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Potential effect of monolaurin and nisin on the growth of three aerobic spore-forming bacteria isolated from Tunisian milk

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ABSTRACT

Antibacterial capacities of monolaurin and nisin on the growth of vegetative cells of three spore-forming bacteria isolated from cow milk collected from Tunisia (*Terribacillus aidingensis, Bacillus sporothermodurans* and *Paenibacillus sp* and) were evaluated. Nisin or monolaurin, at different concentrations, can inhibit the growth of three *Bacillus*. 500 IU/mL and 25 µg/mL corresponded to the minimum inhibitory concentrations of nisin and monolaurin, respectively. The growth of the tested species in the presence of sub-lethal concentrations of monolaurin and nisin was monitored in UHT milk for 7 days at three different temperatures (4°C, 37°C and 25°C). Nisin (250 IU/mL) was able to induce an immediate reduction (after 3 h of incubation at 4°C) of *Bacillus* species growth (5 log for *B. sporothermodurans*, 4 log for *Paenibacillus sp* and 3.2 log for *T. aidingensis*). Also, monolaurin (25 µg/mL) reduce the growth, at 4°C of *B. sporothermodurans* by 5 log, *Paenibacillus sp* by 3 log and *T. aidingensis* by 4 log, after 3 h of incubation. In the presence of nisin or monolaurin, it can be observed a low reduction in cell growth followed by a rapid regrowth which can attenuate 10 log, at 37°C and 25°C. The obtained data elucidate the potency of nisin and monolaurin, and the application of these antimicrobial agents at low temperatures (4°C) to decontaminate any product from these spoilage bacteria.

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1. Introduction

Bacteria which able to produce heat-resistant endospores are an emerging problem in food production. These micro-organisms and others bacteria genus (*Staphylococcus aureus*, *Escherichia coli* and *Salmonella*) are susceptible to contaminate the milk products by causing organoleptic damages (1-2).

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The antimicrobials agents possess an important power against the bacteria produced endospore and can, therefore, be used as another dealing in combination with other preservation methods (3).

In this context and as a part of the improvement of sterilization strategies to eliminate these germs, we studied the effect of two antimicrobial agents (nisin and monolaurin) that are used as food additives on the inactivation of three *Bacillus* species: *Bacillus*

sporothermodurans, *Paenibacillus sp* and *Terribacillus aidingensis* isolated from Tunisian milk. The bacteriocins are utilized for long time to inhibit the growth of several food spoilage microorganisms and pathogens (4).

Gram-positive bacteria that caused food spoilage such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* were inhibited by the nisin (5-7). Monolaurin is a natural compound composed of fatty acids and monoglycerides with an important antibacterial activity (8). Eshghjoo et al. (9) studied the growth of *E coli* O157:H7 in the presence of different concentrations of monolaurin in the cheese for several days. The results indicated the potential inhibitory effect of monolaurin on bacterial growth in dairy products. Besides, combination of nisin and monolaurin exerted a bactericidal effect against diverse *Bacillus* species in skim milk, also inhibited their regrowth and sporulation (10) and could represent a promising advance for the microbiological safety and maintenance of sensory properties in dairy products (4). In a previous study, Mansour et al. (11) investigated the inhibitory synergistic effect of nisin and monolaurin used in association with pH on *B. licheniformis* spores in milk. The purpose of the current investigation was to determine, for the first time, the efficacy of nisin-monolaurin on the inhibition of growth of three *Bacillus* species: *B. sporothermodurans*, *Paenibacillus* sp and *T. aidingensis* by determining the minimum inhibitory concentration of the antimicrobial agents. The second purpose of this work was to investigate the effect of storage temperature against the antimicrobial power of nisin and monolaurin against the growth of Bacillus species in UHT milk.

2. Materials and Methods

2.1. Bacterial strains

In the current investigation, three different *Bacillus* species isolated from Tunisian UHT milk by Kmiha et al. (12) were used: (i) *B. sporothermodurans* isolated from raw milk, (ii) *T. aidingiensis* and (iii) *Paenibacillus sp*. The isolation of *Terribacillus* and *Paenibacillus* was performed from UHT-milk on BHI (Brain Heart Infusion) agar-vitB12 medium (Sigma Aldrich). The isolation of *B. sporothermodurans* was carried out after heat treatment of raw milk (100°C for 40 min). This treatment allowed the selection of very heat-resistant organisms

2.2. Determination of the MIC of nisin and monolaurin

The broth dilution method was used to estimate the minimum inhibitory concentrations (MIC) of the used antimicrobial agents as described by Trotter and Marshall (13). In fact, the MIC is defined as the lowest concentration of an antibacterial agent that prevents the visible growth of bacteria. Indeed, suitable quantities of nisin or monolaurin were supplemented to the broth of BHI-vitB12 to obtain the following final concentrations: 50, 100, 500, 1000 and 2000 IU/mL for nisin and 50, 100, 250 and 500 µg/mL for monolaurin using the plate of the vegetative cells. A working solution of each tested strain corresponds to 10^5 cfu/mL. A positive control corresponds to the sterile distilled water inoculated without nisin or monolaurin, as well as a negative control corresponds to the distilled water supplemented with antimicrobial and not inoculated by the bacteria included in all the experiments.

