PREVALENCE OF SHIGA TOXIN-PRODUCING *Escherichia coli* IN SOUTHERN BRAZIL ISOLATED FROM GROUND BEEF AND RAW MILK

Cláudio Dias Timm,[1] Fabrício Rochedo Conceição,[2] Angelo Menin,[1] Rita de Cássia dos Santos da Conceição[1], Odir Antônio Dellagostin[2] e José Antônio Guimarães Aleixo[3,4]

1. Laboratório de Inspeção de Produtos de Origem Animal, Faculdade de Veterinária, UFPel, Pelotas, CEP 96020-900, RS, Brazil. E-mail: inspleit@ufpel.tche.br
2. Laboratório de Biologia Molecular, Centro de Biotecnologia, UFPel, Pelotas, RS, Brazil
3. Laboratório de Imunologia Aplicada, Centro de Biotecnologia, UFPel, Pelotas, RS, Brazil
4. Departamento de Nutrição, Faculdade de Nutrição, UFPel, Pelotas, RS, Brazil

ABSTRACT

The aim of this work was to investigate the occurrence of shiga toxin-producing *Escherichia coli* (STEC) in ground beef and raw milk in Southern Brazil, and to study the fate of STEC isolates from cattle faeces in ground beef and milk, and its resistance in acid and alcoholic media. Among 464 *E. coli* isolated from ground beef and raw milk there were no *stx1* and *stx2* genes. The population of STEC isolates from cattle faeces was quite stable when inoculated in ground beef and increased in inoculated milk along the 120 hours of storage at 8ºC. These STEC isolates were inactivated when exposed to pH 2.5 and 3.0, but they were viable after eight hours at pH 4.0. The STEC isolates did not survive 48 hours in medium containing 12% ethanol. At 6% ethanol, STEC O174:H21, O163:H19 and O112:H2 have shown an increase in population and STEC O91:H21 and O22:H8 did not resist beyond 24 and 48 hours of incubation, respectively. The low prevalence of STEC in foods together with the attributes of the STEC found in Brazilian cattle could be among the reasons for the low prevalence of foodborne diseases caused by STEC in Brazil.

KEY WORDS: Acid, ethanol, ground beef, milk, Shiga toxin-producing *Escherichia coli.*

RESUMO

PREVALÊNCIA DE *Escherichia coli* PRODUTORA DE SHIGA TOXINAS ISOLADA DE CARNE MOÍDA E LEITE CRU NO SUL DO BRASIL

O trabalho teve como objetivo determinar a ocorrência de *Escherichia coli* produtora de toxina Shiga (STEC) em carne moída e leite cru no sul do Brasil e estudar o comportamento de STEC isoladas de fezes de bovinos de corte e leite, verificando sua resistência em meios ácido e alcoólico. Não foram identificados genes *stx1* e *stx2* nas 464 *E. coli* isoladas de carne moída e leite cru. STEC isoladas de fezes de bovinos mantiveram populações estáveis e apresentaram crescimento, respectivamente, em carne moída e em leite experimentalmente contaminados, durante 120 horas a 8ºC. Esses isolados foram inativados quando expostos a pH 2,5 e 3,0, mas permaneceram viáveis após oito horas em pH 4,0. Os isolados de STEC não sobreviveram 48 horas em meio contendo 12% de etanol. Em 6% de etanol, STEC O174:H21, O163:H19 e O112:H2 apresentaram crescimento, ao passo que STEC O91:H21 e O22:H8 não resistiram além de 24 e 48 horas de incubação, respectivamente. A baixa prevalência de STEC em alimentos e as características das cepas encontradas em bovinos podem estar relacionadas com a baixa prevalência de enfermidades de origem alimentar causadas por STEC no Brasil.

PALAVRAS-CHAVES: Ácido, carne moída, etanol, leite, *Escherichia coli* produtora de toxina Shiga.

