USE OF BACTERIOPHAGE AS AN ANTIMICROBIAL IN FOOD PRODUCTS

by

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Abstract

Food recalls and incidence of foodborne disease are on the rise throughout the world. Food products are recalled in the United States almost daily, and typically a large quantity of food is affected. Pathogenic microorganisms are readily invading the food supply and traditional methods and use of antimicrobials are not performing as well as in the past. The microorganisms that prompt the recalls cause symptoms ranging from mild gastroenteritis to death. All humans eat food, therefore all humans have the potential to be exposed to pathogens in food at some point in their life. There is a need for new, more effective antimicrobials for use on food products in order to ensure that consumers have access to a safe food supply. Any new treatments for prevention of pathogenic growth in the food supply should be researched. Phage preparations used as antimicrobials on food products are a novel idea. Phages are advantageous over traditional antimicrobials such as antibiotics, pesticides, and sanitizers in numerous ways. This report presents the history of phage and phage therapy in humans, advantages and disadvantages of phage use over traditional methods, current phage preparations available or under research, and approvals and objections of phage use in the food supply.

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Dedication

This work is dedicated in memory of my mother, Phyllis B. Hagwood (1950 – 1995).

Chapter 1: Introduction and History

Numerous antimicrobial products exist. Bacteriophages have been found in human surroundings for generations, and use of the microorganism to combat bacterial pathogens is a constant area of research. Bacteriophages have the potential to be an effective antimicrobial for use on foods, without some of the side effects present in current methods. Bacteriophage is only one of the numerous methods being considered for use as an antimicrobial in foods.

Definition and Mechanism of Action

Bacteriophage, also known as phage, is a type of virus that infects only bacterial cells. Phages are very tiny and measure 20 to 200 nanometers, which is approximately 100 times smaller than most bacteria. Phage comes from the Greek word *phagin,* which translates "to eat or devour" (Sulakvelidze et al. 2001). Phages are very specific toward their target bacteria; they only infect that particular type or strain, and have no effect on any other type of cell including human, animal, and plant cells. Phages are not living organisms but consist simply of genetic material wrapped in a protein or membrane outer coating (Snyder and Champness 2003). Typically, a phage has a hollow head that contains tightly packed genetic material such as DNA or RNA, and a tail with a binding site specific to their target bacteria (Figure 1). The genetic material is forced into the bacterial cell through the tail and new phages are replicated rapidly, sometimes as great as 3.3 phages/minute. A typical DNA phage injects genetic material into the cell and RNA transcription commences immediately. Retroviruses are a type of RNA phage that uses reverse transcriptase, an enzyme, to transcribe the RNA into DNA inside the host cell.

Other RNA phages use enzymes known as RNA replicases to replicate their RNA and therefore do not transcribe to DNA as an intermediate (Snyder and Champness 2003).

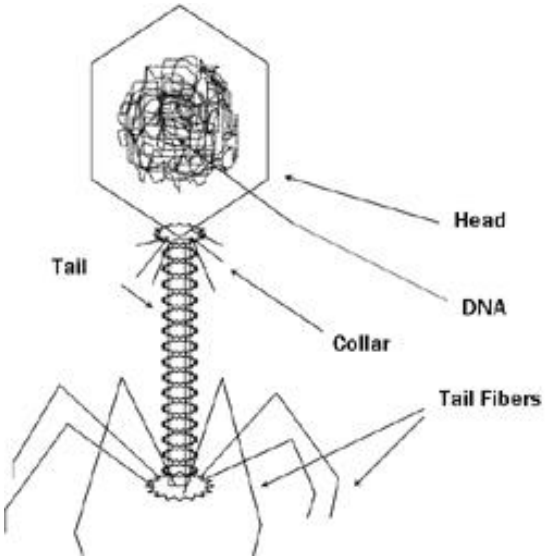

Figure 1. A representative schematic of the structure of a bacteriophage. (taken from Intralytix, Inc. 2006a)

Two types of phages exist: virulent (lytic) and temperate (lysogenic). Virulent phages cause lysis of the cell by interrupting the metabolism of the bacteria, which subsequently causes rupturing of the cell wall and results in death of the cell (Figure 2). Lysis begins with the phage attaching to the cell wall of the target bacteria and injecting its genetic material. Once inside the bacterium, replication begins and eventually the cell bursts to release the daughter phages and the cycle starts over. Temperate phages will typically integrate their genetic material along with that of the host cell to form what is known as a prophage. Prophages use a process known as transduction to transfer the combined bacterial and viral genetic material between host cells. Some temperate phages are capable of lysing the cell. The phage of concern in the area of phage therapy and for use as an antimicrobial on food is the lytic phage.

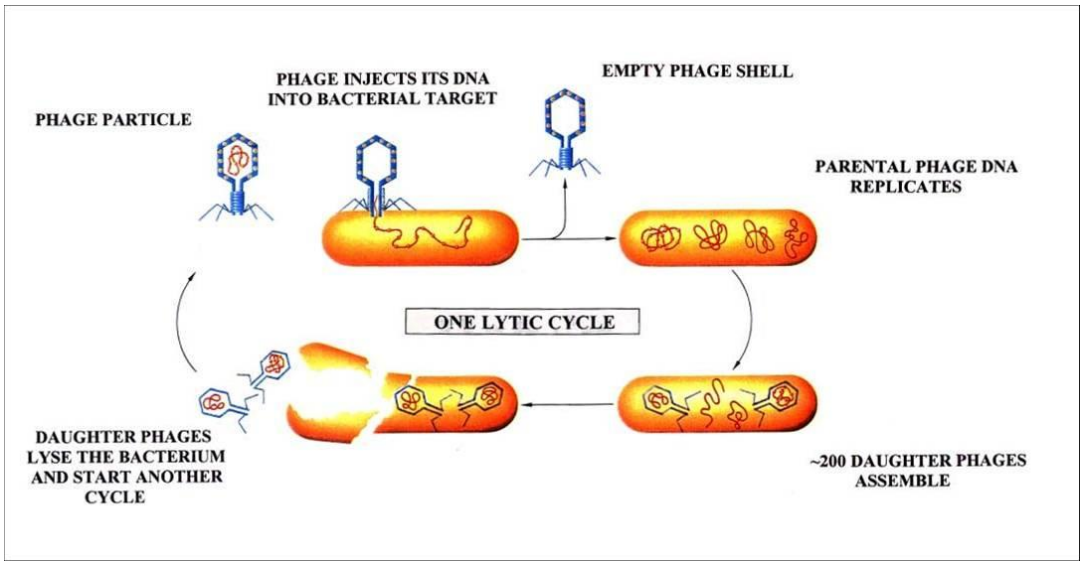

Figure 2. Flow diagram of the lytic cycle of a bacteriophage (≈ 20 min). (taken from EBI Food Safety 2006a)

Bacteriophage Therapy: The Beginning

In 1896, a British bacteriologist by the name of Ernest Hankin, was the first to report on phages when he discovered that sewage water from the Ganges and Jumna rivers in India was an effective antimicrobial against cholera, caused by the bacteria *Vibrio cholerae* (Sulakvelidze et al. 2001). Frederick Twort investigated bacteriophage in 1915, but chose not to fully pursue and understand the meaning of his findings. Felix d'Herelle also discovered the presence of the viruses in 1915 while studying an outbreak of dysentery among French soldiers (Sulakvelidze et al. 2001). During his investigation, one step of the process consisted of plating a mixture containing *Shigella* and fecal samples from the patients onto an agar medium to observe bacterial growth. The cultures on the agar plates showed areas of clearing throughout the bacterial growth, and these areas were attributed to the phages. D'Herelle termed the clearings "plaques" and are still known by that name today (Figure 3). These clearings are currently used to denote

phage growth in laboratory settings. D'Herelle is credited with the official discovery of bacteriophage in 1917 when his research was presented to the French Academy of Sciences and subsequently published.

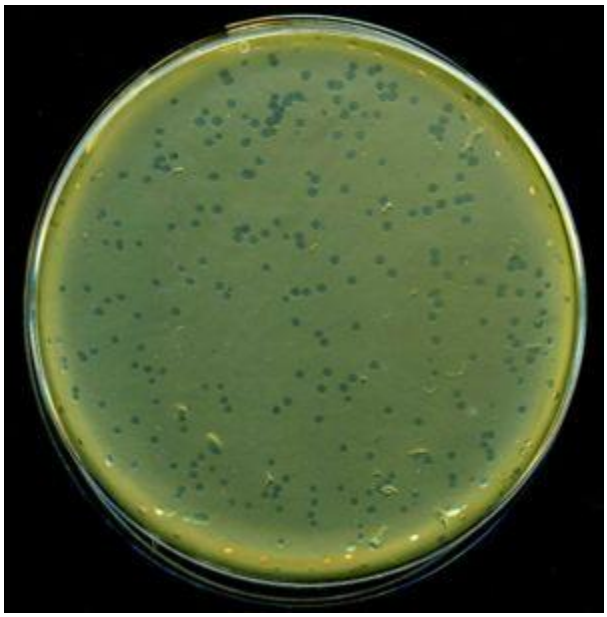

Figure 3. Lysis plaques of lambda phage on *Escherichia coli* bacteria.
(taken from Wikipedia 2007a)

The first clinical studies of phage therapy began in 1919 when d'Herelle used a phage preparation to treat a twelve-year old boy suffering from dysentery. The success was rapidly evident when the boy began to improve within 24 hours after one dose of the phage, and completely recovered within a few days. Subsequently, three more patients suffering from dysentery were treated and began to improve within 24 hours after administration of one dose. In 1921, Richard Bruynoghe and Joseph Maisin reported successful treatment of staphylococcal skin lesions using bacteriophage. The phage was injected into and around the open skin lesions and subsequent improvements in patient conditions were noticed after 24 to 48 hours (Sulakvelidze, et al. 2001).

