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Phages in the global fruit and vegetable industry

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Abstract

From recent papers we have learned that phages can constitute a promising alternative in the food industry to eliminate bacterial pathogens from seedlings in greenhouse and field environments, as well as from fresh-cut food products. The fruit and vegetable industry requires quite a different approach than the meat or dairy industry. Several factors can inhibit efficacy of phage treatment such as plant watering or washing ready-to-eat products (water may dilute therapeutic doses), UV irradiation or extensive spreading of phytopathogens by wind, insects or even humans. Spontaneously occurring anomalous weather conditions in different parts of the world also may have an enormous impact on phage persistence in cultivations and on yields. Despite that, some phage preparations are commercially available and, without doubt, are much safer than chemical treatments. Along with increasing worldwide fruit and vegetable consumption, plant diseases and human foodborne illnesses are becoming a serious economic problem, resulting in a focus on optimization of phage treatment.

Keywords: food safety, agriculture, antimicrobials, bacteriophages, biocontrol

Introduction

These days bacteriophages (commonly called phages), viruses that infect bacteria, are increasingly used as part of the bacteria-fighting arsenal in the food industry. Their antimicrobial potential has been noticed not only by physicians treating bacterial infections in humans (including those that result from consuming microbiologically contaminated food or water, such as dysentery) (Brüssow 2005) but also by researchers trying to control foodborne pathogens before they become a cause of major human diseases. Phages are present in almost every environment (Brüssow and Kutter 2005; Weinbauer *et al.* 2007). They can even be present in municipal water supplies of large European cities, indicating resistance to physico-chemical methods of purification of drinking water (Weber-Dąbrowska *et al.* 2014). This example clearly shows the continuous direct contact of humans with phages. Such widespread and frequent occurrence of phages supports the view that phages are safe for humans and their environment and can be utilized efficiently in agriculture (Meaden and Koskella 2013). Furthermore, phage particles are mostly stable after being suspended in a wide range of neutral solutions such as phosphate buffered saline (PBS). This feature allows phages not to alter the flavor, texture or nutritional value of foods. Phages have also been proposed as agents to eliminate plant pathogens. Katznelson (1937) still before World War II described in his review paper phages isolated from soil or parts of plants against phytopathogenic bacteria (e.g. *Agrobacterium tumefaciens* causing crown gall disease and

Erwinia carotovora responsible for soft rot in many vegetables and fruits) and their therapeutic and prophylactic possibilities. Phages have been considered to be natural and safe plant disease control agents since the 1970s when they were found occurring extensively and continuously in unaffected plant environments (Dunleavy and Urs 1973).

The aim of our review paper is to present some historical as well as recent reports regarding phage application in the fruit and vegetable industry - from the field to the market. Its main focus is on the results, which are sometimes contradictory or unclear, independently of type of investigation, showing that, since the current phage applications still need to be improved and developed, further research in the field of phages needs to be implemented.

Fruit and vegetable industry

Global crop production is closely related to adverse weather, natural disasters and the condition of plants. Such a correlation can be seen clearly in Brazil, the world's largest citrus producer. The US Department of Agriculture states that Brazil's citrus production in 2013 dropped by nearly 20% due to tree stress after extensive harvests in previous years and plant diseases. The same source indicates an 8% increase of Brazil's production at the beginning of 2014 based on favorable weather and higher yields (United States Department of Agriculture 2014). Despite these influencing factors, the global fruit and vegetable market grew by 11.7% in 2011 to reach a value of $1,517.3 billion. In 2016 the value of the industry will reach over $2,700 billion (an increase of 79.2% since 2011) and is forecast to have a volume of 818.1 million tons (MarketLine 2012). The vegetable sector is the largest segment of the global fruit and vegetable industry, accounting for almost 70% of its total value. Also the citrus industry is a large branch of the market in 140 countries, with total world production exceeding 105 million tons per year (Balogh 2006). In a 15-year period, between 1982 and 1997, US consumption of raw fruits and vegetables increased by 18% and 29%, respectively (Sharma 2013). Increasing fruit and vegetable consumption is the result, inter alia, of developing a national strategic framework for public health nutrition in many countries (Catford 2000; Kuriyan *et al.* 2014). In Australia, such a program focuses on education and promotion of a balanced diet as diet-related disease costs Australia at least $2.5 billion per year (at least 10% of the total burden of disease can be attributed to nutrition) (Catford 2000). The authors state that eating insufficient amounts of fruits and vegetables contributes to serious health problems, including cancer (Colón-López *et al.* 2013). In the latest reports (Christian *et al.* 2014; Hert *et al.* 2014) many chronic human diseases are still linked with unhealthy, high-calorie food consumption. This, together with the constantly increasing

human population, certainly will contribute to extensive growth of the global fruit and vegetable industry in the near future.

Phages as biocontrol agents against bacterial phytopathogens and foodborne pathogens

Bacterial plant pathogens (also known as phytopathogens) and foodborne pathogens, like many other bacteria, have quickly become resistant to antibiotics (Flaherty *et al.* 2000). The first report indicating bacterial phytopathogens' resistance to certain antibiotics comes from 1954. During this time, the first concerns about toxicity of chemical pesticides, an alternative substitute to antibiotics, were appearing as well (Agrios 2005a). Restricted application of antibiotics in many countries and increasing bacterial resistance to copper-based bactericides (Flaherty *et al.* 2000; McNeil *et al.* 2001; Jackson and Jones 2004) in connection with soil chemical contamination (Balogh *et al.* 2008) led to a search for new ways to combat bacterial phytopathogens. Phages, as mentioned above, have started to be considered as a natural and potentially safe remedy for bacterial plant diseases (Jones *et al.* 2007). One of the main advantages of using phages to eliminate bacterial pathogens over other treatments is their propagation followed by bacterial lysis (Lim *et al.* 2013).

Nowadays, some commercial phage preparations are available on the market. Listex P100 is one of the most well-known commercially available phage preparations developed by the Dutch company Micreos. Phage Listex P100 has been approved by the United States Food and Drug Administration (FDA) and the United States Department of Agriculture's (USDA) Food Safety and Inspection Service for *Listeria monocytogenes* control in both raw and ready-to-eat food products (Soni *et al.* 2010). Intralytix, a biotech company from Baltimore, USA, offers several phage preparations targeting the most popular bacterial food pathogens (ListShield against *Listeria monocytogenes*, EcoShield against *Escherichia coli* O157:H7 and SalmoFresh against highly pathogenic *Salmonella* serotypes). The latter is FDA-approved as GRAS (Generally Recognized As Safe) and in 2014 has been approved by Health Canada as a processing aid for control of *Salmonella* on, inter alia, fresh and processed fruits and vegetables (Intralytics 2014a). On August 2014, the National Food Service, Ministry of Health in Tel Aviv, Israel, issued "Guidelines: Use of Bacteriophages (bacteria killing viruses) in Food". The guidelines approve the use of all FDA-approved phage preparations for similar applications on food in Israel (Intralytics 2014b). Another US phage company, OmniLytics, has developed the preparation AgriPhage, which consists of phages against *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* for treatment of

bacterial disease in agricultural crops (especially on tomato and pepper plants). The company states that AgriPhage is completely safe for the environment and humans and provides increased yields (OmniLytics 2014).

Unquestionably, phage preparations are becoming more popular in the global food industry together with increasing interest in phage treatment, which is recognized as generally safe and effective.

