An investigation on biocontrol of *Escherichia coli* O157:H7 by a bacteriophage cocktail in pastirma

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Summary: In this study, it was aimed to investigate the biocontrol of *Escherichia coli* O157:H7 in pastirma (a traditional Turkish meat product produced and consumed without heat treatment) by bacteriophages. A cocktail was prepared from two pre-characterized phages which were isolated from cattle slaughterhouse wastewaters and were found to have lytic activity against *E. coli* O157:H7. The phage cocktail at a concentration of 10^9 pfu/ml was applied to pastirma slices which experimentally contaminated at 6,9 x 10^3 cfu/ml and incubated for one week at room temperature (22-24ºC). During the incubation period, reduction effects of bacteriophages on *E. coli* O157:H7 were investigated on certain times and days. As a result, the phage cocktail was able to reduce the bacterial count approximately 2 log cfu/g in the bacteriophage applied group, therefore the number of *E. coli* O157:H7 decreased and remained below the detection limit (< 10 cfu/g) during the experiment. The bacteriophage cocktail consisting of phage M8AEC16 and phage M12BEC16 has been found to be effectively usable for biocontrol of *E. coli* O157:H7 in pastirma.

Keywords: Bacteriophage, biocontrol, *E. coli* O157:H7, pastirma.

Pastırmada *Escherichia coli* O157:H7'nin bakteriyofaj kokteyli ile biyokontrolünün araştırılması

Özet: Bu çalışmada, ısıl işlem görmeden üretilen ve tüketilen geleneksel bir Türk et ürünü olan pastırmada *Escherichia coli* O157:H7'nin bakteriyofajlarla biyokontrolünün araştırılması amaçlanmıştır. Bu amaçla, sığır mezbaha atık sularından izole edilen ve *E. coli* O157:H7'ye karşı litik etkinliğe sahip olduğu önceden belirlenmiş iki bakteriyofajdan oluşan bir faj kokteyli hazırlandı. 10^9 pob/ml konsantrasyondaki faj kokteyli, deneysel olarak 6,9 x 10^3 kob/ml düzeyinde kontamine edilen pastırma dilimlerine uygulandı ve bir hafta süresince oda sıcaklığında (22-24ºC) inkübasyona bırakıldı. İnkübasyon süresince, belirli saat ve günlerde yapılan *E. coli* O157:H7 sayımları ile fajların bakteri sayısında meydana getirdikleri redüksiyon değerleri belirlendi. Sonuç olarak, bakteriyofaj uygulanan grupta faj kokteylinin bakteri sayısını yaklaşık 2 log kob/g azalttığı, bu nedenle de deney süresince faj uygulanan grupta *E. coli* O157:H7 sayısının tespit sınırının (< 10 kob/g) altında kaldığı tespit edildi. Bu çalışma neticesinde, M8AEC16 ve M12BEC16'dan oluşan faj kokteylinin, pastırmada *E. coli* O157:H7'nin biyokontrolü amacıyla etkin bir şekilde kullanılabilir olduğu ortaya konuldu.

Anahtar sözcükler: Bakteriyofaj, biyokontrol, *E. coli* O157:H7, pastırma.

Introduction

As a cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), *Escherichia coli* O157:H7 is seen as an important public health problem in many regions of the world. *Stx₁* and *stx₂* shiga-like toxins are the main virulence factors produced by *E. coli* O157:H7 that are very virulent, acid-resistant and have low minimal infection dose. These cytotoxins can cause HC, HUS and subsequent death in infected people, especially in children and in elderly (24). Ruminants, particularly cattle, are the main reservoir of *E. coli* O157:H7. During the slaughter process, meat can be contaminated with pathogens from skin and intestinal contents of the animal. Consuming such raw/inadequately cooked meat or preparing foods with these kinds of meat products or meats play important role in foodborne infections (20). Although there is no monitoring program for determining the prevalence of *E. coli* O157:H7 in Turkey, there are number of local studies showing that prevalence of the bacteria is between 1,4% and 7,1% (2, 5, 6, 13).

