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Evaluation of the efficacy of Ultra-High Pressure Homogenization technology to improve the safety and quality of liquid foods and especially of orange juice

Doctoral Thesis

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HACEN CONSTAR: que Rita María Velázquez Estrada ha realizado, bajo se dirección, el trabajo titulado “Evaluation of the efficacy of Ultra-High Pressure Homogenization technology to improve the safety and quality of liquid foods and especially of orange juice” que presenta para optar para optar al grado de Doctor en Ciencia y Tecnología de los Alimentos.

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Summary

The main objective of this thesis has been to evaluate the capacity of the ultra-high pressure homogenization (UHPH) technology as alternative to the conventional pasteurization in ensuring the safety and quality of liquid foods.

To test the effectiveness of the technology to guaranty the microbial safety of foods we inoculated strains of *Listeria monocytogenes* and/or *S. enterica* serovar Senftenberg 775W into different liquid foods such as whole egg, milk and fruit juices (orange and grape). They were submitted to a single cycle of UHPH treatment at 150, 200 and 250 MPa for liquid whole egg and at 200, 300, and 400 MPa for milk and fruit juices. The effectiveness of the UHPH treatments over low (3 log CFU/ml) and high (7 log CFU/ml) bacteria loads was evaluated in both liquid whole egg and milk. Moreover, the influence of milk fat content (0.3, 3.6, 10, and 15% of fat) in the *Listeria monocytogenes* inactivation by UHPH treatments was also studied. Samples counts were followed during of storage at 4ºC over 20 days for liquid whole egg and 15 days for milk and fruit juices. Viable and injured bacterial counts were evaluated by means of a differential plating method using tryptone soy agar enriched with yeast extract and the same medium supplemented with salt. Additionally, with the purpose to explain some of the results of the survival of pathogens inoculated in fruit juices, the effectiveness of the response acid tolerance (ATR) on the protection in the exponential or stationary phase of *Salmonella* Seftenberg and *L. monocytogenes* inoculated in orange and grapefruit juice during their conservation at 4 °C and 25ºC was studied. In liquid whole egg, UHPH treatments at 250 MPa effectively reduced *Salmonella enteritidis* serovar Senftenberg 775W to similar levels than reported for thermal pasteurization. Surviving Salmonella counts decreased below the detection limit during the storage of the liquid egg at 4ºC, although Salmonella was immunologically detected during all the storage period. In milk samples which a low fat concentration (0.3 and 3.6 %), pressures of 300 and 400 MPa damaged considerably *L. monocytogenes* cells but they were able to recover and grow up during the subsequent cold storage. Nevertheless, higher lethality values were achieved in milk with the highest fat content (15 and 10%). In fruit juices, UHPH treatments at 400 MPa inactivated completely *Salmonella enteritidis* serovar Senftenberg 775W, being this strain more sensitive than *L. monocytogenes* to the UHPH treatments applied. However, in grape juice *L. monocytogenes* viable counts were

undetectable at the end of storage in both control and pressurized samples, which could be attributed to the presence of natural compounds with antilisterial effect. Acid tolerance response (ATR) was induced in *L. monocytogenes* and *Salmonella enteritidis* serovar Senftenberg 775W to assess if it was able to protect cells from a most severe acid stress. Cells in stationary phase, used on the UHPH experiment with juices, showed a natural resistance to low pH values.

Taking orange juice as food model, we also evaluated the effect of UHPH treatments on enzymatic activity (pectin methylesterase PME) and microbiological (mesophilic aerobic bacteria, psychrotrophic aerobic bacteria, lactic-acid bacteria and yeast) spoiling activity, as well as on physical attributes (cloud stability and particle size distribution), bioactive properties (L-ascorbic, carotenoid and polyphenol content), antioxidant activity and other general quality parameters (color, pH, °Brix, titratable acidity, reducing sugars and non-enzymatic browning index). In this case the UHPH treatments used consisted in combinations of two inlet temperatures (10 or 20ºC), three pressures (100, 200 or 300 MPa) and two holding times (≤ 0.7 or 30 seconds). Results were compared with two thermal pasteurization treatments (1 or 2 min at 90 ºC). UHPH treatments above 200 MPa were as effective as pasteurization to control both PME activity and spoilage bacteria in orange juice. Neither pectin methyl esterase activity nor microbial counts increased significantly after 50 days of storage at 4ºC. UHPH treated juices showed a better particle size distribution and cloudiness values than the pasteurized juices. In particular, the smallest particles were observed in samples treated at 300 MPa. L-ascorbic acid and carotenoid retention of orange juice depended on the high pressure used and more specifically on the maximum temperature achieved during the UHPH treatment. Content of flavonoids in orange juice increased after the UHPH treatments, achieving the maximum concentration in the samples treated at 200 and 300 MPa. Moreover, UHPH treated juices showed higher levels of trolox equivalent antioxidant capacity (TEAC) values than did fresh and heat-treated samples. Additionally, a preliminary sensorial test of preference and acceptability was made with the orange juice treated at 20ºC of inlet temperature and 300 MPa. Moreover with these samples a shelf-life test was carried out by 90 days at 6ºC and 20ºC. In the consumer acceptability study, the UHPH samples in term of color obtained the lowest score. Microbial counts of both UHPH treated and pasteurized samples kept below of the detection limit (1 Log CFU/ml) during the 90 days of storage at 6ºC.

Resumen

El principal objetivo de esta tesis ha sido evaluar la capacidad de la tecnología de ultra alta presión de homogeneización (UHPH) como alternativa a la pasteurización convencional para garantizar la seguridad y calidad de los alimentos líquidos.

Para probar la eficacia de la UHPH y garantizar la seguridad microbiana de los alimentos se inoculó *Listeria monocytogenes* y/o *S. enterica* serovar Senftenberg 775W en diferentes alimentos líquidos como son huevo entero líquido, leche y zumo de frutas (naranja y uva). y se trataron a 150, 200 y 250 MPa en el caso del huevo liquido y a 200, 300 y 400 MPa en el de leche y zumos de frutas, realizándose la evaluación de su supervivencia durante el almacenamiento en refrigeración a 4ºC durante 20 días en las muestras de huevo y 12 días en las muestras de leche y zumo de frutas. Los recuentos de células viables y dañadas se realizaron utilizando agar triptona soya enriquecida con extracto de levadura y el mismo medio suplementado con sal. Para evaluar los factores que pudieran afectar a la inactivación bacteriana en la eficacia del tratamiento UHPH, se estudió la influencia de la concentración bacteriana baja (3 log CFU/ml) y alta (7 log CFU/ml) en huevo y leche, así como la influencia del contenido de grasa en leche (0.3, 3.6, 10, y 15% de grasa). Adicionalmente, con la finalidad de explicar algunos de los resultados de supervivencia de los patógenos inoculados en los zumos de frutas se estudió la eficacia a la respuesta de ácido tolerancia (ATR) en la protección de *Listeria monocytogenes* y *S. enterica* en fase exponencial o estacionaria en zumo de naranja y uva conservados a 4 y 25ºC. En huevo, el tratamiento a 250 MPa disminuyo los recuentos de *Salmonella enteritidis* serovar Senftenberg 775W a niveles similares a los obtenidos en la pasteurización térmica, produciéndose una disminución posterior por debajo de los límites de detección durante el almacenamiento 4ºC, aunque se detecto su presencia. En las muestras de leche (0.3 and 3.6%), se observó que 300 y 400 MPa dañaban considerablemente a *L. monocytogenes*, pero durante el almacenamiento fue capaz de recuperarse y desarrollarse. No obstante, los mayores valores de letalidad se consiguieron en las muestras de leche con un contenido de 15 y 10% de grasa. En las zumos, el tratamiento a 400 MPa inactivó completamente *Salmonella enteritidis* serovar Senftenberg 775W, este cepa se mostró mas sensible que *L. monocytogenes* a los tratamientos UHPH aplicados. Sin embargo, al finalizar el periodo de almacenamiento del zumo de uva control y homogenizado no se detectaron recuentos de *L. monocytogenes*, lo que podría ser atribuido a la presencia de compuestos naturales de la

uva con efecto antilisteria. La respuesta de ácido tolerancia se indujo a *L. monocytogenes* y *Salmonella enteritidis* serovar Senftenberg 775W para evaluar si se presentaba un efecto protector ante estrés por acidez. Se observó que las células en fase estacionaria mostraban una resistencia natural a pH bajos.

Tomando como modelo al zumo de naranja, se evaluó el efecto de la UHPH en la actividad enzimática (pectin metilesterasa) y microbiológica (bacterias aerobias mesófilas, psicotrófas, ácido lácticas y levaduras), en los atributos físicos (turbidez y distribución del tamaño de partícula), en las propiedades bioactivas (contenido de L-acido ascórbico, carotenoides y polifenoles), en la actividad antioxidante y en otros los parámetros generales de calidad (color, pH, °Brix, acidez titulable, azúcares reductores y índice de oscurecimiento no enzimático). Los tratamientos UHPH aplicados consistieron de dos temperaturas de entrada (10 o 20ºC), tres niveles de presión (100, 200 y 300 MPa) y dos tiempos de retención (≤ 0.7 o 30 segundos). Los resultados se compararon con los resultados obtenidos por pasteurización térmica (1 o 2 min a 90 ºC). Los tratamientos de UHPH iguales o superiores a 200 MPa fueron igualmente efectivos que la pasteurización para el control de la actividad enzimática y de las bacterias alterantes del zumo no apreciándose actividad enzimática ni incremento de los recuentos microbianos tras 50 días de almacenamiento a 4ºC. Los zumos tratados por UHPH tuvieron la mejor distribución de tamaño de partícula y los mejores valores de turbidez en comparación con los zumos pasteurizados, obteniendo una mayor reducción de del tamaño de partícula en las muestras tratadas a 300 MPa. La retención de L-acido ascórbico y carotenoides dependió de la presión aplicada y específicamente de la temperatura alcanzada durante el tratamiento. El contenido de flavonoides se incrementó en el zumo con los tratamientos por UHPH, obteniendo el mayor contenido a 200 y 300 MPa. Además, los zumos tratados por UHPH mostraron niveles de capacidad equivalente antioxidante trolox (TEAC) mayores que los de los zumos frescos o pasteurizados. Además, se realizó un estudio sensorial de preferencia y aceptabilidad en zumo homogeneizado en las condiciones consideras de elección (20ºC de temperatura de entrada y 300 MPa) y se evaluó la vida útil a 6ºC y 20ºC por 90 días. En el estudio de aceptabilidad, la muestras tratada por UHPH obtuvieron la menor puntuación en términos de valoración de color con respecto a la muestra pasteurizada. Durante los 90 días de almacenamiento a 6ºC los recuentos de las muestras tratadas por UHPH así como las pasteurizadas se mantuvieron por debajo del límite de detección (1 Log CFU/ml).

List of papers

The thesis is based on the following papers referred to in the text by their respective Roman numerals. The papers are appended at the end of the thesis.

I. Inactivation of *Salmonella enterica* Serovar Senftenberg 775W in Liquid Whole Egg by Ultra high Pressure Homogenization

II. Fat content increases the lethality of ultra-high-pressure homogenization on *Listeria monocytogenes* in milk

III. Inactivation of *Listeria monocytogenes* and *Salmonella enterica* serovar Senftenberg 775W inoculated into fruit juice by means of ultra high pressure homogenisation

IV. Acid Tolerance Response Induced by organic acids on *Listeria monocytogenes* and *Salmonella enterica* serovar Senftenberg can enhance their survival in fruit juices.

V. Impact of ultra high pressure homogenization on pectin methylesterase activity and microbial characteristics of orange juice: A comparative study against conventional heat pasteurization

VI. Influence of ultra high pressure homogenization on physicochemical properties of orange juice in comparison with traditional thermal processing

VII. Influence of ultra high pressure homogenization processing on bioactive compounds and antioxidant activity of orange juice

Content

1. Theoretical background 1
1.1 Food safety issues 1
1.1.1 Fruit juices 1
1.1.2 Egg products 3
1.1.3 Milk 5
1.2 Citrus and orange world production 6
1.2.1 Citrus juices and quality parameters 7
1.3 Food processing 10
1.3.1 Thermal pasteurization 10
1.3.2 Non-thermal food pasteurization 12
1.4 Ultra high pressure homogenization 14
1.4.1 Characteristics of high pressure homogenization equipments 15
1.4.2 UHPH and microorganisms 16
1.4.3 Factors affecting microbial inactivation by UHPH 20
1.4.4 UHPH and physicochemical, nutritional, sensorial properties of food 24
2. Objectives 29
3. Material and methods 31
3.1 Main experimental design 31
3.2 Description of the procedures done to evaluate the effect of UHPH on pathogenic microorganism (paper I, II and III) 32
3.2.1 Microorganisms used 32
3.2.2 Preparation of inoculates 33
3.2.3 UHPH treatments 33
3.2.4 Microbiological analysis 34
3.3 Experiment performed with organic acids to evaluate the acid tolerance response (ATR) of Salmonella Senftenberg 775W and Listeria monocytogenes in fruit juices (paper IV) 35
3.3.1 Bacterial strains 35
3.3.2 Acid adaptation of strains in TSB at different pHs 35
3.3.3 Induction of ATR 35
3.3.4 Acid tolerance response in commercial juices 36
3.4 Description of the procedures done to evaluate the effect of the UHPH on the quality, physical, microbiological and sensory properties orange juice (paper V and VI) 36
3.4.1 Orange juice processing 36
3.4.2. PME Activity Measurement 37
3.4.3 pH and Titratable acidity (TA) 38
3.4.4 Total Soluble Solids (TSS) 38
3.4.5 Determination of total and reducing sugars 38
3.4.6 Non-enzymatic browning index 38
3.4.7 Cloudiness determination and particle size analysis 38
3.4.8 Color measurements 39
3.4.9 Microbiological analysis 39
3.4.10 Sensory evaluations 40
3.5 Description of the procedures done to evaluate the effect of the UHPH on the bioactive compounds and antioxidant activity orange juice (paper VII) 40
3.5.1 Determination of L-Ascorbic acid 40
3.5.2 Determination of carotenoids and vitamin A 40

3.5.3 HPLC Analysis of flavonoid compounds 41
3.5.4 Determination of total polyphenol content 42
3.5.5 Measurement of antioxidant capacity 42
3.6 Statistical Analysis 42
4. Results and discussion 43
4.1 Effect of UHPH on pathogenic microorganisms 43
4.2 Pectin methylesterase activity, microbial and physicochemical stabilization of orange juice processed by UHPH 50
4.3 Bioactive compounds content and antioxidant activity of orange juice processed by UHPH 55
5. Conclusions 61
6. References 63

Table Index

Table1. Food Safety and Inspection Service (FSIS) pasteurization requirements for different egg products 4
Table 2. Effect of UHPH on native microbiota of different food 17
Table 3. Effect of UHPH on specific microorganisms inoculated into different food 18
Table 4. Temperature and pressure changes of orange juice during UHPH processing. 37
Table 5. Effect of UHPH on specific microorganisms inoculated into different food matrices 43
Table 6. Quality characteristics of fresh, pasteurized and UHPH treated orange juice. 54
Table 7. Effect of thermal pasteurization and UHPH treatments on bioactive compounds and antioxidant activity of orange juice. 59

Figure Index

Figure 1. Schematic representation of Stansted high pressure homogenizing valve from Floury, et al. (2004a). 16
Figure 2. Diagram of the procedure followed to evaluate the effect of UHPH application on different liquid food matrices inoculated with pathogenic microorganisms. 31
Figure 3. Diagram of the procedure followed to evaluate the effect of the UHPH on the enzymatic, physical, microbiological, bioactive and sensory properties of orange juice. 32
Figure 4. Evolution of viable counts of *Salmonella* Senftenberg 775W in liquid whole egg stored at 4ºC. 46
Figure 5. Evolution of viable counts of *L. monocytogenes* in milk with different fat content and stored at 4ºC.. 47
Figure 6. Evolution of viable counts of *L. monocytogenes* (A and B) and of *Salmonella* Senftenberg 775W (C and D) in fruit juices stored at 4ºC. 49
Figure 7. Survival (log CFU/ml) of *Listeria monocytogenes* (A and B) and of *Salmonella* Senftenberg 775W (C and D) in fruit juices stored at 4ºC and 25ºC. 50
Figure 8. Residual activity of PME in UHPH and pasteurized orange juice..... 51
Figure 9. Particle size distribution of fresh, pasteurized and UHPH treated orange juice.. 54

1. THEORETICAL BACKGROUND

1.1 Food safety issues

1.1.1 Fruit juices

Although spoiling bacteria, yeasts and molds dominate the microbiota of fruits and vegetables, the occasional presence of foodborne pathogens associated with these foods has been recognized for many years (Francis, et al. 1999). Outbreaks of illness associated with consumption of fruit juices has stimulated new research aimed at achieving a better understanding of the factors affecting the behavior of pathogens in fruits and fruit products and how the safety of these products can be improved (Centers for Disease Control and Prevention. 1996, 1997 and 1999). The process used to prevent biological contamination must result in at least a 5 log reduction in the pathogen of concern to public health, usually *E. coli* O157:H7 or *Salmonella* (Vojdani, et al. 2008).

The emergence of juice-associated outbreaks also caused the USA Food and Drug Administration altered labeling laws, requiring manufacturers of unpasteurized juices to include warnings on product labels describing the risk of exposure to pathogenic microorganisms (Burnett and Beuchat. 2001). Several intrinsic and extrinsic factors are known to influence the survival and growth of foodborne pathogens in juices during their intended shelf-life. A better understanding of these factors would facilitate accurate assessment of the potential of juices to serve as vehicles in outbreaks of foodborne illness.

Sources of contamination and potential for pathogen internalization. There are several routes by which fruits and fruit juices may become contaminated with pathogens. A contributing factor is that these products are raw agricultural commodities, which may become contaminated by animal or human waste and consumed without a processing step that would kill or remove associated pathogens. A pathogen that has become internalized within a fruit or vegetable must be able to survive in the product until it reaches the consumer in order to become a public health hazard. Preliminary studies carried out by the FDA show that *Salmonella enterica* serovar Hartford and *Escherichia coli* O157:H7 can infiltrate oranges that are visually intact, and once the pathogenic organism infiltrates, it can survive for at least five days, and in some cases

grow. During juicing operation such microorganisms can be transferred into the orange juice resulting in a contaminated product (Food and Drug Administration.1999a and b).

Adaptation of pathogen cells. Many foodborne pathogenic bacteria exhibit stress responses, which enhance their survival in adverse environmental conditions. One stress commonly encountered in foods is an acidic environment, where enhanced survival can involve induction of an acid tolerance response (ATR) (Greenacre, et al. 2003). ATR is a phenomenon by which microorganisms show an increased resistance to environmental stress after the exposure to a moderate acid environment (Hsin-Yi and Chou. 2001). The effectiveness of this response appears to be critically dependent of the pH of the adaptive exposure and the duration of the adaptive period (Davis, et al. 1996). ATR is also dependent on the strain and the kind of acid used. The lethal effects of these weak acids are not only concentration dependent, but are also related to the pH of the environment and to the dissociation constant of the chemical. The unionized (protonated) form of a weak acid is more permeable to cell membranes than the ionized form. Thus, the more acid the environment, the greater the proportion of undissociated weak acid is available to penetrate a cell. After the unionized form is inside the cell, the more alkaline intracellular environment (pH, 7.5 to 7.8) causes dissociation of the weak acid and acidification of the cytoplasm (Foster. 1995). Growing phase is another important factor to consider on ATR. Stationary phase cells are naturally more resistant than exponential phase cells (O'Driscoll, et al. 1996, Buchanan and Edelson. 1999). Foster (1995) discovered that induction of the ATR will protect against the lethal effects of organic acids, especially at low pH (pH 4.4). Previous studies with different pathogens have demonstrated that ATR can significantly enhance their survival in acidic foods (Leyer and Johnson. 1992, Gahan, et al. 1996, Gahan and Hill. 1999, Phan-Thanh, et al. 2000). In fact, induction of the acid tolerance response also protects pathogens against the effects of other environmental stresses. Acid-adapted cells demonstrate increased resistance to heating, osmotic stress, lactoperoxidase crystal violet, ethanol and gamma radiation (Leyer and Johnson. 1993, O'Driscoll, et al. 1996, Buchanan and Edelson. 1999).

Outbreaks of illness associated with juice consumption have been suspected or confirmed to have been caused by a variety of different pathogens, including *E. coli* O157:H7, other Shiga-like toxin–producing *E. coli*, several *Salmonella enterica*

serotypes and *Cryptosporidium parvum*. Data from both outbreak investigations and laboratory research suggest that many of these pathogens can survive in juice for prolonged periods. *E. coli* O157:H7, for example, can survive at 8 ºC for 20 days in apple cider (pH 3.6 to 4.0) that did not contain preservatives (Besser, et al. 1993). In another study, *E. coli* O157:H7 showed that could survive in apple cider (pH 3.56 to 3.98) and orange juice (pH 3.82 to 3.86) held at 5 or 25 ºC for up to 42 days (Ryu and Beuchat. 1998). Some strains of *Salmonella* also exhibit unexpected tolerance to acidic conditions in juices. Parish (1997) observed that *Salmonella*, initially at approximately 6 log CFU/ml, survived in orange juice at pH 3.5, 3.8, 4.1, and 4.4 for 24,39, 57, and 70 days, respectively, at 4 ºC. *Salmonella* Poona increased by 5 log CFU/ml in cantaloupe juice (pH 6.3) stored at 20 ºC for 24 h and by 8 log CFU/ml within 48 h (Richards, et al. 2004), and *Salmonella* Baildon grew well in tomato juice (pH 4.8) (Weissinger, et al. 2000).

Regulations of fruit juices. In the absence of known specific pathogen-product associations, the National Advisory Committee on Microbiological Criteria in Foods (NACMCF) recommends the use of *E. coli* O157:H7 or *Listeria monocytogenes* as target organisms. They are two of the most difficult organisms to control (i.e., by juice acidity or heat lethality) thus by controlling them, other pathogenic organisms will likely be also controlled. The Food and Drug Administration (FDA) has proposed a 5 log reduction in the target pathogens, as the NACMCF recommended, as a necessary step in a HACCP plan for juice (Food and Drug Administration. 1998).

1.1.2 Egg products

Egg is a multifunctional food due to the ability of their components to coagulate when heated, to act as emulsifiers in oil and water formulations and to form foams when whipped. Moreover, whole egg is an excellent source of high quality protein, vitamins and trace minerals (Stadelman and Schimieder. 2002). Unfortunately, most human cases of Salmonellosis are mainly linked to consumption of eggs and egg products. Between 2004 and 2007, 3511 foodborne outbreaks were notified in Spain, 1688 of which were caused by *Salmonella*, the 55.7% of these were associated with egg and derivatives (Martínez, et al. 2008). In the USA foodborne *Salmonella* are estimated to cause approximately 1.3 million illnesses, with 500 deaths each year, being 80% of these

infections associated with the consumption of contaminated egg products (Anonymous. 2005).

About one-third of all eggs produced in the USA are marketed as egg products (i.e., whites, yolks, or whole eggs). These eggs are sent to processing plants where they are cracked open. The internal contents of these eggs are accumulated in large vats and subsequently pasteurized. USDA periodically samples pasteurized egg products and has occasionally found evidence of *Salmonella* contamination in these samples. Such results suggest that current pasteurization practice has not been completely effective at eliminating *Salmonella* from all egg products. Current pasteurization regulations specify times and temperatures for treating egg products (Table 1).

Table1. Food Safety and Inspection Service (FSIS) pasteurization requirements for different egg products

Products	Minimum temperature (°C)	Minimum holding time
Albumen (without use of chemicals)	56.7 55.6	3.5 min 6.2 min
Whole egg	60	3.5 min
Whole egg blends (<2% added non-egg ingredients), sugar whole egg (2 to 12% sugar added), and plain yolk	62.2 61.1	3.5 min 6.2 min
Fortified whole egg and blends (24 to 38% egg solids, 2 to 12% added non-egg ingredients)	63.3 62.2	3.5 min 6.2 min
Spray-dried albumen	54.4	7 days
Pan-dried albumen	51.7	5 days

Adapted from Code of Federal Regulations (2005 a and b).

Heat destroys bacterial cells, but it must not cook the egg material to the point at which its usefulness as a foodstuff is affected. Different combinations of pasteurization time and temperature result in different levels of effectiveness in destroying *Salmonella*. If pasteurization is effective, then the probability that *Salmonella* will survive this process is low. If pasteurization is ineffective, then *Salmonella* can survive the process and grow in numbers, and consumers may eventually become exposed. After pasteurization, the processor, wholesaler, retailer, and consumer may store egg products. Surviving *Salmonella* may grow in the egg products during these times. The amount of growth

depends on the combination of storage times and temperatures (NACMCF. 2006). However, due to egg proteins coagulation temperature and holding times are very limited. According to the data about heat resistance of *Salmonella* in liquid whole egg reported in the literature, recommended heat pasteurization treatments at 60 °C for 3.5 min (USA) or at 64 °C for 2.5 min (UK) should provide a 5–9 log cycles reduction of the most frequent *Salmonella* serotypes: *S. enterica* serovars Typhimurium and Enteritidis (NACMCF. 2006). However, other serotypes of *Salmonella*, such as *S. enterica* serovar Senftenberg 775 W have been isolated from foods and traditional pasteurization treatments would not cause more than 1–4 log cycles reduction (Mañas, et al. 2003).

1.1.3 Milk

Milk is a good source of nutrients and edible energy, not only for mammals but for numerous microorganisms, which thus can grow in milk. These microorganisms are primarily bacteria, but some moulds and yeast can also grow in milk. Water activity and ionic strength of milk are never limiting, and pH is so only for a few organisms. But redox potential and O_2 pressure are mostly such that strictly anaerobic bacteria cannot grow. Milk also contains natural inhibitors. Some bacteria do not grow in milk despite the presence of sufficient nutrients and suitable conditions. The presence of several species of microorganisms in raw milk is undesirable, either because the organisms can be pathogenic, or because their growth results in undesirable transformation in the milk (Marth and Steele. 2001, Walstra, et al. 2006). A variety of microbes with human pathogenic potential, including *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Mycobacterium tuberculosis*, can sometimes be found in raw milk. Pathogens generally do not grow very well in milk, but milk may merely act as a carrier. Outbreaks of listeriosis have been particularly associated with the consumption of raw milk or milk products elaborated with raw milk in the recent years (De Buyser, et al. 2001, Marth and Steele. 2001).

Because of the central role of milk in the food supply and its ease of microbial contamination, production and processing of milk is subject to tight regulation in most developed countries. In the USA, most milk is regulated according to Grade A Pasteurized Milk Ordinance, a document that sets the standards for all aspects of milk

production and processing. From a microbiological standpoint, the pasteurized milk ordinance is important primarily in setting the standards for acceptable numbers of viable microorganisms in milk before and after pasteurization. The ordinance sets limits for microbial counts in raw milk for pasteurization at 1 x 10^5CFU/ml of milk from an individual producer and 3 x 10^5CFU/ml for commingled milk from multiple producers (Marth and Steele. 2001). Similarly, current European regulations set limits for aerobic mesophilic counts in raw milk for pasteurization at 1 x 10^5 CFU/ml (Anonymous, 2004).

Sources of bacterial contamination of raw milk can be divided into three general categories: environment, udder, and milk equipment. Environmental sources, which include water, soil, vegetation, and bedding material, vary in the numbers and types of organisms that can be introduced into raw milk. Poor pre-milking udder hygiene that fails adequately to clean dirty udders can result in the introduction of vegetation, soil, and bedding material and their associated microorganisms into the milk. Common contamination sources associated with milking equipment include milking machines, milk pipelines, bulk tanks, and transport tankers (Marth and Steele. 2001).

1.2 Citrus and orange world production

World citrus production and consumption has witnessed a period of strong growth since the mid-1980s. Production of oranges, tangerines, lemons and limes has all expanded rapidly. Larger production levels have enabled higher levels of total as well as per capita consumption of citrus. Even, faster growth has been realized for processed citrus products as improvements in transportation and packaging have lowered costs and improved quality. Regards to oranges, world consumption of oranges grew at a compound rate of 3.5 percent over the period ranging from1986-88 to 1996-98. While consumption of fresh oranges grew at an annual rate of 2.9 percent, this was superseded by growth in processed orange consumption, which grew 4.2 percent per annum. Increased consumption of processed oranges in Europe was one of the primary forces supporting expanded world consumption. Even though per capita consumption of fresh oranges in the EU declined from 13 to 9.7 kg, per capita processed orange consumption nearly doubled to 30 kg (fresh fruit equivalent). Per capita consumption of processed oranges also grew in Canada and the USA, offsetting decreases in fresh orange

consumption. Fresh orange consumption is declining in the developed countries due to it is being replaced by orange juice consumption and with advancements in transportation and storage, fresh citrus is now confronted with more competition from other fruits such as bananas, grapes and strawberries (Spreen. 2010).

1.2.1 Citrus juices and quality parameters

Citrus is a product that offers many advantages in the lifestyles of people who are health conscious, demand convenience, and place a premium on food safety. In fact, citrus juices are the most consumed fruit juices in the world and are a very important sector in the food industry. The characterization and analysis of citrus juices are an important aspect in the citrus processing. The principal characteristics, which juices are object of routine analysis during quality control program, are the total soluble solid content, acidity and pulp content. Citrus juices content a huge diversity of chemical compounds but the most abundant are sugars and organic acids, representing about 80% and 10% respectively, of the total soluble solid content. The density of juice is one of the most important control quality parameters in the citrus industry. Because the carbohydrate concentration of citrus juices is high, the density can be determined through methods and scales apply to pure sugar solutions. However, because no everything in soluble material is exclusively carbohydrate, it is used the expression of soluble solid content. The ºBrix value indicates the percentage of soluble solids and it has converted in the reference scale for measuring the juice concentration in the fruit industry. The acidity of citrus juices is the second most important quality factor after the ºBrix. Acids provide to citrus products their acid or sour characteristic taste which makes them recognized as one of most effective beverage to quench thirst. The principal of these acids is the citric acid or tricarboxilyc acid. Each specific variety has a different taste because of different composition of oils, aromas and essences present in juice. Although some components contribute more than others, the exact flavour reproduction after processing requires a delicate equilibrium of multiple components. It has achieved to return about 87% of orange juice flavour to concentrated orange juice by the addition of d-limonene, ethyl butyrate, citral and acetaldehyde (Kimball. 2001).

In general, consumers prefer filtrated and clarified juices as example apple, grape or berries juice. However, in the case of citrus juices, they prefer them with a cloudy and

opaque aspect. The cloud particles impart the characteristic flavor, color and mouthfeel to orange juice (Croak and Corredig. 2006). Cloud in citrus is composed of finely divided particles of pectin, cellulose, hemicellulose, proteins and lipids in suspension (Irwe and Olsson, 1994; Klavons, et al. 1994). The loss of cloudiness and concentrates gelation a short time after squeezing has been associated to the PME activity. PME is present in all citrus fruits as a cell wall-bound enzyme forming a complex with pectin through electrostatic interactions. During the juice extraction process the enzyme is released into the juice hydrolysing the pectin (methyl esters of homogalacturonan) and transforming it gradually to low methoxy pectin and pectic acids, which may then form insoluble complexes with calcium ions, leading to precipitation of the pectins and cloud loss (Basak and Ramaswamy. 1996). Thermal pasteurization of juice is based on the PME inactivation level above 90% because its thermo tolerance is higher than the majority of spoilage microorganisms found naturally in this type of product (Tribess and Tadini. 2006). Severe conditions (90 °C, 1 min or 95 °C, 30 s) are necessary to inactivate orange PME (Cameron, et al. 1998, Do Amaral, et al. 2005).

Another parameter to consider is the microbiological contamination to which juices are exposed. Many organisms, particularly acid-tolerant bacteria and fungi (yeasts and molds), can use fruit as substrate and cause spoilage, producing off-flavors and odors and product discoloration. In citrus juices, the microbial activity is generally harmful, because besides affecting the product composition can have pathological consequences. Historically, acid foods such as fruit juices have been considered safe; however, unprocessed orange juice has been recognized as vehicle of foodborne diseases (Parish. 1997, Parish. 1998b, Burnett and Beuchat. 2001). These problems have led an effort to develop more stringent strategies and measures to control the microbiological quality and safety of juices, resulting in several guidelines being published by national food standard agencies. The inactivation of spoilage and pathogenic microorganisms as well as the inactivation of endogenous pectin methylesterase (PME) are prerequisites for the extension of the shelf-life of the juice (Katsaros, et al. 2010). Nowadays, refrigerated juices which are not obtained from concentrates and have been subjected to mild pasteurization, partly satisfies the requirements of higher quality demanded by consumers. The shelf-life of these juices ranges between 28 and 45 days in refrigeration and their quality approaches that of freshly squeezed juices (Esteve and Frígola. 2007). Although, these types of orange juices remain the most common, nowadays, the

consumers are demanding more natural products, in consonance with their increasing concern about the wholesomeness of foodstuffs in general. Thus, at present, there is a renewed interest within the citrus industry in the development of innovative practices to meet the demand of orange juices of the highest quality, as a consequence of which a noticeable rise in the consumption of direct orange juices not subjected to thermal treatments has taken place in the last years (Shomer, et al. 1999, Farnworth, et al. 2001, Meléndez-Martínez, et al. 2007).

On the other hand, scientific evidences have demonstrated that an adequate intake of fruit and vegetable is relevant in the prevention of health problems and in the reduction of chronic disease risks (Steinmetz and Potter. 1996, van't Veer, et al. 2000, Southon. 2000, Ford and Mokdad. 2001). Bioactive compounds are held responsible for the benefits, e.g. dietary fibre, vitamin C, carotenoids and components such as glucosinolates, folic acid and (iso)flavonoids (Steinmetz and Potter. 1991). Natural vivid colors of fruit juices have been considered traditionally as one of the principal advantages over others food products. The main carotenoids responsible for the orange color of orange and mandarin juices are α-carotene and β-carotene, zeta-antheraxanthin (yellow), violaxanthin (yellow), β-citraurine (reddish orange) and β-cryptoxanthin (orange) (Kimball. 2001). The color of orange juice is one of the parameters assessed for their commercial classification in the US, so the legislation in this country attaches great importance to the measurement of such parameter (Meléndez-Martínez, et al. 2005). Oranges and orange juice may be highlighted as an important source of vitamin C and polyphenolic compounds (Klimczak, et al. 2007), however the industry do not realize routine analysis of vitamin C, due to its stability and content is generally uniform (Kimball. 2001). Nevertheless, it is know that ascorbic acid is a typically heat sensitive nutrient (Saguy, et al. 1978). Orange juice is a major source of antioxidant flavanones in the diet of developed countries (Gil-Izquierdo, et al. 2001). Flavonoid has a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease, anti-spasmolytic, anti-inflammatory, antioxidative, anti-tumor and antimicrobial activities, among others (Harborne and Williams. 2000). Moreover, flavonoids may contribute to juice quality in many ways, influencing the appearance, the taste and the nutritional value of the product (Shomer, et al. 1999). It is important to assess the effect of minimal processing on phenolic

compounds and their antioxidant capacity, which is known to be influenced by processing and storage (Gil-Izquierdo, et al. 2001, Klimczak, et al. 2007).

1.3 Food processing

Based on NACMCF (2006) evaluation, the term pasteurization can be defined as: Any process, treatment, or combination thereof that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage.

Historically, pasteurization has had two roles, one being public health protection and the other being shelf-life extension. However, new microbial hazards might occur due to pasteurization treatments based on guidance from the NACMCF:

- **Eliminating competition**. Pasteurization will inactivate many nonpathogenic organisms that may grow faster than pathogens and therefore limit pathogen growth. Once products are pasteurized, therefore, they need to be protected against recontamination.

- **Selecting for more resistant organisms**. Microorganisms with greater resistance than microorganism target can survive the pasteurization process. For example, pathogenic sporeformers may germinate and grow unless proper controls, such as refrigeration or formulation, are applied.

- **Sublethal injury.** If organisms are injured rather than inactivated during processing, assurance must be provided that they do not repair and grow during the product shelf life (NACMCF. 2006).

1.3.1 Thermal pasteurization

Thermal pasteurization are predominantly used in the food industry for being the most efficient and economical process to achieve microbial inactivation in several perishable liquid foods. Excessive heat treatment may, however, cause undesirable protein denaturation, non-enzymatic browning and loss of vitamins and volatile flavor compounds (Lavigne, et al. 1989, Farnworth, et al. 2001).

Fruit juices: FDA has for many years had a regulation on pasteurized orange juice (Code of Federal Regulations, 2005e). However, this regulation is a standard of identity, and the pasteurization process is for quality (to reduce substantially the enzymatic activity and the number of viable microorganisms). In April 1997, the NACMCF made recommendations for control of pathogens in fresh juices that were subsequently accepted by FDA and incorporated into regulations (Code of Federal Regulations, 2005c). In these regulations, FDA requires that juice to be used in a beverage must be processed under HACCP. These plans must include control measures that will consistently produce, at a minimum, a 5 log reduction of the most resistant microorganism of public health significance in the product (Code of Federal Regulations, 2005d).

Milk: The first federal standard for milk pasteurization, 61.7ºC for 30 min, was established in 1924 based on destruction of *Mycobacterium tuberculosis* (Meanwell. 1927). In 1956, *Coxiella burnetii* was recognized as the most resistant organism of concern, leading to an increase in the minimum batch pasteurization temperature to 63ºC for 30 min and establishment of HTST pasteurization of 72 ºC for 15 s (Enright, et al. 1957). The effectiveness of milk pasteurization was reexamined in the 1980s after several milk-associated *L. monocytogenes* outbreaks (Bunning, et al. 1988).

Egg products: Under the Egg Products Inspection Act (Act. 2001), egg products are, with few exceptions, pasteurized before they leave the official plant. The term ‘‘egg product’’ means any dried, frozen, or liquid eggs, with or without added ingredients. The term ‘‘pasteurize’’ means the subjecting of each particle of egg products to heat or other treatments to destroy harmful viable microorganisms by specific processes prescribed by regulations. Egg products must be free of viable *Salmonella* microorganisms (Code of Federal Regulations, 2005f). The pasteurization requirement for liquid whole eggs (60ºC for 3.5 min) is expected to achieve a relative 8.75 log reduction of *Salmonella* (US Department of Agriculture, 1969).

1.3.2 Non-thermal food pasteurization

In addition to traditional thermal pasteurization, other technologies can satisfy the definition of pasteurization for certain foods: ohmic heating, microwave heating, steam and hot water treatments, high-pressure processing (HPP), UV radiation, irradiation, pulsed electric field (PEF), and chemical treatments. Other technologies, such as filtration, infrared and high voltage arc discharge, may also have the potential to be used alone or in combination with other treatments (Mañas and Pagán. 2005, Chen, et al. 2006).

Irradiation: The irradiation process involves the application of electromagnetic waves or electrons to foods. Radiation sources are either gamma rays from cobalt-60 or cesium-137, electron beams or X-rays, and the amount of irradiation absorbed by a food is measured in kGy (Mañas and Pagán. 2005). In the United States, governmental agencies have approved irradiation (gamma energy, high-energy electrons, X rays) for various food items. When food irradiation is approved, the upper treatment level (referred to as ‘‘dose’’) generally is limited to 10 kGy, with the exception of spice treatment in the United States and some countries where higher doses are approved. The effect of the irradiation process on food safety needs to be equivalent to that of thermal pasteurization and result in no changes in the food that present a public health risk. The Joint Expert Committee on Food Irradiation representing the United Nations/International Atomic Energy Agency (FAO/IAEA) and the World Health Organization (WHO) concluded that irradiation of any food up to 10 kGy caused no toxicological hazards and introduced no nutritional or microbiological problems (Kuo, et al. 1997, Wemekamp-Kamphuis, et al. 2004). The Codex Alimentarius Commission adopted a standard in 2003 for irradiated foods that accepts the use of doses higher than 10 kGy for food products to achieve specific technical purposes (Codex. 2003). Irradiation at doses higher than required for control of sprouting or disinfestation can damage or soften some produce items. The end result depends on the type of produce, variety, harvest practices, and other treatment issues and cannot be generalized.

Ultraviolet (UV): Ultraviolet processing involves the treatment of foods with radiation from the UV region of the electromagnetic spectrum to inactivate microorganisms. Wavelengths in the range of 200- 280 nm inactivate bacteria and viruses (Kuo, et al.

1997, Bintsis, et al. 2000). The effectiveness of the treatment depends on the specific product characteristics. The technology can be used as an alternative to chlorine for disinfection of water and wastewater (Chang, et al. 1985). For microbial inactivation in water, 400 J/m^2 must be achieved in all parts of the fluid (Sommer, et al. 2000).

Ultrasound: Ultrasound is defined as sound waves with frequencies above the threshold for human hearing (>16 kHz). Although ultrasound was initially discarded for food preservation because of its weak lethal action, the application of an external hydrostatic pressure of up to 600 kPa [manosonication (MS)] increases substantially the lethality of the treatment (Mañas and Pagán. 2005).

High hydrostatic pressure (HHP): The HHP involved the application of pressures from 100 to 1000 MPa for few minutes, being capable of inactivating microorganisms. The first studies on the lethal effect of HHP were conducted at the end of the 19th century, but it has been in the nineties when commercial applications of this procedure have started (Mañas and Pagán. 2005). An advantage of HPP is the minimal effect it has on covalent bonds; thus, minimal damage occurs to flavors, aromas, provitamins, and vitamins (NACMCF. 2006).

Pulsed Electric Fields (PEF): this technology consists in the application of short duration (1–100 μs) high electric field pulses (5–50 kV cm^{-1}) to a food placed between two electrodes (Mañas and Pagán. 2005). Energy loss due to heating foods is minimized, reducing detrimental changes of the sensory and physical properties of foods. Destruction of microbial cells is the result of electroporation of cell membranes. The process can be static or continuous. Due to design limitations of current treatment chambers (gap between the electrodes is in the range of 3 mm), the process is currently limited to fluids (NACMCF. 2006).

These alternative preservation processes achieve the equivalent of pasteurization, but not sterilization (Hayakawa, et al. 1994, Grahl and Markl. 1996, Gervilla, et al. 2000). Irradiation, for example can achieve an effect equivalent to thermal pasteurization. While irradiation is useful for a wide variety of foods, its use is limited for some products, e.g., some dairy products, because of negative organoleptic changes that occur following treatment (NACMCF. 2006). Although well suited to the destruction of

vegetative cells, HPP has limited efficacy against sporeformers unless combined with other treatments, such as heat and pH. HPP is very difficult to evaluate because of the multiple combinations of pressure with temperature, number of passes, pH, time, isotonic strength, and content of organic compounds. Some of these relationships are not linear, and although some mathematical projections have been developed, the combined effects listed above may need to be experimentally determined (Lado and Yousef. 2002). Monochromatic pulsed UV has been shown to inactivate bacteria in milk (Smith, et al. 2002); however, its effectiveness against the target organism for traditional milk pasteurization, *C. burnetii*, has not been evaluated, so equivalence to thermal pasteurization is unknown. UV radiation has demonstrated to be capable of delivering a process that achieves an effect equivalent to thermal pasteurization in some foods (e.g., fruit juices). Considerable data have been published that support the adequacy of PEF technology as a feasible pasteurization treatment for fluids (NACMCF. 2006).

1.4 Ultra high pressure homogenization

Homogenization at moderate pressures (20–50MPa) is largely used in the food, pharmaceutical and cosmetics industries to disperse non-miscible phases, stabilize emulsions, and/or prepare products with appropriate rheological properties. In the dairy industry, homogenization is of special interest since it reduces the size of fat globules by shear, turbulence and cavitation forces caused by the pressure difference across, and high speed collisions within, the homogenizing valve, resulting in final globule diameters up to 1 mm, with a concomitant increase in surface area (4–10 fold) (Datta, et al. 2005).

High pressure homogenization (HPH), dynamic high pressure (DHP) or ultra-high pressure homogenization (UHPH) refers to treatments with the same design principle as conventional homogenizers but working at much higher pressure levels (up to 400 MPa, depending on the design of the homogenizer) (Paquin. 1999, Floury, et al. 2002, Datta, et al. 2005, Lacroix, et al. 2005).

During ultra high pressure homogenization process, the fluid is forced through a narrow gap, after which it is subjected to an ultra-rapid depression. In particular, when the local

pressure in a liquid is reduced without temperature change, gas-filled bubbles (or cavities) nucleate and grow within the body of liquid. The collapse of such cavities could transmit several localized forces to surfaces or particles, including the microbial cell (Lanciotti, et al. 1996). Another parameter to considerer is the temperature rise in the reaction chamber. The role of heating on the modifications produced by UHPH is uncertain although the heating is of short duration in the UHPH chamber. This phenomenon is very different from the deliberate application of heat in the industry to heat denature macromolecules over longer times (Paquin. 1999).

UHPH treatments above 200 MPa are expected: (i) to produce very fine and stable emulsions, with mean oil droplets diameters below 0.3–0.4 μm, (ii) to induce significant microbial and/or enzymatic inactivation, even when operating at a moderate temperatures (40–50ºC), and (iii) to modify the rheological and/or coagulation properties of milk or dairy emulsions. Currently available UHP homogenizers can reach pressures up to 350 MPa, with a maximum throughput of 15 L per 1 h at this higher pressure level (Thiebaud, et al. 2003).

