Your enemy's foe is your friend

Listeria in ready-to-eat food products, related outbreaks and usage of bacteriophage technology – a review

Listeria monocytogenes (_Listeria_) is a Gram-positive facultative intracellular opportunistic pathogen which is ubiquitous to the environment. It is often transmitted through contaminated food and causes a rare but serious disease called listeriosis. One fourth of the food poisoning related deaths are caused by _Listeria_. Immunocompromised patients and elderly people as well as pregnant women and neonates are susceptible to listeriosis. Several studies have found that _Listeria_ enters the body through the intestines and can spread to the liver whilst eluding the immune system by entering hepatocytes and proliferating intracellularlly. Infected resident phagocytes often spread through the blood to the brain and uterus. Outbreaks of _Listeria_ are often related to contaminated ready-to-eat foods. The food industry is constantly evolving to minimise the outbreaks. Recently, several companies have received governmental approval for the use of bacteriophage technology to improve or even ensure food safety.

By Henk R. Hoogenkamp

The genus *Listeria* currently includes six species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L welshimeri* and *L. grayi* [56]. Two species, *L. monocytogenes* and *L. ivanovii*, are considered potentially pathogenic. This review will focus on the species *Listeria monocytogenes* and will be referred to as *Listeria* in the following text.

Listeria monocytogenes (*Listeria*) is a Gram-positive bacterium which lacks a capsule and is motile at temperatures between 10 and 25 °C. It is a facultative intracellular pathogen which can induce its own uptake into non-phagocytic cells. It is ubiquitous in the environment and can be predominantly found in mammals, birds, insects, soil, waste water and vegetation [57,43]. Studies suggest that 1 to 10% of the healthy human population may have their gastrointestal tracts transiently colonised by *Listeria* [49].

Infection by *Listeria* is called listeriosis, which involves numerous primary and secondary diseases. After it invades the host's gastrointestinal tract, it often spreads through the blood to the liver, central nervous system and possibly the pregnant uterus (Fig. 1) – termed "invasive listeriosis". Manifestations of listeriosis include bacteraemia, meningitis, encephalitis, meningoencephalitis and pneumonia in the immunocompromised population. In pregnant women, infection can cause a mild fever up to miscarriage, premature birth, stillbirth and neonatal diseases. Surviving children of Fetomaternal Listeriosis may suffer granulomatosis infantiseptica and possible physical retardation [56]. Incubation periods range from a typical two to three weeks up to three months [24]. Symptoms including nausea, diarrhea, vomiting, and fever, often precede more serious forms of listeriosis [51,52].

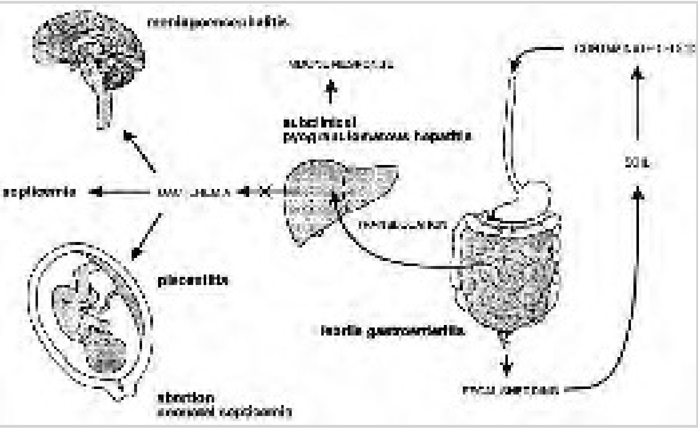

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Fig. 1: Overview of the *Listeria* infection cycle [49]. This diagram illustrates the route of infection by *Listeria* and the most relevant organs. The diseases that can arise are printed in bold.