Bacteriophages were first described as bacteria-eating viruses in the early 1900s. In recent years, they have reemerged as an alternative control method in combating pathogens. Bacteriophages are widely distributed in the environment with a high population (10^{30}-10^{32}) and they can be isolated from sea, fresh water, soil, animal and human gastrointestinal tracts and genitourinary ducts, skin, milk etc. (9). There have been many studies that have investigated antibacterial activity of bacteriophages and their beneficial effects (3, 15, 18, 21, 23). In 2011, FDA approved the use of an *E. coli* O157:H7 specific phage

preparation, EcoShield™, in foods in the United States (28). Studies have shown that *E. coli* O157:H7 specific bacteriophages reduce *E. coli* O157:H7 spread in cattle (23), are lytic for *E. coli* O157:H7 isolated from humans (30), can be used as a biocontrol agent (18), do not show side effects when used for therapy in humans (8) and can be used for decontamination of *E. coli* O157:H7 in foods (1, 22). In this respect, the isolation and characterization of bacteriophages with high lytic activity on the target pathogen bacterium is of great importance in terms of public health. In addition, testing of isolated phages in different and previously unexamined food models is significant for determining effective use areas.

Pastirma (Turkish dry cured beef product) is among the most consumed meat products in Turkey with its typical taste and flavor, can be considered as a risky ready to eat meat product for *E. coli* O157:H7 due to its production process and consumption without heat treatment. Therefore, decontamination of *E. coli* O157:H7 in pastirma without harming the natural structure of the product becomes important for food safety and public health. In this study, it was aimed to investigate the reduction level of *E. coli* O157:H7 in contaminated pastirma food model by applying lytic bacteriophages.

Materials and Methods

Bacterial culture: In the study, *E. coli* O157:H7 ATCC 43895 (EC43895) reference strain was used. To provide antibiotic resistance as a selective feature, EC43895 strain was cultivated with increasing concentrations of nalidixic acid (1, 5, 10, 15, 20 and 25 µg/ml) on Luria Bertani agar (LB; tryptone [pancreatic digest of casein] 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, agar 15 g/l) and was made resistant to 25 µg/ml nalidixic acid (1). Then, nalidixic acid resistant *E. coli* O157:H7 ATCC 43895 (NA-EC43895) was enriched at 37°C in TSB (Tryiptic soya broth, Oxoid CM0129) overnight and the resistance was confirmed by plating and counting the colonies on both sorbitol-MacConkey agar containing 25 µg/ml nalidixic acid (NA-CT-SMAC, Oxoid CM0813; supplemented with 0,05 mg/l sefixime and 2,5 mg/l tellurite) and CT-SMAC (not containing nalidixic acid). Before the experiment setup, in order to prepare the bacterial inoculums in the desired cfu/ml, NA-EC43895 was measured in a spectrophotometer (Shimadzu UV 1700, Japan) at 600 nm optic density (OD_{600}) during the log phase in TSB and cfu/ml correlations were determined by counting on NA-CT-SMAC.

Phage cocktail: In the study, phage cocktail was prepared with M8AEC16 and M12BEC16 phages which were previously classified in *Myoviridae* family by Transmission Electron Microscopy (Figure 1) and were showed broad lytic activity towards many *E. coli* O157:H7 strains (14). Phage dilutions were prepared with SM buffer

(0,05 M Tris-HCl [pH 7.4-7.5], 0,1 M NaCl, 10 mM $MgSO_4$, 1% (w/v) gelatine). Spot tests were done on double-layer LB agar and incubated at 37ºC for 24 hours. At the end of the incubation period, phage titers were determined by counting plaques (clear lysis areas). In order to prepare stock solutions, the host EC43895 strain was enriched overnight in 50 ml TSB at 37°C, then infected with 100 µl of pure phage suspension and vortexed gently. Following overnight incubation at 37°C, 100 µl chloroform ($CHCl_3$, Sigma 288306) was added and centrifuged at 12 000 × *g* for 15 minutes. Supernatants were stored in fresh sterile tubes at +4°C.
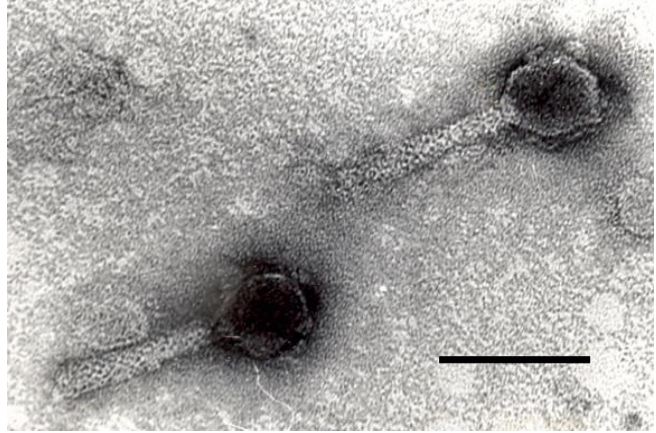

Figure 1. Transmission electron micrograph image of M8AEC16 phage (bar: 100 nm).
Şekil 1. M8AEC16 fajının elektron mikroskop görüntüsü (bar: 100 nm).

