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# Stress Response Kinetics of Two Nisin Producer Strains of *Lactococcus lactis* spp. *lactis*

**Ömer Şimşek · Sencer Buzrul · Nefise Akkoç · Hami Alpas · Mustafa Akçelik**

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**Abstract** The purpose of this study is to determine the survival and nisin production behaviors of two strains of *Lactococcus lactis* under different stress conditions that represent the food ecosystem. In this respect, the survival ratios of two nisin producers were determined under different pH, temperature, NaCl, and bile salt concentrations. Then, nisin production levels of the strains were determined at each stress conditions. Both strains had similar growth or inactivation patterns under the same stress conditions. NaCl and bile salt stresses on the survival ratio of the strains could be successfully described by the exponential decay function, whereas Gaussian function produced good fits for temperature and pH stresses. The nisin activity of two nisin producers (in their mid-exponential and/or early stationary phase) decreased dramatically under all stress conditions, except osmotic (NaCl) and low temperature applications. The results of this study showed that two nisin producers had similar adaptive responses under severe stress conditions, which could be described by appropriate mathematical equations. Moreover, the effect of harsh environment on the nisin activity of *L. lactis* strains depends on the stress factors applied.

**Keywords** *L. lactis* spp. *lactis* · Nisin production · Stress response · Survival · Growth

## Introduction

Lactic acid bacteria consist of a versatile group of microorganisms generally regarded as safe and have profound applications in food fermentation. Apart from their beneficial characteristics, such as acid production, proteolytic activity, and aroma formation—which

Ö. Şimşek
Institute of Biotechnology, University of Ankara, Ankara, Turkey

S. Buzrul · H. Alpas
Food Engineering Department, Middle East Technical University, Ankara, Turkey

N. Akkoç · M. Akçelik (✉)
Department of Biology, Faculty of Science, University of Ankara, Tandoğan, 06100 Ankara, Turkey
e-mail: akcelik@science.ankara.edu.tr

have direct relevance in food fermentation processes—they have an enormous potential to inhibit microorganisms through the production of bacteriocins, mainly nisin [1, 2]. *Lactococcus lactis* is a commercially important member of lactic acid bacteria, since it is widely used in the manufacture of dairy products and production of antimicrobials used as natural food preservatives. A possible application of *L. lactis* is now being extended to the area of medicine as a vaccine delivery vehicle to the gastrointestinal tract [3]. Nisin producer *L. lactis* strains have to overcome some severe stresses, such as acid, heat, osmotic shock, and bile during the industrial processes of fermented foods and during the passage through gastrointestinal tract. However, such stresses may reduce the physiological activity of the cells, which could also be related with nisin production.

Several physiological changes have been observed in bacteria with the changing environment conditions, which are generally referred as stress response [4, 5]. One possible survival mechanism is the adaptive response; when cells are exposed to a moderate level of stress, they acquire increased resistance when they are subsequently exposed to extreme levels of the same stress [6]. In adaptive response, it has been demonstrated that bacteria either produce or modify proteins at the molecular level to improve their adaptation to stress conditions. Therefore, many different proteins, such as DnaK and CpsA, produced by different species of bacteria have been examined [7, 8]. Another common behavior of bacteria under stress conditions is the confinement of metabolic activity, and the most conventional way to accomplish this activity is to eliminate the secondary genetic material, mainly plasmids.

Nisin is synthesized in early active growth phase, and the production rate is maximal toward the end of the exponential growth phase or the early stationary phase. Cells completely stop nisin biosynthesis as they enter the mid- or late-stationary growth phase [9]. It is well known that nisin production at fermentation systems is influenced by many factors, mainly carbon starvation and fermentation conditions. Especially the outcome of fermentation conditions may cause unfavorable conditions for nisin producer *L. lactis* strains that resulted particularly or completely lost nisin production [9, 10]. On the other hand, nisin producer strains have been successfully used as protective cultures in model food systems, mainly in cheese, to inhibit several pathogens. However, the physiological behavior of the nisin producers against several stress conditions (NaCl, pH, and temperature) representative to the food systems is far from clear. Understanding of how nisin producer cells survive is of interest and may lead to the development of strategies for improved application of these cells both industrially and therapeutically.