1.4.1 Characteristics of high pressure homogenization equipments

Since the first homogenizer presented by Auguste Gaulin at the 1900 World Fair in Paris, homogenization technology has evolved over the years. In the early 1980s new technology was introduced for production of fine emulsions, based on a very high pressure capacity as well as on a new reaction chamber design (Paquin. 1999). Different types of equipment in this category now exist, such as Panda GEA Niro-Soavi (Italy-USA), APV-Rannie (Dennmark-UK), APV-Gaulin (USA), Microfluidizer-Microfluidics (USA), Nanojet-Haskel (USA), Emulsiflex-Avestin (Canada) and Stansted Fluid Power (UK).

In most of these equipments the fluid is fed axially into the valve seat, and then is accelerated radially into the gap between the valve and seat. When the fluid leaves the gap, it becomes a radial jet that stagnates on an impact ring before leaving the homogenizer. Nevertheless, as shown in Figure 1, in the Stansted Fluid Power Ltd. valve design, the fluid streams axially under high pressure along the mobile part of the valve and flows with high velocity through the radial narrow gap formed between the

valve seat and the piston, before leaving the valve seat at atmospheric pressure. The size of the slit (h) and the resulting stream velocity and pressure of the liquid ahead of the valve depend on the force acting on the valve piston, which can be adjusted to regulate the homogenizing intensity. The pressure drop of the liquid in the valve is called the homogenizing pressure (ph). Due to that the Stansted homogenizing valve technology consists of ceramic material, which is known to withstand to ultra high pressure levels, it is able to reach much higher pressures than other technologies available (above 350 MPa) (Floury, et al. 2004a and b,).

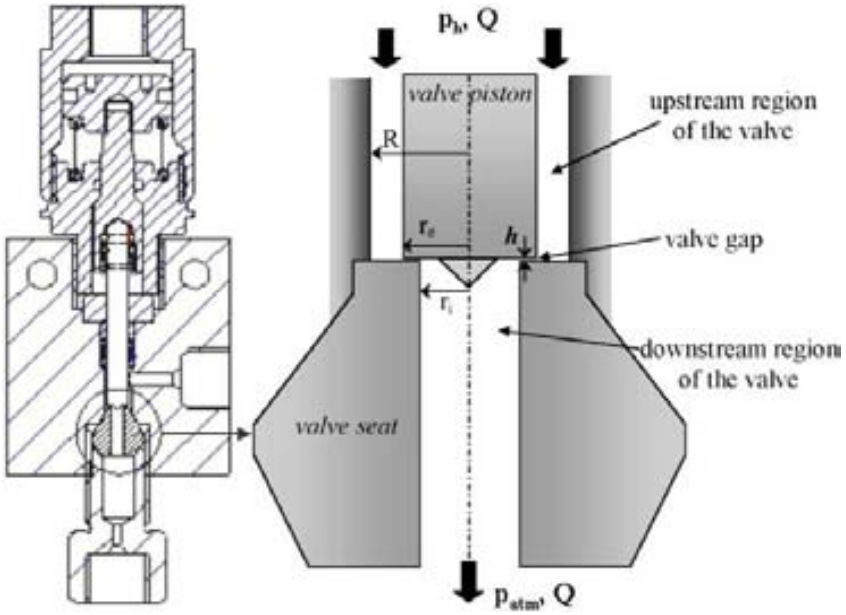

Figure 1. Schematic representation of Stansted high pressure homogenizing valve from Floury, et al. (2004a).

1.4.2 UHPH and microorganisms

Inactivation of microorganisms

Several authors have studied the applicability of UHPH for the inactivation of the native microbiota of different food products (Table 2). There are also some published works that described the effect of UHPH on specific pathogen bacteria (Table 3). The effect of UHPH has also been evaluated on phages. However, the effect of UHPH on a specific microorganism varies depending on different factors, such as the kind of microorganism, matrix composition and viscosity of the fluid, among others.

Table 2. Effect of UHPH on native microbiota of different food

Food	Pressure (MPa)	Inlet temperature (ºC)	Effect of UHPH	Reference
Milk	100-300	4, 14 and 24	At higher pressure and higher inlet temperature, the inactivation of endogenous flora increases. A second pass at 200 MPa attains a double inactivation.	Thiebaud, et al. 2003
Milk	150, 200 and 250	45	Complete inactivation of psychrotrophic bacteria at 200 and 250 MPa and a reduction of 2.73 log cycles at 150 Mpa. Reductions of mesophilic bacteria by 1.3, 1.83 and 3.06 log at 150, 200 and 250 MPa, respectively.	Hayes, et al. 2005
Orange juice	200	25	Significant inactivating effect against spoilage microflora of orange juice.	Tahiri, et al. 2006
Milk	200 and 250	55 and 70	Reduction of total bacteria count, Psychrotrophs, Pseudomonads, Coliforms, *Staphylococcus aureus* and Lactobacilli to non-detectable levels. After storage (14 days at 4ºC) total bacteria count, psychrotroph and pseudomonad counts reached approximately 10^8 CFU/ml.	Smiddy, et al. 2007
Milk	100, 200 and 300	30 and 40	Efficient in a 99.99%, reducing psychrotrophic, lactococci, and total bacteria. Elimination of Coliforms, lactobacilli, and enterococci. Milk with a microbial shelf life between 14 and 18 days, similar to high pasteurized milk.	Pereda, et al. 2007
Soymilk	200 and 300	40	Reductions of total count bacteria by 2.42 and 4.24 log CFU/ml at 200 and 300 MPa, respectively. Reduction of spores by around 2 log CFU/ml and detection of enterobacteria below the detection level in both treatments.	Cruz, et al. 2007
Orange juice	0-250	22, 35 and 45	Both initial temperature and pressure have effect on the microbial load. More pressure was required to inactivate one Log cycle of microorganisms when homogenizing at low initial temperature and vice versa. The higher reduction of the microbial load was around 1.5 log cycles.	Welti-Chanes, et al. 2009
Apple juice	100, 200 and 300	4 and 20	Successful reduction of microbial load at 200 and 300 MPa. Reductions comparable to conventional heat treatment. During 60 days storage (4ºC) mesophilic counts did not change.	Suárez-Jacobo, et al. 2011

Table 3. Effect of UHPH on specific microorganisms inoculated into different food

Food	Microorganism	Pressure (MPa)	Number of passes	Inlet temperature (ºC)	Effect of UHPH	Reference
Milk	*Listeria monocytogenes* and *E. coli* O157:H7	100, 200 and 300	1, 3 and 5	25	Significantly reductions with increasing pressure or number of passes. *E. coli* O157:H7 much more sensitive than *L. monocytogenes*.	Vachon, et al. 2002
Milk	*Listeria innocua*	200	5	5	3 to 4 log cycle reduction of *L. innocua*	Kheadr, et al. 2002
Milk and whey permeate	Lactococcal bacteriophages	100 and 200	1, 3 and 5	25 and 50	Proportional inactivation to both pressure and number of passes. Greater resistances of Lactococcal bacteriophages to DHP in milk than in whey permeate. Inactivation depends on initial phage concentration. Heating milk at 50ºC prior to DHP treatment did not enhance inactivation.	Moroni, et al. 2002
Orange juice	*E. coli* O157:H7	200	1,3 and 5	25	High sensitivity of *E. coli* O157:H7 to UHPH. Complete inactivation after 5 passes.	Tahiri, et al. 2006
Milk and orange juice	*Listeria innocua*	300+30 (first and second valve)	1	6 and 20	Both the inlet temperature and the food matrix influence the inactivation, being higher in whole milk at 20ºC. Few or no sublethal injuries.	Briñez, et al. 2006a
Whole and skimmed milk	*E. coli* and *E. coli* O157:H7	300+30 (first and second valve)	1	6 and 20	The type of milk influences the degree of inactivation in both strains of *E. coli*, being higher at 20 ºC in whole milk. No sublethal injuries.	Briñez, et al. 2006b

Table 3. Continued

Food	Microorganism	Pressure (MPa)	Number of passes	Inlet temperature (ºC)	Effect of UHPH	Reference
Milk and orange juice	*Staphylococcus aureus* and *Staphylococcus carnosus*	300+30 (first and second valve)	1	6 and 20	The inlet temperature, the food matrix and the kind of strain influence the lethality level, being higher for *S. aureus* in whole milk at an inlet temperature of 20 °C. No sublethal injuries	Briñez, et al. 2007
Orange juice	*Saccharomyces cerevisiae* and *Lactobacillus plantarum*	100-300	1	10	Complete elimination of initial loads (1.2×10^7 CFU/ml) of *L. plantarum* and (2.9×10^5 CFU/ml) of *S. cerevisiae*.	Campos et al.2007
Tomato juice	*Fusarium oxysporum, Emericella nidulans* and *Penicillium italicum*	30-150	1, 2 and 3	20	Reduction of the number of conidia with the progressive increase of the pressure. Effect more evident with a multi-step process. Low effectiveness of treatment at a lower fluid viscosity.	Corbo, et al. 2010

Mechanisms of inactivation

When the foodstuff undergoes UHPH, the microbial cells experience a non-specific tearing apart of the cell wall determined by the physical interaction of the cells with the small-gap homogenization valve (Middelberg. 1995). A lot of controversy exists in the literature about the exact cause of cell disruption by high-pressure homogenization. Save, et al. (1994) proposed that cavitation and the shock waves and pressure impulses generated upon cavity collapse are responsible for cell disruption. Engler and Robinson (1981) and Moore, et al. (1990) believed that the impingement and impact of cells against the walls of the homogenization valve are the main causes for cell disruption. Also other mechanisms of cell disruption have been proposed, such as turbulence (Doulah, et al. 1975), rate and magnitude of pressure drop (Brookman. 1974), and viscous shear.

1.4.3 Factors affecting microbial inactivation by UHPH

Inlet temperature

There is evidence that the effectiveness of UHPH is affected by the inlet temperature of the sample. Vachon, et al. (2002) observed that an increase of the sample temperature prior to UHPH treatment resulted in a significant increase in the inactivation of *Salmonella* Enteritidis and *L. monocytogenes*. One study conducted by Wuytack, et al. (2002) showed that when the inlet temperature increased to 45 and 50°C, reductions of 2 and 3 log units, respectively, of *Staphylococcus aureus* were reached. This behavior was confirmed by Briñez, et al. (2006a and b), where inactivation of bacterial cells was significantly higher when increased the inlet temperature.

Temperature markedly affects membrane lipid composition and physical state. At low temperatures (2 to 10ºC), crystallization of phospholipids occurs and cell membranes become more rigid and consequently more sensitive to pressure. At temperatures over 50ºC, hydrogen and hydrophobic bonds may be weakened, making bacterial membranes less resistant to high pressure. The better resistance to pressure at 25ºC reported by Vachon, et al. (2002) and Wuytack, et al. (2002) is attributed to greater membrane flexibility at that temperature.

Pressure level and number of passes

Most of the studies carried out have proved that pressure level and number of cycles have a significant impact on the lethal effect of UHPH, inactivation increasing with increased pressure level and number of cycles (Vachon, et al. 2002, Moroni, et al. 2002, Wuytack, et al. 2002, Thiebaud, et al. 2003, Tahiri, et al. 2006, Lujan Capra, et al. 2009). As example Wuytack, et al. (2002) observed that under homogenization pressure up to 300 MPa and treatment temperature 25 or 45ºC, inactivation of the most resistant organisms (*Staphylococcus aureus*) by a single homogenization treatment remained under 1 log unit, which is far insufficient for applications such as food pasteurization. The level of inactivation increased to almost 4 log units after four rounds of homogenization.

Viscosity of the fluid

An increase in process temperature at a certain pressure level leads to an increase in microbial inactivation by high-pressure homogenization (Thiebaud, et al. 2003, Diels, et al. 2003). Diels, et al. (2004) observed that *Escherichia coli* MG1655 inactivation was positively correlated with the applied pressure (100-300 MPa) and with the initial temperatures (5-50 °C). When samples were adjusted to different concentrations of polyethylene glycol to have the same viscosity at different temperatures below 45 °C and then homogenized at these temperatures, no difference in inactivation was observed. These observations strongly suggest that the influence of temperature on bacterial inactivation by high pressure homogenization is only through its effect on fluid viscosity. At initial temperature up to 45°C, corresponding to an outlet sample temperature below 65°C, the inactivation level was higher than the one predicted on the basis of the reduced viscosity at these temperatures, suggesting that under these conditions heat starts to contribute to cellular inactivation in addition to the mechanical effects that are predominant at lower temperatures. In another work, Diels, et al. (2005) studied the inactivation of *E. coli* MG1655 by high-pressure homogenization (100-300 MPa) in buffered suspensions adjusted to different relative viscosities (1.0, 1.3, 1.7, 2.7 and 4.9) with polyethylene glycol (PEG) and observed that bacterial inactivation decreased with increasing viscosity of the suspensions, an effect that was more pronounced at higher pressures. Also, it was found that inactivation of *E. coli* MG1655 by HPH in skim milk, soy milk and strawberry–raspberry milk drink was the same as in PEG containing buffer of the corresponding viscosity.

Temperature during UHPH processing

Temperature effects have to be necessarily taken into account in UHPH, since, upon homogenization, an important rise of the temperature is observed in fluid downstream of the valve. In the range between 0 and 45°C, where it is not directly responsible for microbial inactivation, the role played by temperature is significant. Temperature effect can be related with viscosity of fluids since viscosity generally decreases with increasing temperature and vice versa. Consequently, temperature effect on microbial inactivation can at least partly be explained by an indirect effect on fluid viscosity (Diels, et al. 2004).

Food matrices

Some studies have demonstrated that the fluid where microorganisms are suspended can also have an influence on the inactivation effectiveness by UHPH. Vachon, et al. (2002) studied the inactivation of *L. monocytogenes* and *E. coli* O157:H7 in PBS and milk, and found that UHPH is less effective in milk than PBS. This difference is attributed to differences in the media composition. Milk constituents may have offered protection against the lethal effects of pressurization. Among the various milk constituents, fat would most likely provide a protective effect for microorganisms against unfavorable conditions. At difference of these authors, Briñez, et al. (2006b) observed clear differences in the lethality obtained in *E. coli* between skim milk and whole milk in favor of whole milk. This appeared to be due to an increase in the temperature of the liquid during UHPH processing where the increment in the viscosity caused by fat content probably contributed to an increase in friction, in turn increasing the temperature reached. In anothers studies, Briñez, et al. (2006a and 2007) observed that the UHPH treatment applied was more efficient against *L. innocua* and *Staphylococcus* when it was applied in whole milk than in orange juice under the same conditions. However, Tahiri, et al. (2006) observed that UHPH was more efficient in PBS than in orange juice reducing microbial load. It is clear that several matrix factors are implied such as nutrients, viscosity, pH, inhibiting agents, and others.

Kind of microorganisms

Some studies have shown changes in cell morphology as well as splits in the cytoplasmic membrane of bacteria submitted to UHPH treatments (Kheadr, et al. 2002). Previous research has also shown that the cellular membrane is the site most damaged

by pressure (Earnshaw. 1992). Made of phospholipids and proteins held together by hydrogen bonds and hydrophobic bonds, the membrane is somewhat rigid and plays a significant role in cellular respiration and transport. Sudden increases in permeability or rupture of the cell membrane, as may occur under pressure, may cause cell death (Cheftel. 1992). Vachon, et al. (2002) and Tahiri, et al. (2006) observed in transmission electron micrographs of ultra high pressure homogenized samples that the treatment caused disruption of the cells.

Experimental evidences indicated that Gram-negative bacteria are more sensitive to UHPH than Gram-positive bacteria (Vachon, et al. 2002, Wuytack, et al. 2002), supporting the widely held belief that UHPH inactivates vegetative bacteria mainly through mechanical disruption, because Gram-positive bacteria have a thicker peptidoglycan layer and henceforth have greater mechanical strength than their Gram-negative counterparts (Feijoo, et al. 1997, Diels, et al. 2005).

Regarding of the bacterial spores, scarce information is available although spores are expected to be highly resistant to UHPH. However, used at mild temperatures or with repeated cycles can significantly increase the inactivation of sporulated bacteria, such as *Bacillus* (Feijoo, et al. 1997, Chaves-López, et al. 2009).

Initial load

There is controversy about whether cell concentration has influence on cell disruption efficiency. Over a wide range of cell concentrations and operating pressures, Vachon, et al. (2002) reported that the inactivation of pathogens depended on the initial bacterial concentration. The highest reduction was obtained when the bacterial load did not exceed 10^5 CFU/ml, using phosphate buffered saline (PBS) as inoculation media. Contrary to the buffer results, the initial bacterial concentration of the milk samples had no impact on the effectiveness of high-pressure homogenization. Moroni, et al. (2002) reported that the effectiveness of UHPH in inactivating lactococcal bacteriophages in PBS is affected by the initial phage concentration; with greater initial load, the treatment become less effective. Also, Tahiri, et al. (2006) reported that the highest degree of inactivation was obtained with the lowest initial bacterial concentration. However, experiments done by Diels, et al. (2005) with *E. coli* shown that cell concentration seems to have no influence on inactivation on these PBS suspensions. Lujan Capra, et

al. (2009) observed that inactivation of lactic acid bacteria and probiotic bacteria phages seems to depend on phage concentration; the higher the initial load, the bigger the reduction achieved.

Sublethal injury

UHPH is a technology that inactivates bacteria by an "all or nothing" physical disruption of the cell wall. Bacteria are either disrupted or not. This situation reflects that UHPH does not induce sublethal injury (Wuytack, et al. 2002).The works of Briñez, et al. (2006a, b and 2007) seems to support the apparent inability of high pressure homogenization to induce sublethal injury in bacteria. Wuytack, et al. (2002) proposed a model that describes cellular inactivation and sublethal injury as a result of the inactivation of one or two targets in the cell. If only one target is present in the cell in a single copy and this target is vital for the cell and is inactivated by the treatment, then every first "hit" in a cell will cause death, and no state of sublethal injury will exist. They suggest the peptidoglycan chain as the sole vital target in the case of UHPH.

1.4.4 UHPH and physicochemical, nutritional, sensorial properties of food

Emulsions are presented in a large variety of foods, from the more natural, e.g. milk, to the more sophisticated, e.g. mayonnaises. Emulsions are dispersions of liquid droplets in a liquid continuous phase. In order to prevent coalescence and to obtain stable emulsions the immiscible phases are homogenized in high pressure homogenizers, in colloid mills or in batch reactors with high-speed blenders.

The study of the effect of UHPH on emulsions shows that there are modifications in the structure and the texture of emulsions with increasing pressure (Floury, et al. 2000, Desrumaux and Marcand. 2002) as well as a reduction of droplet size. However, at high pressure treatments, high shear and relatively high temperature could also damage some constituents and characteristics of food emulsions (Floury, et al. 2000).

Floury, et al. (2003) suggested that deformation and break-up of droplets, adsorption of surfactants on the created interface, and collision and possibly recoalescence of droplets are the main mechanisms that occur during emulsification in a Stansted type ultra high homogenizer. Final emulsion droplet size distribution is the result of an equilibrium

between droplet break-up and recoalescence. This equilibrium strongly depends on homogenizing pressure. Ultra high pressures as high as 350 MPa do not appear to be real benefits on emulsification efficiency, because recoalescence rates are then quite large. However, ultra high pressure homogenizing conditions allow obtaining very fine emulsions (mean droplet diameter inferior to 300 nm) in only one pass through the homogenizing valve. Geometry of the homogenizing valve appears to be very important as regards to the quality of the finished product. The objective of changing valve designs is to find one that gives a better possible product at the lowest pressure, without mechanical damage.

On the other hand, milk is the food where most comprehensively has been studied the effect of UHPH. The reduction of the size of fat globules in milk has a special interest in the dairy industry to prevent creaming and coalescence during shelf storage. It has been observed that an increment of pressure level and inlet temperature in UHPH processing results in a decrement milk fat globule size (Kheadr, et al. 2002, Thiebaud, et al. 2003, Hayes and Kelly. 2003a). Thiebaud, et al. (2003) observed that a second homogenization pass at 200 MPa markedly decreased the size distribution of fat globules. However, some occasions after UHPH processing there are formation of aggregates (Thiebaud, et al. 2003, Pereda, et al. 2007), but without creaming formation in samples (Pereda, et al. 2007). Pressure homogenization processing of milk also results in reductions of casein micelle size (Kheadr, et al. 2002, Sandra and Dalgleish. 2005). Properties of milk such as whiteness and rennet coagulation are unaffected or enhanced, respectively, as homogenization pressure is increased (Hayes and Kelly. 2003a). Moreover, UHPH does not affect the color of milk (Hayes, et al. 2005).

Otherwise, some studies are focused on the manufacturing properties of milk previously subjected to UHPH. In the production of cheese, Guerzoni, et al. (1999) found that the pressure homogenization treatment confers both direct and indirect effects on cheese characteristics and their evolution during ripening. The direct effects are principally linked to the change in water binding capacity of proteins as shown also by the lower whey separation. The indirect effects involve the microbial growth or activity and, particularly, modifications of the population of the lactic acid bacteria that occurred naturally and their evolution as well as a more precocious yeast and mold growth with a consequent rapid rise in pH. Moreover, cheeses homogenized under high pressure

present a more homogeneous microstructure. Kheadr, et al. (2002) observed that cheeses made from pressurized milk retained more moisture and protein and produced higher yield than those from pasteurized milk. Zamora, et al. (2007) found the best rennet coagulation properties when applied a single-stage UHPH at 200 and 300 MPa. However, these properties were negatively affected by the use of the UHPH secondary stage. Increasing the pressure led to higher yields and moisture content of curds. Lanciotti, et al. (2004) studied the effect of homogenization on the proteolytic and lipolytic profiles, of Crescenza, a traditional Italian soft cheese. Results showed that Crescenza obtained using pressurized milk showed evidence of early and significant lipolysis.

Regards to enzymes and UHPH effect on them, studies undertaken by Hayes and Kelly. (2003b), show the effect of high pressure homogenization on alkaline phosphatase activity and plasmin and plasminogen-derived activities in raw whole bovine milk. They observed that inactivation of plasmin and plasminogen-derived activities increased as HPH pressure increased. Two-stage UHPH reduced both activities to a greater extent than single-stage UHPH. Milk inlet temperature had a significant effect on residual plasmin and plasminogen activities of UHPH-treated milk samples. However, all homogenized milk samples retained active alkaline phosphatase, indicating that thermal conditions during UHPH did not equate to that of conventional high temperature short time pasteurization, and that the wide range of forces experienced by milk during UHPH treatment does not inactivate the latter enzyme. Datta, et al. (2005), applying 200 MPa with inlet temperatures up to 45ºC, also observed a reduction of alkaline phosphatase, plasmin and lactoperoxidase activity. Denaturation of β-lactoglobulin was more extensive following UHPH than the equivalent heat treatment. Inactivation of plasmin was correlated with increasing fat/serum interfacial area but was not correlated with denaturation of β-lactoglobulin. In agreement Hayes, et al. (2005) found that the activities of plasmin, alkaline phosphatase and lactoperoxidase in milk were all greatly reduced by UHPH.

Manufacturing properties of milk pressurized to produce yogurt also has been studied. The UHPH processing of milk results a useful tool to obtain yoghurts with a greater variety of textures associated to a high microbiological quality (Lanciotti, et al. 2004). Moreover, yogurts manufactured from UHPH treated milk present higher water-holding

capacity and firmness values compared with the conventional yogurts. However, the disruption of the network from UHPH-treated milk into stirred gels results in yogurts with higher consistency, less syneresis but coarser structure than the conventional ones (Serra, et al. 2009). The suitability for manufacturing soy-yogurts from UHPH treated soy milk was studied by Cruz, et al. (2009). Their results indicated that conventionally heat-treated soymilks and UHPH-treated samples exhibited different behavior to coagulation. Heat-treated soymilk had a shorter onset of gelation, and higher aggregation rate and gel network density than UHPH-treated soymilk. However, physical quality parameters, especially firmness, were much better in UHPH than in conventional heat-treated soy-yogurts. Same authors showed that soy yogurts from UHPH-treated soymilk presented higher values of mechanical parameters related to firmness and G*, and better water holding capacity. Moreover, soy yogurts maintained these positive characteristics during cold storage (Ferragut, et al. 2009).

There are few studies about the effect of UHPH on fruit juices properties. Lacroix, et al. (2005) for example studied the effect of UHPH alone or in combination with pre-warming on pectin methylesterase (PME) activity and opalescence stability of orange juice. UHPH treatment at 170 MPa for five passes decreased PME activity by 20%. Warming the juice (50ºC, 10 min) prior to homogenization significantly increased the effectiveness of UHPH. Moreover, accelerated shelf-life study at 30ºC revealed that opalescence stability can be increased by several days by UHPH treatment, even in the presence of active PME. Welti-Chanes, et al. (2009) evaluated the effect of five pressures (0-250 MPa), three inlet temperatures and five passes on PME activity, cloudy appearance, and vitamin C of orange juice. Reductions of 50.4, 49.4 and 37.8% of PME activity were observed in juice homogenized by one pass at 250 MPa at the initial temperatures of 22, 35, and 45°C, respectively. PME activity in orange juice was reduced as the number of passes increased. After five passes at 100 and 250 MPa the enzyme activity was reduced in more than 30 and 80%, respectively. The vitamin C content remained stable after homogenization at different pressures and number of passes. The cloudy appearance of the homogenized orange juice was maintained for 12 days under low temperature conditions. Betoret, et al. (2009) studied the effect of lower pressures ranking from 0 to 30 MPa on the particle size distribution, color, cloudiness, and flavonoid content of fresh citrus juices. The results showed that homogenization pressure affected the particle size distribution and color of the citrus juices, which made

it possible to define different sample groups on the basis of the applied pressure. In fresh juice the contents of the flavonoids were not affected by homogenization pressure but after five months of storage the content of the flavonoid hesperidin was affected. Recently, Suárez-Jacobo, et al. (2011) have reported the effect of UHPH treatments on the antioxidant capacity, polyphenol composition, vitamin C and provitamin A contents of apple juice. UHPH processing did not change apple juice antioxidant capacity, regards to 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. A high correlation between the antioxidant capacity and total polyphenol content was observed. Moreover, it was found that UHPH-treatment prevented degradation of phenols. Although, vitamin C concentrations did not change in UHPH treated samples, significant losses were observed for provitamin A content.

2. OBJECTIVES

Main objective

To evaluate the UHPH technology as alternative to conventional heat pasteurization of for ensuring the safety and quality of liquid foods, especially of orange juice, which was taken as a model.

Specific objectives:

Safety related objectives:

- To evaluate the effectiveness of the UHPH against different strains of pathogenic microorganisms inoculated into different liquid foods: whole egg, milk and fruit juices.

- To study the influence of the UHPH treatments and the characteristics of the matrix on the capability of these microorganisms to survive, repair and grow during the shelf life of the product, evaluating the possibility to generate sub-lethal injuries.

General quality related objectives: orange juice as a model

- To study the influence of the UHPH treatment variables on the inactivation of pectin methyl-esterase activity, as well as in the cloudiness of orange juice.

- To study these variables on the microbiological stability of orange juice during the shelf-life.

- To determine their influence on the preservation of the orange juice bioactive properties.

- To elucidate the influence of these treatments on the sensorial characteristics with respect to fresh and pasteurized orange juices.

3. MATERIAL AND METHODS

In this section, the experimental design and the methods used are listed and discussed, but more detailed descriptions can be found in papers I-VII.

3.1 Main experimental design

The steps followed to carry out the evaluation of the effectiveness of the UHPH against pathogenic microorganisms on different liquid food products are shown in Figure 2.

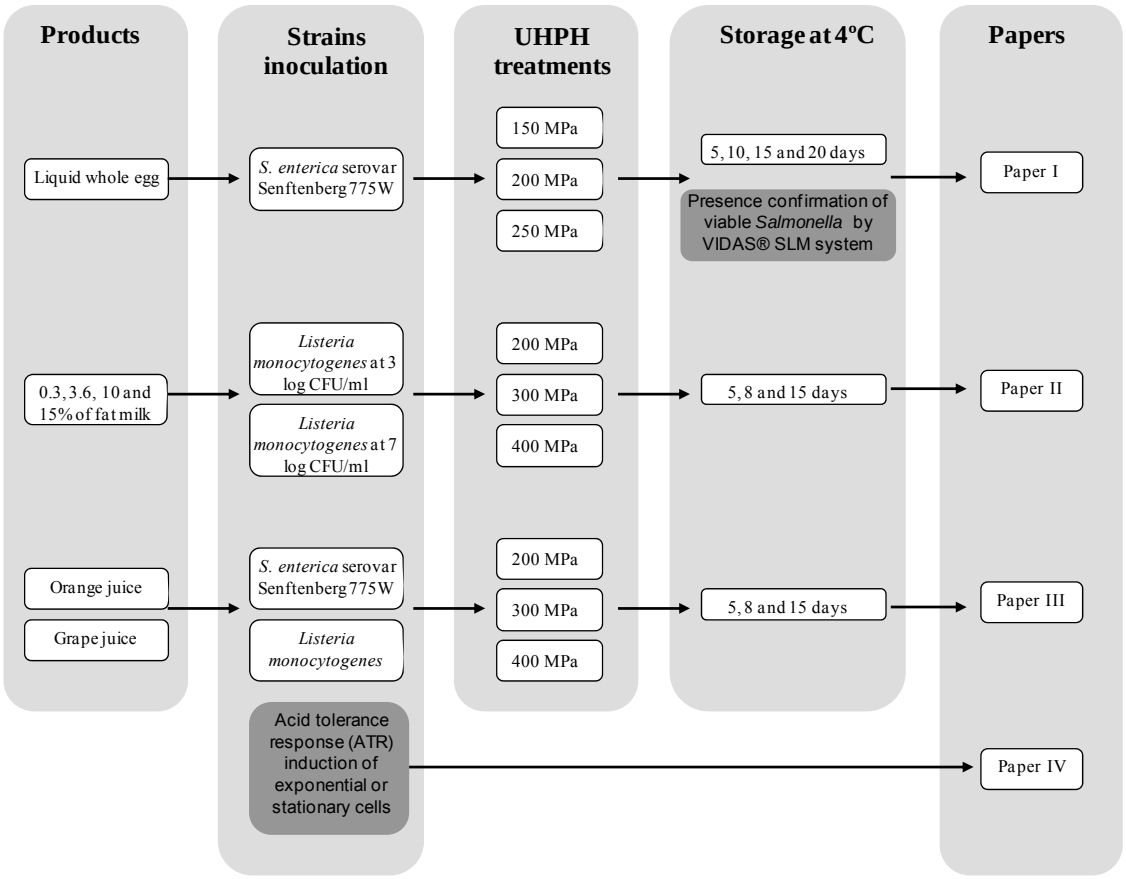

Figure 2. Diagram of the procedure followed to evaluate the effect of UHPH application on different liquid food matrices inoculated with pathogenic microorganisms.

To evaluate the effect of the UHPH on the enzymatic, physical, microbiological, bioactive and sensory properties of foods, we used orange juice as a model. This part of the work was carried out with the financial support of the Spanish Ministry of Education and Science (research project AGL-2006-09607). Figure 3 shows the diagram of the procedure.

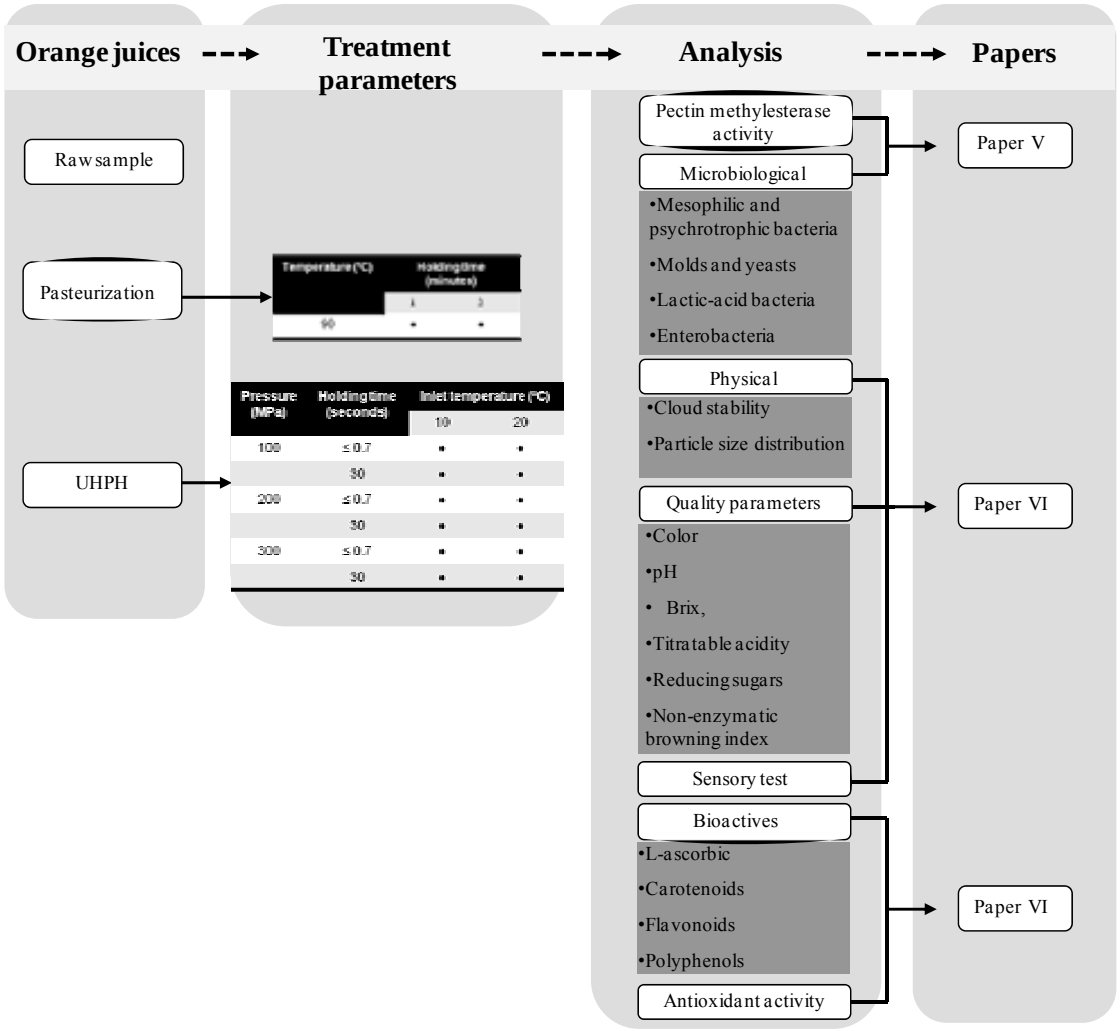

Figure 3. Diagram of the procedure followed to evaluate the effect of the UHPH on the enzymatic, physical, microbiological, bioactive and sensory properties of orange juice.

3.2 Description of the procedures done to evaluate the effect of UHPH on pathogenic microorganism (paper I, II and III)

3.2.1 Microorganisms used

The strains used were supplied by the Culture Collection of the University of Goteborg, Sweden, and by the Spanish Type Culture Collection of the University of Valencia, Spain. Freeze-dried cultures were rehydrated in tryptone soy broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37 ºC for 24 h and subsequently used to inoculate tryptone soy agar plates (Oxoid). Individual colonies were collected to prepare cryobeads (Nalgene System 100TM Laboratories Microkit Iberica S.L., Madrid, Spain) that were kept at -20 ºC to provide stock cultures for the assays.

- *Listeria monocytogenes* (CCUG 15526) was chosen because it is one of the most difficult organisms to control (i.e., by juice acidity or heat lethality) and in the absence of known specific pathogen-product associations, the National Advisory Committee on Microbiological Criteria in Foods (NACMCF) (Food and Drug Administration. 1998).

- *Salmonella enterica* subsp. *enterica* serovar Senftenberg 775W (CECT 4565) is described as one of the most resistant to thermal pasteurization (Mañas, et al. 2003).

3.2.2 Preparation of inoculates

Prior to each experiment, the strains were revived by placing one bead in tryptone soy broth (TSB) and incubated at 37ºC for 18 h. This broth was used to streak the culture on tryptone soy agar plates (TSA, Oxoid) which were incubated for 37ºC for 18 h. Once the purity of the culture was verified, cell suspensions were prepared in 11 ml of tryptone sodium chloride solution (1 g/L of tryptone pancreatic casein digestion, Oxoid) and 8.5 g/L of sodium chloride (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain a final concentration of approximately 9 to 9.5 log CFU/ml. The exact concentration was assessed by determining the optical density at 405 nm using a spectrophotometer (Cecil 9000 series, Cecil Instruments, Cambridge, UK). Later, 10 ml of the cell suspension, depending of the microorganisms of interest, were inoculated into one liter of the food matrix to be tested: pasteurised liquid whole egg, sterile milk (0.3 %, 3.6 %, 10% and 15% of fat content) or fruit juices, to reach a final concentration of 7-7.5 log CFU/ml approximately. Only for liquid whole egg dilutions of the cell suspension were made to obtain a final concentration of approximately 3 log CFU/ml.

3.2.3 UHPH treatments

A benchtop ultra-high pressure homogeniser model/DRG FPG12500 from Stansted Fluid Power Ltd. (Essex, UK) was used for these assays. This high pressure equipment comprises two intensifiers, driven by a hydraulic pump and a high pressure valve made of resistant ceramics able to support 400 MPa. All these components guarantee a constant flow rate of 7.0 L/h during the process. To avoid poor homogenization performance due to temperature increase and rapid expansions or contractions in the processing valve, this was cooled by a constant circulation of water at room temperature in an external jacket built around it. For each experiment, one liter of inoculated sample

was subjected to a single cycle. The pressures applied were: 150, 200 and 250 MPa for liquid whole egg and 200, 300 and 400 MPa for milk and fruit juice samples. Between 80 and 100 ml of the processed samples were taken for analysis. The collected samples were immediately stored at 4 ºC. A specific sanitation program developed for the UHPH machine was applied each UHPH treatment using a 70 % (v/v) ethanol solution.

3.2.4 Microbiological analysis

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

The first microbiological analysis of treated and control samples was performed 2 h after the UHPH treatment, and the rest after 5, 10, 15 and 20 days of storage at 6 ºC for liquid whole egg samples, and after 5, 8 and 15 for milk and fruit juice samples. To assess the lethality and the level of injuries caused by UHPH treatment, decimal dilutions in peptone water (Oxoid) of untreated and treated samples were prepared and plated by duplicate in TSAYE and TSAYE supplemented with 50 g/L of NaCl (Panreac; TSAYE+NaCl) and incubated at 37 ºC for 48 h. The use of this differential plating technique enables injuries to be monitored. Both non-injured and injured cells were able to form colonies on TSAYE whereas only non-injured cells formed colonies in the presence of NaCl (Patterson, et al. 1995). Results were expressed as the logarithm of CFU per ml. Lethality was calculated as the difference between the logarithms of colony counts of the untreated and treated samples (log No- log N).

To confirm total inactivation of *Salmonella* in the liquid whole egg samples with counts under the detection limit, 25 ml of each sample were added into 225 ml of buffered peptone water (Oxoid) and incubated at 37 ºC for 18-24 h. Subsequently, 1 ml of this solution were added into 10 ml of Rappaport-Vassiliadis broth (Biomérieux, Marcy l'Etoile, France) and incubated 6-8 hours at 42ºC, and 0.1 ml were added into 10 ml of Muller-Kauffmann tetrationate (Biomérieux) and incubated at 37ºC for 6-8 hrs. After incubation, 1 ml of each medium was mixed with 10 ml of M-broth (Biomérieux) and

incubated 18-24 hrs at 42ºC. Then 1 ml of the M-broth was heated at 100ºC during 15 min. and after 0.5 ml were allocated in the VIDAS® SLM system (Biomérieux) to confirm presence of viable *Salmonella* (McMahon, et al. 2004).

3.3 Experiment performed with organic acids to evaluate the acid tolerance response (ATR) of *Salmonella Senftenberg* 775W and *Listeria monocytogenes* in fruit juices (paper IV)

3.3.1 Bacterial strains

L. monocytogenes (CECT 4031) and *S. enterica* serovar Senftenberg 775W (CECT 4565) were used in this part since they were studied in paper III, see 3.2.1 for specifications.

3.3.2 Acid adaptation of strains in TSB at different pHs

Stationary phase. TSB was inoculated with a colony of the strain to be tested. After incubation at 37ºC for 24 hr, 100 µl were inoculated in 20 ml of TSB (Oxoid) previously adjusted at pH 7 and then incubated at 37ºC for 24 hr. This bacterial suspension was in stationary phase.

Exponential phase. For obtaining the exponential phase 1 ml of the bacterial suspension in stationary phase was inoculated in 100 ml of TSB (pH 7). Each 10 minutes until complete 8 hours the optical density at 600 mn were measured in Automated Bioscreen C MBR (Oy Growth Curves Ab Ltd, Helsinki, Finlandia), concluding that the exponential phase required 3.5 hours.

Both suspensions were then used to inoculate tubes of TSB at pH 4, 4.5 and 5. Citric acid (30%) or malic acid (Panreac) were added to obtain the different pHs, having a final concentration of citric acid of 0.6, 0.4 and 0.2%, respectively; or 0.4, 0.3 and 0.18% as a final concentration of malic acid. Counts after 0, 2, 3 and 5 hours of incubation at 37ºC were determined in TSA. TSA plates then were incubated at 37ºC for 24 hours. The counts were expressed as log CFU/ml.

3.3.3 Induction of ATR

To evaluate the induction of the ATR both growing phases were obtained as it was described previously. Duplicated samples of each growing phase were centrifuged (15 minutes at 1500g at 4ºC), the resultant pellets re-suspended in 25 ml of TSB with pH adjusted to 5 (pH of acid-adaptation) or to 7 (control) and incubated for 3 hours at 37ºC.

To determine the ATR, acid-adapted and control cells were harvested by centrifugation and re-suspended in 25 ml of TSB adjusted at pH 2.5 and/or 3 (pHs of induction) and incubated at 37ºC. As acid-adaptation the acidification for induction was carried out adding the acid of interest.

To assess the lethality and the level of injuries caused by the acid conditions, counts were determined at 0, 20, 40, 60 and 90 minutes. Decimal dilutions of samples in peptone water were prepared and spiral plated (Eddy Jet, IUL Instruments S.A., Barcelona, Spain) for duplicate in TSAYE and TSAYE+NaCl (NaCl 5%) and incubated at 37 ºC for 24-48 h.

3.3.4 Acid tolerance response in commercial juices

Following the same procedure described in the previous step the acid-adapted and control cells in their exponential or stationary phase were inoculated into orange and grape juices. The final concentration of juices was about 7 log CFU/ml. The pHs of the juices were 3.76 and 3.11 for orange juice and grape juice respectively. To evaluate the ability of acid-adapted strains to survive and/or to grow in juices, viable and injured bacterial counts were obtained from samples after inoculation (initial load) and after 5, 8, 10 and 15 days of storage at 4 ºC and 25ºC.

3.4 Description of the procedures done to evaluate the effect of the UHPH on the quality, physical, microbiological and sensory properties orange juice (paper V and VI)

3.4.1 Orange juice processing

Oranges (*Citrus sinensis* L. var. Valencia) were washed and pressed to obtain juice. To remove the thickest part of orange pulp the juice was filtered using a 2 mm steel sieve. A pilot scale pasteurizer with a tubular heat exchanger (ATI, Granollers, Barcelona, Spain) was used for conventional heat pasteurization (90 °C for 1 and 2 min). UHPH treatments were applied using a Stansted ultra-high-pressure homogenizer (FPG 11300:400 Hygienic Homogenizer Unit, Stansted Fluid Power Ltd., Essex, UK), with a flow rate of 120 L/ h. Juice was pressurized at 100, 200 and 300 MPa. Previously, orange juice was tempered at the required inlet temperatures (10 or 20ºC). Processed juice was cold passing it through a heat exchanger feed with cold water. The inlet temperatures (Ti), the temperature before the homogenization valve (T1), the temperature after the homogenization valve (T2) as well as the final temperature (TF) of

the orange juice after passing through the heat exchanger were monitored throughout the experiment. An increase of temperature during UHPH treatments was observed as a consequence of the adiabatic heating generated in the machine in addition to the high turbulence, shear, and cavitation forces that the fluid suffers in the homogenization valve (Table 4).

Table 4. Temperature and pressure changes of orange juice during UHPH processing. Data are presented as the mean value of three replications ± standard deviation.

