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EXPERIMENTAL ARTICLES

# Interactive Effects of pH and Temperature on the Bacteriocin Stability by Response Surface Analysis[1]

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**Abstract**—The combined influence of pH and temperature on bacteriocins produced by three lactic acid bacteria, *Pediococcus pentosaceus* MMZ26, *Enterococcus faecium* MMZ17 and *Lactococcus lactis* MMZ25, isolated from Tunisian traditional dry fermented meat was studied using a second order orthogonal factorial design and response-surface methodology (RSM). This method allows estimating the interactive effects of pH and temperature on the stability of each bacteriocin. The high heat stability of the three bacteriocins was demonstrated, with optimum values at light acidic pH around 5.0, temperature below 90°C and short incubation times. This study contributes to a better understanding of relation between bacteriocins production and stability in order to enhance their, in situ, application as a food and feed biopreservative in fermented and/or heated food products.

*Keywords*: lactic acid bacteria, bacteriocin, response surface methodology.

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Because there is aversion towards the use of chemical preservatives in food, there is increasing interest in the use of lactic acid bacteria (LAB) as natural preservatives [1, 2]. These food-grade bacteria can improve the safety, shelf life, nutritional value, flavour and quality of the product and may function as probiotics and contribute to the general health of the consumers. These microorganisms can produce a variety of antimicrobial agents including organic acids like lactic and acetic acid, ethanol, carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins [3]. Bacteriocins have been defined as biologically proteinaceous substances exhibiting bactericidal activity against closely related species [4]. Some of these have a relatively broad spectrum of antibacterial activity against food spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* and in some case against gram negative bacteria [5], and thus, may be considered as promising biopreservatives.

In view of a widespread use of bacteriocins in food preservation, an important factor to consider for their application is the cost of their production. In fact, for high and maximum biomass and bacteriocin production at an industrial scale, an adequate experimental design (optimization) must be developed through the use of accurate models describing cell growth and product formation [6, 7]. The influence of certain parameters should be investigated to achieve the optimum experimental conditions for bacteriocin production and stability. Treating each factor separately would be very time consuming; furthermore, if several factors play a role, their interactions would not be discernable even if they were dominant [8]. Hence, the application of an adequate experimental design is the best strategy to obtain maximum information with a minimum number of experiments. The surface response methodology (RSM) can provide an empirical modeling of the activity, as a function of the diverse variables of interest [9]. Already successfully applied in many areas of biotechnology [10–14], RSM is well suited to the study of the main and interaction effects of distinct factors on bacteriocin production and stability [15–17].

In this paper, a multifactorial experimental design and response surface analysis were used to estimate the interactive effects of pH and temperature on the stability of bacteriocins produced by three LAB: *Pediococcus pentosaceus* MMZ26, *Enterococcus faecium* MMZ17 and *Lactococcus lactis* MMZ25 isolated from artisanal Tunisian fermented meat, in order to optimize their production and application.

## MATERIAL AND METHODS

**Microorganisms and media.** The bacteriocins producing strains (*Pediococcus pentosaceus* MMZ26, *Enterococcus faecium* MMZ17 and *Lactococcus lactis*

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**Table 1.** Coded experimental design for the response surface of maximum bacteriocin stability of Pediocin MMZ26, enterocin MMZ17, and lactococcin MMZ25 as a function c combined influence of temperature and pH

| Codified values | Natural values | |
|---|---|---|
| | $T$ (°C) | pH |
| −1.414 | 59.4 | 2.8 |
| −1 | 70 | 3.5 |
| 0 | 95.5 | 5.3 |
| +1 | 121 | 7 |
| +1.414 | 131.6 | 7.7 |

Codification : Vc = (Vn − Vo)/ΔVn.
Decodification: Vn = Vo + (ΔVn*Vc).
Vc : coded value; Vn natural value; Vo : natural value.
In the centre of the experimental domain; ΔVn:
increment of Vn corresponding to one unit of Vc.

