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Antimicrobial effect of nisin in processed cheese - Quantification of residual nisin by LC-MS/MS and development of new growth and growth boundary model for *Listeria monocytogenes*

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ABSTRACT

This study tested the hypothesis that growth of *Listeria monocytogenes* in processed cheese with added nisin can be predicted from residual nisin A concentrations in the final product after processing. A LC-MS/MS method and a bioassay were studied to quantify residual nisin A concentrations and a growth and growth boundary model was developed to predict the antilisterial effect in processed cheese. 278 growth rates were determined in broth for 11 *L. monocytogenes* isolates and used to determine 13 minimum inhibitory concentration (MIC) values for nisin between pH 5.5 and 6.5. To supplement these data, 67 MIC-values at different pH-values were collected from the scientific literature. A MIC-term was developed to describe the effect of pH on nisin MIC-values. An available growth and growth boundary model (doi: https://doi.org/10.1016/j.fm.2019.103255) was expanded with the new MIC-term for nisin to predict growth in processed cheese. To generate data for model evaluation and further model development, challenge tests with a total of 45 growth curves, were performed using processed cheese. Cheeses were formulated with 11.2 or 12.0 ppm of nisin A and heat treated to obtain residual nisin A concentrations ranging from 0.56 to 5.28 ppm. Below 15 °C, nisin resulted in extended lag times. A global regression approach was used to fit all growth curves determined in challenge tests. This was obtained by combining the secondary growth and growth boundary model including the new term for the inhibiting effect of nisin on μ_{max} with the primary logistic growth model with delay. This model appropriately described the growth inhibiting effect of residual nisin A and showed that relative lag times depended on storage temperatures. With residual nisin A concentrations, other product characteristics and storage temperature as input the new model correctly predicted all observed growth and no-growth responses for *L. monocytogenes*. This model can support development of nisin A containing recipes for processed cheese that prevent growth of *L. monocytogenes*. Residual nisin A concentrations in processed cheese were accurately quantified by the developed LC-MS/MS method with recoveries of 83 to 110% and limits of detection and quantification being 0.04 and 0.13 ppm, respectively. The tested bioassay was less precise and nisin A recoveries varied for 53% to 94%.

1. Introduction

Nisin A is an antimicrobial peptide belonging to the lantibiotic class of bacteriocins (Class I), composed of 34 aminoacids and with a molecular mass of 3.5 kDa. Nisin has antimicrobial activity against a wide range of Gram-positive food-borne pathogens and it influences both cell membrane pore formation and cell wall synthesis (Cotter et al., 2005). Commercial nisin preparations have been used for over 30 years and are allowed in foods as an added preservative (FDA, 1988; Thomas and Delves-Broughton, 2005). Nisin is used in processed cheese to control potential growth of spore-forming bacteria. The anaerobic environment, high moisture and pH of 5.4 to 6.0 usually found in processed cheese may allow germination of spores and growth of *Clostridium* spp. or facultative anaerobic *Bacillus* spp. if present in the product and not

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controlled by means of formulation (Delves-Broughton, 2008; Glass et al., 2017). Though nisin has a well-documented use for the control of these spore-forming bacteria in processed cheese formulations, little information is available with regards to its protective effect against vegetative pathogens such as *L. monocytogenes*, that may contaminate these products during consumer handling (Redmond and Griffith, 2003).

The antimicrobial activity of nisin against *L. monocytogenes* has been studied in broth or agar by determination of minimum inhibitory concentration (MIC) (Benkerroum and Sandine, 1988; Ferreira and Lund, 1996; Martínez and Rodríguez, 2005). Furthermore, several nisin bioassays have been developed over the years to measure the antimicrobial activity of nisin. The most widely used bioassay is the horizontal agar diffusion method relying on a test microorganism (Fowler et al., 1975; Papagianni et al., 2006; Tramer and Fowler, 1964). When estimating residual nisin A in foods one has to use a method describing the extraction of nisin A from foodstuffs. However, no information is available about recovery and precision for bioassays when used to estimate residual nisin A in foods (Pongtharangkul and Demirci, 2004; Rogers and Montville, 1991).

In the last years, more advance analytical methods have been developed to quantify nisin A present in foods. An ISO standard has been published for the quantification of nisin A in cheese by liquid chromatography/mass spectrophotometry (LC-MS) and LC-MS/MS (ISO, 2009). This LC-MS/MS method has been used to quantify nisin A in different types of cheeses (Fuselli et al., 2012; Ko et al., 2016; Schneider et al., 2011). However, the relation between concentrations of nisin measured by LC-MS/MS and its antimicrobial activity has not been studied.

The objectives of the present study were to develop and validate a LC-MS/MS method for quantification of residual nisin A in different cheeses and to determine the inhibitory effect of residual nisin A concentrations on growth responses of *L. monocytogenes* in cheese. The antilisterial effect of nisin A was studied by using a predictive food microbiology approach for comparison of observed and predicted growth. Firstly, a nisin MIC-term was developed including the effect of pH on nisin MIC-values and this term was added to an available cardinal parameter growth and growth boundary model. Secondly, processed cheese with added nisin A was studied by challenge testing to quantify growth of *L. monocytogenes* at different constant storage temperatures and product characteristics (pH, a_w, lactic-, acetic-, citric acids, orthophosphate salt and nisin A concentrations). These data was used to develop a simple secondary model for relative lag time (RLT). Lastly, the expanded growth and growth boundary model was evaluated by comparison of observed and predicted growth for *L. monocytogenes* in cheeses when (a) assuming absence of nisin A, (b) using residual nisin A concentrations measured by LC-MS/MS and (c) using concentrations of nisin A added to the cheeses at formulation.

2. Materials and methods

2.1. Nisin and stock solutions

Nisin stock solutions (50 ppm and 500 ppm) were prepared with Nisaplin (2.5% nisin A, Danisco, batch number 4263501621) by adding 0.020 g or 0.200 g to 10 ml of 0.02 N HCl. These stock solutions were stored at 4 °C for a maximum of 24 h.

2.2. Nisin quantification by LC-MS/MS

2.2.1. Nisin extraction method for LC-MS/MS analysis

Methanol:water (1:1 *v*/v) (HPLC grade, VWR chemicals, Søborg, Denmark) with 0.1% formic acid (695076, Sigma-Aldrich, St. Louis, MO, USA) adjusted with 5 M HCl to pH 2.0 was used as solvent to extract nisin A from cheese. Cheese (2.0 g) was homogenized in 30 ml extraction solvent with a T25 Ultra Turrax (IKA, Staufen, Germany). The suspension was sonicated for 10 min. at room temperature and then centrifuged (Eppendorf centrifuge 5810 R, Hørsholm, Denmark) at 4500 rpm for 20 min at 4 °C. The supernatant was transferred to a volumetric flask and the precipitate was washed/extracted twice with 10 ml extraction solvent. Supernatants were combined and supplemented as necessary with extraction solvent to 50 ml and then filtered (0.2 µm Ministart ®, Sartorius Stedim Biotech GmbH) prior to LC-MS/MS analysis.

Table 1
Comparison of LC-MS/MS protocols from the present study and from ISO (2009).

