7th International Conference on Predictive Modelling of Food Quality and Safety

7ICPMF, September 12 — 15th, 2011, Dublin, Ireland

Radisson Blu Royal Hotel, Golden Lane, Dublin 8, Ireland

Conference Proceedings

www.icpmf.org/2011

E. Cummins, J.M. Frías and V.P. Valdramidis (Eds.),
Predictive Modelling of Food Quality and Safety – Conference Proceedings,
UCD, DIT, Teagasc, Dublin, Ireland

Risk assessments of *Listeria monocytogenes* in Dutch-type semihard cheese: incorporating variability in both product parameters and microbial growth parameters

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Abstract

Dutch-type Gouda cheeses made from pasteurized milk have not been associated with growth of *Listeria monocytogenes*, the causative agent of listeriosis. To further validate the microbiological safety of these cheeses, compounds with potential bacteriostatic or bactericidal action against *L. monocytogenes* were identified and the natural variation in concentrations was evaluated (experimentally and through literature review). In addition, the sensitivity of *L. monocytogenes* for these compounds was experimentally determined using a variety of strains and by literature review. The variability of compounds present in Gouda cheese and sensitivity amongst *L. monocytogenes* strains were subsequently incorporated in a risk assessment model to predict the fate of *L. monocytogenes* in Gouda cheese.

Compounds that potentially inhibit *L. monocytogenes* in semi-hard cheese were identified as lactic, acetic, propionic and citric acid, diacetyl, lactoferrin, nitrate, nitrite and nisin and the enzyme lactoperoxidase. Of the potential inhibiting compounds in cheese, undissociated lactic acid has the largest inhibiting effects on *L. monocytogenes*. Additional experiments were performed to assess the efficacy of undissociated lactic acid to inhibit 6 different strains of *L. monocytogenes* at pHs relevant to cheese (pH 4.2-6.0). By taking the variation of both product parameters and microbiological growth parameters into account, critical factors for growth inhibition of *L. monocytogenes* in Gouda cheese were identified. The approach followed is applicable to all bacteria in all kinds of liquid, soft or (semi)hard foods.

Together with pH, temperature and water activity, undissociated lactic acid has a prominent role in inhibition of *L. monocytogenes* in Gouda cheese. Undissociated lactic acid has therefore been incorporated into a predictive model on the fate of *L. monocytogenes* in Gouda cheese in time.

Keywords: *Listeria monocytogenes, Gouda cheese, lactic acid, microbial sensitivity, inhibiting compounds, MIC, critical factors*

Introduction

Listeria monocytogenes is a severe food-borne pathogen as it can cause listeriosis, which is a rare *food*-borne infection with a high case-fatality rate (20%). Listeriosis is mainly a risk for immune-compromised people, but *L. monocytogenes* is ubiquitous and difficult to ban from the food processing environment. Therefore predictive models for the pathogen during food production are necessary. To predict whether *L. monocytogenes* is unable to grow in a food product, simple and more complex predictive models are used. Simple models for growth of *L. monocytogenes* in food incorporate pH, temperature and water activity. More complex models are extended with factors like organic acids, CO_2 and nitrite concentration. Such predictive models do not always incorporate the right critical parameters for growth, as the critical factors differ largely for specific foods. It is essential to determine the right critical growth parameters, as incorporation of the wrong parameters can lead to strong over- or underestimation of risks. This work presents a systematic way to determine the critical factors for growth of *L. monocytogenes* in Gouda cheese in addition to pH, temperature and water activity. Gouda cheese is a semi-hard cheese made from pasteurized milk. Semi-hard cheese is a highly complex product for food modelers, as it is a solid fermented product, made from starter-induced curds. Previous challenge tests show that growth of *L. monocytogenes* is not promoted in Gouda cheese. The critical parameters for this growth inhibition need to be determined in Gouda cheese in order to build a good predictive model for *L. monocytogenes*. The presented approach is applicable for all pathogens in fluid and (semi)solid food products and reduces the chance of over- or underestimation of growth.

Materials and methods

Identification of critical factors for growth of L. monocytogenes in Gouda cheese

A literature research was performed for growth-inhibiting factors that inhibit growth of *L. monocytogenes* in Gouda cheese. First, the components were listed that are present in Gouda cheese which can cause growth inhibition of Gram-positive bacteria. The concentration of these compounds needed for growth inhibition of *L. monocytogenes* was reviewed, and the concentration at which these components are present in cheese was determined as well. A compound was evaluated as critical for growth when the concentrations of the compound present in Gouda were higher or in the same range as the concentration needed for suppression of growth of *L. monocytogenes*, as observed in culture medium. Undissociated lactic acid was evaluated as critical for growth of *L. monocytogenes* and the existing lactic acid data set was limited. Therefore, additional experiments have been performed to determine the undissociated lactic acid concentration needed for growth inhibition of *L. monocytogenes* (MIC) at different pHs.

