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Search for diarrheagenic *Escherichia coli* in raw kibbe samples reveals the presence of Shiga toxin-producing strains

Jacqueline Tanury Macruz Peresi [a, *], Ivete Aparecida Zago Castanheira de Almeida [a], Tânia Mara Ibelli Vaz [b], Rodrigo Tavanelli Hernandes [c], Inara Siqueira de Carvalho Teixeira [a], Sonia Izaura de Lima e Silva [a], Rejane Alexandre Silva Graciano [a], Sandra Regina Pinheiro [b], Luis Fernando dos Santos [b]

[a] Instituto Adolfo Lutz, Centro de Laboratório Regional de São José do Rio Preto, Rua Alberto Sufredine Bertone, 2325, Maceno, São José do Rio Preto, SP, CEP: 15060-020, Brazil
[b] Instituto Adolfo Lutz, Laboratório Central – Centro de Bacteriologia, Av. Dr. Arnaldo, 355, Cerqueira Cesar, São Paulo, SP, CEP: 01246-000, Brazil
[c] Instituto de Biociências, Universidade Estadual Paulista (UNESP), Distrito de Rubião Junior, s/n°, Botucatu, SP, CEP: 18618-970, Brazil

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ABSTRACT

The aim of this study was to investigate the occurrence of diarrheagenic *Escherichia coli* (DEC) strains in raw kibbe samples. For this purpose, 70 samples of raw kibbe were collected from retail establishments and analyzed. Isolated bacterial strains that presented a biochemical profile of *E. coli* were screened by multiplex PCR for the genetic markers defining the main DEC pathotypes. Two strains belonging to O125:H19 and O149:H8 serotypes were positive for *stx* 1c genetic sequence and expressed the gene, being thus classified as Shiga-toxigenic *E. coli* (STEC). These strains were further characterized in respect to other virulence traits and susceptibility to antimicrobial agents. One of them presented the genes *ast*A and lpf_{O113} and the other harbored only lpf_{O113}. One of the strains was susceptible to all the antimicrobials tested but the other presented an intermediate profile of susceptibility to ampicillin. To the best of our knowledge this is probably the first description of O125:H19 and O149:H8 STEC serotypes in refrigerated raw kibbe and foods in general.

1. Introduction

Escherichia coli are a heterogeneous group of typically harmless bacteria. However, throughout the evolutionary process some clones of *E. coli* have become pathogenic to humans due to the acquisition of specific virulence genes (Nataro & Kaper, 1998). Pathogenic *E. coli* are capable of causing intestinal and extra-intestinal infections (Kaper, Nataro, & Mobley, 2004). Strains associated with intestinal infections are designated Diarrheagenic *E. coli* (DEC). Based on their virulence strategies and some epidemiological features, DEC strains are currently divided into six categories or pathotypes: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Shiga toxin-producing *E. coli* (STEC) (Croxen et al., 2013).

Of the currently known foodborne pathogens, STEC strains are regarded as one of the most important (Blanco et al., 2004; CDC, 2012) mainly due to their ability to cause an array of diseases ranging from uncomplicated diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) – an extraintestinal pathology characterized by hemolytic anemia, thrombocytopenia, thrombotic thrombocytopenic purpura (TTP) and acute renal failure (Nataro & Kaper, 1998; Paton & Paton, 1998; Riley et al., 1983).

STEC may produce one or more types of so-called Shiga toxins (Stx), which are antigenically divided into Types 1 and 2 (Stx1 and Stx2) and each type can present a variable number of subtypes. These toxins have similar structures and immune reactivity with the toxin produced by *Shigella dysenteriae* serotype 1 (Melton-Celsa & O'Brien, 1998). In addition to Stx, other virulence factors are known and characterized in STEC, including the EHEC enterohemolysin (Ehx) (Beutin et al., 1989) and a specialized adhesin called intimin (Frankel, Phillips, Rosenshine, Kaper, & Knutton, 1998). Other toxins such as the cytolethal distending toxin V

* Corresponding author.
E-mail addresses: jacquetmp@hotmail.com, jtmperesi@ial.sp.gov.br (J.T.M. Peresi).

