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Assessment of Survival and Production of Shiga Toxins by Enterohemorrhagic *E. coli* in Stored Hamburgers

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Abstract: *E.coli* O_{157}:H_7 was first isolated in 1982 in relation to a hemorrhagic colitis outbreak due to consumption of insufficiently cooked hamburgers. The aim of this work was to assess the effect of different temperatures and storage times on *E.coli* survival and production of Shiga toxins in hamburgers experimentally contaminated with enterohemorrhagic *E.coli*. The hamburgers were stored at 4°C for 7 days and at 0 and-20°C for 30 days. Bacterial cell counts were carried out before and after storage and toxicity was assayed. Similarly, Shiga toxins were determined at different storage temperatures and time intervals. Bacterial survival after storage at 4°C was>60 and at 0°C 65% after 7 days, diminishing to 20% after 30 days of storage. At-20°C survival was 55% after 7 days and 40% after 30 days of storage. In all the cases they maintain its virulence factors (toxin production).These results suggest that hamburgers, although stored at low temperatures, need excellent manufacturing processes. Besides, maintaining the cooling chain uninterrupted is absolutely necessary to assure the hamburgers are safe for consumption.

Key words: Hamburgers-enterohemorrhagic *E.coli*-viability-shiga toxins

INTRODUCTION

Changes in the behavior of food consumption by humans, the processing and distribution of food, globalization as well as adaptations of microorganisms themselves affect the development of new pathogens and the increase in the occurrence of bacteria admitted to be agents of diseases transmitted by food.[1-3]

E.coli O_{157}: H_7 and other enterohemorrhagic serotypes (EHEC) are emerging microorganisms able to produce sporadic cases of food borne diseases such as diarrhea and hemorrhagic colitis in humans and extra-intestinal complications like Hemolytic Uremic Syndrome (HUS) in children and elderly people and thrombocytopenic purpura in adults. Among the virulence factors especially potent cytotoxins, codified by phages called Shiga toxins (Stx_1 and Stx_2) and responsible for the clinical symptoms, stand out.[4,5]

It is well known that EHEC is associated with various types of food from animal origin. Beef cattle represent an important storage place for this microorganism. During the slaughtering process and mainly during the process of cutting up and taking out the guts, *E.coli* strains from the animal's intestines inevitably get to the surface of the entrails. Consequently, contamination of the meat can occur in slaughterhouses, meat processing plants and supermarkets. Ground beef and hamburgers are the principal transmission vehicles. The Center of Investment, Development and Export of Agriculture Produce (IDEA) has detected that 52% of the outbreaks were associated to beef cattle-originated products.[6-8]

Shiga toxin producer *E.coli* O_{157}:H_7 was first recognized as a human pathogen during a hemorrhagic colitis outbreak in the USA in 1982 due to consumption of insufficiently cooked hamburgers. Since then numerous cases have been reported throughout the world, all associated with consumption of food contaminated by this bacterium. The majority of these cases took place in the USA, Canada, Japan and the UK.[9-17]

In the USA, due to outbreaks originated in fast food chains after consumption of insufficiently cooked hamburgers, the Food and Drugs Administration (FDA) established that the temperature inside during cooking of ground beef must be at least 68°C (155°F) to assure the safety of the product.[2,18,19]

It has been demonstrated that the microflora present in meat inhibits reproduction of *E.coli* O_{157}:H_7 in surface layers.[20,21] However, inadequate processing and interruption of the cold chain in the hamburger production line worsen the problem, thus increasing the reproduction capacity of the microorganism. The infective dose is low and only 100 *E.coli* O_{157}:H_7 cfu g^{-1} of food is enough

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to cause disease.[6,22] These microorganisms can reproduce fast in warm environments and growth can be inhibited or stopped by refrigeration or freezing. Although the quality and safety of well-stored and refrigerated products highly depend on the microbiological quality of the raw material, this does not mean that well-stored food products are not exposed to bacterial contamination that can lead to disease.[23]

In Argentina 300 new cases of HUS are reported yearly. Per 100,000 habitants 9.2 cases are estimated in children less than 5 years of age and since 1965 more than 7,000 cases have been described.[6]

E.coli O_{157}:H_7 is endemic in Argentina and therefore people coexist with this microorganism and other Shiga producing EHEC.