Bacterial phytopathogens

Plant pathogens are responsible for enormous losses (hundreds of millions of dollars each year) in cultivated and stored crops and are a major impediment to effective food distribution worldwide (Osusky *et al.* 2000; Stavrinides 2009). The losses are highest in the developing countries, where lack of food is much more noticeable. Plant pathogens have an enormous indirect impact even on stock and poultry farming as plants constitute the dominant diet for animals (Agrios 2005a). Among all plant pathogens (mainly bacteria, viruses and fungi) almost 200 species of bacteria are known. They are widespread in every moist, warm environment, causing disease of almost all kinds of plants (Agrios 2005b). Such an extensive range around the globe is the effect of being easily spread by rain, wind, animals and even by humans (for instance by pruning infected trees) (Ellis *et al.* 2008). Additionally, phytopathogenic bacteria are characterized by high virulence. They can easily adapt to changing environmental conditions and demonstrate simultaneously high plant-colonizing capabilities (Stavrinides 2009). A summary of phage applications in plants under greenhouse and field conditions is presented in Table 1.

Phages against *Xanthomonas* strains affecting citrus, walnuts, tomatoes and peach trees

The genus *Xanthomonas* is characterized by the ability to produce an extracellular polysaccharide called xanthan gum. This feature enables bacterial cells to retain their pathogenic activity during dry weather (Bradbury 1990). These Gram-negative bacteria are widely known as phytopathogens, and their virulence on tomatoes was described almost one hundred years ago (Doidge 1921).

Xanthomonas axonopodis pv*. citri* and *X. axonopodis* pv. *citrumelo* are plant pathogenic bacteria which are responsible for serious plant diseases such as citrus canker and citrus bacterial spot respectively. Citrus canker is known as an extremely contagious disease. Introduced to Florida, USA in 1912, it returned a few times

through the last century, bringing each time economic losses (Balogh *et al.* 2008). Citrus canker infects many citrus species (lime, orange, lemon, pomelo, among others) (Rodriguez-R *et al.* 2012). Infection causes lesions on the leaves, stems, and fruits. The second citrus disease caused by *Xanthomonas axonopodis* pv. *citrumelo*, bacterial spot, unlike citrus canker is endemic only to Florida and infects mainly young plant tissues.

Greenhouse trials with *X. axonopodis* phages were carried out by Balogh *et al.* (2008). Duncan grapefruit plants (highly susceptible to citrus canker) were treated with a phage cocktail consisting of four different phages (10^8 - 10^9 pfu/ml). Phage particles were suspended in sterilized tap water or in 0.75% skim milk solution. The next day, plants were infected with a *X. axonopodis* pv. *citri* (10^6 - 10^8 cfu/ml) sensitive to applied phages. Results were read after 3 to 4 weeks. In nursery trials under field conditions in Argentina grapefruit plants were inoculated with an *X. axonopodis* pv. *citri* pathogenic strain (twice at monthly intervals). One group of tested plants was sprayed twice weekly with a phage cocktail (10^6 pfu/ml in 0.75% skim milk solution), the second group was treated weekly with a chemical biocide based on copper, and, finally, the third one was treated both with phages (twice weekly) and with the copper biocide (once weekly). The investigators found that during the 12 week trials, phage particles suspended in clear water gave the most promising results under greenhouse conditions. Interestingly, phages suspended in skim milk solution did not result in any disease reduction despite their higher persistence on leaf tissue. Under nursery conditions phages significantly ($p<0.001$) reduced mean disease intensity (lesion number per diseased leaf) but were less effective than copper-based treatments, which turned out to be more appropriate even than mixed phage and drug application. It was found that protective formulations (skim milk or skim milk and sucrose) ensure greater survivability of phage particles but may reduce the effectiveness of phage treatment. Also, it was demonstrated that UV irradiation is the most limiting factor for phages as therapeutic agents for cultivated plants (Jones *et al.* 2007, 2012). Similarly, a three-phage cocktail applied on Valencia oranges infected with bacterial spot in a commercial citrus nursery in Florida significantly reduced the disease progress measured as area under the disease progress curve (AUDPC). However, phages were not effective after application on grapefruit plants highly susceptible to pathogen infection ($p=0.159$). In contrast, phage treatment was successful on citrumelo plants, which are extremely susceptible to *X. axonopodis* pv. *citrumelo*. In the experimental nursery, under high disease pressure, phages significantly reduced mean disease incidence as well as mean AUDPC, but copper-based treatment yet again gave the best results (Balogh *et al.* 2008).

Another plant disease incited by *Xanthomonas arboricola* pv. *juglandis* (also known as *X. campestris* pv. *juglandis*) is walnut blight (Buchner *et al.* 2009). After many unsuccessful trials with chemical sprays, McNeil *et al.* (2001) started to consider phages as biocontrol agents. Pathogenic bacterial strains were collected from

infected walnut tissues throughout New Zealand and then were used for isolation and enumeration of phage particles. Six *X. campestris* pv. *juglandis* phages exploited in the experiment were isolated from soil under walnut orchards (from the top 2.5 cm). During greenhouse trials cut branches of Payne walnut trees were sprayed with a phage cocktail. To determine survivability of phage particles they were re-isolated from bud samples 5 hours after application. Unfortunately, phages used during tests showed very poor survival on plant tissues (after 5 hours the titer had dropped from 10^6 pfu/bud to 10^2 pfu/bud). Romero-Suarez *et al.* (2012) reached very similar conclusions. They characterized 26 *X. arboricola* phages isolated from the phyllosphere and rhizosphere from walnut orchards in the South Island of New Zealand. Examined phages showed cold sensitivity and media/chloroform sensitivity, and their titer decreased significantly (from the order of 10^2 to 10^5 pfu/ml) after 12 months in storage. Both groups of authors suggest that *X. arboricola* pv. *juglandis* phages can be helpful in reducing walnut blight disease. However, further trials related to phage survivability should be conducted.

At the turn of the 20th and 21st centuries several attempts to control bacterial spot on tomatoes were performed. This disease, one of the most devastating plant diseases, is caused by a *Xanthomonas campestris* pv. *vesicatoria* strain. In experiments by Balogh *et al.* (2003) greenhouse and field trials were performed. Bacterial strains were isolated directly from infected plants in Florida. Phages specific to those strains were isolated from tomato/pepper plant tissues as well as from soil, field runoff, river or stream waters. Among isolated phages there occurred h-mutants (extended host range mutant phages) (Jones *et al.* 2007) which have the ability to lyse phage-resistant bacterial strains. A phage mixture consisting of six to eight different phages (10^{10} pfu/ml) was used. Under greenhouse conditions tomato seedlings were sprayed with a phage suspension two hours before pathogen application. Disease severity was assessed 14 days after phage inoculation. In field experiments seedlings of tomato were treated with a phage suspension before pathogen inoculation and treatment (twice weekly) was continued until 2 weeks before harvest. A copper-based bactericide was applied once a week.

Unlike previously described experiments performed by Balogh *et al.* (2008) in the greenhouse, phage particles suspended in clear water did not reduce bacterial spot disease on tomato plants. In comparison, phages suspended in skim milk formulation gave the most promising results (79% reduction of disease in the first experiment and 45% in the second one). Remaining protective phage formulations (pregelatinized corn flour and water-soluble casein protein polymer) also significantly reduced disease symptoms. Similarly, in the field

nonformulated phage treatment turned out to be the most ineffective, but all remaining phage treatments (corn flour, skim milk and particularly casein protein polymer formulated phages) significantly reduced disease severity in the majority of the experiments compared with the control. Interestingly, seedlings treated with copper preparation had more disease symptoms than the untreated control group. Skim milk formulation brought the best results under greenhouse conditions, while casein formulation brought the best results in the field. The authors took note of proper timing of phage applications. Evening applications gave better treatment results than morning applications. Similar results were obtained by Obradovic *et al.* (2004) when a phage mixture was applied to tomato plants at dusk and by Iriarte *et al.* (2007) in trials investigating persistence of *Xanthomonas* phages on tomato leaf surfaces. The lower intensity of UV irradiation after dusk is most likely the crucial factor determining better results of the treatment. Of note, good phage treatment results did not coincide with a significant increase in yield. These results do not correspond to data obtained by Flaherty *et al.* (2000), showing that tomato seedlings treated with *X. campestris* pv. *vesicatoria* h-mutant phages produced more extra-large fruit at harvest than seedlings treated with a copper compound or the untreated control group. Also Obradovic *et al.* (2004) observed higher yield from plants treated with phages or phages in combination with chemical compounds than in plots with no phage application. Furthermore, use of phages suspended in skim milk and sucrose was more effective in control of tomato bacterial spot than copper treatment despite copper-sensitive strains being used in this trial.

