International Journal of Food Microbiology 191 (2014) 69–74

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Bacteriophage P22 to challenge *Salmonella* in foods

Paola Zinno [a,b], Chiara Devirgiliis [b], Danilo Ercolini [a], Duncan Ongeng [c], Gianluigi Mauriello [a,*]

[a] Department of Agriculture, University of Naples Federico II, via Università 100, 80055 Portici, Italy
[b] CRA-NUT, Food and Nutrition Research Center, Agricultural Research Council, 00178 Rome, Italy
[c] Faculty of Agriculture and Environment, Gulu University, Uganda

ARTICLE INFO

Article history:
Received 3 March 2014
Received in revised form 18 August 2014
Accepted 19 August 2014
Available online 16 September 2014

Keywords:
Bacteriophage biocontrol
Bacteriophage P22
Salmonella Typhimurium

ABSTRACT

In this study we considered the influence of phage addition on the fate of *Salmonella enterica* serovar Typhimurium in different foods. Phage P22 was applied to the following: liquid eggs, energy drinks, whole and skimmed milk, apple juice, chicken breast and chicken mince all spiked with its host, whose growth was monitored for 24 and 48 h at 4 °C. Appreciable host inactivation, generally in the order of 2 log cycles, was achieved compared to phage-free controls in all food matrices when 10^4 UFC/g host inoculum was used. Furthermore, wild food strains belonging to the serotypes Typhimurium, Enteritidis, Derby Give, Newport, Muenchen and Muenster were assayed towards phage P22. Only isolates of *Salmonella* Typhimurium as well as *Salmonella* Derby and *Salmonella* Enteritidis was inhibited by the presence of P22 phage. Additional challenge experiments were carried out by spiking liquid-eggs, chicken breast and chicken mince with mixes of wild *Salmonella* Typhimurium (at concentration of about 10^4 UFC/g) strains along with their relative phage P22. The results showed a reduction of 2–3 log cycles after 48 h at 4 °C depending on both mix of strains and the specific food. Overall, the results indicate that phages may be useful in the control of food-borne pathogens. The food matrices considered, the liquid more than the solid, do not seem to affect the phage ability of infection compared to similar tests performed *in vitro*.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

The food industry has interest in finding alternative approaches to inactivate bacterial pathogens. As a matter of fact, in recent years the interest for the application of bacteriophages as potential biocontrol agents is widely increasing. Bacteriophages have been recognized as a possible alternative to antibiotics in animal health, as biopreservatives in foods and finally as tools for detecting pathogenic bacteria (Henry and Debarieux, 2012). They are viruses that infect and kill (lytic phages) bacterial cells. Their host range is very diverse, and some of them are specific for a narrow range of bacterial strains while others show a broad host range of bacterial strains. Their application may help prevent an incidence of food-born diseases caused by pathogens like *Salmonella*, *Campylobacter*, *Escherichia*, *Listeria* and others, reducing food processing (e.g. temperature application) and use of chemical additives (e.g. sulphite and nitrate) (Hagens and Loessner, 2010; Guenther and Loessner, 2011).

The approval by FDA of the use of the LMP 102 phage preparation (ListShield™ Intralytix) as an additive to control *Listeria monocytogenes* in ready-to-eat food and later its recognition of GRAS status for its use in all food products has allowed the development and the offering of many phage preparations against pathogens by many companies such as Micreos, OmniLytics, Novolytics, Biophage Inc. Furthermore, the US Environmental Protection Agency (EPA) approved a phage cocktail to control tomato and pepper pathovars of *Pseudomonas putida*, to use for the plants' treatment against bacterial spot disease (Balogh et al., 2010). The application of bacteriophages as biocontrol has been investigated in a variety of foods. The addition of anti-*Salmonella* bacteriophages to cheese milk was shown to reduce the number of *Salmonella* Enteriditis in cheese made both from raw and pasteurized milk (Modi et al., 2001). Garcia et al. (2007) focused their study on anti-staphylococcal bacteriophages employed in curd manufacturing and several studies have focused on the use of phage to eradicate *L. monocytogenes* in soft cheese, mozzarella cheese and other similar dairy products (Carlton et al., 2005; Schellekens et al., 2007). Recently, Hagens and Loessner (2010) reviewed the use of bacteriophage in foods for biocontrol of undesired bacteria. Furthermore, they report some important considerations regarding the application of phages in foods, highlighting the reasons to avoid lysogeny.

In addition to dairy products, other food matrices were used to investigate the potential of bacteriophages to control the growth of foodborne pathogens. For instance, Guenther et al. (2012) described the application of the bacteriophage FO1-E2 to control the growth of *Salmonella* Typhimurium in some ready-to-eat foods such as cooked and sliced turkey breast and egg yolk. Furthermore, alfalfa seeds were artificially contaminated with a *Salmonella* Oranienburg culture and

* Corresponding author at: Via Università 100, 80055 Portici (NA), Italy. Tel.: +39 0812539452; fax: +39 0812539407.
E-mail address: giamauri@unina.it (G. Mauriello).

