Production of nisin-loaded solid lipid nanoparticles for sustained antimicrobial activity

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A B S T R A C T

Nisin is a natural antimicrobial agent that is used as a preservative in heat processed and low pH foods. However, its bioactivity is lost by interaction with food components. Slow release nisin-loaded solid lipid nanoparticles (SLN) were produced by high pressure homogenization to provide protection from the food environment and prolong the biological activity. The optimized conditions for the preparation of Imwitor 900 based SLNs was a pressure of 1500 bars in a high pressure homogenizer for three cycles, with 5% (w/v) poloxamer 188 and 0.125% (w/v) sodium deoxycholate as the surfactant and co-surfactant, respectively. Unloaded SLN produced under this condition had the smallest nanometric particle size (119 ± 15.1 nm) with a narrow polydispersity (0.38 ± 0.03). Nisin-loaded SLNs, prepared from 0.5 to 3.0% (w/w) nisin, were larger than the unloaded SLN, with a size range of 159 ± 6.4–167 ± 8.6 nm, had a zeta-potential of −28.3 ± 0.15 to −29.2 ± 0.12 mV and nisin entrapment efficiency of 69.2 ± 0.04 to 73.6 ± 0.04%, the optimal being at 2% (w/w) nisin. During 28 day of aqueous suspension at 30 °C, the size of the SLNs increased to 214 ± 10.8–245 ± 15.7 nm and zeta-potential decreased to −21.6 ± 0.43 to −25.9 ± 0.34 mV. Scanning electron microscopy (SEM) demonstrated that nanoparticles had platelet shape. In vitro release studies revealed that nisin was released from the SLNs throughout the 25 day period but the release rate decreased as the pH of buffer increased from 2.0 to 7.4 and as the salt concentration increased, up to 0.5 M sodium chloride, whereupon high nisin was released within the first day. The antibacterial activity of nisin-loaded SLNs against Listeria monocytogenes DMST 2871 and Lactobacillus plantarum TISTR 850 was evident for up to 20 and 15 days, respectively, compared to only one and three days, respectively, for free nisin.

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1. Introduction

Nisin, a polypeptide of 34 amino acids, is produced by Lactococcus lactis strains and belongs to the lantibiotic family, which contains lanthionine and methyllanthionine groups. Nisin exists as two variants (A and Z), which differ by a single amino acid at position 27, being histidine in nisin A but asparagine in nisin Z (De Vuyst & Vandamme, 1994). Nisin has received particular attention because of its large antibacterial activity against a wide variety of Gram-positive bacteria (Tjakko, Lothar, & Colin, 1995). The generally accepted mode of action of nisin on vegetative cells involves the formation of pores in the cytoplasmic membrane of target cells leading to the efflux of essential small cytoplasmic components, such as amino acids, potassium ions and ATP (Wiedemann et al., 2001). At present, nisin is the only bacteriocin considered as Generally Recognized as Safe (GRAS), and the World Health Organization has approved its use as a food additive (WHO, 1969). Several forms of concentrated nisin are commercially produced and routinely added into food for controlling pathogenic bacteria in food products, such as milk, pasteurized cheese spreads, sauces and salad dressings (Gálvez, Abriouel, López, & Omar, 2007).

