

Predicting growth of *Listeria monocytogenes* in fresh ricotta

Erica Tirloni^{a,*}, Simone Stella^a, Cristian Bernardi^a, Paw Dalgaard^b, Per Sand Rosshaug^b

^a Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy

^b Technical University of Denmark, National Food Institute (DTU Food), Kemitorvet, Building 202, 2800, Kgs. Lyngby, Denmark



ABSTRACT

Challenge tests with eight brands of fresh ricotta showed rapid growth of *Listeria monocytogenes* and significant variability in physical-chemical characteristics. Thus, two cardinal parameters models were developed for the growth of *L. monocytogenes* in ricotta including, respectively, terms for temperature (Model 1) and temperature and pH (Model 2). Also an extensive, existing growth model including the effect of organic acids (Model 3) was product recalibrated to predict growth of *L. monocytogenes* in ricotta. Interestingly, a lack of anti-listerial effect of organic acids in ricotta was observed in this study. The range of applicability of Models 1 and 2 in ricotta (characterized by absence of competitive microbiota) included storage at temperatures from 4.1 to 20.6 °C, pH from 5.5 to 6.6, moisture contents from 72% to 82%, NaCl from 0.38% to 0.60%, concentrations of acetic acid from 579 to 1559 ppm in the water phase, citric acid from 14,774 to 46,116 ppm in the water phase, and lactic acid from 0 to 4146 ppm in the water phase. Comparing observed and predicted maximum specific growth rates of *L. monocytogenes* in ricotta showed a bias-factor significantly above 1, for existing models developed for broth and these models were thus fail-safe with predicted growth being faster than observed, while typically below 1 for models developed for other food types. The models developed in the present study showed bias-factors of 1.10, 1.06 and 0.78, respectively, for Model 1, 2, and 3. In particular, Model 1 and 2 developed and successfully validated could allow an easy determination of safe shelf-life of ricotta and facilitated the reformulation the product with the aim to increase shelf-life or safety.

1. Introduction

Fresh ricotta is an unripened acid-heat coagulated dairy product originating from Italy. It is an important fresh dairy product in the Italian market and it is also popular internationally (Assolatte, 2014). This product is mainly consumed as it is, without a heat treatment and it can be used for cakes and sweets as well as an ingredient in main courses like pasta.

Ricotta is mainly produced from bovine whey resulting from the production of different cheeses such as mozzarella. Protocols for the manufacture of ricotta varies. In industrial production, the whey is typically acidified by citric acid, and then heated to 65–70 °C. Whole milk and/or skim milk are usually added (5–25% v/v) and milk cream (~1%) can be added at temperatures of 75–80 °C. Then, NaCl (0.5% w/v) is supplemented and heating continues to 85–95 °C. Food-grade organic acids (mainly acetic, citric and lactic acids) are used at this stage to reduce the pH, usually to around 6.0, in order to coagulate the whey proteins. Later, the product is typically hot filled/packaged in basket moulds under vacuum, gas flushed, and finally sealed at high temperatures of 75 °C (Farkye, 2004; Mucchetti and Neviani, 2006). The chilled shelf life of industrial fresh ricotta is typically 20–40 days.

The physical-chemical characteristics of ricotta including high moisture content, low salt content and a relatively high pH value (6.0–6.5), has previously been reported to support the growth of the

pathogenic bacterium *L. monocytogenes* (Genigeorgis et al., 1991). This is important as Italian and international scientific studies found *L. monocytogenes* in fresh and soft dairy products made from raw, thermized and pasteurized milk (De Buyser et al., 2001; Little et al., 2008; Lundèn et al., 2004; Martinez-Rios and Dalgaard, 2018). Also for ricotta-type products the presence of this pathogen has been sporadically reported (RASFF, 2015). Due to the processing including heating to 85–95 °C the presence of *L. monocytogenes* in ricotta is most likely caused by post-heating contamination. However, there have been identified outbreaks of listeriosis associated with the consumption of fresh cheese and the predominant microorganisms responsible include *Listeria monocytogenes* (Zottola and Smith, 1991; Oliver et al., 2005; Little et al., 2008; Martinez-Rios and Dalgaard, 2018).

The EU Regulation no. 2073/2005, and subsequent amendments, sets food safety criteria for *L. monocytogenes* in ready-to-eat (RTE) food. Before the food has left the immediate control of the food manufacturer, the microorganism must be absent in 5×25 g, while, at the point of consumption, it must not exceed 100 CFU g^{-1} . Based on the chemical-physical characteristics of ricotta, in particular pH and water activity, the EU-regulation also categorizes this product as a ready-to-eat food able to support the growth of *L. monocytogenes*. Moreover the EU-regulation recognizes and points at predictive modelling as a method to document safety of RTE foods.

Mathematical predictive models, quantifying microbial responses to

* Corresponding author. Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133, Milan, Italy.
E-mail address: erica.tirloni@gmail.com (E. Tirloni).

Table 1

Challenge tests with *L. monocytogenes* in ricotta and application of data for model calibration, model validation and assessment of MIC (minimum inhibitory concentration) values for organic acids.

Data-set #	Use of dataset	Average storage temperature (°C)	No. Of sampling times ^a	Temperature variability	Product brand code
1	Calibration	4.1	29	Static	A
2	Calibration	8.3	21	Static	C
3	Calibration	8.3	21	Static	E
4	Calibration	13.4	12	Static	D
5	Calibration	20.6	12	Static	B
6	Calibration	14.7	8	Static	G
7	Calibration	14.7	7	Static	G with added acetic acid
8	Validation	8.3	21	Static	A
9	Validation	10.3	20	Static	A
10	Validation	13.4	12	Static	A
11	Validation	14.7	8	Static	G with added citric acid
12	Validation	14.7	8	Static	G with added lactic acid
13	Validation	20.6	12	Static	A
14	Validation	5.6	16	Dynamic 4–8 °C	F
15	Validation	5.6	16	Dynamic 4–8 °C	H
16	Validation	5.9	16	Dynamic 4–8 °C	A
17	Validation	13.5	14	Dynamic 12–16 °C	A
18	Validation	13.5	14	Dynamic 12–16 °C	G
19	Assess MIC value for acetic acid	15.2	2	Static	G with added acetic acid
20		15.2	2	Static	G with added acetic acid
21		15.2	2	Static	G with added acetic acid
22	Assess MIC value for citric acid	15.2	2	Static	G with added citric acid
23		15.2	2	Static	G with added citric acid
24		15.2	2	Static	G with added citric acid
25	Assess MIC value for lactic acid	15.2	2	Static	G with added lactic acid
26		15.2	2	Static	G with added lactic acid
27		15.2	2	Static	G with added lactic acid

^a At each sampling time analyses were performed in triplicate.

physical-chemical characteristics of fresh ricotta, could be used to predict potential growth at intended and at abusive storage conditions and thereby facilitate the determination of safe product shelf-life. Furthermore, a validated model can be used to predict a product reformulation (e.g. changed pH and concentration of organic acids) that inhibit or prevent growth of *L. monocytogenes* as previously shown for other food commodities such as seafood (Mejlholm and Dalgaard, 2009, 2015).

Several mathematical modelling approaches have been proposed to predict microbial growth in food products. Cardinal parameter models are popular due to their parameters with an apparent biological meaning and extensive cardinal parameter models, including the effect of various inhibitory components have previously been developed for *L. monocytogenes* (Augustin and Carlier, 2000a; b; Augustin et al., 2005; Coroller et al., 2005; Mejlholm and Dalgaard, 2009). Validated models that specifically predict growth of *L. monocytogenes* in fresh ricotta are not available, but as mentioned above they could be useful to set formulations and storage conditions leading to *L. monocytogenes* inhibition.

