International Journal of Food Microbiology 217 (2016) 7–13

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Efficacy of two *Staphylococcus aureus* phage cocktails in cheese production

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ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 19 September 2015
Accepted 1 October 2015
Available online 5 October 2015

Keywords:
Phage
Staphylococcus
Cheese
Enterotoxin

ABSTRACT

Staphylococcus aureus is one of the most prevalent pathogenic bacteria contaminating dairy products. In an effort to reduce food safety risks, virulent phages are investigated as antibacterial agents to control foodborne pathogens. The aim of this study was to compare sets of virulent phages, design phage cocktails, and use them in a cocktail to control pathogenic staphylococci in cheese. Six selected phages belonging to the three *Caudovirales* families (*Myoviridae*, *Siphoviridae*, *Podoviridae*) were strictly lytic, had a broad host range, and did not carry genes coding for virulence traits in their genomes. However, they were sensitive to pasteurization. At MOI levels of 15, 45, and 150, two anti-*S. aureus* phage cocktails, each containing three phages, one from each of the three phage families, eradicated a 10^6 CFU/g *S. aureus* population after 14 days of Cheddar cheese curd ripening at 4 °C. The use of these phages did not trigger over-production of *S. aureus* enterotoxin C. The use of phage cocktails and their rotation may prevent the emergence of phage resistant bacterial strains.

1. Introduction

Bacteriophages (or phages) are found everywhere their bacterial hosts are present (Breitbart and Rohwer, 2005; Clokie et al., 2011; Sulakvelidze, 2011). Because of their ubiquitous nature, phages are regularly consumed when eating and drinking (Mahony et al., 2011). Phages can also lyse specific bacterial strains without affecting the remaining microbiota (García et al., 2010). Accordingly, some virulent phages have been successfully tested in different medical and food ecosystems to prevent and control the proliferation of bacterial pathogens (Goodridge and Bisha, 2011; Sulakvelidze, 2013). These phage-based strategies may improve the safety of processed foods, preserve ready-to-eat foods, and reduce the contamination on hard surfaces in industrial environments.

However, not all phages can be used as biocontrol agents. Several desirable properties are needed. First, the phage genome must be free of gene coding for any known virulence factors. Second, the phage must be strictly virulent and thus, lack the ability to integrate its genome into a bacterial genome. Third, the phage should have a broad host range, thereby infecting several strains of the target bacterial species (Goodridge and Bisha, 2011; Hagens and Loessner, 2010). Finally, phage-resistant bacterial clones or derivatives should not be readily selected following phage use (Labrie et al., 2010). In the latter case, the use of a cocktail of phages may reduce the probability of the emergence of phage resistant strains (García et al., 2007; Gill and Hyman, 2010), particularly if they contain taxonomically-distinct virulent phages with overlapping host ranges.

Some phage-based products are already commercially available to prevent, among others, the growth of *Listeria monocytogenes* (Leverentz et al., 2003; Soni and Nannapaneni, 2010), *Escherichia coli* (Carter et al., 2012), and *Salmonella* (Carol, 2013) in foods. Milk products have been among the preferred foods for testing the efficacy of virulent phages as biocontrol agents, likely because this industry is familiar with the destructive effect of virulent phages on the beneficial starter cultures used to make fermented dairy products (Garneau and Moineau, 2011; Samson and Moineau, 2013). Many phages have been tested in dairy products to reduce the occurrence of the above mentioned foodborne pathogens (Guenther and Loessner, 2011; McLean et al., 2013; Modi et al., 2001), as well as *Staphylococcus aureus* (Bueno et al., 2012). A cocktail of two *S. aureus* phages (phiIPLA35 and phiIPLA88) of the *Siphoviridae* family (non-contractile tail and double-stranded DNA genome) was found to synergically reduce *S. aureus* concentration in fresh and hard-type cheeses during the ripening process

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.10.001
0168-1605/

(Bueno et al., 2012; García et al., 2007; García et al., 2009). However, they did not completely eliminate the targeted bacterial population in hard-type cheeses. Phage cocktails have also been designed to target biofilms and chronic infections caused by *S. aureus* (Drilling et al., 2014; Markoishvili et al., 2002; Merabishvili et al., 2009; Rhoads et al., 2009).

We recently characterized and compared the host range of three polyvalent staphylococcal phages (Team 1, K, phi812) belonging to the *Myoviridae* family (contractile tail and dsDNA genome) using a panel of 57 *S. aureus* strains collected from various sources and representing 18 sequence types (ST) and 14 clonal complexes (CC). We only found one *S. aureus* strain (ST 25, CC 25) insensitive to all three polyvalent myophages. This strain was, however, sensitive to phages of the *Podoviridae* family (short tail and dsDNA genome) (El Haddad et al., 2014), highlighting the necessity of using a phage combination that broadens the host range.

Here, we tested two cocktails composed of three phages at different concentrations for their efficacy in reducing *S. aureus* cell population and toxin production in cheeses. Phages were selected according to the different criteria cited above and tested during a small-scale, laboratory-based Cheddar-like cheese production.