In experiments with *Xanthomonas pruni* phages (Civerolo and Keil 1969) used to control bacterial spot of Elberta peach foliage, the sequence of phage and bacterial strain application seemed to be crucial. Treatment was successful when a phage suspension (10^9-10^{10} pfu/ml) was applied on the leaf surface 1 hour or 24 hours before pathogen inoculation, giving 48% and 42% disease reduction respectively. There was no significant effect when phages were applied after bacterial strain inoculation. These data indicate that phages can be used in preventive treatment, as the phage lysate was stable for at least 24 hours after application.

Phages against the genus *Erwinia* causing fire blight and rot diseases in crops

Erwinia amylovora is the main cause of a contagious disease called fire blight which can affect pome fruits, apple and pear trees in North America, Europe, the Mediterranean region and New Zealand (Schnabel and Jones 2001; Gill *et al.* 2003). The possible role of *Erwinia amylovora* phages in the epidemiology of fire blight was

investigated by Erskine in the 1970s (Erskine 1973). Bacterial strains as well as phages specific to them were isolated from infected pear trees and from soil near trees in British Columbia, Canada. An important role in biocontrol of fire blight was performed by a saprophytic bacterium named Y, closely related to a pathogenic strain and obtained from the same trees. Strain Y easily incorporated an *E. amylovora* phage into its own genome and released phages under UV irradiation. The author suggests that use of the saprophytic strain, a natural reservoir of specific phages, to control fire blight disease has some advantages over the use of phage particles alone. These include the ability to relocate within plant tissues and phage protection against inactivation by environmental stresses (the tested *E. amylovora* phage was unstable at temperatures typical of summer and in ultraviolet irradiation). Tests in pear slices confirmed that disease symptoms decreased when the pathogenic bacterial strain together with strain Y were inoculated. Moreover, reduced virulence of phage-resistant mutants of *E. amylovora* strains in comparison to phage-sensitive strains was observed. Investigations on *E. amylovora* phages were also performed by Gill *et al.* (2003). As in the trials by Erskine (1973) the majority of phages were isolated from the soil near infected trees in Ontario, Canada. Such an environment protects phage particles against desiccation and ultraviolet irradiation, which explains why *E. amylovora* phages were unstable with respect to these factors during investigations. Occurrence of phage resistance in nature within *E. amylovora* strains was examined by Schnabel and Jones (2001). Among 40 tested strains 65% showed resistance to at least one phage. All 40 strains were sensitive to phage ΦEa116C, 39 strains were sensitive to phage ΦEa1 and 36 to phage ΦEa7. These three phages were proposed as natural agents for biocontrol of *E. amylovora* strains. The author opined that successful treatment of fire blight disease should use phage application particularly on blossoms (as pathogenic strains multiply primarily on the blossom parts).

Soft rot and stem rot diseases in crops such as Chinese cabbage, potato and tomato are caused by *Pectobacterium carotovorum* subsp. *carotovorum* bacteria (formerly known as *Erwinia carotovora* subsp. *carotovora*). In trials performed by Lim *et al.* (2013), the isolated phage PP1 came from soil samples collected in infected Chinese cabbage fields (South Korea). The phage was considered as highly specific to *P. carotovorum* subsp. *carotovorum* and stable at different ranges of pH. Under greenhouse conditions two-week-old lettuces were sprayed with a phytopathogenic bacterial strain (10^8 cfu/ml) prior to phage application. Development of disease symptoms was recorded for 6 days. After this period 80% of lettuces from the non-phage treated group showed disease symptoms. Seedlings treated with specific phage were almost as healthy as plants from

untreated controls. These results justified further studies on *P. carotovorum* phages as very efficient agents to control rot diseases.

Phages against *Ralstonia solanacearum* causing bacterial wilt in crops

Bacterial wilt affects over 200 plant species, which are economically important in tropical and subtropical areas (Murugaiyan *et al.* 2011; Bae *et al.* 2012). Despite that, the main causative agent, *Ralstonia solanacearum*, is able to survive for a long time in cool weather. Due to its lethality and persistence, biocontrol of *R. solanacearum* is one of the most important challenges in worldwide agriculture. Several lytic phages against *R. solanacearum* have been tested in recent years (Fujiwara *et al.* 2011; Murugaiyan *et al.* 2011; Bae *et al.* 2012). Unfortunately, some of them demonstrate very narrow host ranges.

Fujiwara *et al.* (2011) and Bae *et al.* (2012) conducted research on phages for biocontrol of bacterial wilt of tomato. In experiments by Fujiwara *et al.* (2011) three pathogenic bacterial strains came from the Leaf Tobacco Research Center and from the National Institute of Agrobiological Sciences in Japan, and seeds of the tomato (cultivar Oogata-Fukuju) were obtained from a commercial distributor. Seeds were soaked with a phage suspension (1.3×10^{10} pfu/pot) and after 1 month the same amount of phage was applied again to the pellets. In the next 2 days cut seedlings were washed in bacterial suspension (10^8 cfu/ml) for 30 seconds. After treatment plants were grown in an incubator at 28°C. Symptoms were measured on a scale of 0 to 5 where 0 means no symptoms and 5 signifies plant death. In contrast to those trials, Bae *et al.* (2012) in greenhouse experiments did not focus on the preventive effect of phage application. Commercially obtained seeds of tomato (cultivar Seogun) in South Korea were used in his investigations. Four-week-old seedlings were infected with an *R. solanacearum* strain (10^7 cfu/g of soil). Phage application was carried out in three ways: before and after bacterial treatment and in a few cases together with the bacterial suspension. The disease index was rated using a 0-5 scale, similarly as described above.

Very promising results were obtained by Fujiwara *et al.* (2011). Efficient prevention of bacterial wilt was achieved in all plants treated with phage ϕRSL1 (disease index = 0). In the untreated control group after 18 days all plants showed wilting symptoms indicated by a disease index from 2 to 5. Also, phage ϕRSL1 was quite stable under experimental conditions on plant roots as well as in surrounding soil, showing that it can be used as a preventive agent on seedlings. Interestingly, treating plants with a cocktail of two or three *R. solanacearum* lytic phages (with or without ϕRSL1 phage) gave very poor results. Wilting patterns were observed on every

treated plant after 14 days and several phage-resistant mutants were observed. This suggests that choosing one stable lytic phage characterized by a wide range of bacterial hosts is the most appropriate. Such a stable phage (PE204), despite its narrow host range, was used by Bae *et al.* (2012). Mixed treatment containing phage particles together with host cells did not cause any disease symptoms. Most likely, the presence of host bacteria helped to propagate the phage population. However, preventive phage application 1 day prior to pathogen inoculation resulted in development of disease after 7 days and after 12 days it became as high as in untreated infected plants (disease index = 2). Phage application 1 day after pathogenic strain inoculation was quite effective (the disease index after 16 days was still under 1). Different data obtained in both experiments indicate that phage PE204 was probably less stable in the rhizosphere without host bacteria than phage ϕRSL1. Further studies on *R. solanacearum* phages as biocontrol agents are necessary. Murugaiyan *et al.* (2011) determined host specificity of 15 *R. solanacearum* phages isolated from the rhizosphere. All 9 tested bacterial strains were sensitive to phage PE226 and TM227 and resistant to at least one other phage, with one exception which was sensitive to all 15 phages. DNA sequencing revealed almost 99% similarity of their genome. It may help finding phages with a broad host range in the future based on DNA sequences.

