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Behavior of *Escherichia coli* in Crottin goat's cheese at fluctuating storage temperature

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Summary.

Behavior of *Escherichia coli* in Crottin goat's cheese at fluctuating storage temperature.

In this work wild strains of *E. coli* isolated from Crottin goat's cheese were analyzed to determine their behavior when the cold chain is broken. Cheese samples were inoculated with a mixing of two wild strains and independently with *E. coli* ATCC 25922. These were stored in sterile bags at 5 ºC for five days and then divided into three groups: *C*– control at 5 ºC for 47 days; *E*– kept for 8 h at 25 ºC and then cooled down to 5 ºC; *T*– kept along 24 h at 25 ºC and then cooled down to 5 ºC. *E. coli* ATCC 25922 remained steady in C; E treatment was similar to *C*. In *T* the population increased reaching 8.2 log cfu/g at 15 days of storage, finally decreasing. Wild strains of *E. coli* had similar behavior to the reference strain in *C*. The increase in E was higher than the reference one. In *T* two increase cycles were observed. The population grew rapidly after 24 h at 25 °C and remained unchanged for 9 days. Then they decreased to 5.8 log cfu/g and stayed at this level for 10 days. The return to 5 ºC might have produced a sublethal damage or a change to viable but non-culturable state. Later there was a new increase in the number of cells. Our results imply that cells of *E. coli* isolated from cheese showed a different behavior than *E. coli* ATCC 25922.

Key words:

Bacterial count. Cold storage. *Escherichia coli*. Fluctuating temperature. Goat cheese

Resumen

Comportamiento de *Escherichia coli* en queso de cabra Crottin bajo condiciones fluctuantes de la temperatura de almacenamiento.

En este trabajo se analizó el comportamiento de cepas de *E. coli* aisladas de queso de cabra Crottin cuando se rompe la cadena de frío. Se inocularon muestras de queso con una mezcla de dos cepas salvajes y, en forma independiente, con *E. coli* ATCC 25922. Se almacenaron en bolsas estériles a 5 ºC durante cinco días y se dividieron en tres grupos: *C*-control a 5 ºC durante 47 días, *E*-mantenidas 8 horas a 25 ºC y luego a 5 ºC; *T*-mantenidas 24 h a 25 ºC y luego a 5 ºC. *E. coli* ATCC 25922 se mantuvo estable en el tratamiento *C*. El tratamiento *E* fue similar. En *T* la población aumentó llegando a 8,2 log ufc / g, finalmente disminuyó. Las cepas salvajes de *E. coli* tuvieron un comportamiento similar a la cepa de referencia en *C*. El aumento de la población en *E* fue mayor que con la cepa referencia. En *T* se observaron dos ciclos de aumento. La población creció rápidamente después de 24 horas a 25 °C y se mantuvo durante 9 días. Luego se redujo a 5,8 log ufc / g, y se mantuvo en este nivel 10 días. El retorno a temperatura de refrigeración podría haber producido un daño subletal o un cambio al estado viable pero no cultivable-(VNC). Posteriormente hubo un nuevo incremento en el número de células. Nuestros resultados mostraron que las células de *E. coli* aisladas de queso y expuestas a 25 º C durante 24 h tienen un comportamiento diferente a *E. coli* ATCC 25922.

Palabras clave:

Almacenamiento en frío. *Escherichia coli*. Fluctuaciones en la temperatura. Queso de cabra. Recuento bacteriano. Seguridad alimenticia.

Introduction:

The last (not so few) decades have witnessed a continuous raise in consumers demand for high quality food products, free of antimicrobial compounds and processes. These products commonly rely on refrigerated storage and distribution for their preservation. Nonetheless the storage at refrigeration temperature can not always guarantee the desired safety and quality. Previous studies have shown that retailers are not familiar with the importance of maintaining the cold chain in food trade. The after effect can be a shorter durability of highly perishable foodstuffs and a