MMZ25) were isolated from "Gueddid", an artisanal Tunisian fermented meat [18]. These strains are a producer of pediocin, enterocin, and lactococcin respectively. *Listeria innocua* F was used as the indicator organism for the estimation of bacteriocin activity. Cultures were maintained as frozen stocks at −20°C in Nutrient Broth containing 15% (v/v) of glycerol and were propagated twice in appropriate media before experimentation. Working cultures were prepared monthly from frozen stock cultures and maintained at 4°C on de Man Rogosa Sharpe agar (MRS, Difco, United States) for *Pediococcus pentosaceus* MMZ26 and *Lactococcus lactis* MMZ25 and Brain Heart Infusion BHI (Difco, USA) for *Enterococcus faecium* MMZ17 and *Listeria innocua* F.

**Extraction of antibacterial activity.** All bacteriocin-producing cultures of LAB were grown in 250 mL Erlenmeyer flasks containing 50 mL of medium, on a rotary shaker (Innova 4330, New Brunswick Scientific Co., Inc., New Jersey) (200 rpm at appropriate temperature), until the early stationary phase. All cultivations were started with a 2% (v/v) inoculum of a 12-h culture. After centrifugation at 27200 *g* for 15 min at 4°C, the supernatants containing overall antibacterial activity (bacteriocin extract) were frozen until further use.

**Evaluation of bacteriocin activity.** Quantitative activities of bacteriocin extracts from culture samples of the three LAB under investigation were estimated by using a photometric assay on culture tubes [19]. *Listeria innocua* F was used as target organism. The method consists of the determination of growth inhibition at 700 nm of the target organism caused by serial dilutions of bacteriocin extracts. The method was carried out as follows: bacteriocin extracts were diluted as needed in distilled sterile water (this step eliminated the need to correct the pH of bacteriocin extracts). Two point five mL of the diluted bacteriocin extracts were added in sterile culture tubes. Each tube was inoculated with 2.5 mL of a culture of *L. innocua* F (diluted to an absorbance of 0.2 at 700 nm). Controls consisted of three culture tubes in which the diluted bacteriocin extract was substituted by distilled sterile water. The tubes were incubated for 6 h at 30°C. Growth inhibition was measured spectrophotometrically at 700 nm. Dose-response curves were obtained from these data. Bacteriocin activity was calculated as Bacteriocin Units (BU $mL^{-1}$, 1 BU $mL^{-1}$ = amount of bacteriocin needed to obtain 50% growth inhibition (lethal dose 50 (LD50) Compared to control tubes) [19].

**Experimental design.** A multifactorial composite orthogonal design [20] based on five levels and two variables was used to optimize bacteriocin stability as a function of pH and temperature. The design consisted of 16 experiments with four ($2^2$) factorial points, four axial points to form a central composite design with astar points fixed at ±1.414 to account for orthogonality and five centre points for replication. Once the experiments were performed, the experimental results were fitted with a 2nd order polynomial function:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2.$$

Where, $Y$ is the predicted response; $b_0$ the intercept; $b_1$, $b_2$ the linear coefficient; $b_4$, $b_5$ the squared co-efficient and $b_3$ the interaction co-efficient.

Response surfaces were depicted from the empirical equations derived of design. The range and coding of the two variables are shown in Table 1 and composition of the various runs of the central composite design is shown in Table 2. Bacteriocin extracts from 18 h cultures of the three bacteriocin-producing LAB under investigation were adjusted at different pH values with 5 N HCl or NaOH and incubated for 5, 10 and 20 min at the corresponding temperature, according to the experimental matrix defined by the design used. After incubation the samples were adjusted to pH 6.0 and monitored for remaining activity (% RA). Controls consisted of samples of untreated bacteriocin extracts.

