BEHAVIOUR OF PATHOGENIC GRAM NEGATIVE BACTERIA INOCULATED IN MILK AND MODEL CHEESE TREATED WITH HIGH HYDROSTATIC PRESSURE

SÍLVIA DE LAMO CASTELLVÍ

Departament de Ciència Animal i dels Aliments
Facultat de Veterinària
Universitat Autònoma de Barcelona

BEHAVIOUR OF PATHOGENIC GRAM NEGATIVE BACTERIA INOCULATED IN MILK AND MODEL CHEESE TREATED WITH HIGH HYDROSTATIC PRESSURE

MEMÒRIA PRESENTADA PER OPTAR AL GRAU DE DOCTORA EN CIÈNCIA I TECNOLOGIA DELS ALIMENTS

SÍLVIA DE LAMO CASTELLVÍ
Bellaterra (Cerdanyola del Vallès), 2006

Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA)

MARTA CAPELLAS PUIG, Professora Titular de Tecnologia dels Aliments, i ARTUR XAVIER ROIG SAGUÉS, Professor Titular de Tecnologia dels Aliments de la Universitat Autònoma de Barcelona,

FAN CONSTAR: que la llicenciada en Química i en Ciència i Tecnologia dels Aliments Sílvia De Lamo Castellví ha dut a terme, sota la seva direcció, a l'Àrea de Tecnologia dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, el treball titulat "Behaviour of pathogenic Gram negative bacteria inoculated in milk and model cheese treated with high hydrostatic pressure", que presenta per optar al grau de Doctora.

I perquè així consti, signem el present document a Bellaterra (Cerdanyola del Vallès) a 7 de febrer de 2006.

Marta Capellas Puig Artur Xavier Roig Sagués

I don't believe
there would be
any science at all
without intuition

Rita Levi Montalcini,
1986 Nobel Prize in
Physiology and Medicine

Acknowledgements

Al Ventura per donar-me l'oportunitat de realitzar aquest treball i entendre els meus desitjos.

A l'Artur per tot el teu treball en la direcció d'aquesta tesi i per la teva paciència amb les meves divagacions.

A la Marta pel teu treball en la direcció d'aquesta tesi i més enllà, per estar sempre que t'he necessitat i per tenir la paraula i la idea justa en el moment adequat.

A la Manoli pels teus magnífics consells i per la teva bona disposició tant en els moments bons com en els difícils.

Al Josep, per escoltar-me i ajudar-me. També per l'experiència workshop, inoblidable.

Al Ramón per inspirar-me amb la teva recerca i per la teva bona disposició.

A la Dolors per ser una molt bona jefa del laboratori i perdonar els meus nombrosos tubs bruts.

A la Núria, la Dora i el Manuel, per ser de les millors persones que he conegut i crear aquest màgic vincle.

Al Tomás per compartir aventures i desventures en la realització d'aquest projecte i a vegades les tensions pròpies que comporta aquesta feina.

Al Wilfido per ser un bon company i amic, per saber escoltar i per alegrar el dia a dia de la recerca.

A la Pilar i la Patricia, per ser unes bones conselleres i iniciar la meva immersió en la cultura mexicana i peruana.

Als meus companys de despatx Ibrahima, Betty, Diana, Patricia i Osvaldo per compartir el mini espai del que disposem i més coses.

A la resta de companys de les dues Unitats que m'han facilitat la feina en molts moments i que m'han fet riure quan calia.

A la Inga, la Berta i la Laia per la seva magnífica contribució en la realització d'aquest treball.

To Dr. Frank for giving me the chance to open my eyes to a new research world reality.

To Dr. Toledo for letting me use one of his high pressure machines. You made my dream come true.

To Carl for helping me all the time.

A Danitza, por ser mi mayor apoyo durante mi aventura americana, por ayudarme a salir adelante cuando todo parecía desvanecerse y por creer siempre en mi.

To Linda, for being my main support in the lab but specially, for being my friend.

To Wut, for being the best officemate that I have ever had. I will never forget our salsa lessons in our lunch breaks.

Als meus pares i germans pel vostre afecte, comprensió i ajuda.

Al David per la teva infinita paciència i per entendre i compartir aquesta vida boja que portem els que ens dediquem a recerca.

This study was financially supported by:

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria.
Project: CAL-00-005-C2-1.

Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, which provided Sílvia De Lamo Castellví a fellowship (2002FI-00079) to carry out this research.

Contents

List of Tables

List of Figures

1. Introduction

1. Introduction

Consumers in the 21st century are demanding high quality foods that are free from additives, fresh tasting, microbiologically safe and with an extended shelf-life. One food technology that has the potential to meet these demands is high pressure processing. High pressure processing, also known as high hydrostatic pressure (HHP), uses pressures up to 900 MPa to inactivate many of the microorganisms found in foods (Patterson, 2005).

This technology has been proposed as a viable alternative to conventional heat treatment for preserving food. In contrast to thermal processing, the application of high hydrostatic pressure to food causes negligible impairment of nutritional value, taste, colour or flavour (Smelt, 1998).

It is well known that the effectiveness of any food preservation technique has to be evaluated by its ability to eradicate any pathogenic microorganism to assure product safety and to inactivate spoilage microorganisms to improve the shelf life of the food (McClements et al., 2001). High hydrostatic pressure induces a number of changes to morphology, biochemical reactions and genetic mechanisms, and to cell membrane and wall of microorganisms (Hoover et al., 1989) and is generally effective to inactivate most vegetative bacteria, yeasts, and molds between 300 and 700 MPa (Smelt, 1998). Nevertheless, it is important to remark that there are different factors that affect the resistance of bacteria to high hydrostatic pressure treatments: temperature, magnitude and duration of pressure treatment, the stage of growth and the composition of medium (McClements et al., 2001). Moreover, in food, two effects always determine microbiological safety and stability: the effect of the food matrix during treatment (Patterson and Kilpatrick, 1998), and its effect after treatment, during the repair phase of the microorganisms (Smelt, 1998).

1.1 General principles of high pressure

The thermodynamic term describing the effects of pressure on chemical or biochemical systems is ΔV, the variation of the molar volume between two states (such as the final and the initial states) at constant temperature (Balny and Masson, 1993). High hydrostatic pressure is governed by two principles: the principle of *Le Chatelier* and the isostatic principle. Firstly, the principle of *Le Chatelier* states that any phenomenon (phase transition, chemical reactivity, change in molecular configuration or chemical reaction) accompanied by a decrease in volume will be enhanced by pressure (**Table 1**). An antagonistic effect of temperature is expected from the fact that temperature increase results in a volume increase. Depending on the nature of the product, the initial product temperature and the applied pressure, the adiabatic temperature increase may vary from 3 to 9ºC/100 MPa (**Table 2**) (de Heij et al., 2003). On the other hand, the reaction rate increases with increasing temperature according to Arrhenius' law.

Table 1. *Volume changes accompanying the formation of major interactions and bonds in biosystems.*

Interaction and bonds	
Covalent bonds	[a]$\Delta V^{\neq}$ of –10 ml/mol for the formation of covalent bonds, and nearly zero ΔV values for exchanges in the covalent bonds
Electrostatic interactions	[b]ΔV of – 10 ml/mol for hydratation of a charged group and a positive value of 10-20 ml/mol for the formation of an electrostatic interaction
Hydrophobic interactions	A positive ΔV of 10-20 ml/mol CH_2-groups entering the hydrophobic contact
Stacking of aromatic rings	Nearly zero values of ΔV
Hydrogen bonds	Nearly zero values of ΔV

[a]Differences in the volumes of the activated ($V^{\neq}$) and initial states.
[b]Differences in the volumes of the final and the initial states
Source: Mozhaev et al., 1994.

Table 2. *Temperature changes of selected substances due to compression heating*

Substance	Initial temperature (ºC)	Temperature change (ºC/100 MPa)
Water	20	2.8
	60	3.8
	80	4.4
Steel	20	≈0
Chicken	20	2.9
Cheese (Gouda type)	20	3.4
Milk fat	20	8.5

Source: de Heij et al., 2003.

Secondly, the isostatic principle (isostatic pressure) that shows that the pressure is instantaneously and uniformly transmitted independently of the size and the geometry of the food (Smelt, 1998).

1.2 Effects of pressure on microorganisms

Microorganisms are inactivated when they are exposed to factors that substantially alter their cellular structure or physiological functions. Structural damage includes DNA strand breakage, cell membrane rupture or mechanical damage to cell envelope. Cell functions are altered when key enzymes are inactivated or membrane selectivity is disabled. A preservation technology may cause cell death through multiple mechanisms.

High pressure is well known to produce alterations (**Figure 1**) in biochemical reactions, genetic mechanisms, structural changes in the morphology, cell wall and membranes of microorganisms (Hoover et al., 1989; Cheftel, 1995). However, the mechanisms of microbial inactivation are still not fully understood (Patterson, 2005).

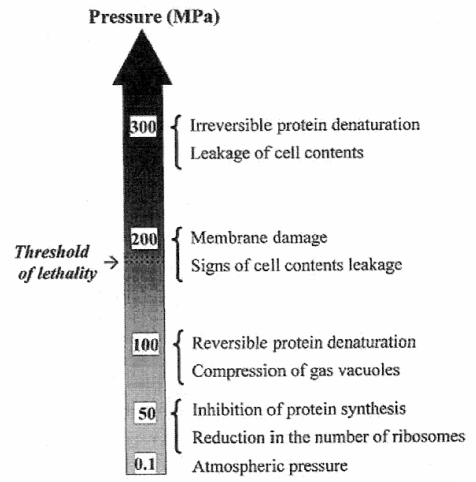


Figure 1. *Structural and functional changes in microorganisms at different pressures.* **Source:** Lado and Yousef (2002).

1.2.1 Effect of pressure on cell membranes

The cell membrane is generally considered to be a primary site of pressure damage in microorganisms (Hoover et al., 1989). The cell membrane is composed by a bilayer of phospholipids with embedded functional proteins that play an important role in transporting ions and other substances across the membrane. The effect of pressure on the membranes might be due to the fact that lipids are particularly sensitive to pressure, being an order of magnitude more compressible than proteins (Fernandes, 2005). It has been observed that lipid bilayers undergo phase transitions under pressure (San Martin et al., 2002). Membrane fluidity it is a multifaceted phenomenon that has contributions from molecular packing (order) and molecular motion (viscosity). The changes in fatty acyl composition may alter either or both of these aspects of fluidity and may exert its influence through control of the ion pumps in the membrane that are essential for maintaining pH homeostasis (Russell, 2002). The composition of lipid membrane is known to be altered in response to variations of pH, external osmolality and low temperature to retain membrane fluidity and functionality (Scheyhing et al., 2004). Kato and Hayashi (1999) suggested that the fluidity of lipid bilayers decreases with an increase in the pressure applied, resulting in functional disorder of membrane associated enzymes, accompanied by a reversible phase transition of the biomembrane, and finally, the lipid bilayer is irreversible fragmented, accompanied by enzyme denaturation. Casadei et al. (2002) reported that membrane fluidity affects the pressure resistance of

exponential and stationary-phase cells in a similar way, but it is the dominant factor in exponential-phase cells whereas in stationary-phase cells, its effects are superimposed on a larger effect of the physiological stationary-phase cell response that is itself temperature dependent. Moreover, loss of membrane-bound ATPase activity and loss of the ability to maintain a transmembrane pH gradient have been shown to occur during inactivation of *Lactobacillus plantarum* by high pressure (Wouters et al., 1998).

Physical damage to cell membrane has been demonstrated as leakage of ATP or UV-absorbing material -in the case of bacteria (Smelt et al., 1994)-, and solubilization and leakage of intracellular substances such as amino acids -in the case of yeasts (Kato and Hayashi, 1999)-, subjected to pressure occurs. A loss of osmotic responsiveness or increased uptake of the fluorescent dyes such as ethidium bromide and propidium iodide that do not normally penetrate the membranes of healthy cells (Benito et al., 1999; Ulmer et al., 2000; Pagán and Mackey, 2000) has also been observed. Benito et al. (1999) found that when *Escherichia coli* O157:H7 strains were exposed to the same pressure for different times, the pressure-sensitive strains took up stain sooner than the more resistant strain. The differences in resistance may be related to susceptibility to membrane damage and possibly to a property associated with the protein component. Mañas and Mackey (2004) reported that exponential-phase cells are inactivated under high pressure by irreversible damage to the cell membrane. In contrast, stationary-phase cells have a more robust cytoplasmic membrane that can better withstand pressure treatments. The retention of an intact membrane appears to allow the stationary-phase cell to repair gross changes in other cellular structures and to remain viable at pressures that are lethal to exponential-phase cells.

1.2.2 Effect of pressure on cell wall

Hoover at el. (1989) reported that pressures of 20-40 MPa can cause larger cells to lyse from mechanical disruption of a stressed cell wall. Kato and Hayashi (1999) suggested that the cell wall structure is altered by high pressure treatment, which leads to solubilization of intra-cellular substances. Malone et al. (2002) found that the pressure treatment at 100 MPa promoted cell wall hydrolase activity in *Lactococcus lactis* subsp.

cremoris MG1363 while treatment at pressures in excess of 300 MPa inactivated the enzyme activity.

1.2.3 Effect on cell morphology

Intracellular damage has been studied for several researchers by observation under a light microscope. Hoover et al. (1989) reported a variety of morphological changes in cells after applying pressure treatments that included longer individual cells, separation of the cell wall from the cytoplasmic membrane, thickened cell walls with no membrane structure, clear zones of spongy or reticular structures in the cytoplasm, and a decrease in number of ribosomes. Ritz et al. (2001, 2002), using scanning electron microscopy, reported the occurrence of bud scars on the surface of *Listeria monocytogenes* cells after 10 min of treatment at 400 MPa in citrate buffer. Intracellular regions of low density were observed in *L. monocytogenes* and *L. lactis* subsp. *cremoris* MG1363 using transmission electron microscopy (Mackey et al., 1994; Malone et al., 2002). These authors suggested that the low-density regions are caused by transient membrane invaginations under pressure that are subsequently reversed upon pressure release, leaving the low-density regions adjacent to the cell membrane. However, these low-density regions were not observed in high pressure treated *Salmonella enterica* serovar Thompson (Mackey et al., 1994), suggesting that only the Gram positive cell wall may be associated with this phenomenon.

Mañas and Mackey (2004) reported that membranes of exponential-phase cells showed physical disruption after pressurization, with the formation of vesicles, areas of engrossment, and also invaginations toward the cytoplasm. These authors suggested that the vesicles observed in pressurized exponential-phase cells could be from outer membrane materials, whereas engrossment areas, which occupied part of the cytoplasm, could be from the cytoplasmic membrane.

1.2.4 Effect on biochemical reactions

The application of pressure enhances reactions that lead to a volume decrease and generally retards reactions involving a volume increase. Most biochemical reactions result in a change in volume. Consequently, biological processes are influenced by pressure application (Hoover, 1989). Pressure acts as a modulator of biochemical processes ranging from inhibition of bacterial growth or viability of viral particles to activation-inactivation of enzymatic reactions. Moreover, high pressure affects the structure and function of enzymes in a complex way by altering intra and intermolecular interactions involved in protein stability (Dallet and Legoy, 1996).

Simpson and Gilmour (1997) found that *L. monocytogenes* intracellular enzymes could be affected by high pressure treatments. The electrophoretic mobility of those enzymes was modified after pressure treatment corresponding to some variations of their conformational forms. In some instances, the activity of those enzymes appeared to decrease as pressure increased.

1.2.5 Effect on genetic mechanisms

Nucleic acids are relatively resistant to high pressures and as the structure of the DNA helix is largely the result of hydrogen bond formation, it is also stable under pressure (Patterson, 2005). However, an extreme condensation of the nuclear material was observed in *L. monocytogenes* and *S. typhimurium* (Mackey et al., 1994). The hypothesis is that at elevated pressures, DNA comes in contact with endonucleases, which cleave DNA. This condensation has been found in many other instances and it is reversible and, presumably, also an enzyme responsible for renaturation is involved. If this enzyme is deactivated by high hydrostatic pressure, the cell is no longer able to multiply (Smelt, 1998).

One of the mechanisms that bacteria have developed to survive in unfavourable conditions is the ability to respond to stress situations. This stress response is mediated by a changed pattern of gene expression that typically results in an increased tolerance of the bacteria for the stress factor that triggered the response and usually also for a

number of other stress factors (Aertsen et al., 2004). Upon temperature stress and other environmental stresses, gene expression adjusts to adapt to such environmental changes through regulations by several DNA-binding proteins (Ishii et al., 2005). These authors studied pressure effects on *E. coli* using DNA microarray analysis and found that heat and cold stress responses were induced simultaneously by the elevated pressure.

1.3 Effects and interactions of treatment variables

The degree of inactivation depends on different factors: type of microorganism, amount of pressure applied, temperature and time of the treatment, pH of the dispersion medium, composition of the medium and the presence of antimicrobial compounds.

1.3.1 ***Type of microorganism***

Generally, yeast and moulds are very sensitive to high pressure, Gram positive microorganisms are the most resistant and Gram negative are moderately sensitive. Yeast viability during hydrostatic pressure treatment decreases with increasing pressure and this effect is more pronounced when cells are submitted to pressures above 100 MPa (Fernandes, 2005). Vegetative bacteria forms are inactivated by pressures between 400 and 600 MPa while bacterial spores are extremely resistant and survive pressures in excess of 1,000 MPa.

Smelt (1998) suggested that bacteria with a more fluid membrane are more resistant to high pressure. Gram positive bacteria are less sensitive than Gram negative probably due to the thicker cell wall (the rigidity of the teichoic acids in the peptidoglycan layer) of the former (Russell, 2002; Lado and Yousef, 2002). Bacteria of small size and coccoid in shape are generally more resistant to high hydrostatic pressure treatments than the large rod-shaped ones (Ludwing and Schreck, 1997). Several authors have suggested that the structure and thickness of the bacterial spores coats could be the reason for their high resistance. The formation of pores in spore coat during treatment at 50-300 MPa may indicate that high hydrostatic pressure induces spore germination. No germination was observed at high pressures, likely because the environmental

conditions were potentially lethal to germinating spores (Smelt, 1998). Spore inactivation by pressure is due to the induction of spore germination by pressure, with the germinated spores then being killed rapidly by pressure (Sojka and Ludwing, 1994; Nakayama et al., 1996; Van Opstal, 2004).

However, there are many exceptions of these general rules. Patterson et al. (1995) studied a clinical isolate of *E. coli* O157:H7 that possesses a pressure resistance comparable to spores. Arroyo et al. (1999) found that the resistance to high pressure was greater in *Saccharomyces cerevisiae* than in bacteria. This finding indicates that other factors than the shape and size of the cell are involved in cell resistance and the nature of the cell membrane influences cell resistance to alternative preservation processes. Paidhungat et al. (2002) reported that although the coats are important in spore resistance to some agents, they are not important in the resistance of spores to high pressure. Moreover, although one or more spore coat proteins are involved somehow in spore germination the spore coats are not important in pressure-induced spore germination.

The conditions and stage of growth of microorganisms play an important role in determining their sensitivity to high pressure. McClements et al. (2001) demonstrated that *L. monocytogenes* strains in stationary phase showed higher inactivation rate when grown at 30ºC than grown at 8ºC. However, for *Bacillus cereus*, stationary-phase cells grown at 30ºC were more resistant than those grown at 8ºC. Actually, bacterial cells in exponential-phase of growth are less pressure resistant than those in stationary phase of growth (Smelt, 1998). In addition, a considerable variation in pressure resistance within strains of the same species has been demonstrated in Gram positive and Gram negative bacteria (Styles et al., 1991; Patterson et al., 1995; Alpas et al., 1999). Benito et al. (1999) suggested that the differences in resistance between *E. coli* O157:H7 strains were related to their susceptibility to membrane damage.

The development of high levels of barotolerance in microorganisms has important implications for the practical application of pressure technology in food preservation. Hauben et al. (1997) found that *E. coli* cells subjected to successive pressurization cycles (18) developed barotolerance. For the parent strain, a pressure treatment of 220 MPa produced a 10% of surviving cells, whereas at this same pressure, 50% of the

barotolerant mutant survived. Fernandes (2005) reported that *S. cerevisiae* cells submitted to a mild sublethal pressure do not acquire resistance to a subsequent rise to severely high hydrostatic pressure. Baroresistance after pressure treatment is only acquired if the yeast cells are incubated at room pressure for a short period of time before the severe high hydrostatic pressure stress. The protective effect is seen after 15 min of incubation at atmospheric pressure and persists for one hour in the presence of severe pressure.

1.3.2 Pressure level and time

Generally, it is assumed that increases in the magnitude of pressure or length of exposure correspond to increasingly more adverse effects, and even death, to the microorganisms (Hoover, 1989). However, Kalchayanand et al. (1998a,b) reported that pressurization for a longer time at low pressure range (to minimize adverse effects on food texture and color) was not a great advantage for microbial inactivation, even if antibacterial compounds were included.

1.3.3 Temperature

Temperature during pressure treatment can have a significant effect on microbial survival. Increased inactivation is usually observed at temperatures above or below 20ºC (Patterson, 2005). Bacterial cells are relatively less sensitive to hydrostatic pressure at 20-35ºC but become more sensitive to pressurization above 35ºC due to phase transition of membrane lipids (Kalchayanand et al., 1998b). Moreover, membrane repair of pressure induced pores could be harder to accomplish when intermolecular forces are weakened by warming (Russell, 2002). Several authors have reported that the effect of temperature on the viability of all strains studied was more pronounced at higher pressures (Hauben et al., 1997). Alpas et al. (2000) found that increasing the pressurization temperature from 25 to 35 and 45ºC and then to 50ºC had a significant effect on the viability loss of all the strains (*Staphylococcus aureus, L. monocytogenes, E. coli* O:157:H7*, S. enteritidis* and *S. thypimurium*) tested.

The effects of temperature are of great practical interest, because combined pressure and temperature processing may cause equivalent microbial inactivation ratios while operating at lower pressure levels and/or for shorter periods of time (Cheftel, 1995). It has been demonstrated that significant reductions in spore survival can be obtained by application of a cyclic process alternating between low and high pressures at moderate temperatures between 40 to 70°C (Wuytack et al., 1998). Cléry-Barraud et al. (2004) showed that the combination of heat and pressure resulted in complete destruction of *B. anthracis* spores, with a D value of approximately 4 min after pressurization at 500 MPa and 75°C, compared to 160 min at 500 MPa and 20°C and 348 min at atmospheric pressure and 75°C.

Refrigeration temperatures can also enhance pressure inactivation. Gervilla et al. (1997a) reported that pressure treatments at 2ºC were more effective inactivating *L. innocua* inoculated in ewe's milk than at room temperature (25ºC), but less effective than at 50ºC. The lower resistance of microorganisms at lower temperature could be also attributable to alteration of the cell membrane with resultant phase transition from liquid into gel (Eze, 1990). Hayakawa et al. (1998) showed that at sub-zero temperatures, the high pressure conditions generated (60-140 MPa) were able to inactivate microorganisms effectively: yeasts (*S. cerevisiae* and *Zygosaccharomyces rouxii*), bacteria (*Lactobacillus brevis* and *E. coli*), and fungi (*Aspergillus niger* and *A. oryzae*). However, *S. aureus* was only partly inactivated under the same conditions.

1.3.4 pH

The application of pressure can alter the pH of a medium, as well as, progressively, narrow the pH range for growth (Hoover et al., 1989; Stippl et al., 2004). Vegetative bacteria are quite sensitive to pressure and low pH and are more sensitive to sub-optimal pH after pressure treatment (Smelt, 1998). Alpas et al. (2000) found that pressurization at 345 MPa of peptone water inoculated with strains of four foodborne pathogens in the presence of either citric or lactic acid increased their viability loss by an additional 1.2–3.9 log (cfu/ml) at pH 4.5 for both acids. Pagán et al. (2001) observed that pressure-damaged *E. coli* O157 cells were more acid sensitive than native cells suggesting that pressure sensitization may involve loss of cell protective or repair

functions. Whereas, yeasts and moulds are quite resistant to low pH and a pH lower than 4.0 hardly sensitises these microorganisms against pressure (Smelt, 1998).

1.3.5 Composition of medium

Several authors have reported that certain foods could protect microorganisms from pressure inactivation with factors such as low water activity, sodium chloride, sucrose and readily available supply of nutrients. Carbohydrates are generally more protective than salts. In general, low water activity protects cells against pressure but microorganisms injured by pressure are generally more sensitive to low water activity (Smelt, 1998).

Salmonella spp. were more sensitive to pressure treatment in phosphate buffer saline (PBS) than in richer media such as chicken baby food (Metrick et al., 1989). UHT milk and raw milk inoculated with *L. monocytogenes* appeared to provide a protective effect and lessened cell death as compared to pressurization in PBS (Styles et al., 1991). Patterson et al. (1995) found that *L. monocytogenes* and *E. coli* were more resistant to pressure treatment in ultra heat treated (UHT) milk than in poultry meat or PBS and *E. coli* O157:H7 inactivation was significantly greater in poultry meat than in milk. In contrast, *S. aureus* was more resistant in milk than in poultry meat (Patterson and Kilpatrick, 1998). Gervilla et al. (1997a) found a baroprotective effect of ewe's milk (6% fat) on *L. innocua.* Black et al. (2005) detected that milk had a clearly protective effect in the inactivation of *Pseudomonas fluorescens* at 250 MPa for 5 min at 20ºC. The reduction in milk was only 4.51 log (cfu/ml) whereas in PBS the cell population was reduced to undetectable levels from an initial number of 1.6×10^8 cfu/ml. The inactivation of *E. coli* by high hydrostatic pressure treatment at up to 550 MPa and 20ºC was studied in potassium phosphate buffer containing high concentrations of sucrose. *E. coli* strain MG1655 was pressure-sensitive in the absence of sucrose, but became highly pressure resistant in the presence of 10% to 50% (w/v) sucrose (Van Opstal et al., 2003). However, Ellenberg and Hoover (1999) reported no notable protective effect when *Aeromonas hydrophila* and Y*ersinia enterocolitica* were pressurized in pork as compared to buffer or tryptic soy broth (TSB), or when they were pressurized in TSB as compared to buffer.

1.3.6 Antimicrobial compounds

Several authors have reported a synergistic effect between high pressure and antimicrobial compounds like nisin (Masschalck et al., 2001; Kalchayannand et al., 2004), lysozyme (López-Pedemonte et al., 2003), lacticin 3147 (Ryan et al., 1996) and lactoperoxidase system (García-Graells et al., 2003).

Nisin is an antibacterial peptide produced by *L. lactis* subsp. lactis and it is active principally against Gram positive bacteria, but Gram negative bacteria, damaged cells in particular, may be sensitive to nisin as well. The primary site of action of nisin against vegetative cells is considered to be the cytoplasmatic membrane, acting as a depolarizing agent in a voltage-depenent fashion (Ray, 1992). Ponce et al. (1998) found that high hydrostatic pressure inactivation of *E. coli* and *L. innocua* inoculated in liquid whole egg was improved significantly with nisin addition at concentrations of 1.25 and 5 mg/l. The combination of nisin and pressure promoted a large reduction in mechanically recovered poultry meat cell counts, specially for psychrotrophe populations (Yuste et al., 1998). The combination of high pressure and nisin has been also used to increase the inactivation of *B. cereus* in model cheese (López-Pedemonte et al., 2003). When the treatment (60 MPa at 30ºC for 210 min to germinate spores followed by 400 MPa at 30ºC for 15 min to eliminate the vegetative cells) was done adding 1.56 mg/l of nisin in the model cheeses the highest inactivation of the spores was obtained (2.4 log [cfu/g]).

Lysozyme is a natural component of egg white and their bactericidal properties were primarily ascribed to its N-acetylmuramoylhydrolase enzymic activity, resulting in peptidoglycan hydrolysis and cell lysis. However, new findings support the existence of a nonenzymic and/or nonlytic mode of action (Masschalck et al., 2002). Gram negative bacteria are normally insensitive to lysozyme by their outer membrane that acts as a physical barrier preventing the access of the enzyme (Masschalck and Michiels, 2003), but, several studies have showed that high hydrostatic pressure treatments can produce membrane permeabilization.

Lacticin 3147 is a broad spectrum bacteriocin produced by *L. lactis* DPC3147, and is inhibitory to a wide range of Gram-positive organisms, including *Listeria*, *Clostridium*,

Enterococcus, *Staphylococcus* and *Streptococcus* (Ryan et al., 1996). Lacticin 3147 elicits a bactericidal effect on sensitive cells first by interacting with the cell membrane. This causes the membrane to become porous for K^+ and inorganic phosphate, which leak out of the cells. In an attempt to reaccumulate these ions, ATP dependent uptake systems lead to hydrolysis of internal ATP. Since ATP is required for the maintenance of essential cellular functions, such as the pH gradient at the cell membrane, cellular functions are disrupted and the cell eventually loses energy and dies. When cells are exposed to pressure in addition to lacticin induced membrane damage, the level of cell damage results in a greater kill than either treatment alone (Morgan et al., 2000). These researchers showed that the combination of 250 MPa (2.2 log [cfu/ml] of reduction) and lacticin 3147 (1 log [cfu/ml] of reduction) resulted in more than 6 log (cfu/ml) of viability loss of *S. aureus* ATCC6538 inoculated in 10% reconstituted skimmed milk.

Lactoperoxidase is a native milk enzyme that catalyzes the oxidation of thiocyanate by peroxide into short-lived reactive oxidation products, such as the hypothiocyanite anion, that, in turn, rapidly oxidizes many biomolecules. Most relevant for microbial inactivation is probably the oxidation of enzymes and other proteins in the bacterial cell membrane that have exposed sulfhydryl groups. The first direct effect of lactoperoxidase action on the cell is membrane damage resulting in loss of pH gradient, K^+ leakage, and inhibition of transport of solutes, such as amino acids and glucose. This enzyme has a broad working spectrum being effective for Gram positive and Gram negative bacteria (García-Graells et al., 2000). These authors detected an strong synergistic interaction of high pressure treatments and lactoperoxidase system for *L. innocua* strains. Inactivation over 7 log (cfu/ml) was achieved for all strains with a mild treatment (400 MPa, 15 min, 20°C), which in the absence of the lactoperoxidase system caused only 2 to 5 log (cfu/ml) of inactivation depending on the strain.

1.4 Injured population

Bacterial cells exposed to different physical and chemical treatments suffer injury that could be reversible in food materials during storage (**Figure 2**). Injury has been observed for many bacterial cells (Bozoglu et al., 2004). Several authors have detected injured cells after high pressure treatments (Metrick et al., 1989; Styles et al., 1991;

Patterson et al., 1995; Simpson and Gilmour, 1997; McClements et al., 2001; Chen and Hoover, 2003), suggesting that, given favourable conditions such as prolonged storage in a suitable substrate, these cells may be able to repair. Further studies have been done to confirm this behaviour. Carlez et al. (1994) found that, although there were not counts of *Pseudomonas* spp. after minced meat was pressurized (400 MPa, 20 min), growth was detected at day 6 of storage at 3ºC. García-Risco et al. (1998) showed that no psychrotrophic bacteria (<10 cfu/ml) were detected immediately after the pressure treatment (400 MPa, 30 min) of raw whole and skimmed milk, but after storage at 7ºC for 15 days the psychrotrophic counts reached levels similar to those of the mesophilic bacteria (5.30 and 5.68 log [cfu/ml]). Black et al. (2005) detected recovery and subsequent growth of *P. fluorescens* in milk during refrigerated storage from days 1 to 10 in samples treated at 250 MPa for 5 min at 20ºC. This potential repairing is problematic and has particular significance for psychrotrophic foodborne pathogens that can survive and grow at refrigeration temperatures (McClements et al., 2001). On the other hand, high hydrostatic pressure induced injury can be advantageous in high acid foods, where lower pressure can be used to produce injured cells that could not repair in acidic medium (Bozoglu et al., 2004). Some authors suggest that high hydrostatic pressure treatments could restrict the pH range that bacteria can tolerate, possibly due to the inhibition of ATPase-dependent transfer protons and cations -or directly their denaturation- or the dislocation of bound ATPase in the membrane, and therefore recovery of sublethally injured cells would be reduced (Pagán et al., 2001; Wouters et al., 1998).

Traditionally, injured cells produced by high pressure treatments have been detected using classic culture techniques (comparing viable counts on non-selective and selective media). Nevertheless, sublethally injured cells generally do not grow on or in selective media. This is because these media contain agents that may inhibit the repair of damaged cells, and so they are not detected. Alternative options have been proposed to these methods. Several researchers have been adding chemical products (sodium chloride, sodium dodecyl sulphate, a mixture of citric acid and Na_2HPO_4, or glucose) in nutritive culture media to act as selective agents and inhibit growth of injured cells (Patterson et al., 1995; Wuytack et al., 2002; O'Reilly et al., 2000). Patterson et al. (1995) detected differences between *E. coli* counts obtained in trypticase soy agar enriched with 6% of yeast extract (TSAYE) and TSAYE supplemented with 2% of

sodium chloride after 15 min of treatment at 600 MPa for UHT milk, poultry meat and PBS. Kang and Fung (1999) proposed the thin agar layer (TAL) method to recover injured microorganisms. This method consists of selective medium overlaid with non-selective medium. During the first few hours of incubation of TAL plates, injured cells recover and start to grow on the non-selective medium top layer, whereas the agents of the selective medium gradually diffuse to the top layer. Then, the target microorganism performs most reactions that it typically does on selective medium, and growth of most other microorganisms is inhibited by the now lower concentration of selective agents (Kang and Fung, 1999, 2000). This method has been effective to recover inoculated pathogens injured by heat, acid, and chemicals treatments (Kang and Fung, 1999, 2000; Wu et al., 2001; Hajmeer et al., 2001; Yuste and Fung, 2002) and new research is trying to find if this method is useful to recover injured microorganisms produced by high hydrostatic pressure treatments or mainly allows differentiation of the inoculated pathogens from background microbiota (Yuste et al., 2003; Yuste et al., 2004).

Bozoglu et al. (2004) reported that there are three states of cells just after pressure treatment: active cells (those that can form visible colonies on selective and non-selective agar), primary injury (those that can form visible colonies on non-selective agar but not on selective agar, but can form colonies on selective agar during prolonged storage) and secondary injury (those that can not form visible colonies on either non-selective or selective agar but can form colonies first on non-selective agar and later on selective agar during prolonged storage). These two types of injuries were observed in high pressure treated cells depending on the species and the level of pressure applied. Moreover, some researchers have detected, using flow cytometry and different fluorescent dyes, the presence of viable subpopulations that did not form colonies and were therefore not detected on agar media (Arroyo et al., 1999). Ritz et al. (2001) reported that high hydrostatic pressure treatments produce heterogeneous cell populations likely due to variations in levels of injury, metabolic activity and viability among the cells and due to this heterogeneity the reversible damage and the cellular repair that may occur under favourable conditions should not be ruled out. Ananta et al. (2004) reported that loss of capacity of forming colonies on agar as a consequence of pressure treatments beyond 500 MPa did not necessarily correlate with the absence of metabolic activity, in particular esterase activity.

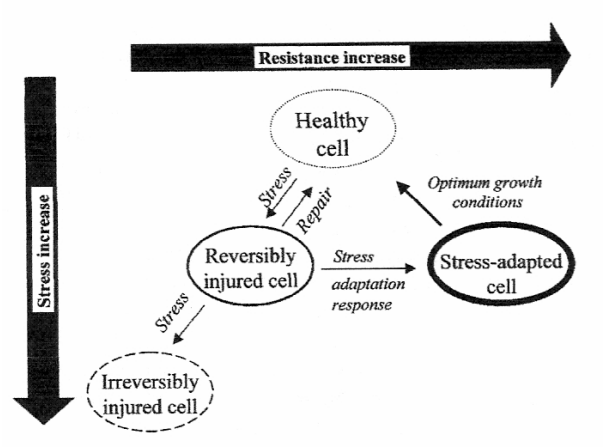


Figure 2. *Microbial stress, injury, adaptation and resistance to processing.*
Source: Lado and Yousef (2002).

1.5 Kinetics of microbial inactivation

The traditional approach to inactivation kinetics is based on the assumption that microorganisms or their spores die exponentially following first-order kinetics (Mussa and Ramaswamy, 1997; Gervilla et al., 1999). It also assumes that cells or spores have identical pressure resistance. Mussa et al. (1998) inoculated raw milk (containing the natural microbiota) with *L. monocytogenes* Scott A. Samples were subjected to different pressure treatments (150 to 400 MPa) for selected holding times of up to 120 min to evaluate kinetics of destruction of this microorganism. These authors found that all the pressures applied followed a first order rate of death in the destruction of *L. monocytogenes* as well as microbiota present in raw milk. Dogan and Erkmen (2004) determined high hydrostatic pressure kinetics of aerobic bacteria and *L. monocytogenes* inactivation in brain heart infusion broth, milk and in peach and orange juice. For this purpose, different pressures (200-700 MPa) at 25ºC for different holding times (25 to 100 min) were applied. The decimal reduction times (D values) that these researchers obtained were about 3.04 and 2.43 min for aerobic bacteria and *L. monocytogenes*, respectively, in milk at 600 MPa, 2.13 and 1.52 min, respectively, in peach juice, and 1.24 and 0.87 min, respectively, in orange juice.