Food model: Pastirma was purchased as whole from a delicatessen in Kirikkale, Turkey. It was sliced into 10 g portions with a sterile knife in the laboratory within the same day and each 10 g slice placed in a sterile petri dish. Before the experiment, pastirma samples were analyzed for the presence of *E. coli* O157 by immunomagnetic separation (IMS) based culture method.

Experimental design: Samples were divided into two groups; phage group in which known stock phage suspension was added (P group) and control group in which the same amount of sterile water was added (C group). Each pastirma slice was contaminated on the entire surface with 1 ml NA-EC43895 at 6,9 x 10^3 cfu/ml using a pipette. After waiting 10 minutes for the bacteria to attach to the surface, 1 ml of 10^9 pfu/ml bacteriophage cocktail was administered to the P group. Then, the samples were incubated at 22–24°C (room temperature) in order to simulate the generally used storage conditions. Addition to the bacterial counting at 0 hour for determining the initial level of contamination, NA-EC43895 enumerations were done at 0,5., 1., 3., 6., 12. hours and on the 1., 2., 3., 4., 5., 6., 7. days. The experiment was performed in duplicate.

Bacterial counts: At the end of the storage, pastirma samples (10 g) were homogenized in stomacher (Labblender 400 Stomacher, London, England) for 1 minute with 90 ml *E. coli* broth (m-EC broth, Oxoid CM0853) containing 20 µg/l of novobiocin. Appropriate dilutions were prepared with peptone water (PS, 0.1% peptone, 1.07214, Merck) and plated onto NA-CT-SMAC. After the incubation at 37°C for 24 hours, colony counts were done and the results were compared with the C group counts. In P group, an enrichment method was needed to detect colonies below the detection limit (< 10 cfu/g), considering that the phage cocktail could reduce the number of bacteria approximately 2 log cfu/g. Therefore, in addition to the colony counting, suspended samples with peptone water were also concentrated by IMS method (anti-*E. coli* O157 Dynabeads, Norway) and then subjected onto NA-CT-SMAC. Randomly selected up to five colonies that observed on Na-CT-SMAC were confirmed by *E. coli* O157 latex agglutination test (Oxoid, DR0620).

Statistical analysis: Two independent replicate experiments were performed. General Linear Models with repeated measures design were used to investigate the differences in *E. coli* O157:H7 reductions over time. For the determination of statistical significance of the effect of time, Independent one-way ANOVA analyses were used. Limit of detection was taken as 1 log cfu/g for statistical analyses. For comparisons, differences were considered with a minimum of 0.05 significance level. Statistical analyses were performed using SPSS®14.1 for Windows.

Results

In the study, the *E. coli* O157:H7 count did not increase in the control group during 7 days of storage at 24°C. The decrease of 0,94 log cfu/g on the fourth day reached 1,84 log cfu/g on the seventh day. However, in the phage added group (P group) after the 30 minute of storage at 24°C, *E. coli* O157:H7 was not detected by counting method on NA-CT-SMAC. Due to the fact that the phage cocktail was able to reduce the bacterial count in the P group, *E. coli* O157:H7 presence was revealed by IMS method. At IMS performed inoculations *E. coli* O157:H7 was detected on NA-CT-SMAC. All the selected colonies were positive with latex agglutination test (Table 1) and no unexpected colonies were observed. Therefore, it was determined that the bacteriophage cocktail consisting of the M8AEC16 and M12BEC16 phages inhibited NA-EC43895 and reduced the bacterial count under the detection limit but not totally eliminated. In addition, regrowth was not observed during 7 days of storage (Table 1).

The significance of reductions over time could not be detected statistically due to the absence of bacterial count in P group after the 30 minutes of storage.

Table 1. NA-EC43895 counts at certain times.
Tablo 1. Belirli sürelerde yapılan NA-EC43895 sayımları.