This study reports an assessment of behavior of two different nisin producer strains of *L. lactis* against NaCl, bile salt, heat, and pH stresses. Results were then formulated by using survival ratio for the growth or inactivation. Furthermore, the nisin production rates of mid-exponential and early stationary phase cells under stress conditions were analyzed.

## Materials and Methods

### Bacterial Strains and Culture Conditions

Nisin A producer strains *L. lactis* spp. *lactis* LL27 (wild-type natural isolate) and *L. lactis* spp. *lactis* ATCC 11454 were used in this study. They were grown at 30°C on M17 medium (Merck, Darmstadt, Germany) containing 0.5% glucose (M17G). The pH of standard M17G (Oxoid) is 6.9, and it contains no bile salt. To determine the nisin activity, *Micrococcus luteus* NCIMB8166 was used as a sensitive indicator that was grown

aerobically in Luria–Bertani medium (Fluka, Steinheim, Germany) at 37°C. For long-term storage, stock cultures were prepared by mixing 8 mL of a fresh culture with 2 mL of glycerol (Difco, Detroit, USA) and then freezing 1 mL aliquots of this mixture at −80°C in 2 mL sterile cryovials (Nalgene, Rochester, NY, USA).

### Stress Treatments

Cultures of nisin producer *L. lactis* spp. *lactis* BLL27 and *L. lactis* spp. *lactis* ATCC11454 grown overnight in M17G broth were harvested by centrifugation at 5,000×*g* for 10 min, washed in 0.85% NaCl, and refreshed with fresh M17G broth. Cells were then inoculated in fresh broth or stress conditioning media as specified below. The cell densities in treatments were adjusted to an optical density at 600 nm ($OD_{600}$) of 0.02.

The medium for the control (no stress treatment) and heat shock treatments were sterile M17G broth. The pH stress medium was sterile M17G broth adjusted between pH 3.0 and 12.0 with 5 N HCl, and the osmotic shock medium was sterile M17G broth supplemented with NaCl to a final concentrations ranging between 1.0% and 9.0% and for bile salt ranging between 0.025% and 0.25%. All cultures except heat shock cultures were incubated at 30°C for 3 h. Heat shock samples were incubated at between 5°C and 45°C. Samples were taken at time 0 and after 3 h, then serially diluted in peptone water and plated on to M17G plates with overnight incubation at 30°C.

### Assessment of Nisin Production of Exponential and Stationary Phase Cultures Under Stress Conditions

Two tubes containing 5 mL of M17G were inoculated at an $OD_{600}$ of approximately 0.02 and then incubated at 30°C until mid-exponential phase (6 h) and early stationary phase (8 h). The cells were then pelleted by centrifugation, and the supernatants were removed. The cells in one tube were resuspended with 5 mL of M17G at pH 6.9 (control culture), and the other tubes were resuspended with 5 mL of M17G adjusted with predetermined stress conditions (3% NaCl, 0.05% bile salt, 20°C, 45°C, pH 4.0, 5.0, and 10.0). A sample was collected at time zero and at 3 h, and CFU was described as before. Tubes were centrifugated, and the supernatant was kept at −20°C for nisin quantification after heating the supernatant at 80°C for 15 min in a new sterile tube.

### Nisin Quantification

Nisin titer was measured by the method of Tramer and Fowler [11] modified with Pontharangkul and Demirci [12]. The samples were adjusted to pH 2.0 using a 10 M HCl solution, heated in water bath for 10 min and cooled to room temperature, then centrifuged at 8,000 rpm for 10 min. The supernatant was appropriately diluted with 0.02 M HCl, and the assay was performed using the agar-diffusing method, using indicator strain *M. luteus* NCIMB8166. A standard curve (50–600 IU/mL) was plotted using a stock solution of 1,000 IU/mL nisin (Sigma; nisin content 2.5% *w*/*w*). Assays were performed in triplicate, and average results are given.

### Model Assessment

For the model assessment study, determination coefficient ($R^2$) and adjusted determination coefficient ($R^2_{adj}$) values were used to investigate the goodness-of-fit of the proposed models

(see below). The parameters of the models were obtained by using SigmaPlot 2000 version 6.00 (Chicago, II, USA).

## Results

### Growth Curves of Two Nisin Producer *L. lactis* Strains

*L. lactis* spp. *lactis* strains were cultivated in batch culture to determine the phases of growth (Fig. 1). It took approximately 6 and 8 h to reach mid-exponential and early stationary phases, respectively, for both strains. These values were required in the assessment of the nisin production levels of the cells that are in the mid-exponential and early stationary phases under stress conditions.

