Absorbent food pads containing bacteriophages for potential antimicrobial use in refrigerated food products

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ABSTRACT

The incessant search by the consumer for products that are ready for consumption and for new technologies aimed at ensuring the safety and quality of food has intensified with the adoption of measures that can reduce these risks. This work shows that an absorbent food pad used in chilled meat trays, containing a mix of six bacteriophages, BFSE16, BFSE18, PaDTA1, PaDTA9, PaDTA10 and PaDTA11 isolated and characterized by the work group, used as biocontrol, has application in the food preservation area. It is an excellent method of extending the shelf life of refrigerated processed foods ready for consumption. The system was evaluated for the ability, in vitro, to reduce the initial count of Salmonella Typhimurium present in the environment. Three different phage concentrations were incorporated in pads that have the ability to reduce 4.36, 3.66 and 0.87 log cycles at 15°C, respectively, and an average 0.55 log cycles at 10°C at the concentrations used. The higher the concentration of this bacteriophage, the better its effect on the host, having a greater capacity for infection. The viable phage remained on the pad during the treatment time of 48 h.

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1. Introduction

There is growing concern in the food industry in the production and distribution of food both to comply the need of reduction risks to consumer health as to comply to the stringent sanitary barriers to international trade. Thus, the search for new technologies that guarantee safety and quality foods has been the subject of much research. (Appendini & Hotchkiss, 2002; Hauser & Wunderlich, 2011; Soares, Silva, Pires, Camilloto, & Silva, 2009).

Traditionally perishable food products such as meat, poultry, fish and vegetables were displayed and sold in bulk form, thereby allowing the purchaser to select the item or items to be purchased. This practice was particularly true for meat and poultry products, commonly sold by a butcher from a display case. More recently, such food products are prepackaged for display and sale. Such packaging, especially with respect to meat and poultry, involves the use of a semi-rigid tray, usually plastic, to contain the food product, and a plastic overwrap to seal the product within the tray, to maintain the freshness of the contents for a finite period (Hansen, Rippi, Mikkkif, & Neuwhirst, 1989).

Meat and poultry, as well as other perishable food products, naturally contain liquids and juices. Occasionally these liquids, with time, will drain from the product. When such food products are packaged within a plastic wrapped tray, these liquids may collect within the tray and may subsequently leak during transportation and handling. Market analysis has also revealed that the consumer considers unappealing free liquid in such packages. Consequently, many such packages today employ an absorbent material in the bottom of the tray to absorb the liquids as they are released from the food. However, whether the liquids from the food products remain free or are absorbed in a pad, such liquids provide a medium for bacterial propagation and transmitting food-borne pathogens which cause disease in humans (Hansen et al., 1989).

Foods of animal origin present the most types of microorganisms, which are part of their normal microbiota. For multiplying, these microorganisms need favorable conditions, represented by multiple factors, including temperature, which during the process of conservation is crucial to inhibit microbial growth and minimize the chemical reactions that cause the spoilage of food (Mürmann, Mallmann, & Dilkin, 2005).

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For example, mesophilic bacteria producing food poisoning or infections can be isolated from chilled meat, including *Salmonella* sp., *Clostridium botulinum*, *C. perfringens*, *Campylobacter* sp., *Escherichia coli* and even *Listeria monocytogenes* (Hoffmann, Mansor, Coelho, & Vinturim, 2002; Soares & Hotchkiss, 1997). Spector and Kenyon (2012) report that the major sources of *Salmonella* for humans are contaminated or infected beef, pork, eggs, poultry, fruit, vegetables or derivatives and by-products of these foods, such as peanut butter, mayonnaise, etc. *Salmonella* sp. are able to adapt, grow and/or survive in a wide range of stressful environments, such as extracellular pH between 3.99 and 9.5, media concentrations up to 4% NaCl and temperatures as high as 54 °C or as low as 2 °C.

