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Effect of the sequence of nisin and pulsed electric fields treatments and mechanisms involved in the inactivation of *Listeria innocua* in whey

Luciana I. Gallo [a], Ana M.R. Pilosof [b,1], Rosa J. Jagus [a,*]

[a] *Laboratorio de Microbiología Industrial, Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires, Pabellón de Industrias, Ciudad Universitaria (1428), Buenos Aires, Argentina*
[b] *Departamento de Industrias, Facultad de Ciencias Exactas y Naturale, Universidad de Buenos Aires, Pabellón de Industrias, Ciudad Universitaria (1428), Buenos Aires, Argentina*

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Abstract

The combination of nisin and pulsed electric fields (PEF) on *Listeria innocua* in liquid whey protein concentrate (LWPC) were studied with the purpose of enhancing nisin antibacterial action.

The efficiency of the combined treatment of nisin and PEF was strongly dependent on the sequence of application. The exposure to nisin after PEF produced an antagonistic effect on *L. innocua* inactivation. This behaviour could be mainly attributed to changes in the cell envelope and to modifications of the medium caused by PEF application. Consequently, the posterior action of nisin was reduced, showing an increase in the resistance of *L. innocua* to nisin. In opposite, the addition of nisin prior to PEF treatment exhibited an additive and slightly synergistic effect, suggesting that the binding of nisin to the cell membrane would increase the susceptibility of the microorganism to PEF treatment.

Keywords: Cheese whey; Nisin; Pulsed electric fields; Combined treatments; *Listeria innocua*

1. Introduction

Cheese whey is a by-product from the cheese industry, generated in great amount, being the ratio whey/cheese 9:1. It is an important source of proteins (primarely α-lactalbumin and β-lactoglobulin), lactose and minerals, which make this product be considered as functional food and a source of valuable nutrients (González-Martínez et al., 2002). Functional properties of whey proteins also make the product interesting for use as a food ingredient. Cheese whey is generally processed by ultrafiltration and spray drying. If the liquid from the membrane process is adequately stabilized, it could be used directly as a liquid whey protein concentrate (LWPC), in the formulation of food products.

Listeria monocytogenes is a food-borne pathogen that can contaminate dairy products (Menéndez, Godinez, Rodriguez-Otero, & Centeno, 1997). Of particular importance is how milk, including that used to manufacture cheese and other culture dairy products, can serve as a primary vehicle for transmission of the organism.

Although *Listeria* is inactivated under normal conditions of pasteurization, problems can arise from post pasteurization contamination. Bacteria can enter cheese at many stages during its processing. The environmental diversity of dairy processing plants provides the microorganism with various sites for colonization. Any pathogen existing in raw milk can potentially make its way into the environment of plant processing cheese and whey (Cotton & White, 1992; Silva, Almeida, Alves, & Almeida, 2003).

* Corresponding author. Tel./fax: +54 11 4576 3241.
E-mail address: rjagus@di.fcen.uba.ar (R.J. Jagus).
[1] Tel./fax: +54 11 4576 3300.

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Listeria innocua, a non-pathogenic gram positive, non-spore forming, rod-shape, psychrotrophic bacteria, able to grow in various low acid foods, is often selected for inactivation studies because of its similar response to physico-chemical parameters and still closely related to *L. monocytogenes* (Kamat & Nair, 1996).

Pulsed electric fields (PEF) represent a promising non-thermal preservation alternative to traditional thermal process, since it is capable of destroying microorganisms and some enzymes, while maintaining the freshness of food (Barsotti, Dumay, Mu, Fernandez Diaz, & Cheftel, 2001; Zhang, Barbosa-Cánovas, & Swanson, 1995). Pulsed electric fields produce structural changes in the membrane, resulting in pore formation and loss of selective permeability properties of the membrane (Hamilton & Sale, 1967).

Nisin is an antimicrobial peptide produced by lactococci and had been used in food products for many years. Although this lantibiotic is inhibitory to microorganisms, it is harmless to humans (Hurst & Hoover, 1993). It is the first antimicrobial peptide with a "generally recognized as safe" status in the United States for use in processed cheese, with a permitted level of 250 μg/g food. In addition, its use in various food products is allowed in several countries (Delves-Broughton, 1990).

Hurdle technology, as a multifactor procedure, can be employed to accomplish food preservation. Each factor, by itself, contributes to the stability and safety of the food product (Leistner, 1992, 1994).