INTRODUCTION

Foods of cattle origin, like undercooked ground beef and dairy products, are among those most frequently implicated in food-borne diseases caused by Shiga toxin-producing *Escherichia coli* (STEC), including diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) (NEIL, 1997; BLANCO et al., 2004). Ground beef can be contaminated all over in the grinding operation, and the risk of transmitting STEC to consumer is further increased because the organism may be protected inside the body of the reformed meat during the cooking process (BARLOW et al., 2006). Outbreaks and sporadic cases of illnesses, including HUS, have been caused by consumption of raw milk and dairy products contaminated by O157 and non-O157 STEC. There are also reports of human infections by visiting dairy farms (HUSSEIN & SAKUMA, 2005).

In spite of the high incidence of HUS in the neighboring Argentina (LÓPEZ et al., 1997), in Brazil only sporadic cases of diarrhea and HUS associated with STEC have been reported (CANTARELLI et al., 2000; GUTH et al., 2002; IRINO et al., 2002; VAZ et al., 2004; NISHIMURA et al., 2005) and there are only a few reports of the occurrence of STEC in foods (CERQUEIRA et al., 1997; BERGAMINI et al., 2004). One reason for such disparity in the STEC prevalence in foods and in human disease in populations having similar eating habits may be differences in the mechanisms of adaptation to stress conditions such as acid resistance or ethanol tolerance. The acid tolerance varies among *E. coli* isolates and significant variability has been observed in the survival of the different strains of STEC when stationary-phase cultures were exposed to low-pH conditions (JORDAN et al., 1999). Ethanol also has been implicated in the low level of survival of STEC in certain foods (MOLINA et al., 2003).

The objectives of this study were to determine: (i) the occurrence of STEC in ground beef and raw milk in southern Brazil; (ii) the fate of non-O157:H7 STEC isolated from cattle faeces in ground beef and milk; (iii) the resistance profile of the cattle isolates exposed to different pH values and ethanol concentrations.

MATERIAL AND METHODS

Sampling

Fifty samples of ground beef were collected from 17 butcher shops that sell meat from cattle raised in the south of the Rio Grande do Sul State, Brazil. Fifty samples of refrigerated raw bulk tank milk were obtained from trucks from collection routes in the same region. The samples were placed in ice boxes and transported under refrigeration to the laboratory.

Isolation

The isolation was made according BLANCO et al. (2007), with modifications. Twenty five grams of ground beef or 25 mL of raw milk from each sample were placed in 225 mL of Buffered Peptone Water (Acumedia, Lansing, Michigan, USA) and incubated at 37ºC for 6 hours. After, 1 mL was added to 9 mL of MacConkey broth (Difco Laboratories, Detroit, USA) and incubated at 37ºC for 18 h. Portions of these cultures were spread onto MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37ºC overnight to obtain isolated colonies. Five lactose-fermenting colonies were transferred to separate tubes with Brain and Heart Infusion (BHI; Merck, Darmstadt, Germany) and incubated at 37ºC for 16 to 18 h. The BHI cultures were mixed with equal volume of glycerol at 80% in phosphate-buffered saline (PBS 0.01 M, pH 7.4) and kept at -70ºC. The isolates were recovered in BHI at 37ºC when necessary.

DNA extraction

The DNA was extracted from the isolates according to SAMBROOK & RUSSEL (2001). Briefly, the pellet obtained by centrifugation of 1 mL of BHI culture was ressuspended in 100 µL of STES buffer [Tris-HCl 0.2 M, NaCl 0.5 M, SDS 0.1% (w/v), EDTA 0.01 M, pH 7.6]. Fifty µL of

glass beads and 100 µL of phenol/chloroform were added. After homogenization for 1 min, the mixture was centrifuged at 13,000 *g* for 5 min, the supernatant collected and precipitated in 2 volumes of absolute ethanol and 0.1 volume of NaCl 5 M at -70ºC for 30 min. A new centrifugation was carried out at 13.000 *g* for 20 min, the supernatant was discarded and the pellet washed with ethanol 70%. After elution in 40 µL of elution buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.4), 1 µL of RNAse (10 µg μL^{-1}) was added. The DNA extracted was stored at -70ºC.

