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PREDICTION OF *LISTERIA MONOCYTOGENES* GROWTH AS A FUNCTION OF ENVIRONMENTAL FACTORS

N. Topic Popovic[a], A. Benussi Skukan[b], P. Dzidara[b], I. Strunjak-Perovic[a], S. Kepec[c], J. Barisic[a]* and R. Coz-Rakovac[a]

[a]Laboratory for Ichtyopathology-Biological Materials, Rudjer Boskovic Institute, Bijenicka cesta 54, 10 000 Zagreb. Croatia
[b]Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10 000 Zagreb. Croatia
[c]Virkom d.o.o, Public Water Supply and Wastewater Services, Kralja Petra Kresimira IV 30, 33 000 Virovitica. Croatia

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Listeria monocytogenes is a bacterium widespread in the environment, which has a capacity to survive and grow under various conditions. The bacterial growth results from interactions when subjected to various temperatures, pH levels, and NaCl concentrations were examined by measurements and predictive modelling. Good correlation across the range of growth conditions was shown among observed and predicted growth values, having similar trends and minimal deflections for pH levels 5.0 and 6.0. The growth condition in the 8% NaCl concentration (pH 7.0, temperature 4 °C) resulted with a growth curve of 1 log interval greater than the fitted curve for all the measurements. In all of the cases, there were consistent increases in the rates and decreases in the lag time when the growth temperature increased. Higher incubation temperatures provided higher growth rates as 30 °C and 35 °C yielded double increase of the fitted rate. Fitted and measured growth rates for salinity conditions were significantly different ($P<0.05$). Comparison of doubling times showed good compatibility, particularly at lower temperatures. Critical use of a model is suggested, although it may enable microbiologists to limit the need of challenge tests and to make rapid and realistic prediction of the growth of *L. monocytogenes* under conditions relevant to a range of aquatic and other products examined.

Keywords: predictive modelling, bacterial growth rates

The causative agent of listeriosis, *Listeria monocytogenes* bacterium, was first isolated from rabbit livers in Sweden in 1911. Murray and co-workers (1926) described a small Gram-positive rod causing 1900 disease cases in laboratory animals. They named the bacterium *Bacterium monocytogenes* due to its clinical features – mononucleosis in the affected animals. It later became apparent that the disease also affects humans, and the rise in the number of human cases in several countries, along with the evidence for food-borne transmission, renewed the interest in listeriosis (McLauchlin et al., 2004). Indeed, *L. monocytogenes* has been recognized as an important foodborne pathogen, what is more, the outbreaks of listeriosis have been associated with seafood, milk, cheese, vegetables, and meat products. Of special concern are *L. monocytogenes* hazards related to urban and natural environments (Sauders et al., 2012), water environments and sewage (Budzinska et al., 2012), wastewaters related to farming (Dungan et al., 2012), and aquatic food products. The organism is particularly problematic for the food industry, because it is widespread in the environment (Giffel & Zwietering, 1999). As a consequence of foodborne listeriosis, numerous studies have

* To whom correspondence should be addressed.
Phone/fax:+385 1 4571 232; e-mail:jbarisic@irb.hr

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determined the extent to which *L. monocytogenes* is present in a variety of foods (Skovgaard & Morgen, 1988; Farber et al., 1989; Vorster et al., 1993; Wang & Muriana, 1994). *L. monocytogenes* was found to be able to grow over a wide range of temperatures (–1.5 to 45 °C) pH levels (4.39 to 9.4), and osmotic pressures (NaCl concentrations up to 10%) (Giffel & Zwietering, 1999). Because of the widespread distribution of *L. monocytogenes* in food products of aquatic origins, it is important to understand the capacity of the bacterium to survive and grow under conditions associated with their processing, storage, and distribution.

In food microbiology, mathematical modelling relies on databases obtained by laboratory observations of growth and death of microorganisms under defined conditions. Assessing the likelihood and extent of growth, in response to a number of key controlling factors acting in combination via mathematical models, is one of several approaches being used by McClure and co-workers (1997). Mathematical analytics has been used to describe growth responses of microorganisms to combinations of factors (Szigeti & Farkas, 2000). Therefore, despite the complexity of many food systems, strategies based on predictive models can simplify problematic areas and allow useful predictions and analyses to be made, giving a rational framework for understanding the microbial ecology of food and water (Ross & McMeekin, 1994).