## RESULTS AND DISCUSSION

The effects of pH, and temperature on the bacteriocin stability of pediocin MMZ26, enterocin MMZ17 and lactococcin MMZ25 were determined by modulating the variables according to a complete second-order orthogonal design. The empirical models obtained with this procedure permitted the evaluation of the stability of the extracts (without the need to assay all the combinations of the variables and to calculate the optimum conditions of stability). In this design, temperature ranged from 59.4 to 131.6°C, and pH from 2.8 to 7.7 units (Table 1). The choice of these values was, first, according to the literature describing the effect of pH and temperature comportment on bacteriocins (thermo-resistance and pH tolerance), and also, in view of the possible applications of bacteriocins as food preservatives regarding usual levels

**Table 2.** Composition of the various runs of the central composite design of the Table 1

| Treatment (run) | pH | | Temperature | |
|---|---|---|---|---|
| | Coded level | Physical value | Coded level | Physical value (°C) |
| 1 | 1 | 7 | 1 | 121.0 |
| 2 | −1 | 3.5 | 1 | 121.0 |
| 3 | 1 | 7 | −1 | 70.0 |
| 4 | −1 | 3.5 | −1 | 70.0 |
| 5 | 0 | 5.3 | +1.414 | 131.6 |
| 6 | 0 | 2.8 | −1.414 | 59.4 |
| 7 | +1.414 | 5.3 | 0 | 95.5 |
| 8 | −1.414 | 5.3 | 0 | 95.5 |
| 9 | 0 | 5.3 | 0 | 95.5 |
| 10 | 0 | 5.3 | 0 | 95.5 |
| 11 | 0 | 5.3 | 0 | 95.5 |
| 12 | 0 | 5.3 | 0 | 95.5 |
| 13 | 0 | 5.3 | 0 | 95.5 |
| 14 | 0 | 5.3 | 0 | 95.5 |
| 15 | 0 | 5.3 | 0 | 95.5 |
| 16 | 0 | 5.3 | 0 | 95.5 |

existing in foods and in their processing operations (for both variables).

