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Modeling the combined effects of enterocins A and B, lactate, and EDTA on the growth of *Salmonella* at different temperatures

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Summary. The effects of enterocins A and B (produced by *Enterococcus faecium* CTC492), lactate, and ethylenediaminetetraacetic acid (EDTA) on the growth of *Salmonella* were modeled together with temperature using the response surface methodology. Six serovars of *Salmonella enterica* were inoculated (ca. 10^3 cells/ml) in brain-heart infusion broth with different levels of the studied factors and then incubated at different temperatures. The results showed that while *Salmonella* growth was affected by all the factors, temperature was the most important factor influencing the time to detection of the pathogen. All factors, including temperature, showed significant two-way interactions. The presence of enterocins A and B, lactate, and EDTA had an inhibitory effect that was enhanced at suboptimal temperatures for growth, thus delaying the time to detection. Moderate-low concentrations of lactate and EDTA increased the inhibitory effect of enterocins A and B. The effectiveness of these bacteriocins was not further enhanced by high concentrations of lactate (>3.6%) or EDTA (>200 mg/l). The mathematical model obtained from these analyses provides a useful tool to assess the effects of natural antimicrobials and their interactions with other growth-related factors on the growth response of *Salmonella*. The results can be applied to determine the most effective combination of hurdles to be used in the preservation of food products. [**Int Microbiol** 2008; 11(1):11-16]

Key words: *Salmonella* · enterocins A and B · lactate · EDTA · bacterial growth · response surface

Introduction

Biopreservation of foods offers an alternative control measure for improving the stability and safety of mildly processed food products and has therefore been the focus of increased attention in the last few years. Biopreservation reduces the amounts of chemical preservatives as well as the intensity of heat treatments, both of which can negatively affect food quality [17,21]. In this context, bacteriocins have been widely studied as natural antimicrobials that delay food spoilage and increase food safety, for example in meat and meat products [4]. The bacteriocins produced by lactic acid bacteria are cationic, hydrophobic or amphiphilic, low-molecular weight peptides [27]. They have an inhibitory effect against closely related microorganisms, mostly gram-positive bacteria such as *Listeria monocytogenes* and *Clostridium botulinum*. However, the inhibition spectrum of bacteriocins can be extended to gram-negative bacteria exposed to hurdles that disrupt the bacterial outer membrane [7,17]. These hurdles include cation-chelating agents, such as ethylenediaminetetraacetic acid (EDTA), and other physicochemical sublethal stresses, such as acidic and osmotic stress, heat or cold shock, and high hydrostatic pressure [2,6,9,18,32].

Before antimicrobial substances can be used for biopreservation purposes, their performance against the target organisms must be evaluated. Several publications have described the beneficial effects of outer-membrane-permeabilizing agents used in combination with nisin to inactivate

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gram-negative bacteria, including *Salmonella* and *Escherichia coli*, in certain food products, e.g., cantaloupe [33], cooked ham and bologna sausages [19], chicken skin [10,26], ground beef [16], and meat homogenate [18]. Another study examined the effects of enterocin AS-48 against *E. coli* in the biopreservation of apple juice [2].

Microbial modeling has proven to be a useful approach to assess and quantify the effects and possible interactions of environmental conditions on the growth of food-related bacteria. This approach has already been applied to investigate the behavior of *L. monocytogenes* exposed to nisin in combination with other antimicrobial factors, including leucocin (a bacteriocin synthesized by *Leuconostoc gelidum*), sodium chloride, and EDTA, as well as temperature [8,11,29], and the response of *E. coli* to reuterin (a growth inhibitor produced by *Lactobacillus reuteri*), salt, pH, and temperature conditions [30].

Several authors have modeled the growth of *Salmonella* spp., mainly as a function of temperature, pH, and water activity [22,28]. However, there are currently no studies that have modeled the effects of bacteriocins when used in combination with other hurdles that may increase the effectiveness of natural antimicrobials against this food-borne gram-negative pathogen. Therefore, the aim of the present study was to model the effects of enterocins A and B (produced by *Enterococcus faecium* CTC492 [3]) when used in combination with lactate and EDTA on the growth response of *Salmonella* at different temperatures. Bacterial growth was measured in terms of "time-to-detection" (TTD, time to the nearest visible growth detection) in culture media. The interactions among the different hurdles were studied since their strategic use may contribute to improving the safety of food products.