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.037
0168-1605/© 2014 Elsevier B.V. All rights reserved.

the phage SSP6 used to control the bacterial growth during sprouting (Kocharunchitt et al., 2009). Another study focused on the use of phages as biocontrol agents of *Salmonella* Typhimurium and *Campylobacter jejuni* in cooked and raw meat (Bigwood et al., 2008). More recently, Oliveira et al. (2014) reported the effectiveness of the bacteriophage Listex P100 in reducing *L. monocytogenes* on fresh-cut fruits and fruit juices.

The aim of this work was to investigate the influence of P22 phage addition on the fate of *Salmonella* Typhimurium, in different food matrices.

2. Materials and methods

2.1. Bacteria and bacteriophage

The microorganisms used were *Salmonella enterica* serovar Typhimurium LT2, host of bacteriophage P22, and 18 isolates of *S. enterica* from chicken samples and belonging to seven different serovars (12 Typhimurium, 1 Derby, 1 Enteritidis, 1 Give, 1 Newport, 1 Muenchen and 1 Muenster). Isolates were provided by Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici, Italy). All the bacteria were routinely grown in Tryptone Soya Broth (TSB, Oxoid, UK) at their optimal growth temperature.

Bacteriophage P22 was amplified adding 0.1 ml of a 10^7 PFU/ml suspension to 10 ml of TSB containing 1:100 dilution of an overnight culture of its host LT2. The mixture was incubated overnight at 37 °C until complete lysis occurred. Then the incubation was prolonged and 1 ml of bacterial culture was added at 1 h intervals for 3–4 times, in order to obtain a further amplification of the phage population. After centrifugation (7000 g for 10 min at 4 °C) the supernatant was filtered through a 0.22 μm pore size filter (Minisart® plus, Sartorius AG, Goettingen, Germany) and the filtrates were stored at either 4 °C until used or at −20 °C for long-term storage. The titre of the phage stock (PFU/ml) was determined by the double-layer plaque titration method (Civerolo, 1990) using Tryptone Soya Agar (TSA, Oxoid, UK) for the bacterial growth.

2.2. Challenge tests with a single microorganism

Seven different foods purchased at local markets, five liquid (energy drink, apple juice, pasteurized whole and skimmed milk, whole egg) and two solid (sliced chicken breast and chicken mince) were selected to cover the spectrum of products frequently found to be contaminated by *Salmonella* Typhimurium. Whole eggs were aseptically broken and transformed in liquid whole eggs by gentle homogenization. All food samples were preliminarily analyzed, according to the standard procedures, in order to check the possible natural contamination by *Salmonella*. Samples (200 ml) of liquid foods, previously conditioned at 4 °C, were contaminated with diluted over-night cultures of *Salmonella* Typhimurium LT2, to reach a cell load of approximately 10^4 CFU/ml. Food samples were incubated at 4 °C for 1 h allowing the bacteria to adapt to the environmental conditions. Afterwards, an aliquot of 100 ml was added of 1 ml of a P22 phage suspension at 10^{12} PFU/ml to reach a multiplicity of infection (MOI) of 10^8. Solid foods, previously conditioned at 4 °C, were treated as following described. Briefly, six slices (about 50.0 cm^2 and 30 g each) of chicken breast were superficially spiked with a 5 ml suspension of *Salmonella* Typhimurium LT2 at 10^5 CFU/ml. After 1 h at 4 °C, 5 ml of a P22 phage suspension at 10^{12} PFU/ml was inoculated, by using a small spray dispenser, on the surface of three slices, to reach a MOI of about 10^7, while the remaining three slices were used as control samples. Furthermore, 300 g of chicken mince was homogeneously contaminated with a 3 ml suspension of *Salmonella* Typhimurium LT2 at 10^6 CFU/ml. After 1 h at 4 °C the sample was divided in two parts of 150 g each; 8 ml of a P22 phage suspension at 10^{12} PFU/ml was homogeneously included into a 150 g fraction and then six balls of about 25 g each were prepared. The remaining part was used to prepare control samples of chicken balls. Bacterial loads of challenged microorganism were monitored by viable count on TSA immediately after phage addition and after 24 and 48 h of storage at 4 °C. Viable count of *Salmonella* Typhimurium LT2 was performed on one slice of chicken breast and a mix of two balls of chicken mince for each time. The experiments were carried out in three separated trials and mean value of control was compared to that of treated samples by t-test (Microsoft Excel 2011 for Mac) to ascertain significant differences ($P < 0.01$).