Phages against *Streptomyces scabies*-infected seed potatoes

Potato scab is a common tuber disease which represents a serious problem for potato growers. In Australia, intensive and repeated cropping may be increasing disease distribution. The main causative agent responsible for the appearance of reddish-brown spots on the tuber surface is *Streptomyces scabies*. This pathogen tolerates a wide temperature range and can survive for long periods in the absence of hosts (Johnson and Lambert 2010). McKenna *et al.* (2001) described the first study consisting of disinfecting seed potatoes with a highly virulent and polyvalent *S. scabies* phage isolated from a potato field in Western Australia. The pathogen isolation was carried out from infected tubers. To evaluate the efficacy of phage treatment small tubers of potato were soaked in pathogen inoculum (1×10^{10} cfu/ml) for 1 hour and planted in soil. After 60 days infected tubers were selected for further treatment. The phage was propagated in a specially designed and constructed bioreactor. The final volume of ∅AS1 phage suspension (1×10^{9} pfu/ml) was 40 l. Selected seed tubers were divided into two groups. One of them was immersed in phage suspension at 28°C for 24 hours. The control group was treated in the same way in sterilized (at 121°C for 20 min) phage suspension. Treated tubers were planted in steam-sterilized soil and were harvested 8 weeks after the experiment began. The severity of disease was assessed as the percentage of lesion surface area using a 0-6 scale where 0 means no lesions and 6 stands for

>50% lesion surface area. Lesion type was rated on a 0-4 scale (0 - no lesions, 4 - deeply pitted lesions). Phage-treated tubers had 3.82 lesions per tube and 1.2% lesion surface area in contrast to 44.23 lesions per tube and 23% lesion surface area in the non-phage-treated group. Data were statistically significant. Furthermore, no differences were noted in tuber weight, size or number between the two tested groups.

Phages against *Pseudomonas* strains causing bacterial blotch disease in mushrooms

Application of phages was also evaluated by researchers in infected mushroom farms. Although mushrooms do not require sunlight to obtain energy for growth, their cultivation requires quite similar conditions as cultivation of vegetables under greenhouse conditions (proper temperature, humidity, pH and growth medium). Bacterial blotch is an economically important disease affecting the commonly consumed champignon mushroom (*Agaricus bisporus*) (Munsch and Olivier 1993). *Pseudomonas tolaasii*, which causes the disease, can produce an extracellular toxin called tolaasin that disrupts eukaryotic membranes (Hutchison and Johnstone 1993). The first trials involving lytic *P. tolaasii* phage TO.1 were performed in the early 1990s (Munsch and Olivier 1993). After spraying phage suspension onto soil in the greenhouse biocontrol efficiency was greater than 50%. Korean investigations consisted of application of *Ps. tolaasii* phages in oyster mushroom crops (Kim *et al.* 2011). Bacterial strains of *P. tolaasii* 6264 were obtained from infected fruits of oyster mushroom. Twenty-one phages were isolated from sewage samples in South Korea. Phages were classified by researchers in three different groups (very toxic, toxic and mildly toxic) depending on plaque morphology. Disease development was measured using the pitting test. Dropping bacterial culture on mushroom caps was visible after a 15-hour incubation period as brown discoloration. Total area of brown spots indicated the degree of disease progress. Dropping very toxic and toxic phage suspensions (10^9 pfu/ml) simultaneously with bacteria suppressed bacterial blotch (almost no bacterial growth was observed). However, decreasing phage titer induced larger blotches on the cap surface. Phage application became less effective in proportion to longer intervals after pathogen inoculation (treatment 6 hours later resulted in bacterial growth which was almost as high as in the control group treated only with bacteria). Phage lysis in experiments was most effective at temperature optimal to host bacteria (20-25°C) while no lysis was observed at 37°C. The authors stated that choosing suitable phage for treatment can be evaluated by plaque morphology (highly virulent phages formed large, clear plaques in contrast to mildly toxic phages which had small or turbid plaques).

Foodborne pathogens

Diseases caused by foodborne pathogens have become a serious problem (Zhao *et al.* 2014). In fact, foodborne pathogens can seriously affect everyone. The U.S. Centers for Disease Control (CDC) estimates that each year nearly 50 million Americans get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. Moreover, the CDC predicts that reducing foodborne illness by 10% would prevent about 5 million Americans from getting sick each year (Centers for Disease Control and Prevention 2011). Among the top five pathogens causing domestically acquired foodborne illnesses resulting in hospitalization two of them (50%) belong to the bacterial genera *Salmonella* and *Campylobacter*. Leading causes of death are *Salmonella* (28%) and *Listeria* (19%) (Scallan *et al.* 2011). Infections caused by *Salmonella* strains cost nearly 3 million euros in the European Union each year (Sillankorva *et al.* 2012). In the United States more than 1.5 million infections are reported per annum (Ye *et al.* 2009). On the other hand, the number of registered salmonellosis cases in Poland in 2011 was the lowest ever recorded (the overall incidence was 22.9/100 000) (Sadkowska-Todys and Czarkowski 2013). However, the authors emphasize that cases of salmonellosis may be underdiagnosed or underreported.

Foodborne pathogens can exist in raw and processed food, both in meat and vegetable products. The FDA mentions at least a few bacterial pathogens that can be found in vegetables (mainly leafy greens, tomatoes, cucurbits, and other fresh produce that make up salads), such as *Staphylococcus aureus*, *Shigella* sp. or *Escherichia coli* (United States Food and Drug Administration 2013). In The Netherlands in the last two months of the year 2000 *Salmonella enterica* serotype Enteritidis phage type 4b infection was associated with consumption of vegetables such as tomato, lettuce, cabbage, bean sprouts, cucumber, paprika, peppers, onion, carrots, parsley and celery (van Duynhoven *et al.* 2002). *Listeria monocytogenes* is another foodborne pathogen which can be found not only on meat products (beef, pork, poultry) but also on salad vegetables and is implicated in outbreaks of foodborne listeriosis (Leverentz *et al.* 2003). The high death rate after listeriosis infections led the FDA to establish a zero-tolerance policy (no detectable level permitted) for *L. monocytogenes* in food, including fruits and vegetables. In particular, *Escherichia coli* O104:H4 is a human pathogen responsible for one of the most well-known outbreaks caused by vegetable consumption in Germany in 2011, which affected almost 4,000 people (Choffnes *et al.* 2012; Balabanova *et al.* 2013). It was proven that sprouts were the vehicle of infection. The greatly increased consumption of fruits and vegetables is not the only reason for foodborne illness. Sharma M. (2013) considers that other factors may also be responsible for the large number of these outbreaks. These factors may be associated with inadequate temperature storage of potentially

hazardous food, poor employee hygiene and microbiologically contaminated equipment or eating food from unsafe sources. A summary of phage applications on fresh-cut vegetables is presented in Table 2.

Phages against *Escherichia coli* O157:H7

E. coli O157:H7 causes a variety of human diseases such as mild diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. The bacterium was identified in 1983 and quickly became one of the most important foodborne pathogens (O'Flynn *et al.* 2004). From 407 food samples obtained from markets in Lima (Peru) 50 of them (12.3%) were contaminated by *E. coli* O157:H7 strain (4 contaminated samples were found among 101 samples of fresh vegetables). The majority of tested strains presented genes encoding Shiga toxins and belonged to the serovars previously associated with outbreaks in Europe and Canada (Mora *et al.* 2007). In the last few years several reported *E. coli* outbreaks were linked with baby spinach and lettuce consumption (Ferguson *et al.* 2013).