Since nisin and PEF, both acts on the membrane, an additive effect might be expected if they are combined. Several works have studied the efficiency of PEF and nisin in a combined treatment on microbial inactivation (Dutreux, Notermans, Gongora-Nieto, Barbosa-Canovas, & Swanson, 2000; Liang, Mittal, & Griffiths, 2002; Pol, Mastwijk, Bartels, & Smid, 2000). However, in these reports, it has not been explored if the sequence of application of each treatment in combination has any effect on the final microbial inactivation. Previously, we have reported no difference on the inactivation of *E. coli* when nisin was applied either before or after PEF technology (Terebiznik, Jagus, Cerrutti, de Huergo, & Pilosof, 2000; Terebiznik, Jagus, Cerrutti, de Huergo, & Pilosof, 2002).

The objective of the present work was to explore the effect of the sequence of application of nisin and PEF in a combined treatment on *L. innocua* in liquid whey protein concentrate.

2. Materials and methods

2.1. Strains and growth conditions

L. innocua (CIP 8011, CCMA 29) was grown in 150 ml Tryptic Soy broth enriched with 0.6% yeast extract (TSBYE), in a continuously agitated temperature-controlled shaker at 28 °C overnight. Five milliliters were inoculated in 150 ml of fresh TSBYE, agitated for 1–2 h to obtain the final desired concentration of cells.

2.2. Nisin preparation

A stock solution (1×10^5 IU/ml) was prepared by dissolving Nisaplin™ (Aplin & Barrett Ltd., Dotset, UK) in sterile distilled water. The pH was adjusted to 2.0 with 0.1 N HCl to ensure high bacteriocin solubility and stored at −20 °C.

When nisin was added to the sample from the stock solution the pH of the medium was not altered.

2.3. Liquid whey protein concentrate preparation

The liquid whey protein concentrated (LWPC), at 8%, was prepared from solid whey protein concentrate 34%. The pH was adjusted by HCl addition. The conductivity was measured with a Conductimeter Antares IV, Parsec.

2.4. Nisin, pulsed electric fields and combined treatments

A *L. innocua* culture in log or stationary phase was centrifuged at $5600 \times g$ for 30 min and resuspended in LWPC.

Nisin treatments were conducted as in Terebiznik et al. (2002). Briefly, the cell suspensions were exposed to the adequate concentration of nisin during 30 min at 7 °C and then were harvested by centrifugation, resuspended and serially diluted in 0.1% peptone water.

According to Terebiznik et al. (2000), PEF treatments were performed as follows: 400 μl of the cells suspension were withdrawn into a 0.2 cm electrode gap electroporation cuvette (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547) and subjected to PEF treatment with exponential decay pulses in a Gene Pulser II Electroporation system (Bio-Rad). Cells were subjected to two different treatments: (1) 60 pulses, at 12 kV/cm of electric field intensity with 3 μF capacitance and 0.2 ms/pulse time decay (τ), (2) 10 pulses at electric field intensity of 10 kV/cm with 50 μF capacitance and 2 ms/pulse time decay, and (3) 60 pulses at electric field intensity of 12 kV/cm with 10 μF capacitance and 0.4 ms/pulse time decay.

The final temperature in every PEF treatment was lower than 10 °C (Scanning thermocouple thermometer, Digi-Senns, Cole-Palmer Instrment). After PEF treatment, cells were serially diluted in 0.1% peptone water.

The combined treatments were performed in different ways: nisin was added before PEF treatment ('NB'), simultaneously with PEF application ('NS') or the cells were treated with nisin immediately after PEF ('NA'). For the assessment of the survival of *L. innocua,* cells were harvested by centrifugation, suspended and serially diluted in 0.1% peptone water.

To evaluate the antagonistic effect observed in the application of the combined treatments, suspensions of LWPC with or without *L. innocua* inoculum, were treated with PEF. After the treatment, pellet and supernatant were separated by centrifugation at $5600 \times g$. Subsequently, several combinations of pellet and supernatants (both or either treated or untreated with PEF, with or without previously

inoculated microorganisms) were performed. After resuspension of pellets, the suspensions were treated with different concentrations of nisin.

After individual or combined treatment, dilution drops (20 μl) of the bacterial suspension in LWPC were spotted in duplicate onto tryptic soy agar enriched with 0.6% yeast extract. The number of cfu/ml was determined after incubation at 37 °C for 48 h. Determinations were made in triplicate.