However, deviations in log-linear models have been detected in form of tails (biphasics) and shoulders (Heldman and Newsome, 2003). Several authors have detected this behaviour. Kinetics of microbial inactivation of *L. monocytogenes* was not linear in time and Patterson et al. (1995) stated that it was not possible to calculate conventional D values as a method to determine relative pressure sensitivities of different organisms. In general, inactivation curves did not follow first-order kinetics but tended to be exponential, with an initial decrease in numbers, during the first 15 min of treatment followed by a tail, suggesting that a small fraction of the population was more pressure resistant. Gervilla (2001) reported that, in some of the microorganisms analysed, the kinetics changed from first order to second order after the first 15-20 min of high hydrostatic pressure treatment. A small group of cells could survive after a considerably long treatment that was expected to be completely lethal. This behaviour suggested that cells had the ability to adapt to the medium in high pressure conditions. However, Metrick et al. (1989) worked with *Salmonella* spp and pressurized the supposed resistant cells a second time. Comparing the resistance to pressure between the original culture and the remaining population, they found no significant changes. Tay et al. (2003) investigated the inactivation kinetics of *L. monocytogenes* Scott A (pressure-sensitive strain) and OSY-8578 (pressure-resistant strain) at 350 and 800 MPa. First-order kinetics was not suitable to describe the inactivation, and extended pressure treatment did not eliminate the tailing phenomenon. Chen and Hoover (2003) reported a strong tailing in the survival curves of *Y. enterocolitica* obtained at four pressure levels (300, 350, 400 and 450 MPa) in PBS and whole milk. They indicated that the best models for predicting pressure inactivation of this organism were the non-linear regression and the Weibull models and suggested that in each food is necessary to develop new models to predict the inactivation kinetics of microorganisms. Moreover, these authors (2004) studied also the survival curves of *L. monocytogenes* Scott A inactivated by high hydrostatic pressure at seven pressure levels (300, 350, 400, 450, 500, 550 and 600 MPa) in UHT whole milk. In these case, the shapes of the survival curves were concave upward or downward depending on the treatment pressure levels and the Weibull model produced also a better fit than the linear model. Guan et al. (2005) studied the inactivation of *S. typhimurium* DT 104 in UHT whole milk by high hydrostatic pressure applying different pressures (350, 400, 450, 500, 550, and 600 MPa) at room temperature and using four modelling methods (linear, Weibull, modified Gompertz and log-logistic models) to fit the results. A strong tailing was observed in all

survival curves and log-logistic model was the model that produced the best fit to survival data.

1.6 Milk and dairy products as a vehicle of foodborne pathogens

Milk is a nutritious medium that presents a favourable environment (near neutral pH) for the multiplication of microorganisms and supports a wide range of spoilage and pathogenic bacteria. Traditionally, raw or unpasteurized milk has been a major vehicle for transmission of pathogens. Dairy products have long been recognized as being susceptible to postprocessing contamination (Donnelly, 1990). Food poisoning outbreaks and other illnesses involving milk and milk products have been reported since the beginning of the dairy industry (**Table 3**). Nowadays, special attention is focused on milk and dairy products contaminated with pathogenic bacteria as *L. monocytogenes*, *Y. enterocolitica*, *Campylobacter jejuni*, *A. hydrophila* and enteropathogenic *E. coli* (Vasavada, 1988; Donnelly, 1990; Klausner and Donnelly, 1991; Jayarao and Henning, 2001).

Table 3. *Some outbreaks associated with milk and dairy products contaminated with pathogenic bacteria*

Date	Location	Product	Pathogen
1982	Arkansas Tennessee Mississippi	Pasteurized milk	*Y. enterocolitica*
1985	Illinois	Low fat milk	*S. typhimurium*
1986	Pennsylvania	Ice cream	*L. monocytogenes*
1997	Washington	Raw milk cheese	*S. typhimurium* DT104
2000	Pennsylvania New Jersey	Pasteurized milk	*S. typhimurium*
2001	France	Cantal cheese	*S. enteritidis*
2002	Canada	Unpasteurized Gouda cheese	*E. coli* O157:H7

Sources: Vasavada, 1988; Villar et al., 1999; Haeghebaert et al., 2003; Olsen et al., 2004; Honish et al., 2005.

Heat treatment is the oldest and most widely used technological process applied to milk (**Table 4**).

Table 4. *Heat treatments more common used in dairy industry*

Treatment	Temperature (ºC)	Time (s or min)
Termization	63-65	15-30 s
Pasteurization		
High Temperature Short Time (HTST)	72-75	15-20 s
Low Temperature Long Time (LTLT)	63-65	30 min
Ultra Pasteurization	125-138	2-4 s
Sterilization		
Ultra High Temperature (UHT)	135-140	2-4 s
In-bottle	115-120	20-30 min

Source: Gervilla, 2001

The principal objective of pasteurization is the elimination of pathogens and the reduction of the natural microbiota that can be present in milk. But it has been reported that pasteurization may not destroy all foodborne pathogens in milk due to pathogens such as *L. monocytogenes* that can survive and thrive in post-pasteurization processing environments, thus leading to recontamination of dairy products (Oliver et al., 2005). Traditionally, the heat treatment usually applied to cheese milk is pasteurization. However, some researchers have found differences between cheeses made from raw and pasteurized milk; cheeses made from raw milk tend to develop a stronger flavour and generally ripen faster than cheeses made from pasteurized, due to inactivation of enzymes and destruction of the heat sensitive microbiota by pasteurization treatment (Grappin and Beuvier, 1997).

1.7 Gram negative bacteria used for this research

1.7.1 Yersinia enterocolitica

Y. enterocolitica is a facultative anaerobic, Gram negative, non-spore forming, short rod-shaped bacterium, currently classified as part of the family *Enterobacteriaceae* (ICMSF, 1998). It has been isolated from animals belonging to various taxonomic groups, from mammals to insects. This microorganism comprises over 60 serotypes but only a few are pathogenic for humans (Kapperud, 1991). This organism has been associated with human gastro-intestinal disease called yersiniosis, but may also cause further infections including skin, eye, muscle and wound abscesses, endocarditis and pharyngitis, and postinfection immunologic consequences in the form of arthritis and its complications, erythema nodosum, and thyroid disorders (Schiemann, 1987). The majority of *Yersinia* infections are food-borne. The spread of the disease is mainly dependent on the degree of hygiene standard at every step of the technological process of food processing, storage and distribution (Stojek, 1999).

Y. enterocolitica has been isolated from humans in many countries, but it seems to be most frequently found in cooler climates (Kapperud, 1991). In Europe, Canada and Japan, the majority of cases are due to *Y. enterocolitica* serogroup O:3 and to O:9 on a lesser extent, and are caused by consumption of pork products. Pigs are regarded as major reservoirs of pathogenic *Y. enterocolitica* since it is often carried in the oral cavity or intestinal tract of healthy exemplars (Tauxe et al., 1987; Doyle, 1990; Andersen et al., 1991). In contrast, in the United States, the prevalent serotype between 1976 and 1982 had been O:8. However, after 1988, several US outbreaks of *Y. enterocolitica* O:3 suggested a serogroup shift (Ackers et al., 2000). In Spain, O:3 is the most frequent serotype identified in human yersiniosis cases (Anonymous, 2001).

The organism has been frequently isolated from raw milk and even from pasteurized milk (Larkin et al., 1991). Milk has been implicated in several outbreaks of yersiniosis. The cases with more people affected have been reported in the United States. In September and October 1976, an outbreak of illness linked to consumption of chocolate milk contaminated with *Y. enterocolitica* resulted in the hospitalization of 36 children. Investigation of this outbreak revealed that *Yersinia* was introduced during hand mixing

of unpasteurized chocolate syrup with pasteurized milk in an open vat (Black et al., 1978). In July 1981, gastrointestinal disorders of varying severity were observed in 239 campers and staff members at a summer camp in New York State. *Y. enterocolitica* serogroup O:8 was isolated from 54% of persons examined. Isolates belonging to the same serogroup and biogroup of human isolates were recovered from dissolved powdered milk and a milk dispenser, amongst others (Morse et al., 1984). In June and July 1982, a large interstate outbreak of yersiniosis occurred in Tennessee, Arkansas and Mississippi. Pasteurized milk was epidemiologically implicated as the vehicle of the infection (Tacket et al., 1984). Although yersiniosis outbreaks from cheese have not been reported, some studies have showed the ability of *Y. enterocolitica* to grow in Brie cheese (Little and Knochel, 1994) and survive in Turkish feta cheese (Erkmen, 1996).

The psychrotrophic nature of this organism is of particular significance in milk and milk products that are normally stored at low temperatures. In raw milk, *Y. enterocolitica* strains could survive in presence of high numbers of competing microorganisms and could maintain the virulence plasmid during extended storage at refrigeration temperatures (Larkin et al., 1991). In pasteurized milk, contamination has been mainly attributed to the inadequate pasteurization or post process contamination (Klausner and Donnelly, 1991; Kushal and Anand, 1999).

1.7.2 Salmonell*a spp.*

Salmonella is ubiquitous in nature and the serotypes that can cause human infections occur in all types of animals, including a significant but unknown number of domestic animals. Infections exhibit a number of clinical presentations, but gastrointestinal disorders are the most common clinical manifestation. The severity and duration of symptoms depend on the type of *Salmonella* present, the amount of food eaten and the susceptibility of the person involved (El-Gazzar and Marth, 1992). The two most prevalent serovars of *Salmonella* currently isolated from foodborne outbreaks in USA and Europe are *S. enterica* serovar *enteritidis* and *S. enterica* serovar *typhimurium* (Mattick et al., 2001; Anonymous, 2003). Moreover, *S. enterica* serovar *enteritidis* is one of the *Salmonella* serotypes most commonly associated with morbidity and mortality in humans (Ahmed et al., 2000).

Raw and pasteurized milk and different types of cheeses have been involved in several outbreaks (CDC, 1985; **Table 3**). Some studies have showed that when milk becomes contaminated with *Salmonella* spp. after pasteurization, the pathogen could survive the cheese making process and persist for several months in the cheese (Leyer and Johnson, 1992). In ripened Cheddar cheese, *Salmonella* counts were detected for up to 7 months at 7ºC. In cold-packed cheeses, cells were found depending on the pH value and preservative used (El-Gazzar and Marth, 1992). Moreover, fat and proteins in cheese can protect foodborne pathogens from gastric acidity, reducing the number of organisms necessary to cause clinical infections (Altekruse et al., 1998). These trends increase the need of detecting low numbers of *Salmonella* cells in cheeses .

1.7.3 Escherichia coli

E. coli is a Gram negative, non-spreforming, rod-shaped organism. Different groups have been recognized, namely, enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative and enterohaemorrhagic (Vasavada, 1988; Nataro and Kaper, 1998; Gonzalez Garcia, 2002).

E. coli O157:H7 has emerged as a foodborne pathogen of major concern for the food industry due to its ability to cause severe illness, in particular haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. Dairy cattle is considered a main reservoir of *E. coli* O157:H7 for human infection (Doyle, 1991) being fecal contamination of milk an important vehicle for its transmission (Borczyk et al., 1987; Gonzalez Garcia, 2002).

This pathogen has been isolated from raw milk (Reitsma and Henning, 1996), and multiple outbreaks of *E. coli* O157:H7 linked to ingestion of raw milk and dairy products have been reported (Altekruse et al., 1998; Honish et al., 2005). In many countries, the fact that raw milk is used for cheese manufacture increases the risk that pathogenic bacteria would contaminate the product (De Buyser et al., 2001). Several authors have studied the ability of *E. coli* O157:H7 to grow and survive in different types of cheese. In fresh cheese, *E. coli* O157:H7 grew 2 log (cfu/g) during cheese

manufacture (Arocha et al., 1992), but total inactivation was obtained during the heat treatment. Reitsma and Henning (1996) found that this microorganism was able to grow during Cheddar cheese manufacture even with an initial inoculum in milk of 1 cfu/ml. *E. coli* O157:H7 also survived the manufacture of Camembert and Feta cheeses (Ramsaran et al., 1998) and reached counts higher than those present in milk after 75 and 65 days of storage, respectively. Moreover, Maher et al. (2001) found that this pathogen was able to survive all stages of smear-ripened cheese production up to 70 days post-manufacture.

1.8 Effect of high hydrostatic pressure on microorganisms in milk

The interest in high hydrostatic pressure for treatment of milk has increased mainly due to the possibility of reducing the microbial number without significant effects on flavour or nutritional components. In fact, several studies have currently examined pressure inactivation of microorganisms that are either naturally present in milk or introduced into the milk artificially.

Styles et al. (1991) studied the effect of high pressure treatments in raw and UHT milk inoculated with 6 log (cfu/ml) of *L. monocytogenes* Scott A. The total inactivation of this microorganism was obtained after treating samples at 340 MPa for 80 min, in the case of UHT milk, and 60 min, in the case of raw milk. López-Fandiño et al. (1996) evaluated the effects of high pressure treatments (from 100 to 400 MPa) applied for different periods (10 to 60 min), on the biochemical and microbiological characteristics of raw milk. Psychrotrophic bacteria were reduced with pressure more quickly than total bacterial counts. After applying 300 MPa for 30 min and 10 min at 400 MPa, no psychrotrophic bacteria were detected by the plate count method (<10 cfu/ml). These authors suggested that this failure in growth initiation could be due to the lower incubation temperature and these microorganisms could have been regenerated under more favourable conditions.

Gervilla et al. (1997b) inoculated ovine milk (6% fat) with *E. coli* and *P. fluorescens* at a rate of 10^6 and 10^7 cfu/ml, respectively and treated the samples with different combinations of pressure (300, 400, 450 and 500 MPa), temperatures (2, 10, 25 and

50ºC) and times (5, 10 and 15 min). Inactivation (over 6 log [cfu/ml]) of both strains was observed at 50ºC for all the pressures and times and the lowest reduction was observed at 10ºC for *E. coli* and at 25ºC for *P. fluorescens*. Mussa and Ramaswamy (1997) used different combinations of pressure (200-400 MPa) and times (5 to 20 min) to inactivate microorganisms present in raw milk. These authors found that milk subjected to a microbial inactivation of 4 times de D value at 350 MPa had a shelf-life of 25 days at 0ºC, 18 days at 5ºC or 12 days at 10ºC.

Patterson and Kilpatrick (1998) used different combinations of pressure, temperatures and times to eliminate the population of *E. coli* O157:H7 and *S. aureus* inoculated in UHT milk and poultry. In UHT milk, population of *E. coli* was reduced 5 log (cfu/ml) when was treated at 400 MPa for 15 min at 50ºC and for *S. aureus*, the reduccion was approximately 6 log (cfu/ml) when it was treated at 500 MPa for 15 min at 50ºC. Mussa et al. (1998) applied different pressures (150-400 MPa) for selected holding times of up to 120 min to raw milk inoculated with 10^6 cfu/ml of *L. monocytogenes* Scott A to study the kinetics of destruction of this microorganism and the natural flora present in the milk. At 400 MPa, the instantaneous pressure kill (destruction achieved due to application of a pressure pulse with no hold-time) was higher for *L. monocytogenes* than the normal flora (2.10 log [cfu/ml]).

The effects of high pressure on the viability and acidifying and peptidolytic activities of *L. lactis* ssp. lactis*, L. casei* ssp. casei and *L. plantarum* strains were studied by Casal and Gómez (1999). Cells, inoculated in 10% reconstituted bovine skim milk, were treated at different pressures (100 to 400 MPa) for 20 min at 20°C. The Lactococci were more sensitive than the Lactobacilli to pressures of 100 to 350 MPa. The pressure-treated cells exhibited lower acidification rates, even with treatments that did not affect cell viability and increased the hydrolytic activity of these microorganisms on the carboxyl-terminal fragment from b-casein (C-peptide), which contributes to bitterness in cheese. Gervilla et al. (1999) inoculated ovine milk with *S. aureus* and *L. helveticus* at a concentration of 10^7 cfu/ml and treated the samples using different combinations of pressures (200, 300, 400, 450 and 500 MPa), temperatures (2, 10, 15, 25 and 50ºC) and times (5, 10 and 15 min). These authors found that *S. aureus* was extremely resistant to pressure and cell reductions above 7 log (cfu/ml) were only achieved after applying 500 MPa at 50ºC for 15 min. For *L. helveticus* the pressure treatments were more effective

at low (2 and 10ºC) and moderately high (50ºC) temperatures than at room temperature (25ºC).

Linton et al. (2001) studied the pressure resistance of a range of pathogenic *E. coli* in skimmed milk applying different pressures (200-700 MPa) for 15 min at 20ºC. A pressure treatment of 500 MPa for 40 min gave only a 4 log (cfu/ml) reduction of the two most resistant strains (NCTC 11601 and NCTC 9706) but after a treatment of 600 MPa for 30 min, no survivors of either strain could be detected (> 7 log [cfu/ml]). Molina-Höppner (2002) reported that high pressure treatments of *L. lactis* spp. *cremoris* MG1363 inoculated in milk buffer, initially affected metabolic activity and subsequently damaged membrane integrity. After a treatment for 5 min at 300 MPa the metabolic activity was 10-12% of the activity of untreated microorganisms and after 12 min of treatment the cells did not show any metabolic activity. During the treatment at 300 MPa cell death was closely followed by the loss of metabolic activity, but cultures retained about 25% of metabolic activity, even after 3 log (cfu/ml) reduction in cell counts.

Bozoglu et al. (2004) studied the effect of high pressure treatments on two Gram positive (*L. monocytogenes* and *S. aureus*) and two Gram negative (*E. coli* O157:H7 and *S. enteritidis*) bacteria inoculated in milk and the formation of injured cells. The pathogens were pressurized at 350, 450 and 550 MPa for 10 min and stored at 4, 22 and 30ºC over a period of four weeks. Except for *L. monocytogenes* where there was survival at 350 MPa (colony formation on both selective and non-selective agars), pathogens were either inactivated or injured at all pressures studied. Gao et al. (in press) employed response surface methodology (RSM) to create a quadratic equation to predict the optimum process parameters for a 6 log (cfu/ml) reduction of *L. monocytogenes* inoculated in milk buffer. The conditions found to obtain this reduction with this microorganism were a pressure treatment at 448 MPa for 11 min at 41°C.

Moreover, several researchers have studied the potential of combining high hydrostatic pressure treatments with antimicrobial compounds to reduce the pressures required for microbial inactivation in milk. Morgan et al. (2000) evaluated the combination of high pressure (150-600 MPa, 30 min, 25ºC) and lacticin 3147 (10,000 AU/ml) to improve the inactivation of *S. aureus* ATCC6538, inoculated in 10% reconstituted skimmed milk,

and *L. innocua* DPC1770, inoculated in 20% reconstituted demineralised whey powder. The results showed a more than additive effect when both treatments were used in combination for both bacterial species. In the case of *S. aureus*, up to 200 MPa little or no effect on viability was observed. Above this pressure, a rapid decline in viability occurred, with approximately a 6 log (cfu/ml) kill being detected at 300 MPa. *L. innocua* demonstrated results similar to those observed for the other strain up to 200 MPa. At 275 MPa, a 3 log (cfu/ml) reduction in viable cell numbers was observed and, at 325 MPa, viable cells were not detectable and the reduction in population was greater than 6 log (cfu/ml).

García-Graells et al. (2003) studied the inactivation of different bacteria (*E. coli* MG1655 and LMM1010, *S. typhimurium*, *P. fluorescens*, *S. aureus*, *Enterococcus faecalis*, *L. innocua* and *L. plantarum*) by high pressure in skimmed milk supplemented with the lactoperoxidase-hydrogen peroxide-thiocyanate (LP) system at naturally concentration. In absence of pressure treatment, the effect of LP system varied with the bacterial strain from bactericidal (*P. fluorescens*) to bacteriostatic or inhibitory (*E. coli* MG1655, *L. innocua*, *S.aureus*, *L. plantarum* and *E. faecalis*), or no effect (*S. typhimurium* and *E. coli* LMM1010). The presence of the LP system affected inactivation by high pressure in a cell density dependent manner. At low concentration (10^6 cfu/ml), the LP system strongly increased high pressure inactivation except *E. coli* LMM1010 (pressure resistant strain) and at high concentration (10^9 cfu/ml) only inactivation of *L. innocua*, *E. faecalis* and *L. plantarum* were enchanced at relatively mild pressures (400-450 MPa). The behaviour of Gram negative (*E. coli* and *P. fluorescens*) and Gram positive bacteria (*L. innocua* and *L. viridescens*) associated with milk, in response to application of high pressure (250–500 MPa, 5 min, 20ºC) combined with nisin (0, 250, or 500 IU/ml) was evaluated by Black et al. (2005). These authors found that the combination of high pressure and nisin not only showed a synergistic effect to enhance the level of inactivation for bacteria in milk but also an effective effect in sensitising *E. coli* and *P. fluorescens* to this bacteriocin.

1.9 Effect of high hydrostatic pressure on microorganisms in cheese

In cheese, differences in the degree of microbial inactivation obtained applying high hydrostatic pressure could be due to the species and the quantity of starter cultures used as well as cheese acidity and composition (O'Reilly et al., 2001). Several authors have studied the effect of high hydrostatic pressure alone or combined with bacteriocins on microorganisms and spores indigenous or inoculated in different types of cheese to control microbial growth.

Capellas et al. (1996) elaborated fresh cheese inoculated with *E. coli* CECT 405. Samples were treated using combinations of pressure (400-500 MPa), temperature (2, 10 or 25ºC) and time (5, 10 or 15 min) and subsequently stored at 2-4 ºC. No survival of *E. coli* cells were detected 1 day after pressurisation, except in samples treated for 5 min at 25ºC at pressures of 400-450 MPa while *E. coli* in the untreated control cheese remained at a concentration around 10^8 cfu/g. No surviving *E. coli* were detected in cheese treated at 400-500 MPa for 5-15 min at 2, 10 or 25ºC after 15, 30 or 60 days of storage. O'Reilly et al. (2000) studied the effect of high pressure on the inactivation of three microbial contaminants (*E. coli*, *S. aureus* and *Penicillium roqueforti*) in Cheddar cheese, cheese slurry inoculated together at a level of 10^7 and 10^6 cfu/g, respectively. Cheese slurries (combination of 100 g of Cheddar cheese curd chips and 42 g of 10% sterile saline solution) were subjected to pressures of 50 to 800 MPa for 20 min at temperatures of 10, 20 and 30ºC. At 400 MPa, the viability of *P. roqueforti* in cheese slurry decreased over 2 log (cfu/g) at 10ºC and around 6 log (cfu/g) at temperatures of 20 and 30ºC. *S. aureus* and *E. coli* were not detected after high pressure treatments in cheese slurries at pressures higher than 600 MPa at 20ºC and 400 MPa at 30ºC, respectively. Cheddar cheeses were treated at 100 to 500 MPa for 20 min at 20ºC and they found that the organisms were more sensitive to pressure in cheese than in slurry, specially *E. coli*.

The effectiveness of high pressure treatment alone or combined with the addition of nisin or lysozyme in reducing levels of *Bacillus cereus* in model cheeses elaborated with raw milk has been evaluated for López-Pedemonte et al. (2003). For this purpose, model cheeses were submitted to a germination cycle of 60 MPa at 30°C for 210 min or/and to a vegetative cells destruction cycle of 300 or 400 MPa at 30°C for 15 min.

These authors found that the combination of both cycles improved the efficiency of the whole treatment and the highest inactivation (2.4 log [cfu/g]) was obtained with the presence of nisin when cheeses were treated at 400 MPa. Arqués et al. (2005) reported a synergistic effect of high hydrostatic pressure treatments combined with bacteriocins (lacticin 481, nisin Z, nisin A, non-characterized bacteriocin TAB 57, non-characterized bacteriocin TAB 7, enterocin I and enterocin AS-48) to inactivate *S. aureus* inoculated in cheese elaborated with raw milk and ripened for 60 days at 12ºC. For this purpose, cheeses were treated on day 2 or day 50 at 300 MPa (10ºC, 10 min) or 500 MPa (10ºC, 10 min). Rodríguez et el. (2005) found similar effects using the same bacteriocins that Arqués et al. (2005) but, in this case, studying *E. coli* O157:H7.

Some authors have studied the effect of high hydrostatic pressure treatments on acid lactic bacteria. O'Reilly et al. (2002) worked with cheese starters (*L. lactis* strains 303, 223, 227 and AM2) and subjected them to high pressure (100–400 MPa , 25ºC, 20 min) either in citrate buffer (pH 5.3), the same buffer containing 4.5% NaCl, or in cheese manufactured using each strain individually as a starter. Inactivation and cell lysis (release of lactate) of each strain were examined post-pressurisation. These authors found that starter bacteria were more pressure tolerant in cheese than in buffer and the application of pressures over 200 MPa caused significant inactivation of starter cultures in Cheddar cheese (differing between strains at 400 MPa) but without inducing autolysis. Wick et al. (2004) studied the effect of high pressure to control the ripening process of Cheddar cheese. For this purpose, 1 and 4 months old Cheddar cheeses were subjected to pressures ranging from 200 to 800 MPa for 5 min at 25ºC. The number of viable *L. lactis* (starter) and *Lactobacillus* (non-starter) cells decreased as pressure increased. For the pressure treated cheeses at 300, 500 and 800 MPa after 1 month of aging, *L. lactis* population did not change significantly during incubation (24 weeks). But in the case of untreated and cheeses treated at 200 MPa the population of *L. lactis* began to decrease after 84 days of keeping cheeses at 10ºC. Viable *Lactobacillus* cells in 500 and 800 MPa treated cheeses were below the detection limit of 10 cfu/g throughout the sampling period. For cheeses pressurized at 4 months of age, all the pressure treatments (500, 600, 700 and 800 MPa) resulted in significant reductions in starter culture levels. Slight increase in cell numbers occurred over time in moderate pressure treatments (500 and 600 MPa), while no changes occurred in the highest

pressure treatments (700 and 800 MPa). Pressure treatments of 500, 600, 700 and 800 MPa reduced the *Lactobacillus* population to undetectable levels (detection limit of 10 cfu/g) where it remained during incubation at 10ºC. Counts just above the detection limit were found in the 500 and 700 MPa samples after 24 weeks. Non-starter bacteria can have a positive effect on cheese flavour; thus, the early inactivation of *Lactobacillus* in Cheddar cheese ripening (after applying pressures over 400 MPa) may cause a dramatic change in cheese flavour development and proteolysis during ripening.

Moreover, some researchers applied high hydrostatic pressure treatments directly on milk before making cheese. Drake et al. (1997) made Cheddar cheese with with raw, pasteurized (72ºC, 15 s) and high pressure treated milk. Microbiological quality of pressurized milk and cheese was comparable to pasteurized milk and cheeses. However, a small number of coliforms were only detected in pressurized milk suggesting the authors that these coliforms could have been injured and unable to survive the cheesemaking process. Buffa et al. (2001) made cheese with raw, pasteurized (72ºC, 15 s) and high pressure treated (500 MPa, 20ºC, 15 min) goat milk to compare their bacteriological quality during ripening time (45 days). The cell counts obtained for the groups studied (total bacteria, psychrotrophic bacteria, Enterobacteriaceae, Lactobacilli, Lactococci, Enterococci, Micrococcaceae) in cheese samples made with pasteurized and pressure treated milk were similar.

2. Aim of this dissertation and Sampling protocol

2.1 Aim of this dissertation

The objective of this dissertation was to evaluate the effect of high hydrostatic pressure and the ability for survival, repair and growth after the treatment of three human pathogenic microorganisms (*Y. enterocolitica, S. enterica* and *E. coli*) inoculated in skimmed milk (only in the case of *Y. enterocolitica* strains) and in model cheese produced with or without starter culture. Moreover, the variation in pressure resistance between different serotypes of the same organism and the kinetics of microbial inactivation (only in milk, for one of the most baroresistant strains of *Y. enterocolitica*) were determined.

2.2 Sampling Protocol

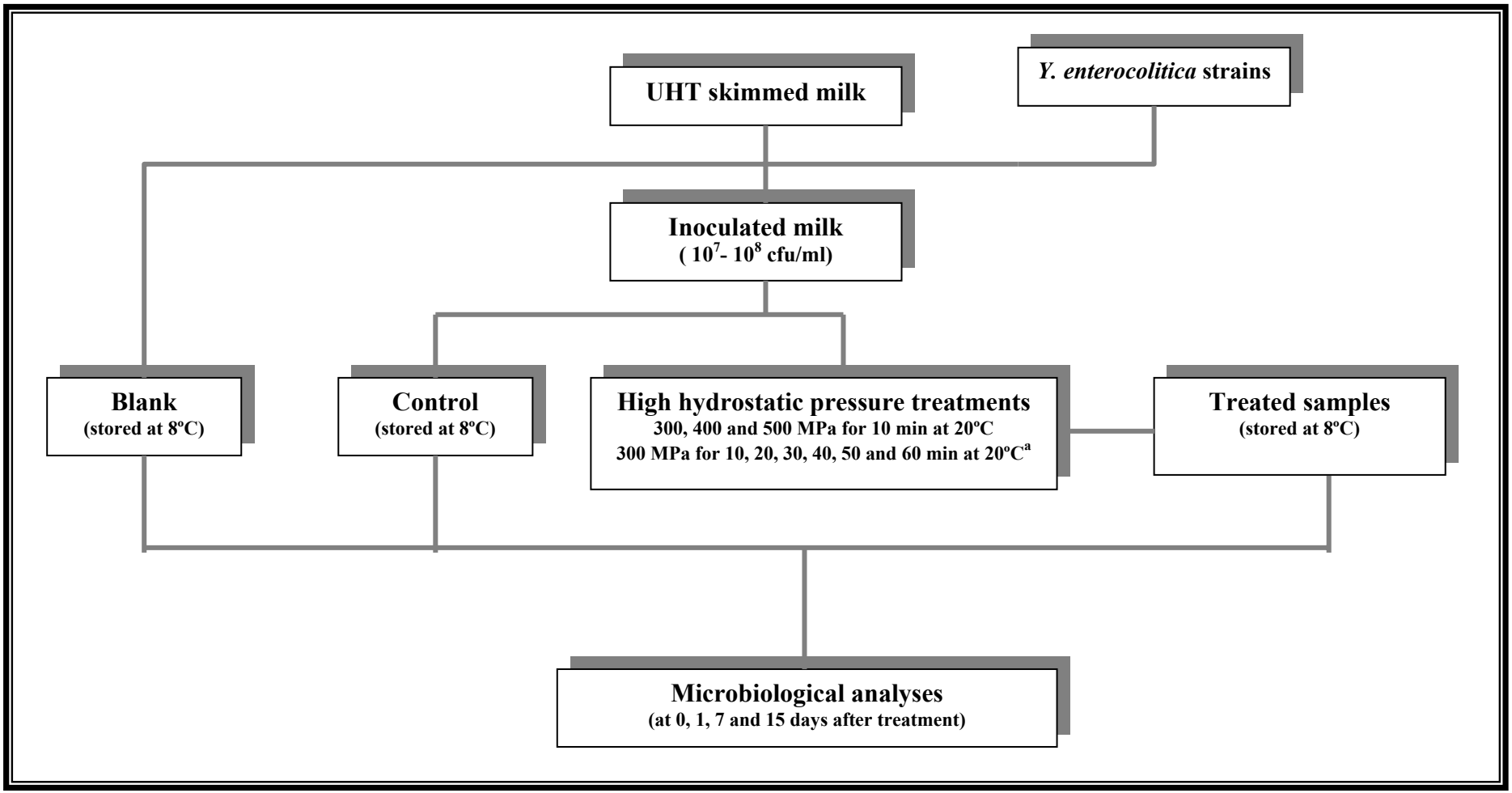
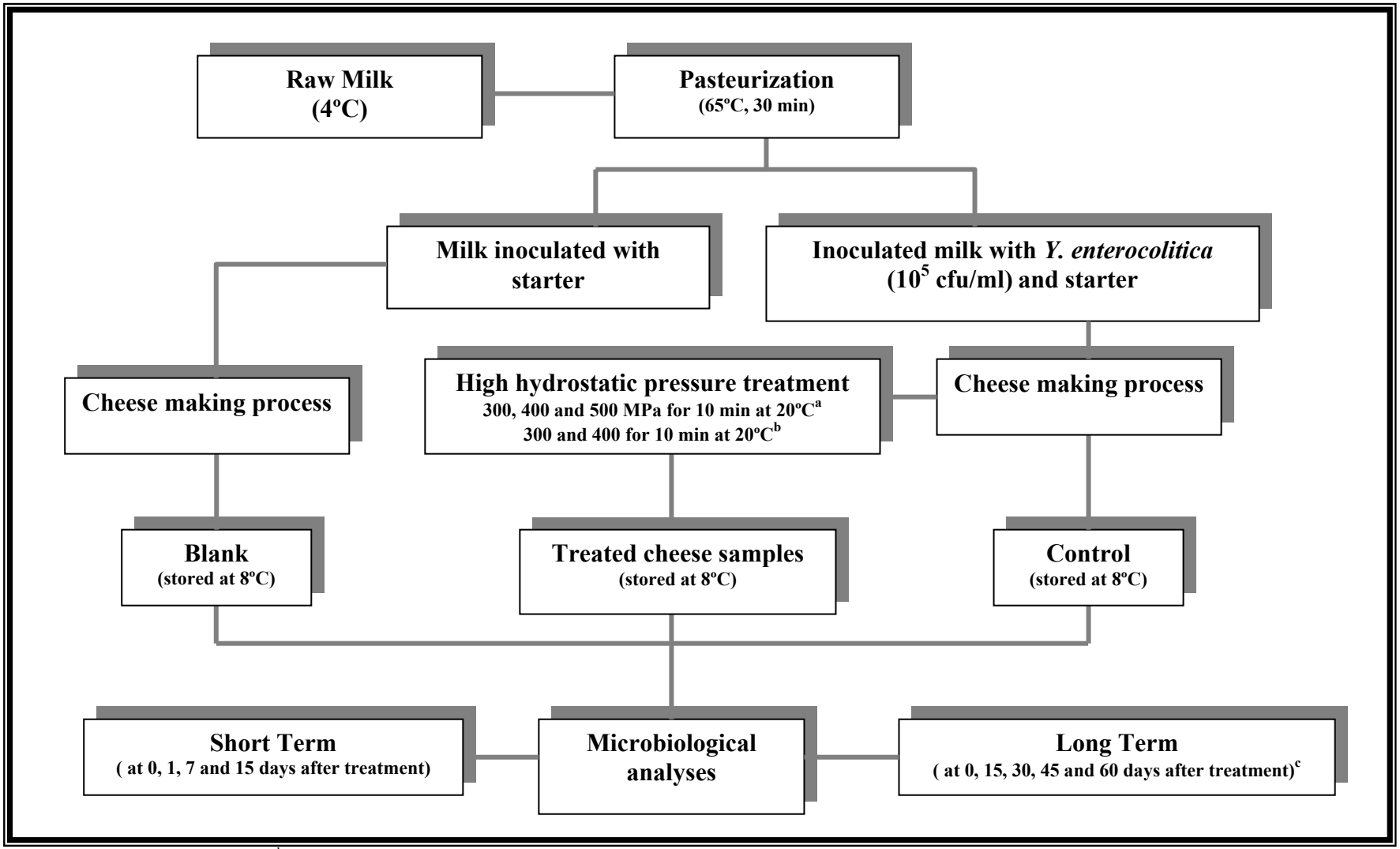
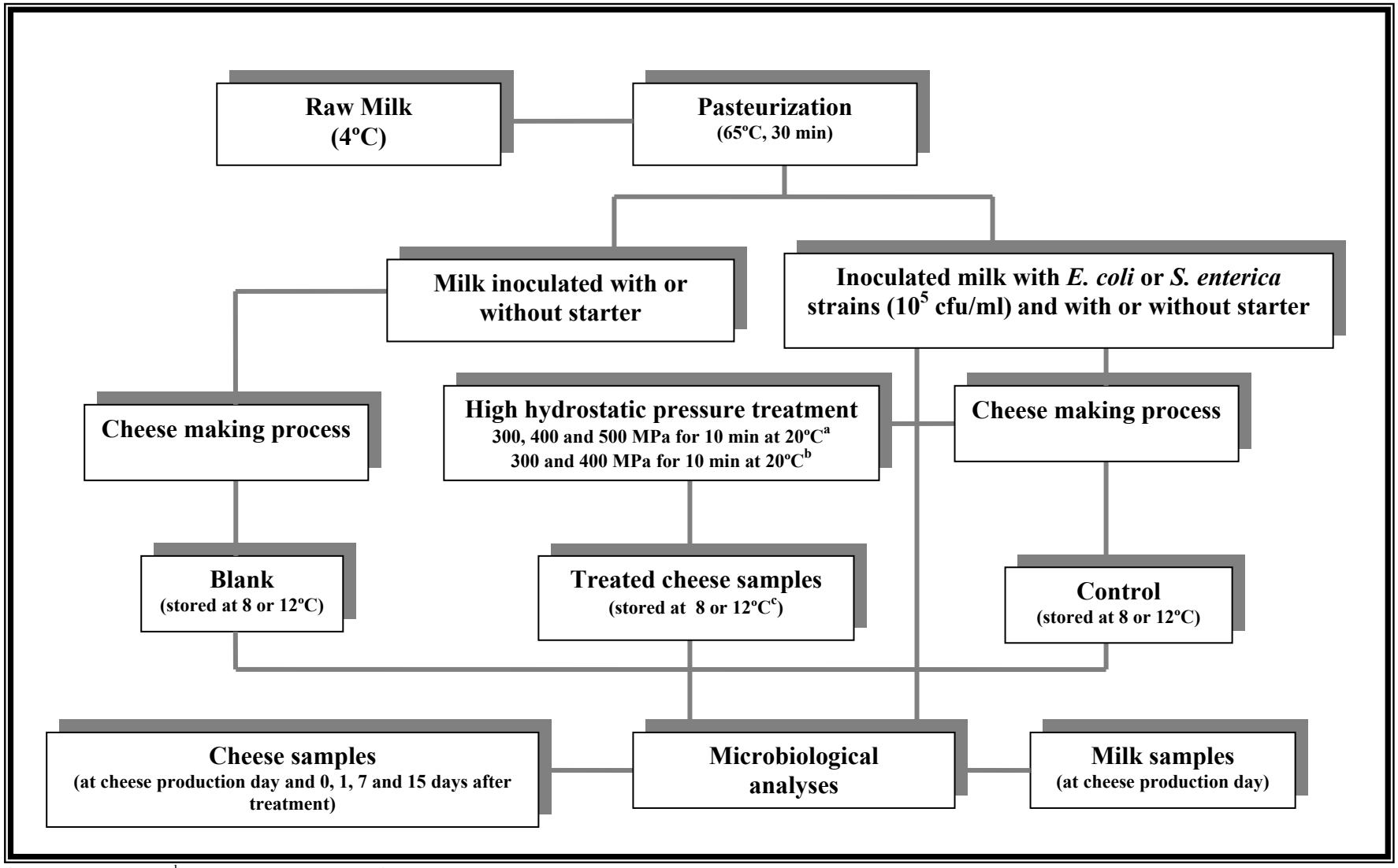


Figure 3. *Milk experiments.* [a]For kinetic study



[a] For short term experiments; [b] For long term experiments; [c]Only with CECT 4055 strain (serotype O:3).

