Combined Effect of Nisin and Pulsed Electric Fields on the Inactivation of *Escherichia coli*

MAURICIO R. TEREZBIZNIK,1,* ROSA J. JAGUS,2 PATRICIA CERRUTTI,1 MARTA S. DE HUERGO,1 AND ANA M. R. PILOSOF3

1Departamento de Ingeniería Química and 2Departamento de Ingeniería Industrial, F.I., and 3Departamento de Industrias, F.C.E., N., Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina

ABSTRACT

The Doehlert design was applied in order to investigate the combined effect of nisin and high voltage pulsed electric fields (PEF) on the inactivation of *Escherichia coli* in simulated milk ultrafiltrate media. Nisin alone was totally inactivated by PEF, but in the presence of bacterial cells a protective effect was observed. However, the effectiveness of nisin was still decreased when bacterial cells were subjected to the combined treatment. In spite of this phenomenon, an almost additive response emerged as a consequence of the combined treatment. A 4-log cycle reduction may be accomplished with around 1,000 IU/ml (7.15 μM) of nisin and three pulses of 11.25 kV/cm or 500 IU/ml for five pulses of the same intensity. The observed efficacy arising from the combination of both treatments suggests the possibility of using PEF for improving the action spectrum of natural antimicrobials.

The bacteriocin nisin, a 3.5-kDa peptide produced by *Lactococcus lactis* subsp. *lactis*, has many biotechnological applications in the control of pathogenic gram-positive bacteria in foods (16). However, nisin inhibition of gram-negative bacteria is particularly problematic due to the protective outer membrane that provides an efficient barrier function against hydrophobic solutes and macromolecules (17). Nevertheless, Carneiro de Melo et al. (5) reported that *Escherichia coli* growth could be inhibited in the presence of very high doses of nisin. This is not always commercially feasible because of regulatory limits and high costs. However, the sensitivity of gram-negative bacteria against nisin was found to be increased if pulsed electric fields (PEF), heating and freezing treatments that induce sublethal injuries, were applied (12, 13). It has been emphasized that sublethal injury can be usefully applied in a “hurdle” approach to achieve effective food preservation (14).

Inactivation of microorganisms exposed to high-voltage PEF is a promising nonthermal food preservation technology that has mainly been applied to liquid foods or to pumpable food pastes. Lethal and sublethal injury is induced in bacterial cells by PEF and is mainly caused by an increase in cell membrane permeability due to compression and poration (2).

We report herein our results on the combined effect of PEF and nisin on *E. coli* inactivation in simulated milk ultrafiltrate media.

The Doehlert (7) design was applied in order to optimize electric field intensity (E), number of pulses (P), and nisin concentration (N) for inactivating *E. coli*.

MATERIALS AND METHODS

Strain and growth condition. *E. coli* NRRL B-3704 (National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, IL 61604) was grown in 150 ml of Luria–Bertani medium enriched with 0.22% (wt/vol) glucose (5) in a continuously agitated temperature-controlled shaker at 37°C for 2 h to obtain a population density of approximately 3 × 108 CFU/ml corresponding to an optical density of 0.2 at 630 nm.

Nisin preparation. Aplin & Barrett Ltd. (Dorset, England) kindly provided pure nisin. A stock solution of 10 mM nisin (1.4 × 108 IU/ml) was prepared by dissolving 175 mg of pure nisin in distilled water. The pH was adjusted to 4.5 with 0.1 N HCl, which ensures high bacteriocin solubility (19). The stock solution was sterilized by membrane filtration and stored at −20°C.

PEF and nisin combined treatment. Forty milliliters of *E. coli* culture were centrifuged at 10,000 rpm for 20 min and suspended in simulated milk ultrafiltrate (18). Aliquots of the *E. coli* suspension (900 μl) were transferred to microcentrifuge tubes and an appropriate volume of solution was added to reach the indicated concentrations in each case; finally, simulated milk ultrafiltrate was added up to a final volume of 1 ml. For performing PEF treatment, 400 μl of this bacterial suspension was withdrawn into a 0.2-cm electrode gap electroporation cuvette (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547) and subjected to PEF in a Gene Pulser II Electroporation system (Bio-Rad). Cells were exposed to one, three, and five pulses at electric fields intensities of 5.00, 6.25, 7.50, 8.75, 10.00, 11.25, and 12.5 kV/cm with 50 μF capacitance. The temperature elevation was avoided by allowing 5- to 10-s time intervals between pulses. The PEF-treated samples were kept in an ice-water bath during all of the procedures. For the enumeration of survival, *E. coli* cells were harvested by centrifugation (20 min at 10,000 rpm), suspended, and serially diluted in 0.1% peptone water. Dilution drops (20 μl) were spotted in triplicate onto Luria–Bertani agar plus 0.22% wt/vol...
TABLE 1. Real and coded values for the studied variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Real and coded values</th>
<th>Number of levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (kV/cm)</td>
<td>5 (-0.866); 6.25 (-0.5774) 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5 (-0.2887); 8.75 (0); 10 (0.2887); 11.25 (0.5774) 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.5 (0.866) 7</td>
<td></td>
</tr>
<tr>
<td>N (IU)</td>
<td>0 (-1); 3.57 (-0.5); 7.15 (0) 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.72 (0.5); 14.30 (1) 5</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>1 (-0.8165); 3 (0); 5 (0.8165) 3</td>
<td></td>
</tr>
</tbody>
</table>