The aim of the present study was to identify or develop and validate predictive models to accurately predict the potential growth of *L. monocytogenes* in fresh ricotta. Firstly, a physical-chemical and microbiological characterization of fresh ricotta from eight major Italian producers was performed. Secondly, challenge tests with *L. monocytogenes* in the eight brands of fresh ricotta at different static and dynamic storage temperatures were conducted including also challenge tests with modified concentrations of organic acids. Finally, the collected information and data were used to (i) either develop or calibrate selected models to growth rates in fresh ricotta and (ii) to evaluate the performances of existing or new predictive models for ricotta.

2. Materials and methods

2.1. Microbiological screening of fresh ricotta

Fresh ricotta samples from eight different producers (A-H) were obtained on the day of production. The weight of the samples varied from 100 to 450 g, depending on the producer and the declared commercial shelf-life was 20–40 days at 4 °C and under aerobic conditions (residual oxygen was determined as $18.84 \pm 1.21\%$ by using a Dansensor gas analyser). During transport, samples were kept in ice and at the laboratory they were stored at 4 °C and analysed within 24 h. Microbiological analyses were carried out at three different storage times: beginning (T0), middle (T1) and end of the shelf-life of 20–40 days as indicated by the producers (T2). All analyses were performed at least in triplicate.

For microbial counts, 10 g of each sample were homogenized in 90 ml of pre-chilled sterile diluent solution (0.85% NaCl and 0.1% peptone), for 60 s in a Stomacher 400 (Seward Medical, London, UK) and serial 10-fold dilutions were prepared. Total Viable Count (TVC) was determined using spread plating on Plate Count Agar (Oxoid, Basingstoke, UK, CM0325) according to the ISO 4833:2013 method. Lactic acid bacteria (LAB) were enumerated on de Man-Rogosa-Sharpe agar (Oxoid, CM0361), at pH 5.5; plates were incubated at 30 °C for 48 h under anaerobic conditions (AnaeroGen, Oxoid, AN0025). Qualitative detection of *L. monocytogenes* in 25 g of ricotta was performed according to the AFNOR, 2001 AFNOR BRD 07/4–09/98 method, including enrichment in Half fraser broth followed by plating onto ALOA agar.

2.2. Challenge tests with inoculated fresh ricotta

A series of challenge tests was carried out with the purpose of

establishing data to calibrate and validate growth models of *L. monocytogenes* in ricotta. Growth of *L. monocytogenes* in eight different brands of ricotta was determined in 18 challenge tests with 13 at constant storage temperatures and five at dynamic temperatures (Table 1). Seven challenge tests used for model calibration were performed at 4.1–20.6 °C using different brands of ricotta with varying pH and concentrations of organic acids (See 2.2.1). Model calibration means finding the best model curve through observations by adjusting model parameter values so that model predictions conform to observed data as well as possible. It is here done by minimizing sums of squares of residuals (see details in 2.3.2). Validation data from eleven challenge tests were used to evaluate the performance of the novel predictive model for ricotta and of these, six were performed at constant temperature and five were performed at dynamic temperatures (Table 1). However, all 13 data sets conducted at static temperature were used to validate existing models ability to predict growth rates, because these models were not derived from the calibration data in this study. The five challenge tests conducted at dynamic temperature were used to evaluate how the models predicted bacterial growth over time during dynamic temperature conditions. The challenge tests included treatments with organic acids added to ricotta in the laboratory (Table 1). All microbiological analyses were performed in triplicate. Thus, the effect of temperature (4.1–20.6 °C), pH (5.49–6.61) and organic acids concentrations on microbial growth was reflected in the calibration data sets (Table 1; Table 2). In three series of challenge tests organic acids were added to the brand of ricotta characterized by lower organic acids concentration (G) to observe more clearly the responses of *L. monocytogenes* to acetic, citric, and lactic acids in ricotta (see Table 1; data set # 19–27 and section 2.2.4).

2.2.1. Physical-chemical characterization

Before inoculation of ricotta, moisture (Bradley and Vanderwarn, 2001), water activity (a_w) (RotronicHygromer Aw-DIO, Basserdorf, CH), pH (Amel Instrument, 334-B, Milan, I) and salt content (Pearson, 1973) were determined. Concentrations of organic acids were determined by HPLC (Tormo and Izco, 2004). Briefly, 1.0 g of fresh ricotta was diluted to 5.0 ml in water and vigorously shaken by using a vortex during 20 s. After centrifugation (3000 × g; 15 min) the supernatant was filtered through a 0.45 µm cellulose membrane. The HPLC system consisted of two pumps (Waters 510, Milan, Italy), an auto-sampler (Waters 717 plus) and a UV-VIS detector (Waters 484) set at 210 nm. The separation was performed on a Rezex ROA column 300 mm × 7.8 mm, 8 µm (Phenomenex, Torrance, USA). The mobile phase (0.5 ml min⁻¹ in isocratic mode) was 0.005 N sulphuric acid. External standards were used for identification and quantification of acetic, citric and lactic acids. The limit of detection (LOD: 0.076, 0.23 and 0.24 mM for acetic, citric and lactic acid respectively) and limit of quantification (LOQ: 0.39, 0.43 and 0.77 mM for acetic, lactic and citric acid respectively) were determined. LOD and LOQ were calculated by the signal-to-noise approach (ICH, 1995).

2.2.2. Bacterial strains and inoculation

Ricotta samples used for challenge tests were inoculated with a three strains cocktail of *L. monocytogenes* including a human isolate (ATCC 7644), an isolate from a fresh dairy product (strain MS12209), provided by DTU Food and a strain previously involved in a dairy associated outbreak (LM 6), supplied by Arla Strategic Innovation Center (ASIC, Stockholm, Sweden). The strain stocks were kept frozen at -80 °C in Microbank Cryogenic vials (Pro-Lab Diagnostics U.K., Merseyside, UK). From each stock culture, a loop was transferred to Brain Heart Infusion broth (BHI) (Oxoid, CM1135) with pH 7.2 and incubated at 37 °C for 24 h. In order to pre-adapt the cells to the environmental conditions of each of the challenge tests, cultures were subsequently re-inoculated in BHI broth with a pH around 6 (adjusted with HCl) according to the characteristics of the product to be inoculated and then incubated at different temperatures (4, 8, 12, 20 °C)

depending on the storage temperature of the challenge test (Table 1). The cultures were harvested in late exponential growth phase, defined as a relative change in absorbance of 0.05–0.2 at 540 nm (Jenway 6105, Staffordshire, UK). Cell concentrations of these pre-cultures were determined by microscopy at 1000x magnification (Motic, B310, Wetzlar, Germany), considering that one cell per field of view corresponded to a concentration around 10⁶ CFU ml⁻¹ (Adams and Moss, 2000). Finally, pre-cultures of individual isolates were diluted in sterile saline water (0.85% NaCl) to obtain the same concentrations and mixed together in equal volumes (Mix-Lm). The Mix-Lm culture was diluted with sterile saline in order to reach an initial concentration of 3–4 log CFU g⁻¹ after inoculation of the product. To minimize changes in product characteristics, the inoculum volume did not exceed 1% of the weight of fresh ricotta samples. Thus, the inoculum was between 1 and 4.5 mL depending on the total volume of ricotta (from 100 to 450 g). The inoculated samples were then incubated at selected temperature conditions (Table 1).

