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 nonthermal 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 or lysozyme, or both, 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 arbitrary units/mL of AS-48 and 20 IU/mL of nisin were added to the milk, and it was treated with HIPEF for 800 μ s, 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.

Key words: milk, high-intensity pulsed-electric field, enterocin AS-48, nisin

INTRODUCTION

The interest of the food industry in food preservation through nonthermal methods arises, in general, from

the fact that they make it possible to guarantee the microbiological qualities of a product while, at the same time, minimizing the effect 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 nonthermal techniques for treating liquid foods for 2 reasons: first, 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, 2006).

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

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(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 because they are often related with a large number of fermentations in the food industry. In particular, nisin is a peptide produced by *Lactococcus lactis* ssp. *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 feasible applications in food such as sausages (Ananou et al., 2005b), vegetable sauces (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 bacteriocin 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 (Biokar Diagnostics, Beauvais, France) at 4°C until it was used.

Strain growth was performed by incubating cultures on tryptone soy broth at 35°C 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 4°C (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 25°C 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-Aldrich, Steinheim, Germany), filtered through 0.22- μ m pore size low-protein binding filters (Millex GV, Millipore Corp., Belford, MA) 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

Table 1. Inactivation of *Staphylococcus aureus* in milk by combining added enterocin AS-48 and high-intensity pulsed-electric field (HIPEF)

Assay number ¹	Point type	Enterocin AS-48 dose (AU/mL)	HIPEF treatment time ² (μ s)	Milk pH	Microbial inactivation ($-\log s^3$)
1	Factorial	28.0	1,200	5.0	2.8
2	Factorial	28.0	120	6.8	1.6
3	Factorial	3.5	1,200	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	1,200	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

¹Assay number does not correspond to the order of processing.

²HIPEF treatment was set at 35 kV/cm, 6 μ s of pulse width, and 75 Hz of pulse frequency at bipolar mode.

³ $-\log s$ = microbial inactivation as mean of 2 replicates.

HIPEF unit (Ohio State University, Columbus) 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 cm³. Electrical parameters were set at 35 kV/cm of electric field strength, 6 μ s of pulse width, and 75 Hz of pulse frequency in bipolar mode in accordance with previous studies. Treatment temperature was kept under 25°C 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 μ s. Each trial was performed in triplicate.

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) and lysozyme (L 2879, 43,560 IU of lysozyme/mg of solid, Sigma-Aldrich) 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 μ s).

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 of nisin and 120 to 1,200 μ s of 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 μ s. 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 μ s of HIPEF treatment time and prolonging exposure

Table 2. Microbial death of *Staphylococcus aureus* suspended in milk with added 28 AU/mL of enterocin AS-48 and treated under different conditions of nisin dose and high-intensity pulsed-electric field (HIPEF) treatment time

Assay number ¹	Point type	Independent variables		
		Nisin dose (IU/mL)	HIPEF treatment time ² (μs)	Microbial inactivation (−log s ³)
1	Factorial	1.0	120	2.7
2	Factorial	20.0	120	3.2
3	Factorial	1.0	1,200	4.3
4	Factorial	20.0	1,200	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	1,200	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

¹Assay number does not correspond to the order of processing.²HIPEF treatment was set at 35 kV/cm, 6 μs pulse width, and 75 Hz of pulse frequency at bipolar mode.³−log s = microbial inactivation as mean of 2 replicates.

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 4°C and 22°C. Population of *Staph. aureus* in the stored samples was evaluated after 24 and 48 h. Assays were performed in triplicate.

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:

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

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 nonsignificant 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, MN) was used in all RSM analyses and generated plots.

Microbial Inactivation of *Staphylococcus aureus*

The untreated and treated samples were serially diluted in peptone saline solution, plated on slants of plate count agar, and incubated for 48 h at 35°C. 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 before 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 22°C. On the contrary, the *Staph.*

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 temperatures¹

Storage (h)	4°C			22°C		
	Untreated	Treated		Untreated	Treated	
		5.0 pH	6.8 pH		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

¹Survivors as log N. Mean ± standard deviation.

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 22°C or at natural milk pH than at 4°C 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 22°C. 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, whereas on the other hand, when the temperature was

kept at 4°C, the reduction increased progressively up to practically 4 log cycles over the 48 h.

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.

