to iceberg lettuce, *Listeria* cell numbers continuously decreased over the whole time. At day 6 the reduction was 1.6 log units compared to the control ($p<0.05$). This was not significant different from the reduction observed with HPL511, but from the effect with HPL118.

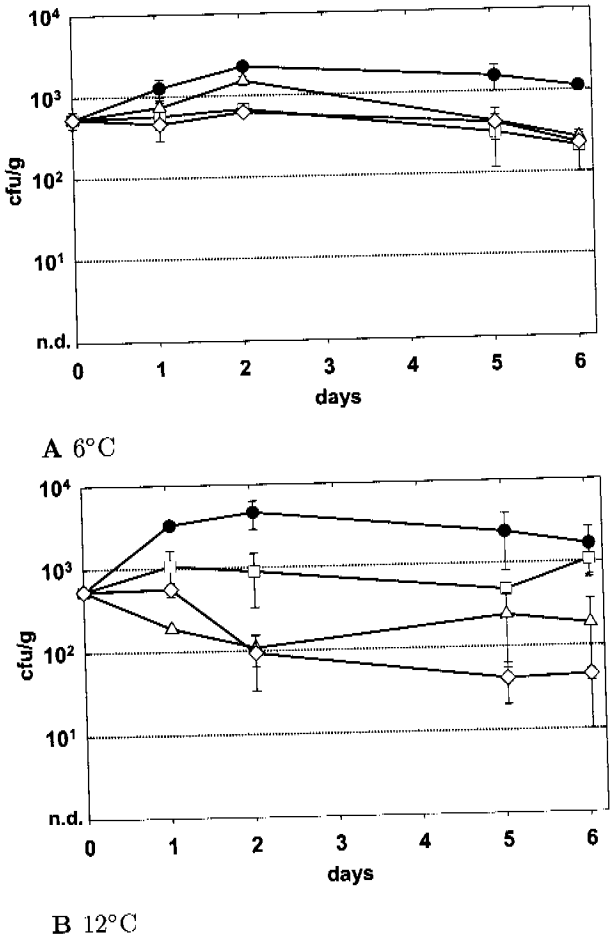


Figure 3.26: Effect of phage endolysins HPL118, HPL511 and the combination of both on growth of *L. monocytogenes* WSLC 1001 on iceberg lettuce during storage at 6°C (A) and 12°C (B) (● control; □ HPL118; △ HPL511; ◇ HPL118+HPL511).

In order to evaluate the efficacy of PLY511 produced by *S. carnosus* (pPSHG7-*ply*511), strain WSLC 1001 was challenged with the concentrated and purified supernatant of the *S. carnosus* strain (Figure 3.27). The supernatant of *S. carnosus* wild type was applied as negative control and endolysin HPL511 from *E. coli* for direct comparison.

Using HPL511, growth reduction of WSLC 1001 was 2.4 log unit compared to the control after 6 days ($p<0.05$). After application of PLY511, *Listeria* cell counts decreased from day 2 on, but increased again on day 6. They were still significantly lower (0.7 log units) than on control samples, but also significantly higher than on samples containing HPL511 ($p<0.05$). On samples containing supernatant of *S. carnosus*

TM300 (wildtpye), no significant growth of WSLC 1001 was observed. Between day 2 and 5, *Listeria* cell numbers differed significantly from the control by 1.5 - 1.7 log units, but not at day 6 ($p>0.05$). Suppression of growth on samples treated with HPL511 or PLY511 was significantly higher than on samples treated with supernatant of *S. carnosus* TM300.

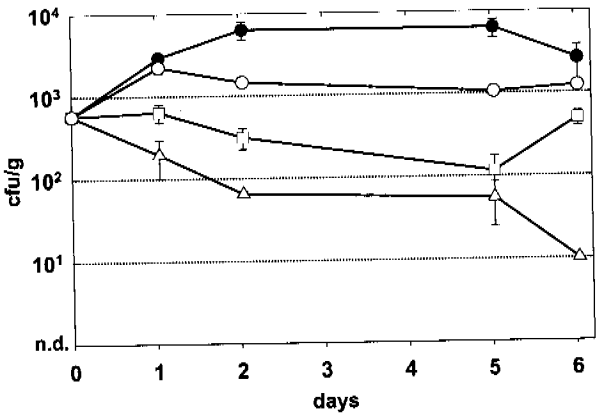


Figure 3.27: Effect of phage endolysins HPL511 and PLY511 on growth of *L. monocytogenes* WSLC 1001 on iceberg lettuce. Supernatant of *S. carnosus* wild type (wt) was used as negative control (● control; △ HPL511; □ PLY511; ○ wt).

When *Listeria* strain Scott A was tested, HPL500, HPL511 and the combination of both were used on iceberg lettuce (Figure 3.28). On control samples, *Listeria* cell numbers increased by more than 4.2 log units after 6 days. Either the single or the combined application of the enzymes led to significant growth inhibition of more than 2 log units compared to the control after 6 days. However, there were no significant differences between the three applications (HPL500, HPL511 or both endolysins).

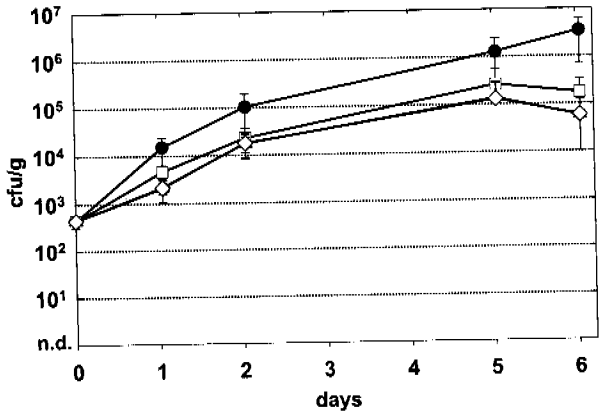


Figure 3.28: Effect of phage endolysins HPL500, HPL511 and the combination of both on growth of *L. monocytogenes* Scott A on iceberg lettuce (● control; □ HPL500; △ HPL511; ◇ HPL500+HPL511).

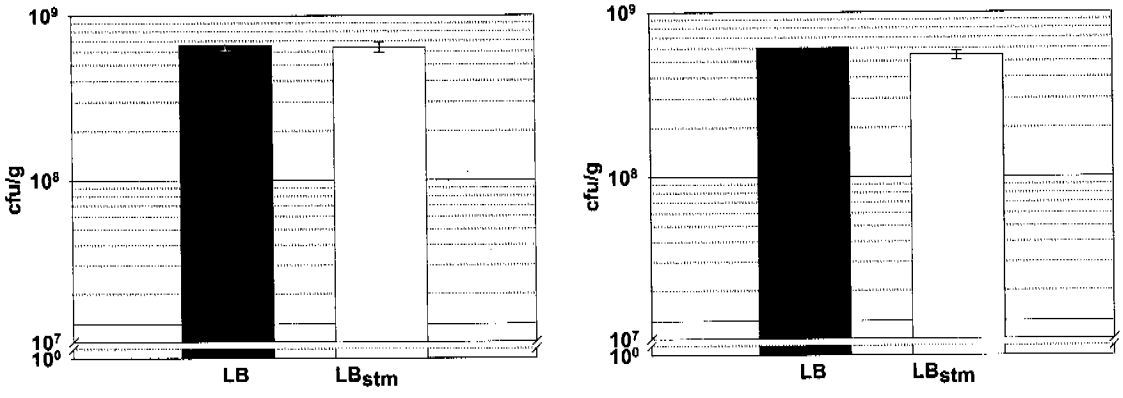
3.2 Effect of bacteriophage on growth of *Salmonella* Typhimurium in foods

3.2.1 Construction of mutants of *Salmonella* Typhimurium for quantification in foods

In order to facilitate enumeration of *Salmonella* cells from inoculated foods, antibiotic resistant mutants of the host bacterium for *Salmonella* phage FO1-E2 were used. They were either selected after spontaneous resistance to streptomycin or were genetically engineered for resistance to chloramphenicol.

3.2.1.1 Resistance against streptomycin

Strain *Salmonella* Typhimurium DB7155 stm^R was isolated after few passages on streptomycin containing media (Chapter 2.5.1.1). For food experiments, it was important to ensure stability of resistance against streptomycin. This was tested in non selective LB medium and on sterilized hot dogs. Figure 3.29 shows the results for both experiments. There were no significant differences between *Salmonella* cell numbers plated on media without (LB) and with streptomycin (LBstm, 500 μg/ml).



A LB medium **B** Hot dogs

Figure 3.29: Cell counts of *S.* Typhimurium DB7155 stm^R after cultivation in non-selective medium for 7 h (A) and on sterile hot dogs for 6 days (B). *Salmonella* cell numbers were determined by plating on selective (LBstm) and non-selective LB agar plates (LB).

Additionally, growth rates of *S.* Typhimurium DB7155 stm^R in full medium (LB) were compared to wild type strain *S.* Typhimurium DB7155 (Figure 3.30). Growth of strain DB7155 stm^R was significantly slower compared to the wild type strain DB7155.

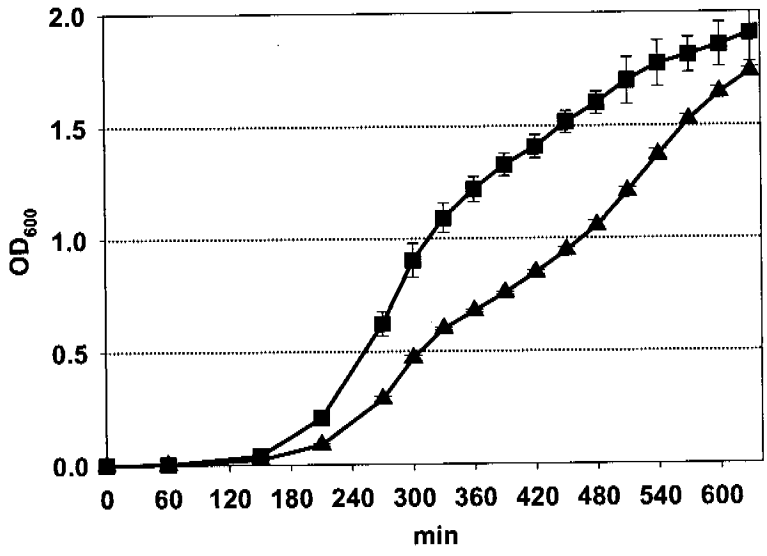


Figure 3.30: Growth of *S.* Typhimurium DB7155 stm^R compared to growth of *S.* Typhimurium DB7155 (wild type) in LB media without streptomycin. Optical density was measured at 600 nm. Data refer to three independent experiments (■ DB7155 wt, ▲ DB7155 stm^R).

3.2.1.2 Resistance against chloramphenicol

Strain *Salmonella* Typhimurium DB7155 was genetically modified using recombinase mediated homologous recombination. A chloramphenicol resistance cassette was integrated into the genome by replacing the pseudogene *stm1666*(Chapter 2.5.1.2).

