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CURING EFFECTS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AND *ESCHERICHIA COLI* O157:H7 IN CAMEL MEAT USING MOST PROBABLE NUMBER-POLYMERASE CHAIN REACTION METHOD

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ABSTRACT

MPN-PCR method was used to determine *Escherichia coli O157:H7* and *Listeria monocytogenes* in cured camel meat samples during nine days of storage. The MPN-PCR is based on the combination between the conventional Most Probable Number and the Polymerase Chain Reaction technique targeting the *Stx1*and *Stx2* gene for *Ecoli O157:H7* and *hlyA* gene for *L. monocytogenes*.Growth of both bacteria in the cured and control experimental groups was initially subjected to a spectrophotometric MPN assay using a microplate reader followed by confirming by two PCR assays. It was shown that in the inoculated samples, the population of both microorganisms was significantly increased at all the test times. However, a significant reduction in the populations of both microorganisms was shown in the cured meat compared to the inoculated -but not- cured group. Our results suggested that curing can improve the microbial quality of camel meat.

Keywords: *Listeria monocytogenes, Escherichia coli O157:H7, Curing, Camel meat*

INTRODUCTION

One of the major concerns in the food industry is the preservation of meat and meat products **(Drosinos *et al.*, 2006)**. Highly perishable foods such as meat provide excellent conditions for the growth of hazardous microorganisms. *Listeria monocytogenes and Escherichia coli O157H7* are the most important food-borne pathogens of humans **(Adzitey and Huda, 2010; Park *et al.*, 1999)**. *L. monocytogenes* is a Gram-positive and ubiquitous bacterium responsible for foodborne disease (Listeriosis). The bacterium can contaminate several categories of foods. Multiplying at low temperatures makes *L. monocytogenes*a difficult pathogen to control in refrigerated foods that are consumed without further lethality treatments, such as reheating. **(Delia and Silvia, 2009)**. *E. coli* O157:H7 is a foodborne pathogen with a low infectious dose that has been associated with meat and meat products **(Sivapalasingam *et al.*, 2004).**The infection can lead to hemolytic uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, and renal injury **(Banatvala *et al.*, 2001).** The bacterium has the ability to survive at refrigeration temperatures **(Perez Rodriguez *et al.*, 2011).** Several methods such as cooking, fermenting, drying and curing have been used to preserve meat. Curing is one of the oldest methods for preserving and protecting meat from spoilage. Meat curing is the application of salt, color fixing ingredients, and seasoning to inform unique properties to the product. Salt and nitrite are two main ingredients used to cure meat **(Gheisari and Danesh, 2012).**

Camel is one of the most popular domestic animals in the dry and semi dry regions **(Knoess, 1977)**. Due to lower fat and cholesterol content and relatively higher polyunsaturated fatty acids, camel meat is healthier than beef **(Kadim*et al.*, 2008; Dawood and Alkanhal, 1995)**. Camel meat has a good capability for curing and additionally, mixed methods of curing resulted in more tender and higher quality than other methods **(Gheisari and Danesh, 2012)**. There are no reports on the microbiological safety of the cured camel meat. Therefore, we determined the survival of *E. coli O157:H7* and *L. monocytogenes* during the process of curing. Several methods have been employed to detect and enumerate the spoilage producing bacteria in foods. PCR is the most sensitive of rapid tests to detect microbial pathogens in samples **(Yamamoto, 2002)**. In addition, the combination of MPN and PCR techniques (MPN-PCR procedure) is a rapid detection test with advantages of less work load and less material consumption **(Chang *et al.*, 2013).**

MATERIAL AND METHODS

Bacterial cultures

A proven strain of *L. monocytogene*s (ATCC 19115) and nalidixic acid-resistant strain of *E. coli* O157:H7 (ATCC 43895) were provided by Department of Microbiology, School of Veterinary Medicine, Shiraz University. To confirm nalidixic acid-resistant *E. coli O157:H7*, the bacteria was cultred onto the McConkey agar (MERK, Germany) plate containing 400µg ml^{-1} nalidixic acid and incubated at 37°C for 24 h. Appearance of a colony was considered as bacterial resistance.

