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MOLECULAR IDENTIFICATION AND ANTIBIOGRAM OF *Enterococcus* spp. ISOLATED ON ENTEROCOCCUS SELECTIVE DIFFERENTIAL (ESD) MEDIA FROM MEAT, MEAT PRODUCTS AND SEAFOOD IN LIBYA

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ABSTRACT

This study was conducted to investigate the presence of *Enterococcus* spp. in meat, meat products and seafood. A hundred and four samples were randomly collected from different geographic localities in Libya. The samples were subjected to microbiological analysis for enumeration and isolation of *Enterococcus spp.* by conventional cultural and molecular identification using PCR and partial sequencing of 16S rDNA techniques. Out of 104 samples, 73 (70.2%) isolates were found to be enterococci based on their cultural characteristics on ESD medium. However, out of 36 samples subjected to molecular identification, only six isolates were confirmed to be *Enterococcus* spp. using PCR and partial sequencing of 16S rDNA technique. All enterococci strains tested for their antibiotic sensitivity profiles showed high percentage of multi-resistance phenotype. These results can be used for further studies on enterococci as an emerging food borne pathogen and its role in human infection in Libya and would suggest that meat, meat products and seafood might play a role in the spreading of enterococci through the food chain with antimicrobial resistance characteristics.

Keywords: 16S rDNA, antibiogram, enterococci, food, Libya

INTRODUCTION

Enterococcus spp. is a genus of lactic acid bacteria of the phylum Firmicutes that possess Lancefield group D antigen as some of streptococci. Enterococci are Gram-positive cocci, often occur in pairs (diplococci) or short chains bacteria of the gastrointestinal tract of healthy human intestinal flora (**Aarestrup *et al.*, 2001**). Enterococci are able to survive in extremes of temperature (5 to 60 °C), pH (4.6 to 9.9) and high sodium chloride (6.5% w/v) (**Murray, 1990**). They are capable of growth in the presence of bile salts (40% w/v) (**Fisher and Phillips, 2009**) and they commonly occur in foods, especially those of animal origin such as meat and milk (**Giraffa, 2003**).

Previously, all streptococci of fecal origin that produce group D antigen were considered as enterococci **(Hartman *et al.*, 2001**). Molecular biology studies (including oligonucleotide cataloging of 16S rRNA, DNA-DNA and DNA-rRNA hybridization), combined with physiological studies showed more detailed classification (**Schleifer and Kilpper-Bälz, 1987**). Members of this genus are: *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. malodoratus* and *E. mundtii* (**Hartman *et al.*, 2001**).

Previous studies have shown that meat and meat products represent a continuous supply of commensal bacteria, including enterococci **(Choi and Woo, 2013; Sharifi *et al.*, 2013; Sparo *et al.*, 2013**). *E. faecalis* and *E. faecium* are common commensal organisms in the intestines of humans were shown to be the predominant isolates in raw meat (beef and pork carcasses) (**Knudtson and Hartman, 1993**). Meanwhile, *E. faecalis* was the most frequent isolate among the Gram-positive cocci found in chicken meat (**Turtura and Lorenzelli, 1994**). In processed meat, the presence of enterococci reflects the extent of initial fecal contamination (**Holley *et al.*, 1988**).

Enterococci are recognized as opportunistic human pathogens and lately have distinguished themselves as major nosocomial pathogens causing bacteremia, endocarditis, urinary tract, central nervous system, intra-abdominal and pelvic infections (**Franz *et al.*, 1999**). In addition, enterococci can be also used as an enteric contamination indicator (**Foulquie Moreno *et al.*, 2006**).

Enterococci are also known for their capability to exchange genetic information by conjugation (**Dunny, 2007**) and may spread antibiotic resistance genes among non-pathogenic organisms (**Cocconcelli *et al.*, 2003; Fisher and Phillips, 2009**). Thus, there is a concern about their presence in uncooked fermented meats because of the contribution they may have to the baseline level of antibiotic resistance in other genera and the potential for transfer of antibiotic resistant bacteria from the indigenous animal microflora to the human gastrointestinal tract (**Mathur and Singh, 2005**), also leading causes of highly antibiotic-resistant and hospital-acquired infection (**Aarestrup *et al.*, 2001**). Enterococci are recognized as opportunistic human pathogens, and as indicator for fecal contamination. Due to lack of good hygienic practice in the Libyan slaughterhouses and meat retail markets, therefore, the objectives of this study were to evaluate the presence of enterococci in meat, meat products of different animal species and seafood from different Libyan localities and for their antibiotic resistance profiles.