Materials and methods

Bacterial strains. Six serovars of *Salmonella enterica* were used. The strains from our culture collection (CTC, formerly, Centre Tecnologia Carn; currently, IRTA Food Technology) were originally isolated from meat products and included serovars London (strain CTC1003), Schwarzergrund (CTC1015), and Derby (CTC 1022). The serovars Enteritidis (strain GN-3), Typhimurium (GN-6) and Rissen (GN-13) were of animal origin and kindly provided by the Center for Health Animal Research (CReSA, Autonomous University of Barcelona, Spain). The strains were obtained from –80ºC stock cultures and precultured twice in brain heart infusion (BHI, from Difco, Detroit, MI, USA) broth at 37ºC for 18 h under aerobic conditions. The cultures were appropriately diluted with BHI and a standardized inoculum was prepared by mixing together all six *Salmonella* serovar cultures.

Experimental design and analytical measurements. A cocktail of the six *Salmonella enterica* serovars was inoculated (ca. 10^3 cells/ml) in BHI broth containing different concentrations of enterocins A and B, extracted as described in Aymerich et al. [3]. The enterocins were added at concentrations of 0, 1000, 2000, 3000, and 4000 AU (arbitrary units)/ml; potassium lactate (Purac Biochem, Gorinchem, Netherlands) at 0, 1.2, 2.4, 3.6, and 4.8% (w/v), and EDTA (Merck, Darmstadt, Germany) at 0, 100, 200, 300, and 400 mg/ml. The cultures were incubated under oxic conditions at five different temperatures (6, 14, 22, 30, and 38ºC). These environmental conditions were combined according to a central composite design (CCD) [NIST/SEMATECH. (2003). e-Handbook of statistical methods. http://www.itl.nist.gov/div898/handbook/]. Six central points were chosen, which was sufficient to allow estimation of the experimental error. Additional combinations at the extreme temperatures were also assessed.

Changes in optical density (OD) at 560 nm were periodically measured in a microplate reader (EMax, Molecular Devises, BioNova Científica, Madrid, Spain) and the TTD was recorded as the nearest time to visible growth detection [5]. Six independent trials were carried out.

Mathematical modeling. The data were processed using the statistical package Statistica for Windows (Statsoft, Tulsa, OK, USA), which generated the polynomial equations describing the effects of the environmental factors, as individual and quadratic terms, and their interactive effects on the TTD. A stepwise linear regression procedure was applied. The goodness of fit of the obtained model was evaluated using the multiple determination coefficients (R^2), the Fisher F-test, and the derived *P*-values as well as the observed versus predicted values plot. In order to draw the corresponding response surface plots as a function of two independent variables, the other independent variables remained constant at a fixed level (i.e., the central value among those considered in the CCD). Some mathematical transformations (i.e., log, ln, square root) of the dependent variable were explored.

Results

The TTDs of the pooled six serovars of *Salmonella enterica* obtained from each combination of conditions established by the CCD were statistically analyzed by linear regression. The best-fitting ($r > 0.98$) and most significant ($P < 0.00001$) second-order polynomial equation was selected for the TTD model as a function of temperature, enterocins A and B, lactate, and EDTA. Through the stepwise procedure of the regression analysis, only the terms of the polynomial equation significantly different from zero ($P < 0.05$) were retained in the model. Mathematical transformations of the dependent variable (TTD) did not improve the result and therefore were withdrawn.

The growth of *Salmonella* was found to be affected by all of the studied environmental factors. Thus, temperature, enterocins A and B, lactate, and EDTA contributed with statistically significant ($P < 0.05$) coefficients to the final model for the TTD of the six pooled serovars of *S. enterica*. The polynomial model describing these effects is given in Eq. (1):

$$\begin{aligned} TTD = {} & 157.6133 - 10.6286[T] + 0.0060[B] + 4.6470[L] + 0.0526[E] \\ & - 0.0001[T \times B] - 0.3079[T \times L] - 0.0023[T \times E] \\ & - 0.0009[B \times L] - 0.00001[B \times E] + 0.0144[L \times E] \\ & + 0.1834[T^2] + 1.7592[L^2] + 0.0001[E^2] \end{aligned} \quad \text{(Eq. 1)}$$

where *TTD* is the time to detection (measured in hours), *T* is the growth temperature (ºC), *B* is the concentration of enterocins A

