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Characterization and Antimicrobial-Resistance Profile of Escherichia coli O157 and O157: H7 Isolated from Modified Atmosphere Packaged Meat Samples

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A B S T R A C T

Shiga-like toxin producing *Escherichia coli* is still an important public issue which causes extremely dangerous health problems. This study was planned in order to examine the inhibitory effect of Modified Atmosphere Packaging application on *E. coli* O157 and O157: H7. The purposes of the present study were to detect *E. coli* O157 and O157: H7 strains from ground and cubed beef. A total of 100 MAP cattle meat products (50 minced meat, 50 meat cubes) were collected from the markets and butchers in Samsun province between May and October 2013. According to results, 1(1/50-2%) *E. coli* O157 and 1(1/50-2%) *E. coli* O157: H7 strains isolated from 50 ground beef samples, while 1 (1/50-2%) *E. coli* O157 strain was identified from 50 cubed beef samples. It was determined that *E. coli* O157 isolate obtained from the MAP ground beef carried $stx_{1,}$ stx_2 genes; *E. coli* O157: H7 isolate carried $stx_{1,}$ $stx_{2,}$ *eaeA* and *hylA* genes while *E. coli* O157 isolate obtained from the MAP cubed meat only carried the stx_2 gene. In antibiogram test, both *E. coli* O157 isolates were resistant to streptomycin and one *E. coli* O157: H7 isolate was resistant to streptomycin, cephalothin and tetracycline. As a consequence; in order to protect public health, products should be kept in proper hygienic and technical conditions during sale and storage and use of uncontrolled antibiotics should be avoided.

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Modifiye Atmosfer Paketli Et Örneklerinden İzole Edilen *Escherichia coli* O157 ve O157: H7'nin Karakterizasyonu ve Antibiyotik Direnç Profili

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Ö Z E T

Bu çalışmada 2013 yılında Mayıs ve Ekim ayları arasında Samsun ilinde satışa sunulan Modifiye Atmosfer Paketli (MAP) sığır kıyma ve kuşbaşı örneklerinde *Escherichia coli* O157 ve O157: H7'nin klasik kültür tekniği kullanılarak belirlenmesi, PCR ile doğrulanması, genomik karakterizasyonu ve fenotipik antibiyotik dirençlilik profillerinin ortaya konulması amaçlanmıştır. Araştırmada toplam 100 (50 sığır kıyma-50 sığır kuşbaşı) adet MAP sığır et ürünü materyal olarak kullanılmıştır. Analiz edilen 50 MAP sığır kıyma örneğinin 1'inin (1/50-%2) *E. coli* O157, diğerinin (1/50-%2) *E. coli* O157: H7 ile, 50 adet MAP sığır kuşbaşı örneğinin 1'inin (1/50-%2) ise *E. coli* O157 ile kontamine olduğu belirlenmiştir. Yapılan genotiplendirme sonucunda MAP sığır kıyma örneklerinden elde edilen *E. coli* O157 izolatının *stx1, stx2, E. coli* O157: H7 izolatının ise *stx1, stx2, eaeA* ve *hlyA* gen bölgelerini taşıdığı, MAP sığır kuşbaşı örneklerinden elde edilen *E. coli* O157 izolatının ise sadece *stx2* gen bölgesini taşıdığı belirlenmiştir. Fenotipik antibiyotik profil analizinde *E. coli* O157 izolatlarının her ikisinin yalnızca streptomisine karşı dirençli olduğu bununla beraber *E. coli* O157: H7 izolatının ise streptomisin, sefalothin ve tetrasiklin antibiyotiklerine karşı dirençli olduğu saptanmıştır. Sonuç olarak halk sağlığının korunması amacıyla ürünlerin uygun teknik ve hijyenik şartlarda satışa sunulması ve muhafaza edilmesine dikkat edilmesi, ayrıca izolatların antibiyotiklere direnç göstermesi nedeniyle kontrolsüz antibiyotik kullanımının önlenmesi önerilmektedir.