First, purified plasmid pKD46 (6,329 bp) encoding the red-recombinase system was transformed in *S.* Typhimurium DB7155. Plasmid identity was confirmed by restriction enzyme analysis. The linear DNA construct containing the resistance cassette flanked by homologous regions (1,114 bp) was constructed by PCR-based methods. This construct was transformed into *S.* Typhimurium DB7155 (pKD46) by electroporation. Transformants were confirmed by colony PCR using oligonucleotides spanning the modified region in the genome and chromosomal DNA of the mutants and the wildtype strain. The target region corresponds to 381 bp in case of the wild type strain DB7155. Allelic replacement of the wildtype gene *stm1666* with the chloramphenicol resistance cassette increased this size to 1,178 bp, as shown in Figure 3.31. Genetic modification was further verified by subsequent sequencing of the target site of one mutant.

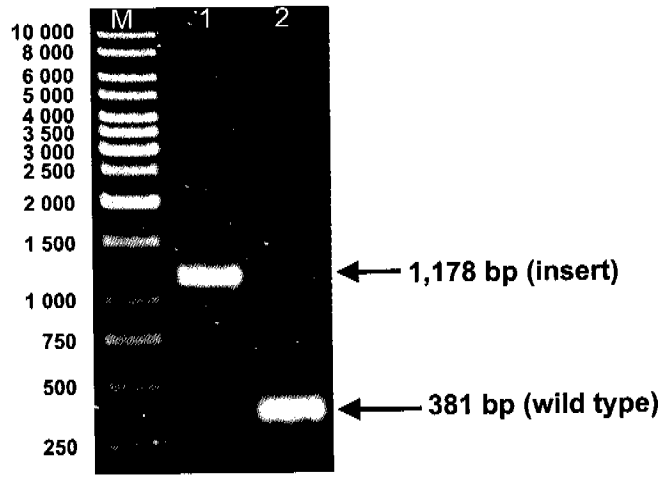
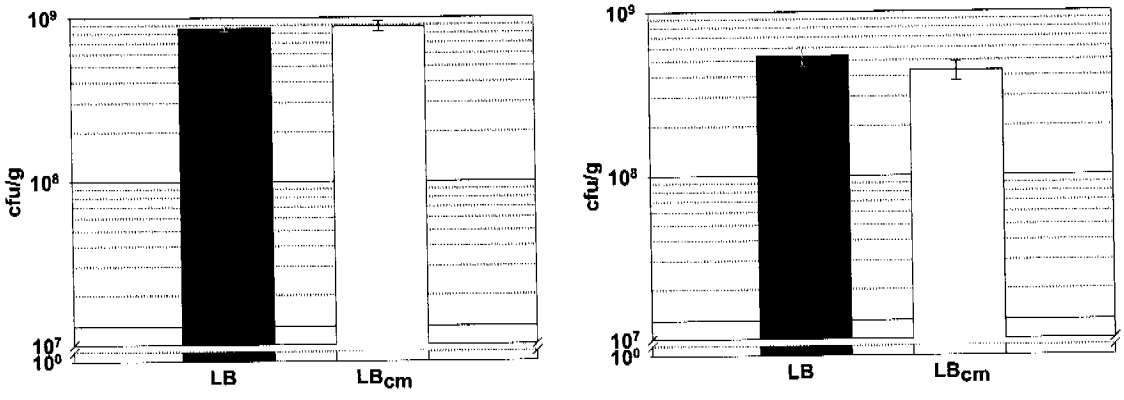


Figure 3.31: Verification of the allelic replacement of *stm1666* by the chloramphenicol resistance cassette in *S.* Typhimurium: lane M: DNA ladder Mix; lane 1: mutant *Salmonella* Typhimurium DB7155 cm^R (insertion of the chloramphenicol resistance cassette leads to 1,178 bp); lane 2: wild type strain *Salmonella* Typhimurium DB7155 (381 bp).

Stability of resistance against chlorampenicol was tested in non selective LB medium and on sterilized hot dogs. Figure 3.32 shows the results for both experiments. There were no significant differences between *Salmonella* cell numbers plated on media without (LB) and with chloramphenicol (LBcm, 20 μg/ml).



A LB medium

B Hot dogs

Figure 3.32: Cell counts of *S.* Typhimurium DB7155 cm^R after cultivation in non-selective medium for 7 h (A) and on sterile hot dogs for 6 days (B). Cell counts were determined by plating on selective (LBcm) and non-selective LB agar plates (LB).

Growth rates of *Salmonella* Typhimurium DB7155 cm^R were also compared to wild type strain *S.* Typhimurium DB7155 in full medium without chloramphenicol (LB). There were no significant differences (Figure 3.33).

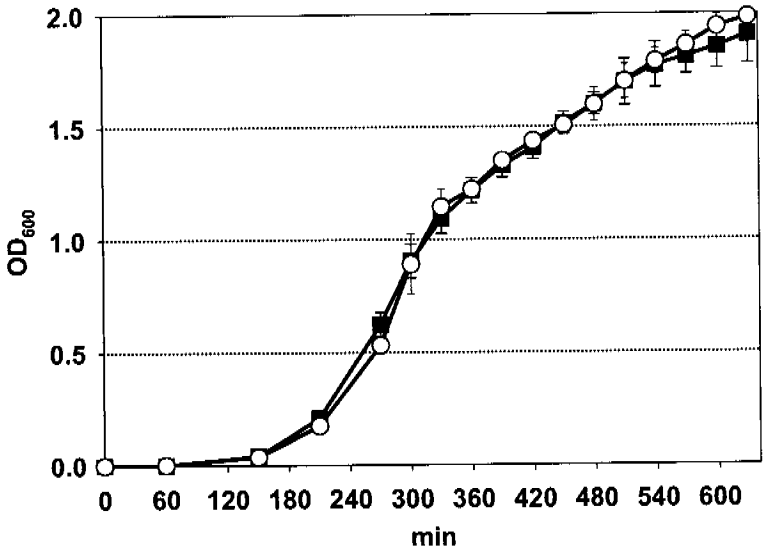


Figure 3.33: Growth of *S.* Typhimurium DB7155 cm^R compared to growth of *S.* Typhimurium DB7155 (wild type) in LB media without chloramphenicol. Optical densitiy was measured at 600 nm. Data refer to three independent experiments (■ DB7155 wt, ○ DB7155 cm^R).

3.2.2 Control of *Salmonella* Typhimurium on foods

Stock suspensions with titers of 1.5×10^{11} pfu/ml were prepared for *Salmonella* phage FO1-E2. Using a modified method (Chapter 2.2), higher phage concentrations could be produced, and 10 instead of 40 agar plates of semi-confluent lysis were needed.

The effect of *Salmonella* phage FO1-E2 was studied on six different foods (hot dogs, sliced turkey breast, mixed seafood, chocolate milk, egg yolk, mung bean sprouts), at two different temperatures (8°C and 15°C). In experiments with solid foods, the detection limit by direct plating was 10 cfu/g, and with liquid foods 1 cfu/ml, with respect to one single measurement. If no *Salmonella* cell was detected by direct plating, the result was valuated as negative. Three samples were analyzed: (i) the negative control, (ii) the positive control with *S.* Typhimurium, and (iii) the phage treated sample containing *S.* Typhimurium and phages. In general, negative control samples did not yield any antibiotic resistant bacterial colonies. Statistical analysis confirmed that most of the results were significant based on an α level of 5 % ($p<0.05$). Exceptions are indicated in the text.

3.2.2.1 Food experiments at 8°C

Experiments at 8°C were performed on hot dogs, mixed seafood and in chocolate milk (Figure 3.34). In general, both strains *S.* Typhimurium DB7155 stm^R and DB7155 cm^R did not grow on control samples at this temperature. On hot dogs, cell counts of DB7155 stm^R even decreased by 0.9 units. For strain DB7155 cm^R this reduction was 1.4 log units on hot dogs, 0.5 log units on mixed seafood, and 0.8 log units in chocolate milk after 6 days. On all foods treated with phage FO1-E2, no colony of both *Salmonella* strains could be detected after 1 day by direct plating. In addition to direct plating, selective enrichment was performed according to ISO 6579:2002 [13] in experiments with DB7155 cm^R. This was done at day 5 or 6. *Salmonella* was detected in all phage treated samples.

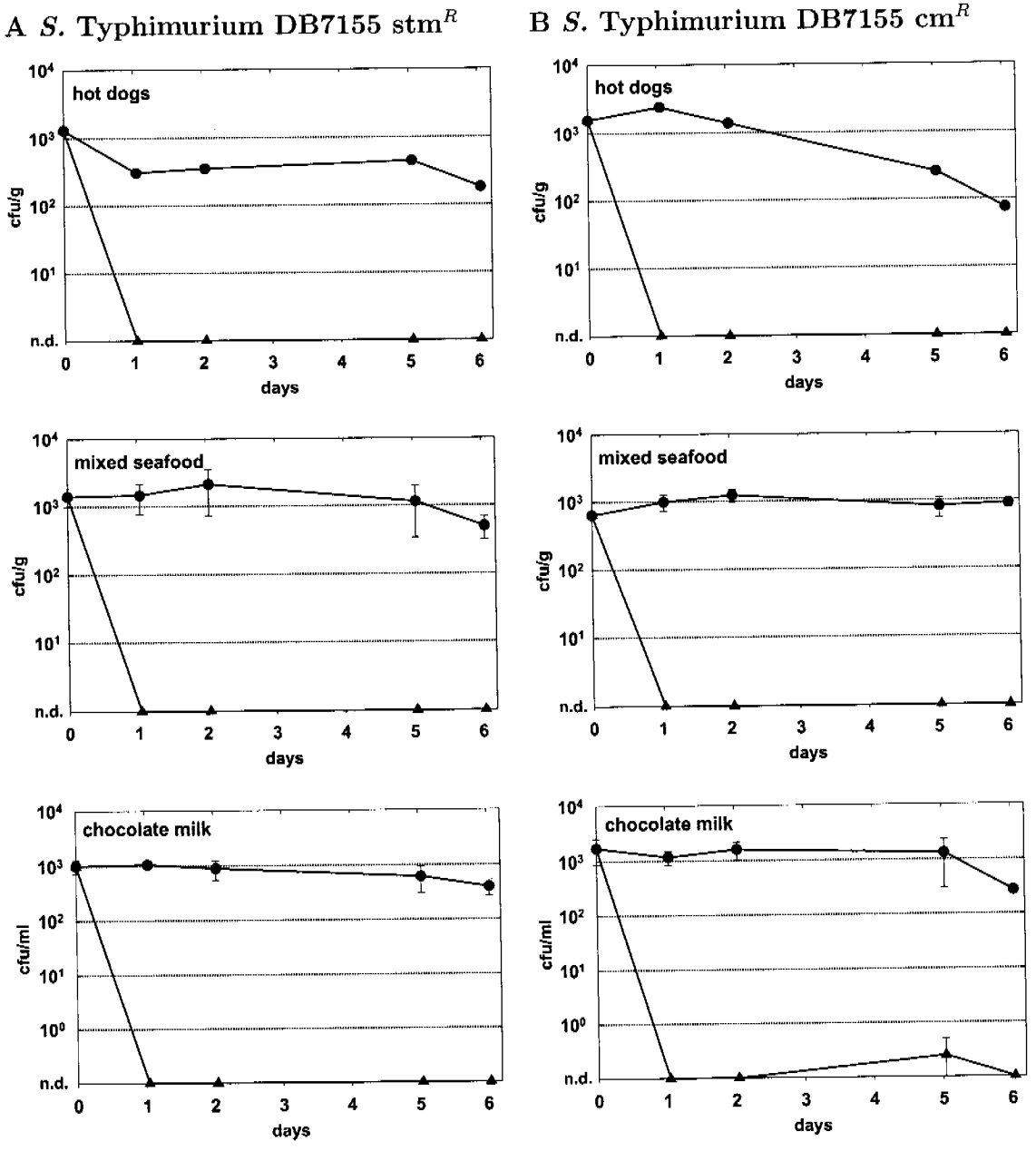


Figure 3.34: Effect of phage FO1-E2 on growth of *S.* Typhimurium DB7155 stm^R (Column A) and DB7155 cm^R (Column B) on different foods at 8°C during 6 days (●control; ▲+FO1-E2). Phage FO1-E2 was applied in concentrations between 1.0×10^8 pfu/g and 6.0×10^8 pfu/g after 1 hour. Data of hot dogs correspond to only 1 experiment.