Two of the first reports on the effectiveness of *E. coli* O157:H7 phages in reducing pathogen contamination of vegetables were published in the years 2008-2009 (Abuladze *et al.* 2008; Sharma *et al.* 2009). In experiments by Abuladze *et al.* (2008), the effectiveness of a phage preparation called ECP-100 was evaluated. ECP-100 is a cocktail of three lytic phages (ECML-4, ECML-117 and ECML-134) suspended in phosphate-buffered saline (PBS). The phage cocktail lysed 90% of the 111 *E. coli* O157:H7 strains obtained from various research and public health laboratories in the USA and from the collection of the Microbial Evolution Laboratory at Michigan State University. Furthermore, ECP-100 reduced bacterial contamination (710 cfu/ml of *E. coli* O157:H7/gram) on broccoli samples by 99.5%, 99% and 97% during storage at 10°C for 24 hours, 120 hours and 168 hours respectively. Similarly, it was equally successful in reduction of a pathogenic strain on tomato slices and spinach samples. All results were statistically significant ($p<0.05$) in comparison to the PBS-treated control group. The authors also reported that bacterial colonies surviving after phage treatment were still sensitive to ECP-100, in contrast to chemical sanitizers (such as calcium hypochlorite), which can induce development of bacterial resistance to them (Abuladze *et al.* 2008). ECP-100 turned out to be an effective solution after application on contaminated cantaloupe slices (application with a pipette) and fresh-cut lettuce (treatment by spraying) stored at refrigeration temperature (4°C) (Sharma *et al.* 2009).

In experiments by Viazis *et al.* (2011) artificially contaminated organic baby spinach and baby romaine lettuce leaves purchased at a local market in the US were treated with a mixture of eight lytic *E. coli* O157:H7 specific

phages (10^7 pfu/ml) at different multiplicity of infection (MOI) levels (1, 10 and 100) and under various conditions (at 8, 23 and 37°C for 10 minutes, 1 hour and 24 hours). Treatment significantly ($p<0.05$) reduced the number of *E. coli* O157:H7 cells. The higher the temperature and MOI applied, and the longer the incubation period, the greater the observed level of bacterial inactivation. The authors suggest that phage treatment combined with trans-cinnamaldehyde (a natural essential oil whose components exhibit antimicrobial activity) may become the most environmentally friendly way to reduce bacterial contamination of food and is more effective than the phage treatment alone.

Several papers have described attempts to control *E. coli* O157:H7 with the ready-to-use commercial preparation EcoShield, developed by Intralytix, Inc. Boyacioglu *et al.* (2013) and Ferguson *et al.* (2013) investigated application of this preparation on fresh-cut leafy greens. EcoShield consists of three different *E. coli* O157:H7 specific phages and recently has been approved by the FDA and USDA Food Safety and Inspection Service for use on meats and on food contact surfaces. In one of the performed experiments (Ferguson *et al.* 2013) leaves of iceberg lettuce obtained from a grocery store were immersed in phage solution (10^8 - 10^9 pfu/ml for 30 seconds or 2 minutes). After drying 50 µl of bacterial suspension (10^5 cfu/ml) was applied to the surface of leaves. Pieces of lettuce were stored for up to 7 days at 4°C, which is a typical storage period at grocery stores. Boyacioglu *et al.* (2013) treated purchased fresh spinach and romaine lettuce first with an *E. coli* O157:H7 dilution (10^7 cfu/ml), and after 20 minutes an EcoShield suspension (10^7 pfu/ml) was sprayed onto cut leaves. The leaf pieces were stored for up to 7 days at 4°C and 10°C.

In both cases, phage application significantly ($p<0.05$) reduced the pathogen population with one exception. Preventive usage of the phage cocktail at a lower titer (10^8 pfu/ml) and for a shorter duration (30 seconds) brought no significant results in the Ferguson *et al.* (2013) experiments. In addition, preventive phage application did not cause reduction of *E. coli* cells quickly after pathogen application and was most successful after several days of storage at 4°C. In the Boyacioglu *et al.* (2013) investigations the effect of the phage treatment after pathogen inoculation on the leaf surface was observed as early as 30 minutes after EcoShield spraying and was maintained over the seven-day storage period of spinach at 4°C and 10°C as well as through a 48 h incubation period at 4°C for romaine lettuce. Carter *et al.* (2012) investigated whether EcoShield is able to reduce bacterial contamination at much lower concentrations as high moisture content might dilute the original dose of phage preparation. It turned out that artificially contaminated lettuce leaves after EcoShield treatment (1×10^6 pfu/ml and 1×10^5 pfu/ml) for 5 minutes showed a significant ($p<0.05$) reduction of pathogen

contamination on their surface. However, a lower phage titer (10^5 pfu/ml) slightly reduced the efficacy of the treatment. The presented results indicate that EcoShield has the potential to inhibit growth of E. coli O157:H7 on ready-to-eat green vegetables and can be used as a biocontrol tool in the food industry.

Phages against *Salmonella* species

Salmonellosis is often associated with consumption of contaminated food. Typical symptoms in humans range from diarrhea to systemic typhoid fever (McGhie *et al.* 2009). *Salmonella* outbreaks have been linked to seed sprouts, cantaloupes, unpasteurized fruit juice, watermelons, mango and tomatoes (Ye *et al.* 2009).

Since *Salmonella* strains were associated with foodborne illness outbreaks after consumption of sprouts, attempts to use phages as biocontrol agents were evaluated (Kocharunchitt *et al.* 2009; Ye *et al.* 2010). Phage application seems to be ideal chemical-free treatment for sprouting seeds as they are considered as healthy, organic food. Two *Salmonella* phages (SSP5 and SSP6) were used by Kocharunchitt *et al.* (2009) to control *Salmonella* Oranienburg on experimentally contaminated alfalfa seeds as chemical disinfectants were not satisfactory. Alfalfa seeds, purchased from a local store in Australia, were immersed in 25 ml of bacterial suspension (10^7 cfu/ml) for 1 hour. Next, dried seeds were soaked in a phage suspension for 12 hours at 25°C to give an MOI of approximately 70. Seeds, rinsed twice daily at 25°C, were sprouting within 5 days. Although continous bacterial resistance to applied phages did not occur and phages survived the first 12 h after their application, the number of *Salmonella* cells was not considerably reduced in the phage-treated group. Possible explanations include changes in the environment or host cells, or high levels of background microbiota on the seeds, which may offer alternative phage attachment sites. Those hypotheses seems to be admissible, as the *Salmonella* growth during simple *in vitro* tests was two orders of magnitude lower in comparison to the trials on sprouting seeds.

Ye *et al.* (2010) conducted biocontrol of *Salmonella* on sprouting mung bean and alfalfa seeds using lytic phages together with the *Enterobacter asburiae* JX1 strain, which exhibits antagonistic activity against *Salmonella*. Numerous *Salmonella* serovars obtained from the Public Health Agency of Canada-Guelph (Ontario) were selected based on their association with previous foodborne illness. Phages were isolated from pig or cattle manure effluent. Beans and seeds after being soaked in an *E. asburiae* JX1 suspension (10^6 cfu/ml) and in a phage cocktail (10^6 pfu/ml) for 20 minutes were sprouting under very similar conditions (temperature, humidity) as previously described (Kocharunchitt *et al.* 2009). Combined treatment significantly ($p<0.05$)

inhibited the growth of the pathogen on mung bean sprouts and alfalfa sprouting seeds. The number of *Salmonella* cells on alfalfa sprouts was reduced to below the detectable level. Furthermore, in both cases no difference in the yield was observed. However, after single (phage or *E. asburiae* JX1) treatment *Salmonella* grew to high levels on sprouting mung beans, which corresponds to data obtained previously by Kocharunchitt *et al.* (2009). Quite different results were observed during *in vitro* studies. While Kocharunchitt *et al.* (2009) achieved better *in vitro* than *in vivo* results, phage treatment directly on sprouts brought more satisfactory results compared with in-broth culture in trials by Ye *et al.* (2010). Most likely, naturally occurring endogenous sprout microflora takes part in inhibition of *Salmonella* growth. The presence of such endogenous microflora has not been observed by Kocharunchitt *et al.* (2009). Understanding those environmental factors during *in vivo* tests will contribute achieving better treatment results in the future.

