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Research Article

Bacteriological Status and Prevalence of Shiga Toxin Producing *E. coli* in Bovine Meat Products with a Reduction Trial Using Acetic and Citric Acids

Zohair S. Mulla[1*], Mostafa M. Abdelhafeez[2]

[1]Department of Public Health, College of Veterinary Medicine, King Faisal University, Saudi Arabia; [2]Food Control Department, Faculty of Veterinary Medicine, Misurata University, Libya.

Abstract | The current investigation was performed to examine the bacteriological status of three bovine meat products namely, ground beef, beef burger, and beef sausage retailed in Saudi Arabia. Bacteriological examinations involved the estimation of total bacterial count (TBC), total psychrophilic count (TPsC), most probable number (MPN) of coliforms, and MPN of *E. coli*. In addition, isolation, and identification of shiga toxin producing *E. coli* were further conducted. An experimental trial was done for improvement of the bacteriological status of the ground beef using diluted acetic acid, citric acid, and their mixture containing equal volumes of both acetic and citric acids at 0.5%, 1%, and 2%. The achieved results in the current study declared high microbial counts for the tested meat products, particularly for the ground beef. *E. coli* was isolated from the examined meat products at 40% for ground beef, 20% for beef burger, and 10% for beef sausage, respectively. The recovered *E. coli* were further identified into six serotypes, namely *E. coli O2:H6, O26:H11, O55:H7, O78:H-, O86:H11,* and *O119:H4*. Detection of shiga toxin producing genes (*stx1* and *stx2*) in the recovered serotypes was followed using multiplex PCR. A significant decrease in the microbial loads was recorded after dipping of the formulated ground beef as meat balls in the acid solutions, particularly at the acid cocktail 2%. To the best of our knowledge, this is the first study to report isolation and identification of shiga toxin producing *E. coli* from retailed meat products in Al-Ahsa, Saudi Arabia.

Keywords | Ground beef, Sausage, Burger, Microbial status, *E. coli*, Organic acids

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***Correspondence** | Zohair S Mulla, Department of Public Health, College of Veterinary Medicine, King Faisal University, Saudi Arabia; **Email**: zmulla@kfu.edu.sa
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INTRODUCTION

Bovine meat products including ground meat, beef burger, and beef sausage are among the primary sources of essential amino acids. In addition, they can provide humans with many minerals such as phosphorus, and iron; vitamins such as vitamin B group, and polyunsaturated fatty acids. Meat products' industry had increased worldwide, particularly in Saudi Arabia (Hessain et al., 2015).

The hygienic practices followed during preparation and manufacture of meat products control the bacterial contamination in such meat products. Such hygienic practices additionally control the shelf life of the meat products. Therefore, isolation of foodborne pathogens is a direct reflection of the poor microbial quality of food. Microbial contamination of meat products might occur during the manufacture process or due to the use of contaminated raw ingredients (Darwish et al., 2015). Therefore, there is a large need for continuous monitoring of the bacteriological status of the meat products intended for human consumption (Aberle et al., 2001; Darwish et al., 2018).

Escherichia coli (E. coli) is a normal inhabitant of the intestinal tracts of humans and animals. Therefore, its detection in animal products indicates fecal contamination. Besides, *E. coli* is one of the foodborne pathogens linked to the occurrence of several human illness outbreaks (Xia et al., 2010). Shiga toxin producing *E. coli* (STEC) is responsible for many cases of human hospitalizations worldwide (Darwish et al., 2015). Several *E. coli* pathotypes are classified as STEC. Of these, *E. coli O157* causes more than 50% of *E. coli*-related human food poisoning cases, while other pathotypes such as *O26, O45, O103, O111, O121,* and *O145* have been associated with *E. coli*- related foodborne infections (Scallan et al., 2011). There is a scarce information about the prevalence of shiga toxin producing *E. coli* in retailed meat products in Saudi Arabia.