Host susceptibility

The groups at risk for contracting listeriosis are, as previously mentioned, pregnant women and their neonates, immunocompromised adults (e.g. acquired immune deficiency syndrome and organ transplant patients) and the elderly (60 years and older). There is controversy in the classification of *Listeria*. Host susceptibility plays a major role in the presentation of clinical disease upon exposure to *Listeria*. Most cases of listeriosis involve patients, which have a physiological or pathological defect that affects cell-mediated immune response. This reasoning justifies the classification of *Listeria* as an opportunistic pathogen. Nevertheless, European and North American governmental safety regulations still regard *Listeria* as a pathogenic organism and a threat to overall public health [50]. This issue will be elaborated upon later in this review.

As mentioned earlier, severe infections during pregnancy can lead to pre-mature birth or miscarriage, and meningitis in newborn children. However, in immunocompromised adults and the elderly, an infection can cause septicemia and meningitis [57]. Approximately 2,500 cases of serious listeriosis and 500 deaths, in the United States alone, are reported each year [44]. This number is assumptively lower than the actual number of infections due to the frequent misdiagnosing by physicians. *Listeria* is often overlooked as a possible cause of illness due to several important factors. It is difficult to detect due to its unique growth capabilities. It grows best at refrigeration temperatures (1 to 10 °C). Even when detected, phenotypic characteristics resemble that of Streptococci and other diphteroids which are then often discarded as harmless contamination by microbiology lab technicians [49]. Nearly one-fourth of all estimated food-borne disease-related deaths are contributed to *Listeria* [43]. Nevertheless, *Listeria* infection is relatively uncommon in comparison with other food borne pathogens (*Salmonella* Typhimurium and *Escherichia coli*). Unique to *Listeria* is the unusually high fatality rate which can be as high as 40% among at-risk people [24].

Food industry

Certain features make *Listeria* especially problematic for the food industry. It can proliferate (respectively) at temperatures from 1 to 45 °C and survive at extremely low to extremely high temperatures for a certain time (–20 to +80 °C). *Listeria* is also halotolerant, meaning that it can survive and grow in salt solutions where other bacteria cannot [57]. Even relatively low pH values, frequently encountered in food processing, seem to have little effect against *Listeria*. Affected products include

- fish, shellfish and fish products;

- raw meat, poultry and their products, including hot dogs and pâté;
- raw and processed vegetables;
- ripened soft cheeses;
- ice cream;
- retail cook-chill meats;
- salads including coleslaw; raw and partially pasteurised milk as well
- raw and liquid egg [49].

Ready-to-eat (RTE) products are especially vulnerable to contamination by *Listeria* since the products are not always re-heated before consumption. Post-pasteurisation processed products, e.g. sliced and repackaged deli meats, have been deemed by the United States Department of Agriculture (USDA) as high risk for the susceptible population. Outbreaks of *Listeria* in the United States have lead to several nationwide recalls of RTE products [59].

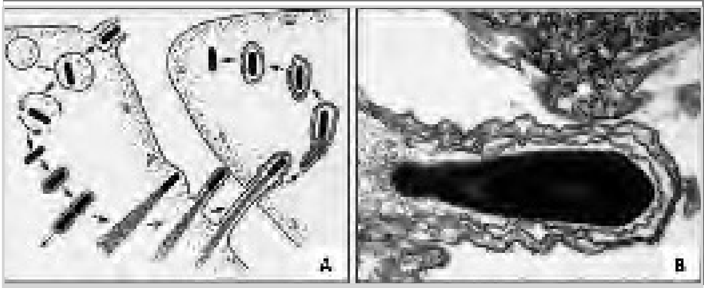

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Fig. 2: A) Schematic representation of the intracellular proliferation and spread cycle. B) TEM image of *Listeria* exiting a host cell. [49]

Fighting *Listeria*

As indicated by the high fatality rates, *Listeria* infection cannot be treated effectively [24]. At this moment there is no known vaccine and is, due to the low occurrence, very unlikely that pharmaceutical companies are interested in developing one. Due to the lack of feedback from patient to source, there is a limited opportunity for *Listeria* to develop into (extremely) resistant strains. However, the globally increasing risk of resistant bacteria has the medical world worried about the limited resources of combating listeriosis. The food industry on the contrary, has plentiful means to prevent *Listeria* contamination. Countless food grade listeriocidal agents and anti-listeral processes are available. This will be elaborated upon later in this review. The majority of the food producing companies cannot make use of these techniques due to product formulation and lack of financial means. The food industry, like the medical society, is searching for a silver bullet which will improve the odds when fighting *Listeria*.