2.2.3. Microbial sampling

Inoculated fresh ricotta samples were periodically analysed and *L. monocytogenes* counts were determined in triplicate during the storage period of challenge tests. Each challenge test included from 8 to 29 times of repeated samplings. At each sampling time, 10 g of ricotta were 10-fold diluted in pre-chilled sterile saline (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made with pre-chilled sterile saline. *L. monocytogenes* was enumerated by spread plating on Palcam Agar (Oxoid, CM0877) added Palcam Selective Supplement (Oxoid, SR0150) and incubated at 37 °C for 48 h.

During storage of ricotta, temperature was regularly recorded by data loggers (Escort iLog, Escort Data Logging System Ltd, Aesch Bei Birmensdorf, CH) and pH was measured at each sampling time in triplicate by using a pH meter (Amel Instruments, Milan, I) (See 2.2.1).

2.2.4. Effect of organic acids

Specific challenge tests with ricotta were carried out to establish the range of Minimum Inhibiting Concentrations (MIC) for undissociated acetic, citric, and lactic acids on *L. monocytogenes*. Ricotta samples from the brand showing the lowest organic acids concentrations (as described in section 2.2.1) were obtained. Considering the organic acids present in ricotta and resulting from industrial processing, nine treatments with different contents of organic acids were generated by adding three different quantities for each of the three food grade organic acids (Table 1; data set # 19–27). The resulting total concentrations of organic acids for samples of the nine treatments were estimated with the HPLC method described in section 2.2.1. Each of the three organic acid was added to obtain a final concentrations for acetic acid (code 1005706, Sigma Aldrich, Steinheim, Germany) of 37.03 mM, 74.96 mM and 173.1 mM, for lactic acid (code 252476, Sigma Aldrich) of 35.25 mM, 111.0 mM and 213.7 mM, and for citric acid (code 251275, Sigma Aldrich) of 1764 mM, 2803 mM and 3915 mM. The pH was adjusted to a level of 5.0–5.1 with HCl/NaOH, in order to obtain high undissociated amount of organic acid. The undissociated concentrations of organic acids in the nine ricotta challenge tests were estimated with Equation (1):

$$\text{Undissociated organic acid (mM)} = \frac{\text{organic acid (mM)}}{1 + 10^{(pH - pK_a)}} \quad (1)$$

The applied pKa values were 4.76, 3.13, and 3.86 for acetic acid, citric acid, and lactic acid, respectively. The highest concentrations of undissociated acid reached were 68.4 mM for acetic acid, 42.5 mM for citric acid, and 14.7 mM for lactic acid. All the samples were then inoculated with *L. monocytogenes* (approximately 3–4 log CFU g⁻¹) (see section 2.2.2) and bacterial enumeration was immediately performed in triplicate as described in section 2.2.3. The samples were then

Table 2
Observed kinetics parameters of *L. monocytogenes* in ricotta and environmental conditions measured for each challenge test.

Dataset #	Product brand code	Temperature (°C) (AVG ± SD)	pH T0 ^a (AVG ± SD)	pH T final (AVG ± SD)	Moisture content (%) (AVG ± SD)	WPS ^b (%)	a _w	Organic acid concentration in the water phase of the products (ppm) (AVG ± SD)			Lactic acid	Citric acid	Acetic acid	μ _{max} (h ⁻¹); AVG ± SD)	Lag time (h)	N _{max} (log CFU g ⁻¹); AVG ± SD)
								Acetic acid	Citric acid	Lactic acid						
1	A	4.1 ± 0.2	6.07 ± 0.08	5.95 ± 0.02	74.6 ± 0.54	0.41	0.997	1559 ± 62	46,116 ± 1314	3187 ± 349	0.034 ± 0.000	NS ^c	8.62 ± 0.02			
2	C	8.3 ± 0.2	6.52 ± 0.08	6.43 ± 0.11	78.7 ± 0.19	0.20	0.998	1210 ± 241	32,536 ± 3308	2425 ± 471	0.077 ± 0.001	NS ^c	8.73 ± 0.07			
3	E	8.3 ± 0.2	6.49 ± 0.10	6.38 ± 0.06	79.3 ± 0.51	0.42	0.997	1331 ± 105	32,909 ± 937	2316 ± 186	0.073 ± 0.001	NS ^c	8.62 ± 0.04			
4	D	13.4 ± 0.2	6.22 ± 0.07	5.61 ± 0.09	82.1 ± 0.87	0.37	0.998	921 ± 301	30,820 ± 13,875	3878 ± 794	0.162 ± 0.005	NS ^c	8.33 ± 0.08			
5	B	20.6 ± 0.2	5.95 ± 0.11	5.78 ± 0.04	72.5 ± 0.52	0.36	0.998	1345 ± 150	42,491 ± 2643	2929 ± 502	0.310 ± 0.038	NS ^c	8.30 ± 0.02			
6	G	14.7 ± 0.1	6.61 ± 0.06	6.53 ± 0.03	77.5 ± 0.30	0.60	0.996	660 ± 14	14,774 ± 245	< 69.4	0.197 ± 0.012	NS ^c	8.48 ± 0.00			
7	G ^d	14.3 ± 0.1	5.69 ± 0.18	5.91 ± 0.12	77.3 ± 0.65	0.60	0.996	1153 ± 401	17,867 ± 7029	641 ± 411	0.156 ± 0.107	59.2 ± 37.4	7.58 ± 1.39			
8	A	8.3 ± 0.2	6.15 ± 0.07	5.94 ± 0.08	75.5 ± 0.53	0.41	0.997	1513 ± 96	36,119 ± 1750	3270 ± 300	0.064 ± 0.001	NS ^c	8.64 ± 0.03			
9	A	10.3 ± 0.2	6.12 ± 0.07	5.82 ± 0.02	74.4 ± 0.13	0.41	0.997	1582 ± 225	49,272 ± 5263	2534 ± 795	0.093 ± 0.001	NS ^c	8.54 ± 0.06			
10	A	13.4 ± 0.2	6.14 ± 0.12	5.84 ± 0.04	75.0 ± 0.35	0.41	0.997	2021 ± 74	49,351 ± 2850	4004 ± 136	0.150 ± 0.004	NS ^c	8.25 ± 0.02			
11	C ^e	14.7 ± 0.1	5.70 ± 0.15	5.73 ± 0.15	77.9 ± 2.79	0.60	0.996	579 ± 22	34,994 ± 2296	396 ± 199	0.168 ± 0.003	NS ^c	7.99 ± 0.17			
12	G ^f	14.7 ± 0.1	5.49 ± 0.18	5.61 ± 0.11	78.2 ± 1.00	0.60	0.996	668 ± 45	15,903 ± 2787	4146 ± 1825	0.151 ± 0.019	NS ^c	7.85 ± 0.08			
13	A	20.6 ± 0.2	6.05 ± 0.14	5.58 ± 0.04	75.0 ± 0.35	0.41	0.997	1725 ± 221	46,779 ± 5446	3414 ± 587	0.320 ± 0.099	NS ^c	8.20 ± 0.08			
14	F	5.6 ± 1.9	6.03 ± 0.10	5.90 ± 0.06	78.4 ± 0.21	0.48	0.997	1710 ± 175	39,687 ± 1002	2911 ± 310	- ^g	- ^g	- ^g			
15	H	5.6 ± 1.9	6.56 ± 0.10	6.27 ± 0.11	77.2 ± 0.45	0.38	0.998	1051 ± 71	27,456 ± 463	2600 ± 178	- ^g	- ^g	- ^g			
16	A	5.9 ± 1.9	6.12 ± 0.09	6.17 ± 0.05	74.5 ± 0.10	0.41	0.997	1696 ± 452	48,956 ± 569	3352 ± 752	- ^g	- ^g	- ^g			
17	A	13.5 ± 2.1	6.07 ± 0.11	5.68 ± 0.03	74.4 ± 0.17	0.41	0.997	1977 ± 77	50,862 ± 967	4138 ± 316	- ^g	- ^g	- ^g			
18	G	13.5 ± 2.1	6.61 ± 0.11	6.14 ± 0.12	72.2 ± 0.24	0.60	0.996	852 ± 66	20,465 ± 917	306 ± 256	- ^g	- ^g	- ^g			

^a Average of values in exponential growth phase.

