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CONSERVACIÓN DE ALIMENTOS MEDIANTE TRATAMIENTOS POR ALTA PRESIÓN HIDROSTÁTICA

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CONSERVACIÓN DE ALIMENTOS MEDIANTE TRATAMIENTOS POR ALTA PRESIÓN HIDROSTÁTICA

FOOD PRESERVATION BY HIGH HYDROSTATIC PRESSURE TREATMENTS

Memoria para optar al grado de Doctor

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HACEN CONSTAR: Que el trabajo expuesto en la presente Tesis Doctoral: **"Conservación de alimentos mediante tratamientos por alta presión hidrostática"** presentado por **Dª. Mª Julia Toledo del Árbol** ha sido realizado bajo nuestra dirección y supervisión, cumpliendo todas las exigencias para su presentación y defensa para optar al Grado de Doctor en la modalidad de Mención Internacional. Parte del trabajo presentado ha sido realizado durante la estancia de la doctoranda en el "Department of Agricultural Sciences, Division of Microbiology" de la Universitá degli studi di Napoli Federico II (Nápoles), por un periodo de tres meses.

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SUMMARY

The presence and activity of microorganisms in foods may lead to a number of undesirable changes in the food matrix, modifying food rheological and organoleptic characteristics and the production of secondary metabolites, making the food product no longer suitable for human consumption and, in some cases, detrimental to human health. Microbial food spoilage results in removal of large amounts of spoiled products from the food market, which also results in significant economic losses. Transmission often occurs when microorganisms are introduced in food preparation areas and are allowed to multiply in food, for example, due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) food.

In the present study, both fresh and processed vegetables were studied in order to gain insights into the survival ability of foodborne pathogens and the effect of alternative food processing technologies on microbial inactivation in these food systems. Ready-to-eat foods may be contaminated with human pathogenic bacteria from the vegetable raw ingredients used for its preparation. Fresh produce have been implicated in foodborne outbreaks caused mainly by *Salmonella enterica* and *Escherichia coli* O157, but also by Methicillin-resistant *Staphylococcus aureus*. Lactic Acid Bacteria (LAB) are usually found in low numbers on vegetable surfaces, but they tend to proliferate during food storage at the expense of nutrients released during processing. Some of them may cause food spoilage. Control of foodborne pathogens in the food chain requires a careful selection of treatments and hurdles compatible with product characteristics and production processes. One of the methods proposed to control microorganisms in foods is High Hydrostatic Pressure (HHP) treatments. Among the available food preservation methods, HHP has emerged as a non-thermal food processing technology that is used by the food industry to inactivate pathogenic and spoilage bacteria, thus improving the food quality and safety, as well as extending the shelf-life of these food products. Applied at room temperature, high pressure destroys vegetative bacterial cells that spoil the foodstuffs and inactivates certain enzymes, with minimal changes on the organoleptic properties and nutrients.

The efficacy of HHP treatments can improve in combination with other antimicrobial substances, including natural antimicrobials such as bacteriocins or essential oils. Enterocin AS-48 is a circular bacteriocin with a broad bactericidal spectrum against

Gram-positive bacteria. When added at high concentration, it may also inhibit some Gram-negative bacteria. Its spectrum of inhibition can be extended at low bacteriocin concentrations added in combination with outer membrane-permeabilizing treatments. This bacteriocin has been widely investigated for biopreservation of foods from meat, dairy, seafood and vegetable origin.

One of the purposes of the present study was to determine is HHP treatments (applied singly or in combination with natural antimicrobials) could be applied to avoid transmission of antibiotic resistant bacteria through the food chain. Methicillin-resistant *Sataphylococcus aureus* (MRSA) was chosen as model microorganism since MRSA may cause life-threatening infections in hospitals and are also becoming widely spread in the food chain associated with food products derived from animals. Inactivation of MRSA by high hydrostatic pressure (HHP) treatments applied singly or in combination with natural antimicrobials (nisin, enterocin AS-48, cinnamon oil and clove oil) was investigated in rice pudding. Treatments at 600 MPa for 10 min reduced initial populations of staphylococci (7.9 log CFU/g) below detectable levels of 1 log CFU/g in the puddings. Treatments at 500 MPa for 5 min (achieving a 2.9-log reduction of viable counts) were investigated singly or in combination with nisin (200 and 500 IU/g), enterocin AS-48 (25 and 50 μg/g), cinnamon oil (0.2%, vol/wt) or clove oil (0.25% vol/wt). The combined treatment of enterocin AS-48 (50 μg/g) and HHP caused a non-significant reduction of 0.4 to 0.6 log cycles compared to HHP alone. Additional reductions of 0.87, 1.3 and 1.8 log cycles were recorded for the combined HHP treatments with nisin (500 IU/g), cinnamon oil (0.2%) and clove oil (0.25%), respectively. During refrigeration storage for one week, viable counts in puddings from combined treatments were significantly lower compared to the single HHP treatments, eg. 1.5 to 2.7 log cycles for HHP-nisin (500 IU/g), 1.1 to 1.3 log cycles for HHP-AS-48 (50 μg/g) or approx. 1.5 log cycles for HHP-cinnamon oil (0.2%). These results suggest that the time and intensity of HHP treatments required for inactivation of methicillin-resistant *S. aureus* in puddings can be reduced when HHP is applied in combination with selected natural antimicrobials.

Another zoonotic foodborne pathogen of concern in the food chain is *Salmonella enterica*. This pathogen has been widely investigated on eggs, meat products and dairy products, but not so much in vegetable foods or in foods containing a variety of

ingredients from animal and vegetable origin. As a matter of fact, the fate of *S. enterica* in Spanish omelette (*tortilla de patatas*) treated or not by HHP had never been studied before. Omelettes may often appear undercooked in the central parts, increasing the risk for survival and transmission of this pathogenic bacterium. Furthermore, omelettes can be sold in supermarkets as a ready-to eat food, and may become exposed to temperature abuse conditions before consumption. The effect of high hydrostatic pressure treatments on a cocktail of four *Salmonella* Enteritidis strains inoculated in commercial Spanish potato omelette was studied. Inactivation of *Salmonella* was influenced by treatment time and pressure. Treatments at 600 MPa for 5 or 8 min reduced the counts of *Salmonella* on selective medium (XLD agar) by 5.9 and 6.5 log cycles, respectively. Investigation of sublethally injured cells by growth on triple agar layer (TAL) plates or in non-selective medium (TSA) revealed that the population of sublethally injured cells able to grown on TSA could be 2.5 to 3.0 log cycles higher (depending on treatment) compared to non-injured cells. The TAL method only allowed recovery of a fraction of sublethally injured cells, suggesting different degrees of cell damage. In addition, tailings were observed in survival curves for treatments above 500 MPa both for selective and non-selective determinations. Omelette samples inoculated with *Salmonella* and treated at 500 MPa (5 min) or 680 MPa (8 min) were stored at 6 ºC for 15 days. Counts of survivors on selective medium increased at day 3 of storage for the less intense HHP treatment (suggesting repair of sublethally injured cells), but not until day 10 for the 680 MPa treatment. Temperature abuse of the treated omelettes (6 h at 22ºC) applied at day 3 of storage increased the concentrations of cells able to grow on non-selective as well as on selective media, an effect that was more pronounced in the samples treated at 500 MPa for 5 min. The results suggest that temperature abuse may compromise the efficacy of HHP treatments against *Salmonella* in omelettes.

Another traditional food that seemed interesting as model to study the effects of HHP treatments was salmorejo, a tomato-based creamy product. Since salmorejo is not heat-processed, there is a risk of contamination with foodborne pathogens from raw materials. Even though bacterial growth in salmorejo is strongly inhibited because of its acidic pH (close to 3.9), the growth and survival of foodborne pathogens in this food has not been studied before. In the present study, three cocktails consisting of *Escherichia coli* O157, *Salmonella enterica* serovar Enteritidis and *Listeria monocytogenes* strains were inoculated in freshly-prepared salmorejo. The food was treated by high hydrostatic pressure (HHP) at

400, 500 or 600 MPa for 8 min, or left untreated, and stored at 4 ºC for 30 days. Viable cell counts were determined on selective media and also by the triple-layer agar (TAL) method in order to detect sublethally-injured cells. In control samples, *L. monocytogenes* viable cells decreased by 2.4 log cycles at day 7 and were undetectable by day 15. *S. enterica* cells decreased by 0.5 or 2.4 log cycles at days 7 and 15 respectively, but still were detectable at day 30. *E. coli* O157 cells survived much better in salmorejo, decreasing only by 1.5 log cycles at day 30. Treatments at pressure of 400 MPa or higher, reduced viable counts of *L. monocytogenes* and *S. enterica* to undetectable levels. HHP treatments significantly ($P < 0.05$) reduced *E. coli* counts by approx. 5.2 to 5.4 log cycles, but also yielded surviving cells that apparently were sublethally-injured. Only samples treated at 600 MPA for 8 min were devoid of detectable *E. coli* cells during storage. Results from the present study indicate that HHP treatments provide an effective barrier against possible accidental transmission of foodborne pathogens from contaminated raw materials used in the preparation of salmorejo.

In spite of the fact that HHP treatments have been investigated mainly for inactivation of classical foodborne pathogens, they may be equally effective in the control of emerging opportunistic pathogens and spoilage bacteria. One example are the Lactic Acid Bacteria (LAB). LAB are usually found in low numbers on vegetable surfaces, but they tend to proliferate during food storage at the expense of nutrients released during processing. Some species of genus *Leuconostoc* (such as *Leuconostoc mesenteroides*, *Leuconostoc gelidum* and *Leuconostoc gasicomitatum*) are able to proliferate during storage and can spoil food products, especially refrigerated foods packed under vacuum or under modified atmosphere. As they are intrinsically vancomycin-resistant bacteria, are recognized as emerging pathogens that can cause severe infections, particularly in immunocompromised patients. One of the purposes of this thesis was to investigate the capacity of leuconostocs to survive and proliferate in cherimoya pulp and to determine the viability of using HHP treatments singly or in combination with enterocin AS-48 for inactivation of these bacteria, and also to determine if the added bacteriocin would act as a protective hurdle against proliferation of survivors during storage. A cocktail of *Leuconostoc mesenteroides, Leuconostoc gasicomitatum* and *Leuconostoc gelidum* inoculated on autoclaved cherimoya pulp were treated by high hydrostatic pressure (HHP; 400, 500 or 600 MPa for 8 min) and with enterocin AS-48 (35 μg/g), singly or in combination. After treatments, samples were stored at 4 ºC for 30 days or at 22 ºC for 10 days. HHP treatments at 400 or 500 MPa reduced viable cell

concentrations by 4.3 and 4.9 log cycles, respectively. No survivors were detected at 600 MPa. Bacteriocin addition in combination with HHP achieved an additional reduction of 0.6 to 0.9 log cycles after treatments at 400 or 500 MPa, and also reduced viable counts for most treatments during storage. In samples treated by HHP, regrowth of leuconostocs was delayed to days 15 (400 or 500 MPa) or 30 (600 MPa) of refrigeration storage, or day 3 at 22 ºC (all treatments). Results from the study suggest that treatments at 600 MPa (8 min) can be useful to inactivate leuconostocs in cherimoya pulp.

Cherimoya fruit pulp preparations with an extended shelf life could find new markets as functional foods and contribute to solve the problem of seasonal excess production and decrease in market price. However, cherimoya pulp spoils easily, and its shelf life may be severely limited by surface contaminating bacteria reaching the pulp during processing. In our study, pulp obtained from cherimoya pulp (*Annona cherimola*) was inoculated with epiphytic microbiota collected from cherimoya fruits, and supplemented or not with enterocin AS-48 (50 μg/g) and then packed under vacuum. Samples supplemented or not with enterocin were treated by high hydrostatic pressure (600 MPa, 8 min) and then stored at 5 ºC for 30 days. The single AS-48 treatment only delayed microbial growth non-significantly ($p > 0.05$). HHP treatment reduced microbial counts by 5 log cycles, but it did not prevent further growth of survivors by day 7. The combined treatment (AS-48 + HHP) was the most effective, keeping bacterial cell densities at $\leq$ 1.5 log CFU/g for up to 15 days. 16S rRNA gene pyrosequencing analysis was done on amplicon libraries from the growth on TSA plates seeded with ten-fold dilutions of pulp suspensions and incubated at 22 ºC for 24 h. The results obtained are limited by the experimental conditions used in the study, and only concern the bacterial fraction that was selected by the TSA and growth conditions used. *Pantoea* (*Pantoea agglomerans, Pantoea vagans*) were the operational taxonomic units (OTUs) detected at highest relative abundance in bacterial biomass grown from control samples for the first 7 days of storage, followed by *Enterococcus gallinarum* and *Leuconostoc mesenteroides* during late storage. The single HHP treatment significantly reduced the relative abundance of OTUs belonging to *Pantoea* and strongly increased that of endosporeformers (mainly *Bacillus firmus* and *Bacillus stratosphericus*) early after treatment, although *Pantoea* became again the predominant OTUs during storage. Samples singly treated with enterocin AS-48 revealed a strong inhibition of *E. gallinarum* as well as an early decrease in the relative abundance of *Pantoea* and an increased relative abundance of OTUs belonging to other Gram-negative species (mainly from genera *Serratia* and *Pseudomonas*).

The strong microbial inactivation achieved by the combined treatment with enterocin and HHP reduced the levels of viable cells below detectable limits at days 0 and 1, and survivors recovered on TSA at day 7 were represented in > 99% by *B. firmus* OTU. OTUs from endosporeformers were no longer detected during prolonged incubation, displaced by *Pantoea* spp., *Erwinia billingiae* and leuconostocs. Results from the present study indicate that HHP in combination with enterocin AS-48 is more effective in preserving the microbiological quality of cherimoya pulp during storage than the single HHP treatment.

One additional model chosen to study the effects of HHP was green asparagus. The spears from green asparagus possess a variety of biological properties, such as being antioxidants, immunostimulants, anti-inflammatory, antihepatotoxic, antibacterial, antioxytocic, and reproductive agents, but at the same time are highly perishable. Treatment by HHP could possibly be a non-thermal process for preservation of green asparagus. The application of high hydrostatic pressure (HHP, 600 MPa, 8 min) on brined green asparagus and the changes in bacterial diversity after treatments and during storage at 4 ºC (30 days) or 22 ºC (10 days) were studied. HHP treatments reduced viable cell counts by 3.6 log cycles. The residual surviving population did not increase during storage at 4 °C. However, bacterial counts significantly increased at 22 ºC by day 3, leading to rapid spoilage. The microbiota of green asparagus was composed mainly by *Proteobacteria* (mainly *Pantoea* and *Pseudomonas*), followed by *Firmicutes* (mainly *Lactococcus* and *Enterococcus*) and to a less extent *Bacteroidetes* and *Actinobacteria*. During chill storage of untreated asparagus, the relative abundance of *Proteobacteria* as well as *Enterococcus* and *Lactococcus* decreased while *Lactobacillus* increased. During storage of untreated asparagus at 22 ºC, the abundance of *Bacteroidetes* decreased while *Proteobacteria* increased during late storage. The HHP treatment determined a reduction of the *Proteobacteria* both early after treatment and during chill storage. In the HHP treated samples stored at 22 ºC, the relative abundance of *Pseudomonas* rapidly decreased at day 1, with an increase of *Bacteroidetes*. This was followed by a marked increase in *Enterobacteriaceae* (*Escherichia*) simultaneously with increase in viable counts and spoilage. Results from the study indicate that the effect of HHP treatments on the viability of microbial populations in foods also has an impact on the dynamics of microbial populations during the storage of the treated foods.

INTRODUCCIÓN

1. *BIOTECNOLOGÍA Y MICROBIOLOGÍA ALIMENTARIA.*

Madrid *et al.* (1994) definen la Biotecnología Alimentaria como el conocimiento y la utilización de los microorganismos para producir más y mejores alimentos, además de la manipulación genética en plantas y animales con los mismos fines. Los avances en el campo de la biotecnología proporcionan un conjunto de herramientas para mejorar la variedad, la productividad y la eficacia en la obtención de alimentos, por lo que también se consideran aquellos procesos industriales que implican el uso de sistemas biológicos (Blanco *et al.,* 2006).

Aunque es muy reciente el uso de la palabra biotecnología en la industria alimentaria, hace miles de años que el ser humano experimentó la necesidad de conservar los alimentos. Por ejemplo, utilizaba la sal como conservante para la carne y el pescado. También utilizaba los microorganismos para la obtención de determinados alimentos y bebidas, con ejemplos clásicos como: la fermentación del mosto de la uva para la obtención de vino (levaduras presentes en el hollejo de la uva); la fermentación de cerveza a partir de cebada malteada junto con lúpulo, agua y levaduras; la acidificación de la leche por bacterias lácticas para obtener yogur, cuajadas y quesos; la maduración de los productos cárnicos, etc. (Madrid *et al.,* 1994; Morcillo *et al.,* 2005). La industria alimentaria del siglo XX y XXI se fundamenta en el conocimiento científico y técnico para proporcionar alimentos seguros y con una mayor vida útil. El tipo de operaciones que intervienen en la elaboración de un alimento y la forma de aplicarlas determina las características del producto final. Las aplicaciones de la Biotecnología a la Tecnología de los Alimentos son muy diversas, quizás las fermentaciones son las que más se han beneficiado, pues la ingeniería genética ofrece una alternativa a los métodos tradicionales de selección de microorganismos (Blanco *et al.,* 2006). Con la tecnología y el procesado de los alimentos se pretende aumentar la capacidad de conservación de los alimentos y transformarlos con el fin de mejorar su calidad sensorial, incrementar su valor nutritivo, facilitar su consumo, diversificar la oferta y abaratar los precios, utilizar técnicas respetuosas con el medio ambiente, etc. Estos avances pueden ser mediante la optimización de tecnologías ya existentes, el desarrollo de nuevas técnicas emergentes o por combinación de diferentes técnicas, para conseguir lo que se llama el "efecto barrera".

Las enfermedades transmitidas por alimentos contaminados (incluyendo el agua potable) constituyen uno de los problemas de salud más comunes y son una importante causa en la reducción de la productividad económica. Las prácticas de mayor riesgo sanitario que favorecen la proliferación de microorganismos y la contaminación de los alimentos suelen ser la conservación de los productos a temperatura ambiente o con refrigeración insuficiente, la intervención de manipuladores portadores de alguna infección, la preparación de los alimentos en grandes cantidades y con mucha antelación a su consumo, el cocinado insuficiente de alimentos contaminados, una descongelación defectuosa, contaminaciones cruzadas, insuficiente limpieza y desinfección de equipos y utensilios de cocina, etc.

Existen dos tipos principales de enfermedades relacionadas con los alimentos:

i. Las infecciones alimentarias producidas como consecuencia de la ingestión de un microorganismo patógeno con el alimento o bebida. Una vez en el organismo, continúa el crecimiento de los patógenos y la invasión de los tejidos del cuerpo, o la liberación de toxinas, o ambas a la vez.
ii. Las intoxicaciones alimentarias se producen por el crecimiento microbiano en los alimentos con la liberación de toxinas. Los síntomas se producen tras la ingestión de dichas toxinas. Son ejemplos *Staphylococcus aureus* y *Clostridium* spp.

1.1. Microorganismos de riesgo en la industria alimentaria.

Son muchos los microorganismos que pueden contaminar los alimentos y bebidas y causar enfermedades de diversa gravedad, casi todos ellos se asocian a deficientes prácticas de higiene en alguna de las etapas de producción o procesado de los alimentos y/o mala calidad de las materias primas. Se puede hacer una distinción en dos categorías:

Microorganismos patógenos.

Son aquellos que causan enfermedades tanto en animales como en humanos, cuyos vehículos de transmisión son los alimentos y el agua, aunque el crecimiento de estos microorganismos sobre los alimentos no suele alterar sus propiedades organolépticas. La mayoría de los casos desembocan en una gastroenteritis aguda, con episodios de diarrea y vómitos, que suele producirse a las pocas horas de haber ingerido el producto contaminado. Una de las patologías más comunes es la salmonelosis, producida *Salmonella*

enterica como consecuencia de la ingestión de carnes, aves de corral, huevos y productos lácteos contaminados con la bacteria. También son muy comunes las infecciones ocasionadas por *Campylobacter jejuni* a través del consumo de aves de corral, y por determinadas cepas de *Escherichia coli*, siendo hoy en día especialmente grave el serotipo enterohemorrágico O157:H7. Otras enterobacterias patógenas son *Yersinia enterocolitica*, cuyo principal reservorio son los cerdos, y *Shigella* spp., cuyo reservorio natural es el intestino humano. Las especies patógenas del género *Vibrio* también son importantes en determinados tipos de alimentos; esta bacteria tiene como reservorio las aguas marinas y está presente en alimentos de origen marino (moluscos, crustáceos y pescado crudo). *Listeria monocytogenes*, agente etiológico de la listeriosis, es una bacteria ubicua asociada al consumo de leche cruda, vegetales frescos y productos cárnicos, y *Staphylococcus aureus*, presente en la epidermis de humanos y animales, es un productor de enterotoxinas. Las bacterias formadoras de endosporas también deben ser tenidas en cuenta, ya que sus endosporas pueden sobrevivir durante el procesado de los alimentos y germinar posteriormente, con el consiguiente riesgo de producción de enterotoxinas que pueden ocasionar intoxicaciones graves del individuo que los ingiere. Las más importantes son las anaerobias *Clostridium botulinum* y *Clostridium perfringens*, y las aerobias *Bacillus cereus*.

Microorganismos alterantes.

Son capaces de crecer sobre los alimentos y degradar sus componentes, modificando sus propiedades organolépticas y reduciendo su vida útil, haciéndolos inaceptables para su consumo. Entre ellos se encuentran bacterias psicrótrofas (*Pseudomonas* spp. y *Aeromonas hydrophila*, entre otras) procedentes de diversos ambientes y capaces de sobrevivir en alimentos almacenados en refrigeración; bacterias fermentadoras no esporuladas, como son las bacterias del ácido láctico (pertenecientes a los géneros *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* y *Enterococcus*); bacterias formadoras de endosporas (principalmente *Bacillus* spp.). También debemos tener en cuenta los mohos capaces de crecer a pH bajo y contaminar tanto productos lácteos como vegetales (*Penicillium, Aspergillus*, *Fusarium*, *Mucor*, *Botrytis*, *Rhizopus*, etc.) y algunas levaduras que confieren olor afrutado y la formación de gas en productos lácteos.

Mejorando las condiciones de envasado (reducción del oxígeno, almacenaje en frío, adición de sustancias antimicrobianas, etc.) se consigue evitar la proliferación de estos microorganismos en los alimentos y minimizar los daños que puedan ocurrir en los

mismos. Debido a ello, la tecnología alimentaria contribuye al desarrollo de métodos alternativos de conservación que mejor se adapten a cada tipo de producto y evitar así enfermedades transmitidas por los alimentos entre los individuos de una población a la vez que prolongar la vida útil del producto.

2. *LA CONSERVACIÓN DE LOS ALIMENTOS.*

Los alimentos constituyen un medio de cultivo ideal para el crecimiento de microorganismos y son muchas las causas que pueden influir negativamente sobre su calidad, ya sea por factores intrínsecos del alimento debido a su contenido en nutrientes, la disponibilidad de agua, el pH, etc., o por factores extrínsecos como la temperatura de almacenamiento, la humedad relativa, la exposición a la luz solar y el aire, la manipulación y el procesado de las materias primas, etc. Las bacterias, junto con hongos filamentosos y levaduras son, generalmente, los contaminantes más frecuentes causantes de la alteración de los alimentos (Morcillo *et al.,* 2005). La contaminación se puede originar en cualquier punto de la cadena alimentaria. La higiene y seguridad alimentaria se basa, pues, en la detección y el control de los microorganismos patógenos y alterantes que son responsables de la descomposición de dichos alimentos y evaluar si un producto es apto o no para su consumo.

La conservación de los alimentos persigue como objetivo primordial mantener un producto en perfectas condiciones higiénicas y preservar sus cualidades reológicas y organolépticas (Casp y Abril, 2003). Para ello debe impedir o minimizar el crecimiento o la actividad de los microorganismos, proporcionar niveles aceptables de seguridad higiénica, así como alargar y mejorar la vida útil de los alimentos. Existen numerosos métodos empleados para cumplir estos objetivos. Desde hace siglos nuestros antepasados griegos, romanos, fenicios, egipcios y chinos utilizaban técnicas de conservación basadas en el secado, la salazón y el ahumado de carnes y pescados. Estas técnicas se han seguido manteniendo a lo largo de la historia de forma tradicional junto a otros métodos como son el escabechado, el encurtido, el adobo, el marinado, el confitado y el manejo de las fermentaciones. Los avances en este campo permiten agrupar la forma de conservar los alimentos mediante métodos físicos, químicos o biológicos y sus diversas combinaciones.

2.1. Métodos físicos de conservación.

Existe una amplia diversidad de métodos físicos aplicables a la conservación de los alimentos. Muchos de ellos se basan en el empleo de distintas temperaturas y en la reducción de la actividad de agua de un alimento para inhibir, destruir o eliminar los microorganismos indeseables. La **conservación en frío** (refrigeración y congelación), el tratamiento por calor (pasteurización y esterilización) y la deshidratación (desecación y liofilización) de los alimentos son los métodos más empleados para evitar o ralentizar el crecimiento de microorganismos. En la refrigeración, la temperatura del producto se mantiene entre -1 y 8ºC, y se utiliza con frecuencia en combinación con otras operaciones de conservación (fermentación, irradiación, pasteurización) o con conservantes químicos (como la sal o los ácidos orgánicos) para reducir la velocidad de las transformaciones microbianas y bioquímicas que tienen lugar en el alimento y para prolongar la vida útil de alimentos sometidos a procesos de conservación poco drásticos (Fellows, 1994). Los **tratamientos térmicos** tienen como objetivo principal inactivar a los microorganismos de riesgo en el alimento, bien reduciendo la carga microbiana (como en el caso de la pasterización) o consiguiendo su total inactivación como es el caso de los métodos de esterilización por calor.

En las últimas décadas se han desarrollado nuevas tecnologías y sistemas de envasado para cumplir estos objetivos sin la necesidad de aplicar un tratamiento térmico al alimento. Los métodos no térmicos de conservación han generado un gran interés en la industria alimentaria dado que los consumidores de hoy en día demandan que un producto fresco también sea de calidad, por lo que se está evaluando su potencial como una alternativa o como un proceso complementario a los métodos clásicos de conservación (Casp y Abril, 2003). A modo de ejemplo, en determinados alimentos líquidos se suele emplear la filtración por membrana; con ello se consigue reducir la carga microbiana y además conserva las propiedades sensoriales y nutricionales del alimento.

La **irradiación** es un tratamiento que emplea radiaciones ionizantes, generalmente electrones de alta energía u ondas electromagnéticas (rayos X o gamma), para mejorar la seguridad microbiológica de ciertos alimentos, ya que provoca daños en el ADN impidiendo su replicación y paraliza la división celular. Se usa también para impedir la

germinación de granos y retardar la maduración de frutas tropicales. Pero a pesar de que este método para inactivar microorganismos patógenos no induce radioactividad en los alimentos ni en los envases, continúa siendo objeto de polémica y con frecuencia rechazo entre científicos y consumidores.

Otros tratamientos que también han demostrado resultados prometedores para inactivar los microorganismos de los alimentos son los **tratamientos por altas presiones hidrostáticas** y por **campos eléctricos pulsantes**. En el tratamiento de los alimentos por altas presiones se aplican presiones elevadas (entre 100 y 1000 MPa) de forma continua durante varios minutos. De este método, objeto de la presente tesis, se hablará en el siguiente apartado. En el tratamiento por pulsos eléctricos (Heinz *et al.*, 2001; Ross *et al.*, 2003) se crea un campo eléctrico con dos electrodos, donde se aplican descargas de alto voltaje (entre 10 y 80 kV/cm) en pulsos muy rápidos (milisegundos o microsegundos) a través de un alimento líquido. La lisis de las células microbianas se debe a una electroporación irreversible de la membrana celular, es decir, produce cambios conformacionales en las membranas que dan lugar a incremento transitorio en el potencial electro-químico entre el citoplasma y el medio extracelular (potencial transmembrana), originando la formación irreversible de poros en la membrana celular. Esto origina un aumento drástico en la permeabilidad, por lo que la célula afectada pierde su integridad y se destruye.

Otros tratamientos son los **campos magnéticos oscilantes** y la **luz pulsada** (Casp y Abril, 2003). Los campos magnéticos, ya sean estáticos –con intensidad magnética constante donde el tiempo y la dirección del campo son siempre iguales– u oscilantes –se aplican en forma de pulsos, invirtiendo la carga en cada pulso– causan cambios en la orientación de las biomoléculas y las biomembranas, influyen en la dirección de migración y alteran el crecimiento y proliferación de los microorganismos. Por otra parte, la luz pulsada se basa en la aplicación de pulsos intensos de luz blanca y de corta duración. Se emplea para esterilizar las superficies de los alimentos y de los materiales de procesado y envasado. Son de interés en la industria alimentaria porque se puede reducir o eliminar la carga microbiana de las superficies sin la necesidad de usar desinfectantes químicos.

2.2. Métodos químicos de conservación.

Se basan en el uso de aditivos químicos; son sustancias químicas carentes de toxicidad capaces de evitar el crecimiento de microorganismos y se añaden a los alimentos para mejorar su calidad de conservación, mantener su valor nutritivo y adaptarlos al uso al que van destinados. Para ello cumplen una serie de funciones: evitar la descomposición por el crecimiento microbiano, mantener las condiciones físico-químicas del producto y ayudar en su procesamiento o fabricación, evitar la oxidación del alimento, potenciar el sabor, endulzar en sustitución de los azúcares naturales, mejorar el sabor, color, textura, aspecto, estabilidad y comodidad del consumidor. Entre los diferentes tipos de aditivos que se pueden incorporar a los alimentos se encuentran los colorantes, conservantes, antioxidantes, espesantes, estabilizantes, aromatizantes, emulsionantes, edulcorantes, acidulantes, etc. La lista de aditivos va incrementándose con el tiempo, pero están regulados cualitativa y cuantitativamente por las autoridades de cada país para asegurar que el consumo total de cada aditivo en los alimentos de una dieta diaria esté dentro de un margen de seguridad, además estos compuestos deben figurar en el etiquetado de los alimentos. En los países de la Unión Europea, los aditivos alimentarios autorizados (incluyendo los conservantes) se designan mediante un número de código que comienza con la letra E seguida de una cifra de 3 o 4 dígitos.

Los conservantes químicos pueden ser orgánicos o inorgánicos, obtenidos a partir de fuentes naturales o de síntesis química. Muchos se encuentran en la naturaleza, como la sal, nitritos, fosfatos, sulfitos, así como los ácidos orgánicos y sus ésteres (ácidos benzoico, sórbico, láctico, acético, propiónico,…). Gran parte de los conservantes orgánicos se pueden encontrar en extractos de origen vegetal, como algunas especias y sus aceites esenciales (eugenol, aldehído cinámico, carvacrol, timol, etc.) que provienen de plantas como el clavo, canela, orégano, tomillo, salvia o romero, entre otras (Burt, 2004). También existen sustancias de naturaleza proteica, en su mayoría de origen animal, cuya actividad antibacteriana puede ser explotada en la conservación de alimentos (lisozima, lactoferrina, etc.). Los conservantes procedentes de fuentes biológicas se conocen en conjunto como **bioconservantes.** Debido a que los nuevos métodos de procesado y distribución de los alimentos crean también nuevas oportunidades para el desarrollo de microorganismos patógenos o alterantes, y que los consumidores muestran una clara preferencia por los alimentos con menos conservantes químicos y estabilizados mediante métodos menos

agresivos (Gálvez *et al.,* 2007; Lucera *et al.*, 2012), la industria alimentaria trata de encontrar alternativas basadas en estos compuestos antimicrobianos naturales para prevenir el crecimiento microbiano.

La industria alimentaria también emplea otro tipo de sustancias conocidas como **desinfectantes** o **biocidas** (McDonnell y Russell, 1999) en las operaciones rutinarias de limpieza y desinfección de maquinarias y equipos, y a veces también para reducir la carga microbiana en la superficie de las materias primas como puede ser el caso de frutas y verduras. Se trata de compuestos químicos que atacan a moléculas esenciales para el metabolismo microbiano o la estructura celular mediante interacciones químicas específicas. Los productos biocidas se regularon por primera vez en el ámbito europeo por medio de la Directiva 98/8/CE, del Parlamento Europeo y del Consejo, que los definía como sustancias activas o mezclas de ellas destinadas a destruir, contrarrestar o neutralizar cualquier organismo nocivo, impedir su acción o ejercer sobre él un efecto de control que no sea una acción física o mecánica.

2.3. Métodos biológicos de conservación.

La demanda de alimentos naturales y cada vez más seguros llevó en su momento a proponer el uso intensivo de métodos de bioconservación (Stiles, 1999), basados en el empleo de la propia microbiota del alimento de forma natural o controlada, e incluso sus productos antibacterianos. Para favorecer la conservación de los alimentos se incluyen, por un lado, la fermentación, esencialmente láctica y alcohólica –depende de los microorganismos implicados y las condiciones de almacenamiento para no provocar la descomposición del producto– y, por otro lado, el uso de **bacteriocinas**, que son péptidos o proteínas con actividad antimicrobiana, producidas por diferentes grupos de bacterias, generalmente del grupo de las bacterias del ácido láctico (BAL (Klaenhammer, 1993).

Las bacteriocinas producidas por las BAL ofrecen una serie de características que las hacen ser adecuadas para su uso como conservantes alimentarios:

(i) son de naturaleza proteica, y tras ser ingeridas se degradan por los enzimas proteolíticos del tracto digestivo;

(ii) muchas de ellas poseen un amplio espectro de acción antimicrobiano;

(iii) la ausencia de toxicidad y escasa inmunogenicidad;

(iv) carecen de actividad en eucariotas, por lo que no presentan riesgos para la salud;

(v) son termoestables, por lo que generalmente conservan su actividad antimicrobiana tras someter el alimento a los tratamientos térmicos de pasterización;

(vi) los genes de la mayoría de las cepas productoras suelen encontrarse en plásmidos, lo que facilita su manipulación genética.

Se ha demostrado que las bacteriocinas tienen un efecto bactericida sobre muchas bacterias patógenas, toxicogénicas o alterantes de los alimentos, destacando entre ellas *Listeria monocytogenes* (Cleveland *et al.,* 2001; Guinane *et al.,* 2005; Gálvez *et al.*, 2007, 2014). Son activas a bajas concentraciones frente a la mayoría de bacterias Gram-positivas, aunque también actúan sobre Gram-negativas que previamente han sido subletalmente dañadas.

La nisina es la principal bacteriocina utilizada como bioconservante. Se trata de un polipéptido producido por cepas de *Lactococcus lactis* y ha sido aprobada como un aditivo alimentario con el estatus de organismo GRAS (*Generally Recognized As Safe*, es decir, generalmente reconocido como seguro) en más de 50 países de todo el mundo (Lucera *et al.*, 2012). Además de la nisina, existen otras preparaciones comerciales (en este caso bajo la categoría de aditivos alimentarios) que contienen péptidos antibacterianos como pediocinas o lacticinas, e incluso preparados con actividad antifúngica como la natamicina.

La enterocina AS-48 es una bacteriocina que ha sido ampliamente estudiada como potencial bioconservante. Es un péptido cíclico de amplio espectro producido por una cepa de *Enterococcus faecalis* subsp. *liquefaciens* S-48 (Gálvez, 1989a) y su mecanismo de acción consiste en atacar la membrana citoplasmática bacteriana y permeabilizarla, produciéndose un colapso en el potencial de membrana debido a la formación de canales iónicos y poros que permiten la difusión de solutos, y la posterior lisis (Gálvez *et al.*, 1991). Es estable frente al calor y el pH de ciertos alimentos como frutas y verduras (Grande *et al.*, 2005) y se ha comprobado que actúa frente a bacterias alterantes, patógenas, formadoras de endosporas y productoras de toxinas (Gálvez, 1989b; Maqueda *et al.*, 2004; Grande *et al.*, 2014), aunque resulta ser más eficaz frente a bacterias Gram-positivas que frente a Gram-negativas, debido a que la membrana externa de estas últimas actúa como una barrera protectora. La enterocina AS-48 ha sido ensayada bajo condiciones de laboratorio en

diferentes tipos de productos (cárnicos, lácteos, vegetales) sola o en combinación con conservantes químicos (Grande *et al.*, 2014)

El uso de bacteriocinas en la industria alimentaria puede suplir la adición de aditivos químicos e incluso facilitar y/o mejorar el uso de otros mecanismos de barrera, como por ejemplo, reducir la intensidad de los tratamientos térmicos, para conseguir de este modo que los alimentos sean frescos, mínimamente procesados y que conserven sus propiedades nutricionales.

3. *ALTA PRESIÓN HIDROSTÁTICA.*

3.1. Interés aplicado de los tratamientos por alta presión hidrostática.

La creciente demanda de alimentos con características lo más parecidas al producto fresco, que sean mínimamente procesados, seguros y que conserven sus cualidades nutricionales y organolépticas, ha impulsado el desarrollo de nuevas alternativas de conservación para alargar la vida útil y preservar la calidad de los productos para así satisfacer los gustos y necesidades del consumidor. Los métodos no térmicos para la conservación de alimentos están siendo actualmente objeto de un gran número de investigaciones para evaluar su potencial como una alternativa o como un proceso complementario a los métodos tradicionales de conservación (Considine *et al.,* 2008).

Una de las tecnologías más prometedoras es el tratamiento por **Alta Presión Hidrostática** (**APH**, en inglés **High Hydrostatic Pressure** o **HHP**), también llamada presurización, pascalización o simplemente alta presión. Es una de las tecnologías más aceptadas (reconocida por el *Codex Alimentarius*) y permite conservar de forma más efectiva que los tratamientos térmicos la calidad (sabor, aroma, color y vitaminas) de determinados alimentos frescos e inactivar microorganismos, esporas y enzimas, incrementando su vida útil (en combinación con la refrigeración) y facilitando su comercialización. Esta tecnología destaca, por tanto, sobre los procesos térmicos (Knorr, 1993), pues estos últimos causan inevitablemente una pérdida de nutrientes y sabores.

El proceso térmico es el método tradicionalmente utilizado para lograr la estabilidad microbiológica de los alimentos y dar lugar a productos estables y seguros, siendo el más usado en la industria alimentaria. Aunque esta tecnología es efectiva, económica y está ampliamente disponible en diversas formas dentro de la industria alimentaria, en muchos casos su aplicación ocasiona una pérdida importante en la calidad de los alimentos, pues, en algunos de ellos, la alta termotolerancia de enzimas y microorganismos (principalmente endosporas bacterianas) hace necesaria la aplicación de tratamientos térmicos extremos, los cuales afectan a las propiedades nutricionales y organolépticas de los alimentos (Raso y Barbosa-Cánovas, 2003). En cambio, el procesamiento por APH a temperaturas de refrigeración, ambiental o con calentamiento moderado, permite la inactivación de microorganismos patógenos y alterantes en los alimentos con cambios mínimos en su textura, color y sabor en comparación con el efecto que tienen los tratamientos térmicos convencionales (Torres y Velázquez, 2005; Velázquez *et al.,* 2002; Cheftel, 1995; Knorr, 1993).

El tratamiento por APH consiste en someter un alimento a elevados niveles de presión hidrostática (magnitudes de 100-1000 MPa) de forma continua durante un cierto tiempo –varios minutos– (Herrero y Romero de Ávila, 2006); con esto se reduce la carga microbiana prolongando su vida útil para conseguir un producto con mejores características organolépticas y con una conservación más extendida en el tiempo. El atractivo principal de esta tecnología es que, al poderse realizar el tratamiento a temperatura ambiente, se conservan los parámetros de calidad del producto original. Como tecnología de conservación, la utilización de APH permite obtener reducciones decimales de al menos 5 unidades logarítmicas en patógenos importantes para la conservación de los alimentos, incluyendo *Salmonella* Typhimurium*, S. enteritidis, L. monocytogenes, S. aureus* y *Vibrio parahaemolyticus* (Velázquez *et al.,* 2005). La APH provoca la inactivación de las células microbianas sin alterar la calidad sensorial ni los nutrientes de los alimentos (Cheftel, 1995).

La APH ofrece una serie de ventajas, aunque posee algunos inconvenientes y limitaciones (Téllez-Luis *et al.,* 2001):

Ventajas:

- Evita la deformación de los alimentos, debido a que la alta presión se transmite de forma uniforme e instantáneamente, es decir, cumple el principio isostático y, por

lo tanto, no hay gradientes. A diferencia de lo que ocurre con los procesos térmicos, el tratamiento APH es independiente del volumen y de la forma de la muestra, con lo que se reduce el tiempo requerido para procesar grandes cantidades de alimento (Cheftel, 1995).

- No se altera el sabor natural, ni la coloración del alimento, pues las altas presiones no favorecen la reacción de Maillard o de pardeamiento no enzimático.
- No deteriora los nutrientes termolábiles, como las vitaminas. No destruye la vitamina C en los zumos, frente a los métodos tradicionales de pasteurización (Kimura *et al.,* 1994); tampoco en patata (Eshtiaghi y Knorr, 1993), ni altera otros compuestos de bajo peso molecular, especialmente los responsables del aroma y sabor.
- No produce residuos, se trata de una energía limpia y de bajo gasto energético, de conformidad con las políticas medioambientales actuales.
- No requiere la incorporación de aditivos alimentarios.
- Mejora y, en muchos casos, induce la aparición de propiedades funcionales en los alimentos.
- Mayor flexibilidad y simplicidad en su limpieza. Permite procesar distintos tipos de alimentos sin necesidad de limpiar la cámara tras cada operación y sin peligro de re-contaminación, se reduce el riesgo de contaminación del alimento por los propios lubricantes de la máquina (Téllez-Luis *et al.,* 2001).

Desventajas:

- El elevado coste de inversión inicial, aunque ya se están desarrollando equipos cada vez más baratos. Además, se ahorra energía debido al bajo consumo energético de esta técnica.
- Modificación de la textura y propiedades reológicas de algunos alimentos.
- La desconfianza del consumidor a decidirse a comprar un producto presurizado por ser algo novedoso y desconocido. A pesar de ello, los productos presurizados se consumen cada vez más en Japón, E.E.U.U. y en algunos países europeos.

Limitaciones:

- No todas las bacterias son igualmente sensibles, y con frecuencia aparecen células parcialmente inactivadas o con daños subletales. El estrés subletal induce mecanismos de reparación celular, lo cual provoca que los microorganismos puedan adaptarse y sobrevivir a varios tratamientos combinados, aunque su

recuperación y proliferación se evita conservando el alimento en frío o manteniendo en alimento a un pH bajo.