3.2.2.2 Food experiments at 15°C

In order to evaluate the effect of phage FO1-E2 on *S.* Typhimurium, when bacteria were enabled for stronger growth, further experiments were performed at 15°C (Figure 3.36). On control samples of hot dogs, sliced turkey breast and mung bean sprouts, *Salmonella* DB7155 stm^R increased by 2.3 to 3.5 log units after six days. On mixed seafood, in chocolate milk and egg yolk, this increase was 4.5 and 5.7 log units and therefore higher compared to the other foods.

When phage was added to the foods, growth of *S.* Typhimurium was inhibited, or bacteria were reduced by 1 to more than 2 log units on all foods in the first two days, except for mung bean sprouts. After day 2, however, re-growth of *Salmonella* was observed. Reduction of viable cell numbers in chocolate milk and sliced cooked turkey breast were highest compared to the control after 6 days (> 5 log units). In the case of chocolate milk, however, re-isolated colonies were insensitive to FO1-E2 at day 6 (EOP ¡1 0^{-7}). On hot dogs, growth suppression was 3.0 log units, and on mixed seafood 1.9 log units compared to the control. In egg yolk and on mung bean sprouts, no significant differences to control samples were detected after 6 days. However, on day 2, contamination levels of *Salmonella* in egg yolk significantly differed by 2.3 log units from the control. In experiments with mung bean sprouts the negative control sample was not included and contamination by other bacteria than *Salmonella* resistant to streptomycin could not be excluded.

Concentrations of FO1-E2 were monitored throughout the duration of the experiments. In general, no significant change in levels of *Salmonella* phage FO1-E2 was observed in foods over 6 days at 8°C or 15°C (Figure 3.35).

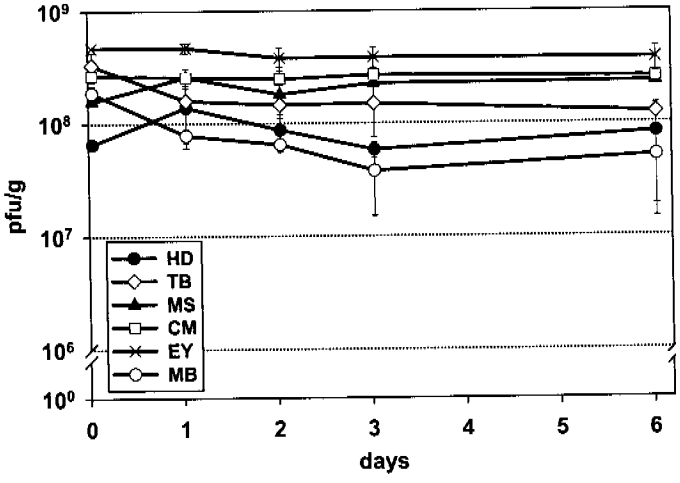


Figure 3.35: Concentration of *Salmonella* phage FO1-E2 on different foods at 15°C over 6 days. HD = hot dogs, TB = sliced turkey breast, MS = mixed seafood, CM = chocolate milk, EY = egg yolk, MB = mung bean sprouts.

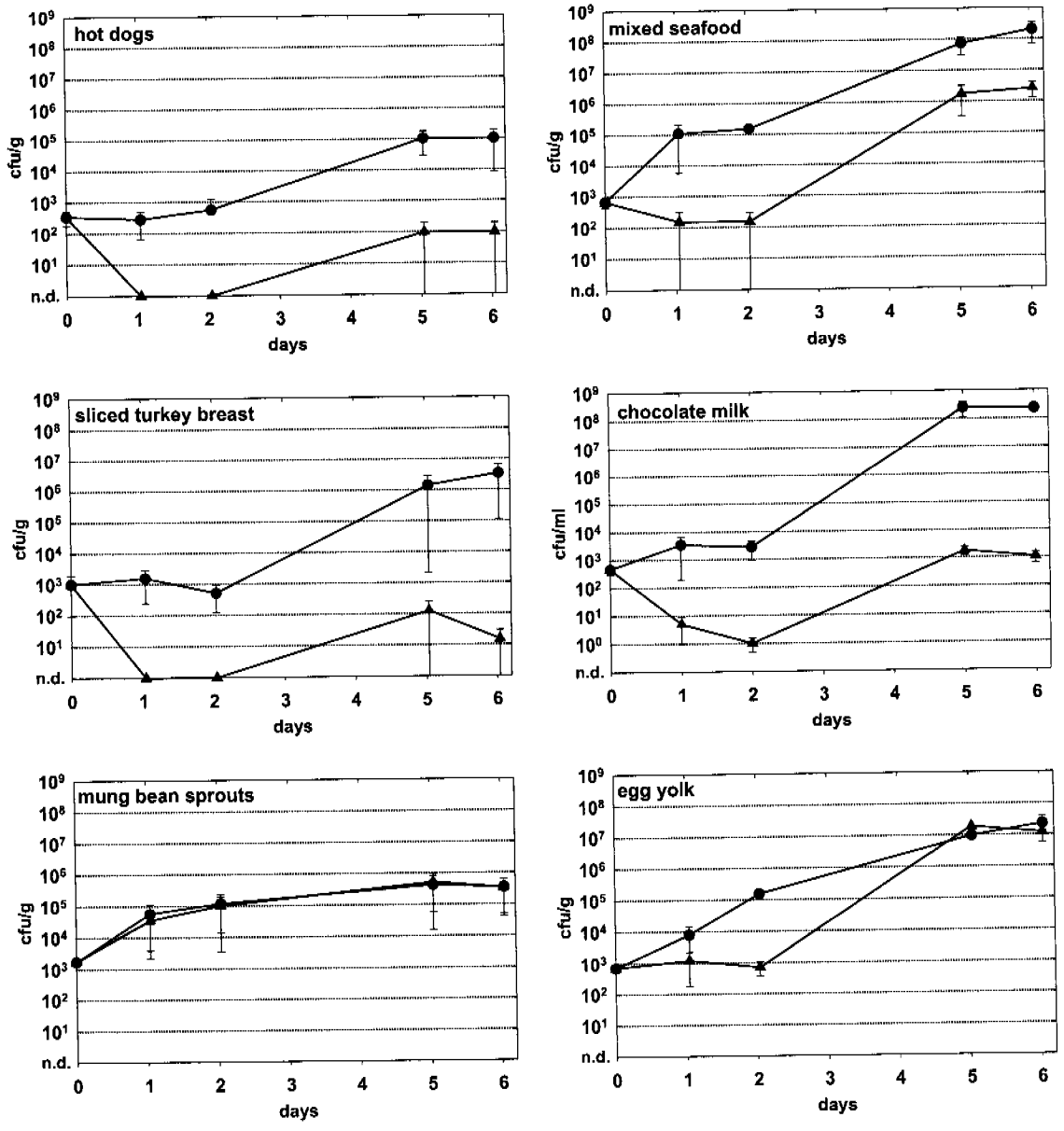


Figure 3.36: Effect of phage FO1-E2 on growth of *S.* Typhimurium DB7155 stm^R on different foods at 15°C during 6 days (● control; ▲ +FO1-E2). Phage FO1-E2 was applied in concentrations between 1.0×10^8 pfu/g and 5.0×10^8 pfu/g after 1 hour. Data of egg yolk at day 5 correspond to only 1 experiment.

4 Discussion

Food safety presents challenging tasks as indicated by high case numbers of infections and the enormous economic burden involved [14] (Chapter 1.1.3, 1.2.3). Although hygiene is on a very high level in most industrial countries, control of pathogenic bacteria in foods can still not be considered satisfactory. In the last decades, several factors have contributed to this issue. These include changes in human behavior and demographics, microbial adaptation, increase in international travel and trade, and especially changes in food production and distribution.

Consumer behaviors have changed. There is an increased consumption of ready-to-eat, pre-packed foods, or meals in food service establishments [14, 95, 232]. Improper preparation or storage of foods provides opportunities for contamination, growth, or survival of pathogens. Besides, consumers rather prefer foods without chemical preservatives or other food additives. Furthermore, the proportion of susceptible persons is increasing due to aging or underlying medical conditions. Elderly or immunocompromised people have weak defense mechanisms against foodborne pathogens and infections often lead to severe diseases with a high mortality rate [14, 220]. Additionally, international travel and transport may contribute to global distribution of bacteria, as travelers may become infected by pathogens uncommon in their home country.

In the last decades, other infectious agents have been newly described or the role of food for their transmission has been recognized. Examples are enterohemorrhagic *E. coli* (EHEC), Bovine Spongiform Encephalopathy (BSE), or *Listeria monocytogenes*. Another concern is the emergence of multi drug resistant bacteria that makes treatment of disease more difficult [220, 232].

Furthermore, production and distribution of foods have changed. Contamination of more widely distributed foods causes rather diffuse and widespread outbreaks instead of local ones, and are more difficult to identify [219]. Besides, consequences of a failure on industrial scale today are tremendous as many consumers will be involved.

Therefore, efforts for improvement of food safety are required, from governments, food industry, and the consumers. For this purpose, research and evaluation of new approaches for improved control of foodborne pathogens are important. A novel approach such as the application of alternative antibacterial substances has to fulfill certain criteria for usage in food. The application must be harmless to humans and must not influence product quality. It has to be sustainable, simple to apply, cost-effective, and contribute to existing food safety strategies like Hazard Analysis Critical Control Point (HACCP).

In recent years, research on bacteriophages has undergone a revival. Due to the increased occurrence of antibiotic resistant bacteria, clinical phage therapy is studied again. In addition, bacteriophages are candidates for biocontrol of food. Phages can not infect eucaryotic cells, generally have a very specific host range, and are the most abundant self-replicating units in the environment. They are of natural origin and have been isolated from a broad variety of foods (Chapter 1.3.5). However, there are also some critical issues regarding the application of bacteriophages. One important example is the emergence of phage resistance (addressed in detail in Chapter 4.4). Furthermore, virulent phages are more suitable for use as natural antimicrobials, since many temperate phages can integrate their genome into the host bacterial genomes. This can be accompanied by lysogenic conversion, that means undesired changes of the phenotype, e.g., contribution to virulence, antibiotic resistance or other undesirable features of the host bacteria [33, 214]. This is never the case for virulent phages, because they lack the genetic information required for integration. They are strictly lytic, and will always kill the infected bacteria. Furthermore, phages should not be able for transduction. Last but not least, consumer perception of adding viruses to foods has to be taken into account.