Figure 4. *Model cheese experiments with* Y. enterocolitica *strains.*



[a]For *E. coli* strains; [b] For *S. enterica* strains; [c]8ºC for *E. coli* strains and 12ºC for *S. enterica* strains.

Figure 5. *Model cheese experiments with* E. coli *and* S. enterica *strains*

3. Materials and Methods

3.1 Bacterial strains

The bacteria used in this research are shown in **Table 5.**

Table 5. *Bacterial strains*

Microorganisms	Serotype	Culture	Source
Yersinia enterocolitica	O:1	[a]CECT 559	chinchilla
Yersinia enterocolitica	O:3	CECT 4055	blood culture
Yersinia enterocolitica	O:8	CECT 4054	blood of human patient
Yersinia enterocolitica	O:9	CECT 754	human faeces
Salmonella enterica	Enteritidis	[b]ATCC 13076	-
Salmonella enterica	Thypimurium	ATCC 13311	human faeces
Escherichia coli	O59:H21	CECT 405	-
Escherichia coli	O157:H7	CECT 5947	-

[a]CECT: Spanish Type Culture Collection, Universidad de Valencia, Valencia, Spain.
[b]ATCC: American Type Culture Collection, University Boulevard, Manassas, USA.

All the strains were moisturized following the protocol provided by CECT or ATCC and were kept in cryobeads (Nalgene® System 100™, Microkit Iberica S.L., Madrid, Spain) at –20ºC.

3.2 Inocula preparation

Strains were revived by placing one cryobead in 10 ml brain heart infusion broth (BHI, Oxoid, Basingtoke, UK) supplemented with 0.6% of yeast extract (BHIYE, Oxoid) and incubated for 24 h at 30ºC in the case of *Y. enterocolitica* CECT 4055 and CECT 4054 strains and at 37ºC for the rest of the strains. One ml of the first culture was transferred to 10 ml of BHIYE and incubated for an additional 18 h at the same temperatures, to allow the culture to reach the stationary phase. Then, cells were centrifuged at 1,250 x g for 15 min at room temperature, washed once in 10 mM phosphate-buffered saline (PBS, Oxoid) and the pellets were resuspended in PBS to a final concentration of about 10^8-10^9 cfu/ml.

3.3 Starter culture preparation

A mixture of commercial lyophilized strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ezal MAO 11, Rhodia Iberia S.A., Madrid, Spain), that is known as a non-bacteriocin producer, was used as a starter culture for the washed-curd model cheese manufacture. The culture was revived inoculating 0.015 g of mixture in 1,000 ml of commercial sterilized skimmed milk and incubated at 30ºC for 24 h. A volume of 50 ml was used to prepare a subculture in 200 ml of sterilized skimmed milk, which was incubated under the same temperature and time conditions.

3.4 Sample preparation

3.4.1 Skimmed milk

These experiments were performed only with *Y. enterocolitica* strains. An aliquot of the inoculum was added to UHT skimmed milk to obtain a final concentration near 10^7-10^8 cfu/ml. Thirty milliliters of milk were dispensed into low density polyethylene bottles (Azlon, Bibby Sterilin Ltd, Staffordshire, UK) avoiding the formation of air bubbles and the caps were sealed with Parafilm. Bottles were twice vacuum packaged in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) before applying the high hydrostatic pressure treatment.

3.4.2 Model cheese manufacture

The protocol for model cheese production (López-Pedemonte et al, 2003; **Figure 6**) has been adapted from the procedures proposed by Shakeel-Ur-Rehman et al. (1998) and Hynes et al. (2000). Raw cow's milk obtained from a local farm was transported and stored at 4ºC. Before inoculation, milk was pasteurized at 65ºC for 30 min and cooled to 32ºC in an ice and water bath. Two per cent (vol/vol) of starter culture and 0.01% (vol/vol) of a 35% $CaCl_2$ dilution (Arroyo, Santander, Spain) were added. Milk was kept in a water bath at 32ºC for 20 min and then inoculated with approximately 1% (vol/vol) of initial inoculum (except blanks). 0.02% (vol/vol) of liquid rennet extract of bovine origin (520 mg/l of active chymosin, Arroyo, Santander, Spain) was added as coagulating agent. Centrifugation bottles (Nalgene®, Nalge Nunc International, Rochester, USA) were filled with 225 ml of inoculated milk and placed in a bath at 32ºC for 40 min until curd was formed. Curd was cut into small pieces and kept in the water bath while temperature was increased to 37ºC for over 5 min. The bottles were kept in the bath at 37ºC for further 15 min. Curds were washed to avoid excessive acidification, substituting 40% (vol/vol) of whey with sterile water. Bottles were then centrifuged at 7,000 *x* g for 40 min at 20ºC and kept in their containers in a water bath at 37ºC until pH 5.5 was reached. When pH of the resulting cheeses dropped to 5.5 (only in the case of cheese made with starter), whey was completely removed from the bottles by decantation and cheeses were salted by adding, in each bottle, 100 ml of 20% (wt/vol) NaCl sterile brine for 15 min. After this, cheeses were removed from the centrifugation bottles using sterile pincers and dried on sterile paper. They had a final weight of approximately 23 g with 55% of dry matter and 1.5% of salt in moisture content. In the case of model cheeses produced without starter culture, the same protocol without adding starter culture was followed. Cheeses were twice vacuum packed in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) and stored overnight at 8ºC (in the case of *Y. enterocolitica* and *E. coli* strains) and at 12ºC (for *Salmonella* strains) before high hydrostatic pressure treatment.

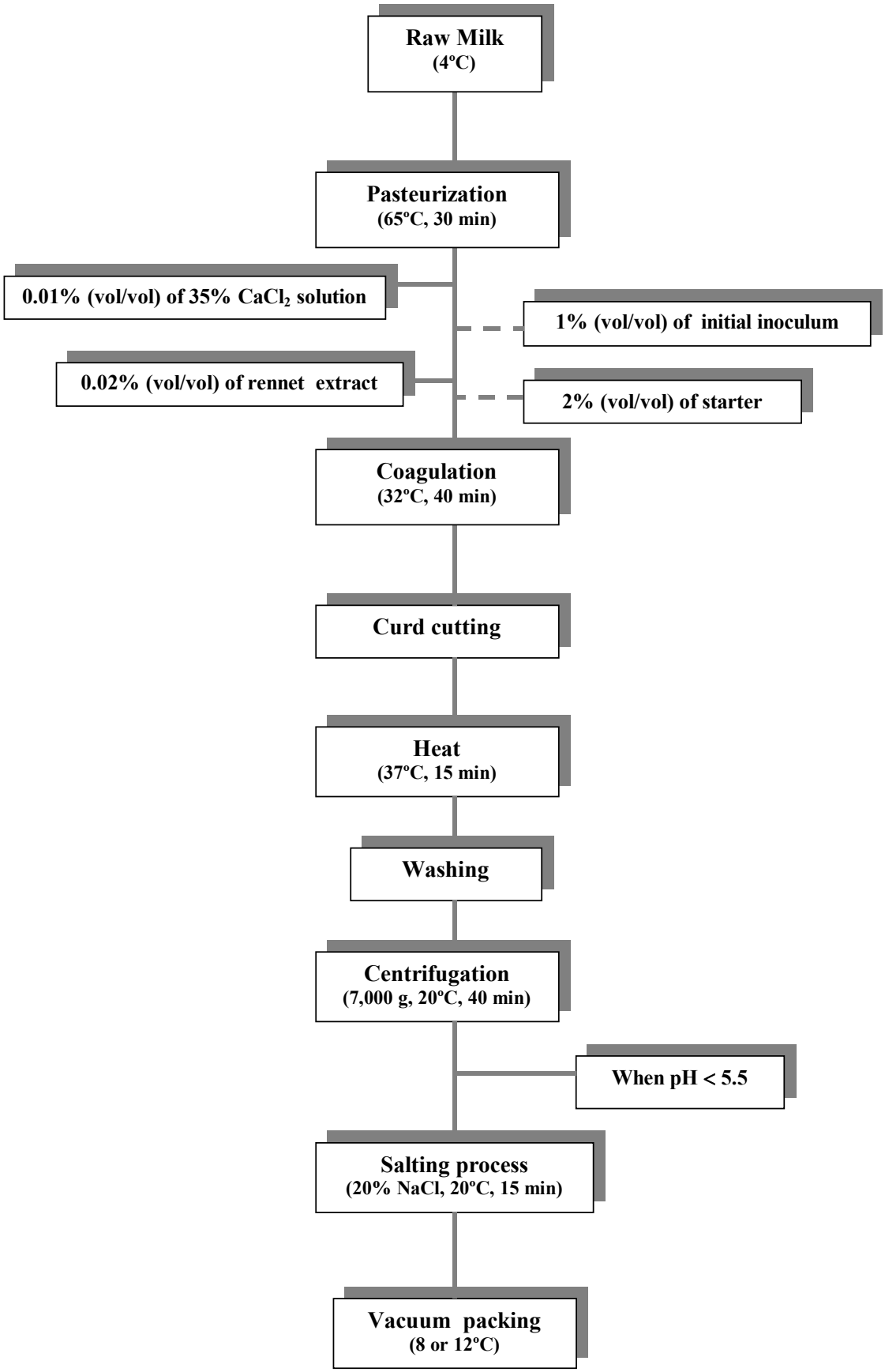


Figure 6. *Cheese making process.*

3.5 High pressure treatment

The details of the three high pressure equipments used in this research and the conditions of the treatments applied in milk and cheese samples are showed in **Table 6**.

3.6 Microbiological analyses

3.6.1 Skimmed milk

Ten-fold dilutions from each sample of skimmed milk were made in maximum recovery diluent (Oxoid, MRD). One milliliter of the appropriate dilution was plated into brain heart infusion agar (Oxoid, BHI) plates containing 0.6% of yeast extract (BHIAYE), to count both injured and non-injured cells, and into BHIAYE plates supplemented with 2% NaCl (Panreac, Panreac Química S.A, Montcada i Reixac, Spain), to count only non-injured cells (Patterson et al., 1995). Plates were incubated at 32ºC for 48 h. Representative colonies (two from each plate) were picked and inoculated onto a BHIYE plate and incubated at 32ºC for 24 h before being identified using biochemical tests (API 20E, Bio-Mérieux, Marcy-L'Etolie, France).

3.6.2 Model cheese

Ten g of each sample were homogenized in 90 ml of MRD for 1 min using an electromechanical blender (BagMixer®, Interscience, St Nom, France) at room temperature. Ten-fold serial dilutions were produced in MRD and 1 ml of appropriate dilution was plated into Selective culture medium (SCM) to count non-injured cells. A modification of the thin agar layer method (TAL_m) (Kang and Fung, 1999) was used to count both injured and non-injured cells to a detection level of 10 cfu/g. After a selective medium layer, the first layer (7 ml) of nutritive medium was added. Then, 1 ml of appropriate dilution of cheese sample and a second layer (7 ml) of nutritive medium was added.

The culture media and the incubation conditions used for cheese samples are shown in **Table 7.**

To check the presence of injured cells in cheese samples that did not show colonies growing in both culture media, an enrichment procedure was used. For this purpose, the first dilution of each sample obtained after high hydrostatic pressure treatment and after 1, 7 and 15 days of storage in the case of *E. coli* and *S. enterica* strains, and after 15, 30, 45 and 60 days in the case of *Y. enterocolitica* strains, was stored at the right temperature for 24 h. A loopful of this culture was streaked onto a plate of selective culture medium and incubated at the appropriate temperature for 24 h.

3.7 Expression of results

Results are expressed as the logarithm of colony-forming units (cfu) per ml or g, in milk and cheese, respectively. Increase value during cheese manufacture was calculated as the difference between the logarithms of colony counts of control cheese samples (N_c) and milk samples (N_m) analysed at the end of cheese production (log N_c- log N_m). Decrease value was calculated as the difference between the logarithms of colony counts of the control (N_0) and treated samples at day 0 (log N_o- log N). All the experiments were run three times with duplicate analyses each time.

Lethality is the common word to express the reductions obtained after applying high pressure treatments (the difference between the logarithms of colony counts of the control and treated samples at day 0). Several authors (including ourselves) have used this term, but we are now persuaded that it is more appropriate to express reductions in cell counts with the expression decrease value, because traditional culture media are not able to recover all the viable population present in the samples due to the formation of injured cells.

3.8 pH measurement

Model cheese pH was determined using a penetration electrode (Crison, Crison Instruments S.A., Alella, Spain). pH values were based on the mean of nine measurements.

3.9 Statistical analyses

Analysis of variance was performed using the General Linear Models Procedure of SAS® software (SAS® System for Windows™, 8.02, 1999, SAS Institute, Inc., Cary, North Carolina, USA). Tukey and Student-Newman-Keuls tests were used to obtain paired comparisons among sample means. Level of significance was set at $P < 0.05$.

3.10 Kinetic study of microbial inactivation

The decimal reduction time (D value) was calculated from the absolute value of the inverse of the slope of the linear-regression equations by plotting log numbers of survivors *versus* time (FDA/CFSAN, 2000).

Table 6. *Details of high hydrostatic pressure equipments and the conditions of the treatments applied.*

Equipment	Pressure chamber		Microorganisms	Samples	Treatments
	Diameter (cm)	Length (cm)			
ACB (Nantes, France)	10	25	*Y. enterocolitica* strains	Milk	A) 300, 400 and 500 MPa for 10 min at 20ºC B) 300 MPa for 10, 20, 30, 40, 50 and 60 min at 20ºC
			E. coli strains	Cheese	A) 300, 400 and 500 MPa for 10 min at 20ºC
Stansted (Stansted, UK)	3.5	12	*Y. enterocolitica* strains	Cheese	A) 300, 400 and 500 MPa for 10 min at 20ºC
ACB (Nantes, France)	30	70	*S. enterica* strains	Cheese	A) 300 and 400 MPa for 10 min at room temperature

Table 7. *Culture media and incubation conditions used for cheese samples analyses.*

Microorganisms	Selective culture medium (SCM)	Nutritive culture medium (NCM)	Conditions
Y. enterocolitica	*Yersinia* cefsulodin-irgasan-novobiocin agar (Oxoid, CIN)	BHIAYE[a]	SCM 32ºC 48 h TAL_m[b] 32ºC 48 h
E. coli	Sorbitol McConkey agar containing selective cefixime tellurite supplement (Oxoid, SMC)	BHIAYE	SCM 37ºC 24 h TAL_m 37ºC 24- 48 h
S. enterica	*Salmonella* Chromogenic agar base containing *Salmonella* Selective Supplement (Oxoid, SC)	BHIAYE	SCM 37ºC 24 h TAL_m 37ºC 24- 48 h
Lactococcus lactis	M17 agar (Oxoid, M17) supplemented with 5% (wt/vol) of lactose	-	SCM 30ºC 48 h

[a]BHIAYE, brain heart infusion agar supplemented with 0.6% of yeast extract
[b]TAL_m, modification of thin agar layer method

4. Papers included in the dissertation

4.1 Survival and growth of *Yersinia enterocolitica* strains inoculated in skimmed milk treated with high hydrostatic pressure

Sílvia De Lamo-Castellví, Artur X. Roig-Sagués, Marta Capellas, Manuela Hernández-Herrero and Buenaventura Guamis

International Journal of Food Microbiology (2005), 102:337-342.

Available online at www.sciencedirect.com

SCIENCE DIRECT®

International Journal of Food Microbiology 102 (2005) 337–342

INTERNATIONAL JOURNAL OF Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Short communication

Survival and growth of *Yersinia enterocolitica* strains inoculated in skimmed milk treated with high hydrostatic pressure

Sílvia De Lamo-Castellví, Artur X. Roig-Sagués*, Marta Capellas, Manuela Hernández-Herrero, Buenaventura Guamis

Centre Especial de Recerca Planta de Tecnologia dels Aliments, CeRTA, XiT, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària Edifici V, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain

Received 25 July 2004; received in revised form 24 September 2004; accepted 30 November 2004

Abstract

Four human pathogenic strains of *Yersinia enterocolitica* (serotypes O:1, O:3, O:8, and O:9) were inoculated (7–8 log CFU/ml) in UHT skimmed milk and treated at 300, 400, and 500 MPa for 10 min at 20 °C, and then kept at 8 °C to assess their evolution for 15 days. Treatments at 400 and 500 MPa caused the highest lethality, generally reaching counts below detection level (1 CFU/ml) in the culture media. At 300 MPa, the most baroresistant serotypes were O:3 and O:8. After 15 days of storage at 8 °C, *Y. enterocolitica* showed growth over 8 log (CFU/ml) in all treatments. Kinetic study of microbial inactivation in skimmed milk was performed with serotype O:8 at 300 MPa, showing a tailing after 35 min of pressure treatment.

Keywords: *Yersinia enterocolitica*, skimmed milk, high hydrostatic pressure; Sublethally injured cells

1. Introduction

Yersinia enterocolitica has been associated with a human gastro-intestinal disease called yersiniosis (Schiemann, 1987). In Europe, Canada, and Japan, the majority of cases are due to *Y. enterocolitica* serotype O:3 and, to a lesser extent, to serotype O:9 (Andersen et al., 1991). In the United States, recent outbreaks suggested a serotype shift from O:8 to O:3 (Ackers et al., 2000). In Spain, O:3 is the only serotype identified in human yersiniosis cases (Anonymous, 2001).

Although pigs are regarded as major reservoirs of pathogenic *Y. enterocolitica*, this organism has been frequently isolated from raw milk, which is being implicated in several outbreaks of yersiniosis (Tacket et al., 1984; Larkin et al., 1991). The psychrotrophic nature of this organism is of particular significance in milk and milk products that are normally stored at low temperatures. In raw milk, *Y. enterocolitica* strains were able to survive in the presence of high numbers

* Corresponding author. Tel.: +34 93 581 14 60; fax: +34 93 581 14 94.
E-mail address: ArturXavier.Roig@uab.es (A.X. Roig-Sagués).

0168-1605/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2004.11.025

of competing microorganisms and were able to maintain the virulence plasmid during extended storage at refrigeration temperatures (Larkin et al., 1991).

High hydrostatic pressure (HHP) is currently considered as an attractive non-thermal process for preserving food. HHP could induce a number of changes to morphology, biochemical reactions, and genetic mechanisms, and to cell membrane and the wall of microorganisms, but do not cause negligible impairment of organoleptical and nutritional value (Hoover et al., 1989). However, a considerable variation in pressure resistance within strains of the same species has been demonstrated (Patterson et al., 1995). Several authors have detected sublethally injured cells after high-pressure treatments (Metrick et al., 1989; Patterson et al., 1995; Simpson and Gilmour, 1997; McClements et al., 2001; Chen and Hoover, 2003), suggesting that these cells may be able to repair themselves under favourable conditions. This potential repairing is problematic and has particular significance for psychrotrophic foodborne pathogens that can survive and grow at refrigeration temperatures (McClements et al., 2001).

The main aim of this study was to investigate the response to HHP of different strains of *Y. enterocolitica* in skimmed milk belonging to different serotypes involved in human diseases. The ability of this pathogen for survival, repair, and growth in refrigerated storage after pressure treatment was also examined. One of the most baroresistant strains was selected to estimate its kinetics of microbial inactivation in milk.

2. Materials and methods

2.1. Inocula preparation

Four strains of *Y. enterocolitica* were obtained from the Spanish Type Culture Collection (CECT, Universitat de Valencia, Valencia, Spain): CECT 559 (serotype O:1), CECT 4055 (serotype O:3), CECT 4054 (serotype O:8), and CECT 754 (serotype O:9). Strains were revived in 10 ml of Brain Heart Infusion broth (BHI; Oxoid, Unipath, Basingtoke, UK) containing 0.6% yeast extract (BHIYE, Oxoid) and incubated at 30 °C (strains CECT 4055 and CECT 4054) or 37 °C (strains CECT 559 and CECT 754) for 24 h. 1 ml of the first culture was transferred to 10 ml of BHIYE and incubated for an additional 18 h at the same temperatures to let the culture reach the stationary phase. Then cells were centrifuged at 1250×*g* for 15 min at room temperature, washed once in 10 mM phosphate-buffered saline (PBS; Oxoid), and the pellets were resuspended in PBS to a final concentration of approximately 10^8–10^9 CFU/ml.

2.2. Sample preparation

An aliquot of the inoculum was added to commercial sterilized (UHT) skimmed milk to obtain a final concentration about 10^7–10^8 CFU/ml. 30 ml volumes of milk were dispensed into low-density polyethylene bottles (Azlon, Bibby Sterilin, Staffordshire, UK), avoiding air bubbles, and caps were sealed with Parafilm (Pechiney Plastic Packaging, Neenah, USA). Bottles were double vacuum-packed in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) before applying high-pressure treatment.

2.3. High-pressure treatment

Milk samples were pressurized in a discontinuous isostatic press (ALSTOM, Nantes, France) with a pressure chamber of 10 cm diameter and 23 cm length. The time needed to achieve maximum pressure (500 MPa) was 2 min. The pressure chamber and the pressurization medium inside were adjusted to 20 °C with a constant flow of water. Samples were held in a water bath at 20 °C for 10 min to reach the treatment temperature. The temperature of the pressurization fluid was measured by a thermocouple. Skimmed milk samples were pressurized at 300, 400, and 500 MPa for 10 min at 20 °C and skimmed milk samples for kinetic studies were treated at 300 MPa and 20 °C for 10, 20, 30, 40, 50, and 60 min.

2.4. Sampling protocol

Three groups of skimmed milk samples were analysed: non-inoculated (blank samples); inoculated with *Y. enterocolitica* but not treated by HHP (control samples); and inoculated and HHP-treated (pressurized samples). All samples were stored at 8 °C after high-pressure treatment. Microbiological analyses

were performed at 0 (just after HHP treatment), 1, 2, 4, 7, 10, and 15 days after treatment. For kinetic study of microbial inactivation, samples were analysed at day 0. Experiments were run three times with duplicate analysis in each replicate.

2.5. Microbiological analyses

Tenfold dilutions from each sample of skimmed milk were made in Maximum Recovery Diluent (MRD; Oxoid). 1 ml of the appropriate dilution was plated into Brain Heart Infusion Agar (BHIA; Oxoid) plates containing 0.6% yeast extract (BHIAYE, Oxoid) to count both injured and non-injured cells, and into BHIAYE plates supplemented with 2% NaCl (Panreac, Panreac Quimica SA, Montcada i Reixac, Spain) to count only non-injured cells (Patterson et al., 1995). Plates were incubated at 32 °C for 48 h. Representative colonies (two from each plate) were picked and inoculated onto a BHIYE plate and incubated at 32 °C for 24 h before being identified using biochemical tests (API 20E, BIO-Mérieux, Marcy-L'Etoile, France). Results are expressed as the logarithm of colony-forming units (CFU) per ml.

2.6. Statistical analyses

Analysis of variance was performed using the General Linear Models Procedure of SAS® software (SAS® System for Windows™, 8.02, 1999; SAS Institute, Cary, North Carolina, USA). Tukey and Student–Newman–Keuls tests were used to obtain

Table 1
Mean (n=6) and standard deviation of counts in BHIAYE and BHIAYE supplemented with 2% of NaCl of four strains of *Yersinia enterocolitica* inoculated in skimmed milk and pressurized at 20 °C for 10 min

Time (days)	Treatment (MPa)	O:1		O:3		O:8		O:9	
		BHIAYE	BHIAYE+2% NaCl	BHIAYE	BHIAYE+2% NaCl	BHIAYE	BHIAYE+2% NaCl	BHIAYE	BHIAYE+2% NaCl
0	0	7.72±0.14	7.76±0.19	7.88±0.15	7.48±0.20	8.10±0.19	8.04±0.14	7.97±0.43	7.49±0.21
	300	3.71±0.82	3.43±0.72	5.25±1.45	4.22±2.06	5.24±0.43	4.92±0.35	3.85±0.56	3.31±0.77
	400	0.72±1.12	n.d.	0.37±0.58	n.d.	0.20±0.31	n.d.	0.59±0.92	0.53±0.87
	500	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1	0	8.26±0.05	7.41±0.64	8.62±0.28	8.37±0.15	8.61±0.28	8.24±0.39	8.68±0.29	8.22±0.19
	300	4.40±0.55	4.09±0.63	5.81±1.40	5.47±1.73	5.80±0.58	5.63±0.54	4.75±0.34	4.24±0.35
	400	1.21±1.65	0.90±1.39	1.20±1.10	n.d.	0.43±0.87	0.51±0.57	0.99±1.53	n.d.
	500	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	0	–	–	–	–	–	–	–	–
	300	5.37±0.72	5.23±0.98	6.56±1.51	6.65±1.73	6.78±0.66	7.00±0.95	5.30±0.53	5.46±0.55
	400	2.96±1.05	2.70±0.99	1.20±1.39	0.87±1.01	1.34±1.55	1±1.07	1.93±2.23	1.18±2.05
	500	n.d.	n.d.	n.d.	n.d.	0.90±1.03	0.61±1.05	n.d.	n.d.
4	0	–	–	–	–	–	–	–	–
	300	7.93±0.54	7.71±0.74	8.25±0.74	8.04±0.65	8.52±0.17	8.29±0.36	8.31±0.36	7.64±0.95
	400	5.23±1.98	5.06±1.94	3.38±0.22	3.10±0.17	4.88±1.48	4.60±1.53	4.00±2.03	4.41±2.76
	500	0.95±1.09	0.62±0.97	0.65±0.73	0.43±0.60	1.41±2.63	1.58±2.86	0.73±1.14	0.31±0.97
7	0	–	–	–	–	–	–	–	–
	300	8.99±0.04	8.74±0.03	8.96±0.11	8.75±0.12	8.98±0.12	8.68±0.07	8.99±0.10	8.74±0.10
	400	7.63±0.94	7.17±1.45	6.99±0.73	6.58±0.55	8.04±0.51	7.68±0.73	7.75±0.78	7.43±1.06
	500	1.46±2.26	1.18±1.84	2.14±0.28	1.18±0.93	3.55±4.03	3.45±4.08	1.46±2.17	1.18±1.84
10	0	–	–	–	–	–	–	–	–
	300	8.83±0.12	8.74±0.12	8.90±0.13	8.84±0.13	9.01±0.14	8.98±0.15	8.88±0.11	8.44±0.62
	400	8.64±0.43	8.51±0.38	8.58±0.29	8.21±0.46	8.73±0.10	8.46±0.59	8.87±0.37	8.60±0.51
	500	2.58±4.00	2.56±3.97	5.69±0.95	5.13±1.06	4.96±3.45	4.64±3.33	4.05±3.48	3.52±3.48
15	0	9.11±0.21	8.84±0.10	8.70±0.44	9.07±0.13	9.02±0.14	8.89±0.16	8.93±0.16	8.26±0.37
	300	8.84±0.05	8.80±0.11	8.99±0.10	8.96±0.10	8.94±0.09	8.98±0.04	8.89±0.12	8.58±0.14
	400	8.99±0.10	8.86±0.10	9.03±0.25	9.00±0.21	9.09±0.21	9.04±0.19	9.10±0.11	8.81±0.11
	500	5.69±4.40	5.54±4.43	8.82±0.21	8.72±0.32	8.55±0.29	8.79±0.28	5.81±4.51	5.68±4.41

n.d.=not detected. Level of detection=1 CFU/ml.

paired comparisons among sample means. Level of significance was set at $P<0.05$. Three replicate experiments were run, with duplicate analysis in each of them.

2.7. Kinetic study of microbial inactivation

The decimal reduction time (D value) was calculated from the absolute value of the inverse of the slope of the linear regression equations by plotting log numbers of survivors versus time (FDA/CFSAN, 2000). R^2 value was the criterion used to compare the two mathematical models. The higher the R^2 value, the better the adequacy of the model to describe data (Chen and Hoover, 2003).

3. Results and discussion

3.1. High-pressure effect at day 0

Treatments at 400 and 500 MPa proved to be effective for all the studied strains (Table 1). Although there were no significant differences ($P>0.05$) between these two treatments, a few viable cells were still observed in the culture medium after 400 MPa treatment. Statistical differences ($P<0.05$) were observed in the lethality values of the four strains treated at 300 MPa. Serotypes O:3 and O:8 were clearly more baroresistant than O:1 and O:9. This variation in pressure resistance within strains of the same species has already been reported in other Gram-negative bacteria (Patterson et al., 1995; Benito et al., 1999).

3.2. Behaviour of Y. enterocolitica after HHP

Skimmed milk samples treated at 300 MPa showed growth over 8 log units after approximately 4 days of storage at 8 °C, reaching stationary phase. 400 MPa treatment caused more lethality than 300 MPa, but it is important to point out that although in three strains (O:1, O:8, and O:9) two out of three of the replicates did not offer counts at day 0, cells started to grow between days 1 and 4. At 500 MPa, cells started to grow between days 1 and 10 (mostly at day 4), except in one replicate of strains O:1 and O:9 that only started growing after day 15.

There were no significant differences ($P>0.05$) between *Y. enterocolitica* counts in BHIAYE and BHIAYE supplemented with 2% NaCl in any treat-

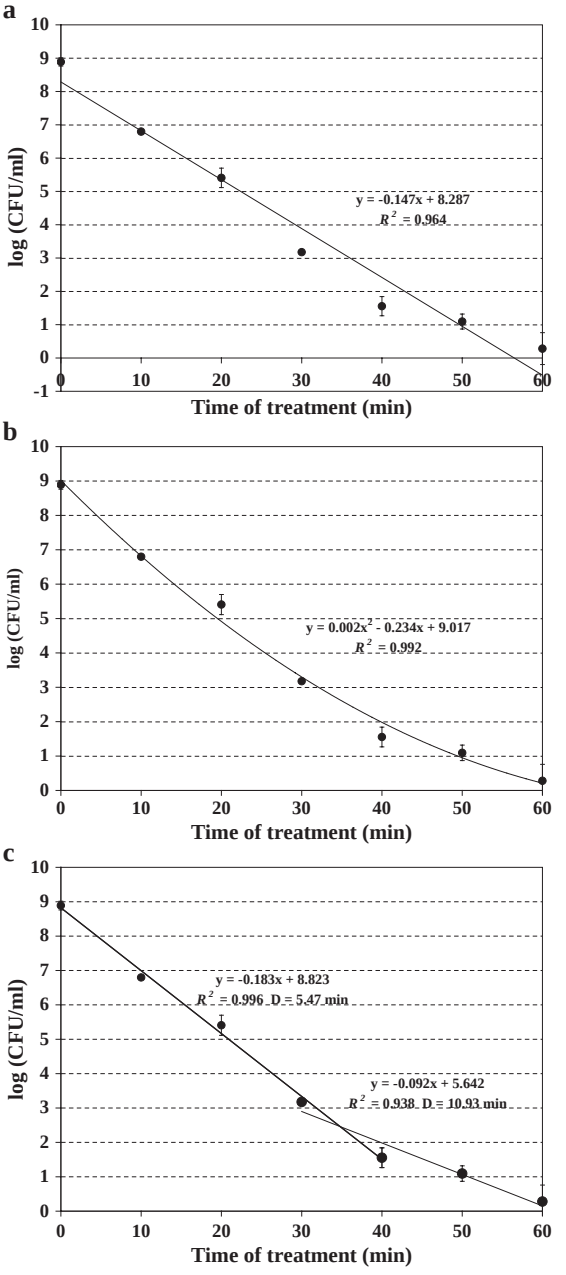


Fig. 1. Mathematical models of kinetics of microbial inactivation of *Y. enterocolitica* serotype O:8 at 300 MPa and 20 °C: (a) linear regression; (b) quadratic adjustment; and (c) simplification of quadratic adjustment with D values.

ment. This would suggest that the treatment at 300 MPa did not cause a significant number of injured cells in any of the strains studied. Nevertheless, at 500 MPa, the fact that cells started to grow after some days suggests that the treatment had injured them seriously and they needed time to repair themselves. The considerably high repair and growth abilities of *Y. enterocolitica* in milk may be due to the nutritive nature of the medium and its liquid nature combined with the psychrotrophic behaviour of the pathogen. Metrick et al. (1989) reported that *Salmonella* spp. strains were able to repair themselves in chicken but not in a buffer with the same conditions of pressure treatment and storage. Apparently, the lack of available nutrients in buffer prevented the cells from repairing. Moreover, Ellenberg and Hoover (1999) reported that growth of pressure-treated *Y. enterocolitica* stored at 30 °C was more rapid in TSB than in pork and suggested that injured cells may obtain nutrients more easily and quickly from liquid media. The presence of injured cells that needed time to repair and grow has also been observed in heat-treated milk (Kushal and Anand, 1999).

3.3. Kinetic study of microbial inactivation

One of the most baroresistant strains in milk (serotype O:8) was selected to determine the kinetics of population reduction and to determine the *D* value. Two mathematical models were compared: linear regression and quadratic adjustment. Quadratic adjustment was the mathematical model that better fitted the results (R^2=0.992). In order to calculate *D* values, quadratic adjustment could be represented by two equations obtained by linear regression. In consequence, two *D* values were established (Fig. 1).

Some authors have already described this kinetic trend (Gervilla, 2001; Chen and Hoover, 2003), naming it as tailing (Metrick et al., 1989) and suggesting that it is due to stabilization of the remaining population. Patterson et al. (1995) stated that for that reason, it was not possible to calculate conventional *D* values as a method to determine relative pressure sensitivities of different organisms. Chen and Hoover (2003) reported a strong tailing in the survival curves of *Y. enterocolitica* obtained at four pressure levels (300, 350, 400, and 450 MPa) in PBS and whole milk. They indicated that the best models for predicting pressure inactivation of this organism were the nonlinear regression and the Weibull models. Although this behaviour may suggest that cells had the ability to adapt to the medium in HHP conditions, being more pressure-resistant, Metrick et al. (1989) did not find significant differences after HHP treatment when comparing the resistance to pressure between the remaining population and the original culture of *Salmonella* spp.

Acknowledgements

This study was supported financially by CAL-00-005-C2-1 project (Instituto Nacional de Investigaciones Agrarias) and by the *Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya*, which provided S. De Lamo-Castellví a fellowship to carry out this investigation. We would like to thank the Spanish Type Culture Collection for providing *Y. enterocolitica* strains.

References

Ackers, M.L., Schoenfeld, S., Markman, J., Smith, G., Nicholson, M.A., DeWitt, W., Cameron, D.N., Griffin, P.M., Slutsker, L., 2000. An outbreak of *Yersinia enterocolitica* O:8 infections associated with pasteurized milk. J. Infect. Dis. 181, 1834–1837.

Andersen, J.K., Sørensen, R., Glensbjerg, M., 1991. Aspects of the epidemiology of *Yersinia enterocolitica*: a review. Int. J. Food Microbiol. 13, 217–231.

Anonymous, 2001. *Yersinia enterocolitica*. Casos notificados al Sistema de Información Microbiológica. Años 1989–2001. BOE 9, 305–312.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat and other stresses. Appl. Environ. Microbiol. 65, 1564–1569.

Chen, H., Hoover, D.G., 2003. Pressure inactivation kinetics of *Yersinia enterocolitica* ATCC 35669. Int. J. Food Microbiol. 87, 161–171.

Ellenberg, L., Hoover, D.G., 1999. Injury and survival of *Aeromonas hydrophila* 7965 and *Yersinia enterocolitica* 9610 from high hydrostatic pressure. J. Food. Saf. 19, 263–276.

FDA/CFSAN: Food and Drug Administration Center for Food Safety and Applied Nutrition. June 2, 2000. Overarching principles: kinetics and pathogens of concern for all technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-over.html.

Gervilla, R., 2001. Estudio de los tratamientos por alta presión hidrostática en la leche de oveja. Departament Ciència Animal i dels Aliments. Universitat Autònoma de Barcelona, Barcelona.

Hoover, D.G., Metrick, C., Papineau, A.M., Farkas, D.F., Knorr, D., 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol. 43, 99–107.

Kushal, R., Anand, S.K., 1999. Repair and recovery of thermally injured cells of *Yersinia enterocolitica* in milk. J. Food Prot. 62, 1203–1205.

Larkin, L.L., Vasavada, P.C., Marth, E.H., 1991. Incidence of *Yersinia enterocolitica* in raw milk as related to its quality. Milchwissenschaft 46, 500–502.

McClements, J.M., Patterson, M.F., Linton, M., 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. J. Food Prot. 64, 514–522.

Metrick, C., Hoover, D.G., Farkas, D.F., 1989. Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. J. Food Sci. 54, 1547–1564.

Patterson, M.F., Quinn, M., Simpson, R., Gilmour, A., 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. J. Food Prot. 58, 524–529.

Schiemann, D.A., 1987. *Yersinia enterocolitica* in milk and dairy products. J. Dairy Sci. 70, 383–391.

