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BIOCONTROL OF SHIGA TOXIGENIC *ESCHERICHIA COLI* O157:H7 IN TURKISH RAW MEATBALL BY BACTERIOPHAGE

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ABSTRACT

With an *Escherichia coli* O157:H7 virulent bacteriophage, M8AEC16, biocontrol efficiency of phages on a highly risky, ready-to-eat, traditional delicacy food called "raw meatball" under different storage conditions was investigated. Phage, belonging to the *Myoviridae* family, was isolated from the wastewater of a local slaughterhouse and showed a broad lytic activity toward many *E. coli* O157:H7 strains with high efficiency of plating and O157 specificity. Our experimental study provided favorable results, with 0.69–2.09 log colony-forming unit (cfu)/g *E. coli* O157:H7 reductions in the first 5 h of the replica trials. Major reductions of viable *E. coli* O157:H7 counts were observed in the beginning of the storage period, reaching up to 1.85 log cfu/g. Although a significant reduction in *E. coli* O157:H7 was observed with increased phage concentration, storage conditions had minor effect on efficiency of phage biocontrol. This is the first study in Turkey that investigates applicability of phage biocontrol for a traditional food model.

PRACTICAL APPLICATIONS

Phage addition in preparation stage of a very complex food model, ready-to-eat Turkish raw meatball, is a promising application in decontamination of *Escherichia coli* O157:H7. Although investigation of its genomic characteristics along with its stability to different food matrices must be completed for further use of the model phage M8AEC16, findings of this work were encouraging, as phages are valuable in biocontrol of important foodborne in this ready-to-eat Turkish delicacy.

INTRODUCTION

Shiga toxigenic *Escherichia coli* (STEC) O157:H7 is one of the leading causes of hemorrhagic colitis and hemolytic uremic syndrome around the globe, mainly resulting from direct or indirect contamination of water, food or food products and ingredients by feces of asymptomatic ruminant carriers of the pathogen. Primarily, cattle, and to a lesser extent other ruminants such as sheep, can carry the agent in their gastrointestinal tracts and contamination of meat at slaughter produce on fields and drinking water by reservoir feces have been linked to many human cases worldwide (Doyle *et al.* 2006).

Raw meatball (çig köfte) is a Turkish, ready-to-eat appetizer delicacy generally made of a thorough mixture of raw lean ground beef, fine bulgur, salt, red and black pepper, cumin, tomato paste, onion, garlic, sunflower seed oil and water, in which spices may differ from recipe to recipe. Kneading with hand, meatball dough is prepared by addition of these ingredients. Without any additional heating process, aliquots are formed into small meatballs and served, generally being consumed within a few hours following preparation, while the leftovers are generally refrigerated. The safety of the product is primarily dependent on the initial quality of the ground meat and other ingredients included. Previous investigations on the presence of *E. coli* O157:H7 in ground beef and bovine carcasses showed a considerable prevalence for this bacteria (Sarimehmetoglu *et al.* 2009; Ayaz *et al.* 2014). Furthermore, constituents such as spices might harbor enteric pathogens, as the source of a

multistate outbreak that affected 272 people in U.S.A. was traced back to *Salmonella* Montevideo contamination of black and red pepper (CDC 2010).

The lack of heating process and the subsequent potential risk they possess resulting from often found fecal indicator bacteria (Cetin *et al.* 2008), microbiological quality and decontamination of raw meatballs with a variety of techniques had been in focus of previous works (Gezgin and Gunes 2007; Dikici *et al.* 2013). Although pronounced efficacies were reported, drawbacks resulting from loss of sensory characteristics of the product and overall acceptance remain to be thoroughly elucidated. Hence, other methods that would not deteriorate the characteristics of the raw meatballs must be investigated. Because of their host specificity, inertia toward animal and plant cells, and self-replicating nature, bacteriophages (phages in short), obligate parasitic predators of bacteria, are extensively being exploited for this purpose and promising reductions in *E. coli* O157:H7 counts in an array of food models have been reported (Abuladze *et al.* 2008; Sharma *et al.* 2009; Anany *et al.* 2011; Viazis *et al.* 2011; Carter *et al.* 2012; Hudson *et al.* 2013; Tomat *et al.* 2013).

Here, screening a local slaughterhouse wastewater for a 2 year period, we isolated a number of phages virulent to *E. coli* O157:H7, determined their host ranges and efficiency of plating (EoP) and with one selected model phage, coded M8AEC16, investigated the decontamination efficiency of phage application in a highly risky and very complex raw meatball model.

MATERIALS AND METHODS

Isolation and Purification of Phages

Using a total of five different host strains, selective enrichment-based phage isolation technique was used to increase the variety of phages to be isolated from the local slaughterhouse's wastewater efflux (Table 1) (Oot *et al.* 2007; Twest and Kropinski 2009). For this purpose, with monthly visits to the slaughterhouse, 50 mL of wastewater samples was collected in sterile bottles, between July 2011 and June 2013, over a 2 year period. Samples were brought to laboratory on ice and analyzed in less than 2 h.

