

Fate of *Escherichia coli* enteroinvasive, enteropathogenic and enterohemorrhagic in pasty *dulce de leche**

Comportamento de *Escherichia coli* enteroinvasiva, enteropatogênica e entero-hemorrágica em doce de leite pastoso

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Abstract

Dulce de leche is the product obtained by concentrating milk and adding sucrose. This sweet can be contaminated by improper practices during the manufacturing process, or in the consumer's home. If manipulation is not performed in a hygienic manner the *dulce de leche* can be a vehicle of pathogenic bacteria, like some strains of *Escherichia coli*, to man. The aim of this paper was evaluate the survival capacity and the fate of *Escherichia coli* Enteroinvasive, Enteropathogenic and Enterohemorrhagic in pasty *dulce de leche*. Aliquots of this sweet were experimentally contaminated with these pathogenic microorganisms at 10^2 bacterial cells per gram, and later analyzed to evaluate microorganism count after storage for 0, 1, 2, 3, 5, 10, 20 and 30 days. EIEC could be recovered up to 20 days post inoculation in one of the reps, count EIEC on the third day reached 1,500 MPN per g. The strains of EPEC and EHEC, did not show growth, as EIEC, therefore, seem to be more sensitive to the adversities of the medium. The survival of pathogens for long periods in this food highlights the need for strict care in the manufacture and handling of *dulce de leche*.

Keywords: food pathogens, dairy products, food safety.

Resumo

O doce de leite é um alimento obtido por concentração do leite adicionado de sacarose. Este alimento pode ser contaminado por práticas inadequadas durante o processamento. Caso a manipulação não seja realizada de maneira higiênica o alimento pode ser veículo de bactérias patogênicas, como algumas cepas de *Escherichia coli*, para o homem. O objetivo desse trabalho foi avaliar a capacidade de sobrevivência e o comportamento de EIEC, EPEC e EHEC em doce de leite pastoso. Alíquotas deste doce foram experimentalmente contaminadas com esse micro-organismo patogênico na concentração 10^2 células por grama e posteriormente analisados para avaliar a contagem bacteriana após 0, 1, 2, 3, 5, 10, 20 e 30 dias de estocagem. EIEC pôde ser recuperada até 20 dias após a inoculação em duas das três repetições. Em uma das repetições, a contagem de EIEC no terceiro dia atingiu 1.500 NMP por g. As cepas de EPEC e EHEC, não apresentaram crescimento, como EIEC, portanto, parecerem ser mais sensíveis às adversidades do meio. A sobrevivência desses patógenos durante dias neste alimento evidencia a necessidade de rigorosos cuidados na fabricação e manuseio do doce de leite pastoso.

Palavras-chave: patógenos alimentares, produtos lácteos, segurança alimentar.

Introduction

Dulce de leche is the product obtained by concentrating milk and adding sucrose (Brasil, 1997). It is a sweet of wide acceptance in Latin America, where is common fractionating it into portions to be sold by retail. Due to the low water activity and high concentration of sugar, it is assumed that this food has barriers which reduce the likelihood of contamination and viability of pathogenic microorganisms. However, Hentges et al. (2010) showed that *Escherichia coli* O157: H7 is able to remain viable for up to five days in *dulce de leche* after contamination with the concentrations of 10^1 bacteria per gram, and up to 30 days after

contamination with the concentrations of 10^3 bacteria per gram of this food. This sweet can be contaminated by improper practices during the manufacturing process, distribution and storage, or in the consumer's home. If manipulation is not performed in a hygienic manner and with health care, the food can be a vehicle of pathogenic bacteria to man (Doyle e Buchanan, 2012).

E. coli belongs to the Family *Enterobacteriaceae*. The pathogenic strains from this specie are sub-classified in pathotypes, once the bacteria use different mechanisms of virulence (Kaper et al., 2004). *E. coli* from the pathotype Enteroinvasive (EIEC) invades the intestinal cells and multiplies inside of them. Also affects the adjacent cells causing colon ulceration, resulting in bloody

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diarrhea (Nataro e Kaper, 1998; Kaper et al., 2004). Most strains of EIEC have several biochemical characteristics that make them different from the others, among these is the inability to ferment lactose or ferment it late (Franco e Landgraf, 2003). *E. coli* Enteropathogenic (EPEC) is a causative agent of diarrhea in children and is more common in developing countries (Torres et al. 2005). It is capable, mediated by an adherent factor called EAF, to adhere to the intestinal cells and cause destruction of the microvilli (Franco e Landgraf, 2003). *E. coli* Enterohemorrhagic (EHEC) has as a virulence factor the production of cytotoxins, of the Shiga Toxin family, that can cause hemorrhagic colitis, hemolytic-uremic syndrome and death (Nataro e Kaper, 1998; Trabulsi et al., 2004)

The consequences of *E. coli* infection justify the importance of determining their ability survival and behavior in food, even in those who do not have the ideal conditions for its multiplication and development, how *dulce de leche* and ambrosia (Ferrasso et al., 2014). After experimental contamination of ambrosia, Ferrasso et al. (2014) concluded that EIEC, EPEC and EHEC showed ability to survive in ambrosia, representing potential danger to the health of consumers. The aim of this paper was evaluate the fate of EIEC, EPEC and EHEC in pasty *dulce de leche*.

Methodology

The *dulce de leche* was prepared from standardized milk containing 3% milk fat with the addition of 200g sucrose to each liter of milk. The ingredients were kept at 100-105°C and stirred for 2 hours and 30 minutes.

