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Inhibitory effects of nisin and potassium sorbate alone or in combination on vegetative cells growth and spore germination of *Bacillus sporothermodurans* in milk

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ABSTRACT

The inhibitory activities of nisin or/and potassium sorbate on spores and vegetative cells of *Bacillus sporothermodurans* LTIS27, which are known to be a contaminant of dairy products and to be extremely heat-resistant, were investigated. First, the tested concentrations of nisin or potassium sorbate inhibited vegetative cell growth; with the minimum inhibitory concentrations were 5×10^3 IU/ml and 2% (w/v), respectively. Then, the behaviour of vegetative cells and spores in presence of sub-lethal concentrations of nisin (50 UI/ml) or/and potassium sorbate (0.2%), in milk at 37 °C for 5 days, were evaluated. In the absence of inhibitors, strain grew and sporulated at the end of the exponential phase. Nisin (50 UI/ml) was able to inhibit spore outgrowth but didn't affect their germination. It induced an immediate and transitory reduction ($1.6\log_{(10)}$ after 1 h and $2.8\log_{(10)}$ after 6 h of incubation) of vegetative cell growth which reappeared between 10 h and 24 h. Potassium sorbate (0.2%) had a durable bacteriostatic effect ($1.1\log_{(10)}$ after 6 h), on vegetative cells, followed by a slower regrowth. It was able to inhibit both germination and outgrowth of spores. Association of nisin and potassium sorbate, at sub-lethal concentrations, showed a synergistic effect and resulted in a total inhibition of cells growth after 5 days. The results illustrate the efficacy of nisin and potassium sorbate in combination, and the commercial potential of applying such treatment to decontaminate any product that has a problem with persistence of bacterial spores.

1. Introduction

Mesophilic aerobic spore-forming bacteria of the genus *Bacillus*, often present in raw milk, play an important role in the bacterial deterioration of milk and its products (Criedly et al., 1994). To control the growth of *Bacillus* species, various kinds of heat treatments are used. The use of ultrahigh-temperature (UHT) processing should result in fluid milk products with a long shelf-life without refrigeration. Although these processes are designed to result in commercially sterile products, problems regarding the sterility of UHT-milk products have been reported (Hammer et al., 1995; Klijn et al., 1997; Scheldeman et al., 2006). This non-sterility appeared to be caused by the presence of highly heat-resistant spores (HRS), belonging to *Bacillus sporothermodurans* species, described for the first time by Pettersson et al. (1996). These spores can germinate and grow to 10^5 cfu/ml in stored UHT milk and therefore cause its spoilage and reduce its shelf-life (Hammer et al., 1995; Klijn et al., 1997; Montanari et al., 2004).

The use of antibacterial agents has been proposed as an interesting approach for the inactivation of bacterial spores or destruction of vegetative cells in food systems (Mansour et al., 1998; Ukuku and Fett, 2004; Black et al., 2008). For example, nisin is produced by *Lactococcus lactis* subsp. *lactis*. It is the only bacteriocin that has been approved as a food additive since 1969 (Food and Drug Administration, 1998). It is used to inhibit the outgrowth of *Clostridium* and *Bacillus* spores in the production of processed cheeses, canned vegetables, various pasteurised dairy products (Delves-Broughton et al., 1996) and milk (Black et al.,

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2008). Potassium sorbate has several advantages as food preservative. It has been investigated as an antimicrobial agent for use in all food products (Sofos and Busta, 1981, 1993). It is able to inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes* (González-Fandos and Dominguez, 2007) or *Bacillus* and *Clostridium* spores (Oleyede and Scholefield, 1994; Seward et al., 1982).

The emergence of resistant strains to antimicrobial agents justifies the search for new antibacterial strategies (Mazzotta and Montville, 1999). For this purpose, the combination of some antimicrobials has been evaluated with the aim at improving the destruction of spores and vegetative cells. Nisin has been extensively used in combination with antimicrobial agents such as lactoperoxidase system or reuterin (Arquès et al., 2008), monolaurin (Tokarskyy and Marshall, 2008) or other treatments such as high pressure and heat treatment (Aouadhi et al., 2013a). The combination of potassium sorbate with heat treatment and NaCl (Oleyede and Scholefield, 1994) or sodium lactate (Ukuku and Fett, 2004) has been tested. The combined effect of nisin–potassium sorbate on *Bacillus licheniformis* spores in milk has been examined by Mansour et al. (1998). These authors showed the synergistic inhibitory effect of these antimicrobials, used in association, on spores. Moreover, this combination has also been employed to reduce *Salmonella* in whole and fresh cut cantaloupe (Ukuku and Fett, 2004).

The inactivation of *B. sporothermodurans* by high pressure alone or in combination with nisin or thermal treatment has been studied (Aouadhi et al., 2013a), but the effect of such treatment on the vegetative cell growth and spore germination of *B. sporothermodurans* has not previously been investigated. The aims of our study were to evaluate the inhibitory activity of nisin or potassium sorbate on germination and outgrowth of spores and vegetative cell growth of *B. sporothermodurans.* In addition, the effects of sub-lethal concentrations of nisin or/and potassium sorbate on the behaviour of vegetative cells and spores of *B. sporothermodurans* in skim milk at 37 °C for 5 days, were monitored.