However, nisin is eventually exhausted from the foods due to its interactions with the food components, such as proteins and lipids, inactivation by enzymatic degradation, or uneven distribution of nisin molecules within the food matrix (Aasen et al., 2003; Jung, Bodyfelt, & Daeschel, 1992; Rayman, Malik, & Hurst, 1983). In order to overcome these problems, the loading of nisin into micro/nano-particles has been investigated (Benech, Kheadr, Lacroix, & Fliss, 2002; Colas et al., 2007; Lariati et al., 2003; Salmaso, Elavassore, Bertucco, Lante, & Caliceti, 2004). An enhanced stability and efficacy of nisin Z in inhibiting target bacteria during...
cheddar cheese ripening when encapsulated in liposomes, compared to nisin produced in situ, has been reported (Benen et al., 2002). Moreover, the encapsulation efficiency and physico-chemical properties of the liposomes were reportedly affected by proliposomes, the pH and concentration of the nisin solution and liposome stability (Laridi et al., 2003). In addition, the nisin release rate was noted to depend on the environmental conditions, such as the presence of bivalent ions and other medium components (Laridi et al., 2003). Nisin-loaded polymeric nanoparticles produced by semi-continuous compressed CO₂ anti-solvent precipitation were also found to prolong the antimicrobial activity of nisin compared to that seen with free nisin. Although this formulation provided a slow nisin release rate and sustained antimicrobial activity, the use of an organic solvent in this method might cause a cytotoxic effect and may also modify the protein structure by alteration of the hydrophobic/hydrophilic interactions (Salmaso et al., 2004). In addition, the large scale production of polymeric nanoparticles is troublesome (Wissing, Kayser, & Muller, 2004). Hence, alternative materials for production of sustained-release nisin from nanoparticles were studied.

Solid lipid nanoparticles (SLN) represent one such alternative colloidal drug delivery system, and the use of solid lipids as matrix materials for drug delivery is already well-known from the use of lipid pellets for oral delivery. In addition, nanoparticles made from solid lipids instead of lipid oil have gained increasing attention during recent years (Mehnert & Mader, 2001), since the drug mobility in solid lipids is considerably lower than that in liquid oils (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003). SLNs can be employed for various purposes for which nanoparticles have distinct advantages (Wissing et al., 2004). These advantages include the possibility of incorporating drugs for controlled drug release, the low cytotoxicity due to its composition of physiological compounds, the possibility for loading both lipophilic and hydrophilic drugs into the solid matrix and for its likely large scale production. Also, the solid matrix has been shown to protect the incorporated active ingredients, such as lysozyme, tamoxifen and cyclosporine, against chemical degradation (Almeida, Runge, & Muller, 1997; Muller et al., 2008; Reddy, Vivek, Bakshi, & Murthy, 2006).

The present study aims to prepare stable long lasting antibacterial nisin-loaded SLNs, which can be used as food preservatives for a diverse array of foods with different physical consistencies. Thus, the particle morphology, nisin release profile and in vitro biological properties of the nisin-loaded SLNs were characterized in order to verify the reproducibility of nisin-loaded SLN production and to determine the feasibility to use nisin-loaded SLNs as a food preservative.

2. Materials and methods

2.1. Materials

Nisin powder (2.5% (w/w) pure nisin in denaturated milk solids, approximately 1,000,000 IU/g) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The solid lipids (Cetyl palmitate, Softisan 378, Softisan 154, Immun 900 and Witsepol EBS) were obtained from Sasol, Germany. The surfactant Poloxamer 188 was bought from BASF, Germany and the co-surfactant sodium deoxycholate was purchased from Fluka (Fluka Biochemika, Buchs, Switzerland). Brain heart infusion (BHI) and Lactobacilli MRS medium were obtained from Difco (Sparks, MD, USA). All other chemicals used in the study were of analytical grade.

2.2. Bacterial strains

Listeria monocytogenes DMST 2871 and Lactobacillus plantarum TISTR 850 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). L. monocytogenes DMST 2871 was grown in BHI broth at 37 °C for 24 h, whilst L. plantarum TISTR 850 was grown in MRS broth for 24 h at 30 °C.

2.3. Nisin solubility in melted solid lipids

The nisin solubility studies were carried out to identify the solid lipid composition that conferred a better solubility for nisin. Four different amounts of nisin powder (0.5%, 1%, 2%, and 3% (w/w)) were dissolved in each of the five different melted solid lipids at 80 °C with stirring. Then, the solubility was determined by visual observation.