2.3. P22 phage host range

The overlay method (Zinno et al., 2010) was used to determine the host range of P22 phage within the group of 18 *Salmonella* isolates. Briefly, 0.5 ml of a *Salmonella* culture at mid exponential growth phase was added to 5 ml of soft agar, mixed by gentle swirling and then poured on a TSA agar base plate. Ten microliters of a P22 bacteriophage suspension at 10^7 PFU/ml was spotted onto the bacterial lawn and the plate incubated at 37 °C for 24 h. The presence of a clear growth inhibition halo (lysis zone) in correspondence of spots indicated the susceptibility of the microorganism to the P22 bacteriophage. In this experiment *Salmonella* Typhimurium LT2 was used as positive control.

2.4. Challenge tests with undefined cocktails of Salmonella Typhimurium in liquid eggs

Three different challenge tests were carried out using a different cocktail of four *Salmonella* Typhimurium isolates, in order to use all twelve isolates. Experiments were performed as follows: 200 ml of liquid eggs, previously conditioned at 4 °C, was contaminated with a cocktail of four isolates of *Salmonella* Typhimurium to reach 10^4 CFU/ml of each one. Food sample was incubated at 4 °C for 1 h allowing the bacteria to adapt to the environmental conditions. One hundred milliliters of sample was added of P22 phage solution to reach a MOI of 10^5. The remaining 100 ml of contaminated liquid eggs was treated as control sample. Bacterial load was monitored by viable count on TSA immediately after phage addition and after 24 and 48 h of storage at 4 °C.

The experiment was carried out in three separated trials and mean values were compared by a t-test (Microsoft Excel 2011 for Mac) to ascertain significant differences.

2.5. Strain typing of Salmonella isolates

Total DNA was extracted, according to Marmur (1961) protocol, from 10 ml of overnight *Salmonella* cultures showing susceptibility towards the P22 bacteriophage. After the extraction, the DNA was quantified by using the Nanodrop 1000 (Thermo Scientific, Milano, Italy). DNA samples were stored at −20 °C. The visualization by ethidium bromide staining was performed according to standard protocols (Sambrook and Russell, 2001).

For REP-PCR the primers REP1 (5′-IIIGCGCCGICATCAGGC-3′) and REP2 (5′-ACGTCTTATCAGGCCTAC-3′) were used (Albufera et al., 2009). ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used for ERIC-PCR as reported by de la Puente-Redondo et al. (2000). All PCR reactions were performed in a 25 μl solution containing 125 μM deoxynucleotide triphosphate, 5 μM of the primers, 2.5 U of *Taq* DNA polymerase (Invitrogen, Milano, Italy), *Taq* buffer (20 mM Tris–HCl pH 8.4, 1.5 mM magnesium chloride, 50 mM potassium chloride), and 60 ng of template DNA. The conditions of REP-PCR amplifications were set as follows: 1 cycle at 95 °C for 5 min; 30 cycles at 90 °C for 30 s, at 50 °C for 30 s, at 52 °C for 1 min, and at 72 °C for 1 min; and 1 cycle at 72 °C for 8 min; for ERIC-PCR 1 cycle at 95 °C for 3 min; 30 cycles at 90 °C for 30 s, at 40 °C for 1 min, and at 72 °C for 1 min; and 1 cycle at 72 °C for 8 min. PCR products were separated by electrophoresis in a

Table 1
Viable count (log CFU/ml) of *Salmonella* Typhimurium LT2 after 0, 24 and 48 h at 4 °C, alone (control, C) and in contact (treated, T) with the bacteriophage P22 in different liquid foods.

Time (h)	Foods									
	Energy drink		Apple juice		Whole milk		Skimmed milk		Liquid egg	
	C	T	C	T	C	T	C	T	C	T
0	4.06^{aA}	4.17^{aA}	4.46^{aA}	4.58^{aA}	4.57^{aA}	4.36^{aA}	4.62^{aA}	4.21^{aA}	4.11^{aA}	4.10^{aA}
24	4.16^{aA}	2.03^{bB}	3.68^{aB}	1.23^{bB}	4.15^{aA}	2.42^{bB}	4.07^{aA}	2.10^{bB}	4.19^{aA}	3.39^{bB}
48	4.23^{aA}	2.14^{bB}	3.18^{aB}	1.12^{bB}	4.45^{aA}	0^{bC}	4.32^{aA}	0^{bC}	3.22^{aB}	2.26^{bC}

Mean values of three different trials for each challenge test. Different superscripts (lowercase) comparing control (C) and treated (T) sample at the same time within the same food indicate a significant difference ($P < 0.01$). Different superscripts (uppercase) comparing different times within each sample indicate a significant difference ($P < 0.01$).