Organic acids such as acetic, citric, lactic, and propionic, acids are regarded as safe interventions that used largely by the meat industry sector to reduce microbial contamination on carcass surfaces. Such organic acids were found to be effective for reducing several foodborne pathogens such as *Salmonella typhimurium*, and *Listeria monocytogenes* (Fabrizio et al., 2002). However, the effects of organic acids such as acetic and citric acids against shiga toxin producing *E. coli* received less attention.

This study was conducted to investigate the bacteriological status of ground beef, beef burger, and beef sausage retailed in Saudi markets. Evaluation of the microbial status of such meat products were done via estimation of total bacterial count (TBC), total psychrophilic count (TPsC), most probable number (MPN) of coliforms and MPN of *E. coli*. Furthermore, prevalence of shiga toxin producing *E. coli* in such meat products was further conducted. Detection of shiga toxin coding genes (*stx1*, and *stx2*) was additionally screened. A trial for improving the bacteriological status of the bovine ground meat formulated as meat balls was done using diluted acetic, citric acids, and their acid mixture containing equal volumes of both acids.

MATERIALS AND METHODS

Collection of Samples

Ninety random samples were collected from three bovine meat products marketed at retail shops in Al-Ahsa, Saudi Arabia. The examined meat products were ground beef, beef burger, and beef sausage (n = 30 each, each sample weighs 50-100 g). The collected samples were transferred cooled directly without undue delay to the laboratory for bacterial isolation and identification.

Organoleptical examinations

Sensory evaluation of the examined samples was done according to Varnam and Sutherland (1995). Samples with brick red color, fresh odor, and firm consistency were considered normal as used in the present investigation.

Bacteriological examinations

Samples were prepared for bacteriological examination according to the protocol recommended by APHA (2001). In brief, to prepare a dilution of 10^{-1} from the sample homogenate, twenty-five grams of each sample were homogenized with 225 ml of sterile buffered peptone water 0.1% for 1-2 minutes at 2000 rpm using sterile homogenizer (Precyzina, Poland). One ml from the prepared dilution (10^{-1}) was aseptically transferred to another sterile tube containing 9 ml of sterile 0.1% buffered peptone water and further tenfold decimal serials dilution were prepared.

Total bacterial Count (TBC)

Total bacterial count was estimated using plate count agar and according to the method of APHA (2001). After 48 h incubation of the culture plates at 35 ± 2 °C, all colonies including pinpoint size colony forming units were recorded.

TBC/g = average No. of colonies × reciprocal of dilution

Counted colonies expressed as log cfu/g.

Determination of total psychrophilic count (TPsC)

The pour plate technique was adopted using standard plate count agar medium and incubated at 7 °C for 10 days (APHA, 2001). Results were recorded in the same way as TBC. Counted colonies expressed as log cfu/g.

Determination of most probable number (MPN) of Coliforms:

The three tubes method was used for determination of MPN of coliforms (APHA, 2001). One mL of each prepared dilution was inoculated into three test tubes containing MacConkey broth with inverted Durham's tubes followed by incubation at 37 °C for 24-48 hrs. Positive tubes with acid and gas production were recorded. The most probable number of coliforms was calculated according to the recommended tables.

Determination of MPN of *E. coli*

Loopfuls from positive tubes showing acid and gas productions on MPN of coliforms experiments were inoculated into tubes containing 7 ml of *E. coli* (EC) broth (Himedia, Mumbai) and incubated at 44.5° C for 24-48 hrs (APHA, 2001). Positive tubes, showing acid and gas production, were used for calculation of MPN of *E. coli* according to the recommended tables.

Isolation of *Escherichia coli*

Eosin Methylene blue (EMB) agar was used for isolation of *E. coli* using the protocol of APHA (2001). From

Table 1: Primer sequences of shiga toxin producing genes

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614	Dhanashree and Mallya (2008)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779	Dhanashree and Mallya (2008)
stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		

each positive tube (acid and gas) of EC broth, a loopful was streaked onto EMB agar. The inoculated plates were incubated at 37 ˚C for 24 hrs. Typical colonies of *E. coli* appeared as metallic greenish with dark purple center. Suspected colonies were purified and subcultured onto nutrient agar slope and incubated for further investigations. Identification of isolates was done based on staining and biochemical tests (APHA, 2001).