^b NaCl in the water phase (WPS).

^c Not significant (NS).

^d Acetic acid added.

^e Citric acid added.

^f Lactic acid added.

^g Not applicable.

incubated at 15.2 °C during 7 days and then *L. monocytogenes* was enumerated again in triplicate. An increase of +0.5 Log CFU g⁻¹ was used to discriminate growth and no growth in the product. pH and organic acids concentrations were measured at the beginning and at the end of the trial (see section 2.2.1).

2.3. Modelling

2.3.1. Estimation of kinetic parameters from challenge tests by primary modelling

The initial cell concentration (log N_0 , log CFU g⁻¹), lag time (λ , h), maximum specific growth rate (μ_{max} , h⁻¹) and maximum population density (log N_{max} , log CFU g⁻¹) of *L. monocytogenes* were determined by fitting a primary model to growth kinetics obtained in each challenge test (Table 1). The Baranyi and Roberts (1994) model was fitted to the log transformed growth data of *L. monocytogenes* (log CFU g⁻¹) by using the Excel Add-in DMFit of IFR (Institute of Food Research, Reading, UK). A conversion factor of ln 10 was multiplied with the DMfit estimated growth rates, as the bacterial concentrations applied in DMfit were log transformed. Kinetic parameters were estimated for 13 challenge tests conducted at constant temperatures in the present study (Table 1, data set # 1 to 13).

2.3.2. Development and calibration of secondary growth rate models for *L. monocytogenes* in ricotta

Two secondary gamma concept μ_{max} -models were developed based on seven experimentally obtained μ_{max} -values for growth of *L. monocytogenes* in fresh ricotta (see 2.2). A simple Ratkowsky et al., 1982 Ratkowsky-type temperature model (Model 1) was fitted to μ_{max} values from the calibration challenge tests (Table 1; Equation (2)).

$$\mu_{max} = \mu_{ref\ 1} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \quad (2)$$

where T is the storage temperature (°C), T_{min} (°C) the theoretical minimum temperature for growth of *L. monocytogenes*, T_{ref} (°C) the reference temperature of 25 °C and $\mu_{ref\ 1}$ the maximum specific growth rate at T_{ref} . Equation (2) was fitted to data sets #1–7 in order to determine the $\mu_{ref\ 1}$ - and T_{min} -values by using the Add-in Solver of Microsoft Excel to minimize a measure for goodness-of-fit: root mean square error (RMSE).

Secondly, a temperature and pH model (Presser et al., 1997) with interaction between the two environmental parameters (ξ ; Le Marc et al., 2002) was used (Model 2; Equation (3))

$$\mu_{max} = \mu_{ref\ 2} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \cdot (1 - 10^{(pH_{min} - pH)}) \cdot \xi \quad (3)$$

where pH_{min} is the theoretical minimum pH for growth of *L. monocytogenes*. Equation (3) was fitted as above to determine $\mu_{ref\ 2}$ - and pH_{min} -values.

The T_{min} value fitted in equation (2) was reused directly in equation (3).

Finally, the existing and extensive *L. monocytogenes* μ_{max} -model of Mejlholm and Dalgaard (2009) was calibrated by fitting its $\mu_{ref\ 3}$ parameter-value as reported for Model 1 above (Model 3; Equation (4)). Importantly, all other parameter values in the Mejlholm and Dalgaard (2009) model were kept unchanged when the $\mu_{ref\ 3}$ -value was calibrated.

$$\begin{aligned} \mu_{max} = \mu_{ref\ 3} \cdot & \left(\frac{T + 2.83}{T_{ref} + 2.83} \right)^2 \cdot \frac{a_w - 0.923}{1 - 0.923} \cdot (1 - 10^{(4.97 - pH)}) \\ & \cdot \left(1 - \frac{LAC_U}{3.79} \right) \cdot \frac{32.0 - P}{32.0} \cdot \left(\frac{350 - NIT}{350} \right)^2 \\ & \cdot \frac{3,140 - CO_2\ equilibrium}{3,140} \cdot \left(1 - \sqrt{\frac{DAC_U}{4.8}} \right) \\ & \cdot \left(1 - \sqrt{\frac{AAC_U}{MIC_U\ acetic\ acid}} \right) \cdot \left(1 - \frac{BAC_U}{MIC_U\ benzoic\ acid} \right) \\ & \cdot \left(1 - \frac{CAC_U}{MIC_U\ citric\ acid} \right) \cdot \left(1 - \frac{SAC_U}{MIC_U\ sorbic\ acid} \right) \cdot \xi \end{aligned} \quad (4)$$

where $\mu_{ref\ 3}$ is equal to μ_{max} at the reference temperature (T_{ref}) of 25 °C; T is the temperature (degrees Celsius); a_w the water activity calculated from the concentration of NaCl in the water phase of the product; P and NIT are the concentrations (ppm) of smoke components (phenol) and nitrite, respectively; CO_2 equilibrium is the concentration (ppm) of dissolved CO_2 at equilibrium; $[LAC_U]$, $[DAC_U]$, $[AAC_U]$, $[BAC_U]$, $[CAC_U]$, and $[SAC_U]$ are the concentrations (mM) of undissociated lactic acid, diacetate, acetic acid, benzoic acid, citric acid, and sorbic acid, respectively; and $[MIC_U\ acetic\ acid]$, $[MIC_U\ benzoic\ acid]$, $[MIC_U\ citric\ acid]$, and $[MIC_U\ sorbic\ acid]$ are the fitted MICs (mM) of undissociated acetic, benzoic, citric, and sorbic acids, respectively, that prevent growth of *L. monocytogenes*. The interaction between environmental parameters (ξ) was modeled as previously described using the Le Marc approach Le (Marc et al., 2002).

For fitting of Model 1, 2 and 3 seven data sets for calibration including six brands of ricotta were used (Table 1; data set # 1–7).

2.3.3. Prediction of *L. monocytogenes* growth in ricotta over time

The differential form of the Baranyi and Roberts (1994) primary growth model was applied in combination with growth rate models to predict growth of *L. monocytogenes* over time. Studied growth rate models were equation (2), equation (3), Model 3 and the Augustin et al. (2005) model for liquid dairy products (see section 2.3.4).

The Runge-Kutta 4th order numerical method (Press et al., 2007) was used for numerical integration of the Baranyi and Roberts (1994) model to predict growth over time under both constant and dynamic temperature storage. The time step in the simulations was set to a fixed value of 600 s and the calculations were conducted in Microsoft Excel. For simplicity the so called adjustment function $\alpha(t)$ of the Baranyi and Roberts (1994) model was set to 1 in case the challenge tests as expected showed no significant lag due to the pre-adaptation of the cells. The curvature parameter m in the Baranyi and Roberts (1994) model was fixed and set to 1 in the present study.

2.3.4. Model evaluation

Model validation is an essential part of model evaluation and conducted to investigate the validity of a model by testing its performance on different data sets than the model was derived from. The indices of model performance bias- (Bf) and accuracy- (Af) factors (Ross, 1996) were calculated for Model 1, Model 2 and Model 3 on the basis of μ_{max} -values from 11 validation challenge tests at constant temperatures (Table 1; data set #8–18). A Bf-value of 1.0 indicates an unbiased model, whereas a Bf-value above 1.0 corresponds to a biased model that predicts growth rates to be higher than observed in challenge tests. Bf-values from 0.95 to 1.11 indicates good model performance, whereas Bf-values of 1.11–1.43 and 0.87–0.95 corresponds to acceptable model performance. Bf-values < 0.87 or > 1.43 corresponds to unacceptable model performance (Ross, 1999). Af-values > 1.5 indicate incomplete models or systematic deviation between observed and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013).