Growth curve of bacteria

L. monocytogenes and *E. coli O157:H7* were cultured in Tripticase Soy Broth (TSB) (Merck, Germany) and incubated at 37°C for 24 h. Bacterial suspension were then centrifuged at 3000g for 5 minutes. 10 ml of sterilized peptone water was added to the sediment. The process was repeated twice. Finally, 10 ml of TSB was added to the sediment, serial decimal dilutions (10^{-1}, 10^{-2}, 10^{-3}, $10^{-4}$and 10^{-5}) were prepared and the absorbance of each dilution was measured by spectrophotometer (JENWAY, U.K). 100 µL of each dilution was subsequently cultured to TSA medium and incubated at 37°C for 24 h. Number of colonies was then recorded. Bacterial growth curve was plotted based on the absorbance and number of colonies for each concentration**.**

Sampling procedure

Six male, adult Iranian dromedary (one-humped camel), were slaughtered at local slaughterhouse. In order to complete the rigor mortis, the carcasses were stored refrigerated for 24h . The *Longissimus dorsi* muscles were dissected and trimmed off external fat and connective tissue. After that, meats were chopped into pieces about 1 cm. In order to evaluate each of the bacterium, camel meat samples were divided into the following experimental groups:
Group 1: No treated samples (Negative Control).
Group 2: No curing meat containing the bacteria (*E.coli O157:H7*and/or *L. monocytogenes*) (Positive Control).
Group 3: Cured meat containing the bacteria (*E. coli O157:H7*and/or *L. monocytogenes*).
All treatments were performed in triplicate.

Meat curing

Two kilograms of meats were mixed with curing compounds [NaCl (22 g), sodium nitrite (0.18 g), soy protein isolate (15 g), starch (50 g), spices (15 g), phosphate (26 g), water and ice (400 g) and ascorbic acid (0.2 g) for 1 kg meat] before being stored at 4 °C for 9 days. Microbial count was recorded at the days 1, 3,5 and 9. All the experiments were performed in triplicate.

Inoculation of the bacteria

Each bacterium was inoculated in TSB medium and following a 24h incubation at 37°C, the number of colonies were determined according to the growth curve of bacteria. After that, 10^4cfu/g of each bacterium was added to the meat samples (Groups 2 and 3) and mixed well.

Bacterial counting using MPN-method

Different experimental groups (containing bacteria) were homogenized to prepare a 10-fold serial dilutions using peptone water up to 10-5. 96-well microplates were used for MPN test. For detection and enumeration of *E-coli* O157:H7 and *L. monocytogenes*, double-strength McConkey broth contained nalidixic acid and Buffered Listeria Enrichment Broth (BLEB) were respectively used. 100 µL of each dilution of the homogenate was transferred into culture wells containing 100µL of growth medium.
For each sample, 3 replicate MPN assays were set up for each dilution step, and the microplates were incubated in the 37°C for 24h. After incubation, the turbid wells (*L. monocytogenes*) and/or yellow discoloration (*E-coli O157:H7*) were considered as positive. The pattern of growth was then read from the MPN table to provide the most probable number and 95% confidence interval.To confirm the MPN results, each positive MPN well was used for the surface plating on the selective agar medium. Palcam agar (Merck, Germany) and CTS-Mac (AQUALAB, Germany) were respectively used for the isolation of *L.monocytogenes* and *E-coli O157:H7*. Cultured plates were incubated at 37°C for 24h. The positive MPN results were then subjected to PCR for the detection of *Stx1*and *Stx2* genes specific for *E. coli O157:H7* and *hlyA* gene for *L. monocytogenes*.