2.3. Individual effects of nisin or monolaurin on *Bacillus* growth in milk

This experiment aimed to follow the behavior of three *Bacillus* species in the presence of two concentrations

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(1/2 of the MIC and the MIC) of nisin or monolaurin in UHT milk and with variable temperatures, which could correspond to the milk storage conditions. These temperatures have been set at + 4°C (refrigerator), 25°C and 37°C (temperature similar to those encountered in summer). Three types of trials (nisin, monoalurin and no antibacterial) were performed for each strain.

Bacterial strains (10^5 cfu/mL) which served as inoculum in these assays were obtained by the growth of the tested strain in BHI-broth incubated for 24 h at 37°C.

Samples were taken immediately after adding the antimicrobial agent, and then every 24 h for 7 days. The vegetative cells were counted by culture on BHI agar, after incubation for 24 at 37°C h.

2.4. Reproducibility of data

Experiments were carried out three times. The obtained results are presented as mean values +/- standard deviation.

3. Results

3.1. Determination of the minimum inhibitory concentration of the antimicrobial agents

The different concentrations of nisin and monolaurin were tested for their antibacterial capacities on the vegetative growth of *T. aidingensis, B. sporothermodurans*, and *Paenibacillus sp.* (Table 1). After incubation at 37°C for 24 h, the obtained results show that nisin or monolaurin generates a remarkable reduction in the viable cell count. The minimum inhibitory concentration of monolaurin was 25 µg/mL. While for nisin, it corresponds to 500 IU/mL.

3.2. Power of nisin and monolaurin on growth of *Bacillus* species

3.2.1. Effect of nisin

The obtained results showed that the antagonistic power of nisin on *B. sporothermodurans* depends on the incubation temperature. At 4°C, a 5-log reduction was noted after 3 h (Fig. 1A). At 25°C, a 4-log reduction in the initial count of the vegetative cells was detected after 3 h of incubation. Nevertheless, after 24 h of incubation, the bacterial growth restarted and reached up to 8 logs after incubation for 7 days (Fig. 2B). Finally, at 37°C, the reduction rate is of the order of 3.7 log. After 2 days of incubation, an increase in bacterial growth was observed and could reach 10 log at 7 days of incubation (Fig. 1C). The inhibition of the growth of *Paenibacillus sp* was able to achieve a remarkable reduction of 4 logs during the 7 days at 4°C (Fig. 2A). At 25°C, the reduction was 0.5 log in the presence of 500 IU/mL after 3 h of incubation, however, an exponential growth was noted later and it could reach up to 9 log (Fig. 2B). Whereas, at 37°C, the bacterial multiplication was increased to 10 log (Fig. 2C). For *T. aidingensis* and at 4°C, there is a reduction in the order of 3.2 log. This inhibitory effect was observed for both nisin and positive control cultures, which may suggest that this is due to the effect of cold and not nisin (Fig. 3A). At 25°C, a reduction rate of around 3 log was observed, after 3 h, in the presence of 250 IU/mL or 500 IU/mL of nisin in comparison with the positive control. Then, there was a restart of the growth up to 9 log (Fig. 3B). At 37 °C, a reduction of 3 log of growth was observed, which decreased after a few hours and then the bacterial multiplication resumed to gain up to 9 log (Fig. 3C).

Table 1. Effect of different concentrations of two antimicrobial agents on the growth of three *Bacillus* species

	Inhibition index (II) of vegetative cell growth		
	B.sporothermodurans	*Paenibacillus sp.*	*Terribacillus aidingensis*
T+	0	0	0
Nisin (UI/mL)			
10	0.019	0.01	0.03
50	0.34	0.013	0.1
100	0.55	0.14	0.3
500	0.58	0.93	0.98
1000	0.6	0.9	0.97
2000	0.6	0.91	0.83
Monolaurin (µg/mL)			
50	0.92	0.97	0.9
100	0.91	0.94	0.87
250	0.85	0.91	0.86
500	0.81	0.88	0.8

T + (positive control ; culture without antimicrobial agent)

