Escherichia coli O157:H7 and *Listeria monocytogenes* in raw meatballs and phage control of *L. monocytogenes*

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Summary

Raw meatball (cig kofte, CK) is a traditional ready-to-eat product that is commonly consumed in Turkey. This appetizer poses a great risk in terms of containing foodborne pathogens due to the ingredients contained, such as ground beef. In this study, *Escherichia coli* O157:H7 and *Listeria monocytogenes* strains previously isolated from CK were characterized. Out of 27 *E. coli* O157 isolates, 13 were identified as *E. coli* O157:H7. In all of *E. coli* O157:H7 isolates, *eaeA*, *hly*, *fliCh7*, *espA*, $lpfA_{1\text{-}3}$ and *stx* genes were detected. All *L. monocytogenes* isolates ($n = 4$) were identified as serotype 1/2a or 3a. In addition to strain characterization, efficacy of a cocktail of lytic bacteriophages on *L. monocytogenes* in CK food model was investigated in order to demonstrate an alternative method to combat this pathogen. Samples contaminated with 2.30 log CFU·g^{-1} in the phage-applied group, *L. monocytogenes* was not detected from the very first hour of incubation. The highest reduction was observed as 2.35 log CFU·g^{-1} after incubation of 3 h when the multiplicities of infection was 4.14 log PFU·CFU^{-1}g^{-1}. This study showed that *L. monocytogenes* counts could be reduced to acceptable levels by bacteriophage application in a complex food like CK.

Keywords

bacteriophage; biocontrol; *Escherichia coli* O157:H7; *Listeria monocytogenes*; raw meatball; ready-to-eat

Raw meatball (cig kofte, CK) is a traditional ready-to-eat (RTE) product, which is commonly consumed and sold in markets and restaurants in Turkey, as well as in Middle East countries, Russia and in some parts of Asia. Although the ingredients of the product may vary from recipe to recipe, it is generally made of ground beef (optional), fine bulgur, paprika powder, onion, powdered garlic, tomato paste, salt, red pepper, black pepper, cumin, pomegranate syrup, water and vegetable oils. The mixture is obtained by kneading with hand or machine without applying any heat treatment. Then the dough is shaped into small meatballs (dimensions approximately 7 cm × 2 cm × 2 cm, weight approximately 30 g) and served with lettuce. Although CK is mostly consumed immediately after preparation, leftovers are stored refrigerated at 4 °C. The microbiological quality of CK depends on the initial contamination level of the raw ground beef and other components used. In Turkey, there are approximately 10 000 places from which CK can be purchased. The product can be sold as pre-packaged or unpackaged wraps, portions or balls in many retail outlets such as grocery stores, buffets, street corner shops, fast food restaurants or regular restaurants [1, 2]. Poor hygiene conditions during production of CK may result in contamination with foodborne pathogens, which are frequently associated with ingredients [3]. Among these foodborne pathogens, *Escherichia coli* O157:H7 and *Listeria monocytogenes* belong to the most important microorganisms since previous studies showed that raw meatballs can be contaminated with these bacteria [2, 4, 5].

Being natural predators of bacteria; bacteriophages have quite high host specificity and only kill target bacterial cells. The phage applications in foods are based on the elimination of specific pathogenic microorganisms and do not cause

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damage to other bacteria in the food environment, which is considered a wished feature of the antimicrobial substances used in foods and helps to maintain product quality. Lytic phages are mostly preferred phage types for biocontrol purposes because their lytic cycle ends up with the burst of the target cells-in a very short time [6, 7].

In this study, we aimed to characterize *E. coli* O157:H7 and *L. monocytogenes* strains previously isolated from CK. In addition to characterization of the strains, we also investigated the effect of a lytic bacteriophage cocktail on *L. monocytogenes* in CK food model in order to demonstrate an alternative method to combat this pathogen.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