- La exposición repetida a la alta presión puede seleccionar mutantes altamente resistentes, lo que conlleva un riesgo de proliferación en los alimentos presurizados. La capacidad de adaptación a las altas presiones, sin embargo, no es un fenómeno generalizado.
- Las endosporas bacterianas son resistentes a los tratamientos por APH convencionales, y pueden germinar en el alimento en ausencia de otras barreras selectivas.

3.2. Funcionamiento de los sistemas de tratamiento por alta presión hidrostática.

En el mercado existen diferentes tipos de equipos para tratamiento por altas presiones, tanto a escala piloto como a escala industrial. En nuestro caso se utilizó un equipo Stansted Fluid Power High Pressure, modelo "Iso-Lab System *FPG9400.*922", U.K. Este modelo trabaja a una presión máxima de 700 MPa (7000 Bar). El equipo (Figura 1) se compone de una canasta para muestras de 80 mm de diámetro × 50 cm de altura, donde se introducen las muestras para su tratamiento por alta presión; un cilindro de presión de doble cara y de acero forjado en níquel, donde se aloja la solución acuosa estabilizante; una cámara de descompresión; una bomba de precarga intensificadora Stansted TC10W-0812 y otra bomba electro-hidráulica Stansted TC40H-1412, un fluido conductor de la bomba hidráulica Shell Tellus 22T. Los fluidos de trabajo constituyen el medio acuoso, permitiendo la distribución de la presión a lo largo de todo el sistema. El agente presurizante utilizado fue agua destilada adicionada de propilenglicol al 5%, que sirve como anticorrosivo y a la vez lubricante (Téllez-Luis *et al.,* 2001). La mezcla sirve como estabilizante para evitar que el agua destilada se congele en el interior de la máquina y ocasione problemas. El equipo trabaja en un rango de temperatura ambiente entre 23 y 27ºC.

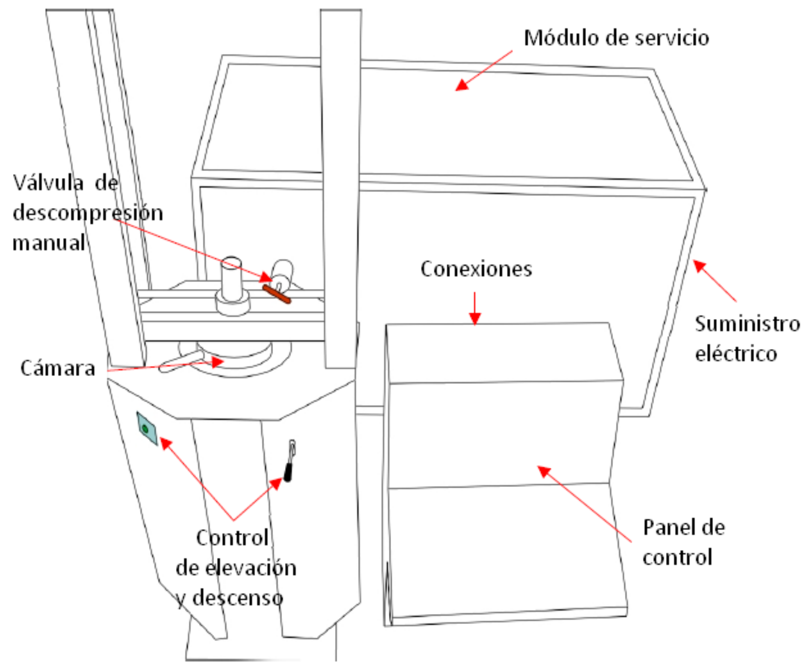

Figura 1. *Esquema del equipo piloto utilizado en nuestro laboratorio de investigación*

El tratamiento que se le ha dado a las muestras se ha realizado con ciclos de presión escalonados (velocidad de subida de 75 MPa/min) hasta llegar a la presión seleccionada, manteniendo la presión durante 8 minutos, seguida de una inmediata y rápida descompresión (Figura 2).

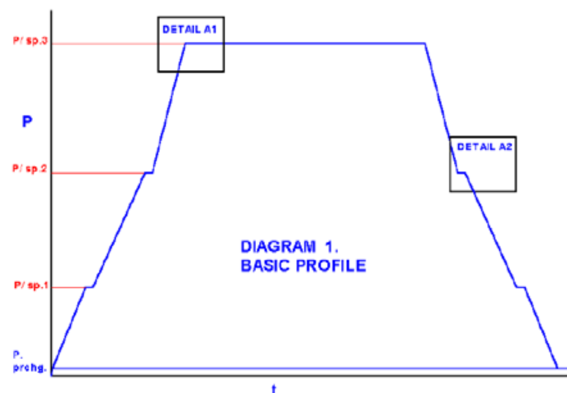

Figura 2. *Perfil básico de funcionamiento de un sistema de alta presión.*

En la actualidad se utilizan con frecuencia equipos de funcionamiento discontinuo, donde los alimentos envasados (líquidos o sólidos) se colocan en el interior de la cámara de presurización. El sistema de bombeo irá sustituyendo el aire de la cámara por el fluido de presurización hasta su total llenado y posteriormente incrementará la presión hasta los

niveles establecidos. Una vez alcanzada la presión deseada, una válvula que cierra el circuito permite su mantenimiento sin necesidad de aporte adicional de energía durante el tiempo. El fluido transmisor de la presión suele ser el agua, de ahí el nombre de alta presión hidrostática, pero existen otros fluidos, los más frecuentemente utilizados son el aceite de silicona, soluciones de benzoato sódico, propilenglicol y etanol.

Fundamento del sistema.

Este método consiste en someter un alimento a ciertos niveles de presión hidrostática (magnitudes de 100-1000 MPa) de forma continua durante un cierto tiempo (varios minutos e incluso horas). En el Sistema Internacional de Medidas, la unidad de presión es el Pascal o N/m2, pero es una unidad muy pequeña para medir presiones altas, por lo que se utilizan escalas mayores (el MegaPascal). Las principales equivalencias en las distintas medidas de presión son: 100 MPa = 1 kbar = 987 atm. La utilización de altas presiones hidrostáticas se rige fundamentalmente por dos principios (Cano *et al.,* 2006; Herrero y Romero de Ávila, 2006):

i. Principio de *Le Chatelier*: enuncia que cualquier fenómeno (reacciones químicas, cambios moleculares, etc.) que va acompañado de una disminución de volumen está favorecido por un aumento de la presión, y viceversa.
ii. Ley de Pascal o Principio Isostático: una presión externa aplicada a un fluido confinado se transmite de forma uniforme e instantánea en todas las direcciones y por todo el espesor de la muestra. De acuerdo con este principio, esta tecnología puede aplicarse directamente a alimentos líquidos o a cualquier producto envasado al vacío y sumergidos en un fluido de presurización de baja compresibilidad (Herrero y Romero de Ávila, 2006).

A diferencia de los procesos térmicos y otras tecnologías de conservación, los efectos de la APH son uniformes y casi instantáneos a través del alimento, permitiendo un tratamiento isostático (Figura 3) y, por lo tanto, son independientes de la geometría, tamaño y volumen del producto procesado (Herrero y Romero de Ávila, 2006; Velázquez *et al.,* 2005).

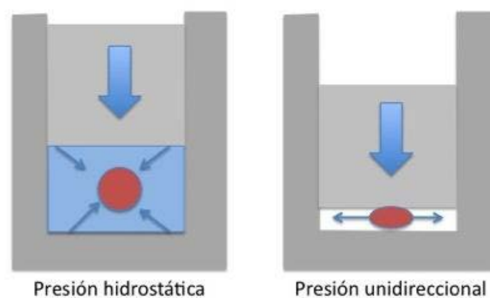

Figura 3. *Principio Isostático de presión.*

Descripción del proceso de presurización en un sistema discontinuo.

El alimento se coloca en un recipiente de plástico estéril, se sella al vacío y se introduce en la cámara de presurización para su procesamiento. El envase de los alimentos a presurizar debe ser flexible y deformable; ha de tolerar reducciones de volumen de hasta un 15% (Herrero y Romero de Ávila, 2006). El material plástico suele ser una película de alcohol de polivinilo (PVOH) y películas copoliméricas de alcohol de etileno y vinilo (EVOH). No hay posibilidad de deformación del producto porque la presión ejercida es uniforme (no hay gradientes).

La cámara de presurización, donde se introduce el alimento envasado, se cierra y se llena con el medio transmisor de la presión. La presión aplicada comprime el medio transmisor alrededor del alimento, provocando una pequeña disminución del volumen que varía según la presión y temperatura aplicadas (Barbosa-Cánovas *et al.,* 1998; Herrero y Romero de Ávila, 2006). A temperatura ambiente, esta reducción es del orden del 4% a 100 MPA, del 7% a 200 MPa, del 11,5% a 400 MPa y del 15% a 600 MPa (Barbosa-Cánovas *et al.,* 1998; Téllez-Luis *et al.,* 2001) y el hecho de que se comprima tan poco volumen hace que el proceso sea poco peligroso, de esta forma se mantiene homogéneo y sin zonas sobretratadas. Al finalizar el tiempo, la cámara se descomprime y se extrae el alimento tratado para colocar una nueva carga de alimento en la cámara de presurización y así se inicia otro ciclo (Barbosa-Cánovas *et al.,* 1998; Casp y Abril, 2008). Es importante la evacuación de los gases del interior para evitar que su compresión reduzca la eficacia de la presurización. Los equipos de alta presión hidrostática empleados en el procesado de alimentos están formados por una cámara de presurización cilíndrica de acero (de elevada resistencia), un generador de la presión (generalmente un sistema de bombeo constituido

por una bomba hidráulica y un sistema multiplicador de presión) y un sistema de control de temperatura. Como sistema de conservación, se han conseguido resultados equivalentes a una pasterización térmica en diversos productos con tratamientos de 400–500 MPa durante varios minutos. También se han probado distintas estrategias para incrementar la eficacia combinando procesos de presurización (> 400 MPa) con tratamientos térmicos suaves (esterilización a baja temperatura) y/o agentes como bacteriocinas (lisozima, nisina) y lactoferrina (Herrero y Romero de Ávila, 2006).

3.3. Efectos biológicos de los tratamientos por alta presión hidrostática.

El efecto de la alta presión sobre la viabilidad de los microorganismos es una combinación de varias acciones (Farr, 1990):

(i) se originan cambios en la morfología de la célula, los cuales son reversibles a bajas presiones (< 200 MPa) pero irreversibles a presiones altas (> 300 MPa);

(ii) provoca la desnaturalización de proteínas a presiones altas debido al desplegamiento de las cadenas peptídicas;

(iii) se originan daños que afectan a la permeabilidad de la membrana celular.

Estos efectos varían en función de la presión aplicada: a 200 MPa hay una clara influencia sobre la cinética enzimática, con una modificación de las propiedades físicas de las proteínas y la alteración de la membrana de los microorganismos. A 300 MPa se produce una inactivación enzimática irreversible y la muerte de ciertos microorganismos. A 400 MPa causa la gelificación del almidón y desnaturalización de las proteínas (Doyle y Beuchat, 2007). Y a partir de los 500 MPa se pueden inactivar enzimas y algunas endosporas bacterianas. Algunos de los efectos mencionados (desnaturalización de proteínas y otras moléculas, inactivación de enzimas) también son extensibles a los componentes del alimento.

3.3.1. Efectos sobre las células microbianas.

La muerte celular se asocia a daños estructurales y/o disfunciones fisiológicas; entre los daños estructurales más frecuentes se citan la interrupción de las envolturas celulares, los cambios conformacionales del ADN, las alteraciones de los ribosomas o la agregación

de proteínas. También se describen alteraciones fisiológicas que provocan la muerte celular, tales como alteraciones en la permeabilidad selectiva de la membrana o la pérdida de las funciones enzimáticas (Gould, 1989). Se sabe que varias de estas lesiones pueden ocurrir al mismo tiempo cuando las células se someten a un tratamiento y es difícil atribuir la pérdida de la viabilidad de la célula a un solo tipo de lesión (Mañas y Pagán, 2005). Se cree que la inactivación es el resultado de los daños múltiples ocasionados por el tratamiento. Por ejemplo, el calor provoca daños en la membrana, la pérdida de nutrientes e iones, la agregación de los ribosomas, la ruptura de la cadena de ADN, la inactivación de enzimas esenciales, la coagulación de proteínas, etc. (Gould, 1989). La presión modifica las reacciones químicas y bioquímicas (Casp y Abril, 2008). Existen numerosas investigaciones publicadas sobre los cambios que ocurren en las células microbianas inducidos por tratamientos de presión, incluyendo modificaciones en la membrana celular, la morfología de la célula, los efectos en las proteínas, incluidas las enzimas, y los efectos sobre la maquinaria genética de los microorganismos. Sin embargo, estos mecanismos todavía no son totalmente conocidos (Pagán y Mackey, 2000).

La alta presión produce cambios morfológicos en las células vegetativas como compresión del gas de las vacuolas, el alargamiento de las células, la formación de filamentos, la separación de la membrana celular de la pared celular, la formación de poros por contracción de la pared celular, modificaciones del citoesqueleto, núcleos y orgánulos intracelulares (en el caso de células eucariotas), la coagulación de la proteína citoplasmática y liberación de constituyentes intracelulares fuera de la célula. Al mismo tiempo, provoca modificaciones bioquímicas y genéticas al inactivar enzimas implicadas en la replicación y transcripción del ADN (Téllez-Luis *et al.,* 2001). Una de las estructuras biológicas más importantes es la membrana celular (Escriu y Mor-Mur, 2009) y se ha demostrado que juega un papel importante en la supervivencia de las células sometidas a alta presión. Los microorganismos que contaminan los alimentos son sensibles a las altas presiones, principalmente debido a que su membrana celular, rica en lípidos y proteínas, se ve afectada por la sensibilidad de estos componentes (Casp y Abril, 2008). Además, las células dañadas no siempre son capaces de crecer en medios selectivos; por esa razón es conveniente utilizar tanto medios de cultivo selectivos como medios generales de crecimiento a la hora de determinar los efectos de los tratamientos por APH en la viabilidad celular. A continuación se citan algunas modificaciones:

Membranas: Mañas y Pagán (2005) señalan que una membrana citoplasmática intacta es esencial para el mantenimiento de la homeostasis bajo condiciones ambientales desfavorables. Las células que se encuentran en fase estacionaria son normalmente más resistentes a la presión que las células en fase exponencial. Mañas y Mackey (2004) propusieron que, durante la fase exponencial, las células se inactivan a alta presión por el daño irreversible en su membrana celular. Por el contrario, en la fase estacionaria, las células tienen una membrana citoplasmática más gruesa y pueden soportar mejor el tratamiento a presión. Esta propuesta se basaba en el hecho de que durante la fase exponencial las células mostraban cambios en sus envueltas celulares que no fueron vistos en la fase estacionaria. Estos cambios incluyen perturbaciones físicas en la estructura de la envoltura celular, la pérdida en la capacidad de respuesta osmótica y la pérdida de proteínas y ARN al medio extracelular.

Morfología celular: La pared celular se ve menos afectada por la alta presión que la membrana celular y, por lo general, no se producen cambios morfológicos que puedan ser observados con un microscopio óptico, aunque el daño intracelular sí se puede observar mediante microscopía electrónica (Patterson, 2005).

Mecanismos genéticos: Los ácidos nucleicos (Patterson, 2005) son relativamente resistentes a las altas presiones, y como la estructura de la doble hélice de ADN es en gran parte el resultado de la formación de enlaces de hidrógeno, también es estable bajo presión. Sin embargo, la actuación de las enzimas implicadas en las etapas de replicación y transcripción del ADN se interrumpe. Se ha descrito que la presión provoca una condensación del material nuclear en *L. monocytogenes* y *S.* Typhimurium (Mackey *et al.* 1994). Chilton *et al.* (1997) postularon que a presiones elevadas, el ADN entra en contacto con endonucleasas que fragmentan el material genético.

3.3.2. Efectos sobre biomoléculas: proteínas, enzimas y vitaminas.

A una intensidad adecuada, un tratamiento por alta presión consigue inactivar un número importante de microorganismos. Sin embargo, en algunos casos también puede afectar las propiedades sensoriales de los alimentos, alterando la estructura de proteínas y polisacáridos, causando cambios en la textura, la apariencia física y la funcionalidad de los alimentos (Knorr, 1993; Williams, 1994). También puede desnaturalizar las proteínas y

producir radicales libres que pueden afectar negativamente al sabor de frutas o alimentos con alto contenido en grasa (Williams, 1994). El caso es que las altas presiones, en los rangos de presión y de temperatura utilizados en la industria alimentaria, sólo actúan sobre los enlaces químicos débiles, pues la energía que interviene es insuficiente para romper los enlaces covalentes. Por tanto, no hay aparición de pequeñas moléculas ni productos de degradación procedentes de la ruptura de grandes moléculas.

Los **azúcares** simples y las **vitaminas** son pequeñas moléculas en las que no intervienen enlaces débiles y apenas son alteradas por la presurización. Por el contrario, las macromoléculas, tales como las proteínas y polisacáridos (almidón, pectinas, alginatos, etc.), son ricas en enlaces débiles, y estos enlaces que aseguran su estabilidad o les confieren propiedades texturizantes pueden ser modificados irreversiblemente bajo presión (Casp y Abril, 2008). En el caso de las vitaminas**,** Sancho *et al.* (1999) estudiaron el efecto del tratamiento por alta presión en la retención de las vitaminas hidrosolubles B1, B6 y C de un modelo multivitamínico en yema de huevo y fresas, tras un tratamiento de alta presión, comparándolo con la retención lograda en estas vitaminas a las temperaturas de pasteurización y esterilización. Encontraron que las vitaminas B1 y B6 siempre se mantenían en más de un 99% en todos los procesos, mientras que la vitamina C se retenía menos en los tres procesos, independientemente de la intensidad del tratamiento de presión aplicado.

La alta presión desnaturaliza las **proteínas** actuando principalmente en los enlaces hidrofóbicos y electrostáticos de la molécula. Presiones en el rango de 100-300 MPa provocan la disociación de proteínas oligoméricas, siendo dicha desnaturalización reversible. Por otro lado, presiones superiores a 300 MPa producen la desnaturalización irreversible de proteínas de cadena simple (Barbosa-Cánovas *et al.,* 1998). La desnaturalización depende del tipo de proteína, las condiciones en el procesado y el rango de presión aplicada; en base a estos factores las proteínas pueden disolverse en el medio o precipitar (Rastogi *et al.,* 2007).

Las altas presiones modifican las estructuras terciaria y cuaternaria de las proteínas y, por tanto, también afectan a las **enzimas**. El daño inducido por la presión en las membranas facilita el contacto enzima-sustrato y la reacción resultante puede ser acelerada o retardada por la presión (Rastogi *et al.,* 2007). Se ha observado que presiones

relativamente bajas (100 MPa) activan algunas enzimas de tipo monomérico y presiones más elevadas provocan la inactivación enzimática (Hendrickx *et al.,* 1998). El grado de inactivación depende del tipo de enzima, pH, composición del medio, temperatura, etc. (Balny y Masson, 1993). El efecto de la alta presión varía ampliamente entre las diversas enzimas, probablemente debido a las diferencias en su estructura. En general, combinaciones de presión con temperaturas moderadas aumentan el nivel de inactivación enzimática, pero en algunos casos se ha descrito un incremento en la actividad enzimática (Hendrickx *et al.,* 1998). Por ejemplo, en el caso de la enzima polifenoloxidasa (PPO) en champiñones, se ha comprobado un incremento de su actividad tras una presurización a 400 MPa (San Martín *et al.,* 2002). Temperaturas entre 45 y 55ºC y presiones entre 600 y 900 MPa pueden provocar un grado de inactivación variable de enzimas como pectinesterasa, lipasa, polifenoloxidasa (PPO), lipoxigenasa, peroxidasa (POD), lactoperoxidasa, fosfatasa y catalasa (Raso y Barbosa-Cánovas, 2003; Seyderhelm *et al.*, 1996). La gran resistencia de las enzimas endógenas a los tratamientos por APH o a la combinación de APH con calor pone de manifiesto la necesidad de combinar la presurización con otras técnicas, tales como el almacenamiento a baja temperatura, modificaciones químicas de las enzimas y el uso de enzimas ("killer enzymes" o "enzimas asesinas") de origen natural o inhibidores de proteínas para mantener la calidad de los alimentos y prolongar la vida útil (Ashie *et al.,* 1996).

3.3.3. Inactivación de los microorganismos por altas presiones.

Las **células vegetativas** son bastante sensibles a la presión, siendo inactivadas a presiones entre 300 y 600 MPa, mientras que las endosporas bacterianas son más resistentes y se inactivan a presiones superiores a 1200 MPa (Knorr, 1995). Así pues, las bacterias Gram-negativas (*Salmonella*, coliformes, etc.) son las más sensibles a las altas presiones y, por tanto, más fáciles de destruir, seguidas por levaduras, mohos y bacterias Gram-positivas (estreptococos, estafilococos, *Listeria*, etc.) que requieren un tratamiento de mayor intensidad y, por último, las más resistentes son las endosporas de las bacterias esporuladas, como *Clostridium* y *Bacillus* (Smelt, 1998; Casp y Abril*,* 2008). En términos generales, las bacterias Gram-positivas tienden a ser más resistentes a la presión que las Gram-negativas y los cocos son más resistentes que las bacterias de forma bacilar (Patterson, 2005)

Una de las operaciones más difíciles en la conservación de los alimentos es la inactivación de las **endosporas bacterianas**: bajo un tratamiento de alta presión, las endosporas pueden germinar a células vegetativas y así pueden ser inactivadas. Esta inactivación está fuertemente influida por la temperatura y menos fuertemente por el pH, la actividad de agua y la fuerza iónica (Cano *et al.,* 2006). La inactivación de las endosporas es mayor cuando el pH se acerca a la neutralidad y menor a niveles extremos de pH, ya que la presión a un pH neutro induce la germinación de las endosporas (Hayakawa *et al.,* 1994). La inactivación de endosporas bacterianas con APH, a diferencia de la inactivación de las bacterias vegetativas, ocurre en 2 pasos. Inicialmente, la presión induce la germinación de las endosporas y a continuación, inactiva las formas germinadas (Gould y Sale, 1970; Heinz y Knorr, 2001). El inicio de la germinación e inactivación de endosporas bacterianas por APH son altamente dependientes de la temperatura. En general, las endosporas bacterianas parecen ser resistentes a los tratamientos de APH a temperatura ambiente, se ha descrito que pueden resistir presiones tan altas como los 800 MPa durante varias horas. Sin embargo, presiones tan bajas como 10 MPa pueden iniciar la germinación de las endosporas bacterianas, sensibilizándolas al calor, radiación, agentes químicos e incluso tratamientos de APH (Gould, 1973).

La combinación de APH y calor es especialmente eficaz a temperaturas que permiten la inactivación de endosporas germinadas (> 60ºC), lo que sugiere que las endosporas germinadas por APH son directamente inactivadas por el calor. Sin embargo, recientemente se ha observado que la inactivación de las endosporas de *Bacillus subtilis* y *Geobacillus stearothermophilus* por APH a 70 y 90ºC, respectivamente, no implicaron su germinación. Hayakawa *et al.* (1994), encontraron que la inactivación de endosporas de *G. stearothermophilus* mediante APH en combinación con calor fue más eficaz cuando se aplicaron tratamientos oscilatorios (6 ciclos de 5 minutos). A 70ºC y 600 MPa, se obtuvieron 4 unidades logarítmicas de inactivación con un tratamiento continuo y más de 6 unidades logarítmicas de inactivación con el tratamiento oscilatorio.

Las **levaduras** generalmente no se asocian con enfermedades transmitidas por alimentos pero son importantes en su deterioro, especialmente en alimentos ácidos. Son relativamente sensibles a la presión y es ésta una razón por la cual el tratamiento a presión de productos frutícolas para extender la vida útil resulta particularmente exitoso (Patterson, 2005).

Por otro lado, existe poca información sobre la presión la sensibilidad de los **mohos**, pero se ha demostrado que las formas vegetativas son relativamente sensibles, mientras que las ascosporas son más resistentes (Patterson, 2005). El efecto de la presión sobre las micotoxinas se cree que es limitado, ya que el tratamiento tiene poco efecto en los enlaces covalentes. La inactivación de formas vegetativas de levaduras y mohos en combinación con APH y calor apenas ha sido investigado, probablemente porque los microorganismos implicados son muy sensibles a leves tratamientos de presión a temperatura ambiente (Raso y Barbosa-Cánovas, 2003). Sin embargo, la inactivación de ascosporas en mohos requiere la combinación de APH con temperaturas moderadas de 60-70ºC.

Los **virus** son agentes infecciosos de gran importancia en alimentos que se consumen crudos (como vegetales y productos marinos) y que pueden contaminarse por la ruta fecal-oral. Los virus muestran un grado de sensibilidad variable a las altas presiones (Baert *et al.*, 2009; Kovač *et al.*, 2010). En general, los virus envueltos son más sensibles que los virus desnudos. Los estudios realizados con norovirus muestran que éstos son altamente sensibles a los tratamientos por APH, aunque su inactivación está influenciada por factores como el pH y la temperatura. Por el contrario, el virus de la polio es altamente resistente a los tratamientos por APH.

3.4. Aplicaciones de las altas presiones en alimentos.

Las altas presiones, por todas sus ventajas y características, cuentan con aplicaciones de muy diversa índole en la industria alimentaria, la mayoría de ellas orientadas a la conservación de los alimentos. Japón fue el país pionero en el desarrollo de la alta presión para su utilización en la industria alimentaria, hacia la década de los ochenta, y fue ya en 1992 cuando salieron al mercado los primeros productos tratados por alta presión, seguido por Estados Unidos, Alemania, Francia y España (Guamis *et al.,* 2006). Debido al éxito comercial que supuso la presurización en mermeladas y otros productos confitados, se han comercializado también jaleas y mariscos en Japón, ostras y guacamole en Estados Unidos y jugos de fruta en Francia, México y Reino Unido (Smelt, 1998; Torres y Velázquez, 2005).

En algunos trabajos (Mozhaev *et al.,* 1994; Cheftel *et al.,* 1995; Ledward *et al.,* 1995; Téllez-Luis *et al.,* 2001) se describen todas estas aplicaciones para distintos grupos de alimentos. Entre ellas figuran:

(i) pasteurización y esterilización sin modificar el valor nutritivo ni las propiedades organolépticas de los alimentos;

(ii) inactivación/activación de enzimas para retardar/acelerar procesos de maduración, fermentación u otro tipo de transformaciones enzimáticas deseables en los alimentos;

(iii) modificación de la estructura debido a cambios en la configuración proteica (ablandamiento de textura en carnes y pescados, decoloración de hemoglobina en sangre de animales, inactivación de ciertas toxinas);

(iv) cambios en las transiciones de fase (congelación a temperaturas bajo cero evitando la formación de cristales de hielo, disminución del punto de fusión de lípidos, gelatinización a bajas temperaturas);

(v) extracción de componentes alimentarios (pectinas, pigmentos e incluso agua), (vi) agregación de sólidos o polvos alimentarios para elaborarlos en forma de barras, cubos, tabletas;

(vii) impide el pardeamiento no enzimático en determinados alimentos y evita la oxidación lipídica en ciertos productos.

La APH es bastante suave comparada con el resto de métodos de procesado de alimentos, por tanto, puede inactivar algunos microorganismos y enzimas que producen la alteración de los alimentos almacenándolo a bajas temperaturas sin cambiar la mayoría de las propiedades sensoriales o nutricionales de los mismos (Zhou *et al.,* 2010), pero las altas intensidades del tratamiento requerido para la inactivación de algunos microorganismos pueden causar cambios adversos en las propiedades sensoriales y funcionales de los algunos alimentos (Knorr, 1993; Williams, 1994). A pesar de ello, en la mayoría de los casos, la APH actúa manteniendo y/o mejorando las características organolépticas de los alimentos.

3.4.1. Zumos, frutas y verduras.

La APH se usa principalmente en industrias alimentarias dedicadas al procesado de frutas, verduras y hortalizas para la inactivación de microorganismos y enzimas y para extender la vida media de estos productos, a la vez que mantiene y/o mejora las propiedades organolépticas, sensoriales y nutricionales (Rastogi *et al.,* 2007). Los zumos de cítricos, junto con las confituras y productos derivados del tomate, fueron los primeros alimentos tratados por altas presiones. Su pH ácido (Casp y Abril, 2008) facilita la

destrucción de microorganismos por altas presiones y evita la germinación de endosporas tras el tratamiento. Las altas presiones, al contrario que el calor, permiten conservar el sabor del zumo fresco y su contenido en vitaminas. La vitamina C contenida en los zumos no se destruye incluso con tratamientos de 600 MPa durante 10 minutos a temperatura ambiente. El zumo de pomelo presurizado, a diferencia de los procesos térmicos convencionales, no posee el sabor amargo que le confiere el limoneno; los zumos de otros cítricos tras el tratamiento APH adquieren un sabor fresco, sin pérdida de la vitamina C y con mayor vida útil (Téllez-Luis *et al.,* 2001), mientras que la estructura interna del tomate se endurece con la presurización. Por el contrario, algunas frutas como las peras, manzanas y caquis adquieren una textura más blanda y se produce un rápido oscurecimiento tras la presurización, debido a que la intensidad de la presión aplicada incrementa la actividad de la enzima polifenoloxidasa (Asaka y Hayashi, 1991).

En el caso de las mermeladas y confituras, la APH permite conservar el sabor y el color de la fruta fresca. Kimura *et al.* (1994) afirman que las mermeladas obtenidas por alta presión retienen el sabor y el color de la fruta fresca, a diferencia de las mermeladas convencionales procesadas por calentamiento. Un tratamiento de 400-600 MPa durante 10-30 minutos a temperatura ambiente permite la esterilización del producto y, a su vez, la penetración del azúcar en los trozos de frutas y la formación de geles (Casp y Abril, 2008; Cheftel, 1995). La estabilización se produce sin afectar a las características físico-químicas del producto ni a su valor nutricional, principalmente las vitaminas C y A (Guamis *et al.,* 2006), aunque para conseguir la inactivación de enzimas resistentes a la presión, como enzimas pectinolíticas y polifenoloxidasas, resulta necesario un tratamiento térmico moderado que acompañe a la presión (Farr, 1990).

3.4.2. Leche y derivados lácteos.

La aplicación de la APH en la leche comenzó a investigarse como una alternativa a los procesos de pasteurización para la inactivación de microorganismos (tales como *L. monocytogenes, S. aureus* y *L. innocua*) bien presentes en la leche de forma natural o introducidos por contaminación cruzada (Rastogi *et al.,* 2007). Los tratamientos de la leche por altas presiones (Guamis *et al.,* 2006) provocan la desintegración de la estructura micelar y la desnaturalización de proteínas séricas que precipitan a pH ácido junto a la fracción caseínica de la leche (Felipe *et al.,* 1997). En contrapartida, las propiedades de coagulación

de la leche por el cuajo animal y la capacidad de retención de agua se ven incrementadas. Las consecuencias son una mejora importante en el rendimiento quesero, así como de las propiedades de la cuajada.

Los usos de la APH en estos productos son muy variados: se utiliza para reducir la carga microbiana de la leche y el queso, el desarrollo del aroma en la maduración acelerada del queso suizo y el queso Cheddar, y también se aplica en la producción de yogur para prevenir la acidificación del yogur después del envasado. Con tratamientos entre 200-300 MPa durante 10 minutos a 20ºC se evita la acidificación del yogur tras el envasado a la vez que se mantiene la población de bacterias lácticas, mientras que a partir de los 300 MPa los recuentos de estos microorganismos disminuyen (Guamis *et al.,* 2006).

3.4.3. Carnes y derivados cárnicos.

La APH aumenta y alarga la vida útil de la carne y productos cárnicos almacenados bajo refrigeración. Además de la mejora microbiológica en carnes y derivados cárnicos presurizados, también se producen otros efectos de especial interés. La alta presión rompe la membrana de los lisosomas y las proteasas contenidas en ellos se liberan en el citoplasma (Homma *et al.,* 1994). Como consecuencia de la desnaturalización causada por la presión, las proteínas son más sensibles a las proteasas sin que su valor biológico se vea alterado; esto mejora la digestibilidad y además influye positivamente en el aroma y sabor de la carne. Los tratamientos por alta presión también ablandan los tejidos cárnicos en *pre-rigor mortis*, mejorando su textura. Sin embargo, la carne y productos cárnicos no curados que han sido presurizados tienen un aspecto más pálido, lo que puede provocar el rechazo por parte del consumidor.

La presurización también se aplica en carnes recuperadas mecánicamente y en la obtención de geles y carnes reestructuradas (que sirven como ingredientes en las formulaciones de productos cárnicos y avícolas). La alta presión, a pH y temperaturas adecuados, puede aumentar la cohesión entre partículas y en productos picados o emulsiones, reduciendo considerablemente la cantidad de NaCl y polifosfatos a añadir (Guamis *et al.,* 2006). Los geles obtenidos por presurización son más uniformes y presentan mejor aspecto, textura y exudan menos que los obtenidos por calor.

3.4.4. Huevos y ovoproductos.

Las altas presiones pueden también permitir mejorar la calidad microbiológica de huevos y ovoproductos, pero la yema de huevo coagula a 400 MPa y la clara a 600 MPa, es decir, el huevo se cuece en frío, por lo que aparentemente no tiene gran futuro en la industria alimentaria (Casp y Abril, 2008). Donde sí se obtienen buenos resultados es mediante la presurización del huevo líquido haciendo ciclos de subida y bajadas de presión que permitan la permanencia del producto durante un corto tiempo a la presión máxima; así se consigue disminuir la coagulación del producto mientras se destruyen los microorganismos contaminantes -las salmonellas y coliformes son particularmente sensibles-. La presión actúa eficazmente en la destrucción de microorganismos patógenos y alterantes del huevo líquido, de manera que en condiciones de refrigeración se puede obtener una vida útil de 30 días tras la aplicación de tratamientos cíclicos de presión: 450 MPa/3 ciclos de 5 min (Ponce *et al.,* 1999). Este procedimiento es interesante para las industrias que utilizan los ovoproductos como materia prima, puesto que las propiedades tecnológicas de los huevos se modifican poco por las altas presiones. Además, los huevos sometidos a altas presiones no tienen el sabor y el olor sulfuroso característico provocado por el calentamiento (Téllez-Luis *et al.,* 2001).

3.4.5. Pescado y mariscos.

El pescado, y en particular los moluscos bivalvos, son vehículos de muchas enfermedades de origen alimentario, por lo que se están implantando los tratamientos por APH para reducir los riesgos asociados al consumo de marisco crudo como, por ejemplo, en el procesamiento comercial de ostras. Se ha constatado que el patógeno *Vibrio* sp. (*V. vulnificus*, *Vibrio parahaemolyticus*) es relativamente sensible a un tratamiento moderado de APH durante varios minutos. El tratamiento por APH favorece la inactivación de *Vibrio* sp. en ostras, a la vez que mejora su apariencia, textura y sabor (Murchie *et al.*, 2005), además de facilitar la apertura y separación de la concha, lo que permite una mejor comercialización de las ostras listas para su consumo.

La APH se también se utiliza en la producción de surimi y kamaboko, productos tradicionales japoneses elaborados con carne picada de pescado, debido a que el tratamiento por alta presión induce la gelificación, proporcionando una textura más suave y

uniforme y conservando el color y sabor del pescado, en comparación con el tratamiento térmico.

En la industria alimentaria también se emplea la APH para el pelado de crustáceos, facilitando la separación de la concha y la carne en langostas y cangrejos (de ese modo se evitan los daños físicos por el pelado con cuchillo), mejorando el rendimiento y reduciendo los costes en la producción. Generalmente se aplican presiones entre 250 y 400 MPa con tiempos de exposición cortos, de 1 a 3 minutos, los que mejora la calidad microbiológica y permite comercializar el marisco fresco sin necesidad de usar calor. Además de la destrucción de microorganismos patógenos y la mejora de la calidad del producto, la APH se está abriendo camino en nuevos mercados, particularmente en la industria de sushi, siendo una apuesta comercial prometedora.

3.4.6. Vinos.

En enología apenas se ha estudiado la aplicación de las altas presiones para la elaboración y conservación del vino. Este proceso sólo se ha probado en algunas ocasiones para la estabilización microbiológica de mostos y vinos (Guamis *et al.,* 2006), incluido el efecto de la APH sobre la inactivación de la enzima polifenoloxidasa, la estabilidad del color y la calidad organoléptica de los mostos. Uno de los objetivos es conseguir la inertización microbiológica sin necesidad de incorporar sulfitos y demás aditivos que pueden provocar cambios organolépticos que empobrecen o empeoran la calidad del producto final (Guamis *et al.,* 2006). La eliminación de sulfitos aportaría también otras ventajas desde el punto de vista normativo además de disminuir el riesgo de aparición de alergias.

3.4.7. Otras posibles aplicaciones.

Aunque las altas presiones todavía no se han puesto en práctica a gran escala en la industria agroalimentaria en muchos países, hoy en día es posible pascalizar zumos de frutas, confituras y yogures con tratamientos entre 350 y 600 MPa durante 5-30 minutos a temperatura ambiente o con un calentamiento moderado (Casp y Abril, 2008). Así mismo, se han propuesto muchas otras aplicaciones, algunas de las cuales se están utilizando

también a escala industrial mientras que otras no han pasado de la fase experimental (Hoover, 1993; San Martin *et al.*, 2002; Rendueles *et al.,* 2011):

(i) el desarrollo de nuevos productos a base de frutas (coulis, salsas, etc.);

(ii) la pascalización de platos preparados, sopas, etc.;

(iii) la gelatinización de almidones: la alta presión provoca la gelatinización del almidón en harinas de trigo a partir de los 400 MPa (Gomes *et al.,* 1998), por lo que esta alteración de la estructura del almidón y de la proteína se podría utilizar en el arroz para cocerlo en pocos minutos mediante la alta presión (Hoover *et al.,* 1989). La estructura cristalina del almidón de maíz y arroz se destruyen por presiones de 500 MPa, mientras que en patata no se ve afectada (Téllez-Luis *et al.,* 2001);

(iv) la conservación de alimentos a temperaturas inferiores a 0°C sin congelación: tanto la textura como la estructura de las zanahorias mejoran cuando se exponen a presiones de 200-400 MPa a temperaturas de -20ºC (Téllez-Luis *et al.,* 2001), porque la densidad del agua en estas condiciones es mayor que la densidad a presión atmosférica;

(v) la descongelación acelerada a baja temperatura.

OBJETIVOS (AIMS)

El tratamiento mediante alta presión hidrostática se considera una tecnología con un elevado potencial para la conservación de los alimentos, por su eficacia frente a diferentes tipos de microorganismos. Ha sido bien aceptada por los consumidores, dado que el producto tratado conserva mejor sus propiedades organolépticas y nutritivas en comparación con los tratamientos térmicos convencionales, y evita o disminuye el empleo de conservantes químicos. Por ello resulta de gran interés para el procesado no térmico de alimentos con propiedades funcionales, y para la mejora en general de la calidad higiénico-sanitaria de los alimentos. También podría ser de gran interés para evitar la transmisión de patógenos emergentes o con resistencias a antimicrobianos a lo largo de la cadena alimentaria. Las altas presiones se pueden aplicar como único tratamiento sobre el producto ya envasado, o como parte de la tecnología de barreras, en combinación con otros agentes antimicrobianos. En este contexto, existe un gran interés en mejorar la eficacia de los tratamientos por alta presión mediante combinación con antimicrobianos naturales como pueden ser las bacteriocinas o los aceites esenciales.

Un fenómeno al que se ha prestado relativamente poca atención en los tratamientos por alta presión es la presencia de una fracción superviviente de bacterias, que posteriormente podrían multiplicarse en el alimento. También es conocido que las altas presiones provocan daños subletales que algunas células bacterianas son capaces de reparar si encuentran las condiciones adecuadas. La mayoría de los estudios previos sobre la fracción superviviente a los tratamientos por alta presión se han realizado sobre microorganismos modelo (generalmente los patógenos de origen alimentario más importantes), pero el fenómeno es igualmente extrapolable al resto de poblaciones microbianas presentes en el alimento a tratar. Conocer el impacto que tienen los tratamientos por altas presiones sobre la microbiota total de los alimentos es algo que sólo ha sido posible tras el desarrollo de avances tecnológicos como la secuenciación masiva de ADN, la creación de bases de datos genéticos y la puesta en funcionamiento de herramientas bioinformáticas que permitan manejar la ingente cantidad de información generada.

Considerando los antecedentes arriba mencionados, el presente estudio se planteó con los siguientes objetivos:

1. Conocer el potencial de los tratamientos por alta presión, sola o con antimicrobianos naturales (bacteriocinas nisina y enterocina AS-48, y aceites esenciales) en la inactivación de *Staphylococcus aureus* resistentes a meticilina como modelo de bacteria portadora de resistencia a antibióticos.
2. Conocer el potencial de las altas presiones en el control de *Salmonella enterica* en un alimento tradicional listo para consumo con un pH próximo a neutralidad como la tortilla de patatas, y determinar la capacidad de recuperación de las células de *Salmonella* con daños subletales durante el almacenamiento del alimento tratado.
3. Conocer la capacidad de supervivencia de tres patógenos transmisibles por alimentos (*Listeria monocytogenes, Salmonella enterica* y *Escherichia coli*) en un alimento tradicional listo para consumo con un pH ácido (salmorejo), y determinar la eficacia de los tratamientos por alta presión en su inactivación.
4. Conocer la eficacia de los tratamientos por altas presiones solos o en combinación con enterocina AS-48 sobre la carga microbiana total y sobre bacterias alterantes del género *Leuconostoc* en pulpa de chirimoya.
5. Conocer el potencial de los tratamientos por altas presiones y/o enterocina AS-48 en la conservación de pulpa de chirimoya y determinar su impacto sobre la diversidad bacteriana y la dinámica de las poblaciones supervivientes durante el almacenamiento de las muestras durante su almacenamiento.
6. Conocer el potencial de los tratamientos por altas presiones para la conservación de espárragos en salmuera y determinar su impacto sobre la carga microbiana total, diversidad bacteriana y dinámica de las poblaciones bacterianas en las muestras durante su almacenamiento.