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Introduction

Escherichia coli (STEC) that produces Shiga toxin is still on the agenda as an important public health issue that results from zoonosis. Asymptomatic cattles are considered as primary source for *E. coli* O157: H7 infections. In previous surveys carried out, it is known that the dominant prevalence in cattle is between 1% and 71% (Meng et al. 1995; Hussein and Sakuma 2005). The terminal recto-anal junction (RAJ) becomes colonized in carrier animals, and it is found at a level of 10-100 CFU/g in faeces. It is known that in most cases the infection is formed as a result of consuming undercooked meat contaminated with cattle faeces. The effects of *E. coli* serotype with STEC character lead to bloody diarrhea among non-complicated diarrheal cases in people (Hemorrhagic Colitis) and haemolytic uremic syndrome (HUS) that reaches a point that threatens human life in advanced cases. Five major STEC serotypes have been determined so far, and these are known as O26: H11, O103: H2, O111: H8, O145: H28, O157: H7. It is reported that major virulence factors of *E. coli* O157: H7 are the production of *stx1*, *stx2* or their variants that are coded by lysogenic and lambdoid bacteriophages. Another important virulence factor is *eae* gene that localized in the chromosome and coding the intimine (*E. coli* bonding and adherence protein). These proteins ensure the adherence of the factor on the surface of intestinal epithelium (Eklund 2005). At the same time, it is reported in the studies carried out in recent years, *E. coli* O157: H7 develops multiple resistances to several antibiotics and this resistance mechanism developed contributes to virulence of the bacteria. Multiple antibiotic resistance mechanism of the agent is generally explained by mutation, selective repression and genetic transformation (Schroeder et al. 2002). The modified atmosphere packaging is a food preservation technique widely used in many food products to increase the shelf life. With the MAP technology, it is aimed to reduce the speed of respiration, slow down oxidative and enzymatic deteriorations, delay microbial deterioration as much as possible by inhibiting or stopping microbial reproduction, and thus, increase the shelf life of products. Oxygen (O_2), carbon dioxide (CO_2) and nitrogen (N_2) are widely used gas components in order to change the atmosphere composition in the package (Cooksey 2014).

In this research, it was objectived to i) determine the presence of *E. coli* O157 and O157:H7 using the classical culture technique, ii) confirm the isolates and associated genes by PCR, and iii) reveal the phenotypic antibiotic resistance profiles of the isolates obtained from Modified Atmosphere Packaged (MAP) beef minced meat and meat cube samples.

Material and Methods

A total of 100 MAP cattle meat products (50 minced meats, 50 meat cubes) were collected from the markets and butchers in Samsun province between May and October 2013. All samples were transported to the laboratory in the shortest time possible under the cold chain and analyzed immediately. The samples were selected from different parties, and the packages were at least 500 g.

Isolation and Identification of E. coli *O157 and* E. coli *O157: H7*

In the enrichment process, Modified Tryptone Soy Broth (mTSB- Merck 1.09205) containing 225 ml of novobiocin was added to 25 g of the sample taken to sterile bags and incubated at 41.5ºC for 18-24 h. A loop of suspension was taken to Tellurite (2.5 mg/l) Cefixime (0.05 mg/l)-Sorbitol Mac Conkey (CT-SMAC) agar (Oxoid-CM 813, Supl. SR 172 E) and the plates were incubated at 37ºC for 24 h. Following the incubation, 5 suspected colonies that did not fermentate sorbitol reproduced in the plates were chosen, subcultured in Yeast Extract-Trypticase Soy Agar (TSA-YE) (Oxoid-CM 131-L21), and the plates were incubated at 37ºC for 24-48 h. The suspected isolates were streaked onto Sorbitol Mac Conkey Agar (SMAC-MUG Supl. Oxoid-BR 071 E) containing 4-methylumbelliferly-B-D-Glucuronide (MUG) by the drawing method the plates were incubated at 37ºC for 24 h. In addition, indole and motility tests were performed. The colonies that did not represent fluorescent light under UV light (at 366 nm wavelength) were assessed as MUG-negative. Isolates were then inoculated on Purple Broth Base (Difco-0227-01-6) containing cellobiose and incubated at 37°C for 24 h. Consequently; non-sorbitol fermenting, indole positive, motile, MUG negative, non-cellobiose fermenting colonies were selected and agglutination test was performed to these colonies with O157 antiserum (DR0620M; Oxoid, UK). Agglutination positive isolates were considered as *E. coli* O157. Afterwards, *E. coli* O157 isolates were tested with H7 antiserum and agglutination positive strains were considered as *E. coli* O157: H7 (Cagney et al. 2004; Dontorou et al. 2004).