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questionable safety for consumers [19] Furthermore there is the risk of microbes inhabiting contact and environmental sites in a food process, which are capable of contaminating the product on first contact [15, 26]. Due to the stresses inherent to the environments where bacteria live this could add up to a surprising predisposition to survive and even multiply in food the moment they enter in contact with it. This in spite of the presence of several preservative barriers, such as low temperature, freezing conditions and other physical and chemical stresses [1, 3, 32]. To account for such proposition consider milk and milk products, which are regarded as potentially hazardous; even pasteurized products have been implicated in outbreaks. Different illnesses were associated with the consumption of cheese [2, 7, 10]. *Escherichia coli* was isolated from yoghurt and several kinds of cheeses [5, 11, 20, 21]. Rey et al. [24] confirmed that dairy products in Spain are an important reservoir of Shiga toxin-producing *E. coli*, pathogenic for humans. Altogether, spoilage or pathogen microorganisms can cause serious economical losses in the food industry. Even though both kinds of microorganisms are exposed to the same sort of stresses, spoilage organisms often outnumber food pathogens and tend to be more resistant to harsh environmental conditions [25].

In this work wild strains of *E. coli* isolated from Crottin goat's cheese were analyzed to determine their behavior in cheese during chill storage and when the cold chain is broken for different time intervals.

Material and methods

Bacterial strains: three strains of *E. coli* were used. One of these was used as reference (ATCC 25922); the other two were wild strains isolated at the end of the shelf life of Crottin goat cheese (*E coli* LAM 5 y 7). For the experimental inoculation the strains were individually grown in 50 ml of Tryptone Soya Broth (Britania, Argentina) at 25 ºC for 24 h. The two wild strains were mixed to make an inoculum containing equal quantity of cells of each strain.

Experimental design: using aseptic techniques cheese samples of 5 g each (composed of inner material and exposed surface) were placed in sterile bags (Whirl-Pack, Nasco, USA). Samples were taken from as many units as was necessary. Each sample was inoculated with *E coli* ATCC 25922 (reference) or with the mixing of *E coli* LAM 5 y 7. Aliquots of the inoculum's dilution of each (reference strain or mixing) were spread over the surface of each individual cheese sample to obtain a cell concentration of approximately 10^5 cfu/g of cheese.

The inoculated samples were stored at 5 ºC for 5 days and were later divided into three groups, each with its own treatment: *C*- control at 5 ºC for a maximum period of 47 days; *E*- kept along 8 h at 25 ºC and then taken to 5 ºC until the end of the experiment; *T*- kept along 24 h at 25 ºC and then taken to 5 ºC until the end of the experiment. Numbers of *E. coli* were determined by 5 g of homogenized cheese in 45 ml of sterile 0,1 % peptone water with 0,1 % Tween 80. Appropriate dilutions were pour plated on Violet Red Bile Agar and incubated at 35-37 ºC for 24 h. Determinations were made in duplicate. Non inoculated samples were analyzed for *E. coli* [8].

Data analysis: for the data analysis the count of colony forming units (cfu) was transformed to log cfu/g. The behavior of the strains with time was drawn using Origin 6.1 (OriginLab Corp.)

Results

E. coli ATCC 25922 (reference): the growth/survival kinetic of reference strain is shown in Fig.1. In the control treatment (5ºC), the inoculated population remained stable throughout the storage period. In treatment *E* there was a slight increase (0.2 log cfu/g) after exposing the population to 25 ºC for 8 h, then stabilized at similar densities to those of the initial inoculation showing no substantial differences with *C*. In treatment *T*, where the product was exposed for 24 h to 25 °C, the population increased progressively reaching 8.2 log cfu/g after 15 days of storage, then decreased to levels similar to those of inoculation which persisted until the end of the experiment.

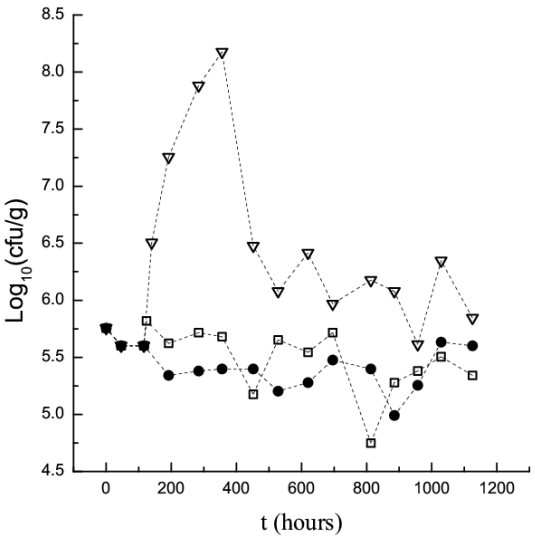

Fig.1
Effect of break in the cold chain for *E. coli* 25922. Control treatment (black circle); break during 8 h at 25 ºC (square), break during 24 h at 25 ºC (triangle). Dashed lines are only to guide the eye.