AIMS

High hydrostatic pressure is considered to be a food technology with a strong potential for application in food preservation against different types of microorganisms. This food processing technology is well accepted by consumers, since it allows better preservation of the food product organoleptic properties and nutritional value in comparison to conventional heat treatments, while at the same time decreasing or avoiding the use of chemical preservatives. Therefore, it seems very interesting for non-thermal processing of foods with functional properties as well as to improve the food sanitary quality. It could also be of interest as treatment against transmission of emerging foodborne pathogens or strains carrying antimicrobial resistance traits. High hydrostatic pressure processing can be applied as a single treatment directly on the packaged food product, but it can also be applied as part of hurdle technology in combination with other antimicrobials. In this context, there is great interest to improve the efficacy of high hydrostatic pressure treatments in combination with natural antimicrobials such as bacteriocins or essential oils.

Comparatively less attention has been paid in past research to the fraction of bacterial populations surviving high hydrostatic pressure treatments, an issue of outmost importance since surviving bacterial cells could further proliferate in the processed food. It is also known that high hydrostatic pressure treatments can yield sublethally-injured cells, which may be able to repair cell damage under suitable conditions. Most studies on the surviving fraction after high hydrostatic pressure treatments have been carried out on models based on the most common foodborne pathogens, but the phenomenon of survival could well be extrapolated to the remaining bacterial populations that may be present in the food to be treated. Investigation of the global effects of high hydrostatic pressure treatments on the food microbiota has only been possible after implementation of advances in other technologies such as massive DNA sequencing, the generation of strong and robust DNA sequence databases, and the development of bioinformatic tools that facilitate the analysis of the huge amount of data generated.

Considering the existing background of information as briefly summarized above, the present study included the following main aims:

1. To determine the potential of high hydrostatic pressure treatments applied singly or in combination with natural antimicrobials (including the bacteriocins nisin and enterocin AS-48 and essential oils) for inactivation of methicillin-resistant *Staphylococcus aureus* as a model foodborne bacterium carrier of antimicrobial resistance.
2. To determine the potential of high hydrostatic pressure treatments in the control of *Salmonella enterica* in Spanish omelette (a traditional ready-to-eat food having a pH close to neutrality), and to determine the capacity for recovery of sublethally-injured *Salmonella* cells during storage of the treated food.
3. To determine the capacity of three common foodborne pathogens (*Listeria monocytogenes, Salmonella enterica* and *Escherichia coli*) to survive in a traditional ready-to-eat food having an acidic pH (*salmorejo*), and to determine the efficacy of high hydrostatic pressure treatments for inactivation of these foodborne pathogens in the food.
4. To determine the potential of high hydrostatic pressure treatments applied singly or in combination with enterocin AS-48 on the microbial load and also on spoiling bacteria of genus *Leuconostoc* in cherimoya pulp.
5. To determine the potential of high hydrostatic pressure treatments and/or enterocin AS-48 in the preservation of cherimoya pulp and to determine the impact of treatments on the bacterial diversity in the pulp and the dynamics of bacterial populations during storage.
6. To determine the potential of high hydrostatic pressure treatments in the preservation of brined green asparagus as well as the impact of treatments on the microbial load, bacterial diversity and dynamics of bacterial populations during storage of asparagus.

TRABAJO EXPERIMENTAL Y RESULTADOS

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Bactericidal effects of high hydrostatic pressure treatment singly or in combination with natural antimicrobials on *Staphylococcus aureus* in rice pudding

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Inactivation of *Staphylococcus aureus* strains by high hydrostatic pressure (HHP) treatments applied singly or in combination with natural antimicrobials (nisin, enterocin AS-48, cinnamon oil and clove oil) was investigated in rice pudding. Treatments at 600 MPa for 10 min reduced initial populations of staphylococci (7.9 log CFU/g) below detectable levels of 1 log CFU/g in the puddings. Treatments at 500 MPa for 5 min (achieving a 2.9-log reduction of viable counts) were investigated singly or in combination with nisin (200 and 500 IU/g), enterocin AS-48 (25 and 50 μg/g), cinnamon oil (0.2%, vol/wt) or clove oil (0.25% vol/wt). The combined treatment of enterocin AS-48 (50 μg/g) and HHP caused a non-significant reduction of 0.4–0.6 log cycles compared to HHP alone. Additional reductions of 0.87, 1.3 and 1.8 log cycles were recorded for the combined HHP treatments with nisin (500 IU/g), cinnamon oil (0.2%) and clove oil (0.25%), respectively. During refrigeration storage for one week, viable counts in puddings from combined treatments were significantly lower compared to the single HHP treatments, e.g. 1.5–2.7 log cycles for HHP-nisin (500 IU/g), 1.1–1.3 log cycles for HHP-AS-48 (50 μg/g) or approx. 1.5 log cycles for HHP-cinnamon oil (0.2%). These results suggest that the time and intensity of HHP treatments required for inactivation of *S. aureus* in puddings can be reduced when HHP is applied in combination with selected natural antimicrobials.

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1. Introduction

Staphylococcus aureus is found in the nostrils as well as on the skin and hair of warm-blooded animals, and up to 30–50% of human population are carriers (Le Loir, Baron, & Gautier, 2003). It has been isolated from several foods including meat and meat products, chicken, milk and dairy products, fermented food items, salads, vegetables, fish products, etc. (Jørgensen et al., 2005; Seo & Bohach, 2007; Tamarapu, McKillip, & Drake, 2001; Wieneke, Roberts, & Gilbert, 1993). Staphylococcal food poisoning is among the most common causes of reported food-borne diseases (Bean, Goulding, Matthew, & Angulo, 1997; EFSA, 2010; Le Loir et al., 2003; Mead et al., 1999; Tirado & Schmidt, 2001; WHO, 2002), requiring hospital attention by up to 19.5% of the affected individuals (EFSA, 2010). Most strains are capable of producing one or more heat stable enterotoxins (Balaban & Rasooly, 2000; Ortega, Abriouel, Lucas, & Gálvez, 2010) which are the cause of the gastrointestinal symptoms observed during intoxications (Tamarapu et al., 2001). *S. aureus* is also widely disseminated in nosocomial infections, where it poses a threat due to its acquired resistance to most common antimicrobials. Methicillin-resistant *S. aureus* (MRSA) strains are of particular concern (Ippolito, Leone, Lauria, Nicastri, & Wenzel, 2010). The presence of enterotoxin-producing antibiotic-resistant *S. aureus* strains in foods is an additional risk for dissemination of antibiotic resistance through the food chain and also for exposure of immunocompromised individuals to more virulent strains.

One of the methods proposed to control staphylococci in foods is high hydrostatic pressure (HHP) treatments (Ananou et al., 2010; Erkmen & Karataş, 1997; Fioretto et al., 2005; Gervilla, Ferragut, & Guamis, 2000; López-Pedemonte, Roig-Sagués, De Lamo, Gervilla, & Guamis, 2007; Raghubeer, Dunne, Farkas, & Ting, 2000; Tassou, Galiatsatou, Samara, & Mallidis, 2007). HHP has emerged as a non-thermal process that is becoming widely used to inactivate microorganisms in foods (Rastogi, Raghavaro, Balasubramaniam, Niranjan, & Knorr, 2007; Rendueles et al., 2011). Applied at ambient temperature, HHP destroys vegetative bacterial cells and inactivates certain enzymes, with minimal changes on the product

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organoleptic properties and nutrients. However, the resistance of microorganisms to HHP is highly variable, depending on the type of microorganism, its physiological state, and the food matrix. The presence of fat, proteins, minerals and sugars serves as a protector and increases microbial resistance to pressure (Black, Huppertz, Kelly, & Fitzgerald, 2007). The efficacy of HHP treatments can improve in combination with other antimicrobial substances such as bacteriocins, lysozyme, or essential oils (Corbo et al., 2009; Evrendilek & Balasubramaniam, 2011; García-Graells, Van Opstal, Vanmuysen, & Michiels, 2003; Masschalck, Van Houdt, & Michiels, 2001; Somolinos, García, Pagán, & Mackey, 2008; Vurma, Chung, Shellhammer, Turek, & Yousef, 2006). Nisin is widely used as a licensed food preservative (Thomas, Clarkson, & Delves-Broughton, 2000). Enterocin AS-48 is a cyclic antimicrobial peptide that has been tested singly and on combination with other hurdles such as food preservatives or pulsed electric fields for inactivation of bacteria in foods (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Maqueda et al., 2004). Given the increasing number of reports on the incidence of MRSA in food production animals (such as mastitis in dairy cows) as well as in foods (Argudín et al., 2012; Vanderhaeghen, Hermans, Haesebrouck, & Butaye, 2010), the present study was designed to evaluate the effects of HHP treatment on inactivation of a cocktail of MRSA strains by HHP in rice pudding as a model dairy food, singly or in combination with natural antimicrobials (nisin, enterocin AS-48, clove oil and cinnamon oil).

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

S. aureus strains CCUG 31966, CCUG 35601 and CCUG 41879 were obtained from the Culture Collection of the University of Göteborg, Sweden. All strains were resistant to methicillin (MRSA). Strain CCUG 31966 expresses high level methicillin-resistance and produces enterotoxin B, while strain CCUG 35601 has a minimum inhibitory concentration (MIC) of 256 mg/l for methicillin. Strains were grown in brain–heart infusion broth (BHI broth, Merck) or BHI-agar (Merck) and stored at 4 °C for routine use or as stocks in 30% glycerol at −80 °C.

For preparation of inocula, staphylococcal strains were grown overnight (20 h) in BHI broth at 37 °C. Cultures from each strain (10 ml each) were mixed in a 50 ml sterile plastic tube to prepare the cocktail of strains. The mixture was centrifuged (4.500 × g, 15 min) and the sediment resuspended in sterile saline solution (ca. 9.8 log CFU/ml). This mixed suspension of strains was used for inoculation of rice pudding.

2.2. Sample inoculation and HHP treatment

Commercial rice puddings (Dia, Spain; pH 6.65 ± 0.08) were purchased from a local supermarket as a refrigerated ready to eat food. Upon arrival to the laboratory, puddings were stored at 4 °C until use (not more than 24 h). Puddings contained milk (70%), rice (10%), milk fat, grated lemon peel, corn starch, ground cinnamon and other flavouring ingredients. Puddings were inoculated at ambient temperature with the cocktail of *S. aureus* strains at final cell densities of 7.9, 6.8 or 5.7 log CFU/g, thoroughly mixed and distributed in 25 g aliquots into vacuum-sealed polyethylene–polyamide plastic bags. The bags were incubated in a water bath (Memmert) at 22 °C for 30 min and treated by HHP at pressures of 0, 300, 400, 500 and 600 MPa for 10 min. Treatments were done in duplicate (two pudding samples per treatment). HHP treatments were carried out by using a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) suited with a 2.5 l vessel capable of operating in a pressure range of 0–700 MPa, under non-thermal conditions. Come-up speed was 75 MPa/min. Decompression was immediate. Pressurization fluid was water with added 5% propyleneglycol. The temperature inside the vessel during treatments ranged between 23 and 27 °C. The temperature of pudding samples was 24 °C at the end of treatment.

After treatments, portions of rice pudding (25 g) in duplicate were mixed with 25 ml of ice-cold buffered peptone water (yielding a 1:1 wt/vol dilution) and serially diluted with the same solution. Dilutions were plated in triplicate onto Trypticase Soya Agar (Scharlab, Barcelona). Plates were incubated at 37 °C for 48 h. The average numbers of colonies per plate were used to calculate the sample viable cell concentration, expressed as the decimal logarithm of colony forming units (log CFU) per gram of sample. The detection limit was 1.0 log CFU/g. Control puddings were processed in the same way in order to discard the presence of background microbiota.

2.3. Combined treatments with bacteriocins and essential oils

A stock solution of nisin (10^5 IU/ml) was prepared by dissolving commercial nisin 10^6 IU/g (Sigma Chemical Co., Madrid, Spain) in 100 mg/ml of sterile 0.02 N HCl. The solution was heated at 80 °C for 7 min, and kept at −20 °C until use. Nisin was added to obtain final concentrations of 0, 200 and 500 IU/g of pudding.

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Bacteriocin concentrates were diluted 20–50-fold in the puddings in order to achieve the desired final bacteriocin concentrations of 25 or 50 μg/g.

Cinnamon and clove oils commercial solutions (Sigma) were added to the puddings at subinhibitory concentrations of 0.2% and 0.25% (vol/wt) respectively.

Following addition of bacteriocins, essential oils, or their combinations, puddings were inoculated with the cocktail of *S. aureus* strains (at approx. 6 log CFU/g), vacuum-sealed in polyethylene–polyamide plastic bags as described above, and treated by HHP at 0 or 500 MPa for 5 min. After treatment, samples were processed for viable cell counts as described above.

In order to determine changes in the populations of survivors during storage, samples treated with nisin (200 or 500 IU/g), enterocin AS-40 (50 μg/g) or cinnamon oil (0.2%, vol/wt) in combination with HHP at 0 or 500 MPa for 5 min were placed immediately on ice and then incubated in a refrigeration chamber at 4 °C for 7 days. Viable counts were determined at desired intervals of incubation as described above.

2.4. Statistical analysis

All experiments were carried out in duplicate, and the average data ± standard deviations were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA), in order to determine the statistical significance of data.

3. Results and discussion

3.1. Effect of HHP treatment on inactivation of staphylococci in pudding

The bactericidal effect of HHP treatment on staphylococci in rice pudding increased with pressure intensity. Microbial inactivation

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during 10 min treatments increased from approx. 0.8 log cycles at 300 MPa to 2.3 or 4.2 log cycles at 400 or 500 MPa, respectively. At 600 MPa, viable cell concentrations were reduced to below the detection limit of one log CFU/g (Fig. 1A). For initial cell concentrations of 7.9 log CFU/g this means a reduction close to 7 log cycles. As expected, when puddings were inoculated at lower cell densities of 6.8 or 5.7 log CFU/g, the residual numbers of viable cells after treatments also decreased at lower pressure values (Fig. 1B, C), being in some cases below the detectable levels.

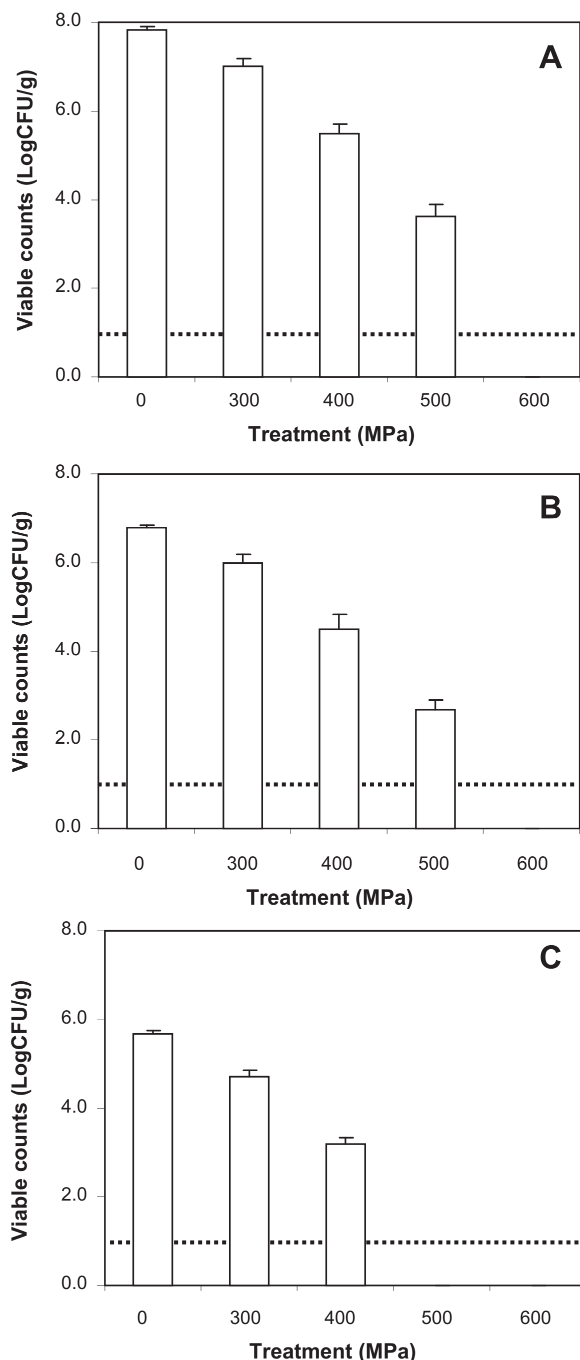

Fig. 1. Effect of HHP intensity on inactivation of a cocktail of *Staphylococcus aureus* strains in rice pudding. Samples (A to C) were inoculated with decreasing cell concentrations of *S. aureus* (7.9, 6.8 and 5.7 log CFU/g) and treated by HHP for 10 min. The dotted line denotes the detection limit.

Our results on inactivation of the cocktail of MRSA strains for 10 min treatments are similar to those obtained by Gervilla et al. (2000) for *S. aureus* CECT 534 in ovine milk (15 min treatments at 400 or 500 MPa and 25 °C) and by López-Pedemonte et al. (2007) in cheeses. Similarly, pressures above 500 MPa were needed to obtain significant log reductions (e.g., 3.5 log units with 500 MPa at 25 °C for 10 min) for *S. aureus* ATCC 25923 in pork slurries (Shigehisa, Ohmori, Saito, Taji, & Hayashi, 1991). Higher pressure treatments (e.g. 600 MPa) induce much greater damage to staphylococci, and our results are in agreement with those by Guan, Chen, Ting, and Hoover (2006), who reported a reduction of 7.3 log cycles for *S. aureus* ATCC 12600 in UHT milk after treatment at 600 MPa for 8 min at 21 °C.

3.2. Combined effects of HHP, bacteriocins, and essential oils on inactivation of staphylococci in pudding

Antimicrobials were tested in combination with HHP treatments of lower intensity (500 MPa, 5 min). The single HHP treatments achieved viable cell reductions of approx. 2.9 log cycles in puddings inoculated with 6 log CFU/g (Fig. 2). Addition of nisin or enterocin AS-48 without HHP treatment caused some non-significant reduction of viable counts (Fig. 2A, B). Nisin improved inactivation of staphylococci in pudding by HHP. The reduction of viable counts increased significantly ($P < 0.05$) by 0.87 log cycles for the 500 IU/g nisin-HHP combination compared to the single HHP treatment (Fig. 2A). Enterocin AS-48 did not improve inactivation of staphylococci by HHP remarkably. The combination of enterocin AS-48 and HHP reduced viable counts by an additional 0.4 to 0.6 log cycles, but this reduction was non-significant ($P > 0.05$) (Fig. 2B). The bactericidal effects of bacteriocins in food systems are greatly influenced by interaction of bacteriocin molecules with food constituents and also by many other factors such as incubation time and temperature (Gálvez, Abriouel, Lucas López, & Ben Omar, 2006). The very low effects demonstrated by nisin and enterocin AS-48 applied singly on staphylococci in puddings would also predict a low interaction with HHP treatments. In previous studies, enterocin AS-48 (148 AU/g, equivalent to 42 μg/g) in combination with HHP (400 MPa, 5 min) did not decrease the viability of *S. aureus* in low acid fermented sausages (Ananou et al., 2010). Nevertheless, other bacteriocins such as nisin, sakacin K and pediocin AcH slightly improved the effect of HHP treatments (400 MPa, 10 min) against *S. aureus* strains in a meat model system (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002), and nisin improved considerably the effect of HHP treatment (600 MPa, 5 min) against a cocktail of staphylococci (CTC1008, CTC1019, CTC1021) in ham (Jofré, Garriga, & Aymerich, 2008). There are scarce reports on the combined action of HHP and bacteriocins against staphylococci in dairy foods, however in one study lacticin 3147 (10.000 AU/ml) increased the efficacy of HHP (250 or 275 MPa for 30 min) against *S. aureus* ATCC6538 in demineralized whey (Morgan, Ross, Beresford, & Hill, 2000).

The effect of HHP treatment against MRSA was enhanced significantly by cinnamon and clove oils at concentrations where none of the oils tested had any effect on the viability of staphylococci. Viable counts obtained for the combined treatments of HHP and cinnamon oil (Fig. 2C) or clove oil (Fig. 2D) were significantly lower ($P < 0.05$) by 1.3 and 1.8 log cycles, respectively, compared to the single HHP treatment. In spite of being the most active, clove oil conferred an unpleasant odour to the puddings. However, the combined treatment of cinnamon oil and HHP at 500 MPa for 5 min had a similar effect as the single treatments at 500 MPa for 10 min, and this combination could be applied to shorten the duration of HHP treatments in puddings. Inactivation of staphylococci increased by some non-significant 0.4 to 0.5 log cycles when

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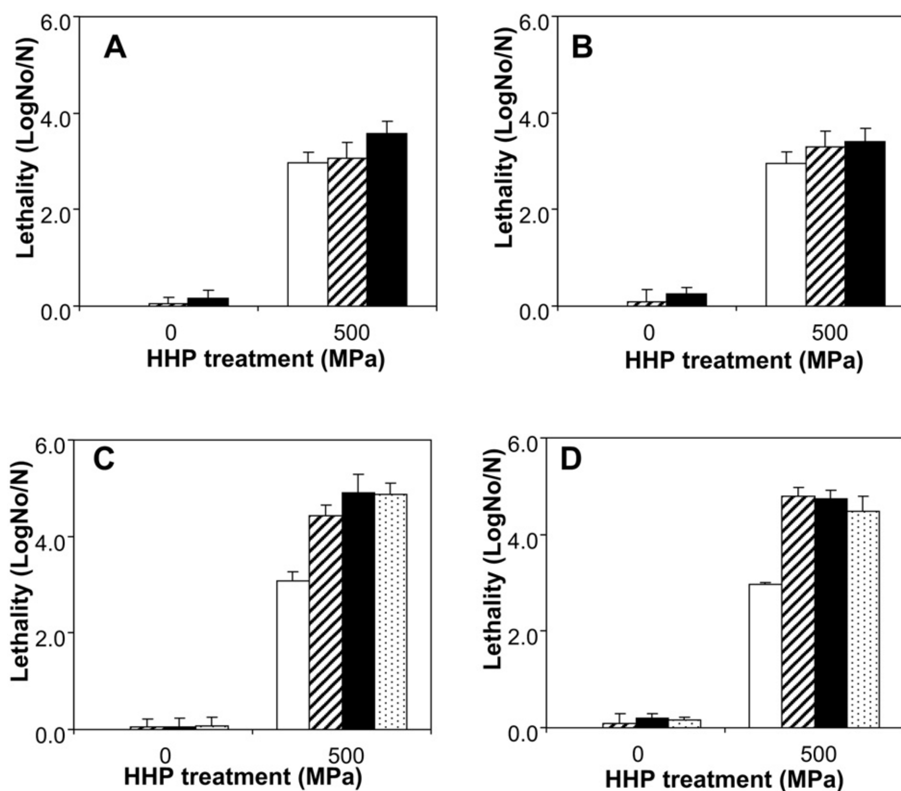

Fig. 2. Effect of treatments by HHP (0 or 500 MPa, 5 min) on *Staphylococcus aureus* cocktail of strains in rice pudding singly (open bars) or in combination with nisin (A) at 200 IU/g (striped bars) or 500 IU/g (closed bars), enterocin AS-48 (B) at 25 μg/g (striped bars) or 50 μg/g (closed bars), 0.2% cinnamon oil (C) or 0.25% clove oil (D). In C and D, treatments consisted of HHP plus essential oils (striped bars), HHP plus essential oil plus 500 IU/g nisin (closed bars) or HHP plus essential oil plus 50 μg/g AS-48 (dotted bars).

cinnamon oil plus nisin or enterocin AS-48 were combined with HHP (Fig. 2C,D). These results indicate a very low contribution of these two bacteriocins in the inactivation of staphylococci by combined treatments in pudding. The activity of bacteriocins can be potentiated by essential oils (Gálvez et al., 2006), yet there are few reports on the combination of essential oils and HHP treatments (Evrendilek & Balasubramaniam, 2011; Somolinos et al., 2008; Vurma et al., 2006), none of them dealing with *S. aureus*. This could be an interesting field for future research in order to improve the effects of HHP treatments and also to decrease the impact of essential oils on the food organoleptic properties.

3.3. *Effect of bacteriocins and cinnamon oil on survival of staphylococci in pudding during storage*

Nisin was tested singly or in combination with HHP treatment (500 MPa, 5 min) against staphylococci in pudding during one week storage at 4 °C. The single addition of nisin significantly ($P < 0.05$) reduced viable counts of staphylococci in rice pudding (Fig. 3A). At 200 IU/g nisin, viable counts were reduced gradually during storage, being the greatest reduction obtained at day 7 (1.57 log cycles). At 500 IU/g, reduction of viable counts was faster and significantly higher ($P < 0.05$) compared to 200 IU/g at day 3 of storage. However, at day 7 viable counts of samples treated with 200 or 500 IU/g nisin did not differ significantly. For the combined treatments, nisin improved inactivation of staphylococci by HHP and decreased the concentrations of survivors during storage of the samples, especially during the first 3 days of storage (Fig. 3A). Best results were obtained during storage of samples treated with HHP and 500 IU/g nisin, which reduced the population of staphylococci below detectable levels at day 3. In addition, viable counts of samples treated with 500 IU/g nisin in combination with HHP were significantly lower ($P < 0.05$) than the singly HHP treated samples by 1.2 to 1.4 log cycles at days 0 and 7.

Enterocin AS-48 addition (50 μg/g) reduced viable counts very slowly during storage of samples (Fig. 3B). At day 7, viable counts of samples treated with AS-48 alone were significantly lower ($P < 0.05$) compared to controls. For the combined treatments with HHP, the reduction obtained right after treatment was non-significant compared to the single HHP treatment. However, enterocin AS-48 improved inactivation of staphylococci during storage of the treated samples. Viable counts of samples treated with HHP-AS-48 became significantly lower ($P < 0.5$) compared to the single HHP treatment at days 3 and 7 of storage, with additional reductions ranging from 1.1 to 1.3 log cycles. In a previous study, enterocin AS-48 had no effect on survival or proliferation of a cocktail of *S. aureus* strains (CTC1010, CTC1011, CTC1034) in pressurized ham during storage at room temperature or at 7 °C (Ananou et al., 2010). Similar negative results were also reported for enterocins A and B in low acid fermented sausages (Jofré, Aymerich, & Garriga, 2009). In contrast, nisin improved considerably the effect of HHP treatment (600 MPa, 5 min) against staphylococci in ham during storage (Jofré et al., 2008). These results illustrate the great variability in the efficacy of bacteriocins when tested in different food systems singly or in combination with other hurdles and the needs to validate combined treatments in each particular food system.

Addition of 0.2% cinnamon oil in combination with HHP improved the inactivation of staphylococci significantly ($P < 0.05$) by approx. 1.4 to 2.0 cycles compared to the single HHP treatment, both after treatment and during storage (Fig. 3C), while the single addition of cinnamon oil did not improve microbial inactivation remarkably. After 7 days of storage, the viable counts in puddings treated by HHP in combination with 0.2% cinnamon oil were similar to those achieved by the combination of HHP and 500 IU/ml nisin,

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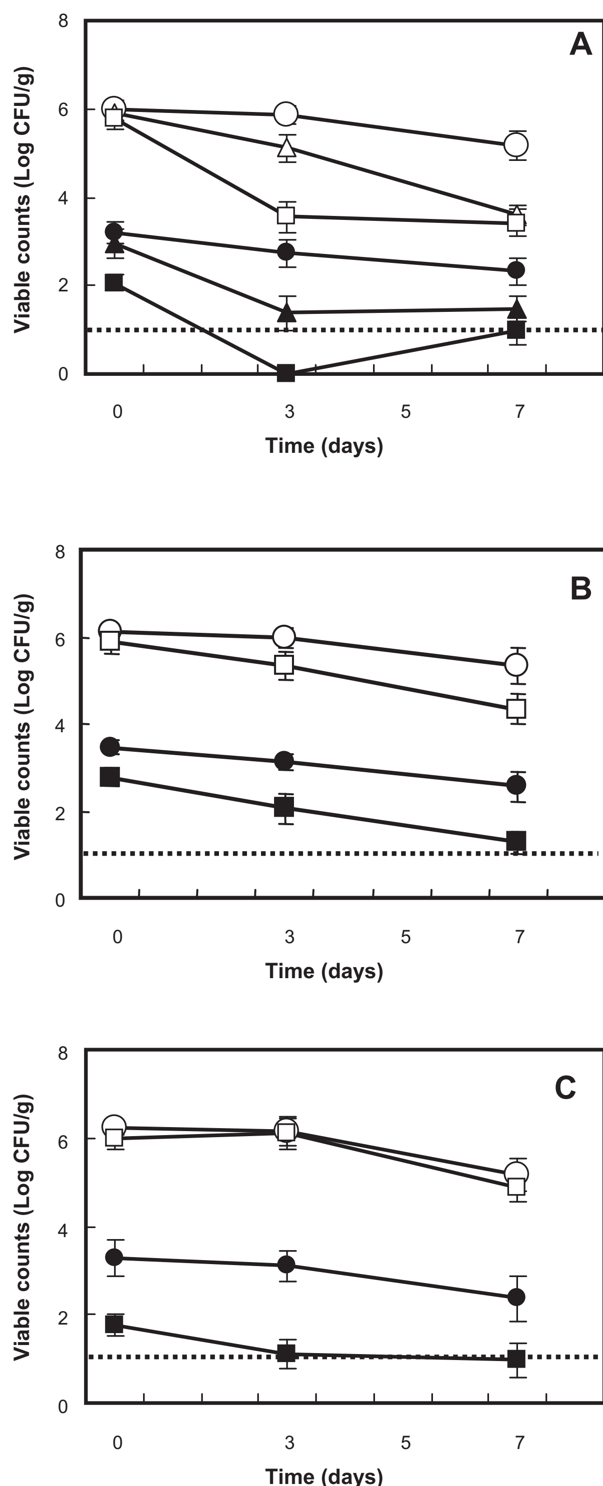

Fig. 3. Survival of the *Staphylococcus aureus* cocktail of strains in puddings in combination with nisin (A), enterocin AS-48 (B) or cinnamon oil (C). A, samples with 0 (○), 200 IU/g (△) or 500 IU/g nisin (□). B, samples with 0 (○) or 50 μg/g enterocin AS-48 (□). C, samples with 0% (○) or 0.2% cinnamon oil (□). Samples were pressurized at 500 MPa for 5 min (closed symbols) or not (open symbols) and then stored at 4 °C. The dotted line denotes the detection limit.

and just slightly lower compared to the combined treatment with enterocin AS-48. Therefore, the three combinations seem adequate for inactivation of staphylococci in puddings during storage.

Although cinnamon oil achieved a greater reduction of viable counts, nisin produced a faster inactivation of staphylococci. Bacteriocins do require a minimum incubation period to cause cell damage and for this reason their bactericidal effects in combination with HHP are more pronounced during storage of the treated samples. The use of bacteriocins in combined treatments with HHP should be recommended because residual bacteriocin molecules still show inhibitory effects in the food, inactivating and preventing growth of surviving cells. The anti-staphylococcal activity of cinnamon and clove oils has been reported in previous studies (Nunes Barbosa et al., 2009; Smith-Palmer, Stewart, & Fyfe, 1998). Combination of HHP treatments with other hurdles such as bacteriocins or essential oils could improve the efficacy of HHP treatments by possibly decreasing treatment parameters such as pressure and/or treatment time. Inactivation of staphylococci by HHP treatments in combination with essential oils should be investigated in other food systems where HHP has a higher impact on the food physico-chemical properties such as the meat or seafood products.

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ARTÍCULO 2

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Inactivation of *Salmonella enterica* cells in Spanish potato omelette by high hydrostatic pressure treatments

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ABSTRACT

The effect of high hydrostatic pressure treatments on a cocktail of four *Salmonella* Enteritidis strains inoculated in commercial Spanish potato omelette (*tortilla de patatas*) was studied. Inactivation of *Salmonella* was influenced by treatment time and pressure. Treatments at 600 MPa for 5 or 8 min reduced the counts of *Salmonella* on selective medium (XLD agar) by 5.9 and 6.5 log cycles, respectively. Investigation of sublethally injured cells by growth on triple agar layer (TAL) plates or in non-selective medium (TSA) revealed that the population of sublethally injured cells able to grow on TSA could be 2.5 to 3.0 log cycles higher (depending on treatment) compared to non-injured cells. The TAL method only allowed recovery of a fraction of sublethally injured cells, suggesting different degrees of cell damage. In addition, tailings were observed in survival curves for treatments above 500 MPa both for selective and non-selective determinations. Omelette samples inoculated with *Salmonella* and treated at 500 MPa (5 min) or 680 MPa (8 min) were stored at 6 °C for 15 days. Counts of survivors on selective medium increased at day 3 of storage for the less intense HHP treatment (suggesting repair of sublethally injured cells), but not until day 10 for the 680 MPa treatment. Temperature abuse of the treated omelettes (6 h at 22 °C) applied at day 3 of storage increased the concentrations of cells able to grow on non-selective as well as on selective media, an effect that was more pronounced in the samples treated at 500 MPa for 5 min. The results suggest that temperature abuse may compromise the efficacy of HHP treatments against *Salmonella* in omelettes.

Industrial relevance: The efficacy of HHP treatments depends greatly on the food substrate and microorganisms. Therefore, the degree of protection afforded by HHP treatments for each foodborne pathogen and food system need to be determined experimentally. So far, there are no previous studies addressing the efficacy of HHP treatments on inactivation of *Salmonella* in Spanish omelettes. This type of RTE food is widely consumed not only in Spain, but also in many other countries, especially as an appetizer. Industrial application of HHP treatments on omelettes could improve their safety while at the same time enhancing the perception of this food product as a safer food.

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1. Introduction

In 2008, salmonellosis was the second most often reported zoonotic disease in humans in the EU, accounting for 131,468 confirmed human cases, of which 3833 occurred in Spain (EFSA, 2010). *Salmonella* was the most frequently reported cause of food-borne outbreaks (35.4% of all outbreaks), with eggs and products made with raw eggs being the most important food vehicles in these outbreaks (23.1%). In foodstuffs, *Salmonella* was most often detected in fresh broiler, turkey and pig meat, on average at levels of 5.1%, 5.6% and 0.7%, respectively. *Salmonella* was rarely detected in other foodstuffs, such as dairy products, fruit and vegetables. However, in sprouts, herbs and spices some higher incidences of contamination were reported. The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which result in a variety of foodstuffs covering both food of animal and plant origin as sources of infections. *S.* Enteritidis and *S.* Typhimurium are the serovars most frequently associated with human illness in the EU, with human *S.* Enteritidis cases being most commonly associated with the consumption of contaminated eggs and poultry meat (EFSA, 2010). Transmission often occurs when microorganisms are introduced in food preparation areas and are allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) food.

Control of foodborne pathogens in the food chain requires a careful selection of treatments and hurdles compatible with product characteristics and production processes. High hydrostatic pressure (HHP) treatment has emerged as a non-thermal process that is becoming widely used to inactivate microorganisms in foods (Rastogi, Raghavaro, Balasubramaniam, Niranjan, & Knorr, 2007; Rendueles et al., 2011). Applied at ambient temperature, HHP destroys vegetative bacterial cells and inactivates certain enzymes, with minimal changes on the

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product organoleptic properties and nutrients. The effectiveness of treatment depends primarily on the pressure applied and on the holding time (Cheftel, 1995; Hoover, 1993). However, the resistance of microorganisms is highly variable, depending on the type of microorganism, its physiological state, and the food matrix. The presence of fat, proteins, minerals and sugars serves as a protector and increases microbial resistance to pressure (Black, Huppertz, Kelly, & Fitzgerald, 2007). The efficacy of HHP also decreases with reduced water activity (a_w) and when the pH of the food is closer to neutral values (Alpas, Kalchayanand, Bozoglu, & Ray, 2000; Black et al., 2007). Therefore, results obtained in model systems using artificial substrates cannot be directly compared to real foods and should be validated (Claeys, Van Loey, & Hendrickx, 2003). Indeed, the degree of protection afforded by HHP treatments for each foodborne pathogen and food system needs to be determined experimentally.

In the present study, the effect of HHP treatment was tested on Spanish potato omelette. Spanish potato omelette is made from eggs and potatoes. It is very popular in Spanish bars (as "tapas"), restaurants and catering services, but it is also frequently sold at supermarkets for consumption at home as a convenient refrigerated RTE food. The purpose of the study was to determine the effect of HHP treatments on *Salmonella enterica* inoculated in potato omelette and to evaluate the fate of bacterial cells surviving HHP treatment during cold storage as well as during temperature abuse conditions.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Salmonella enterica serovar Enteritidis strains UJ3449 and UJ3197 were isolated from foods implicated in domestic outbreaks of human salmonellosis (Spanish omelette, grilled pork). Strains S62 and S64 were isolated from contaminated foods (chicken hamburger, mayonnaise). Strains were stored in 30% glycerol at −80 °C. Strains were propagated at 37 °C in Brain Heart Infusion Broth (BHI, Scharlab, Barcelona) or BHI agar slants. For preparation of inocula, strains were grown overnight in BHI broth. One milliliter from each culture was transferred to a sterile test tube and mixed to make the cocktail of the four strains (cell density, ca. 9.7 log CFU/ml). The cocktail was used directly or further diluted with sterile saline solution before inoculation of omelettes.

2.2. Sample inoculation and HHP treatment

Commercial omelettes (17 cm diameter, 500 g; pH 6.95 ± 0.08) were purchased from a local supermarket as a refrigerated RTE food. Upon arrival to the laboratory, they were stored at 4 °C until use (for no more than 24 h). Omelettes were manufactured from fried potatoes (56%), pasteurized egg (40%) and minor ingredients (sunflower and olive oil, salt, garlic, pepper, stabilizers and antioxidants). Before inoculation with salmonellae, omelettes were cut into square portions (25 ± 0.5 g per portion). Each portion was inoculated at two separate points with a total volume of 200 μl of the *Salmonella* cocktail (at a final cell density of ca. 7.6 log CFU/g) by using a sterile hypodermic needle. After inoculation, portions were vacuum-packaged in polyethylene-polyamide plastic bags and kept on ice before they were treated by HHP. Control omelette samples without inoculation with *Salmonella* were processed in the same way. All treatments were done in duplicate (two samples per treatment). Treatments (at pressures of 300, 400, 500, 600 and 680 MPa and holding times of 5 and 8 min) were carried out with a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) suited with a 2.5 l vessel capable of operating in a pressure range of 0 to 700 MPa, under non-thermal conditions. Come-up speed was 75 MPa/min. Decompression was immediate. Pressurization fluid was water with added 5% propylenglycol. The initial temperature inside the vessel was 21 °C. During treatments, temperature increased to between 24 and 28 °C (depending on pressure value). During decompression, temperature decreased to initial values. The temperature of omelette samples was between 5 and 7 °C at the moment of being introduced into the HHP equipment and between 15 and 17 °C at the end of treatments. After application of treatments, samples were processed for determination of survivors as described below.

2.3. Sample analysis

Following HHP treatments, the 25 g omelette portions (in duplicate) were transferred aseptically to stomacher bags, mixed with 25 ml ice-cold buffered peptone water (yielding a 1:1 wt/vol dilution) and pummeled for 30 seconds in a Stomacher 80 (Seward, London, UK). Homogenized samples were serially diluted with ice-cold buffered peptone water. Dilutions were plated in triplicate on Xylose Lysine Deoxycholate agar (XLD; Scharlab, Barcelona) and also on Trypticase Soy Agar (TSA, Scharlab) and on XLD-TSA by the thin agar layer (TAL) method in order to investigate recovery of sublethally injured cells. TAL method was performed as described by Kang and Fung (2000). Briefly, after solidification of XLD agar on a Petri dish (8.5 cm diameter; 25 ml agar added) 7 ml of melted TSA (50 °C) was added and allowed to solidify for a few minutes. Then, a second layer of melted TSA (7 ml) was added. Sample dilutions were spread-plated on top of freshly-prepared TAL plates. All plates were incubated at 37 °C for 48 h. For XLD and TAL plates, colonies with typical *Salmonella* morphology were counted. Colonies grown on TSA plates were tested by Gram staining and growth on XLD agar for confirmation. The average numbers of colonies per plate were used to calculate the sample viable cell concentration, expressed as $\log_{10}$ CFU/g of sample. The detection limit was 1.0 log CFU/g.

2.4. Changes in Salmonella counts during storage of omelettes

Omelettes were inoculated with the cocktail of *Salmonella* cells (at 6.6 log CFU/g) and treated by HHP at 500 MPa for 5 min or at 680 MPa for 8 min. The treated samples were placed immediately on ice and then incubated in a refrigeration chamber at 6 °C for 15 days. At day 3, half of the samples were subjected to a temperature abuse treatment at 22 °C for 6 h in a refrigerated incubation chamber (Memmert, Schwabach, Germany), and then stored back at 6 °C. *Salmonella* counts were determined on TSA, XLD agar and TAL plates, both before and after HHP treatment, during storage, and right before and after the temperature abuse treatment. The identity of colonies grown on TSA plates was investigated as described in previous paragraph. The pH of samples was measured with a Crison pH meter equipped with a surface pH electrode.

2.5. Statistical analysis

All experiments were carried out in duplicate, and the average data ± standard deviations were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA), in order to determine the statistical significance of data.

3. Results

3.1. Influence of treatment parameters on microbial inactivation

Injury of *Salmonella* Enteritidis in omelettes depended on pressure values and duration of treatment. When residual non-injured cells were investigated by plating on XLD agar, lowest inactivation was obtained for 5 min treatments (Fig. 1A), with reductions of 1.2 to 6.2 log CFU/g for pressure treatments in the range of 300 to 680 MPa. Longer time treatments (8 min) improved inactivation of

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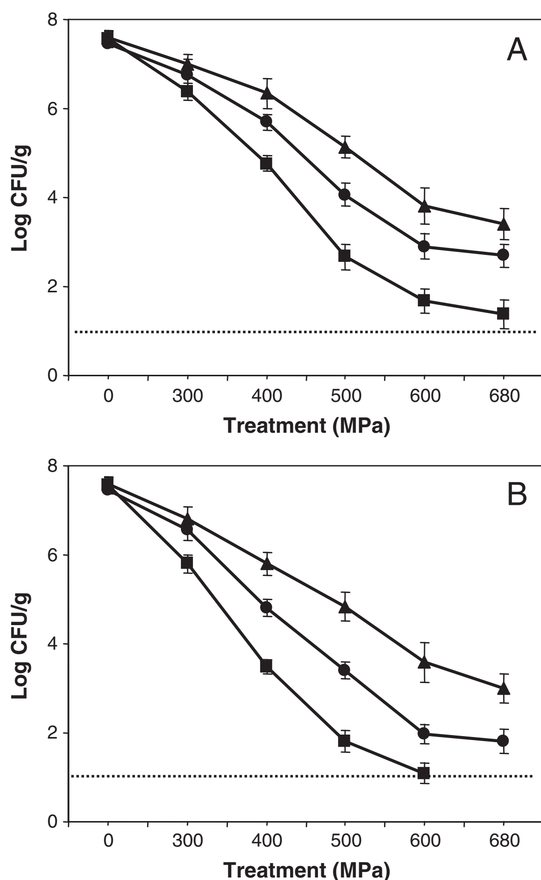

Fig. 1. Effect of inoculum density and treatment time on the microbial inactivation of *Salmonella enterica* cells in Spanish omelette. Treatment times were of 5 min (**A**) or 8 min (**B**). Omelettes were inoculated with a cocktail of *Salmonella* cells at initial cell densities of ca. 7.6 log CFU/g. Cell counts were performed on XLD agar (squares), Trypticase Soy agar (triangles) and triple agar layer plates (circles). The dotted line denotes the detection limit (1.0 log CFU/g).

salmonellae, with reductions ranging from 1.8 to 6.5 log cycles for treatments at 300 to 600 MPa (Fig. 1B). No viable salmonellae were detected on XLD agar in samples treated at 680 MPa for 8 min. The logarithmic reductions of viable counts obtained for 8 min treatments were significantly higher ($p<0.05$) compared to 5 min treatments for pressures of 300, 400 and 500 MPa. The differences obtained at 600 or 680 MPa were non-significant ($p>0.05$) due to the higher error obtained for counts close to the detection levels and also to the presence of tailing effects.