The aim of this study was to determine the accuracy and reliability of the predictive results of the food micromodel, FMM (Food MicroModel Ltd., Randalls Road, Leatherhead, Surrey, KT22 7RY, UK), screening the growth responses of *L. monocytogenes* in culture medium adjusted to various pH and NaCl levels, and stored at wide temperature ranges. The compatibility of bacterial growth in culture medium was compared with bacterial growth predicted by the FMM, having in mind the need for reduction of the challenge tests for securing microbiologically unharmful food of various origins.

1. Materials and methods

The ATCC–7644 (Oxoid 3970) strain of *Listeria monocytogenes* was used for this study. Tryptic soy broth (TSB, Biolife, 402155) was used for optimal growth. Batches were made of 300 ml volume sterile media in Erlenmeyer flasks, where concentrations of NaCl and pH levels were adjusted. The pH level was adjusted using 2 M HCl (Kemika 1824301) or 2 M NaOH (Kemika 1452506) before making up to final volume. The NaCl (Kemika 1417506) concentration in broth ranged from 5–80 g l^{-1}.

The pH value of prepared TSB broths was measured by a pH-meter (Iskra MA 5835). The NaCl concentration in TSB broths was measured using the AOAC potentiometric method III (1990).

1.1. Experimental procedure

Into 300 ml batches of TSB, a 24-h *L. monocytogenes* culture was inoculated to the expected initial number of cells of 10^2–10^3 ml^{-1}. Immediately after inoculation, enumeration was conducted using the colony count technique in accordance with the ISO standard (ISO, 1991) in sight of its specified principle, culture media and the grade the fluid was diluted, apparatus and glassware, sampling, preparation of the test sample, procedure, expression of results and test report. Viable numbers in each batch were determined by plating 20 µl volumes on duplicate plates of tryptone soya agar (TSA, Biolife, 402155) immediately after dispensing.

Decimal dilutions were made from separate bottles of TSB and 100 μl of each was plated onto duplicate plates of TSA. After incubation for 48 h at the temperature of 35 °C, the mean number of colonies on the duplicate plates was determined and the number of colony-forming units (CFU) per ml was calculated and expressed as log10 CFU ml^{-1}.

The experiment was conducted with the combination of the following conditions, in which *L. monocytogenes* may grow, after a preliminary screening to determine the growth limits of the strain used:

Storage temperature (°C): 4, 20, 30, 35.

NaCl (g l^{-1}): 5, 20, 40, 60, 80.

pH: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.4.

1.2. Statistical modelling

For statistical evaluation of data a model based on the function of Baranyi and Roberts (1994) was used, assuming the function of specific bacterial growth by Richards (1959). The Baranyi model is an alternative to the tri-linear primary growth model by Pouillot and Lubran (2011). The food micromodel FMM is a polynomial model used to predict the growth of various pathogenic microorganisms used as a function of controlling growth factors (e.g. temperature, pH, NaCl). FMM pathogen models are principally based upon research sponsored by the U.K. Ministry of Agriculture, Fisheries and Food. The data used to generate the models were obtained from extensive experiments performed on microbiological culture media by Giffel and Zwietering (1999). Both experimental and modelled data for doubling times were compared with the ComBase Predictor, an on-line tool for predicting the response of pathogens and spoilage microorganisms to key environmental factors (available at: http://modelling.combase.cc/ComBase_Predictor.aspx).