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(Cdt-V) and the STEC subtilase toxin (SubAB) (Janka et al., 2003; Paton, Srimanote, Talbor, Wang, & Paton, 2004) and adhesins, such as, the long polar fimbriae (Lpf_{O113}), the Enterohemorrhagic *E. coli* (EHEC) factor for adherence (Efa), the STEC autoagglutinating adhesin (Saa), the Iron regulated homologue adhesin (Iha) and the protein ToxB (Doughty et al., 2002; Nicholls, Travis, & Robins-Browne, 2000; Paton, Srimanote, Woodrow, & Paton, 2001; Tarr et al., 2000; Tatsuno et al., 2001) may also be present in STEC strains.

It is estimated that STEC causes 265,000 cases of disease in the United States annually, with more than 3600 hospitalizations and 30 deaths (Scallan et al., 2011). In Brazil, most infections due to this pathogen are of a sporadic nature and generally occur, with or without bloody diarrhea, in children and HIV-positive patients. However, cases of HUS have also been reported in Brazil (Dos Santos, unpublished; Guth, Lopes, Vaz, & Irino, 2002; Souza, Carvalhaes, Nishimura, Andrade, & Guth, 2011).

Ruminant animals, especially cattle and sheep are considered to be a natural reservoir for STEC. Thus, through the consumption of foods of animal origin, in particular meat and milk, STEC enters the food chain and infects humans (Blanco et al., 2004). Recently, an increasing number of different kinds of foods have been associated with STEC transmission, including cheese, salami, unpasteurized juices, melon, different kinds of vegetables and even water (CDC, 1995; Gyles, 2007; Karmali, Gannon, & Sargeant, 2010; Sandrini, Pereira, Brod, Carvalho, & Aleixo, 2007).

Among meat products, ground beef is the main ingredient in the preparation of kibbe, a dish from Lebanese cuisine appreciated worldwide. This kind of food can be consumed either cooked or raw. When raw, it has several intrinsic characteristics, such as water content, high levels of nutrients and a pH close to neutral, which favor the multiplication of different microorganisms, including DEC. Moreover, during its preparation, the handling process exposes the product to other sources of contamination thereby further increasing the risk of the transmission of enteropathogens (Nascimento, Valle, Boari, Alcântara, & Vieira, 2002).

Few studies have investigated the presence of DEC in raw kibbe samples (Cerqueira, Tibana, & Guth, 1997). So, the objective of this study was to investigate the possible occurrence of DEC strains in raw kibbe samples marketed in the northwestern region of São Paulo State, Brazil.

2. Material and methods

2.1. Raw kibbe samples: isolation and identification of E. coli

In the period between January 2010 and July 2011, one sample of 500 g of refrigerated raw kibbe was collected from different commercial establishments selected among the leading producers of raw kibbe in the city of São José do Rio Preto, which is located in the northwestern region of the São Paulo State, Brazil. Of the total samples (70), 32 were collected from supermarkets and 38 from butchers. These establishments were distributed in five distinct geographic regions of the city: central (14 establishments/samples), north (15 establishments/samples), south (14 establishments/samples), east (14 establishments/samples) and west (13 establishments/samples).

Right after the sampling procedure samples were transferred to a refrigerated box and maintained in this condition until the moment they were processed. The investigation of *E. coli* in general was performed by the Most Probable Number (MPN) technique as described in the Compendium of Methods for the Microbiological Examination of Foods (Kornacki & Johnson, 2001). After the isolation of thermotolerant coliforms, *E. coli* identification was achieved by standard presumptive biochemical tests (Kornacki & Johnson, 2001; Pessoa & Silva, 1974). All biochemically identified *E. coli* isolates were subcultured on Mac Conkey-Sorbitol Agar (SMAC) plates and sorbitol positive and/or negative colonies were further analyzed for virulence.

2.2. Virulence characterization of the isolated E. coli strains

2.2.1. Genotypic assays

DNA extraction: One to ten colonies of each raw kibbe sample positive for *E. coli* from SMAC plates (403 in total) were grown overnight in Luria-Bertani (LB) broth at 37 °C. After the incubation period, an aliquot of 100 uL of each bacterial inoculum were diluted in 900 μL of ultrapure sterile water and subjected to lysis at 100 °C in a dry bath. The resulting bacterial suspension was centrifuged at 10,000 × g for 5 min and the supernatant was collected and used directly in genotypic assays.