Investigation of sublethally injured cells by the TAL method and by plating on TSA revealed substantial differences in cell counts compared to the values obtained on XLD agar. Since omelettes are not a sterile product, we first tested control omelette samples (not inoculated with salmonellae) before and after HHP treatments by plating on selective and on non-selective media. Only a few colonies were observed when control omelette samples before HHP treatment were plated on TSA, but not on XLD or TAL plates. Similar results were observed after the HHP treatments (even at 680 MPa), suggesting that they belonged to bacterial endospores. The numbers of colonies were always below the established detection levels.

When omelettes inoculated with *Salmonella* and treated by HHP were plated on TSA, viable counts were significantly higher ($p<0.05$) compared to counts obtained on XLD agar for samples treated during 5 min at 400 to 680 MPa and for samples treated for 8 min at 300 MPa or above. The greatest differences in counts (2.5 to 3.0 log CFU/g) as well as in count reductions (also 2.5 to 3.0 log cycles) were observed in the samples treated at 500 MPa for 5 as well as for 8 min treatments (Fig. 1A, B). All colonies grown on TSA plates were confirmed to grow on XLD agar plates as typical *Salmonella* and were Gram-negative rods by microscopic examination. Furthermore, the numbers of salmonellae that were able to repair sublethal damage on TSA after being treated at 600 to 680 MPa for 5 or 8 min ranged from 3.0 to 3.6 log CFU/g of omelette. Viable counts obtained on TAL plates showed intermediate values between XLD and TSA counts, suggesting that this method only recovered a fraction of the sublethally injured population. Counts obtained on TAL plates were significantly lower ($p<0.05$) than TSA counts for 5 min treatments at 400 or 500 MPa and for 8 min treatments at 400 to 680 MPa and significantly higher ($p<0.05$) than XLD counts for all treatments (5 or 8 min) at 400 MPa or above.

3.2. Survival of Salmonella during storage of treated samples

The viability of survivors after treatments was determined in samples (inoculated at 6.6 log CFU/g) treated at 500 MPa for 5 min or 680 MPa for 8 min and stored at 6 °C for 15 days (Fig. 2). In the control samples (inoculated with *Salmonella*, without HHP treatment), counts of *Salmonella* remained stable for the complete storage period, with minimal decreases between days 7 and 15 (Fig. 2A). In the samples treated at 500 MPa, the concentration of *Salmonella* able to grow on XLD agar increased by 1.3 log CFU/g at day 3 of storage, suggesting repair of sublethal damage (Fig. 2A). After that point, the counts remained at stable levels with only a slight decrease by the end of storage period. A similar evolution was observed for counts of sublethally injured cells able to grow on TAL plates, however in this case the increase in viable counts obtained at day 3 was much lower (0.5 log CFU/g). From days 3 to 15, counts on TAL plates were slightly higher than counts on XLD agar, but the differences were not statistically significant ($p>0.05$). These results would suggest that a fraction of the sublethally injured cells had repaired cell damage by day 3 and were now able to grow both on TAL plates and on XLD agar. Viable counts obtained on TSA right after HHP treatment were much higher compared to counts on XLD agar or TAL plates, but did not increase significantly ($p>0.05$) during the first 3 days of storage. All colonies grown on TSA plates during the first 3 days of storage behaved as typical *Salmonella* on XLD agar and by Gram-staining. The differences observed at day 3 between viable counts obtained on TSA and on TAL plates or XLD agar would suggest a residual population of sublethally injured cells that still were unable to grow on selective media and had not repaired cell damage during cold storage. During further storage (days 7 to 15), counts on TSA did not increase significantly, but the plates contained a mixture of *Salmonella* and other bacteria, and for that reason the data were discarded.

For the samples treated at 680 for 8 min, no viable salmonellae were detected on XLD agar for the first 7 days of storage, but viable counts of 1.0 to 1.2 log CFU/g were detected at days 10 and 15 (Fig. 2C). Nevertheless, sublethally injured salmonellae were recovered on TAL plates at levels between 1.0 and 1.3 log CFU/g for the first 7 days (Fig. 2C). Counts on TAL plates increased slightly to 2.0 log CFU/g at day 10 and at that point were significantly ($p<0.05$) higher than counts on XLD agar, suggesting that a fraction of the population still had not repaired sublethal damage. In addition, the bacterial population able to grow on TSA was significantly higher ($p<0.05$) by 1.0 log CFU/g for the first 3 days of storage compared to the population able to grow on TAL plates which also suggests a fraction of the population unable to repair sublethal damage during cold storage.

A temperature abuse event (6 h at 22 °C) was simulated at day 3 of storage (Fig. 2B, D). During the temperature abuse period, counts of *Salmonella* in the untreated controls increased by 0.8 to 1.0 log cycles, and then decreased slightly during further storage. For the samples treated at 500 MPa, counts on TSA plates increased non-significantly ($p>0.05$) by 0.5 log cycles, while counts on TAL plates and on XLD agar plates increased significantly ($p<0.05$) by 0.9 and 2.0 log cycles, respectively (Fig. 2B). These increments were non-significantly higher

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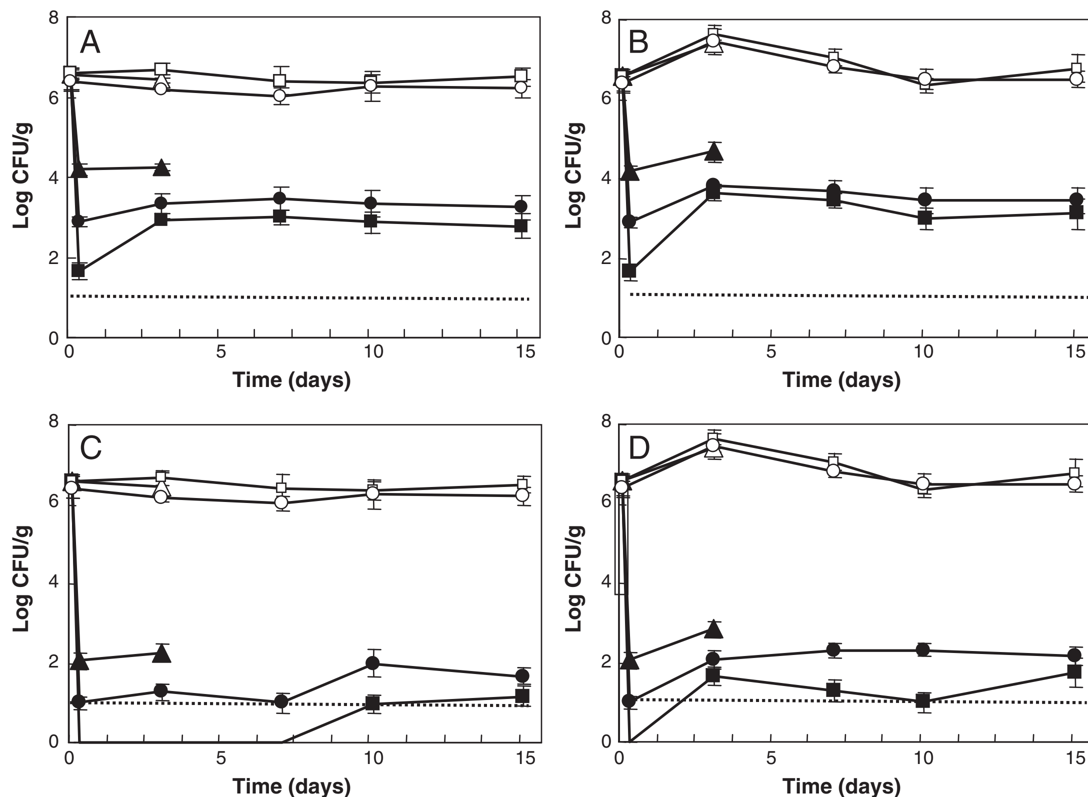

Fig. 2. Changes in the surviving fraction of *Salmonella enterica* cells (inoculated at ca. 6.6 log CFU/g) after HHP treatment in omelettes stored at 6 °C. Samples were pressurized at 500 MPa for 5 min (A, B) or 680 MPa for 8 min (C, D). In B and D samples were exposed to temperature abuse at 22 °C for 6 h at day 3 of storage and then placed back under refrigeration for the remaining storage period. Cell counts were performed on XLD agar (squares), Trypticase Soy agar (triangles) and triple agar layer plates (circles). Open symbols denote untreated samples, while closed symbols correspond to pressurized samples. The dotted line denotes the detection limit (1.0 log CFU/g).

($p > 0.05$) by 0.5 log cycles in the case of TAL plates and significantly higher ($p < 0.05$) by 0.7 log cycles in the case of XLD agar counts when compared to samples without temperature abuse, and could be explained by multiplication of the non-injured cell fraction but also by an additional damage repair by sublethally injured cells during the temperature abuse event. In the storage period of days 3 to 15, viable counts obtained on TAL plates were slightly higher compared to counts obtained on XLD agar, but the differences were non-significant ($p > 0.05$).

Following temperature abuse, viable counts on TSA and TAL plates of samples treated at 680 MPa for 8 min were significantly higher ($p < 0.05$) by 0.8 and 1.0 log cycles respectively compared to counts obtained on the same media right after treatment (Fig. 2D). However, the differences observed at day 3 between temperature-abused and non-abused samples (0.5 log cycles for TSA or 0.8 log cycles for TAL plates) were not statistically significant ($p > 0.05$). Nevertheless, counts on XLD agar increased from below detection levels to 1.65 log cycles upon temperature abuse. As in the 500 MPa treatments, the observed increases in viable counts could be attributed to damage repair by sublethally injured cells but also to cell multiplication. During storage, counts on XLD agar were significantly lower than TAL counts only for days 7 and 10 (Fig. 2D).

4. Discussion

Eggs and egg-containing foods contaminated with bacterial human pathogens have been implicated in numerous foodborne outbreaks leading to costly recalls. Spanish potato omelette is a convenient refrigerated RTE food made from eggs and potatoes. It is frequently sold at supermarkets but is also very popular in food and beverage services. Eggs stand among the raw materials of animal origin with higher risks for transmission of *Salmonella.* In addition, omelettes thicker in size (such as potato omelettes, which can be between 2 and 3 cm high) may often appear undercooked in the central parts, increasing the risk for survival and transmission of this pathogenic bacterium. Since HHP technology is becoming more popular at industrial scale for treatment of RTE foods, it could also be applied for inactivation of *Salmonella* in potato omelettes.

In previous studies carried out with liquid egg, pressure treatments in the low range of 400–450 MPa were applied for inactivation of *Salmonella* with negligible effects on the food functional properties (Ponce, Pla, Sendra, Guamis, & Mor-Mur, 1999). Potato omelettes will tolerate much higher pressures since egg proteins are already coagulated during the cooking process. High-pressure treatments could be applied on omelettes to significantly reduce the levels of possible *Salmonella* contamination. However, there are no previous studies concerning inactivation of *Salmonella* in omelettes by HHP treatment. HHP has been tested for inactivation of *Salmonella* cells in many other food systems such as meat and meat products (Ananou et al., 2010; Ananth, Dickson, Olson, & Murano, 1998; Kruk et al., 2011; Morales, Calzada, Rodríguez, De Paz, & Nuñez, 2009; Shigehisa, Ohmori, Saito, Taji, & Hayashi, 1991), whole egg (Bari, Ukuku, Mori, Kawamoto, & Yamamoto, 2008; Ponce et al., 1999), seafood (Malicki, Sysak, & Bruzewicz, 2005), whole milk (Guan, Chen, & Hoover, 2005), cheese (De Lamo-Castellví et al., 2007), fruit juices (Nakimbugwe, Masschalck, Anim, & Michiels, 2006; Whitney, Williams, Eifert, & Marcy, 2007), and seeds for seed sprout production (Neetoo & Chen, 2010; Wuytack, Diels, Meersseman, & Michiels, 2003), with variable results. In one example, treatment at 400 MPa for 15 min achieved a 4.8 log reduction in chicken breast fillets (Morales et al., 2009). In liquid egg, combinations of pressure intensity, treatment time and heat were tested to achieve inactivation of *Salmonella* without causing egg coagulation. Treatment at 450 MPa for 5 min at 20 °C achieved a reduction of 4 log cycles in *Salmonella* Enteritidis (Ponce et al., 1999), while in another study treatment at 400 MPa and 25 °C required a treatment time of up to 40 min to achieve a 6 log reduction (Bari et al., 2008).

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Results on the inactivation of microbes in foods may depend greatly on the procedure applied for estimation of survivors. Since most foods are not sterile products, the use of selective media may be considered for enumeration of foodborne pathogens. In our study, treatments in the range of 400 to 600 MPa for 5 or 8 min reduced viable counts of *Salmonella* in omelettes by 2.8 to 6.5 log cycles when a selective medium (XLD agar) was used for enumeration. However, when sublethally injured cells were investigated in a non-selective medium (TSA) or by the TAL method, the concentrations of survivors were considerably higher especially for the 400 to 680 MPa treatments. The TAL method was proposed by Kang and Fung (2000) for selective recovery of *Salmonella* Typhimurium injured by heat. During the first hours of incubation, injured salmonellae repaired their cell damage and started to grow in the TSA while the selective agents from XLD diffused gradually to the TSA top layer part. This procedure yielded slightly lower but not statistically different counts compared to TSA for sublethal heat injury and also improved the recovery of acid-injured salomonellae (Kang & Fung, 2000; Wu, Fung, Kang, & Thompson, 2001). In our study with salmonellae pressurized at 300 MPa, the differences between TAL method and TSA counts were not statistically significant. However, the observed differences were much greater for treatments of higher intensities, indicating that the fraction of injured cells not recovered by the TAL method can be significantly high under more severe stressing conditions. Bozoglu, Alpas & Kaletunç (2004) suggested two types of sublethal injury (I1 and I2) in foodborne pathogens after HHP treatment. In their study on *Salmonella*, they described a primary injury (I1) that was seen by colony formation only on non-selective agar and recovery of the capacity to grow on selective medium after one day storage. The results from our study would suggest different levels of cell damage, e.g., cells unable to grow on XLD agar but still able to repair damage within a few hours and grow on TAL plates and cells requiring longer storage under non-selective conditions for damage repair (such as cells growing on TSA but not on TAL plates or XLD agar).

In the present study, treatments of 600 or 680 MPa for 8 min were required to achieve microbial reductions of 6.5 log cycles (considering non-injured cells as survivors) or a maximum of 4.7 log cycles considering sublethally injured cells. The observed higher resistance of *Salmonella* in omelettes compared to other foods could be attributed to the neutral pH of omelettes (a factor known to decrease the efficacy of HHP treatments; Alpas et al., 2000) and perhaps also to the semi-solid structure of the food substrate (as compared to liquid foods). Other factors such as inoculum preparation, inoculum density, and differences in strain sensitivity should also be taken into consideration. For example, formation of cell aggregates at high cell density inocula may have a protective effect against HHP treatment. So does the possible growth of salmonellae and formation of microcolonies or biofilms (as may occur in the food processing lines or in the food during accidental cold chain breakage). It has been reported that in many foods, preservation by HHP processing requires pressure levels above 600 MPa for inactivation of pressure-resistant pathogens (Balasubramaniam, Farkas, & Turek, 2008). In omelettes inoculated with high concentrations of salmonellae (simulating a worst-case scenario), survival curves showed tailings at 600 to 680 MPa. The tailing effect often observed in survival curves has been attributed to a fraction of the population with higher pressure resistance or to damage repair of sublethally injured bacterial cells (Chen, 2007; San Martín, Barbosa-Cánovas, & Swanson, 2002) or even to both factors, as seems the case of the present study where tailings were observed for both selective and non-selective enumeration of survivors.

Although omelettes are sold under refrigeration, there is a risk that *Salmonella* cells sublethally injured by HHP treatments could repair cell damage during storage and/or proliferate in omelettes under temperature abuse conditions. Therefore, it is important to evaluate the recovery levels and proliferation potential of surviving fractions during storage of the HHP treated omelettes. In our study, recovery of sublethally injured cells was observed within the first 3 days of refrigeration storage for omelettes treated at 500 MPa for 5 min (as shown by increases in viable counts obtained on TAL plates and on XLD agar), while recovery of cells treated at 680 MPa was delayed to day 10 of storage. Treatments of at least 680 MPa for 8 min should be recommended in order to reduce the concentrations of intact cells and to delay recovery of sublethally injured *Salmonella* in omelettes. Nevertheless, upon a temperature abuse event recovery of sublethally injured cells was observed at day 3 also for the higher intensity treatment, although the recovered fraction was approx. 2 log cycles lower compared to the 500 MPa treated samples under the same temperature abuse conditions. In conclusion, although HHP treatments of high intensity may reduce significantly the populations of *Salmonella* in omelettes, temperature abuse episodes may still compromise the safety of the treated food product.

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ARTÍCULO 3

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Survival and High-Hydrostatic Pressure Inactivation of Foodborne Pathogens in Salmorejo, a Traditional Ready-to-Eat Food

Julia Toledo Del Árbol, Rubén Pérez Pulido, Mª José Grande, Antonio Gálvez, and Rosario Lucas

Abstract: Salmorejo is a traditional tomato-based creamy product. Because salmorejo is not heat-processed, there is a risk of contamination with foodborne pathogens from raw materials. Even though bacterial growth in salmorejo is strongly inhibited because of its acidic pH (close to 3.9), the growth and survival of 3 foodborne pathogens in this food has not been studied before. In this study, 3 cocktails consisting of *Escherichia coli* O157, *Salmonella enterica* serovar Enteritidis, and *Listeria monocytogenes* strains were inoculated in freshly prepared salmorejo. The food was treated by high hydrostatic pressure (HHP) at 400, 500, or 600 MPa for 8 min, or left untreated, and stored at 4 °C for 30 d. Viable cell counts were determined on selective media and also by the triple-layer agar method in order to detect sublethally injured cells. In control samples, *L. monocytogenes* viable cells decreased by 2.4 log cycles at day 7 and were undetectable by day 15. *S. enterica* cells decreased by 0.5 or 2.4 log cycles at days 7 and 15 respectively, but still were detectable at day 30. *E. coli* O157 cells survived much better in salmorejo, decreasing only by 1.5 log cycles at day 30. Treatments at pressures of 400 MPa or higher reduced viable counts of *L. monocytogenes* and *S. enterica* to undetectable levels. HHP treatments significantly ($P < 0.05$) reduced *E. coli* counts by approximately 5.2 to 5.4 log cycles, but also yielded surviving cells that apparently were sublethally injured. Only samples treated at 600 MPA for 8 min were devoid of detectable *E. coli* cells during storage.

Keywords: *Escherichia coli* O157, foodborne pathogens, high hydrostatic pressure, *Listeria monocytogenes* serotypes 4a, 4b, salmorejo, *Salmonella* Enteritidis

Practical Application: Salmorejo is a traditional, vitamin-rich food, usually produced on a small scale. HHP treatment at 600 MPa for 8 min can be an efficient nonthermal method for industrial-scale preparation of preservative-free salmorejo with improved safety against transmission of foodborne pathogens *L. monocytogenes* serotyes 4a and 4b, *S. enterica* serovar Enteritidis, and *E. coli* O157.

Introduction

Salmorejo is a tomato-based, typical Andalusian ready-to-eat traditional food (Barrenechea and Koehler 2005). The cream is made from raw tomatoes, together with garlic, salt, vinegar, olive oil, and bread. Other raw vegetables such as cucumber, green pepper, and onion may also be optionally included in the recipe. Salmorejo is rich in vitamins, minerals, and antioxidant compounds, and is often taken as a 1st dish, or as refreshing appetizer. The heat pasteurization of salmorejo has a negative impact on the taste and flavor of the freshly made food. Therefore, alternative food processing methods such as high hydrostatic pressure (HHP) may provide a fresh-tasting product with longer shelf life.

Salmorejo may be contaminated with human pathogenic bacteria from the vegetable raw ingredients used for its preparation. Tomato and other types of fresh produce have been implicated in foodborne outbreaks caused mainly by *Salmonella enterica* and *Escherichia coli* O157, and to a much less extent also by *Listeria monocytogenes* (Sivapalasingam and others 2004; Berger and others 2010; Francis and others 2012; Hoelzel and others 2012; Callejón and others 2015). In the United States, numerous outbreaks of salmonellosis associated with the consumption of fresh products, especially round tomatoes, have been reported (Fatika and Schneider 2011). Before serving, salmorejo is often topped with pieces of Iberian ham or boiled egg, which may be additional sources of bacteria. There is also a trend in innovative cuisine to introduce other ingredients in salmorejo such as peeled shrimps, increasing the risks for contamination.

As salmorejo is not heat-processed and is served as a cold dish, it is important to know the survival ability of foodborne pathogens and the effect of alternative food processing technologies like HHP on microbial inactivation in this food. The development of nonthermal methods suitable for inactivation of pathogens and preservation of salmorejo could enhance the market for this ready-to-eat food. Treatments by HHP have become very popular for preservation of different kinds of ready-to-eat foods, including meats, seafoods, and fruit and vegetable products (Rastogi and others 2007; Considine and others 2008; Oey and others 2008; Rendueles and others 2011; Ortega-Rivas and Salmerón-Ochoa 2014). The inactivation of bacteria by HHP depends on several factors, such as the intensity of treatment, pH, and the food matrix (Alpas and others 2000). The presence of sublethally injured cells as well as the recovery and multiplication of survivors in the treated food during storage is a matter of concern. There are no previous published studies on the survival of human pathogenic bacteria

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in salmorejo or the potential application of HHP treatments for preservation of this traditional food. Therefore, the purpose of this study was to investigate the inactivation by HHP of 3 major human pathogenic bacteria (*S. enterica, E. coli,* and *L. monocytogenes*) in salmorejo, and their survival during refrigeration storage.

Materials and Methods

Bacterial strains and cultivation conditions

The *S. enterica* serovar Enteritidis (*S.* Enteritidis) strains UJ3449 and UJ3197 were isolated from foods implicated in domestic outbreaks of human salmonellosis (Spanish omelette, grilled pork), whereas *S.* Enteritidis strains S62 and S64 were isolated from contaminated foods (chicken hamburger, mayonnaise). The *E. coli* strains CCUG 47553 and CCUG 47557 were from the Culture Collection of the Univ. of Göteborg (Sweden). Both strains are reported by CCUG to agglutinate with O157 serum, but are negative for verotoxin VT1 and VT2 production. The *L. monocytogenes* strains CECT 4032 (serovar 4b), CECT 934 (serovar 4a), and CECT 5672 (serovar 4b) were obtained from the Spanish Type Cultures Collection (CECT, Burjasot, Valencia). All bacterial strains were stored in 30% glycerol at −80 °C and were propagated at 37 °C in brain heart infusion broth (BHI, Scharlab, Barcelona). For each bacterial species, a cocktail of strains was prepared before inoculation in salmorejo by mixing 1 mL from overnight, stationary-phase cultures of the corresponding strains. The cell densities of the species cocktails ranged from approximately 8.7 $\log_{10}$ CFU/mL for *Listeria* to approximately 9.4 $\log_{10}$ CFU/mL for *Salmonella* and *E. coli*.

Sample preparation and application of high hydrostatic pressure treatments

Salmorejo was prepared with the following ingredients: ripened tomatoes, 900 g; garlic, 10 g; white bread, 70 g; olive oil, 30 mL; wine vinegar, 15 mL; salt, 5 g. Tomatoes, garlic, and bread were cut into small pieces with a sterile knife. The mixture of ingredients was beaten with a domestic blender. Four batches of salmorejo were used. One was used as negative control, and the other 3 were individually inoculated (1%, v/v) with cocktails of strains prepared as described in section "Bacterial strains and cultivation conditions," yielding final cell concentrations of 5.7, 6.35, and 6.42 $\log_{10}$ CFU/mL for *L. monocytogenes, S.* Enteritidis, and *E. coli* O157, respectively. Twenty grams of the inoculated salmorejo was packed under vacuum in polyethylene-polyamide bags and HHP treated. Untreated inoculated bags as well as bags without bacterial inoculation were prepared as control samples.

Samples were treated by HHP with a 2.5 L capacity Stansted Fluid Power LTD HHP system (SFP, Essex, UK). Samples were pressurized at 400, 500, or 600 MPa for 8 min. The come-up speed was 75 MPa/min, and decompression was immediate. The pressure transmitting fluid consisted of 5% propylenglycol in water. The temperature inside the vessel before treatment was 21 °C, and increased to between 24 and 27 °C during treatment (depending on pressure value). In the process of decompression, temperature decreased to initial value. The temperature of salmorejo was between 5 and 8 °C before treatments, and between 16 and 18 °C after treatments. After treatment, the samples were stored at 4 °C for 30 d.

Directly after the HHP treatments and after 1, 7, 15, and 30 d, duplicate bags from controls and from HHP-treated samples were withdrawn for viable cell counts. The content of each bag was homogenized with 20 mL of ice-cold sterile-buffered peptone water in a stomacher 80 (Seward, UK) for 1 min at maximum speed, and serially diluted with the same diluent. Samples were plated in triplicate on nonselective medium (trypticase soya agar, TSA, Scharlab) as well as on the selective media xylose lysine deoxycholate agar (XLD, Scharlab) for *Salmonella*, eosine methylene blue agar (EMB, Scharlab) for *E. coli* or PALCAM agar with added supplement (Scharlab) for *L. monocytogenes*. Sublethally injured cells were investigated by the triple-layer-agar (TAL) method (Kang & Fung, 2000). Briefly, after solidification of the selective media (XLD agar, EMB agar or PALCAM agar) on a Petri dish (8.5-cm diameter; 25 mL agar added) 7 mL of melted TSA (50 °C) was added and allowed to solidify for a few minutes. Then, a 2nd layer of melted TSA (7 mL) was added. Sample dilutions were spread-plated on top of freshly prepared TAL-XLD, TAL-EMB, or TAL-PALCAM plates, depending on the specific pathogen. All plates were incubated at 37 °C for 48 h. Colonies with typical morphology corresponding to each selective medium were counted. Colonies from TAL plates selected at random were spotted on their corresponding selective media for confirmation. The average numbers of colonies per plate were used to calculate the sample viable cell concentration, expressed as $\log_{10}$ CFU/g of sample. The detection limit was 1.0 $\log_{10}$ CFU/g. The pH of samples was measured with a Crison pH meter.

Statistical analysis

The average data from duplicate experiments ± the standard deviations were calculated with Microsoft^{R} Excel programme (Microsoft Corp., U.S.A.). In order to compare controls and treated samples at each sampling point, a paired *t*-test was performed at the 95% confidence interval by using Statgraphics Plus version 5.1 (Statistical Graphics Corp., U.S.A.).

Results

Effect of HHP treatments on background microbiota

Viable cell counts in control salmorejo not challenged with pathogens remained between 4.2 and 4.4 $\log_{10}$ CFU/g at day 0 and 1, but then decreased significantly ($P < 0.05$) during storage to reach a stable basal level close to 2.3 $\log_{10}$ CFU/g from day 7 to 30 (Figure 1). HHP treatments reduced viable cell counts significantly ($P < 0.05$) by 2.0 log cycles at 400 MPa and 2.6 log cycles at 600 MPa (Fig. 1). The surviving fraction after HHP treatments remained fairly stable (about 2 $\log_{10}$ CFU/g) during storage of the treated samples. Viable counts obtained during storage of samples treated at 500 or 600 MPa were nonsignificantly ($P > 0.05$) lower than counts from samples treated at 400 MPa. During storage, viable counts from all pressurized samples were significantly lower than initial counts at day 1, and only samples treated at 600 MPa showed viable counts significantly lower than controls at days 7 and 15. The pH of salmorejo remained in the interval of 3.82 at the beginning to 3.77 after 30 d both in controls and in samples treated by HHP.

Effect of HHP treatments on inoculated pathogens

E. coli O157 was able to survive in untreated salmorejo during storage, decreasing in viable counts by not more than 1.5 log cycles after 30 d as determined by plating on selective medium (Table 1). The observed viability loss was even lower when plating was done on TAL-EMB or in TSA. After treatments at 400, 500, or 600 MPa, no viable *E. coli* O157 cells were detected on EMB agar. However, viable cell counts on TAL-EMB were close to 1.0 $\log_{10}$ CFU/g for all 3 treatments, (Table 1), suggesting the

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Table 1–Effect of HHP treatments on a cocktail of *Escherichia coli* O157 strains inoculated in salmorejo. Samples were stored at 4 °C for 30 d.

	Viable cell counts ($\log_{10}$ CFU/g)								
	Control			400 MPa		500 MPa		600 MPa	
Storage time (d)	TSA	TAL-EMB	EMB	TAL-EMB	EMB	TAL-EMB	EMB	TAL-EMB	EMB
0	6.42 ± 0.14	6.36 ± 0.21	6.34 ± 0.27	1.15 ± 0.21	<1.00	1.00 ± 0.14	<1.00	1.00 ± 0.31	<1.00
1	6.30 ± 0.14	6.25 ± 0.21	6.33 ± 0.18	<1.00	<1.00	1.20 ± 0.21	<1.00	<1.00	<1.00
7	6.24 ± 0.24	6.25 ± 0.35	6.01 ± 0.28	1.38 ± 0.39	1.00 ± 0.14	1.00 ± 0.14	1.00 ± 0.14	<1.00	<1.00
15	6.08 ± 0.32	5.92 ± 0.17	5.74 ± 0.21	1.47 ± 0.42	1.10 ± 0.14	<1.00	<1.00	<1.00	<1.00
30	5.74 ± 0.35	5.44 ± 0.48	4.86 ± 0.23	1.00 ± 0.28	1.23 ± 0.39	<1.00	<1.00	<1.00	<1.00

Data are the average from duplicate samples ± standard deviation. The detection limit was 1.0 $\log_{10}$ CFU/g.

presence of sublethally injured cells. During storage, viable cells were still detected after plating on EMB and/or TAL-EMB for up to 30 d in samples treated at 400 MPa (Table 1) and for up to 15 d in the samples treated at 500 MPa (Table 1), but not in any of the samples treated at 600 MPa (Table 1).

When *S.* Enteritidis was inoculated in salmorejo without HHP treatment, viable cell counts on selective medium decreased by 2.5 log cycles after 15 d of storage or by 5.3 log cycles after 30 d (Table 2). *S.* Enteritidis inoculated in salmorejo was very sensitive to HHP treatments at 400 MPa, as no viable cells were detected on any of the selective conditions tested (TAL-XLD or XLD) right after treatments and also during storage (Table 2). Identical results were obtained after treatments at 500 or 600 MPa (Table 2).

The ability of *L. monocytogenes* to survive in salmorejo was much more limited compared to enterobacteria, decreasing by 2.3 log cycles until day 7 of storage (Table 3). Furthermore, no viable *L. monocytogenes* were detected under any of the selective conditions tested (TAL-PALCAM or PALCAM) until day 15 or 30. HHP treatments were highly effective in the inactivation of *L. monocytogenes*. In the samples treated at 400 MPa (Table 3), *L. monocytogenes* only were detected at day 7 of storage. In the samples treated at 500 or MPa or 600 MPa (Table 3), no viable *L. monocytogenes* were detected after treatments or during storage.

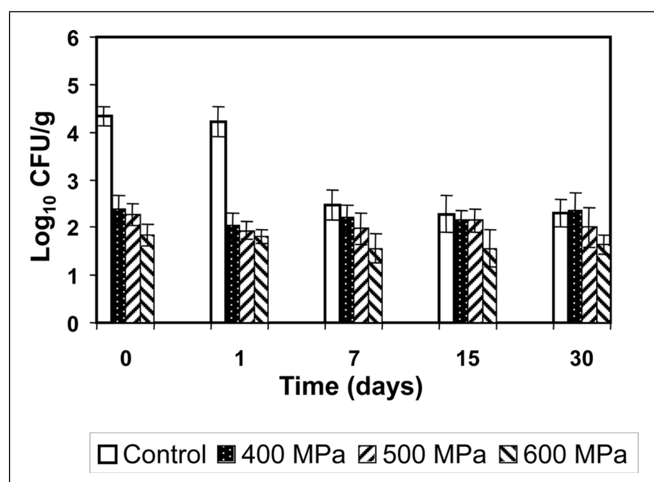

Figure 1–Effect of HHP treatments on background microbiota from salmorejo not inoculated with pathogens. Viable cell counts from untreated controls and from samples treated for 8 min at 400, 500, or 600 MPa were determined at different points during refrigeration storage (4 °C). Data are the average from duplicate samples ± standard deviation.

Discussion

Results from this study indicate strong differences in the ability of foodborne pathogens *E. coli* O157, *S. enterica* serovar enteritidis, and *L. monocytogenes,* to survive in salmorejo during refrigerated storage. Among them, *L. monocytogenes* was the bacterium with the lowest ability to survive in salmorejo as shown by the complete inactivation observed after 15 d of refrigerated storage. However, due to the fact that viable cell concentrations decreased by only 2.3 log cycles during 7 d storage, if initial numbers were high, there could be a microbiological risk especially to susceptible populations. Previous studies, however, have shown that *L. monocytogenes* is remarkably resistant to acidic conditions and survives well under refrigeration in different sustrates, including tomato salsa (Ahamad and Marth 1989; Walker and others 1990; Glass and Doyle 1991; Raghubeer and others 2000). In salmorejo, *S.* Enteritidis was more resistant than the *L. monocytogenes* tested, decreasing only by 2.4 log cycles after 15 d. *E. coli* O157 was even more resistant, and decreased by only 1.5 log cycles after 30 d of storage. These results are in agreement with previous studies showing that some strains of *E. coli* O157 are acid-resistant and can survive for long periods in acid foods, especially at low temperature (Glass and others 1992; Miller and Kaspar 1994; Weagant and others 1994; Zhao and Doyle 1994). A number of factors such as the low storage temperature, the low pH of salmorejo, and possibly also the antibacterial activities of acetic acid and the antimicrobial compounds present in garlic, could account for the observed growth inhibition and decreased viability of the foodborne pathogens as well as the background microbiota in salmorejo.

Inactivation of microorganisms by HHP treatments greatly depends on the food matrix and the pH of the food product (Alpas and others 2000). Acid stress may enhance tolerance to HHP, but at the same time organic acids may act synergistically with HHP (Alpas and others 2000). Maitland and others (2011) reported logarithmic reductions for *Salmonella* between 1.44 and 3.67 log CFU/g in whole tomatoes or between 2.25 and 3.35 in diced tomatoes after treatments at 450 or 550 MPa (120 s), respectively. Stewart and others (1997) reported an additional 3 log CFU/g reduction in *L. monocytogenes* when pressurized in buffer at pH 4.0 as compared with pH 6.0 at 353 MPa, 45 °C for 10 min. Other studies also showed that *E. coli* and *Salmonella* cells were more pressure-sensitive under acidic pH conditions compared to neutral pH values (Garcia-Graells and others 1998; Alpas and others 2000; Pagan and others 2001; Teo and others 2001; Whitney and others 2007).

In this study, *E. coli* O157 cells were highly sensitive to 600 MPa treatments for 8 min. Taking compression rate into account, the compression period (8 min) could also have an effect on microbial inactivation. However, *E. coli* O157 cells were more tolerant to

Table 2–Effect of HHP treatments on a cocktail of *Salmonella enterica* serovar Enteritidis strains inoculated in salmorejo. Samples were stored at 4 °C for 30 d.

	Viable cell counts ($\log_{10}$ CFU/g)								
	Control			400 MPa		500 MPa		600 MPa	
Storage time (days)	TSA	TAL-XLD	XLD	TAL-XLD	XLD	TAL-XLD	XLD	TAL-XLD	XLD
0	6.35 ± 0.21	6.50 ± 0.14	6.26 ± 0.07	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
1	6.27 ± 0.38	6.26 ± 0.23	6.24 ± 0.34	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
7	6.03 ± 0.32	5.75 ± 0.21	5.74 ± 0.42	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
15	5.03 ± 0.48	4.27 ± 0.45	3.82 ± 0.45	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
30	2.63 ± 0.47	1.17 ± 0.28	1.00 ± 0.14	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00

Data are the average from duplicate samples ± standard deviation. The detection limit was 1.0 $\log_{10}$ CFU/g.

Table 3–Effect of HHP treatments on a cocktail of *Listeria monoytogenes* serovars 4a and 4b strains inoculated in salmorejo. Samples were stored at 4 °C for 30 d.

	Viable cell counts ($\log_{10}$ CFU/g)								
	Control			400 MPa		500 MPa		600 MPa	
Storage time (d)	TSA	TAL-PALCAM	PALCAM	TAL-PALCAM	PALCAM	TAL-PALCAM	PALCAM	TAL-PALCAM	PALCAM
0	5.66 ± 0.38	5.57 ± 0.21	5.58 ± 0.21	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
1	5.58 ± 0.14	5.51 ± 0.14	5.43 ± 0.32	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
7	3.67 ± 0.06	3.38 ± 0.53	3.24 ± 0.38	1.00 ± 0.21	<1.00	<1.00	<1.00	<1.00	<1.00
15	2.12 ± 0.33	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
30	2.15 ± 0.42	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00

Data are the average from duplicate samples ± standard deviation. The detection limit was 1.0 $\log_{10}$ CFU/g.

treatments at 400 and 500 MPa compared to *S.* Enteritidis or *L. monocytogenes*. One explanation for this could be a higher pressure resistance of the *E. coli* strains used. The *E. coli* strains tested in this study belonged to O157 serogroup, although they were not verotoxin producers. It has been reported that some *E. coli* strains are relatively pressure sensitive and can be easily inactivated at pressures as low as 200 MPa (Robey and others 2001; Buckow and others 2010), although others, such as *E. coli* strains belonging to the O157-Group are among the most pressure-resistant vegetative cells (Benito and others 1999). Muñoz and others (2007) reported reductions of 3.44 log cycles for *E. coli* strain CECT 515 (serotype O1:K1[L1]:H7) in gazpacho (a tomato soup) after treatment at 350 MPa (22 °C, 15 min), but Jordan and others (2001) reported great differences in sensitivity to HHP treatments in the range of 350 to 500 MPa (22 °C, 5 min) between an *E. coli* O157 strain and the type-strain *E. coli* ATCC11775. The same authors also reported that *E. coli* pressure inactivation was greater in tomato and apple juices compared to orange juice. In addition to strain-dependent differences in pressure resistance, a relationship between acid tolerance and pressure resistance has been shown in *E. coli* O157:H7 (Benito and others 1999). Simultaneous survival to acid stress and HHP treatments has strong implications for food safety, because it has been reported that acid stress response mechanisms in *E. coli* protect against acid stress involved in food processing and facilitate the low infectious dose characteristic of *E. coli*, significantly contributing to the pathogenesis of this organism (Richard and Foster 2003; Kanjee and Houry 2013).

In salmorejo samples inoculated with *E. coli* O157, a small fraction of the population was able to repair sublethal damage and grow on TAL-EMB after treatments at 400 as well as 500 MPa. Survivors able to grow directly on selective medium were also detected at later storage sampling times. A previous study by Muñoz and others (2007) showed that HHP treatments up to 350 MPa yielded injured *E. coli* cells in the acidic foods orange and apple juice and gazpacho, although these authors did not investigate the fate of survivors during storage. The ability to repair sublethal damage depends not only on the bacterial species and strain but also on environmental conditions that facilitate bacterial growth and energy generation. An acidic pH would be expected to inhibit recovery of sublethally injured cells, as shown in one study with orange juice as an example, in which *E. coli* cells sublethally injured by HPP died rapidly in the acidic food environment (Linton and others 1999).

In conclusion, results from this study suggest that HHP at 600 MPa for 8 min can be an effective treatment for inactivation of foodborne pathogens *E. coli* O157, *S. enterica* serovar Enteritidis, and *L. monocytogenes* in salmorejo, and also to reduce the load of endogenous microbiota. This nonthermal treatment could be applied to improve the safety of commercial salmorejo, preventing the accidental transmission of foodborne pathogens through the finished product.

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Conflicts of interest

The authors declare no conflict of interest.

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ARTÍCULO 4

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Inactivation of leuconostocs in cherimoya pulp by high hydrostatic pressure treatments applied singly or in combination with enterocin AS-48

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ABSTRACT

A cocktail of *Leuconostoc mesenteroides, Leuconostoc gasicomitatum* and *Leuconostoc gelidum* inoculated on autoclaved cherimoya pulp were treated by high hydrostatic pressure (HHP; 400, 500 or 600 MPa for 8 min) and with enterocin AS-48 (35 μg/g), singly or in combination. After treatments, samples were stored at 4 °C for 30 days or at 22 °C for 10 days. HHP treatments at 400 or 500 MPa reduced viable cell concentrations by 4.3 and 4.9 log cycles, respectively. No survivors were detected at 600 MPa. Bacteriocin addition in combination with HHP achieved an additional reduction of 0.6–0.9 log cycles after treatments at 400 or 500 MPa, and also reduced viable counts for most treatments during storage. In samples treated by HHP, regrowth of leuconostocs was delayed to days 15 (400 or 500 MPa) or 30 (600 MPa) of refrigeration storage, or day 3 at 22 °C (all treatments). Results from the study suggest that treatments at 600 MPa (8 min) can be useful to inactivate leuconostocs in cherimoya pulp.