1.2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide and visualized under UV illumination.

2.6. Challenge test with a two-strain cocktail of Salmonella Typhimurium in liquid eggs

Two hundred milliliters of liquid eggs were contaminated with a double-strain cocktail of *Salmonella* Typhimurium to reach 10^4 CFU/ml of each one. Salmonella strains in the cocktail were chosen because of the different strains according to the results of the strain typing. The food sample was incubated at 4 °C for 1 h allowing the bacteria to adapt to the environmental conditions. One hundred milliliters of sample was added of P22 phage solution to reach a MOI of 10^5. The remaining 100 ml of contaminated liquid eggs was used as a control. Bacterial load was monitored by viable count on TSA immediately after phage addition and after 24, 48 and 72 h of storage at 4 °C. Finally, at each time 30 colonies were randomly picked-up from countable plates and used for fingerprinting analysis in order to verify the efficacy of P22 phage towards each strain of *Salmonella* in the couple used. The experiment was carried out in three separated trials and mean values were compared by a t-test (Microsoft Excel 2011 for Mac).

3. Results and discussion

3.1. Challenge tests with a single microorganism

The results of challenge tests performed against a single microorganism in liquid foods are reported in Table 1. The cell loads at the initial time of each microorganism in the different treated foods do not show significant differences ($P > 0.01$) compared to control samples. On the contrary, a significant difference ($P < 0.01$) in the mean value of viable count at 24 and 48 h was shown comparing control and treated sample of all liquid foods. However, it is interesting to highlight the dissimilar behavior of the microorganism in different foods both with and without phage treatment. In particular, results showed that in apple juice and liquid egg there was a significant reduction ($P < 0.01$) of the bacterial population of about 1 log cycle after 48 h, while in other foods the cell loads were not significantly affected ($P > 0.05$). On the other hand, the presence of P22 phage determined the total loss of the bacterial population in milk, the reduction of 3 log cycles in apple juice and 2 log cycles in energy drink and liquid egg. Therefore, the maximum effect of the bacteriophage against *Salmonella* Typhimurium LT2 was registered in milk, while the minimum effect in liquid egg. While milk was used in other similar works, apple juice and liquid egg were, to the extent of our knowledge, previously used only by Oliveira et al. (2014) and Guenther et al. (2012), respectively. Oliveira et al. (2014) tested Listex P100 against a mix of three strains of *L. monocytogenes* in apple juice, while Guenther et al. (2012) used the bacteriophage FO1-E2 to control the growth of *Salmonella* Typhimurium in different ready to eat foods and liquid egg yolk. Guenther et al. (2012) registered a difference of 2.6 log units between control and treated sample after two days. However, they stressed the environmental condition with an incubation temperature of 15 °C that led to a growth of untreated population. Instead, the presence of the phage did not reduce the cell load but held constant the population level within two days. Considering the differences in the experimental conditions, we can assert that our results are consistent with that of Guenther et al. (2012). Furthermore, consistently with the results of these authors, we registered the minimum effect of the bacteriophage in liquid egg. They hypothesize that the highly viscous matrix of egg could reduce diffusion and homogeneous distribution of the phage particles. This hypothesis was corroborated by our findings on counting of PFU/ml during the storage period (data not shown). In fact, we registered an increase of PFU/ml from 10^{12} to 10^{14} in all foods with the exception of liquid egg, where a dramatic reduction of PFU/ml was observed. This result could be actually ascribable to the viscous matrix of liquid egg. On the other hand, the increase of PFU/ml gave evidence that P22 infected bacterial cells by a lytic cycle and excluded a "lysis from without" mechanism (Abedon, 2011).