Five milliliters of wastewater was filtered through 0.20 μm syringe filter (Minisart 16523K, Sartorius Stedim Biotech GmbH, Gottingen, Germany) and 500 μL of aliquots was added to 1 mL cation-adjusted tryptone soya broth (cTSB; Oxoid CM0129, Hampshire, UK) supplemented with 2 mM $CaCl_2$ (Merck Millipore 102379, Darmstadt, Germany) that was spiked with 200 μL of each reference host strain's overnight culture at 37C, then incubated at 37C for 18 h for selective enrichment of phages. Following incubation, 100 μL of chloroform (Sigma-Aldrich 288306, St. Louis, MO) was added to each Eppendorf tube, vortexed and centrifuged (Beckman Coulter 22 R Centrifuge, Irvine, CA) at 3,000× g for 15 min and dilutions of up to 10^{-8} were prepared from the supernatant in SM buffer (0.05M Tris-HCl [pH 7.4–7.5, Srlchem 204991, Mumbai, India], 0.1 M NaCl [Merck Millipore 567440], 10 mM $MgSO_4$ [Merck Millipore 105886], 1% w/v gelatin [Lobachemie 0392000500, Mumbai, India]) and spotted on lawns of corresponding isolation hosts in 3×10 μL aliquots. For spotting assays, bacterial lawns were prepared by spreading 3 mL of tryptone top agar (TTA; 10 g/L tryptone [Sigma T7293], 10 g/L NaCl, 7 g/L agar [Oxoid LP0013], 1 g/L glucose [Merck Millipore 108342], pH 7.2) that was tempered to 45C and spiked with 100 μL of overnight host cultures on tryptone soya agar (TSA; Oxoid CM0131) (Carlson 2005). Following drying in a hood for 30–45 min, overlay agar plates were spotted (10 μL) and incubated at 37C for 18 h. Up to five clear plaques, which showed different plaque morphologies, were taken for purification with a sterile filter tip and inoculated into 200 μL cTSB. Then 1 mL of corresponding host strain's overnight culture was added. These steps were repeated for a total of three times for purification of the phages.

Preparation of Phage Stocks and Determination of Phage Titers

Following the final purification, a single plaque was inoculated into 200 μL of cTSB, which was then added into a 10 mL log phase culture of the isolation host strain in TSB, and finally was incubated overnight at 37C. The following day, 500 μL of chloroform was added to lyse the culture. The lysed culture was then vortexed and centrifuged (Hermle Labortechnik GmbH Z326K, Wehingen, Germany) at 3,000× g for 15 min. Supernatants were filter sterilized and kept at 4C under refrigeration as master stocks (Carlson 2005).

To determine the stock phage titers that were isolated on Shiga toxin-negative host strains, 100 μL of 10-fold dilutions of phage stock in SM buffer and 100 μL of corresponding log phase isolation host were mixed and left at 37C for 15 min. Following the incubation for proper phage adsorption, phage–host strain mixture was added to 3 mL of TTA, poured on TSA and incubated at 37C for 18 h (Sambrook and Russell 2001). Following incubation, plaques were counted and titers were calculated. Stock phage titers, isolated on Shiga toxigenic host strains, were determined by spot assay to simplify the counting of the plaques as described earlier. All titers were determined in duplicate.

TABLE 1. BACTERIAL STRAINS USED IN THIS STUDY

Strain	Serotype	Relevant information	Source	Susceptibility to M8AEC16
Isolation host strains				
Escherichia coli NCTC 12900 (EC00)	O157:H7	*stx1*$^-$, *stx2*$^-$	N.D. Ayaz	+
E. coli RSKK 16 (EC16)	O157:H7	*stx1*$^+$, *stx2*$^+$	RSKK	+
E. coli ATCC 35150 (EC50)	O157:H7	*stx1*$^+$, *stx2*$^+$	J.E. Olsen	+
E. coli ATCC 43888 (EC88)	O157:H7	*stx1*$^-$, *stx2*$^-$	J.E. Olsen	+
E. coli ATCC 43895 (EC95)	O157:H7	*stx1*$^+$, *stx2*$^+$	N.D. Ayaz	+
EoP determination			Ayaz *et al*. (2014)	
E. coli 3RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 3KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 19RA	O157	*stx1*$^-$, *stx2*$^-$		+
E. coli 25KA*	O157:H7	*stx1*$^-$, *stx2*$^-$, SF$^+$		R
E. coli 34RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 36KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 44RA	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli 44KA	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli 68RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 69RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 91KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 120RA	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli 120KA	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli 120KC	O157:H7	*stx1*$^+$, *stx2*$^-$		+
E. coli 135RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 143RA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 143KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 163KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 168KA	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli 210KB	O157	*stx1*$^-$, *stx2*$^-$		+
E. coli 219RA	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli 236KB	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli M1A	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli M1C	O157:H7	*stx1*$^-$, *stx2*$^-$		+
E. coli M14A	O157:H7	*stx1*$^-$, *stx2*$^-$		+
E. coli M17A	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli M18A	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli M21A	O157:H7	*stx1*$^-$, *stx2*$^-$		+
Host range analysis			J.E. Olsen	
E. coli	O1			R
E. coli	O2			R
E. coli	O5			R
E. coli	O8			R
E. coli	O9			R
E. coli	O12			R
E. coli	O14			R
E. coli	O15			R
E. coli	O18			R
E. coli	O20			R
E. coli	O35			R
E. coli	O36			R
E. coli	O53			R
E. coli	O78			R
E. coli	O81			R
E. coli	O83			R
E. coli	O102			R
E. coli	O103			R
E. coli	O115			R
E. coli	O116			R
E. coli	O132			R
E. coli c3380	O147			R
E. coli 330	O149:K88			R
E. coli MS1675	O157:H7	*stx1*$^-$, *stx2*$^+$		+
E. coli MS2219	O157:H7	*stx1*$^+$, *stx2*$^+$		+
E. coli MS6269	O157:H7	*stx1*$^-$, *stx2*$^-$		+
E. coli MS6698	O157:H7	*stx1*$^-$, *stx2*$^-$		+
Salmonella Typhimurium 23M5519		human isolate		R
S. Typhimurium SL1344		cattle isolate		R
S. Typhimurium 43481/15		chicken isolate		R
S. Enteritidis E2187		PT4, human isolate		R
S. Enteritidis dv3202		chicken isolate		R
S. Enteritidis 9740280-3		chicken isolate		R
S. Enteritidis E2331		PT1, human isolate		R
Enterococcus faecalis ATCC 29212			ATCC	R
Staphylococcus aureus ATCC 25923			ATCC	R

* Sorbitol positive on CT-SMAC (Ayaz *et al*. 2014).
+, susceptible; EoP, efficiency of plating; R, resistant.