Recent publications have re-introduced bacteriophages as possible anti-listeral agents [29,50]. Bacteriophages are inanimate obligate parasites which have a broad or specific range of prokaryotic target organisms. Natural bacteriophages are the most abundant microorganisms in our environment and are present in high numbers in water and foods of various origins. They contribute to the natural bacterial homeostasis in nature.

This comprehensive review will firstly cover the pathophysiology of *Listeria*. Secondly the epidemiology in relation with the food industry will be explored. The last part of this article will focus on possible prevention of *Listeria* contamination with a 'touch and go' on Bacteriophage technology itself.

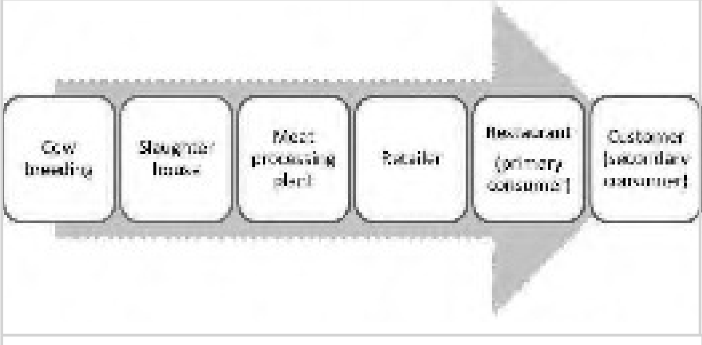
Pathophysiology

Since the discovery of *Listeria* in 1924, scientists have been trying to identify the virulence factors and mechanism of disease. Researching this topic is a tedious task due to *Listeria's* two-faced characteristic. It is a ubiquitous non-spore-forming bacterium which can be found in a variety of environmental niches where it exhibits non-pathogenic traits [29]. However, *Listeria* can facilitate its survival within mammalian host cells and has even been isolated from insects and amoebas [24,38]. Relatively little information is available about the peaceful existence of *Listeria*, unlike the numerous publications regarding its pathogenic character. The majority of the studies have focused on *Listeria* in relation to invasion, survival and pathogenesis in mammalian hosts.

Infection and primary disease progression

Listeria must firstly endure the harsh environment of the upper digestive tract. It has to pass through the highly acid stomach before an opportunity arises to invade the host. Generally accepted, *Listeria* enters the body through the epithelial cells of the intestines, but the exact mechanism and point of entry is still controversial and largely unknown [48]. More recent studies have presented evidence which suggested that *Listeria* uses the M-cell epithelium, which line so-called Peyer's patches, as a point of entry [29,41,53]. If the *Listeria* cells remain in the intestines and endothelium febrile gastroenteritis can occur as a consequence.

Once *Listeria* has crossed the intestinal barrier it is translocated by the blood or lymph fluids to the spleen, lymph nodes or most importantly, the liver [47,50]. During translocation, macrophages in the blood rapidly remove and kill most of the *Listeria* cells. The surviving cells, which arrive in the liver, are likely to be captured and killed by Kupffer cells which are resident macrophages of the liver [44,53]. Several publications suggest that Kupffer cells initiate anti-listeral immunity by inducing the antigen dependent proliferation of T lymphocytes and the secretion of cytokines [44,53]. The *Listeria* cells, which still elude the different macrophages, will invade hepatocytes and start to proliferate thus entering the intracellular infectious cycle.



Source: Hoogenkamp Fleischwirtschaft International 2/2009

Fig. 3: Different parties involved from cow to consumer. After meat processing plants the risk of (re-) contamination increases.

Intracellular infectious (life) cycle

Listeria's defense mechanism against the humoral and innate immune responses seems more an offensive technique – by inducing their own internalisation into various types of cells including those, which are not usually capable of phagocytosis (hepatocytes, fibroblasts, nerve, epithetlial and endothelial cells) [11,13,46,47,58,59].