	Present study	ISO (2009)
Equipment	UHPLC (Dionex Thermo scientific) EVOQ EliteTM triple quadrupole MS (Bruker)	LC-MS/MS
Column	Zorbax 300 SB-C18 (2.1 x 50 mm) (Agilent Technologies)	PLRP-S (2.1 x 150 mm) (Polymer Lab Ltd.)
Extraction solvent	0.1% formic acid, methanol/water (1:1), pH 2.0 with HCL	Formic acid/water (1:200)
Parent ion	839	672
Fragment ions	1081, 811	811

2.2.2. LC-MS/MS analysis

For the LC-MS/MS analysis (Table 1), an EVOQ Elite™ triple quadrupole mass spectrometer (Bruker, Frederikssund, Denmark), equipped with an electrospray ionization (ESI) source was coupled to an UltiMate 3000 ultra-high-performance liquid chromatography system (UHPLC) from Dionex Thermo scientific (Waltham, MA, USA). The UHPLC consisted of a pump, autosampler and column compartment. Chromeleon Xpress (Thermo Fisher Scientific, Waltham, USA) and MS Workstation 8 (Agilent, Santa Clara, USA) were used to perform system control and data acquisition on the UHPLC and mass spectrometer, respectively. Chromatographic separation was performed using a Zorbax 300 SB-C18 column (Table 1).

The mobile phase consisted of an aqueous solution with 0.1% formic acid (mobile phase A) and 100% acetonitrile (mobile phase B; Chromasolv™ LC-MS, Honeywell, Seelze, Germany). A linear gradient was used as follows: 0–2 min 80% A; 2–6 min 20% A; 6–7 min 80% A, and held for 3 min to equilibrate the column. The column was maintained at 40 °C. The flow rate was 0.3 ml/min and the injection volume for the nisin standards (0.1, 0.5, 1, 2.5, 5, 10 ppm) or samples was 10 µl. The diverter valve was used to eliminate NaCl interferences, directing the flow into the discharge for the first 3 min of each run, avoiding contamination of the ion source. The parent ion $[M+4H]^{4+}$ was identified as 839.0 (average mass, 3354). The fragment ions of nisin A were selected as 1081 (quantitative ion) and 811 (qualitative ion). Diagnostic ions were found using an infusion experiment. A standard solution of nisin A (1 ppm) was infused directly into the ion source without applying collision gas and collision energy to identify parent ions. Fragment ions were likewise identified with infusion of standard solution and collision gas and collision energy applied.

2.2.3. Calculation of nisin A concentrations

Content of nisin A in cheese samples was calculated using Eq. (1).

$$Nisin\ A\ (\text{ppm}) = \frac{H_t \cdot \rho_{nA,s} \cdot V_t}{H_s \cdot m_t} \quad (1)$$

where H_t is the test sample peak area, $\rho_{nA,s}$ is the concentration in ppm of the standard solution used to construct the calibration curve, V_t is the test sample volume (50 ml), H_s is the peak area of $\rho_{nA,s}$ and m_t is the mass (g) of cheese sample.

2.3. Nisin quantification by bioassay

The method described in this section combined a simple nisin extraction method and quantification by a plate diffusion assay with an

indicator organism (Tramer and Fowler, 1964).

2.3.1. Nisin extraction for the bioassay

Cheese (2.0 g) was macerated in a 50 ml glass beaker using 10 ml of 0.02 N HCl with pH 2.0 and magnet stirring for 30 min. Thereafter the mixture was place on a hot plate (pre-heat to 100 °C) with magnetic stirring and boiled for 5 min. 40 ml of 0.02 N HCl was used to wash the beaker and the sample was transfer to a 50 ml falcon tube and centrifuged at 4500 rpm for 20 min at 4 °C (Eppendorf centrifuge 5810 R, Hørsholm, Denmark). The supernatant with nisin was analysed as described below.

2.3.2. Indicator organism and pre-culture conditions

Preliminary studies with both *Micrococcus luteus* DSM 1790 and *Lactobacillus sakei* ATCC 15521 as indicator organisms were performed. Quantification of nisin did not differ between the two indicator organisms and *M. luteus* was retained as its yellow colonies produced a more clear inhibition zone. *M. luteus* DSM 1790 was transferred from storage at −80 °C to Nutrient broth (NB) (CM001, Oxoid, Hampshire, UK) and incubated for 24 h at 30 °C. Subsequently, the culture with *M. luteus* was streaked on NB agar (1.3%) plates and incubated for 48 h at 30 °C. 200 ml NB containing a magnet was inoculated with a single colony and incubated at 30 °C with stirring (200 rpm) for 24 h. The cell concentration was then determined by direct phase contrast microscopy at 1000× magnification considering that one cell per field of view corresponded to a concentration around 10^6 cfu/ml (Adams et al., 2016).

2.3.3. Agar diffusion bioassay

Molten NB (1.3%) agar tempered at 48 °C was supplemented with 2% of a 1:1 mixture of Tween 20 (P9416, Sigma-Aldrich, St. Louis, USA) and sterile water, previously held for 30 min at 48 °C, and thoroughly mixed. The medium agar (200 ml bottles) were held for 30 min at 46 °C and then inoculated with the indicator organism to reach a final concentration of 10^6 cfu/ml. 20 ml of inoculated medium was placed in each sterile Petri dish (100 × 20 mm). After agar solidification at room temperature, wells of 5 mm diameter were made with a cork borer. 30 µl of nisin standard solutions (0.1, 0.5, 1, 2.5, 5, 10 ppm; pH 2.0) were placed in each well and the plates were incubated at 30 °C for 72 h. The diameter of inhibition zones around wells with nisin were measured on images of the plates by using an automated image analyser software (ImageJ Basics, v. 1.38, 2008). Four zones were measured for each nisin concentration and assays were performed in triplicate on three different days ($n = 4 \times 3 \times 3$).

2.4. Evaluation of methods for quantification of nisin

The quantification methods were evaluated for linearity, specificity, recovery and precision (NMKL, 2009). The only difference in the evaluation of the methods studied was the used of two matrixes (PC and SPC) for LC-MS/MS or exclusively one (SPC) for the evaluation of the bioassay. Standard curves with four concentrations (including zero) (x-axis) and the corresponding peak areas determined by the LC-MS/MS method (y-axis) were developed for both PC and SPC. Likewise, standard curves for the bioassay were developed with four concentrations and the corresponding inhibition zone but exclusively for SPC. Linearity was evaluated using two replicates for each nisin concentration, in three different days. LC-MS/MS linearity of the response was further studied by plotting residuals against concentrations of standard curves performed for the two types of cheeses. LC-MS/MS specificity was studied by analysing samples of each matrix, to evaluate the possibility for interference of some endogenous compounds. The results were assessed by checking the presence of interfering substances with the same retention time as nisin A, in comparison to cheese samples fortified with nisin A. The recovery and precision (repeatability and reproducibility) of both, the LC-MS/MS and bioassay method were determined by spiking experiments where PC and SPC samples were fortified with 0.1, 0.5 and 1 ppm of nisin A before and after the extraction procedure. Precision with limits of acceptability <16% for CV and 80–110% for recovery were based on duplicate analysis on three different days for each concentration level. Precision was the degree of agreement between independent analysis results obtained in different days and was calculated by the standard deviation of the method's repeatability and reproducibility. Data analyses were performed using the Microsoft Excel software. The limit of detection (LOD) was calculated as three times the within sample standard deviation for reproducibility (S_R) and the limit of quantification (LOQ) was calculated as 10 times S_R. Matrix effects (suppression or enhancement of signal due to other substances in the sample) were calculated by comparing slopes for two types of calibration curves. Curve type I, was prepared by spiking extraction solvent with nisin A to reach 0.1, 0.5 and 1 ppm. Curve type II, was prepared by spiking cheese samples with nisin A to reach 0.1, 0.5 and 1 ppm. Matrix effect was calculated by division of the slope of curve type II with the slope of curve type I.