Serodiagnosis of *E. coli*

The confirmed *E. coli* isolates were exposed to serological identification using specific *E. coli* antisera sets (Difco, Detroit, USA) (Kok et al., 1996).

DNA preparation

DNA extraction from each of glycerol stock *E. coli* isolates was done according to the method described before (Darwish et al., 2015).

Detection of shiga toxin producing genes in the identified isolates

A multiplex polymerase chain reaction (multiplex PCR) method was used for detection of shiga toxin producing genes in the identified *E. coli* isolates. Primer sequences and amplified products sizes were shown in Table 1. The amplification was performed on a Thermal Cycler (Eppendorf, Hamburg, Germany). PCR assays were carried out using the method of Dhanashree and Mallya (2008). Amplification conditions consisted of an initial 95 ˚C for 3 min as a denaturation step, followed by 35 cycles of 95 ˚C/20 sec, 58 ˚C/40 s, and 72 ˚C/ 90 sec. A final cycle was done at 72 ˚C for 5 min. The reference strains were *E. coli* O157:H7 Sakai and *E. coli* K12DH5α were used as positive and negative strains, respectively.

Amplified DNA products were analyzed and visualized on 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with Ethedium bromide. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used as a DNA marker.

Improvement of the bacteriological status of ground beef using diluted organic acids

A reduction trial for the bacterial load of the ground beef samples was conducted using diluted acids. Ground beef samples were formulated as meat balls and grouped into three groups. Group 1: Meat balls (n = 5 for each treatment) were immersed in acetic acid 0 (DDW), 0.5%, 1.0%, and 2.0%. Group 2: Meat balls (n = 5 for each treatment) were immersed in citric acid 0 (DDW), 0.5%, 1.0%, and 2.0%. Group 3: Meat balls (n = 5 for each treatment) were immersed in acid cocktail (equal parts of acetic acid and citric acid 1:1) 0 (DDW), 0.5%, 1.0%, and 2.0%. Such reduction trials were conducted to investigate the effect of different concentrations of the diluted acids on TBC, TPsC, MPN of coliforms, and MPN of *E. coli*. Five meat ball samples (50 g/each) were used in each exposure. Sensory evaluation and bacteriological examination were conducted as mentioned before.

Statistical analysis

All values are expressed as means SD. Statistical significance was evaluated using the Tukey–Kramer HSD test. In case of reduction experiments, measurements were compared with that of the control (DDW) using Dunnett's test. In all analyses, $P < 0.05$ was used to indicate statistical significance using JMP statistical package, SAS Institute Inc., Cary, NC.

RESULTS

Sensory evaluation of the examined samples revealed that all samples had normal organoleptic characteristics (Data are not shown). Microbiological examination of the meat product samples in the current study declared that the mean values of TBC were 5.25 ± 0.28, 3.59 ± 0.15 and 6.50 ± 0.21-log cfu/g in the examined ground beef, beef burger, and beef sausage, respectively (Fig. 1A). The average concentrations of TPsC in the examined ground beef, beef burger, and beef sausage were 3.69 ± 0.11, 2.60 ± 0.22 and 4.69 ± 0.18-log cfu/g, respectively (Fig. 1B). Most probable number of coliforms (MPN) was evaluated in the examined samples, the recorded results demonstrated that the mean values of MPN of coliforms were 4.21 ± 0.14, 3.47 ± 0.25 and 4.85 ± 0.21-log MPN/g in the examined ground beef, beef burger, and beef sausage, respectively (Fig. 2A). By the use of EC broth, MPN of *E. coli* was additionally estimated. The recorded results showed that MPN of *E. coli* in the examined samples were 3.25 ± 0.18, 2.11 ± 0.22 and 4.15 ± 0.19-log MPN/g in the examined ground beef, beef burger, and beef sausage, respectively (Fig. 2B). Figure 3A showed the prevalence rates (%) of *E. coli* in the exam