aN, nisin; E, electric field strength; P, number of pulses.

Values in parentheses.

vol glucose. The number of CFU/ml was determined after incubation at 37°C for 24 h.

**Experimental design.** According to preliminary studies based on the survival of *E. coli* after different treatments, a Doehlert uniform shell design for three factors (7) was selected and analyzed using the software Statgrafic 5.1.

Processing variables investigated were nisin concentration (N), electric field strength (E), and number of pulses (P).

The real and coded values and number of levels for each variable are shown in Table 1. The response (Y) was expressed as the log of the survival fraction of *E. coli* (N/N₀), where N and N₀ are the number of CFU/ml after and before each treatment, respectively.

The Doehlert plan and experimental responses are shown in Table 2. Three replicates of the central point allowed calculation of the pure error of the method.

A full quadratic model containing 10 coefficients, including interactions terms, was used to describe the response observed to fit the following equation:

\[
Y = b₀ + b₁X₁ + b₂X₂ + b₃X₃ + b₄X₁² + b₅X₂² + b₆X₃² + b₇X₁X₂ + b₈X₁X₃ + b₉X₂X₃
\]

where bᵢ are the regression coefficients given by the model.

Contour plots were generated as a function of two variables (N and E) while the other (P) was kept constant.

**RESULTS AND DISCUSSION**

The effect of nisin on the survival fraction of *E. coli* is shown in Figure 1. The treatment of *E. coli* with 14.3 μM nisin, corresponding to 2,000 IU, produced a 4-log reduction in bacterial viability. This result indicates that nisin is able to produce gram-negative bacterial inactivation. However, the efficiency of nisin against *E. coli* was significantly inferior as compared to that reported for gram-positive cells. In fact, Bruno et al. (4) observed that 100 IU/ml were enough to produce a 5-log cycle reduction in *Listeria monocytogenes*. In agreement with these results, Carneiro de Melo et al. (5) showed that around one order more of nisin was required for the total growth inhibition of *E. coli* with respect to *Staphylococcus aureus*.

Figure 2 shows the effect of increasing voltage in three-pulse PEF treatments from 5 to 12.5 kV/cm. *E. coli* viability was significantly reduced by electric field intensities above 8.75 kV/cm. In agreement with these results, Pothakamury et al. (18) reported that *E. coli* could be inactivated by PEF strengths greater than 12 kV/cm.

Different combined treatment conditions of PEF and nisin resulted in a significant decrease in the survival fraction of *E. coli* (Table 2). Estimated regression coefficients for the dependent variable were obtained by multiple linear regression analysis (Table 3). The regression coefficients for the survival fraction showed that only E and N had significant linear and quadratic effects. Significant interaction was observed between EP, NP, and NE. The largest values of estimated coefficients for E (-1.6622) and EP (-1.2291) showed that they were the most important variables influencing the response. The linear effects of E and
TABLE 3. Model coefficients estimated by multiple linear regression analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-2.8461</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>-1.00182(^{b})</td>
</tr>
<tr>
<td>E</td>
<td>-1.66222(^{b})</td>
</tr>
<tr>
<td>P</td>
<td>(0.0137375)</td>
</tr>
<tr>
<td>Quadratic</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.0351(^{b})</td>
</tr>
<tr>
<td>E</td>
<td>-0.199245(^{b})</td>
</tr>
<tr>
<td>P</td>
<td>(-9.33008 \times 10^{-3})</td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>0.365473(^{b})</td>
</tr>
<tr>
<td>NP</td>
<td>-0.795698(^{b})</td>
</tr>
<tr>
<td>EP</td>
<td>-1.22919(^{b})</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.974316</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>NS(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\) Values in parentheses are not significant.
\(^{b}\) Significant \((P < 0.05)\).
\(^{c}\) NS, nonsignificant.

