Universitat de Lleida

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El presente trabajo se ha realizado en la planta piloto y en el laboratorio de Noves Tecnologies de Processat d'Aliments del Departament de Tecnologia d'Aliments de la Universitat de Lleida. Para su realización se ha contado con la colaboración de la sección de microbiología del Departamento de Ciencias de la Salud de la Universidad de Jaén.

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RESUMEN

La leche se ha procesado tradicionalmente mediante el uso de calor para asegurar su calidad microbiológica y su estabilidad enzimática. Sin embargo, los cambios organolépticos y la pérdida de componentes nutritivos inducidos por el calentamiento podrían minimizarse con el empleo de tratamientos no térmicos, como los pulsos eléctricos de alta intensidad de campo (PEAIC). El presente trabajo tuvo como objetivo general estudiar la efectividad del tratamiento con PEAIC sobre la supervivencia del patógeno *Staphylococcus aureus* en leche, así como el efecto resultante de su combinación con substancias antimicrobianas.

La inactivación de *Staph. aureus* debida a la aplicación de PEAIC se estudió mediante un diseño experimental de superficie de respuesta en el que las variables independientes fueron el número, anchura y polaridad de pulso, la intensidad de campo y el porcentaje de grasa de la leche. De modo similar, se evaluó el efecto individual y conjunto de la adición de nisina, lisozima, enterocina AS-48 o de sus combinaciones con la aplicación de PEAIC. En este último caso, se determinó el grado de destrucción en función del tiempo de tratamiento, la intensidad de campo, la concentración y tiempo de exposición al antimicrobiano, el pH, la temperatura de almacenamiento y la secuencia de tratamiento. Se determinó la vida útil en refrigeración (4ºC) de leche tratada con PEAIC y antimicrobianos a partir de la evolución de la flora mesófila, enterobacterias, coliformes y los patógenos *Escherichia coli*, *Listeria* spp., *Salmonella* spp. y *Staphylococcus aureus*. Se comparó también la evolución de la flora de la leche pasteurizada con la de leche sometida simultáneamente a un calentamiento moderado, la adición de antimicrobianos y la aplicación de PEAIC.

En general, pulsos bipolares o una mayor intensidad de campo, número o anchura de pulsos se tradujo en un menor número de células viables de *Staph. aureus*. Si bien, se obtuvo un máximo de inactivación que osciló entre 6.3 y 7.1 µs de anchura de pulso en el rango de 50 a 150 pulsos a 35 kV/cm. Al mismo tiempo, la interacción entre la anchura de pulsos y el número de pulsos o la intensidad de campo definió distintas combinaciones de las variables con igual nivel de destrucción microbiana. La combinación de PEAIC con la adición a dosis subletales de nisina o enterocina AS-48 actuó de forma sinérgica en la inactivación de *Staph. aureus* en leche a su pH natural. Este efecto fue aún mayor cuando se combinaron dos antimicrobianos, como nisina y lisozima o nisina y enterocina AS-48. La combinación de 300 IU/mL de lisozima y 1 IU/mL de nisina redujo la población hasta 6.4 ciclos logarítmicos cuando se aplicaron 1,200 µs con pulsos bipolares de 6 µs de anchura y 35 kV/cm. El tratamiento de leche con PEAIC o PEAIC junto a la adición de antimicrobianos alargó su vida útil en refrigeración. La adición 1 IU/mL de nisina y 300 IU/mL de lisozima la aumentó hasta 7 días si se procesaba con pulsos de 6 µs de anchura y 1,200 µs de tiempo de tratamiento a 35 kV/cm, no detectándose, además, la presencia de los patógenos en estudio. La combinación de PEAIC, calentamiento moderado a 55ºC durante 16 s y adición de antimicrobianos en leche cruda consiguió inactivar a enterobacterias y coliformes durante 4 días y prolongar su almacenamiento hasta 8 días. En el caso particular de aumentar la temperatura hasta 65ºC, la vida útil (26 días) y la calidad microbiológica de muestras con 20 IU/mL de nisina y tratadas con PEAIC superó y mejoró respecto a la de la leche pasteurizada a 75ºC durante 16 s.

En consecuencia, el tratamiento simultáneo de leche con antimicrobianos, tales como nisina, lisozima, enterocina AS-48 o sus combinaciones, mediante PEAIC y/o un calentamiento moderado del medio podría mejorar la calidad organoléptica de los productos lácteos a la par que asegurar su calidad microbiológica.

RESUM

La llet s'ha processat tradicionalment mitjançant mètodes tèrmics que permeten assegurar la seva qualitat microbiològica i estabilitat enzimàtica. Malgrat això, els canvis organolèptics i la pèrdua de components nutritius induïts pel mateix escalfament podrien minimitzar-se gràcies a la utilització de mètodes no tèrmics de tractament, com els polsos elèctrics d'alta intensitat de camp (PEAIC). L'objectiu general d'aquest treball va ser l'estudi de l'efectivitat del tractament de polsos elèctrics d'alta intensitat de camp sobre el microorganisme patogen *Staphylococcus aureus* en llet, així com l'efecte resultant de la seva combinació amb l'addició d'agents antimicrobians.

La inactivació de *Staph. aureus* deguda a l'aplicació de PEAIC es va estudiar mitjançant un disseny experimental de superfície de resposta en el que les variables independents van ésser el nombre, l'amplada i la polaritat de pols, la intensitat de camp i la quantitat de grassa de la llet. De mode similar, s'avaluà l'efecte de l'addició de nisina, lisozima, enterocina AS-48 i les seves combinacions juntament amb l'aplicació de PEAIC. En aquest darrer cas, es va observar el grau de destrucció en funció del temps de tractament, la intensitat de camp, la concentració i temps d'exposició a l'antimicrobià, el pH, la temperatura d'emmagatzemament i la seqüència dels tractaments. Es va determinar la vida útil en refrigeració (4ºC) de llet tractada amb PEAIC i antimicrobians a partir de l'evolució de la flora mesòfila, d'enterobactèries, de coliforms i dels patògens *Escherichia coli*, *Listeria* spp., *Salmonella* spp. i *Staphylococcus aureus*. Es va comparar també l'evolució de la flora de llet pasteuritzada amb la de la llet sotmesa simultàniament a un escalfament moderat, l'addició d'antimicrobians i l'aplicació de PEAIC.

En general, polsos bipolars o una major intensitat de camp, nombre o amplada de pols es va traduir en un menor nombre de cèl·lules viables de *Staph. aureus*. Ara bé, es va obtenir un màxim d'inactivació que oscil·là entre 6.3 i 7.1 µs d'amplada de pols en el rang de 50 a 150 polsos de 35 kV/cm. Al mateix temps, la interacció entre l'amplada i el nombre de polsos o la intensitat de camp van proporcionar diferents combinacions de les variables amb igual nivell de destrucció microbiana. La combinació de PEAIC amb l'addició a dosis subletals de nisina o enterocina AS-48 actuà de forma sinèrgica a la inactivació de *Staph. aureus* en llet al seu pH natural. Aquest efecte va ser encara superior quan es combinaren dos antimicrobians, com la nisina i la lisozima o la nisina i l' enterocina AS-48. La mescla de 300 IU/mL de lisozima y 1 IU/mL de nisina va produir prop de 6.4 cicles logarítmics de reducció quan s'aplicaren 1,200 µs de polsos bipolar de 6 µs d'amplada i 35 kV/cm. El tractament de llet amb PEAIC o PEAIC simultàniament amb l'addició d'antimicrobians va augmentar la seva vida útil en refrigeració. L'addició d'1 IU/mL de nisina i 300 IU/mL de lisozima va allargar la vida útil de la llet fins a 7 dies si es processava amb polsos de 6 µs d'amplada i 1,200 µs de temps de tractament a 35 kV/cm, no detectant-ne la presència dels patògens en estudi. La combinació de PEAIC, escalfament moderat a 55ºC durant 16 s i l'addició d'antimicrobians en llet crua va inactivar les enterobactèries i coliforms durant 4 dies i allargà l'emmagatzemament fins a 8 dies. En el cas particular d'augmentar la temperatura fins a 65ºC, la vida útil (26 dies) i la qualitat microbiològica de les mostres amb 20 IU/mL de nisina i tractades amb PEAIC va superar i millorar a la de la llet pasteuritzada a 75ºC durant 16 s.

En conseqüència, el tractament simultani de llet amb antimicrobians, com la nisina, la lisozima, l'enterocina AS-48 o les seves combinacions, mitjançant PEAIC i/o un escalfament moderat del medi podria millorar la qualitat organolèptica de múltiples productes làctics a la vegada que asseguraria la seva qualitat microbiològica.

SUMMARY

Milk has been traditionally processed by thermal treatments that assure their microbiological acceptance and enzimatical stability. However, changes of sensory properties induced by heating may be minimized by using nonthermal treatments, such as the use of high-intensity pulsed-electric field (HIPEF). Thus, the main purpose of this work was to study the lethal effect of applying high-intensity pulsed-electric field against *Staphylococcus aureus* inoculated in milk, as well as the effect of combining HIPEF with the addition of antimicrobial compounds.

Cell death of *Staph. aureus* was studied by a response surface methodology, in which the independent variables were pulse number and width, pulse polarity, electric field intensity and fat content. Similarly, the joint effect of the addition of nisin, lysozyme, enterocin AS-48 or their combination with the application of HIPEF was also considered by varying the HIPEF treatment time, electric field intensity, concentration and exposure time to the antimicrobials, pH, storate temperature and the sequence of application. Shelf-life of milk treated by HIPEF and added antimicrobial compounds was determined. A microbial screening of mesophilic bacteria, enterobacteria, coliforms and the pathogens *Escherichia coli*, *Listeria* spp., *Salmonella* spp. and *Staphylococcus aureus* was performed periodically during refrigerated storage. Fate of survivors of pasteurized milk was compared to that of milk submitted to mild heating, HIPEF and added antimicrobials.

In general, the highest microbial inactivation was shown setting bipolar pulses or by increasing field intensity or pulse number and width. However, a maximum on cell death was observed depending on pulse width, which varied from 6.3 to 7.1 µs when pulse number ranged from 50 to 150 pulses, respectively, at 35 kV/cm of field intensity. Likewise, different levels of pulse width and pulse number or pulse width and field intensity reached the same level of inactivation. The combination of HIPEF with the addition of antimicrobial peptides (nisin, enterocina AS-48, nisin with lysozyme and nisin with enterocina AS-48) at sublethal doses acted synergistically against *Staph. aureus* in milk at its natural pH. A reduction over 6.4 log cycles was registered when 300 IU/mL of lysozyme and 1 IU/mL of nisin were added to milk prior to treating by HIPEF (1,200 µs treatment time and 6 µs pulse width at 35 kV/cm). Shelf-life of milk processed by HIPEF or by combining HIPEF and the addition of antimicrobial compounds was prolonged under refrigeration conditions. Milk with added 300 IU/mL of lysozyme and 1 IU/mL of nisin and treated by HIPEF (1,200 µs treatment time and 6 µs pulse width at 35 kV/cm) was stored for 7 days before reaching the limit of microbial acceptance. Moreover, absence of the considered pathogens was found on the latter treated samples. Counts of enterobacteria and coliforms remained below the detection limit during 4 days of storage at 4ºC and shelf-life prolonged for 8 days when milk was submitted to HIPEF, mild heating at 55ºC for 16 s and the addition of antimicrobial compounds. In the particular case of increasing temperature up to 65ºC, shelf-life (26 days) and microbial counts of milk with 20 IU/mL was longer and lower than milk pasteurized at 75ºC for 16 s.

In conclusion, milk and dairy products simultaneously processed by the addition of antimicrobial peptides, such as nisin, lysozyme, enterocina AS-48 or their combination, mild heating and HIPEF may improve their sensory properties while microbial acceptance is assured.

ÍNDICE

INTRODUCCIÓN

Parte I

Potential of High-Intensity Pulsed Electric Field Technology for Milk Processing

A. Sobrino-López, O. Martín-Belloso

Food Engineering Reviews (accepted with minor revision)

ABSTRACT

Among non-thermal treatments, High-Intensity Pulsed Electric Field (HIPEF) has received special attention on account of its potential use in treating liquid foods and its feasible application in continuous flow processing. Improving the food quality of milk and dairy products with HIPEF processing may be a relevant consideration in product development research. However, the future implementation of HIPEF equipment on an industrial scale may require more studies into the effects of the process parameters on the shelf-life of milk, the optimization of the HIPEF process with specific emphasis on the degree of microbial inactivation and energy consumption, more accurate design of the HIPEF equipment used for milk processing and a sensorial and nutritive evaluation of the treated product.

INTRODUCTION

The commercialization of food products is subject to strict microbiological and chemical controls in order to assure their safe consumption. Thermal treatments are commonly used by the food industry as methods of preservation, but applying heat to food often causes cooked flavor and undesirable changes in its components, such as protein denaturation and loss of vitamins (Wirjantoro et al. 1997). In this respect, the nonthermal processing of food may play an important role in improving food quality by maintaining its microbiological acceptance while only minimally affecting its positive sensory and health-related properties.

Among nonthermal treatments, the application of high-intensity pulsed electric fields (HIPEF) has been gaining interest over the last decade due to the feasibility of applying this technique mainly to liquid foods. Though, treatments of mild or moderate pulsed-electric field intensity are currently under study to prospect their potential of permeabilising tissue structures, thus allowing the implementation in the context of already existing operations such as extraction or drying (Toepfl et al., 2006). Moreover, pumpable media containing solid particles, such as juices, milk products, chocolate milk (Evrendilek et al. 2001a) or starch solutions (van den Bosch, 2007), and even viscous liquids, such as yoghourt, syrups, soup stuff and liquid eggs (Fang et al., 2006) , are susceptible of being treated by pulsed-electric field. Likewise, HIPEF research has fostered the development of studies into different aspects of this technology, such as the mechanism involved in its lethal effects on microorganisms (Rowan et al. 2000; Aronsson et al. 2005; García et al. 2007), its effects on food components (Barsotti et al. 2002; Bendicho et al. 2002; Van Loey et al. 2002) and food quality (Yeom et al. 2004; Zárate-Rodríguez et al. 2000; Cortés et al. 2005; Aguilar-Rosas et al. 2007), the modeling of its microbial and enzyme inactivation (Elez-Martínez et al. 2005; Sobrino-López et al. 2006a and 2006b; Giner et al. 2005), and, finally, the feasibility of scaling up this technique (Fiala et al. 2001; Góngora-Nieto et al. 2003; Roodenburg et al. 2005a and 2005b).

Milk and milk-based products are part of the human diet and suitable to HIPEF treatment, which may offer the advantage on retaining their fresh appearance and health-related properties. The main purpose of this paper was to gather information relating to the main issues associated with advances in this technique and the potential drawbacks associated with its application in milk.

ENGENIEERING ASPECTS OF HIPEF PROCESSING

Although the HIPEF treatment has mainly been studied in laboratory units, scaling up HIPEF technology in a pilot plant would be the first step towards the development of industrial continuous-flow HIPEF systems. Analysis of HIPEF engineering implies such aspects as controlling and measuring the induced electric field, the effect of liquid-media flow in a continuous system, the efficiency of the electrodes, and the control of temperature. However, in contrast to diluted or buffered media, the singular and complex composition of milk may require the specific study of these items and how they are affected by milk handling and processing. Certain substances that naturally occur in milk, such as fat and proteins, may interact with the electric field and also affect the uniformity of the process itself (e.g. a variation in pH may facilitate the formation of aggregates). The design of the equipment should therefore foresee and seek to overcome any potential drawbacks and improve the efficacy of this technology for processing milk and suitable dairy products for being treated by HIPEF.

The circulation mode

Two different fluid handling systems allow the treatment of liquid foods in HIPEF laboratory devices. The liquid can be repeatedly pumped through the system, as many times as needed, to apply the desired treatment time in what is called the stepwise circulation mode (SWM) (Evrendilek et al., 2004) (Fig. 1). On the other hand, the liquid can also pass through the system and return to the container without interruption in what is called the recirculation mode (RM) (Evrendilek et al., 2004). For technical reasons, the SWM fluid handling system needs to be cleaned after each step, which is time-consuming. Even so, treatment of the whole liquid is assured when this system is used. In contrast, the RM fluid handling system would seem more appropriate when a long treatment time is performed and a relatively small amount of liquid is likely to be wasted in comparison with the SWM system. The use of several treatment chambers in series was suggested as a way of improving the microbial inactivation efficiency of HIPEF regardless of the circulation mode (Abram et al. 2003). With regard to the effectiveness of the two modes, Evredinlek et al. (2004) observed a non-significant difference in microbial inactivation of *S. aureus* in skim milk, but Martin et al. (1997) found that the difference in the inactivation of *E. coli* in pea soup was 0.5-log units smaller for RM than SWM.

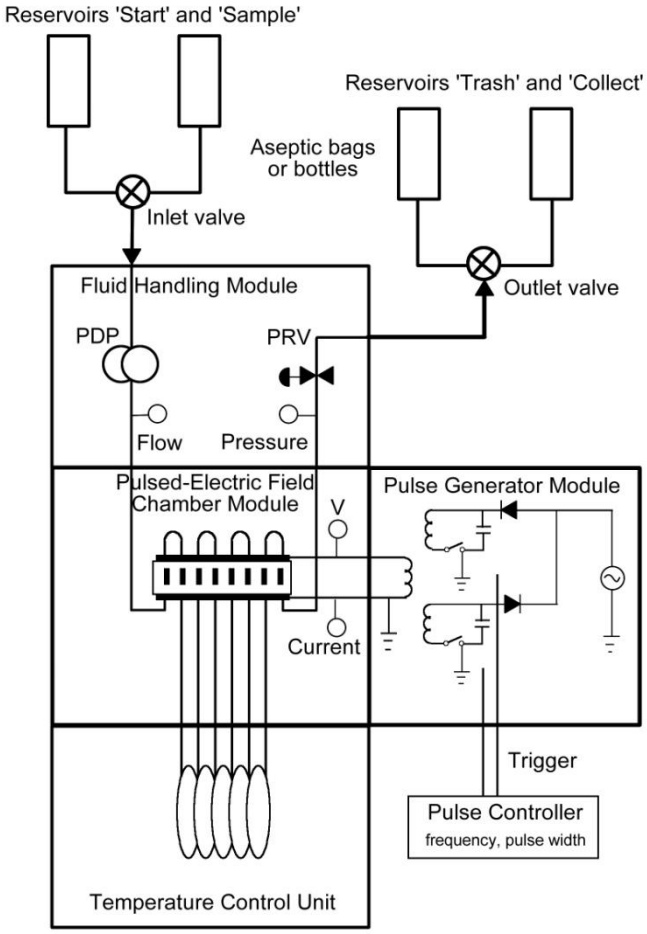

Figure 1. Configuration of a stepwise circulation mode (OSU-4F laboratory scale PEF unit, Ohio State University, Columbus, Ohio, USA)

The treatment chamber

The design of the treatment chamber is relevant in the appropriate application of the electric pulses to the product. Once they have been generated, it is desirable to establish a spatially uniform field distribution throughout the treatment chamber and, especially, among the electrodes (Fiala et al. 2001). Some research must therefore be directed towards modeling, fitting and comparing the distribution of the electric field for different electrode shapes and chamber designs (Zhang et al., 1995)(Fig. 2). The optimization of the treatment chamber should also include measures to control the residence time distribution and variations in temperature on account of their indirect effect. Attending the chamber design, turbulent flow through the treatment chamber may be desirable since residence time distribution in turbulent flow is more homogeneous than in laminar flow (Heinz et al., 2002). Temperature can modify the electrical conductivity, density, viscosity, and thermal conductivity of the milk media and, as a consequence, electric field distribution and product flow are inherently coupled to variations in temperature. Fiala et al. (2001) proposed computerized modeling of a co-field continuous flow-through chamber based on the mutual influence of the electric field induced and the product flow. As a result, regulating both the degree of uniformity in food processing and the time during which the product remains resident in the chamber would seem possible as a result of fitting and changing the diameter and position of the probes fitted in the pipe. Lindgren et al. (2002) improved the approach developed by the previously cited authors by adding the effect and distribution of temperature to their model. Analyzing the geometry of different continuous-flow HIPEF chambers, these authors concluded that the electrical field could be more homogeneous if the insulator and electrodes intersected at angles close to 90º and if a cooling system could be incorporated in order to minimize heating of the product close to the pipe wall, where the flow velocity is low. These two models agree on the need to eliminate recirculation regions in order to reduce the time during which the fluid product remains in the system, since product remaining trapped in the treatment chamber may dissipate less energy in a given volume of product and, consequently, increasing its temperature. However, thermal equilibration by heat removal across the electrodes may be complicated and even insufficient, despite suitable dimension of electrode area and gap, due to inhomogenities in the electric field and the boundary layer of the flow (Heinz et al., 2002). The possibility of two or more chambers in series may facilitate heat dissipation by inserting a cooling system after a determined number of chambers, as well as multiple pulses can be applied (Abram et al., 2003).

The presence of air bubbles

Any optimization of the treatment chamber for milk processing should also consider the punctual heterogeneity of the fluid, including the possible presence of air bubbles, and localized enhancements to the electric field, such as voltage breakdown and partial discharges. The presence of air bubbles embedded in the milk media may act as a limiting factor for HIPEF processing. In fact, equipotential lines concentrate in the cavity of the bubble, distorting the electric field within the gap, and developing voltage potentials that get higher as the gap distance gets shorter and/or the bubble gets bigger (Góngora-Nieto et al. 2003). The unexpected electrical gradient gives rise to one of two possibilities: i) the sample is under-treated and the effectiveness of the treatment is reduced if breakdown is disregarded (Góngora-Nieto et al. 2003), or ii) there is an immediate breakdown and

subsequent arcing. Since the breakdown strength of gases is much lower than that for liquids (Góngora-Nieto et al. 2003), arcs may cause the coagulation and/or evaporation of milk and the formation of solid deposits on stainless steel surfaces. The impact of these air bubbles can be minimized by shortening the pulses (≤5 μs) or by simple pressurization and/or degasification. In the first case, there would be no time for the ionization process to take place; in the second, an increase in pressure could lead to a reduction in bubble diameter (Góngora-Nieto et al. 2003).

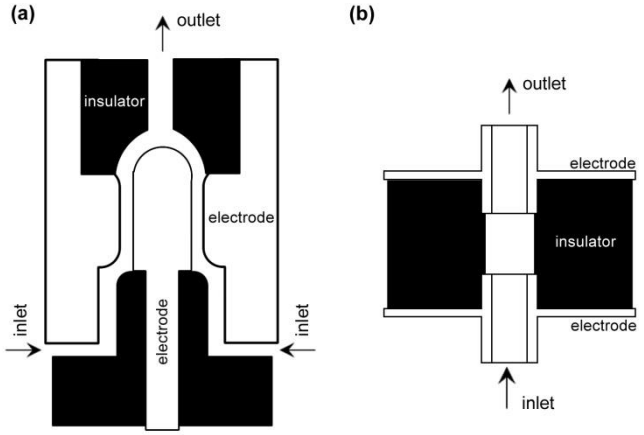

Figure 2. Schematic geometry of different treatment chambers: (a) concentric-electrode geometry from Zhang et al. (1995), (b) OSU-geometry chamber from Yin et al. (1997)

Electrolysis and the release of metals

Electrochemical interaction between the electrodes and the food media could be one immediate result of electrical processing. Despite the fact different studies concluded that HIPEF-induced changes in food products are negligible and do not affect product safety (Dunn, 1996; Lelieveld et al., 2001), the result of electrochemical reactions depends on the medium considered. Since milk can be considered an electrolyte, the electric field between the two electrodes permits the movement of charged molecules and electrolysis reactions in the interfacial region. Charged particles, such as ions, proteins, and living cells, have net electric charge and can migrate during the application of successive pulses. The deposition of these particles on the electrodes can consequently provoke electric field distortion due to differences in conductivity and food electrolysis (Evrendilek et al. 2005). In this sense, the use of bipolar pulses has been reported to reduce the deposition of solids over the electrode surface and to also reduce food electrolysis (Evrendilek et al. 2005).

Moreover, the strict food legislation suggests not ruling out the possibility of metal release from the electrode to the fluid media. Taking into account that fact, various studies produced conclusions stating that quantities of metal contained in certain dilute solutions treated by HIPEF remained below maximum permitted values. Roodenburg et al. (2005a) studied the concentration of dissolved metals (iron, chromium, nickel, and manganese) in a sodium chloride solution and observed that the monopolar mode resulted in low metal concentrations. These studies of metal dissolving therefore allow us to predict the life expectancy of the electrodes, which in the previous study was estimated

at around 760 h, or approximately 276,000 L of treated orange juice. Extending the working life of the electrodes could be feasibly achieved by increasing the pulse voltage in line with the loss of metal and by preventing the degradation of the electrodes by applying a polish treatment to the stainless steel surfaces (Roodenburg et al. 2005b). However, the use of special electrode materials, such as carbon or metals with particular metallurgical contents, cannot be disregarded as complement to well-designed HIPEF-systems (Matser et al., 2007).

Monitoring the HIPEF treatment

The generation of each pulse requires suitable and appropriate control throughout the treatment time. These tasks may be hindered by the design of the treatment chamber, which may limit the placement of sensors, and also by the intrinsic drawbacks of measuring a high-voltage field applied for a very short time. Góngora-Nieto et al. (2002) designed and validated a metrology system for measuring and acquiring electrical data from a HIPEF pilot plant device. The evaluation of the system was performed using a saline solution with an electrical conductivity close to that of milk flowing through the treatment chamber. As a result, the system implemented provided a user-friendly solution, with real-time monitoring and data recording, which was capable of offering precise analysis for quality control and energy efficiency. Moreover, the importance of the methodology developed in the previous study lies in the possibilities of extrapolating results and applying them to an industrial scale.

ENERGY REQUIREMENTS

Although most of the studies were performed at the laboratory scale, and estimations of energy requirements did not therefore correspond to real industrial processing, the application of HIPEF either alone or in combination with other preservation methods, such as classical thermal treatments, could reduce the amount of energy used during processing.

The total energy consumption required for HIPEF processing of milk averaged 107-201 kJ/L, depending on the target microorganism. This was considerably lower than the 300 kJ/L of energy used for heating in the HTST milk pasteurization process (Fernández-Molina et al. 2006). A more in-depth analysis of the HIPEF effect through the variables involved and their relationships indicated that it was possible to improve energy efficiency by several different strategies, though it was necessary to take into account the fact that: i) the number of viable bacteria decreased with increasing energy input (Picart et al. 2002; Sampedro et al. 2007) and ii) the number of microorganisms fell sharply at the start of the treatment, though microorganisms became more resistant as the treatment continued (Rivas et al. 2006; Sampedro et al. 2007). Electrical optimization of the HIPEF process may therefore be feasible, with either the application of higher electric field strengths permitting a reduction in both the number of pulses and the total amount of energy dissipated in the sample (Picart et al. 2002; Sampedro et al. 2007), or the establishment of a combination of different field strengths, pulse lengths and numbers of pulses that would produce a maximum level of inactivation at the lowest possible level of energy input (Abram et al. 2003). Furthermore, an increase in the initial treatment temperature may also offer an opportunity to save

energy. For a given level of microbial death, the heating of a milk-based beverage from 35 to 55ºC reduced the amount of energy applied from890 kJ/L to 352 kJ/L, implying a saving of up to 60% (Sampedro et al. 2007). Based on energy requirements and energy usage, Hoogland et al. (2007) compared the cost of a HIPEF process with that of traditional heating. They estimated a total HIPEF saving of 1.5 €/100 L due to a cost reduction of downtime, maintenance and equipment cleaning.

BIOLOGICAL AND QUALITY ASPECTS OF HIPEF TREATMENT OF MILK

It is generally accepted that microbial inactivation by HIPEF depends on the process parameters and product properties (Wouters et al. 2001). Though, interest and feasibility of industrial implementation of a preservation method as HIPEF also entails the effect over specific compounds related to physical and sensory quality of the product and viability in terms of the shelf-life extension.

Microbial inactivation

Incidence of milk media

The complex composition of milk may explain the decrease in the effectiveness of HIPEF in this medium in comparison with its action in dilute solutions and fruit juices (Bendicho et al. 2002; Picart et al. 2002; Rivas et al. 2006). HIPEF proved to be more effective and energy efficient when applied to media with low electrical conductivity (Zhang et al. 1995), since the osmotic forces and subsequent pressures in their respective membranes are much higher as the difference in conductivity between the cytoplasms of their respective microorganisms and the medium increase (Sampedro et al. 2006). In milk media, conductivity is mainly determined by its charged compounds, which include mineral salts consisting of chlorides, phosphates, citrates, carbonates and bicarbonates of potassium, sodium, calcium, and magnesium (Mabrook et al. 2003). However, the electrical charge of milk can be altered by factors such as fat content, pH, and temperature. The presence of fat is known to hinder or reduce the conductivity of milk by impeding the mobility of conducting ions, as well as the globule size of the milk fat may be reduced by the application of HIPEF (Zulueta et al., 2007). pH was seen to modify the electrical behavior of milk by solubilization and release of free conducting ions. Finally, heating milk causes higher conductivity due to a reduction in the resistivity of the liquid (Fernández-Molina et al. 2006; Picart et al. 2002). As a result of increasing conductivity, pulse width is shortened and the degree of microbial survivability may be affected (Fernández-Molina et al. 2006).

Unfortunately, there is no general agreement in the published data found about the exact effect of milk conductivity on microbial reduction owing to variations of the fat content. On the one hand, Sobrino-López et al. (2006b) and Michalac et al. (2003) found no differences between microbial inactivation of whole and skim milk and Mañas et al. (2001) reported that the fat content did not modify the lethal effect of HIPEF processing of dairy cream. On the other hand, Martin et al. (1997) and Craven et al. (2008) claimed that the complex composition of milk and high content of protein and fat may act as shield to protect microorganisms from the lethal effect of HIPEF, which largely confirms the general proposal stated by Zhang et al. (1995). In accordance with that, a higher resistance of microorganism was observed processing cream with 18% (Otunola et al., 2008) and 20%

of fat content (Picart et al., 2002). To explain these results, Zhang et al. (1995) compared fat globules with air bubbles trapped in the treatment chamber, concluding that these globules do not act as effective insulators, either because they are dispersed as small droplets in a continuous aqueous and conductive phase or because electrically-charged casein molecules are adsorbed at the oil/water interface.

The acidification of milk may influence the efficiency of the HIPEF treatment by directly affecting the survival of bacterial growth and by provoking changes in the electrical properties of milk (Mabrook et al. 2003). A decrease in pH causes the gradual solubilization of the colloidal salts connected to the casein micelles and, thereby, an increase in the final conductivity of milk (Mabrook et al. 2003). The acidity of milk media should therefore be considered as a major factor in determining the final microbial resistance. However, many of these studies used different buffered media, instead of acidified milk, in order to avoid difficulties in processing pH-adjusted milk and to improve isolation of the pH effect. Sobrino-López et al. (2006a) reported no difference in either the inactivation of *S. aureus* in milk at its natural pH or in acidification up to 5.0 pH. Furthermore, Smith et al. (2002) and Sobrino-López et al. (2006b) reported similar levels of microbial death among different target bacteria in milk samples treated at different pH values. In contrast, Aronsson et al. (2001b) observed that the effectiveness of the HIPEF process was significantly enhanced following the inactivation of *E. coli*, which is Gram-negative, by decreasing pH from 7.0 to 4.0. However, no significant differences were registered in *P. fluorescens* inoculated in milk at different concentrations of acetic acid (Fernández-Molina et al. 2005a). The effects of fat content and pH are unclear, although the use of different HIPEF devices, setting different conditions for the HIPEF variables, the influence of unknown or uncontrolled environmental factors, and the use of different media and/or target microorganisms, may make it difficult to compare the observed results.

Influence of the process variables

As reported in other studies conducted in diluted solutions, microbial death increased when process variables were set at higher values. A further study of HIPEF variables suggested that their simultaneous action and/or the influence of other environment, such as treatment temperature and pH, or uncontrolled factors, such as variability of natural flora and protein content of the media, may modify the pattern and final count of microbial destruction after the treatment in milk. The effects of pulse width and frequency are not fully understood, but they may be influenced by the type of microorganism involved and the composition of the milk.

It has been established that a rise in pulse width causes a fall in the microbial population due to the high level of energy applied (Martin et al. 1997). Moreover, the effect of pulse duration can be intensified by increasing the number of pulses and/or the strength of the electric field, enabling the possibility of a greater reduction in the microbial population (Aronsson et al. 2001a). In fact, Sobrino et al. (2006a) observed that the combined effect of pulse width and the number of pulses or the electric field strength caused a greater reduction in the population of *S. aureus* in milk than the lethality achieved for each level of the variables when they were studied separately. However, Sobrino-López et al. (2006a) reported that while higher levels of microbial inactivation were reached when the pulses were wider, after a certain point, an increase in pulse duration did not produce any further increase in the lethality of the treatment. Sampedro et al. (2006) also verified that lengthening pulse width produced no variation in microbial enumeration of *Lactobacillus plantarum*

in a juice-milk beverage. The role in cell destruction in other electrical variables, such as pulse frequency, received very little attention. In whole milk, an increase in pulse frequency markedly enhanced *L. innocua* inactivation, although the final reduction was dependent on fat content (Picart et al. 2002).

Other authors have reported enhancement of the HIPEF effect led by certain combinations of the process variables. In particular, increasing milk temperature improved the lethal activity of HIPEF. Heating skim milk from 13 to 33ºC accelerated the inactivation of *Pseudomonas fluorescens* and *Listeria innocua* as electric field strength, treatment time and/or energy input increased (Fernández-Molina et al. 2006). This behavior is attributed to changes of the electrical parameters during processing and inherent cell-membrane causes. First, heating may produce a variation of the sample conductivity that could leads to changes in the actual electric field intensity, pulse width and total treatment time applied and, consequently, to a higher reduction of the survival fraction (Raso et al., 2000). Second, thermal injury may diminish the electrical resistance of cells by decreasing the transmembrane potential of the cell membrane (Jeyamkondan et al.,1999). Sampedro et al. (2006) added that this thermal injury perturbed the physical stability and fluidity of the cell membrane. As a result, membranes may lose their elastic properties as temperature increases in accordance with the phospholipid phase transition from gel to liquid-crystalline (Stanley, 1991).

Nutritional and sensory properties of HIPEF-treated milk

Increasing consumer concern over healthy habits has prompted research into the incidence of HIPEF processing on the nutritional content and sensory quality of milk and the functional compounds present in it. HIPEF has been shown to minimally alter the sensory properties of food and to preserve its nutritional components. Michalac et al. (2003) reported no significant variation in different physicochemical properties of raw skim milk, such as its density, viscosity, electrical conductivity, pH, protein and total solids content, after processing with HIPEF (35 kV/cm, 188 μs) without exceeding the outlet temperature of 52ºC . Furthermore, the concentrations of different fractions of whey proteins in whole milk treated by HIPEF (35.5 kV/cm, 1000 μs) at a temperature below 40ºC were slightly reduced with respect to untreated samples, while the lowest values of whey protein content were observed in milk treated by traditional heat pasteurization (75ºC, 15 s) (Odriozola-Serrano et al. 2006). Zulueta et al. (2007) concluded that changes of the total concentration of fatty acids and pH of a juice-milk beverage were negligible processing by HIPEF (35-45 kV/cm, 40-180 μs, temperature < 65ºC) and only the ºBrix varied after a treatment time of 90 μs at 40 kV/cm.

With regard to vitamin contents, different authors observed either no or only minor variations in the case of HIPEF processing of milk and milk-based beverages. Bendicho et al. (2002) reported no modifications in thiamine, riboflavin, cholecalciferol and tocopherol contents in skim milk under any of the tested HIPEF conditions (18.3-27.1 kV/cm, 0-400 μs) and milk temperature (20-25ºC or 50-55ºC) evaluated, whereas retention of ascorbic acid followed a first-order kinetic model. In a further study, Rivas et al. (2007) reported no changes in the content of riboflavin, folic acid, biotin and pantothenic acid in a milk-based juice within the established range of electrical variables (15-40 kV/cm, 40-700 μs) and maintaining the treatment temperature below 55ºC, while under storage conditions, the stability of vitamins at 4ºC was similar, irrespective of the treatment and technology

applied. The only exception in this last case was with riboflavin, whose concentrations remained high in HIPEF-treated samples after 15 and 60 days of storage at 4ºC. The latter authors also reported no variation in the inhibitory activity of angiotensin-I-converting enzyme (ACE), an important bioactive peptide.

Taking into account that heat alters sensory properties of milk and HIPEF seems to keep nutritional content and sensory properties, HIPEF may be applied to retain the quality attributes of milk destined for dairy-product manufacture, such as cheeses, yogurt and milk beverages. In relation to cheese-making, Sepulveda-Ahumada et al. (2000) compared the texture of Cheddar cheese made from HIPEF(35 kV/cm and 30 pulses)-treated milk with that made by heating milk at 63ºC for 30 min (LTLT) or 72ºC for 15 s (HTST) in terms of adhesiveness, cohesiveness, hardness, and springiness. In their study, cheese made from LTLT milk resulted quite differently, as compared to cheese obtained from milk treated by either HIPEF or HTST. Unfortunately, although no discrepancies on the textural properties were found between cheese made by applying HIPEF and HTST, the sensory evaluation of aroma showed that cheese made from HTST milk were the most similar to cheese made from untreated milk. Likewise, different authors evaluated the effect of HIPEF on yogurt. Yeom et al. (2004 and 2007) studied the quality of a fruity based-yogurt beverage treated by HIPEF (30 kV/cm and 32 μs treatment time), mild heat (60ºC for 30 s) and their combination. Color, soluble solids and pH were only minimally affected by the combined treatment and sensory evaluation gave the processed products a rating indicating good acceptability. Likewise, Evrendilek et al. (2001b) processed a yogurt drink by HIPEF and heat under the same conditions proposed by the latter authors. No variation was observed between the untreated and the treated samples in terms of color, soluble solids, and PH. Finally, Evrendilek et al. (2001a) reported that milk with chocolate treated by HIPEF (35 kV/cm and 45 μs treatment time) combined with heat (112 and 105ºC for 33 s) remained without any browning or change in color retention during the storage period.

Shelf-life of HIPEF-treated milk

Extending the shelf-life of milk and dairy products implies ensuring their microbiological safety and maintaining an acceptable level of quality, but also meeting customer expectations (Fernández-Molina et al. 2005b). The commercialization of milk product should also be practicable within the period of extended shelf-life. Skim milk processed by HIPEF (40 kV/cm, 60 μs; 36 kV/cm, 84 μs) achieved 14 days of shelf-life at 4ºC with a level of acidity that was lower than that of heated milk (Fernández-Molina et al. 2005c). In contrast, Odriozola-Serrano et al. (2006) reported 5 days of shelf-life after processing whole milk with HIPEF (35.5 kV/cm, 1,000 μs) and no changes in acidity throughout storage at 4ºC for either HIPEF-treated or heated samples. The discrepancy between these results may have been due to factors including: different HIPEF conditions and/or equipment, the presence of fat, and the use of different criteria for establishing the end of shelf-life.

From a hurdle concept, the combination of HIPEF with a mild thermal treatment has received much attention, since the simultaneous effect is greater than any of the individual effects in terms of both microbial and storage stability (Odriozola-Serrano et al. 2006). Fernández-Molina et al. (2005b) processed skim milk by heat (80ºC, 6s) before applying HIPEF (30 kV/cm, 60 μs) and prolonged the shelf-life of milk by up to 30 days, which was equivalent to doubling the shelf-life associated with any individually applied treatment. Moreover, similar extensions to the shelf-life of skim milk were

achieved when a mild thermal treatment (65ºC, 21s) was applied after HIPEF (36 kV/cm, 84 μs) (Fernández-Molina et al. 2005c). In another case, shelf-life was extended beyond 60 days when a thermal treatment (72ºC, 15 s) was applied prior to HIPEF (35 kV/cm, 11.5 μs) (Sepulveda et al. 2005).

The synergistic effect of combining HIPEF and heating to extend shelf-life has also been observed in other dairy product. Yeom et al. (2004) used a HIPEF treatment (30 kV/cm, 32 μs treatment time) in combination with a mild thermal treatment (60ºC, 30 s) to process flavored yogurt-based products. The acceptability of the products tested was prolonged for 90 days at 4ºC, which was 3 times longer than for an untreated sample. Yeom et al. (2007) also reported that shelf life of flavored yogurt treated by HIPEF (30 kV/cm, 32 μs treatment time), heat (65ºC, 30 s) and stored at 4ºC and 22ºC was extended up to 4 weeks more with respect to the control. In a different study, chocolate milk was processed by HIPEF (35 kV/cm, 45 μs) prior to heating at 105 and 112ºC for 31.5 s (Evrendilek et al. 2001a). Samples were then stored and exhibited 119 days of shelf-life at storage temperatures of 4, 22 and 37ºC.

DEFINING HIPEF PROCESS CONDITIONS THROUGH MODELING MICROBIAL INACTIVATION

Due to the natural flora present in milk, including a wide range of spoilage and pathogenic bacteria, the modeling and prediction of microbial inactivation is of great interest when seeking to optimize the treatment conditions and efficiency of the process and develop suitable equipment. Different models have been proposed for studying the kinetics of microbial inactivation by HIPEF processing, but the relationship between the log of the survival fraction does not always follow a lineal trend within the whole range of the treatment time and the consequent presence of tails and shoulders makes it difficult to establish a fit. Furthermore, any model should include, or at least reflect, the mechanism by which inactivation takes place, in order to facilitate the comparison of such factors as differences in the resistance of target microorganisms to HIPEF and in the efficacy of the HIPEF equipment.

Similarly to the modeling of microbial death in thermal treatments, cell death due to HIPEF can be fitted using a first-order kinetic model in which microbial inactivation is dependent on the HIPEF treatment time of each of the values established for the electric field strength (Raso et al. 2000). Based on the resistance of microbial populations to thermal treatments, Cole et al. (1993) described a log-logistic model for fitting survivor curves. This model was adapted to the HIPEF treatment and validated by Raso et al. (2000) and finally compared with the first-order kinetic, whose fit with their data was unsatisfactory (Table 1). However, later models restricted their focus to response patterns as a function of the controlled variables of the process, without involving media-related variables or those depending on the microorganism. Based on the minimum electrical values required to induce cell death, Hülsheger et al. (1981) fitted the survival fraction using a potential equation (Table 1) including two parameters, t_c and E_c, which respectively corresponded to the critical values for treatment time and electric field strength. To achieve a better adjustment of survival fraction around the critical values, Peleg (1995) proposed a model based on Fermi's equation which also included the characteristic parameters of the microorganism (Table 1). A recent modification of the Weibull distribution was used to fit survival curves meeting a need to include the pH of the media as the main factor (Gómez et al. 2005) (Table 1). In that case, a quadratic polynomial model and the Gompertz

Table 1. Mathematical models used in fitting the antimicrobial effect of HIPEF by different process variables

Model	Target microorganism	Food media	Range of the variables		Reference
			E (kV/cm)	t (μs)	
$s = k_1 \cdot e^{-k_2 t}$	*Listeria innocua* *Pseudomonas fluorescens*	Skim milk	30-50	60	Fernandez-Molina et al. (2001)
	Listeria innocua *Escherichia coli*	Phosphate buffer	46	200	Picart et al. (2002)
$s = \left(\frac{t}{t_c}\right)^{-\frac{E-E_c}{k}}$	*Escherichia coli*	Buffer media	6-20	36-1,080	Hülsheger et al. (1981)
$s = \frac{1}{1+e^{\frac{E-E_d(n)}{c(n)}}}$ $E_d(n) = E_{d0} \cdot e^{-k_1 \cdot n}$ $c(n) = c_0 \cdot e^{-k_2 \cdot n}$	*Candida albicans* *Listeria monocytogenes* *Pseudomonas aeruginosa*	Buffer media	0-20	36-1,080	Peleg (1995)
$-\log s = \frac{\alpha + (\omega - \alpha)}{1+e^{\frac{4\sigma(\tau - \log t)}{\omega - \alpha}}}$	*Salmonella senfterberg*	Citrate-phosphate McIlvaine buffer	12-28	600	Raso et al. (2000)
$-\log s = -\left(\frac{1}{2.303}\right)\left(\frac{t}{a}\right)^p$ $p = f(pH)$ $a = f(E, pH)$	*Listeria monocytogenes*	Apple juice	15-28	600	Gómez et al. (1995)

Table 1 (cont.). Mathematical models used in fitting the antimicrobial effect of HIPEF by different process variables

$\ln s = -\left(\frac{t}{a}\right)^{0.233}$ $a = f(E)$ $\bar{t}_{cw} = a \cdot \Gamma(1 + p^{-1})$	*Escherichia coli*	Juice-milk base beverage	15-40	700	Rivas et al. (2006)
$log(-\log s) = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ j=1}}^{n} \beta_j X_i^2$	*Lactobacillus plantarum*	Phosphate buffer	0-40	60	Abram et al. (2003)
$-\log s = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ j=i+1}}^{n} \beta_{ij} X_i X_j$	*Staphylococcus aureus*	Milk	25-35	1,200	Sobrino-López et al. (2006a)
	Staphylococcus aureus	Skim milk	35	2,400	Sobrino-López et al. (2006b)

α, upper asymptote (log CFU/ml)
β_i, parameter of the equation
σ, maximum slope of the inactivation curve
τ, log time at which σ is reached
ω, lower asymptote (log CFU/ml)
Γ, gamma function
a, scale parameter (µs)
c, steepness parameter (V/m)
E, electrical field strength (V/m)
E_c, E_d, critical value of the electrical field strength (V/m)
k, k_1, k_2, E_{d0}, c_0, constant parameters of the equation
n, number of pulses
N_0, initial concentration of the microorganism (CFU/ml)
N, concentration of the microorganism (CFU/ml)
p, shape parameter
s, survival fraction calculated as N/N_0
t, HIPEF treatment time (µs)
t_c, critical HIPEF treatment time (µs)
$\bar{t}_{cw}$, mean time (µs)
X_i, variable

function were applied to describe variations in the scale and shape parameters, *α* and *p*, respectively, in function of electric field strength and media pH. Interestingly, this model appears to fit well with experimental data when tails are less apparent, as in the case of high field intensities (Rivas et al. 2006), although has not already been applied in a milk media. In a detailed study, the Weibull model was modified by replacing parameter *α* with a more suitable kinetic parameter, $\bar{t}_{wc}$, associated with the resistance of the microorganism over time (Rivas et al. 2006) (Table 1).