Simpson, R.K., Gilmour, A., 1997. The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. Food Microbiol. 14, 567–573.

Tacket, C.O., Narain, J.P., Sattin, R., Lofgren, J.P., Konigsberg, C.J., Rendtorff, R.C., Rausa, A., Davis, B.R., Cohen, M.L., 1984. A multistate outbreak of infections caused by *Yersinia enterocolitica* transmitted by pasteurized milk. J. Am. Med. Assoc. 251, 483–486.

4.2 Behavior of *Yersinia enterocolitica* strains inoculated in model cheese treated with high hydrostatic pressure

Sílvia De Lamo-Castellví, Marta Capellas, Tomás López-Pedemonte,
M. Manuela Hernández-Herrero, Buenaventura Guamis
and Artur X. Roig-Sagués.

Journal of Food Protection (2005), 68:528-533.

Journal of Food Protection, Vol. 68, No. 3, 2005, Pages 528–533

Behavior of *Yersinia enterocolitica* Strains Inoculated in Model Cheese Treated with High Hydrostatic Pressure

SILVIA DE LAMO-CASTELLVÍ, MARTA CAPELLAS,* TOMÁS LÓPEZ-PEDEMONTE, M. MANUELA HERNÁNDEZ-HERRERO, BUENAVENTURA GUAMIS, AND ARTUR X. ROIG-SAGUÉS

Centre Especial de Recerca Planta de Tecnologia dels Aliments (CeRTA), Xarxa Innovació Technològica, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària Edifici V, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

MS 04-272: Received 16 June 2004/Accepted 25 September 2004

ABSTRACT

The effects of high hydrostatic pressure treatment and the ability for survival, repair, and growth of three human pathogenic serotypes (O:1, O:3, O:8) of *Yersinia enterocolitica* were investigated in washed-curd model cheese made with pasteurized bovine milk. Samples were treated at 300, 400, and 500 MPa for 10 min at 20°C and analyzed at 0, 1, 7, and 15 days to assess the viability of the *Yersinia* population. A long-term study (up to 60 days of ripening after high hydrostatic pressure treatment) was also undertaken. Treatments at 400 and 500 MPa caused maximum lethality, and only the treatment at 300 MPa showed significant differences ($P < 0.05$) between serotypes; the most baroresistant was O:3. Ability to repair and grow was not observed after 15 days of storage at 8°C. *Yersinia* counts in untreated cheese samples also decreased below the detection limit at day 45 in the long-term study. These results suggest that the cheese environment did not allow recovery of injured cells or growth. A primary contributing factor to this effect seemed to be the low pH resulting from the production of lactic acid during cheese ripening.

High hydrostatic pressure (HHP) is viewed as one of the more promising nonthermal methods for inactivation of microorganisms in food. HHP induces a number of changes to morphology, biochemical reactions, genetic mechanisms, cell membrane, and wall of microorganisms *(7).* Gram-negative bacteria and cells in exponential growth phase are, respectively, more sensitive than gram-positive bacteria and stationary-phase cells *(1).* The effect of the matrix during treatment *(15, 20, 21, 25)* and its effect after treatment during the recovery of the microorganisms *(24)* determine microbiological safety and stability in food. Sublethally injured cells have been detected after high pressure treatments *(14, 15, 21, 23, 25),* suggesting these cells may be able to recover given favorable conditions, such as prolonged storage in a suitable substrate.

Yersinia enterocolitica is considered to be a foodborne pathogen associated with the human disease called yersiniosis, gastroenteritis being the most frequently clinical manifestation *(22, 27).* This organism has been classified into approximately 60 serotypes, but pathogenic significance in humans is mainly associated with just a few of them: O:3, O:9, O:8, and O:5,27. In Europe, Canada, and Japan, the majority of cases of yersiniosis are caused by *Y. enterocolitica* serotype O:3 and to a lesser extent by O:9. In the United States the most frequently isolated serotype is O:9, followed by O:5,27 *(10).*

The psychrotrophic nature of this organism is of particular significance in milk and milk products that are normally stored at low temperatures. Yersiniosis outbreaks from cheese have not been reported, although milk has been implicated in several outbreaks of yersiniosis *(2, 16, 26),* and some studies have shown the ability of *Y. enterocolitica* to grow in Brie cheese *(12)* and survive in Turkish Feta cheese *(6).*

In some studies, the effect of high hydrostatic pressure on inoculated or indigenous microorganisms and spores in different types of cheese (slurries, fresh, and ripened) has been evaluated *(17),* but more research needs to be undertaken in human pathogens like *Y. enterocolitica.* We chose to work with washed-curd model cheeses *(13, 28).* This allowed us to produce cheeses inoculated with pathogenic microorganisms in our laboratory under controlled bacteriological conditions.

The objectives of this study were to determine the response to HHP of three human pathogenic serotypes (O:1, O:3, O:8) of *Y. enterocolitica* in model cheese, as well as the variation in pressure resistance between different serotypes of the same organism, and the ability of this pathogen for survival, repair, and growth in refrigerated storage after pressure treatment.

MATERIALS AND METHODS

Bacterial strains. Three strains of *Y. enterocolitica* were obtained from the Spanish Type Culture Collection (CECT; Universidad de Valencia, Valencia, Spain): CECT 559 (serotype O:1), CECT 4055 (serotype O:3), and CECT 4054 (serotype O:8).

The following protocol has been adapted from the one proposed by Patterson et al. *(21).* Strains were revived by placing one cryobead (Nalgene System 100, Microkit Iberica S.L., Madrid, Spain) in 10 ml of brain heart infusion broth (Oxoid, Basingstoke, UK) containing 0.6% yeast extract (Oxoid). Strains CECT 4055 and CECT 4054 were incubated at 30°C and CECT 559 at 37°C for 24 h. One milliliter of the first culture was trans-

* Author for correspondence. Tel: (34) 93 581 14 46; Fax: (34) 93 581 14 94; E-mail: Marta.Capellas@uab.es.

ferred to 10 ml of brain heart infusion broth containing 0.6% yeast extract and incubated for an additional 18 h at the same temperatures to allow the culture to reach the stationary phase. Cells were centrifuged at 1,250 × *g* for 15 min at room temperature and washed once in 10 mM phosphate-buffered saline (Oxoid), and the pellets were resuspended in phosphate-buffered saline to a final concentration of about 10^8 to 10^9 CFU/ml.

Starter preparation. A mixture of commercial liofilized strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ezal MAO 11, Rhodia Iberia S.A., Madrid, Spain) was used as a starter culture for the model cheese manufacture. The culture was revived inoculating 0.015 g of starter in 1,000 ml of commercial sterilized skimmed milk and incubated at 30°C for 24 h. A volume of 50 ml was used to prepare a subculture in 200 ml of sterilized skimmed milk, which was incubated under the same temperature and time conditions.

Model cheese manufacture. The protocol for model cheese production was developed by López-Pedemonte et al. *(13).* Raw cow's milk obtained from a local farm was transported and stored at 4°C. Before inoculation, milk was pasteurized at 65°C for 30 min and cooled to 32°C in an ice and water bath. We added 2% (vol/vol) of starter culture and 0.01% (vol/vol) of a 35% $CaCl_2$ dilution (Arroyo, Santander, Spain). Milk was kept in a water bath at 32°C for 20 min and then inoculated with 1% (vol/vol) of *Y. enterocolitica* inoculum (except blanks). We added 0.02% (vol/vol) of liquid rennet extract of bovine origin (520 mg/liter of active chymosin, Arroyo) as a coagulating agent. Centrifugation bottles (Nalge Nunc International, Rochester, N.Y.) were filled with 225 ml of inoculated milk and placed in a bath at 32°C for 40 min until curd was formed. Curd was cut into small pieces and kept in the water bath, and the temperature was increased to 37°C over 5 min. The bottles were kept in the bath at 37°C for 15 min more. Curds were washed to avoid excessive acidification, substituting 40% (vol/vol) of whey with sterile water. Bottles were then centrifuged at 7,000 × *g* for 40 min at 20°C and kept in their containers in a water bath at 37°C until pH 5.5 was reached. When the pH of the resulting cheeses dropped to 5.5, whey was completely removed from the bottles by decantation and cheeses were salted by adding, in each bottle, 100 ml of 20% (wt/vol) NaCl sterile brine for 15 min. After this, cheeses were removed from the centrifugation bottles using sterile pincers and dried on sterile paper. They had a final weight of approximately 23 g with 55% of dry matter and 1.5% of salt in moisture content. Cheeses were twice vacuum packed in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) and stored at 8°C for 15 to 18 h before high pressure treatment.

High pressure treatment. Cheese samples were pressurized in a discontinuous isostatic press (Stansted Fluid Power Ltd., Stansted, UK) with a pressure chamber measuring 3.65 cm in diameter and 24 cm in length. The pressure chamber and the pressurization medium inside were adjusted to 20°C with a constant flow of water. Samples were placed in a water bath at 20°C for 10 min to reach the treatment temperature. Cheese samples were pressurized at 300, 400, and 500 MPa for 10 min at 20°C, and samples for long-term analysis were treated at 300 and 400 MPa for 10 min at 20°C.

Sampling protocol. Three groups of cheese samples were analyzed: noninoculated (blank samples), inoculated with *Y. enterocolitica* but not treated by HHP (control samples), and inoculated and treated (pressurized samples). All samples (treated and untreated) were stored at 8°C. Microbiological analyses were performed at 0 (immediately after HHP treatment), 1, 7, and 15 days after treatment. Long-term samples were analyzed at day 0 (immediately after HHP treatment), 15, 30, 45, and 60 days. Experiments were run three times with duplicate analysis each time.

Microbiological analyses. Ten grams of each sample was homogenized in 90 ml of maximum recovery diluent (Oxoid) for 1 min using an electromechanical blender (BagMixer, Interscience, France) at room temperature. Tenfold serial dilutions were prepared in maximum recovery diluent (Oxoid), and 1 ml of appropriate dilution was plated into *Yersinia* cefsulodin-irgasan-novobiocin agar (CIN, Oxoid) to count noninjured cells. A modification of the thin agar layer method (TAL) *(9)* was used to count both injured and noninjured *Y. enterocolitica* cells to a detection limit of 10 CFU/g. After a selective medium (CIN) layer, the first layer of nutritive medium (brain heart infusion broth containing 0.6% yeast extract) was added. Then, 1 ml of appropriate dilution of cheese sample and a second layer of nutritive medium was added. All plates were incubated at 32°C for 48 h. Representative colonies of *Y. enterocolitica* (two from each plate) were picked and inoculated onto brain heart infusion broth containing 0.6% yeast extract plates and incubated at 32°C for 24 h before identification using a biochemical test (API 20E, Bio-Mérieux, Marcy-L'Etoile, France).

Results are expressed as log CFU per gram. Lethality was calculated as the difference between the logarithms of colony counts of the untreated and treated samples ($\log N_0 - \log N$).

An enrichment procedure was used to check the presence of injured *Y. enterocolitica* cells in cheese samples for long-term analyses. First dilution of cheese was stored at 30°C for 24 h. A loopful of this culture was streaked onto a plate of CIN and incubated at 32°C for 24 h. Growing colonies were identified using biochemical tests (API 20E).

pH measurement. Model cheese pH was determined using a penetration electrode (Crison, Crison Instruments S.A., Alella, Spain). pH values were based on the mean of nine measurements.

Statistical analyses. Analysis of variance was performed using the general linear models procedure of SAS software (SAS System for Windows, 8.02, 1999, SAS Institute, Inc., Cary, N.C.). Tukey and Student-Newman-Keuls tests were used to obtain paired comparisons among sample means. Level of significance was set at $P < 0.05$.

RESULTS

High pressure effect at day 0. Lethality results obtained immediately after HHP treatments in model cheeses with *Y. enterocolitica* serotypes O:1, O:3, and O:8 are shown in Table 1. Treatments of 400 and 500 MPa caused the highest lethality, reaching counts below the detection level (10 CFU/g) in all serotypes. Thus, lethality values of both treatments depended on the level of *Y. enterocolitica* in the samples at that moment. It is important to point out that control samples at day 0 showed different counts (Table 1), although the initial inocula used during cheese manufacture were similar in all samples (approximately 5 log CFU/ml). The only treatment that allowed the detection of differences ($P < 0.05$) between strains was at 300 MPa, where serotype O:3 appeared to be more baroresistant than O:1 and O:8.

Behavior of *Y. enterocolitica* after HHP treatment. Any of the *Y. enterocolitica* strains inoculated in samples treated at 400 and 500 MPa showed repair ability after 15

TABLE 1. *Counts in CIN and lethality values of three strains of* Yersinia enterocolitica *inoculated in model cheese and pressurized at 300, 400, and 500 MPa at 20°C for 10 min at day 0*

Serotype	Treatment (MPa)	Counts (log CFU/g)[a]		Lethality (log N_0 − log N)	
		Mean	CI	Mean[b]	CI
O:1	0	4.36	±0.41	—	—
	300	ND	—	≥3.36 B	±0.41
	400	ND	—	≥3.36 B	±0.41
	500	ND	—	≥3.36 B	±0.41
O:3	0	6.03	±0.10	—	—
	300	4.09	±0.50	1.95 C	±0.45
	400	ND	—	≥5.03 A	±0.17
	500	ND	—	≥5.03 A	±0.17
O:8	0	5.28	±0.40	—	—
	300	1.80	±0.51	3.48 B	±0.17
	400	ND	—	≥4.28 A	±0.40
	500	ND	—	≥4.28 A	±0.40

[a] CI, confidence interval; ND, not detected. Level of detection: 1 log CFU/g.
[b] Means ($n = 6$) with different letters are significantly different ($P < 0.05$).

days of storage at 8°C, their counts remaining below the detection level in both culture media (CIN and TAL). Serotype O:1 treated at 300 MPa (Fig. 1a) was not able to repair, and serotypes O:3 and O:8 treated at 300 MPa (Fig. 1b and 1c) showed a decrease in their cell counts after day 0 in both cases. None of the controls had the ability to grow after 15 days of ripening at 8°C (Fig. 1a, 1b, and 1c).

The comparison of the behavior of control counts in the two culture media used to evaluate injured and noninjured *Y. enterocolitica* cells (Fig. 1a, 1b, and 1c) showed different sensitivity at low pH between serotypes (Table 2). In serotypes O:1 and O:3, cell counts of control samples in TAL were significantly ($P < 0.05$) higher than in CIN. Moreover, serotype O:8 seemed to be more resistant to acid conditions because there were not differences between CIN and TAL cell counts and control counts remained stable during the 15 days of storage at 8°C (Fig. 1c), while serotypes O:3 and especially O:1 were sensitive to acid conditions and control counts decreased over time (Fig. 1a and 1b).

To confirm this decreasing trend, a long-term study was conducted (60 days). The most baroresistant serotype in cheese at day 0 (O:3) was selected for this purpose (Fig. 2). Samples treated at 300 and 400 MPa did not show repair ability during the 60 days of ripening at 8°C. *Y. enterocolitica* counts decreased below the detection level after 15 and 30 days in CIN and TAL culture media, respectively, in cheeses treated at 300 MPa. However, one of three replicates showed growth (after the enrichment treatment) at day 45. Control samples did not show ability to grow, and their cell counts progressively decreased, reaching the detection level after 45 and 60 days in CIN and TAL, respectively.

DISCUSSION

It is clear from the lethality values obtained that 400 and 500 MPa were effective treatments to reduce the initial population of *Y. enterocolitica* in all serotypes. Other studies have evaluated the effect of HHP on inoculated microorganisms in cheese. Capellas et al. *(3)* elaborated fresh cheese inoculated with *Escherichia coli* CECT 405. Samples were treated using combinations of pressure (400 to 500 MPa), temperature (2, 10, or 25°C), and time (5, 10, or 15 min) and subsequently stored at 2 to 4°C. No survival of *E. coli* was detected 1 day after pressurization, except in samples treated for 5 min at 25°C at pressures of 400 to 450 MPa.

The differences detected in pressure resistance values between serotypes had already been reported in other gram-negative bacteria *(21)*, but it is important to point out that, in our experiment, these differences would be due mainly to one factor: the low pH of cheese. The matrix was continuously in evolution due to the conversion of lactose in lactic acid by lactic acid bacteria. The three serotypes used in this experiment showed different sensitivity to low pH, and these differences were detected through the variations in control sample cell counts in the two culture media (TAL method and CIN) used in the experiment to evaluate the presence of injured and noninjured cells. Wu et al. *(29)* found that TAL was an adequate method to enumerate acid injured *Y. enterocolitica.*

In a previous survey *(4)*, we examined extensively the effect of HHP treatment on the same serotypes of *Y. enterocolitica* used in this study and also the effect together with serotype O:9 inoculated in skimmed milk. Comparing lethality values of each serotype at day 0 in milk and cheese we only found differences among matrices in serotype O:3, which was more baroresistant in cheese than in milk. Several authors have reported that certain foods could protect microorganisms from pressure inactivation with factors such as a more favorable osmotic pressure and readily available supply of nutrients: *Salmonella* spp. were more sensitive to pressure treatment in phosphate buffer than in richer media such as chicken baby food *(15).* Ultrahigh-temperature milk and raw milk provide a protective effect and lessen cell death to *Listeria monocytogenes* when compared with phosphate-buffered saline after HHP treatments *(25).* Patterson et al. *(21)* found as well that *L. monocyto-*

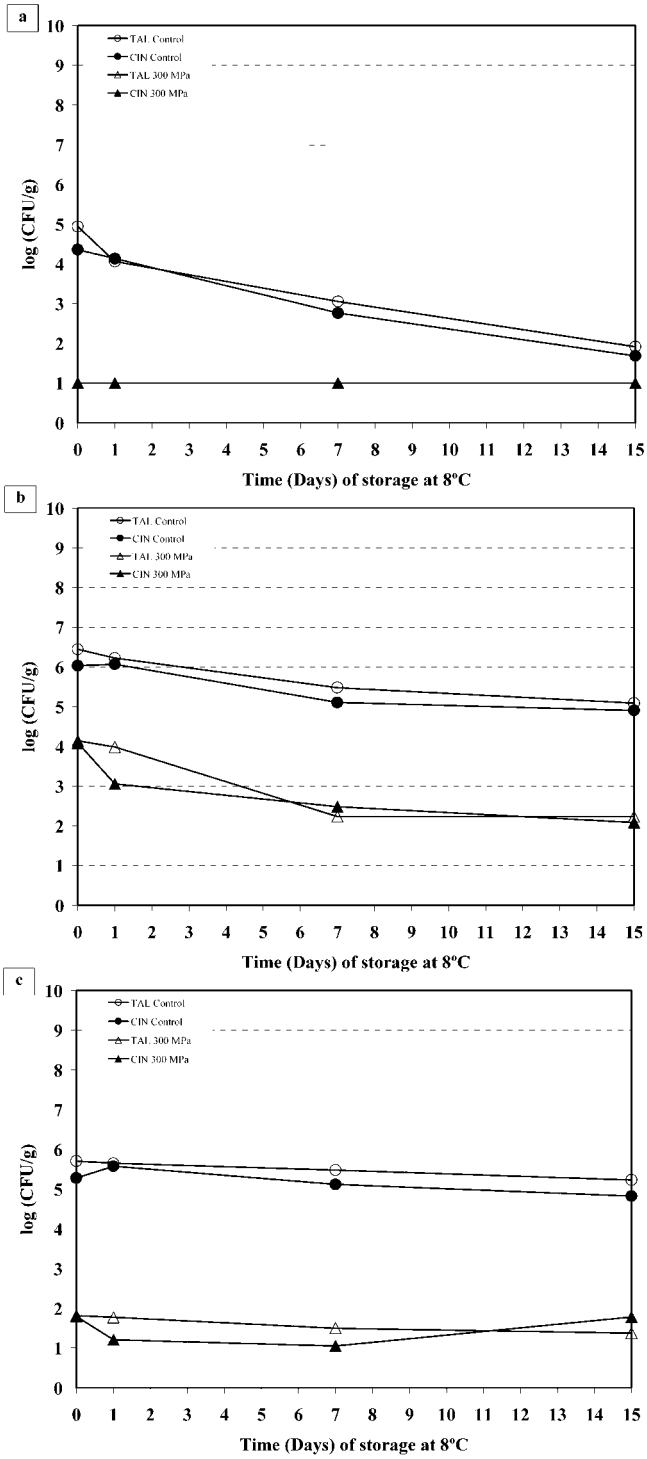


FIGURE 1. *Behavior of* Y. enterocolitica *inoculated in model cheese after HHP treatments at 300 MPa for 10 min at 20°C: (a) serotype O:1, (b) serotype O:3, and (c) serotype O:8.*

TABLE 2. *Evolution of pH in model cheese ripened for 60 days at 8°C*

	pH					
	Blank		Control		300 MPa	
Day	Mean[a]	CI[b]	Mean	CI	Mean	CI
0	5.40 A	0.01	—	—	—	—
30	4.79 D	0.01	4.77 D	0.03	5.13 B	0.03
45	4.68 E	0.02	4.66 E	0.01	4.96 C	0.01
60	4.65 E	0.02	4.68 E	0.02	4.97 C	0.01

[a] Means ($n = 6$) with different letters are significantly different ($P < 0.05$).
[b] CI, confidence interval.

genes and *E. coli* were more resistant to pressure treatment in ultrahigh-temperature milk than in poultry meat or phosphate-buffered saline, and *E. coli* O157:H7 inactivation was significantly greater in poultry meat than in milk. In contrast, *Staphylococcus aureus* was more resistant in milk than in poultry meat *(12).* However, Ellenberg and Hoover *(5)* reported no notable protective effect when *Aeromonas hydrophila* and *Y. enterocolitica* were pressurized in pork compared with buffer or tryptic soy broth (TSB) or when they were pressurized in TSB compared with buffer.

Some studies have demonstrated the ability of different species of pathogenic microorganisms to grow during the cheese-making process when they have been added to pasteurized milk after being grown in favorable conditions *(8).* In our experiment, cells were inoculated in stationary phase and could have started to grow during the first stages of cheese manufacture. When pH decreased, growth would have stopped due to the acid condition and, therefore, a stationary phase would have been reached again. This suggests that serotype O:3 cells were in stationary phase when the HHP treatment was applied, and this could explain their greater baroresistance, since bacterial cells in exponential phase are less pressure resistant than those in stationary phase *(14, 24).* When serotype O:1 was pressurized, death phase could have been already present due to its high sensitivity to low pH, and the lethality observed could be a combined result of pH and HHP treatment. On the other hand, when *Y. enterocolitica* serotype O:8 was pressurized, exponential phase could have been present in the samples due to its greater resistance to acid conditions. This could explain the higher sensitivity to HHP of serotype O:8. This would be in accordance with the observations of O'Reilly et al. *(18),* who studied the effect of high pressure on the inactivation of microbial contaminants in Cheddar cheese and slurries (*E. coli, S. aureus,* and *Penicillium roqueforti*). These authors found greater HHP sensitivity of microorganisms in Cheddar cheese than in the cheese slurry system. They suggested that this result may be explained by two factors: acid injury to the bacteria during fermentation and log phase of growth. Thus, the matrix effect could be understood as an indirect protective effect because it could generate a phase change that would be directly responsible for the greater baroresistance of serotype O:3.

The analysis of the evolution of cell counts after the treatment suggests that the cheese matrix did not allow recovery of injured cells or growth. The main contributing factor to this effect seems to be low pH. Pressure sensitization may involve loss of cell protective or repair functions *(19).* These authors observed that pressure-damaged *E. coli* O157 cells were more acid sensitive than native cells. Karaioannoglou et al. *(11)* found that when the pH of Feta cheese dropped to 4.6 or lower within 48 h after

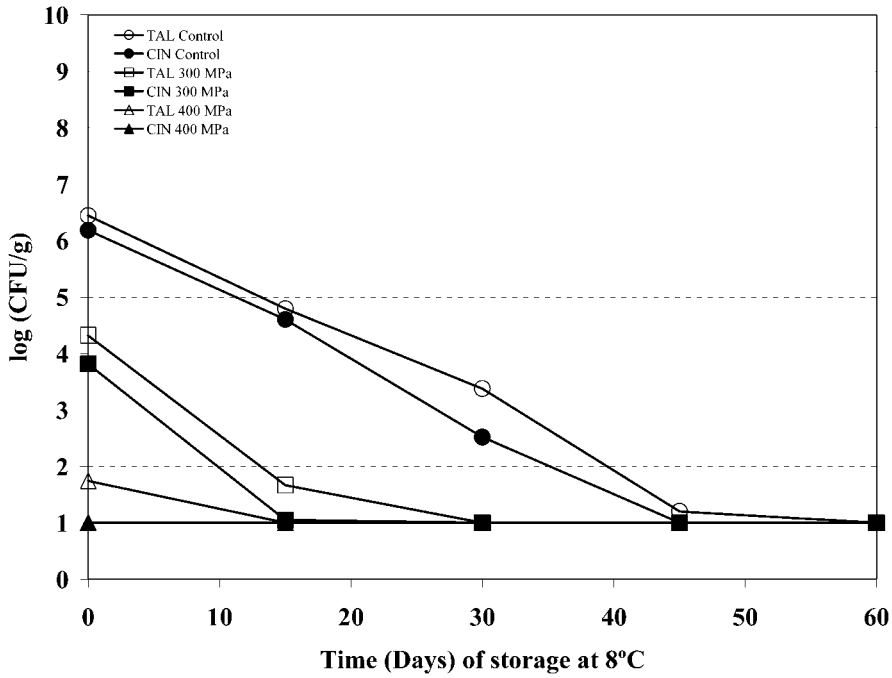


FIGURE 2. *Behavior of* Y. enterocolitica *serotype O:3 inoculated in model cheese treated at 300 and 400 MPa for 10 min at 20°C and ripened for 60 days at 8°C.*

curdling, the numbers of *Y. enterocolitica* (serotype O:9) decreased between 1 and 6 log (CFU/g) within 48 to 96 h and were not detectable within 72 to 120 h. Erkmen *(6)* reported that *Y. enterocolitica* counts in Turkish Feta cheese declined 5.60 log (CFU/g) after salting to day 75. Salt concentration (5.83 to 7.28% from 22 h to 75 days) and pH (4.7 to 6.2 from 7 h to 75 days) seemed to be the major factors responsible for the reduction in the viable number of cells. Nevertheless, *Y. enterocolitica* has been reported to grow in cheese, for instance, in Brie cheese at 4 and 8°C for up to 35 days *(12).* Moreover, in previous experiments *(4),* we found that the same serotypes of *Y. enterocolitica* used in this research, inoculated in milk and treated at 400 and 500 MPa, were able to grow in 1 to 4 days and in 1 to 10 days, respectively, after not having found cell counts in the same day of the treatment. In this case, the considerably high repair and growth abilities of *Y. enterocolitica* in milk may be due to the nutritive nature of the medium and its liquid nature, combined with the psychrotrophic behavior of the pathogen.

We could conclude that HHP treatments are useful to accelerate the reduction of initial levels of *Y. enterocolitica* in complex food, model cheese. Furthermore, this study reaffirms the importance of applying HHP treatments on food because the baroresistance of *Y. enterocolitica* strains and the recovery ability of damaged cells after the treatment may depend, among other factors, on the nature of the food.

ACKNOWLEDGMENTS

This study was supported financially by CAL-00-005-C2-1 project (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria) and by Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, which provided S. De Lamo-Castellví a fellowship to carry out this investigation. We thank the Spanish Type Culture Collection for providing *Y. enterocolitica* strains.

REFERENCES

1. Alpas, H., N. Kalchayanand, F. Bozoglu, A. Sikes, C. P. Dunne, and B. Ray. 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol.* 65:4248–4251.
2. Black, R. E., R. J. Jackson, T. Tsai, M. Medvesky, M. Shayegani, J. C. Feeley, K. I. MacLeod, and A. M. Wakelee. 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *N. Engl. J. Med.* 298:76–79.
3. Capellas, M., M. Mor-Mur, E. Sendra, R. Pla, and B. Guamis 1996. Populations of aerobic aesophils and inoculated *E. coli* during storage of fresh goat's milk cheese treated with high pressure. *J. Food Prot.* 59:582–587.
4. De Lamo-Castellví, S., M. Capellas, A. X. Roig-Sagues, and B. Guamis. 2002. Effect of high hydrostatic pressure on different strains of inoculated in skimmed milk, p. 116. *In* AZTI Fundación (ed.), XIII Congreso de Microbiología de los Alimentos, Bilbao, Spain.
5. Ellenberg, L., and D. G. Hoover. 1999. Injury and survival of *Aeromonas hydrophila* 7965 and *Yersinia enterocolitica* 9610 from high hydrostatic pressure. *J. Food Sci.* 19:263–276.
6. Erkmen, O. 1996. Survival of virulent *Yersinia enterocolitica* during the manufacture and storage of Turkish Feta cheese. *Int. J. Food Microbiol.* 33:285–292.
7. Hoover, D. G., C. Metrick, A. M. Papineau, D. F. Farkas, and D. Knorr. 1989. Biological effects of high hydrostatic pressure on food microorganisms. *Food Technol.* 43:99–107.
8. Johnson, E. A., J. H. Nelson, and M. Johnson. 1990. Microbiology safety of cheese made from heat-treated milk, part II. Microbiology. *J. Food Prot.* 53:519–540.
9. Kang, D. H., and D. Y. C. Fung 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes. J. Food Prot.* 62: 1346–1349.
10. Kapperud, G. 1991. *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.* 12:53–65.
11. Karaioannoglou, P., P. Koidis, D. Papageorgioti, and A. Mantis. 1985. Survival of *Yersinia enterocolitica* during the manufacture and storage of Feta cheese. *Milchwissenschaft* 40:204–206.
12. Little, C. L., and S. Knochel. 1994. Growth and survival of *Yersinia enterocolitica, Salmonella* and *Bacillus cereus* in Brie stored at 4, 8, and 20°C. *Int. J. Food Microbiol.* 24:137–145.
13. López-Pedemonte, T. J., A. X. Roig-Sagués, A. J. Trujillo, M. Ca-

pellas, and B. Guamis. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *J. Dairy Sci.* 86:3075–3081.
14. McClements, J. M., M. F. Patterson, and M. Linton. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. *J. Food Prot.* 64:514–522.
15. Metrick, C., D. G. Hoover, and D. F. Farkas. 1989. Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. *J. Food Sci.* 54:1547–1564.
16. Morse, D. L., M. Shayegani, and R. J. Gallo. 1984. Epidemiologic investigation of a *Yersinia* camp outbreak linked to a food handler. *Am. J. Public Health* 74:589–592.
17. O'Reilly, C. E., A. L. Kelly, P. M. Murphy, and T. P. Beresford. 2001. High pressure treatment: applications in cheese manufacture and ripening. *Food Sci. Technol.* 12:51–59.
18. O'Reilly, C. E., A. L. Kelly, P. M. O'Connor, A. L. Kelly, T. P. Beresford, and P. M. Murphy. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Appl. Environ. Microbiol.* 66:4890–4896.
19. Pagán, R., S. Jordan, A. Benito, and B. Mackey. 2001. Enhanced acid sensitivity of pressure-damaged *Escherichia coli* O:157 cells. *Appl. Environ. Microbiol.* 67:1983–1985.
20. Patterson, M. F., and D. J. Kilpatrick. 1998. The combined effect of high hydrostatic pressure and mild heat on inactivation of pathogens in milk and poultry. *J. Food Prot.* 61:432–436.
21. Patterson, M. F., M. Quinn, R. Simpson, and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *J. Food Prot.* 58:524–529.
22. Schiemann, D. A. 1987. *Yersinia enterocolitica* in milk and dairy products. *J. Dairy Sci.* 70:383–391.
23. Simpson, R. K., and A. Gilmour. 1997. The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. *Food Microbiol.* 14:567–573.
24. Smelt, J. P. P. M. 1998. Recent advances in the microbiology of high pressure processing. *Trends Food Sci. Technol.* 9:152–158.
25. Styles, M. F., D. G. Hoover, and D. F. Farkas. 1991. Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. *J. Food Sci.* 56:1404–1407.
26. Tacket, C. O., J. P. Narain, R. Sattin, J. P. Lofgren, C. J. Konigsberg, R. C. Rendtorff, A. Rausa, B. R. Davis, and M. L. Cohen. 1984. A multistate outbreak of infections caused by *Yersinia enterocolitica* transmitted by pasteurized milk. *J. Am. Med. Assoc.* 251:483–486.
27. Tauxe, R. V. 2002. Emerging foodborne pathogens. *Int. J. Food Microbiol.* 78:31–41.
28. Ur-Rehman, S., P. F. Fox, P. L. H. McSweeney, S. A. Madkor, and N. X. Farkye. 2001. Alternatives to pilot plant experiments in cheese-ripening studies. *Int. J. Dairy Technol.* 54:121–126.
29. Wu, V. C. H., D. Y. C. Fung, D. H. Kang, and L. K. Thompson. 2001. Evaluation of thin agar layer method for recovery of acid-injured foodborne pathogens. *J. Food Prot.* 64:1067–1071.

4.3 Fate of *Escherichia coli* strains inoculated in model cheese produced with or without starter and treated by high hydrostatic pressure

Sílvia De Lamo-Castellví, Marta Capellas, Artur X. Roig-Sagués, Tomás López-Pedemonte, M. Manuela Hernández-Herrero and Buenaventura Guamis.

Submitted to be published in *Journal of Food Protection*

Running head: *E. coli* in model cheese treated with high hydrostatic pressure

Fate of *Escherichia coli* strains inoculated in model cheese produced with or without starter and treated by high hydrostatic pressure

SÍLVIA DE LAMO-CASTELLVÍ, MARTA CAPELLAS*, ARTUR X. ROIG-SAGUÉS, TOMÁS LÓPEZ-PEDEMONTE, M. MANUELA HERNÁNDEZ-HERRERO AND BUENAVENTURA GUAMIS.

Centre Especial de Recerca Planta de Tecnologia dels Aliments, CeRTA, XIT, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària Edifici V, Universitat Autònoma de Barcelona, 08193 Bellaterra (Spain).

*Author for correspondence: Marta Capellas Tel.: (34) 93 581 14 46; Fax: (34) 93 581 14 94

e-mail address: Marta.Capellas@uab.es

Abstract

The aim of this research was to study high hydrostatic pressure (HHP) inactivation of two strains of *Escherichia coli* (*E. coli* O59:H21 CECT 405 and *E. coli* O157:H7 CECT 5947) inoculated in washed-curd model cheese produced with and without starter culture, and the ability of these strains for survival, recovery and growth. Samples were treated at 300, 400 and 500 MPa for 10 min at 20ºC and analysed after the treatment and after 1, 7 and 15 days of storage at 8ºC to study the behaviour of *Escherichia* population**.** Cheese samples produced with starter culture showed the maximum lethality at 400 and 500 MPa and no significant differences in the baroresistant behaviour of both strains were detected, except in the case of *E. coli* O157:H7 at 400 MPa in TAL_m medium, where decrease value was significantly lower. In cheese produced without starter culture the highest decrease value was observed at 500 MPa, except for *E. coli* O59:H21 in selective culture medium, where it was also found at 400 MPa. Ability to repair and grow was not observed in model cheese made with starter culture as cell counts of treated samples decreased after 15 days of storage at 8ºC. Whereas, in cheese produced without starter culture, all pressurized samples showed the trend to repair and grow during the storage period in both strains. These results suggest, that the presence of starter culture and low pH are the main factors to control the ability to recover and grow of *Escherichia* strains inoculated in this type of cheese and treated by HHP.

Escherichia coli O157:H7 has emerged as a foodborne pathogen of major concern for the food industry due to its ability to cause severe illness, in particular haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. Dairy cattle is considered a main reservoir of *E. coli* O157:H7 for human infection (*14*), being fecal contamination of milk an important vehicle for its transmission (*6*, *17*). The pathogen has been isolated from raw milk (*35*), and multiple outbreaks of *E. coli* O157:H7 linked to ingestion of raw milk and dairy products have been reported (*2*, *19*). In many countries, the fact that raw milk is used for cheese manufacture increases the risk that pathogenic bacteria would contaminate the product (*11*). Several authors have studied the ability of *E. coli* O157:H7 to grow and survive in different types of cheese. In fresh cheese, *E. coli* O157:H7 increased 2 log (CFU/g) during cheese manufacture (*3*) but total inactivation was obtained during the heat treatment. Reitsma and Henning (*35*) found that this microorganism was able to grow during Cheddar cheese manufacture even with an initial inoculum in milk of 1 CFU/ml. *E. coli* O157:H7 also survived the manufacture of Camembert and Feta cheeses (*34*) and reached counts higher than those present in milk after 75 and 65 days of storage, respectively. Moreover, Maher et al. (*26*) found that this pathogen was able to survive all stages of smear-ripened cheese production up to 70 days post-manufacture.

High hydrostatic pressure (HHP) is being investigated as a non-thermal processing technique to destroy foodborne pathogens in order to enhance safety and shelf life of perishable food (*20*, *24*). HHP treatments allow the destruction of most vegetative cells and –at high levels and multiple pressure cycles– the destruction of most bacterial spores without decreasing the nutritional value nor changing the sensory characteristics of the products (*38*). HHP is know to cause morphological, biochemical and genetic alterations in microorganisms (*10*) being the membrane damage the main cause of cell inactivation. Pressure sensitivity of microorganisms is highly dependent on the food matrix (*33*). Bacteria that have been considered as pressure-sensitive based on studies in buffers can become highly pressure resistant in complex matrices such as milk or cheese (*32*, *17*, *30*). Moreover, it is well established that HHP treatments produce sublethally injured cells (*18*, *7*), which in favourable conditions, are able to recover (*9*, *13*). Several authors have studied the effect of high hydrostatic pressure, alone or combined with bacteriocin, on microorganisms and spores in different types of cheese (*31*, *25*, *12*, *36*) but it is necessary to obtain further data about *E. coli* O157:H7.