2.5. Development and evaluation of growth and growth boundary model for Listeria monocytogenes

2.5.1. Bacterial strains and pre-culture conditions

Seven dairy related strains of *L. monocytogenes* were provided by Arla Foods or Martínez and Rodríguez (2005). The strains were used as a cocktail (*Lm-mix* including SLU 92, 612, LM 19 and 6) or individually (LM 13, LM 4, LM 4R) to determine μ_{max}-values in broth and for inoculation of challenge tests. Strain LM 4R was a spontaneous nisin resistant derivate from LM 4. Each strain was transferred from storage at −80 °C to Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Hampshire, UK) and incubated for 24 h at 25 °C. Subsequently, for broth studies the strains were pre-cultured at 7 °C or 20 °C in BHI broth adjusted with HCl to pH 5.5, 6.0 or 6.5. For challenge tests the individual strains (SLU 92, 612, LM 19, 6) were pre-cultured at a temperature ranging from 8 °C to 20 °C in BHI broth with 1% NaCl and pH 5.5 (HCl) to simulate conditions in processed cheese. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). *Lm-mix* used in broth and challenge tests was obtained by mixing different volumes of individually pre-cultured strains depending in the absorbance measurement. The *Lm-mix* concentration was determined by direct phase contrast microscopy at 1000× magnification considering that one cell per field of view corresponded to a concentration around 10^6 cfu/ml (Adams et al., 2016).

2.5.2. Cardinal parameter term for nisin and expansion of growth and growth boundary model

The effect of 24 different nisin A concentrations (0–10 ppm nisin, Nisaplin®, Danisco, Braband, DK in 0.02 N HCl) on μ_{max}–values of *Lm-mix* or individual strains were determined in duplicate by automated absorbance measurements at 540 nm (BioScreen C, Labsystems, Helsinki, Finland) at 7 °C or 20 °C using BHI broth adjusted to pH 5.5, 6.0 or 6.5 with HCl and sterilized by filtration (0.2 µm, Minisart®, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Detection times, defined as the incubation time necessary to observe an increase in absorbance of 0.05 from the lowest absorbance measured in the beginning of incubation were determined for each absorbance growth curve. μ_{max}-values were determined from absorbance detection times for serially diluted inoculation levels of 10^2, 10^3, 10^4, 10^5 and 10^6 cfu/ml as previously described (Dalgaard and Koutsoumanis, 2001). A total of 278 μ_{max}-values of *L. monocytogenes* were determined experimentally in BHI-broth. Nisin MIC-values, μ_{ref^*}, n1 and n2 were estimated by fitting Eq. (2) to square root transformed μ_{max}-values obtained for the studied nisin concentrations.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref^*} \cdot \left(1 - \left(\frac{nisin}{MIC_{nisin}}\right)^{n_1}\right)^{n_2}} \quad (2)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) and μ_{ref^*} is the reference maximum specific growth rate for the studied temperature. When fitting Eq. (2), n1 was set to 0.5 or 1 and n2 was set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately and this was determined from root mean square error (RMSE) values. The "n" values are specific for the combination of the microorganism and the inhibitory compound. A simple equation was used to model and predict the effect of pH on log-transformed nisin MIC-values, log $MIC_{nisin} = a + b * pH$.

A total of 67 nisin MIC-values, for different *L. monocytogenes* strains and determined in broth or agar with different pH values (relevant for processed cheese), were extracted from literature. Four MIC-values were obtained at pH 5.0, 39 MIC-values at pH 5.5 and 24 MIC-values at pH 6.8 for a total of 29 different strains (Ferreira and Lund, 1996; Taylor, 2009).

The growth and growth boundary model of Martinez-Rios et al. (2019) (Supplementary Table 1) previously validated for processed cheese and containing terms for the inhibitory effect of temperature, a_w, pH, lactic, acetic and citric acids as well as phosphate melting salts was expanded with a nisin-term (Eq. (2)) to predict growth of *L. monocytogenes* in processed cheese containing nisin (Eq. S3 in Supplementary Table 4).

2.5.3. *L. monocytogenes growth in processed cheese with added nisin*

To generate data for model development and evaluation, growth of *L. monocytogenes* in commercial PC and laboratory produced SPC was determined in 15 challenge tests in triplicate resulting in 45 maximum specific growth rates, lag times and initial and maximum cell concentrations at constant temperatures from 3.6 °C to 19.5 °C.

2.5.3.1. Production, inoculation and microbiological analysis of processed cheese. Arla Foods supplied PC cheese formulated with 11.2 ppm of added nisin A and SPC cheese formulated with 12 ppm of nisin A was produced in the laboratory. SPC was produced by mixing and cooking the ingredients (Supplementary Table 2) in a Themomixer (TM5, Vorwerk, USA). The butter was melted at 40 °C for 4 min, then the water was added and the mixture was blended for four more minutes at 40 °C. Subsequently, the dry ingredients were added and blended for an additional 4 min at 40 °C. At this point, the pH of SPC was adjusted (5.4–5.9) with 50% (*w/v*) citric acid monohydrate. Lastly, the mixture was blended at 2000 rpm and cooked with different temperature/time profiles (Supplementary Table 3) as recorded by data loggers (TinytagPlus, Gemini Data Loggers Ltd., Chichester, UK). To increase the creaminess of SPC, the mixture was blended at 3100 rpm for 3 min. Both PC and SPC cheeses were inoculated 24 h after production with 0.1% (*v/w*) of *Lm-mix* appropriately diluted in chilled saline water (0.85% NaCl) to obtain an initial concentration between 1 and 2 log cfu/g. After inoculation, cheeses were packaged and stored in containers with 50 ± 1 g cheese. Storage temperature was recorded by data loggers (TinytagPlus). At each time of sampling, 10 g of cheese from three different containers were diluted 10-fold with chilled physiological saline (0.85% NaCl and 0.10% Bacto-peptone), homogenized for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK) and then 10-fold serial dilutions were performed. Viable counts of *L. monocytogenes* were determined by surface plating on PALCAM agar base (CM0877, Oxoid, Hampshire, UK) with PALCAM selective supplement (SR0150, Oxoid, Hampshire, UK) and incubation at 37 °C for 48 h.

To estimate the kinetic growth parameters for each growth curve the primary integrated and log transformed logistic model with delay (four parameter model) or without delay (three parameter model) (Eq. (3); Rosso et al., 1996) was fitted to all individual growth curves of *L. monocytogenes* obtained in challenge tests at constant temperatures. Fitted parameter values for initial cell concentration (Log N_0, Log cfu/g), lag time (t_{lag}, h), maximum specific growth rate (μ_{max}, h^{-1}) and maximum population density (Log N_{max}, Log cfu/g) were determined using the MS-Excel Solver Add-in (Microsoft Corp. Redmond, WA., USA). Data was reported as average ± standard deviation for each challenge tests. An F-test was used to determine if the lag time was significant.

$$\begin{aligned} \text{Log}\,(N_t) &= \text{Log}\,(N_0) && \text{if } t < t_{lag} \\ \text{Log}\,(N_t) &= Log\left(\frac{N_{max}}{1+\left(\left(\frac{N_{max}}{N_0}\right)-1\right)\cdot exp\left(-\mu_{max}\cdot\left(t-t_{lag}\right)\right)}\right) && \text{if } t \geq t_{lag} \end{aligned} \quad (3)$$

where t is the storage time (h) and N_t is the cell concentration (cfu/g) at time t. Other parameters were indicated above.