Listeria recognises many different eukaryotic receptors: E-cadherin, several different complement fraction receptors, hepatocyte growth factor recptor and extracellular matrix components such as heparan sulfate and fibronectin [39,48,57]. The detection of these receptors by listerial ligands such as internalins A (InlA) and B (InlB), actin-polymerising protein ActA and hydrolase P60 induce adhesion to the cell surface and uptake into the eukaryotic cell [51]. *Listeria* is engulfed by the plasma membrane and enters the cell still being surrounded by the host's membrane. The vacuole (phagosome) is inhibited from maturation to a phagolysosome by the *Listeria* cell to ensure survival. The phagosome is eventually broken down by hemolysins and phopholipases which lead to the release of the *Listeria* cells into the cytoplasm [15]. Studies show that within 2 hours 50 % of the intracellular bacterial population is free and starts proliferating with an approximate 1 hour doubling time [55]. The cytoplasm permits listeral growth and infection has shown to upregulate genes involved in nucleic acid synthesis (purH, purD and pyrE) [39].

After a short acclimatisation period in the cytoplasm, *Listeria* cells are surrounded by actin filaments, which phenotypically resemble a bacterial capsule. After a certain time point, the filament arranges in an actin tail at one pole of the bacterium. The rearrangement and assembly of the actin tail propel the cell randomly in the cytoplasm and gradually towards the host's cell membrane. By chance the bacterium can push the plasma membrane towards a neighbouring cell, which in turn can phagocytose the unusual structure. The *Listeria* cell is now surrounded by a double membrane from which it escapes once again (Fig. 2). This simultaneously marks the end and beginning intracellular proliferation and intracellular spread.

Controlling *Listeria* infections

The adaptive immune system has several ways of controlling *Listeria* infections. CD8+ T-cell response plays a major role in disrupting the intracellular proliferation and intracellular spread cycle [32]. CD8+ T-cells are the principal T-cell effectors and regulate lysis of infected host cells [26,35]. Direct or indirect activation of CD8+ T-cell's two potential bactericidal mechanisms depend on MHC class-I and class-II recognition of peptide antigens expressed on target cells [46]. The T-cell's arsenal includes perforin-dependant lysis and Fas-L/Fas induced apoptisis [58]. The Perforin-dependent mechanism inserts perforin into the cell membranes of infected cells creating pores which allow enzymes such as granzymes and proteases to enter and initiate apoptosis [55]. Fas-L/Fas interactions mechanisms appear to be more important during late primary infection. Studies using mice, which are deficient in both perforin and Fas, are highly susceptible to listeriosis [28].

Bacteremia and secondary disease progression

Secondary diseases involving the invasion of the blood, uterus, and the brain, usually occur after the colonisation of the liver. Inadequate immune response to a *Listeria* infection can lead to sepsis (blood poisoning). However, sepsis symptoms rarely go unnoticed and can be treated at an early stage. Fast spreading infections can lead to severe sepsis which cause organ dysfunction and eventually septic shock.

Numerous studies have shown that *Listeria* can gain access to the fetus through the placenta [56]. T-cell-dependent elimination of *Listeria* from the circulation of the pregnant woman is inefficient. Low levels of certain cytokines (e.g. INF-γ) and high levels of female hormones may explain this inefficiency. Additionally, local depression of the cellular immune response in the placenta which prevents the rejection of the fetus. This may also contribute to the susceptibility of the uterus to listeric infection [50]. The penetration mechanism is haematogenous, which indicates that infected cells passively transport the bacterial cell in the blood stream to the placental barrier. The *Listeria* cells cause inflammatory reactions which eventually lead to small abscesses in the placenta. This provides an opportunity for the bacterium to translocate to the fetal bloodstream. Unfortunately, the infection will generally lead to severe complications and subsequent death, due to the lack of a cell mediated immune response in the neonate.