Confirmation of Isolates with Multiplex PCR and Determination of stx1, stx2, eaeA and hlyA Genes

Confirmation of *E. coli* O157 and *E. coli* O157: H7 isolates were performed using mPCR. The presence of *stx1, stx2, eaeA* and *hlyA* genes was carried out according to the procedure described by Maurer et al. (1999) and Fratamico et al. (2000). List of primers used was shown in Table 1. *E. coli* O157: H7 ATCC 43895 and *E. coli* O157: H7 ATCC 35150 were used as reference strains.

DNA Extraction

The genomic DNA extraction of isolates was performed with boiling method. Briefly, the isolates were incubated at 37°C for 24 h in Brain Heart Infusion Broth (BHI-Oxoid CM 0225), then 1 ml suspension was transferred to sterile eppendorf tubes and centrifuged for 5 min at 10,000×g (Hettich Universal 320R, Germany). Afterwards, the supernatant was discarded and the pellet was resuspended in 500 µL of PBS and kept in a water bath at 95°C for 10 min. Then centrifuged again for 5 min at 10,000×g. The extracted DNA was stored at -20ºC until analysis.

mPCR and Electrophrosesis

PCR mixture was prepared in a total volume of 50 µL containing 1X PCR Buffer (Sigma P2317), 1.5 mM $MgCl_2$ (Sigma M8787), 0.1 mM dNTP (Sigma DNTP100A), 0.5 U Taq-Polymerase (Sigma D4545), 1 µM of each primer and 5 µL of target DNA.

Table 1 Characteristics primers used for detection of *E. coli* O157: H7 and virulence genes

Primer	Sequence (5'- 3')	Product size (bp)	Reference
rfbO157 F *rfbO157* R	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	420	(9)
fliCh7 F *fliCh7* R	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	(9)
stx1 F *stx1* R	TGTAACTGGAAAGGTGGAGTATACA GCTATTCTGAGTCAACGAAAAATAAC	210	(9)
stx2 F *stx2* R	GTTTTTCTTCGGTATCCTATTCC GATGCATCTCTGGTCATTGTATTAC	484	(9)
eaeA F *eaeA* R	ATTACCATCCACACAGACGGT ACAGCGTGGTTGGATCAACCT	397	(9)
hlyA F *hlyA* R	ACGATGTGGTTTATTCTGGA CTTCACGTCACCATACATAT	166	(9)

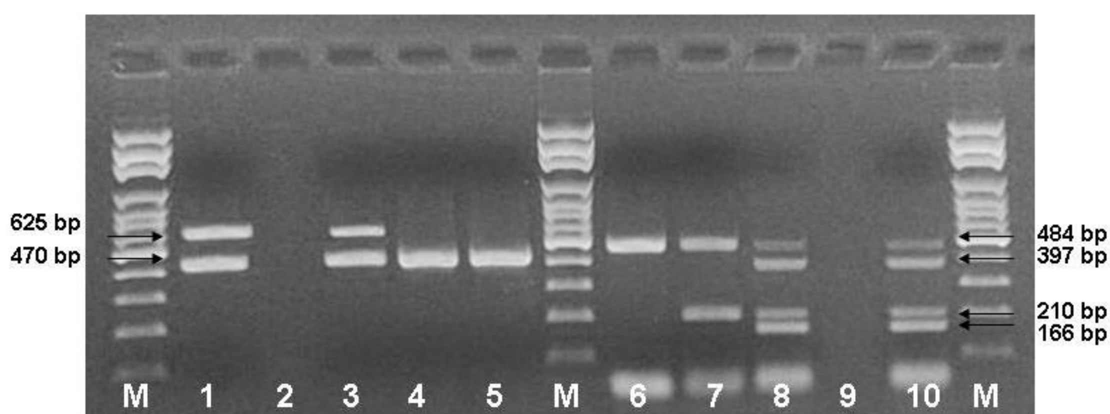

Figure 1 Electrophorese Image of *rfbo157*, *flich7*, *stx*1, *stx*2, *eaea* and *hlya* Genes of Isolates by Multiplex PCR.
[M: 100 bp DNA marker, lane 1: Positive control for *rfbO157* and *fliCh7* genes (*E. coli* O157:H7 ATCC 43895), lane 2: Negative control, lane 3: *rfbO157* and *fliCh7* positive isolate, lane 4-5: *rfbO157* positive isolates lane 6: *stx2* gene positive isolate, lane 7: *stx1*, *stx2* gene positive isolates, lane 8: *stx2*, *eaeA*, *stx1* and *hlyA* genes positive isolates, lane 9: Negative control, lane 10: Positive control for *stx2, eaeA, stx1* and *hlyA* genes (*E. coli* O157:H7 ATCC 35150)]