2.4. Preparation of nisin-loaded SLNs

The nisin-loaded SLNs were prepared using hot high pressure homogenization. Briefly, a lipid phase composed of 10% (w/w) of the solid lipid (hereon this was Immun 900) was melted at approximately 5–10 °C above its melting point. The melted phase was then mixed with the aqueous surfactant solution containing 2.5% or 5.0% (w/v) poloxamer 188 and 0.125% (w/v) sodium deoxycholate, pre-heated to the same temperature. The pre-emulsion was produced by high speed stirring using an Ultra-Turrax T25 (Jahneke and Kunkel GmbH, Germany) for 1 min at 8000 rpm. Then, the resulting pre-emulsion was homogenized at the melting temperature by using an Emulsiflex C3 (Avestin, Canada) at pressure of 500, 1000 or 1500 bars with three homogenization cycles. Finally, the nanoemulsion was recrystallized to SLN by cooling down to room temperature.

2.5. Nisin encapsulation efficiency

The amount of nisin loaded into the SLNs was determined by suspending 50 mg of nanoparticles in 1.0 ml of methanol and then extracting the loaded nisin with 1 ml of 0.02 M hydrochloric acid. The mixture was centrifuged at 13,000 × g for 10 min and the supernatant was then analyzed for nisin content by reverse-phase HPLC using a C-18 column eluted with water/0.05% (v/v) TFA (eluent A) and acetonitrile/0.05% (v/v) TFA (eluent B) (gradient: 0–5 min, 20% eluent A; 5–20 min from 20% eluent A to 80% eluent A) with UV detection at 220 nm. The amount of nisin was calculated by means of a calibration curve, derived from standards of nisin.

2.6. In vitro nisin release studies

Nisin release experiments were performed by suspending 50 mg of 2.0% (w/w) nisin-loaded SLNs into 1 ml of 0.02 M phosphate buffer (pH 5.0) containing 0.05, 0.1, 0.2 or 0.5 M NaCl, or in 1 ml of 0.15 M NaCl/0.02 M phosphate buffer at pH 2.0, 3.0, 5.0, 6.0 or 7.4. The suspensions were stirred and then sonicated for 5 min and maintained at room temperature under shaking at 150 rpm. At the indicated time, 1 ml of sample was withdrawn from the suspension and loaded into an Ultra-filtration tube (M.W. cutoff of 30 kDa) and centrifuged at 3000 × g for 10 min. The nisin content in the buffer was determined by reverse-phase HPLC, as previously described. All experiments were performed in triplicate.

2.7. In vitro nisin biological activity

The biological activity of the nisin-loaded SLNs was evaluated by determination the number of bacteria colony forming units (CFU) after incubation of a 1% (v/v) suspension of either L. plantarum TISTR 850 cells in MRS medium at 30 °C or L. monocytogenes DMST 2871 in BHI medium at 37 °C, both for 24 h, with nisin-loaded SLNs at one of four concentrations (0.5%, 1%, 2% and 3%). In addition,
the assays were set up in parallel with the equivalent amount of free nisin for comparison. Phosphate buffer and unloaded SLN were used as the positive and negative controls, respectively. All experiments were performed in duplicate.

2.8. Stability, size measurement and morphological characterization of SLNs

Unloaded and nisin-loaded SLNs were stored at 30 °C for 14 and 28 days, respectively. Thereafter the average particle size, zeta-potential and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) using a Zetasizer NanoZS apparatus (Malvern Instruments, UK), and analyzed by the CONTIN method in the MALVERN software. Size measurements were performed immediately after preparation of the SLNs by dispersing 20 μl of each nanoparticle suspension in 5 ml of distilled water. Then, the suspensions were sonicated for 3 min and filtered through a 0.45 μm membrane filter prior to particle size determination. The morphology of the dried nisin-loaded SLNs was examined by scanning electron microscopy (SEM) (S-3400 N, Hitachi, Japan). Nanoparticle suspensions were dried and then stuck onto a brass stub with double-sided adhesive tape and were sputtered with gold under high vacuum. Samples were then observed under low vacuum at an accelerating voltage of 20 kV. Photographs were taken at magnifications ranging from 1000 to 25,000×.