MATERIAL AND METHODS

Collection and preparation of samples

A total of 104 samples (Table 1) included: raw meat samples (51), meat products (30) and seafood (23), were randomly collected from different cities in Libya (Tripoli, Regdalin, Janzour and Tobruk). The samples were packed in sterile plastic bags, stored in an insulated icebox and transferred as quickly as possible to Food Hygiene and Control Laboratory Department, Faculty of Veterinary Medicine, University of Tripoli. All samples were subjected to *Enterococcus* spp. microbiological enumeration and isolation techniques. Decimal dilutions, culturing and enumeration techniques were performed according to the methods described by the American Public Health Association (APHA) (**Downes *et al.*, 2001**). Briefly, 25 g from each sample was aseptically transferred into a sterile stomacher bag (Seward Medicals, UK) and homogenized (Stomacher 400, Seward Medicals, UK) with 225 mL of sterile peptone water 0.1% (w/v) (Park Scientific, UK) at 230 rpm for 2 min.

Enumeration and isolation of *Enterococcus* spp.

Enumeration and isolation of enterococci were performed using enterococci selective differential agar medium (ESD) (**Efthymiou *et al.*, 1974**). ESD plates were seeded by surface spreading of 0.1 mL of appropriate tissue homogenate serial dilutions and then incubated at 37 °C for 24 h. ESD plates were examined for the presence of either magenta, round, 2-3 mm diameter colonies (*E. faecalis*), or white, round, 2-3 mm diameter colonies (*E. faecium*), or pink, round, 2-3 mm diameter colonies (*E. intermediate*). Isolates were identified to the species level by using API 20 Strep system (bioMérieux®, France).

Identification of enterococci by PCR and partial sequencing of 16S rDNA
DNA extraction and amplification of 16S rDNA

DNA extraction of enterococci isolates was performed by GF-1 bacterial DNA extraction kit (Cat. # GF-BA-100, Vivantis, Malaysia) as described in a previous study (**Azwai *et al.*, 2016**). The 16S rDNA was amplified using the universal oligonucleotides primers forward: S-D-Bact-0341-b-S-17 5′-CCTACGGGNGGCWGCAG-3′ and Reverse: S-D-Bact-0785-a-A-21 5′-GACTACHVGGGTATCTAATCC-3′ (**Herlemann *et al.*, 2011**).

Electrophoresis, gel extraction and DNA sequencing

The amplified 16S rDNA PCR fragment (464 bp) was excised from the gel and the DNA was purified using GF-1 Ambi Clean kit (Cat. # GF-GC-100, Vivantis, Malaysia) as described in previously (**Azwai *et al.*, 2016**). The purified 16S rDNA amplicons underwent cycle sequencing with Big Dye® Terminator v1.1 kit (AB Applied Bioscience, TECHNE, TC-512, USA) and were sequenced on four capillary ABI PRISM® 3130-Avant Genetic Analyzer at IZSLER Istituto Zooprofilattico Sperimentale Della Lombardia e dell'Emilia Romagna, Brescia, Italy. Sequences were assembled and edited using the SeqMan module within Lasergene package, (DNA Star Inc., Madison, WI, USA). The obtained consensus sequences were subjected to BLAST search both at NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and at 16S bacterial cultures Blast Server for the identification of prokaryotes (http://bioinfo.unice.fr/blast/).

Antibiogram of isolated strains

Inoculum Preparation

Upon confirmation by PCR and partial sequencing of 16S rDNA gene isolated strains of enterococci were preserved by freezing at -80 °C in vials containing Brain Heart Infusion broth (BHI, Difco, Michigan, USA) supplemented with 30% (v/v) glycerol. To propagate the culture, frozen vial was thawed at room temperature, and 0.5 mL of thawed culture was transferred to 5 mL of BHI broth and incubated for 24 h at 37 °C. The inoculum was prepared from the second transfer of that culture (0.5 mL) to another 5 mL of BHI broth and incubated for 16 – 18 h at 37 °C. After the overnight incubation Muller Hinton agar plates (Oxoid, Hampshire, UK) were surface swabbed, then the selected antibiotic discs were dispensed and lightly pressed onto the inoculated agar surface according to (**Coyle, 2005**) then incubated at 37 °C for 24 h.