Ti (ºC)	Homogenization pressure (Mpa)	T1 (ºC)	T2 (ºC)	TF (ºC)
9.0 ± 1.4	112.1 ± 11.7	20.0 ± 6.4	41.8 ± 2.4	20.0 ± 2.7
9.3 ± 1.5	218.2 ± 4.6	23.4 ± 5.6	67.7 ± 0.7	24.0 ± 2.2
9.7 ± 1.3	319.8 ± 11.5	27.5 ± 4.2	91.9 ± 2.0	23.0 ± 2.4
18.9 ± 1.4	108.7 ± 13.1	31.3 ± 3.9	48.3 ± 2.3	20.0 ± 2.9
19.0 ± 1.9	209.8 ± 5.4	34.3 ± 4.0	72.1 ± 1.5	23.8 ± 1.6
19.0 ± 2.5	310.4 ± 7.3	37.8 ± 4.1	95.3 ± 2.8	22.3 ± 2.9

Ti = inlet temperature; T1 = temperature before the first homogenization valve; T2 = temperature before the second homogenization valve. All values are means (± standard deviations) of data from 3 independent experiments.

3.4.2. PME Activity Measurement

Residual PME activity was determined using the method described by Rouse and Atkins (1955) using an automatic titrator (Titrando model 842, Metrohm AG, Herisau, Switzerland). PME activity was evaluated by titration of free carboxyl groups at pH 7.5. Briefly, 5 ml of orange sample was added to 50 mL of 1% citrus pectin (Sigma-Aldrich, St. Louis, USA) solution containing 0.3 M of NaCl (Panreac). The pectin-juice mixture was adjusted to pH 7.5 with 0.02 N NaOH (Panreac). Then when a stable pH was reached, the consumption of NaOH during a 30 min reaction time was recorded. One unit of PME activity (UPE) was defined as the liberation of 1 μmol of carboxyl groups/minute at pH 7.5. Enzyme activity was calculated according to the following formula:

$$UPE/ml = \frac{(ml.NaOH)(NaOH.normality)(1000)}{(ml.orangejuice)(\min)}$$

PME activities may vary distinctly among juice production lots (Basak and Ramaswamy. 1996, Collet, et al. 2005), for that values were converted to % residual

activity with respect to the unprocessed orange juice sample which represents a PME activity of 100%.

3.4.3 pH and Titratable acidity (TA)

pH and TA determination were measured using an automatic titrator (model Titrando 842, Metrohm, Herisau, Switzerland). The fruit juice was titrated with 0.1M NaOH (Panreac) to endpoint of pH 8.1. Results were expressed as grams of citric acid per 100 ml orange juice (IFJJP. 1996).

3.4.4 Total Soluble Solids (TSS)

TSS was measured by using a Spectronic Instruments refractometer (Rochester, NY, USA) at 20 ºC. Results were reported as ºBrix.

3.4.5 Determination of total and reducing sugars

Total and reducing sugars were determined using the Luff-Schoorl method. Results were calculated as grams of glucose in 100 ml of sample (IFJJP. 1985).

3.4.6 Non-enzymatic browning index

Five ml of ethyl alcohol (95%, Panreac) was added to 5 ml of orange juice sample, and then this mix was centrifuged at 7800 g for 10 minutes. The absorbance of the supernatant was read at 420 nm (Cecil Instruments, Cambridge, UK). The value obtained was considered as the non-enzymatic browning index NEBI described by (Meydav, et al. 1977)

3.4.7 Cloudiness determination and particle size analysis

Orange juice samples were centrifuged at 1500 rpm for 10 min at room temperature. Cloudiness was measured as supernatant absorbance at 660 nm (Cecil Instruments, Cambridge, UK) at 660 nm (Krop. 1974).

The particle size distribution in orange juice samples was determined using a Mastersizer Micropulus 2.15 (Malvern Instruments, Inc., Worcs, UK). Orange juice samples were diluted in distilled water to reach appropriated laser obscuration (12%). An optical model based on the Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index = 1.520; refractive index of fluid (water) = 1.330. The size distribution was characterized by the diameter below which 50 or 90% of the volume of particles are found (d0.5 and d0.9, respectively), the

Sauter diameter (surface-weighted mean diameter, $D_{3,2}$), and the volume-weighted mean diameter ($D_{4,3}$) value.

3.4.8 Color measurements

Color values of orange juice samples were determined using a Hunter Lab colorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, VA). Color coordinates were measured with an illuminant of D65 and a standard observer of 10° and the colorimeter was calibrated against white and black tile standards. Fifty milliliters of each orange juice sample was warmed to 20°C before analysis. The Commission Internationale de l'Eclairage (CIE) L*, a*, and b* values were measured in triplicate. The L* value represents the lightness with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser; the a* and b* axes have no specific numerical limits and represent chromatic components. Positive values of a* are red and negative values are green, whereas positive values of b* are yellow and negative ones are blue. The total color difference was calculated by applying the formula:

$$\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$$

3.4.9 Microbiological analysis

To establish the shelf-life of orange juice were performed two assays:

Assay 1 (paper V): Microbiological analyses of samples were performed 2 h after UHPH and pasteurization treatments and every week for a total of 7 weeks of storage at 4ºC.

Assay 2 (paper VI): Either pasteurized or processed by UHPH (20ºC/300 MPa) orange juices were stored at 6 and 20ºC. Microbiological analyses of samples were performed 2 h after treatments and after 5, 10, 30, 45, 60, 75 and 90 days of storage.

For both assays, samples were diluted appropriately in sterile peptone solution (Oxoid) and plated on total plate count agar (PCA, Oxoid) and incubated at 30ºC for 24 h and 10ºC for 5 days for mesophilic and psychrotrophic bacteria counts, respectively; on rose bengal (RB, Oxoid) incubated at 25ºC for 5 days for molds and yeasts count; on Man Rogosa Sharpe (M.R.S., Oxoid) incubated at 30ºC for 5 days for lactic-acid bacteria; on violet red bile glucose (VRBG, Oxoid) for enterobacteria and on Coli ID for coliforms and *E.coli* (Biomérieux), both incubated at 37ºC for 24 h. Results were expressed as the logarithm of colony forming units per ml of orange juice (log CFU/ml).

3.4.10 Sensory evaluations

For evaluating consumer acceptability 25 assessors without any training were randomly selected from volunteer personnel working at the Veterinary Medicine School of the *Universitat Autònoma de Barcelona*. The panelists compared pilot-scale pasteurized and UHPH processed juices in terms of product-specific color, acidity, sweetness, bitterness, strange flavors and overall acceptability on an 11 point hedonic scale (0 was dislike extremely and 10 was like extremely). Prior to sensory evaluation orange juices were tempered at 15ºC, randomly coded and served (50 ml). Finally, panelists were asked about their preferences among the samples of orange juice pasteurized and UHPH treated used in this study and a commercial orange juice pasteurized.

3.5 Description of the procedures done to evaluate the effect of the UHPH on the bioactive compounds and antioxidant activity orange juice (paper VII)

3.5.1 Determination of L-Ascorbic acid

L-Ascorbic acid [L-AA] was determined by HPLC with the method described by (Sanchez-Moreno, et al. 2003) using a Summit x2 dual gradient HPLC system (Diodex, Idstein, Germany). Separation of ascorbic acid was performed using a reversed-phase column Waters Spherisorb 5 μm ODS2 (4.6x150 mm) (Waters, Massachusetts, USA). The solvent system used was an isocratic gradient of a 0.01% H_2SO_4 (Fluka, Chemie Buchs, Switzerland). The flow rate was fixed at 1.0 ml/min. Detection was performed at 245 nm, using a L-AA (Sigma-Aldrich) calibration curve in the range of 0.5-5 mg/100ml.

3.5.2 Determination of carotenoids and vitamin A

To determine the carotenoid content in orange juice the method described by (Stracke, et al. 2009b) was used. For extracting carotenoids from orange juice samples, 1 g of juice was mixed with 2 ml of a methanolic 5% KOH solution containing 0.01% butylated hydroxytoluene (BHT) (Merck, Darmstadt, Germany). After 1 hour of incubation the methanol was evaporated and then 2 ml of a saturated NaCl solution was added. Samples were homogenised in 50μl 100mM-EDTA (Merck) and 5ml acetone (Merck) containing 0.01% BHT using an Ultra Turrax T25 (IKA, Staufen, Germany). The homogenate was centrifuged at 3000 g for 5 min at 4ºC and the organic extracts were collected in a separate tube. Extraction was repeated until the organic extracts were colourless. After washing the combined organic extracts with 10 ml of a saturated NaCl solution the organic solvent was removed. The remaining aqueous phase was

extracted three times with 2 ml n-hexane (Merck) containing 0.01% BHT and the combined organic extracts were evaporated to dryness under a stream of N_2 gas. Prior HPLC analysis, the residue was dissolved in 2 ml of acetone and 50 µl were used for HPLC analysis. HPLC analysis was performed on a low-pressure gradient system from Shimadzu (Duisburg, Germany) equipped with an autoinjector, column oven and photodiode array detector. The autoinjector was set to 10ºC and the column oven to 27ºC. Separation was carried out on a 250 x 4.6 mm internal diameter, 5 µm, YMC 'Carotenoid' S5 reversed-phase C30 column with a corresponding 10 x 4.0 mm internal diameter guard column (YMC Europe GmbH, Dinslaken, Germany). Solvent A consisted of *tert*-butyl methyl ether (Merck), solvent B of methanol (Merck) and solvent C of water. A linear gradient was used starting with 20% A, 60% B and 20% C going to 80% A, 20% B and 0% C within 50 min. The flow rate was 1 ml/min and the detection wavelength was set to λ = 450 nm. Quantification was performed by external calibration using reference compounds. Calibration curves of different carotenoids were performed in the range of 0.025–25 mmol/L, in which the linearity of the response was given. The recovery for all carotenoids was greater than 95%. The CV of the method was below 5% (intra-assay). The vitamin A value has been expressed as retinol equivalents (RE) (Trumbo, et al. 2003). To calculate RE on the basis of carotenoids, the following conversion has been employed:

RE = µg of β-carotene/12 + µg of other provitamin A carotenoid (β-cryptoxanthin + β-carotene)/24.

3.5.3 HPLC Analysis of flavonoid compounds

The HPLC analysis was determined following the procedure described by (Stracke, et al. 2009a). Samples of orange juice were centrifuged at 4700 g for 15 min. Then 90 µl of the supernatant was injected. HPLC analysis was performed with a high-pressure gradient system from LaChrom (Duisburg, Germany) equipped with an autoinjector, a photodiode array detector, and a fluorescence detector. Separation was carried out with a ProntoSIL (150 mm x 4.0 mm internal diameter, particle size = 3 µm) reversed-phase column (Bischoff, Leonberg, Germany). Solvent A consisted of 0.1% formic acid (Merck) in water (pH 3) and solvent B of acetonitrile (Merck). A linear gradient was used: from 15 to 30% B in 50 min, from 30 to 50% B in 10 min, and from 50 to 56.5% B in 20 min. The flow rate was set to 0.8 ml/min, and the injection volume was 90 µL.

The eluent was recorded with diode array detection at 280 nm. Quantification was performed by external calibration using commercially available reference compounds. Calibration curves for the different polyphenols were in the range of 0.05-100 μM in which the linearity of the response was given.

3.5.4 Determination of total polyphenol content

Total polyphenols were determined by the Folin–Ciocalteu method (Singleton and Rossi Jr. 1965), on a spectrophotometer (Cecil Instruments, Cambridge, UK) at 750 nm and using gallic acid (Sigma-Aldrich) as standard. Calibration curve was in the range of 1-20 mg/100 mL. Results were expressed as mg of gallic acid equivalent (GAE) per 100 mL of juice.

3.5.5 Measurement of antioxidant capacity

The antioxidant capacity of the orange juices was evaluated by two spectrophotometric methods: 1) Trolox equivalent antioxidant capacity (TEAC), method described by (Van den Berg, et al. 2000) using a spectrophotometer (Cecil Instruments, Cambridge, UK) at 734 nm and 2) Ferric reducing antioxidative power assay (FRAP), a modification of the method of Benzie and Strain. (1996), using a microplate. Briefly, 5 μl of the sample, 15 μL of water and 150 μl of the FRAP reagent were mixed in a microplate. After 30 min at 37ºC, Absorbance was measured at 595 nm (ATTC 340, SLT Labinstruments, Salzburg, Austria). To prepare the FRAP reagent, a mixture of 2.5 ml of a TPTZ [2,4,6-tri(2-pyridyl)-s-triazine] solution (10 mmol/L, Sigma-Aldrich) in hydrochloric acid (40 mmol/L, Sigma-Aldrich), 2.5 ml of a FeCl3 solution (20 mmol/L, Panreac) and 25 ml of a acetate buffer (0.3 mol/L, pH 3.6) was made. Trolox [(±)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid] (Sigma-Aldrich) was used as standard compound in both methods. Calibration curve was in the range of 100-1000 μM.

3.6 Statistical Analysis

Statistical analyses were performed with either Statistical Package for the Social Sciences 14.0.1 software (SPSS Inc, Chicago, ILL) or SAS® System (Statical Analysis Systems Version 9.2, SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$. In papers is described the model applied to the results.

4. RESULTS AND DISCUSSION

4.1 Effect of UHPH on pathogenic microorganisms

In this section, a brief discussion about the main effects of UHPH on pathogenic microorganisms is presented. The UHPH effectiveness on them depends on different factors such as the kind of microorganism, its initial load, the composition and the viscosity of the matrix, among others.

Results obtained confirm that the level of inactivation of pathogenic microorganisms significantly increases with the pressure level (Table 5). This behaviour is in agreement with the results reported in previous studies (Vachon, et al. 2002, Moroni, et al. 2002, Wuytack, et al. 2002, Thiebaud, et al. 2003, Tahiri, et al. 2006, Lujan Capra, et al. 2009).

Table 5. Effect of UHPH on specific microorganisms inoculated into different food matrices

Food matrix	Microorganism	Pressure (MPa)	Effect of UHPH	Reference
Liquid whole egg	*Salmonella enterica* serovar Senftenberg 775W	150, 200 and 250	At 7 log CFU/ml initial load 250 MPa treatment was as effective (3.2 log CFU/ml) as thermal pasteurization. At 3 log CFU/ml initial load apparently total inactivation was achieved after 250 MPa treatment.	Paper I
Milk (0.3, 3.6, 10 and 15% of fat milk)	*Listeria monocytogenes*	200, 300 and 400	The higher the fat content was, the higher the lethality achieved at same treatment conditions.	Paper II
Orange and grape juice	*Salmonella enteica* serovar Senftenberg 775W and *Listeria monocytogenes*	200, 300 and 400	Pressure level had a significant impact on the lethal effect of UHPH. More resistance of *L. monocytogenes* than *S. enterica* serovar Senftenberg 775 W to the UHPH treatments in both juices.	Paper III

UHPH is a technology which produces mainly physical and structural damages on the cell. Some authors have described the complete rupture of the cell and the consequent loss of the cytoplasmic content due to UHPH treatments on transmission electron

micrographs (Vachon, et al. 2002, Tahiri, et al. 2006). In accordance with previous studies (Vachon, et al. 2002, Wuytack, et al. 2002, Briñez, et al. 2006a, b and 2007), it was observed that UHPH treatments caused no significant sublethal injuries (Paper I, II and III). This is the main difference between UHPH and high hydrostatic pressure, which can cause accumulation of sublethal injuries and lead to subsequent recovery of the cells, depending on the conditions of treatment and subsequent storage (Wuytack, et al. 2002, De Lamo-Castellví, et al. 2005). In fact, Wuytack, et al. (2003) have proposed a model which describes cellular inactivation and sublethal injury as a result of the inactivation of one or two targets in the cell. If only one target is presented in the cell in a single copy and this target is vital for the cell and it is inactivated by the treatment, then every first "hit" in a cell will cause death, and no state of sublethal injury will exist. These authors suggest the peptidoglycan chain as the sole vital target. Experimental evidence indicates that Gram-negative bacteria are more sensitive to UHPH than Gram-positive bacteria, supporting that UHPH kills vegetative bacteria mainly through mechanical disruption, because Gram-positive bacteria have a thicker peptidoglycan layer and henceforth have greater mechanical strength than their Gram-negative counterparts (Vachon, et al. 2002, Wuytack, et al. 2002). The higher resistance of *L. monocytogenes* at 400 MPa observed in fruit juices (Paper III) can be explained by the cell wall composition.

On the other hand, there is controversy whether cell load has influence on cell disruption efficiency, over a wide range of cell loads and operating pressures. The Institute of Food Technologists (Heldman and Newsome. 2003) has recommended using lower loads (2 or 3 log CFU/ml) of bacteria to test the efficacy of the treatments. Taking this into consideration (Paper I and II), a study with both high and low cell loads were evaluated. In liquid whole egg, no significant differences were observed in the lethality values obtained at lowest *Salmonella* Senftenberg 775W load with respect to those obtained with the highest. In milk samples, a more realistic load approximately 3.0 log CFU/ml of *Listeria monocytogenes* was inoculated to confirm the real efficiency of the treatment in samples with 0.3 and 3.6% fat. Surprisingly, although in the 300 and 400 MPa treatments the lethality was closer to the detection limit of the method, a complete inactivation was not achieved as expected. Vachon, et al. (2002) found contradictory results about the effect of the initial load. In phosphate buffered saline solution (PBS) the highest reduction was obtained when the bacterial load did not

exceed 10^5 CFU/ml. However, in milk samples the initial bacterial load had no impact on the effectiveness of high-pressure homogenization. In addition, Diels, et al. (2005) observed that initial cell load seems to have no influence on *E. coli* inactivation in PBS suspensions. Nevertheless, Tahiri, et al. (2006) reported that in PBS the highest degree of inactivation was obtained with the lowest initial bacterial concentration (10^4 CFU/ml).

Food matrix is another factor to consider in the UHPH processing. Microbial resistance is usually higher in foods than in buffers, but the mechanisms by which foods protect bacterial cells are not clear in most cases. In this work we used three different kinds of food to evaluate this.

Liquid whole egg (Paper I) was selected due to the fact that its heat pasteurization is very limited to low temperature and long holding times to avoid coagulation. We have chosen *S. enterica* serovar Senftenberg 775 W as a target because literature data showed that this strain was one of the most heat-resistant serotypes of *Salmonella* that has been isolated from foods (Mañas, et al. 2003). UHPH treatment at 250 MPa showed to be at least as effective as conventional thermal pasteurization, achieving a lethality of 3.2 log CFU/ml. In fact, Mañas, et al. (2003) observed reductions of less than 2 log CFU/ml in counts of this strain when conventional industrial pasteurization treatments were applied to liquid whole egg (60ºC for 3.5 min and 64ºC for 2.5 min) and less than 4 log CFU/ml when an ultra-pasteurization treatment was applied (70ºC for 1.5 min). Even though, the maximum temperature achieved at 250 MPa was 64.5ºC, which is closer to the temperatures used for egg pasteurization, the egg remained at this temperature less than a second. These results encourage further investigation of UHPH processing of liquid whole egg, in order to assay higher pressures and inlet temperatures. Moreover, we observed a decrease in the bacterial counts during the storage of pressurized samples (Figure 4A). This behavior was also observed in both control and pressurized samples inoculated with the lowest cell load (Figure 4B). This decrement could be explained by the effect of the inhibiting agents naturally present in the egg (e.g., lysozyme) and the low storage temperature which could affect the viability of the cells (Ibrahim, et al. 1996, Kijowski, et al. 2000).

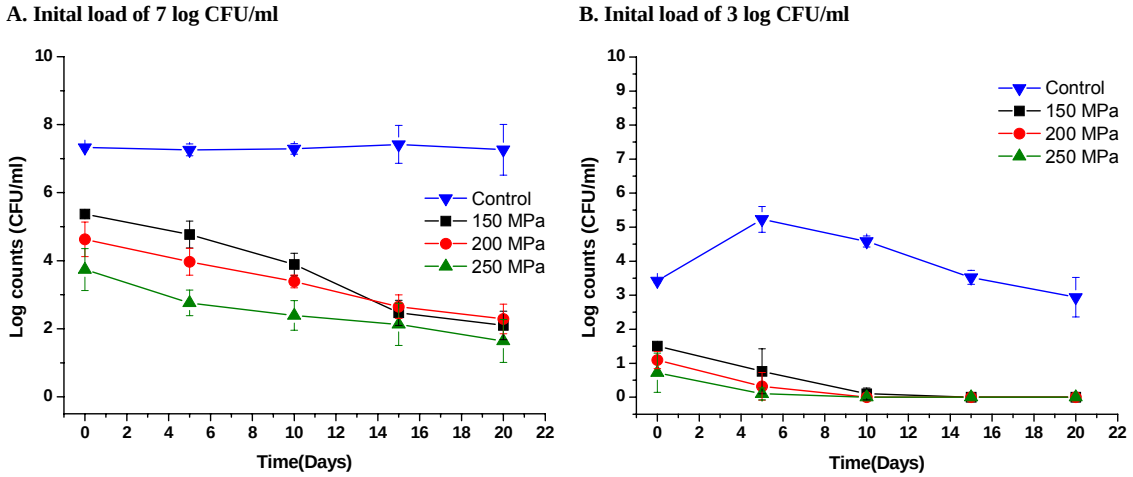

Figure 4. Evolution of viable counts of *Salmonella* Senftenberg 775W in liquid whole egg stored at 4ºC. Counts were obtained in TSAYE medium. Data are presented as the mean value of three replications ± standard deviation.

In order to study the influence of the fat content of milk on the effectiveness of the *L. monocytogenes* inactivation by UHPH (Paper II), milk with different percentages of fat (0.3, 3.5, 10 and 15 %) were used. Overall, it was clear that the higher the fat content was, the higher the lethality achieved for the same treatment conditions. Previously, Briñez, et al. (2006b) reported clear differences in the lethality obtained in *E. coli* between skimmed milk and whole milk in favor of whole milk. A possible explanation is that the level of pressure and the friction arising when the sample passes through the narrow gap of the valves causes an increase in the temperature of the liquid, which would be dependent on the viscosity and the inlet temperature of the sample and on the pressure applied. The increment in the viscosity caused by fat content probably contributed to an increase in friction, in turn increasing the temperature reached. Diels, et al. (2004) suggests that the effect of temperature can be related with viscosity of fluids since viscosity generally decreases with increasing temperature and vice versa. Consequently, temperature effect on microbial inactivation can at least partly be explained by an indirect effect on fluid viscosity. Moreover, Gervilla, et al. (2000) in ovine milk with different percentages of fat (0, 6, and 50%), but treated by high hydrostatic pressure (500 MPa for 2 min), observed as well that the amount of fat appeared to increase the piezosensibility of the microorganisms. The cause might be the increase of the concentration of certain liposoluble substances that have an antimicrobial effect in the fat content, which causes interchanging of triglycerides of milk with lipoproteins of the cellular membrane, altering its permeability. During

storage, it was observed that *L. monocytogenes* was able to recover the initial counts (approximately 7 log CFU/ml) in both skimmed (Figure 5A) and 3.6% fat milk (Figure 5B) but in milk samples with 10% (Figure 5C) and 15% fat (Figure 5D) *L. monocytogenes* did not recover the initial counts, confirming that fat content significantly influences the degree of damage caused to cells during the UHPH treatments and reduces the chance of these cells to recover.

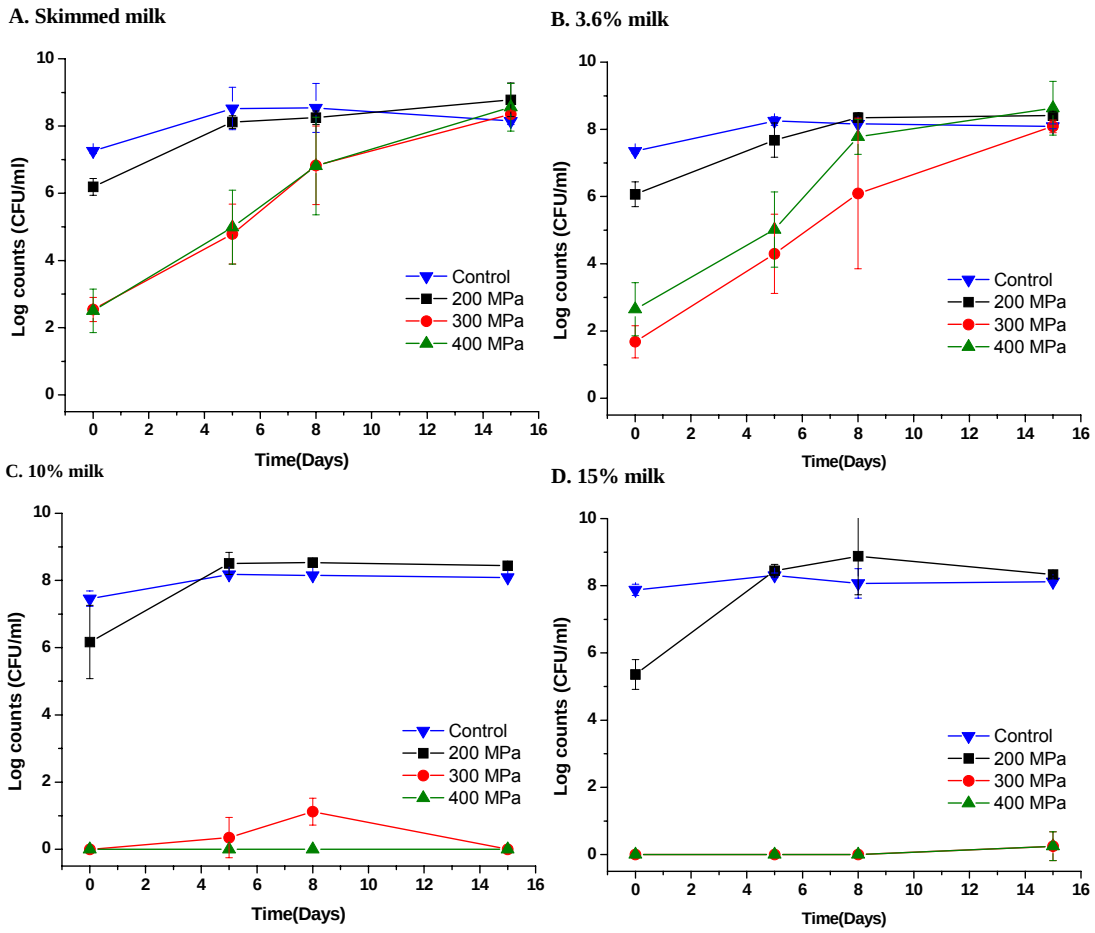

Figure 5. Evolution of viable counts of *L. monocytogenes* in milk with different fat content and stored at 4ºC. Counts were obtained in TSAYE medium. Data are presented as the mean value of three replications ± standard deviation.

Regarding fruit juices, orange and grape juice were used as a model of food with acidic environment (Paper III) for testing the UHPH effect, and *Salmonella enterica* serovar Senftenberg 775W and *Listeria monocytogenes* were used as targets. In the UHPH treated samples it was observed a significant reduction of the surviving cell counts, which increased during the later storage at 4ºC (Figures 6A, 6B, 6C and 6D). The decrement in viable cell counts during storage at 4ºC showed evidence that the

inoculated strains were not able to survive and grow in both food matrixes. This was more emphasized for *L. monocytogenes* when inoculated in grape juice, which counts diminished until undetectable in both, pressurized and control samples (Figure 6C). The reduction in viable cell counts showed evidence of the inhibitory effect of the matrix compounds. Briñez, et al. (2006a) observed different behavior of *L. innocua* inoculated in milk and orange juice along the storage time after the UHPH treatments. In milk counts increased by approximately 2 logarithmic units from the first to the 9th days of storage, whereas in orange juice counts diminished by approximately 2.5 logarithmic units from the first to the 18th day. The authors supposed that the whole milk stimulated the growth of *L. innocua* due to the favorable characteristics of milk (pH close to neutrality and large presence of nutrients) while the low pH of orange juice could have caused interferences in the recovery and grow of bacterial cells. Previous studies done in orange juice have shown that the death rates of *Salmonella* (serovars Gaminara, Hartford, Rubislaw and Typhimurium) are inversely correlated with pH (pH 3.5, 3.8, 4.1 and 4.4) (Parish, et al. 1997). For *L. monocytogenes,* Phan-Thanh, et al. (2000) observed that the bacteria began to die when the pH of the medium descended below 4. However, the great reduction of bacterial counts observed in grape juice samples could be also due to the presence of phenolic compounds. The antilisterial activity of selected phenolic acids has been demonstrated in different studies (Wen, et al. 2003). Rhodes, et al. (2006) also found that grape juice in particular had a great inhibitory effect against *L. monocytogenes* and other *Listeria* species. In fact, Mullen, et al. (2007) evaluating the phenolic content of fruit juices and fruit drinks observed that grape juices had considerable high contents of individual phenolic compounds such as flavan-3-ols, anthocyanins, and hydroxycinnamates.

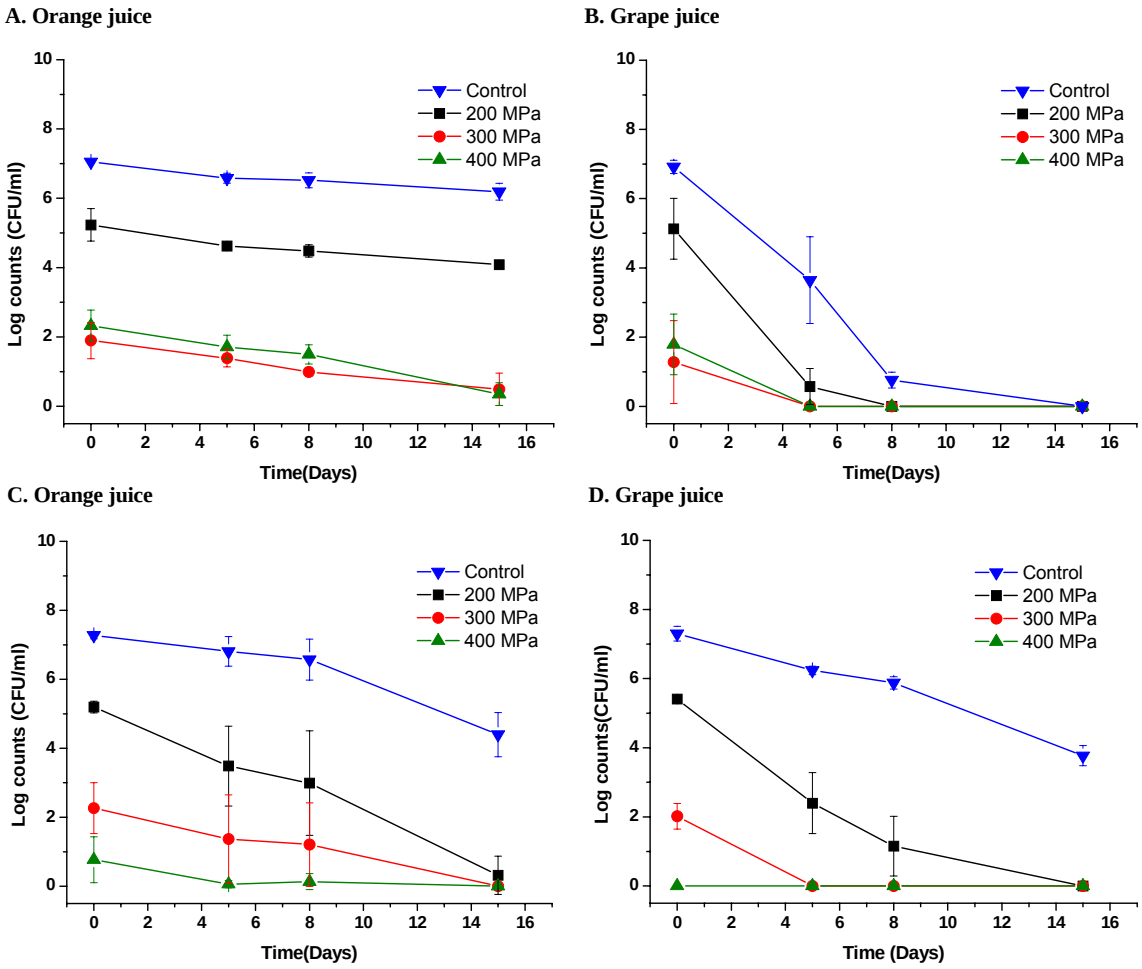

Figure 6. Evolution of viable counts of *L. monocytogenes* (A and B) and of *Salmonella* Senftenberg 775W (C and D) in fruit juices stored at 4ºC. Counts were obtained in TSAYE medium. Data are presented as the mean value of three replications ± standard deviation.

In order to determine the role of the organic acids present in fruit juices as responsible to inhibit *Salmonella enterica* serovar Senftenberg 775W and *Listeria monocytogenes*, the acid tolerance resistance (ATR) of these strains with different organic acids (citric and malic) was induced and evaluated (paper IV). ATR is a phenomenon by which microorganisms show an increased resistance to environmental stresses after being exposed to a moderate acid environment (Hsin-Yi and Chou. 2001). *L. monocytogenes* and *S. enterica* exhibited adaptive ATR after 3 hours of exposure to mild acid (pH 5) and were able to resist more severe acid stresses (pH 3 and 3.5 in citric and malic acid medium, respectively). Overall, cells in stationary phase showed a natural resistance to low pH and solely in exponential cells ATR induction was required. In orange and grape juices (Figure 7), *Listeria monocytogenes* showed to be more sensitive than

Salmonella. Storage temperature at 25ºC resulted very deleterious for every type of cells over time. In fruit juices held at 4ºC, the exponential phase cells of both strains previously acid adapted showed remarkable ATR. These results confirm that ATR may influence the survival of acid adapted pathogens in fruit juices. The results observed in this part are in agreement with the ones observed in paper III, where *Listeria* was more sensitive than *Salmonella* during cold storage. Further, we used cell in stationary phase to evaluate the UHPH effect.

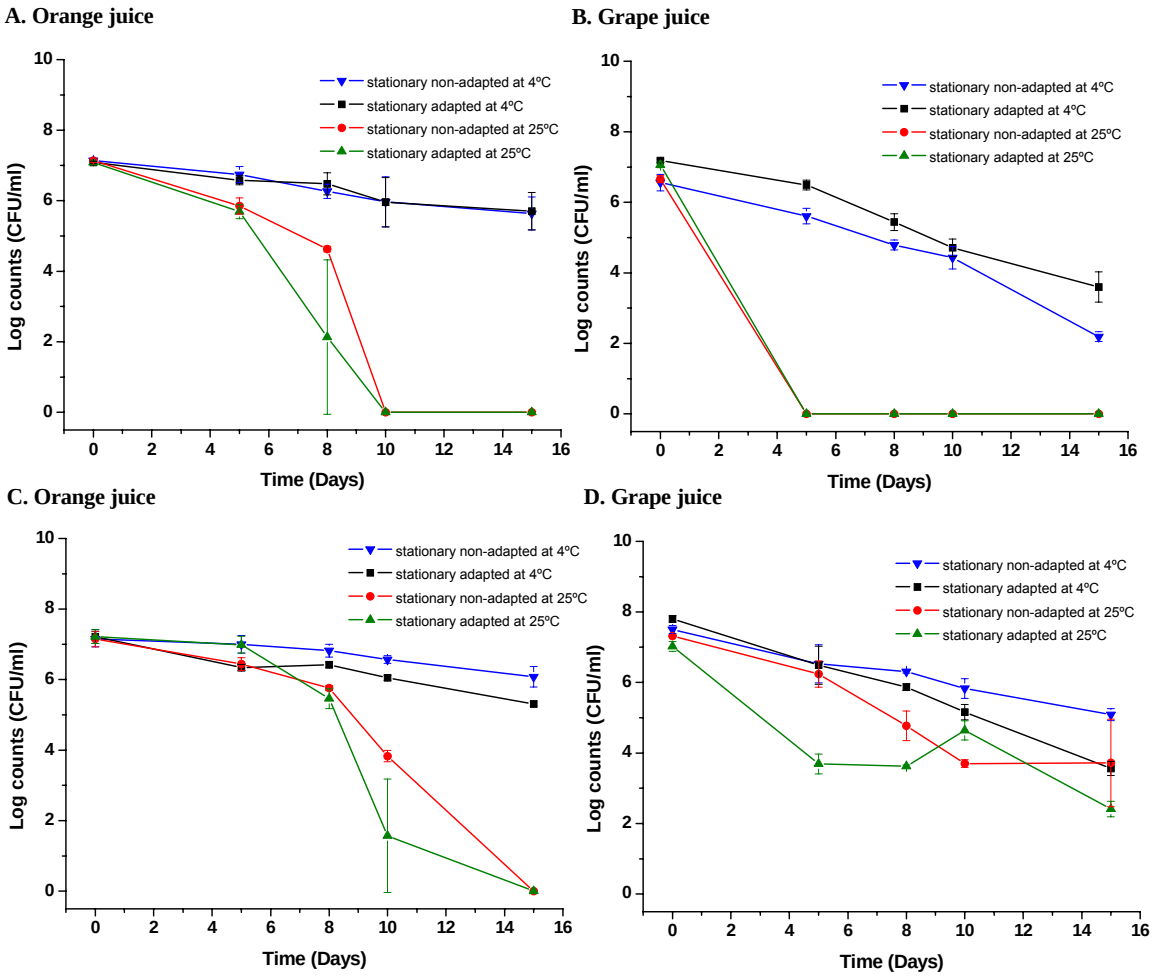

Figure 7. Survival (log CFU/ml) of *Listeria monocytogenes* (A and B) and of *Salmonella* Senftenberg 775W (C and D) in fruit juices stored at 4ºC and 25ºC.

4.2 Pectin methylesterase activity, microbial and physicochemical stabilization of orange juice processed by UHPH

This section shows how the characteristics of orange juice can be affected by the UHPH processing. A common problem associated with freshly orange juice is the loss of cloudiness and concentrate gelation which has been directly related to the activity of

pectin methylesterase enzyme (PME). Thermal pasteurization is very effective for inactivating undesirable enzymes in fruit juices, but this treatment affects intrinsic quality regarding their nutritional and sensory values (Farnworth, et al. 2001). Paper V was focusing on inactivate the orange juice PME applying different UHPH treatments. It was observed that the decrease of PME activity was higher as the pressure applied increased, achieving the maximum reduction in the samples treated at 200 and 300 MPa (Figure 8). Previous studies reported a lower effectiveness of UHPH treatments against PME (Lacroix, et al. 2005, Welti-Chanes, et al. 2009).

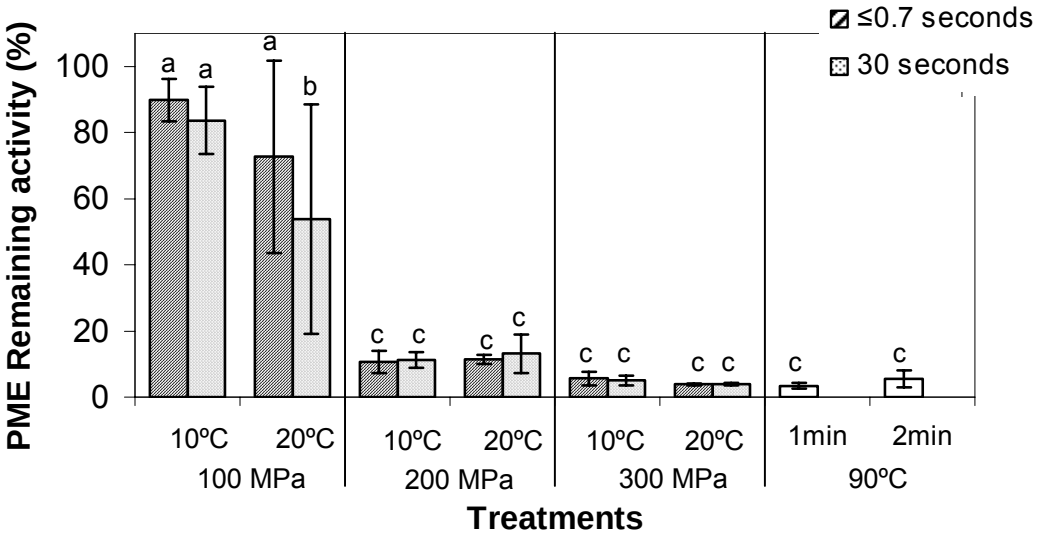

Figure 8. Residual activity of PME in UHPH and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation. Bars with different superscript letter are significantly different ($P < 0.05$).

In this report, UHPH showed to be as efficient as thermal pasteurization inactivating PME. The most probable reason is that the effect of UHPH on the PME is not only dependent on the pressure, but also on the temperature achieved due to heat dissipation of kinetic energy in the high-pressure valve and as a consequence of the exposition to the other physical effects generated when the sample passes through the valve (Lopez-Pedemonte, et al. 2006).

The inactivation of spoilage and pathogenic microorganisms is also a prerequisite to evaluate the orange juice shelf-life extension (Katsaros, et al. 2010). In paper V, the shelf-life of both pressurized and pasteurized juices was studied, evaluating the evolution of the spoilage microorganisms of orange juice during storage at 4ºC for fifty

days. The mean value of mesophilic bacteria counts in raw juice was 4.75 log CFU/ml. After 15-22 days of cold storage, the amount increased to 6 log CFU/ml, as did yeasts and lactic-acid bacteria. In the samples pressurized at 100 MPa the behaviour of these microbial groups was quite similar, but in samples pressurized at 200 and 300 MPa the microbial changes after 50 days of storage were minimal (all counts remained below the 3 log CFU/ml). No significant differences were observed between these samples and the pasteurized ones. The best results were observed in samples processed at 200 MPa when the juice was prewarmed at 20ºC and in samples processed at 300 MPa at any inlet temperature (10 or 20 ºC). With regard to the PME activity during shelf-life, it was not observed any recovery in the PME activity during the 50 days of storage in the samples where the PME was inactivated by the UHPH treatment and the juice showed always an homogeneous cloudy appearance. The juice cloudiness, which is composed of finely divided particles of pectin, cellulose, hemicellulose, proteins and lipids in suspension (Irwe, et al. 1994), is considered a desirable characteristic of orange juice due to the fact that particles impart the characteristic flavor, color and mouthfeel of orange juice (Croak and Corredig. 2006). It is well known that the visual appearance related with cloudiness and color influences considerably the consumers' preference for this kind of juice.

The influence of UHPH on physical attributes (cloud stability and particle size distribution) as well as general quality parameters (color, pH, °Brix, titratable acidity (TA), reducing sugars and non-enzymatic browning index (NEBI) of orange juice was also investigated (Paper VI). The values obtained for pH, total and reducing sugars, TA and °Brix of both UHPH treated and untreated juices (Table 6) were within the recommended values for orange juice described in the literature (Floyd and Rogers. 1969, Esteve, et al. 2005).

The pasteurization treatment (90 °C for 1 min) reduced significantly the ºBrix value from 12.12 of fresh juice to 10.47, probably due to the reduction in the content of reducing sugars (from 7.16 to 5.36) and TA (from 1.35 to 1.04). No significant differences in the pH and NEBI were observed between juices treated by either pasteurization or UHPH and the fresh (untreated) juice.

With respect to the physical attributes, the particle size of the fresh orange juice was significantly higher than the particle size of the pasteurized and UHPH ($P < 0.05$). The smallest particles were observed in the samples treated at 300 MPa (Figure 9). The particle distribution in the pasteurized juices was bimodal and very similar to that found in freshly squeezed juice. As a consequence of UHPH-treatment, the distribution became monomodal and with a noticeable decrease on the particle size. Betoret, et al. (2009) also reported that the mean $D_{4,3}$ values decreased in citrus juices as the homogenization pressure increased, although the highest pressure applied was only 30 MPa.

The visual turbidity is originated from a suspension of pectin particles ranging from 0.4 to 2 μm. PME causes cloud instability in orange juice by de-esterification of pectin as a consequence of a series of events initiated by PME; thus, a thermal process is applied to inactivate this enzyme (Leizerson and Shimoni. 2005). Nevertheless, by homogenization large coarse particles, tending to settle by gravity, are fragmented into smaller particles, (under 2 μm) which are mainly responsible for cloud stability (Betoret, et al. 2009). The particle suspension stability of orange juice depends not only on PME activity, but is also related to the conformation of the enzyme substrate or pectin, to enzyme–substrate interactions and to the size of the particles in suspension (Lacroix, et al. 2005). In this study, the cloudiness of the UHPH treated juices were around 5 times higher than those of control juice. Homogenization converts part of the suspended pulp into colloidal pulp, the size reduction probably contributed to the delay in clarification by decreasing the particle Stokes radius and thereby slowing down sedimentation (Lacroix, et al. 2005, Leizerson and Shimoni. 2005).

Concerning the juice color, UHPH treated orange juice showed brighter color than the fresh and the heat pasteurized orange juices. UHPH slightly increased the lightness probably as result of decreasing the particle size. After UHPH processing, the decrease in the a^* value was greater than the increase in b^* value. These variations indicate that the orange juice treated with UHPH shows a greater tendency towards the yellow color and a lesser tendency towards the red, with respect to the fresh and the pasteurized orange juices.

Table 6. Quality characteristics of fresh, pasteurized and UHPH treated orange juice.