Determining Target Host Range and EoP

Host ranges of isolated phages were determined by spot assay on a variety of Shiga toxigenic and non-Shiga toxigenic *E. coli* O157:H7$^+$/H7$^-$ strains ($n = 28$) collected at the same period (Ayaz *et al.* 2014), isolation host strains ($n = 5$) and other strains ($n = 36$) (Table 1). Following incubation at 37C for 18 h, phage titers on corresponding strains were calculated as plaque-forming unit (pfu)/mL and compared with that of the isolation host, which resulted in EoP.

Screening of Virulence Factor Genes in Phage M8AEC16 by Polymerase Chain Reaction

Detection of Shiga toxin genes *stx1* and *stx2* and major *E. coli* O157:H7 associated virulence factors *eaeA* and *hly* in M8AEC16 propagated on *E. coli* O157:H7 NCTC12900 (EC00) was performed using polymerase chain reaction (PCR; Fratamico *et al.* 2000). *E. coli* ATCC 43895 (stx_1^+, stx_2^+, $eaeA^+$, hly^+) as Shiga toxin positive, *E. coli* O157:H7 NCTC 12900 (stx_1^-, stx_2^-, $eaeA^+$, hly^+) as Shiga toxin negative and ultra pure water as negative control were used in PCR analyses.

Transmission Electron Microscopy Analysis

Phages from master stock were concentrated and purified in ammonium acetate solution as described (Ackermann 2009). Purified samples were placed on formvar/carbon coated 300 mesh copper grids, stained with 2% uranyl acetate and micrographs were obtained using JEO 100CX-II Transmission Electron Microscope (JEOL) at a magnification of 225,000.

Bacterial Strain and Application of the Inoculum

E. coli O157:H7 ATCC 43895 (EC95) was used as the model bacteria in decontamination trials of raw meatballs. In order to add a selective advantage, nalidixic acid resistance in EC95 was induced by serial passages of increasing concentrations of nalidixic acid (Applichem A1894, Darmstadt, Germany) on LB agar (10 g/L tryptone, 5 g/L yeast extract [Oxoid LP0021B], 5 g/L NaCl, 15 g/L agar) with a final concentration of 100 μg/mL (Abuladze *et al.* 2008). EC95 that went on five serial passages on LB agar with 100 μg/mL was determined to be nalidixic acid resistant (naEC95) and used in raw meatball trials.

A single colony was transferred to 50 mL of naTSB (TSB supplemented with 100 μg/mL of nalidixic acid) and incubated for 18 h at 37C. Broth cultures were centrifuged at 5,000× g for 7 min, and pellet was suspended in sterile physiological saline (PS; 0.9% NaCl). After three washing steps, pellet was resuspended in PS and an optical density (Shimadzu UV1700, Tokyo, Japan) at 600 nm (OD_{600}) of 0.2 (~10^7 colony-forming unit [cfu]/mL) was achieved. Then, 10-fold dilutions of the washed naEC95 stock were prepared in PS and 1 mL of the corresponding dilutions (as described next) were carefully inoculated on 25 g of raw meatball aliquots residing in sterile filtered plastic bags, letting the raw meatballs soak the inoculum.

Experimental Design of Raw Meatball Decontamination with M8AEC16

For three independent trials, in order to establish the uniformity of the raw meatballs, a commercially available raw meatball mixture kit that consists of fine bulgur, paprika powder, black pepper, cumin, all spice, salt, mild pepper powder, tomato powder, onion and garlic granulate, and dried parsley was used. One and a half kilograms of lean ground beef was purchased from a local supermarket, sampled for analysis of *E. coli* O157 presence (see below), aseptically divided into 150 g aliquots and placed in sterile plastic bags to be frozen at −20C until use. Aliquots of ground beef were moved to 4C for thawing 18 h before the trial was initiated. In each trial, two different groups (F [phage group] and K [control group]) of raw meatball dough were prepared for three different contamination levels (i.e., ~10^2, ~10^4 and ~10^6 cfu/g) to achieve three different multiplicities of infections (MoIs).

Each dough was aseptically formed, consisted of 200 g of raw meatball mixture, 150 g of lean ground beef, 25 g of canned tomato paste, 50 mL of sunflower seed oil and 250 mL of distilled water, and was kneaded with sterile gloves on a sterile stainless steel surface. In the F group, however, rather than pure distilled water, 175 mL of distilled water and 75 mL of phage M8AEC16 stock (~10^{10} pfu/mL) were used. Following a settling time of 15 min at room temperature, 25 g of aliquots from F and K groups was aseptically weighed in filtered sterile plastic bags. After inoculation of the bacteria as described earlier, spiked raw meatballs were thoroughly kneaded over their plastic bags and distributed to their corresponding storage conditions.

In order to simulate generally used storage conditions of raw meatballs, experimental samples were stored either in refrigerator at 4C or in an incubator at 22C. For samples that were stored under refrigeration, enumerations were carried out at 3, 5, 24 and 48 h, whereas for samples stored at room temperature they were carried out at 1, 3, 5 and 24 h. In each trial, for every group and contamination levels, an initial enumeration of naEC95 and M8AEC16 (for F group) was performed. pH values of experimentally produced raw meatballs were measured using a mobile pH

meter with a penetration probe (WTW GmbH 330/Set-1, Oberbayern, Germany) and observed in declining values between pH 6.1 and 4.1.