2. Results and discussion

Laboratory growth curves of *L. monocytogenes* measured under conditions of constant salinity (0.5%) and temperature (4 °C), over ten days, with different pH levels (pH=5.0, 6.0, and 7.0) are presented in Fig. 1, with initial counts of 8×10^2, 6×10^2 and 3×10^2 of CFU ml^{-1}, respectively, and compared with predicted values as calculated by the model. In general, good compatibility across the range of growth conditions was shown between observed and predicted growth values, having similar trends and minimal deflections for pH levels 5.0 and 6.0, while for pH 7.0, the growth curve of observed and predicted growth differed up to 2 log intervals by the end of the day 13 (figure demonstrating a 10-day curve). The curve at 6.0 pH level, although higher than the predicted curve at all data points, probably due to the observed initial count of 6×10^2 versus a predicted 5×10^2 count, was in agreement with the work of Begot and co-workers (1997). The initial count of 7×10^2 CFU ml^{-1} *L. monocytogenes* in 1 ml of TSB (with close to optimal growth conditions at pH 7.0, NaCl 0.5%, and temperature 20 °C) increased to 9×10^8 CFU ml^{-1} after a 5-day period. With the increased NaCl concentration of 4% (pH 7.0, temperature 4 °C), the count of *L. monocytogenes* in 1 ml broth grew from 4×10^2 to only 7×10^6 CFU ml^{-1} in 20 days, due to the lowered temperature and unfavourable NaCl concentration. It was in good compatibility with the predicted curve up to the 7th day, while by the end of a 20-day period it was up to 1 log over the fitted curve. A further increase of the NaCl concentration to 6% and 8%, led to the count of 10^7 and 2×10^6 CFU ml^{-1}, respectively, in 20 days. Although the predicted curve for the 6% salinity resembled the

previous predicted curve for 4% salinity, the 8% NaCl growth condition (pH 7.0, temperature 4 °C) resulted a growth curve 1 log higher than the fitted curve for all measurements. In all of the cases, there was a consistent increase in the rates and decrease in the lag time when the growth temperature increased.

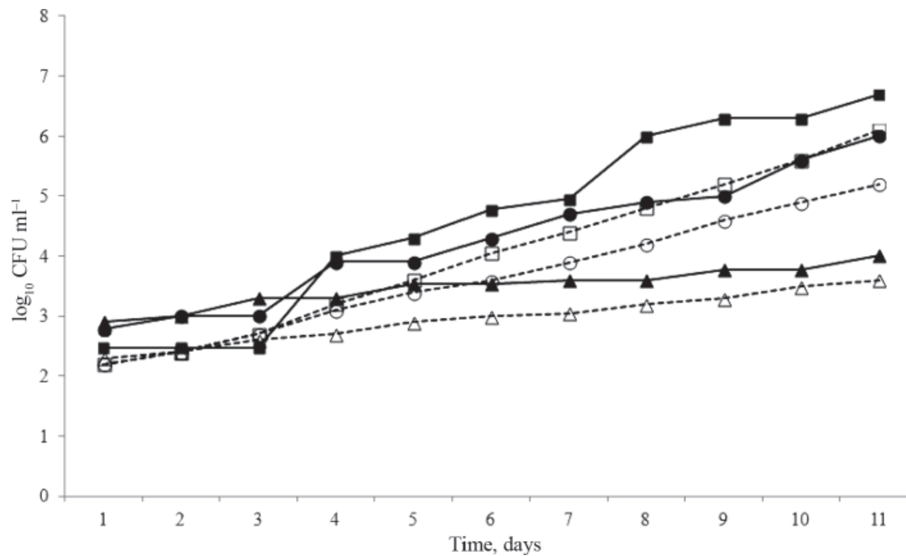

Fig. 1. Observed (full line) and predicted (dashed line) growth curve for *L. monocytogenes* in various pH levels, with constant salinity (0.5%) and temperature (4 °C) conditions, over a 10-day period. (pH 5.0: ▲ △; pH 6.0: ● ○; pH 7.0: ■ □).

Although bacterial growth was significantly slower at 4 °C than at higher temperatures, it was clearly measurable, as opposed to the findings of PARK and co-workers (2005) who detected no growth of *L. monocytogenes* at the storage temperature of 4 °C for 3 weeks in presence of all experimental variables (pH, NaCl). Numerous studies have evaluated the effect of environmental factors such as oxygen, temperature, and level of mixed microbial population on microbial growth (ROSS et al., 2000; SHIMONI & LABUZA, 2000). However, the most important factor for controlling microbial growth remains temperature. Growth predictions under suboptimal conditions, such as low temperature and high salinity, are relevant for the food industry, as this situation most likely occurs in food production (GIFFEL & ZWIETERING, 1999). While slightly elevated salt concentration may inhibit growth rate, it has also been reported to increase the high temperature tolerance of many bacterial species, though the effect is not universal (ROSS et al, 2000).