A number of 27 strains *E. coli* O157 and 4 strains *L. monocytogenes* isolated from 168 CK samples (24 beef and 144 vegetarian) in a previous study [2] were characterized. *E. coli* O157:H7 ATCC 43895 (stx_1^+, stx_{1c}^+, stx_2^+, $eaeA^+$, $eae\gamma_1^+$, hly^+, $lpfA1\text{-}3^+$, $espA^+$, $fliC_{h7}$), *E. coli* O157:NM 137/98 (stx_{2c}^+), *E. coli* O62:H$^-$ 551/98 (stx_{2d}^+), *E. coli* O139:K12 107/86 (stx_{2e}^+), *E. coli* O$^-$:H18 214/125 (stx_{2f}^+) and *E. coli* O2:H25 S86 (stx_{2g}^+) were used as positive controls, while *E. coli* O157:H7 NCTC 12900 (stx_1^- and stx_2^-) was used as a negative control for the characterization of *E. coli* O157 isolates by polymerase chain reaction (PCR). For serotyping of *L. monocytogenes* isolates by multiplex PCR, *L. monocytogenes* ATCC 19111 (serotype 1/2a), *L. monocytogenes* N7144 (serotype 1/2b), *L. monocytogenes* ATCC 7644 (serotype 1/2c) and *L. monocytogenes* RSKK 475 (serotype 4b) reference strains were used as positive controls. In the study, five bacteriophages encoded LMF-M61, LMF-M83, LMF-M117, LMF-M119 and LMF-M135 that were lytic to *L. monocytogenes* [8] were used to prepare a phage cocktail for the biocontrol experiment of *L. monocytogenes* in CK.

DNA extraction

DNA was extracted using Chelex 100 resin (Bio-Rad, Hercules, California, USA). The isolates that had been stored at –80 °C (MDF-U5186S, Sanyo, Osaka, Japan) were grown in tryptic soy broth (TSB, Oxoid, Basingstoke, United Kingdom) at 37 °C for 24 h. One millilitre of each culture was centrifuged at 12000 ×*g* for 3 min (5417R, Eppendorf, Hamburg, Germany). Resuspension of the pellets was done by adding 200 μl of 6 % Chelex 100 and then 2 μl of proteinase K (20 mg·ml^{-1}; AppliChem, Darmstadt, Germany). The suspensions were incubated at 55 °C for 40 min and then at 95 °C for 8 min in a thermo-shaker MSC-100 (Hangzhou Allsheng Instruments, Hangzhou, China). Subsequently, the resulting supernatants were centrifuged at 12000 ×*g* for 3 min and kept at –20 °C until use for the characterization analyses for two weeks.

Detection of genes in *E. coli O157*

Detection of virulence genes stx_1, stx_2, *eaeA*, *hly*, $fliC_{h7}$ [9], *espA* [10] and *lpfA*1-3 [11] was performed with verified and identified *E. coli* O157 colonies using simplex and multiplex PCR. Intimin gene (*eae*) variants of α1, α2, β, β1, β2, γ1 and γ2/θ were tested in isolates carrying *eaeA* gene by previously published primer pairs and PCR conditions [12]. For determination of stx_1 variants (stx_{1c} [13], stx_{1d} [14]) and stx_2 variants (stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f} [15], stx_{2g} [16]) in *E. coli* O157:H7 isolates (stx_1^+ and/or stx_2^+), consecutive simplex and multiplex PCR assays were used.

PCR-based serotyping of *L. monocytogenes*

Multiplex PCR-based protocol was used with lmo0737 (691 bp), lmo1118 (906 bp), ORF2819 (471 bp) and ORF2110 (597 bp) primers (Integrated DNA Technologies, IDT, Leuven, Belgium) as described by Doumith et al. [17] (Tab. 1). PCR products were separated by electrophoresis and DNA fragments indicated the serotype:

- 691 bp fragment indicated serotype 1/2a or 3a,
- 471 bp fragment indicated serotype 1/2b or 3b,
- 906 bp fragments indicated serotype 1/2c or 3c,
- 597 bp fragments indicated serotype 4b, 4d or 4e.

Preparation of biocontrol experiment

In order to add a selective advantage, *L. monocytogenes* ATCC 19111 was made resistant to nalidixic acid (100 μg·ml^{-1}) by serial passages of increasing concentrations of the antibiotic

Tab. 1. Nucleotide sequences of primer sets used in this study [17].

Gene target	Primer sequence (5'-3')	Product size [bp]
lmo0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691
lmo1118	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906
ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471
ORF2110	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597

(25 $\mu g \cdot ml^{-1}$, 50 $\mu g \cdot ml^{-1}$, and 100 $\mu g \cdot ml^{-1}$) in TSB. Fifty millilitres of broth culture was centrifuged at 5000 $\times g$ for 7 min and pellet was resuspended in physiological saline solution (PS, 0.9% NaCl). Then, tenfold dilutions of the suspension was prepared in PS for inoculation of 10 g of CK in sterile sample containers. Phages LMF-M61, LMF-M83, LMF-M117, LMF-M119 and LMF-M135 were individually enriched in overnight cultures of nalidixic acid-resistant *L. monocytogenes (*NA-LM) in TSB at 30 °C statically. After 18 h of incubation, chloroform was added, the solution was centrifuged (3000 $\times g$, 15 min) and supernatants filtered through a syringe filter (pore size 0.22 μm; Corning, New York, New York, USA). Phage suspensions were mixed just before use in bacterial trials, and phage titre was determined by soft agar overlay technique using the host strain [1].