Processing treatment	pH	Soluble solids (ºBrix at 20ºC)	Reducing sugars (g of glucose/100 ml)	Total sugars (g of glucose/100 ml)	Titratable acidity (g of citric acid/100 ml)	NEBI (at 420 nm)
Fresh	3.188 ± 0.054a	12.120 ± 0.198a	7.167 ± 0.406a	13.973 ± 0.751a	1.346 ± 0.065ab	0.148 ± 0.014a
Pasteurized	3.249 ± 0.061a	10.467 ± 0.580b	5.359 ± 0.440b	10.969 ± 0.753b	1.040 ± 0.096d	0.119 ± 0.006a
10ºC/100 MPa	3.160 ± 0.057a	12.738 ± 0.859a	7.443 ± 0.746a	13.005 ± 1.064ab	1.382 ± 0.009a	0.130 ± 0.010a
20ºC/100 MPa	3.174 ± 0.064a	12.256 ± 0.194a	6.818 ± 0.566ab	13.393 ± 0.830ab	1.193 ± 0.060abcd	0.138 ± 0.008a
10ºC/200 MPa	3.167 ± 0.059a	12.040 ± 0.542a	7.182 ± 0.708a	12.703 ± 0.877ab	1.342 ± 0.025ab	0.126 ± 0.007a
20ºC/200 Mpa	3.170 ± 0.063a	12.276 ± 0.224a	7.086 ± 0.107a	13.563 ± 0.958ab	1.154 ± 0.052bcd	0.133 ± 0.009a
10ºC/300 MPa	3.177 ± 0.055a	11.700 ± 0.558ab	6.488 ± 0.523ab	11.318 ± 0.753ab	1.268 ± 0.027abc	0.130 ± 0.012a
20ºC/300 MPa	3.174 ± 0.066a	11.680 ± 0.484ab	6.072 ± 0.483ab	13.436 ± 0.615ab	1.084 ± 0.073cd	0.122 ± 0.008a

Data are presented as the mean value of three replications ± standard error. Columns with different letters are significantly different (P <0.05).

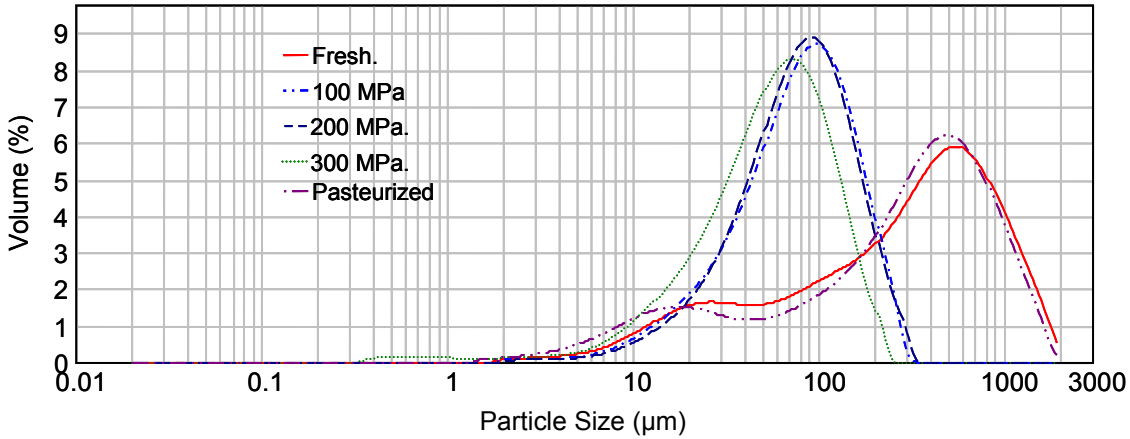

Figure 9. Particle size distribution of fresh, pasteurized and UHPH treated orange juice. Curves do not consider the inlet temperature.

The shelf-life of refrigerated juices, which have been subjected to mild pasteurization, is between 28 and 45 days in refrigeration and partly satisfies the requirements of high quality demanded by consumers (Esteve, et al. 2005). Considering this, an inlet temperature of 20ºC and a pressure level of 300 MPa were selected as UHPH treatment parameters for the orange juice to evaluate the microbial shelf-life and sensory attributes study. This selection was mainly based on the pectin methyl esterase and microbial inactivation achieved (paper V). The mean initial counts of mesophilic, psychrotrophic, lactic-acid bacteria, yeast and enterobacteria counts were 3.04, 2.55, 2.85, 2.16 and 1.88 log CFU/ml, respectively, which is normal for a fresh orange juice (Parish. 1998a). Just after UHPH processing, microbial counts were reduced under the detection limit (1 log CFU/ml). The same was observed in the pasteurized juice used to compare UHPH treatments efficiency. A microbial growth of approximately 2 log CFU/ml for the mesophilic, psychrotrophic and lactic-acid bacteria counts was observed in the pasteurized samples over the 75^{th} day of storage at 20ºC. However, this value is within the recommended acceptable limit for orange juice during storage (Kimball. 2001). However, microbial groups counts of both UHPH treated and pasteurized samples remained below of the detection limit (1 log CFU/ml) during the 90 days of storage at 6ºC.

The results of the consumer acceptability study showed no significant differences ($P \geq 0.05$) between the pasteurized and the UHPH samples in terms of bitterness, acidity, sweetness, strange flavors and overall acceptability. However, in term of color the UHPH samples obtained the lowest score. The lowest acceptability about the color attribute probably could be related to the greatest tendency towards the yellow color observed on the UHPH-treated orange juices.

4.3 Bioactive compounds content and antioxidant activity of orange juice processed by UHPH

Changes in the consumer habits due to the desire to maintain a diet that promotes better health have increased the demand of juices preserving their natural nutritive value. In the particular case of orange juice, pasteurization treatment used to inactivate the most heat resistant PME (90ºC for 1 min) reduces “freshness”, affecting nutritional characteristics (Yeom, et al. 2000). The main objective of the work described on paper

VII was to evaluate the effect of UHPH processing on quality loss of orange juice, in terms of bioactive compounds and antioxidant activity (Table 7).

Regards L-ascorbic acid (L-AA), UHPH treated orange juices retained a significantly higher content than the heat pasteurized samples ($P < 0.05$). Samples treated at 100 and 200 MPa showed ascorbic acid losses of approximately 2% and 5%, respectively, while samples treated at 300 MPa had a significant loss of 11 %. A possible explanation could be the high temperature achieved during the UHPH treatments, being the maximal around 45, 70 and 94ºC for 100, 200 and 300 MPa treatments, respectively. Nevertheless, it is important to highlight that the processing time during which the product remains at the maximum temperature is less than 0.7 s and result in a minimal heat damage in comparison with the at least 1 min of heat pasteurization. Our results differs from those reported by Welti-Chanes, et al. (2009) who observed in orange juice pre-warmed at 22ºC and treated by UHPH from 50 to 250 MPa that the vitamin C content was not affected. Suárez-Jacobo, et al. (2011) also described that the content of ascorbic acid did not change as result of UHPH or thermal treatment applied on apple juice but a significant decrement on the vitamin C (ascorbic acid plus dehydroascorbic acid) content (88% of original value) was found after thermal pasteurization.

Another quality indicator for orange juice is carotenoids, as they contribute both to the color and to the nutritional value of the juice. The main compounds of this group found in orange juice were *β*- and *α*-carotene (43%), while β-cryptoxanthin, zeaxanthin and lutein accounted for about 10, 4 and 2% of total carotenoids, respectively. Total carotenoid content in fresh juices was around 147 μg/100 ml, decreasing after pasteurization to 87 μg/100 ml (35% of loss). Cortes, et al. (2006) reported a lower reduction (12.6%) in the total carotenoid concentration in orange juice pasteurized at 90 °C for 20 s and Gama and de Sylos. (2007) found reductions of 13% after pasteurizing orange juice at 95–105 °C for 10 s. In the same way that L-AA content, UHPH treated orange juices retained a significantly ($P< 0.05$) higher amount of carotenoids than the heat-pasteurized did. This retention depended significantly on the pressure used during UHPH-processing of orange juices ($P< 0.05$) and more specifically of the maximal temperature achieved after UHPH treatment. Total carotenoid content found on samples treated at 100, 200 and 300 MPa were around 120, 136 and 106 μg/100 ml, respectively. Changes in total carotenoids were especially related to

provitamin A compounds (α-carotene and β-carotene), with a slight ($P>0.05$) decrease on the vitamin A concentration. These results are in agreement with those obtained by Suárez-Jacobo, et al. (2011) who observed a decrease of vitamin A content after UHPH-processing of apple juice.

Contrary to results obtained for L-AA and carotenoids, high temperature did not affect the flavonoid content. In our survey, pasteurization did not modify the content of flavonol and flavanones in orange juice as it has been reported in a previous study (Gil-Izquierdo, et al. 2002). However, UHPH treatment increased the flavanone content of orange juice, concretely of hesperidin, achieving the highest content on the samples treated at 200 and 300 MPa. The reason could be that UHPH treatment improved the extractability of flavonoids, decreasing the flavanone content of the cloud fraction and increase the soluble flavanones. Nevertheless, Suárez-Jacobo, et al. (2011) observed in apple juice that the amount of total phenolic compounds was not affected by the UHPH treatments.

About the effect of UHPH on polyphenols, no significant differences were observed between fresh and UHPH orange juice samples on total polyphenol content; however significant differences were detected in comparison with pasteurized orange juice samples, which showed lower polyphenols content.

Two different methods were used to determine the antioxidant activity of the juice, the trolox equivalent antioxidant capacity (TEAC) and the ferric reducing anti-oxidative power assay (FRAP). Each assay is based on a different principle and is therefore necessary to use a combination of methods in order to express the total antioxidant capacity of a sample (Ryan and Prescott. 2010). Moreover, individual antioxidants may, in some cases, act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources (Prior, et al. 2005). The FRAP and TEAC value of fresh juices were 9.7 and 6.0 mM, respectively, and decreased after pasteurization, a fact that can be mainly attributed to ascorbic acid and carotenoids losses. The reaction between L-AA and the ABTS radical cation used on TEAC assay occurs practically instantaneously (Re, et al. 1999). After UHPH treatments, orange juice showed higher levels of TEAC values than did fresh and heat-

treated orange juices. The variation in L-AA retention due to UHPH was not reflected in a TEAC values depletion. Suárez-Jacobo, et al. (2011) also observed that UHPH did not change the antioxidant capacity in apple juice, according to the FRAP and TEAC assays. Among compounds exhibiting antioxidant activity in orange juice, L-AA is the most important, contributing 50–90% of the total antioxidant activity of orange juice (Gardner, et al. 2000, Sánchez-Moreno, et al. 2003). However, in addition to vitamin C, it is necessary to take into account the possible synergistic effect of other phytochemicals as flavonoids and carotenoids, the effect of which will depend on their structure, interaction mode, and concentration (Plaza, et al. 2011).

Table 7. Effect of thermal pasteurization and UHPH treatments on bioactive compounds and antioxidant activity of orange juice.

	Processing treatments				
	Fresh	**100 MPa**	**200 MPa**	**300 MPa**	**Pasteurized**
L-AA (mg/100mL)	54.21 ± 5.38	53.28 ± 6.80	51.74 ± 6.44	48.41 ± 5.08	43.32 ± 5.01
Polyphenols (mg/100mL)	77.10 ± 12.23	76.50 ± 10.82	75.91 ± 9.68	72.00 ± 10.30	62.43 ± 17.20
FRAP (mM)	9.70 ± 1.88	8.59 ± 1.66	8.67 ± 1.79	8.11 ± 1.48	8.71 ± 1.36
TEAC (mM)	6.04 ± 0.92	6.33 ± 0.92	6.35 ± 0.98	6.12 ± 0.91	5.58 ± 0.84
β-carotene (μg/100mL)	53.74 ± 21.95	44.90 ± 23.98	51.77 ± 26.00	37.01 ± 26.35	32.79 ± 13.01
Total carotenoid content (μg/100mL)	147.39 ± 54.03	120.73 ± 49.40	136.29 ± 67.48	106.83 ± 66.52	87.30 ± 33.60
Vitamin A (RE/100mL)	7.15 ± 2.45	5.66 ± 2.49	6.53 ± 2.99	5.00 ± 3.16	4.32 ± 1.53
Hesperidin (mg/100mL)	7.84 ± 2.00	9.81 ± 2.35	17.47 ± 6.62	18.76 ± 7.57	10.10 ± 3.19
Total flavonoid content (mg/100mL)	12.28 ± 2.09	14.02 ± 2.71	20.98 ± 7.41	22.98 ± 8.31	14.08 ± 3.76

Data are presented as the mean value of three replications ± standard deviation.

5. CONCLUSIONS

UHPH technology is effective to eliminate food borne pathogenic microorganisms like *Salmonella enterica* or *Listeria monocytogenes*, but their efficacy depend greatly on the properties of the matrix employed.

UHPH at 250 MPa is as effective as conventional thermal pasteurization to eliminate *Salmonella enterica* from liquid whole egg. Even though surviving *Salmonella enterica* can be detected after the treatments, they cannot multiply during the shelf life of the product.

In milk, the efficacy of the UHPH treatments to eliminate *Listeria monocytogenes* increases with the fat content. The amount of fat also limits the capability of the surviving cells to recover and grow during the subsequent cold storage (4ºC).

In grape and orange juices, UHPH treatments above 300 MPa are also efficient in reducing *Listeria monocytogenes* and *Salmonella enteritidis*. *Listeria monocytogenes* is less able to survive in grape juice than *Salmonella enterica* probably due to a greater acid stress effect and/or to the presence of natural compounds with antilisterial effect described in grape. Nevertheless, when cells are in stationary phase or when the acid tolerance response is previously induced, both *L. monocytogenes* and *Salmonella enterica* are less sensitive to the acid stress caused by pH of the fruit juices.

In orange juice, UHPH treatments above 200 MPa are as effective as the thermal pasteurization to control the activity of both pectin methylesterase and the spoiling bacteria, which are not able to recover significantly during storage. UHPH treated juices also show better particle size distribution and cloudiness values. All these have as a consequence a better stability of the juice during the shelf life period.

UHPH treated orange juices retain a significant higher content of L-ascorbic acid and carotenoids than the pasteurized ones, although the amount of the remaining reduces when the highest pressure are used (300 MPa). Nevertheless, UHPH treatments do not affect the total polyphenols content and increases the content of flavonones with respect

to the fresh squeezed orange juice. All this may be the cause of the higher antioxidant activity observed in the UHPH treated juices when it is expressed as TEAC.

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Paper I

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Research Note

Inactivation of *Salmonella enterica* Serovar Senftenberg 775W in Liquid Whole Egg by Ultrahigh Pressure Homogenization

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ABSTRACT

Two batches of samples of liquid whole egg were inoculated with a load of approximately 3 and 7 log CFU/ml, respectively, of *Salmonella enterica* serovar Senftenberg 775W and submitted to different ultrahigh pressure homogenization (UHPH) treatments at 150, 200, and 250 MPa. The inlet temperature of the samples was 6°C. Counts of viable and injured *Salmonella* cells were obtained 2 h after the UHPH treatments and after 5, 10, 15, and 20 days of storage at 4°C. The level of pressure applied influenced the lethality attained significantly ($P < 0.05$). In the samples with an initial load of approximately 7 log CFU/ml, the highest lethality value of 3.2 log CFU/ml was obtained at 250 MPa, and it is similar to those values reported in other surveys for thermal pasteurization with this same *Salmonella* strain. When the initial load was approximately 3 log CFU/ml, total inactivation was apparently obtained after the 250-MPa treatment (2.7 log CFU/ml). After 10 days of storage at 4°C, *Salmonella* counts decreased in UHPH-treated samples, and colonies were not observed in tryptone soy agar and yeast extract medium. Nevertheless, presence of viable *Salmonella* cells was detected with the VIDAS *Salmonella* immunoassay method during the entire storage period. These results encourage further investigation of UHPH processing of liquid whole egg, assaying the possibility of using higher pressures and fluid inlet temperatures.

Eggs are a multifunctional food because of the ability of their components to coagulate when heated, to act as emulsifiers in oil and water formulations, and to form foams when whipped. Moreover, whole egg is an excellent source of high-quality protein, vitamins, and trace minerals *(18).* Unfortunately, most human cases of salmonellosis are mainly linked to consumption of eggs and egg products. Between 1993 and 2002, 9,364 foodborne outbreaks were reported in Spain, 4,944 (52%) of which were caused by *Salmonella* and 3,546 of which were associated with egg products *(7).* In the United States, foodborne *Salmonella* is estimated to cause approximately 1.3 million illnesses, with 500 deaths each year and with 80% of these infections associated with the consumption of contaminated egg products *(4).*

Pasteurization of liquid whole egg is limited to low temperature and long holding times because of egg protein coagulation. According to data reported in the literature about heat resistance of *Salmonella* in liquid whole egg, recommended heat pasteurization treatments at 60°C for 3.5 min (United States) or at 64°C for 2.5 min (United Kingdom) should provide a 5- to 9-log cycle reduction of the most frequent *Salmonella* serotypes: *S. enterica* serovars Typhimurium and Enteritidis *(14).* However, other serotypes of *Salmonella,* such as *S. enterica* serovar Senftenberg 775W, have been isolated from foods, and traditional pasteurization treatments would not cause a reduction of more than 1 to 4 log cycles *(12).*

Some alternative treatments to inactivate microorganisms have been assayed in egg products. These are based on high hydrostatic pressure, gamma radiation, pulsed electric fields, and ultrasound or their combination *(8, 16).* Another emerging technology that is currently under investigation as an alternative to thermal pasteurization processing is ultrahigh pressure homogenization (UHPH) *(1, 2, 20).* The principle of this technology is similar to conventional homogenization used in the dairy industry but implies the use of considerably higher pressures (100 to 400 MPa). The effects of UHPH on bacterial cells are not completely known, but microorganisms are probably disrupted by the sudden pressure drop, by the torsion and shear stresses, and by cavitation shock waves resulting from imploding gas bubbles *(5, 11, 17).* Although previous studies *(1, 2, 19, 20)* have demonstrated the effectiveness of UHPH in reducing foodborne pathogens in some food matrices, such as milk and orange juice, no data exist about the effect of this technology on other food matrices such as egg products.

The purpose of this study was to evaluate the effectiveness of different UHPH treatments of liquid whole egg against strain 775W of *S. enterica* serovar Senftenberg, described as one of the most resistant to thermal pasteurization *(12).* We assayed two different initial loads (~3 and 7 log CFU/ml) of cells to determine the maximum lethality

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achieved and to asses the real efficiency of the treatment to achieve total destruction of the microorganism, taking into consideration the recommendations of the Institute of Food Technologists *(6).* We also evaluated the possibility to generate sublethal injuries, as well as the ability of the surviving cells to grow during cold storage.

MATERIALS AND METHODS

Culture and sample preparation. The strain 775W of *S. enterica* serovar Senftenberg was obtained freeze-dried from the Spanish Type Culture Collection (University of Valencia, Spain). The strain was rehydrated in tryptone soy broth (Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37°C for 18 h. This broth was used to inoculate tryptone soy agar plates (Oxoid), which were incubated at 37°C for 18 h. Subsequently, cell suspensions were prepared in 11 ml of tryptone sodium chloride solution (1 g/liter tryptone pancreatic casein digestion; Oxoid) and 8.5 g/liter sodium chloride (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain a concentration of approximately 9 to 9.5 log CFU/ml. The concentration was assessed by determining the optical density at 405 nm with a spectrophotometer (Cecil 9000 series, Cecil Instruments, Cambridge, UK). Later, 10 ml of this cell suspension was inoculated into 1 liter of pasteurized liquid whole egg to reach a final concentration of ~7 log CFU/ml. Also, dilutions of the cell suspension were made to obtain a final concentration of ~3 log CFU/ml in pasteurized liquid whole egg. The inoculated samples were placed in a water bath before the UHPH treatments. The liquid whole egg inlet temperature (temperature of the liquid whole egg samples before they are fed into the UHPH machine and pass through the intensifiers) was 6°C.

UHPH treatment of liquid whole egg samples. UHPH treatments were applied to the liquid whole egg samples with a high-pressure bench-top homogenizer (FPG12500, Stansted Fluid Power Ltd., Essex, UK). This high-pressure machine is composed of two intensifiers with a peak pressure of 400 MPa. The flow rate in the machine was 7 liters/h. To avoid excessive temperature increase, an external jacket with constant circulation of ethylene glycol at 2°C was located immediately after the high-pressure valve. For each experiment, 2.0 liters of inoculated liquid whole egg was subjected to a single cycle of UHPH treatment at 150, 200, and 250 MPa. During pressurization, maximum temperatures registered were 46, 55, and 64.5°C, respectively. After the treatments, the homogenized samples were cooled rapidly and stored immediately at 4°C. After each assay, the machine was cleaned and disinfected to avoid cross-contamination: first the machine was cleared with circulating sterile distilled water at 70°C, and then it was cleaned with a 5% (vol/vol) solution of neutral detergent for 20 min. The remaining detergent was withdrawn with further sterile distilled water, and finally, the machine was disinfected by circulating a 70% ethanol solution (Panreac) for 120 min.

Microbiological analysis. To determine the load of cells in the inoculated samples before treatment, decimal dilutions were prepared in peptone water prepared with 10 g/liter peptone (Oxoid) and 5 g/liter NaCl. Subsequently, 1 ml of these dilutions was plated in TSAYE (tryptone soy agar plus 0.6 g/liter yeast extract; Oxoid) and incubated at 37°C for 48 h. Microbiological analysis of treated samples and control samples (inoculated but untreated samples) were performed 2 h after the UHPH treatments and after 5, 10, 15, and 20 days of storage at 4°C. To assess the lethality achieved and the level of injuries caused by the UHPH treatments, decimal dilutions were prepared in peptone water (Oxoid) and plated in duplicate in TSAYE and TSAYE+NaCl (the TSAYE medium supplemented with 50 g/liter NaCl) and then incubated at 37°C for 48 h. The use of this differential plating technique (TSAYE and TSAYE+NaCl) enables sublethal injuries to be monitored. Both noninjured and injured cells were able to form colonies on TSAYE, whereas only noninjured cells could form colonies in the presence of NaCl *(15).* Results were counts of *S. enterica* serovar Senftenberg 775W in whole egg (log CFU per milliliter), and lethality was calculated as the difference between the logarithms of the colony counts of the untreated (control) and the treated samples ($\log N_0 - \log N$).

To confirm total inactivation of *Salmonella* in samples with counts under the detection limit, 25 ml of each sample was added to 225 ml of buffered peptone water (Oxoid) and incubated at 37°C for 18 to 24 h. Subsequently, 1 ml of this solution were added to 10 ml of Rappaport-Vassiliadis broth (bioMérieux, Marcy l'Etoile, France) and incubated 6 to 8 h at 42°C. Other 0.1-ml samples were added to 10 ml of Muller-Kauffmann tetrathionate (bioMérieux) and incubated at 37°C for 6 to 8 h. After incubation, 1 ml of each medium was mixed with 10 ml of M-broth (bioMérieux) and incubated 18 to 24 h at 42°C. Then, 1 ml of the M-broth was mixed and heated at 100°C for 15 min. Finally, 0.5 ml was allocated in the VIDAS SLM system (bioMérieux) to confirm presence of viable *Salmonella (13).*

Statistical analysis. All experiments were repeated three times with duplicate analysis in each replicate. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed with the Statistical Package for the Social Sciences 14.0.1 software (SPSS Inc, Chicago, Ill.).

RESULTS AND DISCUSSION

The mean count of the samples inoculated with the highest load of *S. enterica* serovar Senftenberg 775W was 7.3 log CFU/ml before the UHPH treatments. Lethality values ($\log N_0 - \log N$) increased with the pressure used in the treatment, obtaining mean reductions of 1.7, 2.2, and 3.2 log CFU/ml for 150, 200, and 250 MPa treatments, respectively (see Fig. 1, time 0). No previous references exist about the effect of the UHPH on *Salmonella* in egg products. Wuytack et al. *(22)* reported a reduction of less than 3 log CFU/ml of *S. enterica* serovar Typhimurium after an UHPH treatment at 200 MPa with an inlet temperature of 25°C in PBS, and Vachon et al. *(20)* reported a reduction of more than 4 log CFU/ml on *S. enterica* serovar Enteritidis at the same treatment conditions and more than 8 log CFU/ml at 300 MPa. However, in both cases, it should be taken into consideration than the inlet temperature, the matrix, and the serotype of *Salmonella* used in these works were different from that used by us, which could greatly influence the lethality achieved. Compared with milk, PBS is less protective to microorganisms *(20),* and an inlet temperature of 25°C would probably increase the final temperature of the treatments, increasing the lethality, as was previously described by Briñez et al. *(1).*

Lethality values achieved are far from the 9 log cycles recommended by the FDA for the pasteurization of egg products *(14).* However, this value was proposed considering the thermal resistance of the most frequently isolated *S. enterica* serovars, such as Enteritidis and Typhimurium; however, heat-resistant strains such as *S. enterica* serovar

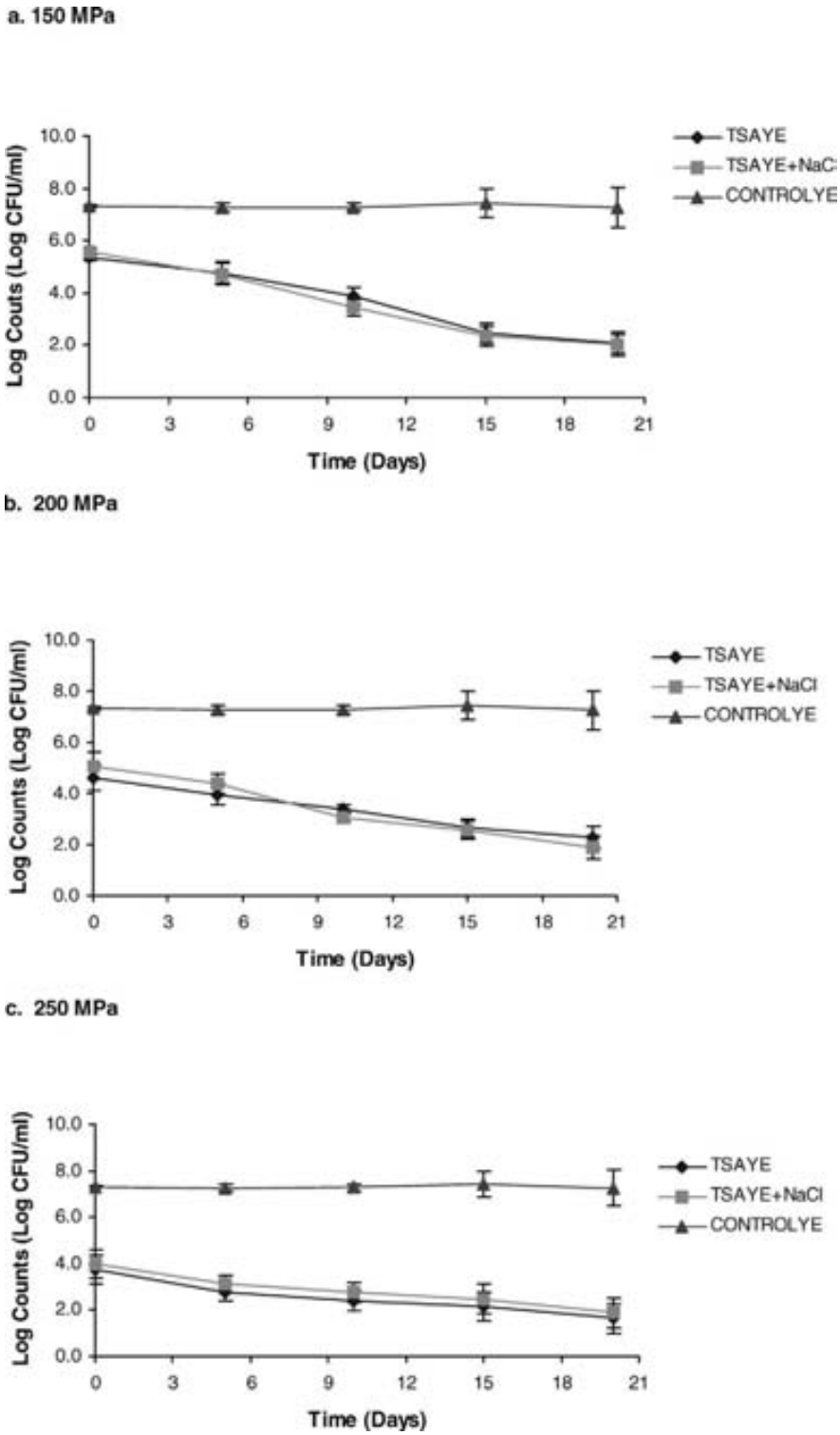

FIGURE 1. *Change over time of viable counts of* Salmonella enterica *serovar Senftenberg 775W in liquid whole egg stored at 4°C with an initial load of ~7 log CFU/ml. Counts obtained in TSAYE and TSAYE+NaCl are presented as the mean ± SD of three replications for samples pressurized at (a) 150, (b) 200, and (c) 250 MPa.*

Senftenberg 775W are much less sensitive to thermal treatments, making it difficult to reach this goal. In fact, Mañas et al. *(12)* observed reductions of less than 2 log CFU/ml when conventional industrial pasteurization treatments were applied to liquid whole egg (60°C for 3.5 min and 64°C for 2.5 min) and less than 4 log CFU/ml when an ultra-pasteurization treatment was applied at 70°C for 1.5 min with the same *Salmonella* strain. Considering this, we can conclude that a 250-MPa UHPH treatment was at least as effective as conventional thermal pasteurization against *S. enterica* serovar Senftenberg 775W. The maximum temperature achieved at this pressure was 64.5°C, which is closer to the temperatures used in pasteurization of egg products, except the egg remained at this temperature for less than 1 s.

Figure 2 shows the inactivation of *S. enterica* serovar Senftenberg 775W inoculated with an initial load of ~3 log CFU/ml. The mean initial load of the samples before the UHPH treatments was 3.4 log CFU/ml. Apparently, total inactivation was only achieved after the 250-MPa treatment

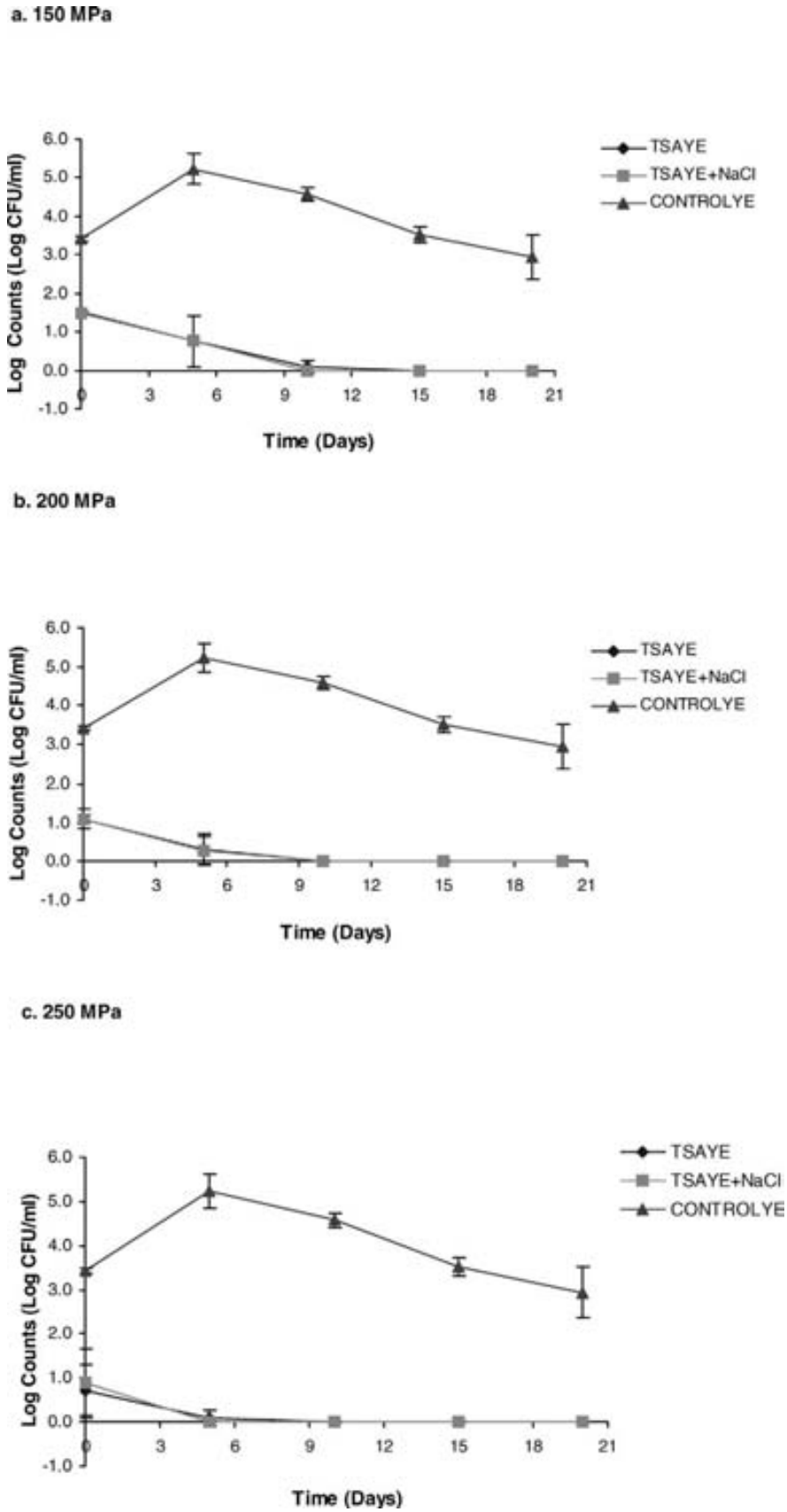

FIGURE 2. *Change over time of viable counts of* Salmonella enterica *serovar Senftenberg 775W in liquid whole egg stored at 4°C with an initial load of ~3 log CFU/ml. Counts obtained in TSAYE and TSAYE+NaCl are presented as the mean ± SD of three replications for samples pressurized at (a) 150, (b) 200, and (c) 250 MPa.*

(2.7 log CFU/ml), whereas reductions at 150 and 200 MPa were only 1.9 and 2.3 log CFU/ml, respectively. No significant differences were observed in the lethality values obtained with respect to those obtained with the highest *Salmonella* Senftenberg 775W load. Similarly, Diels et al. *(3)* did not find that cell concentration was an influential variable on the inactivation of *E. coli.* No significant differences were observed when comparing the counts

of *Salmonella* Senftenberg 775W between TSAYE and TSAYE+NaCl media, concluding that the UHPH treatment caused no significant sublethal injuries as reported previously by Briñez et al. *(1, 2)* in milk and orange juice inoculated with *Listeria innocua* and *E. coli* O157:H7.

Colony counts in samples inoculated with the highest load of *Salmonella* Senftenberg 775W decreased to only 2 log CFU/ml after 20 days of storage under refrigeration (Fig. 1), although in the control samples, *Salmonella* counts remained constant during the entire storage time. When the initial load was only 3 log CFU/ml (Fig. 2), colony counts increased in the control samples during the first 5 days of storage (up to 5.2 log CFU/ml), but the counts decreased thereafter. In pressurized samples, counts decreased approximately 1.5 and 1 log CFU/ml from day 0 to day 10 in samples treated at 150 and 200 MPa, respectively, and were undetectable after 10 days of storage, whereas in samples treated at 250 MPa, bacterial counts were undetectable from day 5. No significant differences were observed either between counts in TSAYE and TSAYE+NaCl at any time during this storage period, indicating that no sublethal injuries were caused by either UHPH treatments or refrigeration conditions. Despite the undetectable levels found after 5 or 10 days of storage in the UHPH-treated samples, the VIDAS test revealed the presence of surviving *Salmonella* cells during the entire storage time in most of the samples (data no shown). The different behavior observed in both control and treated samples might be due to *Salmonella* cell damage caused by the UHPH treatments, but it was not severe enough to be detected by the differential plating medium method used. However, the inhibiting agents naturally present in the egg (e.g., lysozyme) and the low storage temperature could have acted synergistically, affecting the viability of the cell. It has been long believed that the action of lysozyme could only be attributed to its catalytic effect on certain gram-positive bacteria *(10).* Nonetheless, it has also been reported that heat denaturation of lysozyme caused by increasing temperatures results in a promotion of the antimicrobial action toward gram-negative bacteria *(9).* In our work, we used pasteurized whole egg as a matrix in which lysozyme could already have been denatured, but the UHPH treatment can also contribute to increase this inhibitory effect. Vannini et al. *(21)* observed that a synergism took place, increasing viability losses of *Lactobacillus helveticus, Lactobacillus plantarum,* and *Listeria monocytogenes* when the combination of high-pressure homogenization (up to 130 MPa) and lysozyme was evaluated. In the case of gram-negative bacteria, Diels et al. *(3)* observed that, above 150 MPa, *E. coli* became more sensitive to lysozyme when this compound was added before the UHPH treatment compared with adding the enzyme after the treatments, indicating that the UHPH treatment increases the effectiveness of lysozyme.

From our results, we conclude that the application of UHPH can be a promising alternative to thermal pasteurization to guarantee the safety of whole egg. Although viable but not cultivable cells of *Salmonella* Senftenberg 775W were still found in the final samples, it must be considered that the strain assayed has been described as one of the most resistant to thermal treatments. No previous references were found concerning the effect of high pressures on this strain, but the degree of lethality achieved by our treatments was equivalent to those reported by thermal pasteurization in a previous report by Mañas et al. *(12).* In this report, the effectiveness of the treatment was only determined by means of plate counts, and the presence on viable but not cultivable cells of *Salmonella* was not determined as we did. Moreover, in our study, we did not use all the possibilities currently offered by this technology. For instance, we limited the maximum temperature achieved to avoid the risk of product coagulation by refrigerating the pipes located immediately after the high-pressure valve, but with adequate control, and considering the short residence time of the product at the maximum temperature (<1 s), it can be assumed that the thermal tolerance would be greater. Increasing the temperature at this point would increase the lethal effect of the treatment, as has been observed in previous surveys with other matrices *(1, 2).* Another point that would increase the effectiveness is increasing the pressure. We used up to 250 MPa, but the current UHPH machines can supply up to 400 MPa. To confirm definitively the potential applicability of this technology for the whole-egg manufacturing industry, further studies with several *Salmonella* strains currently found in egg products should be pursued, especially in comparing the results with those of pasteurization treatments. The effects of this technology on the technological and sensorial properties of whole egg must also be evaluated.

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Paper II

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Fat content increases the lethality of ultra-high-pressure homogenization on *Listeria monocytogenes* in milk

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ABSTRACT

Listeria monocytogenes CCUG 15526 was inoculated at a concentration of approximately 7.0 $\log_{10}$ cfu/mL in milk samples with 0.3, 3.6, 10, and 15% fat contents. Milk samples with 0.3 and 3.6% fat content were also inoculated with a lower load of approximately 3.0 $\log_{10}$ cfu/mL. Inoculated milk samples were subjected to a single cycle of ultra-high-pressure homogenization (UHPH) treatment at 200, 300, and 400 MPa. Microbiological analyses were performed 2 h after the UHPH treatments and after 5, 8, and 15 d of storage at 4°C. Maximum lethality values were observed in samples treated at 400 MPa with 15 and 10% fat (7.95 and 7.46 $\log_{10}$ cfu/mL), respectively. However, in skimmed and 3.6% fat milk samples, complete inactivation was not achieved and, during the subsequent 15 d of storage at 4°C, *L. monocytogenes* was able to recover and replicate until achieving initial counts. In milk samples with 10 and 15% fat, *L. monocytogenes* recovered to the level of initial counts only in the milk samples treated at 200 MPa but not in the milk samples treated at 300 and 400 MPa. When the load of *L. monocytogenes* was approximately 3.0 $\log_{10}$ cfu/mL in milk samples with 0.3 and 3.6% fat, complete inactivation was not achieved and *L. monocytogenes* was able to recover and grow during the subsequent cold storage. Fat content increased the maximum temperature reached during UHPH treatment; this could have contributed to the lethal effect achieved, but the amount of fat of the milk had a stronger effect than the temperature on obtaining a higher death rate of *L. monocytogenes.*
Key words: *Listeria monocytogenes*, ultra-high-pressure homogenization, fat

INTRODUCTION

Listeria monocytogenes is an intracellular pathogen that can cause invasive diseases in both humans and animals. Approximately 99% of human listeriosis infections appear to be foodborne, though the disease process is complex with multiple routes of infection (Lunden et al., 2004; McLauchlin et al., 2004). The incidence of listeriosis varies between 0.1 and 11.3 per 1,000,000 people, depending on the country, and has an average case-fatality rate of 20 to 30% despite adequate antimicrobial treatment (Swaminathan and Gerner-Smidt, 2007).

This zoonotic foodborne pathogen is especially troublesome for the food industry because of its ubiquitous distribution in nature and its ability to grow at low temperatures in the presence of high salt concentrations and relatively acid pH (ICMSF, 1998). Outbreaks of listeriosis are often related to the consumption of milk and dairy products such as ripened soft cheeses (including blue cheeses), Mexican-style soft cheese, chocolate milk, and butter, which support its growth and have long shelf lives at refrigerating temperatures (Waak et al., 2002; Carminati et al., 2004; Lunden et al., 2004). Its presence in milk-based products can be a result of either raw-milk or postprocessing contamination (Waak et al., 2002; Borucki et al., 2004; Carminati et al., 2004). Because *Listeria* spp. have been found in different locations in the environment of dairy plants and may also survive for a long time in milk products, large numbers of *L. monocytogenes* may accumulate during storage, potentially resulting in foodborne illness (Gray et al., 2004). The incidence of *Listeria* in raw milk varies from 0.4 to 12.6% and is maximal in cold months because of the introduction of silage into feed. If raw milk is contaminated, it is usually at low levels (<1 cfu/mL of milk) but may be much higher (10^4–10^6 cfu/mL) where there are cases of mastitis (Linton et al., 2008).

Heat treatments are the most commonly chosen preservation method for milk and other perishable liquid foods. Thermal processing has a long tradition in food preservation because it is economical and efficient for achieving microbial inactivation, but it cannot be used to treat heat-labile compounds. High temperatures may lead to undesirable effects in milk such as off-flavors, nonenzymatic browning, and denaturation of certain vitamins and proteins (Vachon et al., 2002; Diels et

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al., 2005). The growing trend for fresher, high-quality convenience food has generated an increasing interest in nonthermal processing alternatives such as high-pressure technologies, which are considered to be the most promising emerging food processing technologies because of recent advances in high-pressure machinery and the successful introduction of pressure-processed foods (Gervilla et al., 2000; Kheadr et al., 2002; Diels et al., 2005). Ultra-high-pressure homogenization (**UHPH**; also called dynamic high-pressure in the literature) is based on the same design principles as the conventional homogenization process used in the dairy industry for reducing the size of fat globules but works at significantly higher pressures (>200 MPa), resulting in the destruction of large quantities of microorganisms (Vachon et al., 2002; Thiebaud et al., 2003). Consequently, this technology appears to be an important means of lowering the initial microbial load while helping to minimize product damage from unnecessary heat stress (Popper and Knorr, 1990). The effects of UHPH on bacterial cells are not yet well known, but some studies on UHPH have shown changes in bacterial cell morphology as well as splits in the cytoplasmic membrane. These sudden increases in permeability or ruptures of the cell membrane that occur under pressure may cause cell death (Guerzoni et al., 1999; Kheadr et al., 2002).

There is scarce information concerning the effect of the matrix on the degree of inactivation shown by the microorganisms. Previous studies have examined the effectiveness of UHPH in reducing different foodborne pathogens inoculated in different matrices such as PBS, orange juice, and milk after passing the sample one or more times through the machine. Diels et al. (2005) proposed that viscosity is the major environmental parameter effecting bacterial inactivation by UHPH (as opposed to water activity or product composition), observing that bacterial inactivation decreases with increasing viscosity of the suspension, this effect being more pronounced at higher pressures. Vachon et al. (2002) observed that *L. monocytogenes* was more resistant to pressure treatments in milk than in PBS buffer. However, more recently, Diels et al. (2005) and Briñez et al. (2006a) observed that *Escherichia coli* presented a higher inactivation ratio when it was UHPH-treated in whole milk than in skimmed milk. Similar results were observed by Gervilla et al. (2000) in ovine milk with different percentages of fat (0, 6, and 50%) but treated by high hydrostatic pressure. They suggested that the fat content of milk would increase the piezosensibility of the microorganisms in contrast with thermal treatments, in which fat is assumed to have a protective effect (MacDonald and Sutherland, 1993). The cause might be the increase of the concentration of certain liposoluble substances that have an antimicrobial effect caused by the increase in the fat content, which causes interchanging of triglycerides of milk with lipoproteins of cellular membrane, altering the permeability of microorganisms.

The main objective of this work was to determine how the fat concentration of milk influences the effectiveness of the UHPH treatments on *L. monocytogenes* and to assess its influence on the ability of the surviving microorganisms to recover and grow during the subsequent cold storage.

MATERIALS AND METHODS

Preparation of Cell Suspension

Listeria monocytogenes CCUG 15526 was obtained as freeze-dried cultures in thermosealed vials from the Culture Collection of the University of Goteborg (Sweden). Freeze-dried cultures were rehydrated in tryptone soy broth (Oxoid Ltd., Basingstoke, UK) at 37°C for 24 h and subsequently used to inoculate tryptone soy agar plates (Oxoid Ltd.). Individual colonies were collected to prepare cryobeads (Nalgene System 100 Laboratories, Microkit Iberica S.L., Madrid, Spain) that were kept at −20°C to provide stock cultures for the assays.