2.5.3.2. Product characteristics. Product characteristics of cheeses were determined in triplicate at the start of each challenge test. In addition, pH was measure every day of microbiological analysis by a PHC725 SN probe (Hach, Brønshøj, Denmark) after 1 h stirring of 5 g sample in 25 ml of distilled water. NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Hesisau, Switzerland) and a_w was measured by a water activity meter (Aqua Lab model CX-2, Decagon devices Inc., Pullman, US). Concentrations of lactic-, acetic- and citric acids were determined by HPLC using external standards for identification and quantification (Østergaard et al., 2014). Concentration of orthophosphate salt was determined by Eurofins (test method QA02S) using ion chromatography. Nisin concentrations were measured by LC-MS/MS (see Section 2.2).

2.5.4. *Evaluation of existing predictive models including the effect of nisin*

Three predictive models including the inhibiting effect of nisin were evaluated to assess their ability to predict growth responses of *L. monocytogenes* in processed cheese containing nisin. The studied models included the models of Bouttefroy et al. (2000), Boziaris and Nychas (2006) and Parente et al. (1998). These models were developed to predict the effect of nisin on survival or growth inhibition of *L. monocytogenes* in broth and did not consider the distinction between residual and added nisin A. Nevertheless, these models were evaluated by using the concentrations of added nisin A and the residual concentrations of nisin A in the studied cheeses. Evaluation was performed by comparison of predicted and observed growth and no-growth responses with calculation of the percentage of all samples that were correctly predicted. Incorrect predictions were described as fail-safe (growth predicted when no growth was observed) or as fail-dangerous (no growth predicted when growth was observed).

2.6. *New growth and growth boundary model for L. monocytogenes in processed cheese containing nisin*

The effect of temperature on lag times of *L. monocytogenes* in processed cheese containing nisin (see Section 2.5.3.1) was assessed by applying the relative lag time (RLT) concept ($RLT = t_{lag} \cdot \mu_{max}/\text{Ln}(2)$) (Mellefont and Ross, 2003). It was evaluated by using an F-test if RLT-values were constant ($RLT = k_1$) or dependent on storage temperature (Eq. (4)).

$$RLT = k_1 + k_2/T^3 \quad (4)$$

In a similar way, it was evaluate if log (N_{max}) was constant (k_3) or dependent on storage temperature (Eq. (5))

$$Log\,(N_{max}) = k_3 + k_4 \cdot T \quad (5)$$

Values of the parameters μ_{ref} in Eq. (S3), k_1 and k_2 (Eq. (4)) as well as k_3 and k_4 (Eq. (5)) were estimated by using both a classical two-step predictive microbiology approach and a global regression procedure (Hereu et al., 2014; Jewell, 2012). For the classical two-step approach μ_{ref} in Eq. (S3) was calibrated using μ_{max}-values from challenge tests

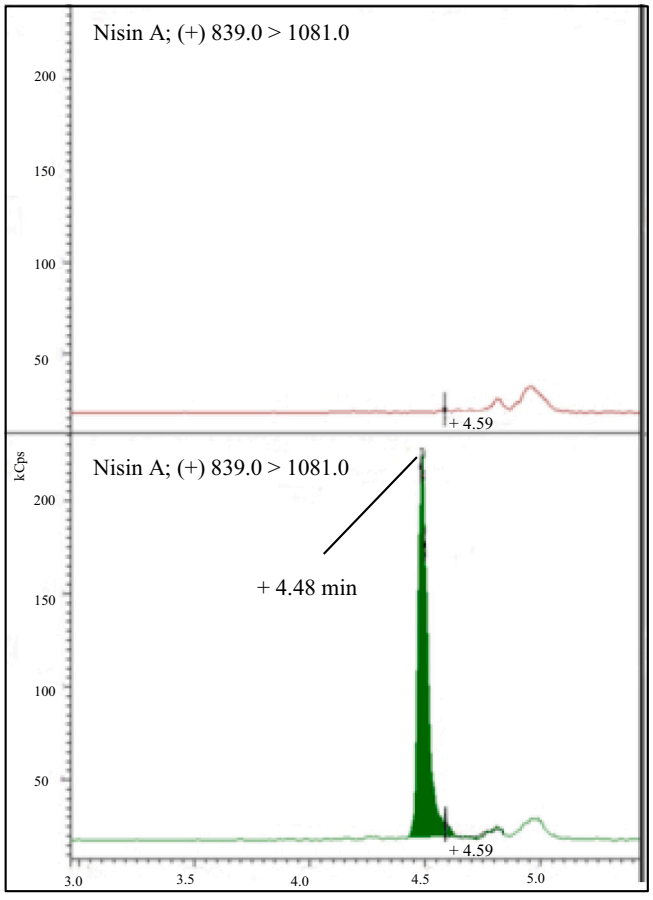

Fig. 1. Chromatogram obtained after LC-MS/MS analysis of cheese which was non-spiked (top) and spiked (bottom) with nisin A (0.5 ppm).

(Table 4). Calibration allowed finding a μ_{ref}–value so that the model predictions corresponded to observed data as well as possible. Likewise, k_1 and k_2 in Eq. (4) as well as k_3 and k_4 in Eq. (5) were independently estimated from *RLT*- and log (N_{max})-values observed in challenge tests with growth of *L. monocytogenes* in processed cheese containing nisin A. Global (one-step) regression was used to determine values of μ_{ref}, k_1, k_2, k_3 and k_4 by combining the growth and growth boundary model (Eq. (S3)) with the RLT model (Eq. (4)), the Log (N_{max}) model (Eq. (5)) and the primary logistic growth model with delay (Eq. (6)). Eq. (6) was fitted in one regression to the entire data set with 45 growth curves and including, respectively, 157 and 417 cell concentrations (log cfu/g) for PC and SPC. Statistical performance of the classical two-step approach and the global regression procedure were compared by calculation of RMSE-values.

$$\text{Log}\,(N_t) = \text{Log}\,(N_0) \quad if\ t < t_{lag}$$

$$\text{Log}\,(N_t) = Log\left(\frac{10^{k3+k4\cdot T}}{1+\left(\left(\frac{10^{k3+k4\cdot T}}{N_0}\right)-1\right)\cdot exp\left(-(\mu_{max}Eq.(S3))\cdot\left(t-\left(\frac{(k_1+k_2/T^3)\cdot ln(2)}{\mu_{max},Eq.(S3)}\right)\right)\right)}\right) \quad if\ t \geq t_{lag} \tag{6}$$

Table 2
Recovery and precision for determination of nisin A by LC-MS/MS and bioassay.

Method	Matrix	Conc. of nisin A (ppm)	Recovery (%)	CV (%)
LC-MS/MS	Processed cheese (PC)	0.1	99	13
		0.5	108	2
		1	110	4
	Spreadable processed cheese (SPC)	0.1	83	14
		0.5	95	11
		1	100	9
Bioassay	Spreadable processed cheese (SPC)	0.1	53[a]	46[a]
		0.5	94	35[a]
		1	93	33[a]

[a] Result outside acceptability range being: CV < 16 % and recovery within 80 - 110%

2.7. Evaluation of new growth and growth boundary model

Performance of the new growth and growth boundary model including the effect of nisin (Eq. (S3)) was evaluated by comparing observed and predicted growth and no-growth responses for the 45 growth responses determined in challenge tests with *L. monocytogenes* (See Section 2.5.3). The percentage of correct, fail-safe and fail-dangerous predictions were calculated (See Section 2.5.4). For each experiment with corresponding product characteristics and storage temperature, growth of *L. monocytogenes* was predicted for three scenarios: (a) assuming absence of nisin, (b) using the measured residual nisin concentration and (c) using the concentration of nisin added to the cheeses at formulation.