Decontamination of raw meatballs with bacteriophage cocktail

A procedure previously described by GENCAY et al. [1] was used. Briefly, a commercially available CK mixture kit (containing tomato powder, paprika powder, salt, black pepper, cumin, onion and powdered garlic, allspice, mild pepper powder, bulgur and dried parsley) was added to distilled water, sunflower seed oil and canned tomato paste to prepare CK without meat. Two different groups, phage (P) and control (C), were formed for three different bacterial contamination levels of approximately 10^2 $CFU \cdot g^{-1}$ (A), 10^4 $CFU \cdot g^{-1}$ (B) and 10^6 $CFU \cdot g^{-1}$ (C) to achieve three different multiplicities of infection (*MOI*). In group P, 1 ml of phage cocktail stock (approximately 10^{10} $PFU \cdot g^{-1}$) was used. Twenty-five grams of raw meatballs from P and C groups were aseptically weighed in filtered sterile plastic bags. One millilitre of bacterial suspensions of three different concentrations were inoculated to raw meatballs and kneaded thoroughly over the plastic bags. The samples were incubated at 4 °C and NA-LM counting was carried out at the initial minute and then after 1 h, 3 h, 6 h and 24 h on modified Oxford agar (Merck, Darmstadt, Germany) supplemented with 100 $\mu g \cdot ml^{-1}$ nalidixic acid (NA-MOX). Immunomagnetic separation (IMS)-based cultivation technique [18] was also performed for the detection of NA-LM, which produced results below the limit of detection (*LOD*) in the test samples. The test portions in the experiment were analysed in three replicates.

Statistical analysis

For each bacterial contamination level of raw meatballs, three independent experiments were performed. To analyse the reduction of *L. monocytogenes* over time and the interactions between time and *MOI*, General linear models with repeated measures design were used. To reveal statistical significance of effect of time at each *MOI*, independent one-way ANOVA analyses were used. For statistical analyses, *LOD* was taken as 1.0 log $CFU \cdot g^{-1}$ (1.0×10^1 $CFU \cdot g^{-1}$) and significance level of 0.05 was used. SPSS 14.1 for Windows (IBM, New York, New York, USA) was used for all statistical analyses.

RESULTS AND DISCUSSION

Over the years, several studies have evaluated the microbiological quality of CK in terms of presence of *E. coli* O157 and *L. monocytogenes* [2, 4, 5], but none of them extended their studies to genomic characterization level. In our previous study [2], the microbiological quality of 168 CK samples (24 beef and 144 vegetarian) commercially available in Turkey was evaluated, and it was determined that the prevalence of *E. coli* O157 and *L. monocytogenes* in the CK was 16.1 % (27 of 168 samples) and 2.4 % (4 of 168 samples), respectively. In general, the prevalence of *E. coli* O157:H7 was lower in vegetarian CK than in beef CK. In the present study, out of 27 *E. coli* O157 isolates, 13 (7.7 %, 13 of 168 samples) were identified as *E. coli* O157:H7 by the detection of *fliC*$_{h7}$ (Tab. 2). Prevalence of *E. coli* O157:H7 was found to be 12.5 % (3 of 24 samples) and 6.9 % (10 of 144 samples) in beef and vegetarian CK samples, respectively. Our results support the idea that the primary source of these two pathogens is raw or undercooked ground beef and beef products.

It is well known that the pathogenesis of *E. coli* O157 depends on specific virulence genes, such as *stx$_1$, stx$_2$, eae, hly, lpf* and *espA*. In this study, *eaeA*, *hly, fliC*$_{h7}$, *espA*, *lpfA$_{1-3}$* and at least one of the shiga toxin-encoding genes were detected in all of *E. coli* O157:H7 isolates. Genes *stx$_{1d}$, stx$_{2c}$* and *eae* γ_1 were found as variants of the related genes (Tab. 2). The main virulence factors are known as shiga toxins encoded by *stx1* and *stx2* genes, which are responsible for the illness progression to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [19, 20]. Our PCR analysis confirmed the presence of *stx$_1$* and *stx$_2$* genes. In six isolates, both *stx$_1$* and *stx$_2$* were detected, while in seven isolates only *stx$_1$* was detected by multiplex PCR. Compared to the *stx$_1$*-harbouring strains, isolates harbouring *stx$_2$* are known to be more virulent. Studies based on epidemiology demonstrated that *stx$_2$*-harbouring strains are more com-

monly responsible for HUS than *stx1*-harbouring strains, and *stx2* is the most important virulence factor associated with severe illness [21, 22]. These data indicate that strains isolated from CK were highly virulent and could cause human infection with manifestations of HC and/or HUS.