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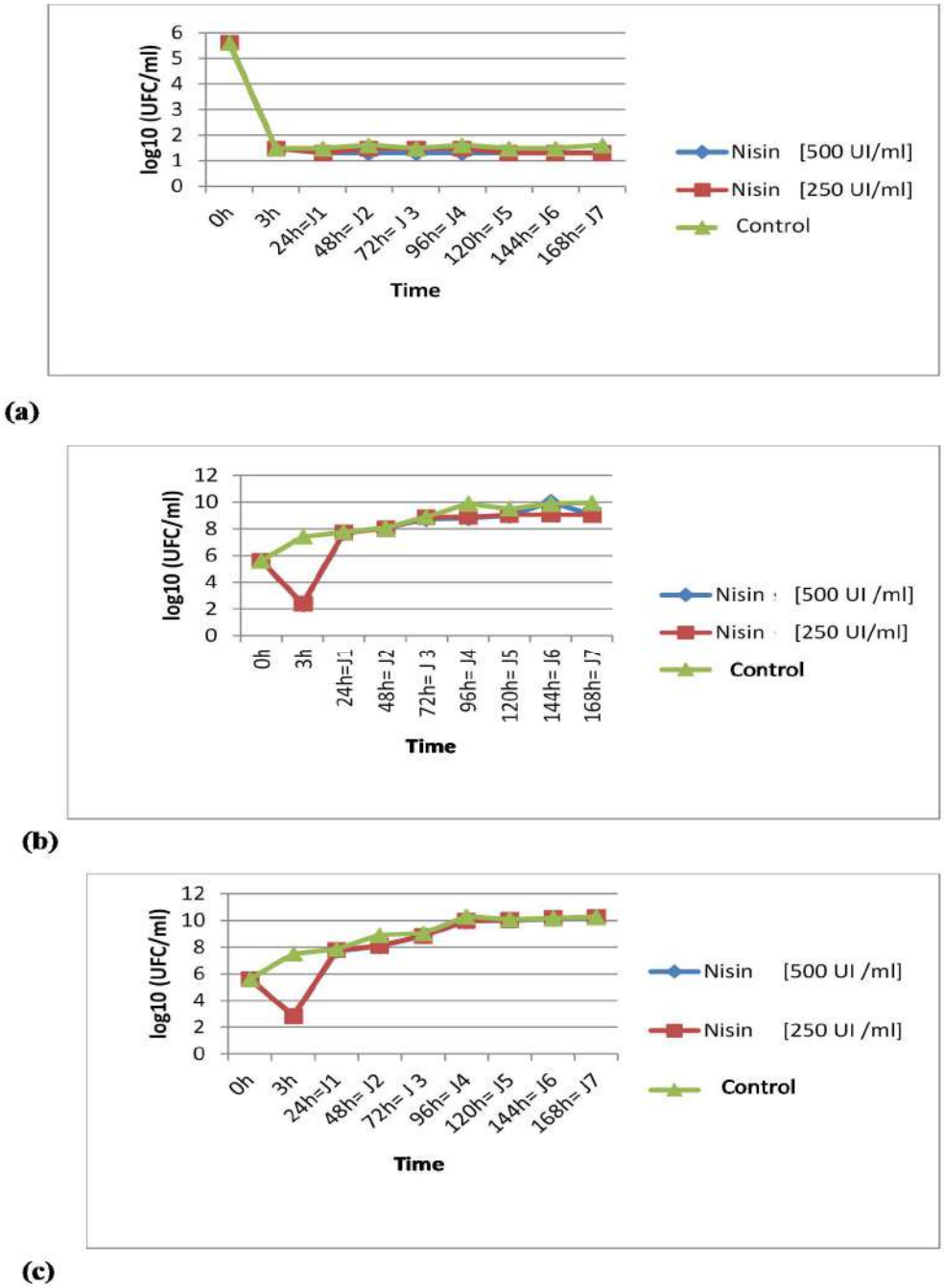
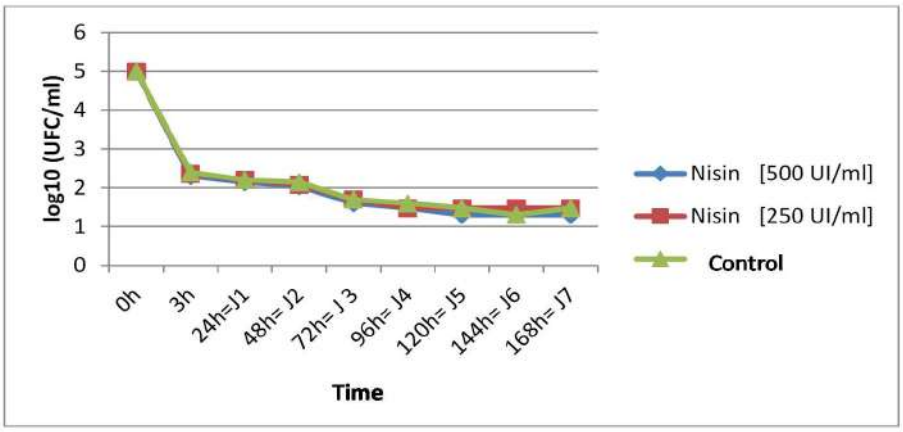


Figure 1. Behavior of vegetative cells of *B.sporothermodurans* in the presence of nisin in UHT milk incubated a): at 4°C; (b): at 25°C and (c): at 37°C)



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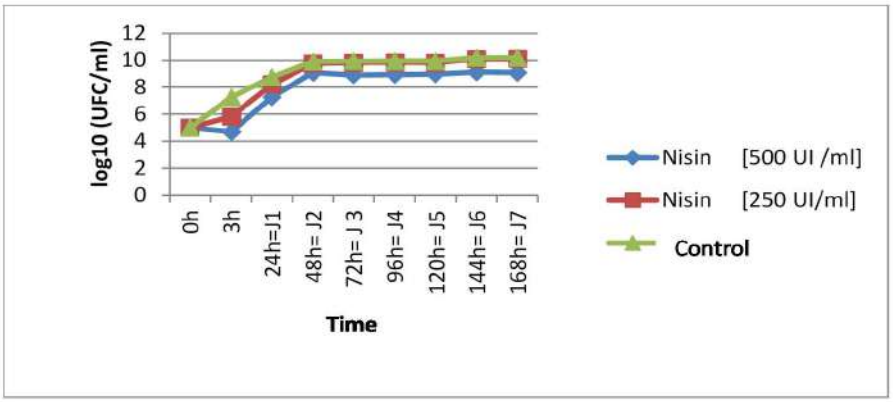