Furthermore, the ability of 10 other secondary models to predict the growth rates of *L. monocytogenes* in fresh ricotta was evaluated by calculation of Bf and Af values from μ_{max} -values in all the 13 calibration and validation challenge tests at constant temperatures (Table 1). The studied existing growth rate models were: the web-based version of

Table 3
Fitted model parameters.

Model #	Parameter values	95% Confidence interval	RMSE/R ²
1	$\mu_{ref\ 1} = 0.433\ h^{-1}$ $T_{min} = -4.06\ ^\circ C$	0.415–0.451 h^{-1}	0.009/0.988
2	$\mu_{ref\ 2} = 0.471\ h^{-1}$ $pH_{min} = 4.91$	0.451–0.491 h^{-1}	0.005/0.997
3	$\mu_{ref\ 3} = 0.808\ h^{-1}$	0.774–0.842 h^{-1}	0.040/0.794

ComBase (2012), two web-based models of the PMP software developed for broths (aerobic and anaerobic conditions, respectively), the model by Mejlholm and Dalgaard (2009) previously validated for meat and seafood products and included in the FSSP, 1995 FSSP software, the two cardinal parameter models for respectively liquid dairy and cheese products by Augustin et al. (2005), the two models proposed by Østergaard et al. (2014) for cottage cheese with respectively fresh and cultured cream dressing, the model by Rosshaug et al. (2012) developed for blue-white cheese and the model by Gougouli et al. (2008) validated for ice cream. The characteristics of each ricotta brand, obtained from section 2.1 and the storage conditions measured during each of the challenge tests, were used as input to evaluate the existing models (Tables 1 and 2).

Moreover, the acceptable simulation zone (ASZ) approach was used to evaluate the models ability to predict bacterial growth over time and it was done by comparing observed and predicted bacterial concentrations ($\log\ CFU\ g^{-1}$). The acceptable zone was defined as $\pm 0.5\ \log\ CFU\ g^{-1}$ from the predicted growth curve. The model performances of equation (2), equation (3), the recalibrated Mejlholm and Dalgaard (2009) model and the Augustin et al. (2005) model for liquid dairy products were evaluated for independent growth data of *L. monocytogenes* obtained from 11 validation challenge tests conducted at both constant and dynamic storage temperatures (Table 1, dataset # 8 to 18). These data were not used to fit or calibrate the evaluated models. When at least 70% of the observed cell concentrations ($\log\ CFU\ g^{-1}$) in challenge tests were within ASZ, the prediction was considered acceptable (Møller et al., 2013; Oscar, 2005; Østergaard et al., 2014).

3. Results

3.1. Microbiological screening of fresh ricotta

The microbiological characterization of fresh ricotta from eight different brands showed that *L. monocytogenes* was absent in all the samples before inoculation and other types of bacteria were present in low concentrations throughout shelf life of the products. TVC at the beginning of storage (T0) were $< 2.7\ \log\ CFU\ g^{-1}$ for all the eight studied brands of fresh ricotta and 37 of the 42 TVC values were below the detection limit of $2.0\ \log\ CFU\ g^{-1}$. At half of the shelf-life (T1), four of the eight brands showed TVC between 2.0 and $3.5\ \log\ CFU\ g^{-1}$, while the others were below the detection limit. At the end of shelf-life (T2) all the brands had TVC $< 4.0\ \log\ CFU\ g^{-1}$. LAB at T1 and T2 were $< 2.0\ \log\ CFU\ g^{-1}$.

3.2. Physical-chemical characterization

The studied fresh ricotta brands showed variability in physical-chemical characteristics (Table 2). The observed average pH values during storage for the different brands varied between 5.49 and 6.61. Variations were also observed for the concentrations of acetic acid (579–2021 ppm), citric acid (14,774 to 49, 351 ppm) and lactic acid (LOQ = < 69.4 to 4146 ppm). Due to the product pH, approximately 84–99% of the total acetic acid, 99.6–99.7% of total citric acid and 97.3–99.8% of total lactic acid were in the dissociated form. Un-

dissociated average concentrations of organic acids were 0.8 mM acetic acid, 0.2 mM citric acid, and 0.1 mM lactic acid at a specific pH of 6.2 in ricotta. This is important, as the inhibitory effects of organic acids mainly are due to their un-dissociated form (Eklund, 1985; Houston et al., 1994). Fresh ricotta showed little variability in water activity among brands (0.996–0.998), despite some variability in moisture contents (72.21–82.05%).

3.3. Growth in challenge tests

μ_{max} -values in the challenge tests were, as expected, markedly influenced by the storage temperature (Table 2). The observed μ_{max} values increased $\sim 941\%$ from $0.034\ h^{-1}$ to $0.320\ h^{-1}$ when the temperature increased from $4.1\ ^\circ C$ to $20.6\ ^\circ C$. The ability of *L. monocytogenes* to grow at chill temperature was also confirmed in this study as a T_{min} -value of -4.06 was determined (Table 3). The variability in pH between the investigated fresh ricotta brands was sufficient to cause detectable differences in observed μ_{max} values of *L. monocytogenes* in the product. At $14.7\ ^\circ C$ the observed μ_{max} values dropped $\sim 23\%$ from $0.197\ h^{-1}$ to $0.151\ h^{-1}$ when pH decreased from 6.61 to 5.49 (Table 2). This was also specifically observed for brand A, C and E at $8.3\ ^\circ C$.

The observed $\log\ N_{max}$ -values in challenge tests showed little variability with an average value of $8.46 \pm 0.24\ \log\ CFU\ g^{-1}$. In the present study, due to the adaptation of pre-cultures, *L. monocytogenes* grew without a significant lag phase in challenge tests, except for the product where acetic acid was added in the laboratory (Table 2; dataset #7).

3.4. Effect of organic acids

Our experiments (Table 4) carried out to identify the growth boundary (the concentration separating Growth/No-Growth conditions for undissociated organic acid concentrations) in ricotta showed that at $15.2\ ^\circ C$ ricotta with an undissociated concentration of acetic acid of 8.8 mM (or more) do not support growth of *L. monocytogenes* within 7 days. Ricotta with an undissociated concentration of citric acid of 24.0 mM supported growth of *L. monocytogenes*, and therefore the MIC value for citric acid was above 24 mM (Table 4). The tested higher levels of citric acid unfortunately had pH values too close to the growth boundary of pH for *L. monocytogenes* according to model 2, and therefore dataset # 23 and #24 (Table 1) cannot be used to conclude anything about the effect or citric acid on growth of *L. monocytogenes* in ricotta. Ricotta with an undissociated concentration of lactic acid of 5.1 mM supported growth of *L. monocytogenes*, but not the level of 14.7 mM, even though growth should be possible at this pH value.

3.5. Model evaluation

3.5.1. Evaluation of existing models

Several existing predictive models developed in broths or for other dairy products were evaluated for ricotta and some of these were acceptable as shown in Table 6. Augustin et al. (2005) calibrated an extensive cardinal parameter model to *L. monocytogenes* μ_{max} -values in liquid dairy products. When applied to fresh ricotta, this model resulted in an acceptable Bf value of 0.88 (Table 6). The acceptable performance of this model was confirmed for storage of different brands of ricotta at constant and dynamic temperature, when using the ASZ method for model evaluation. A mean of 83% of the challenge test data were within the ASZ (Table 7).

