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Original article

Inactivation of two strains of *Escherichia coli* inoculated into whole and skim milk by ultrahigh-pressure homogenisation

Wilfido José BRIÑEZ, Artur X. ROIG-SAGUÉS*,
M. Manuela HERNÁNDEZ-HERRERO, Buenaventura GUAMIS-LÓPEZ

Centre Especial de Recerca-Planta de Tecnologia dels Aliments (CERPTA), XiT, Departament de Ciencia Animal i dels Aliments, Facultat de Veterinària, edifici V, Universitat Autònoma de Barcelona, 08193, Bellaterra (Barcelona), Spain

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Abstract – The inactivation by ultrahigh-pressure homogenisation (UHPH) of *Escherichia coli* ATCC 10536 and *Escherichia coli* O157:H7 CCUG 44857 inoculated into whole and skim milk was investigated. Samples of UHT whole and skim milk inoculated at a concentration of approximately 7.0 Log_{10} (cfu·mL^{-1}) were pressurised in a two-valve system UHPH machine at 300 MPa at the primary homogenising valve and at 30 MPa at the secondary valve. Inlet temperatures of milk were 6 ºC and 20 ºC. Viable and injured bacterial counts were measured 2 h after UHPH treatment and after 3, 6 and 9 days of storage at 4 ºC. The type of milk significantly influenced ($P < 0.05$) the degree of inactivation reached in both strains of *E. coli,* being higher at 20 ºC in whole milk. The level of inactivation was similar for *E. coli* ATCC 10536 and *E. coli* O157:H7 CCUG 44857, reaching lethality values of 4.30 and 3.94 Log_{10} cfu·mL^{-1}, respectively, at an inlet temperature of 20 ºC. No sublethal injuries were detected after treatments. The changes in cultivable cells during storage at 4 ºC were similar in whole and skim milk although the *E. coli* O157:H7 CCUG 44857 strain showed significant differences, with a decreasing tendency of approximately 0.3 logarithmic units between 0 and 9 days of storage.

ultrahigh-pressure homogenisation / lethality / milk / *Escherichia coli* O157:H7 / sublethal injury

摘要 – 超高压均质对接种在全脂和脱脂牛乳中两株大肠杆菌 灭活效果的研究。本文研究了超高压均质技术对接种到全乳和脱脂乳中的大肠杆菌 ATCC10536 和大肠杆菌 O157:H7 CCUG44857 的灭活效果。两株大肠杆菌接种到经超高温处理过的全脂和脱脂牛乳样品中，接种量约为 1.0×10^7 cfu·mL^{-1}，利用一个双阀系统的超高压均质机对样品加压处理，第一个均质阀的压力为 300 MPa，第二个均质阀的压力为 30 MPa。控制入口的牛乳温度为 6 ºC 和 20 °C。经超高压处理后的牛乳在 2h 内测定活菌数和受损菌数，并将处理后的样品在 4 °C 贮存，分别在 3， 6 和 9d 测定贮存样品的活菌数和受损菌数。牛乳的类型对两株大肠杆菌的灭活效果均有明显的影响 ($P < 0.05$)，在入口温度为 20 °C 的全脂乳中两株大肠杆菌的灭活效果均较高。对于入口温度为20 °C的样品，经超高压均质后样品中大肠杆菌ATCC 10536 和大肠杆菌 O157:H7 CCUG 44857 的灭活程度相似，致死率分别为 2.0×10^4 和 8.7×10^3 (cfu·mL^{-1})。经超高压均质处理后没有检测出亚致死性损伤的大肠杆菌。在 4 ºC 下贮藏期间内，全脂和脱脂牛乳中培养细胞的整体变化情况数基本相同，只是 *E. coli* O157:H7 CCUG 44857 在 0 ～ 9d 的贮藏期内菌数呈现出下降的趋势，约有 0.3 对数单位的减少。