Investigation of markers specific for the known DEC categories: All the isolates identified as *E. coli* were initially subjected to multiplex polymerase chain reaction (PCR) in order to screen for genetic markers associated with DEC. Reactions were performed in a Gene Amp PCR System (Applied Biosystems) employing the following amplification conditions: 1 cycle at 50 °C (2 min), 1 cycle at 95 °C (5 min), 40 cycles at 95 °C (40 s), 58 °C (1 min) and 72 °C (2 min) and a final extension step at 72 °C for 7 min. The set of primers used in these reactions as well as amplicon sizes are described in Table 1.

Other putative virulence genes related to toxins, adhesins and autotransporter proteins: Additional virulence genes associated with DEC in general including the STEC pathotype and coding for toxins other than Stx, putative adhesins and autotransporter proteins were investigated by single PCR reactions. Sequences sought were: *ehxA*, sub_{AB}, *ast*A, Ipf_{O113}, *saa*, *iha*, *toxB* and *esp*P. The primers used in the reactions are described in Table 2.

Subtyping of stx genes: The subtyping of the *stx* genes was determined according to the recently proposed method of Scheutz et al. (2012).

2.2.2. Phenotypic assays

Serotyping: O:H serotypes were determined by using the method of Ewing (1986). Tube agglutination assays were performed employing absorbed antisera to somatic (O1 to O181) and flagelar (H1 to H56) antigens, prepared at the Instituto Adolfo Lutz, São Paulo, Brazil. In the case of the somatic antigen a strain was considered to belong to a given serogroup when it presented an agglutination title equal or higher than the title of the reference strain employed in the antisera production for the considered O group.

Vero cells cytotoxic assay: the ability to express *stx* genes was investigated in Vero cells cultivated *in vitro* similarly to Konowalchuk, Speirs, and Stavric (1977). Briefly, strains to be tested were cultivated at 37 °C under agitation in Penassay media for 48 h. After this period cultures were pelleted and the supernatants were sterilized by filtration and inoculated in the cells. An amount of 50 μl of the filtered supernatant was employed. Cells were observed daily for the characteristic cytotoxic effect of Shiga toxins, for a maximum of 96 h. Positive and negative strains for Stx production as well as filtered bacteria-free media were included in the assays as experimental controls. Assays were run in three independent times.

Enterohemolysin production: production of EHEC enterohemolysin (Ehx) was investigated using the method described by Beutin et al. (1989) in washed blood sheep agar plates. A drop of an overnight bacterial culture growth in Luria-Bertani broth was placed in the plate and incubated at 37 °C. A reading after 4 h of incubation was made for the investigation of α hemolysin production. The ascertainment of Ehx production was made after

Table 1
Set of primers composing the multiplex PCR assay used in this study for the search of DEC in raw kibbe samples.

Target gene	DEC pathotype	Primers sequence[a](5′-3′)	Amplicon size	Reference
stx_1	STEC	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	Paton & Paton, 1998
stx_2		GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	
eae	EPEC	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	384	López-Saucedo et al., 2003
*ipa*H	EIEC/*Shigella*	CTCGGCACGTTTTAATAGTCTGG GTGGAGAGCTGAAGTTTCTCTGC	933	Vidal et al., 2005
elt	ETEC	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	450	Aranda, Fagundes-Neto, & Scaletsky, 2004
est		ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT	190	
*aat*A (CVD432)	EAEC	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	

[a] M, A/C; R, A/G.

Table 2
Primers employed in PCR assays for the detection of additional virulence genes in the characterization of STEC isolates from raw kibbe samples.