### Evaluation of Stress Conditions for Nisin-Producing *L. lactis* Strains

The survival ratio of two nisin producer *L. lactis* spp. *lactis* strains decreased as NaCl concentration increased (Fig. 2a,b). However, both of the *L. lactis* spp. *lactis* strains could grow up to 3.0% concentration of NaCl, and thereafter, inactivation occurred. Similar behavior (growth or reduction) were also observed for bile salt application. Cell growth was observed between 0% and 0.05% and inactivation started from this concentration forward (Fig. 3a,b).

The decrease of the survival ratio (decrease of growth, transition from growth to inactivation, and initiation of inactivation) as NaCl and bile salt concentration increased (Figs. 2 and 3) can be described by an exponential decay function (Eq. 1):

$$S(t) = \frac{N(t)}{N_0} = I.\exp(-k.C) \tag{1}$$

where $S(t)$ is the survival ratio, i.e., $S(t)=N(t)/N_0$, [$N(t)$ and $N_0$ are the number of survivors after an exposure time $t$ and initial number of microorganisms, respectively]. If $S(t<1$, this means the number of microorganism is reduced, i.e., inactivation occurs due to a lethal

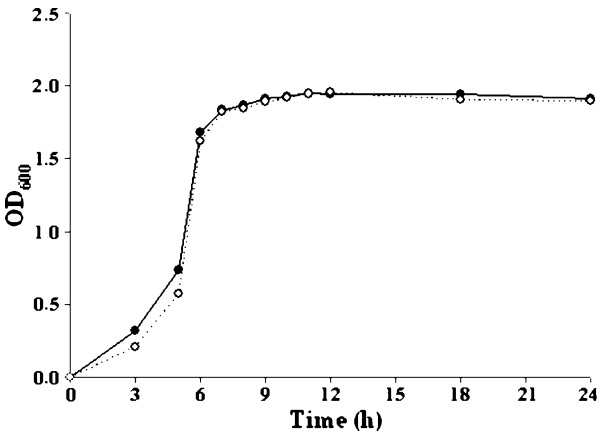


**Fig. 1** Growth of nisin producer *L. lactis* spp. *lactis* strains in batch culture. *Black circles* represent BLL27; *white circles* represent ATCC11454. Data were the mean of the two replicates