Analysis of *E. coli* O157 Presence and Enumeration of naEC95 and M8AEC16

E. coli O157 presence in purchased lean ground beef was analyzed by the immunomagnetic separation (IMS)-based cultivation technique (ISO 2001). Twenty-five grams of lean ground beef was selectively enriched in 225 mL of mEC broth (Oxoid CM0853, supplemented with 20 mg/L of novobiocin [Sigma N-1628]) at 37C after homogenization (Seward Laboratory, Labblender 400 stomacher, London, UK), and 1 mL of the enrichment was subjected to IMS with anti-*E. coli* O157 Dynabeads (Invitrogen 710-04, Oslo, Norway), plated on cefixime-tellurite (Oxoid SR0172) supplemented Sorbitol MacConkey agar (Oxoid CM0813) (CT-SMAC) in duplicate, and incubated at 37C for 18 h. Suspected sorbitol-negative colonies were tested for the presence of O157 antigen by latex agglutination (Oxoid DR0620).

For enumeration of naEC95 in experimentally contaminated raw meatballs, 225 mL of mEC broth was added to each 25 g of aliquots of raw meatballs at the time of enumerations, and then the mixtures were thoroughly homogenized. After homogenization, 10-fold dilutions were prepared in PS and appropriate dilutions were double serially plated on na-CT-SMAC (CT-SMAC supplemented with 100 μg/mL of nalidixic acid). After all of the plated liquids were dried, plates were incubated at 37C for 18 h. Sorbitol-negative colonies that were positive with latex agglutination test were counted, and the mean values were calculated.

For enumeration of M8AEC16, 225 mL of SM buffer was added to 25 g of aliquots of F group initial raw meatball samples and the mixture was then thoroughly homogenized. Following filter sterilization of an aliquot (c.a. 2 mL), 10-fold dilutions were prepared and 100 μL of M8AEC16 dilutions were mixed with 100 μL of log phase culture of EC00 in duplicate and plated as described earlier.

Statistical Analyses

All statistical analyses were executed within the R computing environment (R version 3.1.0; http://www.r-project.org/). Three independent replicate experiments were performed for every contamination level of raw meatballs and all bacterial enumerations were converted to log cfu/g. For calculation of MoIs, mean initial pfu/g counts were divided by mean initial cfu/g counts for each achieved contamination level. Limit of detection was taken as log 0.95 (<10 cfu/g) for statistical analyses. Initially, fixing the MoI, time and temperature, an unpaired *t*-test was executed to investigate the significance of reduction between control (K) and phage (F) groups. Then, mean values from replica trials were taken for each enumeration time and differences between K and F groups were calculated. Unpaired *t*-test was used for comparison of each viable *E. coli* O157:H7 reductions under room temperature (22C) and refrigerated (4C) storage conditions for every achieved MoIs (log 5.59, 4.17 and 2.38). Independent one-way analyses of variance (ANOVAs) were used to establish statistical significance in reduction levels over time for a fixed MoI and storage temperature. For establishment of statistical significance of effect of time and temperature in each of the achieved MoIs, independent two-way ANOVAs were used while the effect of varying MoIs was integrated with a three-way ANOVA. Tukey's honest significant difference test was used as post hoc analyses where a statistical significance was observed. The level of statistical significance was $P \leq 0.05$.

RESULTS

Isolation of Phages from Slaughterhouse Wastewater Efflux

Using reference *E. coli* O157:H7 strains as host isolation strains, virulent phages with clear plaques were isolated, from 9 of 24 (37.5%) slaughterhouse wastewater samples collected over a 2 year period. Lytic phages with clear plaques were isolated from five samples collected in warm season (5/12 [41.7%], May–October) while the remaining were isolated from four samples that were collected in cold season (4/12 [33.3%], November–April). A total of 31 putative phages were isolated and purified throughout the study (Table 2).

Host Range Analysis and EoP Determination

Host range analysis and EoP determination of 31 putative phages were performed on isolation host strains, cattle and slaughterhouse wastewater isolates (Fig. 1), and 12 representative phage isolates showing variance in their host range, EoP or isolation host strains are given in Table 3. Out of 31 putative phages, 10 different host range distributions were observed (Table 3). Phages M8AEC16, M9AEC95, M11AEC16 and M12AEC50 showed the broadest activity toward all of the tested strains ($n = 33$), being able to form clear plaques with varying morphologies on 32 strains tested, while *E. coli* O157:H7 isolates 25KA, 3KA, 3KA and M14A were found to be resistant, respectively.

Selection of M8AEC16 as the Model Phage

Of the four phages showing the broadest host ranges, M8AEC16 was the only phage that showed activity toward

TABLE 2. ISOLATED BACTERIOPHAGES AND THEIR ISOLATION HOSTS

Sample no.	Date	Isolation host*	Bacteriophage
M1	July 2011	EC88	M1AEC88
		EC00	M1AEC00
			M1CEC00
M4	October 2011	EC88	M4AEC88
			M4BEC88
M8	February 2012	EC16	M8AEC16
			M8BEC16
			M8CEC16
			M8DEC16
			M8EEC16
M9	March 2012	EC95	M9AEC95
			M9BEC95
			M9CEC95
			M9DEC95
			M9EEC95
M11	May 2012	EC16	M11AEC16
			M11BEC16
			M11CEC16
			M11DEC16
M12	June 2012	EC00	M12AEC00
			M12BEC00
		EC16	M12AEC16
			M12BEC16
		EC50	M12AEC50
M18	December 2012	EC00	M18AEC00
			M18BEC00
			M18CEC00
M21	March 2013	EC00	M21AEC00
M23	May 2013	EC16	M23AEC16
			M23BEC16
			M23CEC16

* See Table 1.

all of the typical sorbitol fermentation negative (SF^-) *E. coli* O157:$H7^+$/$H7^-$ isolates with a high EoP, while the accidental strain SF^+ *E. coli* O157:H7 25KA (Ayaz *et al*. 2014) showed resistance toward this phage. Furthermore, M8AEC16 virulence was found to be *E. coli* O157:H7 specific in comparison with M9AEC95, as M9AEC95 was also virulent toward *E. coli* strains of serotype O14 and O102, when both were screened with an array of *E. coli* serotypes and strains of other species (Table 1). Additionally, M8AEC16's propagation on EC00 yielded higher titers of phage ($\geq 10^{10}$ pfu/mL) showing high EoP for most of the *E. coli* O157:H7 strains. Also by PCR, M8AEC16 was found to lack major virulence genes (*stx1*, *stx2*, *eaeA* and *hly*) typical for *E. coli* O157:H7.