E. coli LAM 5 y 7 (wild strains): the behavior of wild strains is shown in Fig.2. In the control treatment the population remained stable during all

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47 days of storage at 5 ºC. In treatment *E* the population increased 0.67 log cfu/g (approximately 3 times more than with *E. coli* ATCC 25922) after exposure to 25 ºC for 8 h, and declined later in day 15 to inoculation levels for the remainder of the experiment. In treatment *T* two increase cycles in the number of viable cells were observed. First, the population increased rapidly by 2.4 log cfu/g in 24 h, reaching approximately 7.4 log cfu/g and then remaining steady for 9 days. Afterward there was a decrease in the number of viable cells to approximately 5.8 log cfu/g, remaining at these levels for 10 days. Finally there was a late increase in the number of viable cells, but the reached density peak was lower than the former (approximately 6.8 log cfu/g).

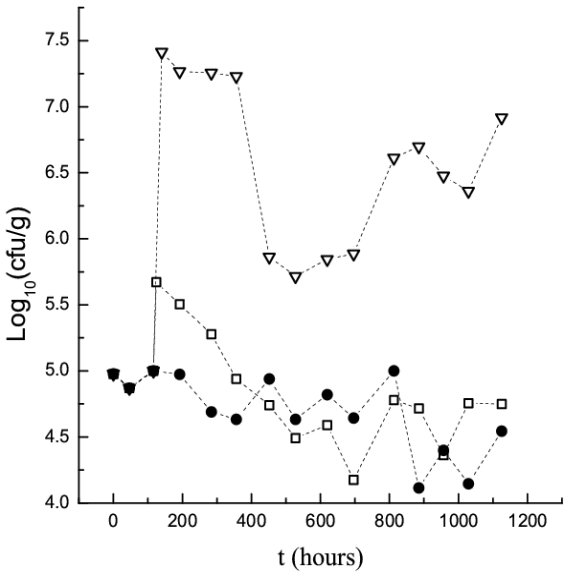

Fig.2
Effect of break in the cold chain for *E. coli* LAM 5 y 7 (wild strains). Control treatment (black circle); break during 8 h at 25 ºC (square), break during 24 h at 25 ºC (triangle). Dashed lines are only to guide the eye.

Discussion

The operations that are applied in food processing offer several opportunities for the settling of contaminating microorganisms**,** sometimes leading to organoleptic defects. The types of microorganisms (spoilage and/or pathogens) and their effects depend on intrinsic and extrinsic factors of the food they colonize. Epidemiological data shows that food implicated in outbreaks of foodborne diseases have often been handled incorrectly or improperly stored. Non-sterile refrigerated foods could change over time by the growth of certain microorganisms Therefore, without optimal working and storage temperatures throughout the entire production and transportation processes, the alteration thereof could be massive. Food processing methods can leave some of the surviving microorganisms in a damaged or injured condition. After a sublethal treatment, such as refrigeration, different populations of microorganisms are likely to include viable, dormant and dead cells. Dormancy is defined as a reversible state of metabolic shutdown, which reflects an absence of activity. It consists of cells that have ceased growth due to injury and of viable but non-culturable cells. Cells of a population under a particular kind of injury don't necessarily respond identically to the same injury**,** nor each type of injury produces identical damage on them [12, 17]. Cells struggling to survive in a changing environment must maintain homeostasis in a variety of vital parameters such as pH or internal fluidity of their membranes. The ability of a cell to maintain homeostasis may determine their success in colonizing food [9].