Multiplex PCR

The DNA was analyzed by PCR for the presence of the rDNA *16S* (internal control), *stx1*, *stx2* and *eaeA* genes, with the primers shown in Table 1. The amplification protocol reported by WANG et al. (2002) was used, with modifications. Briefly, each reaction of 25 µL contained 200 µM dNTP, buffer 1x (Tris-HCl 10 mM, KCl 50 mM, pH 8.3), 1.5 mM $MgCl_2$, 2.5 U of recombinant *Taq* DNA polimerase (AmpliTaq Gold; Applied Biosystems), 20 ng of DNA and 0.1 µM of primer 16S (E16S), 0.75 µM of primer EAE e 0.5 µM of primers Stx1 and Stx2. The amplification was carried out in a Perkin-Elmer thermocycler 2400 using an initial denaturation step at 95ºC for 8 min, followed by 30 cycles of 95ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s and final extension at 72ºC for 7 min. DNA of *E. coli* O157:H7 and *Klebsiella pneumoniae* were used as positive and negative controls, respectively.

TABLE 1. Primers used in this study

Primer	Sequence (5' to 3')	Target gene	Localization within gene	Size of PCR amplicon (bp)	Reference
Stx1-a	TCTCAGTGGGCGTTCTTATG	*stx1*	777-796	338	WANG et al. (2002)
Stx1-b	TACCCCCTCAACTGCTAATA		1114-1095		
Stx2-a	GGCACTGTCTGAAACTGCTCC	*stx2*	603-623	255	PATON & PATON (1998)
Stx2-b	TCGCCAGTTATCTGACATTCTG		857-837		
EAE-a	ATGCTTAGTGCTGGTTTAGG	*eaeA*	132-151	248	WANG et al. (2002)
EAE-b	GCCTTCATCATTTCGCTTTC		379-360		
E16S-a	CCCCCTGGACGAAGACTGAC	*E. coli* 16S rRNA	1682-1701	401	WANG et al. (2002)
E16S-b	ACCGCTGGCAACAAAGGATA		2082-2063		

STEC isolates

Five isolates of STEC of serotypes previously associated with STEC strains that cause HUS, isolated from faecal samples of cattle in southern Brazil in a previous study (TIMM et al., 2007), were used in the studies with experimentally contaminated foods and media. The characteristics of the isolates are showed in table 2. One strain of the O157:H7 serotype, a gentle gift from Dr. T. Yano (Unicamp, Campinas, Brazil), was used for comparison purposes.

TABLE 2. Characteristics of STEC strains used in the present study (TIMM et al., 2007).

Serotype	Genotypic profile	Source
O174:H21	*stx1- stx2+ eae-*	Beef cattle
O22:H8	*stx1- stx2+ eae-*	Beef cattle
O163:H19	*stx1+ stx2+ eae-*	Dairy cattle
O112:H2	*stx1- stx2+ eae-*	Dairy cattle
O91:H21	*stx1- stx2+ eae-*	Dairy cattle

Induction of nalidixic acid resistance

The STEC isolates and the *E. coli* O157:H7 strain were successively cultured in Plate Count Agar (PCA; Difko Laboratories, Detroit, Michigan, USA) containing increasing concentrations of nalidixic acid until growth in PCA with 100 µg of nalidixic acid per mL of medium. The nalidixic acid-resistant (NAR) isolates were cultured in BHI at 37ºC for 16 to 18 h, mixed with equal volume of 80% glycerol in PBS and kept at -70ºC.

Fate in experimentally contaminated ground beef

Ground beef purchased from a local supermarket was used. The bacterial population on ground beef was determined in PCA and 0.1 mL of the 10^{-1} dilution in sterile saline was plated onto MacConkey agar with 100 µg of nalidixic acid per mL (MCNA) to confirm the absence of bacteria naturally resistant to nalidixic acid.

The NAR isolates were cultured in BHI at 37ºC for 16 to 18 h until reaching stationary phase. The BHI cultures were diluted in saline, added to 100 g of ground beef to obtain a final concentration of 10^2 to 10^3 CFU g^{-1} and homogenized in a stomacher (BagMixer 400, Interscience, St. Nom, France) for 10 min. The contaminated ground beef was kept under refrigeration at 8ºC and bacterial counts were made after 0, 24, 48, 96 and 120 h of storage.