Due to the fact that storage procedures for the cooling chain are not always followed strictly, the aim of the present work was to assess the effect of different temperatures and storage times on the survival of EHEC and the production of Shiga toxins (Stx_1 and/or Stx_2) in experimentally contaminated hamburgers.

MATERIALS AND METHODS

Strains: The EHEC strains used in this study were: M1: Stx_2 producing *E.coli* O_{157}:H_7 (933W) and M2: Stx_1 and Stx_2 producing *E.coli* O_{111}: H⁻. Both strains were obtained from the Carlos Malbran Institute, Buenos Aires. 24 h Cultures were used to prepare suspensions with sterile physiological solution at a turbidity of 0.5 at the McFarland scale (10^8 cfu mL^{-1}). A 10^3-fold dilution was made until reaching a final concentration of 10^5 cfu mL^{-1}.

Preparation and contamination of the hamburgers: 14 batches of hamburgers were prepared which were later stored at different temperatures and time intervals. Each batch consisted of three hamburgers (30 g each) of lean ground beef, supplemented with 1.5 g NaCl. One of the three hamburgers was contaminated with M1, another with M2 and a third was used as negative control (no contamination with *E.coli*). The meat samples were contaminated with 100 μL of the bacterial suspension and perfectly homogenized to distribute the inoculum throughout the meat and to obtain a final concentration of 3.3 10^3 cfu g^{-1} of hamburger.

Temperature and storage time: The hamburgers were stored at 4°C for 3 days and at 0°C and-20°C for 30 days.

Survival of EHEC: Assessment of *E.coli* survival was carried out as follows. Each hamburger from the different batches was resuspended in 60 mL McConkey broth and homogenized for 5 min. Bacterial cell counts of hamburgers stored at 4°C were carried out at T_0 and after 1, 2 and 3 days of storage. Those stored at 0°C and-20°C were thawed at room temperature for 2h before resuspending them in broth. Bacterial cell counts were carried out at T_0 and after 1, 7, 15 and 30 days of storage. Quantification of viable *E.coli* O_{111}:H-was carried out on McConkey and McConkey-sorbitol agar (Difco) and *E.coli* O_{157}:H_7 on McConkey-sorbitol+cefixime+telurite agar (Oxoid) and Chrome agar (Chrome agar Microbiology), using the method by Clark *et al.*[24]

Detection of Shiga toxins: Shiga toxins were detected using 2 methods: Cytotoxicity assaying in cell cultures and the passive reverse agglutination test (VTEC-RPLA: Toxin Detection Kit, Oxoid).

Extracts: Hamburgers were resuspended in McConkey broth and then incubated at 35°C for 24 h. Supernatant was first gauze-filtered and then centrifuged at 10,000g for 10 min at 4°C. The pellet was washed twice with physiological solution and resuspended in 1 mL of 0.85% NaCl containing 5,000 U/mL of polymyxin B. Suspensions were incubated at 37°C for 30 min on a shaker and then centrifuged at 4,000 g for 20 min at 4°C. The supernatant was membrane-filtered (Millipore 0.22 μm) and the extract was used to determine the Shiga toxin production using both aforementioned methods.

Cytotoxicity assaying: Cytotoxic activity was assayed in Vero cells (African Green Monkey kidney cells) in minimum essential Eagle medium (MEM) with Earle salts (Gibco, Life Technologies), supplemented with 7% fetal bovine serum (FBS, Gibco, Life Technologies), 0.03 M glutamine, gentamicin (50 μg mL^{-1}) and fungizone (2.5 μg mL^{-1}). Quantitative studies were carried out with serial dilutions (10^{-1} to 10^{-8}) of the enzyme extract. Each dilution was processed fourfold, sowing 50 μL on a monolayer of Vero cells. Toxins obtained from reference strains M1 and M2 were used as positive controls. Plates were incubated under a CO_2 atmosphere in a humid chamber for 24h. Vero cells were examined daily under a microscope to determine the cytotoxic effect produced by the Stx toxins, observing rounding and refringent of the cells, which leads to destruction of the cell tissue. After the readings the micro plates were dyed with 0.75% crystal violet in 40% methanol for 3 min and then destained with running tap water to obtain a better impression of the effect (vital staining).[25]