超高压均质 / 致死率 / 牛乳 / 大肠杆菌 O157:H7 / 亚致死性损伤

* Corresponding author (通讯作者): arturxavier.roig@uab.es

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Résumé – Inactivation par homogénéisation à très haute pression de deux souches d'*Escherichia coli* inoculées au lait entier ou écrémé. L'inactivation par homogénéisation à très haute pression (UHPH) d'*Escherichia coli* ATCC 10536 et d'*Escherichia coli* O157:H7 CCUG 44857 inoculées au lait entier et au lait écrémé a été étudiée. Des échantillons de lait entier ou écrémé UHT ont été inoculés avec des souches à une concentration approximative de 7,0 Log_{10} (cfu·mL^{-1}) et ont ensuite été mis sous pression dans une machine de UHPH composée de deux pistons, le premier avec un système de soupape homogénéisant à 300 MPa, le second avec une soupape à 30 MPa. Les températures initiales du lait étaient de 6 ºC et 20 ºC. Le comptage des microorganismes vivants et ceux ayant subi des dommages a été fait 2 heures après le traitement UHPH et au 3e, 6e, et 9e jours de conservation à 4 ºC. La nature du lait a influencé significativement ($P < 0,05$) le degré d'inactivation atteint pour les deux souches d' *E. coli*, et de manière plus importante dans le lait entier pour une température de 20 ºC. Le niveau d'inactivation était similaire pour *E. coli* ATCC 10536 et *E. coli* O157:H7 CCUG 44857, atteignant respectivement les valeurs létales de 4,30 et 3,94 Log_{10} cfu·mL^{-1} à une température initiale de 20 ºC. Aucun dommage subletal n'a été détecté sur les souches après les traitements. L'évolution des microorganismes vivants durant la conservation à 4 ºC était similaire dans le lait entier et le lait écrémé. La souche *E. coli* O157:H7 CCUG 44857 a montré une tendance à la baisse de 0,3 unités logarithmiques et des différences significatives entre le 1er jour et le 9e jour de conservation.

homogénéisation à très haute pression / inactivation / lait / *Escherichia coli* O157:H7 / blessure sublétale

1. INTRODUCTION

The nutritional attributes of milk which make it an important part of the human diet are the same components that support the growth of many pathogenic bacteria associated with milk and dairy products [25]. A broad spectrum of microbial pathogens can contaminate human food and cause illnesses when they or their toxins are consumed [21]. Contaminated milk and dairy products have been associated with foodborne outbreaks caused by *Salmonella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 [1, 11, 19, 25]. Consumption of raw milk contaminated with *Escherichia coli* O157:H7 is probably the most likely source of infection in diverse outbreaks which have occurred during the last decade [15, 18, 19].

Heat treatment is the most commonly chosen preservation method for milk and other perishable liquid foods. Thermal processing has a long tradition in food preservation because it is economical and efficient at achieving microbial inactivation, but it cannot be used to treat heat-labile compounds. Furthermore, high temperatures may lead to undesirable effects in milk such as off-flavours, nonenzymatic browning and denaturation of certain vitamins and proteins [5, 7, 23].

The growing trend for fresher, high-quality convenience food has generated an increasing interest in nonthermal processing alternatives, such as high-pressure technologies, which are considered to be the most promising emerging food-processing technologies due to recent advances in high-pressure machinery and the successful introduction of pressure-processed foods [5, 7, 8, 12, 14]. Ultrahigh-pressure homogenisation UHPH (also called dynamic high pressure in the literature) is based on the same design principles as conventional homogenisation processes that are used in the dairy industry for reducing the size of fat globules [10, 22, 23], but working at significantly higher pressures (> 200 MPa), resulting in the destruction of large quantities of microorganisms. Consequently, this technology appears to be an important means of lowering the initial microbial load while helping to minimise product damage from unnecessary heat stress [17]. The effects of UHPH on bacterial cells are not yet well known, but some studies on UHPH have shown changes in bacterial cell morphology, as well as splits in the cytoplasmic membrane. Sudden increases in permeability or rupture of the cell membrane, such as may occur under pressure, cause cell death [9, 12, 13, 23]. Vannini et al. [24] and Vachon et al. [23] reported that UHPH treatments induced cell damage whose