Secondary models were developed for specific growth rates as a function of temperature, salinity and acidic to neutral pH. Specific growth rates, μ h^{-1}, of *L. monocytogenes* predicted with mathematical analytics and calculated from earlier laboratory measurements for different pH levels (5.0, 6.0, 7.0), NaCl concentrations (0.5, 2.0, 4.0, 6.0, 8.0%) and temperature conditions (4, 20, 30, 35 °C) are presented in Fig. 2. Higher incubation temperatures between 30 °C and 35 °C, which yielded a double increase of the fitted rate, provided higher growth rates, due to a very short doubling time in its optimal conditions. Fitted and measured growth

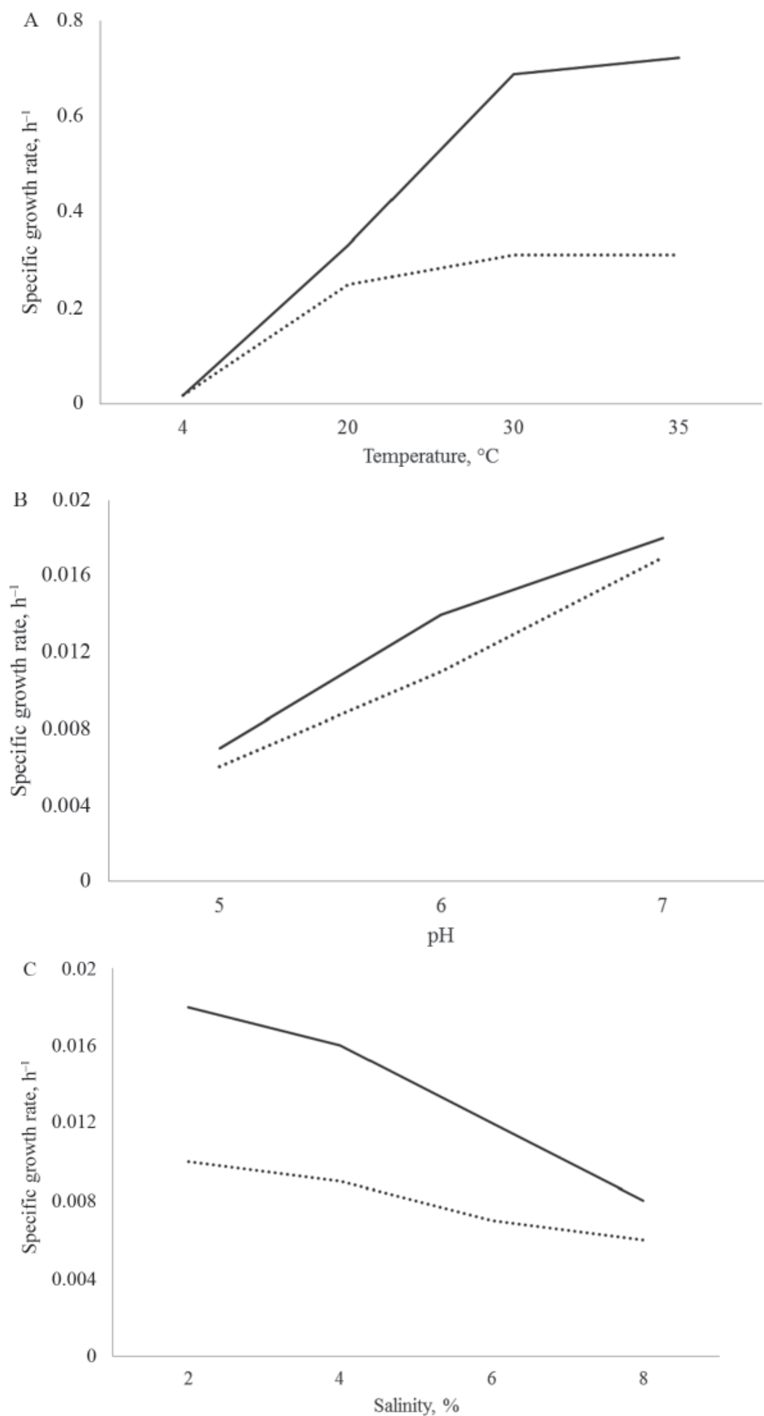

Fig. 2. Specific growth rates, μ h^{-1} of *L. monocytogenes* predicted with mathematical model and calculated from laboratory measurements for different temperature conditions (A), pH levels (B) and NaCl concentrations (C). Full line=fitted curve, dotted line=measured curve.