The finding that *stx2* strains were less frequently detected than the *stx1* ones is similar to that of OSAILI et al. [21]. In contrast, SALLAM et al. [22] reported that *E. coli* O157 isolates harbouring *stx2* were more common in raw beef than those harbouring *stx1*. On the other hand, the *eae* gene, which encodes intimin (an outer membrane protein), *hly* gene, which encodes for EHEC hemolysin, *lpf* gene encoding for long polar fimbria and *espA* gene, which encodes for secreted protein A of *E. coli*, are also important to the pathogenicity of *E. coli* O157:H7 [19, 20]. Here in this study, *eae, hly, lpf* and *espA* genes were detected in 13 of 27 isolates. This result corresponds to SALLAM et al. [22], OSAILI et al. [21] and AYAZ et al. [19] showing that almost all of the isolated *E. coli* O157: H7 strains were positive for *eae, hly, lpf* and *espA* genes. In the present study, the presence of the six virulence genes (*stx1, stx2, eae, hly, lpf* and *espA*) in almost all of the isolates may link CK, and its beef-containing variant in particular, with a high potential health risk. Furthermore, out of 27 *E coli* O157 isolates, 13 were identified as *E. coli* O157:H7 in the present study. Researchers have not found *E. coli* O157:H7 in raw meatball samples in previous studies, only *E. coli* O157 was detected in a study conducted by CADIRCI et al. [18].

In general, prevalence of *L. monocytogenes* was lower in vegetarian CK than in beef CK in this study. Since the primary contamination comes from raw or undercooked ground beef or beef products, it is seen that our results confirm this. The prevalence of *L. monocytogenes* in beef CK was 8.3 % (2 of 24 samples), while it was 1.4 % (2 of 144 samples) in vegetarian CK. According to a study conducted previously in Turkey [23], the prevalence of *L. monocytogenes* in ground beef was 41.9 %. Furthermore, *L. monocytogenes* was isolated from 45.6 % of beef samples collected from carcasses after slaughtering in beef cattle slaughterhouses [24], 53.3 % of samples of retail beef in Iran [25] and 6 % of RTE meat products in Estonia [26]. Recent researches also showed that *L. monocytogenes* can be found in RTE foods. In a study conducted by LUCHANSKY et al. [27], 19 out of 1689 raw cut vegetable samples and 16 out of 6488 meat samples were found positive for *L. monocytogenes.*

In the present study, 4 *L. monocytogenes* isolates were identified as serotype 1/2a or 3a using multiplex PCR. Prior studies [28] showed that virulence of *L. monocytogenes* strains is related to serotype type to a certain extent. Most human listeriosis outbreaks are caused by *L. monocytogenes* serotypes 4b, 1/2b or 3b, whereas serotypes 1/2a, 1/2c, 3a and 3c were found to relate to sporadic cases [29].

Biofilm formation at low water activity and high salt concentration makes disinfection regarding *L. monocytogenes* difficult. Recently, many studies on the use of bacteriophages in the biocontrol of *L. monocytogenes* in food were published. Various food models, such as raw salmon fillet tissue [30], soft cheeses [31] or raw beef [32] were

Tab. 2. Molecular characteristics of *E. coli* O157:H7 isolates.

Isolate	*fliC*$_{h7}$	*stx1*	*stx2*	*eae*	*hly*	*lpf*	*espA*
Vegetarian cig kofte isolates							
1	+	*stx1d*	*stx2c*	*eae γ1*	+	+	+
3A	+	*stx1d*	*stx2c*	*eae γ1*	+	+	+
6	+	*stx1d*	*stx2c*	*eae γ1*	+	+	+
8A	+	*stx1c*	–	*eae γ1*	+	+	+
9A	+	*stx1d*	–	*eae γ1*	+	+	+
15A	+	*stx1c*	–	*eae γ1*	+	+	+
16A	+	*stx1c*	–	*eae γ1*	+	+	+
17	+	*stx1c*	–	*eae γ1*	+	+	+
18	+	*stx1c*	–	*eae γ1*	+	+	+
19A	+	*stx1c*	–	*eae γ1*	+	+	+
Beef cig kofte isolates							
Etli 2	+	*stx1d*	*stx2c*	*eae γ1*	+	+	+
Etli 8A	+	*stx1c*	*stx2c*	*eae γ1*	+	+	+
Etli 10A	+	*stx1c*	*stx2c*	*eae γ1*	+	+	+

(+) – related gene positive, (–) – related gene negative.