Table 2: Improvement effects of acetic acid, citric acid, and their acid cocktail on the microbial load of ground beef

	Acetic acid	Citric acid	Acid cocktail
Reduction (%) in total bacterial count			
0%	0	0	0
0.5%	10.62	12.50	17.81
1%	21.23	24.66	38.36
2%	40.07	45.21	58.22
Reduction (%) in total Psychrophilic count			
0%	0	0	0
0.5%	11.11	16.67	22.22
1%	27.78	33.33	38.89
2%	44.44	50	55.56
Reduction (%) in MPN of coliforms			
0%	0	0	0
0.5%	13.05	17.39	23.91
1%	30.43	34.78	45.65
2%	39.13	45.65	60.87
Reduction (%) in MPN of *E. coli*			
0%	0	0	0
0.5%	13.54	19.31	27.95
1%	25.07	27.95	42.36
2%	36.59	42.36	56.77

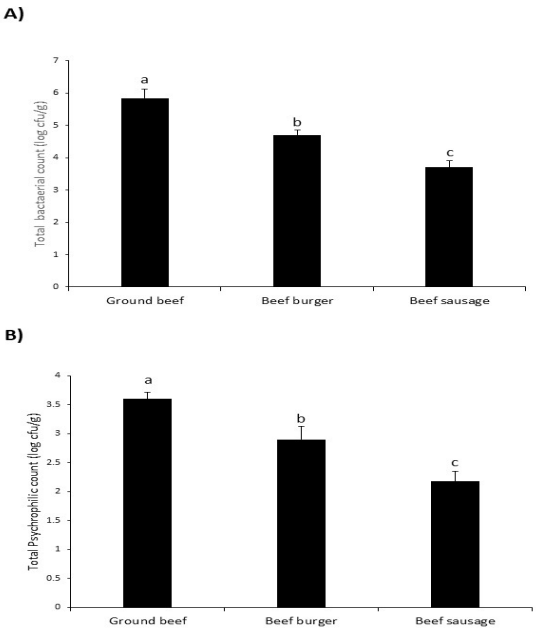

Figure 1: Bacterial counts of the examined meat product samples. Values represent means SD (Log cfu/g) of A) total bacterial count, B) total psychrophilic count in in the examined ground beef, beef burger, and beef sausage. Columns with different letter are significantly different at $p < 0.05$.

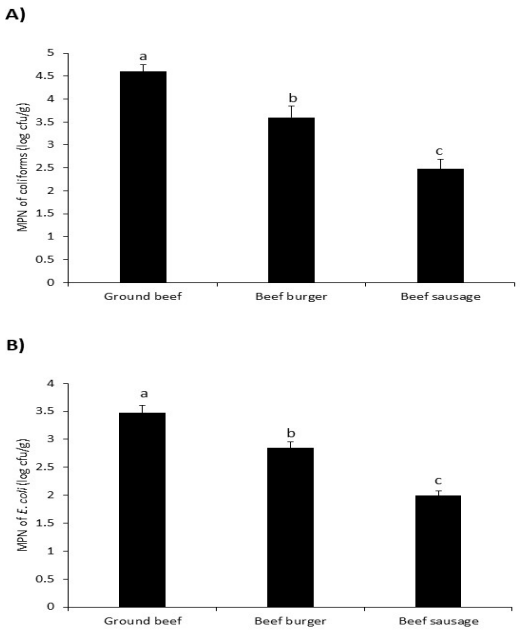

Figure 2: Coliforms and *E. coli* counts of the examined meat product samples. Values represent means SD (Log cfu/g) of A) Coliforms, B) *E. coli* counts in in the examined ground beef, beef burger, and beef sausage. Columns with different letter are significantly different at $p < 0.05$.