3. Results

3.1. Nisin solubility in melted solid lipids

Due to its hydrophobic nature, SLNs may be more appropriate to incorporate relatively lipophilic molecules that can easily dissolve in melted lipids (Almeida et al., 1997). Thus, the solubility of the amphiphilic peptide nisin in each of the five solid lipids was first evaluated out by dissolving known amounts of nisin into each of the five melted solid lipids (i.e. cetylpalmitate, Softisan 378, Softisan 154, Imwitor 900 and Witepsol E85) and then stirring at 80 °C for 15 min. The suspensions were filtered prior to particle size determination in the presence of 0.125% (w/v) sodium deoxycholate provided the smallest average size of SLN (119 ± 15.1 nm) with the smallest change in zeta-potential (−34.0 to −30.2 mV) during the 14 day observation period. Moreover, the SLNs produced under this condition had a polydispersity index (PDI) of 0.38 ± 0.03, indicating a good homogeneity of the nanoparticles. Therefore, this condition was used for the preparation of nisin-loaded SLNs.

3.2. Stability test of unloaded SLN dispersions

For the production of nisin-loaded SLNs, both the surfactant concentration and the homogenization pressure were varied by preparing a pre-emulsion with either 2.5% or 5.0% (w/w) poloxamer 188 as the surfactant in the presence of 0.125% (w/v) sodium deoxycholate and subjecting this mixture to a homogenization pressure of 500, 1000 or 1500 bars. With a poloxamer 188 concentration of 2.5% (w/v), as the homogenization pressure increased from 500 to 1000 bars, the mean particle size of SLNs remarkably decreased and zeta-potential slightly increased at 0 and 7 days. Increasing the pressure further to 1500 bars had no significant effect on the diameter or zeta-potential of particles stored for seven days, or the particle size of fresh samples, but significantly decreased the particle size of SLNs stored for 14 days (Fig. 1).

Aside the higher zeta-potential of freshly prepared nanoparticles with 1500 bars, broadly similar trends were obtained with the three homogenization pressures when the concentration of the poloxamer 188 was increased from 2.5% to 5.0% (w/v). The decrease in SLN diameter was more marked at 14 days for samples made at 1000 bar and, as the pressure increased from 1000 to 1500 bar, the significant increase in zeta-potential was also observed in fresh sample and storage up to 7–14 days (Fig. 2).

Thus, with respect to particle size, as measured by photon correlation spectroscopy (PCS), using a homogenization pressure of 1500 bars and a 5.0% (w/w) poloxamer 188 surfactant concentration in the presence of 0.125% (w/v) sodium deoxycholate provided the smallest average size of SLN (119 ± 15.1 nm) with the smallest change in zeta-potential (−34.0 to −30.2 mV) during the 14 day observation period. Moreover, the SLNs produced under this condition had a polydispersity index (PDI) of 0.38 ± 0.03, indicating a good homogeneity of the nanoparticles. Therefore, this condition was used for the preparation of nisin-loaded SLNs.

3.3. Characterization of nisin-loaded SLNs

The dispersions of freshly prepared nisin-loaded SLNs had the appearance of homogeneous cream with white color when observed macroscopically. Particle size measurements showed that freshly prepared nisin-loaded SLNs had a size range of

Table 1 Solubility study of nisin at 3% (w/w) in various types of melted solid lipid at 80 °C

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Time (min)</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetylpalmitate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Softisan 154</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Softisan 378</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Imwitor 900</td>
<td>M</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Witepsol E85</td>
<td>1</td>
<td>1</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
</table>

S, soluble; M, moderately soluble; I, insoluble.

Fig. 1. (A) Particle size and (B) zeta-potential of unloaded SLN prepared from Imwitor 900 and either 2.5 (w/v) poloxamer 188 and a homogenization pressure of 500, 1000 or 1500 bars, during 14 day incubation at 30 °C in the phosphate buffered saline. Data are expressed as the mean ± SD, and are derived from three independent repeats.
6.4 e 167/C6 8.6 nm for 0.5 e 3% (w/w) nisin (Fig. 3A) and a zeta-potential ranging from 28.3 ± 0.15 to 29.2 ± 0.12 mV (Fig. 3B). Incorporation of nisin into SLNs therefore resulted in a slight increase in the average particle size with a slight decrease in zeta-potential. After 28 days of storage at 30°C, the 0.5%, 1.0%, 2.0% and 3.0% nisin-loaded SLNs had all significantly increased their average sizes from 160 ± 5.3, 159 ± 6.4, 161 ± 5.5 and 167 ± 8.6 nm to 220 ± 10.7, 214 ± 10.8, 224 ± 9.1 and 245 ± 15.7 nm, respectively, with overall decrease in zeta-potential (Fig. 3A and B). The morphological characteristics of nisin-loaded SLNs, as examined using SEM, were demonstrated to be in the nanometric size range with a platelet-like shape (Fig. 4).