Types of Bacteriophage

In 2005, United States Environmental Protection Agency (US EPA) estimated that approximately 1,030 different phages existed throughout the world. These bacteriophages have been characterized into several distinct families (Table 1). Understanding the family to which a phage belongs can provide valuable information regarding shape, genetic material, and similar phages. A commonly researched phage is T4 of the *Myoviridae* family; this phage infects *E. coli* bacteria. T4 phage contains more than 200 genes and its DNA genome is approximately 10μm in length (Snyder and Champness 2003). Nobel Prize winning scientists Max Delbrück, Alfred Hershey, Salvador Luria, Francis Crick, and James D. Watson used phage T4 in their research. According to Snyder and Champness (2003) an advantage of analyzing T4 replication is that rather than using the host cell replication proteins, T4 encodes many of its own including "DNA polymerase, sliding clamp, clamp-loading proteins, primase, replicative helicase DNA ligase, etc." The characterization of phage is especially useful to researchers developing new industrial phage products or applying the genetic information to understand the phage mechanism.

Table 1. Chart of the twelve distinct groups of bacteriophage

Family or Group:	Genera:	Type Member:	Particle Morphology:	Envelope:	Genome:
Corticoviridae	*Corticovirus*	PM2	isometric	No	supercoiled d/s DNA
Cystoviridae	*Cystovirus*	Ø6	isometric	Yes	3 segments d/s RNA
Inoviridae	*Inovirus*	coliphage fd	rod	No	circular s/s DNA
	Plectrovirus	Acholeplasma phage			
Leviviridae	*Levivirus*	coliphage MS2	icosahedral	No	1 (+)strand RNA
	Allolevirus	coliphage Qbeta			
Lipothrixviridae	*Lipothrixvirus*	Thermoproteus phage 1	rod	Yes	linear d/s DNA
Microviridae	*Microvirus*	coliphage ØX174	icosahedral	No	circular s/s DNA
	Spirovirus	Spiroplasma phages			
		Mac-1 phage			
Myoviridae		coliphage T4	tailed phage	No	linear d/s DNA
Plasmaviridae	*Plasmavirus*	Acholeplasma phage	pleiomorphic	Yes	Circular d/s DNA
Podoviridae		coliphage T7	tailed phage	No	linear d/s DNA
Siphoviridae	lambda phage group	coliphage lambda	tailed phage	No	linear d/s DNA
Sulpholobus shibatae virus		SSV-1	lemon-shaped	No	circular d/s DNA
Tectiviridae	*Tectivirus*	phage PRD1	icosahedral	No	linear d/s dna

Source: taken from Tulane University 1999

Phage Production on a Commercial Scale

D'Herelle had a commercial laboratory in Paris, France that produced five phage preparations in the early 1900's. In the United States, Eli Lilly produced seven phage

preparations during the 1940's. However, once mass production of penicillin began in the 1940's, the focus of the medical field shifted from phage therapy to antibiotic therapy. The high, exclusive usage of antibiotics contributed to the drug resistant bacteria of today.

Currently, several companies conducting phage research exist throughout the world. The most well-known phage therapy research and treatment center is the Phage Therapy Center located in Tbilisi, Republic of Georgia in the former Soviet Union. Phage research has been ongoing in Republic of Georgia for over 80 years. Currently the Phage Therapy Center (2005) can provide phages against many gram-positive bacteria including *Staphylococcus* spp. such as Methicillin Resistant *Staphylococcus aureus* (MRSA), *Streptococcus* spp., *Pseudomonas aeruginosa*, *Enterococcus* spp., and numerous gram-negative bacteria such as *Proteus, E. coli,* and *Klebsiella pneumoniae.* The focus of the Phage Therapy Center is primarily on patients dealing with an infection that is chronic, located in an area with poor circulation, or caused by a bacterium that is antibiotic resistant. Phage International Inc. is located in California, United States and was merged with Phage Therapy Center in 2005.

GangaGen, Inc. (2007) is a biotechnology company based in India that is currently researching phage therapy in the areas or recurring urinary tract infections and prophylactic therapy for patients entering a hospital setting. Biophage Pharma, Inc. (2006) is a biopharmaceutical company in Canada that is developing therapeutic and diagnostic phage-based products. Several other companies, primarily in the United States, are focusing their phage research on food products in order to prevent food-borne illness prior to infection of the victim.

Benefits of Phage Use

Numerous benefits of phages over antibiotics exist. Notable examples include killing of the target bacteria, whereas antibiotics sometimes only inhibit growth; very specific to target

bacteria so no adverse effects on beneficial microflora; lower risk of bacteria mutating to resist the phage (Table 2). According to Phage Biotech Ltd (2007), the cost of producing phage is low.

Table 2. Side-by-side comparison of the prophylactic and/or therapeutic uses of phages and antibiotics.

Bacteriophages	Antibiotics	Comments
Very specific (i.e., usually affect only the targeted bacterial species); therefore, dysbiosis and chances of developing secondary infections are avoided.	Antibiotics target both pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections.	High specificity may be considered to be a disadvantage of phages because the disease-causing bacterium must be identified before phage therapy can be successfully initiated. Antibiotics have a higher probability of being effective than phages when the identity of the etiologic agent has not been determined.
Replicate at the site of infection and are thus available where they are most needed .	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection.	The “exponential growth” of phages at the site of infection may require less frequent phage administration in order to achieve the optimal therapeutic effect.
No serious side effects have been described.	Multiple side effects, including intestinal disorders, allergies, and secondary infections (e.g., yeast infections) have been reported.	A few minor side effects reported for therapeutic phages may have been due to the liberation of endotoxins from bacteria lysed in vivo by the phages. Such effects also may be observed when antibiotics are used.
Phage-resistant bacteria remain susceptible to other phages having a similar target range.	Resistance to antibiotics is not limited to targeted bacteria.	Because of their more broad-spectrum activity, antibiotics select for many resistant bacterial species, not just for resistant mutants of the targeted bacteria.
Selecting new phages (e.g., against phage-resistant bacteria) is a relatively rapid process that can frequently be accomplished in days or weeks.	Developing a new antibiotic (e.g., against antibiotic-resistant bacteria) is a time-consuming process and may take several years.	Evolutionary arguments support the idea that active phages can be selected against every antibiotic-resistant or phage-resistant bacterium by the ever-ongoing process of natural selection.

Source: taken from Sulakvelidze 2001

Phages have an increased capability of killing bacteria that has become antibiotic resistant. Phages are beneficial over anti-microbial sanitizers because they target only one bacterium as opposed to a wide spectrum of bacteria. Traditional anti-microbial sanitizers tend to kill all bacteria exposed to them, and in turn bacteria mutate and build resistance to the sanitizer.

Safety

Phages are ubiquitous in the environment and may be found in soil, water, and food, making contact with these microorganisms inevitable. Bergh et al. (1989) determined concentration of phages to be up to 2.5×10^8 plaque forming units (pfu)/ml of natural unpolluted water. Research shows that the majority of known viruses do not have the ability to penetrate human tissues and cause disease (Omnilytics 2006). A historical study conducted by Whitman and Marshall (1971) proved that phages are found in refrigerated food products. Quantities and types of food products containing phage included:

- 11 of 17 total samples of ground beef
- 4 of 7 samples of pork sausage
- 4 of 8 samples of chicken
- 2 of 5 samples of raw milk

According to the USEPA (2005) in the Federal Register, as many as 1.4×10^6 pfu/g of the phage that targets *Propionibacterium freundenreichii* has been found in Swiss cheese.

Research conducted by Bruttin and Brussow (2005) at Nestle showed the apparent safety of oral administration of *E. coli* phage T4. Fifteen adult volunteers participated in a blind study and were given a low dose (10^3 pfu/ml), a high dose (10^5 pfu/ml), and a placebo in alternating sequences. The doses were administered in drinking water and no serious adverse effects were noted.

Danisco is a worldwide company that provides cultures for use in meat and yogurt products. Currently they are conducting research to determine how to control phages that are killing their cultures and resulting in reduced yields (Danisco 2007). In March 2007, a breakthrough was announced in which scientists at Danisco found the genomic avenue to

promote phage resistance in bacterial cultures. Therefore, consumers are already exposed to "good" bacteria and phages through products that utilize cultures.