Morphology of M8AEC16

Having a contractile tail, M8AEC16 can be classified in family *Myoviridae* under the order *Caudovirales* with the A1 morphotype because of its isometric head with 57 ± 1 nm in height and 56 ± 2 nm in width (Fig. 2) (Ackermann 2001). The length of the tail of M8AEC16 was 104 ± 4 nm with a width of 20 ± 1 nm.

Effect of M8AEC16 on *E. coli* O157:H7 in Raw Meatballs

M8AEC16 was found to be effective in the decontamination of *E. coli* O157:H7 in raw meatballs. The mean reductions of viable *E. coli* O157:H7 varied between 0.69 and 2.09 log cfu/g in the first 5 h of the replica trials, and were presented in Table 4 and Fig. 3. The colony counts for initial 5 h of refrigeration and 3 h of room temperature storage conditions were undetectable. At higher MoIs, such as 5.59 and 4.17 log, the observed reductions at certain time and temperatures showed statistical significance ($P \leq 0.05$), while at low MoI (2.38 log), reductions were not significant ($P > 0.05$) (Table 4). However, differences of reductions were neither statistically significant ($P > 0.05$) between different storage conditions (4 and 22C) nor among different enumeration times (3, 5 and 24 h). Nonetheless, when the initial MoI was the highest, efficacy of M8AEC16 in reduction of *E. coli* O157:H7 was significantly higher ($P \leq 0.05$) than any other achieved MoI irrelevant of the storage conditions and enumeration times. A significant effect of MoI

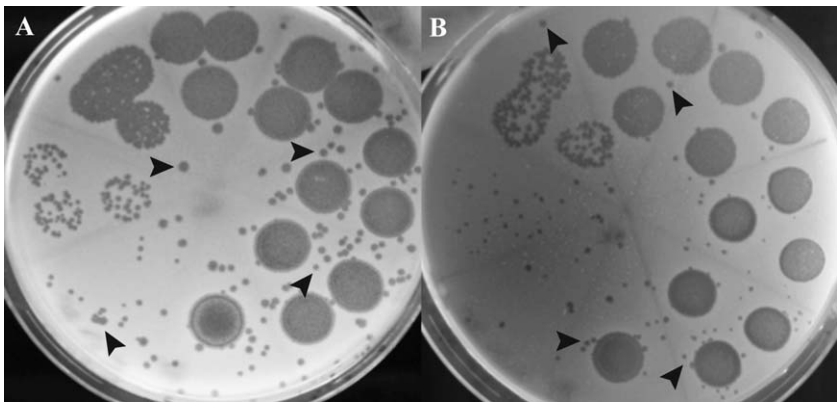

FIG. 1. REPRESENTATIVE OVERLAY PLATES IN EFFICIENCY OF PLATING ANALYSIS OF 10-FOLD DILUTIONS OF M9AEC95 ON *ESCHERICHIA COLI* O157:H7 25KA (A) AND M8AEC16 ON *E. COLI* O157:H7 M1A (B) Arrows show peripheral plaques.