rate for salinity conditions were significantly different (P<0.05). Although the growth rates of *L. monocytogenes* was somewhat similar through the different pH levels, it was steadily decreased by the increase of NaCl concentration in TSB media stored at 4 °C. Bacterial growth in this assay was observed for all combinations studied, even at low pH, although PETRAN and ZOTTOLA (1989) found that the minimum pH for *L. monocytogenes* growth was at or above pH 4.5 at 30 °C. GEORGE and co-workers (1988) reported that the maximum pH levels for no growth at 20 °C and 30 °C ranged from 4.2 to 4.43, depending on the strain tested, while at 4 °C none of the strains grew at pH below 5.03.

Comparison of doubling times for *L. monocytogenes* under laboratory conditions and as predicted by the model under various temperature and salinity conditions, grouped according to different pH levels, is presented in Table 1. Doubling times in this study at pH 5.0 and 4 °C temperature varied slightly with NaCl concentrations, but were observed between 41.28 and 43.92 h, while the model predicted up to a 7 h increase in doubling times, except for the 2% salinity, where they corresponded with the measured time. Comparison of doubling times generally showed good agreement, particularly at lower temperatures, whereas at 30 °C and 35 °C the model mostly predicted slower doubling times than observed. When close to optimal growth conditions (low salinity, pH neutral) temperature related differences in predicted and observed doubling times were the most pronounced, doubling times being equal at low temperatures, and parting at higher temperatures. Under all conditions, when noted, most of the discrepancies between modelled and measured doubling times were related to predicting slower growth than actually observed. The observed and predicted doubling times were close to the line of equivalence, having 85.7% of data on the fail-safe side of the plot (data not shown). Higher temperatures at the same pH level significantly shortened the doubling times, both measured and predicted. When both measured and modelled doubling times were compared with those predicted by the ComBase Predictor, discrepancies were noted in relation to the NaCl concentrations irrespective of the pH level or temperature. In particular, a good correlation was found at higher salinities between the FMM and the ComBase, while low salinities, particularly of 0.5 and 2% NaCl, yielded a better correlation between the measured times and the ComBase than with the FMM. The inconsistent reports regarding the growth limits for *L. monocytogenes* may suggest that there are important strain differences, that there may be specific nutritional requirements for growth at lower temperatures, or that the physiological state of the inoculums used in challenge studies influences the minimum growth temperature (ROSS et al., 2000).

Table 1. Comparison of doubling times (h) for *L. monocytogenes* under laboratory conditions and as predicted by the FMM model, grouped according to pH levels: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.4.

Temperature (°C)	NaCl (%)	Doubling times (h)	
		Measured	Model
		pH=4.5	
20	0.5	4.08	6.72
20	2	3.84	3.84
20	4	4.32	8.4
20	6	5.76	5.28
20	8	9.12	11.52
30	0.5	1.92	4.08

Table 1. continued

Temperature (°C)	NaCl (%)	Doubling times (h)	
		Measured	Model
30	2	1.92	4.08
30	6	3.12	5.52
30	8	5.04	12.96
35	0.5	1.92	4.08
35	2	1.92	4.08
35	6	3.12	4.82
35	8	5.04	13.44
		pH=5.0	
4	0.5	43.92	50.4
4	2	41.28	42
4	4	43.92	50.4
20	0.5	2.4	2.4
20	2	2.4	2.4
20	4	2.64	3.6
20	6	3.6	2.88
20	8	5.52	4.32
30	0.5	1.2	2.64
30	2	1.2	2.64
30	4	1.44	2.88
30	6	1.92	2.88
30	8	3.12	4.56
35	0.5	1.2	1.68
35	2	1.2	2.4
35	4	1.44	2.4
35	6	1.92	2.4
35	8	3.12	4.08
		pH=5.5	
4	0.5	28.8	32.4
4	2	27.36	26.64
4	4	29.52	25.44
4	6	37.92	38.16
20	8	3.84	10.8
		pH=6.0	
4	0.5	21.36	21.60
4	2	20.64	13.76
4	4	22.56	21.6