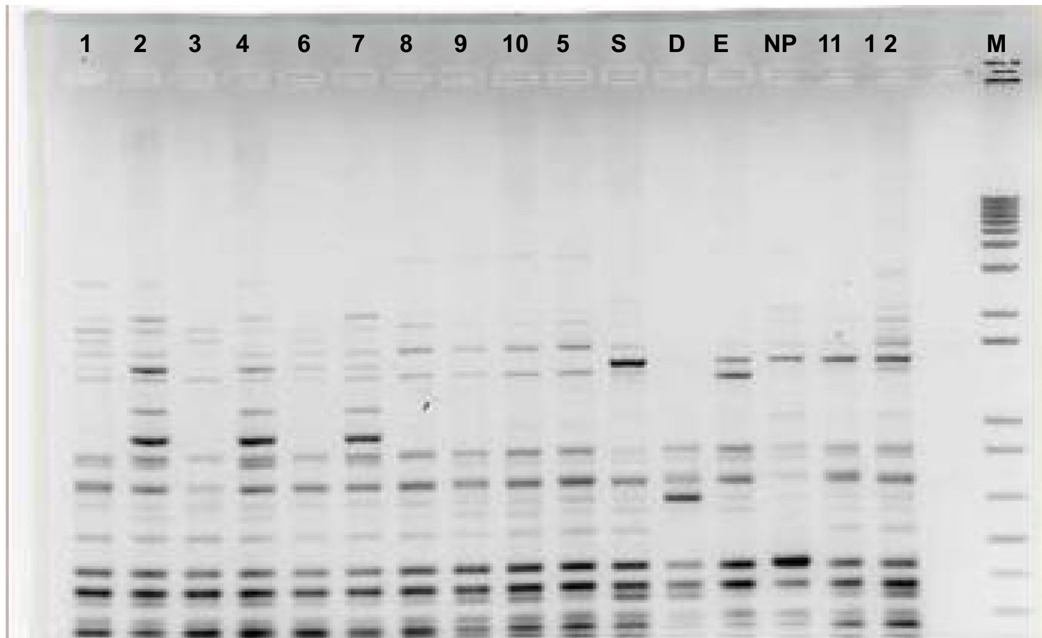
The results of challenge tests performed against *Salmonella* Typhimurium LT2 in chicken meat are reported in Table 2. The bacterial population remained significantly unchanged ($P > 0.05$) after 48 h in both control foods while at the same time a significant reduction ($P < 0.01$) of about 1 and 2 log cycles was observed in phage treated sliced chicken breast and treated chicken mince, respectively. Interestingly, the phage P22 affected the cell load of sliced chicken breast also immediately after the treatment so that a significant difference ($P < 0.01$) with the respective control sample was observed. A significant difference ($P < 0.01$) of about 2 log comparing treated and control samples after 48 h in both chicken foods was registered. However, no significant difference between treated chicken breast samples after 24 and 48 h was found and a difference of only 0.7 log between treated chicken mince samples after 24 and 48 h was registered. This result could suggest the presence of a sub-population less susceptible to phage attack. However, cultures from single colonies after 48 h of incubation showed to be lysed after contact with phage P22 (data not shown). Our result was in contrast with Guenther et al. (2012), who found after 6 days of contact colonies insensitive to bacteriophage FO1-E2.

Table 2
Viable count (log CFU/g) of *Salmonella* Typhimurium LT2 after 0, 24 and 48 h at 4 °C alone (control, C) and in contact (treated, T) with P22 bacteriophage in sliced chicken breast and chicken mince.

Time (h)	Foods			
	Sliced chicken breast		Chicken mince	
	C	T	C	T
0	4.04^{aA}	3.27^{bA}	4.24^{aA}	4.17^{aA}
24	4.16^{aA}	2.43^{bB}	4.22^{aA}	2.83^{bB}
48	4.19^{aA}	2.36^{bB}	4.18^{aA}	2.14^{bC}

Mean values of three different trials for each challenge test. Different superscripts (lowercase) comparing control (C) and treated (T) sample at the same time within the same food indicate a significant difference ($P < 0.01$). Different superscripts (uppercase) comparing different times within each sample indicate a significant difference ($P < 0.01$).