TABLE 3. HOST RANGE AND EOP DISTRIBUTION OF A SELECTED NUMBER OF BACTERIOPHAGES ISOLATED IN THIS STUDY

	Bacteriophage											
Bacterial strains	M1AEC88	M1AEC00	M4AEC88	M8AEC16	M9AEC95	M11AEC16	M12AEC00	M12AEC16	M12AEC50	M18AEC00	M21EC00	M23AEC16
EC00*	8.1	8.3	6.6	10.4	9.0	10.3	9.1	8.9	10.3	9.3	8.3	6.0
EC16*	6.6	7.0	R	10.4	9.4	10.4	8.2	7.2	10.0	7.0	7.0	6.7
EC50*	7.4	7.0	R	10.2	9.1	10.5	8.1	10.0	7.3	8.0	7.0	6.0
EC88*	8.7	6.3	7.3	9.0	9.2	10.3	9.0	8.0	10.0	R	6.3	6.9
EC95*	R	7.0	R	10.2	9.2	10.7	9.8	8.5	10.2	7.0	7.0	6.1
3GA	6.0	6.0	R	9.6	7.5	9.3	7.0	6.0	9.7	7.7	6.3	R
3KA	7.0	6.0	6.0	6.0	R	R	7.5	6.0	4.0	4.0	R	7.0
19RA	7.0	7.0	5.0	8.0	6.0	8.0	7.0	6.0	9.7	7.0	5.0	R
25KA†	R	R	R	R	8.6	10.3	7.0	7.0	7.0	R	R	R
34RA	9.7	8.9	7.4	10.6	8.3	10.4	8.8	7.9	9.9	9.1	7.1	7.2
36KA	6.1	9.0	6.0	10.2	8.0	10.4	8.7	7.3	9.9	8.9	6.0	7.0
44RA	R	8.8	R	10.3	10.3	10.5	9.2	8.3	10.0	9.1	7.0	R
44KA	R	7.0	R	10.3	10.0	10.4	9.0	7.3	10.0	8.0	6.2	R
68RA	7.3	9.3	6.7	10.6	9.9	10.4	9.1	9.9	9.9	9.4	6.7	7.3
69RA	8.3	9.0	4.0	10.3	9.8	10.6	8.9	8.0	10.0	9.2	6.7	6.0
91KA	7.0	8.5	6.7	10.0	8.6	10.3	8.8	7.7	10.0	9.8	6.7	6.0
120RA	R	7.0	R	10.1	10.0	10.6	8.6	R	10.2	8.0	6.0	R
120KA	R	7.0	R	10.2	9.6	10.4	8.2	7.2	10.3	8.0	5.8	R
120KC	R	7.0	R	10.3	8.0	10.6	8.6	7.0	10.1	8.0	5.0	R
135RA	7.7	9.4	7.0	10.7	10.0	10.5	8.7	7.4	10.2	9.4	6.3	6.6
143RA	7.4	9.0	R	10.5	9.0	10.4	9.0	7.3	10.8	9.2	6.2	R
143KA	7.3	9.1	R	10.4	9.1	10.2	9.1	7.2	10.6	9.1	6.3	R
163KA	R	7.9	R	10.2	9.0	10.2	8.3	7.6	10.0	8.7	5.8	5.0
168KA	R	8.8	R	10.4	9.0	10.3	8.6	8.0	10.2	9.3	6.2	R
210KB	R	R	R	6.9	6.0	9.0	R	R	9.5	7.0	R	R
219RA	R	9.0	R	10.1	9.0	10.2	8.5	7.5	10.0	9.0	6.1	R
236KB	R	6.0	R	9.5	8.2	10.3	7.0	7.0	9.5	8.3	6.0	R
M1A	7.7	7.0	R	10.0	9.2	10.2	9.6	9.6	10.0	8.9	5.9	7.3
M1C	6.0	7.0	R	9.8	9.0	9.7	9.4	9.9	10.0	8.0	5.0	5.0
M14A	8.7	8.0	R	10.4	9.7	7.5	R	R	R	9.7	6.5	6.7
M17A	R	8.0	R	10.1	9.5	11.7	8.9	7.6	10.3	8.9	6.2	2.0
M18A	R	8.0	R	10.4	9.4	10.7	9.3	8.1	10.3	9.6	6.9	2.4
M21A	R	7.0	R	8.7	8.6	10.0	9.5	8.4	9.7	7.0	5.0	7.0

* See Table 1.

† Sorbitol positive on CT-SMAC (Ayaz *et al.* 2014).

EoP, efficiency of plating; R, resistant.

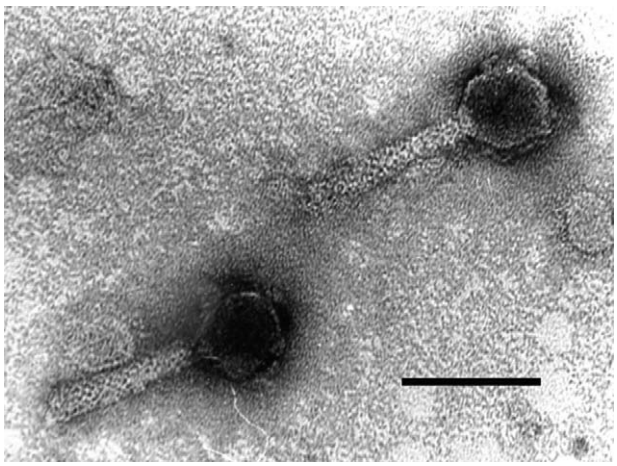

FIG. 2. ELECTRON MICROGRAPH OF M8AEC16; BAR, 100 NM

(three-way ANOVA, $P = 1.61e\text{-}07$), but neither of storage conditions nor time, was detected for bacterial counts. And no interaction effects were present. In 24 h and at room temperature storage, an increase in *E. coli* O157:H7 count was observed for phage applied raw meatball groups. These colonies were atypical in morphology as being smaller or rougher, yet positive with latex agglutination (Fig. 4) and indicated emergence of bacteriophages-insensitive mutants (BIMs) as none such colonies were observed in the control groups.

DISCUSSION

Phages, in this study, were isolated from slaughterhouse wastewater using five different host strains, suggesting their common presence in slaughterhouse wastewater, as it was also determined from cattle and sheep-associated environments (Kudva *et al.* 1999; Callaway *et al.* 2006; Raya *et al.* 2006; Oot *et al.* 2007). Although many of the cloudy plaque-forming phages were discarded due to their likeliness of being temperate phages, a total of 31 phages with a variety of plaque morphologies were isolated from 9 of 24 samples. Furthermore, analysis of two samples, M1 and M12, enabled us to isolate two and three different groups of phages showing different host ranges (Table 2), respectively, indicating the importance of using more than one isolation host strain for yielding a variety of phages addressing diverse phage receptors and, hence, showing different host ranges.

A noteworthy observation during the EoP determination of certain phages on some of the tested bacterial strains (data not shown) would be not spill out-associated morphologically different plaques that were seen on peripheries of spot zones, especially when the applied phage titer was higher (Fig. 1). By their plaque morphologies and peripheral positioning, these not spot-associated plaques could be easily distinguished. Induction of lambdoid Shiga toxin harboring prophages of STEC might occur either spontaneously (Livny and Friedman 2004) or in response to environmental stimuli such as UV, antimicrobials, NaCl or H_2O_2 (Kimmitt *et al.* 2000; Los *et al.* 2009). Furthermore, it was shown that 12 of 18 seemingly remnant prophages, including *stx*-carrying phages, possessed by enterohemorrhagic *E. coli* O157:H7 strain Sakai could be induced either spontaneously or with treatment of mitomycin C and potentiate dissemination of virulence determinants through recombination and inter-prophage interactions (Asadulghani *et al.* 2009). These observed peripheral plaque-forming phages (Fig. 1) might be induced in response to certain concentrations of diffused intracellular contents that were liberated from lysed *E. coli* O157:H7 cells after phage predation. Even though further evaluations must be carried out in the formation of these plaques and inducing factors of phage liberation, it is enticing to speculate that phage predation might be a contributing factor of phage liberation in neighboring cells and thus transfer of virulence determinants.