Fate in experimentally contaminated milk

Pasteurized milk containing 3% fat was used. The bacterial population of the milk was determined in PCA and 0.1 mL of milk was plated on MCNA to confirm the absence of bacteria naturally resistant to nalidixic acid.

The NAR isolates were cultured in BHI at 37ºC for 16 to 18 h until reaching stationary phase. The BHI cultures were diluted in saline and added to 100 mL of milk to obtain a final concentration of 10^2 to 10^3 CFU mL^{-1}. The contaminated milk was kept under refrigeration at 8ºC and counts on MCNA were made after 0, 24, 48, 96 and 120 h of storage.

Resistance in acid environment

Overnight BHI cultures of each STEC isolate studied were diluted to 10^{-5} with saline and 0.2 mL were added to 20 mL of BHI acidified with tartaric acid until pH 2.5, 3, 4, and 5, and incubated at 37ºC. The bacterial population was determined by plating onto PCA after 0, 2, 4, and 6 h of incubation.

Resistance in ethanol

Overnight BHI cultures of each STEC isolate studied were diluted to 10^{-5} with saline and 0.2 mL were added to 20 mL of BHI containing 6% and 12% of ethanol and incubated at 37ºC. The bacterial population was determined by plating onto PCA after 0, 3, 6, 12, 24 and 48 h of incubation.

RESULTS

Occurrence of STEC in ground beef and raw milk

There were no positives among the 464 *E. coli* isolates from ground beef and from raw milk tested for the presence of *stx1* or *stx2* genes, and only four isolates from a sample of ground beef harboured the *eaeA* gene (Figure 1).

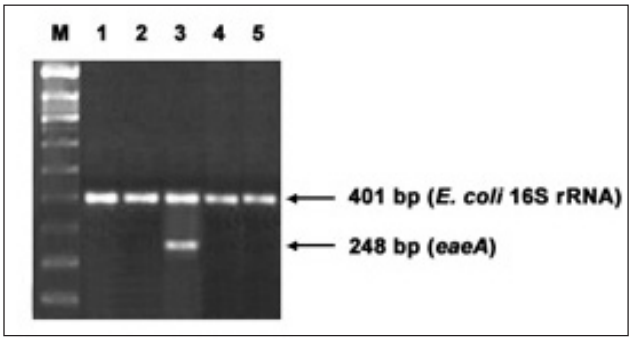


FIGURE 1. Agarose gel electrophoresis of products from multiplex PCR of isolates from ground beef and raw milk. Lanes: M, DNA size marker; 1, 2, 4, 5, *E. coli stx1- stx2- eae-*; 3, *E. coli stx1- stx2- eae+*.

Fate of STEC in ground beef

Counts of the NAR STEC isolates in the ground beef were quite stable along the 120 hours period of storage at 8ºC, although in general a small decrease was observed between the initial and final counts (Figure 2). The initial bacterial population of the ground beef was 3.4 x 10^5 CFU mL^{-1} and bacteria naturally resistant to nalidixic acid were not isolated.

Fate of STEC in milk

The counts of the NAR STEC isolates in milk increased by about 0,5 log CFU mL^{-1} along the 120 hours period of storage at 8ºC, with exception of the isolates from serotypes O174:H21 and O22:H8 that grew 1.04 and 2.08 log CFU mL^{-1}, respectively (Figure 3). The initial bacterial population in milk was 2.9 x 10^4 CFU mL^{-1} and bacteria naturally resistant to nalidixic acid were not isolated.

Resistance of STEC in acid environment

The STEC isolates were immediately inactivated when exposed to pH 2.5, and no one survived exposure to pH 3.0 along 8 h (Figure 4). Although there was a decrease in population, all STEC isolates were viable after exposure to pH 4.0 for 8 h. Only STEC O157:H7 was not recovered after exposure to this experimental condition. All STEC isolates tested grew at pH 5.0.