For the survival evaluation assays the strains of *E. coli* ATCC 43893 (EIEC) , Fiocruz 00182 (EPEC), and ATCC 43895 (EHEC) were used. The strains were recovered in Brain Heart Infusion broth (BHI, Acumedia, Lansing, Michigan) and incubated at 37°C for 24 hours. Then, from them and new cultures were performed for more 24 hours. Counting were made on standard Plate Count Agar (PCA, Acumedia, Michigan, USA) to establish the population obtained in these culture conditions. Serial dilutions, in saline solution 0.85% (SS), were prepared in order to obtain an inoculum with a concentration of approximately 10⁴ bacterial cells per ml.

Twenty-five gram of *dulce de leche* fractions were experimentally contaminated with 0.25mL of the inoculum from the serial dilutions, to obtain final concentrations of about 10² bacterial cells per gram of sweet. 25g of non-experimentally contaminated *dulce de leche* were used for negative control.

The samples were kept at a temperature of about 25°C, and analyzed after 0, 1, 2, 3, 5, 10, 20 and 30 storage days. The viability of *E. coli* was verified by the counting technique of Most Probable Number (MPN) as recommended by the US Food and Drug Administration. Following incubation, dilutions that exhibited gas formation were resuspended to test tubes with 10ml of Brilliant Green Bile Broth (VB, Acumedia, Lansing, Michigan) with Durham tubes, and incubated at 37°C for 48 hours. The tubes of each dilution were counted considering the gas formation and the results were obtained using a table to MPN. To count EIEC, was necessary an adaptation of this method that considers the presence of gas in the Durham tubes, once

EIEC does not ferments lactose, not forming gas. To confirm the presence of the micro-organism in the tubes, aliquots of the tubes were transferred to MacConkey agar plates (MC, Acumedia, Lansing, Michigan), when the visualization of bacterial growth was possible the tube were considered positive.

The experiment was performed in triplicate.

Results and discussion

EIEC could be recovered 20 days post inoculation in one of the three replicates, which indicates that this pathotype is able to remain viable for long periods in pasty *dulce de leche*. According Silveira et al. (2012), *Salmonella* Typhimurium and *Salmonella* Enteritidis also have the ability to remain viable for up to 20 days in *dulce de leche* initially infected with the same concentration used in this study. There was no growth in the control sample even when were transferred to MacConkey agar plates. Despite the low water activity and high concentration of sucrose, *dulce de leche* proved to be a suitable environment for the survival of pathogens.

In one of the reps, the EIEC count on the third day reached 1,500 MPN per g, after the *dulce de leche* was contaminated with average 125 bacterial cells per gram of sweet on zero-day, demonstrating not only the bacteria adaptation to sweet, but also their ability to multiply in this environment. The infective dose of EIEC is high, about 10⁶ micro-organisms to healthy humans, but there are reports of infections with less than 10 micro-organisms doses (Todd et al., 2008). The results of our study demonstrate that EIEC, contaminating *dulce de leche*, can reach populations capable of causing disease in humans.

Table 1: *E. coli* counts of *dulce de leche* experimentally contaminated along 30 days of storage*

Storage days	EIEC (SD)	EPEC (SD)	EHEC (SD)
0	1.99 ^A (0.31)	1.79 ^A (0.18)	1.53 ^A (0.73)
1	1.45 ^{AB} (0.13)	2.33 ^A (1.01)	1.37 ^A (0.78)
2	1.23 ^{AB} (0.71)	1.99 ^A (1.51)	2.03 ^A (1.33)
3	2.63 ^B (0.38)	1.37 ^A (1.63)	1.40 ^A (1.09)
5	1.65 ^B (0.25)	NR	0.76 ^A (0.57)
10	1.30 ^B (0.09)	NR	NR
20	0 ^a	NR	NR
30	NR ^b	NR	NR

* = expressed as log MPN per g (average of three assays)

SD = Standard Deviation

^{A,B} Average with different letters in the same column differed on the Tukey test (P <0.05)

^a Log 0 = Most Probable Number per gram (MPN) less than 1.

^b NR = *E. coli* was not recovered in any repetition

Values in bold = *E. coli* was not recovered from all repetitions.

Population decline of EPEC and EHEC was similar between them, however, different from EIEC (Table 1). The different behavior of the tested strains indicates that the adaptations to

the environment provided by the *dulce de leche* are different between EPEC/EHEC and EIEC. Hentges et al. (2010) evaluated the survival of *E. coli* O157: H7 in *dulce de leche* and found that even in the initial bacterial concentration of 10^1 cells per g of sweet, the bacteria can be isolated from this until 10 days after inoculation. In the present study, the bacterial strains studied survived for shorter periods, despite the slightly higher initial concentration of 10^2 cells per g. The strains of EPEC and EHEC, although showed no growth as EIEC, appear to be more sensitive to the adversities of the environment, cells remained viable in the food up to four and five days of storage, respectively. This fact, coupled with infective dose of EHEC, which can be as low as 10 bacterial cells (Food and Drug Administration, 2009), emphasizes

the importance to public health of investigation the behavior of these pathogens in pasty *dulce de leche*.

Conclusions

Escherichia coli Enteroinvasive, Enteropathogenic and Enterohemorrhagic are able to remain viable in the environment provided by the pasty *dulce de leche*, wherein EIEC can remain viable for 20 days in this sweet and increase its population in the first days after contamination. The survival of pathogens for long periods in this food highlights the need for strict care in the manufacture and handling of *dulce de leche*.

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