The objectives of the current work were to determine the effect of HHP treatments on two strains of *Escherichia coli* inoculated in washed-curd model cheese made with or without starter culture and to provide information about the behaviour of both strains in the two types of cheese during a storage period of 15 days at 8ºC.

Materials and Methods

Bacterial strains. *E. coli* O59:H21 (CECT 405) and *E. coli* O157:H7 (CECT 5947, which virulence factor had been deleted), were obtained from Spanish Type Culture Collection (CECT, Universidad de Valencia, Valencia, Spain) and were kept in cryobeads (Nalgene® System 100™, Microkit Iberica S.L., Madrid, Spain) at -20ºC.

Inocula preparation. Strains were revived by placing one cryobead in 10 ml brain heart infusion broth (BHI, Oxoid, Basingtoke, UK) containing 0.6% yeast extract (BHIYE, Oxoid) and incubated at 37ºC for 24 h. 1 ml of the first culture was transferred to 10 ml of BHIYE and incubated for an additional 18 h at the same temperature, to allow the culture to reach the stationary phase. Then, cells were centrifuged at 1,250 X g for 15 min at room temperature, washed once in 10 mM phosphate-buffered saline (PBS, Oxoid) and the pellets were resuspended in PBS to a final concentration of about 10^8-10^9 CFU/ml.

Starter culture preparation. A mixture of commercial lyophilized strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ezal MAO 11, Rhodia Iberia S.A., Madrid, Spain) that is known as a non-bacteriocin productor, was used as a starter culture for the washed-curd model cheese manufacture. The culture was revived inoculating 0.015 g of mixture in 1,000 ml of commercial sterilized skimmed milk and incubated at 30ºC for 24 h. A volume of 50 ml was used to prepare a subculture in 200 ml of sterilized skimmed milk, which was incubated under the same temperature and time conditions.

Model cheese manufacture. Raw cow's milk obtained from a local farm was transported and stored at 4ºC. Before inoculation, milk was pasteurized at 65ºC for 30 min and cooled to 32ºC in an ice and water bath. Two *per cent* (vol/vol) of starter

culture and 0.01% (vol/vol) of a 35% $CaCl_2$ dilution (Arroyo, Santander, Spain) were added. Milk was kept in a water bath at 32ºC for 20 min and then inoculated with 1% (vol/vol) of *E. coli* O59:H21 or *E. coli* O157:H7 inoculum (except blanks). 0.02% (vol/vol) of liquid rennet extract of bovine origin (520 mg/l of active chymosin, Arroyo, Santander, Spain) was added as coagulating agent. Centrifugation bottles (Nalgene®, Nalge Nunc International, Rochester, USA) were filled with 225 ml of inoculated milk and placed in a bath at 32ºC for 40 min until curd was formed. Curd was cut into small pieces and kept in the water bath and the temperature was increased to 37ºC over 5 min. The bottles were kept in the bath at 37ºC for further 15 min. Curds were washed to avoid excessive acidification, substituting 40% (vol/vol) of whey with sterile water. Bottles were then centrifuged at 7,000 *X g* for 40 min at 20ºC and kept in their containers in a water bath at 37ºC until pH 5.5 was reached. When the pH of the resulting cheeses dropped to 5.5 (only in the case of cheese made with starter), whey was completely removed from the bottles by decantation and cheeses were salted by adding, in each bottle, 100 ml of 20% (wt/vol) NaCl sterile brine for 15 min. After this, cheeses were removed from the centrifugation bottles using sterile pincers and dried on sterile paper. They had a final weight of approximately 23 g with 55% of dry matter and 1.5% of salt in moisture content. Cheeses were twice vacuum packed in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) and stored overnight at 8ºC before high pressure treatment.

In the case of model cheeses produced without starter culture, the same protocol without adding starter culture was followed.

High pressure treatment. Cheese samples were pressurized in a discontinuous isostatic press (ACB, Nantes, France) with a pressure chamber of 10 cm diameter and 23 cm length. The time needed to achieve the treatment pressure was between 1.45 and 2.55 min, depending on the pressure applied and the decompression time was around 18 s (Fig. 1 and Fig. 2). The pressure chamber and the pressurization medium inside were adjusted to 20ºC with a constant flow of water. The temperature of the pressurization fluid was measured by a thermocouple. Cheese samples were pressurized at 300, 400 and 500 MPa for 10 min at 20ºC.

Sampling protocol. Milk samples inoculated with *E. coli* O59:H21 or *E. coli* O157:H7 were analysed to count the number of these microorganisms prior to the

cheese making process. Three groups of cheese samples were analysed: Non-inoculated (blank cheese samples), inoculated with *E. coli* O59:H21 or *E. coli* O157:H7 but not treated by HHP (control cheese samples), and inoculated, and treated (pressurized cheese samples). All samples were stored at 8ºC. At the end of the cheese production, control samples were analysed to assess the population of *E. coli* O59:H21 or *E. coli* O157:H7. Microbiological analyses were performed at 0 day (immediately after HHP treatment) and at 1, 7 and 15 days after treatment for all samples. Experiments were run three times with duplicate analyses each time.

Microbiological analyses. Ten grams of each sample were homogenized in 90 ml of maximum recovery diluent (MRD, Oxoid) for 1 min using an electromechanical blender (BagMixer®, Interscience, St Nom, France) at room temperature. Ten-fold serial dilutions were produced in maximum recovery diluent (MRD, Oxoid) and 1 ml of appropriate dilution was plated into Sorbitol McConkey agar (SMC, Oxoid) containing selective cefixime tellurite supplement (Oxoid) to count non-injured cells of E. coli O59:H21 and E. coli O157:H7. A modification of the thin agar layer method (TAL_m) (23) was used to count both injured and non-injured cells to a detection level (10 CFU/g). After a selective medium (SMC) layer, the first layer of nutritive medium (BHIAYE) was added. Then, 1 ml of appropriate dilution of cheese sample and a second layer of nutritive medium was added. All plates were incubated at 37ºC for 24 h.
Results are expressed as the logarithm of colony-forming units (CFU) per g. Increase value was calculated as the difference between the logarithms of colony counts of control cheese samples and milk samples analysed at the end of cheese production ($\log N_c - \log N_m$). Decrease value was calculated as the difference between the logarithms of colony counts of the untreated and treated samples ($\log N_o - \log N$).
At 0, 1, 7 and 15 days, an enrichment procedure was used to check the presence of injured cells in cheese samples that did not show colonies growing in both culture media. The first dilution of each sample (10 g of cheese diluted in 90 ml of MRD) was stored at 37ºC for 24 h. A loopful of this culture was streaked onto a plate of SMC and incubated at 37ºC for 24 h.
Growing colonies of *E. coli* O59:H21 were identified using a biochemical test (API 20E, Bio-Mérieux, Marcy l'Etoile, France) and colonies of *E. coli* O157:H7 were confirmed using a latex agglutination kit (LA, Oxoid) which is based on the detection of antigens specific to this microorganism.

pH measurement. Model cheese pH was determined using a penetration electrode (Crison, Crison Instruments S.A., Alella, Spain). pH values were based on the mean of nine measurements

Statistical analyses. Analysis of variance was performed using the General Linear Models Procedure of SAS® software (SAS® System for Windows™, 8.02, 1999, Sas Institute, Inc., Cary, North Carolina, USA). Student-Newman-Keuls test was used to obtain paired comparisons among sample means. Level of significance was set at $P < 0.05$.

Results and Discussion

Behaviour of initial inocula during model cheese production. Microbial counts of inoculated milk and control cheese made from inoculated milk and produced with and without starter culture and their increase values during cheese production are shown in Table 1. Both strains of *E. coli* increased their counts during the production process of cheese produced with and without starter culture. Several studies have reported this behaviour with *E. coli* in hard and fresh cheese (*39*, *8*) and some authors suggest that it could be produced by a combination of a physical entrapment in the curd and growth of the initial population during cheese manufacture (*26*). Moreover, it is important to remark that no significant differences were detected in the increase values of different strains and in the cell counts obtained in both culture media (Table 1).

High Pressure effect at day 0. Decrease values obtained after HHP treatments in washed-curd model cheese inoculated with and without starter culture and with *E. coli* O59:H21 or *E. coli* O157:H7 are shown in Table 2. In cheese samples produced with starter culture, the most effective treatments to reduce the initial population of *Escherichia* were 400 and 500 MPa. Whereas, in cheese samples produced without starter culture, the most effective treatment was 500 MPa, except for *E. coli* O59:H21 in selective culture medium, where it was also 400 MPa. Nevertheless, it is worth to mention that in the cases where cell counts obtained after treatment were below the detection level, decrease values depended on the initial concentration of these

microorganisms in cheese prior to the treatment so they could have been higher. It is clear from the reported results that in cheese produced without starter culture it was necessary to apply more pressure to obtain the same reduction than in cheese produced with starter culture. Differences in pH between the two types of cheese (Table 3) and the presence of starter culture could be the reason for this behaviour. In fact, several studies have demonstrated that bacteria could become much more pressure-sensitive at low pH and an efficient inactivation of even the most pressure-resistant vegetative bacteria could be produced at relatively mild pressure in acidic foods (*15*, *1*, *21*). In a preliminary research (data not shown), we analysed the effect of HHP on the starter population in model cheese inoculated with *S. enteritidis* or *S. typhimurium* and treated at 300 and 400 MPa. We found that the effect of HHP treatment at 300 MPa was minimum, with a reduction of 0.23-0.26 log (CFU/g), and close to 3 log (CFU/g) at 400 MPa. Molina-Höppner (*29*) reported that during the treatment at 300 MPa, *Lactococcus lactis* spp. *cremoris* retained about 25% of metabolic activity, even after 3 log (CFU/ml) reduction in cell counts.

No significant differences between decrease values obtained from both strains and from both culture media were found, except in the case of *E. coli* O157:H7 in cheese made with starter culture at 400 MPa in TAL_m, where decrease value was significantly lower. Thus, we could not detect differences in pressure resistance behaviour between strains in both types of cheese. Nevertheless, in cheese and other food matrices, some authors have reported different pressure resistance behaviour between strains of the same microorganism (*32*, *5*, *12*).

Behaviour of *E. coli* O59:H21 and *E. coli* O157:H7 after HHP treatments in model cheese produced with starter culture. None of the *E. coli* O59:H21 or *E. coli* O157:H7 cells inoculated in control samples had the ability to grow after 15 days of storage at 8ºC (Fig. 3 a, b, c and d) and showed a decrease trend in their counts after 1 day of storage. This trend indicates that the low pH and the presence of the starter culture had an effect on the survival of cells not treated by HHP. *Escherichia* strains inoculated in model cheese treated at 400 and 500 MPa -only for *E. coli* O59:H21- were not able to repair and grow after 15 days of storage at 8ºC (Fig. 3 a and b) and kept their counts below the level of detection (10 CFU/g) in both culture media. In this research, a selective culture medium (SMC) and a modification of TAL method were used to detect injured *Escherichia* population produced by the cheese making process,

HHP treatments and the low pH of cheese (Table 3). Wu et al. (*40*) reported that TAL was an adequate method to enumerate acid injured *E. coli* O157:H7 cells. Moreover, in previous experiments carried out on the same washed-curd model cheese, we found that the combination of selective and nutritive culture media was an appropriate system to recover *Yersinia enterocolitica* injured cells (*12*). Nevertheless, in the present case, TAL_m medium was not able to recover the total population of injured cells of *E. coli* O59:H21 and *E. coli* O157:H7.

But after applying the enrichment process (24 h at 37ºC), cells of *E. coli* O59:H21 and *E. coli* O157:H7 were detected for 7 days (at days 0, 1 and 7) -in samples treated at 500 MPa- and for 15 days (at days 0, 1, 7 and 15) -in samples treated at 400 MPa- stored at 8ºC (Table 4). At 300 MPa, cheese samples inoculated with *E. coli* O59:H21 and *E. coli* O157:H7 decreased their counts after 1 day of storage (Fig. 3 a, b, c and d) without reaching the level of detection in both culture media. Several authors have reported that HHP treatments produce sublethally injured cells (*27*, *7*, *13*), which in a stressful environment, are not able to recover and grow. Arroyo et al. (*4*) suggested that, in most cases, pressurized cells did not behave like dead or live cells. Cells that survive HHP treatments could become injured and develop sensitivity to physical and chemical environments to which the normal cells are resistant to (*18*, *22*). It is clear from the data obtained that cheese matrix did not allow recovery and growth of injured cells being low pH and the presence of the starter culture the most important factors determining this behaviour (Table 3 and Fig. 3). Moreover, in a previous study (*12*), we made washed-curd model cheeses inoculated with *Y. enterocolitica* (serotypes O:1, O:3 and O:8) and starter culture, and we treated them with HHP at 300, 400 and 500 MPa for 10 min at 20ºC. None of the *Y. enterocolitica* strains inoculated in cheese samples treated at 400 and 500 MPa showed repair ability after 15 days of storage at 8ºC remaining their cell counts below the detection level (1 log [CFU/g]).

Behaviour of *E. coli* O59:H21 and *E. coli* O157:H7 after HHP treatments in model cheese produced without starter culture. Control samples inoculated with *E. coli* O59:H21 and *E. coli* O157:H7 did not show significant differences ($P > 0.05$) in their cell counts during the time of storage in any culture media (Fig. 4). *E. coli* O59:H21 and *E. coli* O157:H7 inoculated in model cheese and treated at 300, 400 and 500 MPa showed an increase trend in their counts (ability to repair and grow) during the storage period at 8ºC (Fig. 4). Nevertheless, no significant differences ($P > 0.05$) –

except for *E. coli* O59:H21 at 500 MPa- in cell counts were found in both culture media. These results could be explained by the high variability found between replicates. Ritz et al. (*36*) reported that HHP treatments produce heterogeneous cell populations likely due to variations in levels of injury, metabolic activity and viability among the cells. Using vital staining with fluorescent dyes showed the presence of viable subpopulations that did not form colonies and were therefore not detected on agar culture media. Moreover, these authors suggested that due to this heterogeneity the reversible damage and the cellular repair that may occur under favourable conditions should not be ruled out.

At 500 MPa, both strains began to grow after 1 day of storage in both culture media (Fig. 4 b) reaching counts close to 3 and 6 log CFU/g in selective medium and TAL_m, respectively, at the end of the process. Repair of foodborne pathogens during storage is important for HHP treated low acid foods because it can cause overestimation of safety during storage (*7*). Generally, nutritionally rich media allow a relatively rapid repair for a high proportion of injured cells (*41*). Several authors have reported this behaviour after applying HHP treatments in different matrices. Metrick et al. (*28*) reported that *Salmonella* spp. strains were able to repair in chicken but not in buffer under the same conditions of pressure treatment and storage. Apparently, the lack of available nutrients in buffer prevented the cells from repairing. Carlez et al. (*9*) found that, although there were no counts of *Pseudomonas* spp. after minced meat was pressurized (400 MPa, 20 min), growth was detected at 6 days of storage at 3ºC. After this initial lag, the growth rate of the repaired organisms was similar to that of the controls. Bozoglu et al. (*7*) showed that after pressure treatment at 550 MPa, initially colony formation of *Listeria monocytogenes* was not observed in selective or non-selective agar, suggesting that all cells were dead. However, at 4ºC after 6 days and at 22 and 30ºC after 1 day, colony formation occurred in both selective and non-selective agar. Moreover, in previous experiments (*13*), we found that three strains of *Y. enterocolitica* inoculated in skimmed milk and treated at 400 and 500 MPa, were able to grow in 1-4 days and in 1-10 days respectively after not having found cell counts the same day of the treatment.

HHP treatments applied in this research are useful to reduce *Escherichia* population in this model cheese, but the presence of the starter culture is necessary to inhibit the recovery and growth and to increase the death rate of the injured population.

Acknowledgements

This study was supported financially by CAL-00-005-C2-1 project (*Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria*) and by *Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya* which provided S. De Lamo-Castellví a fellowship to carry out this investigation. We would like to thank the Spanish Type Culture Collection for providing *E. coli* strains.

References

1. Alpas, H., N. Kalchayanand, F. Bozoglu and B. Ray. 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *Int. J. Food Microbiol.* 60:33–42.

2. Altekruse, S. F., B. B. Timbo, J. C. Mowbray, N. H. Bean and M. E. Potter. 1998. Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: sanitary manufacturing practices protect consumers. *J. Food Prot*. 61:1405-1407.

3. Arocha, M. M., M. Mc Vey, S.D. Loder, J.H. Rupnow and L. Bullerman. 1992. Behaviour of enterohaemorrhagic E. coli O157:H7 during manufacture of cottage cheese. *J. Food Prot.* 55: 379–381.

4. Arroyo, G., P. D. Sanz and G. Prestamo. 1999. Response to high-pressure, low-temperature treatment in vegetables: determination of survival rates of microbial populations using flow cytometry and detection of peroxidase activity using confocal microscopy. *J. Appl Microbiol.* 86:544-556.

5. Benito, A., G. Ventoura, M. Casadei, T. Robinson and B. Mackey. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat and other stresses. *Appl. Environ. Microbiol*. 65:1564-1569.

6. Borczyk, A. A., M. A. Kamali,, H. Lior and L. M. C. Duncan. 1987. Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* 1:98.

7. Bozoglu F., H. Alpas and G. Kaletunc. 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. *FEMS Immunol Med Microbiol.*40:243-247.

8. Capellas, M., M. Mor-Mur, E. Sendra, R. Pla and B. Guamis 1996. Populations of aerobic aesophils and inoculated *E. coli* during storage of fresh goat's milk cheese treated with high pressure. *J. Food Prot*. 59:582-587.

9. Carlez, A., J. P. Rosec, N. Richard and J.C. Cheftel. 1994. Bacterial growth during chilled storage of pressure-treated minced meat. *Lebensm. Wiss. Technol*. 27:48-54.

10. Cheftel, J. C. 1995. Review: high pressure, microbial inactivation and food preservation. Food *Sci. Technol. Int.* 1:75:90.

11. De Buyser, M. L., B. Dufour, M. Maire and V. Lafarge. 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int J. Food Microbiol.* 67:1-17.

12. De Lamo-Castellví, S., M. Capellas, T. López-Pedemonte, M. M. Hernández-Herrero, B. Guamis and A. X. Roig-Sagués. 2005a. Behavior of *Yersinia enterocolitica* strains inoculated in model cheese treated with high hydrostatic pressure. *J. Food Prot.* 68:528-533.

13. De Lamo-Castellví, S., A. X. Roig-Sagués, M. Capellas, M. M. Hernández-Herrero and B. Guamis. 2005b. Survival and growth of *Yersinia enterocolitica* strains inoculated in skimmed milk treated with high hydrostatic pressure. *Int. J. Food Microbiol.* 102:337-342.

14. Doyle, M. P. 1991. *Escherichia coli* O157:H7 and its significance in foods. *Int J. Food Microbiol.* 12:289-301.

15. García-Graells, C., K. J. A. Hauben and C.W. Michiels. 1998. High Pressure inactivation and sublethal injury of pressure-resistant *Escherichia coli* mutants in fruit juices. *Appl. Environ. Microbiol*. 64:1566–1568.

16. García-Graells, C., B. Masschalck and C. W. Michiels. 1999. Inactivation of *Escherichia coli* in milk by high-hydrostatic-pressure treatment in combination with antimicrobial peptides. *J. Food Prot.* 62:1248–1254.

17. Gonzalez Garcia, E. A. 2002. Animal health and foodborne pathogens: enterohaemorrhagic O157:H7 strains and other pathogenic *Escherichia coli* virotypes (EPEC, ETEC, EIEC, EHEC). *Pol. J. Vet. Sci*. 5:103-115.

18. Hauben, K. J. A., E. Y. Wuytack, C. C. F. Soontjens and C. W. Michiels. 1996. High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. *J. Food Prot*. 59:350-355.

19. Honish, L., G. Predy, N. Hislop, L. Chui, K. Kowalewska-Grochowska, L. Trottier, C. Kreplin and I. Zazulak. 2005. An outbreak of *E. coli* O157:H7 hemorrhagic colitis associated with unpasteurized gouda cheese. *Can J Public Health.* 96:182-184.

20. Hoover, D. G., C. Metrick, A. M. Papineau, D. F. Farkas, and D. Knorr. 1989. Biological effects of high hydrostatic pressure on food microorganisms. *Food Technol.* 43:99-107.

21. Jordan, S. L., C. Pascual, E. Bracey and B. M. Mackey. 2001. Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. *J. Appl. Microbiol.* 91:463–469.

22. Kalchayanand, N., A. Sikes, C. P. Dunne and B. Ray. 1998. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. *J. Food Prot.* 61:425-431.

23. Kang, D. H. and D. Y. C. Fung 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. *J. Food Prot*. 62:1346-1349.

24. Knorr, D. 1993. Effects of high-hydrostatic pressure processes on food safety and quality. *Food Technol.* 47:156:161.

25. López-Pedemonte, T. J., A. X. Roig-Sagués, A. J. Trujillo, M. Capellas and B. Guamis. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *J. Dairy Sci*. 86:3075-3081.

26. Maher, M. M., K. N. Jordan, M. E. Upton and A. Coffey. 2001. Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *J. Appl. Microbiol.* 90:201-207.

27. McClements, J. M., M. F. Patterson and M. Linton. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. *J. Food Prot*. 64:514-522.

28. Metrick, C., D. G. Hoover and D. F. Farkas. 1989. Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. *J. Food Sci.* 54:1547-1564

29. Molina-Höppner, A. 2002. Physiological response of *Lactococcus lactis* to high-pressure. M. S Thesis, Technische Universität München, München.

30. O'Reilly, C. E., A. L. Kelly, P. M. O'Connor, A. L. Kelly, T. P. Beresford and P. M. Murphy. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Appl. Environ. Microbiol.* 66:4890-4896.

31. O'Reilly, C. E., A. L. Kelly, P. M. Murphy and T. P. Beresford. 2001. High pressure treatment: applications in cheese manufacture and ripening. *Food Sci. Technol.* 12:51-59.

32. Patterson, M. F., M. Quinn, R. Simpson and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *J. Food Prot*. 58:524-529.

33. Patterson, M. F. and D. J. Kilpatrick. 1998. The combined effect of high hydrostatic pressure and mild heat on inactivation of pathogens in milk and poultry. *J. Food Prot*. 61:432-436.

34. Ramsaran, H., J. Chen, B. Brunke, A. Hill and M. W. Griffiths. 1998. Survivial of bioluminescent *Listeria monocytogenes* and *Escherichia coli* O157:H7 in soft cheeses. *J. Dairy Sci*. 81:1810-1817.

35. Reitsma, C. J. and D. R. Henning,. 1996. Survival of enterohaemorrhagic *E. coli* O157:H7 during the manufacture and curing of Cheddar cheese. *J. Food Prot.* 59:460-464.

36. Ritz M., J. L. Tholozan, M. Federighi and M. F. Pilet. 2001. Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. *Appl. Environ. Microbiol*. 67:2240–2247.

37. Rodríguez, E., J. L. Arqués, M. Nuñez, P. G. and M. Medina. 2005. Combined Effect of High-Pressure Treatments and Bacteriocin-Producing Lactic Acid Bacteria on Inactivation of *Escherichia coli* O157:H7 in Raw-Milk Cheese. *Appl. Environ. Microbiol*. 71:3399–3404.

38. Smelt, J.P.P.M.. 1998. Recent advances in the microbiology of high pressure processing. *Trends Food Sci. Technol.* 9:152-158.

39. Spahr, U. and B. Url. 1994. Behaviour of pathogenic bacteria in cheese-a synopsis of experimental data. *Butlletin of IDF*. 298.

40. Wu, V. C. H., D. Y. C. Fung, D. H. Kang and L. K. Thompson. 2001. Evaluation of thin agar layer method for recovery of acid-injured foodborne pathogens. *J. Food Prot.* 64:1067-1071.

41. Wuytack, E. Y., L. D. T. Phuong, A. Aertsen, K. M. F. Reyns, D. Marquenie, B. De Ketelaere, B. Massachlck, I. Van Opstal, A. M. J. Diels and C. W. Michiels. 2003. Comparision of sublethal injury induced in *Salmonella enterica* serovar *Typhimurium* by heat and by different nonthermal treatments. *J. Food Prot.* 66:31-37..

TABLE 1. *Cell counts and increase values of* E. coli *strains inoculated in milk (initial inoculum) and in model cheese at cheese production day*

Strains	Sample	Counts in SMC log (CFU/g)		Increase values ($\log N_c - \log N_m$)		Counts in TAL_m log (CFU/g)		Increase values ($\log N_c - \log N_m$)	
		Mean[a]	SD[b]	Mean	SD	Mean	SD	Mean	SD
With starter									
E. coli O59:H21	milk	5.08^{A}	±0.33	-	-	5.19^{A}	±0.32	-	-
	cheese	7.24^{B}	±0.53	2.12^{I}	±0.22	7.34^{B}	±0.46	2.14^{I}	±0.17
E. coli O157:H7	milk	5.28^{A}	±0.15	-	-	5.35^{A}	±0.19	-	-
	cheese	6.88^{B}	±0.29	1.60^{I}	±0.15	7.01^{B}	±0.24	1.72^{I}	±0.12
Without starter									
E. coli O59:H21	milk	5.04^{A}	±0.19	-	-	5.05^{A}	±0.20	-	-
	cheese	6.92^{B}	±1.31	1.88^{I}	±1.13	7.17^{B}	±1.01	2.11^{I}	±0.83
E. coli O157:H7	milk	5.03^{A}	±0.24		-	5.06^{A}	0.22	-	-
	cheese	6.86^{B}	±1.15	1.83^{I}	±1.00	7.08^{B}	±0.95	2.02^{I}	±0.83

[a] Means (n=6) with different letters and numbers are significantly different ($P < 0.05$)

[b] Standard Deviation

TABLE 2. *Cell counts and decrease values of inoculated in model cheese produced with and without starter culture and pressurized at 300, 400 and 500 MPa at 20ºC for 10 min at day 0.*

Strains	Treatment (MPa)	Counts in SMC log (CFU/g)		Decrease values [log(No) – log(N)]		Counts in TAL_m log (CFU/g)		Decrease values [log(No) – log(N)]	
		Mean[a]	SD[b]	Mean	SD	Mean	SD	Mean	SD
With starter									
E. coli O59:H21	0	6.89	±0.35	-	-	7.14	±0.52	-	-
	300	4.24	±0.46	2.65^{A}	±0.25	4.89	±0.03	2.25^{A}	±0.54
	400	n.d	-	$\geq 5.89^{D}$	±0.35	1.08	±0.19	6.06^{D}	±0.63
	500	n.d	-	$\geq 5.89^{D}$	±0.35	n.d	-	$\geq 6.14^{D}$	±0.52
E. coli O157:H7	0	7.08	±0.11	-	-	7.23	±0.16	-	-
	300	5.12	±0.26	1.96^{A}	±0.18	5.31	±0.14	1.96^{A}	±0.18
	400	1.32	±0.50	5.76^{D}	±0.61	2.55	±0.51	$4.68^{B,C}$	±0.61
	500	n.d	-	$\geq 6.08^{D}$	±0.11	1.05	±0.12	$\geq 6.23^{D}$	±0.20
Without starter									
E. coli O59:H21	0	6.36	±1.57	-	-	7.03	±0.68	-	-
	300	4.44	±1.08	1.92^{A}	±0.49	4.76	±1.31	2.27^{A}	±1.31
	400	1.75	±0.87	$4.61^{B,C}$	±0.70	2.75	±1.05	4.28^{B}	±0.38
	500	n.d	-	$\geq 5.36^{B,C}$	±1.57	n.d	-	$\geq 6.03^{D}$	±0.68
E. coli O157:H7	0	6.97	±1.05	-	-	7.19	±1.01	-	-
	300	4.03	±0.39	2.94^{A}	±0.66	4.26	±0.66	2.92^{A}	±0.36
	400	2.78	±1.12	4.19^{B}	±0.10	3.14	±0.67	4.05^{B}	±0.36
	500	n.d	-	$\geq 5.97^{D}$	±1.05	n.d	-	$\geq 6.19^{D}$	±1.01

n.d.: not detected. Detection level: 1 log (CFU/g)

[a] Means (n=6) with different letters are significantly different ($P < 0.05$)

[b] Standard Deviation

TABLE 3. *Values of pH in blank cheese samples produced with and without starter culture and stored at 8ºC for 15 days.*

Time (Days)	With starter		Without starter	
	Mean[a] (n=6)	SD[b]	Mean (n=6)	SD
cpd	5.08[A]	0.06	6.64[D]	0.02
0	5.08[A]	0.03	6.69[E]	0.03
1	5.02[B]	0.03	6.57[F]	0.03
7	4.82[B]	0.01	6.48[G]	0.02
15	4.78[C]	0.03	6.46[G]	0.01

cpd: cheese production day

[a] Means with different letters are significantly different ($P < 0.05$)

[b]Standard Deviation

TABLE 4. *Results of the enrichment process (24 h at 37ºC) applied to selected cheese samples produced with starter culture (those which showed cell counts below the detection level [10 CFU/g] during the storage time at 8ºC).*

Sample	Time (Days)							
	0		1		7		15	
	E. coli O59:H21	*E. coli* O157:H7	*E. coli* O59:H21	*E. coli* O157:H7	*E. coli* O59:H21	*E. coli* O157:H7	*E. coli* O59:H21	*E. coli* O157:H7
Blank	-[a]	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
400 MPa	+[b]	+	+	+	-	-	-	-
	+	+	+	+	+	+	+	+
	+	+	+	+	-	-	-	-
500 MPa	+	+	+	+	-	-	-	-
	+	+	+	+	+	+	-	-
	+	+	+	+	-	-	-	-

[a] Negative growth

[b] Positive growth

FIGURE 1. Water pressure variation in the chamber as a function of time during the treatment at 20ºC.

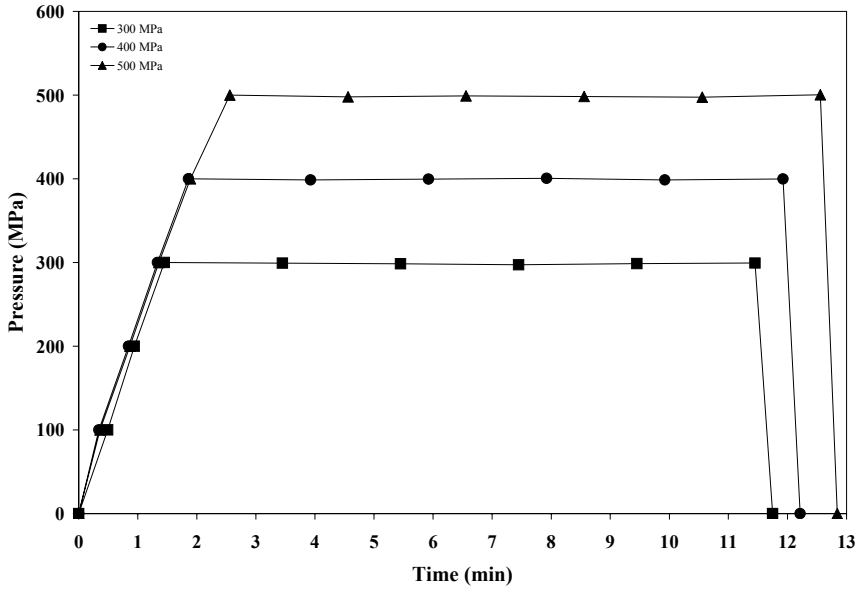


FIGURE 2. Water temperature variation in the chamber as a function of time during the treatment at 20ºC and 300, 400 and 500 MPa of pressure.

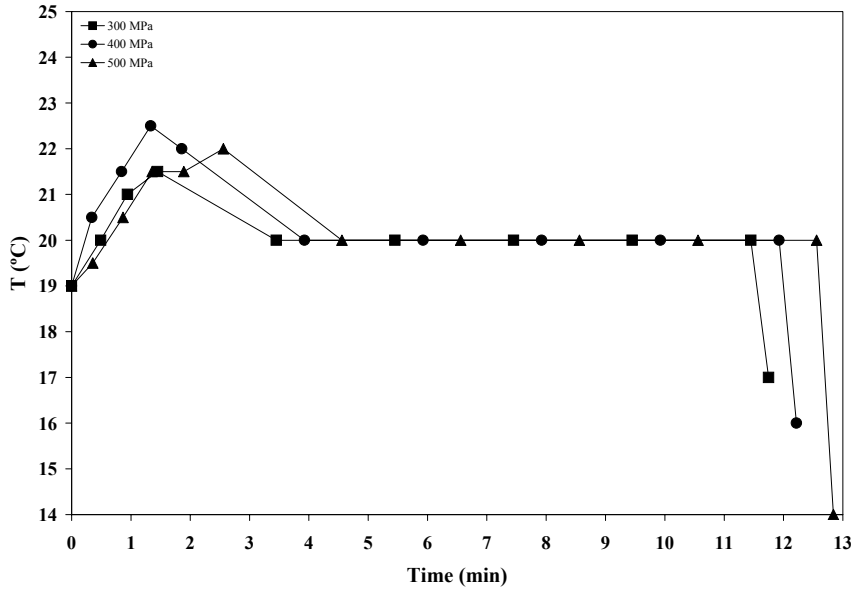


FIGURE 3. Behaviour of *E coli* O59:H21 – (a) in TAL_m and (b) in SMC media – and *E. coli* O157:H7 –(c) in TAL_m and (d) in SMC media – inoculated in model cheese produced with starter culture and treated at 300 MPa, 400 and 500 MPa for 10 min at 20ºC.

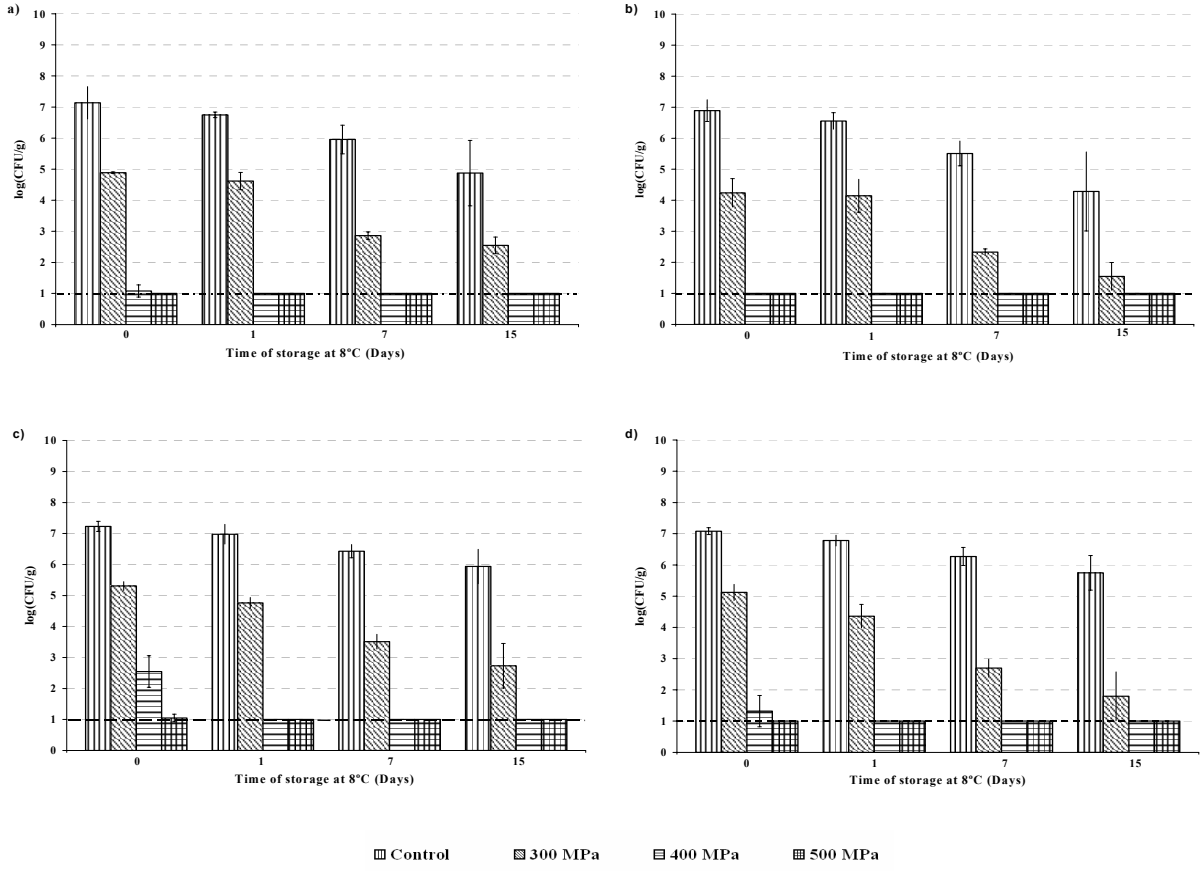
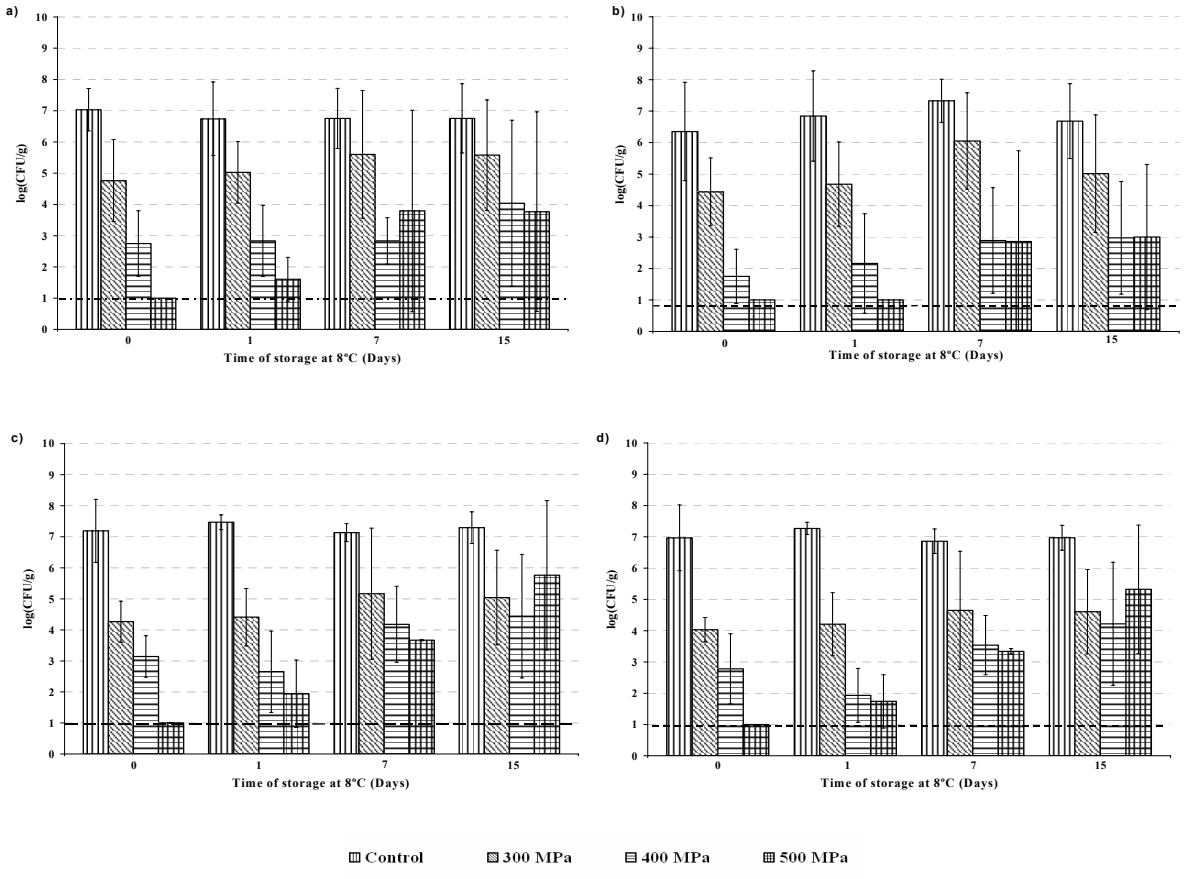


FIGURE 4. Behaviour of *E coli* O59:H21 – (a) in TAL_m and (b) SMC media – and *E. coli* O157:H7 – (c) in TAL_m and (d) SMC media – inoculated in model cheese produced without starter culture and treated at 300 MPa, 400 and 500 MPa for 10 min at 20ºC.