Applying phages to raw meatballs in this study showed favorable results. In general, the efficacy of M8AEC16 was slightly higher at 22C than at 4C, while more pronounced differences in efficacy were shown in previous studies (Viazis *et al.* 2011; Tomat *et al.* 2013; Hudson *et al.* 2015).

TABLE 4. MEAN REDUCTION OF VIABLE NaEC95 IN RAW MEATBALLS EXPERIMENTALLY CONTAMINATED WITH DIFFERENT LEVELS OF BACTERIA

Initial MoI (log [pfu/cfu]/g)	Initial bacterial count (log cfu/g)	Storage temperature (C)	Mean reduction (log cfu/g)				
			1 h	3 h	5 h	24 h	48 h
5.59	2.81	4	–	1.79*	1.85*	1.96*	1.72*
		22	1.85*	1.82*	2.09*	1.41*	–
4.17	4.51	4	–	1.00*	1.02*	0.87	1.11
		22	0.74*	1.52	1.47	0.91	–
2.38	6.30	4	–	0.70	0.69*	0.05	0.84
		22	0.73	0.82	0.91	0.98	–

* Statistically significant reduction observed between K and F groups when MoI, time and temperature were fixed ($P \leq 0.05$).
cfu, colony-forming unit; MoI, multiplicities of infection; pfu, plaque-forming unit.

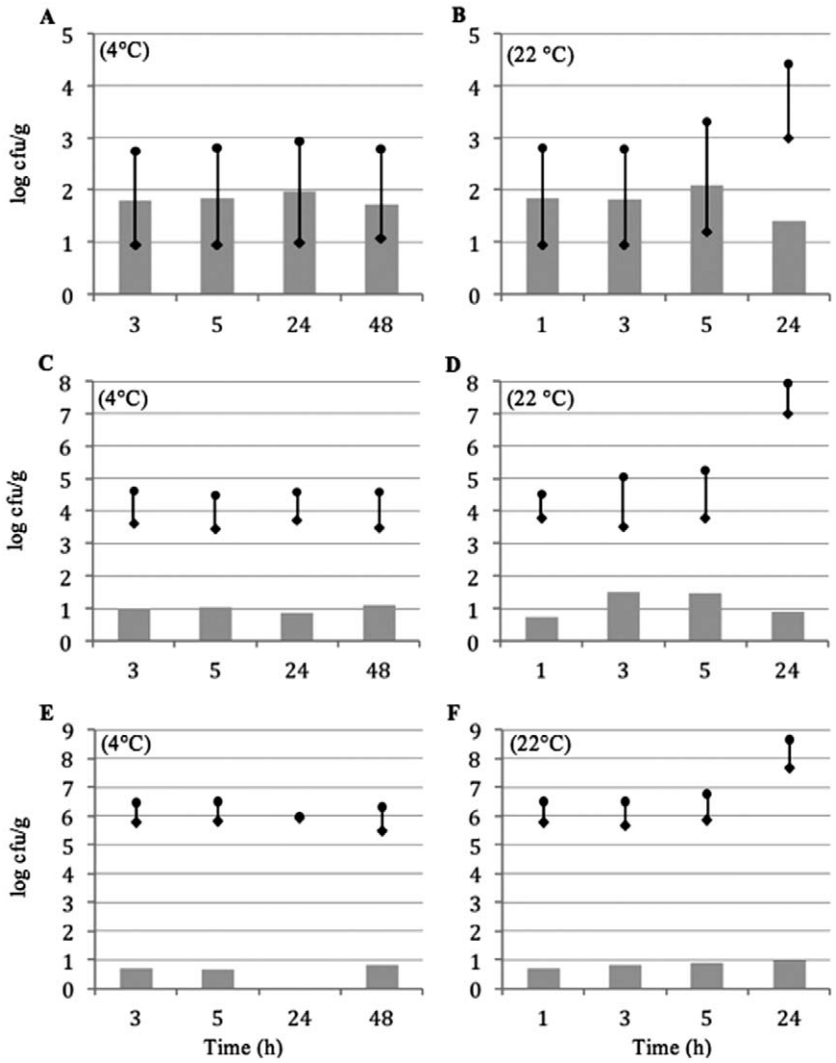

FIG. 3. MEAN *ESCHERICHIA COLI* O157:H7 COUNTS IN CONTROL (●) AND PHAGE M8AEC16 APPLIED (◆) RAW MEATBALLS WITH MULTIPLICITIES OF INFECTION (MOI): LOG 5.59 (A AND B), MOI: LOG 4.17 (C AND D) AND MOI: LOG 2.38 (E AND F)
Temperatures are given and bars indicate observed mean reductions.

Complex and dry matrix of raw meatball might have been a limitation of a much higher efficiency at room temperature storage, as efficiently replicated progeny phages would be curbed in their diffusion capability. When the contamination level of raw meatball was low (2.81 log cfu/g), in which a higher MoI (5.59 log) was achieved, there were no detectable bacterial counts (<10 cfu/g) for the first 3 h of room temperature. Because preparation of raw meatballs by kneading with hand is a time-consuming process, observing a significant 1.85 log reduction in a more realistic contamination level was indeed favorable (Table 4). However, the subsequent increase observed at 5 and 24 h showed the lack of total *E. coli* O157:H7 elimination (Fig. 3). Likewise, *E. coli* O157:H7 counts were not detectable for the first 5 h under refrigerated storage condition of low contamination raw meatball group. Based on our results, it can be observed that an increase in MoI results in a significance in reduction. Hence, it is enticing to speculate that regardless of storage temperature, applying an even higher phage titer would result in a higher reduction level in this very complex raw meatball matrix.