A



B

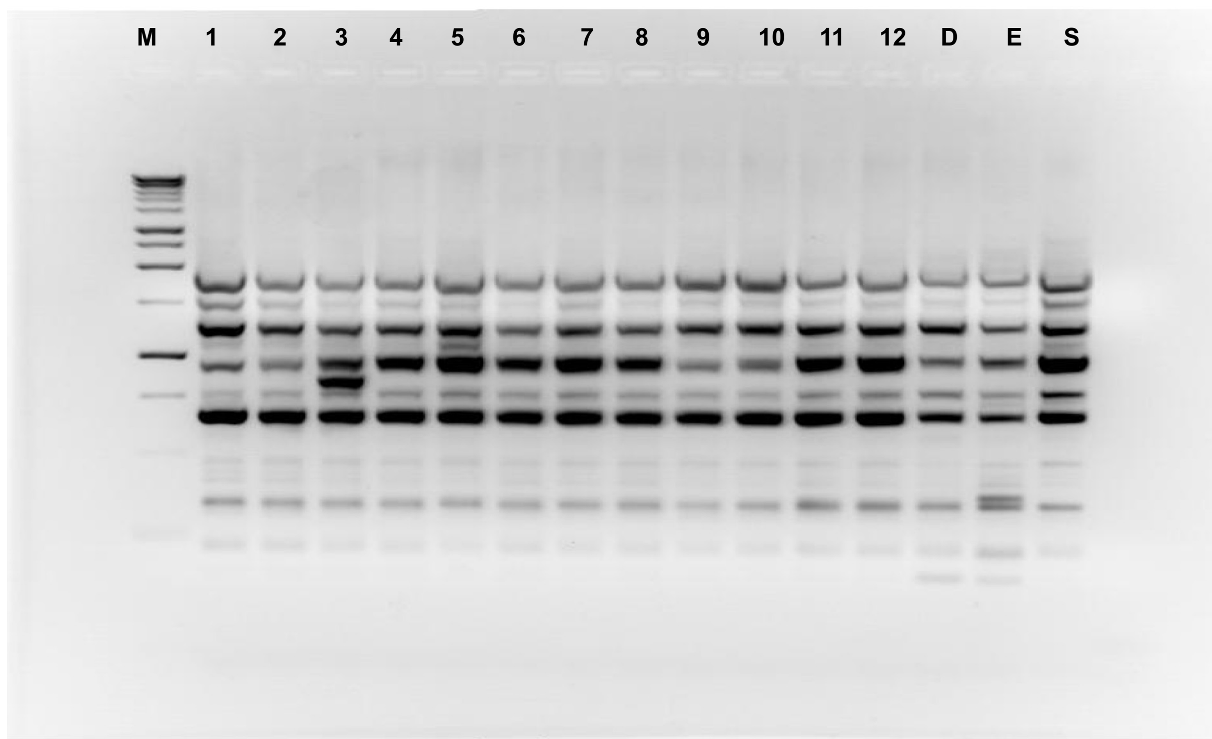


Fig. 1. A. REP-PCR DNA fingerprints of *Salmonella* isolates (lanes 1–12). Lane D: *Salmonella* Derby; lane E: *Salmonella* Enteritidis; lane S: *Salmonella* Typhimurium LT-2; lane NP: *Salmonella* New Port. Lane M: 1 kb plus ladder. B. ERIC-PCR DNA fingerprints of *Salmonella* isolates (lanes 1–12). Lane D: *Salmonella* Derby; lane E: *Salmonella* Enteritidis; lane S: *Salmonella* Typhimurium LT-2. Lane M: 1 kb ladder.

Salmonella is the microorganism mainly involved in food consumption related illness and the major sources of salmonellosis outbreaks include eggs, meats and dairy products (EFSA, 2011); although, poultry and poultry products are the major vehicles for food-borne salmonellosis (Singh et al., 2011). In this context we focused the follow experiments on *Salmonella* and liquid whole egg.

3.2. P22 phage host range

The results of susceptibility of *Salmonella* isolates towards the P22 bacteriophage showed that all the 12 isolates of *Salmonella* Typhimurium as well as *Salmonella* Derby and *Salmonella* Enteritidis were inhibited by the presence of P22 phage. On the contrary, the

Table 3
Viable count (CFU/ml) of *Salmonella enterica* population of three different undefined four-isolate cocktails (A, B and C) after 24 and 48 h, alone (control, C) and in contact with the bacteriophage P22 (treated, T) in liquid eggs.

Time (h)	Cocktail A		Cocktail B		Cocktail C	
	C	T	C	T	C	T
0	4.11^{aA}	4.10^{aA}	4.22^{aA}	4.18^{aA}	4.55^{aA}	4.10^{aA}
24	4.08^{aA}	2.83^{bB}	4.07^{aA}	2.5^{bB}	4.52^{aA}	2.50^{bB}
48	4.13^{aA}	2.69^{bB}	4.08^{aA}	2.1^{bB}	4.56^{aA}	2.54^{bB}

Mean values of three different trials for each challenge test. Different superscripts (lowercase) comparing control (C) and treated (T) sample at the same time within the same food indicate a significant difference ($P < 0.01$). Different superscripts (uppercase) comparing different times within each sample indicate a significant difference ($P < 0.01$).

growth of the microorganisms belonging to the serotypes Give, Newport, Muenchen and Muenster was not affected by the presence of the P22 phage. As well known, P22 is a *S. enterica* serovar Typhimurium temperate bacteriophage (Zinder and Lederberg, 1952) whose total genome was definitively completed by Pedulla et al. (2003). Furthermore, it is reported that the O-antigenic repeating units of the *Salmonella* cell surface lipopolysaccharides serve as receptor for phage P22 and that only the serotypes A, B and D are susceptible to the attachment (Steinbacher et al., 1997). In fact, Thouand et al. (2008) proposed a method using P22 for rapid detection of the serotypes A, B and D of *S. enterica*. Among the microorganisms used in this study, Typhimurium and Derby belong to serotype B and Enteritidis to the serotype D1. Instead, Newport and Muenchen belong to the serotype C2 and Give and Muenster to the serotype E. In this scenario, our findings are in agreement with the reported susceptibility of *Salmonella* towards P22 phage. Although the use of temperate bacteriophages for biocontrol of foodborne pathogens is not recommended (Hagens and Loessner, 2010), we used P22 phage as a model to prove the ability of bacteriophages to control the bacterial growth in foods. On the other hand, in all our experiments the P22 phage behaves towards the susceptible *Salmonella* isolates as a virulent phage.