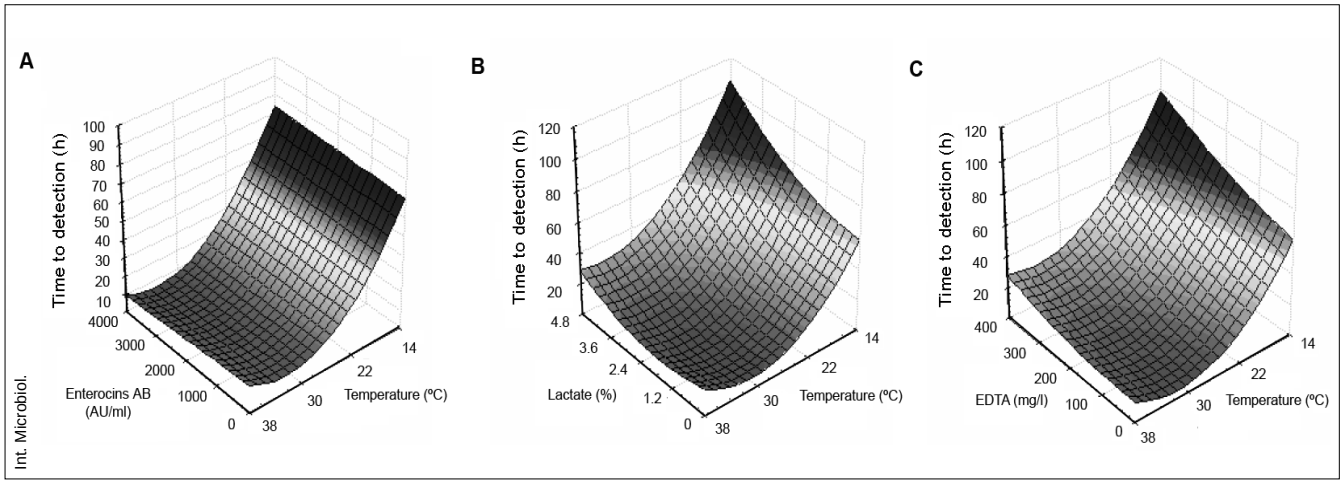

Fig. 1. Response surface plots of the combined effect of temperature and enterocins A and B (**A**), lactate (**B**), or EDTA (**C**) on the time to detection of *Salmonella enterica*.

and B (AU/ml), *L* is the concentration of lactate (% w/v), and *E* is the concentration of EDTA (mg/l).

The response surface plots derived from the TTD model are shown in Fig. 1 and are presented as a function of temperature and the concentrations of the other environmental factors. Figure 2 shows the response surface plots and the combined effect of enterocins A and B, lactate, and EDTA. In each figure, the independent variable not present was kept constant with respect to the central value of the experimental design (i.e., 2000 AU/ml enterocin A and B, 2.4% lactate, 200 mg/l EDTA, 22ºC growth temperature).

Temperature was the most important factor modulating *Salmonella* growth, as shown by the beta values from the regression analysis. Beta values are the standardized coefficients that permit comparative evaluation of the magnitude of the effects of the tested factors. Both the individual (beta = –3.71) and quadratic (beta = 3.36) terms concerning temperature were 10-fold-higher than those of the other factors included in the model (with beta values < 0.35). In fact, after 30 days at 6ºC, no growth was detected at all.

All the studied factors, except for enterocins A and B, had statistically significant quadratic terms, which was also evident by the nonlinear shape of the surface plots. Thus, the delay in *Salmonella* growth increased exponentially with decreasing temperature (especially below 30ºC), increasing lactate concentration (especially ≥2.4%) and, to a lesser extent, EDTA concentration.