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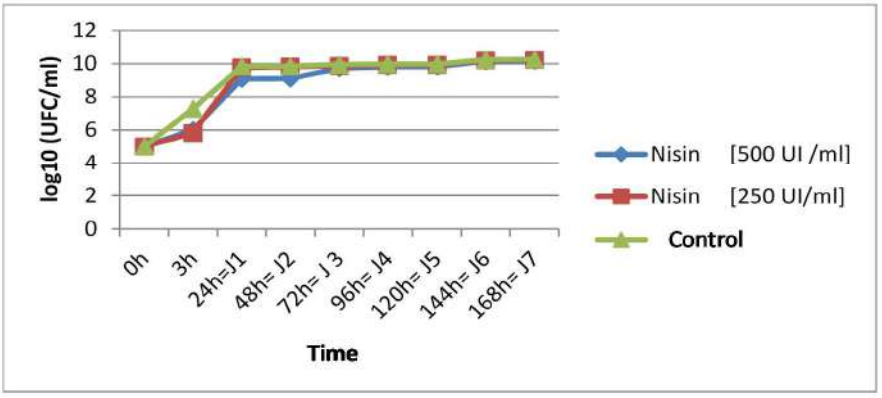

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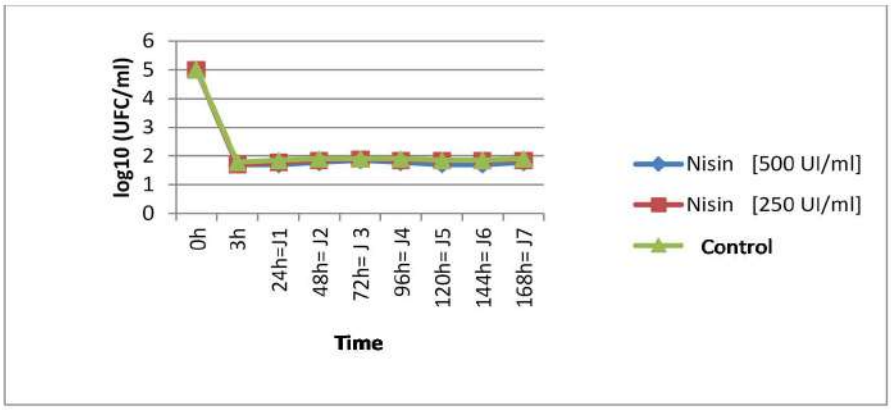
Figure 2. Behavior of vegetative cells of *Paenibacillus sp* in the presence of nisin in UHT milk incubated (a): at 4°C; (b): at 25°C and (c): at 37°C



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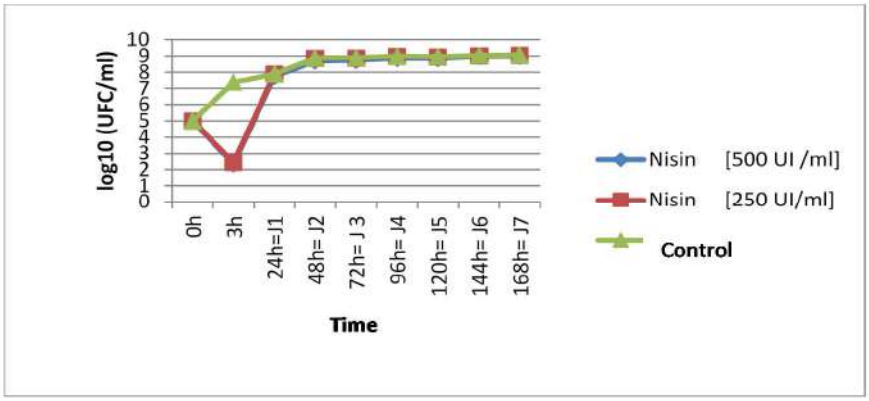

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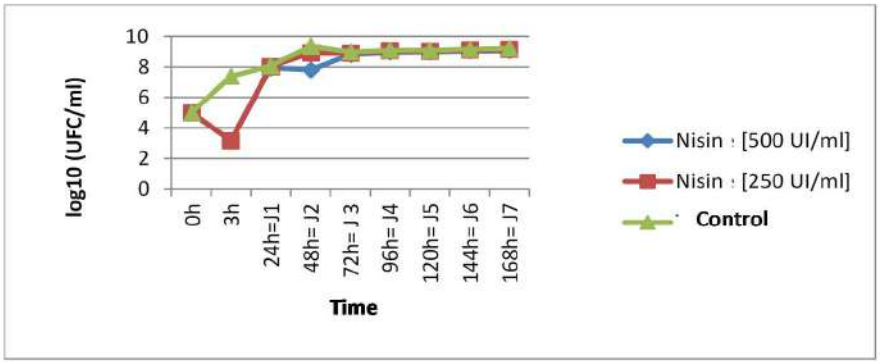