3. Results and discussion

3.1. Quantification of nisin in cheese by LC-MS/MS and bioassay

A clearly distinguishable peak with retention time of 4.48 min for nisin A was observed (Fig. 1). The LC-MS/MS method showed linear responses for nisin A concentrations ranging from 0.1 to 10 ppm ($r^2 >$ 0.98). Residuals of LC-MS/MS responses at different nisin concentrations were randomly distributed, indicating variance homogeneity (Results not shown). The LOD for nisin A in cheese was 0.04 ppm, and the LOQ was 0.13 ppm. No matrix interference was observed for nisin A detection in the tested cheeses (PC and SPC). Precision was <16% and nisin A recoveries from the two studied cheeses ranged from 83 to 110% (Table 2). Recovery of nisin A in SPC tended to increase with nisin concentrations (Table 2).

The bioassay showed linear responses for nisin A concentrations ranging from 0.1 to 1 ppm with a correlation coefficient $r^2 > 0.88$. Residuals were randomly distributed, indicating variance homogeneity (Results not shown). The LOD for nisin A in cheese was 0.07 ppm, and the LOQ was 0.25 ppm. Precision was >16% and nisin A recoveries from SPC ranged from 53 to 94% (Table 2).

Clearly, LC-MS/MS measurement of residual nisin A in processed cheese was reproducible and provided a more precise quantifications of nisin A compared to the studied bioassay. The LC-MS/MS method developed in the present study provided recovery values similar to those

Table 3
Experimentally determined minimum inhibitory concentration (MIC) values of nisin A for different *L. monocytogenes* strains

Strain	pH	Temp. (°C)	μ_{ref}, h^{n1} (AVG ± SE)	MIC, ppm (AVG ± SE)	Duration of experiments (days)
LM 4	6.5	7	0.205 ± 0.01	4.9 ± 0.4	20
LM 4R	6.5	7	0.081 ± 0.00	24.9 ± 3.1	14
LM13	6.5	7	0.193 ± 0.01	8.1 ± 0.7	14
Lm-mix[a]	6.5	7	0.225 ± 0.00	9.1 ± 0.3	5
LM4R	6.5	20	0.406 ± 0.01	27.0 ± 3.4	3
6	6.5	20	0.455 ± 0.02	6.4 ± 1.0	13
LM13	6.5	20	0.474 ± 0.01	14.1 ± 0.9	20
Lm-mix[a]	6.5	20	0.527 ± 0.01	15.5 ± 0.6	14
Lm-mix[a]	6.0	20	0.431 ± 0.01	8.8 ± 1.2	4
LM 4R	5.5	20	0.353 ± 0.01	3.3 ± 0.1	13
612	5.5	7	0.138 ± 0.00	0.55 ± 0.05	64
612	5.5	20	0.417 ± 0.02	0.75 ± 0.04	13
Lm-mix[a]	5.5	20	0.415 ± 0.02	0.95 ± 0.08	11

[a] The cocktail *Lm-mix* included the isolates SLU 92, 612, LM 19 and 6.

previously obtained by Ko et al. (2016); however the present study used an extraction solvent without NaCl as it can interfere with ionization, cause ion suppression and broader peaks in the LC-MS/MS (Sterling et al., 2010). Residual concentrations of nisin A quantified in the present study using LC-MS/MS (0.56–5.28 ppm) were lower than the added concentrations used in the cheese formulations (11.2–12.0 ppm). These differences are in agreement with other studies on the stability of nisin in broth or foods after heat treatments (Davies et al., 1998). For instance, Schneider et al. (2011) found the nisin A concentrations determined by LC-MS/MS in processed cheese to be reduced more than 92% by a heat treatment at 120 °C during 30 min. Ko et al. (2016) also quantified low nisin A concentrations in processed cheese by LC-MS/MS (0.34–0.64 mg/kg) but no information on nisin A concentrations in recipes were provided.

3.2. Effect of nisin on growth rates of L. monocytogenes in laboratory media

The experimentally determined nisin MIC-values at pH 5.5 and at 7 °C or 20 °C ranged between 0.55 ± 0.05 ppm and 3.3 ± 0.1 ppm. At pH 6 and at 20 °C the nisin MIC-value was 8.8 ± 1.2 ppm and at pH 6.5 the values were between 6.4 ± 1.0 and 24.9 ± 3.1 (Table 3; Fig. 2). Markedly, higher MIC-values were determined for *L. monocytogenes* LM 4R compared to the other strains studied, including its wild type LM4 (Table 3). In order to best fit the μ_{max}-values by Eq. (2) both n1 and n2 were found to be 1 for all strains studied. Nisin MIC-values for *L. monocytogenes* increased significantly ($p < 0.05$) with increase of pH and this was observed for data from the present study as well as for data from the scientific literature (Fig. 3). To predict MIC-values of nisin depending on pH a nisin MIC-term based on a simple regression model was developed (Eq. (7))

$$log(MIC_{nisin}, ppm) = -(3.08 \pm 0.32) + (0.64 \pm 0.05 \cdot pH) \quad R^2 = 0.65 \qquad (7)$$

Several studies found nisin to be more effective in acidic environments (Khan et al., 2015; Thomas and Wimpenny, 1996) and this is in agreement with Eq. (7). This simple model was developed including both experimental and literature nisin A MIC-values and reflected variability among 29 *L. monocytogenes* strains; an aspect also previously discussed by Katla et al. (2003). Eq. (7) can estimate nisin A MIC-values for products with pH values outside the average values (5.4–6.0) for processed cheese and therefore support new formulations with altered pH values. Furthermore, it can support the development of other foods products with added nisin A such as dairy, meat and fish products with higher pH than processed cheese (Davies et al., 1997; Nilsson et al., 1997; Oshima et al., 2014; Ruiz et al., 2010; Schillinger et al., 2001). Importantly, this simple model predicts with reasonable precision (RMSE of 0.54) the MIC-values of nisin A (32–37 ppm) at pH 7.2–7.3 when compared to 53 MIC-values (average of 17 ppm) extracted from the literature for various isolates of *L. monocytogenes* (Campion et al., 2013; Hara et al., 2009; Martínez and Rodríguez, 2005; Neetoo et al.,

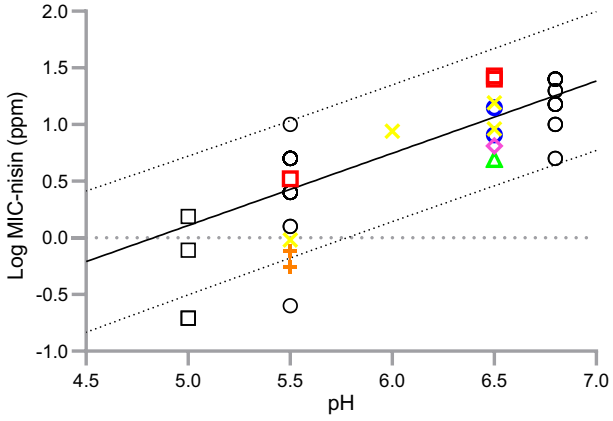

Fig. 3. Effect of pH on nisin A MIC-values for *L. monocytogenes* as determined in the present study and from literature. Data for a cocktail of *L. monocytogenes* strains [*Lm-mix*: SLU 92, 612, LM19, 6 (×)], individual strains [LM 4 (△), LM 4R (□), LM 13 (○), 6 (◊), 612 (+)], Taylor et al. (2009) (□) and Ferreira and Lund (1996) (○). Solid line (–) and dashed line (—) represent, respectively, the fitted model (Eq. (6)) and confidence interval (95%).