N and its negative values indicate that the survival fraction decreases with increasing E and N values. The negative quadratic term for E suggests that the survival fraction reaches a maximum as a function of this variable, reflecting the experimental behavior shown in Figure 2, in which there is a zone of maximum \(N/N_0\), and then it decreases as E increases. The positive quadratic term for N indicates that \(N/N_0\) reaches a minimum in function of this variable. The opposite was observed for the quadratic N term. The negative value of EP suggests a positive interaction effect between those variables. By contrast, the positive correlation coefficients for NE and NP indicate a negative interaction between nisin and electric field variables (E and P).

The coefficient of determination \((R^2)\) and lack of fit tested the model adequacy. The \(R^2 = 0.975\) indicated that only 2.5% of the total variation was not explained by the model. Furthermore, the lack of fit was not significant, which means that the model included all appropriate functions of the independent variables.

The predicted results by the response surface methodology were validated experimentally. As shown in Figure 3, a good correlation between experimental and predicted results was found \((R^2 = 0.80, P < 0.05)\). Thus, we conclude that the Doehlert approach and derived model allows a good prediction of the effect of the combination of the studied variables on \(E. coli\) viability.

Contour plots were developed for the log \(N/N_0\) as a function of E and N, with the number of pulses fixed at 1, 3, or 5 (Fig. 4A through C). For all the levels of nisin evaluated, increasing E intensity decreased \(E. coli\) survival, this effect being more significant as P increased. Simpson et al. (21) pointed out that increasing the electric field strength and number of pulses resulted in increasing inactivation of both \(L. monocytogenes\) and \(Salmonella Typhimurium\). As was shown in the analysis of the interaction coefficients, the electric field intensity and number of pulses affected the response of \(E. coli\) survival against nisin concentration. When only one pulse was applied (Fig. 4A) increasing values of nisin produced a continuous reduction of the survival fraction. However, when three pulses (Fig. 4B) were applied, the linear effect was partially lost, reaching a plateau for nisin values above 7.15 \(\mu\text{M}\). The regression model predicted this plateau as a minimum followed by a slight increase in the curve trend, which is a consequence of the significance of a positive N quadratic term. A further increment of P (Fig. 4C) caused the loss of the linear response with nisin. The regression model predicted that the effectiveness of nisin decreased when PEF was applied, this phenomenon being clear evidence for electric fields that are not effective per se. Actually, for an electric field of 5 kV/cm, the combined treatment showed an \(E. coli\) inactivation lower than would be expected for the nisin doses alone (Fig. 1), this effect being enhanced by increasing the number of pulses (Fig. 4). This behavior suggests that nisin could be inactivated by PEF. Many authors have outlined the inactivation of different enzymes or the occurrence of structural changes leading to increased sensitivity of protein to proteases after PEF treatment (3). Figure 5 shows the activity of 7.15 \(\mu\text{g}\) of nisin during combined treatments with an electric field intensity of 5 kV/cm as a function of the number of pulses applied. Only one pulse of 5 kV/cm reduced the activity of nisin by approximately 15%, but 50% of the initial activity was lost when five pulses were applied. However, nisin, first subjected to one, three, or five pulses of 5 kV/cm and assessed on \(E. coli\), showed no activity and attained a degree of inactivation larger than would be expected from the combined treatment. Consequently, in the presence of bacterial cells nisin appeared to be protected partially against PEF.

The possibility of improving the combined treatments by avoiding nisin inactivation was evaluated by adding the nisin after the \(E. coli\) were subjected to PEF treatment. Nevertheless, the results obtained showed no differences from the response predicted by the regression model (Fig. 6). Furthermore, addition of nisin just before the PEF treatment did not change the lethality response (data not shown). Thus, the loss of activity could not be attributed to the action of PEF over nisin, but it seems to be a consequence of the application of PEF over the bacterial cells.

Nisin adsorption on food components is a well-known...
FIGURE 4. Combined effect of different nisin doses and electric field intensities on the survival fraction of E. coli. Isoresponse lines show the bacterial inactivation expressed as the logarithmic of survival fraction for PEF treatments of one (A), three (B), and five (C) pulses.
E. coli inactivation by combined pulsed electric fields and nisin

FIGURE 5. Residual activity of nisin after combined treatments at one, three, and five pulses from 5 kV/cm. The 100% activity corresponds to the log of survival fraction of E. coli given by 14.3 μM nisin.

phenomenon. When it is added to complex food products, such as meat or milk, it undergoes a slow but steady decay in its antimicrobial activity (10, 23). The decreased nisin activity in foods has been mainly attributed to adsorption onto proteins and lipid particles due to the strong cationic and hydrophobic character of nisin (6, 20). The application of PEF produces bacterial cell permeabilization, leading to the leakage of bacterial cell components. Simpson et al. (21) reported that increasing the field strength and number of pulses resulted in increasing levels of leakage of UV-absorbing substances from L. monocytogenes and Salmonella Typhimurium, and this was also observed for PEF treatments without lethal effects. Such leakage of cellular debris and its putative interaction with nisin could be the cause of the loss of antimicrobial activity observed in the present work.