For biocontrol of foodborne pathogens in foods, concentration of bacteriophages has to be high enough to eliminate even sparse populations of bacteria by infection or lysis from without. Therefore, initial phage concentration seems to be crucial for successful control of the pathogens. Treatments are further influenced by phage adsorption rate, phage persistence, sensitivity of the host bacteria, and especially interactions with the environment.

The aim of this work was therefore a comprehensive evaluation of the use of virulent bacteriophages for control of *L. monocytogenes* and *Salmonella* Typhimurium in foods under various conditions.

4.1 Bacteriophages for control of *Listeria monocytogenes* in foods

The results presented in this study clearly show that *Listeria* bacteriophages are qualified for the control of *Listeria monocytogenes* in different foods. The effect on cell growth is mainly influenced by the kind of food, the type of phage and the amount of phages applied. To a minor extent it is dependent on the *Listeria* strain, the storage temperature and the storage time.

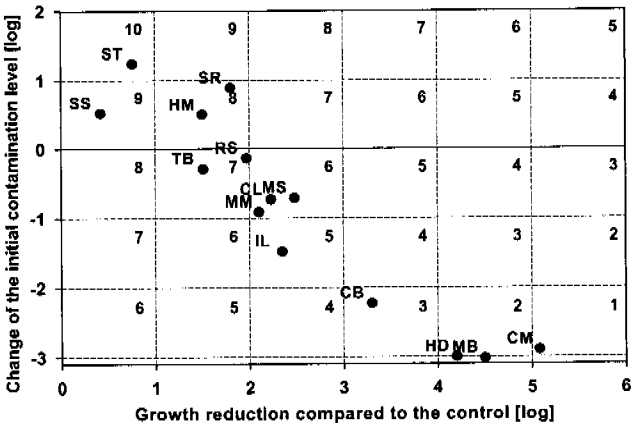
In most foods, the initial killing of *L. monocytogenes* by phage A511 contributed mostly to the overall control effect. Phages were applied to foods in high concentrations (3×10^8 pfu/g) and bacterial cells were destructed immediately by primary infection

or lysis from without. Killing of the remaining bacteria afterward may have been more difficult due to the non-motility of the phages and diffusion barriers in foods. The highest reduction levels compared to non phage treated foods were mostly achieved at the end of the experiments. This is due to the fact, that *L. monocytogenes* could well multiply on non treated foods, but not or only to a minor extend on phage containing foods. Leverentz *et al.* [135, 136] also showed that major reductions of the initial contamination level of *L. monocytogenes* or *Salmonella* occurred mostly immediately after phage application. In comparison to control samples, growth inhibition was also best at the end of the experiment (after 7 days).

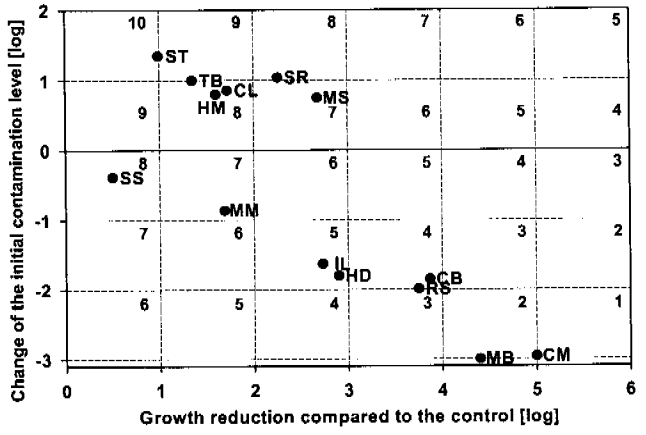
4.1.1 Control of *Listeria monocytogenes* during the storage of different foods

4.1.1.1 Efficacy of phage A511 on growth of *Listeria monocytogenes* is dependent on the type of food

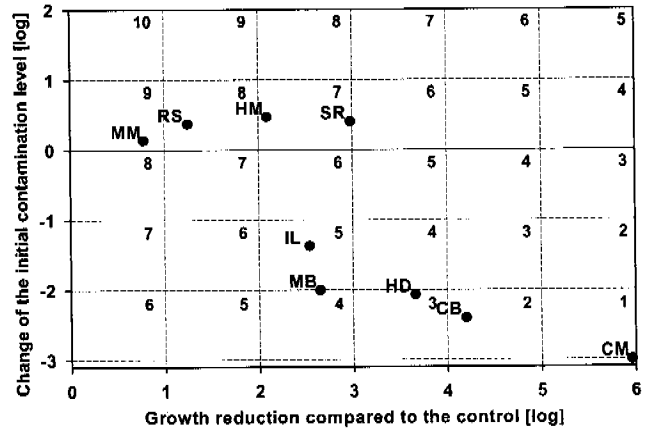
For the evaluation of the efficacy of phage A511 on development of *L. monocytogenes* in foods, two criteria were used. First, the reduction of the initial contamination level, and second, the growth reduction compared to control samples at the end of the experiment. The two criteria were then plotted against each other for each single food and *Listeria* strain (Figure 4.1). The plot was then divided into different areas corresponding to certain "effect values". The best effect was rated with 1, the second best with 2, etc. In general, the higher reduction of the initial contamination level and the higher the difference to the control, the better the effect of phage A511 against *L. monocytogenes* on the appropriate food. For example, in the experiment with hot dogs contaminated with WSLC 1001 (Figure 4.1 A), the initial contamination level was reduced by 3.0 log units after 6 days (y-axis). Reduction of *Listeria* cell numbers compared to the control was then 4.2 log units (x-axis). Here, an "effect value" of 2 was assigned to the control effect of phage A511 against WSLC 1001 on hot dogs. The reduction of the initial contamination level as well as the growth difference compared to the control was therefore high. Effect values were assigned to all foods contaminated with each of the 3 *Listeria* strains.



A WSLC 1001



B Scott A



C CNL 103/2005

Figure 4.1: Efficacy of phage A511 on growth of *L. monocytogenes* WSLC 1001 (A), Scott A (B) and CNL 103/2005 (C) on different foods after 6 days at 6°C. HD = hot dogs, MM = minced meat, TB = sliced turkey breast, HM = ham, SS = smoked salmon, RS = raw salmon, ST = smoked trout, SH = shrimps, CL = calamari, MS = mixed seafood, CM = chocolate milk, MB = mozzarella cheese brine, IL = iceberg lettuce, CB = cabagge. Explanation see text.

Furthermore, the values of the individual foods for each *Listeria* strain were averaged between the three different strains (Table 4.1). The best results were achieved in chocolate milk and mozzarella cheese brine. This is reasonable, because phage distribution in liquids is more homogeneous, and diffusion is better compared to solid foods. High control effects were also achieved on hot dogs, iceberg lettuce and cabbage. Hot dogs have a very smooth and moist surface that also promotes good distribution and diffusion. Growth reduction of *L. monocytogenes* on the two vegetables was quite high, although the surface is large and phages were less stable (Chapter 4.3).

On most meat products and seafoods, reduction of the initial contamination level was 1 log unit at the most, and the difference to control samples between 1 and 3 log units after 6 days. The control effect of phage A511 on these foods can be rated as average.

Efficacy of A511 on the two smoked fishes, salmon and trout, was low. The surface of these products is very uneven and provides niches for bacterial cells restricting interaction with phages. Besides, phages could be immobilized by binding to other constituents, e.g. fish proteins. Other ingredients such as salt or smoke components may also adversely affect phage adsorption to *Listeria* on the food. This possibility is also suggested by the fact that phage was more effective against WSLC 1001 or Scott A on raw salmon compared to smoked salmon (Chapter 3.1.1.2). Raw salmon is furthermore one of the few examples where control *L. monocytogenes* was dependent on the strain: control of strain Scott A (value 3.5) was significantly better than that of WSLC 1001 (value 7) or CNL 103/2005 (value 8).

Table 4.1: Rating of foods according to the applicability of A511 against different strains of *L. monocytogenes*

Rank	**Food**	**Value (mean±SD)**
1	Chocolate milk	1.17±0.24
2	Mozzarella cheese brine	2.67±1.31
3	Cabbage	3.00±0.82
4	Hot dogs	3.17±1.43
5	Iceberg lettuce	5.00±0.00
6	Raw salmon	6.17±1.93
7	Mixed seafood	6.50±0.50
8	Calamari	7.00±1.00
9	Minced meat	7.33±1.25
10	Cooked ham	7.67±0.47
10	Shrimps	7.67±0.47
12	Sliced turkey breast	7.75±0.75
13	Smoked salmon	8.50±0.50
14	Smoked trout	9.75±0.25

4.1.1.2 Influence of the initial contamination level on the efficacy of phage application

To determine whether the initial contamination level of *L. monocytogenes* had an influence on the efficacy of treatment with phages, growth inhibition was evaluated at two initial concentrations (10^3 and 10^2 cfu/g). On 3 out of 4 foods, control of *L. monocytogenes* WSLC 1001 by phage A511 was comparable (iceberg lettuce) or better (mozzarella cheese brine, smoked salmon) at lower initial contamination levels than at higher counts. This is in accordance with Leverentz *et al.* [136] who observed a higher decrease of the population of *L. monocytogenes*, when the bacterial inoculum (25 μl) was decreased from 10^6 cfu/ml to 10^5 cfu/ml on honey dew melon slices. In another study, Greer [88] measured a higher increase in shelf life of beef after application of *Pseudomonas* phages to beef contaminated with 5×10^3 cfu/cm^2 instead of 5×10^5 cfu/cm^2. However, this was not the fact with 5 cfu/cm^2 instead of 5×10^3 cfu/cm^2. Ellis *et al.* [62], in contrast, reported a significant effect of homologous phage on growth of *Pseudomonas fragi* in milk only at a high contamination level (10^5 cfu/ml), but not at lower ones (10^3 cfu/ml). In this case, however, low phage concentrations of maximal 10^3 pfu/ml were applied at the beginning. Phage titer increased to more than 10^8 pfu/ml in samples contaminated with 10^5 cfu/ml, but only to 10^4 pfu/ml in samples contaminated with 10^3 cfu/ml. Therefore, the effect was dependent on phage propagation during the experiment. In this study, however, high phage concentrations were applied which were stable independent of the bacterial contamination level.

4.1.1.3 Control of *Listeria monocytogenes* by phage application can be maintained over a longer storage time

Control of three different *Listeria* strains by phage application was maintained over a longer storage period of 13 days. However, in the case of strains WSLC 1001 and CNL 103/2005, insensitivity against phage A511 was observed in 5 out of 16 experiments. Therefore, efficacy was diminished compared to experiments, where no insensitivity was seen. This is discussed in more detail in Chapter 4.4.