For decontamination of foods by means of phage application, encounters of infective phages and of susceptible host remain to be the primary obstacle for any given matrix (Hagens and Loessner 2010). Because diffusion of phage particles on a solid food will be very limited, for a thoroughly mixed food model such as raw meatball, in which the contaminating pathogen might reside in deeper fractions, surface applications such as spotting/inoculation (using a pipette) or spraying as previously applied to fresh produce, fruits or sliced/ground beef (Abuladze *et al.* 2008; Sharma *et al.* 2009; Viazis *et al.* 2011; Carter *et al.* 2012; Hudson *et al.* 2013, 2015; Tomat *et al.* 2013) would have been an important incapacitating factor for phage–host

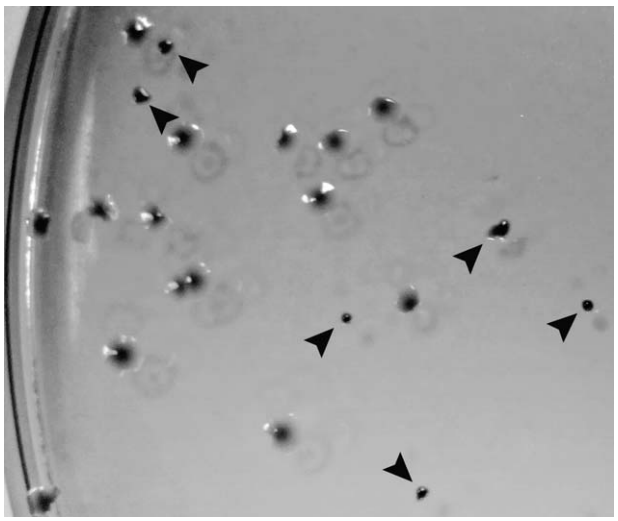

FIG. 4. REPRESENTATIVE BACTERIOPHAGE-INSENSITIVE MUTANTS ON NA-CT-SMAC
Arrows indicate atypical sorbitol fermentation-negative *Escherichia coli* O157:H7 colonies.

encounter. Adding M8AEC16 in the liquid fraction during preparation of raw meatball enabled phages to access deeper fractions of the raw meatball, increasing the likelihood of phage–*E. coli* O157:H7 encounter. Depending on the MoI, significant initial reductions were observed in the current study, yet M8AEC16 failed to show additional reductions following the first enumeration times for any of the MoI/storage temperature combinations (Table 4). This is in accordance with a previous observation as residual phages in ground meat failed to show continuous efficacy after *E. coli* O157:H7 was reintroduced (Carter *et al.* 2012). Limitation of phage diffusion in solid food models indeed appears to be an important factor curbing phage efficacy.

When phage applied raw meatball batches were analyzed for *E. coli* O157:H7 enumeration, morphologically different colonies that suggests emergence of BIMs were observed (Fig. 4). The emergence, stability and reversion frequencies of these BIMs were not evaluated but, likewise, BIM emergence was previously observed on meat or *in vitro* (Tomat *et al.* 2013) for *E. coli* O157:H7. Authors have reported low frequencies of emergence and somewhat variable stability or reversion of BIMs, yet to the best of our knowledge, the virulence of these *E. coli* O157:H7 BIMs that emerged in food models has not been elucidated. In *Campylobacter jejuni*, for example, emergence of resistance in response to phage predation by loss of a recently determined phase variable *O*-methyl phosphoramidate (MeO*P*N) receptor (Sorensen *et al.* 2011) did not affect chicken colonization levels (Sorensen *et al.* 2012) while the MeO*P*N knockout mutants, however, showed increased invasion to Caco-2 cells but decrease serum resistance and adhesion in piglet model (van Alphen *et al.* 2014). Thus, variation in the virulence of the *E. coli* O157:H7 BIMs that might emerge following the phage application in foods is both interesting and needs further investigation should total elimination of the pathogen cannot be ensured.

Biocontrol of foodborne pathogens by means of lytic phage application on food has drawn substantial interest around the globe, and their potential in reduction of predominant agents on a variety of food products was elegantly compiled elsewhere (Hertwig *et al.* 2013). Consecutively, as a result of the success in biocontrol with phages, both in the U.S.A. and in the European Union, there had been a variety of issued approvals for application phages on defined foods (Hagens and Loessner 2010; Hertwig *et al.* 2013; Sharma 2013). However, on the regulatory basis, there had yet been no development in Turkey. This is primarily due to the lack of basic research that investigates the applicability of phages on foods consumed in Turkey. The current study, being the first of its kind (i.e., in which phages were used as a part of a food ingredient rather than applied on the surface of the food), proves that even in a very complex food matrix, phages have the potential to reduce unwanted bacteria.

CONCLUSION

This work showed that inclusion of phage as an ingredient in preparation of ready-to-eat Turkish raw meatball is a promising application in decontamination of *E. coli* O157:H7, as significant reductions were observed, especially when the contamination level was low. This was achieved with a model phage originating from a local slaughterhouse's wastewater, namely M8AEC16, which has a broad and O157-specific lytic activity toward many local and foreign wild-type *E. coli* O157:H7 strains and can be propagated up to efficient titers on a non-pathogen host for application. Although investigation of its genomic characteristics along with its stability must be completed for further use of this model phage as a processing aid, findings of this work were encouraging, as phages might be valuable in biocontrol of some other important foodborne pathogens such as *Listeria monocytogenes* and *Salmonella* spp. in this ready-to-eat Turkish delicacy.

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