ined samples, *E. coli* was isolated at 30%, 20% and 40% in the examined ground beef, beef burger, and beef sausage, respectively (Fig. 3A). The recovered *E. coli* was further identified into six serotypes, namely *E. coli O2:H6*, *E. coli O26:H11*, *E. coli O55:H7*, *E. coli O78:H-*, *E. coli O86:H11*, and *E. coli O119:H4*. The prevalence rates of these serotypes

were 33.33%, 23.81%, 19.05%, 9.52%, 9.52%, and 4.76%, respectively (Fig. 3B). Using PCR, detection of shiga toxin coding genes among the recovered *E. coli* serotypes was screened. The obtained results demonstrated that *stx1* could be detected in three *E. coli* serotypes (*E. coli O2:H6, E. coli O55:H7,* and *E. coli O119:H4*). While *stx2* could be detected in four *E. coli* serotypes (*E. coli* serotypes (*E. coli O2:H6, E. coli O55:H7, E. coli O78:H-*, and *E. coli O119:H4*). *E. coli O86:H11* did not harbor any of the tested genes (Fig. 4). In an improvement trial for the microbial load in ground beef samples formulated as meat balls, diluted acetic acid, citric acid, and acid cocktail were used. The obtained results in Table 2 showed that TBC was significantly ($P < 0.05$) reduced on a concentration-dependent manner achieving the highest reduction at 2% concentration by 40.07% (acetic acid), 45.21% (citric acid), and 58.22% (acid cocktail), respectively. Similarly, TPsC was significantly reduced after immersion in the acid solutions. The highest reduction rates were recorded at 2% acid concentration reaching to 44.44% (acetic acid), 50% (citric acid), and 55.56% (acid cocktail), respectively. For MPN of coliforms these rates were 39.13% (acetic acid), 45.65% (citric acid), and 60.87% (acid cocktail), respectively. For MPN of *E. coli*, the reduction rates were 36.59% (acetic acid), 42.36% (citric acid), and 56.77% (acid cocktail), respectively (Table 2).

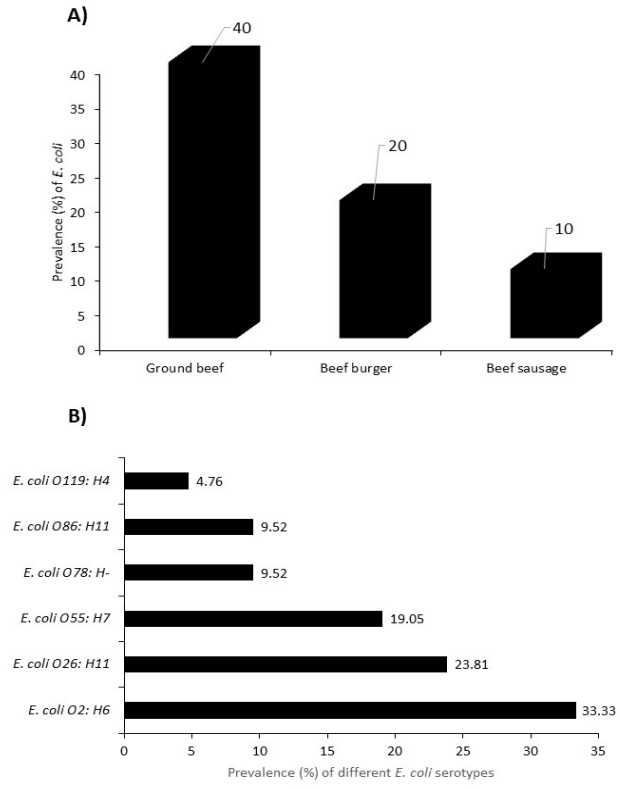

Figure 3: A) Prevalence rates of *E. coli* in the examined ground beef, beef burger, and beef sausage. **B)** Prevalence rates of different *E. coli* serotypes recovered from the examined meat products

Figure 4: Agarose gel electrophoresis of shiga toxins coding genes (*stx1 & stx2*) in the identified *E. coli* serotypes isolated from the examined meat products using PCR.