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1. Introduction

Lactic acid bacteria (LAB) are usually found in low numbers on vegetable surfaces, but they tend to proliferate during food storage at the expense of nutrients released during processing. LAB can spoil food products, especially refrigerated foods packed under vacuum or under modified atmosphere. Leuconostocs have been described as spoilage bacteria in several studies. *Leuconostoc mesenteroides* can multiply and cause sourness, discoloration, or off-flavor in vegetables such as peas and beans prepared for freezing (Sharpe & Pettipher, 1983) and to spoil fresh ready-to-use grated carrots (Carlin, Nguyen-the, Cudennec, & Reich, 1989). The bacterium can also cause souring of cane juice and spoil other under-processed or recontaminated juices and beverages containing juice, leading to formation of slime, CO_2, off-flavors, turbidity, lactic and acetic acid production (reviewed by Björkroth & Holzapfel, 2006). *L. mesenteroides* and *Leuconostoc gelidum* have been isolated from spoiled commercial fresh-cut honeydew melon cubes (Zhang, Samapundo, Pothakos, Sürengil, & Devlieghere, 2013) while *Leuconostoc gasicomitatum* has been reported to cause spoilage of cold-stored, modified-atmosphere-packaged, nutrient-rich foods, including meat products (Björkroth et al., 2000; Vihavainen & Björkroth, 2007) and cooked vegetable sausages packaged under vacuum (Vihavainen, Murros, & Bjorkroth, 2008). *L. gelidum, L. gasicomitatum*, and *L. mesenteroides* are the predominant LAB in the commercial vegetable sausages (Vihavainen et al., 2008). Furthermore, *L. gasicomitatum* and *L. gelidum* were found to dominate in ready-to-eat minimally processed vegetable salads (Pothakos, Snauwaert, De Vos, Huys, & Devlieghere 2014a, 2014b). In spite of their wide incidence in foods, *Leuconostoc* species are intrinsically vancomycin-resistant bacteria now being recognised as emerging pathogens that can cause severe infections, particularly in immunocompromised patients (Albanese, Spanu, Sali, Novegno, D'Inzeo et al., 2006; Florescu, Hill, Sudan, & Iwen, 2008; Nelson, 1999).

Cherimoya (*Annona cherimola* Mill.) is an exotic fruit found in different subtropical areas around the world (Gupta-Elera, Garrett, Martínez, Robison, & O'Neill, 2011). Spain has become the world's largest cherimoya producer (van Zonneveld et al., 2012). Cherimoya fruit was used in traditional medicine as an antimicrobial and insecticide and as an effective treatment for digestive disorders and skin disease (Amoo, Emenike, & Akpambang, 2008). It has

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antioxidant and cytoprotective properties due to its content in phenolic compounds (Loizzo et al., 2012; Roesler, Malta, Carrasco, & Pastore, 2006). These compounds may help to prevent diseases associated with oxidative stress, such as cancer, atherosclerosis and neurodegenerative diseases (La Vecchia, Altieri, & Tavani, 2001; Steinmetz & Potter, 1996; Zibadi et al., 2007). In addition to its claimed health benefits, cherimoya pulp is commercialized for direct consumption or as an ingredient for preparation of different food and beverage products such as drinks, smoothies, sorbets, fruit salad, salad dressing or dessert sauce as examples. Due to the seasonal production of this fruit and to improve the retention of its bioactive components there is an interest in producing lightly-processed, commercially-stable cherimoya pulp preparations. For this purpose, in a previous study we applied high-hydrostatic pressure treatments on cherimoya pulp heavily contaminated with its own epiphytic microbiota and found that HHP treatment applied singly or in combination with the broad-spectrum circular bacteriocin enterocin AS-48 (Grande Burgos, Pérez-Pulido, López Aguayo, Gálvez, & Lucas, 2014) could prolong the shelf life of refrigerated, vacuum-packed pulp (Pérez Pulido, Toledo, Grande, Gálvez, & Lucas, 2014). However, it was also found that leuconostocs were able to proliferate during storage. Because of its high water activity, sugar content and acidic pH (around 4.5), cherimoya pulp stored under refrigeration can be an excellent substrate for the growth of these psychrotrophic spoilage LAB. The purpose of the present study was to specifically determine the effect of HHP treatments singly or in combination with enterocin AS-48 on the inactivation of the most frequent leuconostoc species known to cause food spoilage (*L. mesenteroides, L. gelidum* and *L. gasicomitatum*) inoculated at a high cell density on cherimoya pulp.

2. Materials and methods

2.1. Sample preparation

Cherimoya (*Annona cherimola* Mill.) was purchased from local food stores and kept under refrigeration until processing (for no more than 24 h). The cherimoya pulp was obtained manually under aseptic conditions from fresh cherimoyas after removing the skin and seeds with sterile knife, spoon and fork. Pulp was sterilized by autoclaving in order to avoid interference from background microbiota.

2.2. Bacterial strains and cultivation conditions

L. gasicomitatum CECT 5767 (from spoiled tomato-marinated broiler meat strips packed under modified atmosphere), *L. gelidum* CECT 4026 (from vacuum-packed refrigerated meat) and *L. mesenteroides* CECT 912 (type strain) were cultivated for 48 h in De Man Rogosa Sharpe (MRS) broth (Scharlab) at 22 °C for 48 h. A cocktail was prepared by mixing one ml from each culture, followed by centrifugation (13,000× *g*, 5 min), washing and resuspension with 3 ml sterile saline solution. The cocktail viable cell concentration was 8.54 $\log_{10}$ CFU/ml.

2.3. Preparation of enterocin AS-48

The bacteriocin enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions.

2.4. Antimicrobial treatments

Cherimoya pulp (2000 g) was inoculated (1%, vol/vol) with a cocktail of leuconostocs obtained as described above, at a final cell concentration of 6.54 $\log_{10}$ CFU/ml, and then divided in four batches (A1, A2, B1, B2). The duplicate batches A1 and A2 were supplemented with enterocin AS-48 (35 µg/g final concentration), while the duplicate batches B1 and B2 were not. All batches were distributed in aliquots (10 g each) in polyethylene-polyamide bags and sealed under vacuum for application or not of HHP treatments. Ten bags were prepared for each batch (A1, A2, B1, B2) and HHP treatment: controls without HHP treatment, and treatments at 400, 500 and 600 MPa applied as described below. After treatments, 80 bags (corresponding to five bags from each batch and treatment, adding up 800 g pulp in total) were stored at 4 °C for up to 30 days (simulating a 30-d shelf life period). An equivalent 80 bag set was stored at 22 °C for a maximum of 10 days (simulating a worst-case scenario of temperature abuse).

After treatments and also at desired times during storage, bags from each duplicate batch were opened and their content mixed with 10 ml sterile saline solution and pummeled in stomacher bags for 2 min. The obtained pulp suspension from each batch was serially diluted in sterile saline solution and plated in triplicate on MRS agar (Scharlab, Madrid) and incubated at 22 °C for 48 h. The average number of colonies obtained from duplicate batches was used to calculate the viable cell concentration.

High hydrostatic pressure (HHP) treatments were applied with a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) fitted with a 2.5 l vessel capable of operating in a pressure range of 0–700 MPa. Cherimoya samples (supplemented or not with enterocin AS-48) were pressurized at 400, 500 or 600 MPa for 8 min. Come-up speed was 75 MPa/min. Decompression was almost instantaneous. Pressurization fluid was water with added 10% propylene glycol. The temperature inside the vessel during treatments ranged between 23 and 27 °C.

2.5. Statistical analysis

All experiments were carried out in duplicate. The average Log(N) values from duplicate batches ± standard deviations from viable cell counts were determined with Excel programme (Microsoft Corp., USA). A paired *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp, USA) in order to determine the statistical significance between treatments.

3. Results

The effect of treatments and the changes in viable cell counts during storage at 4 °C of cherimoya pulp inoculated with the cocktail of leuconostocs are shown in Fig. 1. Application of HHP treatments without bacteriocin addition reduced viable cell counts significantly ($P < 0.05$) by 4.3 and 4.9 log cycles for treatments at 400 and 500 MPa (Fig. 1A, B). No viable cells were detected after treatment at 600 MPa (Fig. 1C). The single bacteriocin addition reduced viable cell counts non-significantly ($P > 0.05$) shortly after addition. Reductions of viable cell counts obtained after treatments by HHP in combination with bacteriocin (5.2 and 5.5 log cycles at 400 and 500 MPa, respectively) did not differ significantly ($P > 0.05$) from treatments without bacteriocin (Fig. 1A, B). The effect of bacteriocin addition in the combined treatment at 600 MPa could not be measured, since the HHP treatment alone already inactivated all detectable cells.

During storage of cherimoya pulp samples at 4 °C, a significant ($P < 0.05$) viable cell count reduction of 1.4 log cycles was observed

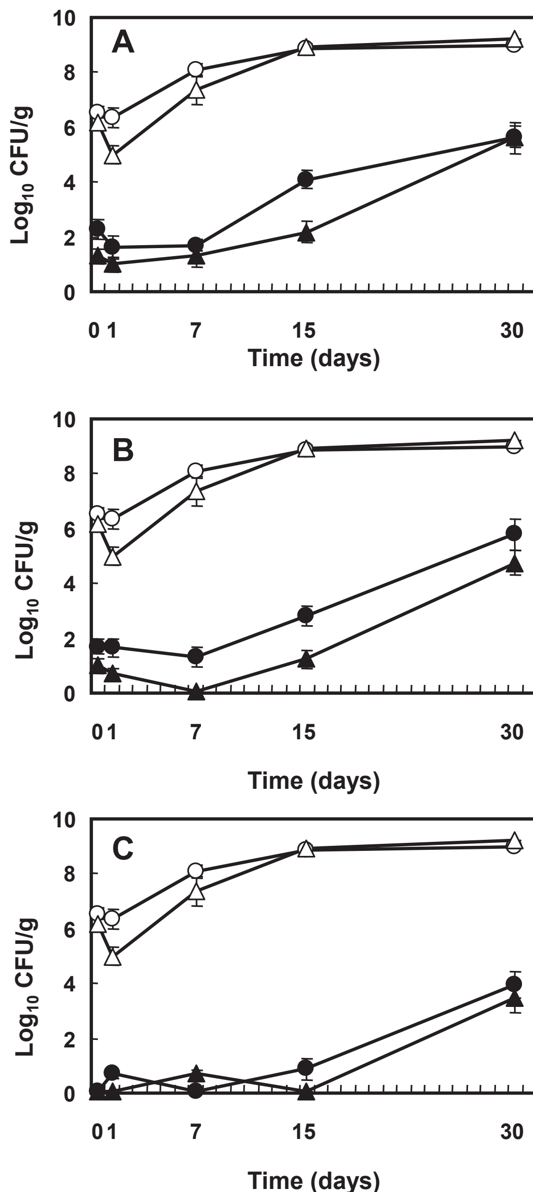

Fig. 1. Effect of HHP treatments at 400 (A), 500 (B) or 600 MPa (C) on a cocktail of leuconostocs inoculated on cherimoya pulp stored at 4 °C. Treatments were applied singly (●) or in combination with 35 µg/g enterocin AS-48 (▲). Untreated controls (○). Samples singly treated with enterocin AS-48 (Δ).

in samples singly treated with bacteriocin compared to untreated controls at day 1, but after that viable cell counts increased both in controls and in samples supplemented with bacteriocin (Fig. 1). Viable cell counts in samples treated by HHP were always significantly lower ($P < 0.05$) than samples not treated by HHP for all storage times and treatments. Regrowth of cultures during storage depended on the intensity of treatment and on addition of bacteriocin. For example, at day 15 of storage, viable cell counts in samples treated at 600 MPa were significantly lower ($P < 0.05$) compared with samples treated at 500 MPa, which in turn showed significantly lower counts ($P < 0.05$) than samples treated at 400 MPa. In samples treated by HHP and bacteriocin, viable cell counts were significantly lower ($P < 0.05$) compared to the single HHP treatment for 400 MPa at day 15 (Fig. 1A) and also for 500 MPa and days 1, 7 and 15 (Fig. 1B). For the HHP treatment at 600 MPa, viable cell counts remained below or close to the detection limit for the first 15 days for both the single and the combined HHP treatment, and regrowth was only observed after 30 days of storage (Fig. 1C).

In the cherimoya pulp samples stored at 22 °C, samples with bacteriocin but no HHP treatment showed a non-significant ($P > 0.05$) growth delay at day 1 compared to the untreated controls (Fig. 2). In samples treated by HHP, viable cell counts remained at low levels until day 1, but regrowth was always observed by day 3 (Fig. 2). Nevertheless, viable cell counts in the HHP-treated samples were significantly lower ($P < 0.05$) compared to the untreated controls for the first 7 days (in samples treated at 400 or 500 MPa) or during the whole storage period in the case of samples treated at 600 MPa. Addition of enterocin AS-48 delayed bacterial growth significantly ($P < 0.05$) at day 3 in samples treated at

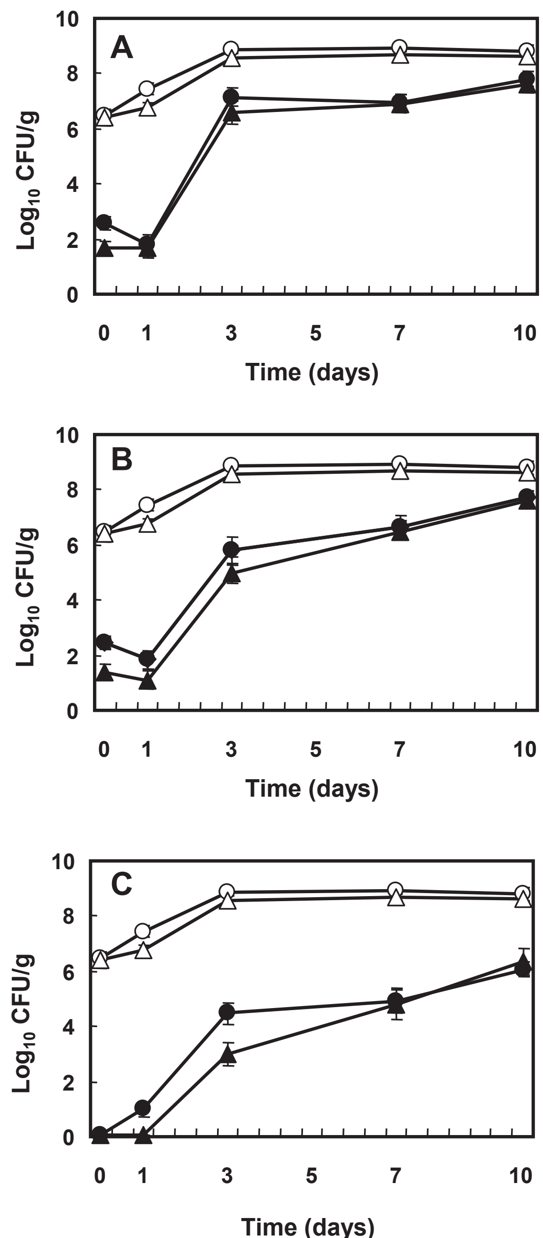

Fig. 2. Effect of HHP treatments at 400 (A), 500 (B) or 600 MPa (C) on a cocktail of leuconostocs inoculated on cherimoya pulp stored at 22 °C. Treatments were applied singly (●) or in combination with 35 µg/g enterocin AS-48 (▲). Untreated controls (○). Samples singly treated with enterocin AS-48 (Δ).

600 MPa (Fig. 2C).

4. Discussion

High hydrostatic pressure can be used as a final processing step applied on packed foods, and it seems a promising non-thermal process for cherimoya pulp. Today, a variety of pressure-treated products such as jams, fruit juices, avocado salad (guacamole), fresh-cut fruit salads, fresh whole oysters, etc. are commercially available in the USA, Europe and Japan (Torres & Velazquez, 2005). HHP treatments have also been applied on different kinds of fruits, such as pomegranate, peach, cashew apple, melon, guava, banana, persimmon fruit, avocado or mango (Jacobo-Velázquez & Hernández-Brenes, 2012; Kaushik, Kaur, Srinivasa Rao, & Mishra, 2014; Rawson et al., 2011; Vázquez-Gutiérrez, Hernández-Carrión, Quiles, Hernando, & Pérez-Munuera, 2012). Compared with food-borne pathogens, inactivation by HHP of spoilage bacteria such as leuconostocs has been studied to a lesser extent. One study reported that HHP treatments at 250 and 500 MPa caused changes in the external surface and internal structure of cells of *L. mesenteroides*, including dechaining and blister formation on the bacterial cell surface together with an increasing denaturation of ribosomes (Kaletunç, Lee, Alpas, & Bozoglu, 2004). Another study reported that mild HHP treatment (345 MPa at 25 °C for 5 min) induced cell lysis of *L. mesenteroides* cell suspensions and reduced cell viability by over 6 log cycles (Kalchayanand, Frethem, Dunne, Sikes, & Ray, 2002). There are no previous studies on the effects of HHP on other spoilage leuconostocs such as *L. gelidum* and *L. gasicomitatum*.

The efficacy of HHP treatments may vary depending on the target bacteria and food product, and therefore each specific food needs to be tested at laboratory or pilot scale before moving to industrial application. In the present study, treatments of at least 600 MPa were necessary to achieve a logarithmic reduction of at least 6 log cycles on the cocktail of leuconostocs inoculated on cherimoya pulp. Addition of enterocin AS-48 at 35 μg/g had a limited effect on inactivation of leuconostocs, probably because of the high bacterial inoculum used and also a possible interaction of the bacteriocin with the food matrix. Previous results on application in different food systems indicate that the efficacy of enterocin AS-48 depends on several factors including the target bacterium and the food system (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010). In a previous study, we showed that the combined treatment of enterocin AS-48 (50 μg/g) and HHP (600 MPA, 8 min) enhanced the inactivation of epiphytic microbiota inoculated on cherimoya pulp and also delayed growth of survivors during storage compared with the single HHP treatment (Pérez Pulido et al., 2014). Application of enterocin AS-48 in combination with HHP treatments improved the inactivation of *Salmonella enterica* in fuet and *Staphylococcus aureus* in rice pudding (Ananou et al., 2010; Pérez-Pulido, Toledo del Árbol, Grande-Burgos, & Gálvez, 2012). Other bacteriocins such as nisin and pediocin PA1/Ach have been shown to potentiate the bactericidal effects of HHP treatments (reviewed by Gálvez, Lucas-López, Abriouel, Valdivia, & Ben Omar, 2008; Kalchayanand, Sikes, Dunne, & Ray, 1994; Kalchayanand, Dunne, Sikes, & Ray, 2004).

This is the first report in which enterocin AS-48 has been tested against leuconostocs in a food system singly or in combination with HHP. Interestingly, although the contribution of enterocin AS-48 to microbial inactivation was small, the added bacteriocin had a positive effect in keeping microbial counts lower compared to the single HHP treatments during storage of samples under refrigeration conditions. Furthermore, when samples were stored at 22 °C to simulate temperature abuse conditions, the delay in bacterial growth was greater in the samples treated at 600 MPa in combination with bacteriocin compared with the single HHP treatment. Altogether, these results highlight the potential of HHP treatments in controlling leuconostocs in cherimoya pulp and suggest a protective effect of added bacteriocin during storage of the HHP-treated foods.

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Pyrosequencing

ABSTRACT

In the present study, pulp obtained from cherimoya pulp (*Annona cherimola*) was inoculated with epiphytic microbiota collected from cherimoya fruits, and supplemented or not with the circular bacteriocin enterocin AS-48 (50 μg/g) and then packed under vacuum. Samples supplemented or not with enterocin were treated by high hydrostatic pressure (600 MPa, 8 min) and then stored at 5 °C for 30 days. The single AS-48 treatment only delayed microbial growth non-significantly ($p > 0.05$). HHP treatment reduced microbial counts by five log cycles, but it did not prevent further growth of survivors by day 7. The combined treatment (AS-48 + HHP) was the most effective, keeping bacterial cell densities at ≤1.5 log CFU/g for up to 15 days. 16S rRNA gene pyrosequencing analysis was done on amplicon libraries from the growth on TSA plates seeded with ten-fold dilutions of pulp suspensions and incubated at 22 °C for 24 h. The results obtained are limited by the experimental conditions used in the study, and only concern the bacterial fraction that was selected by the TSA and growth conditions used. *Pantoea* (*Pantoea agglomerans, Pantoea vagans*) were the operational taxonomic units (OTUs) detected at highest relative abundance in bacterial biomass grown from control samples for the first 7 days of storage, followed by *Enterococcus gallinarum* and *Leuconostoc mesenteroides* during late storage. The single HHP treatment significantly reduced the relative abundance of OTUs belonging to *Pantoea* and strongly increased that of endosporeformers (mainly *Bacillus firmus* and *Bacillus stratosphericus*) early after treatment, although *Pantoea* became again the predominant OTUs during storage. Samples singly treated with enterocin AS-48 revealed a strong inhibition of *E. gallinarum* as well as an early decrease in the relative abundance of *Pantoea* and an increased relative abundance of OTUs belonging to other Gram-negative species (mainly from genera *Serratia* and *Pseudomonas*). The strong microbial inactivation achieved by the combined treatment with enterocin and HHP reduced the levels of viable cells below detectable limits at days 0 and 1, and survivors recovered on TSA at day 7 were represented in >99% by *B. firmus* OTU. OTUs from endosporeformers were no longer detected during prolonged incubation, displaced by *Pantoea* spp., *Erwinia billingiae* and leuconostocs. Results from the present study indicate that HHP in combination with enterocin AS-48 is more effective in preserving the microbiological quality of cherimoya pulp during storage than the single HHP treatment.

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1. Introduction

Annona cherimola Mill. (Annonaceae), commonly known as cherimoya, is a species of exotic fruit found in different subtropical areas around the world (Gupta-Elera et al., 2011). Spain has become the world's largest cherimoya producer (van Zonneveld et al., 2012), where the plant is cultivated intensively in the tropical coast of Granada-Malaga characterized by specific pedoclimatic conditions. Cherimoya fruit was used in traditional medicine as an antimicrobial and insecticide and as an effective treatment for digestive disorders and skin disease (Amoo et al., 2008). It has antioxidant and cytoprotective properties due to its content in phenolic compounds (Loizzo et al., 2012; Roesler et al., 2006). These compounds may help to prevent diseases associated with oxidative stress, such as cancer, atherosclerosis and neurodegenerative diseases (La Vecchia et al., 2001; Steinmetz and Potter, 1996; Zibadi et al., 2007). The local production of cherimoya and its seasonal availability are limitations to a widespread consumption of this fruit. Cherimoya fruit pulp preparations with an extended shelf life could find new markets as functional foods and contribute to solve the problem of seasonal excess production and decrease in market price. However, cherimoya pulp spoils easily, and its shelf life may be severely limited by surface contaminating bacteria reaching the pulp during processing. So far, there are no previous studies on epiphytic bacteria on cherimoya or how contaminating bacteria may proliferate in pulps stored under refrigeration conditions.

Among the available food preservation methods, high hydrostatic pressure (HHP) stands as a non-thermal food processing technology

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that offers ideal performance for preservation of bio-active molecules in foods while at the same time may serve to inactivate pathogenic and spoilage bacteria, thus improving the food safety and product shelf life. HHP is applied to packed food, avoiding further cross-contamination. One of the advantages of HHP is that it may have very low or no effect on the food quality attributes such as colour, flavour, and nutritional value, preserving most of the food bioactive molecules including those found in fruits and vegetables (Oey et al., 2008; Rastogi et al., 2007; Rawson et al., 2011; Rendueles et al., 2011). HHP treatments have been applied on several kinds of fruits, such as pomegranate, peach, cashew apple, melon, guava, banana, persimmon fruit, avocado or mango (Jacobo-Velázquez and Hernández-Brenes, 2012; Rawson et al., 2011; Vázquez-Gutiérrez et al., 2012), but no report has been found on cherimoya in the scientific literature.

HHP treatments can be applied singly or in combination with other hurdles, such as bacteriocins (Gálvez et al., 2008). Bacteriocins are natural antimicrobial peptides produced by many different bacterial groups (Klaenhammer, 1993). Enterocin AS-48 is a circular bacteriocin with a broad bactericidal spectrum against Gram-positive bacteria (Maqueda et al., 2004). At high concentration, it may also inhibit some Gram-negative bacteria. Its spectrum of inhibition can be extended at low bacteriocin concentrations added in combination with outer membrane-permeabilizing treatments (Abriouel et al., 1998; Ananou et al., 2005). This bacteriocin has been widely investigated for biopreservation of foods from meat, dairy, seafood and vegetable origin (Abriouel et al., 2010; Ananou et al., 2014; Gálvez et al., 2011).

The purpose of the present study was to determine the effects of HHP treatments and enterocin AS-48 addition (singly or combined) on cherimoya pulp stored under refrigeration. Cherimoya pulp was inoculated with its own epiphytic microbiota in order to simulate a worst-case surface contamination scenario during processing. In order to determine the impact of HHP and enterocin AS-48 treatments, pyrosequencing analysis of TSA-grown bacterial biomass recovered from control and treated samples was carried out after treatment and during sample storage.

2. Materials and methods

2.1. Sample preparation

Cherimoya (*A. cherimola* Mill.) was purchased from four different local food stores and kept under refrigeration until processing (for no more than 24 h). A microbial suspension from cherimoya surface was obtained by washing 30 representative cherimoya units (3540 g total weight) with buffered peptone water. The obtained suspension was washed with sterile saline solution and stored under refrigeration for no longer than 18 h before it was inoculated into freshly-made cherimoya pulp. The cherimoya pulp was obtained manually under aseptic conditions from fresh cherimoyas after removing the skin and seeds with sterile knife, spoon and fork.

2.2. Preparation of enterocin AS-48

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel et al., 2003). Bacteriocin concentrates were filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions.

2.3. Antimicrobial treatments

Cherimoya pulp was inoculated (1%, vol/vol) with a suspension of epiphytic microbiota obtained as described above (having an inoculum density of 8.4 $\log_{10}$ CFU/ml), and supplemented or not with enterocin AS-48 (50 μg/g final concentration). The resulting cherimoya pulps were divided in four batches each, as follows: batches A and B were used as control pulp without bacteriocin and without further treatment. Batches C and D were used as pulp without bacteriocin for high hydrostatic pressure treatment. Batches E and F were used as bacteriocin-added pulp without HHP treatment, and batches G and H were bacteriocin-added pulp treated by HHP. Five aliquots (10 g each) from each batch were placed in polyethylene–polyamide bags and sealed under vacuum for application or not of HHP treatments.

High hydrostatic pressure (HHP) treatments were carried out by using a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) suited with a 2.5 l vessel capable of operating in a pressure range of 0–700 MPa, under non-thermal conditions. Cherimoya samples (supplemented or not with enterocin AS-48) were pressurised at 600 MPa for 8 min. Come-up speed was 75 MPa/min. Decompression was immediate. Pressurization fluid was water with added 5% propylenglycol. The temperature inside the vessel during treatments ranged between 23 and 27 °C. All samples (treated or not by HHP) were stored at 5 °C for up to 30 days.

After treatments and also at desired times during storage (1, 7, 15 and 30 days), aliquots from each batch were mixed with 10 ml sterile saline solution and pummeled in stomacher bags for 2 min. The obtained pulp suspension was serially diluted in sterile saline solution and plated in triplicate on tryptic soya agar (TSA, Scharlab, Madrid) plates and incubated at 22 °C for 24 h. The pH of pulp suspensions was measured with a pH meter (Crison Instruments, S.A., Barcelona).

2.4. DNA extraction, amplicon library preparation and sequencing

Bacterial biomass was collected with a sterile cotton swab from the TSA plates seeded with the ten-fold diluted cherimoya pulp suspension obtained as described above, and resuspended in 1 ml sterile distilled water. The bacterial biomass suspension so obtained was stored at −20 °C until processing. Aliquots (20 μl) of biomass suspensions were used for extraction of total DNA by using a GenElute™ bacterial genomic DNA kit (Sigma-Aldrich, Madrid). The concentration and quality of the obtained DNA were determined with a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom).

For pyrosequencing, V3–V5 region of the 16S rRNA gene was amplified using key-tagged bacterial primers prepared by Lifesequencing S.L. (Valencia, Spain) based on Sim et al. (2012). PCR reactions were performed with 20 ng of community DNA, 200 μM of each of the four deoxynucleoside triphosphates, 400 nM of each primer, 2.5 U of FastStart HiFi Polymerase, and the appropriate buffer with $MgCl_2$ supplied by the manufacturer (Roche, Mannheim, Germany), 4% of 20 mg/ml BSA (Sigma, Dorset, United Kingdom), and 0.5 M Betaine (Sigma). Thermal cycling consisted of initial denaturation at 94 °C for 2 minutes followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 5 min. To obtain sufficient material, PCR reactions were repeated in triplicate and pooled prior to purification by running the PCR amplicons on 1% (w/v) agarose gels. Amplicons were quantified using the PicoGreen assay (Quant-iT, PicoGreen DNA assay, Invitrogen) and combined in a single tube in equimolar concentrations. The pooled amplicon mixture was purified twice (AMPure XP kit, Agencourt, Takeley, United Kingdom) and the cleaned pool requantified with PicoGreen assay. Amplicons were submitted to the pyrosequencing services offered by Life Sequencing S.L. (Valencia, Spain) where EmPCR was performed and subsequently, unidirectional pyrosequencing was carried out on a 454 Life Sciences GS FLX + instrument (Roche) following the Roche Amplicon Lib-L protocol.

2.5. Bioinformatic analysis

Bioinformatic analysis was carried out by Life Sequencing S.L. services. Raw reads were first filtered according to the 454 processing pipeline. Pyrosequencing reads were filtered with Q20 FASTX_tool_kit version 0.0.14, and reads were excluded from the analysis if they had

an average quality score < Q20 and if there were ambiguous base calls (Ns). Reads were trimmed for adaptors and PCR primers, and only reads greater than 300 nts were retained for analysis. Chimeras were eliminated using the Uchime algorithm under default mode (UCHIME version 4.2.40). The average final lengths of reads ranged from 531 to 585 nt (Table 1). The numbers of total reads ranged from 4054 to 20,526, depending on the sample (Table 1). Sequences were compared with NCBI 16S rRNA database using BLASTN. Operational taxonomic units (OTUs) were defined by a 97% similarity. The read clusters were further assigned to taxonomies using the RDP classifier. Rarefraction curves were obtained for each sample and taxonomical levels were analysed in order to confirm they have reached the plateau and no more taxonomical groups were expected to be found if sequencing was increased. Sequence reads have been deposited in the Short Read Archive Database (http://www.ncbi.nlm.nih.gov/sra, bioproject number PRJNA263914, SRR1616689 to SRR1616705 and SRR1614185).

2.6. Statistical analysis

All experiments were carried out in duplicate with three replicates per test. The average data ± standard deviations from viable cell counts were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp., USA), in order to determine the statistical significance of data corresponding to viable cell counts. Data on the microbial composition of samples for the different treatments applied and at different storage times were analyzed by principal component analysis (PCA) using XLSTAT 2014 evaluation version (2014.1.03, Addinsoft, France). The Pearson correlation coefficient (r) was applied, and correlations were defined as very weak (0.00–0.19), weak (0.20–0.39), moderate (0.4–0.59), strong (0.60–0.79) or very strong (0.80–0.99), with a P significance of <0.05. Only data for genera with relative abundances ≥1.0 were used for the PCA analysis.

3. Results

3.1. Effect of treatments on total viable cell counts

Cherimoya pulp was artificially contaminated with its own microbiota to simulate a worst-case scenario of contamination during pulp preparation. In control samples without any treatment, viable cell counts increased significantly ($P < 0.05$) from approx. 6.4 $\log_{10}$ CFU/g at time 0 to a maximum of 8.5 at day 15 (Fig. 1). Addition of enterocin AS-48 alone caused a non-significant ($P > 0.05$ delay of microbial growth that was noticed only at days 1 and 7. Viable cell counts for

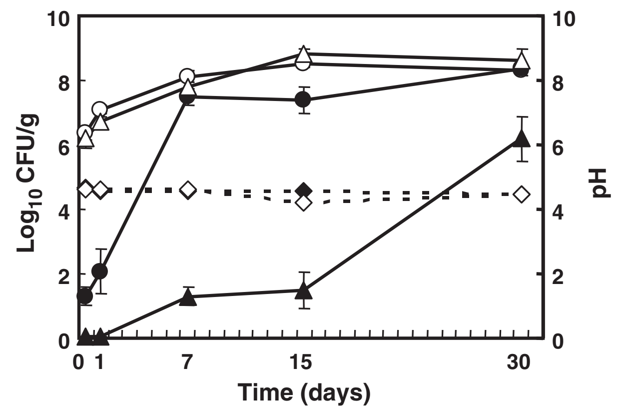

Fig. 1. Effect of high hydrostatic pressure (HHP at 600 MPa for 8 min) and enterocin AS-48 (50 μg/g) singly or combined on total viable cell counts in cherimoya pulp inoculated with its epiphytic microbiota and stored at 5 °C for 30 days. Controls (○); enterocin AS-48 treated samples (△); HHP-treated samples (●); samples treated by HHP in combination with enterocin AS-48 (▲). pH of control (◊) and combined treatment samples (♦).

the single bacteriocin treatment did not differ significantly ($P > 0.05$) from controls for any of the storage times. The single HHP treatment achieved an initial and significant ($P < 0.05$) reduction of viable cell counts of five log cycles. The surviving fraction increased non-significantly ($P > 0.05$) to 2.0 $\log_{10}$ CFU/g at day 1 of storage, and then significantly ($P < 0.05$) to 7.4 $\log_{10}$ CFU/g at day 7, after which time it remained stable (day 15) or increased non-significantly ($P > 0.05$) up to 8.3 $\log_{10}$ CFU/g (day 30). Viable cell counts in the HHP-treated samples were significantly lower than controls ($P < 0.05$) at storage times 0 and 1. The combined treatment of HHP and enterocin AS-48 was the most effective, since it reduced viable cell counts to below detectable levels after treatment and also at day 1 of storage. Furthermore, microbial counts in samples from the combined treatments never increased significantly ($P > 005$) above 1.5 $\log_{10}$ CFU/g between days 7 and 15, indicating the efficacy of the combined treatments in keeping the microbiological quality of cherimoya pulp during storage for at least 15 days. Afterwards, microbial counts increased significantly ($P < 0.05$) up to 6.2 $\log_{10}$ CFU/g at day 30. The concentrations of viable cells recovered from samples corresponding to the combined treatment were always significantly lower ($P < 0.05$) than controls and the single HHP treatment.

The pH of samples gradually decreased slightly from average initial values of 4.64–4.67 to between 4.44 and 4.47 at the end of the storage period (Fig. 1). Differences in pH between control and treatment samples were not statistically significant ($P > 005$) for any of the points.

Table 1
Number of reads and average fragment lengths obtained for the different samples (trimmed, non-chimera data).

Sample	Number of reads	Average length (nt)
Control T0	7150	541,413
Control T1	6363	551,732
Control T7	5920	555,376
Control T15	9559	555,709
Control T30	8721	551,528
HHP T0	13747	554,923
HHP T1	10225	550,753
HHP T7	20526	546,217
HHP T15	5360	531,203
HHP T30	16833	546,189
AS48 T0	7963	554,138
AS48 T1	6821	543,199
AS48 T7	9363	550,379
AS48 T15	5944	547,578
AS48 T30	4054	539,861
AS48-HHP T7	7697	585,299
AS48-HHP T15	4645	534,611
AS48-HHP T30	4957	542,137

3.2. Impact of treatments and storage on microbial diversity

The diversity in bacterial biomass grown on TSA plates seeded with samples right after treatments and during 30 days of storage at 5 °C was determined by pyrosequencing (Fig. 2). Phylum *Proteobacteria* was predominant in bacterial growth recovered from control samples at time 0 (92.21% of OTUs), followed by *Firmicutes* (7.79%). During storage, the proportion of *Firmicutes* increased gradually, while that of *Proteobacteria* decreased. At day 15, *Firmicutes* represented 43.53% of OTUs, while at day 30 they had a relative abundance much greater than *Proteobacteria* (89.79% versus 10.11%). At genus and species level, genus *Pantoea* (represented mainly by OTUs belonging to *Pantoea agglomerans* and *Pantoea vagans* and to a much less extent also by *Pantoea annatis*) was the predominant group detected in growth from the untreated controls from the beginning until day 15 of storage. Genus *Escherichia* was detected at low relative abundance (2.1%) on day 1 (*Esherichia hermannii*) and also at higher relative abundance (10%) at day 30 (*Escherichia fergusonii*). Among the *Firmicutes*, genus *Enterococcus* was second in relative abundance in growth recovered

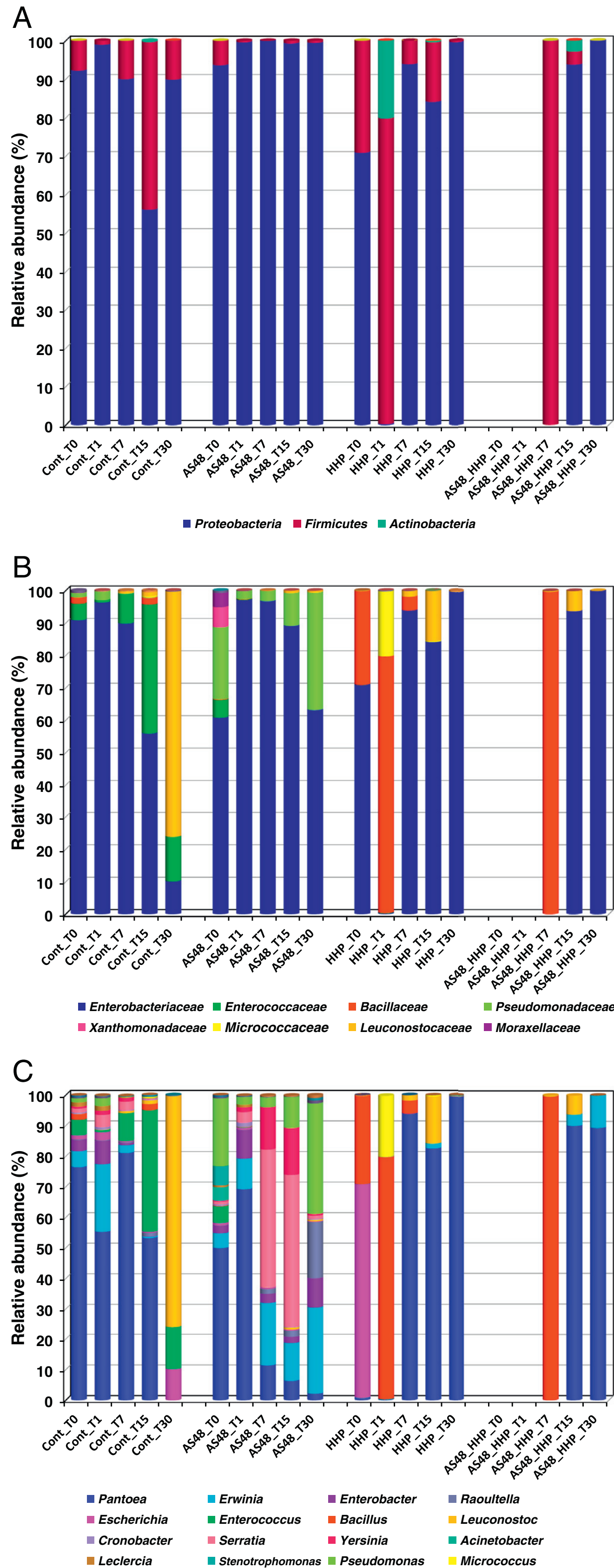

Fig. 2. Relative abundance of OTUs based on 16S rRNA gene pyrosequencing analysis of DNA from viable bacterial cells recovered on TSA from cherimoya pulp. Cherimoya pulp inoculated with epiphytic microbiota without any treatment (Cont), treated with 50 μg/g enterocin AS-48 (AS48), treated by high hydrostatic pressure at 600 MPa for 8 min (HHP) or processed by the same HHP treatment in combination with 50 μg/g enterocin AS-48 (AS48_HHP). Samples were analyzed at T0, 1, 7, 15 and 30 days of storage at 5 °C. OTUs were sorted by *Phylum* (A), *Family* (B), *Genus* (C) and *Species* (D) taxonomic levels.

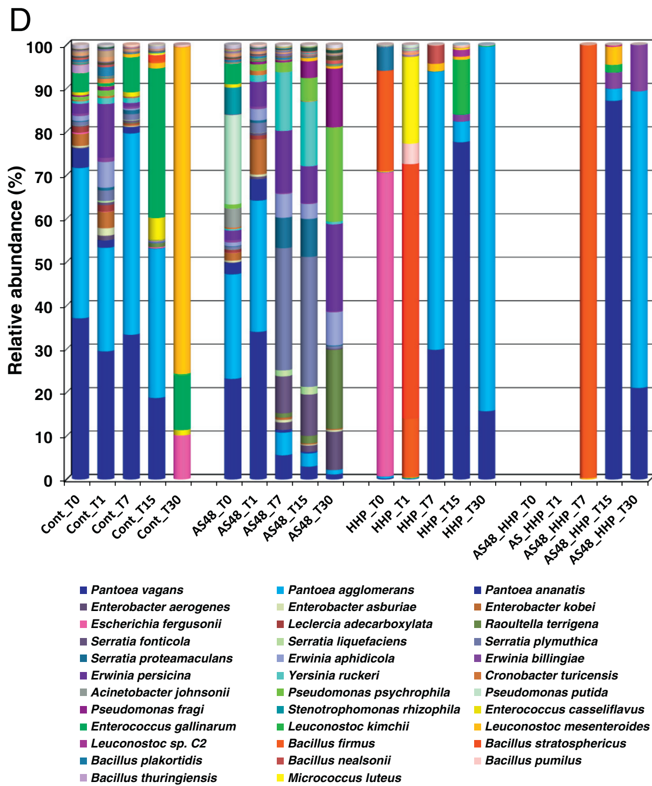

Fig. 2 (*continued*).

from control samples from days 7 to 30. It was represented mainly by OTUs corresponding to the motile species *Enterococcus casseliflavus* and *Enterococcus gallinarum*, which became the most abundant OTU at day 15 (34.4%). Genus *Leuconostoc* was represented mainly by *Leuconostoc mesenteroides*, which displaced enterococci by day 30, becoming the predominant OTU at that point (75.2%). In addition, *Leuconostoc kimchii* was detected at a relative abundance below 1% at day 30. Principal component analysis (PCA) of relative abundances at genus level for untreated controls at different storage times revealed that storage times from 0 to 15 days had strong or very strong ($r = 0.680$–0.996) and significant ($P < 0.05$) positive correlations and similar microbial populations in which genus *Pantoea* was predominant (Fig. 3A). In contrast, storage time 30 was not correlated with the rest of control storage times ($r = -0.041$ to -0.201; $P > 0.05$), indicating strong changes in microbial composition associated to a predominance of genus *Leuconostoc* (Fig. 3A).