Phage could possibly have an adverse effect on humans if used to treat a bacterium that contains toxin, since lysing of the cell would release the toxin. This scenario could occur during phage preparation using a bacterium that produces toxin, if any residue of the bacteria remained in the phage preparation.

Chapter 2: Common Food Pathogens

Bacteria are ubiquitous in the environment and all living things come into contact with these microorganisms daily. Pathogenic bacteria cause diseases in humans and plants, and directly affect the supply and quality of the food chain. There has been an increase in food-borne disease outbreaks in recent years. The majority of these outbreaks can be contributed to a select few pathogenic bacteria that will be discussed in this chapter.

Food-borne Human Pathogens

Numerous bacteria cause food-borne illnesses throughout the world every year. In the United States the Centers for Disease Control and Prevention (CDC) annually tracks reports of all outbreaks, their sources, bacteria involved, and states affected (Table 3). CDC defines an outbreak as "occurrence of two or more cases of a similar illness resulting from the ingestion of a common food" (MMWR 2006). In Europe the European Commission (EC) tracks food-borne outbreaks.

Table 3. Chart showing the number of reported bacterial food-borne disease outbreaks, cases, and deaths in the United States from 1998-2002

Etiology	Outbreaks	Cases	Deaths
Bacillus cereus	37	571	0
Brucella	1	4	0
Campylobacter	**61**	**1440**	**0**
Clostridium botulinum	12	52	1
Clostridium perfringens	130	6724	4
Escherichia coli	**140**	**4854**	**4**
Listeria monocytogenes	**11**	**256**	**38**
Salmonella	**585**	**16821**	**20**
Shigella	67	3677	1
Staphylococcus aureus	101	2766	2
Streptococcus	1	4	0
Vibrio cholerae	3	12	0
Vibrio parahemolyticus	25	613	0
Yersinia enterocolitica	8	87	0
Total	**1182**	**37881**	**70**

Source: adapted from MMWR 2006

CDC (2005a) declared that known pathogens account for 14 million illnesses, 60,000 hospitalizations, and 1800 deaths each year in the United States. The estimation of illnesses, hospitalizations, and deaths due to unknown pathogens or unreported cases is five times higher. Victim costs associated with food-borne outbreaks include hospitalization and other medical costs, lost wages from time off work, and psychological and physical harm. Estimated costs due to salmonellosis alone in the United States approaches $1 billion/year in lost wages and medical expenses (CDC 2005a).

According to Hartford (2002), company costs associated with recalls involving pathogenic organisms include:

- Product Disposition: Removing, returning and disposing of affected product
- Victim lawsuits

- Employees redirected to help with investigation and procedures regarding recall – costs may result from overtime of current employees or outsourcing
- Loss of consumer trust in product
- Damage to company image
- Possible closing of facility if unable to handle financial burden of recall

Listeria monocytogenes

Listeria spp. is a group of gram-positive bacilli that exist in the environment (Figure 4). *Listeria monocytogenes* is the most common strain associated with food-borne illness. *Listeria monocytogenes* is an opportunistic pathogen that has the ability to colonize the human gastrointestinal system and cause listeriosis. Symptoms of listeriosis may consist of nausea, diarrhea, fever, and/or muscle aches. *Listeria monocytogenes* is notorious for infecting pregnant women, which subsequently infects the fetus and frequently results in stillbirth or miscarriage. Immuno-compromised individuals, the elderly, and young children are susceptible to listeriosis. Ingestion of contaminated food is the most likely cause of infection with *L. monocytogenes*. *Listeria* is a psychrotrophic organism therefore easily killed at high temperatures such as 160^{o}F, but able to grow well at lower climates including refrigeration temperatures. Therefore, contaminated food typically results from unsanitary conditions during handling of food after it has been heat processed.

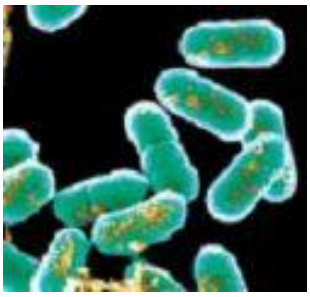

Figure 4. Microscopic image of a *Listeria* bacterium. (taken from Lancaster City Council 2005)

Due to the relatively high fatality rate associated with *L. monocytogenes*, the two United States food regulatory agencies, United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) and Food and Drug Administration (FDA), have imposed a zero tolerance limit for *L. monocytogenes* in Ready-to-Eat (RTE) food products. Zero tolerance means that this pathogen cannot be found at detectable limits in a food.

Foods that are commonly contaminated with *L. monocytogenes* include meat and dairy products such as sliced deli meats and soft cheeses. CDC (2005b) estimates 2,500 people contract a serious form of listeriosis each year in the United States alone and of these, approximately 500 will die. Recent cases of *Listeria* related USDA-FSIS (2007a) recalls include:

- 2,768 pounds of RTE chicken from a Tennessee facility on June 29, 2007
- 6,907 pounds of RTE turkey products from Diestel Turkey Ranch in California on May 1, 2007
- 2.8 million pounds of Oscar Mayer and Louis Rich chicken products from Carolina Culinary Foods in South Carolina on February 28, 2007.

Recent USFDA (2007a) recalls involving *Listeria* include one initiated on June 19, 2007 for one lot of diced yellow onions produced by Gills Onions, LLC in California. The product was distributed to six states, and no illnesses were reported as a result of this recall. Raw milk is frequently associated with recalls due to *Listeria monocytogenes.* In the first half of 2007, three cases of recalled raw milk from three separate dairies were reported (USFDA 2007b).

Europe has its own recalls and cases of listeriosis that are of concern (Table 4). The Commission Regulation of the European Communities has established microbiological criteria guidelines for

Table 4. Chart of the observed cases of listeriosis by European country in recent years.

Country	Year	Observed Cases
Austria	2000	14
Belgium	1999	26
	2000	48
Denmark	2000	6
	2001	38
England and Wales	2000	81
	2001	144
Finland	2001	29
France	2000	148
	2001	187
Germany	2001	220
Greece	2001	3
Iceland	2001	0
Ireland	2001	6
Italy	1999	40
	2001	31
Netherlands	2000	26
	2001	17
Norway	2000	11
	2001	17
Scotland	2001	15
Spain	2000	60
Sweden	2001	67
Switzerland	2000	54

Source: adapted from de Valk et al. 2005

food products in Europe (Eur-Lex 2005). Regarding *L. monocytogenes*, the limits vary depending upon the type of food tested. For RTE foods intended for use by infants or for medical purposes, there is a zero tolerance level. For foods that are able to support growth of *L. monocytogenes*, there is a zero tolerance level unless the manufacturer can prove to the

regulatory agency that the product can maintain a level of 100 cfu/g or less throughout the entire shelf life of the product. For food products that do not readily support growth of *L. monocytogenes*, the tolerance limit is less than 100 cfu/g.

Escherichia coli O157:H7

Escherichia coli are a group of gram-negative enteric bacteria that are commonly found in animals (Figure 5). Some strains exist as normal microflora of the human bowel, but others produce toxins resulting in serious infections. *E. coli* O157:H7 is an enterohemorrhagic *E. coli* (EHEC) that produces a toxin that can cause bloody diarrhea and severe dehydration in humans. According to O'Flynn et al. (2004) infections with *E. coli* O157:H7 may be caused by as few as 10 cells. If the infection is severe enough, *E. coli* O157:H7 causes damage to the kidneys and leads to hemolytic-uremic syndrome (HUS), which is most common in young children, and may result in death. Foods commonly associated with *E. coli* O157:H7 contamination include raw or undercooked ground beef, raw milk, and most recently, fresh produce. Cooking meat to recommended temperatures and complete pasteurization of milk are effective means to kill *E. coli* O157:H7. The most famous report of *E. coli* O157:H7 contamination in food was related to ground beef used in hamburgers at Jack-in-the-Box in 1993. The contaminated hamburgers in question were not cooked to a temperature sufficient to kill *E. coli* O157:H7. The outbreak prompted an increase in testing for *E. coli* O157:H7 at USDA inspected meat establishments, as well as raising awareness of the need to cook ground meat to appropriate temperatures at food service establishments and in the home.

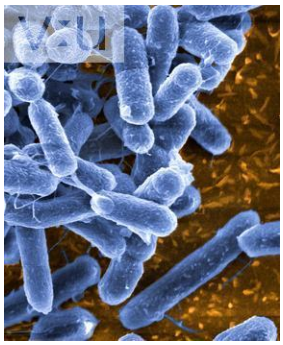

Figure 5. Microscopic image of *Escherichia coli* O157:H7. (taken from Goshen College 2005)

An outbreak of *E. coli* O157:H7 associated with Natural Selections baby spinach in fall of 2006 resulted in 205 confirmed infections and three deaths (USFDA 2007c). Recent cases of USDA-FSIS (2007a) recalls involving *E. coli* O157:H7 contaminated products include:

- 40,440 pounds of ground beef from a Tyson plant in Texas on June 8, 2007
- 26,669 pounds of ground beef from Abbot Meats in Michigan on July 21, 2007
- 5,920 pounds of ground beef and buffalo from Nebraska based Custom Pack, Inc. on July 25, 2007
- 21.7 million pounds of frozen ground beef patties from Topps Meat Company, LLC in New Jersey; initial recall on September 25, 2007 was expanded on September 29, 2007 (USDA-FSIS 2007b).