Table 1. continued

Temperature (°C)	NaCl (%)	Doubling times (h)	
		Measured	Model
4	6	29.28	36.96
20	0.5	1.2	2.4
20	2	1.2	4.08
20	4	1.44	0.96
20	6	1.92	6
20	8	3.12	3.36
30	0.5	0.48	0.96
30	2	0.48	2.16
30	4	0.72	1.44
30	6	0.96	1.92
30	8	1.68	2.64
35	0.5	0.48	0.96
35	2	0.48	2.16
35	4	0.72	1.44
35	6	0.96	2.16
35	8	1.68	2.64
pH=6.5			
4	0.5	18	11.04
4	2	17.28	12.48
4	4	21.16	23.04
20	0.5	0.96	2.16
20	2	0.96	2.16
20	4	1.2	2.88
20	8	2.64	6.72
30	0.5	0.48	2.88
30	2	0.48	2.16
30	4	0.48	1.92
30	6	0.96	2.16
30	8	1.44	2.16
35	0.5	0.48	2.16
35	2	0.48	1.92
35	4	0.48	2.16
35	6	0.96	2.16
35	8	1.44	2.16

Table 1. continued

Temperature (°C)	NaCl (%)	Doubling times (h)	
		Measured	Model
		pH=7.0	
4	0.5	16.80	17.76
4	8	39.12	36.72
20	0.5	0.96	1.2
20	2	0.96	1.2
20	4	1.2	2.4
20	8	2.64	3.36
30	0.5	0.48	0.96
30	2	0.48	0.96
30	6	0.96	1.2
30	8	1.44	1.68
35	0.5	0.48	0.96
35	2	0.48	0.96
35	6	0.96	1.44
35	8	1.44	1.92
		pH=7.4	
4	0.5	17.52	16.56
4	2	17.28	15.84
4	4	19.68	18.72
4	8	42.24	45.36
20	0.5	0.96	1.92
20	2	0.96	2.4
20	4	1.2	1.92
20	6	1.68	1.68
20	8	2.88	4.08
30	0.5	0.48	1.92
30	2	0.48	1.92
30	4	0.72	1.92
30	6	0.96	2.16
30	8	1.68	1.92
35	0.5	0.48	1.92
35	2	0.48	1.92
35	4	0.48	1.92
35	6	0.96	1.92
35	8	1.68	4.08

In this study, although the predictions were close to the observed values, especially at low temperatures, the FMM was generally predicting slower growth than actually observed. However, even when the predictions of doubling times were greater than those observed, they were within an acceptable range and provided biologically plausible parameter estimates. Since most points fell close to the line of equivalence, predicted values were close to the observed values, indicating that the model predicted doubling times similar to those reported in published work. Interestingly, in the most cases for models based on BARANYI and co-workers (1993), predicted doubling times were shorter (i.e. growth rates were faster) than the observed values of MCCLURE and co-workers (1997). This is not unanticipated, since the liquid experimental systems provide optimal growth conditions, apart from the experimental variations of temperature, pH, and NaCl concentration, and such models tend to give fail-safe predictions (GIFFEL & ZWIETERING, 1999). Many published values are very close to the predicted values, supporting the argument that predictive models should be constructed using experimental data in media, which will give the fastest possible growth MCCLURE and co-workers (1997).

3. Conclusions

Currently, no single model includes the effect of all the variables that may be of interest in all tested matters and foods. Users of models, FMM included, must be aware of the predictive limits of the model, both in terms of the range of conditions and variables that it considers (BARANYI et al., 1996; ROSS et al., 2000). The FMM cannot make predictions for the effect of conditions that fluctuate over time, temperature being the factor most likely to change over the storage life of a product. Also, differences between the estimated growth parameters relate to the growth model being used. The use of the Gompertz model (FMM) led to the significant over-estimation of specific growth rates when compared to the Baranyi equation (AUGUSTIN & CARLIER, 2000). Although existing models have been expanded with a higher number of environmental parameters, including organic acids, to assess the *L. monocytogenes* growth rates in shrimp (MEJLHOLM & DALGAARD, 2009), generally applicable predictive microbial model for quantitative risk assessment in sea products is not available. However, they may enable microbiologists to limit the need of challenge tests and to make rapid and realistic prediction of the growth of *L. monocytogenes* in conditions relevant to a range of aquatic and other products examined.

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