severity increased with pressure level and number of cycles to which the sample was submitted. The damage observed was more severe on bacterial cells, including discharges of cytoplasmic content when pressure increased. Information concerning the effect of the matrix on the degree of inactivation showed by the microorganisms is scarce. The available results seem to indicate that UHPH treatments were more effective against microorganisms in saline buffered solutions than in more complex matrices such as milk [7, 23].

The main objective of this work was to study the inactivation of two different strains of *Escherichia coli*: *E. coli* O59:H21 (ATCC 10536) and *E. coli* O157:H7 (CCUG 44857), inoculated into whole and skim milk using a new-generation UHPH machine with two intensifiers and a double-valve system. We also studied the effect of inlet temperature of the milk, type of food matrix and the strain kind on the inactivation rate, the capacity of UHPH treatments to produce sublethal injuries, and the ability of the microorganisms to repair and grow during further refrigerated storage.

2. MATERIALS AND METHODS

2.1. Bacterial strains used and growing conditions

Escherichia coli O59:H21 ATCC 10536 and *Escherichia coli* O157:H7 CCUG 44857 were obtained as freeze-dried cultures in thermosealed vials from the Spanish Type Culture Collection (University of Valencia, Valencia, Spain). The freeze-dried cultures were rehydrated in tryptone soy broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37 ºC for 18 h. Subsequently, these broths were used to inoculate tryptone soy agar plate (Oxoid), and individual colonies were collected to prepare cryobeads (Nalgene® System 100™ Laboratories Microkit Iberica S.L., Madrid, Spain) of each strain. They were then kept at –20 ºC to provide stock cultures for the assays.

2.2. Preparation of cell suspension and inoculation of whole and skim milk

Prior to each experiment one cryobead was inoculated into 10 mL of tryptone soy broth (Oxoid) and incubated at 37 ºC for 20 h. After incubation, the broth was spread using a disposable loop on tryptone soy agar slant (Oxoid) incubated at 37 ºC for 20–24 h. Subsequently, cell suspensions were prepared in 11 mL tryptone sodium chloride solution (TSC) (1 $g \cdot L^{-1}$ tryptone pancreatic casein digestion and 8.5 $g \cdot L^{-1}$ sodium chloride) in order to obtain 9.0 Log_{10} $cfu \cdot mL^{-1}$ to 9.5 Log_{10} $cfu \cdot mL^{-1}$. Thereafter, 1 mL of cell suspension was used to determine the concentration by means of optical density at 405 nm (405 OD_{405}) using a spectrophotometer (Cecil 9000 series, Cecil instruments, Cambridge, England). Later, 10 mL of this cell suspension was inoculated into one L of UHT whole and skim milk at room temperature. The final concentration of cells in milk was 7.0 Log_{10} $cfu \cdot mL^{-1}$ to 7.5 Log_{10} $cfu \cdot mL^{-1}$. The inoculated milk samples were placed for 70 min at 6 and 20 ºC in a water bath to reach pressurisation temperature.