Prior to each experiment, 1 cryobead was inoculated into 10 mL of tryptone soy broth (Oxoid Ltd.) and incubated at 37°C for 20 h. After incubation, the broth was spread using a disposable loop on tryptone soy agar slant (Oxoid Ltd.) and incubated at 37°C for 20 to 24 h. Subsequently, cell suspensions were prepared in 11 mL of tryptone sodium chloride solution consisting of 1 g/L of tryptone pancreatic casein digestion (Oxoid Ltd.) and 8.5 g/L of sodium chloride (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain a 9.0 to 9.5 $\log_{10}$ cfu/mL cell suspension.

Preparation of Milk Samples

Ten milliliters of the cell suspension were inoculated in 1 L of sterilized milk that had previously been adjusted to different percentages of fat content with sterilized 35% fat milk cream when necessary. The milk samples assayed were 0.3% (skimmed milk), 3.6%, 10%, and 15% fat milk. The final concentration of *L. monocytogenes* cells in these samples was approximately 7.5 $\log_{10}$ cfu/mL. The inoculated milk samples were placed in a water bath for 70 min at 6°C to reach the inlet temperature before the UHPH treatments.

UHPH Treatments

A benchtop high-pressure homogenizer (model/DRG FPG7400H:350, Stansted Fluid Power Ltd., Essex,

UK) was used for this study. This machine consisted of 2 intensifiers driven by a hydraulic pump and a high-pressure valve made of resistant ceramics able to support 400 MPa. These components guaranteed a constant flow rate of 7.0 L/h during the process. To avoid poor homogenization performance caused by temperature increase and rapid expansions or contractions in the processing valve, the processing valve was cooled by a constant circulation of water at room temperature in an external jacket built around it. Milk samples were subjected to a single cycle at UHPH of 200, 300, and 400 MPa at an inlet temperature of 6°C. For the experiment, 1.0 L of each type of milk was used with the majority of this volume being processed through the homogenizer to ensure temperature equilibration. Afterwards, between 80 and 100 mL of each sample was taken for analysis. The homogenized samples reached an outlet temperature of 16 to 18°C by means of an external jacket built around the pipeline located between the first and second homogenizing valves and a spiral located in a water bath with cold water after the second valve. The collected samples were immediately stored at 4°C. A specific sanitation program developed for the UHPH machine was applied after each UHPH treatment using a 70% (vol/vol) ethanol solution.

Microbiological Analysis

To determine the initial number of cells in the inoculated samples of milk, 1 mL of each sample was used to prepare decimal dilutions in peptone water (10 g/L of peptone and 5 g/L of NaCl, Oxoid Ltd.). Then, 1 mL of each dilution was placed in duplicate on tryptone soy agar plates supplemented with 6 g/L of yeast extract (**TSAYE**, Oxoid Ltd.) and incubated at 37°C for 48 h. Also, 20 mL of each untreated inoculated sample (control) was placed into sterile tubes and stored at 4°C.

The first microbiological analysis of treated and control samples was performed 2 h after the UHPH treatment and after 5, 8, and 15 d of storage at 4°C. To assess the lethality and the level of injuries caused by UHPH treatment, decimal dilutions in peptone water (Oxoid Ltd.) of untreated and treated milk samples were prepared and plated in duplicate in TSAYE and TSAYE supplemented with 5 g/L of NaCl (Panreac) and incubated at 37°C for 48 h. The use of this differential plating technique enabled injuries to be monitored. Both noninjured and injured cells were able to form colonies on TSAYE whereas only noninjured cells formed colonies in the presence of NaCl (Patterson et al., 1995). Results were expressed as $\log_{10}$ colony-forming units per milliliter. Lethality was calculated as the difference between the logarithms of colony counts of the untreated (N_0) and treated (N_f) samples ($\log_{10} N_0 - \log_{10} N_f$).

Statistical Analysis

All experiments were repeated 4 times with duplicate analysis in each replicate. Data are presented as least squares means of each experiment. ANOVA was performed using the GLM procedure of SAS (version 8, SAS Institute Inc., Cary, NC). Evaluation was based at a level of significance of $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the lethality values obtained for *L. monocytogenes* inoculated in milk samples (0.3, 3.6, 10, and 15% of fat) after being UHPH-treated at 200, 300, and 400 MPa. Best results were observed at 300 and 400 MPa, without significant differences between both treatments. Maximum lethality values (7.95 and 7.46 $\log_{10}$ cfu/mL) were observed in milk with 15 and 10% fat content, respectively. In these cases, no colonies were detected after the treatments; thus, the lethality values match the amount of *Listeria* inoculated. The lowest lethality values were obtained in skimmed milk (4.76 $\log_{10}$ cfu/mL). From these results it is clear that the higher the fat content was, the higher the lethality achieved for the same treatment conditions.

The type of matrix influences the effectiveness of all kinds of treatments. Microbial resistance is usually higher in foods than in buffers, but the mechanisms by which foods protect bacterial cells are still unknown in most cases. Foods are very complex chemical systems in which most components (salts, sugars, protein, fats, and so on) may affect microbial tolerance to heat or pressure, hence the difficulty in studying the protective mechanism of all interacting components (Mañas et al., 2001). Some authors describe that lipids increase bacterial heat resistance (Ababouch et al., 1987; Fain et al., 1991; Ahmed et al., 1995; Kaur et al., 1998). This could be caused by the dehydration of cells immersed in the lipid phase (Jay, 1992; Ahmed et al., 1995), but this mechanism could not explain the protective effect on the whole bacterial population that is sometimes observed in foods (Fain et al., 1991; Ahmed et al., 1995). However, in the case of pressure-based technologies such as high hydrostatic pressure, Gervilla et al. (2000) observed that in ewe's milk with different percentages of fat (0, 6, and 50%) treated during 2 min at 500 MPa, the amount of fat appeared to increase the piezosensibility of the microorganisms. These researchers suggest that the cause might be the increase of the concentration of certain liposoluble substances

Table 1. Lethality values ($\log_{10}$ cfu/mL) of *Listeria monocytogenes* inoculated in milk samples with 0.3, 3.6, 10, and 15% fat content after being treated by ultra-high-pressure homogenization at 200, 300, and 400 MPa with an inlet temperature of 6°C

	0.3%		3.6%		10%		15%	
Pressure (MPa)	TSAYE[1]	TSAYE + NaCl	TSAYE	TSAYE + NaCl	TSAYE	TSAYE + NaCl	TSAYE	TSAYE + NaCl
200	$1.14^{A,a}$	$1.07^{A,a}$	$1.36^{A,a}$	$1.28^{A,a}$	$2.79^{B,a}$	$1.28^{A,a}$	$3.09^{B,a}$	$2.59^{B,a}$
300	$4.74^{A,b}$	$4.73^{A,b}$	$5.78^{A,b}$	$5.67^{A,b}$	$7.46^{B,b}$	$7.46^{B,b}$	$7.95^{B,b}$	$7.95^{B,b}$
400	$4.77^{A,b}$	$4.76^{A,b}$	$5.31^{A,b}$	$4.70^{A,b}$	$7.46^{B,b}$	$7.46^{B,b}$	$7.95^{B,b}$	$7.95^{B,b}$

^{A,B}Different superscript uppercase letters in the same row indicate statistically significant differences ($P < 0.05$).

^{a,b}Different superscript lowercase letters in the same column indicate statistically significant differences ($P < 0.05$).

[1]Tryptone soy agar with yeast extract; manufactured by Oxoid Ltd., Basingstoke, Hampshire, UK.

that have an antimicrobial effect caused by the increase of the fat content; this causes the interchanging of triglycerides of milk with lipoproteins of the cellular membrane of microorganisms, altering its permeability. However, more recently Diels et al. (2005) identified fluid viscosity as a major environmental parameter affecting bacterial inactivation by UHPH. These authors evaluated the inactivation of *E. coli* MG1655 by UHPH at pressures ranging from 100 to 300 MPa in buffered suspensions adjusted with polyethylene glycol to different relative viscosities (1.0, 1.3, 1.7, 2.7, and 4.9) and observed that bacterial inactivation was found to decrease with increasing viscosity of the suspensions, an effect that was more pronounced at higher pressures. However, in our case we observed the opposite behavior because viscosity of milk increased with the fat content (data not shown). Previously, Vachon et al. (2002) observed lower lethality values for *E. coli* O157:H7 when treated in PBS (approximately 6.0 $\log_{10}$ cfu/mL) than when inoculated in raw milk (8.5 $\log_{10}$ cfu/mL) for the same treatment conditions. Briñez et al. (2006a) reported clear differences in the lethality obtained in *E. coli* between skim milk and whole milk in favor of whole milk.

The level of pressure and the friction arising when the sample passes through the narrow gap of the valves causes an increase in the temperature of the liquid, which would be dependent on the viscosity and the inlet temperature of the sample and on the pressure applied. In our survey, the lowest maximum temperature was observed at 200 MPa with the skimmed milk whereas the highest maximum temperature was reached at 400 MPa with the milk containing 15% fat (Table 2). The increment in the viscosity caused by fat content probably contributed to an increase in friction, in turn increasing the temperature reached. The effect of temperature on the bactericidal efficacy of UHPH on *L. monocytogenes* was previously observed by Vachon et al. (2002) after preheating milk (45, 55, and 60°C) before UHPH treatment. These authors did not report the maximum temperature reached during the treatments, but in previous surveys we observed that increasing the inlet temperature also increases the maximum temperature achieved. Briñez et al. (2006b) observed an increased lethality over *Listeria innocua* in milk when the inlet temperatures increased from 6 to 20°C. We used an inlet temperature of 6°C to minimize the thermal effect, but higher lethality would be expected using higher inlet temperatures. It must be taken into account that a sample remains at the maximum temperature for just a fraction of second because, after passing the valve, the temperature drops to approximately 16°C as a result of both the sudden decrease in pressure and the cooling system applied after the valves. Consequently, although temperature may contribute to the lethal effect, it cannot be considered to be the main factor. In fact, treatments at 300 MPa in milk with 15% fat caused a significantly higher lethality than observed in milk with 3.6% fat after a 400 MPa treatment (Table 1) even though the maximum temperatures achieved in both cases were similar (Table 2).

No significant differences were observed in *L. monocytogenes* counts between TSAYE and TSAYE + NaCl media (except for 10% fat milk treated at 200 MPa), indicating that the UHPH treatment caused no significant sublethal injuries. This was also described previously for microorganisms such as *Listeria innocua*, *Staphylococcus aureus, Salmonella enterica* serovar Typhimurium, and *E. coli* (Vachon et al., 2002; Wuytack et al., 2002; Briñez et al., 2006a,b). Wuytack et al. (2003) compared mild-heat treatments with different emerging nonthermal food preservation treatments (high-pressure homogenization, high hydrostatic pressure, pulsed whitelight, and pulsed electric fields), reporting that the highest levels of sublethal injuries among the emerging treatments were observed for high hydrostatic pressure compared with pulsed white light, pulsed electric field, and UHPH, which showed the lowest levels of sublethal injuries. This is the point that clearly makes the effect of this technology different from high hydrostatic pres-

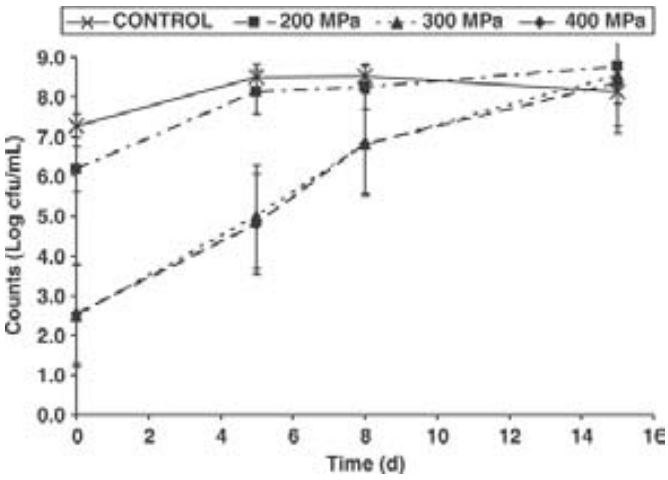

Figure 1. *Listeria monocytogenes* evolution in milk with 0.3% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 7.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

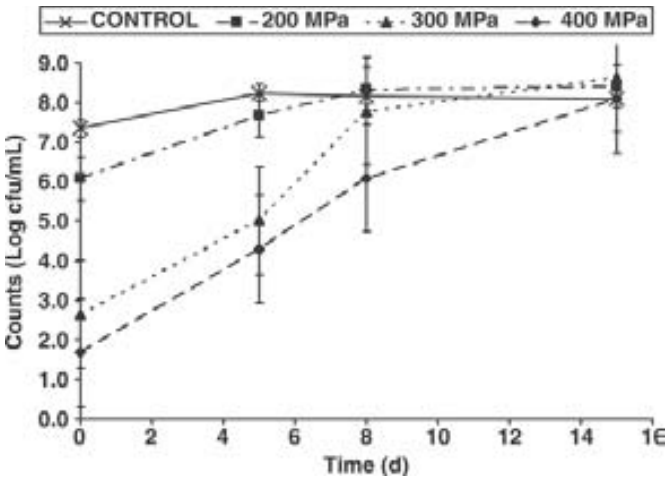

Figure 2. *Listeria monocytogenes* evolution in milk with 3.6% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 7.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

sure, which can cause accumulation of sublethal injuries and lead to subsequent recovery of the cells, depending on the conditions of treatment and subsequent storage (Wuytack et al., 2002; De Lamo-Castellví et al., 2005).

Classic culture technique may underestimate the numbers of truly viable bacteria, especially when cells have been damaged by physical treatments. This fraction of viable but noncultivable cells might remain and grow in processed foods like milk, where these cells are able to repair themselves and replicate if they have enough time. To evaluate this, the evolution of *L. monocytogenes* was monitored during 15 d of storage at 4°C. During this time, *L. monocytogenes* was able to recover the initial counts in both skimmed (Figure 1) and 3.6% fat milk (Figure 2). Nevertheless, in milk samples with 10% (Figure 3) and 15% fat (Figure 4), *L. monocytogenes* recovered the initial counts only in the milk treated at 200 MPa but not in the milk samples treated at 300 and 400 MPa, confirming that fat content significantly influences the degree of damage caused to cells during the UHPH treatments and reduces the chance of these cells to recover.

Usually, lethality tests are performed by inoculating high amounts of the target microorganisms and counting the surviving cells after the treatment, obtaining a numeric value with the formula

$$\text{Lethality} = \log_{10} N_0\ (\text{cfu/mL}) - \log_{10} N_f\ (\text{cfu/mL}).$$

For that reason, in our tests we inoculated more than 7.0 $\log_{10}$ cfu of *L. monocytogenes* per mL of milk. In milk samples with 10% and 15% fat content, no surviving microorganisms were detected in the media after treatment at 300 and 400 MPa or during the subsequent cold storage. This was not the case of milks with 0.3 and 3.6% fat treated at 300 and 400 MPa. In milk samples with 3.6% fat, lethality values above 5.0 $\log_{10}$ cfu/mL were observed in most cases, which is

Table 2. Effect of pressure and fat content on the maximum temperature (°C) reached during the ultra-high-pressure homogenization treatment of milk samples with 0.3, 3.6, 10, and 15% fat content

	Fat content			
Pressure (MPa)	0.3%	3.6%	10%	15%
200	$51.5^{A,a}$	$53.7^{AB,a}$	$57.3^{BC,a}$	$58.7^{C,a}$
300	$63.5^{A,b}$	$65.7^{A,b}$	$72.7^{B,b}$	$77.0^{B,b}$
400	$77.0^{A,c}$	$79.3^{A,c}$	$84.0^{B,c}$	$90.0^{C,c}$

$^{A–C}$Different superscript uppercase letters in the same row indicate statistically significant differences ($P < 0.05$).

$^{a–c}$Different superscript lowercase letters in the same column indicate statistically significant differences ($P < 0.05$).

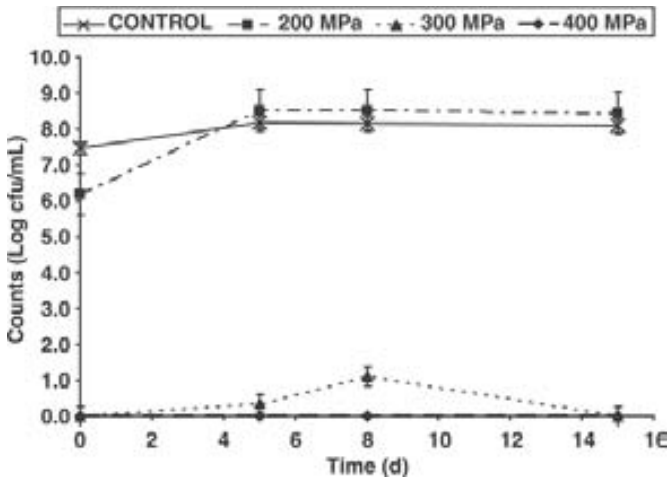

Figure 3. *Listeria monocytogenes* evolution in milk with 10% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 7.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

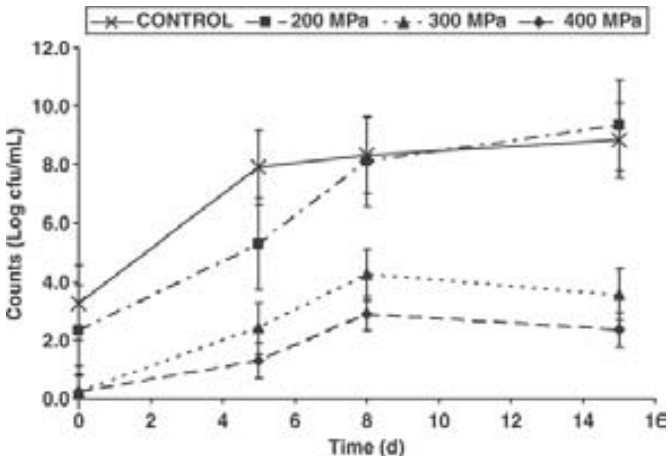

Figure 5. *Listeria monocytogenes* evolution in milk with 0.3% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 3.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

good considering the minimum microbial inactivation required in a thermal pasteurization. However, the Institute of Food Technologists (Heldman and Newsome, 2003) has recommended using lower loads of bacteria to test the efficacy of the treatments. Taking this into consideration, we inoculated a more realistic load of approximately 3.0 $\log_{10}$ cfu/mL to confirm the real efficiency of the treatment in milk samples with 0.3 and 3.6% fat (Figures 5 and 6). Surprisingly, although in the 300 and 400 MPa treatments the lethality was closer to the detection limit of the method, a complete inactivation was not achieved as expected and surviving *Listeria* cells were able to recover and grow during the subsequent cold storage. In this case, no significant differences could be observed in the lethality values achieved in both kinds of milk.

Ultra-high-pressure homogenization has been suggested as an alternative to pasteurization with the aim to extend the microbial and physicochemical shelf life of milk without causing significant changes in its nutritional, organoleptic, or technological properties (Pereda et al., 2007). In the particular case of foodborne pathogens like *L. monocytogenes*, UHPH treatments may reach the objective of inactivating at least 5.0 $\log_{10}$ cfu/

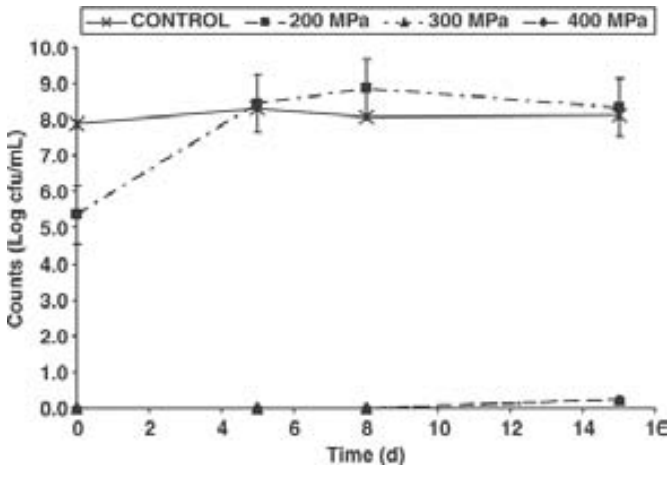

Figure 4. *Listeria monocytogenes* evolution in milk with 15% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 7.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

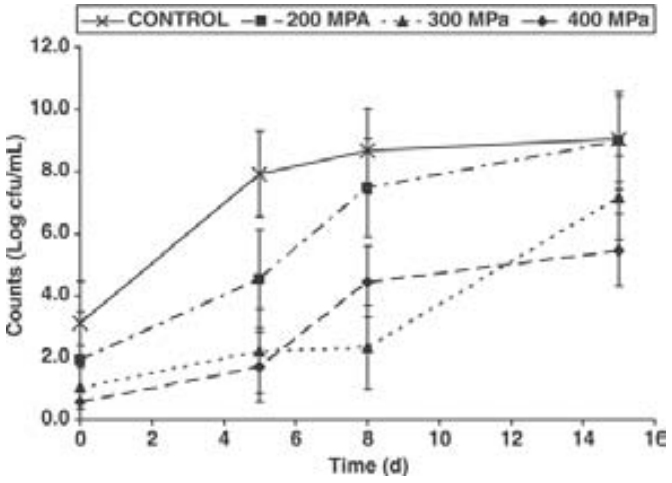

Figure 6. *Listeria monocytogenes* evolution in milk with 3.6% fat during cold storage at 4°C for 15 d. Milk samples were treated with ultra-high-pressure homogenization at 200, 300, and 400 MPa and inoculated at an initial load of approximately 3.0 $\log_{10}$ cfu/mL (mean values ± standard error bars are shown).

mL of *L. monocytogenes* but the efficacy would strongly depend on the amount of fat in the milk. Treatment at 300 MPa would be enough to achieve this goal in whole milk (more than 3.6% fat) and milk cream but would be more difficult in skimmed milk, at least using low inlet temperatures, and growth during the subsequent cold storage could not be prevented if viable cells remain after the treatment.

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Paper III

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Inactivation of *Listeria monocytogenes* and *Salmonella enterica* serovar Senftenberg 775W inoculated into fruit juice by means of ultra high pressure homogenisation

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ABSTRACT

The inactivation of *Listeria monocytogenes* and *Salmonella enterica* serovar Senftenberg 775 W by ultra high pressure homogenisation (UHPH) was evaluated in grape and orange juices inoculated at a concentration of approximately 7 log CFU/ml. The fluid inlet temperature used was 6 °C and the pressure levels assayed were 200, 300 and 400 MPa. Viable and injured bacterial counts were obtained 2 h after the UHPH treatments and after 5, 8, and 15 days of storage at 4 °C. Pressure level had a significant impact on the lethal effect of UHPH and complete inactivation of *S. enterica* serovar Senftenberg 775 W was achieved at 400 MPa. *L. monocytogenes* showed more resistance than *S. enterica* serovar Senftenberg 775 W to the UHPH treatments and no significant differences were observed between 300 and 400 MPa treatments in both juices. Sublethal injuries were not detected in any case. During the storage at 4 °C viable counts of both strains showed a decreasing trend. *L. monocytogenes* viable counts became undetectable in UHPH treated and also in control samples of grape juice which could be attributed to the presence of natural compounds with antilisterial effect.

1. Introduction

Fresh fruit and vegetable juices are recognized as an emerging cause of foodborne illness (Parish, 1997). A contributing factor is that these products are raw agricultural commodities, which may become contaminated by animal or human waste and consumed without a processing step that would kill or remove associated pathogens. Pathogenic agents able to reach the inside of a fruit or a vegetable must be able to survive in the product until it reaches the consumer in order to become a public health hazard. Preliminary studies carried out by the FDA show that *Salmonella enterica* serovar Hartford and *Escherichia coli* O157:H7 can infiltrate oranges that are visually intact, and once inside these pathogens can survive for at least five days, and in some cases grow. During juicing operation such microorganisms can be transferred into the orange juice resulting in a contaminated product (Food and Drug Administration, 1999a,b). In the absence of known specific pathogen-product associations, the National Advisory Committee on Microbiological Criteria in Foods (NACMCF) recommends the use of *E. coli* O157:H7 or *Listeria monocytogenes* as target organisms. These are two of the most difficult organisms to control (i.e., by juice acidity or heat lethality) and thus by controlling them, other pathogenic organisms will also probably be controlled. The Food and Drug Administration (FDA) has proposed a 5-log reduction in the target pathogens, as the NACMCF recommended, as a necessary step in a HACCP plan for juice (Food and Drug Administration, 1998).

Thermal pasteurisation in fruit juice processing has as primary purpose to destroy pathogenic and deteriorative organisms as well as inactivate undesirable enzymes. However, high pasteurisation temperatures impact negatively on the nutritional quality and taste of orange juice (Farnworth, Lagacé, Couture, Yaylayan, & Stewart, 2001). Consumer demands are more and more directed towards high-quality, additive-free, minimally processed, nutritious, and fresh like products. In order to combine efficient microbial reduction with a maximal retention of the desirable properties, food researchers are testing alternative non-thermal treatments. One of these alternative treatments is ultra high pressure homogenisation (UHPH). UHPH refers to a treatment similar to the homogenisation processes were a liquid is forced through a very narrow and adjustable orifice achieving high pressure and high velocity, producing physical changes in the treated product. This treatment differs from static high pressure in that microorganisms are

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subjected to a combination of shear forces (being caught between liquid flow paths of greatly differing velocities), cavitation shock waves (from the collapse of holes formed by vaporization within the liquid) and impingement (striking hard surfaces) (Lacroix, Fliss, & Makhlouf, 2005; Tahiri, Makhlouf, Paquin, & Fliss, 2006). The potential of UHPH as alternative to heat pasteurisation to inactivate foodborne pathogens has been demonstrated in milk (Brinez, Roig-Sagues, Hernandez, & Guamis, 2006a,b; Briñez, Roig-Sagués, Hernández, & Guamis, 2007; Kheadr, Vachon, Paquin, & Fliss, 2002; Vachon, Kheadr, Giasson, Paquin, & Fliss, 2002). Important reductions of *E. coli O157:H7* and *Listeria innocua* have also been reported in orange juice (Brinez, Roig-Sagues, Hernandez and Guamis, 2006a; Tahiri et al., 2006). Taking into account the scarce knowledge of the effect of UHPH treatment on foodborne pathogens when it is applied on different food matrixes and the recent improvements made in UHPH machines, it is possible to process liquid foods at pressures up to 400 MPa. The objective of this work was to evaluate the inactivation induced by UHPH treatments on *L. monocytogenes* and *S. enterica* serovar Senftenberg 775 W inoculated into orange and grape juices and the capability of these strains to survival, repair and grow during storage at refrigerating temperature after UHPH treatment. We selected these microorganisms because of their high incidence of foodborne outbreaks, some of them caused by contaminated fruit juices. We specifically selected the strain 775 W of *S. enterica* serovar Senftenberg because it has been described as one of the most resistant to thermal pasteurisation (Manas, Pagán, Alvarez, & Condón, 2003).

2. Materials and methods

2.1. Bacterial strains

L. monocytogenes (CCUG 15526) was obtained from the Culture collection of the University of Goteborg, Sweden and *S. enterica* serovar Senftenberg 775 W (CECT 4565) from the Spanish Type Culture Collection of the University of Valencia, Spain. Freeze-dried cultures of these microorganisms were re-hydrated in tryptone soy broth (TSB, Oxoid Ltd., Basingstoke, Hampshire, UK) at 37 °C for 18 h. Subsequently, these broths were used to inoculate tryptone soy agar plates (TSA, Oxoid). Individual colonies were collected to prepare cryobeads (Nalgene System 100 Laboratories Microkit Iberica S.L., Madrid, Spain) of each strain and kept at −22 °C to provide stock cultures for the assays.

2.2. Sample preparation

Before each experiment, one cryobead was inoculated into 10 ml of TSB and incubated at 37 °C for 20 h. After incubation, the broth was spread using a disposable loop on TSA plates and incubated at 37 °C for 20–24 h. Subsequently, cell suspensions were prepared in 11 ml of tryptone sodium chloride solution (1 g/L of tryptone pancreatic casein digestion, Oxoid) and 8.5 g/L of sodium chloride (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain 9–9.5 log CFU/ml by means of optical density at 405 nm using a Cecil 9000 spectrophotometer (Cecil Instruments, Cambridge, UK). Later, 10 ml of this cell suspension was inoculated in 1 L of commercial pasteurised orange or grape juice to reach a final concentration of approximately 7 log CFU/ml. Previous to the UHPH treatments inoculated juice samples were placed in a refrigerated water bath to reach 6 °C.

2.3. High-pressure treatment of samples

The equipment used was an UHPH benchtop homogeniser Stansted FPG12500 (Stansted Fluid Power Ltd., Essex, UK) equipped with two intensifiers that allowed a constant flow rate of 7 L per hour and able to achieve a peak pressure of 400 MPa. Samples were adjusted to 6 °C before treatments (inlet temperature). To avoid an excessive increase in the temperature of the juice during the treatments an external jacket with constant circulation of ethylene glycol at 2 °C was located immediately after the high pressure valve. For each experiment 2.0 L of inoculated juice were subjected to a single cycle UHPH treatment at 200, 300 and 400 MPa. The maximum temperatures achieved during the treatments were 74.2 °C at 400 MPa, 62.4 °C at 300 MPa and 51.3 °C at 200 MPa, with a holding time shorter than 0.7 s. After pressure treatments, the juice samples were immediately cooled in a heat exchange coil which was immersed in an ice water bath to decrease the temperature to 11–13 °C. Between 80 and 100 ml of the samples were taken aseptically and were stored at 4 °C before the analysis. After each assay the machine was cleaned and disinfected in order to avoid cross contamination: first the machine was clarified circulating sterile distilled water at 70 °C, and then cleaned with a 5% v/v solution of neutral detergent during 20 min. The remaining detergent was withdrawn with further sterile distilled water and, finally, the machine was disinfected circulating a 70% ethanol (Panreac) solution for additional 120 min.

2.4. Microbiological analysis

To determine the initial load of cells in the inoculated samples 1 ml of each sample was used to prepare decimal dilutions in peptone water (10 g/L of peptone and 5 g/L of NaCl, Oxoid). Subsequently, 1 ml of these dilutions was placed for duplicate in TSAYE (tryptone soy agar and 6 g/L yeast extract, Oxoid) and incubated at 37 °C for 48 h. Also, 20 ml of the untreated inoculated samples were placed into sterile tubes and stored at 4 °C (controls) and analysed to determine the effect of the matrix in the inoculated microorganisms. Microbiological analysis of treated samples and controls were performed 2 h after the UHPH treatment. The rest of treated samples were kept at 4 °C and analysed after 5, 8, and 15 days of storage. To assess the lethality and the level of injuries caused by UHPH treatment, decimal dilutions in peptone water of untreated and treated orange and grape juice samples were prepared and plated for duplicate in TSAYE and TSAYE + NaCl (50 g/L of NaCl) and incubated at 37 °C for 48 h. The use of this differential plating technique enables injuries to be monitored. Both non-injured and injured cells are able to form colonies on TSAYE, whereas only non-injured cells formed colonies in the presence of NaCl (Garcìa et al., 2005). Results were expressed as the logarithm of CFU/ml of orange and grape juice. Lethality was calculated as the difference between the logarithms of colony counts of the untreated and treated samples ($\log N_o - \log N$).

2.5. Statistical analysis

All experiments were repeated three times with duplicate analysis in each replicate. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P < 0.05$. All statistical analyses were performed with Statistical Package for the Social Sciences 14.0.1 software (SPSS Inc, Chicago, ILL).

3. Results and discussion

The level of inactivation of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775 W achieved after the different UHPH treatments in orange and grape juice samples is shown in Fig. 1. The results for *S. enterica* serovar Senftenberg 775 W indicate that lethality values increased with pressure until reaching complete

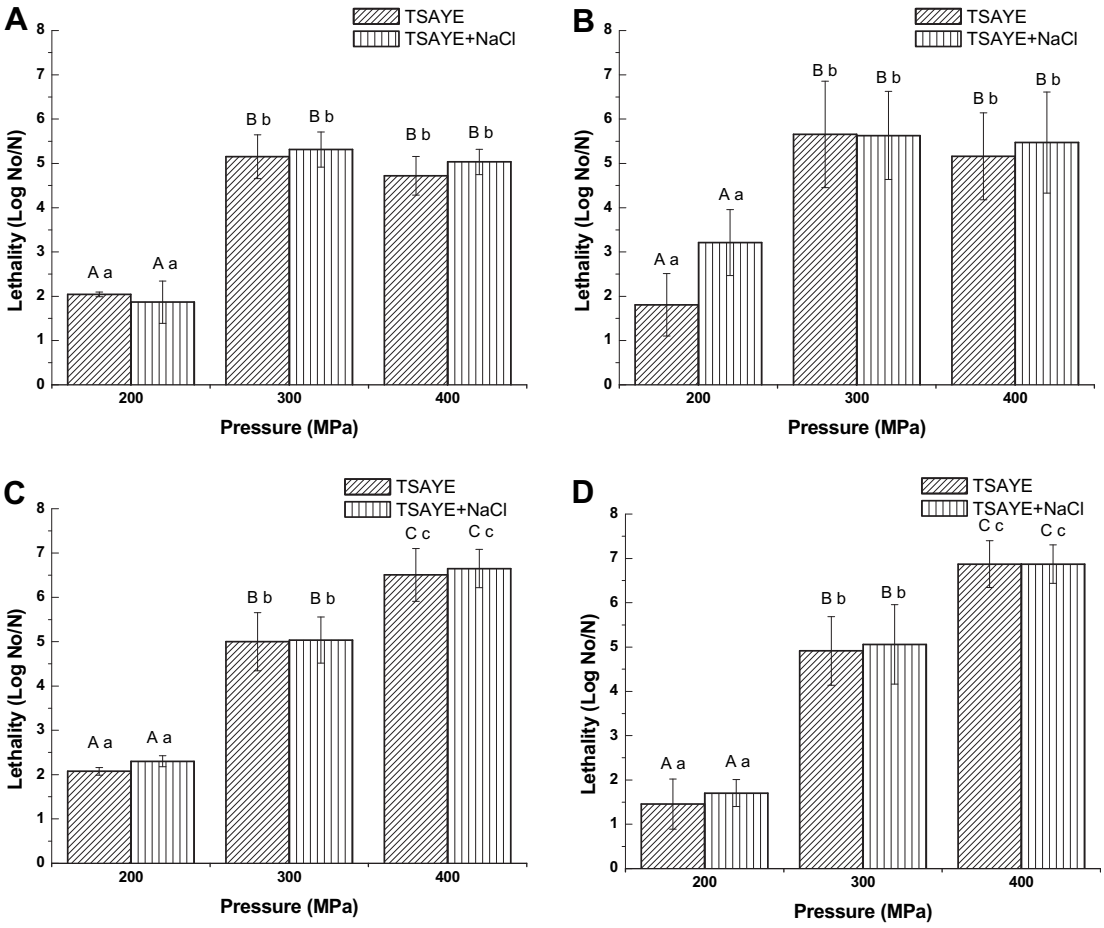

Fig. 1. Inactivation of *L. monocytogenes* in orange juice (A) and grape juice (B), and *S. enterica* serovar Senftenberg 775 W in orange juice (C) and grape juice (D) pressurised at 200, 300, and 400 MPa for one cycle and inlet temperature of 6 °C. Lethality values were obtained in both TSAYE and TSAYE + NaCl medium. Bars with different superscript capital letter are significantly different ($P < 0.05$) for the same matrix. Bars with different superscript small letter are significantly different ($P < 0.05$) for the same strain. Data are presented as the mean value of three replications ± standard deviation.

inactivation of the microorganisms at 400 MPa. *L. monocytogenes* was more resistant to the treatments at 400 MPa than *S. enterica* in both juices. Vachon et al. (2002) reported higher reductions of viable counts of *L. monocytogenes* and *Salmonella* Enteritidis, among other microorganisms in phosphate buffered saline solution (PBS), being *L. monocytogenes* the most resistant to UHPH. The same authors observed similar results in milk. Tahiri et al. (2006) also observed that the higher the pressure applied was, the higher was the reduction observed on different species of spoiling bacteria and *E. coli* O157:H7 inoculated in PBS. Regarding orange juice, Brinez, Roig-Sagués, Hernández Herrero, and Guamis López (2006a) obtained reductions of approximately 3.2 log units of *L. innocua* with 300 MPa UHPH treatments at an inlet temperature of 6 °C. In our study we obtained significantly higher reductions (5.15 log units) of *L. monocytogenes* using similar treatments.

When comparing the lethality values of the strains assessed with TSAYE and TSAYE + NaCl media no significant differences were observed in any case, which could indicate that UHPH treatments did not cause sublethal injuries. This is in agreement with Brinez et al. (2006a,b), Brinez et al. (2007) and Wuytack, Diels, and Michiels (2002)that observed little or no sublethal injuries associated with UHPH treatments. UHPH is a technology that produces mainly physical or structural damage on the cell. Some authors have shown the complete cell rupture and loss of cytoplasmic contents by UHPH treatments on transmission electron micrographs (Tahiri et al., 2006, Vachon et al., 2002). Wuytack et al. (2003) proposed a model that describes cellular inactivation and sublethal injury as a result of the inactivation of one or two targets in the cell. If only one target is present in the cell in a single copy and this target is vital for the cell and is inactivated by the treatment, then every first "hit" in a cell will cause death, and no state of sublethal injury will exist. They suggest the peptidoglycan chain as the sole vital target. The high resistance of *L. monocytogenes* at 400 MPa can be explained by the composition of the cell wall, since Gram positive bacteria have a thicker peptidoglycan than Gram negative.

Figs. 2 and 3 show the evolution of viable counts of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775 W in orange and grape juices stored at 4 °C. No significant differences were observed in the counts during the storage time at 4 °C in any culture media used (TSAYE or TSAYE + NaCl) or sample category indicating that UHPH treatments or juice conditions did not cause persistent sublethally injured cells in any of the strains tested.

For *L. monocytogenes* the counts of UHPH treated samples diminished from day 0 to day 15 of storage at 4 °C by 1.15, 1.40 and 1.98 log units in orange juice treated at 200, 300 and 400 MPa, respectively, untreated (control) samples with an initial load of 7.05 CFU/ml showed a reduction of approximately 0.9 logarithmic unit along the storage (Fig. 2A). The counts of grape juice samples diminished during storage being undetectable from the 5th day in 300 and 400 MPa pressurised samples. In samples pressurised at 200 MPa the counts were undetectable from the 8th day, while in control samples only reached undetectable levels at the end of the storage period (Fig. 2B).

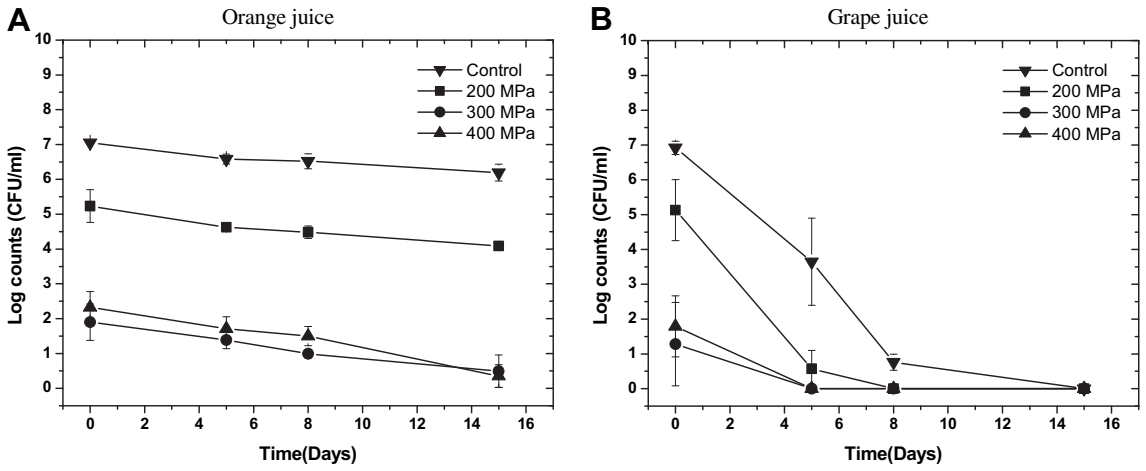

Fig. 2. Evolution of viable counts of *L. monocytogenes* in orange juice (A) and grape juice (B) pressurised at 200, 300 and 400 MPa, and stored at 4 °C. Counts were obtained in TSAYE medium. Data are presented as the mean value of three replications ± standard deviation.

Fig. 3 shows the evolution of viable counts of *S. enterica* serovar Senftenberg 775 W in orange juice and grape juice stored at 4 °C after the UHPH treatments. In orange juice from day 0 to day 15, counts diminished 4.87 and 2.27 logarithmic units for 200 and 300 MPa treatments, respectively; for 400 MPa treated samples counts were undetectable from the 5th day of storage. During storage, counts of *S. enterica* in control samples of orange juice with an initial load of 7.28 CFU/ml diminished up to 3.0 logarithmic units (Fig. 3A). In grape juice, high reductions of approximately 5.3 logarithmic units were obtained from day 0 to day 15 for 200 MPa, while undetectable counts were observed from day 5 of storage for 300 MPa treated samples. In 400 MPa pressurised samples counts were always undetectable. The counts of grape juice controls decreased approximately 3.7 logarithmic units (Fig. 3B) after 15 days at 4 °C.

The changes in viable counts during storage at 4 °C showing significant decreases evidenced that the inoculated strains were not able to survive and grow in both food matrixes. However, in grape juice the reductions were higher than in orange juice for both strains. In grape juice undetectable counts of *L. monocytogenes* were observed in pressurised samples as well as in control samples towards the end of the storage time. These reductions in control samples suggest an inhibitory effect of the matrix compounds. Brinez et al. (2006a) observed different behaviour of *L. innocua* inoculated in milk and orange juice along the storage time after UHPH treatments. In milk counts increased by approximately 2 logarithmic units from day 0–9, whereas in orange juice counts diminished by approximately 2.5 logarithmic units from days 0–18. The authors supposed that the whole milk stimulated the growth of *L. innocua* in refrigerated storage, due to the characteristics of milk such as pH close to neutrality and large presence of nutrients. In orange juice the low pH could have caused interferences in the evolution of viable counts. In our case, we attributed the decrease in counts during storage to the pH of juices (3.63 for orange juice and 3.10 for grape juice). Previous studies in orange juice have shown that the death rates of *Salmonella* (serovars Gaminara, Hartford, Rubislaw and Typhimurium) are inversely correlated with pH (Parish, Narciso, & Friedrich, 1997). For *L. monocytogenes* Phan-Thanh, Mahouin, and Aligé (2000) observed that the bacteria began to die when the pH of the medium descended below 4. Moreover, it is important to consider the effect of organic acids on the cells. As for weak organic acids, they permeate the cell membrane as undissociated molecules through permeases or porins. Once dissociated inside the cell, they cannot diffuse out, thus

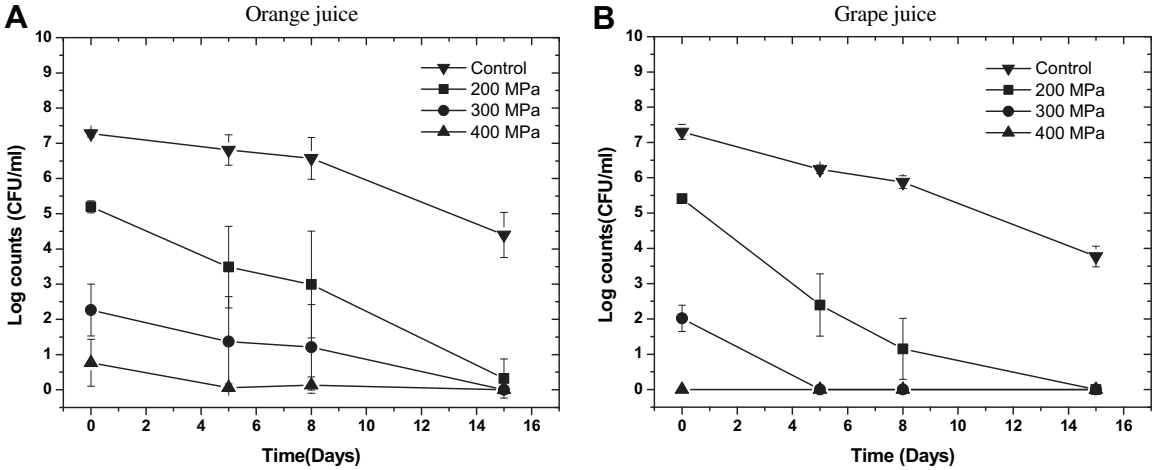

Fig. 3. Evolution of viable counts of *S. enterica* serovar Senftenberg 775 W in orange juice (A) and grape juice (B) pressurised at 200, 300 and 400 MPa, and stored at 4 °C. Counts were obtained in TSAYE medium. Data are presented as the mean value of three replications ± standard deviation.

lowering the intracytoplasmic pH to dramatic values and deregulating the metabolic machinery of the cell (Phan-Thanh et al., 2000).