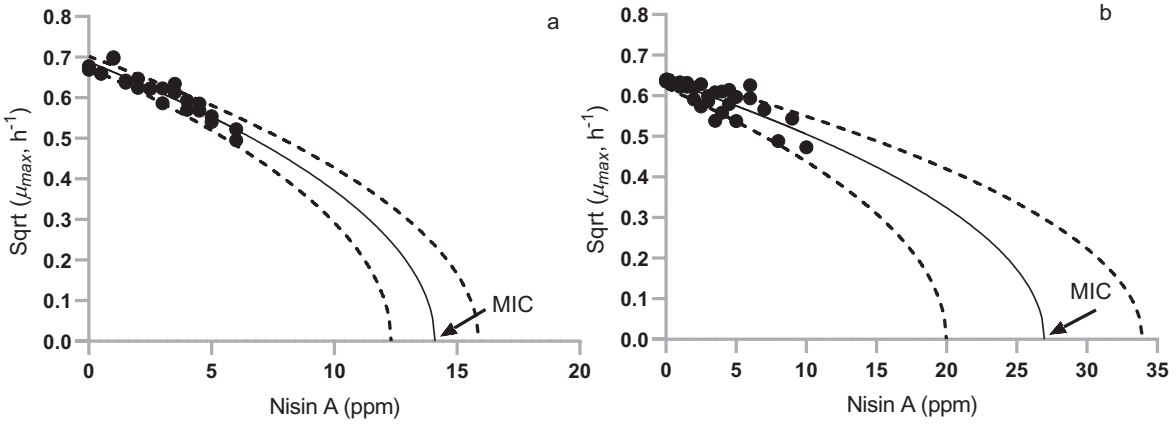

Fig. 2. Effect of nisin A on maximum specific growth rates (μ_{max}, h^{-1}) of *L. monocytogenes* LM13 (a) and LM4R (b) in BHI broth at 20 °C and pH 6.5. MIC-values were determined by fitting Eq. (2) to the observed data (●). Solid and dashed lines represent the fitted values and confidence intervals (95%), respectively.

Table 4
Storage conditions, product characteristics and estimated growth parameters from fitting the logistic model with delay (eq. (3)) to the *L. monocytogenes* counts on processed cheese obtained from challenge tests.

CT [b]	N [c]	Storage Temp. (°C)	Product characteristics (Avg.±SD)[a]								Growth parameter values (Avg. ±SD)[a]			
			pH	a_w	Organic acids in water phase (ppm)			P1[d] in water phase (%)	Residual nisin A (ppm)	t_{lag} (h)	Log N_0 (Log cfu/g)	Log N_{max} (Log cfu/g)	μ_{max} (h^{-1})	Duration of experiments (days)
					Lactic acid	Acetic acid	Citric acid							
1	3	10.0±0.3	5.9±0.1	0.981±0.001	9,415±293	2,471±60	5,030±26	0.9±NA[e]	0.68±0.05	63. 1±16	0.8±0.0	4.2±0.3	0.027±0.0	21
2	3	15.0±0.1	6.0±0.1	0.983±0.003	9,132±147	1,908±685	3,766±1,731	0.9±NA	0.68±0.05	0.0±0.0	0.9±0.1	7.1±0.1	0.050±0.0	19
3	3	19.5±0.1	5.9±0.1	0.982±0.001	9,345± 275	2,384±2	4,947±47	0.9±NA	0.68±0.05	0.0±0.0	1.0±0.0	6.5±0.1	0.0740±0.0	16
4	3	10.0±0.3	6.0±0.1	0.983±0.001	9,869± 46	2,305±81	5,070±282	0.8±NA	0.59±0.01	15.2±22	1.0±0.1	4.6±0.2	0.0240±0.0	21
5	3	15.0±0.1	6.1±0.0	0.981±0.001	9,364± 291	2,317±110	4,710±119	0.8±NA	0.59±0.01	0.0±0.0	1.0±0.1	5.4±0.1	0.0450±0.0	21
6	3	19.5±0.1	6.1±0.0	0.982±0.000	9,679±401	2,397±105	5,234±447	0.8±NA	0.59±0.01	0.0±0.0	1.1±0.1	5.6±0.1	0.0630±0.0	16
7	3	3.6±0.3	5.5±0.0	0.973±0.001	1,101±33	< LOD	19,395±364	2.0±NA	4.21±0.15	ND[f]	1.9±0.1	<1.0±0.0	0.0000±0.0	33
8	3	3.6±0.3	5.7±0.0	0.972±0.001	1,109±33	< LOD	18,196±432	2.0±NA	5.28±0.13	ND	2.0±0.0	2.3±0.2	0.0010 ±0.0	35
9	3	3.6±0.3	5.8±0.0	0.975±0.001	1,152±90	< LOD	17,471±392	2.0±NA	3.35±0.10	ND	2.1±0.0	<1.0±0.0	0.0000 ±0.0	33
10	3	7.1±0.3	5.9±0.1	0.974±0.001	1,028±68	< LOD	15,585±1,164	2.0±NA	0.88±0.6	211±6.3	1.4±0.1	10.5±0.0	0.0203 ±0.0	35
11	3	7.1±0.3	5.4±0.1	0.973±0.002	877±17	< LOD	20,864±438	2.0±NA	3.56±0.19	ND	1.2±0.2	<1.0±0.0	0.0000 ±0.0	32
12	3	7.1±0.3	5.7±0.0	0.976±0.001	958±9	< LOD	17,172±699	2.0±NA	4.38±0.26	ND	1.6±0.3	<1.0±0.0	0.0000 ±0.0	35
13	3	11.3±0.1	5.8±0.0	0.970±0.001	1,067±73	< LOD	17,721±235	2.0±NA	0.98±0.01	31.6±4.3	1.7±0.2	8.7±0.1	0.0508 ±0.0	18
14	3	11.3±0.1	5.6±0.1	0.971±0.001	1,251±476	< LOD	18,660±442	2.0±NA	1.07±0.13	27.1±11	1.2±0.2	8.5±0.1	0.0406 ±0.0	21
15	3	11.3±0.1	5.6±0.0	0.970±0.001	869±23	< LOD	18,340±710	2.0±NA	0.56±0.03	0.0±0.0	0.8±0.1	8.2±0.1	0.0420 ±0.0	21

[a] Avg: average; SD: standard deviation.
[b] Challenge test. Challenge tests 1-6 performed using processed cheese (PC) and 7-15 with spreadable processed cheese (SPC).
[c] Number of growth curves per experiment.
[d] P1: orthophosphate salt.
[e] NA, not available. Information not provided by Eurofins.
[f] ND: not determine.

Table 5
Comparison of observed and predicted growth and no-growth responses of *L. monocytogenes* in processed cheese containing nisin[a].

Models and responsesa	Predicted responses by three available models including the effect of nisin A					
	Parente et al. (1998); Probability of survival in 1 ml		Bouttefroy et al. (2000); Viable population log10 cfu/ml		Boziaris and Nychas (2006); Probability of growth	
Factors included in model	pH, NaCl, EDTA, nisin		pH, NaCl, nisin		T, aw, pH, nisin	
	Added nisin A[b]	Residual nisin A[c]	Added nisin A[b]	Residual nisin A[c]	Added nisin A[b]	Residual nisin A[c]
Correct (%)	14	3	86	86	14	100
Fail-safe (%)	0	11	0	14	0	0
Fail-dangerous (%)	86	86	14	0	86	0

[a] Observed responses included 45 kinetics of which 30 showed growth and 15 showed no-growth.
[b] Added nisin A: 11.2-12.0 ppm.
[c] Nisin A measured by LC-MS/MS: 0.59-5.28 ppm.