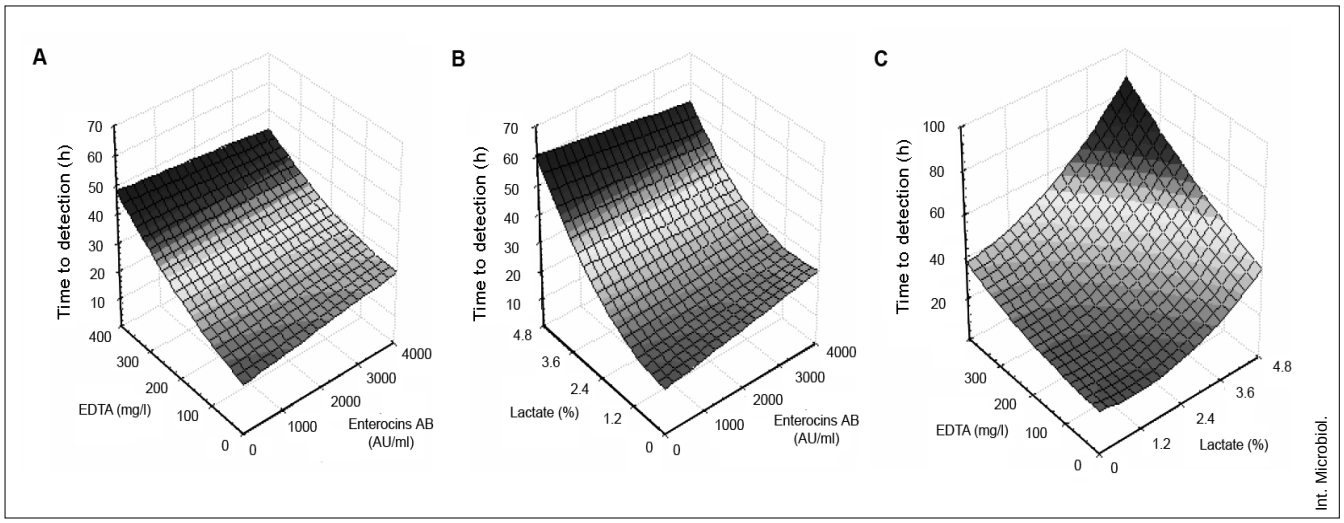

Fig. 2. Response surface plots of the combined effect of enterocins A and B and EDTA (**A**) or lactate (**B**) and EDTA and lactate (**C**) on the time to detection of *Salmonella enterica*.

From the significance of the interaction terms of the model formulated in Eq. (1) and the surfaces shown in Fig. 1, it can be seen that temperature interacted notably with the amount of lactate and EDTA, but much less intensively (although still significantly) with the bacteriocins. At an optimal temperature (around 37ºC), the growth of *Salmonella* was either hardly delayed or not affected at all by the included preservatives. However, decreasing growth temperature magnified the inhibitory effects of lactate and EDTA. When *Salmonella* was exposed to increasing concentrations of bacteriocins, a slight increase in the TTD was only appreciable at suboptimal temperatures (i.e., 14ºC). Two-way interactions between the studied preservatives were also statistically significant and included in the model. For a given concentration of enterocins A and B, the TTD tended to increase with increasing amounts of EDTA and lactate (Fig. 2A,B). Specifically, moderate-low concentrations of lactate and EDTA increased the effect of these bacteriocins. However, higher amounts of lactate (>2.7%) or EDTA (>200 mg/l) did not lead to further improvement in the effectiveness of the enterocins (Fig. 2A,B). Figure 2C shows that the interaction between lactate and EDTA with respect to the delay of *Salmonella* growth was much stronger than the interaction between these factors and the enterocins along the entire range of concentrations tested.

Discussion

Salmonella spp. has been recognized as an important zoonotic pathogen of economic and sanitary significance for animals and humans. In 2005, salmonellosis was the second most frequent food-borne disease within the European Union, after *Campylobacter* [14]. According to the European Food Safety Authority [15], risk mitigation during processing requires the implementation of the principles elaborated in the Hazard Analysis and Critical Control Point (HACCP) system, the maintenance of the cold chain, and the application of the hurdle concept, which states that several inhibitory factors—hurdles—that individually are unable to inhibit microbial growth, will be, nevertheless, effective when combined. Therefore, methods based on the hurdle technology to reduce or inhibit *Salmonella* are of primary importance for the food industry.