One recall that gained an abundance of media coverage involved Taco Bell restaurants in the northeastern United States in 2006. CDC (2006a) believed this case was linked to the lettuce used in the restaurants, and caused illnesses in 71 individuals, of which eight developed HUS. The recent Topps Meat recall was the second largest beef recall in United States history, and 25 illnesses were reported in eight states according to the FSIS Recall Release (USDA-FSIS 2007b).

The massive recall forced the company to permanently close its doors six days after the recall was expanded (Gold 2007).

In Europe, the EC monitors verocytotoxigenic *E. coli* (VTEC) that includes *E. coli* O157. EC has defined several key areas of foods where VTEC poses a particular threat to human health including "raw or undercooked beef and possibly meat from other ruminants; minced and/or fermented beef, and products thereof; raw milk and raw milk products; fresh produce, in particular sprouted seeds, and unpasteurised fruit and vegetable juices, and water" (EC 2003a). Currently, the EC (2003a) does not recommend any appropriate set of microbiological levels for VTEC O157 in foods.

Salmonella

Salmonella spp. is a group of gram-negative enteric bacteria commonly found in animals (Figure 6). *Salmonella* is typically not part of the normal human flora so presence in humans is usually indication of an infection. Certain individuals are carriers of *Salmonella*, which means they do not get sick from the bacteria but they can transmit the bacteria to other people and cause illness. Gastroenteritis, including diarrhea, fever, and abdominal cramps, is a common symptom of infection resulting from ingestion of contaminated foods such as poultry or dairy products. *Salmonella* is easily killed by cooking food to proper temperatures and pasteurizing milk. According to the CDC (2006b), approximately 40,000 cases of *Salmonella* are reported every year in the United States. This number includes only the reported cases, though the actual amount of cases including minor, unreported cases might be much higher. According to the EC (2003b), there were 150,165 cases of human salmonellosis in 17 European regions in the year

2000. According to the Commission Regulation of the European Communities microbiological criterion on *Salmonella*, there is a zero tolerance level across all types of foods (Eur-Lex 2005).

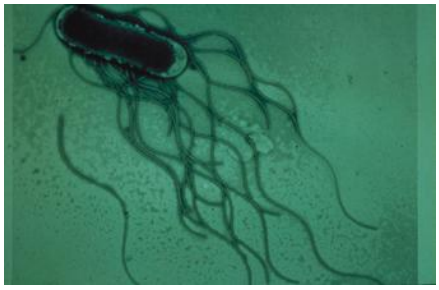

Figure 6. Microscopic image of a *Salmonella* bacterium. (taken from Utah Department of Health 2006)

One recent outbreak of *Salmonella* resulted from peanut butter distributed throughout the United States (USFDA 2007d). ConAgra, a major worldwide food manufacturer, produced the peanut butter under the brand names Peter Pan and Great Value. As of March 7, 2007, CDC had received reports of 425 cases of *Salmonella* infections, involving 44 states, caused by the affected peanut butter. On June 28, 2007, FDA reported that Veggie Booty snacks made by Robert's American Gourmet in New York were being recalled due to *Salmonella* contamination. CDC (2007) identified 60 individuals who became sick associated with this product. People from 19 states were affected and most of the victims were young children. In the first half of 2007, one case of recalled raw milk was associated with *Salmonella* contamination (USFDA 2007b). Major European countries reported numerous cases in recent years; the EC summarized the major outbreaks in 2003 (Table 5).

Table 5. Chart of the major foodborne outbreaks of human salmonellosis by food, year, countries affected, serotype, and cases.

Vehicle	Year	Exporting Country	Importing Country	Serotype	Cases	Deaths
Chocolate	1973	Canada	USA	*Salmonella Eastbourne*	122	0
	1982	Italy	UK	*Salmonella Napoli*	245	0
	1985-86	Belgium	Canada	*Salmonella Nima*	29	0
	1987	Norway	Finland	*Salmonella Typhimurium*	12	0
Alfalfa Sprouts	1994	Australia	Finland & Sweden	*Salmonella Bovismorbificans*	492	0
	1995	Netherlands	USA & Finland	*Salmonella Stanley*	>230	0
	1995-96	Netherlands	Canada & USA	*Salmonella Newport*	150	0
Black Pepper	1982	Brazil	Norway	*Salmonella Oranienburg*	126	1
Pate	1984	France	UK	*Salmonella Gold-coast*	506	0
Aspic Glaze	1984	UK	International	*Salmonella Enteritidis PT4*	766	2
Mung Bean Sprouts	1988	Australia	England	*Salmonella Saint-paul*	143	0
Mustard Cress	1989	Netherlands	England	*Salmonella Gold-coast*	14	0
Cantaloupe	1989-90	Mexico	USA	*Salmonella Chester*	>245	2
Infant Milk Formula	1996	France	UK	*Salmonella Anatum*	>12	0

Source: taken from EC 2003b

Campylobacter jejuni

Campylobacter jejuni is a gram-negative bacterium that causes diarrhea in humans (Figure 7). CDC (2005c) estimates 2.4 million individuals are infected each year in the United States, of which approximately 124 cases result in death of the victim. *C. jejuni* causes an infection known as campylobacteriosis, which is the most common cause of diarrhea due to bacterial infection in the United States (USDA-FSIS 2006a). *Campylobacter* infections can be caused by as few as 400-500 cells (Hagens and Loessner 2007). *Campylobacter jejuni* is found as part of the normal flora in most live chickens and therefore can be found in an estimated 20-100% of raw poultry available in retail (USFDA-CFSAN 1992).

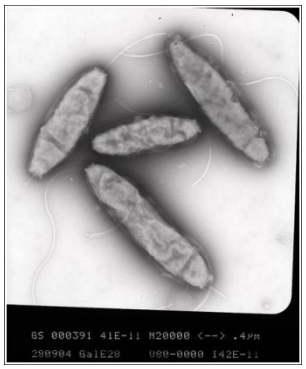

Figure 7. Microscopic image of *Campylobacter jejuni.* (taken from National Research Council Canada 2005)

In the United States, *C. jejuni* is not normally associated with outbreaks and infections are usually sporadic, however a small amount of outbreaks have been reported due to drinking of non-chlorinated water and raw milk. One *Campylobacter* related outbreak was reported among school children in the United States in 1986. USFDA-CFSAN (1992) reported that the company supplying the milk was not using proper time and temperature requirements to pasteurize the milk. Most infections associated with these bacteria can be avoided with proper cooking of meat and poultry, or pasteurization of milk.

According to USFDA-CFSAN (2001a), pasteurization of milk consists of several methods:

- High-Temperature-Short-Time (HTST): heat at 161^{o}F (72^{o}C) for 15 seconds
- Low-Temperature-Long-Time (LTLT): heat at 145^{o}F (63^{o}C) for 30 minutes
- Ultra-High-Temperature (UHT): heat at 230^{o}F (138^{o}C) for a minimum of 2 seconds; this extends the refrigerated shelf life 60-90 days
- Ultrapasteurization: heating at 280-302^{o}F (138-150^{o}C) for 1 to 2 seconds; if the milk is then hermetically sealed in a sterile package, this extends the shelf life to unrefrigerated for 90 days.

The EC reported approximately 156,232 cases of human campylobacteriosis in 13 member states in the year 2001, though the actual number of cases is estimated to be from 8-10 times higher (European Food Safety Authority 2005).

Plant Crop Pathogens

Several plant pathogens harm crops and cause serious financial trouble to farmers. University of Georgia (1998) reported, "crop disease cost Georgia farmers more than $654 million, or about 20 percent of the $3.25 billion total crop value" in 1997. Though these bacteria may not be directly harmful to humans as a pathogen, they affect the quality and cost of food in the human food chain.

Xanthomonas campestris pv. *vesicatoria*

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) causes bacterial spot in tomatoes and pepper plants (Figure 8). Bacterial spot was first noticed in Texas in 1912 and is now widespread throughout the North and South America, Europe, Asia, and Africa (University of Connecticut 1999). *Xcv* is easily spread and causes water-soaked, circular, brown spots on the leaves of the plants (University of Massachusetts Amherst 2005a). Seeds are a primary source of dispersal of *Xcv*, and prime conditions for growth of the bacteria include rainfall and temperatures between 80-90^{o}F. Current treatment recommendations include buying certified seed, rotating fields, and use of chemicals or pesticides. However, pesticides are typically ineffective against *Xcv* if a significant amount of disease is present.