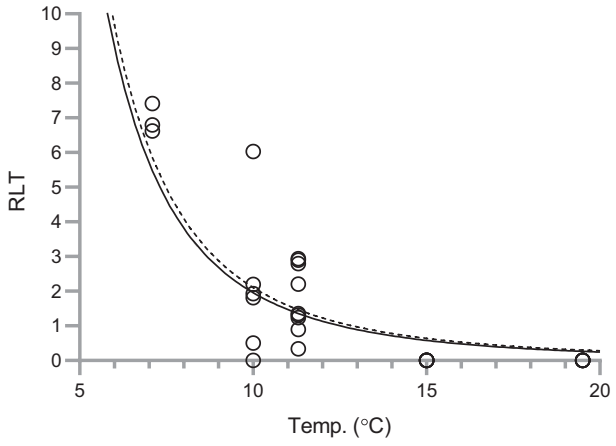

Fig. 4. Effect of storage temperature on observed and fitted relative lag time (RLT) values for *L. monocytogenes* growing in processed cheese containing nisin A. The two-step approach (solid line) and the global regression (dashed line) were used to fit RLT-values (Table 6).

Table 6
Growth kinetic parameters of *L. monocytogenes* in processed cheese containing nisin A and goodness of fit indices for the two-steps (eq. (S3); eq, (4)) and the global (one-step, eq. (5)) regression approaches.

Modelling approaches	Growth and growth boundary model	RLT[a] secondary model		Log (N_{max}, cfu/g)	Goodness of fit	
	μref (h^{-1})	*kl*	*k2*		RSS[b]	RMSE[c]
Classical two-step approach	0.510	0.0	1957	6.60	465	1.21
Global (one-step) regression	0.629	0.0	2087	6.60	393	0.97

[a] RLT: relative lag time
[b] RSS: residual sum of squares
[c] RMSE: root mean square error

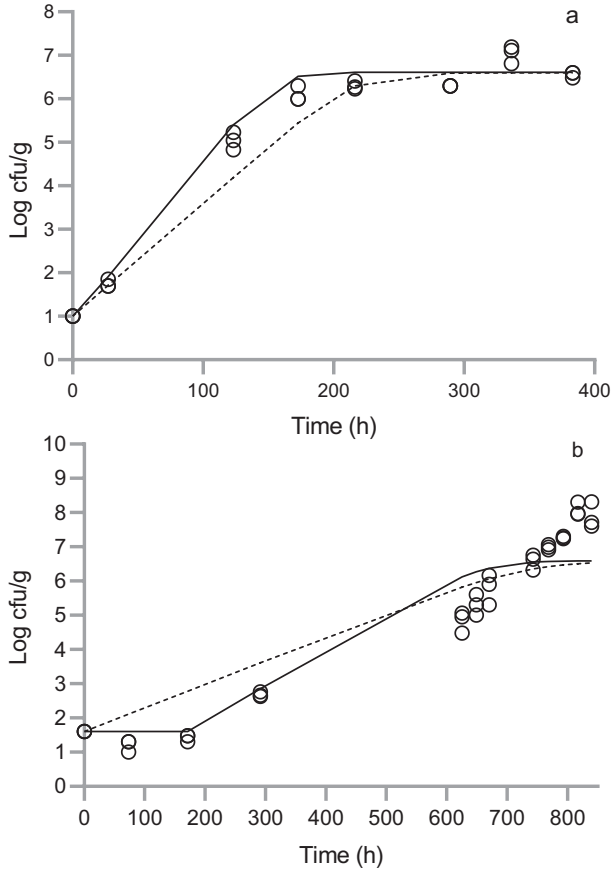

Fig. 5. Growth of *L. monocytogenes* (○) in commercial (a) and laboratory made (b) processed cheese containing nisin and stored at 19.5 °C or 7.3 °C, respectively (CT 3, CT 10; Table 4). Solid lines indicate predictions obtained with the model developed in the present study (Eq. (6) and Table 6) and dashed lines indicate predictions obtained with the model of Martinez-Rios et al. (2019) including (Eq. (S1)).

2008; Popowska et al., 2006; Smith et al., 2016; Taylor, 2009).

3.3. Product characteristics of processed cheese and L. monocytogenes growth kinetics

Commercial PC and laboratory produced SPC showed some variation in initial pH (5.4–6.1) and a_w (0.970–0.983) (Table 4). More variability was observed for water phase concentrations of lactic acid (869–9869 ppm), acetic acid (<LOD–2384 ppm), citric acid (3766–20,864 ppm) and concentration of orthophosphate (0.8–2.00%). The variability observed in residual nisin A concentrations among challenge test (CT7–15) (Table 4) was expected as a result of the different heat treatments used during preparation (Supplementary Table 3).

L. monocytogenes grew in both PC and SPC from 7 to 20 °C, and μ_{max}-values were influenced by storage conditions and product characteristics (Table 4). Residual nisin A concentrations and pH had a pronounced effect on *L. monocytogenes* μ_{max}-values, as suggested by Eq. (6). This is for example seen from challenge tests 7 to 12, which were all formulated with 12 ppm nisin A. However, due to its heat treatment, challenge test 10 had lowest residual nisin A concentration (0.88 ppm) and also

Table 7
Observed and predicted growth and no-growth responses for *L. monocytogenes*

Observed		Predicted	
n[a] Growth [No] growth	Assuming no added Nisin A	Residual nisin A	Nisin A added to formulation
45 30 15	0 ppm	0.59-5.28 ppm	11.2-12 ppm
Correct (%)	86	100	14
Fail-safe (%)	14	0	0
Fail-dangerous (%)	0	0	86

[a] Number of growth curves: a total of 15 challenge tests with triplicate measurements.

highest pH of 5.9 and this resulted a μ_{max}-value of 0.023 h^{-1} compared to 0.000 h^{-1} for the other challenge tests (Table 4). The observed long lag times at low temperatures for processed cheese containing nisin were in agreement with previous studies where researchers reported extended lag times for *L. monocytogenes* and other microorganisms in products containing nisin (Davies et al., 1997; Nilsson et al., 1997; Oshima et al., 2014; Ruiz et al., 2010; Schillinger et al., 2001). For example, Davies et al. (1997) showed that the incorporation of 2.5 ppm of nisin A in ricotta-cheese after production extended the lag times of *L. monocytogenes* by 44 days compared with the same product supplemented with 1.25 ppm when stored between 6 and 8 °C. In the same way, nisin A was found to extend the lag times of *L. monocytogenes* in tofu stored at 10 °C (Schillinger et al., 2001).

3.4. Evaluation of available predictive models including the growth inhibiting effect of nisin A

The effect of nisin on growth of *L. monocytogenes* in combination with other environmental factors including temperature, pH and NaCl/a_w has previously been studied in broth and predictive models were suggested by Bouttefroy et al. (2000), Boziaris and Nychas (2006) and Parente et al. (1998). These models did not consider the distinction between residual and added nisin and they were not previously evaluated for their ability to predict growth responses of *L. monocytogenes* in foods containing nisin. Interestingly, the performance of two out of the three models improved when residual nisin A concentrations rather than added nisin A concentrations in processed cheese (Table 5) were used as model input together with other measured product characteristics and storage conditions (Table 4). Most notable, the percentage of correct predictions increased from 14% to 100% for the model of Boziaris and Nychas (2006) when nisin A concentrations measured by LC-MS/MS were used as model inputs rather than added nisin A concentrations (Table 5). These results suggest residual nisin A concentrations may be more useful than added nisin A concentrations when the objective is to predict the growth responses of *L. monocytogenes* in processed cheese containing nisin A.