The model proposed by Østergaard et al. (2014) for cottage cheese with fresh cream dressing gave acceptable predictions with a slight fail-safe overestimation of growth rates in ricotta (Bf = 1.18) (Table 6). Also the generic Combase model (ComBase, 2012) gave acceptable fail-safe predictions of growth rates in ricotta (Bf = 1.32). These results advised on the potential risks deriving from the use of product specific models for different products.

Table 4
Observed growth or no-growth of *L. monocytogenes* in fresh ricotta with added organic acids.

Dataset #	Product brand code	Average temperature (°C)	pH ^a Initial	pH ^b Final	Moisture content (%)	Total organic acid concentration in the water phase (undissociated conc.) (mM)				Growth during 7 day at 15.2 °C			G/NG ^c
						Acetic acid	Citric acid	Lactic acid	Log N ^d Initial (log CFU g ⁻¹)	Log N ^b Final (log CFU g ⁻¹)	Δ Log N (log CFU g ⁻¹)		
19	G ^d	15.2	5.27	5.57	76.7	37.3 (8.8)	77.5 (0.6)	5.7 (0.2)	4.20	4.11 ± 0.14	-0.09	NG	
20	G ^d	15.2	5.21	5.29	76.7	75.5 (19.8)	88.6 (0.7)	6.3 (0.3)	4.34	2.89 ± 0.40	-1.45	NG	
21	G ^d	15.2	4.95	4.90	74.6	174.4 (68.4)	117.3 (1.7)	12.7 (1.0)	4.45	2.87 ± 0.03	-1.58	NG	
22	G ^e	15.2	4.99	5.04	75.0	7.6 (2.8)	1764.3 (24.0)	1.3 (0.1)	4.20	4.81 ± 0.13	0.61	G	
23	G ^e	15.2	4.90	4.84	75.7	5.2 (2.2)	2802.8 (46.8)	6.0 (0.5)	3.86	2.84 ± 0.09	-1.02	NG	
24	G ^e	15.2	4.92	5.01	76.6	4.9 (2.0)	3914.8 (62.5)	3.6 (0.3)	4.25	< 2.00	< -2.25	NG	
25	G ^f	15.2	5.11	6.03	75.8	4.2 (1.3)	53.1 (0.6)	35.2 (1.9)	3.74	> 6.00	> 2.26	G	
26	G ^f	15.2	5.18	5.13	74.5	9.4 (2.6)	76.7 (0.7)	111.0 (5.1)	4.13	5.08 ± 0.21	0.95	G	
27	G ^f	15.2	4.99	5.02	75.2	14.4 (5.4)	84.7 (1.2)	213.6 (14.7)	4.33	3.05 ± 0.06	-1.28	NG	

^a Before inoculation.^b After 7 days.^c Growth (G) defined as Δ Log N > 0.5 log CFU g⁻¹ with no growth (NG) corresponding to ≤ Δ Log N 0.5 log CFU g⁻¹.^d Acetic acid added.^e Citric acid added.^f Lactic acid added.

Other existing models developed for different substrates were tested and unable to accurately predict the growth of *L. monocytogenes* in fresh ricotta. Some of the models underestimated the growth of this pathogen, showing Bf ranging from 0.25 to 0.64, resulting in fail dangerous estimations: (a) The model of Gougouli et al. (2008) developed for ice-cream, (b) The model for cheese by Augustin et al. (2005), (c) The model for blue-white cheese by Rosshaug et al. (2012), (d) The model for cottage cheese with cultured cream dressing by Østergaard et al. (2014). The extensive growth and growth boundary model of Mejlholm and Dalgaard (2009), validated for various seafood and meat products (Mejlholm et al., 2010) markedly underestimated growth rates in fresh ricotta (Bf = 0.43) highlighting the need for evaluation of models with the same type of products where predictions are needed.

3.5.2. Model calibration

Three models were calibrated to predict growth of *L. monocytogenes* in ricotta. The calibration process resulted in RMSE values of 0.009 for Model 1 with two fitted parameters; 0.005 for Model 2 with two fitted parameters, and 0.040 for the recalibrated Mejlholm and Dalgaard (2009) model with one fitted parameter. The calibrated parameters μ_{ref} 1 and T_{min} for Model 1, μ_{ref} 2 and pH_{min} for Model 2 and μ_{ref} 3 in the recalibrated Mejlholm and Dalgaard (2009) model are reported in Table 3.

3.5.3. Performances of the calibrated models

The calibrated and thereby product specific models resulted in Bf/Af-values of 1.10/1.11 for Model 1, 1.06/1.09 for Model 2 and 0.78/1.31 for the recalibrated model of Mejlholm and Dalgaard (2009) (Table 6). The good performances of Models 1 and 2 were confirmed for storage of different brands of ricotta at constant and dynamic temperature when using the ASZ method for model evaluation (Fig. 1). Mean ASZ scores were 85% for Model 1 with two challenge tests having below 70% of the prediction within the ASZ, 91% for Model 2 with all challenge tests having above 70% of prediction within ASZ and 65% for Model 3, with six of the 11 challenge tests having less than 70% of the prediction within ASZ (Table 7).

4. Discussion

The present study allowed characterizing from a chemical-physical and microbiological point of view several brands of Italian fresh ricotta: until this moment limited information about growth characteristics of *L. monocytogenes* in this product was available in literature (Genigeorgis et al., 1991). This study provided new and quantitative data for the growth potential of *L. monocytogenes* in ricotta depending on storage temperatures and product characteristics. The new data provided allows potential growth of *L. monocytogenes* to be evaluated directly in the eight studied brands of ricotta and in general by using the predictive models successfully validated for this substrate in the present study. This is important for the evaluation of the potential growth of *L. monocytogenes* in this product type and to facilitate product reformulation to reduce or prevent growth of this pathogen. Furthermore, very few models that estimates the growth of this pathogen have been validated for dairy products (Augustin et al., 2005 Østergaard et al., 2014), thus this novel model could possibly be convenient also for other fresh dairy products.

The production process of ricotta includes a thermal treatment that can be monitored by the producer, and a hot packaging process including fast sealing of the packages, when the product temperature is still very high. Therefore it is likely that *L. monocytogenes* is absent when ricotta products leave the dairy with hygienic conditions being maintained. Growth of *L. monocytogenes* in fresh ricotta and the related human health risk, seems particularly important after cross-contamination when the sealed packaging is opened and the product thereafter is handled by consumers including further storage in home-refrigerators. The influence of storage conditions can be critical,

Table 5
MIC values for un-dissociated concentrations of acetic acid, citric acid, and lactic acid in different substrates.

References	MIC Undissociated acid (mM)			Substrate	Temp. (°C)	Species
	Citric acid	Acetic acid	Lactic acid			
Present study	> 24.0	< 8.8	5.1–14.7	Fresh ricotta	15.2	<i>L. monocytogenes</i>
Coroller et al., 2005	0.2–3.6	21.5	6.4	BHI	30	<i>L. innocua</i>
Le Marc et al., 2002	- ^a	20.3	8.0	Modified BHI	20	<i>L. innocua</i>
Mejlholm and Dalgaard, 2009	2.12	10.3	3.79	BHI pH 6.0	8	<i>L. monocytogenes</i>
Wemmenhove et al., 2016	3.8 ± 0.9	19.0 ± 6.5	5.0 ± 1.5	BHI at pH 4.6–5.8	30 °C	<i>L. monocytogenes</i>

^a Not determined.

because they depend on the behaviour of the consumer. It has to be considered that many RTE products (like ricotta) spend most of their shelf life in a domestic refrigerator; a survey published by the EFSA Panel on Biological Hazards (2007), covering data coming from 7 different European countries, showed high frequencies of improper home refrigeration temperatures, with more than 50% of temperatures above 6–8 °C. Sealed products are normally safe, but not always, severe listeriosis outbreaks related to other thermally processed foodstuffs with sealed package have been reported. For example, the consumption of cooked ham in Switzerland caused 9 severe listeriosis cases and in Denmark a deli-meat (*rullepølse*), recently resulted in 17 fatal cases (Hächler et al., 2013; SSI, 2014). For both these outbreaks, a post-process contamination during slicing/packaging and followed by growth of *L. monocytogenes* is likely. This highlights the importance of both predicting the potential growth of *L. monocytogenes* in fresh ricotta and considering product reformulation to reduce potential growth.