However, the use of the described models may mask the complexity of the whole system and also hinder a deeper study of the process. Firstly, these models are limited by their very expression, as this ignores the effects of a large number of factors and also their respective interactions. For instance, the first order and the log-logistic model are expressed by including only one independent variable, usually HIPEF treatment time, while the rest of the controlled variables are held constant. In contrast, the two bivariant models of Hülsheger et al. (1981) and Peleg (1995) were defined with respect to HIPEF treatment time and electric field strength, which improved the study of each effect with respect to previous models. Secondly, the models were generally validated using HIPEF equipment in which these variables could be easily isolated. In one such case, Abram et al. (2003) concluded that pulse length and the number of pulses should be included as variables in a predictive model instead of treatment time, since treatment time is inadequate for describing inactivation kinetics in a continuous-flow HIPEF system. Thirdly, these models do not consider the probable mutual influence of the process variables. Aronsson et al. (2001a) observed that an increase in one parameter, such as electric field strength, intensified the effect of others, such as treatment time. Unfortunately, they did not quantify the degree of this mutual effect. Finally, optimization of the HIPEF process is either seriously limited or effectively impossible when the controlled variables are not simultaneously considered.

Abram et al. (2003) proposed a three-parameter, second-order inactivation model for a continuous flow-through system (Table 1). After validating the model, an optimization of the HIPEF process was devised by combining electric field strength, pulse width and the number of pulses that led to the highest level of inactivation and the lowest amount of applied energy. The most recent, surface response modeling (RSM) (Table 1) appears to offer a better alternative than the models mentioned above when processes are affected by many different variables. Moreover, these types of statistical design allow researchers to reduce the number of assays required for a full factorial design, thereby omitting information that may be unnecessary for statistical conclusions while also permitting optimization of the overall process, which can be also be developed in detail (Myers et al. 2002). Despite RSM being narrowly spread throughout the fitting of cell death caused by HIPEF, Sobrino-López et al. (2006a) satisfactorily performed a response surface design to determine the microbial inactivation of *S. aureus* in milk, considering electric field intensity, pulse number, pulse width, pulse polarity and the fat content of the milk as the process variables. Given the large number of variables involved in HIPEF treatment of milk and the numerous interactions among them, RSM seems a powerful mathematical and statistical tool for modeling and optimizing HIPEF processing.

CONCLUSION

HIPEF is a nonthermal technique that is of great interest because it is capable of reducing changes of sensory properties of liquid foods while ensuring their microbiological acceptance and because of its potential application in the food, and particularly the milk, industry. Significant advances have been achieved on important issues relating to HIPEF processing, but data collected from the laboratory should be compared and checked at the pilot plant scale. Moreover, potential drawbacks and critical points in the handling and treating of milk and dairy products should be tested in advance in order to ensure that industrial HIPEF equipment is better designed. This is important because milk and dairy products may require specific yet different conditions from those used with other liquid foods. From a practical point of view, the possibility of combining HIPEF with classical thermal treatments, or with other novel techniques, should not be discarded, as this may offer an appropriate combination of processes capable of synergistically extending the shelf-life of milk. HIPEF therefore represents a promising nonthermal technique that may be used by the dairy industry to improve the commercialization of fresh-like dairy products.

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Parte II

Use of Nisin and other Bacteriocins for Preservation of Dairy Products

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ABSTRACT

Non-thermal treatments are attracting interest of the food industry due to their capability of assuring the quality and safety of food. Among them, bacteriocins from lactic acid bacteria, such as nisin, pediocin PA-1, lacticin 3147 and enterocins, may be potentially useful for the dairy industry. Although cheese manufacturers have used bacteriocins for years, the combination of bacteriocins with heat and non-thermal treatments, such as high pressure, pulsed electric fields and other antimicrobials, opens innovative possibilities for application in other dairy products in hurdle-type approach. Bacteriocins alone, or combined with other treatments, could represent a promising advance for the microbiological safety and maintenance of sensory properties in dairy products. However, more research is needed to identify drawbacks out that may hinder their future application, such as their complete characterization, influence of food media on their effectiveness, and their microbiological spectra.

INTRODUCTION

Consumer demand today is for natural and minimally-processed foods, with a fresh appearance and taste, ease-to-eat and high safety. As a result, research and development of new products is leading to the reduction or even displacement of heat treatments and traditional preservatives by treatments capable of assuring the sensory and nutritional properties of the product without reducing food safety. Non-thermal preservation methods are thus of growing interest as alternative treatments, especially high-intensity pulsed electric fields (HIPEF), high pressure (HP) and the addition of natural antimicrobial substances (Bendicho et al., 2002).

Natural antimicrobial compounds have been exploited unknowingly for ages due to their effect against several food spoilage microorganisms and pathogens. Common spices and aromatic plants have been used in cooking for their taste, but also for their antibacterial effect. The practical application of these compounds generates changes in the sensory and textural properties foods when they are added. On the other hand, lactic acid bacteria (LAB) have been used in food production as an effective method for extending safe storage of foodstuffs by simple fermentation, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus* and *Carnobacterium* are the genera most commonly used as starter cultures in the fermentation processes of milk, meat and vegetable products (Stiles & Hastings, 1991).

The preservative effect of those bacteria is mainly due to the production of one or more active metabolites with antimicrobial properties, such as organic acids (lactic and acetic acid), that intensify their action by reducing the pH of the media. However, another mechanism was suspected to be involved in killing or at least inhibiting the growing of other related bacteria and even pathogens by LAB. As a result, a large number of bacteriocins produced by LAB have been identified, although their potential application as biopreservatives has not been fully developed.

Bacteriocins are gaining interest because of their wide antibacterial spectrum with feasible application in foods, such as meat and fish products, fruits and vegetables, cereals and beverages (Cleveland et al., 2001). Moreover, LAB-derived bacteriocins are generally recognized as safe (GRAS) and are attractive to the food industry because of their activity against key gram-positive pathogens involved in food-borne illnesses, such as *Listeria monocytogenes* or *Staphylococcus aureus*.

These compounds may be used in three ways (Schillinger et al., 1996): (i) as purified or semi-purified antimicrobial additives, (ii) as bacteriocin-based ingredients from fermented foods, and (iii) through bacteriocin-producing starter cultures. The use of bacteriocins as purified powders and, consequently, as food additives, demands an exhaustive evaluation for toxicological effects before legal acceptance. For that reason, nisin and pediocin PA-1 are the only bacteriocins commercially exploited to date. Unfortunately, although nisin is applied world-wide in dairy products (especially cheesemaking) as well as sausages, canned and packaged meat and brewing, there is no widespread agreement on the maximum level of bacteriocin allowed among those countries where nisin has been approved as a preservative. For instance, nisin can be added to cheese without limit in United Kingdom, while a maximum concentration of 12500 mg g^{-1} in that food is allowed in Spain. Currently, new techniques in bacteriocin application are being steadily directed toward spread of bacteriocin-containing powder (Morgan et al., 2001), bacteriocin-producing strains as fermentation starter cultures (Martínez-Cuesta et al., 2001) or biofilms incorporating the bacteriocin (Mauriello et al., 2005).

Furthermore, recent strategies for controlling spoilage and pathogenic microorganisms tend to apply hurdle technology, whereby different preservation methods are combined to inhibit microbial growth and improve food safety. Synergism has been reported between bacteriocins and traditional and novel treatments: mixtures of LAB bacteriocins (O'Sullivan et al., 2003) or LAB bacteriocins combined with other antibacterial compounds (Kozáková et al., 2005) may enhance their antibacterial effect. An increase in microbial inactivation has been also reported by adding bacteriocins prior to a mild thermal (Penna & Moraes, 2002) or non-thermal (Sobrino-López et al., 2006) treatment.

Although LAB are the main source of bacteriocin-producing bacteria and bacteriocin breakthroughs, few have been studied as potentially applicable in dairy products. Therefore, many bacteriocins have not been fully characterized yet and, consequently, they are not extensively used in food industry. To date, different studies have highlighted particular features of nisin, as well as pediocin PA-1 and lacticin 3147, that make them suitable for promising uses. However, potential uses of nisin and other novel bacteriocins are being studied in a wide variety of food products; meanwhile, their dairy origin also suggests the possibility of improving and enhancing features of those dairy products from which they have been isolated. Hence, the objective of this review is to compile research, applications and drawbacks of LAB-derived bacteriocins, as well as the interactions of bacteriocins of LAB with thermal and non-thermal treatments in dairy products.

NISIN

Nisin is a peptide composed of 34 amino acid residues, with a molecular weight of 3.5 kDa, and is classified as a class Ia bacteriocin or lantibiotic (Hurst, 1981). It is produced by strains of *Lactococcus lactis* subsp. *lactis* isolated from milk and vegetable-based products and its importance is due to its wide spectrum of activity against gram-negative and gram-positive bacteria.

The use of nisin as a biopreservative has been widely investigated in a large variety of fresh and processed foods (Jung et al., 1992). Consequently, it was admitted into the European food additive list, where it was assigned the number E234 (EEC, 1983). Because it was thoroughly studied and formed part of the human diet, nisin was also approved by the Food and Drug Administration (1988) in the USA as GRAS; to date, it is the only bacteriocin that has been approved by the World Health Organization for use as a food preservative and it is commercialized as a dried concentrated powder.

Applications of nisin for fermented dairy products

This peptide has been shown to be effective in the microbial control of a number of dairy products and its use has been widely assessed in cheese manufacturing at low pH. The use of nisin-producing and nisin-resistant starter cultures appears to be a viable means of incorporating and maintaining this bacteriocin through the cheese-making process to control food-borne pathogenic and spoilage bacteria. *L. lactis* subsp. *lactis* TAB50 and its lactase-negative proteinase-negative mutant strain TAB50-M4 have been tested and selected as useful starter cultures or adjuncts in semi-hard cheese from raw or pasteurized milk, providing protection against contamination of milk or curd by *Staph. aureus* (Rodríguez et al., 2000). However, the environmental conditions and processing factors, such

as pH and water activity, required to stimulate nisin production by transconjugant or natural producers should be defined for further and future implementation of this technique. In Cheddar cheese, strains of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* grown in pH-controlled reconstituted skim milk (RSM) produced 2-5 times more nisin than bulk cultures incubated in RSM, although differences in nisin production depended on bacterial strains (Yezzi et al., 1993). When those bulk starter cultures prepared by pH control of the starter media were used to make Cheddar-type cheese, the concentrations of nisin increased by approximately 20% (Yezzi et al., 1993). A threshold of 400 IU nisin g^{-1} were sufficient to protect against spoilage by *C. sporogenes* for more than 90 days in cheese spreads (Roberts & Zottola, 1993). In contrast, the use of a nisin-producing strain to control *L. monocytogenes* in Feta and Camembert cheeses has shown variable results, ranging from partial to non-inhibitory effects (Ramsaran et al., 1998).

The addition of nisin powder to milk for the production of cheese made without a starter culture can control microbial contamination, while nisin concentration remains unaltered after pasteurisation. Addition of nisin at 100 or 500 mg kg^{-1} suppressed total plate and anaerobic spore counts in processed cheese during 3 months of storage at 5 or 21ºC, and even the growth of *B. stearothermophilus*, *B. cereus*, *B. subtilis* were inhibited by 5 mg kg^{-1} nisin (Plockova et al., 1996). Shelf-life analysis of Ricotta-type cheese demonstrated that 2.5 mg L^{-1} nisin inhibited the growth of *L. monocytogenes* for more than 8 weeks, while cheese made without nisin contained unsafe levels of the bacteria within 1 to 2 weeks. In addition, the residual levels of nisin in the cheese after 10 weeks of incubation at 6 to 8ºC indicated a high level of retention, with only 10 to 32% loss of nisin activity (Davies et al., 1997).

Inclusion of nisin in active packaging

Nisin is a highly surface-active molecule that can bind to different compounds, such as fatty acids of phospholipids; this feature makes it suitable for adsorption to solid surfaces and killing bacterial cells which subsequently adhere. Therefore, nisin adsorption may represent a promising advance in the development of active packaging, where the classical protective function of packaging is supported by the antimicrobial action of nisin. Furthermore, the efficacy of the bacteriocin activity could be improved by control of migration of the bacteriocin into the packaged media, enabling its antimicrobial effect to be preserved beyond consumer purchase. To achieve this purpose, packaging materials such as those made of polymers (e.g. cellulose-based packaging or polypropylene) may incorporate, or be coated with, the bacteriocin. .

Mauriello et al. (2005) successfully tested a low-density polyethylene film coated with nisin for inhibition of *Micrococcus luteus* as an indicator strain during the storage of milk. The antimicrobial package retarded microbial growth and lowered the maximum growth levels in raw, pasteurized and UHT milk, although the activity and the release of nisin from the film strongly depended on pH and temperature. Since the solubility and stability of nisin decreases from the optimal pH 2.0 to 6.0 (Hurst, 1981), a lower pH and a higher temperature favoured the migration of the bacteriocin from the film. These results agree with those of Lee et al. (2004), whereby virgin paperboard coated with nisin and/or chitosan in a binder of vinyl acetate-ethylene copolymer was assessed during storage of pasteurised milk at different temperatures. The antimicrobial paperboards retarded the microbial growth of aerobic bacteria and yeasts at 3 and 10ºC, whereas the effect was marginal at 20ºC. Cross-

linked hydroxypropylmethylcellulose (HPMC) films containing nisin also have been found to be active against *M. luteus* (Sebti et al., 2003). However, the temperature reached in the process of cross-linking meant that the heat stability of the bacteriocin, if this was added previously, needed to be taken into account.

Further applications of packaging materials have been focused on the production of inserts placed between portions of sliced products, such as cheese. Cellulose-based packaging including nisin was assayed as an insert interleaved between slices of Cheddar cheese packaged under a modified atmosphere (Scannell et al., 2000). In that case, the population of *L. innocua* and *Staph. aureus* dropped dramatically in the first week of refrigeration conditions and, as a result, the shelf-life of Cheddar cheese was significantly extended.

Use of nisin in combination with thermal treatments

Milk is commonly heated to provide stability during storage and assure microbiological safety to consumers. From a hurdle approach, nisin is known to influence the thermal resistance of microorganisms. The D-value of *B. cereus* in milk was reduced in the presence of nisin by up to 40% at temperatures in the range 80 to 100ºC (Penna et al., 2002), while the apparent D values of *Bacillus stearothermophilus* at 130ºC were reduced by 21% due to the presence of 4000 IU nisin mL^{-1} (Wandling et al., 1999). Consequently, the use of nisin in combination with those heat treatments extended the shelf-life of milk, even with poor refrigeration conditions, making possible the substitution of the current thermal treatment for milder treatments and, consequently, better sensory quality. Rao and Mathur (1996) indicated that higher sterilization of *B. stearothermophilus* spores can be achieved at 121ºC for 5 min when 500 IU mg^{-1} nisin was incorporated into an acidified concentrated buffalo milk model system. In a detailed study, milk containing 40 IU nisin mL^{-1} treated at 72ºC for 15 s showed an increased keeping quality of 7 days compared with the control, and also showed a significantly lower count of *Lactobacillus* when stored at 10ºC (Wirjantoro & Lewis, 1996). Moreover, milk heated at 115ºC for 2 s with or without nisin was microbiologically acceptable after 28 days, although milk with nisin was superior in flavour, with no off-flavors within 32 days. Similarly, in a later study no microbial growth could be detected in milk treated simultaneously with nisin (75 to 150 IU mL^{-1}) and heat (117ºC for 2 s) after storage at 10 or 20ºC for 1 year and, moreover, milk treated in this way was easily distinguishable and preferred to a UHT-heated control in sensory analysis trials (Wirjantoro et al., 2001).

Use of nisin in combination with non-thermal treatments

Current thermal treatments are known to cause undesirable changes in the sensory, nutritional and/or technological properties of milk. Taking advantage of the antimicrobial action of nisin against several spoilage and pathogenic microorganisms, innovative non-thermal food preservation offer the inactivation of microorganisms with minimal impact on quality and nutritional factors. Combining nisin addition with other antimicrobial agents or non-thermal treatments, such as high pressure or high-intensity pulsed electric fields, has acted synergistically in reducing the population of different

microorganisms, including bacterial spores (Table 1). Therefore, each treatment can be optimised by setting lower values of the controlled variables while the goal of microbial reduction is improved.

Use of nisin in combination with other antimicrobial substances

Combining nisin with other antimicrobial compounds, such as monolaurin, the lactoperoxidase system (LPS) or other bacteriocins, can induce the sensitization of resistant spoilage and food-borne microorganisms. Monolaurin, the monoester of lauric acid, has received special attention because of its antimicrobial properties (Wang & Johnson, 1997), which may be intensified when combined with nisin. The combination of monolaurin and nisin has been found to be active against bacilli in milk; in particular, the inhibition by both antimicrobial substances of *Bacillus licheniformis* increased with increasing pH when they were added simultaneously to milk (Mansour et al., 1999). In addition, the combination of both compounds successfully exerted a bactericidal effect against different *Bacillus* species in skim milk and also inhibited their regrowth and sporulation (Mansour & Milliere, 2001). However, a high concentration of monolaurin may produce an unpleasant soapy odour and taste (Bell & Lacy, 1987), which is undesirable in dairy products.

The LPS system in raw milk increases the storage stability of raw milk at ambient temperature (Wolfson & Summer, 1993). The combination of LPS and nisin had a synergistic and long-lasting inhibitory effect on *L. monocytogenes* in reconstituted skim milk and, in addition, its effectiveness did not depend on pH (Boussouel et al., 1999). Curiously, higher antibacterial activity was observed when the inhibitors were added to skim milk in two steps; the effect of the LPS-nisin combination was enhanced when nisin was added to skim milk inoculated with *L. monocytogenes* after the addition of LPS (Zapico et al., 1998). Clearly, the order of addition for maximum inactivation has received insufficient attention.

Although the mechanisms of action are different for LPS and nisin, both antimicrobial agents cause damage to the cytoplasmic membrane, which could explain their synergistic action (Zapico et al., 1998). The primary reaction product of the LPS, hypothiocyanite, is known to react with the thiol groups of various proteins and inactivate crucial enzyme and protein systems (Boots & Floris, 2006). On the other hand, nisin forms a wedge-like pore composed of multiple nisin molecules bound on to the phospholipids of the membrane (Moll et al., 1997) (Figure 1). Both antimicrobials play an important role in depletion of the proton motive force, since the bacterial respiration chain is targeted by the LPS (Boots & Floris, 2006) and nisin pores lead to a collapse of the membrane electrical potential and the pH gradient (Bruno et al., 1992).

Lysozyme, which may be added to cheese milk to prevent blowing and the activity of *Bacillus* spp., displayed an inhibitory effect on different strains of lactobacilli when mixed with nisin, while it did not have any influence on nisin-producing lactococci used as starter (Kozáková et al., 2005). Reuterin is a broad-spectrum antimicrobial compound produced by some strains of *Lactobacillus reuteri* (Axelsson et al., 1989). The combination of reuterin and nisin acted synergistically against *L. monocytogenes* and additively against *Staph. aureus* in milk (Arqués et al., 2004). Recently, activity of nisin and a cell-free supernatant of *Bacillus licheniformis* ZJU12 have also been found to be synergistic against three food-borne bacteria: *Micrococcus flavus*, *B. cereus* and *Staph. aureus* (He & Chen, 2006). The flora of minimally-processed dairy products mainly consists of bacteria, but moulds and yeasts are commonly associated with special ingredients, such as wild blueberries added to yoghurt (Penney et al., 2004). For that reason, yoghurt containing minimally-processed wild

blueberries has an extremely short shelf-life; thus, some phytopreservatives, such as vanillin, have been tested efficiently for fungal inactivation in yoghurt with blueberries, while added nisin did not prevent spoilage (Penney et al., 2004). In the case of yoghurt containing fresh peaches, nisin actually hastened the growth of spoilage microorganisms (Penney et al., 2004). This fact may represent a drawback in nisin use since, particularly; yeast growth might be stimulated by added nisin through two mechanisms: the addition of new carbohydrate substrates, and the suppression of lactic acid bacterial competitors (Penney et al., 2004).

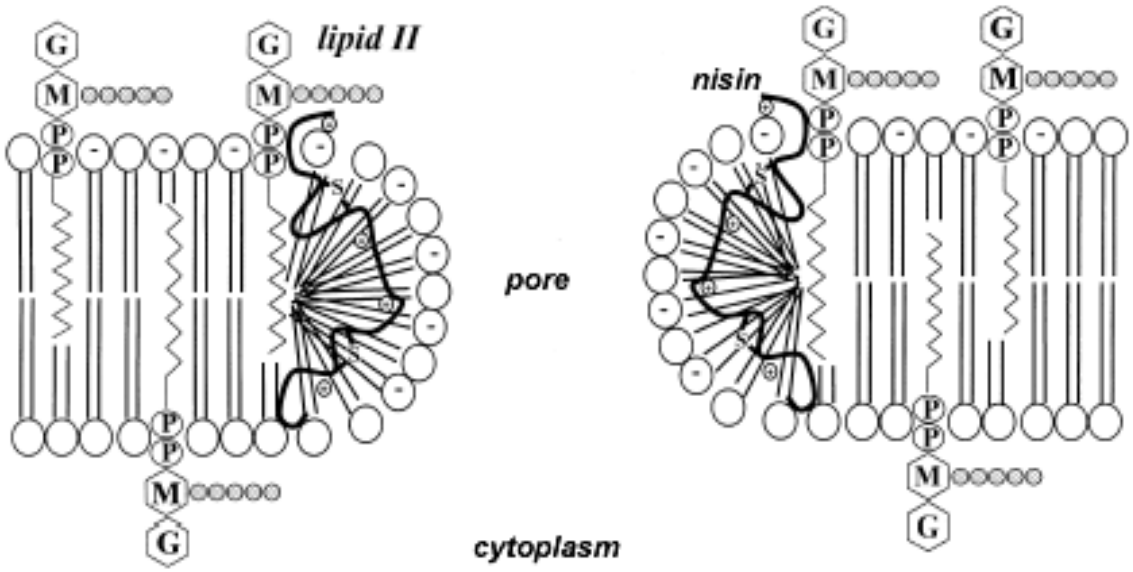

Figure 1. General mode of action of nisin: lipid II serves as a docking molecule which energetically facilitates the formation of pores by binding the molecule of nisin and allowing to adopt the correct position for pore opening (adapted from Brötz & Sahl, 2000).

Use of nisin in combination with high pressure

Nisin has been shown to enhance the microbial reduction achieved by HP, due to sublethal injury and sensitization caused to target cells. Studies carried out on milk demonstrated that Gram-negative bacteria, such as *Pseudomonas fluorescens* or *E. coli*, and Gram-positive bacteria, as *L. innocua*, are inactivated by HP treatment, although Gram-positive bacteria seemed to be rather resistant (Black et al., 2005). Hence, the degree of inactivation achieved by the combined use of HP and antimicrobials such as nisin should be higher than that achieved by the sum of the inactivation achieved by the individual treatments. Treatment at 500 MPa for 5 min in the presence of 500 IU mL^{-1} nisin completely inactivated *P. fluorescens* and *E. coli* and reduced *L. innocua* by more than 8.3 log, whereas those treatments, when applied separately, produced a decrease of only 3.8 and 1.5 log-units, respectively (Black et al., 2005). Working from the hypothesis that the conditions used to destroy pressure-resistant strains would be sufficient to kill less resistant pathogens, Alpas and Bozoglu (2000) found that *Staph. aureus* was the most resistant to HP among the pathogens considered in their study. After that, a biopreservative powder consisting of nisin and pediocin PA-1 at a final level of 5000 Au mL^{-1} was added to milk inoculated with *Staph. aureus* followed by HP treatment (345 MPa, 50ºC, 5 min); a reduction in cell population of *Staph. aureus* of more than 8-log

Table 1. Effect of nisin on viability of different microorganisms when combined with other treatments

Nisin dose	Combined treatment		Product	Target microorganism	Inactivation (log units)	References
	Treatment	Process conditions				
75 IU mL^{-1}	Heat	117ºC, 2s	Milk	Natural flora	>5	Wirjantoro et al. (2001)
1000 IU mL^{-1}	Monolaurin	250 μg mL^{-1}	Skim milk	*Bacillus cereus*	1.5	Mansour et al. (2001)
				B. coagulans	<5	
				B. subtilis	<5	
				B. licheniformis	4	
100 IU mL^{-1}	Reuterin	8 Au mL^{-1}	Milk	*Listeria monocytogenes*	4	Arqués et al. (2004)
				Staphylococcus aureus	<1	
100 IU mL^{-1}	LPS[1]	0.8 ABTSU mL^{-1}	Milk	*L. monocytogenes*	<6	Zapico et al. (1998)
7 IU mL^{-1}	HP[2]	500 MPa, 30 min, 25ºC	Cheese	Aerobic mesophilic bacteria	>2	Capellas et al. (2000)
CNQ[4]	HP[2]	500 MPa, 5 min, 10ºC	Cheese	*Staphylococcus aureus*	2.5-7	Arqués et al. (2005a)
400 IU mL^{-1}	Lysozyme and HP[2]	400 μg mL^{-1} 550 MPa, 15 min, 20ºC	Milk	*Escherichia coli*	4	Garcia-Graells et al. (1999)
20 IU mL^{-1}	HIPEF[3]	35 kV cm^{-1}, 2400 μs	Skim milk	*Staphylococcus aureus*	6	Sobrino-Lopez et al. (2006)
100 IU mL^{-1}	HIPEF[3]	50 kV cm^{-1}, 50 pulses	Skim milk	*Listeria innocua*	<4	Calderon-Miranda et al. (1999a)
38 IU mL^{-1}	Lysozyme and HIPEF[3]	1638 IU mL^{-1} 80 kV cm^{-1}, 50 pulses, 52ºC	Raw skim milk	Natural flora	7	Smith et al. (2002)

[1]LPS: lactoperoxidase system
[2]HP: high pressure
[3]HIPEF: high intensity pulsed electric fields
[4]CNQ: concentration not quantified

cycles was found and no growth was observed for up to 30 days in samples stored at 25ºC. Nevertheless, Garcia-Graells et al. (1999) found that the complex environment of milk exerts a strong protective effect on microorganisms against HP inactivation. In the latter study, an increase in the lethality of pressure-resistant *E. coli* strains in milk was achieved by the addition of lysozyme (400 μg mL^{-1}) and nisin (400 IU mL^{-1}) before HP treatment. As a result, the population decreased by 3 log units in skim milk at 550 MPa, which represented an additional log reduction. However, that reduction level was significantly lower in 1.55% fat and whole milk.

Owing to the microbial reduction achieved by the combination of HP and nisin, particularly on pathogenic bacteria such as *Staph. aureus* or *L. monocytogenes*, the microbiological safety of cheese made from raw milk could also be improved while producing little or no change in its sensory quality. Arqués et al. (2005a) reported 6.7 log units of *Staph. aureus* on day 1 in cheese made from milk inoculated with LAB excluding bacteriocin-producing LAB. In that study, synergistic effects on *Staph. aureus* were recorded in cheese made from milk inoculated with a commercial lactic acid bacterial culture and a nisin-producing LAB submitted to HP treatment after manufacture. When nisin-containing cheese was treated at 500 MPa and 10ºC for 5 min on day 2, counts of *Staph. aureus* dropped sharply by up to 2.5 log-cycles on day 3 and no growth was detected between 20 and 60 days later. Similarly, Arqués et al. (2005b) evaluated the combined effect of HP and nisin-producing LAB in cheese on *L. monocytogenes*; HP treatment at 500 MPa and 10ºC for 5 min proved to be more effective in killing *L. monocytogenes* when applied on day 51 than on day 2. In agreement with the results of Arqués et al. (2005b), applying HP treatment at 500 MPa and 10ºC for 5 min in raw-milk cheese manufactured with nisin-producing LAB led to undetectable counts of *E. coli* on day 50 (Rodríguez et al., 2005). Capellas et al. (2000) applied those combinations of HP and nisin that caused the lowest impact on the sensory characteristics of cheese, and measured more than 2 log reductions in the viability of aerobic mesophilic bacteria when 7 IU mL^{-1} nisin and 500 MPa were combined for 30 min at 25ºC. Furthermore, HP treatment may also improve the efficacy of nisin for inactivation of some spores by increasing the permeability of the spore coat after the germinating process; counts of spores of *B. cereus* in traditional cheese curd were dramatically reduced when the addition of nisin was followed by two HP cycles, a cycle to induce spore germination and a second to destroy vegetative cells (López-Pedemonte et al., 2003).

Two hypotheses may explain the synergism of combining HP and nisin. The first step of the wedge model of nisin pore formation (Moll et al., 1997) is a parallel orientation of the molecule and subsequent binding to the membrane, which could increase sensitisation of the microorganisms to pressure by local immobilization of phospholipids (Ter Steeg et al., 1999). Secondly, synergistic effects have been attributed to sublethal damage by the permeabilization effect of high pressure on the cell wall and/or outer membrane for gram-negative microorganisms that could facilitate the access of bacteriocins to the cytoplasmic membrane (Hauben et al., 1996).

Mechanisms involved in permeabilization of the cell envelope and its subsequent sensitisation to nisin seem to be dependent of process variables such as pressure and treatment time. Diels et al. (2005) studied the effect of nisin on *E. coli* under low pressure and short exposure time conditions and concluded that the outer membrane was permeabilised transiently. This transience only occurred during the treatment within the pressure range of 150 to 300 MPa by mechanical damage, rather than physiological or metabolic damage and, thus, the outer membrane was immediately repaired after the process. In contrast, HP treatment can causes permanent membrane damage due

to a higher pressure applied or a longer exposure time. Black et al. (2005) observed that, although part of the damage sustained during HP treatment (200 MPa for 5 min at 20ºC) is rapidly reversed on depressurisation, a portion of the cells of *P. fluorescens* remained permeabilised and susceptible to nisin, demonstrating that significant cell damage is sustained during and after pressure.

Permeabilization due to HP has been evidenced by leakage of the periplasmic enzyme β-lactamase of *E. coli* (Hauben et al., 1996). This phenomenon may be explained by changes in membrane fluidity following HP treatment (Ter Steeg et al., 1999). HP treatment induces a phase transition of the lipid bilayer membrane, shifting the natural crystalline phase to an initial reversible gel-phase and finally to an irreversible integrated phase, as well as reduced thickness of the bilayers (Kato & Hayashi, 1999).

Membrane composition has been shown to influence the efficacy of nisin-HP treatment with regard to membrane fluidity, as well as treatment temperature. An increased degree of unsaturation of membrane fatty acids was correlated with protection against pressure inactivation, while higher content of lysylphosphatidylglycerol and diphosphatidylglycerol play a key role in increased susceptibility to nisin and/or HP, respectively (Ter Steeg et al., 1999). With respect to temperature, cell membranes far below their growth temperature are in a semicrystalline gel state, which is more rigid and HP-sensitive than those of cells closer to their growth temperature (Ter Steeg et al., 1999). The effect of such changes and damage in the cell envelope results in the disruption of H-bonds, ionic bonds and hydrophobic interactions of the macromolecules (Hoover, 1993), protein denaturation (Hoover et al., 1989) and losses in transmembrane proton gradient and proton motive force (Kalchayanand et al., 2004).

Nevertheless, treatment variables and proposed mechanisms for modes of action of and HP action seem to be strongly interrelated. Surprisingly, the effect of adding nisin to milk of cheese treated by posterior HP was rapidly manifested in killing aerobic mesophilic bacteria within the pH range 6-7, although pH 4-5 is apparently the most favourable for nisin activity (Capellas et al., 2000). Moll et al. (1997) found that acidity of media affected nisin-induced pores in terms of dissipation of the transmembrane pH gradient (ΔpH) and the transmembrane electrical potential (Δψ). The efficiency of nisin in collapsing the Δψ decreases markedly when the pH is lowered from pH 7.0 to 6.0. In addition, dissipation of the ΔpH is only marginally lower at pH 6.0 than 7.0, being the ΔpH the primary target for nisin action at an acidic pH. Thus, pore dissipation changes with regard to pH but pores can keep their functionality. Moreover, higher doses of nisin were incapable of increasing synergism with HP for inactivation of germinated spores of *B. cereus* (López-Pedemonte et al., 2003) and, in addition, poor effectiveness of nisin in reducing *E. coli* population in milk was observed despite the higher concentration of nisin (400 IU mL^{-1}) and high pressure applied (600 MPa for 15 min at 20ºC) (García-Graells et al., 1999). The antagonism between nisin activity and nisin concentration could be due to competition for membrane binding sites, to the formation of hetero-oligomeric pore complexes, or both (Moll et al., 1997). In contrast, Christ et al. (2007) concluded that nisin concentration is not a limit for pore formation. Therefore, the interaction of nisin and HP in causing cell death suggests that mechanisms involved in synergism may need further research for a better understanding.

Use of nisin in combination with high-intensity pulsed electric fields

HIPEF has generated interest as a feasible non-thermal technology for processing liquid foods. Although only a few studies have been focused on microbial inactivation in milk by this treatment, the results of inactivation by combinating HIPEF with other antimicrobials, particularly nisin, bode well for future development. Calderon-Miranda et al. (1999a) found that increasing the electric field intensity, the number of pulses and the nisin concentration acted synergistically for inactivation of *L. innocua* in skim milk. Up to 3.8 log units inactivation of *L. innocua* was observed in that study after exposure to 100 IU nisin mL^{-1} and a HIPEF treatment of 32 pulses at 50 kV cm^{-1}, over 0.6 log units more than an additive effect. The same behaviour was observed for natural flora of raw milk, when nisin added prior to a HIPEF treatment at 80 kV cm^{-1} for 100 μs produced an extra inactivation of 4.42 log cycles (Smith et al., 2002). Similarly, the loss of viability and cell death of *Staph. aureus* in skim milk caused by both treatments applied simultaneously resulted in a remarkable synergistic effect, compared to those treatments applied individually (Sobrino-López et al., 2006), with over 4.0 additional log units inactivation when HIPEF treatment time, pH and nisin were 2400 μs, 6.8 and 20 IU mL^{-1}, respectively. However, the combined effect was found to be pH-dependent, since only 2 extra log cycles inactivation were achieved at pH 5.0 under the same treatment conditions (Figure 2). Based on those results, nisin seemed to be inactivated by HIPEF, although either the presence of bacterial cells (Terebiznik et al., 2000) or insolubilization at neutral pH may exert a protective effect (Sobrino-López et al., 2006). Thus, the interaction between nisin and HIPEF may be affected in a complex manner by treatment variables and food properties.

Synergy between HIPEF treatment and nisin may be further enhanced by a third hurdle, such as a mild thermal treatment or the addition of other antimicrobials. The efficacy of a HIPEF-nisin treatment against vegetative cells of *B. cereus* in milk may be intensified by adding carvacrol, although only when used at a high concentration (1.2 mM) (Pol et al., 2001). The combination of HIPEF treatment at 80 kV cm^{-1} and 50 pulses at 52ºC of raw skim milk with 1638 IU lysozyme mL^{-1} and 38 IU nisin mL^{-1} provided at least 7.0 log reduction of the native microflora (Smith et al., 2002). Therefore, the processing of milk with a combination of HIPEF, mild temperature, nisin and lysozyme may be an effective method for the pasteurization of skim milk (Smith et al., 2002).

However, variables and conditions affecting synergism of the combined treatment or mechanisms by which the cell membranes of microorganisms becomes sensitized are not yet fully understood. From observations of *L. innocua* by transmission electron microscopy, Calderon-Miranda et al. (1999b) noticed marked morphological differences between nisin-HIPEF-treated cells and those treated with nisin and HIPEF alone as electric field intensity increased. Cells treated by nisin-HIPEF exhibited lack of cytoplasmic or holes, cytoplasmic clumping, an increase in the cell wall surface roughness and cell membrane thickness, blebs or separation of the cytoplasm from the cell membrane, and, finally, a slight increase in the cell length. Therefore, the combination of nisin and HIPEF treatment induced damage to the cell wall and cell membrane that inhibited their functionality as a barrier.

As synergism exhibited by the combination of nisin and HP, formation of nonlethal pores in the cell wall or outer membrane by HIPEF could ease the reach of the cytoplasmic membrane to nisin (Terebiznik et al., 2000) and, conversely, using nisin as an adjunct to HIPEF treatment may alter the HIPEF resistance of microorganisms (Dutreux et al., 2000) by reducing the critical field strength required for cell lysis (Ho et al., 1995). However, contradictory results obtained in different studies

apparently suggest that the proposed hypotheses need to be detailed or even rewritten. Dutreux et al. (2000) showed that there was no sublethal injury in *M. luteus* as a result of HIPEF treatment. In agreement with those results, Gallo et al. (2007) studied the sequence of application of nisin and HIPEF and concluded that applying nisin before HIPEF treatment enhanced their simultaneous effect, whereas nisin addition after HIPEF did not modify the final effect. This behaviour suggests that changes on the media and those inflicted to the cell envelope by HIPEF may impede the action of

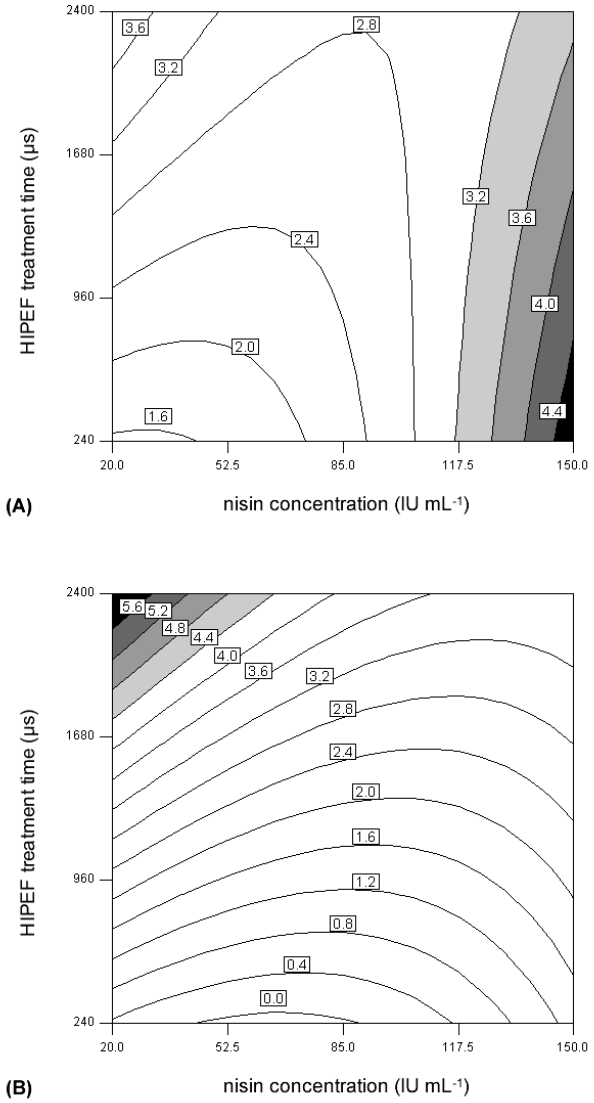

Figure 2. Effect of nisin concentration (IU mL^{-1}) on the survival fraction of *Staphylococcus aureus* in skim milk for different HIPEF treatment times (µs) at different pH values (A: 5.0 pH; B: 6.8 pH) (Sobrino-López et al., 2006).

nisin. In this respect, modification of the soluble components of whey protein concentrate by HIPEF would favour interaction of nisin with the medium instead of the bacteria, and changes in the cell envelope, loss of osmotic response, and reduction of cell surface hydrophobicity may induce cellular resistance to nisin (Gallo et al., 2007). These results partially agree with the mode of action of nisin and its amphipathic character, which allows nisin to interact with phospholipid membranes (Moll et al., 1997), since membrane phospholipids act as target molecule of nisin where it is bound by

electrostatic attraction of their negative charges. In addition, and consistent with the wedge model for pore formation of nisin (Moll et al., 1997), Terebiznik et al. (2000) claimed that no additional effect of nisin would be expected when HIPEF has lethal effects. The shrinkage of the outer and cytoplasmic membranes could allow entrance of nisin into the cytoplasm and, consequently, internalized nisin would not be able to form pores from the cytoplasm due to inadequate $\Delta\psi$ (negative inside) and ΔpH (alkaline inside). Since little research has been performed on membrane changes due to HIPEF and their influence on mode of action of nisin, interaction between nisin and HIPEF in cell inactivation has not been fully explained.

Limitations of using nisin in dairy products

Several limitations curb the use of nisin in dairy products, such as its adsorption to fat and the surface of protein globules, a heterogeneous distribution in dairy product matrices, the inhibition of non-resistant starter cultures, or flavour alteration on incorporation of nisin-producing strains as starters. To overcome these limitations, microencapsulation of nisin in phospholipid vesicles has been tested in Cheddar cheese (Laridi et al., 2003). Vesicles binding nisin could successfully withstand the Cheddar cheese-making temperature cycle and improve nisin stability, efficacy and distribution, although the stability of liposome vesicles is affected by the fat content of milk (Laridi et al., 2003). The appearance of resistant cells in strains sensitive to nisin may constitute another limitation to its use. *Strep. thermophilus* INIA 463 is a nisin-sensitive strain, although it has been shown to become nisin-resistant after exposure in skim milk to subminimal inhibitory concentrations of nisin (1 to 3 IU mL^{-1}) for less than 2 h, by the induction of a resistance mechanism based on changes in the cell wall (Garde et al., 2004). In a similar way, nisin-resistant variants of wild-type *Listeria* isolated from hand-made cheeses commercialized in Spain were able to survive and grow in milk fermented by a nisin-producing *Lactococcus* (Martinez et al., 2005). The exposure of *L. monocytogenes* to acidic conditions in milk enhanced its long-term survival in the presence of nisin in refrigerated conditions (Bonnet & Montville, 2005).