2.3. Ultrahigh-pressure homogenisation treatment of the milk

UHPH treatments were applied to the milk samples using a Stansted high-pressure homogeniser (model/DRG FPG7400H:350, Stansted Fluid Power Ltd, Essex, UK). This high-pressure machine comprises two intensifiers (80 mL useful volume), driven by a hydraulic pump and a high-pressure valve made of resistant ceramics able to support 350 MPa. It also comprises a second pneumatic valve able to support 50 MPa located behind the first one. All these components guarantee a constant flow rate (18 $L \cdot h^{-1}$) during the process. To avoid poor homogenisation performance due to temperature increase and rapid expansions or contractions in the first stage valve, the latter is cooled by constant circulation of water at room temperature in an external jacket built around the valve. Milk was subjected to a single cycle at UHPH of

300 MPa at the primary homogenising valve and 30 MPa at the secondary valve (300 + 30 MPa) at inlet temperatures of 6 and 20 ºC. For the experiment 2.0 L of each type of inoculated milk were used, with the majority of this volume being processed through the homogeniser to ensure temperature equilibration. Afterwards, between 80 and 100 mL of the samples were taken for analysis. The homogenised samples reached an outlet temperature of 16.0 to 18.0 ºC by means of: (i) an external jacket built around the pipeline located between the first and second homogenising valves and (ii) a spiral-type heat-exchanger (BCI/2843 type, Occo Cooller Ltd, Telford, UK) located behind the second valve. The samples were immediately stored at 4 ºC.

A specific sanitation programme developed for the UHPH machine was applied each time immediately after submitting the milk samples to UHPH treatment. A mixture of peracetic acid and hydrogen peroxide (P3-Oxonia Active, Ecolab Hispanic Portuguese, Barcelona, Spain) was used as a disinfectant agent, whose bactericidal efficacy was previously evaluated using a quantitative test of suspension [2]. We also carried out several experiments to adjust the conditions of cleaning and disinfection in the UHPH machine. The sanitation sequence of the machine was applied according to methodology developed by Briñez et al. [3].

2.4. Microbiological analysis

To determine the initial number of cells in the inoculated samples of milk at 6 and 20 ºC, 1 mL of each sample was used to prepare decimal dilutions in peptone water (Oxoid, 10 $g \cdot L^{-1}$ peptone and 5 $g \cdot L^{-1}$ NaCl). Subsequently, 1 mL of these dilutions was placed in duplicate in tryptone soy agar plate (Oxoid) supplemented with 6 $g \cdot L^{-1}$ yeast extract (Oxoid, TSAYE) and incubated at 37 ºC for 48 h. Also, 20 mL of the untreated inoculated samples (controls) were placed into sterile tubes and stored at 4 ºC.

The first microbiological analysis of treated and control samples was performed 2 h after the UHPH treatment. Afterwards samples were kept at 4 ºC and analysed after 3, 6 and 9 days of storage. To assess the lethality and the level of injuries caused by UHPH treatment, decimal dilutions in peptone water (Oxoid) of untreated and treated milk samples were prepared and plated in duplicate in TSAYE, and TSAYE supplemented with 20 $g \cdot L^{-1}$ of NaCl (Panreac, Moncada i Reixac, Barcelona, Spain; TSAYE+NaCl) and incubated at 37 ºC for 48 h. The use of this differential plating technique enables injuries to be monitored. Both noninjured and injured cells were able to form colonies on TSAYE, whereas only noninjured cells formed colonies in the presence of NaCl [16]. Results were expressed as the logarithm of $cfu \cdot mL^{-1}$. Lethality was calculated as the difference between the logarithms of colony counts of the untreated and treated samples ($Log_{10} N_0 – Log_{10} N$).

2.5. Statistical treatment of data

All experiments were repeated four times with duplicate analysis in each replicate. Data are presented as least square means of each experiment. Analysis of variance was performed using the General Lineal Models Procedure (GLM) of SAS® System [20]. Evaluation was based at a level of significance of $P < 0.05$.