Of the existing models the most accurate predictions (Bf/Af = 0.88/1.13) were obtained by a cardinal parameter model previously calibrated to *L. monocytogenes* μ_{max} -values in liquid dairy products and including the effect of temperature, pH and water activity as well as other factors including nitrite, phenol and CO₂ without relevance for ricotta in the present study (Table 6, Augustin et al., 2005). The acceptable performance of the model calibrated to liquid dairy products suggests food structure has little influence on *L. monocytogenes* growth in ricotta. This is noteworthy as structure has been reported to limit bacterial growth in gels and model soft cheese structured with agar (Wilson et al., 2002; Noriega et al., 2008; Antwi et al., 2008). A likely explanation is that the heterogeneous food structure of fresh ricotta contains aqueous pores and cracks where planktonic growth can take place contrary to homogeneous gels based on agar.

Table 6
Performance evaluation of growth models for *L. monocytogenes* applied for fresh ricotta.

Reference/Software	n ^a	Substrate	Variables included ^b	Bf	Af
PMP (aerobic) (USDA, 2013)	13	Broth	<i>T, pH, NaCl, (NIT)</i>	1.86	1.86
PMP (anaerobic) (USDA, 2013)	13	Broth	<i>T, pH, NaCl, (NIT)</i>	2.01	2.01
ComBase (2012)	13	Broth/Food	<i>T, pH, Aw,</i>	1.32	1.32
Gougouli et al., 2008	13	Ice cream	<i>T</i>	0.64	1.56
Rosshaug et al., 2012	13	Blue-white cheese	<i>T, pH, LAC, NaCl</i>	0.37	2.71
Østergaard et al., 2014	13	Cottage cheese-fresh cream	<i>T, pH, Aw, LAC, (SAC)</i>	1.18	1.28
Østergaard et al., 2014	13	Cottage cheese-cultured cream	<i>T, pH, Aw, LAC, (SAC)</i>	0.56	1.79
Augustin et al., 2005, model #5	13	Cheese	<i>T, pH, Aw, (NIT, P, CO₂)</i>	0.25	3.96
Augustin et al., 2005, model #5	13	Liquid dairy products	<i>T, pH, Aw, (NIT, P, CO₂)</i>	0.88	1.13
Mejlholm and Dalgaard, 2009	13	Meat and fish products	<i>T, pH, Aw, LAC, AAC, CAC, (NIT, P, CO₂, SAC, DAC, BAC)</i>	0.43	2.34
Model 1, two fitted parameters:	6	Fresh ricotta	<i>T</i>	1.10	1.11
Model 2, two fitted parameters:	6	Fresh ricotta	<i>T, pH</i>	1.06	1.09
Recalibrated Mejlholm and Dalgaard, 2009 model; one fitted parameter	6	Fresh ricotta	<i>T, pH, Aw, LAC, AAC, CAC, (NIT, P, CO₂, SAC, DAC, BAC)</i>	0.78	1.31

Factors included in the specific models but not taken into account for ricotta are reported in brackets ().

^a n = Number of growth curves evaluated.

^b T: temperature; Aw: water activity; pH: pH value; NIT: nitrite; P: phenol; CO₂: carbon dioxide; NaCl: sodium chloride; LAC: lactic acid; SAC: sorbic acid; AAC: acetic acid; CAC: citric acid; DAC: diacetate; BAC: benzoic acid; NaLac: sodium lactate; NaAcet: sodium acetate; KSorb: potassium sorbate; PLAC: potassium lactate.

Table 7
Comparison of model performances by using the Acceptable Simulation Zone (ASZ) approach.

Product brand code	Temperature (°C)	Augustin et al. (2005), model #5 (liquid dairy)	Percentages of measurements within a $\pm 0.5 \log \text{CFU g}^{-1}$ band along the simulated values		
			Model 1	Model 2	Recalibrated Mejlholm and Dalgaard (2009) model
A	8.3	95	87	84	97
A	10.3	98	100	97	97
A	13.4	100	100	100	83
A	20.6	100	94	94	94
A	4–8	77	83	83	29
F	4–8	56	92	92	21
H	4–8	29	65	81	90
A	12–16	90	100	100	48
G	12–16	98	93	98	55
G with added lactic acid	14.7	88	33	83	38
G with added citric acid	14.7	88	92	96	63
Mean ASZ score (%)		83	85	91	65

for a quick simple prediction of growth of *L. monocytogenes* in typical ricotta products, but it does not enable the user to assess the effect on shelf-life of a possible reformulation of product characteristics. In our study the influence of temperature on the growth of *L. monocytogenes* in ricotta was clearly defined, but also pH played a role in growth inhibition in this substrate. The variability in pH between the investigated fresh ricotta brands was sufficient to cause detectable differences in observed μ_{\max} values of *L. monocytogenes* in the product. However, the pH values measured in this study never prevented growth in most of the conducted challenge tests, except for datasets 19, 20, 21, 23, 24 and 27 where additional organic acids were added. Model 2, including the effects of temperature and pH, and with Bf/Af of 1.06/1.09 was successfully validated for to predict growth of *L. monocytogenes* in fresh ricotta. Model 2 did not included terms to describe the inhibiting effect of organic acids in fresh ricotta (Equation (2)). The inhibiting effect of organic acids in fresh ricotta was therefore lumped into the fitted parameters in Model 2 and this may explains the relatively high observed parameter value for pH_{\min} of 4.91 describing the combined effect of pH and organic acids in the studied fresh ricotta.

The estimated cardinal parameter values ($T_{\min} = -4.06 \text{ °C}$; $pH_{\min} = 4.91$) corresponded partially to literature values. The pH_{\min} value was very similar to the value obtained by Østergaard et al. (2014) (4.87 ± 0.01), and higher than the values observed by Augustin et al. (2005) from a model for liquid microbiological media, both with hydrochloric (4.26 ± 0.24) or lactic acid as acidulant (4.71 ± 0.28). Considering the T_{\min} values, the result obtained in our study was evidently lower than those obtained in the two studies cited (-2.01 ± 0.40 and $-1.72 \pm 2.55 \text{ °C}$, respectively). These results suggested the specificity of ricotta substrate, with a strict pH range for *L. monocytogenes* growth and a less evident inhibition of growth rate when temperatures are getting lower.

The successfully validated Model 2 and the model of Augustin et al. (2005) can be used to assess the safe shelf life of ricotta depending on storage temperature and product pH and finally to reformulate the product. Considering, as an example, an open package of ricotta (pH ~ 6.2) contaminated with *L. monocytogenes* to a concentration of 1 CFU g^{-1} and stored in a household refrigerators (7 °C), to avoid exceedance of the limit of 100 CFU g^{-1} or 2 log CFU g^{-1} (EC, 2005), shelf life should not exceed 3–4 days (Table 8). A reduction of the product pH to 5.8, combined with a decrease in storage temperature (4 °C) allowed to extend the predicted safe shelf life up to 6–10 days. These results confirmed the importance of good practices for consumers requiring a correct refrigeration and consumption of ricotta in few (two-three) days after opening of the packaging; labels providing consumers instructions on how long a RTE product like ricotta could be stored and when to discard after opening the package are helpful and well established (Lenhart et al., 2008).