The development and efficacy of the use of nisin as a biopreservative in processed or minimally-processed foods may depend on a wide range of physicochemical properties of the molecule itself and its behaviour in the medium. Milk is a complex mixture of different substances, such as water, proteins and fat, so that the effectiveness of nisin in processed milk may be affected by composition. Several studies have reported an interaction between milk fat and nisin activity, which may limit its application in fat-containing dairy products. Jung et al. (1992) found that activity of nisin against *L. monocytogenes* decreased as the milk fat concentration increased. Bhatti et al. (2004) also found a maximum antilisterial effect of nisin in skim milk and a reduced effect in milk with ≥2% fat. Moreover, Bhatti et al. (2004) verified that homogenisation of milk reduced the antilisterial activity of nisin. Zapico et al. (1999) reported a loss of up to 64% in the effectiveness of the bacteriocin against *Listeria innocua* in homogenised whole milk, which may be due to the binding of nisin to milk fat globules, and thus may be prevented by minimizing its adsorption to the globules' surface. For example, emulsifiers have been shown to be useful in maintaining nisin activity; polyoxyethylene sorbitan monooleate, known as Tween 80, was tested in half-whole milk and shown to retain 43.4% of the original activity of nisin after 2 h at 37ºC, while only 19.6% was detected without the emulsifier (Jung et al., 1992). Although the mechanism by which emulsifiers reverse the loss of the

antilisterial effects of nisin in homogenized milk is not known at this time, Bhatti et al. (2004) suggested that peptides such as nisin can be displaced from an interface by the Tween 80 and be available to adhere to bacterial cells.

PEDIOCIN PA-1

Pediocin PA-1, a plasmid-encoded peptide produced by *Pediococcus acidilactici*, is commercially exploited as a bacteriocin-containing fermentate powder. Although this antimicrobial compound is mainly used in meat products, the extension of its application to dairy products is being evaluated due to its antilisterial activity, but also due to its stability in aqueous solutions, its wide pH range for activity and the fact that it is unaffected by heating or freezing (Nes et al., 1996). However, as far as is known, the effect of adding the pediocin-containing powder in milk has not yet been studied. Instead, the production of pediocin in heterologous hosts is currently considered an attractive alternative in milk and dairy products, since *P. acidilactici* is not suitable for the production of dairy products from both metabolic and technological points of view. The antilisterial activity of recombinant *L. lactis* MM217 as a pediocin-producing starter culture has been successfully tested in Cheddar cheese without affecting its technological properties (Buyong et al., 1998). In a recent study, strains of *L. lactis* ESI 153 and *L. lactis* ESI 515, isolated from hand-made raw milk cheese and transformed into pediocin producers, were identified as likely candidates for food-grade bacteriocin-producing strains (Reviriego et al., 2005). In addition, *Lactobacillus plantarum* WHE 92, which grows particularly well in cheese, especially Munster-type, has been revealed as a spontaneous pediocin-producer at a high enough concentration and pH range to be used in the industrial manufacture of cheeses (Ennahar et al., 1996).

LACTICIN 3147

Although lacticin 3147 has not been commercially exploited, many studies have suggested this bacteriocin as being potentially suitable for many applications. More particularly, interest in lacticin 3147 has steadily risen owing to its activity against a broad range of organisms of importance in foods. Lacticin was isolated from an Irish kefir grain used for making buttermilk; *L. lactis* subsp. *lactis* DPC3147, identified as this bacteriocin producer (McAuliffe et al., 1998), is not a nisin producer. Lacticin 3147 is a two-component bacteriocin which is hydrophobic in nature (McAuliffe et al., 1998) even though it has been classified as belonging to class I (Cintas et al., 2001). Like nisin, properties of lacticin 3147 are pH-dependent; its activity increases in acidic media, as well as its stability to thermal treatments. Lacticin 3147 was inactivated when treated at 121ºC for 10 min at 9.0 pH, while it only lost 50% of its activity at pH 5.0 (Ryan et al., 1996).

Potential use of lacticin 3147 in fermented dairy products

Research on use of lacticin 3147 has been led to both a commercial-lacticin-3147 powder and the generation of lacticin 3147-producing transconjugant starters. Since production of, and immunity to, lacticin 3147 are plasmid-encoded traits, this plasmid can be conjugally transferred to commercial

starters (Ryan et al., 1996). To date, more than 30 lacticin-3147-producing transconjugant starters have been generated for possible application in cheese-making (Coakley et al., 1997). From a technological point of view, lacticin 3147 has shown antimicrobial effects, but it may be applied to achieve control of cell lysis of adjuncts or LAB and flavour formation in cheese as a lytic compound. Martínez-Cuesta et al. (2001) proved that the constructed lacticin-3147-producing transconjugant, *L. lactis* IFPL3593, improved sensory properties of semi-hard cheese, such as taste, after 27 days of maturation. However, transconjugants, such as *L. lactis* DPC4275 are generally less efficient producers of lacticin 3147, and, as a result, there is not a sufficiently high bacteriocin concentration to inhibit spoilage by NSLAB (Ryan et al., 2001).

The industrial viability of lacticin 3147-based powder is being evaluated as a biopreservative, so that it may be applied as a food ingredient in a variety of foods. Morgan et al. (2001) found that a 10% lacticin 3147 powder was extremely effective for the inhibition of *Listeria* in yoghurt and cottage cheese. Within 60 min of adding lacticin 3147 powder, no viable cells of *Listeria* remained in the yoghurt and, in the case of cottage cheese, counts showed 85% of non-viable cells after 120 min; the kill rate was more rapid at higher temperature. In spite of lacticin 3147 powder being effective as an antimicrobial, two serious setbacks may prevent its commercial use: its stability in food media and heat-sensitivity. A concentrated powdered product containing lacticin 3147 lost 75% of its activity after 5 months at room temperature, while full activity was retained for 5 months at 4ºC (Morgan et al., 2001). The effect of autoclaving (121ºC for 15 min) a lacticin 3147 powder and a resuspended preparation in whey also resulted in considerable loss of activity (Morgan et al., 2001). In contrast, Morgan et al. (1999) reported that pasteurization had no effect on a lacticin 3147-enriched demineralized whey powder. Assessment of the inhibitory activity of the bioactive powder demonstrated that it is capable of inhibiting both *L. monocytogenes* and *Staph. aureus* at pH 5 and 7 and effectively inactivated 99% of *L. monocytogenes* Scott A added to infant formula within 3 h.

Use of lacticin 3147 in combination with other bacteriocins

From a hurdle concept, a cheap method of introducing bacteriocins to foods could be the use of cultures that produce multiple bacteriocins. Although co-production of some bacteriocins, such as pediocin PA-1 and nisin, does not improve their antimicrobial activity, others, such as lacticin 3147 and 481, have a more inhibitory effect than either of the individual bacteriocins. O'Sullivan et al. (2003) demonstrated that it is relatively straightforward to construct food-grade lactococcal strains which co-produce the lantibiotics lacticin 3147 and lacticin 481 by conjugating the lacticin 3147 genetic determinants into a 481-producing recipient. As an alternative to inefficient transconjugant strains, resistant starter strains of *Lactobacillus* have been isolated to evaluate their potential use as adjuncts of bacteriocin-producers. *Lactobacillus paracasei* DPC5337, incorporated in combination with bulk starters and lacticin 3147-producing starter culture, improved Cheddar cheese flavour up to commercial grade in comparison addition of the bacteriocin producer alone (Ryan et al., 2001).

Use of lacticin 3147 in combination with non-thermal treatments

The combination of two or more antimicrobial treatments at a suboptimal concentration is more effective than one at the optimal level. In the same way as nisin, adding lacticin 3147 following HP treatment at 150 - 275 MPa acted synergistically on the inactivation of *Staph. aureus* in reconstituted skim milk (Morgan et al., 2000); interestingly, lacticin 3147 activity remained stable and even increased after the HP treatment. Although lacticin 3147 has been mainly used in cheese production by incorporation of the starter producer, its characteristics open new possibilities for its application in minimally-processed or refrigerated food products and for additional strategies for control of growth and survival of pathogens. Since lacticin 3147 should be broken down easily by digestive enzymes, it does not represent a threat to humans, making it a safe and economically viable alternative to chemical preservatives currently used in the food industry (Guinane et al., 2005). Furthermore, industrial exploitation of this bacteriocin is expected to be given legislative approval in different countries.

ENTEROCINS

Several bacteriocin-producing strains of enterococci have been isolated from sausage, fish, vegetables and dairy products, specifically cheese, where they naturally occur. Enterococcal bacteriocins have been characterized as substances with strong activity towards *L. monocytogenes*, although many of those bacteriocin-like compounds have not yet been identified. Enterocin CCM 4231, produced by *Enterococcus faecium* CCM 4231, has shown great inhibitory effects against *Staph. aureus* in skim milk and yoghurt and *L. monocytogenes* in yoghurt, indicating possibilities for further application in dairy products (Lauková et al., 1999). Alvarado et al. (2005) atributed the antilisterial activity of *E. faecium* UQ31, a strain isolated from hand-made Mexican-style cheese, to one bacteriocin-like inhibitory substance. That compound has not yet been completely characterized, although *E. faecium* UQ31 has been suggested as a feasible culture for the preservation of dairy products. In an extensive study, the bacteriocin of *E. faecium* 7C5, added as an adjunct with a thermophilic culture in soft-cheese, led to complete death of *L. monocytogenes* and *L. innocua* without altering the acidifying activity of the starter culture (Giraffa et al., 1995). Recently, a bacteriocinogenic *E. faecium* F58 strain was isolated from goat's Jben cheese and, when added as adjunct culture, caused a sharp decrease in the number of viable *L. monocytogenes*, which were undetectable after 1 week of cheese storage at 22ºC (Achemchem et al., 2006). The strains *E. faecium* M241 and 249 obtained from raw goat milk produced bacteriocins especially active towards *L. monocytogenes* and *C. butyricum*, while other species of LAB were not affected (Cocolin et al., 2007). The production of enterocin AS-48 by *Enterococcus faecalis* A-48-32 as an adjunct in milk or fermented cheese was also found to be persistent and effective against *B. cereus* without modifying the growth of starter cultures (Munoz et al., 2004). Incubation of *L. monocytogenes* in raw milk in the presence of *E. faecalis* INIA 4, which releases enterocin 4, also revealed low counts of the pathogen after 24 h (Rodríguez et al., 1997).

OTHER BACTERIOCINS

Little research has been focused on the use of other bacteriocins in dairy products; even so, their technological properties, their bactericidal or bacteriostatic effect, and their implication on some sensory mechanisms suggest both potential usefulness and promising advances in dairy product. Among them, pediocin 5, a bacteriocin produced by *P. acidilactici* UL5, sharply reduced viable counts of *L. monocytogenes* in milk (Huang et al., 1994). Reuterin, which is produced by *Lb. reuteri*, inhibited growth of *L. monocytogenes* and *E. coli* in both cottage cheese and milk when added as a lyophilized powder (El-Ziney & Debevere, 1998); in contrast to nisin, efficacy of reuterin activity did not depend on fat content.

Most research on the application and the effectiveness of new bacteriocins is being directed towards the use of the bacteriocinogenic strains as protective cultures in different milk products. Hence, the activity of the bacteriocin-producing strain and its suitability for the fermented milk product should also be evaluated. Recently, a proteinaceous compound produced by a *Lb. paracasei* subsp. *paracasei* strain used as a starter for Bulgarian yellow cheese was shown to be active against some yeast species, such as *Candida albicans* and *Saccharomyces cerevisiae* (Atanassova et al., 2003). The non-identified antimicrobial peptide of the thermophilic starter *Strep. thermophilus* was active against *L. monocytogenes* and *Staph. aureus* during fermentation and refrigerated storage (Benkerroum et al., 2002). *Strep. macedonicus* ACA-DC 198, a strain isolated from Greek Kasseri cheese, produces an anticlostridial bacteriocin called macedocin. This strain is of particular interest for the production of hard-cooked cheeses, due to its thermophilic character, but also for its contribution to aroma and flavor development (Van den Berghe et al., 2006). Moreover, the persistence of macedocin upon prolonged incubation suggested that the protective effect may be present during the maturation period of cheese (Van den Berghe et al., 2006).

Alternatively, the use of bacteriocins to accelerate cheese maturation and to control flavour development could represent a novel approach to their potential usefulness. A *L. lactis* subsp. *lactis* strain encoding production of lactococcins A, B and M was used to satisfactorily enhance the sensory characteristics of Cheddar cheese (Morgan et al., 1997). Proteolysis and flavour in semi-hard cheese was likewise increased by a bacteriocin-producing strain of *E. faecalis* INIA 4 (Martínez-Cuesta et al., 2000). Lacticin 481, a bacteriocin produced by *L. lactis* subsp. *lactis* strain DPC5552, was shown to cause membrane permeabilization of starter cultures in Cheddar cheese (O'Sullivan et al., 2002); lacticin-481-producing strains allowed the target strain to continue to grow, with a simultaneous release of intracellular enzymes involved in development of cheese flavour. Therefore, strain DPC5552 may provide improved adjuncts for delivering intracellular lactococcal enzymes into the cheese matrix and thus improve cheese quality and flavour. In a later study, the lacticin-481-producing strain *L. lactis* CNRZ481, used as an adjunct for Cheddar cheese manufacture, achieved improved flavour and a reduction in defects in the final product owing to the prevention of NSLAB proliferation (O'Sullivan et al., 2003).

Unfortunately, many bacteriocin producers are poorly adapted to the milk environment, such as probiotic lactobacilli in yoghurt, which may hinder their use as starter cultures. Avons et al. (2004) found that some probiotic lactobacilli of intestinal origin showed only slight growth in milk. *Lb. casei* strains showed the best growth in milk, whereas best growth and bacteriocin production was observed with *Lb. acidophilus* when milk was supplemented with yeast extract. The addition of a

growth factor, co-cultivation or fermentation in another medium and subsequent addition to the milk were suggested in order to overcome the problem.

Recently, a number of studies have pointed out the antimicrobial activity of new substances from bacteria, especially potential bacteriocins that still remain to be defined, classified or characterized, although their incidence in the dairy industry may be also promising. Rodríguez et al. (2000) studied the natural flora of milk and found non-identified bacteriocins to be produced by 16 LAB strains isolated from raw milk. Those bacteriocins were, in general, heat-resistant, and in some cases showed a broad inhibitory spectrum, especially those from *L. lactis* subsp. *lactis* biovar diacetylactis or some strains of *E. faecalis*. Additionally, an antimicrobial substance from *B. subtilis* has exhibited a broad inhibitory spectrum against Gram-positive, Gram-negative bacteria and moulds (Bie et al., 2006). This antimicrobial compound enhanced milk preservation and the sensory acceptance of pasteurized milk. *B. cereus* isolated from raw milk and other dairy products, ice cream or milk powder, has been found to be a producer of bacteriocins, which were stable at pH 3-10 and after heating at 75ºC for 2 min (Torkar & Matijasic, 2003).

CONCLUSIONS

Studies of the antimicrobial activity and behaviour of LAB bacteriocins in the milk environment suggest that they have the potential to ensure microbiological safety and to control quality of dairy products. Their practical use in dairy products may lie both in their incorporation into the finished product, such as adhered bacteriocins on active packaging, and in their addition or inclusion during food manufacture. In the latter case, great effort is being directed towards selecting bacteriocin-producers as potential adjuncts in fermentation processes or even as starter cultures. Moreover, bacteriocins have been shown to improve synergistically the inhibitory effects of thermal and non-thermal treatments, such as HIPEF and HP. In this way, the combination of bacteriocins with heat may facilitate the application of mild thermal treatments, which may diminish the typical cooked flavour of milk and reduce the cost of the heating operation. In particular, the enhancement of the lethal effect of HIPEF and HP when combined with bacteriocin addition may provide a suitable alternative for traditional thermal treatments since it causes minimal alteration to sensory properties of the product. However, a few drawbacks may curb the use and practical application of bacteriocins. Firstly, the use of bacteriocin producers in fermented products demands sensory evaluation of such products, while the commercialization of bacteriocins as food preservatives needs to be regulated by strict requirements of food legislation, with nisin being the only bacteriocin exploited as a food additive to date. Secondly, results in the laboratory have to be scaled up to a food industry process to evaluate the effectiveness of bacteriocins during processing and storage. The use of bacteriocins is thus still limited in dairy products, although their potential applications suggest they may be industrially exploited in the medium term.

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OBJETIVOS

El presente trabajo tuvo como objetivo general estudiar la viabilidad de la aplicación de pulsos eléctricos de alta intensidad de campo (PEAIC) en la inactivación del microorganismo patógeno *Staphylococcus aureus* en leche, y su combinación con otros métodos no térmicos de tratamiento, como el uso de compuestos antimicrobianos de origen natural. Para la consecución del mismo se establecieron los siguientes objetivos específicos:

- Determinar el efecto de las distintas variables del tratamiento de PEAIC, intensidad de campo eléctrico, anchura de pulso, tiempo de tratamiento y polaridad, y el efecto de la presencia de grasa sobre *Staph. aureus* inoculado en leche.

- Evaluar el efecto combinado de PEAIC con la adición de bacteriocinas, nisina y enterocina AS-48, y otros compuestos antimicrobianos de origen natural, como la lisozima, en la inactivación de *Staph. aureus* en leche.

- Determinar la vida útil y la estabilidad microbiológica en leche cruda procesada mediante la aplicación individual de PEAIC o la combinación de PEAIC con la adición de compuestos antimicrobianos de origen natural.

- Comparar la evolución microbiológica de la flora natural de la leche tras un tratamiento de pasteurización con la conseguida mediante la combinación de un calentamiento moderado, la adición de substancias antimicrobianas y el procesado de PEAIC.

MATERIAL Y MÉTODOS

LECHE

Se utilizó leche UHT desnatada, semidesnatada o entera (Puleva, Mollerussa, Lleida, España) inoculada con *Staphylococcus aureus* para evaluar el efecto letal de la aplicación de pulsos eléctricos de alta intensidad de campo (PEAIC) y la adición de antimicrobianos. Se empleó leche cruda (Puleva, Mollerussa, Lleida, España) para determinar su vida útil tras la aplicación de los distintos tratamientos planteados. La leche se mantuvo en refrigeración a 4ºC hasta su utilización.

Staphylococcus aureus

Se utilizó la cepa *Staphylococcus aureus* CECT 240 (Departamento de Tecnología de Alimentos, Universidad de Lleida, Lleida, España) como microorganismo de referencia. La cepa se mantuvo en tubos de ensayo con agar inclinado (PCA; Biokar diagnostics, Beauvais, Francia) a 4ºC hasta el momento de su utilización. Se multiplicó la cepa inoculando caldo de triptona y soja (TSB; Biokar diagnostics, Beauvais, Francia) e incubando con agitación durante 6 h a 200 rpm y 35ºC, alcanzando así una concentración de aproximadamente 10^9 UFC/mL y una absorbancia a 620 nm entre 0.60 y 0.70.

PREPARACIÓN DE LAS MUESTRAS

Se utilizó leche en su pH natural o se ajustó a 5.0 ó 5.9 utilizando ácido láctico (L(+)-lactic acid, Panreac, Barcelona, Spain) controlándose con la ayuda de un pH-metro (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, España). Para los distintos valores de acidez, se midió la conductividad eléctrica de la leche utilizando un conductímetro (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Alemania). Seguidamente, se inoculó la cepa de *Staph. aureus* hasta alcanzar una concentración aproximada de 10^7 ufc/mL.

OBTENCIÓN DE LA SOLUCIÓN DE ENTEROCINA AS-48

La solución de enterocina AS-48 se obtuvo por concentración mediante cromatografía de intercambio catiónico de caldo inoculado con la cepa productora *Enterococcus faecalis* A-48-32 (Abriouel et al., 2003) según el método descrito por Martínez-Bueno et al. (1990). La solución concentrada se desaló utilizando tubos de diálisis de 2,000 Da (Sigma-Aldrich, Steinheim, Alemania) y filtró a través de filtros de baja retención proteica de 0.22 µm (Millex GV; Millipore Corp., Belford, MA, EE.UU.) en condiciones estériles. La actividad de la solución de enterocina AS-48 resultante se determinó con el método de difusión en agar propuesto por Gálvez et al. (1986). La concentración final fue de 3,500 AU/mL de enterocina AS-48 que se diluyó convenientemente en agua desionizada según la actividad final deseada en las muestras a tratar.

ADICIÓN DE AGENTES ANTIMICROBIANOS

El efecto de la adición de agentes antimicrobianos sobre *Staph. aureus* se determinó añadiendo nisina (N 5764, 2.5% de nisina, 1,000,000 IU/mg, Sigma-Aldrich, Steinheim, Alemania), lisozima (L 2879, 43,560 IU de lisozima/mg sólido, Sigma-Aldrich, Steinheim, Alemania) o enterocina AS-48 a la leche inoculada con el mencionado microorganismo en las cantidades oportunas para alcanzar concentraciones en los rangos 0-100 IU/mL, 0-3,000 IU/mL y 0-28 AU/mL, respectivamente. La influencia del tiempo de exposición, el pH, la presencia de grasa y la temperatura también fueron consideradas.

EQUIPO Y TRATAMIENTO DE PULSOS ELÉCTRICOS DE ALTA INTENSIDAD DE CAMPO

Para desarrollar este estudio se utilizó un equipo de tratamiento continuo de PEAIC OSU-4F (Ohio State University, Columbus, Ohio, USA). El dispositivo descarga pulsos eléctricos de forma cuadrada a través de 8 cámaras colineales de electrodos paralelos. La distancia entre sus electrodos es de 0.29 cm y cada cámara de tratamiento dispone de 0.012 cm^3 de volumen. La intensidad de campo (kV/cm), la anchura (µs) y polaridad del pulso y el número de pulsos fueron las variables eléctricas controladas, prefijando la frecuencia a 75 ó 100 Hz. La temperatura del medio se mantuvo por debajo de 25ºC usando un baño termostático.

EQUIPO Y TRATAMIENTO TÉRMICO

Se utilizó un intercambiador de calor tubular de 2.16 mm de diámetro interior y 1,100 cm de longitud. El caudal se mantuvo a 152 cm^3/min mediante una bomba para conseguir un tiempo de tratamiento de 16 s. Como fluido térmico se empleó agua caliente para elevar la temperatura de la leche desde 4ºC hasta 55, 65 ó 75ºC. Tras el calentamiento, la temperatura se redujo nuevamente a 4ºC mediante un baño termostático.

DISEÑOS EXPERIMENTALES DE SUPERFICIES DE RESPUESTA

Se propusieron modelos de superficie de respuesta para el estudio del efecto sobre *Staph. aureus* de las distintas variables independientes. Los ensayos de cada diseño se llevaron a cabo aleatoriamente y por duplicado, a su vez, cada una de las muestras tratadas se sembró por triplicado. La inactivación microbiana observada, -log *s*, se ajustó mediante un modelo polinomial de segundo orden cuya expresión general se muestra en la ecuación (1)

$$-\log s = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (1)$$

en el que β_i son los coeficientes de regresión y X_i representa cada una de las variables propuestas. El modelo se optimizó por iteración omitiendo aquellos términos no significativos (P>0.05) del análisis de varianza. El intervalo de confianza fue del 95% en todos los procedimientos. El programa Design Expert 6.0.1 (Stat Ease Inc., Minneapolis, USA) se utilizó para todos los análisis de superficie de respuesta y los subsiguientes gráficos.

EFECTO DE LA COMBINACIÓN DE MÉTODOS NO TÉRMICOS DE TRATAMIENTO

En primer lugar, se valoró la efectividad de la adición individual de cada uno de los agentes antimicrobianos a dosis subletales con el tratamiento de PEAIC. Después, se comprobó el efecto cuando, previamente al procesado mediante PEAIC, se incorporaba una mezcla de dos antimicrobianos, nisina y lisozima, nisina y enterocina AS-48 o enterocina AS-48 y lisozima.

INACTIVACIÓN DE *Staphylococcus aureus*

Se preparó un banco de diluciones con peptona salina con alícuotas de las muestras tratadas y no tratadas y se sembró por superficie en placas estériles con PCA, que se incubaron a 35ºC durante 48 h. El número de células viables de *Staph. aureus* tras el tratamiento se expresó como fracción de supervivientes, *s*, calculada con el cociente entre la concentración de microorganismos tras cualquiera de los tratamientos, *N*, y la concentración inicial antes del tratamiento, N_0. La inactivación microbiana se calculó como –log *s*.

VIDA ÚTIL DE LECHE

Se determinó la vida útil a 4ºC de leche cruda tratada mediante aquellas combinaciones de métodos que proporcionaron mayor nivel de inactivación de *Staph. aureus*. Además, se comparó la inactivación de la flora natural de la leche tras la aplicación simultánea de la adición de antimicrobianos, procesado mediante PEAIC y calentamiento moderado (55 o 65ºC durante 16 s) con el tratamiento de pasteurización (75ºC durante 16 s). Los recuentos de mesófilos, enterobacterias, coliformes y los patógenos *Escherichia coli*, *Listeria* spp., *Salmonella* spp. y *Staphylococcus aureus* fueron los análisis microbiológicos considerados y practicados por triplicado según las normas ISO 4833:1991, ISO 7402:1993, ISO 4832:1991, NF V 08-53:1993, ISO 11290-2:1998, ISO 6579:1993 e ISO 6888-1:1999, respectivamente. El límite de aceptación microbiológica fue el indicado en la Directiva 92/46/EEC de la U.E. (1992).

ANÁLISIS ESTADÍSTICO EN EL ESTUDIO DE VIDA ÚTIL DE LECHE

El análisis de varianza y el procedimiento de la menor diferencia significativa de Fisher se emplearon para discriminar entre medias significativamente diferentes (P< 0.05) de cada una de las combinaciones planteadas. Se determinaron los tratamientos que proporcionaron resultados diferentes mediante un análisis de covarianza respecto al tiempo de almacenamiento. Tales análisis

estadísticos se resolvieron con Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MD, EE.UU.).

PUBLICACIONES

CAPÍTULO I

High intensity pulsed electric field variables affecting *Staphylococcus aureus* inoculated in milk

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ABSTRACT

Staphylococcus aureus is an important milk-related pathogen that is inactivated by High Intensity Pulsed Electric Fields (HIPEF). In this study, inactivation of *Staph. aureus* suspended in milk by HIPEF was studied using a response surface methodology, in which electric field intensity, pulse number, pulse width, pulse polarity and the fat content of milk were the controlled variables. It was found that the fat content of milk did not significantly affect the microbial inactivation of *Staph. aureus*. A maximum value of 4.5 log reductions was obtained by applying 150 bipolar pulses of 8 μs each at 35 kV/cm. Bipolar pulses were more effective than those applied in the monopolar mode. An increase in electric field intensity, pulse number or pulse width resulted in a drop in the survival fraction of *Staph. aureus*. Pulse widths close to 6.7 μs lead to greater microbial death with a minimum number of applied pulses. At a constant Treatment time a higher number of shorter pulses achieved a better inactivation than those treatments performed at a lower number of longer pulses. The combined action of pulse number and electric field intensity followed a similar pattern, indicating that the same fraction of microbial death can be reached with different combinations of the variables. The behavior and relationship among the electrical variables suggest that the energy input of HIPEF processing might be optimized without decreasing the microbial death.

INTRODUCTION

The percentage of people suffering from foodborne diseases each year has been reported to be up to 30%. In contrast to traditional outbreaks, current bursts of foodborne diseases often spread over a wide geographic area involving a potentially high number of patients (Rocourt et al., 2003). In the United States of America, for instance, around 76 million cases of food borne diseases, resulting in 325,000 hospitalizations and 5,000 deaths, are estimated to occur each year (WHO, 2002). Among the identified microorganisms causing outbreaks and illnesses, *Staphylococcus aureus* is one of the main foodborne pathogens (Buzby et al., 1997). *Staph. aureus* represented the highest attack (66%) within the annual foodborne outbreaks of infectious intestinal disease in England and Wales from 1992 to 1993 (Cowden et al., 1995).

Staphylococcal food poisoning is a typical intoxication resulting from the ingestion of food containing one or more preformed staphylococcal enterotoxins (SE). Symptoms usually develop quite rapidly, usually 1-6 hours after ingestion, are of relatively short duration, and have no lasting effects. Because of the mild symptoms and rapid recovery, a doctor is seldom consulted and many cases are not reported. Although *Staph. aureus* is a poor competitor and is often overgrown by other microorganisms, milk has been implicated in outbreaks of Staphylococcal intoxication. One of the largest outbreaks ever recorded occurred in June-July 2000 in the Kansai District in Japan. There were 13,420 victims although the primary vehicle food was reconstituted milk from powdered skim milk. An SE-producing strain of *Staph. aureus* was the etiologic agent. Symptoms appeared in 83.4% of interviewed victims within 6 hours, with 3-4 h being the peak period (Jay et al., 2005). Up to 1,000 food borne outbreaks are registered every year in Spain, 8% of which are related to dairy products, while *Staph. aureus* is believed to cause almost 6% of them (Hernández-Pezzi et al., 2004).

Since milk is one of the most important foods in human nutrition susceptible to both spoilage and pathogenic microorganisms, a mandatory pasteurization is required. However, preformed enterotoxins will not be destroyed even though pasteurization is capable of destroying the microorganism (ICMSF, 1998). One outbreak occurred in Kentucky in 1985 where 860 schoolchildren became ill after drinking 2% chocolate milk. The milk was inadvertently kept for several hours at a warm temperature before pasteurization. No staphylococci were isolated, but SE-A was detected in the pasteurized milk (Everson et al., 1988).

Furthermore, thermal pasteurization is involved in causing undesirable changes in the organoleptic qualities, nutritional and/or technological properties of milk. Generation of a “cooked” flavor is the most obvious sensory change in milk processed by heat (Wirjantoro et al., 1997), whereas degradation of its nutritional value, such as protein denaturation and the loss of vitamins, are only detected with analytical procedures.

The increasing demand for fresh-like quality products has promoted the effort for developing innovative non-thermal food preservation methods. Among them, High Intensity Pulsed Electric Field (**HIPEF**) processing is a non-thermal treatment that offers the advantage of inactivating microorganisms with a minimal impact on quality and nutritional factors (Sampedro et al., 2005). Inactivation of different pathogens, such as *Escherichia coli* (Martin et al., 1997; Evrendilek et al., 2005), *Salmonella dublin* (Sensoy et al., 1997) or *Staph. aureus* (Sobrino et al., 2006), by applying a HIPEF treatment in milk has been demonstrated by several authors. However, most of the studies in regard to the effects of HIPEF on microorganisms have been performed in model solutions (Bendicho

et al., 2002) so that their results exclude the influence of the food composition on microbial resistance. For instance, HIPEF research on milk has been developed in milk ultrafiltrate (SMUF) despite milk being a complex food in which proteins and fat are present. Martin et al. (1997) found that inactivation of *E. coli* in milk was more limited than in a buffer solution, due to milk proteins. Furthermore, there is no agreement with the possible influence of fat content on HIPEF inactivation. Moreover, the only process parameters considered in those studies were field strength and treatment time, even though the relationship among the different process parameters acting simultaneously may be important in optimizing the inactivation of microorganisms by HIPEF.

As there is not enough information referring to the microbial death of *Staph. aureus* in milk by HIPEF treatment and the resistance of this pathogen to the different parameters involved in the treatment, the purpose of this research was to study the individual or combined effect of *Staph. aureus* inactivation in milk due to HIPEF, in which the controlled variables were electric field intensity, pulse number, pulse width, pulse polarity and fat content of milk.

MATERIAL AND METHODS

Staphylococcus aureus culture

Staphylococcus aureus CECT 240 (Dept. of Food Technology, University of Lleida, Spain) was used as a target microorganism and maintained on slants of plate count agar (PCA; Biokar diagnostics, Beauvais, France) at 4ºC throughout the experiments. *Staph. aureus* cells were grown by inoculation and incubation in tryptone soy broth (TSB) up to approximately 10^9 cfu/ml with orbital agitation at 200 rpm, 35ºC and 6 h.

Treatment Media

Homogenized UHT milk was supplied by a dairy plant and stored at 4ºC (Granja Castelló, Mollerussa, Lleida, Spain). The pH of the milk was 6.68±0.02 at 25ºC and a pH-meter was used for the measurement (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain). The electrical conductivity of the milk was measured at 25ºC and determined with a conductivity meter (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Germany). Table 1 shows the electrical conductivity of the milk with different fat contents.

Table 1. Electrical conductivity of milk at 25ºC

Fat content	Electrical conductivity [a] (mS/cm)
Whole milk	5.50±0.01
1.5% fat milk	5.85±0.01
Skim milk	6.03±0.01

[a] mean of three measurements±SE

Sample Preparation

Prior to HIPEF treatment, samples of *Staph. aureus* were prepared by inoculating milk with cells of the microorganism suspended in TSB at the mid-exponential phase to a final concentration of approximately 10^7 cfu/ml. Air bubbles of the sample were removed with a diaphragm vacuum pump (Vacuubrand, Wertheim, Germany).

HIPEF Equipment

A continuous-flow HIPEF system was used to carry out this study. The treatment device was a OSU-4F HIPEF unit (Ohio State University, Columbus, Ohio, USA) that discharges square-shaped pulses within eight co-field flow chambers. Gap distance and volume in each chamber were 0.29 cm and 0.012 cm^3, respectively. The pulse frequency was set to 100 Hz. The treatment temperature was kept under 25ºC using a cooled water bath to rule out thermal effects.

Survival Fraction of *Staphylococcus aureus*

The untreated and treated samples were serially diluted in peptone water, spread-plated on PCA plates and incubated for 48 h at 30ºC. The number of viable cells of *Staph. aureus* after applying a HIPEF treatment was expressed as survival fraction, *s*, which was calculated as N/N_0, where N_0 was the initial count in samples prior to the HIPEF treatment, and N was the count after each treatment. Microbial inactivation was calculated as –log *s*.

Experimental Design

A response surface analysis was utilized to evaluate the effect of the different variables of the HIPEF treatment on the survival fraction of *Staph. aureus* in milk. A face-centered, central composite design with five factors was the proposed experimental design (Myers et al., 2002). The independent variables were electric field intensity, pulse number, pulse width, pulse type and fat content of milk, and were set at 25 or 35 kV/cm, 50 or 150 pulses, 4 or 8 μs, in the monopolar or bipolar mode and with 0 or 3% fat, respectively. Variable levels were chosen according to previous studies. The experimental design along with each experimental condition is shown in Table 2. A duplicate was performed, resulting in two blocks of experiments. The order of assays within each block was randomized and performed in triplicate.

The effect of the five independent variables was modeled using a polynomial response surface. The second-order response function was predicted by the following equation:

$$-\log s = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{eq. 1}$$

where factor *X* represents the encoded values of the variables and β are the constant coefficients. The non-significant terms ($p \leq 0.05$) were deleted from the second-order polynomial model after an

analysis of variance (ANOVA), and then a new ANOVA was recalculated to obtain the coefficients of the final equation for better accuracy.

To perform this study Design Expert 6.0.1 software (Stat Ease Inc., Minneapolis, USA) was used in all analyses and generated plots. A 95% confidence interval was used for all procedures.

Table 2. Central composite response surface design and microbial inactivation of *Staphylococcus aureus* suspended in milk and submitted to different combinations of fat content and HIPEF variables

Assay number[a]	Point type	Uncoded variables					
		Electric field intensity (kV/cm)	Pulse number	Pulse width (μs)	Fat content (%)	Pulse type	
						monopolar	bipolar
						Response variable[b] (-log *s*)	
1	Factorial	35	150	8	0.0	3.55	4.48
2	Factorial	35	150	4	0.0	2.43	3.48
3	Factorial	35	50	8	3.0	1.38	2.70
4	Factorial	25	150	4	3.0	0.40	0.96
5	Factorial	35	50	4	3.0	1.83	1.92
6	Factorial	25	50	8	0.0	0.22	0.58
7	Factorial	25	150	8	3.0	1.39	1.52
8	Factorial	25	50	4	0.0	0.00	0.17
9	Axial	25	100	6	1.5	0.78	1.54
10	Axial	35	100	6	1.5	3.36	3.90
11	Axial	30	50	6	1.5	1.33	1.56
12	Axial	30	150	6	1.5	2.36	2.34
13	Axial	30	100	4	1.5	1.51	1.11
14	Axial	30	100	8	1.5	2.33	2.23
15	Axial	30	100	6	0.0	1.74	2.54
16	Axial	30	100	6	3.0	2.10	2.86
17	Center	30	100	6	1.5	1.14[c]	2.97[c]

s: survival fraction

[a] Order of assays were randomized

[b] Data shown are the mean of two treatment repetitions, each assay was performed in triplicate

[c] Data shown are the mean of five repetitions

HIPEF treatment was set at 100 Hz

RESULTS AND DISCUSSION

Checking of the fitted model

In order to determine the effect of HIPEF treatment on microbial death of *Staph. aureus* inoculated in milk, a response surface design was performed. The process variables were electric field intensity, pulse number, pulse width, pulse polarity and the fat content of the milk. Table 2 shows the microbial inactivation of *Staph. aureus* achieved for each combination of the controlled variables. No

microbial death of Staph. aureus inoculated in skim milk was observed with either monopolar or bipolar pulses when a HIPEF treatment at 25 kV/cm electric field intensity and 50 pulses of 4 μs pulse width were applied. Conversely, a HIPEF treatment at 35 kV/cm of electric field intensity and 150 pulses of 8 μs pulse width reached 4.5 log reductions of *Staph. aureus* in skim milk when bipolar pulses were used, and 3.6 log units were registered in the case of monopolar pulses with the same variable combination.

Table 3. Analysis of variance for the response surface quadratic model of *Staphylococcus aureus* in milk

Source	Mean square	F-value	Prob>F
Quadratic model	4.1260	21.1649	< 0.0001 [s]
E	12.2007	62.5855	< 0.0001 [s]
N	1.6259	8.3402	0.0056 [s]
τ	4.3068	22.0923	< 0.0001 [s]
F	0.2331	1.1958	0.2790
Pp	7.4844	38.3922	< 0.0001 [s]
E^2	0.3340	1.7131	0.1961
n^2	1.0194	5.2290	0.0262
τ^2	1.8064	9.2663	0.0036 [s]
f^2	0.0847	0.4347	0.5125
$E \cdot n$	0.7673	3.9362	0.0524 [s]
$E \cdot \tau$	0.0091	0.0467	0.8297
$E \cdot f$	0.1194	0.6124	0.4373
$E \cdot pp$	0.3811	1.9548	0.1678
$n \cdot \tau$	0.9167	4.7022	0.0345 [s]
$n \cdot f$	0.2626	1.3470	0.2509
$n \cdot pp$	0.0229	0.1176	0.7330
$\tau \cdot f$	0.0973	0.4989	0.4830
$\tau \cdot pp$	0.1351	0.6931	0.4088
$f \cdot pp$	0.0229	0.1175	0.7331
Pure error	0.081		
Cor. Total	88.92		
Standard Deviation	0.44		
Mean	1.92		
R-squared	0.88		
Adjusted R-Squared	0.84		

[s] Significant at 95% Confidence Interval

E: electric field intensity; *n*: pulse number; τ: pulse width; *f*: fat content of milk; *pp*: pulse polarity

Table 3 shows the ANOVA for the response surface model. The second-order response function showed a significant fit with the data ($P < 0.0001$) and the determination coefficient, R^2, was 0.88, meaning that the model is adequate for predicting the response across the design space. The variables electric field intensity, pulse number and pulse width affected microbial inactivation linearly, whereas only the quadratic term of pulse width resulted significant. There were also differences in the survival fraction of *Staph. aureus* achieved by monopolar and bipolar pulses. The

combined action of pulse number with electric field intensity and pulse width was included in the model as interaction terms. Coefficients of the fitted model are shown in Table 4. The fat content of the milk produced no statistical effect on the inactivation of *Staph. aureus*.

Effect of fat content of milk

The fat content of the milk did not modify the resistance of *Staph. aureus* to a HIPEF treatment. However, the influence of this variable on the HIPEF inactivation of microorganisms is still unclear. Grahl et al. (1996) indicated that the fat content of the medium is inversely related to microbial inactivation and claimed that fat particles of milk seemed to protect bacteria against electric pulses. Their results could be explained by variation of milk conductivity, since this variable decreases as the percentage of fat increases. However, as reflected in the statistical analysis (Table 3), the variation in fat content did not significantly modify the counts of *Staph. aureus* suspended in milk after applying a HIPEF treatment even though the electrical conductivity of whole and skim milk varied from 5.50 to 6.03 mS/cm, respectively (Table 1). Coinciding with our results, some authors found no difference when comparing samples treated by HIPEF with a different fat content. Reina et al. (1998) inoculated *Listeria monocytogenes* in whole and skim milk samples, which were treated at 30 kV/cm for 100 to 600 μs, and no significant differences were found. Michalac et al. (2003) also reported that electrical conductivity showed no effect on the inactivation of different bacteria in the HIPEF-processed milk.

Table 4. Significant regression coefficients of the quadratic model for the survival fraction of *Staphylococcus aureus* suspended in milk

Factor	Coefficient estimate	Low	High	Standard error
Intercept:				
-monopolar pulses	-7.607	-10.524	-3.980	1.636
-bipolar pulses	-6.971	-10.304	-3.560	1.686
E	0.144	0.110	0.178	0.017
N	-0.020	-0.045	-0.0053	0.013
τ	1.337	2.032	0.265	0.442
τ^2	-0.112	-0.162	-0.060	0.026
$E \cdot n$	$7.02 \cdot 10^{-4}$	$7.60 \cdot 10^{-5}$	$1.32 \cdot 10^{-3}$	$3.11 \cdot 10^{-4}$
$n \cdot \tau$	$1.69 \cdot 10^{-3}$	$1.30 \cdot 10^{-4}$	$3.30 \cdot 10^{-3}$	$7.93 \cdot 10^{-4}$

95% Confidence Interval

E: electric field intensity; n: pulse number; τ: pulse width

Effect of pulse polarity

Since pulse polarity is a categorical variable, the microbial inactivation of *Staph. aureus* suspended in milk due to a HIPEF treatment using monopolar pulses can be fitted through equation 2, and in the case of bipolar pulses the survival fraction of *Staph. aureus* can be modeled by equation 3:

$$-\log s = -7.607 + 0.144 \cdot E - 0.020 \cdot n + 1.337 \cdot \tau - 0.112 \cdot \tau^2 + \\ + 7.02 \cdot 10^{-4} \cdot E \cdot n + 1.693 \cdot 10^{-3} \cdot n \cdot \tau \qquad \text{eq. 2}$$

$$-\log s = -6.971 + 0.144 \cdot E - 0.020 \cdot n + 1.337 \cdot \tau - 0.112 \cdot \tau^2 + \\ + 7.02 \cdot 10^{-4} \cdot E \cdot n + 1.693 \cdot 10^{-3} \cdot n \cdot \tau \qquad \text{eq. 3}$$

where *s* is the survival fraction, *E* is the electric field intensity (kV/cm), *n* the pulse number and τ the pulse width (μs).

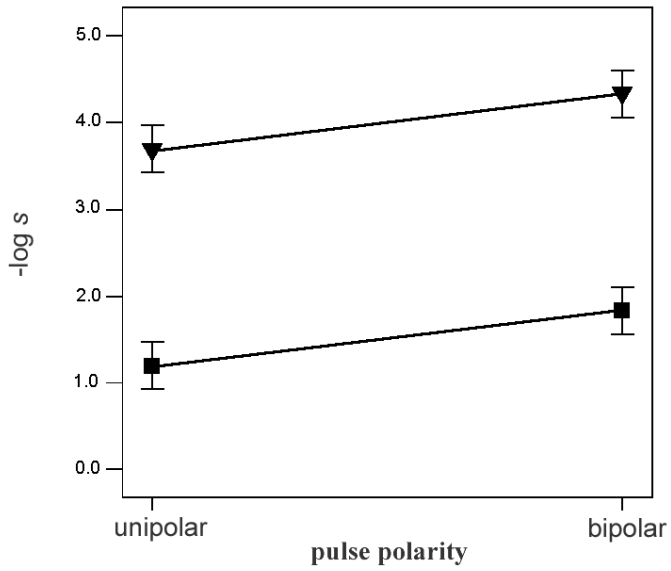


Figure 1. Effect of pulse polarity on the survival fraction (*s*) of *Staphylococcus aureus* inoculated in milk when the HIPEF treatment was set at (■) 25 or (▼) 35-kV/cm electric field intensity of 150 pulses of 8 μs each.