In recent years, researchers have dedicated themselves to studies of packaging that not only acts passively, but also interacts with food. These studies have named the subject 'active packaging' (Kerry, O'Grady, & Hogan, 2006). Active packaging with antimicrobial agents have been developed in order to increase the shelf life of food and reduce microbial risk potential (Hauser & Wunderlich, 2011). The antimicrobial agents can be added to the food surface and thus reduce the microbial counts. The antimicrobial be released into the food slowly and steadily extending the antimicrobial activity taking place more intensively and where it is necessary (Melo et al., 2012). There are several antimicrobial agents that can be added and/or incorporated into packaging. But among the main advantages of incorporating antimicrobial on the package is that the antimicrobial be released into the food slowly and steadily extending the antimicrobial activity taking place more intensively and where it is necessary (Melo et al., 2012).

The use of bacteriophages to control pathogens is promising and is becoming a reality. Although the practice of primary bacteriophage therapy has been performed with a view to the treatment of bacterial infections in humans, the concept of removing undesirable bacterial populations using bacteriophages can be extended to animals, plants, foodstuffs and other domains (Gill & Young, 2011). Many authors report an absorbent pad containing antimicrobial activity that is the antimicrobial be released into the food slowly and steadily extending the antimicrobial activity taking place more intensively and where it is necessary (Melo et al., 2012).

2. Material and methods

2.1. Microorganisms used

The bacteriophages used were BFSE16, BFSE18, PaDTA1, PaDTA9, PaDTA10, and PaDTA11. Bacteriophages were isolated from chicken feces, poultry exudates and swine feces and characterized in the laboratory. The bacterial strain used as the host target was *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028.

2.2. Qualitative in vitro simulation of absorbent food pads in contact with a surface containing the bacteria

The phage mix was added to the absorbent food pad (Techno-paper, Brazil) by being dripped onto their surface. The amount used was 20%, 50%, and 80% (volume used in each pad) solution in bacteriophage of 10⁶ PFU mL⁻¹ concentration, in accordance with the weight of the pads mass. The pads were fixed on the inside of the lid of the Petri dishes, and the base plates contained an agar base (Tryptic Soy Broth + 1.2% agar - Himedia, India) containing the bacteria previously activated (incubated at 35 °C ± 2 °C for 18 h) and inoculated using smear swab. The plates were kept in an incubator (Greenhouse Incubator for B.O.D - biochemical oxygen demand - Biosystems, Brazil) using two different temperatures (15 °C and 10 °C) for seven days. The antimicrobial activity of the absorbent food pad was determined qualitatively, by identifying the area with an absence of bacteria and checking the spread of bacteriophage to a solid medium, simulating the contact of the pad with food. The experiment was conducted in three experimental repetitions for each temperature.

2.3. In vitro activity of absorbent food pads using different phage concentrations

The absorbent food pad with their added concentrations of the mix of bacteriophages (10⁶, 10⁸, and 10⁹ PFU mL⁻¹) were fixed to the base within the plates. It was added to the pad 12 ml of TSB broth (Tryptic Soy Broth - Himedia, India) containing pre-activated bacteria at a concentration of 10⁶ CFU mL⁻¹, showing a bacteriophage infection capacity of 1, 2, and 3 MOI (multiplicity of infection). Control plates were added with only 12 ml of broth containing the bacteria at a concentration of 10⁶ CFU mL⁻¹ under the same conditions, and for the control zero time was considered. The experiment was conducted in three experimental repetitions for each temperature.

At times of 6, 12, 24, 36, and 48 h the bacterial growth counts were performed. For that, 10 ml of SM buffer solution (50 mmol L⁻¹ Tris–HCl (Vetec, Brazil) [pH 7.5], 0.1 mol L⁻¹ NaCl (Vetec, Brazil), 8 mmol L⁻¹ MgSO₄·7H₂O (Vetec, Brasil), 0.01% gelatin (Himedia, India)) was added to the plates (treatment and control) to rinse the pads. The solution was collected in centrifuge tubes and centrifuged at 13,000 g for 5 min at 15 °C, the supernatant was used to assess the presence of bacteriophages in the same period. The pellet was resuspended in saline solution (NaCl 0.85% w/v sterile) and serial dilutions were made and plated on specific medium SS (Salmonella–Shigella Agar – Himedia, India). The plates were incubated at 35 °C ± 2 °C for 18–24 h. After the incubation time the colonies were counted, and the efficacy of treatment was analyzed.