A generic predictive model that can be used for many food products is interesting, easier to use and may be more valuable than a product specific model where accurate predictions can be restricted to a particular food. However, the extensive Mejlholm and Dalgaard (2009) model that has been successfully validated for a range of seafood and meat products (Mejlholm et al., 2010) markedly under-predicted growth rates of *L. monocytogenes* in ricotta (Bf = 0.43) highlighting the need for product specific predictive models (Table 6). With μ_{ref} recalibrated to *L. monocytogenes* μ_{\max} -values in fresh ricotta this model performed better (Bf = 0.78) but it was still not acceptable for this product. Calibrating μ_{ref} and MICs for acetic, citric and lactic acids in the Mejlholm and Dalgaard (2009) model to ricotta datasets # 1–13 (Table 1) and with μ_{\max} -values as shown in Table 2 resulted in a μ_{ref} -value of 0.523, RMSE of 0.008 and MIC-value of above 1000 mM of all three undissociated acids (Results not shown). This confirms that there is practically no inhibiting effect against growth of *L. monocytogenes* for the three organic acids in fresh ricotta as shown above by the ability to predict growth accurately by simple models not including the inhibiting effects of acetic, citric and lactic acids (Tables 6 and 7). This also explains the poor performance of the original Mejlholm and Dalgaard (2009) model (Bf = 0.43) as the acetic, citric and lactic acid concentrations in fresh ricotta results in reduced predicted μ_{\max} -values for *L. monocytogenes*.

MIC values determined in the present study for undissociated acetic and lactic acids were similar to those reported in literature (Table 5, Coroller et al., 2005; Le Marc et al., 2002; Mejlholm and Dalgaard, 2009; Wemmenhove et al., 2016). However, a much higher MIC value was found for citric acid (Table 5) this could be related to differences in substrate when determining these values: the MIC-value suggested by Le Marc et al. (2002), Coroller et al. (2005) and Mejlholm and Dalgaard (2009) were measured in broths, while our data were obtained from ricotta substrates.

For lactic acid bacteria a previous study reported that MICs determined from challenge tests in real foods were 2–3 times higher than MICs determined for broth (Mejlholm and Dalgaard, 2013). Importantly, they found MIC values determined from challenge tests with brined shrimps resulted in a good model performance whereas this was not the case for MICs determined by using APT broth. We have found no previous reports of very high MIC values as suggested by the present study for acetic, citric and lactic acid in fresh ricotta. It remains to be determined why MIC values apparently can depend on the type of substrate as also previously pointed out for brined shrimps (Mejlholm and Dalgaard, 2013). Future studies should be performed in order to understand mechanisms of action and the quantitative influence of temperature on MICs, that remains uncertain.

In conclusion, the present study provided new data and validated predictive models to define the growth potential of *L. monocytogenes* in

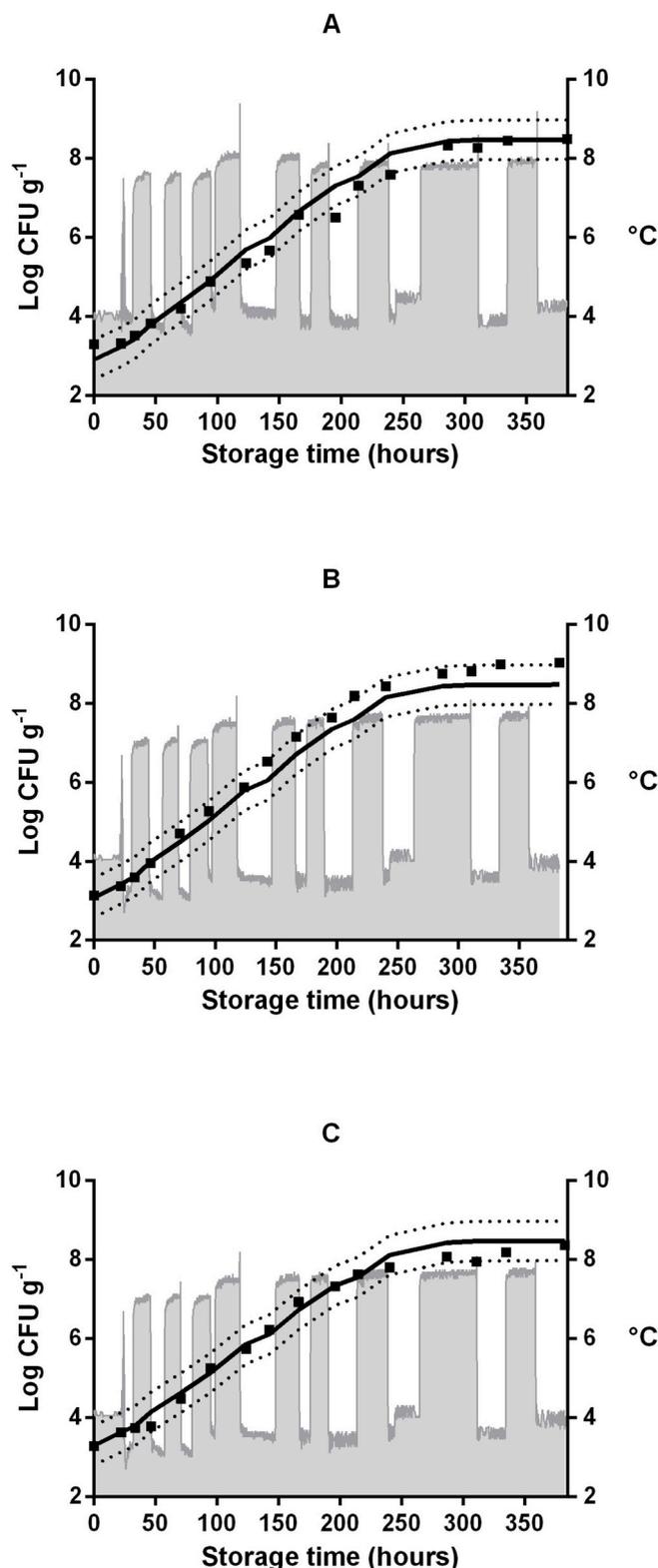


Fig. 1. Comparison of observed (■) and predicted (---) growth for *L. monocytogenes* in three brands of fresh ricotta. Data set #16 (A), data set #15 (B) and data set #14 (C). Predictions and the acceptable simulation zone (.....) were obtained by using Model 2 including the effect of temperature and pH. Temperature profiles are illustrated with grey lines.

fresh ricotta as relevant for safe shelf-life and to reformulate the product for increased shelf-life or safety. This would be extremely advantageous especially in the reformulation of ricotta by the

Table 8

Predicted time to obtain a potential 2 log CFU g⁻¹ growth of *L. monocytogenes* in fresh ricotta. Calculated as $\frac{2 \ln 10}{\mu_{max}}$.

Temperature (°C)	pH	Predicted time for growth of 2 Log cfu g ⁻¹ (d) Model 2	Augustin et al. (2005, model #5) liquid dairy
7	6.2	3.0	3.8
6	6.2	3.6	4.8
5	6.2	4.4	6.3
4	6.2	5.6	8.7
4	5.8	6.1	9.9
5	5.9	4.7	6.9
5	5.5	5.6	8.4
4	5.6	6.7	10.9
4	5.1	67.2	52.7

manufacturers, with important social and economic returns.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2018.10.004>.

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