Among HIPEF processing parameters, pulse polarity was one of the most important factors (P < 0.0001) in *Staph. aureus* inactivation (Table 3). In comparison with the monopolar mode, bipolar pulses enhance HIPEF treatment. As the intercept terms of equation 1 and 2 shows, inactivation due to bipolar pulses remained within the defined space over microbial death due to monopolar pulses. A difference of over 0.64 log units was observed between the mono and bipolar mode when HIPEF treatment was set at 150 pulses of 8 μs each and 35 kV/cm (Fig. 1). It is generally assumed that microbial cells are affected by pulse polarity, even though there is a lack of information about the effect of this variable on the inactivation of several microorganisms in milk or in other complex media. However, it is believed that bipolar pulses cause stress and fatigue on the cell membrane and facilitate its electric breakdown due to alternating changes in the movement of charged molecules (Chang, 1989) and by reducing the deposition of solids on the electrode surface, which decreases food electrolysis (Qin et al., 1994). Evrendilek and Zhang (2005) reported that bipolar pulses produced 1.88 log units of inactivation of *E. coli* in skim milk whereas monopolar pulses only produced 1.27 log units. However, they found no significant difference between the bipolar and

monopolar mode for the inactivation of *E. coli* in apple juice in the same study. In contrast, Elez-Martínez et al. (2005) observed an extra 0.9 log reductions in the inactivation of *L. brevis* in orange juice by the bipolar mode in comparison with the monopolar mode at 35 kV/cm and 1000 μs Treatment time.

Effect of electric field intensity

As seen in Figure 2, the higher the electric field intensity, the greater the inactivation of the *Staph. aureus* population achieved. Among treatment variables, electric field intensity resulted in quite a marked effect on *Staph. aureus* ($P < 0.0001$), although the value of 35 kV/cm could not be exceeded without the electrical breakdown of milk in our experimental conditions. Other authors have also reported electric field intensity as being among the main parameters determining microbial destruction (Elez-Martínez et al., 2004; Martin et al., 1997). Cell death of *Staph. aureus* increased from 1.8 to 4.3 log units when *E* rose from 25 to 35 kV/cm, respectively, with the other variables set at 150 pulses, 8 μs pulse width and in the bipolar mode (Fig. 5). Similar microbial reductions were obtained by other authors, such as Evrendilek et al. (2004) who found that a HIPEF treatment of 450 μs treatment times at 35 kV/cm reduced the population of *Staph. aureus* suspended in skim milk by 3.7 log units. Raso et al. (1999) reported up to 4.0 log reductions when *Staph. aureus* suspended in milk was treated with 40 kV of input voltage and 40 pulses. However, no cell death was achieved below treatments at 25 kV/cm with 100 pulses of 4 μs. This result is consistent with those obtained by Evrendilek et al. (2004), who determined a critical or threshold value of electric field intensity of at least 20 kV/cm for *Staph. aureus* suspended in skim milk. In contrast, a critical electric field intensity of 11.7 kV/cm was reported by Damar et al. (2002) when the HIPEF treatment was carried out in Peptone Saline (PS). That low value in their study can be explained by the protective properties of milk versus PS media, since milk is a complex food material meanwhile PS media is simply a dilute solution. Nevertheless, *Staph. aureus* appears to be more resistant than other bacteria to a HIPEF treatment in milk. The *B. cereus* population suspended in skim milk was reduced by more than 2 log units after a HIPEF treatment of 90 μs at 35 kV/cm (Michalac et al., 2003), while less than 1.8 log reductions of *Staph. aureus* were achieved with the same treatment values. *E. coli* was inactivated by 1.88 log reductions in skim milk when submitted to a bipolar HIPEF treatment of 24 kV/cm and 141 μs (Evrendilek et al, 2005), whereas no microbial destruction of *Staph. aureus* was observed with a HIPEF treatment with equal values of the same variables. In addition, the critical electric field intensity of *E. coli* inoculated in skim milk was close to 14 kV/cm, and the minimal pulse number ranged from 1.9 to 5.4 pulses, which corresponded to a treatment time of 4.8 and 9.7 μs, respectively (Martin et al., 1997).

Effect of pulse number

The coefficient of the variable pulse number, *n*, was negative, meaning that the counts of *Staph. aureus* should decrease linearly with *n* (eq. 2 and 3). However, the expected behavior of *n* was apparently not clearly expressed according to equation 2 and 3, as is reflected in Figure 3. At 35 kV/cm and 8 μs pulse width, only 2.5 log reductions were registered with 50 pulses, whereas 4.3 log units were obtained with 150 pulses. Hence the single negative effect of *n* may be masked by the

positive effect in either of the interactions, $E \cdot n$ or $n \cdot \tau$ (eq. 2 and 3). Therefore, these results agree with those generally accepted, whereby the microbial population decreases as the pulse number rises. The survival fraction of *Staph. aureus* suspended in milk ultrafiltrate (SMUF) was reduced by more than 2 log units when applying a HIPEF treatment of 50 pulses at 16 kV/cm (Pothakamury et al., 1995). In similar conditions, only a 0.5 log reduction was observed when 60 pulses at 20 KV/cm were applied to samples of *Staph. aureus* inoculated in peptone saline (Damar et al., 2002). As for other microorganisms, samples of milk were treated with 30 pulses at 25 kV/cm and up to 2.0 log reductions in the *E. coli* population were obtained (Martin et al., 1997). The lethal effect of increasing the pulse number has been related to the resulting decrease in the critical potential difference of the cell membrane (Barbosa-Canovas et al., 1999).

Effect of pulse width

The inactivation of *Staph. aureus* depended on the negative quadratic term of pulse width, τ (eq. 2 and 3). Hence an increment in the value of τ, while the other variables remained constant, resulted in lower increments in the microbial inactivation of this microorganism. The number of log reductions increased from 3.3 to 4.3 log units when pulse width grew up from 4 to 8 μs, respectively, applying 150 bipolar pulses at 35 kV/cm (Fig. 4). However, the maximal destruction of *Staph. aureus* under these conditions was reached at approximately 7.1 μs pulse width while the survival fraction decreased slightly from 7.1 to 8.0 μs. Under a HIPEF treatment of 50 bipolar pulses at 35 kV/cm, the maximum was observed at 6.3 μs pulse width (Fig. 4). This phenomenon has received little attention since traditional research has been performed with, at the most, two values of pulse width. Martín et al. (1997) noticed that the application of 25 pulses of 0.7 μs each at 25 kV/cm reduced the survival fraction of *E. coli* inoculated in skim milk by less than 1 log cycle, but that under the same conditions

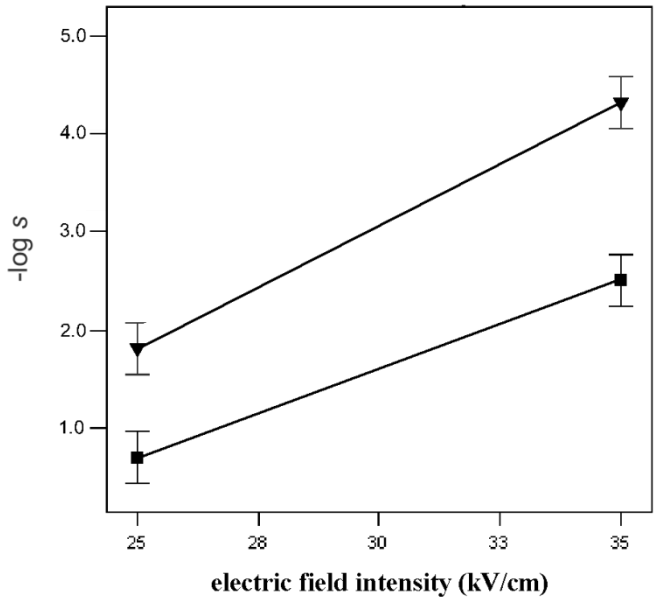


Figure 2. Effect of electric field intensity on survival rate (*s*) of *Staphylococcus aureus* suspended in milk at bipolar mode, 8 μs pulse width and (■) 50 or (▼) 150 pulse number.

and with 1.8 μs pulse width a reduction of more than 2 log cycles was achieved. These authors explained that the increase in the microbial death depended on the higher energy applied in each pulse, despite the fact that no wider pulses were considered.

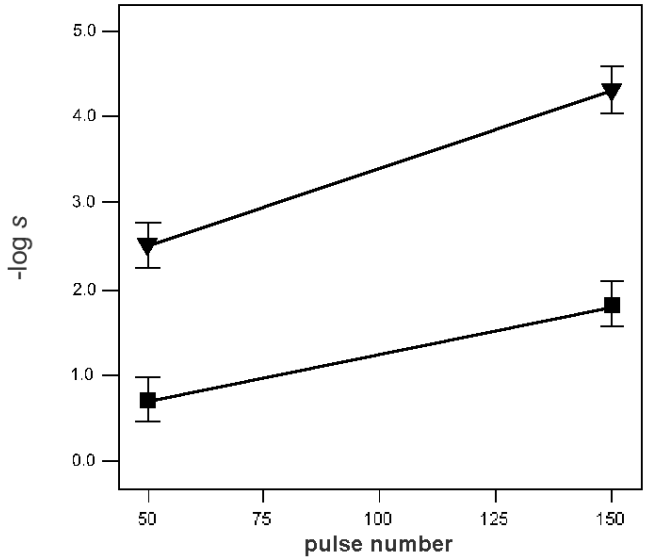


Figure 3. Effect of pulse number on survival rate (*s*) of *Staphylococcus aureus* suspended in milk when HIPEF treatment was set at bipolar mode, 8 μs pulse width, and (■) 25 or (▼) 35-kV/cm electric field intensity.

Effect of the combined action of pulse number and pulse width

Pulse width and pulse number exert a reciprocal influence, as revealed by the significance of the interaction term $n \cdot \tau$ (Table 3). The product of these two variables defines the discrete variable treatment time, t (μs) (eq. 4). Treatment time is also considered to be one of the main variables affecting cell inactivation (Elez-Martínez et al., 2005) since the longer the electric field intensity is applied the more damage to the membrane is found. Different authors agree that the inactivation of different microorganisms was greater when the treatment time increased (Elez-Martínez et al., 2005; Martin et al., 1997). A rise from 2.2 to 4.3 log reductions was observed between samples treated for 200 μs (50 pulses of 4 μs pulse width) and 1200 μs (150 pulses of 8 μs), respectively, when electric field intensity was 35 kV/cm and bipolar pulses were used (Fig. 5). In comparison with the electrical conditions set in this study, Evrendilek et al. (2004) determined that skim milk inoculated with *Staph. aureus* and subjected to HIPEF for 450 μs at 35 kV/cm resulted in a significant decrease of a 3 log reduction. Raso et al. (1999) observed that *Staph. aureus* and coagulase negative *Staphylococcus* sp. in milk could be inactivated over 4 and 2 log cycles, respectively, after a HIPEF treatment of 40 pulses at 40 kV/cm.

$t = n \cdot \tau$ eq. 4

As shown in Fig. 5, the combined effect of the variables *n* and τ followed a non-linear curve with the microbial destruction of *Staph. aureus*. Two different aspects affecting the survival fraction and the HIPEF process application were derived from the interaction $n \cdot \tau$. On the one hand, it is possible to exchange different combinations of the variables *n* and τ to achieve the same level of microbial inactivation. In this way, an inactivation of 4 log reductions was observed with either 150 pulses of 5.2 μs each, which resulted in a Treatment time of 780 μs, or with 125 pulses of 6.8 μs each, resulting in a 850 μs Treatment time, when the HIPEF treatment was carried out in both cases at 35 kV/cm and in bipolar mode (Fig. 5). Moreover, energy input in each treatment chamber, *W* (J), can be calculated as:

$W = k_1 \cdot k_2 \cdot E \cdot I \cdot f \cdot t = k_1 \cdot k_2 \cdot E \cdot I \cdot f \cdot n \cdot \tau$ eq. 5

where k_1 is a HIPEF device constant, k_2 is a processing constant, *E* is the electric field intensity in kV/cm, *I* is the current in A, *f* is the frequency in Hz and *t* is the treatment time in μs (Evrendilek et al., 2004). Thus the combination of a higher number of shorter pulses, 150 pulses of 5.2 μs each, reduced the energy input in comparison with the treatment of 125 pulses of 6.8 μs each. Consequently, it could be feasible to minimize the energy requirements of the HIPEF treatment by selecting the lowest Treatment time while maintaining the process objective of inactivation. Furthermore, as is shown in Figure 5, the use of a higher number of shorter pulses resulted in a higher microbial death than a treatment performed with a smaller number of longer pulses when the Treatment time was equal. Considering a Treatment time of 600 μs, a treatment of 150 pulses of 4 μs pulse width reduced the population of *Staph. aureus* by 3.3 log units whereas a treatment of 75 pulses of 8 μs each only achieved 2.9 log reductions (Fig. 5).

On the other hand, every curve from the contour plot shows a minimum on the survival fraction of *Staph. aureus* (Fig. 5). If the microbial death was kept at 4.0 log reductions, for instance, the combination of 125 pulses of 6.8 μs width at 35 kV/cm led to a minimum at this level of destruction, whereas the minimum at 3.0 log units corresponded to the combination of 60 pulses of 6.5 μs each. Pulse width and pulse number of each minimum decreased as microbial inactivation dropped, even though pulse width seemed to be included in a narrow range of values. Pulse width of the optimized points followed the equation $\tau = 7.56 \cdot 10^{-3} \cdot n + 5.97$ (eq. 6), which can be obtained by the derivation of equation 1 or 2, and varied from 6.4 to 7.0 μs when the pulse number was 50 and 150, respectively. Therefore, pulse widths close to 6.7 μs were shown to be more effective on microbial inactivation of *Staph. aureus* independently of the pulse number and electric field intensity applied.

The effect of combining pulse width and pulse number on the microbial death has not been clearly explained owing to the contradictory results obtained by different authors. Elez-Martínez et al. (2005) reported that shorter pulses might be considered more effective in destroying *Lactobacillus brevis* in orange juice. On the other hand, Abram et al. (2003) observed that longer pulse widths resulted in higher inactivation of *L. plantarum* suspended in a buffer solution than shorter pulse widths at a constant treatment time and electric field intensity. HIPEF equipment, the flow mode of

processing, process conditions, microorganism resistance or sample media could explain the lack of coincidence of the results achieved in different studies.

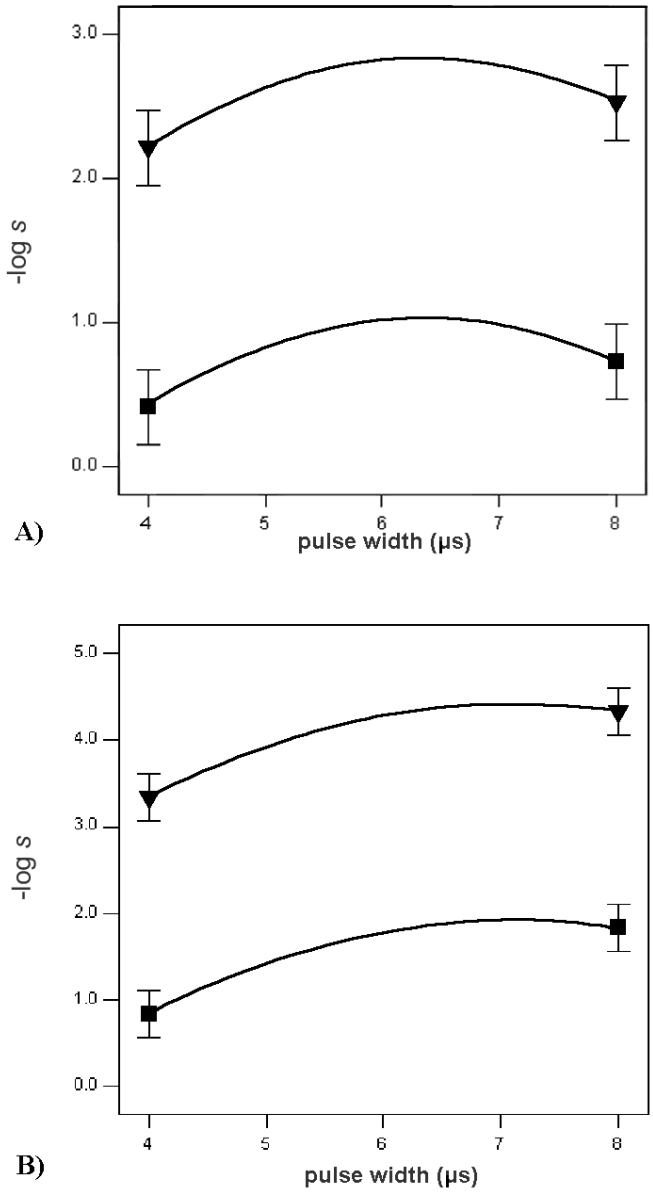
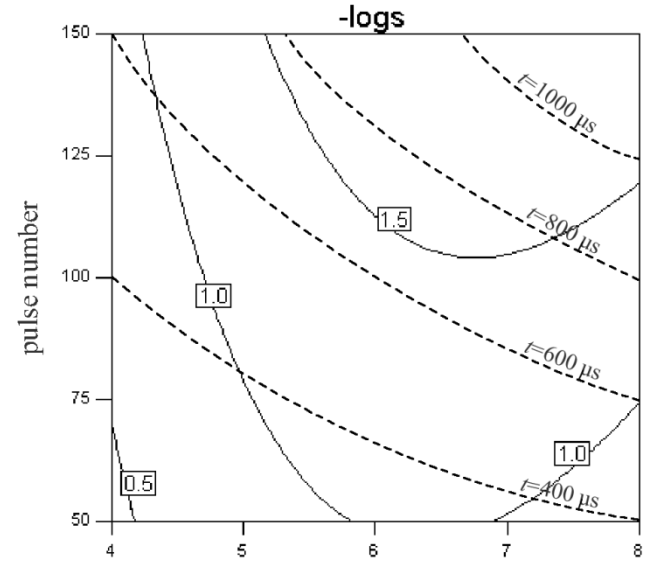


Figure 4. Effect of pulse width on survival rate (*s*) of *Staphylococcus aureus* inoculated in milk when HIPEF treatment was carried out at bipolar mode, and (■) 25 or (▼) 35-kV/cm electric field intensity (A: 50 pulses; B: 150 pulses).

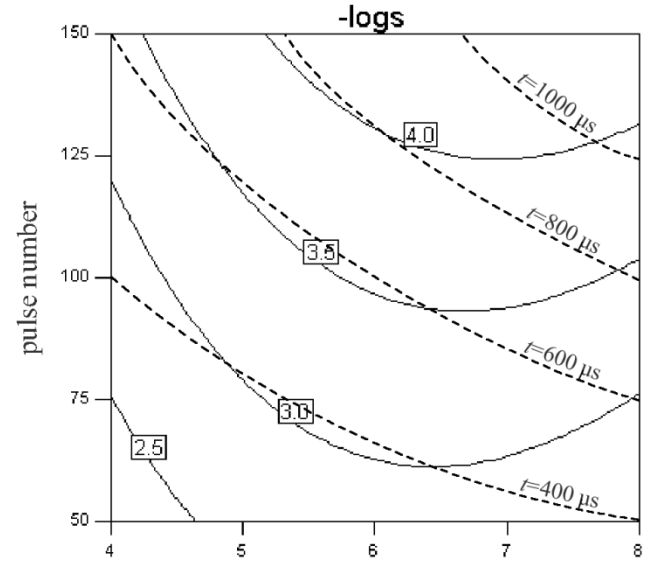
Effect of the combined action of pulse number and electric field intensity

The survival fraction of *Staph. aureus* was affected by the combined effect of electric field intensity and pulse number which was included in the response model as the interaction $E{\cdot}n$ (eq. 2 and 3). The positive value of its coefficient suggests that higher inactivation can be achieved by an increase in any or both variables. Considering bipolar pulses of 8 μs pulse width, the survival fraction decreased from 0.7 to 4.3 log units when *E* and *n* moved from 25 kV/cm and 50 pulses towards 35 kV/cm and

150 pulses, respectively (Fig. 6). Therefore, the simultaneous increase in the two variables resulted in an increment of microbial inactivation of 3.6 log units, whereas the sum of single increments in each variable while the other one was kept constant was only 2.7 log reductions. Electric field intensity and pulse number, expressed as the Treatment time, have been signaled as the major factors determining microbial inactivation in HIPEF processing (Elez-Martínez et al., 2005; Martin et al., 1997). Reina et al. (1998) found that at short treatment times (100 μs) there was no difference between 25 or 35 kV/cm of electric field intensity, but that at 300 and 600 μs treatment time, a higher electric field intensity resulted in a greater reduction in viable cells. However, the effect of



A)



B)

Figure 5. Effect of the combination of the variables pulse number and pulse width at bipolar mode on survival rate (*s*) of *Staphylococcus aureus* (*t*: Treatment time; A: 25 KV/cm; B: 35 kV/cm).

both variables acting simultaneously have not been already clearly pointed out, although Barbosa-Canovas et al. (1999) concluded that as the pulse number increased, the critical potential difference of the cell membrane decreased, resulting in a higher susceptibility of microorganisms to HIPEF.

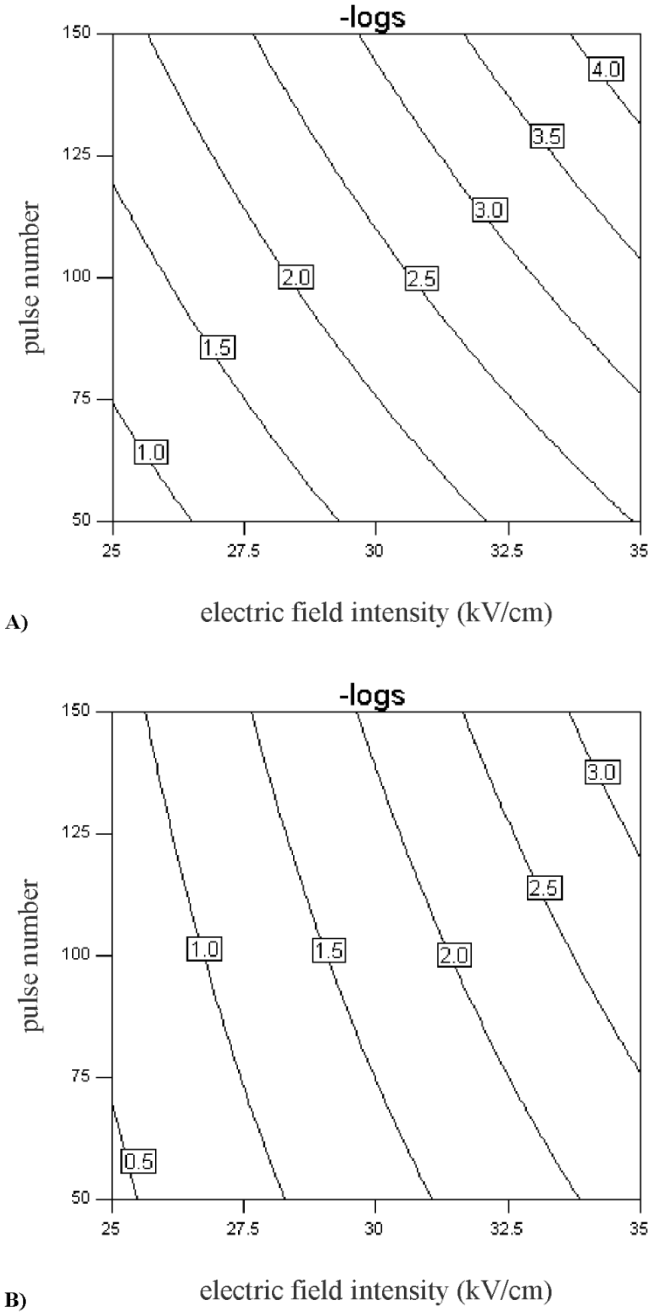


Figure 6. Effect of the combined action of the variables electric field intensity and pulse number on survival rate (*s*) of *Staphylococcus aureus* (polarity: bipolar; A: 8 μs; B: 4 μs).

Moreover, *E·n* interaction allows different (*E*, *n*) coordinates with the same inactivation level to be exchanged. As observed in Figure 6, a bipolar HIPEF treatment of 8 μs pulse width at 35 kV/cm and

80 pulses was equivalent to a HIPEF process at 30 kV/cm and 150 pulses, while microbial inactivation was held at 3.0 log units. Therefore, the interaction *E·n* suggests that the same damage value in the cell membrane could occur after carrying out different combinations of the treatment variables. Since the temperature increment in a co-field flow treatment chamber depends on the pulse number and the quadratic term of electric field intensity (Evrendilek et al., 2004), the non-thermal character of the treatment is also inferred from the decreasing electric field intensity despite the increase in the value of pulse number.

CONCLUSIONS

Staph. aureus was efficiently inactivated in milk by HIPEF treatment, although this microorganism seemed to be more resistant than others. A maximum inactivation of 4.5 log units was observed when HIPEF treatment was set at 150 bipolar pulses of 8 μs each at 35 kV/cm. Among the studied variables, polarity, pulse number, pulse width, electric field intensity and the combined action of pulse number with pulse width or electric field intensity significantly affected the microbial death of *Staph. aureus*, and this can be modeled by a second-order equation. The fat content of milk did not modify microbial inactivation.

An increment on pulse number, pulse width or electric field intensity resulted in higher microbial inactivation, although the effect of pulse width on microbial inactivation was non-linear. The use of bipolar pulses raised the survival fraction less within the defined ranges of the variables than that obtained by applying the HIPEF treatment in the monopolar mode. The combined action of pulse number and pulse width revealed that pulses within 6.3 to 7.1 μs showed higher microbial inactivation with a lower number of applied pulses. Furthermore, a HIPEF treatment of a higher number of narrow pulses resulted in a higher microbial death than a HIPEF treatment with a lower number of wide pulses for an equal value of Treatment time. Different combinations of pulse number and electric field intensity also achieved equivalent microbial inactivation. The response of the electrical variables suggested that it is possible to optimize the HIPEF treatment by reducing the amount of energy input and, consequently, the temperature increase during the process while microbial inactivation is kept constant. In consequence, the death of *Staph. aureus* in milk was solely due to cell damage caused by the application of electric fields and not to a possible temperature increment. Variables affecting the HIPEF process showed that microbial inactivation was not only led by the single variables but also by their combination. Hence further research is needed in order to clearly define the variables affecting the process, and/or the mechanisms involved in cell death.

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CAPITULO II

Enhancing inactivation of *Staphylococcus aureus* in skim milk by combining high-intensity pulsed-electric fields and nisin

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ABSTRACT

High Intensity Pulsed Electric Fields (HIPEF) is a non-thermal preservation method that is believed to enhance the effect of nisin on microorganisms, such as *Staphylococcus aureus*. The survival fraction of *Staph. aureus* by adding nisin, applying a HIPEF treatment or a combination of nisin-HIPEF treatment was evaluated by inoculating the microorganism in skim milk. Nisin dose, milk pH and HIPEF treatment time were the controlled variables that were set up at 20-150 ppm, 5.0-6.8 pH and 240-2400 μs, respectively. Electric field strength and pulse width of HIPEF were kept constant at 35 kV/cm and 4 μs. No microbial reduction of *Staph. aureus* was observed in skim milk at its natural pH treated with nisin but 1.1 log cycles were registered after 90 min at pH 5.0 and 150 ppm of nisin. A reduction in viable count of 0.3 and 1.0 log cycles of *Staph. aureus* in skim milk treated by HIPEF at its natural pH was observed at 240 and 2400 μs, respectively. Nisin-HIPEF treatment design was performed by a response surface methodology. The combined effect of nisin and HIPEF was clearly synergistic. However, synergism depended on pH. A maximum microbial inactivation of 6.0 log units was observed at 6.8 pH, 20-ppm nisin dose, and 2400 μs of HIPEF treatment time whereas over 4.5 log units was achieved when pH, nisin concentration and HIPEF treatment times were set at 5.0, 150 ppm and 240 μs, respectively.

INTRODUCTION

Staphylococcus aureus is an important gram-negative pathogen related to milk that produces a enterotoxin. The minimal concentration of *Staph. aureus* required to produce enough enterotoxin to cause food poisoning is believed to be about 10^7 cfu/g of food (*25*). Its pH range for growth is between 4.2 and 9.3 with an optimum value of 7.0 and even though *Staph. aureus* is sensitive to thermal treatments, such as pasteurization processes, heat resistance is increased in high-fat and high-salt foods. In fact, it has been found that D_{60} can reach more than 50 min in salty foods (*6*).

Non-thermal treatments are being developed in order to reach safe foods with minimal loss of sensory characteristics and bioactive compounds (*9*). The possibility of achieving fresh-like taste in milk products has generated interest in nonthermal treatments such as High Intensity Pulsed Electric Fields (HIPEF). However, *Staph. aureus* inactivation by HIPEF has been little studied (*43*, *44*, *46*, *47*, *60*) while most research has paid more attention to other microorganisms, such as *Lactobacillus* (*1*, *20*, *49*), *Listeria* (*2*, *22*, *48*) or *Escherichia* (*23*, *59*). Moreover, only a few studies have focused on microbial inactivation in milk by HIPEF (*22*, *45*, *47*, *48*, *50*), whereas other media, such as model solutions, have been widely used (*9*, *41*). Model solutions are useful in determining the susceptibility of microorganisms to HIPEF treatment when the process parameters must be isolated from interactions with milk composition. But results of microbial inactivation in milk by HIPEF are very different from microbial death achieved in model solutions when exposed to similar treatment conditions of field strength and HIPEF treatment time. The inactivation of *L. monocytogenes* in McIlvaine citrate-phosphate buffer exposed to a HIPEF treatment set at 28 kV/cm field strength and 600 μs treatment time (*2*) was over 1 log units higher than this microorganism treated at 30 kV/cm and 600 μs treatment time in whole milk (*48*).

On the other hand, nisin is a polypeptide, forming part of class I bacteriocins and type A lantibiotics, produced by certain strains of *Lactococcus lactis*, which has a broad anti-microbial spectrum (*18*) including *Staph. aureus* (*39*). Furthermore, nisin has been internationally accepted since 1969 (*55*) as a food preservative in many countries for the control of pathogenic bacteria in foods because of its relatively long history of safe use and its documented effectiveness. However, its efficacy and activity in foods may be affected by several factors, such as protein protective effect, temperature, chemical composition of the medium (*16*) and, particularly, pH (*28*) and fat content of milk (*8*). Nisin activity decreased by about 33% when it was added to skim milk and by over 50% when added to milk containing 1.29% fat (*30*) and it was completely inactivated if pH was higher than 7.0 (*28*).

It has been emphasized that sublethal injury can be usefully applied in a hurdle approach to achieve effective food preservation (*34*). In fact, the sensitivity of bacteria to nisin was found to be enhanced whether it was combined with other treatments (*31*, *32*, *39*). A synergistic action has been described by combining nisin and High Hydrostatic Pressure (*24, 31*, *33*, *57*), nisin and other antimicrobials (*3*, *4*, *56*) or nisin and HIPEF (*12*, *41*, *42*, *50*). Dutreux *et al*. (*17*) reported that the application of nisin in milk clearly enhanced the lethal effect of HIPEF on *Micrococcus luteus* with up to 5.2 log reduction. Since these treatments have previously been shown to be effective against other pathogens in milk, the combination of HIPEF treatment with addition of nisin could be a way of achieving enough microbial inactivation of *Staph. aureus* while fresh-like appearance is retained in foods. In general, cell-death mechanism has not been clearly explained, although it is believed that many stresses impose sublethal injuries to microbial cells, which become more sensitive to different physical and chemical

environments and, in particular, *Staph. aureus* inactivation by HIPEF is not very well known. The purpose of this research was to study *Staph. aureus* inactivation and sensitization in skim milk due to HIPEF in the presence of nisin at different pH.

MATERIAL AND METHODS

Skim milk

Homogenized UHT skim milk was obtained from a dairy plant and stored at 4ºC (Granja Castelló, Mollerussa, Lleida, Spain). Natural pH of milk was 6.79±0.02 and was measured by a pH-meter (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain). The electrical conductivity of the skim milk was 5.54±0.042 mS/cm at 6.8 pH and 6.98±0.134 mS/cm at 5.0 pH. The measurement was performed at 25ºC and determined with a conductivity meter (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Germany).

Staphylococcus aureus culture

Staphylococcus aureus CECT 240 (Food Technology Department, University of Lleida, Spain) was used as target microorganism and was maintained on slants of plate count agar (PCA; Biokar diagnostics, Beauvais, France) at 4ºC until it was used.

Strain growth was performed by incubating cultures serially. Firstly, a subculture on a slant of PCA was prepared and incubated at 30ºC for 24h. Petri dishes were inoculated with the microorganism suspended in peptone solution (PS) (Biokar diagnostics, Beauvais, France) obtained from scraping off the slant and incubated at 30ºC for 48h. Then, the microorganisms were scraped off by a Drigalsky handle and kept up in tubes containing PS. Inoculum concentration was determined by optical measurement. A population density of approximately 10^8 cfu/ml matches an absorbance value between 0.1 and 0.2 at 620 nm.

Sample preparation

Samples of *Staph. aureus* were prepared by inoculating the microorganism in skim milk to a final concentration of approximately 10^7 cfu/ml. Samples were treated by HIPEF, by adding nisin or by combining HIPEF and nisin.

HIPEF equipment

A continuous-flow HIPEF system was used to carry out this study. The treatment device was a OSU-4F HIPEF unit (Ohio State University, Columbus, Ohio, USA) that discharges square-shape pulses within eight collinear chambers, in which gap distance was 0.29 cm and each treatment chamber volume

was 0.012 cm^3. Electrical parameters were set at 35 kV/cm of electric field strength, bipolar pulses of 4 μs of pulse width and 100 Hz of pulse frequency. The flow rate of the process was adjusted to 90 ml/min and controlled by a variable speed pump (model 75210-25, Cole Palmer; Vernon Hills, Illinois, USA). Treatment temperature was kept under 25ºC using a cooled water bath to rule out thermal effects.

Microbial inactivation of *Staphylococcus aureus* induced by nisin treatment

The antimicrobial effect of nisin (N 5764, Sigma-Aldrich, Steinheim, Germany) against *Staph. aureus* was measured by exposing samples to different nisin concentrations, pH values and exposure time. Two pH values were selected, 5.0 and 6.8. The latter was the natural pH of skim milk. The 5.0 pH value was adjusted by adding lactic acid (Merck Eurolab, Briare Le Canal, France) to skim milk samples. This 5.0 pH value is frequently found in many acidic dairy products and avoids precipitation of caseins since their isoelectric point is 4.6. Then, powder nisin was added up to concentrations of 20 or 150 ppm on pH-adjusted samples and they were subjected to 30, 60, 90 and 120 min exposure time. Three replications were performed for each assay and temperature was maintained at 25ºC. Statgrafic plus 2.1 performed this statistical analysis (Statistical Graphics Co., Rockville, MD, USA).

Microbial inactivation of *Staphylococcus aureus* induced by HIPEF treatment

Samples of inoculated skim milk were treated by HIPEF in order to determine its effect on microbial inactivation of *Staph. aureus*. HIPEF was applied to milk samples at pH of 6.8 and 5.0, setting the field strength at 35 kV/cm and varying the HIPEF treatment time from 240 to 2400 μs, calculated as the product of number of pulses and pulse width (4 μs). Treatment conditions were selected considering sample and equipment limitations. Three replications were performed for each assay. Statgrafic plus 2.1 performed this statistical analysis.

Combined effect of nisin and HIPEF treatment

A response surface methodology was performed to observe the effect of the combined nisin-HIPEF treatment on the survival fraction of *Staph. aureus* in skim milk. A central composite design with three factors and faced centered was the proposed experimental design (*38*). The independent variables were pH (5.0-6.8), nisin concentration (20-150 ppm) and HIPEF treatment time (240-2400 μs). Variable levels were chosen according to previous studies. In this experimental design, there were three coded factor levels: -1, 0, +1 in which –1 corresponds to the lowest level of each factor, +1 to the highest level and 0 to the mid-level. The encoded and uncoded values for each variable are shown in Table 1. The experimental design shown in table 1 was performed in duplicate resulting two blocks of experiments. The order of assays within each block was randomized.

The effect of the three independent variables was modeled using a polynomial response surface. The second-order response function was predicted by the following equation:

$$-\log s = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \qquad \text{eq. 1}$$

where factor *X* represents the encoded values of the variables and β are the constant coefficients. The non-significant terms ($p \leq 0.05$) were deleted from the second-order polynomial model after calculating an analysis of variance (ANOVAs), and then new ANOVAs were recalculated to obtain the coefficients of the final equation.

Table 1. Central composite response surface design and survival fraction of *Staph. aureus* suspended in skim milk and submitted to different combinations of nisin concentration, pH and HIPEF treatment time

Assay number[a]	Point type	Variables						Response variable
		Coded			Uncoded			
		X_1	X_2	X_3	Nisin concentration (ppm)	pH	HIPEF treatment time (μs)	-log s
1	Factorial	+1	+1	-1	150	6.8	240	1.2
2	Factorial	+1	-1	+1	150	5.0	2400	3.4
3	Factorial	-1	+1	+1	20	6.8	2400	6.0
4	Factorial	-1	-1	-1	20	5.0	240	1.4
5	Center	0	0	0	85	5.9	1320	3.2
6	Center	0	0	0	85	5.9	1320	3.1
7	Center	0	0	0	85	5.9	1320	3.0
8	Axial	-1	0	0	20	5.9	1320	4.0
9	Axial	+1	0	0	150	5.9	1320	4.4
10	Axial	0	-1	0	85	5.0	1320	2.7
11	Axial	0	+1	0	85	6.8	1320	2.1
12	Axial	0	0	-1	85	5.9	240	1.8
13	Axial	0	0	+1	85	5.9	2400	4.1
14	Axial	0	0	0	85	5.9	1320	3.0
15	Center	0	0	0	85	5.9	1320	3.5
16	Center	0	0	0	85	5.9	1320	3.5

[a] This does not correspond to the order of processing

X_1: Nisin concentration

X_2: pH

X_3: HIPEF treatment time

HIPEF treatment was set at 35 kV/cm, bipolar pulses of 4 μs pulse width and 100 Hz

To perform this study Design Expert 6.0.1 software (Stat Ease Inc., Minneapolis, USA) was used in all analyses and generated plots. A 95% confidence interval was used for all these procedures.

Survival fraction of *Staphylococcus aureus*

The untreated and treated samples were serially diluted in PS, plated on slants of PCA and incubated for 72 h at 30ºC. The number of viable cells of *Staph. aureus* after applying a HIPEF-nisin treatment was expressed as survival fraction, *s*, which was calculated as N/N_0, where N_0 was the initial count in samples prior to any of the treatments, nisin, HIPEF or nisin-HIPEF, N was the count after each treatment. Microbial inactivation was calculated as –log *s*.

RESULTS AND DISCUSSION

Effect of nisin on survival fraction of *Staphylococcus aureus*

The effect of nisin on *Staph. aureus* in skim milk was evaluated by exposing microbial cells to 20 or 150-ppm nisin, 5.0 or 6.8 pH and exposure times from 30 to 120 min. No microbial reduction was observed at pH 6.8 in any case. The solubility and stability of nisin decrease from optimal pH 2.0 to 6.0, and it is irreversibly inactivated at pH 7.0 (28). Therefore, no microbial inactivation should be expected at high pH, since nisin remains insoluble in milk. Smith et al. (50) also reported that nisin did not significantly reduce the natural flora in raw skim milk when 100 ppm was added.

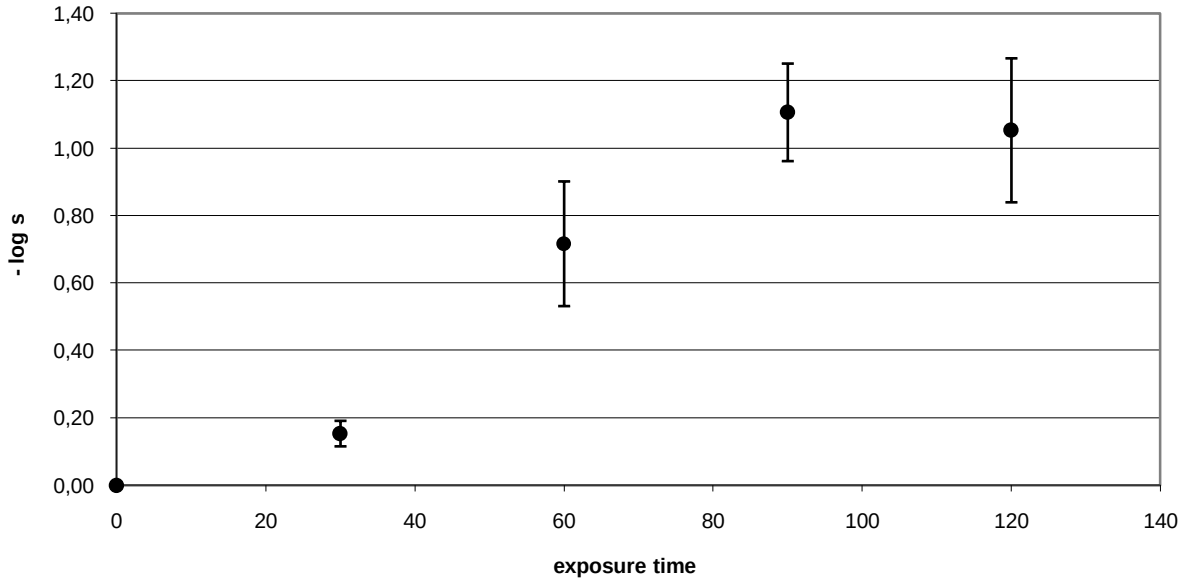

Figure 1. Effect of exposure time of 150-ppm added nisin on the survival fraction of *Staphylococcus aureus* in skim milk at 5.0 pH.

However, adjusting the pH of skim milk to 5.0 caused microbial death when 150 ppm of nisin were added but 20 ppm of nisin did not significantly ($p<0.05$) produce microbial inactivation. Figure 1 shows the microbial inactivation of *Staph. aureus* in skim milk at pH 5.0 at different exposure times

when nisin concentration was 150 ppm. Nisin caused a maximal microbial death of 1.1 log units after sample had rested for 90 min whereas no extra reduction was observed after 120 min. These results were similar to those obtained by Arques *et al*. (*4*) who reported a count on *Staph. aureus* of 0.23 log reduction after 24 h when 100 ppm of nisin was added to milk samples. Furthermore, the antimicrobial activity of nisin on other microorganisms in other media has shown approximately similar values. Jung *et al*. (*30*) found that the viable count of *L. monocytogenes* decreased to 0.3 log cycles in skim milk with 50 ppm added nisin after 2 h from inoculation. Dutreux *et al*. (*17*) reported a reduction of 1.4 log units on *M. luteus* in a salt solution when held for 120 min with a 100-ppm nisin dose. Calderón-Miranda *et al.* (*14*) also observed over 0.70-log unit inactivation on *L. innocua* if 100-ppm nisin was included in the agar medium.

Effect of HIPEF treatment on the survival fraction of *Staphylococcus aureus*

HIPEF at 35 kV/cm, 100 Hz and 4 μs pulse width caused a reduction in viable count of 0.3 and 1.0 log cycles of *Staph. aureus* in skim milk at its natural pH when HIPEF treatment time was 240 μs and 2400 μs, respectively. In comparison with the electric values set in this study, Evrendilek *et al*. (*21*) determined that skim milk inoculated with *Staph. aureus* and subjected to HIPEF for 450 μs at 35 kV/cm and 250 Hz resulted in a significant decrease of 3 log reduction. Raso *et al*. (*47*) observed that *Staph. aureus* and coagulase negative *Staphylococcus* sp. in milk could be inactivated over 4 and 2 log cycles, respectively, after a HIPEF treatment at 40 kV/cm, 40 pulses and 3.5 Hz. Although *Staph. aureus* inactivation by HIPEF has not been sufficiently studied in skim milk, some research focusing on cell death of *Staph. aureus* by HIPEF has been performed in different media or model solutions. HIPEF treatment at 20 kV/cm with 60 pulses resulted in an almost 2 log reduction in viable cell counts of *Staph. aureus* suspended in peptone solution (*15*). Pothakamury *et al*. (*44*) reported between 3 and 4 log inactivation cycles when cells of *Staph. aureus* in a model solution were subjected to a HIPEF treatment at 12-16 kV/cm and 200-300 μs. In the case of simulate milk ultrafiltrate (SMUF) inoculated with *Staph. aureus*, its population could be reduced up to 5 log cycles after subjecting the samples to 40 pulses at 60 kV/cm (*46*). However, microbial death of *Staph. aureus* achieved in a model solution is obviously higher than death observed in milk even if a HIPEF treatment is carried out. These differences between the effectiveness of HIPEF on the survival fraction in milk and other media may be due to the more complex composition of milk, its low resistivity, and the presence of proteins (*9*), so inactivating micro-organisms is more difficult in complex food materials, such as milk, than in dilute buffer solutions (*58*).