VTEC-RPLA: This methodology uses latex particles labeled with anti-stx_1 and anti-stx_2 antibodies. 25 μL

previously membrane-filtered (Millipore 0.22 μm) samples of each supernatant were successively diluted with medium and pooled with 25 μL of an anti-stx_1 and anti-stx_2 rabbit IgG suspension to examine presence of either or both toxins. Solutions were incubated for 24 h. A positive reaction was observed as a diffuse pale pink precipitation and a negative reaction (absence of an antigen-antibody reaction) as a puntiform precipitation.

RESULTS AND DISCUSSION

The results of *E.coli* survival are shown in Fig. 1 to 3: 4°C: After 2 and 3 days the *E.coli* strains demonstrated survival of over 60%. *E.coli* $O_{111}H$-viability was slightly higher than that of *E.coli* $O_{157}:H_7$ (Fig. 1). 0°C: A recovery of approximately 65% was observed for both strains after 7 days; after 30 days this was 20% (Fig. 2).-20°C: Survival was about 55% and 40% after 7 and 30, respectively for *E.coli* $O_{157}:H_7$; survival for *E.coli* $O_{111}H$-was 30% after 30 days (Fig. 3).

Initial cell counts (T_0) of hamburgers corresponding to the non-contaminated batch after being stored at different temperatures for different time intervals were negative.

Extracts obtained from contaminated hamburgers with strains M1 and M2 showed positive cytotoxicity at all temperatures and time intervals assayed, using both methods: Cell cultures and VTEC-RPLA Fig 1, 2 and 3).

Present results demonstrate a reduction in the initial inoculum (T_0) of about 30 and 40% for M1 and M2, respectively.

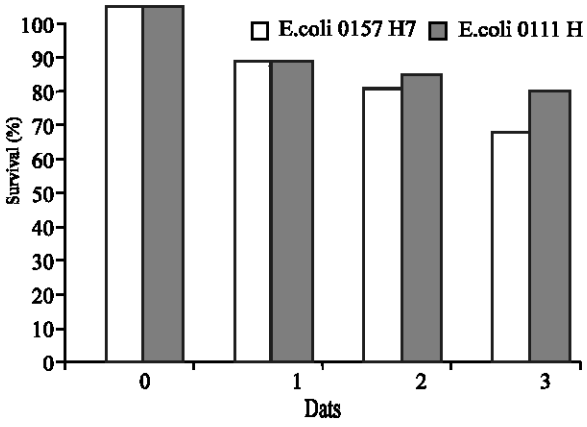


Fig. 1: Survival of Stx-producing *E.coli* (M1 and M2) after 0, 1, 2 and 3 days in contaminated hamburgers stored at 4°C

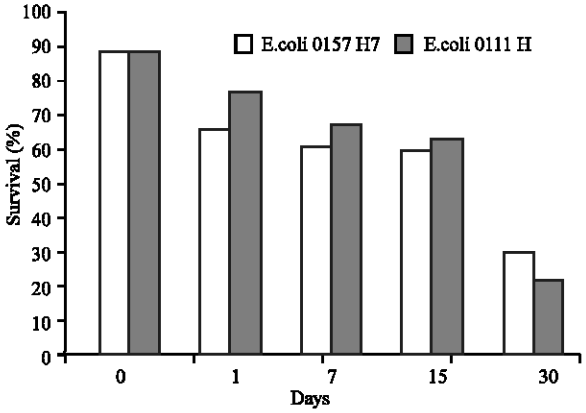


Fig. 2: Survival of Stx-producing *E.coli* (M1 and M2) after 0, 1, 7, 15 and 30 days in contaminated hamburgers stored 0°C