Mutual activity of *E. asburiae* JX1 and a lytic phage cocktail against *Salmonella* Javiana was evaluated by the same researchers on tomato fruits and plants (Ye *et al.* 2009). The authors did not note synergistic activity of the *E. asburiae* JX1 strain and phage mixture on tomato fruits. Differences between the trials result from various conditions among stored tomatoes and sprouting mung beans (temperature, incubation period, humidity, nutrient composition of sprouts and tomatoes, remnants of antimicrobial agents on tomato surface). Magnone *et al.* (2013) found that another type of combined treatment of fresh vegetables (phage application before storage at 10°C and levulinic acid produce wash after storage at 10°C) was more successful in reduction of bacterial count (*E. coli* O157:H7, *Shigella* ssp. and *Salmonella*) in cases where one-step treatment did not bring satisfactory results.

As in previously described trials based on reduction of *E. coli* O157:H7 in lettuce, similar attempts with *Salmonella enterica* Enteritidis and Typhimurium serovars were made (Spricigo *et al.* 2013). Fresh-cut romaine lettuce was purchased from a local store in Spain. Pieces of lettuce were contaminated by *S. enterica* Enteritidis or by *S. enterica* Typhimurium (10^5 cfu/ml for 5 minutes). During three-phage cocktail treatment at room temperature (10^9 pfu/ml) the number of bacterial cells was evaluated after 30 and 60 minutes. In all examples the phage cocktail significantly ($p<0.05$) reduced *Salmonella* concentration, which corresponded with a decreasing number of *E. coli* O157:H7 cells on lettuce after specific phage application.

Cutting fresh fruits for commercial purposes deprives them of peel and rind, which constitute a natural barrier against bacterial pathogens. Leverentz *et al.* (2001) state that *Salmonella enterica* Enteritidis populations can survive on fresh-cut melons and apples, showing increased growth with increasing temperature. The fruit slices after contamination with 25 µl of *Salmonella* suspension (10^6 cfu/ml) were treated with 25 µl of a phage mixture (2 x 10^{10} pfu/ml, diluted before application to 10^8 pfu/ml) consisting of four lytic phages obtained from Intralytix, Inc. Slices were incubated at 5, 10 and 20°C and the number of *Salmonella* cells was measured at 0, 3, 24, 48, 120 and 168 hours after phage inoculation. During examination phage persistence was much higher on melon slices and decreased to a nondetectable level after 24 hours on apple slices. Further investigation showed that low pH of apples (4.2) was a possible factor inhibiting phage survivability. In contrast, the *Salmonella* strain survived at all pH and temperature regimes. Moreover, at 20°C its population started increasing 3 hours after inoculation on both melon and apple slices. Phages were able to significantly reduce *Salmonella* populations only on melon slices. Overall, phages seemed to be pH sensitive during treatment.

Phages against *Listeria monocytogenes*

Listeria monocytogenes is another pathogen linked with foodborne outbreaks after consumption of red bell peppers, romaine lettuce, sprouts, apple slices and processed and mixed fruits and vegetables (Leverentz *et al.* 2004). *Listeria monocytogenes* can survive at low temperatures on refrigerated food products, which constitutes a serious problem in the food industry. Listeriosis can be dangerous especially in pregnant women, neonates, the elderly and in people with immune malfunctions (Vázquez-Boland *et al.* 2001). The disease is characterized by a high mortality rate ranging from 20 to 30% (Choffnes *et al.* 2012).

Preventive usage of a phage cocktail on honeydew melon pieces was tested by Leverentz *et al.* (2004). A mixture of six *Listeria* phages, named LMP-102, was provided by Intralytix, Inc. The phage suspension was diluted to various concentrations and sprayed on slices. Application of the phage cocktail before bacterial contamination (5 x 10^5 cfu/ml) or at the same time was most effective. Phage treatment after contamination resulted in increasing growth of bacteria by the fifth day of storage at 10°C. Phage titers over 10^6 pfu/ml successfully inhibited growth of bacteria but 10^8 pfu/ml concentration reduced the number of bacterial cells to nondetectable levels. This indicates that use of higher phage doses and phage treatment immediately after bacterial contamination (for example during cutting slices and packaging) is the most suitable way to control pathogen growth. The same authors also investigated efficacy of phage treatment depending on various

numbers of bacterial cells contaminating fresh-cut products (the smaller the counts of bacteria, the greater the suppression of the *Listeria* population by phages on melon slices after 5 and 7 days of storage at 10°C) (Leverentz *et al.* 2003). Combined treatment of phage cocktail and nisin (bacteriocin produced by lactic acid bacteria) was more efficient than single treatment on melon as well as on apple slices, similar to results of combined treatment (phage application and levulinic acid produce wash) described by Magnone *et al.* (2013). The authors recommend using low-pH-tolerant phage mutants or high phage doses on fresh-cut products with low pH.

Oliveira *et al.* (2014) evaluated practical application of Listex P100 preparation on melon, pear and apple products (juices and slices). The fruit slices were inoculated with Listex P100 by pipetting a phage suspension (10^8 pfu/ml) onto cored wells in slices after bacterial inoculation. Samples of juices were mixed with the phage and bacteria suspension to obtain a final concentration of 10^5 cfu/ml and 10^8 pfu/ml. The pH of apple products showed the lowest levels (3.70-3.76), followed by pear (4.61-4.91). The highest pH value was measured in melon slices (5.77-5.92). In harmony with these results the largest statistically significant reduction of *L. monocytogenes* was observed on melon slices during all 8 days of the storage period. The phage titer decreased on apple slices below a detectable level. A similar effect was observed in fruit juices, where no significant reduction of bacterial counts in apple juice was noted. However, phage application was more efficient in juices as a liquid environment may contribute to better contact with bacteria host cells. These results, together with results related to pH-sensitive *Salmonella* phages obtained previously by Leverentz *et al.* (2001), confirm the idea that phage persistence under various environmental conditions is an important parameter when considering phages as biocontrol agents. Too low pH can constitute a limiting factor for phage treatment of fruits, especially those stored at higher temperatures (above 5°C).

Positive and negative implications of exploiting phages in the fruit and vegetable industry

Phages can play an important role in agriculture and the food industry and their role is not restricted to elimination of pathogenic bacterial strains. An advantage of their use is the increasing number of genetically well-characterized phages. This enables researchers to identify and eliminate genes responsible for lysogeny or bacterial virulence (Frampton *et al.* 2012). It was shown that phages can be responsible for high virulence of bacterial phytopathogens as they can be involved in horizontal gene transfer causing exchange of virulence

factors between bacterial genomes (Stavrinides 2009). On the other hand, ongoing revisions in bacterial taxonomy imply the necessity of isolation and full characterization of new phages highly specific to bacterial pathogenic strains (Jones *et al.* 2012). However, high specificity is an important feature which can be exploited in the field. Phages generally do not lyse rhizobia, bacteria involved in biological nitrogen fixation, inseparably connected with the *Fabaceae* family represented by important agricultural species such as soybean, beans, pea, chickpeas, and alfalfa. Moreover, in experiments by Basit *et al.* (1992), a specific phage coating of soybean seed increased nodulation by the superior *Bradyrhizobium japonicum* strain USDA 110 from 48 to 82%. A deleterious effect of phages on rhizobacteria was investigated as well. Svircev *et al.* (2010) describe an experiment where large populations of phages introduced into the rhizosphere of clover plants reduced the *Rhizobium trifolii* population.