The amplification of the *rfbO157, fliCh7, stx1, stx2, eaeA* and *hlyA* genes was performed in Thermal Cycler (Bio-Rad MJ mini Gradient CA-USA), as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 20 seconds, annealing at 54°C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min (Fratamico et al. 2000). The acquired amplicons were run on 2% agarose gel (Sigma A9539; Tris-Borate-EDTA, Sigma T4415) at 90V for 90 min (BioRad Power Pac-Basic, Singapore) containing ethidium bromide (5 µg/ml). 100 bp DNA ladder was used as DNA size marker in in the process. The PCR products were visualized under UV illumination for *rfbO157, fliCh7, stx1, stx2, eaeA* and *hylA* genes at 420 bp, 625 bp, 210 bp, 484 bp, 397 and 166 bp respectively (Wise-UV-Wuv-L50, Grafstal, Germany) (Figure 1).

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed using the disc diffusion method on Mueller-Hinton agar (Oxoid, UK). For this purpose, ten antibiotic disks were chosen as follows: Sulphamethoxazole-Trimethoprim (Oxoid-CT 0052B, 25 µg), Ampicillin (Oxoid-CT 0003B, 10 µg), Amoxicillin (Oxoid-CT 0223B, 30 µg), Cephalothin (Oxoid CT0010B, 30 µg), Chloramphenicol (Oxoid-CT0013B, 30 µg), Tetracycline (Oxoid CT0054B, 30 µg), Ofloxacin (Oxoid-CT0446B, 10 µg), Ciprofloxacin (Oxoid-CT1615B, 10 µg), Streptomycin (Oxoid-CT0047B, 10 µg), Gentamycin (Oxoid-CT0024B, 10 µg). Briefly, 24 h fresh colonies were grown at 35 °C for 24 h in tryptic soy broth (Merck 1.05459) with yeast extract (Oxoid LP0021). After incubation, the turbidity of colonies was adjusted to 0.5 McFarland (10^8 CFU/ml) using a McFarland densitometry (Biosan, DEN-1, Latvia). 1 ml of the bacteria suspension was spread onto MHA (Oxoid CM 337) using a sterile swab and plates were dried for 10-15 min at room. Then plates were incubated at 35°C for 18-24 h by placing antibiotic discs. At the end of the incubation, the diameters of the inhibition zones around antibiotic discs were measured, and the isolates were classified as sensitive, intermediate or resistant (Wayne 2007).

Results and Discussion

It is determined that 1 of 50 (1/50-2%) MAP beef minced meat samples analyzed is contaminated with *E. coli* O157, the other (1/50-2%) is contaminated with *E. coli* O157: H7, and 1 of 50 (1/50- 2%) MAP beef meat cube samples (1/50- 2%) is contaminated with *E. coli* O157 (Table 2).

All around the world, the studies carried out in order to show the importance of contamination frequency, the risk level incurred and importance in the sense of public health in terms of *E. coli* O157: H7 serotype are similar to the findings of this research. Doyle and Schoeni (1987) isolated the agent in 6 of 164 beef meat (3.7%), 4 of 264 pork (1.5%), 4 of 263 poultry (1.5%), and 4 of 205 lamb