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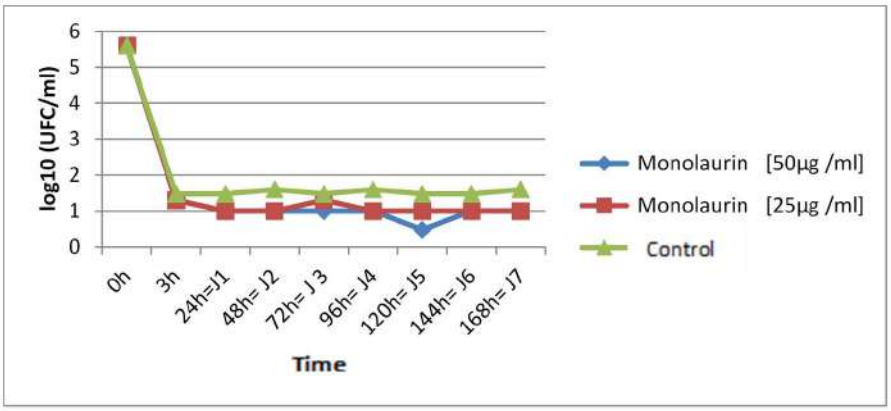
Figure 3. Behavior of vegetative cells of *Terribacillus aidingensis* in the presence of nisin in UHT milk incubated a): at 4°C; (b): at 25°C and (c): at 37°C)



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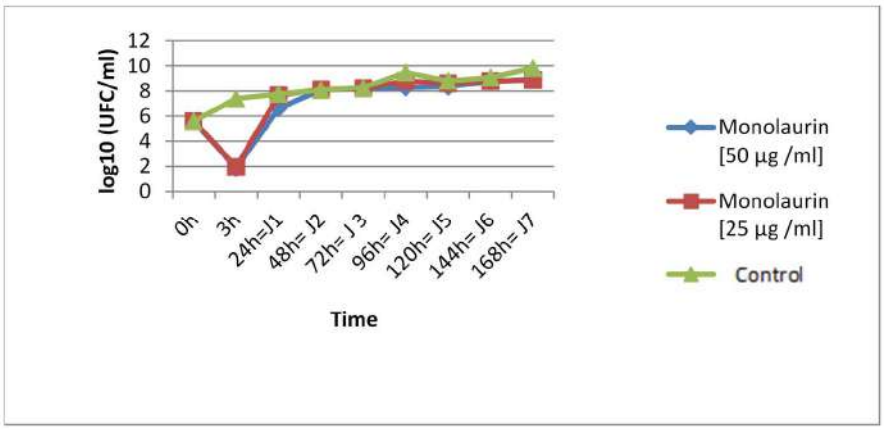

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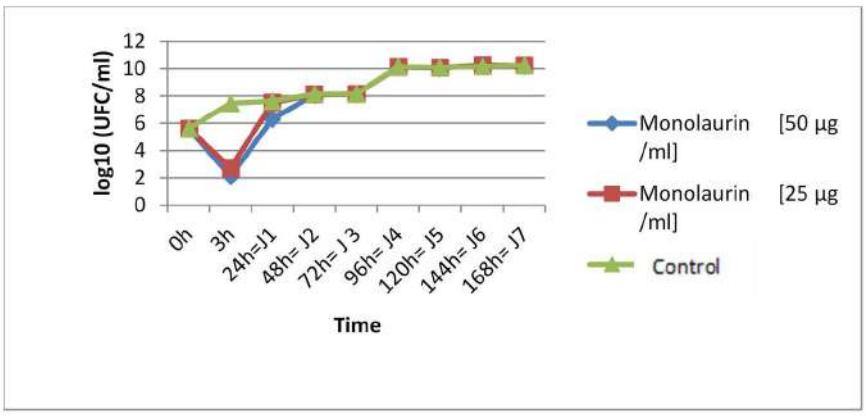

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Figure 4. Behavior of vegetative cells of *B. sporothermodurans* in the presence of monlaurin in UHT milk incubated a): at 4°C; (b): at 25°C and (c): at 37°C)

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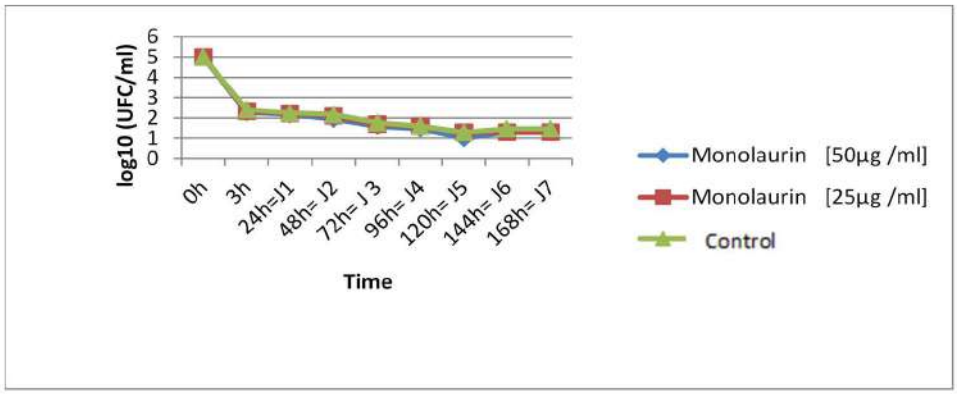