Resistance of STEC in ethanol

The STEC isolates did not survive in medium containing 12% ethanol along the 48 h incubation period (Figure 5). At a 6% ethanol, STEC O174:H21, O163:H19 and O112:H2 have shown an increase in population after 12 or 24 h of incubation. On the contrary, STEC O22:H8, O91:H21 and O157:H7 did not resist to 6% ethanol beyond 24 or 48 h of incubation.

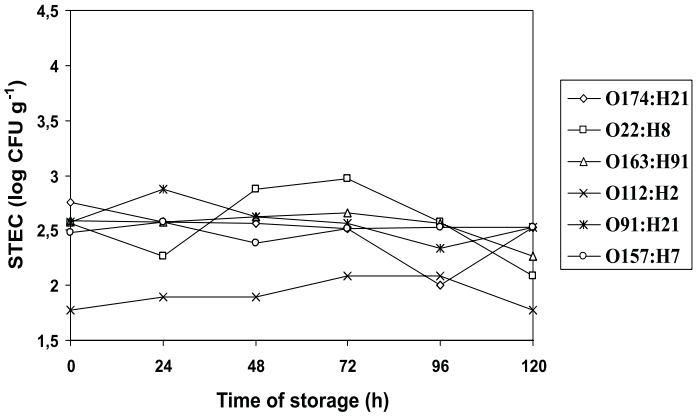


FIGURE 2. STEC counts in artificially contaminated ground beef stored at 8ºC.

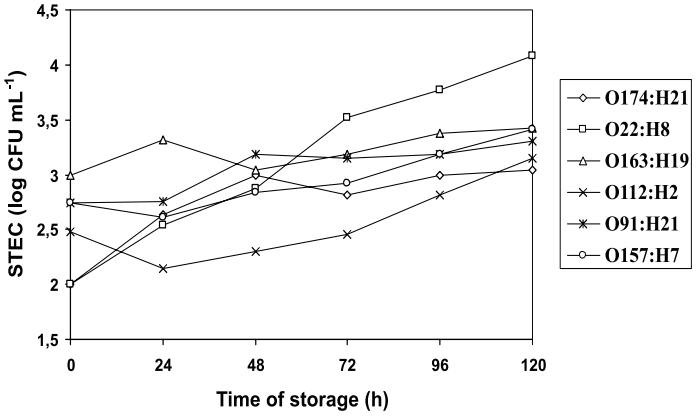
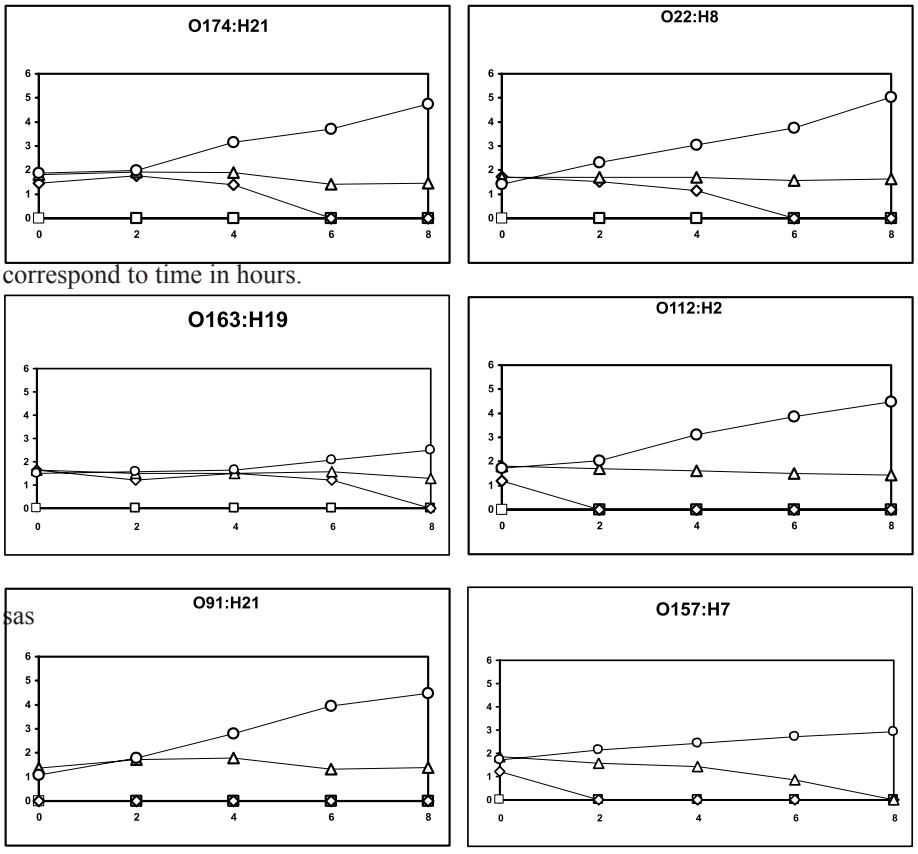