3.5. Modelling growth of L. monocytogenes in processed cheese containing nisin

RLT-values significantly ($p < 0.05$) increased at low storage temperatures and this effect was appropriately described by Eq. (4) (Fig. 4). In contrast, storage temperature had no significant effect ($p > 0.05$) on log (N_{max}).

The model of Martinez-Rios et al. (2019) was expanded with a nisin term that included MIC_{nisin}-values as described by Eq. (7). For this new growth and growth boundary model (Eq. (S3)), the μ_{ref}-value of 0.419 in the original model, increased markedly (Table 6) when the model was calibrated to describe the data for growth of *L. monocytogenes* in processed cheese containing nisin A (Table 4). Importantly, all parameter values in Eq. (S3), other than μ_{ref}, were kept constant during this product calibration of the growth and growth boundary model. The global (one-step) regression approach described the growth data (log cfu/g) slightly better than the two-step approach and resulted in the highest calibrated μ_{ref}-value of 0.629 (Table 6).

The long lag times (Table 4) and corresponding high RLT-values at low storage temperatures (Figs. 4, 5b) observed together with increased growth rates (μ_{ref}–value; Table 6) of *L. monocytogenes* growing in processed cheese containing nisin A are in agreement with previous studies. In fact, increased μ_{max}-values in different foods were reported when the pathogen resumed growth after extended lag times (Davies et al., 1997; Nilsson et al., 1997; Ruiz et al., 2010). As shown in Fig. 5b, the model of Martinez-Rios et al. (2019) was unable to describe the RLT-values observed at low temperatures in challenge tests (Table 4). However, combining simple models for the effect of pH on MIC_{nisin}-values (Eq. (7)) and for the effect of temperature on RLT-values (Eq. (4)) with an extensive growth and growth boundary model (Eq. S3) allowed this growth pattern to be described (Table 6) as shown in Fig. 5b.

3.6. Evaluation of the new growth and growth boundary model

The new growth and growth boundary model (Eq. S3) correctly predicted 100% of the growth or no-growth responses for *L. monocytogenes* in processed cheese with nisin A. This positive result was obtained when using residual nisin A concentrations, determined by the developed LC-MS/MS method, as model input (Table 7). It is interesting that the same model was less performant both when nisin A concentrations added to products and when no nisin were used as model input together with other measured product characteristics and storage conditions (Tables 4, 7). This supports the approach of the present study that accurate measurement of residual nisin A concentrations is important to predict growth of *L. monocytogenes* in foods that contain nisin. Furthermore, these results represent a successful product validation of the new growth boundary model (Eq. (S3)) for processed cheese with nisin A. However, when used to predict growth of *L. monocytogenes* in processed cheese with nisin, further evaluation of this model with additional data is needed. This is the case as model parameters related to lag time and growth rates were calibrated to data in Table 4, and these data therefore cannot be used to evaluate and validate the same model. The range of applicability, where the new model has been successfully validated for prediction of growth and no-growth responses include processed cheese with residual nisin A concentrations from 0.56 ppm to 5.28 ppm together with other product characteristics and storage condition as shown in Table 4.

86% correct and 14% fail-safe predictions of growth or no-growth responses were obtained by assuming no added nisin A in challenge tests (Table 7). These predictions correspond to using the model of Martinez-Rios et al. (2019) that does not include a nisin term (Eq. (S1)). This model however, include terms for the melting salts di-phosphate (P2) and tri-phosphate (P3) and these terms may be included in the new growth and growth boundary model to expand its range of applicability to processed cheese containing mixtures of different phosphate melting salts. As the developed nisin A term (Eq. (7)) reasonably predicted MIC_{nisin}-values at pH as high as 7.2–7.3 (see Section 3.3) it is also likely that the new model can be validated above the pH of 5.4–6.1 currently studied for processed cheese (Table 4). The logit model of Boziaris and Nychas (2006), including the effect of temperature, a_w, pH and nisin, also correctly predicted 100% of the observed growth and no-growth responses (Table 5). Compared to that model, the new model has the advantage of including the effect of more environmental factors relevant for processed cheese such as ortho-phosphate salt and organic acids as discuss below. Ψ is a quantitative measurement for the distance between specific environmental conditions and the growth boundary of *L. monocytogenes* (ψ = 1) (see Supplementary Table 5 for details about calculation of the ψ-value). Compared with logit models were a probability response of growth/no-growth is obtained; the new model

provides a mathematical value (ψ) representing how far the developed formulation is from the growth boundary of *L. monocytogenes*. The new model (Eq. (S3)) included the combined effect of eight environmental factors on the growth boundary of *L. monocytogenes* in processed cheese. As previously described and discussed in several studies such extensive models can be developed by variations of the cardinal parameter approach, which is much less data demanding than for example polynominal models or logistic regression (logit) models. Due to their demand for data, polynominal and logit models most often are limited to include the combined effect of between 4 and 7 environmental factors even when studied in liquid laboratory media and the calibration of these model for specific food remains a challenge (Dalgaard and Mejlholm, 2019; Le Marc et al., 2002; Ross and Dalgaard, 2004; Razavilar and Genigeorgis, 1998).

The developed extensive growth boundary model (Eq. (S3)) included the combined effect of eight environmental factors which will facilitate its use for product development and re-formulation. As an example if a processed cheese is contaminated by a consumer after opening a package, the new growth and growth boundary model will predict growth of *L. monocytogenes* with a ψ-value of 0.4 at 5 °C when the cheese contain 1.0 ppm of residual nisin A, pH 6.0, a_w of 0.980 and water phase concentrations of lactic acid (4000 ppm), acetic acid (1000 ppm), citric acid (10,000 ppm) and orthophosphate (0.8%). By lowering the pH of this product to 5.75 with water phase concentrations of lactic acid (6000 ppm) and acetic acid (2000 ppm), *L. monocytogenes* will be predicted to be at its growth boundary with a ψ-value of 1.0. However, if the residual nisin A concentration in the processed cheese with pH 5.75 is increased from 1.0 to 2.5 ppm then the predicted ψ-value will become 2.2 and the product will be stabilized against growth of *L. monocytogenes* during the open shelf-life with a solid margin to its growth boundary (Dalgaard and Mejlholm, 2019). Clearly, practical application of the new growth and growth boundary model to support product development and re-formulation of processed cheeses, stabilized against growth of *L. monocytogenes*, during the open shelf-life requires information about product characteristics. Residual nisin A concentrations in processed cheese can be measured by LC-MS/MS (see Section 3.1) and further information about added nisin A in formulations, applied heat processing and stability of nisin A during storage is likely to be required to obtain desired levels of residual nisin A (Supplementary Table 3). In conclusion, the present study quantified and modelled the effect of pH on nisin A MIC-values for *L. monocytogenes* and included the effect in a cardinal parameter growth and growth boundary model that can predict the effect of residual nisin A concentrations on growth/no-growth responses in cheese. The new cardinal parameter growth and growth boundary model is useful to predict growth/no-growth and consequently can support product development when used together with LC-MS/MS to determine residual nisin A concentrations. Further studies to determine the precision and range of applicability for the growth part of the new growth boundary model is desirable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108952.

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