PCR

DNA extraction

On days 0, 2, 4 and 8, 200µL of turbid broth, were stored at -20°C for further use. DNA extraction was carried out using the boiled-cell method as was previously described **(Chai *et al*., 2007)**, with some modification. Briefly, the samples were thawed and centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded; the pellet was re-suspended with 50 µL of deionized water, vortexed and boiled in a water bath (100 ^{o}C) for 10 minutes to lyses the cells followed by cooled on ice for 2 minutes. Samples were then centrifuged at 10,000 rpm for 10 minutes. Aliquot of the supenatant which contained the template DNA was transferred to a sterile tube and stored at -20°C until further use.

MPN-PCR for Listeria monocytogenes

One pair of primers F and R with sequence of 5'-CGGAGGTTCCGCAAAAGATG-3' and 5'- CCTCCAGAGTGATCGATGTT-3' was designed to amplify *hlyA* gene at 485bp region. PCR reaction was carried out in 25 ml reaction mixture consisting 2.5 µL PCR buffer, 1.5 µL $MgCl_2$, 0.5µL dNTPs mix, 0.5µL TaqPolymerase (PROMEGA, USA), 0.5 µL of each primer, 1.5µL DNA templates and17.5µL sterile distilled water. Amplification of DNA segment was performed in thermal cycler (APPLIED BIOSYSTEMS, USA) using thethermo-cycling conditions of initial denaturation (94^{o}C for 2 minutes), 35 cycles of denaturation (94^{o}C for 45 seconds), annealing (64^{o}C for 45seconds), extension (72^{o}C for 1 minute) followed by final extension (72^{o}C for 5 minutes). PCR products were loaded in 1.5% agarose gel stained with ethidium bromide (1mg. ml^{-1}) and were electrophoresis at 50mA for one hour. Gel was visualized under UV light using and photograph (SYNGENE, USA).

MPN-PCR for *E. coli O157:H7*

Two pairs of primers used in MPN-PCR were *Stx1* and *Stx2* primer. Sequences of primers used are shown in table 1. PCR reaction was carried out in 21.5 ml reaction mixture consisting 2.5µL PCR buffer, 1.25µL $MgCl_2$, 1µL dNTPs mix, 0.5 µL TaqPolymerase (Promega, USA), 0.5 µL of each primer, 1.5 µL DNA templates and 13.75µL sterile distilled water. Amplification of DNA segment was performed in thermal cycler using the thermo-cycling conditions of initial denaturation (94 ^{o}C for 2 minutes), 35 cycles of denaturation (95 ^{o}C for 60 seconds), annealing (64 ^{o}C for 60seconds), and extension (72^{o}C for 90seconds) followed by final extension (72 ^{o}C for 7minutes). PCR products were analyzed on gels of 1.2% agarose.

Table 1 Sequence and size of primers based on the coding from the following genes used for *E. Coli* O157H7 .

Primer name	Gene	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
Stx$_1$F	*Stx*$_1$	ATAAATCGCCATTCGTTGACTAC	480
Stx$_1$R	*Stx*$_1$	AGAACGCCCACTGAGATCATC	
Stx$_2$F	*Stx*$_2$	GGCACTGTCTGAAACTGCTCC	510
Stx$_2$R	*Stx*$_2$	TCGCCAGTTATCTGACATTCTG	

Statistical analysis

The data were analyzed using the Repeated Measure ANOVA test of SAS 9/1 statistical software. Duncan's post hoc test was used to assess differences between groups. Differences were considered significant at values of P<0.05.