Listeria seems to have a preference for nerve tissue (neurotropism) [15]. Infection of the central nerve system (CNS) usually leads to a form of meningitis (infection of protective membranes covering the CNS). Lesions in the brain, brain stem and spinal cord are frequently observed. This can lead to symptoms like headaches, stiff necks, fever, photophobia and even seizures. Unlike the acute phase of sepsis, infections of the brain are usually systemic and are nearly impossible to treat effectively [24].

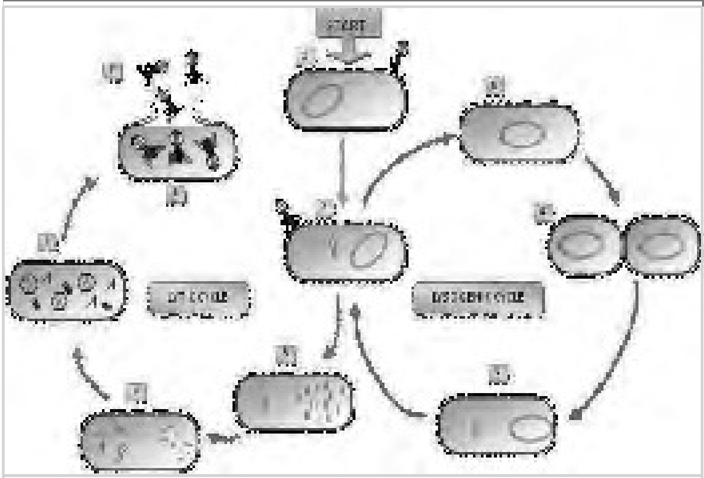
Epidemiology

Due to the ubiquity in nature, opportunities to come into contact with *Listeria* are abundant. It has been isolated from many different niches including soil and silage. Domesticated and feral animals, which frequently come into contact with the natural environment of *Listeria*, can carry and spread it in many different ways. At least 37 mammalian species as well as 17 avian species and some species of fish have been found to carry *Listeria*.

Listeriosis has been associated with food transmitted *Listeria* for a long time, but until 1979 has never been documented as an outbreak. In 1979, 23 patients in a Boston hospital contracted a form of listeriosis. Evidence from an investigation suggested a correlation with infected patients and contaminated vegetables prepared in the hospital kitchen [24,27].

Outbreaks from 1979 onward have been well documented and several excellent publications and reviews have arisen [38,41,45,49]. In Europe a well known outbreak occurred in 1997. A total of 1,566 people in Italy were infected by *Listeria* and reported symptoms related to mild non invasive listeriosis. The symptoms mainly included fever and abdominal pain and no fatalities were reported [43]. An important research paper was recently published, which investigated the incidence of listeriosis from 1999 to 2006 predominantly in France and alos 8 other European countries [41]. The researchers determined that the increase from 3.5 to 4.7 cases per million persons occurred in the population above the age of 60. They rule out an outbreak related with contaminated foods but cannot explain otherwise. The implication of this paper results in a background occurrence at which listeriosis occurs without epidemiological aspects.

In comparison to Europe, more outbreaks seem to surface in North America. Large food related outbreaks in North America have lead to invasive listeriosis of hundreds of people with an average 20% mortality rate. Often the large companies, which own several different smaller food producing businesses, are affected. In 1998, an outbreak occurred in which *Listeria* was spread via hot dogs and deli meats produced by BelMar Foods, a subsidiary of the food retail giant Sara Lee Foods. Over 120 people showed clinical listeriosis of which 20 died. An estimated thirty-five million pounds of meat were recalled.



In the first steps (1, 2) the phage attaches to the bacterium and injects its genetic material. Here the phage can enter the lytic or lysogenic cycle. The lytic cycle starts when the host DNA is digested by restriction enzymes (3). From the digested host DNA new viral DNA is formed (4). The new viral DNA is transcribed by the host cell machinery forming new phage particles (5). The phage is assembled, and a phage endoded enzyme causes the cell to lyse. In the lysogenic cycle the phage DNA integrates into the bacterial genome and becomes a noninfective prophage (3). The host cell can replicate with the integrated prophage DNA (4). The prophage DNA may in some cases separate from the genome and enter the lytic cycle (5).