2.4. Evaluation of the presence of the phage

The supernatant collected above containing the bacteriophage was diluted in SM buffer and plated according to the methodology described by Sambrook and Russell (2001). On an agar overlay (TSB + 0.6% agar–agar – Himedia, India) were placed 100 μL of the dilution of phage and 100 μL of pre-activated bacteria, and poured on plates containing agar base (TSB + 1.2% agar–agar – Himedia, India). The plates were incubated at 35 °C ± 2 °C for 6–18 h; after the incubation period, were observed the formation of lysis plaques (PFU - Plaque Forming Units).

2.5. Statistical analysis

To evaluate the spread of bacteriophage of pad to the mix the solid medium containing the bacteria was used DIC (completely randomized design) in factorial scheme (3 × 2) three concentrations (20%, 50%, and 80%) and two temperatures (15 °C and 10 °C). The result was analyzed qualitatively.

From the experiment arranged in a randomized design, a block diagram for evaluating the in vitro activity of pads containing the bacteriophage was arranged in a factorial arrangement (3 × 5) with three concentrations of bacteriophages (10⁶, 10⁸, and 10⁹ PFU mL⁻¹) and five incubation times (6; 12; 24; 36, and 48 h). Two
temperatures (15 °C and 10 °C) were tested at different times. Concentrations of bacteriophages were characterized by treatments T6, T8 and T9, respectively.

The data were analyzed at 5% probability by analysis of variance (ANOVA). Differences between means were analyzed by Tukey’s HDS test using “MINITAB” 16 (Minitab Inc., USA) as a statistical resource.

3. Results and discussion

3.1. Qualitative simulation of absorbent food pads

Figs. 1 and 2 show the spread of bacteriophage in the medium containing the Salmonella Typhimurium culture at 15 °C and 10 °C, respectively. The plates containing absorbent food pads with the mix of bacteriophages were analyzed after seven days of incubation. In both temperatures, the bacteriophage could diffuse into the solid medium containing the bacterium (Figs. 1 and 2). It was observed that in place around the pad touches the medium there is no growth of the cells, indicating lysis caused by the phage. In the control pad, in contrast, there was no cell lysis around the pad. When bacteriophage is in contact with the bacteria, the bacteriophage adsorbs in the cell wall of bacteria and infects the host causing cell lysis and death. This can be observed around the control pad in contact with the medium containing the bacteria where there was no cell death.

In the plates incubated at 10 °C bacterial growth was less significant because the bacterial metabolism is slower at lower temperatures (Forsythe, 2002; Malheiros, Paula, & Tondo, 2007; Spector & Kenyon, 2012). One can also notice that there was no difference in relation to inhibition of the pathogen between the quantities of bacteriophage added to the pad. All quantities added to the mix of bacteriophages in pad 20%, 50% and 80% have the same effect when visually observed.

3.2. In vitro activity of absorbent food pads in liquid medium

In Figs. 3 and 4 the antimicrobial effect of bacteriophage on the Salmonella cell count can be seen. We observe that the treatment with the concentration of $10^9$ PFU mL$^{-1}$ of bacteriophage on the pad (T9) and the concentration of $10^8$ PFU mL$^{-1}$ of bacteriophage (T8) were more efficient. The results were more significant at the temperature of 15 °C than at 10 °C for the first 12 h of treatment, and the log cycle reduction was more significant at 15 °C. It reached 4.36 log cycle reductions for treatment T9, 3.66 log cycles for treatment T8 and 0.24 log cycles for treatment T6 (bacteriophage concentration in pad of $10^6$ PFU mL$^{-1}$). After 24 h the bacteria began to grow both in the control and in the treatment, but kept the...
reductions compared to the control after 48 h of contact.