FIGURE 3. STEC counts in artificially contaminated milk stored at 8ºC.



correspond to time in hours.

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FIGURE 4. Growth of STEC at different pH. Ordinates correspond to bacterial population in log CFU mL^{-1} and abscis

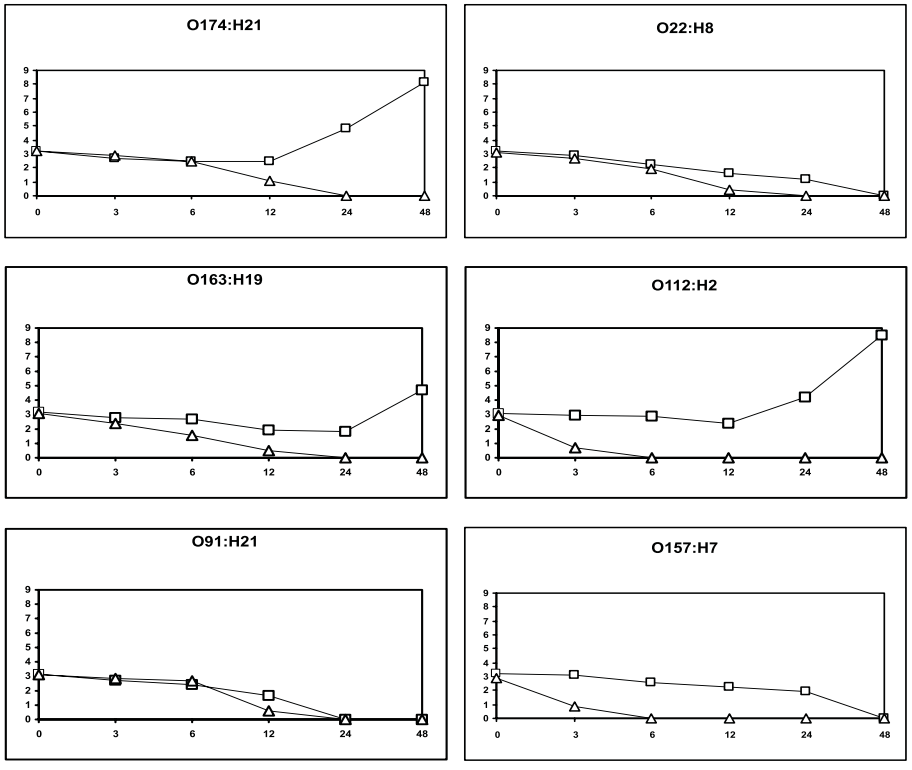

FIGURE 5. Growth of STEC in medium containing 6% and 12% ethanol. Ordinates correspond to bacterial population in log CFU mL^{-1} and abscissas correspond to time in hours.

DISCUSSION

Four hundred sixty four *E. coli* isolates obtained from 50 samples of ground beef and 50 of raw milk in Southern Brazil were surveyed for the presence of STEC using molecular detection of virulence markers such as the shiga toxins and the adhesin intimin. None of the isolates tested possessed the shiga toxin genes and only a few carried the intimin gene. This was the first investigation on the occurrence of STEC in foods of animal origin in the extreme South of Brazil, a region near the border of Argentina and Uruguay, countries where STEC has been isolated from meat and dairy products (PARMA et al., 2000; CHINEN et al., 2001; GOMEZ et al., 2002; BOSILEVAC et al., 2007).