DISCUSSION

Bovine meat products such as ground beef, beef burger, and beef sausage contribute largely to the national economy in several Arab countries, particularly in Saudi Arabia. In addition, meat products are preferred by a large section of the population, specifically among children because of their specific aroma and flavor, and their easy and fast preparation (El-Ghareeb and Ismail, 2021). The hygienic status of the retailed meat products reflects the sanitary measures adopted during handling, and manufacturing of such products and affect both the microbiological quality and the shelf-life of the end products (Tang et al., 2020). In the current study, the hygienic status of the retailed bovine meat products in Saudi markets were evaluated via investigation of several microbial indicators for hygiene such as TBC, TPsC, MPN of coliforms, and MPN of *E. coli*. These indicators are recommended to give a correct idea about the hygienic practices followed during handling and processing of the end meat products (Mossel et al., 1995; Darwish et al., 2018).

The recorded results in the present study demonstrated high microbial loads in the examined ground beef, beef burger, and beef sausage samples. As declared by the high mesophilic, psychrophilic, MPN of coliforms and *E. coli* counts. In particular, ground beef had significantly ($P < 0.05$) the highest counts followed by beef burger and beef sausage, respectively. These results go in agreement with AL-Dughaym and Altabari (2010) who examined microbiologically 10 samples for each of chicken breast fillet and nuggets collected from Al-Ahsa markets, Saudi Arabia. They recorded high total mesophilic count (6.79 and 4.43-log cfu/g) in the chicken fillet and nuggets respectively. Furthermore, examination of meat samples from Karachi, Pakistan revealed high aerobic count. The average aerobic count log10 cfu/g of chicken, mutton and beef samples was 6.67, 6.38 and 7.05, respectively (Zafar et al., 2016). Similarly, El-Ghareeb and Ismail (2021) reported high

TBC, TPsC, MPN of coliforms in the retailed camel meat products such as mince, and sausage in Saudi Arabia. The high bacterial counts in the ground beef compared with the burger and sausage as reported in the current investigation seem reasonable as the process of meat mincing might lead to increasing the microbiological load of the produced mince. As the mincing machine is considered as a potential source of transferring food-borne organisms from contaminated meat to non-contaminated ones (Papadopoulou et al., 2012). Presence of indicator organisms in ground beef, beef burger, and sausage reflects the unsatisfactory hygienic measures adopted during the manufacture process of such meat products.

E. coli is a primary foodborne pathogen responsible for cases of foodborne infections worldwide. *E. coli* is responsible for several cases of hospitalization and deaths especially among children and elderly. For instances, enteroaggregative *E. coli O104:H4* outbreak occurred in Germany during Many 2011 leading to about 3000 human infection cases with 50 deaths (Frank et al., 2011). Furthermore, 12 persons were infected with shiga toxin-producing *E. coli O157:H7* in four of United States due to ingestion of contaminated ground beef during 2014 (CDC, 2014). In the current study, *E. coli* was isolated from the examined ground beef, beef burger, and beef sausage at 40%, 20%, and 10%, respectively. Serological identification of the recovered *E. coli* isolates revealed six serotypes namely *E. coli O2:H6, E. coli O26:H11, E. coli O55:H7, E. coli O78:H-, E. coli O86:H11,* and *E. coli O119:H4* at variable rates. Interestingly, all identified E. coli serotypes harbored at least one shiga toxin coding gene (either *stx1*, *stx2*, or both) except for *E. coli* O86:H11 which did not harbor any of the screened genes. In agreement with the results of the present study, Dambrosio et al. (2007) mentioned that *E. coli O2, O26, O103,* and *O111* are among the most *E. coli* serotypes of public health significance of non-O157 serogroups, particularly *E. coli O26* that able to cause wide range of human illnesses. Besides, Konishi et al. (2011) reported that the major enterotoxigenic *E. coli* serogroups isolated from outbreaks in Tokyo, Japan were *O6, O27, O148,* and *O159*. In Jeddah, Saudi Arabis, Iyer et al. (2013) isolated *E. coli* from cattle meat at a higher rate (65%). While in Riyadh, Saudi Arabia, Hessain et al. (2015) isolated shiga toxin producing *E. coli O157:H7* from ground beef, beef burger, beef sausage, ground chicken, and chicken burgers were 5%, 10%, 0.0%, 5% and 0.0%, respectively. Likely, Darwish et al. (2018) isolated shigatoxigenic *E. coli* from cattle meat retailed in Egypt. They could identify *E. coli O111:H4, O26:H11* which harbored *stx1* and *stx2*; *E. coli O86,* and *O114:H21* which harbored only *stx1*; *E. coli O55:H7* that harbored only *stx2.* Contamination of bovine meat products with *E. coli* could be attributed to mishandling of animal carcasses during dressing, and evisceration, cross contamination from the abattoir and processing plants environments including soil, contaminated water, cutting instruments, utensils, and equipment and improper personal hygiene (Chang et al., 2013).