Antibiotic assay

The selection of antibiotics was based on their common use in food animal practice and included: (oxytetracyclin (30 µg), streptomycin (10 µg) and vancomycin (30 µg)). The antibiotic discs were purchased from Oxoid with the exception of the enrofloxacin (5 µg), amoxicillin (25 µg) obtained from Arcomex Arab (Medical Diagnostics CO., Amman, Jordan), while colistin (10 µg), doxycycline (30 µg), gentamycin (10 µg), erythromycin (10 µg), were obtained from Mast Diagnostics (Mast group ltd., Merseside, UK). The clear zones around antibiotic discs that has no growth, referred to as the zone of inhibition, were measured and scored as sensitive, intermediate (reduced susceptibility) or resistant according to the Clinical and Laboratory Standards Institute guidelines (**CLSI, 2015**).

RESULTS AND DISCUSSION

Isolation and enumeration of *Enterococcus* spp.

One hundred and four samples from various regions of Libya comprising raw meat (51), meat products of different species (30) and seafood (23) were tested for the presence of *Enterococcus* spp. by using ESD medium (Table 1). *Enterococcus* spp. were isolated from the samples of raw meat: beef 12/17 (70.5%), camel 13/22 (59%) and chicken meat 11/12 (91.6%) respectively, and from the samples of seafood: fish 5/13 (38.4%) and shrimp 3/6 (50%) respectively, with counts ranged from 8.7x10 to $4.2x10^4$ CFU/g and the most common isolate was *E. faecalis*. No isolate was detected from clam samples. As for meat products, isolation rate of *Enterococcus* spp. on ESD agar plates from 30 samples of meat products of different animal species was 100%, except for beef burger, that was 87.5% (7/8) with counts ranged from $7x10^3$ to $6.8x10^6$ CFU/g. The maximum mean count of enterococci was recorded in chicken burger $3.8x10^6$ CFU/g; while the minimum mean count was in shrimp $1.1x10^3$ CFU/g (Table 1). The occurrence of *Enterococcus* spp. was 87.5% in beef burger with counts ranging from $1.7x10^5$ to $1.4x10^6$ CFU/g and the mean counts was $7.6x10^5$CFU/g, meanwhile, in beef kebab the isolation rate was 100% with counts ranging from $2x10^4$ to $1.8x10^5$ CFU/g and the mean count was $9x10^4$ CFU/g (Table 1). Detection of enterococci in chicken burger was 100% with counts ranging from $7.7x10^5$ to $6.8x10^6$ CFU/g with a mean counts $3.8x10^6$ CFU/g. While, in ground chicken the rate was 100% with counts ranging from $9x10^3$ to $8x10^4$ CFU/g and the mean counts was $4.5x10^4$ CFU/g.

Table 1 Comparison between growth on ESD medium and partial sequencing of 16S rDNA technique for identification of *Enterococcus* spp.

Type of Sample	No. of Samples	No. of Suspected *Enterococcus* spp. Growth on ESD (%)	Average Count (*CFU/g*) of *Enterococcus* spp. on ESD	No. of Sequenced Isolates	No. of Positive *Enterococcus* spp. by 16S rDNA Sequencing
Raw meat					
Beef	17	12 (70.5)	2.2×10^4	4	None
Camel meat	22	13 (59)	1.6×10^4	4	None
Chicken meat	12	11 (91.6)	4×10^3	4	None
Clam	4	0	-	-	-
Fish	13	5 (38.4)	4.4×10^3	2	None
Shrimp	6	3 (50)	1.1×10^3	2	None
Meat products					
Chicken burger	8	8 (100)	3.8×10^6	4	3
Chicken kebab	2	2 (100)	9×10^4	2	None
Chicken sausage	2	2 (100)	9×10^4	2	None
Beef burger	8	7 (87.5)	7.6×10^5	4	1
Beef kebab	2	2 (100)	9×10^4	2	1
Beef sausage	2	2 (100)	8×10^4	2	None
Ground beef	2	2 (100)	9×10^3	2	None
Ground chicken	4	4 (100)	4.5×10^4	2	1
Total	**104**	**73 (70.2)**		**36**	**6**