2. Materials and methods

2.1. Bacterial strain and spore preparation

B. sporothermodurans strain LTIS27, isolated from UHT milk produced in Tunisia (Aouadhi et al., 2014), was used in this study. Cells were grown in brain-heart infusion agar supplemented with 1 mg/l vitamin B_{12} (Sigma–Aldrich) (BHI–B_{12}), at 37 °C. Twenty-four hours before use, cultures were held in BHI–B_{12} broth on a rotary shaker at 37 °C to obtain a working culture. Spores were prepared according to method previously reported by Aouadhi et al. (2013a).

2.2. Preparation of inhibitors

Nisin and potassium sorbate were obtained from Sigma–Aldrich. Standard stock solution of nisin containing 1×10^4 IU/ml was prepared by dissolving 10 mg of nisin in 1-ml sterile 0.02 N HCl. Potassium sorbate stock solution (4% w/v), was prepared using distilled water and was filter sterilised using a 0.20 μm membrane filter.

2.3. Effect of nisin or potassium sorbate on vegetative cells

The minimum inhibitory concentrations (MICs) of nisin and potassium sorbate were determined using broth dilution method (Trotter and Marshall, 2003). Appropriate quantities of nisin or potassium sorbate solutions were added to BHI–B_{12} broth to get final concentrations of nisin and potassium sorbate ranging from 50 to 5×10^3 UI/ml and 0.2 to 2% (w/v), respectively. Overnight broth culture was diluted in peptone water (0.1% v/v) to obtain a working culture (10^5 CFU/ml). One ml aliquot was aseptically transferred into each antibacterial agent-containing broth. The MIC corresponds to the lowest concentration of the potential antibacterial agent that completely inhibits microbial growth in BHI–B_{12} broth, as determined after incubation at 37 °C for 24 h by optical density measurements at 625 nm and viable cell enumeration.

Positive control consisted of inoculated BHI–B_{12} broth without nisin or potassium sorbate. Negative control consisted of uninoculated BHI–B_{12} broth.

The obtained results were expressed as the inhibition index or I.I as determined using the following equation (Chaibi et al., 1997):

$$I.I = 1 - (\text{Change in } OD_{625} \text{ of the experimental culture/Change in } OD_{625} \text{ of the control culture})$$

The I.I equal 0 for no inhibition and 1 for total inhibition; an I.I value <0 indicates multiplication of vegetative cells greater than the control and I.I >1 indicates cell lysis.

2.4. Effect of nisin and potassium sorbate on spore germination

The influence of nisin or potassium sorbate on the germination of *B. sporothermodurans* LTIS27 spores was examined by adding various concentrations of the both antimicrobials to the germination medium as described by Aouadhi et al. (2013b).

After preparation and heat-activation (100 °C, 30 min), spores were suspended in the germination medium. Then, appropriate quantities of nisin or potassium sorbate stock solution were added to the medium. A negative control, without addition of inhibitors, was included in every experiment. The optical density at 580 nm (OD_{580}) of the spore suspension was monitored after 60 min. The germination rate is expressed as the maximum rate of loss of OD_{580} of the spore suspension, relative to the initial value. The data are presented as the percent reduction in OD, with the initial reading taken as 100% (Aouadhi et al., 2013b).

2.5. Individual or combined effect of nisin and potassium sorbate on vegetative cell and spore

The behaviours of vegetative cells and spores of *B. sporothermodurans* in the presence of sub-lethal concentrations of nisin or/and potassium sorbate, in skim milk at 37 °C for 5 days, were studied. Four antimicrobial combinations (nisin, potassium sorbate, nisin–potassium sorbate and no addition) were tested, in presence of vegetative cells or spores.

The spore growth was first evaluated in the absence of inhibitors in skim milk, supplemented with 100 mM L-alanine (Sigma–Aldrich) and 1 mM D-glucose (Sigma–Aldrich) as a germination promoter. Spores, after preparation, were heat-activated (100 °C, 30 min) before inoculation at a final concentration of 5×10^4 spores/ml. Antimicrobial concentrations in final volumes were 50 UI/ml for nisin and 0.2% for potassium sorbate, individually or in combination. Selected antimicrobial concentrations were chosen based on minimum inhibitory concentrations experiments that allow growth of strain LTIS27 yielding measurable turbidity.

For enumeration of total cells and spores, samples were removed immediately after the addition of antimicrobials and at intervals until 5 days. The presence of spores was checked before inoculating the skim milk and throughout the experiment. The total cells and spores were enumerated before and after heat treatment (100 °C, 30 min) on BHI–B_{12} agar after serial decimal dilutions in peptone water (0.1%). This treatment ensured that all vegetative