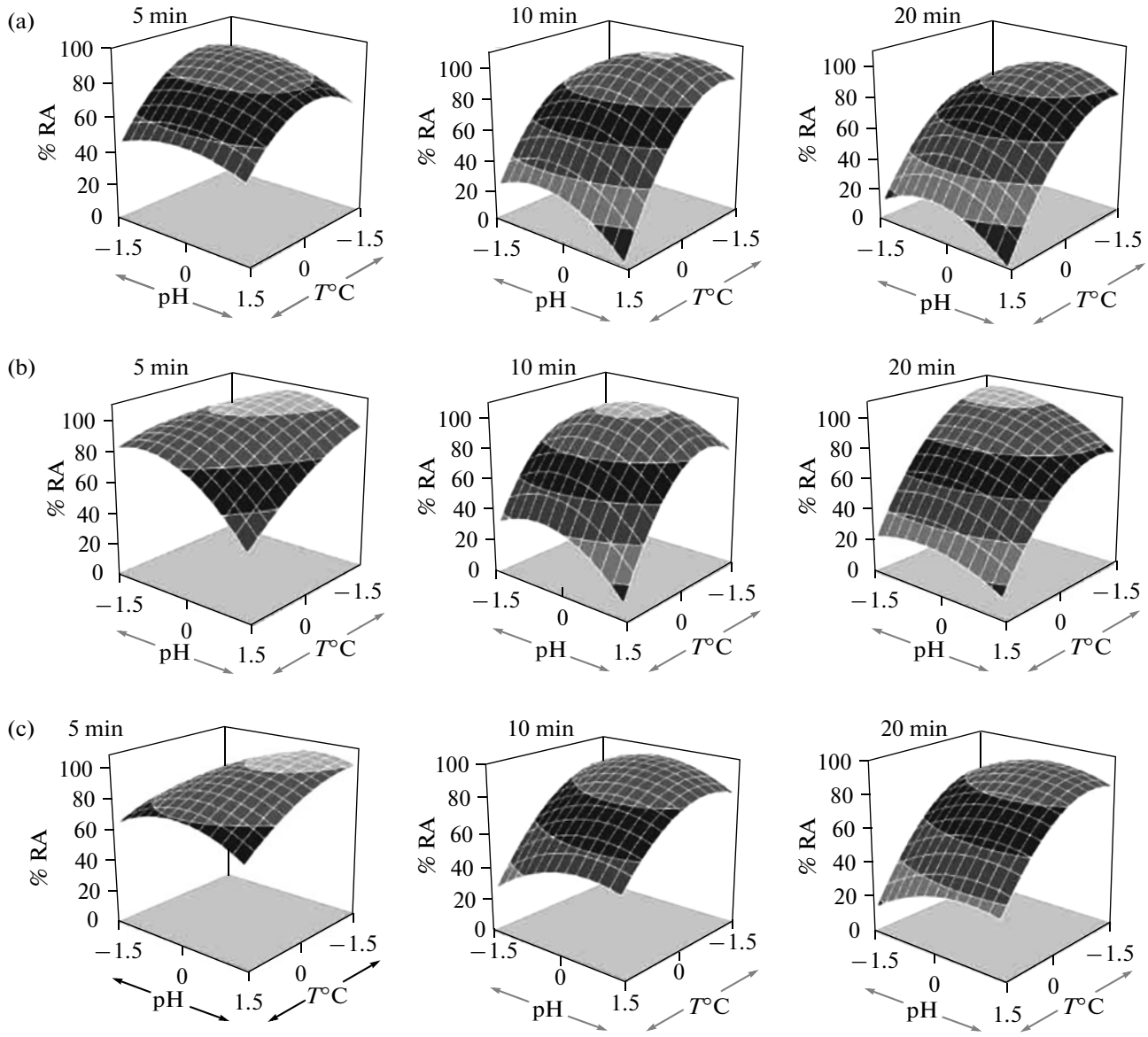
Statistical analysis of results showed that, in the range studied, the two variables (pH/$T$°C) have a significant effect on bacteriocin stability. The empirical equations obtained for the residual activity (RA) (Table 3) were highly consistent, being the coefficients acceptable according to the Student's $F$-test ($\alpha = 0.05$) and the validity of the equations according to the Fisher $F$-test ($\alpha = 0.05$). Despite the quadratic effect of the temperature was most significant, interactions between the two variables (pH/$T$°C) were observed for: lactococcin MMZ25 after 5, 10 and 20 min of incubation, enterocin MMZ17 after only 5 min of incubation, and pediocin MMZ26 after 5 and 10 min of incubation (Table 3). In all cases, the pH range (2.8–7.7) had no considerable influence on the response by comparison to the effect of temperature. The response surfaces of the % residual activity (% RA) after 5, 10, and 20 min of pH/$T$°C treatment are depicted in figure. According to the assayed experimental domains (figure), enterocin MMZ17 showed a high mean stability, especially after 5 and 10 min of incubation, whereas, pediocin MMZ26 was the most sensitive bacteriocin, also after 5 and 10 min of incubation. Moreover, after 20 min of incubation, the effect of the temperature was considerable, especially in the upper limit (131°C) which caused the lost of bacteriocin activity despite a high resistance of the enterocin MMZ17. According to the Table 4, which summarizes the maxima of stability (pH/$T$°C) after different times of incubation, the stabilities of the three bacteriocins were maximal at a light acid pH around 5.0, and temperature below 90°C.

## DISCUSSION

The effect of some relevant crucial factors, prevailing in the food environment, such as temperature, pH, media composition, availability of certain essential compounds, presence of inhibitory compounds, etc... on bacteriocin production and stability has been reported by several works [9, 17, 21–26]. In this study, a second-order rotatable design as a function of temperature and pH and their interaction, led to the identification of key factors from which response surfaces where constructed and bacteriocin stability could be modeled by a two variable polynomial function. The response surface methodology used in this study indicated an interaction between temperature and pH and showed that, in the range studied, the two variables have significant effects on bacteriocin stability. The quadratic effect of the temperature was the more significant effect because there was a steep hill response with a marked curvature along the temperature axis (figure). In all cases, the overall initial activity was al-