Determination of MIC of undissociated lactic acid for growth inhibition of L. monocytogenes.

L. monocytogenes strains Scott A (4b, milk isolate), EGDe (1/2a, rabbit isolate), 1F (1/2a, cheese isolate), 2F (1/2a, cheese isolate), 6E (1/2a, cheese equipment isolate) and L4 (1/2b, milk isolate) were used (NIZO culture collection). The strains were cultivated overnight (18 hours) in BHI. The cells in the overnight culture were harvested and resuspended in BHI of the desired pH and added to 96 wells plates with BHI and lactic acid at set pHs, with final *L. monocytogenes* concentrations of $1.8*10^6$ cfu/ml. *L. monocytogenes* strains were exposed to lactic acid independently and in three-fold and experiments were reproduced in an independent experiment.
The minimal undissociated lactic acid concentration needed for growth inhibition (MIC) was determined in BHI in triplicate in 2 independent experiments for 6 *L. monocytogenes* strains at small increments of lactic acid (0-1.67 M) and pH (4.2-6.0) (Table 1) and 12 and 30°C. The MIC was evaluated by optical density measurements and enumeration of viable numbers (comparison of optical density and viable numbers after 30 days incubation at 30°C to inoculum). The MIC values were calculated by the Henderson-Hasselbalch equation:

$$Undissociated\ acid = \frac{Total\ acid\ concentration}{1+10^{pH-pKa}} \quad (1)$$

Lactic acid was set at the target pH by use of predetermined molarities of lactic acid and potassium lactate (experiment 1). In addition, the interaction in growth inhibition of lactic and acetic acid was studied at 30°C by a combination of 0, 5 and 10 mM undissociated lactic and acetic acid (experiment 2).
MIC values of *L. monocytogenes*, as determined in BHI at pH 5.0-5.6 were compared with literature MICs in broth with cumulative frequency distributions based on Monte Carlo simulations (Microsoft Excel with the @Risk add in for Excel, Version 5.5.0, Palisade Corporation, New York, USA).

Table 1: Intervals of total lactic and acetic acid concentrations and pH chosen in experiment 1 (lactic acid), experiment 2 (lactic & acetic acid).

Experiment	pH	Lactic acid concentration (M)	Acetic acid (mM) concentration
1	4.2-4.4-4.6-4.8-5.0-5.2-5.6-5.8-6.0	0-0.02-0.03-0.04-0.06-0.08-0.1-0.12-0.15-0.19-0.24-0.37-0.43-0.67-1.06-1.67	-
2	5.2-5.6	0-0.11-0.23	0-0.02-0.04

Results and discussion

Determination of critical factors for growth of L. monocytogenes in Gouda cheese

Compounds that potentially inhibit *L. monocytogenes* in semi-hard cheese were identified as lactic, acetic, propionic and citric acid (Table 2), diacetyl, lactoferrin, nitrate, nitrite and nisin and the enzyme lactoperoxidase (Table 3). The concentrations of the compounds as found in Gouda were divided by the concentration at which growth was inhibited by the compound. When this resulted in values larger than 1, the compound was evaluated as critical for growth of *L. monocytogenes*. In Table 3 it is shown that diacetyl, nitrate and nitrite do not have an inhibitory effect on *L. monocytogenes*. Nisin is not present in Gouda, but could have an inhibitory effect of *L. monocytogenes* in other types of semi-hard cheeses that contain nisin-producing starter cultures. At the concentration present in cheese, lactoferrin could inhibit growth of *L. monocytogenes* based on broth experiments, but in milk higher concentrations lactoferrin were needed for growth inhibition, so lactoferrin is not expected to inhibit growth of *L. monocytogenes* in Gouda cheese; Calcium could possibly counteract the inhibiting activity of lactoferrin. Lactoperoxidase is not completely inactivated after pasteurisation, but as it is known to only increase the lag time of *L. monocytogenes* in milk, lactoperoxidase will not prevent growth of *L. monocytogenes* in semi-hard cheese. As concentrations of diacetyl, lactoferrin, nitrate, nitrite, nisin and lactoperoxidase present in Gouda are much lower than the concentration needed for inhibition, no further study on the variation of the concentrations present and the sensitivity of *L. monocytogenes* has been performed.

Table 2 Identification of critical parameters for growth of L. monocytogenes by dividing the present concentrations of organic acids in Gouda cheese at pH 5.0-5.6 by the MIC (minimal concentration of the compound needed for inhibition of growth of *L. monocytogenes*).