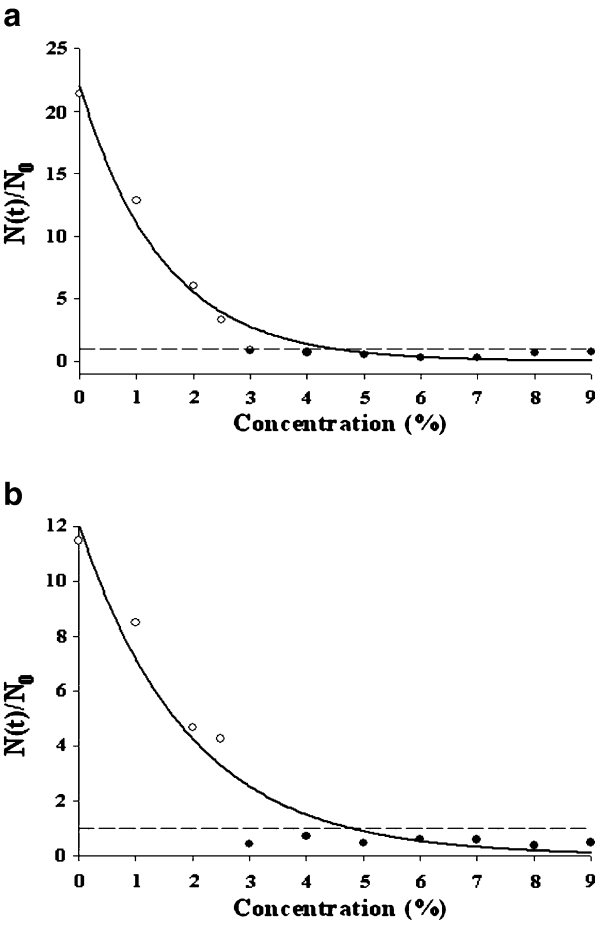


**Fig. 2** $S(t)$ vs. salt concentration plots for *L. lactis* spp. *lactis* strains BLL27 (**a**) and ATCC11454 (**b**). Incubation temperature and period were 30°C and 3 h, respectively, at each concentration value. *Dashed line* indicates the inactivation–growth interface i.e., $S(t)=1$. Data below this line (inactivation) were shown as *black circles* and above this line (growth) were shown as *white color*. *Solid line* indicates the fitting of Eq. 1 ($R^2$ and $R^2_{adj}$ values were both 0.98 and 0.94 for *L. lactis* spp. *lactis* strains BLL27 and ATCC11454, respectively). Model parameters were obtained as $I=12.1\pm0.9$a; $k=0.5\pm0.07$a for BLL27 and $I=22.0\pm1.0$a; $k=0.7\pm0.05$a for ATCC11454, where a is the standard error

event or condition. If $S(t)>1$, this means microorganisms grow. If it is equal to 1, then neither inactivation nor growth occurs. $I$ (intercept value) and $k$ are the model parameters. $R^2$ and $R^2_{adj}$ were both equal to 0.99 for bile salt stress, while they were above 0.94 for NaCl stress conditions. From the model fits, the transition from growth to inactivation [the intersection between the model and $S(t) \sim 1$] occurs between 4.0% and 5.0% of NaCl concentration for both strains (Fig. 2). Similarly, transitions from growth to lethality were determined as between 0.03% and 0.05% and about 0.05% for BLL27 and ATCC11454, respectively (Fig. 3).

Data obtained from both *L. lactis* spp. *lactis* strains indicated that, at temperatures between 5°C and 20°C, very slight inactivation occurred that $S(t)$ was close to 1 (Fig. 4a,b). However, when the incubation temperature was increased to 25°C, accelerated cell growth was observed up to 40°C. The only difference between the two nisin producers was that cell growth of BLL27 started from 20°C (Fig. 4a). Inactivation was observed in the highly acidic and basic conditions for both strains, while neutral pH conditions favored the cell growth (Fig. 5a,b).

The survival ratios of nisin producer *L. lactis* spp. *lactis* strains (plotted against the different temperature and pH levels) could be described by the Gaussian function; equations

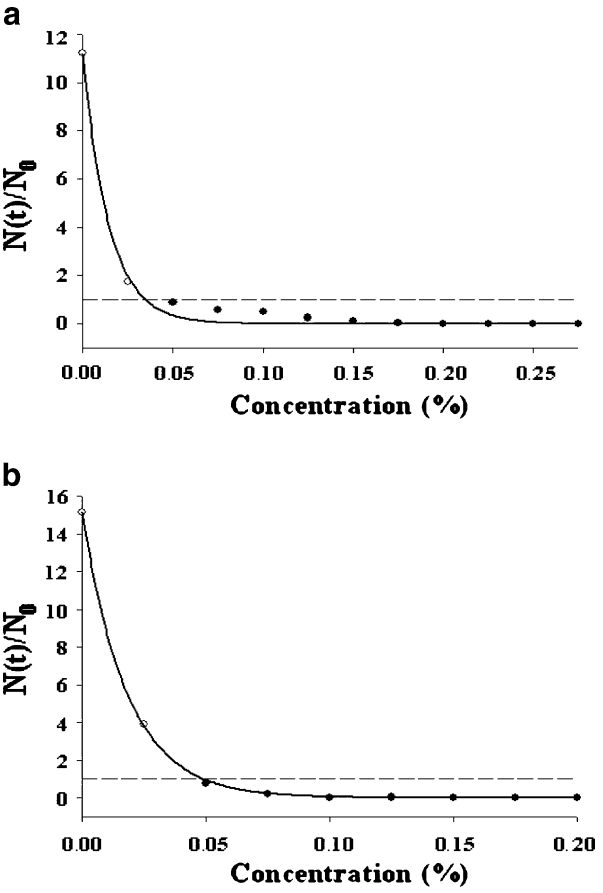


**Fig. 3** $S(t)$ vs. bile salt concentration plots for *L. lactis* spp. lactis strains BLL27 (**a**) and ATCC11454 (**b**). Incubation temperature and period were 30°C and 3 h, respectively, at each concentration value. *Dashed line* indicates the inactivation–growth interface i.e., $S(t)=1$. Data below this line (inactivation) were shown as *black circles* and above this line (growth) were shown as *white color*. *Solid line* indicates the fitting of Eq. 1 ($R^2$ and $R^2_{adj}$ values were both 0.99 and 0.99 for *L. lactis* spp. *lactis* strains BLL27 and ATCC11454, respectively). Model parameters were obtained as $I=12.2\pm0.3a$; $k=69.1\pm5.9a$ for BLL27 and $I=15.2\pm0.08a$; $k=55.3\pm0.7a$ for ATCC11454, where a is the standard error