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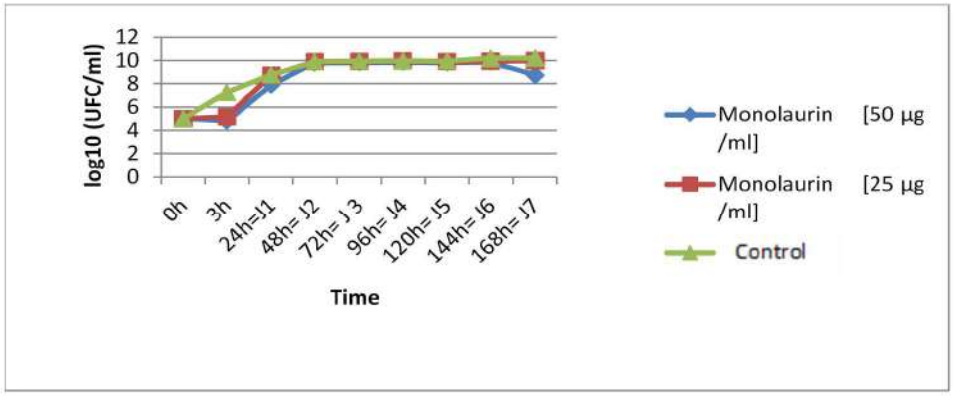

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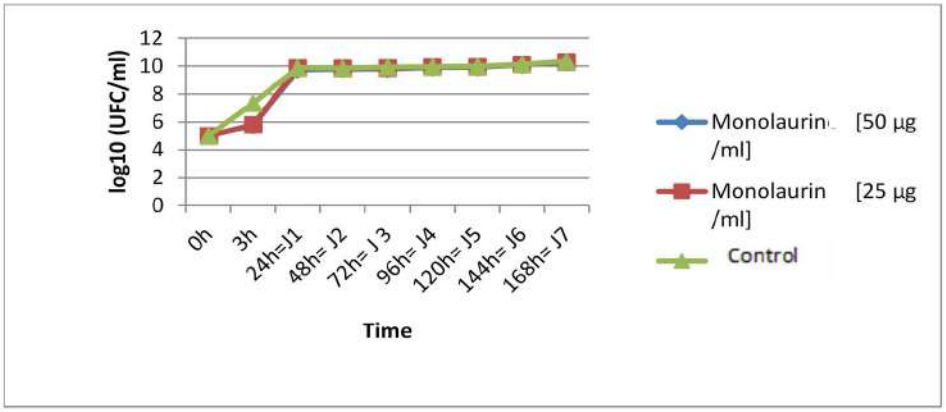
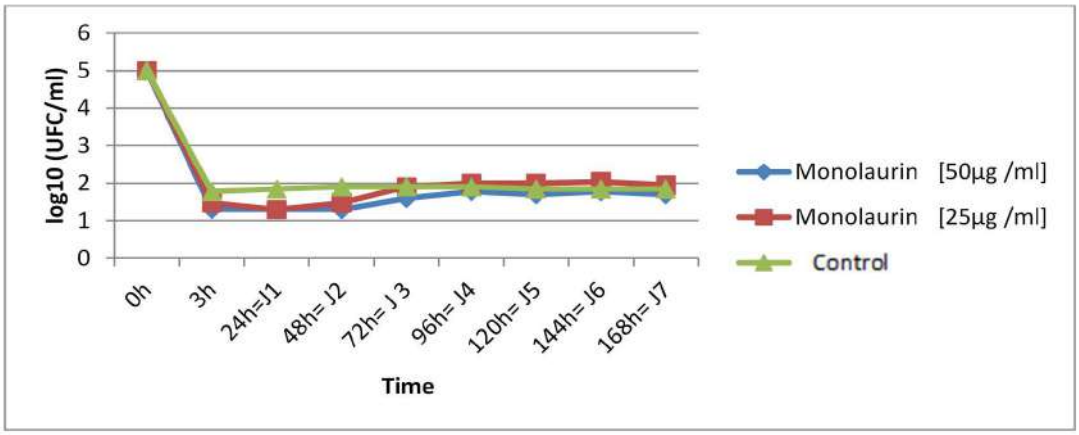


Figure 5. Behavior of vegetative cells of *Paenibacillus sp* in the presence of monlaurin in UHT milk incubated a): at 4°C; (b): at 25°C and (c): at 37°C)