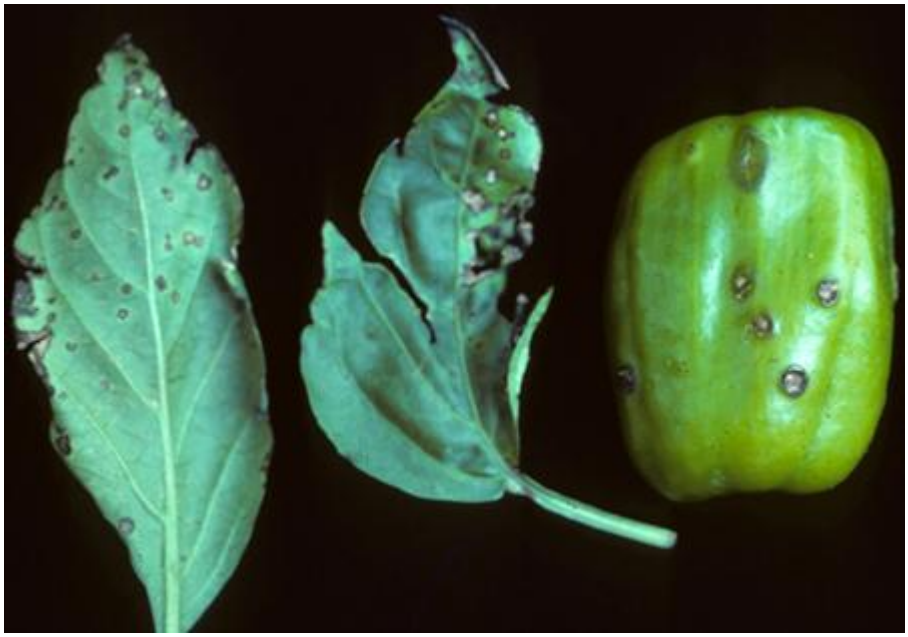

Figure 8. Effects of *Xanthomonas*-causing bacterial spot on pepper fruits and leaves. (taken from University of Massachusetts Amherst 2007)

Pseudomonas syringae pv. *tomato*

Psuedomonas is a species of bacteria that are gram negative. *Psuedomonas syringae* is pathogenic to numerous plants and their fruits. *Pseudomonas syringae* pv. *tomato* causes bacterial speck on tomato fruits. Bacterial speck was first noted in Taiwan in 1929 and in the United States in 1933 (University of Connecticut 1999). Optimum growth conditions for this bacteria include low temperatures of 64-75^{o}F and high moisture environments (University of Connecticut 1999). The bacterium causes small, dark spots or specks on the tissue of the fruit (University of Massachusetts Amherst 2005b). Current treatment recommendations include use of disease-free seeds and chemicals or pesticides. However, pesticides are typically ineffective against *Pseudomonas.*

Chapter 3: Current Methods for Control of Bacteria in Food

Sanitizers, lethality treatments, and pesticides are common tools currently used to combat pathogens in the food industry. Each method is regulated by government agencies FDA, USDA, and/or EPA for compliance in areas of concentration, exposure time, and toxicity. This chapter will discuss use of these current methods as antimicrobials on food products and equipment in the food industry.

Sanitizers

Numerous sanitizers are used in production facilities to prevent and control pathogenic bacteria. Quaternary ammonium (quat) is a commonly used chemical that is approved for usage in food production facilities (Figure 9). Quat is a cationic surface acting agent commonly used as a microbial sanitizer. Quat acts on the cell membrane of bacteria, and causes loss of structural organization of the target cell (McDonnell and Russell 1999). One example of a liquid quat sanitizer is Saniwise Six, manufactured by Supply Systems for use in USDA inspected establishments. Saniwise Six contains the four active ammonium ingredients octyl decyl dimethyl ammonium chloride, didecyl dimethyl ammonium chloride, dioctyl dimethyl ammonium chloride and alkyl dimethyl benzyl ammonium chloride, hence the term "quaternary ammonium" (Supply Systems 2003a). At strength of 200 ppm exposure for 60 seconds, Supply Systems (2003a) claims Saniwise Six eliminates 99.999% of bacteria such as *C. jejuni, E. coli* O157:H7, *L. monocytogenes*, and various *Salmonella* species, among other pathogens. Quat compounds may cause eye and skin irritation upon contact and are harmful if swallowed (Supply

Systems 2003b). USDA-FSIS (2006b) claims quat, chlorine compounds, and compounds containing periacetic acid are most effective against *L. monocytogenes*. USDA-FSIS requires all sanitizers used in federally inspected meat facilities be food-grade and approved by FDA. FDA defines sanitizers as "chemical or physical agents that reduce microorganism contamination levels present on inanimate environmental surfaces" (USFDA-CFSAN 2001b). According to FDA, sanitizers are for use on inanimate objects that indirectly touch food, and not for use on the actual food itself. Therefore, the equipment will not contaminate the food. FDA details approved sanitizer solutions and concentrations in 21 CFR 178.1010 (US Government Printing Office 2000). USDA requires federally inspected establishments to have procedures for sanitizer use on file. These are known as Sanitation Standard Operating Procedures (SSOPs). Establishments are also required to keep records of all sanitizers used and strengths. Often, sanitizers must be rotated on a regular basis to discourage bacterial resistance.

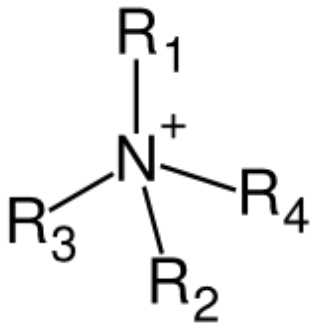

Figure 9. Diagram of a quaternary ammonium ion in which R represents alkyl groups.
(taken from Wikipedia 2007b)

Lethality Treatments

USDA has strict guidelines that its federally inspected establishments are required to follow in meat preparation. Requirements include combinations of time and temperature depending upon the type of meat (beef, pork, or poultry) and the type of finished product (Table 6). USDA requires inspected establishments to have a validated Hazard Analysis and Critical Control Point (HACCP) plan in effect that addresses cooking time and temperature requirements and keep records that show proof of those requirements being met. USDA has established guidelines for consumers when handling and cooking raw products, though a large majority of the population is still unaware of correct handling techniques for raw meat and vegetables, as well as cooking times and temperatures. USDA-FSIS (2006c) recommends consumers wash hands often when preparing foods, keep foods separate to prevent cross-contamination, cook foods to proper internal temperatures, and chill the food immediately.

Table 6. Processing temperatures for meeting USDA lethality performance standards for meat products.

Minimum Internal Temperature		Minimum processing time in minutes or seconds after minimum temperature is reached	
Degrees Fahrenheit	Degrees Centigrade	6.5-$\log_{10}$ Lethality	7-$\log_{10}$ Lethality
130	54.4	112 min.	121 min.
131	55.0	89 min.	97 min.
132	55.6	71 min.	77 min.
133	56.1	56 min.	62 min.
134	56.7	45 min.	47 min.
135	57.2	36 min.	37 min.
136	57.8	28 min.	32 min.
137	58.4	23 min.	24 min.
138	58.9	18 min.	19 min.
139	59.5	15 min.	15 min.
140	60.0	12 min.	12 min.
141	60.6	9 min.	10 min.
142	61.1	8 min.	8 min.
143	61.7	6 min.	6 min.
144	62.2	5 min.	5 min.
145	62.8	4 min.*	4 min.*
146	63.3	169 sec.	182 sec.
147	63.9	134 sec.	144 sec.
148	64.4	107 sec.	115 sec.
149	65.0	85 sec.	91 sec.
150	65.6	67 sec.	72 sec.
151	66.1	54 sec.	58 sec.
152	66.7	43 sec.	46 sec.
153	67.2	34 sec.	37 sec.
154	67.8	27 sec.	29 sec.
155	68.3	22 sec.	23 sec.
156	68.9	17 sec.	19 sec.
157	69.4	14 sec.	15 sec.
158	70.0	0 sec.**	0 sec.**
159	70.6	0 sec.**	0 sec.**
160	71.1	0 sec **	0 sec.**

* Past regulations have listed the minimum processing time for roast beef cooked to 145°F as "Instantly." However, due to their large size, most of these roasts dwell at 145°F, or even at higher temperatures, for at least 4 minutes after the minimum internal temperature is reached. FSIS has revised this time/temperature table to reflect this and emphasizes that, to better ensure compliance with the performance standard, establishments should ensure a dwell time of at least 4 minutes if 145°F is the minimum internal temperature employed.

**The required lethalities are achieved instantly when the internal temperature of a cooked meat product reaches 158°F or above.

(Source: taken from USDA-FSIS Appendix A 1999)

Pesticides

Pesticides are defined by EPA (2007a) as "substance or mixture of substances intended for:

- preventing,
- destroying,
- repelling, or
- mitigating any pest"

to include insecticides, herbicides, fungicides, and any other product that controls pests. Pesticides are typically used on crops and EPA regulates use of pesticides in the United States. In 2006, EPA implemented a new program known as registration review, to ensure review of a pesticide's active ingredients every 15 years (EPA 2007b). EPA has other programs in place including re-registration, tolerance reassessment, and special review. EPA is very strict in their testing requirements for a pesticide to become approved for use. Key areas tested include hazards to humans and domestic animals, environmental toxicity, residues, and accumulation studies, as well as product performance studies to assess effectiveness (EPA 2007c). Once approved, levels are tightly regulated and enforced to ensure that guidelines are met and toxicity to animals, humans, or the environment is kept at a minimum. According to Iowa State University (2003) pesticides have advantages and disadvantages.