given below represent the survival ratios of nisin producing strains under different incubation temperature (Eq. 2) and pH (Eq. 3) values.

$$S(t)=\frac{N(t)}{N_0}=A.\exp\left[-0.5\left(\frac{T-T_{opt}}{b}\right)^2\right] \tag{2}$$

$$S(t)=\frac{N(t)}{N_0}=A.\exp\left[-0.5\left(\frac{pH-pH_{opt}}{b}\right)^2\right] \tag{3}$$

where $A$ is the peak (maximum) value of $S(t)$, $T_{opt}$, and $pH_{opt}$ are the optimum (growth) temperature and pH values, respectively, and $b$ is the parameter that determines the width of the right function half. $R^2$ and $R^2_{adj}$ values of Eq. 2 were both equal to 0.99 (for both strains); however, lower values ($R^2_{adj}=0.77$ and 0.70, respectively) were obtained for *L. lactis* spp. *lactis* strains BLL27 and ATCC11454 due to the scatter of the data.

Model fits for temperature (Fig. 4) indicated that the transition from lethality to growth starts above 20°C for both strains, while the growth starts thereafter and reaching its maximum at about 31°C and 33°C for BLL27 and ATCC11454, respectively. Increasing the

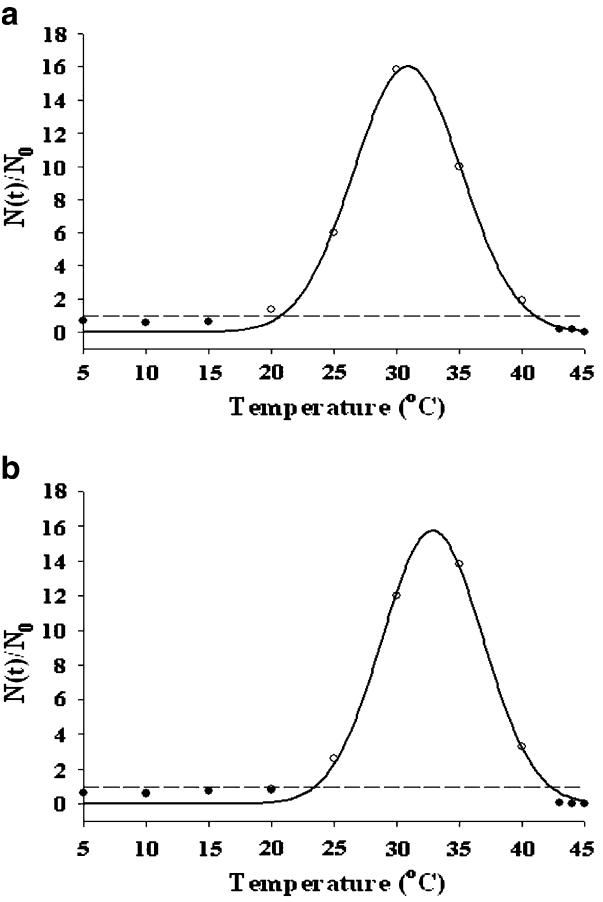


**Fig. 4** $S(t)$ vs. temperature plots for *L. lactis* spp. *lactis* strains BLL27 (**a**) and ATCC11454 (**b**). Incubation temperature and period are 30°C and 3 h, respectively, at each concentration value. *Dashed line* indicates the inactivation–growth interface i.e., $S(t)=1$. Data below this line (inactivation) were shown as *black circles* and above this line (growth) were shown as *white color*. *Solid line* indicates the fitting of Eq. 2 ($R^2$ and $R^2_{adj}$ values were both 0.99 and 0.99 for *L. lactis* spp. *lactis* strains BLL27 and ATCC11454, respectively). Model parameters were obtained as $A=16.0\pm0.5$a; $T_{opt}=30.9\pm0.2$a; $b=4.3\pm0.1$a for BLL27 and $A=15.8\pm0.6$a; $T_{opt}=32.9\pm0.2$a; $b=4.0\pm0.2$a for ATCC11454, where a is the standard error

temperature decreased the growth as expected, and transition from growth to lethality occurred above 40°C.