Source: Hoogenkamp — Fleischwirtschaft International 2/2009

Fig. 4: Lysogenic and lytic cycles of a bacteriophage [adapted from 54].

In 2000, a multistate outbreak of listeriosis in the United States was identified [45]. A total of 29 illnesses were spread over 10 states. Among the 29 infected, 21 nonperinatal and 8 perinatal cases resulted in four deaths and three miscarriages. After tenuous investigation, several companies were implicated, 6 months after the first case. Four days after the report was released, the turkey product subdivision of Cargill, a large agricultural company, voluntarily recalled all turkey and chicken deli meats that might have been contaminated [42].

The most recent outbreak dates from September 2008 in Canada. 52 cases have been linked to Maple Leaf Foods, where 20 people have died from severe listeriosis. The company recalled 191 products and has lead to millions dollars of damage. No research data is available from this outbreak [52].

Listeria epidemics are not limited to contamination during food production and processing. Several cases have been reported where small groups of people have been infected by mild listeriosis at company outings such as pick nicks or in hospital kitchens [24, 33].

Food contamination

Listeria infections can be sporadic due to contact with animals and agricultural materials. Veterinarians are often confronted with listeriosis in animals and are thus more likely to develop sporadic listeriosis. However, the majority of listeriosis cases are related to the consumption of infected foods. Raw meat products are likely to be contaminated with food-borne pathogens, but are practically always heated to temperatures which kill the bacteria before consumption. The risk products are those which fall in the category ready-to-eat. Vegetables which have been fertilised by natural manure and that can be eaten raw are highly susceptible to transmit the pathogen. The focus, however, lies on meat and dairy products because of the amount of processing the products generally undergo. Each processing step increases the risk of contamination.

Several considerations for usage possible

Tab.: Bacteriophage approach to the control of food borne bacteria [29]

	Advantages	Disadvantages
1	Self-perpetuating	Limited host ranges
2	Selective modification of bacterial flora	Possible phage-resistant bacterial mutants
3	Stable in foods and able to survive processing	Requires relatively large numbers of target bacteria
4	Natural	Barriers in food environments
5	Ubiquitous	Tansduction of undesirable characteristics
6	Ease of preparation and application	Lysogenic conversion to temperate phages
7	Nontoxic to eurkaryotic cells	Antigenicity
8	No effect on food quality	Consumer perception

Source: Hoogenkamp — Fleischwirtschaft International 2/2009

Beef deli meats

Take for example a beef deli meat (roast beef). The process from cow to sandwich can involve as many as five to six different parties and countless different processes before actual consumption (Fig. 3). Beef products are cured with traditional brines which determine the product specificities. Modern methods require that the brine be injected into the beef through automated syringes. This process can force the pathogen from the surface deeper into the meat. After processing all meats undergo a common listericidal process: cooking. When the beef is cooked (pasteurised), it should be free from *Listeria*. Once the roast beef exits the oven, it is prone to re-contamination from environmental sources (e.g. contaminated workers, aerosols and condense drip). When the meat is packaged, certain companies may apply a post-pasteurisation anti-listerial process like infrared heat treatment of the product surface before packaging. After packaging, the beef is stored at refrigeration temperatures until shipping. Large companies will often apply post-packaging pasteurisation steps, which often include a hot water bath. The beef will stay at refrigeration temperatures until it reaches the primary or secondary consumer. As mentioned before; *Listeria* can and will proliferate at refrigeration temperatures. This poses an additional risk without even considering the possible temperature fluctuation of the beef or the abuse of the packaging to which it might undergo during transportation.

Deli meats are especially vulnerable to recontamination, because they are often sliced and repackaged by the retailer. If recontamination occurs during the slicing process, the *Listeria* cells can and probably will systemically spread to all slices of the consecutive batches until the slicing machine is cleaned.

In summary, there are many possible sources of recontamination, of which not all are included in the preceding text. Processed and unprocessed ready to eat foods are vulnerable in which some might have more sources than others.