2.5. Hydrophobicity test

Cell surface hydrophobicity was determined with the hydrocarbon/buffer two phase system of Rosenberg, Gutnick, and Rosenberg (1980). Briefly, a suspension containing approximately 10^8 cfu/ml in 1.2 ml of 0.15 mol/l NaCl (OD_{600} of between 0.6 and 0.8 [A_0]) was vortexed for 60 s with 0.2 ml of xylene (Sigma) or hexadecane (Sigma). The mixture was held for 15 min to ensure complete separation of the two phases before a sample (1 ml) was carefully removed from the aqueous phase and the OD_{600} was measured (A). The percentage of cells in the solvent was subsequently calculated as: $[1 - (A/A_0) \times 100]$. Each experiment was performed in triplicate on two independent cultures.

2.6. Osmotic response

According to Pagan and Mackey (2000), the suspensions of PEF-treated and untreated bacterial cells were centrifuged and washed once with buffer phosphate, pH 7 (PBS). Washed cells were resuspended in 0.4 ml of PBS, after which 100 μl of this suspension was added in triplicate to (i) 1.0 ml of PBS and (ii) 1.0 ml of PBS containing 0.75 mol/l NaCl. The OD_{680} of these suspensions were measured 4 min after mixing. The increase in OD_{680} was calculated by substracting the mean value of the three measurements in the PBS from the mean value of the three measurements in PBS containing 0.75 mol/l NaCl. The OD increase was expressed as a percentage of the mean value obtained with PBS alone. Determinations were made in triplicate.

2.7. Statistical analysis

Statistics were generated using Microsoft Excel™ and Instat statistical functions (Anova and *t*-Student).

3. Results and discussion

L. innocua suspended in LWPC was treated with pulsed electric fields (PEF) and nisin (Fig. 1). The PEF process conditions were fixed at 12 kV/cm of electric field intensity, 3 μF of capacitance and 60 pulses and nisin concentration to 25 or 50 IU/ml. Under these conditions, *L. innocua* was less sensitive to PEF than to nisin treatment (Fig. 1, 'Nisin alone' compared to 'PEF alone') ($P < 0.05$). Next, nisin and PEF were combined in different sequences to achieve an enhanced microbial inactivation of *L. innocua* in LWPC. The NB combination (described in Section 2) exhibited an additive or slightly synergistic effect on the inactivation of *L. innocua*, being more pronounced at lower nisin concentration. The combination of 50 IU/ml of nisin prior to PEF showed an additive effect, defined as the sum of the reductions obtained with individual treatments. If the nisin was reduced to 25 IU/ml, the effect was synergistic, with a microbial reduction superior to the sum of reductions observed for each individual treatment ($P < 0.05$). This response was observed even at nisin concentration as low as 6.25 IU/ml (data not shown). When the effect of nisin was tested simultaneously with the PEF treatment (NS combination) the observed response was not additive. Contrarily, when the cells were treated with NA combination, an antagonistic response with respect to nisin treatment was observed if 50 IU/ml of nisin was applied. At a lower concentration of nisin (25 IU/ml), the inactivation by NA combination and that produced by nisin alone did not differ ($P > 0.05$). Additionally, if 100 or even 500 IU/ml of nisin were used in the NA combination, an increase in the antagonistic response was observed (Fig. 2).

The results obtained indicate that the sequence of the treatment strongly influenced the final microbial inactivation, showing a clear benefit of NB combination on the inactivation of *L. innocua*.

Lee, Heinz, and Knorr (2003) reported that the addition of nisin prior to high hydrostatic pressure treatment (HHP) significantly increased the lethal effect of HHP against *L. seeligeri* up to five cycles in liquid whole egg. Our results are in agreement with these observations, probably as a consequence of the changes produced by nisin in the cell envelope, enhancing cells' sensitivity to PEF treatment, as described for HHP. In opposite, if the microorganism is not sensible to nisin, e.g. *E. coli* (Terebiznik et al., 2000), no difference could be found in the response to different sequences of the combined treatments.

The observed non-additive or even antagonistic behaviour, described as the decrease in nisin action by the previous application of PEF (NA combination), could be attributed to different mechanisms: (a) modifications by PEF treatment in the whey components, (b) interactions of nisin molecules with intracellular components released by PEF treatment; and/or (c) modifications in the cell envelope produced in response to PEF application.