Target gene	Virulence factor	Primer sequence (5′-3′)	Amplicon size	Reference
*ehx*A	EHEC[a] enterohemolysin	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1551	Schmidt, Beutin, & Karch, 1995
sub_{AB}	STEC subtilase toxin	GTACGGACTAACAGGGAACTG GCAAAAGCCTTCGTGTAGTC	1823	Paton et al., 2004
*ast*A	EAEC enterotoxin 1	CCATCAACACAGTATATCCGA GCGAGTGACGGCTTTGT	111	Yamamoto & Echeverria, 1996
lpf_{O113}	Long polar fimbriae	CTGGCAAAATCGGTAACGGT CCACCGGAAGAACCGAT	573	Doughty et al., 2002
saa	STEC autoagglutinating adhesin	CGTGATGAACAGGCTATTGC ATGGACATGCCTGTGGCAAC	119	Paton & Paton, 2002
iha	IrgA[b] homologue adhesin	CAGTTCAGTTTCGCATTCACC GTATGGCTCTGATGCGATG	1305	Toma et al., 2004
*esp*P	Extracellular serine protease	AAACAGCAGGCACTTGAACG GGAGTCGTCAGTCAGTAGAT	1830	Beutin, Kaulfuss, Herold, Oswald, & Schmidt, 2005
toxB	Plasmidal full adherence required protein	ATA CCT ACC TGC TCT GGA TTG A TTC TTA CCT GAT CTG ATG CAG C	602	Toma et al., 2004

[a] Enterohemorrhagic *Escherichia coli.*
[b] Iron regulated adhesin.

overnight incubation of the plate, as this kind of hemolysin can be detected only after extend periods of incubation.

Adhesion assays: adhesion assays were performed as described by Cravioto, Gross, Scotland, and Rowe (1979). Briefly, HeLa cells were cultivated under the surface of glass coverslips in 24-well tissue culture plates for 24 h. After this period, semi-confluent cell monolayers were infected with 20 μL of bacteria previously grown in LB broth. After 3 h of incubation, unattached bacteria were removed by washing the monolayers six times with 1 mL of sterile phosphate-buffered saline solution. The coverslips were then stained and observed under light microscopy. If non-adherent bacteria were observed or the adherence pattern was undefined (UND), the assay was repeated extending to 6 h the total period of bacteria–cells interaction, with a washing step after the initial 3 h. By the end of the incubation time coverslips were stained and observed under light microscopy (Rodrigues et al., 1996).

Antimicrobial susceptibility: Antimicrobial susceptibility tests were determined using the disk diffusion method (Bauer, Kirby, Sherris, & Turk, 1966). The following drugs were tested following the recommendations of the Clinical and Laboratory Standards Institute, CLSI (2009): nalidixic acid (30 μg), amikacin (30 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), chloramphenicol (10 μg), streptomycin (10 μg), gentamicin (10 μg), imipenem (10 μg), tetracycline (30 μg) and trimethoprim-sulfamethoxazole (1.25/23.75 μg).

3. Results and discussion

The presence of bacteria with a biochemical profile of *E. coli* could be detected in 61 (87.1%) of the 70 raw kibbe samples tested. Amongst the isolated *E. coli* strains (403 in total) two were positive for *stx*1 genes by PCR. Based on the notion that each DEC pathotype is defined by specific genetic virulence markers, and that the mere presence of *stx* genes define the STEC pathotype (Croxen et al., 2013), both of these *E. coli* isolates could be classified as STEC. These strains, one belonging to serotype O125:H19 (7008) and the other to serotype O149:H8 (7776) had the stx_{1c} subtype and were able to express the correlate gene. The other virulence features found and the antimicrobial susceptibility profile of the two STEC isolates are shown in Table 3. Both strains had the lpf_{O113} gene and strain 7008 had additionally the EAST-1 toxin related gene *ast*A. Although, none of them produced the EHEC enterohemolysin (Ehx), they demonstrated the ability to interact with HeLa cells cultivated *in vitro*. Strain 7008 exhibited an aggregative pattern of adherence, while strain 7776 adhered in an undefined pattern.

This study aimed to investigate the occurrence of pathogenic *E. coli* strains in raw kibbe samples marketed in a large urban center in the northwest of São Paulo State, the most populated and economically relevant state in Brazil. Previous works have demonstrated that raw kibbe can be a source of infectious agents

Table 3
Virulence characteristics of the two STEC strains isolated from raw kibbe samples in the northwestern region of São Paulo State, Brazil.