Bacteriophages depend on host metabolic function for their morphogenesis and replication process. Because the phage depends on the metabolic function of the host, at a temperature of 10°C to cell lysis was less efficient. Under optimal growth conditions, the host has a higher metabolic rate, favoring the replication process of bacteriophage and cell lysis. Physiological changes in the microorganism due to the variation in temperature and nutritional conditions can affect the infection of the bacteriophage by the host (Hungaro, Mendonça, Gouvêa, Vanetti, & Pinto, 2013; Kim, Klumpp, & Loessner, 2007).

Studies with Pseudomonas fluorescens showed a greater concentration of proteins and RNA in the bacteria’s optimum growth temperature when this temperature was changed. Furthermore, there was also a change in the behavior of bacteriophage (Guillou & Guespin-Michel, 1996; Sillankorva, Neubauer, & Azeredo, 2008a). These authors report that optimal temperatures for growth of the bacterial cells are higher, which probably explains a higher adsorption of phage on the surface and consequently a greater effect of lysis. One of the important factors in phage infection is the presence of receptors on the cell wall that allow its adsorption. When the temperature is not the optimum temperature for growth of the microorganism, it may influence the presence of such receptors in the cell wall of bacteria (Sillankorva, Neubauer, & Azeredo, 2008b).

The release rate of the bacteriophage is higher with cells in exponential phase than in stationary phase cells (Sillankorva, Oliveira, Vieira, Sutherland, & Azeredo, 2004a) and this is seen in the charts during the first 12 h of infection, when the bacterial cell is in exponential phase. When the cells become older (stationary phase), the infection efficiency decreases. There is a long latency period when the cells are in the decline phase. In this aspect inhibition of cellular protein synthesis can occur, which may increase the latency period of the bacteriophage. The release rate of the bacteriophage may occur with an increase in the growth rate of the microorganism (Abedon, 2014; Miller & Day, 2008). The transition from exponential phase to stationary, results in a dramatic change in cell morphology, in rates of macromolecular synthesis and degradation, and in the formation and surface characteristics of the cell wall (Abedon, 2014; Rossi & Aragno, 1999).

Therefore, the physiology of the cell influences the adsorption rate of the bacteriophage, and may cause a longer latency period when the cells are in the decline phase. Host cells only release new viral particles when they grow back after cell division. This decreases the rate of cell lysis and release of the bacteriophage.

Bacteriophages have an important part as regulators of microbial growth, provided that they are present in high-density environments (Miller & Day, 2008), under favorable conditions (Moisture, Water Activity, Nutrients, etc.) that allow them to meet the bacteria and infect them, because bacteriophages are non-motile. The efficiency of the bacteriophages can be affected by the nutritional and physiological state of the host bacterium, and this infection may determine the parameters and their efficiency. TSB medium favors the growth of bacteria, affecting the latency

![Fig. 2. Bacteriophage quantity in the solid medium containing the Salmonella Typhimurium at a temperature of 10°C.](image)

(a) control; (b) 20% (v/w); (c) 50% (v/w); and (d) 80% (v/w), respectively.
period and the cell lysis time, since the medium is rich in nutrients that can directly affect the phage receptor, the ATP levels and cyclic AMP, or even enhanced expression of genes characteristic of lyticogenic replication (Miller & Day, 2008). This may explain the growth of bacteria after 12 h of incubation, as shown in Figs. 3 and 4.

According to Hungaro, Lopez, Albino, and Mendonça (2014), inactivation of microorganisms in food is only achieved by using a high bacteriophage-host relationship (multiplicity of infection or MOI). When this inactivation occurs at temperatures below the minimum, lysis of host growth can occur from the outside through a large number of phages bound to the cell wall of the cell. This was reported in the inactivation of *Salmonella* in melons stored at 5°C and at a MOI of 200 (Leverentz et al., 2001). However, some evidence suggests that lysis from the outside and infection can occur simultaneously, and contribute to cell death (Kim et al., 2007; Kutter & Sulakvelidze, 2005).