Without any doubt, the ability of phages to penetrate plant tissues and to lyse some phytopathogens needs further investigations. For example, extracellular polysaccharides produced by *Ralstonia solanacearum* may inhibit phage adsorption to host cells on plants (Fujiwara *et al.* 2011). Unfavorable environmental conditions within the rhizo- and phyllosphere (drought, pH variation, temperature, UV irradiation) constitute a challenge for researchers working on optimal phage formulations and investigating phage-host interactions (Kocharunchitt *et al.* 2009). Although it was revealed that some phages may be extremely resistant to a dry environment and may survive in extreme temperatures (Jończyk *et al.* 2011), it seems such conditions make phage infection and replication impossible. Furthermore, phages can strongly bind to clay particles via electrostatic interactions, resulting in loss of their lytic activity [Ye *et al.* 2009]. The still common chemical treatment under field and greenhouse conditions (i.e. ionic copper, surfactants, iron chelators) additionally affects phages (Bae *et al.* 2012). Wang and Sabour (2010) describe development of protective formulations (encapsulation) of phages for food animal production. As phage application under greenhouse or field conditions encounters quite different obstacles than the food animal industry (lack of difficulties connected with different gastrointestinal fluids reducing phage efficacy), slow release of phage particles may be a promising solution for phage survivability under unfavorable environmental conditions.

Occurrence of phage resistance within bacterial populations is another problem which can limit phage application in the food industry. Using a combination of phages did not always reduce the probability of resistant mutants appearing (Ye *et al.* 2010). Of note, phages are able to kill bacterial cells without carrying out a lytic cycle in a process called "lysis from without" (Ferguson *et al.* 2013). This attribute is probably responsible for reducing the number of bacteria on fresh products at refrigeration temperature where the lytic cycle is hard to perform. The occurrence of numerous phage particles adsorbing to the host cell results in cell

wall damage. The number of bacterial hosts is no less significant during phage treatment. In nature, under unfavorable conditions, the bacterial population density required for phage replication at a high level can be too low (Wiggins and Alexander 1985).

On the other hand, there are concerns about the safety of phage release into the environment as they can disrupt the naturally occurring ratio between phages and bacteria and disrupt nutrient cycling (Svircev *et al.* 2011; Meaden and Koskella 2013). Furthermore, some phages may infect strains used to promote plant growth and to suppress fungal plant diseases such as *Pseudomonas fluorescens* CHA0 lysed by ΦGP100 phage (Keel *et al.* 2002).

Some sources indicate that prophages can have a favorable effect on pathogenic bacteria (Bondy-Denomy and Davidson 2014). They are able to inhibit other phages in a process called "superinfection exclusion" through, for example, blocking the cell surface adsorption of superinfecting phages T1, Φ80 and N15. Prophages may also increase the fitness of bacterial cells as they are involved in controlling sporulation, inducing exopolysaccharide production and long-term survivability of *Bacillus anthracis* in soil. Novel *in situ* phage induction and collection methods revealed that circa 80% of the soil strains were lysogenic. A very interesting phenomenon was observed in aphids – well-known pests on cultivated plants. Prophage-encoded toxins produced by the symbiotic bacterium *Hamiltonella defensa* are responsible for protection of the aphid host from attack by a parasitoid wasp.

Other possibilities of phage application in vegetable production

The importance of phages in the fruit and vegetable industry is not limited to biocontrol of pathogenic bacterial strains. Phages are present in the process of vegetable fermentation, although their role remains uncertain (Yoon *et al.* 2002; Lu *et al.* 2003a, 2003b, 2010, 2012). However, a possible negative role of phages in industrial food fermentation was described as well. Kleppen *et al.* (2012) draw a conclusion that phages may be responsible for affecting bacterial starter cultures in vegetable fermentation, such as lactic acid bacteria *Weissella cibaria* involved in fermentation of kimchi, a traditional Korean dish made from Chinese cabbage and radish.

Phages may also be involved in detection of phytopathogens and fecal food contamination. Schofield *et al.* (2012, 2013) described recombinant phages used as diagnostic tools for the detection of phytopathogens. Endley *et al.* (2003a, 2003b) investigated application of coliphages as a fecal contamination indicator on fresh carrots and herbs. It appears to be an important feature in the food industry as most food-borne illnesses are preventable (Choffnes *et al.* 2012). Endley *et al.* (2003b) note that phages are more useful as fecal contamination indicators than bacterial indicators because they can be resistant to commonly used disinfection measures in water. This feature was confirmed later by Weber-Dąbrowska *et al.* (2014). The bacterium *E. coli* was also more susceptible to sanitizers such as chlorine bleach, hydrogen peroxide, peroxyacetic acid and sodium bicarbonate than coliphage MS2 during experiments performed on leafy salad vegetables (Allwood *et al.* 2004). The positive and negative impacts of phages on the fruit and vegetable industry are presented in Figure 1.

Conclusions

In the last few years several authors have evaluated the potential of using phages in biocontrol of bacterial phytopathogens as well as in biocontrol of human foodborne pathogens. These applications have a long history and in both instances many areas linked with phage application still need to be intensively investigated. Interactions between phages and their bacterial hosts still remain largely unexplored. Differences in the outcomes of several investigations clearly affirm this fact. Greater availability of molecular techniques and decreasing costs of research definitely facilitate the undertaking of further studies. Together with better recognition of phage biology, morphology, ultrastructure and genetics, attempts to evaluate phage preparations are becoming more frequent.

As mentioned above, the importance of phages in the fruit and vegetable industry is not limited only to biocontrol of pathogenic bacterial strains. Both the positive and negative roles of phages in the environment provide a vivid picture of their importance for humanity. Popularization of this knowledge is no less significant than focusing on research, and has to be done for wider public acceptance.

Conflict of interest

No conflict of interest declared.

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Table 1. Phage applications in plants under greenhouse and field conditions

Target organism	Phage symbol	Plant	Type of investigation	Application method	Result
Xanthomonas axonopodis pv. *citri*	CP2, ΦXac2005-1, ccΦ7, ccΦ13	grapefruit, cultivar Duncan	greenhouse trial	the emerging foliage was treated [10^8-10^9 pfu/ml] in the evening prior to bacterial infection [10^6-10^8 cfu/ml]	phages suspended in clear water caused an average 59% reduction in disease severity. [Balogh *et al.* 2008]
	ΦXaacA1	grapefruit, cultivar Duncan	nursery trial	plants were sprayed with phage suspension [10^6 pfu/ml] after bacterial infection	phage solution in skim milk powder significantly reduced lesion number per diseased leaf from 2.6 and 3.1 to 2.0 and 2.1 in both tests [Balogh *et al.* 2008]
Xanthomonas axonopodis pv. *citrumelo*	ΦXacm2004-4, ΦXacm2004-16, ΦX44	grapefruit, cultivar Duncan*, orange, cultivar Valencia†	nursery trial	phages [10^8 pfu/ml] in a suspension of 0.75% skim milk powder were sprayed at dawn to control a naturally occurring bacterial pathogen population	three-phage cocktail significantly (48% and 35%) reduced the disease only in oranges [Balogh *et al.* 2008]
	ccΦ13, α-MME, Φ5536	citrumelo plant*	nursery trial	phages [10^8 pfu/ml] suspended in 0.75% skim milk solution were applied in the evenings after bacterial infection [10^7 cfu/ml]	phage solution significantly reduced mean disease incidence (from 22.2% to 8,9%) and area under the disease progress curve (from 24.3 to 14.9) [Balogh *et al.* 2008]
Xanthomonas arboricola pv. *juglandis*	mixture of six soil phages	walnut, cultivar Payne	greenhouse trial	the buds were sprayed with a mixture of phages [10^7 pfu/ml] suspended in water to control a naturally occurring bacterial pathogen population	phages showed very poor survivability and their number was not significantly different from untreated branches [McNeil *et al.* 2001]
Xanthomonas campestris pv. *vesicatoria*	Agriphage, OmniLytics Inc. (six to eight different phages)	tomato, cultivar Bonny Best	greenhouse trial	the plants were treated with a phage mixture [10^{10} pfu/ml] two hours prior to bacterial infection [10^8 cfu/ml]	phages significantly reduced the lesion numbers on plants by 30-62% [Balogh *et al.* 2003]