Strain	Phenotypic characteristics				Genotypic characteristics	
	Serotype	Cytotoxic activity[a]	Hemolysin production	Antimicrobial susceptibility[b]	*stx* subtype	Virulence genes[c]
7008	O125:H19	+++	–	S	1c	lpf_{O113}, *ast*A
7776	O149:H8	+++	–	AMP (I)	1c	lpf_{O113}

[a] Determined in Vero cells; +++: An intense cytotoxic effect with more than 50% of the cells lysed and detached could be seen after 48 h of incubation.
[b] S: Sensible for all antibiotics tested; AMP (I): intermediate susceptibility to ampicillin.
[c] The genes *eae*, *ehx*A, *toxB*, sub_{AB}, *esp*P, *saa* and *iha* were also investigated, but both strains were negative for them.

such as the parasite *Sarcocystis* (Pena, Ogassawara, & Sinhorini, 2001).

Although this study tested for all the main DEC pathotypes, only STEC strains were found. This is not so surprising as the main ingredient of kibbe is ground beef which is considered one of the most important vehicles for the transmission of STEC (Llorente, Barnech, Irino, Rumi, & Bentancor, 2014; Mora et al., 2007). However, the presence of STEC strains in kibbe samples is of great concern because this type of food is usually eaten raw, and thus could lead to human contamination.

To the best of our knowledge this is probably the first report that links the serotypes O125:H19 and O149:H8 to the STEC pathotype in food. Both strains only carried the *stx*1 genetic sequence and demonstrated the ability to express this gene in Vero cells assays. Some studies have reported that *stx*2-producing STEC are more often linked to severe human illness including HUS than *stx*1-producing strains (Boerlin et al., 1999; Friedrich et al., 2002). However, there are publications that demonstrated that *stx*1-producing STEC can also cause HUS (Rüssmann et al., 1995; Zhang et al., 2007). Bearing this in mind we can suppose that the strains of this study may have the potential to cause more serious diseases, including HC and HUS, but additional virulence studies are needed to give support to our hypothesis. The *stx*1c subtype was found in both of the strains studied. This *stx* subtype was also detected in different serotypes of STEC isolated from clinical sources in Germany (Friedrich et al., 2003; Zhang, Bielaszewska, Kuczius, & Karch, 2002). Friedrich et al. (2003) detected this subtype in 17.0% of STEC isolates from humans with diarrhea and demonstrated that a prominent feature of *stx*1c-producing STEC is the absence of intimin encoded by the *eae* gene, indicating the absence of the locus of enterocyte effacement (*LEE*), which harbors *eae*, in these strains. This finding is in agreement with the current study as the two strains reported here were also devoid of the *eae*.

The two strains of this study showed the capacity to adhere to epithelial cells cultured *in vitro* in 6 h interaction assays (Fig. 1). Strain 7008 exhibited an aggregative adhesion pattern (AA), while an undefined adhesion pattern was seen for strain 7776. Only the sequence for LpfO113 fimbria was found in the two strains of the present study, suggesting that the set of adhesins they express can be limited. It is worth to mention that only the most common adhesin genes so far described in the literature and which are linked more specifically with STEC pathotype was investigated in this study. STEC strains lacking *eae* gene may have, apart from LpfO113, a diverse repertoire of other fimbrial and afimbrial adhesins and it is postulated that an efficient adhesion in these strains would be the result of the synergistic action of several adhesins (Doughty et al., 2002; Paton et al., 2001; Tarr et al., 2000). Such strains also do not normally exhibit a typical pattern of adhesion to cultured epithelial cells (Dytoc et al., 1994), as it can be seen in *eae*-harboring strains where the localized or localized-like adhesion pattern (ALL) prevail (Pitondo-Silva et al., 2015). So, the fact that the AA pattern was observed in strain 7008 could suggest that this strain can harbor unknown adhesion structures which could be responsible for the pattern of interaction observed. This hypothesis justifies the need for further studies to gain a better understanding into the mechanisms involved in the adhesion abilities of *eae* negative STEC strains as the ones we characterized. Anyways, considering that the two strain here studied presented the capacity of adhering, the risk they represent is of concern, and it must be reminded that the infective dose for STEC is usually low; with fewer than 10 viable cells being capable of causing infection in the human host or persist viable in the environment (Griffin et al., 1994; Paton & Paton, 1998).