3.3. Strain typing of Salmonella

The results of REP and ERIC PCR allowed the identification of 2 different types of fingerprints (Fig. 1). In particular, within the 12 *Salmonella* Typhimurium isolates, REP PCR distinguished isolates 2, 4 and 7 from the others while ERIC PCR distinguished only isolate 3 from the others (Fig. 1). The strain differentiation obtained was a suitable premise to use this type of fingerprinting for a strain-specific monitoring of the effect of the phage treatment on mixtures of wild strains during challenge tests in spiked foods. Accordingly, isolates 2 and 3 were chosen to be used in the challenge tests.

Table 4
Viable count (CFU/ml) of *Salmonella enterica* population of a cocktail of two strains after 24, 48 and 72 h, alone (control, C) and in contact with the bacteriophage P22 (treated, T) in liquid eggs.

Time (h)	Samples	
	C	T
0	4.1^{aA}	4.1^{aA}
24	4.05^{aA}	2.83^{bB}
48	4.18^{aA}	2.69^{bB}
72	$4{,}08^{aA}$	2.07^{bC}

Mean values of three different trials for each challenge test. Different superscripts (lowercase) comparing control (C) and treated (T) sample at the same time within the same food indicate a significant difference ($P < 0.01$). Different superscripts (uppercase) comparing different times within each sample indicate a significant difference ($P < 0.01$).

3.4. Challenge test with cocktails of Salmonella strains in liquid eggs

The effect of P22 bateriophage against three undefined and one defined cocktail of *Salmonella* in liquid eggs is shown in Table 3 and Table 4, respectively. In both the experiments the control population remained unchanged during the time, in contrast with the results obtained in the previous challenge test carried out with the strain *Salmonella* Typhimurium LT2 alone. In fact, liquid eggs showed to reduce the control population of this strain by approximately 1 log cycle after 48 h (Table 1). The inconsistency with the previous results could be due to the presence in the cocktails of different strains with diverse susceptibility to the environmental conditions of liquid egg at 4 °C. The phage treatment of the undefined cocktail caused a reduction of bacterial population ranging between 1.27 and 2.08 log cycles, in the cocktail A after 24 h and the cocktail B after 48 h, respectively (Table 3). No significant difference was found between 24 and 48 h (Table 3), suggesting the main effect of P22 phage on the bacterial cells within the first hours of contact. A similar result was registered in chicken samples, and in this case colonies picked at the end of the incubation period were sensitive towards phage P22. Generally, slight difference in sensitivity was registered among the three cocktails.

Although the P22 phage seems to reduce the *Salmonella* population by 1.5–2.0 log cycles, both in the experiment with the single strain and in the experiment with undefined cocktail, comparing the treated and control bacterial population we registered a significant ($P < 0.01$) difference ranging between 1.5 and 2.0 log in the experiment with undefined cocktail (Table 3) and only 1 log in the experiment with the single strain LT2 (Table 1). Similar results were registered in the experiment with the two-strain cocktail where the phage treatment led to a reduction of about 1.5 log after 24 and 48 h and of about 2 log after 72 h. There was also a significant ($P < 0.01$) difference of approximately 1.5 log between the treated and control sample after 48 h, which increased to 2 log after 72 h. This result could be a further confirmation of the lytic activity of P22 against all the microorganisms tested in this study.

In order to investigate a possible preference of P22 phage within the couple of *Salmonella* strains, 30 colonies were picked from countable plates of each sample (control and treated) at each time (24, 48 and 72 h) and analyzed by REP and ERIC PCR. Results showed that in liquid eggs the phage P22 did not infect preferentially one of the two *Salmonella* strains used. In fact, in all the cases both strains were recovered at the same level at each time of sampling as shown by identification of their PCR profiles.

4. Conclusions

In conclusion, phages could be used in food technology as a hazard control system, within a more complex and desirable application of hurdle technology. In fact, our results showed that phage application was not enough to inactivate the entire population of bacteria. Therefore, combination of phage with other natural antimicrobials (e.g. bacteriocins or essential oils) should be encouraged. We tested a contamination level of 10^4 CFU/ml of *Salmonella* but experiments with lower contamination levels (e.g. 10^2 CFU/ml) are needed to resemble a more realistic scenario. However, our experimental conditions showed that bacteriophages are a promising tool to control food pathogens both in liquid and solid foods.

Acknowledgments

This study was supported by a grant from "Ministero della Salute" within the project no. IZS ME03/11 RC.

References

Abedon, S.T., 2011. Lysis from without. Bacteriophage 1, 46–49.