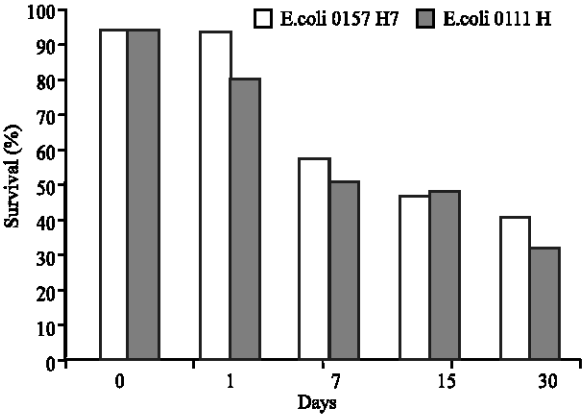
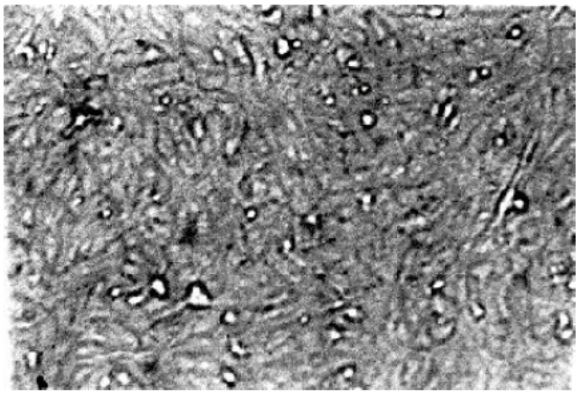


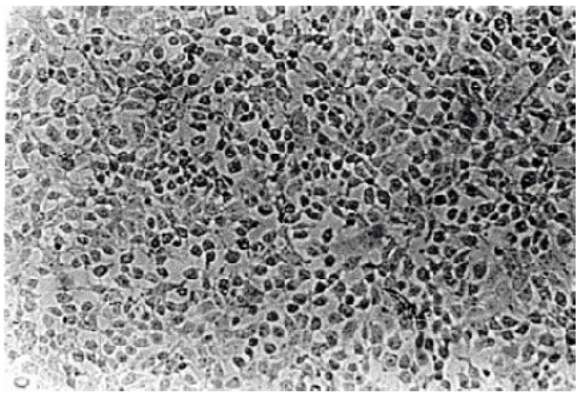
Fig. 3: Survival of Stx-producing *E.coli* (M1 and M2) after 0, 1, 7, 15 and 30 days in contaminated hamburgers stored-20°C

Some researchers believe that survival of pathogenic bacteria in food varies highly. Consequently, *E.coli* $O_{157}:H_7$ shows prolonged survival when it is kept refrigerated.[13,15,26] Although frozen food presents an acceptable record referring safety it may be exposed to deterioration by microorganisms.[7] Temperature control during processing and storage of meat is important to prevent excess development of *E.coli* $O_{157}:H_7$. It is essential to adopt strict sanitary habits and excellent manufacturing processes, as they prevent and reduce contamination of meat with this microorganism.[15,26]

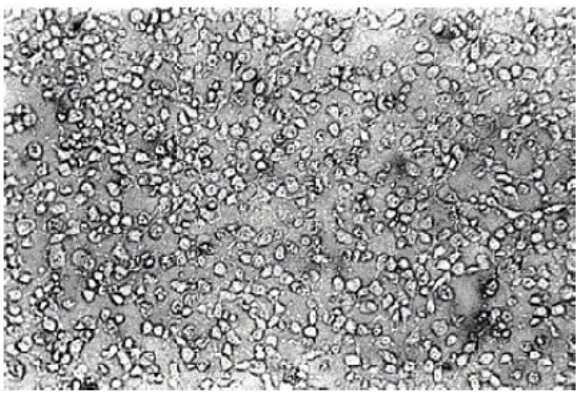
A group of researchers studied in 1986 the effect of low temperatures on growth of food-transmitted bacteria. Similarly, Palumbo *et al.* observed that enterohemorrhagic *E.coli* strains grew at temperatures as low as 8°C, reaching maximum population when incubated at 12°C