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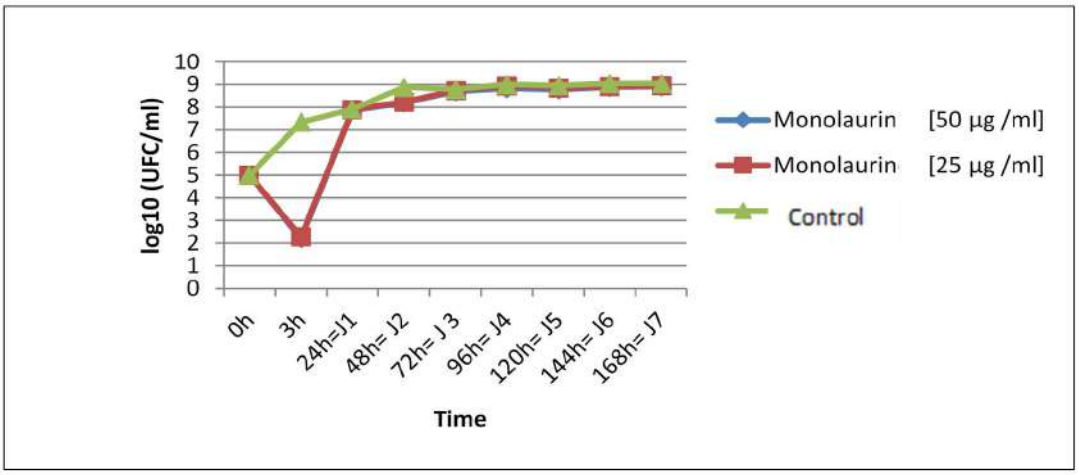

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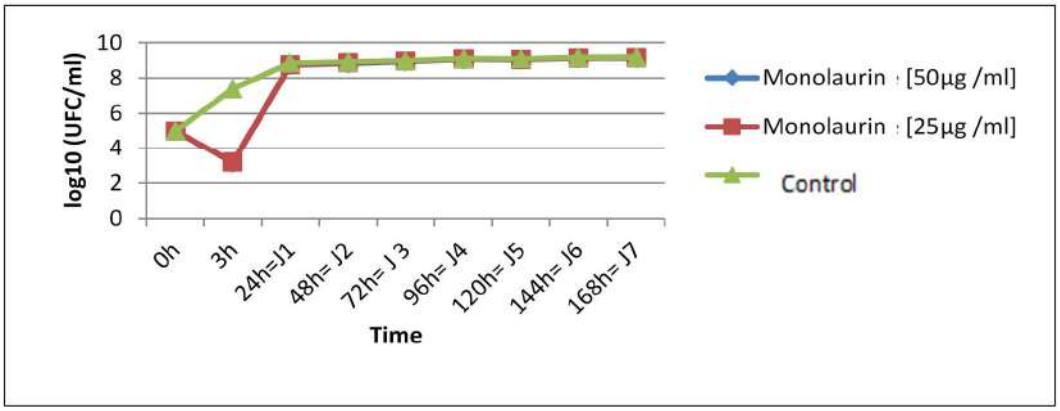

(c)

Figure 6. Behavior of vegetative cells of *T. aidingensis* in the presence of monlaurin in UHT milk incubated a): at 4°C; (b): at 25°C and (c): at 37°C)

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3.2.2. Effect of monolaurin

In a second part, we studied the influence of monolaurin (half-MIC = 25 µg/mL and MIC = 50 µg/mL) in skimmed UHT milk inoculated by one of the three species; *B. sporothermodurans*, *Paenibacillus sp*. or *T. aidingensis* and incubated for 7 days under the above three temperature conditions.

For *B. sporothermodurans*, at 4°C, we had a 5 log reduction for the positive control (without additive) (1.5 log) and in the presence of monolaurin (MIC or a half MIC) until reaching 1 log of vegetative propagation (Fig. 4A).

On the other hand, we noticed a reduction rate of 4 log after 3 h incubation at 25°C. However, after 24 h there was an acceleration of bacterial growth to 9 log (Fig. 4B). The difference between the reduction in the presence of MIC or a half-MIC of monolaurin is not remarkable. At 37°C, the reduction is 4 log. After 3 days, we noted a resumption of growth up to 8 logs to reach 10 log of growth after 5 days (Fig. 4C). Vegetative multiplication is always less important in the presence of monolaurin.

For *Paenibacillus sp,* no reduction of initial count was observed at 25°C and 37°C. Indeed, *Paenibacillus sp* growth could reach up to 10 log (Fig. 5B and 5C). On the other hand, a rate of 3 log of growth reduction was noted at 4°C. Nevertheless, the effect of monolaurin cannot be accurately detected in this condition since this reduction rate was also noted for the control culture (without additive), which makes the role of cold (4°C) on slowing bacterial growth (Fig. 5A). By studying the effect of monolaurin on *T. aidingensis*, we noticed a 4 log reduction at + 4°C (Fig. 6A).

Furthermore, a reduction of 3 log at 25°C after 3 h of incubation was noted, and then the growth was resumed from 12 h until reaching 8 log (Fig. 6B). Finally, after 3 h, at 37°C, the reduction is 2 log. After 2 days, the vegetative cells begin to multiply and they have been able to gain up to 9 log of growth (Fig. 6C).