In order to elucidate the causes of this response, the following experiments were performed. Combinations of PEF-treated and PEF-untreated *L. innocua* pellets were resuspended in different supernatants from PEF-treated or PEF-untreated whey. Fig. 3 shows the nisin action in samples of PEF-untreated and PEF-treated pellets in contact with supernatants from PEF-untreated, PEF-treated whey and from PEF-treated whey, which has been previously inoculated with *L. innocua*. As a control of the nisin treatment (without the influence of PEF effects), nisin was

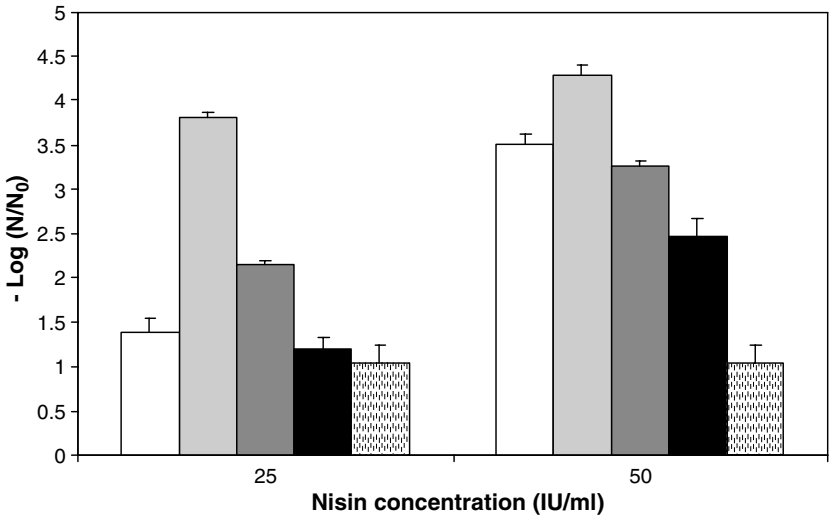

Fig. 1. Influence of the sequence of PEF and nisin treatment. Treatment conditions: electric field strength = 12 kV/cm, capacitance = 3 μF and 60 pulses; pH = 6.0; $N_0 = 2 \times 10^8$ cfu/ml. Treatment sequence: nisin alone (□); 'NB' nisin before PEF treatment (▨); 'NS' nisin simultaneously PEF treatment (▩); 'NA' nisin after PEF treatment (■); PEF alone (▨).

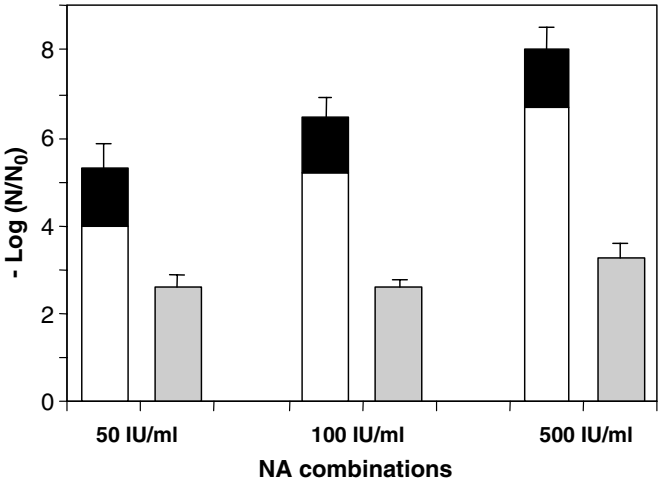

Fig. 2. Effect of nisin action on *Listeria innocua* after the application of PEF treatment: (□) nisin alone; (■) PEF alone; (▨) combined treatment (NA). Treatment conditions: PEF—electric field strength = 10 kV/cm, capacitance = 50 μF and 10 pulses.

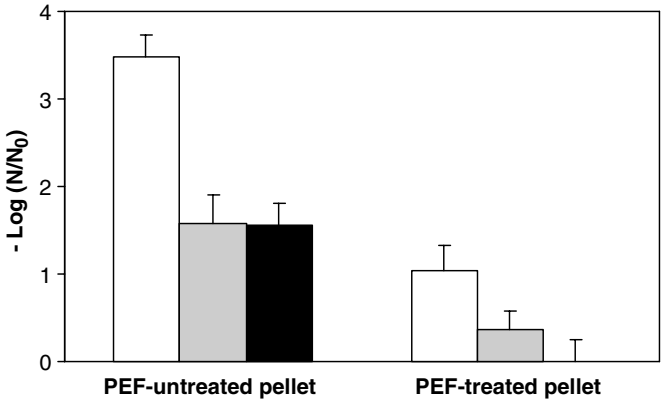

Fig. 3. Effect of nisin action on *Listeria innocua* after the combination of PEF-treated and PEF-untreated pellets with different supernatants: (□) supernatant from PEF-untreated samples; (▨) supernatant from PEF-treated samples; (■) supernatant from PEF-treated inoculated samples. Treatment conditions: PEF—electric field strength = 10 kV/cm, capacitance = 50 μF and 10 pulses. Nisin = 50 IU/ml.

added to a suspension of PEF-untreated cells (pellets) resuspended in PEF-untreated whey. Nisin effectiveness was higher ($P < 0.05$) when applied to the control samples.