Compound	Concentration present in Gouda cheese (mM) at pH 5.0-5.2-5.4-5.6 / Inhibiting concentration (MIC)	Critical parameter for growth?
Lactic acid	0.6 – 13.8	Yes
Acetic acid	0.07-0.7	No
Propionic acid	0	No
Citric acid	0	No

Table 3 Identification of critical parameters for growth of *L. monocytogenes*, next to organic acids, by dividing the present concentrations in Gouda cheese at pH 5.0-5.6 by the MIC (minimal concentration of the compound needed for inhibition of growth of *L. monocytogenes*).

Compound	Concentration present in Gouda cheese (mM) at pH 5.0-5.2-5.4-5.6 / Inhibiting concentration (MIC)	Critical parameter for growth?
Diacetyl	0.012	No
Lactoferrin	<0.6 with decrease in time	No
Nitrate	<0.2	No
Nitrite	<0.01	No
Nisin	0 in Gouda (0.01-280 in semi-hard cheeses with nisin-producing starters, but decrease of effect in time)	No

Lactic acid was evaluated as critical for growth of *L. monocytogenes* in Gouda cheese (Table 2), as the concentration undissociated lactic acid present in Gouda cheese was larger or in the same range as the concentration needed for inhibition of growth of *L. monocytogenes*, as was observed in experiments with culture medium. Experimental MIC values were lower than literature data, probably due to our experimental setup in which the stepwise intervals of pH and lactic acid were smaller (Figure 1). The variability between our strains, however, was larger. Additional experiments show no synergistic effects with acetic acid and no influence of temperature (although at 12°C the lag time is increased, the MIC values were the same at 12 and 30°C).

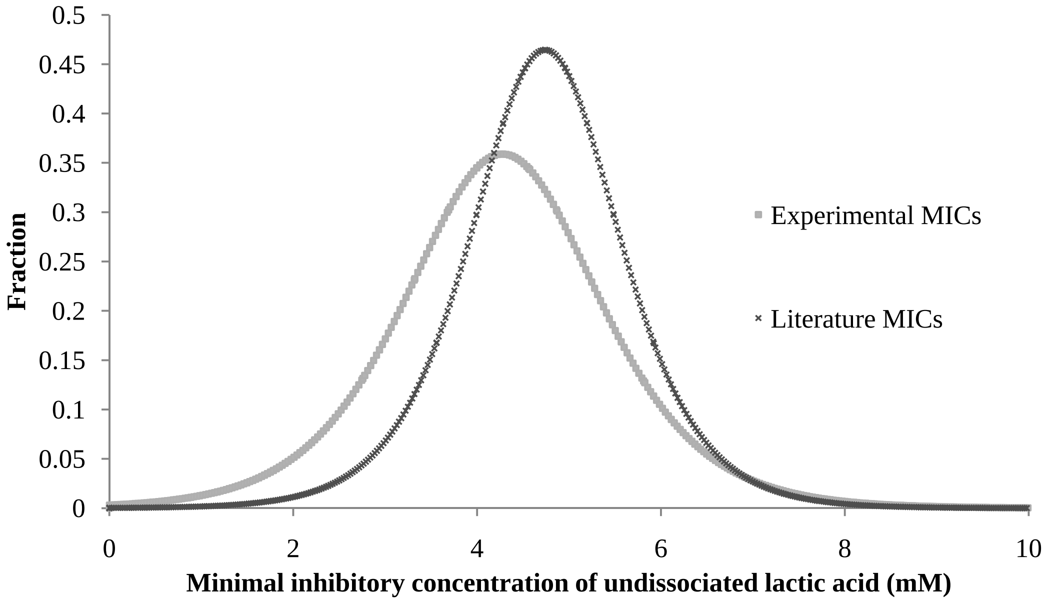

Figure 1: Comparison of minimal inhibitory concentration of lactic acid needed for inhibition of growth of *L. monocytogenes*, with a Monte Carlo simulation of the experimental and literature data.

Conclusion

Literature and experimental data on concentrations needed for growth inhibition have been compared to concentrations that are present in Gouda cheese to determine which compounds in Gouda cheese are critical for growth of *L. monocytogenes* in Gouda. Of all parameters reviewed, lactic acid was identified as the most critical growth factor and for that reason this factor was included into a *L. monocytogenes* model for Gouda cheese that incorporates variation, together with water activity, temperature and pH. Growth-inhibiting effects of calcium and sodium have not been taken into account yet. Preliminary experiments show a slight increase in MIC values when calcium and sodium are added to lactic acid at relevant concentrations for Gouda cheese, which could lower the growth-inhibiting capacity of lactic acid. The influence of calcium and sodium will be further explored.