However, in spite of this, much evidence suggests that PEF appears to enhance bacterial sensitivity against the remaining active nisin. This fact became evident by comparing combined treatments carried out at electric field intensities that, applied alone, had quite similar effects. For instance, a combined treatment with 7.15 μM nisin and three pulses exhibited an increment in lethality (around 1.3-log cycles) if electric field intensity was increased from 5 to 8.75 kV/cm (Fig. 4B), with the effect of those PEF treatments alone being around −0.5-log cycles (Fig. 2). In addition, for electric field strengths effective per se (i.e., over the abovementioned range), a bacterial inactivation higher than that expected from each treatment separately (i.e., 1,000 IU of nisin in combination with one, three, or five pulses from 10 kV/cm) could be observed. However, the difficulty of assessment of the amount of active remaining nisin that takes part in a combined treatment makes it difficult to state whether a synergistic or an additive positive effect arises from its combinations with PEF.

The described phenomena were more evident when the number of pulses were increased from one to three, but due to the improvement in PEF lethality, they were not seen at five pulses (Fig. 4).

Kalchayanand et al. (13) reported that PEF alone (12.5 kV/cm) applied to E. coli O157:H7 reduced bacterial viability by 4-log cycles. However, this treatment in combination with nisin further reduced viability by only 1 more log cycle. Our results support the minimal effects of nisin observed by this author at 12.5 kV/cm.

The primary target of nisin in gram-positive bacteria is the cytoplasmic membrane, resulting in an efflux of essential small cytoplasmic components, depletion of both components of proton motive force (the transmembrane electric potential Δψ, and ΔpH), and cessation of biosynthesis (1, 3). Nisin has been shown to act on several species of gram-negative bacteria, provided that the integrity or barrier function of the outer membrane is first disrupted (12). The nature of the positive interaction between nisin and electric field strength observed at E values where PEF itself does not have a lethal effect may be explained by the formation of nonlethal pores in the outer membrane of the gram-negative bacteria allowing nisin to reach the cytoplasmic membrane. Similar results were obtained by Hauben et al. (11) using another nonthermal methodology. E. coli strain MG1655 treated with high pressure became sensitive to nisin as a consequence of outer membrane disruption, as evidenced by leakage of the periplasmic enzyme β-lactamase.

It has been reported that nisin forms pores in the cytoplasmic membrane only when the transmembrane electrical potential (Δψ), negative inside, is high enough (8) or when the ΔpH inside the cell is alkaline (9, 15). This mechanism is consistent with the wedge model (8) that assumes an oriented interaction of nisin with the membrane relative to ΔpH. This model for pore formation by nisin might explain the lack of positive interaction between nisin and PEF at electric field strengths where PEF alone has lethal effects (Fig. 4C).

When subjecting E. coli cells to electric fields that cause a lethal effect, shrinkage of the outer and cytoplasmic membranes could allow entrance of nisin into the cytoplasm. Internalized nisin would not be able to form pores from the cytoplasm due to inadequate transmembrane electrical potential and ΔpH (inverted), according to the wedge model for pore formation. Furthermore, if the damage caused by PEF was important enough to produce a Δψ and ΔpH dissipation, no additional effect of nisin would be expected.

Shrinkage of the cytoplasmic membrane away from the outer membrane (2) with crenations in the outer membrane indicative of this phenomenon has been shown in micro-
graphs of thin sections of E. coli ATCC 11229 suspended in simulated milk ultralfiltrate and subjected to PEF.

As a consequence of the positive interaction of nisin and PEF, several combined conditions of E, N, and P levels that produce a significant E. coli survival reduction could be selected from Figures 4A through C. A 4-log cycle reduction may be accomplished with around 1,000 IU/ml (7.15 μM) of nisin and three pulses of 11.25 kV/cm or 500 IU/ml for five pulses of the same intensity. Taking into account that the amount of effective active nisin in the above mentioned examples was indeed much less than that added initially, we speculate that a synergistic effect is possible that takes place during combined treatments.

Hence, these results illustrate the operational advantage that arises from the proposed methodology and how its potential for nisin adsorption to cellular debris or foodborne components could be reduced. In this sense, Wei and Hansen (22) suggested the possibility of obtaining nisin analogues that will differ in properties such as solubility and chemical activity.

The proposed synergistic effect suggests the possibility of using PEF as a practical tool for enhancing the effect of natural antimicrobials as well as broadening its operative range. Regarding these results, PEF performance could also be increased by its combination with antimicrobial treatments as an alternative to the use of high-intensity PEF.

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REFERENCES