When the supernatant was obtained from PEF-treated whey (without inoculated microorganisms), the nisin effectiveness diminished. This response could be attributed to the interaction of nisin with whey components modified by PEF treatment and thus, to the decrease of available nisin in the media (mechanism a).

If the PEF-untreated pellet was combined with supernatant from PEF-treated whey previously inoculated with *L. inoccua*, the possible leakage of intracellular material do not introduce an additional detrimental effect on the antimicrobial activity of nisin (mechanism b).

If the pellets were previously treated with PEF, the effects of PEF on the microorganism could be studied. The nisin action in samples with PEF-treated pellet in combination with supernatants from PEF-treated whey which have or not been inoculated with microorganisms is also shown in Fig. 3. The PEF-treated pellet with PEF-untreated supernatant showed only the effects of the changes produced in the microorganism (pellet) by PEF treatment on nisin activity (mechanism c).

The PEF-treated pellet in contact with PEF-treated supernatant (without inoculated microorganisms) showed the combination effects of the changes produced in the microorganism (pellet) and in the medium (supernatant) by the application of PEF treatment on nisin action. Again, the modifications of the LWPC soluble components

produced by PEF would favor nisin interaction with the media and not with the bacteria, reducing even more the nisin action (mechanisms a and c).

The proposed modifications produced in the cell envelope as a consequence to PEF treatment (mechanism c) can be evidenced in Fig. 3. If PEF-untreated supernatant is combined with PEF-treated pellet (cells) in the presence of nisin, the inactivation observed was lower than that observed in the combination with PEF-untreated pellet, suggesting that the cells were affected by PEF treatment. In addition, if the severity of PEF treatment was increased, the antagonic effect was more pronounced with the severity of PEF treatment (Fig. 4).

Due to the amphiphilic properties of nisin molecule, the peptide inserts its hydrophobic side into the bacterial membrane. Henning, Metz, and Hammes (1986) proposed that hydrophobic interaction between the membrane phospholipids was responsible for interactions between nisin and membranes. On the other hand, the electrostatic attraction between nisin molecules and negatively charges phospholipids has also been considered as the primary bactericidal mechanism of action of nisin. In both cases, phospholipids of bacterial cell membranes were strongly implicated as the target of nisin (Bauer & Dicks, 2005).

The hydrophobicity of PEF-treated and untreated cells of *L. innocua* in LWPC (measured as percentage of adhesion at the non-polar solvent) were compared in our study. We found that PEF-treated cells in LWPC had a lower hydrophobicity than that of PEF-untreated cells. If hexadecane was used for the partition, the cell hydrophobicity observed was minimally reduced from 73% to 70% ($P = 0.05$) when the bacteria were treated with PEF. When another non-polar solvent as xylene was used, the hydrofobicity was reduced from 58% to 38% ($P < 0.05$). In accordance with these results, Ming and Daeschel (1995) observed that the cell surface of cells of *L. monocytogenes* Scott A resistant to nisin was less hydrophobic compared to sensitive cells. In this respect, the effects of the application of PEF to *L. innocua* produced a reduction in its cell surface hydrophobicity, which could explain the decrease in its sensibility to nisin action.

Some authors related the nisin effectiveness with membrane integrity. Loss of the physical integrity of the cytoplasmic membrane was measured as loss of osmotic response. Fig. 5 shows the osmotic response of PEF-treated and heat-treated cells. *L. innocua* cells exposed to PEF treatment and placed in hypertonic solution, showed a change in optical density (OD) with respect to the PEF-untreated cells, indicating that PEF treatment produced changes in the osmotic response of the cells. The increment in the severity of the treatment (heat treatment as the most severe) produced a reduction in the microbial count in concordance with the loss of osmotic response.