However, the great reduction of bacterial counts observed in grape juice samples, in both control and pressurised samples, could be due to the presence of substances such as phenolic compounds. Mullen, Marks, and Crozier (2007) evaluated the phenolic content of 13 commercial fruit juices and fruit drinks. The largest content of individual phenolic compounds and the highest concentration of total phenolics were found in purple grape juice. The main components were flavan-3-ols, anthocyanins, and hydroxycinnamates, which accounted for 93% of the total phenolic content. In contrast, white grape juice, which contained principally hydroxycinnamates, had the lowest total phenolic content. On the other hand, the antilisterial activity of selected phenolic acids has also been evaluated and hydroxycinnamic acids demonstrated to have a bactericidal effect at pH 4.5 and a bacteriostatic activity at higher pH values (Wen, Delaquis, Stanich, & Toivonen, 2003). Rhodes, Mitchell, Wilson, and Melton (2006) found that commercial dark grape juice had inhibitory effect against *L. monocytogenes* and other *Listeria* species, decreasing *L. monocytogenes* number from 6 to 7 log CFU/ml to no detectable colonies within 10 min. We observed undetectable counts including for control samples which had an initial load of about 7 log CFU/ml. This reduction was presumably caused by the presence of the phenolic compounds described above.

Considering the results obtained in this study we can conclude that UHPH is presumably a technology with a great potential to be used in the industry of fruit juices, since it effectively inactivates pathogens such as *L. monocytogenes* and *S. enterica* serovar Senftenberg 775 W increasing their safety level. Further studies will be conducted to evaluate its effect on both the organoleptic and the nutritional properties of juices.

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Paper IV

Acid Tolerance Response Induced by organic acids on *Listeria monocytogenes* and *Salmonella enterica* serovar Senftenberg can enhance their survival in fruit juices.

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Abstract

Listeria monocytogenes and *S. enterica* serovar Senftenberg were acid adapted with either citric acid or malic acid during either exponential or stationary phase and subsequently inoculated into orange and grape juices. The effectiveness of the acid tolerance response (ATR) induced phenomenon in protecting cells from low pH was evaluated. Viable and injured bacterial counts were obtained by means of a differential plating method using Tryptone Soy Agar enriched with yeast extract (TSAYE) and the same medium supplemented with 5% of salt. Samples were analyzed after 5, 8 10 and 15 days of storage at 4ºC and 25ºC. *L. monocytogenes* and *S. enterica* exhibit adaptive ATR after remaining 3 hours in a mild acid medium (pH 5), which is capable to protect cells from a most severe acid stress (pH 3 and 3.5 for citric and malic acid, respectively). Stationary phase cells showed a natural resistance to low pH, while exponential phase cells required previous ATR induction to survive. In fruit juices held at 4ºC, the acid adapted cells in exponential growing phase of both strains showed a remarkable ATR. The data suggest that previous ATR may greatly influence the survival of pathogens in fruit juices.

Introduction

Nowadays consumption patterns are focused on minimally processed foods, which present a high quality since they are nutritious, with freshly flavor, texture and color. Unfortunately, increases in numbers of produce-related outbreaks on an international scale have been attributed to a higher per capita consumption of fresh or minimally processed fruits and vegetables (Francis et al., 1999). In fruit juices, unprocessed orange juice has been recognized as vehicle of food-borne diseases, although it was previously considered too acidic to serve as vehicle for pathogenic bacteria (Parish et al., 1997; Parish 1997; Parish 1998; Burnett et al., 2001).

Many food-borne pathogenic bacteria exhibit stress responses which enhance their survival to adverse environmental conditions. One stress commonly encountered in foods is an acidic environment where enhanced survival can involve induction of an acid tolerance response (ATR) (Greenacre et al., 2003). ATR is a phenomenon by which microorganisms show an increased resistance to environmental stresses after being exposed to a moderate acid environment (Hsin-Yi et al., 2001). The effectiveness of this response appears to be critically dependent several factors: the pH and the duration of the adaptive exposure are two of them (Davis et al., 1996), but ATR is also dependent on the strain and on the kind of acid used. Phan-Thanh et al. (2000) observed that organic volatile acids exerted a more deleterious effect on *L. monocytogenes* than inorganic acids, because weak acids infer a lower intracytoplasmic pH. The lethal effects of these weak acids are not only concentration dependent, but are also related to the pH of the environment and to the dissociation constant of the chemical. The unionized (protonated) form of a weak acid is more permeable to cell membranes than the ionized form. Thus, the lower the pH of the environment, the greater the proportion of undissociated weak acid is available to penetrate into the cell. After the unionized form is inside the cell, the more alkaline intracellular environment (pH 7.5 to 7.8) causes dissociation of the weak acid and acidification of the cytoplasm (Foster, 1993). Despite of the fact that food-borne pathogenic bacteria are more commonly exposed to weak organic acids either as by-products of bacterial metabolism in fermentation processes or as deliberate additions to foods as preservatives, the majority of published studies on ATR has been performed using minerals acids, such as hydrochloric acid (Greenacre et al., 2003).

Growing phase is another important factor to consider on ATR. Stationary phase cells are naturally more resistant than exponential phase cells (O'Driscoll et al., 1996; Buchanan et al., 1999). Foster (1993) discovered that induction of the ATR will protect bacterial cells against the lethal effects of organic acids, especially at a low pH (pH 4.4). Weak acids tested included acetic, propionic, and benzoic. The ATR protected log phase cells against all three, whereas ATR only worked with propionic acid for the stationary phase cells. These data suggest that different weak acids have different effects on the bacterial cells and the protection afforded by the ATR to stationary phase cells is different from that of the log phase cells.

Previous studies with different pathogens have demonstrated that ATR can significantly enhance their survival in acidic foods (Leyer et al., 1992; Leyer et al., 1995; Gahan et al., 1999; Phan-Thanh et al., 2000). In fact, induction of the acid tolerance response also protects pathogens against the effects of other environmental stresses. Acid-adapted cells demonstrate an increased resistance to heating, osmotic stress, lactoperoxidase crystal violet, ethanol and gamma radiation (Leyer et al., 1993; O'Driscoll et al., 1996; Buchanan et al., 1999; Buchanan et al., 2004).

The objective of this work was to evaluate acid adaptation of *Listeria monocytogenes* and *Salmonella enterica* in both exponential and stationary growing phase using citric acid and malic acid as acidulant, since they are the principal acids present in orange and grape juice, respectively. It is important to take into consideration the ability of food-borne pathogens to initiate an ATR and surviving in food with a low pH. Specifically, the main objective of this study was to determine differences in ATR between acid adapted and non-adapted cultures, as affected by the strain and the acid challenge conditions (pH and type of acidulant) of the fruit juices tested. Also, we intended to study the capability of the strain to survive, repair and grow during storage at refrigerating temperature.

Materials and methods

Bacterial strains

L. monocytogenes (CECT 4031) and *S. enterica* serovar Senftenberg 775W (CECT 4565) were obtained from the Spanish Type Culture Collection of the University of Valencia, Spain. Freeze-dried cultures of these microorganisms were re-hydrated in

tryptone soy broth (TSB, Oxoid Ltd., Basingstoke, Hampshire, UK) at 37ºC for 18 h. Subsequently, these broths were used to inoculate tryptone soy agar plates (TSA, Oxoid). Individual colonies were collected to prepare cryobeads (Nalgene System 100 Laboratories Microkit Iberica S.L., Madrid, Spain) of each strain and kept at -22ºC to provide stock cultures for the assays. Before each experiment, one cryobead was inoculated into 10 ml of TSB and incubated at 37ºC for 20 h. After incubation, the broth was spread using a disposable loop on TSA slants and incubated at 37ºC for 20 to 24 h.

Preparation of media

Tryptic soy broth (TSB) and tryptic soy agar plus 6% of yeast extract (TSAYE) were prepared according to the manufacturers' instructions. TSAYE supplemented with 5% sodium chloride was also prepared.

Acid adaptation of strains in TSB at different pHs

Both stationary and exponential phases were tested for acid-adaptation of the strains.

Stationary phase. TSB was inoculated with a colony of the strain to be tested. After incubation at 37 ºC for 24 hr, 100 µl were inoculated in 20 ml of TSB previously adjusted at pH 7 and then incubated at 37ºC for 24 hr. This bacterial suspension was in stationary phase.

Exponential phase. For obtaining the exponential phase 1 ml of the bacterial suspension in stationary phase was inoculated in 100 ml of TSB (pH 7). Each 10 minutes until complete 8 hours the optical density at 600 mn were measured in Automated Bioscreen C MBR (Oy Growth Curves Ab Ltd, Helsinki, Finlandia), concluding that the exponential phase required 3.5 hours.

Both suspensions were then used to inoculate tubes of TSB at pH 4, 4.5 and 5. Citric acid (30%) or malic acid (Panreac, Montcada i Reixac, Barcelona) were added to obtain the different pHs, having a final concentration of citric acid of 0.6, 0.4 and 0.2%, respectively; or 0.4, 0.3 and 0.18% as a final concentration of malic acid. Counts after 0, 2, 3 and 5 hours of incubation at 37ºC were determined in TSA. TSA plates then were incubated at 37ºC for 24 hours. The counts were expressed as log CFU/ml.

Induction of ATR

To evaluate the induction of the ATR both growing phases were obtained as it was described previously. Duplicated samples of each growing phase were centrifuged (15

minutes at 1500g at 4ºC), the resultant pellets re-suspended in 25 ml of TSB with pH adjusted to 5 (pH of acid-adaptation) or to 7 (control) and incubated for 3 hours at 37ºC. To determine the ATR, acid-adapted and control cells were harvested by centrifugation and re-suspended in 25 ml of TSB adjusted at pH 2.5 and/or 3 (pHs of induction) and incubated at 37ºC. As acid-adaptation the acidification for induction was carried out adding the acid of interest.

To assess the lethality and the level of injuries caused by the acid conditions, counts were determined at 0, 20, 40, 60 and 90 minutes. Decimal dilutions of samples in peptone water were prepared and spiral plated (Eddy Jet, IUL Instruments S.A., Barcelona, Spain) for duplicate in TSAYE and TSAYE+NaCl (NaCl 5%) and incubated at 37 ºC for 24-48 h. The use of this differential plating technique enables injuries to be monitored. Both non-injured and injured cells are able to form colonies on TSAYE, whereas only non-injured cells formed colonies in the presence of NaCl (Garcia et al., 2005).

Acid tolerance response in commercial juices

Following the same procedure described in the previous step the acid-adapted and control cells in their exponential or stationary phase were inoculated into orange and grape juices. The final concentration of juices was about 10^7 CFU/ml. The pHs of the juices were 3.76 and 3.11 for orange juice and grape juice respectively. To evaluate the ability of acid-adapted strains to survive and/or to grow in juices, viable and injured bacterial counts were obtained from samples after inoculation (initial load) and after 5, 8, 10 and 15 days of storage at 4 ºC and 25ºC.

Statistical analysis

All statistical analyses were performed with Statistical Package for the Social Sciences 14.0.1 software (SPSS Inc, Chicago, ILL). All experiments were repeated three times with duplicate analysis in each replicate. Results were evaluated by analysis of variance (ANOVA) using the Generalized Linear Model (GLM) and the Student-Newman-Keuls (SNK) test for comparing the means. Differences were considered to be significant at $P<0.05$.

Results and discussion

Acid adaptation of strains

In order to exhibit an increased ATR, an adaptation of *L. monocytogenes* and *Salmonella* Senftenberg were made. Ryu et al. (1998) defined the acid adapted cells as those which have been exposed to a gradual decrease in environmental pH and suggest that response of acid adapted cells may depend on the type of acidulant used to induce adaptation. In this study citric acid (Figure 1) and malic acid (Figure 2) were used as acidulants. Organic acids are generally considered more effective against food borne pathogens than mineral acids. This increased antimicrobial activity is associated with the anion portion of the molecule and varies among the various organic acids (Buchanan et al., 2004). In fact, Phan-Thanh et al. (2000) noted that organic acids were more lethal at low pH than inorganic acids. This appeared to be due to their ability to alter the internal pH (pHi) of *L. monocytogenes*. There is evidence that the adaptation time may be linked to maintain the pHi of the cell. Acid adapted cells are better able to maintain their pHi, and consequently survive better in acidic environments (Greenacre et al., 2003). In the pH range between 4.0 and 5.5, Buchanan et al. (1999) and Buchanan et al. (2004) found that among five acidulants (lactic, acetic, citric, malic, and hydrochloric acids) tested, lactic acid consistently had the greatest activity against enterohemorrhagic *E. coli* and HCl had the least.

In the present work, the period in which the microorganisms were in an early exponential growth phase was considered to select the optimum time and pH of exposure to promote ATR. For both strains on study, 3 hours were the optimum time and 5 the optimum pH for either citric acid and malic acid, independently of the growing phase (Figure1-2). Greenacre et al. (2003) observed that the ATR optimization occurred after exposure of *L. monocytogenes* for 3 hours to pH 5.5 poised with acetic acid and for 2 hours to pH 5.5 with lactic acid. For *S. enterica* serovar Typhimurium 2 hours of exposure to pH 5.5 poised with acetic acid and 3 hours to pH 5.5 with lactic where the optimum, concluding that the adaptation times differed according to the acidulant used to adapt the cells. Phan-Thanh et al. (2000) confirmed that the extent of acid tolerance acquired depended on the duration of acid adaptation, describing that a maximum acid tolerance of *L. monocytogenes* was obtained after 2-3 hours of adaptation, but after a longer time (24 hours) resulted the tolerance to acids was weaker. In agreement, Caggia et al. (2009) obtained acid adapted cells of *L. monocytogenes* after

maintaining them in TSB acidified with lactic acid at pH 5.7 for 3 hours. Our results of acid adaptation with citric and malic acid showed some accordance with these works. However, O'Driscoll et al. (1996) found that *L. monocytogenes* exhibited a significant adaptive tolerance response following a 1 hour exposure to pH 5.5, using as acidulant lactic acid.

Effectiveness of ATR induction

In accordance with previous surveys (Foster, 1993; Davis et al., 1996; O'Driscoll et al., 1996; Gahan et al., 1999; Hsin-Yi et al., 2001; Greenacre et al., 2003; Caggia et al., 2009) pH values of 3 and 3.5 were selected to assess the effectiveness of the ATR induction in protecting cells from low pH. Figure 3 shows the ATR of acid adapted and non-adapted *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W in exponential or stationary phase by exposure to acidified broth at pH 3 with citric acid. It can be observed for both strains that exponential and stationary acid adapted cells were the most acid tolerant. Overall, pathogens increase their ATR upon exposure to acid pH of sublethal levels while they are growing exponentially (Foster, 1993; Davis et al., 1996, O'Driscoll et al., 1996; Phan-Thanh et al., 2000). *L. monocytogenes* cultures in stationary phase have demonstrated a natural resistance to the challenge pH (pH 3.5) (O'Driscoll et al., 1996). Although *L. monocytogenes* cells showed sublethal injuries, counts remained until the end of the exposure (90 minutes), except the exponential non-adapted cells. It is known that *Listeria monocytogenes* is a pathogen capable of surviving at low pH as well as in environments of high salt content (Farber et al., 1991). Data show that a substantial injury was caused in *Salmonella* cells by the acid conditions. It has been previously described in *E. coli* O157:H7 that enhancement of acid tolerance is accompanied by a decreased tolerance to sodium chloride (Ryu et al., 1998). Non-adapted *Salmonella* cells in stationary phase were also injuried, but unlike of the other ones, counts were detected in all time points along the 90 minutes. This result confirms the finding of Lin et al., (1995) who observed that *Salmonella thyphimurium*, *Shigella flexneri* and *Escherichia coli* expressed a pH-independent general stress resistance system that contributed to acid survival of cells during stationary phase.

On the other hand, the *L. monocytogenes* cells which showed major sensibility were the non-adapted cells in exponential phase followed by the non-adapted in stationary phase.

For *Salmonella* the most sensitive were the non-adapted cells in both stationary and exponential phase.

Figure 4 shows the ATR of acid adapted and non-adapted *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W in exponential or stationary phase by exposure to acidified broth at pH 3.5 with malic acid. A pH of 3 was initially selected to confirm the ATR of the strains. However this pH resulted very deleterious for all cells (data not shown), then the pH selected was pH 3.5. Likewise in this case *L. monocytogenes* showed a major acid tolerance than *Salmonella*, whose acid adapted cells at stationary and exponential phase showed to be the most acid tolerant, as expected. For both strains the most sensitive cells were the exponential non-adapted, although sublethal injuries were observed in all cells as it was observed with citric acid.

Our results confirm that the ATR increase considerably when the cells have been exposed previously to mild acid for some time, protecting them from normally lethal acid stress (O'Driscoll et al., 1996; Ryu et al., 1998; Greenacre et al., 2003). The ATR involves the synthesis of an important number of proteins in a multistage cascade (Foster, 1993; Davis et al., 1996; O'Driscoll et al., 1996). The bacteria required more stress proteins to face severe acidic conditions. Indeed, confronted with acidic conditions, the bacteria cell attempts to resist by maintaining its pHi homeostasis (Phan-Thanh et al., 2000).

ATR in fruit juices

Acid adaptation, which is normally transient, has become a major concern with regard to food safety because it can enhance resistance of *E. coli* O157:H7 (Leyer et al., 1995), *Salmonella* (Leyer et al., 1992) and *L. monocytogenes* (Gahan et al., 1996) to acidic foods. In fact, the ability to induce the ATR could be a significant factor in predicting the fate of pathogens in acidic food.

Surviving *L. monocytogenes* and *Salmonella* exposed to acid adaptation with organic acids (citric and malic acid) in either stationary or exponential phase were challenged against orange juice (Table 1 and 2) and grape juice (Table 3 and 4) stored at 4ºC and 25ºC for up to 15 days. The acid adaptation in stationary phase of the *Listeria* and

Salmonella seems not to exhibit an increase of ATR, which is in agreement with the results previously mentioned in ATR induction.

As regards the cells in exponential phase, the acid adapted cells of both strains showed a remarkable ATR to the low pH of both juices when were kept at 4ºC. Furthermore, in orange juice kept at 25ºC acid adapted *Listeria* and *Salmonella* with respect to its counterpart non adapted showed counts until 8th day and until 15th day, respectively. For acid adapted and non adapted *E. coli* O157:H7 it has been reported a higher viability at 5ºC than at 25ºC (Ryu et al., 1998).

In both juices *Listeria monocytogenes* showed to be more sensitive than *Salmonella*. However, in grape juice its sensitivity was greater at 25ºC and a complete elimination for any type of cell was observed. Comparing the counts at 25ºC of *Salmonella* in orange juice and grape juice, it can be observed that cells are most sensitive in orange juice. In general, in the juices at 25ºC it was observed a decrement of counts and a significant level of sublethal injury for every type of cell over time. It is known that the antimicrobial activity of organic acids in fruit juices becomes more apparent as temperature increases from refrigeration to room temperature (Parish et al., 1997). On the other hand, Ryu et al. (1998) suggested that different nutrient and sugar contents in apple cider or orange juice may influence the rate of death and growth as well as the tolerance of *E. coli* O157:H7 to low pH. In fact, the severest reduction of *L. monocytogenes* observed in grape juice samples could be explained by the presence of substances like phenolic compounds. The antilisterial activity of selected phenolic acids has been previously described (Wen et al., 2003). Rhodes et al. (2006) found that commercial dark grape juice had inhibitory effect against *L. monocytogenes* and other *Listeria* species, decreasing the *L. monocytogenes* number from 6-7 log CFU/ml to not detectable within 10 min.

Although *L. monocytogenes* presented a better ATR in a low pH medium (3 or 3.5, for citric or malic acid, respectively) in fruit juices demonstrated to have a lower ATR, this could be due to different compound content of juices which may have effect on cells. However, in general we can conclude that the exponential acid adapted cells could survive in fruit juices preserved at low temperatures.

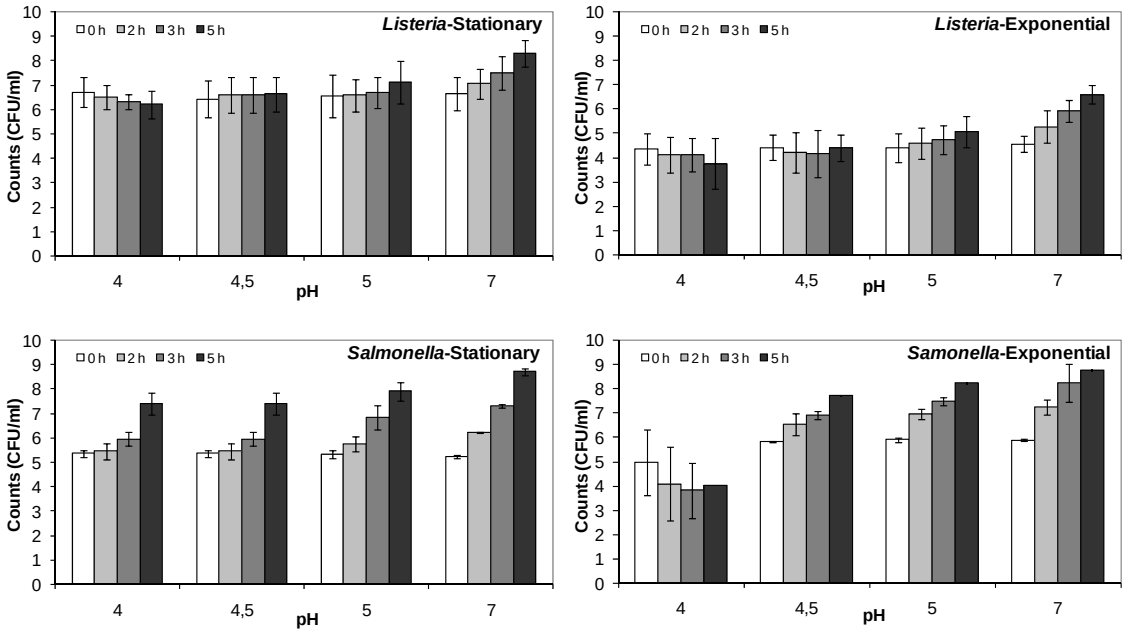

Figure 1. Counts after acid adaptation of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W in their exponential or stationary phase using citric acid as acidulant.

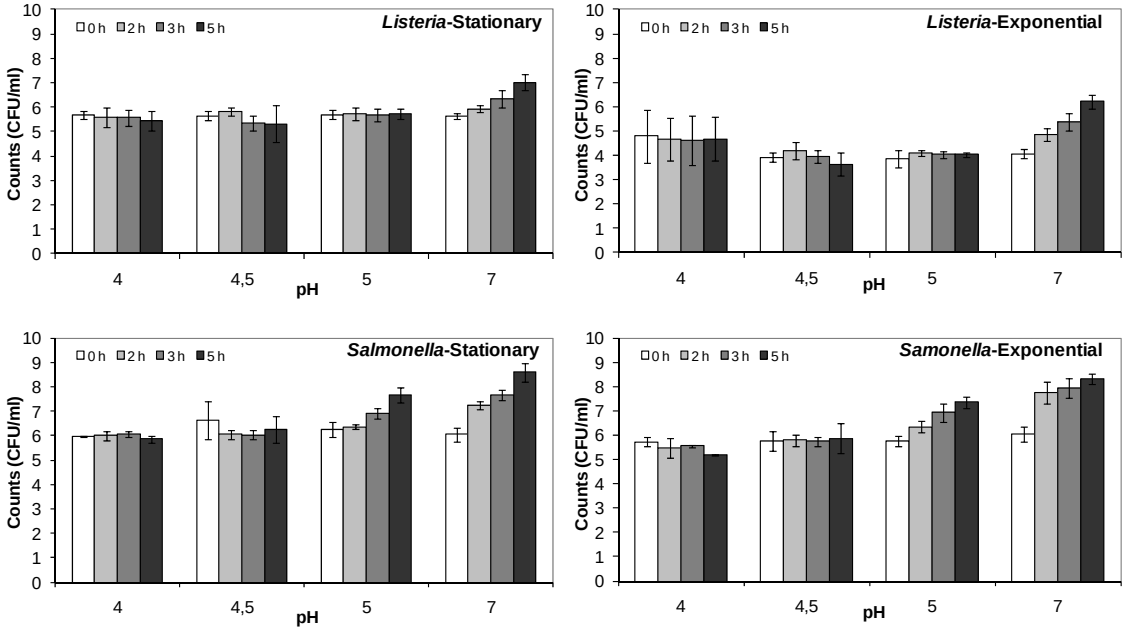

Figure 2. Counts after acid adaptation of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W in their exponential or stationary phase, using malic acid as acidulant.

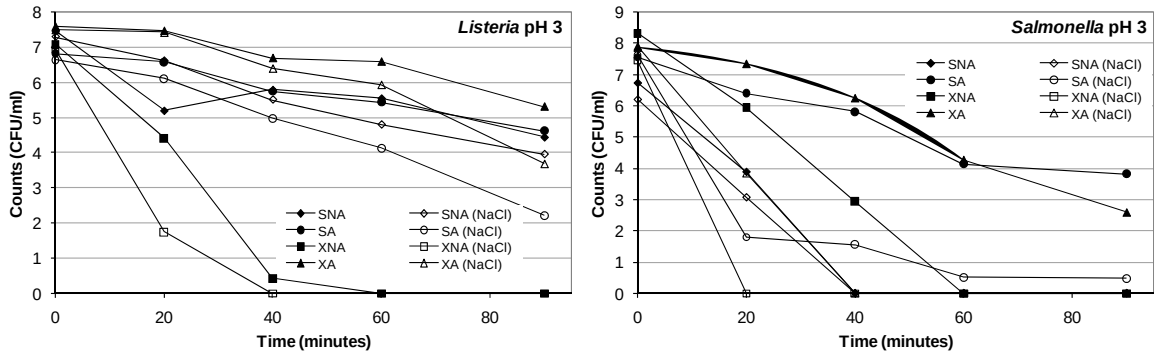

Figure 3. Acid tolerance resistance (ATR) of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W, using citric acid as acidulant.

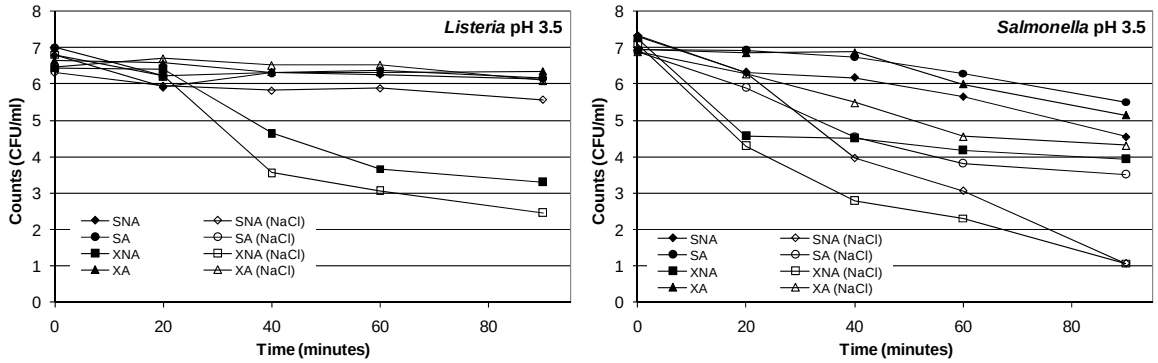

Figure 4. Acid tolerance resistance (ATR) of *L. monocytogenes* and *S. enterica* serovar Senftenberg 775W, using malic acid as acidulant.

Table 1. Survival (log CFU/ml) of *Listeria monocytogenes* in orange juice incubated at 4ºC and 25ºC.

Growing phase/adaptation	Time (days)	4ºC		25ºC	
		TSAYE	**TSAYE+sal**	**TSAYE**	**TSAYE+sal**
Stationary/non-adapted	**0**	7.14±0.06^{aA}	6.77±0.09^{bA}	7.14±0.06^{aA}	6.77±0.09^{bA}
	5	6.74±0.23^{aB}	6.29±0.12^{bB}	5.85±0.23^{cB}	1.27±2.24^{dB}
	8	6.27±0.20^{aC}	6.00±0.12^{bC}	4.63±0.08^{cC}	1.34±1.37^{dB}
	10	5.97±0.71^{aD}	5.75±0.68^{bD}	*ND*^{cD}	*ND*^{cB}
	15	5.64±0.47^{aE}	5.48±0.28^{bE}	*ND*^{cD}	*ND*^{cB}
Stationary/adapted	**0**	7.09±0.03^{aA}	6.95±0.10^{aA}	7.09±0.03^{aA}	6.95±0.10^{aA}
	5	6.58±0.13^{aB}	6.31±0.14^{bB}	5.69±0.20^{cB}	*ND*^{dB}
	8	6.48±0.31^{aB}	6.31±0.19^{aB}	2.13±2.19^{bC}	*ND*^{cB}
	10	5.96±0.70^{aC}	5.87±0.79^{aC}	*ND*^{bD}	*ND*^{bB}
	15	5.70±0.53^{aC}	5.66±0.42^{aC}	*ND*^{bD}	*ND*^{bB}
Exponential/non-adapted	**0**	6.63±0.10^{aA}	6.59±0.05^{aA}	6.63±0.10^{aA}	6.59±0.05^{aA}
	5	6.68±0.60^{aA}	6.13±0.37^{aB}	*ND*^{bB}	*ND*^{bB}
	8	6.03±0.18^{aB}	5.84±0.16^{bC}	*ND*^{bB}	*ND*^{bB}
	10	5.73±0.44^{aC}	5.67±0.51^{aCD}	*ND*^{cB}	*ND*^{cB}
	15	5.63±0.47^{aC}	5.47±0.23^{bD}	*ND*^{cB}	*ND*^{cB}
Exponential/adapted	**0**	6.69±0.06^{aA}	6.71±0.03^{aA}	6.69±0.06^{aA}	6.71±0.03^{aA}
	5	6.58±0.25^{aA}	6.39±0.18^{aB}	3.92±2.36^{bB}	*ND*^{cB}
	8	6.40±0.27^{aA}	6.33±0.32^{aB}	3.34±0.30^{bB}	*ND*^{cB}
	10	5.88±0.81^{aB}	5.95±0.80^{aC}	*ND*^{bC}	*ND*^{bB}
	15	5.75±0.54^{aB}	5.74±0.46^{aC}	*ND*^{bC}	*ND*^{bB}

$^{a-c}$: Rows with different superscript small letter are significantly different ($p < 0.05$).

$^{A-E:}$ Columns with different superscript capital letter are significantly different ($p < 0.05$) for each phase.

ND: No detected (detection limit < 1 CFU/ml)

Table 2. Survival (log CFU/ml) of *Salmonella* Senftenberg in orange juice incubated at 4ºC and 25ºC.

Growing phase/adaptation	Time (days)	4ºC		25ºC	
		TSAYE	**TSAYE+sal**	**TSAYE**	**TSAY+sal**
Stationary/non-adapted	**0**	7.15 ± 0.22^{aA}	7.00 ± 0.13^{aA}	7.15 ± 0.22^{aA}	7.00 ± 0.13^{aA}
	5	7.00 ± 0.25^{aB}	6.85 ± 0.22^{aA}	6.44 ± 0.18^{bB}	5.57 ± 0.33^{cB}
	8	6.82 ± 0.18^{aC}	6.78 ± 0.20^{aA}	5.76 ± 0.07^{bC}	4.08 ± 0.51^{cC}
	10	6.57 ± 0.11^{aD}	6.53 ± 0.15^{aB}	3.83 ± 0.16^{bD}	ND^{cD}
	15	6.08 ± 0.29^{aE}	5.43 ± 0.59^{bC}	ND^{cE}	ND^{cD}
Stationary/adapted	**0**	7.22 ± 0.20^{aA}	6.87 ± 0.15^{aA}	7.22 ± 0.20^{aA}	6.87 ± 0.15^{aA}
	5	6.34 ± 0.09^{aB}	6.53 ± 0.09^{aB}	6.99 ± 0.24^{aA}	5.17 ± 0.40^{bB}
	8	6.42 ± 0.08^{aC}	6.25 ± 0.15^{aC}	5.46 ± 0.28^{bB}	1.77 ± 1.81^{cC}
	10	6.05 ± 0.07^{aD}	6.06 ± 0.06^{aD}	1.57 ± 1.61^{bC}	ND^{cD}
	15	5.31 ± 0.06^{aE}	5.30 ± 0.29^{aE}	ND^{bD}	ND^{bD}
Exponential/non-adapted	**0**	7.38 ± 0.54^{aA}	7.18 ± 0.84^{aA}	7.38 ± 0.54^{aA}	7.18 ± 0.84^{aA}
	5	6.86 ± 0.44^{aB}	6.89 ± 0.56^{aB}	6.58 ± 0.22^{bB}	5.34 ± 0.27^{cB}
	8	6.80 ± 0.43^{aB}	6.74 ± 0.35^{aB}	6.08 ± 0.03^{bC}	3.75 ± 0.32^{cC}
	10	6.55 ± 0.13^{aC}	6.29 ± 0.23^{bC}	4.49 ± 0.22^{cD}	ND^{dD}
	15	5.71 ± 0.23^{aD}	4.63 ± 0.56^{bD}	ND^{cE}	ND^{cD}
Exponential/adapted	**0**	7.87 ± 0.24^{aA}	8.03 ± 0.07^{aA}	7.87 ± 0.24^{aA}	8.03 ± 0.07^{aA}
	5	7.11 ± 0.27^{aB}	7.26 ± 0.28^{aB}	6.61 ± 0.41^{bB}	5.74 ± 0.34^{cB}
	8	7.17 ± 0.22^{aB}	7.27 ± 0.23^{aB}	6.22 ± 0.21^{bC}	4.85 ± 0.41^{cC}
	10	7.23 ± 0.41^{aB}	6.77 ± 0.20^{aC}	4.87 ± 0.06^{bD}	1.68 ± 1.77^{cD}
	15	6.39 ± 0.40^{aC}	5.68 ± 0.61^{bD}	1.12 ± 1.14^{cE}	0.41 ± 0.73^{dE}

$^{a-c}$: Rows means with different superscript small letter are significantly different ($p < 0.05$).

$^{A-E:}$ Columns with different superscript capital letter are significantly different ($p < 0.05$) for each phase.

ND: No detected (detection limit < 1 CFU/ml)

Table 3. Survival (log CFU/ml) of *Listeria monocytogenes* in grape juice incubated at 4ºC and 25ºC.

Growing phase/adaptation	Time (days)	4ºC		25ºC	
		TSAYE	**TSAYE+sal**	**TSAYE**	**TSAYE+sal**
Stationary/non-adapted	**0**	6.56 ± 0.23^{aA}	5.35 ± 0.23^{bA}	6.66 ± 0.10^{aA}	5.36 ± 0.16^{bA}
	5	5.61 ± 0.22^{aB}	4.24 ± 0.07^{bB}	ND^{cB}	ND^{cB}
	8	4.79 ± 0.14^{aC}	3.53 ± 0.19^{bC}	ND^{cB}	ND^{cB}
	10	4.43 ± 0.32^{aC}	2.68 ± 0.10^{bD}	ND^{cB}	ND^{cB}
	15	2.19 ± 0.14^{aD}	1.60 ± 0.33^{bE}	ND^{cB}	ND^{cB}
Stationary/adapted	**0**	7.18 ± 0.05^{aA}	6.41 ± 0.22^{bA}	7.06 ± 0.08^{aA}	6.07 ± 0.29^{cA}
	5	6.49 ± 0.14^{aB}	5.16 ± 0.13^{bB}	ND^{cB}	ND^{cB}
	8	5.44 ± 0.24^{aC}	4.03 ± 0.39^{bC}	ND^{cB}	ND^{cB}
	10	4.71 ± 0.25^{aD}	3.41 ± 0.08^{bD}	ND^{cB}	ND^{cB}
	15	3.60 ± 0.43^{aE}	1.85 ± 0.11^{bE}	ND^{cB}	ND^{cB}
Exponential/non-adapted	**0**	5.89 ± 0.17^{aA}	5.32 ± 0.23^{bA}	5.73 ± 0.14^{aA}	4.34 ± 0.23^{cA}
	5	ND^{B}	ND^{B}	ND^{B}	ND^{B}
	8	ND^{B}	ND^{B}	ND^{B}	ND^{B}
	10	ND^{B}	ND^{B}	ND^{B}	ND^{B}
	15	ND^{B}	ND^{B}	ND^{B}	ND^{B}
Exponential/adapted	**0**	7.21 ± 0.09^{aA}	6.76 ± 0.19^{bcA}	7.07 ± 0.16^{abA}	6.52 ± 0.39^{cA}
	5	6.45 ± 0.13^{aB}	5.29 ± 0.43^{bB}	ND^{cB}	ND^{cB}
	8	5.10 ± 0.04^{aC}	3.93 ± 0.28^{bC}	ND^{cB}	ND^{cB}
	10	4.63 ± 0.21^{aD}	2.33 ± 0.05^{bD}	ND^{cB}	ND^{cB}
	15	3.63 ± 0.55^{aE}	2.01 ± 0.20^{bD}	ND^{cB}	ND^{cB}

$^{a-c}$: Rows means with different superscript small letter are significantly different ($p < 0.05$).

$^{A-E:}$ Columns with different superscript capital letter are significantly different ($p < 0.05$) for each phase.

ND: No detected (detection limit < 1 CFU/ml)

Table 4. Survival (log CFU/ml) of *Salmonella* Senftenberg in grape juice incubated at 4ºC and 25ºC.

Growing phase/adaptation	Time (days)	4ºC		25ºC	
		TSAYE	**TSAYE+sal**	**TSAYE**	**TSAY+sal**
Stationary/non-adapted	**0**	7.50 ± 0.11^{aA}	7.36 ± 0.12^{aA}	7.32 ± 0.01^{aA}	6.94 ± 0.12^{bA}
	5	6.53 ± 0.54^{aB}	6.32 ± 0.38^{aB}	6.24 ± 0.37^{aB}	5.78 ± 0.29^{aB}
	8	6.31 ± 0.04^{aB}	5.95 ± 0.08^{aB}	4.77 ± 0.42^{bC}	4.24 ± 0.47^{cC}
	10	5.83 ± 0.28^{aC}	4.54 ± 0.07^{bC}	3.70 ± 0.11^{cD}	3.35 ± 0.09^{dD}
	15	5.09 ± 0.17^{aD}	4.38 ± 0.43^{abC}	3.72 ± 1.24^{bD}	2.57 ± 0.43^{cE}
Stationary/adapted	**0**	7.80 ± 0.09^{aA}	7.54 ± 0.23^{aA}	7.02 ± 0.14^{bA}	6.28 ± 0.19^{cA}
	5	6.49 ± 0.54^{aB}	5.87 ± 0.25^{bB}	3.69 ± 0.28^{cC}	3.63 ± 0.07^{cB}
	8	5.87 ± 0.09^{aC}	4.90 ± 0.29^{bC}	3.62 ± 0.04^{cC}	3.45 ± 0.08^{cB}
	10	5.16 ± 0.22^{aD}	3.15 ± 0.10^{dD}	4.64 ± 0.27^{bB}	3.45 ± 0.03^{cB}
	15	3.56 ± 0.20^{aE}	1.96 ± 0.77^{bE}	2.41 ± 0.22^{bD}	2.42 ± 0.12^{bC}
Exponential/non-adapted	**0**	7.62 ± 0.13^{aA}	7.35 ± 0.13^{bA}	6.81 ± 0.13^{cA}	5.54 ± 0.19^{dA}
	5	6.34 ± 0.36^{aB}	6.33 ± 0.54^{aB}	5.89 ± 0.44^{aB}	5.68 ± 0.49^{aA}
	8	5.50 ± 0.16^{aC}	4.90 ± 0.11^{abC}	5.20 ± 0.52^{abC}	4.56 ± 0.52^{bB}
	10	4.51 ± 0.25^{aD}	3.55 ± 0.10^{bD}	3.55 ± 0.23^{bD}	3.68 ± 0.20^{bC}
	15	1.73 ± 1.15^{bE}	ND^{cE}	4.65 ± 0.22^{aE}	4.16 ± 0.27^{aB}
Exponential/adapted	**0**	8.05 ± 0.19^{aA}	7.97 ± 0.20^{aA}	7.07 ± 0.07^{bA}	5.84 ± 0.13^{cA}
	5	6.48 ± 0.61^{aAB}	6.37 ± 0.57^{aB}	4.64 ± 0.19^{bAB}	4.44 ± 0.16^{bB}
	8	6.48 ± 0.06^{aAB}	5.78 ± 0.23^{bB}	4.56 ± 0.16^{cAB}	4.61 ± 0.16^{cB}
	10	6.25 ± 0.20^{aAB}	5.64 ± 0.41^{bB}	4.22 ± 0.09^{cAB}	4.34 ± 0.08^{cB}
	15	3.08 ± 2.41^{aB}	1.58 ± 1.83^{aC}	3.21 ± 1.31^{aB}	3.30 ± 0.80^{aC}

$^{a-c}$: Rows means with different superscript small letter are significantly different ($p < 0.05$).

$^{A-E:}$ Columns with different superscript capital letter are significantly different ($p < 0.05$) for each phase.

ND: No detected (detection limit < 1 CFU/ml)

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Paper V

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Impact of ultra high pressure homogenization on pectin methylesterase activity and microbial characteristics of orange juice: A comparative study against conventional heat pasteurization

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ABSTRACT

The effect of different ultra high pressure homogenization (UHPH) treatments on pectin methylesterase (PME) activity and on the contaminating microorganisms of orange juice was investigated. The treatments consisted in combinations of two inlet temperatures (10 or 20 °C), three pressures (100, 200 or 300 MPa) and two holding times (≤0.7 or 30 s). Results were compared with two thermal pasteurization treatments (1 and 2 min at 90 °C). Shelf-life of treated juices was evaluated for a period of 50 days at 4 °C. Mean bacterial counts in raw orange juice were: mesophilic aerobic bacteria (MAB) 4.75 ± 0.48 Log CFU/ml; psychrotrophic aerobic bacteria (PAB) 4.58 ± 0.30 Log CFU/ml; lactic-acid bacteria (LAB) 4.69 ± 0.40 Log CFU/ml; yeasts 4.26 ± 0.16 Log CFU/ml. UHPH treatments using 200 and 300 MPa reduced significantly the counts of all the microbial groups up to 4.38 Log CFU/ml for MAB; to 4.43 Log CFU/ml for PAB; to 4.69 Log CFU/ml for LAB and to undetectable from the rest of the group. No significant differences were observed with the thermal pasteurization. These treatments also reduced the PME activity above the 96% of its initial activity. The effect of increasing the inlet temperature from 10 to 20 °C, or the holding time (time during which the sample remains at the maximum temperature achieved) did not increase the efficacy of treatments above 200 MPa. During the later 50 days of storage at 4 °C neither the microbial count nor the PME activity increased their values and no differences were observed with the pasteurized samples during this period.

Contents

1. Introduction . . . 0
2. Material and methods . . . 0
2.1. Orange juice processing . . . 0
2.2. PME activity measurement . . . 0
2.3. Microbiological analyses . . . 0
2.4. Statistical analysis . . . 0
3. Results and discussion . . . 0
3.1. Effect of UHPH on PME activity . . . 0
3.2. Effect of UHPH on microorganisms . . . 0
3.3. Effect of UHPH on the shelf-life of the orange juice . . . 0
Acknowledgments . . . 0
References . . . 0

1. Introduction

Today, one must consider the change in consumer habits, the increasing demand for fresh juices and the desire for them to be free of chemical preservatives. Thus, fruit juice industries have directed their studies to finding alternative processing technologies to produce

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foods with a minimum of nutritional, physicochemical, or organoleptic changes induced by the technologies themselves. This concept of minimal processing is currently becoming a reality with conventional technologies (mild pasteurization) and non-thermal technologies (Esteve & Frígola, 2007; Tribst, Sant'Ana, & de Massaguer, 2009).

The inactivation of spoilage and pathogenic microorganisms as well as the inactivation of endogenous pectin methylesterase (PME) are prerequisites for the extension of the shelf-life of the juice (Katsaros, Tsevdou, Panagiotou, & Taoukis, 2010). Many organisms, particularly acid-tolerant bacteria and fungi (yeasts and molds), can use fruit as substrate and cause spoilage, producing off-flavors and odors and product discoloration. If the contaminating microorganisms are pathogens, they could also cause human illness. Historically, acid foods such as fruit juices have been considered safe; however, unprocessed orange juice has been recognized as vehicle of foodborne diseases (Burnett & Beuchat, 2001; Parish, 1997, 1998a).