3. RESULTS AND DISCUSSION

UHPH treatments applied at 300 + 30 MPa in a single cycle at inlet temperatures of 6 ºC and 20 ºC were able to reduce significantly the counts of *E. coli* ATCC 10536 and *E. coli* O157:H7 CCUG 44857. No statistical differences between strains were found when the treatment was applied in the same matrix and at the same inlet temperature (Fig. 1). Either the inlet temperature of the sample or the type of food matrix significantly influenced ($P < 0.05$) the degree of inactivation reached. The highest lethality values were observed for the strain *E. coli* ATCC 10536 inoculated into whole milk at an inlet temperature of 20 ºC (4.30 Log_{10} $cfu \cdot mL^{-1}$). The inoculated samples of skim milk treated at 6 ºC showed

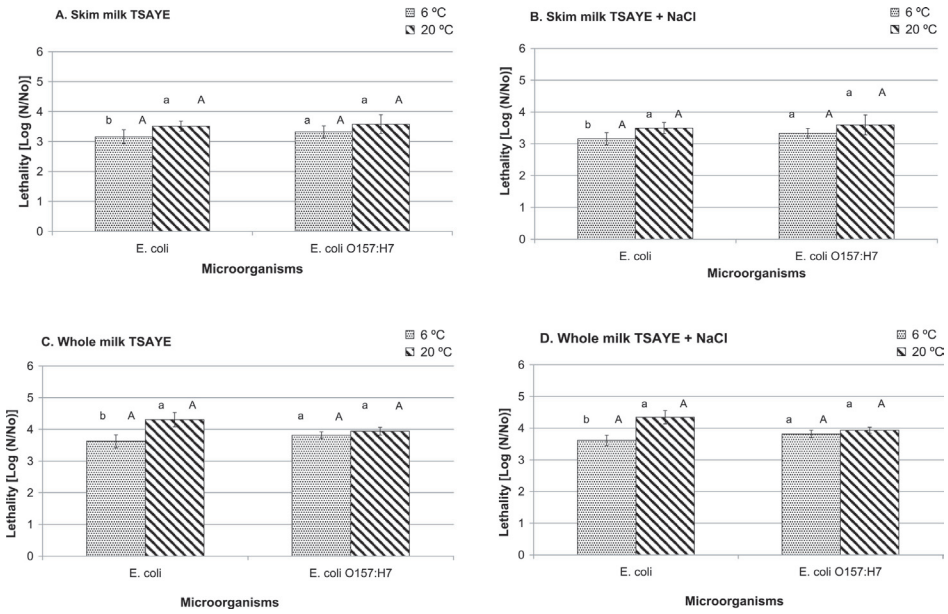

Figure 1. Inactivation of *Escherichia coli* (ATCC 10536) and *Escherichia coli* O157:H7 (CCUG 44857), in skim (A, B) and whole milk (C, D) treated with ultrahigh-pressure homogenisation (300 + 30 MPa) using inlet temperatures of 6 and 20 ºC. Survivors were enumerated using tryptone soy agar with yeast extract (TSAYE) and TSAYE with 20 g of salt added per litre (TSAYE + NaCl) with incubation at 37 ºC for 48 h. Bars with different superscript small letters are significantly different ($P \leq 0.05$) for the same microorganism. Bars with different superscript capital letters are significantly different ($P \leq 0.05$) for the same inlet temperature. Data are presented as the mean values of four replications ± confidence intervals.

the lowest values of lethality for *E. coli* ATCC 10536 and *E. coli* O157:H7 CCUG 44857 (3.15 and 3.32 Log_{10} cfu·mL^{-1}, respectively).