### 3.3. Presence of phage on the pad

One of the main challenges in the development of phage-based antimicrobial packaging materials is the stability of the phage materials. Most of the current understanding of phage stability is based on the analysis of phage in an aqueous medium, while there is limited knowledge of phage stability in material formulations (Jepson & March, 2004; Schaper, Durán, & Jofré, 2002).

The viability of the bacteriophage on the pad during the exposure time at temperatures of 15°C and 10°C is shown in a simple linear regression graph (Figs. 5 and 6). Each time of analysis, the bacteriophage remained constant relative to its initial concentration for T9 and T8 treatments. For the T6 treatment we observe an increase in bacteriophage titer, reaching levels of 10^7 PFU mL^-1 at 15°C after 48 h incubation. This occurs due to the increase in bacterial cells and, consequently, a greater phage infection demonstrated by the increase of viral cells. At 10°C the concentration remained practically constant for all three treatments. Most of the current understanding of factors that contribute to the stability of viral particles is largely based on empirical observations. Based on these observations, the main factors that can increase the stability of viral particles include preventing and/or limiting oxidative damage to DNA/RNA and viral capsid components and stabilization of the structure of the viral capsid proteins (Wigginton, Pecson, Sigstam, Bosshard, & Kohn, 2012).

Vonasek, Le, and Nitin (2014) evaluated the release of encapsulated bacteriophages in edible films of whey protein (WPI) in an aqueous medium and on the surface of foods. It was observed that the release in aqueous medium phage was greater than on the food surface. It can be noted that in an aqueous medium there was an increase in the concentration of phage after 6 h.

According to Sillankorva, Oliveira, Vieira, Sutherland, and Azeredo (2004b) the effect of temperature has no effect on phage growth. The bacteriophage remains viable during the treatment time, which can be seen in the graph. However, the temperature...
Fig. 4. Phage viability compared in the reduction of *Salmonella Typhimurium* at 10°C. (a) CFU log Control (---) and log CFU of *Salmonella Typhimurium* in the presence of a bacteriophage mix at the concentrations of 10⁹ (T9), 10⁸ (T8), and 10⁶ PFU mL⁻¹ (T6); (b) CFU log Control (---), PFU log T9 (-----), and log CFU *Salmonella Typhimurium* in the presence of T9 (at bacteriophage concentration 10⁹) (x3); (c) CFU log Control (---), PFU log T8 (-----), and log CFU *Salmonella Typhimurium* in the presence of T8 (at bacteriophage concentration 10⁸) (x3); and (d) CFU log Control (---), PFU log T6 (-----), and log CFU *Salmonella Typhimurium* in the presence of T6 (at bacteriophage concentration 10⁶) (x3).

Fig. 5. Phage viability evaluation in absorbent food pad over time at 15°C. (T9 — bacteriophage treatment at the initial concentration 10⁹ PFU mL⁻¹ (---); T8 — bacteriophage treatment at the initial concentration 10⁸ PFU mL⁻¹ (-----); and T6 — bacteriophage treatment at the initial concentration 10⁶ PFU mL⁻¹ (x3)).
affects the microbial growth and may interfere with the adsorption of the bacteriophage on the bacterial cell wall, because the bacteria are subjected to temperatures which are not optimal for their growth and consequently influences the adsorption of the phage. At different temperatures, the bacteria may show different behavior in terms of protein production (Hungaro et al., 2014; Abedon, 2014; Sillankorva et al., 2008b; Sulakvelidze, Morris, Alavidze, Pasternak, & Brown, 2004).

4. Conclusions

The antimicrobial action of the mix of bacteriophages BFSE16, BFSE18, PaDTA1, PaDTA9, PaDTA10, and PaDTA11 in the absorbent food pads can be observed both in spreading of the bacteriophage to the solid medium and also to a liquid medium, as represented by the in vitro method used. The mix of bacteriophages added to the absorbent food pad remained viable during the 48 h treatment time and may be a biocontrol alternative packaging for food products. However, incorporation technique of bacteriophage into absorbent food pad should be improved in order to keep it viable in the package for further commercialization. The packages are proposed to complement the existing preservation methods. It is important to report that this will not dispense with the use of good manufacturing practices and hygiene for the food industry.

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