	Agriphage, OmniLytics Inc. (six to eight different phages)	tomato, cultivars Mountain Fresh, BHN 444, and BHN 555	field trial	phage applications [10^{10} pfu/ml] were begun before bacterial inoculation [10^{8} cfu/ml] and were continued until two weeks before harvest	evening applications resulted in better control than morning applications (27% and 13% disease reduction respectively) [Balogh *et al.* 2003]
Xanthomonas pruni	not specified	peach, cultivar Elberta	greenhouse trial	peach seedlings were sprayed with phage lysates [10^{9}-10^{10} pfu/ml] in different combinations	up to 65% disease reduction when phages were applied before pathogen inoculation [10^{8} cfu/ml] [Civerolo and Keil 1969]
Pectobacterium carotovorum subsp. *carotovorum*	PP1	lettuce	greenhouse trial	seedlings were sprayed with a high-titer phage suspension with addition of $MgCl_2$ one day after pathogen inoculation [10^{8} cfu/ml]	over 80% of phage-treated seedlings did not show disease symptoms [Lim *et al.* 2013]
Ralstonia solanacearum	ΦRSA1, ΦRSB1, ΦRSL1	tomato, cultivar Oogata-Fukuju	greenhouse trial	plants were soaked with a phage solution [10^{10} pfu/ml] before bacterial infection [10^{8} cfu/ml]	plants treated only with phage ΦRSL1 had no wilting symptoms. [Fujiwara *et al.* 2011]
	PE204	tomato, cultivar Seogun	greenhouse trial	phage solution [10^{8} pfu/ml] in SM buffer was applied to tomato plants in different combinations	phage application together with bacterial cells [10^{7} cfu/g of soil] completely inhibited bacterial wilt symptoms [Bae *et al.* 2012]
Streptomyces scabies	ΦAS1	potato, cultivar Kennebeck	greenhouse trial	diseased [10^{10} cfu/ml] tubers were immersed in phage suspension [10^{9} pfu/ml] for 24 hours	phage-treated tubers had a significantly reduced number of lesions per tuber (from 44.23 to 3.82) and lesion surface area (from 23% to 1.20%) [McKenna *et al.* 2001]

* Highly susceptible to bacterial pathogen

† Moderately susceptible to bacterial pathogen

Table 2. Phage applications on fresh-cut vegetables

Target organism	Phage symbol	Vegetable product	Application method	Result
Escherichia coli O157:H7	ECP-100 (cocktail of three lytic phages ECML-4, ECML-117, ECML-134)	broccoli and spinach samples, tomato slices	contaminated food samples (710 cfu/g of broccoli, 650 cfu/g of tomato, 14 000 cfu/g of spinach) were sprayed with ECP-100 phage cocktail [10^8-10^{10} pfu/ml]	bacterial contamination was reduced by over 90% during storage at 10°C for 24, 120 and 168 hours [Abuladze *et al.* 2008]
	BEC8 (mixture of eight lytic phages)	baby spinach, baby romaine lettuce leaves	phages [10^7 pfu/ml] were applied on top of the leaf spots previously inoculated with bacterial strains at three different MOI levels (1, 10 and 100)	reduction of at least one log cfu in the number of *E. coli* O157:H7 cells after 24 h at all temperatures tested above 8°C [Viazis *et al.* 2011]
	EcoShield (mixture of three lytic phages)	iceberg lettuce leaves	cut lettuce pieces were immersed in phage solution [10^8-10^9 pfu/ml] before pathogen inoculation (2.38 cfu/cm^2)	after six days of incubation at 4°C the population of *E. coli* O157:H7 was reduced to an undetectable level [Ferguson *et al.* 2013]
	EcoShield (mixture of three lytic phages)	green leaf lettuce and spinach	leafy greens were sprayed with EcoShield [10^7 pfu/ml] after pathogen inoculation [10^7 cfu/ml]	application of EcoShield reduced EHEC populations after 30 min and 2 h of storage at 4°C and 10°C by 2.38-3.28 log cfu/cm^2 [Boyacioglu *et al.* 2013]
Salmonella Oranienburg	SSP5, SSP6	alfalfa seeds	artificially contaminated seeds were immersed in phage solution to give an MOI of approximately 70	only 10^1 cfu/g reduction of viable *Salmonella* 3 h after phage application at 25°C [Kocharunchitt *et al.* 2009]
Salmonella cocktail consisted of several serovars	cocktail of the six selected bacteriophages (F01, P01, P102, P700, P800, and FL 41)	mung beans, alfalfa seeds	beans or seeds were soaked in *Salmonella* cocktail [10^6 cfu/ml], then in suspension of *E. asburiae* JX1 [10^6 cfu/ml] and bacteriophage cocktail [10^6 pfu/ml]	combined treatment reduced the level of *Salmonella* by 6.72 log cfu/g on sprouting mung beans after 4 days at room temperature. Similar results were obtained for alfalfa sprouts. [Ye *et al.* 2010]
Salmonella Javiana	cocktail of five lytic bacteriophages	red tomatoes	ripened tomatoes were immersed in a pathogen suspension [10^6 cfu/ml] and then in a suspension of *E. asburiae* JX1 [10^6 or 10^3 cfu/ml] with bacteriophage	combined treatment had a negligible impact on the final populations of the pathogen. [Ye *et al.* 2009]

			cocktail [10^6 or 10^3 pfu/ml]	
Salmonella Enteritidis and Typhimurium	cocktail of three lytic bacteriophages (UAB_Phi 20, UAB_Phi78, and UAB_Phi87)	romaine lettuce	pieces of lettuce were contaminated by a pathogen suspension [10^5 cfu/ml], then the phage cocktail was applied [10^9 pfu/ml]	phage cocktail reduced the number of bacterial cells over 3 log cfu/g in *S.* Typhimurium and ca. 2 log cfu/g of lettuce in *S.* Enteritidis at room temperature [Spricigo *et al.* 2013]
Salmonella Enteritidis	SCPLX-1 (cocktail of four lytic bacteriophages)	Red Delicious apples, Honeydew melons (slices)	artificially contaminated [10^6 cfu/ml] fruit slices were treated with phage cocktail [10^8 pfu/ml]	number of bacterial cells in melon slices at 5 and 10°C was 3.5 log units lower than untreated control samples. No significant reduction was observed in apple slices. [Leverentz *et al.* 2001]
Listeria monocytogenes	LMP-102 (cocktail of six lytic bacteriophages)	Honeydew melons	melon pieces were inoculated with bacterial suspension [10^5 cfu/ml]; phage solution was sprayed at different concentrations [10^4 - 10^8 pfu/ml]	phage applications from 1 h before to 4 h after contamination reduced bacterial populations (from 1.5 to 6.9 log cfu/treatment after 7 days of storage at 10°C) [Leverentz *et al.* 2004]
	Listex P100	apples, pears, melons (slices)	the fruit slices were inoculated with phage solution [10^8 pfu/ml] after bacterial inoculation [10^5 cfu/ml]	1.5 log cfu reduction of bacterial populations was observed on melon during the whole storage period at 10°C [Oliveira *et al.* 2014]
	Listex P100	apples, pears, melons (juices)	juices were mixed together with phage [10^8 pfu/ml] and bacterial suspension [10^5 cfu/ml]	ca. 8 log cfu reduction of bacterial populations was observed in melon juice after 8 days of storage at 10°C [Oliveira *et al.* 2014]