4.4 Response of two *Salmonella enterica* strains inoculated in model cheese treated with high hydrostatic pressure

Sílvia De Lamo-Castellví, Artur X. Roig-Sagués, Tomás López-Pedemonte, Manuela Hernández-Herrero, Buenaventura Guamis and Marta Capellas.

Submitted to be published in *International Journal of Food Microbiology*

Response of two *Salmonella enterica* strains inoculated in model cheese treated with high hydrostatic pressure

Sílvia De Lamo-Castellví; Artur X. Roig-Sagués*; Tomás López-Pedemonte; Manuela Hernández-Herrero; Buenaventura Guamis; Marta Capellas.

Centre Especial de Recerca Planta de Tecnologia dels Aliments, CeRTA, XIT, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària Edifici V, Universitat Autònoma de Barcelona, 08193 Bellaterra (Spain).

*Corresponding author: A. X. Roig-Sagués; Tel.: (34) 93 581 1460; Fax: (34) 93 581 1494. e-mail address: ArturXavier.Roig@uab.es.

Abstract

The aim of this work was to determine the response to high hydrostatic pressure (HHP) and the ability for survival, recovery and growth of *S. enteritidis* and *S. typhimurium* inoculated in washed-curd model cheese produced with and without starter culture. Inoculated samples were treated at 300 and 400 MPa for 10 min at room temperature and analysed after the treatment and after 1, 7 and 15 days of storage at 12 ºC to study the behaviour of *Salmonella* population. Cheese samples produced with starter culture and treated at 300 and 400 MPa showed maximum lethality and no significant differences in the baroresistant behaviour of both strains were detected. Nevertheless, when starter culture was not present, the maximum lethality was only observed in cheese samples treated at 400 MPa and only in the case of *S. enteritidis*. Ability to repair and grow was not observed in model cheese made with starter culture and cell counts of treated samples decreased after 15 days of storage at 12 ºC. Whereas, in cheese produced without starter culture, *Salmonella* cells showed the ability to repair and grow during the storage period, reaching counts over 3 log (CFU/mL) in both applied treatments and strains. These results suggest that HHP treatments are effective to reduce *Salmonella* population in this type of cheese, but the presence of the starter culture controls the ability of this microorganism to repair and grow during the storage period.

Keywords: high hydrostatic pressure; injured cell; *Salmonella*; cheese; starter culture.

1. Introduction

Salmonella is ubiquitous in nature and the serotypes that can cause human infections occur in all types of animals, including a significant but unknown number of domestic animals. Infections exhibit a number of clinical manifestations, but gastrointestinal disorders are the most common. The severity and duration of symptoms depend on the type of *Salmonella* present, the amount of food eaten and the susceptibility of the person involved (El-Gazzar and Marth, 1992). The two most prevalent serovars of *Salmonella* currently isolated from foodborne outbreaks in USA and Europe are *S. enterica* serovar *enteritidis* and *S. enterica* serovar *typhimurium* (Mattick et al., 2001, Anonymous, 2003). Moreover, *S. enterica* serovar *enteritidis* is one of the *Salmonella* serotypes most commonly associated with morbidity and mortality in humans (Ahmed et al., 2000).

Raw and pasteurized milk and different types of cheese have been involved in several outbreaks (CDC, 1985; Altekruse et al., 1998). Some studies have showed that when milk becomes contaminated with *Salmonella* spp. after pasteurization, the pathogen could survive the cheese making process and persist for several months in the cheese (Leyer and Johnson, 1992). In ripened Cheddar cheese, *Salmonella* counts were detected for up to 7 months at 7 ºC. In cold-packed cheeses, cells were found depending on the pH value and preservative used (El-Gazzar and Marth, 1992). Moreover, fat and proteins in cheese can protect foodborne pathogens from gastric acidity, reducing the number of organisms necessary to cause clinical infections (Altekruse et al., 1998). These trends reasserts the importance of detecting low numbers of *Salmonella* cells in cheese .

High hydrostatic pressure (HHP) has been proposed as a viable alternative to conventional heat treatment for preserving food. In contrast to thermal processing, the application of HHP to foods causes negligible impairment of nutritional value, taste, colour or flavour (Smelt, 1998). HHP induces a number of changes to morphology, biochemical reactions and genetic mechanisms, and to the cell membrane and wall of microorganisms (Hoover et al., 1989). Several factors are known to affect the resistance of bacteria to HHP: the temperature, magnitude and duration of pressure treatment, the stage of growth and the

composition of medium (McClements et al., 2001). Moreover, in food, two effects always determine microbiological safety and stability: the effect of the food matrix during treatment (Patterson and Kilpatrick, 1998), and its effect after treatment, during the repair phase of the microorganisms (Smelt, 1998).

In cheese, differences in the degree of microbial inactivation obtained applying HHP may be due to the species and the quantity of starter cultures used as well as cheese acidity and composition (O'Reilly et al., 2001). Several authors have studied the effect of high hydrostatic pressure on microorganisms and spores in different types of cheese (O'Reilly et al., 2001; López-Pedemonte et al., 2003 and De Lamo-Castellví et al., 2005a) but it is also necessary to evaluate the behaviour of *Salmonella*. For this purpose, we selected a washed-curd model cheese following the protocol proposed by López-Pedemonte et al. (2003) which allowed us to work under controlled microbiological conditions.

The aim of this study was to evaluate the effect of HHP in two strains of *Salmonella enterica* (*S. enteritidis* and *S. typhimurium*) inoculated in washed-curd model cheese produced with or without starter culture and to analyse the behaviour of both pathogens during a storage period of 15 days at 12 ºC.

2. Materials and methods

2.1. Bacteria culture preparation

Salmonella enteritidis (ATCC 13076) *and S. typhimurium* (ATCC 13311) were obtained from Spanish Type Culture Collection (CECT, Universidad de Valencia, Valencia, Spain) and were kept in cryobeads (Nalgene® System 100™, Microkit Iberica S.L., Madrid, Spain) at –20 ºC. Initial bacterial culture was obtained by inoculating a cryobead in 10 ml of Brain Heart Infusion broth (BHI, Oxoid, Basingtoke, UK) containing 0.6% yeast extract (BHIYE, Oxoid) and incubated at 37 ºC for 24 h. One ml of this first culture was transferred to 10 ml of BHIYE and incubated for 18 h at the same temperature to allow the culture to reach the stationary phase. Cells were centrifuged at 1,250 *x* g for 15 min at room temperature,

washed once in 10 mM phosphate-buffered saline (PBS, Oxoid) and the pellets were resuspended in PBS to a final concentration of about 8-9 log (CFU/ml).

2.2. Starter culture preparation

A mixture of commercial lyophilized strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ezal MAO 11, Rhodia Iberia S.A., Madrid, Spain), that is known as a non-bacteriocin producer, was used as a starter culture for the washed-curd model cheese manufacture. The culture was revived inoculating 0.015 g of mixture in 1,000 ml of commercial sterilized skimmed milk and incubated at 30 ºC for 24 h. A volume of 50 ml was used to prepare a subculture in 200 ml of sterilized skimmed milk, which was incubated under the same temperature and time conditions reaching a final concentration of about 9 log (CFU/ml).

2.3. Model cheese manufacture

Raw cow's milk obtained from a local farm was transported and stored at 4 ºC. Before inoculation, milk was pasteurized at 65 ºC for 30 min and cooled to 32 ºC in an ice and water bath. Two per cent (vol/vol) of starter culture and 0.01% (vol/vol) of a 35% $CaCl_2$ dilution (Arroyo, Santander, Spain) were added. Milk was kept in a water bath at 32 ºC for 20 min and then inoculated with 1% (vol/vol) of *S. enteritidis* or *S. typhimurium* inoculum (except blanks). 0.02% (vol/vol) of liquid rennet extract of bovine origin (520 mg/l of active chymosin, Arroyo, Santander, Spain) was added as coagulating agent. Centrifugation bottles (Nalgene®, Nalge Nunc International, Rochester, USA) were filled with 225 ml of inoculated milk and placed in a bath at 32 ºC for 40 min until curd was formed. Curd was cut into small pieces and kept in the water bath while temperature was increased to 37 ºC for over 5 min. The bottles were kept in the bath at 37 ºC for further 15 min. Curds were washed to avoid excessive acidification, substituting 40% (vol/vol) of whey with sterile water. Bottles were then centrifuged at 7,000 *x* g for 40 min at 20 ºC and kept in their containers in a water bath at 37 ºC until pH 5.5 was reached. When pH of the resulting cheeses dropped to 5.5, whey was completely removed from the bottles by decantation and cheeses were salted by adding, in each bottle, 100 ml of 20% (wt/vol) NaCl sterile brine for 15 min. After this, cheeses were removed from the centrifugation bottles using sterile

pincers and dried on sterile paper. They had a final weight of approximately 23 g with 55% of dry matter and 1.5% of salt in moisture content. Cheeses were twice vacuum packed in plastic bags (Cryovac Packaging, Sant Boi de Llobregat, Spain) and stored overnight at 12 ºC before high pressure treatment.

In the case of model cheeses produced without starter culture, the same protocol without adding starter culture was followed.

2.4. High pressure treatment

Cheese samples were pressurized in a discontinuous isostatic press (ACB, Nantes, France) with a pressure chamber measuring 30 cm of diameter and 70 cm of length. The time needed to achieve maximum pressure (400 MPa) was 4 min. The temperature of the pressurization fluid (water) was measured by a thermocouple. Cheese samples were pressurized at 300 and 400 MPa for 10 min at room temperature.

2.5. Sampling protocol

Pasteurized milk samples inoculated with *S. enteritidis* or *S. typhimurium* were analysed to count the population of these microorganisms prior to the cheese making process. Three groups of cheese samples were analysed: Non-inoculated (blank cheese samples), inoculated with *S. enteritidis* or *S. typhimurium* but not treated by HHP (control cheese samples), and inoculated and treated (pressurized cheese samples). All samples were stored at 12 ºC. At the end of the cheese production, control samples were analysed to assess the population of *S. enteritidis* and *S. typhimurium*. Microbiological analyses were performed at 0 day (immediately after HHP treatment) and at 1, 7 and 15 days after treatment for all samples. Experiments were run three times with duplicate analyses each time.

2.6. Microbiological analyses

Ten g of each sample were homogenized in 90 ml of maximum recovery diluent (MRD, Oxoid) for 1 min using an electromechanical blender (BagMixer®, Interscience, St Nom, France) at room temperature. Ten-fold serial dilutions were produced in MRD and 1 ml of appropriate dilution was plated into *Salmonella* Chromogenic agar base (SC, Oxoid) containing *Salmonella* Selective Supplement (Oxoid) to count non-injured cells of *S.*

enteritidis and *S. typhimurium*. A modification of the thin agar layer method (TAL_m) (Kang and Fung, 1999) was used to count both injured and non-injured cells to a detection level of 10 CFU/g. After a selective medium (SC) layer, the first layer of nutritive medium (Brain Heart Infusion agar supplemented with 0,6% yeast extract, BHIAYE, Oxoid) was added. Then, 1 ml of appropriate dilution of cheese sample and a second layer of nutritive medium were added. All plates were incubated at 37 ºC for 24 h.

Besides this, 1 ml of appropriate dilution was plated into M17 agar (M17, Oxoid) supplemented with 5% (wt/vol) of lactose (Oxoid) at 20% (wt/vol) to evaluate the count of *Lactococcus* in model cheeses produced with starter culture. All plates were incubated at 30 ºC for 48 h.

Results are expressed as the logarithm of colony-forming units (CFU) per g. Increase value was calculated as the difference between the logarithms of colony counts of control cheese samples (N_c) and milk samples (N_m) analysed at the end of cheese production ($\log N_c - \log N_m$). Lethality was calculated as the difference between the logarithms of colony counts of the control (N_0) and treated samples at day 0 ($\log N_o - \log N$).

To check the presence of injured *S. enteritidis* and *S. typhimurium* cells in cheese samples that did not show colonies growing in both culture media, an enrichment procedure was used. For this purpose, the first dilution of each sample obtained after HHP treatment and after 1, 7 and 15 days of storage, consisting of 10 g of cheese diluted in 90 ml of MRD, was stored at 37 ºC for 24 h. A loopful of this culture was streaked onto a plate of SC and incubated at 37 ºC for 24 h.

2.7. pH measurement

Model cheese pH was determined using a penetration electrode (Crison, Crison Instruments S.A., Alella, Spain). pH values were based on the mean of nine measurements.

2.8. Statistical analyses

Analysis of variance was performed using the General Linear Models Procedure of SAS® software (SAS® System for Windows™, 8.02, 1999, SAS Institute, Inc., Cary, North Carolina, USA). Student-Newman-Keuls tests were used to obtain paired comparisons among sample means. Level of significance was set at $P<0.05$.

3. Results and Discussion

3.1. Behaviour of initial inocula during model cheese production

Cell counts of *S. enteritidis* and *S. typhimurium* inoculated in milk and in model cheese produced with and without starter culture and their increase values are shown in Table 1 and Table 2. It is clear from the results obtained that *S. enteritidis* and *S. typhimurium* increased their cell counts during cheese making process in both types of cheese. Several authors have reported an increase of *Salmonella* counts during Cheddar cheese manufacture process (Wood et al., 1984; Modi et al., 2001). This increase in regard to inoculated milk could be considered a consequence of physical concentration and growth of the inocula during cheese production. Spahr and Url (1994) found that the increase of 1 log (CFU/g) in bacterial counts during the first steps of cheese making process is due to the physical concentration phenomenon resulting from the syneresis of the curd rather than to bacterial growth. Also, some studies have demonstrated the ability of different species of pathogenic microorganisms to grow during the cheese making process when they have been added to pasteurized milk after being grown in favourable conditions (Johnson et al., 1991). Moreover, it is important to remark that no significant differences ($P>0.05$) were detected in the increase values of different strains and culture media obtained during the production of cheese produced with starter culture (Table 1). However, in cheese made without starter culture, the increase value of *S. enteritidis* and *S. typhimurium* observed during cheese manufacture was highly significant ($P<0.05$) in TAL_m, but not in selective medium. No differences were detected between strains (Table 2).

A population of bacteria after physical preservation treatment may contain three physiological types of cells: non-injured cells, that are capable of growth and multiplication

both in selective and non-selective culture medium; injured cells, that are capable of multiplication in a non-selective medium but not in a selective medium; and dead cells, which are incapable of multiplication under any conditions (Wuytack et al., 2003). In this research, a selective culture medium (SC) and a modification of TAL_m were used to detect injured *Salmonella* population produced by the cheese making process, HHP treatments and low pH of cheese (Table 6). Wu et al. (2001) reported that TAL was an adequate method to enumerate acid injured *S. typhimurium* cells. In previous experiments carried out on the same washed-curd model cheeses, we found that the combination of both culture media was an appropriate method to detect *Yersinia enterocolitica* injured cells (De Lamo-Castellví et al., 2005a). In the present case, the population of injured cells of *S. enteritidis* and *S. typhimurium* induced by cheese making and the low pH of cheese was not able to recover and grow in TAL_m medium. Other authors have reported this behaviour: Brashears et al. (2001) found that *Salmonella* cells subjected to stress by lactic acid (pH 3.5 after 18 h of incubation at 4 ºC) were not able to repair themselves using another recovery media method: stressed cells were put on a 5 ml thin layer of tryptic soy agar, incubated for 2 h at room temperature and then overlayed with Xylose Lysine $Tergitol_4$ agar.

3.2. High pressure effect at day 0 in model cheese

Lethality results obtained immediately after HHP treatments in washed-curd model cheese inoculated with and without starter culture and with *S. enteritidis* or *S. typhimurium* are shown in Table 3 and Table 4. In the case of cheese produced with starter culture, 300 and 400 MPa treatments did not produce any significant differences in lethality values in both strains and culture media ($P>0.05$). Thus, we could not detect any differences in the pressure resistance behaviour between strains. Nevertheless, it is important to point out that at 400 MPa -in the case of *S. enteritidis* and *S. typhimurium*- and 300 MPa -in the case of *S. typhimurium* in SC culture medium- lethality values depended on the concentration of these microorganisms in cheese prior to the treatment and could have been higher. In cheese made without starter culture and inoculated with *S. enteritidis*, the treatment that caused the highest lethality was 400 MPa and it is worth to mention that this value was more significant ($P<0.05$) in TAL_m than in selective medium. No significant differences were detected in the lethality values obtained after applying 300 and 400 MPa in model cheese

made without starter culture and inoculated with *S. typhimurium* except in the case of 400 MPa in TAL_m, that was highly significant ($P<0.05$).

HHP treatments used in this work were effective to reduce the initial counts on selective medium and TAL_m of *S. enteritidis* and *S. typhimurium* inoculated in cheese produced with and without starter culture. Other studies have evaluated the effect of high hydrostatic pressure on inoculated microorganisms in cheese. Capellas et al. (1996) made fresh cheese inoculated with *Escherichia coli* CECT 405. Samples were treated using different combinations of pressure (400-500 MPa), temperature (2, 10 or 25 ºC) and time (5, 10 or 15 min) and subsequently stored at 2-4 ºC. No survival of *E. coli* cells were detected 1 day after pressurisation, except in samples treated for 5 min at 25 ºC at pressures of 400-450 MPa. O'Reilly et al. (2000) did not detect cell counts of *Staphylococcus aureus* and *E. coli* inoculated in cheese slurries after applying HHP treatments higher than 600 MPa at 20 ºC and 400 MPa at 30 ºC, respectively. In a previous survey (De Lamo-Castellvi et al., 2005a) we reported that HHP treatments at 400 and 500 MPa for 10 min at 20 ºC were effective to reduce the initial population of *Y. enterocolitica* inoculated in model cheese produced with starter culture.

3.3. Behaviour of S. enteritidis *and* S. typhimurium *after HHP treatment in model cheese produced with starter culture*

No significant differences ($P>0.05$) between cell counts of *S. enteritidis* and *S. typhimurium* were detected in both culture media, except in cheese samples inoculated with *S. enteritidis* and *S. typhimurium* and treated at 300 MPa ($P<0.05$). *Salmonella* strains inoculated in model cheese treated at 400 MPa were not able to repair after 15 days of storage at 12 ºC (Fig. 1 a and b) and kept their counts below the detection level (10 CFU/g) in both culture media. Moreover, after the enrichment process (24 h at 37 ºC), *S. enteritidis* and *S. typhimurium* cells were only detected at day 1 of storage at 12 ºC (data not shown). At 300 MPa, cheese samples inoculated with *S. enteritidis* decreased their cell counts during the time of storage (Fig. 1 a) without reaching the level of detection, and *S. typhimurium* decreased their cell counts below the level of detection after 7 days of storage (Fig. 1 b) in

both culture media. In this case, it is worth noting that cell counts of *S. typhimurium* were found after the enrichment process from 0 to 15 days of storage.

It is well established that HHP treatments produce sublethally injured cells (McClements et al., 2001; Bozoglu et al., 2004), which in a stressful environment are not able to repair. The analysis of the behaviour of cell counts after the treatment suggests that the cheese matrix did not allow recovery and growth of injured cells. The main contributing factor to this effect seems to be low pH mainly produced by the conversion of lactose in lactic acid and also by the presence of the starter culture (Table 6 and Fig. 6). Several authors have reported that when pH is low, most microbes become more susceptible to HHP inactivation and recovery of sublethally injured cells is reduced (Pagán et al., 2001). In a preliminary research (De Lamo-Castellví et al., 2005a), we made washed-curd model cheeses inoculated with three strains of *Y. enterocolitica* (serotypes O:1, O:3 and O:8) and starter culture, and we treated them with HHP at 300, 400 and 500 MPa for 10 min at 20 ºC. In this case, none of the *Y. enterocolitica* strains inoculated in samples treated at 400 and 500 MPa showed repair ability after 15 days of storage at 8 ºC: their cell counts remained below the detection level (1 log [CFU/g]). Serotype O:1 treated at 300 MPa was not able to repair, and serotypes O:3 and O:8 treated at 300 MPa showed a decrease in their cell counts during storage.

None of *S. typhimurium* or *S. enteritidis* cells inoculated in control samples had the ability to grow after 15 days of ripening at 12 ºC (Fig. 1 a and b) and showed a decrease trend in their cell counts after 1 and 7 days of storage, respectively. This trend was more accentuated in the case of *S. typhimurium* (Fig. 1 b) and showed counts close to the detection level (10 CFU/g) after 15 days of storage at 12 ºC. This decrease trend detected in *Salmonella* cell counts of control samples proves that both strains are affected by low pH and starter culture and this effect was more important in the case of *S. typhimurium*. Modi et al. (2001) reported that *Salmonella* can survive during the manufacture and ripening of Cheddar cheese made from raw milk at least 60 days at temperature greater than 2 ºC and mainly the pH and starter culture had an effect on its survival.

3.4. Behaviour of S. enteritidis *and* S. typhimurium *after HHP treatment in model cheeses produced without starter culture*

S. enteritidis and *S. typhimurium* inoculated in model cheese and treated at 300 and 400 MPa had the ability to repair and grow during the storage period at 12 ºC (Fig. 2 b and c). Nevertheless, depending on the HHP treatment, the time necessary to detect this behaviour and the level of *Salmonella* population after the storage period were different. At 300 MPa, both strains began to grow after 1 day of storage in both culture media (Fig. 2 b) reaching counts close to 4 and 8 log (CFU/g) in selective medium and TAL_m, respectively, at the end of the process. At 400 MPa, *S. enteritidis* and *S. typhimurium* increased their cell counts after 1 and 7 days of storage in TAL_m and selective medium (Fig. 2 c). At the end of storage period, both strains showed cell counts over 3 and 7 log (CFU/g) in selective medium and TAL_m, respectively.

Control samples inoculated with *S. enteritidis* and *S. typhimurium* did not show significant differences ($P<0.05$) in their cell counts during the time of storage in any culture media (Fig. 2 a), except for *S. typhimurium* in TAL_m ($P<0.05$). Nevertheless, significant differences ($P<0.05$) between cell counts of *S. enteritidis* and *S. typhimurium* were detected in both culture media and in all treated cheese samples analysed during the storage period at 12 ºC (Fig. 2 a, b and c).

Sublethally injured cells are usually capable of repairing their injury in a non-selective environment. Generally, nutritionally rich media allow a relatively rapid repair of a high proportion of injured cells (Wuytack et al., 2003). Several authors have reported this behaviour after applying HHP treatments in different matrices. Metrick et al. (1989) reported that *Salmonella* spp. strains were able to repair in chicken but not in buffer under the same conditions of pressure treatment and storage. Apparently, the lack of available nutrients in buffer prevented the cells from repairing. Carlez et al. (1994) found that, although there were no counts of *Pseudomonas* spp. after minced meat was pressurized (400 MPa, 20 min), growth was detected at 6 days of storage at 3 ºC. After this initial lag, the growth rate of the repaired organisms was similar to that of the controls. Moreover, in previous experiments (De Lamo-Castellví, 2005b), we found that three strains of *Y. enterocolitica* inoculated in skimmed milk and treated at 400 and 500 MPa, were able to

grow in 1-4 days and in 1-10 days respectively after not having found cell counts in the same day of the treatment.

3.5. High pressure effect at day 0 in starter culture

At 300 MPa, the effect of HHP treatment on starter population was minimum, whereas it was close to 3 log (CFU/g) at 400 MPa (Table 5). Moreover, no significant difference ($P>0.05$) in pressure resistance behaviour of starter culture was detected between cheeses inoculated with *S. enteritidis* or *S. typhimurium*.

Molina-Höppner (2002) reported that HHP treatments of *Lactococcus lactis* spp. *cremoris* MG1363 inoculated in milk buffer, initially affected metabolic activity and subsequently damaged membrane integrity. After a treatment for 5 min at 300 MPa the metabolic activity was 10-12% of the activity of untreated microorganisms and after 12 min of treatment the cells did not show any metabolic activity. During the treatment at 300 MPa cell death was closely followed by the loss of metabolic activity, but cultures retained about 25% of metabolic activity, even after 3 log (CFU/ml) reduction in cell counts. Nevertheless, the addition of high concentration of salts and solutes showed a protective effect during HHP treatment. The composition of the medium where the starter culture is dispersed in at the moment of pressurization significantly influences the efficiency of inactivation. Loss of viability is lower in food systems than in PBS (Kalchayanand et al., 1998 a).

3.6. Behaviour of starter culture after HHP treatments in model cheeses inoculated with S. enteritidis and S. typhimurium

Starter population treated by HHP showed a decrease trend in its cell counts in cheese samples inoculated with *S. enteritidis* and *S. typhimurium* during the storage time (Fig. 3 a and b). At 300 MPa, cell counts began to decrease during the same day of the treatment, in the case of *S. enteritidis,* and after 1 day of storage at 12 ºC, in the case of *S. typhimurium*. At 400 MPa, this trend was observed after 1 day keeping cheese samples under refrigerated conditions. In control samples, this behaviour was observed after 7 days.

Some authors suggest that HHP could restrict the pH range that bacteria can tolerate, possibly due to the inhibition of ATPase-dependent transfer protons and cations -or directly their denaturation- or the dislocation of bound ATPase in the membrane, and therefore

recovery of sublethally injured cells would be reduced (Pagán et al., 2001; Wouters et al., 1998). Moreover, Molina-Höppner, (2002) found that *L. lactis* cell counts on GM17 supplemented with 3% of NaCl were generally lower than cell counts on GM17 indicating a formation of sublethally injured population during the application of HHP treatments.

We conclude that HHP treatments can be useful to accelerate the reduction of initial levels of *Salmonella* in cheese, but it is necessary to combine this technology with the presence of the starter culture to inhibit the recovery and growth and to increase the death rate of the injured population.

Acknowledgements

This study was supported financially by CAL-00-005-C2-1 project (*Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria*) and by *Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya* which provided S. De Lamo-Castellví a fellowship to carry out this investigation. We would like to thank the Spanish Type Culture Collection for providing *S. enteritidis* and *S. typhimurium* strains.

References

Ahmed, R., Soule, G., Demczuk, W. H., Clark, C., Khakhrria R., Ratnam, S., Marshall, NG L., Woodward, D. L., Johnson, W. M., Rodgers, F. 2000. Epidemiologic typing of *Salmonella enterica* serotype enteritidis in a Canada-wide outbreak of gastroenteritis due to contaminated cheese. J Clin Microbiol. 38, 2403-2406.

Altekruse, S. F., Timbo, B. B., Mowbray, J. C., Bean, N. H., Potter, M. E. 1998. Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: Sanitary Manufacturing Practices Protect Consumers. J. Food Prot. 61, 1405-1407.

Anonymous. 2003. Análisis de las cepas de *Salmonella* spp. aisladas de muestras clínicas de origen humano en España. Año 2001 (II). BES. 13, 145-156.

Bozoglu, F., Alpas, H., Kalentunç, G. 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. 2004. FEMS Immunology and Medical Microbiology. 40, 243-247.

Brashears, M. M., Amezquita, A., Stratton, J. 2001. Validation of methods used to recover *Escherichia coli* O157:H7 and *Salmonella* spp. subjected to stress conditions. J. Food Prot. 64, 1466-1471.

Capellas, M., Mor-Mur, M., Sendra, E., Pla, R., Guamis, B. 1996. Populations of aerobic mesophils and inoculated *E. coli* during storage of fresh goat's milk cheese treated with high pressure. J. Food Prot. 59, 582-587.

Carlez, A., Rosec, J. P., Richard, N., Cheftel, J.C. 1994. Bacterial growth during chilled storage of pressure-treated minced meat. Lebensm. Wiss. Technol. 27, 48-54.

Center for Disease Control. 1985. Milk-borne salmonellosis-Illinois. Morbi. Mortal. Weekly Rep. 34, 200.

De Lamo-Castellví, S., Capellas, M., López-Pedemonte, T., Hernández-Herrero, M. M., Guamis, B., Roig-Sagués, A. X. 2005a. Behaviour of *Yersinia enterocolitica* strains inoculated in model cheese treated with high hydrostatic pressure. J. Food Prot. 68, 528-533.

De Lamo-Castellví S., Roig-Sagués, A. X., Capellas, M., Hernández-Herrero, M. M., Guamis, B. 2005b. Survival and growth of *Yersinia enterocolitica* strains inoculated in skimmed milk treated with high hydrostatic pressure. Int. J. Food Microbiol. 102, 337-342.

El-Gazzar, F. E., Marth, E. H. 1992. *Salmonellae*, Salmonellosis, and dairy foods: a review. J. Dairy Sci. 75, 2327-2343.

Fontaine, R. E., Cohen, M. L., Martin, W. T., Vernon, T. M. 1980. Epidemic salmonellosis from Cheddar cheeses: surveillance and prevention. Am. J. Epidemiol. 111, 247.

Hoover, D. G., Metrick, C., Papineau, A. M., Farkas, D. F., Knorr, D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol. 43, 99-107.

Johnson, E. A., Nelson, J. H., Johnson, M. 1990. Microbiology safety of cheese made from heat-treated milk, part II. Microbiology. J. Food Prot. 53, 519-540.

Kalchayanand, N., Sikes, A., Dunne, C. P., Ray, B. 1998a. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. J. Food Prot. 61, 425-431.

Kang, D. H., Fung, D. Y. C. 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. J. Food Prot. 62, 1346-1349.

Leyer, G. J., Johnson, E. A. 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. 58:, 2075-2080.

López-Pedemonte, T. J., Roig-Sagués, A. X., Trujillo, A. J., Capellas, M., Guamis, B. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J. Dairy Sci. 86, 3075-3081.

Mattick, K. L., J⌀rgensen, F., Legan, J. D., Lappin-Scott, H. M., Humphrey, T. J. 2001. Improving recovery of *Salmonella enterica* serovar Typhimurium DT104 cells injured by heating at different water activity values. J. Food Prot. 64 , 1472-1476.

McClements, J. M., Patterson, M. F., Linton, M. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. J. Food Prot. 64, 514-522.

Metrick, C., Hoover, D. G., Farkas, D. F. 1989. Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. J. Food Sci. 54, 1547-1564.

Modi, R., Hill, Y., Griffiths, M. W. 2001. Effect of phage on survival of *Salmonella enteritidis* during manufacture and storage of Cheddar cheese made from raw and pasteurised milk. J. Food Prot. 64, 927-933.

Molina-Höppner, A. 2002. Physiological response of *Lactococcus lactis* to high-pressure. M. S Thesis, Technische Universität München, München.

O'Reilly, C. E., O'Connor, P. M., Kelly, A. L., Beresford, T. P., Murphy, P. M. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl. Environ. Microbiol. 66, 4890-4896.

O'Reilly, C. E., Kelly, A. L., Murphy, P. M., Beresford, T. P. 2001. High pressure treatment: applications in cheese manufacture and ripening. Food Sci. Technol. 12, 51-59.

Pagán, R., Jordan, S., Benito, A., Mackey, B. 2001. Enhanced acid sensitivity of pressure-damaged *Escherichia coli* O:157 cells. Appl. Environ. Microbiol. 67, 1983-1985.

Patterson, M. F., Kilpatrick, D. J. 1998. The combined effect of high hydrostatic pressure and mild heat on inactivation of pathogens in milk and poultry. J. Food Prot. 61, 432-436.

Smelt, J.P.P.M. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci. Technol. 9, 152-158

Spahr, U., Url, B. 1994. Behaviour of pathogenic bacteria in cheese-a synopsis of experimental data. Butlletin of IDF 298.

Wood, D. S, Collins-Thompson, D. L., Irvine, D. M., Myhr, A. N. 1984. Source and persistence of *Salmonella muenster* in naturally contaminated Cheddar cheese. J. Food Prot. 47, 20-22.

Wouters, P. C., Glaasker, E., Smelt, J. P. P. M. 1998. Effects of high-pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64, 509-514.

Wu, V. C. H., Fung, D. Y. C., Kang, D. H., Thompson, L. K. 2001. Evaluation of thin agar layer method for recovery of acid-injured foodborne pathogens. J. Food Prot. 64, 1067-1071.

Wuytack, E. Y., Phuong, L. D. T., Aertsen, A., Reyns, K. M. F., Marquenie, D., De Ketelaere, B., Massachalck, B., Van Opstal, I., Diels, A. M. J., Michiels, C. W. 2003. Comparision of sublethal injury induced in *Salmonella enterica* serovar Typhimurium by heat and by different nonthermal treatments. J. Food Prot. 66, 31-37.

Table 1

Cell counts and increase values of *S. enteritidis* and *S. typhimurium* inoculated in milk (initial inoculum) and in model cheese produced with starter culture (at the end of cheese production).

		Counts in SC log (CFU/g)		Increase ($\log N_c - \log N_m$)		Counts in TAL_m log (CFU/g)		Increase ($\log N_c - \log N_m$)	
Strains	Sample	Mean[†] (n=6)	SD[‡]	Mean (n=6)	SD	Mean (n=6)	SD	Mean (n=6)	SD
S. enteritidis	milk	5.40^{a}	±0.45	-	-	5.61^{a}	±0.34	-	-
	cheese	6.45^{b}	±0.26	0.95^{1}	±0.18	6.81^{b}	±0.17	1.20^{1}	±0.46
S. typhimurium	milk	5.68^{A}	±0.28	-	-	$5.92^{A,B}$	±0.13	-	-
	cheese	$6.03^{A,B}$	±0.79	0.35^{1}	±0.61	6.77^{B}	±0.61	0.84^{1}	±0.50

[†]Means with different letters and numbers are significantly different ($P<0.05$)

[‡]Standard Deviation

Table 2

Cell counts and increase values of *S. enteritidis* and *S. typhimurium* inoculated in milk (initial inoculum) and in model cheese produced without starter culture (at the end of cheese production).

		Counts in SC log (CFU/g)		Increase ($\log N_c - \log N_m$)		Counts in TAL_m log (CFU/g)		Increase ($\log N_c - \log N_m$)	
Strains	Sample	Mean† (n=6)	SD‡	Mean (n=6)	SD	Mean (n=6)	SD	Mean (n=6)	SD
S .enteritidis	milk	4.79[a]	±0.21	-	-	5.37[b]	±0.34	-	-
	cheese	5.36[b]	±0.33	0.57[1]	±0.43	7.50[c]	±0.55	2.13[2]	±0.29
S. typhimurium	milk	4.09[A]	±0.60	-	-	4.93[A]	±0.68	-	-
	cheese	4.81[A]	±0.47	0.72[1]	±0.69	7.32[B]	±0.67	2.38[2]	±0.10

†Means with different letters and numbers are significantly different ($P<0.05$)

‡Standard Deviation

Table 3

Cell counts and lethality values of *S. enteritidis* and *S. typhimurium* inoculated in model cheese produced with starter culture and pressurized at 300 and 400 MPa at room temperature for 10 min at day 0.