The juice cloud, which is composed of finely divided particulates of pectin, cellulose, hemicellulose, proteins and lipids in suspension (Irwe & Olsson, 1994; Klavons, Bennett, & Vannier, 1994), is considered a desirable characteristic of orange juice; however, it shows a loss of cloudiness and concentrates gelation a short time after squeezing, which has been associated to the PME activity. PME is present in all citrus fruits as a cell wall-bound enzyme forming a complex with pectin through electrostatic interactions. During the juice extraction process the enzyme is released into the juice hydrolysing the pectin (methyl esters of homogalacturonan) and transforming it gradually to low methoxy pectin and pectic acids, which may then form insoluble complexes with calcium ions, leading to precipitation of the pectins and cloud loss (Basak & Ramaswamy, 1996). Several PME isoenzymes are present in oranges, with both the heat-labile and heat-stable forms (Versteeg, Rombouts, Spaansen, & Pilnik, 1980). Thermal pasteurization of juice is based on the PME inactivation level of >90% because its thermotolerance is higher than the majority of spoilage microorganisms found naturally in this type of product (Tribess & Tadini, 2006). Severe conditions (90 °C, 1 min or 95 °C, 30 s) are necessary to inactivate orange PME (Cameron, Baker, & Grohmann, 1998; Do Amaral, De Assis, & De Faria Oliveira, 2005). The required heat treatment to inactivate the heat-stable isoenzymes may result in flavour and aroma changes that reduce the fresh-like attributes of the juice (Farnworth, Lagacé, Couture, Yaylayan, & Stewart, 2001), hence there is much interest in the use of non-thermal processing technologies for the inactivation of PME in citrus fruit juices such as high hydrostatic pressures or pulsed electric fields (Bull et al., 2004; Sampedro, Geveke, Fan, & Zhang, 2009).

Ultra high pressure homogenization (UHPH) is an emergent technology based on the application of high pressures, but that allows to process in continuous fluid foods and that has been proposed as an alternative to the pasteurization for foods with heat-sensitive properties by its germicidal effect. Principles of UHPH are similar to conventional homogenization processes, where a liquid is forced through a valve with a very narrow and adjustable gap achieving high pressure and high velocity and bringing physical changes about in the treated product. The potential of UHPH to inactivate pathogenic and spoilage microorganisms has been previously demonstrated in milk (Briñez, Roig-Sagués, Hernández, & López, 2006a; Briñez, Roig-Sagués, Hernández, & López, 2006c; Briñez, Roig-Sagués, Hernández, & López, 2007; Kheadr, Vachon, Paquin, & Fliss, 2002; Vachon, Kheadr, Giasson, Paquin, & Fliss, 2002), in whole egg (Velázquez-Estrada, Hernandez-Herrero, López-Pedemonte, Guamis-López, & Roig-Sagués, 2008) and in orange juice (Briñez, Roig-Sagues, Hernández, & López, 2006b; Briñez et al., 2006a, 2007; Campos & Cristianini, 2007; Tahiri, Makhlouf, Paquin, & Fliss, 2006; Velázquez-Estrada et al., 2011).This technology has also been tested against enzymes of milk (Hayes & Kelly, 2003) and orange juice (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009), but in the last case the UHPH treatments used were not able to inactivate the PME permanently. In order to achieve better results, it has been suggested the possibility of passing the samples two or more times through the valve, which increases the efficacy of the treatments, but this implies to lose the advantage of the continuity of the process. Recently, several improvements introduced in the design and materials of UHPH equipments allowed to increase the performances of these equipments in order to bring them closer to the industry requests. Among these improvements, is worth to mention the possibility to process higher volumes of product at pressures up to 400 MPa where it can be controlled the processing temperatures and extended the effect of the pressure. Nevertheless the real effectiveness of these improvements on the product properties has not been extensively evaluated to date.

In this study we evaluated the potential of newly designed UHPH equipment for orange juice processing as an alternative to pasteurization. To investigate whether the combination of different conditions could be able to inactivate PME or spoilage microorganisms of orange juice, we assessed two inlet temperatures (10 and 20 °C), three levels of pressure (100, 200 and 300 MPa) and two holding times (≤0.7 and 30 s). We compared the results of these treatments with those of two pasteurization treatments 90 °C for 1 and 2 min commonly used in the juice industry. Shelf-life was also compared during storage at 4 °C for fifty days.

2. Material and methods

2.1. Orange juice processing

Raw orange juice from *Citrus sinensis* var. Valencia was obtained from a local juice manufacturer. Most of the pulp was removed using a 2-mm steel sieve before processing.

Two heat pasteurization treatments (90 °C for 1 and 2 min) were applied to raw juice using pilot scale pasteurizer with a tubular heat exchanger (ATI, Granollers, Barcelona, Spain).

UHPH treatments were applied to raw orange juice using a Stansted ultra high pressure homogenizer (FPG 11300:400 Hygenic Homogenizer Unit, Stansted Fluid Power Ltd., Essex, UK), with a flow rate of 120 L h^{-1}. Previously, orange juice was tempered at the required inlet temperatures (10 or 20 °C), and then was pressurized at 100, 200 and 300 MPa, with ≤0.7 or 30 s holding time. Processed orange juice was cold passing it through a heat exchanger fed with cooled water. The inlet temperatures (Ti) as well as the temperature before the homogenization valve (T1) and the temperature after the homogenization valve (T2), and the final temperature (TF) of the orange juice after passing through the heat exchanger were monitored throughout the experiment. UHPH processed orange juice samples were collected in sterile bottles and stored at 4 °C until being analyzed.

2.2. PME activity measurement

Residual PME activity was carried out using the method described by Rouse and Atkins (1955) using an automatic titrator (Titrando model 842, Metrohm AG, Herisau, Switzerland). PME activity was evaluated by titration of free carboxyl groups at pH 7.5. Briefly, 5 ml of orange sample was added to 50 ml of 1% citrus pectin (Sigma-Aldrich) solution containing 0.3 M of NaCl. The pectin-juice mixture was adjusted to pH 7.5 with 0.02 N NaOH. Then when a stable pH was reached, the consumption of NaOH during a 30-min reaction time was recorded. One unit of PME activity (UPE) was defined as the liberation of 1 μmol of carboxyl groups/minute at pH 7.5. Enzyme activity was calculated according to the following formula:

$$UPE/ml = \frac{(ml.NaOH)(NaOH.normality)(1000)}{(ml.orangejuice)(\min)}$$

PME activities may vary distinctly among juice production lots (Collet, Shigeoka, Badolato, & Tadini, 2005; Hirsch, Förch, Neidhart, Wolf, & Carle, 2008), for that values were converted to % residual

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activity with respect to the unprocessed orange juice sample which represents a PME activity of 100%.

2.3. Microbiological analyses

Microbiological analyses of samples were performed 2 h after treatments and every week for a total of 7 weeks of storage at 4 °C. Samples were diluted appropriately in sterile 0.1% peptone solution (Oxoid, Basingstoke, UK) and plated on total plate count agar (PCA, Oxoid) and incubated at 30 °C for 24 h and 10 °C for 5 days for mesophilic and psychrotrophic bacteria counts, respectively; on rose bengal agar (RB, Oxoid) incubated at 25 °C for 5 days for molds and yeasts count; on de Man Rogosa Sharpe agar (M.R.S., Oxoid) incubated at 30 °C for 5 days for lactic acid bacteria; on violet red bile glucose agar (VRBG, Oxoid) for enterobacteria and on Coli ID agar for both coliforms and *E. coli* (Biomérieux, Marcy l'Etoile, France), incubated at 37 °C for 24 h. Results were expressed as the logarithm of colony-forming units per ml of orange juice (Log CFU/ml).

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered to be significant at $P<0.05$. All statistical analyses were performed with Statistical Package for the Social Sciences 14.0.1 software (SPSS Inc, Chicago, IL). All experiments were repeated three times with duplicated analysis in each replicate.

3. Results and discussion

3.1. Effect of UHPH on PME activity

As shown in Fig. 1, PME activity decreasing was higher as the pressure applied increased, achieving the maximum reduction in the samples treated at 200 and 300 MPa, where no significant differences were observed with respect to samples pasteurized at 90 °C (reduction higher than 96%). No inactivation was found in orange juice treated at 100 MPa, observing reductions between 10 and 28% in the samples prewarmed at 10 and 20 °C. However reductions increased significantly to 47% in samples prewarmed at 20 °C and with a holding time of 30 s. The inactivation level in chilled pasteurized orange juices can be considered acceptable if only 10% of the initial PME activity in the fresh juice remains according to Irwe and Olsson (1994). Although in orange juice samples treated at 200 MPa the residual activity was slightly higher, the stability of the opalescence of the juice remained during storage. These results are very promising since the inactivation of PME activity avoids the loss of turbidity and therefore improves the commercial value of the juice. Previous surveys reported less effective UHPH treatments against PME. Lacroix et al. (2005) reported just a 20% reduction of PME activity after 170-MPa UHPH treatments, and after five passes. Welti-Chanes et al. (2009) reduced 50.4, 49.4 and 38% the PME activity in samples treated at 250 MPa with inlet temperatures of 22, 35 and 45 °C, respectively, after one single pass, and needed to pass the samples 5 times to achieve a 80% of reduction. Although UHPH-treated juices contained residual PME activities, they were significantly more stable. This effect was attributed to modifications of the structure of pectin, making the substrate less available to PME, and also due to particle size reduction resulting from the homogenization treatment (Lacroix et al., 2005). It is known that orange juice contains at least three different forms of PME. Two of the isozymes, PME I and II, are thermolabile (TL-PME) and represent 90% of the total activity. The third form, which is thermostable (TS-PME) has a higher molecular weight and contributes 5–10% of the total activity (Versteeg et al., 1980). Heating at temperatures ≥72 °C inactivate the thermo-labile PE isoenzymes almost completely (Hirsch, Alexandra, Carle, & Neidhart, 2011). According to several authors (Do Amaral et al., 2005; Sampedro et al., 2009) in our study, approximately 4–5% of residual activity probably corresponding to thermostable PME remained after the 90 °C treatments. This form probably destabilized juice cloud under cold storage (Cameron et al., 1998).

On instead of in PME is described as a pressure-tolerant enzyme, being stable at pressures up to 400 MPa when other pressure-based treatments, such as high hydrostatic pressure (HHP), are used. Combinations of high pressures with high temperatures (55 °C and 700 MPa for 2 min) have been suggested as necessary to inactivate PME (Sampedro, Rodrigo, & Hendrickx, 2008). Considering this, it would be assumed that at the pressures we used (up to 300 MPa) the treatment would be insufficient to achieve a significant reduction in the PME activity. In our case UHPH showed to be as efficient as pasteurization inactivating PME. The probable reason is that the effect of UHPH on the PME is not only dependent on the pressure, but also on the consequences of the exposition to hydrodynamic cavitations, impingement against static surfaces, high turbulences and fluid shears generated when the sample passes through the valve. All these have as a consequence, among others, a temperature increase due to heat dissipation of kinetic energy in the high-pressure valve (López-Pedemonte, Briñez, Roig-Sagués, & Guamis, 2006). Table 1 shows the temperatures of different stages during the UHPH treatment. The increase of temperature during UHPH treatments is a consequence of the adiabatic heating generated in the machine in addition to the high turbulence, shear, and cavitation forces that the fluid suffers in the homogenization valve (Hayes & Kelly, 2003; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003). Nevertheless, the holding time at the higher temperature is very short (≤0.7 s) and this imply minimum adverse effects on flavour and nutrients occasioned by heating. The T2 temperature (maximum temperature reached by the sample just after having passed through the valve) even at minimum holding time and in combination with pressure would probably explain the increased inactivation of PME with respect to HHP, but would not explain the differences with respect to other similar UHPH

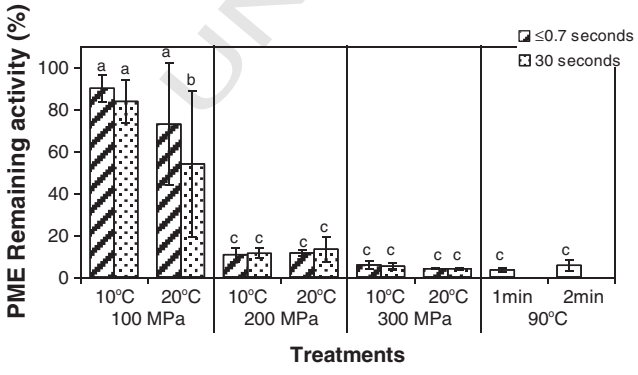

Fig. 1. Residual activity of PME in UHPH and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation. Bars with different superscript letter are significantly different ($P<0.05$).

Table 1 t1.1
Temperature and pressure changes of orange juice during UHPH processing. Data are presented as the mean value of three replications ± standard deviation.

Ti (°C)	Homogenisation pressure (Mpa)	T1 (°C)	T2 (°C)	TF (°C)
9.0 ± 1.4	112.1 ± 11.7	20.0 ± 6.4	41.8 ± 2.4	20.0 ± 2.7
9.3 ± 1.5	218.2 ± 4.6	23.4 ± 5.6	67.7 ± 0.7	24.0 ± 2.2
9.7 ± 1.3	319.8 ± 11.5	27.5 ± 4.2	91.9 ± 2.0	23.0 ± 2.4
18.9 ± 1.4	108.7 ± 13.1	31.3 ± 3.9	48.3 ± 2.3	20.0 ± 2.9
19.0 ± 1.9	209.8 ± 5.4	34.3 ± 4.0	72.1 ± 1.5	23.8 ± 1.6
19.0 ± 2.5	310.4 ± 7.3	37.8 ± 4.1	95.3 ± 2.8	22.3 ± 2.9

t1.2 t1.3 t1.4 t1.5 t1.6 t1.7 t1.8 t1.9

Ti = inlet temperature; T1 = temperature before the first homogenization valve; T2 = temperature before the second homogenization valve. All values are means (± standard deviations) of data from 3 independent experiments. t1.10

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Table 2
Microbial counts of unprocessed orange juice and after UHPH and pasteurization treatment. Data are presented as the mean value of three replications ± standard deviation.

Treatment	Counts (Log CFU/ml)						
	Mesophilic	Psychrotrophic	Lactic-acid bacteria	Yeasts	Molds	Enterobacteria	Coliforms
Unprocessed juice	4.75 ± 0.48 a	4.58 ± 0.30 a	4.69 ± 0.40 a	4.26 ± 0.16 a	2.77 ± 0.58 a	2.06 ± 0.67 a	0.35 ± 0.36 ab
100 MPa/10 °C/0 s	4.78 ± 0.39 a	4.30 ± 0.04 a	4.50 ± 0.14 ab	4.08 ± 0.13 ab	2.56 ± 0.70 a	2.14 ± 0.13 a	0.23 ± 0.29 ab
100 MPa/10 °C/30 s	4.48 ± 0.51 a	4.16 ± 0.13 a	4.27 ± 0.22 b	3.93 ± 0.07 bc	2.48 ± 0.52 a	2.12 ± 0.17 a	0.42 ± 0.51 a
100 MPa/20 °C/0 s	4.32 ± 0.55 a	3.94 ± 0.62 a	4.45 ± 0.37 ab	3.70 ± 0.19 c	2.31 ± 0.37 a	1.93 ± 0.21 a	ND b
100 MPa/20 °C/30 s	3.88 ± 0.24 a	3.69 ± 0.34 a	3.86 ± 0.31 c	3.07 ± 0.67 d	2.11 ± 1.35 a	1.36 ± 1.06 b	ND b
200 MPa/10 °C/0 s	0.89 ± 1.04 b	0.37 ± 0.74 cd	ND d	ND e	ND b	ND c	ND b
200 MPa/10 °C/30 s	1.47 ± 1.69 b	1.61 ± 1.86 b	ND d	ND e	ND b	ND c	ND b
200 MPa/20 °C/0 s	0.85 ± 0.91 b	0.37 ± 0.68 cd	ND d	ND e	ND b	ND c	ND b
200 MPa/20 °C/30 s	0.59 ± 0.92 b	0.41 ± 0.66 cd	0.14 ± 0.35 d	ND e	ND b	ND c	ND b
300 MPa/10 °C/0 s	0.37 ± 0.74 b	0.25 ± 0.50 cd	0.50 ± 0.58 d	ND e	ND b	ND c	ND b
300 MPa/10 °C/30 s	1.34 ± 1.55 b	1.40 ± 1.62 bc	ND d	ND e	ND b	ND c	ND b
300 MPa/20 °C/0 s	1.05 ± 1.15 b	0.20 ± 0.57 cd	ND d	ND e	ND b	ND c	ND b
300 MPa/20 °C/30 s	0.43 ± 0.67 b	0.17 ± 0.41 cd	ND d	ND e	ND b	ND c	ND b
90 °C/1 min	0.71 ± 0.92 b	0.15 ± 0.47 cd	ND d	ND e	ND b	0.16 ± 0.34 c	ND b
90 °C/2 min	ND b	ND d	ND d	ND e	ND b	ND c	ND b

Columns with different letters are significantly different ($P<0.05$).

treatments reported in other surveys. It is clear that the different design and performances of the equipment would have been determinant to justify these differences in some cases. One of the new features included in our equipment is the possibility of keeping the maximum temperature for up to 30 s before cooling the sample, in order to magnify the thermal effect of UHPH the treatment, but this showed to be partially useful only at the lowest pressure (100 MPa), when the sample was prewarmed at 20 °C. In that case, the T2 was closer to 50 °C. At pressures above 200 MPa no significant effects were observed probably because the combined effect of the pressure with the T2 (above 67 °C) would have been enough to achieve an almost complete and irreversible inactivation of the enzyme.

3.2. Effect of UHPH on microorganisms

Table 2 shows the microbial counts of the unprocessed (raw) orange juice, as well as the remaining microbial counts after applying the UHPH and pasteurization treatments on the juice. The mean value of mesophilic bacteria counts observed in the raw juice was 4.75 Log CFU/ml. Among them, the predominant microbial groups were probably LAB (4.69 Log CFU/ml) and yeasts (4.26 Log CFU/ml), which is in accordance with previous surveys reported in the literature (Elez-Martinez, Escola-Hernandez, Soliva-Fortuny, & Martin-Belloso, 2005; Parish, 1998b). Microbial counts of samples treated at 100 MPa were not significantly different from those of raw juice. Nevertheless, UHPH treatments at 200 and 300 MPa showed to be more effective against all the bacterial groups, reaching reductions above 4 Log CFU/ml in most of the cases and no significant differences were observed between these samples and the pasteurized ones. As the reduction observed in the microbial load was the maximum possible, we could not evaluate whether the higher inlet temperatures or the 30 s holding time increased the effectiveness of the UHPH treatments, except for the 100 MPa treatments, where a slightly higher reduction were observed for the LAB, yeasts and enterobacteria counts (0.83, 1.19 and 0.7 Log CFU/ml, respectively). In that case, application of a 30 s retention time showed to be more effective than increasing the inlet temperature. In previous surveys of our Group (Briñez et al., 2006a, 2006b, 2007), performed with orange juice inoculated with different foodborne pathogen strains (*Escherichia coli,* including serotype O157:H7, *Listeria innocua* and *Staphylococcus aureus*) using UHPH treatments at 300 MPa with inlet temperatures of 6 and 20 °C, they observed count reductions between 3 and 4 Log CFU/ml. Tahiri et al. (2006) evaluated the effectiveness of UHPH treatments with pressures ranging from 100 to 200 MPa against different strains of spoilage bacteria in orange juice (*Saccharomyces cerevisiae, Lactobacillus plantarum, L. mesenteroides* and *Penicillium* spp.) and determined that at least three passes through the valve were required to achieve a reduction of the microbial load not higher than 3 Log CFU/ml (except for *Penicillium* spp.). In this survey the inlet temperature used (25 °C) was even higher than that we used. More recently, Welti-Chanes et al. (2009) tested the effectiveness of

Table 3
Mesophilic bacteria counts (Log CFU/ml) of unprocessed; UHPH pressurized and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation.

Processing treatments	Days				
	8	15	22	36	50
Unprocessed juice	5.51 ± 0.31a	6.36 ± 0.62a	6.81 ± 0.32a		
100 MPa/10 °C/0 s	5.01 ± 0.05ab	5.96 ± 0.43a	6.50 ± 0.33a		
100 MPa/10 °C/30 s	4.31 ± 0.62ab	5.31 ± 0.04a	6.44 ± 0.46a		
100 MPa/20 °C/0 s	5.33 ± 0.99a	4.69 ± 0.71a	6.05 ± 0.53a		
100 MPa/20 °C/30 s	4.06 ± 0.96b	5.04 ± 1.57a	5.42 ± 1.89a		
200 MPa/10 °C/0 s	1.14 ± 1.31cde	0.80 ± 1.13bc	2.67 ± 3.09b	3.24 ± 3.03a	1.56 ± 1.18a
200 MPa/10 °C/30 s	1.57 ± 1.30 cd	2.13 ± 0.82b	2.05 ± 0.86bc	2.34 ± 0.75ab	2.12 ± 1.13a
200 MPa/20 °C/0 s	0.50 ± 0.78cde	1.22 ± 1.38bc	0.71 ± 0.98c	1.16 ± 1.33ab	1.20 ± 1.35a
200 MPa/20 °C/30 s	0.57 ± 0.89cde	0.65 ± 1.01bc	0.65 ± 1.01c	0.94 ± 1.07ab	0.89 ± 1.02a
300 MPa/10 °C/0 s	1.50 ± 0.59cde	1.16 ± 1.34bd	1.98 ± 0.78bc	1.93 ± 0.47ab	1.53 ± 1.10a
300 MPa/10 °C/30 s	1.83 ± 0.52c	1.72 ± 0.68bc	1.39 ± 1.60bc	2.10 ± 0.72ab	1.60 ± 1.34a
300 MPa/20 °C/0 s	0.66 ± 1.03cde	0.99 ± 1.09bc	0.64 ± 0.72c	1.07 ± 1.22ab	0.99 ± 1.18a
300 MPa/20 °C/30 s	0.41 ± 0.66cde	0.51 ± 0.81bc	0.38 ± 0.85c	0.22 ± 0.53b	0.55 ± 0.85a
90 °C/1 min	0.13 ± 0.35de	0.64 ± 0.83bc	0.30 ± 0.42c	0.96 ± 1.25ab	1.19 ± 1.76a
90 °C/2 min	NDe	NDc	NDc	NDb	0.15 ± 0.30a

Columns with different letters are significantly different ($P<0.05$).

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Table 4
Psychrotrophic bacteria counts (Log CFU/ml) of unprocessed; UHPH pressurized and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation.

Processing treatments	Days				
	8	15	22	36	50
Unprocessed juice	5.42 ± 0.37a	6.57 ± 0.94a	6.76 ± 0.30a		
100 MPa/10 °C/0 s	4.95 ± 0.08ab	6.13 ± 0.38ab	6.58 ± 0.25a		
100 MPa/10 °C/30 s	4.38 ± 0.46ab	5.42 ± 0.09abc	6.39 ± 0.39a		
100 MPa/20 °C/0 s	5.32 ± 0.99a	4.32 ± 0.66c	6.07 ± 0.59a		
100 MPa/20 °C/30 s	3.88 ± 0.86b	5.01 ± 1.57bc	5.40 ± 1.92a		
200 MPa/10 °C/0 s	1.19 ± 1.37 cd	1.15 ± 0.21de	3.09 ± 2.87b	3.13 ± 3.06a	0.77 ± 0.89a
200 MPa/10 °C/30 s	2.07 ± 0.98c	2.15 ± 1.16 d	1.61 ± 1.86c	1.86 ± 1.43ab	1.34 ± 1.55a
200 MPa/20 °C/0 s	0.28 ± 0.69 d	0.13 ± 0.35e	NDc	0.37 ± 0.68b	0.63 ± 1.17a
200 MPa/20 °C/30 s	0.55 ± 0.85 d	0.38 ± 0.60e	0.38 ± 0.60c	0.48 ± 0.76b	NDa
300 MPa/10 °C/0 s	1.06 ± 1.22 cd	1.06 ± 1.22de	1.40 ± 1.11c	1.41 ± 1.03ab	0.37 ± 0.74a
300 MPa/10 °C/30 s	1.35 ± 1.56 cd	1.32 ± 1.52de	1.32 ± 1.53c	1.32 ± 1.53ab	0.74 ± 0.85a
300 MPa/20 °C/0 s	0.25 ± 0.60 d	NDe	NDc	0.60 ± 0.66b	0.35 ± 0.64a
300 MPa/20 °C/30 s	NDd	NDe	NDc	NDb	NDa
90 °C/1 min	NDd	NDe	0.26 ± 0.55c	0.77 ± 1.10b	0.83 ± 1.75a
90 °C/2 min	NDd	NDe	NDc	NDb	NDa

Columns with different letters are significantly different ($P<0.05$).

UHPH treatments against mesophiles and yeasts plus moulds at three inlet temperature (22, 35 and 45 °C) and two levels of pressure (200 and 250 MPa), reporting that the microbial counts in orange juice homogenized five times at 100 and 250 MPa were less that 3.0 and 3.3 Log CFU/ml for mesophiles and yeasts plus moulds, respectively. We observed higher reductions using a single pass and with lower inlet temperatures. Probably differences on the design of the valves can explain these differences observed in the effectiveness of the treatments.

3.3. Effect of UHPH on the shelf-life of the orange juice

In order to determine the shelf-life of the UHPH treated orange juice we followed the evolution of the mesophilic and psychtrophic bacteria, yeasts and LAB during cold storage at 4 °C, comparing it with those of the pasteurized and the unprocessed (raw) orange juice samples (Tables 3– 6). After 15–22 days of cold storage, there was an increase of 4 to 6 Log CFU/ml in bacterial counts as well as yeasts and LAB in the unprocessed orange juice samples and samples pressurized at 100 MPa. However, in samples pressurized at 200 and 300 MPa the microbial changes after 50 days of storage were minimal (counts lower than 3 Log CFU/ml). Best results were observed in samples processed at 200 MPa when juice was prewarmed at 20 °C and in samples processed at 300 MPa at any inlet temperature. No significant differences were observed between counts in these samples and those observed in the orange juice pasteurized at 90 °C for 1 or 2 min. The possible effect of the 30 s holding time on the counts could be only appreciated in the samples pre-warmed at 20 °C and treated at 100 MPa, were the final counts at day 22 were slightly ($P<0.05$) lower (about 5 Log CFU/ml).

Patrignani, Vannini, Kamdem, Lanciotti, and Guerzoni (2009) evaluated the survival and growth of *S. cerevisiae* in apricot and carrot juices during the storage at 4 °C for 216 h after a 100 MPa UHPH treatment and several passes. They observed that in all cases *S. cerevisiae* was able to recover and grow up, even after 8 passes treatment in apricot juice, although they observed that the higher was the number of passes applied the lower was the final count observed. Our results are difficult to compare since we used only one pass through the valve at 100 MPa, and this pressure showed to be insufficient to avoid the spoilage of the orange juice. However, we can conclude that increasing the pressure in 100 MPa is much more effective in preventing the proliferation of the spoilage microbiota of juice and, consequently, in increasing the shelf-life of the product, than increasing the number of passes.

With respect to the PME activity during shelf-life, Welti-Chanes et al. (2009) reported that PME activity of orange juice increased significantly its activity during the storage at 4 °C during 12 days, even in the samples that were previously treated 5 times at 250 MPa at an inlet temperature of 45 °C. These authors pointed the increased PME activity could

Table 5
Lactic acid bacteria counts (Log CFU/ml) of unprocessed; UHPH pressurized and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation.

Processing treatments	Days				
	8	15	22	36	50
Unprocessed juice	5.46 ± 0.32a	6.23 ± 0.64a	6.74 ± 0.41a		
100 MPa/10 °C/0 s	5.01 ± 0.13ab	5.90 ± 0.39a	6.33 ± 0.50a		
100 MPa/10 °C/30 s	4.45 ± 0.54bc	5.09 ± 0.02a	6.31 ± 0.34a		
100 MPa/20 °C/0 s	4.88 ± 0.48ab	4.81 ± 0.49a	6.01 ± 0.49a		
100 MPa/20 °C/30 s	3.96 ± 0.85c	4.86 ± 1.53a	5.36 ± 1.90a		
200 MPa/10 °C/0 s	NDd	2.40 ± 2.78b	2.73 ± 3.15b	3.42 ± 2.79a	0.96 ± 1.11a
200 MPa/10 °C/30 s	NDd	2.06 ± 1.23b	NDc	NDb	1.39 ± 1.61a
200 MPa/20 °C/0 s	0.57 ± 0.89 d	0.13 ± 0.35c	NDc	0.71 ± 1.31b	0.70 ± 1.30a
200 MPa/20 °C/30 s	0.38 ± 0.60 d	0.05 ± 0.12c	0.48 ± 0.76c	NDb	0.64 ± 0.99a
300 MPa/10 °C/0 s	NDd	1.15 ± 1.33bc	0.33 ± 0.65c	NDb	0.92 ± 1.06a
300 MPa/10 °C/30 s	NDd	1.27 ± 1.47bc	NDc	NDb	1.18 ± 1.36a
300 MPa/20 °C/0 s	NDd	NDc	NDc	0.60 ± 1.11b	0.49 ± 0.90a
300 MPa/20 °C/30 s	0.17 ± 0.41 d	NDc	NDc	NDb	NDa
90 °C/1 min	NDd	NDc	NDc	0.52 ± 1.10b	0.85 ± 1.79a
90 °C/2 min	NDd	NDc	NDc	NDb	NDa

Columns with different letters are significantly different ($P<0.05$).

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Table 6
Yeast counts (Log CFU/ml) of unprocessed; UHPH pressurized and pasteurized orange juice. Data are presented as the mean value of three replications ± standard deviation.

Processing treatments	Days				
	8	15	22	36	50
Unprocessed juice	5.18 ± 0.45a	6.09 ± 0.70a	6.64 ± 0.37a		
100 MPa/10 °C/0 s	4.61 ± 0.28ab	5.76 ± 0.30a	5.31 ± 1.26ab		
100 MPa/10 °C/30 s	4.00 ± 0.30b	5.13 ± 0.02ab	6.03 ± 0.79ab		
100 MPa/20 °C/0 s	4.44 ± 0.67ab	4.17 ± 0.87b	5.85 ± 0.83ab		
100 MPa/20 °C/30 s	2.76 ± 2.29c	4.35 ± 2.37b	4.72 ± 2.76b		
200 MPa/10 °C/0 s	NDd	2.44 ± 2.82c	2.79 ± 3.22c	2.90 ± 3.35a	NDa
200 MPa/10 °C/30 s	NDd	NDd	0.15 ± 0.30 d	NDb	NDa
200 MPa/20 °C/0 s	NDd	NDd	NDd	NDb	NDa
200 MPa/20 °C/30 s	NDd	NDd	NDd	NDb	NDa
300 MPa/10 °C/0 s	NDd	NDd	NDd	NDb	NDa
300 MPa/10 °C/30 s	NDd	NDd	NDd	NDb	NDa
300 MPa/20 °C/0 s	NDd	NDd	NDd	NDb	NDa
300 MPa/20 °C/30 s	NDd	NDd	NDd	NDb	NDa
90 °C/1 min	NDd	NDd	NDd	0.52 ± 1.10b	0.84 ± 1.76a
90 °C/2 min	NDd	NDd	NDd	NDb	NDa

Columns with different letters are significantly different ($P<0.05$).

be due to isoenzymes arising throughout the storing of orange juice. Nevertheless, we did not observe any recovery in the PME activity after the 50 days of storage in the samples where the PME was inactivated by the UHPH treatment; these samples showed a homogeneous cloudy appearance. Lacroix et al. (2005) reported that the stability of orange juice after UHPH processing could be associated to the inactivation of the enzyme pectin methylesterase and to the dispersion of particles caused by the homogenization. In our study, we observe that when the pressure of the treatment increased, the size of the particle diminished considerably; while no significant differences were appreciated between the particle size of raw and pasteurized juices (data not shown).

This study confirms that UHPH is effective as a treatment for the control ~~of~~ both PME activity and spoilage bacteria of orange juice. Treatments at 200 MPa, prewarming the samples at 20 °C, or treatments at 300 MPa would be good alternatives to thermal pasteurization. Nevertheless, it would be necessary to determine the advantage of this technology in preserving other important characteristics of the orange juice, such as the sensorial, functional and nutritive properties, before proposing UHPH as a real alternative to thermal pasteurization.

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Paper VI

Influence of ultra high pressure homogenization on physicochemical properties of orange juice in comparison with traditional thermal processing

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Key words: UHPH, orange juice,

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Abstract

The effect of different ultra high pressure homogenization (UHPH) treatments on the physical attributes (cloud stability and particle size distribution) as well as on general quality parameters (color, pH, °Brix, titratable acidity, reducing sugars and non-enzymatic browning index) of orange juice was studied. The UHPH treatments consisted in combinations of two inlet temperatures (10 or 20ºC) and three level pressures (100, 200 or 300 MPa). Results were compared with thermal pasteurized samples (90 ºC, 1 min). The non-enzymatic browning index and pH of the UHPH treated orange juice did not significantly differ from either fresh or pasteurized juice. UHPH treated juices showed the best particle size distribution as well as cloudiness values. The smallest particles were observed in samples treated at 300 MPa. UHPH treated orange juice showed apparently a brighter color than the fresh and the heat-pasteurized orange juices. In the consumer acceptability study, however, the UHPH samples obtained the lowest score in terms of color.

INTRODUCTION

Orange juice is probably the most popular fruit juice worldwide owing to its high nutritional value and desirable sensory characteristics. Sugars and organic acids are among the major components of orange juice representing about 80% and 10%, respectively, of the total soluble solid content (Kimball, 2001). It is well known that the visual appearance related with cloudiness and color of orange juices influences considerably the consumers' preferences. The cloud particles impart the characteristic flavor, color and mouthfeel to orange juice (Croak et al., 2006). The color of orange juice is one of the parameters assessed for their commercial classification in the US, so the legislation in this country attaches great importance to the measurement of this parameter (Melendez-Martinez et al., 2005). Cloud in citrus consists of four major components. These particles are chromoplastids (colored, smooth surface, 1 μm diameter), pulp (rag-like, 2 to 10 μm) or cell wall fragments, spherical oil droplets (~1 μm), and needle-like crystals of hesperidin (0.5 to 3 μm long, 0.05 to 0.2 μm thick). Crystals present in cloud can be made either of hesperidin or naringin, depending on the citrus specie, and may be a minor component except in orange varieties (Beveridge, 2002). The pectin methyl esterase (PME) induced instability is caused by a sequence of events resulting in the interactions of the protein, carbohydrate, and hesperidin crystal components of cloud, resulting in complex formation and precipitation (Shomer et al., 1999).

Commercial orange juice is thermally processed to inactivate PME and the spoilage microorganisms (Eagerman et al., 1976; Ackerley et al., 2002) extending its shelf-life, but this also lowers notably its vitamin content and deteriorates somehow its flavor, aroma, and color (Parish, 1998; Farnworth et al., 2001). Although thermal-treated orange juices remain as the most common, consumers are demanding nowadays a more natural product in consonance with their increasing concern about the wholesomeness of foodstuffs in general. Thus, at present, there is a renewed interest within the citrus industry in the development of innovative practices to meet the demand of higher quality orange juices, as a consequence of which a noticeable rise in the consumption of direct orange juices not subjected to thermal treatments has taken place in the last years (Shomer et al., 1999; Farnworth et al., 2001; Meléndez-Martinez et al., 2007). Due to technological development, high-hydrostatic pressure (HHP) processing, pulsed electric

fields (PEF) (Sanchez-Moreno et al., 2003; Meléndez-Martinez et al., 2007) ultrasound (Valero et al., 2007), ohmic heating (Leizerson et al., 2005), dense phase carbon dioxide (DPCD) (Leizerson et al., 2005; Niu et al., 2010) and ultra-high pressure homogenization (UHPH) processing (Welti-Chanes et al., 2009) have received increased attention.

Recently, several improvements introduced in the design and materials of UHPH equipments allowed to increase the performances of these equipments in order to bring them closer to the industry requests. UHPH is an emergent technology based on the application of high pressures that allows to process in continuous fluid foods and that has been proposed as an alternative to the pasteurization for foods with heat-sensitive properties by its germicidal effect. Principles of UHPH are similar to conventional homogenization processes, were a liquid is forced through a valve with a very narrow and adjustable gap achieving a high pressure and high velocity and bringing physical changes about in the treated product. The potential of UHPH to inactivate pathogenic and spoilage microorganisms and enzymes have been previously demonstrated in orange juice (Tahiri et al., 2006; Briñez et al., 2006a; Briñez et al., 2006b; Briñez et al., 2007; Lacroix et al., 2005, Campos et al., 2007; Welti-Chanes et al., 2009; Velázquez-Estrada et al., 2011a; Velázquez-Estrada et al.2011b). Some reports about the effect of high pressure homogenization on the juice stability has been performed (Lacroix et al., 2005) but there was none about the effect of UHPH processing on the juice particles size distribution, which also are greatly significant for the cloud stability of orange juice (Croak et al., 2006).

Our research was focused on evaluating the effect of different UHPH treatments on the physicochemical attributes of orange juice, especially on the particle size distribution and cloudiness value. In addition, general quality parameters, such as color, °Brix, titratable acidity and non-enzymatic browning index were also investigated. After selecting the best UHPH treatment (inlet temperature=20, pressure= 300 MPa), microbiological shelf-life during 90 days of storage at 6 and 20ºC as well as the sensory attributes in comparison with their pasteurized counterpart were evaluated.

MATERIAL AND METHODS

Product and processing treatments

Oranges (*Citrus sinensis* L. var.Valencia) were washed and pressed to obtain juice. To remove the thickest part of orange pulp the juice was filtered using a 2 mm steel sieve. A pilot scale pasteurizer with a tubular heat exchanger (ATI, Granollers, Barcelona, Spain) was used for conventional heat pasteurization (90 °C for 1 min). UHPH treatments were applied using a Stansted ultra-high pressure homogenizer (FPG 11300:400 Hygienic Homogenizer Unit, Stansted Fluid Power Ltd., Essex, UK), with a flow rate of 120 L h^{-1}. Juice was pressurized at 100, 200 and 300 MPa. Previously, orange juice was tempered at the required inlet temperatures (10 or 20ºC). Processed juice was cold passing it through a heat exchanger feed with cold water. The inlet temperatures (Ti), the temperature before the homogenization valve (T1), the temperature after the homogenization valve (T2) as well as the final temperature (TF) of the orange juice after passing through the heat exchanger were monitored throughout the experiment. An increase of temperature during UHPH treatments was observed as a consequence of the adiabatic heating generated in the machine in addition to the high turbulence, shear, and cavitation forces that the fluid suffers in the homogenization valve (Table 1).

Table 1. Temperature and pressure changes of orange juice during UHPH processing. Data are presented as the mean value of three replications ± standard deviation.

Ti (ºC)	Homogenization pressure (MPa)	T1 (ºC)	T2 (ºC)	TF (ºC)
9.0 ± 1.4	112.1 ± 11.7	20.0 ± 6.4	41.8 ± 2.4	20.0 ± 2.7
9.3 ± 1.5	218.2 ± 4.6	23.4 ± 5.6	67.7 ± 0.7	24.0 ± 2.2
9.7 ± 1.3	319.8 ± 11.5	27.5 ± 4.2	91.9 ± 2.0	23.0 ± 2.4
18.9 ± 1.4	108.7 ± 13.1	31.3 ± 3.9	48.3 ± 2.3	20.0 ± 2.9
19.0 ± 1.9	209.8 ± 5.4	34.3 ± 4.0	72.1 ± 1.5	23.8 ± 1.6
19.0 ± 2.5	310.4 ± 7.3	37.8 ± 4.1	95.3 ± 2.8	22.3 ± 2.9

Ti = inlet temperature; T1 = temperature before the first homogenization valve; T2 = temperature before the second homogenization valve. All values are means (± standard deviations) of data from 3 independent experiments

Methods of analysis

pH and Titratable acidity (TA). pH and TA determination were measured using an automatic titrator (model Titrando 842, Metrohm, Herisau, Switzerland). The fruit juice was titrated with 0.1M NaOH (Panreac, Montcada i Reixac, Barcelona, Spain) to endpoint of pH 8.1. Results were expressed as grams of citric acid per 100 ml orange juice (IFJJP, 1996).

Total Soluble Solids (TSS). TSS were measured by using a Spectronic Instruments refractometer (Rochester, NY, USA) at 20 ºC. Results were reported as ºBrix.

Determination of total and reducing sugars

Total and reducing sugars were determined using the Luff-Schoorl method. Results were calculated as grams of glucose in 100 ml of sample (IFJJP, 1985).

Non-enzymatic browning index

Five ml of ethyl alcohol (95%, Panreac) was added to 5 ml of orange juice sample, and then this mix was centrifuged at 7800 g for 10 minutes. The absorbance of the supernatant was read at 420 nm (Cecil Instruments, Cambridge, UK). The value obtained was considered as the non-enzymatic browning index NEBI described by (Meydav et al., 1977).

Cloudiness determination and particle size analysis

Orange juice samples were centrifuged at 1500 rpm for 10 min at room temperature. Cloudiness was measured as supernatant absorbance at 660 nm (Cecil Instruments, Cambridge, UK) at 660 nm (Krop, 1974).

The particle size distribution in orange juice samples was determined using a Mastersizer Micropulus 2.15 (Malvern Instruments, Inc., Worcs, UK). Orange juice samples were diluted in distilled water to reach appropriated laser obscuration (12%). An optical model based on the Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index = 1.520; refractive index of fluid (water) = 1.330. The size distribution was characterized by the diameter below which 50 or 90% of the volume of particles are found (d0.5 and d0.9, respectively), the

Sauter diameter (surface-weighted mean diameter, $D_{3,2}$), and the volume-weighted mean diameter ($D_{4,3}$) value.

Color measurements

Color values of orange juice samples were determined using a Hunter Lab colorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, VA). Color coordinates were measured with an illuminant of D65 and a standard observer of 10° and the colorimeter was calibrated against white and black tile standards. Fifty milliliters of each orange juice sample was warmed to 20°C before analysis. The Commission Internationale de l'Eclairage (CIE) L*, a*, and b* values were measured in triplicate. The L* value represents the lightness with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser; the a* and b* axes have no specific numerical limits and represent chromatic components. Positive values of a* are red and negative values are green, whereas positive values of b* are yellow and negative ones are blue. The total color difference was calculated by applying the formula, $\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$.

Microbiological analysis

For the shelf-life study, either pasteurized or processed by UHPH (20ºC/300 MPa) orange juices were stored at 6 and 20ºC. Untreated juice was used as control parameter. Microbiological analyses of samples were performed 2 h after treatments and after 5, 10, 30, 45, 60, 75 and 90 days of storage. Samples were diluted appropriately in sterile peptone solution (Oxoid, Basingstoke, UK) and plated on total plate count agar (PCA, Oxoid) and incubated at 30ºC for 24 h and 10ºC for 5 days for mesophilic and psychrotrophic bacteria counts, respectively; on rose-bengal chloramphenicol agar (RB, Oxoid) incubated at 25ºC for 5 days for molds and yeasts count; on de Man Rogosa Sharpe agar (MRS, Oxoid) incubated at 30ºC for 5 days for lactic-acid bacteria count and on violet red bile glucose agar (VRBG, Oxoid) incubated at 37ºC for 24 h for enterobacteria count. Results were expressed as the logarithm of colony forming units per ml of orange juice (log CFU/ml).

Sensory evaluations

For evaluating consumer acceptability 25 assessors without any training were randomly selected from volunteer personnel working at the Veterinary Medicine School of the

Universitat Autònoma de Barcelona. The panelists compared pilot-scale pasteurized and UHPH processed juices in terms of product-specific color, acidity, sweetness, bitterness, strange flavors and overall acceptability on an 11 point hedonic scale (0 was dislike extremely and 10 was like extremely). Prior to sensory evaluation orange juices were tempered at 15ºC, randomly coded and served (50 ml). Finally, panelists were asked about their preferences among the samples of orange juice pasteurized and UHPH treated used in this study and a commercial orange juice pasteurized.

Statistical Analysis

Significant differences between the results were calculated by Analysis of variance (ANOVA) using the General Lineal Models Procedure and the Friedman rank and Spearman test was used in sensory analysis of SAS® System (Statiscal Analysis Systems Version 9.2, SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$. The correlations between a pair of variables were also evaluated.

Results and discussion

Effect of UHPH and heat on pH, sugars, titratable acidity, Brix and Browning Index

The values obtained for titratable acitdity (TA), pH, reducing sugars and °Brix for control samples were 1.35 %, 3.2, 7.2 % and 12.1, respectively (Table 2), which were within the values previously reported (Floyd et al., 1969; Yeom et al., 2000; Esteve et al., 2005).

No significant differences were observed in the pH and NEBI values between the orange juices treated by both pasteurization and UHPH, and the fresh (untreated) orange juice. Bull et al. (2004) reported at initial time a browning index of 0.096 and 0.050 for fresh Valencia and Navel juice, respectively, which is much lower than the NEBI mean value of the fresh orange juice we used in our survey (0.148). During the pasteurization and the UHPH treatments several colorless compounds are probably formed, which do not contribute to a noticeable increase in the absorbance giving mean values lower than 0.150.