Prevention

Not all hope is lost. There are many ways of preventing recontamination of *Listeria*. This review will not go into detail about these techniques but will give a general idea of what is being done to prevent outbreaks. Depending on the type of product and formulation limitations, a food processing company can opt for several different approaches. There are three general concepts of prevention:

- mechanical pasteurisation,
- physical containment and
- (bio-)chemical treatment.

Mechanical pasteurisation mainly involves high temperature processing, but can also make use of UV radiation and high pressure. Physical containment is usually a bacteriostatic process where the materials and machinery used for packaging prevent proliferation of possible pathogens. Modified atmospheric packaging is a widely used technique, which removes all the oxygen and adds nitrogen to inhibit growth of aerobic bacteria. Solutions which require new machinery often consume relatively large amounts of space and require high production capacities. These limitations are often related to unfavourable return of investment or lack of financial means.

The last category is the use of bactericidal or bacteriostatic (bio-)chemicals. Many of which are techniques from antiquity, like the use of acids and high salt concentrations. The use of salts and acids are an integral part of most meat formulations. These traditional components can hinder the use of many agents which might be pH specific. New age (bio-)chemicals like highly purified herbs and smoke extracts are often very effective but can possibly affect the quality of the product (e.g. taste, texture and colour). This issue will mostly affect small/medium sized businesses with traditional product formulations. There is need for an universal product which evolves along with the bacterium in question [25].

Bacteriophage technology

Bacteriophage technology is an old, yet new emerging technique to deal with *Listeria*. Bacteriophages are viruses infecting bacteria. They are inanimate obligate intracellular parasites and lack their own metabolism. Phages are usually extremely host-specific and are the natural enemies of all sorts of bacteria. Listeriophages are, like *Listeria*, ubiquitous to the environment and can be isolated from the same niches where their host resides. A rather difficult concept to understand is the unusual mechanism of lytic phages. Once infected by a phage, the cell can still function normally depending on which cycle the phage enters (lytic or lysogenic, Fig. 4 [49]). A phage-infected *Listeria* cell will not lyse immediately due to the phage life cycle. Thus, a lytic phage-infected cell is technically still alive, depending on the aggressiveness of the phage and the activity of the host's metabolism. A higher host metabolism (correlated with optimal growth conditions) will lead to quicker lysis of the cell and *vice versa*.

Only lytic in comparison to lysogenic phages are useful to the food industry due to obvious reasons [54]. Companies (EBI food safety, Wageningen, the Netherlands and Intralytix, Baltimore, United States) have successfully isolated and improved several lytic phages specific for different pathogens including *Listeria*. Depending on the product, one can opt for a phage mix, which helps prevent the development of resistant target pathogens. The phage specificity can be optimised to target certain strains or even strains which have become resistant.

Selected bacteriophages can act as a listericidal and listeriostatic agent, by merely spraying a high titre solution on virtually any given product. There are several considerations for applying bacteriophage technology, which have been carefully listed by Greer in an excellent review (Tab.) [29].

Future prospects and concluding remarks

In Europe bacteriophages have been approved as a food grade processing aid. They are currently being used in the cheese industry, mainly for the production of soft cheeses made from unpasteurised milk. In the United States, bacteriophage technology has been awarded the GRAS status (generally regarded as safe) [25,29,50]. The use of this technology is being limited by labelling issues with respect to consumer preference. An important factor to consider is educating the public about bacteriophages. Until then, the producers of RTE products could combine efforts to persuade the national regulatory agencies to classify bacteriophage technology as a processing aid, circumventing consumer related labelling issues. However, the increasing risk of bacterial resistance and the change of the political/financial environment can eventually lead to the wide spread use of bacteriophages in the United States and other countries.

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References

Literature references can be downloaded at *www.fleischwirtschaft.com/literature* and requested from the author or the editorial office, respectively.

Author's address

Henk R. Hoogenkamp, Groteloef 36, 6581JG, Malden, The Netherlands

Henk R. Hoogenkamp studied biochemistry in Life science at the HAN University Nijmegen. He is working as Research Technician at the Nijmegen Centre for Molecular Life Science, Radboud University Nijmegen Medical Centre.