The conductivity of skim milk increased from 5.54 to 6.98 mS/cm as pH decreased from 6.8 to 5.0, respectively (Table 2). The addition of acid decreases the milk pH and results in gradual solubilization of the colloidal salts connected to the casein micelles, so conductance of milk rises with acidification (*37*). Wouters *et al.* (*54*) concluded that low conductivity could enhance HIPEF antimicrobial effect and other authors have reported that pH also seems to be related to the capacity of cells to repair sublethal injuries (*5*). However, analysis of variance of the microbial inactivation by HIPEF of *Staph. aureus* on samples at pH 5.0 did not significantly ($p<0.05$) differ in this study from that at 6.8 when 240 or 2400 μs of HIPEF treatment time and 35 kV/cm were considered, since electrical and pH conditions or differences in microbial inactivation might not have been broad enough to have statistically significant meaning. But these results agreed with Hülsheger *et al*. (*27*) or Smith *et al*.

(*50*), who reported that there was no significant difference ($p<0.05$) in microbial inactivation of natural flora of raw milk at pH 5.0 or at its natural pH of 6.7 after HIPEF treatment, and concluded that the cell membranes of the microorganisms present in milk are not adversely affected by changes of pH in this range.

Table 2. Electrical conductivity of skim milk at different pH values and fat content

pH	skim milk conductivity[a] (mS/cm)
6.8	5.54±0.042
5.9	6.01±0.057
5.0	6.98±0.134

[a]Results are the mean±SD of three measurements

Effect of nisin combined with HIPEF treatment on the survival fraction of *Staphylococcus aureus*

A response surface methodology was used to study the survival fraction, *s*, of *Staph. aureus* applying nisin and HIPEF treatment. The process variables were nisin concentration, pH and HIPEF treatment time. Conductivity of milk was not affected by adding nisin. Table 1 shows the results obtained for each experimental condition on *Staph. aureus* inactivation. The maximum inactivation achieved, 6.0 log reductions, corresponded to a HIPEF treatment of 2400 μs and 35 kV/cm, 20 ppm added nisin and a pH 6.8. In contrast, the combination of 240 μs of HIPEF treatment time, 150-ppm nisin and pH 6.8 was found to give the minimum inactivation value, which was 1.2 log reductions.

Analysis of variance revealed that the second-order model was well adjusted to the experimental data (Table 3). Determination coefficient, R^2, was 0.99 and the lack-of-fit did not result in a significant value, indicating that the model is sufficiently accurate for predicting the response. Survival fraction, transformed as –log *s*, was affected by treatment time, quadratic terms of nisin concentration and pH, and all the interactions among the variables in the study. Coefficients of the fitted model are shown in Table 4.

The response was represented by the following polynomial quadratic equation in terms of actual factors:

$$-\log s = \begin{aligned} & 0.000210n^2 - 1.11p^2 + 0.0365n + 12.4p - 0.00302t - \\ & - 0.0000125n \times t + 0.000872p \times t - 0.00902n \times p - 32.9 \end{aligned}$$

where *s* is the survival fraction, *n* is the nisin concentration (ppm), *p* the pH value and *t* the HIPEF treatment time (μs). Lineal terms of the variables *n* and *p* were included in order to retain hierarchy of the effects.

The positive value of the quadratic term for nisin concentration ($p<0.0006$), n^2, indicated that inactivation of *Staph. aureus* reached a minimum value within the range of *n*. On the other hand, the negative coefficient of p^2 indicates an enhancement of the treatment related to a *p* increase although microbial death might decrease if *p* increased further.

Table 3. Analysis of variance for the response surface quadratic model of *Staph. aureus* in skim milk

Source	Mean square	F-value	Prob>F
Model	2.31	55.16	0.0002[s]
n	0.05	1.13	0.3369
p	0.17	3.98	0.1024
t	2.63	62.88	0.0005[s]
n^2	2.38	56.91	0.0006[s]
p^2	1.73	41.28	0.0014[s]
t^2	0.20	4.88	0.0781
$n \times p$	0.37	8.86	0.0309[s]
$n \times t$	1.03	24.64	0.0042[s]
$p \times t$	0.96	22.86	0.0050[s]
Lack of fit	0.02	0.44	0.5454
Pure error	0.047		
Cor. Total	21.16		
Standard Deviation	0.20	R-squared	0.99
Mean	3.12	Adjusted R-Squared	0.97
Coefficient of variation	6.50		

[s] Significant at 95% Confidence Interval
n: Nisin concentration
p: pH
t: HIPEF treatment time

The lineal term of HIPEF treatment time, *t*, was negative ($p<0.0005$) so that the higher the HIPEF treatment time was set, the lower the survival fraction that was observed. Moreover, significance of the interaction terms $n \times t$ and $p \times t$ showed that *t* was clearly influenced by nisin concentration, *n*, and pH, *p*. The coefficient sign of $n \times t$ was negative so that they acted in opposite directions (Fig. 2). The number of log reductions at 5.0 pH and 20 ppm of *n* increased quickly from over 1.6 up to 4 log units when *t* rose from 240 to 2400 μs, respectively. In contrast, if *n* was augmented to 150-ppm, microbial death decreased from over 4.5 to 3.5-log reduction when *t* increased from 240 to 2400 μs. Considering the natural pH of milk, microbial inactivation tended to increase when *n* decreased and *t* increased, even though the opposite behavior was not observed as in the previous case. Survival fraction at 20-ppm fell from 6.0 log reductions at 2400 μs to less than 0.5 log cycles at 240 μs. The term $p \times t$, whose coefficient was positive, showed that an increase in *p* and/or *t* acted efficiently on

Staph. aureus inactivation (Fig. 3). Therefore, as higher values of *t* were approached, a rise in *p* at 20 ppm became more effective on *Staph. aureus* inactivation; otherwise, microbial death at 150 ppm was higher when *t* and *p* moved towards their lower values. The last term $n \times p$ was also negative. The higher inactivation at 240 μs was reached considering low *p* and high *n* values (Fig. 4). More than 4.5 log cycles were registered at 240 μs when 150 ppm were added and *p* was 5.0 as opposed to less than 0.5 log units at 20 ppm and 6.8 *p*. However, the survival fraction decreased from 3.5 log units at 150 ppm *n* and 5.0 *p* to over 6.0 log cycles at 20-ppm and 6.8 *p* when 35 kV/cm and 2400 μs were applied.

Table 4. Significant regression coefficients of the quadratic model for the survival fraction of *Staph. aureus* related to the uncoded variables nisin concentration (*n*), pH (*p*) and HIPEF treatment time (*t*)

Factor	Coefficient estimate	Standard error	Low	High
Intercept	-32.9	9.6	-56.5	-10.2
n^a	0.0365	0.0312	-0.0386	0.111
p^a	12.4	2.8	5.91	19.1
t	-0.00302	0.00151	-0.00663	0.000622
n^2	0.00021	0.00004	0.00012	0.00030
p^2	-1.11	0.20	-1.59	-0.642
$n \times p$	-0.00902	0.00395	-0.0185	0.000496
$n \times t$	-0.0000125	0.0000033	-0.0000205	-0.00000456
pxt	0.000872	0.000238	0.000298	0.00144

95% Confidence Interval

[a] Non-significant terms included because of hierarchy

Viability loss and cell death of *Staph. aureus* were achieved by combining HIPEF and nisin treatment but both treatments carried out simultaneously resulted in a remarkable synergistic effect. Maximal microbial destruction by applying HIPEF at 35 kV/cm and 2400 μs in inoculated skim milk at 6.8 or 5.0 pH was 1.0 log reduction and the microbial destruction achieved by adding 150-ppm nisin in 5.0 adjusted pH skim milk was over 1.1 log inactivation cycles, but the two treatments applied simultaneously reported 3.5 log cycles. However, the maximal cell death reached by combining HIPEF and nisin was over 6.0 log units when HIPEF treatment time, pH and nisin were 2400 μs, 6.8 and 20 ppm, respectively. So, combining both treatments resulted in a reduction of approximately 4.0 log units more than the sum of the best microbial death counted in the single treatments. Smith *et al*. (*50*) also reported that nisin added to raw skim milk prior to a HIPEF treatment at 80 kV/cm and 100 μs of HIPEF treatment time produced an extra inactivation of 4.42 log cycles of the natural flora of milk. The population of *L. innocua* as a result of exposure to 100-ppm nisin after HIPEF at 30, 40 and 50 kV/cm and 32 pulses was reduced 2.3, 2.9 and 3.6 log units according to Calderon-Miranda *et al*. (*12*). Other authors have reported synergism between nisin and HIPEF, although those studies were performed in a model solution. Pol *et al*. (*40*) also observed a synergistic effect of HIPEF and nisin treatment for vegetative cells of *Bacillus cereus* in a buffer solution whose population was reduced 1.8 log units more than the sum of each single treatment (16.7 kV/cm and 100 μs of HIPEF treatment and 60 ppm of nisin). The inactivation of *M. luteus* in phosphate buffer caused by exposure to 32.5 kV/cm and 100 μs of HIPEF treatment time and 100-ppm nisin was 1.4 log units greater than that

obtained separately (*17*). However, this synergy was not seen when germinating spores of *B. cereus* were used (*41*) or in the microbial count of yeasts in unclarified apple juice treated by HIPEF (90 kV/cm, 40 pulses) in the presence of 100-ppm nisin (*35*). In contrast, an additive effect was observed on *L. innocua* in liquid whole egg for all HIPEF treatment time values and nisin concentration except for an electric field intensity of 50 kV/cm, in which none of the pulses or nisin concentrations showed synergism (*14*).

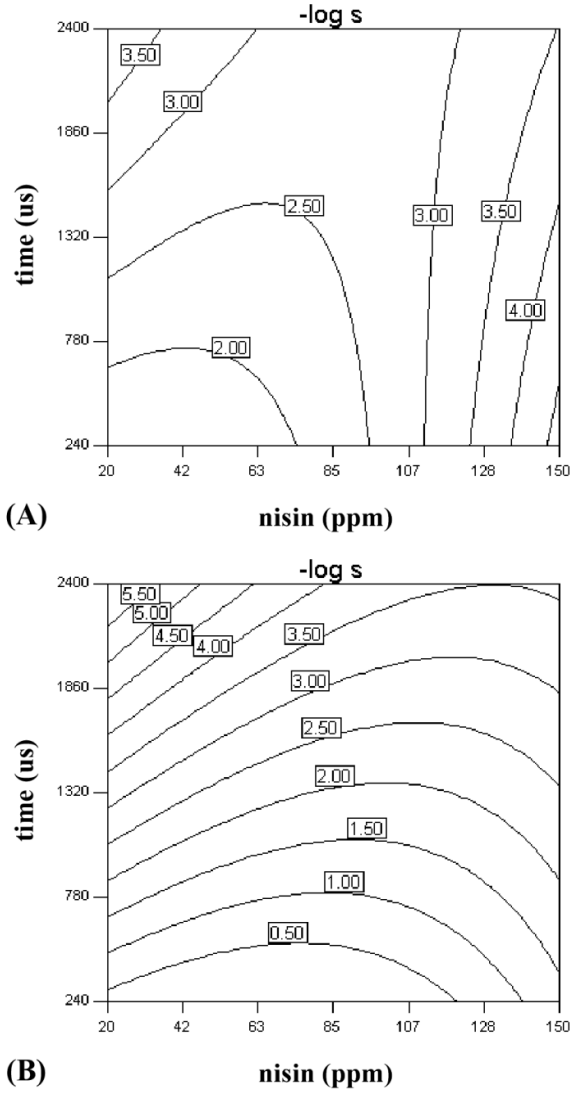

Figure 2. Effect of nisin concentration (ppm) on the survival fraction of *Staphylococcus aureus* in skim milk vs. HIPEF treatment time (μs) at different pH (A: 5.0 pH; B: 6.8 pH).

The mechanisms entailed in HIPEF-nisin synergism are not yet clearly understood although different reasons could explain this phenomenon. On the one hand, synergism may be a consequence of the sensitization induced by HIPEF to nisin due to an increase in the permeability of the cell membrane (*36*). Membrane electropermeabilization may be irreversible or reversible depending on the degree of membrane organizational changes (*53*) so that electric field strength may frequently result in

sublethal injuries (*32*). In addition, HIPEF is involved in different membrane permeabilization mechanisms that cause changes in the cell membrane, such as thinner or ruptured cell walls or cytoplasm disorganization, as well as leak out of some cytoplasmatic material (*19*, *20*, *43*). Calderon-Miranda *et al*. (*13*) concluded that cells of *L. innocua* exposed to HIPEF in skim milk with nisin exhibited an increased cell wall thickness in comparison to cells subjected to the single HIPEF treatment. Indeed, HIPEF could facilitate the incorporation of nisin into the cytoplasmatic membrane resulting in larger pores or pores with a longer lifetime (*40*). Ho *et al.* (*26*) also found that the critical field strength required for cell lysis was reduced by inducing additional stress, so adding nisin could result in a lower critical field of HIPEF treatment. On the other hand, this synergism in term of log cycles reduction was affected by the pH value. As was shown in the analysis of the interaction terms (Fig. 2), when 150 ppm nisin doses were added and only 240 μs HIPEF treatment time was applied, over 4.5 log unit reductions were reached at 5.0 pH and, moreover, an increase of HIPEF treatment time at that point produced an increase in the survival fraction. So, HIPEF seemed to inactivate nisin further than the growth of the synergistic effect. This observation agreed with those obtained by Therebiznik *et al*. (*51*), who also suggested that nisin could be inactivated by HIPEF since many authors have outlined the inactivation of different enzymes (*10*, *11*) or the occurrence of structural changes leading to increased sensitivity of protein to proteases after HIPEF (*7*). However, results showed that the combined treatment at 20-ppm nisin, 6.8 pH, 35 kV/cm and 2400 μs HIPEF treatment time achieved over 6.0 log reduction, which meant an increment of over 1.5 log units in relation to the previous case. This phenomenon was completely opposed to what could be expected for nisin activity. Firstly, nisin remains insoluble at pH 6.8 but not inactivated and, secondly, HIPEF seemed to reduce nisin activity. As far as we know, no references have been found about this behavior in the literature.

Thus, the unexpected behavior of nisin-HIPEF interaction at neutral pH could be explained by local pH changes on the surrounding membrane cell. Damage and rupture of the cell membrane have been confirmed in relation to HIPEF treatment, i.e., membrane permeability is modified by electropermeabilization. Normally, the membrane is relatively impermeable to H^+ and OH^- ions (*29*), but its permeability increases due to the formation of pores during HIPEF treatment. Thereby, the rate of transportation of H^+ increases (*5*) and leakage of cellular content is possible. So that local pH changes close to the cell membrane may dissolve a part of the nisin due to local acidification. For this reason, nisin, which is insoluble at pH 6.8, might recover its antimicrobial activity just beside the membrane injury. Moreover, sublethal injuries caused by HIPEF could improve the nonspecific binding of nisin to cellular debris or the emergence of new binding sites in or from cells (*52*). Otherwise, solubilized nisin, which remained active and free in the milk sample without being linked up, could be unprotected against the HIPEF effect. In fact, this argument could also explain why microbial inactivation is reduced when nisin concentration and HIPEF treatment time increases at neutral pH.

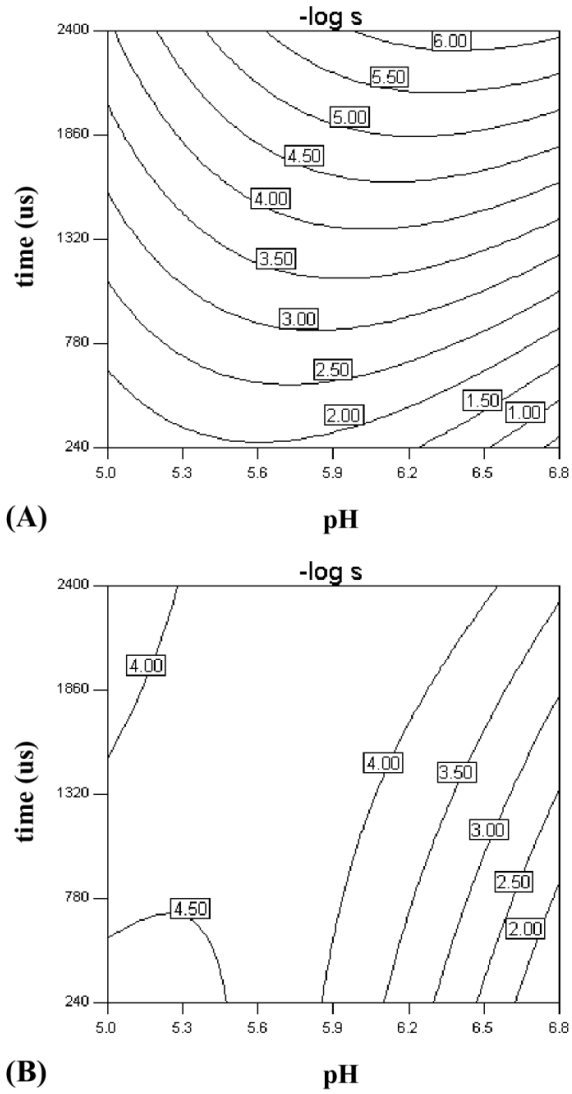

Figure 3. Effect of pH on the survival fraction of *Staphylococcus aureus* in skim milk vs. HIPEF treatment time (μs) at different nisin concentration (ppm) (A: 20 ppm; B: 150 ppm).

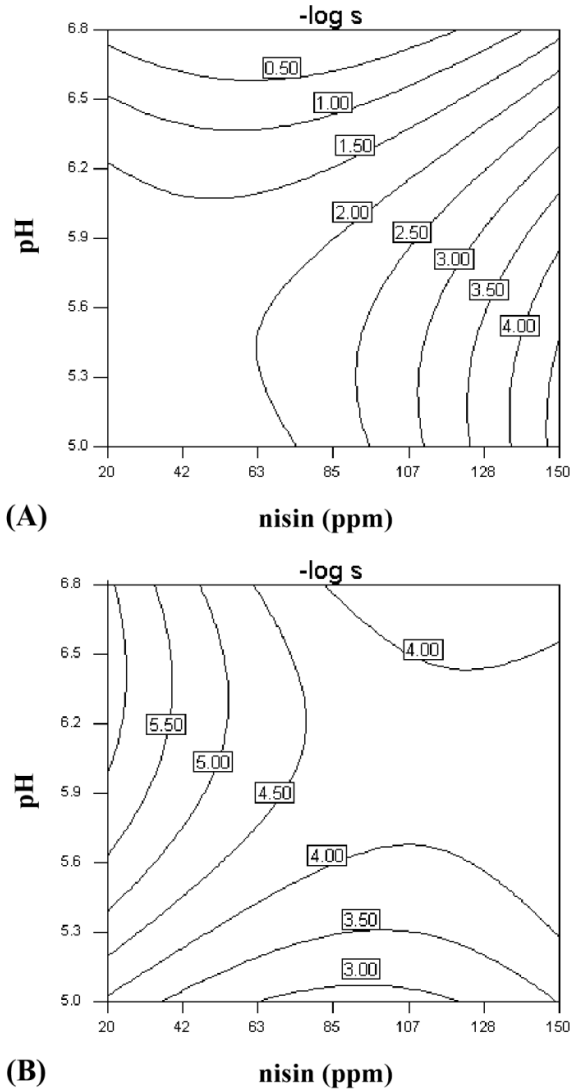

Figure 4. Effect of nisin concentration (ppm) on the survival fraction of *Staphylococcus aureus* in skim milk vs. pH at different HIPEF treatment time (μs) (A: 240 μs; B: 2400 μs).

CONCLUSIONS

A surface response methodology was performed to determine the combined effect of nisin and HIPEF where pH, HIPEF treatment time and nisin concentration were the independent variables. HIPEF and nisin acted synergistically probably due to sublethal injuries caused by HIPEF that enhanced nisin activity. However, synergism was affected by the pH. A microbial inactivation over 4.5 log units was achieved when pH, nisin concentration and treatment time were set at 5.0, 150 ppm and 240 μs, respectively, when applying a 35 kV/cm HIPEF. In this case, nisin seemed to be inactivated by HIPEF treatment since the higher the HIPEF treatment, the less the survival fraction dropped. On the other hand, a maximum microbial inactivation of 6.0 log units was observed at pH 6.8, 20-ppm nisin dose, 35 kV/cm and 2400 μs of HIPEF treatment time. This behavior of nisin might be explained by local changes of pH around the cell membrane caused by electropermeabilization that might dissolve nisin whereas nisin was kept aware of HIPEF inactivation. Synergism found

between nisin and HIPEF opens new possibilities for using this combination as a mild preservation method for milk and other dairy products but more research is needed in order to explain the nisin-HIPEF combination effect on the survival fraction.

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CAPITULO III

Enhancing the lethal effect of high-intensity pulsed-electric field in milk by antimicrobial compounds as combined hurdles

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ABSTRACT

High intensity pulsed electric field (HIPEF) is a non-thermal treatment studied for its wide antimicrobial spectrum on liquid food, including milk and dairy products. Moreover, the antimicrobial effect of HIPEF may be enhanced by combining different hurdles. Nisin and lysozyme are natural antimicrobial compounds that could be used in combination with HIPEF. So the purpose of this study was to determine the effect of combining HIPEF with the addition of nisin and lysozyme to milk inoculated with *Staphylococcus aureus* with regard to different process variables. The individual addition of nisin and lysozyme did not produce any reduction on cell population within the proposed range of concentrations, while their combination resulted in a pH-dependent microbial death of *Staph. aureus*. The addition of nisin and lysozyme to milk combined with HIPEF-treatment resulted to be synergistic. Applying 1,200 μs HIPEF treatment time to milk at pH 6.8 containing 1 IU/ml nisin and 300 IU/ml lysozyme resulted in a reduction of more than 6.2 log-units of *Staph. aureus* population. Final counts owing to adding nisin and lysozyme and applying HIPEF strongly depended on both the sequence of application and milk pH. Thus, more research is needed to elucidate the mode of action of synergism as well as the role of different process variables, although the use of HIPEF in combination with antimicrobial compounds, such as nisin and lysozyme, is shown to be potentially useful in processing milk and dairy products.

INTRODUCTION

Non-thermal treatments are being studied because of their antimicrobial effect with minimal alteration of sensory properties, as opposed to heat treatments. For that reason, non-thermal treatments, such as high intensity pulsed electric fields (**HIPEF**), together with the use of natural antimicrobial compounds, are gaining importance among novel technologies of food preservation. Since milk and dairy products are part of the human diet, the control of natural flora and bacterial growth is an important issue in milk processing. As liquid foods are suitable for treatment by HIPEF, the dairy industry is focusing its attention on the potential application of HIPEF as an acceptable alternative to classical heat treatments.

The antimicrobial spectrum of HIPEF includes a large number of Gram-positive and Gram-negative bacteria and, among these, the microbial death of pathogenic and spoilage bacteria, such as *Staphylococcus aureus* (Sobrino-López et al., 2006b), *Escherichia coli* (Martín *et al., 1997*), *Listeria monocytogenes* (Chen and Hoover, 2004), *Pseudomonas fluorescens* (Fernández-Molina et al., 2005), and *Lactobacillus brevis* (Grahl and Märkl, 1996), is particularly remarkable. However, the inactivation levels of viable cells recorded in milk as a result of HIPEF is noticeably lower than cell death of microorganisms inoculated in other media, such as fruit juice. According to Bendicho et al. (2002a), these differences in the effectiveness of HIPEF in milk and other media may be due to the more complex composition of milk, its low resistivity, and the presence of proteins. Thus, inactivation of microorganisms is more difficult in complex food materials, such as milk, than in simple solutions.

In recent decades, the use of naturally occurring antimicrobials to inhibit pathogen growth and prevent food spoilage has received special attention (Cleveland et al., 2001). Among these, nisin is considered especially relevant due to its international acceptance as a food additive by the Joint Food and Agriculture Organization/World Health Organization (**FAO/WHO**) Expert Committee on Food Additives (WHO, 1969). Moreover, its potential use in the food industry is based on its dairy origin and antimicrobial activity against pathogens (Chen and Hoover, 2003). On the other hand, lysozyme is a natural and broad-spread antimicrobial compound (Jollès, 1996) also present in milk and commonly classified as a milk enzyme (Chandan et al., 1965). Like nisin, lysozyme was granted a status of generally recognized as safe (**GRAS**) by WHO/FDA (WHO Food Additives Series 30) and, for matured cheese, received the 'quantum satis' status through European directive 95/2/EC on food additives other than colors and sweeteners. As regards its antimicrobial spectrum, gram-positive bacteria are generally sensitive to lysozyme whereas those gram-negative are commonly resistant (Masschalck et al., 2003).

Although HIPEF treatment induces a loss of microorganism viability in general, the protective effect of milk composition implies a drop in HIPEF effectiveness (Martín et al., 1997), which may represent a drawback in its potential industrial implementation. Nevertheless, different studies have pointed out a synergistic effect when combining HIPEF and other non-thermal treatments, particularly antimicrobial compounds, such as nisin and lysozyme. However, the combination of these treatments have scarcely been studied in milk, while process variables, range of concentrations added or effect of milk media have not been analyzed in depth either. Sobrino-López and Martín-Belloso (2006a) reported a synergistic activity in the reduction of *Staph. aureus* population when milk samples were treated by adding 20 ppm nisin before applying 2400 μs treatment time at 35 kV/cm field strength and 100 Hz of pulse frequency in bipolar mode. Smith et al. (2002) found that the

addition of 4250 IU/ml lysozyme and a HIPEF treatment of 50 pulses at 80 kV/cm led to higher microbial inactivation of natural flora of milk than each treatment applied separately. Interestingly, the combination of 1638 IU/ml lysozyme and 38 IU/ml nisin with a HIPEF treatment set at 50 pulses and 80 kV/cm produced an even more lethal effect on total microbial counts of raw milk than their effects when used alone (Smith et al., 2002). Hence, a hurdle approach by combining HIPEF and antimicrobial compounds seems to improve the microbial destruction in milk. However, there is a lack of information about the effect of the combined application of those treatments against milk pathogens as well as little information about the influence of treatment conditions on the final effect. Thus, the purpose of this study was to determine the combined effect of nisin, lysozyme and HIPEF against *Staph. aureus* in milk affected by different treatment conditions, such as pH of milk, nisin and lysozyme concentrations, and the sequence of the combined treatment.

MATERIALS AND METHODS

Skim milk

Homogenized UHT skim milk was obtained from a dairy plant (Puleva, Mollerussa, Lleida, Spain) and stored at 4ºC. Natural pH of milk was 6.8±0.02 and was measured by a pH-meter (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain). The electrical conductivity of the skim milk was 5.55±0.04 mS/cm at pH 6.8 and 6.99±0.13 mS/cm at pH 5.0. The measurement was performed at 25ºC and determined with a conductivity meter (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Germany).

Staphylococcus aureus culture

Staphylococcus aureus CECT 240 (Food Technology Department, University of Lleida, Spain) was used as the target microorganism. It was maintained on slants of plate count agar (PCA; Biokar diagnostics, Beauvais, France) at 4ºC until used.

Strain growth was performed by incubating cultures on tryptone soy broth (TSB) at 35ºC for 6 h. Inoculum concentration was determined by optical measurement. A population density of approximately 10^9 cfu/ml matches an absorbance value between 0.60 and 0.70 at 620 nm.

Sample preparation

Samples with *Staph. aureus* were prepared by inoculating the microorganism in skim milk to a final concentration of approximately 10^7 cfu/ml. The milk pH was adjusted by adding lactic acid (L(+)-lactic acid, Panreac, Barcelona, Spain) to the desired value. Then, samples were treated by adding nisin and/or lysozyme or by combining the addition of the mentioned antimicrobial compounds before or after applying HIPEF.

HIPEF equipment and treatment conditions

A continuous-flow HIPEF system was used to carry out this study. The treatment device was an OSU-4F HIPEF unit (Ohio State University, Columbus, Ohio, USA) that discharges square-shape pulses within eight collinear chambers, in which gap distance was 0.29 cm and each treatment chamber volume was 0.012 cm^3. Electrical parameters were set at 35 kV/cm of electric field strength, 6 μs of pulse width and 75 Hz of pulse frequency in bipolar mode according to previous studies (Sobrino-López et al, 2006b). Treatment temperature was kept always under 25ºC, using a cooled water bath to rule out thermal effects.

Table 1. Inactivation of *Staphylococcus aureus* by combining added nisin and lysozyme at different pH of milk

Assay number[1]	Point type	Nisin dose (IU/ml)	lysozyme dose (IU/ml)	pH	Microbial inactivation -log s^2
1	Factorial	5	3000	5.0	0.73
2	Factorial	5	300	6.8	1.6
3	Factorial	1	3000	6.8	0.37
4	Factorial	1	300	5.0	0.07
5	Axial	1	1650	5.9	0.08
6	Axial	5	1650	5.9	0.72
7	Axial	3	300	5.9	0.36
8	Axial	3	3000	5.9	0.32
9	Axial	3	1650	5.0	0.18
10	Axial	3	1650	6.8	0.72
11	Center	3	1650	5.9	0.26
12	Center	3	1650	5.9	0.25
13	Center	3	1650	5.9	0.15
14	Center	3	1650	5.9	0.25
15	Center	3	1650	5.9	0.25

[1] Assay number does not correspond to the order of processing
[2] –log *s*, microbial inactivation as mean of two replicates

Effect of the individual and simultaneous addition of nisin and lysozyme

The antimicrobial effects of nisin (N 5764, 2.5% nisin, 1,000,000 IU/mg, Sigma-Aldrich, Steinheim, Germany) and lysozyme (L 2879, 43,560 IU lysozyme/mg solid, Sigma-Aldrich, Steinheim, Germany) against *Staph. aureus* was measured by exposing milk samples to different concentrations of nisin (0 to 5 IU/ml) or lysozyme (0 to 3,000 IU/ml) at two different pH (5.0 to 6.8). Exposure time was prolonged for 1 h in accordance with previous studies (Sobrino-Lopez et al., 2006a).

A response surface design was set to study the effect of adding nisin and lysozyme (Table 1). On the central composite and faced centered design, nisin ranged from 1 to 5 IU/ml, lysozyme from 300 to 3,000 IU/ml and milk pH from 6.8 to 5.0, while exposure time was set at 1 h. Assays were replicated

twice and the experimental order randomized within each block. The effect of the independent variables was modeled by using a second-order response function:

$$-\log s = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{(Eq. 1)}$$

where factor *X* represents the encoded values of the variables and β are the constant coefficients. Confidence interval was set at 95% for all procedures.

Table 2. Central composite response surface design and microbial inactivation of *Staphylococcus aureus* suspended in milk treated under different conditions of nisin and lysozyme dose, HIPEF treatment time and pH of milk

Assay number[1]	Point type	Independent variables				Microbial inactivation
		Nisin dose (IU/ml)	Lysozyme dose (IU/ml)	HIPEF treatment time[2] (μs)	pH	-log *s*[3]
1	Factorial	5	3000	1200	5.0	4.8
2	Factorial	5	3000	120	5.0	2.0
3	Factorial	5	300	1200	6.8	5.3
4	Factorial	1	3000	120	6.8	2.3
5	Factorial	5	300	120	6.8	3.0
6	Factorial	1	300	1200	5.0	3.5
7	Factorial	1	3000	1200	6.8	4.8
8	Factorial	1	300	120	5.0	1.9
9	Axial	1	1650	660	5.9	4.9
10	Axial	5	1650	660	5.9	5.4
11	Axial	3	300	660	5.9	4.6
12	Axial	3	3000	660	5.9	3.5
13	Axial	3	1650	120	5.9	2.6
14	Axial	3	1650	1200	5.9	4.7
15	Axial	3	1650	660	5.0	3.1
16	Axial	3	1650	660	6.8	4.6
17	Center	3	1650	660	5.9	4.8
18	Center	3	1650	660	5.9	4.2
19	Center	3	1650	660	5.9	4.1
20	Center	3	1650	660	5.9	4.5
21	Center	3	1650	660	5.9	4.3

[1] Assay number does not correspond to the order of processing

[2] HIPEF treatment was set at 35 kV/cm, 6 μs pulse width, 75 Hz of pulse frequency in bipolar mode

[3] –log *s*, microbial inactivation as mean of two replicates

Combined effect of nisin, lysozyme and HIPEF treatment

The antimicrobial effect of HIPEF on *Staph. aureus* inoculated in skim milk at its natural pH or pH 5.0 was evaluated by setting a HIPEF treatment time of 1,200 μs. The assay was performed in triplicate. The response surface methodology was performed to observe the effect of the addition of nisin and lysozyme followed by HIPEF treatment on the microbial inactivation of *Staph. aureus* in skim milk. A central composite design with four factors and faced centered was the proposed experimental design. The independent variables were nisin concentration (1 to 5 IU/ml), lysozyme concentration (300 to 3,000 IU/ml), HIPEF treatment time (120 to 1,200 μs) and pH (5.0 to 6.8). The values for each variable and combination are shown in Table 2. The experimental design was performed in duplicate, providing two blocks of experiments and each assay in triplicate. The order of assays within each block was randomized.

The effect of the independent variables was modeled by using a second-order response function (Eq. 1). The non-significant terms ($p \leq 0.05$) were deleted from the second-order polynomial model after calculating an analysis of variance (**ANOVAs**), and then new ANOVAs were recalculated to obtain the coefficients of the final equation. Design Expert 6.0.1 software (Stat Ease Inc., Minneapolis, USA) was used in all analyses and generated plots. A 95% confidence interval was considered for all these procedures.

The sequence of addition of the antimicrobial compounds was also evaluated by applying a HIPEF treatment prior to the addition of nisin and lysozyme. In that case, exposure time of the HIPEF-treated sample to nisin and/or lysozyme lasted 1 h. Each assay was performed in triplicate.

Microbial inactivation of *Staphylococcus aureus*

The untreated and treated samples were serially diluted in Peptone Solution, plated on slants of Plate Count Agar and incubated for 72 h at 30ºC. The number of viable cells of *Staph. aureus* after applying a treatment was expressed as survival fraction, *s*, which was calculated as N/N_0, where N_0 was the initial count in samples prior to any of the treatments, addition of nisin and lysozyme, application of HIPEF or the proposed combinations, and N was the count after each treatment. Microbial inactivation was calculated as –log *s*.

RESULTS AND DISCUSSION

Effect of the addition of nisin and/or lysozyme

The addition of nisin alone up to 5 IU/ml to milk caused no cell death of *Staph. aureus* within milk-pH and exposure time ranges. These results are in agreement with different studies that have reported high doses of nisin to be lethal on *Staph. aureus* in milk and other media. Sobrino-López and Martín-Belloso (2006a) found that concentrations lower than 20 IU/ml had no lethal effect on *Staph. aureus* population in milk, while Smith et al. (2002) observed a reduction of 0.8-log units on natural flora of milk at 100 IU/ml nisin. Moreover, doses of lysozyme up to 3,000 IU/ml remained beneath the

minimal bactericidal concentration within the conditions set up in this study. The susceptibility of other pathogenic and lactic acid bacteria to lysozyme has been shown to be significant (Branen and Davidson, 2004) at higher concentrations than the latter concentration. A lysozyme concentration of 4,250 IU/ml showed a drop of only 0.2-log cycles on total microbial counts in milk (Smith et al., 2002) and Chung and Hancock (2000) reported no cell death of *Staph. aureus* at 12,000 IU/ml. The resistance of *Staph. aureus* to lysozyme has been explained through the cell envelope composition of the microorganism and the singular action mode of the compound. The inhibition mechanism of lysozyme is based on both lytic and nonlytic activity. While peptidoglycan hydrolysis of the bacterial cell wall is brought on by its muramidase activity, the conformation of the protein probably plays an important role in its nonlytic mechanism of inactivation (Masschalck et al., 2003). Consequently, gram-negative bacteria are believed to be resistant because of the outer membrane (Hughey and Johnson, 1987), while inert activity against some gram-positive bacteria may be due to peptidoglycan modifications, such as *O*-acetylation (Clarke and Dupont, 1992). Thus, the resistance of *Staph. aureus* to lysozyme is believed to be based on the *O*-acetylation of its cell wall, which ranges from 35 to 90% depending on the strain and the growth conditions (Clarke and Dupont, 1992).

The combined effect of nisin and lysozyme addition was studied by response surface modeling. Microbial inactivation achieved by combining the addition of both antimicrobial compounds is shown in Table 1. A maximal inactivation of 1.6-log units was observed when 5 IU/ml nisin and 300 IU/ml lysozyme were added to milk at pH 6.8, whereas almost no reduction on cell population was seen with the combination of 1 IU/ml and 300 IU/ml of nisin and lysozyme at pH 5.0, respectively. Microbial inactivation of *Staph. aureus* was satisfactorily fitted by a second order equation (Eq. 1) with a determination coefficient, R^2, of 0.995 and no significant lack of fit. Cell death of *Staph. aureus* was affected by nisin and lysozyme doses as well as by milk pH and their interaction (Table 3). Microbial inactivation was expressed by the following polynomial quadratic equation:

$$-\log s = 7.2 - 0.45 \cdot n + 5.4 \cdot 10^{-4} \cdot l - 2.7 \cdot p + 0.039 \cdot n^2 + 5.1 \cdot 10^{-8} \cdot l^2 + \\ + 0.25 \cdot p^2 + 0.064 \cdot n \cdot p - 1.2 \cdot 10^{-4} \cdot l \cdot p \qquad \text{(Eq. 2)}$$

where -log *s* is the microbial inactivation, *n* is the nisin concentration (IU/ml), *l* is the lysozyme concentration (IU/ml), and *p* is the pH of milk.

Although the additions of nisin and lysozyme alone did not affect the viability loss of *Staph. aureus* within the studied ranges, the simultaneous combination of these antimicrobial compounds at sublethal doses acted synergistically in cell inactivation. However, the effect of the mixture clearly depended on the milk pH as well as the bacteriocin doses used at each pH (Fig. 1). Firstly, the resistance of *Staph. aureus* against the nisin and lysozyme combination sharply diminished at natural pH of milk. Secondly, the activity against *Staph. aureus* of adding nisin and lysozyme at acidic milk pH increased as the nisin and lysozyme concentration rose, whereas lower doses of lysozyme enhanced microbial inactivation when adding nisin at natural milk pH. Results reported by Smith et al. (2002) showed a decrease of 1.2-log units on total microbial counts when 38 IU/ml and 1638 IU/ml of nisin and lysozyme, respectively, were added to raw skim milk. The final counts of the latter study may diverge from our results in that the microbial target of the treatment was the natural flora of milk

and a higher nisin concentration was added. Chung and Hankock (2000), who studied the effect of different mixtures of nisin and lysozyme in *Staph. aureus* population by fluorescence assay, observed that a combination of 12.5 μg/ml nisin and 900 IU/ml lysozyme had a greater and more dramatic effect compared to other mixtures with higher lysozyme concentration. A similar behavior of an increase of cell death at any nisin dose if lysozyme concentration drops was shown in our results at natural milk pH, whereas increasing the lysozyme dose acted efficiently only at acidic pH. Our results may differ from those of Chung and Hankock (2000) because of both the higher concentration of the antimicrobial compounds they used and the different media, whose pH was not determined. In this sense, Nattress et al. (2001) also found that the greater the lysozyme concentration in the mixture, the longer the antimicrobial efficacy was when evaluating the ability of lysozyme and nisin to control meat spoilage bacteria.

Table 3. ANOVA and significant regression coefficients for the response surface model of microbial inactivation of *Staphylococcus aureus* in milk by combining nisin and lysozyme addition

Source[1]	Mean square	F-value	Prob>F	Regression coefficients[3]
Model	0.262	156.10	< 0.0001[2]	
intercept				7.2±1.6
n	0.203	120.80	< 0.0001 [2]	-0.45 ±0.14
l	0.0009	0.54	0.4887 [2]	$5.4 \times 10^{-4} \pm 2.0 \times 10^{-4}$
p	0.472	280.92	< 0.0001 [2]	-2.7 ± 0.5
n^2	0.063	37.20	0.0009 [2]	0.039 ± 0.006
l^2	0.022	13.35	0.0107 [2]	$5.1 \times 10^{-8} \pm 1.4 \times 10^{-8}$
p^2	0.110	65.66	0.0002 [2]	0.25 ± 0.03
$n \times p$	0.018	10.50	0.0177 [2]	0.064 ± 0.020
$l \times p$	0.029	17.40	0.0059 [2]	$-1.2 \times 10^{-4} \pm 3 \times 10^{-5}$
Lack of fit	0.00045	0.20	0.8292	
Pure error	0.0023			
Standard deviation	0.0410			
Mean	0.418			
Coefficient of variation	9.801			
R^2	0.995			
Adjusted R^2	0.989			

[1] *n*, nisin concentration (IU/ml); *l*, lysozyme concentration (IU/ml); *p*, milk pH
[2] Significant at 95% confidence interval
[3] Mean ± standard deviation

The synergistic mode of action exerted by the combination of nisin and lysozyme is still not well understood. With regard to morphological changes on the cell envelope, Chung and Hankock (2000) proved, by scanning electron microscopy of treated cells of *Lb. sake*, that nisin produced surface ruffling and alterations at the division septum, while lysozyme caused the production of small balls of material all over the surface. Interestingly, their combination displayed holes or craters in the cell surface, drastic abnormalities at the position septa, and the release of large pieces of material. Thus, sensitization of bacteria to any of the two compounds may be enhanced by easing the access to their respective targets or intensifying and accelerating their action. Therefore, lysozyme may promote

nisin pore formation and, conversely, nisin may improve the murasidase activity or the nonlytic mode of action of lysozyme. Consequently, the mutual influence and simultaneous action seem to be further and complexly affected by media conditions and process variables.

Combined effect of HIPEF, nisin, and lysozyme

The individual effect of HIPEF treatment of 1,200 μs treatment time was performed in skim milk at its natural pH and pH 5.0. A microbial reduction on counts of *Staph. aureus* of 3.8±0.4-log units was induced in milk at its natural pH, while no difference on cell destruction was shown at pH 5.0. Few studies have considered pH as a variable process of HIPEF treatment. Some authors reported that pH had no effect on the inactivation achieved by HIPEF treatment (Smith et al., 2002; Sobrino-López et al., 2006a), while others claimed that a modification of milk conductivity (Wouters et al., 2001) and cell capacity for recovering from sublethal injuries caused by HIPEF treatment (Aronsson et al., 2001; Liang et al., 2002) are directly affected by pH.

Microbial inactivation achieved by applying HIPEF after adding nisin and lysozyme in milk is shown in Table 2. ANOVA for the second order model fits well the data obtained, with a determination coefficient, R^2, 0.982 and an insignificant lack of fit (Table 4). The microbial inactivation was adjusted by the following quadratic polynomial equation:

$$-\log s = -31 + 0.7 \cdot n - 3 \cdot 10^{-4} \cdot l + 5.1 \cdot 10^{-3} \cdot t + 9.9 \cdot p + 0.18 \cdot n^2 - 2.0 \cdot 10^{-7} \cdot l^2 - 2.6 \cdot 10^{-6} \cdot t^2 - \\ - 0.68 \cdot p^2 + 1.4 \cdot 10^{-4} \cdot n \cdot l - -0.32 \cdot n \cdot p + 2.4 \cdot 10^{-7} \cdot l \cdot t$$

(Eq. 3)

where –log *s* is the microbial inactivation, *n* is the nisin concentration (IU/ml), *l* is the lysozyme concentration (IU/ml), *t* is the HIPEF treatment time (μs), and *p* is the pH of milk.