meat (2.0%). Willshaw et al. (1994) detected *E. coli* O157 not forming verotoxin in 5 (1.6%) of 134 minced meat, 52 sausage and 124 hamburger samples in the UK. Abdul-Raouf et al. (1996) reported that they isolated *E. coli* O157:H7 in 3 (6%) of 50 beef minced meat samples. In Switzerland, Fantelli and Stephan (2001) reported 2.3% prevalance of *E. coli* O157: H7 in 211 ground beef samples. Guyon et al. (2001) reported that they isolated only one *E. coli* O157: H7 only from 225 beef samples. In Turkey, the prevalence of the agent was investigated in various studies. Temelli et al. (2012) detected the prevalence of the agent 5.55% from 106 meat samples. In a study they conducted on 100 hamburger and İnegöl meatballs, Sarımehmetoğlu et al. (1998) reported that 5% of İnegöl meatballs and 2% of hamburgers were contaminated with *E. coli* O157, and all of the isolates were of verotoxigenic character. Alişarlı and Akman (2004), detected *E. coli* O157 in 4.6% (7/150) of 150 minced beef meat, and in 2% (3/150) of 150 minced mutton meat samples. In the study conducted by Keleş et al. (2006), researchers detected the pathogen in 1 of minced meat samples, 3 of cooled İnegöl meatballs, 1 of frozen İnegöl meatballs, and 3 of frozen hamburger meatballs examined. In the study conducted by Cadirci et al. (2010), researchers analysed 100 ground beef and 100 raw meatball samples by immunomagnetic separation and PCR methods and detected 1 *E. coli* O157 from ground beef, and 4 *E. coli* O157 from raw meatballs. Researchers also indicated that these strains were negative for H7.

As a result of the PCR analysis conducted, it was determined that the *E. coli* O157 isolate obtained from MAP beef minced meat samples contained the gene zones *stx*1, *stx2*; *E. coli* O157: H7 isolate contained the gene zones *stx1, stx2, eaeA* and *hlyA*; and *E. coli* O157 isolate obtained from MAP beef meat cube samples contained only the *stx*2 gene region (Table 2). The pathogenesis of the agent depends on several virulence factors such as *stx1* and *stx2*, intimin and enterohemolysin. The disease caused by STEC is an important public health issue. It is reported that a great majority of the strains of these microorganisms contain *stx2*, some of them contain *stx1* and *stx*2, and just a very small portion contain only the *stx1* gene (Law 2000). In line with the literature, it has been determined in this study that the most frequently detected toxin gene is *stx2*. Cagney et al. (2004) examined 1533 beef minced meat and burger samples and it was determined that 43 isolates they obtained had *eaeA*, *hlyA* and *fliCh7* genes, and 41 isolates had stx_1 and stx_2 genes. The presence of these two virulence factors (*eaeA* and *hlyA*) bears a resemblance to this study.

Varela-Hernandez et al. (2007), collected 27 *E. coli* O157: H7 (n=11) and *E. coli* O157 (n=16) in total from 258 carcasses and tested them in terms of virulence factors using the multiplex PCR process. The *stx2*, *eaeA* and *hyl*A genes were detected only in 1 *E. coli* O157: H7 isolate. In the study, they carried out for the purpose of determining the presence of *E. coli* O157: H7 and virulence genes, Ertaş et al. (2013), examined a total of 500 samples consisting of meat cubes, minced meat, hamburgers, raw cow milk and cheese made from raw cow milk; and the presence of *E. coli* O157: H7 was determined in 5 (1%) samples in total, 2 samples of meat cubes, 1 sample of minced meat and 2 samples of raw milk, among 500 samples. They reported that 3 of these isolates (3/5-60%) were positive in terms of the *stx1*, *stx2* and *hlyA* genes. However, 2 isolates isolated from raw milk products carried the *stx1*, *eaeA* and *hlyA* genes.

Antimicrobial susceptibility testing results showed that both of *E. coli* O157 isolates are resistant only to streptomycin while *E. coli* O157: H7 isolate is resistant to streptomycin, cephalothin and tetracycline antibiotics (Table 3). Multiple antimicrobial resistance is a widespread case among *E. coli* O157: H7 strains isolated from different sources. Schroeder et al. (2002), have detected that 17% of *E. coli* O157 isolates in the US are resistant to one antimicrobial, 7.5% are resistant to two antimicrobials, 8% are resistant to three antimicrobials, 5% are resistant to four, 2% are resistant to five, and 0.1% is resistant to six different antimicrobials. Similarly, Zhao et al. (2001), have determined that 4 of 29 E. coli O157: H7 isolates obtained from human, animal and food resources in the US show resistance to 5 different antibiotics, these being tetracycline, ampicillin, streptomycin, kanamycin, and sulfamethoxazole. They reported that 2 of these 4 isolates were of human origin while the other 2 were of minced meat origin.