Addition of enterocin AS-48 caused a decrease in the relative abundance of *Firmicutes* in the surviving fraction recovered on TSA plates. This was specially so for OTUs belonging to members of genus *Enterococcus* (*E. casseliflavus, E. gallinarum*), which only were detected at the beginning of storage period (Fig. 2). Among *Proteobacteria,* Genus *Pantoea* (*P. agglomerans*, *P. vagans*) had highest relative abundance early after AS-48 treatment, and PCA analysis (Fig. 3B) revealed a significant ($P < 0.05$) very strong correlation ($r = 0.893$) for storage times 0 and 1 associated with the predominance of this genus. Enterocin AS-48 treatment was also significantly ($P < 0.05$) and very strongly correlated ($r = 0.906$–0.968) with controls at storage times 0 and 1. However, the microbial composition in the cultured fraction from AS-48 treated samples did change in a different way than controls during storage, and *Pantoea* was displaced at days 7 and 15 of storage by genus *Serratia* (mainly *Serratia pymuthica*) followed by genera *Erwinia* (mainly *Erwinia persicina* and to a less extent *Erwinia aphidicola*) and *Yersinia* (*Yersinia ruckeri*). These two storage times also showed very strong correlation ($r = 0.959$) according to PCA analysis (Fig. 3B). Storage time 30 did not correlate with previous samplings ($r = 0.076$ to -0.121) and showed strong changes in microbial composition: OTUs with highest relative abundance belonged to genera *Pseudomonas* (represented mainly by *Pseudomonas psycrophila*), followed by *Erwinia* (mainly *E. persicina*), *Raoultella* (*Raoultella terrigena*), and *Enterobacter* (*Enterobacter aerogenes*).

The HHP treatment greatly decreased the relative abundance of OTUs belonging to *Proteobacteria* compared to *Firmicutes* after treatment and also at day 1 (Fig. 2), in which *Firmicutes* were the predominant OTUs detected (followed by *Actinobacteria*) from growth on TSA plates. Genus *Escherichia* (*E. fergusonii*) was the OTU with highest relative abundance (69.9%) at time 0, but it was not detected at other sampling points. Genus *Bacillus* was clearly the predominant OTU from the HHP treated samples on day 1 of storage (*Bacillus stratosphericus, Bacillus firmus*), followed by genus *Micrococcus* (represented by *Micrococcus luteus*). PCA analysis (Fig. 3C) revealed a very weak ($r = 0.140$) and non-significant ($P > 0.05$) correlation between storage times 0 and 1 (with genera *Bacillus* and *Escherichia* as predominant associated OTUs). For storage times 0 and 1, HHP treatment did not correlate with untreated controls ($r = -0.140$) or with AS-48 treatments ($r = -0.244$), indicating that this treatment had a strong impact on the bacterial community. However, the microbial composition of HHP-treated samples changed from day 7 on, and the relative abundance of *Firmicutes* decreased while *Proteobacteria* became the predominant group. Genus *Pantoea* (which was found at levels below 1% at days 0 and 1) became the predominant OTU in growth recovered from the treated samples from days 7 to 30 after treatment. OTUs with highest relative abundance from this genus were *P. agglomerans* and *P. vagans*, switching in order of relative abundance during late storage. Genus *Leuconostoc* (mainly *L. kimchii*) was also a relevant group in samples from day 15. PCA analysis revealed very strong ($r = 0.993$–0.999) and significant ($P < 0.05$) correlation for storage times 7–30 (associated with genus *Pantoea*), and also strong or very strong significant ($P < 0.05$) correlations ($r = 0.992$ or 0.750) with untreated controls at days 7 and 15, indicating recovery of bacterial populations initially affected by treatment.

For the combined treatments of AS-48 and HHP, no DNA could be recovered from sampling points at time 0 and day 1 since viable cells at these two points were below the detectable levels of 10 CFU/g. On day 7 of storage, *Firmicutes* were the main group detected (Fig. 2). These were represented in more than 99% by OTUs from genus *Bacillus* (*B. firmus*). However, after days 15 and 30, no OTUs belonging to genus *Bacillus* were detected, and *Proteobacteria* became the predominant group detected from growth on TSA plates. *Proteobacteria* were represented at day 15 mainly by *P. vagans, Erwinia billingiae* and *P. agglomerans* (by order of relative abundance), shifting in order at day 30 (*P. agglomerans, P. vagans, E. billingiae*). Some leuconostocs (mainly *L. mesenteroides*, followed by *L. kimchii* in relative abundance) were also detected at day 15, but altogether they represented less than 1% of OTUs by day 30. PCA analysis (Fig. 3D) clearly revealed a very strong ($r = 0.992$) and significant ($P < 0.05$) correlation for storage times 15 and 30 (associated with genus *Pantoea*) as opposed to storage time 7 (associated with genus *Bacillus*). The combined treatment HHP-AS-48 had strongest impact on bacterial populations after treatment and early during storage, as shown by the negative correlations observed at day 7 with controls and with the single treatments ($r = -0.069$ to -0.244). However, the combined treatment correlated very strongly ($r = 0.992$–0.993) and significantly ($P < 0.05$) with the single HHP treatment at storage times 15 and 30, indicating that the late recovery of bacterial populations in the combined treatments was not affected by bacteriocin.

4. Discussion

Surface microbiota is often the main source of microbial contamination of processed fruits and vegetables. In the present study, cherimoya

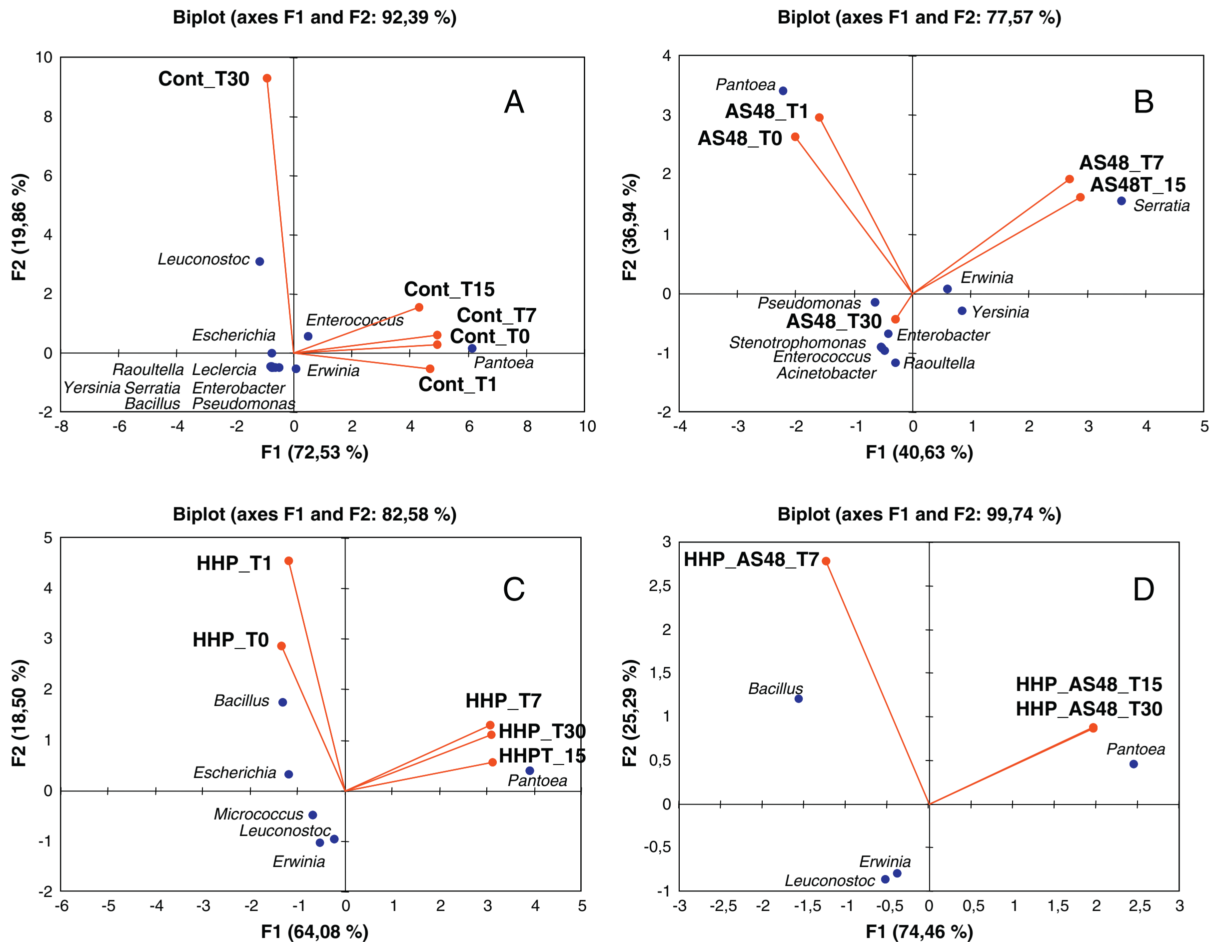

Fig. 3. Biplot principal component analysis of sample variables (storage times) and scores (genus relative abundance) of untreated controls (A) and samples treated with enterocin AS-48 (B), high hydrostatic pressure (C) or AS-48 in combination with high hydrostatic pressure (D).

pulps were inoculated with surface-recovered microbiota at 6.4 $\log_{10}$ CFU/g final cell concentration to simulate a worst-case scenario of contamination during pulp preparation and also to ensure that logarithmic reductions of at least five log cycles could be measurable after application of treatments. A high density inoculum could also facilitate the study of changes in the culturable surviving fraction during storage of the treated samples. Nevertheless, in our experience, microbial load in pulps under normal contamination conditions would be much lower, of approximately between 2 and 4 $\log_{10}$ CFU/g (unpublished results).

In the present study, we used 16S rRNA gene pyrosequencing to evaluate the microbial diversity in the surviving fraction able to grow on TSA plates from cherimoya pulp artificially contaminated with surface microbiota, both after treatments by HHP, enterocin AS-48 or a combination of the two, and during storage. Clearly, the results obtained from pyrosequencing are limited by the experimental conditions used in the study, since only the bacterial fraction that was selected by the TSA and growth conditions could be analysed. Therefore, the results can be biased by growth conditions and should not be extrapolated to microbial composition in samples. Keeping in mind that this approach does not necessarily reflect the abundance of taxa in the original samples, it overcomes the problem of interference from DNA of dead cells after treatments. Pyrosequencing is becoming widely used for analysis of microbial communities in spite of the limitations of using short gene fragments for identification at species levels. Nevertheless, the sequence similarities obtained in the present study were in the range of 97–100%. Phylum *Proteobacteria* was the main bacterial group represented in the microbiota from cherimoya pulp recovered on TSA plates, followed by *Firmicutes*. *Actinobacteria* were only detected at relatively high abundances in two treated samples. OTUs belonging to *Bacteroidetes* and *Cyanobacteria* only appeared in a few samples, and always had very low relative abundances not higher than 0.08%. Remarkably, *Enterobacteriaceae* were the main bacterial group recovered from the artificially contaminated cherimoya pulp. Members of Fam. *Enterobacteriaceae* include saprophytic, commensal, opportunistic pathogens as well as pathogens causing severe infections on humans and animals. One recent study also based on pyrosequencing showed that Fam. *Enterobacteriaceae* had the highest relative abundances in the microbiota of various vegetable foods including bean and alfalfa sprouts, spinach, lettuce, tomato, pepper and strawberries (Leff and Fierer, 2013). The main representatives of *Enterobacteriaceae* recovered from the untreated cherimoya pulp belonged to genus *Pantoea*. Putative *Pantoea* sp. was also a particularly abundant OTU on many of the produce types harboring large proportions of *Enterobacteriaceae* (such as bean sprouts, spinach and pepper). *Pantoea* spp. are frequently isolated from a wide range of ecological niches and have various biological roles, as plant epi- or endophytes, biocontrol agents, plant-growth promoters or as pathogens of both plant and animal hosts including humans (De Maayer et al., 2012). In particular, *P. agglomerans* is an unusual cause of human disease typically associated with thorn prick injuries, contaminated parenteral fluids and debilitating patient conditions (Lalas and Erichsen, 2010; Shubov et al., 2011). Nevertheless, two commercial preparations based on *P. agglomerans* (BlossomBless™ and Bloomtime™) and one based on *P. vagans* (BlightBan C9-1™) are registered as biocontrol agents. The high abundance detected for OTUs from these two species in cherimoya pulp from the present study could possibly be due a natural adaptation of pantoeae to this tropical fruit

and/or their intended use as biocontrol agents in farming operations. There is an ongoing debate on the hazards to human health of *Pantoea* strains used as biocontrol agents (which are included in the biosafety level 2 as opportunistic pathogens), although it also seems that many of the clinical isolates may have been misclassified as *Pantoea* (Rezzonico et al., 2009). The presence of *Pantoea* in foods has also been questioned because some isolates may carry antibiotic or biocide resistance traits (Aibinu et al., 2012; Blaak et al., 2014; Schwaiger et al., 2011; Fernández-Fuentes et al., 2014). Although sequences identities obtained for *Pantoea* species in our study were very high (97 to 100%), additional work needs to be carried out involving isolation and confirmative identification of the putative *Pantoea* species reported here and on their antibiotic susceptibility background.

Human enteropathogens such as *Escherichia coli* or *Salmonella enterica* were not detected as significant OTUs in the TSA-grown bacterial biomass from control pulps, and only *E. fergusonii* was detected at day 30 at a rather high abundance. OTUs belonging to other members of *Enterobacteriaceae* (*E. aphidicola, E. persicina, Enterobacter kobei*, *Y. ruckeri*, *Serratia plymuthica* and *Leclercia adecarboxylata*) also detected at low percentages in early stages did not increase during storage, although they illustrate the broad microbial diversity that can be found on cherimoyas. Interestingly, enterococci (mainly *E. gallinarum* and to a much less extent also *E. casseliflavus*) could greatly increase in relative abundance in the cultured microbiota from untreated cherimoya pulp during storage. The motile enterococcal species *E. gallinarum* and *E. casseliflavus* have been known to be associated with plants and fruits (Micallef et al., 2013; Ong et al., 2014), but also can be found in the intestine of animals and on meats (Byappanahalli et al., 2012).

One of the objectives of the present study was to determine the effect of treatments with enterocin AS-48 and high hydrostatic pressure (HHP) singly or in combination on the TSA-culturable microbial populations in cherimoya pulp and their possible influence on microbiota changes during storage. Enterocin AS-48 has a wide inhibitory spectrum against Gram-positive bacteria, but in the absence of other stress factors it has very low effect on Gram-negatives (Maqueda et al., 2004). This difference in inhibitory activity was clearly seen when cherimoya pulp was supplemented with AS-48, since it inhibited proliferation of the main *Firmicutes* detected in controls during storage (mainly enterococci). As a matter of fact, plate count assays indicated that the single enterocin AS-48 treatment did not inhibit proliferation of the microbiota, and the differences in viable cell counts between controls and enterocin-treated samples were not statistically significant. However, the samples supplemented with AS-48 did show differences in the predominant OTUs from *Proteobacteria* during storage compared with the untreated controls (mainly at days 7, 15 and 30). This could be an indirect effect of the inhibition of enterococci by bacteriocin, or maybe the bacteriocin also has growth inhibitory effects on some of the Gram-negatives found in the inoculated cherimoya pulp. In a previous study carried out by using denaturing gel electrophoresis (DGGE) analysis of the microbial community, it was shown that application of a washing treatment with enterocin AS-48 induced changes in the microbial community of soybean sprouts that involved a decrease in the population of *Pantoea* sp., *E. hermannii* and *Enterobacter* sp., and an increase in the populations of *Serratia* sp. and *S. plymuthica, Enterococcus* sp. and *Leuconostoc inhae* (Cobo Molinos et al., 2009). These changes clearly resemble the ones reported in the present study, at least for *Pantoea* sp. and *S. plymuthica.* Altogether, these results illustrate how the effect of adding bacteriocins in foods systems affects the whole microbial community and not just the target bacteria. Considering that foods are complex ecosystems in which the different microbial populations interact with each other (by means of cooperation, competition for the nutrients, ammensalism, etc.), factors that influence single microbial populations are also expected to have an impact on the whole microbial community.

Although HHP treatment reduced viable cell counts of samples by 5fivelog cycles, not all microbial populations detectable on TSA under the incubation conditions tested in our study seemed to be affected equally. Notably, the increase in relative abundance of endosporeformers observed early after treatments could be explained by the fact that bacterial endospores are resistant to the HHP treatment applied. Furthermore, endospores can be induced to germinate by HHP treatments, and, in the absence of competitors, may proliferate and become a dominant population after treatment (Rastogi et al., 2007; Rendueles et al., 2011). Species of genus *Bacillus* are known for their capacity to produce an array of antimicrobial substances (Abriouel et al., 2011) and extracellular enzymes, which may altogether facilitate substrate utilization and displacement of competitors. Interestingly, among the main OTUs detected for endosporeformers were *B. firmus* and *Bacillus plakortidis,* both of them alkali-tolerant bacteria that have also been isolated from seawater environments (Borchert et al., 2007; Geng et al., 2014). The tropical coast of Southern Spain is known for the intensive farming of cherimoya trees. It could be speculated that endospores of these bacteria could be transported to cherimoya plantations by sea winds, but the possibility that they may also live as epiphytic bacteria cannot be ruled out. Endospore-forming bacilli known to cause food poisoning like *Bacillus cereus* were not detected. Nevertheless, the potential toxin production by *B. firmus* has been described (Taylor et al., 2005). During further storage of the treated samples and increase in viable cell counts, it could also be observed that the residual populations of *Pantoea* sp. surviving HHP treatments were able to rapidly overgrow endosporeformes, and that surviving leuconostocs also proliferated during late storage. Leuconostocs are well adapted to ferment vegetable substrates and grow at low temperatures. Under proper selective conditions, leuconostocs displace epiphitic microbiota and carry out a lactic acid fermentation. However, the results obtained clearly indicated that under cold storage of cherimoya pulp epiphitic *Pantoea* were far more competitive than leuconostocs. This was also apparent in the control cherimoya pulp, in which leuconostocs only proliferated at the end of storage period, when nutrients for *Enterobacteriaceae* were most likely exhausted.

Results from the present study indicate application of HHP treatment in combination with enterocin AS-48 was the most effective treatment for improving the microbiological quality and safety of cherimoya pulp, since it had a much more pronounced effect than the single treatments in keeping viable cell concentrations at low levels during storage for at least 15 days and reducing the populations of both Gram-positive and Gram-negative bacteria. In previous studies, application of enterocin AS-48 in combination with HHP treatments improved the inactivation of *S. enterica* in fuet and *Staphylococcus aureus* in rice pudding (Ananou et al., 2010; Pérez-Pulido et al., 2012). Synergistic effects between other bacteriocins and HHP treatments have been reported in several studies (reviewed by Gálvez et al., 2008; Kalchayanand et al., 1994). The mechanism of action of enterocin AS-48 relies on permeabilization of the bacterial cytoplasmic membrane, leading to a rapid collapse of the cytoplasmic membrane potential (Gálvez et al., 1991). Bacteriocin addition also has other secondary effects like induction of cell autolysis. The synergistic activities reported in previous studies as well as those observed for enterocin AS-48 in the present study could be due to the damaging effect of bacteriocins on the cell wall and cell membrane in the sensitive bacteria together with an increased sensitivity of the cells, injured by pressurization, to bacteriocins.

Altogether, results from the present study clearly indicate the complexity of microbial populations derived from cherimoya surfaces and how these populations may change during storage of contaminated cherimoya pulp. Clearly, food preservation treatments induce changes in the food microbial populations that depend on the type of treatment.

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Changes in microbial diversity of brined green asparagus upon treatment with high hydrostatic pressure

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ABSTRACT

The application of high hydrostatic pressure (HHP, 600 MPa, 8 min) on brined green asparagus and the changes in bacterial diversity after treatments and during storage at 4 °C (30 days) or 22 °C (10 days) were studied. HHP treatments reduced viable cell counts by 3.6 log cycles. The residual surviving population did not increase during storage at 4 °C. However, bacterial counts significantly increased at 22 °C by day 3, leading to rapid spoilage. The microbiota of green asparagus was composed mainly by Proteobacteria (mainly *Pantoea* and *Pseudomonas*), followed by *Firmicutes* (mainly *Lactococcus* and *Enterococcus*) and to a less extent *Bacteroidetes* and Actinobacteria. During chill storage of untreated asparagus, the relative abundance of Proteobacteria as well as *Enterococcus* and *Lactococcus* decreased while *Lactobacillus* increased. During storage of untreated asparagus at 22 °C, the abundance of *Bacteroidetes* decreased while Proteobacteria increased during late storage. The HHP treatment determined a reduction of the Proteobacteria both early after treatment and during chill storage. In the HHP treated samples stored at 22 °C, the relative abundance of *Pseudomonas* rapidly decreased at day 1, with an increase of *Bacteroidetes*. This was followed by a marked increase in Enterobacteriaceae (*Escherichia*) simultaneously with increase in viable counts and spoilage. Results from the study indicate that the effect of HHP treatments on the viability of microbial populations in foods also has an impact on the dynamics of microbial populations during the storage of the treated foods.

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1. Introduction

Asparagus (*Asparagus officinalis* L.), belonging to the Liliaceae family, is a vegetable whose edible organ is the immature stem called spear (Kanno and Yokoyama, 2011). Spears are originated from an underground root crown and then harvested when emerge from the ground. Asparagus are achieving more popularity because of their unique taste and texture (Lau et al., 2000). They have diuretic properties by their content of potassium and asparagine, and stimulate intestinal transit due to their high cellulose concentration. Asparagus are rich in proteins, sulfur and volatile essential oils and are a source of folate and B vitamins. They also contain flavonoids and other phenolic compounds (Makris and Rossiter, 2001; Nindo et al., 2003; Rodríguez-Arcos et al., 2002) and possess a variety of biological properties, such as being antioxidants, immunostimulants, anti-inflammatory, antihepatotoxic, antibacterial, antioxytocic, and reproductive agents (Negi et al., 2010).

The spear from green asparagus has a high percentage of water, is actively growing and has a high respiration rate, thus it can deteriorate rapidly after harvest (King et al., 1990) depending on storage temperature (Brash et al., 1995; King et al., 1988). Due to green asparagus growth on soil surface, it is prone to high microbial contamination from the soil, irrigation water and fertilizers. There are few studies of the bacterial epiphyte microbiota in asparagus, as most diseases are caused by the fungus *Fusarium* sp.

In terms of food quality and safety, high hydrostatic pressure treatment (HHP) has emerged as an alternative non-thermal process in food preservation. HHP is widely used by the food industry, including the processing of fruits and vegetables for the inactivation of pathogens and spoilage microorganisms and certain enzymes, as well as extending the shelf-life of these products (Balasubramaniam et al., 2008; Rastogi et al., 2007; Rendueles et al., 2011). Applied at room temperature, high pressure destroys the bacterial vegetative cells that spoil the foodstuffs, with minimal changes in the organoleptic properties (Zhou et al., 2010). However, resistance to HHP treatments is variable, depending on the type of microorganism, the physiological state and the food matrix. One main concern is regrowth of bacterial populations surviving the HHP-treated samples during storage. Storage and temperature are two critical parameters affecting growth of microorganisms and therefore

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limiting the shelf-life of the treated foods. Therefore, the study of the changes in the microbial populations upon HHP treatment and during storage of the treated products can be very important to understand the power of the treatment and to work out the best storage conditions. The population dynamics can be monitored with a high-level of sensitivity by sequencing-based, culture-independent approaches (Ercolini, 2013) in order to look at dominant as well as minor microbial populations. The use of culture-independent high-throughput sequencing (HTS) enables the study of the microbial ecology and taxonomic diversity at a high resolution and can potentially reveal unculturable microbiota (Ercolini, 2013; Cocolin and Ercolini, 2015). HTS can provide insights into the microbiota of raw materials and sources of contamination, and help us to better understand dynamic microbial processes such as food fermentation, spoilage, or changes in microbial populations during the shelf life of processed foods (Cocolin and Ercolini, 2015). HTS has been applied to study the bacterial communities associated with the surfaces of fruits and vegetables, including apples, grapes, lettuce, mushrooms, peaches, peppers, spinach, sprouts, strawberries and tomatoes (Leff and Fierer, 2013) or spinach (Lopez-Velasco et al., 2013), to investigate the presence of opportunistic pathogens on the phyllosphere of rucula (Berg et al., 2014), or in the study of microbial dynamics during storage of cherimoya pulp processed under different conditions (Pérez Pulido et al., 2015).

The purpose of the present study was to determine the effect of high hydrostatic pressure treatment on the microbiological quality of green asparagus immersed in a brine, stored under refrigeration and at room temperature. The spears were inoculated with their own epiphytic microbiota to simulate a critical incident of contamination during handling and food processing. Changes in microbial diversity during storage of the treated samples were monitored by pyrosequencing in order to gain insights into the microbial ecology of this food during storage.

2. Materials and methods

2.1. Sample preparation

A total of 10 bundles of green asparagus (250 g each) from different Andalusian harvests (provinces of Jaén, Córdoba and Granada) were purchased at ten different stores in the province of Jaén and stored refrigerated until processed (no more than 24 h). For each bundle, the 10 cm distal part (which is harder and closer to the ground) was cut and discarded, and the remaining 10–15 cm apical and central part was introduced into a sealable plastic bag and mixed by hand for 2 min with 10 ml of sterile buffered peptone water (0.5 M) in order to recover its surface microbiota. The obtained bacterial cell suspension was centrifuged in 50 ml Falcon test tubes at 4000 $\times g$ for 30 min, and the resulting pellets were collected together in one Eppendorf tube, centrifuged at 12,000 rpm for 10 min. From this washing suspension, serial dilutions were plated on TSA to calculate the containing cell concentration. One-milliliter aliquots of the obtained cell suspension were mixed with 20% of glycerol and stored at −80 °C.

Before application of HHP treatments, spears from fresh, unwashed green asparagus were cut into pieces of 4 cm in length with a sterile knife under aseptic conditions, having fragments that contain the apical area and others that contain the central soft area. For each sample contained in a zip lock bag, 4 pieces of cut spears (2 with apex and 2 with intermediate soft zone) were introduced, with an average weight of 8 g each sample and immersed in 8 ml of sterile brine composed by distilled water supplemented with 2% NaCl and 0.15% citric acid (pH 2.7). This brine has the same composition as brine used for industrial canning of green asparagus. Aerobic mesophilic counts in the unwashed green asparagus were in the range of 5 to 6 log CFU/g. In order to simulate a worst-case scenario of heavy contamination, spears immersed in brine were inoculated (1%, vol/vol) with the pool of epiphytic microbiota obtained as described above, to provide a final cell concentration of 6.8–7.0 $\log_{10}$ CFU/ml in brine. Four batches of asparagus samples (each one in duplicate) inoculated with epiphytic microbiota as described above were used for treatments: two served as controls and the other two were treated by high hydrostatic pressure (HHP). Two of the batches (A1, untreated controls and A2, treated by HHP, all of them in duplicate) were stored at 4 °C. Sampling for viable cell counts and DNA extraction was done on days 0 (immediately after treatment), 1, 7, 15 and 30. The remaining two batches (consisting also of untreated controls –B1– and samples treated by HHP –B2–, in duplicate) were stored at 22 °C, with sampling at days 0 (immediately to treatment), 1, 3, 7 and 10. The pH of brines was measured with a pH meter (Crison Instruments, S.A., Barcelona). Gas production (swelling of bags) or abnormal odor was annotated at each sampling point.

2.2. HHP treatment

Application of HHP treatment was done with a Stansted Fluid Power Ltd HHP equipment (SFP, Essex, UK) suited with a 2.5 l vessel and provided with a hydraulic pump system, capable of operate up to 700 MPa under non-thermal conditions (temperature range is between 23 and 27 °C). Pressurization fluid consisted of distilled water supplemented with 10% propyleneglycol. Asparagus samples were pressurized at 600 MPa for 8 min at room temperature. This was the pressure/time combination that achieved greatest microbial inactivation in preliminary trials. The come-up speed was 75 MPa/min and decompression after the process was immediate.

2.3. Sample analysis

At each sampling point, duplicate bags of controls and HHP-treated asparagus were homogenized in a stomacher for 30 s at maximum speed. Then, 1.5 ml from each bag was transferred to a sterile Eppendorf test tube. A 0.5 ml portion of this suspension was serially-diluted in sterile 0.85% NaCl saline solution and plated in duplicate on Tryptone Soy Agar (TSA, Scharlab, Madrid) plates. The plates were then incubated for 24 h at 30 °C for viable cell counts. The remaining cell suspension was used for DNA extraction and analysis as described below.

2.4. Treatment with propidium monoazide and DNA extraction

Duplicate bacterial cell suspensions (1 ml each) recovered from samples as described above were centrifuged at 12,000 rpm for 10 min and the resulting pellets were resuspended with sterile saline solution and mixed (1 ml final volume). Samples were treated with Propidium Monoazide (PMA™, Biotium, UK) to block subsequent PCR amplification of the genetic material from dead cells as described by Elizaquivel et al. (2012). Briefly, a 20 mM PMA stock solution dissolved in 20% dimethylsulfoxide (DMSO) and stored at −20 °C in the dark, was added to the samples at 50 μM final concentration. Following PMA addition, samples were incubated for 5 min in the dark, at room temperature, with occasional mixing to allow reagent penetration. Thereafter, samples were exposed to light for 15 min using a photo-activation system (Led-Active Blue, Ingenia Biosystems, Barcelona, Spain). After photo-induced cross-linking, the cells were centrifuged at 12,000 rpm for 10 min, washed twice with sterile saline solution and once with sterile molecular-grade water. The resulting sediment of PMA-treated cells was used for DNA preparation. Briefly, total DNA was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid), following instructions provided by the manufacturer. DNA recovered from duplicate samples was then pooled into a single sample. DNA concentration and quality was measured with a NanoDrop spectrophotometer (Thermo Scientific, U.K.).

2.5. Amplicon library preparation and sequencing

DNA samples were analyzed to study the microbial diversity by a model culture-independent amplification technique, consisting on a

pyrosequencing of the variable regions V1–V3 of the 16S rRNA gene. Amplification was carried out by using primers Gray28f and Gray519r amplifying a 520 bp fragment (Ercolini et al., 2012). In forward primer, 454-sequencing adaptors (Roche, U.S.A.) were included, followed by a Multiplex Identifier (MID), a 10 bp specific sequence for each sample.

Each PCR mixture (final volume of 50 μl) containing template DNA at 50 ng/μl concentration, 25 μM of each of four deoxynucleotide triphosphates (dCTP, dGTP, dATP and dTTP), 0.4 mM of each primer, 2.5 mM $MgCl_2$, 5 μl of 10× PCR buffer and 2.5 U of native *Taq* polymerase (Invitrogen, Milan, Italy). The following PCR conditions were used: an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 45 s and extension at 72 °C for 5 min, and a final extension at 72 °C for 7 min. To verify the amplified fragment, PCR products were run on an agarose gel (1.5% agarose) with a molecular weight marker of 1 kb, for 30 min at 100 V.

After agarose gel electrophoresis, PCR products were purified twice with Agencourt AMPure purification kit (Beckman Coulter, Milan, Italy), and quantified using the technique QuantiFluor™ (Promega, Milano, Italy) with PicoGreen. DNA amplicons were combined in a single tube and pooled in equimolar concentration of each sample. Pyrosequencing was carried out on a GS Junior platform (454 Life Sciences, Roche Diagnostics, Italy), according to manufacturer's instructions by using Titanium chemistry.

2.6. Bioinformatics and data analysis

Raw reads were first filtered according to 454 processing pipeline. The resulting sequences were analyzed using the QIIME 1.8.0 software (Caporaso et al., 2010). In order to ensure a higher level of accuracy in terms of Operational Taxonomic Unit (OTU) detection, *denoising* was applied after the split_library Script performed by QIIME. Sequences were excluded from analysis if they had an average quality score below 25, if they were shorter than 300 bp, if they had ambiguous bases to remove overlapping or defective sequences, and the singletons were excluded. OTUs defined by a similarity of 97% were picked using the *Uclust* method (Edgar, 2010), and the representative sequences were submitted and classified to obtain the taxonomy assignment and the relative abundance of each OTU, using the Greengenes 16S rRNA gene database (McDonald et al., 2012). The alpha- and beta-diversity were evaluated through QIIME as previously described (De Filippis et al., 2013, 2014). To test for significant differences in alpha diversity parameters, the compare_alpha_diversity.py script by QIIME was employed.

3. Results

3.1. Effect of HHP treatment on total viable cell counts

Asparagus inoculated with its epiphytic microbiota, treated or not by HHP, were stored at 4 °C for 30 days or at 22 °C for 10 days to simulate a worst-case scenario of temperature abuse. In controls (not treated by HHP) stored at 4 °C, viable cell counts increased gradually from 6. 8 log CFU/ml at day 0 to 8.2 log CFU/ml at day 15 or 9 log CFU/ml at day 30 (Fig. 1A). The HHP treatment significantly ($P < 0.05$) reduced viable counts by 3.6 log cycles, and the surviving fraction did not increase significantly ($P > 0.05$) during the whole storage period (Fig. 1A). The pH of untreated controls increased from 2.7 at time 0 to pH 3.0 at day 1, and then gradually to pH 5.0 at the end of storage period (Fig. 1A). In samples treated by HHP, pH increased to 3.1 at day 1, but then it remained quite stable and never increased above 3.9 during storage. Compared with untreated controls, the pH of HHP-treated samples was significantly lower ($P < 0.05$) for days 15 and 30 of storage.

Storage of asparagus samples at 22 °C resulted in much faster microbial growth, reaching 8.2 log CFU/ml at day 1 and 9.9 log CFU/ml at day 10 (Fig. 1B). In samples treated by HHP, viable cell counts were significantly lower than the untreated controls for the first three days of

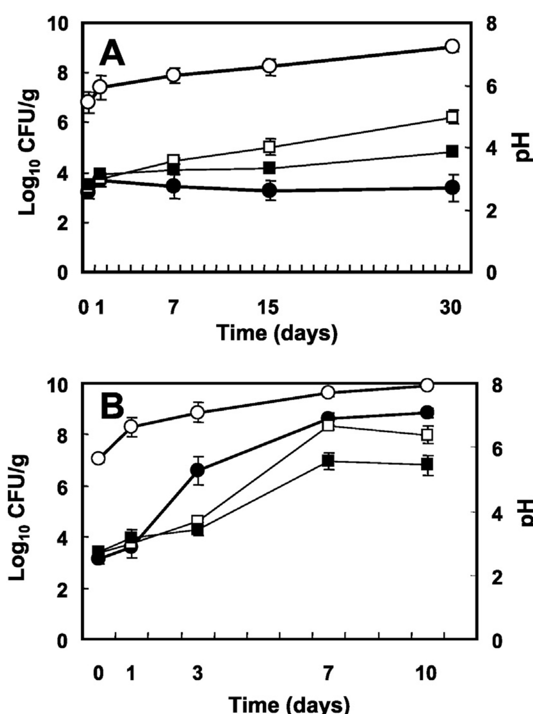

Fig. 1. Total viable cell counts of green asparagus stored in brine at 4 °C (A) or 22 °C (B). Viable counts (circles) and pH (squares) of controls (open symbols) and samples pressurized at 600 MPa for 8 min (closed symbols) are shown.

incubation, and non-significantly lower at days 7 and 10 (Fig. 1B). The pH of untreated controls increased moderately (from 2.7 to 3.7 within the first three days of storage, but it raised considerably to 6.7 at day 7 or 6.4 at day 10 (Fig. 2). The pH of HHP-treated samples was not significantly different ($P > 0.05$) from the untreated controls for the first three sampling points, but it was significantly lower ($P < 0.05$) at days 7 and 10. Clear signs of spoilage (gas formation and offensive odor) were observed in the untreated controls by day 3, while in the HHP-treated samples spoilage was observed at day 7 and afterwards.

3.2. Impact of HHP treatment and storage temperature on bacterial diversity

3.2.1. Bacterial diversity of green asparagus

The bacterial diversity recovered from brined asparagus, pressurized or not at 600 MPa and stored at different temperatures, was determined by pyrosequencing of the V1–V3 variable regions of the 16S rRNA gene. The number of OTUs, the Chao1 and Shannon indices, and Good's estimated sample coverage (ESC) are reported in Table 1. A total of 93,506 sequence reads were obtained after the filtering protocol, with an average fragment length of 491 bp calculated after primer removal and an average number of reads per sample of 4675. The estimated sample coverage was of at least 98%. The lowest diversity indices were observed in samples treated by HHP during late storage at 22 °C (Table 1).

After OTU assignment, those with a relative abundance lower than 1% were not considered further. The results obtained indicated that green asparagus had a diverse microbiota (Fig. 2). In control samples without pressurization, the phylum Proteobacteria had highest relative abundance (60.88%), followed by *Firmicutes* (29.02%) and *Bacteroidetes* (9.5%).

The most abundant taxonomic group was Gammaproteobacteria, in which family Enterobacteraceae had the highest relative abundance (48.67%). The main genera identified were *Pantoea* (*Pantoea ananatis*), *Rahnella*, *Erwinia*, *Raoultella* and *Serratia*. Second in abundance within Gammaproteobacteria was Fam. Pseudomonadaceae (7.44%), represented by genus *Pseudomonas*. Most *Firmicutes* belonged to class *Bacilli*

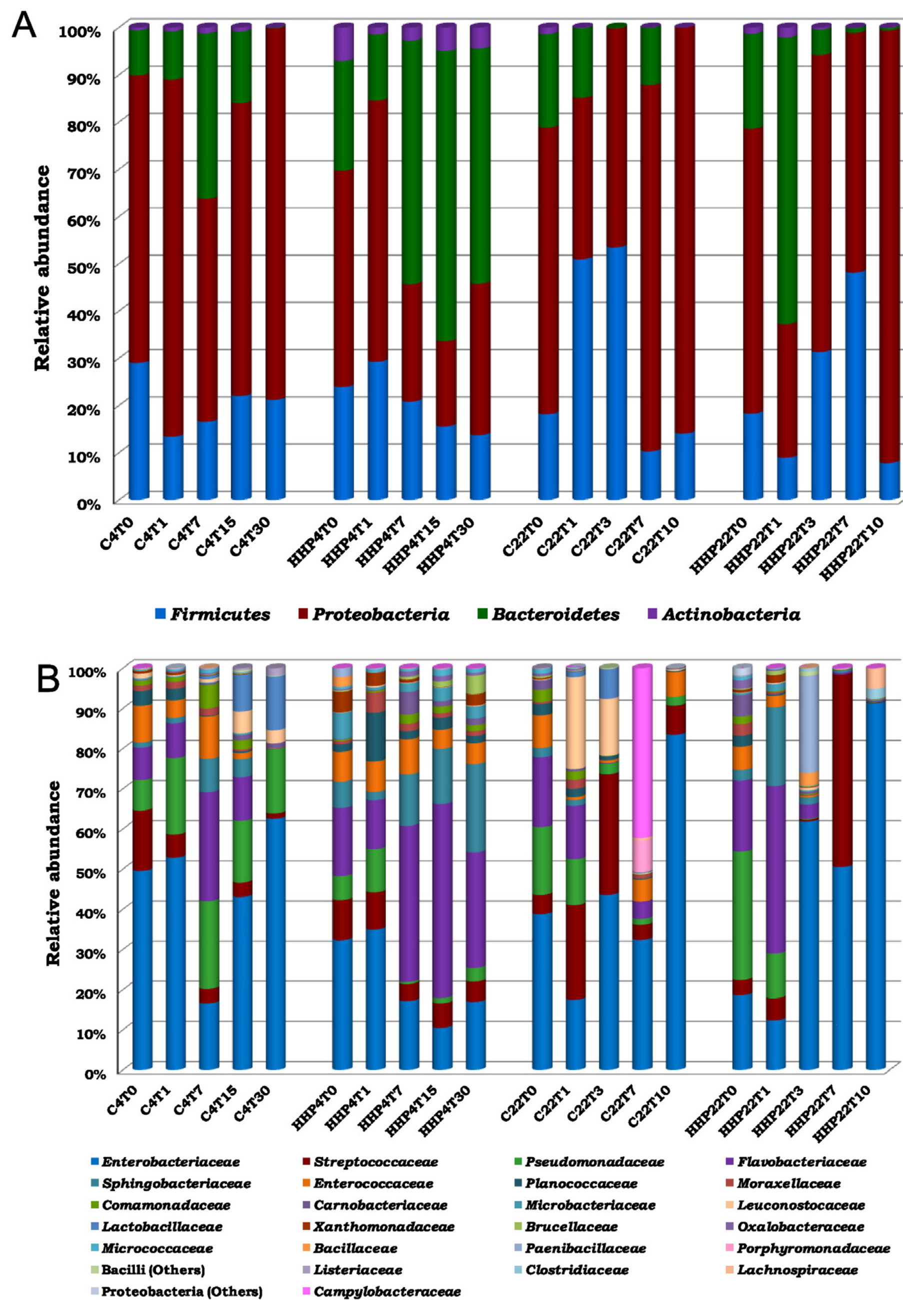

Fig. 2. Relative abundance of OTUs at *Phylum* (A), *Family* (B) or *Genus* (C) taxonomic levels based on 16S rRNA gene pyrosequencing analysis of DNA from green asparagus inoculated with its own epiphytic microbiota and packed in brine. The packed asparagus were treated or not by high hydrostatic pressure at 600 MPa for 8 min (HHP) and stored at 4 °C for 30 days or at 22 °C for 10 days.

(28.90%), mainly to Fam. Streptococcaceae (14.70%). The main genera detected in class *Bacilli* were *Lactococcus* (with *Lactococcus lactis*, *Lactococcus garvieae* and *Lactococcus raffinolactis* as identified species), *Enterococcus*, *Kurthia* (*Kurthia gibsonii*) and *Leuconostoc*. *Bacteroidetes* were represented mainly by Fam. Flavobacteriaceae (7.91%), and Sphingobacteriaceae (1.29%).