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

Table 4. Analysis of variance 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 high-intensity pulsed-electric field (HIPEF; 35 kV/cm, 6 µs of pulse width, and 75 Hz of frequency at bipolar mode)

Source ¹	MS	F-value	Probability > F	Regression coefficients ²
Model	1.09	14.83	0.0010 ³	
Intercept				-21.1 ± 8.6
<i>e</i>	0.62	8.44	0.0228 ³	-0.0635 ± 0.0195
<i>t</i>	3.68	50.09	0.0002 ³	-0.00266 ± 0.00340
<i>p</i>	0.45	6.15	0.0422 ³	8.04 ± 2.64
<i>e</i> ²	0.71	9.59	0.0174 ³	0.00346 ± 0.00117
<i>t</i> ²	1.10	14.92	0.0062 ³	$-2.22 \times 10^{-6} \pm 5.7 \times 10^{-7}$
<i>p</i> ²	0.90	12.18	0.0101 ³	-0.722 ± 0.207
<i>t</i> × <i>p</i>	0.45	6.10	0.0428 ³	0.00119 ± 0.00048
Lack of fit	0.12	3.20	0.1456	
Pure error	0.038			
SD	0.27			
Mean	2.72			
CV	9.97			
R^2	0.94			
Adjusted R^2	0.88			

¹*e* = enterocin AS-48 concentration (AU/mL); *t* = HIPEF treatment time (µs); *p* = milk pH.

²Mean ± standard deviation.

³Significant at 95% confidence interval.

Figure 1. Effect of increasing enterocin AS-48 concentration (arbitrary units/mL) and high-intensity pulsed-electric field (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 of pulse width, and 75 Hz at bipolar mode).

and a nonsignificant 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 4°C 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 22°C.

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 nonsignificant 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:

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

where n is the nisin concentration (IU/mL) and t is 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 with 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 4°C, 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 22°C.

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

Table 5. Analysis of variance 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 high-intensity pulsed-electric field (HIPEF; 35 kV/cm, 6 μ s of pulse width, and 75 Hz of frequency at bipolar mode)

Source ¹	Mean square	F-value	Probability > F	Regression coefficients ²
Model	4.06	71.77	<0.0001 ³	
Intercept				1.50 $\pm$ 0.26
<i>n</i>	1.07	18.93	0.0024 ³	-0.0658 $\pm$ 0.0231
<i>t</i>	4.96	87.72	<0.0001 ³	0.0103 $\pm$ 0.0005
<i>n</i> ²	0.62	10.96	0.0107 ³	0.00525 $\pm$ 0.00158
<i>t</i> ²	10.01	176.83	<0.0001 ³	-6.53 $\times 10^{-6}$ $\pm$ 4.91 $\times 10^{-7}$
Lack of fit	0.079	2.28	0.2218	
Pure error	0.035			
SD	0.24			
Mean	4.69			
CV	5.08			
R ²	0.97			
Adjusted R ²	0.96			

¹*n* = nisin concentration (IU/mL); *t* = HIPEF treatment time (μ s).

²Mean $\pm$ standard deviation.

³Significant at 95% confidence interval.

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

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 4° or 22°C, 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 μ g/mL. That result explains the sublethal or non-existent effect of the concentration of AS-48 used in this study (8 μ g/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-López 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 Chun 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 22°C. 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 h of storage. However, temperature is the factor that acts as an accelerator or decelerator of the activity during that period of time. Thus, a temperature of 22°C during storage accelerated and intensified the joint activity of the peptides during the first 24 h, although that activity stopped during the following 24 h, with a consequent regrowth in the population of *Staph. aureus*. On the contrary, a temperature of 4°C first slowed the inhibiting effect of the bacteriocins, but prolonged the decrease in the population up to 48 h 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 the 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 with 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 μ s (Figure 2), and both lower and higher times were less effective in reducing the population. Similar behavior was described by Sobrino-López 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 4°C up to 48 h. 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 4° and at 22°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 electroporeabilization 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.

In conclusion, the treatment of milk with added AS-48 or nisin, or both, at sublethal doses with HIPEF acted

Figure 2. Microbial inactivation of *Staphylococcus aureus* in milk by combining nisin (1 to 20 IU/mL) and enterocin AS-48 (28 arbitrary units/mL) addition before applying high-intensity pulsed-electric field (HIPEF) treatment (35 kV/cm, 6 μ s of pulse width, and 75 Hz of frequency at bipolar mode).

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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