Experiments lasting for 13 days showed that growth reduction (i) was either maintained (ham) or (ii) was even higher after 13 days (hot dogs, chocolate milk and mozzarella cheese brine). One exception was *Listeria* strain WSLC 1001 in mozzarella cheese brine. Here, growth inhibition was lower after 13 days than after 6 days. However, in this case, viable cell numbers were also lower in control samples and therefore differences became smaller. On hot dogs (Scott A) and in chocolate milk (CNL 103/2005), a high variability between the single experiments was observed. These might be due to differences in the food samples, phage concentrations or the emergence of phage insensitivity. However, phage A511 was stable in all experiments.

Besides, no phage resistant isolates were detected with the methods used in this study. But there was a certain possibility for false-negative results (Chapter 4.4). In general, storage time seems not to be a limiting factor for phage application in foods.

Only little information is available about the effect of bacteriophages against pathogens in foods over a longer storage time. Most studies lasted between 24 h and 72 h [62, 179, 243] or for 7 days [88, 135, 136, 138]. In one report, *Listeria* phages were applied on vacuum-packaged beef contaminated with *L. monocytogenes* and stored for 4 weeks [60]. However, according to the authors, there was no significant cell count reduction at all, when phages were applied, alone or in combination with the bacteriocin nisin.

4.1.1.4 Increase of storage temperature has a minor influence on the effect of phage A511

When incubation temperature was increased from 6°C to 20°C (i) the initial contamination level was less reduced or even increased, but (ii) the difference to the control was higher after 6 days. The initial reduction effect in the first two days was comparable between the two temperatures. Afterward, however, *Listeria* cells multiplied more strongly at 20°C than at 6°C. At higher temperatures growth of *L. monocytogenes* was enhanced, and bacterial multiplication rate seemed to be higher than phage infection rate. However, growth on phage samples was still slower compared to controls and caused higher reduction levels at 20°C after 6 days. Similar observations were done in experiments with *Salmonella* (Chapter 4.2.2). According to these results, it may be concluded that control of *L. monocytogenes* by phage A511 also works at higher temperatures. However, these temperatures are not applicable anyways for the storage of foods.

4.1.1.5 The higher the initial phage concentration the better the control of *Listeria monocytogenes*

The influence of the initial phage concentration was evaluated by testing three different phage concentrations (3×10^6 pfu/g, 3×10^7 pfu/g or 3×10^8 pfu/g) on three different foods with *Listeria* strain WSLC 1001. Each led to a significant reduction of *Listeria* cell numbers compared to control samples after 6 days. The initial contamination level was decreased in most cases, except in experiments with chocolate milk and cabbage using 3×10^6 pfu/g. Best results were obtained with the highest phage concentration. However, this effect was dependent on the type of food. For example, the difference between application of 3×10^6 pfu/g to 3×10^7 pfu/g was significantly lower on hot dogs than in chocolate milk (Chapter 3.1.1.8).

Enhancement of phage control by increasing the amount of phages was also observed

by others [38, 82, 88, 113, 138, 237]. Best effects were usually achieved with highest phage concentrations. It was supposed that the optimal phage/bacteria ratio depends on the organisms used [138]. Greer [88] observed a maximum increase in shelf life of beef when approximately 10^8 pfu/cm^2 were added to samples contaminated with *Pseudomonas* spp. Huff *et al.* [113] reported effective phage treatment against infection with *E. coli* in broiler chicken only when the highest titer of 10^8 pfu was used.

In conclusion, phage concentrations of at least 10^8 pfu/g are required for sufficient control of pathogens in foods. However, the optimal concentration must be determined for every specific application.

4.1.1.6 Other *Listeria* phages are also suitable for control of *Listeria monocytogenes*

Efficacy of phage P100 against *L. monocytogenes* on foods was comparable to phage A511. On hot dogs, effect of phage A511 was better, but on mixed seafood and cabbage, P100 caused higher reduction levels. However, these differences of growth inhibition were negligible. The similar efficacy could be expected, as the two phages are closely related. They belong to the same family (*Myoviridae*), are strictly virulent and have a broad, only slightly different host range within *Listeria* [251]. There are extensive nucleotide sequence homologies between both phages [38, 58, 148].

Control effects of phage P35 were significantly smaller in two out of four food experiments than effects of phage A511. This can be either attributed to variabilities of food compositions. However, it also may indicate that efficacy of phage P35 is lower compared to A511 on foods. For definite conclusions, more experiments comparing the application of phage P35 with A511 should be performed.

Overall, these results demonstrate that other virulent *Listeria* phages than A511 may also be suitable for application at least in some foods.

4.1.1.7 Optimized application of phage A511 for control of *Listeria monocytogenes* on smoked salmon

When phage concentrations of 3×10^8 pfu/g were used on sliced smoked salmon, growth of *L. monocytogenes* could not sufficiently be reduced. Therefore, it was important to improve the effect on growth of *L. monocytogenes* by phage A511, also because ready-to-eat smoked salmon is often contaminated with *Listeria monocytogenes* [61, 100, 116].

Application of bacteriophages before the bacterial contamination of salmon did not increase the efficacy of phage A511. In contrast, Leverentz *et al.* [138] observed an increase of reduction levels of *L. monocytogenes*, when a *Listeria* phage cocktail was applied 1 h before contamination instead of 1 h thereafter. Results were further improved by an application immediately before contamination. The authors stated

that the later phage addition occurred the smaller the effect. However, exact timing of phage application seems to be more complex and seems to be influenced by different factors such as the *Listeria* strain, the phage, and especially the type of food.

Control of *L. monocytogenes* on smoked salmon could be improved, when smoked salmon pieces were dipped into phage suspension. This indicates that the type of application influences efficacy of phage treatment. Clearly, distribution of the phages must be optimized for each food.

Repeated application of phage to sliced smoked salmon also improved growth reduction. However, the single application of a higher initial phage concentration (2×10^9 pfu/g) increased phage efficacy even more.

Slicing of smoked salmon had also some effect on reduction of viable cell numbers of strain Scott A. When solid blocks of smoked salmon were used, growth suppression was significantly higher than on sliced pieces. Slicing increases the surface and also affects the fish texture. This may provide more niches for infiltration by *Listeria* cells, and therefore may restrict phage-host interactions. Interestingly, there was no influence of slicing observed for strain WSLC 1001. This may be due to strain specific properties. Overall, for sufficient control of *L. monocytogenes* on smoked salmon, phage concentrations of 10^9 pfu/g appear to be required. Besides, slicing should be performed after the phage treatment.

There are several studies regarding the control of *L. monocytogenes* on smoked salmon, by using chemical preservatives (sodium diacetate, sodium or potassium lactate, sodium nitrite) [184, 185, 234, 249], or lactic acid bacteria and/or bacteriocins [120, 172, 173]. Results indicate that chemical preservatives or bacteriocins can inhibit growth of *L. monocytogenes* on smoked salmon at least in the first days of storage at temperatures between 4°C and 10°C. However, these approaches do not significantly decrease the initial contamination level on smoked salmon [120, 172, 173, 234, 249]. Reductions were only achieved in smoked salmon juice [172, 173, 234]. However, this model does not mimic realistic conditions, because reduction in *Listeria* cell numbers was lower on whole smoked salmon than in homogenized salmon or in salmon juice [172, 173, 234].

In conclusion, efficacy of a high dose phage application on growth of *L. monocytogenes* on smoked salmon was higher compared to effects reported for any other antilisterial substances. Bacteriophages should therefore be considered as a potential tool for the control of *Listeria monocytogenes* in the industrial production of smoked salmon.

4.1.2 Control of *Listeria monocytogenes* during the ripening of soft cheeses

Cheese is considered to be one of the most frequent foods contaminated by *Listeria monocytogenes*. About 30% of the larger food related outbreaks of *L. monocytogenes* were traced back to contaminated cheese (see Table1.1, page 6). Several investigations regarding the occurrence of *L. monocytogenes* on cheese have been conducted, and the incidence of *L. monocytogenes* on cheese was found to be in a range of 1% to 22%, with an average of 7.4% [11, 28, 63, 65, 152, 178, 186, 195, 221]. Soft cheeses seem to be more frequently contaminated with *L. monocytogenes* than semi-hard or hard cheeses [195, 196], although there are contrary reports [152]. However, until now, only soft cheeses have been implicated in major foodborne outbreaks. Soft cheeses provide appropriate growth conditions for *Listeria* [25, 199]. The bacteria tolerate the high salt content on the cheeses and the low temperatures during ripening. This tolerance provides an advantage compared to other bacteria [161]. The subsequent pH increase during ripening additionally enhances growth of *Listeria*.

Due to the high relevance of soft cheese for foodborne listeriosis, the efficacy of phage application during ripening of white mold cheeses and red smear cheeses was studied. To ensure that ripening processes in the laboratory were appropriate for these cheeses, the pH was monitored throughout the whole experiments. Development of pH was typical for the ripening of white mold cheeses or red smear cheeses in all trials [64, 144, 198, 238]. Control and phage-treated cheeses were indistinguishable from each other in odor, texture, and appearance and comparable to commercially manufactured cheeses. Additionally, no influence on the total aerobic plate counts were determined. Therefore, laboratory conditions are suitable to simulate cheese ripening.

Development of *Listeria* strains Scott A or CNL 103/2005 on control cheeses were closely reflecting the changes in pH, a critical factor to enable growth of *L. monocytogenes* [25, 64, 144, 198]. Growth of strain Scott A was lower compared to CNL 103/2005. The latter had been isolated from a white mold soft cheese in 2005 [31]. It may be better adapted to the growth conditions on the cheese surface compared to strain Scott A which is a clinical isolate [72]. Similar cell concentrations and differences between strains of *L. monocytogenes* were also observed in other cheese-related studies [25, 38, 64, 144, 198].

On white mold soft cheeses, phage treatment resulted in elimination of Scott A in the first days, but re-growth occurred when pH was above pH 6. However, growth was slower compared to control samples. Repeated application or application of a higher dose of phage A511 did further improve reduction of the *Listeria* population in the first 8 to 10 days compared to the single low dose. Additionally, the single high dose resulted in best growth suppression after 20 days, compared to the single or repeated application

of the low dose. However, differences between the three application protocols were small and not significant after 20 days. Thus impression that a single high dose application exceeds the effect of repeated lower doses can not be statistically confirmed.

On red smear cheeses, significant decrease of *Listeria* cell numbers was observed over the first 6 to 9 days. When the pH rose above pH 6, re-growth occurred. Double application of A511 significantly improved control of *L. monocytogenes* between days 8 to 15, and a triple application extended this to days 6 to 18. The killing effect was more than 3 log units after 22 days, and thus higher than on white mold cheeses. This may also be due to the fact that phage A511 was more stable on red smear cheeses. Therefore, phage concentrations may not have been the limiting factor, compared to white mold cheeses. The type of cheese is therefore important for the stability of the phage. When strain CNL 103/2005 was tested, similar reduction effects were achieved on red smear cheeses, although growth of CNL 103/2005 was higher compared to Scott A, and some isolates were insensitive to phage A511 (Chapter 4.4).