Diels et al. [7] also reported reductions of around 3.5 Log_{10} cycles in *E. coli* (strain MG1655) when inoculated into skim milk pressurised at 300 MPa in one single cycle, but using a higher inlet temperature (25 ºC). Inlet temperature may influence the maximum temperature reached during the process, and consequently, may affect the lethality reached. In our work the lethality increased by 0.13–0.70 Log_{10} cycles depending on the matrix type when the inlet temperature increased from 6 ºC to 20 ºC. However, these lethalities are still far away from the ones described by Vachon et al. [23] who reported reductions of approximately 8 Log_{10} cycles in cultivable cells in another strain of *E. coli* (*E. coli* O157:H7 ATCC 35150) when inoculated into whole raw milk pressurised at 300 MPa with an inlet temperature of 25 ºC, using the same model of UHPH machine employed by Diels et al. [7] (Emusiflex C5, Avestin, Ottowa, Canada). They also reported that increasing the milk temperature from 55 ºC to 60 ºC brought an additional reduction of 1.2 logarithmic units in cultivable cells of *E. coli* O157:H7. This seems to indicate that the type of strain used (*E. coli* MG1655 or *E. coli* O157:H7 ATCC 35150) and pressurisation conditions (control of the rise in temperature during and after the pressurisation) may influence the results, although in our study, we did not detect significant differences between the two strains of *E. coli.* An inlet temperature of 6 ºC would be interesting for the milk industry since milk could

be treated just after being unloaded from the containers without breaking out of the refrigeration conditions. However, 3 Log_{10} units of reduction could be not sufficient, depending on the microbiological quality of the raw milk.

The other factor that greatly influences the efficacy of the treatments is the kind of matrix. If we compare the reductions shown by Vachon et al. [23] with the ones reported by Diels et al. [7] at the same pressure and inlet temperature (300 MPa, 25 ºC), a clear difference between skim milk and whole milk is observed in favour of whole milk; results that agree with ours, although they used slightly higher temperatures (25 ºC). Moreover, Vachon et al. [23] also observed lower lethality values for *E. coli* O157:H7 when treated (300 MPa at 25 ºC of inlet temperature and one pass) in phosphate-buffered saline (6 Log_{10} cfu·mL^{-1} approximately) than when it was inoculated into raw milk (8.5 Log_{10} cfu·mL^{-1}). Similar results were previously reported by Gervilla et al. (2000) [8] in milk with different percentages of fat (0, 6 and 50%), but treated with high hydrostatic pressure (400 MPa, 15 minutes). It seems that fat increases the piezosensibility of the microorganisms, in contrast with heat treatments where fat is assumed to have a protective effect. These researchers suggested that the cause might be the increase in the concentration of certain liposoluble substances with an antimicrobial effect caused by the increase in the fat content, which causes interchanging of triglycerides of milk with lipoproteins of the cellular membrane, altering the permeability of microorganisms. However, more recently Diels et al. [6, 7] identified fluid viscosity as a major environmental parameter affecting bacterial inactivation by UHPH.

No significant differences were observed in the lethality values of either strain between TSAYE and TASAYE + NaCl media within the same food matrix and inlet temperature, indicating that the UHPH treatment caused no sublethal injuries. This was previously observed for other microorganisms such as *Yersinia enterocolitica*, *Staphylococcus aureus* and *Salmonella enterica* serovar *typhimurium* [26, 27]. Wuytack et al. [27] compared five different treatments, reporting that high levels of sublethal injuries were observed for high hydrostatic pressure and heat treatments compared with treatment with pulsed white light, pulsed electric field and UHPH, which showed very low levels or even no sublethal injury. In a previous investigation by our group [3] we observed that UHPH treatment caused few or no sublethal injuries in *Listeria innocua* inoculated into whole milk and orange juice. This is the point that clearly makes the effect of this technology different from high hydrostatic pressure, which can cause accumulation of sublethal injuries, leading to subsequent recovery of the cells, depending on the conditions of treatment and later storage [4].

The evolution of cultivable cells of *E. coli* O157:H7 (CCUG 4485) during the later storage at 4 ºC was similar in all samples, showing a slight but significant ($P < 0.05$) decrease of approximately 0.3 logarithmic units between days 0 and 9 of storage (Fig. 2). However, in the case of the strain ATCC 10536 their counts did not significantly differ during the whole storage (Fig. 3).