Temperature is a cardinal factor controlling the rate of microbial growth. Risk of pathogens as a result of the exposure of chilled foods to abusive temperature may differ between food previously stored at ≤ 2 ºC and food stored above 2 ºC [14]. On the light of this facts consider that contamination of milk and milk products may occur after pasteurization, as minor faults in the process are always expected to some extent [16]. Evidence abounds: dairy products are considered an important reservoir of Shiga toxin-producing *E. coli* [24]. Vernozy-Rozand et al. [31] indicate that *E. coli* O157:H7 survives the lactic goat cheese manufacturing process. *E. coli* was isolated from Port Salut Argentine cheese kept for 10 days at refrigeration temperature (4ºC) and also after a temperature abuse of 20ºC. This clearly suggests poor manufacturing practices [11]. Lekkas et al. [18] have shown that these bacteria survived in Galotyri cheese at 4 and 12 ºC. In soft mold-ripened cheeses a rise in pH due to the activity of the mold can allow the multiplication of bacteria, including pathogens, to very high numbers.

Control treatment, cheese storage at 5 ºC: the behavior of *E. coli* ATCC 25922 and wild strains inoculated into cheese and stored at 5 ºC showed no substantial differences, both survived but didn't grow throughout the storage period. In previous experiments performed with *E. coli* LAM 5 and 7 we analyzed their behavior in culture medium at 5 ºC by measurement of OD. There was a lag phase which lasted nearly 35 h**;** after that the population increased with a value of $\mu = 0.009\ h^{-1}$. These results could be attributed to an augmented cell size and growth due to the absence of adverse conditions**,** giving as a result a slight increase in OD values [29]. This is supported by the work of Jones et al. [13] who found that in cultures of *E. coli* incubated at 7 ºC, the cell's optical absorbance and length increased.

It should be taken into consideration that the population analyzed by inoculating cheese corresponded to viable cells in a food matrix with a competing flora and non-optimal initial pH values.

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Such conditions are likely to restrict the strain reproduction. Similar behavior was observed by Sims et al. [27] with strains of *E. coli* that survived but failed to grow during storage at 7 ºC into cottage cheese varieties.

Treatments with 8 and 24 h of temperature abuse: when the temperature abuse (25 ºC) was maintained for 8 h there was a boosted increase in the population of viable cells in the samples inoculated with wild strains, which was somewhat larger (almost 3 times more) than that of *E. coli* ATCC 25922. The latter only increased significantly in number after exposure to 25 ºC for 24 h. Upon returning to the cold storage the population declined gradually over time to the same levels of viable cells observed at the time of inoculation. The population of viable cells of wild strains subjected to treatment *T* reached its maximum after 24 h at 25 ºC. After that the return to refrigerator temperature would have produced a sublethal damage or a change in cellular status of the population to viable non-culturable cells (VNC). The switch to the VNC stage has been described and documented for several bacterial species, including *E. coli* [4, 22, 23]. Viable but non-culturable stage is reversible and can be viewed as an example of a programmed mechanism for survival in environmental conditions not suitable for cell division. Foodborne pathogens in nutritionally rich media can enter into non-culturable state when they are exposed to refrigeration temperatures. When they do, cells could take several days to recover or resuscitate [6]. Tashiro et al., [30] have recently demonstrated the induction of ReIE-mediated dormancy by high *E. coli* cell density. In that work a population-based dormancy mechanism is proposed to help explain *E. coli* survival in stressful environment.

While the physiologycal, biochemical and genetic mechanisms of many of the stress responses have not yet been delineated, it is clear that when exposed to mild doses of stress microorganisms may adapt to it, thus developing tolerance or resistance to stronger doses of that stress [3]. Our results show that when the population of viable cells of *E. coli* LAM 5 and 7 were exposed to 25 ºC for 24 h, they showed a different behavior than *E coli* ATCC 25092 even after the return to 5 ºC, regardless of the cellular processes that allowed us to observe the fluctuations. As to the fluctuations, the wild strains**,** contrary to the *E. coli ATCC* 25922 maintained a high cellular density even after returning to the refrigerator.

General observations: microbiological tests on cheeses have an important place in quality control, but these tests can not ensure the microbiological safety of the product. Contamination of refrigerated foods would cause great damage, especially if strains are adapted to the dairy farm [28]. Altogether, when microorganisms undergo sublethal injuries some cellular changes may occur. If optimal temperatures during storage and transportation of chilled foods are not ensured, its microbial alteration can be greatly accelerated. There is a need for both producers and consumers to implement not only Good Manufacturing Practices**,** but also to comply with the transport and distribution requirements of various food products. Thus, an understanding of how *E. coli* adapts to changes is of major importance in food industry, where prevention of bacterial contamination is imperative.

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