There are several studies about bio-control of *L. monocytogenes* in cheese and partial or complete elimination of *Listeria* from the surface of Camembert [158, 215, 238] or red smear soft cheese [38, 64, 144, 177] has been reported. In most studies "protective cultures" or bacteriocins were used. One report exists about phage application for control of *L. monocytogenes* on the surface of red smear cheeses [38]. There, reduction effects were better, when a single high dose of the *Listeria* phage P100 was applied onto the cheese surface, instead of repeated low doses. The single high dose did eliminate *Listeria* from the cheeses [38]. In this referenced work, however, the initial *Listeria* contamination levels were approximately two log units lower (2×10^1 cfu/cm^2) than in this study (1×10^3 cfu/cm^2). This may affect phage efficacy, because lower cell counts may be easier eliminated. Furthermore, phages were added to the contaminated washing/brining solution that were added to the cheeses. Therefore, interactions between phage and host cells were possible in the smearing solution before being added to the cheeses. In the study presented here, phages were added to the contaminated cheese surfaces in order to simulate a setting where *Listeria* contamination of the cheeses has already occured. Furthermore, the initial contamination level was 2 log units lower compared to the study presented here. Lower initial *Listeria* contamination values might result in better control effects as has already been observed here for other foods (Chapter 4.1.1.2). This should be further tested also because natural contamination levels found on cheese are considered to be low [152, 195].

In general, it appears difficult to compare results from different experimental setups also using various types of cheeses. The contamination level, the *Listeria* strain, the type of phage or bacteriocin, the time point of application, the type of cheese, and the ripening conditions all are important parameters, which have an impact on the results.

Moreover, cheese is a very complex system with differences not only in biochemical and physical but also in microbiological properties [197]. It can be concluded that the efficacy of phage A511 seems to be comparable to or better than the efficacy of other antilisterial substances or cultures on soft cheeses, and phage application has to be adapted to every specific cheese production.

4.1.3 *Listeria* phage endolysins can contribute to the control of *Listeria monocytogenes* in foods

Phage enzymes have the potential for a broad application. Due to high substrate specificity, targeted killing of Gram-positive bacteria is possible without effecting the surrounding microbial flora. There are some literature reports about medical applications and plant protection [32, 70, 71, 147]. Furthermore, endolysins can be directly applied to foods or can be produced by fermenting bacteria as reported for *Lactococcus lactis* [74] or *Lactobacillus* spp. [228]. In the case of *Lactococcus lactis* it was shown that active listerial endolysin PLY511 could be secreted [74]. Besides, expression systems of enzymes that destroy the cell wall of the own producer strain have also been developed. An example are *Lactococcus lactis* starter strains which may accelerate cheese ripening by controlled lysis of the ripening culture [53].

Until now, however, these approaches have been limited to in vitro experiments and no data are available about the effect of phage endolysins against bacterial pathogens in foods. In this study, different bacteriophage endolysins were tested for control of *L. monocytogenes* on iceberg lettuce. In most cases a significant reduction in viable cell numbers was achieved. Application at 12°C was more successful than at 6°C. Temperature and pH are among the most important parameters that influence enzymatic activity. Cell lysis is best at 30°C to 37°C and is slowed down with decreasing temperatures. Besides, the pH optimum was found to be around pH 8.0 [228] for HPL511 and even higher of around pH 9.0 for HPL118 and HPL500 [235]. On foods, pH values of pH 6 to pH 7 or even lower are common. Enzymatic activities in this pH range are reduced by 30 to 80% [235]. These observations suggest that conditions on many foods seem not to be optimal for endolysin application. Furthermore, endolysins are "one-use" enzymes and multiple molecules are required for digesting the cell wall. In contrast, one single phage is able to destroy the cell after successful binding. These factors may explain that efficacy of phage endolysins was lower than that of phage application on iceberg lettuce. Nevertheless, a significant cell count reduction could be observed.

Comparing the effect of different endolysins, HPL118 seems to have weaker effects on growth of *L. monocytogenes* compared to HPL511. However, in these experiments, enzyme concentrations were kept constant [μg/g], instead of the enzymatic activities

[U/g]. In fact, activity of HPL118 was measured in vitro to be about 50% of the activity of HPL511 (Chapter 3.1.4). Therefore, to improve comparability between the two enzymes on foods, these experiments should be repeated and identical enzymatic activities should be used. This was done in the experiments with strain Scott A using HPL511 and HPL500. No differences in the effect of HPL511 or HPL500 could be detected. Combined application of different enzymes did not improve killing effects. In vitro, synergistic effects were also not observed when different *Listeria* phage endolysins were combined (Mathias Schmelcher, personal communication). The used *Listeria* phage endolysins were either peptidases (HPL118, HPL500) or amidases (HPL511) and attack neighboring cleavage sites in the peptidoglycan (Chapter 1.3.3). Synergistic effects can probably be expected with combination of enzymes having completely different activity or cleavage sites. Loeffler and Fischetti [143] have observed a synergistic lethal effect of a combination of two different phage endolysins on *Streptococcus pneumoniae* strains in vitro. They used pneumococcal bacteriophage endolysins that have similar binding but different catalytic sites: Pal, an amidase, and Cpl-1, a phage muramidase. Therefore, combination of *Listeria* endolysins with other enzymes such as lysozymes or even phages may lead to synergistic effects.

In addition to recombinant endolysins produced by *E. coli*, PLY511 secreted by *S. carnosus* (pPSHG7-*ply*511) was tested in food experiments. *S. carnosus* is used for fermentation of sausage, fish and soy, and has a long history of safe use in foods [189]. In the experimental setup, the supernatant was concentrated either by ammonium sulfate precipitation or ultrafiltration. For large scale production, tangential flow filtration might be applicable. On iceberg lettuce, application of PLY511 led to similar growth suppression of *L. monocytogenes* compared to other recombinant endolysins. When the concentrated supernatant of the control strain was used, antilisterial activity was also observed, but the effect was lower compared to the endolysins. *Staphylococcus* spp. is known to produce antilisterial substances [171]. Moreover, acid production of *S. carnosus* may inhibit *L. monocytogenes*.

In general, reproducibility of experiments on iceberg lettuce was poor, resulting in extensive standard deviations. This was mainly due to differences in growth of *L. monocytogenes* on iceberg lettuce.

In conclusion, phage endolysins have an impact on growth of *Listeria monocytogenes* on foods. However, application seems to be restricted to certain foods, because enzyme activity is strongly dependent on temperature and pH. Besides, production and purification of endolysins is rather complex and cost-intensive. Further research is necessary, including other foods such as hot dogs and especially soft cheeses. First results indicate higher effects on hot dogs than on iceberg lettuce (data not shown).

4.2 Bacteriophages for control of *Salmonella* Typhimurium in foods

4.2.1 Optimized quantification of *Salmonella* cell concentrations

In this study, the emergence of spontaneous mutation was used for selection of a streptomycin resistant strain of *S.* Typhimurium DB7155. Resistance to streptomycin is based on preventing the antibiotic from binding to its ribosomal target, and from inhibiting protein biosynthesis. This can be mediated by genes that encode streptomycin-modifying enzymes [97]. More often, however, the resistance is due to spontaneous mutation of genes coding for ribosomal subunit proteins, such as *rpsL* for subunit S12 [211]. Resistance was found to be sufficiently stable in vitro and also in foods. Growth rate was lower compared to the wild type strain when tested in vitro. However, on foods, sufficient growth was observed at 15°C and sufficient survival at 8°C. Strain DB7155 stm^R was even more stable on foods at 8°C than strain DB7155 cm^R that showed no different growth behavior compared to the wildtype strain, in vitro. Phage sensitivity was indistinguishable from that of the wild type strain. In most experiments, determination of *Salmonella* cell numbers was reliable, because the concomitant microbial flora on the foods could be sufficiently suppressed by streptomycin. On mung bean sprouts and especially minced meat, however, exact measurement of *Salmonella* cell counts was difficult. A high background of microorganisms other than *Salmonella* made exact quantification very difficult.

A second approach was the use of site-directed mutagenesis based on homologous recombination resulting in antibiotic resistance [51]. The gene encoding chloramphenicol acetyl transferase was inserted into the genome of *S.* Typhimurium by replacing the pseudogene *stm1666*. *S.* Typhimurium DB7155 cm^R was suitable for food experiments. Stability of resistance to chloramphenicol was confirmed in vitro and on foods. Growth of DB7155 cm^R was not different from the wildtype strain in vitro, and phage sensitivity was indistinguishable from that of the wild type strain. Again, however, in the case of mung bean sprouts, a high concomitant flora was observed that could not be fully repressed by chlorampenicol. A combination of selective media such as XLD and chloramphenicol could improve selectivity and overcome this problem.

Similarly, determination of target bacteria on sprouts was also difficult with *L. monocytogenes*. When sprouts were plated on Oxford agar, growth of other bacteria was not fully suppressed, and evaluation was not possible. Therefore, selectivity of detection media has to be improved or, otherwise, sprouts should be grown under aseptic laboratory conditions.

4.2.2 Bacteriophage FO1-E2 is suitable for control of *Salmonella* Typhimurium

Food experiments with *S.* Typhimurium and bacteriophage FO1-E2 were performed at two storage temperatures: 8°C and 15°C. The temperature of 8°C was chosen to mimic conditions often found in household refrigerators [131]. Experiments at 15°C allowed testing the limits of phage application in a situation where growth of *Salmonella* was possible. Storage temperature had a strong impact on growth of *Salmonella* DB7155 stm^R on different foods. At 8°C, both *Salmonella* strains could not multiply, but survive. On hot dogs, cell count even decreased by approximately 1 log unit. At 15°C, however, *Salmonella* started to multiply immediately following the contamination.

Phage application at 8°C did significantly reduce *Salmonella* contamination, below the direct detection limit. However, the bacteria could not be completely eliminated from the foods, as *Salmonella* was detected by enrichment of the food samples. These results are comparable to the findings of Leverentz *et al.* [135] who observed growth inhibition of *Salmonella* on honeydew melon slices at low temperatures and additional reduction by phage application.

At 15°C, reduction of *Salmonella* cell numbers was similar or even higher after six days compared to experiments at 8°C. However, re-growth of *Salmonella* could not be prevented. Leverentz *et al.* [135] reported similar observations. Reduction levels of *Salmonella* on phage treated honeydew melons compared to non-treated slices were similar at 5°C, 10°C or 15°C during 48 h or even 7 days. Therefore, they claimed that efficacy of phage application for control of *Salmonella* on honeydew melon slices is temperature independent. However, absolute cell concentrations on phage samples were significantly higher at higher incubation temperatures. This is also in accordance to the results for *L. monocytogenes* (Chapter 4.1.1.4).

In egg yolk and mung bean sprouts, even no significant control effect was observed after six days. Only after 2 days, inhibition effect by phage FO1-E2 was significant in egg yolk. In case of experiments with sprouts, results have to be carefully interpreted due a high background of microorganisms other than *Salmonella* that made exact quantification very difficult as mentioned before (Chapter 4.2.1).