In general, the population of *Staph. aureus* decreased dramatically at higher HIPEF treatment time. A drop of over 2.6-log units in counts of *Staph. aureus* was observed when HIPEF treatment time was lowered from 1,200 to 120 μs in milk with 1 IU/ml nisin and 3,000 IU/ml lysozyme added at its natural pH. Interestingly, milk pH seems to have a strong influence on the inactivation effect of the combined treatment, since acidic milk pH reduced the susceptibility of the microorganism to the combined treatment in comparison to milk at its natural pH (Fig. 2). The difference between the maximal inactivation at natural milk pH and pH 5.0 was over 1.2-log cycles when HIPEF treatment time was set at 1200 μs. Thus, the combination of HIPEF with antimicrobial compounds may involve a pH-dependent and complex mode of action. Therefore, milk pH modified the final effect of the addition of nisin and lysozyme followed by the application of HIPEF. In this way, maximal inactivation at acidic pH was obtained by high doses of nisin and lysozyme, whereas low nisin and lysozyme concentration displayed better results on cell death at natural milk pH. Maximal inactivation of *Staph. aureus* was nearly 6.4-log cycles if 1 IU/ml nisin and 300 IU/ml lysozyme were added to milk at pH 6.8 before applying 1,200 μs HIPEF treatment time. Other authors obtained similar values on

microbial death, although it has to be pointed out that different media, extreme HIPEF conditions, and higher antimicrobial compound doses make comparisons difficult and, in addition, the process temperature set in these studies may have had a relevant effect. A greater than 7.0-log reduction on natural flora of milk was registered when skim milk was added to 38 IU/ml nisin and 1638 IU/ml lysozyme and submitted to HIPEF treatment at 80 kV/cm and 50 pulses at 52ºC (Smith et al., 2002). Wu et al. (2005) observed a microbial reduction of 5.9-log units on natural spoilage flora of grape juice when 20 IU/ml nisin and 6,550 IU/ml lysozyme were added and mixed for 2 h before applying 20 pulses of 65 kV/cm at 50ºC. Similarly, a microbial reduction of over 6.5-log on population of *S. typhimurium* was produced by a mixture of 27.5 IU/ml nisin and 690 IU/ml lysozyme combined with 30 pulses of 90 kV/cm at 45ºC (Liang et al, 2002).

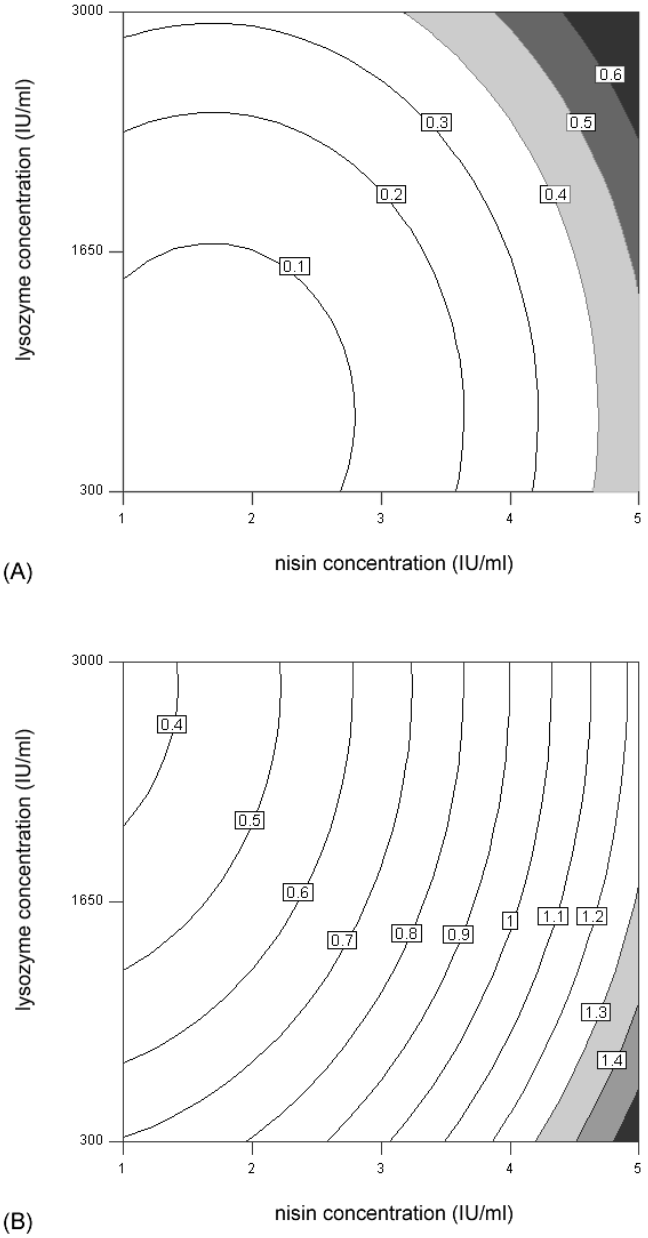

Figure 1. Microbial inactivation of *Staphylococcus aureus* in milk by combining nisin (IU/ml) and lysozyme (IU/ml) addition at different pH values: A, 5.0; B, 6.8.

Table 4. ANOVA and significant regression coefficients for the response surface quadratic model of microbial inactivation of *Staphylococcus aureus* in milk by combining nisin and lysozyme addition

Source[1]	Mean square	F-value	Prob>F	Regression coefficients[3]
Model	2.08	44.71	< 0.0001 [b]	
intercept				-31±7
n	1.01	21.70	0.0012 [2]	0.7±0.8
l	0.58	12.37	0.0065 [2]	$-3 \times 10^{-4} \pm 4 \times 10^{-4}$
t	12.70	272.42	< 0.0001 [b]	$5.1 \times 10^{-3} \pm 6 \times 10^{-4}$
p	1.19	25.53	0.0007 [2]	9.9±2.1
n^2	1.37	29.37	0.0004 [2]	0.18±0.03
l^2	0.33	7.17	0.0253 [2]	$-2.0 \times 10^{-7} \pm 7 \times 10^{-8}$
t^2	1.49	31.87	0.0003 [2]	$-2.6 \times 10^{-6} \pm 5 \times 10^{-7}$
p^2	0.79	16.87	0.0026 [2]	-0.68±0.17
$n \times l$	0.24	5.21	0.0484 [2]	$1.4 \times 10^{-4} \pm 6 \times 10^{-5}$
$n \times p$	0.53	11.47	0.0080 [2]	-0.32±0.09
$l \times t$	0.24	5.26	0.0476 [2]	$2.4 \times 10^{-7} \pm 1.0 \times 10^{-7}$
Lack of fit	0.0348	0.5665	0.7274	
Pure error	0.0614			
Standard deviation	0.216			
Mean	3.935			
Coefficient of variation	5.486			
R^2	0.982			
Adjusted R^2	0.960			

[1] *n*, nisin concentration (IU/ml); *l*, lysozyme concentration (IU/ml); *t*, HIPEF treatment time (μs); *p*, milk pH

[2] Significant at 95% confidence interval

[3] Mean ± standard deviation

The combination of nisin and lysozyme enhanced the HIPEF effect synergistically, which agrees with results presented by other authors (Liang et al., 2002; Smith et al., 2002; Wu et al., 2005). In particular, the application of 1,200 μs HIPEF treatment time on milk at pH 6.8 containing 1 IU/ml nisin and 300 IU/ml lysozyme produced an extra inactivation of *Staph. aureus* over 2.5-log units with respect to the cell death achieved by each treatment applied separately, and 1.8-log units more than the sum of the microbial inactivation induced by HIPEF treatment alone and the simultaneous combination of nisin and lysozyme. However, the combined effect was additive or only slightly synergistic at acidic pH. Only an extra 0.7-log unit was observed when 5 IU/ml nisin and 1,650 IU/ml lysozyme were added to milk before applying HIPEF treatment. However, Smith et al. (2002) indicated that HIPEF combined with nisin alone is almost as effective as the combination of HIPEF with nisin and lysozyme on the natural flora of milk. The addition of lysozyme has a remarkable effect on the activity of nisin, allowing a reduction of 10-fold in nisin concentration, in comparison with a previous nisin-HIPEF effect on *Staph. aureus* reported by Sobrino-López and Martín-Belloso (2006a).

The mechanism of the nisin-lysozyme-HIPEF synergism is not fully understood. It is believed that HIPEF and nisin have a mutual influence in inactivating microorganisms, and the inclusion of a third preservation hurdle, lysozyme in this case, may intensify their reciprocal action. HIPEF may facilitate the binding of nisin to their target on the cell membrane causing sublethal injuries to the cell

envelope (Calderon-Miranda et al., 1999; Terebiznick et al., 2000), and, simultaneously, nisin may diminish the resistance of the target cell lowering its critical field strength (Ho et al., 1995), while lysozyme may also facilitate and accelerate nisin binding (Chung et al., 2000).

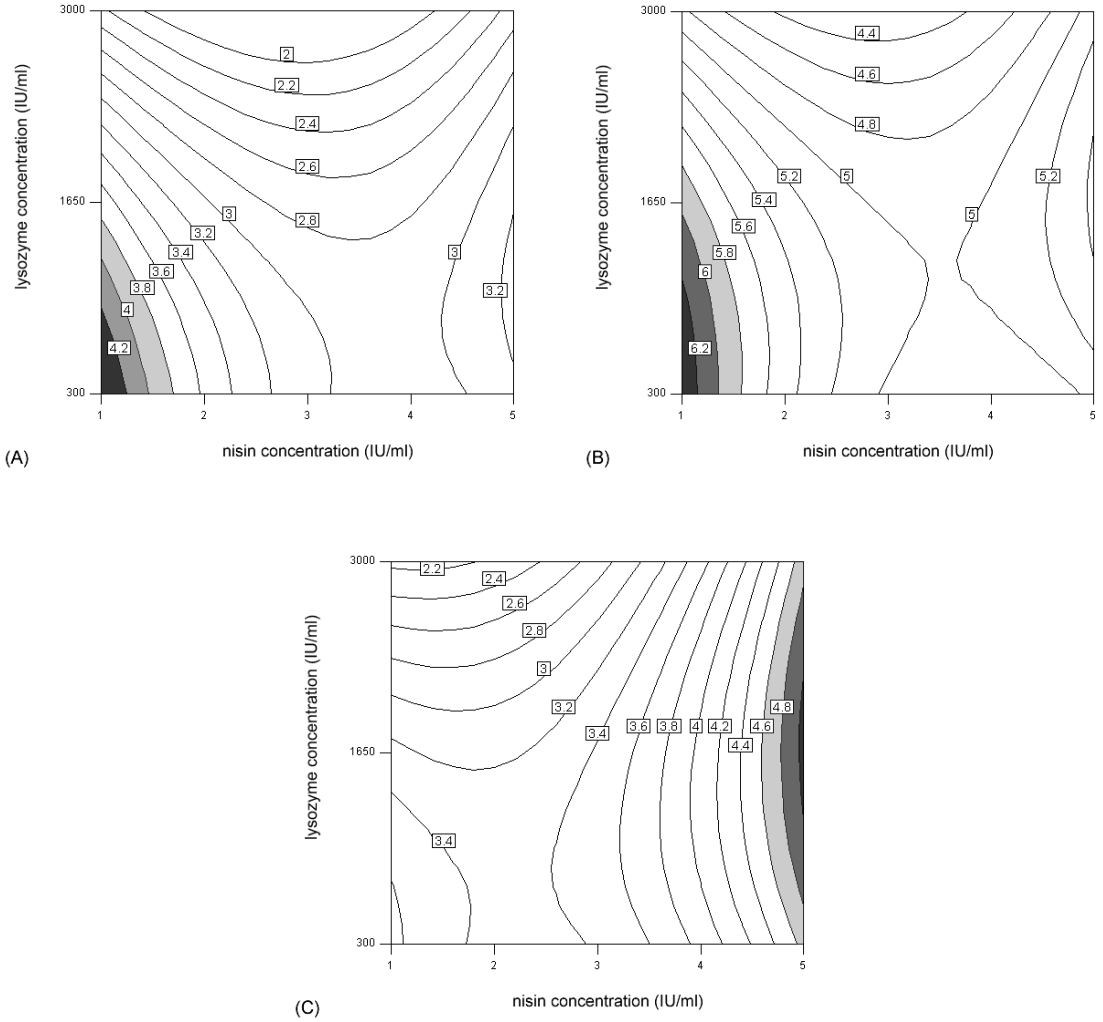

Figure 2. Microbial inactivation of *Staphylococcus aureus* in milk by combining nisin (IU/ml) and lysozyme (IU/ml) addition before applying HIPEF treatment (35 kV/cm, 6 µs pulse width, 75 Hz frequency, and bipolar mode) at different milk pH (A, pH 6.8 and 120 µs HIPEF treatment time; B, pH 6.8 and 1,200 µs HIPEF treatment time; C, pH 5.0 and 1,200 µs HIPEF treatment time).

Surprisingly, increasing lysozyme concentration failed to achieve lower counts of *Staph. aureus* in milk at its natural pH followed by HIPEF treatment. In contrast, acidic milk pH led to a higher antimicrobial effect as nisin and lysozyme concentration increased, although the final inactivation goal reached lower values than those at natural pH. Two hypotheses may explain these phenomena. In this respect, it has been suggested that this behavior is related to the loss of activity caused by exposure to HIPEF. Furthermore, HIPEF is shown to inactivate bacteria but also enzymes (Bendicho et al., 2002a), to cause feasible structural changes on proteins (Bendicho et al., 2002b), and to alter some soluble components of the media (Gallo et al., 2007). In a previous work, the variation of nisin

solubility at different pH, and leakage of intracellular content due to pore formation were proposed as possible causes of the loss of nisin activity depending on milk pH (Sobrino-López and Martín-Belloso, 2006a). Nevertheless, since lysozyme activity is stable within acidic and neutral pH, more evidence is needed to clarify the susceptibility of nisin to HIPEF and, above all, the influence and resistance of lysozyme to HIPEF. As a second hypothesis, nisin pore formation and its functionality may explain differences in nisin activity at acidic and neutral pH. Nisin molecules may compete for the same target site on the cell membrane at high nisin concentration, which may lead to activity decay (Moll et al., 1997). In our case, lysozyme and nisin bind to the phospholipids of the cell membrane and may compete for them. On the other hand, the low efficiency of nisin pore in dissipating the transmembrane electrical potential may explain the loss of nisin activity and a lower final microbial inactivation at acidic pH (Moll et al., 1997), even when combined with lysozyme.

Different concentrations of nisin and lysozyme were added to HIPEF-treated milk inoculated with *Staph. aureus* in order to better understand the behavior and mode of action of the synergism exerted by nisin-lysozyme-HIPEF treatment. As seen in Table 5, microbial death as a result of the combined treatment did not depend on the sequence of application at acidic pH of milk. This result disagrees with those of Gallo et al. (2007), who observed that nisin addition after HIPEF treatment did not modify the final effect. In contrast, García et al. (2007) concluded that damage to the cell envelope by HIPEF depended on the bacterial species and, particularly, on the treatment medium pH. In this sense, the degree of permanent membrane permeabilization or the inability to reseal due to HIPEF may collaborate in the final microbial death achieved once the mixture of nisin and lysozyme is added to milk at acidic pH.

Table 5. Effect of the combined addition of nisin and lysozyme in the microbial inactivation of *Staphylococcus aureus* inoculated in milk before and after applying a HIPEF treatment (6 μs pulse width, 1200 μs HIPEF treatment time, 75 Hz frequency, and bipolar mode)

pH	Nisin [2] (IU/ml)	Lysozyme [2] (IU/ml)	Microbial inactivation -log *s*	
			HIPEF after	HIPEF before [1]
6.8	1	300	6.30	3.09
6.8	5	300	5.05	5.40
5.0	5	3,000	4.70	4.70
5.0	5	1,650	5.00	5.10

[1] Mean of three trials

[2] Exposure time to nisin and lysozyme after HIPEF treatment was 1 h

Interestingly, the nisin dose affected the final microbial counts at natural pH of HIPEF-treated milk with 300 IU/ml lysozyme (Table 5). On the one hand, neither synergism nor any additive effect was observed when 1 IU/ml nisin and 300 IU/ml lysozyme were added to milk, which it is opposed to an inactivation level of nearly 6.4-log units achieved by applying HIPEF after addition of both antimicrobial compounds. This result suggests the simultaneous interaction between nisin and lysozyme at low doses and transient injuries inflicted to the cell membrane by HIPEF. In fact, Calderon-Miranda et al. (1999) classified some abnormalities on the cell envelope due to HIPEF treatment, which were augmented when HIPEF was combined with added nisin. These results are in

agreement with transient and reversible permeabilization of the cell envelope due to HIPEF (García et al., 2007). While the simultaneous action of nisin, lysozyme and HIPEF may inflict lethal and permanent damage to the cell membrane, the addition of the antimicrobial compounds at low concentration after applying HIPEF may not be capable of avoiding the resealing of transient permeabilization. On the other hand, increasing nisin to 5 IU/ml resulted in over 0.4-log units more reduction in cell population than by adding both antimicrobial compounds at the same doses before HIPEF treatment. In this case, the formation of permanent sublethal injuries and the sensitization of the cell membrane (Terebiznik et al., 2000; García et al., 2007) may b the cause of an increase in the death level with respect to the addition of both antimicrobials before HIPEF treatment, while loss of activity of nisin and/or lysozyme in milk at its natural pH may occur during the application of HIPEF treatment due to possible inactivation of those peptides. As regards nisin pore formation (Moll et al., 1997), nisin bound to the cell membrane would not be able to form pores if the damage caused by HIPEF is strong enough to alter the pH gradient or the transmembrane electrical potential (Terebiznik et al., 2000). Thus, more research is needed to clarify the role of pH on nisin-lysozyme-HIPEF treatment as well as the mechanism leading to the final effect of the combined hurdles.

CONCLUSIONS

In conclusion, no cell death was observed when nisin and lysozyme were added individually to milk up to 5 IU/ml and 3,000 IU/ml, respectively. However, the simultaneous addition of nisin and lysozyme to milk at sublethal doses acted synergistically in inactivating *Staph. aureus*. In general, acidic milk pH acted to strengthen the resistance of *Staph. aureus* against the mixture of nisin and lysozyme and, consequently, to diminish the level of inactivation. Nevertheless, the optimal concentration of each compound was also pH-dependent, in such a way that a lower lysozyme concentration enhanced more the nisin activity against *Staph. aureus* at natural milk pH than at acidic pH.

The use of a third preservation method, such as HIPEF, in combination with the addition of nisin and lysozyme to milk at different pH effectively inactivated *Staph. aureus*. The effect of the combined treatment was clearly synergistic at natural milk pH when low doses of nisin (1 IU/ml) and lysozyme (300 IU/ml) were added and 1,200 µs HIPEF treatment time were applied. However, a higher concentration of both antimicrobial compounds was needed at acidic milk pH to observe synergism, which was less efficient in terms of microbial inactivation than that at natural pH. In addition, the combination of the addition of nisin and lysozyme with HIPEF treatment made it possible to obtain similar cell death compared to nisin-HIPEF treatment but with a reduced nisin concentration, which may represent an advantage from an economic point of view.

The mode of action of combining added nisin and lysozyme with HIPEF has been little studied. However, the results derived from applying HIPEF treatment after or before the addition of the antimicrobial compounds suggest that the loss of nisin activity may occur during HIPEF due to a possible inactivation of the bacteriocin. Despite that, the mechanism of synergism seems to be complexly influenced by process variables. Moreover, more evidence is needed to explain the role of lysozyme in the synergism as well as the possible effect of HIPEF on its activity. Hence, the application of the hurdle concept through the application of non-thermal technologies, such as the

combination of HIPEF treatment with the addition of nisin and lysozyme, opens up a feasible possibility of preserving milk and dairy products with minimal loss of their sensory properties.

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CAPITULO IV

The effect of adding antimicrobial peptides to milk inoculated with *Staphylococcus aureus* and processed by high-intensity pulsed-electric field

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ABSTRACT

The use of high-intensity pulsed-electric field (**HIPEF**) and antimicrobial substances of natural origin, such as enterocin AS-48 (**AS-48**), nisin, and lysozyme, are among the most important non-thermal preservation methods. Thus, the purpose of this study was to evaluate the combined effect on milk inoculated with *Staphylococcus aureus* of the addition of AS-48 with nisin and/or lysozyme, together with the use of HIPEF. Synergy was observed in the reduction of *Staph. aureus* counts with the following combination methods: i) addition of AS-48 and nisin; ii) addition of AS-48 plus use of HIPEF; and iii) addition of AS-48 and nisin plus use of HIPEF. Specifically, when 28 AU/mL AS-48 and 20 IU/mL nisin were added to the milk, and it was treated with HIPEF for 800 μs, over 6 log reductions were observed in the microorganism. In general, *Staph. aureus* inactivation was dependent on HIPEF treatment time, antimicrobial doses, and medium pH. During storage of the treated milk, survivor population was related to peptide concentration and temperature. Final cell viability was influenced by the sequence in which the treatments were applied: the addition of AS-48 or AS-48 and nisin was more effective before than after HIPEF treatment. The results obtained indicate that the combination of HIPEF and antimicrobials could be of great interest to the dairy industry, although it is necessary to study further the way in which the combined treatments act.

INTRODUCTION

The interest of the food industry in food preservation through non-thermal methods arises, in general, from the fact that they make possible to guarantee the microbiological qualities of a product while, at the same time, minimizing the impact on its sensory properties. Of these methods, the use of high-intensity pulsed-electric field (**HIPEF**) and of antimicrobial compounds of natural origin are of special interest because of their great potential as preservation treatments in a wide range of food.

High-intensity pulsed-electric field is one of the most important non-thermal techniques for treating liquid foods for 2 reasons: firstly, because it is possible to use it industrially on continuous-flow processing lines, specifically before reaching the aseptic packaging equipment, and, second, because of its wide spectrum of microorganism inactivation. However, the resistance of different microorganisms to HIPEF treatment is dependent on many factors, such as food media (Bendicho et al., 2002), electrical variables (Sobrino-López et al., 2006) and type of microorganism, species and even strains (MacGregor et al., 2000). On the basis of a hurdle approach, the combination of HIPEF with other preservation methods can improve the final effectiveness of the treatment. Thus, it has been observed that the simultaneous combination of HIPEF with certain added antimicrobial substances results in a synergistic effect in the cell death (Wu et al., 2005; Sobrino-López and Martín-Belloso, 2006a).

Among antimicrobial compounds of natural origin, we should differentiate between those that form part of the intrinsic composition of the food, such as enzymes capable of cell-membrane damages, and those that are formed during its processing, such as antimicrobial substances of bacterial origin released during fermentation of the product. Lysozyme is a component usually present in milk whose muramidase activity focuses above all on gram-positive microorganisms, although certain gram–negative bacteria have also been identified as being sensitive to this substance (Masschalck and Michiels, 2003). As a preservative agent, lysozyme is generally recognized as safe by the Joint Food and Agriculture Organization-World Health Organization (WHO Food Additives Series 30), and as quantum satis by European directive 95/2/EC on food additives other than colors and sweeteners.

With regard to the natural compounds added during the food processing, bacteriocins are substances with antimicrobial activity produced by bacteria (Gálvez et al., 2007). Especially important are those bacteriocins produced by acid-lactic bacteria, since they are often related with a large number of fermentations in the food industry. In particular, nisin is a peptide produced by *Lactococcus lactis* subsp. *lactis*. Its lethal activity covers a wide spectrum of Gram-positive pathogenic and food-spoilage bacteria (Rodríguez et al., 2000) and it can be used in a wide variety of foods, including dairy products (Jung et al., 1992). Because of its history as a preservative agent, nisin was accepted as a food additive, E-234, in the European Union (EEC, 1983) and, later, was qualified as generally recognized as safe by the FDA (1988).

In contrast to the long-use history of nisin, other bacteriocins have been identified and characterized only recently, although some authors have already pointed out the great potential of some of them to be used in the food industry. Enterocin AS-48 (**AS-48**) is a circular peptide, which is stable over a wide pH range, heat-resistant, and displays a broad antimicrobial spectrum. It is produced by *Enterococcus faecalis* (Gálvez et al., 1986) and shows feasibly applications in food such as sausages (Ananou et al., 2005b), vegetable sauces (Cobo-Molinos et al., 2005), fruit juices (Grande et al., 2005), and dairy products (Muñoz et al., 2007). However, there is still little or no information on the

effectiveness of this bacteriocins and its degree of microorganism inactivation when combined with HIPEF, or even with other bacteriocins. Because *Staphylococcus aureus* is an important milk- and dairy product-related pathogen, the purpose of this study was to evaluate the effect of enterocin AS-48 added alone to milk inoculated with *Staph. aureus* or in combination with nisin or lysozyme, and processed simultaneously with HIPEF treatment. The influence of processing parameters, the storage conditions of the samples treated and the sequence of application of each treatment were also studied.

MATERIALS AND METHODS

Staphylococcus aureus Culture

Staphylococcus aureus CECT 240 (Food Technology Department, University of Lleida, Spain) was the target microorganism. The strain was maintained routinely on slants of plate count agar (PCA; Biokar diagnostics, Beauvais, France) at 4ºC until it was used.

Strain growth was performed by incubating cultures on tryptone soy broth at 35ºC for 6 h. Inoculum concentration was determined by optical measurement. A population density of approximately 10^8 cfu/mL matches an absorbance value between 0.6 and 0.7 at 620 nm.

Skim Milk

Homogenized UHT skim milk was obtained from a dairy plant and stored at 4ºC (Puleva, Mollerussa, Lleida, Spain). Natural pH of milk was 6.80±0.02 measured by a pH-meter (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain). The electrical conductivity of the skim milk was 5.55±0.04 mS/cm. The measurement was performed at 25ºC and determined with a conductivity meter (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Germany).

Preparation of Enterocin AS-48 Solution

Enterococcus faecalis A-48-32 (Martínez-Bueno et al*.,* 1990) was used as enterocin AS-48 producer. The bacteriocin solution was obtained by purification of cultured broths of the producer strain after concentration by cation-exchange chromatography in accordance with the method described by Abriouel et al. (2003). Bacteriocin concentrates were desalted through 2,000 Da cut-off dialysis tubing (Sigma), filtered through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. The AS-48 activity was determined by the agar well diffusion method (Gálvez et al., 1986). The enterocin AS-48 preparation resulted in a concentration of 3,500 arbitrary units per mL (AU/mL) which was 10-fold diluted with demineralized water to obtain the final solution.

Sample Preparation

Samples with *Staph. aureus* were prepared by inoculating the microorganism in skim milk to a final concentration of approximately 10^7 cfu/mL. According to the experimental design, pH of milk was adjusted by adding lactic acid (L(+)-lactic acid, Panreac, Barcelona, Spain). When acidity was adjusted to 5.0 pH, electrical conductivity of milk was 6.99±0.13 mS/cm.

HIPEF Equipment and Treatment

A continuous-flow HIPEF system was used to carry out this study. The treatment device was an OSU-4F HIPEF unit (Ohio State University, Columbus, Ohio, USA) that discharges square-shape pulses within 8 collinear chambers, in which gap distance was 0.29 cm and each treatment chamber volume was 0.012 cm^3. Electrical parameters were set at 35 kV/cm of electric field strength, 6 μs of pulse width and 75 Hz of pulse frequency in bipolar mode in accordance with previous studies. Treatment temperature was kept under 25ºC using a cooled water bath to rule out thermal effects. The antimicrobial action of HIPEF treatment against *Staph. aureus* was determined by treating samples at their natural pH and pH 5.0 up to a final treatment time of 1,200 μs. Each trial was performed in triplicate.

Table 1. Inactivation of *Staphylococcus aureus* in milk by combining added enterocin AS-48 and HIPEF

Assay number[a]	Point type	Enterocin AS-48 dose (AU/mL)	HIPEF treatment time[b] (μs)	milk pH	Microbial inactivation –log *s*[c]
1	Factorial	28.0	1200	5.0	2.8
2	Factorial	28.0	120	6.8	1.6
3	Factorial	3.5	1200	6.8	3.3
4	Factorial	3.5	120	5.0	1.2
5	Axial	3.5	660	5.9	3.1
6	Axial	28.0	660	5.9	4.3
7	Axial	15.8	120	5.9	1.8
8	Axial	15.8	1200	5.9	3.3
9	Axial	15.8	660	5.0	2.2
10	Axial	15.8	660	6.8	3.0
11	Center	15.8	660	5.9	2.8
12	Center	15.8	660	5.9	3.0
13	Center	15.8	660	5.9	3.1
14	Center	15.8	660	5.9	2.6
15	Center	15.8	660	5.9	2.9

[a] Assay number does not correspond to the order of processing
[b] HIPEF treatment was set at 35 kV/cm, 6 μs pulse width, 75 Hz of pulse frequency at bipolar mode
[c] –log *s* = microbial inactivation as mean of 2 replicates

Enterocin AS-48 in Combination with Nisin or Lysozyme

The single effect of enterocin AS-48, nisin (N 5764, 2.5% nisin, 1,000,000 IU/mg, Sigma-Aldrich, Steinheim, Germany) and lysozyme (L 2879, 43,560 IU of lysozyme/mg of solid, Sigma-Aldrich, Steinheim, Germany) against *Staph. aureus* was measured by exposing samples at their natural pH and pH 5.0 to a bacteriocin concentration up to 28 AU/mL, 20 IU/mL and 5,000 IU/mL, respectively. The effectiveness of AS-48 was also evaluated when added together with nisin or lysozyme and holding samples for 1 h at room temperature. All assays were performed in triplicate.

Application of HIPEF Treatment in Combination with Added Enterocin AS-48

The susceptibility of *Staph. aureus* against the combination of added enterocin AS-48 and HIPEF was studied by adding the bacteriocin to milk samples and then applying the HIPEF treatment. A response surface methodology (**RSM**) was set to study the effect of the combined treatment (Table 1). Process variables were AS-48 concentration (3.5 to 28 AU/mL), pH of milk (5.0 to 6.8), and HIPEF treatment time (120 to 1,200 µs).

Application of HIPEF Treatment in Combination with Added Enterocin AS-48 and Nisin or Lysozyme

Resistance of *Staph. aureus* to the addition of AS-48 together with nisin before carrying out HIPEF treatment was also evaluated by a RSM (Table 2). Levels of the process variables were 1 to 20 IU/mL nisin and 120 to 1,200 µs HIPEF treatment time. Similarly, samples with added AS-48 and lysozyme were also treated by HIPEF and setting a RSM, in which lysozyme varied from 300 to 5,000 IU/mL and HIPEF treatment time from 120 to 1,200 µs. Enterocin AS-48 concentration was kept constant at 28 AU/mL in both cases.

Sequence of Application

The sequence of application of each treatment was evaluated by comparing the final microbial inactivation of adding the antimicrobial compounds before applying HIPEF with that of applying HIPEF after. Therefore, a mixture of 28 AU/mL of AS-48 and 20 IU/mL of nisin or 5,000 IU/mL of lysozyme was added to HIPEF-treated samples at natural pH of milk by setting 1,200 µs HIPEF treatment time and prolonging exposure to the antimicrobial mixture for 1 h at room temperature. Assays were performed in triplicate.

Storage Conditions of Treated Samples

Samples with single and combined additions of AS-48, nisin and lysozyme or those treated with a combination of HIPEF and different mixtures of the antimicrobial compounds were stored at 4ºC and 22ºC. Population of *Staph. aureus* in the stored samples was evaluated after 24 and 48 h. Assays were performed in triplicate.

Response Surface Methodology (RSM)

Response surface methodology was performed to study the simultaneous effect of HIPEF and added AS-48 and nisin or lysozyme (Tables 1 and 2). In each experimental model, a central composite and faced centered design was selected (Myers and Montgomery, 2002). Assays were replicated twice and the experimental order randomized within each block. Each trial was performed in triplicate. The effect of *n* independent variables was modeled by using a second-order polynomial equation:

$$-\log s = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad [1]$$

where –log *s* is the microbial inactivation of *Staph. aureus*, β_i are the regression coefficients and factor X_i represents the encoded values of the variables. Regression coefficients were estimated by model reduction, as such omitting non-significant terms (P>0.05) from ANOVA. A 95% confidence interval was set for all procedures. Design Expert 6.0.1 software (Stat Ease Inc., Minneapolis, USA) was used in all RSM analyses and generated plots.

Table 2. Microbial death of *Staphylococcus aureus* suspended in milk with added 28 AU/ml enterocin AS-48 and treated under different conditions of nisin dose and HIPEF treatment time

Assay number[a]	Point type	Independent variables		Microbial inactivation
		Nisin dose (IU/mL)	HIPEF treatment time[b] (μs)	–log *s*[c]
[1]	Factorial	1.0	120	2.7
2	Factorial	20.0	120	3.2
3	Factorial	1.0	1200	4.3
4	Factorial	20.0	1200	5.1
5	Axial	1.0	660	5.4
6	Axial	20.0	660	6.6
7	Axial	10.5	120	2.7
8	Axial	10.5	1200	4.6
9	Center	10.5	660	5.4
10	Center	10.5	660	5.3
11	Center	10.5	660	5.5
12	Center	10.5	660	5.0
13	Center	10.5	660	5.2

[a] Assay number does not correspond to the order of processing
[b] HIPEF treatment was set at 35 kV/cm, 6 μs pulse width, 75 Hz of pulse frequency at bipolar mode
[c] –log *s* = microbial inactivation as mean of 2 replicates

Microbial Inactivation of *Staphylococcus aureus*

The untreated and treated samples were serially diluted in peptone saline solution, plated on slants of PCA and incubated for 48 h at 35ºC. The number of viable cells of *Staph. aureus* after applying a treatment was expressed as survival fraction, *s*, which was calculated as N/N_0, where N_0 was the initial count in samples prior to any of the treatments and *N* was the count after each treatment. Microbial inactivation was calculated as –log *s*.

RESULTS

Effect of Adding AS-48, Nisin or Lysozyme, or AS-48 with lysozyme

The addition of 28 AU/mL of AS-48, 20 IU/mL of nisin or 5,000 IU/mL of lysozyme alone, and the combination of enterocin AS-48 (28 AU/mL) with lysozyme (5,000 IU/mL) caused no variation in survival of *Staph. aureus* in milk at its natural pH and pH 5.0 after 1 h of exposure at room temperature, nor after 24 and 48 h of storage at 4 and 22ºC. On the contrary, the *Staph. aureus* population fell when 28 AU/mL of AS-48 was combined with 20 IU/mL of nisin, although the level of inactivation depended on exposure time, milk pH and temperature. Thus, after 1 h of exposure to the 2 bacteriocins, log reductions of 1.8 and 1.1 in *Staph. aureus* were observed at 6.8 and 5.0 pH, respectively (Table 3). During storage, the activity of the 2 bacteriocins, in general, led to an even greater reduction in *Staph. aureus* population, although the number of survivors was lower at 22ºC or at natural milk pH than at 4ºC or at 5.0 pH. Up to 6 log reductions of the microorganism were observed when the milk was kept at its natural pH with 28 AU/mL of AS-48 and 20 IU/mL of nisin for 24 h at 22ºC. However, this tendency was blocked when storage was lengthened to 48 h. On the one hand, the recovery in *Staph. aureus* counts to the initial inoculated level at both of the 2 pHs under study revealed the ending of the bactericide effect of the 2 antimicrobials, while on the other hand, when the temperature was kept at 4ºC, the reduction increased progressively up to practically 4 log cycles over the 48 hours.

Table 3. Fate of *Staphylococcus aureus* inoculated in milk treated by enterocin AS-48 (28 AU/mL) and nisin (20 IU/mL) addition and storage at different temperatures

	Storage temperature					
	4ºC			22ºC		
		Treated [a,b]			Treated [a,b]	
Storage (h)	Untreated [a,b]	5.0 pH	6.8 pH	untreated	5.0 pH	6.8 pH
0	6.9±0.3	6.9±0.3	6.9±0.3	6.9±0.3	6.9±0.3	6.9±0.3
1	6.9±0.3	6.3±0.3	6.1±0.3	7.0±0.3	5.8±0.3	5.1±0.3
24	6.8±0.2	5.6±0.4	3.8±0.2	8.7±0.3	4.6±0.2	0.7±0.3
48	6.8±0.4	5.3±0.2	3.1±0.4	10.4±0.2	6.9±0.3	5.0±0.2

[a] Survivors as log *N*

[b] Mean ± standard deviation

Effect of HIPEF in Combination with Enterocin AS-48

To determine the greatest level of *Staph. aureus* inactivation by HIPEF, different samples were processed at natural milk pH and at pH 5.0, with a field intensity of 35 kV/cm, a frequency of 75 Hz in bipolar mode, and a treatment time of 1,200 μs. This treatment resulted in log reductions in counts of *Staph. aureus* of 3.5±0.3, with no significant differences in the level of destruction between the 2 pH levels studied.

Table 4. ANOVA and significant regression coefficients for the response surface model of microbial inactivation of *Staphylococcus aureus* in milk at different pH by combining enterocin AS-48 and HIPEF (35 kV/cm, 6 μs pulse width, 75 Hz frequency, and bipolar mode)

Source[a]	Mean square	F-value	Prob>F	Regression coefficients[c]
Model	1.09	14.83	0.0010 [b]	
intercept				-21.1 ± 8.6
e	0.62	8.44	0.0228 [b]	-0.0635 ± 0.0195
t	3.68	50.09	0.0002 [b]	-0.00266 ± 0.00340
p	0.45	6.15	0.0422 [b]	8.04 ± 2.64
e^2	0.71	9.59	0.0174 [b]	0.00346 ± 0.00117
t^2	1.10	14.92	0.0062 [b]	$-2.22 \times 10^{-6} \pm 5.7 \times 10^{-7}$
p^2	0.90	12.18	0.0101 [b]	-0.722 ± 0.207
$t \times p$	0.45	6.10	0.0428 [b]	0.00119 ± 0.00048
Lack of fit	0.12	3.20	0.1456	
Pure error	0.038			
Standard deviation	0.27			
Mean	2.72			
Coefficient of variation	9.97			
R^2	0.94			
Adjusted R^2	0.88			

[a] *e* = enterocin AS-48 concentration (AU/mL); *t* = HIPEF treatment time (μs); *p* = milk pH
[b] Significant at 95% confidence interval
[c] Mean ± standard deviation

The effect of the addition of enterocin AS-48 to milk before HIPEF treatment was studied in accordance with the proposed RSM. The inactivation achieved for each combination of variables is shown in Table 1. The ANOVA revealed that the results fit a second-order model, with a coefficient of determination (R^2) of 0.94 and a non-significant lack of fit (Table 4). The reduction in *Staph. aureus* population could be fitted by means of the following quadratic equation:

$$-\log s = -21.1 - 0.0635 \cdot e - 0.00266 \cdot t + 8.04 \cdot p + 0.00346 \cdot e^2 - 2.22 \cdot 10^{-6} \cdot t^2 - 0.722 \cdot p^2 + 0.00119 \cdot t \cdot p, \quad [2]$$

where –log *s* represents the number of log reductions, *e* the AS-48 concentration (AU/mL), *t* the treatment time (μs), and *p* the pH of milk. On the basis of this model, maximum destruction of *Staph. aureus,* of 4.5 log reductions, was achieved when 28 AU/mL of AS-48 was added to milk at its natural pH, which was then treated with HIPEF for 1,200 μs (Figure 1). This level of destruction represented 1.3 log reductions more than the cell death achieved by summing the inactivation obtained by each of the treatments applied separately. However, counts of *Staph. aureus* in the samples at pH 5.0 treated simultaneously with AS-48 and HIPEF were no different, in the best of cases than those obtained when using only HIPEF. The number of viable microorganisms in samples at pH 6.8 with 28 AU/mL of AS-48 processed with HIPEF for 1,200 μs remained constant during storage at 4ºC for 48 h,

whereas a growth in the population up to the initial inoculated level was observed in the same samples after 48 h if storage was held at 22ºC.

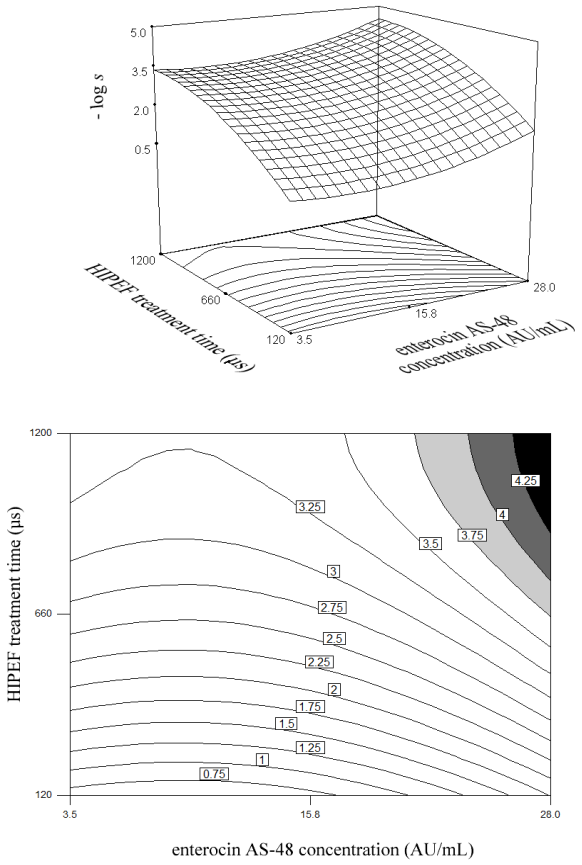

Figure 1. Effect of increasing enterocin AS-48 concentration and HIPEF treatment time on the microbial inactivation of *Staphylococcus aureus* in milk at its natural pH (HIPEF set up at 35 kV/cm, 6 µs pulse width, and 75 Hz of frequency at bipolar mode).

Effect of HIPEF in Combination with Added Enterocin AS-48 and Nisin or Lysozyme

The inactivation achieved by combining nisin and AS-48 (28 AU/mL) in milk samples which were then treated with HIPEF is shown in Table 2. As can be seen in the ANOVA (Table 5), the model fitted the response variable with a determination coefficient (R^2) of 0.97 and a non-significant lack of fit. The regression coefficients are shown in Table 4. The number of log reductions (–log *s*) was fitted by means of the following polynomial equation:

$$-\log s = 1.50 - 0.0658 \cdot n + 0.0103 \cdot t + 0.00525 \cdot n^2 - 6.53 \cdot 10^{-6} \cdot t^2 \qquad [3]$$

where *n* is the nisin concentration (IU/mL), and *t* the HIPEF treatment time (µs). The greatest effect achieved by combined treatment with nisin, AS-48 and HIPEF was a log reduction of 6.3, which was obtained by combining 28 AU/mL of AS-48, 20 IU/mL of nisin, and HIPEF treatment for 800 µs at pH 6.8. This level of inactivation is, on the one hand, almost double that obtained when summing the maximum levels achieved by each of the treatments if applied separately and, on the other, shows

an increase of approximately 1 log reduction compared to the level achieved by summing the maximum effect obtained by HIPEF and that achieved with the combined addition of AS-48 and nisin. Furthermore, after 48 h of storage at 4ºC, viable microorganism counts in the samples treated with the previous combination of variables remained stable, although a growth in the *Staph. aureus* population was observed in the same samples stored at a temperature of 22ºC.

The addition of lysozyme to samples with AS-48 previously treated with HIPEF neither enhanced nor modified the level of inactivation achieved compared to the same samples treated without lysozyme.