3.4. Entrapment efficiency

SLN dispersions were prepared with a constant Imwitor 900 lipid concentration of 10.0% (w/w), whilst the nisin concentrations were varied from 0.5% to 3.0% (w/w) with respect to the lipid. The results showed that increasing the concentration of the nisin from 0.5% to 2.0% (w/w) led to a slight increase in the nisin entrapment efficiency to a maximum of 73.6 ± 0.04% (Table 2).

3.5. In vitro nisin release studies

The release of entrapped nisin from the nisin-loaded SLNs was evaluated by incubating the SLNs in phosphate buffered saline of various pH values or sodium chloride concentrations. A slow but incomplete nisin release into the media from the nisin-loaded SLNs were observed throughout the 25 day assay period, but both the total amount of released nisin and the release rate depended on the solution pH (Fig. 5) and salt concentration (Fig. 6).

With respect to the pH, nisin release could be detected throughout the 25 day period, but as the medium pH was decreased, both the accumulative amount of released nisin and the

<table>
<thead>
<tr>
<th>Concentration of loaded nisin (% (w/w))</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>72.3 ± 0.12</td>
</tr>
<tr>
<td>1.0</td>
<td>70.1 ± 0.08</td>
</tr>
<tr>
<td>2.0</td>
<td>73.6 ± 0.04</td>
</tr>
<tr>
<td>3.0</td>
<td>69.2 ± 0.04</td>
</tr>
</tbody>
</table>
release rate increased. However, at each pH, the release rate decreased dramatically after the second day onwards (Fig. 5) indicating a slow nisin release from the SLNs after 5–25 days of suspension.

With respect to the salt concentration, as it decreased then so both the accumulated nisin level in the medium and the release rate also increased (Fig. 6), with the exception of at 0.5 M NaCl, where a burst release was observed on the first day of experiment that resulted in an immediate loss of nisin accumulation in the medium afterward.

3.6. In vitro nisin biological activity

The biological activity of free nisin and nisin-loaded SLNs are shown in Fig. 7. Free nisin displayed antimicrobial activity against _L. monocytogenes_ DMST 2871 and _L. plantarum_ TISTR 850 for three and one day, respectively, and thereafter the bacterial growth rebounded, suggesting no significant antibacterial activity persisted beyond these time points. In contrast, the inhibitory effect of nisin against _L. monocytogenes_ DMST 2871 from 0.5%, 1%, 2% and 3% loaded SLNs was prolonged for at least up to 20 days. However, in the case of _L. plantarum_ TISTR 850, nisin-loaded SLNs could prolong their antibacterial activity to 15 days. In contrast, unloaded SLN (a negative control) did not show any antibacterial activity.

4. Discussion

The preparation of nisin-loaded SLNs, in an attempt to prolong the antibacterial activity of nisin, was achieved by hot and high pressure homogenization. This method requires a suitable solid lipid matrix and the optimization of production conditions to

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**Fig. 5.** In vitro release kinetics of nisin from SLN suspended in 0.15 M NaCl/0.02 M phosphate buffer at the indicated pH. Data are shown for (A) the accumulated total amount of nisin released during 25 days and (B) the nisin release rate during the first 25 days. Data are expressed as the mean of duplicate experiments.

**Fig. 6.** In vitro release kinetics of nisin from non-loaded SLN suspended in 0.02 M phosphate buffer at pH 5.0 containing NaCl at the indicated concentrations. Data are shown for (A) the accumulated total amount of nisin released during the 25 days and (B) the nisin release rate during the first 25 days. Data are expressed as the mean of duplicate experiments.