3.2.2. Changes observed during chill storage

During storage of non-pressurized asparagus at 4 °C, Proteobacteria were still the most abundant, changing from 47.05% at day 7 to 78.59% at day 30 (Fig. 2A). Fam. Enterobacteriaceae was also the most important in this group during storage, with *Rahnella* and *Raoultella* increasing and *Pantoea* decreasing with the time. Remarkably, *Pseudomonas* showed an increase in relative abundance and persisted during storage (Fig. 2B). *Bacteroidetes* increased (mainly Flavobacteriaceae) in relative abundances at days 7 and 15 of storage and it is worth noting that at day 15 such increase corresponded to a decrease in Enterobacteria. The proportion of *Firmicutes* did not change considerably during storage. However, endospore formers (*Bacillus* and *Clostridium*) were found in very low proportion of less than 0.1% during storage at 4 °C.

The HHP treatment induced changes in the relative abundance of the different bacterial groups compared with the untreated controls (Fig. 2). In fact, the relative abundance of Proteobacteria decreased, while *Bacteroidetes* and Actinobacteria increased. This early change observed right after treatments was important also during storage, when the relative abundance of Enterobacteriaceae (mainly *Raoultella* and *Rahnella*) as well as *Pseudomonas* further decreased, while *Bacteroidetes* increased. Among *Firmicutes*, *Lactococcus* was the most stable in terms of relative abundance during chill storage of the HHP-treated samples, and *Kurthia* (*K. gibsonii*) increased in relative abundance early during storage.

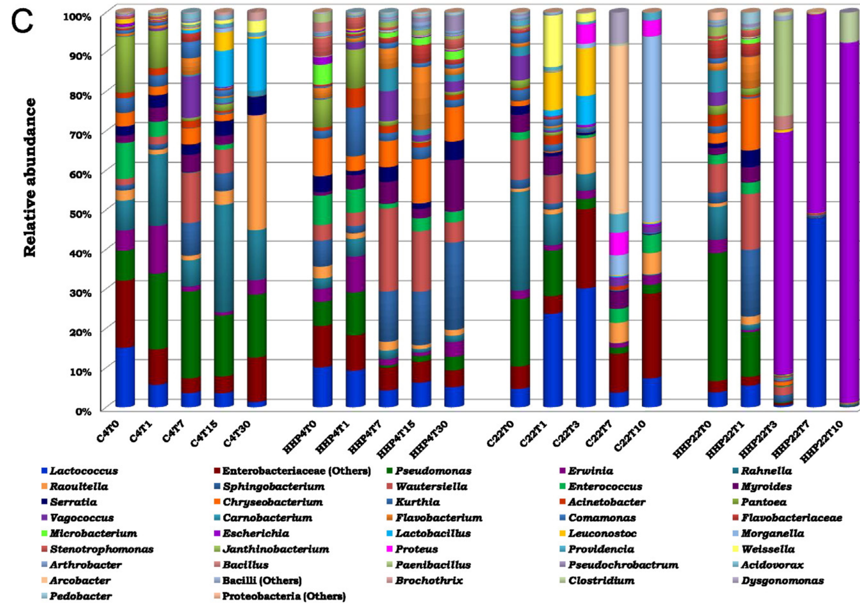

Fig. 2 (*continued*).

3.2.3. Changes observed during storage at 22 °C

In control samples stored at 22 °C, Pseudomonadaceae and Flavobacteriaceae decreased markedly after day 1 (Fig. 2C). Late during storage, there was a marked increase in the relative abundance of Enterobacteriaceae (*Morganella–Morganella morganii*–, *Raoultella* and Others) at day 10 and a singular increase of Campylobacteraceae (represented by *Arcobacter butzleri*) at day 7. *Firmicutes* showed highest relative abundances at days 1 and 3, mainly for members Leuconostocaceae (*Leuconostoc* and *Weissella*) and Streptococcaceae (Gen. *Lactococcus*—mainly *L. lactis*). Remarkably, OTUs belonging to the specie *L. lactis* had a high relative abundance between days 1 and 3 (21.34% and 29.15%, respectively). The relative abundance of *Bacteroidetes* decreased during storage at 22 °C.

Table 1
Number of sequences (reads), observed diversity and sample estimated coverage for 16S rRNA amplicons analyzed in this study. Shannon index, Chao1 and Goods coverage (ESC) were calculated by QIIME at distance level of 3%.

Sample	No of Reads	Shannon index	Chao1 index	OTUs	ESC (%)
Control 4 °C T0	5177	5.73	318.00	251	99
Control 4 °C T1	5414	5.18	322.38	255	99
Control 4 °C T7	3955	5.37	255.89	215	99
Control 4 °C T15	4806	5.14	315.54	250	99
Control 4 °C T30	3522	4.08	133.00	108	99
HHP treatment 4 °C T0	6906	6.08	443.32	312	98
HHP treatment 4 °C T1	6421	5.61	353.08	276	99
HHP treatment 4 °C T7	4361	5.64	340.36	254	98
HHP treatment 4 °C T15	3947	5.76	335.83	239	98
HHP treatment 4 °C T30	3678	5.68	281.29	243	98
Control 22 °C T0	6475	5.09	310.22	266	99
Control 22 °C T1	6158	5.05	310.38	243	99
Control 22 °C T3	5173	4.45	214.40	157	99
Control 22 °C T7	3077	4.04	188.24	130	99
Control 22 °C T10	2675	3.64	194.10	108	98
HHP treatment 22 °C T0	4654	5.31	313.67	252	98
HHP treatment 22 °C T1	5243	5.67	352.57	265	98
HHP treatment 22 °C T3	5303	2.59	161.05	114	99
HHP treatment 22 °C T7	2780	1.74	48.00	35	99
HHP treatment 22 °C T10	3781	1.43	72.30	47	99

Abbreviations:
OTU: Operational Taxonomic Unit (species observed).
ESC: estimated sample coverage.

In the HHP-treated samples stored at 22 °C, there was a remarkable increase in the relative abundance of *Bacteroidetes* at day 1 of storage (Fig. 2), including both Flavobacteriaceae (mainly *Wautersiella*, *Chryseobacterium*, *Flavobacterium* and *Myroides*, by order), and Sphingobacteriaceae (mainly *Sphingobacterium–Sphingobacterium faecium*). The relative abundance for all these groups decreased remarkably during further incubation, coincidently with an increase of *Firmicutes* at days 3 (*Paenibacillus*) and 7 (*Lactococcus—L. garvieae*) and Proteobacteria at days 3 to 10 (*Escherichia*). Clostridia (*Clostridium* sp. and *Clostridium butyricum*) were also detected in samples from storage day 10.

3.2.4. Beta-diversity analysis

HHP treatment and storage temperature significantly influenced the composition of bacterial communities ($P < 0.05$). In particular, Enterobacteriaceae dominated in untreated asparagus samples both at 4 °C and 22 °C and in the HHP-treated asparagus stored for 3 or more days at 22 °C. Pseudomonadaceae persisted through the whole storage period only in control samples stored at 4 °C, but they greatly decreased in relative abundance after day 1 of storage for all other samples. Flavobacteriaceae also showed significantly higher relative abundances in samples stored at 4 °C compared to samples stored at 22 °C. Such differences were also supported by the beta-diversity analysis performed by UniFrac. A comparative analysis of the abundance of species detected in asparagus samples as a function of HHP treatment is shown in Fig. 3A for samples stored at 4 °C and in Fig. 3B for samples stored at 22 °C. In both cases, differences between the untreated controls and samples treated by HHP were observed. At time 0, diversity did not vary between controls and pressurized batches, but as the storage period increased, the differences in species diversity became greater.

4. Discussion

The resident microbiota on the surface of fruits and vegetables is often the main source of microbial contamination during processing, leading to microbial spoilage during storage. The initial microbial load in fresh green asparagus has been investigated by several authors by culture-dependent methods (García-Gimeno et al., 1998; Sothornvit and Kiatchanapaibul, 2009), with total aerobic mesophilic and psychrophilic bacteria counts close to 5 log CFU/g. In our studies, the microbial load of green asparagus was between 5 and 6 log CFU/g (results not

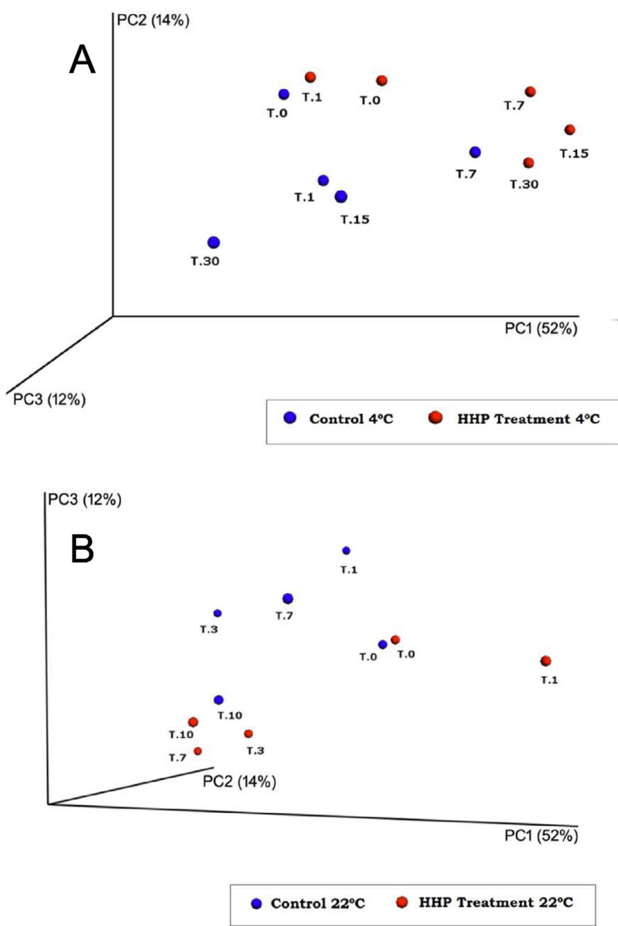

Fig. 3. Principal Coordinates Analysis of weighted UniFrac distances for 16S rRNA gene sequence data of brined green asparagus stored at 4 °C for 30 days or at 22 °C for 10 days. Asparagus were pressurized at 600 MPa for 8 min (red) or not (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown). An increase in these levels indicates the beginning of food spoilage. Since the presence and number of microorganisms on vegetable foods depends on the type of crop, agronomic practices, geographical area and climatic conditions (Brackett, 1999), it is necessary to develop techniques that minimize pollution and prevent the growth of pathogens and spoilage microorganisms. In spite of the fact that high hydrostatic pressure (HHP) treatments are placed on the top among non-thermal methods for food processing, there are no previous reports on application of HHP treatments on green asparagus. According to results from the present study, HHP treatments at 600 MPa for 8 min could stabilize brined green asparagus for up to one month under refrigeration under a worst-case scenario of heavy contamination close to 7 log CFU/g. Compared to thermal processes currently being used, HHP treatments offers a potential as a mild-treatment for preservation of brined, fresh green asparagus. Results from storage experiments carried out at 22 °C suggested that the treated asparagus could also withstand a limited exposure to temperature abuse conditions of at least one day, but not for longer periods of time due to proliferation of survivors and abolition of the acidic pH barrier. Therefore, it is important to study the dynamics of microbial populations during storage of foods, including those stabilized by HHP treatment, in order to evaluate the main bacterial groups that can proliferate after treatments and become involved in spoilage.

In the present study, a culture-independent approach based on pyrosequencing of the 16S rRNA gene was applied in order to investigate the microbiota from green asparagus surface and to determine the impact of HHP treatment and the changes in the surviving fraction during cold and room temperature storage. Pyrosequencing is widely employed for microbial community analysis and for identification to specie level. Nevertheless, only a very limited number of studies have applied this technology to study the microbial diversity from foods treated by HHP (Pérez Pulido et al., 2015). In the present study, samples were treated with propidium monoazide (PMA) before DNA extraction in order to avoid interference of DNA from dead cells in amplification steps for pyrosequencing. Pre-treatment of a sample with PMA has been reported to prevent amplification of DNA from dead cells, allowing PCR methodology to quantitatively discriminate between live and dead cells (Nocker et al., 2006, 2007).

Among the OTUs detected from green asparagus artificially contaminated with its own surface microbiota, Enterobacteriaceae was the most abundant bacterial group, represented mainly by members of genera *Rahnella*, *Raoultella*, *Pantoea*, *Erwinia*, *Serratia* and *Escherichia*. These bacteria also predominated during all stages of storage, in both pressurized and control samples, although with different relative abundances. The family Enterobacteriaceae is a heterogeneous group of Gram-negative bacteria, comprising mesophilic microbial saprophytes, commensal and opportunistic pathogens, and is widely distributed in soil, water, vegetation and animal and human guts (Dworkin et al., 2006). It is also present at high levels in minimally processed vegetables (Abadias et al., 2008; Li et al., 2001; Oliveira et al., 2010), being part of the initial microbiota. Enterobacteriaceae levels in lettuce may vary between 3 and 7 $\log_{10}$ CFU/g, as described in a previous work by Oliveira et al. (2010), which is common in raw vegetables and not been necessarily associated with a fecal contamination. A recent study by Leff and Fierer (2013), based on pyrosequencing, also demonstrates that Enterobacteriaceae has a high relative abundance in most of the analyzed vegetables (bean and alfalfa sprouts, strawberries, spinach, lettuce, tomato and pepper) being *Pantoea* sp. the most represented taxon. In the present study on green asparagus, in the initial days of storage at 4 °C OTUs belonging to the species *P. ananatis* showed greatest relative abundance, both in control and pressurized asparagus. *Pantoea* was also the predominant OTU detected from the microbiota of cherimoya (Pérez Pulido et al., 2015). Species from genus *Pantoea* are frequently isolated from vegetal material as part of the epiphytic or endophytic microbiota of the plant, although they can occupy different ecological niches. Some strains act as plant growth promoters (De Maayer et al., 2014), while others are pathogenic to plants and humans, causing disease and bacteremia. *Pantoea* is also being used as a biological control agent due to its capacity to produce antimicrobial substances (Coutinho and Venter, 2009; Walterson et al., 2014).

A culture-dependent study carried out by García-Gimeno et al. (1998) indicated that alteration of packed green asparagus correlates with high levels of psychrotrophic bacteria, mostly belonging to lactic acid bacteria and the genus *Pseudomonas*. *Pseudomonas* sp. is a very heterogeneous group of saprophytic bacteria found in soil, decomposing organic matter, vegetation and water, besides being opportunistic pathogens of plants, animals and humans (Cornelis, 2008). In the present study, the relative abundance of OTUs belonging to *Pseudomonas* remained at relatively high values during storage of control samples under refrigeration, but not in control samples stored at 22 °C or in any of the samples treated by HHP. Pseudomonads group (which includes psychrotrophic species) would be expected to have a greater capacity to survive during cold storage, but they would not be expected to grow at acidic pH and would be displaced upon establishment of fermentative conditions by the faster growth of Enterobacteria at 22 °C.

Members of Fam. Enterobacteriaceae seemed to be the main bacterial group involved in spoilage of the brined green asparagus. The observed reduction in the relative abundance of Enterobacteriaceae by HHP treatments could be associated with a reduction of spoilage and would suggest that members of this group were more sensitive to the HHP treatments than *Bacteroidetes* and *Firmicutes*. Interestingly, the population of Enterobacteriaceae would not recover during refrigeration

storage of the HHP treated samples, leaving *Bacteroidetes* (mainly Flavobacteriaceae and to a less extent Sphingobacteriaceae) as the predominant group. Flavobacteria and Sphingobacteria are natural inhabitants of soil and water equipped with a variety of exocellular enzymes important for decomposition of biopolymers (Bernardet and Nakagawa, 2006). Nevertheless, their metabolic activity in the cold-stored asparagus would be limited by the low temperature, acidic pH and low oxygen availability. A relatively stable fraction of *Firmicutes* from Fam. Enterococcaceae and Fam. Streptococcaceae was also detected in the HHP treated samples during cold storage. These *Firmicutes* are well adapted to acidic environments, but do not multiply or grow very slowly during refrigeration. In contrast, when HHP-treated samples were stored at 22 °C, the relative abundance of OTUs belonging to Enterobacteriaceae (mainly *Escherichia*) and to a less extent also *Firmicutes* increased at the same time as viable cell counts increased by 3.5 log cycles. Interestingly, the biodiversity of samples was reduced considerably in HHP treated samples during late storage at 22 °C, indicating that only a few microbial groups surviving the HHP treatments were able to proliferate.

Endospore formers (such as the detected *Paenibacillus*, *Bacillus* and *Clostridium*) would be expected to survive HHP treatments, since bacterial endospores are usually abundant in plant materials and are resistant to the pressure treatments applied in the present study. Furthermore, they are important in the production of enzymes for breakdown of polymeric substances and the release of fermentable carbohydrates. The relative abundance of *Clostridium* sp. did not exceed 5% in pressurized asparagus stored at room temperature. The spores found in food must be carefully investigated because, being present in soils, usually are an indicator that there has been insufficient hygiene or improper handling during processing. Although some *Clostridium* species are associated in incidents of food poisoning due to the production of neurotoxins, other are involved in spoilage of food products, causing changes in the sensory quality (production of butyric acid, putrid odors, etc.), which results in large economic losses (Brown, 2000). The presence of LAB in the brined asparagus, together with the acidic pH of the brine used, may create an environment difficult for germination of clostridial endospores, as has been suggested in general for low-pH foods (Black et al., 2007).

In conclusion, results from the study indicate that reductions of microbial loads in green asparagus by HHP treatment at 600 MPa for 8 min extend the shelf life of the brined asparagus during storage at chill temperature. The microbial load of green asparagus reported by other studies is at least 2 log cycles lower compared to samples artificially contaminated used in the present study. Therefore, we would expect a much lower surviving fraction after HHP treatment of conventional (non-inoculated) asparagus. Although not all bacterial populations in green asparagus are affected equally by HHP treatments, selected groups of Enterobacteriaceae have a greater capacity for proliferation in the treated asparagus stored at 22 °C, leading to a rapid spoilage.

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DISCUSIÓN GENERAL

En la actualidad, los consumidores son cada vez más conscientes de la necesidad de consumir alimentos frescos, saludables y mínimamente procesados, carentes de conservantes químicos sintéticos, y que sean seguros desde el punto de vista microbiológico. Sin duda, las demandas del consumidor se orientan hacia los nuevos alimentos que son beneficiosos para la salud y fáciles de mantener. Determinados factores, tales como el estilo de vida y los hábitos alimenticios, han impulsado el desarrollo de nuevas tecnologías para la elaboración, la conservación y el control de los alimentos (Señorans *et al.*, 2003). La probabilidad de crecimiento de microorganismos patógenos y alterantes en los alimentos supone un riesgo para la salud humana, por lo que un aspecto importante de la seguridad alimentaria es la necesidad de detectar rápidamente estos microorganismos con el fin de evitar brotes que puedan afectar a grandes poblaciones (Tienungoon *et al.,* 2000; Morcillo *et al.,* 2005), y diseñar estrategias basadas en la tecnología de barreras para impedir o retrasar su crecimiento.

La intoxicación alimentaria estafilocócica es una de las causas más comunes de enfermedades transmitidas por los alimentos. La mayoría de las cepas de *Staphylococcus aureus* son capaces de producir enterotoxinas termoestables, siendo además resistentes a los antibióticos, lo cual aumenta el riesgo de infección. Uno de los métodos propuestos para el control de los microorganismos en los alimentos es el tratamiento mediante alta presión hidrostática (APH) y el uso de sustancias antibacterianas naturales (Cleveland *et al.*, 2001; Gálvez *et al.*, 2007). Sin embargo, es necesario realizar más estudios a pequeña escala para comprender mejor el funcionamiento y la efectividad de estos tratamientos en diversos tipos de productos alimentarios, antes de ser aplicados a escala industrial.

En nuestro primer estudio se ha podido comprobar que el efecto bactericida del tratamiento APH sobre los estafilococos en arroz con leche aumenta con la intensidad de la presión. La inactivación microbiana fue mayor en tratamientos de 10 minutos a 600 MPa, donde se consiguió una reducción de células viables cercana a los 7 ciclos logarítmicos, permaneciendo los supervivientes por debajo del límite de detección. Nuestros resultados sobre la inactivación de un cóctel de cepas de *S. aureus* resistentes a la meticilina (MRSA) durante tratamientos de 10 minutos son similares a los obtenidos por Gervilla *et al.* (2000) para *S. aureus* CECT 534 en leche de oveja y por López-Pedemonte *et al.* (2007) en quesos. Del mismo modo, se necesitan presiones superiores a 500 MPa para obtener reducciones logarítmicas significativas para *S. aureus* ATCC 25923 en carne de cerdo emulsionada

(Shigehisa *et al*., 1991). Tratamientos a presiones más elevadas inducen un daño mucho mayor a los estafilococos. Nuestros resultados están en concordancia con los de Guan *et al.* (2006), quienes obtuvieron una reducción de 7.3 ciclos logarítmicos para *S. aureus* ATCC 12600 en leche UHT tras el tratamiento a 600 MPa durante 8 minutos a 21ºC.

En el presente estudio intentamos mejorar la inactivación de los estafilococos empleando antimicrobianos (bacteriocinas nisina y enterocina AS-48, y aceites esenciales de canela y clavo) en combinación con tratamientos de alta presión de menor intensidad (500 MPa, 5 minutos). La nisina mejoró la inactivación de los estafilococos por APH en arroz con leche y la combinación APH-nisina tuvo una reducción significativamente mayor comparada con el tratamiento individual de presión. La enterocina AS-48 no mejoró sustancialmente la inactivación de los estafilococos por APH y, aunque la combinación de enterocina AS-48 y APH sí consiguió una reducción logarítmica adicional, aunque dicha reducción no fue estadísticamente significativa.

Los efectos bactericidas de las bacteriocinas en los sistemas alimentarios están muy influenciados por la interacción de las moléculas de bacteriocina con los constituyentes de los alimentos y también por muchos otros factores, como son el tiempo de incubación y la temperatura (Gálvez *et al*., 2006). Los escasos efectos demostrados por la nisina y la enterocina AS-48 aplicadas de forma individual sobre los estafilococos en arroz con leche también podrían predecir una interacción muy baja con los tratamientos por APH. En anteriores estudios, la enterocina AS-48 en combinación con APH no disminuyó la viabilidad de *S. aureus* en salchichas fermentadas de baja acidez (Ananou *et al*., 2010). No obstante, otras bacteriocinas como la nisina, sakacina K y pediocina AcH, mejoraron ligeramente el efecto de los tratamientos por APH contra las cepas de *S. aureus* en un producto cárnico (Garriga *et al*., 2002) y la nisina mejoró considerablemente el efecto del tratamiento APH contra un cóctel de estafilococos (CTC1008, CTC1019, CTC1021) en jamón cocido (Jofré *et al*., 2008). Existen escasos informes sobre la acción combinada de APH y bacteriocinas contra estafilococos en productos lácteos, sin embargo, en un estudio se observó que la adición de lacticina 3147 incrementó la eficacia de la alta presión contra la cepa *S. aureus* ATCC 6538 en suero de leche desmineralizado (Morgan *et al*., 2000).

El efecto de los tratamientos APH contra estafilococos resistentes a meticilina mejoró con la adición de aceites de canela y clavo a concentraciones donde ninguno de los aceites ensayados tuviera efecto alguno sobre la viabilidad de los estafilococos. Los

recuentos viables obtenidos mediante la combinación de los tratamientos por APH y aceite de clavo fueron significativamente menores, comparados con el tratamiento individual de alta presión. A pesar de ser el más activo, el aceite de clavo confirió un olor desagradable en el arroz con leche. Sin embargo, el tratamiento con aceite de canela y APH durante 5 min. tuvo un efecto similar a los tratamientos individuales por presión durante 10 min, lo que sugiere que el tratamiento combinado se podría aplicar para reducir el tiempo de tratamiento en arroz con leche. La inactivación de los estafilococos aumentó de manera no significativa cuando el aceite de canela fue utilizado junto con nisina o enterocina AS-48 en combinación con APH. Estos resultados indican una muy baja contribución de estas dos bacteriocinas en la inactivación de los estafilococos por los tratamientos combinados en arroz con leche. La actividad de las bacteriocinas puede ser potenciada por aceites esenciales (Gálvez *et al.*, 2006) y, aunque aún hay pocos informes sobre la combinación de aceites esenciales y tratamientos por APH (Vurma *et al.*, 2006; Somolinos *et al.*, 2008; Evrendilek y Balasubramaniam, 2011), ninguno de ellos trata sobre *S. aureus*. Este campo puede resultar de interés para futuras investigaciones, con el fin de mejorar los efectos de los tratamientos APH y también para disminuir el impacto de los aceites esenciales sobre las propiedades organolépticas de los alimentos.

A fin de comprobar su efecto protector durante la vida útil del producto, la nisina fue ensayada individualmente o en combinación con un tratamiento por APH contra los estafilococos en arroz con leche almacenado durante una semana a 4ºC. La adición individual de nisina redujo significativamente los recuentos viables de estafilococos en arroz con leche. A mayor concentración de nisina, la reducción de los recuentos viables fue más rápida. Para los tratamientos combinados, la nisina mejoró la inactivación de los estafilococos por APH y disminuyó la concentración de supervivientes durante el almacenaje de las muestras por debajo del nivel de detección en el día 3, obteniéndose mejores resultados que en las muestras tratadas individualmente con APH.

La adición de la enterocina AS-48 redujo muy lentamente los recuentos viables durante el almacenaje de las muestras, comparada con las muestras control. Para los tratamientos combinados con APH, la reducción obtenida justo tras el tratamiento no fue significativa, comparada con el tratamiento individual por presión. No obstante, la enterocina AS-48 mejoró la inactivación de los estafilococos durante el almacenaje de las muestras tratadas. En un estudio previo, la enterocina AS-48 no tuvo efecto sobre la supervivencia o proliferación de un cóctel de cepas de *S. aureus* (CTC 1010, CTC 1011,

CTC 1034) en jamón presurizado durante su almacenaje a temperatura ambiente o a 7ºC (Ananou *et al.*, 2010). De forma similar, también se han descrito resultados negativos para las enterocinas A y B en salchichas fermentadas de baja acidez (Jofré *et al.*, 2009). Por el contrario, la nisina mejoró considerablemente el efecto del tratamiento por APH contra los estafilococos en jamón durante su almacenamiento (Jofré *et al.*, 2008). Estos resultados muestras la gran variabilidad en la eficacia de las bacteriocinas cuando se prueban en diferentes productos alimentarios, solas o en combinación con otros métodos de barrera, y la necesidad de validar tratamientos combinados en cada producto alimentario en particular.

La adición de un 0.2% de aceite de canela en combinación con APH mejoró significativamente la inactivación de los estafilococos en comparación con el tratamiento individual de alta presión, tanto después del tratamiento como durante el almacenamiento, mientras que la adición sola de aceite de canela no mejoró considerablemente la inactivación microbiana. Tras 7 días de almacenaje, los recuentos viable en el arroz con leche tratado por APH en combinación con un 0.2% de aceite de canela fueron similares a los logrados por la combinación de APH y 500 IU/g de nisina, y justo ligeramente inferior comparado con el tratamiento combinado con enterocina AS-48. Por lo tanto, las tres combinaciones parecen adecuadas para la inactivación de los estafilococos durante el almacenaje del arroz con leche. Aunque el aceite de canela logra una mayor reducción de los recuentos viables, la nisina produce una inactivación más rápida de los estafilococos.

Las bacteriocinas requieren de un mínimo periodo de incubación para causar daño celular y, por esta razón, sus efectos bactericidas en combinación con APH son más pronunciados durante el almacenamiento de las muestras tratadas. El empleo de bacteriocinas en combinación con tratamientos APH puede tener un gran interés aplicado, dado que las moléculas residuales de bacteriocina todavía muestran efectos inhibitorios en los alimentos, inactivando y previniendo el crecimiento de células supervivientes. Por otra parte, dado que la actividad anti-estafilocócica de los aceites de canela y clavo empleados de forma individual ha sido descrita en estudios previos (Smith-Palmer *et al.*, 1998; Nunes-Barbosa *et al.*, 2009), los tratamientos combinados con APH también pueden ser interesantes. La inactivación de estafilococos por tratamientos APH en combinación con aceites esenciales debe ser investigado en otros alimentos, donde la APH tiene un mayor impacto sobre las propiedades físico-químicas del alimento, tales como la carne, pescado, marisco o derivados cárnicos y productos del mar. Cabría esperar, por tanto, que los

tratamientos combinados incluyendo aceites esenciales permitiesen reducir la intensidad de las altas presiones, minimizando su impacto en las propiedades del alimento.

Los huevos y alimentos preparados a base de huevo contaminados con bacterias patógenas humanas también han estado implicados en numerosos brotes de origen alimentario, dando lugar a costosas retiradas del producto. La tortilla de patatas española es un alimento refrigerado listo para su consumo, muy cómodo de servir, elaborado a partir de huevos y patatas. Se vende con frecuencia en los supermercados, aunque también en muy popular en bares, restaurantes y servicios de catering e incluso en locales de comidas y bebidas para llevar. Los huevos destacan entre las materias primas de origen animal con mayor riesgo de transmisión de *Salmonella*. Además, en las tortillas de tamaño más grueso (tales como tortillas de patatas, que pueden tener entre 2 y 3 cm de alto) a menudo pueden aparecer zonas poco cocinadas en la parte central, lo que aumenta el riesgo de supervivencia y transmisión de esta bacteria patógena. Dado que la tecnología APH es cada vez más popular a escala industrial para el tratamiento de alimentos listos para su consumo, se podría aplicar también para conseguir la inactivación de *Salmonella* en tortilla de patatas.

En estudios previos llevados a cabo con huevo líquido, se aplicaron tratamientos de presión en un rango bajo (entre 400 - 450 MPa) para la inactivación de *Salmonella,* con un impacto mínimo sobre las propiedades funcionales de los alimentos (Ponce *et al*., 1999). Las tortillas de patatas tolerarían presiones mucho mayores, pues las proteínas del huevo se coagulan durante el proceso de cocción. Los tratamientos de alta presión se podrían aplicar en tortillas para reducir significativamente los niveles de una posible contaminación por *Salmonella*. Sin embargo, no hay estudios previos relativos a la inactivación de *Salmonella* en tortillas mediante tratamiento por altas presiones. La APH ha sido ensayada para la inactivación de las células de *Salmonella* en muchos otros sistemas alimentarios, tales como carne y productos cárnicos (Shigehisa *et al*., 1991; Ananth *et al*., 1998; Morales *et al*., 2009; Ananou *et al*., 2010; Kruk *et al*., 2011), huevo entero (Ponce *et al*., 1999; Bari *et al*., 2008), marisco (Malicki *et al*., 2005), leche entera (Guan *et al*., 2005), queso (De Lamo-Castellví *et al*., 2007), jugos de frutas (Nakimbugwe *et al*., 2006; Whitney *et al*., 2007), y semillas para la producción de brotes germinados (Wuytack *et al*., 2003; Neetoo y Chen, 2010), con resultados variables. Por ejemplo, el tratamiento a 400 MPa durante 15 minutos logró una reducción de 4.8 unidades logarítmicas en filetes de pechuga de pollo (Morales *et al*., 2009). En huevo líquido, fueron ensayadas combinaciones entre la intensidad de la presión, el

tiempo de tratamiento y la aplicación de calor para lograr la inactivación de *Salmonella* sin causar la coagulación del huevo. Tratamientos a 450 MPa durante 5 minutos a 20ºC lograron una reducción de 4 ciclos logarítmicos en *Salmonella* Enteritidis (Ponce *et al.*, 1999), mientras que en otro estudio con un tratamiento a 400 MPa y 25ºC fue requerido un tiempo de tratamiento de hasta 40 minutos para lograr una reducción de 6 logaritmos (Bari *et al.*, 2008).

Los resultados sobre la inactivación de microorganismos en los alimentos dependen en gran medida del procedimiento aplicado para la estimación de los supervivientes. Puesto que la mayoría de los alimentos no son productos estériles, se puede considerar el uso de medios selectivos para la enumeración de patógenos transmitidos por los alimentos. En nuestro estudio, los tratamientos en un rango de 400 a 600 MPa durante 5 u 8 minutos redujeron los recuentos viables de *Salmonella* en las tortillas de 2.8 a 6.5 ciclos logarítmicos cuando se usó un medio selectivo (XLD agar) para su enumeración. Sin embargo, cuando las células subletalmente dañadas fueron investigadas en un medio no-selectivo (TSA) o por el método de la Triple Capa de Agar (*triple agar layer,* TAL), las concentraciones de los supervivientes fueron considerablemente más altos, especialmente para los tratamientos de 400 a 680 MPa. El método TAL fue propuesto por Kang y Fung (2000) para la recuperación selectiva de *Salmonella* Thyphimurium dañada por calor. Durante las primeras horas de incubación, las salmonelas lesionadas repararon su daño celular y empezaron a crecer en el medio TSA, mientras que los agentes selectivos del medio XLD difundían gradualmente a la parte superior hacia la capa de TSA. Este procedimiento produjo un rendimiento ligeramente inferior, pero no unos recuentos estadísticamente diferentes comparados con TSA para el daño subletal por calor, y también mejoró la recuperación de las salmonelas dañadas por ácidos (Kang y Fung, 2000; Wu *et al.*, 2001). En nuestro estudio con *Salmonella* presurizada a 300 MPa, las diferencias entre los recuentos por el método TAL y con TSA no fueron estadísticamente significativas. Sin embargo, las diferencias observadas fueron mucho mayores para los tratamientos de intensidades más altas, lo que indica que la fracción de células dañadas no recuperadas por el método TAL puede ser significativamente superior bajo condiciones más severas de estrés. Bozoglu *et al.* (2004) sugirieron dos tipos de lesiones subletales (I1 e I2) en patógenos transmitidos por alimentos tras el tratamiento APH. En su estudio sobre *Salmonella*, describieron una lesión primaria (I1) que reconocible por la formación de colonias sólo en agar no-selectivo y por la recuperación en la capacidad de crecer en medio selectivo tras un día de almacenamiento. Los resultados de nuestro estudio sugieren diferentes niveles de daño celular, por ejemplo,

células incapaces de crecer en XLD agar pero que aún pueden reparar su daño al cabo de pocas horas y crecer en placas de TAL, y células que requieren un almacenamiento más prolongado bajo condiciones no selectivas para reparar su daño celular (tales como aquellas células capaces de crecer en TSA pero no en las placas de TAL o XLD agar).

En nuestro estudio, se requirieron tratamientos de 600 o 680 MPa durante 8 minutos para lograr reducciones microbianas de 6.5 ciclos logarítmicos (considerando las células no dañadas como supervivientes) o un máximo de 4.7 ciclos logarítmicos considerando las células subletalmente dañadas. La alta resistencia de *Salmonella* observada en tortillas, comparada con otros alimentos, puede ser atribuida al pH neutro de las tortillas (un factor ya conocido que disminuye la eficacia de los tratamientos APH; Alpas *et al.*, 2000) y quizás también a la estructura semi-sólida del substrato alimentario (en comparación con los alimentos líquidos). Otros factores también deben ser tomados en consideración, tales como la preparación del inóculo, la densidad del inóculo y las diferencias entre la sensibilidad de las cepas. Por ejemplo, la formación de agregados celulares en inóculos de alta densidad celular puede tener un efecto protector contra el tratamiento APH. Lo mismo sucede con el posible crecimiento de *Salmonella* y la formación de microcolonias o biopelículas (como puede ocurrir en las líneas de procesado de alimentos o en el propio alimento durante la rotura accidental de la cadena de frío). En muchos alimentos se ha informado de que la conservación mediante el procesado por APH requiere niveles de presión por encima de 600 MPa para la inactivación de patógenos resistentes a la presión (Balasubramaniam *et al.*, 2008). En tortillas inoculadas con altas concentraciones de salmonelas (simulando un escenario de contaminación en el peor de los casos), las curvas de supervivencia mostraron colas a 600 y 680 MPa. El efecto de asimetría de las colas, observadas a menudo en las curvas de supervivencia, ha sido atribuido a una fracción de la población con gran resistencia a la presión o a la reparación del daño en células bacterianas subletalmente lesionadas (Chen, 2007; San Martín *et al.*, 2002) o incluso en ambos factores, como parece ser el caso de este estudio, en el que las colas fueron observadas tanto para la enumeración de supervivientes en medios selectivos como en no-selectivos.

Aunque las tortillas se venden bajo condiciones de refrigeración, existe el riesgo de que las células de *Salmonella*, subletalmente dañadas por tratamientos APH, puedan reparar el daño celular durante el almacenamiento y/o proliferar en tortillas bajo condiciones de abuso de temperatura. Por lo tanto, es importante evaluar los niveles de recuperación y el potencial de proliferación de las fracciones supervivientes durante el almacenamiento de las

tortillas tratadas por alta presión. En nuestro estudio se observó una recuperación de las células subletalmente dañadas en los 3 primeros días de almacenaje en refrigeración para las tortillas tratadas a 500 MPa durante 5 minutos (mostrado por el incremento de los recuentos viables obtenidos en las placas de TAL y XLD agar), mientras que se retrasó la recuperación de las células tratadas a 680 MPa al día 10 de almacenaje. Se recomiendan tratamientos de al menos 680 MPa durante 8 minutos para reducir las concentraciones de células intactas y retrasar la recuperación de salmonelas subletalmente dañadas en tortillas. No obstante, se observó también una recuperación de las células subletalmente dañadas cuando se aplicaron condiciones de abuso de temperatura en el día 3 para el tratamiento de mayor intensidad, aunque la fracción recuperada fue aproximadamente 2 ciclos logarítmicos menos, comparada con las muestras tratadas a 500 MPa bajo las mismas condiciones de abuso de temperatura. A pesar de que los tratamientos por APH de alta intensidad pueden reducir significativamente las poblaciones de *Salmonella* en tortilla de patata, los episodios de abuso de temperatura todavía pueden comprometer la seguridad del producto alimentario tratado.

El sector de elaboración de platos preparados de cuarta y quinta gama abarca un amplio y variado abanico de ingredientes, recetas y tecnologías de procesado y de envasado, e incluye una gran diversidad de productos alimenticios, entre ellos, gazpachos, cremas, caldos, etc., siendo necesario mantener la cadena de frío hasta el momento de su utilización y consumo.

El salmorejo es una crema tradicional andaluza elaborada a partir de tomates maduros y otros ingredientes (Barrenechea, 2005); por lo tanto, puede contaminarse con bacterias patógenas que afectan a los humanos a partir de estas materias primas vegetales usadas para su elaboración. Antes de ser servido, el salmorejo se suele cubrir con trozos de jamón Ibérico o huevo cocido, pudiendo ser una fuente adicional de bacterias. También hay una tendencia en la cocina creativa de introducir otros ingredientes en el salmorejo, como las gambas peladas, lo que aumenta los riesgos de contaminación. Dado que el salmorejo no está sometido a un proceso térmico y se sirve como un plato frío, es importante conocer la capacidad de supervivencia en este sustrato de los patógenos que se transmiten por los alimentos y el efecto de las tecnologías alternativas de procesado de alimentos, como la alta presión hidrostática, sobre la inactivación microbiana en este producto.

Nuestros resultados indicaron que había grandes diferencias en la capacidad de los patógenos alimentarios investigados (*E. coli* O157, *S. enterica* serovar Enteritidis y *L. monocytogenes*) para sobrevivir en salmorejo durante el almacenamiento en refrigeración. De todos ellos, *L. monocytogenes* fue la bacteria que mostró menor capacidad para sobrevivir en el salmorejo control, tal y como se muestra por la completa inactivación observada tras 15 días de almacenamiento refrigerado. Sin embargo, dado que las concentraciones de células viables decrecieron solamente en 2,3 ciclos logarítmicos durante 7 días de almacenaje, si la carga inicial fuese alta, podría existir un riesgo microbiológico, especialmente para aquellas poblaciones de consumidores más susceptibles. No obstante, estudios anteriores han demostrado que *L. monocytogenes* es notablemente resistente a condiciones de acidez y sobrevive bien bajo condiciones de refrigeración en distintos sustratos, incluyendo salsa de tomate (Ahamad y Marth, 1989; Walker *et al.*, 1990; Glass y Doyle, 1991; Raghubeer *et al.*, 2000). En salmorejo, *S.* Enteritidis resultó ser más resistente que *L. monocytogenes*, disminuyendo solamente 2.4 ciclos logarítmicos tras 15 días. *E. coli* O157 fue incluso más resistente y disminuyó solo 1.5 ciclos logarítmicos tras 30 días de almacenamiento. Estos resultados coinciden con estudios previos, mostrando que algunas cepas de *E. coli* O157 son ácido-resistentes y pueden sobrevivir durante largos periodos en alimentos ácidos, especialmente a baja temperatura (Glass *et al.*, 1992; Miller y Kaspar, 1994; Weagant *et al.*, 1994; Zhao y Doyle, 1994). Diversos factores, tales como una baja temperatura de almacenamiento, el bajo pH del salmorejo y posiblemente también las actividades antibacterianas del ácido acético y los componentes antimicrobianos presentes en el ajo, podrían ser los responsables de la inhibición del crecimiento observada en este trabajo y la disminución en la viabilidad de los patógenos de origen alimentario, así como la propia microbiota residente en el salmorejo.

La inactivación de los microorganismos por tratamientos APH depende en gran medida de la matriz del alimento y del pH de ese producto alimentario (Alpas *et al.*, 2000). El estrés ácido puede mejorar la tolerancia a la APH pero, al mismo tiempo, los ácidos orgánicos pueden actuar sinérgicamente con la APH (Alpas *et al.*, 2000). Los resultados obtenidos en el presente estudio indican que tanto *L. monocytogenes* como *S. enterica* inoculadas en salmorejo fueron altamente sensibles a los tratamientos por APH a 400 MPa y no se detectaron supervivientes o células dañadas subletalmente. Maitland *et al.* (2011) obtuvieron reducciones logarítmicas para *Salmonella* entre 1.44 y 3.67 log CFU/g en tomates enteros o entre 2.25 y 3.35 en tomates cortados en dados después de tratamientos a 450 o 550 MPa (120 segundos), respectivamente. Stewart *et al.* (1997) obtuvieron una

reducción adicional de 3 log CFU/g en *L. monocytogenes* cuando se presurizó en un tampón a pH 4.0, en comparación con un pH 6.0 a 353 MPa y 45ºC durante 10 minutos. Otros estudios también mostraron que las células de *E. coli* y *Salmonella* eran más sensibles a la presión bajo condiciones ácidas de pH, comparadas con valores de pH neutro (García-Graells *et al*., 1998; Alpas *et al*., 2000; Pagán *et al*., 2001; Teo *et al*., 2001; Whitney *et al*., 2007).