Table 5. ANOVA and significant regression coefficients for the response surface quadratic model of microbial inactivation of *Staphylococcus aureus* in milk by combining the addition of enterocin AS-48 (28 AU/mL) and nisin with HIPEF (35 kV/cm, 6 μs pulse width, 75 Hz frequency, and bipolar mode)

Source[a]	Mean square	F-value	Prob>F	Regression coefficients[c]
Model	4.06	71.77	< 0.0001 [b]	
intercept				1.50 ± 0.26
n	1.07	18.93	0.0024 [b]	-0.0658 ± 0.0231
t	4.96	87.72	< 0.0001 [b]	0.0103 ± 0.0005
n^2	0.62	10.96	0.0107 [b]	0.00525 ± 0.00158
t^2	10.01	176.83	< 0.0001 [b]	$-6.53 \times 10^{-6} \pm 4.91 \times 10^{-7}$
Lack of fit	0.079	2.28	0.2218	
Pure error	0.035			
Standard deviation	0.24			
Mean	4.69			
Coefficient of variation	5.08			
R^2	0.97			
Adjusted R^2	0.96			

[a] *n* = nisin concentration (IU/mL); *t* = HIPEF treatment time (μs)
[b] Significant at 95% confidence interval
[c] Mean ± standard deviation

Effect of the Sequence of Application of HIPEF and Bacteriocins AS-48 and/or Nisin

The addition of AS-48 to samples treated previously by HIPEF did not improve the lethality achieved by processing the samples with HIPEF alone or by processing them with HIPEF after the addition of AS-48. Likewise, after storage for 24 or 48 h at 4º or 22ºC, no differences in the counts were observed among the samples treated with HIPEF before or after the addition of AS-48. Similarly, no differences were observed in the number of log reductions in samples with AS-48 (28 AU/mL) and nisin (20 IU/mL), whether treated previously with HIPEF or not, or even in the survivor counts during storage.

DISCUSSION

The addition to milk of each of the antimicrobials AS-48, nisin or lysozyme on their own did not lead to any reduction in the *Staph. aureus* population within the range of concentrations and pH levels tested. Among the most important features of AS-48 is its stability under different pH and temperature conditions, as well as its wide antimicrobial spectrum (Diaz et al., 2003). In the particular case of *Staph. aureus,* Muñoz et al. (2007) found that the minimum dosage to succeed in inhibiting growth of the CECT 976 strain in milk was 50 μg/mL. That result explains the sub-lethal or non-existent effect of the concentration of AS-48 used in this study (8 μg/mL, equivalent to 28 AU/mL) against the target strain of *Staph. aureus*. Similarly, other authors determined that the minimum lethal concentrations of nisin and lysozyme are higher than the maximums used in this study. Sobrino-Lopez and Martín-Belloso (2006) observed that the concentration of nisin necessary to achieve a lethal effect on *Staph. aureus* in milk was 20 IU/mL, and Chung and Hancock (2000) detected a reduction in the population of the microorganism above 12,000 IU/mL of lysozyme. In the case of this latter antimicrobial, the modifications in the peptidoglycans of the *Staph. aureus* cell membrane, consisting of *O*-acetylation, may also explain the low activity of lysozyme against this microorganism (Clarke and Dupont, 1992).

Although the activity of each of the antimicrobials separately was not sufficient to inhibit the growth of *Staph. aureus* in the ranges of concentrations used, the combination of AS-48 (28 AU/mL) and nisin (20 IU/mL) did manage to reduce the population by 1.8 log cycles after an hour of exposure and almost up to detection level when the milk samples, at their natural pH, were exposed for 24 h at 22ºC. These results suggest that the 2 peptides together act synergistically to destroy *Staph. aureus* even when their individual concentrations are at sublethal levels. However, the joint, simultaneous activity of the 2 peptides seems to be intrinsically dependent on pH, temperature and exposure time to the 2 substances. Contrary to what might be expected, higher acidic levels in the milk reduced the combined activity of AS-48 and nisin, when their activity separately is higher precisely at low pH levels (Ananou et al., 2004). Abriouel et al. (2001) proposed that certain changes in the oligomerization of the AS-48 molecule, together with a change in the surface electrical charge of the target bacteria, could alter the activity of the bacteriocin at low pH conditions. This fact could probably explain the loss of synergy between the 2 molecules when the milk pH was 5.0 within the range of concentrations studied. Until now no other study has highlighted the synergistic effect of the simultaneous addition of enterocin AS-48 and nisin to milk, although it has been observed that the bactericide effect of AS-48 increases when it is combined with organic acids, such as benzoic, sorbic, and lactic (Ananou et al., 2007), or chelating agents, such as sodium tripolyphosphate and EDTA (Ananou et al., 2005a).

Temperature and, above all, exposure time seem to indicate the level of involvement of each molecule in the inhibiting effect. On the one hand, it was observed that nisin acts immediately or in a very short period of time (Hyde et al., 2006), thus the reduction observed during the first hour is directed and controlled principally by this molecule. On the other hand, the action of AS-48 is mainly responsible for the inhibition achieved after 24 or 48 hours of storage. However, temperature is the factor that acts as accelerator or decelerator of the activity during that period of time. Thus, a temperature of 22ºC during storage accelerated and intensified the joint activity of the peptides

during the first 24 hours, although that activity stopped during the following 24 hours, with a consequent re-growth in the population of *Staph. aureus*. On the contrary, a temperature of 4º C first slowed the inhibiting effect of the bacteriocins, but prolonged the decrease in the population up to 48 hours and, second, may have acted as a third treatment in itself with a bacteriostatic effect, given the thermophilic nature of the bacteria under study.

As can be seen in Figure 1, the number of log reductions achieved by HIPEF treatment of milk, at natural pH and with enterocin AS-48, increased as treatment time and AS-48 concentration reached their maximum values. The combination of HIPEF and AS-48 in inactivating *Staph. aureus* was synergistic. The loss of cell viability as a result of HIPEF treatment is basically due to cell damage that leads to permanent or transitory permeabilization of the membrane (Garcia et al., 2007). Thus, enterocin AS-48 may act with the induced electrical field in 3 possible ways: i) by maintaining and preventing the resealing of transient pores; ii) by enabling the formation of pores and disruption of the cell membrane by previous sensitization; and iii) by aiding in the extension of the permeabilization of the cell membrane above the critical level of cell viability. However, this pattern was not observed when milk pH was 5.0. Although the involvement of pH in the overall effectiveness of the treatment is not clear, a different conformation of the AS-48 molecule, together with a change in the sensitivity of the cell under these conditions (Abriouel et al., 2001; Ananou et al., 2007), or the ability of bacteria to recover from possible sublethal damage inflicted by HIPEF treatment (Aronsson et al., 2001) could explain the results observed at acidic pH values.

From a hurdle concept, the more preservation methods that are combined, the greater the inactivation that may be achieved. Although the addition of lysozyme to milk with added AS-48 and then treated with HIPEF did not improve the level of reduction of *Staph. aureus* compared to the same treatment without lysozyme, the addition of nisin to milk with added AS-48 (28 AU/mL), at natural pH and then treated with HIPEF, did noticeably reduce the population of *Staph. aureus.* In this case, the number of log reductions depended on the concentration of nisin and the treatment time. Specifically, maximum destruction was observed for a HIPEF treatment time of 800 μs (Figure 2), and both lower and higher times were less effective in reducing the population. Similar behavior was described by Sobrino-Lopez and Martín-Belloso (2006) when milk inoculated with *Staph. aureus* and with added nisin was treated with HIPEF. In this case, an increase in the treatment time decreased the effectiveness of the combined treatment. In spite of the high level of initial destruction, the microbial population in the samples treated remained constant during storage at 4º C up to 48 hours. This result contrasts with the behavior observed at the same temperature when only the 2 bacteriocins were added, when decrease in the population was gradual (Table 3).

With regard to the sequence of application, the addition of AS-48, or of AS-48 combined with nisin, to milk previously treated with HIPEF did not enhance the lethal effect of the treatment as compared with when HIPEF was applied after the addition of the bacteriocins. Likewise, no increase was observed in the counts of viable microorganisms when the samples were stored at 4º and at 22º C. This loss of effectiveness, contrary to what was to be expected (i.e. an increase in the lethality of *Staph. aureus* after the addition of the bacteriocins caused by the prior HIPEF damage to the cell), could be due to 2 reasons. Different authors have observed that the electropermeabilization of the cell membrane may be reversible depending on the degree of structural damage caused and its recovery occurs immediately after the ending of the treatment (Tsong, 1990; García et al., 2007). Thus, the sensitivity induced in the membrane would be lost before the bacteriocins could act. The

second reason is that the changes caused in the membrane by the action of the electrical field (Calderon-Miranda et al., 1999) may prevent or hinder the adhesion of the peptides, which would trigger an apparent increase in the resistance of the microorganism.

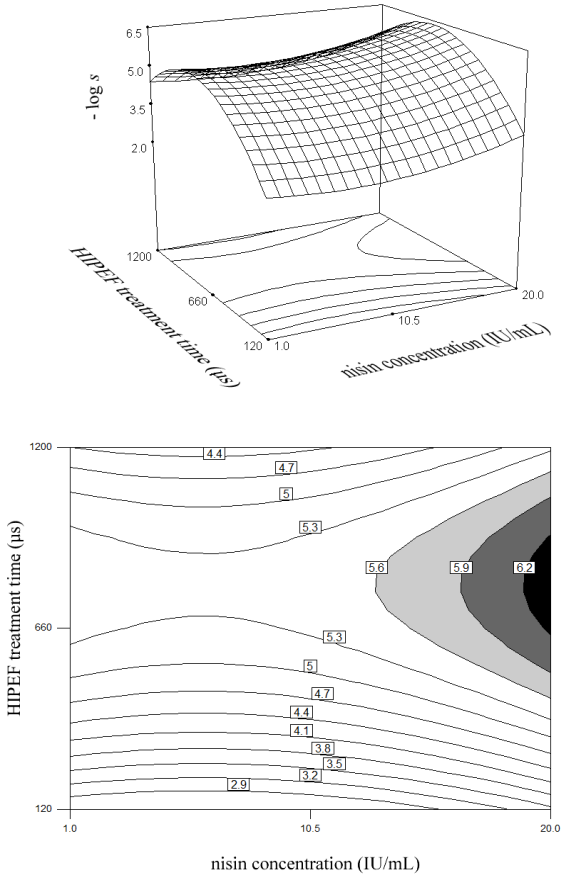

Figure 2. Microbial inactivation of *Staphylococcus aureus* in milk by combining nisin (1-20 IU/mL) and enterocin AS-48 (28 AU/mL) addition before applying HIPEF treatment (35 kV/cm, 6 µs pulse width, and 75 Hz of frequency at bipolar mode).

In conclusion, the treatment of milk with added AS-48 or nisin, or both, at sublethal doses with HIPEF acted in a synergistic way to destroy *Staph. aureus*. However, the possible application of the combined treatment should be studied on acidic dairy products, due to its loss of effectiveness under these pH conditions. It is also necessary to study in greater depth the joint mode of action of the 2 bacteriocins combined with HIPEF and to evaluate the effect of the application of HIPEF on the activity of the bacteriocins. However, the degree of destruction achieved with the combination of the above treatments, that is, the addition of AS-48 or nisin, or both, together with the application of HIPEF, and the prolongation of the inhibiting or bactericide effect during later storage could be of great interest for the preservation of both milk and other dairy products.

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CAPITULO V

Shelf-life of milk processed by combining the addition of antimicrobial compounds, high-intensity pulsed electric field and a mild thermal treatment

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ABSTRACT

Novel technologies such as the use of high-intensity pulsed-electric field (HIPEF) or antimicrobial compounds of natural origin are of special interest for processing liquid foods. Moreover, the combination of different preservation methods in the form of hurdles may induce greater microbial inactivation than each treatment applied separately. In this study, the shelf life and microbial stability of raw milk with nisin (1-20 IU/mL), or with nisin and lysozyme (300 IU/mL) or enterocin AS-48 (28 AU/mL), and also processed by HIPEF (35 kV/cm, 1,200 μs) and/or a mild thermal treatment (55-65ºC, 16 s), was evaluated during storage.

In general, milk with any of the antimicrobials and also processed by HIPEF and/or mild heat showed lower microbial counts during storage and longer shelf life than the samples subjected to a single treatment. The shelf life of milk with 1 IU/mL nisin and 300 IU/mL lysozyme, and also processed by HIPEF, was prolonged 4 days more compared with that of untreated milk. Samples with 20 IU/mL nisin and 28 AU/mL enterocin AS-48, and also treated by HIPEF and thermally processed at 65ºC, achieved the lowest counts and had a shelf life of 1 day more than pasteurized milk. Enterobacteria and the coliform population remained below detection level and no presence of pathogenic bacteria was detected among samples processed by a mild thermal treatment, HIPEF and antimicrobials.

The combination of nisin, combined or not with lysozyme, HIPEF and/or a mild thermal treatment may offer an alternative to classical heating to obtain long-lasting milk while maintaining microbiological acceptance.

INTRODUCTION

Milk and dairy products are commonly processed by heat to assure their microbiological acceptance and lengthen their shelf life. However, thermal treatments induce the generation of a cooked flavor and changes in, or loss of, the nutritive content of milk (*32*). Since trends in consumer demands are leaning towards minimally processed foods, research on novel preservation methods are focusing on the use of non-thermal treatments, such as High-intensity pulsed-electric field (HIPEF) and the addition of antimicrobial substances.

HIPEF seems to minimally alter the organoleptic properties of liquid foods and to exert an antimicrobial effect against a wide range of spoilage and pathogenic bacteria (6). Moreover, the use of antimicrobial peptides may play an important role in food preservation due to their natural origin and wide antibacterial spectrum, while their application in the food industry is feasible (*29*). Among these, bacteriocins, such as nisin and the novel enterocin AS-48 (AS-48), together with lysozyme, are arousing special interest. In general, these antimicrobial peptides have been thoroughly studied in a large variety of food products and have shown antimicrobial activity against many food spoilage and pathogenic bacteria (*10*, *15*, *18*). Moreover, the importance of lysozyme and nisin lie both in their status as GRAS (Generally Recognized As Safe) substances and the fact that they have been approved by the World Health Organization (*14*) and the European Community (*4*) for use as food preservatives.

From a hurdle perspective, the combination of different preservation methods in the form of hurdles was proved to enhance their lethality against diverse microorganisms. In the case of non-thermal treatments, the processing of milk by HIPEF (35 kV/cm, 1,200 μs) acted synergistically with the previous addition at sublethal doses of 20 IU/mL nisin (*28*) or 1 IU/mL nisin combined with 300 IU/mL lysozyme (*30*) in reducing the population of *Staphylococcus aureus* up to 6.0 log cycles in each case. Similar behavior against *Salmonella enterica* was also reported by Martínez-Viedma et al. (*17*) when HIPEF (35 kV/cm, 1,000 μs) was combined with the addition of 210 AU/mL enterocin AS-48. The combination of HIPEF with a thermal treatment has also received attention due to the fact that it achieved a greater effect than any of the individual treatments applied alone (*20*). Fernández-Molina et al. (*8*) processed skim milk by heat (80ºC, 6s) before applying HIPEF (30 kV/cm, 60 μs) and in that way prolonged the shelf life of milk by up to 30 days. Sepulveda et al. (*25*) used a mild thermal treatment at 72ºC for 15 s followed by HIPEF processing at 35 kV/cm for 11.5 μs treatment time, which extended the shelf life of milk beyond 60 days.

Most of the studies on the lethal effect of HIPEF, combined or not with antimicrobial compounds, have focused attention on its immediate lethal activity against specific spoilage or pathogenic microorganisms related to the food media considered. In addition, the joint effect of HIPEF and heat has focused basically on the fate of aerobic-bacteria survivors, whereas quality standards for milk usually consider a wide microbial screening for their acceptance. Thus, the main purpose of this research was to evaluate the shelf life of milk stored under refrigeration conditions after being treated by different combinations of HIPEF, antimicrobials and mild heating. The fate of mesophilic bacteria, enterobacteria, coliforms and the pathogens *Escherichia coli*, *Listeria* sp., *Salmonella* sp., and *Staphylococcus aureus*, was also taken into account.

MATERIALS AND METHODS

Sample preparation

Whole raw milk was obtained from a dairy plant (Puleva, Mollerussa, Lleida, Spain) and stored at 4ºC before processing. The physical properties as well as the initial microbial screening of the whole milk are summarized in Table 1.

Table 1. Physical properties and microbiological screening of whole raw milk

		Value[1]
Physical properties	Fat content	3.5%
	Density	1029 kg/m^3
	pH	6.7±0.02
	Electrical conductivity	5.50±0.02 mS/cm
	Specific heat	3.97±0.02 J/g K
Microbial screening	Mesophilic bacteria	$2.8 \cdot 10^4 \pm 4.0 \cdot 10^3$
	Enterobacteria	$6.0 \cdot 10^2 \pm 8.0 \cdot 10^1$
	Coliforms	$4.9 \cdot 10^1 \pm 1.3 \cdot 10^1$
	Staphylococcus aureus	$1.7 \cdot 10^2 \pm 1.7 \cdot 10^1$
	Escherichia coli	$4.6 \cdot 10^1 \pm 1.2 \cdot 10^1$
	Salmonella spp.	No presence
	Listeria spp.	No presence

[1]Values are the mean±standard deviation of three measurements

Thermal treatment

Raw milk was subjected to conventional pasteurization (75ºC, 16 s) or different mild thermal treatments according to the experimental design (Table 2). A stainless-tubular heat exchanger with 2.16 mm inner diameter and 1,100 cm length was used to treat the milk. Flow rate was maintained at 152 cm^3/min by a gear pump providing a residence time of 16 s for all treatment conditions. Hot water was used as heat transfer fluid to increase the temperature of the raw milk from an initial temperature of 4ºC to exit temperatures of 55, 65 or 75ºC. After heating, the milk was immediately cooled down to 4ºC by a cooling water bath.

HIPEF processing

A continuous-flow HIPEF system was used to carry out this study. The treatment device was an OSU-4F HIPEF unit (Ohio State University, Columbus, Ohio, USA) that discharges square-shape pulses within eight collinear chambers, in which gap distance was 0.29 cm and chamber volume was 0.012 cm^3. Electrical parameters were set at 35 kV/cm electric field strength, 6 μs pulse width, 75 Hz pulse frequency in bipolar mode and 1,200 μs treatment time according to previous studies. Treatment temperature was kept under 25ºC using a cooling water bath to rule out thermal effects.

Table 2. Experimental design of milk subjected to different combinations of non-thermal and/or mild thermal treatments

HIPEF treatment time[1] (μs)	Nisin (IU/mL)	Lysozyme (IU/mL)	Enterocin AS-48 (AU/mL)	Mild thermal treatment[2] (ºC)	Abbreviation
-	1	300	-	-	N1L
1,200	1	300	-	-	HN1L
1,200	1	300	-	55	HN1L+T55
1,200	1	300	-	65	HN1L+T65
1,200	20	-	-		HN20
1,200	20	-	-	55	HN20+T55
1,200	20	-	-	65	HN20+T65
-	20	-	28	-	N20A
1,200	20	-	28	-	HN20A
1,200	20	-	28	55	HN20A+T55
1,200	20	-	28	65	HN20A+T65

[1] HIPEF was set at 35 kV/cm, 6 μs pulse width, 75 Hz frequency and bipolar mode
[2] Mild thermal treatment was prolonged for 16 s at each temperature

Preparation of enterocin AS-48 solution

Enterococcus faecalis A-48-32 (*16*) was used as enterocin AS-48 producer. The bacteriocin solution was obtained by purification of cultured broths of the producer strain after concentration by cation-exhange chromatography in accordance with the method described by Abriouel, Valdivia, Martínez-Bueno, Maqueda and Gálvez (*2*). Bacteriocin concentrates were desalted through 2,000 mw cut-off dialysis tubing (Sigma) and filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. AS-48 activity was determined by the agar well diffusion method (*11*). The enterocin AS-48 preparation resulted in a concentration of 3,500 AU/ml which was 10-fold diluted with demineralized water to obtain the final solution.

Raw milk treated by antimicrobial compounds and their combination with HIPEF and/or mild thermal treatment

The single effect of enterocin AS-48, nisin (N 5764, 1,000,000 IU/mg, Sigma-Aldrich, Steinheim, Germany) and lysozyme (L 2879, 43,560 IU lysozyme/mg solid, Sigma-Aldrich, Steinheim, Germany) on microbial inactivation was evaluated by adding a concentration of 28 AU/ml, 20 IU/ml and 300 IU/ml to whole raw milk, respectively. The combined treatments are summarized in Table 2. The antimicrobial action of combining added nisin with AS-48 and nisin with lysozyme was considered. The simultaneous effect of combining HIPEF or mild thermal treatment with different mixtures of the antimicrobial compounds was studied by applying a HIPEF treatment of 1,200 μs or heating up to 55 or 65ºC for 16 s, respectively. Samples with different combinations of the peptides were also jointly processed by mild heating and HIPEF. Doses of the antimicrobial compounds as well as the choice of each combination were selected in accordance with previous studies.

Microbiological analysis

Counts of mesophilic bacteria, enterobacteria, coliforms and the pathogens *Escherichia coli*, *Listeria* spp., *Salmonella* spp., and *Staphylococcus aureus* were carried out periodically following the spread plate method and guidelines ISO 4833:1991, ISO 7402:1993, ISO 4832:1991, NF V 08-53:1993, ISO 11290-2:1998, ISO 6579:1993, and ISO 6888-1:1999, respectively. Each treatment was performed in triplicate as well as each assay.

The log number of viable cells was expressed following ISO 4833. Microbial inactivation was calculated as –log *s*, where the survival fraction, *s*, was calculated as the quotient of the count in treated samples, *N*, and the initial count prior to any treatment, N_0.

Criteria for shelf life

Microbiological acceptance of raw and treated milk was determined in accordance with the quality standards described in Council Directive 92/46/EEC (*3*) of the European Community (Table 3).

Table 3. Microbiological criteria of Council Directive 92/46/EEC for milk-based products and drinking milk (*3*)

	Raw milk	Pasteurized milk
Mesophilic bacteria	<50,000 UFC/mL	<50,000 UFC/mL
Coliforms	-	<5 UFC/mL
Pathogenic microorganisms:		
Listeria monocytogenes	Absent in 25 g	Absent in 25 g
Salmonella spp.	Absent in 25 g	Absent in 25 g
Staphylococcus aureus	<500 UFC/mL	-
Escherichia coli	-	-

Statistical analysis

Analysis of variance (ANOVA) and Fisher's least significant differences (LSD) procedure were used to discriminate among significantly different ((P<0.05) mean values of each treatment combination. Differences among treatments throughout storage time were analyzed using covariance analysis (ANCOVA). To perform this study, Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MD, USA) was used in all statistical analyses.

RESULTS

Microbial stability of raw milk treated by heat, HIPEF, and/or adding antimicrobial compounds

The population of mesophilic bacteria in raw milk increased rapidly over the acceptance limit of $5.0 \cdot 10^5$ cfu/mL after 2 days under refrigeration conditions (Fig. 1), while counts of coliforms and enterobacteria reached $2.4 \cdot 10^4$ and $7.4 \cdot 10^3$ cfu/mL at that point, respectively. During storage of raw milk, no *Salmonella* or *Listeria* were detected, while the population of *E. coli* remained invariable at a level of $4.6 \cdot 10^1$ cfu/mL, and counts of *S. aureus* at $1.7 \cdot 10^2$ cfu/mL. The single addition of the antimicrobial compounds, nisin, lysozyme and AS-48, and their combinations, N20A and N1L, produced no inactivation in the microbial population of raw milk. Therefore, no difference was found between the fate of survivors in these samples and the untreated milk (P<0.05).

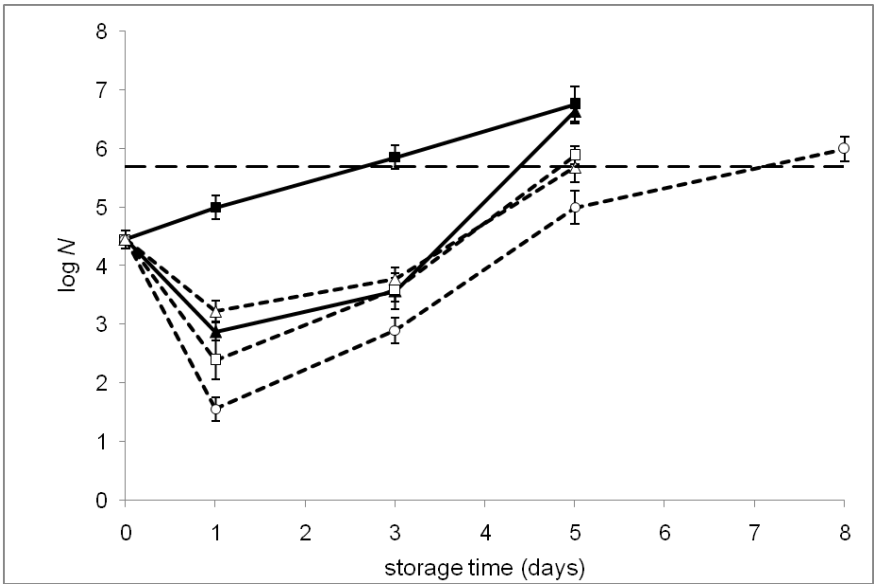

Figure 1. Fate of mesophilic bacteria in milk treated by high-intensity pulsed-electric field (HIPEF) or HIPEF combined with added antimicrobials (HIPEF: 35 kV/cm, 6 µs pulse width, 75 Hz at bipolar mode and 1,200 µs treatment time; *N*, microbial count; - — -, bacterial acceptance limit; —■—, whole raw milk; —▲—, HIPEF; -□-, HIPEF with 20 IU/mL nisin; -○-, HIPEF with 1 IU/mL nisin and 300 IU/mL lysozyme; -△-, HIPEF with 20 IU/mL nisin and 28 AU/mL enterocin AS-48).

The application of HIPEF to raw milk led to over 1.5 log units in microbial inactivation of mesophilic bacteria (Fig. 1), coliforms and enterobacteria and, moreover, no presence of *E. coli* and *S. aureus* was detected. As a result, the shelf life of the HIPEF-treated samples was extended by up to 5 days, which was 3 days longer than that of the untreated samples. With regard to the effect of classic pasteurization, milk thermally processed at 75ºC for 16 s reduced mesophilic counts by more than 2.0 log units the within the first 5 days of storage at 4ºC, while beyond that day a gradual increase of the population was observed (Fig. 3), resulting in a shelf life of 25 days. No growth of coliforms, enterobacteria and the pathogens *E. coli* and *Staph. aureus* was detected in the heated samples.

In general, the addition of antimicrobial compounds to milk before applying HIPEF improved their effect synergistically. A microbial reduction ranging from 1.0 to 2.0 log cycles was observed by applying HN20 and HN20A, while almost 3.0 log units was recorded with HN1L (Fig. 1). This level of inactivation was equal to the destruction seen in pasteurized milk. Despite the initial drop in microbial counts, the number of survivors in HN20/HN20A-processed samples rose dramatically beyond the third day of storage. As a consequence, the shelf life of HN20/HN20A-treated milk did not differ from that of milk treated by HIPEF (5 days), whereas milk treated by HN1L lasted up to 7 days.

Table 4. Log survivors during storage at 4ºC of natural flora of milk submitted to the combination of a thermal treatment at 65ºC for 16 s with the addition of antimicrobial compounds

Microorganism	day	Thermal treatment				
		Antimicrobial compounds[1,2]				
		N20	L300	A28	N1+L300	N20+A28
Mesophilic bacteria	1	NP	NP	NP	NP	NP
	3	1.7±0.2^{a}	3.0±0.3^{b}	3.0±0.2^{b}	2.0±0.3^{a}	2.9±0.2^{b}
	6	4.5±0.2^{a}	4.8±0.2^{ab}	5.0±0.2^{b}	4.8±0.2^{ab}	5.5±0.2^{c}
	10	7.5±0.3^{a}	7.2±0.3^{a}	7.4±0.3^{a}	7.2±0.2^{a}	7.6±0.3^{a}
Enterobacteria	1	NP	NP	NP	NP	NP
	3	NP	NP	NP	NP	NP
	6	0.7±0.2^{a}	1.1±0.2^{b}	1.4±0.2^{b}	1.2±0.2^{b}	0.6±0.2^{a}
	10	2.6±0.3^{a}	1.1±0.2^{b}	1.5±0.2^{c}	1.0±0.2^{b}	0.6±0.2^{d}
Coliforms	1	NP	NP	NP	NP	NP
	3	NP	NP	NP	NP	NP
	6	1.4±0.2^{a}	2.2±0.2^{b}	2.0±0.2^{b}	1.9±0.3^{b}	1.6±0.2^{a}
	10	2.6±0.2^{a}	2.7±0.3^{a}	2.4±0.2^{ab}	2.2±0.2^{ab}	1.4±0.2^{c}

[1] N20, 20 IU/mL nisin; L300, 300 IU/mL lysozyme; A28, 28 AU/mL enterocin AS-48; N1, 1 IU/mL nisin
NP, no presence
[2] Different superscript letters in the same row indicate significant differences (P<0.05)

Survivors in milk treated by combining non-thermal treatments and mild heating at 55ºC

A mild thermal treatment at 55ºC prolonged the shelf life of milk by up to 3 days (Fig. 2) owing to an initial decrease in coliform and enterobacteria population, although mesophilic bacteria and coliforms both regained their ability to grow. The addition of antimicrobial substances before mild heating at 55ºC did not result in any advantage in reducing the microbial counts in milk or in extending its shelf life in comparison with mild thermal treatment. In contrast, the combination of heat at 55ºC with HIPEF produced 2.0 log reductions in mesophilic bacteria (Fig. 2). Moreover, the addition of antimicrobials to milk before processing by heat at 55ºC and HIPEF resulted in higher microbial destruction than in milk treated by combining only mild thermal treatment and HIPEF. Hence, the joint effect of thermally processing milk at 55ºC with HIPEF, HN20, HN20A or HN1L acted synergistically on microbial death. A maximum of over 4.0 log units reduction in mesophilic bacteria was observed in the case of heating at 55ºC and HN20. This reduction was approximately 1.5 log cycles more than when only heating at 55ºC and processing by HIPEF. Interestingly, the number of cells of coliforms and enterobacteria fell below the detection limit for the first 4 days of refrigerated

storage when milk was heated at 55ºC and processed with HIPEF, HN20, HN20A, and HN1L (Fig. 2). Beyond that point, the number of survivors of coliforms and enterobacteria increased rapidly. The shelf life of milk combining a mild thermal treatment at 55ºC with HIPEF, HN20, HN20A and HN1L was 6, 8, 6 and 8 days, respectively. No growth of the tested pathogens was observed in these treated samples.

Survivors in milk treated by combining non-thermal treatments and a mild heating at 65ºC

Milk heated at 65ºC for 16 s lasted up to 16 days (Fig. 3). Almost 2 log cycles reduction in counts of mesophilic bacteria were obtained after heating, although the microbial population rose steadily throughout refrigerated storage.

The addition of nisin, lysozyme, AS-48, or their combinations (N20A, N1L), to raw milk, which was then processed by heat at 65ºC, resulted in a general shelf life of approximately 7 days (Table 4). Mesophilic bacteria in these processed samples remained below detection limit after 1 day of storage at 4ºC. The same pattern was also followed by coliforms and enterobacteria, but only up to 3 days.

The combination of HIPEF, HN20, HN1L and HN20A with heating at 65ºC produced null counts of all microbial screenings for 4, 19, 7, and 19 days of refrigeration, respectively (Fig. 3). Beyond that point, the microbial population suddenly reached the limit of acceptance criteria, resulting in a shelf life of 10, 26, 17 and 26 days for the treatments HIPEF, HN20, HN1L and HN20A combined with thermal treatment at 65ºC, respectively. No significant differences (P<0.05) were found between survivors in milk treated by HN20 and HN20A and heated at 65ºC. Moreover, no coliforms, enterobacteria, and pathogens were registered during storage.

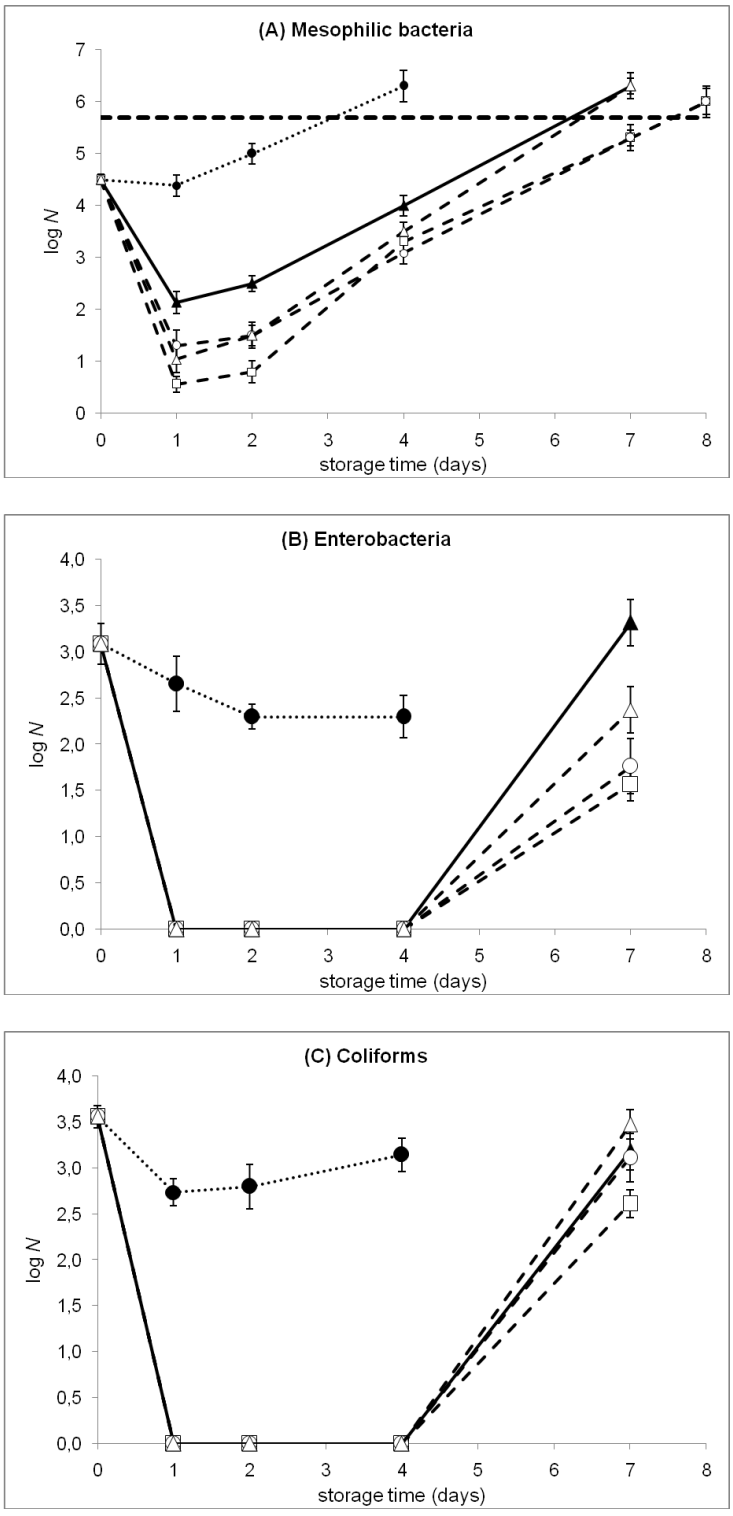

Figure 2. Fate of survivors in milk treated by combining a mild thermal treatment (T55) with added antimicrobials and high-intensity pulsed-electric field (HIPEF) (HIPEF: 35 kV/cm, 6 µs pulse width, 75 Hz at bipolar mode and 1,200 µs treatment time; A, mesophilic bacteria; B, enterobacteria; C, coliforms; *N*, microbial count; – — –, bacterial acceptance limit; T55, heat at 55ºC for 16 s; *N*, microbial count; – — –, microbial acceptance limit; ···●···, T55; —▲—, HIPEF and T55; – -□- –, HIPEF with 20 IU/mL nisin and T55; – -○- –, HIPEF with 1 IU/mL nisin, 300 IU/mL lysozyme and T55; – -△- –, HIPEF with 20 IU/mL nisin, 28 AU/mL enterocin AS-48 and T55).

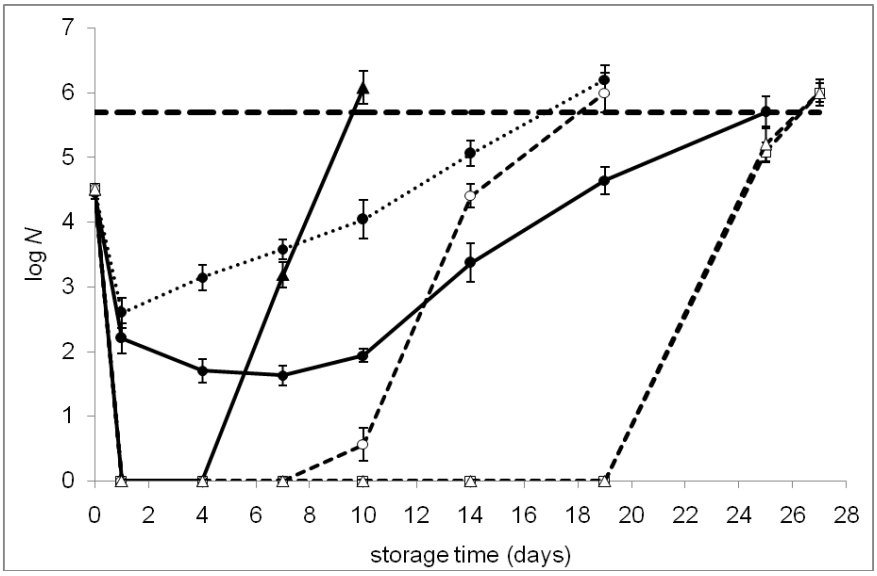

Figure 3. Fate of mesophilic bacteria in milk treated by thermal pasteurization (T75) and by a combination of antimicrobials, a mild thermal treatment (T65) and high-intensity pulsed-electric field (HIPEF) (HIPEF: 35 kV/cm, 6 µs pulse width, 75 Hz at bipolar mode and 1,200 µs treatment time; T75, heat at 75ºC for 16 s; T65, heat at 65ºC for 16 s; *N*, microbial count; - — -, bacterial acceptance limit; —●—, T75; ···●··, T65; —▲—, HIPEF and T65; -□-, HIPEF with 20 IU/mL nisin and T65; -○-, HIPEF with 1 IU/mL nisin, 300 IU/mL lysozyme and T65; -△-, HIPEF with 20 IU/mL nisin, 28 AU/mL enterocin AS-48 and T65).

DISCUSSION

No microbial death was observed when nisin, lysozyme, AS-48 or the proposed combinations of the antimicrobial substances were added to raw milk. These data are in accordance with other studies that found no bacterial inactivation within the studied range of concentrations, which are beneath minimal bactericidal concentrations (*19*, *27*, *28*, *30*).

Milk is traditionally processed by heat to assure its microbial safety. Shelf life of milk pasteurized at 75ºC for 16 s was sustained for 25 days. Although some authors state that commercially high-temperature short-time (HTST) pasteurized milk usually lasts between 2 and 3 weeks before spoiling (*22*), others claim that the shelf life of HTST-pasteurized milk can be lengthened to between 30 and 90 days (*5*, *25*). In contrast to pasteurized milk, the shelf life of HIPEF-treated milk stored under refrigeration conditions was 5 days. This result agrees with the storage time recorded by Odriozola-Serrano et al. (*20*) when raw milk was treated by similar HIPEF conditions (35.5 kV/cm and 1,000 µs treatment time) before refrigeration. In contrast, Fernandez-Molina et al. (*8*) concluded that raw skim milk processed at 40 kV/cm for 60 µs HIPEF treatment time had a shelf life greater than 14 days. That result may be explained by the high bacterial limit of 7 log units established in their study, the low initial degree of bacterial contamination, and sample handling. Likewise, Sepulveda et al. (*26*) showed that HIPEF-treated (35 kV/cm, 5 exponential decaying pulses of 2.3 µs width) milk lasted 24

days, although temperature reached by the milk during HIPEF processing (65ºC) may suggest that milk was also treated thermally.

Coliforms and enterobacteria were completely inactivated during storage by heat pasteurization, whereas, when HIPEF was applied to milk, their numbers recovered after day 1. Therefore, final spoilage of pasteurized milk was due to growth of psychrotrophic bacteria during storage and, conversely, growth of coliforms and enterobacteria in HIPEF-processed milk accounted for the main fraction of the increase in total mesophilic counts. This may reveal the difference in the nature of the mechanism of cell death caused by each preservation method. On the one hand, heat irreversibly destabilizes the cell envelope by inducing phase transition to a rigid semicrystalline gel state (*31*). On the other hand, HIPEF affects the integrity of the cell envelope by i) provoking multiple structural changes leading to permeabilization (*33*) and breakdown with leak-out of intracellular content (*7*), or ii) causing transient injuries to the cell envelope and reversible permeabilization (*12*, *24*). The latter case may explain the short lag time needed by microorganisms to recover from sub-lethal injuries inflicted by HIPEF.

The combined action of HIPEF with added nisin or nisin with lysozyme acted synergistically in decreasing microbial counts in raw milk (Fig. 1). The addition of nisin and AS-48 to milk prior to applying HIPEF reduced the mesophilic population initially but only a bacteriostatic effect was shown against enterobacteria during storage. In spite of this, all the sets of combinations of HIPEF with antimicrobial substances reached maximal microbial tolerance after 4 days storage, except the HNL combination, which prolonged shelf life up to 7 days, 3 days more than that of the HIPEF-treated samples. The variable degrees of resistance and multiple microorganisms in the natural flora of milk may explain the lower lethality observed in comparison with studies on the simultaneous action of HIPEF and antimicrobial substances against one or several inoculated microorganisms, such as those of Sobrino et al. (*29*, *30*) and Martínez-Viedma et al. (*17*). Since the lethal effect of HIPEF and the combinations of HIPEF and antimicrobials assayed was basically studied immediately after the processing of the sample, little is known about the pattern followed by survivors during storage.

To improve control of the microbial population during storage, milk treated with non-thermal methods was also processed by mild heating. As depicted in Figure 2, heating at 55ºC offered no further advantage for the microbial stability of milk. However, heating milk under the same conditions affected cell viability synergistically when HIPEF, HN, HNL and HNA were also used as hurdles. This synergism was exerted via i) decreasing the mesophilic counts beneath the sum of microbial inactivation recorded when applying those treatments alone, and ii) producing nil counts of enterobacteria and coliform cells during storage. Therefore, the combination of heat, HIPEF and the proposed antimicrobial agents was more effective in maintaining the number of mesophilic, enterobacteria and coliform cells as compared to any of the treatments alone. The same pattern of microbial counts was seen when the temperature of the mild thermal treatment was increased to 65ºC. However, microbial inactivation at 65ºC was clearly higher than that recorded when combining non-thermal treatments and heating at 55ºC, and storage time was also lengthened even beyond the shelf life of pasteurized milk (Fig. 3). A similar trend in the fate of survivors was noted in other studies carried out on milk subjected to heat and HIPEF simultaneously, but no reference was found to the combination of heat, HIPEF and the antimicrobial compounds used in the present research. Fernández-Molina et al. (*8*, *9*), Sampedro et al. (*23*), and Sepulveda et al. (*26*) observed that increasing the milk-media temperature when treating by HIPEF resulted in higher inactivation of

viable cells. Moreover, Sampedro et al. (*23*) concluded that the same degree of inactivation could be achieved by reducing the energy consumed by HIPEF while the temperature of the milk-media rises.

In the present experiment, the temperature selected for mild thermal treatment and storage time seemed to affect the later bactericidal or bacteriostatic activity of the antimicrobial compounds added. Among the different combinations proposed, the single addition of nisin to milk treated by HIPEF and heated at 65ºC achieved the best values of shelf life and microbial inactivation. From a hurdle perspective, the sequence of application of each treatment may explain the loss of microbial resistance. Firstly, the addition of antimicrobial compounds at sub-lethal doses to milk before heating may lower the thermal resistance of microorganisms. During heating, the temperature and consequent changes in the cell envelope may facilitate bonding and the activity of the antimicrobial compounds. Secondly, injured membranes may diminish their electrical transmembrane potential, thus facilitating the later electroporation by HIPEF. And, thirdly, the presence of the antimicrobial compounds used in this study, among the main features of which is thermoresistance (*1*, *19*, *21*), may impede a later recovery of damaged cells during storage. After their activity has ceased, the repair of the cell envelope and the resumption of metabolic activity may allow growth until the end of shelf life of the treated samples. However, the mix of AS-48 with nisin, HIPEF and heat seemed to impede or accelerate the mutual lethal activity of both bacteriocins depending on the temperature set during thermal processing and, in this way, a shorter lag phase and shelf life were noted than for treated samples treated without AS-48.