2. Materials and methods

2.1. Bacteriophages

The seven phages used in this study are stored at the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca) and belong to the *Caudovirales* order (tailed phages) (Table 1). Tryptic-Soy Agar (TSA) and TSB soft agar (7.5 g/L agar) were used for phage titration. Phages used in cheesemaking assays were propagated on *S. aureus* SMQ-1320 in TSB, concentrated using PEG precipitation and purified using two CsCl gradients (Sambrook and Russell, 2011). Purified phages were recovered by ultracentrifugation using a Beckman SW41 Ti rotor at 35,000 rpm (210,053 ×*g*) for 3 h, followed by a second ultracentrifugation using a Beckman NVT65 rotor at 60,000 rpm (342,317 ×*g*) for 18 h. Phages were then dialyzed in phage buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 8 mM $MgSO_4$) and stored at 4 °C until use.

2.2. Bacterial strains

Fifty-seven strains of *S. aureus* were used to determine the host ranges of the phages used in this study. The strains were previously clustered into 14 groups according to their clonal complexes and sequence types using MLST (El Haddad et al., 2014). These strains were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses, the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, the National Microbiology Laboratory of the Public Health Agency of Canada, and the Canadian Bovine Mastitis Research Network. Strains were routinely grown in TSB at 37 °C. Baird-Parker with egg yolk-tellurite (BD™) was used for cell counts. Strains were stored at −80 °C in TSB broth with 150 g/L glycerol.

Lactococcus lactis subsp. *cremoris* strain CUC-222 (Cargill, Inc.) was used as a starter culture in the Cheddar cheese detailed in Section 2.7.

2.3. Host range

The host range of two podophages (P68, 44AHJD) and three siphophages (LH1-MUT, phi2, and M^{SA}) were determined on 57 *S. aureus* strains. The host ranges of the myophages Team1 and phi812 were previously reported (El Haddad et al., 2014). Bacterial strains were grown at 37 °C to an $OD_{600\ nm}$ of 0.1 and 10^5 phages per mL were added to the medium. The phage-infected cultures were incubated at 37 °C until complete bacterial lysis, and the resulting lysate was filtered (0.45 μm). The host ranges were determined by spotting 5 μL of the phage lysate serially diluted (10^{-1} to 10^{-8}) in phage buffer on TSB soft agar containing 100 to 200 μL of an overnight culture of the staphylococcal strain. At least, two biological and two technical repetitions were done per phage-host. Efficiency of plaquing (EOP) values were calculated by dividing the phage titer on the tested strain by the phage titer on its host strain.

2.4. Phage resistance to pasteurization treatment

The seven *S. aureus* phages were tested for their resistance to an extended pasteurization treatment by exposing them to 73 °C for 20 s in milk as follows. Commercial micro-filtered pasteurized milk (2% fat) was heated in tubes at 73 °C. Once the temperature inside the tube reached 73 °C, each phage was added at a concentration of 10^7 PFU per mL of heated milk and incubated for 20 s. After treatment, milk samples were put on ice and the phages were enumerated. The titers of the seven phages were compared in milk before and after the heat treatment to quantify the phage count reduction, expressed as $\log_{10}$ PFU/mL. Phage titers were obtained on TSA overlaid with TSB soft agar containing 100 μL of the *S. aureus* host strain and 100 μL of the phage dilution. The bacterial strains employed to calculate the respective EOPs of the phages were used for phage counting.

2.5. Phage stability in a model Cheddar cheese curd

As a semi-solid medium with a low pH, cheese curd may hinder the resistance of phages and thus, their efficiency. Phages Team1, phi812, P68, 44AHJD, LH1-MUT, and phi2 were tested for their survival in a Cheddar curd model under 390 g/kg humidity and 17.7 g/kg salt. First, a lyophilized Cheddar-like curd (Lacroix et al., 2010) was acidified using lactic acid (pH 2.6 obtained with 85% lactic acid) in order to obtain a final pH of 5.2. Phages were added at a concentration of 10^7 PFU/mL and mixed manually with the model curd for 5 min. Ten grams of reconstituted curd was removed and initial (T = 0) phage counts were determined. The remaining curd was divided into 10-gram aliquots, vacuum-packed at 95% of nitrogen, and sampled after 14 days of ripening at 4 °C. Each 10-gram curd sample was homogenized in 90 mL of sterile 20 g/L sodium citrate solution pre-warmed to 45 °C, using a stomacher (Seward Stomacher 400 Circulator; London England). Dilutions ranging from 10^0 to 10^{-5} were made in phage buffer. Phage

Table 1
Phages used in this study.

Name	Classification	Origin of isolation	Accession number (if applicable)	Reference
Team1	*Myoviridae*, Twort-like	Wound infection	KC012913	El Haddad et al. (2014)
Phi812	*Myoviridae*, Twort-like	Clinical infection	Separated CDS (incomplete genome published online)	Pantucek et al. (1998)
P68	*Podoviridae*, 44AHJD-like	Unknown	NC_004679.1	Vybiral et al. (2003)
44AHJD	*Podoviridae*	Unknown	NC_004678.1	Vybiral et al. (2003)
LH1-MUT	*Siphoviridae*	Raw milk, natural mutant of LH1	JX174275	El Haddad & Moineau (2013)
phi2	*Siphoviridae*	Raw milk	KT186243	This study
M^{SA}	*Siphoviridae*	Clinical infection, natural mutant of W^{SA}	N/A	Capparelli et al. (2007)