Model fits for pH (Fig. 5) indicated that transition pH's from lethality to growth were about 4 and 6.5 for BLL27 and ATCC11454, respectively, whereas they were about 9.5 from growth to lethality for both strains. Maximum growth occurred at pH's 6.8 and 7.9 for BLL27 and ATCC11454, respectively. However, it should be noted that the proposed model (Eq. 3) did not produce very good fits for both strains, which may affect the transition and optimum pH values. Nevertheless, the chosen model (Eq. 3) was the best in terms of goodness-of-fit among the other models tried (data not shown).

## Nisin Production of Mid-exponential and Early Stationary Phase Cultures of *L. lactis* Strains Under Stress Conditions

*L. lactis* spp. *lactis* strains (BLL27 and ATCC11454) in their mid-exponential and early stationary phases were encountered with the stress conditions of 3.0% NaCl, 0.05% bile salt, pHs 4.0 (for ATCC11454), 5.0 (for BLL27), and 10.0, and 20°C and 45°C temperatures. These were the transition values [$S(t) \sim 1$] from growth to lethality or vice versa (see Figs. 2, 3, 4, and 5).

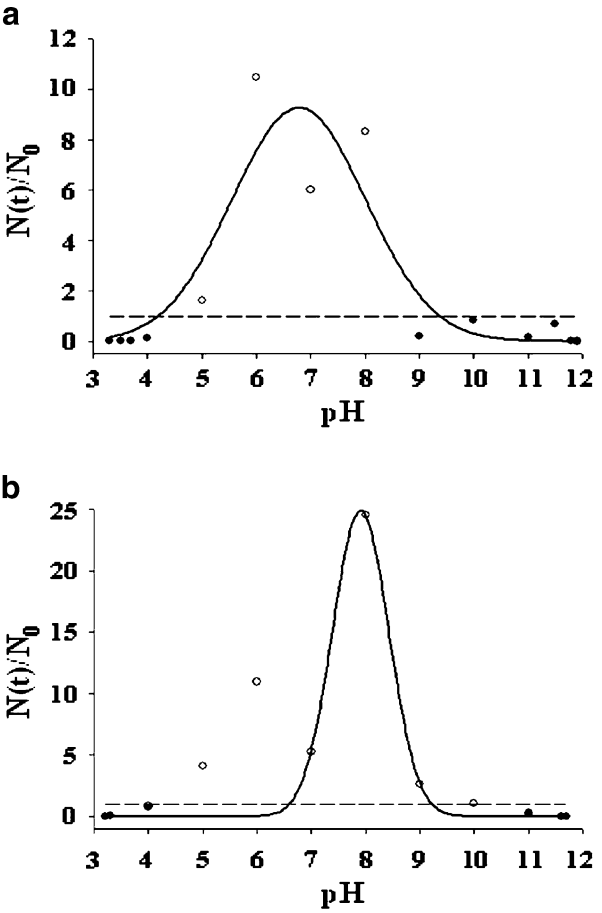


**Fig. 5** $S(t)$ vs. pH plots for *L. lactis* spp. *lactis* strains BLL27 (**a**) and ATCC11454 (**b**). Incubation temperature and period are 30°C and 3 h, respectively, at each concentration value. *Dashed line* indicates the inactivation–growth interface, i.e., $S(t)=1$. Data below this line (inactivation) were shown as *black circles* and above this line (growth) were shown as *white color*. *Solid line* indicates the fitting of Eq. 3 ($R^2$ and $R^2_{adj}$ values were 0.80 and 0.77 for *L. lactis* spp. *lactis* strains BLL27 and 0.76 and 0.70 for ATCC11454, respectively). Model parameters were obtained as $A=9.3\pm1.4$a; $pH_{opt}=6.8\pm0.2$a; $b=1.2\pm0.2$a for BLL27 and $A=24.9\pm4.3$a; $pH_{opt}=7.9\pm0.2$a; $b=0.5\pm0.1$a for ATCC11454, where a is the standard error

Among the stress applications, both strains in their mid-exponential phases produced nisin as much as control when they were encountered with 3.0% NaCl and 20°C temperature stresses, indicating that osmotic and low temperature (low compared to their optimum temperature values) stresses have not any suppressing effect on nisin production (Figs. 6 and 7). However, at 5°C, nisin production levels decreased 82% and 87% for BLL27 and ATCC11454, respectively (data not shown).