The low temperature of storage did not exert any significant effect on the behaviour of cultivable cells of *E. coli* O157:H7 in the treated samples, showing a similar evolution to the control samples (Fig. 2). No other references were found to the evolution of *E. coli* treated by UHPH and stored at low temperatures. Vannini et al. [24] reported an increment in the cultivable cells of *E. coli* of approximately 3.5 logarithmical units in skim milk after being treated at 130 MPa, but after 33 hours of incubation at 37 ºC. In our work, the changes in cultivable cells remained steady or, in the case of the *E. coli* O157:H7 CCUG 44857 strain, showed a slightly decreasing tendency after 9 days of storage at 4 ºC although the physical-chemical characteristics of milk (pH of 6.7 close to neutrality and a large presence of nutrients) are very favourable to this microorganism. De Lamo-Castellví et al. [4] reported that various strains of *Yersinia enterocolitica* were able to increase their counts in skim milk by about 8 Log_{10} cycles after 15 days of storage at 8 ºC after being submitted to a high hydrostatic-pressure treatment of 500 MPa that apparently

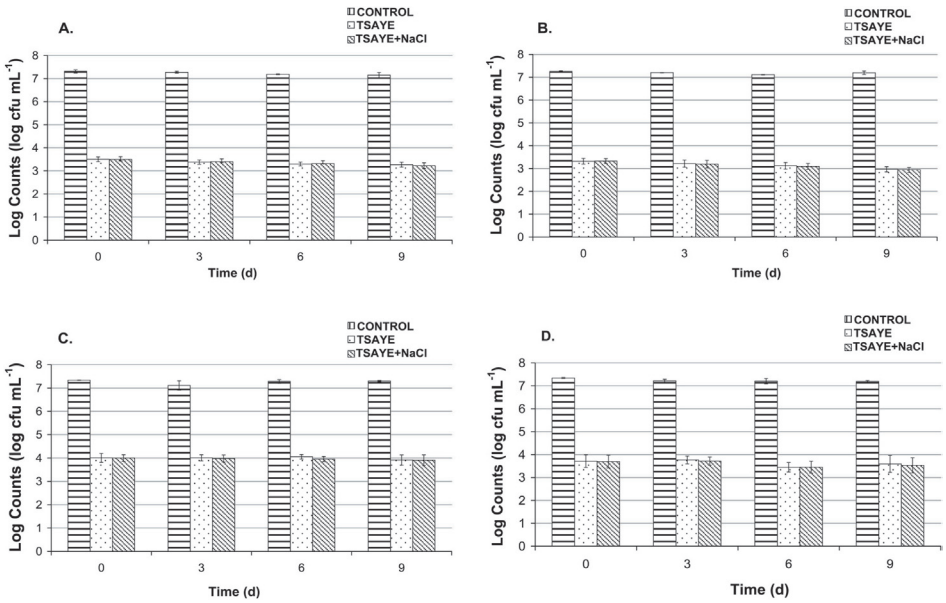

Figure 2. Changes in cultivable cells of *Escherichia coli* O157:H7 (CCUG 44857), in whole and skim milk pressurised at 300 + 30 MPa and stored at 4 ºC. (A) Whole milk pressurised at inlet temperature of 6 ºC. (B) Whole milk pressurised at inlet temperature of 20 ºC. (C) Skim milk pressurised at inlet temperature of 6 ºC. (D) Skim milk pressurised at inlet temperature of 20 ºC. The data are presented as the mean value of four replications ± 95% confidence interval.

caused its complete inactivation. *E. coli* is not a psychrotrophic bacterium and an increase in its count in milk at 4 ºC is not expected, but we could have expected a greater sensitivity of surviving cells to unfavourable growing conditions, such as low temperature. We also observed in studies with another strains that *Staphylococcus aureus* significantly decreased their counts during cheese ripening after submitting the inoculated milk to a similar UHPH treatment prior to cheese elaboration; meanwhile, in cheeses elaborated from untreated milk their counts remained steady during the ripening period (data not shown). This indicated that although surviving cells can grow in culture media, they showed a greater sensibility to unfavourable environment conditions (low pH and salt) than cells from the untreated samples. If the cells that survive UHPH treatment are not injured, as was previously discussed, it can be supposed that they will not be more sensitive to unfavourable growing conditions, such as low temperature. In our trials, no significant differences were observed in cultivable cells between TSAYE and TASAYE + NaCl at any day of storage for samples at the same conditions and no differences were detected in the evolution of either strain between the treated ones and the controls (Figs. 2 and 3), confirming that UHPH treatments did not increase the sensitivity of the cell to low temperatures of storage.