4. Discussion

Firstly, the MICs of nisin and monolaurin obtained in presence of tested three *Bacillus* species are in concordance with the finding of Jill et al. (14) which demonstrated that the monolaurin inhibited the growth of *L. monocytogenes* with the MIC was 25 µg/mL. Whereas, Roberts and Zottola (15) showed that a 400 IU/mL of nisin was adequate to preserve cheese spreads against the deterioration caused by *C. sporogenes*. Recently, Chen et al. (16) showed that the MIC of nisin against *P. aeruginosa* was >8 mg/mL. The MIC value of monolaurin against *B. burgdorferi is* situated between 75 µg/mL and 150 µg/mL (17). The inhibition indices, in the presence of these concentrations, are 0.58; 0.93 and 0.98 for *B. sporothermodurans, Paenibacillus sp.* and *T. aidingensis* with nisin, respectively. Whereas with monolaurin, the inhibition indices were 0.92, 0.97 and 0.9, respectively (Table 1). *T. aidingensis* appears to be the most sensitive to nisin followed by *Paenibacillus sp*. Whereas the most resistant strain is *B. sporothermodurans*. With monolaurin, *Paenibacillus sp* is the most sensitive strain, followed by *B. sporothermodurans,* whereas the *Terribacillus* strain appears to be the most resistant.

Then, the growth of the vegetative cells of three species in UHT milk for 7 days at three different temperatures was evaluated in the presence a weak concentrations of monolaurin and nisin. In fact, nisin (250 IU/mL) was able to induce an immediate reduction (after 3 h of

incubation at 4°C) of *Bacillus* species growth. The reduction rates were 5 log, 4 log and 3.2 log for *B. sporothermodurans*, *Paenibacillus sp* and *T. aidingensis*, respectively. Also, at 4°C, monolaurin (25 µg/mL) decreases the growth of *B. sporothermodurans* (5 log-reduction), *Paenibacillus sp* (3 log-reduction) and *T.aidingensis* (4 log-reduction), after 3 h of incubation. Both at 37°C and at 25°C, nisin or monolaurin generated a low reduction in cell growth followed by a rapid regrowth which can attenuate 10 log. Thus, it can be demonstrated that temperature has an important effect on the vegetative cells growth in the presence of antimicrobial agents. Each antibacterial agent has a specific mechanism, as shown by other studies for the inhibitory effect of nisin on vegetative cells or spores of certain species such as *B. cereus* (18), *B. anthracis* (19), *Bacillus stearothermophilus* and *Clostridium butyricum* (20). The cytoplasmic membrane is the target structure in the vegetative cells forms of bacteria in presence of nisin. The proton motive force was destructed in presence of nisin followed by the pH imbalance and ion leakage causing the hydrolysis of ATP and cell death (21). Besides, Jofrés et al. (22) demonstrated the antagonistic effect of nisin against *Listeria* in cooked ham at 6°C. Concerning the monolaurin, it able to changes the permeability and fluidity of bacteria when incorporated into the membrane. Moreover, the monlaurin can inhibit the cellular respiration (23). Lori et al. (24) showed that monolaurin reduced the number of *B. cereus* (10^5 cfu/mL) in BHI to 2 log after incubation for after 4 h at 25°C. Another study showed that the most rapid inactivation of *L monocytogenes* in presence of monolaurin occurred with the highest temperature (35°C) (25). Even if Wang and Johnson (26) signaled that the reduction of number of *L. monocytogenes* by monolaurin (200 µg/mL) in dairy products detained at 4°C, the anti-listeria activity was the most important in 2% and whole milk than in skim and 1% of milk. Moroever, monoloaurin at 30°C doesn't affect the growth of *L. monocytogenes*. The inhibition of *L. monocytogenes* in presence of monolaurin was lower at 23°C than at 4°C. Jill et al. (14) elucidated that, at the tested concentrations (250-1000 µg/mL), *E. coli* or *L. monocytogenes* in UHT milk were resistant to the action of monolaurin. They suggested that this can be due to binding or solubilization of monolaurin by milk fat. So, the efficacy of bacteriocins was influenced by environment factors (pH and storage temperature), their stability, food composition, and their interaction with the composition of food (27).

5. Conclusion

The current study suggested that nisin and monolaurin have an inhibitory effect at tolerated concentrations in milk. In addition, storage temperature has an obvious effect on the effectiveness of these antibacterial substances on the *Bacillus* inactivation. Thus, it is recommended to conserve the UHT milk at low temperatures (4°C) to preserve its organoleptic as well its alimentary qualities and to prevent its contamination by these spore-forming bacteria, as well as their spores.

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Authorship contribution

Souhir Kmiha

Conceptualization; Data curation; Investigation; Software; Supervision; Visualization; Writing - original draft.

Chedia Aouadhi and Khouloud Aziz Methodology; Formal analysis; Validation; review & editing. Abderrazak Maaroufi Funding acquisition; Project administration; Validation; review & editing.

Declaration of competing interest

The authors declare no conflict of interest

Data availability

Data included in the article/referenced in the article.

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