Similarly, nisin production levels of *L. lactis* spp. *lactis* strains were dramatically decrease at other stress conditions [nisin production level of BLL27 decreased about 29%, 62%, and 69% under bile salt (0.05%), low pH (5.0), and high temperature (45°C) stress applications, respectively, where the reductions for ATCC11454 were determined as 88%, 67%, and 92%, respectively], whereas nisin activity completely disappeared at high pH environment (pH 10.0). Although similar nisin production patterns were observed between the nisin producer *L. lactis* spp. *lactis* strains under the applied stress conditions, higher amount of decreases were observed for ATCC11454 than for BLL27. This indicated that the nisin production level may be affected by the stress resistance of the strains.

The influence of stress conditions on the nisin production of early stationary phase cultures of *L. lactis* spp. *lactis* strains found to be similar with the nisin production level of

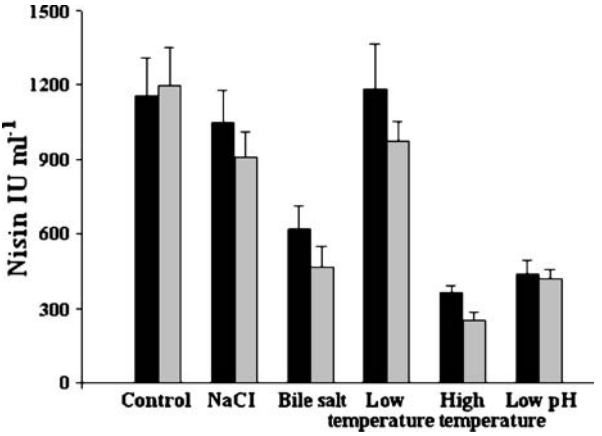


**Fig. 6** Nisin production levels of mid-exponential (*black color*) and early stationary phase (*gray color*) cultures of *L. lactis* spp. *lactis* BLL27 under stress conditions. After incubation of 6 h in M17G medium, cells were incubated 3 h in the conditions of 3% NaCl, 0.05% bile salt, at 20°C, at 45°C and pH 5 for NaCl, bile salt, low temperature, high temperature, and low pH stresses separately. Nisin activity completely disappeared at pH 10.0

mid-exponential phase cultures of the same strains (Figs. 6 and 7). However, nisin productions of both *L. lactis* spp. *lactis* strains at their early stationary phase were found less than the cells at their mid-exponential phase.

## Discussion

In this study, the adaptive response and nisin production ability of *L. lactis* spp. *lactis* strains under heat, NaCl, bile salt, and pH stresses was examined. The applied stress conditions in this study were the most common hurdle factors encountered for lactococci that both at its natural niche and industrial processes, for example, in the gastrointestinal tract and during starter handling. Therefore, these strains must resist the adverse conditions encountered in industrial processes for their reliability of starter culture functions not only in terms of quality and functional properties but also in terms of growth performance and robustness [13–16]. In this study, two nisin producer *L. lactis* spp. *lactis* strains (BLL27 and ATCC11454) originated from different sources showed similar growth/inactivation patterns under all applied stress parameters, indicating that the adaptive response is stable for these