One principal task of the food technologists and food industry sector is to confirm safety of the final meat products distributed to consumers and to try to improve the microbiological quality and extend the shelf life of such meat products. In this direction, a trial to reduce the microbial load in ground beef was carried out using diluted acetic, citric, and acid cocktails. All used acid concentrations did not affect the sensory characteristics of the formulated balls. Besides, a clear reduction on the bacterial load was observed as TBC, TPsC, MPN of coliforms, and MPN of *E. coli* were significantly reduced. The used acid cocktail 2% achieved the highest reduction in all bacterial counts tested in the present study. In correspondence with this result, Menconi et al. (2013) recorded significant antibacterial activities for acetic, citric, and propionic acids using raw chicken skin as a food subject. One explanation for the reduction in the microbial counts of the ground beef using diluted acid solutions is possibly via producing slightly acidic medium in the ground beef which interferes with the multiplication of the bacteria (Sallam et al., 2020). Similarly, Koutsoumanis et al. (2006) demonstrated that lowering pH of meat has significant antibacterial effect as declared on the reduced growth of *Pseudomonads,* and *Enterobacteriaceae*.

CONCLUSION

To the best of our knowledge, this is the first study to isolate and identify shiga toxin producing *E. coli* in meat products retailed in Al-Ahsa, Saudi Arabia. As *E. coli* was isolated at 30%, 20% and 40% in the examined ground beef, beef burger, and beef sausage, respectively. Six *E. coli* serotypes, namely *O2:H6, E. O26:H11, O55:H7, O78:H-, O86:H11,* and *O119:H4* were recovered. The prevalence rates of these serotypes were 33.33%, 23.81%, 19.05%, 9.52%, 9.52%, and 4.76%, respectively. In addition, this study is also among the first reports to investigate the inhibitory effects of acetic and citric acids against E. coli using ground beef as a food matrix. Therefore, the principles of hygiene are highly recommended the manufacture steps of meat products starting from slaughter of the animal, selection of meat with high quality, processing, transportation, and distribution in retail markets. In addition, the use of diluted acid solutions such as acetic and citric acids by the suitable concentrations is of significant value in reducing the microbial load of the final products.

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CONFLICT OF INTEREST

There are no conflicts of interest.

NOVELITY STATEMENT

This study is the first study that describe the hygienic status of meat products retailed in Al-Ahsa, Saudi Arabia, and to isolate *E. coli* from such products. In addition, the inhibitory effects of acetic and citric acids were investigated against *E. coli* using ground beef as a food matrix.

AUTHORS' CONTRIBUTIONS

Zohair S. Mulla, Mostafa M. Abdelhafeez equally designed the study plan and experimental work. Zohair S. Mulla collected the samples and performed the microbiological analysis. Mostafa M. Abdelhafeez performed PCR work and collected and performed data analysis. Both authors wrote the manuscript and approved the authors' order.

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