Identification of enterococci spp. by PCR and sequencing of partial 16S rDNA gene

A total of 36 (16 raw meat samples and 20 meat products samples) randomly selected isolates (36 out of 73 isolates were found to be enterococci based on their cultural characteristics on ESD medium) were sent for partial sequencing of 16S rDNA (464 bp) of enterococci strains using the universal oligonucleotides primers (FOR.: S-D-Bact-0341-b-S-17 and REV.: S-D-Bact-0785-a-A-21) (Fig. 1). Only six isolates (16.6%) (Table 2) were identified as *Enterococcus* spp. These isolates of enterococci were all isolated from meat products (beef burger, beef kebab, ground chicken and chicken burger) (Table 3).

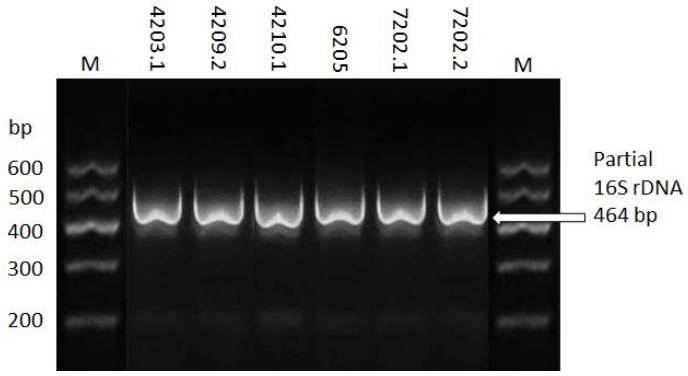

Figure 1 Representative gel of partial amplification of 16S rDNA (464 bp) products of isolated Enterococci strains using the universal oligonucleotides primers. First and last lanes contain DNA marker (M).

Table 2 Convential and molecular identification of suspected *Enterococcus* spp. in different meat products samples (CFU/g)

Type of Sample	Suspected Growth of *Enterococcus spp.* on ESD	No. of Suspected Isolates Growth on ESD	No. of Sequenced Isolates	No. of Positive *Enterococcus* spp. by 16S rDNA Sequencing
Chicken burger	*E. intermediate*	4	2	0
	E. faecalis	4	2	3
Chicken kebab	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Chicken sausage	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Beef burger	*E. intermediate*	3	2	0
	E. faecalis	4	2	1
Beef kebab	*E. intermediate*	1	1	1
	E. faecalis	1	1	0
Beef sausage	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Ground beef	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Ground chicken	*E. intermediate*	2	1	0
	E. faecalis	2	1	1
Total		**29**	**20**	**6**

Table 3 Identity of suspected isolate after sequencing by blast NCBI

Blast NCBI	Identity (%)	Isolate Code	Suspected Isolate on ESD	Type of Sample	Storage Condition	Source
Enterococcus durans	100	4203.1	Enterococci	Beef burger	Frozen	Suqaljuma, Tripoli
Enterococcus faecium	100	4209.2	Enterococci	Chicken burger	Frozen	Salaheldin Tripoli
Enterococcus durans	100	4210.1	Enterococci	Beef kebab	Chilled	Salaheldin Tripoli
Enterococcus faecalis	100	6205	Enterococci	Ground chicken	Chilled	Suqaljuma, Tripoli
Enterococcus faecium	100	7202.1	Enterococci	Chicken burger	Frozen	Abusetta, Tripoli
Enterococcus durans	100	7202.2	Enterococci	Chicken burger	Frozen	Abusetta, Tripoli

Antibiotics Resistant Phenotype

The results (Table 4) showed testing of the six confirmed enterococci isolates from meat products against nine antimicrobial agents (amoxicillin, colistin, doxycycline, enrofloxacin, erythromycin, gentamycin, oxytetracyclin, streptomycin and vancomycin). Antibiotic resistance profile showed that, *E. durans* found in beef burger and *E. faecium* found in chicken burger were resistant to five out of nine antibiotics (55.5%). Meanwhile, *E. durans* from beef kebab and *E. faecalis* from chicken burger both were resistant to seven out of nine antibiotics (77.7%). On the other hand *E. faecalis* from ground chicken showed resistance to eight out of nine (88.8%), lastly *E. durans* from chicken burger was resistant to six out of nine (66.6%). In conclusion, enterococci isolates exhibited resistance to at least five out of nine (55.5%) of the tested antibiotics. All six enterococci isolates (100%) were resistant to colistin. While five out of six tested isolates (83.3%) were resistant to amoxicillin, enrofloxacin, erythromycin and streptomycin. Resistance to oxytetracyclin and doxycycline was recorded among 66.6% of the isolates. However, only two isolates (33.3%) were resistant to gentamycin and vancomycin (Table 4).