Although the occurrence of STEC in meat and dairy products from several countries has been reported (HUSSEIN & SAKUMA, 2005; HUSSEIN, 2007; PERELLE et al., 2007), in Brazil there are only a few reports on the presence of STEC in raw beef products (CERQUEIRA et al., 1997; BERGAMINI et al., 2004). A recent survey on the presence of STEC in 80 beef carcasses from an abattoir in the State of São Paulo found only one *E. coli* harbouring *stx2* gene (RIGOBELO et al., 2006). Also in São Paulo, *stx* sequences were detected only in 3.3% of 30 milk samples collected from dairy farms (VICENTE et al., 2005).

STEC of serotypes associated with cases of HUS had been previously isolated from cattle (TIMM et al., 2007). These isolates were used to study their behavior in foods of animal origin and in stress conditions they are exposed in the environment. The behavior of the STEC isolates in experimentally contaminated ground beef was similar. The numbers of viable cells were stable along the 120 h storage period, with final counts being very close to that of the moment of contamination. In contrast, all isolates grew in experimentally contaminated milk albeit at different ratios. The STEC O22:H8 increased 2.08 log CFU mL^{-1} along the 120 h period, showing that it is adapted to milk environment. The presence of concurrent natural bacterial population did not prevent the STEC survival and growth in ground beef and milk, respectively.

The ability of a STEC strain to survive stress conditions encountered in foods and in the stomach is indispensable to be considered a human pathogen. We found that the pH 2.5 was improper for survival of the STEC isolates tested, and that at pH 3.0 their behavior was variable. STEC O91:H21 did not survive at this pH value and STEC O112:H2 and O157:H7 could not be recovered after 2 h exposure. On the other hand, the STEC isolates tolerate pH 4.0, except STEC O163:H19 that, like STEC O157:H7, was not able to survive 8 h of exposure to this pH.

A study on the acid tolerance of STEC isolates from Argentina found all STEC tolerant to pH as low as 2.5, and an STEC O91:H21 was the isolate most resistant to pH decrease (MOLINA et al., 2003). Differently, in our study the STEC O91:H21 studied was the isolate with less acid resistance. This different behavior can be relevant to the incidence of human illness caused by serotype O91:H21 in the two countries. STEC O22:H8 isolates from other countries were also able to survive more than 5 h at pH 2.5 and 3.0 (BENJAMIN & DATTA, 1995). The STEC O22:H8 used in this study did not survive 6 h at pH 3.0. A wide variety of stress responses is controlled by alternative σ factors which replace the primary σ factor reversibly associated with RNA polymerase and co-ordinately express genes involved in diverse functions (HENGGE-ARONIS, 2002; PAGET & HELMANN, 2003). The low resistance of STEC O157:H7 to acid stress can be due to a particular attribute of the strain used, as not all O157:H7 strains have the same capability to survive in a low-pH environment (LARGE et al., 2005).

The ethanol content of beverages can inactivate or reduce the bacterial population of these foods. SEMANCHEK & GOLDEN (1996) demonstrated that alcoholic fermentation of fresh cider is an effective means of destroying STEC O157:H7. In the present study, the viability of the STEC isolates was lost within 24 h in culture broth with 12% ethanol. However, at 6% ethanol the STEC O174:H21, O163:H19 and O112:H2 isolates grew after 12 or 24 h of incubation. In a previous study by MOLINA et al. (2003) the STEC O91:H21 was the isolate with the highest ethanol

resistance. In our study, the O91:H21 isolate tested did not survive more than 24 h at 6% ethanol.

Although some of the STEC isolates used in our study were more resistant to acid and alcoholic stresses than the STEC O157:H7 strain tested for comparison purposes, their overall resistance was lower than that observed with STEC isolates from Argentina and other countries (BENJAMIN & DATTA, 1995; MOLINA et al., 2003). A test side-by-side including STEC isolates from Brazil and Argentina would be necessary to check if this is really true. The low prevalence of STEC in foods together with the attributes of the STEC found in Brazilian cattle could be among the reasons for the low prevalence of foodborne diseases caused by STEC in Brazil.

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