Our results show an increase on the changes in the osmotic response with the increase on the severity of treatments. Pagan and Mackey (2000) examined the relationship between membrane damage and loss of viability following pressure treatment in *E. coli*. They found that the loss of viability correlated with loss of osmotic response. Gram positive bacteria also can be made to shrink in salt solutions, even though they are not readily plasmolized. Marquis (1968) indicated that the salt induced contraction of Gram positive *Bacillus megaterium* cells did not primarily involve osmotic plasmolysis, but that was caused mainly by electrostatic contraction of anphoteric, polyionic, cell wall polymers.

Collectively, these observations suggest that PEF treatment produced changes in the cell envelope enhancing resistance to nisin action. For these reasons, the

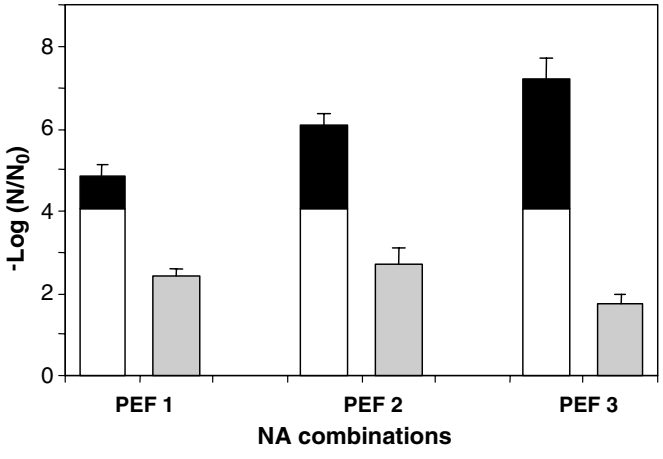

Fig. 4. Effect of nisin action on *Listeria innocua* after the application of PEF treatment: (□) nisin alone; (■) PEF alone; (▨) combined treatment (NA). Treatment conditions: PEF treatment: PEF_1—electric field strength = 12 kV/cm, capacitance = 3 μF and 60 pulses; PEF_2—electric field strength = 10 kV/cm, capacitance = 50 μF and 10 pulses; PEF_3—electric field strength = 12 kV/cm, capacitance = 10 μF and 60 pulses. Nisin: 50 IU/ml.

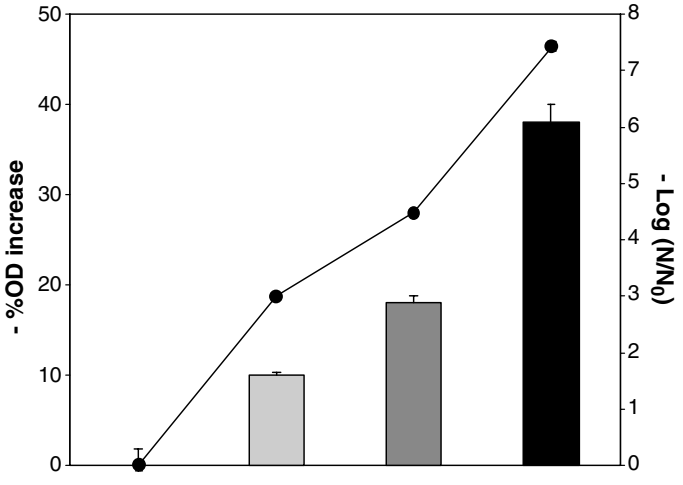

Fig. 5. Osmotic response of PEF-treated and heat-treated cells, measured as the change in optical density (OD) of cells suspensions, placed in 0.75 M NaCl and cell inactivation (—●—). Treatment conditions: (□) PEF-untreated cells. (▨) Electric field strength = 12 kV/cm, capacitance = 3 μF and 60 pulses; $\log N/N_0 = 3.5$. (▩) Electric field strength = 10 kV/cm, capacitance = 50 μF and 10 pulses; $\log N/N_0 = 4.5$. (■) Temperature = 65 °C, time = 10 min; $\log N/N_0 = 7.4$.

application of the sequence 'nisin followed by exposure to PEF treatment' (NB) exhibited the best performance for the treatment of *L. innocua* in LWPC.

From our previous and actual research, we could propose that if the microorganism is not sensitive to nisin, like the case of *E. coli* (Terebiznik et al., 2000), no difference in the response to different sequences of the combined treatment could be found. On the contrary, if the microorganism is sensitive to nisin, like *L. innocua* (in this work), the addition of nisin prior to PEF treatment would increase the susceptibility of the microorganism to PEF treatment. It could be interesting to evaluate this response for other antimicrobials.

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