Similar control effects of *Salmonella* in foods using bacteriophages were reported by other authors. For example, a decrease of about 2 log units compared to control samples was achieved 24 h after phage treatment of chicken frankfurters at 22°C [243]. After application of *Salmonella* phages on mustard seeds cell numbers differed by 1.4 log units from untreated seed after 24 h [179]. However, these studies lasted only for 24 h.

In conclusion, as also observed for the application of *Listeria* phages, the initial

killing effect was crucial for overall growth control. When bacterial growth was enhanced, control of *S.* Typhimurium DB7155 stm^R by phage FO1-E2 was different on various kind of foods, similar to *L. monocytogenes.* Important for the efficacy of phages is a homogeneous distribution and high diffusion of the phages such as, e.g., in liquid foods. Furthermore, adsorption and infection can be influenced by food ingredients [121]. Until now this has not been extensively studied. Furthermore, the emergence of phage resistant mutants have an influence on growth control of bacteria in foods (Chapter 4.4).

In experiments with *Salmonella*, qualitative detection by selective enrichment was included in addition to quantitative determination by direct plating at certain time intervals. This indicates that selective enrichment of *Salmonella* is possible, even when a high concentration of bacteriophages is present in the enrichment culture. There are some reports about the influence of bacteriophages on the outcome of bacterial enrichment broths [46, 81, 167]. However, this influence was observed when the initial contamination level of the food was high ($> 10^4$ cfu/g), but not when the contamination level was low (50 - 100 cfu/g), similar to this study.

4.3 Stability of bacteriophages in foods

Concentrations of phages A511, P100, P35 and FO1-E2 were stable in foods of animal origin during storage over 6 up to 13 days. On vegetables, however, phage counts decreased significantly by 1 to 2 log units. This decrease may have been due to the presence of secondary plant-derived compounds that are known to be able to inactivate viruses [5, 54, 212]. During ripening of red smear cheeses, concentration of phage A511 was more stable than on white mold cheeses. A possible explanation for this difference could be a different proteolytic activity of starter cultures which may inactivate phages. Proteolytic inactivation of enteroviruses has been reported [45], and it is also assumed to be responsible for the deactivation of bacteriocins on the surface of cheeses [238].

Carlton *et al.* [38] reported no significant change of *Listeria* phage P100 titer on the surface of soft cheese after six days. This was also observed for *Salmonella* or *Campylobacter jejuni* phages on chicken skin for at least 48 h [82]. Leverentz *et al.* [135, 136] observed a rapid decrease of *Listeria* phage concentrations on apple slices as well as of *Salmonella* phage counts on honeydew melon and apple slices. The authors thought this was probably due to a lower acid tolerance of the phages. In light of the data available today, also plant compounds seem to influence phage stability on foods. Increase of phage titer was also observed on foods [62, 87, 166]. In these cases, bacterial concentrations were high and phage counts low. Therefore, proliferation after infection was detectable. In this study, however, high phage concentrations were used

to eliminate low levels of pathogens from foods. Therefore, not the proliferation of phages, but the stability of high phage concentrations is one of the key factors for successful control of bacterial growth.

This is the first study where the stability of high phage titer has been evaluated on different kind of foods during storage and ripening. All four bacteriophages tested showed high stability on most foods except for vegetables and one type of cheese. Therefore, phages may be suitable for longer-term application in foods. However, when phage concentrations are expected to decrease over time, higher initial phage concentrations may be used.

4.4 Emergence of insensitivity to bacteriophages in foods

A critical issue regarding bacteriophage application for control of pathogens in foods is the emergence of phage resistance. Soon after the discovery of bacteriophages, the emergence of bacterial mutants resistant to phages has been observed [216]. There are different strategies of bacteria to prevent phage infection. In the case of superinfection exclusion, replication of injected phage DNA is inhibited by repressor molecules encoded by the prophage present in the cell [216]. Phage infection can furthermore be interrupted by prophage- or plasmid-induced abortive infection [59]. Superinfection exclusion and abortive infection can be interpreted as a kind of phage-directed resistance [216]. Further strategies are modifications of specific phage binding receptors of the bacterial cell. They can be altered either in their location, quantity, accessibility or configuration. Furthermore, injection of phage DNA can be inhibited [163, 239] or phage DNA might be degraded by restriction mechanisms of the host cell. Restriction enzymes can cut foreign DNA, but not host DNA, because it is protected by methylation [22]. Resistance often emerges due to spontaneous mutations in bacterial genes, for example, in genes involved in phage adsorption. These mutations occur independently of the presence or action of the phages [155]. There are few studies addressing mutation rates for phage resistance; values between 10^{-6} and 10^{-8} per generation have been observed [78, 104, 124, 155, 174].

In this work, none of the *Listeria* Scott A re-isolates from foods showed resistance or reduced sensitivity. With strain WSLC 1001, the emergence of A511 insensitive isolates was observed in 3 out of 8 experiments under long-term storage. Re-growth was observed, consequently phage efficacy was lower. When strain CNL 103/2005 was used, phage resistant cells were identified in several experiments. In 2 out of 5 experiments with chocolate milk at 6°C, in both experiments with hot dogs under long-term storage, and on red smear cheese. In the case of *S.* Typhimurium DB7155 stm^Ralso phage

insensitive colonies were detected.

The results indicate that development of phage resistance on foods seems to be variable and hardly predictable. If there are no negative effects on cell fitness or growth rate, resistant bacteria will probably gain an advantage over non-resistant bacteria, and therefore will dominate the bacterial population on phage treated foods. However, if costs for phage resistance are too high, resistant cells will remain only as a smaller minority. Similar explanations were suggested by Levin and Bull for in vitro observations [139].

Phage insensitivity can emerge during the application of phages on foods, and strategies to overcome this problem have to be developed. One possibility is the application of a mixture of different bacteriophages. The probability for resistance decreases with the amount of applied phages [89, 140]. Moreover, a mixture of different phages combines and extends the host range. It was shown in this study that isolates resistant to phage A511 could still be infected by other phages such as P100, P35 or P40. Therefore, application of different phages in a rotating scheme may be advisable to avoid the build-up of resistance.

Additionally, phages themselves are permanently evolving and may have the potential to overcome bacterial resistance [36]. Furthermore, stability of resistance under non-selective pressure - in the absence of phage - might be weak as seen in the experiment with hot dogs and strain CNL 103/2005. O'Flynn *et al.* [174] also observed that phage resistant cells of *E. coli* reverted to phage sensitivity after propagation through 50 generations in vitro.

In several other studies, no phage resistance of different bacteria was detected: *Salmonella* Enteritidis on fresh-cut fruit during 7 days [135], *Listeria monocytogenes* on cheese during 21 days [38], and *Campylobacter jejuni* on chicken skin during 10 days [23]. However, Greer and Dilts [90] reported mediated emergence of resistant bacteria during phage control of *Brochothrix thermosphacta* spoilage of pork adipose tissue. They found that 20 to 65% of the bacteria isolated after 8 days from phage treated samples were resistant [90].

The procedure for detection of insensitivity used in this study could detect stable resistance, but transient insensitivity might have been overseen. For plaque assays, overnight cultures and subsequent subcultures of re-isolated colonies were prepared. Cells were propagated through several generations (approximately 15 to 20) in absence of the phage, thus without selective pressure. Transient insensitivities might be lost during this procedure, and would not be detectable. Therefore, this method may be modified or supplemented by other techniques. A kind of "online" monitoring would be plating the food homogenate on phage agar plates. A sufficient number of phages (about 10^9 pfu) has to be spread over the whole selective agar plate before plating the

homogenate with the bacteria. Bacteria can only grow, if they are resistant to the phage. A simplification of this technique would be streaking re-isolated colonies from common selective agar plates on phage agar plates [36]. However, in this case, a certain probability for reverting resistance is given again.

The here presented data indicate that emergence and development of phage resistance seems to be strain dependent, and is a rather rare event on food. However, the possibility for development of phage resistance exists. To minimize the risk for phage insensitivity, mixtures of different virulent phage cultures should be used [135, 218] and reasonable application protocols should be developed.

4.5 Conclusion and outlook

The aim of this study was a comprehensive evaluation of the use of virulent bacteriophages for control of *Listeria monocytogenes* and *Salmonella* Typhimurium in several foods under different conditions. It could be shown that application of virulent bacteriophages and their endolysins can contribute to the combat against pathogens in foods.

The effect of phages on *L. monocytogenes* or *S.* Typhimurium was mainly influenced by the kind of food, the amount of phages applied and the stability of phages on the food as well as the kind of phage. To a minor extend it was dependent on the bacterial strain, the initial contamination level, the storage temperature and time. Furthermore, increase of the initial phage concentration seemed to improve efficacy of phage application more than repeated application.

It was shown that growth of three different *Listeria* strains could be controlled by the application of phage A511 and two other phages, P100 and P35, on various foods during storage at 6°C for 6 or 13 days. Bacterial cell numbers could not be completely eliminated on most foods, but existing methods are rather expected to reduce the microbial load in order to minimize the risk of transmission [191]. Efficacy of phages is dependent on homogeneous distribution and high diffusion of the phages such as, e.g., in liquid foods. Furthermore, adsorption and infection can be influenced by food ingredients and the food matrix. Growth control was also possible at a higher temperature of 20°C. However, re-growth of bacterial cells could not be prevented. With respect to absolute bacterial cell count levels on foods, phage application should be used in combination with low storage temperatures. Similar observations were done in experiments with *S.* Typhimurium. Therefore, effective control of foodborne pathogens and insurance of food safety requires the combination of compatible control mechanism. This is in consistence with the hurdle technology of Leistner [133].

For sufficient control of pathogens in foods, phage concentrations of at least 10^8 pfu/g were required. For smoked salmon it was at least 10^9 pfu/g. Therefore, the exact and optimal phage concentration has to be determined for every specific application. There still is a need for further research, especially regarding phage treatment of soft cheeses. Here, an increase in the initial phage concentration might improve growth control. Furthermore, phages may be more effective when cheeses are contaminated with lower cell count levels, as would be the case for naturally contaminated samples. Improved efficacy was already shown for at least some of the foods, when the initial contamination level of *L. monocytogenes* was decreased. Further experiments using even lower cell numbers could give more information about the question of a certain threshold of bacterial host cells for phage effectiveness as it is discussed for at least in vitro experiments [118, 181–183, 240, 246].

In addition to bacteriophages, phage endolysins also have an effect on growth of *L. monocytogenes* on foods. However, application seems to be restricted to certain foods, because enzymatic activity is dependent on temperature and pH. Besides, production and purification of endolysins is more complex and cost-intensive.

This is the first study where the stability of phage has been evaluated in and on different kind of foods, during storage and ripening. Each of the four bacteriophages showed high stability on most foods except for vegetables and one kind of cheese. Therefore, phages appear suitable even for long-term applications in foods. In foods, where phage concentrations will decrease over time, higher initial phage concentrations are required.

In conclusion, bacteriophages represent a novel, effective and environmentally safe way to achieve production of safer food. Different bacteriophage products have recently received GRAS status by the Food and Drug Administration (FDA) or the approval for use as food-additives in particular foods. However, due to the possibility of the emergence of phage insensitive bacteria, measures should be considered to minimize this potential draw back. For instance, mixtures of different virulent phages can be used, and application protocols should be developed so that phage can contribute to existing hurdle technologies.