The pasteurization treatment reduced significantly the ºBrix value from 12.12 of fresh juice to 10.47, probably due to the depletion of the reducing sugars content (from 7.16 to 5.36) and TA (from 1.35 to 1.04). Moreover, although treatment of freshly squeezed orange juice with UHPH, irrespective of pressure and inlet temperature, did not cause significant differences ($P < 0.05$) in the ºBrix and the amount of reducing sugars, juices treated at 300 MPa, which reached a temperature above 90ºC during the treatment (T2, Table 1), showed the lowest values of both parameters. However, titratable acidity (TA) was mostly influenced by the inlet temperature (Ti) than by the level of pressure achieved. UHPH treatments at 20ºC significantly decreased the TA value of juices; bring it closer to the values of the pasteurized juice, which showed the lowest ones. A negative correlation was observed between the pH and the TA values. The slight decrease in the acidity might be partly due to the copolymerization of the organic acids with products from the browning reactions. It has also been suggested that organic acids can react with the reducing sugars to produce brown pigments (Lozano, 2006). However, our result differs from these reported by Akinyele et al. (1990) and Rivas et

al. (2006) who observed an increase of the total titratable acidity after orange juice pasteurization.

Effect of UHPH and heat on the particle size, cloudiness and color

Table 3 shows the cloudiness and particle size values whereas color parameters are presented in Table 4. The particle size of the fresh orange juice was significantly higher than the particle size of both the pasteurized and the UHPH processed juices ($P < 0.05$), whereas surface area mean diameters, $D_{3,2}$, and volume-weighted mean diameters, $D_{4,3}$ of pasteurized orange juice were also significantly higher than the levels obtained in the UHPH treated juices, in which the smallest particles sizes were observed in the samples treated at 300 MPa. High levels of $D_{3,2}$ value indicate a higher potential for interaction between particles in the pasteurized orange juice, thus leading more easily to aggregation. Additionally, high $D_{4,3}$ values indicate that the particles in the conventionally pasteurized orange juice are heavier than those in UHPH juice; consequently, these particles may tend to settle more rapidly during storage (Leizerson et al., 2005). The effect of the UHPH processing on the particle size can be better observed in Figure 1. The distribution of the particles in the pasteurized juices was bimodal and very similar to that observed in the freshly squeezed juice, while as a consequence of the UHPH-treatment the distribution became monomodal and with a noticeable decrease on the particle size. Betoret et al. (2009) also reported that the mean $D_{4,3}$ values decreased in citrus juices as the homogenization pressure increased although the highest pressure applied was only 30 MPa. At this pressure the decrease of the $D_{4,3}$ value was up to 123 μm. In other foodstuffs, such as milk, it has been observed that an increment of the pressure level and the inlet temperature of the UHPH processing resulted in a decrement of the milk fat globule size (Kheadr et al., 2002; Hayes et al., 2003; Thiebaud et al., 2003). However, also it has been observed a markedly presence of larges particles at 300 MPa, indicating the later possible formation of aggregates (Thiebaud et al., 2003; Pereda et al., 2007). Due to that, Cruz et al. (2007) found highest values of particle size parameters in soymilk UHPH treated at 300 MPa.

Table 2. Effect of UHPH and pasteurization treatments on the physicochemical characteristics of orange juice.

Processing treatment	pH	Soluble solids (ºBRIX at 20ºC)	Reducing sugars (g of glucose/100 ml)	Total sugars (g of glucose/100 ml)	Titratable acidity (g of citric acid/100 ml)	NEBI (at 420 nm)
Fresh	3.188 ± 0.054a	12.120 ± 0.198a	7.167 ± 0.406a	13.973 ± 0.751a	1.346 ± 0.065ab	0.148 ± 0.014a
Pasteurization (90ºC/1 min)	3.249 ± 0.061a	10.467 ± 0.580b	5.359 ± 0.440b	10.969 ± 0.753b	1.040 ± 0.096d	0.119 ± 0.006a
10ºC/100 MPa	3.160 ± 0.057a	12.738 ± 0.859a	7.443 ± 0.746a	13.005 ± 1.064ab	1.382 ± 0.009a	0.130 ± 0.010a
20ºC/100 MPa	3.174 ± 0.064a	12.256 ± 0.194a	6.818 ± 0.566ab	13.393 ± 0.830ab	1.193 ± 0.060abcd	0.138 ± 0.008a
10ºC/200 MPa	3.167 ± 0.059a	12.040 ± 0.542a	7.182 ± 0.708a	12.703 ± 0.877ab	1.342 ± 0.025ab	0.126 ± 0.007a
20ºC/200 Mpa	3.170 ± 0.063a	12.276 ± 0.224a	7.086 ± 0.107a	13.563 ± 0.958ab	1.154 ± 0.052bcd	0.133 ± 0.009a
10ºC/300 MPa	3.177 ± 0.055a	11.700 ± 0.558ab	6.488 ± 0.523ab	11.318 ± 0.753ab	1.268 ± 0.027abc	0.130 ± 0.012a
20ºC/300 MPa	3.174 ± 0.066a	11.680 ± 0.484ab	6.072 ± 0.483ab	13.436 ± 0.615ab	1.084 ± 0.073cd	0.122 ± 0.008a

Data are presented as the mean value of three replications ± standard error. Columns with different letters are significantly different (P <0.05).

Table 3. Effect of UHPH and pasteurization treatments on cloudy and the particle size parameters of orange juice

Processing treatment	Cloudiness (at 660 nm)	d (0.5) (μm)	d (0.9) (μm)	Volume weighted mean $D_{4,3}$ (μm)	Surface weighted mean $D_{3,2}$ (μm)
Fresh	0.46±0.05d	298.92 ±23.67a	985.39±45.74a	409.24±22.73a	53.52±2.66a
Pasteurization (90ºC/1 min)	1.56 ± 0.23c	264.42±23.51a	870.20±57.41b	362.61±26.09b	42.23±3.15b
10ºC/100 MPa	2.53 ± 0.58ab	71.61±2.46b	240.50±2.18c	109.32±6.45c	25.15±3.94c
20ºC/100 MPa	2.87 ± 0.10a	79.45±5.13b	275.69±17.61c	117.87±7.98c	14.36±3.06d
10ºC/200 MPa	2.72 ± 0.39a	92.94±8.94b	298.49±48.66c	137.14±19.55c	16.08±2.05d
20ºC/200 Mpa	2.47 ± 0.11ab	81.15±5.14b	368.06±49.41c	141.62±11.11c	7.03±1.18e
10ºC/300 MPa	2.25 ± 0.22abc	39.72±3.96c	103.65±5.71d	48.05±3.69d	6.46±1.72e
20ºC/300 MPa	1.82 ± 0.10bc	21.57±3.43c	90.20±8.65d	35.58±3.93d	1.84±0.22e

Data are presented as the mean value of three replications ± standard error. Columns with different letters are significantly different (P <0.05).

Table 4. Effect of UHPH and pasteurization treatments on the color parameters of orange juice

Processing treatment	L*	a*	b*	ΔE*
Fresh	58.71±2.63a	2.28±1.64a	67.59±4.12ab	0.00
Pasteurization (90ºC/1 min)	58.85±2.21a	2.21±1.77a	66.21±3.95ab	2.32±0.89a
10ºC/100 MPa	63.32±2.21b	0.80±0.73b	70.81±1.98b	5.19±1.51cd
20ºC/100 MPa	62.56±1.13b	0.69±0.77b	69.12±2.93bc	4.62±0.83cd
10ºC/200 MPa	63.23±2.59b	0.17±0.69b	68.43±1.36abc	5.28±0.69d
20ºC/200 Mpa	62.45±1.57b	0.28±0.68b	66.76±3.86ab	3.69±0.89bc
10ºC/300 MPa	62.74±2.58b	-0.18±0.49b	66.97±1.14ab	5.81±0.34d
20ºC/300 MPa	61.22±2.09b	-0.16±0.74b	63.50±3.86a	4.20±1.73bc

Data are presented as the mean value of three replications ± standard error. Columns with different letters are significantly different (P <0.05).

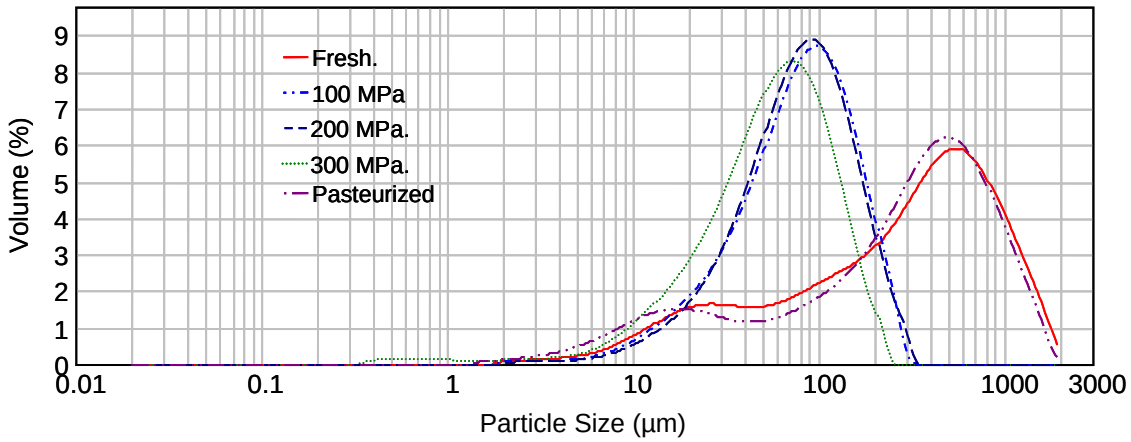

Figure 1. Particle size distribution of fresh, pasteurized and UHPH treated orange juices. Curves do not considerer the inlet temperature.

The visual turbidity originated from a suspension of pectin particles ranging from 0.4 to 2 µm. PME causes cloud instability in orange juice by de-esterification of pectin as a consequence of a series of events initiated by pectin-methyl esterase (PME); thus, a thermal process is applied to inactivate this enzyme (Leizerson et al., 2005). Nevertheless, during UHPH treatments large and coarse particles tending to settle by gravity are fragmented into smaller particles as consequence of the homogenization, increasing the proportion of particles of size under 2 µm, which are the main responsible of cloud stability (Betoret et al., 2009). In the present study, the cloudiness of the UHPH treated juices was around 5 times higher than this of control juice. On the other side, the cloudiness of the pasteurized juice was slightly lower (less turbid) than that of the UHPH treated juices. Homogenization converts part of the suspended pulp into colloidal pulp and the size reduction probably contributed to the delay in the clarification by decreasing the particle Stokes radius and thereby slowing down the sedimentation (Lacroix et al., 2005; Leizerson et al., 2005). In a previous survey we observed

that the PME activity of juices UHPH treated at 100 MPa was reduced from 10 % to 28 % (Velázquez-Estrada et al. 2011b), although they presented cloud stability. Results presented in this study are in agreement with the findings of Lacroix et al. (2005). They reported that the particle suspension stability of the orange juice depends not only on the PME activity, but is also related to the conformation of the enzyme substrate or pectin, to the enzyme–substrate interactions and to the size of the particles in suspension. A similar phenomenon was also found in juices treated with pulsed electric fields (Yeom et al., 2000) and dense phase carbon dioxide (Niu et al., 2008).

Concerning the effect of the treatments on the color, in the fresh and the pasteurized orange juices the whiteness (L*) was significantly higher than in the UHPH treated juices. Consequently, the UHPH treated orange juices showed an apparently brighter color than the fresh and the pasteurized juices. Several authors (Lee et al., 2003; Sánchez-Moreno et al., 2005) obtained similar results when they compared the color of the fresh and the pasteurized orange juice. The parameter L* presented a positive correlation with cloudiness and a negative correlation with the $D_{4,3}$ and $D_{3,2}$ values. Thus, UHPH could increase only slightly the lightness of juice as result of decreasing the particle size. After UHPH processing, the decrease in the a^* value was greater than the increase in the b* value. These variations indicate that the orange juice treated with UHPH shows a greater tendency towards the yellow color and a lesser tendency towards the red color compared with the untreated and pasteurized orange juices. The total differences on the color value ΔE* indicates the magnitude of the color difference between the fresh and the processed orange juices. The highest ΔE* value was observed in the UHPH orange juices treated at 10ºC, with values of ΔE* higher than 5 while on the pasteurized juices and the UHPH-treated juices at 20ºC the ΔE* value was higher than 3. It has been considered that a ΔE of 2 would imply a noticeable visual difference (Gullett et al., 1972). Cortes et al. (2006) and Lee et al. (2003) reported similar results when they studied fresh and pasteurized orange juices. Different distribution of carotenoids between the serum and the pulp as well as modification of the pulp size during processing may be the responsible for the color modification, but this point still needs further investigation (Niu et al., 2008).

Effect of UHPH and heat on the microbiogical stability during storage

Currently, refrigerated juices which are not obtained from concentrates and have been subjected to mild pasteurization partly satisfy the requirements of higher quality demanded by consumers. The shelf-life of these juices ranges between 28 and 45 days in refrigeration and their quality approaches that of freshly squeezed juices (Esteve et al., 2005). In our survey, samples for the shelf-life study were subjected to a 20ºC pre-warming and UHPH treated at 300 MPa. This UHPH processing conditions were chosen based on the PME inactivation achieved in a previous survey (Velázquez-Estrada et al., 2011b). The mean initial counts of the mesophilic and psychrotrophic microorganisms, lactic-acid bacteria (LAB), yeast and enterobacteria shown in Table 5 coincident with results reported in the literature (Parish, 1998), corresponding the highest counts to the aerobic mesophilic microorganisms (3.04 log CFU/ml) and LAB (2.85 CFU/ml). After 5 days of storage at 20ºC mesophilic, psychrotrophic and LAB counts of fresh orange juice increased to approximately 8 log CFU/ml, while the juices stored at 6ºC reached counts above 6 log CFU/ml just after 30 days of storage (Table 5). After being thermal or UHPH processed all the microbial counts were reduced to below the detection limit (1 CFU/ml), although mesophilic, psychrotrophic and LABs were able to grow achieving counts of approximately 2 log CFU/ml on the 75th day of storage at 20ºC in pasteurized juice, which is an acceptable limit for an orange juice during storage (Kimball, 2001).

Table 5. Evolution of microbial counts in raw orange juice stored at 6 and 20ºC.

Days	Counts (log CFU/ml) at 6ºC of storage				
	Mesophilic	Psychrotrophic	Lactic-acid bacteria	Yeasts	Enterobacteria
0	3.04 ± 0.21	2.55 ± 0.07	2.85 ± 0.17	2.16 ± 0.53	1.88 ± 0.16
5	4.79 ± 0.76	4.85 ± 0.60	2.90 ± 0.26	1.75 ± 1.01	1.01 ± 0.59
10	6.48 ± 0.09	6.74 ± 0.06	2.39 ± 1.38	2.48 ± 1.30	ND
30	6.11 ± 0.01	6.27 ± 0.04	6.06 ± 0.02	2.00 ± 2.00	ND
	Counts (log CFU/ml) at 20ºC of storage				
5	7.92 ± 0.01	7.61 ± 0.02	7.95 ± 0.03	5.83 ± 0.02	ND
10	7.92 ± 1.01	7.19 ± 0.39	7.30 ± 0.30	7.78 ± 0.04	ND
30	6.75 ± 0.05	6.23 ± 0.05	6.20 ± 0.01	ND	ND

Data are presented as the mean value of three replications ± standard error.

Effect of the pasteurization and UHPH treatments on sensory attributes

The results of the consumer acceptability study are shown in Figure 2. No significant differences ($P \geq 0.05$) were detected by the panelists between the pasteurized and the UHPH samples in terms of bitterness, acidity, sweetness, strange flavors and overall acceptability. However, in term of color the UHPH samples obtained the lowest score. The lowest

acceptability about the color attribute, that could probably be related to a greater tendency towards the yellow color observed in the UHPH-treated orange juice samples. On the basis of the responses given by the panelists the commercial samples (50%) showed a major preference ($P < 0.05$) than either pilot-scale pasteurized (30%) or UHPH processed ones (20%).

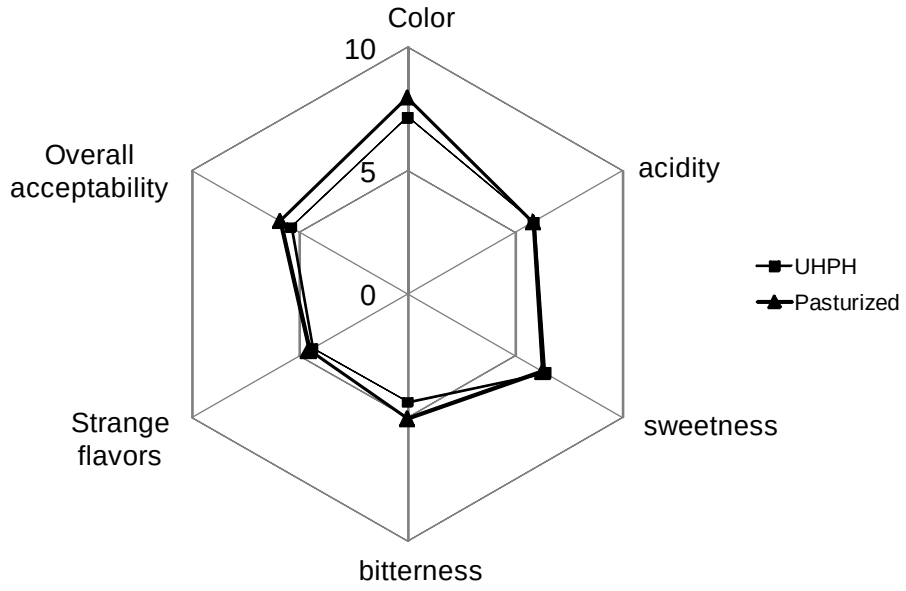

Figure 2. Sensory evaluation of pasteurized on pilot-scale (90ºC/1 min) and UHPH (20ºC/300 MPa) processed orange juice.

Conclusion

Orange juice treated by UHPH showed better characteristics, such as particle size and cloudiness, than either raw or pasteurized samples. The orange juice treated at an inlet temperature of 20ºC and a pressure level of 300 MPa presented a best shelf-life more than 75 days in comparison to pasteurized juice. The evaluation of the sensory quality of the orange juice processed with UHPH or pasteurized resulted in similar overall consumer acceptability, although the color acceptability was lower.

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Paper VII

Influence of ultra high pressure homogenization processing on bioactive compounds and antioxidant activity of orange juice

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Abstract

The purpose of this study was to evaluate the effect of UHPH on the bioactive compounds and antioxidant activity of orange juice. The juices were processed at 100, 200 and 300 MPa at two inlet temperatures 10 and 20ºC. UHPH treated orange juices retained a significantly higher content of L-ascorbic acid than the pasteurized one ($P < 0.05$). As L-ascorbic acid, the carotenoid retention depended significantly on the high pressure used during UHPH processing of orange juices ($p < 0.05$) and more specifically on the maximal temperature achieved during the UHPH treatment although the inlet temperature did not significantly influenced in any of the parameters evaluated. UHPH treatments increased the flavanone content of orange juice, concretely of hesperidin, achieving the highest content on the samples treated at 200 and 300 MPa. However, no significant differences were observed on the total polyphenol content between fresh and UHPH treated orange juice samples. After UHPH treatments, orange juice showed higher levels of TEAC values than did fresh and heat-treated orange juices.

INTRODUCTION

Fruits and vegetables are essential parts of human nutrition, especially the large amount of micronutrients make them valuable in terms of nutritional physiology. A large number of surveys conducted in the last years appear to reveal that diets rich in vegetables and fruits may be protective against certain human diseases, some of them, such as cancer or cardiovascular disease, being especially serious (Steinmetz et al., 1996; Southon, 2000; van't Veer et al., 2000; Ford et al., 2001). These studies suggest that this protection may be due to the intake of antioxidants, so more attention is being paid to the assessment of potentially antioxidant species present in those sources, such as vitamin C, polyphenols, and carotenoids, among others (Steinmetz et al., 1991). Current government recommendations promote the consumption of any five portions of fruit or vegetables per day in order to facilitate good health. However, though lacking in the fiber content of whole fruits, fruit juices are an excellent source of antioxidants (Melendez-Martinez et al., 2007a; Seeram et al., 2008).

Orange juice is an excellent dietary source of bioactive compounds which possess antioxidant properties. In addition to vitamin C, it contains carotenoids and flavanones, among other phytochemicals (Sanchez-Moreno et al., 2005; Klimczak et al., 2007). Orange juice in general is a complex source of carotenoids (Meléndez-Martínez et al., 2008). Color is one of the most important attributes of orange juice and the characteristic color of both the peel and the pulp of most varieties of ripe oranges is mainly due to carotenoid pigments (Fratianni et al., 2010). They are also important, from a nutritional point of view, because some of them have provitamin A (α-carotene, β-carotene, and β-cryptoxanthin). There is evidence that these pigments may be scavengers of reactive species, thus showing antioxidant activity (β-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin, lutein and zeaxanthin) (Krinsky, 2001). Due to the potential health benefits mentioned above carotenoids, showing or not vitamin A activity, are becoming increasingly important (Melendez-Martinez et al., 2007a; Fratianni et al., 2010). In addition, orange juice, contain mostly flavanones (mainly hesperidin and narirutin) (Gattuso et al., 2007). In fact, orange juice is a major source of antioxidant flavanones in the diet of developed countries (Gil-Izquierdo et al., 2001). Flavonoid has a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease, anti-spasmolytic, anti-inflammatory, antioxidative, anti-tumor and antimicrobial activities, among others (Harborne et al., 2000). Moreover, flavonoids may contribute to juice quality in many ways, influencing the

appearance, the taste and the nutritional value of the product. In orange juice, for instance, hesperidin can contribute to the formation of sediments which result in undesirable cloudiness (Shomer et al., 1999).

Non-pasteurized fresh orange juice has a limited shelf-life (Velázquez-Estrada et al. 2011). To prolong orange juice shelf-life, the most common method to inactivate microorganisms and enzymes is thermal processing (Eagerman et al., 1976). Commercial orange juice pasteurization is designed to inactivate pectin methylesterase (PME) activity since this enzyme is responsible for the hydrolysis of pectin that causes loss of fresh juice cloudiness (Tajchakavit et al., 1997), since PME is more thermal resistant than spoilage microorganisms (yeasts and lactic acid bacteria) (Fratianni et al., 2010). However, heat causes irreversible losses of nutritional compounds, undesirable changes in physicochemical properties and alteration of their antioxidant properties (Plaza et al., 2006). In the last years, degradation of bioactive compounds has received increased attention (Gil-Izquierdo et al., 2002; Torregrosa et al., 2005; Gama et al., 2007). The ascorbic acid degradation during thermal processing of orange juice has been described (Lima et al., 1999) as well as a relatively large loss of provitamin A and carotenoids (Lessin et al., 1997).

Changes in the consumer habits due to the desire to maintain a diet that promotes better health have increased the demand of juices that preserve their natural nutritive and organoleptic values, thus fruit juice industries have directed their studies to find alternative processing technologies which cause the minimal damage on these properties (Melendez-Martinez et al., 2007a). This concept of minimal processing is currently becoming a reality with non-thermal technologies such as high-pressure (HP) processing, pulsed electric fields (PEF) (Plaza et al., 2011), ultrasound (Valero et al., 2007), and ultra-high pressure homogenization processing (Welti-Chanes et al., 2009).

Ultra high pressure homogenization (UHPH) is an emerging technology which is currently under investigation. The principle of this technology is similar to conventional homogenization used in the dairy industry, but implies the use considerable higher pressures (up to 400 MPa). UHPH allows to process in continuous fluid foods and has demonstrated its high potential to inactivate pathogenic and spoilage microorganisms in fruit juices (Campos et al., 2007; Velázquez-Estrada et al., 2011). Besides its ability to lowering the initial microbial load, UHPH also minimises heat stress during the treatment while reducing the adverse

effects of heat on food properties or constituents. In addition, inhibition of indigenous enzymes of food by UHPH, such as pectin methylesterase of orange juice, has also been described (Hayes et al., 2003b; Lacroix et al., 2005; Welti-Chanes et al., 2009; Velázquez-Estrada et al., 2011). Recently, Suárez-Jacobo et al. (2011) evaluated the effect of UHPH treatments on quality characteristics of apple juice, such as antioxidant capacity, polyphenol composition, vitamin C and provitamin A contents, and reported that UHPH-treatment prevented degradation of most of these compounds compared with pasteurized samples. However, no references are available in the literature concerning these aspects related to orange juice. Therefore, the aim of this work was to evaluate the effect of different UHPH treatments on the bioactive compounds and antioxidant activity of orange juice in comparison with the conventional pasteurization treatments.

MATERIAL AND METHODS

Product and processing treatments

Raw orange juice from *Citrus sinensis* var. Valencia was obtained from a local juice manufacturer. The thickest part of the pulp was removed using a 2 mm steel sieve before processing. To compare the effect of the thermal processing on the orange juice properties one batch of pressed juice was pasteurized at 90ºC for 2 min, using a pilot scale pasteurizer with a tubular heat exchanger (ATI, Granollers, Barcelona, Spain). UHPH treatments were applied using a Stansted ultra-high-pressure homogenizer FPG 11300:400 (Stansted Fluid Power Ltd., Essex, UK) with a flow rate of 120 L h−1. The high-pressure system consisted of two intensifiers, which were driven by a hydraulic pump and a ceramic valve able to withstand up to 400 MPa. Orange juice was pre-warmed at 10 and 20ºC (inlet temperatures) before being pressurized at 100, 200 or 300 MPa.

Methods of analysis

Chemicals

All chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, USA), Merck (Darmstadt, Germany) or Panreac (Montcada i Reixac, Barcelona, Spain).

Determination of L-Ascorbic acid

L-Ascorbic acid (L-AA) was determined by HPLC with the method described by (Sanchez-Moreno et al., 2003) using a Summit x 2 dual gradient HPLC system (Diodex, Idstein,

Germany). Separation of ascorbic acid was performed using a reversed-phase column Waters Spherisorb 5 µm ODS2 (4.6x150 mm) (Waters, Massachusetts, USA). The solvent system used was an isocratic gradient of a 0.01% H_2SO_4 solution. The flow rate was fixed at 1.0 ml/min. Detection was performed at 245 nm, using a L-AA calibration curve in the range of 0.5-5 mg/100ml.

Determination of carotenoids and vitamin A

The HPLC method described by Stracke et al. (2009a) was used. HPLC analysis was performed on a low-pressure gradient system from Shimadzu (Duisburg, Germany) equipped with an auto-injector, column oven and photodiode array detector. The auto-injector was set to 10ºC and the column oven to 27ºC. Separation was carried out on a 250 x 4.6 mm internal diameter, 5 µm, YMC 'Carotenoid' S5 reversed-phase C30 column with a corresponding 10 x 4.0 mm internal diameter guard column (YMC Europe GmbH, Dinslaken, Germany). Calibration curves of different carotenoids were performed in the range of 0.025–25 mmol/l, in which the linearity of the response was given. The recovery for all carotenoids was greater than 95%. The CV of the method was below 5% (intra-assay). The vitamin A value has been expressed as retinol equivalents (RE) (Trumbo et al., 2003). To calculate RE on the basis of carotenoids, the following conversion has been employed: RE = µg of β-carotene/12 + µg of other provitamin A carotenoid (β-cryptoxanthin + β-carotene)/24.

HPLC Analysis of flavonoid compounds

HPLC analysis was performed with a high-pressure gradient system from LaChrom (Duisburg, Germany) equipped with an auto-injector, a photodiode array detector, and a fluorescence detector. Separation was carried out with a ProntoSIL (150 mm x 4.0 mm internal diameter, particle size= 3µm) reversed-phase column (Bischoff, Leonberg, Germany). Flavonoid compounds quantification was performed by external calibration using commercially available reference compounds. Calibration curves for the different flavonoids were in the range of 0.05-100 µM in which the linearity of the response was given (Stracke et al., 2009b).

Determination of total polyphenol content

Total polyphenols were determined by the Folin–Ciocalteu method (Singleton et al., 1965), on a spectrophotometer (Cecil Instruments, Cambridge, UK) at 750 nm and using gallic acid

as standard. Calibration curve was in the range of 1-20 mg/100 mL. Results were expressed as mg of gallic acid equivalent (GAE) per 100 mL of juice.

Measurement of antioxidant capacity

The antioxidant capacity of the orange juices was evaluated by two spectrophotometric methods: 1) Trolox equivalent antioxidant capacity (TEAC), method described by (van den Berg et al., 2000), using a spectrophotometer (Cecil Instruments, Cambridge, UK) at 734 nm and 2) Ferric reducing antioxidative power assay (FRAP), method carried out as (Benzie et al., 1996). Use of microplate was a modification of the procedure. Absorbance was measured at 595 nm (ATTC 340, SLT Labinstruments, Salzburg, Austria). Trolox [(±)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was used as standard compound in both methods. Calibration curve was in the range of 100-1000 μM.

Statistical Analysis

Significant differences between the results were calculated by Analysis of variance (ANOVA) using the General Lineal Models Procedure of SAS® System (Statistical Analysis Systems Version 9.2, SAS Institute Inc., Cary, NC, USA). All experiments were repeated three times. Differences were considered to be significant at $P < 0.05$. The correlations between a pair of variables were also evaluated.

Results and Discussion

Effect of UHPH and heat on L-Ascorbic acid

The content of L-ascorbic acid (L-AA) in fresh orange juice has been widely studied and the results obtained in the present work (Table 1) are in the range of those published in the literature, which varied from 25 mg/100 ml to 68 mg/100 ml (Sanchez-Moreno et al., 2005; Elez-Martinez et al., 2007; Klimczak et al., 2007). With the thermal pasteurization application (90 °C, 1 min), the retention levels of L-AA were 82.6%. These results are in agreement with those obtained by (Elez-Martinez et al., 2007) for pasteurized orange juice (90ºC 1 min), in which vitamin C reduced significantly (82.4%). Nevertheless, but lower than those described by (Sanchez-Moreno et al., 2005) who observed that the retention level of L-AA was about 92%.

UHPH treated orange juices retained a significantly higher content of L-AA than the pasteurized one ($P < 0.05$). This retention depended significantly on the level of pressure used during the UHPH processing of orange juices ($P < 0.05$). Samples treated at 100 and 200 MPa showed a decrease in the ascorbic acid content of about 2% and 5%, respectively, while samples treated at 300 MPa had a significantly higher decrease of 11 %. During UHPH treatments there was observed an increase of the temperature of juice as a consequence of the adiabatic heating generated in the machine in addition to the high turbulence, shear, and cavitation forces that the fluid suffers in the homogenization valve (Hayes et al., 2003a; Thiebaud et al., 2003).. For 100, 200 and 300 MPa treatments the maximal temperatures achieved were around 45ºC, 70ºC and 94ºC, respectively, which can explain why losses of L-AA were higher in samples treated at 300 MPa. Nevertheless, it is worth to point out that the time of processing during which the product remains at the maximum temperature is < 0.7 s, resulting in a minimal heat damage, and this explain the differences observed with respect to the pasteurized orange juice samples. However, the inlet temperature did not cause any significant effect on the L-AA content. In general, high pressure and especially high temperature tended to show the higher decrease in the content of L-AA because heat is known to speed the oxidation process of ascorbic acid (Gahler et al., 2003).

Our results differs from these reported by (Welti-Chanes et al., 2009) who observed that vitamin C content of orange juice was not affected by UHPH treatments (50-250 MPa, 22ºC). (Suárez-Jacobo et al., 2011) did not observed either any significant change on the content of

ascorbic acid as result of UHPH when applied to apple juice, but a significant decrement on the total vitamin C content (88% of original value) was found after thermal pasteurization. In other non-thermal technologies, such as high hydrostatic pressure or pulsed electric fields, there have been reported decreases of the L-AA content between 5 % and 10 % (Sanchez-Moreno et al., 2005; Plaza et al., 2006)although the depletion of L-AA after combined treatments were dependent mainly on the temperature achieved (Sanchez-Moreno et al., 2003; Sanchez-Moreno et al., 2005).

Effect of UHPH and heat on carotenoids

Table 2 shows the individual carotenoid content, total carotenoids and vitamin A value in untreated (freshly squeezed) and treated (UHPH and pasteurized) orange juices. The main compounds found were *β*- and *α*-carotene (43%), while β-cryptoxanthin, zeaxanthin and lutein, accounted for about 10%, 4% and 2% of the total carotenoids, respectively. Considering that the amount of carotenoids increases according to fruit maturity and varies with the variety of orange, the values obtained for the individual carotenoid compounds in the fresh orange juice was in reasonable agreement with those described by (Melendez-Martinez et al., 2007b), who described that the levels of β-carotene and α-carotene were within the range 1-81 μg/100 mL and 2-24 μg/100 mL, respectively. However, in our study the levels of luteine, zeaxanthine and β-cryptoxanthin were lower than those reported by other authors (Gama et al., 2007; Melendez-Martinez et al., 2007b; Cinquanta et al., 2009; Plaza et al., 2011)

Total carotenoid content was around 147 μg/100 mL in fresh juices, decreasing after pasteurization (87 μg/100 mL), thus resulted in a 35% loss. Cortes et al., 2006 found a lower reduction of 12.6% in the total carotenoid concentration for pasteurized orange juice (90 °C, 20 s), and Gama et al. (2007) found reductions of 13% in total carotenoids after pasteurizing orange juice at 95–105 °C for 10 s. Each carotenoid compound showed a different level of depletion in comparison with the fresh (untreated) orange juice: α-carotene (44%), β-carotene (38%), β-cryptoxanthin (32%), zeaxanthin (23%), and lutein (21%). Changes in the total carotenoids content were especially related to pro-vitamin A compounds (α-carotene, β-carotene, β-cryptoxanthin), with a depletion on the vitamin A concentration of about 40%. These results were in agreement with Lessin et al. (1997), who observed losses of about 36% in the concentrations of carotenoids with provitamin A activity in pasteurized orange juice (80ºC 2 min). However, Lee et al. (2003) reported no significant losses in provitamin A

activity after thermal pasteurization (90 °C, 30 s), although they detected losses on the content of the most labile xanthophylls (lutein and zeaxanthin). Cortes et al. (2006) and Zulueta et al. (2007) reported a reduction of 15.6% and 11% in vitamin A, respectively.

On the same way of L-AA content, UHPH treated orange juices retained a significantly higher content of carotenoids than did the pasteurized one ($P < 0.05$). This retention depended significantly on the high pressure used during UHPH processing of orange juices ($P < 0.05$) and more specifically of the maximal temperature achieved after UHPH treatment, although as described for L-AA, the inlet temperature did not cause any significant effect on the L-AA content either. Total carotenoid content found on samples treated at 100, 200 and 300 MPa were around 120, 136 and 106 μg/100 ml, respectively. Changes in total carotenoids were especially related to provitamin A compounds (α-carotene and β-carotene), with a significant ($P>0.05$) influence on the vitamin A depletion from the 7.15 μg/100 mL of the fresh orange juice to 5.7 and 6.5 μg/100 mL of samples treated at 100 and 200 MPa, respectively, while the treatments at 300 MPa caused a depletion of the 30% of the original content. These results are in agreement with those obtained by (Suárez-Jacobo et al., 2011) who observed a decrease of the vitamin A content after UHPH-processing apple juice at 100, 200 and 300 MPa of 18%, 20% and 33%, respectively. In general, the higher the pressures (and consequently, the temperatures), the higher the depletion observed in the content of carotenoids, probably because UHPH processing causes the instability of the polyene chain of the carotenoids. As consequence, these compounds may undergo processes of geometric isomerization (promoted by heat, light and acids) and oxidation (stimulated by light, heat, metals, enzymes and peroxides) which are the main causes of carotenoid degradation described in the literature (Rodríguez-Amaya, 1997; Melendez-Martinez et al., 2007b).

Effect of UHPH and heat on flavonoids

Table 3 shows the flavonoid content of the untreated (freshly squeezed) and treated (UHPH and pasteurized) orange juices. The concentrations of the flavanone glycosides were similar to those cited by several authors for narirutin (0.5-14 mg/100 ml), hesperidin (3.5-55) and eriocitrin (0.1-0.7) (Gattuso et al., 2007; Rapisarda et al., 2008). The dominant flavonone glycosides in sweet oranges (*C. sinensis*), regardless of variety, are by far hesperidin followed by narirutin (Peterson et al., 2006; Gattuso et al., 2007). Flavanove aglycones (hesperitin, naringinin and eriodictyol) were found in lower contents in oranges. All orange-type citrus

fruits contain the flavanone aglycones, but they occur less frequently in juices (Gattuso et al., 2007).

Contrary to results obtained on L-AA and carotenoids, high temperature did not affect the flavonoid content. Pasteurization did not modify the content of flavonol (kaempferol) and flavanones in orange juice as it has been previously reported by (Gil-Izquierdo et al., 2002). However, UHPH treatment increased the flavonoid content of orange juice, concretely of hesperidin and kaempferol, achieving the highest content on the samples treated at 200 and 300 MPa. Flavanones in orange juice are both soluble and precipitated; hesperidin forms crystals which interact with proteins, pectins, or other carbohydrates to be included in the cloud (Cameron et al., 1997). It has been reported that processing techniques used to manufacture the orange juice, such as squeezing, decreases the content of soluble flavanones and increases the flavanone content of the cloud fraction and, as the precipitated flavanones in the cloud particles, can be less accessible to the action of the intestinal microbial enzymes than the soluble ones (Gil-Izquierdo et al., 2002). In the case of UHPH, the rheological phenomena associated to the process, such as cavitation, friction, shear and turbulence (Lacroix et al., 2005) results in a decrement of the cloud fraction size. Consequently, UHPH treatment could improve the extractability of flavonoids from the cloud fraction increasing the amount of soluble flavanones. (Suárez-Jacobo et al., 2011) observed in apple juice that the amount of total phenolic compounds was not affected by the UHPH treatments, but the juice used in that survey was clarified before the UHPH survey and consequently the extractive effect of the UHPH treatments on the flavonones of the cloud fraction less obvious.

Effect of UHPH and heat on total polyphenol content and antioxidant activity

The content of phenol compounds in fresh orange juice (Table 1) are in the range of those published in the literature, which varied from 50 to 75 mg/100 mL (Rapisarda et al., 1999; Gardner et al., 2000). No significant differences were observed between fresh and UHPH orange juice samples on the total polyphenol content; however, significant differences were detected between these and the pasteurized juice samples. This observation can be mainly related to the loss of ascorbic acid and carotenoids described previously. There is evidence that the Folin-Ciocalteu method overestimates the polyphenol content. This is explained by the lack of selectivity of the Folin–Ciocalteu reagent, which reacts not only with phenols but also with other reducing compounds such as carotenoids, amino acids, sugars and vitamin C

(Vinson et al., 2001). Actually, the correlation coefficients for the total polyphenol content and the vitamin C and vitamin A content were $r = 0.940$ and 0.895 ($P < 0.05$), respectively.

The identification and measurement of all the individual antioxidant components in different fruits and vegetables is impractical, costly and inefficient, giving several antioxidant compounds synergistic affect each others. Four of the most popular laboratory methods used are ORAC (oxygen radical absorbance capacity), DPPH (free radical 2, 2-diphenyl-1-picrylhydrazyl) scavenging method, TEAC and FRAP. Different assays produce different results as each assay is based on a different principle. It is therefore necessary to use a combination of methods in order to elucidate the total antioxidant capacity of a sample (Ryan et al., 2010). Moreover, individual antioxidants may, in some cases, act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources (Prior et al., 2005). In our study, the antioxidant activity of orange juice was evaluated using both TEAC and FRAP assays. The mean FRAP value of the fresh juice was 9.7 mM and decreased significantly after pasteurization and UHPH treatments. The fresh juice that showed a higher antioxidant activity also contained a higher concentration of vitamin C and carotenoid compounds. The antioxidant activity of orange juice measured by TEAC was 6 mM and decreased after pasteurization, a fact that can be mainly attributed to the L-ascorbic acid and carotenoids losses. The reaction between the L-ascorbic acid and the ABTS radical cation used on TEAC assay occurs practically instantaneously (Re et al., 1999). After the UHPH treatments, orange juice showed a higher level of TEAC values than did the fresh and the heat-treated orange juices. The variation in the L-ascorbic acid retention due to UHPH was not reflected in a depletion of the TEAC values. Among the compounds exhibiting antioxidant activity in orange juice, L-ascorbic acid is the most important, contributing with 50–90% of the total antioxidant activity (Gardner et al., 2000; Sanchez-Moreno et al., 2003). However, in addition to vitamin C, it is necessary to take into account the possible synergistic effect of other phytochemicals as flavonoids and carotenoids, the effect of which will depend on their structure, interaction mode, and concentration (Plaza et al., 2011).

As a conclusion, UHPH treated juice showed to have a better retention of bioactive compounds and antioxidant activity in comparison with the orange juice subjected to thermal pasteurization. The retention of L-AA and vitamin A as well as the increment of flavanones

depended on the pressure treatment. UHPH treatments at 200 MPa led to higher contents of vitamin A, a high retention of L-AA and a higher increment of flavanones content on the orange juice just after the treatments, but further research is required to elucidate the effects of UHPH-processing parameters on the bioactive compounds of orange juice during the storage period.

Table 1. Effect of processing treatments on the L-AA, polyphenols, FRAP and TEAC content of orange juice.

Processing treatments	L-AA (mg/100 mL)	Polyphenols (mg/100 mL)	FRAP (mM)	TEAC (mM)
Fresh	54.21 ± 5.38a	77.10 ± 12.23a	9.70 ± 1.88a	6.04 ± 0.92a
100 MPa	53.28 ± 6.80a	76.50 ± 10.82a	8.59 ± 1.66b	6.33 ± 0.92a
200 MPa	51.74 ± 6.44a	75.91 ± 9.68a	8.67 ± 1.79b	6.35 ± 0.98a
300 MPa	48.41 ± 5.08b	72.00 ± 10.30a	8.11 ± 1.48b	6.12 ± 0.91a
Pasteurization (90ºC/1 min)	43.32 ± 5.01c	62.43 ± 17.20b	8.71 ± 1.36b	5.58 ± 0.84b

Data are presented as the mean value of three replications ± standard deviation. Columns with different letters are significantly different (P <0.05).

Table 2. Effect of processing treatments on the carotenoid content of orange juice.

Processing treatments	Carotenoid content (μg/100 mL)						Vitamin A (RE/100 mL)
	Lutein	Zeaxanthin	β-Cryptoxanthin	α-carotene (as β-carotene)	β-carotene	Total	
Fresh	2.35 ± 0.63a	4.75 ± 1.10a	11.82 ± 1.41a	52.40 ± 17.04a	53.74 ± 21.95a	147.39 ± 54.03a	7.15 ± 2.45a
100 MPa	2.28 ± 0.57a	4.52 ± 1.41ab	10.29 ± 2.57a	35.76 ± 17.60ab	44.90 ± 23.98ab	120.73 ± 49.40ab	5.66 ± 2.49ab
200 MPa	2.10 ± 0.81a	4.53 ± 1.36ab	10.96 ± 3.24a	42.32 ± 24.24ab	51.77 ± 26.00ab	136.29 ± 67.48ab	6.53 ± 2.99ab
300 MPa	2.31 ± 0.82a	4.29 ± 1.35ab	10.29 ± 2.12a	35.58 ± 23.25ab	37.01 ± 26.35ab	106.83 ± 66.52ab	5.00 ± 3.16ab
Pasteurization (90ºC/1 min)	1.84 ± 0.85a	3.61 ± 1.03b	8.00 ± 1.59b	30.14 ± 11.68b	32.79 ± 13.01b	87.30 ± 33.60b	4.32 ± 1.53b

Data are presented as the mean value of three replications ± standard deviation. Columns with different letters are significantly different (P <0.05).

Table 3. Effect of processing treatments on the flavonoids content of orange juice.

Processing treatments	Flavonoid content (mg/100 mL)							
	Narirutin	Hesperidin	Eriocitrin	Eriodictyol	Naringenin	Hesperetin	Kaempferol	Total
Fresh	2.04 ± 0.33a	7.84 ± 2.00a	0.16 ± 0.07a	0.08 ± 0.02a	1.88 ± 0.56a	0.28 ± 0.07a	0.53± 0.18a	12.28 ± 2.09a
100 MPa	2.04 ± 0.35a	9.81 ± 2.35a	0.18 ± 0.04a	0.05 ± 0.01a	1.65 ± 0.70a	0.28 ± 0.14a	0.63 ± 0.21a	14.02 ± 2.71a
200 MPa	1.72 ± 0.33a	17.47 ± 6.62b	0.12 ± 0.06a	0.09 ± 0.04a	1.38 ± 0.61a	0.21 ± 0.06a	0.81 ± 0.42ab	20.98 ± 7.41b
300 MPa	1.91 ± 0.35a	18.76 ± 7.57b	0.16 ± 0.07a	0.08 ± 0.02a	1.82 ± 0.65a	0.25 ± 0.08a	1.13 ± 0.44b	22.98 ± 8.31b
Pasteurization (90ºC/1 min)	1.82 ± 0.31a	10.10 ± 3.19a	0.15 ± 0.09a	0.07 ± 0.02a	1.70 ± 0.48a	0.24 ± 0.08a	0.74 ± 0.20a	14.08 ± 3.76a

Data are presented as the mean value of three replications ± standard deviation. Columns with different letters are significantly different (P <0.05).

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