4. CONCLUSION

The results indicate than even low inlet temperatures (6 and 20 ºC) affect the lethality values in UHPH treatments only for the *Escherichia coli* ATCC 10536 strain. These temperatures were efficient at reducing the cultivable cells in whole and skim milk treated at 300 + 30 MPa, which is interesting since pre-heating milk implies an increase in the treatment costs. Pre-heating

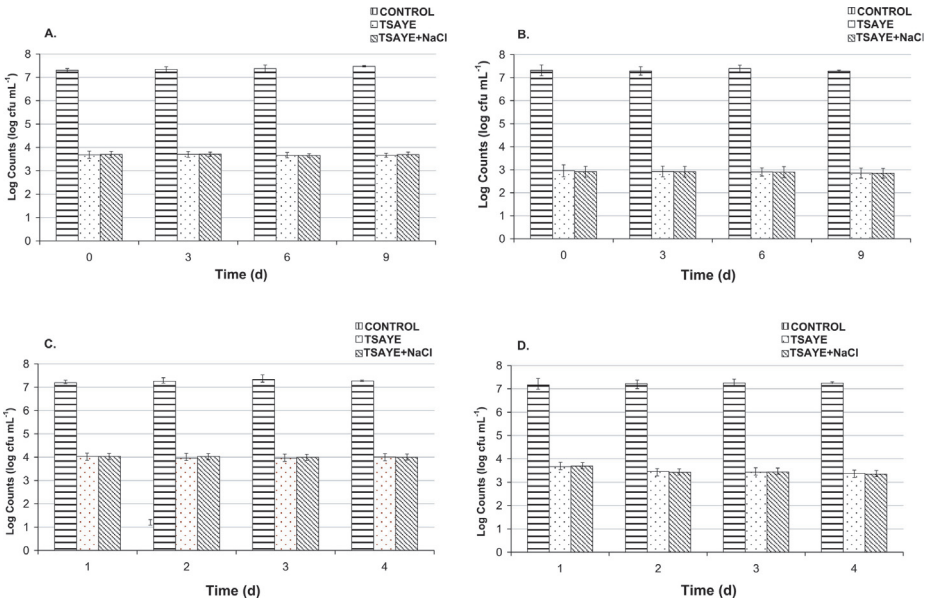

Figure 3. Changes in cultivable cells of *Escherichia coli* (ATCC 10536), in whole and skim milk pressurised at 300 + 30 MPa and stored at 4 ºC. (A) Whole milk pressurised at inlet temperature of 6 ºC. (B) Whole milk pressurised at inlet temperature of 20 ºC. (C) Skim milk pressurised at inlet temperature of 6 ºC. (D) Skim milk pressurised at inlet temperature of 20 ºC. The data are presented as the mean value of four replications ± 95% confidence interval.

milk to higher temperatures would probably increase the lethality values after UHPH treatment. Fat content did not increase the resistance of the microorganism to UHPH; on the contrary, the presence of fat significantly increased the efficacy of the treatment and the further survival capability during the subsequent storage period of *E. coli*. The role of fat in the baro-resistance of microorganisms in comparison with heat treatments needs further research. Considering that UHPH treatment did not cause sublethal injuries, this technology may offer a promising alternative to the pasteurisation of milk and others liquid foods, supported by the possibility of increasing the inlet temperature of the sample before the process.

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