Advantages include:

- effective against a broad range of pests
- potential for rapid action
- lower cost compared to other methods

Disadvantages include:

- potentially hazardous to applicator and environment
- repeated exposure can lead to pest resistance
- dependence requires annual costs
- potential to cause harm to beneficial pests
- potential to contaminate groundwater
- potential adverse health effects from long term exposure to pesticides

Other Antimicrobial Methods

Rivera (2005) in his report "A review of chemical disinfection methods for minimally processed leafy vegetables" discussed chemical antimicrobial methods. In his review of intervention strategies he discussed methods including ozone treatments, wash solutions, organic acid treatments, irradiation, and modified atmosphere packaging (MAP). Other current methods in use for reduction of microorganisms in food include use of antioxidants in processed foods, and dehydration to reduce water activity.

Chapter 4: Effectiveness of Bacteriophage on Foods

Bacteriophages exhibit beneficial effects when used on foods. Research has been conducted to prove the effectiveness. This chapter will discuss the benefits of bacteriophage as well as research in the area of bacteriophage effects when used on food products.

Benefits of Bacteriophages

Bacteriophages can be applied directly to food and be eaten by the consumer with no harm to the properties of the food or to the human (USFDA-CFSAN 2006a). Whereas sanitizers and pesticides typically consist of chemicals that are closely regulated by United States government under 21 CFR 178.1010 to prevent overuse and adulteration in foods (US Government Printing Office 2000). Bacteriophages are specific for a particular pathogen, so phage could be selected based on the type of food and the environment to which the food was exposed. For example, a phage specific for *E. coli* O157:H7 could be used on raw ground beef to ensure that mishandling or undercooking does not contribute to a human infection. *Listeria* specific phage could be used on RTE foods after a lethality process and subsequent packaging where pathogens such as *Salmonella* and *E. coli* O157:H7 are not an issue. *Campylobacter* and *Salmonella* specific phage (Figure 10) could be used on poultry products and possibly be adapted for use in milk products in the future. The specificity of bacteriophage would decrease chances of bacterial mutations for resistance, as is common with non-specific sanitizers. If bacteria mutate to resist the bacteriophage, the phage will in turn mutate to continue to infect the bacterial host. Phage need their bacterial host to survive therefore mutation alongside the bacteria is crucial to the phage's existence.

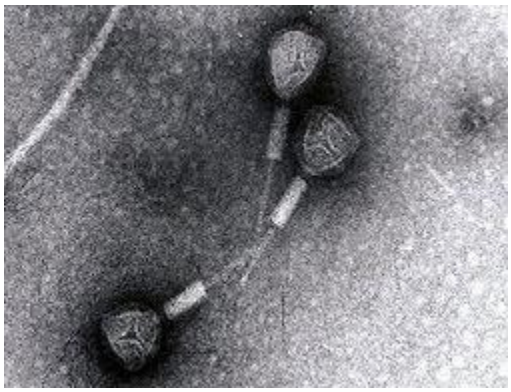

Figure 10. Electron micrograph of a *Salmonella* phage isolated from the Inner Harbor in Baltimore, Maryland (K9514-5).
(taken from USDA-ARS 2005)

Research

Various experiments were conducted to determine effectiveness of phage use both preharvest and postharvest. Preharvest measures in animals include experiments attempting to control or reduce *Campylobacter* in broiler chickens (Wagenaar et al. 2005). Bacteriophages 69 & 71 from the National Collection of Type Cultures (NCTC) in the UK were used in this experiment because they are both lytic for *Campylobacter jejuni*. Results showed that treatment with phage reduced *Campylobacter* colonization in the broiler chickens to levels ten times lower than the control group, and no adverse side effects were noted in the chickens. A significant decline occurred immediately after phage administration, but stabilized thereafter. This could be a beneficial effect if used on broiler chickens just prior to slaughter, since rates of *Campylobacter* would possibly be at the lowest levels. A study by Atterbury et al. (2005) showed a significant reduction in *Campylobacter* in broiler chickens when bacteriophage was present. In fact, *Campylobacter* was undetectable in 71% of the phage-containing chickens.

Raya et al. (2006) discovered a phage, CEV1, in sheep that is effective against 17 of 19 tested strains of *E. coli* O157:H7. CEV1 is similar to phage T4, which was studied extensively and used safely in numerous applications for at least 50 years (Raya et al. 2006). The new phage

could be used to treat sheep preharvest. O'Flynn et al. (2004) used a cocktail of three phages (e11/2, e4/1c, pp01) on meat to determine effectiveness against *E. coli* O157:H7. The phages effectively lysed 12 of the 14 strains of *E. coli* O157:H7 against which they were tested. After contaminated meat was treated with the phage, 7 of the 9 samples had undetectable levels of *E. coli* O157:H7 (O'Flynn et al. 2004). The two remaining samples were at the low end of the threshold at <10 cfu/ml. O'Flynn et al. (2004) believe the phage cocktail could be useful as a control measure for *E. coli* O157:H7 during slaughter.

Research relating to company specific phage will be discussed in the next chapter.

Chapter 5: Commercially Available Products

Phage therapy in humans is a proven and common treatment for bacterial infections in areas of the world such as Russia. Several companies throughout the United States and Europe are focusing research on use of phages as antimicrobials in food. Some companies have government approvals on their products and are researching other product lines as well. The companies conducting research and developing products for use on foods will be discussed in this chapter.

Intralytix, Inc.

Intralytix, Inc. is a phage research facility located in Baltimore, Maryland in the United States. Intralytix, Inc. was founded in 1998 to "address growing problems in the control and treatment of disease-causing bacteria" (Intralytix 2006b). Currently, their focus is on use of phage to enhance food safety, sanitation, and as therapy for bacterial diseases in humans and

animals. LMP-102™ is a phage preparation produced by Intralytix that targets *Listeria monocytogenes*, and consists of six bacteriophages that are found naturally occurring in the environment. LMP-102™ is manufactured using *L. monocytogenes* as the host, but the phages are later purified and no residue of the strain remains in the finished product. LMP-102™ is currently targeted for use on RTE products, and does not contain any preservatives or allergens. LMP-102™ does not alter the taste, color, or odor of the meat product (Intralytix 2006c). According to Intralytix, Inc. (2006c) internal research, when used on foods contaminated with *L. monocytogenes*, LMP-102 ™ is effective in reducing the bacterial load 100-1000 fold. LMP-102™ is also available for use on food processing equipment.

ECP-100 ™ is a phage preparation similar to LMP-102™ except that it targets *E. coli* O157:H7 (Intralytix 2006c). ECP-100 ™ is currently in the final testing stages and Intralytix, Inc. predicts effective use on ground beef, fruits, and vegetables.

Intralytix, Inc. has licensed phage preparations for veterinary use preharvest in animals intended for use in the human food chain. Currently, they have three products licensed for use with poultry including SPLX-1™ and PLSV-1™ against *Salmonella*, and INT-401™ against *Clostridium perfringens* (Intralytix 2006d). Currently under development is a phage targeting *E. coli* O157:H7 for use in cattle preharvest.

Phage SCPLX-1 contains four lytic phages specific for *Salmonella Enteritidis*. Leverentz et al. (2001) studied the phage cocktail effectiveness on apples and honeydew melons. *Salmonella* was reduced as much as 3.5 logs on the honeydew melons, whereas no significant difference was observed between the control apples and the apples treated with phage.

LMP-102 ™ has been tested on produce including red delicious apples and honeydew melons. Leverentz et al. (2003) showed a significant decrease of *L. monocytogenes* on

honeydew melons when phage was applied, but minimal decrease on the apple slices. In both the *Salmonella* and *Listeria* experiments, the researchers believed that the apple slices showed little to no reduction in target bacteria and decreased levels of phage titers due to the acidity of the apples (typical pH 4.2).

EBI Food Safety

EBI Food Safety is a phage research facility located in Wageningen, The Netherlands. EBI Food Safety's focus is on use of bacteriophage in foods, and one goal of the company is for use of bacteriophage as an antimicrobial to become a food industry standard (EBI Food Safety 2006b). Phage P100 was originally isolated from a wastewater sample taken from a dairy plant in Germany in 1997 (Carlton et al. 2005). Listex^{TM} P-100 (Figure 11) is a phage preparation derived from Phage P100 that targets *Listeria monocytogenes* and a few other species of *Listeria* in foods without affecting flavor, taste, or odor. EBI Food Safety (2006b) claims 100% effectiveness when Listex^{TM} P-100 is brought in contact with the host strain. Listex^{TM} P-100 is cultivated using *Listeria innocua* as the host.