Table 4 Sensitivity of six strains of enterococci to nine antibiotics

Enterococci Strains	Isolate Codes	Antibiotic Discs (mm)									R%	S%
		E (10 µg)	CO (10 µg)	AMO (25 µg)	S (10 µg)	ENR (5 µg)	DOX (30 µg)	Gen (10 µg)	Van (30 µg)	OT (30 µg)		
Enterococcus durans	4203.1	20 (S)	(R)	(R)	(R)	9 (R)	25 (S)	12 (R)	20 (S)	21 (S)	**55.5**	**44.5**
Enterococcus faecium	4209.2	12 (R)	(R)	(R)	(R)	12 (R)	23 (S)	16 (S)	19 (S)	21 (S)	**55.5**	**44.5**
Enterococcus durans	4210.1	11 (R)	(R)	(R)	(R)	13 (R)	10 (R)	15 (S)	17 (S)	(R)	**77.7**	**22.3**
Enterococcus faecalis	6205	(R)	(R)	16 (S)	(R)	16 (R)	(R)	(R)	(R)	(R)	**88.8**	**11.2**
Enterococcus faecium	7202.1	(R)	(R)	(R)	(R)	13 (R)	10 (R)	15 (S)	17 (S)	(R)	**77.7**	**22.3**
Enterococcus durans	7202.2	(R)	(R)	(R)	20 (S)	20 (S)	(R)	23 (S)	(R)	(R)	**66.6**	**33.4**
R%		**83**	**100**	**83**	**83**	**83**	**66.6**	**33.3**	**33.3**	**66.6**		
S%		**17**	**0**	**17**	**17**	**17**	**33.4**	**66.6**	**66.6**	**33.4**		

(S): Sensitive, (R): Resistant, E: erythromycin, CO: colistin, AMO: amoxicillin, S: streptomycin, ENR: enrofloxacin, DOX: doxycycline, Gen: gentamycin, Van: vancomycin, and OT: oxytetracyclin.

DISCUSSION

Enterococcus spp. are widely distributed in nature and are associated with the spoilage of meat and meat products (**Hugas *et al.*, 2003**). Current study was conducted to isolate *Enterococcus* spp. from 104 samples of different meat, meat products of different animal species and seafood, collected from various geographical places in Libya. This study reported the presence of *Enterococcus* spp. in most meat and all local un-heat treated meat products samples except one sample of beef burger by conventional cultural method. Generally, the incidence of *Enterococcus* spp. all over the collected samples of raw meat was 70.5% (36/51) and seafood was 34% (8/23). However, the incidence rate of enterococci in meat products was 96.6% (29/30); this high incidence in meat products could be attributed to low hygienic practice and cross contamination during preparation of such products. The results showed that the contamination with *Enterococcus* spp. in meat of different animal species and meat products was higher than that in seafood. The higher values could be as a result of contamination from the processing area, equipment used, also the means of transportation which was used in bringing the produce to the market centers and the hygienic practice employed by meat sellers and butchers. The meat during its preparation remains in the ground for a long time which creates a good environment for microbial pathogens to proliferate on it. On the other hand, seafood were sold at the seafood market freshly with better hygienic conditions that reduce the possibility from being contaminated (**Franz *et al.*, 2003**).

The occurrence of *Enterococcus* spp. in meat of different animal species and seafood (73) was in beef, camel, chicken, fish and shrimp 70.5%, 59%, 91.6%, 38.4 and 50% respectively, with counts ranging from $1.5x10^4$ to $6.8x10^6$ CFU/g (Table 1). The average counts of *Enterococcus* spp. in camel meat was $1.6x10^4$ $\pm 1.2x10^4$ CFU/g. **Hugas *et al.* (2003)** reported that the numbers of viable count of enterococci in contaminated beef, poultry and pork are usually in the range of 10^2 – 10^4 CFU/g. Meanwhile, our study did not detect enterococci among four examined samples of clam (bivalve shellfish). In contrary to **Montiel *et al.* (2013)** who found enterococci in all samples of clam examined with their densities generally higher in clams than sediment and water. Our result could be due to *Enterococcus* spp. were removed from hard shell clams by depuration occurred at the fish market where the samples were collected (**Love *et al.*, 2010**).