RESULTS AND DISCUSSION

Moisture, protein, fat and ash percents of camel meat samples were 73.82 ± 2.63, 23.57 ± 0.64, 4.16 ± 0.72, 1.22 ± 0.35, respectively. Microbial contamination can reduce the quality of fresh meat, shorten its shelf- life and cause economic losses and health hazards. Minimizing product contamination and delaying or inhibiting growth of spoilage and pathogenic organisms in the product are major keys for improving fresh meat shelf life and increasing consumer safety. While general cleanliness and proper sanitation are very effective, other means of controlling microbial growth in the meat products may be proven useful.The contamination of camel meat with *E. coli* O157:H7 and *L. monocytogenes* was detected by using a combination of culture method and MPN-PCR technique. *E. coli* O157:H7 was revealed as clear colony occasionally with yellow-orange halo on CTS-Mac agar and *L. monocytogenes* exhibited as gray-green colony with black halo on PALCAM agar. Results of the bacterial count by MPN method are shown in tables 2 and 3. Even though, an initial contamination to *L. monocytogenes* was obvious in our samples as shown in table 3, the curing was effective enough to significantly reduce the microorganisms in all the experimental groups**.**
Both microorganisms in all the experimental groups (except for *E.coli* O157:H7) increased during the storage time but the curing was dramatically reduced the number of them. All of the turbid samples in Buffered *Listeria* Enrichment Broth were shown a DNA fragment of the expected size **(**485bp) for *Listeria* spp. Positive samples in MPN test for *E. coli* O157:H7 produced a DNA band at 480 and 510 bp regions (figure 1).

Table 2 Details of the MPN values for *E. coli* O_{157}-H_7 (cfu/g) during 9 days of storage at 4 ºC.

Group	Days of storage			
	1	3	5	9
Group 1	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}
Group 2	833.5 ± 201.64 ^{cA}	5143 ± 1197.18 ^{cB}	42475 ± 9933.06 ^{cC}	110000 ± 12306.38 ^{cD}
Group 3	317.5 ± 62.99 ^{bA}	445 ± 17.32 ^{bB}	670 ± 115.38 ^{bC}	3300 ± 471.31 ^{bD}

Values are mean ± standard deviation. Group 1: control; Group 2: No curing meat containing the bacteria; Group 3: Cured meat containing the bacteria. The different small letters in the same column indicate significant differences between groups and different large letters in the same row indicates significant differences between the experimental days (P < 0.05).

Table 3 MPN results of *L. monocytogenes* (cfu/g) during 9 days of storage at 4 ºC.

Group	Days of storage			
	1	3	5	9
Group 1	240 ± 11.54 ^{bA}	2375 ± 50 ^{cB}	59350 ± 11981.63 ^{cC}	40500 ± 4489.80 ^{cC}
Group 2	555.75 ± 133.53 ^{cA}	1582.5± 248.01^{bB}	4100 ± 707.55 ^{bC}	19950 ± 4015.67 ^{bD}
Group 3	33 ± 4.71 ^{aA}	45.5± 13.48 ^{aA}	23± 9.76 ^{aA}	189 ± 37.95 ^{aB}

Values are mean ± standard deviation. Group 1: control; Group 2: No curing meat containing the bacteria; Group 3: Cured meat containing the bacteria. The different small letters in the same column indicate significant differences between groups and different large letters in the same row indicates significant differences between the experimental days (P < 0.05).

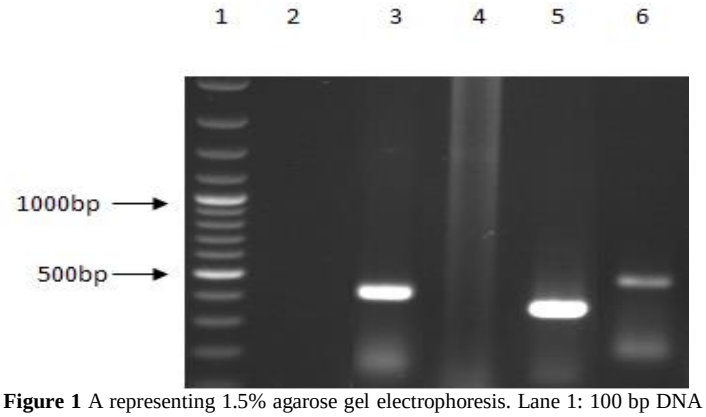

Figure 1 A representing 1.5% agarose gel electrophoresis. Lane 1: 100 bp DNA ladder; Lane 2: negative control (no template); Lane 3: 485 bp fragment corresponding to the *hly* gene of *L. monocytogenesis*; Lane 4: negative sample; Lane 5 & 6 480 bp and 510 bp fragments corresponding to the *Stx1* and *Stx2* genes of *E. coli* O157: H7