Preliminary experiments with the same pool of six serovars of *S. enterica* revealed that enterocins A and B alone (4000 AU/ml) did not significantly inhibit bacterial growth, since growth at 25ºC and 15ºC was detected in less than 24 h (data not shown). Similarly, exposure of *S. enterica* to lactate (≥5%) and EDTA (≥400 mg/l) resulted in only a slight delay in growth, which was detected in less than 48 h and 4 days at 25ºC and 15ºC, respectively. In view of these results, in the present work we decided to study, through a quantitative approach, the possibility of enhancing the effects of enterocins A and B when they are combined with lactate and EDTA.

Among the environmental factors studied, the only one that totally affected the growth of *S. enterica* serovars after the 30-day experiment was temperature: the lowest temperature applied (i.e., 6ºC), which was very close to the minimum growth temperature (5.2ºC) reported for *Salmonella* [20], inhibited growth of the bacterium. In fact, maintenance of the cold chain during the storage and distribution of foods is a major control measure to prevent the growth of *Salmonella*. However, low temperatures are not always applied correctly [23,24,31] and the use of other hurdles is recommended.

Interactions (of either additive or synergistic nature) among hurdles were demonstrated by the quantitative approach followed in this work. The interaction factors involving temperature (i.e., $T \times L$, $T \times E$) were more intense than those of the preservatives alone (i.e., $L \times E$, $B \times E$, $B \times L$). In the food chain, low temperatures are more often mandated for preservation and storage than room temperatures. Our results showed that the interactions of preservatives can be enhanced at low temperatures. In fact, chilling temperatures have been reported to sensitize gram-negative bacteria against nisin [10,13], which is in agreement with the fact that the inhibitory effect of enterocins A and B against *S. enterica* occurred at suboptimal temperatures, although only to a limited extent. It has been postulated that cold-shock modifies the nature of the outer membrane through the crystallization of phospholipids, resulting in damage to the permeable barrier, which in turn allows the bacteriocin to penetrate the bacterial cytoplasmic membrane. Sensitivity may vary between different strains and species depending on the fatty-acid composition of the outer membrane [10]. Boziaris and Adams [10] found that the addition of EDTA enhances the reduction in *Salmonella* growth after combined chilling plus nisin treatments of chicken skin. However, the reduction is much less than that in *Pseudomonas*. *Salmonella* has been reported to be more resistant to antimicrobial combinations of bacteriocins and sensitizing agents than other gram-negative bacteria [9,12,18,25].

In the present work, in addition to the temperature interactions, statistically significant interactions also occurred between enterocins A and B, EDTA and lactate, which contributed to the global model for *Salmonella* TTD. This model proposes an enhanced effect when the potentially inhibitory factors are combined.

The mathematical model developed in this work can be used to estimate the optimal combination of factors that max-

imally delay the growth of *S. enterica*. The surface response plots indicated that TTD can be delayed by the enterocins A and B in combination with moderate or low concentrations of other preservatives, for example <2.7% lactate and <200 mg/l EDTA. At higher levels of lactate and EDTA, the TTD remained fairly constant, even in the presence of increasing concentrations of bacteriocins. In contrast, the delaying trend of *Salmonella* TTD was exponential when lactate and EDTA were combined, even at high concentrations, thus suggesting a synergetic mechanism.

The effectiveness of bacteriocins is lower when these compounds are applied to food products, due to their binding to food constituents [1]. In the above-described experiment of Boziaris and Adams [10], the reduction in the growth of *Salmonella* in chicken skin was less than that obtained in broth. In the present work, the mathematical model was obtained from laboratory broth experiments and, although it is a step forward in this field, the results must be further validated in food samples.

In conclusion, the mathematical model developed in the present work can be used to assess the effect of natural antimicrobials (bacteriocins and lactate) and, e.g., EDTA and temperature on the growth response of *Salmonella.* The model also allows the evaluation of interactions between the factors assayed, thus assisting in the selection of the most effective amounts and combinations of hurdles to be applied in food processing. Our findings will assist in the design of experiments aimed at validating the feasibility of enterocins A and B as biopreservatives of food products. The end result should be the optimal application of hurdle strategies to improve food quality and safety.

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