The presence of the EAST-1 toxin gene, *ast*A, in one of the strains of this study confirms that this gene, first described in the EAEC pathotype, is not exclusive nor represents a critical virulence factor for a particular DEC category (Savarino et al., 1996; Sousa & Dubreuil, 2001). Studies have shown that the *ast*A gene may be present at high frequencies in ETEC (Gonzales et al., 2013) and EPEC (Scaletsky, Aranda, Souza, Silva, & Morais, 2009) strains associated

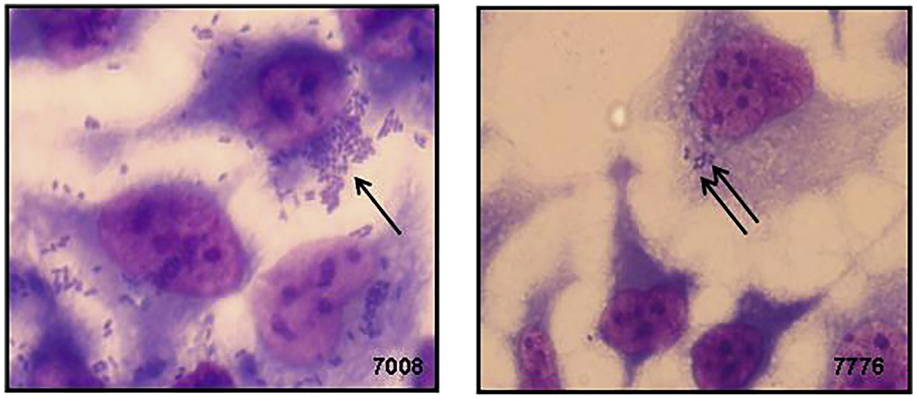

Fig. 1. *In vitro* adhesion assays of the STEC strains isolated from raw kibbe samples in the northwestern region of São Paulo State, Brazil. The arrow indicates the occurrence of the aggregative adhesion pattern (AA) in strain 7008 after a total of 6 h of contact with cells and an undefined adhesion pattern in strain 7776 (double arrows).

with diarrhea. This gene can also be found in a relatively high frequency in the STEC pathotype (Dos Santos, Irino, Vaz, & Guth, 2010), although the precise role of EAST-1 in pathogenesis of the STEC disease is still to be clarified. Interestingly the presence of *ast*A was the only difference in terms of virulence markers between the two strains of this study.

There are reports, even in Brazil, on the occurrence of antimicrobial resistance in STEC isolated from different kinds of foods (Paneto, Schocken-Iturrino, Macedo, Santo, & Marin, 2007; Rodolpho & Marin, 2007) and drinking water (Lascowski et al., 2012). This prompted us to analyze the *in vitro* susceptibilities of the STEC strains of this study in relation to drugs commonly used in clinical and food animal settings. None of them showed a resistance pattern to the antimicrobials tested, but considering the fact that we investigated only two strains, a comparison of the present results with previous data is difficult to be done.

After the large outbreak that occurred in Europe in 2011 caused by an unusual O104:H4 STEC strain transmitted by fenugreek sprouts (Buchholz et al., 2011), there is currently a growing interest in knowing the antigenic and virulent heterogeneity as well as the ways of transmission of different STEC serotypes beyond the classic ones such as O157:H7. In this regard, the results of the present work should contribute to increase the knowledge of the STEC strains diversity.

4. Conclusion

In this study two STEC strains belonging to serotypes O149:H8 and O125:H19, probably described for the first time in refrigerated raw kibbe and foods in general were isolated and characterized in terms of putative virulence traits. The findings herein presented points out the fact that some kinds of ethnical foods, such as kibbe, which can be eaten raw, may be vehicles for the transmission of STEC strains, including highly virulent serotypes, representing thus a risk for human health. So, the consumption uncooked foods especially those prepared with raw meat should be discouraged.

Conflict of interest

No conflict of interest to be declared.

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