LWT - Food Science and Technology 67 (2016) 50–54

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Resistance of foodborne pathogen coliphages to additives applied in food manufacture

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ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form
5 November 2015
Accepted 17 November 2015
Available online 23 November 2015

Keywords:
Bacteriophage
Phage viability
Food additive
Escherichia coli

ABSTRACT

In order to evaluate whether coliphages can be used in combination with food additives, six lytic phages against pathogenic *Escherichia coli* strains were tested for their resistance to additives commonly used in the dairy and meat industries. All the phages evaluated were completely inactivated after a 1-min incubation at 25 °C when exposed to acetic and lactic acids at 4% v/v without pH adjustment, whereas phage viability remained unchanged when pH was adjusted to 5.0 (acetic) and 4.5 (lactic). Likewise, the six phages proved to be highly resistant to both acetate and lactate (4%; sodium salts) after a 24-h incubation. When phage viability was evaluated at 25 °C in Tris-Magnesium-Gelatin buffer supplemented with nitrite (0.015% w/v), phage titers were never below 7–8 $\log_{10}$ PFU ml^{-1} for all the phages tested. Regarding the influence of additives added to dairy products on phage viability, each phage (10^{7-8} PFU ml^{-1}) challenged with nisin (0.25 mg ml^{-1}) remained viable after a 24 h-incubation. In addition, phage viability was either slightly affected or not affected at all when phages were exposed to chymosin. These results proved that phages can be used against pathogenic *E. coli* strains along with other additives as an additional hurdle in order to improve food safety.

1. Introduction

Foodborne diseases caused by pathogenic *Escherichia coli* (*E. coli*) strains are a serious and growing problem. This pathogen has been responsible for hemolytic uremic syndrome cases since 1980 (Karmali et al., 1985). Foodborne bacteria can contaminate food products at any point along the chain production - during slaughtering, milking, storage or packaging (García, Martínez, Obeso, & Rodríguez, 2008). Therefore, several food additives such as weak acids (Ouattara, Simard, Holley, Piette, & Bégin, 1997), nitrite (Honikel, 2008), and nisin (Gharsallaoui, Joly, Oulahal, & Degraeve, 2015) are used at different stages of production in order to ensure food quality and safety. Regarding the maximum concentration allowed in foodstuff, most of these additives are strictly regulated (FDA, 2000; CAA, 2010) as they may be toxic, *e.g.* nitrite (Honikel, 2008), cause alteration of the organoleptic characteristics of food, *e.g.* weak acids at higher concentrations (Kotula & Thelappurate, 1994) or by the activity needed to achieve a high quality product, *e.g.* chymosin (Vallejo, Ageitos, Poza, & Villa, 2012). Although these additives are widely used and accepted, and numerous publications have documented the effectiveness of food preservatives against *E. coli* (Yoder et al., 2012) and other pathogens (Glass et al., 2002; Michaelsen, Sebranek, & Dickson, 2006), novel strategies, such as the use of phages, are needed to fulfill consumer demands for food with lower amounts of chemical compounds. Furthermore, additives are less specific than phages, affecting both foodborne pathogens as well as the normal microflora of food due to their nonspecific mechanisms of action (Kin et al., 2011).

The application of bacteriophages in food safety has been extensively documented against pathogenic *E. coli* strains as well as other foodborne pathogens such as *Salmonella*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Enterobacter sakazakii*, and *Staphylococcus aureus* (Tomat, Mercanti, Balagué, & Quiberoni, 2013a).

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http://dx.doi.org/10.1016/j.lwt.2015.11.031
0023-6438/

However, the action of food additives on phage viability was not assessed in most of the studies carried out *in situ*, namely in the food matrix.

There are studies analyzing the effect of acetic acid on phage expression (Wallin-Carlquist et al., 2010), and the effect of lactic acid on phage viability (García, Madera, Martinez, & Rodriguez, 2007), as well as other works where phages are characterized by their acid resistance (Coffey et al., 2011). However, articles reporting the effect of food additives on the infectivity of coliphages are scarce. Several authors have studied different combination of antimicrobials such as bacteriocins and phages (Leverentz et al., 2003; Ly-Chatain, Moussaoui, Vera, Rigobello, & Demarigny, 2013), bacteriocins and endolysins (Schmelcher, Powell, Becker, Camp, & Donovan, 2012), and bacteriocins and essential oils (Bajpai, Yoon, Bhardwaj, & Kang, 2014). However, most of these studies were focused on phages of LAB (Ly-Chatain et al., 2013), *L. monocytogenes* (Leverentz et al., 2003) and *S. aureus* (Martínez, Obeso, Rodríguez, & García, 2008). Regarding studies on phages affecting pathogenic *E. coli* strains, Ly-Chatain et al. (2013) were the only authors who analyzed the antiviral activity of several cationic compounds, specifically nisin, against the bacteriophage MS2, a phage infecting *E. coli* strains, and found a weak antiviral effect (1 $\log_{10}$ reduction after 10 min) only at the highest concentration of nisin (100,000 IU) tested.