		Counts in SC log (CFU/g)		Lethality (log N_0 – log N)		Counts in TAL_m log (CFU/g)		Lethality (log N_0 – log N)	
Strains	Treatment (MPa)	Mean (n=6)	SD	Mean (n=6)	SD	Mean (n=6)	SD	Mean (n=6)	SD
S. enteritidis	0	5.98	±0.74	-	-	6.46	±0.27	-	-
	300	1.79	±1.22	4.19[a]	±1.41	2.87	±1.10	4.13[a]	±0.98
	400	n.d	-	≥4.98[a]	±0.74	n.d	-	≥5.46[a]	±0.27
S. typhimurium	0	5.27	±0.73	-	-	6.37	±0.35	-	-
	300	n.d	-	≥4.27[a]	±0.73	1.46	±0.56	4.91[a]	±0.82
	400	n.d	-	≥4.27[a]	±0.73	n.d	-	≥5.37[a]	±0.35

n.d.: not detected. Detection level: 1 log (CFU/g)

[†]Means with different letters are significantly different ($P<0.05$)

[‡]Standard Deviation

Table 4

Cell counts and lethality values of *S. enteritidis* and *S. typhimurium* inoculated in model cheese produced without starter culture and pressurized at 300 and 400 MPa at room temperature for 10 min at day 0.

Strains	Treatment (MPa)	Counts in SC log (CFU/g) Mean[†] (n=6)	SD[‡]	Lethality ($\log N_0 - \log N$) Mean (n=6)	SD	Counts in TAL_m log (CFU/g) Mean (n=6)	SD	Lethality ($\log N_0 - \log N$) Mean (n=6)	SD
S. enteritidis	0	5.78	±0.34	-	-	7.75	±0.22	-	-
	300	3.80	±0.15	1.89^{A}	±0.32	5.93	±0.17	2.14^{A}	±0.28
	400	n.d	-	$\geq 4.78^{C}$	±0.34	2.03	±0.41	6.34^{D}	±0.35
S. typhimurium	0	4.50	±1.17	-	-	7.69	±0.12	-	-
	300	2.59	±0.43	$2.66^{A,B}$	±0.31	5.05	±0.10	$3.07^{A,B}$	±1.02
	400	n.d	-	$\geq 3.50^{B}$	±1.17	n.d	-	6.69^{D}	±0.12

n.d.: not detected. Detection level: 1 log (CFU/g)

[†]Means with different letters are significantly different ($P<0.05$)

[‡]Standard Deviation

Table 5

Cell counts in M17 and lethality values of starter culture inoculated in model cheese and pressurized at 300 and 400 MPa at room temperature for 10 min at day 0.

Strains	Treatment (MPa)	Counts in SC log (CFU/g) Mean[†] (n=6)	SD[‡]	Lethality (log N_0 – log N) Mean (n=6)	SD
S. enteritidis	0	9.21	±0.10	-	-
	300	8.95	±0.10	0.26[a]	±0.12
	400	6.37	±0.64	2.84[b]	±0.71
S. typhimurium	0	9.11	±0.15	-	-
	300	8.89	±0.10	0.23[a]	±0.18
	400	5.90	±0.86	3.30[b]	±0.88

[†]Means with different letters are significantly different ($P<0.05$)

[‡]Standard Deviation

Table 6

Behaviour of pH in blank cheese samples produced with and without starter culture and stored at 12ºC.

Time (Days)	With starter		Without starter	
	Mean[†] (n=6)	SD[‡]	Mean (n=6)	SD
cpd	4.89[a]	0.05	6.60[c]	0.06
0	4.89[a]	0.02	6.56[c,d]	0.11
1	4.79[b]	0.04	6.53[d,e]	0.05
7	4.75[b]	0.10	6.49[e,f]	0.02
15	4.79[b]	0.04	6.46[f]	0.01

cpd: cheese production day

[†]Means with different letters are significantly different ($P<0.05$)

[‡]Standard Deviation

Fig. 1. Behaviour of *S. enteritidis* (a) and *S. typhimurium* (b) inoculated in model cheese produced with starter culture after HHP treatments at 300, 400 MPa for 10 min at room temperature.

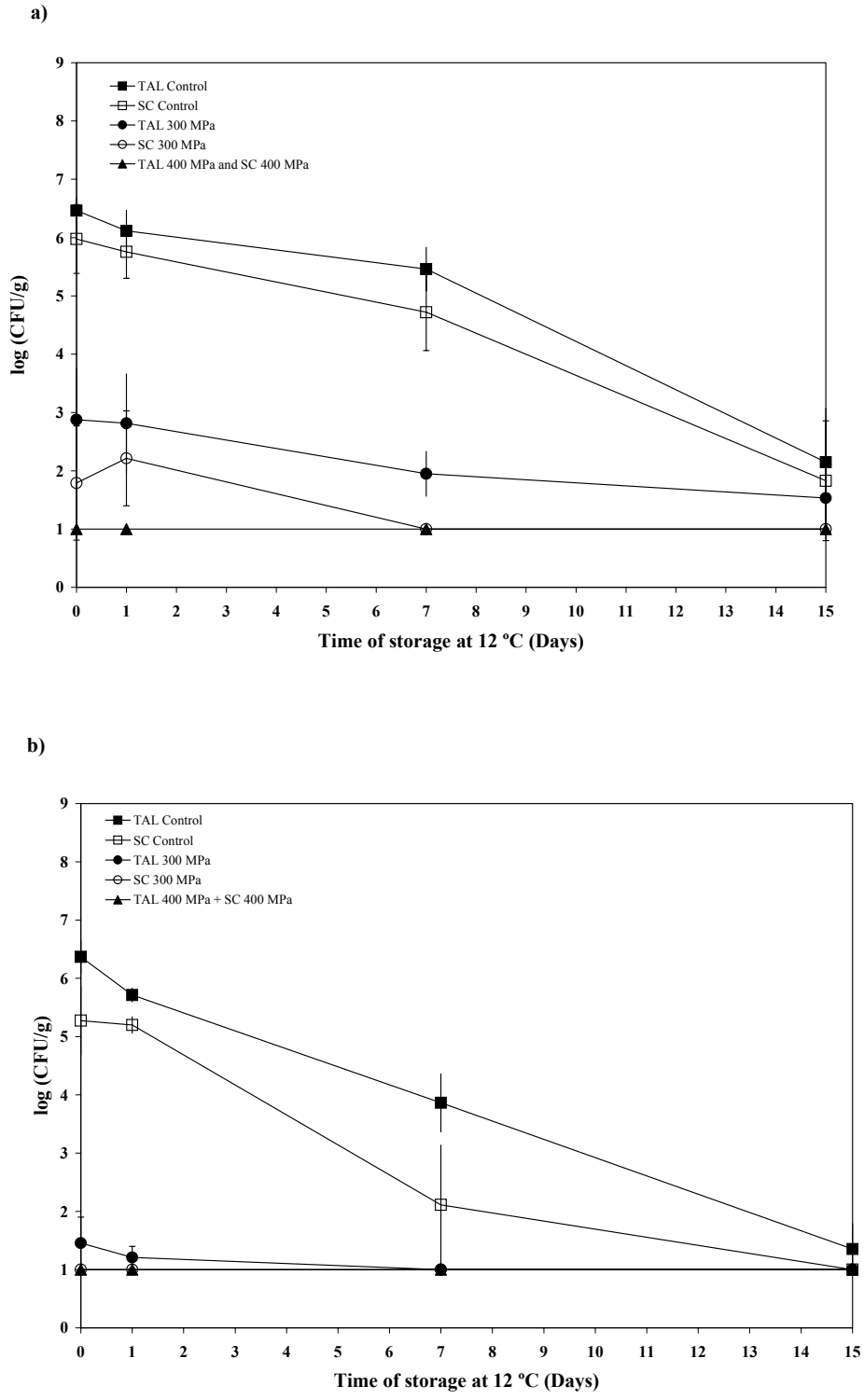


Fig. 2. Behaviour of *S. enteritidis* and *S. typhimurium* inoculated in model cheese produced without starter culture and non-treated (a) and after treated at 300 MPa (b) and 400 MPa (c) for 10 min at room temperature.

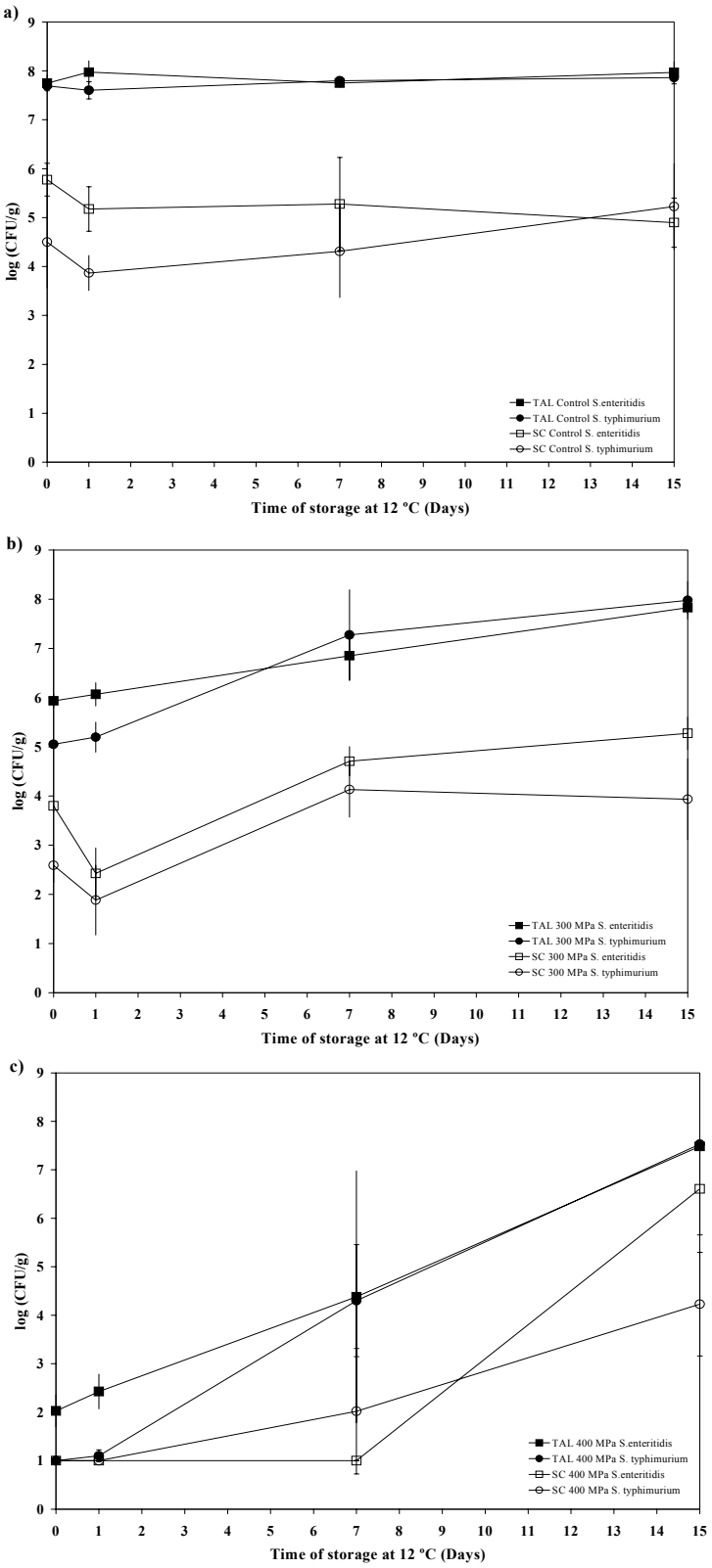
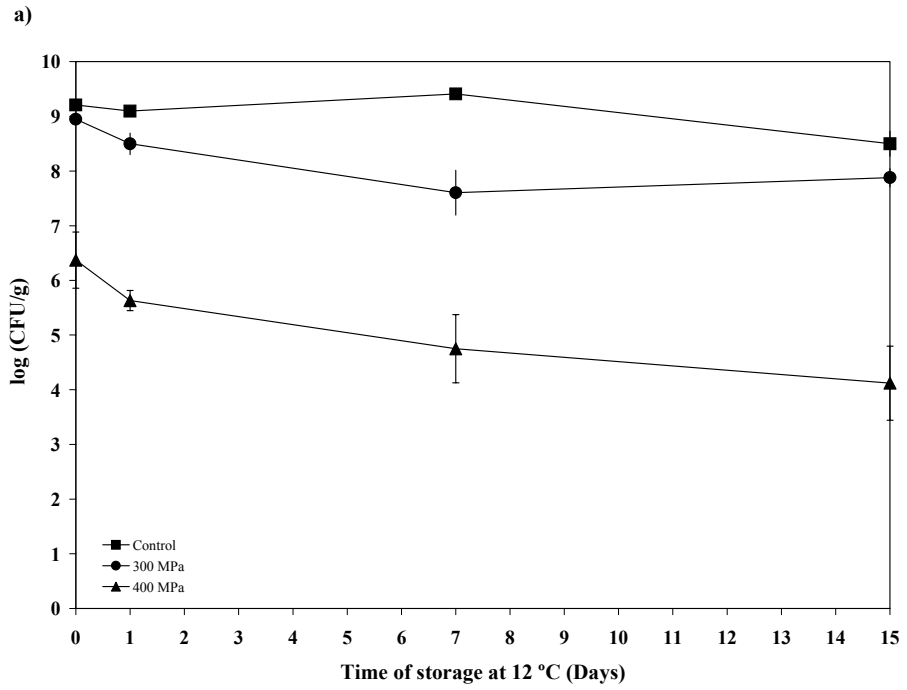
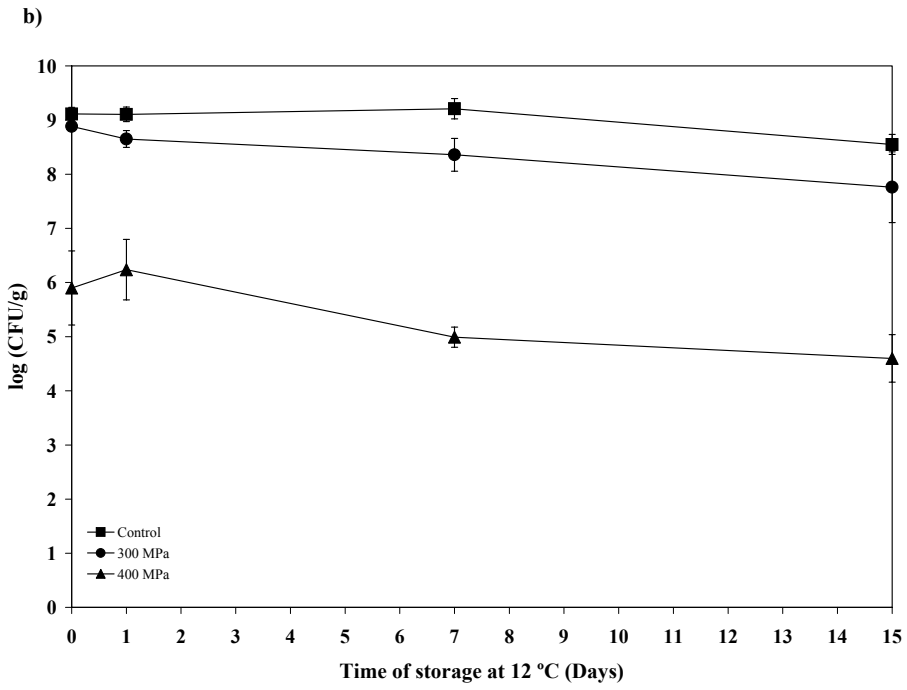


Fig. 3. Behaviour of starter culture in cheese samples inoculated with *S. enteritidis* (a) and *S. typhimurium* (b) after HHP treatment at 300 MPa and 400 MPa for 10 min at room temperature.





5. Results and Discussion

5.1. Skimmed milk [1]

5.1.1 High Pressure effect at day 0

Cell counts obtained at day 0 showed that the most effective treatments for all *Y. enterocolitica* strains studied were 400 and 500 MPa (*Table 1*, **paper 1**). Pressure treatment at 300 MPa was the only that showed significant differences in the decrease values of the four strains used in this research. Serotypes O:3 and O:8 were clearly more pressure resistant than O:1 and O:9. This variation in baroresistance within strains of the same species has already been reported in other Gram negative bacteria (Patterson et al., 1995; Benito et al., 1999).

5.1.2 Behaviour of **Y. enterocolitica** *after high hydrostatic pressure treatments*

Y. enterocolitica strains inoculated in skimmed milk and treated at 300, 400 and 500 MPa had the ability to repair and grow during the storage period at 8ºC. Nevertheless, the time necessary to detect this behaviour and the concentration of *Yersinia* population after the storage period were different. At 500 MPa cells started to grow between day 2 and 10 (mostly at day 4), except in one replicate of *Y. enterocolitica* serotypes O:1 and O:9, that only started to grow after 15 days of storage (**Table 8**, this chapter).

The fact that cells treated at 500 MPa only showed colony formation in culture media after certain days of storage at 8ºC suggests that the treatment injured them seriously and they needed time to repair. After this initial lag, the growth rate of the repaired organisms was similar to that of the controls. The considerably high repair and growth abilities of *Y. enterocolitica* in milk may be due to the nutritive nature of the medium and its liquid nature combined with the psychrotrophic behaviour of the pathogen.

[1] Related to the first paper

Table 8. *Cell counts at 0 and 15 days of* Y. enterocolitica *inoculated in milk and pressurized at 500 MPa at 20ºC for 10 min.*

Serotype	Pressure (MPa)	Counts in BHIAYE after HHP treatment log (cfu/ml)		Starting day of increase of cell counts	Counts in BHIAYE after 15 days of storage log (cfu/ml)	
		Mean (n=6)	SD[a]		Mean (n=2)	SD
O:1	500	n.d	-	4	8.54	±0.07
				10	8.51	±0.02
				-	-	-
O:3	500	n.d	-	4	8.96	±0.03
				4	8.55	±0.09
				7	8.95	±0.05
O:8	500	n.d	-	2	8.26	±0.26
				10	8.35	±0.12
				4	8.85	±0.01
O:9	500	n.d	-	4	9.06	±0.26
				7	8.38	±0.03
				-	-	-

n.d.: not detected. Detection level: 1 cfu/ml
[a]Standard Deviation

5.1.3 Kinetic study of microbial inactivation

Kinetic study of microbial inactivation in skimmed milk was performed with one of the most pressure resistant *Y. enterocolitica* strains in milk (serotype O:8) at 300 MPa and 20ºC. A strong tailing was detected in the survival curves of this microorganism after applying the pressure treatment for 35 min (*Figure 1*, **paper 1**). Several authors have reported this kinetic trend (Metrick et al., 1989; Patterson et al., 1995; Gervilla, 2001) and different mathematical models have been proposed to explain these deviations in log-linear models (Chen and Hoover, 2003; Guan et al., 2005). In this case, quadratic adjustment was the mathematical model that better fitted the results and, in order to calculate D values, two equations obtained by linear regression were used. This simplification allowed us to obtain two D values (5.47 and 10.93 min) to explain the

different kinetic behaviour that this strain showed depending on the duration of the pressure treatment.

5.2. Model cheese [2]

5.2.1 Behaviour of initial inoculum during model cheese production

In this research, a selective culture medium and a modification of the thin agar layer method (TAL_m) were used to detect injured bacteria produced by the cheese making process, high hydrostatic pressure treatments and/or low pH of cheese. Wu et al. (2001) reported that TAL was an adequate method to enumerate acid injured *Y. enterocolitica*, *E. coli* O157:H7 and *S. typhimurium* cells. Nevertheless, in this application, only in the case of *Y. enterocolitica* (cheese produced with starter culture) and *S. enterica* (cheese produced without starter culture) strains, TAL_m medium was able to recover injured cells. Ritz et al. (2001) reported that high hydrostatic pressure treatments produce heterogeneous cell populations likely due to variations in levels of injury, metabolic activity and viability among the cells. Using vital staining with fluorescent dyes, they showed the presence of viable subpopulations that did not form colonies and were therefore not detected on agar culture media.

E. coli and *S. enterica* strains increased their cell counts during cheese making process in both types of cheese (*Table 1*, **paper 3**; *Table 1* and *Table 2*, **paper 4**). Several studies have reported this behaviour with *E. coli* in hard and fresh cheese (Spahr and Url, 1994; Capellas et al., 1996) and with *Salmonella* during Cheddar cheese manufacture process (Wood et al., 1984; Modi et al., 2001). This increase, in regard to inoculated milk, could be produced by a combination of a physical entrapment in the curd (Spahr and Url, 1994) and growth of the initial population during cheese making process (Maher et al., 2001). Moreover, it is important to remark that no significant differences were detected in the increase values of different strains and in the cell counts

[2] Related to the second, third and fourth paper

obtained in selective medium and the medium designed for repairing (TAL_m), excepting for *Salmonella* strains in TAL_m, for cheese samples produced without starter culture.

5.2.2 High Pressure effect at day 0 in pathogenic bacteria

High pressure treatments used in this research were effective to reduce the initial counts of *Y. enterocolitica, E. coli* and *S. enterica* strains inoculated in cheese produced with and without starter culture, and the analysis of decrease values at the same day of the treatment showed that the most effective pressure treatments were the highest pressures applied (*Table 1*, **paper 2**; *Table 2*, **paper 3**; *Table 3* and *Table 4*, **paper 4**). In cheese samples produced with starter culture, these pressures were 400 and 500 MPa, -in the case of *Y. enterocolitica* (serotypes O:3 and O:8)*, E. coli* O59:H21, *E. coli* O157:H7 (only in selective culture medium) and 300 and 400 MPa, -in the case of *S. enterica* (*S. enteritidis* and *S. typhimurium*)-. Nevertheless, it is important to remark that in cheese produced without starter culture, it was necessary to apply more pressure to obtain the same reduction than in cheese produced with starter culture (**Figure 7**, this chapter). In fact, the most effective treatment was mainly 500 MPa, for *E. coli* strains and 400 MPa for *S. enteritidis* and *S. typhimurium* -in this case only in TAL_m-. Differences in pH between the two types of cheese (**Table 9**, this chapter) and the presence of starter culture could be the main reasons for this behaviour. In fact, several studies have demonstrated that bacteria could become much more pressure-sensitive at low pH and an efficient inactivation of even the most pressure-resistant vegetative bacteria could be produced at relatively mild pressure in acidic foods (García-Graells et al., 1998; Alpas et al., 2000; Jordan et al., 2001).

In model cheese produced with starter culture, 300 MPa was the only treatment that allowed us to detect differences between baroresistant behaviour in *Y. enterocolitica* strains. Serotype O:3 was more pressure resistant than serotype O:1 and O:8. The different sensitivity to low pH generated during cheese manufacture and changes in the log phase of growth of *Yersinia* cells could explain these results. Whereas, no differences in the pressure resistance behaviour between *E. co*li and *S. enterica* strains were detected in both types of cheese.

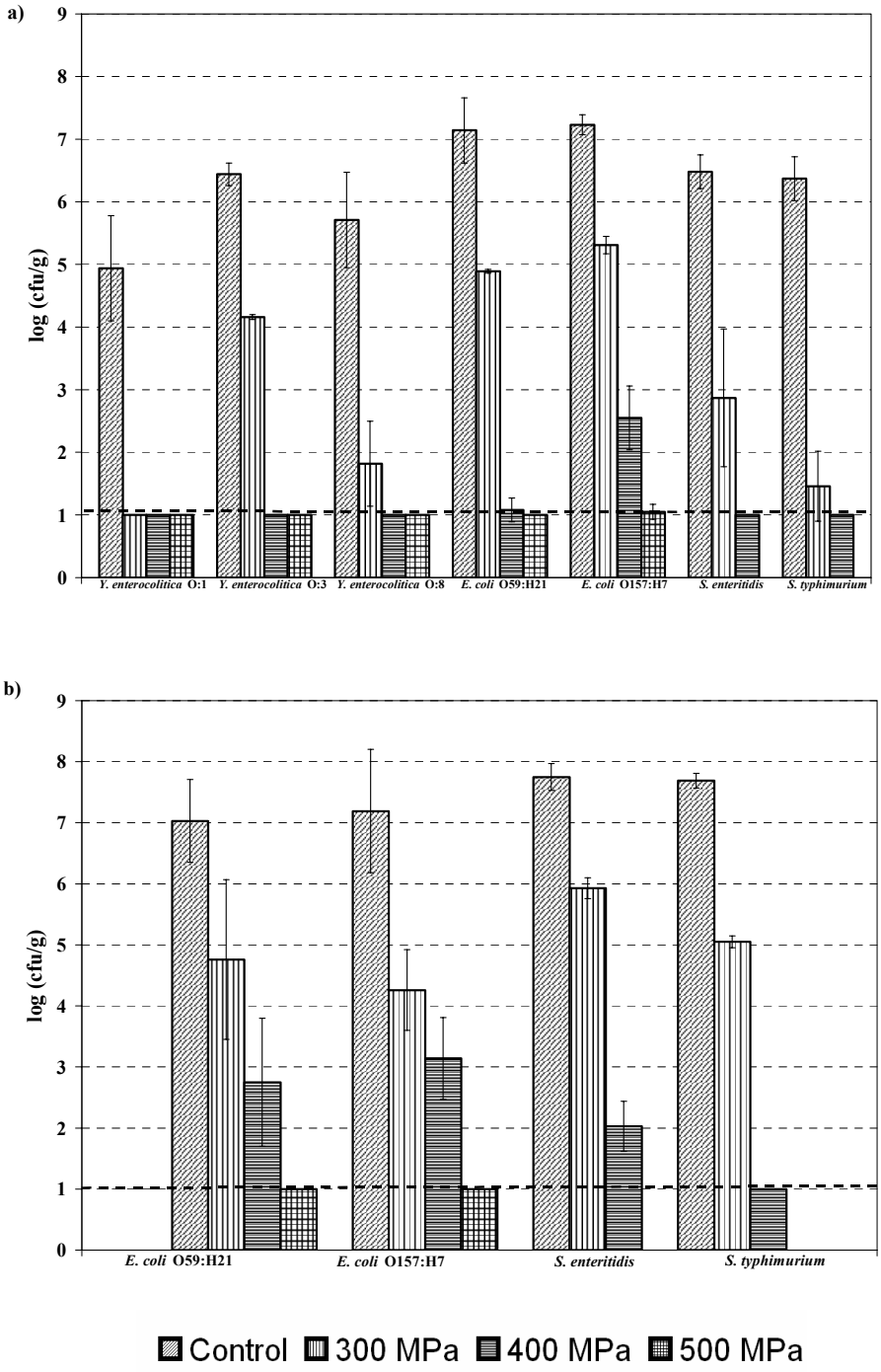


Figure 7. *Cell counts in TAL_m at day 0 of pathogenic bacteria inoculated in model cheese produced (a) with and (b) without starter culture and treated at 300, 400 or 500 MPa for 10 min at 20ºC.*

Table 9. *Values of pH in blank cheese samples (non inoculated and untreated) produced with and without starter and stored at 8 or 12ºC for15 or 60 days.*

Experiments	Time (Days)	With starter		Without starter	
		Mean (n=9)	SD[a]	Mean (n=9)	SD
Yersinia	0	5.40	0.01	-	-
	30	4.79	0.01	-	-
	45	4.68	0.01	-	-
	60	4.65	0.01	-	-
E. coli	cpd	5.08	0.06	6.64	0.02
	0	5.08	0.03	6.69	0.03
	1	5.02	0.03	6.57	0.03
	7	4.82	0.01	6.48	0.02
	15	4.78	0.03	6.46	0.01
Salmonella	cpd	4.89	0.05	6.60	0.06
	0	4.89	0.02	6.56	0.11
	1	4.79	0.04	6.53	0.05
	7	4.75	0.10	6.49	0.02
	15	4.79	0.04	6.46	0.01

cpd: cheese production day
[a]Standard Deviation

5.2.3 Behaviour of pathogenic bacteria after high hydrostatic pressure treatment in model cheese produced with starter culture

Cheese samples inoculated with *Y. enterocolitica* or *E. coli* strains and treated at 400 and 500 MPa did not show repair ability after 15 days of storage at 8ºC and kept their counts below the detection level (10 cfu/g) in both culture media (selective medium and TAL_m). Moreover, *Salmonella* strains inoculated in model cheese treated at 400 MPa were not able to repair either after 15 days of storage at 12ºC (**Table 10**, this chapter). But after applying the enrichment process (24 h at 37ºC), cells of *E. coli* O59:H21 and *E. coli* O157:H7 were detected for 7 days -in samples treated at 500 MPa- and for 15 days -in samples treated at 400 MPa- stored at 8ºC. Whereas, cells of *S. enteritidis* and *S. typhimurium* were only detected at day 1 of storage at 12ºC (**Table 11**, this chapter).

At 300 MPa, all bacteria showed a decreasing trend in their cell counts during the storage time and were not able to repair and grow after the treatment. In the case of *Y. enterocolitica* serotype O:8 and O:3, cells decreased their counts after 0 and 7 days of storage at 8ºC, respectively. For *E. coli* and *S. enterica* strains, the decreasing trend in their cell counts happened after 1 and 7 days of keeping the samples at 8 and 12ºC, respectively (**Table 10**). Moreover, it is important to remark that none of the cell counts of control samples (inoculated but non-treated cheese samples) had the ability to increase after 15 days of storage and their cell counts decreased with the time depending on their sensitivity to low pH.

To assess this decreasing trend, a long term study was conducted (60 days). The most baroresistant *Y. enterocolitica* strain in cheese at day 0 (serotype O:3) was selected for this purpose. Samples treated at 400 MPa did not show repair ability during the 60 days of ripening at 8ºC. Cheese samples treated at 300 MPa decreased their cell counts below detection level at day 15 (**Table 10**, this chapter). However, one of three replicates showed growth (after an enrichment treatment) at day 45 (**Table 11**, this chapter). Controls decreased below detection level at day 45, but one of three replicates grew at day 60 after the enrichment process.

It is well established that high hydrostatic pressure treatments produce sublethally injured cells (Patterson et al., 1995; Simpson and Gilmour, 1997; Kalchayanand et al.,

1998b; McClements et al., 2001; Bozoglu et al., 2004), which in a stressful environment, are not able to repair. The analysis of the behaviour of cell counts after the treatment suggests that the cheese matrix did not allow recovery and growth of injured cells. The main contributing factor to this effect seems to be low pH (**Table 9**, this chapter) mainly produced by the conversion of lactose in lactic acid and also the presence of the starter culture. Several authors have reported that when pH is low, most microbes become more susceptible to high pressure inactivation (Wouters et al., 1998; Alpas et al., 2000; Pagán et al., 2001) and recovery of sublethally injured cells is reduced.

5.2.4 Behaviour of pathogenic bacteria after high hydrostatic pressure treatment in model cheese produced without starter culture

E. coli and *S. enterica* strains inoculated in model cheese without starter culture and treated at 400 and 500 MPa or 300 and 400 MPa, respectively, had the ability to repair or/and grow during the storage period reaching similar levels of cell population. Nevertheless, depending on the high hydrostatic pressure treatment applied, the time necessary to detect this behaviour was different (**Table 12**, this chapter). Control samples inoculated with *E. coli* or *S. enterica* strain*s* did not show significant differences in their cell counts during storage time in any culture media except for *S. typhimurium* in TAL_m.

Sublethally injured cells are usually capable of repairing their injury in a non-selective environment. Generally, nutritionally rich media allow a relatively rapid repair for a high proportion of injured cells (Wuytack et al., 2003). Several authors have reported this behaviour after applying high hydrostatic pressure treatments in different matrices (Metrick et al., 1989; Carlez et al., 1994; García-Risco et al., 1998; Alpas et al., 2000; Black et al., 2005). Bozoglu et al. (2004) showed that after pressure treatment at 550 MPa, initially colony formation of *L. monocytogenes* was not observed in selective or non-selective agar, suggesting that all cells were dead. However, at 4ºC after 6 days and at 22 and 30ºC after 1 day, colony formation occurred in both selective and non-selective agar.

Table 10. *Cell counts at 0 and 15 or 60 days of pathogenic bacteria inoculated in cheese produced with starter culture and pressurized at 300, 400 or 500 MPa at 20ºC for 10 min.*

Strain	Pressure (MPa)	Counts in TAL_m after HHP treatment log (cfu/g)		Counts in TAL_m after 15 or 60 days of storage log (cfu/g)	
		Mean	SD[a]	Mean	SD
Y.enterocolitica O:1	300	n.d	-	n.d	-
	400	n.d	-	n.d	-
	500	n.d	-	n.d	-
Y.enterocolitica O:3	300	4.14[b]	±0.03	2.23	±1.10
		4.32[c]	±0.25	n.d	-
	400	n.d[b]	-	n.d	-
		1.74[c]	±0.19	n.d	-
	500	n.d	-	n.d	-
Y.enterocolitica O:8	300	1.82	±0.68	1.38	±1.38
	400	n.d	-	n.d	-
	500	n.d	-	n.d	-
E. coli O59:H21	300	4.89	±0.03	2.55	±0.27
	400	1.08	±0.19	n.d	-
	500	n.d	-	n.d	-
E. coli O157:H7	300	5.31	±0.14	2.73	±0.72
	400	2.55	±0.51	n.d	-
	500	1.05	±0.12	n.d	-
S. enteritidis	300	2.87	±1.10	1.53	±0.59
	400	n.d	-	n.d	-
S. typhimurium	300	1.46	±0.56	n.d	-
	400	n.d	-	n.d	-

n.d.: not detected. Detection level: 1 log (cfu/g)
[a]Standard Deviation
[b]Results of short term experiments (15 days)
[c]Results of long term experiments (60 days)

Table 11. *Results of the enrichment process (24 h at 37ºC or 24 h at 32ºC) applied to selected cheese samples produced with starter culture (those which showed cell counts below the detection level [10 cfu/g] during the storage time).*

Strain	Pressure (MPa)	Time (Days)						
		0	1	7	15	30	45	60
Y. enterocolitica (serotype O:3)	300	+[a]			+	-[b]	-	-
		+			+	+	+	+
		+			+	-	-	-
		-			-	-	-	-
	400	-			-	-	-	-
		-			-	-	-	-
E. coli O59:H21	400	+	+	-	-			
		+	+	+	+			
		+	+	-	-			
		+	+	-	-			
	500	+	+	+	-			
		+	+	-	-			
E. coli O157:H7	400	+	+	-	-			
		+	+	+	+			
		+	+	-	-			
		+	+	-	-			
	500	+	+	+	-			
		+	+	-	-			
S. enteritidis	400	+	-	-	-			
		+	+	-	-			
		+	-	-	-			
S. typhimurium	300	+	+	+	+			
		+	+	+	+			
		+	+	+	+			
		+	-	-	-			
	400	+	+	-	-			
		+	+	-	-			

[a]Positive growth
[b]Negative growth

Table 12. *Cell counts at 0 and 15 days of pathogenic bacteria inoculated in cheese produced without starter culture and pressurized at 300, 400 or 500 MPa at 20ºC for 10 min.*

Strain	Pressure (MPa)	Counts in TAL_m after HHP treatment log (cfu/g)		Starting day of increase of counts	Counts in TAL_m after 15 days of storage log (cfu/g)	
		Mean (n=6)	SD[a]		Mean (n=6)	SD
E. coli O59:H21	300	4.76	±1.31	-[b]	5.58	±1.77
	400	2.75	±1.05	-	4.04	±2.66
	500	n.d	-	1	3.77	±3.20
E. coli O157:H7	300	4.26	±0.66	-	5.04	±1.52
	400	3.14	±0.67	-	4.44	±1.99
	500	n.d	-	1	5.76	±2.41
S. enteritidis	300	5.93	±0.17	1	7.83	±0.12
	400	2.03	±0.41	1	7.48	±0.10
S. typhimurium	300	5.05	±0.10	1	7.98	±0.39
	400	n.d	-	1	7.53	±0.10

n.d: not detected. Detection level: 1 log (cfu/g)
[a]Standard Deviation
[b]No significant differences ($P > 0.05$) in cell counts were found during storage

5.2.5 Behaviour of starter culture in model cheese inoculated with **Y. enterocolitica** *serotype O:3 (long term experiments) or with* **S. enterica** *strains (short term experiments)*

At 300 MPa, the effect of high hydrostatic pressure treatment on starter population in cheese inoculated with *S. enteritidis* or *S. typhimurium* was minimum, whereas it was close to 3 log (cfu/g) at 400 MPa (*Table 5*, **paper 4**). Moreover, no significant differences in pressure resistance behaviour of starter culture were detected between cheese samples inoculated with *Salmonella* strains. Some authors have studied the effect of high pressure treatments on *Lactococcu*s obtaining similar decrease values. Saldo (2002) found that a treatment of 400 MPa for 5 min reduced in 3 log (cfu/g) the presence of lactic bacteria in cheese. It is clear from these results that when we apply high hydrostatic pressure treatments in cheese to reduce the population of pathogen microorganisms we are also inactivating part of the starter population and maybe affecting its role during the ripening process. Nevertheless, Molina-Höppner (2002) reported that during the treatment at 300 MPa, *Lactococcus lactis* spp. *cremoris* (inoculated in milk buffer) retained about 25% of metabolic activity, even after 3 log (cfu/ml) reduction in cell counts. Moreover, this author found that the addition of high concentration of salts and solutes showed a protective effect on *Lactococcus* population during high hydrostatic pressure treatment.