Table 2 Prevalence of *stx1, stx2, eaeA* and *hlyA* genes in *E. coli* O157 and H7 isolates from MAP minced and cubed meat samples

Samples	Number of *E. coli* O157 and H7 positive samples (%)	Number of *E. coli* O157 and H7 positive isolates (*rfbO157* and *fliCh7* genes)			Virulence genes			
		Isolate code	*E. coli* O157 (*rfbO157*)	*E. coli* O157:H7 (*fliCh7*)	*stx1*	*stx2*	*eaeA*	*hlyA*
Minced Meat (n:50)	2 (4%)	33-1	+	-	+	+	-	-
		72-1	+	+	+	+	+	+
Cubed Meat (n:50)	1 (2%)	60-1	+	-	-	+	-	-

Table 3 Antibiotic resistance profiles of *E. coli* O157 and H7 from MAP minced and cubed meat samples.

C	Results of Disc Diffusion Test																															
	1E	2E	ST			AM10			AM30			CE30			CH30			TE30			OX10			CI10			ST10			GE10		
			R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
33-1	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+
72-1	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+	+	-	+	-	-	+	-	-	+	+	-	-	-	-	+
60-1	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+

C: Code, 33-1: MAP minced meat, 72-1: MAP minced meat, 60-1: MAP cubed meat, 1E: *E. coli* O157, 2E: *E. coli* O157:H7, ST: Sulphamethoxazole- Trimethoprim (25 µg), AM10: Ampicillin (10 µg), AM30: Amoxicillin (30 µg), CE30: Cephalothin (30 µg), CH30: Chloramphenicol (30 µg), TE30: Tetracycline (30 µg), OX10: Oxfloxacin (10 µg), CI10: Ciprofloxacin (10 µg), ST10: Streptomycin (10 µg), GE10: Gentamycin (10 µg)

All *E. coli* O157: H7 isolates tested in this study were found sensitive to tetracycline in parallel to 3 isolates of ewe's milk, fresh sausage and pig intestines obtained in the study carried out by Dontorou et al. (2004), in Greece. In another study, it was reported that *E. coli* O157: H7 isolates isolated from cattle were moderately resistant to ampicillin, sulfamethoxazole and cefoxitin, at the rates of 63.6%, 63.6% and 9.1%, respectively, with the disc diffusion method (Goncuoglu et al., 2010).

N_2 and CO_2 are the two most frequently used gases in MAP technology, and the latter is liable for actual bacteriostatic effect. O_2 helps to conserve the red colour of fresh meat by delaying the formation of metmyoglobin that gives the meat brown color (Farber 1991). Uyttendaele et al. (2001), reported that there was a reduction of 0.93 log CFU/g at the level of *E .coli* O157: H7 as a result of storing the packaged beef meat slices in the atmosphere containing 40% CO_2 at 4°C for one week. In the study carried out by Nissen et al. (2000), it was seen that the storage of minced meat in the environments containing 30% and 60% CO_2 at 10°C was not affected by CO_2 concentration and the development of *E. coli* O157: H7 was nearly completely inhibited.

Barrera et al. (2007), have reported that 100% CO_2 and 35% CO_2- 35% O_2 and 30% N_2 gas mixtures ensure inhibition on *E. coli* O157: H7 at the levels of 0.8 log CFU/g and 0.45 log CFU/g, respectively, but there is no full eradication. Ramamoorthi et al. (2009), reported that the inhibition of the gas mixtures used in MAP packaged meat products hardly became 1 log on the 28th day, and MAP packaging did not ensure full inhibition on *E. coli* O157: H7 of aerobic character.

Conclusions

Since there are no sufficient studies on the effect of the gases used in MAP technology which explains the development and survival of the pathogenic bacteria in meat products, this study was planned in order to examine the inhibition effect of MAP application. According to the results obtained from this research, it has been determined that there are pathogenic *E. coli* factors that may constitute a very serious danger in terms of human health in MAP packaged meat samples. It is considered that performing both the preparation and conservation processes of MAP packaged meat samples in accordance with hygienic processes is very important in terms of preventing the development of the infection due to the fact that *E. coli* O157: H7 may lead to extremely important infections in terms of human health, such as hemorrhagic colitis or haemolytic uremic syndrome, even at quite low titres (<10 cells).

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