En nuestro estudio, las células de *E. coli* O157 fueron altamente sensibles a los tratamientos de 600 MPa durante 8 minutos. Sin embargo, las células de *E. coli* O157 fueron más tolerantes a los tratamientos a 400 y 500 MPa en comparación con *S.* Enteritidis o *L. monocytogenes*. Una explicación para este hecho podría ser una mayor resistencia a la alta presión de las cepas de *E. coli* utilizadas para este trabajo. Las cepas de *E. coli* ensayadas en el presente estudio pertenecían al serogrupo O157, aunque no eran productoras de verotoxina. Se ha descrito que algunas cepas de *E. coli* son relativamente sensibles a la presión y pueden ser fácilmente inactivadas a presiones tan bajas como 200 MPa (Robey *et al*., 2001; Buckow *et al*., 2008), mientras que otras, tales como las cepas de *E. coli* pertenecientes al serogrupo O157, se encuentran entre las células vegetativas más resistentes a la presión (Benito *et al*., 1999). Muñoz *et al.* (2007) lograron reducciones de 3.44 ciclos logarítmicos para la cepa CECT 515 de *E. coli* (serotipo O1:K1(L1):H7) en gazpacho tras un tratamiento a 350 MPa (22ºC, 15 minutos), aunque Jordan *et al.* (2001) encontraron grandes diferencias en la sensibilidad a los tratamientos por APH en el rango de 350 a 500 MPa (22ºC, 5 minutos) entre una cepa de *E. coli* O157 y la cepa *E. coli* ATCC 11775. Los mismos autores también revelaron que la inactivación de *E. coli* por alta presión fue mayor en zumos de tomate y manzana, comparada con el zumo de naranja. Además de las diferencias entre cepas en cuanto a resistencia a la presión, se ha mostrado una relación entre la tolerancia a los ácidos y resistencia a la presión en *E. coli* O157:H7 (Benito *et al*., 1999). La supervivencia simultánea al estrés ácido y a tratamientos APH tiene importantes implicaciones para la seguridad alimentaria, puesto que se ha descrito que los mecanismos de respuesta al estrés ácido en *E. coli* protegen a la bacteria durante el procesado de los alimentos y a la vez activan sus mecanismos de virulencia, facilitando la baja dosis infectiva característica de *E. coli* y contribuyendo significativamente a la patogénesis de este microorganismo (Richard y Foster, 2003; Kanjee y Houry, 2013).

Los tratamientos APH aplicados sobre *L. monocytogenes* o *S. enterica* no dieron lugar a la aparición de células supervivientes dañadas subletalmente. En las muestras de salmorejo

inoculadas con *E. coli* O157, una pequeña fracción de la población fue capaz de reparar el daño subletal y crecer en el medio TAL-EMB tras los tratamientos a 400 MPa, así como a 500 MPa. Los supervivientes capaces de crecer directamente sobre el medio selectivo también fueron detectados a tiempos posteriores de almacenaje de las muestras. Un estudio previo realizado por Muñoz *et al.* (2007) mostró que los tratamientos APH hasta los 350 MPa ocasionaban lesiones subletales en células de *E. coli* en alimentos ácidos, como los zumos de naranja y manzana y en gazpacho, aunque estos autores no investigaron la tasa de supervivientes durante el almacenamiento. La capacidad para reparar el daño subletal no solo depende de las especies bacterianas y las cepas, sino también de las condiciones ambientales que facilitan el crecimiento bacteriano y la generación de energía. Se podría esperar que un pH ácido inhibiese la recuperación de las células subletalmente dañadas, como se demostró en un estudio con zumo de naranja, en el cual las células de *E. coli* subletalmente dañadas por APH murieron rápidamente bajo las condiciones de pH ácido del zumo (Linton *et al.*, 1999).

Los resultados de este estudio sugieren que la aplicación de APH a 600 MPa durante 8 minutos puede ser un tratamiento efectivo para la inactivación de los patógenos de origen alimentario *E. coli* O157, *S. enterica* serovar Enteritidis y *L. monocytogenes* en salmorejo, y también para reducir la carga de la microbiota endógena. Este tratamiento no-térmico podría aplicarse para mejorar la seguridad comercial del salmorejo, previniendo la transmisión accidental de patógenos de origen alimentario a través del producto ya terminado.

La alta presión hidrostática se puede utilizar como una etapa final en el procesado de alimentos, aplicada sobre los productos ya envasados, y parece ser un proceso no-térmico prometedor para la pulpa de chirimoya. Hoy en día hay una gran variedad de productos de origen vegetal presurizados, tales como mermeladas, zumos de frutas, pasta de aguacate (guacamole), ensaladas de frutas frescas troceadas, etc., y se encuentran comercialmente disponibles en E.E.U.U., Europa y Japón (Torres y Velázquez, 2005). Los tratamientos APH también han sido aplicados en diferentes tipos de frutas, como la granada, melocotón, anacardo, melón, guayaba, plátano, caqui, aguacate o mango (Jacobo-Velázquez y Hernández-Brenes, 2012; Kaushik *et al.*, 2014; Rawson *et al.*, 2011; Vázquez-Gutiérrez *et al.*, 2012). Comparada con los patógenos transmitidos por los alimentos, la inactivación mediante APH de las bacterias alterantes, tales como los leuconostocs, ha sido estudiada en

menor medida. Un estudio previo demostró que tratamientos por APH a 250 y 500 MPa causaban cambios en la superficie externa y la estructura interna de las células de *Leuconostoc mesenteroides*, incluyendo rotura de las cadenas de cocos, formación de vesículas en la superficie celular, y un incremento progresivo en la desnaturalización de los ribosomas (Kaletunç *et al*., 2004). Otro estudio indicó que un tratamiento leve por APH (345 MPa a 25ºC durante 5 minutos) inducía la lisis celular en una suspensión de *L. mesenteroides* y provocaba una pérdida de viabilidad celular superior a 6 ciclos logarítmicos (Kalchayanand *et al*., 2002). No hay estudios previos sobre los efectos de la APH en otros leuconostocs alterantes de alimentos, tales como *L. gelidum* y *L. gasicomitatum*.

La eficacia de los tratamientos por APH puede variar dependiendo de la bacteria diana y la matriz alimentaria y, por lo tanto, cada alimento específico necesita ser ensayado en el laboratorio o a escala piloto antes de pasar a la aplicación industrial. En el presente estudio fueron necesarios tratamientos de al menos 600 MPa para lograr una reducción logarítmica de al menos 6 ciclos logarítmicos en un cóctel de leuconostocs inoculados en pulpa de chirimoya. La adición de enterocina AS-48 a 35 μg/g tuvo un efecto limitado en la inactivación de los leuconostocs, probablemente debido a la alta carga bacteriana del inóculo utilizado y también por una posible interacción de la bacteriocina con la matriz del alimento. Resultados anteriores sobre la aplicación en diferentes productos alimentarios indican que la eficacia de la enterocina AS-48 depende de varios factores, incluyendo el tipo de bacteria y el alimento (Abriouel *et al*., 2010). El tratamiento combinado de enterocina AS-48 (50 μg/g) y APH (600 MPa, 8 minutos) mejoró la inactivación de la microbiota epífita inoculada en la pulpa de chirimoya y también retrasó el crecimiento de los supervivientes durante el almacenamiento, comparado con el tratamiento simple de presión (Pérez-Pulido *et al*., 2015). Así mismo, la aplicación de la enterocina AS-48 en combinación con tratamientos por APH mejoró la inactivación de *Salmonella enterica* en fuet y de *Staphylococcus aureus* en arroz con leche (Ananou *et al*., 2010; Pérez-Pulido *et al*., 2012). Otras bacteriocinas, tales como la nisina y la pediocina PA1/Ach han demostrado que potencian los efectos bactericidas de los tratamientos por APH (revisado por Gálvez *et al*., 2008; Kalchayanand *et al*., 1994; Kalchayanand *et al*., 2004).

Este ha sido el primer trabajo en el que se ha ensayado la enterocina AS-48 frente a leuconostocs en un producto alimentario, sola o en combinación con APH. Curiosamente, aunque la contribución de la enterocina AS-48 en la inactivación microbiana fue pequeña, la bacteriocina añadida tuvo un efecto positivo en el mantenimiento de unos recuentos

bacterianos inferiores comparados con los tratamientos simples de presión durante el almacenamiento de las muestras bajo condiciones de refrigeración. Además, cuando las muestras se almacenaron a 22ºC para simular unas condiciones de abuso de temperatura, el retraso en el crecimiento bacteriano fue mayor en las muestras tratadas a 600 MPa en combinación con la bacteriocina, comparado con el tratamiento simple de presión. En conjunto, estos resultados sugieren que la bacteriocina añadida ejerce un efecto protector durante el almacenaje de los alimentos presurizados. La pulpa de chirimoya podría ser considerada como un alimento funcional debido a sus componentes fenólicos bioactivos (Loizzo *et al.*, 2012; Roesler *et al.*, 2006). Por lo tanto, parece interesante estabilizar el producto elaborado por procesos no-térmicos, como la APH, con el fin de preservar mejor sus componentes bioactivos. El control de los leuconostocs por tratamientos APH, solos o en combinación con bacteriocina, también podría ser un interesante enfoque para proporcionar alimentos seguros a una población de alto riesgo, susceptible a las infecciones oportunistas como las que se han descrito de forma esporádica por esta bacteria.

A menudo, la principal fuente de contaminación microbiana de las frutas y hortalizas procesadas es la propia microbiota superficial residente en estos vegetales, dando lugar a su deterioro durante el almacenamiento. Debido a sus propiedades funcionales y beneficiosas para la salud, en nuestro estudio utilizamos la chirimoya, cuyo cultivo intensivo se extiende por la costa tropical española y su consumo es muy habitual entre la población, aunque esta fruta se deteriora fácilmente y su vida útil es muy limitada. Hasta el momento no hay estudios previos sobre la microbiota epífita de la chirimoya o sobre las bacterias contaminantes que pueden proliferar en esta fruta almacenada bajo condiciones de refrigeración, por lo que resultó de gran interés profundizar en este ámbito.

En nuestro trabajo, las pulpas de chirimoya se inocularon con la microbiota recuperada de su superficie, a una concentración celular final de 6.4 log_{10} CFU/g, para simular un escenario de elevada contaminación durante la preparación de la pulpa y también para garantizar que se podían medir reducciones logarítmicas de al menos 5 unidades tras la aplicación de los tratamientos. Un inóculo de alta densidad también nos podía facilitar el estudio de los cambios en la fracción superviviente cultivable durante el almacenamiento de las muestras tratadas. Sin embargo, según nuestra experiencia, la carga microbiana en las pulpas bajo condiciones normales de contaminación podría ser mucho menor, de aproximadamente entre 2 y 4 log_{10} CFU/g. En nuestro trabajo utilizamos la

pirosecuenciación del gen 16S rRNA para evaluar la diversidad microbiana de la fracción superviviente capaz de crecer en las placas de TSA a partir de pulpa de chirimoya artificialmente contaminada con su microbiota de superficie, tanto después de los tratamientos por APH, con enterocina AS-48, o con una combinación de los dos, como durante el almacenamiento. Evidentemente, los resultados de pirosecuenciación obtenidos están limitados por las condiciones experimentales utilizadas en el estudio, ya que solo podía ser analizada la fracción bacteriana que fue seleccionada a partir de las condiciones de crecimiento en placas de TSA. Por lo tanto, los resultados pueden estar sesgados por las condiciones de crecimiento y no deben extrapolarse a la composición microbiana total de las muestras. No obstante, aunque este enfoque no refleja necesariamente la abundancia de los taxones en las muestras originales, permite eliminar el problema de la interferencia del ADN de las células muertas tras los tratamientos.

La pirosecuenciación figura entre las técnicas de secuenciación masiva más ampliamente utilizadas para el análisis de las comunidades bacterianas, a pesar de las limitaciones de usar fragmentos cortos de genes para la identificación a nivel de especies. No obstante, las similitudes de las secuencias obtenidas en este estudio estaban en un rango del 97–100%, con las correspondientes secuencias depositadas en bases de datos. El phylum *Proteobacteria* fue el principal grupo bacteriano representado en la microbiota de la pulpa de chirimoya recuperada de las placas de TSA, seguido por *Firmicutes*. El phylum *Actinobacteria* solamente fue detectado en abundancia relativamente alta en dos muestras tratadas. Las unidades taxonómicas operativas (OTUs) pertenecientes a *Bacteroidetes* y *Cyanobacteria* solo aparecieron en algunas muestras, y siempre tuvieron abundancias relativas muy bajas, no superiores al 0.08%. Sorprendentemente, *Enterobacteriaceae* fue el principal grupo bacteriano recuperado a partir de la pulpa de chirimoya artificialmente contaminada. Los miembros de la familia *Enterobacteriaceae* incluyen patógenos oportunistas, saprófitos, comensales, así como patógenos que causan severas infecciones en humanos y animales. Un reciente estudio, también basado en la pisosecuenciación, mostró que la familia *Enterobacteriaceae* tenía las mayores abundancias relativas en la microbiota de varios alimentos vegetales, incluyendo brotes de soja y alfalfa, espinaca, lechuga, tomate, pimiento y fresas (Leff y Fierer, 2013). Los principales representantes de *Enterobacteriaceae* recuperados de la pulpa de chirimoya no tratada pertenecían al género *Pantoea*. La putativa especie *Pantoea* sp. también fue una OTU particularmente abundante en muchos estos tipos de productos que albergan una gran proporción de *Enterobacteriaceae* (tales como brotes de soja, espinaca y pimiento). *Pantoea* spp. se aísla frecuentemente de una amplia variedad de

nichos ecológicos y tiene diversas funciones biológicas, tales como epífitas o endófitas de plantas, agentes de control biológico, promotores del crecimiento vegetal o como patógenos de plantas y animales, incluyendo los seres humanos (De Maayer *et al.*, 2012). En concreto, *P. agglomerans* es una causa poco frecuente de enfermedad en humanos, típicamente asociada a lesiones producidas por pinchazos con espinas, fluidos parenterales contaminados y condiciones debilitantes en los pacientes (Lalas y Erichsen, 2010; Shubov *et al.*, 2011). No obstante, se han registrado dos preparaciones comerciales basadas en *P. agglomerans* (BlossomBless™ y Bloomtime™) y otra basada en *P. vagans* (BlightBan C9-1™) que se utilizan como agentes de biocontrol. En este estudio, la alta abundancia detectada para las OTUs de estas dos especies en la pulpa de chirimoya podría deberse a la natural adaptación de *Pantoeae* a esta fruta tropical y/o a su intencionado uso como agentes de control biológico en las operaciones agrícolas.

Hay un debate abierto acerca de los riesgos para la salud humana de las cepas de *Pantoea* utilizadas como agentes de control biológico (las cuales se incluyen como patógenos oportunistas en el nivel 2 de bioseguridad), aunque también parece ser que muchos de los aislados clínicos podrían haber sido clasificados de forma errónea como *Pantoea* (Rezzonico *et al.*, 2009). También ha sido cuestionada la presencia de *Pantoea* en los alimentos, debido a que algunas cepas pueden portar caracteres de resistencia a antibióticos o biocidas (Aibinu *et al.*, 2012; Blaak *et al.*, 2014; Schwaiger *et al.*, 2011; Fernández-Fuentes *et al.*, 2014). A pesar de que las identidades de las secuencias obtenidas para las especies *Pantoea* de nuestro estudio fueron muy altas (de 97 a 100%), se necesita llevar a cabo un trabajo adicional que implique el aislamiento y la identificación confirmativa de las teóricas *Pantoea* aquí descritas y confirme su susceptibilidad a los antibióticos. Las OTUs correspondientes a enteropatógenos humanos, tales como *Escherichia coli* o *Salmonella enterica*, no fueron detectadas con una abundancia relativa significativa en la biomasa bacteriana crecida sobre TSA a partir de las pulpas control, y solamente se detectó *Escherichia fergusonii* en el día 30 a una abundancia relativamente alta. Las OTUs pertenecientes a otros miembros de *Enterobacteriaceae* (*Erwinia aphidicola*, *Erwinia persicina*, *Enterobacter kobei*, *Yersinia ruckeri*, *Serratia plymuthica* y *Leclercia adecarboxylata*), detectados también en bajos porcentajes en las primeras etapas, no aumentaron durante el almacenamiento, aunque ilustran la amplia diversidad microbiana que se puede encontrar en las chirimoyas. Curiosamente, los enterococos (principalmente *Enterococcus gallinarum* y, en menor medida, *Enterococcus casseliflavus*) pudieron aumentar considerablemente en abundancia relativa durante el periodo de almacenaje según se deduce del crecimiento bacteriano recuperado partir de la

pulpa de chirimoya no tratada. Se conoce que las especies de enterococos móviles *E. gallinarum* y *E. casseliflavus* están asociadas con las plantas y los frutos (Micallef *et al*., 2013; Ong *et al*., 2014), pero también se pueden encontrar en el intestino de los animales y en las carnes (Byappanahalli *et al*., 2012).

Uno de los objetivos de este estudio era determinar el efecto de los tratamientos con enterocina AS-48 y alta presión hidrostática, solos o combinados, en las poblaciones microbianas de la pulpa de chirimoya y su posible influencia sobre los cambios en la microbiota durante el almacenamiento. La enterocina AS-48 posee un amplio espectro inhibitorio contra bacterias Gram-positivas pero, en ausencia de otros factores, tiene un muy bajo efecto sobre bacterias Gram-negativas (Maqueda *et al*., 2004). Esta diferencia en la actividad inhibitoria se observó claramente cuando la pulpa de chirimoya se suplementó con AS-48, ya que inhibió la proliferación de los principales *Firmicutes* detectados en los controles durante el almacenaje (fundamentalmente enterococos). De hecho, los ensayos de los recuentos en placa indicaron que la enterocina AS-48 sola no inhibió la proliferación de la microbiota y las diferencias en los recuentos de las células viables entre los controles y las muestras tratadas con enterocina no fueron estadísticamente significativas. Sin embargo, las muestras suplementadas con AS-48 sí mostraron diferencias en las OTUs predominantes de *Proteobacteria* durante el almacenaje, comparadas con los controles no tratados (especialmente en los días 7, 15 y 30). Esto podría ser un efecto indirecto de la inhibición de enterococos por la bacteriocina, o quizás la bacteriocina también tenga efectos inhibitorios en el crecimiento de algunos Gram-negativos encontrados en la pulpa de chirimoya inoculada. En un estudio anterior, el análisis de las comunidades microbianas, llevado a cabo mediante el uso de electroforesis en gel desnaturalizante en gradiente (DGGE), demostró que la aplicación de un tratamiento de lavado con enterocina AS-48 inducía cambios en la comunidad bacteriana de los brotes de soja, lo que implicó un descenso en la población de *Pantoea* sp., *Eschrichia hermannii* y *Enterobacter* sp., y un incremento en las poblaciones de *Serratia* sp. y *Serratia plymuthica*, *Enterococcus* sp. y *Leuconostoc inhae* (Cobo Molinos *et al*., 2009). Estos cambios se asemejan con claridad a los obtenidos en nuestro estudio, al menos para *Pantoea* sp. y *S. plymuthica*.

En general, nuestros resultados revelan cómo el efecto de añadir bacteriocinas en los sistemas alimentarios afecta a toda la comunidad microbiana y no solo a las bacterias diana. Teniendo en cuenta que los alimentos son ecosistemas complejos en los cuales las diferentes poblaciones microbianas interactúan entre sí (por medio de la cooperación,

competencia por los nutrientes, amensalismo, etc.), se espera que los factores que influyen en las poblaciones microbianas individuales también tengan un impacto sobre la comunidad microbiana entera.

Aunque el tratamiento por APH redujo los recuentos de células viables de las muestras en 5 unidades logarítmicas, no todas las poblaciones microbianas detectadas parecían estar afectadas por igual. Cabe destacar que el aumento en la abundancia relativa de las formadoras de esporas, observada tempranamente tras los tratamientos APH, podría explicarse por el hecho de que las endosporas bacterianas son resistentes a los tratamientos por APH aplicados. Además, la germinación de las endosporas puede ser inducida mediante los tratamientos por APH y, en ausencia de competidores, pueden proliferar y llegar a ser la población dominante después del tratamiento (Rastogi *et al.*, 2007; Rendueles *et al.*, 2011). Las especies del género *Bacillus* son conocidas por su capacidad para producir una gran variedad de sustancias antimicrobianas (Abriouel *et al.*, 2011) y enzimas extracelulares, lo que puede facilitar la utilización de los diferentes nutrientes del sustrato y el desplazamiento de los competidores. Resulta interesante que entre las principales OTUs de formadores de endosporas detectadas se encontrasen *Bacillus firmus* y *Bacillus plakortidis*, ambas bacterias tolerantes a la alcalinidad pero que también han sido aisladas de ambientes de agua marina (Borchert *et al.*, 2007; Geng *et al.*, 2014). La Costa Tropical del sur de España es bien conocida por el cultivo intensivo de chirimoyos. Se podría especular que las endosporas de estas bacterias pudieran ser transportadas hasta las plantaciones de chirimoya a través de los vientos marinos, pero no se puede descartar la posibilidad de que también puedan vivir como bacterias epífitas. En este trabajo no fueron detectados bacilos formadores de esporas causantes de intoxicación alimentaria, como *Bacillus cereus*. Sin embargo, se ha descrito el potencial de la producción de toxinas por *B. firmus* (Taylor *et al.*, 2005). Durante el posterior almacenaje de las muestras y dado el aumento de los recuentos de células viables, también pudo observarse que las poblaciones residuales de *Pantoea* sp. supervivientes a los tratamientos APH fueron capaces de crecer rápidamente y sobrepasar a las formadoras de esporas, y que los leuconostocs supervivientes también proliferaron durante la última etapa de almacenaje. Los leuconostocs están bien adaptados para fermentar substratos vegetales y crecer a bajas temperaturas. En condiciones selectivas adecuadas, los leuconostocs desplazan a la microbiota epífita y llevan a cabo una fermentación ácido-láctica. Sin embargo, los resultados obtenidos indicaron claramente que bajo el almacenaje en frío de la pulpa de chirimoya, *Pantoea* fue mucho más competitiva que los leuconostocs. Esto también fue evidente en la pulpa de chirimoya control, en la que los

leuconostocs solo proliferaron al final del periodo de almacenamiento, probablemente cuando se habían agotado los nutrientes para *Enterobacteriaceae*.

Nuestros resultados indican que la aplicación del tratamiento por APH en combinación con enterocina AS-48 fue el tratamiento más eficaz para mejorar la calidad y seguridad microbiológica de la pulpa de chirimoya, pues tenía un efecto mucho más pronunciado que los tratamientos individuales en el mantenimiento de las concentraciones de células viables a bajos niveles durante un almacenamiento de al menos 15 días y en la reducción de las poblaciones de ambas bacterias Gram-positivas y Gram-negativas. En estudios anteriores, la aplicación de enterocina AS-48 en combinación con tratamientos por APH mejoró la inactivación de *S. enterica* en fuet y de *Staphylococcus aureus* en arroz con leche (Ananou *et al.*, 2010; Pérez-Pulido *et al.*, 2012). Los efectos sinérgicos entre otras bacteriocinas y los tratamientos por APH han sido descritos en diversos estudios (revisado por Gálvez *et al.*, 2008; Kalchayanand *et al.*, 1994). El mecanismo de acción de la bacteriocina AS-48 se basa en la permeabilización de la membrana citoplasmática bacteriana, dando lugar a un rápido colapso del potencial de membrana (Gálvez *et al.*, 1991). La adición de bacteriocinas también tiene otros efectos secundarios, como la inducción de la autolisis celular. Las actividades sinérgicas mencionadas en los anteriores estudios, así como aquellas observadas para la enterocina AS-48 del presente estudio, podrían deberse al efecto de las bacteriocinas sobre la pared celular y la membrana celular en las bacterias sensibles, junto con un aumento en la sensibilidad hacia las bacteriocinas de las células dañadas por la presurización.

Nuestros resultados indican claramente la complejidad de las poblaciones microbianas derivadas de la superficie de las chirimoyas, y cómo estas poblaciones pueden cambiar durante el almacenamiento de la pulpa de chirimoya contaminada. Es evidente que los tratamientos para la conservación de los alimentos inducen cambios en las poblaciones microbianas de esos alimentos y esto depende del tipo de tratamiento aplicado.

Además del cultivo de chirimoya, España es uno de los mayores países productores de espárragos. Esta hortaliza es bastante susceptible a las condiciones de almacenamiento (temperatura, humedad, tasa de respiración, etc.), por lo que es altamente propensa al deterioro durante la post-cosecha. Dado que los espárragos crecen sobre la superficie del suelo, están sujetos a una alteración y contaminación microbianas que los hacen vulnerables

en el almacenaje, donde la mayoría de enfermedades son producidas por hongos. Al igual que en el trabajo anterior, hay pocos estudios sobre la microbiota epífita residente en espárragos. La carga microbiana inicial en espárragos verdes frescos ha sido investigada por varios autores mediante métodos dependientes de cultivo (García-Gimeno *et al*., 1998; Sothornvit y Kiatchanapaibul, 2009), reflejando recuentos de aerobios mesófilos totales y bacterias psicrófilas cercanos a los 5 log CFU/g. Un aumento en estos niveles indica el comienzo de la alteración de los alimentos. Dado que la presencia y el número de microorganismos en los alimentos vegetales depende del tipo de cultivo, las prácticas agrícolas, el área geográfica y las condiciones climáticas (Brackett, 1999), es necesario desarrollar técnicas que minimicen la contaminación y prevengan el crecimiento de microorganismos patógenos y alterantes. A pesar de que los tratamientos por alta presión hidrostática se sitúan en la cima entre los métodos no-térmicos par el procesado de alimentos, no hay estudios previos sobre el efecto de la aplicación de los tratamientos APH en espárragos verdes. De acuerdo con los resultados del presente estudio, los tratamientos por APH a 600 MPa durante 8 minutos podrían estabilizar los espárragos verdes en salmuera hasta un mes bajo condiciones de refrigeración en un escenario de intensa contaminación, en el peor de los casos, cercana a los 7 log CFU/g. En comparación con los procesos térmicos que se utilizan actualmente, los tratamientos por APH ofrecen el potencial de ser un método suave para la conservación del espárrago verde fresco en salmuera. Los resultados de los experimentos de almacenamiento realizados a 22ºC sugieren que los espárragos tratados también podrían soportar una exposición limitada a condiciones de abuso de temperatura de al menos un día, pero no para periodos más largos de tiempo, debido a la proliferación de los microorganismos supervivientes. Por ello, es importante estudiar la dinámica de las poblaciones microbianas durante el almacenaje de los alimentos, incluyendo aquellos estabilizados por el tratamiento APH, con el fin de evaluar los principales grupos bacterianos que pueden proliferar tras los tratamientos e involucrarse en su alteración.

En este estudio volvimos a aplicar un método independiente de cultivo, basado en la pirosecuenciación del gen 16S rRNA, con el fin de investigar la microbiota de la superficie del espárrago verde y determinar el impacto del tratamiento APH y los cambios en la fracción superviviente durante el almacenamiento en frío y a temperatura ambiente. La pirosecuenciación se utiliza ampliamente para el análisis de las comunidades microbianas y para la identificación a nivel de especie. Sin embargo, solamente un número muy limitado de estudios han aplicado esta tecnología para estudiar la diversidad microbiana de los

alimentos tratados por APH (Pérez-Pulido *et al.*, 2015). En el presente estudio, las muestras fueron tratadas con Monoazida de Propidio (PMA) antes de la extracción del DNA, con el fin de evitar la interferencia del DNA de las células muertas en las etapas de amplificación para la posterior pirosecuenciación. Se ha descrito que el PMA penetran en las céllas muestras, uniéndose al ADN y bloqueando su posterior amplificación por PCR, lo que en definitiva permite discriminar cuantitativamente entre células vivas y muertas (Nocker *et al.*, 2006, 2007).

De entre las OTUs detectadas a partir de espárragos verdes artificialmente contaminados con su propia microbiota de superficie, *Enterobacteriaceae* fue el grupo bacteriano más abundante, representado esencialmente por miembros del género *Rahnella*, *Raoultella*, *Pantoea*, *Erwinia*, *Serratia* y *Escherichia*. Estas bacterias también predominaron durante todas las etapas de almacenaje, tanto en muestras presurizadas como en las muestras control, aunque con distintas abundancias relativas. La familia *Enterobacteriaceae* es un grupo heterogéneo de bacterias Gram-negativas, que comprende microbios mesófilos saprófitos, comensales y patógenos oportunistas, y está ampliamente distribuido en suelos, agua, vegetación e intestinos de animales y humanos (Dworkin *et al.*, 2006). También se encuentra presente en altos niveles en vegetales mínimamente procesados (Oliveira *et al.*, 2010; Abadias *et al.*, 2008; Li *et al.*, 2001), siendo parte de la microbiota inicial. Los niveles de *Enterobacteriaceae* en lechuga pueden variar entre 3 y 7 log_{10} CFU/g, tal y como se describe en un trabajo previo por Oliveira *et al.* (2010), algo que es común en los vegetales crudos y no necesariamente está asociado con una contaminación fecal. Un reciente estudio por Leff y Fierer (2013), basado en la pirosecuenciación, también demuestra que *Enterobacteriaceae* tiene una abundancia relativamente alta en la mayoría de los vegetales analizados (brotes de soja y alfalfa, fresas, espinaca, lechuga, tomate y pimiento), siendo *Pantoea* sp. el taxón más representado. En el presente estudio sobre espárragos verdes, durante los primeros días de almacenamiento a 4ºC, las OTUs pertenecientes a la especie *Pantoea ananatis* mostraron una mayor abundancia relativa, tanto en los espárragos control como en los presurizados. *Pantoea* también fue detectada como la OTU predominante en la microbiota de la chirimoya (Pérez- Pulido *et al.*, 2015). Las especies del género *Pantoea* se aíslan frecuentemente a partir del material vegetal como parte de la microbiota epífita o endófita de la planta, aunque pueden ocupar diferentes nichos ecológicos. Algunas cepas actúan como promotores del crecimiento vegetal (De Maayer *et al.*, 2014), mientras que otras son patógenas de plantas y seres humanos, causando enfermedades y bacteriemia.

Pantoea está siendo utilizada como agente de control biológico, debido a su capacidad de producir sustancias antimicrobianas (Coutinho y Venter, 2009; Walterson *et al*., 2014).

Un estudio dependiente de cultivo, realizado por García-Gimeno *et al.* (1998) indicó que la alteración de los espárragos verdes envasados se correlaciona con altos niveles de bacterias psicrótrofas, en su mayoría pertenecientes a bacterias del ácido láctico y al género *Pseudomonas*. *Pseudomonas* sp. es un grupo muy heterogéneo de bacterias saprófitas que se encuentra en el suelo, la materia orgánica en descomposición, la vegetación y el agua, además de ser patógenos oportunistas de plantas, animales y humanos (Cornelis, 2008). En el presente estudio, la abundancia relativa de las OTUs pertenecientes a *Pseudomonas* se mantuvo en valores relativamente altos durante el almacenamiento en refrigeración de las muestras control, pero no en las muestras control almacenadas a 22ºC o en cualquiera de las muestras tratadas por APH. Sería de esperar que el grupo *Pseudomonas* (que incluye especies psicrótrofas) tuviera una mayor capacidad para sobrevivir durante el almacenaje en frío, aunque no se esperaba que creciese a pH ácido, y podría ser desplazado al establecerse unas condiciones fermentativas a 22ºC por el rápido crecimiento de enterobacterias.

Los miembros de la familia *Enterobacteriaceae* parecían ser el principal grupo bacteriano implicado en el deterioro de los espárragos verdes en salmuera. La reducción en la abundancia relativa observada en *Enterobacteriaceae* tras los tratamientos APH podría estar asociada a una reducción de la alteración del producto y sugeriría que los miembros de este grupo eran más sensibles a los tratamientos APH que *Bacteroidetes* y *Firmicutes.* Curiosamente, la población de *Enterobacteriaceae* no se recuperaría durante el almacenamiento en refrigeración de las muestras tratadas por APH dejando a *Bacteroidetes* (principalmente *Flavobacteriaceae* y, en menor medida, *Sphingobacteriaceae*) como el grupo predominante. *Flavobacteria* y *Sphingobacteria* son habitantes naturales del suelo y del agua, equipados con una variedad de enzimas exocelulares, importantes para la descomposición de biopolímeros (Bernardet y Nakagawa, 2006). No obstante, su actividad metabólica en los espárragos almacenados en frío podría estar limitada por la baja temperatura, el pH ácido y la baja disponibilidad de oxígeno. Una fracción relativamente estable de *Firmicutes* de la familia *Enterococcaceae* y la familia *Streptococcaceae* también fue detectada en las muestras tratadas por APH durante el almacenamiento en frío. Estos *Firmicutes* se adaptan bien a entornos ácidos, pero no se multiplican, o bien, crecen muy lentamente durante la refrigeración. Por el contrario, cuando las muestras tratadas por APH se almacenaron a 22ºC, aumentó la abundancia relativa de las OTUs pertenecientes a *Enterobacteriaceae*

(especialmente *Escherichia*) y, en menor medida, también *Firmicutes*, al mismo tiempo que el recuento de células viables se incrementó en 3.5 ciclos logarítmicos. Sorprendentemente, la biodiversidad de las muestras se redujo considerablemente en aquellas muestras tratadas por APH durante el último periodo de almacenaje a 22ºC, lo que indica que sólo unos pocos grupos microbianos que sobrevivieron a los tratamientos APH fueron capaces de proliferar en los espárragos.

Se podría esperar que los formadores de esporas (como los *Paenibacillus*, *Bacillus* y *Clostridium* detectados) sobreviviesen a los tratamientos por APH, ya que las endosporas bacterianas son, generalmente, abundantes en los materiales vegetales y son resistentes a los tratamientos de presión aplicados en el presente estudio. Además, son importantes en la producción de enzimas para la descomposición de sustancias poliméricas y la liberación de los carbohidratos fermentables. La abundancia relativa de OTUs correspondientes a *Clostridium* sp. no superó el 5% en espárragos presurizados almacenados a temperatura ambiente. La presencia de endosporas en los alimentos deben ser cuidadosamente investigadas porque, al estar presentes en suelos, generalmente son un indicador de que ha habido una higiene insuficiente o un manejo inadecuado durante el procesamiento. Aunque algunas especies de *Clostridium* se asocian con incidentes de intoxicación alimentaria debido a la producción de neurotoxinas, otras están implicadas en la alteración de alimentos, provocando cambios en la calidad sensorial (producción de ácido butírico, olores pútridos, etc.), lo que se traduce en grandes pérdidas económicas (Brown, 2000). La presencia de las BAL en espárragos en salmuera, junto con el pH ácido de la salmuera utilizada, puede crear un ambiente difícil para la germinación de las endosporas de *Clostridium*, como se ha sugerido de un modo más general para los alimentos de bajo pH (Black *et al.*, 2007).

Nuestros resultados indican que las reducciones de las cargas microbianas en espárragos verdes mediante un tratamiento por APH a 600 MPa durante 8 minutos, prolongan la vida útil de los espárragos en salmuera durante el almacenamiento bajo refrigeración. La carga microbiana de los espárragos verdes obtenida en otros estudios es, al menos, 2 ciclos logarítmicos inferior comparada con las muestras contaminadas artificialmente utilizadas en el presente estudio. Por lo tanto, se esperaría una fracción superviviente mucho menor tras el tratamiento APH en espárragos convencionales (no inoculados). A pesar de que en los espárragos verdes no todas las poblaciones bacterianas se ven igualmente afectadas por los tratamientos mediante APH, determinados grupos de

Enterobacteriaceae tienen una mayor capacidad de proliferación en los espárragos tratados almacenados a 22ºC, dando lugar a un rápido deterioro.

A lo largo del estudio realizado hemos podido comprobar que existen diferentes oportunidades para mejorarla eficacia de los tratamientos por APH, mediante el uso combinado con sustancias antimicrobianas naturales. Estas combinaciones pueden ser útiles no solo para la inactivación de los patógenos clásicos, sino también para patógenos emergentes y para combatir la transmisión de resistencias a antimicrobianos en la cadena alimentaria. Sin embargo, se conoce muy poco sobre el impacto que tienen los tratamientos por APH sobre el conjunto de las poblaciones microbianas que se pueden encontrar en los alimentos. Este aspecto puede ser fundamental para predecir la evolución de la microbiota superviviente durante la vida útil del producto, como ha quedado demostrado en los estudios que hemos realizado con pulpa de chirimoya y con espárragos. Cabe esperar que en un futuro este tipo de estudios se extiendan a otras categorías de alimentos, lo que permitiría obtener una visión mucho más exacta de la complejidad y la dinámica de las poblaciones microbianas en los sistemas alimentarios.

CONCLUSIONES

1. Los tratamientos por alta presión a 600 MPa durante 10 minutos provocaron una reducción superior a 7 unidades logarítmicas en las concentraciones de estafilococos meticilina-resistentes inoculados en arroz con leche.

2. Las combinaciones de tratamientos subletales por altas presiones con nisina, enterocina AS-48 o aceite de canela redujeron las concentraciones de estafilococos resistentes a meticilina en arroz con leche de forma no significativa tras la aplicación del tratamiento y de forma significativa durante el almacenamiento del alimento.

3. El tratamiento por presión a 680 MPa durante 8 minutos fue el más eficaz para la inactivación de *Salmonella enterica* en tortilla de patata y también el que más retrasó la recuperación de las células con daños subletales durante el almacenamiento.

4. Los patógenos transmisibles por alimentos *Listeria monocytogenes*, *Salmonella enterica* y *Escherichia coli* difieren considerablemente en su capacidad de supervivencia en salmorejo, siendo ésta muy baja en el primero y muy elevada en el último de ellos.

5. El tratamiento por presión a 680 MPa durante 8 minutos puede ser un método no térmico eficaz para la preparación a escala industrial de salmorejo libre de conservantes y con una mayor seguridad frente a la posible transmisión de patógenos como *Listeria monocytogenes*, *Salmonella enterica* y *Escherichia coli*.

6. El tratamiento por presión a 680 MPa durante 8 minutos aplicado de forma individual sobre pulpa de chirimoya inoculada con su microbiota epifita reduce la carga microbiana en 5 unidades logarítmicas, pero no previene su posterior crecimiento tras 7 días de almacenamiento en frío.

7. El tratamiento combinado por presión (600 MPa, 8 minutos) junto con enterocina AS-48 a 50 μg/g fue el más eficaz, evitando el crecimiento de la fracción bacteriana superviviente durante al menos 15 días de almacenamiento en frío.

8. El análisis de la biodiversidad microbiana procedente de pulpa de chirimoya reveló que *Pantoea* es la unidad taxonómica operativa con mayor abundancia relativa durante los 7 primeros días de almacenaje de la pulpa.

9. El tratamiento individual por alta presión redujo inicialmente de forma significativa la abundancia relativa de *Pantoea* e incrementó fuertemente la de formadores de endosporas aerobios.

10. El crecimiento bacteriano tardío observado en la pulpa sometida a tratamientos combinados por alta presión y enterocina AS-48 se debió a una sucesión de poblaciones de formadores de endosporas aerobios, enterobacterias y leuconostocs.

11. La aplicación de tratamientos a 600 MPa durante 8 minutos en espárragos verdes colocados en salmuera redujo los recuentos de células viables en 3,6 unidades logarítmicas. La fracción superviviente residual no incrementó durante el almacenaje a 4 ºC, pero sí lo hizo a 22ºC, alterando el producto rápidamente.

12. La microbiota de los espárragos verdes estaba compuesta principalmente por miembros de *Proteobacteria*, seguido de *Firmicutes* y, en menor grado, *Bacteroidetes* y *Actinobacteria*.

13. El tratamiento por altas presiones redujo la abundancia relativa de *Proteobacteria* respecto a *Bacteroidetes* justo después del tratamiento así como durante el almacenamiento en frío.

14. En conjunto, los resultados del estudio indican que los tratamientos por altas presiones no solo reducen la viabilidad de las poblaciones microbianas presentes en los alimentos, sino que también tienen un impacto en las dinámicas de las poblaciones supervivientes durante el almacenamiento de los productos tratados.

CONCLUDING REMARKS

1. High hydrostatic pressure treatments at 600 MPa for 10 min caused inactivation of methicillin-resistant *Staphylococcus aureus* (MRSA) greater than 7 log cycles in rice pudding.

2. Combinations of sublethal HHP treatments with nisin, enterocin AS-48 or cinnamon oil reduced viable counts of MRSA non-significantly after treatment and significantly during storage.

3. A HHP treatment at 680 MPa for 8 min was most effective in the inactivation of *Salmonella enterica* in Spanish omelette and delayed recovery of sublethally-injured cells during storage.

4. The foodborne pathogens *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli* differ greatly in their capacity to survive in salmorejo, ranging from very low survival in the first case to very high in the latest.

5. High hydrostatic pressure treatment at 600 MPa for 8 min can be an efficient nonthermal method for industrial-scale preparation of preservative-free salmorejo with improved safety against transmission of foodborne pathogens *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli*.

6. High hydrostatic pressure treatment at 600 MPa for 8 min applied singly on cherimoya pulp artificially contaminated with its own epiphytic microbiota reduced viable counts by 5 log cycles, but it did not prevent further growth of survivors by day 7 during refrigeration storage.

7. The combined treatment of HHP at 600 MPa for 8 min in combination with enterocin AS-48 at 50 μg/g was far more effective, preventing regrowth of survivors in cherimoya pulp for at least 15 days of cold storage.

8. Microbial biodiversity analysis revealed that *Pantoea* were the operational taxonomic units (OTUs) detected at highest relative abundance in bacterial biomass grown from control cherimoya pulp samples for the first 7 days of storage.

9. The single HHP treatment of cherimoya pulp significantly reduced the relative abundance of OTUs belonging to *Pantoea* and strongly increased that of aerobic endosporeformers early after treatment.

10. The late bacterial growth observed from cherimoya pulp after application of combined treatments by HHP and enterocin AS-48 was due to a succession of microbial populations belonging to endosporeformers, enterobacteria and leuconostocs.

11. High hydrostatic pressure treatments at 600 MPa for 8 min applied on brined green asparagus reduced viable cell counts by 3.6 log cycles. The residual surviving population did not increase during storage at 4 °C, but it did grow and cause rapid spoilage in samples stored at 22 ºC.

12. The microbiota of green asparagus was composed mainly by *Proteobacteria*, followed by *Firmicutes* and to a less extent *Bacteroidetes* and *Actinobacteria*.

13. The HHP treatment reduced the relative abundance of *Proteobacteria* compared to *Bacteroidetes* both early after treatment and during chill storage.

14. Overall, results from the study indicate that HHP treatments not only reduce the viability of microbial populations in foods but also have an impact on the dynamics of microbial populations during the storage of the treated foods.

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