Albufera, U., Bhugaloo-Vial, P., Issack, M.I., Jaufeerally-Fakim, Y., 2009. Molecular characterization of *Salmonella* isolates by REP-PCR and RAPD analysis. Infect. Genet. Evol. 9, 322–327.

Balogh, B., Jones, J.B., Iriate, F.B., Momol, M.T., 2010. Phage therapy for plant disease control. Curr. Pharm. Biotechnol. 11, 48–57.

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G.V., Heinemann, J.A., 2008. Phage inactivation of foodborne pathogens on cooked and raw meat. Food Microbiol. 25, 400–406.

Carlton, R.M., Noordman, W.H., Biswas, B., de Meester, E.D., Loessner, M.J., 2005. Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. Regul. Toxicol. Pharmacol. 43, 301–312.

Civerolo, E.L., 1990. Bacteriophages. In: Klement, Z., Rudolph, K., Sands, D.C. (Eds.), Methods in Phytobacteriology. Akademiai Kiado, Budapest, Hungary, pp. 205–213.

de la Puente-Redondo, V.A., del Blanco, N.G., Gutierrez-Martin, C.B., Garcia-Pena, F.J., Ferri, E.F.R., 2000. Comparison of different PCR approaches for typing of *Francisella tularensis* strains. J. Clin. Microbiol. 38, 1016–1022.

European Food Safety Authority, 2011. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. EFSA J. 9.

Garcia, P., Madera, C., Martinez, B., Rodriguez, A., 2007. Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. Int. Dairy J. 17, 1232–1239.

Guenther, S., Loessner, M.J., 2011. Bacteriophage biocontrol of *Listeria monocytogenes* on soft ripened white mold and red-smear cheeses. Bacteriophage 1, 94–100.

Guenther, S., Herzig, O., Fielser, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol. 154, 66–72.

Hagens, S., Loessner, M.J., 2010. Bcteriophage for biocontrol of foodborne pathogens: calculations and considerations. Curr. Pharm. Biotechnol. 11, 58–68.

Henry, M., Debarieux, L., 2012. Tools from viruses: bacteriophage successes and beyond. Virology 434, 151–161.

Kocharunchitt, C., Ross, T., McNeil, D.L., 2009. Use of bacteriophages as biocontrol agents to control *Salmonella* associated with seed sprouts. Int. J. Food Microbiol. 128, 453–459.

Marmur, J., 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3, 208–218.

Modi, R., Hirvi, Y., Hill, A., Griffiths, M.W., 2001. Effect of phage on survival of *Salmonella* Enteriditis during manufacture and storage of cheddar cheese made from raw and pasteurized milk. J. Food Prot. 64, 927–933.

Oliveira, M., Viñas, I., Colàs, P., Anguera, M., Usall, J., Abadias, M., 2014. Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. Food Microbiol. 38, 137–142.

Pedulla, M.L., Ford, M.E., Karthikeyan, T., Houtz, J.M., Hendrix, R.W., Hatfull, G.F., Poteete, A.R., Gilcrease, E.B., Winn-Stapley, D.A., Casjens, S.R., 2003. Corrected sequence of the bacteriophage P22 genome. J. Bacteriol. 185, 1475–1477.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, Third ed. Cold Spring Harbor Laboratory Press, New York, NY, USA.

Schellekens, M.M., Wouters, J., Hagens, S., Hugenholtz, J., 2007. Bacteriophage P100 application to control *Listeria monocytogenes* on smeared cheese. Milchwiss. Milk Sci. Int. 62, 284–287.

Singh, A., Korasapati, N.R., Juneja, V.K., Subbiah, J., Froning, G., Thippareddi, H., 2011. Dynamic predictive model for the growth of *Salmonella* spp. in liquid whole egg. J. Food Sci. 76, M225–M232.

Steinbacher, S., Miller, S., Baxa, U., Weintraub, A., Seckler, R., 1997. Interaction of *Salmonella* phage P22 with its O-antigen receptor studied by X-ray crystallography. Biol. Chem. 378, 337–343.

Thouand, G., Vachon, P., Liu, S., Dayre, M., Griffiths, M.W., 2008. Optimization and validation of a simple method using P22::luxAB bacteriophage for rapid detection of *Salmonella enterica* serotypes A, B, and D in poultry samples. J. Food Prot. 71, 380–385.

Zinder, N.D., Lederberg, J., 1952. Genetic exchange in *Salmonella*. J. Bacteriol. 64, 679–699.

Zinno, P., Janzen, T., Bennedsen, M., Ercolini, D., Mauriello, G., 2010. Characterization of *Streptococcus thermophilus* lytic bacteriophages from mozzarella cheese plants. Int. J. Food Microbiol. 138, 137–144.