Figure 11. Picture of a Listex^{TM} P100 product bottle. (taken from EBI Food Safety 2006a)

Carlton et al. (2005) conducted a study on P100 to determine the effectiveness against *L. monocytogenes* on food and its toxicity to animals. The toxicity study was performed using rats, and the food of choice was surface-ripened red smear cheese also known as Muenster cheese. The toxicity study consisted of a test group of ten rats given high doses (5×10^{11} pfu/ml) of P100 suspended in phosphate buffered saline orally for five days. A control group of ten rats was given a placebo orally for five days. Results of short-term phage use showed no evidence of changes in behavior, physical attributes, or signs of toxicity in the group given P100 (Carlton et al. 2005). However, additional research is needed to determine long-term effects of toxicity related to phage use.

The cheese used in the study was inoculated at a known level (2×10^{1} cfu/cm^2) with *L. monocytogenes*, and subjected to either a repeated high dose or a low dose of P100, or a single high dose of the bacteriophage (Carlton et al. 2005). According to this study, effects of P100 on the target bacteria were dose-dependent. A repetitive low dose concentration of 1.5×10^{8} pfu/ml

obtained a 2-3 log decrease in the levels of *Listeria*. When a repeated high dose concentration of 3 x 10^9 pfu/ml was used, results indicated absence of *Listeria*. This was further confirmed by culturing the cheese using a selective enrichment and plating for *Listeria*, in which no presence of the target bacteria was found. When a single high dose (6 x 10^8 pfu/ml) was used, a uniform distribution of P100 was achieved on the surface of the cheese, and *Listeria* cells were undetectable (Figure 12).

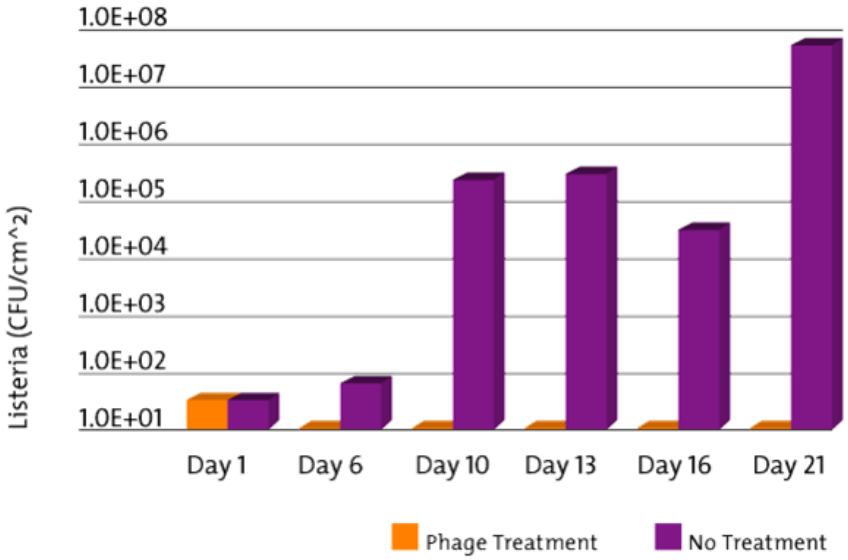

Figure 12. Graph showing Listex™ P100 can irradicate *Listeria* in cheese.
The graph demonstrates recent results of successful eradication of *Listeria* in a cheese production environment. Day 0, *Listeria monocytogenes* inoculation 7 CFU/ cm^2 cheese surface (taken from EBI Food Safety 2006a).

The Chief Scientific Officer at EBI Food Safety, Dr. Steven Hagens, conducted a study on the effectiveness of Listex™ P-100 on fish fillets and found that *Listeria monocytogenes* was drastically reduced (EBI Food Safety 2007a). When used on fish fillets, EBI recommends dipping or spraying the fillets immediately following the filleting process.

Omnilytics, Inc.

Omnilytics, Inc. is a biotechnology company whose emphasis is on bacteriophage technology. Locations include Salt Lake City, Utah, United States with an official presence in Honduras, Ecuador, and El Salvador. One of their main focuses is the use of bacteriophage in agriculture settings such as on farm crops. AgriPhage™ is one of their products useful for controlling bacteria such as *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato,* among others that are known to cause bacterial spot and speck respectively in tomato and pepper plants. The active ingredient in AgriPhage™ is four billion phage per milliliter (Omnilytics 2004a). AgriPhage™ is meant to be used on farms at the preharvest level, and should be diluted prior to dispersal and applied using drip irrigation, ground or aerial spray equipment. A unique service that Omnilytics provides growers is to periodically receive and analyze field samples and based on the results, they tailor the formulation for that grower and particular crop.

Omnilytics has two products that target bacteria on animal hides prior to slaughter. Both products are termed BacWash™ and target *Salmonella* and *E. coli* O157:H7 (Omnilytics 2007a, b). BacWash™ can be applied as a wash, mist, or spray directly to the live animal. Future potential uses of the BacWash™ line of products include treating animal holding areas, transportation equipment and containers, and living areas.

Omnilytics has numerous other products under research in the areas of food and water, industrial, defense, pharmaceuticals, as well as a large variety of crops (Omnilytics 2004b). The main food safety areas of research include *E. coli* O157:H7, *Salmonella, Listeria, Campylobacter, and Pseudomonas aeruginosa* for RTE foods, water, and processing facilities. Areas of research for crops include bacteria such as *Burkholderia cepacia,* and various strains of

Xanthomonas, Erwinia, and Clavibacter for use on common fruit and vegetable crops (Table 6). Importance from a food safety viewpoint exists because many of these crops are typically harvested and sold to consumers in raw form without further processing in many cases. Though many of the target bacteria are not necessarily pathogenic to humans, they are harmful to the crops and an outbreak can result in reduced crop yield and therefore drive up prices for consumers, as well as diminishing the quality of the available crops. The potential exists to develop bacteriophage applications for use on crops against bacteria pathogenic to humans. Application in this area would be very beneficial as is evident from recent outbreaks of *E. coli* O157:H7 and *Salmonella* in fresh produce.

Table 7. Chart of Omnilytics current areas of bacteriophage research in agriculture.

Crop	Pathogen	Disease
Peach, Apricot, Cherry	*Xanthomonas campestris pv pruni*	Bacterial Leaf Spot
Citrus	*Xanthomonas campestris pv citrumelo*	Bacterial Leaf Streak
Citrus	*Xantomonas campestris pv citri*	Bacterial Canker
Walnut, Almond, Grape	*Agrobacterium tumefaciens*	Crown Gal
Walnut, Almond, Grape	*Xanthomonas campestris pv juglandis*	Walnut Blight
Apple, Pear	*Erwinia amylovora*	Fire Blight
Tomato	*Xanthomonas perforans*	Bacterial Leaf Spot
Pepper	*Xanthomonas euvesicatoria*	Bacterial Leaf Spot
Tomato	*Xanthomonas campestris pv vesicatoria*	Bacterial Leaf Spot
Strawberry	*Xanthamonas fragariae*	Angular Leaf Spot
Watermelon	*Acidovorax avenae subsp. Citrulli*	Fruit Blotch
Tomato	*Clavibacter michiganensis pv michiganensis*	Bacterial Canker
Onion	*Burkholderia cepacia*	Sour Skin and Bulb Rot
Lettuce	*Xanthomonas vitians*	Bacterial Leaf Spot
Cabbage	*Xanthomonas campestris pv campestris*	Blackrot
Potato	*Erwinia carotova*	Soft Rot
Snap Beans	*Xanthamonas campestris pv phaseoli*	Bacterial Blight
Cucumber, Squash, Pumpkins	*Pseudomonas syringae pv lachrymans*	Leaf Spot
Potato	*Clavibacter michiganensis pv sepedonicus*	Ring Rot
Geraniums	*Xanthomonas campestris pv pelargonii*	Bacterial Blight
Tobacco	*Ralstonia (Pseudomonas solanacearum)*	Bacterial Wilt

Source: adapted from Omnilytics 2004b

Luna Innovations, Inc.

Luna Innovations has six facilities located throughout the state of Virginia in the United States. Luna claims on their website to be currently working on bacteriophage solutions. Their work with food is in the research and development phase, as evidenced on the website http://www.lunainnovations.com/research/phages.htm and confirmed through email contact with an employee at the facility (Luna Innovations 2007).

Chapter 6: Government Approvals

United States and European government agencies have given approval or no objection to some of the phage products manufactured for use in foods and food products. The approvals and special status given to bacteriophage products for use in foods are discussed in this chapter.

United States Environmental Protection Agency

Omnilytics obtained final rule for EPA registration of AgriPhage™ product on December 28, 2005 (Table 8). A unique characteristic of this registration includes an exemption from the requirement of tolerance levels for the bacteriophage included in the product (USEPA 2005). The exemption means there is no maximum allowable level when using the product as a bactericide on tomato and pepper plants. EPA is only allowed to provide this exemption if the product is deemed 'safe.' According to Section 408(c)(2)(A)(ii) of the United States Federal Food, Drug, and Cosmetic Act (FFDCA) the definition of 'safe' means "there is a reasonable

certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information" (US EPA 2005). With this final rule, AgriPhage™ became the first phage-based product to be formally approved by any United States government regulatory agency.