On the other hand, 30 samples of meat products revealed an incidence of 100% *Enterococcus* spp., except in beef burger was 87.5% (Table 1). The mean counts of enterococci were $2.2x10^4$ CFU/g in beef, $3.8x10^6$ CFU/g in chicken burger, $9x10^4$ CFU/g in chicken kebab and beef Kebab, chicken sausage and ground beef, $8x10^4$ CFU/g in beef sausage, and $4.5x10^4$ CFU/g in ground chicken. Our study revealed that, the highest enterococci count was in chicken burger $3.8x10^6$ CFU/g, however, the lowest count $1.1x10^3$ CFU/g was recorded in shrimp. The most common enterococci recorded in our investigation in meat products were *E. durans*, *E. faecalis* and *E. faecium*, while, (**Jahan *et al.*, 2013; Sadeghifard *et al.*, 2015**) reported *E. faecalis* as a predominant isolate in all meat samples. In agreement with our findings, **Naas *et al.* (2009a,b)** recorded high enumeration of enterococci in all tested samples that included beef burger and beef sausage at rate of $2x10^7$ and $9x10^6$ CFU/g respectively. As for molecular confirmation only six out of 36 randomly selected enterococci isolates were identified and confirmed by partial sequencing of 16S rDNA, (8.2%) were confirmed as *Enterococcus* spp. in particular *E. durans*, *E. faecalis* and *E. faecium* (Table 3).

Enterococci raise major concern during the last decades, as they are becoming one of the most important nosocomial infections causing serious illnesses in human. The presence of *Enterococcus* spp. in foods may act as reservoir of antibiotic resistance genes (**Valenzuela *et al.*, 2009**). The susceptibility of enterococci isolates to different antibiotics was tested (Table 4) and the highest incidence of resistance was recorded to colistin (100%), colistin is a last-resort antibiotic in both animals and humans, this antibiotic is used against particularly dangerous types of multi resistant bacteria that can withstand many other antibiotics. The existence of such isolates in the food chain of humans is of a great concern not only to public health but also because of the ease of resistance gene transfer to other bacteria. Lower resistance rates (83%) were recorded against erythromycin, amoxicillin, streptomycin and enrofloxacin while it was (66.6%) to oxytetracyclin and doxycyclin. Only 33.3% of the isolates were resistant to vancomycin and gentamicin, similar results were recorded by **Jahan *et al.* (2013)**. Vancomycin resistant enterococci (VRE) are nosocomial pathogens that have been detected in environmental habitats including soil, water and wildlife faces. The spread of opportunistic pathogens harboring VR genes beyond hospitals into community is a potential threat to public health as vancomycin is used as last-resort against many infections. Most of the isolated enterococci strains were resistant to more than five antibiotics out of nine (55.5%). In the contrary to **Fracalanzza *et al.* (2007)** who found overall percentages of antimicrobial resistant of isolates were: 31.2% to tetracycline, 23.8% to erythromycin, 11.3% to streptomycin, 4.3% to chloramphenicol, 3.9% to gentamicin, 1.4% to enrofloxacin and 0.4% to ampicillin. In another work, **Klibi *et al.* (2013)** studied enterococci strains isolated from meat samples that showed 14% resistance to streptomycin and 100% to streptomycin and tetracycline.

CONCLUSION

In conclusion, our findings demonstrated the presence of *Enterococcus* spp. in meat, meat products of different animal species and seafood. Vancomycin resistant enterococci were also isolated from local meat products sold in different cities in Libya. Moreover, conventional cultural methods on ESD medium were less significant than using the molecular techniques as partial sequencing of 16S rDNA techniques for identification of enterococci. Only six enterococci isolates cultured on ESD medium were confirmed to be *Enterococcus* spp. by PCR and partial sequencing of 16S rDNA. The occurrence of resistant strains of enterococci in food of animal origin should be considered as important threat to public health.

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