Al-Bachir and Zeinoun (2009) finde 10^6 CFU/g total (mesophilic aerobic) plate count and 10^3 CFU/g coliform count in the camel meat on day 0. This microbial load was high enough to indicate that the meat had been heavily contaminated during slaughtering and fabrication operations or alternatively, that the meat had been stored for an unknown length of time before being purchased for the study. Except for *E. coli* O157H7 in the negative control group, the bacterial populations were increased during 9 days of storage at 4 °C. The MPN method is the most advantageous to detect lower levels of microorganisms in food samples (10-100 MPN/g) **(Martin *et al*., 2004)**. Recently, the MPN-PCR method is widely used for detection and enumeration of food-borne pathogens in various foods **(Chai *et al*., 2007; Lee *et al*., 2009).** It was also noticeable that this method is more useful and effective for detecting microorganisms such as *L. monocytogenes* and *E-coli* O157:H7 than the plating method. Thus, application of the MPN-PCR method in the present study suggest for more accurate and reliable results. Recently, more attention is paid to the nutritional value of the camel meat **(Ulmer *et al*., 2004)**. The three most common methods use for camel meat preservation and processing are thermal processing, smoking and curing **(Kalalou*et al*., 2004; Zegeye, 1999).**

Salt has a variety of effects on both food tissues and microbial cells which are responsible for its preservation action such as interactive enzyme systems vital to the cells, stopping or slowing the growth and drawing water out the cells due to osmotic pressure. Sodium chloride (NaCl) has been traditionally used in curing processes. One of the functions of NaCl in meat products is to extract myofibrillar proteins. Extraction and solubilization of these muscle proteins contributes to meat particle binding, fat emulsification, and water-holding capacity, and thus, it reduces cook losses and improves quality and texture **(Sofos, 1986).** The role of nitrite in cured meat is four-fold: i) providing the characteristic pink-red cured-meat color to the lean tissue; ii) inhibiting the growth of a number of bacteria that cause food poisoning or spoilage; iii) contributing to the distinctive flavour of cured meats; and iv) retarding the oxidative rancidity in processed meat products, principally through a process of metal chelation **(Honikel, 2008).** Results of this study showed that curing may significantly reduce the growth of *L. monocytogenes* and *E. Coli* O157: H7 in camel meat. From the first day, number of the bacteria in the treated group was less than the controls. Low pH is an important factor contributing to the inhibition growth of foodborne pathogens. Several factors including pH associate with the survival of *E. coli* and *L.monocytogenes* during fermentation of sausage **(Cho *et al*., 2011). Gheisari and Danesh (2012)** report that curing of camel meat will reduce pH values of meat. We hypothesize that change in pH contributed to the reduction of *E. coli* and *Listeria* populations during the curing of camel meat. Additionally, in a work conducted by **Castano *et al.* (2002)** the counts of Enterobacteriaceae in the sausages manufactured industrially decrease continuously from the second day after the sausage mass is stuffed.This decrease is probably due to the addition of curing salt to the samples. **Kajak and Kołożyn-Krajewska (2006)** reported that the addition of 60 ppm sodium nitrite to meat was significantly inhibited the growing of microorganisms. While **Yu and Chou (1997)** reported that sodium nitrite at the concentrations between 0.07 and 0.15 g kg-1 have no significant effect on the reduction of *E coli* O157: H7 in sausage. Camel meat is a potential source of meat particularly in the arid tropics. As we found here, curing was considerably reduced the risk of microbial contamination.

CONCLUSION

Camel meat is a potential source of meat particularly in the arid tropics. If it was cured, it would be less susceptible to the microbial contamination. However, more research work in the areas of meat production, technology, marketing, and social awareness is needed to exploit the potential of camels as a source of meat and related products**.**

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