Tab. 3. Mean nalidixic acid-resistant *L. monocytogenes* counts of three test portions.

Storage time	Mean *L. monocytogenes* counts [log CFU·g^{-1}]					
	Control groups			8.78 log PFU·g^{-1} phage threated groups		
	A	B	C	A	B	C
Initial	2.30	4.63	6.34			
1 h	2.50	4.64	6.30	nd	2.78	4.70
3 h	2.50	4.65	6.30	nd	2.30	4.52
6 h	2.20	4.47	6.20	nd	2.25	4.20
24 h	2.90	4.64	6.20	nd	2.78	4.34

Contamination level: A – 2 log CFU·g^{-1}, B – 4 log CFU·g^{-1}, C – 6 log CFU·g^{-1}.
nd – not detected (limit of detection is 1 log CFU·g^{-1}).

Tab. 4. Reduction of viable nalidixic acid-resistant *L. monocytogenes* in experimentally contaminated cig kofte.

Initial *MOI* [log PFU·CFU^{-1}g^{-1}]	Initial bacterial counts [log CFU·g^{-1}]	Storage temperature [°C]	Mean reduction [log CFU·g^{-1}]			
			1 h	3 h	6 h	24 h
6.47	2.30	4	nd	nd	nd	nd
4.14	4.63	4	1.86	2.35	2.22	1.86
2.43	6.34	4	1.60	1.78	2.00	1.86

MOI – multiplicities of infection, nd – not detected (limit of detection is 1 log CFU·g^{-1}).

used to investigate biocontrol of *L. monocytogenes*. The use of bacteriophages in *L. monocytogenes* biocontrol in the food model of CK was first investigated in this study. Application of bacteriophage cocktail significantly ($p < 0.05$) decreased counts of viable *L. monocytogenes* to undetectable levels. Thus, it was shown that *L. monocytogenes* could survive in CK. It was also reported that the product supports vitality of *L. monocytogenes* in studies investigating the presence of *L. monocytogenes* in CK sold in Turkey [33].

Due to the development of bacterial resistance to multiple antibiotics, studies on the use of bacteriophages as biocontrol agents have been increasing. However, efficacy of bacteriophages against target bacteria can vary considerably. Environmental conditions, such as type of food matrix, strongly influence the killing of host cells by bacteriophage infection. Some studies showing that higher bacteriophage counts give better results support this idea [1, 6, 7]. Soni and Nannapaneni [30] reported that phage concentrations of 10^4 PFU·ml^{-1}, 10^6 PFU·ml^{-1} and 10^8 PFU·ml^{-1} were equally effective in inhibiting *L. monocytogenes* growth in the samples. In our study, approximately 10^{10} PFU·ml^{-1} of phage cocktail (LMF-M61, LMF-M83, LMF-M117, LMF-M119 and LMF-M135) was used. In samples contaminated with 2.30 log CFU·g^{-1} bacteria in the phage-applied group, *L. monocytogenes* was not detected (*LOD* was 1 log CFU·g^{-1}) from the very first hour of incubation. However, by IMS it was revealed that the bacteria were not completely eliminated (Tab. 3). The highest reduction was observed in 4.63 log CFU·g^{-1} bacteria containing group, with 2.35 log CFU·g^{-1} after 3 h. In the group contaminated with 6.34 log CFU·g^{-1}, reductions were seen almost at the same level and reached to 2.00 log CFU·g^{-1} in 6 h. Bacteriophage count reached 9.3 log PFU·g^{-1}in phage-treated groups during 6 h incubation at 4 °C (Tab. 4). The initial phage count applied in this study was calculated according to the count of bacteriophages per *L. monocytogenes* (*MOI*). Initial *MOI* values are shown in Tab. 4.

In this study, our aim was to reduce the counts of *L. monocytogenes* to the acceptable limit for RTE foods as specified in European Commission Regulation (EC) No 2073/2005 [34]. According to some studies, when the initial *MOI* is high, the decrease in bacterial counts is more prominent [7]. On the other hand, some studies reported that a high initial bacterial inoculation reduces the efficacy of bacteriophages in foods [31], as in our study.

The present study showed that the level of contamination in CK with *L. monocytogenes* can be reduced by bacteriophage treatment to 10^2 CFU·g^{-1} even at high contamination levels. Furthermore, risk analysis and legislation of some countries, such as Canada, indicate that the tolerable limit for *L. monocytogenes* contamination in RTE foods is 10^2 CFU·g^{-1} [35]. These results further demonstrate that bacteriophage cocktails are suitable for biocontrol applications for *L. monocytogenes* in RTE foods like CK.

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