**Table 3.** Empirical equations obtained for remaining activity (% RA) of bacteriocin samples after pH and heat treatment

| Bacteriocin samples | Empirical equations |
|---|---|
| Enterocin MMZ17 | % RA (5 min) = $98.67 - 7.39T + 3.22\,\mathrm{pH} - 2.97T\,\mathrm{pH} - 3.75T^2 - 5.77\,\mathrm{pH}^2$ |
| | % RA (10 min) = $86.64 - 14.37T + 2.04\,\mathrm{pH} + \underline{0T\,\mathrm{pH}} - 7.15T^2 - 4.53\,\mathrm{pH}^2$ |
| | % RA (20 min) = $81.9 - 19.97T + 2.75\,\mathrm{pH} + \underline{0T\,\mathrm{pH}} - 8.4T^2 - 4.04\,\mathrm{pH}^2$ |
| Pediocin MMZ26 | % RA (5 min) = $99.92 - 8.67T - 6.06\,\mathrm{pH} - 4.96T\,\mathrm{pH} - 3.14T^2 - 7.64\,\mathrm{pH}^2$ |
| | % RA (10 min) = $99.77 - 16.44T - 3.59\,\mathrm{pH} - 3.17T\,\mathrm{pH} - 15.03T^2 - 8.08\,\mathrm{pH}^2$ |
| | % RA (20 min) = $86.36 - 23.3T - 6.64\,\mathrm{pH} + \underline{0T\,\mathrm{pH}} - 9.77T^2 - 4.35\,\mathrm{pH}^2$ |
| Lactococcin MMZ25 | % RA(5 min) = $87.96 - 8.73T - 3.39\,\mathrm{pH} - 2.11T\,\mathrm{pH} - 9.03T^2 - 3.54\,\mathrm{pH}^2$ |
| | % RA (10 min) = $91.56 - 21.72T - 0.93\,\mathrm{pH} - 4.24T\,\mathrm{pH} - 12.92T^2 - 7.34\,\mathrm{pH}^2$ |
| | % RA (20 min) = $79.01 - 21.93T - 0.93\,\mathrm{pH} - 2.59T\,\mathrm{pH} - 9.99T^2 - 7.66\,\mathrm{pH}^2$ |




Response surface graph showing % residual activity (% RA) after both pH and temperature treatment of pediocin MMZ26 (a), enterocin MMZ17 (b), and lactococcin MMZ25 (c) samples for 5, 10, and 20 min, according to the experimental design and model in Tables 1 and 2. Maximum pH, $T$°C = 1.5; minimum pH, $T$°C = −1.5.

most retained for temperatures below 90°C, low pH (around 5.0) and short incubation times. The same results was found by [17] when they tested the effect of pH/$T$ °C on stability of pediocin and nisin produced respectively by *P. acidilactici* NRRL-B-5627 and *Lc. lactis* subsp. *lactis* CECT 539.

In this study, the residual activity of the three bacteriocin tested was maxima at acid pH around 5.0, and

**Table 4.** Optimum temperature and pH values for each bacteriocin samples

| Time of incubation | Pediocin MMZ26 | | | Enterocin MMZ17 | | | Lactococcin MMZ25 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 min | 10 min | 20 min | 5 min | 10 min | 20 min | 5 min | 10 min | 20 min |
| $T$°C | 58.86 | 81.87 | 67.04 | 67.72 | 69.87 | 65.17 | 81.37 | 3.27 | 67.1 |
| pH | 5.37 | 5.04 | 5.01 | 5.93 | 5.64 | 5.84 | 4.91 | 5.58 | 5.47 |
| Maximum RA (%) | 102.64 | 104.12 | 98.55 | 103.08 | 94.09 | 94.32 | 91.5 | 100.2 | 91.16 |

temperature below 90°C. This heat-stability at slightly acid pH has been described for most bacteriocins [17, 27]. According to the literature, maximum specific production has been registered mostly in the pH range 4.5–5.5 [28–30]. Moreover, most of the inhibitory compounds are unstable above neutral pH, with some exceptions. This behavior is attributed to their high content of glycine and to the formation, at a molecular level, of globular structures and strong hydrophobic interactions in the molecule [31]. These results suggests that the bacteriocins described inthis study can withstand the conditions normally encountered in food processing, so would remain effective during processing and might have applied interest in medium-acid fermented food products whose final pH values fall in such range, this including a number of fermented and ripened dairy and meat products.

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