Time	Bacterial count (log cfu/g)		IMS based culture technique	Latex agglutination
	Control group	Phage group		
0. minute	2,84	2,84		
0,5. hour	2,84	-	+	+
1. hour	2,84	-	+	+
3. hour	2,84	-	+	+
6. hour	2,47	-	+	+
12. hour	2,30	-	+	+
1. day	2,36	-	+	+
2. day	2,36	-	+	+
3. day	2,17	-	+	+
4. day	1,90	-	+	+
5. day	1,90	-	+	+
6. day	1,90	-	+	+
7. day	1,00	-	+	+

-: Not detected (detection limit: 1 log cfu/g). The significance of reductions over time could not be detected statistically due to the absence of bacterial count in P group after the 30 minutes of storage.

Discussion and Conclusion

In this study, we used a cocktail consisting M8AEC16 and M12BEC16 phages, which were isolated from one of our previous studies and identified to have specific lytic effect in many Shiga toxigenic and non-Shiga toxigenic *E. coli* O157:H7 strains, including *E. coli* O157:H7 ATCC 43895 strain (14). Phage cocktails are being used instead of a single phage in many studies where biocontrol of pathogens are being investigated (3, 19, 22). While it has been stated that the use of more than one phage in phage therapies has controlled or delayed the bacteria becoming resistant to phages (11, 26), in biocontrol studies this is especially preferred in order to provide a broad host spectrum (17).

Pastirma is a cured and dried meat product (27). Salt, nitrite, nitrate, spices and other additives that are used in the curing process of pastirma give the product a peculiar flavor and aroma as well as bacteriostatic properties. Especially the high salt concentration causes pastirma to have a very low water activity, which suppresses or completely prevents the reproduction of microorganisms (4). In the study, the number of bacteria in the control group did not increase during 7 days of storage at 24°C, moreover a decrease of around 1 log cfu/g was observed on the 4th day in the same group. Thus, it was shown that pastirma did not support *E. coli* O157:H7 growth due to the properties of the product but it also did not provide a

reduction in the current level of contamination. In studies investigating the presence of *E. coli* O157 in pastirma sold in Turkey, it has also been reported that the product does not support *E. coli* O157 growth (7, 10).

On the other hand, in the phage applied group, the number of bacteria decreased from 2,84 log cfu/g to the undetectable level from the first 30 minutes. Studies show that when the initial MOI (multiplicity of infection) is high, the decrease in the number of bacteria increases (15, 18, 29).

Phages are being applied in many foods for biocontrol of various pathogens. Among the food models for *E. coli* O157:H7 biocontrol there are foods such as raw meat (16, 22), tomato, spinach, broccoli, ground beef (1), lettuce, cantaloupe (25) and raw meatball (14). However, there is no phage study for pastirma. Considering that cattle are the reservoir of *E. coli* O157:H7 (12) and that pastirma are consumed without any heat treatment, it is feasible to use phages to prevent *E. coli* O157:H7 contamination from raw material or during production of pastirma.

One of the studies on beef, Hudson et al. (16) applied various phage concentrations to thinly sliced beef contaminated with *E. coli* O157:H7 and indicated that the best result was achieved at the highest MOI. It was stated that at about 4,5 log pob/kob MOI, the number of bacteria decreased below the detection limit from the 1st hour and could not reproduce again similar to our study. O'Flynn et al. (22) contaminated raw steak meat with 2 x 10^3 cfu/ml *E. coli* O157:H7 and then applied a phage cocktail containing three phages at MOI 10^6 pfu/cfu. After the incubation at 37°C for 1 hour, the researchers enriched the samples in a liquid media for 2 hours and found out that in 7 out of 9 samples, the number of bacteria was below the detection limit (10 cfu/ml).

In conclusion, it was determined that the phage cocktail reduced the number of *E. coli* O157:H7 in pastirma samples contained 2,84 log cfu/g bacteria, and it lowered the pathogen under the detection limit (< 10 cfu/g). In the light of this information, it can be concluded that the bacteriophage cocktail consisting of M8AEC16 and M12BEC16 could be effectively used for biocontrol of *E. coli* O157:H7 in pastirma. In this context, in order to gain a phage cocktail preparation to the food industry, different MOI levels studies, in-vivo acute toxicity tests and characterization studies should be carried out in the future.

Acknowledgement

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