Thermization of milk is defined as heating of milk between 57 and 68ºC for 15 s (*3*). The milk used in the manufacture of milk-based products, such as cheese, has habitually undergone this mentioned initial heat treatment, although thermization of fermented dairy products should not be rejected for improving their longevity, such as in yoghourt. Thermization of milk is not full pasteurization and it can only be stored at 7ºC for up to 3 days (*13*). In our study, better microbial levels than in thermized milk were achieved when milk was processed by thermal treatment at 55 or 65ºC, with HIPEF and any mix of the antimicrobial substances. In particular, enterobacteria and coliforms were not detected for the first 4 days of storage in samples treated with the combinations of HIPEF, antimicrobials and mild heating at 55ºC. Moreover, the addition of nisin to milk heated at 65ºC and HIPEF led to a longer shelf life than that of pasteurized milk, and microbial contamination during storage time was non-detectable.

CONCLUSIONS

The results obtained in this research suggest that the combined treatment of HIPEF and added antimicrobial substances (nisin and lysozyme) resulted in longer shelf life and better microbial quality during storage than for raw milk and HIPEF-treated milk. When raw milk with added antimicrobial agents was processed by a mild thermal treatment and HIPEF, the effectiveness of the treatment in inactivating natural flora was synergistic. As a result, no enterobacteria and coliforms were detected for 3 days of storage in samples with added antimicrobials treated by heat at 55ºC; for 4 days when heat at 55ºC was combined with HIPEF and antimicrobials; and throughout the whole of the storage time when HIPEF-treated milk with antimicrobials was heated at 65ºC. Therefore, milk with added antimicrobials processed by heat at 55ºC and HIPEF may comply with microbiological criteria of milk-based products and even improve their microbial acceptance. The shelf life and microbial screening

of milk with added nisin and enterocin AS-48, heated at 65ºC and processed by HIPEF was comparable to and offered better microbial stability than that of pasteurized milk. In addition, the combined treatments tested in this study were shown to inactivate pathogenic bacteria, such as *E. coli* and *S. aureus*. Thus, hurdle technology may enable the intensity of each preservation method to be diminished, especially thermal processing in this case, while resulting in better organoleptic properties, microbiological safety and longer-lasting products. The use of non-thermal methods such as HIPEF and added antimicrobial substances with a mild thermal treatment offers an excellent way to increase the marketability and quality of a great variety of dairy products.

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DISCUSIÓN GENERAL

Tradicionalmente, los tratamientos térmicos se han empleado en la conservación de alimentos para lograr tanto la estabilidad microbiológica como la inactivación enzimática. Sin embargo, el empleo de nuevos tratamientos no térmicos de procesado de alimentos, como la aplicación de pulsos eléctricos de alta intensidad de campo (PEAIC), podría consolidarse como una de las alternativas a los métodos clásicos de conservación de alimentos líquidos. Su interés radica, principalmente, en su capacidad para no alterar o minimizar los cambios de las características organolépticas del producto tratado en relación al producto fresco y, al mismo tiempo, reducir las pérdidas de componentes nutritivos.

El efecto de los PEAIC se ha estudiado principalmente en zumos, y existen relativamente pocos estudios sobre su efectividad sobre microorganismos patógenos en el procesado de un alimento complejo como la leche. Por este motivo, y en primer lugar, se estudió la influencia de las distintas variables implicadas, tanto del medio como del procesado por PEAIC, en la inactivación de *Staphylococcus aureus* inoculado en leche. A continuación, se evaluó la letalidad de los PEAIC sobre *Staph. aureus* en combinación con la adición individual o conjunta de distintos antimicrobianos de origen natural: lisozima, nisina y enterocina AS-48. Finalmente, se almacenó, en condiciones de refrigeración, leche procesada mediante PEAIC y distintas combinaciones de los antimicrobianos citados para determinar su estabilidad microbiológica y compararla con la conseguida mediante tratamientos térmicos convencionales.

EFECTO DEL PROCESADO CON PEAIC SOBRE *Staphylococcus aureus* INOCULADO EN LECHE

Staph. aureus es un microorganismo patógeno habitual en la flora natural de la leche. Y, aunque algunos estudios han comprobado el efecto inactivador de los PEAIC en esta bacteria (Evrendilek et al., 2004; Raso et al., 1999) y otros patógenos (Bendicho et al., 2002; Élez-Martínez et al., 2004), la mayoría se han llevado a cabo en medios diluidos, como zumos y soluciones modelo y tamponadas. El número de variables de proceso y del propio medio implicadas dificultan la extrapolación de los resultados observados en medios diluidos a un medio complejo como la leche.

Con el fin de optimizar el efecto letal de los PEAIC sobre *Staph. aureus* en leche, se planteó un diseño experimental basado en superficies de respuesta cuyas variables independientes fueron la intensidad de campo eléctrico (25-35 kV/cm), el número (50-150), anchura (4-8 μs) y polaridad de pulso y el contenido en grasa de la leche (0-3%). La combinación de 35 kV/cm, 150 pulsos de 8 μs en modo bipolar registró el valor máximo de inactivación de 4.5 reducciones logarítmicas en leche desnatada. Sin embargo, no se observó variación en la fracción de supervivientes o fue mínima, apenas 0.2 reducciones logarítmicas, cuando la leche desnatada se procesó a 25 kV/cm, con 50 pulsos de 4 μs en modo monopolar y bipolar, respectivamente. Estos datos se modelizaron satisfactoriamente utilizando un polinomio multivariante de segundo orden, en el que la falta de ajuste no fue significativa y el coeficiente de determinación, R^2, de 0.88. El modelo, en el caso de pulsos monopolares, correspondió al de la ecuación (2)

$$-\log s = -7.607 + 0.144 \cdot E - 0.020 \cdot n + 1.337 \cdot \tau - 0.112 \cdot \tau^2 + 7.02 \cdot 10^{-4} \cdot E \cdot n + \\ + 1.693 \cdot 10^{-3} \cdot n \cdot \tau \qquad (2)$$

y, en el caso de pulsos bipolares, al de la ecuación (3)

$$-\log s = -6.971 + 0.144 \cdot E - 0.020 \cdot n + 1.337 \cdot \tau - 0.112 \cdot \tau^2 + 7.02 \cdot 10^{-4} \cdot E \cdot n + \\ + 1.693 \cdot 10^{-3} \cdot n \cdot \tau \quad (3)$$

en las que *s* representa la fracción de supervivientes, *E* la intensidad de campo eléctrico (kV/cm), *n* el número de pulsos y τ la anchura de pulso (μs).

De las variables estudiadas, el porcentaje de grasa en la leche no afectó a la destrucción final alcanzada. Tal tendencia ha sido también observada por autores como Reina et al. (1998) al comprobar que el número de células viables de Listeria monocytogenes en leche tras el tratamiento fue independiente de si se empleaba leche desnatada o entera. Otros, sin embargo, hallaron una correlación positiva entre la letalidad del tratamiento de PEAIC y el contenido en grasa de la leche (Grahl et al., 1996). Este comportamiento se ha atribuido a la disminución de la conductividad eléctrica del medio al aumentar el número de glóbulos grasos (Rivas et al., 2006; Zhang et al. 1995). Si bien, la influencia de la cantidad de grasa en la leche ha sido todavía muy poco estudiada y variables como el pH, la temperatura, la cantidad y tipo de sales e, incluso, la propia sensibilidad del microorganismo en estudio podrían modificar la respuesta final conseguida.

En general, el empleo de pulsos bipolares, el aumento de la intensidad de campo o del número de pulsos condujo a un incremento de la inactivación microbiana. De este modo, manteniendo constante la anchura en 8 μs y el número de pulsos bipolares en 150, una intensidad de campo de 25 kV/cm provocó una disminución de la población de *Staph. aureus* de 1.8 ciclos logarítmicos, mientras que a 35 kV/cm fue de 4.3 ciclos. Por el contrario, incrementando el número de pulsos de 50 a 150 la destrucción aumentó de 2.5 a 4.3 reducciones logarítmicas, respectivamente, cuando la intensidad de campo fue de 35 kV/cm y la anchura de pulso de 8 μs en modo bipolar. Este comportamiento coincide con el observado por otros autores en el rango de los valores de las variables establecido (Evrendilek et al., 2004) e, incluso, cuando los medios y/o microorganismos de referencia fueron diferentes (Élez-Martínez et al., 2005; Evrendilek et al., 2005; Michalac et al., 2003).

Sin embargo, se observó que, aplicando pulsos más anchos, la mortalidad no era necesariamente superior, obteniéndose un punto máximo para esta variable en función del número de pulsos y la intensidad de campo. Derivando cualquiera de las dos ecuaciones, (2) o (3), se consigue la relación entre la anchura (τ) y número de pulsos (*n*) que optimiza la inactivación microbiana a una intensidad de 35 kV/cm [Ecuación (4)]

$$\tau = 7.56 \cdot 10^{-3} \cdot n + 5.97 \quad (4)$$

De la expresión (4) se deduce que la anchura óptima osciló entre 6.3 y 7.1 μs en el rango de 50 a 150 pulsos, respectivamente.

Teniendo en cuenta que el producto de la anchura (τ) por el número de pulsos (n) corresponde a la variable tiempo de tratamiento (t). Se observó que a tiempos de tratamiento más largos ($t = \tau \cdot n$) el número de supervivientes descendía. Mientras se consiguieron sólo 2.2 reducciones logarítmicas aplicando 200 µs de tiempo de tratamiento (50 pulsos de 4 µs), se registraron hasta 4.3 reducciones si el tiempo fue de 1,200 µs (150 pulsos de 8 µs) siendo la intensidad de 35 kV/cm, en ambos casos. Este comportamiento ha sido también señalado por otros autores como Evrendilek et al. (2004) y Raso et al. (1999) utilizando también leche inoculada con *Staph. aureus*. De un modo similar, la interacción positiva entre la anchura y la intensidad de tratamiento (E) indica que un incremento simultáneo de las dos variables da lugar a un mayor grado de inactivación. Existen pocas referencias en las que se refleje la interacción entre la anchura y el número de pulsos o entre la anchura de pulso y la intensidad de campo, ya que la limitación técnica de los equipos empleados y/o el mismo diseño experimental dificultaba establecer diversas anchuras de pulso.

El modelo planteado y, en particular, el análisis de las interacciones, proporciona herramientas para la optimización del proceso de PEAIC en función del objetivo marcado. Por un lado, es posible alcanzar el mismo grado de muerte microbiana mediante distintas combinaciones del par formado por la anchura y el número de pulsos o por el par anchura e intensidad de campo. Así, se puede alcanzar un valor de inactivación de 3.5 ciclos logarítmicos aplicando 150 pulsos de 4.2 µs o 100 pulsos de 7.6 µs con una intensidad de 35 kV/cm. O bien, el nivel de destrucción anterior se podría conseguir también si se combinan 110 pulsos a 35 kV/cm o 150 pulsos a 32 kV/cm de 8 µs en ambos casos. Además, a igual tiempo de tratamiento, las combinaciones de series largas de pulsos cortos resultaron más efectivas en la destrucción de *Staph. aureus* que las combinaciones de pocos pulsos anchos. En este sentido, sería posible optimizar la energía consumida en función de la intensidad de campo, del tiempo de tratamiento, del número de pulsos o de la anchura de pulso para un valor preestablecido de inactivación microbiana.

EFECTO INDIVIDUAL Y COMBINADO DE LA ADICIÓN DE AGENTES ANTIMICROBIANOS SOBRE *Staphylococcus aureus* INOCULADO EN LECHE

Tras estudiar el efecto de las distintas variables de proceso sobre la inactivación de *Staph. aureus* en leche, se procedió al estudio del efecto individual y combinado de la adición de agentes antimicrobianos sobre el mencionado microorganismo. Se seleccionaron un antimicrobiano presente de forma natural en leche, lisozima, y dos bacteriocinas, nisina y enterocina AS-48. Tras observar el efecto individual de cada uno de los antimicrobianos, se estudió la actividad de las combinaciones siguientes: nisina con lisozima, enterocina AS-48 con lisozima y, finalmente, enterocina AS-48 con nisina.

Efecto de la adición de nisina, lisozima o enterocina AS-48

El efecto letal de cada uno de los antimicrobianos utilizados se evaluó añadiendo distintas concentraciones a leche inoculada con *Staph. aureus* a su pH natural o ajustándolo a 5.0 ó 5.9. Además, en el caso particular de la enterocina AS-48 se tuvo en cuenta el almacenamiento a

temperatura controlada de 4 y 22ºC. Las concentraciones máximas empleadas de nisina, lisozima y enterocina AS-48 fueron 150 IU/mL, 5,000 IU/mL y 28 AU/mL, respectivamente.

En general, no se observó efecto bactericida de los antimicrobianos a las dosis, tiempo de exposición, pH y temperaturas de almacenamiento planteadas. Branen et al. (2004) y Smith et al. (2002) comprobaron que eran necesarias concentraciones superiores a 5,000 IU/mL de lisozima para detectar muerte celular en leche, aunque la dosis letal parece depender de la cepa y condiciones de crecimiento de *Staph. aureus* (Clarke et al., 1992). De modo similar, Muñoz et al. (2007) necesitaron añadir una concentración hasta 6 veces superior de enterocina AS-48 a la utilizada en este estudio para inhibir el crecimiento de *Staph. aureus* en leche. No obstante, se detectó que la adición de nisina a una concentración de 150 IU/mL redujo la población de *Staph. aureus* 1.1 ciclos logarítmicos tras 90 min de exposición en leche a pH 5.0. Si bien, tiempos de exposición inferiores a 30 min en las condiciones anteriores y concentraciones inferiores a 150 IU/mL y/o un pH superior a 5.0 no causó inhibición alguna en este microorganismo. La ausencia de muerte microbiana observada en muestras de leche a su pH natural coincide con la dependencia de la actividad de esta bacteriocina con el pH del medio, ya que su solubilidad aumenta conforme disminuye la acidez (Hurst, 1981). Arques et al. (2004) contabilizaron sólo 0.23 reducciones logarítmicas al añadir 100 IU/mL de nisina a leche inoculada con *Staph. aureus*.

Efecto de la adición combinada de antimicrobianos

Para estudiar el efecto combinado de la adición de nisina y lisozima se propuso un diseño experimental de superficie de respuesta en el que la concentración de ambos compuestos y el pH de la leche fueron las variables independientes. Combinando 5 IU/mL de nisina y 300 IU/mL de lisozima se alcanzó la mayor disminución de la población de *Staph. aureus*, 1.6 ciclos logarítmicos, en leche a su pH natural. Por el contrario, se observó un máximo de sólo 0.7 reducciones logarítmicas con 5 IU/mL de nisina y 3,000 IU/mL de lisozima a pH 5.0. Los datos conseguidos se ajustaron mediante un modelo de segundo orden, cuyo coeficiente de determinación, R^2, fue de 0.995 y la falta de ajuste no significativa [Ecuación (5)]

$$-\log s = 7.2 - 0.45 \cdot n + 5.4 \cdot 10^{-4} \cdot l - 2.7 \cdot p + 0.039 \cdot n^2 + 5.1 \cdot 10^{-8} \cdot l^2 + \\ + 0.25 \cdot p^2 + 0.064 \cdot n \cdot p - 1.2 \cdot 10^{-4} \cdot l \cdot p \qquad (5)$$

y en el que –log *s* representa el número de reducciones logarítmicas de la población, *n* la concentración de nisina (IU/mL), *l* la concentración de lisozima (IU/mL) y *p* el pH.

Aunque las dosis máximas de ambos compuestos establecidas en este estudio fueran dosis subletales, su acción conjunta fue claramente sinérgica en la disminución de la población. La mortalidad estuvo fuertemente determinada por el pH de la muestra. El número de células viables de *Staph. aureus* fue muy superior cuando se ajustó el pH de la leche a 5.0 que a 6.8, el pH natural de la leche. Por otro lado, la pérdida de viabilidad aumentó a mayor concentración de ambos antimicrobianos a pH ácido, mientras que, al pH natural de la leche, concentraciones moderadas de

lisozima y concentraciones altas de nisina mejoraron la letalidad del tratamiento. Este comportamiento no ha sido descrito con anterioridad en leche, aunque Chung et al. (2000) observaron una tendencia similar empleando mayores concentraciones de ambos antimicrobianos en medios diferentes.

Aunque *Staph. aureus* fue resistente a la combinación de lisozima y enterocina AS-48, la adición simultánea de enterocina AS-48 (28 AU/mL) y nisina (20 IU/mL) actuó sinérgicamente en función del tiempo de exposición, pH de la leche y temperatura de almacenamiento de las muestras. Un pH de la leche de 5.0 mitigó el efecto letal de ambas bacteriocinas cuando su actividad individual es óptima a pH ácidos. La temperatura de almacenamiento, además, condicionó la velocidad de destrucción microbiana, ralentizándola en refrigeración y acelerándola a 22ºC. De este modo, la población de *Staph. aureus* en leche a su pH natural a 22ºC decreció hasta 1.8 ciclos logarítmicos tras 1 h de exposición, a las 24 h, en esas condiciones de temperatura, prácticamente no se detectó crecimiento microbiano (0.7 ciclos logarítmicos) y la población creció rápidamente tras 48 h. A 4ºC, el número de supervivientes decreció paulatinamente hasta las 48 h en leche a su pH natural, alcanzando casi 4 reducciones logarítmicas.

El mecanismo de acción del sinergismo observado entre las distintas combinaciones de antimicrobianos, nisina y lisozima o nisina y enterocina AS-48, no se conoce con exactitud. Tampoco se han descrito las causas exactas por las que al acidificar la leche hasta un pH de 5.0 se reduce notablemente el efecto sinérgico en cualquiera de las dos combinaciones planteadas, mientras que, contrariamente a lo que cabría esperar, los mejores resultados de inactivación se observan al pH natural de la leche. Sin embargo, las múltiples alteraciones a nivel de membrana provocadas por cualquiera de las dos bacteriocinas, nisina o enterocina AS-48, y la actividad murasidásica de la lisozima, según el caso, podrían verse afectadas por oligomerizaciones de las moléculas y/o cambios de la carga eléctrica de la membrana cuando disminuye el pH del medio (Abriouel et al., 2001).

EFECTO COMBINADO DE PEAIC Y ADICIÓN DE ANTIMICROBIANOS

Seguidamente, y teniendo en cuenta los resultados obtenidos, se procesó leche mediante PEAIC con agentes antimicrobianos, añadidos individualmente (nisina, lisozima o enterocina AS-48) o combinados entre sí (nisina con lisozima, enterocina AS-48 con nisina o enterocina AS-48 con lisozima). Se plantearon diseños de superficie de respuesta en los que se determinaron distintos niveles de concentraciones subletales para los distintos antimicrobianos, pH del medio y tiempo de tratamiento con PEAIC. Teniendo en cuenta los resultados previos, la aplicación de PEAIC se efectuó utilizando leche desnatada y manteniendo una intensidad de campo de 35 kV/cm.

Efecto combinado de PEAIC y adición de nisina

Para estudiar el efecto combinado de la adición de nisina a leche procesada mediante PEAIC se planteó un diseño de superficie de respuesta en el que la concentración de la bacteriocina (20-150 IU/mL), el pH (5.0-6.8) y el tiempo de tratamiento (240-2400 µs) fueron las variables controladas. El modelo de segundo orden que mejor satisfizo los datos, con un coeficiente de determinación, R^2, de 0.99 y una falta de ajuste no significativa, fue el que se muestra en la ecuación (6)

$$-\log s = \quad 0.000210n^2 - 1.11p^2 + 0.0365n + 12.4p - 0.00302t - \\ - 0.0000125n \times t + 0.000872p \times t - 0.00902n \times p - 32.9 \qquad (6)$$

y en el que –log *s* representa el número de reducciones logarítmicas de la población, *n* la concentración de nisina (IU/mL), *p* el pH y *t* el tiempo de tratamiento (µs).

La aplicación simultánea de ambos tratamientos actuó de modo sinérgico en la destrucción de *Staph. aureus*, siendo el pH de la leche determinante en el comportamiento de las variables y en el número de supervivientes resultante. En general, a menor valor de pH se observó un mayor efecto protector sobre la bacteria inoculada, al mismo tiempo que la diferencia entre los valores máximo y mínimo de inactivación a un determinado pH fue menor conforme disminuyó el pH de la leche. Además, esta última variable influyó en el efecto final de la concentración de nisina y del tiempo de tratamiento. Por un lado, el mayor nivel de inactivación se alcanzó a tiempos elevados de tratamiento y dosis bajas de nisina cuando se procesó leche a su pH natural y, por el contrario, tiempos de tratamientos breves y concentraciones altas de nisina fueron más efectivos si el pH fue ácido. El mayor grado de inactivación, 6.0 ciclos logarítmicos, se registró cuando leche con 20 IU/mL a su pH natural se procesó durante 2,400 µs de tratamiento. Este último valor supuso 4 reducciones logarítmicas más que las obtenidas aplicando cada método de conservación por separado. Por otro lado, dado un nivel de destrucción y cantidad de nisina constantes, es posible encontrar un par de valores óptimos de las variables pH y tiempo de tratamiento. Para una concentración de 20 IU/mL de nisina y un nivel de 5.0 reducciones logarítmicas, el tiempo de tratamiento mínimo para lograr ese grado de destrucción fue de aproximadamente 1,900 µs a un pH de 6.2.

El efecto sinérgico entre el procesado con PEAIC y la adición de nisina ha sido resaltado por otros autores, aunque en sus estudios se han utilizado otros medios, microorganismos de referencia y, además, distintas condiciones de procesado. Smith et al. (2002) contabilizaron 4.4 reducciones logarítmicas en la flora natural de leche con nisina cuando aplicaron 100 µs de 82 kV/cm. Y, Caldrón-Miranda et al. (1999), observaron una disminución de 3.6 ciclos logarítmicos si se añadían 100 ppm de nisina a leche tratada a 50 kV/cm durante 32µs con PEAIC.

El mecanismo de acción conjunto de ambos tratamientos no se ha definido exactamente aunque existen diferentes teorías. Brevemente, el campo eléctrico generado durante el tratamiento de PEAIC induce desde múltiples alteraciones de la membrana celular (Élez-Martínez et al., 2004 y 2005) hasta la formación de poros cuyo carácter puede ser reversible o irreversible (Tsong et al., 1990). La nisina, en cambio, parece adherirse a la membrana, en la que no se han identificado receptores específicos, para después formar poros (Abee, 1995). De este modo, la presencia de nisina a concentraciones subletales podría sensibilizar la membrana de la célula frente a PEAIC (Ho et al., 1995) o viceversa (Pol et al., 2000), para formar un número mayor de poros, de mayor dimensión o aumentar el número de poros permanentes. Sin embargo, esta hipótesis no explica el mejor rendimiento de la aplicación de PEAIC y nisina al pH natural de la leche (6.8). Partiendo de la diferencia de solubilidad y, en consecuencia, de la variación de actividad de la molécula de nisina frente al pH, la formación de

poros y la secreción de contenido intracelular debido a la acción de PEAIC podría causar una acidificación local alrededor del poro que favorecería la solubilización de la molécula de nisina (Aronsson et al., 2001). Además, las alteraciones causadas en la membrana podrían aumentar el número de puntos para la adhesión de la bacteriocina (Therebiznik et al., 2002). Aún así, la diferencia entre la inactivación lograda al pH natural de la leche (con dosis bajas de nisina y tiempo de tratamiento largo) y la inactivación a pH ácido (con dosis altas de la bacteriocina y tiempo de tratamiento breve) sugiere también que el campo eléctrico podría alterar la actividad y efectividad de la nisina.

Efecto combinado de PEAIC y adición de enterocina AS-48

El efecto de la combinación de la adición de enterocina AS-48 y aplicación de PEAIC se estudió mediante un diseño de superficie de respuesta en el que las variables independientes fueron la concentración de enterocina AS-48 (3.5-28 AU/mL), el tiempo de tratamiento (120-1,200 µs) y el pH de la leche (5.0-6.8). La inactivación microbiana, expresada como –log *s*, se ajustó a un modelo de segundo orden, con un coeficiente de determinación, R^2, de 0.94 y una falta de ajuste no significativa, mediante la ecuación (7)

$$-\log s = -21.1 - 0.0635 \cdot e - 0.00266 \cdot t + 8.04 \cdot p + 0.00346 \cdot e^2 - 2.22 \cdot 10^{-6} \cdot t^2 - \\ - 0.722 \cdot p^2 + 0.00119 \cdot t \cdot p \qquad (7)$$

en la que *e* representa la concentración de enterocina AS-48 (AU/mL), *t* el tiempo de tratamiento (µs) y *p* el pH de la leche.

La combinación de PEAIC y enterocina AS-48 fue sinérgica en la inactivación de *Staph. aureus*. El incremento de la concentración de enterocina AS-48 y/o del tiempo de tratamiento dio lugar a un descenso de la población microbiana cuando la leche se procesó a su pH natural. Con la aplicación de 1,200 µs y con la adición de 28 AU/mL de enterocina AS-48 a leche a su pH natural se consiguieron hasta 4.5 reducciones logarítmicas. La población superviviente se mantuvo constante en muestras tratadas en las condiciones anteriores y almacenadas a temperatura de refrigeración durante las 48 h siguientes, mientras que, si permanecían a 22ºC, la población recuperó su concentración inicial tras ese mismo periodo de almacenamiento. Sin embargo, no se observó más inactivación combinando PEAIC y enterocina AS-48 en aquellos ensayos efectuados a pH 5.0 en comparación con ensayos en los que se procesó leche a ese mismo pH únicamente mediante PEAIC.

Aunque la acción conjunta entre esta bacteriocina y PEAIC no se ha estudiado con anterioridad en leche, se ha confirmado el sinergismo cuando se combina la adición de enterocina AS-48 con otros agentes antimicrobianos (Ananou et al., 2007) y, de modo particular, frente a *Salmonella enterica* cuando se trató zumo de manzana con esta bacteriocina y PEAIC (Viedma-Martínez et al., 2008).

Estos últimos autores observaron un descenso de hasta 4.5 ciclos logarítmicos cuando al zumo de manzana con 60 µg/mL de bacteriocina e inoculado con *S. enterica* se le aplicaron PEAIC durante un total de 1,000 µs con pulsos de 4 µs de anchura y 35 kV/cm. La efectividad de ambos tratamientos aplicados simultáneamente podría deberse a los daños que tanto la bacteriocina (Garcia et al., 2007) como el campo eléctrico infringen a la membrana. El modo de acción conjunto podría explicarse de dos formas diferentes según el papel del antimicrobiano. Por un lado, la bacteriocina podría impedir la reparación de los poros formados en la membrana, que en su ausencia serían transitorios, o, por otro, la actividad del antimicrobiano podría aumentar la sensibilidad de la membrana al campo eléctrico, agravando los daños y las lesiones de la membrana.

Efecto combinado de PEAIC y la adición de nisina y lisozima

La pérdida de viabilidad de *Staph. aureus* en leche debido a la adición de nisina y lisozima y el tratamiento con PEAIC se evaluó a través de un diseño de superficie de respuesta cuyos niveles de las variables fueron 1-5 IU/mL de nisina, 300-3,000 IU/mL de lisozima, 120-1,200 µs de tiempo de tratamiento y 5.0-6.8 de pH. La inactivación microbiana, -log *s*, se expresó mediante la ecuación polinómica (8), cuyo coeficiente de determinación, R^2, y falta de ajuste fueron de 0.995 y no significativa, respectivamente,

$$-\log s = -31 + 0.7 \cdot n - 3 \cdot 10^{-4} \cdot l + 5.1 \cdot 10^{-3} \cdot t + 9.9 \cdot p + 0.18 \cdot n^2 - 2.0 \cdot 10^{-7} \cdot l^2 - \\ - 2.6 \cdot 10^{-6} \cdot t^2 - 0.68 \cdot p^2 + 1.4 \cdot 10^{-4} \cdot n \cdot l - 0.32 \cdot n \cdot p + 2.4 \cdot 10^{-7} \cdot l \cdot t \quad (8)$$

en la que *n* representa la concentración de nisina (IU/mL), *l* la concentración de lisozima (IU/mL), *t* el tiempo de tratamiento (µs) y *p* el pH.

En general, un incremento del tiempo de tratamiento se tradujo en una disminución del número de supervivientes. Sin embargo, el nivel de destrucción estuvo condicionado por el pH, que determinó, a su vez, el comportamiento de la respuesta de las dosis de antimicrobianos utilizadas. Por un lado, la acción combinada fue sinérgica al pH natural de la leche y a pH ácido, sin embargo, la respuesta fue sinérgica únicamente a elevados tiempos de tratamiento. A una concentración de 300 IU/mL de lisozima y 1 IU/mL de nisina, se observaron casi 4.4 reducciones logarítmicas aplicando 120 µs y cerca de 6.4 ciclos logarítmicos cuando se aumentó hasta 1,200 µs en leche a su pH natural. En este último caso, la diferencia respecto a la inactivación conseguida aplicando cada tratamiento por separado fue de 2.5 ciclos logarítmicos más. A pH 5.0, la mayor destrucción, aproximadamente 5.0 ciclos logarítmicos, se obtuvo aplicando 1,200 µs de tiempo de tratamiento y añadiendo 5 IU/mL de nisina y 1,650 IU/mL de lisozima. Cabe señalar, además, que al pH natural de la leche, dosis bajas de nisina y lisozima proporcionaron mayor efectividad que dosis elevadas independientemente del tiempo de tratamiento aplicado. Por el contrario, la letalidad aumentó a pH ácido si se incrementaba la dosis de nisina y lisozima, alcanzándose un punto óptimo a tiempos de tratamiento elevados.

Aunque se ha apuntado la acción sinérgica entre nisina, lisozima y PEAIC, el papel del pH sobre el efecto final de los tres tratamientos aplicados simultáneamente no se ha estudiado. Smith et al.

(2002) contabilizaron hasta 7.0 reducciones logarítmicas de la flora natural de la leche añadiendo 38 IU/mL de nisina, 1,638 IU/mL de lisozima y aplicando 50 pulsos de 80 kV/cm a 52ºC. El mecanismo implicado en la sinergia entre los tres tratamientos todavía no se ha detallado. No obstante, y teniendo en cuenta las hipótesis formuladas para la interacción entre los PEAIC y la nisina, la presencia de lisozima podría facilitar la adhesión de la molécula de nisina a la membrana y así potenciar el efecto conjunto (Chung et al., 2000). Sin embargo, la relación entre el pH y el resto de variables parece afectar de modo sustancial al efecto y actividad finales de cada uno de los tratamientos. Considerando la diferencia entre la fracción de supervivientes debida exclusivamente a la adición de nisina y lisozima y la debida a la aplicación de PEAIC en combinación con la adición de ambos péptidos, caben dos posibles hipótesis para tal comportamiento. Por un lado, y teniendo en cuenta que la solubilidad de la nisina aumenta con la acidez del medio, su actividad podría verse afectada por el campo eléctrico, ya que se ha comprobado que los PEAIC son capaces de inactivar enzimas (Bendicho et al., 2002). Y, por otro lado, ambos antimicrobianos podrían competir por las mismas dianas en la membrana de la célula a la vez que la eficiencia del poro formado por nisina para disipar el potencial eléctrico transmembrana podría disminuir con el descenso del pH (Moll et al., 1997).

Efecto combinado de PEAIC y la adición de enterocina AS-48 con nisina o lisozima

Manteniendo una concentración constante de enterocina AS-48 de 28 AU/mL se evaluó la adición de nisina o lisozima en leche a su pH natural y tratada posteriormente con PEAIC. La adición conjunta de lisozima (300-5,000 IU/mL) y enterocina AS-48 en leche procesada mediante PEAIC no disminuyó la fracción de supervivientes en comparación con el mismo tratamiento sin la adición de lisozima. Sin embargo, la inactivación microbiana, -log *s*, lograda combinando enterocina AS-48, nisina y PEAIC se modelizó mediante la ecuación (9) de segundo orden (coeficiente de determinación, R^2, de 0.97 y falta de ajuste no significativa)

$$-\log s = 1.50 - 0.0658 \cdot n + 0.0103 \cdot t + 0.00525 \cdot n^2 - 6.53 \cdot 10^{-6} \cdot t^2 \qquad (9)$$

en la que *n* representa la concentración de nisina (IU/mL) y *t* el tiempo de tratamiento (µs).

En general, el grado de inactivación aumentó tanto más cuanto mayores fueron el tiempo de tratamiento y la dosis de nisina, aunque se obtuvo un óptimo a partir del cual un incremento del tiempo disminuyó la efectividad del tratamiento. El valor máximo, 6.3 ciclos logarítmicos, se registró a 800 µs de tiempo de tratamiento y 20 IU/mL de nisina. Además, el número de supervivientes de esas muestras se mantuvo constante tras 48 h de almacenamiento en refrigeración. Este resultado contrasta con el observado en muestras tratadas únicamente con ambas bacteriocinas y almacenadas en las mismas condiciones. Añadiendo 28 AU/mL y 20 IU/mL de enterocina AS-48 y nisina, respectivamente, la población descendió paulatinamente cuando la temperatura de almacenamiento fue 4ºC. Esta diferencia sugiere la posibilidad de que la actividad de una o ambas

bacteriocinas pueda verse afectada por la aplicación de campos eléctricos y que, por tanto, cese la acción bactericida durante el almacenamiento.

Efecto de la secuencia de aplicación de PEAIC y adición de antimicrobianos

Las distintas combinaciones de antimicrobianos se añadieron antes o después de procesar la leche mediante PEAIC. En general, la adición de los antimicrobianos o de sus combinaciones (enterocina AS-48 con nisina o nisina con lisozima) después del procesado con PEAIC dio lugar a un menor nivel de destrucción respecto al mismo tratamiento con adición previa de los antimicrobianos. Sin embargo, en el caso concreto de la combinación de nisina, lisozima y PEAIC, no hubo diferencias entre las fracciones de supervivientes aplicando antes o después que los PEAIC en leche a pH 5.0. Esta tendencia concuerda con la citada por Gallo et al. (2007), en cuyo estudio no se detectaron diferencias entre la secuencia de aplicación de PEAIC y nisina.

Aunque sería presumible un mayor rendimiento de los antimicrobianos sobre células dañadas previamente por PEAIC, diversas teorías podrían justificar los resultados obtenidos. Por un lado, se ha comprobado la reversibilidad y temporalidad de los daños infringidos por el campo eléctrico (García et al., 2007; Terebiznik et al., 2000) y, por otro, los daños sobre la membrana debidos al campo eléctrico podrían impedir o dificultar la adhesión de las moléculas de antimicrobianos, en particular, de la nisina (Calderón-Miranda et al., 1999; Terebiznik et al., 2000). De este modo, estando presente el antimicrobiano en el momento en que se aplica el campo eléctrico permitiría un ataque más rápido a la célula dañada a la par que la propia actividad del antimicrobiano podría aumentar la sensibilidad de esa membrana al campo. En el caso opuesto, la célula podría disponer de tiempo suficiente para recuperar su integridad si el antimicrobiano no se halla presente, incluso, se dificultaría el mecanismo de acción de los agentes antimicrobianos por no poder alcanzar a tiempo la membrana dañada o alterada. Por consiguiente, la resistencia aparente de las células a los péptidos empleados y al tratamiento con PEAIC aumentaría.

VIDA ÚTIL DE LECHE TRATADA MEDIANTE MÉTODOS COMBINADOS DE PEAIC Y AGENTES ANTIMICROBIANOS

Se trató leche cruda para evaluar el efecto de la aplicación individual y conjunta de PEAIC, agentes antimicrobianos (nisina, lisozima y enterocina AS-48) y sus combinaciones (nisina con lisozima y nisina con enterocina AS-48) sobre la flora natural. Las muestras tratadas se almacenaron refrigeradas para comparar la evolución de mesófilos, enterobacterias, coliformes y patógenos (*Salmonella* spp., *Listeria* spp., *Escherichia coli* y *Staph. aureus*) con la de muestras procesadas con métodos térmicos convencionales. Además, se estudió el efecto combinado de un calentamiento moderado de la leche (55 y 65ºC), adición de antimicrobianos y procesado con PEAIC.

Efecto del tratamiento de PEAIC y agentes antimicrobianos

La vida útil de la leche cruda almacenada en refrigeración fue de 2 días. No hubieron diferencias entre los recuentos efectuados en leche cruda y leche con antimicrobianos a dosis subletales (20 IU/mL de nisina, 300 IU/mL de lisozima y 28 AU/mL de enterocina AS-48) o sus combinaciones (1 IU/mL de nisina con 300 IU/mL de lisozima o 20 IU/mL de nisina con 28 AU/mL de enterocina AS-48). Sin embargo, el procesado mediante PEAIC (1,200 μs de tiempo, 6 μs de anchura de pulso bipolar de 35 kV/cm y 75 Hz) alargó la vida útil de la leche en refrigeración hasta 5 días no detectándose, además, presencia de *E. coli* y *Staph. aureus*. Este resultado de vida útil coincide con los obtenidos por Odriozola-Serrano et al. (2006) en leche tratada en condiciones similares. Aunque Fernández-Molina et al. (2005) registraron tiempos de almacenamiento más largos, las condiciones de tratamiento de PEAIC así como el límite microbiológico establecido dificulta establecer comparaciones con sus resultados.

A pesar de que la inactivación microbiana de leche procesada mediante las distintas combinaciones de antimicrobianos y PEAIC fue superior a la de leche tratada únicamente con PEAIC, no se observaron diferencias entre la vida útil alcanzada en unos casos y otros. La similitud en la vida útil registrada podría deberse a la reversibilidad de los daños infringidos y, por tanto, a la corta fase de latencia de la población de supervivientes (1 día). Sólo la combinación de PEAIC con 1 IU/mL de nisina y 300 IU/mL de lisozima produjo más de 3.5 reducciones logarítmicas de la población de mesófilos y alargó la vida útil de la leche hasta 7 días. En todas las muestras procesadas no hubo recrecimiento de los microorganismos patógenos seleccionados.

La diferencia entre la destrucción microbiana alcanzada en leche cruda respecto a la registrada en leche inoculada con *Staph. aureus* podría deberse a la variedad de microorganismos de la flora natural y, consecuentemente, a los múltiples grados de resistencia de esas células frente a cada uno de los tratamientos utilizados.

Efecto del tratamiento de PEAIC, agentes antimicrobianos y calentamiento moderado

El calentamiento de leche hasta 55ºC durante 16 s y procesada con PEAIC o con las distintas combinaciones de PEAIC y antimicrobianos actuó sinérgicamente tanto en la inactivación de la población de mesófilos como de enterobacterias, coliformes y patógenos. De este modo, se observaron hasta más de 4 ciclos logarítmicos en la reducción de mesófilos cuando se combinó el calentamiento a 55ºC con la aplicación de PEAIC y 20 IU/mL de nisina. En general, no se detectaron enterobacterias y coliformes durante los primeros 4 días de almacenamiento, siendo la vida útil de muestras tratadas con nisina o con nisina y lisozima, calentadas a 55ºC y procesadas con PEAIC de hasta 8 días. La adición de los antimicrobianos o sus combinaciones a leche calentada hasta 65ºC durante 16 s proporcionó resultados similares, si bien, hubo ausencia de enterobacterias y coliformes durante los 3 primeros días y de mesófilos durante el primer día. Los resultados conseguidos combinando antimicrobianos, tratamiento térmico (55ºC, 16 s) y PEAIC fueron mejores que los observados en leche termizada utilizada en productos lácteos tales como los quesos (Hinrichs et al., 1995).

Cuando la temperatura del tratamiento térmico aumentó hasta 65ºC (16 s) y se procesó con PEAIC previa adición de los agentes antimicrobianos, se observó un efecto sinérgico que se tradujo en ausencia de microorganismos durante gran parte del periodo de almacenamiento en todos los recuentos realizados y una vida útil más larga. La adición de 20 IU/mL de nisina a leche cruda calentada después a 65ºC y procesada con PEAIC alcanzó una vida útil de 26 días, mejorando la calidad microbiológica y superando la vida útil de las muestras pasteurizadas (75ºC, 16 s) refrigeradas. El menor valor de vida útil, 17 días, correspondió a leche con 1 IU/mL de nisina, 300 IU/mL de lisozima, calentada a 65ºC y procesada con PEAIC. En todos estos casos, la sensibilidad de la membrana a los antimicrobianos y a los PEAIC podría verse intensificada y mantenida por la acción del calor, tal como reflejan algunos estudios tras comprobar la sinergia entre temperatura del medio y PEAIC (Fernández-Molina et al., 2005; Sampedro et al., 2007). Además, la presencia de los antimicrobianos durante el almacenamiento podría ejercer un efecto, al menos, bacteriostático.

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CONCLUSIONES

La conclusión general del presente estudio fue que la combinación de pulsos eléctricos de alta intensidad de campo (PEAIC), un calentamiento moderado y la adición de agentes antimicrobianos (nisina, nisina y lisozima o nisina y enterocina AS-48) actuó sinérgicamente en la inactivación de la flora natural de la leche, llegándose a mejorar la calidad microbiológica y superar la vida útil en condiciones de refrigeración (4ºC) de la leche pasteurizada (75ºC durante 16 s) en el caso concreto de procesar con PEAIC, calentar hasta 65ºC y usar nisina.

De los resultados obtenidos en el presente estudio y de su interpretación se pueden extraer las siguientes conclusiones particulares:

- Se obtuvo mayor grado de inactivación de *Staph. aureus* en leche tratada con PEAIC cuando se usaron pulsos bipolares y a mayor valor de las variables número y anchura de pulso e intensidad de campo eléctrico.
- El porcentaje de grasa de la leche procesada mediante PEAIC no influyó en la supervivencia de *Staph. aureus*.
- Para un determinado valor de intensidad de campo, existe un valor óptimo de anchura de pulso con el que se alcanza un valor máximo de inactivación de *Staph. aureus* aplicando un mínimo número de pulsos. Al procesar con PEAIC con una intensidad de campo de 35 kV/cm, se consiguieron 4.4 reducciones logarítmicas de *Staph. aureus* con 150 pulsos de anchura 7.1 µs.
- Distintas combinaciones de anchura y número de pulsos o anchura e intensidad de campo permiten alcanzar un mismo nivel de destrucción al tratar con PEAIC leche inoculada con *Staph. aureus*.
- La adición simultánea de nisina y lisozima o nisina y enterocina AS-48, así como la combinación de PEAIC con la adición de i) nisina, ii) nisina y lisozima o iii) nisina y enterocina AS-48 actuaron sinérgicamente en la destrucción de *Staph. aureus* en leche.
- La disminución del pH aumentó la resistencia aparente de *Staph. aureus* respecto a las muestras en su pH natural cuando se trató leche con antimicrobianos (nisina, lisozima, enterocina AS-48 o sus combinaciones) y PEAIC.
- El procesado mediante PEAIC podría afectar a la actividad de los distintos antimicrobianos utilizados (nisina, lisozima y enterocina AS-48), de tal forma que se reduciría el efecto sinérgico durante la aplicación conjunta del tratamiento y/o durante el almacenamiento de las muestras procesadas.
- La leche procesada con PEAIC o con PEAIC y la adición de i) nisina, ii) nisina y lisozima o iii) nisina y enterocina AS-48 alargó su vida útil en refrigeración, registrándose hasta un máximo de 7 días en el caso de tratar con PEAIC (1,200 µs de tiempo de tratamiento, 6 µs de anchura de pulso bipolar de 35 kV/cm y 75 Hz de frecuencia) y añadir 1 IU/mL de nisina y 300 IU/mL de lisozima.
- La combinación de un calentamiento moderado, PEAIC y adición de nisina, nisina y lisozima o nisina y enterocina AS-48 mejoró la calidad microbiológica y prolongó el almacenamiento de las muestras tratadas. Cuando leche con 20 IU/mL de nisina se calentó a 65ºC durante 16 s y se procesó con PEAIC se alcanzó una vida útil de 26 días, superior a la de la leche pasteurizada (75ºC durante 16 s).