United States Food and Drug Administration

On August 18, 2006 FDA announced approval of the use of bacteriophage as an antimicrobial food additive targeting *Listeria monocytogenes* on RTE meat and poultry products (USFDA CFSAN 2006a). The approval was based on a petition regarding LMP-102™ submitted by Intralytix, Inc. FDA based its approval of LMP-102™ on review of several safety factors including *L. monocytogenes* residues remaining from manufacture of the product, potential human infection with phage, and lytic versus lysogenic phages. FDA reviewed each of these areas with respect to LMP-102™ and concluded that no potential residues exist, no potential for human infection exists, and the phages used in the cocktail are lytic, not lysogenic. Therefore, FDA concluded use of LMP-102™ on RTE meat and poultry products is safe, when used at levels up to 1 ml/500 cm^2 on the surface of a food product immediately prior to packaging (USFDA CFSAN 2006a). Definitions of RTE meat products and control of *Listeria monocytogenes* on RTE meat products may be found in the Federal Code of Regulations in sections 9 CFR 430.1 and 430.4 respectively (GPO Access 2007).

9 CFR 430.1 defines:

> "***Antimicrobial agent.*** A substance in or added to an RTE product that has the effect of reducing or eliminating a microorganism, including a pathogen such as

L. monocytogenes, or that has the effect of suppressing or limiting growth of *L. monocytogenes* in the product throughout the shelf life of the product."

"Ready-to-eat (RTE) product. A meat or poultry product that is in a form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes. RTE product is not required to bear a safe-handling instruction (as required for non-RTE products by 9 CFR 317.2(l) and 381.125(b)) or other labeling that directs that the product must be cooked or otherwise treated for safety, and can include frozen meat and poultry products."

9 CFR 430.4 specifies approved methods for controlling *L. monocytogenes* on RTE meat products (GPO Access 2007). However, USDA-FSIS, not FDA, regulates the United States meat supply so this approval simply opens the door for USDA to approve LMP-102^{TM} for use on meat products. Until USDA evaluates and approves the product within its own agency, the product will not be permitted use on meat products under USDA inspection. Once USDA approves LMP-102^{TM}, users of the phage will be required to declare it as an ingredient on the label of the food product on which it was used.

On October 17, 2006 FDA approved Listex^{TM} P100, manufactured by EBI Food Safety, as Generally Recognized as Safe (GRAS). The approval is based solely on EBI Food Safety's research and GRAS determination panel consisting of qualified and experienced scientists, and not based on FDA research (USFDA CFSAN 2006b). The GRAS panel discussed issues pertaining to the identities of the phage and the target organism, the manufacturing process, specifications, approximate dietary intake when used on cheese, and studies concerning the

safety of the phage preparation. The panel discussed research on potential pathogenicity, allergenicity, and virulence factors. The GRAS panel concluded Listex™ P-100 is GRAS when used as an antimicrobial on cheese at levels not exceeding 1 x 10^9 pfu/gram. EBI claims Listex™ P-100 to be GRAS when used to control *Listeria monocytogenes* in cheeses that are typically aged and/or ripened such as Brie, Swiss, and Cheddar (USFDA CFSAN 2006b). According to EBI Food Safety (2007b), in July 2007 FDA extended GRAS status for Listex™ P-100 to cover all food products, not just cheese.

United States Department of Agriculture

In 2007, USDA issued separate no objection letters for use of bacteriophage targeting *Salmonella* and *E. coli* O157:H7, also known as Omnilytic's BacWash product line, on animal hides prior to slaughter (Omnilytics 2007a,b). These no objection letters open the door for livestock processors who wish to reduce the microbial load of the animals prior to slaughter. Use of BacWash targeting *E. coli* O157:H7 may reduce the number of positive samples on trim and save slaughter and processing facilities extra traceability paperwork that is required when selling or processing trim that is positive or presumptive for *E. coli* O157:H7.

EU Law

In June 2007, The Public Inspection Authority of The Netherlands confirmed organic status of Listex™ P-100 as organic according to EU Regulation (EEC) nr. 2092/91 Annex VI Section B (EBI Food Safety 2007c). Therefore, Listex™ P-100 is available for use in certified

organic products. Article 14 (2) Regulation (EC) No 178/2002 defines unsafe foods and any product considered under this provision that is deemed unsafe or unfit for human consumption may not be placed on the market. Therefore, according to EU Law bacteriophage may be allowed use on food products without prior authorization if adequate proof of safety is provided confirming it does not make the food unsafe (von Jagow and Teufer 2007).

Table 8. Overview of phage approvals by government food regulatory agencies.

Company	Product	Target Bacteria	Approvals	Date of Approval
Intralytix	LMP-102	*Listeria monocytogenes*	FDA 21 CFR Part 172	August 18, 2006
	ECP-100	*E.coli* O157:H7	N/A	N/A
EBI Food Safety	Listex P-100	*Listeria monocytogenes*	FDA-GRAS GRN No. 000198; EU Law	October 17, 2006 N/A
Omnilytics	Agriphage	*Xanthamonas campestris* & *Pseudomonas syringae*	EPA Reg. No. 67986-1	December 28, 2005
	BacWash	*E. coli* O157:H7	FSIS Case No. 06-NT-0239-N-A	December 2006
	BacWash	*Salmonella*	FSIS Case No. 07-NT-0253-N-A	December 2006

Chapter 7: Objections

A few consumer advocacy groups have filed objections to use of phage in food with government regulatory agencies or seek to warn consumers via their websites. This chapter will discuss the objections and the reasoning behind the objections.

Food and Water Watch

Food and Water Watch is a consumer advocacy group that aims for environmentally friendly practices and criticizes use of technologies deemed as risky or questionable when it comes to dealing with the public food and water supply. On September 18, 2006 Food and

Water Watch submitted a formal objection to the provision allowing use of bacteriophages as a food additive. Their main concern deals with the safety of the consumer during use of or after application of these new technological advances on food products. Food and Water Watch (2006) does not feel that FDA sufficiently evaluated the potential risks. Food and Water Watch (2006) based their objections on several factors:

- FDA did not follow its own procedures for assessing safety when it comes to food additives
- FDA relied on assumptions of safety when it came to evaluating residues of Listeriolysin O (LLO), one of *L. monocytogenes* virulence factors
- Efficacy studies did not meet zero tolerance requirements set for *Listeria monocytogenes* in RTE foods
- Research used to support FDA's decision was conducted by the company seeking product approval
- FDA did not make the information regarding this rule available to the public, as noted when Food and Water Watch requested certain data
- FDA failed to provide the required 30 day period for third parties to place objections to the rule

Truth in Wellness

Truth in Wellness is an advocacy group devoted to providing consumers with accurate health information. In an article titled " The FDA Approves Viral Adulteration of Our Food Supply" Byron Richards (2006) of Truth in Wellness attacks the FDA approval of bacteriophage as a food additive. Richards (2006) uses the more well-known, and often fear-invoking term "viruses" to discuss the phage in his article. Richards warns the general public by asking, " How do you like the idea of buying virus-infested food for your family?"

He accuses FDA of several atrocities including:

- Adding more opportunity for adulteration to the food supply rather than addressing the problem of bacterial contamination at the source
- Allowing a battle between two infectious organisms to fight each other in the human digestive tract
- Not conducting adequate safety tests to ensure the long-term safety of use of phage as a food additive
- Failing to prove without a doubt that the phage preparation will attack only the target organism and not mutate to recognize and attack human cells and other bacteria
- Failing to satisfactorily address concerns over release of endotoxins when the phage causes the bacterial cell to lyse
  (Richards 2006)

Richards (2006) encourages Americans to "boycott viral tainted foods" and "quit buying poor quality toxic food."

Other

Capparelli et al. (2007) reported objections to use of phage as therapy in humans due to evidence of "rapid clearance in the spleen, an inability to kill intracellular bacteria, and stimulation of neutralizing antibodies."

Chapter 8: Conclusion

Numerous reports of the safety of phage use in humans exist; specifically the effectiveness of phage therapy in humans over the past 80 years is very encouraging. The fact

that bacteriophages only target specific bacterial organisms is a valid and proven justification for allowing phage preparations to come into contact with foods designated for human consumption. However the few doubts raised show the need for further research to prove the safety of phage use in foods beyond a reasonable doubt.

If use of phage in RTE products becomes a widespread practice, consumers deserve to be notified that the products they are buying contain phage preparations as an additive since the use of any unknown substance in the food supply raises concern among consumers. Therefore consumers should be educated on the issue of phage use so they can make an informed decision as to whether they want to consume food products treated with phage preparations.

Use of phage at any phase of the food chain, from farm to fork, is not a substitute for sanitary conditions under any circumstance. Government food regulatory agencies such as USDA and FDA should be required to ensure food companies continue to adhere to sanitary guidelines to prevent adulteration of the food supply and not rely solely on use of phage as a 'quick fix.' Phages have the potential to be very effective in eliminating pathogenic bacteria on food products and in the future current research can be broadened to provide products targeting a greater range of bacterial pathogens.

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