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A Composition of media (in alphabetical order)

ANC enrichment broth
basic medium: TSB (trypticase soya broth)
17.0 g peptone from caseine
3.0 g NaCl
2.5 g K_2HPO_4
2.5 g D(+)glucose
6.0 g yeast extract
add 1000 ml purified water, pH 7.3±0.2, portions of 175 ml in 500 ml bottles, 15 min at 121.1°C

supplement: ANC, added before use

1.0 ml	**A**criflavin	(230 mg dissolved in 100 ml purified water)
0.2 ml	**N**alidixin acid sodium salt	(460 mg dissolved in 10ml 0.05N NaOH)
0.5 ml	**C**ycloheximid	(230 mg dissolved in 10ml 40% EtOH)

B medium
10.0 g peptone from caseine
5.0 g yeast extract
5.0 g NaCl
5.0 g D(+)glucose
1.0 g $K_2HPO_4x3H_2O$
12.0 g agar
add 1000 ml purified water, pH 7.2±0.2, 15 min at 121.1°C

BBL^{TM} CHROMagar^{TM} Salmonella
ready-to-use agar plates from Becton Dickinson, USA

BHI broth (brain heart infusion)
37.0 g of BHI ready-to-use substrate (biolife, Milan, Italy), containing:
27.5 g Brain-heart infusion, peptone
2.0 g D(+)glucose
5.0 g NaCl
2.5 g Na_2HPO_4
pH 7.4±0.2
add 1000 ml purified water, 15 min at 121.1°C

BHI 1/2 medium
18.5 g of BHI ready-to-use substrate (biolife, Milan, Italy)
add 1000 ml purified water, 15 min at 121.1°C

BHI agar plates
37.0 g of BHI ready-to-use substrate (biolife, Milan, Italy)
12.0 g agar
add 1000 ml purified water, 15 min at 121.1°C, poured into sterile petri dishes

BHI soft agar
37.0 g of BHI ready-to-use substrate (biolife, Milan, Italy)
4.0 g agar
add 1000 ml purified water, test-tubes of 4-5 ml, 15 min at 121.1°C

BPW (Buffered peptone water)
10.0 g Casein
5.0 g NaCl
9.0 g $Na_2HPO_4x12H_2O$
1.5 g KH_2PO_4
add 1000 ml purified water, portions of 225 ml in 500 ml bottles , 15 min at 121.1°C

LB medium (Luria Bertani)
10.0 g peptone from caseine
5.0 g yeast extract
10.0 g NaCl
add 1000 ml purified water, pH 7.0±0.2, 15 min at 121.1°C

LB soft agar
10.0 g peptone from casein
5.0 g yeast extract
10.0 g NaCl
4.0 g agar
add 1000 ml purified water, pH 7.0±0.2, test-tubes of 4-5 ml, 15 min at 121.1°C

LB agar
10.0 g peptone from casein
5.0 g yeast extract
10.0 g NaCl
12.0 g agar
add 1000 ml purified water, pH 7.0±0.2, 15 min at 121.1°C, poured into sterile petri dishes

LB-PE medium (protein expression)
15.0 g peptone from casein
8.0 g yeast extract
5.0 g NaCl
add 1000 ml purified water, pH 7.8±0.2, 15 min at 121.1°C

LC agar

basic medium:

10.0 g peptone from casein
5.0 g yeast extract
10.0 g NaCl
1.0 g D(+)glucose
12.0 g agar

add 1000 ml purified water, pH 7.5±0.2, 15 min at 121.1°C

supplement:

5 ml 1M $CaCl_2$
5 ml 1M $MgSO_4$

added to basic agar subsequent after chilling to 50°C, poured into sterile agar dishes

MKTTn (Muller-Kauffmann tetrahionate novobiocin broth)

Basic medium:

4.3 g Meat extract
8.6 g Casein
2.6 g NaCl
38.7 g $CaCO_3$
47.8 g $Na_2S_2O_3x5H_2O$
4.78 g Ox bile
9.6 g Brilliantgreen

add 1000 ml purified water, dissolved for 5 min during boiling, pH 8.2±0.2, stored for 4 weeks at 3±2°C

Iodine-Iodide solution:

20.0 g iodine
25.0 g KI

add 100 ml purified, sterilized water

Novobiocin solution:

0.04 g novobiocin sodium salt

add 5 ml purified water, filter sterilized, aliquots at -20°C

MKTTn: 1000 ml basic medium, 20 ml iodine-iodide solution and 5 ml novobiocin solution, aseptically mixed and filled 10 ml in test-tubes, used at day of production

Oxford agar plates
basic medium: 55.5 g of *Listeria* selective agar base (Oxford formulation; Oxoid, Basingstoke, England), containing:

39.0 g	Columbia-agar-base
1.0 g	Esculine
0.5 g	Iron (III) ammonium citrate
15.0 g	Lithium chloride

pH 7.0±0.2
additionally 2.0 g agar
add 1000 ml purified water, 15 min at 121.1°C
supplement: 2 portions of Oxford selective supplement (Oxoid, Basingstoke, England):

200.0 mg	Cycloheximide
10.0 mg	Colistinsulphate
2.5 mg	Acriflavine
1.0 mg	Cefotetan
5.0 mg	Fosfomycin

dissolved in 5.0 ml EtOH (70 % in purified water) each, added to Oxford agar subsequent after chilling to 50°C, poured into sterile agar dishes

PC3+ agar plates (plate count + 3% NaCl)

5.0 g	peptone from caseine
2.5 g	yeast extract
1.0 g	D(+)glucose
30.0 g	sodium chloride
12.0 g	agar (Oxoid, Basingstoke, England)

add 1000 ml purified water, pH 7.0±0.2, 15 min at 121.1°C, poured into sterile petri dishes

RVS (Rappaport Vassiliadis medium with soy)
55 g of XLD ready-to-use substrate (Oxoid, Basingstoke, England), containing:

5.0 g	Soya
8.0 g	NaCl
1.4 g	KH_2PO_4
0.2 g	K_2HPO_4
40.0 g	$MgCl_2x6H_2O$
0.4 g	Malachite green

add 1000 ml purified water, pH 5.2±0.2, test-tubes of 10 ml, 15 min at 121.1°C, storage at 2 - 8°C

Yeast extract medium

45.0 g	yeast extract
50 ml	1M sodium phosphate buffer pH 8.0
5.0 g	D(+)glucose
10.0 g	glycerol

add 1000 ml purified water, pH 7.0±0.2, 15 min at 121.1°C

XLD agar (Xylose-lysin-desoxycholate agar)

55 g of XLD ready-to-use substrate (Merck, Germany), containing:

3.0 g	yeast extract
5.0 g	NaCl
3.75 g	D(+)xylose
7.5 g	lactose
7.5 g	sucrose
5.0 g	L(+)lysine
6.8 g	sodium thiosulfate
0.8 g	ammonium iron(III) citrate
0.08 g	phenol red
1.0 g	sodium desoxycholate
12.0 g	agar

add 1000 ml purified water, pH 7.4±0.2, solved under stirring until boiling, immediately cooled down to 50°C, avoiding overheating and prolonged storage in water bath, poured into sterile petri dishes, storage 5 days at 3±2°C

B Composition of buffers (in alphabetical order)

Buffer A
500 mM NaCl
50 mM Na_2HPO_4
5 mM Imidazole(1,3-Diaza-2,4-cyclopentadien)
0.1 % Tween 20 (before or after autoclaving)
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C, degased, filter sterile

Buffer B
500 mM NaCl
50 mM Na_2HPO_4
250 mM Imidazole (1.3-Diaza-2.4-cyclopentadien)
0.1 % Tween 20 (before or after autoclaving)
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C, degased, filter sterile

Citrate buffer
58 mM trisodium citrate dihydrate
add 1000 ml purified water, pH 7.5, portions of 45 ml, 50 ml, 90 ml or 180 ml, 15 min at 121.1°C

Dialysis buffer
100 mM NaCl
50 mM Na_2HPO_4
250 mM Imidazole (1.3-Diaza-2.4-cyclopentadien)
0.005 % Tween 20 (before or after autoclaving)
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C

PBS buffer (phosphate buffered saline solution)
120 mM NaCl
50 mM Na_2HPO_4
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C

PBSm buffer (phosphate buffered saline solution modified)
100 mM NaCl
20 mM Na_2HPO_4
add 1000 ml purified water, pH 7.3, 15 min at 121.1°C

PBST buffer (phosphate buffered saline solution containing Tween20)
120 mM NaCl
50 mM Na_2HPO_4
0.1 % Tween20 (before or after autoclaving)
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C

SM buffer

stock solution:

121.1 g Tris
100 ml 25 % HCl
add 1000 ml purified water, pH 7.4, 15 min at 121.1°C

working solution:

50 ml SM stock solution
5.5 g NaCl
2.0 g $MgSO_4$ x $7H_2O$
add 1000 ml purified water, pH 7.4, 15 min at 121.1°C

Sodium phosphate buffer pH 8.0

932 ml 1M Na_2HPO_4
68 ml 1M NaH_2PO_4
add 1000 ml purified water, pH 8.0, 15 min at 121.1°C

C Composition of other solutions (in alphabetical order)

Ampicillin stock solution 100 mg/ml
2.0 g ampicillin sodium salt (Applichem, Darmstadt, D)
add 20 ml purified water, filter sterilized, aliquots of 1 ml stored at -20°C

L(+) arabinose stock solution 1M
3.0 g L(+) arabinose
add 20 ml purified water, filter sterilized

Chloramphenicol stock solution 1.5 mg/ml
30 mg chloramphenicol (Sigma-Aldrich, USA)
add 20 ml 70% ethanol, filter sterilized, stored at -20°C

Chloramphenicol stock solution 20 mg/ml
400 mg chloramphenicol (Sigma-Aldrich, USA)
add 20 ml 70% ethanol, filter sterilized, stored at -20°C

Galactose stock solution 20%
20 g galactose
add 100 ml purified water, filter sterilized, aliquots of 1ml stored at -20°C

Glycerol 5%
5.0 g glycerol
add 100 ml purified water, 15 min at 121.1°C

Glycerol 10%
10.0 g glycerol
add 100 ml purified water, 15 min at 121.1°C

Glycerol 85%
85.0 g glycerol
add 100 ml purified water, 15 min at 121.1°C

IPTG stock solution 840 mM
2.0 g Isopropyl-β-D-Thiogalactopyranosid (Sigma-Aldrich, USA)
add 10 ml purified water, filter sterilized, aliquots of 1ml stored at -20°C

Sodium chloride solution 4.3%
43.0 g NaCl
add 1000 ml purified water, 15 min at 121.1°C

Sodium chloride solution 7.95%
79.5 g NaCl
add 1000 ml purified water, 15 min at 121.1°C

Streptomycin stock solution 50 mg/ml
1.0 g streptomycin sulfate salt (Sigma-Aldrich, USA)
add 20 ml purified water, filter sterilized, aliquots of 1ml stored at -20°C

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Curriculum vitae

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Zürich, May 2007 Susanne Günther