Starter population treated by high hydrostatic pressure and population of control samples showed a decrease trend in their cell counts during the storage time at 8 or 12ºC. In cheese treated at 300 MPa, cell counts began to decrease during the same day of the treatment in the case of *S. enteritidis*, after 1 day of storage at 12ºC in the case of *S. typhimurium* (**Figure 3**, *paper 4*) and after 15 days in the case of *Y. enterocolitica* serotype O:3 (**Figure 8**). At 400 MPa, this trend was observed after 1 day in cheese samples inoculated with *S. enterica* strains and after 15 days for cheese samples inoculated with *Y. enterocolitica* serotype O:3. For control samples, this behaviour was observed after 7 and 30 days, in the case of *S. enterica* strains and *Y. enterocolitica*, respectively. Wick et al. (2004) found that the population of *L. lactis* began to decrease after 84 days of keeping untreated and treated (200 MPa) Cheddar cheeses at 10ºC. Nevertheless, Saldo (2002) reported that *Lactococcus* inoculated in cheese increased their cell counts after being treated at 400 MPa for 5 min at 14ºC reaching the same

level of cell counts that showed control cheese samples (≈7 log [cfu/g]) after three weeks due to the fact that control cell counts decreased during the storage period (60 days).

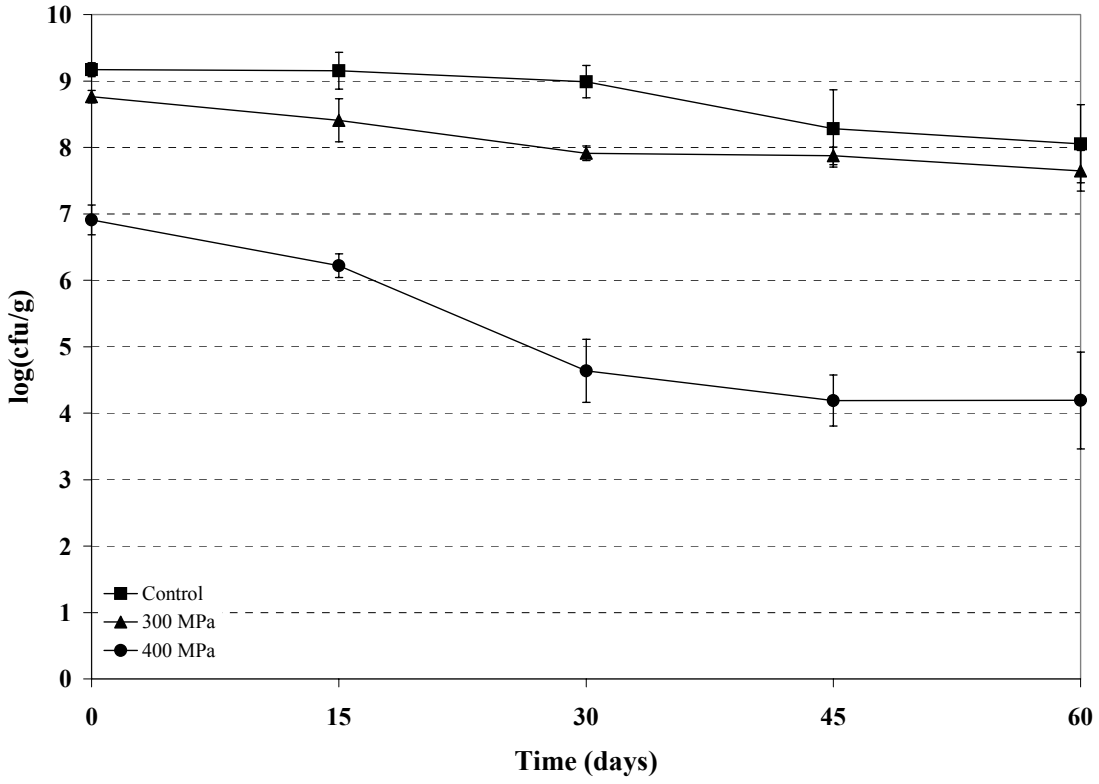


Figure 8. *Behaviour of starter culture in cheese samples inoculated with* Y. enterocolitica (*serotype O:3*) *and treated at 300 and 400 MPa for10 min at 20ºC and ripened for 60 days at 8ºC.*

6. Conclusions

This study reasserts the importance of assessing the efficacy of high hydrostatic pressure treatments directly on food and analysing the ability of injured cells to repair and grow after the treatment.

The kinetic study of microbial inactivation shows that there is a tailing effect that makes difficult to calculate a unique D value.

The behaviour of *Y. enterocolitica, E. coli* and *S. enterica* strains in model cheese produced with starter culture suggests that 400 MPa is an adequate pressure to guarantee its microbiological safety.

This research confirms that high hydrostatic pressure treatments are useful to accelerate the reduction of initial levels of *Y. enterocolitica*, *E coli* and *Salmonella* strains in this type of cheese, but it is necessary to combine this technology with the presence of the starter culture to inhibit the recovery and growth and to increase the death rate of the injured population.

In skimmed milk and in model cheese made without starter culture, 400 MPa and or 500 MPa treatments produced an adequate lethality at the same day of the treatment, but the combination of the reported phenomena (repair ability and growth) indicates that these treatments are not suitable to prevent *Y. enterocolitica*, *E. coli* and *S. enterica* strains from growing during storage at 8 or 12ºC.

Classic culture techniques used in this work to count injured and non-injured cells seem to be inefficient to detect all the population of viable cells. Other techniques, like flow cytometry, should be tested to detect more efficiently this population.

7. References

Ackers, M.L., S. Schoenfeld, J. Markman, G. Smith, M.A. Nicholson, W. DeWitt, D.N. Cameron, P.M. Griffin, and L. Slutsker. 2000. An outbreak of *Yersinia enterocolitica* O:8 infections associated with pasteurized milk. *J. Infect. Dis*., 181:1834-1837.

Aertsen, A., K. Vanoirbeek, P. De Spiegeleer, J. Sermon, K. Hauben, A. Farewell, T. Nyström and C.W. Michiels. 2004. Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Appl. Environ. Microbiol*., 70:2660-2666.

Ahmed, R., G. Soule, W.H. Demczuk, C. Clark, R. Khakhrria, S. Ratnam, N.G.L. Marshall, D.L. Woodward, W.M. Johnson and F. Rodgers. 2000. Epidemiologic typing of *Salmonella enterica* serotype *enteritidis* in a Canada-wide outbreak of gastroenteritis due to contaminated cheese. *J. Clin. Microbiol*., 38:2403-2406.

Alpas, H., N. Kalchayanand, F. Bozoglu, A. Sikes, C.P. Dunne and B. Ray. 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol.*, 65:4248-51.

Alpas, H., N. Kalchayanand, F. Bozoglu and B. Ray. 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodboorne pathogens. *Int. J. Food Microbiol.,* 60:33-42.

Altekruse, S.F., B.B. Timbo, J.C. Mowbray, N.H. Bean and M.E. Potter. 1998. Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: Sanitary Manufacturing Practices Protect Consumers. *J. Food Prot.*, 61:1405-1407.

Ananta, E., V. Heinz and D. Knorr. 2004. Assessment of high pressure induced damage on *Lactobacillus rhamnosus* GG by flow cytometry. *Food Microbiol*., 21:567-577.

Andersen, J.K., R. Sørensen and M. Glensbjerg. 1991. Aspects of the epidemiology of *Yersinia enterocolitica*: a review. *Int. J. Food Microbiol.*, 13:231-217.

Anonymous. 2001. *Yersinia enterocolitica*. Casos notificados al Sistema de Información Microbiológica. Años 1989-2001. *B.E.S.*, 9:305-312.

Anonymous. 2003. Análsis de las cepas de *Salmonella* spp. aisladas de muestras clínicas de origen humano en España. Año 2001 (II). *B.E.S.*, 13:145-156.

Arocha, M.M., M. McVey, S.D. Loder, J.H. Rupnow and L. Bullerman. 1992. Behaviour of enterohaemorrhagic *E. coli* O157:H7 during manufacture of cottage cheese. *J. Food Prot.*, 55: 379-381.

Arqués, J.L., E. Rodríguez, P. Gaya, M. Medina, B. Guamis and M. Núñez. 2005. Inactivation of *Staphylococcus aureus* in raw milk cheese by combinations of high-pressure treatments and bacteriocin-producing lactic acid bacteria. *J. Appl. Microbiol*., 98:254-260.

Arroyo, G., P.D. Sanz and G. Préstamo. 1999. Response to high-pressure, low-temperature treatment in vegetables: determination of survival rates of microbial populations using flow cytometry and detection of peroxidase activity using confocal microscopy. *J. Appl. Microbiol.*, 86:544-556.

Balny, C. and P. Masson. 1993. Effects of high pressure on proteins. *Food Rev. Intern.*, 9:611-628.

Benito, A., G. Ventoura, M. Casadei, T. Robinson and B. Mackey. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Appl. Environ. Microbiol*., 65:1564-1569.

Black, R.E., R.J. Jackson, T. Tsai, M. Medvesky, M. Shayegani, J.C. Feeley, K.I. MacLeod and A.M. Wakelee. 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *N. Engl. J. Med.,* 298:76-79.

Black, E.P., A.L. Kelly and G.F. Fitzgerald. 2005. The combined effect of high pressure and nisin on inactivation of microorganisms in milk. *Innov. Food Sci. Emerg. Technol.*, 6:286–292.

Borczyk, A.A., M.A. Kamali, H. Lior and L.M.C. Duncan. 1987. Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* 1:98.

Bozoglu, F., H. Alpas and G. Kalentunc. 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. 2004. *FEMS Immunol. Med. Microbiol*., 40:243-247.

Buffa, M., B. Guamis, M. Pavia and A.J. Trujillo. 2001. Lipolysis in cheese made from raw, pasteurized or high-pressure-treated goats' milk. *Int. Dairy J*., 11:175-179.

Capellas, M., M. Mor-Mur, E. Sendra, R. Pla and B. Guamis. 1996. Populations of aerobic mesophils and inoculated *E. coli* during storage of fresh goat's milk cheese treated with high pressure. *J. Food Prot.*, 59:582-587.

Carlez, A., J.P. Rosec, N. Richard and J.C. Cheftel. 1994. Bacterial growth during chilled storage of pressure-treated minced meat. *Lebensm. Wiss. Technol.*, 27:48-54.

Casadei, M. A., P. Mañas, G. Niven, E. Needs and B.M. Mackey. 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. *Appl. Environ. Microbiol*., 68:5965-5972.

Casal, V. and R. Gómez. 1999. Effect of High Pressure on the viability and enzymatic activity of mesophilic lactic acid bacteria isolated from caprine cheese. *J. Dairy Sci.*, 82:1092-1098.

CDC. Center for Disease Control. 1985. Milk-borne salmonellosis-Illinois. Morbi. Mortal. *Weekly Rep*. 34:200.

Cheftel, J.C. 1995. Review: high pressure, microbial inactivation and food preservation. *Food Sci. Technol. Int.*, 1:75-90.

Chen, H. and D.G. Hoover. 2003. Pressure inactivation kinetics of *Yersinia enterocolitica* ATCC 35669. *Int. J. Food Microbiol.*, 87:161-171.

Chen, H. and D.G. Hoover. 2004. Use of Weibull model to describe and predict pressure inactivation of *Listeria monocytogenes* Scott A in whole milk. *Innov. Food Sci. Emerg. Technol.*, 5:269-276.

Cléry-Barraud, C., A. Gaubert, P. Masson and D. Vidal. 2004. Combined effects of high pressure and temperature for inactivation of *Bacillus anthracis* spores. *Appl. Environ. Microbiol*., 70:635-637.

Dallet, S. and M.D. Legoy. 1996. Hydrostatic pressure induces conformational and catalytic changes on two alcohol dehydrogenases but no oligomeric dissociation. *Biochim. Biophys. Acta,* 1294:15-24.

De Buyser, M.L., B. Dufour, M. Maire and V. Lafarge. 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int. J. Food Microbiol.*, 67:1-17.

de Heij, W.B.C., L.J.M.M. Van Schepdael, R. Moezelaar, H. Hoogland, A.M. Master and R.W. van den Berg. 2003. High-pressure sterilization: maximizing the benefits of adiabatic heating. *Food Technol*., 57:37-41.

Dogan, C. and O. Erkmen. 2004. High pressure inactivation kinetics of *Listeria monocytogenes* inactivation in broth, milk, and peach and orange juices. *J. Food Process Eng.*, 62:47-52.

Donnelly, C.W. 1990. Concerns of microbial pathogens in association with dairy foods. *J. Dairy Sci*., 73:1656-1661.

Doyle, M.P. 1990. Pathogenic *Escherichia coli*, *Yersinia enterocolitica*, and *Vibrio parahaemolyticus*. *The Lancet*, 336:1111-1115.

Doyle, M.P. 1991. *Escherichia coli* O157:H7 and its significance in foods. *Int. J. Food Microbiol.*, 12:289-301.

Drake, M.A., S.L Harrison, M. Asplund, G. Barbosa-Canovas, and B.G. Swanson. 1997. High pressure treatment of milk and effects on microbiological and sensory quality of Cheddar cheese. *J. Food Sci.*, 62:843-845, 860.

El-Gazzar, F.E. and E.H. Marth. 1992. *Salmonellae*, Salmonellosis and dairy foods: a review. *J. Dairy Sci.*, 75:2327-2343.

Ellenberg, L. and D.G. Hoover. 1999. Injury and survival of *Aeromonas hydrophila* 7965 and *Yersinia enterocolitica* 9610 from high hydrostatic pressure. *J. Food Sci.*, 19:263-276.

Erkmen, O. 1996. Survival of virulent *Yersinia enterocolitica* during the manufacture and storage of Turkish Feta cheese. *Int. J. Food Microbiol.,* 33:285-292.

Eze, M.O. 1990. Consequences of the lipid bilayer to membrane associated reactions. *J. Chem. Educ*., 67:17-20.

FDA/CFSAN: Food and Drug Administration Center for Food Safety and Applied Nutrition. June 2, 2000. Overarching principles: kinetics and pathogens of concern for all technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-over.html.

Fernandes, P.M.B. 2005. How does yeast respond to pressure*? Braz. J. Med. Biol. Res.*, 38:1239-1245.

Fontaine, R.E., M.L. Cohen, W. T. Martin and T.M. Vernon. 1980. Epidemic salmonellosis from Cheddar cheeses: surveillance and prevention. *Am. J. Epidemiol.,* 111:247.

Gao, Y.L., X.R. Ju and H.H. Jiang. In press. Statistical analysis of inactivation of *Listeria monocytogenes* subjected to high hydrostatic pressure and heat in milk buffer. *Appl. Microbiol. Biotechnol*.

García-Graells, C., K.J.A. Hauben and C.W. Michiels. 1998. High pressure inactivation and sublethal injury of pressure-resistant *Escherichia coli* mutants in fruit juices. *Appl. Environ. Microbiol.*, 64:1566-1568.

García-Graells, C., C. Valckx and C.W. Michiels. 2000. Inactivation of *Escherichia coli* and *Listeria innocua* in milk by combined treatment with high hydrostatic pressure and the lactoperoxidase system. *Appl. Environ. Microbiol*., 66:4173-4179.

García-Graells, C., I. Van Opstal, S.C.M. Vanmuysen and C.W. Michiels. 2003. The lactoperoxidase system increases efficacy of high-pressure inactivation of foodborne bacteria. *Int. J. Food Microbiol.,* 81:211-221.

García-Risco, M.R., E. Cortés, A.V. Carrascosa and R. López-Fandiño. 1998. Microbiological and chemical changes in high-pressure-treated milk during refrigerated storage. *J. Food Prot*., 61:735-737.

Gervilla, R., M. Capellas, V. Ferragut and B. Guamis. 1997a. Effect of high hydrostatic pressure on *Listeria innocua* 910 CECT inoculated into ewe's milk. *J. Food Prot*., 60:33-37.

Gervilla, R., X. Felipe, V. Ferragut and B. Guamis. 1997b. Effect of high hydrostatic pressure on *Escherichia coli* and *Pseudomonas fluorescens* strains in ovine milk. *J. Dairy Sci*., 80:2297-303.

Gervilla, R., E. Sendra, V. Ferragut and B. Guamis. 1999. Sensitivity of *Staphylococcus aureus* and *Lactobacillus helveticus* in ovine milk subjected to high hydrostatic pressure. *J. Dairy Sci*., 82:1099-1107.

Gervilla, R. 2001. Estudio de los tratamientos por alta presión hidrostática en la leche de oveja. Departament Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, Barcelona.

Gonzalez Garcia, E.A. 2002. Animal health and foodborne pathogens: enterohaemorrhagic O157:H7 strains and other pathogenic *Escherichia coli* virotypes (EPEC, ETEC, EIEC, EHEC). *Pol. J. Vet. Sci*., 5:103-115.

Grappin, R. and E. Beuvier. 1997. Possible implications of milk pasteurization on the manufacture and sensory quality of ripened cheese-proteolytic enzymes and cheese ripening-proteolytic enzymes and their relation to cheese ripening and flavor: an overview. *Int. Dairy J.*, 7:751-761.

Guan, D., H. Chen and D.G. Hoover. 2005. Inactivation of *Salmonella typhimurium* DT 104 in UHT whole milk by high hydrostatic pressure. *Int. J. Food Microbiol.,* 104:145-153.

Haeghebaert, S., P. Sulem, L. Deroudille, E. Vanneroy-Adenot, O. Bagnis, P. Bouvet, F. Grimont, A. Brisabois, F. Le Querrec, C. Hervy, E. Espie, H. de Valk and V. Vaillant. 2003. Two outbreaks of *Salmonella enteritidis* phage type 8 linked

to the consumption of Cantal cheese made with raw milk, France, 2001. *Euro. Surveill*., 8:151-156.

Hajmeer, M.N., D.Y.C. Fung, J.L. Marsden and G.A. Milliken. 2001. Effects of preparation method, age, and plating technique of thin agar layer media on recovery of *Escherichia coli* O157:H7 injured by sodium chloride. *J. Microbiol. Methods*, 47:249-253.

Hauben, K.J.A., D.H. Bartlett, C.C.F. Soontjens, K. Cornelis, E.Y. Wuytack and C.W. Michiels. 1997. *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. *Appl. Environ. Microbiol*., 63:945-950.

Hayakawa, K., Y. Ueno, S. Kawamura, T. Kato and R. Hayashi. 1998. Microorganism inactivation using high-pressure generation in sealed vessels under sub-zero temperature. *Appl. Microbiol. Biotechnol.*, 50:415-418.

Heldman, D.R. and R.L. Newsome. 2003. Kinetic models for microbial survival during processing. *Food Technol.*, 57:40-46.

Honish, L., G. Predy, N. Hislop, L. Chui, K. Kowalewska-Grochowska, L. Trottier, C. Kreplin and I. Zazulak. 2005. An outbreak of *E. coli* O157:H7 hemorrhagic colitis associated with unpasteurized gouda cheese. *Can. J. Public Health,* 96:182-184.

Hoover, D.G., C. Metrick, A.M. Papineau, D.F. Farkas and D. Knorr. 1989. Biological effects of high hydrostatic pressure on food microorganisms. *Food Techno.,* 43:99-107.

Hynes, E., J.C. Ogier and A. Delacroix-Buchet. 2000. Protocol for the manufacture of miniature washed-curd cheeses under controlled microbiological conditions. *Int. Dairy J.*, 10:733-737(5).

ICMSF: International Comission on Microbiological Specifications for Foods. 1998. *Yersinia enterocolitica*. In: Microorganismos de los alimentos. Acribia, Zaragoza. pp:541-563.

Ishii, A., T. Oshima, T. Sato, K. Nakasone, H. Mori and C. Kato. 2005. Analysis of hydrostatic pressure effects on transcription in *Escherichia coli* by DNA microarray procedure. *Extremophiles*, 9:65-73.

Jayarao, B.M. and D.R. Henning. 2001. Prevalence of foodborne pathogens in bulk tank milk. *J. Dairy Sci.,* 84:2157-2162.

Jordan, S.L., C. Pascual, E. Bracey and B.M. Mackey. 2001. Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. *J. Appl. Microbiol*., 91:463-469.

Kalchayanand, N., A. Sikes, C.P. Dunne and B. Ray. 1998a. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. *J. Food Prot.,* 61:425-431.

Kalchayanand, N., A. Sikes, C.P. Dunne and B. Ray. 1998b. Factors influencing death and injury of foodborne pathogens by hydrostatic pressure-pasteurization. *Food Microbiol*., 15:207-214.

Kalchayanand, N., P. Dunne, A. Sikes and B. Ray. 2004. Viability loss and morphology change of foodborne pathogens following exposure to hydrostatic pressures in the presence and absence of bacteriocins. *Int. J. Food Microbiol.*, 91:91-98.

Kang, D.H. and D.Y.C. Fung. 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. *J. Food Prot*., 62:1346-1349.

Kang, D.H. and D.Y.C. Fung. 2000. Application of thin agar layer method for recovery of injured *Salmonella typhimurium*. *Int. J. Food Microbiol.*, 54:100-104.

Kapperud, G. 1991. *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.*, 12:53-65.

Karaioannoglou, P., P. Koidis, D. Papageorgioti and A. Mantis. 1985. Survival of *Yersinia enterocolitica* during the manufacture and storage of Feta cheese. *Milchwissenschaft*, 40:204-206.

Kato, M. and R. Hayashi. 1999. Effects of high pressure on lipids and biomembranes for understanding high-pressure-induced biological phenomena. *Biosci. Biotechnal. Biochem*., 63:1321-1328.

Klausner, R.B. and C. W. Donnelly. 1991. Environmental sources of *Listeria* and *Yersinia* in Vermont dairy plants. *J. Food Prot*., 54:607-611.

Kushal, R. and S. K. Anand. 1999. Repair and recovery of thermally injured cells of *Yersinia enterocolitica* in milk. *J. Food Prot*., 62:1203-1205.

Lado, B.H. and A.E. Yousef. 2002. Alternative food-preservation technologies: efficacy and mechanisms. *Microbes Infect.,* 4:433-440.

Larkin, L.L., P.C. Vasavada, and E.H. Marth. 1991. Incidence of *Yersinia enterocolitica* in raw milk as related to its quality. *Milchwissenschaft*, 46:500-502.

Leyer, G.J. and E.A. Johnson. 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. *Appl. Environ. Microbiol.*, 58:2075-2080.

Linton, M., J.M.J. McClements and M.F. Patterson. 2001. Inactivation of pathogenic *Escherichia coli* in skimmed milk using high hydrostatic pressure. *Innov. Food Sci. Emerg. Technol*., 2:99-104.

Little, C.L. and S. Knochel. 1994. Growth and survival of *Yersinia enterocolitica*, *Salmonella* and *Bacillus cereus* in Brie stored at 4, 8, and 20ºC. *Int. J. Food Microbiol.,* 24:137-145.

López-Fandiño, R., A.V. Carrascosa and A. Olano. 1996. The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. *J. Dairy Sci.*, 79:929-936.

López-Pedemonte, T.J., A.X. Roig-Sagués, A.J. Trujillo, M. Capellas and B. Guamis. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *J. Dairy Sci.,* 86:3075-3081.

Ludwing, H. and C. Schreck. 1997. The inactivation of vegetative bacteria by pressure. In: A high pressure research in the biosciences and biotechnology. Leuven University Press, Belgium. pp:221-224.

Mackey, B.M., K. Forestiere, N.S. Isaacs, R. Stenning and B. Brooker. 1994. The effect of high hydrostatic pressure on *Salmonella thompson* and *Listeria monocytogenes* examined by electron microscopy. *Lett. Appl. Microbiol*., 19:429-430.

Maher, M.M., K.N. Jordan, M.E. Upton and A. Coffey. 2001. Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *J. Appl. Microbiol,*. 90:201-207.

Malone, A.S., T.H. Shellhammern and P.D. Courtney. 2002. Effects of high pressure on the viability, morphology, lysis, and cell wall hydrolase activity of *Lactococcus lactis* subsp. cremoris. *Appl. Environ. Microbiol*., 68:4357-4363.

Masschalck, B., R. Van Houdt and C.W. Michiels. 2001. High pressure increases bactericidal activity and spectrum of lactoferrin, lactoferricin and nisin. *Int. J. Food Microbiol.,* 64:325-332.

Masschalck, B., D. Deckers and C.W. Michiels. 2002. Lytic and nonlytic mechanism of inactivation of Gram-positive bacteria by lysozyme under atmospheric and high hydrostatic pressure. *J. Food Prot.,* 65:1916-1923.

Masschalck, B. and C.W. Michiels. 2003. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit. Rev. Microbiol*., 29:191-214.

Mattick, K.L., F. Jørgensen, J.D. Legan, H.M. Lappin-Scott and T.J. Humphrey. 2001. Improving recovery of *Salmonella enterica* serovar *typhimurium* DT104 cells injured by heating at different water activity values. *J. Food Prot.,* 64:1472-1476.

Mañas, P. and B.M. Mackey. 2004. Morphological and physiological changes induced by high hydrostatic pressure in exponential and stationary-phase cells of *Escherichia coli*: relationship with cell death. *Appl. Environ. Microbiol*., 70:1545-1554.

McClements, J.M., M.F. Patterson and M. Linton. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. *J. Food Prot.,* 64:514-522.

Metrick, C., D.G. Hoover and D.F. Farkas. 1989. Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. *J. Food Sci.,* 54:1547-1564.

Modi, R., Y. Hill and M.W. Griffiths. 2001. Effect of phage on survival of *Salmonella enteritidis* during manufacture and storage of Cheddar cheese made from raw and pasteurised milk. *J. Food Prot.,* 64:927-933.

Molina-Höppner, A. 2002. Physiological response of *Lactococcus lactis* to high-pressure. M. S Thesis, Technische Universität München, München.

Morgan, S.M., R.P. Ross, T. Beresford and C. Hill. 2000. Combination of hydrostatic pressure and lacticin 3147 causes increased killing of *Staphylococcus* and *Listeria*. *J. Appl. Microbiol*., 88:414-420.

Morse, D.L., M. Shayegani and R.J. Gallo. 1984. Epidemiologic investigation of a *Yersinia* camp outbreak linked to a food handler. *Am. J. Public Health.,* 74:589-92.

Mozhaev V.V., K. Heremans, J. Frank, P. Masson and C. Balny. 1994. Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends in Biotechnol.*, 12:493-501.

Mussa, D.M. and H.S. Ramaswamy. 1997. Ultra high pressure pasteurisation of milk: kinetics of microbial destruction and changes in physico-chemical characteristics. *Lebensm.-Wiss. u.-Technol*., 30:551-557.

Mussa, D.M., H.S. Ramaswamy and J.P. Smith. 1998. High pressure (HP) destruction kinetics of *Listeria monocytogenes* Scott A in raw milk. *Food Res. Intern.*, 31:343-350.

Nakayama, A., Y. Yano, S. Kobayashi, M. Ishikawa and K. Sakai. 1996. Comparison of pressure resistance of spores of six *Bacillus* strains with their heat resistances. *Appl. Environ. Microbiol.*, 62:3897-3900.

Nataro, J.P. and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev*., 11:142-201.

Oliver, S. P., B.M. Jayarao and R.A. Almeida. 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne Pathog. Dis*., 2:115-129.

Olsen, S.J., M. Ying, M.F. Davis, M. Deasy, B. Holland, L. Iampietro, C.M. Baysinger, F. Sassano, L.D. Polk, B. Gormley, M.J. Hung, K. Pilot, M. Orsini, S. Van Duyne, S. Rankin, C. Genese, E.A. Bresnitz, J. Smucker, M. Moll and J. Sobel. 2004. Multidrug-resistant *Salmonella typhimurium* infection from milk contaminated after pasteurisation. *Emerg. Infect. Dis*., 10:932-935.

O'Reilly, C.E., P.M. O'Connor, A.L. Kelly, T.P. Beresford and P.M. Murphy. 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. *Appl. Environ. Microbiol*., 66:4890-4896.

O'Reilly, C.E., A.L. Kelly, P.M. Murphy and T.P. Beresford. 2001. High pressure treatment: applications in cheese manufacture and ripening. *Food Sci. Technol.,* 12: 51-59.

O'Reilly, C.E., P.M. O'Connor, P.M. Murphya, A.L. Kelly and T.P. Beresford. 2002. Effects of high-pressure treatment on viability and autolysis of starter bacteria and proteolysis in Cheddar cheese. *Int. Dairy J*., 12:915-922.

Pagán, R., S. Jordan, A. Benito and B. Mackey. 2001. Enhanced acid sensitivity of pressure-damaged *Escherichia coli* O:157 cells. *Appl. Environ. Microbiol.,* 67:1983-1985.

Pagán, R. and B. Mackey. 2000. Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential- and stationary-phase cells and variation among strains. *Appl. Environ. Microbiol.,* 66:2829-2834.

Paidhungat, M., B. Setlow, W.B. Daniels, D. Hoover, E. Papafragkou and P. Setlow. 2002. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Appl. Environ. Microbiol.*, 68:3172-3175.

Patterson, M.F., M. Quinn, R. Simpson and A. Gilmour. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *J. Food Prot*., 58:524-529.

Patterson, M.F. and D.J. Kilpatrick. 1998. The combined effect of high hydrostatic pressure and mild heat on inactivation of pathogens in milk and poultry. *J. Food Prot.*, 61:432-436.

Patterson, M.F. 2005. A review. Microbiology of pressure-treated foods. *J. Appl. Microbiol.,* 98:1400-1409.

Ponce, E., R. Pla , E. Sendra, B. Guamis and M. Mor-Mur. 1998. Combined effect of nisin and high hydrostatic pressure on destruction of *Listeria innocua* and *Escherichia coli* in liquid whole egg. *Int. J. Food Microbiol.,* 43:15-19.

Ramsaran, H., J. Chen, B. Brunke, A. Hill and M.W. Griffiths. 1998. Survival of bioluminescent *Listeria monocytogenes* and *Escherichia coli* O157:H7 in soft cheeses. *J. Dairy Sci*., 81:1810-1817.

Ray, B. 1992. Nisin of *Lactococcus lactis* spp *lactis* as a food preservative. In: Food biopreservatives of microbial origin. CMS Press, Boca Raton, FL. pp. 207-264.

Ryan, M.P., M.C. Rea, C. Hill and R.P. Ross. 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl. Environ. Microbiol.,*62:612-619.

Reitsma, C.J. and D.R. Henning. 1996. Survival of enterohaemorrhagic *E. coli* O157:H7 during the manufacture and curing of Cheddar cheese. *J. Food Prot.*, 59:460-464.

Ritz, M., J.L. Tholozan, M. Federighi and M.F. Pilet. 2001. Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. *Appl. Environ. Microbiol*., 67:2240-2247.

Ritz, M., J.L. Tholozan, M. Federighi and M.F. Pilet. 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. *Int. J. Food Microbiol.*, 79:47-53.

Rodríguez, E., J.L. Arqués, M. Nuñez, P.G. and M. Medina. 2005. Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli* O157:H7 in raw-milk cheese. *Appl. Environ. Microbiol*., 71:3399-3404.

Russell, N.J. 2002. Bacterial membranes: the effects of chill storage and food processing. An overview. *Int. J. Food Microbiol.*, 79:27-34.

Ryan, M.P., M.C. Rea, C. Hill and R.P. Ross. 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl. Environ. Microbiol*., 62:612-619.

Saldo, J. 2002. Cambios en las características de un queso de leche de cabra sometido a alta presión hidrostática. Aceleración de la maduración. Departament Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, Barcelona.

San Martín, M.F., G.V. Barbosa-Cánovas and B.G. Swanson. 2002. Food processing by high hydrostatic pressure. *Crit. Rev. Food Sci. Nutr.*, 42:627-645.

Scheyhing, C.H., S. Hörmann, M.A. Ehrmann and R.F. Vogel. 2004. Barotolerance is inducible by preincubation under hydrostatic pressure, cold-, osmotic- and acid-stress conditions in *Lactobacillus sanfranciscensis* DSM 20451T. *Lett. Appl. Microbiol.*, 39:284-289.

Schiemann, D.A. 1987. *Yersinia enterocolitica* in milk and dairy products. *J. Dairy Sci*., 70:383-391.

Shakeel-Ur-Rehman, P., L.H. McSweeney and P.F. Fox. 1998. Protocol for the manufacture of miniature cheese. *Le Lait*, 78:607-620.

Simpson, R.K. and A. Gilmour. 1997. The effect of high hydrostatic pressure on the activity of intracellular enzymes of *Listeria monocytogenes*. *Lett. Appl. Microbiol.*, 25:48-53.

Smelt, J.P.P.M. 1998. Recent advances in the microbiology of high pressure processing. *Trends Food Sci. Tech.,* 9:152-158.

Smelt, J.P.P.M., A.G.I. Rijke and A. Hayhurst. 1994. Possible mechanism of high-pressure inactivation of microorganisms. *High Pressure Re.* 12:1999-2003.

Sojka, B. and H. Ludwig. 1994. Pressure-induced germination and inactivation of *Bacillus subtilis* spores. *Pharmaz. Ind.*, 56:660-663.

Spahr, U. and B. Url. 1994. Behaviour of pathogenic bacteria in cheese-a synopsis of experimental data. *Butlletin of IDF* 298.

Stippl, V.M., A. Delgado and T.M. Becker. 2004. Development of a method for the optical in-situ determination of pH value during high-pressure treatment of fluid food. *Innov. Food Sci. Emerg. Technol.*, 5:285-292.

Stojek, N.M. 1999. Seroepidemiologic study on the occurrence of antibodies against *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *A.A.E.M.*, 6:57-61.

Styles, M.F., D.G. Hoover and D.F. Farkas. 1991. Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. *J. Food Sci.,* 56:1404-1407.

Tacket, C.O., J.P. Narain, R. Sattin, J.P. Lofgren, C.J. Konigsberg, R.C. Rendtorff, A. Rausa, B.R. Davis and M.L. Cohen. 1984. A multistate outbreak of infections caused by *Yersinia enterocolitica* transmitted by pasteurized milk. *J. Am. Med. Assoc.*, 251:483-486.

Tauxe, R.V. 2002. Emerging foodborne pathogens. *Int. J. Food Microbiol.,* 78:31-41.

Tauxe, R.V., J. Vandepitte, G. Wauters, S.M. Martin, V. Goossens, P. De Mol, R. Van Noyen and G. Thiers. 1987. *Yersinia enterocolitica* infections and pork: the missing link. *The Lancet*, 1:1129-1132.

Tay, A., T.H. Shellhammer, A.E. Yousef and G.W. Chism. 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *J. Food Prot.,* 66:2057-2061.

Ulmer, H.M., M.G. Gänzle and R.F. Vogel. 2000. Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1460. *Appl. Environ. Microbiol*., 66:3966-3973.

Van Opstal, I., S.C. Vanmuysen and C.W. Michiels. 2003. High sucrose concentration protects *E. coli* against high pressure inactivation but not against high pressure sensitization to the lactoperoxidase system. *Int. J. Food Microbiol.*, 88:1-9.

Van Opstal, I., C.F. Bagamboula, S.C.M. Vanmuysen, E.Y. Wuytack and C.W. Michiels. 2004. Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *Int. J. Food Microbiol.*, 92:227-234.

Vasavada P.C. 1988. Pathogenic bacteria in milk. A review. *J. Dairy Sci.,* 71:2809-16.

Villar, R.G., M.D. Macek, S. Simons, P.S. Hayes, M.J. Goldoft, J.H. Lewis, L.L. Rowan, D. Hursh, M. Patnode and P.S. Mead. 1999. Investigation of multidrug-resistant *Salmonella* serotype *typhimurium* DT104 infections linked to raw-milk cheese in Washington State. *J.A.M.A.*, 281:1811-6.

Wick, C., U. Nienaber, O. Anggraeni, T.H. Shellhammer and P.D. Courtney. 2004. Texture, proteolysis and viable lactic acid bacteria in commercial Cheddar cheeses treated with high pressure. *J. Dairy Res.,* 71:107-115.

Wood, D.S, D.L. Collins-Thompson, D.M. Irvine and A.N. Myhr. 1984. Source and persistence of *Salmonella muenster* in naturally contaminated Cheddar cheese. *J. Food Prot.,* 47:20-22.

Wouters, P.C., E. Glaasker and J.P.P.M. Smelt. 1998. Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. *Appl. Environ. Microbiol*., 64:509-514.

Wu, V.C.H., D.Y.C. Fung, D.H. Kang and L.K. Thompson. 2001. Evaluation of thin agar layer method for recovery of acid-injured foodborne pathogens. *J. Food Prot*., 64:1067-1071.

Wuytack, E.Y., S. Boven and C.W. Michiels. 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. *Appl. Environ. Microbiol*., 64:3220-3224.

Wuytack, E.Y., A.M.J. Diels and C.W. Michiels. 2002. Bacterial inactivation by high-pressure homogenisation and high hydrostatic pressure. *Int. J. Food Microbiol.*, 77:205-212.

Wuytack, E.Y., L.D.T. Phuong, A. Aertsen, K.M.F. Reyns, D. Marquenie, B. De Ketelaere, B. Massachalck, I. Van Opstal, A.M.J. Diels and C.W. Michiels. 2003. Comparision of sublethal injury induced in *Salmonella enterica* serovar Typhimurium by heat and by different nonthermal treatments. *J. Food Prot.,* 66:31-37.

Yuste, J., M. Mor-Mur, M. Capellas, B. Guamis and R. Pla. 1998. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. *Food Microbiol*., 15:407-414.

Yuste, J. and D.Y. Fung. 2002. Inactivation of *Listeria monocytogenes* Scott A 49594 in apple juice supplemented with cinnamon. *J. Food Prot.*, 65:1663-1666.

Yuste, J., M. Capellas, R. Pla, S. Llorens, D.Y.C. Fung and M. Mor-Mur. 2003. Use of conventional media and thin agar layer Method for recovery of foodborne pathogens from pressure-treated poultry products. *J. Food Sci.,* 68:2321-2324.

Yuste, J., M. Capellas, D.Y.C. Fung and M. Mor-Mur. 2004. Inactivation and sublethal injury of foodborne pathogens by high pressure processing: Evaluation with conventional media and thin agar layer method. *Food Res. Int*., 37:861-866.