(A)



(B)



(C)

Photo 1: Cytotoxicity assaying of M1 (Stx_2 producing *E.coli* O_{157}:H_7) and M2 (Stx_1 and Stx_2 producing *E.coli* $O_{111}H^-$), using Vero cells: A) monolayer of intact Vero cells (converging tissue), B) monolayer of partially destroyed Vero cells and C) monolayer of completely destroyed Vero cells due to the cytotoxic effect.

for 5 days and 15°C for 3 days. The same authors also demonstrated that the number of viable bacteria in milk kept at 5°C maintained stable during 12 days.[20,23,27]

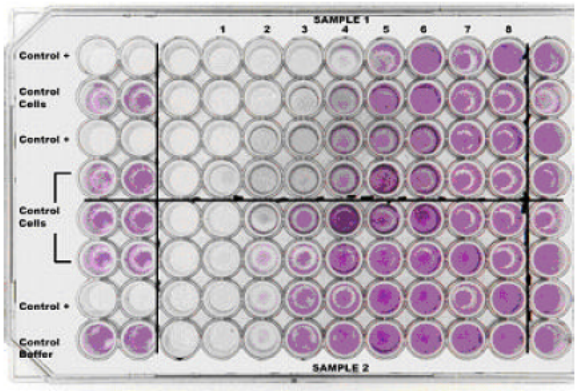


Photo 2: Visualization of the cytotoxic effect on Vero cells. Vital staining.

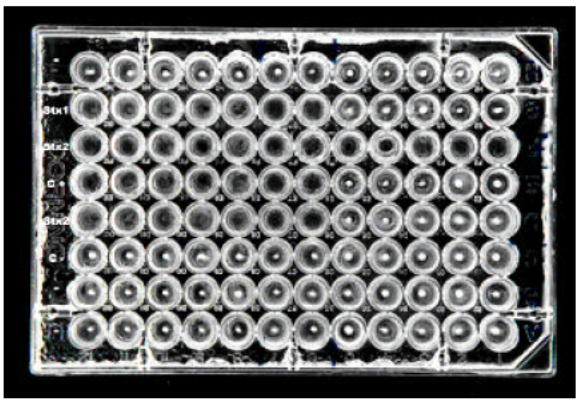


Photo 3: Detection of Stx toxin using VETEC-RPLA.

Márquez *et al.* studied survival of *E.coli* O_{157}:H_7 in fruit pulp kept at 4°C, demonstrating that this microorganism survived until 4 days at pH values between 2.51 and 3.26.[28] This supports our results because if this microorganism is able to survive at such low pH values it will do so all the more in a protecting environment as is ground beef. Hence, it will not only be able to keep viability but also maintain its virulence factors (toxin production).

Cheville *et al.*[29] Miller and Kaspar and Mark *et al.*[30,31] studied the impact of the storage temperature on survival of *E.coli* O_{157}:H_7 and they observed that a temperature of 4°C favored survival of this microorganism at varied pH.[29-31]

Present results have clearly demonstrated survival of *E.coli* O_{157}:H_7 in ground beef samples, previously treated with NaCl and stored at different temperatures. A decrease in the number of viable microorganisms was observed, which coincides with results obtained by Cheville *et al.* and Miller and Kaspar.[29,30]

Even though the behavior of both strains assayed was not exactly alike, storage at 0 and-20°C diminished the number of viable microorganisms without a change in toxin production.

Palumbo *et al.*[27] maintain that meat seems to be a good substrate for Shiga toxin production and that toxin production in milk at the same storage temperature is less.[24]

Our observations suggest that a hamburger contaminated with EHEC maintains to be a risk if the food is stored at 4°C, even under ideal refrigeration conditions, because the bacterial cells maintain viable, even if they do not reproduce themselves.

Due to the fact that in meat products, stored at low temperatures, EHEC maintains viable with the ability to produce Shiga toxins, we would like to emphasize the importance of excellent manufacturing processes and maintaining the cooling chain uninterrupted in order to avoid bacterial contamination and propagation, thus assuring the safety of hamburger consumption.

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