In previous studies, phages have proved to be efficient biocontrol agents of pathogenic *E. coli* strains (Tomat, Migliore, Aquili, Quiberoni, & Balagué, 2013b; Tomat, Mercanti, Balagué, & Quiberoni, 2013c; Tomat, Quiberoni, Mercanti, & Balagué, 2014) and to be highly resistant to thermal and physicochemical treatments (Tomat, Balagué, Casabonne, Verdini, & Quiberoni, 2015). Studies on the interaction (*e.g.* challenges) of coliphages with food additives, such as weak acids and their sodic salts, nitrite, and chymosin, have not yet been carried out. The aim of this study was to evaluate the influence of additives added to meat and dairy products on phage viability in order to determine whether they can be used simultaneously as a hurdle technology in the biocontrol of pathogenic *E. coli* strains.

2. Materials and methods

2.1. Bacterial strains and phages

E. coli DH5α was used as the sensitive host strain to propagate all the bacteriophages used in this study. DH5α was maintained as frozen (−80 °C) stock in Hershey broth (8 g l^{-1} Bacto nutrient broth, 5 g l^{-1} Bacto peptone, 5 g l^{-1} NaCl and 1 g l^{-1} glucose) (Difco, Detroit, Michigan, USA) (Cicarelli, San Lorenzo, Santa Fe, Argentina) supplemented with 15% (v/v) glycerol and routinely reactivated overnight at 37 °C in Hershey broth.

Bacteriophages DT1, DT2, DT3, DT4, DT5 and DT6 were isolated from stool samples of patients with diarrhea treated at the Centenary Hospital, Rosario (Tomat et al., 2013c). High-titre phage suspensions were prepared as previously described (Tomat et al., 2013c). Namely, Hershey-Mg broth was inoculated (1%, v/v) with an overnight culture of DH5α, aliquots (100 μl) of phage stocks were added, incubated (37 °C) with shaking until complete lysis. Next, chloroform was added (0.1 ml) and cultures centrifuged at 4000 g for 10 min. Phage stocks were stored at 4 °C and enumerated (plaque-forming units per millilitre; PFU ml^{-1}) by the double-layer plaque technique. Briefly, aliquots of 100 μl of phage stocks were mixed with 100 μl of recipient strain culture (OD_{600} = 1.0), then added with three ml of Hershey-Mg soft agar (Hershey-Mg with 0.7% agar, w/v) at 45 °C. The mixture was poured into plates with Hershey-Mg agar (1.4%, w/v) and incubated overnight at 37 °C (Jamalludeen et al., 2007).

2.2. Viability studies - additives applied in meat products -

2.2.1. Influence of acetic and lactic acid

Phages ($10^7 - 10^8$ PFU ml^{-1}) were suspended in Tris-magnesium gelatin (TMG) buffer (10 mM Tris-Cl, 10 mM $MgSO_4$ and 0.1% (w/v) gelatin) supplemented with acetic (pH 2.72) and lactic (pH 2.28) acid at 4% v/v without pH adjustment. In addition, further assays were carried out with acetic (pH 5.0) and lactic (pH 4.5) acid at 4% v/v with pH adjusted to the same values which result from treating meat with each acid in *in-vitro* preliminary studies.

After each incubation time, namely without (1 and 5 min) and with (1, 8 and 24 h) pH adjustment, at 25 °C, phage suspensions were enumerated by the double-layer plate titration method (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.2.2. Influence of acetate and lactate (sodium salts)

The influence of acetate and lactate on phage ($10^7 - 10^{-8}$ PFU ml^{-1}) viability was investigated by incubation at 25 °C in TMG buffer supplemented with sodium acetate (4% w/v) or sodium lactate (4% v/v) with the pH adjusted to 5.7, which represents the natural pH of meat. After incubation for 1, 8 and 24 h, phage viability was determined as described above (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.2.3. Influence of nitrite

The influence of nitrite (sodium salt) on phage ($10^7 - 10^{-8}$ PFU ml^{-1}) viability was investigated by incubation at 25 °C in TMG buffer supplemented with nitrite (0.015% w/v; maximum concentration allowed) (CAA, 2005). After incubation for 1, 8 and 24 h, phage viability was determined as described above (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.3. Viability studies - additives applied in dairy products -

2.3.1. Influence of nisin

The influence of nisin on phage ($10^7 - 10^8$ PFU ml^{-1}) viability was investigated by incubation at 25 °C in TMG buffer supplemented with nisin (Nisaplin, nisin 2.5% w/w, 1 million IU g^{-1}) at 0.25 mg ml^{-1} (maximum concentration allowed; FDA, 2001). After incubation for 1, 8 and 24 h, phage particles were enumerated as described above (Jamalludeen et al., 2007) and the counts were compared to those at control (TMG) conditions. Assays were carried out in triplicate.

2.3.2. Influence of chymosin

Phages ($10^7 - 10^8$ PFU ml^{-1}) were suspended in TMG buffer supplemented with chymosin (Maxiren 150, 100% chymosin, rennet strength 150,000 IMCU ml^{-1}) at 8.0 mg ml^{-1}. The suspension was incubated at 25 °C. After incubation for 1, 8 and 24 h, phage particles were enumerated as described above (Jamalludeen et al., 2007) and the counts were compared to those at control (TMG) conditions. Assays were carried out in triplicate.

2.4. Statistical analysis

Means (three determinations) were compared using the one-way ANOVA procedure followed by Duncan's multiple range tests at $p < 0.05$.

3. Results and discussion

3.1. Influence of additives applied in meat products on phage viability

Food additives such as weak acids are widely used in the meat