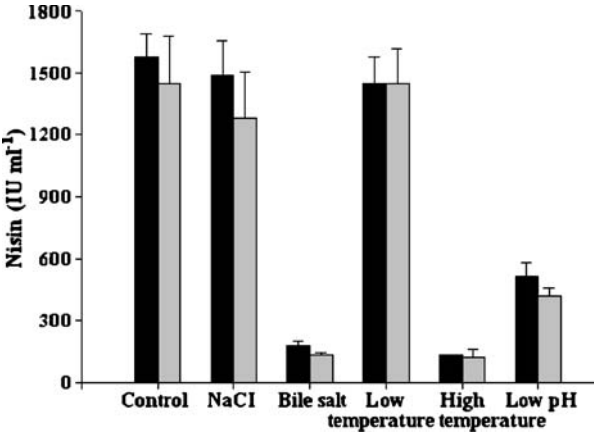


**Fig. 7** Nisin production levels of mid-exponential (*black color*) and early stationary phase (*gray color*) cultures of *L. lactis* spp. *lactis* ATCC11454 under stress conditions. After incubation of 6 h in M17G medium, cells were incubated 3 h in the conditions of 3.0% NaCl, 0.05% bile salt, at 20°C and at 45°C, and pH 4.0 for NaCl, bile salt, low temperature, high temperature, and low pH stresses separately. Nisin activity completely disappeared at pH 10.0

strains and showed no variations among strains. This is also supported by the previous results obtained from three non-nisin producer strains of *L. lactis* spp. *lactis*. Likewise, in the same study, it was concluded that three *L. lactis* spp. *cremoris* lack adaptive response to similar stresses other than *L. lactis* spp. *lactis* strains [4]. Although it has been known that nisin producers are slow acid producers [9], the results of two individual nisin producer strains proved that these strains are capable of displaying adaptive response to stress conditions as well as non-nisin producer *L. lactis* spp. *lactis* strains can resist to stressful fermentation environment to be robust.

Since the results of nisin producers showed similar pattern, the survival ratios (either growth or inactivation) were described by the empirical equations. These equations (Eqs. 1, 2, and 3) produced reasonable fits under each stress conditions, indicating that they could be used to describe the survival ratios of the nisin producers in such stress conditions. Although the stress response of *L. lactis* spp. *lactis* strains has been described extensively at molecular level [8, 17, 18], to the best of our knowledge, there is no modeling study for the stress response of *L. lactis* spp. *lactis* strains. Therefore, these equations could be beneficial for estimating the transition from lethality to growth or vice versa and survival of *L. lactis* strains in the food systems under different stresses.

Bacteriocins, such as nisin, are generally considered primary metabolites, meaning that their production is associated with growth of producer strains. Therefore, conditions that promote higher cell density usually result in high production [19, 20]. However, the production of a bacteriocin under laboratory conditions does not necessarily mean a sufficient and effective bacteriocin production and bioavailability in the food ecosystem. Severe limiting factors may interfere with bacteriocin production capacity of the starter cultures or co-cultures used [16]. When $S(t) \sim 1$, cell growth or inactivation is at their minimum level and the cell number is constant. Therefore, in this state, the effect of each stress condition on the nisin production level could be determined effectively. The nisin activity of two nisin producers decreased dramatically (both in their mid-exponential and early stationary phases) under applied stress conditions with exceptionally 3.0% of NaCl and 20°C applications. Generally high concentrations of salt inversely affect growth and bacteriocin by lactic acid bacteria, such as *Enterococcus faecium* CTC492 [13], *Lactobacillus amylovorous* DCE471 [21] and *Lactobacillus sakei* CTC494 [14]. In contrast, Uguen et al. [22] reported an increased lacticin 481 production when the osmolarity of the growth medium increased due to added NaCl. Also for plantaricin S, the highest production is observed at NaCl concentration of 2.5% [23]. Leroy et al. [15] reported that salt stress decreases both the cell growth and bacteriocin production of *E. faecium* RZS C5. However, in the same study, moderate levels of sodium chloride improved bacteriocin activity of *E. faecium* RZS C5. Evidences for this complex observation have not been currently reported, but it is speculated that the salt molecules interfere with the bacteriocin regulation mechanism by the binding of induction factor to its receptor, which results as inhibition of bacteriocin production [13]. The evidence that the osmotic stress (NaCl) does not suppress the lantibiotic production, for instance nisin (in this study) and lacticin 481 [22], might propose that this above foresight is not acceptable for the lantibiotics regulation.

As a conclusion, it has been shown that two nisin producers in this study had similar adaptive responses under severe stress conditions representing the food ecosystem. These responses of *L. lactis* strains were described by mathematical equations, which could be used to estimate the behaviors of these strains under stress conditions. Furthermore, the effect of harsh environment on the nisin activity of *L. lactis* strains depends on the stress factors that are applied. However, the hopeful results of osmotic and low-temperature

stresses on nisin production of *L. lactis* strains were found significant for cheese technology, where salting and maturation at low temperature processes is the major hurdle for starter cultures.

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