**Fig. 7.** Antibacterial activity of non- and nisin-loaded SLNs against (A) _Listeria monocytogenes_ DMST 2871 and (B) _Lactobacillus plantarum_ TISTR 850. Data are expressed as the mean of duplicate experiments.
obtain nisin-loaded SLNs. Therefore, a preliminary study of the solubility of nisin in the melted solid lipids, and the preparation of unloaded SLN, were first evaluated. Nisin could dissolve to ~3% (w/v) in Imwitor 900 but not in the other four tested lipids, presumably since Imwitor 900 contains a relatively high level of monoglyceride (Soukharev, 2007). In the case of unloaded nanoparticles, a decrease in particle size was achieved with increasing homogenization pressures leading to the development of shear forces that broke down the nanoparticle structure to a smaller size (Reddy et al., 2006). Moreover, the use of a higher surfactant concentration (5% w/w) poloxamer 188 could help reduce the surface tension and facilitated particle partition during homogenization, giving a higher zeta-potential that resulted in electrostatic repulsion to prevent nanoparticle aggregation (Olbrich & Muller, 1999). The reduction in the zeta-potential of the nanoparticle surface could otherwise lead to aggregation upon storage condition. In this study, nanoparticle aggregation was accelerated by high storage temperatures, probably due to the destabilization of SLN dispersions as a consequence of kinetic energy input to a level above the critical point that would favor the collision of SLNs and the loss of electrostatic repulsion (Freitas & Muller, 1998). With respect to the nisin entrapment efficiency, a 2.0% (w/v) nisin loading led to saturation of the lipid matrix and attained an entrapment efficiency of 73.6 ± 0.04%, whilst the higher nisin loading concentration of 3% (w/w) just resulted in an increased level of free nisin rather than nisin encapsulation inside the lipid matrix. Peptides themselves might be difficult to be encapsulated, especially due to the hydrophobic nature of the lipid component of the carrier system, and thus peptides have the tendency to adsorb onto the surfaces (Hu, Hong, & Yuan, 2004). Such adsorption could lead to distinct losses in the amount of peptide available for delivery (Muller et al., 2008). In addition, the physicochemical properties of the peptide, as well as the preparation process, will determine the amount of peptide that can be incorporated into the delivery system (Hu et al., 2004).

Nisin release from the nisin encapsulated SLNs was studied by incubation of the SLNs in media at different pH values and NaCl concentrations. Lower pH values accelerated the hydrolysis of the SLNs and so resulted in a higher level of nisin being released into the medium (Zimmermann & Muller, 2001), whilst the increasing hydrophobic interactions of nisin molecules at lower pH values might lead to entrapment of nisin within the solid matrix (Liu & Hansen, 1990). A high salt concentration in the medium can dehydrate the surfactant layer of poloxamer 188 and thereby further reduce its thickness and stabilizing effect, leading to nanoparticle aggregation and a reduced surface area of particles (Zimmermann & Muller, 2001).

The biological activity (antibacterial) and nisin release studies demonstrated that nisin was entrapped in the nanoparticles in an active form, and thus that the hot homogenization did not substantially deteriorate the nisin activity. Nisin showed a sustained release rate from nanoparticles and could inhibit the growth of L. monocytogenes DMST 2871 and L. plantarum TISTR 850 over at least 15–20 days, compared to less than three days for free nisin in solution. However, nisin-loaded SLNs did not show the inhibitory effect against bacterial growth during the first two days of incubation, presumably because the nisin concentrations in the media released from the nanoparticles were still insufficient to completely inhibit the growth of the indicator strains. However, a short-term antibacterial action of free nisin has previously been observed to contribute to the development of nisin tolerance (Chi-Zhang, Yam, & Chikindas, 2004), or else a loss of antagonist activity from an insufficient concentration and/or proteolytic degradation of nisin (Huang et al., 2009; da Silva Malheiro, Daroit, da Silveira, & Brandelli, 2010).}

**References**


